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STUDY OF THE ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS AND THEIR ASSOCIATIONS IN THE CONTROL OF MASTITIS IN CATTLE

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ABSTRACT

Essential oils (EOs) are volatile, oily compounds that can be extracted from plants. Several constituents of EOs have broad-spectrum antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, and yeasts, making essential oils a promising alternative to conventional treatments, often associated with adverse effects and microbial resistance. Mastitis, an inflammation of the mammary gland caused mainly by bacterial infections, in various ways, harms the entire production system, causing a significant negative impact on milk production. This disease is categorized into clinical and subclinical mastitis, both of which affect the quantity and quality of milk produced. The study of the antibacterial properties of EOs is of growing interest, as alternative drug therapies are welcomed to combat infections caused by antibiotic-resistant strains. Thus, this study aimed to study the antimicrobial properties of the EOs of Syzygium aromaticum, Thymus vulgaris and Pogostemon cablin and the possibility of using them in future research in the treatment of bovine mastitis.

Keywords: Alternative Training. Bacterium. Bioactive Products.

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INTRODUCTION

Pharmacologically, certain plant species have anti-inflammatory, antimicrobial, spasmolytic, sedative, analgesic, and local anesthetic properties, among other properties (TSUCHIYA, 2017; BATIHA et al., 2020). In aromatic plants, it is possible to extract EOs, which have the characteristic of being volatile and oily, extracted from plant materials such as seeds, flowers, leaves, buds, branches, bark, wood, fruits and roots (BRENES; ROURA, 2010). The pharmacological activities of plant species are attributed to their phytoconstituents, such as glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes, with applications in various medical areas (MUJEEB et al., 2014). Some constituents of EOs, such as carvacrol and thymol present in thyme, offer broad-spectrum antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, and yeasts (ROLLER; SEEDHAR, 2002, ABBASZADEH et al., 2014).

The study of the antibacterial properties of EOs is of increasing interest, as alternative drug therapies are welcomed to combat infections caused by antibiotic-resistant strains. In human medicine, they are already used against bacterial, viral, and fungal infections (OJAH, 2020; SILVA et al., 2020; ALJAAFARI et al., 2021). In veterinary medicine, in addition to combating a range of pathogens (VALDIVIESO-UGARTE et al., 2019), other problems could be solved by the use of EOs, such as the presence of antibiotic residues in foods of animal origin and in the environment (PIKKEMAAT et al, 2016). Although the *in vitro* antimicrobial activity of EOs has often been demonstrated in studies conducted with bacterial and fungal strains of different origins, information on their efficacy in the treatment of infections in animals is still scarce (EBANI; MANCIANTI, 2020).

Bovine mastitis is the most important disease affecting dairy herds worldwide, causing direct impacts on farm profitability and food safety issues. The prevention and treatment of this pathology is done especially through antimicrobials, but the increase in antimicrobial resistance of pathogens to this disease can affect the efficiency of conventional drugs. In addition, antimicrobial residues in milk and the environment are a potential threat to human health, as it increases the resistance of bacteria in the long term (LOPES et al., 2020).

Thus, the use of plant extracts and EOs may become a promising alternative for the control of bovine mastitis. Antimicrobial properties present in several plants are already well described. Plant extracts and EOs are often considered safe for animals, humans, and the environment. Thus, this study aimed to study and prove whether there are antimicrobial

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properties of the EOs of *Syzygium aromaticum, Thymus vulgaris and Pogostemon cablin* and the possibility of using them in future research in the treatment of bovine mastitis.

BOVINE MASTITIS AND BACTERIAL RESISTANCE

Brazilian livestock is among the top 5 powers in relation to milk productivity, with its production in 2021 being 35,305,047 liters, which highlights the importance of treating bovine mastitis in a sustainable way (IBGE, 2021). The state that produces the most milk in Brazil is currently the state of Minas Gerais with 9,611,706 thousand liters, with Paraná with a production of 4,415,634 thousand liters (IBGE, 2021).

Bovine mastitis is an inflammatory response of udder tissue in the mammary gland caused by physical trauma or infections by microorganisms. It is considered the most common disease that leads to economic losses in the dairy industry due to reduced yield and poor milk quality (GOMES; HENRIQUES, 2016). On average, the total cost of failure due to bovine mastitis is estimated at 28% per cow per year, mainly due to milk production losses and culling, which represents 11% to 18% of the gross margin per cow per year (HOGEVEEN et al, 2019). In mammary tissue, damage that leads to reduced milk production is responsible for 70% of total losses in the herd (CHEN et al., 2016).

Bovine mastitis can be classified into 3 classes based on the degree of inflammation, namely clinical, subclinical, and chronic mastitis. Clinical bovine mastitis is evident and easily detected by visible abnormalities such as red, swollen udder and fever in dairy cows. Cow's milk is watery with the presence of flakes and clots (KHAN; KHAN, 2006). Clinical mastitis can be subdivided into hyperacute, acute, and subacute, depending on the degree of inflammation (KIBEBEW, 2017). Severe cases of clinical mastitis can also be fatal (GRUET et al., 2001).

Unlike clinical mastitis, subclinical mastitis has no visible abnormalities in the udder or milk, but milk production decreases and somatic cell count (SCC) increases (ABEBE et al., 2016). The loss contributed by subclinical mastitis is very difficult to quantify, but experts agree that it represents more financial losses in the herd than clinical cases (CHEN et al., 2016; ROMERO et al., 2018). On the contrary, chronic mastitis is an inflammatory process that lasts for several months, with clinical outbreaks occurring at irregular intervals.

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RISK FACTORS

There are several risk factors known to be associated with the incidence of bovine mastitis that play a significant role, including some pathogens, bovine genetics, and environmental factors. All these factors should be considered in the implementation of mastitis control programs (KLAAS; ZADOKS, 2018). In the interest of this study, we will describe only the pathogens that cause bovine mastitis.

Intramammary bacterial infection is considered the main cause of bovine mastitis. Many bacterial species have been identified as causative agents of bovine mastitis. These bacterial infections can be classified into 2 types based on bacterial origin – contagious and environmental [LAKEW, 2019]. Contagious mastitis refers to mastitis that can be transmitted from cow to cow, especially during milking (SCHREINER; RUEGG, 2002).

Contagious pathogens such as *S. aureus* and *Streptococcus agalactiae*, and less common species such as *Mycoplama bovis* and *Corynebacterium*, live on the cow's udder and teat skin, colonizing and growing in the teat canal (KIBEBEW, 2017). These are capable of establishing subclinical infections, usually with elevation in the SCC. SCC is a useful indication of IMI infection (intramammary infection) and consists of leukocytes (i.e., neutrophils, macrophages, lymphocytes, and erythrocytes) and epithelial cells (SHARMA et al., 2011). Contagious infections can be controlled by reducing contact between reservoirs and uninfected cows. Therefore, proper maintenance of milking equipment, post-milking teat, disinfection, culling, and dry cow therapy (DCT) are important to prevent contagious infections (LANGE et al., 2017).

Unlike contagious pathogens, environmental pathogens do not usually live on the skin of the cow's udder and teat; instead, they exist in the herd's bedding and housing. They are best described as opportunistic pathogens, looking for the chance to cause an infection. For example, they can enter the teat during milking due to teat cup slippage, or when the cow's natural immunity is weak, causing clinical mastitis. Environmental pathogens such as *Escherichia coli* or *Streptococcus uberis* invade and multiply in the cow's udder, induce a host immune response, and are rapidly eliminated (BRADLEY et al., 2002).

A wide range of bacterial species have been reported to cause environmental mastitis, namely *Streptococcus* spp., coliform species (e.g., *E. coli*, *Klebsiella spp*., *Enterobacter spp*., *Pseudomonas spp*., etc. (BOGNI et al., 2011). Environmental infection control can be achieved by reducing teat end exposure to environmental pathogens and

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increasing cow resistance to IMI by antibiotic intervention and vaccination (LANGE et al., 2017).

BACTERIAL RESISTANCE

Antibiotics are widely used in the dairy industry to fight disease and improve animal performance. Antibiotics such as penicillin, cephalosporin, streptomycin, and tetracycline are used for the treatment and prevention of diseases affecting dairy cows caused by a variety of gram-positive and gram-negative bacteria. Antibiotics are often routinely given to entire herds to prevent mastitis during the dry season. An increase in the incidence of disease in a herd often results in increased antimicrobial use, which in turn increases the potential for antibiotic residues in milk and the potential for increased bacterial resistance to antimicrobials (OLIVER; MURINDA, 2012).

Effective treatment of bovine mastitis depends on the antimicrobial susceptibility of the pathogens, the type of mastitis, the breed of cattle, and the treatment regimen (BARKEMA et al., 2006). The emergence of drug resistance is a serious challenge for mastitis control, as resistance profiles are often herd-specific (SILVEIRA-FILHO et al., 2014). The combination of more than one synergistic antimicrobial agent may be more effective than the use of a single drug and may achieve a high cure rate (OLIVER et al., 2011; LAVEN et al., 2014; VAKKAMÄKI et al., 2017).

Rapid identification and understanding of the diversity of pathogens associated with mastitis is essential for effective prevention and control. However, treatment is expected to become problematic in the near future due to the rapid increase in antibiotic-resistant pathogens (VAKKAMÄKI et al., 2014). Transmission of antimicrobial-resistant mastitis pathogens and foodborne pathogens to humans can occur if unpasteurized milk is consumed (ABRAJMSÉN et al., 2013; OLIVE TREE; RUEGG, 2014; BEYENE et al., 2017).

The widespread use of antibiotics in the control of mastitis greatly increases the risk of installation and transmission of antibiotic resistance to consumers. This possibility is constantly in the attention of animal health and public health authorities, requiring a scientifically based redefinition of antibiotic therapies taking into account the intersection of animal welfare with social concerns (RUEGG 2009; STEVENS et al. 2016).

Among the pathogens that cause mastitis, *S. aureus* is the most prevalent gram-positive pathogen known to be associated with several forms of clinical and subclinical mastitis (VASUDEVAN et al., 2003). The fundamental reservoir of *S. aureus* is chronically infecting mammary gland, so maintaining udder hygiene and milking can protect the healthy cow from the infected cow, thereby reducing infection (RAINARD et al., 2018). *S. aureus* does not trigger an immune response in the cow as strong as *E. coli* or endotoxin, so S. *aureus* infection is always milder, leading to chronic mastitis that lasts a few months (GILBERT et al., 2013). S. aureus does not cause abnormalities or fatality, however, it produces degradative enzymes and toxins that irreversibly damage the milking tissue, decreasing milk production (VASUDEVAN et al., 2003).

Treatment of *S. aureus* is done by the use of antibiotics. However, Rainard et al (2018), demonstrated that the antibiotic is not an efficient method due to the resistance developed by the pathogen against β-lactam antibiotics, i.e., methicillin. Such strains of *S. aureus* are known as methicillin-resistant *S. aureus* (MRSA), which have a mecA gene that confers resistance (HAMID et al., 2017). In addition, the ability of S. aureus to produce biofilm and adapt to the host's environment makes it an even more difficult target for the treatment of this infection (OLIVEIRA et al., 2011; SCALI et al., 2015).

ESSENTIAL OILS

The history of EOs began in the East, as the distillation process, which is the technical basis for obtaining EOs, was conceived and employed for the first time in this region, especially in Egypt, Persia, and India. Although the religious use of EOs was recorded as early as 6,000 BC, the therapeutic use of EOs only began to grow after the 19th century, when lavender EO was successfully used in the treatment of burns (GUENTHER, 2017; MANION; WIDDER, 2017).

EOs are composed of lipophilic and highly volatile compounds, secondary plant metabolites, reaching a mass below a weight of 300, which can be physically separated from other plant components or membranous tissue. As defined by the International Organization for Standardization (ISO), the term essential "oil" is reserved for a product obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by mechanical process, or by dry distillation, i.e., by physical means only. Thus, most of the EOs available on the market are obtained by hydrodistillation (GRASSMANN; ELSTNER, 2003; SCHMIDT, 2010; SELL, 2010).

They are substances isolated by physical processes from an odorous plant. The oil takes its name from the plant from which it is derived, for example, rose oil or peppermint

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oil. These oils were called essential because they were thought to represent the very essence of odor and flavor (GUENTHER, 2017; HAAGEN-SMIT, 2017).

Distillation is the most common method for the isolation of EOs, but other processes, enfleurage (extraction using fat), maceration, solvent extraction, and mechanical pressing are used for certain products. Younger plants produce a greater amount of oil than older ones, but older plants are richer in more resinous and darker oils due to the continuous evaporation of the lighter fractions of the oil (GUENTHER, 2017; HAAGEN-SMIT, 2017, MANION; WIDDER, 2017).

The first records of EOs come from ancient India, Persia, and Egypt, and both Greece and Rome carried out extensive trade in oils and odorous ointments with the countries of the East. Most likely these products were extracts prepared by placing flowers, roots, and leaves in fatty oils. In most ancient cultures, odorous plants or their resinous products were used directly. Only with the arrival of the golden age of Arab culture was a technique for the distillation of EOs developed. The Arabs were the first to distill ethyl alcohol from fermented sugar, thus providing a new solvent for the extraction of EOs in place of the fatty oils that were probably used several millennia ago (URDANG, 2017). EOs have complex chemical constituents, which vary according to the amount of rain and daylight to which the plants are exposed, and the soil conditions, humidity, altitude, even the time of day when the plants are harvested (GOBBO-NETO; LOPES, 2007), and no bacterial resistance was detected in the use of these oils (BECERRIL et al., 2012; WILLING et al., 2018).

EOs are used in the pharmaceutical industry as active ingredients or constituents of medicines, soaps, shampoos, perfumes and cosmetics. They are also used as preservatives for food products in the food industry (PAULI, 2001; CHOUHAN et al., 2017).

Therefore, interest in EOs and other plant extracts as sources of natural products has been increasing in recent years. They are selected for their potential uses as alternative medicines for the treatment of many infectious diseases (KRÓL et al., 2013).

BIOLOGICAL ACTIVITIES OF THE EOS

EOs have long been recognized for possessing several different biological activities. Several of these plant secondary metabolites have marked antimicrobial effects that have made their use as an antiseptic and/or preservative in foods well known since ancient times (MANCIANTI; EBANI, 2020).

In food production, inhibiting the growth of microorganisms through the use of socially acceptable preservatives is a serious problem. Society's reluctance to use synthetic antibiotics and preservatives, such as benzoic acid, sorbic acid, lactic acid, propionic acid, acetic acid and its derivatives, inorganic parabens or sulfates, nitrites, and nitrates, necessitates finding alternative solutions (SHARIFI-RAD et al., 2018). This may be an application for EOs, especially since chemical preservatives cannot eliminate various pathogenic bacteria, such as *Listeria monocytogenes*, in food products or slow the growth of spoilage microorganisms. In addition, natural products are inherently better tolerated in the human body and generally with fewer side effects (LIU et al., 2017).

Most of the EOs have already been tested for their antibacterial/antifungal activity *in vitro* and some microorganisms were chosen, due to their perspective of a practical application to face different situations in the field. The EO of *Clausena lausium* showed potent activity against *Candida* yeasts, corroborating the efficacy of these traditional remedies in Chinese folk medicine (HE et al., 2019), while the EOs of *Cymbopogon flexuosum, Litsea cubeba,* and *Citrus bergamia* were shown to be active against *Saprolegnia parasitica* (NARDONI et al., 2019). However, special care should be given to the *in vivo administration* of these products, and some EOs have been shown to exert strong antimicrobial effects in animal practice, where they would have toxic effects damaging the liver, kidney, or gastrointestinal tissues (HORKY et al., 2019).

Although the use of EOs as antimicrobials has been reported not to induce relevant changes in sensitivity to antibacterial drugs (LEITE de SOUZA, 2016), a new insight into the mechanisms of action of *S. aureus* resistance against active individual constituents such as carvacrol, citral, and (+)-limonene oxide has been proven by Berdejo et al. (2019). Selected microorganisms, exposed to sub-inhibitory doses of such compounds, showed greater tolerance to lethal treatments by individual constituents or heat.

In addition to the multiple and well-documented antimicrobial and antifungal properties in human and veterinary medicine, this group of compounds also have distinct antiviral properties. EOs are able to suppress viruses in different ways. They can inhibit its replication or can prevent its spread from cell to cell (BASER; BUCHBAUER, 2009). Benencia et al. (1999) published their results on the antiviral activity of sandalwood oil (*Santalum album*) against Herpes simplex virus type 1 and type 2, these authors found that EO inhibited the replication of the viruses. HSV-1 was more influenced than HSV-2 depending on the dose used.

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Due to the mode of extraction, mainly by distillation of aromatic plants, EOs contain a variety of volatile molecules, such as terpenes and terpenoids, phenol-derived aromatic components, and aliphatic components. In vitro physicochemical assays characterize most of them as antioxidants. However, recent studies show that in eukaryotic cells, EOs can act as pro-oxidants that affect internal cell membranes and organelles, such as mitochondria. Depending on the type and concentration, they exhibit cytotoxic effects on living cells, but are generally not genotoxic. In some cases, changes in intracellular redox potential and mitochondrial dysfunction induced by EOs may be associated with their ability to exert anti-genotoxic effects. These findings suggest that, at least in part, the beneficial effects found in EOs are due to pro-oxidant effects at the cellular level (BAKKALIA et al., 2008; BASER.; BUCHBAUER, 2009; APONSO et al., 2021).

The antiparasitic activity was also evaluated *in vitro* by EOs originating in India, against flagellated protozoa. In particular, the EO of *Endlicheria bracteolata* has been shown to be active against both stages in amastigote and promastigote culture of *Leishmania amazonensis* (MARGATTO ROTTINI et al., 2019), while some EOs originating in Vietnam have been tested against *Trypanosoma brucei brucei.* Among them, *Curcuma longa* was effective, and in particular, its main component, curlone, appeared as a promising anti-trypanosal candidate (BINH LE et al., 2019).

And finally, since ancient times, EOs have been widely used for bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, medicinal, and cosmetic applications, especially currently in the pharmaceutical, sanitary, cosmetic, agricultural, and food industries.

OES-PRODUCING PLANTS WITH ANTIMICROBIAL ACTIVITIES

Syzygium aromaticum (L.) Merr. & L.M. Perry (Myrtaceae), popularly known as clove (Figure 1), is a shrubby species that grows mainly in the tropical moist biome native to Maluku and introduced in Borneo, Caroline Is., Comoros, Gulf of Guinea Is., Madagascar, Nicobar Is., Seychelles, Tanzania, and Trinidad-Tobago as described in Figure 2 (POWO, 2024).

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Figure 1: Syzygium aromaticum

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Figure 2: Geographic Distribution of Syzygium aromaticum **(L.)**

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This species has 8 synonyms, four of which are classified as homotypic: Caryophyllus aromaticus L, Eugenia aromatica (L.), Syzygium aromaticum (Spreng.), Myrtus caryophyllus Spreng.; and four classified as heterotypic: Caryophyllus hortensis, Caryophyllus silvestris Teijsm. ex Hassk, Eugenia caryophyllata Thunb., Jambosa caryophyllus (Thunb.) Nied. (POWO, 2024).

Syzygium aromaticum is popularly used as a condiment in cooking, due to its characteristic odor, as well as to treat ailments and mask bad oral odors (AFFONSO et al., 2012).

Within its composition, eugenol is the constituent with the highest plant contents, however other substances such as β-caryophyllene also have significant amounts in their structure (AFFONSO et al., 2012). It has proven activity against gram-negative bacteria (REICHLING et al., 2009).

The species *Thymus vulgaris* L. (Lamiaceae) popularly known as thyme, is a subshrub that grows mainly in the temperate biome. It occurs naturally from western

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Europe to southeastern Italy, encompassing the Balearic Islands, France, Italy, and Spain, and has been introduced in Algeria, Belgium, Cuba, Czechoslovakia, Dominican Republic, Haiti, Leeward Is., New Zealand South, Puerto Rico, Switzerland as seen in Figure 4 (POWO, 2024).

Figure 3: Thymus vulgaris L.

Source: Plants of the World Online, Royal Botanic Gardens, Kew. 2024. Licensed under Creative Commons Attribution CC BY). Accessed on: https://powo.science.kew.org/.

Figure 4: Geographic Distribution of Thymus vulgaris L.

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This species has 2 homotypic synonyms: Origanum thymus Kuntze and Thymus collinus Salisb.; and two accepted infraspecific synonyms, these being Thymus vulgaris subsp. mansanetianus and Thymus vulgaris subsp. vulgaris (POWO, 2024).

In folk medicine, it is used as an astringent, expectorant, digestion stimulant, against spasms, in addition to having antiseptic, antifungal, antioxidant and antimicrobial properties (LORENZINI; MATOS, 2002).

Within its composition there is mainly the presence of thymol, which has very high levels within the plant, and in the vast literature its biological activities are reported (SALGADO et al. 2012). Other substances that also have biological activities and that are present in their EO are ρ-cymene and carvacrol in smaller quantities, which have higher or lower levels depending on the particularities found in the cultivation of each plant (NUNES et al. 2016). It is a spice, has action against *S. aureus*, coagulase-negative and Streptococcus sp. (IMELOUANE et al., 2009).

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The species *Pogostemon cablin* (Blanco) Benth. (Lamiaceae), popularly known as Patchouli (Figure 5), is native to Sri Lanka, W. & Central Malesia, Papua New Guinea. It was introduced in China Southeast, Fiji, Hainan, Samoa, Taiwan, Thailand, Tonga, Trinidad-Tobago, Vietnam (Figure 6). It is a perennial or subshrubby plant and grows mainly in the seasonally dry tropical biome (POWO, 2024).

Figure 5: Pogostemon cablin

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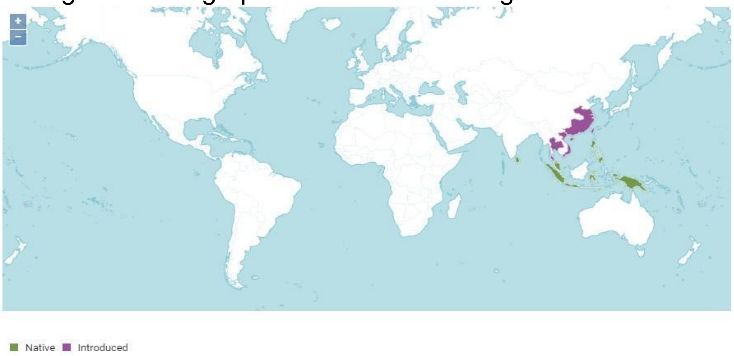
Figure 6: Geographic Distribution of Pogostemon cablin



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This species has 14 synonyms, one homotypic: Mentha cablin Blanco, and thirteen heterotypic: Mentha auricularia Blanco; Pogostemon battakianus Ridl.; Pogostemon comosus Miq.; Pogostemon heyneanus var. patchouly (Pellet.- Saut.) Kuntze; Pogostemon hortensis Backer ex K. Heyne; Pogostemon javanicus Backer ex Adelb.; Pogostemon mollis Hassk.; Pogostemon nepetoides Stapf; Pogostemon nepetoides var. glandulosus Merr.; Pogostemon patchouly Pellet.- Saut.; Pogostemon patchouly var. suavis (Ten.) Hook.f.; Pogostemon suavis Ten.; Pogostemon tomentosus Hassk.

Its leaves, when extracted, have a unique woody odor, which has been used since ancient times as one of the most important EOs to make perfumes (BEEK, 2017). It has action against *S. aureus* and *Escherichia coli* (ABDULLAH et al., 2011).

CONCLUSION

Antibiotic resistance is making it increasingly difficult to treat mastitis in dairy cows. Through this study, it was possible to verify that essential oils become an option to control microorganisms that cause this important disease, thus contributing to the health of the animal.

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