

# Antimicrobial Investigation and Structure-Activity Analysis of Natural Eugenol Derivatives Against Several Oral Bacteria

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## Abstract

**Background:** Essential oils isolated from plants are rich with phenolic compounds which have exhibited promising antimicrobial activity against various oral bacteria.

**Objective:** Herein, the antimicrobial activity of methyl eugenol, eugenol and hydroxychavicol were analyzed against several oral bacteria. A correlation between molecular structure variation and antimicrobial activity was also investigated.

**Materials and Methods:** Minimum inhibitory concentrations were determined using a serial microdilution method in a 96-well plate and by colorimetric analysis with resazurin dye. Aliquots from each well within the MIC range were placed onto suitable agar plates and the minimum bactericidal concentration was determined as the lowest concentration with no observed colony growth. Methyl eugenol, eugenol, and hydroxychavicol were tested against a total of ten oral bacteria.

**Results:** Hydroxychavicol exhibited the lowest inhibitory and bactericidal concentrations against all bacteria tested with MIC values as low as 25-50 µg/mL and MBC values as low as 37.5-50 µg/mL. A structure-activity analysis indicates that free hydroxyl groups attached to the benzene ring of the molecular structure increase the antibacterial effectiveness of these compounds.

**Conclusions:** This study provides insight into the mechanism of antibacterial activity of phenolic extracts against oral bacteria and can be used for the synthesis of more potent analogs for oral health treatment.

**Keywords:** MIC; MBC; Eugenol; Hydroxychavicol; Oral health

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## Introduction

The therapeutic and medicinal properties of plant extracts have been known and practiced for centuries in ancient medicine around the world. There has been much research focused on the identification and isolation of biologically active essential oil extracts, which validates these traditional methods [1,2]. While the essential oil constituents of these plant extracts vary across species, high concentrations of phenolic compounds are ubiquitous. In particular, eugenol (2) (**Figure 1**) is a major component in extracts from clove buds, betle leaves, cinnamon bark and tulsii leaves [3].

Eugenol has shown promising therapeutic activity in a wide variety of applications including: as an analgesic, antioxidant, anesthetic, antibacterial, anticonvulsant, antiviral, anti-inflammatory and anti-cancer agent [3-5]. As many of the above-mentioned natural sources of eugenol are ingested orally, eugenol has specifically

been studied against oral bacteria including *Streptococcus mutans* [6], *Streptococcus sanguinis* [7], *Staphylococcus epidemidis* [8,9] and *Corynebacterium xerosis* [10]; as well as for the treatment of periodontal disease [11]. Due to the antibacterial activity of eugenol, it is even used commercially in the form of Zinc Oxide Eugenol (ZOE) as a temporary cement filling and root canal sealer in dentistry [12,13].

Owing to the vast research surrounding eugenol, we turned our attention to two phenolic structural variants of eugenol: methyl eugenol (1) and hydroxychavicol (3) (**Figure 1**). Both compounds are often isolated from natural sources together with eugenol, albeit in diminished concentrations. The bacterial inhibition of methyl eugenol and hydroxychavicol against oral bacteria has also been investigated, with hydroxychavicol exhibiting encouraging results [14,15]. However, the breadth of oral bacteria analyzed in conjunction with hydroxychavicol has so far been limited

Due to the promising results of hydroxychavicol against some oral bacteria strains, we were prompted to further investigate its antimicrobial potential against various heretofore unexamined strains of oral bacteria including *Streptococcus salivarius*, *Streptococcus sobrinus*, *Corynebacterium pseudodiphtheriticum*, *Lactobacillus salivarius*, *Rothia dentocariosa*, and *Neisseria subflava*. The structural similarities of eugenol, methyl eugenol and hydroxychavicol (**Figure 1**) also warrant a study of their structure-activity relationship as it pertains to antimicrobial activity. To this end, we measured the Minimum Inhibitory Concentration (MIC) as well as the Minimum Bactericidal Concentration (MBC), for compounds 1-3, against each of the above-mentioned oral bacteria.

## Materials and Methods

### Analytes methyl eugenol (1), eugenol (2) and hydroxychavicol (3)

Methyl eugenol (>98% Aldrich W247502) and eugenol (>98% Aldrich W246719) were purchased from Aldrich and used without further purification. Hydroxychavicol was synthesized from eugenol following the demethylation procedure reported by Arifin and coworkers [16]. Resulting spectroscopic characterization was in agreement with known published data. Stock solutions of all three compounds were made using a minimal amount of dimethyl sulfoxide (>99.9% Aldrich 473201) to dissolve each compound and diluted with growth media to give final concentrations of 1000 µg/mL.

### Bacterial strains and culture methods

All antimicrobial methods were performed in compliance with the published Clinical and Laboratory Standards Institute, 2012 [17].

*Streptococcus salivarius* (Wards 470179-174), *Streptococcus mutans* (Wards 470179-170), *Streptococcus sanguinis* (Wards 470179-176), *Streptococcus sobrinus* (ATCC 33478), *Staphylococcus epidermidis* (Wards 470176-542), *Corynebacterium pseudodiphtheriticum* (Carolina 155010), *Corynebacterium xerosis* (Carolina 155015), *Lactobacillus salivarius* (ATCC 11741), *Rothia dentocariosa* (ATCC 17931), and *Neisseria subflava* (ATCC 49275) were initially grown in either Brain Heart Infusion (BHI) (Himedia mv210) or Mueller-Hinton (MH)(Remel R112474) broth under aerobic conditions at 37°C and 240 rpm. *S. salivarius*, *S. sanguinis*, *C. pseudodiphtheriticum* and *N. subflava* required 5% lysed, defibrinated horse blood (LHB) (Fisher 50-863-758) for growth.

Growth on either BHI or MH agar plates at 37°C yielded single colonies which were grown, in respective broth media, to the visual 0.5 McFarland standard using a white background with black lines. For bacteria grown in broth supplemented with 5% LHB, bacteria pellets were formed through centrifugation at 2000 rpm and the supernatant was discarded before resuspension in broth to 0.5 McFarland standard.

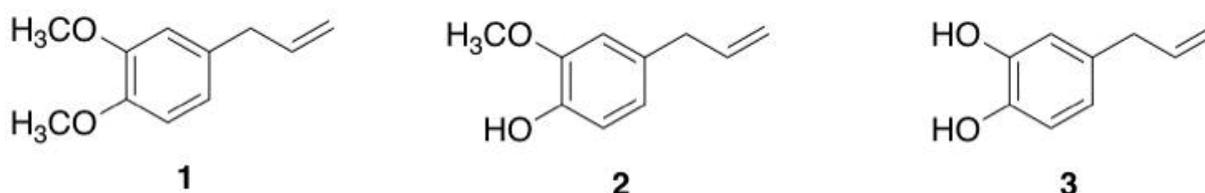
### Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

Minimum inhibitory concentrations were determined using a serial microdilution method in a 96-well plate with resazurin dye (Aldrich R7017) as a growth indicator as described by Elshikh M et al. [18]. Each well contained a total volume of 100 µL, consisting of appropriate growth media (BHI or MH broth), inoculum and analyte concentrations of compounds 1-3. Wells in columns 1-9 contained analyte concentrations of compounds 1-3 of 1000, 800, 600, 500, 400, 300, 200, 100 and 50 µg/mL respectively. Wells in column 10 contained broth and 1000 µg/mL analyte solution as a negative control for sterility. Wells in columns 11 and 12 were both used as positive controls. Wells in column 11 contained broth and dimethyl sulfoxide, and wells in column 12 contained only broth. After inoculation of each well, the 96-well plate was incubated at 37°C for 24 hours. 30 µL of 0.015% resazurin dye was then added to each well and the plate was incubated at 37°C for 1-4 hours. MIC values were determined by colorimetric analysis with a color change from blue to pink indicating the growth of bacteria. The MIC was determined as the lowest concentration at which the resazurin dye in the well did not change colors.

Minimum bactericidal concentrations were obtained by placing 20 µL from each well within the MIC range onto suitable agar plates (either BHI or MH) and incubated overnight at 37°C. The MBC was determined as the lowest concentration with no observed colony growth.

## Results and Discussion

As shown in **Table 1**, it is apparent that there is a structure-activity relationship with regard to antimicrobial activity. Methyl eugenol (1), with no free hydroxyl groups attached to the benzene ring (**Figure 1**), proved fairly ineffective against the oral bacteria tested, exhibiting only mild inhibition against *S. sobrinus* (**Table 1**, entry 3). MBC values for methyl eugenol were accordingly above 1000 µg/mL.



**Figure 1** Chemical structures of (1): methyl eugenol; (2): eugenol and (3): hydroxychavicol.

**Table 1** Summary of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) for Methyl Eugenol, Eugenol and Hydroxychavicol Against Oral Bacteria.

Entry	Bacteria	MIC ( $\mu\text{g}/\text{mL}$ )			MBC ( $\mu\text{g}/\text{mL}$ )		
		Methyl Eugenol (1)	Eugenol (2)	Hydroxychavicol (3)	Methyl Eugenol (1)	Eugenol (2)	Hydroxychavicol (3)
1	<i>S. salivarius</i>	>1000	>1000	400-600	>1000	>1000	500-700
2	<i>S. mutans</i>	>1000	600-1000	25-50	>1000	>1000	37.5-50
3	<i>S. sobrinus</i>	600-800	400-600	100-200	800-1000	600-800	200-300
4	<i>S. sanguinis</i>	>1000	800-1000	50-100	>1000	>1000	200-300
5	<i>S. epidermidis</i>	>1000	800-1000	100-200	>1000	800-1000	200-300
6	<i>N. subflava</i>	>1000	>1000	100-400	>1000	>1000	800-1000
7	<i>C. pseudodiphtheriticum</i>	>1000	>1000	100-300	>1000	>1000	400-500
8	<i>C. xerosis</i>	>1000	500-800	100-200	>1000	800-1000	200-300
9	<i>L. salivarius</i>	>1000	>1000	400-500	>1000	>1000	500-800
10	<i>R. dentocariosa</i>	>1000	300-400	<50	>1000	800-1000	100-300

Eugenol (2), having one free hydroxyl group attached to the benzene ring (**Figure 1**), exhibited mild inhibitory activity against most of the bacteria, with the lowest MIC value observed for *R. dentocariosa* of 300-400  $\mu\text{g}/\text{mL}$  (**Table 1**, entry 10). Although initial inhibition was observed with eugenol against many of the bacteria, MBCs were much higher, above 1000  $\mu\text{g}/\text{mL}$ .

Hydroxychavicol (3), having two free hydroxyl groups attached to the benzene ring (**Figure 1**), inhibited the growth of every oral bacteria tested and in low concentrations. Of special note are the low MICs of hydroxychavicol against *S. mutans* (**Table 1**, entry 2) and *S. sanguinis* (**Table 1**, entry 4), two bacteria linked to oral plaque and cavity formation. *R. dentocariosa*, another bacterium associated with plaque and dental caries, was also inhibited effectively by hydroxychavicol (**Table 1**, entry 10). Hydroxychavicol began inhibiting *S. mutans* and *R. dentocariosa* growth at concentrations under 50  $\mu\text{g}/\text{mL}$ , and inhibiting *S. sanguinis* at 50-100  $\mu\text{g}/\text{mL}$ . While MBC values for hydroxychavicol against *R. dentocariosa* and *S. sanguinis* were observed at higher concentrations than their respective MIC values, the low MBC value for hydroxychavicol against *S. mutans* demonstrates its effective potency against this bacterium. Hydroxychavicol also exhibited good inhibitory activity against *S. sobrinus*, *S. epidermidis*, *N. subflava* and *Corynebacteria pseudodiphtheriticum* and *xerosis* (**Table 1**, entries 3, 5-8). MBC values for these bacteria proved to be more spread out, with lower concentrations of 200-300  $\mu\text{g}/\text{mL}$  for *S. sobrinus*, *S. epidermidis* and *C. xerosis*, and higher concentrations of 400-500  $\mu\text{g}/\text{mL}$  and 800-1000  $\mu\text{g}/\text{mL}$  for *C. pseudodiphtheriticum* and *N. subflava* respectively. Both genera of salivarius (**Table 1**, entries 1 and 9) required higher concentrations of hydroxychavicol for

inhibition, with correspondingly higher MBCs.

From this data, we propose that the hydroxyl groups attached to the benzene ring in eugenol (2) and hydroxychavicol (3) (**Figure 1**) are responsible, and indeed imperative, for the observed antibacterial activity of these phenolic compounds. Eugenol, having only one hydroxyl group exhibits mild antimicrobial activity, whereas hydroxychavicol, with two hydroxyl groups, exhibits a pronounced increase in antimicrobial activity. This observed trend serves to help elucidate the mechanism by which these compounds inhibit bacterial growth.

## Conclusion

Phenolic compounds are prevalent in essential oils isolated from various plants and have been studied for their effectiveness against various oral bacteria. Through our findings, two phenolic compounds; eugenol, and hydroxychavicol, show very promising antimicrobial activity against several oral bacteria which are known to cause dental cavities and other oral maladies. A structure-activity analysis shows that free hydroxyl groups are necessary for effective antibacterial inhibition and bactericidal potency. These findings indicate the potential of hydroxychavicol as an oral health additive and possible preventative treatment for oral cavity formation. The reported structure-activity analysis provides vital information into the future synthesis of phenolic derivatives for oral health care treatment and prevention.

## Conflict of Interest

The authors report no conflict of interest.

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