In vitro antimicrobial activities of cinnamon bark oil, anethole, carvacrol, eugenol and guaiazulene against *Mycoplasma hominis* clinical isolates

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Aims. The aim of this study was to evaluate the antimicrobial effects of five natural substances against 50 clinical isolates of *Mycoplasma hominis*.

Methods and Results. The *in vitro* activity of selected natural compounds, cinnamon bark oil, anethole, carvacrol, eugenol and guaiazulene, was investigated against 50 *M. hominis* isolates cultivated from cervical swabs by the broth dilution method. All showed valuable antimicrobial activity against the tested isolates. Oil from the bark of *Cinnamomum zeylanicum* (MBC $_{90} = 500 \ \mu g/mL$) however was found to be the most effective. Carvacrol (MBC $_{90} = 600 \ \mu g/mL$) and eugenol (MBC $_{90} = 1000 \ \mu g/mL$) also possessed strong antimycoplasmal activity.

Conclusions. The results indicate that cinnamon bark oil, carvacrol and eugenol have strong antimycoplasmal activity and the potential for use as antimicrobial agents in the treatment of mycoplasmal infections.

Key words: Mycoplasma hominis, antimicrobial agents, minimal inhibitory concentration, natural substances

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INTRODUCTION

Mycoplasma (M.) hominis, the first human mycoplasma species¹, has been isolated in the case of a variety of diseases, including bacterial vaginosis, pelvic inflammatory disease and pyelonephritis. In recent years, it has been reported with increasing frequency to cause complicated pregnancies, chorioamnionitis, postabortion and postpartum fevers²⁴. In addition, it has been implicated in extragenital infections such as postoperative wound infections, arthritis, prosthetic valve endocarditis, respiratory and other infections in immunosuppressed patients⁵. In neonates, M. hominis has been associated with neonatal meningitis, pneumonia and skin abscesses⁶⁻⁹.

Mycoplasmas are naturally resistant to antibiotic compounds that are directed to inhibition of cell wall synthesis, such as penicillins and cephalosporins^{2,7}. Moreover, they are generally resistant to polymyxins, rifampicin and sulphonamides⁷. Therefore, treatment options for mycoplasmal infections are limited to relatively few classes of antimicrobial agents, tetracyclines, macrolides, quinolones and related antibiotics.

Increasing incidence of antimicrobial resistance as a result of overprescription and misuse of traditional antibiotics is a growing concern in medical, food and sanitation areas. To reverse high resistance frequencies, substantial resources have been invested in the research of new antimicrobial substances, mainly of microbial and plant origin. To our knowledge, few studies of the antimicrobial susceptibility of *M. hominis* to natural substances have been reported. The antimycoplasmal effects of hydroxytyrosol and oleuropein (major phenolic compounds

identified in olive oil) and of tea tree oil have been determined ¹⁰⁻¹³.

The aim of this study was to examine the *in vitro* susceptibilities of *M. hominis* clinical isolates to several natural substances derived from plants.

MATERIALS AND METHODS

Bacterial strains

The *M. hominis* isolates studied included 50 strains cultivated from the cervical swabs of randomly selected women between 2003 and 2004. *M. hominis* strains were stored at -80 °C. Before performing experiments, strains were thawed and retrieved by transfer into PPLO broth at 37 °C for 24 h. The bacterial inoculum of 10⁵-10⁷ CFU/mL was prepared as described previously¹⁴.

Culture media

M. hominis strains were cultivated in PPLO broth. The following composition is given for the preparation of one litre of medium: 21 g BD Difco™ PPLO broth (BD Diagnostics), 200 mL horse serum (LabMediaServis, Jaromer, Czech Republic), 100 mL freshly-prepared yeast extract, 8 mL 10% thallium acetate (Sigma-Aldrich), 1 g ampicillin (Biotika Bohemia, Prague, Czech Republic), 5 g L-arginine and 2 mL 1% phenol red (both Sigma-Aldrich) and 700 mL of purified water. Antimicrobial susceptibility testing was performed in PPLO broth without inhibitors (ampicillin and thallium acetate). PPLO agar was prepared from 35 g of BD Difco™ PPLO agar (BD Diagnostics) and supplemented the same way as PPLO broth.

Natural substances

All natural substances (anethole, carvacrol, cinnamon bark oil, eugenol and guaiazulene) were purchased from Sigma-Aldrich and were extended in 24% ethanol to obtain stock solutions.

Antimicrobial susceptibility testing

The antimicrobial effects of the selected substances were determined by the broth dilution method. Serial twofold dilutions of anethole, cinnamon bark oil, eugenol and quaiazulene in a concentration range 62.5 - 4000 µg/mL and carvacrol in a concentration range 18.75 - 1200 µg/ mL were prepared (Table 1). Each tube with 1 mL PPLO broth containing tested substances was inoculated with 100 µL of the standardized inoculum and incubated aerobically at 37 °C. A positive growth control consisting of organisms in broth and a negative sterility control consisting of uninoculated broth were included for each assay. Inoculated tubes were examined daily until a colour change was detected in the positive control tube. The minimal inhibitory concentration (MIC₉₀) was defined as the lowest concentration of natural substance in which the metabolism of 90% of tested organisms was inhibited. This was evidenced by a lack of a colour change in the medium at the time when the positive growth control first showed a colour change. For M. hominis, growth was usually evident in the positive control after 24-48 h of incubation. Substances that showed inhibitory activity were further submitted to a subculture of the broth media on PPLO agar in order to evaluate bacterial growth. The concentration at which there was no bacterial growth of 90% tested strains after inoculation on PPLO agar was taken as the minimal bactericidal concentration (MBC_{oo}). When the MIC_{90} was greater than the highest tested concentration, the strain was considered as resistant.

RESULTS

The MIC_{90} and MBC_{90} results of all natural substances were determined (Table 1). Cinnamon bark oil, carvacrol, eugenol and anethole all were variously able to inhibit the growth of M. hominis clinical isolates (Fig.1A-E). Cinnamon bark oil was found to be the most effective

natural substance tested against M. hominis (MIC $_{90}$ = MBC $_{90}$ = 500 µg/mL). Carvacrol also possessed strong antimicrobial activity against M. hominis strains in this study with MIC $_{90}$ and MBC $_{90}$ equal to 600 µg/mL. A somewhat lower value was determined for eugenol (MIC $_{90}$ = MBC $_{90}$ = 1000 µg/mL). The antimycoplasmal activity of anethole was one of the lowest (MIC $_{90}$ = MBC $_{90}$ = 4000 µg/mL). The least effective natural substance was found to be guaiazulene with as much as 50% of resistant strains to the concentration 4000 µg/mL which was the highest concentration tested.

DISCUSSION

A variety of medicinal plants and plant extracts have been screened for their antimicrobial activity but to our best knowledge, the inhibitory effects of only oleuropein, hydroxythyrosol and tea tree oil have been tested against *M. hominis* strains^{10-13,15}. In contrary to these studies we have determined lower antimycoplasmal activities of natural substances used in our work.

In the present study, M. hominis strains were highly sensitive to cinnamon bark oil with MIC $_{90}$ = MBC $_{90}$ equal to 500 µg/mL. As described previously, (E)-cinnamaldehyde is the major component (97.7% w/w) of the oil derived from Cinnamomum zeylanicum 16 . A number of biological activities including antioxidant, antipyretic, analgesic, antifungal and antibacterial have been attributed to this substance. The antimicrobial effect of cinnamaldehyde is related to its ability to bind to proteins preventing the action of amino acid decarboxylases 17 . It has been demonstrated that Cinnamomum zeylanicum oil has an inhibitory effect against a large variety of pathogenic microorganisms, including Haemophilus influenzae, Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus aureus and Escherichia coli 18 .

Strong antimicrobial activity (MIC $_{90}$ = MBC $_{90}$ = 600 µg/mL) was also determined for carvacrol, a phenolic compound isolated from leaves and flowers of *Thymus vulgaris* L. and *Origanum vulgare*. Carvacrol has several biological properties, particularly antioxidant and antimicrobial activity. The antimicrobial action of carvacrol is similar to other phenolic substances: damage of the cyto-

Table 1. In vitro antimicrobial activity of selected natural substances against M. hominis clinical isolates (n = 50).

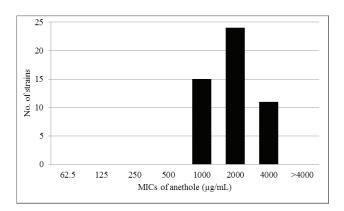
Natural substances	Concentration range	MIC and MBC ^a		
		MIC ₉₀ ^b	MBC ₉₀ °	Range
Anethole	62.5-4000	4000	4000	1000-4000
Eugenol	62.5-4000	1000	1000	1000
Guaiazulene	62.5-4000	>4000	>4000	2000->4000
Carvacrol	18.75-1200	600	600	300-600
Cinnamon oil	62.5-4000	500	500	250-1000

 $[^]a\,MIC_{90}$ and MBC_{90} values are in $\mu g/mL$

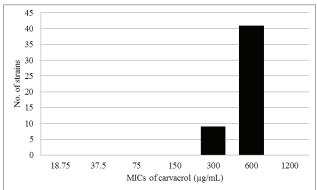
^b MIC₉₀ is the lowest concentration of antimicrobial agent inhibiting metabolism of 90% tested strains

^cMBC₉₀ is the lowest concentration of antimicrobial agent inhibiting growth of 90% tested strains

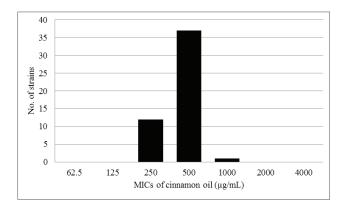
A Anethole MIC distribution



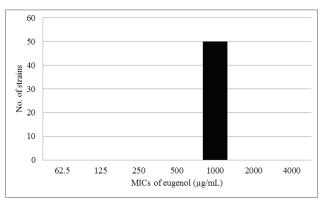
B Carvacrol MIC distribution



C Cinnamon oil MIC distribution



D Eugenol MIC distribution



E Guaiazulene MIC distribution

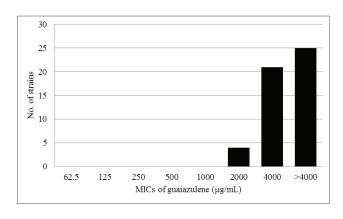


Fig. 1A-E. Distributions of MICs of anethole (A), carvacrol (B), cinnamon oil (C), eugenol (D) and guaiazulene (E) for *M. hominis* clinical isolates (n = 50).

plasmic membrane, disruption of the proton motive force, electron flow, active transport and coagulation of cell contents¹⁷. Carvacrol has been reported to possess inhibitory activity against *Pseudomonas fluorescens*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*^{19,20}.

Eugenol, a major ingredient of the essential oil isolated from Syzygium aromaticum L., showed a lower efficiency than carvacrol (MIC $_{90}$ = MBC $_{90}$ = 1000 µg/mL). Eugenol has pronounced analgesic properties. In recent years, additional biological properties of this substance have been determined including fungicidal, bactericidal, antioxidant

and anti-inflammatory properties²¹. The mechanism of the antimicrobial effect of eugenol has been studied recently. It was observed that treatment of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* with this agent inhibited generation of adenosine triphosphate and caused cell membrane disruption²².

In the present study, anethole and guaiazulene were the least effective natural substances against *M. hominis* strains. The antimicrobial properties of these substances were probably affected by their poor solubility in ethanol.

The critical aspect of mycoplasma infections is their problematic eradication either due to the acquired resist-

ance or due to lack of bactericidal activity. Tetracyclines have been the drug of choice for treatment of *M. hominis* urogenital infections. However, tetracycline resistance associated with transposon-borne *tet*M determinant occurs in 19-30% of *M. hominis* strains and seems to be increasing, making their therapeutic activities no longer predictable^{7,23,24}. *M. hominis* is generally resistant to 14- and 15-membered macrolides, but is susceptible to josamycin, a 16-membered macrolide^{25,26}. Fluoroquinolones, which are frequently used for the treatment of upper genital tract infections, represent an effective alternative because they are the only bactericidal drugs against mycoplasmas. In recent years, *M. hominis* strains resistant to fluoroquinolones have also been reported²⁷.

The results of our study indicate that cinnamon bark oil, carvacrol and eugenol possess bactericidal activity against *M. hominis* and could be used to treat mycoplasmal infections.

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