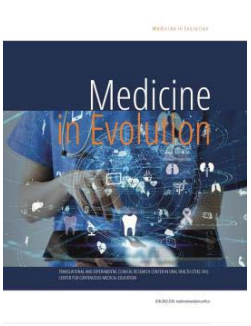
Bacteria, biofilm and cholesteatoma - perspectives of innovative therapeutic approaches



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Abstract

Nowadays, bacterial infections still represent a problem of global interest due to the emergence of antibiotic resistance. Also, microbial infection are the basis of cholesteatoma being known for the production of chronic inflammation, the collection of cellular debris and the increase in the viscosity of the secretions, the dysfunction of the Eustachian tube, the invasion of the cells of the immune system and epithelial hyperplasia Aim and objectives: To evaluate the antibacterial effect of epigallocatechin gallate (ECGC), in association with an antibiotic (ciprofloxacin) related to the possible synergistic effect. Material and methods: The disk diffusion method was employed to test the sensitivity of *S. aureus* and *P. aeruginosa* in the presence of ECGC and ciprofloxacin, at different concentrations (25–100 µg/mL) as such or in combination. Results: The results demonstrated that ECGC has an antibacterial effect on the selected strains, but the strongest activity was observed following the association of the natural compound with the antibiotic, with a better effect on the *S. aureus* strain. Conclusions: The association of natural compounds with antibiotics can represent an alternative to antibiotic resistance, being a possible effective option in combating infections and even pathologies of the middle ear, such as cholesteatoma.

Keywords: ECGC, antibacterial effect, biofilm, cholesteatoma, synergism

INTRODUCTION

Cholesteatoma is a non-cancerous condition characterized by the abnormal growth of squamous epithelial cells in the middle ear. Two types of cholesteatoma are known, the congenital one, which rarely develops, and the acquired cholesteatoma [1].

The causes of cholesteatoma development are not fully known, but a number of factors are responsible for the formation of this medical condition. Among these factors we find the microbial infection that leads to chronic inflammation, the collection of cellular debris and the increase in the viscosity of the secretions, the dysfunction of the Eustachian tube, the invasion of the cells of the immune system and epithelial hyperplasia [2,3]. This pathology is generally manifested by pain and the presence of a smelly liquid at the level of the infected ear and up to the loss of hearing [4]. From a histological point of view, this non-cancerous lesion contains keratin remnants covered with keratinized squamous epithelium [5].

Cholesteatoma has a high tendency to erode the ossicles and the temporal bone that supports the neural structures, which can lead to complications such as vertigo, paralysis of the facial nerve and hearing loss [6]. Also, an amplified inflammatory response determines the development of cholesteatoma and bone erosion, as well as the formation of biofilm is associated with the production of cholesteatomas [7]. The importance of biofilms in otolaryngological diseases is more and more known. In recent years, this subject has been intensely debated, involving *in vitro* studies, the vast majority of which are focused on the complications involving medical implants [8]. The formation of biofilms leads to impaired clearance and chronic middle ear infection that triggers the inflammatory process. Thus, inflammatory mediators such as IL-1, TNF-alpha and PAF induce hyperproliferation of keratinocytes and epithelial cells, increased secretion of mucin and bone resorption by stimulating osteoclasts and collagenases [2].

A large number of Gram-negative and Gram-positive bacterial agents as well as fungal agents were isolated from cholesteatoma tissues [1]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common bacteria observed in cholesteatomatous otitis media [9]. It is considered that the formation of the biofilm keeps the pathogens in the middle ear, thus maintaining the inflammation and developing the cholesteatoma formation. In general, the treatment is based on surgical intervention, possibly including the removal of the inflamed bones; in combination with the administration of antimicrobial medication. However, the partial removal of pathogens or biofilms formed can precipitate the reappearance of the condition [1,6,10].

The ability of pathogens to form biofilms facilitates their survival in unfavourable conditions, allowing them to proliferate and colonize host tissues as well as inert surfaces such as implants, producing negative reactions on human health and resistance to antimicrobial drugs [11]. More controversial is the situation in which biofilms are polymicrobial. A common example of co-infection is that between *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which can aggravate the disease and hinder the choice of antibiotic therapy, the person's recovery being slower [11,12].

Biofilm formation can be considered one of the main causes by which bacteria develop resistance to several drugs. The unreasonable use of antibiotics has led to the development of multi-resistant microorganisms. Natural products derived from plants but also microorganisms and marine species represent an invaluable source of anti-biofilm agents. The compounds isolated from the plants as well as the extracts proved to have important antimicrobial and anti-biofilm effects. The anti-biofilm properties of natural products refer to inhibiting the formation of the polymer matrix, reducing the production of virulence factors,

and suppressing cell adhesion, thus blocking the communication between bacterial cells and the development of biofilm [13].

Biofilm inhibitors as well as quorum sensing (QS) formation inhibitors are being studied as alternatives to current antibiotics due to their small possibility of developing resistance, focusing on products of vegetable origin that have a complex composition [14].

Green tea, an extract from the leaves of *Camellia sinensis* L., is known for its beneficial effects on health due to the wide range of phytochemicals in the composition. It contains numerous constituents, including catechins, caffeine, amino acids, chlorophyll, volatile compounds, minerals. About 30% of its total composition is represented by polyphenols, especially catechins, of which about 65% are represented by epigallocatechin-3-gallate (EGCG) [15,16].

Green tea polyphenols have demonstrated the ability to inhibit acyl homoserine lactone-mediated QS and to inhibit biofilm formation in *Pseudomonas aeruginosa* [15,17].

The inhibitory effect of EGCG against biofilm formation and cholesteatoma development is shown in Figure 1.

Several studies have been carried out to evaluate whether green tea and its main phytocompound, epigallocatechin-3-gallate, have antimicrobial properties. The data obtained showed that both the aqueous and alcoholic extracts and ECGC are effective against *S. aureus* and *P. aeruginosa*. The results were presented using the minimum inhibitory concentration (MIC) and the size of the inhibition zones (IZ). A summary of the studies is shown in Table 1.

Due to the massive acceleration of the development of bacterial resistance at the global level and the lack of new antimicrobial substances, new strategies to eradicate infectious diseases are needed. An alternative would be to combine antibiotics with each other or antibiotics with natural compounds to extract potential synergistic effects, knowing that plants are known for their antimicrobial effects [18].

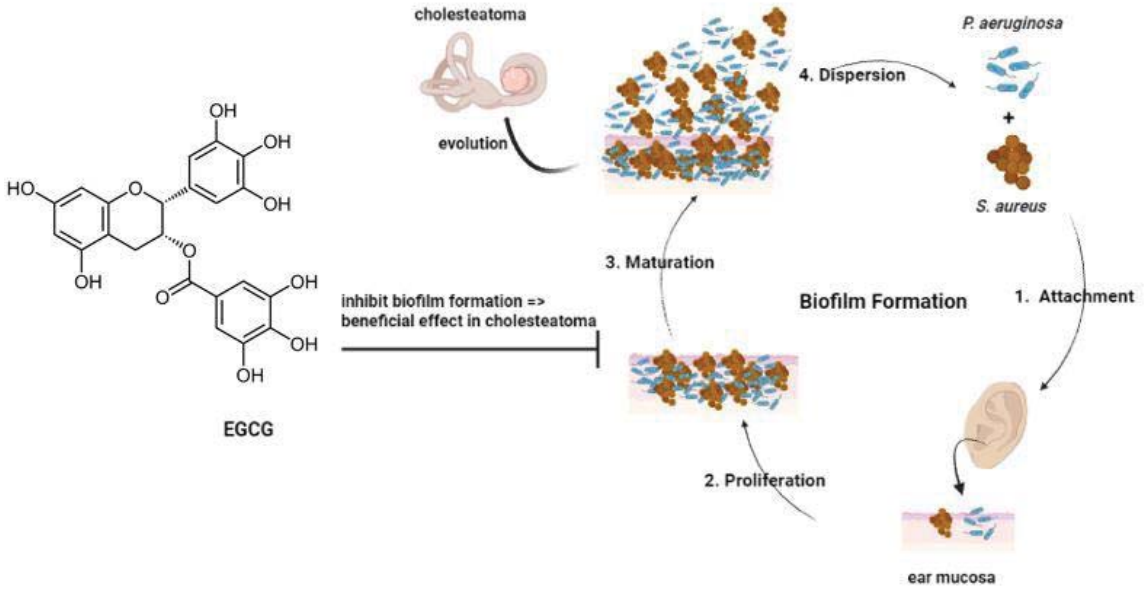

Figure 1. The inhibitory effect of epigallocatechin-3-gallate (EGCG) against biofilm formation and cholesteatoma development

Table 1. Antimicrobial activity of green tea and epigallocatechin-3-gallate (ECGC)

Source -Active compound	Bacterial strain	Zone of inhibition (IZ)	Minimum inhibitory concentration (MIC)	References
Green tea extract (water extract)	***S. aureus***	18.970±0.287 mm	400 µg/mL	[19]
	MRSA	19.130±0.250 mm	400 µg/mL	
	MRSA		0.78 mg/mL	[20]
	S. aureus		0.28 mg/mL	[21]
	MRSA		50–180 µg/mL	[22]
Green tea extract	***S. aureus*** **MDR-*S. aureus***		125 µg/mL	[23]
Green tea extract (alcohol extract)	***S. aureus***		20 µg/mL	[24]
Green tea extract (methanol extract)	***S. aureus***		0.8 mg/mL	[25]
Green tea extract (ethanol extract)	***S. aureus***	12 mm		[26]
EGCG	***S. aureus***		100 mg/mL	[27]
EGCG	**MDR-*S. aureus***		625 µg/mL	[23]
Green tea extract (water extract)	***P. aeruginosa***	17.550±0.393 mm	800 µg/mL	[19]
	MDR-*P. aeruginosa*	17.670±0.398 mm	800 µg/mL	
Green tea extract (ethanol extract)	***P. aeruginosa***	10 mm		[26]
EGCG	***P. aeruginosa***		500 mg/mL	[27]
EGCG	***P. aeruginosa***		200 µg/mL	[28]

Aim and objectives

The objective of this study was to evaluate the antibacterial effect of epigallocatechin-3-gallate (ECGC) on the bacterial strains *S. aureus* and *P. aeruginosa* compared to ciprofloxacin and then to analyze the effect of the association between the natural compound and the antibiotic to assess the synergistic activity obtained; synergism that is necessary to fight infections, even those from the middle ear, and cholesteatoma, a pathology of interest to our research group.

MATERIAL AND METHODS

Epigallocatechin-3-gallate (E4143) and ciprofloxacin (17850) were purchased from Sigma Aldrich (Germany). The antibacterial effect of EGCG, Cip and their association was evaluated against *Staphylococcus aureus* (ATCC 25923™) and *Pseudomonas aeruginosa* (ATCC 27853™), strains acquired from ATCC (American Type Culture Collection, Microbiologics, France). The Disk diffusion method for susceptibility testing, in accordance with the SRAST

(Standard Rules for Antimicrobial Susceptibility Testing) using impregnated disks was employed. The experimental protocol was conducted as presented in literature [23,27,28]. Ciprofloxacin and EGCG solutions were obtained in a wide range of 25–100 µg/mL, samples that were used as such or in combination against *P. aeruginosa* and *S. aureus*. The plates were incubated in standard conditions (at 37°C) and evaluated after 24 h. Data are presented as inhibition zone expressed in mm. All the tests were realized in triplicate.

RESULTS

Regarding the antibacterial activity, in the figures 2 and 3 are exposed the data obtained after the evaluation of ciprofloxacin, epigallocatechin-3-gallate and their association on two bacterial strains, namely *S. aureus* and *P. aeruginosa*. The results showed that Cip, used at low concentrations (25-75 µg/mL) exerted a slight antimicrobial effect compared with the highest concentration (100 µg/mL) tested on *S. aureus* while EGCG at the same concentration (100 µg/mL) presented a weaker effect. EGCG_Cip association showed a higher antimicrobial activity on *S. aureus* bacterial strain compared to the individually tested substances (figure 2).

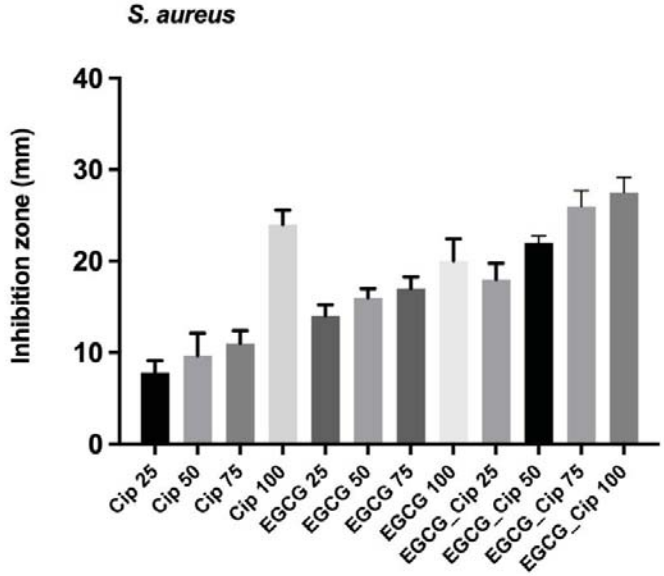

Figure 2. The effect of ciprofloxacin (Cip), epigallocatechin-3-gallate (EGCG) and their association at different concentrations (25, 50, 75, and 100 µg/mL) on *S. aureus* bacterial strain

Regarding the activity on *P. aeruginosa* bacterial strain, EGCG tested at the highest concentration showed a stronger effect compared to ciprofloxacin tested at the highest concentration (figure 3). The association between EGCG and antibiotic leads to a better effect but not as pronounced as in the case of *S. aureus*.

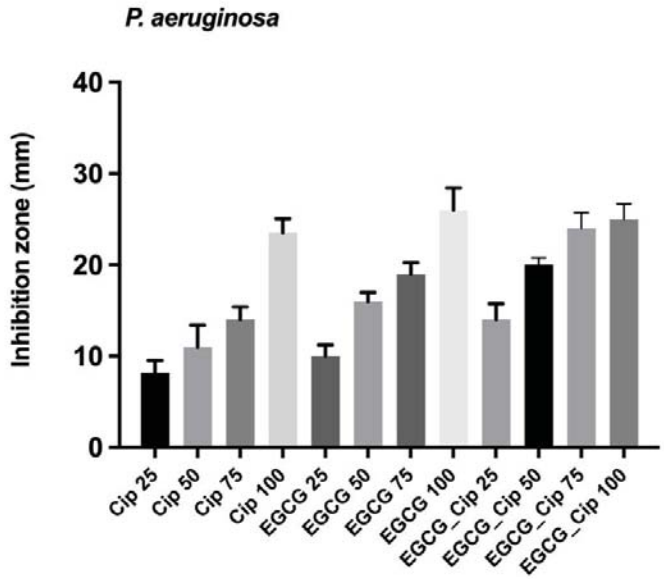

Figure 3. The effect of ciprofloxacin (Cip), epigallocatechin-3-gallate (EGCG) and their association at different concentrations (25, 50, 75, and 100 µg/mL) on *P. aeruginosa* bacterial strain

DISCUSSIONS

The pathogenesis of acquired cholesteatoma has become an intensively studied subject. A first step was the study carried out by Chole and Faddis in which the presence of biofilms was reported in 16 of the 24 clinical cases of cholesteatoma (66%) [29]. Another group led by Lampikoski identified biofilm formation in 3 out of 5 cases of cholesteatoma (60%) [30]. Also, Kaya et al. obtained results like those existing in the specialized literature, more precisely in 8 out of 13 cases the formation of biofilm was observed at the level of the middle ear mucosa (61.5%) [8]. Considering the data presented, it was found that bacteria can infect the keratin matrix and form biofilms that further lead to chronic infections. After an *in vitro* study, it was concluded that biofilm formation was responsible for the containment of more than 50% of the pathogens isolated from cholesteatoma tissues at the level of the ossicles [31].

The formation of biofilms has been shown to represent a main role in the evolution of cholesteatoma. Normally, the middle ear cavity is colonized by bacterial flora, this aspect can make it difficult to differentiate between harmless and pathogenic microbial agents. Therefore, the inflammatory process generated by the wide variety of pathogenic agents has a greater relevance in the production of infection, in the development of cholesteatoma, than the responsible bacterial species itself [1].

The creation of biofilms led to the emergence of resistance to antibiotics and in general to antimicrobial medication, putting great obstacles in the effective treatment of infections among the population. Thus, in recent years, the intensive study of natural products has been started in terms of their efficiency as antimicrobial and antibiofilm agents; there are promising data in this regard.

Most products of natural origin work by inhibiting bacterial growth or by reducing their pathogenicity, acting on specific genes that manage the decisive factors of virulence. In addition, besides these mechanisms, certain compounds including EGCG have been investigated for their action as QS inhibitors [32,33]. The study conducted by Yin et al. pointed out that the polyphenols extracted from the leaves of *Camellia sinensis* L. inhibit the QS system of *Chromobacterium violaceum* not through the production or degradation of acyl homoserine lactones and most likely interfere with acyl homoserine lactone receptors. In

addition, polyphenols extracted from green tea can inhibit the production of elastase, total protease, biofilm formation and motility of *Pseudomonas aeruginosa*, but without slowing its growth. The tea extract showed that it may be able to significantly decrease the biofilm formation of *Pseudomonas aeruginosa* at concentrations between 49 µg/mL-159 µg/mL through QS modulation [15]. The main polyphenolic compound in tea, EGCG at the concentration of 40 µg/mL, demonstrated that it can inhibit biofilm formation by 30% and can decrease the swarming ability of *Burkholderia cepacia* [34].

According to Yang's results, it was shown that epigallocatechin-3-gallate has a high binding affinity to the enoyl-acyl carrier protein reductase of *Pseudomonas aeruginosa*, being an effective quorum-quenching candidate [35].

Further studies on the isolation of bioactive compounds, as well as their activities, are necessary to know the complexity of the beneficial effects of green tea extract.

Monotherapy is often ineffective, the combination of several antibacterial agents is optimal for eradicating infections, such as those with *P. aeruginosa* and *S. aureus*. The main advantages of the combined therapy are the inhibition of the emergence of bacterial resistance and the broadening of the spectrum of antibacterial action compared to the use of monotherapy [36].

Our study highlighted the antibacterial effect of epigallocatechin-3-gallate at concentrations between 25-100 µg/mL compared to a fluoroquinolone, ciprofloxacin, against the bacterial strains *S. aureus* and *P. aeruginosa*. The association of ECGC with ciprofloxacin led to a stronger effect, especially against *S. aureus*, which pointed out the synergistic effect of the two molecules. In the specialized literature, the antibacterial effect of ECGC in association with other antibiotics was also studied, and the synergy was also observed. The study by Shanmugapriya et al. showed that an antibiotic from the class of cephalosporins, such as cefepime, associated with epicatechin 3-gallate of natural origin, induced a synergistic eradication effect against the resistant isolate of *P. aeruginosa*. Thus, combining the antibiotic with ECGC allowed the use of lower concentrations of ECGC and cefepime than when each substance would be administered alone. The minimum inhibitory concentration for the natural compound was reduced to 4, 2, 1 and 0.5 µg/mL in the presence of the antibiotic at a concentration of 0.5, 1, 2 and 4 µg/mL [37].

This synergistic activity between antibiotics and ECGC was also observed in the study conducted by Zhao. Epicatechin 3-gallate in low concentrations demonstrated a synergistic effect with β-lactam antibiotics such as penicillin and oxacillin against methicillin-resistant *S. aureus* (MRSA). ECGC dose-dependently inhibited the growth of both methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA) and reduced tolerance of bacteria to high ionic strength and low osmotic pressure in their external atmosphere [38]. Hu's research also reported the superior effect of EGCG against MRSA when combined with ampicillin [39].

All these results confirm the synergism between antibiotics and phytocompounds, more precisely the effect of their association is greater than the effect of the compounds administered individually or the sum of the effects of the individual compounds; synergism that produced an impressive antibacterial effect.

CONCLUSIONS

Following this study and the review of specialized literature, we concluded that plants and natural compounds extracted from plants have a significant antibacterial role and represent an alternative to antibiotic resistance. In addition, it can be concluded that the association of natural compounds, in this case ECGC, with antibiotics potentiates the antibacterial effect due to the resulting synergy. Thus, this combination can be an effective

option for fighting infections and even ear infections and cholesteatoma, a pathology that can seriously affect the population.

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