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IN VITRO COMPARATIVE EVALUATION OF CEFALEXIN AND THE PHYTOTHERAPY MEDICINES CALENDULA OFFICINALIS, UNCARIA TOMENTOSA, ROSMARINUS OFFICINALIS AND ANACARDIUM OCCIDENTALE IN THE TREATMENT OF MASTITIS IN CATTLE

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Abstract: Bovine mastitis has a high prevalence in dairy herds, and its allopathic treatment, despite being effective when effectively carried out, culminates in microbial resistance, as well as causing financial losses, possible cellular and teat microbiota changes, as well as a risk to the health of the animal. animal. When used in inadequate doses, it can cause animal poisoning, resistance to the medicine, and, if the withdrawal period is not respected, antibiotic residues can be ingested in the milk, which poses a threat to human health. In an attempt to find an alternative therapeutic methodology, this project sought to evaluate the in vitro activity of four herbal medicines with antimicrobial, anti-inflammatory and antioxidant potential: Calendula Officinalis (calendula), Anacardium occidentale (``cajuzinho-do-cerrado``), Uncaria Tomentosa (nail cat) and Rosmarinus Officinalis (rosemary), compared with the action of a first-generation cephalosporin, cephalexin, whose use is wide and indiscriminate on dairy farms. The secondary metabolites of these plants, such as tannins, saponins and flavonoids, are some of the compounds capable of, individually or in synergy, promoting medicinal effects, providing an associative alternative for reducing the use of allopathic medicines. The project was separated into two stages, first evaluating the action of the four herbal medicines against the selected allopathic medicine, obtaining relevant antimicrobial results regarding C. officinalis and A. occidentale. The second stage, in turn, tested these plants separately and in combination, comparing the three extracts with cephalexin. The result obtained showed an inhibitory effect for A. occidentale, when compared to the antibiotic, which showed an attenuating effect, which confirmed bacterial resistance to this allopathic agent and the antimicrobial potential of the plant.
Keywords: Bovine mastitis; Phytotherapeutics; Integrative veterinary medicine.

INTRODUCTION

Bovine mastitis has a high prevalence in Brazil, in addition to being the disease that most impacts the national cattle industry, generating economic losses due to issues such as reduction in milk production, loss in milk quality, disposal of milk with antimicrobial residues, treatment costs and animal disposal (EMBRAPA, 2012). This negative impact is socially important as it represents a risk to public health, due to the presence of pathogens and their toxins in the milk to be consumed, in addition to possible traces of antimicrobials (COSTA, 1998 apud EMBRAPA, 2012).

Mastitis consists of inflammation of the mammary glands of the udder, which can occur through the direct interaction of the cow with the pathogen, or through its contact with humans and the environment, and the microorganisms commonly accused as the cause of mastitis are *Staphylococcus spp, Staphylococcus aureus, Staphylococcus* negative and positive coagulase, E. coli, S. agalactiae, Streptococcus spp (ACOSTA, 2016; EMBRAPA, 2012). Due to the variety and complexity of possible pathogens causing the condition, treatment with broad-spectrum antimicrobials, systemic and/or locally acting, is indicated, with a view to increasing efficacy (EMBRAPA, 2012). The dose used in the treatment is usually inadequate, so that the cow may become intoxicated and develop microbial resistance, highlighting two problems: one, animal welfare; the other, public health (EMBRAPA, 2012; GANDA et al, 2016).

In this sense, there is a growing need to search for alternative therapies to treat bovine mastitis, in addition to the growth of the organic products market, which prohibits the use of antimicrobials (PIRES et al, 2004 apud JESUS et al, 2018). Given the characteristics of low toxicity, reduced side effects and low production cost, there was a growth in the

herbal medicine market (BRAZ FILHO, 2010; NIERO et al., 2003), which has been expanded to rural areas, with studies aimed at research of extracts capable of acting in the treatment of bovine mammary gland disease (EMBRAPA, 2012).

Medicinal plants such as Calendula officinalis (marigold), Rosmarinus officinalis (rosemary), Uncaria tomentosa (Cat's Claw) and Anacardium occidentale (``cajuzinho do cerrado``) present actions that, separately or in combination, have potential for treating bovine mastitis.

Currently, there is a need to complement studies on the standardization of extracts and their production, taking into consideration, the intended use and active ingredients, and it is also necessary to verify the effectiveness of each extract against microorganisms in vitro (CELIKTAS et al., 2007; GACHKAR et al, 2007; GAZOLA et al., 2014; INTANI et al., 2019.

GOALS

MAIN GOAL

To evaluate, comparatively, the use of cephalexin and the herbal medicines Calendula Officinalis, Anacardium occidentale, Uncaria Tomentosa and Rosmarinus Officinalis, in vitro, in the treatment of cattle diagnosed with mastitis.

SPECIFIC OBJECTIVE

- Identify cases of subclinical mastitis, using the California Mastitis Test (CMT);
- To evaluate the in vitro efficiency of cephalexin and the use of extracts from the herbal medicines Calendula officinalis, Anacardium occidentale, Rosmarinus officinalis and Uncaria tomentosa in the treatment of mastitis and verify their effectiveness based on the analysis of the disk diffusion test;
- To compare the effectiveness of the allopathic antibiotic cephalexin and the herbal medicines Calendula officinalis, Anacardium occidentale, Uncaria tomentosa and Rosmarinus officinalis, using the disk diffusion test for the observed pathogens.

THEORETICAL FOUNDATION

Mastitis is a disease of high magnitude and transcendence in Brazilian cattle farming, causing economic losses due to reduced milk production, animal disposal, disposal of milk with antibiotic residues and treatment costs, in addition to having a high prevalence (EMBRAPA, 2012; JESUS et al, 2018; SANTOS et al, 2017).

Timely diagnosis can help preserve the animal's tissues and mammary gland, so that the quality of pre-infection milk can be restored (SANTOS, 2017). Likewise, the effectiveness of the treatment increases, reducing financial and health damage to the herd, by preventing the spread of the disease (SANTOS, 2017).

Subclinical mastitis, as it does not present clinical signs, is the one that causes the most harm to the producer, due to the delay in diagnosis (LANGONI et al., 2017; DELLA LIBERA et al, 2011). Thus, the implementation of forms of diagnosis in the field proves to be allies in reducing cases of the disease in the herd and also helps to obtain better quality milk (DELLA LIBERA et al, 2011; DIAS, 2007 apud SANTOS, 2017).

Since the indiscriminate use of antimicrobials can lead to the development of bacterial resistance and harmful effects on the animal, and that mastitis is highly prevalent on Brazilian properties, there is a need to search for alternative medicines to treat this disease (JESUS et al, 2018). As one of the main arguments, there is the issue that antibiotics release residues in milk, which constitutes a

public health problem, in addition to being a monetary problem for the producer (PIRES et al, 2004 apud JESUS et al, 2018). According to Ganda et al (2016), 80% of medications prescribed for dairy cows are used to treat mastitis. Furthermore, there is the issue of a growing search for organic products, leading to the use of more alternative therapies, such as homeopathy and phytotherapy (PIRES et al, 2004 apud JESUS et al, 2018).

According to the Biodynamic Institute (2004), for the treatment of mastitis in organic production, medicinal plants with antiseptic action could be used, such as Calendula officinalis. It is known that the active principles of medicinal plants are the result of their secondary metabolism, that is, that which is not essential to keep them alive, and that other plants with medicinal action also have their action investigated, as is the case of Uncaria tomentosa, with its antioxidant and anti-inflammatory properties and Rosmarinus officinalis, whose action is antimicrobial and antioxidant (BOZIN et al, 2007; VALENTE, 2006).

Studies indicate the antimicrobial efficiency of Rosmarinus officinalis, and show that S. aureus and E. coli, some of the main pathogens causing bovine mastitis, are susceptible to this extract, due to the formation of a halo in in vitro observations (Celiktas, 2007; Gachkar et al, 2007; Inatani et al, 2005; Mangena & Muyima, 1999).

For C. officinalis, research shows anti-inflammatory, antioxidant and antimicrobial effects, also helping to heal skin conditions, due to the action of secondary metabolism products of the herbal medicine, such as flavonoids, saponins, tannins and polyphenols (DEUSCHLE et al., 2015).

Cat's claw was used to improve the immune system in research on fish, due to the presence of S. agalactiae, another common microorganism that causes diseases of the mammary gland in cattle (YUNIS-AGUINAGA et al, 2015). Uncaria tomentosa, in addition to being immunostimulant, has anti-inflammatory properties, with a low rate of cell death (PERO, 2000; AKESSON et al, 2005;), so that it began to gain commercial value worldwide, with high demand for its therapeutic properties (HUGHES & WORTH, 1999; REVILLA, 2001).

Anacardium occidentale or ``*cajuzinho do cerrado*``, presented antimicrobial, antiviral, anti-inflammatory and antiplatelet potential, with different parts of the plant being able to be used, such as the stem, bark, leaves and flowers, for the production of extracts, with each of these parts having different secondary metabolites, such as phenolic compounds, organic acids, flavonoids and tannins, capable of acting against bacteria and fungi (SILVA, 2012 apud ANDRADE JÚNIOR et al., 2018). Due to this activity, studies show evidence of an inhibitory effect on Staphylococcus aureus and E. coli, pathogens widely found in cases of mastitis (SILVEIRA, 2012 apud ANDRADE JÚNIOR et al., 2018).

METHOD

The experiment began with a trip to Fazenda Cavalcante Alencar, to collect material from 11 cows in the lactation period, moments before milking began. After hand hygiene with 0.05% sodium hypochlorite and drying with paper towels, milking was carried out in a screened mug with a black bottom, with 4 jets from each teat, looking for lumps, and to observe the sensory characteristics of the product, which must be in accordance with Normative Instruction number 62 of December 29, 2011, standardized by the Ministry of Agriculture, Livestock and Supply, which determines the Technical Regulation of Identity and Quality of cow's milk, and must comply with the conditions of homogeneous white liquid, free from foreign flavors and

odors (BRAZIL, 2011). After the tests, it was found that everything was complying, followed by pre-dipping, in order to proceed to the California Mastitis Test (CMT), to check the possibility of a case of subclinical mastitis. To this end, 3 jets of milk were collected from each of the teats separately. The equipment was previously sanitized with the Alcalan Plus solution. In the test tray, there is 1 slot designated for the allocation of samples from each breast quarter. Then, the CMT reagent was added to the mark indicated in each of the 4 slots with milk. After proper homogenization, the characteristics of the liquid obtained were observed, and, if any sample indicated positive for subclinical mastitis, the corresponding ceiling would be chosen for material collection. In the experiment, all animals showed at least one positive ceiling in the CMT, with gel formation corresponding to a cross (+). In cows with more than a quarter positive, the one with the greatest gel formation in the CMT was chosen.

With *swabs* of the Olen ® brand, with Stuart transport means, a sample was collected from the selected teats, which were stored in a Styrofoam box until they were properly placed in a refrigerator in the UniCEUB laboratory. All bottles were duly identified with the name of the animal, as well as the arrangement of the ceiling where the collection was carried out.

The positive control of choice was the antibiotic cephalexin, in paper discs of 6.35 mm in diameter, at the concentration specified according to the standards of the Brazilian pharmacopoeia (1988) and CLSI (2016, 2021). To obtain the cat's claw extract, 100µLof the vegetable drug, which was diluted in 900µL of sterile distilled water, resulting in a 1:10 ratio. The same dilution was carried out with the Calendula mother tincture. The ``Cajuzinho do Cerrado`` and rosemary extracts were in the same concentration, and were obtained by donation from the guiding teacher Francislete. The resulting herbal medicines were packaged separately in eppendorf tubes to ensure conservation and avoid contamination.

After obtaining the materials, laminar flow was used to manipulate the samples, which would be inoculated into Petri dishes in Mueller Hinton culture medium (MHA). Subsequently, they were placed in an oven at 37ºC for 48 hours to grow the bacterial culture.

The use of 22 plates was stipulated to carry out preliminary tests on the effectiveness of the extracts used, using the diffusion method, with division of the plate into quadrants: positive control (cephalexin), negative control (sterile water), extract 1 or 2 (cashew or marigold), extract 3 or 4 (cat's claw or rosemary). For standardization purposes, cashew and rosemary were applied to the same plate, while the two other medicinal herbs formed another set of plates. In both cases, positive and negative controls were applied. Finally, the sample from each animal was spread across two different plates, to encompass all primary variables.

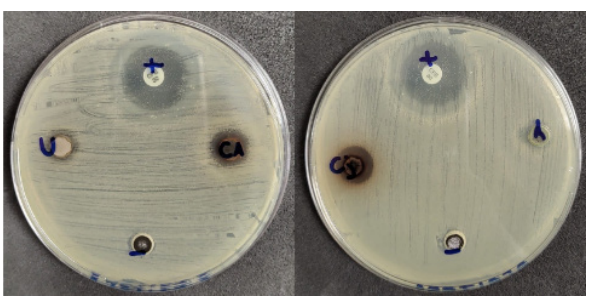


Figure 01: diagram of the division of extracts in Petri dishes for the same cow, in the first experiment: cat's claw; CA: Calendula officinalis; CJ: ``*cajuzinho do cerrado*``; A: rosemary; (+): positive control (cephalexin); (-): negative control (water).

Source: own elaboration

After 48 hours, the inhibition halos formed were measured, considering the methodology recommended by CLSI (2021). A dark cloth was placed as a background, to facilitate the

visualization of the plates and, with a caliper, placed against the bottom of the inverted plate, from the back, any space with any alteration, to the naked eye, generated in the colony was considered, being inhibition or attenuation effect.

In the second phase, once the ``*Cajuzinho do Cerrado*`` and calendula extracts were defined as the herbal medicines that showed the best bacterial inhibition or attenuation, the same samples were again inoculated into new Petri dishes, with MHA. This time, 6 repetitions of the same treatment were performed for each animal, totaling 66 plates.

After inoculation, each plate was separated into 5 parts, for division into positive control (cephalexin), negative control (sterile water), cashew extract, calendula extract and, in the center, the mixture of these two extracts. The dilution of ``*Cajuzinho do Cerrado*`` and calendula remained the same as in the first experiment. These extracts were combined in a 1:1 ratio.

Four wells were drilled to allocate extracts and water, using a sterile tip measuring 0.4 millimeters in diameter. In the wells identified for the respective samples or for the negative control, 30µL of each substance were poured, with the positive control positioned at the top of the plate. 6 replications were made for each sample, which remained in ovens for 48 hours at 37°C. After the period, all samples were collected for analysis and measurement of halos using a caliper.

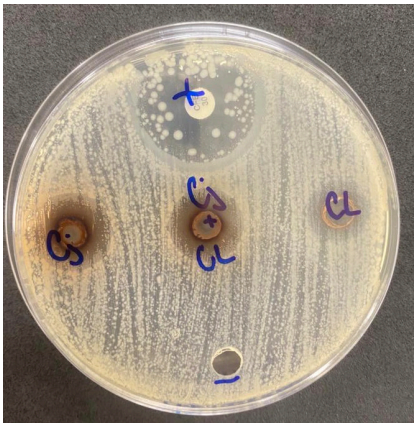


Figure 02: scheme of the division of extracts in Petri dishes in the second experiment.CL: Calendula officinalis; CJ: ``*cajuzinho do cerrado*``; CJ + CL: ``Cajuzinho do Cerrado`` and marigold; (+): positive control (cephalexin); (-): negative control (water).

Source: own elaboration

Then, the data were distributed in a table, and the average halos obtained in each treatment, per animal, were calculated (table 01).

TREATMENT / ANIMAL	Cajuzinho (CJ)	Calendula (CL)	CJ + CL	Control (+)	Control (-)
Varanda	10	3.83	9	28	–
Ametista	10.5	9.17	13	18	–
Rolinha	9.7	–	9.5	ND	–
Maria	4.83	–	4.5	15.3	–
Graúna	6.7	–	5.2	20.5	–
Dama	4.7	–	4	12.7	–
Princesa	11.7	8	11.5	19.3	–
Bela	4.3	–	3.6	20.6	–
Frida	11.8	8.8	12.1	19.2	–
Coreia	11.5	7.3	11.5	6.8	–
Cebola	4.6	–	4.1	16.6	–

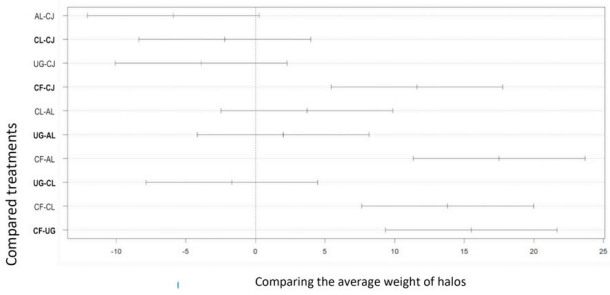
Table 01: Average values (mm) of inhibition or attenuation halos for each cow, per treatment.

Source: own elaboration

RESULTS

After the preliminary test and measurement of halos, the extracts *Rosmarinus officinalis* and Uncaria tomentosa, it was possible to determine that they did not show significant inhibition or attenuation capacity, and were discarded for continuation of the experiment. The result obtained with Anacardium occidentale, at a concentration of 1:10, was the extract that showed the best results, presenting halos with inhibition, while the halo of the positive control (cephalexin) on the same plate showed attenuation for the pathogens present. The Calendula officinalis extract in commercial tincture, as well as Anacardium occidentale, showed inhibition for the pathogens present, however, with smaller halos, compared to the positive control (figure 1).

For biostatistical analysis, the post-hoc method was used in the RStudio ® 2023.06.1 software, combining ANOVA with the Tukey test, which compares the treatment means two by two, considering a significance level of 0.05 (α= 5%).The results obtained in the primary tests indicated that there was a significant difference between all treatment pairs that included cephalexin and an extract, therefore covering all extracts compared to the positive control.



Graph 01: Tukey's 95% Confidence Interval Chart for experiment 1, comparing, two by two, the extracts Calendula (CL), ``*Cajuzinho do Cerrado*`` (CJ), Rosemary (AL), Cat's Claw (UG) and cephalexin (CF) with the size of the inhibition or attenuation halos.

Source: own elaboration

When calculating the *p-value* of the averages between the positive control and each of the herbal medicines (CF-CJ, CF-AL, CF-CL, CF-UG), the values remained belowα (table 02). For values less than or equal to p-value = 0.05, the null hypothesis that there is no significant difference between the inhibition of the herbal medicine and the positive control is rejected (MORETTIN & BUSSAB, 2017).

Average	CF-CJ	CF-AL	CF-CL	CF-UG
P-value	2.8*10-5	0	9*10-7	1*10-7

Table 02: P-value of each herbal medicine, compared with the positive control. Extracts of Calendula (CL), ``Cajuzinho do Cerrado`` (CJ), Rosemary (AL), Cat's Claw (UG) and cephalexin (CF).

Consider α = 0.05.

Source: own elaboration

In this sense, it can be seen that three of the four herbal medicines show significant differences in inhibition in relation to the positive control (graph 01 and table 02). For the rosemary (*p-value*=0), as it resulted in a very low level of significance, it is inferred that there was no difference.

Calendula and ``Cajuzinho do Cerrado`` were selected for the second stage of the test because they presented cases of formation of inhibition halos, while, on the same plates, cephalexin only managed to attenuate the growth of microorganisms (figure 03).

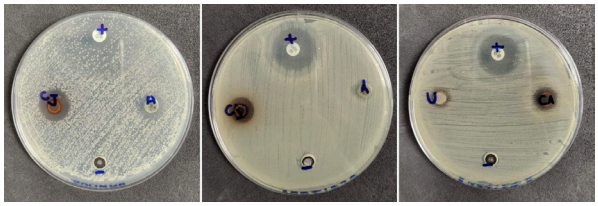
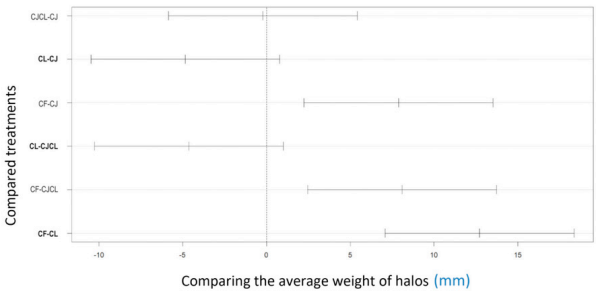


Figure 03: Plates in which the extracts of ``Cajuzinho do Cerrado`` (CJ) or calendula (CA) obtained results of inhibition of microbial growth, and cephalexin (+) showed only attenuation.

Source: own elaboration

When considering the data obtained at this stage, there is, once again, a significant difference in the treatments of each of the extracts evaluated, compared to cephalexin (graph 02 and table 03).



Graph 02: Tukey's 95% Confidence Interval Chart for experiment 2, comparing, two by two, the extracts Calendula (CL), ``Cajuzinho do Cerrado`` (CJ), the combination of the two herbal medicines (CJCL) and cephalexin (CF) with the size of the inhibition or attenuation halos.

Source: own elaboration

The *p-value* for each significant test it was detailed in table 03.

Average	CF-CJ	CF-CJCL	CF-CL
P-value	3*10-3	2.2*10-3	2.4*10-6

Table 03- P-value of each herbal medicine, compared with the positive control. Extracts of Calendula (CL), ``Cajuzinho do Cerrado`` (CJ), the combination of both (CJCL) and cephalexin (CF).

Consider α = 0.05.

Source: own elaboration

Finally, an inhibitory potential of cajuzinho extracts and the combination of cajuzinho and marigold (1:1) was repeatedly verified, while the positive control was only capable of attenuating the microorganisms (figure 04).

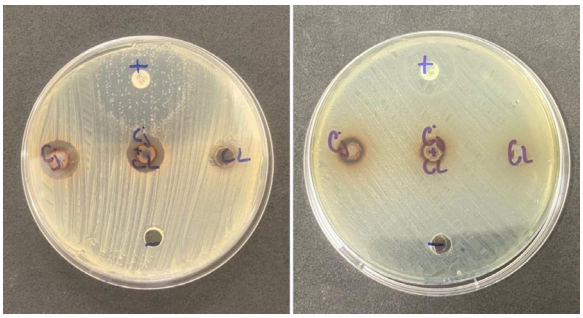


Figure 04: Plates in which the extracts of ``Cajuzinho do Cerrado`` (CJ) and/or the mixed herbal medicine (CJCL) obtained results of inhibition of microbial growth, and cephalexin (+) only showed attenuation.

Source: own elaboration

DISCUSSION

In view of the biostatistics analyzed, the relevance of the action of extracts of cashew (1:10), calendula (1:10) and the 1:1 (v/v) mixture of cashew and marigold was observed, compared to cephalexin. Rosemary and cat's claw, although they showed a p-value lower than 0.05, did not present such divergent results compared to the positive control.

The positive effects of ``Cajuzinho do Cerrado`` against pathogenic bacteria in cattle were confirmed by the in vitro study by Pereira et al. (2010), by inhibiting the growth of S. aureus. Gonçalves et al. (2005) and Silva et al. (2007) also obtained results of inhibition of the growth of S. aureus and Staphylococcus spp., carrying out in vitro tests on human samples.

The bactericidal property of calendula is at odds with Diaz et al. (2010), which did not determine significant antimicrobial activity when using this extract, even at different concentrations. However, Rozwalka et al. (2008) obtained positive results regarding the bactericidal property of C. officinalis in vitro.

Regarding the mixture of the herbal medicines A. occidentale and C. officinalis, more research is required with different concentration levels, in order to determine

whether the metabolites of both acts separately or whether there are synergistic effects, so that the combination of extracts would allow an amplified action (SILVA, 2012 apud ANDRADE JÚNIOR et al., 2018).

The high occurrence of attenuation-only results, generated by the positive control, can be justified by the wide and indiscriminate use of cephalosporins to treat bovine mastitis, especially first-generation ones, such as cephalexin, as they are low cost and have a broad spectrum (LANGONI et al., 2017; GANDA et al., 2016). Even when evaluating the effect of third-generation cephalosporins in the treatment of bovine mastitis, as they are slightly more specific for gram-positive bacteria, Ganda et al. (2016) attests that there was no significance regarding the clinical cure, nor the bacteriological cure of the treated animals, compared to those that did not receive treatment (GANDA et al., 2016). However, Lago et al. (2011), managed to demonstrate high efficacy in treatments using first-generation cephalosporins. To this end, the author implemented bacteriological cultures in the field, so that the antibacterial was selected according to the microbiological profile of the sample from the mastitic animal. The comparison between the results highlights the problem generated by the use of inappropriate drugs for treatment, which can lead to microbial resistance (EMBRAPA, 2012).

FINAL CONSIDERATIONS

Significant results of bacterial inhibition when using *in vitro* of A. occidentale and C. officinalis, show the feasibility of inserting phytotherapy into daily veterinary treatments, associated with conventional antibiotic therapy, aiming to reduce allopathic dosage and treatment time. This way, one can focus on carrying out more assertive treatments and with less economic losses, by implementing a field culture protocol, together with integrative veterinary medicine.

To broadly verify the activity of herbal medicines, however, there is a need to standardize the extracts, as well as carry out tests at different concentrations, which would even allow the evaluation of treatments that rely on the direct mixture of antibiotics with herbal medicines.

It is also worth mentioning that the raw material of the extracts undergoes changes in its composition, depending on the location in which its extraction is carried out, since the production of secondary metabolites is induced by environmental stress factors, for example.

Finally, observation of the effects *in vitro* of the extracts obtained is relevant, in order to verify possible interference from factors inherent to the animal and the environment.

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