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Antimicrobial activity of extracts from GRAS plant species against oral pathogenic microorganisms

Ph.D. Thesis

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Certification

I, Jindřiška Pilná, hereby declare that this dissertation thesis and its intellectual content is my own work submitted for the Ph.D. degree at the Czech University of Life Sciences Prague, unless otherwise referenced. I also affirm that the document has not been submitted for qualifications at any other academic institution.

Prague, October 6, 2015

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Abstract

Microbial oral diseases such as caries and periodontitis are among the most frequent human infections. Conventional chemical antiseptics used for their treatment and prevention often produce adverse side-effects, which restrict their long-term use. Although plants are considered as perspective sources of novel antimicrobial compounds, little is still known about their inhibitory properties against oral pathogens and about their safety while used on a daily basis. The objective of this study was to investigate *in vitro* growth-inhibitory effects of ethanol Generally Recognised as Safe (GRAS) plant and supercritical CO_2 hop extracts on planktonic cultures of cariogenic, periodontal and candidal human pathogens, namely *Aggregatibacter actinomycetemcomitans*, *Bifidobacterium dentium*, *B. longum*, *Candida albicans*, *Eikenella corrodens*, *Fusobacterium nucleatum* subsp. *vincentii*, *Lactobacillus salivarius* subsp. *salivarius*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *S. salivarius* subsp. *salivarius* and *S. sobrinus* using the broth microdilution method. The findings showed that ethanol extracts of all 109 GRAS plant species inhibited the growth of at least one microorganism under study with minimum inhibitory concentration (MIC) equal or lower than 4096 μg/mL. However, only six of them possessed very interesting antiseptic potential against the oral pathogens with MIC < 100 μg/mL. Additionally, three GRAS plant species showed good inhibitory activity with MIC = 128 μg/mL. As far as the particular results are concerned, the best antiseptic effect was observed for both *Humulus lupulus* CO_2 supercritical and ethanol extracts that inhibited the growth of all microorganisms at MICs ≥ 8 μg/mL and MICs ≥ 16 μg/mL, respectively. Hence, the CO_2 supercritical extraction proved to be superior for extraction of active constituents of *H. lupulus*. Further, the ethanol extracts of *Capsicum annuum* and *C. frutescens* showed significant antiseptic potential against *S. sobrinus* and *S. salivarius* (MICs 64 – 128μg/mL). The oral streptococci were also inhibited by *Zanthoxylum clava-herculis* (MIC ≥ 64 μg/mL), *Helichrysum angustifolium* and *Myristica fragrans* (MIC ≥ 128 μg/mL) which further showed the antimicrobial activity against *F. nucleatum* (MIC = 64 μg/mL). Interesting inhibitory effects exhibited an extract of *Punica granatum* against *C. albicans* (MIC = 128 μg/mL) and *F. nucleatum* (MIC = 64 μg/mL). Moreover, the growth of *F. nucleatum* was inhibited by extracts of *Pimenta officinalis* and *Thea sinensis*

(MIC = 128μg/mL). The chemical analysis of the CO_2 supercritical *H. lupulus* extracts revealed that *α*- and *β*-bitter acids represented the two major groups of constituents. Cohumulone was the predominant compound of the *α*-acids, whereas colupulone was the prevalent constituent of the *β*-acids. Our results suggest that the six GRAS plant species, namely *C. annuum, C. frutescens, H. lupulus, M. fragrans, P. granatum* and *Z. clava-herculis* have good potential to become new safe antiseptic agents that might be used for incorporation into oral care products such as toothpastes and mouthrinses.

Keywords: dental caries; periodontal disease; candidiasis; microdilution method; inhibitory properties; *Humulus lupulus*; CO_2 supercritical extract

Abstrakt

Onemocnění dutiny ústní způsobené mikrobiální infekcí, například zubní kazy a parodontóza, se řadí mezi nejčastější lidské infekce. Chemické antiseptické přípravky běžně využívané pro jejich léčbu a prevenci jsou však často spojeny s negativními vedlejšími účinky, což omezuje především jejich dlouhodobou aplikaci. Třebaže rostliny považujeme za perspektivní zdroj nových antimikrobiálních látek, víme stále jen málo o jejich inhibičních vlastnostech proti ústním patogenům, stejně tak jako o jejich bezpečnosti z hlediska každodenního používání. Cílem této práce bylo prozkoumat *in vitro* antimikrobiální aktivitu etanolových extraktů rostlin ze seznamu „Všeobecně považované za bezpečné“ (GRAS) a superkritických CO_2 chmelových extraktů proti planktonickým kulturám patogenů způsobujících zubní kaz, parodontózu a kandidózu, jmenovitě proti *Aggregatibacter actinomycetemcomitans*, *Bifidobacterium dentium, B. longum, Candida albicans, Eikenella corrodens, Fusobacterium nucleatum* subsp. *vincentii, Lactobacillus salivarius* subsp. *salivarius, Porphyromonas gingivalis, Streptococcus mutans, S. salivarius* subsp. *salivarius* a *S. sobrinus*, za použití bujónové mikrodiluční metody. Výsledky ukázaly, že všechny etanolové extrakty 109 GRAS rostlinných druhů inhibovaly růst alespoň jednoho z testovaných mikroorganismů na minimální inhibiční koncetraci (MIK) rovné nebo nižší než 4096 μg/mL. Pouze 6 z nich ale vykazovalo velmi zajímavé antiseptické účinky proti ústním patogenům na MIK < 100 μg/mL. Tři další druhy pak prokázaly dobrou inhibiční aktivitu na MIK = 128 μg/mL. Co se týká konkrétních výsledků, nejlepší antiseptický efekt byl pozorován u CO_2 superkritického a etanolového extraktu druhu *Humulus lupulus*, které inhibovaly růst všech mikroorganismů na MIK ≥ 8 μg/mL a MIK ≥ 16 μg/mL. Metoda CO_2 superkritické extrakce tak byla stanovena jako účinnější pro extrakci účinných složek *H. lupulus*. Etanolové extrakty druhů *Capsicum annuum* a *C. frutescens* ukázaly významný antiseptický účinek proti *S. sobrinus* a *S. salivarius* (MIK 64 − 128μg/mL). Orální streptokoky byly také inhibovány *Zanthoxylum clava-herculis* (MIK ≥ 64 μg/mL), *Helichrysum angustifolium* a druhem *Myristica fragrans* (MIK ≥ 128 μg/mL), který dále prokázal antimikrobiální aktivitu vůči *F. nucleatum* (MIK = 64 μg/mL). Zajímavý inhibiční efekt vykázal také extrakt druhu *Punica granatum* proti *C. albicans* (MIK = 128 μg/mL) a *F. nucleatum* (MIK = 64 μg/mL). Růst

F. nucleatum byl dále inhibován extrakty druhů *Pimenta officinalis* a *Thea sinensis* (MIK = 128μg/mL). Chemická analýza CO_2 superkritických *H. lupulus* extraktů ukázala, že *α*- a *β*-hořké kyseliny představují 2 hlavní skupiny látek. Převládající *α*-hořkou kyselinou byl kohumulon, zatímco kolupulon byl hlavní složkou *β*-hořkých kyselin. Naše výsledky prokázaly, že 6 rostlinných druhů GRAS, jmenovitě *C. annuum, C. frutescens, H. lupulus, M. fragrans, P. granatum* a *Z. clava-herculis*, má dobrý potenciál stát se novými bezpečnými antiseptickými prostředky, které by mohly být používány při výrobě přípravků ústní hygieny, jako jsou například zubní pasty a ústní vody.

Klíčová slova: zubní kaz, parodontální onemocnění, kandidóza, mikrodiluční metoda, inhibiční vlastnosti, *Humulus lupulus*; CO_2 superkritický extrakt

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List of abbreviations

ATCC – American Type Culture Collection

CCM – Czech Culture of Microorganisms

DMSO – Dimethyl sulfoxide

DSM – German Resource Centre for Biological Material

FDA – U.S. Food and Drug Administration

GRAS – Generally Recognised as Safe

HPLC – High-Performance Liquid Chromatography

MIC – Minimum Inhibitory Concentration

SFE – Supercritical Fluid Extraction

WHO – World Health Organization

Introduction

Every tooth in a man's head is more valuable than a diamond.

(Miguel de Cervantes, Don Quixote)

Eating, chewing, smiling and communication are basic activities of humans and all of them are linked to mouth and teeth. That is why maintaining healthy teeth, gums and fresh breath is so important, as every oral problem and pain directly influence people's daily lives and well-being. Moreover, the oral health is integral to general health; no one can be healthy without oral health. Unfortunately, despite the amazing progress in the dental medicine, vast majority of world's population has still some experience with oral diseases such as caries and periodontal disease (WHO, 2004). Oral diseases can be thus called "silent epidemic" (U.S. Department of Health and Human Services, 2000) and considered to be one of the major public health problems worldwide often leading to premature tooth loss and affecting significantly the quality of life (Loesche and Grossman, 2001; Quivey, 2006). Even though great effort has been made to reduce the extent and severity of these diseases (Shalala, 2000), it is expected that the global dental caries and periodontal disease incidence will even increase in the near future as a result of growing consumption of sugars and tobacco in developing countries (Petersen *et al*., 2005).

Traditional curative dental care is not only extremely costly, as it consumes up to 10 % of public health expenditure in most industrialized countries (U.S. Department of Health and Human Services, 2000; Widström and Eaton, 2004), but it is often unavailable for the population of developing countries where oral health services are offered only in regional or central hospitals of urban centers (Petersen *et al*., 2005). Furthermore, the treatment of oral bacterial diseases is becoming increasingly difficult due to pathogen resistance or lower susceptibility to the antibiotic drugs usually prescribed by dentists (Al Haroni *et al*., 2008; Sweeney *et al*., 2004). The cure may be also often ineffective because the oral bacterial diseases are polymicrobial (LeBlanc *et al*., 2006) and reinfection by naturally occurring oral microflora is therefore usually inevitable (Quirynen *et al*., 2005).

Last but not the least problem is related to possible side effects of common oral antiseptic agents used for preventive and therapeutic purposes (e.g. chlorhexidine, stannous fluoride, hexetidine). These active antimicrobial compounds have been reported to cause various harms such as dentine hypersensitivity, increased supragingival calculus, taste disturbance or extrinsic dark discolouration of the teeth (Addy, 2008; Eley, 1999; Ellingsen, 1982; Flötra, 1973; Flötra *et al*., 1971, Watts and Addy, 2001).

All of aforementioned disadvantages linked to prevention and curative treatment of oral diseases indicate the need for further research of natural safe antibacterial agents specifically targeting oral pathogenic microorganisms. It is known that plants, which are used since time immemorial for their healing properties, present a good source of novel antimicrobial molecules (Cowan, 1999; Lam, 2007). On the other hand, botanical active compounds are not benign molecules as plants use them generally as chemical defenses against their enemies (Gupta *et al*., 1998; Gurib-Fakim, 2006). Therefore, it would be naïve to believe that these are always safe for human consumption and that herbal remedies are free from any adverse effects. Little is still known about the relative safety of herbal medicines (Ernst, 1998). To overcome this problem, the Food and Drug Administration (FDA), an agency of the U.S. Department of Health and Human Services, has established a new system of food and drug substances which are generally recognized as safe (GRAS) for consumer consumption (Burdock, 2000; Burdock *et al*., 2004; Degnan, 1991; Knudsen and Ovesen, 1995; U.S. FDA, 2014). Various studies evaluating antimicrobial activity of some GRAS plant species or their constituents have previously been published (Hammer *et al*., 1999; Prabuseenivasan *et al*., 2006), but only few have been directly focused on their anti-oral pathogen activity (Anand *et al*., 2008; Bhattacharya *et al*., 2003; Botelho *et al*., 2007; Iauk *et al*., 2003; Tsai *et al*., 2007).

Keeping in mind the verity of the opening quotation and all the above mentioned facts, we decided to focus this study on antimicrobial properties of GRAS plant species against oral pathogenic bacterial and yeast species. The results of the present study may have use in developing new safe herbal-based antiseptic ingredients, compatible with manufacturing requirements for conventional oral hygiene vehicles such as toothpastes, mouthrinses, chewing gum and confectionary products.

1. Literature overview

1.1 World situation of dental health

Microbial diseases of the oral cavity such as caries and periodontitis are among the most frequent infections of humans (Lamont *et al*., 2006; Stingu *et al*., 2012). Even though we live in era of high technologies and great medicinal advances, these dental diseases still remain common and widespread worldwide, affecting nearly everyone at some point in the life span. With our populations aging and living longer lives, oral health management and disease prevention become increasingly important (Avila *et al*., 2009).

Dental caries is a major health problem in most industrialized countries and in some countries of Latin America, where it affects the nearly 100 % of the adult population and 60-90 % of school-aged children (Bratthall, 2011; U.S. Department of Health and Human Services, 2000; WHO, 2004). On the other hand, levels of dental caries experience are much lower in the developing countries of Africa and Asia (WHO, 2012). Nevertheless, these numbers are now expected to grow in these regions of world, mainly due to increasing consumption of sugars and inadequate caries prevention or treatment (Baelum *et al*., 2007; Petersen *et al*., 2005). This is also a probable scenario for lower socioeconomic groups and new immigrants in Europe and the United States whose poor oral hygiene habits and high-sugar diet lead to the increase of the tooth decay incidence in these countries (Bagramian *et al*., 2009).

The situation is quite similar regarding periodontal health, albeit there is not such difference between developed and developing countries of the world. Severe periodontitis, which may result in tooth loss, can be found in 5-20 % of most world adult populations (Albandar *et al*., 1999). Furthermore, most populations in all regions have signs of gingivitis, the initial stage of periodontitis, and slight to moderate forms of periodontitis (Albandar *et al*., 1997; Petersen *et al*., 2005). Significant disparities appear to exist in the level of periodontitis among different race-ethnicity group, as African ethnicity seem to have the highest prevalence of periodontitis, followed by Hispanics and Asians, which indicates the role of genetic factors in the development of periodontitis (Albandar and Rams, 2002). As the prevalence of periodontal disease increases with age (Brown and Loe,

2000) and as more people live longer and retain more teeth, also the number of people with periodontal diseases will increase in the next decades (Baelum *et al*., 2007; Loesche and Grossman, 2001).

1.1.1 Costs of oral diseases

Traditional treatment of oral diseases is extremely expensive as they rank among four most costly diseases to treat (Petersen *et al*., 2005). In most industrialized countries, the curative dental care consumes 5-10 % of public health expenditures (Baelum *et al*., 2007). For example, according to U.S. Department of Health and Human Services (2000), expenditures spent on oral health in the United States in 1998 were $53.8 billion, which is 4.7 percent of the $1.1 trillion spent on all health care in the United States that year. Similarly, the spendings of the European Union on oral health care in the year 2000 were 54 billion € (Widström and Eaton, 2004). In most developing countries, investment in oral health prevention and treatment is negligible as all financial resources are primarily allocated to the emergency oral care. Several previous studies showed that if medical oral cure were commonly available, the costs of the treatment in children alone would greatly exceed the total health care budget (Manji and Sheiham, 1986; Yee and Sheiham, 2002). The situation in developing countries is even more complicated, in view of the fact that oral health services are mostly offered at regional or central hospitals of urban centres, making them almost inaccessible to majority of rural populations. The low-income countries also have to face a shortage of oral health personnel (Baelum *et al*., 2007). For instance, in Africa, the dentist to population ratio is estimated to be 1:150 000 whereas the ratio in most industrialized countries is approximately 1:2000 (Petersen, 2003).

1.1.2 Relationships between oral and general health

It is important to realize that oral microbial diseases do not only represent threat to oral health but that they have also great influence on general health state of human. It has been proved that oral infections and poor oral health can provoke the introduction of oral microorganisms into the bloodstream or the lymphatic system, which can lead up to bacteremia and consequently to metastatic or immunological injury (Destefano *et al*., 1993; Gendron *et al*., 2000; Mattila *et al*., 1989). The oral health is thus integral to the general health (Petersen, 2003) (Fig. 1). Specific oral bacterial species have been implicated in several systematic diseases, such as diabetes, bacterial endocarditis,

aspiration pneumonia, preterm low birth weight and cardiovascular disease (Aas *et al*., 2005). The World Health Organization's International Classification of Diseases and Stomatology currently lists more than 120 specific diseases that have manifestations in the oral cavity (e.g. HIV/AIDS), often in form of severe periodontitis or increased susceptibility to dental caries (WHO, 1992).

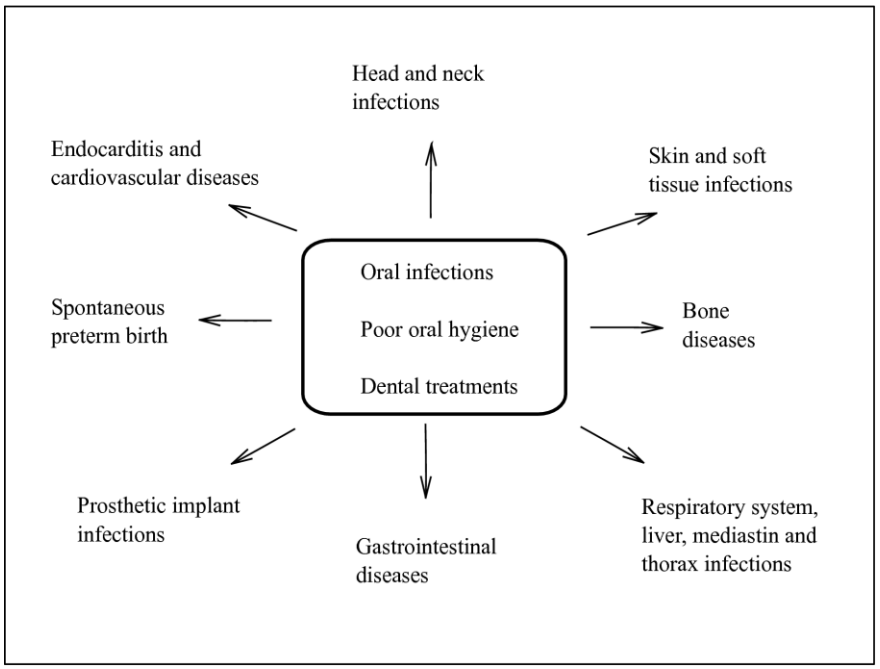


Figure 1 Systemic consequences associated with oral infections, poor oral hygiene and dental treatment (according to Gendron *et al*., 2000)

1.2 Bacterial composition of the oral cavity

The bacterial community in the oral cavity is one of the most complex mixtures of bacteria known (Wade, 2010). At present, mainly thanks to modern molecular methods of microbial detection, it is estimated that over 750 species have the potential to inhabit the human oral cavity (Aas *et al*., 2005; Moore and Moore, 1994; Paster *et al*., 2006; Stingu *et al*., 2012; Wade, 2010). More than a half of those microbes have not been cultivated and new bacterial species are continuing to be discovered (Leys *et al*., 2006; Mager *et al*., 2003; Scannapieco, 2006). The major bacterial taxa found in the mouth are listed in Table 1. More than 100 species are likely to be found in any oral site, although the bacterial

composition varies greatly from person to person, depending on the local environment and the current oral health state (Aas *et al*., 2005; Paster *et al*., 2006).

Table 1 Commonest oral bacterial taxa (according to Scannapieco, 2006)

Spirochetes:	*Treponema denticola, T. maltophilum, T. medium, T. Socranskii*
Fusobacteria:	*Fusobacterium animalis, F. naviforme, F. nucleatum, Leptotrichia buccalis*
Actinobacteria:	*Actinomyces israelii, A. naeslundii, A. odontolyticus, Atopodium, Bifidobacterium dentium, Corynebacterium matruchotii, Propionibacterium propionicus, Rothia denticriosa*
Firmicutes:	Class "Bacilli": *Abiotrophia adiacens, A. defectiva, Gemella haemolysans, Streptococcus anginosus, S. constellatus, S. gordonii, S. intermedius, S. mitis, S. mutans, S. oralis, S. parasanguis, S. salivarius, S. sanguis, S. Sobrinus*
	Class "Mollicutes": *Mycoplasma, Solobacterium moorei*
	Class "Clostridia": *Catonella morbi, Dialister, Eubacterium brachy, E. saburreum, E. saphenum, Megasphaera, Peptostreptococcus anaerobius, P. micros, Selenomonas sputigena , Veillonella dispar, V. Parvula*
Proteobacteria:	*Aggregatibacter actinomycetemcomitans, Campylobacter rectus, C. gracilis, C. concisus, Eikenella corrodens, Haemophilus parainfluenzae, Neisseria mucosa, Pseudomonas aeruginosa*
Bacteroidetes:	*Bacteroides forsythus, Capnocytophaga ochracea, C. gingivalis, Porphyromonas endodontalis, P. gingivalis, Prevotella denticola, P. oris, P. Tannerae*

1.2.1 Uniqueness of oral cavity

The oral cavity represents the entry portal for both the respiratory tract and the gastrointestinal tract, and as such, it is subject to unique ecological constraints (Cole *et al*., 2006; Lamont *et al*., 2006). Significant fluctuations in oral environmental parameters, such as temperature, pH changes and variability in the composition and frequency of exposure to dietary components, as well as the fact that oral tissues are bathed in saliva, which provides physical cleansing by virtue of fluid flow and dilution effects, influence greatly the ecological condition of the mouth (Bowden and Hamilton, 1998; Burne, 1998; Scannapieco, 2006; Sbordone *et al*., 2003). Moreover, the microbial inhabitants of mouth have to cope with host immune and non-immune defence factors that together have also profound consequences to their ability and chance to survive (Lydyard *et al*., 2006).

Therefore, the microorgamisms living in this ecosystem differ from those found anywhere else in the body or, indeed, anywhere else in the world (Lamont *et al*., 2006). The mouth possesses a diverse variety of surfaces, including teeth, the buccal and vestibular mucosa, hard palate, tongue, and the floor of mouth, all of which provide unique habitats for microbial colonization and adhesion (Sbordone *et al*., 2003; Scannapieco, 2006). The growth of bacteria in the mouth occurs as compositionally and structurally complex communities of species adhering to a surface. These types of populations are generally known biofilm or dental plaque (Burne, 2006; Wade, 2010).

1.2.2 Dental plaque formation

Oral biofilm appears to have a highly ordered structure of degradative consortia of organisms that exist there in very close association with one another and that cooperate to make nutrients available and to regulate their overall numbers (Burne, 2006; Paster *et al*., 2006; Rosan and Lamont, 2000; Wade, 2010).

Health-associated bacterial plaque is generally immature and composed of mainly Gram-positive cocci (*Streptococcus* spp.: *S. mutans*, *S. mitis*, *S. sanguis*, *S. oralis*; *Rothia dentocariosa*; *Staphylococcus epidermidis*), followed by some Gram-positive rods and filaments (*Actinomyces* spp.: *A. viscosus*, *A. israelis*, *A. gerencseriae, A. naeslundii*; *Corynebacterium* spp.) and a very few Gram-negative cocci (*Veillonella parvula*, *Neisseria* spp.) (Aas *et al*., 2005; Diaz *et al*., 2006; Li *et al*., 2004; Listgarten, 1976; Nyvad and Kilian, 1987; Sbordone, 2003; Slots 1977). These initial or early plaque colonizers are pioneer species able to colonize tooth enamel by adhering to glycoproteins in salivary pellicle (Scannapieco, 2006). Once the primary colonizers have adhered to the tooth surface, they are followed by secondary plaque formers such as *Fusobacterium nucleatum* that have the capability of co-aggregating with them as well as with a range of other genera and species and thus play a central bridging role in plaque formation (Bradshaw *et al*., 1998; Kolenbrander *et al*., 1989; Wade, 2010).

At the same time as dental plaque is maturing through bacterial interactions and as the number of plaque layers increases, its properties are changing (Paster *et al*. 2006; Aas *et al*., 2005). Nutritional and atmospheric gradients are gradually created, the oxygen level decreases and the obligate anaerobes, such as *Veillonella* spp., *Prevotella* spp. and spirochaetes, are allowed to establish their colonies in lower plaque layers (Bradshaw *et*

al., 1998; Cook *et al*., 1998; Jenkinson *et al*., 2006; Lamont *et al*., 1998; Scannapieco, 2006; Wade, 2010).

The normally benign microbial populations that colonize all the surfaces of the mouth are important contributors to tissue homeostasis, and thus health, in the oral cavity. The oral microbial constituents establish shortly after birth, persist throughout the individual's lifetime, and they are generally compatible with oral health (Burne, 2006). Unless there is significant perturbation in the homeostatic mechanisms of oral biofilm from an adverse environmental stress - such as dietary changes, diminished salivary flow or hormonal changes during puberty or pregnancy - there is little or no obvious damage to the colonized tissues (Gendron *et al*., 2000; Marsh and Percival, 2006; Wade, 2010). Only the ecological imbalance between the components of the resident microbiota and the host immune response results in selective advantage to one microbial group, in other words the microbiota change from health-associated to disease-associated (Cole *et al*., 2006; Jenkinson *et al*., 2006; Scannapieco, 2006).

It has to be mentioned that the human mouth is also host to a diverse collection of viruses, fungi and protozoa (Wade, 2010; Lamont *et al*., 2006), but they are not discussed here as they are not target organisms of this study.

1.3 Dental caries

The incidence of caries in large populations has closely paralleled the industrialization of nations. As wealth has increased, so has sugar consumption that is considered as one of the major causes of oral diseases (Quivey, 2006). Nowadays, dental caries, also known as tooth decay, is, as it has already been mentioned before, one of the most prevalent chronic diseases in the world (U.S. Department of Health and Human Services, 2000). People are susceptible to this disease throughout their lifetime and it is the primary cause of uncomfortable feelings, significant oral pain and premature tooth loss (Anusavice, 2002). Furthermore, the burden of dental caries lasts a lifetime because once the tooth structure is destroyed it usually needs repeated restoration and additional

maintenance (Selwitz *et al*., 2007). A person's risk of caries varies with time and is related to one's lifestyle as oral hygiene and dietary habits play the main roles in disease development (Rodrigues *et al*., 2011; Sheiham, 2001; Touger-Decker and van Loveren, 2003).

1.3.1 Definition of caries

Caries is an oral disease that starts with microbiological shifts within dental biofilm (Marsh, 1994) and is affected by many factors. These factors are mainly salivary flow and composition, exposure to fluoride, diet composition and content of dietary sugars, and preventive behaviour of people (Selwitz *et al*., 2007). Technically speaking, dental caries is localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates (Quivey, 2006).

First, it affects either enamel (the outer covering of the crown) or cementum (the outermost layer of the root), but as decay progresses deeper into a tooth, it affects also dentine, the tissue beneath both enamel and cementum (Kidd *et al*., 2004).

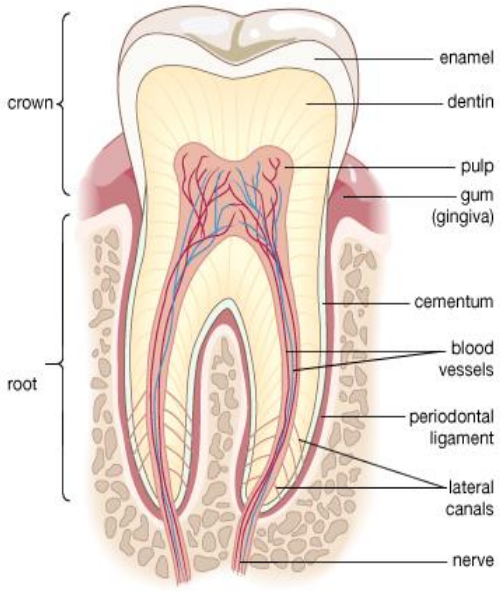


Figure 2 Cross-section of a tooth (according to Scannapieco, 2006)

Depending on the affected tooth part, coronal or root caries of either primary or permanent teeth can be recognized (Rodrigues *et al*., 2011). The term dental caries is used

for all disease states, i.e. for tooth destruction that ranges from sub-surface changes at the molecular level to lesions with dentinal involvement that can be seen both on smooth as well as pitted and fissured tooth surfaces (Featherstone, 2004). Unless proper care and medical treatment is provided caries is not self-limiting and although progresses slowly in most people, may lead up to a complete tooth destruction (Fejerskov *et al*., 2008a).

1.3.2 Pathogenesis

The dental caries results from the ecological imbalance in the physiological equilibrium between oral endogenous microbiota living on tooth surfaces and tooth minerals (Scheie and Peterson, 2004). The mechanisms of the caries development are similar for both coronal and root caries on all types of surfaces (Fejerskov *et al*., 2008b). Endogenous bacteria of dental plaque (e.g. *Streptococcus mutans*, *S. sobrinus*, *Lactobacillus* spp.) produce weak organic acids (such as lactic acid) as a by-product of metabolism of fermentable carbohydrates, in particular of sucrose (Caufield and Griffen, 2000). These acids cause a decrease in local pH values below a critical value (around pH 5.5) resulting in demineralization of tooth tissues, i.e. diffusion of calcium, phosphate and carbonate out of the tooth, and subsequent formation of cavitation (Quivey, 2006). Fortunately, demineralization can be reversed in its early stages through absorption of calcium, phosphate, and fluoride. Fluoride acts here as a catalyst for the diffusion of calcium and phosphate into the tooth, which remineralizes the crystalline structure in the lesion (Selwitz *et al*., 2007). The process of demineralization and remineralization of tooth tissue takes place almost permanently during the day. Whether this process ends up with cavitation within the tooth or repair of the lesion, or maintenance of status quo, depends on many factors of which some are mentioned above (Featherstone, 2004).

Resting plaque pH of healthy subjects is slightly alkaline, approximately around 7.2. In case that the plaque acidification is allowed to continue, the conditions of decreased resting pH values encourage the emergence of aciduric bacteria at the expense of bacteria that are less acidogenic and less acid-tolerant (Burne, 1998). It has been proved that strongly cariogenic species like *S. mutans* and *Lactobacillus casei* can withstand pH values well below pH 4.0, while more weakly cariogenic bacteria such as *A. naeslundii* and *S. sanguis* stop growing at pH 5.0 (Bender, 1986). The constant acidification of oral biofilm

is thus a major cause of ecologic shifts in plaque composition and progress in tooth decay (Quivey, 2006).

1.3.3 Bacteria of dental caries

Regarding the microbial composition of dental caries and its major etiological agents, it is important to repeat that oral microflora is highly specific in every individual and differs from site to site (Aas *et al*., 2005; Paster *et al*., 2006). However, some similarities can be found in bacterial species composition of oral diseases and only a few specific species are believed to cause dental caries (Anand *et al*., 2008). According to Becker *et al*. (2002), the prevalent species associated with early childhood caries are mainly *S. mutans*, other *Streptococcus* spp., *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp., and *Lactobacillus fermentum*.

Diverse bacterial community has been found in dental caries in adults. The initiation of the disease is related with low-pH non-mutans streptococci (e.g. *S. parasanguinis, S. salivarius*), *Prevotella* spp. and *Actinomyces* spp. Where demineralization and caries lesion develops, oral biofilm matures and its composition gradually changes with predominance of acid-tolerant bacteria (Nakajo *et al*., 2010). *Bifidobacterium* spp., *Lactobacillus* spp., *Propionibacterium* spp., S*elenomonas* spp., Streptococcus anginosus, *S. mutans, S. sobrinus* and *Veillonella* spp. are then the most frequently determined species in advanced caries lesions (Aas *et al*., 2005; Chhour *et al*., 2005; Munson *et al*., 2004; Preza *et al*., 2008; Quivey, 2006).

1.3.4 Prevention of caries

A plaque-free tooth does not develop caries; therefore, regular removal of dental biofilm is regarded as a key factor in caries prevention. The main method of home plaque removal is tooth-brushing using a toothbrush and interdental means of cleansing such as dental floss or interdental brushes. According to recent research, brushing the teeth twice a day seems to be sufficient for most individuals, even though the immature plaque requires 8 hours after tooth-brushing to fully re-establish (Ellwood *et al*., 2008). Hence, the tooth-brushing three times a day should be more appropriate (Rodrigues *et al*., 2011).

The methods and means of teeth cleaning and potential side effects related to them are discussed in detail in chapter 1.6.

1.4 Periodontal disease

Periodontal diseases, namely gingivitis and periodontitis, are biofilm-associated inflammatory disorders of periodontium (Lang *et al*., 2009). Accumulation of supragingival plaque and its gradual development from a simple monolayer of Gram-positive coccoid bacteria colonizing the enamel surface and the marginal gingiva (Aas *et al*., 2005) to a complex microbial plaque dominated by Gram-negative anaerobic cocci, filaments and spirochetes seems to be the key factor in development of gingivitis and subsequent progress to periodontitis (Paster *et al*., 2006). Nevertheless, as also other underlying not fully understood risk factors (e.g. gene polymorphism, smoking, endocrine changes associated with puberty, pregnancy and diabetes) appear to affect significantly the outbreak and severity of periodontitis, the mechanism of development, progress and the role of bacteria in it still remain pretty unclear (Albandar and Rams, 2002; Amarasena *et al*., 2002; Tonetti, 1998).

1.4.1 Gingivitis

Plaque-induced gingivitis is the most frequently occurring gingival disease manifested as a reversible inflammation in the marginal periodontal tissues associated with dental plaque (Haake *et al*., 2006). The initial plaque development, that one that stays beyond the inception of gingivitis, starts in the niche created where gingival margin meets the tooth surface. The close proximity of bacteria to crevice epithelium leads to inflammatory response of host. It is a direct result of microbial release of toxins, lipopolysaccharides and enzymes, which triggers the patient's cellular inflammatory systems (Lamont *et al*., 2002). The initial lesion is therefore an acute inflammatory response characterized by vascular changes, collagen degradation, alternation in the appearance of epithelial cells, and infiltration of the tissues by neutrophils (Lang *et al*., 2009). As soon as these inflammation processes pervade the marginal gingiva, the developing gingival lesion matures into chronic gingivitis with massive accumulations of cells and fluid, with the clinical signs of gingival redness, edema, bleeding, changes in tissue contour, loss of tissue adaptation to the teeth, and increased flow of an inflammatory exudate termed gingival crevicular fluid (Haake *et al*., 2006).

Because the symptoms of chronic gingivitis described above are usually rather vague and painless, it leaves most patients unaware of the disease and so the disorder is generally underestimated (Lang *et al*., 2009). The untreated gingivitis has thus the chance to progress into periodontitis, a severe inflammatory disease that leads to eventual loss of the tooth and is much more expensive to cure (Darveau, 2010). Nevertheless, it is noteworthy that not all gingivitis lesions progress to periodontitis. The probability and the factors causing the conversion of gingival lesion to periodontitis have still not been well understood (Lang *et al*., 2009).

1.4.2 Periodontitis

Gingivitis appears to precede the development of periodontitis, as no data indicate that the onset of periodontitis occurs without gingival inflammation (Lang *et al*., 2009). Periodontitis is an inflammation-based infection of the supporting structures of the teeth characterized by progressive destruction of the periodontal ligament and alveolar bone (Baltacioglu *et al*., 2007). Although periodontitis seems to be multifactorially conditioned (genetic, systemic diseases, smoking etc.), the main cause is definitely bacteria living within the dental plaque biofilm (Darveau, 2010).

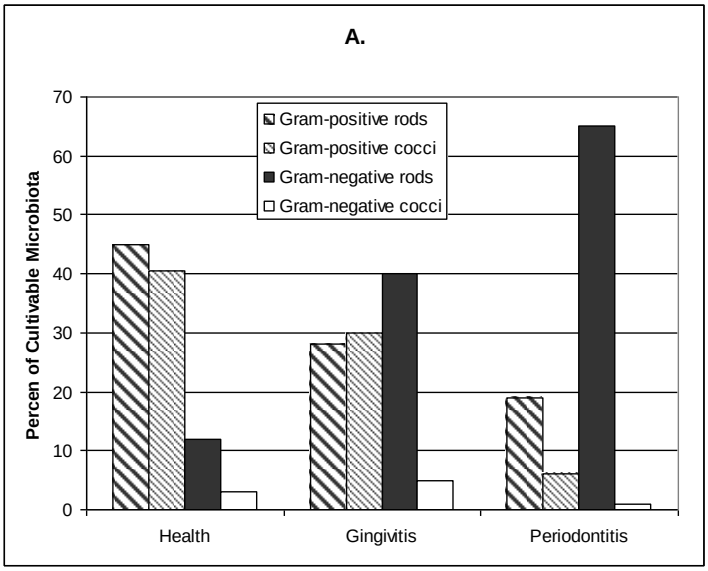
Periodontitis can occur in three distinct forms, the main being aggressive and chronic periodontitis (Page and Komman, 1997a). The third one is periodontitis connected with systemic conditions, such as genetic or hematological disorders (e.g. leukemia) that greatly compromise the host response to bacterial infection (Loesche and Grossman, 2001). Chronic periodontitis is the most common form of periodontitis, found prevalently in adult population. It progresses slowly in tissue destruction and the severity of disease is consistent with local factors of plaque (Concolino *et al*., 2007). Aggressive periodontitis can be easily distinguished from chronic one as the severity of disease is not consistent with occurrence of the local factors of plaque and the progression of tissue destruction is fast (Page *et al*., 1997b). There is often a hereditary occurrence of the illness, which is considered as evidence of the genetically conditioned disease susceptibility (Haake *et al*., 2006).

The clinical hallmark of periodontitis and the most alarming symptom is the destruction of the connective tissue attachment to the root surface and resorption of alveolar bone, with accompanying migration of the epithelial attachment toward the apex

of the tooth. The affected gingival tissue is predominated by plasma cells; moreover, there is further loss of connective tissue elements and osteoclastic bone loss, which can ends up even with teeth loss (Baltacioglu *et al*., 2007; Loesche and Grossman, 2001). Periodontitis is, as well as gingivitis, considered to be a "silent" disease because pain is late manifestation of its progress and it is not generally observed until significant loss of an alveolar bone and connective tissue attachment has occurred (Jeffcoat, 1994).

1.4.3 Bacteria of periodontal diseases

Albeit hundreds of bacterial species have been identified in dental plaque (Moore and Moore, 1994; Paster *et al*., 2006), destructive periodontal diseases are associated with a relatively small number of bacteria. Bacterial pathogens being suspected to cause the diseases are predominantly Gram-negative anaerobic species. These species are also found in healthy subjects, but in incomparably lower levels that in disease states (Fig. 3) (Haake *et al*., 2006).



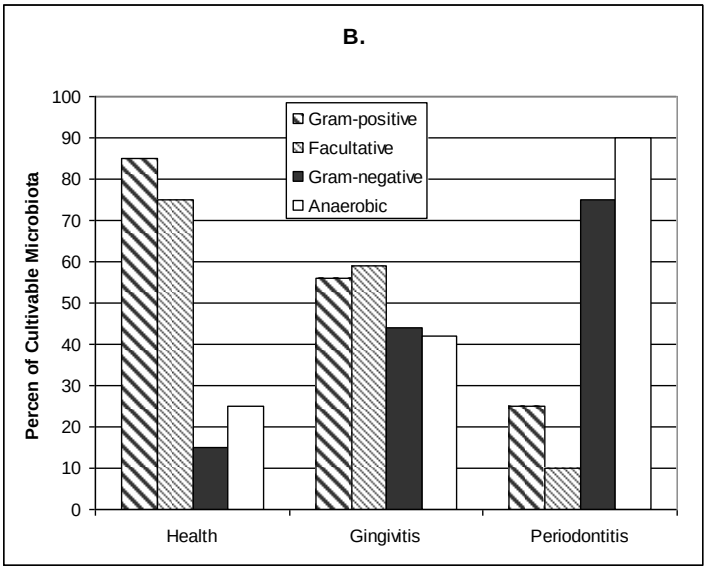


Figure 3 Cultivable subgingival bacteria in periodontal health and disease: A. Analysis of bacteria by morphological groupings demonstrate increases in Gram-negative rod-shaped bacteria in gingivitis and periodontitis as compared to health; B. Gram-positive facultative species predominate in health sites while Gram-negative anaerobic species predominate in periodontitis sites (according to Haake *et al*., 2006)

Only when the plaque-host equilibrium is disturbed (e.g. through pH changes), a shift in the proportions of resident microorganisms occurs and the Gram-negative anaerobic species exceed in number the commensal plaque bacteria (Jenkinson *et al*., 2006).

It is important to say that despite considerable research focusing on identifying periodontal pathogens, many of bacterial strains and clones are still identified and the knowledge about the complexity of pathogenesis of periodontal diseases appears to be still incomplete (Jenkinson and Lamont, 2005). Key reasons for this include the inability to culture microorganisms related with the diseases through common cultivation technique, difficulties in identifying and culturing sites with actively progressing disease and the presence of suspected pathogens in healthy individuals (Haake *et al*., 2006; Paster *et al*., 2006). In spite of these culturing and identifying constrains, the major bacteria that are to blame for causing the periodontal diseases have been recognized. According to literature (Haake *et al*., 2006; Moore and Moore, 1994; Stingu *et al*., 2012), two periodontal microorganisms are considered as likely primary pathogens in periodontitis:

Aggregatibacter actinomycetemcomitans (in aggressive periodontitis) and *Porphyromonas gingivalis* (in chronic periodontitis). Besides these species, several others have been implicated as co-pathogens in periodontal diseases, such as *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens* and oral spirochetes (e.g. *Treponema denticola*, *T. socranskii*) (Colombo *et al*., 2006; Dorn *et al*., 1998; Han *et al*., 2000; Peters *et al*., 1999).

The major bacteria of periodontal disorders are sometimes ranged into "red" and "orange" bacterial groups (two of five major groups which have been defined in human dental biofilm, each consisting of bacterial species that are found in association with one another) (Fig. 4).

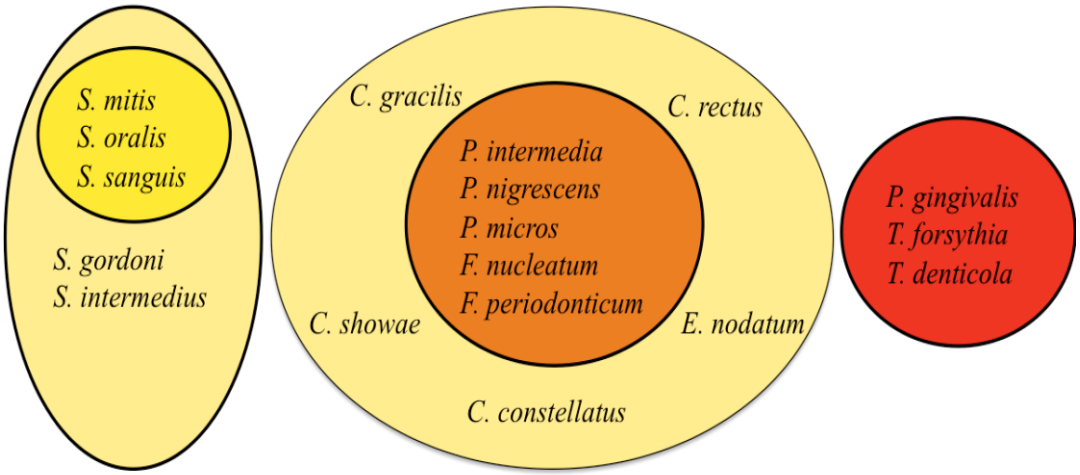


Figure 4 Microbial complexes detected in dental plaque (according to Haake *et al*., 2006)

The red complex includes the key pathogens regarding chronic periodontitis (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*), while orange complex of microorganisms contains less pathogenic organisms (such as *Prevotella intermedia*, *P. nigrescens* and *Fusobacterium nucleatum*) which are nevertheless still considered to assist in causing periodontal diseases as mentioned above (Socransky *et al*., 1998).

1.4.4 Prevention and cure of periodontal diseases

As the periodontal diseases are primarily induced by bacterial dental plaque, the damage to the gingival tissues due to gingivitis and periodontitis can be substantially reversed by removal of dental plaque bacteria from the tooth surface (Haake *et al*., 2006). Adoption of better oral hygiene (such as tooth brushing and flossing) has therefore notable impact on the occurrence and progress of periodontal diseases (West and Moran, 2008). Smoking cessation can also greatly decrease the risk and the severity of periodontitis and thus increase the tooth retention and longer life expectancy (Albandar and Rams, 2002; Amarasena *et al*., 2002).

Once the periodontal disease occurs, the treatment is quite demanding. In order to reduce pathogenic bacteria and related plaque, traditional debridement procedures, i.e. scaling and root planning, are implemented (Berezow *et al*., 2011; Darveau, 2010). However, the effectiveness of these treatments is arguable, as after initial reduction in the bacterial numbers, the measure of pathogens return shortly to predebridement levels and the inflammatory process begins anew. It is mainly because of the deep periodontal pockets which are not fully accessible and where pathogens can still survive in spite of treatment (Loesche and Grossman, 2001).

Antibiotic agents are often prescribed by dentists to accomplish the eradication of periodontal pathogens from patient's mouth. Unfortunately, antibiotics usually yield only modest results (Herrera *et al*., 2008). It is because, as have been already said before, periodontal diseases are biofilm-associated ones (Aas *et al*., 2005), and biofilms are notoriously difficult to treat with antibiotics (Berezow *et al*., 2011). One reason is that the identity of many oral bacteria which may contribute to these disorders is still unknown and those would be unaffected by the drug treatment (Haake *et al*., 2006). Furthermore, different species have different antimicrobial susceptibility, which makes of the antimicrobial therapy a demanding challenge (Darveau, 2010). The main drawback relating to patient is that the antibiotics are often used in combinations to affect more bacterial species; but taking multiple antibiotics simultaneously has often severe systemic adverse effects (Haffajee *et al*., 2003). Moreover, it may leave a patient susceptible to developing an oral yeast infection or provoke a variety of allergy reactions (Berezow *et al*., 2011; Trombelli and Tatakis, 2003).

1.5 Less prevalent oral diseases

1.5.1 Endodontic infection

The dental pulp tissue (undifferentiated connective tissue) is located within the dentin chamber (see Fig. 2) and extends into the root canal. Under normal conditions, intact enamel and dentin provide protection to the pulp and act as a physical barrier to microbial penetration; enclosed vital pulp is thus a sterile tissue (Rosan and Rossman, 2006). However, when the dentine and/or enamel is damaged, usually as a consequence of extension of caries, periodontal disease or traumatic injury, bacteria normally living in dental plaque can intrude into the pulp tissue and produce the infection (Siqueira *et al*., 2007). This pulpal inflammatory diseases are sometimes called "endogenous opportunistic infections" as bacteria causing it are considered to be endogenous oral microbiota and their virulence is usually only expressed when the opportunity occurs (Rosan and Rossman, 2006).

The composition of bacterial species isolated from root canal infection is straight dependent to the source of infection (Chugal *et al*., 2003). Invasion of the pulp as a result of caries is probably one of the most common routes of infection. Studies indicate in these cases the predominance of cariogenic bacteria such as streptococci and lactobacilli. Mechanical exposures of pulp through trauma or restorative dental procedures seem to be another major cause of infection. It may leads to pulpal inflammation by endogenous commensal oral bacteria leading up to pulpal necrosis (Rocas *et al*., 2001). In patients with periodontal disease, plaque bacteria gain the access to the pulp tissue via canals on the lateral surfaces of the tooth (Siqueira *et al*., 2007). The infection results again in inflammation that usually ends up with tooth necrosis (Rocas *et al*., 2001). The prevalent species from root canal infections, no matter the route of infection, are listed in Table 2.

Unless appropriately treated, the necrotic tissue in canals remains infected and itself becomes the reservoir of the pathogenic agents (Lin *et al*., 1992). Untreated infection can hence cause a chronic inflammation, which is accompanied by excruciating pain and leads sometimes up to tooth extraction (Rosan and Rossman., 2006). However, the endodontic treatment is rather complicated because it consists of complete disinfection of the infected

pulp space, which is difficult to reach, accompanied by locally administrated antibiotics (Lin *et al*., 1992). The success of the treatment is also highly conditioned by root canal anatomy and their accessibility for filling (Chugal *et al*., 2003). The failure of the treatment inevitably results in tooth extraction (Sjögren *et al*., 1990).

Table 2 Prevalent bacterial genus isolated from root canal infection (according to Rosan *et al*., 2006)

Genus or group	**Frequency of isolation (%)**
Bacteroides	70
Prevotella	60
Lactobacillus	51
Oral streptococci	41
Clostridium	36
Fusobacterium	33
Propionibacterium	29
Peptostreptococcus	25
Corynebacterium	25
Bifidobacterium	21
Eubacteria	20
Capnocytophaga	17
Actinomycetes	16
Leuconostoc	13
Porphyromonas	10
Candida	10

1.5.2 Halitosis

Halitosis, also known as bad breath, is an unpleasant problem which causes not only discomfort but also many embarrassing situations and a severe social handicap to people who suffer from it (Van den Broek *et al*., 2007). Nevertheless, the number of affected people is not negligible; the statistics state that up to 30 % of the world population suffers from it regularly, while most adults face up to it occasionally (Liu *et al*., 2006; Miyazaki *et al*., 1995). Hundreds of millions of American dollars are spent worldwide every year to combat this disorder (Attia and Marshall, 1982).

Halitosis, as modern science has it, is obvious malodour with intensity beyond socially acceptable level. It is a disorder with a various sources of origin (food/beverages,

smoking, disorders of respiratory or gastrointestinal tract, systemic diseases), but in a majority of cases the source of this breath malodour is the oral cavity (van den Broek *et al*., 2007). It is produced through the putrefactive action of microorganisms on organic substrates, such as glucose, peptides, and proteins present in saliva, crevicular fluid, and retained debris (Kazor *et al*., 2003; Salako and Philip, 2011). While microbial degradation may occur in everyone's mouth, the process is accentuated when degenerative and inflammatory disorders are present. Poor oral hygiene, extensive caries and periodontal diseases are usually the main causes of severe halitosis (Attia and Marshall, 1982; Lu, 1982). Proteins containing sulphurous amino acids like cysteine and methionine are causative substrates. These amino acids are degraded by bacteria partly into volatile sulphur-containing compounds (e.g. hydrogen sulphide, methyl mercaptan, and dimethyl sulphide), which are the major components of halitosis (Roldan *et al*., 2003; Salako and Philip, 2011; Van den Broek *et al*., 2007). In the absence of bacteria, the odoriferous substances are not generated (McNamara *et al*., 1972).

No single type of microorganism is responsible for the oral malodour. Organisms responsible for the production of volatile sulphur-containing compounds include mainly proteolytic obligate anaerobes, especially the Gram-negative species (Roldan *et al*., 2003). These bacterial species include *Aggregatibacter actinomycetemcomitans*, *Actinomyces* spp., *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella* spp., *Tannerella forsythia* and *Veillonella* spp. (Table 3; Kazor *et al*., 2003; Loesche and Kazor, 2002; Van den Broek *et al*., 2007). They have been isolated predominantly from periodontal pockets, and from dorso-posterior region of the tongue and subgingival plaque of periodontitis free patients (Bosy *et al*., 1994; Yaegaki and Coil, 2000). The species diversity found in halitosis samples suggests that this disorder is probably the result of complex interactions between several bacterial species. In addition, the role of uncultivable bacteria may be important in contributing to this process (Van den Broek *et al*., 2007).

Table 3 Most active producers of volatile sulphur compounds (according to Loesche and Kazor, 2002)

H_2S from cysteine:	*Peptostreptococcus anaerobius*	*Bacteroides*spp.	*Selenomonas artermidis*
CH_3SH from methionine:	*Fusobacterium* spp.	*Bacteroides* spp.	*Eubacterium* spp.
H_2S from serum:	*Prevotella* spp.	*Porphyromonas gingivalis*	*Treponema denticola*
CH_3SH from serum:	*Treponema denticola*	*Porphyromonas gingivalis*	*Porphyromonas endodontalis*

Oral halitosis, when it is not secondary to a specific disease (such as leukemia or hepatic failure), can usually be overcome with proper dental and periodontal care and appropriate oral hygiene, which includes brushing of the teeth and tongue, so that bacteria and products of their metabolisms are removed (Attia and Marshall, 1982; Tonzetich *et al*., 1976). The debridement that should be practiced at least twice a day and is usually combined with the use of antimicrobial mouth rinses based on chlorhexidine, essential oils and triclosan (Kazor *et al*., 2003; Van den Broek *et al*., 2007).

1.5.3 Candidiasis

Oral candidiasis is the most prevalent human fungal disease (Cannon and Firth, 2006). It is an opportunistic infection of the oral cavity caused by an overgrowth or infection of *Candida* species. The commonest is *C. albicans*, followed by *C. tropicalis*, *C. glabrata*, *C. pseudotropicalis* and *C. krusei* (Akpan and Morgan, 2002).

C. albicans is a normal commensal of the mouth (found predominantly on the tongue, palate and buccal mucosa) and generally causes no problems in healthy people. In the general population, oral carriage rate for this species is high, with nearly one half of the healthy population harbouring it (Lynch, 1994). The incidence of *C. albicans* found in oral cavity depends significantly on age, the highest being in infants (up to 65 %) and elderly

people, especially those wearing dentures (also up to 65 %) (Akpan and Morgan, 2002; Abu-Elteen and Abu-Elteen, 1998).

As indicated previously, *C. albicans* lives in harmony with other members of the oral microbial flora and is considered to be an opportunistic pathogen. There is a variety of factors, however, that can alter this oral microbial equilibrium and induce a candidal infection (Lynch, 1994). The predisposing factors are mainly impaired salivary gland function, administration of drugs (antibiotics, corticosteroids etc.), denture wearing, carbohydrate-rich diet, vitamin deficiencies, and smoking (Lucas, 1993). Oral candidiasis can be also a syndrome of systemic disease (e.g. diabetes mellitus, malignancies), and is common among immunocompromised patients (Dupont *et al*., 1992; Cannon and Firth, 2006).

Regardless of its cause, oral candidiasis can be manifested through a variety of clinical forms (Lynch, 1994), with white plaque (removable or fixed) and redness of tissue being the most common ones (Cannon and Firth, 2006). The most prevalent problems of patients suffering from oral candidiasis are discomfort, an altered taste sensation and slow recovery; oral candidiasis is usually not related with pain. On the other hand, when it spreads through the bloodstream or upper gastrointestinal tract, it can lead to severe infections with significant mortality rate of up to 79 % (Fraser *et al*., 1992).

Proper oral hygiene and periodic oral examination is usually adequate for preventing oral candidiasis in healthy or recovered people (Akpan and Morgan, 2002). For treating uncomplicated oral candidiasis, the use of topical antifungals is recommended; chlorhexidine, nystatin and amphotericin in form of oral rinse, pastille, and suspension are the most widely used for the treatment (Cannon and Firth, 2006). Where systemic antifungal therapy is appropriate (e.g. at high risk of developing systemic infections), fluconazole or itraconazole are the commonest choice of dentists (Akpan and Morgan, 2002). However, several constraints are related to the treatment. Not only potential side effects of such antifungal agents (vomiting, discolouration of dentures and natural dentition, diarrhea) (Gupta *et al*., 1994), but also increasing resistance of *C. albicans* to antifungals have to be taken into account (Rex *et al*., 1995). For example, the resistant *Candida* spp. to triazole antifungals (e.g.) fluconazole has been frequently reported in

recent years, mainly as a consequence of long-term treatment of candidiasis with those medications (Cannon and Firth, 2006).

1.6 Oral diseases prevention and treatment

It is obvious that prevention of oral diseases is easier than cure. As all oral bacterial diseases are related to dental biofilm (Avila *et al*., 2009), proper control and remove of the dental plaque seems to be a precondition to maintain all benefits of oral health throughout one's life (Allaker and Douglas, 2009). These benefits include not only functional dentition and aesthetic values such as appearance and freshness of breath, but also reduced risk of any severe complications related to oral diseases (e.g. bacteremia) and lower expenditures spent on oral treatment and restorative interventions (Claydon, 2008). Although there is a large range of oral hygiene products, all fall within one of a small number of categories (such as toothbrushes, mouth rinses and dentifrices) and can be classified either as mechanical or chemical means of dental plaque removers (Addy, 2008).

1.6.1 Mechanical means of oral hygiene

In general, tooth brushing with manual or electric toothbrush combined with interdental cleaning is arguably the most common oral hygiene habit in developed countries (Addy, 2008). These self-care methods provide optimal prevention of caries and periodontal diseases if performed properly for an adequate duration of time (Fischman, 1997). However, despite their widespread use, the majority of the population does not clean the teeth thoroughly (Nyvad, 2008). The prevalent technique of tooth brushing used by uninstructed individuals is a horizontal scrubbing motion that fails to prevent plaque accumulation and its maturation on all tooth surfaces (Claydon, 2008). Since tooth brushing alone is considered to be capable of cleaning only the flat surfaces of the teeth and leaves the proximal and interdental areas almost untouched (Löe, 2000), interdental cleaning by interdental brushes or floss is essential within the daily oral hygiene programme for maximum plaque removal (West and Moran, 2008). However, since interdental hygiene is technically demanding and time consuming, it is performed on a daily basis only by approximately 10 % of the population (Petersilka *et al*., 2002).

Finally, it should not be omitted to mention that a large proportion of the world's population (primarily in developing countries) uses chewing sticks for regular removal of dental plaque and food deposits instead of toothbrushes. These chewing sticks are prepared from twigs, stems or roots from a variety of plant species (Wu *et al*., 2001; particular species are listed in chapter 1.7.1) and their cleaning potential appears to be comparable with tooth brushing with toothpaste (Norton *et al*., 1989).

Despite well recognized positive effects of mechanical oral hygiene products, they may have the potential for producing harm to consumers, some of which are more serious and long-lasting than others (West and Moran, 2008). Tooth brushing with toothbrush alone appears to have little or no effect on intact enamel and dentine (Addy and Hunter, 2003). However, in patients with enamel softened by acid erosion, tooth brushing can cause its abrasion and worsening of dentine hypersensitivity, a problem affecting about 15 % of the population worldwide (Addy and Hunter, 2003; West and Moran, 2008). As tooth brushing alone leads to tooth staining, which develops in short time period of this practice, it is thus recommended to combine it with the use of toothpaste (Richards, 2009). Nevertheless, this combination can have an abrasive effect on dentine and/or enamel and cause the teeth hypersensitivity especially when tooth brushing follows an erosive challenge (such as consumption of acidic beverages) (Addy and Hunter, 2003). The side effects of chewing sticks have not been reported yet, but it is considered that they would cause similar and limited wear, as do toothbrushes, on enamel and dentine (Addy, 2008).

The effects of toothbrushes on gingival tissues are less well documented. However, there is little doubt that, during any brushing cycle, some scratching of the gingival tissues occurs (Jepsen, 1998). Count of such incidents seems to be related to the stiffness of a toothbrush; the harder the brush the more excoriations and even evidence of gingiva recession can be observed (Knocht *et al*., 1993). Moreover, abusive tooth brushing can lead up to permanent gingival recession at one or more sites; but such abusive tooth brushing is uncommon and is usually related with some psychological disorder (Addy, 2008). Damage to the gums from interdental cleaning devices is occasionally reported and it is usually associated with improper flossing technique (Gow and Kelleher, 2003). There is also a possibility that some interdental cleaning devices break during use and become lodged between the teeth; the majority of such incidences are related to interdental brushes

and wood sticks. In conclusion, it must be emphasized that, in spite of all the negative effects linked to mechanical plaque removal, the benefits of tooth brushing and interdental cleaning alone on gingival health far outweigh any downside in respect of tooth wear and gingival recession (Addy, 2008).

1.6.2 Chemical means of oral hygiene

Oral care by using chemical agents involves control of plaque via removal of existing plaque or preventing the formation of new plaque by oral topical administration of antiseptic compounds (Munro and Grap, 2004). Furthermore, these chemicals incorporated in toothpastes and mouthrinses facilitate also the mechanical removal process and have usually other value added such as breathe freshening (Addy, 2008; Forward *et al*., 1997). There are a number of dentifrices and mouthrinses available commercially on the market today; however, they contain only a few antimicrobial agents that proved to satisfy several strict criteria for such oral anti-plaque ingredients (Eley, 1999). The oral antimicrobial agents have to be safe for consumers, possess clinical efficacy against oral pathogens while preserving microbial homeostasis in the mouth, have to be easily formulated into dental products and the long-term use should not lead to the development of microbial resistance (Marsh, 1992). Currently used oral antimicrobial agents include bisbiguanides (e.g. chlorhexidine), quaternary ammonium compounds (e.g. cetylpyridinium chloride, hexetidine), metal salts (e.g. zinc, stannous, copper) and phenols (e.g. triclosan) (Eley, 1999) (Table 4). Although many of these agents exhibit good antimicrobial activity in the laboratory, they display variable results in clinical practice of controlling oral plaque (Marsh, 1992). This is mainly due to the short time period (being approximately from seconds up to a few minutes) when the active ingredient is available for the antimicrobial action (Addy, 2008). After this time, a large proportion of the agent is lost by spitting out and swallowing. Subsequent effects are then dependent on the concentration of the agent retained on oral surfaces (Marsh, 1992).

Table 4 Classes of main antimicrobial agents used for plaque control (according to Marsh, 1992)

Class of agent	Example	Main Delivery Vehicle
Bisbiguanides	Chlorhexidine	Mouthwash
Quaternary ammonium compounds	Cetylpyridinium chloride	Mouthwash
	Hexetidine	Mouthwash
Metal salts	Zinc	Mouthwash, toothpaste
	Copper	Mouthwash, toothpaste
	Tin	Mouthwash, toothpaste
Phenols	Triclosan	Mouthwash, toothpaste

A number of bisbiguanides have anti-plaque properties and kill a wide range of microorganisms by damaging the cell wall, but chlorhexidine has been shown to be the most effective agent *in vivo* with a broad spectrum of antimicrobial activity against relevant oral Gram-positive and Gram-negative bacteria (Hennessy, 1973). It has proved to reduce significantly dental plaque (Gjermo *et al*., 1973; Hoffmann *et al*., 2001; Jenkins *et al*., 1994; Löe and Schiott, 1970) and thus consequently caries and gingivitis in humans on concentrations between 0.1-0.2 % (Charles *et al*., 2004). It is hence considered the gold standard among oral anti-plaque agents (Jones, 1997; Moran, 2008; West and Moran, 2008). The great advantage over the other active compounds is its ability to adsorb to oral surfaces and be retained there for extended period of time (Mohammadi and Abbott, 2009). On the other hand, chlorhexidine is notorious for its unpleasant taste (Eley, 1999) and adverse side effects, which prevent it from being used constantly for a long time (Moran, 2008). These side effects include chiefly altered taste sensation (Autio-Gold, 2008), extrinsic brown staining on the teeth and tongue (Watts and Addy, 2001) and increased supragingival calculus (Charles *et al*., 2004), which discourages many patients from using any products containing chlorhexidine (Sanz *et al*., 1994; Yates *et al*., 1993). Therefore, chlorhexidine preparations are used mainly for short-term plaque control (up to 2 weeks) when mechanical oral hygiene procedures are difficult, e.g. in the post-operative period after periodontal surgery (Charles *et al*., 2004). Another drawback related with its production is the limited option of delivery as chlorhexidine is generally incompatible with conventional dentifrice formulations (Eley, 1999) and can be only incorporated into mouthrinses (Marsh, 1992). Other bisbiguanides with anti-plaque activity include alexidine and octenidine which are, however, not widely used as ingredients of dental products.

Quaternary ammonium compounds used in formulating beneficial oral antimicrobial products are mainly cetylpyridinium chloride and hexetidine (Marsh, 1992). Cetylpyridinium chloride possesses equivalent antimicrobial activity as chlorhexidine in laboratory tests, but produces little anti-plaque benefit in humans, as it is inactivated when adsorbed to surfaces (Moran and Addy, 1984). On the other hand, hexetidine is retained in the mouth much longer than cetylpyridinium chloride, but it has only moderate anti-plaque and antimicrobial activity and little effect on gingivitis (Roberts *et al*., 1981). The antibacterial activity of hexetidine has been enhanced by combining it with metal ions such as zinc or copper (Scheie, 1989). The adverse effect limiting the use of quaternary ammonium compounds is tooth staining when used for prolonged period of time (Eley, 1999). Moreover, hexetidine at concentrations greater than 0.1 % can cause oral ulceration (Bergenholz and Hanstrom, 1974).

Several ions of metal salts are also reported to have some anti-plaque and antimicrobial activity, especially zinc, copper and tin (Addy, 1990; Eley, 1999). They have been incorporated into a number of dental products, and sometimes, especially zinc, are used in combination with a number of other antimicrobial agents (such as hexetidine, triclosan, and sanguinarine) in dental products (Scheie, 1989). Additionally, zinc has been reported to be retained by dental plaque and inhibits its regrowth without disrupting the oral ecology. On the contrary, both copper and tin suffer from the local side effect of staining and are not used very often in dental dentifrices and mouthrinses (Eley, 1999; Jackson, 1997).

Phenols are broad-spectrum antimicrobial agents, and triclosan (2,4,4’ trichloro-2’-hydroxydiphenyl ether) is their major representative in oral hygiene products. It has been found to have only moderate anti-plaque properties (Jenkins *et al*., 1994) but it does not cause any side effects (Saxton, 1986). The main drawback is its poor substantivity in the mouth tissues and hence limited action time. Therefore, triclosan is often incorporated into dentifrices by manufacturers for reducing plaque formation and preventing gingivitis in combination with a co-polymer, which increases its oral retention (Marsh, 1992).

It is surprising that more than three decades chlorhexidine remains the best anti-plaque agent regardless its severe side effects (Jones, 1997) and that after all these years, equivalent or superior efficacy for alternative chemical products has not been found yet.

Taking this into consideration together with all the pros and cons of the common means of oral hygiene mentioned above and given the fact that people are increasingly aware of possible side effects of the antimicrobial agents used in oral cleaning products (Moran, 2008), the development and use of new safe herbal-derived antimicrobial additives deserves more attention (Alviano *et al*., 2008; Hammer *et al*., 1999). These new active agents should have improved anti-plaque and antimicrobial properties, be safe for consumers and preserve oral microbial equilibrium (Cummins, 1997; Marsh, 1992).

1.7 Plants with antimicrobial activity against oral pathogens

Medicinal preparations derived from natural sources, particularly from plants, have been widely used since the time immemorial (Raskin *et al*., 2002). Archaeological finds from many prehistoric excavation sites as well as ancient texts from India and China provide us with exhaustive evidence of the use of a variety of plant-derived medications (Cowan, 1999; Jones, 1996). Up to now, traditional medicine based on plant remedies remains the main source of alleviation illnesses for a large proportion of the world population (Harvey, 2000), particularly in the developing world (Ahmad *et al*., 2006).

Plants present a unique pool of novel antimicrobial molecules due to the variety and chemical complexity of their constituents (Turchetti *et al*., 2005). However, it is estimated that less than 10 % of such molecules have been isolated and even fewer substances have been tested for their biological activity (Harvey, 2000). Therefore, plants still offer a great possibility for novel phytochemicals and their research represents an important challenge for scientists in 21st century (Cowan, 1999).

1.7.1 Traditional use of plants against oral pathogens

Oral diseases such as dental caries and periodontal disorders have accompanied human race since the dawn of its existence (Jackson, 1997). It is thus not surprising that people always in history sought relief through many ways of which application of herbal therapy proved to be the most reliable and effective one (Fischman, 1997). In dentistry, phytomedicine has been always used by humans as anti-inflammatory, antimicrobial, analgesic, sedative agent (Groppo *et al*., 2008) and has been ever before closely associated with oral hygiene and dental therapeutic practices (Kumar *et al*., 2009; More *et al*., 2008).

They have been predominantly used for topical treatment and not for systemic consumption, even though some of the preparations have been taken in the form of infusions (Rao *et al*., 2012).

The evidence of use of plants for curing oral diseases can be found in several ancient texts. For instance, Bible mentions usage of mastic, plant resin from *Pistacia lentiscus*, for freshening of breath and tooth cleaning, whereas Talmud suggests the use of peppercorns (*Piper nigrum*) for the same reason. Not only resin was harvested for oral cure from mastic tree (Sterer, 2006), but it also provided effective toothpicks used even already by Ancient Greek and Romans (Fischman, 1997). Folk remedies for bad breath and oral disorders abound and in general, many of them are still in use in natural healing (Rao *et al*., 2012). These folk cures include for example cinnamon (*Cinnamomum* spp.), parsley (*Petroselinum hortense*), mint (*Mentha* spp.), clove (*Syzygium aromaticum*), and guava peels (*Psidium* spp.) for curing offensive mouth odour. Folk medicines describe various recipes for mouth rinsing as an adjunct to mechanical cleansing (Kumar *et al*., 2009). These include for example mixture of honey, olive oil (*Olea europea*) and beer or a combination of dill (*Anethum graveolens*), anise seed (*Pimpinella anisum*), myrrh (oleoresin from genus *Commiphora*), and pure white wine. Rinsing the mouth with vinegar and rose water has been widely popular in many cultures and remains still a custom in Arabic world (Fischman, 1997).

Dentifrices have been utilized throughout the ages as well. A mixture of pounded rose leaves (*Rosa* spp.), plant galls (outgrowth of plant tissues caused by various parasites) and myrrh or barley flour (*Hordeum vulgare*) mixed into a paste with vinegar and honey has been advised for maintaining healthy teeth and removing stains from the teeth (Fischman, 1997). For the same reason, powdered parts of sweet flag (*Acorus calamus*) and yellow dock (*Rumex crispus*) have been used in Europe and North America. Similarly, the powdered bark of *Cinchona officinalis* is still used in South East Asia and Americas. Ash of burned branches of vinegrape (*Vitis vinifera*) was utilized as dentifrice in England (Muhammad and Lawal, 2010), whereas skin of a sun dried horse radish (*Armoracia rusticana*) might have been added to the dentifrice as well (Fischman, 1997).

For treating and alleviation of periodontal diseases, propolis (resinous mixture produced by bees), clove oil, rosemary (*Rosmarinus officinalis*), flowers of tamarisk

(*Tamarix* spp.), coriander (*Coriandrum sativum*), mauve seed (*Abutilon hulseanum*), green tea infusion (*Camellia sinensis*), betel (*Piper betle*) and bamboo concretion (species from tribe *Bambuseae*) have been recommended in Mediterranean and Asian local medicines (Bissa *et al*., 2007; Fischman, 1997; Kumar *et al*., 2009; Palombo, 2011). The use of chamomile (*Matricaria chamomilla*), calendula (*Calendula officinalis*), turmeric powder (*Curcuma longa*), liquorice root (*Glycyrrhiza glabra*), or grapefruit seeds (*Citrus* x *paradisi*) can be also found in texts of folk medicines for elimination of inflammation and infection associated with periodontal diseases (Elvin-Lewis, 1980; Palombo, 2011). *Aloe vera* gel is reported to be used as a traditional way how to sooth gum tissue and relieve pain and discomfort associated with gum diseases (Kumar *et al*., 2009).

It is important to say that there is a large number of plant species suggested for prevention and treatment of oral infective disorders in every folk medicine in the world and abovementioned species are just illustrating examples. Many of these plants have shown lower toxic potential and good pharmacological activity but there are just a few studies investigating these plant species directly against oral microorganisms (Botelho *et al*., 2007; Cai and Wu, 1996; Feres *et al*., 2005; Groppo *et al*., 2008; Iauk *et al*., 2003; Otake *et al*., 1991; Razak and Rahim, 2003; Sterer, 2006; Tichy and Novak, 1998).

Naturally, also the systems of old traditional medicines such as Chinese medicine or Ayurveda offer many recommendations and recipes for treatment of oral diseases where many plant species and parts are included (Hebbar *et al*., 2004; Singh *et al*., 2007; Surathu and Kurumathur, 2011). The exhaustive lists of such therapies can be found in herbal textbooks concerning with these ancient medicinal systems. Recently, some of these medicinal prescriptions have been evaluated concluding that traditional Chinese and Ayurvedic herbal compositions used for oral disorders effectively inhibit bacterial and yeast growth without causing adverse effects (Chan *et al*., 2003; Cao and Sun, 1998; Franzblau and Cross, 1986; Hu *et al*., 2000; Tsao *et al*., 1982). One of the common oral hygiene practice recommended by Ayurveda is the utilization of chewing sticks (Surathu and Kurumathur, 2011). The cleaning of oral cavity through the chewing sticks is an ancient pre-Islamic custom as they were used by the Babylonians as early as 3500 BC (Wu *et al*., 2001), and they are still widely used in many African, Arabic and Asian countries as an oral hygiene aid in place of a toothbrush (Asadi and Asadi, 1997; Bos, 1993; More *et*

al., 2008; Norton *et al*., 1989; Sarita and Tuominen, 1992). Although they may have been used with toothpowders or rose extract, they are most commonly used as a sole cleansing agent (Fischman, 1997). The chewing sticks come from a wide range of plant species as well as from their different parts, and within one stick the chemically active components may be heterogeneous (Akpata and Akinrimisi, 1977). Which plant species is used for the preparation of chewing stick depends on the availability in given region, on the presence of long bristle-like tissue fibres and often on the pleasant taste (Wu *et al*., 2001). *Salvadora persica* is the most preferred species as it is widely distributed (Gupta *et al*., 1968) and its root, twigs and stems have been used as oral hygiene tools for centuries (Al-Bayati and Salaiman, 2008; Sofrata *et al*., 2008; Wu *et al*., 2001). In areas where *S. persica* is absent, the chewing sticks are prepared from other plants. Lime tree (*Citrus aurantifolia*), orange tree (*Citrus sinensis*), senna (*Cassia* spp.), neem tree (*Azadirachta indica*), *Diospyros lycioides*, *Euclea natalensis*, nut tree (*Juglans regia*) or *Sassafras albidum* can be given as examples (Almas and Allafi, 1995; Elvin-Lewis, 1980; Hadissa and Jean-Pierre, 2005; More *et al*., 2008). Many of these plants tested in *in vitro* studies have shown to inhibit both Gram-positive and Gram-negative bacteria (Akpata and Akinrimisi, 1977; Al-Bayati and Salaiman, 2008; Almas and Zeidi, 2004; Cai *et al*., 2000; Homer *et al*., 1990; More *et al*., 2008; Ndukwe *et al*., 2004; Pai *et al*., 2003; Sofrata *et al*., 2008). Moreover, several clinical studies have revealed that the chewing sticks, when used properly, can be as efficient as toothbrushes in removing dental plaque due to the combined effect of mechanical cleaning, the release of beneficial chemicals, and enhanced salivation (Norton *et al*., 1989; Olsson, 1978; Rahmani and Radvar, 2005; Sote, 1987; Wu *et al*., 2001). Hence, the World Health Organization has supported their use as an effective tool for oral hygiene (WHO, 1987).

1.7.2 Modern herbal dental care

Mainstream medicine is increasingly receptive to the use of the antimicrobials derived from plant sources, as traditional antimicrobial agents become ineffective against both existing and new microbial diseases (Allaker and Douglas, 2009; Botelho *et al*., 2007). This renewed interest together with rising demand for alternative natural products influence the market with oral hygiene products. Thus, the herbal antimicrobial substances are incorporated into mouthrinses and toothpastes to enhance their antiseptic properties and

to offer more natural oral care for consumers (Allaker and Douglas, 2009; Cohan and Jacobsen, 2000; Groppo *et al*., 2008; Marsh, 1992; Moran, 2008; Ozaki *et al*., 2006).

Undoubtedly, the category of natural plant-derived ingredients used in oral hygiene products most frequently and investigated widely is category of essential oils and their components. Among essential oils or their components used worldwide in products for oral hygiene, dominate those derived from tea tree (*Melaleuca alternifolia*), eucalyptus (*Eucalyptus globulus*), peppermint (*Mentha x piperita*), calendula, lavender and rosemary (Lauten *et al*., 2005). All these essential oils have been tested for inhibitory activity against cariogenic and periodontopathic bacteria in several studies and they have been shown to inhibit the growth of these oral pathogenic bacteria (Lauten *et al*., 2005; Longstaff, 1987; Maruzzella and Henry, 1958; Meeker and Linke, 1988; Saeki *et al*., 1989; Shapiro *et al*., 1994). Moreover, tea tree oil was shown to inhibit adhesion of *P. gingivalis* and together with the abovementioned oils inhibited adhesion of *Streptococcus mutans* (Cox *et al*., 2000; Saxer *et al*., 2003; Takarada *et al*., 2004). In studies determining the minimal inhibitory and bactericidal concentrations of essential oil components towards pathogenic oral bacteria, carvacrol, cinnamaldehyde, menthol and thymol were found to be the most potent against both Gram-positive and Gram-negative bacteria (Bhattacharya *et al*., 2003; Didry *et al*., 1994; Shapiro *et al*., 1994).

Concerning the essential oil components, thymol, eucalyptol, methyl salicylate and menthol are the active ingredients included in probably the most famous mouthrinse: Listerine^{TM} (Pfizer Inc., Morris Plains, NJ, USA), which has been in widespread use for many years worldwide. Thymol and eucalyptol have a role of antimicrobial agents, whereas methyl salicylate and menthol act as cleaning agents and local anesthetic respectively (Gordon *et al*., 1985). Several *in vitro* and *in vivo* studies have demonstrated the potential of Listerine^{TM} essential oil mouthrinse in the control of plaque-related oral diseases (Albert-Kiszely *et al*., 2007; Fine *et al*., 2000; Fine *et al*., 2005; Gordon *et al*., 1985; Moran *et al*., 1997; Riep *et al*., 1999; Ross *et al*., 1989, Stoeken *et al*., 2007). It has been proved to effectively kill bacteria through cell wall disruption and inhibition of enzyme activity (Fine, 1988) and has also capacity for preventing aggregation of bacteria within the process of plaque maturation and slowing replication of bacteria, and act to reduce endotoxin release from Gram-negative pathogens (Allaker and Douglas, 2009).

Moreover, clinical results have showed that Listerine™ produces significant reduction in *Streptococcus mutans* in plaque, whereas streptococci from the mitis group (commensals) seem to be much less susceptible to it (Fine *et al*., 2000). A few clinical studies have compared the efficacy of essential oil mouthrinse and chlorhexidine one (Charles *et al*., 2004; Haffajee *et al*., 2009; Santos, 2003; Singh *et al*., 2012; Van Leeuwen *et al*., 2011). The results revealed that essential oil mouthrinse inhibit supragingival plaque by up to 56 % and gingivitis by up to 36 %, while chlorhexidine decreases plaque by up to 61 % and reduces gingivitis by up to 80 %. However, in comparison to chlorhexidine, mouthrinse based on essential oils does not cause staining, alter taste perception or promote calculus formation. Another study comparing the effectiveness of rinsing with an essential oil mouthrinse with that of dental floss showed that the essential oil mouthrinse is equally good as dental floss for the control of interproximal gingivitis (Bauroth *et al*., 2003; Sharma *et al*., 2002). All these results indicate that the essential oil-based mouthrinse can be used daily for an effective reduction of caries, gingival inflammation and periodontitis. Hence, it may have an important place in the oral home-care routine, even though it does not reach the efficacy of chlorhexidine and it should be considered only as an adjunct to regular mechanical oral care (Claffey, 2003; Groppo *et al*., 2008; Vlachojannis *et al*., 2012b).

Among other worldwide available oral care products containing plant-derived ingredients, Parodontax®, Corsodyl Daily® (both GlaxoSmithKline, Brentford, UK) and Cervitec® product lines (Ivoclar Vivadent Inc., Schaan, LI) have been tested in several *in vitro* and clinical studies (Groppo *et al*., 2008; Ledder *et al*., 2014; Verkaik *et al*., 2011). Parodontax and Corsodyl Daily dentifrices both contain, besides sodium bicarbonate and sodium fluoride, several herbal components for which medicinal properties are claimed. Rhatany extract (*Krameria triandra*) provides astringent and antimicrobial effects, chamomile extract has proved anti-inflammatory properties, purple coneflower extract (*Echinacea purpurea*) stimulates immune response, sage extract (*Salvia officinalis*) has antihemorrhagic properties, myrrh extract is a natural antiseptic, and mint is an analgesic, antiseptic and anti-inflammatory agent (Pistorius *et al*., 2003; Scholz and Rimpler, 1989; Shapiro *et al*., 1994). The studies conclude that both Parodontax and Corsodyl have a potential to reduce significantly plaque re-growth and thus preventing oral infectious diseases; however, the exact mechanisms of antimicrobial activities of the included herbal

components remain unclear (Ozaki *et al*., 2006; Pannuti *et al*., 2003; Saxer *et al*., 1994; Verkaik *et al*., 2011; Yankell *et al*., 1993). Chlorhexidine-thymol mouthrinse Cervitec® had also a significant inhibiting effect on the levels of oral pathogenic bacteria, mainly the mutans streptococci, and on reduction of caries and supragingival dental plaque formation in previous clinical studies (Araujo *et al*., 2002; Bratthall *et al*., 1995; Ogaard *et al*., 1997; Petersson *et al*., 1991; Weiger *et al*., 1994). However, no conclusions have been made which of the two antimicrobial agents plays more important role in the bacterial inhibition. Nevertheless, it is noteworthy that no negative side effects have been reported in aforementioned clinical trials on Parodontax, Corsodyl or Cervitec, even though there have been several complaints about unpleasant taste of these products. As far as other herbal-based oral care products are concerned, in a study of Lee *et al.* (2004), the antimicrobial potential of 14 natural herbal dentifrices was evaluated. Some of them were highly effective in inhibiting common oral pathogenic bacteria. From the products tested, the most effective were Healthy Mouth produced by Jason Natural Cosmetics (Lake Success, USA) containing tee tree and neem oil with addition of grapefruit seed extract and Dental Gel (NutriBiotic, Lakeport, USA) with content of sanguinarine extract. However, the composition of the latter product had to be changed after several reports on sanguinarine-related oral leukoplakia. The case is discussed further below.

Generally, there are a huge number of herbal oral products available today in national and international markets. The active ingredients used the most besides those mentioned previously are for example aloe vera gel, cinnamon and clove oil, fennel (*Foeniculum vulgare*), geranium (*Geranium* spp.), ginger (*Zingiber officinale*) and juniper (*Juniperus communis*) and nettle (*Urtica dioca*) extract, together with lavender and spearmint oil (*Mentha spicata*), as well as Chinese herbal products and Ayurvedic herbal preparations (Goldstein and Epstein, 2000).

Even though the oral hygiene products containing various herbal additives are thought to be adequate alternative to established commercially available mouthrinses and dentifrices, various questions concerning the equivocal effects of some of these products seen both on plaque and gingivitis have arisen (Arweiler *et al*., 2000; Carvalho *et al*., 2011; Saxer *et al*., 2003). Moreover, several side effects have been reported from the use of such products (Rao *et al*., 2012; Vlachojannis *et al*., 2012b). Strong taste and subsequent

discomfort in oral cavity often associated with essential oil mouthrinses (Cohan and Jacobsen, 2000) together with their low pH, which may have the potential for erosion (Pontefract *et al*., 2001) or allergic reactions to phytochemicals (Rao *et al*., 2012) can be named among others. The issue of toxicity has to be also taken into account when introducing novel antimicrobial plant-derived constituent into oral hygiene products. In this place, it is important to point out that herbs, contrary to common belief, are not necessarily harmless (Ernst, 1998). Contrarily, many plants are toxic and may be extremely dangerous (Elvin-Lewis, 2001; Lai and Roy, 2004). Such evidence about harmful effects of various plant compounds in traditional medicine is wide and many medicinal herbs are therapeutic at one dose and toxic at another (Fugh-Berman, 2000).The question of safety of such ingredients is even more critical for preventive home oral care because it consists usually from long-term and regular everyday usage (Aggarwal and Kunnamakkara, 2009). The past negligence of the toxicity problem related with long-term use of oral hygiene products may be well illustrated on the case of sanguinarine.

Sanguinarine is a benzophenanthridine alkaloid derived from many medicinal plants, especially from Canadian bloodroot plant (*Sanguinaria canadensis*), and plume poppy (*Macleya cordata*) (Grenby, 1996; Kosina *et al*., 2010; Mackraj *et al*., 2008; Marsh, 1992; Psotova *et al*., 2006). It has a broad spectrum of activity against both Gram-positive and Gram-negative oral bacteria, particularly species associated with periodontal diseases, which was confirmed by several *in-vitro* and clinical studies (Cullinan *et al*., 1997; Dzink and Socransky, 1985; Harper *et al*., 1990; Hong *et al*., 2005; Kurbad *et al*., 1990; Wennstrom and Lindhe, 1985). It contains the chemically reactive iminium ion that is probably responsible for such activity as it inhibits multiplication of bacteria and affects the membrane permeability of bacterial cells (Harkrader *et al*., 1990; Schmeller *et al*., 1997). For this reason, it was recommended for long-term control of periodontal diseases, and consequently, it was incorporated in a number of toothpastes and mouthrinses in various concentrations (Tenenbaum *et al*., 1999; Vlachojannis *et al*., 2012a). Nevertheless, the bloodroot extract containing sanguinarine had to be removed from all oral products, such as Viadent® product line (Colgate Oral Pharmaceuticals Inc., NY, USA), as soon as several reports describing possible connection between the use of sanguinarine and precancerous oral leukoplakia were published (Anderson *et al*., 2005; Damm *et al*., 1999; Damm and Fantasia, 2002; Eversole *et al*., 2000; Vlachojannis *et al*., 2012a).

To avoid the risk of consumers' intoxication by food and drug ingredients Food and Drug Administration of US Government (FDA) created in 1958 a database of substances and dietary supplements that are generally recognized as safe (later to be known by an acronym GRAS) (U.S. FDA, 2014). All ingredients that belong to the GRAS system have been approved by experts and are therefore assumed to be safe for human consumption and use (Burdock, 2000). A substance can become GRAS either by the process of expert evaluation through scientific procedures or for those substances used prior to January 1, 1958, through history of common use (Burdock *et al*., 2004). Nowadays, the GRAS database lists more than 130 plant records (U.S. FDA, 2014).

Hence, one option how to avoid adverse effects of plant-derived antimicrobial agents is to focus the research on the GRAS plant species. Many of them or their constituents have been already tested for their antimicrobial properties (Table 5 in chapter Material and methods) but only a few of these studies were conducted directly against oral pathogenic bacteria (Iauk *et al*., 2003; Prabuseenivasan *et al*., 2006; Smullen *et al*., 2007). The majority of studies was aimed at various bacterial strains, with food-borne pathogens (such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp.) being the most frequently tested. The only organism selected for our research with wide evidence about antimicrobial susceptibility tests is *Candida albicans*. On the other hand, according to the literature available, the antimicrobial activity of a couple of plants recognized to be GRAS has been never evaluated before.

2. Hypothesis

Taking into consideration that numerous plant species from the GRAS list have been proved to possess significant inhibitory effects against various human pathogenic microorganisms and that some of them have been used for such properties since the time immemorial, it is possible to suppose that they could have also promising antimicrobial effects on oral cariogenic and periodontal bacteria and yeast.

3. Objectives

The main objective of this thesis is the comprehensive investigation of *in vitro* antimicrobial properties of the extracts from GRAS plant species against selected spectrum of oral pathogenic microorganisms and chemical analysis of the most antimicrobially effective extracts.

The specific aims are as follows:

i. The identification of the most effective extracts according to the results of the *in vitro* growth-inhibitory tests against the major cariogenic and periodontal pathogenic microorganisms

ii. Comparison of antimicrobial properties of the extracts from the most prospective species prepared through distinct extraction methods from different varieties or cultivars

iii. Chemical analysis of the most effective extracts and identification of their main constituents

4. Materials and methods

4.1. Plant material

The study included 109 plant species selected on the basis of the articles §182.10 (Spices and other natural seasonings and flavourings) and §182.20 (Essential oils, oleoresins and natural extractives) of the GRAS database available in the form of Electronic Code of Federal Regulations. The correct scientific plant names were determined according to the International Plant Names Index (2010). The samples have been collected from March 2010 up to September 2012. The samples were provided by Czech botanical gardens (Botanical garden of Czech University of Life Sciences in Prague; Botanical garden of Charles University in Prague) or were purchased in commercial market from various producers (Avokado, CZ; Coffee Source, CZ; F-Dental, CZ; Fytoimex Bohemia, CZ; Golden Way, CZ; Great Tea Garden, CZ; Hadapeon, CZ; Herbata, CZ; Igel, CZ; Koreni od Samuela, CZ; Kotanyi, Austria; Longberry Espresso, CZ; Mountain Rose Herbs, USA; Natura, CZ; Perun, CZ; Poex, CZ; VanHouten, Belgium; Variant, CZ). *Humulus lupulus* cultivars used for comparison of antimicrobial activity of different hop cultivars and extracts were provided by the Hop Research Institute (Saaz, CZ). All tested plant species together with their origin and examples of some previous antimicrobial studies are listed in Table 5.

4.2. Chemicals

Ampicillin, chlorhexidine, nystatin, penicillin G, tetracycline were purchased Sigma-Aldrich (Prague, CZ). HPLC grade methanol, water and phosphoric acid were purchased from the same company as well. Dimethyl sulfoxide (DMSO) and ethanol used as solvents were obtained from Lach-Ner (Neratovice, CZ).

Table 5 List of tested GRAS species, examples of some previous antimicrobial studies, plant parts used in this research and origin of the plant material

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Agropyron repens P.Beauv.	Couch grass	Poaceae	*Staphylococcus aureus*	Dilution method	Quave *et al*., 2008	Roots	Herbata Ltd. (Horni Kruty, CZ)
Allium cepa L.	Onion	Amaryllidaceae	*Escherichia coli, Bacillus subtilis Candida tropicalis, Rhodotorula glutinis, Saccharomyces cerevisiae, Staphylococcus aureus*	Dilution method	Ye *et al*., 2013	Bulbs	Herbata Ltd. (Horni Kruty, CZ)
Allium schoenoprasum L.	Chive	Amaryllidaceae	*Bacillus cereus, Campylobacter jejuni, clostridium botulinum, Escherichia coli, Listeria monocytogenes, Salmonella enterica, Staphylococcus aureus, Vibrio cholerae*	Broth microdilution method	Rattanachaikunsop *et al*., 2008	Aerial part	Kotányi GmbH (Wolkersdorf, AT)
Alpinia officinarum Hance	Galangal	Zingiberaceae	*Staphylococcus aureus*	Microdilution method	Lee *et al*., 2008	Roots	Herbata Ltd. (Horni Kruty, CZ)
Amomum melegueta Rosc.	Grains of paradise	Zingiberaceae	*Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Staphylococcus aureus*	Diffusion method	Oladunmoye *et al*., 2007	Seeds	Koreni od Samuela (Chomutov, CZ)
Angelica archangelica L.	Garden Angelica (wild celery)	Apiaceae	*Alcaligenes faecalis, Aspergillus niger, Bacillus cereus, Enterobacter cloacae, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Rhizopus oligosporus, Staphylococcus aureus, Streptococcus faecalis*	Disc assay method	Chao *et al*., 2000	Roots	Herbata Ltd. (Horni Kruty, CZ)
Anthemis nobilis L.	Roman camomile	Asteraceae	*Candida albicans*	Dilution method	Duarte *et al*., 2005	Flowers	Herbata Ltd. (Horni Kruty, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Anthriscus cerefolium Hoffm.	Chervil	Apiaceae	*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus haemolyticus*	Disc diffusion method	Izzo *et al*., 1995	Leaves	Herbata Ltd. (Horni Kruty, CZ)
Apium graveolens L.	Celery	Apiacea	*Staphylococcus aureus, Listeria* spp.	Macrodilution method	Misic *et al*., 2008	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Armoracia lapathifolia Gilib.	Horseradish	Brassicaceae	*Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enteridis, Staphylococcus aureus*	Disc volatization method	Nedorostova *et al*., 2009	Roots	Koreni od Samuela (Chomutov, CZ)
Artemisia dracunculus L.	Tarragon	Asteraceae	*Candida albicans, Cryptococcus neoformans, Escherichia coli, Fonsecaea pedrosoi, Microsporum* spp., *Staphylococcus spp., Trichophyton rubrum*	Diffusion method	Lopes-Lutz *et al*., 2008	Leaves	Herbata Ltd. (Horni Kruty, CZ)
Brassica alba Boiss.	White mustard	Brassicaceae	*Bacillus spp., Candida* spp., *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus aureus*	Dilution method	Sousa *et al*., 2008	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Brassica nigra Koch	Black mustard	Brassicaceae	*Helminthosporium solani, Verticillium dahliae*	Diffusion method	Olivier *et al*., 1999	Seeds	Hadapeon Ltd. (Prague, CZ)
Calendula officinalis L.	Pot marigold	Asteraceae	*Actinomyces odontolyticus, Capnocytophaga gingivalis, Eikenella corrodens, Fusobacterium nucleatum, Peptostreptococcus micros, Porphyromonas* spp., *Prevotella* spp., *Veillonela parvula*	Microdilution method	Iauk *et al*., 2003	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Cananga odorata Hook.f. & Thomson	Ylang-ylang	Annonaceae	*Candida albicans, Rhodotorula glutinis, Saccharomyces cerevisae, Schizosaccharomyces pombe, Yarrowia lypolitica*	Dilution method	Sacchetti *et al*., 2005	Leaves	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Capparis spinosa L.	Caper bush	Capparaceae	*Bacillus cereus, Candida albicans, Escherichia coli, Fussarium oxysporum, Salmonella typhimurium, Staphylococcus aureus*	Diffusion method	Mahasneh *et al*., 1996	Aerial part	Botanical Garden of CULS (Prague, CZ)
Capsicum annuum L.	Pepper	Solanaceae	*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus* spp.	Dilution method	Erturk, 2006	Fruit	Variant Ltd. (Vsetaty, CZ)
Capsicum frutescens L.	Chilli pepper	Solanaceae	*Bacillus* spp., *Candida albicans*	Diffusion method	Cichewicz *et al*., 1996	Fruit	Herbata Ltd. (Horni Kruty, CZ)
Carum carvi L.	Caraway	Apiaceae	*Lactobacillus* spp.	Dilution method	Damasius *et al*., 2007	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Carya illinoinensis (Wangenh.) K.Koch	Hickory pecan	Juglandaceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enterica, Staphylococcus aureus*	Microdilution method	Do Prado *et al*., 2014	Seeds	Encinger Ltd. (Bratislava, SK)
Ceratonia siliqua L.	Carob tree (St.John's bread)	Leguminosae	*Listeria monocytogenes, Pseudomonas fragi, Salmonella enteritidis, Shewanella putrefaciens, Staphylococcus aureus*	Diffusion method	Tassou *et al*., 1997	Fruit	Herbata Ltd. (Horni Kruty, CZ)
Chimaphila umbellata (L.) Nutt	Pipsissewa	Ericaceae	*Malassezia* spp., *Saccharomyces cerevisiae*	Microdilution method	Galvan *et al*., 2008	Aerial part	Mountain Rose Herbs (Eugene, USA)
Cichorium intybus L.	Chicory	Asteraceae	*Salmonella typhi*	Diffusion method	Rani *et al*., 2004	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Cinnamomum burmannii (Ness & T.Ness) Blume	Padang cassia	Lauraceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella anatum, Staphylococcus aureus*	Dilution method	Shan *et al*., 2007	Bark	Mountain Rose Herbs (Eugene, USA)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Cinnamomum cassia D.Don	Chinese cinnamon	Lauraceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella infantis, Staphylococcus aureus*	Dilution method	Alzoreky *et al*., 2003	Bark	Herbata Ltd. (Horni Kruty, CZ)
Cinnamomum zeylanicum Nees	Ceylon cinnamon	Lauraceae	*Bacillus* spp., *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus*	Diffusion method	Singh *et al*., 2007	Bark	F-Dental Hodonín Ltd. (Hodonin, CZ)
Citrus aurantiifolia (Christm.) Swingle	Key lime	Rutaceae	*Mycobacterium tuberculosis*	Dilution method	Camacho-Corona *et al*., 2008	Fruit	Botanical Garden of CULS (Prague, CZ)
Citrus aurantium L.	Bitter orange	Rutaceae	*Trichoderma harzianum, Verticillium fungicola*	Macro- and microdilution method	Sokovic *et al*., 2006	Pericarp	F-Dental Hodonín Ltd. (Hodonin, CZ)
Citrus limon (L.) Burm.	Lemon	Rutaceae	*Enterobacter* spp., *Listeria monocytogenes, Salmonella enteric, Stahpylococcus aureus*	Disc diffusion method	Settanni *et al*., 2014	Pericarp	Herbata Ltd. (Horni Kruty, CZ)
Citrus paradisi Macfad.	Grapefruit	Rutaceae	*Staphylococcus aureus*	Disk diffusion method	Adukwu *et al*., 2012	Pericarp	Mountain Rose Herbs (Eugene, USA)
Citrus reticulata Blanco	Mandarin orange	Rutaceae	*Helicobacter pylori*	Dilution method	Li *et al*., 2005	Leaves	Botanical Garden of CULS (Prague, CZ)
Citrus sinensis Osbeck	Sweet orange	Rutaceae	*Paenibacillus larvae*	Microdilution method	Fuselli *et al*., 2008	Pericarp	Herbata Ltd. (Horni Kruty, CZ)
Coffea arabica L.	Coffee	Rubiaceae	*Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Staphylococcus* spp.	Diffusion method	Arora *et al*., 2009	Seeds	Great Tea Garden Ltd. (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Coffea robusta L.	Robusta coffee	Rubiaceae	*Aspergillus* spp., *Bacillus* spp., *Candida versatilis, Escherichia coli, Fusarium* spp., *Listeria monocytogenes, Saccharomyces cerevisiae, Staphylococcus aureus, Streptococcus* spp., *Yersinia enterocolitica*	Diffusion method	Murthy *et al*., 2009	Seeds	Coffee source Ltd (Prague, CZ)
Cola acuminata Schott & Endl.	Kola tree	Sterculiaceae	*Bacillus subtilis, Candida albicans, Escherichia coli, Klebsiella pneumonae, Pseudomonas aeruginosa, Staphylococcus aureus*	Diffusion method	Adam *et al*., 2011	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Coriandrum sativum L.	Coriander	Apiaceae	*Bacillus* spp., *Candida albicans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus*	Dilution method	Matasyoh *et al.*, 2009	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Crocus sativus L.	Saffron	Iridaceae	*Acinetobacter lwoffi, Alcaligenes faecalis, Bacillus* spp., *Candida albicans, Enterobacter cloacae, Escherichia coli, Klebsiella* spp., *Listeria monocytogenes, Proteus* spp., *Providencia alcaliaciens, Pseudomonas* spp., *Saccharomyces* spp., *Salmonella* spp., *Staphylococcus* spp., *Streptococcus pyogenes*	Disc diffusion method	Sengul *et al*., 2009	Stigmas	F-Dental Hodonín Ltd. (Hodonin, CZ)
Cuminum cyminum L.	Cumin	Apiaceae	*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus* spp.	Dilution method	Erturk, 2006	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Curcuma longa L.	Turmeric	Zingiberaceae	*Staphylococcus aureus*	Dilution method	Kim *et al*., 2005	Roots	Herbata Ltd. (Horni Kruty, CZ)
Curcuma zedoaria (Bergius) Roscoe	Zedoary	Zingiberaceae	*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Proteus mirabilis, Staphylococcus aureus*	Dilution method	Wilson *et al*., 2005	Rhizome	Herbata Ltd. (Horni Kruty, CZ)
Cymbopogon citratus Stapf.	Lemon grass	Poaceae	*Candida albicans, Cryptococcus neoformans, Malssezia pachydermatis, Pseudomonas* spp., *Staphylococcus* spp.	Dilution method	Koba *et al*., 2004	Leaves	Herbata Ltd. (Horni Kruty, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Daucus carota L.	Carrot	Apiaceae	*Bacillus cereus*	Dilution method	Kumarasamy *et al*., 2002	Roots	Hadapeon Ltd. (Prague, CZ)
Elettaria cardamomum Maton	Cardamom	Zingiberaceae	*Bacillus cereus, Citrobacter freundii, Enterobacter cloacea, Escherichia coli, Klebsiella pneumoniae, Lactobacillus delbrueckii, Listeria monocytogenes, Morganella morganii, Proteus* spp., *Salmonella enteridis, Shigella sonnei, Staphylococcus aureus, Yersinia enterolitica*	Dilution method	El Malti *et al*., 2007	Fruit	Natura Ltd. (Decin, CZ)
Erythroxylum catuaba P.Browne	Catuaba	Erythroxylaceae	*Escherichia coli, Staphylococcus aureus*	In vivo experiment	Manabe *et al*., 1992	Bark	F-Dental Hodonín Ltd. (Hodonin, CZ)
Ferula assa-foetida L. (*Ferula ssp.*)	Asafoetida	Apiaceae	*Aspergillus* sp., *Penicillium digitatum, Trichoderma* sp., *Helminthosporium oryzae*	Diffusion method	Siddiqui *et al*., 1996	Latex	F-Dental Hodonín Ltd. (Hodonin, CZ)
Foeniculum vulgare Hill.	Fennel	Apiaceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella infantis, Staphylococcus aureus*	Dilution method	Alzoreky *et al*., 2003	Fruit	Golden Way Ltd. (Pilsen, CZ)
Galipea officinalis J.Hancock	Angostura	Rutaceae	*Mycobacterium tuberculosis*	Dilution method	Houghton *et al*., 1999	Bark	Fytoimex Bohemia Ltd. (Prague, CZ)
Helichrysum angustifolium Pers.	Curry plant	Asteraceae	*Malassezia furfur*	Disk diffusion method	Lee *et al*., 2010	Aerial part	Botanical Garden of CUNI (Prague, CZ)
Hibiscus moschatus Salisb.	Abelmosk	Malvaceae	*Bacillus subtilis, Candida albicans, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica, Staphylococcus aureus*	Diffusion method	Gul *et al*., 2011	Seeds	F-Dental Hodonín Ltd. (Hodonin, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Humulus lupulus L.	Hop	Cannabaceae	*Streptococcus mutans, S. salivarius, S. sanguis*	Turbidity assay	Bhattacharya *et al*., 2003	Female flowers	Hop Research Institute Co., Ltd. (Saaz, CZ)
Hyssopus officinalis L.	Herb hyssop	Lamiaceae	*Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella oxytoca, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus*	Dilution method	Manzzanti *et al.*, 1999	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Ilex paraguariensis A.St.-Hil.	Yerba mate	Aquifoliaceae	*Escherichia coli, Staphylococcus aureus*	Dilution method	Burris *et al*., 2011	Leaves	Herbata Ltd. (Horni Kruty, CZ)
Illicium verum Hook.f.	Star anise	Illiciaceae	*Actinomyces odontolyticus, Capnocytophaga gingivalis, Eikenella corrodens, Fusobacterium nucleatum, Porphyromonas* spp., *Prevotella* spp.	Microdilution method	Iauk *et al.*, 2003	Seeds	Golden Way Ltd. (Pilsen, CZ)
Jasminum officinale L.	Jasmine	Oleaceae	*Aspergillus niger, Bacillus subtilis, Escherichia coli, Fussarium oxysporum, Pseudomonas aeruginosa, Staphylococcus aureus*	Dilution method	Ngan *et al*., 2008	Flowers	F-Dental Hodonín Ltd. (Hodonin, CZ)
Juniperus communis L.	Common juniper	Cupressaceae	*Helicobacter pylori*	Dilution method	Nakanishi *et al*., 2005	Fruit	Golden Way Ltd. (Pilsen, CZ)
Laurus nobilis L.	Bay laurel	Lauraceae	*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus* spp.	Dilution method	Erturk, 2006	Leaves	Botanical Garden of CULS (Prague, CZ)
Lavandula officinalis Chaix	True lavender	Lamiaceae	*Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Ralstonia picketti, Staphylococcus aureus*	Diffusion method	Muyima *et al*., 2002	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Majorana hortensis Moench	Marjoram	Lamiaceae	*Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enteridis, Staphylococcus aureus*	Disc volatization method	Nedorostova *et al.*, 2009	Aerial part	Hadapeon Ltd. (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Marrubium vulgare L.	White horehound	Lamiaceae	*Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus* spp.	Dilution method	Masoodi *et al*., 2008	Aerial part	F-Dental Hodonín Ltd. (Hodonin, CZ)
Matricaria chamomilla L.	Camomile	Asteraceae	*Aspergillus* spp., *Fussaruim moliforme*	Diffusion method	Soliman *et al*., 2002	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Medicago sativa L.	Alfalfa	Leguminosae	*Acinetobacter baumani, Bacillus* spp., *Blastomyces capitatus, Candida* spp., *Cryptococcus laurentii, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus aureus*	Microdilution method	Avato *et al*., 2006	Aerial part	F-Dental Hodonín Ltd. (Hodonin, CZ)
Melissa officinalis L.	Lemon balm	Lamiaceae	*Actinomyces odontolyticus, Capnocytophaga gingivalis, Eikenella corrodens, Fusobacterium nucleatum, Peptostreptococcus micros, Porphyromonas* spp., *Prevotella* spp., *Veillonela parvula*	Microdilution method	Iauk *et al*., 2003	Aerial part	Botanical Garden of CULS (Prague, CZ)
Mentha crispa L.	Garden spearmint	Lamiaceae	*Bacillus megaterium, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Staphylococcus aureus, Steptococcus faecalis*	Disc diffusion method	Keskin and Toroglu, 2011	Leaves	F-Dental Hodonín Ltd. (Hodonin, CZ)
Mentha x piperita L.	Peppermint	Lamiaceae	*Candida albicans*	Dilution method	Duarte *et al*., 2005	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Mentha spicata L.	Spearmint	Lamiaceae	*Bacillus subtilis, Candida albicans, Enterococcus faecium, Escherichia coli, Pseudomonas aeruginosa, Rhodococcus equi, Salmonella choleraesuis, Staphylococcus* spp.	Dilution method	Sartoratto *et al*., 2004	Aerial part	Mountain Rose Herbs (Eugene, USA)
Monarda punctata L.	Horsemint	Lamiaceae				Aerial part	Herbata Ltd. (Horni Kruty, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Myristica fragrans Houtt.	Nutmeg	Myristicaceae	*Salmonella typhi*	Microdilution method	Rani *et al*., 2004	Seeds	Golden Way Ltd. (Pilsen, CZ)
Nigella sativa L.	Black cumin	Ranunculaceae	*Bacillus spp., Bacteroides fragilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* subsp. *pneumoniae, Pseudomonas aeruginosa, Staphylococcus* spp., *Streptococcus* spp.	Microdilution method	Havlik *et al.*, 2006	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Ocimum basilicum L.	Basil	Lamiaceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Slmonella infantis, Staphylococcus aureus*	Dilution method	Alzoreky *et al*., 2003	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Ocimum minimum L.	Dwarf basil	Lamiaceae	*Candida zeylanoides, Listeria innocua, Pseudomonas* spp., *Saccharomyces cerevisiae, Serratia marcenscens*	Microdilution method	Alves-Silva *et al*., 2013	Aerial part	Botanical Garden of CULS (Prague, CZ)
*Origanum vulgare*L.	Oregano	Lamiaceae	*Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enteridis, Staphylococcus aureus*	Disc volatization method	Nedorostova *et al*., 2009	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Papaver somniferum L.	Opium poppy	Papaveraceae	*Staphylococcus aureus*	Dilution method	Quave *et al*., 2008	Seeds	Poex Ltd. (Velke Mezirici, CZ)
Pelargonium graveolens L´Hér.	Geranium	Geraniaceae	*Aspergillus flavus*	Thompson method	Singh *et al*., 2007	Aerial part	Sukulenty Husner (Hyskov, CZ)
Pelargonium odoratissimum L´Her.	Apple geranium	Geraniaceae	*Aspergillus* spp., *Escherichia coli, Staphylococcus aureus*	Disc diffusion method	Andrade *et al*., 2011	Aerial part	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Petroselinum crispum (Mill.) Nyman	Parsley	Apiacea	*Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus* spp.	Diffusion method	Ojala *et al*., 2000	Roots	Herbata Ltd. (Horni Kruty, CZ)
Pimenta officinalis Lindl.	Allspice	Myrtaceae	*Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus*	Dilution method	Oussalah *et al*., 2007	Fruit	Golden Way Ltd. (Pilsen, CZ)
Pimpinella anisum L.	Anise	Apiaceae	*Candida albicans, Citrobacter koseri, Enterobacter aerogenes, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus* spp., *Streptococcus pneumoniae*	Disc diffusion method	Gulcin *et al.*, 2003	Seeds	Golden Way Ltd. (Pilsen, CZ)
Piper nigrum L.	Black pepper	Piperaceae	*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus spp.*	Dilution method	Erturk, 2006	Fruit	Kotányi GmbH (Wolkersdorf, AT)
Prunus amygdalus Batsch	Almond	Rosaceae	*Aspergillus niger, Candida albicans, Escherichia coli, Penicillium notatum, Pseudomona aeroginosa, Salmonella typhi, Staphylococcus aureus*	Diffusion method	Neogi *et al*., 2008	Seeds	Poex Ltd. (Velke Mezirici, CZ)
Prunus serotina Ehrh.	Black cherry	Rosaceae	*Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Mycobacterium phlei, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus*	Disc diffusion method	Omar *et al*., 2000	Bark	Mountain Rose Herbs (Eugene, USA)
Prunus spinosa L.	Blackthorn	Rosaceae	*Staphylococcus aureus*	Dilution method	Quave *et al*., 2008	Flower	Herbata Ltd. (Horni Kruty, CZ)
Psidium cattleianum Sabine	Cattley guava	Myrtaceae	*Streptococcus mutans*	Dilution method	Brighenti *et al*., 2008	Leaves	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Punica granatum L.	Pomegranate	Lythraceae	*Candida albicans, C. dubliniensis, C. parapsilosis, C. tropicalis*	Microdilution method	Hofling *et al.*, 2010	Fruit	F-Dental Hodonín Ltd. (Hodonin, CZ)
Rosa centifolia L.	Provence rose	Rosaceae	*Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Salmonella abony, Staphylococcus aureus*	Broth dilution method	Wanner *et al.*, 2010	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Rosa damascena Mill.	Damask rose	Rosaceae	*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*	Disk diffusion method	Aridogan *et al*., 2002	Flowers	Mountain Rose Herbs (Eugene, USA)
Rosmarinus officinalis L.	Rosemary	Lamiaceae	*Streptococcus sobrinus*	Microdilution method	Tsai *et al*., 2007	Aerial part	Botanical Garden of CULS (Prague, CZ)
Saccharum officinarum L.	Sugarcane	Poaceae				Stems	Botanical Garden of CULS (Prague, CZ)
Salvia lavandulifolia Vahl	Spanish sage	Lamiaceae	*Listeria monocytogenes*	Microdilution method	Rota *et al*., 2004	Aerial part	Botanical Garden of CUNI (Prague, CZ)
Salvia officinalis L.	Common sage	Lamiaceae	*Aeromonas* spp., *Bacillus* spp., *Citrobacter* spp., *Escherichia coli, Klebsiella oxytoca, Proteus mirabilis, Pseudomonas* spp., *Salmonella typhimurium, Serratia marcescens, Staphylococcus* spp.	Microdilution method	Delamare *et al.*, 2007	Leaves	Herbata Ltd. (Horni Kruty, CZ)
Salvia sclarea L.	Clary sage	Lamiaceae	*Candida albicans, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus* spp.	Microdilution method	Kuzma *et al*., 2007	Leaves	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Sambucus nigra L.	Elderberry	Caprifoliaceae	*Staphylococcus aureus*	Dilution method	Quave *et al*., 2008	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Satureja hortensis L.	Summer savory	Lamiaceae	*Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus*	Dilution method	Oussalah *et al*., 2007	Aerial part	Herbata Ltd. (Horni Kruty, CZ)
Satureja montana L.	Winter savory	Lamiaceae	*Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus*	Dilution method	Oussalah *et al*., 2007	Aerial part	Mountain Rose Herbs (Eugene, USA)
Sesamum indicum L.	Sesame	Pedaliaceae	*Lactobacillus acidophilus, Streptococcus mutans*	Diffusion method	Anand *et al*., 2008	Seeds	F-Dental Hodonín Ltd. (Hodonin, CZ)
Schinus molle L.	Peruvian pepper	Anacardiaceae	*Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas* spp., *Salmonella* spp., *Staphylococcus aureus*	Dilution method	Hayouni *et al*., 2008	Fruit	Fytoimex Bohemia Ltd. (Prague, CZ)
Tamarindus indica L.	Tamarind	Leguminosae	*Bacillus cereus, Escherichia coli, Micrococcus flavus, Pseudomonas aeruginosa, Streptococcus auratus*	Diffusion method	Al-Fatimi *et al.*, 2007	Fruit	Ajurveda v dennim zivote Ltd. (Prague, CZ)
Taraxacum officinale [Weber.]	Dandelion	Asteraceae	*Acinetobacter lwoffi, Alcaligenes faecalis, Bacillus* spp., *Candida albicans, Enterobacter cloacae, Escherichia coli, Klebsiella* spp., *Listeria monocytogenes, Proteus* spp., *Providencia alcaliaciens, Pseudomonas* spp., *Saccharomyces* spp., *Salmonella* spp., *Staphylococcus* spp., *Streptococcus pyogenes*	Disc diffusion method	Sengul *et al*., 2009	Leaves	Herbata Ltd. (Horni Kruty, CZ)
Thea sinensis L.	Tea	Theaceae	*Bacillus* spp., *Enterobacter* spp., *Escherichia coli, Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Vibrio cholerae*	Dilution method	Bandyopadhyay *et al*., 2005	Leaves	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Theobroma cacao L.	Cacao tree	Sterculiaceae	Streptococcus mutans	Dilution method	Smullen *et al*., 2007	Seeds	VanHouten Ltd. (Vebbeke-Wiese, BE)
Thymus serpyllum L.	Wild thyme	Lamiaceae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella infantis, Staphylococcus aureus*	Dilution method	Alzoreky *et al*., 2003	Aerial part	F-Dental Hodonín Ltd. (Hodonin, CZ)
Thymus vulgaris L.	Common thyme	Lamiaceae	*Candida albicans*	Dilution method	Duarte *et al*., 2005	Aerial part	Avokado-Unimex Ltd. (Prague, CZ)
Thymus zygis	Spanish thyme	Lamiaceae	*Candida albican, Haemophilus influenza, Helicobacter pylori, Listeria monocytogenes, Salmonella enterica, Staphylococcus aureus*	Diffusion method	Dandlen *et al*., 2011	Aerial part	Botanical Garden of CUNI (Prague, CZ)
Tilia cordata Mill.	Small-leaved lime	Tiliaceae	*Bacillus cereus, Candida albicans, Escherichia coli, Sarcina lutea, Staphylococcus aureus*	Diffusion method	Fitsiou *et al*., 2007	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Trifolium pratense L.	Red clover	Leguminosae	*Clostridium sticklandii*	Diffusion method	Flythe *et al*., 2010	Flowers	Herbata Ltd. (Horni Kruty, CZ)
Trigonella foenum-graecum L.	Fenugreek	Leguminosae	*Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella infantis, Staphylococcus aureus*	Dilution method	Alzoreky *et al*., 2003	Seeds	Herbata Ltd. (Horni Kruty, CZ)
Vanilla planifolia Andrews	Vanilla	Orchidaceae	*Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Listeria monocytogenes, Salmonella* spp., *Staphylococcus* spp., *Yersenia enterolitica*	Diffusion method	Mourtzinos *et al.*, 2009	Fruit	Golden Way Ltd. (Pilsen, CZ)
Viola odorata L.	Sweet violet	Violaceae	*Escherichia coli, Pseudomonas aeruginosa, Salmonella* spp., *Shigella flexneri, Staphylococcus aureus*	Dilution method	Arora *et al*., 2004	Flowers	Botanical Garden of CULS (Prague, CZ)

Scientific name	English name	Family	Microorganisms tested previously	Previous antimicrobial assay	References	Part/-s tested in this study	Source
Zanthoxylum clava-herculis L.	Hercules' club	Rutaceae	*Staphylococcus aureus*	Microdilution method	Gibbons *et al.*, 2003	Bark	Mountain Rose Herbs (Eugene, USA)
Zingiber officinale Roscoe	Ginger	Zingiberaceae	*Candida albicans, Rhodotorula glutinis, Saccharomyces cerevisae, Schizosaccharomyces pombe, Yarrowia lypolitica*	Dilution method	Sacchetti *et al.*, 2005	Rhizome	Kotányi GmbH (Wolkersdorf, AT)

4.3. Preparation of plant extracts

Raw samples gathered from botanical gardens were dried at 40°C for 24 hours. 15 g of these dried samples and samples purchased from commercial market were homogenized in Grindomix mill GM100 (Retsch GmbH, Haan, DE) and macerated for 24 hours in 450 ml 80% ethanol (Lach-Ner, Neratovice, CZ) at room temperature using laboratory shaker GFL3005 (GFL, Burgwedel, DE). The extracts were then filtered and concentrated using rotary evaporator R-200 (Büchi Labortechnik, Flawil, CH) *in vacuo* at 40°C. Dried residues were subsequently diluted in DMSO in order to obtain stock solution of the final concentration 51.2 mg/mL and were stored at -20°C until their use. The yields (%) of dry residues are given in Table 6 (Results).

4.4. Carbon dioxide supercritical fluid extraction

The CO_2 supercritical fluid extraction (SFE) of two *H. lupulus* cultivars was performed using a full-scale apparatus as described previously by Forster (1994). The stainless extractor was loaded with 100 kg of *H. lupulus* pellets. The extraction was carried out at a constant pressure of 29 MPa and temperature of 50°C. The specific solvent flow related to pellets was 19 kg CO_2/1 kg per hour. Extraction time was 4 hours. The obtained saturated solution of *H. lupulus* components in supercritical CO_2 was expanded to atmospheric pressure and the extract was collected into steel drums.

4.5. High-performance liquid chromatography

The HPLC analysis of the two SFE *H. lupulus* extracts was carried out according to the analytical procedure prescribed by European Brewery Convention in laboratories of Hop Research Institute (Saaz, CZ) on Shimadzu LC 20A liquid chromatograph (Kyoto, JP) equipped with LC 20AD binary gradient pump and Shimadzu Sil 20A Autosampler. *H. lupulus* bitter acids were analysed on chromatographic column Nucleosil with C-18 Hop reversed phase (particle size 5 μm, 250 x 4.0 mm; Macherey Nagel, Düren, DE). The separation was in isocratic mode and the mobile phase was a mixture of 850 ml of methanol, 150 ml of water and 5 ml of phosphoric acid. The column temperature was set to 40°C, the flow rate was 0.8 mL/min and the injection volume was 10 μL. The bitter acids

were detected at the wavelength of 314 nm with SPD-M20A diode array detector. An external standard ICE 3 (Labor Veritas AG, Zürich, CH) exclusively used for determination of α- and β-acid content (International Hop Standard Committee, 2010) was used for the method calibration.

4.6. Microorganism cultures and media

The inhibitory activity of GRAS plant species extracts was evaluated against the major oral bacteria associated with caries and periodontal diseases comprising both Gram-positive and Gram-negative strains and against one pathogenic yeast. *Aggregatibacter actinomycetemcomitans* (DSM 8324), *Fusobacterium nucleatum* subsp. *vincentii* (DSM 19507) and oral streptococci, namely *Streptococcus mutans* (DSM 6178), *S. salivarius* subsp. *salivarius* (DSM 20560) and *S. sobrinus* (DSM 20381) were obtained from German Collection of Microorganisms and Cell Cultures of Leibniz Institute (Braunschweig, DE), whereas oral bifidobacteria, namely *Bifidobacterium dentium* (CCM 7206), *B. longum* (CCM 4990) together with *Lactobacillus salivarius* subsp. *salivarius* (CCM 7561) were purchased from Czech Collection of Microorganisms (Brno, CZ). *Eikenella corrodens* (ATCC 23834), *Porphyromonas gingivalis* (ATCC 33277) and the yeast, *Candida albicans* (ATCC 10231), were obtained from American Type Culture Collection through Oxoid (Basingstoke, UK).

Cation-adjusted Mueller-Hinton broth (Oxoid) equilibrated with Tris-buffered saline (Sigma-Aldrich) was used for aerobic cultivation of *C. albicans* at 37°C for 24 h. The bifidobacteria and lactobacilli were grown in Wilkins-Chalgren Anaerobe broth (Oxoid) supplemented with 5 g/L of soya peptone and 0.5 g/L of cysteine (both Oxoid), whereas the streptococci were grown in the Brain Heart Infusion (Oxoid). For stimulation of growth of *A. actinomycetemcomitans*, *E. corrodens* and *P. gingivalis*, 10 % of bovine calf serum (Sigma-Aldrich) and 1 % of laked horse blood (Oxoid) was added into Brain Heart Infusion. *F. nucleatum* subsp. *vincentii* was cultured in Schaedler Anaerobe Broth (Oxoid). All bacteria were incubated anaerobically at 37°C for 48 h.

4.7. *In vitro* determination of minimum inhibitory concentration (MIC)

The antimicrobial assays were performed by the broth microdilution technique using 96-well micro-plates as described by Hecht (1999) and Jorgensen *et al*. (1999). The

method was modified according to the recommendations proposed for more effective assessment of anti-infective potential of natural products (Cos *et al*., 2006). All steps related with the bacteria were done anaerobically (10 % CO_2, 10 % H_2, 80 % N_2) in Bugbox anaerobic chamber (BioTrace, Bracknell, UK), whereas susceptibility of the yeast was tested under aerobic conditions in laboratory laminar Airflow Cabinet BHC 2004 (BDSL, Dreghorn, UK).

For the initial stage I of the antimicrobial activity testing, fourfold dilution of plant extracts at concentrations ranging from 16 to 4096 μg/mL was prepared in appropriate medium. Subsequently, a stage II and stage III of twofold dilution of the most promising plant extracts were carried out at the concentration range from 1 to 512 μg/mL. Tested wells were then inoculated with 5 μL of microbial suspension at density of 10^5 colony-forming units/mL determined by the McFarland scale. The density of the inoculums was adjusted by the densitometer Densi-La-Meter (Lachema, Neratovice, CZ). After that, the bacterial strains were incubated at 37°C for 48 h in anaerobic jars (Oxoid) using Oxoid anaerobic system, while the yeast was incubated aerobically at 37°C for 48 h. The growth of microorganisms was evaluated spectrophotometrically as turbidity at 405 nm using a Multiscan Ascent Microplate Reader (Thermo Fisher Scientific, Waltham, USA). The MICs were defined as the lowest concentration of a compound inhibiting growth of the test microorganisms by ≥80 % compared with compound-free growth control. The tests were performed in triplicate in three independent experiments and median values were used for MIC calculation. Fourfold dilution of DMSO starting at concentration 8% was tested as the negative control in the stage I, positive control (chlorhexidine) was tested at the concentration range 1 – 512 μg/mL in the stage II.

5. Results

5.1. Antimicrobial activity of GRAS plant species

5.1.1. Preliminary antimicrobial screening of extracts

In the first stage of the study, preliminary screening of the *in vitro* inhibitory effects of all 109 GRAS plant species extracts against the oral pathogenic microorganisms under study was made. The results of the screening revealed that all 109 GRAS extracts possessed inhibitory activity against one or more microorganisms at MIC ≤ 4096 μg/mL. All MICs of the GRAS plant species from this first stage of the study are summarized in Table 6. The best results were obtained for the *H. lupulus* extract as it showed an antimicrobial effect against all pathogenic strains at MIC ≤ 1024 μg/mL. Besides this extract, other 24 species, namely *Capsicum annuum*, *Capsicum frutescens*, *Cinnamomum cassia*, *Coffea arabica*, *Coffea robusta*, *Cola acuminata*, *Curcuma zedoaria*, *Ferula assa-foetida*, *Helichrysum angustifolium*, *Medicago sativa*, *Mentha crispa*, *Myristica fragrans*, *Pimenta officinalis*, *Prunus spinosa*, *Punica granatum*, *Rosa centifolia*, *Sambucus nigra*, *Sesamum indicum*, *Taraxacum officinale*, *Thea sinensis*, *Thymus serpyllum*, *Tilia cordata*, *Trifolium pratense* and *Zanthoxylum clava-herculis*, possessed significant inhibitory properties with MIC < 256 μg/mL against at least one pathogen. From the Gram-positive bacteria, the most susceptible strain was *S. salivarius* subsp. *salivarius* with MICs ≥ 16 μg/mL, followed by other oral streptococci (MICs ≥ 64 μg/mL), *B. dentium* and *L. salivarius* subsp. *salivarius* (MICs ≥ 256 μg/mL), whereas *B. longum* was the most resistant Gram-positive bacteria with MICs being ≥ 1024 μg/mL. From the Gram-negative bacteria, *F. nucleatum* subsp. *vincentii* was the most susceptible one with the MICs ≥ 16 μg/mL, followed by *P. gingivalis*, *A. actinomycetemcomitans* with the MICs ≥ 64 μg/mL and ≥ 256 μg/mL, respectively. *E. corrodens* with the MICs for all plant extracts being ≥ 1024 μg/mL was the most resistant Gram-negative bacteria. The MICs obtained for *C. albicans* were ≥ 256 μg/mL. There was no growth inhibition caused by DMSO (negative control) in the given concentration range of this stage. The sensitivity of all microbial strains to the positive control ranged from 0.125 to 2 μg/mL for specific antibiotic tested.

Table 6 Origin and antimicrobial activities of ethanol extracts from GRAS plant species (first stage of the study): [a] Bd, *Bifidobacterium dentium*; Bl, *Bifidobacterium longum*; Ls, *Lactobacillus salivarius*; Sm, *Streptococcus mutans*; Ssa, *Streptococcus salivarius*; Sso, *Streptococcus sobrinus*; Aa, *Aggregatibacter actinomycetemcomitans*; Ec, *Eikenella corrodens*; Fn, *Fusobacterium nucleatum*; Pg, *Porphyromonas gingivalis*; Ca, *Candida albicans*; [b] not active (> 4096 µg/mL); [c] A, Ampicillin (for *L. salivarius*); N, Nystatin (for *C. albicans*); P, Penicillin G (for *E. corrodens*); and T, Tetracycline (for *B. dentium, B. longum, S. mutans, S. salivarius, S. sobrinus, F. nucleatum* and *P. gingivalis*) were used as positive controls

Species or reference compound	Yield (%)	Microorgamisms[a] / Minimum inhibitory concentration (µg/mL)										
		Gram-positive bacteria						Gram-negative bacteria				Yeast
		Bd	Bl	Ls	Sm	Ssa	Sso	Aa	Ec	Fn	Pg	Ca
Agropyron repens	14.84	4096	4096	-[b]	-	-	1024	4096	-	4096	-	4096
Allium cepa	75.40	4096	4096	-	-	-	-	-	4096	4096	-	4096
Allium schoenoprasum	50.60	-	-	-	-	-	-	-	-	4096	-	-
Alpinia officinarum	19.46	4096	4096	-	4096	1024	-	1024	-	256	-	256
Amomum melegueta	5.93	4096	4096	-	1024	1024	256	1024	-	1024	4096	1024
Angelica archangelica	49.80	4096	4096	-	4096	-	1024	-	-	1024	-	1024
Anthemis nobilis	31.20	4096	4096	-	-	-	1024	256	-	64	4096	-
Anthriscus cerefolium	41.00	-	4096	-	-	-	-	4096	-	1024	-	256
Apium graveolens	61.91	4096	4096	-	4096	-	1024	1024	1024	256	4096	256
Armoracia lapathifolia	23.33	-	4096	-	-	-	1024	-	-	4096	-	-
Artemisia dracunculus	27.30	4096	-	-	1024	-	1024	-	4096	1024	4096	256
Brassica alba	48.04	4096	-	-	-	-	4096	-	-	1024	-	1024
Brassica nigra	14.33	-	-	-	-	-	1024	-	-	4096	-	-
Calendula officinalis	39.00	-	-	-	-	-	1024	4096	-	1024	-	1024
Cananga odorata	42.90	-	-	-	-	-	1024	-	4096	1024	-	1024
Capparis spinosa	20.00	-	-	-	-	-	256	-	4096	4096	-	-
Capsicum annum	46.33	4096	4096	-	1024	256	64	256	4096	16	4096	-
Capsicum frutescens	35.20	-	4096	-	1024	64	256	4096	-	1024	-	256
Carum carvi	14.70	-	4096	-	4096	4096	1024	1024	1024	1024	-	256

Species or reference compound	Yield (%)	Microorgamisms[a] / Minimum inhibitory concentration (µg/mL)										
		Gram-positive bacteria						Gram-negative bacteria				Yeast
		Bd	Bl	Ls	Sm	Ssa	Sso	Aa	Ec	Fn	Pg	Ca
Carya illinoinensis	11.60	4096	4096	4096	-	-	-	-	-	4096	-	1024
Ceratonia siliqua	63.87	4096	4096	-	-	-	4096	4096	-	1024	-	4096
Cichorium intybus	11.66	4096	-	-	4096	-	1024	4096	-	1024	-	1024
Cinnamomum burmannii	34.00	4096	4096	-	-	4096	-	1024	1024	1024	-	256
Cinnamomum cassia	31.00	4096	4096	-	-	-	4096	4096	1024	16	-	4096
Cinnamomum zeylanicum	93.15	-	4096	-	-	4096	-	1024	-	256	-	1024
Citrus aurantifolia	50.40	4096	4096	-	-	-	-	-	-	4096	-	4096
Citrus aurantium	52.93	4096	4096	-	-	-	4096	4096	-	1024	-	-
Citrus limon	34.66	4096	4096	-	4096	-	1024	-	-	4096	-	4096
Citrus paradisi	49.60	-	-	-	-	-	4096	4096	-	4096	-	4096
Citrus reticulata	28.50	-	-	-	4096	4096	1024	-	-	4096	4096	4096
Citrus sinensis	45.00	-	4096	-	4096	-	4096	-	-	4096	-	4096
Coffea arabica	20.13	4096	4096	-	-	-	-	256	1024	16	-	4096
Coffea robusta	23.47	4096	-	-	-	-	4096	1024	1024	64	4096	4096
Cola acuminata	23.73	4096	4096	-	-	-	-	-	-	64	-	4096
Coriandrum sativum	7.30	4096	-	-	4096	4096	4096	1024	-	256	4096	1024
Crocus sativus	59.50	4096	-	-	4096	-	-	-	4096	1024	-	4096
Cuminum cyminum	35.69	-	4096	-	-	-	1024	-	-	1024	4096	1024
Curcuma longa	16.40	4096	-	256	1024	1024	-	4096	4096	256	-	256
Curcuma zedoaria	10.87	-	4096	-	-	4096	-	4096	-	64	-	4096
Cymbopogon citratus	24.20	4096	4096	-	4096	-	256	1024	4096	1024	4096	256
Daucus carota	60.27	-	4096	-	-	-	-	4096	-	4096	-	4096
Elettaria cardamomum	15.73	4096	-	-	-	-	-	-	-	1024	-	1024
Erythroxylum catuaba	20.13	-	-	-	-	-	-	-	-	4096	-	4096
Ferula assa-foetida	28.00	4096	-	-	4096	-	256	256	1024	16	-	4096
Foeniculum vulgare	17.60	-	-	-	4096	4096	4096	-	-	1024	-	256

Species or reference compound	Yield (%)	Microorgamisms[a] / Minimum inhibitory concentration (µg/mL)										
		Gram-positive bacteria						Gram-negative bacteria				Yeast
		Bd	Bl	Ls	Sm	Ssa	Sso	Aa	Ec	Fn	Pg	Ca
Galipea officinalis	32.93	4096	4096	-	-	-	256	1024	-	1024	4096	-
Helichrysum angustifolium	32.85	1024	4096	4096	256	64	256	256	-	256	64	4096
Hibiscus abelmoschus	15.33	-	4096	-	-	-	-	4096	-	4096	-	4096
H. lupulus 'Žatecký poloraný červeňák'	40.00	256	1024	1024	64	16	64	256	1024	256	64	256
Hyssopus officinalis	13.33	4096	-	-	-	-	-	4096	4096	256	-	4096
Chimaphila umbellata	46.40	4096	4096	4096	-	4096	4096	-	-	1024	-	1024
Ilex paraguariensis	39.00	4096	-	4096	4096	-	1024	-	-	256	4096	4096
Illicium verum	28.93	-	-	-	-	-	-	-	-	256	4096	-
Jasminum officinale	39.93	4096	4096	-	4096	-	1024	-	-	256	4096	-
Juniperus communis	54.53	4096	4096	-	256	256	256	4096	-	256	1024	4096
Laurus nobilis	25.20	4096	-	-	-	4096	1024	4096	-	256	-	1024
Lavandula officinalis	27.33	4096	-	-	-	-	4096	-	-	1024	-	256
Majorana hortensis	28.13	4096	-	-	-	-	1024	1024	1024	1024	-	4096
Marrubium vulgare	20.47	-	-	-	4096	4096	1024	-	-	256	-	4096
Matricaria chamomilla	30.26	4096	-	-	-	-	1024	1024	-	256	4096	4096
Medicago sativa	19.07	4096	-	-	4096	-	1024	-	-	64	-	4096
Melissa officinalis	27.90	4096	-	-	-	-	1024	4096	-	1024	-	1024
Mentha crispa	23.90	4096	-	-	4096	4096	1024	4096	-	16	-	-
Mentha spicata	25.80	4096	-	-	4096	-	1024	1024	-	256	-	4096
Mentha x piperita	14.73	4096	-	-	1024	4096	256	1024	4096	256	4096	256
Monarda punctata	21.27	4096	-	-	-	-	4096	-	-	1024	4096	-
Myristica fragrans	16.13	1024	4096	4096	256	256	-	1024	-	64	1024	1024
Nigella sativa	37.93	4096	4096	-	4096	4096	4096	-	-	4096	-	1024
Ocimum basalicum	13.93	4096	-	-	-	4096	4096	4096	-	256	4096	-
Ocimum minimum	14.41	-	-	-	-	-	1024	4096	4096	1024	-	4096
Origanum vulgare	16.00	-	-	-	-	-	1024	-	-	1024	4096	4096

Species or reference compound	Yield (%)	Microorgamisms[a] / Minimum inhibitory concentration (µg/mL)										
		Gram-positive bacteria						Gram-negative bacteria				Yeast
		Bd	Bl	Ls	Sm	Ssa	Sso	Aa	Ec	Fn	Pg	Ca
Papaver somniferum	10.27	-	-	-	4096	4096	1024	4096	4096	256	-	1024
Pelargonium graveolens	9.87	4096	4096	4096	4096	-	4096	-	-	4096	-	4096
Pelargonium odoratissimum	16.89	4096	4096	4096	-	-	-	4096	-	256	-	1024
Petroselinum crispum	35.27	-	-	-	-	-	-	4096	-	4096	-	4096
Pimenta officinalis	30.27	4096	4096	4096	-	1024	1024	1024	-	64	-	4096
Pimpinella anisum	23.53	4096	1024	-	4096	4096	4096	4096	-	256	4096	4096
Piper nigrum	13.93	4096	4096	-	-	4096	1024	1024	-	256	-	256
Prunus amygdalus	11.33	-	-	-	-	-	4096	-	-	4096	-	4096
Prunus serotina	17.13	4096	4096	-	-	-	-	-	4096	4096	-	4096
Prunus spinosa	27.40	4096	4096	-	4096	4096	4096	4096	1024	64	4096	4096
Psidium cattleyanum	37.67	4096	-	4096	-	-	1024	4096	-	256	-	256
Punica granatum	52.20	4096	4096	-	4096	4096	1024	1024	-	64	4096	1024
Rosa centifolia	29.93	4096	4096	-	4096	4096	4096	4096	-	64	4096	256
Rosa damascena	30.67	4096	4096	4096	4096	4096	-	1024	1024	256	-	1024
Rosmarinus officinalis	71.26	4096	4096	-	4096	1024	4096	4096	-	1024	4096	1024
Saccharum officinarum	52.40	4096	4096	-	-	-	1024	-	-	4096	-	4096
Salvia lavandulifolia	31.53	4096	4096	4096	1024	1024	1024	-	-	1024	-	4096
Salvia officinalis	28.73	4096	4096	-	1024	1024	-	-	-	256	-	4096
Salvia sclarea	35.75	4096	-	-	-	-	1024	4096	4096	1024	-	4096
Sambucus nigra	31.53	4096	4096	-	4096	4096	1024	-	-	64	4096	4096
Satureja hortensis	22.53	4096	-	-	1024	-	1024	-	-	256	-	4096
Satureja montana	23.67	4096	-	-	-	4096	1024	4096	-	1024	-	256
Sesamum indicum	12.30	4096	4096	-	4096	-	1024	4096	4096	64	-	-
Schinus molle	46.33	-	4096	-	-	-	4096	4096	4096	4096	-	-
Tamarindus indicus	49.80	-	-	-	-	-	-	-	4096	4096	-	4096
Taraxacum officinale	21.60	4096	4096	-	4096	-	1024	4096	-	64	4096	-

Species or reference compound	Yield (%)	Microorgamisms[a] / Minimum inhibitory concentration (µg/mL)										
		Gram-positive bacteria						Gram-negative bacteria				Yeast
		Bd	**Bl**	**Ls**	**Sm**	**Ssa**	**Sso**	**Aa**	**Ec**	**Fn**	**Pg**	**Ca**
Thea sinensis	30.80	4096	-	-	4096	4096	1024	4096	4096	64	-	256
Theobroma cacao	13.20	4096	-	-	-	-	-	-	-	1024	-	-
Thymus serpyllum	19.40	4096	4096	-	4096	4096	4096	-	-	64	-	4096
Thymus vulgaris	22.67	-	-	-	4096	-	1024	-	-	256	-	4096
Thymus zygis	28.05	4096	-	-	4096	4096	4096	1024	4096	4096	4096	256
Tilia cordata	20.87	4096	4096	-	-	-	4096	-	-	64	4096	4096
Trifolium pratense	21.27	4096	4096	-	-	-	-	-	-	64	-	1024
Trigonella foenum-graecum	22.13	4096	4096	-	1024	4096	1024	4096	-	256	4096	4096
Vanilla planifolia	45.33	4096	-	-	-	-	-	-	-	256	-	4096
Viola odorata	32.75	4096	4096	-	4096	4096	1024	-	-	4096	-	4096
Xanthoxylum clava-herculis	23.60	4096	4096	-	1024	64	64	256	1024	256	4096	256
Zingiber officinale	17.73	-	4096	-	-	-	-	4096	-	256	-	4096
DMSO	-	-	-	-	-	-	-	-	-	-	-	-
A/N/P/T[c]	-	0.5	1	1	1	0.25	1	0.125	2	0.125	0.25	2

5.1.2. Detailed antimicrobial evaluation of the most potent extracts

The most promising 25 GRAS plant species extracts with MICs < 256 μg/mL to at least one microorganism from the first part of the study were subsequently tested in the second stage in order to determine more precisely their antimicrobial activities. These GRAS species were *C. annuum, C. frutescens, C. cassia, C. arabica, C. robusta, C. acuminata, C. zedoaria, F. assa-foetida, H. angustifolium, H. lupulus, M. sativa, M. crispa, M. fragrans, P. officinalis, P. spinosa, P. granatum, R. centifolia, S. nigra, S. indicum, T. officinale, T. sinensis, T. serpyllum, T. cordata, T. pratense* and *Z. clava-herculis*. Additionally, we included six different *H. lupulus* cultivars in this second stage, namely *H. lupulus* 'Agnus', *H. lupulus* 'Harmonie', *H. lupulus* 'Kazbek', *H. lupulus* 'Premiant', *H. lupulus* 'Saaz Late'and *H. lupulus* 'Žatecký poloraný červeňák' since the *H. lupulus* extract exhibited the most significant antibacterial properties against oral pathogens in the first stage. Therefore, we decided to compare the inhibitory properties of different *H. lupulus* cultivars. All 30 extracts were tested as in the prior preliminary screening against six Gram-positive bacteria (*B. dentium, B. longum, L. salivarius* subsp. *salivarius, S. mutans, S. salivarius* subsp. *salivarius, S. sobrinus*), four Gram-negative bacteria (*A. actinomycetemcomitans, E. corrodens, F. nucleatum* subsp. *vincentii, P. gingivalis*) and one yeast strain (*Candida albicans*). The inhibitory growth effects of the 24 GRAS plant extracts together with the MICs of the extracts of six *H. lupulus* cultivars are summarized in Table 7.

The best results from this part of study were obtained for *H. lupulus*, as all its cultivars inhibited at least one oral pathogen at concentration ≤ 64 μg/mL. The research revealed that the extract from cultivar 'Harmonie' possessed the strongest antimicrobial activity with MICs ≥ 16 μg/mL, followed by cultivars 'Kazbek', 'Premiant', 'Saaz Late'and 'Žatecký poloraný červeňák' (MICs being ≥ 32 μg/mL). The cultivar 'Agnus' showed the weakest inhibitory effect against the microorganisms tested as its MICs were ≥ 64 μg/mL. Generally, the Gram-positive bacteria were more susceptible to the *H. lupulus* extracts (MICs ranging from 16 to 256 μg/mL) than were Gram-negative ones (MICs being ≥ 32 μg/mL) and the yeast (MICs being ≥ 256 μg/mL). From the Gram-positive

bacteria, *S. salivarius* subsp. *salivarius* was the most susceptible (MICs 16 – 64 μg/mL), followed by *S. sobrinus* (MICs 16 – 128 μg/mL), *B. longum* and *S. mutans* (MICs 64 – 128 μg/mL) and *L. salivarius* subsp. *salivarius* (MICs 64 – 256 μg/mL). *B. dentium* possessed the highest resistance to the *H. lupulus* extracts with MICs being from 128 to 256 μg/mL. *P. gingivalis* was the most susceptible Gram-negative bacteria to the *H. lupulus* extracts (MICs 64 – 128 μg/mL), followed by *F. nucleatum* subsp. *vincentii* (MICs 256 – 512 μg/mL), whereas *A. actinomycetemcomitans* and *E. corrodens* were the most resistant to all *H. lupulus* cultivars (MICs being ≥ 512 μg/mL). The MICs of all *H. lupulus* extracts for *C. albicans* were ≥ 256 μg/mL.

Table 7 Antimicrobial activities of ethanol extracts of the most promising 25 GRAS plant species including six different cultivars of *H. lupulus* (second stage of the study): [a] Bd, *Bifidobacterium dentium*; Bl, *Bifidobacterium longum*; Ls, *Lactobacillus salivarius*; Sm, *Streptococcus mutans*; Ssa, *Streptococcus salivarius*; Sso, *Streptococcus sobrinus*; Aa, *Aggregatibacter actinomycetemcomitans*; Ec, *Eikenella corrodens*; Fn, *Fusobacterium nucleatum*; Pg, *Porphyromonas gingivalis*; Ca, *Candida albicans*; [b] not active (> 512 μg/mL); [c] A, Ampicillin (for *L. salivarius*); N, Nystatin (for *C. albicans*); P, Penicillin G (for *E. corrodens*); and T, Tetracycline (for *B. dentium, B. longum, S. mutans, S. salivarius, S. sobrinus, F. nucleatum* and *P. gingivalis*) were used as positive controls.

Species or reference compound	**Microorgamisms[a] / Minimum inhibitory concentration (μg/mL)**										
	Gram-positive bacteria						**Gram-negative bacteria**				**Yeast**
	Bd	**Bl**	**Ls**	**Sm**	**Ssa**	**Sso**	**Aa**	**Ec**	**Fn**	**Pg**	**Ca**
Humulus lupulus 'Agnus'	256	128	128	128	64	64	-[b]	-	256	128	256
H. lupulus 'Harmonie'	128	64	64	64	16	16	-	-	256	64	256
H. lupulus 'Kazbek'	256	128	128	128	32	64	-	-	512	128	-
H. lupulus 'Premiant'	256	64	128	64	32	64	-	512	256	128	512
H. lupulus 'Saaz Late'	128	64	128	128	32	128	-	-	512	128	512
H. l. 'Žatecký poloraný červeňák'	256	128	256	128	32	64	-	-	512	128	-
Capsicum annuum	-	-	-	256	64	64	-	-	-	512	-
Capsicum frutescens	-	-	-	256	128	64	-	-	-	512	-
Cinnamomum cassia	-	512	-	-	-	-	-	512	256	512	-
Coffea arabica	-	-	-	-	-	-	-	-	256	-	-
Coffea robusta	-	-	-	-	-	-	-	-	256	-	-
Cola acuminata	-	-	-	-	-	-	-	-	256	-	-
Curcuma zedoaria	-	-	-	-	-	512	-	-	256	512	-
Ferula assa-foetida	-	-	-	-	-	512	-	-	256	256	512
Helichrysum angustifolium	-	-	-	256	128	512	-	-	-	-	-
Medicago sativa	-	-	-	-	-	512	-	-	256	-	-
Mentha crispa	-	-	-	-	-	512	-	-	256	-	-

Species or reference compound	Microorgamisms[a] / Minimum inhibitory concentration (μg/mL)										
	Gram-positive bacteria						Gram-negative bacteria				Yeast
	Bd	**Bl**	**Ls**	**Sm**	**Ssa**	**Sso**	**Aa**	**Ec**	**Fn**	**Pg**	**Ca**
Myristica fragrans	-	-	-	256	256	128	512	512	64	512	512
Pimenta officinalis	-	512	-	-	-	512	-	512	128	-	-
Prunus spinosa	-	-	-	-	-	512	-	-	256	-	-
Punica granatum	-	-	-	-	-	-	-	-	64	-	128
Rosa centifolia	-	-	-	-	-	-	-	-	256	-	256
Sambucus nigra	-	-	-	-	-	512	-	-	256	-	-
Sesamum indicum	-	-	-	-	-	-	-	-	256	-	-
Taraxacum officinale	-	-	-	-	-	512	-	-	256	-	-
Thea sinensis	-	-	-	-	-	512	-	-	128	-	-
Thymus serpyllum	-	-	-	-	-	512	-	-	256	-	-
Tilia cordata	-	-	-	-	-	512	-	-	256	-	-
Trifolium pratense	-	-	-	-	-	512	-	-	256	-	-
Zanthoxylum clava-herculis	-	-	-	256	128	64	-	-	512	512	-
Chlorhexidin	8	4	16	2	2	<1	8	8	4	4	4
A/N/P/T[c]	0.5	1	1	1	0.25	1	0.125	2	0.125	0.25	2

As far as other species are concerned, *C. annuum* and *C. frutescens* were found to possess significant antimicrobial potential against *S. salivarius* subsp. *salivarius* with the MIC of 64 and 128 μg/mL, respectively, and against *S. sobrinus* (MIC = 64 μg/mL). *H. angustifolium* exhibited the antibacterial activity against *S. salivarius* subsp. *salivarius* with MIC = 128 μg/mL. The growth of *S. salivarius* subsp. *salivarius* was also inhibited by the extract of *Z. clava-herculis* (MIC = 128 μg/mL). Moreover, *Z. clava-herculis* possessed the antimicrobial properties against *S. sobrinus* at MIC = 64 μg/mL. Interesting inhibitory effects showed also the extract of *M. fragrans* against *S. sobrinus* (MIC = 128 μg/mL) and against *F. nucleatum* subsp. *vincentii* (MIC = 64 μg/mL), whereas *P. granatum* exhibited the antimicrobial potential against *C. albicans* (MIC = 128 μg/mL) and against *F. nucleatum* subsp. *vincentii* (MIC = 64 μg/mL). The growth of *F. nucleatum* subsp. *vincentii* was further inhibited by the extracts of *P. officinalis* and *T. sinensis* at MIC of 128 μg/mL.

The 16 remaining GRAS plant extracts showed no antimicrobial activity with MIC values ≥ 256 μg/mL. Generally, the most resistant microorganisms to the extracts from the second stage were *A. actinomycetemcomitans* and *E. corrodens* with MICs ≥ 512 μg/mL.

In contrast, *S. salivarius* subsp. *salivarius* and *S. sobrinus* were the most susceptible ones with MICs ≥ 16 μg/mL. The sensitivity of all microbial strains to the positive controls ranged from <1 to 16 μg/mL for chlorhexidine and from 0.125 to 2 μg/mL for specific antibiotic tested.

5.2. Antimicrobial activity and chemical analysis of CO_2 supercritical *H. lupulus* extracts

After obtaining the results of the interesting antimicrobial properties of all *H. lupulus* ethanol extracts against Gram-positive and some of the Gram-negative bacteria in the second part of the study, we decided to make an evaluation of an effect of different extraction method on these properties. Thus, we prepared two CO_2 supercritical *H. lupulus* extracts from two *H. lupulus* cultivars, namely 'Herkules' and 'Hallertau Magnum' and tested their inhibitory activity against the same widerange of oral pathogenic microorganisms as in the previous stages, i.e against six Gram-positive bacteria (*B. dentium, B. longum, L. salivarius* subsp. *salivarius, S. mutans, S. salivarius* subsp. *salivarius, S. sobrinus*), four Gram-negative bacteria (*A. actinomycetemcomitans, E. corrodens, F. nucleatum* subsp. *vincentii, P. gingivalis*) and one yeast strain (*Candida albicans*). The results of this evaluation of the inhibitory properties of two CO_2 supercritical *H. lupulus* extracts can be seen in Table 8.

Table 8 Antimicrobial activities of CO_2 supercritical *H. lupulus* extracts (third stage of the study): [a] Bd, *Bifidobacterium dentium*; Bl, *Bifidobacterium longum*; Ls, *Lactobacillus salivarius*; Sm, *Streptococcus mutans*; Ssa, *Streptococcus salivarius*; Sso, *Streptococcus sobrinus*; Aa, *Aggregatibacter actinomycetemcomitans*; Ec, *Eikenella corrodens*; Fn, *Fusobacterium nucleatum*; Pg, *Porphyromonas gingivalis*; Ca, *Candida albicans*; [b] A, Ampicillin (for *L. salivarius*); N, Nystatin (for *C. albicans*); P, Penicillin G (for *E. corrodens*); and T, Tetracycline (for *B. dentium, B. longum, S. mutans, S. salivarius, S. sobrinus, F. nucleatum* and *P. gingivalis*) were used as positive controls.

Hop cultivar or reference compound	**Microorgamisms[a] / Minimum inhibitory concentration (μg/mL)**										
	Gram-positive bacteria						**Gram-negative bacteria**				**Yeast**
	Bd	**Bl**	**Ls**	**Sm**	**Ssa**	**Sso**	**Aa**	**Ec**	**Fn**	**Pg**	**Ca**
H. lupulus 'Hallertau Magnum'	64	32	32	16	8	16	512	>512	128	32	512
H. lupulus Herkules	64	32	32	32	8	16	512	512	256	32	512
Chlorhexidin	8	4	16	2	2	<1	8	8	4	4	4
A/N/P/T[b]	0.5	1	1	1	0.25	1	0.125	2	0.125	0.25	2

Among all *H. lupulus* samples included in the whole study, the CO_2 supercritical extracts of cultivars 'Hallertau Magnum' and 'Herkules' showed the strongest antimicrobial activity with MICs from 8 to ≥ 512 μg/mL and from 8 to 512 μg/mL, respectively. Gram-positive bacteria were once again more susceptible to these extracts (MICs ranging from 8 to 64 μg/mL) than Gram-negative ones (MICs being ≥ 32 μg/mL) and the yeast (MIC for both extracts was 512 μg/mL). Among the Gram-positive bacteria, the growth of *S. salivarius* subsp. *salivarius* was inhibited the most (MICs = 8 μg/mL), followed by *S. sobrinus* (MICs = 16 μg/mL), *S. mutans* (MICs from 16 to 32 μg/mL), *B. longum* and *L. salivarius* subsp. *salivarius* (MICs = 32 μg/mL), while *B. dentium* was of the highest resistance with MICs being 64 μg/mL. From the group of Gram-negative bacteria, *P. gingivalis* was the most susceptible one (MICs = 32 μg/mL), followed by *F. nucleatum* subsp. *vincentii* (MICs from 128 to 256 μg/mL). On the other hand, *A. actinomycetemcomitans* together with *E. corrodens* were the most resistant bacteria to the SFE *H. lupulus* extracts (MICs ≥ 512 μg/mL). Similarly, *C. albicans*, the only yeast strain in the study, exhibit the highest resistancy to these extracts with MICs = 512 μg/mL.

Consequently, after showing such interesting growth inhibition of all Gram-positive and two Gram-negative bacteria, the bitter acids of the CO_2 supercritical *H. lupulus* extracts were analysed though the HPLC. Results of the analysis are given in Table 9.

Table 9 HPLC analysis of the *H. lupulus* CO_2 supercritical extracts.

Hop cultivar or reference compound	**Alpha acids (w/w)**	**Beta acids (w/w)**	**Cohumulone (% rel.)**	**Colupulone (% rel.)**
H. lupulus ‚Herkules'	55.2	18.3	38.7	57.2
H. lupulus ‚Hallertau Magnum'	46.9	22.9	24.9	44.2

Two main groups of constituents were identified (Fig. 5 and 6). *α*-acids were the major compounds being present by 55.2 w/w in the cultivar 'Herkules' and by 46.9 w/w in 'H. Magnum'. *β*-acids were the second predominant group of bitter acids, which were found by 18.3 w/w in the cultivar 'Herkules' and by 22.9 w/w in the cultivar 'H. Magnum'. The main prevalent *α*-acid in the CO_2 supercritical extracts was cohumulone that represented 38.7 % of *α*-acids in the cultivar 'Herkules' and 24.9 % in 'H. Magnum'. Colupulone was the dominant constituent of *β*-acids occurring in 57.2 % in 'Herkules' and in 44.2 % in 'H. Magnum'.

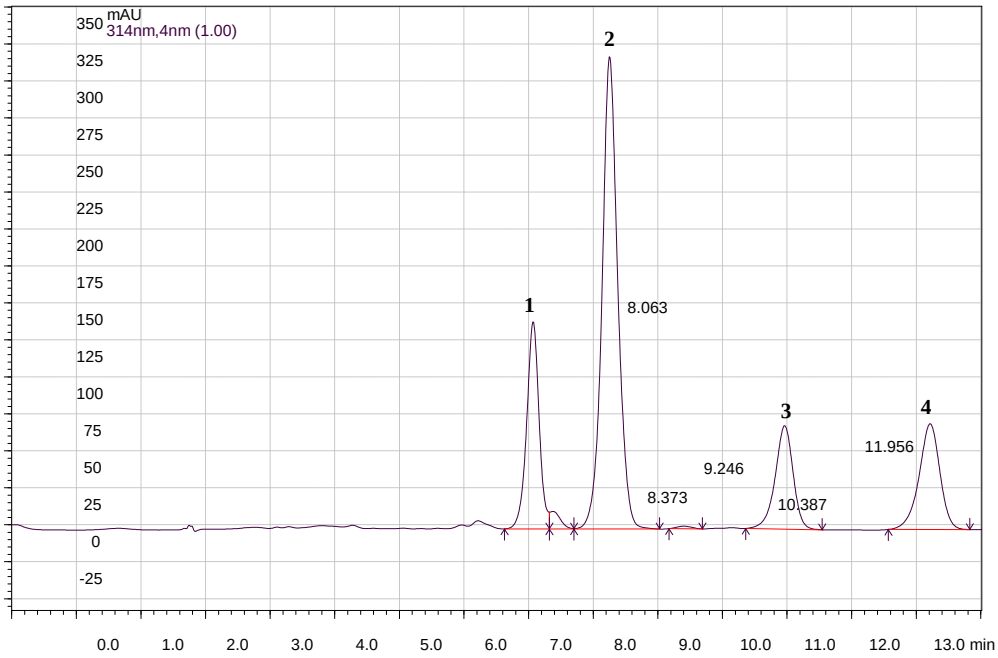


Figure 5 HPLC chromatogram of *H. lupulus* 'Hallertau Magnum'. *α*-acids: peak 1 (cohumulone) and peak 2 (n-humulone and adhumulone); *β*-acids: peak 3 (colupulone) and peak 4 (n-lupulone and adlupulone)

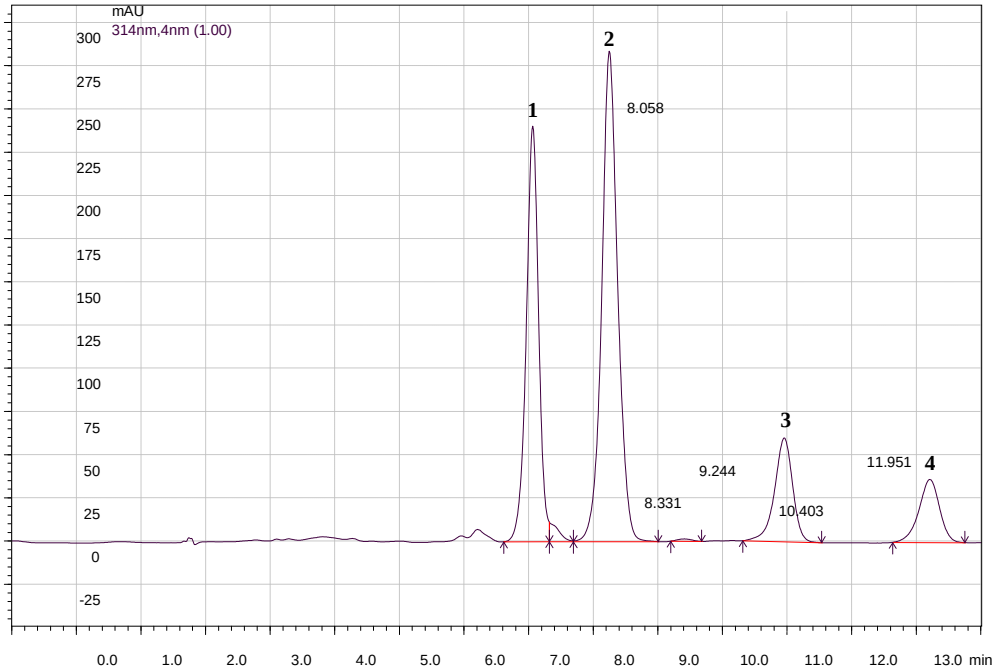


Figure 6 HPLC chromatogram of *H. lupulus* 'Herkules'. *α*-acids: peak 1 (cohumulone) and peak 2 (n-humulone and adhumulone); *β*-acids: peak 3 (colupulone) and peak 4 (n-lupulone and adlupulone)

6. Discussion

The majority of the GRAS plant species or their constituents have been already tested on their antimicrobial activity against various human pathogenic microorganisms as can be seen from the Table 7 (Material and methods). Among the species widely investigated for their antiseptic properties are *Cinnamomum* spp., *Citrus* spp., *Cymbopogon citratus*, *Foeniculum vulgare*, *Laurus nobilis*, *Matricaria chamomilla*, *Melissa officinalis*, *Rosmarinus officinalis*, *Salvia* spp. and *Thymus vulgaris*. On the other hand, only a few of the plant species qualified as GRAS, such as *Calendula officinalis*, *Mentha* spp., and *Thea sinensis*, have been tested for their inhibitory properties against some of the cariogenic and periodontal bacteria or candidal yeast (Iauk *et al*., 2003; Lauten *et al*., 2005; Shapiro *et al*., 1994; Smullen *et al*., 2007; Takarada *et al*., 2004). Moreover, to the best of our knowledge, no similar study evaluating their inhibitory effects against these microorganisms has hitherto been led.

Although in the first part of the study (in the preliminary antimicrobial screening) all of the ethanol extracts of the GRAS plant species included in the research exhibited to some extent the antiseptic effects against the microbes tested, only plant species with the MICs lower than 256 μg/mL could be considered for further research. This is based on one of the rule of thumb for anti-infective potential of natural products (Cos *et al*., 2006; Ríos and Recio, 2005) which defines the activity of an extract below 100 μg/mL to be very promising and worthy of deeper investigation. Thus, from 109 GRAS species only 25 showing the inhibitory effects with MIC lower than 256 μg/mL against at least one microorganism under the study were tested through two-fold dilution with starting extract concentration of 512 μg/mL in order to obtain more precise MIC of each species.

The results from the second part revealed that in correspondence with abovementioned recommendations proposed for a more effective *in vitro* antimicrobial assessment of natural products (Cos *et al*., 2006; Ríos and Recio, 2005), 6 GRAS plant species may be considered as very promising species for further research focused on development of novel herbal-based oral care preparations, namely *C. annuum*, *C. frutescens*, *H. lupulus*, *M. fragrans*, *P. granatum* and *Z. clava-herculis*. Furthermore, three other species were very

close to the MIC level of 100 μg/mL, namely *H. angustifolium, P. officinalis* and *T. sinensis*, and might be also interesting for the future research of antibacterial agents in oral hygiene products.

It is noteworthy that the MICs obtained for these nine extracts against some bacteria and yeast seem to be lower than the MICs previously reported for essential oils used frequently in oral hygiene products such as mouthrinses and toothpastes. For example, Chung *et al*. (2006) tested eucalyptol, thymol, menthol and methyl salicylate against *Streptococcus mutans* using dilution method. Eucalyptol exhibited the strongest antibacterial activity from all compounds with MIC of 250 μg/mL, followed by thymol and menthol with MIC of 500 μg/mL. On the other side, methyl salicylate showed only weak inhibitory properties with MIC being 1000 μg/mL. Also Didry *et al.* (1994) tested the antimicrobial activity of four antiseptic compounds used in oral diseases, namely thymol, carvacrol, cinnamaldehyde and eugenol through broth microdilution method on eigth oral bacteria (*S. mutans, S. sanguis, S. milleri, S. mitis, Peptostreptococcus anaerobius, Prevotella buccae, P. oris, P. intermedia*). The MICs reported for these compounds were in range from 31.25 to 500 μg/mL for cinnamaldehyde, from 125 to 500 μg/mL for thymol and carvacrol, and from 150 to 1000 μg/mL for eugenol depending on microbe tested. The results of our research are thus very interesting considering the fact that we tested complex extracts and no purified substances as in those previous studies.

Concerning the antiseptic properties of the particular effective species, *H. lupulus* extracts were found to have the broadest activity spectrum because all of them were effective in inhibiting the growth of Gram-positive bacteria and to some extend the growth of Gram-negative bacteria. These findings are in accordance with those published in literature as *H. lupulus* and its compounds are known to produce growth-inhibitory effect against human pathogenic bacteria, mainly the Gram-positive ones (Van Cleemput *et al*., 2009; Zanoli and Zavatti, 2008). Futhermore, its antibacterial actions against several oral pathogenic streptococci have been already studied. For example, Bhattacharya *et al*. (2003) reported the antimicrobial activities of *H. lupulus* constituents (purified *β*-acids, xanthohumol, iso-*α*-acid, tetra iso-*α*-acid) on three streptococcal strains (*S. mutans, S. salivarius, S. sanguis*). All compounds inhibited the growth with MICs from 2 to 50 μg/mL

depending on the bacterial strain and the phytochemical tested. It is quite noteworthy that these MICs of the compounds are fully comparable with our MICs obtained for entire *H. lupulus* extracts. *H. lupulus* bract polyphenols were also reported to have the inhibitory effect on cellular adherence of cariogenic streptococci, i.e. *S. mutans* and *S. sobrinus* (Tagashira *et al*., 1997). Moreover, our results agree with findings of several issued patents on oral preparations containing *H. lupulus* acids, which suggest that oral streptococci and other undesirable oral Gram-positive bacteria can be successfully inhibited by *H. lupulus* constituents at concentrations from 0.0001 % to 0.3 % (Barney *et al*., 1994; Haas, 1976; Trivedi *et al*., 2005). The antimicrobial potential of *H. lupulus* to reduce the dental plaque regrowth was confirmed by a recent double-blind crossover clinical trial (Shinada *et al*., 2007). The study showed that rinsing with a mouthrinse containing 0.1 % hop bract polyphenols lowered significantly the number of mutans streptococci and so, the dental biofilm reformation. In our research, the *H. lupulus* extracts inhibited to some extent also two of four Gram-negative bacteria, namely *F. nucleatum* subsp. *vincentii* and *P. gingivalis*. On the other hand, they exhibited only a slight effect on the resting two Gram-negative bacteria, i.e. *A. actinomycetemcomitans* and *E. corrodens*. Similar findings were described by Natarajan *et al*. (2008). They observed some antimicrobial action of *H. lupulus* constituents (lupulone and xanthohumol) against several human pathogenic Gram-negative bacteria (*Pseudomonas aeruginosa, Serratia marscenes*) but not against others (*E. coli, Proteus mirabilis*). Ohsugi *et al*. (1997) also reported antibacterial activity of *H. lupulus* methanol extract against various strains of Gram-negative bacterium *Helicobacter pylori* with MICs from 63 to 130 μg/mL. Contrarily, Barney *et al*. (1994), Srinivasan *et al*. (2004), nor Teuber (1970) observed any inhibitory effect of various *H. lupulus* compounds (e.g. colupulone, humulone, tetrahydroisohumulone) against Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*. The phenomenon of greater susceptibility of Gram-positive bacteria to *H. lupulus* constituents might be caused by the presence of the phospholipid containing outer membrane in Gram-negatives as *H. lupulus* bitter acids, the major antimicrobial compounds, are inactivated by serumphosphatides (Sacks and Humphreys, 1951). In our study, *C. albicans* was inhibited only slightly by all hop extracts with MICs ranging from 256 to ≥ 512 μg/mL. More potent anticandidal activity of *H. lupulus* constituents was reported in previous study (Mizobuchi and Sato, 1985) where

MICs ranged from 6.25 to ≥ 200 μg/mL. In contrast to that research, Barney *et al*. (1994) observed no inhibitory effect of *H. lupulus* compounds on *C. albicans*.

To the best of our knowledge, this is the first study evaluating the growth-inhibitory effect of ethanol and SFE extracts of different *H. lupulus* cultivars against broader spectrum of oral pathogens. The previous comparative study of antimicrobial activities of chloroform extracts prepared from 11 *H. lupulus* cultivars against human pathogenic microbes, namely *Bacillus subtilis, C. albicans, E. coli, Staphylococcus aureus* (Langezaal *et al*., 1992) showed that all extracts exhibited some antibacterial activity against the tested strains, primarily against Gram-positive bacteria. Similarly, all eight cultivars included in our study possessed certain degree of antimicrobial action; however, the SFE extracts showed more potent antimicrobial activities than the ethanol extracts. Such findings are in correspondence with previous reports comparing various extraction methods of *H. lupulus*. These studies concluded that supercritical CO_2 is much more effective solvent for selective isolation of *H. lupulus* bitter acids than ethanol, which besides them extracts also polyphenols, hard resins, fats, waxes and pigmented materials (Helmja *et al*., 2007; Langezaal *et al*., 1990; Laws *et al*., 1977; Mukhopadhyay, 2000).

The main class of antiseptically active compouds from *H. lupulus* cones have been identified as bitter acids (as mentioned above), which are phloroglucinol derivatives usually classified as *α*- and *β*-bitter acids (Simpson and Smith, 1992; Teuber and Schmalreck, 1973). The bitter acids are present in hops as a complex mixture of variable composition and concentrations. The main *α*-acids are humulone (35–70 % of total *α*-acids), cohumulone (20–65 %) and adhumulone (10–15 %); the corresponding *β*-acids are lupulone (30–55 % of total *β*-acids), colupulone (20–55 %) and adlupulone (5–10 %; Verzele, 1986). The mechanism of activity of the *H. lupulus* bitter acids has been described in several previous studies (Schmalreck and Teuber, 1974; Simpson, 1993; Teuber and Schmalreck, 1973). They have been reported to affect the transmembrane pH and cause primary membrane leakage due to the interaction of hydrophobic parts of the molecules with bacterial cell wall. This activity results in inhibition of active transport of sugars and amino acids and in inhibition of cellular respiration, which led subsequently to cell death.

Also the antibacterial actions of the other two promising species from our research, namely *M. fragrans* and *P. granatum* and their constituents against several oral pathogens have been already studied (Howell and D'Souza, 2013; Paul *et al*., 2013). Contrarily to the findings of Shafiei *et al*. (2012), who reported weak inhibitory properties of an ethanol extract from *M. fragrans* seed against several oral pathogenic bacteria (*A. actinomycetemcomitans, F. nucleatum, P. gingivalis, Streptococcus mitis, S. mutans, S. salivarius*) with MIC values ranging from 5 to 20 mg/mL, our results revealed that the *M. fragrans* seed extract possessed good antimicrobial potential against oral streptococci and particularly against *F. nucleatum* subsp. *vincentii*. The distinct results might be caused by different methodology since they used water stock solutions sterilized by membrane filtration, which might have influenced the amount of active principles. The major antibacterial compounds in *M. fragrans* seeds have been identified in previous studies as macelignan, malabaricones B and C, myristic acid, myristicin and trimyristin. All of them effectively inhibited the growth of both Gram-positive and Gram-negative human pathogens including oral bacteria with MIC range from 0.6 to 31.3 μg/mL (Chung *et al.*, 2006; Narasimhan and Dhake, 2006; Orabi *et al*., 1991; Shinohara *et al*., 1999). Such findings indicate that these compounds might contribute to the antiseptic properties of the extracts recorded in our research. Besides the antimicrobial effect of *M. fragrans* and its constituents, also the anti-plaque formation action has been previously described (Namba *et al*., 1985). In that study *M. fragrans* methanol extract potently inhibited the adherence of viable cells of *S. mutans* to smooth surfaces with IC_{50} of 10 – 30 μg/mL.

The antibacterial properties of an ethanol extract of *P. granatum* pericarp against *S. mutans* and *P. gingivalis* have been reported with MIC of 62.5 and 125 μg/mL, respectively (Rosas-Piñón *et al*., 2012). In contrast to those results, we observed significant inhibitory effect of the *P. granatum* whole fruit extract only against *F. nucleatum* subsp. *vincentii*, which might be because of different part used. Nevertheless, it is for the first time that such activity against *F. nucleatum* is described. On the other side, we observed a good anticandidal action of the *P. granatum* extract against *C. albicans*, which is in accordance with conclusions of a recent study describing a potent antifungal activity of *P. granatum* hydro-alcoholic extract against *C. albicans* (Endo *et al*., 2010). Furthermore, the potential of *P. granatum* to suppress effectively candidiasis has been confirmed by a

clinical trial (Vasconcelos *et al*., 2003). The positive effect of mouthrinsing with *P. granatum* standardised extract of 30% punicalagins and *P. granatum* hydro-alcoholic extract on reducing the risk of gingivitis and numbers of dental plaque bacteria has been also reported previously in other clinical studies (DiSilvestro *et al*., 2009; Menezes *et al*., 2006). Such results together with ours raise the possibility of effective use of *P. granatum* extracts in oral health products. Concerning the active principles of *P. granatum*, ellagic and gallic acids together with punicalagin have been determined as compounds possessing the major antimicrobial properties and are probably responsible for the inhibitory effects observed in our study (Endo *et al*., 2010; Ismail *et al*., 2012; Naz *et al*., 2007).

Even though the antimicrobial properties of *C. annuum, C. frutescens* and *Z. clava-herculis* against human pathogenic microorganisms such as *Bacillus cereus, Clostridium tetani, S. aureus* or *S. typhimurium* have been reported (Cichewicz and Thorpe, 1996; Dorantes *et al*., 2000; Gibbons *et al*., 2003, Koffi-Nevry *et al*., 2012), there is complete absence of studies investigating their inhibitory effects towards cariogenic or periodontal bacteria. Hence, the significant antibacterial activity of these species against the oral streptococci is reported for the first time. Among compounds isolated from *Capsicum* sp., capsaicinoids (such as capsaicin and dihydrocapsaicin), chrysoeriol, cinnamic and m-coumaric acids showed previously *in vitro* antimicrobial activity (Dorantes *et al*., 2000; Kim and Ryeom, 1979; Nascimento *et al*., 2014). Based on these reports, we assume that the aforementioned constituents may significantly contribute to the *C. annuum* and *C. frutescens* inhibitory effects noted in our study. As far as the antiseptic properties of *Z. clava-herculis* are concerned, they are probably caused by the presence of an alkaloid chelerythrine that was the major antimicrobially effective principle in the previous and only study on *Z. clava-herculis* inhibitory properties with MICs ranging from 4 to 16 μg/mL (Gibbons *et al*., 2003). Such findings agree with the recent research of related species, *Z. rhoifolium* and *Z. monophyllum*, where alkaloids (e.g. chelerythrine and dihydrochelerythrine) showed the potent antibacterial activity with MICs from 0.15 to 12.5 μg/mL (Rodriguez-Guzman *et al*., 2011; Tavares *et al*., 2014).

The remaining three species, namely *H. angustifolium, P. officinalis* and *T. sinensis* exhibited good antibacterial properties with MIC of 128 μg/mL against *S. salivarius* subsp.

salivarius and *F. nucleatum* subsp. *vincentii*, respectively. The observed inhibitory activity of *H. angustifolium* against oral streptococci is in accordance with a study of Nostro *et al.* (2004) who reported similar effect of an ethanol extract of flowering tops against *S. mutans, S. sanguis* and *S. sobrinus* with MICs from 31.25 to 62.5 μg/mL. The stronger activity might have been caused the fact that the *H. angustifolium* active principles (such as geraniol, geranyl acetate and helichrysine) are more concentrated in the flowering tops than in the aerial part used in our study (Chinou *et al*., 1996; Nostro *et al*., 2001; Taglialatela-Scafati *et al*., 2013). Concerning the inhibitory properties of *P. officinalis*, there is a lack of scientific reports about such activity against oral pathogens. Hence, its effect on *F. nucleatum* subsp. *vincentii* is described for the first time. According to the available literature, the major constituents of *P. officinalis* essential oil (e.g. eugenol, myrcene and geraniol) might be probably the antimicrobial principles as their inhibitory action have been noted previously (Bara and Vanetti, 1996; Hammer *et al*., 1999; Höferl M. *et al*., 2009; Friedman *et al*., 2002). Many studies have focused on the anticariogenic activity of *T. sinensis* or it constituents (Cho *et al*., 2010; Hamilton-Miller, 2001; Jeon *et al*., 2011). However, the MICs values obtained for oral streptococci varied greatly, from 31 to 1000 μg/mL for *T. sinensis* compounds (Sakanaka *et al*., 1989; Tamura *et al*., 2011; Xu *et al*., 2010) and from 1 up to 64 mg/mL for various *T. sinensis* extracts (Smullen *et al*., 2007). These findings are in conformity with the fact that with did not observe any significant inhibitory effect of *T. sinensis* extract on cariogenic bacteria within the given concentration range. On the other side, it showed good antibacterial activity against *F. nucleatum* subsp. *vincentii*, which is the first report of such activity as there has been no previous research on inhibitory properties of *T. sinensis* extract against this periodontal pathogen. Polyphenols, such as catechin, epicatechin and epigallocatechin, might be the responsible for the noted antiseptic effect as they showed the antimicrobial properties in recent studies (Tamura *et al*., 2011; Xu *et al*., 2010).

When considering the future use of the promising antimicrobial agents, it is important to realize that not only antimicrobial efficiency contributes to final product acceptance, but that the flavour of oral care preparations is also an important factor. Unpleasant or sharp taste can discourage a significant portion of consumers from daily use of such products (Yamamoto *et al*., 2012), whereas pleasant non-irritating flavour makes

the acceptability of a new oral hygiene product more feasible (Kim *et al*., 2013; Ota *et al*., 2013). Some constituents of the effective species from our study are known for their strong astringent, sharp or bitter taste (e.g. capsacionids, ellagic acid, myristicin) (Arapitsas, 2012; Ku *et al*., 2012); however, other antimicrobially active compounds (e.g. humulone, lupulone, cohumulone, colupulone, malabaricone C) are tasteless or very slightly bitter (Palamand and Aldenhoff, 1973; Shinohara *et al*., 1999). Thanks to the potent antiseptic properties of the effective extracts, they might be added to oral care products in very small concentrations (0.01 – 0.1%), which may not influence the final product flavour when using common sweeteners and flavourings in the same way as the bitter taste of chemical antiseptics such as chlorhexidine is masked (Löe, 1973). Additionally, there were no complaints about sensory properties of *H. lupulus* or *P. granatum* extract mouthrinse, the only species from the most effective ones tested in clinical studies. It is also worth noticing that their use during the aforementioned trials did not cause any discoloration of teeth or other unexpected side effects (Shinada *et al*., 2007; DiSilvestro *et al*., 2009; Vasconcelos *et al*., 2003).

7. Conclusion

In summary, it was found that out of 109 ethanol extracts of GRAS plant species tested, 25 of them, namely *C. annuum, C. frutescens, C. cassia, C. arabica, C. robusta, C. acuminata, C. zedoaria, F. assa-foetida, H. angustifolium, H. lupulus, M. sativa, M. crispa, M. fragrans, P. officinalis, P. spinosa, P. granatum, R. centifolia, S. nigra, S. indicum, T. officinale, T. sinensis, T. serpyllum, T. cordata, T. pratense* and *Z. clava-herculis* showed good inhibitory action against one or more oral pathogenic microorganisms under the study with the MIC lower than 256 μg/mL. Out of these 25 species, 6 of them, namely *C. annuum, C. frutescens*, *H. lupulus*, *M. fragrans*, *P. granatum* and *Z. clava-herculis* possessed significant antimicrobial properties against some of the oral pathogens with MIC lower than 100 μg/mL. Furthermore, 3 other plants, namely *H. angustifolium, P. officinalis* and *T. sinensis,* exhibited a good inhibitory effect on the cariogenic and periodontal bacteria with MIC = 128 μg/mL. The research focused on the evaluation of antiseptic activity of different *H. lupulus* cultivars revealed that the cultivar 'Harmonie' had the strongest inhibitory effect on the microorganisms tested. The results also showed the effect of extraction methods on the antimicrobial action of *H. lupulus* as the SFE *H. lupulus* extracts possessed stronger antibacterial action on the oral bacterial strains than the ethanol ones. The subsequent HPLC analysis revealed that *α*- and *β*-bitter acids are predominant constituents of the SFE *H. lupulus* extracts.

Our findings showed that *C. annuum, C. frutescens, H. lupulus, M. fragrans, P. granatum* and *Z. clava-herculis* possess potent growth inhibitory effect on oral pathogenic microorganisms and may be therefore considered for the development of oral care products effective against microbial infections. The fact that these species have been used for a long time safely and are declared as GRAS supports fully their applicability as new natural ingredients of oral and dental hygiene preparations, such as toothpastes and mouthrinses. However, further research focused e.g. on effectiveness of such ingredients against biofilm formation can be recommended before the evaluation of their clinical efficacy and sensory acceptability.

8. References

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9. Appendices

9.1. Appendix A: Photographic illustration of GRAS plant species with the highest antimicrobial activity against oral pathogenic microorganisms

Capsicum annuum (Feiertag, 2014)

Punica granatum (Vilmorin, 2014)

Humulus lupulus (Burnham, 2008)

Myristica fragrans (Warrington, 2014)

Capsicum frutescens (Horizon Herbs, 2014)

Zanthoxylum clava-herculis (Floridian, 2014)

9.2 Appendix B: List of author's publications (J. Pilná, born Čepková)

9.2.1 Publication in scientific journal

- **Pilná J**., Vlková E., Krofta K., Nesvadba V., Rada V., Kokoška L., 2015: In vitro growth-inhibitory effect of ethanol GRAS plant and supercritical CO_2 hop extracts on planktonic cultures of oral pathogenic microorganisms. FITOTERAPIA (IF 2.345).

- Lulekal E., Rondevaldová J., Bernášková E., **Čepková J.**, Asfaw Z., Kelbessa E., Kokoška L., Van Damme P., 2014: Antimicrobial activity of traditional medicinal plants from Ankober District, North Shewa Zone, Amhara Region, Ethiopia. PHARMACEUTICAL BIOLOGY; 52: p. 614-620 (IF 1.337).

9.2.2 Conference contributions

- **Čepková J.**, Huml L., Kokoška L., 2014: In vitro growth-inhibitory effects of extracts from GRAS plants against oral pathogenic microorganisms. Book of Abstracts; The Australian Society for Microbiology, Annual Scientific Meeting, 6 – 9 July 2014, Melbourne (Australia): p. 164.

- **Čepková J.**, Kokoška L., 2013: In-vitro antimicrobial activity of extracts from GRAS plants against oral pathogens. Book of Abstracts; 5th International Conference on Environmental Industrial and Applied Microbiology – BioMicroWorld 2013, 2-4 October 2013, Madrid (Spain): p. 465.