



## Influence of plant extracts on the growth of oral pathogens *Streptococcus mutans* and *Candida albicans* in vitro

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**Abstract.** The objective of this study was to investigate the effects of ten plant extracts, six juices, and propolis and their combinations on the in vitro growth of oral pathogens *Streptococcus mutans* and *Candida albicans*. Agar-well diffusion and broth dilution methods were used. Triple and quadruple combinations were tested with the most active extracts. All of the tested 70% ethanolic extracts inhibited the growth of *S. mutans* and *C. albicans*. Cloves, cinnamon, propolis, lavender, and sage were the most active inhibitors. Apple, black chokeberry, black elderberry, cranberry, Japanese quince, and lemon juice demonstrated little activity. Mixed in double 1 : 1 combinations, 8 extract combinations expressed synergistic action and 11 combinations expressed antagonistic action to the inhibition of the growth of *C. albicans*. Chamomile, liquorice, marigold, and lavender were involved both in synergistic and antagonistic interactions depending on the second component of the mixture. Propolis, cinnamon, and cloves were involved only in particular synergistic interactions, while sweet flag, dog rose, and oregano related only to some antagonistic interactions. The most active quadruple combination consisted of cloves, cinnamon, propolis, and lavender. Moreover, it demonstrated activity and synergistic action against both microorganisms. Propolis and all the studied plant extracts may be of great interest for inhibiting the growth of oral pathogens *S. mutans* and *C. albicans*.

**Key words:** microbiology, plant extract, antimicrobial activity, synergistic action, *Candida albicans*, *Streptococcus mutans*.

### INTRODUCTION

Development of a range of digestive organ diseases, for example, helicobacteriosis [1], dental caries [2], and oral candidiasis [3], depends on the diet. Oral health is an integral part of general well-being and an indicator of the quality of life. The relationship of oral health to systemic diseases has also been demonstrated [4].

The lactic acid bacterium *Streptococcus mutans* and the yeast *Candida albicans* belong to the most common microorganisms found in the oral cavity. The bacterium *S. mutans* has an important role in the pathogenesis of dental caries [5]. The bacteria not only metabolize sugars to produce lactic acid that attacks the

dental enamel, but also synthesize extracellular polysaccharides, mainly dextran, a component of the plaque, thus reinforcing cariogenicity of easily assimilated carbohydrates [6]. It is not only the growing consumption of sugar-containing products (sweets, sugar-sweetened beverages, fast food) that promotes the distribution of caries. There are also reports of the resistance of *S. mutans* to antibacterial agents used in oral care products [7]. This fact is considered to be one of the causes of caries distribution nowadays. In most industrialized countries, the prevalence of dental caries in school-aged children is 60–90% and the majority of adults are also affected [8].

Intraoral *C. albicans* is found in 40% of healthy humans [9]. Use of antibiotics [10] and cellular immunodeficiencies [11] contribute to the development

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of candidiasis. Experience shows that application of synthetic antimicrobial substances in oral hygiene is not always justified, because *C. albicans* biofilms produce tolerant cells [12]. Left untreated, candidiasis can lead to a generalized process with serious consequences [13,14].

Candidiasis is combated with propolis [15] as well as with lavender and sage [16,17]. The influence of cloves [17,18] and cinnamon [17,19] on *C. albicans* is also well known. Studies of the influence of plant extracts on *S. mutans* often refer to cloves [20], garlic [21], and liquorice [22]. It is reasonable to expect the highest efficiency from a combination of herbal products [23]; however, this has been poorly studied, particularly with regard to different oral pathogens.

The objective of this study was to investigate the effects of ten plant extracts, six juices, and propolis and their combinations on the growth of oral pathogens *Streptococcus mutans* and *Candida albicans* in vitro.

## MATERIALS AND METHODS

### Tested substances, juices, and extracts

Propolis, six juices, and ten plant extracts were tested. The tested herbs were obtained from the manufacturing plant FitoBALT (IBTI, Latvia). The following herbs were used: flowers of marigold *Calendula officinalis*, flowers of chamomile *Matricaria recutita*, leaves of sage *Salvia officinalis*, bark of cinnamon *Cinnamomum verum*, cloves of *Syzygium aromaticum* buds, root of liquorice *Glycyrrhiza glabra*, flowers of lavender *Lavandula angustifolia*, leaves of oregano *Origanum vulgare*, fruits of dog rose *Rosa canina*, and rhizome of sweet flag *Acorus calamus*.

Four grams of dry plant material was extracted with 20 mL of distilled water or 70% ethanol. The suspensions were stored at room temperature for 24 h and then centrifuged (Eppendorf, 3000 rpm, 15 min). The obtained extracts were filtered through filter paper and stored in the refrigerator at 4°C until use.

Dry extract of propolis (Stanchem, UK) was dissolved in water in the proportion 1:5 (v/v). Pasteurized juices of black chokeberry *Aronia melanocarpa* and Japanese quince *Chaenomeles japonica* were produced by “Lases” (Latvia). Black elderberry *Sambucus nigra* pasteurized juice was produced by “Meldri E.B.” (Latvia). Lemon *Citrus medica* (grown in Spain) non-pasteurized juice and cranberry *Vaccinium macrocarpon* (grown in “Gundegas”, Latvia) non-pasteurized juice were produced by IBTI, Latvia. Apple *Malus domestica* juice was made from juice concentrate (A. Sakalausko, Lithuania). All non-pasteurized juices were centrifuged (Eppendorf, 3000 rpm, 20 min), filtered through filter paper, and sterilized by filtering through 0.2 µm membrane filters. To test the effective-

ness of combinations of extracts, extracts were mixed in a ratio 1:1.

### Microorganisms and culture conditions

Antimicrobial assays were performed on two species of microorganisms maintained in the Microbial Strain Collection of Latvia (MSCL). The following strains isolated from human oral mucosa were used: yeast *Candida albicans* MSCL 378 and bacterium *Streptococcus mutans* MSCL 1174. Malt extract agar (Becton Dickinson, USA) was used for cultivating *C. albicans* but *S. mutans* was cultivated on Columbia blood agar (Oxoid, UK) at a temperature of 37°C.

### Agar-well diffusion method

An agar diffusion test was performed on Columbia blood agar for *S. mutans* and on malt extract agar for *C. albicans*, 25 mL of the medium per every Petri dish. Fresh inoculums of approximately 10<sup>6</sup> colony-forming units (CFU) per mL of tested microorganisms were used. Aliquots of 100 µL of each test sample solution and control (distilled water or 70% ethanol) were applied into 6.0 mm diameter wells. After incubation at 37°C for 24 h the inhibition zone corresponding to the halo formed from the well edge to the beginning of the zone of microbial growth was measured. The tests were performed in triplicate and the final results were presented as the arithmetic average.

### Broth dilution assay

Mueller–Hinton broth (BD Difco™) for *S. mutans* and RPMI-1640 with HEPES and L-glutamin and without NaHCO<sub>3</sub> (Sigma, UK) for *C. albicans* were used. Test strains were suspended in broth to obtain a final density of 10<sup>6</sup> CFU/mL. The test was performed using five concentrations of each extract (0.3%, 1.7%, 3.3%, 16.7%, and 33.3%, v/v) in test tubes, including growth (in water or 70% ethanol dilutions) and sterility controls. Tubes were incubated at 37°C for 24 h. After incubation, the mixtures were subjected to successive 10-fold serial dilutions, mixed with a vortex shaker to ensure dispersion, and quantitatively cultivated in duplicate onto agar plates to determine the number of viable microorganisms. Viable counts were expressed as CFU/mL and, if applicable, the minimum inhibitory concentration (MIC<sub>80</sub>) according to Qaiyumi [24] was evaluated.

### Statistics

Statistical analysis was done by analysis of variance;  $p < 0.05$  was considered statistically significant. Each experiment was repeated three times.

## RESULTS

### Activity against *Candida albicans*

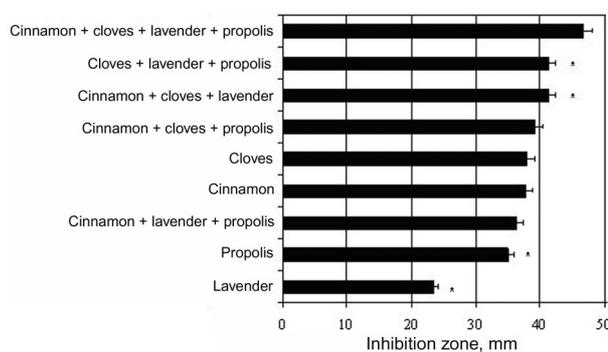
Aqueous extracts of cinnamon and cloves showed antifungal activity in the agar-well diffusion method with 12.8 mm and 20.8 mm diameter inhibition zones, respectively. Other plant extracts as well as propolis did not demonstrate activity against *C. albicans*. Therefore, 70% ethanol extracts were used in the following experiments. All the tested ethanolic extracts were found to show antifungal action (Table 1). The lowest activity was exhibited by chamomile (15.7 mm) and liquorice (16.4 mm) and the highest by cloves (38.0 mm), cinnamon (37.7 mm), and propolis (35.0 mm). When extracts were mixed in various combinations 1 : 1, the highest activity was demonstrated by lavender with cloves (38.7 mm). In total, eight combinations expressed synergistic action and 11 combinations expressed antagonistic action (Table 1). An antagonistic effect was stated if the combination gave less inhibition ( $p < 0.05$ ) than either of the pair alone. Chamomile, liquorice, marigold, and lavender were involved both in synergistic and antagonistic interactions depending on the other component of the mixture. Propolis, cinnamon, and cloves were involved only in particular synergistic interactions, while sweet flag, dog rose, and oregano related only to some antagonistic interactions.

The tested juices (i.e. apple, black chokeberry, black elderberry, cranberry, Japanese quince, and lemon juice) demonstrated little activity. Japanese quince had the highest activity, which resulted in a 17.5 mm inhibition zone diameter in the agar-well diffusion assay.

Triple and quadruple combinations were tested with the more active extracts, i.e., lavender, propolis,

cinnamon, and cloves. The triple combination of cinnamon, cloves, and propolis showed that cinnamon and propolis did not contribute significantly ( $p > 0.05$ ) to the activity of cloves (Fig. 1). The triple combination of cinnamon, cloves, and lavender showed the same activity as the combination of propolis, cloves, and lavender. The quadruple combination demonstrated the greatest activity and synergy.

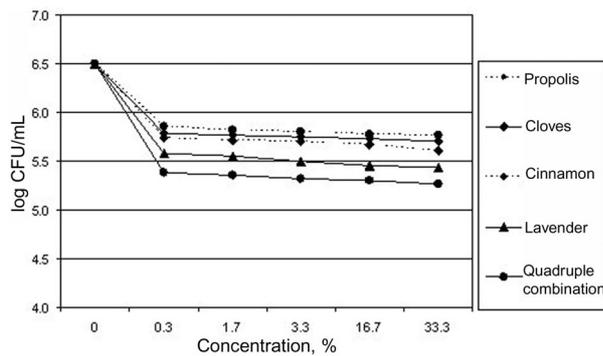
Tested by a broth dilution method, aqueous extracts of cinnamon, cloves, lavender, and propolis demonstrated antifungal activity individually as well as synergistically in the quadruple combination (Fig. 2) with MIC  $\leq 0.3\%$ . Increasing the concentration of extracts from 0.3% to 33.3% had little effect on promoting their activity.



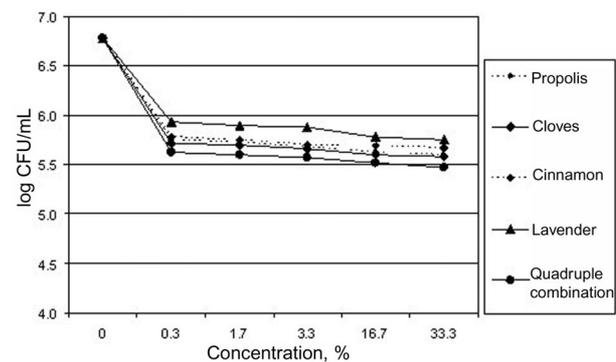
**Fig. 1.** Antimicrobial activity of 70% ethanolic extracts of plants and propolis individually and in mixed triple 1 : 1 : 1 and quadruple 1 : 1 : 1 : 1 combinations against *Candida albicans*. Inhibition zone diameters in mm  $\pm$  SD. \* – significant difference ( $p < 0.05$ ) in comparison with cloves.

**Table 1.** Antimicrobial activity of 70% ethanolic extracts of plants and propolis individually and in mixed double 1 : 1 combinations against *Candida albicans*. Inhibition zone diameters in mm. Values are the means of three replicates. Standard deviation did not exceed 0.5. Synergistic effects are highlighted with light shading and mixed extracts with dark shading. Antagonistic effects, i.e. values significantly ( $p < 0.05$ ) lesser than the value of either of the pair alone, are underlined

Extract	Chamomile	Liquorice	Sweet flag	Dog rose	Oregano	Marigold	Sage	Lavender	Propolis	Cinnamon	Cloves
Chamomile	15.7	23.0	<u>12.0</u>	18.0	<u>15.0</u>	22.0	16.0	20.0	26.0	22.7	23.0
Liquorice	23.0	16.4	<u>14.0</u>	<u>14.0</u>	<u>13.0</u>	17.0	16.1	24.7	21.0	23.7	25.3
Sweet flag	<u>12.0</u>	<u>14.0</u>	17.6	<u>16.0</u>	<u>15.0</u>	<u>15.0</u>	19.0	19.3	27.3	25.0	30.3
Dog rose	18.0	<u>14.0</u>	<u>16.0</u>	17.9	18.0	<u>13.0</u>	18.6	19.7	32.0	22.3	31.2
Oregano	<u>15.0</u>	<u>13.0</u>	<u>15.0</u>	18.0	18.3	<u>15.0</u>	18.4	16.3	29.7	23.3	30.0
Marigold	22.0	17.0	<u>15.0</u>	<u>13.0</u>	<u>15.0</u>	19.0	21.0	27.7	25.7	23.0	23.0
Sage	16.0	16.1	19.0	18.6	18.4	21.0	22.3	25.7	33.3	30.7	27.3
Lavender	20.0	24.7	19.3	19.7	16.3	27.7	25.7	23.5	36.7	30.0	38.7
Propolis	26.0	21.0	27.3	32.0	29.7	25.7	33.3	36.7	35.0	38.3	34.7
Cinnamon	22.7	23.7	25.0	22.3	23.3	23.0	30.7	30.0	38.3	37.7	28.3
Cloves	23.0	25.3	30.3	31.2	30.0	23.0	27.3	38.7	34.7	28.3	38.0



**Fig. 2.** Antimicrobial activity of aqueous extracts of individual plants and their quadruple combination 1:1:1:1 against *Candida albicans* estimated by broth dilution assay.

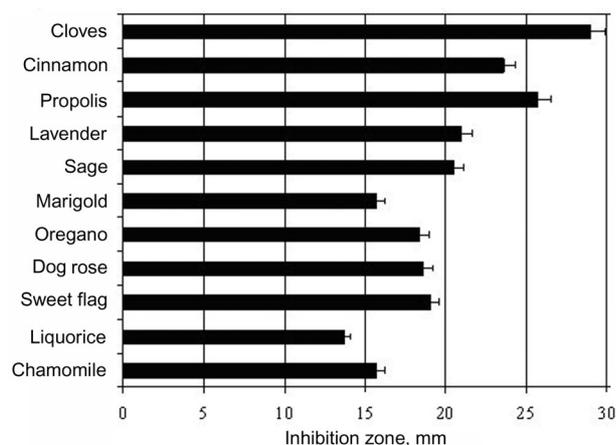


**Fig. 4.** Antimicrobial activity of aqueous extracts of individual plants and their quadruple combination 1:1:1:1 against *Streptococcus mutans* estimated by the broth dilution assay.

### Activity against *Streptococcus mutans*

No aqueous extract of plants and propolis showed any antibacterial activity, but all ethanolic extracts demonstrated antibacterial activity against *S. mutans* in the agar-well diffusion method (Fig. 3). Cloves, propolis, cinnamon, and lavender extracts were the most active. Antibacterial action of aqueous extracts of these plants and propolis was found in the broth dilution assay (Fig. 4). The obtained value of MIC was  $<0.3\%$  in all cases. The greatest effect was shown by a combination of the four extracts.

All six of the tested juices demonstrated insignificant activity. Lemon juice had the highest activity (14.5 mm inhibition zone diameter) and cranberry juice had the lowest activity (13.5 mm) in the agar-well diffusion assay.



**Fig. 3.** Antimicrobial activity of 70% ethanol extracts of plants against *Streptococcus mutans* estimated by the agar-well diffusion method.

### DISCUSSION

In recent years, researchers have focused on the fighting of a variety of gastrointestinal diseases with herbal extracts. In particular, the use of plant extracts against oral pathogens *Candida albicans* and *Streptococcus mutans* has generated great interest.

In our study all six tested juices, i.e., apple, black chokeberry, black elderberry, cranberry, Japanese quince, and lemon juice, demonstrated lower activity in comparison with plant extracts. The difference was especially marked against *S. mutans*.

Our experiments proved a stronger antimicrobial effect of ethanolic extracts than of aqueous extracts when tested with the agar-well diffusion method. The yeast *C. albicans* was more susceptible to the action of ethanolic extracts than the bacterium *S. mutans* (Table 1, Fig. 3). The weak activity of aqueous extracts has been mentioned in several studies [25,26].

Literature data on the effectiveness of plant extracts are inconsistent probably because of differences in extract preparation methods. Most often ethanolic extracts are positioned as more active than aqueous extracts [27]. Probably many biologically active substances are better extracted in this solvent [15]. Ethanol is the most commonly used organic solvent, as the finished products can be relatively safely used [28]. Moreover, nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, and they are most often obtained through initial ethanol or methanol extraction [29].

Tested with a broth dilution assay, an antimicrobial activity was found for aqueous extracts of the most active plants. All of our tested ethanolic extracts inhibited the growth of *C. albicans* and *S. mutans*. Cloves, cinnamon, propolis, lavender, and sage were the most active inhibitors of both microorganisms (Table 1, Fig. 3). The

most active combination, which consisted of cloves, cinnamon, propolis, and lavender, demonstrated activity and synergistic action against both microorganisms. Inhibitory activity against *C. albicans* has been described individually for propolis [30], cinnamon, cloves, and lavender [17], and activity against *S. mutans* has been described for propolis [30], cloves [20], and cinnamon [31]. According to our knowledge, no activity of lavender extract against *S. mutans* has been found previously [32] but lavender oil possesses moderate antimicrobial activity [33,34]. Propolis and all the studied plant extracts may be of great interest for the inhibition of the growth of oral pathogens *Streptococcus mutans* and *Candida albicans*.

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### Taimeekstraktide mõju suuõõne patogeenide *Streptococcus mutans* ja *Candida albicans* *in vitro* kasvu vastu

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Artikli eesmärk on uurida kümne taimse tinktuuri, kuue mahla ja taruvaigu ning nende kombinatsioonide efekti *Candida albicans*'i ja *Streptococcus mutans*'i tüvele. Antibakteriaalse toime määramiseks kasutati agardifusiooni- ja seerialahjendusmeetodit. Kõige aktiivsemad olid taruvaik ja nelgi-, kaneeli-, lavendli- ning salveiekstrakt. Mahlade aktiivsus oli minimaalne. Mõlema mikroobi kasvu inhibeeris kõige tugevamini sünergiline kombinatsioon, mis koosnes nelgist, kaneelist, lavendlist ja taruvaigust. Taruvaik ja uuritud ekstraktid pakuvad huvi seoses suuõõne patogeenidega *S. mutans* ning *C. albicans*.