INTRODUCTION

Intracanal medications assist in root canal system decontamination of teeth with pulp necrosis (1, 2). Calcium hydroxide (CH) paste is commonly selected for this purpose because of its antimicrobial properties and ability to stimulate tissue repair (3). However, pathogens such as Enterococcus faecalis can resist the action of this intracanal medication, especially in the form of bacterial biofilm (4, 5), given that the polysaccharide layer of biofilm can prevent the medication from exerting its action completely, even when associated with biologically active vehicles, such as paramonochlorophenol (6).

N-acetylcysteine (NAC) is a potent antioxidant agent used to treat lung diseases and can modulate pro-inflammatory cytokines and disrupt bacterial biofilms. Furthermore, it inhibits bacterial...
adhesiveness, reduces extracellular polysaccharides production, and decreases cell viability. This action of NAC on bacterial biofilm has prompted the hypothesis that its use alone or associated with intracanal drugs could increase bacterial susceptibility to antimicrobial agents (7, 8).

Ambroxol hydrochloride (AMB) is also used in pulmonology and interferes directly in the composition of mucus by reducing its viscosity and aiding in its elimination (9, 10). However, no published study on its use in combating endodontic bacterial biofilm exists in the literature. Therefore, the present study was conducted with the prospect of using AMB as a mucolytic substance that can act directly on the polysaccharide layer of endodontic biofilm, optimising the action of intracanal medication and irrigating solutions.

Therefore, this study aimed to evaluate the solubility, pH, antimicrobial action against Enterococcus faecalis biofilm, and cytotoxicity of intracanal pastes based on NAC and AMB, compared with CH paste. The null hypothesis was that the two tested substances would show a behaviour similar to that of CH.

MATERIALS AND METHODS
The local animal research ethics committee approved this study (0012019).

Paste preparation
An amount of 500 mg of each compound tested (NAC, AMB, and CH) was weighed on a digital analytical scale and added to 0.5 mL of 500 mg/0.5 mL propylene glycol. Each mixture was manipulated with a glass plate with a sterile endodontic spatula (Endo IntraFlexfill; SSWhite, Rio de Janeiro, RJ, Brazil) inside a laminar flow chamber (VecoFlow, Campinas, SP, Brazil) until a firm and granule-free consistency was obtained.

Solubility test
Thirty root canals of artificial maxillary central incisors made of acrylic resin and having standardised apical foramina with a diameter of 250 μm were used in this study. Ten root canals (n=10) were filled with each experimental paste immediately after manipulation with a Lentulo #25 instrument (VDW, Munich, Germany) at 10,000 rpm, followed by sealing of the access opening with distilled water by abundant irrigation, and the experimental pastes were placed in direct contact with the biofilm formed on the surface of the blocks. The blocks were then kept in an incubator at 37°C and at constant humidity for 7 days. After biofilm maturation, the detached cells were removed using CTAn v 1.11.10.0 software (SkyScan), and the baseline volume (mm³) of each paste was obtained using CTAn v 1.11.10.0 software (SkyScan). The acrylic teeth were stored individually in plastic containers, fully immersed in 10 mL of deionised water at 37°C. After 7 days, the teeth were removed from the containers, scanned, and reconstructed, and the final volume of the pastes was arrived at in the same way as at baseline. The percentage of solubilisation of the paste used in each specimen was calculated according to the difference between the initial and final volumes, divided by the initial volume, and then multiplied by 100.

pH assessment
Immediately after inserting the pastes into the root canals, the teeth were immersed in the containers, and the water was agitated for 30 s. The concentration of hydroxyl and calcium ions released was measured with a pH-meter (Model 371; Micronal, São Paulo, SP, Brazil) previously calibrated and standardised with buffer solutions at pH levels 4, 7 and 12. Assessments were repeated after 3 hours, 3 days, and 7 days, at which the teeth were placed in new plastic containers and immersed in new ultra-pure water. The pH of the ultra-pure water alone was used as a control.

Antimicrobial activity
Ten bovine incisors with complete roots were selected. The teeth were sectioned with a 4.0 mm diameter trephine drill (Harte Prec Grip, Ribeirão Preto, SP, Brazil) coupled to an electric micromotor operating at a speed of 1200 rpm and positioned perpendicularly to the teeth, following a mesiodistal direction, thus obtaining 4 cylindrical dentine blocks per tooth. The dentine surfaces were polished with felt discs (Fortel, São Paulo, SP, Brazil). The blocks were then immersed in a 1% sodium hypochlorite solution (Rioquímica, São José do Rio Preto, SP, Brazil) for 15 minutes and treated with 17% ethylene-diaminetetraacetic acid (Biodyomar, Ibiporã, PR, Brazil) for 5 minutes, to remove any debris remaining after the sectioning procedure. The dentine blocks were stored in individual plastic containers and sterilised in an autoclave. Enterococcus faecalis biofilm formation was induced on the surface of each block for 21 days, as described by Guerreiro-Tanomaru et al. (11).

After biofilm maturation, the detached cells were removed with distilled water by abundant irrigation, and the experimental pastes were placed in direct contact with the biofilm formed on the surface of the blocks. The blocks were then kept in an incubator at 37°C and at constant humidity for 7 days. Finally, all the microbiological procedures were performed aseptically in a laminar flow chamber (VecoFlow).

Antimicrobial action was evaluated using the live/dead staining method (Invitrogen Molecular Probes, Eugene, OR, USA) under an inverted confocal microscope (Leica, Mannheim, Germany). The dentine blocks were washed with 100 μL of phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO, USA) to remove any drug residues and then flushed with 50 μL of SYTO 8 (green colouration) and propidium iodide (Sigma-Aldrich) for 10 minutes. The acquired images were analysed using biolmage L software (www.biolmageL.com; NIH, Bethesda, MD, USA) to calculate the percentage of live-cell volume remaining after the experimental treatments.

Cell viability test
Fibroblast-like L929 cells were grown in Dulbecco Modified Eagle's Medium supplemented with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg, MD, USA), streptomycin (50 g/mL) and a 1% antibiotic/antimycotic cocktail (300 U/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B; Gibco Laboratories) under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO²).

Serial dilutions (undiluted, 1/2, 1/4, and 1/8) of fresh extracts of the experimental AMB, NAC and CH pastes were prepared using culture media, according to the method proposed by Pedano et al. (12).
According to the manufacturer’s instructions, cell viability was determined using the neutral red test (Sigma-Aldrich, San Luis, MO, USA). Fibroblast-like L929 cells were seeded in 96-well plates (105 cells/well) and incubated for 24 hours, after which the cultures were exposed to the aforementioned serial dilutions. The cells cultivated but not exposed to any extracts were used as controls. The neutral red solution was added to each cell batch after 6, 24, and 48 hours. After incubation for 4 hours (37°C, 5% CO₂), the solution was removed, and the cultures were quickly washed with 1% calcium chloride, followed by 1% formaldehyde. Then, the neutral red eluent (EtOH: H₂O:acetic acid at a 50:49:1 ratio) was added to extract the neutral red from the cells. After stirring the solution for 10 minutes at room temperature, the absorbance of the culture medium was read at 540 nm. Each condition was analysed in triplicate.

Statistical analysis
Data normality was analysed using the Shapiro-Wilk test. The volumetric solubility, pH, and cytotoxicity data showed normal distribution, whereas the antimicrobial data did not. The pH, volumetric solubility, and cytotoxicity data were compared using the ANOVA and Tukey tests, and the antimicrobial data were compared using the nonparametric Kruskal-Wallis and Dunn tests. The significance level was set at 5% for all the tests. The statistical analyses were conducted with GraphPad 6.0 software (GraphPad, San Diego, CA, USA).

RESULTS
The solubilisation percentage levels of all the pastes were significantly different after 7 days (P<0.05), where the highest values were for NAC, followed by AMB, and then CH (Table 1). Likewise, The pH values were all significantly different, where NAC and AMB were below 6 at all time points, whereas that of CH was above 11 (P<0.05). Intragroup pH levels were stable for CH throughout all the experimental periods, whereas NAC decreased significantly after 7 days, and AMB varied significantly over all the experimental periods (P<0.05; Table 2).

The antimicrobial action of AMB was significantly higher (P<0.05) than that of CH or that of water (control), whereas that of NAC was significantly higher than that of water (P<0.05; Table 3). A confocal image representative of the results observed for the experimental and control groups after 7 days is shown in Figure 1.

The cell viability associated with all the tested pastes was significantly lower than that associated with the control group after all the periods evaluated, except for the ½ dilution of CH paste, for which cell growth increased significantly after 48 hours (P<0.05). The cell viability associated with CH was significantly higher than that associated with the AMB and NAC extracts for the ½, ¼, and ⅛ dilutions at all timepoints (P<0.05), and no significant differences were detected between AMB and NAC (P>0.05) for any dilution at any time point. Exposure to higher dilutions of CH promoted higher cellular metabolism levels than the undiluted and ½ dilution extracts of the same paste after all the periods evaluated (P<0.05; Fig. 2).

### Table 1. Mean and standard deviation values of initial paste volume, final paste volume, and percentage of volumetric reduction after 7 days, as a result of paste solubilisation

| Paste                   | Initial volume (mm³) | Final volume (after 7 days; mm³) | Paste solubilisation (% volume reduction) |
|-------------------------|----------------------|---------------------------------|------------------------------------------|
| Ambroxol hydrochloride   | 7.54±2.88b          | 5.63±2.42b                      | 25.80±10.86a                             |
| N-acetylcysteine        | 5.74±1.55b          | 1.28±0.84b                      | 77.36±12.29b                             |
| Calcium hydroxide       | 6.92±1.59a          | 6.03±1.17b                      | 12.00±5.74c                              |

Different lowercase letters indicate statistically significant differences (P<0.05) among the pastes; different capital letters indicate statistically significant intragroup differences (P<0.05) between the initial and final paste volumes after 7 days.

### Table 2. Mean and standard deviation values of pH level exhibited by the pastes after 3 hours, 72 hours, and 7 days

| Paste                     | 3 hours | 72 hours | 7 days  |
|---------------------------|---------|----------|---------|
| Ambroxol hydrochloride    | 5.52±0.13AA | 4.75±0.23ab | 5.77±0.13AC |
| N-acetylcysteine          | 3.12±0.10BA | 3.02±0.10aB | 2.88±0.07bB |
| Calcium hydroxide         | 11.45±0.22aA | 11.51±0.25aC | 11.61±0.28aC |
| Control (water)           | 6.21±0.06aA | 6.22±0.07aA | 6.21±0.08aA |

Different lowercase letters indicate statistically significant differences (P<0.05) among the pastes at each time point; different capital letters indicate statistically significant intragroup differences (P<0.05) among the different time points.

### Table 3. Median and minimum and maximum percentage values of live cells in Enterococcus faecalis biofilm after treatment

| Paste                      | Median (%) | Min-max (%) |
|---------------------------|------------|-------------|
| Ambroxol hydrochloride    | 20.05      | 0-94.35a    |
| N-acetylcysteine          | 59.74      | 2.76-98.19ab|
| Calcium hydroxide         | 81.79      | 23.33-99.42bc|
| Control (water)           | 93.31      | 76.53-99.87c|

Different lowercase letters indicate statistically significant differences (P<0.05) among the pastes.

DISCUSSION
Significant differences were observed between the NAC and CH pastes, and between the AMB and CH pastes, with respect to all the properties tested; therefore, the null hypothesis was rejected. The use of CH paste in endodontics is now well established due to its biological and antimicrobial action (13). However, CH’s low penetration level into dentinal tubules and certain factors related to bacterial resistance may compromise its effectiveness (14).

An intracanal medication paste should be capable of filling the entire root canal space, preventing bacterial growth, and preventing recontamination (13). From this perspective, low paste solubility is a desirable property. In the present study, the solubility level of NAC (77.6%) was higher than that of both CH (12%) and MBA (25.8%). In addition, the larger particles of the NAC paste made it challenging to obtain a homogenised
mixture during manipulation; this may have been the reason for its higher degree of dissolution.

The pH levels for NAC and AMB were acidic, whereas that of CH was alkaline throughout the experimental periods. Likewise, both AMB and NAC showed better results than the control group with respect to the percentage of living cells in the experimental biofilm. These results suggest that the pH level was not the main factor in the antimicrobial action against Enterococcus faecalis biofilm. The AMB paste contained smaller particles, which probably favoured more effective penetration into the biofilm matrix, dissolution of the extracellular polysaccharide matrix, and hence greater antimicrobial action (15). As to the effect of pH on the host tissue, a recent study showed that moderately
The use of the AMB and NAC mucolytic agents as intracanal dressings was tested in the present study to find a way to make direct contact with bacterial cells. This approach seemingly increased the antimicrobial effect of the pastes formulated with them. In addition, the properties of NAC have proven to help decrease the ability of bacteria to adhere to a substrate (17, 18) and to produce extracellular polysaccharides (19, 20). Furthermore, it has antioxidant and inflammation-modulatory effects (21). Nonetheless, to the best of our knowledge, NAC has not been studied as an intracanal medication.

AMB has demonstrated satisfactory antimicrobial effects (7, 8) by acting at different stages of biofilm formation, such as aggregation and maturation. In the present study, AMB produced higher rates of microbial reduction in biofilm than NAC (by 39%), although this difference was not statistically significant. Therefore, it has been hypothesised that AMB can promote a higher level of antisepsis and greater action against the exopolysaccharide layer (22, 23). In addition, the mucolytic properties of AMB and NAC could render them capable of acting even in the presence of purulent exudation.

CH’s low action level against biofilm confirms the findings of Ordinola-Zapata (5). When exposed to stress, Enterococcus faecalis resorts to a mechanism that allows it to maintain internal homeostasis by passive means, which reduces membrane permeability, or by active means, whereby it transports ions through its membrane to buffer its cytoplasm (proton pump; 2). The continuous growth of Enterococcus faecalis in contact with CH may also be related to its resistance to an alkaline medium (7) and its strong adherence to dentine when in the form of biofilm (6, 7).

In the present study, L929 fibroblast-like cells were chosen because they are commonly used in cell-culture studies, are easy to control, and produce a precise cytotoxic response (24, 25). The AMB and NAC pastes showed a higher level of cytotoxicity than CH paste for all the dilutions tested. De Menezes et al. (26) showed that the effect of CH depended on the level of dilution, in that cell viability was impaired at higher dilutions. Our results also confirm that NAC affected cell cycle regulation and apoptosis by acting as a cell viability inhibitor and a modulator of gene or protein levels (27). Furthermore, Sunkari et al. (28) also demonstrated a cell-type dependent effect of AMB, in that no significant reduction in the viability of murine macrophages below 2.5 μM was identified, whereas a reduction in the viability of keratinocytes was observed up to 5 μM. In contrast, Yamaya et al. (29) found no difference in the cell viability of human tracheal epithelial cells treated with AMB.

Within the limitations of the methods used in the present study, the NAC and AMB pastes proved effective against Enterococcus faecalis biofilm. In addition, the AMB paste showed lower solubility, a pH closer to neutral, and better antimicrobial action than NAC and CH pastes. Therefore, biocompatibility and clinical studies are suggested to better understand ambroxol’s behaviour as an alternative intracanal medication, particularly in persistent infections where CH has failed to produce the desired outcome.

CONCLUSION
When diluted, the ambroxol hydrochloride and N-acetylcysteine pastes showed lower pH values, higher antimicrobial action, higher solubility, and higher cytotoxicity than the CH paste.

Disclosures
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REFERENCES
1. Vera J, Siqueira JF Jr, Ricucci D, Loghin S, Fernández N, Flores B, et al. One-vs-two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod 2012; 38(8):1040–52.
2. Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB, et al. Antimicrobial activity and physicochemical properties of calcium hydroxide pastes used as intracanal medication. J Endod 2016; 42(12):1822–8.
3. Holland R, Gomes JF Filho, Cintra LTA, Queiroz IOA, Estrela C. Factors affecting the periapical healing process of endodontically treated teeth. J Appl Oral Sci 2017; 25(5):465–76.
4. Evans M, Davies JK, Sundqvist G, Figg D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2002;35(3):221–8.
5. Ordinola-Zapata R, Bramante CM, Minotti PG, Cavagnagio BC, Garcia RB, Bernardinelli N, et al. Antimicrobial activity of triantibiotic paste, 2% chlorhexidine gel, and calcium hydroxide on an intraoral-infected dentin biofilm model. J Endod 2013; 39(1):115–8.
6. Siqueira JF, Rôças IN, Ricucci D. Biofilms in endodontic infection. Endod Topics 2010; 22(1):33–49.
7. Jhajharia K, Parolla A, Shetty K, Mehta L. Biofilm in endodontics: A review. J Int Soc Prev Community Dent 2015; 5(1):1–12.
8. Toker H, Ozdemir H, Eren K, Ozer H, Sahin G. N-acetylcysteine, a thiol antioxidant, decreases alveolar bone loss in experimental periodontitis in rats. J Periodontol 2009; 80(4):672–8.
9. Leite B, Gomes F, Teixeira F, Souza C, Pizzolitto E, Oliveira R. Staphylococcus epidermidis biofilms control by N-acetylcysteine and rifampicin. Am J Ther 2013; 20(4):322–8.
10. Hull JD, Lyon RA. In vitro pharmacology of ambroxol: Potential serotonergic sites of action. Life Sci 2018; 197:67–72.
11. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod 2013; 39(3):346–50.
12. Pedano MS, Li X, Li S, Sun Z, Cokic SM, Putzeys E, et al. Freshly-mixed and setting calcium-silicate cements stimulate human dental pulp cells. Dent
Mater 2018; 34(5):797–808.

13. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999; 32(5):361–9.

14. Wang JD, Hume WR. Diffusion of hydrogen ion and hydroxyl ion from various sources through dentine. Int Endod J 1988; 21(1):17–26.

15. Nakajo K, Komori R, Ishikawa S, Ueno T, Suzuki Y, Iwami Y, et al. Resistance to acidic and alkaline environments in the endodontic pathogen Enterococcus faecalis. Oral Microbiol Immunol 2006; 21(5):283–8.

16. Saghiri MA, Asatourian A, Morgano SM, Wang S, Sheibani N. Moderately acidic pH promotes angiogenesis: an in vitro and in vivo study. J Endod 2020; 46(8):1113–9.

17. Riise GC, Qvarfordt I, Larsson S, Eliasson V, Andersson BA. Inhibitory effect of N-acetylcysteine on adherence of Streptococcus pneumoniae and Haemophilus influenzae to human oropharyngeal epithelial cells in vitro. Respiration 2000; 67(5):552–8.

18. Costa F, Sousa DM, Parreira P, Lamghari M, Gomes P, Martins MCL. N-acetylcysteine-functionalized coating avoids bacterial adhesion and biofilm formation. Sci Rep 2017; 7(1):17374.

19. Quah SY, Wu S, Lui JN, Sum CP, Tan KS. N-acetylcysteine inhibits growth and eradicates biofilm of Enterococcus faecalis. J Endod 2012; 38(1):81–5.

20. Choi YS, Kim C, Moon JH, Lee JY. Removal and killing of multispecies endodontic biofilms by N-acetylcysteine. Braz J Microbiol 2018; 49(1):184–8.

21. Faghfouri AH, Zarezadeh M, Tavakoli-Rouzbahani OM, Radkhah N, Faghfouri E, Kord-Vankaneh H, et al. The effects of N-acetylcysteine on inflammatory and oxidative stress biomarkers: A systematic review and meta-analysis of controlled clinical trials. Eur J Pharmacol 2020; 884:173368.

22. Cataldi M, Sbendorio V, Leo A, Piazza O. Biofilm-dependent airway infections: a role for ambroxol? Pulm Pharmacol Ther 2014; 28(2):98–108.

23. Zhang Y, Fu Y, Yu J, Ai Q, Li J, Peng N, et al. Synergy of ambroxol with vancomycin in elimination of catheter-related Staphylococcus epidermidis biofilm in vitro and in vivo. J Infect Chemother 2015; 21(11):808–15.

24. International Organization for Standardization. ISO 10993-5:2009 – Biological evaluation of medical devices – part 5: Tests for in vitro cytotoxicity. Available at: https://www.iso.org/standard/36406.html. Accessed Aug 6, 2022.

25. Thonemann B, Schmalz G, Hiller KA, Schweikl H. Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. Dent Mater 2002; 18(4):318–23.

26. de Menezes JV, Takamori ER, Bijella MF, Granjeiro JM. In vitro toxicity of MTA compared with other primary teeth pulpotomy agents. J Clin Pediatr Dent 2009; 33(3):217–21.

27. Parasassi T, Brunelli R, Bracci-Laudiero L, Greco G, Gustafsson AC, Krasnowska EK, et al. Differentiation of normal and cancer cells induced by sulfhydryl reduction: biochemical and molecular mechanisms. Cell Death Differ 2005; 12(10):1285–96.

28. Sunkari S, Thatikonda S, Pooladanda V, Challa VS, Godugu C. Protective effects of ambroxol in psoriasis like skin inflammation: Exploration of possible mechanisms. Int Immunopharmacol 2019; 71:301–12.

29. Yamaya M, Nishimura H, Nadine LK, Ota C, Kubo H, Nagatomi R. Ambroxol inhibits rhinovirus infection in primary cultures of human tracheal epithelial cells. Arch Pharm Res 2014; 37(4):520–9.