Ambroxol's potential as an anti-biofilm against biofilm-forming microorganisms: in vitro and in vivo studies

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Antimicrobial resistance is a growing concern in modern medicine, necessitating innovative approaches to combat biofilm-related infections. This systematic review explores the potential of ambroxol, a mucolytic agent, as an anti-biofilm agent both in vitro and in vivo. Ambroxol's diverse applications, including inhibiting biofilm formation, disrupting quorum sensing, and enhancing antibiotic efficacy, are investigated across various microbial species. This research used the Preferred Reporting Items for Systematic Reviews and Meta-analyses method to process the articles obtained. Articles were collected from 2012 to 2022 through various searches such as Scopus, ScienceDirect, and PubMed. Nine articles that met the inclusion and exclusion criteria were obtained. Results indicate that ambroxol's versatility inhibits biofilm formation, improves antibiotic effectiveness, and disrupts established biofilms. These findings suggest that ambroxol holds promise as a valuable tool in the ongoing battle against biofilm-associated infections, offering new treatment and management strategies.

Keywords:
ambroxol, anti-biofilm activity, antimicrobial biofilm forming, CLSM, ELISA Reader, MTT

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Summary of work done by the contributors.

Contributor 1 is tasked with conducting searches and collecting articles according to the theme to be reviewed.

Contributors 1–4 jointly reviewed several articles that had been selected based on inclusion and exclusion criteria.

Introduction

Despite persistent efforts of medicine and the pharmaceutical industry, the escalating issue of antimicrobial resistance among microorganisms toward commonly used antibiotics has emerged as a critical problem in contemporary medicine [1]. Antibiotic resistance, which is prevalent due to the excessive use of antibiotics, has developed in odontology. Breakpoint concentration analysis has shown a higher prevalence of resistant strains in Spain, including F. nucleatum exhibiting resistance to penicillin, amoxicillin, and metronidazole, Prevotella intermedia showing resistance to tetracycline and amoxicillin, and A. actinomycetemcomitans displaying resistance to amoxicillin and azithromycin [2]. The lack of novel alternatives to effectively treat multidrug-resistant pathogenic bacteria represents a pressing issue, underscoring the urgent need to develop new broad-spectrum drugs to combat antimicrobial resistance.

Ambroxol, chemically known as 2-amino-3, 5-dibromo-N-(trans hydroxycyclohexyl) benzylamine, is a mucolytic agent primarily used in the treatment of chronic bronchitis [3]. Its pharmacological effects are characterized by its ability to regulate mucus production in gland cells [4]. In addition, ambroxol (AMB) possesses antioxidative properties [5] and anti-inflammatory attributes, leading to a reduction in the release of inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-2, IL-1, IL-4, IL-13, and interferon-γ. These effects have been observed in bronchoalveolar macrophages, monocytes, and granulocytes [6]

This systematic review aims to assess the potential of ambroxol as an antimucolytic agent based on its documented effectiveness in various in vitro and in vivo studies published in selected journals.

Data process

Conducting a systematic review entails a methodical and structured procedure. The first, determine the inclusion and exclusion criteria for articles to be reviewed. Subsequently, an exhaustive and systematic

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search is executed across multiple databases to identify all potentially relevant articles. A rigorous screening procedure is implemented following the selection of studies based on predetermined criteria, and data extraction is subsequently performed from the selected studies. Subsequent stages involve a rigorous evaluation of study quality and assessment of risk of bias using appropriate assessment tools. Ultimately, the synthesis of findings is conducted, either narratively or through statistical analysis, culminating in the formulation of substantiated conclusions and a discussion of implications for practice and future research. This systematic methodology ensures a transparent, evidence-based aggregation of extant research on a specific subject matter. This systematic review's three primary phases were executed to accomplish the intended goals. First, identifying and selecting relevant studies on the topic. Second, selecting studies based on predefined inclusion criteria. Lastly, a review and data extraction for each study was conducted.

Identification study

The literature search was conducted in March 2022 using several databases and search engines, including Science Direct and PubMed, for English language articles published within the last 10 years from 2012 to 2022. The keywords used for the article search included 'ambroxol as anti-biofilm,' 'effect of ambroxol as anti-biofilm on bacteria and fungi,' and 'combination of ambroxol and antibiotics as anti-biofilm.' Articles were selected based on the predefined inclusion and exclusion criteria outlined in Table 1.

Data processing

Fifty-two articles about ambroxol were extracted from the ScienceDirect and PubMed databases through the data search. Eight articles met the inclusion and exclusion criteria. A total of 43 articles were excluded because they were in the form of review articles, case reports, and books that did not specifically address ambroxol as an anti-biofilm agent or contained duplicate articles. The Preferred Reporting Items for Systematic Reviews and Meta-analyses are complete and detailed stages for conducting a literature review. Five stages are used to conduct a literature review: defining eligibility criteria, defining information sources, selecting literature, collecting data, and selecting data items. A diagram of the selected studies is shown in Fig. 1.

Data extraction

The authors extracted the data obtained. The data extraction form included the methodology and research results of the articles. The authors analyzed articles on the effects of ambroxol as an anti-biofilm agent on various microbes and tissues in animal models, both as a single agent and in combination with antibiotics.

Data quality evaluation

The author assesses the quality of the data from the research based on the suitability of the methods used and the completeness of the data presented.

Searching of literature

The systematic review was conducted to identify all relevant studies. Fifty-two articles were obtained from the combined search on PubMed and ScienceDirect. After further screening, nine articles met the inclusion criteria.

In this study, nine articles meeting the inclusion criteria were systematically reviewed. These articles explore the potential of ambroxol as an anti-biofilm agent based on its activity under various in vitro and in vivo conditions against various types of microorganisms, including both bacteria and pathogenic microscopic fungi. The explanation of ambroxol as an anti-biofilm is explained in detail below

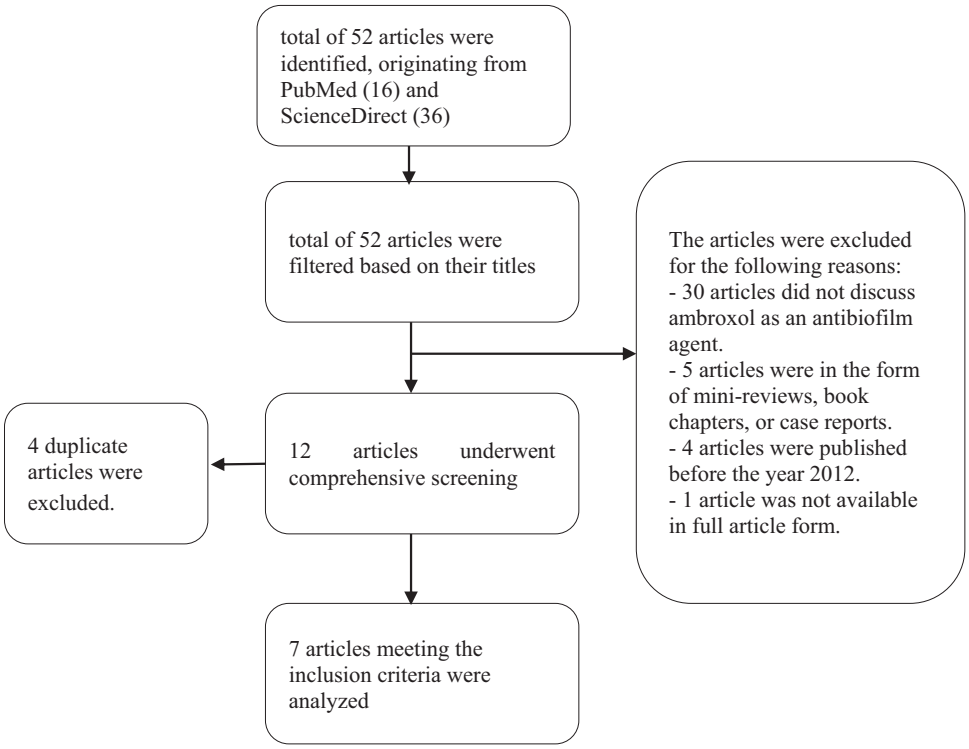
Ambroxol inhibits biofilm-forming bacteria by various mechanisms and increases the effect of antibiotics

For this theme, six articles were obtained for ambroxol-inhibiting biofilm bacteria, which will be discussed. The first study, an article titled 'Ambroxol inhibits the mucoid conversion of *Pseudomonas aeruginosa* and contributes to the bactericidal activity of ciprofloxacin against mucoid *Pseudomonas aeruginosa*

Table 1 Inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
Articles in the English language.9	Review articles, case reports, and books.
Published between 2012 and 2022.	Theme related to diabetic foot ulcers with complications.
Original research articles.	
Theme related to ambroxol as an antibiofilm agent, the effects of ambroxol as an antibiofilm agent on bacteria and fungi, and the combination of ambroxol and antibiotics as antibiofilm agents.	Articles that are not available in full text.

Figure 1



Preferred Reporting Items for Systematic Reviews and Meta-analyses diagram of selected studies.

biofilms' conducted by Wang *et al.* in 2016 [7] focuses on the challenge posed by mucoid *Pseudomonas aeruginosa* infections, especially in cystic fibrosis patients. Mucoid *Pseudomonas aeruginosa* is highly resistant to antibiotics and forms biofilms that protect against the host immune system. The study investigates ambroxol, a mucolytic agent with antioxidant properties, to inhibit mucoid conversion in *Pseudomonas aeruginosa*. The results indicate that ambroxol inhibits mucoid conversion by reducing oxidative stress and enhances the bactericidal activity of ciprofloxacin against mucoid *Pseudomonas aeruginosa* biofilms. This research suggests that ambroxol may be a potential treatment option for combating mucoid *Pseudomonas aeruginosa* infections, particularly in cystic fibrosis patients [8].

The significance data is in the table 'Fluorescence intensity of live bacteria in mucoid *Pseudomonas aeruginosa* biofilms in the presence of various concentrations' (you can read this article in full at DOI: https://doi.org/10.1111/apm.12542). This study indicates a synergistic antimicrobial efficacy between ambroxol and ciprofloxacin. Furthermore, although bacteria cannot be completely eliminated, ambroxol seems to reduce the severity of lung infections caused by mucoid *Pseudomonas aeruginosa* biofilms. However, it should be noted that the biofilm-destruction effect of ambroxol results in the release of bacterial cells, which is likely to increase the bacterial burden in the lungs during the treatment period. Therefore, the combination of anti-biofilm agents, such as ambroxol, with antibiotics seems to be an effective strategy for treating biofilm-associated infections [9].

The second study of research in an article titled 'Ambroxol interferes with *Pseudomonas aeruginosa* quorum sensing' by Q. Lu *et al.* in 2010 [10]. In this study, Ambroxol, as a mucolytic agent, has been found to possess the ability to disrupt the formation of biofilms derived from *Pseudomonas aeruginosa* in addition to reducing alginate production by undefined mechanisms. Since quorum sensing (QS) is a key regulator of virulence and biofilm formation, this research examined the effects of ambroxol on the bacterial clearance rates of wild-type P. aeruginosa PAO1, adhesion profiles, and biofilm formation in comparison to the quorum sensing-deficient, double-mutant strains lasR rhlR and lasI rhlI. The data presented in this study demonstrated that ambroxol treatment dose dependently reduced the survival rates of the double-mutant strains compared with the wild-type strain, even though the double mutants exhibited increased adhesion in the presence of ambroxol compared with the wild-type strain. The PAO1

wild-type strain produced a significantly thicker biofilm compared with the biofilms produced by the lasR rhlR and lasI rhlI isolates. Ambroxol treatment reduced biofilm thickness, increased areal porosity, and decreased the average diffusion distance and textual entropy of both wild-type and double-mutant strains. However, changes observed in the wild-type strain were more pronounced compared with double-mutant strains. Finally, ambroxol exhibited significant antagonistic quorum-sensing properties, suggesting its potential clinical use in the treatment of cystic fibrosis and in reducing biofilm formation and colonization of indwelling devices [11]

To further investigate the effects of ambroxol on biofilm structure, the images obtained using CLSM in this study were analyzed using an image structure analysis program (ISA), you can see the details in the DOI https://doi.org/10.1016/j.ijantimicag.2010.05.007. This analysis revealed that, for all tested strains of Pseudomonas aeruginosa, ambroxol significantly reduced biofilm thickness, average diffusion distance, and textual entropy (TE) in a dose-dependent manner, while increasing area proportion (AP. The changes in PAO1 (wild-type) levels were significantly greater compared with the lasR rhlR and lasI rhlI double mutants ($P<0.05$). Although ambroxol had similar effects on the mutant strains, variations in biofilm thickness, average diffusion distance, area proportion, and textual entropy were not significant [11–14].

Cataldi *et al.* (2014)) [15] explain that ambroxol (ABX) is extensively used for its potential in addressing respiratory tract infections associated with biofilm formation. The studies have demonstrated that Ambroxol possesses the capability to disrupt the structural integrity of bacterial biofilms, as observed in *in-vitro*-produced *Pseudomonas aeruginosa* biofilms. Ambroxol also influences several stages of biofilm development, including reversible and irreversible attachment, maturation, and bacterial detachment from the biofilm [16]. In this article, you can show the DOI: https://doi.org/10.1016/j.pupt.2013.11.002 the effect of ABX on biofilms formed by mucoid Pseudomonas aeruginosa *in vitro*. The panel on the left shows the biofilm formed by control Pseudomonas aeruginosa after 7 days *in vitro*, whereas on the right a micrograph of a culture treated with ABX (3.75 mg/ml for 8 h) is reported. In addition, ambroxol has been shown to interfere with fungal biofilm formation. These collective effects make ambroxol an intriguing candidate in the effort to prevent and treat biofilm-associated respiratory tract infections [17].

The fourth study in this research is an article titled 'Synergy of ambroxol with vancomycinin elimination of catheter-related *Staphylococcus epidermidis* biofilm in vitro and in vivo' by Y. Zhang *et al.* in 2015 [18]. In this study, the researchers investigated the antibiofilm properties of ambroxol in combination with vancomycin for the treatment of catheter-related *Staphylococcus epidermidis* biofilm infections. The researchers used a strain of *Staphylococcus epidermidis* and New Zealand white rabbits for their experiments. In vitro studies involve biofilm formation and treatment with different conditions, including nontreatment, ambroxol, vancomycin, and vancomycin plus ambroxol. The researchers assessed biofilm viability using an XTT reduction assay and visualized biofilms using confocal laser scanning microscopy, you can see this article with DOI https://doi.org/10.1016/j.jiac.2015.08.017.

Structural parameters of biofilms were analyzed using specialized software. In vivo studies included the development of biofilms on catheters and antibiotic lock therapy using heparin, ambroxol, vancomycin, or vancomycin plus ambroxol. Bacterial counts were measured on the catheter and in various tissues, and histopathological analysis was conducted. The results demonstrated that ambroxol enhanced the bactericidal effect of vancomycin on *Staphylococcus epidermidis* biofilms, both in vitro and in vivo, and showed potential for the treatment of catheter-related infections [19–22].

The 50 study in this research is an article titled 'Ambroxol blocks swarming and swimming motilities and inhibits biofilm formation by *Proteus mirabilis* isolated from diabetic foot infection' by Abbas in 2013 [23]. In this study, the authors investigated the potential of ambroxol as an antibiofilm agent against Proteus mirabilis isolated from diabetic foot infections. They found that ambroxol, at subinhibitory concentrations, effectively blocked the swarming and swimming motilities of the bacteria in a dose-dependent manner, with complete inhibition observed at 0.9 mg/ml. In addition, ambroxol significantly inhibited biofilm formation by these bacteria, reducing it by 90.25% to 100% at 0.9 mg/ml. Furthermore, ambroxol demonstrated the ability to eradicate preformed biofilms, with removal rates ranging from 78.38% to 83.77% at the same concentration. These findings suggest that ambroxol could be a promising treatment option for diabetic foot infections caused by Proteus mirabilis, as it hinders tissue invasion and effectively disrupts biofilm formation and preexisting biofilms.

Based on the criteria set by Stepanovic *et al.* [24], all five isolates of *Proteus mirabilis* in this study exhibited strong biofilm-forming abilities. There is an inhibitory effect observed with subinhibitory concentrations of ambroxol on both biofilm formation and the eradication of preformed biofilms, indicating that ambroxol can inhibit biofilm formation and effectively remove previously established biofilms, with a significant dose-dependent increase. You can find this full article with DOI: https://doi.org/10.1016/S0167-7012(00)00122-6 [25].

Ambroxol inhibits biofilm formation of *yeast*

For this theme, three articles were obtained for ambroxol-inhibiting biofilm yeast, which will be discussed. The article titled 'Effects of ambroxol on Candida albicans growth and biofilm formation' by Hernandez-Delgadillo *et al.* in 2013 [26] aimed to investigate the effects of ambroxol (AMB) on Candida albicans growth and biofilm formation. Candida albicans is associated with various diseases, including oral candidiasis. Research was conducted in vitro to assess AMB's fungicidal and antibiofilm activity. They found that AMB exhibited a higher fungicidal activity than the commonly used antifungal terbinafine, with a minimal inhibitory concentration of 1 mg/ml for AMB.

AMB effectively inhibited the formation of *Candida albicans* biofilms and could eliminate fungal cells within established biofilms, you can see the full article with DOI: https://doi.org/10.1111/myc.12147. This study suggests that AMB can be used as a therapeutic alternative in treating oral candidiasis and other fungal infections to reduce antimicrobial resistance. This research sheds light on the multifaceted properties of AMB, highlighting its promise as a mucolytic agent and an antifungal treatment for biofilm-related fungal infections, such as oral candidiasis [27]

The article titled "*In vitro* effects of ambroxol on *Cryptococcus* adherence, planktonic cells, and biofilms" by Kong *et al.* In 2017 [28]. In this study, the authors conducted a series of experiments to assess the impact of ambroxol (Amb) on *Cryptococcus.* The authors determined the minimum inhibitory concentration of Amb against *Cryptococcus.* planktonic cells and evaluated its synergistic effect in combination with fluconazole. Time-killing tests were performed to monitor the growth of *Cryptococcus.* strains treated with Amb and FLU. An agar disk diffusion test visualized the antifungal effects of Amb, while adhesion assays examined its influence on cell adherence. Finally, Amb's anti-biofilm effects were assessed using an XTT reduction assay. Overall, the study's methods aimed to comprehensively investigate Amb's antifungal properties against *Cryptococcus.* in various contexts.

In this passage, the authors investigated the effects of fluconazole (FLU) on the metabolic activity of mature biofilms formed by Cryptococcus neoformans, you can find the full article with DOI: https://doi.org/10.1111/apm.12698. They used an XTT reduction assay to assess the biofilm's response to FLU. The results showed that FLU at a concentration of 64 μg/ml was insufficient to kill the biofilm and exhibited an activity similar to the control. Consequently, the study suggests that FLU is not recommended for the treatment of biofilm-related infections. In addition, the study observed that Cryptococcus gattii strain ZY. C03 with a mucoid phenotype formed weaker biofilms compared with smooth phenotype counterparts. The findings indicated that fluconazole may not effectively target mature biofilms, and the study explored the potential use of ambroxol as an alternative treatment option [29,30].

The review results regarding ambroxol's potential as an antibiofilm made it the first step for researchers to conduct clinical research, where the ambroxol solution was applied directly to the patient's diabetic ulcers. Researchers hope that the ambroxol solution applied to patients can also act as an antibiofilm in diabetic ulcers and as cotherapy with antibiotics to inhibit and eradicate bacteria-forming biofilms. Of these nine articles, all discuss the ability of ambroxol to inhibit and eradicate microbes, namely bacteria, plants, and fungi, with a combination of various antibiotics and antifungals. Several reagents detect biofilm-forming microbes, namely crystal violet, MTT, and XTT. Several methods were used: Elisa Reader, SEM, and CLSM.

Conclusion

Ambroxol has excellent potential as an anti-biofilm on microbes. Apart from that, ambroxol can help antibiotics inhibit and eradicate biofilm-forming bacteria. Further research can be carried out in vivo and applied to patients so that ambroxol can be formulated for topical use in patients with infections whose bacteria form biofilms

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Conflicts of interest
The authors declare that they have no conflict of interest.

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