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Original Article

Evaluation of the antibacterial activity of trans-anethole against *Enterococcus cloacae* and *Enterococcus faecalis* strains of food origin

Avaliação da atividade antibacteriana do trans-anetol contra cepas de *Enterobacter cloacae* e *Enterococcus faecalis* de origem alimentar

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Abstract

The present study sought to evaluate the antibacterial activity of trans-anethole against food-borne strains of *Enterobacter cloacae* and *Enterococcus faecalis*. The study was performed using Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) methods, in addition, disc diffusion technique was used to evaluate the association of trans-anethole with synthetic antimicrobials. Minimum Inhibitory Concentration for Adherence (MICA) testing was also performed. The results revealed that trans-anethole presents no antibacterial activity at any of the concentrations used against the *E. cloacae* strains tested. However, trans-anethole presented antibacterial effect against five of the six *E. faecalis* bacterial strains tested, with MIC values ranging from 500 µg/mL to 1000 µg/mL. Further, when analyzing the MBC results against *E. faecalis*, it was observed that the compound presented values ranging from 500 µg/mL to 1000 µg/mL. As for the associations, it was observed that trans-anethole when combined with the antimicrobials ampicillin, gentamicin, ciprofloxacin, and ceftriaxone presented synergistic effect against most strains of *E. faecalis*. However, both trans-anethole and the control chlorhexidine (0.12%) presented no antibiofilm effects against strains of *E. faecalis*. In short, trans-anethole presented potential antibacterial against *E. faecalis* strains of food origin, and may upon further study, it may be used alone or in association with synthetic antimicrobials to combat infections caused by this bacterium.

Keywords: antimicrobials, bacteria, phenylpropanoid, phytotherapy.

Resumo

O presente estudo procurou avaliar a atividade antibacteriana do trans-anetol contra cepas de *Enterobacter cloacae* e *Enterococcus faecalis* de origem alimentar. O estudo foi realizado utilizando métodos de Concentração Inibitória Mínima (CIM), e Concentração Bactericida Mínima (CBM), além disso, foi utilizada a técnica de difusão de disco para avaliar a associação do trans-anetol com antimicrobianos. O teste de Concentração Inibitória Mínima de Aderência (CIMA) também foi realizado. Os resultados revelaram que o trans-anetol não apresentou atividade antibacteriana em nenhuma das concentrações utilizadas contra as cepas de *E. cloacae* testadas. No entanto, o trans-anetol apresentou efeito antibacteriano contra cinco das seis cepas bacterianas de *E. faecalis* testadas, com valores de CIM variando de 500 µg/mL a 1000 µg/mL. Além disso, ao analisar os resultados da CBM contra *E. faecalis*, observa-se que o composto apresentou valores variando de 500 µg/mL a 1000 µg/mL. Quanto às associações, observou-se que o trans-anetol quando combinado com os antimicrobianos ampicilina, gentamicina, ciprofloxacino, e ceftriaxona apresentou efeito sinérgico contra a maioria das cepas de *E. faecalis*. No entanto, tanto o trans-anetol quanto o controle clorexidina (0,12%) não apresentaram efeito antibiofilme contra a cepa de *E. faecalis*. Em suma, o trans-anetol apresentou potencial antibacteriano contra cepas de *E. faecalis* de origem alimentar, podendo, mediante estudos mais aprofundados, ser utilizado isoladamente ou em associação com antimicrobianos sintéticos para combater infecções causadas por esta bactéria.

Palavras-chave: antimicrobianos, bactérias, fenilpropanóide, fitoterapia.

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1. Introduction

Foodborne pathogens are a leading cause of disease, posing a threat to food safety and causing serious harm to human health (Qi et al., 2021; Tomao et al., 2020). Among these pathogens, bacteria play a considerable role, representing a growing worldwide public health concern (Fung et al., 2018).

The *Enterobacter* genus is responsible for various infections, including nosocomial and urinary tract infections, respiratory infections, osteomyelitis, endocarditis and soft tissue infection (Ramirez and Giron, 2022). *Enterobacter cloacae*, a Gram-negative bacterium belonging to the Enterobacteriaceae family, is found in terrestrial and aquatic environments (Davin-Regli and Pagès, 2015). The bacterium has also been isolated in foods, such as raw cow´s milk (Alves et al., 2015) and meat (Dubey et al., 2018).

Another food-important bacterium is *Enterococcus faecalis*, which is isolated in many foods, including retail meats (Tyson et al., 2018). *E. faecalis* is a Gram-positive bacterium distributed in soil and plants, and commensally resides in the gastrointestinal tract of humans and animals (Guzman Prieto et al., 2016). Despite its commensal nature, the bacterium has emerged as a clinically relevant pathogen, with the potential to cause many enterococcal infections in humans (García-Solache and Rice, 2019). Further, it is worth noting that this bacterium forms biofilms on many substrates (Guerreiro-Tanomaru et al., 2013), and is thus resistant to many antimicrobials (Kim et al., 2018).

The development of antimicrobial resistance is one of the greatest public health challenges faced in contemporary times, and the inappropriate or indiscriminate use of antimicrobials is one of the main factors contributing to this problem (Muñoz Madero et al., 2016). To effectively address antimicrobial resistance, the "One Health" concept was proposed by the WHO, and specifically addresses environment, human, and animal health factors (Robinson et al., 2016; Collignon and McEwen, 2019).

Thus, in view of the increases in bacteria resistance to our current antimicrobials, the need for new agents with antibacterial activity is explicit (Khadake et al., 2021), and phytotherapie emerge as a source of natural remedies that can both treat disease and promote well being (Falzon and Balabanova, 2017).

Essential oils are natural substances derived from plants which possess various biologically active components, usually with biological activities (Khorshidian et al., 2018; Stefanakis et al., 2013). Among the wide variety of components of essential oils, stands out trans-anethole, one of the main constituent of the essential oil of *Foeniculum vulgare* Mill. (fennel) (Nojadeh et al., 2020), and *Pimpinella anisum* (anise seed) (Vieira et al., 2018), has been shown to present biological activity, including antibacterial (Auezova et al., 2020), antifungal (Huang et al., 2010), neuroprotective (Ryu et al., 2014), and anti-inflammatory effect (Kim et al., 2017).

Given the need for new compounds with antibacterial activity, and the scarcity of studies on trans-anethole in the fight against bacteria that contaminate food, our study aimed to evaluate the antibacterial activity of trans-anethole against *E. cloacae* and *E. faecalis* strains of food origin.

2. Materials and Methods

2.1. Study location

The study was performed at the Microbiology Laboratory of the Federal University of Campina Grande (UFCG) at the Centre for Health and Rural Technology (CSTR).

2.2. Test substances

The phenylpropanoid trans-anethole was purchased from Sigma-Aldrich® Industry (São Paulo-SP). To perform the pharmacological assays, the compound was solubilized in dimethylsulfoxide (DMSO) and diluted in distilled water. The concentration of DMSO used was less than 0.1% v/v.

2.3. Bacterial strains

Food-borne bacterial strains of *Enterobacter cloacae* (Ecl 41, Ecl 42, Ecl 43, Ecl 44 and Ecl 45), and *Enterococcus faecalis* (Ef 46, Ef 47, Ef 48, Ef 49, Ef 50) were used. In addition to these, the standard strain *E. faecalis* (ATCC 29212) was also used.

All strains were maintained on Muller Hinton Agar (MHA) at 4°C. Inoculates were obtained from overnight cultures on MHA at 35 ± 2 °C; diluted in sterile saline to obtain final concentrations of approximately 1.5×10^8 Colony Forming Units per mL (CFU/mL), adjusted by turbidity comparing with the McFarland 0.5 tube scale (Bona et al., 2014).

2.4. Culture media

The culture media used in the assays were liquid Mueller Hinton broth and solid Mueller Hinton Agar medium. The culture media were purchased from Difco® and prepared according to the manufacturer's instructions.

2.5. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC was determined using microdilution technique in a 96-well plate with a U-shaped bottom. Initially, in each well, 100 µL of Mueller Hinton broth, doubly concentrated, and 100 µL of the studied compound (trans-anethole) were added to the plate performing a serial dilution (ratio of two), obtaining the concentrations of 1000, 500, 250, 125, 62.5, and 31.2 µg/mL. Determination of the MIC was conducted using 10 µL of the microorganism suspension in each well, being approximately 1.5×10^8 CFU/mL. In the penultimate well, the sterile control was performed containing 100 µL of Muller Hinton broth alone, and in the final well, the growth control was performed, containing only 10 µL of the microorganism suspension in 100 µL of broth. The assay was performed in duplicate. The plates were incubated at 35 ± 2 °C for 24 hours, and after this appropriate bacterial incubation time, the first reading of the results was performed. 20 µL of sodium resazurin solution (SIGMA) was then added, being previously solubilized in sterilized distilled water, at a concentration of 0.01% (w/v). Resazurin is well recognized as a colorimetric oxide-reduction indicator for bacteria. Afterwards, a new incubation at 35 ± 2 °C was performed. The reading was done visually for the absence or presence of microorganism

growth through formation of a cluster of cells (button), as well as observing changes in solution color, from blue to pink to indicate growth. The MIC was determined as the lowest concentration of the compound inhibiting visible growth of the microorganism, as verified through the change of the solution color, from blue to pink, which indicates microorganism growth (Palomino et al., 2002; Ostrosky et al., 2008; CLSI, 2012; Bona et al., 2014).

2.6. Determination of the Minimum Bactericidal Concentration (MBC)

To perform the MBC, inoculations (10 µL) of dilutions from the MIC were performed in Mueller Hinton broth medium (100 µL/cavity) in a sterile microdilution plate, and subsequently, incubation was performed at 35 ± 2 °C for 24 hours. After incubation, 20 µL of resarzurin was added, and a new incubation was performed at 35 ± 2 °C to confirm the concentration capable of total growth inhibition of the bacterial species, which would be verified by no indicator dye color change (Ncube et al., 2008; Guerra et al., 2012).

2.7. Associations of trans-anethole with synthetic antimicrobials

The trans-anethole association studies using synthetic antimicrobials was performed by disc diffusion technique on solid medium with ampicillin, gentamicin, ciprofloxacin, and, ceftriaxone filter paper discs (Bauer et al., 1966; Oliveira et al., 2006). Using a sterile swab, a volume of approximately 1mL of each bacterial suspension was seeded onto the solid surface of the Muller Hinton agar (MHA) contained in flat sterile plates. Subsequently, the paper discs (impregnated with the antimicrobials) were applied onto MHA with the bacterial suspension. Soon afterwards, a 20µL aliquot (MIC) of the tested compound was transferred to the discs containing the antimicrobials. A negative control containing only the bacterial suspension with antimicrobials discs was also performed. The plates were incubated at 35 ± 2 °C for 24-48h, followed by reading. The effect was considered synergistic if the microbial growth inhibition halo formed by the association (compound + antimicrobial) presented a diameter ≥ than 2mm, when compared to the inhibition halo formed by the action of the antimicrobial alone. When the formation of the inhibition halo resulting from the combined action (compound + antimicrobial) was smaller in diameter than that developed by the isolated action of the antimicrobial, it was considered an antagonistic effect. The effect was considered indifferent when the halo of inhibition resulting from the combined application (compound + antimicrobial) presented the same diameter as that resulting from the isolated application of the antimicrobial (Cleeland and Squires, 1991; Oliveira et al., 2006). All tests were performed in duplicate.

2.8. Determination of the Minimum Inhibitory Concentration for Adherence (MICA)

The Minimum Inhibitory Concentration for Adherence (MICA) of the compound was determined in the presence of sucrose (5%), according to Albuquerque et al. (2010) with modifications, using compound dilutions of up to 1:128. From the bacterial growth, the bacterial strain was grown at 35 ± 2 °C in Mueller Hinton broth (DIFCO, Michigan, United States). A 0.9 mL of the subculture was then distributed into test tubes and 0.1 mL of the solution corresponding to the compound dilutions was added. Incubation was performed at 35 ± 2 °C for 24 hours with the tubes tilted at 30°. The reading was performed by visual observation of bacteria adherence to the walls of the tube after shaking. The procedure was performed in duplicate. The same procedure was performed for the positive control, 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive Company, New York, USA). The MICA was considered the lowest concentration of the agent in contact with sucrose which prevented adherence to the glass tube.

3. Results

3.1. Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration results for trans-anethole against *E. cloacae* and *E. faecalis* strains are presented in Tables 1 and 2. According to the results, trans-anethole presented no antibacterial activity at any concentration used against the strains of *E. cloacae*. In relation to *E. faecalis*, trans-anethole presented antibacterial effect against five of the six strains, with MIC values ranging from 500 µg/mL to 1000 µg/mL.

Table 1. Minimum Inhibitory Concentration (MIC) in µg/mL of trans-anethole against strains of *Enterobacter cloacae*.

	Trans-anethole				
	Ecl 41	**Ecl 42**	**Ecl 43**	**Ecl 44**	**Ecl 45**
1000 µg/mL	-	-	-	-	-
500 µg/mL	-	-	-	-	-
250 µg/mL	-	-	-	-	-
125 µg/mL	-	-	-	-	-
62.5 µg/mL	-	-	-	-	-
31.25 µg/mL	-	-	-	-	-

Legend: (+) Inhibited bacterial growth (-) Did not inhibit bacterial growth.

3.2. Minimum Bactericidal Concentration (MBC)

The results for the Minimum Bactericidal Concentration of trans-anethole against *Enterococcus faecalis* are presented in Table 3. Analyzing the results, it is observed that the compound presented MBC values ranging from 500 µg/mL to 1000 µg/mL.

3.3. Associations of trans-anethole with synthetic antimicrobials

The inhibition halos (mm) resulting fromy the associations of trans-anethole with synthetic antimicrobials for *E. faecalis* strains are presented in Table 4. Analyzing the results, it can be seen that trans-anethole when

Table 2. Minimum Inhibitory Concentration (MIC) in µg/mL of trans-anethole against strains of *Enterococcus faecalis*

	Trans-anethole					
	ATCC 29212	**Ef 46**	**Ef 47**	**Ef 48**	**Ef 49**	**Ef 50**
1000 µg/mL	+	+	+	-	+	+
500 µg/mL	+	-	-	-	-	-
250 µg/mL	-	-	-	-	-	-
125 µg/mL	-	-	-	-	-	-
62.5 µg/mL	-	-	-	-	-	-
31.25 µg/mL	-	-	-	-	-	-

Legend: (+) Inhibited bacterial growth (-) Did not inhibit bacterial growth.

Table 3. Minimum Bactericidal Concentration (MBC) in µg/mL of trans-anethole against *Enterococcus faecalis.*

	Trans-anethole				
	ATCC 29212	**Ef 46**	**Ef 47**	**Ef 49**	**Ef 50**
1000 µg/mL	+	-	+	+	-
500 µg/mL	+	-	-	-	-
250 µg/mL	-	-	-	-	-
125 µg/mL	-	-	-	-	-
62.5 µg/mL	-	-	-	-	-
31.25 µg/mL	-	-	-	-	-

Legend: (+) Inhibited bacterial growth (-) Did not inhibit bacterial growth.

Table 4. Inhibition halos (mm) of trans-anethole / synthetic antimicrobial associations for *E. faecalis* strains.

		Trans-anethole			
Bacterial strains	**Association**	**AMP**	**GEN**	**CIP**	**CRO**
ATCC 29212	AIH	24 mm	14 mm	24 mm	10 mm
	AIH w/ TA	22 mm (↓)	18 mm (↑)	24 mm (*)	14 mm (↑)
Ef 46	AIH	24 mm	12 mm	26 mm	12 mm
	AIH w/ TA	30 mm (↑)	14 mm (↑)	28 mm (↑)	18 mm (↑)
Ef 47	AIH	22 mm	12 mm	22 mm	12 mm
	AIH w/ TA	32 mm (↑)	14 mm (↑)	28 mm (↑)	14 mm (↑)
Ef 48	AIH	10 mm	12 mm	24 mm	14 mm
	AIH w/ TA	18 mm (↑)	32 mm (↑)	26 mm (↑)	20 mm (↑)
Ef 49	AIH	26 mm	10 mm	20 mm	10 mm
	AIH w/ TA	38 mm (↑)	20 mm (↑)	32 mm (↑)	14 mm (↑)
Ef 50	AIH	28 mm	10 mm	26 mm	16 mm
	AIH w/ TA	34 mm (↑)	20 mm (↑)	26 mm (*)	28 mm (↑)

AIH: Antimicrobial Inhibition Halo. TA: Trans-anethole. Synergistic effect (↑); antagonistic effect (↓); indifferent effect (*); AMP: ampicillin; GEN: gentamicin; CIP: ciprofloxacin; CRO: ceftriaxone.

Table 5. Minimum Inhibitory Concentration for Adherence (MICA) in µg/mL of trans-anethole and 0.12% chlorhexidine digluconate against *Enterococcus faecalis* strain (Ef 49).

Trans-anethole								
µg/mL	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
	+	**+**	**+**	**+**	**+**	**+**	**+**	**+**
0.12% Chlorhexidine digluconate								
µg/mL	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
	+	**+**	**+**	**+**	**+**	**+**	**+**	**+**

Legend: (+) Adhesion to the tube wall (-) No adhesion to the tube wall.

combined with the antimicrobials ampicillin, gentamicin, ciprofloxacin, and ceftriaxone presented synergistic effect against most of the strains.

3.4. Minimum Inhibitory Concentration for Adherence (MICA)

Table 5 presents the Minimum Inhibitory Concentration for Adherence (MICA) results of trans-anethole, as well as a comparison with the positive control (chlorhexidine 0.12%) against the *Enterococcus faecalis* strain (Ef 49). Analyzing the results, it is observed that in the proportions used, neither trans-anethole nor chlorhexidine (0.12%) presented antibiofilm effects against the studied strain.

4. Discussion

For years, the emergence and spread of microorganisms resistant to market available synthetic antimicrobials has been reported, and the need for new alternatives which present antimicrobial activity is clear (Mendes et al., 2011). In this context, natural products, being both culturally accepted and accessible are an effective therapeutic alternative (Bezerra et al., 2017).

For antimicrobial activity to be considered strong, it must have an MIC of up to 500 µg/mL, MICs of 600 to 1500 µg/mL are considered moderate, and weak activity presents MICs above 1500 µg/mL (Sartoratto et al., 2004). According to the results found in this study, trans-anethole presented moderate antimicrobial activity against *Enterococcus faecalis* strains, since the MIC 90 was 1000 µg/mL.

For a compound to be considered bactericidal or bacteriostatic according to its MBC, its concentration should be, respectively, equal to or twice the MIC or greater than twice the MIC (Hafidh et al., 2011). Thus, our results show that trans-anethole presents bactericidal potential against *E. faecalis* ATCC 29212, Ef 47, and Ef 49, since respectively the MBC against these strains were 500 µg/mL, 1000 µg/mL, and 1000 µg/mL. The strains Ef 46 and Ef 50 presented bacteriostatic potencial, since both presented an MBC >1000 µg/mL.

Although trans-anethole was significantly effective against *E. faecalis* strains, it is worth noting that the compound did not present antibacterial activity against *Enterobacter cloacae*. Donati et al. (2014) also reported disappointing results, in that trans-anethole presented no antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* or *Escherichia coli*.

We note that in the literature it has already been reported that trans-anethole indeed presents antibacterial potential. Orhan et al. (2012) found that trans-anethole presents antibacterial activity against various bacteria, including *E. faecalis*. Their results corroborate our findings, in which trans-anethole presented antibacterial activity against *E. faecalis* of food origin.

Other natural products have presented relevant results against *E. faecalis*. An example is Janani et al. (2019), who observed that oregano essential oil presents antimicrobial activity against *E. faecalis*.

Further, according to our results, trans-anethole presented efficacy in inhibiting microbial growth when associated with synthetic antimicrobials, with synergistic effects for most strains of *E. faecalis*. Many natural products when associated with synthetic antimicrobials present synergistic effects. For instance, Santana et al. (2021) analyzed *Lavandula hybrida* Grosso essential oil, which in association with cephalothin, presented synergistic effect against pathogenic strains of *S. aureus*.

Kwiatkowski et al. (2019a) analyzed the influence of essential oil compounds on the antibacterial activity of mupirocin against strains of methicillin resistant *Staphylococcus aureus* (MRSA) susceptible to mupirocin (^{MupS}), and induced low-level mupirocin resistant (^{MupRL}). According to the authors, trans-anethole presented additive effect with mupirocin against the MRSA ^{MupRL} strain, and indifferent effect against the MRSA ^{MupS} strain.

It is also worth noting that trans-anethole presents other functions, such as contributing to antibiofilm effects. Previous studies reveal that mupirocin in association with 2% trans-anethole was able to significantly decrease *S. aureus* biofilms (Kwiatkowski et al. 2019b). However, in the present study trans-anethole alone was unable to inhibit *E. faecalis* biofilm.

Differently, experiments have been conducted in which other natural products were tested with other bacteria regarding the anti-adherent actitity and pertinent results were found such as that of Ramalho et al. (2020), who observed that *Eucalyptus globulus* oil presents anti-adherent activity equivalent to chlorhexidine digluconate 0.12% against *Klebsiella pneumoniae*, both compunds inhibited the formation of biofilm in a ratio of 1:8.

5. Conclusion

We conclude that trans-anethole is effective in inhibiting the growth of the Gram-positive bacteria *E. faecalis*, yet when tested against the Gram-negative bacteria *E. cloacae* no antibacterial action was observed. Trans-anethole also potentiated the antibacterial activity of synthetic antimicrobials against *E. faecalis*, presenting synergistic effects against most strains. However, trans-anethole did not effectively inhibit *E. faecalis* biofilm under our methodology.

In short, trans-anethole presented potential antibacterial against *E. faecalis* strains of food origin, and may upon further study, it may be used alone or in association with synthetic antimicrobials to combat infections caused by this bacterium.

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