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# *IN VITRO* ANTIMICROBIAL ACTIVITIES OF ANETHOLE, CARVACROL, CINNAMON BARK OIL, EUGENOL, GUAIAZULUNE AND THYMOL AGAINST GROUP G STREPTOCOCCI

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*The present study describes the antimicrobial activity of various natural substances against group G streptococci. A total of 50 strains of group G streptococci was included in this study. The minimum bactericidal concentrations of 6 substances — anethole, eugenol, guaiazulene, carvacrol, cinnamon bark oil from Cinnamomum zeylanicum and thymol were obtained by the broth microdilution method. The substances were extended either directly in brain heart infusion broth or in 24 % ethanol. Carvacrol was found to be the most effective substance with the highest antimicrobial activity, which inhibited the growth of 90 % of group G streptococci strains at the concentration of 236.7 μg ml$^{-1}$. The minimum bactericidal concentrations of eugenol, oil from the bark of Cinnamomum zeylanicum, and thymol were 1000 μg ml$^{-1}$. Anethole and guaiazulene were the least effective natural substances in our study.*

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## Introduction

Group G beta-haemolytic streptococci (GGS) were first reported in association with puerperal sepsis in 1935 [1]. Later, it was found that GGS form a heterogeneous group that includes minute colony formers from humans (*Streptococcus (S.) anginosus* group) and large colony formers from humans (*S. dysgalactiae* ssp. *equisimilis*) and animals (*S. canis*).

GGS are usually regarded as commensals, because they are often isolated from human and animal pharynx, skin, gastrointestinal and urogenital tract. Recently, they have been reported with increasing frequency as a cause of a variety of human infections, such as pharyngitis, skin and soft tissue infections, puerperal and neonatal infections, endocarditis, meningitis [2-4]. The bactaeremia associated with GGS has been related to underlying conditions including malignancy, alcoholism, diabetes, intravenous drug abuse or skin injuries [5]. GGS infections have manifestations similar to those caused by *S. pyogenes*. They express several virulence factors previously described only in *S. pyogenes*, such as M-protein, streptolysins O and S, C5a peptidase, streptococcal pyrogenic exotoxins and hyaluronic acid capsule [6,7].

The first choice in treatment of GGS infections is still penicillin. However, unlike *S. pyogenes*, GGS reaction to penicillin therapy is slow [8]. Macrolides are a common substitute for treatment of streptococcal infections in patients with a penicillin allergy (up to 10 %). In recent years, the resistance of GGS strains to erythromycin has been reported with increasing frequency (3-43.8 % of resistant strains) [9-11]. Tetracyclines have been widely used as the second option; however, increasing resistance (up to 81.3 %) has limited its use [10-13].

Increasing resistance of streptococci to antimicrobial agents has triggered the search for new substances with antimicrobial effect mainly of plant origin. To our knowledge, no studies of antimicrobial effects of natural substances against GGS have been reported. However, effects of eugenol, carvacrol, cinnamaldehyde and thymol were determined for other streptococcal species [14,15].

The aim of our study was to examine the *in vitro* susceptibilities of group G streptococci to several natural substances derived from plants.

## Materials and Methods

### Natural Substances

All the natural substances (anethole, carvacrol, cinnamon bark oil, eugenol, guaiazulene and thymol) were purchased from Sigma-Aldrich spol. s r.o. (The Czech Republic) and were extended in brain heart infusion (BHI) broth (HiMedia s.r.o., Czech Republic) or 24% ethanol (Sigma-Aldrich spol. s r.o., the Czech Republic) to obtain stock solutions.

### Bacterial Strains

The total of 50 GGS isolates included in this study were recovered from children and adult patients (20 and 30 isolates, respectively) at the microbiological laboratory of Pardubice regional hospital. The isolates were collected from April 2005 through July 2005 from various clinical specimens with following frequency: 34 throat swabs, 6 ulcer swabs, 5 skin swabs, 3 pus and 2 vaginal swabs. Serotyping for group antigen was performed using the ITEST STREPTO GROUP kit (ITEST plus s.r.o., the Czech Republic). The biotype of each GGS strain was determined by the STREPTOtest 16 identification system using the Identification program TNW lite 7.0 (ERBA-Lachema s.r.o., the Czech Republic). All GGS strains were identified as *S. dysgalactiae* ssp. *equisimilis*.

The GGS strains were stored at the temperature of –20 ºC, thawed and activated by transfer into BHI broth at 37 ºC for 24 hours. The bacterial suspension equal to a McFarland standard of 0.5 prepared in BHI broth was used to inoculate prepared microplates (Fisher Scientific, spol. s.r.o., the Czech Republic).

### Antimicrobial Susceptibility Testing

Antimicrobial effects of the selected substances were determined by broth microdilution method. Serial twofold dilutions of natural substances in BHI broth were performed. Each well was inoculated with 5 μl of the standardized inoculum and incubated at 37 °C for 48 hours. A positive control (growth) consisting of organisms in broth and a negative control (sterility) consisting of uninoculated broth were included for each assay. Each assay was performed in triplicates.

The results were analyzed visually each 24 hours and classified according to the following patterns: turbidity with bacterial colonies being deposited and total growth inhibition. The substances that showed inhibitory activity were submitted to a subculture of the broth media on blood agar (HiMedia s.r.o., the Czech Republic) in order to evaluate bacterial growth. The concentration at which there was no bacterial growth after inoculation on blood agar was taken as the minimum bactericidal concentration (MBC).

## Results and Discussion

The systematic screening of antibacterial plant extracts represents a continuous effort of many laboratories to find new compounds with the potential to replace antibiotics. Long-term use of antibiotics (in particular penicillins, macrolides, vancomycin and quinolones) causes bacterial resistance to these drugs, which is a growing health problem in general. Recent evidence has also shown the antimicrobial potential of combinations of antimicrobial agents (especially penicillins, cephalosporines or macrolides) with some natural substances such as terpenes [16].

MBCs of all the natural substances tested are shown in Table I. GGS strains were found to be highly susceptible to carvacrol ($MBC_{90}$ = 236.7 μg $ml^{-1}$), a phenolic compound isolated from leaves and flowers of *Thymus vulgaris* L. or *Origanum vulgare*. Mode of antimicrobial action of carvacrol is similar to other phenolic substances: damage of the cytoplasmic membrane, disruption of the proton motive force, electron flow, active transport and coagulation of cell contents [17,18]. Carvacrol was found to possess antimicrobial activity against several respiratory pathogens such as *Haemophilus influenzae*, *Branhamella catarrhalis* (both MIC = 62.5 μg $ml^{-1}$) and *S. pneumoniae* (MIC = 125 μg $ml^{-1}$) [14]. Other oral streptococci (*S. mutans*, *S. sanguinis* and *S. milleri*) were reported to be susceptible to carvacrol in the concentration of 125-250 μg $ml^{-1}$ [19].

Table I *In-vitro* antimicrobial activity of natural substances against GGS clinical isolates determined by microdilution method (*n* = 50)

| Natural substance | Range<br>μg $ml^{-1}$ | $MBC_{50}$ ** = $MBC_{90}$ ***<br>μg $ml^{-1}$ |
|---|---|---|
| Anethole | 500-32 000 | > 32 000 |
| Carvacrol | 7.39-473.4 | 236.7 |
| Cinnamon oil* | 62.5-4 000 | 1000 |
| Eugenol | 62.5-4 000 | 1000 |
| Guaiazulene | 500-32 000 | > 32 000 |
| Thymol | 31.25-2 000 | 1000 |

*Cinnamon oil from bark of *Cinnamomum zeylanicum*, ** The minimum bactericidal concentration at which 50 % of strains were killed, *** The minimum bactericidal concentration at which 90 % of strains were killed

The strong antimicrobial activity ($MBC_{90}$ = 1000 μg $ml^{-1}$) was determined for cinnamon bark oil. Several biological activities, such as antioxidant, antipyretic, analgesic, antifungal and antibacterial have been attributed to this substance. The antimicrobial effect of cinnamaldehyde, a major constituent of cinnamon bark oil, is related to its ability to bind to proteins preventing the action of amino acid decarboxylases [18]. It has been demonstrated that cinnamon bark oil and *trans*-cinnamaldehyde have an inhibitory effect against a large variety of pathogenic microorganisms, including *Haemophilus influenzae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* [20,21]. *trans*-Cinnamaldehyde showed also inhibitory activity against other streptococcal species, such as *S. milleri* (MIC = 31.25 μg $ml^{-11}$) [19].

Eugenol, a major component of essential oil isolated from *Syzygium aromaticum* L., exerted also good effect on GGS ($MBC_{90}$ = 1000 μg $ml^{-1}$). In last years, biological properties of this substance have been determined including fungicidal, bactericidal, antioxidant and anti-inflammatory properties [22]. The mechanism of antimicrobial effect has been studied recently, and it was observed that eugenol inhibited the generation of adenosine triphosphate and caused cell membrane disruption [23]. Eugenol performed satisfactory antimicrobial activity against airborne microbes [21]. It was observed that oral streptococci (*S. mutans*, *S. sanguinis* and *S. milleri*) were susceptible to carvacrol in the concentrations of 125-500 μg $ml^{-1}$ [19].

Antimicrobial activity of thymol, a predominant ingredient of *Thymus vulgaris* L. essential oil, was equal to 1000 μg $ml^{-1}$. Recent studies showed that the antimicrobial effect of thymol is partially due to cell membrane disruption resulting in alterations of membrane permeability and in leakage of intracellular materials [24]. The inhibitory concentration of thymol against *S. pyogenes* was reported to be 100 μg $ml^{-1}$ [15]. Thymol showed also good effect against other oral streptococci (MIC = 125-250 μg $ml^{-1}$) [19].

In the present study, anethole and guaiazulene were the least effective natural substances against GGS streptococci. Antimicrobial properties of these substances were probably affected by their poor solubility both in BHI broth and ethanol.

Differences in methodology such as extraction technique, solvent used, culture medium, incubation period, method of susceptibility testing are factors that make published data somewhat difficult to compare. However the results indicate that carvacrol, cinnamon bark oil, eugenol and thymol possess bactericidal activity against GGS and could have potential therapeutic significance in treating streptococcal infections.

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