UNIVERSIDAD AUTÓNOMA DE ESTADO DE HIDALGO
INSTITUTO DE CIENCIAS AGROPECUARIAS

DOCTORADO EN CIENCIAS AGROPECUARIAS

TESIS DOCTORAL

ACTIVIDAD ANTIBACTERIANA DE FITOCOMPLEJOS Y SU ADICIÓN A UNA BIOPELÍCULA PROTECTORA PARA LA PREVENCIÓN DE MASTITIS BOVINA

Para obtener el grado de
Doctora en Ciencias Agropecuarias

PRESENTA

M en C. Ana Lizet Morales Ubaldo

Director
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Santiago Tulantepec de Lugo Guerrero, noviembre del 2025

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Universidad Autónoma del Estado de Hidalgo
Instituto de Ciencias Agropecuarias
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Área Académica de Medicina Veterinaria y Zootecnia.
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I. RESUMEN GENERAL

La continua aparición de cepas bacterianas multirresistentes ha intensificado la necesidad de estrategias terapéuticas alternativas. Entre estas, el uso de biopelículas enriquecidas con ingredientes funcionales ha ganado creciente atención debido a sus propiedades antibacterianas. El objetivo del presente estudio fue determinar la actividad antibacteriana de fitocomplejos y su actividad protectora sobre bacterias asociadas a mastitis bovina para prevenir infecciones intramamarias y reducir la incidencia de mastitis bovina. Se formuló un fitocomplejo a partir de la mezcla de los extractos hidroalcohólicos de *Larrea tridentata y Caesalpinia Coriaria,* y de los aceites esenciales de *Lippia graveolens* y *Syzygium aromaticum*. La actividad antibacteriana se evalúo a través de la determinación de la Concentración mínima Inhibitoria y la Concentración mínima Bactericida frente a 12 cepas bacterianas asociadas con mastitis bovina. Se realizaron ensayos de cinética de muerte temporal y de integridad de membrana celular para elucidar el mecanismo de acción. Se llevaron a cabo análisis cromatográficos para caracterizar el fitocomplejo. La citotoxicidad se evaluó mediante el ensayo de letalidad en *Artemia salina.* Se obtuvo una biopelícula a base de almidón de chayotextle adicionada con el fitocomplejo por medio de la técnica de casting. La biopelícula fue caracterizada mediante Microscopía Electrónica de Barrido (SEM) y Espectroscopía Infrarroja por Transformada de Fourier (FTIR). La actividad antibacteriana se determinó mediante ensayos de difusión en disco y pruebas de inhibición del crecimiento bacteriano frente a *Escherichia coli* y *Staphylococcus aureus*, utilizadas como cepas indicadoras. Además, se utilizó un modelo estructural físico para evaluar la reducción del crecimiento bacteriano. El fitocomplejo presentó actividad inhibitoria a concentraciones de entre 0.39 mg/Ml y 6.34 mg/mL, mientras que las concentraciones mínimas bactericidas oscilaron entre 0.79 mg/mL y 25.38 mg/mL, ejerciendo su efecto bactericida durante los primeros 30 minutos de contacto, provocando la liberación de proteínas y ácidos nucleicos, evidenciando daño irreversible en la membrana citoplasmática. La actividad antibacteriana se asoció con la presencia de rutinosido de luteolina, rutinosido de apigenina, *nor* 3´-demetoxiisoguaiacina, ácido gálico, ácido elágico, eugenol y timol. El fitocomplejo no presentó citotoxicidad. La biopelícula adicionada con el fitocomplejo mostró zonas de inhibición de 10.25 mm a 24.75 mm, con mayor actividad frente a *S. aureus*. Se determinó una reducción del crecimiento de *S. aureus y E. coli*. La actividad antibacteriana se atribuyó a reordenamientos estructurales dentro de la matriz polimérica y la formación de interacciones entre los grupos funcionales presentes en el polisacárido y en el fitocomplejo. La biopelícula a base de almidón de chayotextle adicionada con el fitocomplejo demostró actividad antibacteriana *in vitro* frente a bacterias asociadas a mastitis bovina.

Palabras clave: Actividad antibacteriana, fitocomplejos, biopelícula funcionalizada, mastitis bovina

II. GENERAL ABSTRACT

The continuous emergence of multidrug-resistant bacterial strains has intensified the need for alternative therapeutic strategies. Among these, the use of biofilms enriched with functional ingredients has gained increasing attention due to their potential antimicrobial properties. The aim of the present study was to determine the antibacterial activity of phytocomplexes and their protective effect against bacteria associated with bovine mastitis, to prevent intramammary infections and reduce the incidence of the disease. A phytocomplex was formulated from a mixture of the hydroalcoholic extracts of *Larrea tridentata* and *Caesalpinia coriaria*, and the essential oils of *Lippia graveolens* and *Syzygium aromaticum*. The antibacterial activity was evaluated by determining the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration against 12 bacterial strains associated with bovine mastitis. Time-kill kinetics and cell membrane integrity assays were conducted to elucidate the mechanism of action, while chromatographic analyses were performed to characterize the phytocomplex. Cytotoxicity was assessed using the brine shrimp lethality assay. A chayotextle starch-based biofilm functionalized with the phytocomplex was obtained using the casting technique. The biofilm was characterized by Scanning Electron Microscopy and Fourier Transform Infrared Spectroscopy. Antibacterial activity was evaluated through disk diffusion and bacterial growth inhibition assays against *Escherichia coli* and *Staphylococcus aureus* as indicator strains. Additionally, a physical structural model was used to assess bacterial growth reduction. The phytocomplex exhibited inhibitory activity at concentrations ranging from 0.39 mg/mL to 6.34 mg/mL, while bactericidal concentrations ranged from 0.79 mg/mL to 25.38 mg/mL, showing bactericidal effects within the first 30 minutes of contact. The release of intracellular proteins and nucleic acids indicated irreversible damage to the cytoplasmic membrane. The antibacterial activity was associated with the presence of luteolin rutinoside, apigenin rutinoside, *nor* 3´-demethoxyisoguaiacin, gallic acid, ellagic acid, eugenol, and thymol. The phytocomplex was found to be non-cytotoxic. The biofilm functionalized with the phytocomplex exhibited inhibition zones ranging from 10.25 mm to 24.75 mm, showing greater activity against *S. aureus* and growth reduction of both *S. aureus* and *E. coli*. The antibacterial activity was attributed to structural rearrangements within the polymeric matrix and the formation of interactions between the polysaccharide functional groups and the compounds present in the phytocomplex. The chayotextle starch-based biofilm functionalized with the phytocomplex demonstrated in vitro antibacterial activity against bacteria associated with bovine mastitis.

Keywords: Antibacterial activity, Phytocomplexes, functionalized biofilm, bovine mastitis

1. INTRODUCCIÓN GENERAL

La mastitis bovina es considerada una de las principales enfermedades dentro de los hatos lecheros alrededor del mundo. Se define como la inflamación de la glándula mamaria en las vacas. La enfermedad es causada principalmente por infecciones bacterianas. A lo largo de los años se han establecido regímenes terapéuticos principalmente basados en el uso de antibacterianos, sin embargo, actualmente debido al fenómeno de resistencia a antimicrobianos no son eficaces, estudios recientes afirman que la eliminación bacteriológica suele ser inferior al 60% (Caneschi, Bardhi, Barbarossa, & Zaghini, 2023; Sharun et al., 2021).

La resistencia a los antimicrobianos es una amenaza creciente, con mecanismos de resistencia nuevos y emergentes que aparecen y se propagan globalmente. A medida que los antibacterianos se vuelven menos efectivos, ciertas infecciones se vuelven más difíciles, e incluso imposibles, de tratar, en este sentido la presencia de bacterias patógenas en la leche se considera un problema de seguridad alimentaria a través del consumo de leche cruda(Naranjo-Lucena & Slowey, 2023).

Por lo tanto, es necesario buscar tratamientos alternativos basados en productos de origen natural, especialmente cuando se enfrentan a bacterias resistentes a múltiples fármacos. El uso de extractos de plantas está documentado en la medicina tradicional a nivel mundial y existe un creciente interés en su aplicación para el tratamiento de la mastitis bovina. Ya se emplean en este campo muchas plantas medicinales con diferentes propiedades biológicas, aunque son escasos los ensayos in vivo que reporten su eficacia, farmacocinética y farmacodinamia (Caneschi et al., 2023; Kher, Sheth, & Bhatt, 2019).

Actualmente la adopción de terapias combinadas tal es el caso de fitocomplejos, en las que se emplean múltiples componentes activos ofrecen mayor eficacia que dosis equivalentes de ingredientes activos individuales o hierbas cuando se usan solas, lo que resalta la importancia de la acción sinérgica en las terapias herbales frente a enfermedades complejas, incluidas las infecciones bacterianas (Zhou et al., 2016), sin embargo los compuestos contenidos en ellas suelen ser susceptibles de degradación y cambios estructurales, por lo que ha surgido la necesidad de protegerlos.

Una alternativa altamente viable su adición a películas biopoliméricas, dadas las ventajas que su uso representa, puesto que actúan como vehículo del ingrediente funcional, dan protección al ingrediente funcional de degradación química o biológica, durante el proceso de almacenamiento y utilización, ya que, durante estos

estados, el ingrediente debe permanecer activo, además son capaces de controlar la liberación del ingrediente funcional (Solano-Doblado, Alamilla-Beltrán, & Jiménez-Martínez, 2018)

Estudios previos han demostrado que el uso de biopolímeros son una alternativa viable para el tratamiento de la enfermedad, tal es el caso de óxidos de polietileno, la hidroxipropilmetilcelulosa, la carboximetilcelulosa, el alginato de sodio y la goma xantana, debido a su capacidad de formar geles no tóxicos con buena biocompatibilidad (Bhattarai, Perumal, Rathbone, Bunt, & Alany, 2021)

Partículas poliméricas a base de las diferentes combinaciones de alginato, quitosano y tripolifosfato de sodio adicionadas con ácido mercaptosuccínico mostraron actividad antibacteriana potencial sobre *S. aureus* y *E. coli* (CMI = 125 µg/mL a 250 µg/mL (Cardozo et al., 2014). En un estudio similar se reportó que el quitosano presentó efectos tanto inhibitorios como bactericidas frente a *S. aureus* y *S. xylosus* (Felipe et al., 2019).

La adición de quitosano a la cloxacilina aumentó la eficacia del antibacteriano al reducir la concentración requerida para presentar su efecto farmacológico, además de inhibir la formación de biopelículas bacterianas y eliminar las ya formadas (Breser et al., 2018), de manera similar la cloxacilina benzatínica encapsulada en nanopartículas de poli-ε-caprolactona a una concentración de 600 mg eliminó el 100% de *Corynebacterium* spp. y *S. uberis in vivo* (Araújo et al., 2019)

Con respecto a la actividad antibacteriana asociada a casos de mastitis bovina (Bhattarai, Alany, Bunt, Abdelkader, & Rathbone, 2015) determinaron que insertos a base de óxido de polietileno actuaron como barrera física frente a algunos patógenos que invaden el canal del pezón de las vacas, además poder actuar como vehículos para la liberación de fármacos.

Por su parte (Kamaruzzaman et al., 2017) reportaron que la polihexametilen biguanida, un polímero antimicrobiano, mostró efectos positivos, matando al 99,9% de *S. aureus* intracelular a 15 mg/L; a la misma concentración, la masa de la biopelícula bacteriana se redujo 37%. Este estudio indicó que este compuesto fue tolerado por las células huésped a altas concentraciones. Otro estudio determinó que este polímero es efectivo en el tratamiento de *Prototheca* spp., asociada a mastitis, determinándose efectos a concentraciones más bajas (≥1,0 a ≥4,0 µg/mL) (Fidelis et al., 2023). El uso y aplicación de biopelículas biodegradables enriquecidas con ingredientes antimicrobianos se consideran como alternativa de tratamientos puesto que ofrecen actividad antibacteriana mejorada.

2. PLANTEAMIENTO DEL PROBLEMA

La mastitis bovina de origen bacteriano es una de las enfermedades de mayor importancia en el sector lácteo, debido a su impacto en la productividad y la calidad de la leche. Los patógenos involucrados tienen la capacidad de colonizar el canal del pezón y la glándula mamaria, provocando infecciones, principalmente subclínicas, que reducen el rendimiento y generan pérdidas económicas significativas para los productores.

Las estrategias actuales de control se basan en buenas prácticas de manejo y en el uso de antibacterianos; sin embargo, el incremento en la resistencia bacteriana ha puesto en evidencia la necesidad de desarrollar alternativas sostenibles para su prevención y tratamiento.

En este contexto, las tendencias recientes en investigación resaltan el valor de un enfoque multifuncional para el diseño de nuevas terapias. La formulación de fitocomplejos representa una estrategia prometedora, ya que permite desarrollar tratamientos más específicos y eficaces frente a enfermedades complejas como la mastitis bovina. Asimismo, los biopolímeros naturales destacan por su biocompatibilidad y capacidad para actuar como vehículos de liberación controlada de diversos compuestos bioactivos, incluidos los de origen vegetal, ofreciendo efectos prolongados en el sitio de infección y reduciendo los efectos secundarios sistémicos.

Por lo tanto, es fundamental evaluar si la adición de fitocomplejos en una biopelícula protectora puede disminuir la colonización de los patógenos asociados a la mastitis bovina y, con ello, contribuir a la prevención de esta enfermedad de manera segura y sostenible.

3. JUSTIFICACIÓN

En México la ganadería dirigida a la producción de leche, tiene un importante papel en la nutrición, los medios de vida del ser humano y la seguridad alimentaria, de acuerdo con organismos internacionales como la FAO, nuestro país podría experimentar un significativo crecimiento en la producción láctea, sin embargo, son muchos los desafíos a los cuales este sector se enfrenta, tal es el caso de las enfermedades infecciosas de carácter bacteriano, como lo es la mastitis bovina, enfermedad asociada con importantes pérdidas económicas debido al aumento en costos de producción, aunado a lo anterior, el uso inadecuado de los antimicrobianos ha propiciado que los agentes bacterianos asociados a la enfermedad generaran resistencia a los antimicrobianos indicados para su control y tratamiento, situación que actualmente se considera alarmante para la salud pública a nivel mundial.

Dicho fenómeno ha incentivado el interés por desarrollar alternativas de tratamiento eficaces, destacando de entre ellas el uso de extractos vegetales, sin embargo, el uso de extractos vegetales en muchas ocasiones no es adecuados para su uso como agentes antimicrobianos debido a las elevadas dosis que se necesitan para su aplicación *in vivo,* por lo que actualmente el uso de extractos vegetales en combinación ha demostrado tener potenciales efectos sobre diversos agentes bacterianos de importancia clínica, a pesar de ello, es sabido que estos compuestos bioactivos son susceptibles de degradación y cambios estructurales por lo que es necesario protegerles, en este sentido el uso de biopelículas resulta altamente viable para dicho propósito.

Dichas alternativas resultan altamente viables para su uso dentro de los sistemas de producción pecuaria, debido a debido a sus aportes benéficos, tales como bajo impacto ambiental, bajo costo y menores efectos adversos sobre los individuos bajo tratamiento. En México la presencia de microorganismos resistentes y multirresistentes a antimicrobianos en el sector pecuario se asocia con elevadas pérdidas económicas, por lo que el control de dichos agentes a través de la evaluación de la actividad antibacteriana de una película protectora adicionada con extractos vegetales para el control de bacterias multirresistentes asociadas a mastitis bovina permitirá proponer una nueva y funcional alternativa de tratamiento que tenga menor impacto al medio ambiente y a la salud pública.

4. HIPÓTESIS

La biopelícula adicionada con fitocomplejos no permite la colonización de la glándula mamaria

5. OBJETIVOS

5.1 Objetivo general

Determinar la actividad antibacteriana de fitocomplejos y su actividad protectora sobre bacterias asociadas a mastitis bovina para prevenir infecciones intramamarias y reducir la incidencia de mastitis bovina.

5.2 Objetivos específicos

1. Evaluar *in vitro* la actividad antibacteriana y el posible mecanismo de acción de fitocomplejos sobre bacterias asociadas a mastitis bovina.
2. Determinar la citotoxicidad del fitocomplejo con mejor actividad antibacteriana en un modelo *in vivo* sobre *Artemia salina*
3. Elaborar una biopelícula protectora adicionada con el fitocomplejo con mejor actividad antibacteriana
4. Evaluar *in vitro* la actividad antibacteriana de la biopelícula protectora adicionada con el fitocomplejo más activo

6. CONCLUSIÓN GENERAL

Se determinó que la adición del fitocomplejo constituido por los extractos hidroalcohólicos de *Larrea tridentata* y *Caesalpinia coriaria* y los aceites esenciales de *Lippia graveolens* y *Syzygium aromaticum*, en una matriz polimérica de almidón de chayotextle confiere actividad antibacteriana *in vitro* sobre bacterias asociadas a mastitis bovina, por lo que se considera una alternativa altamente viable para la prevención de infecciones mamarias y la reducción de la incidencia de mastitis bovina. Sin embargo, serán necesarios estudios *in vivo* para validar su eficacia bajo condiciones reales de producción.

CAPÍTULO I: Mastitis bovina, una enfermedad de impacto mundial: Prevalencia, resistencia a antimicrobianos y estrategias alternativas viables

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Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches

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ABSTRACT

Bovine mastitis is globally considered one of the most important diseases within dairy herds, mainly due to the associated economic losses. The most prevalent etiology are bacteria, classified into contagious and environmental, with *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Escherichia coli and Klebsiella pneumoniae* being the most common pathogens associated with mastitis cases. To date these pathogens are resistant to the most common active ingredients used for mastitis treatment. According to recent studies resistance to new antimicrobials has increased, which is why developing of alternative treatments is imperative. Therefore the present review aims to summarize the reports about bovine mastitis along 10 years, emphasizing bacterial etiology, its epidemiology, and the current situation of antimicrobial resistance, as well as the development of alternative treatments for this pathology. Analyzed data showed that the prevalence of major pathogens associated with bovine mastitis varied according to geographical region. Moreover, these pathogens are classified as multidrug-resistant, since the effectiveness of antimicrobials on them has decreased. To date, several studies have focused on the research of alternative treatments, among them vegetal extracts, essential oils, or peptides. Some other works have reported the application of nanotechnology and polymers against bacteria associated with bovine mastitis. Results demonstrated that these alternatives may be effective on bacteria associated with bovine mastitis.

1. Introduction

Milk and its derivatives are sources of important nutrients to people, which is why the dairy industry continues to consolidate into larger farms, However, this industry has been facing challenges, dealing with demands of accountability for animal welfare and product safety. In this sense identifying diseases is key to recognizing the multifactorial nature of almost all diseases of importance in dairy cattle and redefining them more broadly, to include subclinical conditions, such as bovine mastitis (LeBlanc *et al.*, 2006; Petersson-Wolfe *et al.*, 2018; Fetrow *et al.*, 2020; Nguyen; Briggs et al., 2022).

Bovine mastitis, defined as the inflammation of the mammary, is one of the most critical pathologies within dairy herds worldwide, due to its economic impact, causing huge losses not only reflected in decreased production but also in culling rates (Azooz *et al.*, 2020; Sharun *et al.*, 2021).

Bovine mastitis is mainly classified according to clinical (or subclinical) features and etiology (noninfectious and infectious). Infectious causes are most common, and in several cases, infections associated with bacteria are the most prevalent presentation within herds. Bacterial pathogens are also classified into different categories: Contagious, environmental, and opportunistic bacteria (Ndahetuye *et al.*, 2019). According to several studies, the pathogens most frequently present in mastitis cases are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Escherichia coli and Klebsiella pneumoniae* (Klaas & Zadoks, 2018; Ashraf & Imran, 2020; Cadona-Hernandez et al., 2021).

In addition, bacterial resistance has become a rising threat since mechanisms of resistance are spreading globally. To date, traditional

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antibiotic treatments easy easily cause the apparition of resistant strains (Peng *et al.*, 2022). Resistance to antimicrobials such as, penicillin, amoxicillin, tetracycline, Amikacin, gentamicin, or erythromycin has been widely reported. Nevertheless, according to recent studies resistance to new antimicrobials has increased; bacterial profiles indicated resistance to piperacillin, ceftazidime, cefquinome, tigecycline, colistin and vancomycin. Moreover, the problem of drug residue is also increasing. (Carvalho-Castro *et al.*, 2017; Monistero *et al.*, 2021; Campos et al.;, 2022; Vidal-Amaral *et al.*, 2022; Bonardi *et al.*, 2023).

Due to the abovementioned concerns, the search for therapeutic alternatives that are not invasive and do not cause resistance is necessary (Junior *et al.*, 2023). Research shows that herbal or plant medicine in livestock production has been used as health promoters and for treating of diseases (Kuralkar & Kuralkar, 2021). Recently, new alternatives have been proposed including peptides, nanoparticles, nanocolloids or polymers, and more recently, phototherapy, all of which have demonstrated effectiveness (Gruet et al., 2001; Omara, 2017; Marques-Bastos *et al.*, 2022; Fidelis *et al.*, 2023; Saeed *et al.*, 2023).

In this respect the present review aims to summarize the reports over 10 years, emphasizing bacterial etiology, its epidemiology, and the current situation of antimicrobial resistance, as well as the development of alternative treatments for this pathology.

2. Methodology

A comprehensive search was conducted in the following databases: Google Scholar, PubMed, Scopus and ScienceDirect for studies published from 2013 to 2023. The following headings and keywords were used: "Bovine mastitis", "antimicrobial resistance" and "alternative treatments". In addition, the phrase "antibacterial activity" was employed to generate data for the biological activity discussed in the review. The methods used in this review were as follows: All the considered papers were analyzed according to each section of the review, and the most relevant information was taken and synthetized to obtain concrete information. Duplicate papers were removed, the data were screened, irrelevant work was excluded, and full-text documents were then screened. Inclusion criteria including original articles or reviews and work on natural or chemical alternative treatments. Exclusion criteria were inadequate methods and the lack of access to the full text.

3. Bovine mastitis classification

3.1. Clinical features

Mastitis can be classified according to clinical features, into clinical and subclinical mastitis. The first is characterized by the presence of flakes, clots, or watery secretions in milk. Generally the infected quarters are swollen, hot and painful. In some acute clinical cases, general signs can be found (hyperthermia, anorexia, and depression). The consequences are severe, causing cow's death or agalactia, which leads to premature culling (Cobirka *et al.*, 2020) Subclinical mastitis is more difficult to diagnose, because of the lack of evident signs in milk or animals. Its major symptoms are related to increased somatic cell count and decreased milk production. Subclinical duration lasts longer than clinical and infection allows the spread of pathogens within the herd (Cobirka *et al.*, 2020).

3.2. Noninfectious causes

Mechanical injuries associated with machine milking can induce severe damages to quarters, which makes them more vulnerable, ascending into infections due to damage to keratin or mucous membranes lining the teat sinus (Schlafer & Foster, 2016; Ashraf & Imran, 2020). Poor hygienic practices are highly related, such as unclean udder at the start of milking. Moreover, Holstein cows are more susceptible to mastitis than other breeds (Ramírez *et al.*, 2014). Dietary imbalances are also related to mastitis cases (Bludau *et al.*, 2014). In addition, Perez-Morales *et al.* (2022), determined that cows with seven or more calving showed a higher prevalence of mastitis. Lactation days can also increase the prevalence of disease's prevalence.

3.3. Infectious causes

Etiology is not completely described; to date almost 200 microorganisms are associated with mastitis, and new pathogens are continuously detected and reported, including yeast, fungi, viruses, and bacteria (Benić *et* al., 2018;Ashraf & Imran, 2020).

3.3.1. Yeast and fungal mastitis

In recent years, higher morbidity rates caused by mycotic mastitis in cattle have been reported. A recent study detected zoonotic yeasts, including *Candida albicans* and *Kodamaea ohmeri* (Awandkar *et al.*, 2021). Other *Candida* species were reported *C. guilliermondii, C. famata, C. tropicalis, C. colliculosa, C. krusei, C. rugosa, C. glabrata, C. parapsilosis, C. inconspicua; Trichosporon* sp., *Rhodotorula glutinis, Saccharomyces fragilis; Pichia kudriavzevii, Cyberlindnera rhodanensis*; mold species were also found *Aspergillus amstelodami, A. fumigatus* and *Geotrichum candidum* (Hayashi *et al.*, 2013; Zhou *et al.*, 2013; Ksouri *et al.*, 2015; Dalanezi *et al.*, 2018) *Prototheca zopfii* and *Prototheca blaschkeae* (yeast-like algae) could also be related (Ricchi *et al.*, 2013).

3.3.2. Viral mastitis

It is probable that virus-induced immunosuppression underlies mastitis. It has been reported that bovine herpesvirus 1, foot and mouth disease virus, and parainfluenza virus caused clinical mastitis, and bovine herpesvirus 4 cause subclinical mastitis. On the other hand teat lesions associated with bovine herpesvirus 2, cowpox, pseudo cowpox virus, foot and mouth disease, vesicular stomatitis virus, papillomavirus, and bovine leukemia virus indirectly contribute to mastitis (Herlekar *et al.*, 2013; Martinez Cuesta *et al.*, 2019; Cuesta et al., 2020).

3.3.3. Bacterial mastitis

Bacteria are the most common and prevalent etiologic agents associated with mastitis. More than 150 Gram-positive and Gram-negative bacteria are identified as mastitis pathogens, which can be divided into contagious (spread from other infected quarters) and environmental (surrounding environment; Ruegg, 2017; Ndahetuye *et al.*, 2019, Ashraf & Imran, 2020; Cobirka *et al.*, 2020).

The udder serves as the reservoir of contagious pathogens transmitted from infected to uninfected teats during the milking process. They mainly include *Mycoplasma bovis, Streptococcus agalactiae*, and *Staphylococcus aureus* (Cvetnić *et al.*, 2016; Ashraf & Imran, 2020; Cobirka *et al.*, 2020).

A higher incidence of *S. agalactiae* isolated from mastitic cows was reported. Due to this many countries consider this pathogen predominant among other contagious pathogens (Zhang *et al.*, 2018; Abd El-Aziz *et al.*, 2021; Cadona *et al.*, 2021). Despite this, a diverse number of reports still consider *S. aureus* the most prevalent contagious agent associated with mastitis, as this bacterium is persistent inside the udder (Lamari *et al.*, 2021;Rainard *et al.*, 2018). Another less common contagious pathogen is *M. bovis*, although few studies report its prevalence. Nevertheless, outbreaks due to *M. bovis* are becoming common and regarded as the most prevalent mycoplasma species causing bovine mastitis worldwide (Liu *et al.*, 2020; Gelgie *et al.*, 2022). On the other hand, environmental pathogens are transmitted through feces, the indoor environment, and pastures, the most common being *E. coli, Klebsiella pneumoniae* and *Streptococcus uberis* (Klaas & Zadoks, 2018).

E. coli is the most common Gram-negative coliform responsible for causing environmental clinical mastitis (Alawneh *et al.*, 2020; Campos *et al.*, 2022), mainly due to high genotypic variability (Bag *et al.*, 2021). *K. pneumoniae* is the second most common cause of bovine mastitis and, is considered the most detrimental in decreasing milk production and

quality, as well as in economic terms. Although it is classified as an environmental pathogen, it has also been found to be transmitted from infected to healthy cows (Cheng *et al.*, 2021; Fu *et al.*, 2022). *Streptococcus uberis* causes about one-third of all intramammary infection cases in cows worldwide, invading the teat channel after milking or after damage (Abureema *et al.*, 2014; Monistero *et al.*, 2021). A study reported mycobacteria as causative agents of mastitis, two strains were identified as *Mycobacterium fortuitum* II and *Mycobacterium margeritense,* which are resistant to clarithromycin Cvetnić et al. (2022).

Kotzamanidis *et* al. (2021), stated that it is necessary to understand the population structure, transmission, virulence characteristics, and pathogenicity of pathogens associated with mastitis to develop strategies for reducing the pathogen's spread among herds in a specific geographical region. Epidemiological data (Table 1), virulence characteristics and resistance profiles (Table 2) of bacterial contagious and environmental pathogens associated with mastitis are given in the tables below. Regarding antimicrobial resistance, the existence of patterns between frequently used antimicrobials and increasingly resistant bacteria has been stated; these patterns could help to implement strategies to control mastitis and find opportunities for further reduction (Kovačević et al., 2022b).

4. Financial losses

Economic losses due to mastitis can be defined as a reduction of output due to this disease and an absence of benefits that would otherwise be accrued in the absence of mastitis. Costs are classified into direct costs (veterinary services, additional labor requirements, and discarded milk during treatment) and indirect costs (reduced milk yield and quality premiums, premature culling) (Azooz *et al.*, 2020; Kovačević et al., 2022a).

In their study, Azooz *et al.* (2020) determined the financial impact of mastitis in Egypt. Several criteria were included in this study. Milk yield losses per year associated with subclinical mastitis were 20,563, 656.4822 LE; lower costs were calculated when clinical mastitis cases presented (326,814 LE). Quality premium losses ascended to 1369, 602.12 LE, and the other two most important costs were associated with premature culling (736,000 LE) and discarded milk (100.172 LE). In Ethiopia the average failure cost associated with mastitis was 4765 Ethiopian Birr (ETB) or $213.94 per farm per year, while per lactating cow per farm per year costs were 1961 ETB ($88.04); (Mekonnen *et al.*, 2019).

In Thai dairy farms affected with mastitis, an average of $557 for three months was calculated; 10.4% of losses were caused by the reduction in raw milk price, and the remaining 89.6% was attributed to discharged milk due to clinical mastitis testing positive (Dejyong *et al.*, 2022). India estimated a cost of INR1390 per lactation, from which half of the costs were associated with the loss of milk. Furthermore, veterinary expenses represented 37% of the total costs, and higher losses are associated with crossbred cows (Sinha *et al.*, 2014). In China, the economic impact across large dairy farms from seven provinces ranged from $15,000 to $76,000 farm/month (He *et al.*, 2020).

Through a deterministic partial budget model, the direct and indirect costs of mastitis in the US were calculated. The obtained data showed that during the first 30 days of lactation, the total economic cost was $444. Direct costs included diagnostics ($10), therapeutics ($36), non-saleable milk ($25), veterinary service ($4), labor ($21), and death loss ($32), totaling $128.00. For indirect costs, premature culling and replacement were estimated at more than $180, milk production loss occupied second place with a loss of $125, and future reproductive loss was $9, totaling of $316 (Rollin *et al.*, 2015). Recently, using a Monte Carlo simulation model, costs associated with chronic mastitis were estimated, obtained data showed an average of € 118. In addition, the costs of mastitis per generic intramammary infections case were estimated at € 230 (Bonestroo *et al.*, 2023).

In Canada, mastitis costs represent reductions from $386 to $779 (Puerto *et al.*, 2021). A study performed in an important dairy region of Colombia determined that the impact due to milk losses was over $800.000 per year and $70.3 per cow per year. According to the authors, the impact was greater in small- and medium-sized farms than in large farms, because large farms are more homogeneous in their management of subclinical mastitis (Romero *et al.*, 2018).

A pharmacoeconomic analysis was conducted to determine the cost and effectiveness of conventional bovine mastitis in Serbia, determining a total of € 80.32 (Kovačević et al., 2022a). In Dutch farms the average total cost of mastitis was €240/ lactating cow per year; failure costs (€120) was attributed mainly to milk production losses (€32), discarded milk (€20), and culling (€20), while in preventive costs (€120/ lactating cow per year), labor costs were the main contributor (€82), followed by consumables and investments, €34 and €4, respectively(van Soest *et al.*, 2016).

The study conducted by Hadrich *et al.* (2018), estimated financial losses in 10 months. Daily losses in the first month were $1.20/cow per day, in the 10th month losses increased to $2.06/cow per day. Another study estimated losses associated with pregnancy; the results showed that during the first 75 days when clinical mastitis cases were present, the impact was $148.99 per case (Dahl *et al.*, 2018).

In terms of milk loss production, *S. aureus* infections represent losses

Table 1
Prevalence of bacterial contagious and environmental pathogens associated with bovine mastitis.

Continent	Country	*S. agalactiae*	*S. aureus*	*M. bovis*	*E. coli*	*K. pneumoniae*	*S. uberis*	References
Africa	Cameroon	–	–	–	7.0	2.4	–	(Abegewi *et al.*, 2022)
	Egypt	–	–	–	–	13.59	20.59	(Abd El-Aziz *et al.*, 2021; Tartor *et al.*, 2021)
	Ethiopia	10.3	46.5–72.3	–	6.18–13.31	1.6	–	(Abebe *et al.*, 2016; Seyoum *et al.*, 2018; Tesfaye & Abera, 2018; Lakew *et al.*, 2019; Belay *et al.*, 2022)
	Tunisia	–	–	–	–	20.0	–	(Saidani *et al.*, 2018)
	Zimbabwe	–	16.3	–	–	–	–	(Katsande *et al.*, 2013)
Asia	Bangladesh	–	74.0	–	35.8	–	–	(Hoque *et al.*, 2018; Bag *et al.*, 2021)
	China	–	–	–	11.1	3.0–51.0	74.40	(Bi *et al.*, 2016; Gao *et al.*, 2019; Yu *et al.*, 2020; Cheng *et al.*, 2021; Yang *et al.*, 2021; Zeng *et al.*, 2022)
	Iran	–	20.4	–	–	–	–	(Jamali *et al.*, 2014)
	Japan	–	–	3.8	–	12.30	–	(Murai & Higuchi, 2019; Taniguchi *et al.*, 2021)
	Pakistan	17.54	21.5		17.54–19.40	–	–	(Sadaf *et al.*, 2016; Ali *et al.*, 2021)
America	Canada	0.1	9.9	–	9.0	1.9	0.6	(Levison *et al.*, 2016)
	Argentina	4.4–5.5	21.3–28.1	–	2.1		0.4–31.8	(Dieser *et al.*, 2014; Neder et al., 2015; Srednik *et al.*, 2018)
	Brazil	–	–	3.0	6.9	–	71.0	(Junqueira *et al.*, 2020; Martins *et al.*, 2021; de Oliveira *et al.*, 2022)
	Mexico	–	–	–	7.5–9.0	5.0	–	(León-Galván *et al.*, 2015; Olivares-Pérez *et al.*, 2015)
Europe	Serbia	–	4.57–6.09	–	12.12–26.82	1.51	1.51–6.06	(Kovačević et al., 2022a)
Oceanica	Australia	–	–	6.2–76.0	–	–	16.0–39.2	(Al-Farha *et al.*, 2017; Hazelton *et al.*, 2020; Chung *et al.*, 2021; Dyson *et al.*, 2022)

Table 2
Virulence characteristics and resistance profiles of bacterial contagious and environmental pathogens associated with bovine mastitis.

Agent	Virulence characteristics	Antimicrobial resistance profile	Antimicrobial resistance genes	References
S. agalactiae	Biofilm formation, *Cps, cylE*, CAMP, cfa/cfb, hylB, cylE, iagA, bac, fb, fbsA, fbsB, PI-2a, PI-2b, PI-1, pau-A, *α enolase*, hyaluronate lyase	Amoxicillin Ceftazidime Ceftriaxone Penicillin Piperacillin	mprF, mreA, *TEM*	(Carvalho-Castro *et al.*, 2017; Miranda *et al.*, 2018; Abd El-Aziz *et al.*, 2021; Han *et al.*, 2022; Parasana *et al.*, 2022; Vidal-Amaral *et al.*, 2022)
S. aureus	Biofilm formation, capsules, Panton-Valentine leucocidin, Toxic shock syndrome toxin-1, Immune evasion cluster genes, type E, slime production	Amoxicillin Cefoxitin Ciprofloxacin Clindamycin Erythromycin Gentamicin Oxacillin Oxytetracycline Penicillin Sulfamethoxazole Tetracycline Trimethoprim Vancomycin	blaZ, tetM, tetK, tetL, mecA, mecC, spa fragment, icaA, icaD	(Jamali *et al.*, 2014; Bhattacharyya *et al.*, 2016; Hoque *et al.*, 2018; Seyoum *et al.*, 2018; Srednik *et al.*, 2018; Wang *et al.*, 2018; Zaatout *et al.*, 2020; Chen *et al.*, 2021; Cvetnić *et* al., 2021; Saidi *et al.*, 2021; Crespi *et al.*, 2022)
M. bovis	Adhesines, avoidance of phagocytosis, biofilm formation, hydrogen peroxide production	Kanamycin Oxytetracycline Tilmicosin Tylosin Florfenicol Tiamulin Enrofloxacin	–	(Kawai *et al.*, 2014; Bokma *et al.*, 2020; García-Galán *et al.*, 2020; Gelgie *et al.*, 2022)
E. coli	LpfA, Iss, astA, f17A, irp2, iucD, colV, papC	Amikacyn Ampicillin Carbenicillin Cefoxitin Ceftriaxone Cephalothin Chloramphenicol Ciprofloxacin Doxycycline Erythromycin Gentamicin Lincomycin Nitrofurantoin Oxacillin Oxytetracycline Penicillin Streptomycin Sulfamethoxazole Tetracycline Vancomycin	tetM, tetL, tetA, blaZ, blaEc, ampC, aadA, sul2, strA, strB, sul1, sul3, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, $bla_{CTX-M-28}$, $bla_{CTX-M-14}$	(Blum & Leitner, 2013; Liu *et al.*, 2014; Olivares-Pérez *et al.*, 2015; Fazel *et al.*, 2019; Nüesch-Inderbinen *et al.*, 2019; Alawneh *et al.*, 2020; Ali *et al.*, 2021; Bag *et al.*, 2021; Bhandari *et al.*, 2021; Shafiq *et al.*, 2021; Campos *et al.*, 2022; de Oliveira *et al.*, 2022)
K. pneumoniae	EntB, FimH, Kfu, MrkD, β-D-lacZ, ituA, allS	Amoxicillin Ampicillin Cefazolin Cefotaxime Cefoxitin Cefquinome Ceftazidime Ceftriaxone Cephalothin Chloramphenicol Colistin Enrofloxacin Fosfomycin Gentamicin Kanamycin Neomycin Piperacillin-tazobactam Streptomycin Spectinomycin Sulfisoxazole Sulfonamides Tetracycline Tigecycline Trimethoprim Tylosin	TetA, TetB, OqxAB, qnrB1, sul1, sul2, strA, strB, aadA, blaCTX-M-15, AmpH, blaSHV-12, blaTEM, fosA, fosA5, fosA6, mcr-10, dfrA14	(Timofte et al., 2014; Koovapra *et al.*, 2016; Saidani *et al.*, 2018; Massé *et al.*, 2020; Cheng *et al.*, 2021; Tartor *et al.*, 2021; Yang *et al.*, 2021; Abegewi *et al.*, 2022; Bonardi *et al.*, 2023)
S. uberis	Biofilm production, gapC, oppF, pauA (skc), sua, hasC, mtuA	Amikacin Amoxicillin Ceftazidime Ceftriaxone Cephalothin	LinB, ErmB, tetS	(Chung *et al.*, 2021; Kabelitz *et al.*, 2021; Monistero *et al.*, 2021; Han *et al.*, 2022; Zeng *et al.*, 2022)

(*continued on next page*)

Table 2 (*continued*)

Agent	Virulence characteristics	Antimicrobial resistance profile	Antimicrobial resistance genes	References
		Enrofloxacin Erythromycin Gentamicin Lincomycin Penicillin Piperacillin Rifampin Spectinomycin Streptomycin Tetracycline		

of up to 2.30 kg/day. In the case of non-*aureus* staphylococci, losses range from 1.0 to 1.8 kg/day. Regarding, Gram negatives the most harmful pathogen was *E. coli* (3.5 kg/day), followed by *Corynebacterium bovis* (2.4 kg/day), *S. uberis* (2.1 kg/day), and *S. dysgalactiae* (2.0 kg/day; Heikkilä *et al.*, 2018). Information reported by Hogeveen and Van Der Voort (2017) indicated the costs of clinical mastitis due to *Klebsiella* spp.: $477; *E. coli*: $361; *Staphylococcus aureus*: $266; *Streptococcus* spp.: $174; and *Staphylococcus* spp.: $135. Likewise, *Mycoplasma* had on-farm economic consequences like common conventional mastitis pathogens (Al-Farha *et al.*, 2017). In accordance with the afore mentioned, Fu *et* al. (2022) stated that mastitis caused by Gram-negative pathogens is more expensive ($211.03) than Gram-positive bacterial cases ($133.73). Some authors have stated that economic losses due to clinical, subclinical and even chronic mastitis may vary among countries, factors such as prices, and veterinary services cost also influx (Heikkilä, Liski et al., 2018).

5. New approaches for the development of bovine mastitis treatments

As shown in table 2, conventional treatments based on chemical antimicrobials have lost their effectiveness. Thus, many recent research studies have proposed alternatives to antibiotic therapy that are not invasive and do not cause resistance. In the same sense, other authors have stated that protocols should be accurate, easy to perform, cost-effective, sabe, certifiable, and applicable in different areas of a country (Maćešić *et al.*, 2022). Authors such as Tomanić et al. (2023b), consider that novel and effective agents have potential to reduce the use of antibiotics, increasing productivity and environmental protection. In the following sections, some of the treatment alternatives for bovine mastitis will be addressed.

5.1. Medicinal plants

5.1.1. Vegetal extracts

The antibacterial activity of vegetal or plant extracts has been widely reported due to interest increasing in recent years, plants and their bioactive compounds have antibacterial effects, contributing to the treatment of mastitis (Šukele *et al.*, 2022).

The antibacterial activity of different extracts of *Quercus robur* bark and *Calluna vulgaris* herb was determined against common mastitis pathogens, S. *aureus, E. coli, S. agalactiae, S. uberis*, and *S. liquefaciens*. The *Q. robur* broadest spectrum was detected from 3.08 to 24.68 mg/mL, while *C. vulgaris* were effective from1.14 to 73.06 mg/mL; activity was attributed to the content of total phenolic compounds (*Q. robur*= 4374 mg/100 g GAE; *C. vulgaris*= 2755 mg/100 g GAE; Šukele *et al.*, 2022).

MIC (minimal inhibition concentration) values of ranging from 3.9 to 31.2 μg/mL and MBC (minimal bactericidal concentration) values ranging from 15.6 to 500 μg/mL were determined for *Ocimum tenuiflorum,* Ricinus *communis* inhibited bacterial growth at concentrations from 3.12 to 200 mg/mL and killed bacteria in a range of concentrations from 25 to 200 mg/mL against some major mastitis pathogens (Kebede & Shibeshi, 2022; Srichok *et al.*, 2022).

Prapaiwong *et al.* (2021) determined that the hydrolysable tannin extract showed inhibitory activity at concentrations from 27.3 −190 mg/mL and bactericidal activity at range of concentrations from 58.8 −235 mg/mL. Moreover, antibacterial activity was comparable to penicillin and gentamicin at concentrations over 630 mg/mL. The sorghum phenolic extract at a concentration of 4000 μg/mL inhibited *S. aureus* (7.7 mm), *K. pneumoniae* (0.8 mm) and *E. faecalis* (6.5 mm). No activity was detected against *E. coli* (Schnur *et al.*, 2021).

Ethyl acetate extract of fruits from *Terminalia chebula* (100, 500, and 100 μg/mL) was evaluated against *S. aureus, B. megaterium, E. coli* and *P. aeruginosa*, 1000 μg/mL generated growth inhibition zones from 32.06 to 39.02 mm (Kher *et al.*, 2019). *Knema retusa* wood extract had inhibitory activity and bactericidal effects against the Staphylococcal isolates ranging from 32 - 256 μg/mL and 64 - 512 μg/mL, respectively. Biofilm inhibition of *S. aureus* and *S. haemolyticus* was also determined; its major compound was identified as endo-2-hydroxy-9,9-(ethylenedioxy)−1-carbethoxy bicyclononane (Chuprom *et al.*, 2022).

Antibiofilm activity of the hydroalcoholic extracts of *Eucalyptus globulus*, alone and in combination with *Juglans regia*, against *S. aureus* isolates from bovine mastitis was tested, MIC values of *E. globulus* ranged from 0.19 to 0.39 mg/mL and from 0.78 to 1.56 mg/mL for *J. regia*. Extracts mixtures were more effective than singly used (Gomes et al., 2019a). MIC values from 500 to 3000 μg/mL were reported for the methanolic extract of underground parts of *Aquilegia fragrans* and its five pure compounds (2, 4-dihydroxyphenylacetic acid methyl ester, β-sitosterol, Aquilegiolide, Glochidionolactone-A, and Magnoflorine) against major pathogens associated with mastitis (Mushtaq et al., 2016a).

Rhodomyrtus tomentosa leaves extract was evaluated against multidrug-resistant *S. aureus* strains. MIC and MBC values ranged from 16−64 μg/mL and 64−128 μg/mL, respectively (Mordmuang and Voravuthikunchai, 2015b; Mordmuang *et al.*, 2019). *Clinacanthus nutans* can be considered an alternative treatment for bovine mastitis since it exhibits both antibacterial and anti-apoptosis activities (Panya *et al.*, 2020). *Mentha pulegium, Nepeta cataria, Melissa officinalis, Agastache foeniculum, Lavandula angustifolia, Origanum vulgare, Althaea officinalis, Plantago lanceolata, Artemisia absinthium, Populus nigra* and *Evernia prunastri* were evaluated on 14 Gram negative and 18 Gram positive bacterial strains, isolated from bovine mastitis cases. MIC values varied between 0.09 and 12.5 mg/mL, with bactericidal concentrations from 0.39 to 50 mg/mL. Better results were recorded for *Evernia prunastri, Artemisia absinthium* and *Lavandula angustifolia* (Pașca *et al.*, 2017).

Aquatic plants, such as salvinia auriculata has been shown to possess promising properties for the treatment of Staphylococcus aureus bovine mastitis. The hexane extracts of roots inhibited *S. aureus* at concentrations from 0.04 to 2.50 mg/mL. Moreover, these extracts inhibited the biofilm formation of three strains, the antibacterial activity was attributed to the presence of stigmast-22-ene-3,6-dione, β-sitosterol and octadecanoic acid. Inhibitory activity was detected at concentrations from 0.01 to 0.50 mg/mL. In addition, cytotoxicity was determined. According to the obtained data, the extract was considered as safe. Herbal antiseptic, an ex-vivo assay, demonstrated that the extract caused a log reduction of approximately 4.0, similar to some commercial

antimicrobials (Purgato *et al.*, 2021).

A bio-guided study reported the antibacterial activity of the hydro-alcoholic extract, aqueous and ethyl acetate fractions from L. *tridentata*. Among these treatments, ethyl acetate showed better activity since MIC and MBC, from 0.04 to 3.12 mg/mL and 0.09 and 6.25 mg/mL, respectively, had lower values than those obtained with the other treatments. Antibacterial activity was associated with the presence of lignan *nor* 3́demethoxiisoguaiacin Morales-Ubaldo et al. (2022).

The compounds Manool, *ent*-kaurenoic acid, and *ent*-copalic acid, from *Salvia officinalis* leaves exhibited inhibitory effects (MIC= 1.56 to >400 μg/mL) on *S. aureus, E. coli, S. epidermidis, S. agalactiae* and *S. dysgalactiae*. Being the most active *ent*-copalic acid, this compound was not cytotoxic at concentrations up to 62.5 μM (Fonseca *et al.*, 2013). In a similar study *nor* 3′ demethoxyisoguaiacin from L. *tridentata* inhibited bacterial growth in a range of concentrations from 10 to 780 μg/mL. Moreover, bactericidal effects were determined at concentrations from 20 μg/mL to 3.12 mg/mL against a wide range of mastitis bacteria; most promissory results were determined against *S. aureus* (Morales-Ubaldo *et al.*, 2022). Three alkaloids were isolated from the roots of *Thalictrum minus*, compounds identified as 5′-Hydroxythalidasine, thalrugosaminine, and O-Methylthalicberine inhibited the growth of common mastitis pathogens such as *S. aureus, K. pneumoniae, E. coli*, and other less common agents (*S. xylosus, S. lentus, S. equorum, E. faecalis* and *P. agglomerans*; MIC= 64- 500 μg/mL; Mushtaq et al., 2016b).

The combination of *Angelica dahurica* and *Rheum officinale* was an effective antibacterial treatment in a bovine model; effectiveness was attributed to the presence of emodin, rhein, and polysaccharides (Yang *et al.*, 2019). Similarly, tea saponin, a mixture of saponin from the seeds, leaves, or roots of the tea tree, was reported as an antibacterial against *S. agalactiae*. Furthermore, the proposed treatment inhibited biofilm formation, bacteria exhibited looser structure and lower density, possible mechanism of action propose that tea saponin down-regulate the transcription of genes *srtA, fbsC, neuA*, and *cpsE* associated with biofilm (Shang *et al.*, 2020). 7-epiclusianone, extracted from the *Rheedia brasiliensis* fruit, in combination with copper, named as 7-epiclusianone-copper complex was active against *S. agalactiae* and *S. uberis*; MIC= 7.8 μg/mL and MBC= 31.3 μg/mL. Furthermore, no cytotoxicity was observed in bovine mammary alveolar cells (de Barros *et al.*, 2017). The antibacterial activities of vegetal extracts and their related bioactive compounds are summarized in Table 3.

5.1.2. Essential oils

Essential oils from the medicinal aromatic plants, *Mentha pulegium, Nepeta cataria* and *Melissa officinalis* were tested over two major pathogens associated with mastitis. Volumes of 30 μL and 20 μL generated inhibition zones of 23 mm and 19 mm, respectively, against S. *aureus*. Lower activity was determined for *E. coli* since halos of 5.0 - 7.0 mm were generated (10 μlL; Arbab *et al.*, 2022).

The antibacterial effect of *Thymus vulgaris, Thymus serpyllum* and *Origanum vulgare* was reported in, the inhibitory and bactericidal effects on *Proteus mirabilis* and *Serratia marcescens* (MIC= 1.56 −3.12 mg/mL; MBC= 3.12 - 6.25 mg/mL). Thymol and carvacrol were the predominant compounds contained in the three essential oils (Tomanić *et al.*, 2022). Similarly, *Thymus vulgaris* and *Thymus serpyllum* showed both inhibitory and bactericidal effects on 11 different bacteria associated with mastitis; MIC values ranged from 0.39 mg/mL to 6.25 mg/mL and MBC values from 0.78 mg/mL to 12.50 mg/mL. The main bioactive compounds were thymol, *p*-cymene and γ -terpinene (Kovačević et al., 2021b).

In another study, *Origanum vulgare* and *Satureja montana* essential oils were evaluated on bacteria from clinical or subclinical mastitis cases. The obtained data showed that inhibitory activity presented at concentrations from 0.39 to 6.25 and bactericidal effects at concentrations from 0.78 to > 12.50 mg/mL, *p*-cymene thymol, carvacrol γ-terpinene, α-thujene, *trans*-β-caryophyllene and β-bisabolene were the main compounds (Kovačević et al., 2021a).

Table 3
Antibacterial activities of vegetal extracts and their related bioactive compounds against pathogens associated with bovine mastitis.

Plant-extract	Mechanisms of action	Associated compounds	Reference
Quercus robur, Calluna vulgaris, ethanolic and acetonic	Antimicrobial activity on S. *aureus, E. coli, S. agalactiae, S. uberis, S. liquefaciens*	Phenolic compounds	(Šukele *et al.*, 2022)
Ocimum tenuiflorum, Ricinus communis	Bacteriostatic and bactericidal effects	–	(Kebede & Shibeshi, 2022; Srichok *et al.*, 2022)
Hydrolysable tannin extract	Bacteriostatic and bactericidal effects	Tannins	(Prapaiwong *et al.*, 2021)
Sorghum phenolic extract	Growth inhibitory effects	Phenolics	(Schnur *et al.*, 2021)
Terminalia chebula, ethyl acetate	Growth inhibitory effects	–	(Kher *et al.*, 2019)
Knema retusa wood extract	Inhibitory and bactericidal effects, biofilm inhibition on Staphylococcal isolates	endo-2-hydroxy-9,9 (ethylenedioxy)−1-carbethoxy bicyclononane	(Chuprom *et al.*, 2022)
Eucalyptus globulus, Juglans regia, hydroalcoholic extracts	Antibiofilm activity on *S. aureus*	–	(Gomes et al., 2019a)
Aquilegia fragrans underground parts, methanolic	Inhibitory effects on major pathogens of mastitis and on *Staphylococcus xylosus, Staphylococcus equorum, Enterococcus faecalis and Pantoae*	2, 4-dihydroxyphenylacetic acid methyl ester, β-sitosterol, Aquilegiolide, Glochidonolactone-A and Magnoflorine	(Mushtaq et al., 2016b)
Rhodomyrtus tomentosa leaves extract	Inhibitory and bactericidal effects on multidrug-resistant *S. aureus*	–	(Mordmuang et al., 2015a; Mordmuang *et al.*, 2019)
Clinacanthus nutans	Antibacterial and anti-apoptosis activities	–	(Panya *et al.*, 2020)
salvinia auriculata, hexane	Antibacterial activity on *S. aureus*, biofilm inhibition formation	stigmast-22-ene-3,6-dione, β-sitosterol and octadecanoic acid	(Purgato *et al.*, 2021)
Larrea tridentata, ethyl acetate fraction	Bactericidal activity on pathogens associated with mastitis	*nor* 3demethoxiisoguaiacin	(Morales-Ubaldo *et al.*, 2022)
Salvia officinalis	Inhibitory effects on *S. aureus, E. coli, S. epidermidis, S. agalactiae* and *S. dysgalactiae*	Manool, *ent*-kaurenoic acid, and *ent*-copalic acid	(Fonseca *et al.*, 2013)
Thalictrum minus	Growth inhibition of inhibited growth of *S. aureus, K. pneumoniae, E. coli*, and other less common agents	Alkaloids5′-Hydroxythalidasine, thalrugosaminine and O-Methylthalicberine	(Mushtaq et al., 2016b)
Angelica dahurica, Rheum officinale	*In vivo* antibacterial activity	emodin, rhein and polysaccharides	(Yang *et al.*, 2019)
Tea tree	Inhibtion of biofilm formation, down-regulation of genes *srtA, fbsC, neuA*, and *cpsE*	Saponins	(Shang *et al.*, 2020)
Rheedia brasiliensis	Antibacterial activity on *S. agalactiae* and *S. uberis*	7-epiclusianone	(de Barros *et al.*, 2017)

Minthostachys verticillata essential oil and limonene exerted inhibitory effects on *S. uberis* from 14.3 mg/mL to 114.5 mg/mL and bactericidal effects at 114.5 mg/mL and 229 mg/mL. Concerning limonene, lower concentrations were determined; MIC 3.3 mg/mL to 52.5 mg/mL and MBC = 210 mg/Ml. Both compounds affected biofilm formation (Montironi *et al.*, 2016). Tea tree essential oil (*Melaleuca alternifolia*) and thymol and carvacrol extracts alone and in combination were evaluated against bacteria isolated from clinical mastitis cases. Tea tree oil + thymol and thymol + carvacrol combinations were the most effective treatments since additive effects were determined (Corona-Gómez *et al.*, 2022).

Recent studies determined that thymol, carvacrol, and trans-cinnamaldehyde highly inhibited bacterial growth at concentrations of 0.38 - 1.32 mg/mL. Carvacrol and octanoic acid, in combination, exhibited better inhibitory effects. Changes in cell morphology, leakage of electrolytes, and macromolecules were observed within 1–2 h after treatment (Rani et al., 2022a). The important activities of some of the aforementioned treatments were determined in multi-drug resistant bacteria associated with mastitis (Rani et al., 2022b).

The effect of *Syzygium aromaticum* and *Cinnamomum zeylanicum* and their major components, eugenol and cinnamaldehyde, were evaluated on *S. aureus* biofilm formation. The MIC value of *S. aromaticum* was 0.237 mg/mL, while its main compound eugenol, was active at a higher concentration (0.392 mg/mL), in contrast to that determined for *C. zeylanicum* since the MIC value was higher (0.243 mg/ mL) compared with cinnamaldehyde (0.199 mg/mL). *S. aromaticum* was considered the most effective treatment for the inhibition of biofilm formation (Budri *et al.*, 2015). Table 4 contains details of the antibacterial activities of essential oils.

5.1.3. Combination of plant treatments and antimicrobials

E. globulus and penicillin G in combination, demonstrated synergy against *S. aureus* strains isolated from bovine mastitis (Gomes et al., 2019b), *Ocimum tenuiflorum* exhibited synergistic activity with penicillin G and amoxicillin against *S. aureus*, coagulase-negative Staphylococci, *S. agalactiae*, and *E. coli*, and additive effects when the extract was combined with cefazolin and gentamicin Srichok et al. (2022). *Plectranthus ornatus* extract exhibited a synergistic effect with ampicillin, kanamycin, and gentamicin, reducing eight-fold in the MIC value. Similar results were determined for *Salvia officinalis* and *Senna macranthera*, activity was determined on *S. aureus* (Silva *et al.*, 2019). The efficacy of *Melaleuca armillaris* as an adjuvant of erythromycin was reported, and the obtained data showed a synergistic effect on *S. aureus*; this combination was considered bactericidal (Buldain *et al.*, 2022).

The two active compounds of guttiferone-A and 7-epiclusianone, from the fruits of *Garcinia brasiliensis*, important potentiation was observed when 7-epiclusianone was combined with ampicillin. The MIC values of ampicillin alone were 31.25 μg/mL and 125.0 μg/mL against *S. agalactiae* and *S. uberis*. When an active compound was added, MIC values decreased between 15.63 μg/mL and 62.50 μg/mL. Similar effects were observed for the combination 7-epiclusianone- gentamicin. Regarding guttiferone-A, this compound exhibited better effects on both bacteria in combination with gentamicin Maia et al. (2018). Table 5 summarizes the main results of the combination of plant treatments and antimicrobials.

5.1.4. Plant based-products

A soap with added *Salvina auriculata* extract inhibited the total growth of *S. aureus*. The bioactive compounds were identified as stigmasterone, stigmasterol, and friedelinol (Lima *et al.*, 2013). *S. aureus* was highly inhibited by a suspension of herbal soap (1%), with added *Senna mactanthera* extract. The active compounds were the anthraquinone compounds emodine, physione, and chrysophanol (Inoue-Andrade *et al.*, 2015).

Two herbal gels based on: aqueous extract of propolis, alcoholic extracts of brewers gold and Perle hops, plum lichen, common mallow, marigold, absinthe wormwood, black poplar buds, lemon balm, and essential oils of oregano, lavender, and rosemary, were administrated intramammary in a 10 mL volume to 20 cows diagnosed with clinical

Table 5
Combination of plant treatments and antimicrobials against pathogens associated with bovine mastitis.

Combination	Mechanisms of action	Reference
E. globulus + penicillin G	Synergistic effect against *S. aureus* strains	(Gomes et al., 2019b)
Ocimum tenuiflorum + Penicillin G *Ocimum tenuiflorum* + amoxicillin *Ocimum tenuiflorum*+ cefazolin *Ocimum tenuiflorum*+ gentamicin	Synergistic and additive effects against *S. aureus*, coagulase-negative Staphylococci, *S. agalactiae*, and *E. coli*	(Srichok *et al.*, 2022)
Plectranthus ornatus+ Ampicillin *Plectranthus ornatus*+Kanamycin *Plectranthus ornatus*+ gentamicin	Synergistic effects on important pathogens of bovine mastitis	(Silva *et al.*, 2019)
Melaleuca armillaris + erythromycin	Bactericidal effects on *S. aureus*	(Buldain *et al.*, 2022)
Guttiferone-*A* + gentamicin 7-epiclusianone + ampicillin 7-epiclusianone + gentamicin	Synergistic effects on *S. agalactiae* and *S. uberis*	(Maia *et al.*, 2018)

Table 4
Antibacterial activities of essential oils and its related bioactive compounds against pathogens associated with bovine mastitis.

Essential oil origin	Mechanisms of action	Bioactive compounds	Reference
Mentha pulegium, *Nepeta cataria* and *Melissa officinalis*	Antimicrobial activity on *S. aureus* and *E. coli*	–	(Arbab *et al.*, 2022)
Thymus vulgaris, *Thymus serpyllum* and *Origanum vulgare*	inhibitory and bactericidal effects on *Proteus mirabilis* and *Serratia marcescens*	Thymol and Carvacrol	(Tomanić *et al.*, 2022)
Thymus vulgaris, *Thymus serpyllum*, *Origanum vulgare* and *Satureja montana*	Inhibitory and bactericidal effects	Thymol, *p*-cymene and γ -terpinene, carvacrol γ-terpinene, α-thujene, *trans*-β-caryophyllene and β-bisabolene	(Kovačević et al., 2021a; Kovačević et al., 2021b)
Minthostachys verticillate and limonene	Inhibitory and bactericidal effects on *S. uberis*, inhibition of biofilm formation	–	(Montironi *et al.*, 2016)
Melaleuca alternifolia and thymol	Antimicrobial activity on bacterial strains from clinical mastitis cases	–	(Corona-Gómez *et al.*, 2022)
Carvacrol, trans-cinnamaldehyde	Inhibitory effects, changes in cell morphology, leakage of electrolytes and macromolecules	–	(Rani et al., 2022a)
Syzygium aromaticum and *Cinnamomum zeylanicum*	Inhibition of *S. aureus* biofilm formation	Eugenol and cinnamaldehyde	(Budri *et al.*, 2015)

and subclinical mastitis. The results showed a decreasing number of bacteria (*S. epidermidis, S. aureus, S. schleiferi, S. hominis, Micrococcus* spp., *streptococcus* spp., *Aerococcus* spp., *Bacillus* spp.,*and Corynebacterium* spp.) and somatic cells after treatment (Pașca *et al.*, 2020).

In a similar study, a homeopathic preparation containing natural compunds was administered intramammary, and the obtained data showed a 75% cure rate of clinical mastisis. Moreover, on Day 7 of treatment, healing rates were 51.85% for subclinical mastitis, increasing to 59.29% on Day 14 (Mimoune *et al.*, 2021).

A pharmaceutical formulation (Phyto-Bomat) composed of essential oils (*Thymus vulgaris, Thymus serpyllum Origanum vulgare, Satureja montana*) in combination with oil macerates *(Calendula officinalis, Hypericum perforatum)* was effective on diverse pathogens associated with bovine mastitis. The results indicated that MIC values ranged from 22.72 mg/mL to 45.40 mg/mL, and MBC values from 45.40 mg/mL to 90.09 mg/mL, with thymol and carvacrol being the main constituents (Kovačević et al., 2022c). The antibacterial activity of this product was confirmed in a very recent study, since the resolution of symptoms post-treatment and prevention of clinical mastitis development in cases with subclinical mastitis were determined (Tomanić et al., 2023a). Table 6 contains information about natural compounds-based products.

5.2. Nanotechnology

S. aureus and *S. epidermidis* were sensitive to silver nanoparticles added with *R. tomentosa* ethanolic extract and the liposomal encapsulated rhodomyrtone (MIC= 2–8 μg/mL and MBC= 8–32 μg/mL). Moreover, treatments decreased the adhesion of the bacterial cells to the mammary gland tissues (Mordmuang et al., 2015a). Silver-nanoparticle-decorated quercetin nanoparticles exhibited important antibacterial and antibiofilm activities over a multi-drug resistant strain of *E. coli* isolated from a mastitis case (Yu *et al.*, 2018). Gold nanoparticles added with *Dodonaea angustifolia* extract and honey exhibited both, inhibitory and bactericidal effects over methicillin-resistant and vancomycin-resistant *S. aureus* strains (Omara, 2017).

Zinc-oxide nanoparticles were effective on some major pathogens associated with bovine mastitis; MIC values ranged from 1.0 mg/mL to 20.0 mg/mL, while bactericidal effects were determined at concentrations from 1.0 mg/mL to 30.0 mg/mL (Hozyen *et al.*, 2019). Chitosan -nanoparticles exerted bacteriostatic and bactericidal effects on *S. xylosus* and *S. aureus* at concentrations from 800 μg/mL to 1600 μg/mL; the inhibition of biofilm formation was also determined (Orellano *et al.*, 2021). The efficacy of graphene oxide was determined, was tested against *S. aureus,* at 100 μg/mL; *S. aureus* biofilm mass was reduced by up to 70%, and at 200 μg/mL, graphene oxide treatment killed 80% of bacteria (Saeed *et al.*, 2023).

Using polyherbal nanocolloids formulated with five different extracts (*Syzygium aromaticum, Cinnamomum verum, Emblica officinalis, Terminalia belerica, Terminalia chebula*, and *Cymbopogon citratus*) showed bacteriostatic, bactericidal, and antibiofilm activity effects on *Acinetobacter junii, Klebsiella pneumoniae, Pseudomonas stutzeri*, and *Acinetobacter baumannii*. Interestingly polyherbal nanocolloid reduced bacterial virulence factors of different bacterial strains (Ranjani *et al.*, 2022).

An intramammary nanosuspension based on α-linolenic acid exhibited benefic effects on cows with subclinical mastitis, after treatment the milk color changed from pale yellow to white. Similarly, consistence turned from watery to thick and pH was normalized; on day 10, the parameters of the clinical mastitis test, Whiteside test, bromothymol blue test and bacterial cell count decreased. Moreover, anti-inflammatory and peripheral analgesic properties were determined (Yadav *et al.*, 2020). Table 7 shows some of the most relevant findings about the application of nanotechnology against bovine mastitis pathogens

5.3. Polymers

To provide alternatives for the treatment and prevention of cattle mastitis, Bhattari *et al.* (2021), evaluated *in vitro* 14 hydrophilic polymers and six solvents determining their cytotoxicity and biocompatibility on bovine mammary epithelial cells for their use as carriers for sustained drug delivery. The obtained data showed that polyethylene oxides, hydroxypropyl methylcellulose, carboxymethyl cellulose, sodium alginate and xanthan gum were safe, since no significant cytotoxicity was determined. On the other hand, polycarbophil, carbopol, glycerin, propylene glycol, polyethylene glycol 400, ethanol, N-methyl-2-pyrrolidone and 2-pyrrolidone showed higher cytotoxicity, some of them in a concentration-dependent way. Nevertheless, according to the authors, all formulations could form nontoxic gels with good biocompatibility.

Polymeric particles composed of alginate/chitosan and chitosan/sodium tripolyphosphate were used to encapsulate mercaptosuccinic acid. Treatments showed potential antibacterial activity on *S. aureus* and *E. coli* (MIC= 125 μg/mL to 250 μg/mL; Cardozo *et al.*, 2014). In another study, it was determined that chitosan at a specific molecular weight exhibited activity on *S. aureus*, molecules of 2.6 kDa inhibited biofilm formation, killed bacteria, and prevented the persistence of *S. aureus*. Moreover, these kinds of molecules showed synergy with macrolides (Asli *et al.*, 2017). Felipe *et al.* (2019) reported that chitosan (60–120 kDa) inhibited *S. aureus* and *S. xylosus* growth at concentrations from

Table 6
Antibacterial activity of plant based-products against pathogens associated with bovine mastitis.

Product	Mechanisms of action	Bioactive compounds	Reference
Salvinia auriculata soap	Inhibition of *S. aureus* growth	Stigmasterone, stigmasterol, friedelinol	(Lima *et al.*, 2013)
Senna mactanthera soap	Inhibition of *S. aureus* growth	Emodine, physione, and chrysophano	(Inoue Andrade *et al.*, 2015)
Herbal gels	Antimicrobial activity on diverse pathogens	–	(Pașca *et al.*, 2020)
Phyto-Bomat	Bacteriostatic and bactericidal effects on pathogens associated with mastitis	Thymol and carvacrol	(Kovačević et al., 2022c; Tomanić et al., 2023a)

Table 7
Application of nanotechnology against pathogens associated with bovine mastitis.

Alternative treatment	Mechanisms of action	Reference
Silver nanoparticles added with *R. tormentosa*/ quercetin	Decreased adhesion of the bacterial cells to the mammary gland tissues, antibacterial and antibiofilm activities	(Mordmuang et al., 2015a; Yu *et al.*, 2018)
Gold nanoparticles added with *Dodonaea angustifolia* extract and Honey	Inhibitory and bactericidal effects over *S. Aureus*	(Omara, 2017)
Zinc oxide nanoparticles	Bacteriostatic and bactericidal effects	(Hozyen et al., 2019)
Chitosan nanoparticles	Bacteriostatic, bactericidal and antibiofilm effects on *S. xylosus* and *S. aureus*	(Orellano *et al.*, 2021)
Graphene oxide	Reduction of biofilm mass, bactericidal activity against *S. aureus*	(Saeed *et al.*, 2023)
polyherbal nanocolloids formulated with extracts	Bacteriostatic, bactericidal and antibiofilm activity, reduction of bacterial virulence factors	(Ranjani *et al.*, 2022)
Nanosuspension, α-linolenic acid	Exhibited benefic effects on cows with subclinical mastitis	(Yadav *et al.*, 2020)

100 μg/mL to 800 μg/mL, being *S. xylosus* the most sensitive bacterium, bactericidal effects were determined at concentrations of 200 μg/mL against *S. xylosus*, and concentrations of 1600 μg/mL were determined for *S. aureus*. In addition, in a range of concentrations from 100 μg/mL to 1600 μg/mL chitosan inhibited biofilm formation, reduced biofilm viability and disrupted the established biofilms.

The combination of polymers with conventional antimicrobials has been reported. A study evaluated the addition of chitosan to cloxacillin, demonstrating that this combination increased the efficacy of the antibiotic, reducing the required concentration (16 to 64 fold reduction). Moreover, the combination inhibited bacterial biofilm establishment and increased preformed biofilm eradication (Breser *et al.*, 2018). In another study the design of polymeric nanoparticles (poly--ε-caprolactone), which encapsulated cloxacillin benzathine, obtained data showed that in an *in vivo* assay, this treatment at a concentration of 600 mg eliminated 100% of *Corynebacterium* spp. and *S. uberis* (Araújo *et al.*, 2019).

A study stated that polyethylene oxide-based inserts could act as a physical barrier against pathogens invading the teat canal of cows and possibly control the release of drugs for mastitis treatments (Bhattarai *et al.*, 2015). Polyhexamethylene biguanide, an antimicrobial polymer, showed positive effects, killing 99.9% of intracellular *S. aureus* at 15 mg/L; at the same concentration the biofilḿs mass was reduced up to 37%. This study stated this compound was tolerated by host cells at high concentrations (Kamaruzzaman *et al.*, 2017). In a later study, this same compound was evaluated, confirming its efficacy on *S. aureus* (≥0.5 μg/mL). Polyhexamethylene biguanide nanoparticles showed higher effects with a MIC value of 0.03 μg/mL on said bacterium. Another study determined that this polymer is effective in treating of *Prototheca* spp., associated with mastitis, determining effects at lower concentrations (≥1.0 to ≥4.0 μg/mL; Fidelis *et al.*, 2023).

The emerging photodynamic therapy has achieved mastitis prevention and treatment benefits. A recent study reported the efficiency of chlorophyll-rich spinach extract and curcumin, both incorporated into a micellar copolymer (Pluronic® F127). *In vitro* assays showed that treatments had inhibitory and bactericidal effects on *S. aureus* and *E. coli*; the *in vivo assay* demonstrated that the application of formulations considerably decreased bacterial loads and maintained the milk quality (Junior *et al.*, 2023). These findings suggest that polymers have the potential to be used in the treatment and prevention of mastitis-causing pathogens (Table 8).

Table 8
Application of polymers against pathogens associated with bovine mastitis.

Alternative treatment	Mechanism of action	Reference
Hydrophilic polymers	formulations could form biocompatible nontoxic gels	(Bhattarai *et al.*, 2021)
Polymeric particles + mercaptosuccinic acid	Antibacterial activity on *S. aureus* and *E. coli*	(Cardozo *et al.*, 2014)
polyethylene oxide-based inserts	Physical barrier against pathogens of bovine mastitis	(Bhattarai *et al.*, 2015)
poly-ε-caprolacton + cloxacillin benzathine	Bactericidal effects on *Corynebacterium* spp. and *S. uberis*	(Araújo *et al.*, 2019)
Chitosan	Antibiofilm and bactericidal activity on *S. aureus* and *S. xylosus* synergistic effect with antimicrobials	(Asli *et al.*, 2017; Felipe *et al.*, 2019)
polyhexamethylene biguanide	Bactericidal and antibiofilm activity on S. *aureus* Antibacterial activity against *Prototheca* spp.	(Kamaruzzaman *et al.*, 2017; Fidelis *et al.*, 2023)
Pluronic® F127+ spinach extract and curcumin	inhibitory and bactericidal effects on *S. aureus* and *E. coli*	(Junior *et al.*, 2023)

5.4. Peptides

The effect of an *S. aureus* bacterin and nisin on bovine subclinical mastitis was evaluated in the study carried out by Guan *et al.* (2017). Treatment reduced intramammary infections reduced count of somatic cells and increased protein and fat contents. A nisin derivative demonstrated its potential to eradicate and inhibit biofilms of *S. uberis* strains (Pérez-Ibarreche *et al.*, 2021). A peptide named pm11, inhibited the growth of *S. aureus, S. uberis, S. agalactiae* and *E. coli*. MIC values ranged from 0.32 μM to 2.07 μM. Bactericidal effects were determined at concentrations from 2.5 μM to 10 μM. Furthermore, pm11 reduced viable cell counts within 1 to 4 h (Popitool *et al.*, 2022). Aureocin A53, a peptide produced by *S. aureus,* showed to be bactericidal against staphylococci and streptococci strains. The study determined that A53 was not toxic to bovine mammary gland epithelial cells after 24-h exposure. Moreover, the peptide maintained its antimicrobial activity (Marques-Bastos *et al.*, 2022).

The antimicrobial activity of peptide NZ2114 was reported, and the obtained data showed its efficacy against *S. dysgalactiae* strains (0.11–0.45 μM). This peptide was able to eradicate bacterial biofilm; the *in vivo* assays determined that NZ2114 alleviated mammary gland inflammation, inhibited bacterial proliferation, and reduced the number of mammary gland bacteria (Yang *et al.*, 2022). A similar study determined that the NZ2114 derivative peptide H18R (H2) inhibited *S. aureus* growth at concentrations from 0.5 μg/mL to 1.0 μg/mL (Wang *et al.*, 2019).

The recombinant fungal defensin-like peptide- P2 was effective in inhibiting the growth of *S. dysgalactiae, S. agalactiae*, and *S. aureus* (1- 4 μg/mL).The minimal biofilm inhibition and minimal biofilm eradication concentrations were from 8 μg/mL to >512 μg/mL only against *S. dysgalactiae*. The obtained data showed that the plasma membrane of *S. dysgalactiae* was disrupted by P2 in a time and dose-dependent manner; the *in vivo* assays demonstrated that the peptide decreased the number of mammary bacteria and inflammation (Table 9; Zhang *et al.*, 2021).

5.5. Other alternatives

Bacteria are considered sources of diverse antimicrobial agents, in this sense, the antimicrobial activity of crude extracts from actinomycetes was determined on *Staphylococcus aureus, Staphylococcus chromogenes, Streptococcus dysgalactiae*, and *Streptococcus uberis*. Treatments were active at concentrations from ≥ 0.78 μg/mL to 100 μg/mL (Leite *et al.*, 2018). In the same sense, the search for antiseptics is increasing. The *in vitro* and *in vivo* evaluations of wood vinegar from *Eucalyptus urograndis* demonstrate to have benefic effects on major pathogens (multidrug-resistant) associated with mastitis. The MIC and MBC values ranged from 0.781 to 1.562% and from 0.781 to 3.125%, respectively. In

Table 9
Recent studies regarding the application of peptides against bovine mastitis pathogens.

Alternative treatment	Mechanism of action	Reference
S. aureus bacterin	Reduction of intramammary infections, bactericidal and antibiofilm activity	(Guan *et al.*, 2017; Pérez-Ibarreche *et al.*, 2021)
Nisin pm11	bacteriostatic and bactericidal activity	(Popitool *et al.*, 2022)
Aureocin A53	Bactericidal activity	(Marques-Bastos *et al.*, 2022)
NZ2114	Inhibition of bacterial proliferation, reduction of bacterial loads	(Yang *et al.*, 2022)
H18R	Bacteriostatic effects on *S. aureus*	(Wang *et al.*, 2019)
Defensin-like peptide-P2	Inhibitory and antibiofilm activity	(Zhang *et al.*, 2021)

cows treated with this wood vinegar (1%) a decreased number of colony-forming units were present in the mammary gland, and no signs of hyperemia, pain, edema or crusts were detected (da Silva *et al.*, 2023). An antiseptic preparation containing 5% lactic acid with modified rice gel was evaluated on *S. aureus* and *S. epidermidis*, showing potential bactericidal activity (Chotigarpa *et al.*, 2018). Similar results were determined on *E. coli* (Chotigarpa *et al.*, 2019).

An *in vivo* assay demonstrated that an emulgel based on copaiba oil-resin extracted from *Copaifera reticulata* can reduce bacterial loads in bovine mastitis cases. Moreover, anti-inflammatory effects were determined (Campanholi *et al.*, 2023), Quarters with bacterial infection progressively reduced after the subcutaneous injection of an emulsified oil from the rhizome of *Atractylodis macrocephalae* (32 mg), infection was attributed to *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis* and coagulase-negative staphylococci (Xu *et al.*, 2015). Another study demonstrated that dietary supplementation with zeolite clinoptilolite diminished risk of intramammary infections in pregnant cowa aged 3 to 6 years (Samardžija *et al.*, 2017).

6. Conclusion

According to the analyzed epidemiological data, the prevalence of both, contagious and environmental pathogens may vary according to geographical region. Regarding resistance profiles, most of the above-mentioned studies determined that isolated strains were multidrug-resistant,On this note, therapeutic strategies, such as herbal therapy, nanotechnology, polymers or peptides have been proposed as alternatives for mastitis treatments. Both, *in vitro* and *in vivo assays* showed potential results, demonstrating that these alternative may be effective on bacteria associated with bovine mastitis.

Ethical statement

The authors declare that no animals were used.

Declaration of Competing Interest

Authors declare there is no conflicts of interest.

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CAPÍTULO II: Actividad bactericida y potencial modo de acción de una formulación multi-herbal fitoterapeutica contra patógenos asociados con mastitis bovina

Phytochemistry

Bactericidal activity and potential mode of action of a phytotherapeutic multi-herbal formulation against bovine mastitis associated pathogens.

--Manuscript Draft--

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Abstract:	The aim of the present study was to determine the bactericidal activity and elucidate the mechanism of action of multi-herbal formulation against pathogens associated with bovine mastitis. Antibacterial activity was assessed by determining the minimal inhibitory and bactericidal concentrations. Time kill kinetics and cell membrane integrity assays were conducted to investigate the mechanism of action. Chromatographic assays were performed to characterize the multi-herbal formulation. Cytotoxicity was evaluated using the brine shrimp lethality assay. Inhibitory activity was observed at concentrations ranging from 0.39 mg/mL to 6.34 mg/mL, while minimal bactericidal concentrations ranged from 0.79 mg/mL to 25.38 mg/mL. The multi-herbal formulation exerted bactericidal effects within the first 30 minutes contact. Significant damage to the cytoplasmic membrane was detected, as evidenced by the release of intracellular proteins and nucleic acids associated with presence of luteolin rutinoside, apigenin rutinoside, rutin, nor-3'-demethoxyisoguaiacin, gallic acid, ellagic acid, eugenol and thymol. The formulation demonstrated complete bactericidal activity against bovine mastitis associated pathogens within 30 minutes, suggesting that it's mechanism of action may involve membrane disruption. Moreover, the formulation was found to be noncytotoxic. These findings indicate that multi-herbal formulations could represent a promising alternative for the treatment of bovine mastitis treatment, however, in vivo studies are necessary to confirm their efficacy and safety.

Bactericidal activity and potential mode of action of a phytotherapeutic multi-herbal formulation against bovine mastitis associated pathogens.

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Abstract

The aim of the present study was to determine the bactericidal activity and elucidate the mechanism of action of multi-herbal formulation against pathogens associated with bovine mastitis. Antibacterial activity was assessed by determining the minimal inhibitory and bactericidal concentrations. Time kill kinetics and cell membrane integrity assays were conducted to investigate the mechanism of action. Chromatographic assays were performed to characterize the multi-herbal formulation. Cytotoxicity was evaluated using the brine shrimp lethality assay. Inhibitory activity was observed at concentrations ranging from 0.39 mg/mL to 6.34 mg/mL, while minimal bactericidal concentrations ranged from 0.79 mg/mL to 25.38 mg/mL. The multi-herbal formulation exerted bactericidal effects within the first 30 minutes contact. Significant damage to the cytoplasmic membrane was detected, as evidenced by the release of intracellular proteins and nucleic acids associated with presence of luteolin rutinoside, apigenin rutinoside, rutin, *nor*-3'-demethoxyisoguaiacin, gallic acid, ellagic acid, eugenol and thymol. The formulation demonstrated complete bactericidal activity against bovine mastitis associated pathogens within 30 minutes, suggesting that it's mechanism of action may involve membrane disruption. Moreover, the formulation was found to be noncytotoxic. These findings indicate that multi-herbal formulations could represent a promising alternative for the treatment of bovine mastitis treatment, however, *in vivo* studies are necessary to confirm their efficacy and safety.

Keywords: Bovine mastitis, multidrug-resistance, multi-herbal formulation, bactericidal activity, membrane damage.

1. Introduction

Bovine mastitis is the most diagnosed disease of dairy herds worldwide, and is characterized by the swelling of udder, which is associated with multiple factors, such as poor nutrition, and ineffectual management conditions on the farm, however, in several cases bacterial infections are the most prevalent presentation within herds (Ajose et al., 2022; Morales-Ubaldo et al., 2023; Sigmund et al., 2023).

Affected cows can develop sub-clinical or clinical mastitis depending on the virulence of the causative bacterium, the health of the cow, and the efficacy of treatments (Aiemsaard et al., 2023). Antibiotics have long been considered the first line of defense against bacterial infections in the case of mastitis, however, the non-selective use of antibacterials is one of the major reasons for failures in the treatment of mastitis and the emergence of multidrug-resistant strains (Rasooly et al., 2020; Ajose et al., 2022; Paramasivam et al., 2023).

Antimicrobial resistance in bacteria represents a serious public health threat, and evidence shows that resistant strains relevant to humans may emerge in livestock (Filioussis et al., 2020). Promoting, non-invasive therapeutic alternatives that do not promote antimicrobial resistance are essential for the effective management and prevention of bovine mastitis.

To date, alternative treatments for bacterial infections have focused on plant extracts or essential oils, due to their well-documented antibacterial properties (Kovačević et al., 2021; Morales-Ubaldo et al., 2022; Aiemsaard et al., 2023). However, many of these are not suitable for development, as their efficacy is often observed only at concentrations that are not therapeutically viable under *in vivo* conditions (Alibi et al., 2021). In this context, combination therapies, such as multi-herbal formulations have emerged as a promising

approach. These formulations may enhance therapeutic efficacy through synergistic effects and offer potential for safe and long-term use (Suchetha and Bharwani 2013; Ospondpant et al., 2024; Panossian et al., 2024).

In traditional medicine, such as Thai and Chinese, herbal formulations have been widely used. Synergistic interactions between constituents have several advantages, including increased therapeutic efficacy, and decreased toxicity and/or adverse effects (Zhou et al., 2016; Liu et al., 2023; Panossian et al., 2024).

Several studies documented the diverse pharmacological applications of multi-herbal formulations. Sumantran *et al*. (2011) reported the antiarthritic activity of an Ayurvedic formulation composed of *Zingiber officinale*, *Tinospora cordifolia*, *Phyllanthus emblica* and *Boswellia serrata*. In another study, the combination of *Dracaena cochinchinensis* and *Ardisia elliptica* exhibited superior neuroprotective and anti-inflammatory activity compared to individual botanical drugs (Ospondpant et al., 2024). However, the antibacterial potential of multiherbal formulations remains scarcely examined. The aim of the present study was to determine the bactericidal activity and elucidate the mechanism of action of multi-herbal formulation against pathogens associated with bovine mastitis.

2. Results

2.1 Chemical composition of multi-herbal formulation

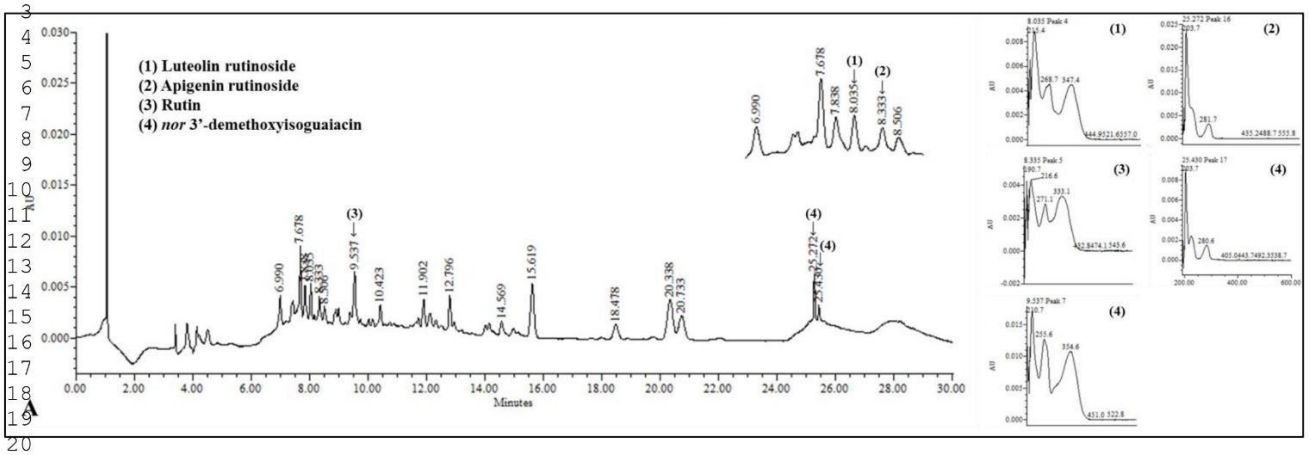
The HPLC chromatographic analysis at 280 nm shows the chemical profile of individual ingredients and their mixture in the multi-herbal formulation.

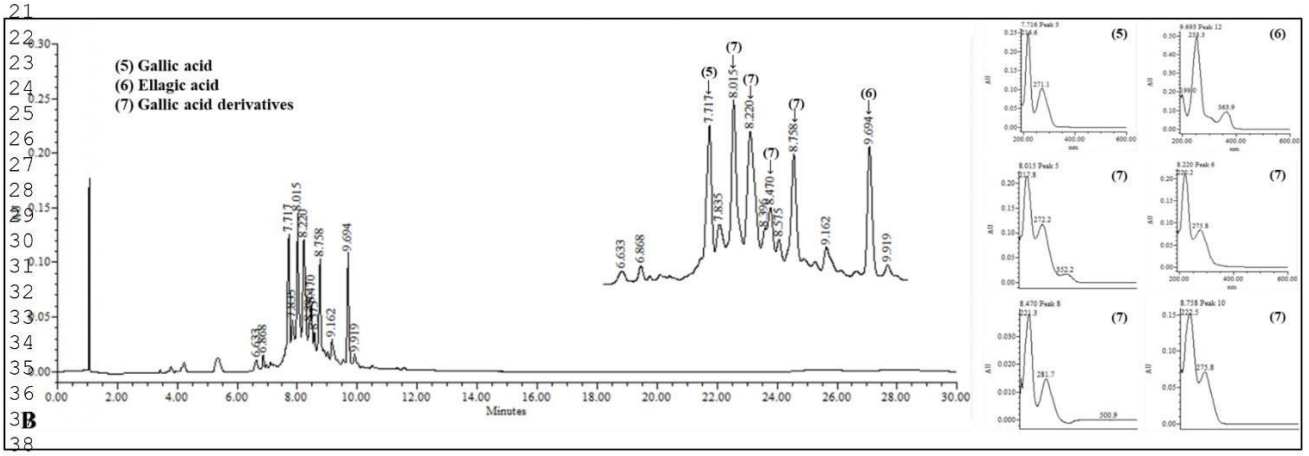
Concerning the hydroalcoholic extract of *L. tridentata* (Fig. 1A) revealed the presence of flavonoid-type compounds, including luteolin rutinoside (**8.0 min**; λmax= 215.4, 268.7,

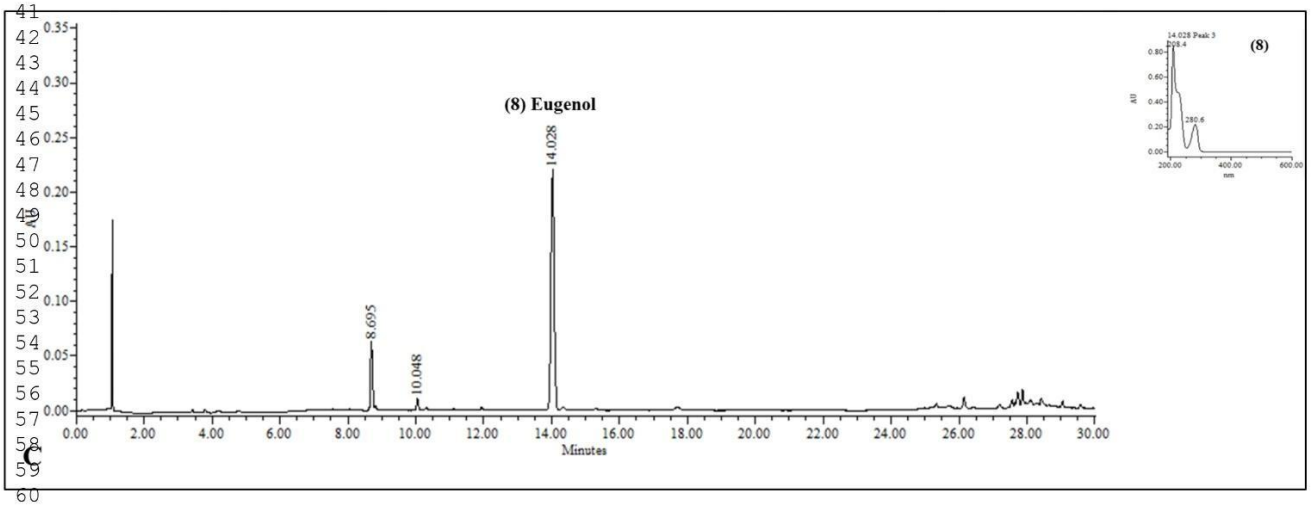
347.4 nm), apigenin rutinoside (**8.3 min**; λmax= 216.6, 271.1, 333.1 nm), and rutin (**9.5 min**; λmax= 210.7, 255.6, 354.6 nm). In addition, the antibacterial lignan *nor*-3'-demethoxyisoguaiacin was detected (**25.2 min**; λmax= 203.7, 281.7 nm and **25.4 min**; λmax= 203.7, 280.6 nm).

For the hydroalcoholic extract of *C. coriaria* (Fig. 1B), the analysis identified gallic acid (**7.7 min**; λmax= 216.6, 271.1 nm) and ellagic acid (**9.6** min; λmax= 199.0, 253.3, 363.9 nm), along with gallic acid derivatives (**8.0** min; λmax= 217.8, 272.2, 352.2 nm; **8.2** min; λmax= 220.2, 275.8 nm; **8.4** min; λmax= 221.3, 281.7 nm; and **8.7** min; λmax= 225.5, 275.8 nm).

Regarding the essential oils, the chromatogram of *S. aromaticum* essential oil (Fig. 1C) showed a major mixture of eugenol and chavibetol (**14.0 min**; λmax= 208.4, 280.6 nm). In the essential oil of *L. graveolens* (Fig. 1D), thymol (**14.0** min; λmax= 204.8, 280.6 nm) was identified as the predominant compound. The multi-herbal formulation exhibited the same compounds identified in the individual extracts and essential oils (Fig. 1E).


(1) Luteolin rutinoside
(2) Apigenin rutinoside
(3) Rutin
(4) nor 3'-demethoxyisoguaiacin
Minutes
A


(5) Gallic acid
(6) Ellagic acid
(7) Gallic acid derivatives
Minutes
B


(8) Eugenol
Minutes
C

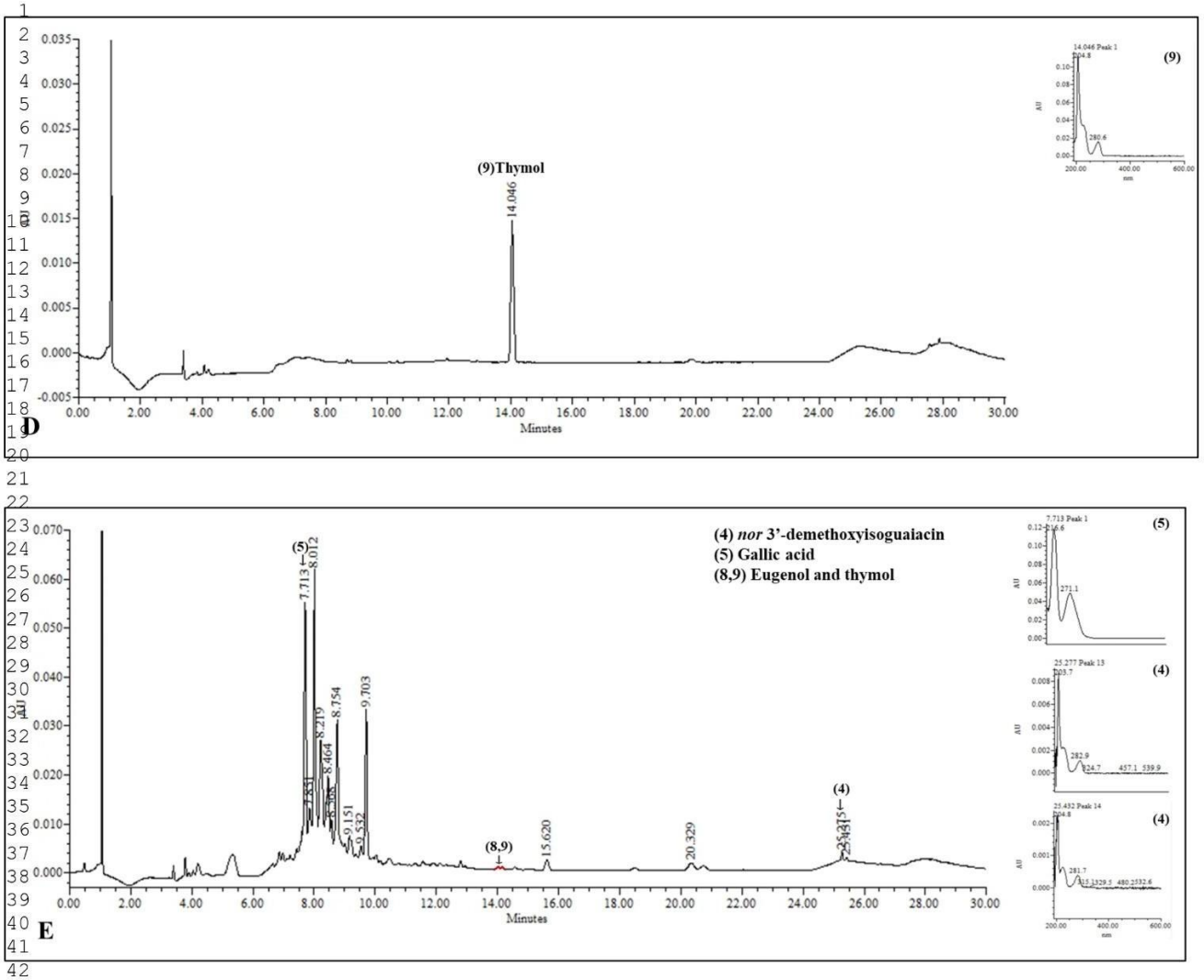


Figure 1. HPLC chromatograms corresponding to the hydroalcoholic extracts of *L. tridentata* **(A)** and *C. coriaria* (**B**); the essential oils of *S. aromaticum* (**C**) and *L. graveolens* (**D**). The multiherbal formulation (**E**). Recorded at 280 nm.

2.2 Minimal inhibitory concentration.

The minimal inhibitory concentrations for the individual extracts are presented in Figure 2. According to the obtained data, when *E. coli* was exposed to individual treatments, *L. tridentata* and *C. coriaria* exhibited antibacterial activity at concentrations of 12.50 mg/mL and 25.0 mg/mL, respectively, whereas *L. graveolens* and *S. aromaticum* were active at 0.03 mg/mL and 0.15 mg/mL. For *S. aureus* inhibition was observed at 0.39 mg/mL for *L. tridentata* and 6.25 mg/mL for *C. coriaria*, and at 0.03mg/mL and 0.31 mg/mL for *L. graveolens* and *S. aromaticum*, respectively.

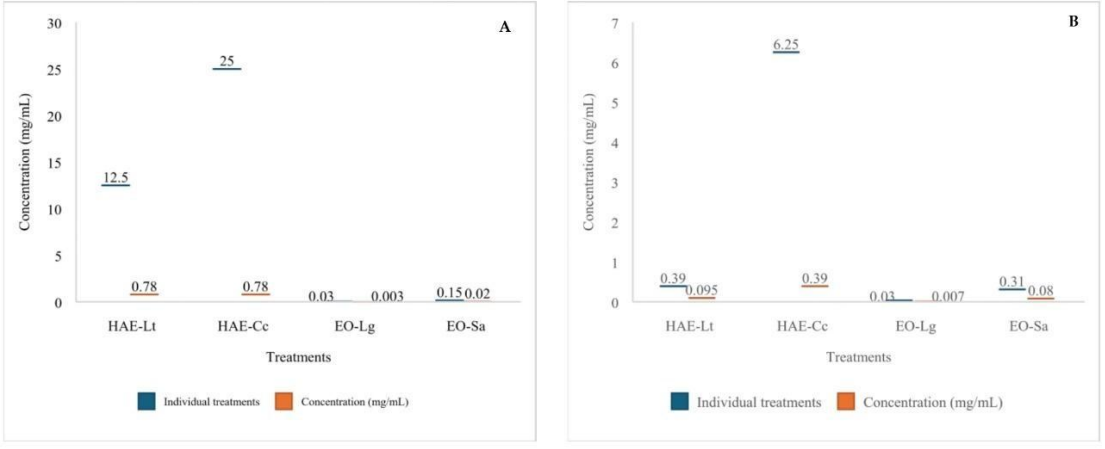


Figure 2. Minimal inhibitory concentrations of individual extracts and multi-herbal formulation against *E. coli* (A) and *S. aureus* (B).

Exposure to the multi-herbal formulation significantly enhanced antibacterial activity, reducing MIC values up to 32-fold for *E. coli* and up to 8-fold for *S. aureus,* demonstrating the polyvalent effect of combining these hydroalcoholic extracts and essential oils.

Once the polyvalent effect of combining the individual plant extracts was confirmed, the efficacy of this formulation against multidrug-resistant bacterial strains associated with bovine mastitis was evaluated.

According to data analysis, the most sensitive bacteria to the multi-herbal formulation were *S. haemolyticus* and *S. maltophilia* (0.39 mg/mL), followed by all *S. aureus* strains, *E. faecium* and *S. chromogenes* (0.57 mg/mL). MIC values of 1.15 and 1.58 mg/mL, were determined against *E. faecalis* and *E. coli*^{35218} respectively. Less sensitive bacteria were *E. coli*^{01} (3.17 mg/mL), *E. coli*^{02} and *P. aeruginosa* (12.34 mg/mL; Table 2).

Table 2. Minimal inhibitory concentration of multi-herbal formulation on bovine mastitis associated pathogens

Bacteria	**Multi-herbal formulation (mg/ml)**	**Kanamycin (µg/ml)**
	Gram negative strains	
E. coli^{35218}	1.58^{d}	0.5
E. coli^{01}	3.17^{e}	4.0
E. coli^{02}	6.34^{f}	2.0
P. aeruginosa	6.34^{f}	4.0
S. maltophilia	0.39^{a}	8.0
	Gram positive strains	
S. aureus^{6538}	0.57^{b}	0.5
S. aureus^{01}	0.57^{b}	2.0
S. aureus^{02}	0.57^{b}	2.0
E. faecium	0.57^{b}	4.0
S. chromogenes	0.57^{b}	0.5

E. faecalis	1.15[c]	2.0
S. haemolyticus	0.39[a]	8.0
P value	0.0001	

[a, b, c] Different literals in the columns indicate significant statistical differences ($p \leq 0.05$)

2.3 Minimal bactericidal concentration.

The minimal bacterial concentrations of the individual extracts are shown in figure 3. *E. coli* was killed by *L. tridentata*, *C. coriaria*, *L. graveolens*, and *S. aromaticum* at 25.0, 50.0, 0.06 and 0.32 mg/mL, respectively, while for *S. aureus* the MBC values were 0.78, 25.0, 0.06 and 0.62 mg/mL. In contrast multi-herbal formulation significantly enhanced bactericidal activity, reducing MBC values (Figure 3).

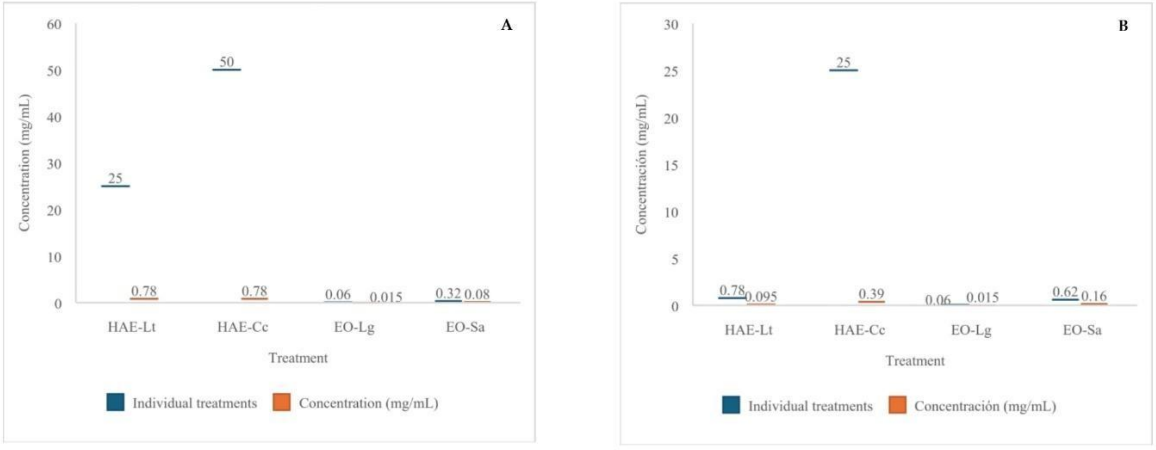


Figure 3. Minimal bactericidal concentrations of individual extracts and multi-herbal formulation against *E. coli* (A) and *S. aureus* (B).

With respect to bactericidal activity of multi-herbal formulation against MDR strains, data analysis showed no significant statistical differences between *S. haemolyticus* and *S. maltophilia* (0.79 mg/mL), similarly to that determined for *E. faecium* and *E. faecalis*, since MBC of 4.60 mg/mL were determined against both pathogens (Table 3).

Formulation showed bactericidal activity at concentration of 1.15 mg/mL against four bacteria, all of them Gram positive, respect to Gram negatives, treatment showed weakness activity since higher values were determined, *E. coli* strains were killed at concentrations from 6.34 to 12.69 mg/mL, being *P. aeruginosa* the less sensitive strain (25.38 mg/mL; Table 3).

With respect to calculations of the MBC/MIC ratio, results showed that multi-herbal formulation exhibited bactericidal effect against all evaluated strains, except for *S. chromogenes*, since value of 8 was determined, considering its effect as bacteriostatic.

Table 3. Minimal bactericidal concentration of multi-herbal formulation on bovine mastitis associated pathogens.

Bacteria	Multi-herbal formulation (mg/ml)	Kanamycin (µg/ml)
Gram negative strains		
E. coli^{35218}	6.34^{d}	4.0
E. coli^{01}	6.34^{d}	8.0
E. coli^{02}	12.69^{e}	4.0
P. aeruginosa	25.38^{f}	32.0
S. maltophilia	0.79^{a}	16.0
Gram positive strains		
S. aureus^{6538}	1.15^{b}	4.0
S. aureus^{01}	1.15^{b}	8.0
S. aureus^{02}	1.15^{b}	4.0
E. faecium	4.60^{c}	32.0
S. chromogenes	1.15^{b}	1.0
E. faecalis	4.60^{c}	4.0
S. haemolyticus	0.79^{a}	16.0
P value	0.0001	

$^{a, b, c}$ Different literals in the columns indicate significant statistical differences ($p \leq 0.05$)

The increasing multidrug resistance among bacterial agents associated with bovine mastitis has significantly limited the effectiveness of conventional treatments, thereby elevating public health risks (Li et al., 2023; Tomanić et al., 2023). Recent studies have reported high

resistance rates among clinical isolates from bovine mastitis cases (Winther et al., 2024; Moradi et al., 2025).

Given the growing complexity of multidrug-resistant (MDR) bacterial infections, both animal and public health authorities have expressed constant concern about their impact and have emphasized the need for multifaceted therapeutic strategies with effective antimicrobial activity. In this context, the search of alternative drugs based on the pharmacological and phytochemical properties of plants became a research priority in livestock health, aiming to reduce the exclusive reliance on conventional antibacterials. (Pinedo et al., 2013; Fratini et al., 2014; Park et al., 2016; Paşca et al., 2017; Soundhararajan et al., 2023; Tomanić et al., 2023).

Among plant-based approaches, multi-herbal formulations have emerged as promising therapeutic options, these mixtures can exert synergistic effects that enhance antimicrobial activity, more over are preferred due to their easy availability and low toxicity (Aladejana, 2023).

In this respect, the present study evaluated multi-herbal formulation against multidrug-resistant mastitis pathogens. The obtained data revealed significant bacteriostatic and bactericidal activity against both Gram-positive and Gram-negative MDR bacteria, with MIC values from 0.39 mg/mL to 6.34 mg/mL, and bactericidal effects observed at concentrations ranging from 0.79 mg/mL to 25.38 mg/mL.

Previous studies have reported antibacterial activity of multi-herbal formulations. The combination of hydroalcoholic extracts from *Eucalyptus globulus* and *Juglans regia* exhibited inhibitory effects against *S. aureus* isolates obtained from bovine mastitis cases

(Gomes et al., 2019). Similarly, a binary mixture of *Satureja montana* and *Thymus vulgaris* essential oils showed stronger inhibitory activity than oils alone, since larger inhibition zones were observed against five *Staphylococcus* strains and against *E. coli*, all associated with bovine mastitis (Fratini et al., 2014). In a related study, Corona-Gómez *et al*. (2022) reported that combinations of tea tree oil with thymol as well as thymol with carvacrol exhibited significant antibacterial activity against *Staphylococcus* spp., *K. pneumoniae* and *E. coli* isolated from mastitis cases, demonstrating additive effects

In antother study Pasca *et al*. (2017) evaluated the antibacterial activity of eight formulations containing different concentrations of aqueous extracts, alcoholic extracts, and essential oils from 11 medicinal plants against 32 bacterial isolates from cows with mastitis, the authors reported that three of their formulations showed strong inhibitroy effect, superior to the activity of individual plant extracts. Similarly do Nascimento *et al*. (2025) formulated two natural products based on the mixtures of extracts, resins and oils obtained from six plants, and demonstrated antibacterial efficacy against *S. aureus* , *S. agalactiae* and *E. coli*, with MIC values as low as 0.097%. Bactericidal activity was also reported agaisnt these pathogens.

2.4 Time-kill kinetics assay

Bacterial growth kinetics of multi-herbal formulation were determined using *E. coli* and *S. aureus*. Fig. 4 shows these results. A rapid decrease in the growth of *E. coli* was observed at concentration 12.69 exhibiting its bactericidal effect after two hours, while at 25.38 mg/mL complete lethality was observed during the first 30 minutes, a similar effect against *S. aureus* was observed for all evaluated doses. Both dose-dependent and time-dependent effect were determined (Fig. 4).

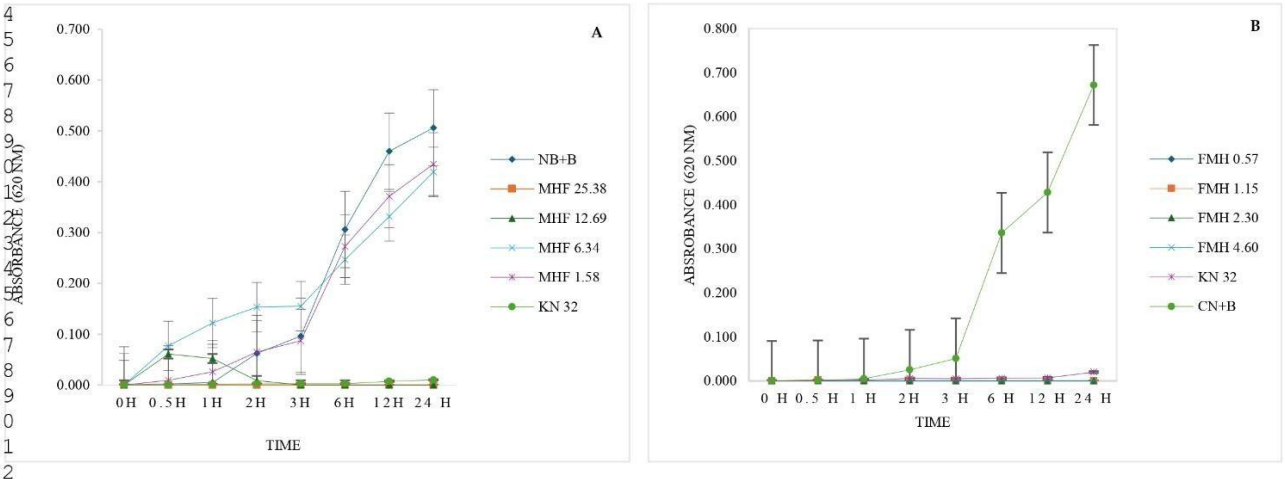


Figure 4. Time-kill assay of the multi-herbal formulation on *E. coli* (A) and S. *aureus* (B) associated with bovine mastitis.

Compared with previous reports, our formulation achieved bactericidal effects in a considerably shorter period. For instance, trans-cinnamaldehyde, eugenol, carvacrol, and thymol required the 12-24 h to reduce the growth of *S. agalactiae*, *S. dysgalactiae*, *S. uberis* *S. aureus*, and *E. coli* associated with bovine mastitis (Baskaran *et al*., 2009). Similarly, *Origanum vulgare* and *Thymus vulgare* essential oils achieved complete inhibition only after 120 minutes (Al-Asmari et al., 2024), and *Morinda citrifolia* essential oil showed inhibition at 6 h and half-maximal inhibition at 36 h against MDR *P. mirabilis*, *P. aeruginosa*, *E. coli* and *S. aureus* (Rajivgandhi et al., 2024). Mobolaji *et al*. (2023), stated that mono-herbal treatments required at least 70 minutes for complete bacterial lethality.

Potential of synergistic phytochemical interactions in this study are suggested, consistent with this Sanguansermsri *et al*. (2024) evidenced that extracts with multiple active constituents exhibited greater bactericidal effects than single component treatments, which evidence that these constituents can act synergistically to enhance antimicrobial potency.

These findings highlight that multi-herbal formulations can achieve faster bactericidal effects. According to Balouiri *et al*. (2016), a bactericidal effect corresponds to 90% lethality at 6 h, and 99.9% at 24 h, further validating the rapid and potent activity observed with our formulation. In this context, bactericidal agents are desirable because they ensure the complete elimination of infections while reducing the likelihood of antimicrobial resistance, by acting on multiple targets through diverse mechanisms of action. Therefore, multi-herbal formulations represent a promising alternative against multidrug-resistant pathogens associated with bovine mastitis (Corona-Gómez et al., 2022).

2.5 Cell membrane integrity

Cell membrane integrity assays were conducted to evaluate the effect of multi-herbal formulation on bacterial membranes. In *E. coli* strongest effect was observed at 12.69 mg/mL, resulting in the release of 56.17 mg/mL of proteins and 2423.70 ng/µL of DNA higher than the values recorded for the positive control (Cell lysis solution; 2.18 mg/mL of proteins and 250.23 ng/µL DNA). Similarly, in *S. aureus* the highest concentration (2.30 mg/mL) induced protein and DNA leakage of 36.49 mg/mL and 1219.70 ng/µL, respectively, whereas the cell lysis solution produced only 1.17 mg/mL proteins and 110.27 ng/µL DNA leakage. These results demonstrate a dose-dependent effect of the multi-herbal formulation on membrane disruption, the robustness of these results is confirmed by the high R^2 values,

with an *r* value of 1.0 for *E. coli* and *r* values ranging from 0.9983 to 0.9996 for *S. aureus*, which demonstrates a strong association between the variables analyzed (Figs. 5 and 6)

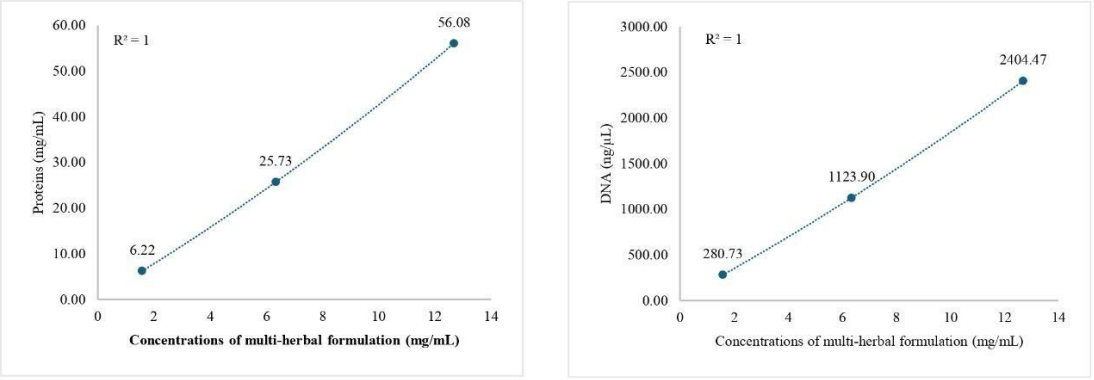


Figure 5. Membrane damage assay of the multi-herbal formulation on *E. coli*. **(A)** Cytoplasmic proteins. **(B)** Nucleic acids.

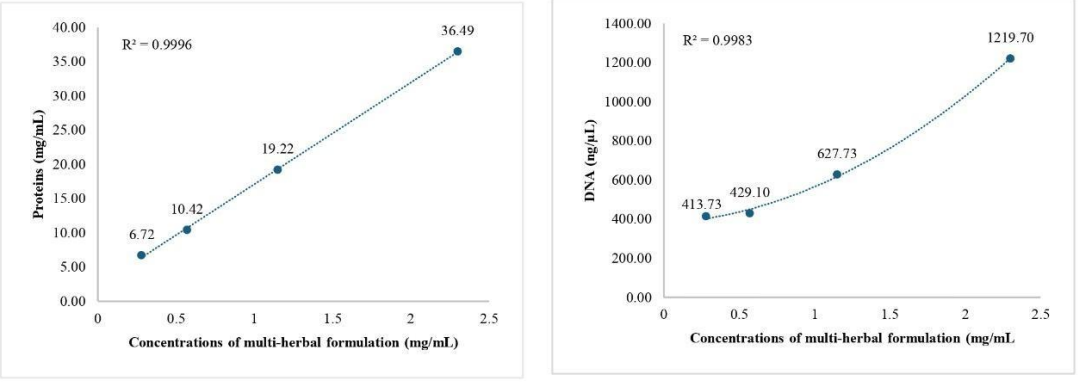


Figure 6. Test on membrane damage of the multi-herbal formulation on *S. aureus*. **(A)** Cytoplasmic proteins. **(B)** Nucleic acids.

Previous studies have reported that plant extracts and essential oils primarily act on the bacterial cell membrane, leading to leakage of proteins and nucleic acids, which indicates irreversible damage to the cytoplasmic membrane (Oussalah et al., 2006; Li et al., 2022; Tang et al., 2023; Zhao et al., 2023).

The phytochemical analysis of the multi-herbal formulation revealed the presence of diverse bioactive compounds, including flavonoid-type compounds, lignans tannins and terpenes, all of which are known to exert antibacterial activity through multiple mechanisms.

In this context the membrane disruption and bactericidal activity observed in our assays are consistent with previous reports. Several authors have suggested that the bacterial cell membrane is the primary target of flavonoids. The role of flavonoids in bacterial leakage has been well documented, for them part, Sulistyani *et al*. (2022) and Zhou *et al*. (2022) reported that bacterial exposure to flavonoids caused a significant leakage of nucleic acid, proteins and DNA, these findings are aligned with those of the present study.

HPLC analysis identified the presence of luteolin rutinoside, apigenin rutinoside and rutin. The antibacterial activity of flavonoids can be enhanced when are combined, rutin potentiate the antibacterial effects of quercetin, morin, kaempferol, myricetin and fisetin, against both Gram-positive and Gram-negative bacteria (Arima et al., 2002), in this context presence of rutin in our formulation may partially explain the significant antibacterial activity observed.

Regarding lignans, some studies have reported that these compounds also act primarily on bacterial cell membranes. For example, cinaguaiacin, a compound structurally similar to the lignan identified in this study, caused significant leakage of proteins and DNA from *S. aureus* and *E. coli* (García-Hernández et al., 2025). Similarly, the antibacterial mode of action of *nor*

3'-demethoxyisoguaiacin has been determined, demonstrating that the lignan's activity is localized at the bacterial cell membrane, affecting ABC transporters (Favela hernandez et al., 2015). This mechanism may contribute to the bactericidal activity observed in the present study as *nor* 3'-demethoxyisoguaiacin has also been reported to be active against multidrug-resistant bacteria associated with bovine mastitis (Morales-Ubaldo et al., 2022)

Concerning tannins, both gallic acid and ellagic acid, as well as their derivatives have been reported to induce bacterial membrane damage. These phenolic compounds bind to membrane components of *E. coli* and *S. aureus* increasing membrane fluidity and permeability, which leads to leakage of proteins and nucleic acids, disrupting membrane integrity (Lee and Je 2013; Zhou et al., 2019; Liu et al., 2022; Pei et al., 2022).

Concerning *L. graveolens* and *S. aromaticum* due to the hydrophobic nature of the essential oil constituents, the cytoplasmic membrane appears to be a primary target for both sensitive and multidrug-resistant bacteria. Compounds such as eugenol and thymol can disrupt membranes of *E. coli* and *S. aureus* leading to the leakage of intracellular components, while other compounds primarily affect only outer structures (Di Pasqua et al., 2007; Nazzaro, et al., 2013; Yadav et al., 2015; Xu et al., 2016; Wang et al., 2017; Zhao et al., 2023). This mode of action is attributed to the hydroxyl groups present in thymol and eugenol, which inactivate target enzymes, causing rupture of the bacterial cell membrane (Guimarães et al., 2019; Qian et al., 2020; Zhang et al., 2024).

This membrane disruption is proposed as the primary mechanism through which the multi-herbal formulation exerts its bactericidal effect. However, some bioactive compounds have also been reported to act through additional mechanisms, including disruption of the phospholipid bilayer, inhibition of the bacterial respiratory chain or ATP synthesis, inhibition

of efflux pumps, inhibition of β-lactamases and suppression of bacterial virulence factors (Yuan et al., 2021; Macêdo et al., 2022; Li et al., 2023; Veiko et al., 2023; Xu et al., 2024).

These findings suggest that bactericidal activity of our multiherbal formulation may be attributed to the combined activity of various phytochemicals. Due to the difficulty in precisely defining and experimentally proving synergy, the interactions among flavonoids, tannins, lignans and terpenes identified in this study can be described as a polyvalent action, this term emphasizes the cooperative and enhanced effects observed in the multi-herbal formulation, highlighting the combined activity of multiple secondary metabolites on different bacterial targets. In this context, multi-herbal formulations may also reduce the risk of resistance development, possibly due to their multiple modes of action that hinder the selection of resistant bacterial strains (Williamson, 2001; Pinedo et al., 2013; Pașca et al., 2017; Yang et al., 2019; Pașca et al., 2020).

2.6 Brine-Shrimp Cytotoxic Assays

The results showed an increasing mortality of *Artemia salina* nauplii as the concentration of multi-herbal formulation increased. The obtained LC_{50} and LC_{90} were 0.186 mg/mL and 0.323 mg/mL, respectively (Fig. 7).

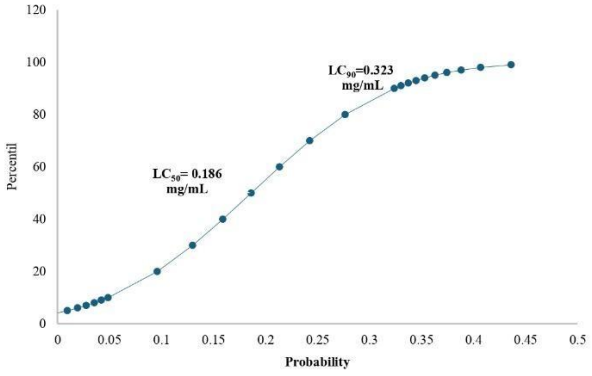


Figure 7. LC_{50} and LC_{90} of the multi-herbal formulation

Although plant extracts and their bioactive compounds are generally considered safe, it is essential to evaluate their cytotoxic potential to ensure safety and therapeutic viability. In this regard the microwell brine shrimp (*A. salina*) assay is an affordable and widely used method for detecting cytotoxic compounds, offering advantages in the standardization and quality control of plant-derived products (Olmedo et al., 2024).

In the present study, a LC_{50} of 0.186 mg/mL (186 µg/mL) was determined, similarly Agbodjento et al. (2020), reported LC_{50} value of 0.166 mg/mL when *A. salina* nauplii were exposed to *Rourea coccinea* extract, which is rich in saponins, tannins, steroids, glycosides, flavonoids, anthraquinone, and alkaloids, and classified the extract as non-toxic. According to Naz *et* al. (2017), extracts are considered non-toxic if the LD_{50} is greater than 100 µg/mL in the brine shrimp lethality assay. Safe and effective therapies from plant-based products depend on the toxicity profiles of the molecules, therefore the concentrations of phytochemicals in a combination and the frequency of use are key factors to achieve optimal benefits (Choudhury, 2022). These findings support the potential use of multi-herbal

formulations as safe alternatives in response to the emergence of bacterial resistance. Nevertheless, further *in vitro* and *vivo* assays are necessary to confirm their selective cytotoxicity against pathogens without affecting host cells (da Costa et al., 2010).

3. Conclusion

Taken together, these findings demonstrate that the multi-herbal formulation exerts potent bactericidal activity against multidrug-resistant pathogens associated with bovine mastitis, mainly through the polyvalent action of flavonoids, lignans, tannins, and terpenes, which primarily disrupt the bacterial membrane. Moreover, the low cytotoxicity of the formulaltion supports its potential safety for future veterinary applications, However, further studies foccusing on *in vivo* assays and elucidation of other the mechanisms of action are necessary to consolidate these findings.

4. Experimental

3.1 Preparation of multi-herbal formulation

Herbal components included different concentrations of *Larrea tridentata, Caesalpinia coriaria*, *Lippia graveolens* and *Syzygium aromaticum*, which were solubilized in 15% dimethyl sulfoxide and 0.5 % Tween 80 ® (SIGMA P1754, St. Louis, MO, USA) solvents. Multi-herbal-formulation data is shown in table 1.

Table 1. Composition of the multi-herbal formulation used in the treatment of bovine mastitis associated pathogens.

Local name	Scientific name	Plant organ	Kind of extract	Bioactive compound	Content Gram negative (mg/mL)	Content Gram positive (mg/mL)
Gobernadora	*Larrea tridentata*	Aerial parts	Hydroalcoholic extract	*nor*-3´demethoxyisoguaiacin (Morales-Ubaldo et al., 2022)	12.50	0.78
Cascalote	*Caesalpinia coriaria*	Fruit	Hydroalcoholic extract	Gallic acid (Olmedo-Juárez et al., 2019)	12.50	3.12
Orégano Mexicano	*Lippia graveolens*	Leaves	Essential oil	Thymol and carvacrol (Bautista-Hernández et al., 2021)	0.06	0.06
Clavo	*Syzygium aromaticum*	Buds	Essential oil	Eugenol (Batiha et al.. 2020)	0.32	0.64
				Total	**25.38**	**4.60**

4.2 Chemical characterization of multi-herbal formulation

Chemical analyses, through HPLC techniques, were conducted for hydroalcoholic extracts (*L. tridentata*, *C. coriaria*) and essential oils (*L. graveolens* and *S. aromaticum*) following methodology described by Olmedo-Juarez *et* al. (2019) and Morales-Ubaldo *et al*. (2022).

High-performance liquid chromatography (HPLC Alliance) was used, consisting of a separation module (Waters e2695 Corp., Milford, MA, EE. UU), a photodiode array detector (Waters 2998), and a Discovery® C18 column (25cm x 4.6 mm, 5 µm). The mobile phase consisted of water with 0.5 % trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The gradient system employed was as follows: 0–1 min, 0% B; 2–3 min, 5% B; 4–8 min, 15% B; 9–20 min, 30% B; 21–23 min, 50% B; 24–25 min, 80% B; 26–27 min, 100% B; and 28–30 min, 0% B. The flow rate was maintained at 0.90 mL/min, and the total run time was 30 min. The sample injection volume was 5 µL. A UV wavelength scan was performed from 200–600 nm. Chromatograms were recorded at 260, 280, and 360 nm to detect flavonoids.

4.3 *Bacterial strains and culture conditions*

Antibacterial evaluations were performed against Gram-positive and Gram-negative bacteria, using reference strains (ATCC) of *Staphylococcus aureus*^{6538} and *Escherichia coli*^{35218}, as well as multidrug-resistant clinical isolates associated with bovine mastitis cases. The Multiple Antibiotic Resistance Index (MARI), which reflects the proportion of antibacterials to which a bacterial isolate is resistant is indicated in parentheses for the following strains *S. aureus*^{01} **(MARI=0.58)** , *S. aureus*^{02} **(MARI=0.66)**, *Staphylococcus chromogenes* **(MARI=0.91)**, *Staphylococcus haemolyticus* **(MARI=0.83)**, *Enterococcus faecium* **(MARI=1.0)**, *Enterococcus faecalis* **(MARI=0.91)**, *E. coli*^{01} **(MARI=0.75)**, *E. coli*^{02}**(MARI=0.58)**,*Pseudomonas aeruginosa* **(MARI=0.83)** and *Stenotrophomonas maltophilia* **(MARI= 0.83)** from the Bacteriology Laboratory of the Academic Area of Veterinary Medicine and Zootechnics of the Autonomous University of Hidalgo State. One colony of each strain was inoculated in nutritive broth (BD Bioxon, Heidelberg, Germany), and incubated under constant agitation (70 RPM) for 24 h at 37 °C.

4.4 Antibacterial activity

Prior to evaluating the antibacterial activity of the multi-herbal formulation, the individual antibacterial activities of *L. tridentata* and *C. coriaria* hydroalcoholic extracts, as well as *L. graveolens* and *S. aromaticum* essential oils were assessed against *E. coli* and *S. aureus* ATCC strains. Multi-herbal formulation was evaluated against both ATCC and multidrug-resistant strains, following the methods described by Morales-Ubaldo *et al*. (2022).

The Minimal inhibitory Concentration (MIC) was determined by the microdilution technique Concentrations ranged from 100 mg/mL to 0.78 mg/mL for individual extracts, 10 mg/mL to

0.15 mg/mL for essential oils, and 25.38 mg/mL-0.19 mg/mL (Gram negative strains) or 4.60 mg/mL- 0.03 mg/mL (Gram positive strains) for the multi-herbal formulation. As negative control nutritive sterile broth was used, while Kanamycin (0.25-32.0 µg/mL) was used as the positive control.

Into a 96-well plate, 100 µL of each concentration were added, followed by the addition of 10 µL of the bacterial suspension adjusted to a 0.5 McFarland standard (Remel, R20421, Lenexa, KS, USA), every treatment was evaluated by triplicate. Plates were incubated at 37°C during 24 at 70 rpm. To determine the concentration at which extracts inhibited bacterial growth, 20 µL of a 0.04% (*w/v*) p-iodonitrotetrazolium solution was added into each well, concentration which turned pinkish was considered as the MIC.

Prior addition of p-iodonitrotetrazolium solution, 5.0 µL from each well was inoculated in Müller–Hinton agar (DIBICO® Mexico City, Mexico) and incubated at 37 °C for 24 h, determining as minimal bactericidal concentration (MBC) the concentration at which treatments killed 99.9% of bacteria.

Bacteriostatic or bactericidal effects of evaluated treatments were determined through calculations of the MBC/MIC ratio, when the MBC/MIC ratio is greater than four, the effect is bacteriostatic and when it is less than or equal to four the effect is bactericidal.

4.5 Time Kill kinetics Assay

The time-kill kinetics assay was used to indicate the bactericidal action of multi-herbal formulation according to the descriptions of Al-Mijalli *et al.*, (2023) with some modifications. The time kill assay was conducted in triplicate using *E. coli*^{35218} and *S. aureus*^{6538} adjusted suspensions. The Final concentrations were as follows MIC, MBC, 2x MBC and 4x MBC of multi-herbal formulation (MHF). Kanamycin (KN) at 32 µg/mL was

used as positive control, while sterile nutritive broth and sterile nutritive broth added with bacteria (NB+B) were used as negative controls.

Into a 96-well plate, 100 µL of each concentration were added, followed by the addition of 10 µL of the bacterial suspension adjusted to a 0.5 McFarland standard. After inoculation plates were incubated at 37° C, the optical density at 620 nm was measured. Measurements were performed considering intervals of 30 min, for three hours, then were performed at 6, 12 and 24 h.

4.6 Cell membrane integrity

Bacteria cells were subjected to treatment with the multi-herbal formulation at MIC, 2x MIC and 4x MIC, saline was used as negative control and cell lysis solution (Wizard® Genomic DNA Purification Kit, Promega, USA) was used as positive control. Samples were incubated for 30 min at 37° C, once the period elapsed, bacteria were separated from the supernatant by centrifugation at 10,000 RPM, for 5 min at 4 ◦C. The concentrations of cytoplasmic proteins were then determined in the supernatant by measuring the optical density at 280 nm. In addition, leakage of nucleic acids was measured (Al-Mijalli et al., 2023).

4.7 Brine-Shrimp Cytotoxic Assays

Cytotoxic activity of the multi-herbal formulation was tested against freshly hatched free-swimming nauplii of *Artemia salina* according to the methodology described by Rangel-López *et al*. (2022). In a 96-well plate, serial dilutions were performed (12.69 mg/mL–0.095mg/mL) in 100 µL of saline; after, 100 µL of saline solution containing between 10 and 15 nauplii were added. Tween®80 (SIGMA P1754, St. Louis, MO, USA) was used as a positive control and saline as a negative. Plate was incubated at 30 °C for 24 h. Once each well was observed under a stereoscopic microscope (Eco SZ-745, Schertz, TX, USA) to

count dead and live nauplii and determine the mortality percentage using the following formula.

$$\%\ Mortality = \frac{number\ of\ dead\ nauplii}{initial\ number\ of\ nauplii}\ x\ 100$$

4.8 Statistical analysis

Obtained data were normalized and analyzed by analysis of variance (ANOVA) and Tukey's comparison of means ($p < 0.05$). The coefficient of determination (R^2) was calculated to determine the strength of association between the concentrations of the multi-herbal formulation and the leakage of intracellular components. Values close to 1.0 were considered indicative of a strong dose-dependent effect. The LD_{50} values of the multi-herbal formulation was determined by Probit analysis. Statistical analysis was performed Minitab 18 software.

CRediT authorship contribution statement

Morales-Ubaldo Ana Lizet: Writing – original draft, conceptualization, methodology, data curation, formal analysis. **Manasés González-Cortazar, Ojeda-Ramirez Deyanira**: Investigation, resources, methodology, writing – review and editing, formal analysis. **Valladares-Caranza Benjamin:** methodology, data curation, formal analysis. **Zamilpa-Álvarez Alejandro, Ramirez-Duran Ninfa**: Investigation, resources, methodology, formal analysis. **Rivero-Perez Nallely, Adrian Zaragoza-Bastida**: Conceptualization, writing – original draft**,** Investigation, resources, methodology, writing – review and editing, supervision, formal, analysis, validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO III: Funcionalización de una película a base de almidón de chayotextle con una formulación multi-herbal que presenta actividad antibacteriana frente a patógenos asociados a mastitis bovina: Potencial uso como sellador de pezones bioactivo

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Functionalization of a chayotextle starch-based film with a multi-herbal formulation exhibiting antibacterial activity against bovine mastitis pathogens: potential use as a bioactive teat sealant.

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Abstract:	A chayotextle starch-based biofilm functionalized with a multi-herbal formulation (MHF) was obtained using the casting method. The biofilm was characterized by Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The antibacterial activity was evaluated through the disk diffusion assay and by determining the percentage of bacterial growth inhibition against Escherichia coli and Staphylococcus aureus. Additionally, a physical structural model was used to assess bacterial growth reduction. The functionalized biofilm exhibited inhibition zones ranging from 10.25 mm to 24.75 mm, showing greater activity against S. aureus. Moreover, it achieved 100% bacterial growth reduction for both strains. The antibacterial activity of the biofilm was attributed to structural rearrangements within the polymeric matrix and the formation of interactions between the polysaccharide functional groups and the phenolic compounds and terpenes present in the MHF. These findings suggest that the incorporation of MHF into a starch-based biofilm enhances its antimicrobial efficacy, likely due to synergistic effects and improved compound stability and retention within the polymeric structure.
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Functionalization of a chayotextle starch-based film with a multi-herbal formulation exhibiting antibacterial activity against bovine mastitis pathogens: potential use as a bioactive teat sealant.

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Abstract

A chayotextle starch-based biofilm functionalized with a multi-herbal formulation (MHF) was obtained using the casting method. The biofilm was characterized by Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The antibacterial activity was evaluated through the disk diffusion assay and by determining the percentage of bacterial growth inhibition against *Escherichia coli* and *Staphylococcus aureus*. Additionally,

a physical structural model was used to assess bacterial growth reduction. The functionalized biofilm exhibited inhibition zones ranging from 10.25 mm to 24.75 mm, showing greater activity against *S. aureus*. Moreover, it achieved 100% bacterial growth reduction for both strains. The antibacterial activity of the biofilm was attributed to structural rearrangements within the polymeric matrix and the formation of interactions between the polysaccharide functional groups and the phenolic compounds and terpenes present in the MHF. These findings suggest that the incorporation of MHF into a starch-based biofilm enhances its antimicrobial efficacy, likely due to synergistic effects and improved compound stability and retention within the polymeric structure.

Keywords: Chayotextle starch, multi-herbal formulation, biofilm, antibacterial activity, bovine mastitis, bioactive, sealant.

1. Introduction

Bovine mastitis is a prevalent disease affecting 40 % of cows in dairy herds, causing significant negative influences on both the health and productivity of animals [1, 2]. Mastitis is characterized by inflammation of the mammary gland and udder, is a multifactorial disease, however, is specially associated with different bacterial agents, including Gram positive cocci and Gram negative bacilli [2, 3].

Among cocci, *Staphylococcus aureus* is a major pathogen of mastitis which is mainly transmitted between cows by the contagious route and is characterized for causing chronic intramammary infections derived in subclinical and clinical mastitis [1, 4] On the other hand, *E. coli*, is one of the most prevalent bacilli in dairy farm environments and is frequently

identified as a pathogen causing mastitis typically is associated with an acute and severe form of mastitis [2, 5].

As bacterial infections stand as the primary cause of bovine mastitis, antibacterials have been the mainstay of treatment for decades, 80 % of the antibacterial agents in dairy herds are used to control or treat mastitis. However, the widespread and often improper use of these antibacterials has become a serious public health concern, primarily due to the emergence of multidrug-resistant bacteria and their potential transmission through the food chain. Therefore, there is urgent need to develop innovative and effective alternatives for the prevention and treatment of bovine mastitis [3, 4, 6, 7].

To date natural biodegradable polymers such as starch have attracted a lot of attention due to their low cost, availability and compostability without generating toxic residues. Starch-based films have been employed in both food and non-food applications; however, starch itself lacks antibacterial properties, therefore the incorporation of antimicrobial agents into starch matrices is necessary to confer such functionality. In this context, the use of natural antimicrobial compounds, particularly those obtained from plant extracts or essential oils has emerged as a promising approach [5, 8-10].

Individual plant-derived antibacterials (essential oils or plant extracts) have been extensively studied, demonstrating that its addition into film formulations increase the antibacterial activity, however, research on the antibacterial efficacy of multi-herbal formulation combining extracts and essential oils is limited in agricultural and livestock sector. These combinations due to their synergistic potential may enhance antibacterial efficacy and broaden the spectrum activity, making them relevant in veterinary medicine or dairy products [11-14].

In this regard, the incorporation of multi-herbal formulations into natural polymeric matrices, such as starch-based biofilms, emerges as a highly promising strategy, as it enables the combination of the synergistic therapeutic action of phytoactive compounds with the physicochemical and functional advantages of starch as a controlled-release system. To date literature does not provide information on the use of multi-herbal formulations added to starch films, nor about its application in the prevention of diseases such as bovine mastitis. Due to this the aim of the present study was to determine antibacterial activity of a Chayotextle starch-based biofilm functionalized with a multi-herbal formulation against *E. coli* and *S. aureus* associated with bovine mastitis.

2. Experimental

2.1 Multi-herbal formulation

The multi-herbal formulation was prepared using *Larrea tridentata* and *Caesalpinia coriaria* hydroalcoholic extracts, *Lippia graveolens* and *Syzygium aromaticum* essential oils. The mixture proportions were established as twice the highest minimal bactericidal concentration (2xMBC) determined within this study to ensure bactericidal efficacy against tested strains.

2.2 Preparation of the film

Chayotextle film functionalized with a multi-herbal formulation (CSF-MHF) was prepared with a concentration of 4% of chayotextle (*Sechium edule*) starch (w/w, dry basis), 2.0 g of glycerol and water (180 mL). The solution was stirred at 125 rpm. The dispersion was heated at 90° C for 10 min until gelatinization, after that it cooled to 45°C and the multi-herbal formulation (MHF) at 2xMBC was added to the solution. After adding MHF, the dispersions were stirred for 10 min at 125 rpm [15]

A control film (CSF-C) was prepared using the same base ingredients, incorporated at identical amounts and proportions to those of the experimental films. The formulation was processed under the same temperature and stirring conditions to ensure comparability. Posteriorly starch suspensions were prepared in a glass container (110 x 110 x 3 mm: length x width x height) and dried in an oven at 40° C for 24 h. After drying, the composites were removed from the glass container and stored in a sealed plastic bag at room temperature until antibacterial evaluation. The chayotextle starch characterization was previously reported by Martínez-Ortíz *et al*. (2017) [15]

2.3 Characterization of films

2.3.1 Scanning Electron microscopy (SEM)

The microscopic morphology of control (CSF-C) and experimental (CSF-MHF) chayotextle starch films was examined using a SEM (Jeol IT-300, Tokyo, Japan). Dried samples were placed in an ion sputter and sprayed with gold coating to increase their conductivity. The film surface and cross-section were observed. [16, 17].

2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The film samples (CSF-C and CSF-MHF) were cut into 10 mm x 10 mm pieces and dehydrated in a desiccator (~0% relative humidity). Subsequently, the samples were analyzed using a Fourier transform infrared spectrophotometer (PerkinElmer, Spectrum Two model, Ohio, USA) equipped with a universal attenuated total reflectance (ATR) accessory. The spectra were recorded in the infrared region with 16 scans, covering a wavenumber range from 400 to 4,000 cm^{-1} [18].

2.4. Antibacterial activity

2.4.1 Bacterial Strains and Culture Conditions

To verify the antibacterial activity, multidrug-resistant *E. coli* and *S. aureus* strains, isolated from bovine mastitis cases, were used as the indicator bacteria. The strains belong to the collection of the Bacteriology Laboratory of the Academic Area of Veterinary Medicine and Zootechnics at the Autonomous University of Hidalgo State.

One colony of each strain was inoculated into nutritive broth (BD Bioxon, Heidelberg, Germany) and incubated under constant agitation (70 rpm) at 37 °C for 6 h, to ensure that the bacteria were harvested during their exponential (log) phase. After incubation, the inoculum was adjusted to 0.5 McFarland turbidity standard (Remel, R20421, Lenexa, KS, USA).

2.4.2 Minimal bactericidal concentration of multi-herbal formulation

To evaluate the bactericidal efficacy of the multi-herbal formulation (MHF), the Minimal Bactericidal Concentration (MBC) was determined. MHF Concentrations ranging from 203.04 mg/mL to 0.79 mg/mL were evaluated against *E. coli* and *S. aureus*. Kanamycin (64.0 to 0.5 µg/mL; AppliChem 4K10421, Darmstadt, Germany) was used as positive control, while sterile nutritive broth was the negative control (BD Bioxon), in accordance with descriptions by Morales-Ubaldo *et al*. (2022) [19].

The broth microdilution technique was employed to assess the antibacterial activity. Serial dilutions of the multi-herbal formulation (MHF) were prepared in nutrient broth within sterile 96-well microplates and 10 µL of bacterial suspension adjusted to a 0.5 McFarland standard was added to each well.

The plates were incubated at 37 °C for 24 h under agitation at 70 rpm. Following incubation, 5 µL from each well were subcultured onto Müller–Hinton agar (BD Bioxon) and incubated

at 37 °C for 24 h. The minimal bactericidal concentration was considered as the lowest concentration of MHF showing no visible bacterial growth on the agar plates.

2.4.3 Zone of inhibition test

The zone inhibition zones were determined by the disk diffusion method in Müller–Hinton agar (BD Bioxon, Heidelberg, Germany) in accordance with described by Rodrigues *et al*. (2020) [20]. Treatments were as follows: MHF (2xMBC), CSF-C and CSF-MHF. Sterile distilled water (SDW) and commercial iodine-based teat sealant (IB-S; OrgaDIP 1.0% DB, Orgachemics, Mexico) were used as negative and positive controls, respectively. Treatments were evaluated in quadruplicates.

A total of 100 µL of each bacterial suspension previously adjusted was inoculated and distributed on Petri plates. Each plate was allowed to dry for 15 min under sterile conditions. and once this period had elapsed, 5 µL of each treatment were placed onto Müller–Hinton agar. The plates were incubated at 37.0° C for 24 hours. The degree of inhibition was expressed as inhibition zone (mm) [20].

The sensitivity was classified according to the descriptions by Imane *et al*. (2020), with some modifications to the sensitivity scale: not sensitive for diameter less than 9.0 mm, sensitive for a diameter of 9.0–15 mm, very sensitive for a diameter of 15–20 mm, and extremely sensitive for diameter larger than 20 mm [21].

2.4.4 Bacterial cell count

The antibacterial activity of the chayotextle starch-based film functionalized with a multi-herbal formulation was tested against the indicator strains according to the methodology proposed by Pagno *et al*., (2015) with some modifications [22].

Evaluated treatments were as follows: MHF (2xMBC), CSF-C and CSF-MHF. The commercial iodine-based teat sealant (IB-S) was used as positive control, while bacterial suspensions (0.5 McFarland) were used as negative controls.

The biofilms were cut into squares of 20 mm^2 and accommodated in Eppendorf tubes. Then, 500 µL of the bacterial suspensions (0.5 McFarland) were added to the evaluated treatments. After incubation for 24 h with gentle agitation, serial dilutions were prepared, 100 µL of the solutions were spread on Müller–Hinton agar plates (BD Bioxon, Heidelberg, Germany) and incubated at 37°C for 10 h. The viable cells on each plate were counted by quantifying the CFUs. Each test was performed in triplicates. The antibacterial effect in each group was calculated as the percentage of inhibition, which was calculated using formula 1.

$$1)\ \%\ \text{inhibition} = \frac{\text{CFU standard-CFU experimental group}}{\text{CFU standard}} * 100$$

2.4.3 Physical-Structural Model

To evaluate the antibacterial activity of the film-forming solution functionalized with the multi-herbal formulation (MHF), 1 mL sterile insulin syringes (27G × 13 mm, DL®, Mexico City, Mexico) was employed. Three treatments were evaluated: **T1:** Syringe + bacterial suspension; **T2:** syringe + commercial iodine-based teat sealant (IB-S); **T3:** syringe + CSF-MHF. As negative control sterile syringes added with nutritive broth were considered. Each treatment was performed in quadruplicate.

500 µL of sterile nutrient broth in Müller–Hinton agar (BD Bioxon, Heidelberg, Germany) was added to each syringe. Once the simulation system was assembled, the syringes were placed on a rack in an inverted position inside an incubator.

For treatment T1, the syringes tips were immersed for 15 seconds in 100 µL of a bacterial suspension (either *Escherichia coli* or *Staphylococcus aureus*), adjusted to a concentration of 100 CFU/mL [23, 24].

For treatments T2 and T3, the syringes were pre-treated by immersion for 60 seconds in the commercial sealant or in the MHF, respectively, ensuring that approximately 5 mm of the syringe tip was fully covered and to allow the formation of an internal seal within the tip. The treatments were then allowed to dry completely. Once dry, the syringes tips were brought into contact with the bacterial suspension under the same conditions described above.

All systems were incubated for 12 hours at 37 °C. After the incubation period, serial dilutions (1:10) were performed for quantification of the bacterial load. The percentage of bacterial growth reduction was calculated by formula 2 as mentioned below [25].

$$2)\ \%\,reduction = \frac{CFU\ standard - CFU\ experimental\ group}{CFU\ standard} * 100$$

2.5 Statistical analysis

Data normality and homoscedasticity were verified prior to analysis. The results were analyzed using a completely randomized design through analysis of variance (ANOVA) with a significance level of $\alpha \leq 0.05$. Differences between means were determined by multiple comparisons using Tukey´s test. All statistical analyses were performed using Minitab software, version 18.

3. Results

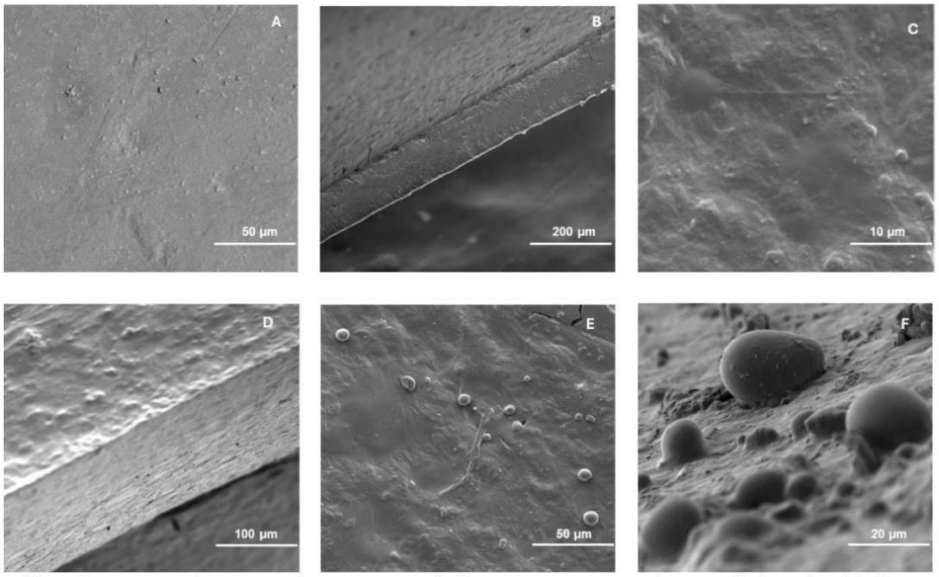
3.1 Characterization of films

3.1.1 Scanning electron microscopy

To characterize the microstructure of the films, scanning electron microscopy (SEM) analysis was performed. The surface and cross-sectional features of the control film and the functionalized film are presented in **Figure 1**.

As observed, the surface morphology of the control film (CSF-C) exhibited a smooth, compact surface without cracks or pores (**Figure 1A**). Similarly, the cross-sectional microstructure showed homogeneous and continuous morphology, with a dense structure free of irregularities (**Figure 1B**).

In contrast, the surface view of the functionalized film (**Figure 1C**) revealed notable changes, including the presence of irregularities that resulted in increased surface roughness. These modifications are attributed to the incorporation of extracts and essential oils into the polymer matrix. Cross-sectional analysis of CSF-MHF (**Figure 1D**) indicated that the addition of active ingredients increased the thickness of the polymer matrix without compromising its uniformity. SEM images of CSF-MHF (**Figures 1E and 1F**) revealed embedded particles



on the surface, which are suggested to correspond to phytocompounds incorporated within the biopolymer matrix.

Figure 1. Scanning electron microscopy (SEM) images showing the microstructure of films. **(A)** Surface morphology of the control chayotextle starch-based film (CSF-C); **(B)** CSF-C cross-section. **(C, E)** Surface morphology of the chayotextle starch-based film functionalized with a multi-herbal formulation (CSF-MHF). **(D, F)** CSF-MHF Cross-section.

The smooth and compact surface, along with the crack and pore-free cross section observed in the control film (CSF-C) are characteristic features of starch-based films without additives. This morphology can be attributed to a homogeneous polymer-plasticizer mixture [26-28].

In contrast, the increased surface roughness and irregularities observed in the functionalized film (CSF-MHF) are associated with the incorporation of extracts and essential oils. Several studies have reported that the inclusion of plant-derived compounds can disrupt the regular surfaces [27, 29, 30].

Comparable findings were reported by Nxumalo *et al*. (2024) who observed that the incorporation of Lippia *javanica*, *Syzygium cordatum* and *Ximenia caffra* extracts into chitosan films altered the microstructure in a similar manner to our study [31].

Riaz *et al*. (2018) found that apple peel polyphenols fused into a chitosan matrix induced morphological modifications in the polymeric matrix [32]. Similarly, Peng and Li (2014) demonstrated that the addition essential oils increased the coarseness of film cross-sections, attributed to phase separation and uneven distribution of the active components in the matrix [33]. Morphological changes may result from spatial organization and the interactions among starch and bioactive compounds during the film formation process [34].

3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used on film samples to investigate the chayotextle starch and MHF interactions. The infrared spectra of the chayotextle starch film and the chayotextle starch film functionalized with a MHF are given in figure 2.

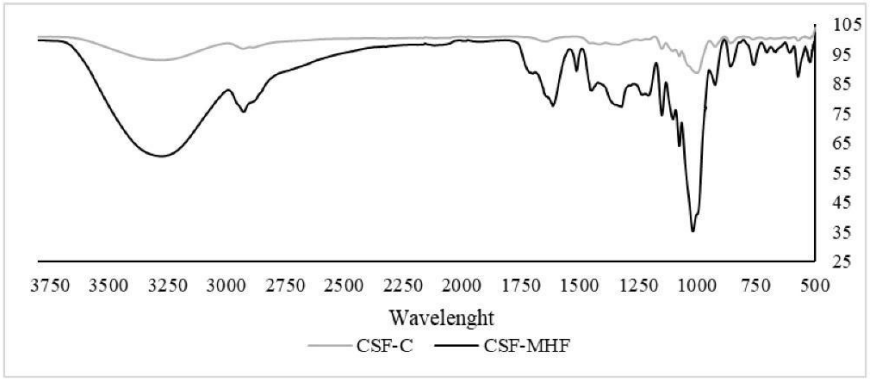


Figure 2. Fourier-transform infrared (FTIR) spectra of starch-based films of the control chayotextle starch-based film (CSF-C) and chayotextle starch-based film functionalized with a multi-herbal formulation (CSF-MHF).

In the O–H stretching region (3750-3000 CM^{-1}), the control film exhibited a broad band corresponding to hydroxyl groups of starch, whereas the functionalized film showed an increase in the intensity of this band, suggesting the formation of hydrogen bonds between chayotextle and the phenolic compounds and terpenes of the multi-herbal formulation.

In the 3000-2800 cm^{-1} region, an increased intensity of the C–H stretching bands was observed, associated with aliphatic structures derived from essential oils incorporated into the matrix. Additionally, in the 1750-1600 cm^{-1} region the CSF-MHF film exhibited a distinct band attributed to carbonyl (C=O) groups from phenolic and aromatic compounds, which was absent in the control film.

The fingerprint region (1200-800 cm^{-1}) of chayotextle starch showed peaks between 1000 and 1150 cm^{-1} (C–O–C) corresponding to carbohydrates rings. In the MHF-enriched film, this band appeared broader, indicating structural rearrangements within the polymeric matrix due to the incorporation of the bioactive components of the formulation.

FTIR spectra further confirmed chemical interactions between the starch matrix and the bioactive compounds.

In the 3750-3000 cm^{-1} region, a broadening and shift of the band was observed in the CSF-MHF film, which is related to the formation of strong hydrogen bonds between starch and phenolic compounds. This interaction results in greater polarization of the O–H bond [35, 36]. This broadening may also be associated with the presence of terpenes, which exhibit a characteristic O–H stretching around 3400 cm^{-1} [37].

The bands at 1614 cm^{-1} are attributed to C=C stretching vibrations of isolated double bonds, characteristic of phenolic compounds and monoterpenes present in plant extracts and essential oils (38, 39). Additionally, the peaks around 1740 cm^{-1}, corresponding to the stretching of the carbonyl group (C=O), are related to esters, ketones, and aldehydes present in essential oils, as well as to flavonoids, tannins, and phenolic acids. This band has proven useful for the differential identification of these compounds [37, 39].

In the fingerprint region (below 1200 cm^{-1}), peaks are generally observed between 1160 and 820 cm^{-1}, which are associated with C–O–C stretching, C–C vibrations, and ring structures characteristic of the main carbohydrate chains. The absorbances at 995 cm^{-1} and 1022 cm^{-1} are commonly used to characterize the structural properties of starch. In our study, the CSF-MHF film showed changes in the definition and intensity of these bands, suggesting a

rearrangement of starch molecules and the formation of interactions between the functional groups of the polysaccharide and the phenolic compounds and terpenes present in the multi-herbal formulation. This evidence structural modifications in the polymeric matrix [35, 36, 38].

3.2 Antibacterial activity

3.2.1 Minimal bactericidal concentration of multi-herbal formulation

According to the obtained results, the multi-herbal formulation exhibited bactericidal activity against at concentrations of 1.15 mg/mL and 12.69 mg/mL against *S. aureus* and *E. coli,* respectively.

In the formulation of antibacterial films, bactericidal activity is desirable to effectively eliminate bacteria, particularly multidrug-resistant strains. The antibacterial efficacy of plant-derived compounds can be enhanced through their combination with non-antibacterial substances, such as starches, since phytochemicals can kill bacteria directly or disrupt cellular functions, thereby reducing the likelihood of antibacterial resistance. Therefore, phytochemicals represent promising candidates for development of novel antibacterial therapies [40, 41].

3.2.2 Zone of inhibition test

According to the statistical analysis, no significant differences were observed between the multi-herbal formulation, and CSF-MHF against *E. coli* ($p=0.0001$), Both treatments were more effective than iodine-based sealant, producing larger inhibition zones (10.00-10.25 mm) compared to the commercial sealant (8.25 mm; Fig 3). The bacterium was classified as

sensitive to all evaluated treatments, except for distilled water and chayotextle starch which were classified as not sensitive.

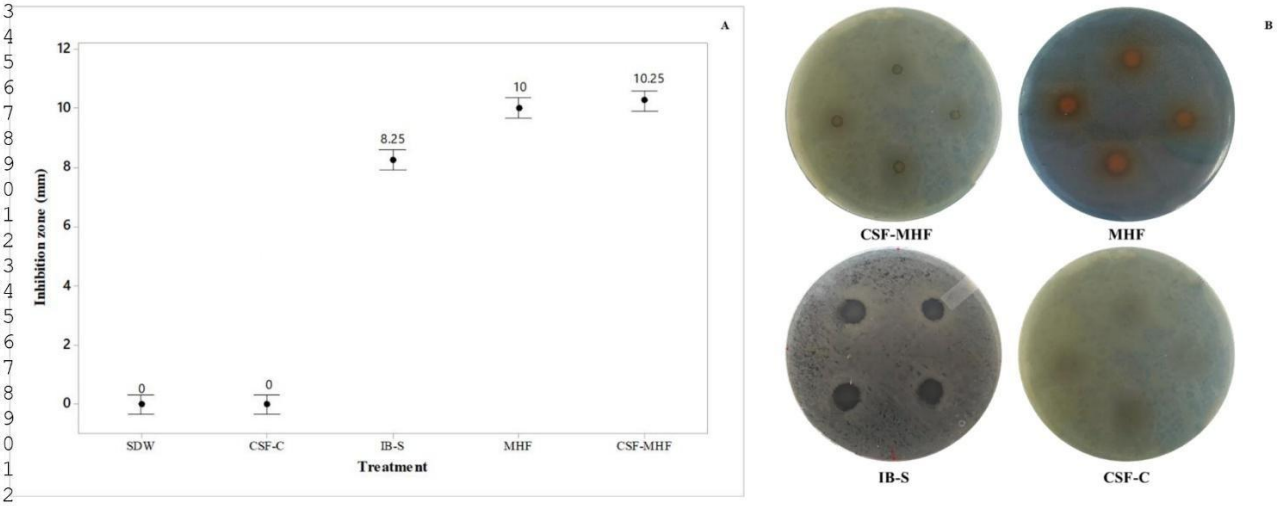


Fig 3. A) 95% confidence interval plot of the evaluated treatments against *E. coli.* The pooled standard deviation was used to calculate the intervals. Different letters indicate significant statistical differences ($p \leq 0.05$) between treatments. B) Inhibition zones of the evaluated treatments against *E. coli.* CSF-MHF, chayotextle starch-based film functionalized with a multi-herbal formulation; CSF-C, chayotextle starch-based film; IB-S, iodine based sealant (positive control); MHF, multi-herbal formulation.

Notably, the biofilm functionalized with MHF (CSF-MHF) exhibited the highest activity against *S. aureus*, producing an inhibition zone of 24.75 mm. In comparison, MHF generated an inhibition zone of 19.57 mm, which statistically was not different to iodine-based sealant. Distilled water and chayotextle starch did not produce any inhibition zones (Fig 4). *S. aureus*

exhibited a graded sensitivity to the treatments tested, classified as very sensitive to the MHF and iodine-based sealant, and extremely sensitive to the CSF-MHF.

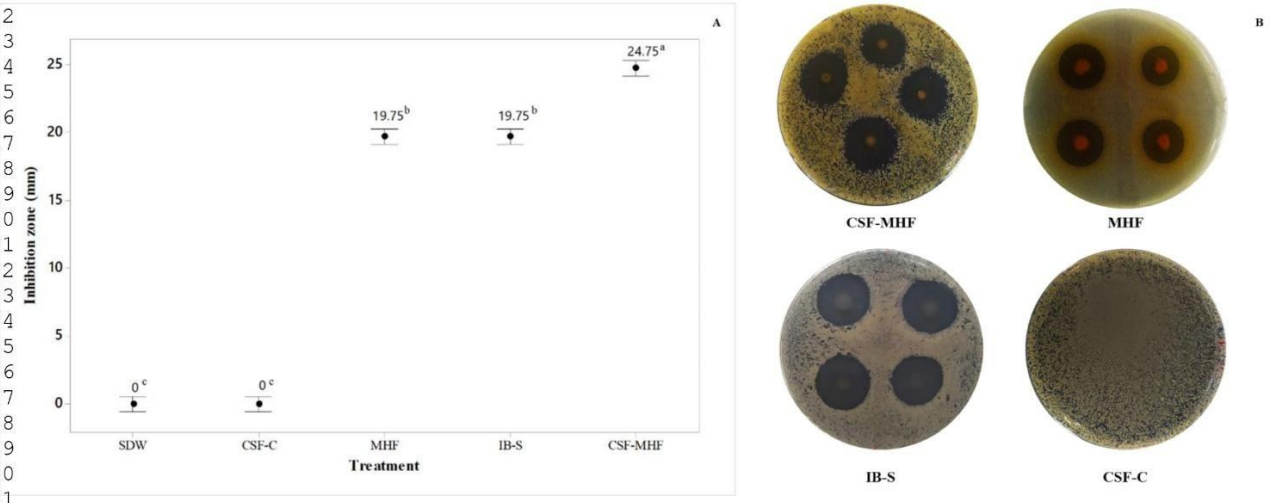


Fig 4. A) 95% confidence interval plot of the evaluated treatments against *S. aureus*. The pooled standard deviation was used to calculate the intervals. Different letters indicate significant statistical differences ($p \leq 0.05$) between treatments. B) Inhibition zones of the evaluated treatments against *S. aureus*. CSF-MHF, chayotextle starch-based film functionalized with a multi-herbal formulation; CSF-C, chayotextle starch-based film; IB-S, iodine based sealant (positive control); MHF, multi-herbal formulation

The incorporation of multi-herbal formulations into natural polymeric matrices, such as starch-based biofilms, represents a highly promising strategy for the addition of antibacterial ingredients.

Previously, Wang *et al*. (2021) reported that the supplementing corn starch-based films with bamboo volatile oil exhibited enhanced their antibacterial activity against Gram positive strains as the concentration of volatile oil increased [42]. Similarly, cassava starch-based films incorporated with geranium essential oil, showed inhibition zones ranging from 99.14 to 126.57 mm^2, exhibiting greater activity against the Gram positive bacteria *S. aureus* and *L. monocytogenes* [7].

In another study, Aaliya *et al*. (2022) evaluated talipot starch films functionalized with different plant extracts (curry tree, neem, tulsi, and Mexican mint) reporting inhibition zones from 18.14–22.13 mm for *S. aureus* and from 17.16–24.83 mm for *E. coli* [43]. Wang *et al*. (2021) observed enhanced antibacterial effects against *S. aureus* and *L. monocytogenes*, Gram positive strains [44].

These findings are consistent with our results, since in the present study, the antibacterial activity of the biofilm also increased significantly after incorporating the multi-herbal formulation into the polymeric matrix, with inhibition zones reaching up to 24.75 mm, particularly against the Gram positive strain.

3.2.3 Bacterial cell count

Table 1 summarizes the antibacterial activity of the evaluated treatments. Except for the CSF-C and the nutrient broth, which exhibited no antibacterial activity and were therefore used as negative controls, all treatments demonstrated antibacterial effects against both bacterial strains. According to the statistical analysis, no significant differences ($p \geq 0.05$) were observed among the CSF-MHF, the MHF, and the iodine-based sealant, as all three treatments achieved 100% inhibition of bacterial growth.

Table 1. Antibacterial activity of chayotextle starch-based film functionalized with a multi-herbal formulation against *E. coli* and S. *aureus*

Treatment	***E. coli***	***S. aureus***
CSF-MHF	100[a]	100[a]
MHF	100[a]	100[a]
IB-S	100[a]	100[a]
CSF-C	0[b]	0[b]
NB-Bact	0[b]	0[b]
p value	0.0001	

CSF-MHF, chayotextle starch-based film functionalized with a multi-herbal formulation; CSF-C, chayotextle starch-based film (control); IB-S, iodine based sealant; MHF, multi-herbal formulation; NB-Bact, nutritive broth inoculated with bacteria. The results represent the average of three determinations. Letters a–b indicate the comparison of means between treatments. Different letters in columns indicate significant statistical differences ($p \leq 0.05$).

3.2.4 Bacterial growth reduction on a physical-structural model

The bacterial growth reduction assay demonstrated that both the iodine-based sealant and CSF-MHF exhibited a significant antibacterial effect, reducing *E. coli* and *S. aureus* cell counts by up to 99.9%, with no statistically significant differences ($p \geq 0.05$) observed between the two treatments (Table 2).

Figure 7 shows the physical structural model used to evaluate antibacterial activity of the chayotextle biofilm functionalized with the MHF. It can be observed that in syringe treated with CSF-MHF formed a larger seal (3.5 mm) inside the tip compared to the commercial sealant (2.0 mm).

Table 2. Percentage of bacterial growth reduction of *E. coli* and *S. aureus* in presence of the chayotextle starch-based film functionalized with a multi-herbal formulation

Treatment	***E. coli***	***S. aureus***
IB-S	99.92%	99.95%
CSF-MHF	99.93%	99.94%
P value	≥ 0.05	

IB-S, iodine-based sealant; CSF-MHF, chayotextle starch-based film functionalized with a multi-herbal formulation

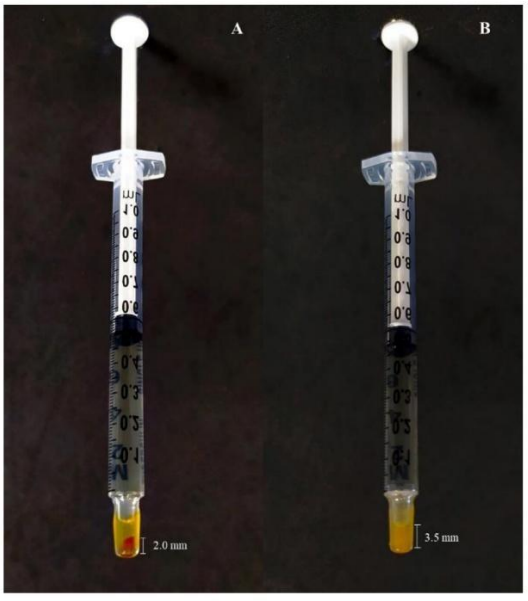


Figure 7. Physical structural model to evaluate antibacterial activity of chayotextle starch-based film functionalized with a multi-herbal formulation. A) Syringe is treated with iodine-based sealant. B) Syringe is treated with CSF-MHF.

The continuous advancement of phytotherapy has underscored the therapeutic potential of multi-herbal formulations, which combine various bioactive compounds for enhanced

therapeutic effects. Trends in pharmaceutical research emphasize the value of a multifunctional approach to the development of these formulations, allowing for more comprehensive and targeted therapeutic strategies. This approach is particularly promising for complex and multifactorial conditions such as bovine mastitis, where conventional treatments may face limitations due to antibacterial resistance and recurrence [45].

On the other hand, natural biopolymers are becoming an increasingly popular choice due to their biocompatibility, biodegradability, and favorable physicochemical properties [46, 47]. Polymers also can act as carrier-assisted delivery of nonantibiotic therapeutic agents, such as antibacterial peptides and plant-derived polyphenols, some authors have been stated that these alternatives can improve the ability to treat antibiotic resistant and recurrent infections, since biomaterials can provide prolonged antibacterial effects at the side of infections, reducing systemic side effects [48, 49].

Caetano *et al*. (2017) reported that films based on cassava starch added with oregano oil and pumpkin residue extract showed antibacterial properties against coliforms and *Salmonella* Enteritidis [50]. Another study determined that the addition of orange essential oil to a commercial corn starch matrix promoted the growth inhibition of the Gram positive strains *S. aureus* and *L. monocytogenes* by up to 66% and 83 %, respectively [51].

Starch chains can interact with phenolic compounds through hydrogen bonds. Moreover, when the hydrogen atom of the hydroxyl group is replaced by other functional groups, modified polysaccharides offer more anchoring sites for drug loading through noncovalent interactions forming intermolecular aggregates making them very promising drug delivery systems since compounds are more stable and bio-accessible [52-56].

Viscous biomaterials such as chayotextle starch can be used in the biomedical field due to their stability in body fluids, their ability to adhere to tissues, and their capacity to release drugs locally and precisely [53].

In our study, we observed a complete (100%) inhibition of bacterial growth of Gram positive and Gram negative strains. This result suggests that the evaluated multi-herbal formulation may offer a better antibacterial effect, likely due to the synergistic action of its combined plant-derived compounds. Additionally, the starch-based matrix appears to have played an important role in enabling the controlled release of these active components [57]. Altogether, our findings point to the potential of this approach to provide more comprehensive and effective antibacterial activity compared to single-agent systems.

4. Conclusion

Taken together, these findings demonstrate that the that the incorporation of the multi-herbal formulation into the chayotextle starch matrix may enhance the antibacterial activity against *E. coli* and *S. aureus* due to structural rearrangements within the polymeric matrix and the formation of interactions between the polysaccharide functional groups and the phenolic compounds and terpenes present in the multi-herbal formulation. These findings highlight the potential of functionalized biopolymers as innovative and sustainable strategies controlling bovine mastitis; however, cytotoxicity assays and mechanism of action studies of the functionalized film are required prior to subsequent *in vivo* evaluations to validate its efficacy under real conditions.

CRediT authorship contribution statement

Ana Lizet Morales-Ubaldo: Writing – original draft, conceptualization, methodology, data curation, formal analysis. Conceptualization, writing – original draft**,** Investigation, resources, methodology. **Aparicio-Burgos José Esteban**: writing – review and editing, formal analysis. **Paula Santana Sepúlveda, Apolonio Vargas-Torres**: Resources, Investigation, methodology. **Adrian Zaragoza-Bastida, Nallely Rivero-Perez**: Conceptualization, writing – original draft**,** Investigation, resources, methodology, writing – review and editing, supervision, formal, analysis, validation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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8. ANEXOS

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Review

Phytochemical Compounds and Pharmacological Properties of *Larrea tridentata*

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Abstract: For centuries, traditional medicine from plants (phytotherapy) was the only treatment for infectious and non-infectious diseases. Although it is still practiced in several countries with excellent therapeutic results, it is frequently underestimated because, unlike Western medicine, it is not based on an empirical scientific foundation. However, interest in the search for plant-based therapeutic resources has been stimulated by disciplines such as phytochemistry and the side effects of conventional pharmacological therapies. For example, *Larrea tridentata* is a perennial shrub used in traditional medicine in northern Mexico and the southern United States to treat infertility, rheumatism, arthritis, colds, diarrhea, skin problems, pain, inflammation and excess body weight. Scientific research has revealed its beneficial effects—antioxidant, antitumor, neuroprotective, regenerative, antibacterial, antiviral, antifungal, anthelmintic, antiprotozoal and insecticidal—although reports indicate that some compounds in *Larrea tridentata* may be hepatotoxic and nephrotoxic. Therefore, the aim of this review was to highlight the updates regarding phytochemical compounds and the pharmacological properties of *Larrea tridentata*.

Keywords: bioactive compounds; pharmacological activities; *Larrea tridentata*

1. Introduction

Larrea tridentata is a perennial shrub of Mexico and the United States that is used to treat a variety of illnesses. This species belongs to the *Zygophyllaceae* family, comprising about 30 genera and 250 species and found mainly in warmer and drier regions (Table 1) [1–3].

L. tridentata is commonly known as chaparral and greasewood in the United States and guamis, fake caper, hediondilla and gobernadora in Mexico. It grows from 0.5 to 3.5 m and has little aroma. The stem has numerous branches with lanceolate green-yellowish leaves. Its flowers are yellow, and the fruit is ovoid with fine white hairs and contains a black seed [2,4].

This plant is well known in both Mexico and the USA for its effectiveness in treating a variety of illness: infertility, rheumatism, arthritis, diabetes, gall and kidney stones, colds, diarrhea, skin problems, overweight, pain and inflammation. It also has uses in industry and as forage. Some studies have centered on its bioactive compounds, mainly to evaluate

its anti-inflammatory, antiviral, antifungal, antibacterial, antioxidant and neuroprotective properties [2,4–8]. The aim of this review was to highlight the updates regarding phytochemical compounds and the pharmacological properties of *Larrea tridentata.*

Table 1. Taxonomic classification of *Larrea tridentata.*

Taxonomy	
Kingdom	*Plantae*
Division	Tracheophyta
Class	Magnoliopsida
Order	Zygophyllales
Family	Zygophyllaceae
Genus	*Larrea*
Species	*Tridentata*

2. Methodology

To carry out the present review, a comprehensive search was performed in the following databases: PubMed, ScienceDirect and Google scholar for studies published from 2010 to 2022, since a previous literature review was published in 2009. The following headings and keywords were used: *Larrea tridentata*, gobernadora, creosote bush, bioactive compounds and biological activities. Duplicate papers were removed, the data were screened, irrelevant work was excluded and full-text documents were then screened. Inclusion criteria included several factors, involving original articles or reviews and work on natural or chemical compounds. Exclusion criteria were inadequate methods and lack of access to the full text.

3. Phytoconstituents

L. tridentata is a species rich in bioactive compounds—tannins, flavonoids, saponins, phytoestrogens and terpenes—and bioactive molecules: ellagic acid, gallic acid, catechins, methyl gallate, cinnamic acid resorcinol, kaempferol, quercetin, nordihydroguaiaretic acid (NDGA), thymol and carvacrol [9–13]. Table 2 shows the main active constituents isolated from *L. tridentata* [14–24].

Table 2. Chemical structure of bioactive molecules isolated from *L. tridentata* from the International Union of Pure and Applied Chemistry (IUPAC).

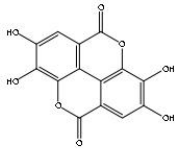
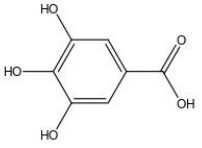
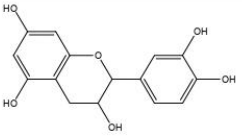
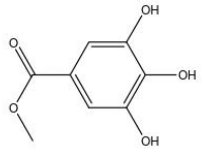
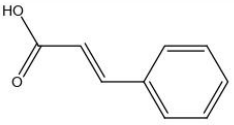
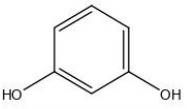
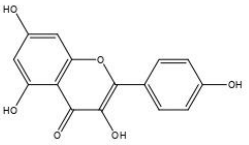
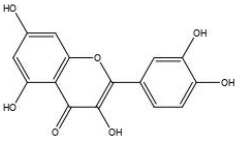
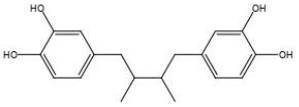
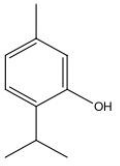
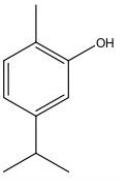
Compound	Class of Compound	IUPAC Name	Chemical Structure
Ellagic acid	organic heterotetracyclic compound, polyphenol	6,7,13,14-tetrahydroxy-2,9-dioxatetracyclo [6.6.2.$0^{4,16}$.$0^{11,15}$]hexadeca-1(15),4,6,8(16),11,13-hexaene-3,10-dione	
Gallic acid	trihydroxybenzoic acid	3,4,5-trihydroxybenzoic acid	
Catechins	Hydroxyflavanoids	2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol	

Table 2. *Cont.*

Compound	Class of Compound	IUPAC Name	Chemical Structure
Methyl gallate	Gallate ester	methyl 3,4,5-trihydroxybenzoate	
Cinnamic acid	Monocarboxylic acid, a styrene	(*E*)-3-phenylprop-2-enoic acid	
Resorcinol	Benzenediol	benzene-1,3-diol	
Kaempferol	Flavonol (tetrahydroxyflavone)	3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one	
Quercetin	Flavonoid	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	
Nordihydroguaiaretic acid (NDGA)	Lignan	4-[4-(3,4-dihydroxyphenyl)-2,3-dimethylbutyl]benzene-1,2-diol	
Thymol	Monoterpene	5-methyl-2-propan-2-ylphenol	
Carvacrol	Monoterpene	2-methyl-5-propan-2-ylphenol	

Isolation of New Compounds

In recent years, many studies have centered on elucidating new compounds. Jitsuno and Mimaki (2010) [25] performed a study that isolated 13 new compounds, identified as triterpene glycosides from the aerial parts of *L. tridentata*. For their part, Yokosuka et al. (2011) [26] isolated two new lignan glycosides called larrealignans. In a study carried out by Favela-

Hernández et al. (2012) [27], a furanoid lignan from the leaves of *Larrea tridentata*, 4-epilarreatricin, was isolated. Schmidt et al. (2012) [28], isolated nine lignans (dibenzylbutanes, epoxylignans and aryltetralins), six flavonoids and one ester.

Recently, two new cyclolignans were elucidated as 4,4′-dihydroxy-3-methoxy-6,7′-cyclolignan and 3,4-dihydroxy-3′,4′-dimethoxy-6,7′-cyclolignan [29]. Table 3 summarizes some of the new isolated compounds.

Table 3. Isolated compounds from *Larrea tridentata*.

Organ-Extract	Compound	Class of Compound
Aerial parts, methanolic extract	3-[(*O*-(4-*O*-sulfo-b-D-glucopyranosyl)-(1→3)-a-L-arabinopyranosyl) oxy]olean-12-en-28-oic acid b-D-glucopyranosyl ester sodium salt 3-[(*O*-(4-*O*-sulfob-D-glucopyranosyl)-(1→3)-*O*-[a-L-rhamnopyranosyl-(1→2)]-a-Larabinopyranosyl)oxy]-30-noroleana-12,20(29)-dien-28-oic acid b-D-glucopyranosyl ester sodium salt	Triterpene glycosides
Aerial parts, methanolic extract	Larrealignans A and B	Lignans
Leaves, chloroformic extract	dihydroguaiaretic acid, 4-epilarreatricin, 3′-demethoxy-6-Odemethylisoguaiacin,	Lignans
Leaves, chloroformic extract	5,4′-dihydroxy-3,7,8,3′-tetramethoxyflavone 5,4′-dihydroxy-3,7,8-trimethoxyflavone 5,4′-dihydroxy-7-methoxyflavone 5,8,4′-trihydroxy-3,7-dimethoxyflavone	Flavonoids
Aerial parts, dichloromethane extract	3,4-dehydrolarreatricin meso-dihydroguaiaretic acid 3-*O*-methyldihydroguaiaretic acid 3-*O*-demethylisoguaiacin	Lignans
Aerial parts, dichloromethane extract	3′-oxohexyl ferulate	Ferulic acid ester
Aerial parts, dichloromethane extract	Naringenin 3′-*O*-methyltaxifolin apigenin-7-methylether Kaempferol-3,7-dimethylether herbacetin-3,7-dimethylether	Flavonoids
Leaves, hexane extract	4,4′-dihydroxy-3-methoxy-6,7′-cyclolignan 3,4-dihydroxy-3′,4′-dimethoxy-6,7′-cyclolignan	Cyclolignans

4. Pharmacological Activities

4.1. Antioxidant Activity

Antioxidant compounds are widely distributed in the plant kingdom, and in this regard, Martins et al. in 2010 evaluated the antioxidant capacity of *L. tridentata* through ferric reducing/antioxidant power (FRAP) and free radical-scavenging capacity techniques. They used 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assays, and the data showed high antioxidant activity, which was attributed to elevated concentration of phenolic compounds and NDGA [30].

In a study performed by Rahman et al. (2011), the modulatory effects of *L. tridentata* and its associated compound NDGA were studied on acute inflammatory and oxidative stress responses in mouse skin induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). They determined that pre-treatment with NDGA before the TPA application mitigated cutaneous lipid peroxidation and inhibited production of hydrogen peroxide. In addition, glutathione levels and antioxidant enzymes were restored, and the activity of myeloperoxidase and xanthine oxidase as well as skin edema formation were lowered [31].

In 2018, Aguirre-Joya et al. reported antioxidant capacity after evaluating $ABTS^{\cdot+}$ radical cation-scavenging activity assay, DPPH, lipid oxidation inhibition (LOI) and FRAP. The antioxidants identified were NDGA, quercetin and kaempferol [32].

Skouta et al. (2018) determined the antioxidant activity of three different extracts of *L. tridentata* (ethanol, ethanol–water and water), through DPPH, ABTS, superoxide, FRAP activity and nitric oxide (NO) assays, determining that ethanol–water (60:40) extract had the most efficient antioxidant properties, with values of 111.7 ± 3.8 μg/mL (DPPH), 8.49 ± 2.28 μg/mL (ABTS), 0.43 ± 0.17 μg/mL (superoxide) and 230.4 ± 130.4 μg/mL (NO). In addition, nine compounds were identified with antioxidant properties, among which were justicidin B and beta peltain [33].

Morán-Santibañez et al. (2019) reported that an ethanol–water extract from *L. tridentata* leaves mitigates cytotoxicity caused by oxidative stress in human cells. In addition, because of its cytoprotective activity against oxidative stress, the extract reduced the levels of different apoptosis hallmarks, thereby showing it to be a natural anti-apoptotic [34].

4.2. Antitumor Activity

The main compound of *Larrea tridentata* (NDGA) showed antitumor effects in bladder T24 cancer cells in vitro. The reactive oxygen species (ROS) levels were evaluated, and after 72 h of incubation, NDGA had reduced T24 cell viability in a dose-dependent manner. Apoptosis also increased at 48 h, and a dose of 20 μM of NDGA promoted mitochondrial stress by inducing oxygen consumption alterations just as in cancer cell death. This suggested that the antitumor effects of NDGA in T24 cells were related to its ability to induce mitochondrial alteration [35].

Probst et al. (2017) reported that lipoxygenase (LOX) inhibitors, such as NDGA, protect acute lymphoblastic leukemia (ALL) cells from RSL3-stimulated lipid peroxidation, reactive oxygen species generation (ROS) and cell death [36].

4.3. Neuroprotective Effects

NDGA has shown protective effects in the acute phase of stroke. In a 2014 transient ischemia rat model study carried out by Zhang et al., NDGA promoted neurogenesis and angiogenesis after 28 days of ischemia and reperfusion by suppressing semaphorin 3A expression [37].

Some studies have also centered on the use of natural products to treat neurodegenerative disorders, such as Alzheimer´s disease (AD) [38]. In this regard, Siddique and Ali (2017) evaluated the effect of NDGA on transgenic *Drosophila* expressing wild-type human Aβ-42 in the brain. Data showed that exposure to doses of 20, 40, 60 and 80 μM of NDGA reduced symptoms, increased life span, delayed the loss of climbing activity and showed a dose-dependent decrease in the activity of caspase 3 and 9 and acetylcholinesterase, which suggested an anti-apoptotic and neuroprotective role. Furthermore, NDGA improved memory loss in flies with AD, demonstrating that this compound reduced neurotoxic, motor and cognitive impairments [39].

4.4. Regenerative Applications

Larrea tridentata and its pure compounds have been widely used in different fields, including tissue engineering. Tovar-Carrillo et al. (2020) analyzed the compatibility of the in vitro and in vivo properties of cellulose hydrogels enriched with *L. tridentata* that had been implanted intramuscularly in female rats. At the end of the in vivo assay (90 days), no evidence of inflammation, toxicity or death was observed; furthermore, it was observed that the addition of *L. tridentata* improved cytocompatibility, demonstrating that enriched hydrogels can be used as regenerative scaffolds [40].

4.5. Hepatoprotective Effect

Del Vecchyo-Tenorio et al. (2016) stated that ethanolic extract of *L. tridentata* is useful in metabolic syndrome (MS) treatment since it was reported that the addition of the extract

in a high fat and cholesterol diet (HFD) in hamsters with signs of MS reduced plasma triglycerides, total cholesterol, insulin and leptin and improved insulin sensitivity. On the other hand, in a standard diet enriched with the same extract, the effects were higher since reduced body and liver weight, glucose concentration, cholesterol, insulin and leptin in serum were increased in addition to insulin sensitivity. According to the authors, these effects were associated with lower lipid peroxidation and increased antioxidant capacity in the liver [41].

Chan et al. (2018), in a murine model with liver injury produced by the American Lifestyle-Induced Obesity Syndrome diet (ALIOS), demonstrated that the coadminstration of NDGA reduced body and epididymal fat weight and levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and triglycerides in the liver. It also improved insulin sensitivity because this compound induced the activation of PPARα, a regulator of fatty acid oxidation, and the mRNA of Cpt1c and Cpt2, genes involved in fatty acid oxidation; furthermore, the NDGA reduced liver stress and the expression of (CASP3), an apoptosis signaling protein, and improved the hepatic expression of antioxidant enzymes and the proteins GPX4 and PRDX3 [42].

In a similar study, Han et al. (2019) evaluated the effect of NDGA (2.5 g/kg of diet) on mice fed a diet high in trans-fat, cholesterol and fructose (HTF) for 16 weeks. The NDGA reduced body and liver weight and the liver-to-body weight ratio in HTF-fed mice also decreased non-esterified fatty acids and serum insulin. The results suggested that NDGA could mitigate liver damage and the accumulation of triglycerides. A glucose tolerance test revealed that mice treated with NDGA showed lower levels of glucose, steatosis and fibrosis. Furthermore, this compound increased fatty acid oxidation and reduced both ER and oxidative stress [43].

4.6. Renal Effects

Some studies have evaluated the renal effects of the major compound of *L. tridentata*. Zuntilde et al. (2012) reported that NDGA prevented renal dysfunction, histological damage and oxidative stress, as well as decreasing the activity of the renal antioxidant enzymes glutathione peroxidase, glutathione reductase, glutathione-S-transferase and catalase. It also affected mitochondrial activity, which is why NDGA was considered to be nephroprotective [44].

Zúñiga-Toalá et al. (2013) [45] reported that pretreatment with NDGA had a protective effect on ischemia–reperfusion renal (I/R) damage. It attenuated tubular epithelium damage since this compound induced, in vivo and in vitro, nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation in rats that had a uni-nephrectomy and I-R damage and apoptosis. The authors of this study suggest that the indirect antioxidant effect of NDGA may have been involved in the cytoprotective effect of the I-R injury, and previous studies support this result. Rojo et al. (2012) reported that NDGA increased the level of the Nrf2 protein and expression of heme oxygenase-1 (HO-1) in kidney cells through the activation of multiple signaling cascades [46].

4.7. Anti-Inflammatory Activity

In an in vivo study carried out by Rahman et al. (2011), mice treated with NDGA (15 and 25 µmol) before a double application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), showed significantly reduced activity of myeloperoxidase, one of the main enzymes related with polymorphonuclear (PMN) activation. It was also observed that animals treated with this compound showed a lower edema response compared with those treated only with TPA. Histological findings showed that the TPA application caused an increase in the epidermal layer, the infiltration of polymorphonuclears (PMNs) and intercellular edema in the skin. It also caused inflammatory responses in the tissue in contrast to those animals that had been pretreated and treated with NDGA, which mitigated inflammation and any histological change [31].

Xue et al. (2013) evaluated the in vivo anti-inflammatory effects of NDGA in spinal cord injury (SCI). Myeloperoxidase (MPO) levels were measured after 3 days of the SCI process, and the results showed that NDGA reduced neutrophil infiltration after injury and infiltration of macrophages–microglia. In this study, NDGA decreased inflammatory factors (IL-1β and TNF-α) associated with spinal cord damage [47].

4.8. Growth Performance

García-López et al. (2018) evaluated the effects of the dietary addition of whole plant, leaves and powdered aqueous extract of *L. tridentata* on the growth, organ weight and serum hepatic enzymes of Cobb broiler chickens. The treatments were added to a basal diet and randomly assigned to 200 Cobb broilers one day old. The authors concluded that those fed the *L. tridentata* aqueous extract had a better performance response; furthermore, the decrease in enzyme hepatic levels means that the extract could be considered a natural growth promoter [48].

In a similar study, dried aerial parts of *L. tridentata* were added to a sheep diet at a rate of 0, 5 and 10% over 60 days. An analysis indicated that the aerial parts contained 85% dry matter, 12% crude protein, 58% neutral detergent fiber and 10% ash, which was similar to hay or conventional silage. The pH values were similar to that of the control group. Despite the data in feed efficiency, there was no significant statistical difference between diets with and without the *L. tridentata* biomass. The study concluded that inclusion of *L. tridentata* in the diet may be suitable for finishing [49].

4.9. Hypoglycemic Effects

Roškar et al. (2016), reported that NDGA demonstrated antidiabetic activity in vivo since this compound inhibited α-amylase, α-glucosidase and dipeptidyl peptidase 4, enzymes associated with postprandial glucose management [50]. The pharmacological activities of *L. tridentata* and its related bioactive compounds are summarized in Table 4.

Table 4. Pharmacological activities of *L. tridentata* its related compounds and mechanism of action.

Activities	Bioactive Compounds	Mechanism of Action	Reference
Antioxidant	NDGA, Quercetin, Kaempferol, Justicidin B and Beta peltain	Mitigation of cutaneous lipid peroxidation and cytotoxicity, inhibition of production of hydrogen peroxide and edema formation, reduction of apoptosis hallmarks	[30–34]
Antitumor	NDGA	Induction of mitochondrial alterations, ferroptosis.	[35,36]
Neuroprotective	NDGA	Promotion of neurogenesis and angiogenesis, anti-apoptotic, reduction of the neurotoxic, motor and cognitive impairments of Alzheimer´s disease	[37–39]
Regenerative	Not indicated	Inhibition of inflammation or toxicity	[40]
Hepatoprotective	NDGA	Lower lipid peroxidation, increase in antioxidant capacity in the liver	[41–43]
Renal effects	NDGA	Decreasing the activity of renal antioxidant enzymes, affection of mitochondrial activities.	[44–46]
Anti-inflammatory	NDGA	Reduction in myeloperoxidase activity, reduced edema response, decrease of inflammatory factors	[31,47]
Hypoglycemic	NDGA	Inhibition of α-amylase, α-glucosidase and dipeptidyl peptidase 4	[50]

4.10. Antibacterial Activity

Seven compounds from the chloroformic *L. tridentata* extract were isolated and identified: dihydroguaiaretic acid; 4-epi-larreatricin and 3′-demethoxy-6-*O*-demethylisoguaiacin (lignans) and 5,4′-dihydroxy-3,7,8,3-tetramethoxyflavone, 5,4′-dihydroxy-3,7,8-trimetho-

xyflavone, 5,4′-dihydroxy-7-methoxyflavone and 5,8,4′-trihydroxy-3,7-dimethoxyflavone (flavonoids). All of these were evaluated through the determination of minimal inhibitory concentration (MIC) against Gram-negative (*Stenotrophomona maltophilia*, *Escherichia coli*, *Acinobacter baumannii*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter cloacae*) and Gram-positive (*Staphylococcus aureus*, *S. aureus* (MR), *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Enterococcus faecalis*) bacteria. The results showed that six of the compounds had antibacterial activity in a range of concentrations from 12.5 to >50 µg/mL, with the most active compound being 3′-demethoxy-6-*O*-demethylisoguaiacin. It showed antibacterial activity against all evaluated bacteria; therefore, it was evaluated against clinical isolates of *E. faecalis*, *S. aureus* and *S. aureus* (MR) and attained MIC values from 12.5 to 50 µg/mL. The mechanism of action of this compound affected the proteins of the ATP-binding cassette (ABC) transport system, thereby causing bacteria death [27,51].

In a study performed by Mendez et al. (2012) [52], different *L. tridentata* leaf extracts (water, ethanol, cocoa butter and lanolin) were evaluated against *E. aerogenes*, *E. coli*, *S. typhi* and *S. aureus*. The results showed that ethanolic extract had the highest growth inhibitory effects on *E. coli* and *S. aureus*. Snowden et al. (2014) evaluated *L. tridentata* leaf and flower extracts against *S. aureus* and obtained an MIC of 60 µg/mL, demonstrating that the extracts had bacteriostatic and bactericidal activity [53].

For their part, Martins et al. (2013) [54] evaluated the antibacterial activity of *L. tridentata* crude methanolic (CME) extract, hexane (H), dichloromethane (DCM), ethyl acetate (EA) and ethanol (Et) fractions and the compound NDGA. Antibacterial activity was determined through agar diffusion, and the results showed that CME, DCM, EA and NDGA were active against Gram-positive bacteria (*S. aureus*, *S. aureus* methicillin-resistant (MRSA), *Staphylococcus saprophyticus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*). An MIC from 31.3 to 125 µg/mL was obtained for the EA fraction, the most active, and 31.3 µg/mL for MRSA, the most sensitive bacterium, which was at a concentration lower than the reference antibiotic, tetracycline (64 µg/mL). In addition, the authors identified three bioactive compounds: quercetin, kaempferol and NDGA, all of which had reported antibacterial activity [54].

The combination of NDGA and conventional antibiotics (gentamicin, neomycin and tobramycin) showed synergistic activity (97–100%) against 200 clinical isolations of methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). In addition, when the MIC values of these combinations were determined, all antibiotics were reduced 2 to 128-fold against MSSA and 2 to 256-fold against MRSA. Moreover in the time-kill assay, NDGA improved the effect of three antibiotics in in vitro and in vivo murine models. According to the authors, the enhancement of antibiotic efficacy was due to the ability of NDGA to permeabilize bacterial membranes [55].

The antibacterial effects of thymol and carvacrol in the *L. tridentata* ethanolic extract were demonstrated in a study carried out by Delgadillo-Ruiz et al. (2017) [12]. Meso-dihydroguaiaretic acid derivatives (esters, ethers and amino-ethers) were tested against Gram-positive and Gram-negative drug-resistant bacterial strains, showing that Gram-positive bacteria (MR *S. aureus*, VR *E. faecium*, LR *S. epidermis* and LR *S. haemolyticus*) were more sensitive, and even two amino-ethers were more active than levofloxacin [56].

A direct comparison of the antibacterial activity against nonantibiotic-resistant *S. aureus* and two different strains of antibiotic-resistant *S. aureus* was performed by Gerstel et al. (2018) [57]. They determined an MIC range of 0.35–15 µg/mL for *L. tridentata* extract. In 2019, Itza-Ortiz stated that *L. tridentata* extract at 30% generated bacterial growth inhibition halos (BGIHs) against a wide range of Gram-negative and Gram-positive bacteria (0.67–1.73 mm). The extract was the most active against *S. aureus* and *S. enterica* when the BGIHs were 1.73 and 1.57 mm, respectively [58].

From the chloroform extract of *L. tridentata*, the compounds 4,4′-dihydroxy-3-methoxy-6,7′-cyclolignan, 3,4-dihydroxy-3′,4′-dimethoxy-6,7′-cyclolignan, meso-dihydroguaiaretic acid, 3′-demethoxyisoguaiacin, 3′-demethoxy-6-*O*-demethylisoguaiacin, nordihydroguaiaretic acid, 5,4′-dihydroxy-3,7,8-trimethoxyflavone and 5,8,4′-trihydroxy-3,7-dimethoxy-

flavone were isolated. These were active against nine multidrug-resistant clinical isolates at concentrations from 6.25 to >50 μg/mL [29]. In a further study, seven amino-ether derivatives from lignan 4,4′-dihydroxy-3-methoxy-6,7′-cyclolignan exhibited antibacterial activity against Gram-positive bacteria [59].

Turner et al. (2021) determined that the ethanolic extract of *L. tridentata* showed bactericidal activity against *S. aureus* (20 μg/mL), *S. pyogenes* (30 μg/mL), *B. cereus* (120 μg/mL), *E. coli and P. aeruginosa* (>1000 μg/mL); moreover, the authors determined that *L. tridentata* extract enhanced the activity of some β-lactam antibiotics, suggesting the presence of a β-lactam-type antibiotic in the extract [60].

Recently, Morales-Ubaldo et al. (2022) evaluated the antibacterial activity of a hydroalcoholic extract, fractions (aqueous and ethyl acetate) and subfractions from organic fractions, all of which were derived from *L. tridentata* aerial parts. When measured against the reference and multidrug-resistant bacterial strains associated with bovine mastitis, the data showed that the antibacterial activity of *L. tridentata* was associated with the pure compound nor 3′-demethoxyisoguaiacin, which exhibited the highest bactericidal effects [61].

4.11. Antimycobacterial Activity

In 2018, according to the World Health Organization (WHO), 1.5 million people died from tuberculosis (TB), one of the top 10 causes of death; moreover, multidrug-resistant TB (MDR-TB) represents a public health threat [62].

In this respect, studies have centered on the search for agents capable of acting against this bacteria. Favela-Hernández et al. (2012) tested seven compounds against both sensitive and MDR *Mycobacterium tuberculosis* strains and obtained an MIC of 12.5 to >50 μg/mL. The compounds responsible for this activity were dihydroguaiaretic acid 4-epi-larreatricin, 3′-demethoxy-6-*O*-demethylisoguaiacin, 5,4′-dihydroxy-3,7,8,3-tetramethoxyflavone and 5,4′-dihydroxy-3,7,8-trimethoxyflavone, [27].

In 2014, Clemente-Soto et al. found that a concentration of 50 μg/mL of meso-dihydroguaiaretic acid (MDGA) inhibited bacterial growth after 48 h [63]; in a similar study, Reyes-Melo et al. (2017) [56] found that MDGA derivatives affected sensitive and multidrug-resistant *M. tuberculosis* strains; MIC values ranged from 3.125 to 50 μg/mL. Furthermore, the authors determined that this compound had no cytotoxic effects. Guzmán-Beltrán et al. (2016) determined that a concentration of 250 μg/mL of NDGA exerted bactericidal activity [64].

The study carried out by Nuñez-Mojica et al. (2021) determined that eight compounds isolated from *L. tridentata* leaves exhibited activity against a susceptible and drug-resistant *M. tuberculosis* strain, and in a further study, amino-ether derivatives from lignan 4,4′-dihydroxy-3-methoxy-6,7′-cyclolignan exhibited antitubercular activity. The most active the compound against the multidrug-resistant *M. tuberculosis* strain was identified as 4C (6.25 μg/mL) [29,59].

4.12. Antiviral Activity

The in vitro antiviral activity of the methylated derivative of NDGA, terameprocol (TMP), was tested to determine if it could inhibit poxvirus (CPXV) growth. The authors performed CPXV plaque-reduction assays containing varied concentrations of TMP: 3.125, 6.25 and 12.5 μM. The results showed a dose-dependent decrease in CPXV plaque size and a reduction in the total number of plaques that could be detected. It was reported that the compound inhibited poxvirus growth in vitro by preventing the efficient spread of virus particles from cell to cell [65].

4.13. Antiprotozoal Activity

In the study carried out by Schmidt et al. (2012), dichloromethane extract from aerial parts of *L. tridentata* was used for antiprotozoal screening against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*, which had

IC_{50} values of 2.8, 14.6, 5.2 and 2.9 μg/mL, respectively. Nine lignans, six flavonoids and one ester of ferulic acid were isolated and evaluated. Lignan meso-nordihydroguaiaretic acid showed the majority of activity obtaining IC_{50} of 4.5, 33.1, 12.0 and 7.7 μM against the above-mentioned parasites, respectively [28].

In their study, Camacho-Corona et al. (2015), evaluated the organic extracts of six plants, including *L. tridentata* against *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis* and obtained IC_{50} values of 100, 116 and 118 μg/mL, respectively [66].

It was also reported that *Entamoeba histolytica*, *Giardia lamblia*, and *Naegleria fowleri* are susceptible to six lignan compounds from *L. tridentata.* Compound 1 (NDGA) showed the highest activity against *G. lamblia* and *N. fowleri* (EC_{50} 36 and 37 μM, respectively), and moderate activity against *E. histolytica (*EC_{50} =103 μM); compound 2 (3′-*O*-methyl-NDGA) showed similar results to compound 1 against *N. fowleri* (EC_{50} 38 μM). In both cases these results were better than the standard drug (miltefosine EC_{50} = 54.5 μM). The other compounds showed activity from 49 to 235 μM. In addition, the authors suggested that the activity of compounds 1 and 2 against *N. fowleri* may be due to the modulation of cysteine protease activity in the trophozoites [67].

4.14. Anthelmintic Activity

Regarding parasitic infections in sheep, the anti-*Haemonchus contortus* properties of *L. tridentata* were reported by García et al. (2018). Sheathed and unsheathed worm larvae of *H. contortus* were incubated with hydro-methanolic extract at concentrations of 12.5, 25, 50, 100 and 200 mg/mL during 24, 48 and 72 h. At the highest concentration, the extract showed weak activity against sheathed larvae (30% mortality), but against unsheathed larvae activity increased to 70%; moreover, the authors found that the compounds identified in their study damaged the larval cuticle and that the worms coiled up and were lethargic [68].

4.15. Antifungal and Antibacterial Activity in Agricultural Crops

L. tridentata-lanolin, cocoa butter and water extracts were evaluated against *Rhizoctonia solani*, an agent of diseases associated with roots and tubers of different crops. The data showed that *L. tridentata*-lanolin extract at 500 ppm of total tannins inhibited 80% of mycelia, but when the tannins were increased to 2000 ppm, 100% inhibition was obtained. Cocoa butter extract required a concentration of 3000 ppm of total tannins to obtain the same inhibition percentage (100%), and water extract required 8000 ppm [69].

In the 2010 study carried out by Osorio et al., polyphenolic extract from *L. tridentata* leaves was evaluated against *Pythium* spp., *Colletotrichum truncatum*, *Colletotrichum coccodes*, *Alternaria. alternata*, *Fusarium verticillioides*, *Fusarium solani*, *Fusarium sambucinum* and *Rhizoctonia solani*, which are all associated with leaf and root diseases. Strong fungicidal activity was observed since the extract inhibited 100% of the fungal strains, except for *F. verticillioides*, for which inhibition was 75%; moreover, the extract was evaluated against 10 different single-spore isolates of *Fusarium oxysporum*. *L. tridentata* inhibited eight of them 100%, and one 75% [70].

Chávez-Solíz et al. (2014) reported that *L. tridentata* leaf extracts at concentrations of 1000 and 5000 mg/L of water, significantly reduced the severity of *Podosphaera xanthii*, one of the causative agents of powdery melon mildew [71]. Galván et al. (2014) reported that the best antifungal activity of *L. tridentata* aqueous extract (10 and 20%) was against *Phytophthora capsici* and *Aspergillus flavus*. After 48, 72 and 96 h, the two extract concentrations caused 100%inhibition in both fungal species [72].

L. tridentata leaf extract alone or in combination with potassium sorbate had positive effects against *A. flavus* in pH conditions 3, 4 and 5. Inhibited growth of 71.91, 69.33 and 70.06% (pH 3, 4 and 5, respectively) was achieved at a concentration of 1000 ppm. It increased to 81.48, 82.82 and 81.43% when the potassium sorbate was added in the same respective pH conditions. The authors determined that, together, both compounds demonstrated synergistic activity [73].

The in vitro antifungal activity of *L. tridentata* water, ethanol, lanolin and cocoa butter extracts against *Phytophthora cinnamomi* Rands was evaluated in a 2015 study by Castillo-Reyes et al. The data showed that ethanol extract caused 100% mycelium inhibition, and lanolin extract caused 80%. When MIC_{50} was determined, a concentration of 6.96 ppm was needed to inhibit 50% of mycelia growth; for lanolin extract, it was 183.6 ppm [74].

Peñuelas-Rubio et al. (2015) [75] reported that *L. tridentata* ethanolic and dichloromethane extracts inhibited fungal growth 75–100% against *Alternaria tenuissima*, *Aspergillus niger*, *Penicillium polonicum* and *Rhizopus oryzae*. In a similar study, ethanolic and dichloromethane extracts inhibited *Fusarium oxysporum radicis-lycopersici* 100%, while methanolic extract achieved 94% [76].

Aguirre-Joya et al. (2018) reported high fungistatic activity by using a bioactive film containing *L. tridentata* polyphenols, which achieved MIC_{50} values of 566, 558, 612 and 579 ppm for *Alternaria alternata*, *Fusarium oxysporum*, *Botrytis cinereal* and *Colletotrichum gloeosporioides*, respectively [32].

Recently, Morales-Ubaldo et al. (2021), reported the antibacterial activity of a hydroalcoholic extract and ethyl acetate fraction of *L. tridentata*, against multidrug-resistant phytopathogenic bacteria (*Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* and *Xanthomonas campestris*). The authors found that the extract showed inhibitory activity at concentrations of 0.39–6.25 mg/mL and bactericidal effects at 0.78–12.5 mg/mL. The concentrations of ethyl acetate fraction were 0.39–3.12 and 0.78–6.25 mg/mL for MIC and MBC, respectively. According to the authors, their study is the first to report the antibacterial activity of *L. tridentata* against multidrug-resistant phytopathogenic bacteria [77].

For their part, Méndez-Andrade et al. (2021) used an aqueous extract from *L. tridentata* leaves as a source for reducing and stabilizing agents to obtain silver nanoparticles, which exert bactericidal activity against *Clavibacter michiganensis*. The authors reported that, at a concentration of 50 mg/L, disease incidence did not exceed 20%, and disease severity was reduced by 36% [78].

4.16. Insecticidal Activity

Pecan black aphids (*Melanocallis caryaefoliae* D.) were exposed to *L. tridentata* (stem and leaf) ethyl acetate, methanol, and water extracts at concentrations of 0.5, 1 and 2%. The authors determined that leaf extracts at 1% concentration showed 80 and 92% mortality in the aqueous and methanol extracts, respectively. Regarding stem extracts, the best effect was obtained when ethyl acetate and aqueous extracts (0.5%) were used. For all extracts, the greatest effect occurred 72 h after the treatment was applied. In addition, the repellent effect was evaluated and showed the best effects after 24 h: 65% repellence from 0.5% methanolic leaf extract; however, 1% ethyl acetate stem extract showed the best activity, repelling 50% of the pecan black aphids [79].

A similar study reported that 20% *L. tridentata* leaf extract reduced the incidence of horn flies (*Haematobia irritans*) on cows [80]. The larvicidal effect of *L. tridentata* extract (1 g/L) on mosquitoes was approximately 50% mortality (L3) [81]. Some of the pharmacological effects of *L. tridentata* related to infectious diseases are shown in Table 5.

Table 5. Pharmacological activities of *Larrea tridentata* related to infectious diseases.

Activities	Bioactive Compound	Mechanism of Action	Reference
Antibacterial activity	Several bioactive Compounds	Affecting proteins of ABC transport system causing bacteria death, bacterial retardation, bacteriostatic, permeabilizing membrane	[12,27,51–56,58–61]
Antimycobacterial activity	Lignans, flavonoids, meso-dihydroguaiaretic acid, NDGA	Growth inhibition, bactericidal	[27,55,58,59,64]

Table 5. *Cont.*

Activities	Bioactive Compound	Mechanism of Action	Reference
Antiviral activity	Terameprocol (TMP)	Inhibition of poxvirus growth	[65]
Antiprotozoal activity	Several compounds	Modulation of cysteine protease activity present in the trophozoites	[28,66,67]
Anthelmintic activity	Hydro methanolic Extracts	Damaging larvae cuticle, coiling up of worms and lethargic movements	[68]
Antifungal activity	Tannins, polyphenolic extracts	Fungi-static and fungicidal effects	[31,69–76]
Insecticidal activity	Different extracts	Repellent effect, causing death of mosquito larvae	[79,81]

5. Side Effects

There are reports stating that the products of *L. tridentata* may be associated with jaundice, cholestatic hepatitis and liver damage, which then progresses to cirrhosis and even fulminant liver failure. In addition, the main compound, NDGA, causes hepatotoxicity and nephrotoxicity in humans and death in mice (LD_{50} = 75 mg/kg). Contact dermatitis has also been attributed to *L. tridentata* [8,82–86].

6. Discussion and Future Prospects

L. tridentata has traditionally been used to treat a variety of diseases. In recent years several investigations have demonstrated the pharmacological properties of this botanical species, especially its antimicrobial activity. Before determining its effects in vitro on bacteria (human, animal, or plant), parasites, fungi and viruses, it is necessary to perform in vivo or in situ tests to support the efficacy of *L. tridentata* as an effective alternative method of treatment. In the same sense, further studies are needed to establish new strategies to improve its pharmacological properties and phytochemical content, such as the study by Nuñez-Mojica et al. (2022), in which the derivation of a known compound from *L. tridentata* led to 11 new antibacterial compounds [59]. In the same sense, is necessary to establish a dose-response relationship for extracts, fractions or pure compounds associated with their toxicological profile and their mechanism of action.

7. Conclusions

This review examined the pharmacological effects of *L. tridentata*, commonly known as gobernadora in Mexico and creosote bush in the USA. It was found that the aerial parts of *L. tridentata* are of great importance in both traditional medicine and pharmaceuticals for treatment of infectious and non-infectious diseases because of their antioxidant, neuroprotective, antitumoral, anti-inflammatory, regenerative, antifungal, insecticidal, anthelmintic, antiprotozoal and antibacterial activities. These are associated with such bioactive compounds as ellagic acid, gallic acid, catechins, methyl gallate, cinnamic acid resorcinol, kaempferol, quercetin, nordihydroguaiaretic acid (NDGA), thymol and carvacrol. However, nephrotoxic and hepatotoxic effects, mainly associated with NDGA, have been reported.

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