UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO" FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS CÂMPUS DE JABOTICABAL

PLANT EXTRACTS AND OILS FOR THE CONTROL OF BOVINE MASTITIS: ANTIMICROBIAL POTENTIAL AND CLINICAL SAFETY

Romário Alves Rodrigues

Médico Veterinário

2026

UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO" FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS CÂMPUS DE JABOTICABAL

PLANT EXTRACTS AND OILS FOR THE CONTROL OF BOVINE MASTITIS: ANTIMICROBIAL POTENTIAL AND CLINICAL SAFETY

Discente: Romário Alves Rodrigues

Orientadora: Profa. Dra. Marita Vedovelli Cardozo

Coorientador: Dr. Lucas José Luduverio Pizauro

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Microbiologia Agropecuária.

2026

R696p

Rodrigues, Romário Alves

Plant extracts and oils for the control of bovine mastitis: antimicrobial potential and clinical safety / Romário Alves Rodrigues. -- Jaboticabal, 2026

93 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

Orientadora: Marita Vedovelli Cardozo

Coorientador: Lucas José Luduverio Pizauro

1. mastite bovina. 2. Bovine mastitis. 3. Plant extracts. 4. Phytotherapy. 5. Dairy cattle. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Dados fornecidos pelo autor(a).

CERTIFICADO DE APROVAÇÃO

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: PLANT EXTRACTS AND OILS FOR THE CONTROL OF BOVINE MASTITIS: ANTIMICROBIAL POTENTIAL AND CLINICAL SAFETY

AUTOR: ROMÁRIO ALVES RODRIGUES
ORIENTADORA: MARITA VEDOVELLI CARDOZO
COORIENTADORA: MARITA VEDOVELLI CARDOZO
COORIENTADOR: LUCAS JOSÉ LUDUVÉRIO PIZAURO

Aprovado como parte das exigências para obtenção do Título de Doutor em Microbiologia Agropecuária, pela Comissão Examinadora:

Profa. Dra. MARITA VEDOVELLI CARDOZO (Participaçao Presencial)
Departamento de Patologia, Reprodução e Saúde Única / UNESP / Câmpus de Jaboticabal - FCAV

Profa. Dra. NATÁLIA MARAMARQUE NESPOLO (Participaçao Virtual)
Departamento de Medicina / Universidade Federal de São Carlos (UFSCar) - São Carlos/SP

Pesquisador Dr. MAURO DE MESQUITA SOUZA SARAIVA (Participaçao Presencial)
Departamento de Patologia, Reprodução e Saúde Única / UNESP / Câmpus de Jaboticabal - FCAV

Prof. Dr. ARGEMIRO SANAVRIA (Participaçao Virtual)
Departamento de Medicina Veterinária / Universidade Federal Rural do Rio de Janeiro (UFRRJ) - Rio de Janeiro/RJ

Profa. Dra. ADOLORATA APARECIDA BIANCO CARVALHO (Participaçao Presencial)
Departamento de Patologia, Reprodução e Saúde Única / UNESP / Câmpus de Jaboticabal - FCAV

Jaboticabal, 09 de julho de 2026.

DADOS CURRICULARES DO AUTOR

ROMÁRIO ALVES RODRIGUES - Nascido em Lassance, MG, em 21 de junho de 1994, filho de Valdemar Alves dos Santos Filho e Vera Lúcia Rodrigues de França Alves, tornou-se médico-veterinário em janeiro de 2018, graduado em Bacharel pelo Instituto Federal do Norte de Minas Gerais – Câmpus Salinas. Em março de 2019, ingressou no Programa de Residência em área Profissional da Saúde – Medicina Veterinária e Saúde, subárea Medicina Veterinária Preventiva, da Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista (FCAV-Unesp) Câmpus de Jaboticabal. Em 2022, concluiu o Curso de Pós-graduação no nível de Mestre e iniciou o curso de Doutorado em Microbiologia Agropecuária pela FCAV – Unesp, Câmpus de Jaboticabal, com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e em 2026 realizou o Doutorado Sanduíche na Université de Montréal, no Canadá.

*“**Resistência** não é apenas ser como o cerne da Aroeira, que suporta todas as pancadas; é também saber **revidar**, **questionar** e **reconstruir-se** sempre que possível.”*

(Romário Alves Rodrigues)

AGRADECIMENTOS

Agradeço primeiramente a Deus, por me guiar nos momentos de angústias e amparar nas horas mais difíceis, no ímpeto do saber de acreditar que sempre haverá esperança.

À professora Dra. Marita Vedovelli Cardozo, pela oportunidade, pela parceria, pelo conhecimento e pelos ensinamentos disponibilizados a mim e a todos que passaram pela FCAV – Unesp, Câmpus de Jaboticabal.

Às amizades antigas, atuais e as que virão, incluindo aqui os amigos e colegas da APG (Associação de Pós-Graduandos da FCAV-Unesp, Câmpus de Jaboticabal) e aqueles presentes nos momentos de lazer.

À minha namorada e parceira, Lara Caveanha Gragnanello, por todo o apoio, incentivo, companheirismo e por acolher minhas emoções, valorizando cada ideia, esforço e atitude ao longo desta caminhada.

Aos meus pais e familiares. A vocês, muito obrigado pelo carinho de sempre!

Aos professores que me apoiaram, acreditaram e confiaram em mim e na minha capacidade.

A todos do Laboratório de Microbiologia (BIOBAC), que de maneira direta ou indireta, auxiliaram na realização desse trabalho.

A todos os profissionais da Pós-Graduação, que não mediram esforços para resolver os problemas frente aos novos desafios. Muito obrigado pelo carinho e compreensão.

Aos pesquisadores de todo o Brasil pela insistência, seriedade, trabalho e paciência frente a tantas dificuldades, questionamentos e notícias falsas propagadas.

Ao Programa de Pós-graduação em Microbiologia Agropecuária, Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES), pela bolsa e apoio prestado.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

SUMÁRIO

Página

CERTIFICADO DA COMISSÃO DE ÉTICA

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de Jaboticabal

ETHICS COMMISSION ON ANIMAL USE

CERTIFICATE

We certify that the research project entitled **"EVALUATION OF THE USE OF PRODUCTS BASED ON PLANT EXTRACTS FOR THE CONTROL AND TREATMENT OF BOVINE MASTITIS"**, protocol nº. 1206/2024, under the responsibility of Dr. FERNANDO ANTONIO DE ÁVILA, which involves the production, maintenance and/or use of animals belonging to the Chordata phylum, vertebrate subfile (except man), for the purposes of scientific research (or teaching) - is in accordance with the precepts of Law no. 11,794, of October 8, 2008, in Decree 6,899, of July 15, 2009, and with the rules issued by the National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on Animal Use (CEUA), from the SCHOOL OF AGRICULTURAL AND VETERINARY SCIENCES, UNESP - JABOTICABAL - SP Campus, at april 17, 2024 an ordinary meeting.

Jaboticabal, April 17, 2024.

Helena C.D Brito

Drª Helena Cristina Delgado Brito
Coordinator – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel: 16 3209-7500 - www.fcav.unesp.br

EXTRATOS E ÓLEOS VEGETAIS NO CONTROLE DA MASTITE BOVINA: POTENCIAL ANTIMICROBIANO E SEGURANÇA CLÍNICA

RESUMO - Os extratos vegetais têm sido investigados pela indústria farmacêutica como alternativas aos agentes antimicrobianos convencionais. O objetivo desse trabalho foi sintetizar as principais evidências sobre o potencial dos extratos vegetais no controle de microrganismos associados à mastite bovina, bem como os efeitos na glândula mamária. Além disso, foi conduzido um estudo para sustentar as observações relatadas na literatura, incluindo os efeitos antimicrobianos *in vitro* e a segurança clínica de uma formulação intramamária composta por extratos e óleos de *Baccharis trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis*, óleo de sementes de *Carapa guianensis, Salvia rosmarinus, Melaleuca alternifolia* e resina de *Copaifera officinalis*. A formulação demonstrou atividade inibitória contra *Escherichia coli, Salmonella* Dublin*, Staphylococcus aureus, Streptococcus agalactiae e Bacillus cereus*, consistente com os perfis antimicrobianos relatados na literatura científica. Os parâmetros hematológicos e bioquímicos séricos permaneceram dentro das faixas esperadas, provavelmente devido às reconhecidas barreiras anatômicas da glândula mamária. Entretanto, observou-se um aumento significativo na contagem de células somáticas do leite e o desenvolvimento de sinais clínicos na glândula mamária, com intensificação dos efeitos na dose duplicada. Essas respostas podem refletir uma reação inflamatória de curto prazo, mas também podem indicar efeitos adversos relacionados a compostos bioativos específicos, sugerindo a necessidade de ajustes de dose ou da remoção de componentes irritantes. Assim, os resultados indicam que, embora a literatura relate resultados favoráveis *in vitro*, os extratos vegetais e seus compostos bioativos podem desencadear um efeito irritante dependente da dose na glândula mamária.

Palavras-chave: Fitoterapia, Mastite bovina, Plantas medicinais, Segurança Clínica

PLANT EXTRACTS AND OILS FOR THE CONTROL OF BOVINE MASTITIS: ANTIMICROBIAL POTENTIAL AND CLINICAL SAFETY

ABSTRACT - Plant extracts have been investigated by the pharmaceutical industry as alternatives to conventional antimicrobial agents. The aim of this study was to synthesize the main evidence regarding the potential of plant extracts in the control of microorganisms associated with bovine mastitis, as well as their effects on the mammary gland. In addition, a study was conducted to support the observations reported in the literature, including *in vitro* antimicrobial effects and the clinical safety of an intramammary formulation composed of extracts and oils from *Baccharis trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis, Carapa guianensis* seed oil, *Salvia rosmarinus*, *Melaleuca alternifolia*, and *Copaifera officinalis* resin. The formulation demonstrated inhibitory activity against *Escherichia coli, Salmonella* Dublin*, Staphylococcus aureus, Streptococcus agalactiae*, and *Bacillus cereus*, consistent with antimicrobial profiles reported for these extracts in previous studies. Hematological and serum biochemical parameters remained within expected ranges, likely due to the well-recognized anatomical barriers of the mammary gland. However, a significant increase in milk somatic cell count and the development of clinical signs in the mammary gland were observed, with effects intensifying at double the dosage. These responses may reflect a short-term inflammatory reaction but could also indicate adverse effects related to specific bioactive compounds, suggesting a need for dose adjustments or the removal of irritant components. Thus, the results indicate that, although the literature reports favorable *in vitro* results, plant extracts and their bioactive compounds may elicit a dose-dependent irritant effect in the mammary gland.

Keywords: Herbal medicine, Clinical safety, Medicinal plants, Phytotherapy

List of Tables

Page

List of Figures

Page

List of Abbreviations

Abbreviation	Description
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
ATCC	American Type Culture Collection
BAS	Basophils
BHI	Brain Heart Infusion
BIOBAC	Laboratório de Microbiologia
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EOS	Eosinophils
GGT	Gamma-Glutamyl Transferase
HCT	Hematocrit
HGB	Hemoglobin
LPS	Lipopolysaccharide
LYMPH	Lymphocytes
MBC	Minimum Bactericidal Concentration
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MIC	Minimum Inhibitory Concentration
MON	Monocytes
NB	Band Neutrophils
NS	Segmented Neutrophils
OD	Optical Density
PLT	Platelets
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SAS	Statistical Analysis System
SCC	Somatic Cell Count
SD	Standard Deviation
WBC	White Blood Cells

1. INTRODUCTION

Mastitis is an inflammatory condition of the mammary gland, including associated anatomical structures such as teats, ducts, and other related tissues. In livestock production, it is commonly described as an inflammation caused by infectious agents; however, it may also be triggered by non-infectious factors, such as physical trauma and exposure to chemical substances (Contreras and Rodríguez, 2011; Cheng and Han, 2020). Bovine mastitis is one of the most costly infectious disease in dairy farming, leading to significant losses such as milk disposal (due to antimicrobial withdrawal periods), reduced milk production, culling of animals, and expenses due to antimicrobial therapy (Morales-Ubaldo et al., 2023). Given that, it is essential to implement management practices for controlling both contagious and environmental microorganisms (Klaas and Zadoks, 2018; Exel et al., 2021).

In this context, the dairy industry faces growing concerns regarding the spread of pathogenic microorganisms and the risk resistance to conventional antimicrobials, which has stimulated the search for alternative therapies, such as phytotherapeutic agents (Kaseke et al., 2023; Tomanić et al., 2024). Many researchers have explored medicinal plants and their natural compounds as potential therapies for diseases and comorbidities. The compounds found in medicinal plants, such as flavonoids, polyphenols, terpenoids, alkaloids, and others, exhibit significant therapeutic potential that may contribute to the control of bovine mastitis (Karimi et al., 2015; Gajarmal et al., 2025). Other studies suggest that some substances do not present significant adverse effects on liver and kidney function, and some compounds also have the ability to reduce mammary inflammation (Hashem et al., 2024; Lv et al., 2024).

Studies have shown that plant extract–based substances exhibit antioxidant, anti-inflammatory, antiproliferative, immunomodulatory, and wound-healing activities (Fachinetto and Tedesco, 2009; Higa et al., 2020). However, several strategies have been tested for the control and treatment of bovine mastitis, as well as for replacing conventional therapies, but none has yet been successfully translated into therapeutic products capable of replacing conventional treatments (Sharun et al., 2021; Gajarmal et al., 2025).

Most studies on medicinal plants focus on their systemic effects, antimicrobial and antioxidant potential, and hepatic and renal impacts. However, little is known about

the intramammary application of these products, including their potential benefits and possible adverse effects. Although the mammary gland is compartmentalized and does not have a direct relationship with systemic circulation, it is essential to investigate the potential systemic and cellular effects resulting from the intramammary application of plant extracts, considering that any injury to this tissue tends to be permanent and irreversible (Feitosa, 2020). It is noteworthy that most plant extracts contain beneficial bioactive compounds; however, certain substances may cause adverse or even toxic effects (Matei et al., 2025; Dos Santos et al., 2018).

In this context, the present work synthesizes the main evidence regarding the potential of plant extracts for controlling microorganisms associated with bovine mastitis, and discusses both the possible mechanisms of action and the safety of formulations tested in dairy cows. Furthermore, a study was conducted to evaluate the *in vitro* antimicrobial activity and clinical safety of an intramammary plant extract-based formulation, thereby providing additional support and context for the evidence discussed in the literature. The intramammary formulation is composed of plant extracts and oils derived from *Baccharis trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis, Carapa guianensis* seed oil, *Salvia rosmarinus*, *Melaleuca alternifolia*, and *Copaifera officinalis* resin.

2. LITERATURE REVIEW

This review was conducted based on previously published scientific evidence through systematic searches of the Web of Science, Scopus, PubMed, and Google Scholar databases, performed between March 2025 and April 2026. For the general review, descriptors and combinations of keywords related to the use of natural products and phytotherapeutics for the treatment of bovine mastitis were employed. Search strategies were developed using the Boolean operators AND and OR. An example of the search strategy was: (bovine mastitis AND (medicinal plants OR phytotherapy OR essential oils)) AND (intramammary therapy OR phytotherapeutic formulations) AND (antimicrobial mechanisms OR clinical safety). This stage aimed to compile and synthesize the available scientific evidence regarding the therapeutic potential of plant extracts, essential oils, and their phytochemicals, focusing on

antimicrobial efficacy, mechanisms of action, clinical safety, and the limitations associated with their intramammary use.

Experimental *in vitro* and *in vivo* studies conducted in cattle or other animal species were included, provided they were directly or indirectly relevant to bovine mastitis or mammary gland physiology. Studies investigating inflammatory processes, antioxidant activity, systemic effects, and toxicological outcomes, particularly those related to hepatic and renal function, were also included to support the safety assessment of phytotherapeutic formulations. Studies unrelated to bovine mastitis, as well as immunological studies without relevance to mammary gland physiology or pathology, were excluded from the review.

In a second stage, a targeted and focused review was conducted, emphasizing plant extracts used in a phytotherapeutic formulation composed of medicinal plant extracts from *Baccharis trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis*, seed oil *from Carapa guianensis, Salvia rosmarinus, Melaleuca alternifolia*, and resin from *Copaifera officinalis*. Evidence obtained from these sources was used to support the discussion regarding the antimicrobial potential and clinical safety, thereby contextualizing the phytotherapeutic formulation evaluated in the present study.

2.1 Therapeutic potential, safety considerations, and cellular responses to plant-derived compounds in the mammary Gland

2.1.1 Therapeutic potential and experimental evidence

Many scientific articles and ethnobotanical studies have highlighted the therapeutic potential of a wide range of medicinal plants for the control of bovine mastitis, originating from diverse regions of the world, including China, Africa, Brazil, Pakistan, Kyrgyzstan, among others (Amber et al., 2018; Gu et al., 2021; Aldayarov et al., 2025; Ma et al., 2025). In addition, other studies emphasize the beneficial effects of dietary supplementation with medicinal plants exhibiting anti-inflammatory and antibacterial properties, resulting in positive impacts on bovine mastitis. These effects are associated with the presence of plant extracts and essential oils rich in bioactive phytocompounds (Kumar et al., 2022; Kumar et al., 2024).

The literature is also rich in review studies that infer the potential use of medicinal plants and essential oils (Lopes et al., 2020; Kumar et al., 2024; Askari et al., 2025 However, few *in vitro* studies have evaluated the cytotoxic effects of these compounds on mammary epithelial tissues; among those available, the findings indicate that the assessed plants were not considered toxic or exhibited low cytotoxicity, predominantly in experimental models using mice (Sserunkuma et al., 2017; Ogbuadike et al., 2023; Masuku et al., 2025). The number of publications is even smaller when considering phytotherapeutic formulations evaluated *in vivo* in cows, which represents one of the main limitations of the available literature (Francoz et al., 2017; Debruyn et al., 2025).

Among the few *in vivo* studies available, an experiment conducted in goats evaluated the potential of *Hymenaea martiana* extract for the treatment of mastitis induced by *Staphylococcus aureus*, demonstrating a significant reduction in total bacterial counts at the end of the study. However, a significant increase in bacterial load was observed immediately after product application, along with an elevation in somatic cell counts (Peixoto et al., 2015). Subsequently, the ability of *H. martiana* extract to reduce *S. aureus* infection was confirmed; however, no clinical evaluations were reported following the application of the intramammary ointment (de Souza et al., 2024).

Another study, also conducted in goats, identified positive effects of *Phyllanthus emblica* fruit extract and Cocos nucifera oil in the treatment of subclinical mastitis when compared with a procaine penicillin–based control. However, the study did not assess the spontaneous cure rate of subclinical mastitis, a parameter generally expected in this type of investigation (Rizwan et al., 2021). In cows, a 10% pectin solution derived from apples showed positive effects in the treatment of clinical mastitis, suggesting efficacy comparable to the antibiotic used as a control and without inducing adverse effects on the udder. However, the same solution, when applied at lower concentrations (2% and 4%), induced inflammatory reactions and increased the severity of mastitis cases, a phenomenon that was not elucidated by the authors (Kocik et al., 2025).

To date, only two phytotherapeutic formulations have demonstrated consistent effects. Among them, propolis, produced by bees from plant resins derived from

different plant species, has shown positive outcomes at the level of mammary epithelial tissue, with no occurrence of adverse effects. Even so, higher concentrations (above 5%) were associated with cytotoxic effects on healthy cells, accompanied by increased somatic cell counts (Pereira et al., 2025). Another formulation, composed of essential oils from common thyme (*Thymus vulgaris L*.), wild thyme (*Thymus serpyllum L*.), oregano (*Origanum vulgare L*.), mountain savory (*Satureja montana L*.), calendula (*Calendula officinalis L*.), and St. John's wort (*Hypericum perforatum L*.), did not induce visible clinical signs of irritation and improved inflammatory indicators. Still, its efficacy was lower than that of the antibiotic used as a control (Tomanić et al., 2024).

Most studies also highlight the antimicrobial effects of various bioactive compounds, as well as of extracts and essential oils from medicinal plants, against both contagious infectious microorganisms (*Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp*., among others) and environmental pathogens (*Escherichia coli, Enterococcus* spp., and coagulase-negative *staphylococci*), which are commonly associated with mastitis cases (Contreras and Rodríguez, 2011; Kowalczyk et al., 2020; Ogbuadike et al., 2023). Each medicinal plant extract, in turn, contains a wide range of bioactive compounds, with even those present in lower proportions potentially playing an important role in antibacterial activity. These compounds may exert synergistic effects when combined but show minimal or no effect when used individually. Moreover, they can also be combined with antibiotics to hinder classical bacterial resistance mechanisms (Vaou et al., 2021). Indeed, it is the presence of this diversity of bioactive molecules that enables the extracts to act through multi-target mechanisms, suppress resistance pathways, and mitigate adverse effects (Wagner and Ulrich-Merzenich, 2009).

On the other hand, many studies are limited to infectious mastitis and do not address the fact that mastitis can also be triggered by non-infectious factors (Contreras and Rodríguez, 2011). Inflammation of the mammary gland may result from trauma or from exposure to chemical or biological agents capable of activating local inflammatory responses, including substances with irritant or cytotoxic potential, among other factors (Sintes et al., 2020; Matouskova et al., 2023; Tang et al., 2022; Zhu et al., 2024). Therefore, understanding the formulations, chemical substances, extracts, and their

various phytocompounds, as well as their interactions with mammary epithelial cells, becomes essential.

2.1.2 Variability of plant bioactives and their effects on mammary epithelium

The mammary gland is highly dynamic and hormone-dependent, characterized by elevated metabolic activity, pronounced cellular plasticity, and marked sensitivity to chemical and oxidative stimuli, which also makes it a particularly relevant target for pharmacological and toxicological studies (Zhou et al., 2025).

The composition of each plant-based extract is highly diverse, containing a range of phytocompounds with distinct chemical and medicinal properties. It can also vary depending on phenotypic and environmental conditions, such as the season, stage of maturation, geographic location, and so on (Asghar et al., 2016; Kumarasinghe et al., 2024). Some studies have already shown that dosage is a critical factor in the use of phytotherapeutics, as high concentrations of certain phytocompounds can induce toxic effects in healthy cells, and are therefore dose-dependent (Yu et al., 2020; Pereira et al., 2025; Saini et al., 2025).

Each medicinal plant contains a range of bioactive compounds with recognized therapeutic potential; however, certain molecules may exhibit antagonistic biological activities or cytotoxic, pro-inflammatory, or dysregulatory effects on the epithelial and myoepithelial cells of the mammary gland, thereby compromising epithelial functional integrity. The main bioactive molecules belong to the groups of phenolic compounds, terpenoids, alkaloids, glycosides, saponins, and polysaccharides, in addition to other less abundant but biologically relevant classes. Most studies on the bioactive compounds within these groups report antibacterial, antifungal, anti-inflammatory, and anticancer properties, as well as other recognized therapeutic effects (Fiordalisi et al., 2016; Han and Bao, 2024).

Phenolic compounds are abundant in many medicinal plant species, with flavonoids and tannins being the most commonly studied. Flavonoids include the subclasses flavones (apigenin, luteolin), flavonols (quercetin, kaempferol, myricetin), flavanones (naringenin, hesperidin), flavanols (catechin, epicatechin), anthocyanins (cyanidin, delphinidin), and isoflavones (genistein, daidzein; common in legumes). Tannins are classified as hydrolyzable (gallotannins and ellagitannins) and

condensed/proanthocyanidins (derivatives of catechins and epicatechins). This group also includes phenolic acids derived from benzoic acid (gallic acid, protocatechuic acid, vanillic acid, syringic acid) and cinnamic acid (caffeic acid, ferulic acid, p-coumaric acid, sinapic acid), as well as other polyphenols such as lignans (secoisolariciresinol and matairesinol) and stilbenes (resveratrol) (Martínez-navarrete et al., 2008; García-Tirado et al., 2012; Kumar et al., 2024).

Terpenoids (or isoprenoids) are classified into monoterpenes (limonene, menthol, α-pinene, β-pinene, binene, α-thujene, α-thujone, γ-terpinene, p-cymene, 1,8-cineole, camphor, borneol, bornyl acetate, thymol, carvacrol, D-verbenol, and L-verbenol), sesquiterpenes (β-caryophyllene, α-humulene, viridiflorene, D-germacrene, γ-eudesmol, β-elemene, cedrol, farnesol, and artemisinin), diterpenes (phytol, carnosic acid, and cafestol), triterpenes (ursolic acid, oleanolic acid, and betulin), and tetraterpenes (β-carotene, lycopene, and lutein) (Kowalczyk et al., 2020; Abdoul-latif et al., 2023; Saini et al., 2025).

In both cases, studies focus on their antioxidant capacity; even so, there is also a growing body of evidence demonstrating that these compounds play a significant role in modulating the inflammatory response and regulating hormonal signaling (Kowalczyk et al., 2020; Yang et al., 2023). The scientific literature has also reported the antiproliferative effects of phenolic compounds and terpenoids against cancer cells, demonstrating their ability to reduce the high rates of cellular proliferation. On the other hand, few studies have investigated the impacts of these compounds on epithelial cells of the mammary gland, which likewise exhibit high cellular proliferation rates, particularly during lactation (Zhou et al., 2020; Saini et al., 2025). This knowledge gap may be significant, as many phytocompounds may not distinguish cancer cells from mammary epithelial cells, and mechanisms often considered beneficial in targeting tumor cells, such as caspase activation, mitochondrial damage, and increased reactive oxygen species, can exert harmful effects on normal epithelial cells, potentially leading to undesired tissue damage.

It is worth noting that the main antitumor mechanism investigated by researchers involves the activation of apoptotic pathways, a classical and tightly regulated strategy widely employed in the clinical use of chemotherapeutic agents to inhibit cell proliferation; however, these drugs also exhibit high cytotoxicity toward

healthy cells. In this context, phytocompounds emerge as a promising alternative to conventional chemotherapeutics, as they demonstrate moderate to low cytotoxicity in normal cells, greater specificity for cancer cells, and the ability to induce apoptosis, making them a viable and potentially safer option for cancer treatment (Jha et al., 2025).

The literature suggests that each isoflavone acts differently on the physiological activity of mammary gland epithelium, affecting milk protein expression and hormonal signaling. They are referred to as phytoestrogens because they can elicit estrogen-like activities and, consequently, influence mammary morphogenesis, potentially impacting milk production (Kumai et al., 2020; Tsugami et al., 2024; Canivenc-Lavier and Bennetau-Pelissero, 2023). Resveratrol, in turn, is commonly found in grape skins and has been reported to exert beneficial effects on the mammary gland. However, no studies have been identified that directly evaluate its effects on mammary epithelium, which may be negative and dose-dependent (Moshiri et al., 2017). The reported beneficial effects are mainly associated with its antioxidant capacity, as well as the active modulation of tight junctions and the stimulation of natural antimicrobial compounds present in milk, which contribute to the prevention of mastitis (Moshiri et al., 2017; Zhou et al., 2020; Tsugami et al., 2024).

Similarly, other molecules, such as carvacrol, thymol, limonene, and artemisinin, among others, exhibit specific anti-inflammatory, antiproliferative, and antioxidant effects; however, they have not yet been individually evaluated via the intramammary route, highlighting the need for targeted studies to investigate the safety, selectivity, and physiological impact of these compounds on the mammary gland (Şehitoğlu et al., 2023).

Plants also contain a wide diversity of alkaloids that exhibit therapeutic potentials similar to those previously described. The main representatives of this group include indole alkaloids (such as reserpine), isoquinoline alkaloids (morphine and berberine), pyrrolidine and piperidine alkaloids (nicotine), tropane alkaloids (tropine and scopolamine), and quinoline alkaloids (quinine) (Chidananda et al., 2025). Also noteworthy is the presence of alkaloids from other structurally relevant subclasses, such as carpaine, sanguinarine, and peiminine, as well as acridone-type alkaloids, including citrusin-I, citratridone-I, 5-hydroxynoracronycine, natsucitrine-I,

glycopholinin, and citrinnovone-III. The latter have been evaluated for their cytotoxic potential, which was found to be more pronounced in cancer cells than in normal human prostate epithelial cells, suggesting that these effects might also be relevant for mammary gland cells (Segun et al., 2018).

Several studies reinforce the potential of this group to interfere with cellular signaling pathways critical for cancer cell survival, as well as to induce apoptosis, as previously observed (Chidananda et al., 2025). In this context, alkaloids such as berberine, sanguinarine, and chelerythrine stand out, with effects that additionally include synergistic actions with classic cytostatic agents, thereby enhancing antitumor activity (Duda-madej et al., 2024).

Sanguinarine has demonstrated chemotherapeutic effects in human breast cancer cells and, in *in vivo* experiments in mice, was shown to be non-toxic to mammary epithelial cells, indicating its potential applicability for the mammary gland (Almeida et al., 2017b; Zheng et al., 2022). Similarly, peiminine exhibited anti-inflammatory effects in lipopolysaccharide-induced mastitis models, while carpaine also demonstrated concentrations that did not elicit toxic effects, further underscoring the relevance of these alkaloids for studies focused on safety and efficacy in mammary tissue (Gong et al., 2018; Arya et al., 2026).

Glycosides (saponins), polysaccharides (pectins, gums, mucilages, and β-glucans such as acemannan and glucomannan), and other secondary metabolites are also equally important, with studies employing approaches similar to those previously described (Podolak et al., 2010; Zhu et al., 2023; Zhang et al., 2025).

In summary, each medicinal plant contains a complex set of bioactive substances and, when used in intramammary formulations, must be carefully evaluated for its chemical composition as well as for its potential beneficial and adverse effects on mammary epithelium. Challenges remain to be addressed, such as optimizing the route of administration and conducting randomized, multicenter clinical trials, with research being essential for understanding these mechanisms and ensuring safe application in clinical practice. (Yang et al., 2023; Fan et al., 2025). Finally, evaluating the diverse extracts and molecules present in each medicinal plant may be important for the identification of compounds with potential cytotoxicity, informing the development of future intramammary formulations.

2.2 Specific considerations

2.2.1 Plant extracts: clinical safety and toxicological considerations

The properties of plant extracts and oils derived from *Baccharis trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis, Carapa guianensis* seed oil, *Rosmarinus officinalis, Melaleuca alternifolia*, and *Copaifera officinalis* resin that are desirable for the control of bovine mastitis and beneficial to the mammary gland, as well as the potential adverse effects and bioactive compounds associated with possible undesirable outcomes, are summarized below (Figure 1).

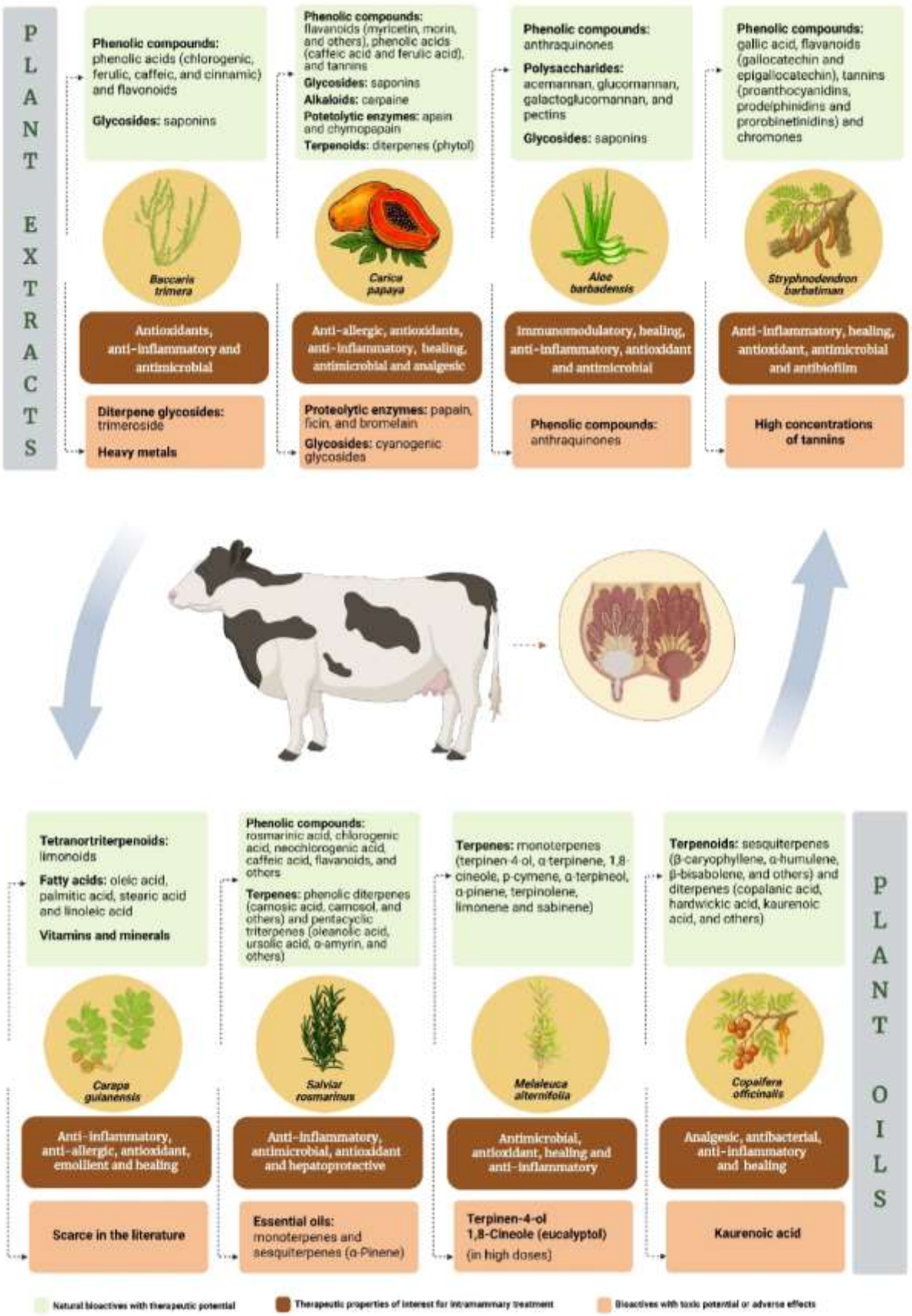

Figure 1: Therapeutic properties of interest for the formulation of intramammary products and bioactive compounds potentially associated with the beneficial and/or adverse effects of plant extracts and vegetable oils from *Baccharis*

trimera, Stryphnodendron barbatiman, Carica papaya, Aloe barbadensis, Carapa guianensis, Rosmarinus officinalis, Melaleuca alternifolia, and *Copaifera officinalis*.

Baccharis trimera

Baccharis trimera exhibits hepatoprotective, antioxidant, anti-inflammatory, and antimicrobial activities (Pádua et al., 2010; da Silva et al., 2018). The phenolic compounds present in its composition, particularly phenolic acids such as chlorogenic, ferulic, caffeic, and cinnamic acids, are associated with its antioxidant properties. Other studies have demonstrated its ability to enhance and restore immune responses, supporting its antioxidant role at the level of neutrophils and macrophages (Da Cruz Pádua et al., 2013; Ximenes et al., 2022). It has also been shown to improve inflammatory and analgesic responses, possibly through the inhibition of prostaglandin biosynthesis (Gené et al., 1996).

Conversely, the oral administration of *Baccharis trimera* in rats has been shown to induce increased urea levels and renal and hepatic histopathological alterations, consistent with acute toxicity (Grance et al., 2008). No evidence of cytotoxic effects on mammary tissue has been reported in the literature; however, if such alterations occur, they may be associated with the presence of trimeroside or heavy metals (cadmium, copper, lead, and zinc) in the plant (Regildo M. G. Silva, 2012; Dos Santos et al., 2018).

Stryphnodendron barbatiman

Stryphnodendron barbatiman exhibits anti-inflammatory, wound-healing, antioxidant, antimicrobial, and antibiofilm properties (Pinto et al., 2015; Domingues et al., 2023; Reis et al., 2025). It contains phenolic compounds such as gallic acid, flavonoids, tannins, and chromones, which are predominantly found in the bark, followed by the leaves and stem, and are responsible for its beneficial effects. At high concentrations of the extract (800 mg/kg), some degree of toxicity has been reported in rats, with hepatic impairment but no significant systemic alterations (Almeida et al., 2017a). The absence or low cytotoxicity in eukaryotic cells also suggests that the

glycolic extract of Stryphnodendron barbatiman does not induce adverse effects on mammary epithelium, although specific studies on this tissue are still needed to confirm this hypothesis (Trevisan et al., 2020; de Melo et al., 2023).

Carica papaya

Carica papaya also contains bioactive compounds with antioxidant, anti-inflammatory, antiallergic, analgesic, antimicrobial, and wound-healing properties. These activities are attributed to the presence of alkaloids, glycosides, tannins, saponins, and flavonoids, as well as the enzymes papain and chymopapain, which are primarily responsible for its wound-healing, antibacterial, antifungal, and antiviral effects (Ayodipupo Babalola et al., 2024; Chaijan et al., 2025; Miah et al., 2025).

Studies have further demonstrated that Carica papaya extract, when administered in the diet, likely due to the action of proteolytic enzymes present in the fruit, exerts immunostimulatory and antioxidant activities, can reduce the occurrence of mastitis, protect the mammary gland against oxidative damage, and enhance the gene expression of proteins, thereby contributing to increased milk production (You et al., 2017; Abouzed et al., 2019; Herawati et al., 2022). On the other hand, the latex, found mainly in the unripe fruit, contains proteolytic enzymes such as papain, ficin, and bromelain. Although there is no evidence that these enzymes cause lesions in the mammary epithelium, papain is widely used for the removal of necrotic tissue, and the consumption of unripe fruit has been associated with risks during pregnancy, suggesting a potential link with toxic effects (Silva et al., 2010; Ayodipupo Babalola et al., 2024).

Similarly, the presence of cyanogenic glycosides, which can release hydrogen cyanide upon activation, may lead to possible implications, although in the fruit they occur at very low levels (Williams et al., 2013; Ruhaizat-Ooi et al., 2022). The literature also warns that the seeds may contain compounds capable of increasing serum levels of hepatic enzymes, as well as causing histological alterations. These effects are dose-dependent and should be taken into account when developing products (Udoh and Udoh, 2005). Furthermore, the phytol present in the leaf extract has also been shown to be non-toxic at high oral doses in rats, without alterations in physiological or

hematological parameters (Singh et al., 2020; Taychaworaditsakul et al., 2024; Alhodieb et al., 2025).

Aloe barbadensis

Aloe barbadensis is one of the most well-known plant extracts due to its wound-healing properties. Its polysaccharides also provide anti-inflammatory, immunomodulatory, antimicrobial, and antioxidant activities. The plant is composed of a wide range of mono- and polysaccharides – acemannan (the most extensively studied bioactive compound), glucomannan, rhamnogalacturonans, homogalacturonans, xyloglucans, arabinogalactans, galactoglucomannans, fructans, cellulose, and hemicellulose; as well as essential and non-essential amino acids, chromones, anthraquinones, vitamins (A, C, and E), minerals (zinc and selenium), trace elements, enzymes (amylase, peroxidase, and catalase), and sterols.

Most of these components are found in the gelatinous inner portion of the leaf, whereas other phenolic compounds, such as the anthraquinones aloin, aloe-emodin, and barbaloin, are concentrated in the outer layer known as the latex. Some of these compounds may cause irritation or allergic reactions in sensitive individuals (Matei et al., 2025). Nevertheless, the potential of Aloe vera cannot be disregarded. Its multiple properties (including antimicrobial activity attributed to the presence of acemannan, phenolic compounds, and saponins) combined with its capacity for tissue regeneration, wound repair, and reduction of scar formation risk, make it a promising extract for the development of phytotherapeutic products aimed at intramammary formulations and mastitis control (Piaia et al., 2022).

Carapa guianensis

The seed oil of *Carapa guianensis*, besides functioning as a lipid vehicle, contains limonoids that provide *in vivo* anti-inflammatory and antiallergic activities by inhibiting platelet activation and inflammatory mediators, consequently reducing T lymphocytes, eosinophils, and mast cells. The seed oil also contains fatty acids (oleic,

palmitic, stearic, and linoleic), vitamins, and minerals, which exhibit emollient and antioxidant properties (Dias et al., 2023; Pereira da Silva et al., 2023). The presence of limonoids has further been associated with accelerated wound healing, stimulation of collagen synthesis, and angiogenesis (Dias et al., 2023; Monteiro et al., 2025). Adverse effects are also scarcely reported in the literature, suggesting that its bioactive compounds are unlikely to cause tissue toxicity, with only mild alterations in hepatic parameters occasionally reported, and little probability of inducing epithelial-level changes (Costa-Silva et al., 2008; Alves de Souza et al., 2017).

Salvia rosmarinus

Rosmarinus officinalis, currently reclassified as *Salvia rosmarinus*, exhibits anti-inflammatory, hepatoprotective, antioxidant, and antimicrobial activities. These effects are primarily attributed to its essential oils, phenolic acids, flavonoids, and terpenes (Ekambaram et al., 2024).

Intraperitoneal administration of *R. officinalis* essential oil in rats was shown to be detrimental to hepatic and renal function, inducing histopathological alterations and changes in biochemical parameters at concentrations above 1%. Conversely, oral administration exhibited antioxidant properties, mitigating induced nephrotoxicity in the same species (El-Demerdash et al., 2021; Motaghi et al., 2021; Babou et al., 2025). Finally, the only identified evidence of toxicity is associated with the essential oil component α-pinene, which demonstrated a potential risk of cytotoxicity in epithelial cells. This effect may be relevant to mammary epithelium; however, as with most plant extracts, clinical trials directly evaluating its action in the mammary gland are still lacking (Huang et al., 2023).

Melaleuca alternifolia

Melaleuca alternifolia oil also displays anti-inflammatory and wound-healing properties, aiding dermal tissue recovery by downregulating pro-inflammatory cytokines and stimulating the production of anti-inflammatory cytokines (Taga et al.,

2012; Caneschi et al., 2023; Carrasco et al., 2024; Vila Nova et al., 2024). Several studies have reported that oral administration of tea tree oil and its bioactive compounds does not induce systemic effects or alterations in hematological, clinical biochemical, or histopathological parameters. Moreover, in a lipopolysaccharide (LPS)-induced mammary cell inflammation model, tea tree oil reduced cytokine expression, suggesting a potential anti-inflammatory role within the mammary gland (Chen et al., 2020; Wei et al., 2021; Yuan et al., 2023). Nevertheless, the toxicity of tea tree oil appears to be associated with the route of administration or the dose employed, with adverse effects reported mainly at high concentrations or during topical applications, such as in corneal epithelial cells (Park and Park, 2024; Sanajou et al., 2024).

Copaifera officinalis

There are, on average, 20 different *Copaifera* species, which share similar properties and are traditionally used as medicinal herbs due to the bioactive compounds present in the oil obtained from their resins. The *Copaifera* genus exhibits analgesic, antibacterial, wound-healing, and anti-inflammatory activities (Menezes et al., 2022; de Mello et al., 2024).

Specific studies on the toxicity of *Copaifera officinalis* and its phytoconstituents have not been identified. However, there are reports that kaurenoic acid, isolated from another *Copaifera* species, may induce cellular damage and genotoxicity when administered at high doses (Cavalcanti et al., 2006; Leandro et al., 2012). Overall, the *Copaifera* genus appears to present systemic safety, as studies to date have not demonstrated significant levels of toxicity or alterations in systemic parameters. Therefore, it represents a promising medicinal plant for the development of topical and intramammary formulations (Frazão et al., 2023).

2.2.2 Antimicrobial effects of phytotherapeutic agents

The efficacy against Gram-negative bacteria is possibly associated with interactions at the plasma membrane, where the bioactive compounds present may

inhibit conventional resistance mechanisms, similar to what has been reported for other medicinal plants tested against bovine mastitis isolates (Vaou et al., 2021; Kaseke et al., 2023). The presence of substances such as alkaloids, phenolic compounds/polyphenols, terpenes, flavonoids, and others ca may confer bactericidal activity against Escherichia coli, Salmonella spp., Staphylococcus spp., Streptococcus spp., and Bacillus spp., (Vaou et al., 2021; Akinboye et al., 2024; Negri Brusamarello et al., 2024). In addition, such extracts exhibit antimicrobial effects through their action on the cell membrane of pathogenic bacteria, being able to reduce bacterial infection in the mammary glands of goats and, therefore, are considered promising candidates for the treatment of bovine mastitis (Kassama and Misir, 2017; Chacón et al., 2019; de Souza et al., 2024)

Baccharis trimera contains flavonoids and other bioactives that may contribute to its ability to inhibit microbial growth. The literature diverges regarding its action against Gram-positive and Gram-negative bacteria; however, reported findings indicate bacteriostatic effects against both groups, including clinical isolates of *E. coli* and *Salmonella* spp. as well as certain ATCC strains of *Staphylococcus* spp. (Aleixo et al., 2013; Negri Brusamarello et al., 2024).

Other plant-derived extracts also possess bioactive compounds with antimicrobial potential, such as the main compound of *Stryphnodendron barbatiman*, tannin, which accounts for the antimicrobial activity against *Staphylococcus aureus*, by interfering with the fatty acid biosynthesis pathway (FAS II) and inhibiting the enzymes FabI, FabG, and FabZ, as well as against *Escherichia coli* and *Citrobacter freundii* (members of the Enterobacteriaceae family) causing cell wall damage and reducing biofilm formation in mixed infections (Souza et al., 2013; Almeida et al., 2017a; Baldivia et al., 2018; de Freitas et al., 2018).

In contrast, the bioactive compound profile of *Carica papaya* varies according to the plant part and may be absent or present in lower concentrations depending on the region of origin (fruit, seed, leaf, latex, and bark) or stage of maturity (Asghar et al., 2016; kumarasinghe et al., 2024). For instance, the seeds contain bioactives with bactericidal and bacteriostatic activity, showing effects against *Staphylococcus aureus* and *Escherichia coli*. The leaves are rich in phytol, which has demonstrated

antimicrobial activity against Salmonella enterica serovar Typhi (Singh et al., 2020; Taychaworaditsakul et al., 2024; Alhodieb et al., 2025).

The antimicrobial activity of *Carapa guianensis* seed oil, in turn, remains poorly prove, its antimicrobial activity remains poorly substantiated, as the few studies available reported no effects against *S. aureus, E. coli*, or *Salmonella*. However, when combined with Cu^{2+} ions in sponge matrices, an antibacterial effect was observed (Leal et al., 2023; Simplício et al., 2025).

On the other hand, the antimicrobial action of *Salvia Rosmarinus* has been demonstrated against both Gram-positive and Gram-negative bacteria, including *S. aureus, C. perfringens, and B. cereus* (spore-forming bacteria), as well as *E. coli* (Ahmad et al., 2022; Gadallah et al., 2024; Soliman et al., 2024; Magtalas and Anapi, 2025; Khan et al., 2025). Additionally, antimicrobial activity has been reported against microalgae and fungi present in milk (Paşca et al., 2015; Ksouri et al., 2017; Kuczyńska et al., 2025).

Similarly*, Melaleuca alternifolia* exhibits activity against a variety of microorganisms, including mastitis isolates and ATCC reference strains of *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, and *Klebsiella pneumoniae* (Corona-Gómez et al., 2022). The most abundant compound of *Melaleuca alternifolia* is terpinen-4-ol, which destabilizes potassium ion gradients, inhibits cellular respiration, and promotes bacterial membrane leakage. Other bioactive components contribute to biofilm reduction, and another species of the *Melaleuca* genus has demonstrated synergistic effects when combined with certain antibiotics.

Ultimately, the antimicrobial effects of *Copaifera* species are primarily directed against Gram-positive microorganisms, such as *Staphylococcus* and *Streptococcus*, making it a promising resource for the formulation of phytotherapeutics as an alternative therapy for the treatment of mastitis in both humans and animals (Pieri et al., 2010; de Faria et al., 2017; Campanholi et al., 2023).

3. MATERIALS AND METHODS

The study evaluated an intramammary formulation based on extracts and oils derived from medicinal plants to investigate its *in vitro* antimicrobial activity, systemic

effects, and response of the mammary gland. All procedures involving animals were approved by the Animal Use Ethics Committee of the School of Agricultural and Veterinary Sciences, São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil (Protocol n° 1206/2024) on 12 June 2024.

3.1 Characteristics of the Phytotherapeutic Formulation

The formulation used in this study had been previously characterized for its physicochemical, phytochemical, and stability properties (confidential data). This phytotherapeutic formulation consists of extracts and oils derived from medicinal plants, as described in Table 1.

Table 1. Centesimal composition of the formulation based on plant extracts, oils, and other components of the formulation.

Popular name/components	**Extracted parts**[1]	**Quantity (%)**	**mg/mL**
Carqueja	*Baccharis trimera* extract	20	200
Barbatimão	*Stryphnodendron barbatiman* glicolic extract	10	100
Mamão	*Carica papaya* extract fruit	3,2	32
Aloe vera or babosa	*Aloe barbadensis* leaf glicolic extract	2,5	25
Andiroba	*Carapa guianensis* seed oil	2,5	25
Alecrim	*Salvia rosmarinus* leaf oil	2,2	22
Melaleuca or tea tree	*Melaleuca alternifolia* leaf oil	1,2	12
Hietelose		0,8	8
Hyaluronic acid		0,3	3
Aminomethyl propanol		0,2	2
Bálsamo de copaíba	*Copaifera officinalis* resin	0,2	2
Methylparaben		0,15	1,5
Propylparaben		0,1	1
Disodium edetate		0,05	0,5
Deionized water q.s.p.		100	100 q.s.p

[1]A list with detailed information on these compounds is available at: https://bibliotecadigital.anvisa.gov.br/jspui/handle/anvisa/11933

3.2 *In vitro* test

For the *in vitro* assays, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the formulated product were determined, following the methodology described by Wiegand et al. (2008), with adaptations. Initially, 200 µL of the formulated product (i.e., the base product containing different concentrations of each extract) were diluted in 100 µL of Mueller-Hinton broth, and subjected to serial two-fold (1:1) dilutions (yielding concentrations of 100%, 50%, 25%, and so forth), in triplicate. Prior to this, the concentration of the microorganisms was determined by spectrophotometry using a Bio Photometer Plus (OD 600), with absorbance values between 0.08 and 0.12, corresponding to a 0.5 McFarland standard (according to CLSI). Additionally, the microorganisms were quantified to ensure that the inoculum reached a concentration of 1×10^6 CFU/mL before dispensing into the ELISA plates, and 5×10^5 CFU/mL after the addition of 100 µL of the bacterial suspension.

The MBC was determined by the absence of visible growth, considering the lowest concentration of the tested product capable of reducing 99.9% of the inoculum's viability after 24 hours of incubation at 36 ± 1 °C (Tao et al., 2022). For MBC determination, each well of the ELISA plate was subcultured using a 10 µL inoculation loop onto Brain Heart Infusion (BHI) agar plates corresponding to the MIC and higher concentrations. The lowest concentration that showed no bacterial growth was considered the MBC, according to established criteria by Negri Brusamarello et al. (2024).

The in vitro assays assessed different bacterial groups, including *Staphylococcus aureus* (ATCC 29213) and *Streptococcus agalactiae* (ATCC 12403), Gram-positive microorganisms commonly associated with contagious bovine mastitis, and *Escherichia coli* (ATCC 25922), a Gram-negative pathogen frequently involved in environmental mastitis. Additionally, the present study evaluated the effects of the phytotherapeutic formulation against other microorganisms in order to investigate the broader spectrum of its antimicrobial activity, including *Salmonella* Dublin (IAL 09/18), an uncommon cause of mastitis with the potential to reach the mammary gland through systemic infection, and *Bacillus cereus*, a Gram-positive spore-forming microorganism.

Three replicates of the formulation were tested for each bacterium, and the mean value was reported as the final result, expressed in mg/mL^{-1}.

3.3 Systemic effects

3.3.1 Eligibility

Thirty cows at peak lactation, randomly selected from dairy farms in the Jaboticabal City, São Paulo State, Brazil, underwent anamnesis and physical examinations. Blood samples were collected for hematological analysis, and milk samples were taken for SCC and standard plate count. Animals showing acute or severe systemic signs of mastitis, evidence of chronic or metabolic diseases, nutritional deficiencies, or any mammary gland abnormalities indicative of pathology were excluded. Cows with abnormal hematological parameters, elevated SCC (≥200,000 cells/mL), or positive microbiological cultures suggestive of intramammary infection were also excluded from the study. The hygienic procedures and milk sample collection for microbiological analysis were performed in accordance with the recommendations established by the National Mastitis Council.

3.3.2 Clinical and toxicological safety

Of the thirty lactating cows, only seventeen were selected, those that met the eligibility criteria and were considered healthy, with each cow treated as an experimental unit. The intramammary formulations were administered into the right fore teat. Animals/teats were allocated into two experimental groups: eight individuals received the recommended dose of the plant-based extract and oil formulation (Treatment 1), while nine received twice the recommended dose (Treatment 2). The protocol for the plant-based formulation (Treatments 1 and 2) consisted of intramammary administration twice daily for three consecutive days, following milking and complete udder emptying. Clinical and toxicological safety were evaluated at three time points: three days before administration (t: -3), 12 hours after the end of the protocol (t: 0.5), and seven days after the end of the protocol (t: 7) (Table 2).

Table 2. Experimental design for the clinical and toxicological safety assessment of different concentrations of a formulation based on extracts and oils derived from medicinal plants using serum biochemical markers and somatic cell count

Groups	Clinical Safety	Animals	Sampling and Testing processing		
			Three days pre-aplication (t: -3)	**12 hours post-aplication (t: 0,5)**	**Seven days post-aplication (t: 7)**
T1	Recommended dose	N= 8	SCC, blood count, AST, GGT, ALP, Urea e Creatinine	SCC, blood count, AST, GGT, ALP, Urea e Creatinine	SCC, blood count, AST, GGT, ALP, Urea e Creatinine
T2	2 x dose	N= 9	SCC, blood count, AST, GGT, ALP, Urea e Creatinine	SCC, blood count, AST, GGT, FA, Urea e Creatinine	SCC, blood count, AST, GGT, ALP, Urea e Creatinine

SCC - Somatic cell count, AST - Aspartate aminotransferase; GGT - Gamma-Glutamyl Transferase; ALP - Alkaline Phosphatase, T1 – Treatment 1, T2 – Treatment 2

3.3.3 Hematological and serum biochemistry evaluation

For the hemogram, blood samples were collected in EDTA-containing tubes to prevent coagulation. Red blood cell, leukocyte, platelet counts, hematocrit, and erythrocyte indices (mean corpuscular volume – MCV, mean corpuscular hemoglobin – MCH, and mean corpuscular hemoglobin concentration – MCHC), were measured using an automated hematology analyzer (pocH – 100 iV Diff, Sysmex Corporation, Kobe, Japan). The differential leukocyte count was performed by counting 100 cells in blood smears stained with modified Rosenfeld stain, under light microscopy (Garcia-Navarro, 2005).

For serum biochemical profile analyses, blood samples were collected in tubes without anticoagulant and centrifuged at 1,000 × g for 10 minutes to obtain 1.5 mL serum aliquots. The aliquots were stored in pre-labeled microtubes and frozen at −20 °C until analysis. Serum activities of aspartate aminotransferase (AST) (kinetic UV-IFCC method), alkaline phosphatase (ALP) (modified Bowers and McComb method), and gamma-glutamyltransferase (GGT) (modified Szasz method) were determined, as well as serum concentrations of creatinine (alkaline picrate – Jaffé method) and urea (enzymatic UV method). Sample readings were performed in a semi-automated spectrophotometer (Labquest, Labtest Diagnóstica S.A., Lagoa Santa, Minas Gerais, Brazil) using wavelength settings appropriate for each assay.

3.4 Effects on the mammary gland

Milk samples were collected at the three evaluation time points (Table 1) for subsequent somatic cell counts (SCC). Clinical signs were evaluated in 18 animals, including 9 cows receiving treatment 1 (recommended dose) and 9 cows receiving treatment 2 (twice the recommended dose). Clinical variables were assessed 12 hours after administration of the last dose of the intramammary formulation.

3.4.1 Somatic Cell Count and clinical assessment

The samples were analyzed at the Clínica do Leite Laboratory (ESALQ/USP, Piracicaba, Brazil) using flow cytometry, according to the recommendations of the International Dairy Federation (IDF/ISO 13366-2, 2006).

3.4.2 Assessment of clinical signs of the udder

Clinical signs were evaluated through inspection and palpation of the udder and teats, as well as visual assessment of milk using a strip cup. Each variable was classified and described as presented in Table 3.

Table 3. Criteria adopted for the evaluation of signs of inflammation, palpation of the mammary gland and teat, and visual inspection of the milk were all assessed individually

Signs of Inflammation	Palpation of the Mammary Gland Parenchyma	Teat Palpation	Milk Consistency
No change	**Normal consistency**: Soft, elastic gland	No change	No change
Tumor (swelling)	**Hardened (firm) consistency:** Increased resistance, uniform	Thickening	Viscous fluid
Heat	**Irregular / nodular consistency**: Presence of nodules, hardened areas	Increased sensitivity	Watery fluid
Redness	**Fluctuant / edematous consistency:** Sensation of fluid or edema	-	Mucous or cheesy
Pain	**Painful (pain) consistency**: Reaction to palpation	-	Slight clots
Loss of function	Fibrous consistency: Very firm, non-elastic	-	Moderate clots
-	**Atrophied consistency**: Reduced volume, flaccidity	-	Severe clots (catarrhal mastitis)

adapted from Feitosa, 2020.

3.5 Statistical analysis

Statistical analyses were performed using SAS software (version 9.4). The Shapiro–Wilk test was applied to assess normality for all variables. Variables that met the assumption of normality (RBC, HGB, HCT, MCV, MCH, MCHC, PLT, BAS, EOS, NS, MON, AST, urea, and creatinine,) were analyzed using a two-way repeated measures ANOVA, with Treatment (levels 1 and 2) and Time (−3, 0.5, and 7 days) as fixed factors. The interaction between Treatment and Time was included to evaluate potential combined effects. When significant effects were detected, Tukey's post-hoc test was applied for multiple comparisons (Supplementary Material 1).

Variables that did not meet the normality assumption (WBC, NB, LYMPH, ALP, GGT, and SCC) were analyzed over time within each treatment using the Friedman test. When the Friedman test was significant, pairwise comparisons between time points were performed using Dunn's post-hoc test. Between-group comparisons at each time point were conducted using the Kruskal-Wallis test (Supplementary Material 1 and 2).

Somatic cell counts were determined by flow cytometry. Readings above 9,999,000 cells/mL corresponded to the upper detection limit of the equipment (right-censoring). To handle ceiling values and assess the robustness of the inferences, two approaches were adopted: (i) a right-censored (Tobit) model considering the limit at 9,999,000 cells/mL, and (ii) sensitivity analyses in which values above the ceiling were replaced with 15,000,000 cells/mL (conservative assumption: 50% above the equipment limit). Because SCC values showed a strong right-skewed distribution, as confirmed by the Shapiro–Wilk test ($W < 0.68$; $p < 0.0001$), data were transformed using the natural logarithm (lnSCC). Between-group comparisons at each time point were performed using the non-parametric Mann–Whitney test applied to lnSCC values. A significance level of $p < 0.05$ was adopted for all analyses (Supplementary Material 2).

Clinical observations were converted into ordinal numerical scores to enable nonparametric statistical analyses. Ordinal variables, including signs of inflammation, mammary gland parenchyma palpation, and milk consistency, were treated according to their natural order rather than as continuous measurements. Comparisons between treatment groups were conducted using the Wilcoxon test for two independent samples

(NPAR1WAY procedure) and confirmed with the Kruskal–Wallis test. Binary variables, specifically teat palpation, were treated as a binary categorical variable (0 = no alteration; 1 = thickening and tenderness) and evaluated for associations with treatment groups using the Chi-square test, with Fisher's exact test applied when expected counts were low (FREQ procedure). Statistical significance was set at $p < 0.05$ (Supplementary Material 3).

4. RESULTS

4.1 In vitro results

The results demonstrated that the formulation was effective at very low concentrations, showing MIC and MBC values below 0.4238 mg/mL (0,09765625% of the original formulation) for all strains tested. The product exhibited greater effectiveness against Gram-negative microorganisms, with *Salmonella* Dublin and *Escherichia coli* requiring the lowest concentration, followed by *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Bacillus cereus*, respectively. In all strains evaluated, the plant extract-based product exhibited bactericidal activity, with only a narrow range of bacteriostatic effect, remaining effective even at extremely diluted concentrations of the original formulation (Figure 2).

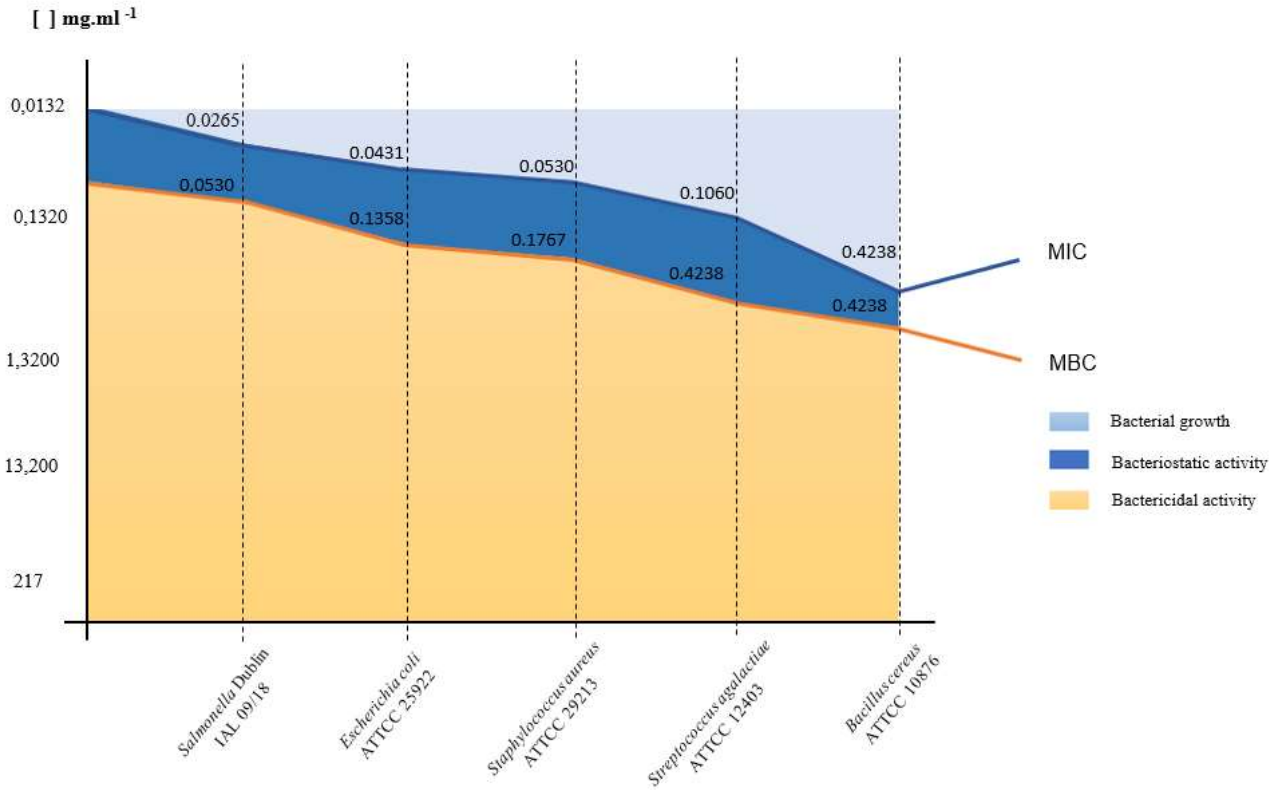

Figure 2. Mean values of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the phytotherapeutic formulation against

Escherichia coli, Salmonella Dublin, Staphylococcus aureus, Streptococcus agalactiae, and *Bacillus cereus*. Results represent the mean of three independent determinations (n = 3). The interval between MIC and MBC values corresponds to the bacteriostatic activity of the formulation.

4.2 Systemic response: hematological and biochemical results

Hematological analyses of the red blood cell series indicated that erythrocyte count (RBC), hemoglobin concentration (HGB), and hematocrit (HCT) were stable over the evaluation period. Although the repeated-measures ANOVA revealed a significant effect of treatment for RBC ($p < 0.0001$), HGB ($p = 0.0004$), and HCT ($p = 0.0008$), no significant effect of time or treatment–time interaction was detected. RBC counts were higher in Treatment 2 at baseline (−3 days; $p = 0.0243$), but differences between groups at other time points were not significant.

Hematimetric indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), showed no significant variations over time or between treatments. While some minor fluctuations in other parameters suggested a trend, post-hoc Tukey analyses did not confirm statistically significant differences, except for platelet count (PLT), which was significantly higher in Treatment 2 at day 7 ($p = 0.0271$). Overall, only erythrocytes and platelets exhibited differences between treatments, while other red blood cell parameters remained stable (Figure 3).

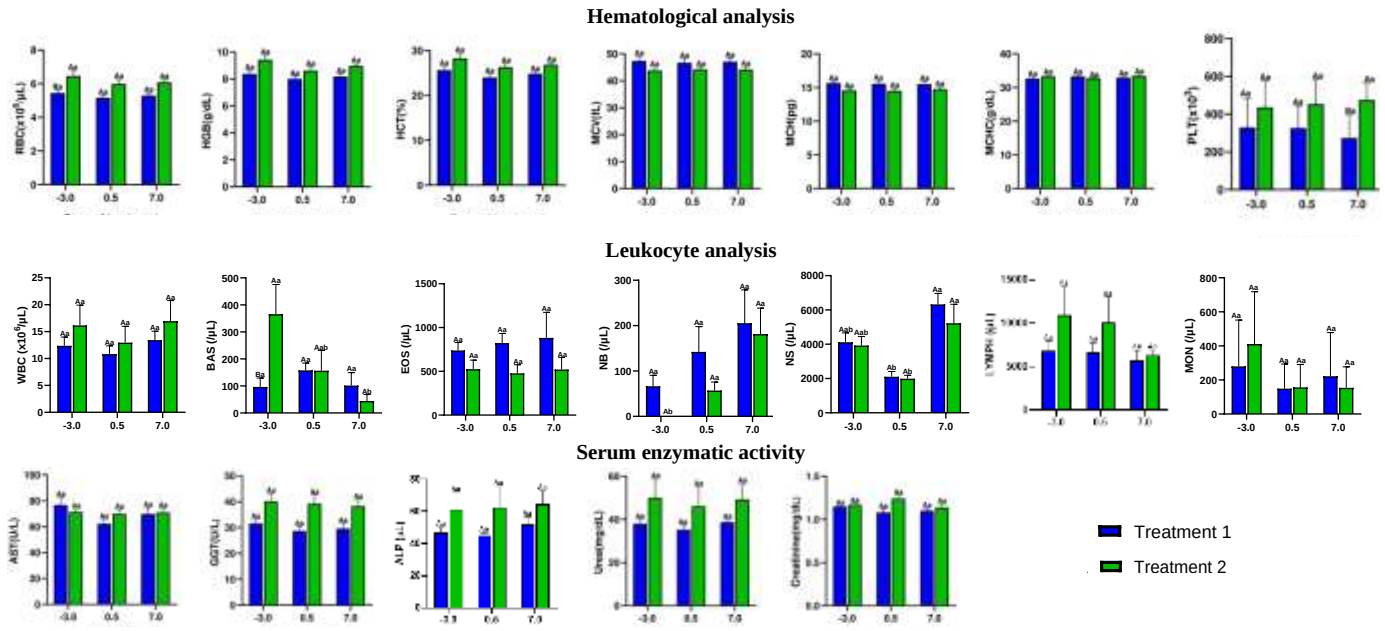

Figure 3. Clinical Safety Assessment: Mean values (± standard error) observed in the analysis of cows treated with a plant extract-based formulation. Treatment 1 (blue line in the graph***)*** received the standard dose, and Treatment 2 (green line) received twice the standard dose. Means (n=9) followed by the same letter do not differ according to Tukey's test (P<0.05). Uppercase letters compare different treatments within each experimental day, while lowercase letters compare the progression of each treatment across different experimental days (–3.0 = 3 days before treatment; 0.5 = 12 hours after treatment; 7.0 = 7 days after treatment). **Hematological analysis**: Red Blood Cells (RBC); Hemoglobin (HGB); Hematocrit (HCT); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC); Platelets (PLT). **Leukocyte analysis**: White blood cells (WBC); Basophils (BAS); Eosinophils (EOS); Band neutrophils (NB); Segmented neutrophils (NS); Lymphocytes (LYMPH); Monocytes (MON). **Serum enzymatic activity**: Aspartate Aminotransferase (AST); Gamma-Glutamyl Transferase (GGT); Alkaline Phosphatase (ALP); Urea; Creatinine.

Leukocyte parameters remained stable over time, with no significant differences between treatments. Although the Cochran-Mantel-Haenszel test indicated a potential trend in WBC counts over time ($p = 0.0183$ for Treatment 1; $p = 0.0010$ for Treatment 2), post-hoc pairwise comparisons did not show statistically significant differences between time points. Basophil counts showed significant effects of Time ($p = 0.0464$) and Group × Time interaction ($p = 0.0252$). Within-group analyses revealed that counts remained stable over time in Treatment 1 (days −3, 0.5, and 7; all $p > 0.98$), whereas Treatment 2 exhibited a significant decrease from day −3 to day 7 ($p = 0.0074$). Between-group comparisons showed higher counts in Treatment 2 at baseline (day −3; $p = 0.0376$), but no significant differences on days 0.5 or 7 ($p = 0.98$–0.99).

These results indicate that basophil dynamics over time differed between treatments, with Treatment 2 showing a more pronounced decline. Eosinophil counts showed a significant overall effect of Group ($p = 0.0237$), but no effect of Time ($p = 0.9033$) or Group × Time interaction ($p = 0.8807$). However, post-hoc pairwise comparisons at individual time points (days −3, 0.5, and 7) did not show significant differences (all $p > 0.45$), indicating that eosinophil levels remained largely stable throughout the study period (Figure 3).

Band neutrophil counts remained stable over time in Treatment 1 (Friedman $\chi^2 = 5.45$, $p = 0.066$), whereas in Treatment 2, counts increased significantly (Friedman $\chi^2 = 9.25$, $p = 0.010$), with paired comparisons showing higher counts at days 0.5 ($p = 0.039$) and 7 ($p = 0.011$) compared to baseline (day −3). No significant differences were observed between treatments at any time point (all $p > 0.05$), indicating that the temporal increase occurred only in Treatment 2. Segmented neutrophil counts increased significantly over time in both Treatment 1 and Treatment 2. Within-group analyses showed that in Treatment 1, counts increased from day 0.5 to day 7 ($p = 0.0005$), while in Treatment 2, counts increased from day 0.5 to day 7 ($p = 0.0090$). Between-group comparisons at individual time points (days −3, 0.5, and 7) did not reveal significant differences (all $p > 0.81$).

Overall, the temporal increase was similar in both groups. Lymphocyte counts remained stable across treatments and time points, with no observable differences between groups, over time, or in the interaction between Group and Time. Pairwise comparisons within and between groups at individual time points also did not reveal any significant changes, indicating that lymphocyte levels were consistent throughout the study period. Monocyte counts showed a significant effect of Time ($p = 0.0259$), but no significant effect of Group ($p = 0.6951$) or Group × Time interaction ($p = 0.3965$). Pairwise comparisons within each group and between groups at individual time points revealed no significant differences (all $p > 0.14$), indicating that monocyte levels fluctuated slightly over time but similarly in both treatments. Statistical analyses indicated that some punctual differences were observed between treatments and over time; however, overall, the hematological parameters remained stable (Figure 3).

Finally, the enzymatic activity results indicated that AST, GGT, and creatinine levels remained stable throughout the evaluation period, with no significant differences between treatments (AST: $p = 0.1825$ for time; $p = 0.3231$ for group–time interaction; GGT: $p = 0.0970$ for Treatment 1 and $p = 0.4724$ for Treatment 2; creatinine: $p = 0.5122$ for time; $p = 0.1606$ for group–time interaction). Alkaline phosphatase (FA) values did not differ significantly between treatments or over time (Treatment 1: $p = 0.3679$; Treatment 2: $p = 0.1690$). Urea levels were slightly higher in Treatment 2 compared to Treatment 1 ($p = 0.0366$), while creatinine levels showed a treatment effect ($p = 0.0286$) but no temporal changes or interactions. Overall, despite minor variations, no

significant alterations in biochemical parameters were detected between groups over time (Figure 3).

4.3 Mammary gland: somatic cell count and clinical assessment

Somatic cell count (SCC) values exhibited substantial variability and a pronounced right skew. In the less conservative sensitivity analysis, in which SCC values exceeding the equipment detection limit were replaced with values 50% higher than the maximum measurable threshold, a significant difference between treatments was observed 12 hours after application, with higher lnSCC values in Treatment 2. Additionally, lnSCC values at this time point were significantly elevated compared to the baseline assessment (–3 days) within the same treatment group. Although SCC decreased by Day 7 post-treatment, levels remained above baseline. These findings suggest a dose-dependent elevation in somatic cell count immediately following treatment administration. This increase may reflect either a transient inflammatory response associated with intramammary intervention or a sustained effect, as values did not return to pre-treatment levels within the observation period (Figure 4).

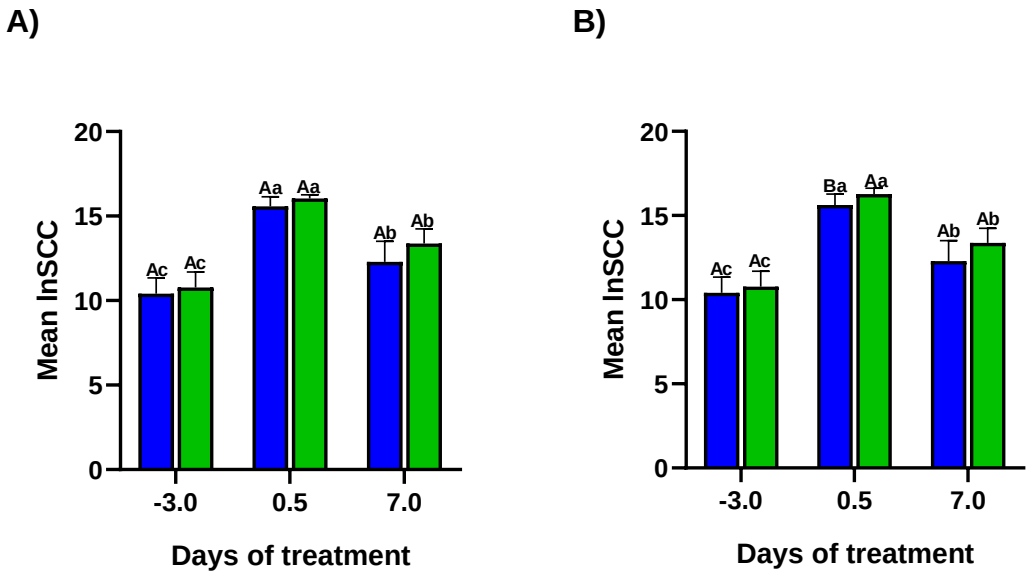

Figure 4. Mean values (± standard error) of somatic cell count (SCC) transformed using the natural logarithm (lnSCC) in cows treated with a plant extract-based formulation. Treatment 1 (blue line in the graph) received the standard dose, and Treatment 2 (green line) received twice the standard dose. Means

followed by the same letter do not differ significantly according to the Kruskal–Wallis test ($p < 0.05$). Uppercase letters compare treatments at each experimental day, whereas lowercase letters compare evaluation days within each treatment. Lowercase letters that are the same do not differ according to Dunn's post hoc test. **(A)** Values above 9.999×10^6 cells/mL were recorded as 9.999×10^6, corresponding to the upper detection limit of the equipment (right-censoring). **(B)** Values above 9.999×10^6 cells/mL were replaced with 15.000×10^6 cells/mL in the sensitivity analysis (conservative assumption: 50% above the equipment limit).

Clinical evaluations revealed qualitative differences between the groups subjected to different treatment doses. In the group treated with the recommended dose, a minority of animals showed no evident inflammatory alterations, with some cases of swelling, heat, or pain in the mammary gland being recorded. In contrast, in the group receiving twice the recommended dose, a higher frequency and intensity of inflammatory signs were observed, including the concomitant presence of swelling, heat, and pain in several individuals (Figure 5).

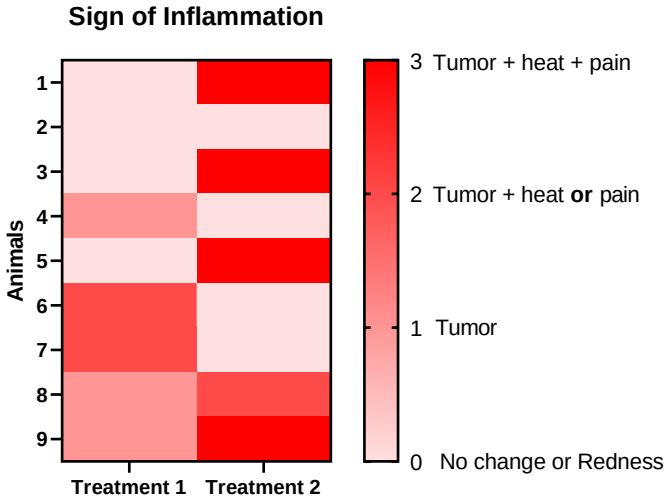

Figure 5. Signs of inflammation in the mammary gland. Each row corresponds to a different animal, and each column represents a treatment applied (Treatment 1, received the standard dose, and Treatment 2, received twice the standard dose). The clinical scores assigned vary according to the severity of signs: 0 = no change or Redness; 1 = tumor; 2 = tumor with heat or pain; 3 = tumor with both heat and pain.

Palpation of the mammary gland parenchyma revealed that, in Treatment 1, animals exhibited signs of firm and edematous or firm and painful consistency,

although in a smaller number of individuals. In Treatment 2, these alterations were more frequent, including cases of firm, edematous, and painful consistency (Figure 6).

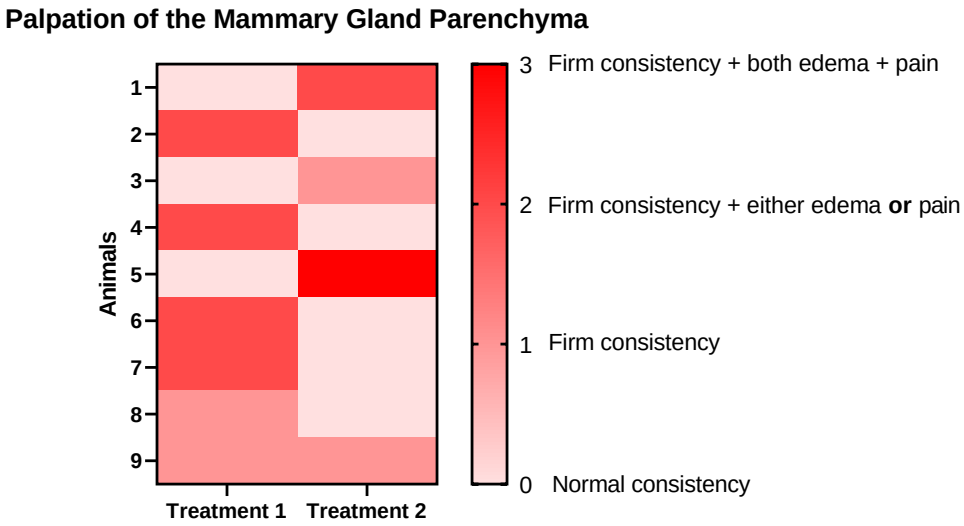

Figure 6. Pappation of the Mammary Gland Parenchyma. Each row corresponds to a different animal, and each column represents a treatment applied (Treatment 1, received the standard dose, and Treatment 2, received twice the standard dose). The clinical scores assigned indicate consistency and associated signs as follows: 0 = normal consistency; 1 = firm consistency; 2 = firm consistency with either edema or pain; 3 = firm consistency with both edema and pain.

Teat palpation was uniform (without thickening or tenderness) in Treatment 1, whereas in Treatment 2, four cases of thickening and tenderness to touch (sensitivity) were recorded. Regarding milk consistency, Treatment 1 showed normal milk or the presence of slight clots, while in Treatment 2, clotting was more pronounced, including moderate and severe cases (Figure 7).

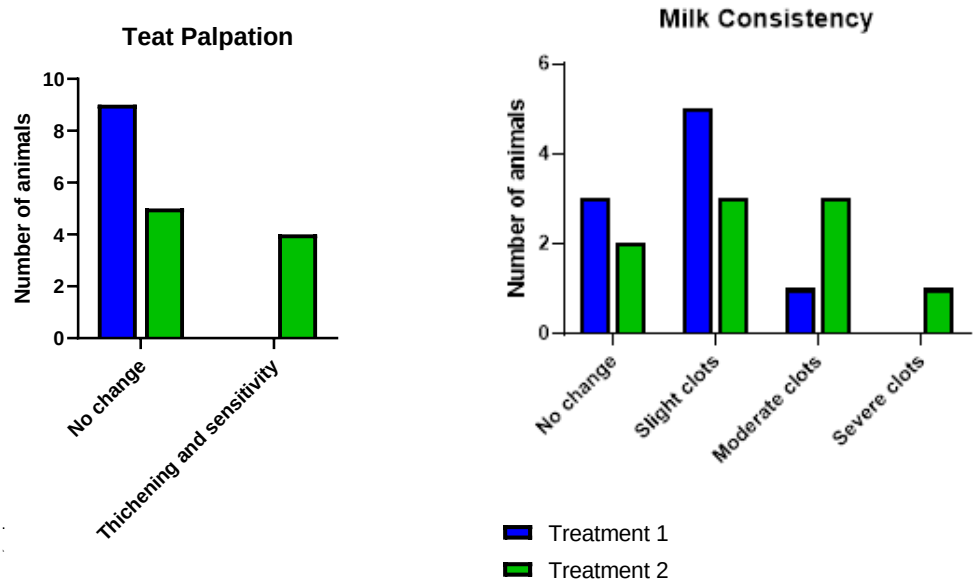

Figure 7. Teat palpation and milk consistency. Number of animals showing alterations in teat palpation and milk consistency in cows treated with a plant extract-based formulation. Treatment 1, shown in blue, received the standard dose, whereas Treatment 2, shown in green, received twice the standard dose.

For “mammary gland parenchyma consistency”, all observations were identical between groups ($p = 1.0$), indicating complete similarity. “Milk consistency” also showed no significant difference ($p = 0.371$). “Inflammatory signs” yielded a p-value of 0.0182 for the likelihood ratio chi-square; however, 100% of the cells had expected counts less than 5, and Fisher’s exact test produced $p = 0.0555$. These results suggest a trend toward greater severity in Treatment 2 (double dose) but are not statistically conclusive due to the small sample size and low expected counts, limiting the reliability of the chi-square test.

For the binary variable “teat palpation”, no changes were observed in Treatment 1, whereas four cases (44.4%) in Treatment 2 presented thickening and tenderness. The Chi-square test indicated a significant association between treatment and this variable ($\chi^2 = 5.14$; $p = 0.0233$). Nevertheless, Fisher’s exact test, which is more appropriate for small sample sizes, indicated only a trend ($p = 0.0824$), preventing rejection of the null hypothesis at the 5% significance level.

5. DISCUSSION

In the analyzed formulation, the combination of different plant extracts did not result in statistically significant systemic complications at the recommended dose. However, signs of local inflammation were observed, and the increase in SCC did not return to baseline levels during the evaluated observation period, suggesting that most animals may exhibit relevant local reactions or adverse effects consistent with non-infectious mastitis, likely triggered by the irritant or cytotoxic properties of certain compounds present in the formulation. The literature indicates that, in experimental models, exposure of mammary tissue to certain phytotherapeutics and antimicrobials can trigger different inflammatory patterns, which vary according to dose, duration of exposure, and the bioactive compounds involved (Chen et al., 2020; Yuan et al., 2023).

The inflammatory response observed is consistent with the physiological defense process of the organism against the introduction of substances into the mammary gland, such as the mobilization of neutrophils and macrophages and, consequently, the temporary increase in somatic cell count (Ezzat Alnakip et al., 2014). Although the response to the recommended dose is not significant, the intensity of the inflammatory clinical signs in the double-dose group may suggest an irritative or toxic effect of one of the formulation's constituents. Exacerbated inflammation may compromise the integrity of the mammary epithelium and, consequently, milk production and quality. Therefore, it is essential to distinguish physiological inflammatory reactions from adverse responses associated with cytotoxicity or the presence of potentially irritating compounds (Sintes et al., 2020). It should be emphasized that the responses observed in the mammary epithelium remain restricted to the mammary gland, due to its anatomical and functional compartmentalization, and do not result in systemic interference or adverse effects in other tissues.

The greater effectiveness against Salmonella Dublin, Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, and Bacillus cereus, in descending order, suggests that the tested formulation is more effective against Gram-negative microorganisms. This observation is possibly related to the plasma membrane, a primary target of bioactive compounds, as observed in previous studies with microorganisms genetically similar to those tested in this study (Vaou et al., 2021; Kaseke et al., 2023).

In addition, it is important to highlight the other components of the formulation, such as hydroxyethylcellulose, hyaluronic acid, aminomethylpropanol, methylparaben, propylparaben, and disodium edetate (EDTA). Hydroxyethylcellulose functions as a thickening agent, emulsion stabilizer, film former, and viscosity control agent, thereby improving the texture and stability of the formulation. Hyaluronic acid primarily acts as a humectant and moisturizer due to its high water-retention capacity; in addition, it accelerates wound healing and tissue regeneration (Bravo et al., 2022). Aminomethylpropanol serves as a pH adjuster, contributing to the physicochemical stability of the formulation. Parabens (methylparaben and propylparaben) act as antimicrobial preservatives widely used for their effectiveness against fungi, yeasts, and Gram-positive bacteria; their combined use enhances preservative activity. EDTA (disodium edetate), in turn, acts as a chelating agent, potentiating the action of preservatives such as parabens, including activity against Gram-negative microorganisms, thus improving the overall efficacy of antimicrobial and stability of formulation (Poddebniak and Kalinowska-Lis, 2024).

The tested formulation contains extracts of Baccharis trimera and Carica papaya fruit, as well as glycolic extracts of Stryphnodendron barbatiman and Aloe barbadensis leaves. The glycolic solvents used in these extracts, such as propylene glycol and glycerin, facilitate the solubilization of plant bioactive compounds and contribute to the physicochemical stability of the formulation (Padmawar and Bhadoriya, 2018; Yu and Goh, 2024). In the evaluated formulation, in addition to plant extracts, oils derived from *Carapa guianensis* seeds, *Rosmarinus officinalis* leaves, *Melaleuca alternifolia*, and *Copaifera officinalis* resin were included.

An important aspect to consider is the clinical safety of each plant extract, as the dosage and, consequently, the concentration of each extract may influence cytotoxicity. In the preliminary study, Stryphnodendron barbatiman, included at a low dose (100 mg) in the formulation, likely contributed to the positive outcomes observed, highlighting its strong potential for application and for the development of formulations targeting mastitis control (Ucella-Filho et al., 2022). However, most extracts still lack studies that adequately establish their clinical safety.

Based on the literature cited, it may be prudent to consider the removal of Baccharis trimera extract due to the potential, yet not scientifically confirmed, risks

associated with the presence of trimerosides and heavy metals. The exclusion of Melaleuca alternifolia may also be considered, given the potential risk to epithelial cells. It is noteworthy, however, that there is no evidence that tea tree oil causes damage to the mammary gland, similar to all the aforementioned extracts, oils, and their bioactive constituents.

Other feasible modifications include directing the use of *Carica papaya* extract from the leaves instead of the fruit, or alternatively, using extract from ripe fruits, to avoid the presence of enzymes predominantly concentrated in the latex. This precaution also applies to *Aloe vera* extract, recommending the use of only the inner gel, free of shell/latex fragments, to prevent high concentrations of anthraquinones, which are potentially irritating and may induce allergic reactions in some individuals. Another strategy for new formulations is the selective extraction of specific bioactive compounds while excluding those with some degree of toxicity. This applies, for instance, to the essential oil component α-pinene present in Rosmarinus officinalis and kaurenoic acid, which may be present in *Copaifera officinalis*. However, it is important to remember that the beneficial effects observed in each extract may be precisely due to the concomitant presence of its bioactive constituents, making the evaluation of their interactions a critical aspect in the development of safe and effective formulations.

There is also concern regarding the presence of contaminants (heavy metals, mycotoxins, etc.) and other biologically active phytochemicals, which may be carcinogenic, mutagenic, genotoxic, or induce other adverse effects. The composition of plant extracts can also vary depending on the extraction method, type of solvent used, and the plant part employed (Kaseke et al., 2023; Negri Brusamarello et al., 2024; Matei et al., 2025). Additionally, these results suggest a trend toward greater severity in Treatment 2 (double dose), but they are not statistically conclusive due to the small sample size, being limited to a preliminary tolerability trial of the tested formulation.

6. CONCLUSION

The present study demonstrates that the evaluated herbal intramammary formulation possesses promising in vitro antimicrobial activity against mastitis-associated pathogens while producing no detectable systemic alterations after intramammary administration. Therefore, a significant increase in somatic cell count and the presence of clinical signs in the mammary gland were observed, with more pronounced effects at twice the recommended dose. Although this response may reflect a transient inflammatory reaction, it may also indicate adverse effects of the bioactive compounds or plant extracts, as well as the need for dose adjustment or the exclusion of specific bioactive compounds from the formulation.

7. ACKNOWLEDGMENTS

The To the Postgraduate Program in Agricultural Microbiology, Universidade Estadual Paulista (UNESP), Faculty of Agricultural and Veterinary Sciences, Jaboticabal, São Paulo state of Brazil.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

8. REFERENCES

Abdoul-latif FM, Ainane A, Aboubaker IH, Mohamed J. Exploring the Potent Anticancer Activity of Essential Oils and Their Bioactive Compounds : Mechanisms and Prospects for Future Cancer Therapy. **Pharmaceuticals** 16(8):1086 26p. Doi: 10.3390/ph16081086.

Abouzed TK, Sadek KM, Ayoub MM, et al. Papaya extract upregulates the immune and antioxidants-related genes, and proteins expression in milk somatic cells of Friesian dairy cows. **J Anim Physiol Anim Nutr (Berl).** 2019;103(2):407-415. doi:10.1111/jpn.13032

Ahmad MM, Chatha SAS, Iqbal Y, Hussain AI, Khan I, Xie F. Recent trends in extraction, purification, and antioxidant activity evaluation of plant leaf-extract polysaccharides. **Biofuels, Bioprod Biorefining.** 2022;16(6):1820-1848. doi:10.1002/bbb.2405

Akinboye AO, Adeyemo RO, Karzis J, Petzer I marie, Mcgaw LJ. South African Journal of Botany Susceptibility patterns of *Escherichia coli* and streptococcal isolates from bovine mastitis cases to antibiotics and selected South African plant extracts with known antibacterial activities. **South African J Bot.** 2024;166:14-26. doi:10.1016/j.sajb.2024.01.014

Aldayarov N, Tulobaev A, Chekirov K. European Journal of Integrative Medicine An ethnobotanical study of plants and traditional herbal therapies in the Kara-Koy Gorge , Kyrgyz Republic. **Eur J Integr Med.** 2025;79(January):102529. doi:10.1016/j.eujim.2025.102529

Aleixo ÁA, Marjorie K, Herrera S, Iara R, Azambuja M De. Antibacterial activity of *Baccharis trimera* (Less .) DC . (carqueja) against bacteria of medical interest 1. Published online 2013:731-734.

Alhodieb FS, Farid M, Sabir M, Nisa S, Sarwar S, Abbas S. Exploring the bioactive compounds of *Carica papaya* leaves: phytol's role in combatting antibiotic-resistant bacteria**. Front Cell Infect Microbiol.** 2025;15(July):1-15. doi:10.3389/fcimb.2025.1564787

Almeida AC, Andrade VA, Fonseca FSA, et al. Acute and chronic toxicity and antimicrobial activity of the extract of *Stryphnodendron adstringens* (Mart.) Coville. **Pesqui Vet Bras.** 2017a;37(8):840-846. doi:10.1590/s0100-736x2017000800010

Almeida IV, Fernandes LM, Biazi BI, Vicentini VEP. Evaluation of the Anticancer Activities of the Plant Alkaloids Sanguinarine and Chelerythrine in Human Breast Adenocarcinoma Cells. **Anticancer Agents Med Chem**. 2017b;17(11):1586-1592. doi:10.2174/1871520617666170213115132

Alves de Souza BA, Braga LA, Oliveira Lopes LR, et al. Effects of Andiroba oil (*Carapa guianensis*) on wound healing in alloxan-diabetic rats. *Int Arch Med*. 2017;10(November). doi:10.3823/2533

Amber R, Adnan M, Tariq A, et al. Saudi Journal of Biological Sciences Antibacterial activity of selected medicinal plants of northwest Pakistan traditionally used against mastitis in livestock. **Saudi J Biol Sci.** 2018;25(1):154-161. doi:10.1016/j.sjbs.2017.02.008

Arya J, Krishnakumar K, George A, Chandran R, Prakash A, John M. Phytochemical characterization and biological evaluation of *Carica papaya* L . leaf alkaloids : In vitro and in silico insights into antioxidant , cytotoxicity , and anti-inflammatory activities.

Asghar N, Naqvi SAR, Hussain Z, et al. Compositional difference in antioxidant and antibacterial activity of all parts of the *Carica papaya* using different solvents. **Chem Cent J.** 2016;10(1):1-11. doi:10.1186/s13065-016-0149-0

Askari S, Rafati A, Aflakian F. Microbial Pathogenesis Alternative treatment candidates to antibiotic therapy for bovine mastitis in the post-antibiotic era : a comprehensive review**. Microb Pathog**. 2025;205(May):107684. doi:10.1016/j.micpath.2025.107684

Babou S, Chakit M, El Gui R, Mesfioui A, Sqalli-Houssaini Y. Acute and Subacute Toxicity Assessment of Ethanolic Extract of *Rosmarinus officinalis* in Female Wistar Rats**. Egypt J Vet Sci.** 2025;56(13):389-402. doi:10.21608/ejvs.2025.369735.2715

Baldivia D da S, Leite DF, de Castro DTH, et al. Evaluation of in vitro antioxidant and anticancer properties of the aqueous extract from the stem bark of *stryphnodendron adstringens*. **Int J Mol Sci.** 2018;19(8):1-23. doi:10.3390/ijms19082432

Bravo B, Correia P, Gonçalves Junior JE, Sant'Anna B, Kerob D. Benefits of topical hyaluronic acid for skin quality and signs of skin aging: From literature review to clinical evidence. **Dermatol Ther.** 2022;35(12):1-9. doi:10.1111/dth.15903

Campanholi K da SS, Junior RC da S, Morais FAP de, et al. Copaiba Oil-Based Emulsion as a Natural Chemotherapeutic Agent for the Treatment of Bovine Mastitis: In Vivo Studies. **Pharmaceutics**. 2023;15(2). doi:10.3390/pharmaceutics15020346

Caneschi A, Bardhi A, Barbarossa A, Zaghini A. Plant Essential Oils as a Tool in the Control of Bovine Mastitis: An Update. **Molecules**. 2023;28(8). doi:10.3390/molecules28083425

Carrasco SBV, Curay RR, Mazon-Velez D, Morales MC, Moreira JM, Bayas-Morejón F. Evaluation of the effectiveness of essential oil tea tree (*Melaleuca alternifolia*) against three bacterial genera isolated from bovine mastitis. **J Med Pharm Chem Res**. 2024;6(7):968-980. doi:10.48309/jmpcr.2024.442412.1116

Cavalcanti BC, Costa-Lotufo L V., Moraes MO, et al. Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in Copaiba oil***. Food Chem Toxicol***. 2006;44(3):388-392. doi:10.1016/j.fct.2005.08.011

Chacón O, Forno N, Lapierre L, Muñoz R, Fresno M. European Journal of

Integrative Medicine E ff ect of Aloe barbadensis Miller (*Aloe vera*) associated with beta-lactam antibiotics on the occurrence of resistance in strains of *Staphylococcus aureus* and *Streptococcus uberis*. **Eur J Integr Med.** 2019;32(September):100996. doi:10.1016/j.eujim.2019.100996

Chaijan S, Chaijan M, Saelee N, et al. Decoding the role of leaf maturity in shaping metabolite landscapes and enhancing bioactive properties of Thai papaya (*Carica papaya L*.): Insights from in vitro analysis*. **Ind Crops Prod.*** 2025;230(April):121095. doi:10.1016/j.indcrop.2025.121095

Chen Z, Zhang Y, Zhou J, et al. Tea Tree Oil Prevents Mastitis-Associated Inflammation in Lipopolysaccharide-Stimulated Bovine Mammary Epithelial Cells. **Front Vet Sci.** 2020;7(August):1-9. doi:10.3389/fvets.2020.00496

Chidananda C, Thakur G, Datta D, Popat K. A comprehensive review of alkaloids in cancer therapy : focusing on molecular mechanisms and synergistic potential of piperine in colorectal cancer. *3* **Biotech.** 2025;15(11):1-29. doi:10.1007/s13205-025-04535-8

Contreras GA, Rodríguez JM. Mastitis: Comparative etiology and epidemiology. **J Mammary Gland Biol Neoplasia.** 2011;16(4):339-356. doi:10.1007/s10911-011-9234-0

Corona-Gómez L, Hernández-Andrade L, Mendoza-Elvira S, Suazo FM, Ricardo-González DI, Quintanar-Guerrero D. In vitro antimicrobial effect of essential tea tree oil(Melaleuca alternifolia), thymol, and carvacrol on microorganisms isolated from cases of bovine clinical mastitis. **Int J Vet Sci Med**. 2022;10(1):72-79. doi:10.1080/23144599.2022.2123082

Costa-Silva JH, Lima CR, Silva EJR, et al. Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliaceae) seed oil. **J Ethnopharmacol.** 2008;116(3):495-500. doi:10.1016/j.jep.2007.12.016

Da Cruz Pádua B, Victor J, Júnior R, et al. Send Orders for Reprints to reprints@benthamscience.net *Baccharis trimera* Improves the Antioxidant Defense System and Inhibits iNOS and NADPH Oxidase Expression in a Rat Model of Inflammation. **Curr Pharm Biotechnol.** 2013;14:975-984.

da Silva ARH, Lopes LQS, Cassanego GB, et al. Acute toxicity and antimicrobial activity of leaf tincture *Baccharis trimera* (Less). **Biomed J.** 2018;41(3):194-201. doi:10.1016/j.bj.2018.04.006

de Faria MJM, Braga CA da SB, de Paula JR, et al. Antimicrobial activity of copaifera spp. Against bacteria isolated from milk of cows with mastitis**. Cienc Anim Bras.** 2017;18(1):1-14. doi:10.1590/1089-6891v18e-39068

de Freitas ALD, Kaplum V, Rossi DCP, et al. Proanthocyanidin polymeric tannins from *Stryphnodendron adstringens* are effective against *Candida* spp. isolates and for vaginal candidiasis treatment. **J Ethnopharmacol.** 2018;216(January):184-190. doi:10.1016/j.jep.2018.01.008

de Mello NP, Carlos Ramos Espinoza F, da Silva Claudiano G, et al. Copaiba oil's bactericidal activity and its effects on health and zootechnical performance for Nile tilapia after oral supplementation. **Sci Rep.** 2024;14(1):1-14. doi:10.1038/s41598-024-66024-x

de Melo RN, de Goes VFF, Canelli AP, et al. Antibacterial activity of barbatimão (*Stryphnodendron adstringens*) against periodontopathogens and cytotoxic effects on fibroblasts. **Lett Appl Microbiol.** 2023;76(6):1-6. doi:10.1093/lambio/ovad054

de Souza MR, Silva AÉVN, de Faria Silva Souza R, et al. Treatment of subclinical mastitis in dairy goats experimentally infected with *Staphylococcus aureus* using natural therapeutic protocol. **Trop Anim Health Prod**. 2024;56(5). doi:10.1007/s11250-024-04026-7

Debruyn E, Zaheer N, Peng J, Kumar H, Gogoi-tiwari J. Research in Veterinary Science Alternative approaches for bovine mastitis treatment : A critical review of emerging strategies , their effectiveness and limitations. **Res Vet Sci.** 2025;185(December 2024):105557. doi:10.1016/j.rvsc.2025.105557

Dias KKB, Cardoso AL, da Costa AAF, et al. Biological activities from andiroba (Carapa guianensis Aublet.) and its biotechnological applications: A systematic review. **Arab J Chem.** 2023;16(4). doi:10.1016/j.arabjc.2023.104629

Domingues N, Ramos L de P, Pereira LM, et al. Antimicrobial action of four herbal plants over mixed-species biofilms of Candida albicans with four different microorganisms. **Aust Endod J.** 2023;49(2):262-271. doi:10.1111/aej.12681

Dos Santos MS, Da Silva J, Menezes APS, et al. Biotoxicological Analyses of Trimeroside from *Baccharis trimera* Using a Battery of in Vitro Test Systems. **Oxid Med Cell Longev**. 2018;2018. doi:10.1155/2018/7804135

Duda-madej A, Viscardi S, Szewczyk W. Natural Alkaloids in Cancer Therapy : Berberine , Sanguinarine and Chelerythrine against Colorectal and Gastric Cancer. Published online 2024.

Ekambaram SP, Paramasivam S, Perumal SS, Dhakshinamurthy SS. Rosmarinus officinalis L.: A Source of Potential Antimicrobial Agents for Combating Antimicrobial Resistance. **Lett Appl NanoBioScience**. 2024;13(1). doi:10.33263/LIANBS131.045

Exel CE, Halasa T, Koop G, et al. A stochastic modelling approach to determine the effect of diverse *Staphylococcus aureus* strains on the economic and epidemiological outcomes of mastitis intervention strategies in dairy cattle. **Prev Vet Med.** 2021;199(April 2021):105566. doi:10.1016/j.prevetmed.2021.105566

Ezzat Alnakip M, Quintela-Baluja M, Böhme K, et al. The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. **J Vet Med.** 2014;2014:1-31. doi:10.1155/2014/659801

Fachinetto JM, Tedesco SB. Atividade antiproliferativa e mutagênica dos extratos aquosos de *Baccharis trimera* (Less.) A. P. de Candolle e *Baccharis articulata* (Lam.) Pers. (Asteraceae) sobre o sistema teste de Allium cepa. **Rev Bras Plantas**

Med. 2009;11(4):360-367. doi:10.1590/s1516-05722009000400002

Fan K qin, Zhang L, Song F, et al. Pharmacological properties and therapeutic potential of berberine: a comprehensive review. **Front Pharmacol.** 2025;16(August):1-26. doi:10.3389/fphar.2025.1604071

FEITOSA, F. L. F. Semiologia da glândula mamária de ruminantes. In: FEITOSA, F. L. F. (org.). Semiologia veterinária: a arte do diagnóstico. 4. ed. Rio de Janeiro: Roca, 2020. Cap. 9, Seção C, p. 293-320.

Fiordalisi SAL, Honorato LA, Loiko MR, et al. The effects of Brazilian propolis on etiological agents of mastitis and the viability of bovine mammary gland explants**. J Dairy Sci.** 2016;99(3):2308-2318. doi:10.3168/jds.2015-9777

Francoz D, Wellemans V, Dupré JP, et al. Invited review : A systematic review and qualitative analysis of treatments other than conventional antimicrobials for clinical mastitis in dairy cows. **J Dairy Sci.** 2017;100(10):7751-7770. doi:10.3168/jds.2016-12512

Frazão DR, Cruz JN, Santana de Oliveira M, et al. Evaluation of the biological activities of Copaiba (*Copaifera* spp): a comprehensive review based on scientometric analysis. **Front Pharmacol.** 2023;14(September):1-14. doi:10.3389/fphar.2023.1215437

Gadallah AH, Hafez RS, Fahim KM, Ahmed LI. Application of rosemary oil nano-emulsion as antimicrobial and antioxidant natural alternative in pasteurized cream and Karish cheese. **Int J Food Microbiol**. 2024;422(July):110823. doi:10.1016/j.ijfoodmicro.2024.110823

Garcia-Navarro, CEK. Manual de Hematologia Veterinária. 2. ed. São Paulo: Varela, 2005. 206 p.

García-Tirado, J., C. Rieger-Reyes, and P. Saz-Peiró. 2012. Efecto de los flavonoides en la prevención del cáncer de pulmón: revisión sistemática. **Med. Clin. (Barc)**. 139:358–363. https://doi.org/10.1016/j.medcli.2012.02.003.

Gené RM, Cartañá C, Adzet T, Marín E, Parella T, Cañigueral S. Anti-inflammatory and analgesic activity of *Baccharis trimera*: Identification of its active constituents. **Planta Med.** 1996;62(3):232-235. doi:10.1055/s-2006-957866

Gong Q, Li Y, Ma H, et al. Peiminine Protects against Lipopolysaccharide-Induced Mastitis by Inhibiting the AKT / NF- κ B , ERK1 / 2 and p38 Signaling Pathways. Published online 2018. doi:10.3390/ijms19092637

Grance SRM, Teixeira MA, Leite RS, et al. Baccharis trimera: Effect on hematological and biochemical parameters and hepatorenal evaluation in pregnant rats. **J Ethnopharmacol.** 2008;117(1):28-33. doi:10.1016/j.jep.2007.12.020

Gu W, Hao X, Wang Z, Zhang J, Huang L. Plant Diversity Ethnobotanical study on medicinal plants from the Dragon Boat Festival herbal markets of Qianxinan ,

southwestern Guizhou , China. **Plant Divers.** 2021;42(6):427-433. doi:10.1016/j.pld.2020.12.010

Han C, Bao H. Therapeutic effects and mechanisms of Inonotus hispidus extract and active monomer compounds in a rat mammary gland hyperplasia model. **J Ethnopharmacol.** 2024;319(P2):117274. doi:10.1016/j.jep.2023.117274

Hashem AE, Elmasry IH, Lebda MA, et al. Characterization and antioxidant activity of nano-formulated berberine and *cyperus rotundus* extracts with anti-inflammatory effects in mastitis-induced rats. **Sci Rep.** 2024;14(1):1-18. doi:10.1038/s41598-024-66801-8

Herawati Y, Kalsum U, Arsana IW, Yuniarti L, Sardjono TW. Carica papaya leaf ethanol extract effect on milk volume, β-casein gene (Csn2) expression, β-casein levels, and milk total protein levels. **Pharm Sci Asia.** 2022;49(2):193-201. doi:10.29090/psa.2022.02.21.158

Higa KC, Limoeiro B, Filho M, et al. Brazilian Journal of health Review Brazilian **Journal of health Review**. Published online 2020:1094-1108. doi:10.34119/bjhrv3n1-085

Huang Y, Xu H, Ding M, et al. Screening of Rosemary Essential Oils with Different Phytochemicals for Antioxidant Capacity, Keratinocyte Cytotoxicity, and Anti-Proliferative Activity. **Molecules**. 2023;28(2). doi:10.3390/molecules28020586

Jha P, Anjum V, Al AA, et al. Plant metabolites and functional foods in metastatic breast cancer : a supportive strategy for management. 2025;(July):1-23. doi:10.3389/fphar.2025.1631232

Karimi A, Majlesi M, Rafieian-Kopaei M. Herbal versus synthetic drugs; beliefs and facts. **J nephropharmacology**. 2015;4(1):27-30. http://www.ncbi.nlm.nih.gov/pubmed/28197471%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5297475

Kaseke TB, Chikwambi Z, Gomo C, Mashingaidze AB, Murungweni C. Antibacterial activity of medicinal plants on the management of mastitis in dairy cows: A systematic review. **Vet Med Sci.** 2023;9(6):2800-2819. doi:10.1002/vms3.1268

Khan NF, Bashir A, Ganaie KA, et al. Chemical composition and anti-microbial potential of essential oils from morphologically distinct Salvia rosmarinus (Spenn.) cultivars from Kashmir, India**. Front Microbiol**. 2025;16(July). doi:10.3389/fmicb.2025.1579383

Klaas IC, Zadoks RN. An update on environmental mastitis: Challenging perceptions. **Transbound Emerg Dis**. 2018;65:166-185. doi:10.1111/tbed.12704

Kowalczyk A, Przychodna M, Sopata S, Bodalska A, Fecka I. Thymol and Thyme Essential Oil—New Insights into Selected Therapeutic Applications. Published online 2020. doi:10.3390/molecules25184125

Ksouri S, Djebir S, Bentorki AA, Gouri A, Hadef Y, Benakhla A. Antifungal activity of

essential oils extract from Origanum floribundum Munby, *Rosmarinus officinalis L*. and *Thymus ciliatus* Desf. against *Candida albicans* isolated from bovine clinical mastitis. **J Mycol Med**. 2017;27(2):245-249. doi:10.1016/j.mycmed.2017.03.004

Kuczyńska M, Kot M, Stocki M, et al. In Vitro Determination of Cytotoxic Effects of Ten Essential Oils on Prototheca bovis, Which Causes Mastitis in Dairy Cows. **Int J Mol Sci.** 2025;26(12):1-31. doi:10.3390/ijms26125451

Kumai A, Tsugami Y, Wakasa H, et al. Adverse Effects of Coumestrol and Genistein on Mammary Morphogenesis and Future Milk Production Ability of Mammary Epithelial Cells. 2020;1900187:1-13. doi:10.1002/adbi.201900187

Kumar T, Buragohain R, Nath R, Kumar T. Phytomedicine Plus Enhanced plasma persistence of ceftriaxone following single intramuscular injection with prior oral dosing of a mammary protective commercial herbal drug as complementary therapy. **Phytomedicine Plus**. 2022;2(2):100228. doi:https://doi.org/10.1016/j.phyplu.2022.100228

Kumar V, Vamsi M, Ausali S, et al. Microbial Pathogenesis Dietary anti-inflammatory and anti-bacterial medicinal plants and its compounds in bovine mastitis associated impact on human life. **Microb Pathog.** 2024;192(April):106687. doi:10.1016/j.micpath.2024.106687

kumarasinghe HS, Kim JH, Kim SL, et al. Bioactive constituents from *Carica papaya* fruit: implications for drug discovery and pharmacological applications. **Appl Biol Chem.** 2024;67(1). doi:10.1186/s13765-024-00962-y

Leal A de B, Hackbart HC dos S, Macueia FBED, Rombaldi CV. Andiroba oil (*Carapa guianensis*): Descriptive analysis and applicability. **Ind Crops Prod.** 2023;202(May):117004. doi:10.1016/j.indcrop.2023.117004

Leandro LM, De Sousa Vargas F, Barbosa PCS, Neves JKO, Da Silva JA, Da Veiga-Junior VF. Chemistry and biological activities of terpenoids from copaiba (*Copaifera* spp.) oleoresins. **Molecules**. 2012;17(4):3866-3889. doi:10.3390/molecules17043866

Lopes TS, Fontoura PS, Oliveira A, Rizzo FA, Silveira S, Felipe A. Research in Veterinary Science Use of plant extracts and essential oils in the control of bovine mastitis. **Res Vet Sci.** 2020;131(October 2019):186-193. doi:10.1016/j.rvsc.2020.04.025

Ma A, Ms B, Vmd SX, Xie H, Ms B. Clinical Application of. *Vet Clin NA Small* **Anim Pract**. 2025;55(6):941-962. doi:10.1016/j.cvsm.2025.06.008

Magtalas TJ, Anapi GR. Evaluation of Bacteriostatic Effect of Rosemary and Oregano Essential Oils Against a Non-Pathogenic Surrogate of Salmonella spp. (*E. coli* ATCC 9637). Published online 2025:7. doi:10.3390/blsf2024040007

Martínez-navarrete N, Mar M, Vidal C, José J, Lahuerta M. Actividad Dietética Los compuestos bioactivos de las frutas y sus efectos en la salud. **Act Diet.** 2008;12(2):64-68. doi:10.1016/S1138-0322(08)75623-2

Masuku NP, Erhabor RC, Mokoka TA, Ramagoma RB, Maqolo K, Mcgaw LJ. South African Journal of Botany South African medicinal plants as natural defences against bovine mastitis : a review. **South African J Bot.** 2025;184(February):1234-1253. doi:10.1016/j.sajb.2025.07.019

Matei CE, Visan AI, Cristescu R. Aloe Vera Polysaccharides as Therapeutic Agents: Benefits Versus Side Effects in Biomedical Applications. **Polysaccharides**. 2025;6(2):36. doi:10.3390/polysaccharides6020036

Matouskova K, Szabo GK, Daum J, et al. Best practices to quantify the impact of reproductive toxicants on development, function, and diseases of the rodent mammary gland. Published online 2023:51-67. doi:10.1016/j.reprotox.2022.06.011

Menezes AC dos S, Alves LDB, Goldemberg DC, de Melo AC, Antunes HS. Anti-inflammatory and wound healing effect of Copaiba oleoresin on the oral cavity: A systematic review. **Heliyon**. 2022;8(2). doi:10.1016/j.heliyon.2022.e08993

Miah K, Nazmul AAHM, Sanzia H, Masud MGM, Sabina P. *Carica Papaya* in Health and Disease : A Review of Its Bioactive Compounds for Treating Various Disease Conditions, Including Anti - Inflammatory and Anti - Arthritic Activities. Vol 33. Springer International Publishing; 2025. doi:10.1007/s10787-025-01780-4

Monteiro I de S, Fonseca ASA, dos Santos CR, et al. The Development and Characterization of an Andiroba Oil-Based Nanoemulsion (*Carapa guianensis*, Aubl.): Insights into Its Physico-Chemical Features and In Vitro Potential Healing Effects. **Pharmaceutics**. 2025;17(4). doi:10.3390/pharmaceutics17040498

Morales-Ubaldo AL, Rivero-Perez N, Valladares-Carranza B, Velázquez-Ordoñez V, Delgadillo-Ruiz L, Zaragoza-Bastida A. Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. **Vet Anim Sci.** 2023;21(July). doi:10.1016/j.vas.2023.100306

Moshiri A, Puppo M, Rossi M, Gherzi R, Briata P. Biochimica et Biophysica Acta Resveratrol limits epithelial to mesenchymal transition through modulation of KHSRP / hnRNPA1-dependent alternative splicing in mammary gland cells. *BBA* **- Gene Regul Mech.** 2017;1860(3):291-298. doi:10.1016/j.bbagrm.2017.01.001

Motaghi S, Shohreh B, Nazem M, Ahmadusefi N. Effects of Long-term Administration of Rosemarinus Officinalis Essential Oil on Blood Factors and Liver and Kidney Cells in Adult Rats Essential Oil on Blood Factors and Liver and Kidney Cells in Adult Rats. **Number 1 Anat Sci**. 2021;18(1):23-30.

Negri Brusamarello M, Brusamarello AP, Alves da Cunha MA, Busso C. Biological and phytochemical potential of *Baccharis trimera* (Less.) DC leaf extract on swine clinical isolates. **Nat Prod Res.** 2024;38(10):1799-1805. doi:10.1080/14786419.2023.2222217

Ogbuadike EC, Nkadimeng SM, Igwe CC, et al. South African Journal of Botany An in vitro study on the potential of selected South African plant extracts to prevent and treat bovine mastitis. **South African J Bot.** 2023;154:98-107. doi:10.1016/j.sajb.2023.01.028

Padmawar AR, Bhadoriya U. Glycol and Glycerin: Pivotal Role in Herbal Industry as Solvent/Co-Solvent. **World J Pharm Med Res.** 2018;4(5):153-155. www.wjpmr.com

Pádua C, Dornela L, Victor J, et al. Antioxidant properties of *Baccharis trimera* in the neutrophils of Fisher rats. 2010;129:381-386. doi:10.1016/j.jep.2010.04.018

Park JH, Park CY. The Effect of Terpinen-4-ol on Human Corneal Epithelium. **Transl Vis Sci Technol.** 2024;13(12):1-9. doi:10.1167/tvst.13.12.18

Paşca C, Al Mărghitaș L, Dezmirean D, et al. The Assessment of the Antibacterial Activity of some Plant Extracts on Normal and Pathogenic Microflora from Milk. **Sci Pap Anim Sci Biotechnol**. 2015;48(1):48.

Peixoto R de M, Araújo R de MP, Bomfim SAG, et al. Treatment of goat mastitis experimentally induced by *Staphylococcus aureus* using a formulation containing Hymenaea martiana extract. 2015;130:229-235. doi:https://doi.org/10.1016/j.smallrumres.2015.07.009

Pereira da Silva V, de Carvalho Brito L, Mesquita Marques A, da Cunha Camillo F, Raquel Figueiredo M. Bioactive limonoids from *Carapa guianensis* seeds oil and the sustainable use of its by-products. **Curr Res Toxicol**. 2023;4(January). doi:10.1016/j.crtox.2023.100104

Pereira M de PM, Januario GB, Fonseca LM da, Assis DCS de, Brito JCM. Propolis as a therapeutic alternative in mastitis treatment : A systematic review of veterinary clinical trials. 2025;197(November). doi:doi.org/10.1016/j.rvsc.2025.105946

Piaia L, Pittella CQP, De Souza SS, Berti FV, Porto LM. Incorporation of *Aloe vera* extract in bacterial nanocellulose membranes. **Polimeros**. 2022;32(1). doi:10.1590/0104-1428.210062

Pieri FA, Mussi MC, Fiorini JE, Schneedorf JM. Efeitos clínicos e microbiológicos do óleo de copaíba (*Copaifera officinalis*) sobre bactérias formadoras de placa dental em cães. **Arq Bras Med Vet e Zootec**. 2010;62(3):578-585. doi:10.1590/S0102-09352010000300012

Pinto SCG, Bueno FG, Panizzon GP, et al. *Stryphnodendron adstringens*: Clarifying Wound Healing in Streptozotocin-Induced Diabetic Rats. **Planta Med**. 2015;81(12-13):1090-1096. doi:10.1055/s-0035-1546209

Poddebniak P, Kalinowska-Lis U. Applied Sciences. **Appl Sci.** 2024;14:1-15. doi:https://doi.org/10.3390/app14041581

Podolak I, Galanty A, Sobolewska D. Saponins as cytotoxic agents : a review. 2010;(June):425-474. doi:10.1007/s11101-010-9183-z

Regildo M. G. Silva. Genotoxic activity and toxicity of *Baccharis trimera* Less. regarding the bioaccumulation of heavy metals. **J Med Plants Res**. 2012;6(26):4321-4327. doi:10.5897/jmpr12.472

Reis TC, Paiva LF d., Santos VHM, Gonçalves CP, Costa FEC, Pereira RM.

Biological activity in hydroethanolic extracts from bark, stem, and leaves of the *Stryphnodendron adstringens* (Mart.) Coville. **Braz J Biol.** 2025;84:e286845. doi:10.1590/1519-6984.286845

Rizwan M, Zameer A, Ahmad T, Saleem S. Comparative therapeutic efficacy of procaine penicillin, Phyllanthus emblica fruit extract and *Cocos nucifera* oil against subclinical mastitis. **Livest Sci**. 2021;251(August):104655. doi:10.1016/j.livsci.2021.104655

Ruhaizat-Ooi IH, Zainal-Abidin RA, Ab Ghani NS, et al. Understanding the Complex Functional Interplay between Glucosinolates and Cyanogenic Glycosides in Carica papaya. **Agronomy**. 2022;12(10). doi:10.3390/agronomy12102508

Saini D, Kumar P, Jitendra C, Chaudhary K, Kaur H, Kumar G. Molecular mechanisms of antiproliferative and pro-apoptotic effects of essential oil active constituents in MCF7 and T24 cancer cell lines : in vitro insights and in silico modelling of proapoptotic gene product- compound interactions. Published online 2025:805-825.

Sanajou S, Ülker Özkan R, Erkekoğlu P, Girgin G, Baydar T. Safety and possible risks of tea tree oil from a toxicological perspective. **İstanbul J Pharm.** 2024;0(0):0-0. doi:10.26650/istanbuljpharm.2024.1434421

Segun PA, Ismail FMD, Ogbole OO, et al. Acridone alkaloids from the stem bark of Citrus aurantium display selective cytotoxicity against breast, liver, lung and prostate human carcinoma cells. **J Ethnopharmacol.** 2018;227(September):131-138. doi:10.1016/j.jep.2018.08.039

Şehitoğlu MH, Öztopuz RÖ, Kılınç N, Ovalı MA, Büyük B, Gulcin İ. Thymol regulates the Endothelin-1 at gene expression and protein synthesis levels in septic rats. **Chem Biol Interact**. 2023;375(February):110426. doi:10.1016/j.cbi.2023.110426

Sharun K, Dhama K, Tiwari R, Bashir M, Iqbal M. Advances in therapeutic and managemental approaches of bovine mastitis : a comprehensive review***. Vet Q.*** 2021;41(1):107-136. doi:10.1080/01652176.2021.1882713

Silva CRD, Oliveira MBN, Motta ES, et al. Genotoxic and cytotoxic safety evaluation of papain (*Carica papaya* L.) using in vitro assays**. J Biomed Biotechnol**. 2010;2010. doi:10.1155/2010/197898

Simplício JLF, Junior MV d. S, Barros JDS, et al. Innovative Polymeric Sponges Loaded with Andiroba Oil and Cu(II) Complex: Synthesis, Characterization, Controlled Release, and Antimicrobial Activity. **ChemistrySelect**. 2025;10(26):1-11. doi:10.1002/slct.202500502

Singh SP, Kumar S, Mathan S V., et al. Therapeutic application of *Carica papaya* leaf extract in the management of human diseases. *DARU,* **J Pharm Sci.** 2020;28(2):735-744. doi:10.1007/s40199-020-00348-7

Sintes GF, Bruckmaier RM, Wellnitz O. Nonsteroidal anti-inflammatory drugs affect

the mammary epithelial barrier during inflammation. **J Dairy Sci.** 2020;103(11):10742-10753. doi:10.3168/jds.2020-18818

Soliman MM, Elsaba YM, Soliman MSA, Ahmed EZ. Composition and antimicrobial activity of Rosmarinus officinalis L. and *Artemisia monosperma* L. leaf essential oils and methanolic extracts from plants grown in normal and saline habitats in Egypt. **Sci Rep.** 2024;14(1):1-10. doi:10.1038/s41598-024-57301-w

Souza NC de, Gomes MN, Maciel RRG, et al. Evaluation of the Antimicrobial Activity of <i>*Stryphnodendron barbatiman*</i> against <i>Citrobacter freundii</i> *Mater Sci Appl*. 2013;04(12):780-785. doi:10.4236/msa.2013.412099

Sserunkuma P, Mcgaw LJ, Nsahlai I V, Staden J Van. South African Journal of Botany Selected southern African medicinal plants with low cytotoxicity and good activity against bovine mastitis pathogens. **South African J Bot**. 2017;111:242-247. doi:10.1016/j.sajb.2017.03.032

Taga I, Lan CQ, Altosaar I. Plant essential oils and mastitis disease: Their potential inhibitory effects on pro-inflammatory cytokine production in response to bacteria related inflammation. **Nat Prod Commun**. 2012;7(5):675-682. doi:10.1177/1934578x1200700534

Tang Y, Zhang X, Wang Y, et al. Dietary ellagic acid ameliorated Clostridium perfringens-induced subclinical necrotic enteritis in broilers via regulating inflammation and cecal microbiota. **J Anim Sci Biotechnol**. 2022;13(1):1-18. doi:10.1186/s40104-022-00694-3

Tao J jun, Xiang J juan, Jiang M, Kuang S fang, Peng X xian. A Microtitre Plate Dilution Method for Minimum Killing Concentration Is Developed to Evaluate Metabolites-Enabled Killing of Bacteria by β -lactam Antibiotics. 2022;9(June):1-9. doi:10.3389/fmolb.2022.878651

Taychaworaditsakul W, Saenjum C, Lumjuan N, et al. Safety of Oral *Carica papaya L*. Leaf 10% Ethanolic Extract for Acute and Chronic Toxicity Tests in Sprague Dawley Rats. **Toxics**. 2024;12(3):1-20. doi:10.3390/toxics12030198

Tomanić D, Božić DD, Kladar N, et al. Clinical Evidence on Expansion of Essential Oil-Based Formulation's Pharmacological Activity in Bovine Mastitis Treatment: Antifungal Potential as Added Value. **Antibiotics**. 2024;13(7). doi:10.3390/antibiotics13070575

Trevisan DAC, da Silva P V., Farias ABP, et al. Antibacterial activity of Barbatimão (Stryphnodendron adstringens) against *Staphylococcus aureus*: in vitro and in silico studies. **Lett Appl Microbiol.** 2020;71(3):259-271. doi:10.1111/lam.13317

Tsugami Y, Nii T, Isobe N. Effects of topical application of resveratrol on tight junction barrier and antimicrobial compound production in lactating goat mammary glands. **Vet Res.** 2024;55(1):20. doi:10.1186/s13567-024-01276-z

Ucella-Filho JGM, Lorenço MS, Souza EC, et al. 20302-75344-1-Pb.

BioResources. 2022;17(4):6578–6587. doi:10.15376/biores.17.4.6578-6587

Udoh F V., Udoh PB. Hepatotoxicity of the methanol extract of *Carica papaya* (Paw-Paw) seeds in wistar rats. **Pharm Biol**. 2005;43(4):349-352. doi:10.1080/13880200590951810

Vaou N, Stavropoulou E, Voidarou C, Tsigalou C, Bezirtzoglou E. Towards advances in medicinal plant antimicrobial activity: A review study on challenges and future perspectives. **Microorganisms**. 2021;9(10):1-28. doi:10.3390/microorganisms9102041

Veleirinho MBR, Filho LCPM, Kuhnen S. The effects of Brazilian propolis on etiological agents of mastitis and the viability of bovine mammary gland explants. **J Dairy Sci.** 2016;99(3):2308-2318. doi:10.3168/jds.2015-9777

Vila Nova BG, Silva L dos S, Andrade M da S, et al. The essential oil of Melaleuca alternifolia incorporated into hydrogel induces antimicrobial and anti-inflammatory effects on infected wounds by *Staphylococcus aureus*. **Biomed Pharmacother.** 2024;173(December 2023). doi:10.1016/j.biopha.2024.116389

Wagner H, Ulrich-Merzenich G. Synergy research: Approaching a new generation of phytopharmaceuticals. **Phytomedicine**. 2009;16(2-3):97-110. doi:10.1016/j.phymed.2008.12.018

Wei SM, Zhao X, Yu J, et al. Characterization of tea tree oil nanoemulsion and its acute and subchronic toxicity. **Regul Toxicol Pharmacol**. 2021;124(July):104999. doi:10.1016/j.yrtph.2021.104999

Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. **Nat Protoc**. 2008;3(2):163-175. doi:10.1038/nprot.2007.521

Williams DJ, Pun S, Chaliha M, Scheelings P, O'Hare T. An unusual combination in papaya (*Carica papaya*): The good (glucosinolates) and the bad (cyanogenic glycosides). **J Food Compos Anal**. 2013;29(1):82-86. doi:10.1016/j.jfca.2012.06.007

Ximenes TVN, Carvalho R, Bonfá IS, et al. *Baccharis trimera* Infusion Reduces Macrophages Activation and High-Fat Diet-Induced Metabolic Disorders in Mice. **Pharmaceuticals**. 2022;15(10):1-15. doi:10.3390/ph15101258

Yang X, Pei X, Zhang H, Zhang W. Mechanisms of single herbs and herbal pairs in the treatment of mammary gland hyperplasia: An integrated review. **Heliyon**. 2023;9(11):e21000. doi:10.1016/j.heliyon.2023.e21000

You Z, Sun J, Xie F, et al. Modulatory Effect of Fermented Papaya Extracts on Mammary Gland Hyperplasia Induced by Estrogen and Progestin in Female Rats. **Oxid Med Cell Longev.** 2017;2017. doi:10.1155/2017/8235069

Yu S, Liu X, Yu D, E C, Yang J. Piperine protects LPS-induced mastitis by inhibiting inflammatory response. **Int Immunopharmacol.** 2020;87(January):106804.

doi:10.1016/j.intimp.2020.106804

Yuan C, Ma X, Jiang M, et al. Effects of Tea Tree Oil on Production Performance, Serum Parameter Indices, and Immunity in Postpartum Dairy Cows. **Animals**. 2023;13(4):1-9. doi:10.3390/ani13040682

Zadoks RN, Middleton JR, McDougall S, Katholm J, Schukken YH. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. **J Mammary Gland Biol Neoplasia.** 2011;16(4):357-372. doi:10.1007/s10911-011-9236-y

Zhang J, Liu K, Yang T, et al. Mechanism of Astragalus Polysaccharide in Alleviating Bovine Mammary Fibrosis Through ROS / NLRP3 Inhibition and EMT Regulation. Published online 2025.

Zheng Z, Zheng Y, Liang X, Xue G, Wu H. Sanguinarine Enhances the Integrity of the Blood–Milk Barrier and Inhibits Oxidative Stress in Lipopolysaccharide-Stimulated Mastitis. **Cells**. 2022;11(22):3658. doi:10.3390/cells11223658

Zhou X, Ullah A, Shi L, et al. Molecular Regulatory Mechanisms of Mammary Gland Development : A Review. Published online 2025.

Zhou Y, Lan R, Xu Y, Zhou Y, Lin X, Miao J. Resveratrol alleviates oxidative stress caused by Streptococcus uberis infection via activating the Nrf2 signaling pathway. **Int Immunopharmacol.** 2020;89(July):107076. doi:10.1016/j.intimp.2020.107076

Zhu M, Sun Y, Bai H, et al. Effects of saponins from Chinese herbal medicines on signal transduction pathways in cancer : A review. 2023;(March):1-17. doi:10.3389/fphar.2023.1159985

Zhu Z, Zhang Y, Wang R, Dong Y, Wu J, Shao L. Ecotoxicology and Environmental Safety Zinc oxide nanoparticles disrupt the mammary epithelial barrier via Z-DNA binding protein 1-triggered PANoptosis. 2024;283(February). doi:doi.org/10.1016/j.ecoenv.2024.116777

APÊNDICES

Supplementary Material 1. Mean, standard deviation, and ANOVA results for hematological parameters, leukocyte profile, serum enzymatic activity.

Hematological parameters: mean and standard deviation

Variable	Treatment 1			Treatment 2		
	-3	0.5	7	-3	0.5	7
RBC (×10⁶/µL)	5.46 ± 0.62	5.16 ± 0.48	5.30 ± 0.50	6.49 ± 0.98	5.98 ± 0.91	6.09 ± 0.46
HGB (g/dL)	8.38 ± 0.86	8.00 ± 0.68	8.19 ± 0.61	9.42 ± 1.17	8.62 ± 0.85	8.97 ± 0.42
HCT (%)	25.68 ± 2.15	24.02 ± 2.20	24.84 ± 1.68	28.27 ± 3.55	26.31 ± 2.62	26.81 ± 1.59
MCV (fL)	47.40 ± 4.73	46.72 ± 4.45	47.11 ± 4.22	43.78 ± 2.79	44.26 ± 2.95	44.17 ± 2.84
MCH (pg)	15.41 ± 1.16	15.56 ± 1.21	15.51 ± 1.18	14.59 ± 0.91	14.51 ± 0.91	14.79 ± 1.01
MCHC (g/dL)	32.60 ± 1.42	33.33 ± 1.05	32.96 ± 1.02	33.34 ± 1.00	32.81 ± 0.76	33.47 ± 1.15
PLT (×10³)	330.11 ± 156.22	326.22 ± 118.50	274.11 ± 117.83	436.44 ± 140.85	453.44 ± 126.78	438.89 ± 141.99

ANOVA results according to treatment, time, and their interaction

RBC

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	10.49844630	10.49844630	22.98	<.0001
Time	2	1.51607778	0.75803889	1.66	0.2010
Treatment * Time	2	0.16249259	0.08124630	0.18	0.8376

Observations: On day −3, the RBC count was significantly higher in treatment 2 compared with treatment 1 (p = 0.0243). On days 0.5 and 7, no statistical differences were observed between treatments.

HGB

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	8.96296296	8.96296296	14.68	0.0004

	Time	2	3.13037037	1.56518519	2.56	0.0876
	Treatment * Time	2	0.41037037	0.20518519	0.34	0.7163

HCT

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	70.26962963	70.26962963	12.73	0.0008
Time	2	30.04111111	15.02055556	2.72	0.0760
Treatment * Time	2	0.87148148	0.43574074	0.08	0.9242

MCV

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	122.4016667	122.4016667	8.73	0.0048
Time	2	0.2100000	0.1050000	0.01	0.9925
Treatment * Time	2	3.0344444	1.5172222	0.11	0.8976

MCH

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	12.06340420	12.06340420	11.53	0.0014
Time	2	0.14743889	0.07371944	0.07	0.9321
Treatment * Time	2	0.36708333	0.18354167	0.18	0.8396

MCHC

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	0.80666667	0.80666667	0.71	0.4038
Time	2	0.51814815	0.25907407	0.23	0.7971
Treatment * Time	2	4.09000000	2.04500000	1.80	0.1765

PLT

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	277814.1295	277814.1295	16.70	0.0002
Time	2	1994.2228	997.1114	0.06	0.9419
Treatment * Time	2	21538.9383	10769.4692	0.65	0.5280

Observations: The two-way repeated measures ANOVA revealed a significant effect of Treatment on platelet count (p = 0.0002), but not of Time (p = 0.94) or their interaction (p = 0.53). According to Tukey's post hoc test, platelet counts on day 7 were significantly higher in the double-dose group compared with the single-dose group (p = 0.0271), while other pairwise comparisons were not statistically different (p > 0.05).

Leukocyte profile: mean and standard deviation

Variable	Treatment 1			Treatment 2		
	-3	0.5	7	-3	0.5	7
WBC (×10³/µL)	12.37 ± 4.88	10.83 ± 4.62	13.43 ± 5.03	16.16 ± 11.08	12.94 ± 9.07	16.94 ± 11.70
BAS (/µL)	96.3 ± 98.7	157.6 ± 81.2	101.7 ± 143.5	365.4 ± 330.0	156.4 ± 225.4	44.3 ± 73.2
EOS (/µL)	738.3 ± 274.9	823.4 ± 329.7	881.8 ± 876.1	524.7 ± 308.6	476.8 ± 296.8	520.2 ± 416.3
NB (/µL)	66.4 ± 72.5	142.2 ± 166.7	205.3 ± 220.3	0.0 ± 0.0	56.9 ± 56.6	181.4 ± 171.6
NS (/µL)	4119.3 ± 1632.8	2104.4 ± 919.8	6320.4 ± 1926.7	3922.4 ± 1573.4	1992.7 ± 600.8	5220.9 ± 3334.7
LYMPH (/µL)	6832.4 ± 3448.9	6596.4 ± 3448.6	5702.6 ± 3538.0	10924.1 ± 9884.6	10105.3 ± 8875.6	6345.4 ± 1759.7

MON (/μL)	279.9 ± 271.5	149.1 ± 144.4	221.6 ± 256.6	410.9 ± 307.3	156.3 ± 134.6	153.9 ± 123.2

ANOVA results according to treatment, time, and their interaction

BAS

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	65169.0955	65169.0955	1.90	0.1743
Time	2	224658.6050	112329.3025	3.28	0.0464
Treatment * Time	2	272789.3783	136394.6892	3.98	0.0252

EOS

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	1223420.340	1223420.340	5.47	0.0237
Time	2	45583.344	22791.672	0.10	0.9033
Treatment * Time	2	56970.335	28485.168	0.13	0.8807

NS

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	2913640.5	2913640.5	0.81	0.3721
Time	2	120946667.1	60473333.6	16.86	<.0001
Treatment * Time	2	2662315.8	1331157.9	0.37	0.6920

MON

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	7467.1296	7467.1296	0.16	0.6951
Time	2	379225.3333	189612.6667	3.95	0.0259
Treatment * Time	2	90596.5926	45298.2963	0.94	0.3965

Serum enzymatic activity: mean and standard deviation

Variable	Treatment 1			Treatment 2		
	-3	0.5	7	-3	0.5	7
AST (U/L)	76.83 ± 18.15	62.20 ± 10.64	69.84 ± 14.81	71.37 ± 6.82	69.84 ± 10.48	71.00 ± 10.18
GGT (U/L)	31.56 ± 4.90	28.69 ± 5.41	29.64 ± 6.38	40.16 ± 8.91	39.21 ± 10.37	38.25 ± 8.18
ALP (U/L)	46.64 ± 9.85	45.01 ± 4.43	51.82 ± 13.11	61.16 ± 29.05	62.65 ± 37.81	64.49 ± 25.14
Urea (mg/dL)	38.04 ± 6.60	35.26 ± 6.44	38.74 ± 3.58	50.03 ± 26.30	46.14 ± 27.02	49.25 ± 22.00
Creatinine (mg/dL)	1.16 ± 0.05	1.08 ± 0.15	1.10 ± 0.10	1.17 ± 0.09	1.24 ± 0.15	1.15 ± 0.13

ANOVA results according to treatment, time, and their interaction

AST

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	16.0921322	16.0921322	0.10	0.7502
Time	2	554.0032355	277.0016177	1.77	0.1825

	Treatment * Time	2	363.3706544	181.6853272	1.16	0.3231

	Source	DF	Type III SS	Mean Square	F Value	Pr > F
UREA	Treatment	1	1605.711755	1605.711755	4.63	0.0366
	Time	2	125.380877	62.690439	0.18	0.8351
	Treatment * Time	2	5.179606	2.589803	0.01	0.9926

	Source	DF	Type III SS	Mean Square	F Value	Pr > F
CREATININE	Treatment	1	0.06829511	0.06829511	5.11	0.0286
	Time	2	0.01815177	0.00907589	0.68	0.5122
	Treatment * Time	2	0.05090562	0.02545281	1.90	0.1606

Friedman test results for non-parametric hematological and biochemical parameters according to treatment

Variable	Treatment	DF	χ^2 Value	p-value
WBC (×10⁶/µL)	1	2	8.0000	0.0183
	2	2	13.8857	0.0010
NB (/µL)	1	2	5.4483	0.0656
	2	2	9.2500	0.0098
LYMPH (/µL)	1	2	5.5556	0.0622
	2	2	4.5714	0.1017
GGT (U/L)	1	2	4.6667	0.0970
	2	2	1.5000	0.4724

ALP (U/L)	1	2	2.0000	0.3679
	2	2	3.5556	0.1690

Observations: All statistics correspond to the Row Mean Scores Differ from Friedman/Cochran-Mantel-Haenszel tests.

Supplementary Material 2. Statistical analyses of somatic cell counts according to treatment and evaluation times.

Comparison of somatic cell count data adjusted using values above 9.999 × 10⁶ cells/mL recorded as 9.999 × 10⁶ (equipment detection limit)

Day	Treatment	N	Median SCC (cells/mL)	Mean lnSCC ± SD	Min–Max SCC (cells/mL)
−3	1	8	37.000	10.41 ± 0.94	12.000 - 177.000
−3	2	9	72.000	10.77 ± 0.92	10.000 - 117.000
0.5	1	8	6.891.000	15.57 ± 0.58	2.967.000 - 9.999.000*
0.5	2	9	9.999.000	16.04 ± 0.22	5.155 - 9.999.000*
7	1	8	117.000	12.29 ± 1.21	81.000 - 1.558.000
7	2	9	484.000	13.11 ± 0.87	361.000 – 4.104.000

Median somatic cell count (SCC; cells/mL), mean natural logarithm of SCC (lnSCC) ± standard deviation (SD), and minimum–maximum SCC values for each treatment group at days −3, 0.5, and 7. Day 0.5 corresponds to the peak measurement after treatment. SCC values were log-transformed for statistical analyses due to their right-skewed distribution. *N* = number of observations per group. *Values above 9.999 × 10⁶ cells/mL were recorded as 9.999 × 10⁶ due to the upper detection limit of the

equipment. Animals that did not meet the eligibility criteria, due to one or more parameters being outside the established limits, were excluded from the analyses.

Comparison between treatments/ Statistical Analysis

Dunn/Conover post-hoc - Variável lnScc9999 -3

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Treatment

Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	64.0	72.0	10.366802	8.000000
2	9	89.0	81.0	10.366802	9.888889

Average scores were used for ties.

Wilcoxon Two-Sample Test	
Statistic	64.0000
Normal Approximation	
Z	-0.7235
One-Sided Pr < Z	0.2347
Two-Sided Pr > |Z|	0.4694
t Approximation	

Wilcoxon Two-Sample Test	
One-Sided Pr < Z	0.2399
Two-Sided Pr > |Z|	0.4798
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	0.5955
DF	1
Pr > Chi-Square	0.4403

Dunn/Conover post-hoc - Variável lnScc9999 0,5

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Treatment					
Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	56.0	72.0	8.375384	7.000000
2	9	97.0	81.0	8.375384	10.777778
Average scores were used for ties.					

Wilcoxon Two-Sample Test	
Statistic	56.0000
Normal Approximation	
Z	-1.8507
One-Sided Pr < Z	0.0321
Two-Sided Pr > |Z|	0.0642
t Approximation	
One-Sided Pr < Z	0.0414
Two-Sided Pr > |Z|	0.0828
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	3.6495
DF	1
Pr > Chi-Square	0.0561

Dunn/Conover post-hoc - Variável lnScc9999 7,0

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable InScc9999 Classified by Variable Treatment					
Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	52.50	72.0	10.385935	6.562500
2	9	100.50	81.0	10.385935	11.166667
Average scores were used for ties.					

Wilcoxon Two-Sample Test	
Statistic	52.5000
Normal Approximation	
Z	-1.8294
One-Sided Pr < Z	0.0337
Two-Sided Pr > |Z|	0.0673
t Approximation	
One-Sided Pr < Z	0.0430
Two-Sided Pr > |Z|	0.0860
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	3.5252
DF	1
Pr > Chi-Square	0.0604

Comparision across Time

TREATMENT 1

Summary Statistics for Timeby lnScc9999

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)

Statistic	Alternative Hypothesis	DF	Value	Prob
1	Nonzero Correlation	1	0.7704	0.3801
2	Row Mean Scores Differ	2	19.5814	<.0001
3	General Association	38	46.0000	0.1748

Total Sample Size = 24

TREATMENT 2

Summary Statistics for Timeby lnScc9999

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)

Statistic	Alternative Hypothesis	DF	Value	Prob
1	Nonzero Correlation	1	3.2232	0.0726

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)				
Statistic	Alternative Hypothesis	DF	Value	Prob
2	Row Mean Scores Differ	2	23.5112	<.0001
3	General Association	38	52.0000	0.0646

Total Sample Size = 27

The SAS System

The NPAR1WAY Procedure

Treatment=1

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	8	41.0	100.0	16.290835	5.1250
0.5	8	164.0	100.0	16.290835	20.5000
7	8	95.0	100.0	16.290835	11.8750
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	19.0963

Kruskal-Wallis Test	
DF	2
Pr > Chi-Square	<.0001

The SAS System

The NPAR1WAY Procedure

Treatment=2

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	9	45.0	126.0	19.191344	5.0
0.5	9	207.0	126.0	19.191344	23.0
7	9	126.0	126.0	19.191344	14.0
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	23.7519
DF	2
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc - Treatment 1, Variável lnScc9999

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Time

Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	8	41.0	100.0	16.290835	5.1250
0.5	8	164.0	100.0	16.290835	20.5000
7	8	95.0	100.0	16.290835	11.8750

Average scores were used for ties.

Kruskal-Wallis Test	
Chi-Square	19.0963
DF	2
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc – Treatment 1, Variável lnScc9999

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: lnScc9999			
Time	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
-3 vs. 0.5	-3.3882	4.7916	0.0020
-3 vs. 7	-2.8377	4.0130	0.0126
0.5 vs. 7	3.3857	4.7880	0.0021

Dunn/Conover post-hoc - Treatment 2, Variável lnScc9999

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc9999 Classified by Variable Time					
Time	**N**	**Sum of Scores**	**Expected Under H0**	**Std Dev Under H0**	**Mean Score**
-3	9	45.0	126.0	19.191344	5.0
0.5	9	207.0	126.0	19.191344	23.0
7	9	126.0	126.0	19.191344	14.0
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	23.7519
DF	2
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc, Variável lnScc9999

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: lnScc9999			
Time	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
-3 vs. 0.5	-3.7421	5.2921	0.0005
-3 vs. 7	-3.5762	5.0576	0.0010
0.5 vs. 7	3.7421	5.2921	0.0005

Comparison of adjusted somatic cell count data by replacing values above 9,999 × 10^6 cells/mL with 15,000 × 10^6 cells/mL in the sensitivity analysis (conservative assumption: 50% above the equipment limit)

Day	Treatment	N	Median SCC (cells/mL)	Mean lnSCC ± SD	Min–Max SCC (cells/mL)
−3	1	8	37.000	10.41 ± 0.94	12.000 - 177.000

−3	2	9	72.000	10.77 ± 0.92	10.000 - 117.000
0.5	1	8	6.891.000	15.62 ± 065	2.967.000 - 15.000.000*
0.5	2	9	15.000.000	16.27 ± 0.36	5.155.000 - 15.000.000*
7	1	8	117.000	12.29 ± 1.21	81.000 - 1.558.000
7	2	9	484.000	13.37 ± 0.87	361.000 - 4.104.000

* Median somatic cell count (SCC; cells/mL), mean natural logarithm of SCC (lnSCC) ± standard deviation (SD), and minimum–maximum SCC values for each treatment group at days −3, 0.5, and 7. Day 0.5 corresponds to the peak measurement after treatment. SCC values were log-transformed for statistical analyses due to their right-skewed distribution. N = number of observations per group. **(*)** Values above 9.999 × 10⁶ cells/mL were replaced with 15.000 × 10⁶ cells/mL in the sensitivity analysis (conservative assumption: 50% above the equipment limit). Animals that did not meet the eligibility criteria, due to one or more parameters being outside the established limits, were excluded from the analyses.

Comparison between treatments:

Dunn/Conover post-hoc - Variável lnScc15000 -3

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Treatment

Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	64.0	72.0	10.366802	8.000000
2	9	89.0	81.0	10.366802	9.888889

Average scores were used for ties.

Wilcoxon Two-Sample Test	
Statistic	64.0000
Normal Approximation	
Z	-0.7235
One-Sided Pr < Z	0.2347
Two-Sided Pr > |Z|	0.4694
t Approximation	
One-Sided Pr < Z	0.2399
Two-Sided Pr > |Z|	0.4798
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	0.5955
DF	1
Pr > Chi-Square	0.4403

Dunn/Conover post-hoc - Variável lnScc15000 0,5

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Treatment					
Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	50.0	72.0	9.936563	6.250000
2	9	103.0	81.0	9.936563	11.444444
Average scores were used for ties.					

Wilcoxon Two-Sample Test	
Statistic	50.0000
Normal Approximation	
Z	-2.1637
One-Sided Pr < Z	0.0152
Two-Sided Pr > |Z|	0.0305
t Approximation	
One-Sided Pr < Z	0.0230
Two-Sided Pr > |Z|	0.0460
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	4.9020
DF	1
Pr > Chi-Square	0.0268

Dunn/Conover post-hoc - Variável lnScc15000 7,0

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Treatment					
Treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	8	52.50	72.0	10.385935	6.562500
2	9	100.50	81.0	10.385935	11.166667
Average scores were used for ties.					

Wilcoxon Two-Sample Test	
Statistic	52.5000
Normal Approximation	
Z	-1.8294

Wilcoxon Two-Sample Test	
One-Sided Pr < Z	0.0337
Two-Sided Pr > |Z|	0.0673
t Approximation	
One-Sided Pr < Z	0.0430
Two-Sided Pr > |Z|	0.0860
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	3.5252
DF	1
Pr > Chi-Square	0.0604

Comparison across time:

TREATMENT 1

Summary Statistics for Timeby lnScc15000

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)				
Statistic	Alternative Hypothesis	DF	Value	Prob
1	Nonzero Correlation	1	0.7390	0.3900
2	Row Mean Scores Differ	2	19.5536	<.0001

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)				
Statistic	Alternative Hypothesis	DF	Value	Prob
3	General Association	40	46.0000	0.2377

Total Sample Size = 24

TREATMENT 2

Summary Statistics for Timeby InScc15000

Cochran-Mantel-Haenszel Statistics (Based on Table Scores)				
Statistic	Alternative Hypothesis	DF	Value	Prob
1	Nonzero Correlation	1	2.8417	0.0918
2	Row Mean Scores Differ	2	23.5867	<.0001
3	General Association	40	52.0000	0.0968

Total Sample Size = 27

The NPAR1WAY Procedure

Treatment=1

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	8	41.0	100.0	16.312172	5.1250
0.5	8	164.0	100.0	16.312172	20.5000
7	8	95.0	100.0	16.312172	11.8750
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	19.0464
DF	2
Pr > Chi-Square	<.0001

The SAS System

The NPAR1WAY Procedure

Treatment=2

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	9	45.0	126.0	19.370874	5.0
0.5	9	207.0	126.0	19.370874	23.0
7	9	126.0	126.0	19.370874	14.0
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	23.3137
DF	2
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc - Treatment 1, Variável lnScc15000

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
-3	8	41.0	100.0	16.312172	5.1250

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Time					
Time	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.5	8	164.0	100.0	16.312172	20.5000
7	8	95.0	100.0	16.312172	11.8750
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	19.0464
DF	2
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc - Treatment 1, Variável lnScc15000

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: lnScc15000			
Time	Wilcoxon Z	DSCF Value	Pr > DSCF
-3 vs. 0.5	-3.3731	4.7703	0.0021

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: lnScc15000			
Time	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
-3 vs. 7	-2.8377	4.0130	0.0126
0.5 vs. 7	3.3706	4.7667	0.0022

Dunn/Conover post-hoc - Treatment 2, Variável lnScc15000

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable lnScc15000 Classified by Variable Time					
Time	**N**	**Sum of Scores**	**Expected Under H0**	**Std Dev Under H0**	**Mean Score**
-3	9	45.0	126.0	19.370874	5.0
0.5	9	207.0	126.0	19.370874	23.0
7	9	126.0	126.0	19.370874	14.0
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	23.3137
DF	2

Kruskal-Wallis Test	
Pr > Chi-Square	<.0001

Dunn/Conover post-hoc - Variável lnScc15000

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: lnScc15000			
Time	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
-3 vs. 0.5	-3.6214	5.1214	0.0009
-3 vs. 7	-3.5762	5.0576	0.0010
0.5 vs. 7	3.6214	5.1214	0.0009

Supplementary Material 3. Interpretation and statistical analysis of the evaluated clinical signs, including signs of inflammation, mammary gland parenchyma palpation, teat palpation, and milk consistency.

Signs of inflammation

Animals	Treatment	Signs of inflammation	Variable number
1	1 x dose	No change	0
2	1 x dose	No change	0
3	1 x dose	No change	0
4	1 x dose	Tumor	1
5	1 x dose	No change	0
6	1 x dose	Tumor, heat	2
7	1 x dose	Tumor, heat	2
8	1 x dose	Tumor	1
9	1 x dose	Tumor	1
10	2 x dose	Tumor, heat, pain	3
11	2 x dose	No change	0
12	2 x dose	Tumor, heat, pain	3
13	2 x dose	No change	0
14	2 x dose	Tumor, heat, pain	3
15	2 x dose	No change	0
16	2 x dose	No change	0
17	2 x dose	Tumor, heat	2
18	2 x dose	Tumor, heat, pain	3

Interpretation of results according to signs of inflammation

1 x dose		2 x dose	
Interpretation of results	Number of observations	Interpretation of results	Number of observations
No change	4	No change	4
Tumor	3	Tumor	0
Tumor + heat or pain	2	Tumor + heat or pain	1
Tumor + heat + pain	0	Tumor + heat + pain	4
Total of Animals	9	Total of Animals	9

Interpretation of results according to signs of inflammation.

Treatment	Category	n	Proportion (%)
Treatment 1 1 x dose	No change	4	44.4%
	Tumor	3	33.3%
	Tumor + heat or pain	2	22.2%
	Tumor + heat + pain	0	0%
Treatment 2 2 x dose	No change	4	44.4%
	Tumor	0	0%
	Tumor + heat or pain	1	11.1%
	Tumor + heat + pain	4	44.4%

Statistical Analysis

The SAS System

The FREQ Procedure

Frequency Percent Row Pct Col Pct	Treatment	No_change	Tumor	Tumor + heat or pain	Tumor + heat + pain	Total
	1_x_dose	4	3	2	0	9
		22.22	16.67	11.11	0.00	50.00
		44.44	33.33	22.22	0.00	
		50.00	100.00	66.67	0.00	
	2_x_dose	4	0	1	4	9
		22.22	0.00	5.56	22.22	50.00
		44.44	0.00	11.11	44.44	
		50.00	0.00	33.33	100.00	
	Total	8	3	3	4	18
		44.44	16.67	16.67	22.22	100.00

Statistics for Table of Treatment by Signs_Inflammation

Statistic	DF	Value	Prob
Chi-Square	3	7.3333	0.0620
Likelihood Ratio Chi-Square	3	10.0439	0.0182
Mantel-Haenszel Chi-Square	1	0.1589	0.6902

Statistic	DF	Value	Prob
Phi Coefficient		0.6383	
Contingency Coefficient		0.5380	
Cramer's V		0.6383	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0043
Pr <= P	0.0555
Sample Size = 18	

Interpretation of Results according Mammary gland Parenchyma Palpation

Animals	Treatment	Palpation of Mammary Parenchyma	Variable number
1	1 x dose	Normal consistency	0
2	1 x dose	Firm consistency, edema	2
3	1 x dose	Normal consistency	0
4	1 x dose	Firm consistency, edema	2
5	1 x dose	Normal consistency	0
6	1 x dose	Firm consistency, edema	2
7	1 x dose	Firm consistency, pain	2
8	1 x dose	Firm consistency	1

9	1 x dose	Firm consistency	1
10	2 x dose	Firm consistency, edema, pain	2
11	2 x dose	Normal consistency	0
12	2 x dose	Firm consisency	1
13	2 x dose	Normal consistency	0
14	2 x dose	Firm consistency, edema, pain	3
15	2 x dose	Normal consistency	0
16	2 x dose	Normal consistency	0
17	2 x dose	Normal consistency	0
18	2 x dose	Firm consisency	1

Interpretation of results according Teat Palpation

1 x dose		**2 x dose**	
Interpretation of results	**Number of observations**	**Interpretation of results**	**Number of observations**
Normal consistency	3	Normal consistency	5
Firm consisency	2	Firm consisency	2
Firm consisency + either edema or pain	4	Firm consisency + either edema or pain	1
Firm consisency + both edema + pain	0	Firm consisency + both edema + pain	1
Total of Animals	9	Total of Animals	9

Interpretation of Results According to Milk Consistency

Treatment	**Category**	**n**	**Proportion (%)**
Treatment 1 1 x dose	Normal consistency	3	33.3%
	Firm consisency	2	22.2%

	Firm consisency + either edema or pain	4	44.4%
	Firm consisency + both edema + pain	0	0%
Treatment 2 2 x dose	Normal consistency	5	55.6%
	Firm consisency	2	22.2%
	Firm consisency + either edema or pain	1	11.1%
	Firm consisency + both edema + pain	1	11.1%

Statistical Analysis

The SAS System

The FREQ Procedure

Frequency
Expected
Percent
Row Pct
Col Pct

Table of Tratamento by Mammary gland parenchyma palpation

Tratamento	Mammary parenchyma				
	Firm + edema + pain	Firm + edema or pain	Firm consistency	Normal consistency	Total
1	0 0.5 0.00 0.00 0.00	4 2.5 22.22 44.44 80.00	2 2 11.11 22.22 50.00	3 4 16.67 33.33 37.50	9 50.00

2	1	1	2	5	9
	0.5	2.5	2	4	
	5.56	5.56	11.11	27.78	50.00
	11.11	11.11	22.22	55.56	
	100.00	20.00	50.00	62.50	
Total	1	5	4	8	18
	5.56	27.78	22.22	44.44	100.00

Statistics for Table of Tratamento by Mammary parenchyma

Statistic	DF	Value	Prob
Chi-Square	3	3.3000	0.3476
Likelihood Ratio Chi-Square	3	3.8191	0.2817
Mantel-Haenszel Chi-Square	1	0.5016	0.4788
Phi Coefficient		0.4282	
Contingency Coefficient		0.3936	
Cramer's V		0.4282	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0346
Pr <= P	0.3896

Statistic	Value	ASE
Gamma	0.3103	0.3464
Kendall's Tau-b	0.1916	0.2219
Stuart's Tau-c	0.2222	0.2548
Somers' D C|R	0.2222	0.2548
Somers' D R|C	0.1651	0.1935
Pearson Correlation	0.1718	0.2407
Spearman Correlation	0.2052	0.2364
Lambda Asymmetric C|R	0.1000	0.2510
Lambda Asymmetric R|C	0.3333	0.2722
Lambda Symmetric	0.2105	0.2268
Uncertainty Coefficient C|R	0.0876	0.0701
Uncertainty Coefficient R|C	0.1530	0.1267
Uncertainty Coefficient Symmetric	0.1114	0.0901

Sample Size = 18

Interpretation of Results According Teat palpation

Animals	Treatment	Teat Palpation	Variable number
1	1 x dose	No change	0
2	1 x dose	No change	0
3	1 x dose	No change	0
4	1 x dose	No change	0
5	1 x dose	No change	0
6	1 x dose	No change	0
7	1 x dose	No change	0
8	1 x dose	No change	0
9	1 x dose	No change	0
10	2 x dose	Thichening and sensitivity	1
11	2 x dose	No change	0
12	2 x dose	Thichening and sensitivity	1
13	2 x dose	No change	0
14	2 x dose	Thichening and sensitivity	1
15	2 x dose	No change	0
16	2 x dose	No change	0
17	2 x dose	No change	0
18	2 x dose	Thichening and sensitivity	1

Interpretation of Results According Teat Palpation

1 x dose		2 x dose	
Interpretation of results	Number of observations	Interpretation of results	Number of observations

No change	9	No change	5
Thichening and sensitivity	0	Thichening and sensitivity	4
Total of Animals	9	Total of Animals	9

Interpretation of Results According Teat palpation

Treatment		Category	n	Proportion (%)
Treatment 1 **x dose**	**1**	No change	9	100%
		Thichening and sensitivity	0	0%
Treatment 2 **x dose**	**2**	No change	5	55.6%
		Thichening and sensitivity	4	44.4%

Statistical Analysis

The FREQ Procedure

Frequency Percent Row Pct Col Pct	Table of Treatment by Teat Palpation		
	Teat Palpation		
Treatment	Thickening and Sensitivity	No_change	Total
1_x_dose	0	9	9
	0.00	50.00	50.00
	0.00	100.00	
	0.00	64.29	
2_x_dose	4	5	9
	22.22	27.78	50.00
	44.44	55.56	

	100.00	35.71	
Total	4	14	18
	22.22	77.78	100.00

Statistics for Table of Treatment by Teat Palpation

Statistic	DF	Value	Prob
Chi-Square	1	5.1429	0.0233
Likelihood Ratio Chi-Square	1	6.7041	0.0096
Continuity Adj. Chi-Square	1	2.8929	0.0890
Mantel-Haenszel Chi-Square	1	4.8571	0.0275
Phi Coefficient		-0.5345	
Contingency Coefficient		0.4714	
Cramer's V		-0.5345	
WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Cell (1,1) Frequency (F)	0
Left-sided Pr <= F	0.0412

Fisher's Exact Test	
Right-sided Pr >= F	1.0000
Table Probability (P)	0.0412
Two-sided Pr <= P	0.0824

Sample Size = 18

Interpretation of Results According to Milk Consistency

Animals	Treatment	Milk Consistency	Variable number
1	1 x dose	No change	0
2	1 x dose	Moderate clots	2
3	1 x dose	No change	0
4	1 x dose	Slight clots	1
5	1 x dose	Slight clots	1
6	1 x dose	No change	0
7	1 x dose	Slight clots	1
8	1 x dose	Slight clots	1
9	1 x dose	Slight clots	1
10	2 x dose	Moderate clots	2
11	2 x dose	Slight clots	1
12	2 x dose	Severe clots	3
13	2 x dose	No change	0

14	2 x dose	Moderate clots	2
15	2 x dose	Slight clots	1
16	2 x dose	Slight clots	1
17	2 x dose	No change	0
18	2 x dose	Moderate clots	2

Interpretation of Results According to Milk Consistency

1 x dose		**2 x dose**	
Interpretation of results	Number of observations	Interpretation of results	Number of observations
No change	3	No change	2
Slight clots	5	Slight clots	3
Moderate clots	1	Moderate clots	3
Severe clots	0	Severe clots	1
Total of Animals	9	Total of Animals	9

Interpretation of Results According to Milk Consistency

Treatment	**Category**	**n**	**Proportion (%)**
Treatment 1 1 x dose	No change	3	33.3%
	Slight clots	5	55.6%
	Moderate clots	1	11.1%
	Severe clots	0	0%
Treatment 2 2 x dose	No change	2	22.2%
	Slight clots	3	33.3%

Moderate clots	3	33.3%
Severe clots	1	11.1%

Statistical Analysis

The FREQ Procedure

Table of Treatment by Milk Consistency

Frequency Percent Row Pct Col Pct	Milk Consistency				
Treatment	slight clots	moderate clots	severe clots	No_change	Total
1_x_dose	5 27.78 55.56 62.50	1 5.56 11.11 25.00	0 0.00 0.00 0.00	3 16.67 33.33 60.00	9 50.00
2_x_dose	3 16.67 33.33 37.50	3 16.67 33.33 75.00	1 5.56 11.11 100.00	2 11.11 22.22 40.00	9 50.00
Total	8 44.44	4 22.22	1 5.56	5 27.78	18 100.00

Statistics for Table of Treatment by Milk Consistency

Statistic	DF	Value	Prob
Chi-Square	3	2.7000	0.4402
Likelihood Ratio Chi-Square	3	3.1395	0.3706
Mantel-Haenszel Chi-Square	1	0.0331	0.8556
Phi Coefficient		0.3873	
Contingency Coefficient		0.3612	
Cramer's V		0.3873	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0461
Pr <= P	0.5738

Sample Size = 18