Current Microbiology (2021) 78:1846–1855
https://doi.org/10.1007/s00284-021-02344-9

Biotechnological Potential of Eugenol and Thymol Derivatives Against *Staphylococcus aureus* from Bovine Mastitis

Daiana O. S. Nunes[1,4] · Rafaelle Vinturelle[1,4] · Francislene J. Martins[1,5] · Thiago F. dos Santos[3] · Alessandra Leda Valverde[2,3] · Carlos Magno R. Ribeiro[3] · Helena C. Castro[1,5] · Evelize Folly[1,4,6]

Received: 15 May 2020 / Accepted: 11 December 2020 / Published online: 16 April 2021
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC part of Springer Nature 2021

Abstract

Bovine mastitis is an infectious disease that affects the mammary gland of dairy cattle with considerable economic losses. *Staphylococcus aureus* is the main microorganism involved in this highly contagious process, and the treatment is only using antibiotics. Currently, the search for new treatment and/or compounds is still in need due to microbial resistance. In this work, we evaluated the potential of eugenol and thymol derivatives against *S. aureus* strains from bovine mastitis. On that purpose, nine derivatives were synthesized from eugenol and thymol (1–9), and tested against 15 strains of *S. aureus* from subclinical bovine mastitis. Initially, the strains were evaluated for the biofilm production profile, and those with strong adherence were selected to the antimicrobial sensitivity determination in the Minimum Inhibitory Concentration (MIC) assays. Herein the compounds toxicity was also evaluated by in silico analysis using Osiris DataWarrior® software. The results showed that 60% of the strains were considered strongly adherent and three strains (*S. aureus* 4271, 4745 and 4746) were selected for the MIC tests. Among the nine eugenol and thymol derivatives tested, four were active against the evaluated strains (MIC = 32 μg mL^{-1}) within CLSI standard values. In silico analysis showed that all derivatives had cLopP < 5, cLogS > − 4 and TPSA < 140 Å2, and similar theoretical toxicity parameters to some antibiotics currently on the market. These molecules also showed negative drug-likeness values, pointing to the originality of these structures and theoretical feasibility on escaping of resistance mechanism and act against resistant strains. Thus, these eugenol derivatives may be considered as promising for the development of new treatments against bovine mastitis and future exploring on this purpose.

Introduction

Bovine mastitis is an infection that presents high economic importance, since it is present in most of productive herds worldwide. It is a disease with multifactorial aspects and caused by the colonization of the secretory tissue of the udder by several types of pathogenic agents [1]. It is characterized as an inflammatory process of the mammary gland that decreases the milk production and alters its composition [2]. It can be classified as clinical mastitis, in which the

✉ Evelize Folly
evelizefolly@yahoo.com.br

Helena C. Castro
hcastrorangel2@gmail.com

[1] Programa de Pós-Graduação em Ciências e Biotecnologia, Instituto de Biologia, Universidade Federal Fluminense, Outeiro de São João Batista s/no, Centro, Niterói, RJ 24020-141, Brazil

[2] Programa de Pós-Graduação em Química, Instituto de Química, Universidade Federal Fluminense, Outeiro de São João Batista s/no, Centro, Niterói, RJ 24020-141, Brazil

[3] Instituto de Química, Departamento de Química Orgânica, Universidade Federal Fluminense, Outeiro de São João Batista s/no, Centro, Niterói, RJ 24020-141, Brazil

[4] Laboratório de Estudos em Pragas e Parasitos (LEPP), UFF, IB, Department of Cellularand Molecular Biology, Instituto de Biologia, Niterói, RJ, Brazil

[5] Laboratório de Antibióticos, Bioquímica, Ensino e Modelagem Molecular (LABIEMOL), Universidade Federal Fluminense, Outeiro de São João Batista s/no, Centro, Niterói, RJ 24020-141, Brazil

[6] Instituto Nacional de Ciências e Tecnologia—Entomologia Molecular (INCT-EM), Rio de Janeiro, Brazil

symptoms are visible to the naked or subclinical eye, but auxiliary tests are necessary for full diagnosis [3].

Several microorganisms have been associated with mastitis by colonizing the mammary gland canal of the infected animal [4, 5]. In terms of economic losses, it is the most common and devastating disease that affects dairy cattle as it reduces not only quality but also quantity of milk produced worldwide. The control and prevention of this infection are a challenge, despite the continuous efforts from the part of ranchers, due especially to resistant strains [6–8].

Bacteria of the genus *Staphylococcus* are considered important agents that cause bovine mastitis, especially the *Staphylococcus aureus* species, which are known to cause contagious mastitis [9, 10]. This disease is transmitted to other animals of the herd, mainly through milking procedures [3–8]. Due to its highly contagious profile, these bacteria cause infection for more than 30 days by colonization in udder wounds, in the hands of milkers and/or in the mammary gland. The production of a thermostable toxin, which remains active even in pasteurized milk, also contributes to the pathogenesis [11–13].

The most common method of treatment of bovine mastitis is the use of antibiotics. However, the inefficiency is observed in approximately 10–30% of the cases, especially for *S. aureus* caused disease [14, 15]. It is worth noting that the presence of antibiotic residues in milk is also an important parameter evaluated in the dairy industry. Thus, the rational use of antimicrobials in dairy herds is essential due to its impact in the final production [16].

In view of its economic impact and the difficulty in treatment due to microbial resistance, it is necessary the search for new therapeutic alternatives [17]. In this context, the products derived from the secondary metabolism of plants have been pointed as biotechnology options due to their therapeutic properties. The current literature showed that molecules derived from plant metabolism such as trans-cinnamaldehyde (TC), eugenol, carvacrol and thymol are effective against microorganisms and present promising results against the major pathogens causing bacterial mastitis. These molecules are consistently pointed as potential alternatives or adjuvant to antibiotics in the prevention and treatment of bovine mastitis [18, 19].

Thus, the objective of this work was to evaluate the biotechnological potential of nine eugenol and thymol derivatives against *S. aureus* strains from bovine mastitis.

Materials and Methods

Compounds

General

All solvents and reagents used were supplied by E. Merck or Aldrich Co. TLC: silica gel 60 F_{254} plates (0.25 mm; Merck). Column chromatography (CC): silica gel 60 (0.040–0.063 mm; Merck). Melting points were determined on a Fischer-Johns apparatus: uncorrected. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra: Varian 7 T instrument in $CDCl_3$ (Me_4Si as internal standard); chemical shifts are in ppm and coupling constants are given in Hz. IR spectra were recorded on films or KBr pellets with a Perkin Elmer 1420 spectrometer. The HRMS/MS analysis was done with mass spectrometer operating in positive ion modes (m/z 100–1000). The capillary voltage applied was 4500 V, end plate offset 500 V, nitrogen was employed as the nebulizer gas (1 bar), dry heater temperature 200 °C, drying gas (8 L/min) and collision cell energy 8 eV. The mass spectrometer was programmed to perform acquisition in auto MS/MS mode (number of precursors 4) in experiments with different collision energy between 18 and 45 eV for all *m/z* range analyzed.

Preparation of Compound 1 (1,3-bis(4-allyl-2-Methoxyphenoxy)Propane)[20]

In a 25 mL flask, 5.0 mL of acetone and 2.2 mmol (304.0 mg) of K_2CO_3 were added, followed by 2.1 mmol (621.3 mg) of eugenol. The reaction mixture was allowed to stir for 5 min, then 1 mmol (295.84 mg) of 1,3-diiodopropane was added dropwise. The reaction was left to reflux with stirring for 9 h. The resulting mixture was evaporated to dryness and the residue (0.378 g) was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:9) as eluent. Compound **1** was obtained in 49% yield (181 mg, 0.5 mmol); M.p. 78 °C; IR (cm^{-1}): 2919, 1513, 1227; ^{1}H-NMR ($CDCl_3$, 500 MHz)δ: 6.85 (d, 2H, $J = 8.26$ Hz), 6.69 (m, 4H), 5.95 (m, 2H), 4.21 (t, 4H, $J = 6.26$ Hz), 3.83 (s, 6H), 3.32 (d, 4H, $J = 6.68$ Hz), 2.32 (quint, 2H, $J = 6.26$ Hz); [21] ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 149.70, 146.96, 137.90, 133.22, 120. 73, 115.78, 113.92, 112.68, 66.27, 56.15, 40.02, 29.59 [21].

Preparation of Compound 2 (1,10-bis(4-Allyl-2-Methoxyphenoxy)Decane [20]

In a 25 mL flask, 10 mL of acetone and 4.4 mmol (609 mg) of K_2CO_3 and then 4.2 mmol (688.8 mg) of eugenol were

added, this mixture was left under stirring for about 10 min, then 2 mmol (576 mg) of 1.10-dibromodecane was added dropwise. The reaction was left to reflux with stirring for 7 h and 30 min. The resulting mixture was evaporated to dryness and the residue (0.8412 g) was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:9) as eluent. Compound **2** was obtained in 58% yield; M.p. > 200 °C; IR (cm^{-1}): 2918, 2852, 1516, 1259, 793; ^{1}H-NMR ($CDCl_3$, 500 MHz)δ: 6.74 (d, 2H, J = 8.52), 6.63 (m, 4H), 5.89 (m, 2H), 4.99 (m, 4H), 3.91 (t, 4H, J = 6.90), 3.78 (s, 6H), 3.26 (d, 4H,J = 6.69), 1.75 (m, 4H), 1.37 (m, 4H), 1.27 (m, 8H); ^{13}C-NMR ($CDCl_3$, 126 MHz)δ: 149.61, 147.17, 137.95, 132.86, 120.66, 115.75, 113.41, 112.62, 69.42, 56.18, 40.03, 29.46, 26.19; HRESI–MS $[M\text{-}Na]^+$*m/z* 489.2971 (calculated. for *m/z* $C_{30}H_{42}NaO_4$, 489.2975).

Preparation of Compound 3 (1,9-bis(4-Allyl-2-Methoxyphenoxy)Nonane) [20]

In a 25 mL flask, 10 mL of acetone and 4.4 mmol (609 mg) of K_2CO_3 and then 4.2 mmol (688.8 mg) of eugenol were added, this mixture was left under stirring for about 10 min, then 2 mmol (576 mg) of 1,9-dibromononane was added dropwise. The reaction was left to reflux for 7 h and 30 min with stirring. The resulting mixture was evaporated to dryness and the residue was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:9) as eluent. Compound **3** was obtained in 32% yield; M.p. > 200 °C; IR (cm^{-1}): 2917, 2849, 1515, 1232, 796; ^{1}H-NMR ($CDCl_3$, 500 MHz)δ: 6.73 (d, 2H, J = 8.47 Hz), 6.63 (m, 4H), 5.8 (m, 2H), 4.99 (m, 4H), 3.91 (t, 4H, J = 6.89 Hz), 3.77 (s, 6H), 3.26 (d, 4H, J = 6.69 Hz), 1.75 (m, 4H), 1.37 (m, 4H), 1.28 (m, 6H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 149.17, 147.17, 137.96, 132.88, 120.67, 115.76, 113.43, 112.63, 69.42, 56.19, 40.03, 29.70, 29.56, 29.46, 26.19; HRESI–MS $[M\text{-}Na]^+$*m/z* 475.2819 (calculated. for *m/z* $C_{29}H_{40}NaO_4$, 475.2818).

Preparation of Compound 4 (*O*-Methyleugenol)[22]

In a 100 mL flask connected to the reflux apparatus containing 10 mmol (1.64 g) of eugenol in anhydrous acetone (60 mL) was added 29 mmol (4.008 g) of K_2CO_3. The mixture was stirred in an oil bath for 1 h at 50–80 °C. Then, 17 mmol (2.413 g) of methyl iodide was added to the reaction medium and refluxed for a further 9 h. The resulting mixture was evaporated to dryness in a rotary evaporator and the residue (1.2722 g) was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound **4** was obtained in 63% yield; yellow oil; IR (cm^{-1}): 2935, 2834, 1638, 1512; [23] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 6.80 (d, 1H, J = 7.97 Hz), 6.72 (m, 2H), 5.96 (m, 1H), 5.07 (m, 2H), 3.87 (s, 3H), 3.85 (s, 3H), 3.33 (d, 2H, J = 6.68 Hz); [24] ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 149.08, 147.57, 137.86, 132.81, 120.57, 115.76, 112.06, 111.46, 56.11, 55.97, 39.97 [23].

Preparation of Compound 5 (*O*-Ethyleugenol) [22]

In a 100 mL flask connected to the reflux apparatus containing 10 mmol (1.64 g) of eugenol in anhydrous acetone (60 mL) was added 29 mmol (4.008 g) of K_2CO_3. The mixture was stirred in an oil bath for 1 h at 50–80 °C. Then, 17 mmol (1.852 g) of ethyl bromide was added to the reaction solution and refluxed for an additional 19 h. The resulting mixture was evaporated to dryness in a rotary evaporator and the residue (1.5056 g) was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound**5** was obtained in 42% yield; yellow oil; IR (cm^{-1}): 3076, 2978, 2904, 1511, 1258, 1230, 911, 802, 747; ^{1}H-NMR ($CDCl_3$, 500 MHz)δ: 6.80 (d, 1H, J = 7.92 Hz), 6.70 (m, 2H), 5.96 (m, 1H), 5.07 (m, 2H), 4.07 (q, 2H, J = 7.00 Hz), 3.85 (s, 3H), 3.33 (d, 2H, J = 6.68 Hz), 1.44 (t, 3H, J = 7.00 Hz); ^{13}C-NMR ($CDCl_3$, 126 MHz)δ: 149.43, 146.82, 137.88, 132.83, 120.58, 115.72, 113.10, 112.34, 64.58, 56.04, 39.98, 15.04 [22].

Preparation of Compound 6 (*O*-Acetyleugenol) [25]

In a 25 mL flask with reflux condenser, were added 6 mmol (0.98 g) of eugenol, 20 mmol (2.04 g) of acetic anhydride and acetic acid (2 mL). Then the mixture was stirred at reflux for 2.5 h. Then it was cooled to room temperature and a mixture of H_2O and ice was added under magnetic stirring. The organic phase was separated and then washed with H_2O (2 × 20 mL), 10% NaOH (2 × 20 mL) and H_2O (2 × 20 mL). The reaction crude was obtained after removal of the solvent in a rotary evaporator, and purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound **6** was obtained in 82% yield; M.p.31 °C; IR (cm^{-1}): 3076, 2840, 1762, 1185; ^{1}H-NMR ($CDCl_3$, 500 MHz)δ: 6.93 (d, 1H, J = 7.98 Hz), 6.79 (s, 1H), 6.76 (d, 1H, J = 7.93 Hz), 5.95 (m, 1H), 5.10 (m, 2H), 3.81 (s, 3H), 3.37 (d, 2H, J = 6.51 Hz), 2.29 (s, 3H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 169.13, 150.84, 138.98, 137.96, 136.99, 122.48, 120.61, 116.14, 112.71, 55.80, 40.04, 20.65 [26].

Preparation of Compound 7 (*O*-Acetylthymol) [25]

In a 25 mL flask with reflux condenser, were added 6 mmol (0.9013 g) of thymol, 20 mmol (2.04 g) of acetic anhydrous and acetic acid (2 mL). Then the mixture was stirred at reflux for 2 h. Then, it was cooled to room temperature and a mixture of H_2O and ice was added under magnetic stirring. The organic phase was separated and then

washed with H_2O (2 × 20 mL), 10% NaOH (2 × 20 mL) and H_2O (2 × 20 mL). The reaction crude was obtained after removal of the solvent in a rotary evaporator, and purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound **7** was obtained in 83% yield; yellow oil; IR (cm^{-1}): 2963, 1759, 1199, 816; [27] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.19 (d, 1H, J = 7.91 Hz), 7.02 (d, 1H, J = 7.94 Hz), 6.80 (s, 1H), 2.97 (m, 1H), 2.31 (s, H, J = 6.94 Hz); [27] ^{13}C NMR ($CDCl_3$, 126 MHz) δ: 169.95, 148.07, 137.19, 136.74, 127.35, 126.64, 122.92, 27.34, 23.22, 21.10, 21.00 [28].

Preparation of Compound 8 (*O*-Methylthymol) [22]

In a 100 mL flask connected to the reflux apparatus, a solution of 10 mmol thymol (1.502 g) in anhydrous acetone (60 mL) was added 29 mmol (4.008 g) of K_2CO_3. The mixture was stirred in an oil bath for 1 h at 50–80 °C. Then, 17 mmol (2.413 g) of methyl iodide was added to the reaction solution and refluxed for an additional 8 h. The resulting mixture was evaporated to dryness in a rotary evaporator and the residue was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound **8** was obtained in 50% yield; colourless oil; IR (cm^{-1}): 2958, 2866, 1502, 1462, 1256, 1191, 1161, 1042;^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.09 (d, 1H, J = 7.66 Hz), 6.74 (d, 1H, J = 7.67 Hz), 6.67 (s, 1H), 3.81 (s, 3H), 3.27 (m, 1H), 2.33 (s, 3H), 1.19 (d, 6H, J = 6.92 Hz); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 156.83, 136.47, 134.20, 125.98, 121.26, 111.59, 55.49, 26.60, 22.99, 21.55 [29].

Preparation of Compound 9 (*O*-Ethylthymol) [22]

In a 100 mL flask connected to the reflux apparatus, a solution of 10 mmol (1.502 g) thymol in anhydrous acetone (60 mL) was added 29 mmol (4.008 g) of K_2CO_3. The mixture was stirred in an oil bath for 1 h at 50–80 °C. Then, 17 mmol (1.852 g) of ethyl bromide was added to the reaction solution and refluxed for an additional 8 h. The resulting mixture was evaporated to dryness in a rotary evaporator and the residue was purified by silica chromatographic column with a mixture of ethyl acetate:hexane (1:5) as eluent. Compound 9 was obtained in 48% yield; colourless oil; IR (cm^{-1}): 2960, 1505, 1255, 1047, 807; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.08 (d, 1H, J = 7.67 Hz), 6.72 (d, 1H, J = 7.72 Hz), 6.65 (s, 1H), 4.02 (q, 2H, J = 6.97 Hz), 3,29 (m, 1H), 2.31 (s, 3H), 1.41 (t, 3H, J = 6.97 Hz), 1.20 (d, 6H, J = 6.91 Hz); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ: 156.16, 136.26, 134.23, 125.92, 121.04, 112.50, 63.59, 26.71, 22.85, 21.42, 15.10 [30].

Bacterial Strains

A total of 15 strains of *S. aureus* from subclinical bovine mastitis isolated from herds of the states of Minas Gerais, São Paulo and Rio de Janeiro (Brazil) were characterized phenotypically and genotypically and provided by the researcher PhD Aparecida Vasconcelos Paiva Brito, from the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). They were designated by 4026, 4027, 4074, 4075, 4098, 4099, 4271, 4272, 4293, 4313, 4384, 4523, 4745, 4746, and 4784.

The strains were evaluated for the susceptibility to antibiotics amicacin, gentamicin, tobramycin, oxacillin, penicillin, sulfonamide, chloramphenicol, oxytetracycline and tetracycline by means of the Disk Diffusion Test, as recommended by Clinical and Laboratory Standards Institute CLSI [31] using 5 mm filter paper discs (Laborclin®, Brazil). For vancomycin, the assay was used to determine the Minimum Inhibitory Concentration (MIC). According to the recommendation, resistant strains have MIC ≥ 16 μg mL^{-1}; with intermediate sensitivity between 4 and 8 μg mL^{-1} and susceptible strains ≤ 2 μg mL^{-1} [32].

Determination of Biofilm Formation

The 15 strains of *S. aureus* were spiked on BHI agar and incubated for 24 h at 37 °C. After this period, one colony from each plate was transferred to test tubes containing Tryptic Soy Broth (TSB) enrichment broth plus 1% glucose. The tubes were kept under vigorous agitation (250–300 rotations per minute) for 20 h at 37 °C until massive growth was achieved. After this time, the cultures were diluted 1: 100 using TSB broth with 1% glucose. Then, 200 μL of these dilutions were transferred to 4 wells of a flat bottom 96 well plate (Nunc, ThermoFisher Scientific, United States of America) and incubated for 20 h at 37 °C without shaking.

The optical density (OD) was first read through the microplate reader (SpectraMax 190, Microplate Reader, Molecular Devices, Sunnyvale, CA) at a wavelength of 540 nm. The supernatant was removed and the wells were washed gently twice with 0.85% saline for non-removal of the adhered cells. Fixation was carried out in an oven at 65–70 °C for about 1 h and 30 min, until the plate was completely dried. Next, 200 μL of violet crystal dye was added to the wells for 1 min. This dye was then discarded and the wells were washed with sterile water and the plates were again dried in an oven. The second OD reading of each well was carried out. The tests were performed in triplicate. The methicillin resistant strain (BMB 9393) was used as a positive control for biofilm production.

The biofilm unit (UB) was calculated according to Amaral et al. [33] and classified as follows:• DO ≤ 0.24—Non-adherent; • 0.24 > OD < 0.46—Weakly adherent; •

0.46 > OD < 0.919—Moderate adherent; • DO ≥ 0.92—Strongly adherent. The strains that were considered to be the strongest adherent were selected for the other tests.

Disc Diffusion Assay

This assay was performed according to the established by CLSI [31]. The antibacterial vancomycin (Sigma-Aldrich®, Germany) was used as a positive control for the *S. aureus* strains. Dimethylsulphoxide (DMSO) was used for the dilution and as negative control under identical conditions to the tested derivatives. The direct inoculation method was carried out for the species of *S. aureus* considered strongly adherent in the previous test. Bacterial inocula corresponding to the 0.5 scale of MacFarland were seeded on Müeller Hinton agar plates, with the aid of sterile swab. Then, the disks impregnated with the compounds at a concentration of 5 mg mL^{-1} were fixed on the surface of this agar. The plates were incubated at 35 °C for 24 h and checked for growth inhibition halo.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

After the identification of the active derivatives by the disc diffusion test, a quantitative analysis (MIC) was performed using broth microdilution test comparing with the positive controls (vancomycin, eugenol and thymol).

The bacterial inoculum was prepared in 0.85% saline solution and adjusted to 1.5×10^8 colony forming units (CFU) mL^{-1} and diluted in Müeller Hinton broth to a final concentration of 5×10^5 CFU mL^{-1}. The experiments were performed in triplicate on a 96 well flat bottom microtiter plate (TPP-Europe, Switzerland) and three controls were used in each experiment, including (a) the culture medium with the compound and without the bacterial inoculum, (b) the culture medium without inoculum or compound to be tested (sterility control of the culture medium) and (c) the culture medium inoculated without the compound (growth control).

Serial dilution of the compounds in Müeller Hinton broth was performed. Whereas derivatives of eugenol and thymol were tested at concentrations of 256 to 2 µg mL^{-1}; the positive controls vancomycin, eugenol and thymol were tested at concentrations of 25 to 2 µg mL^{-1} for the first one and 256 to 2 µg mL^{-1} for the others. Each well of the 96-well plate remained at 100 µL volume, and then added 100 µL aliquot of the appropriately diluted bacterial inoculum.

After 24 h of incubation at 37 °C, MIC was defined as the lowest concentration of the derivative capable of inhibiting visible growth of the bacterial culture. This procedure was performed according to CLSI Protocol M07-A9 [32].

The MBC, the concentration of the drug capable of inhibiting 99.9% of bacterial growth, was carried out after determining the MIC. We added 10 µL of the wells corresponding to the MIC and 2 × MIC concentrations in BHI agar plates. After 24 h of incubation at 37 °C, colonies were counted. Tests were performed in triplicates for the validation of the results.

In Silico Analysis of Toxicological and Physicochemical Parameters

In silico analysis of the derivatives was performed using the Osiris DataWarrior® program from Acetilion Pharmaceuticals Ltda., Version 5.0 available at http://www.organic-chemistry.org/prog/peo/. This program allows the calculation of theoretical physico-chemical and toxicological parameters, aiming to evaluate the tumorogenicity, mutagenicity, effects on reproduction and irritating effects, and parameters such as drug-likeness, which help in the selection of new drug candidates.

Results

Synthesis of Eugenol and Thymol Derivatives

Nine compounds derived from eugenol and thymol were synthesized using classical methods of phenolic alkylation [20, 22]. Compounds designated by **1**, **2**, **3**, **4**, **5**, **6** were derived from eugenol and **7**, **8** and **9** from thymol (Fig. 1). Compounds **2** and **3** were obtained and describe in this work for the first time.

Evaluation of Biofilm Production by Mastitis *S. aureus* Strains

In the assay for evaluation of bacterial biofilm production, optical density values ranged from 0.58, by strain *S. aureus* 4075, to 3.51 for *S. aureus* 4746. Overall, the strains of *S. aureus* 4271, 4745 and 4746 were the strongest biofilm producers, whereas the strains 4074, 4075, 4384 and 4784 showed lower production, when compared to the positive control (Table 1).

Among all the strains tested, approximately 60% ($n = 9$) were classified as strongly adherent whereas 40% ($n = 6$) showed moderate adherence. No non-adherent or poorly adherent strains were observed on the samples tested. The strains of code 4271, 4745 and 4746 were the ones that presented the highest biofilm production among the tested ones, being classified as strongly adherent and presenting values of biofilm production comparable to the values of the strain used as positive control. Thus, these three strains were selected for the subsequent tests.

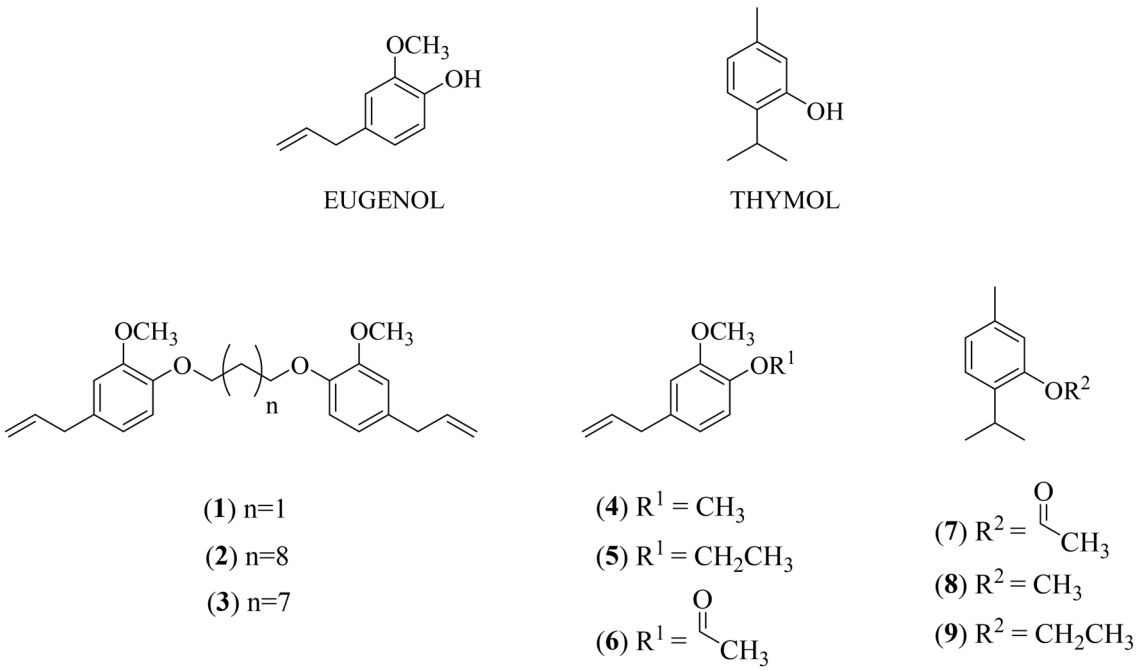

Fig. 1 Eugenol, Thymol and its derivatives **1**–**6** and **7**–**9**, respectively

Table 1 Classification of *S. aureus* strains in the biofilm production according to that described by de Amaral et al. [33]

S. aureus strains	Biofilm unit (BU)	Classification
4026	0.81	Moderate
4027	1.05	Strong
4074	0.59	Moderate
4075	0.58	Moderate
4098	0.90	Moderate
4099	1.46	Strong
4271	3.16	Strong
4272	1.25	Strong
4293	1.19	Strong
4313	2.70	Strong
4384	0.8	Moderate
4523	1.38	Strong
4745	3.22	Strong
4746	3.51	Strong
4784	0.78	Moderate
C +	2.5	Strong

C +, Positive control (*S.aureus* BMB 9393 (MRSA) strain)

Table 2 Evaluation of antibacterial susceptibility by disk diffusion method against strains of *S. aureus* 4271, *S. aureus* 4745 and *S. aureus* 4746

Compounds	Halo (mm)		
	S. aureus 4271	*S. aureus 4745*	*S. aureus 4746*
Thymol	7	6	6
Eugenol	20	16	16
Vancomycin	17	15	15
DMSO	0	0	0
1	0	0	0
2	0	0	0
3	0	0	0
4	21	20	19
5	18	15	15
6	0	0	0
7	10	10	10
8	8	11	10
9	8	10	9

Disc Diffusion Assay

The antibacterial profile was detected for compounds 4, 5, 7, 8 and 9 against *S. aureus* 4271, *S. aureus* 4745 and *S. aureus* 4746 on disc diffusion assays, similar to vancomycin, eugenol and thymol (Table 2). Therefore, these compounds were evaluated in assays for determining Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) and comparison with the controls.

Determination of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The active derivatives were evaluated to determine the lowest concentration of the compound capable of visually inhibiting bacterial growth in culture medium (Table 3). Thus, among the evaluated derivatives, the derivative 4 was the most promising (MIC of 32 μg mL^{-1}) against *S. aureus* 4271, 4745 and 4746 strains; whereas eugenol, thymol, 5, 7, 8 and 9 derivatives were not active within the values of Clinical and Laboratory Standards Institute (CLSI) that provides standards and guidelines for medical professional (Table 3). The MBC for derivative 4 ranged from 32 μg mL^{-1}, against the strains *S. aureus* 4271 and 4746, to 64 μg mL^{-1} against the *S. aureus* 4745 (not shown).

Table 3 Comparison of the Minimal Inhibitory Concentration (MIC) obtained with the synthetic derivatives of eugenol and thymol against strains of *S. aureus* 4271, *S. aureus* 4745 and *S. aureus* 4746

Compounds	MIC (μg mL^{-1})		
	S. aureus 4271	*S. aureus 4745*	*S. aureus 4746*
Thymol	> 256	> 256	> 256
Eugenol	> 256	> 256	> 256
Vancomycin	2	2	2
4	32	32	32
5	> 256	> 256	> 256
7	> 256	> 256	> 256
8	> 256	> 256	> 256
9	> 256	> 256	> 256

MIC Mininal inhibitory concentration

Determination of In Silico Toxicity of Active Compounds

All active derivatives on disc diffusion assays were submitted to the in silico evaluation of toxicological and physical–chemical parameters. According to the Osiris DataWarrior® program, it is estimated that 80% of the drugs on the market have solubility values higher than − 4, which was also observed for all derivatives tested, thus inferring the same biodisponibility (Table 4).

The eugenol and thymol derivatives demonstrated Topographical Polar Surface Area (TPSA) values lower than those observed for vancomycin, close to 4, 5 and 7 values, whereas cLogP values were higher than that found for that antibiotic. Our data suggested that eugenol and thymol derivatives are more hydrophobic than vancomycin and can be easier absorbed.

The OsirisDataWarrior program evaluates the risk of toxicity by locating risk chemical groups within the molecule structure. According our in silico analysis, the derivatives 4 and 5 were theoretically mutagenic whereas 5 was also considered tumorigenic and irritant, 4 have reproductive effects and 7 was also considered irritant. Similarly, Thymol was considered mutagenic, tumorigenic, irritant and likely to cause reproductive effects, whereas Eugenol, also used as positive control, was also pointed as irritant (Table 4). Computational testing help on reducing the time and cost of searching for new antibacterials by applying computational knowledge and somewhat simplifying the process of discovering new active compounds [34]. Vancomycin antibacterial was not associated with the theoretical toxic effects of the program, but is considered in clinical practice as nephrotoxic. This parameter of renal toxicity is not included in the software database [35]. Therefore, far from establishing the real safetyness or risk of these molecules, these theoretical data are useful to prioritize these molecules for future exploring.

Table 4 Evaluation of the toxicological and pharmacokinetic parameters of the eugenol and thymol derivatives analyzed by the Osiris DataWarrior program

Compounds	Toxicological parameters				Physical–chemical parameters			
	Mutagenicity	Tumorigenicity	Effect in reproduction	Irritant	cLogP	cLogS	TPSA (A2)	Molecular weight (Da)
4	+	−	+	−	2.55	− 2.36	18.46	178.23
5	+	+	−	+	2.95	− 2.66	18.46	192.26
7	−	−	−	+	3.18	− 3.13	26.3	192.25
8	−	−	−	−	3.12	− 2.84	9.23	164.224
9	−	−	−	−	3.52	− 3.14	9.23	178.24
EUGENOL	−	−	−	+	2.27	− 2.05	29.46	164.20
THYMOL	+	+	+	+	2.84	− 2.54	20.23	159.22
VANCO	−	−	−	−	− 6.75	− 9.42	530.40	1447.00

Vanco, Vancomycin; −, Negative; +, Positive

The drug-likeness parameters were also calculated based on the scores of the fragments present in these molecules under investigation and compared to the commercially available drugs. Since positive values indicate the similarity of fragments to commercial drugs, the negative values found for these derivatives pointed to the originality of these structures (Fig. 2). Thus, these negative results can be considered as a favorable feature, since these differences may suggest different mechanisms of action for these molecules compared to commercially available drugs. It is noteworthy to mention that various action mechanisms were reported by Zorzet as positive points for new compounds, so that they can delay the emergence of resistance [36].

Discussion

Biofilm production plays an important role in the establishment of infection and colonization of the glandular mammary tissue in bovine mastitis. It is directly related to the establishment and maintenance of infection in host tissues. The biofilm confers resistance to these pathogens against the treatment using antibiotics also allowing the proliferation when it is mature or/and the conditions inside the matrix are unfavorable. The resistance provided by the formation of the biofilm is related to the physical and chemical diffusion barrier formed by the exopolysaccharide matrix, which hinders the antimicrobial action. It also contributes to drug resistance due to: (a) an environment that antagonize antibiotic action, (b) the activation of stress responses that lead to physiological changes in the bacterium, (c) the slower growth of these microorganisms due to nutrient limitation and (d) the absence of antimicrobial targets [37].

Our studies demonstrated a strong biofilm production in 60% of the evaluated *S. aureus* strains. This production is one of the most important mechanisms developed for the survival of this species and is directly related to genetic factors and the production of certain proteins, which help in the adhesion of these bacteria whereas reduces the susceptibility of these organisms to the antibiotics [38, 39].

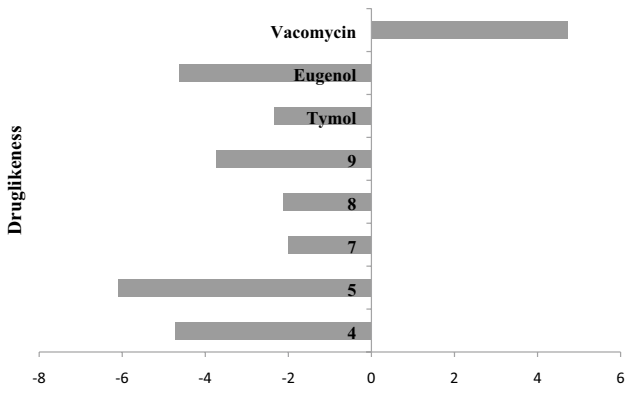

Fig. 2 Evaluation of fragments of the tested derivatives calculated by using Osiris DataWarrior program

According to Aslantas and Demir [40], biofilm formation not only helps in the adhesion of bacteria and colonization of the mammary gland tissue in the avoidance of phagocytosis and adverse conditions in the host, but also in the permanence of the infectious state. These and other authors observed several antibiotic resistance rates among the strains analyzed in their studies with different genes involved [38].

Among the nine derivatives tested, derivative 4 was with the most potential antibacterial profile against the three strains evaluated (MIC = 32 μg mL^{-1} against *S. aureus* 4271, 4745 and 4746), whereas eugenol, thymol and derivatives 5, 7, 8 and 9 were not active within the CLSI values against the *S aureus* strains tested. The structural analysis of compounds 4, 5 and 6 showed that the substitution of a methoxy group for ethoxy in 5 or acetate in 6 reduced the activity of these compounds.

Silva et al. [41] evaluated extracts of *Salvia officinalis* and *Plectranthus ornatus*, which contained eugenol and thymol among their main components. The authors verified MIC values of 300 μg mL^{-1} and 1200 μg mL^{-1} against *S. aureus* 3993 and 4125, for both extracts, respectively, but with values highest and with less potential than our derivative 4. It is worth mentioning that Apolónio et al. [42] did not verify the development of antimicrobial resistance after exposing *S. aureus* ATCC 6838 to eugenol, which may be pointed as an additional, favorable and feasible characteristic for our derivatives.

Silva et al. [43] synthesized eugenol derivatives from addition reactions to the double bond of the allyl or esterification group on the hydroxyl group with carboxylic acids. The compound designated by code 16, obtained by addition reaction to the double bond, exhibited a 10 mm inhibition halo against *S. aureus* and was considered to have a moderate effect to this species. However, this compound was not active under the working conditions in the MIC assay. The authors verified that the reactions of esterification or acetylation in the hydroxyl produced compounds with lower antimicrobial activity than that observed for eugenol, which may justify the results obtained in this research.

The evaluation of toxicological and physical–chemical parameters showed that all derivatives had cLopP < 5, cLogS > − 4 and TPSA < 140 Å2. Derivatives were considered mutagenic (4 and 5); tumorigenic (5); irritating (5 and 7) or with possible effect on reproduction, similar to our antibiotic controls. In addition, all derivatives showed negative drug-likeness values, indicating that they differ from the compounds available on the market with theoretical potential for avoiding resistance bacterial mechanism.

The literature reported that compounds derived from the secondary metabolism of plants may be associated

with antibacterial activitie and some studies highlighted the improvement of this activity when used in synergism. Zheng and colleagues [44] observed that more promising results were observed when using two combinations: thymol and carvacrol and thymol, carvacrol and eugenol. Thus, our derivatives may be more active for use as antimicrobials if combined and it is important to consider that these compounds can also serve as the basis for the synthesis of new substances that may be even more active and less toxic than derivative 4. These perspectives make derivative 4 even more promising to be known and further explored.

Acknowledgements The authors would like to thank Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

Author contributions EFC, HCC, ALV and CMRR designed the experiments and revised the article critically for intellectual content. DOSN, RJVM and FJM wrote the paper with input from the all authors. DOSN, RJVM and TFdS performed the experiments.

References

1. Bandeira FS, Picoli T, Zani JL, da Silva WP, Fischer G (2013) Frequência de *Sthaphylococcus aureus* em casos de mastite bovina subclínica, na região sul do Rio Grande de do Sul. Arq Inst Biol (São Paulo) 80(1):1–6
2. Meade E, Savage M, Garvey P, Slattery MA, Garvey M (2019) Antibiotic resistant zoonotic pathogens of bovine mastitis and possible agents of foodborne disease. CJMI 2(5):1–11
3. Simões TVMD, Oliveira AA (2012) Mastite bovina, considerações e impactos econômicos; Empresa Brasileira de Pesquisa Agropecuária (Brazil). http://www.cpatc.embrapa.br/publicacoes_2012/doc_170.pdf. Accessed 20 Feb 2018
4. Gomes F, Saavedra MJ, Henriques M (2016) Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. Pathog Dis 74(3):1–7
5. Rainard P (2017) Mammary microbiota of dairy ruminants: fact or fiction? Vet Res 48(25):1–10
6. Deb R, Kumar A, Chakraborty S, Verma AK, Tiwari R, Dhama K, Singh U, Kumar S (2013) Trends in diagnosis and control of bovine mastitis: a review. Pak J Biol Sci 16(23):1653–1661
7. Food and Agriculture Organization of the United Nations(2014) Impact of mastitis in small scale dairy production systems, Animal Production and Health Working Paper. http://www.fao.org/3/a-i3377e.pdf. Accessed 01 Apr 2020
8. Alhussien MN, Dang AK (2018) Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: an overview. Vet World 11(5):562–577
9. Bandoch P, Melo LS (2011) Prevalência de mastite bovina por *Staphylococcus aureus*: uma revisão bibliográfica. Biol Health Sci 17:47–51
10. Klibi A, Jouini A, El Andolsi RB, Kmiha R, Hamda CB, Ghedira K, Hamrouni S, Ghram A, Maaroufi A (2019) Epidemiology of β-lactamase-producing *Staphylococci* and gram negative bacteria as cause of clinical bovine mastitis in Tunisia. Bio Med Res Int. https://www.ncbi.nlm.nih.gov/pubmed/31534954. Accessed 21 Jan 2020
11. Contreras A, Sierra D, Sánchez A, Corrales JC, Marco JC, Paape MJ, Gonzalo C (2007) Mastitis in small ruminants. Small Ruminant Res 68:145–153
12. Abebe R, Hatiya H, Abera M, Megersa B, Asmare K (2016) Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res 12(1):270–280
13. Alemu S, Abraha A (2017) Prevalence of bacteria associated with subclinical mastitis in Haramaya University Dairy Cattle, goat and sheep farms. EAJVAS 1(2):61–66
14. Gomes F, Henriques M (2016) Control of bovine mastitis: old and recent therapeutic approaches. Curr Microbiol 72(4):377–382
15. Higham LE, Deakin A, Tivey E, Porteus V, Ridgway S, Rayner AC (2018) A survey of dairy cow farmers in the United Kingdom: knowledge, attitudes and practices surrounding antimicrobial use and resistance. Vet Rec 183:1–9
16. Moudgil P, Bedi JS, Aulakh RS, Gill JPS, Kumar A (2019) Validation of HPLC multi-residue method for determination of fluoroquinolones, tetracycline, sulphonamides and chloramphenicol residues in bovine milk. Food Anal Methods 12:338–346
17. Benhanifia M, Ayad A, Mohamed W (2019) Intramammary honey preparation for treatment of subclinical bovine mastitis: a preliminary study. https://doi.org/10.1007/s13165-019-00244-8. Accessed 1 Dec 2019
18. Baskaran SA, Kazmer GW, Hinckley L, Andrew SM (2009) Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro. J Dairy Sci 92(4):1423–1429
19. Kim SA, Rhee MS (2016) Highly enhanced bactericidal effects of medium chain fatty acids (caprylic, capric, and lauric acid) combined with edible plant essential oils (carvacrol, eugenol, beta-resorcylic acid, trans-cinnamaldehyde, thymol, and vanillin) against *Escherichia coli* O157:H7. Food Control 60:447–454
20. Chen Y, Lan Y, Wang S, Zhang H, Xu X, Liu X, Yu M, Liu B, Zhang G (2014) Synthesis and evaluation of new coumarin derivatives as potential atypical antipsychotics. Eur J Med Chem 74:427–439
21. Stenzenberger H, Koening P (1992) Curable polyimides compositions. Eur Pat Appl, EP 469684 A1 19920205
22. Hamri S, Aboulkas A, El Ammari I, Fadel S, Hafid A, El Haddad M, Noureddine K, Lazar S, Pujol MD, Khouili M (2009) Synthesis of new derivatives of 4,5-dihydro-1h-pyrazole via 4-allyl-2-methoxyphenol. J Mar Chim Heterocycl 8:28–34
23. Baek NI, Hyeyoung KJ, Lee YH, Park JD, Kang KS, Kim SIA (1992) New dehydrodieugenol from *Magnolia officinalis*. Planta Med 58:566–568
24. Maurya R, Ahmad A, Gupta P, Chand K, Kumar M, Rawat JP, Rasheed N, Palit G (2011) Synthesis of novel isoxazolines via 1,3-dipolar cycloaddition and evaluation of anti-stress activity. Med Chem Res 20:139–145
25. Xinlong W, Meigui Y, Quanhai D, Aimin W, Rong X (2012) Design and synthesis of novel pyrethriods containing eugenol moiety. Med Chem Res 21:2827–2830
26. Barbosa JDF, Silva VB, Alves PB, Gumina G, Santos RLC, Sousa DP, Cavalcanti SCH (2012) Structure–activity relationships of eugenol derivatives against *Aedes aegypti* (Diptera: Culicidae) larvae. Pest Manag Sci 68:1478–1483
27. Nagle PS, Pawar YA, Sonawane AE, Bhosale SM, More DH (2012) Synthesis and evaluation of antioxidant and antimicrobial properties of thymol containing pyridone moieties. Med Chem Res 21:1395–1402
28. Morais SM, Vila-Nova NS, Bevilaqua CML, Rondon FC, Lobo CH, Moura AAAN, Sales AD, Rodrigues APR, Figuereido JR, Campello CC, Wilson ME, Andrade HF Jr. (2014) Thymol and eugenol derivatives as potential antileishmanial agentes. Bioorg Med Chem 22:6250–6255

29. Kaur R, Darokar MP, Chattopadhyay SK, Krishna V, Ahmad A (2014) Synthesis of halogenated derivatives of thymol and their antimicrobial activities. Med Chem Res 23:2212–2217
30. Souza LF (1994) Síntese e avaliação da atividade nematicida de derivados do timol e da piperazina. Dissertation. Universidade Federal de Viçosa, Viçosa
31. Clinical and Laboratory Standards Institute (2015) Performance standards for antimicrobial susceptibility testing—twenty-fifth informational supplement, 23, 3. Clinical and Laboratory Standards Institute, Wayne
32. Clinical and Laboratory Standards Institute (2012) Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed. Document M07-A9. Clinical and Laboratory Standards Institute, Wayne
33. Amaral MM, Coelho LR, Flores RP, Souza RR, Silva-Carvalho MC, Teixeira LA, Ferreira Carvalho BT, Figueiredo AM (2005) The predominant variant of the Brazilian epidemic clonal complex of methicillin-resistant *Staphylococcus aureus* has an enhanced ability to produce biofilm and to adhere to and invade airway epithelial cells. J Infect Dis 192(5):801–810
34. Billones JB, Carrillo MC, Organo VG, Sy JB, Clavio NA, Macalino SJ, Emnacen IA, Lee AP, Ko PK, Concepcion GP (2017) In *silico* discovery and in vitro activity of inhibitors against *Mycobacterium tuberculosis* 7,8-diaminopelargonic acid synthase (MtbBioA). Drug Des Devel Ther 2(11):563–574
35. Gupta A, Biyani M, Khaira A (2011) Vancomycin nephrotoxicity: myths and facts. Neth J Med 69:379–383
36. Zorzet A (2014) Overcoming scientific and structural bottlenecks in antibacterial discovery and development. Ups J Med Sci 119(2):170–175
37. Marques VF, Motta CC, Soares BD, Melo DA, Coelho SM, Coelho ID, Barbosa HS, Souza MM (2017) Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. Braz J Microbiol 48(1):118–124
38. Bardiau M, Caplin J, Detilleux J, Graber H, Moroni P, Taminiau B, Mainil JG (2016) Existence of two groups of *Staphylococcus aureus* strains isolated from bovine mastitis based on biofilm formation, intracellular survival, capsular profile and agr-typing. Vet Microbiol 15(185):1–6
39. Khoramrooz SS, Mansouri F, Marashifard M, Malek Hosseini SA, Akbarian COF, Ganavehei B, Gharibpour F, Shahbazi A, Mirzaii M, Darban-Sarokhalil D (2016) Detection of biofilm related genes, classical enterotoxin genes and agr typing among *Staphylococcus aureus* 91 isolated from bovine with subclinical mastitis in southwest of Iran. Microb Pathog 97:45–51
40. Aslantaş Ö, Demir C (2016) Investigation of the antibiotic resistance and biofilm forming ability of *Staphylococcus aureus* from subclinical bovine mastitis cases. J Dairy Sci 99(11):8607–8613
41. Silva DM, da Costa PA, Ribon AOB, Purgato GA, Diaz-Muñoz G, Diaz MAN (2019) Plant extracts display synergism with different classes of antibiotics. An Acad Bras Ciênc 91(2):e20180117
42. Apolónio J, Faleiro ML, Miguel MG, Neto L (2014) No induction of antimicrobial resistance in Staphylococcus aureus and Listeria monocytogenes during continuous exposure to eugenol and citral. FEMS Microbiol Lett 354:92–101
43. Silva FFM, Monte FJQ, Lemos TLG, Nascimento PGG, Costa AKM, Paiva LMM (2018) Eugenol derivatives: synthesis, characterization, and evaluation of antibacterial and antioxidant activities. Chem Cent J 12(34):1–10
44. Zheng L, MinBae Y, Jung KS, Heu S, Lee SF (2013) Antimicrobial activity of natural antimicrobial substances against spoilage bacteria isolated from fresh produce. Food Control 32(2):665–672

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.