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SYNERGISTIC ANTIBACTERIAL ACTIVITY OF PROPOLIS IN COMBINATION WITH *Eucalyptus Globulus* ESSENTIAL OIL AGAINST BACTERIAL STRAINS ISOLATED FROM BOVINE MASTITIS

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Abstract: In this study we evaluate the antibacterial effect of Brazilian Green Propolis (BGP) and Brown Propolis (BP) in combination with *Eucalyptus globulus* (EG) essential oil against bovine mastitis-causing strains. Prior to the evaluation propolis samples were characterized to determine phenolic total (TF), flavonoid content (FC), and antioxidant activity. The phenolic and flavonoid contents of BGP were found to be 3.60 ± 1.2 mg mL^{-1} and 0.58 ± 0.12 mg mL^{-1}, respectively, while for BP, the values ranged from 14.25 ± 0.97 mg mL^{-1} to 5.46 ± 0.12 mg mL^{-1}. BGP exhibited higher antioxidant activity (DPPH 62.74% and ABTS 69.40%) than BP (DPPH 7.27% and ABTS 12.97 %). The antimicrobial activity against pathogens isolated from isolated from bovine mastitis was evaluated using the macrodilution method, including *Escherichia coli, Streptococcus aureus, Staphylococcus dysgalactiae, Staphylococcus agalactiae, Staphylococcus uberis, and Klebsiella oxytoca*. The results for the combination of BGP and EG at 8 and 24-hour intervals revealed a significant inhibitory effect on strains of *S. aureus* (100.58% ± 0.59; 89.35% ± 0.07), *S. agalactiae* (101.46% ± 0.19; 72.80% ± 0.30), *S. dysgalactiae* (100.51% ± 0.19; 72.75% ± 0.19), *S. uberi*s (102.36% ± 0.19; 71.75% ± 0.09), *E. coli* (99.19% ± 0.95; 70.91% ± 2.38), and *K. oxytoca* (97.94% ± 0.23; 69.39% ± 0.23). The results suggested that the approach of combining natural compounds might prove useful in preventing mastitis and could help minimize the overuse of antibiotics to control mastitis.

Keywords: Propolis, antimicrobial, *Eucalyptus globulus*, *Baccharis dracunculifolia*, artepillin C

INTRODUCTION

Bovine mastitis is a disease characterized by severe inflammation of the mammary gland and udder tissue in dairy cattle. The etiology of mastitis is complex, involving multiple factors with bacteria being the most common cause. However, other pathogens such as yeasts, fungi, and algae can also contribute to mastitis [1]. Typical bacterial strains that cause mastitis are *Streptococcus spp., Staphylococcus spp.,* and *Escherichia coli* [2] [3] [4].

Dairy cattle are susceptible to mastitis, which has adverse economic impacts and affects the health of animals. In Brazil, the prevalence and impact of mastitis in dairy cattle have been studied. One study reported that approximately 30% of cattle experienced at least one case of clinical mastitis annually, with an average of 1.02 clinical cases per lactation when repeat cases are included [5]. Another study found a prevalence of 46% for subclinical mastitis, with 18% of uninfected cows developing subclinical mastitis each month [6].

Subclinical mastitis often goes undetected until it has already caused considerable damage, resulting in decreased milk yield and altered milk composition. In dairy farming contexts, the occurrence of mastitis results in significant losses, encompassing production, milk quality, costs related to culling, and expenses for veterinary medications [1] [7].

To control mastitis, preventive measures such as pre- and post-dipping sanitizers containing active substances like iodine, chlorhexidine, and lactic acid are commonly used [8]. Additionally, various antibiotics, including penicillins, sulfonamides, ampicillin, cloxacillin, and aminoglycosides, are frequently used to treat bovine mastitis. As a consequence, the presence of antibiotic residues in milk can disrupt the production of fermented milk products, compromise milk quality, and cause

allergic reactions in consumers. Moreover, these residues contribute to the emergence and proliferation of antibiotic-resistant bacterial strains [9].

The excessive use of medications for the treatment and prevention of bovine mastitis, as well as growing concerns about their long-term effects on the health of the general population and the dairy industry, emphasize the need for sustainable and natural alternatives. Given the aforementioned facts, numerous studies have demonstrated that natural compounds such as essential oils and propolis have antimicrobial properties, suggesting their potential use as effective alternative therapies for mastitis [10] [11] [12] [13] [14] [15].

In this work, we investigate the synergistic potential of combining *Eucalyptus globulus* essential oil with Brazilian Green Propolis (BGP) and Brown Propolis (BP) extracts to inhibit bacterial isolates of clinical bovine mastitis as possible bioactive compounds and natural alternatives to mastitis prevention.

MATERIAL AND METHODS

BGP and BP hydroalcoholic extracts at 12% were donated by Breyer Company from União da Vitória, Paraná, Brazil. The reagents Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) diammonium (ABTS), quercetin, and ascorbic acid, were acquired from Sigma-Aldrich (St. Louis, MO, USA). Solvents and gallic acid were purchased from Dinâmica Química (São Paulo, Brazil). Ferric chloride, sodium carbonate, potassium acetate, metallic zinc, metallic manganese, and aluminum chloride, were provided by Vetec (Rio de Janeiro, Brazil). Oxidase strips were acquired from Laborclin. Bacterial strains isolated from bovine clinical cases were kindly provided by Labvet Animal Pathology from Carambeí, Paraná, Brazil.

THIN-LAYER CHROMATOGRAPHY (TLC)

Silica-gel GF254 plates (0.250 mm) were used in the TLC procedure, employing two mobile phases: 1) toluene-ethyl acetate (95:5), 2) ethyl acetate-ethanol: water (75:15:10). After the TLC run, visual observations were made under short- or long-wave UV light (254 nm and 365 nm), followed by nebulization with an anisaldehyde solution and heating at 100°C for 1 minute [16].

PHYTOCHEMICAL SCREENING

Various qualitative chemical reactions were utilized to identify phytochemical classes in EG essential oil and BGB and BP, following the method recommended by the Brazilian Society of Pharmacognosy [17].

For the Shinoda reaction, a mixture comprising 2 mL of hydroethanolic extract (1:5 v/v), six fragments of metallic magnesium, and 1 mL of hydrochloric acid 37% was prepared. Subsequently, 3 mL of diluted extract (1:5 v/v) in ethanol, metallic zinc (5 portions), and approximately 8 drops of concentrated hydrochloric acid were added. Tannins were detected using 1 mL of propolis extracts (1:10 v/v) mixed with 5 mL of ethanol and 3 drops of a 1% (m/v) aqueous ferric chloride solution. For the reducing sugar reaction, 1 mL of the propolis extracts was mixed with 1 mL of Benedict's reagent in a test tube. The resulting mixture was then heated, and the color change was recorded [18].

ULTRAVIOLET SPECTROSCOPY

The hydroalcoholic extracts of BGP and BP were diluted in ethanol at a ratio of 1:500. The spectra were obtained by using a quartz cuvette with an optical path of 1.0 cm. Scan spectra were acquired using a quartz cuvette with an optical path of 1.0 cm, in the range of 190 to 500 nm, using a Thermo Scientific GENESYS spectrophotometer.

TOTAL PHENOLICS CONTENT

In a 96-well plate, 5 µL of the sample, 25 µL of Na_2CO_3 solution (10% aqueous), and 25 µL of Folin reagent were added, and the volume was adjusted with water to 225 µL. Incubation was carried out for 15 minutes at room temperature. Absorbance was measured at 765 nm using the Synergy™ H1 hybrid microplate reader. The gallic acid analytical curve was prepared to determine the content of phenolic compounds. The results were expressed in gallic acid equivalents (mg/mL).

TOTAL FLAVONOIDS CONTENT

The determination of total flavonoids was based on the methodology proposed by Kosalec and colleges [19] . Briefly, 5 µL of $AlCl_3$ 10% (w/v) was added to 4 µL of potassium acetate (0.1 mmol L–1), and the volume was completed up to 225 µL with methanol. The absorbance was measured at 420 nm using the Synergy™ H1 hybrid microplate reader. The quercetin analytical curve was used to determine the total flavonoid content. The results were expressed as quercetin equivalents (mg/mL).

ANTIOXIDANT CAPACITY

The antioxidant activity of BGP and BP was assessed using the DPPH and ABTS methods, following the methodology of Struiving and colleges [20] with minor modifications. Ascorbic acid was used as a positive control in both tests, at concentrations ranging from 1 to 100 µg mL^{-1}. The calculation of the inhibition percentage was carried out according to equation 1.

Equation 1. Antioxidant activity rate.

$$\%I = \frac{[(A\ sample - A\ blank) * 100]}{A\ control}$$

Where A is Absorbance

In the DPPH reaction, the absorbance was adjusted to 0.837 ± 0.008. An aliquot of 180 µL of methanolic DPPH solution (120 µmol L^{-1}) was mixed with 20 µL of the samples, and the mixture was incubated for 20 minutes. The resulting absorbance was measured in a microplate reader at 518 nm. In the ABTS assay, the reagent solution was prepared by mixing the ABTS solution (140 mmol L^{-1}) with $K_2S_2O_7$ and incubating for at least 12–16 hours before use. For the test, an aliquot of 10 mmol L^{-1} phosphate buffer (pH 7.0) was added to the ABTS solution, resulting in a solution with an absorbance of 0.768 ± 0.005. Next, an aliquot of 190 µL of ABTS radical solution was added to 10 µL of the propolis extracts, followed by incubation for 20 minutes. Then, the absorbance was measured at 734 nm.

DIRECT INFUSION MASS SPECTROMETRY (MS)

MS direct infusion analysis of propolis samples was performed on a Waters Acquity Ultra Performance LC system, consisting of a column manager, heater/chiller, binary solvent manager, and sample manager, coupled to a Waters Xevo TQ-S MS/MS mass spectrometer equipped with electrospray ionization (ESI) (Waters Co., Milford, MA, USA). The operational parameters of the MS detector were as follows: negative mode electrospray ionization, capillary voltage set at 3.50 kV, desolvation temperature at 600°C. Desolvation and cone gas flows were set at 650 L/h and 1 L/h, respectively.

BACTERIA STRAIN IDENTIFICATION

Bacterial strains were isolated from bovine clinical cases and identified according to a specific protocol, strictly following the standards established in the flowchart [21].

ANTIMICROBIAL ACTIVITY

The bacterial strains were incubated on blood agar plates for 24 hours at 35°C. Subsequently, they were inoculated into phosphate-buffered saline and adjusted to a concentration of 10^6 CFU/mL using the McFarland scale. The bacterial suspensions were then transferred to Mueller-Hinton broth (MHB) supplemented with 0.5% polysorbate 80 in a 1:10 ratio. In microtubes, solutions were prepared by combining 850 µL of MHB with 0.5% polysorbate 80, 50 µL of the bacterial inoculum in MHB, and 100 µL of the sample (20 mg/mL for BGP and BP, and 5 mg/mL for EG essential oil). The solutions were incubated for 24 hours at 35°C. Positive and negative controls were carried out under the same experimental conditions. Absorbance readings were taken at a wavelength of 630 nm using the Synergy™ H1 microplate reader. The antibacterial activity was determined using Equation 2, based on the turbidity of the culture medium.

Equation 2 - Antibacterial activity rate.

$$\%AA = 100 - \frac{100 * (A - Ab)}{Apc - Anc}$$

Where AA = antimicrobial activity, A = absorbance of the samples (MHB + propolis + bacteria), Ab = absorbance of the blank (MHB + propolis), Apc = absorbance of the positive control (bacteria + Mueller-Hinton) and Anc = absorbance of the negative control (MHB).

RESULTS

THIN-LAYER CHROMATOGRAPHY (TLC)

Thin-layer chromatography (TLC) was used to characterize the phytochemical profiles of BGP, BP, and EG essential oil. Using an ethyl acetate mobile phase (90:10) and anisaldehyde as a reagent, the EG essential oil showed a blue-violet spot (Rf 0.64) corresponding to the same retention factor as the 1,8-cineole standard spot.

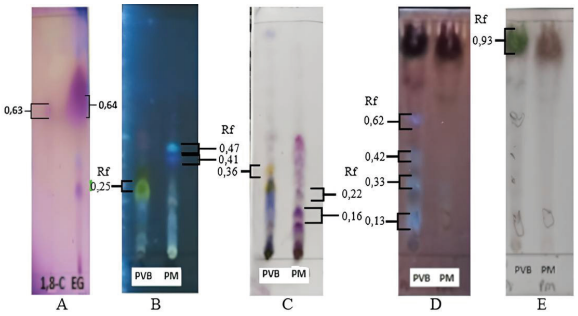


Figure 1 - Thin-Layer Chromatography (TLC) of the *Eucalyptus globulus* essential oil and green and brown propolis.

BGP and BP extracts exhibited distinct profiles, as illustrated in Figures 1B and 1C. The chromatography run was carried out using a mobile phase consisting of toluene and ethyl acetate (95:5). The TLC plate was first visualized at 366 nm (Figure 1B) and then sprayed with acid anisaldehyde (Figure 1C). The BGP extract showed the presence of flavonoids (Rf 0.26 and 0.36), whereas the BP extract revealed the presence of coumarins (Rf 0.41 and 0.47).

Figures 1D and 1E show runs performed using a mobile phase composed of a mixture of ethyl acetate, ethanol, and water (75:15:10). The TLC plate was visualized under 366 nm UV light (Figure 1D) and subsequently sprayed with anisaldehyde sulfuric acid (Figure 1E). The BGP extract revealed the presence of simple coumarins (Rf 0.13, 0.33, 0.42, and 0.62) and tannin (Rf 0.93). In contrast, the BP extract showed a single spot at Rf 0.93.

PHYTOCHEMICAL SCREENING

The chemical screening was conducted to identify different classes of compounds in the BGP and BP propolis samples. The results provided valuable information for distinguishing between the two propolis samples, as summarized in Table 1. The tests

revealed the absence of hydrolysable tannins in both samples, while condensed tannins were present only in BGP. Additionally, flavonoids and reducing sugars were detected in both BGP and BP.

	Assay	BGP	BP
Flavonoids	Shinoda	Blood-red	Reddish-yellow
	Pew	++	+
	Condesnsed	+	-
Tannins	Hydrolysable	-	-
	Reducing sugar	+	++

Table 1 - Analysis of the chemical composition of Brazilian green propolis and Brazilian brown propolis.

ULTRAVIOLET SPECTROSCOPY

The ultraviolet (UV) spectra of propolis samples indicate the presence of flavonoids, which exhibit characteristic absorption bands related to the A and B rings. In the UV spectrum of BGP, two bands are observed at 220 nm and 290 nm. In contrast, BP presents Band I at 275 nm and Band II with a maximum at 210 nm (Figure 2). These UV spectra suggest that BGP and BP have distinct flavonoid compositions, which can influence their respective biological activities and potential applications.

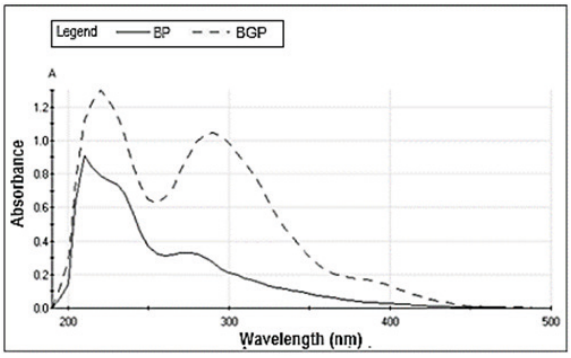


Figure 2 - UV visible spectra of BGP (Brazilian Green Propolis) and BP (Brown Propolis).

PHENOLICS, FLAVONOIDS AND ANTIOXIDANT ACTIVITY

The total phenolic content was determined using the Folin-Ciocalteu reagent, while the total flavonoid content was assessed based on complexation with $AlCl_3$. The analytical curves equations for phenolic compounds ($A = 0.002109x + 0.2926$, $R^2 = 0.998$) and flavonoids ($A = 0.01615x + 0.0338$, $R^2 = 0.9983$) were used to calculate the level of these compounds. The amounts of phenolic and total flavonoids in BGP and BP are in agreement with the criteria of the current Brazilian legislation, which establish minimum concentrations of 0.5% and 0.25% for phenolics and flavonoids, respectively.

As shown in Figure 3, the phenolic content ranged from 3.60 ± 1.2 mg/mL in BGP and 14.25 ± 0.97 mg/mL in BP. The flavonoid content ranged from 0.58 ± 0.12 mg/mL in BGP and 5.46 ± 0.12 mg/mL in BP.

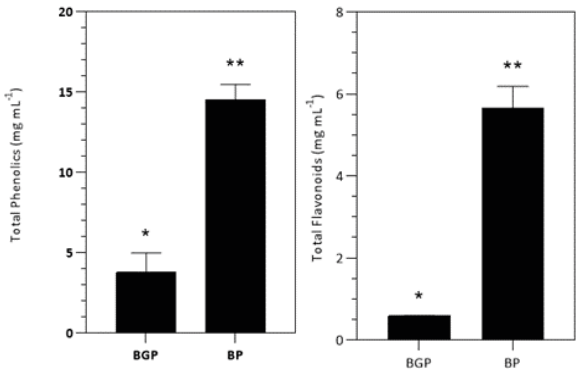


Figure 3 - Total Phenolics and Total Flavonoids. Note: Mean values followed by different symbols are significantly different by the T-test at a 5% probability level.

The antioxidant capacity of the propolis extracts was determined using ABTS and DPPH assays. BGP exhibited higher antioxidant activity (62.74% ± 3.42 and 69.40% ± 2.88) than BP (7.27% ± 0.55 and 12.97% ± 1.69) in both radical scavenging assays (Figure 4).

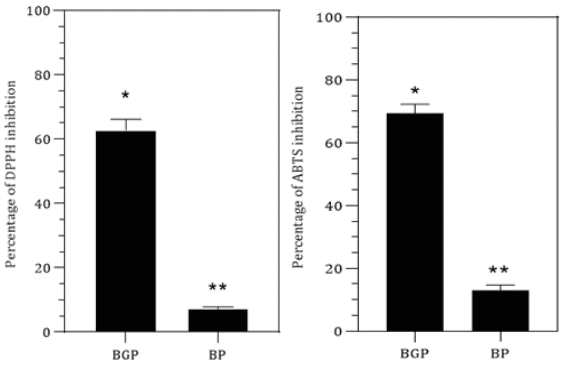
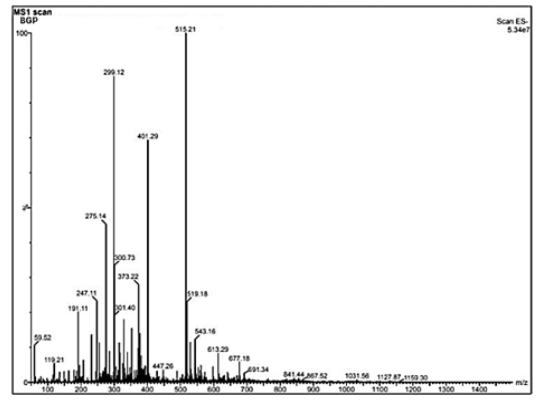


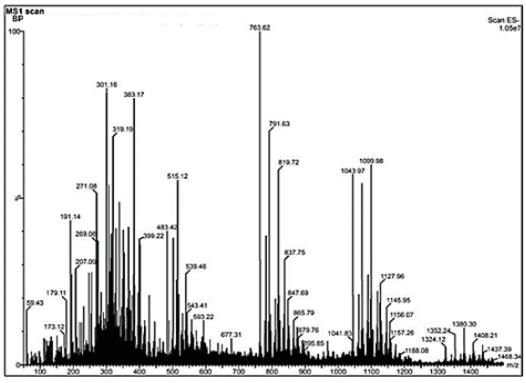
Figure 4 - Antioxidant activity of propolis extracts assessed by DPPH and ABTS assays. Note: Mean values with different symbols indicate significant differences based on the T-test at a 5% significance level.

DIRECT INFUSION MASS SPECTROMETRY

In this study, the composition of propolis samples was analyzed using direct infusion mass spectrometry (MS). This approach is a rapid and efficient method that enables the assessment of a wide spectrum of metabolites without the need to identify each one individually. The mass spectra pattern revealed differences between the chemical compositions of BGP and BP hydroalcoholic extracts. The chemical composition of BGP (Figure 5a) was found to be less complex, with a smaller number of compounds and ions having m/z values below 700, in comparison to BP (Figure 5b). Based on previous studies, the probable components present in BGP and BP extracts were proposed based on the mass-to-charge ratio (m/z), as shown in Table 2.



(a)



(b)

Figure 5 - Negative mode mass spectrometry of Brazilian green propolis (a) and brown propolis (b).

m/z (M^{-1})	BGP	m/z (M^{-1})	BP
163.11	p-coumaric acid	163.27	p-coumaric acid
179.16	caffeic acid	173.12	styrene-acrylic acid
191.11	quinic acid	179.11	caffeic acid
271.2	Naringenin	271.08	Naringenin
285.05	Kaempferol	399.22	3-beta-acetyl-5-cholenic
299.12	artepelin C	483.42	poricoic acid B
301.4	Quercetin	515.12	1,5-dicaffeoylquinic acid
353.05	chlorogenic acid	543.22	Verbenachalcone
401.29	19-nor-10-keto-25-hydroxyvitamin D3	763.62	asprelic acid A
515.21	1,5-dicaffeoylquinic acid		
519.18	daidzein-7-stearate		
577.37	Kaempferitrin		

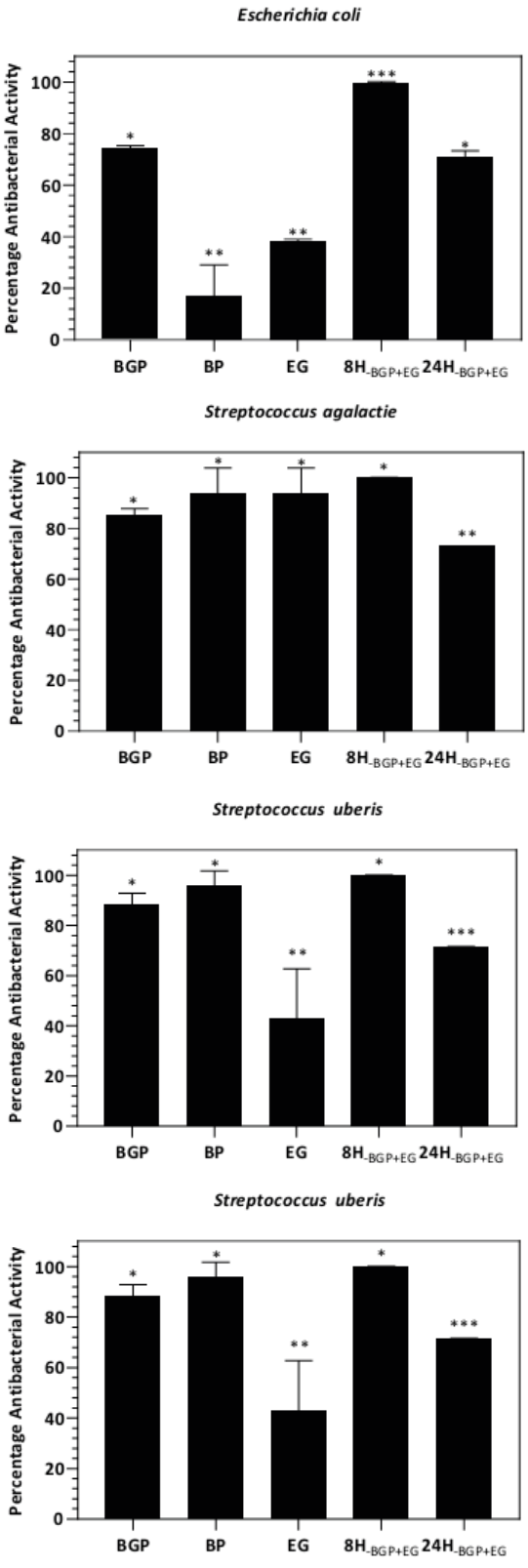
Table 2. Probable compounds in BGP and BP extracts.

BACTERIAL ISOLATES

The bacterial strains isolated from bovine mastitis and used in this work were identified as *Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus agalactiae,* and *Staphylococcus aureus* (Gram-positive cocci), as well as *Escherichia coli* and *Klebsiella oxytoca* (Gram-negative), which belong to the Enterobacteriaceae family.

ANTIMICROBIAL ACTIVITY

Results of antibacterial activity calculated as a function of concentration of the BGP and BP extracts are presented in figure 6. The propolis extracts were tested against six bacterial pathogens associated with clinical and subclinical bovine mastitis. BGP demonstrated inhibitory capacity against species *S. uberis* (87.96% ± 4.86), *S. agalactiae* (85.30% ± 2.43), *E. coli* (73.92% ± 1.34), *S. dysgalactiae* (38.30% ± 3.48), *S. aureus* (21.30% ± 4.97), and *K. oxytoca* (14.71% ± 7.54). On the other hand, BP showed efficacy against species *S. uberis* (96% ± 5.66), *S. agalactiae* (93.76% ± 10.06), *E. coli* (16.72% ± 12.93), *K. oxytoca* (12.09% ± 9.96), *S. dysgalactiae* (2.02% ± 2.23), and *S. aureus* (0.98% ± 0.87). The essential oil exhibited higher efficacy against species *S. agalactiae* (93.89% ± 10.06), *S. aureus* (59.49% ± 9.04), *S. uberis* (43.05% ± 16.61), and *E. coli* (37.93% ± 1.03), whereas it did not show antimicrobial activity against *S. dysgalactiae* and *K. oxytoca*. The combination of BGP and EG essential oil was measured at two different time intervals, 8 and 24 hours, and exhibited microbial activity against *S. aureus* (99.59% ± 0.58; 89.35% ± 0.07), *S. agalactiae* (99.86% ± 0.19; 72.80% ± 0.30), *S. dysgalactiae* (99.86% ± 0.20; 72.75% ± 0.19), *S. uberis* (99.87% ± 0.19; 71.75% ± 0.09), *E. coli* (99.19% ± 0.95; 70.91% ± 2.38), and *K. oxytoca* (97.94% ± 0.23; 69.39% ± 0.23).



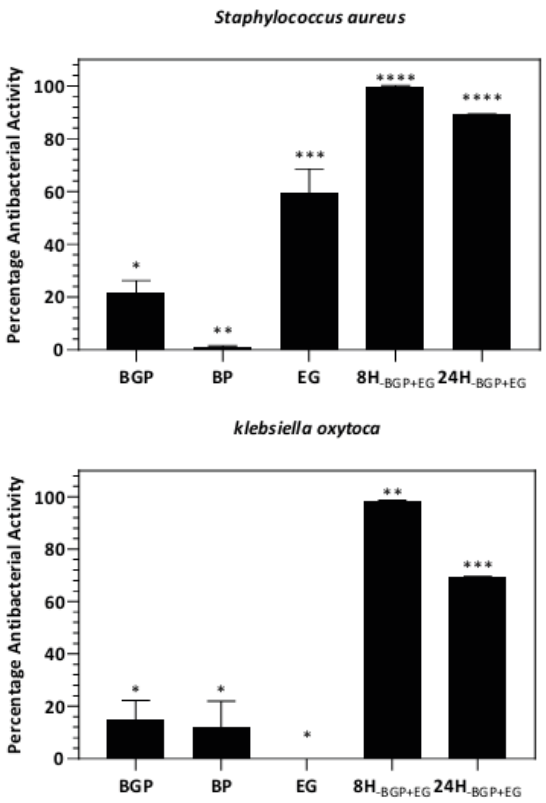


Figure 6 - Antimicrobial activity of BGP, BP, EG essential, and of the combination of BGP and EG essential oil. Note: Mean values followed by different symbols are significantly different by Tukey's test at 5% probability.

DISCUSSION

Bovine mastitis is a major concern for dairy farmers, leading to a continuous search for alternatives to conventional antibiotics. Recently, essential oils and propolis have gained the interest of researchers due to their potential antimicrobial properties and other health benefits [1][3] [22] [23] [24].

Brazilian propolis is classified into 12 distinct types, each with different characteristics. Green and red propolis are particularly interesting in terms of their pharmacological potential [25]. Brazil is the main producer of green propolis, which mainly comes from *Baccharis dracunculifolia* DC, a native medicinal plant popularly known as alecrim do campo. On the other hand, brown propolis is a type that has been little studied. It is produced from botanical sources such as Pinus spp., Eucalyptus spp., and *A. angustifolia* [26] [27].

In the preliminary test using thin-layer chromatography (TLC), we found 1,8-cineole (eucalyptol) as the main compound in *E. globulus* essential oil, confirming previous results [28] [29]. This monoterpene is known for its antibacterial properties, supported by many studies [30] [31]. Although our study didn't find it effective against S. aureus, it did show some activity against E. coli (over 50%). Another study supports the effectiveness of essential oils against bacteria causing mastitis [32].

The local vegetation affects the chemical composition of propolis, influencing its properties [33] [34]. The screening of propolis extracts by TLC reveals flavonoids, coumarins and tannins. Propolis contains over 300 substances, such as caffeoylquinic acid derivatives, p-coumaric acid, flavonols, benzoic acids, dihydroflavonols, terpenes, sesquiterpenes, vitamins, and microelements [27]. Specifically, among other substances, BGP contains compounds like artepillin C, baccharin, and drupanin [35] [36] [37].

Brazilian green propolis (BGP) is recognized for its antibiotic, antioxidant, anti-inflammatory, immunomodulator, and wound-healing properties [34.]. These pharmacological properties are mainly attributed to its high content of flavonoids and phenolic acids. Our results are in agreement with Brazilian legislation, which establishes minimum levels of phenolics (0.5%) and flavonoids (0.25%), respectively.

Antioxidant activity is commonly related to the health benefits of natural compounds. Several studies show significant antioxidant activity in propolis samples [38] [39] [40]. In our study, BGP had lower levels of phenolic compounds and flavonoids but showed higher

antioxidant activity than brown propolis. This result suggests that specific compounds or their combinations might support the capacity to neutralize free radicals.

Mass spectra and UV spectrophotometry results evidenced significant differences between the chemical compositions of BGP and BP. Green propolis shows much less chemical diversity than brown propolis. However, mass spectra show that artepillin C, a p-coumaric acid derivative, is present in BGP extract. This is significant because it has several health benefits [41]. Other compounds, like naringenin, kaempferol, and quercetin, also have antioxidant and antifungal properties. Furthermore, these results suggest that the properties of brown propolis deserve to be further studied.

BGP has been explored for its potential to prevent bovine mastitis. and several studies highlight its effectiveness [10][12][13][42]. Generally, it's more effective against gram-positive bacteria because gram-negative bacteria can neutralize chemical compounds in their outer layers [43]. However, BGP extract inhibited *E. coli* (gram-negative), while BP acted as expected against *K. oxytoca*. Also, the microbiological assay showed that BGP is more effective at inhibiting mastitis-causing bacteria. About 20 mg of BGP extract was enough to inhibit *E. col*i, *S. agalactiae*, and *S. uberis*, showing more than 50% antimicrobial activity.

Based on our microbial inhibition tests and on previous studies that found evidence that propolis can enhance antibacterial effects when combined with other substances [44] [45], we chose BGP to explore its antimicrobial potential combined with EG essential oil. Our results showed that combining BGP and EG essential oil significantly improved antimicrobial activity against the tested strains, suggesting a potential bacteriostatic effect.

CONCLUSION

Our findings indicated that the combination of BGP and the essential oil of *E. globulu*s synergistically inhibited bacteria causing cow mastitis. Also, it is clear that the antimicrobial and antioxidant activities are not solely dependent on high levels of phenolic compounds and flavonoids but are likely attributable to specific bioactive compounds present in BGP. However, further studies should be conducted to better elucidate the mechanism of BGP with EG essential oil as an environmentally friendly alternative to prevent cow mastitis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

REFERENCES

[1] Massote, V. P., Zanateli, B. M., Alves, G. V., Gonçalves, E. S., & Guedes, E. (2019). **Diagnóstico e controle de mastite bovina: uma revisão de literatura.** ***Revista Agroveterinária do Sul de Minas*** *- ISSN: 2674-9661*, *1*(1), 41–54. https://periodicos.unis.edu.br/index.php/agrovetsulminas/article/view/265

[2] Damasceno, M. D., Gomes, A. F. N., Melo, J. P. F., Castro, F. F. A., Guimarães, J. P. F., Lange, C. S., & Souza, G. N. (2022). ***Dinâmica e padrão de infecção dos casos de mastite subclínica e clínica em rebanho bovino mantido em Compost Barn.*** *271*, 44–48.

[3] Almeida, T. V. (2020) **Fatores de risco para mastite bovina e avaliação fenotípica de resistência antimicrobiana.** 2020. Dissertação de Doutorado, Universidade Federal de Goiás.

[4] Perini, S. (2013) **Atividade antimicrobiana de óleos essenciais frente a Staphylococcus aureus e Streptococcus agalactiae isolados de mastite bovina**. Dissertação de Mestrado, Universidade Federal de Lavras.

[5] Oliveira, C. S. F., Hogeveen, H., Botelho, A. M., Maia, P. V., Coelho, S. G., & Haddad, J. P. A. (2015). **Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle**. *Preventive Veterinary Medicine*, *121*(3–4), 297–305. https://doi.org/10.1016/j.prevetmed.2015.08.001

[6] Busanello, M., Rossi, R. S., Cassoli, L. D., Pantoja, J. C. F., & Machado, P. F. (2017). **Estimation of prevalence and incidence of subclinical mastitis in a large population of Brazilian dairy herds**. *Journal of Dairy Science*, *100*(8), 6545–6553. https://doi.org/10.3168/jds.2016-12042

[7] DE SOUZA, E. G.; BERTONCELLO, A. G. (2021) **Conscientização das perdas econômicas decorrentes da mastite em gado leiteiro**. Revista Alomorfia, 5(2), 312-330.

[8] Fitzpatrick, S. R., Garvey, M., Flynn, J., O'Brien, B., & Gleeson, D. (2021). **Evaluating the effectiveness of commercial teat disinfectant products sold in Ireland using the disc diffusion method.** *Irish Journal of Agricultural and Food Research*, *60*(1). https://doi.org/10.15212/ijafr-2020-0130

[9] Tomanić, D., Samardžija, M., & Kovačević, Z. (2023). **Alternatives to antimicrobial treatment in bovine mastitis therapy: A review.** ***Antibiotics*** *(Basel, Switzerland)*, *12*(4), 683. https://doi.org/10.3390/antibiotics12040683

[10] Loguercio, A. P., Groff, A. C. M., Pedrozzo, A. F., Witt, N. M., Silva, M. S. e., & Vargas, A. C. de. (2006). **Atividade in vitro do extrato de própolis contra agentes bacterianos da mastite bovina.** *Pesquisa Agropecuaria Brasileira*, *41*(2), 347–349. https://doi.org/10.1590/s0100-204x2006000200021

[11] Peixoto, M. G. C. D.; Carvalho, M. R. S.; Magalhães, V. M. A. **O leite bovino que produzimos e consumimos**. Embrapa. Brasília, Distrito Federal, 2022.

[12] Saeki, E. K., Peixoto, E. C. T. M., Matsumoto, L. S., Sussumu, L., Marcusso, P., & Monteiro, R. (2011). **Mastite bovina por Staphylococcus aureus: sensibilidade às drogas antimicrobianas e ao extrato alcoólico de própolis**. *Rev. Acta Vet*, *5*(3), 284–290.

[13] Schelles, J. L., Rodrigues, B. M., Pozza, M. S. dos S., & De Lima, L. S. (2021). **Uso de extrato de própolis como agente antisséptico para pré e pós dipping em vacas leiteiras.** *Agrarian*, *14*(51), 95–101. https://doi.org/10.30612/agrarian.v14i51.9164

[14] Castro, R. N., & Salgueiro, F. B. (2016). **Comparação entre a composição química e capacidade antioxidante de diferentes extratos de própolis verde.** *Quimica nova*. https://doi.org/10.21577/0100-4042.20160136

[15] Dalmagro, M., Donadel, G., Moraes Pinc, M., Leuch Stecanella, A., Capello Tominc, G., Miranda, N., Zardeto, G., Alberton, O., Luiz Botelho Lourenço, E., & Hoscheid, J. (2022). **Teste de sinergismo da atividade antimicrobiana dos extratos de eugenia uniflora e própolis por método de checkerboard**. *Revista Multidisciplinar em Saúde*, 1–8. https://doi.org/10.51161/rems/3385

[16] Wagner, H., & Bladt, S. (1996). ***Plant drug analysis: A thin layer chromatography atlas*** (2a ed.). Springer.

[17] ***flavoinoides e antocianos.*** (s/f). Org.br. Recuperado el 30 de setembro de 2023, de http://www.sbfgnosia.org.br/Ensino/flavonoides_e_antocianinos.html

[18] Gonçalves, T. M. (2021). **Determinação de açúcares redutores em alimentos do cotidiano por meio de uma aula prática no ensino médio / determination of reducing sugars in everyday foods through a practical class in high school.** ***Brazilian Journal of Development***, *7*(3), 22940–22955. https://doi.org/10.34117/bjdv7n3-148

[19] Kosalec, I., Bakmaz, M., Pepeljnjak, S., & Vladimir-Knezević, S. (2004). **Quantitative analysis of the flavonoids in raw propolis from northern Croatia**. *Acta Pharm*, *54*(1), 65–72.

[20] Struiving, S., Hacke, A. C. M., Simionatto, E. L., Scharf, D. R., Klimaczewski, C. V., Besten, M. A., Heiden, G., Boligon, A. A., Rocha, J. B. T., Vellosa, J. C. R., Nunes, D. S., Granato, D., & Pereira, R. P. (2020). **Effects of Gender and Geographical Origin on the Chemical Composition and Antiradical Activity of *Baccharis myriocephala* and *Baccharis trimera***. *Foods (Basel, Switzerland)*, *9*(10), 1433. https://doi.org/10.3390/foods9101433

[21] ANVISA (2004). **Manuais de microbiologia clínica. Anvisa**. <https://www.gov.br/anvisa/pt-br/centraisdeconteudo/publicacoes/servicosdesaude/manuais/manuais-de-microbiologia-clinica>

[22] Queiroz, L., & Da, S. (2023). ***Antibacterial action of the essential oil of Origanum vulgare: a review of the literature***. *Research, Society and Development*.

[23] Nazzaro, F., Fratianni, F., Coppola, R., & Feo, V. D. (2017). **Essential oils and antifungal activity.** *Pharmaceuticals (Basel, Switzerland)*, *10*(4), 86. https://doi.org/10.3390/ph10040086

[24] de Moura, R. M. R.; Silva, F. H. M.; Costa, M. C. S.; Vieira, M. D.; de Castro, D. C. C. (2019) **Óleos essenciais: Da extração à utilização**. Editora realize.

[25] Araujo Neto, E. R., Morais, L. S., & Cunha, A. F. S. (2020). **Mapeamento Tecnológico: uma prospecção de patentes e trabalhos científicos relacionados à própolis verde.** *Cadernos de Prospecção*, *13*(1), 268. https://doi.org/10.9771/cp.v13i1.32203

[26] RIBEIRO, V. P, **Estudo fitoquímico, desenvolvimento de método analítico e avaliação biológica de própolis marrom do sudeste brasileiro produzida por Apis mellifera**. doutorado: Faculdade de Ciências Farmacêuticas de Ribeirão Preto, 2022.

[27] Ribeiro, V. P. (2023). **Brazilian Brown Propolis: an Overview About Its Chemical**

Composition, Botanical Sources, Quality Control, and Pharmacological Properties. *Revista Brasileira de Farmacognosia. v*, *33*, 288–299.

[28] Galan, D. M., Ezeudu, N. E., Garcia, J., Gerônimo, C. A., Berry, N. M., Malcolm, B. J. (2020). Eucaliptol (1,8-cineol): um aliado subutilizado nas doenças respiratórias? J. Pesq. Óleos Essenc. 32(2):103-110.

[29] Marinho, T. O., Lucena, H. L. de, Sousa, A. P. de, Silva, F. A. da, Medeiros, T. K. F. de, Souza, O. F. de, Alves, M. de S., Medeiros, M. A. A. de, Brito Junior, L. de, & Oliveira Filho, A. A. de. (2022). **Atividade antiviral do monoterpeno 1,8-cineol: estudo in silico**. *Research, Society and Development*, *11*(4), e31011427363. https://doi.org/10.33448/rsd-v11i4.27363

[30] Rosa, P. V. S., Everton, G. O., Pereira, A. P. M., Fonseca, D., Cunha, J. C. R. da, Mendonça, I. P., Lima, E. C. S., Souza, L. dos S., Souza, L. dos S., Dias, A. A. S., Mouchrek Filho, V. E., & Arruda, M. O. (2020). **Atividade bactericida do óleo essencial e extrato hidroalcoólico das folhas de Eucalyptus globulus.** ***Research, Society and Development***, *9*(7), e804974843. https://doi.org/10.33448/rsd-v9i7.4843

[31] Castillo, M. P. B., Huamán, B. A. L., Castro, A. L. B., Leon, Y. A. G, Salazar, K. F. P., Hilario, C. B. C. (2023). **Eucaliptol: una vista de la medicina tradicional en el siglo XXI**. Rev. Cient. Cienc. Méd. 26(1):52-58.

[32] Lopes, T. S., Fontoura, P. S., Oliveira, A., Rizzo, F. A., Silveira, S., & Streck, A. F. (2020). **Use of plant extracts and essential oils in the control of bovine mastitis**. *Research in Veterinary Science*, *131*, 186–193. https://doi.org/10.1016/j.rvsc.2020.04.025

[33] Menezes, H. (2005). **Própolis: uma revisão dos recentes estudos de suas propriedades farmacológicas.** *Arquivos do Instituto Biologico*, *72*(3), 405–411. https://doi.org/10.1590/1808-1657v72p4052005

[34] Vidal, F. (2021). **Potencial da produção de própolis no Nordeste**. ETENE. 153:1-9.

[35] Machado, J. L., Assunção, A. K. M., da Silva, M. C. P., Reis, A. S. dos, Costa, G. C., Arruda, D. de S., Rocha, B. A., Vaz, M. M. de O. L. L., Paes, A. M. de A., Guerra, R. N. M., Berretta, A. A., & Nascimento, F. R. F. do. (2012). **Brazilian green Propolis: Anti-inflammatory property by an immunomodulatory activity.** *Evidence-Based Complementary and Alternative Medicine: eCAM*, *2012*, 1–10. https://doi.org/10.1155/2012/157652

[36] Costa, P., Almeida, M. O., Lemos, M., Arruda, C., Casoti, R., Somensi, L. B., Boeing, T., Mariott, M., da Silva, R. de C. M. V. de A. F., Stein, B. D. P., Souza, P. de, dos Santos, A. C., Bastos, J. K., da Silva, L. M., & Andrade, S. F. de. (2018). **Artepillin C, drupanin, aromadendrin-4′-O-methyl-ether and kaempferide from Brazilian green propolis promote gastroprotective action by diversified mode of action**. *Journal of Ethnopharmacology*, *226*, 82–89. https://doi.org/10.1016/j.jep.2018.08.006

[37] Arruda, C., Pena Ribeiro, V., Oliveira Almeida, M., Aldana Mejía, J. A., Casoti, R., & Kenupp Bastos, J. (2020**). Effect of light, oxygen and temperature on the stability of artepillin C and p-coumaric acid from Brazilian green propolis**. J. Pharm. Biomed. Anal. 112922. https://doi.org/10.1016/j.jpba.2020.112922

[38] Alves, E.; Kubota, E. H. (2013) **Conteúdo de fenólicos, flavonoides totais e atividade antioxidante de amostras de própolis comerciais.** Revista de Ciências Farmacêuticas Básica e Aplicada, v. 34, n. 1, p. 37-41.

[39] Castro, R. N., Pires, L. de O., Koshiyama, A. S., Bento, K. J. B. (2018). **Biological values of different types of Brazilian Propolis.** ***Greener Journal of Agricultural Science***, *8*(5), 090–099. https://doi.org/10.15580/gjas.2018.5.033118054

[40] Andrade, J. K. S., Denadai, M., de Oliveira, C. S., Nunes, M. L., & Narain, N. (2017). **Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region.** *Food Research International (Ottawa, Ont.)*, *101*, 129–138. https://doi.org/10.1016/j.foodres.2017.08.066

[41] Fonseca, M. W. (2021) **Estudo do potencial antioxidante da própolis verde, do artepelin C e do ácido rosmarínico sobre a funcionalidade mitocondrial em células de *Saccharomyces cerevisia*.** Mestrado em química, Universidade Federal Rural do Rio de Janeiro.

[42] Pinto, M.S., Faria, J.E., Messege, D., Cassini, S.T.A., Gioso, M. M. (2021**) Efeito de extratos de própolis verde sobre bactérias patogênicas isoladas do leite de vacas com mastite**. Braz. J. Vet. Res. Anim. Sci. 38 (6), 2001.

[43] Santos, L. M., Fonseca, M. S., Sokolonski, A. R., Deegan, K. R., Araújo, R. P. C., Umsza-Guez, M. A., Barbosa, J. D. V., Portela, R. D., & Machado, B. A. S. (2020). **Propolis: types, composition, biological activities, and veterinary product patent prospecting**. *Journal of the Science of Food and Agriculture*, *100*(4), 1369–1382. https://doi.org/10.1002/jsfa.10024

[44] Lavigne, J.-P., Ranfaing, J., Dunyach-Rémy, C., & Sotto, A. (2020**). Synergistic Effect of Propolis and Antibiotics on Uropathogenic Escherichia coli.** ***Antibiotics (Basel, Switzerland)*****,** *9*(11), 739. https://doi.org/10.3390/antibiotics9110739

[45] Belmehdi, O., Bouyahya, A., Jekő, J., Cziáky, Z., Zengin, G., Sotkó, G., El Baaboua, A., Senhaji, N. S., & Abrini, J. (2021). **Synergistic interaction between propolis extract, essential oils, and antibiotics against Staphylococcus epidermidis and methicillin resistant Staphylococcus aureus.** *International journal of secondary metabolite*, *8*(3), 195–213. https://doi.org/10.21448/ijsm.947033