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Rosemarinus officinalis and *Thymus eriocalyx* essential oils combat *in vitro* and *in vivo* dental biofilm formation

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ABSTRACT

The antimicrobial activities of *Rosemarinus officinalis* and *Thymus eriocalyx* essential oils and chlorhexidine were assessed against *Streptococcus mutans* and *Streptococcus pyogenes* in particular against a biofilm growth. The oils were analyzed by GC and GC–MS and their antimicrobial activities and biofilm formation preventive properties were studied in vitro and invivo. Male and female volunteers brushed with essential oil blended toothpastes were studied for prevention of dental plaque. Twenty and eighteen compounds were identified in the essential oils of *R. officinalis* and *T.eriocalyx* respectively. Minimal bactericidal concentrations (MBC) of the *R. officinalis* and *T.eriocalyx* oils were found to be 2000 and 4000 ppm and those of chlorhexidine (0.2%) were 8000 and 1000 ppm for both *S.mutans* and *S.pyogenes* respectively. Decimal reduction time of *S.mutans* by *R. officinalis* and *T.eriocalyx* oils at their MBC levels was 2.8 minutes. D value of *S.pyogenes* exposed to *R. officinalis* and *T.eriocalyx* oils and to chlorhexidine were 4.28, 3.6 and 2.8 minutes respectively. Antibacterial and in vivo biofilm preventive efficacies of all the concentrations of rosemary oil were significantly ($p<0.001$) higher. Our results show effective doses of 2 and 8 mg/ml for *R. officinalis* and *T.eriocalyx* oils in planktonic cultures. In vivo experiments indicated that lower concentrations of the oils, in particular the rosemary oil, were high significantly ($p<0.001$) effective during the course of the study as compared to chlorhexidine. In conclusion there may be a potential role for essential oils in the development of novel anticaries treatments.

KEY WORDS: Biofilm, *Streptococcus mutans,* Essential oils, *Rosemarinus officinalis, Thymus eriocalyx*.

INTRODUCTION

Oral diseases, including tooth decay, gingivitis and periodontitis, are among the most prevalent afflictions of humankind. Furthermore, it has been suggested in recent years that oral bacteria are associated with many systemic diseases such as pneumonia and cardiovascular disease (1); therefore, the need for oral care in a systemic health regimen has also been emphasized. Oral biofilms harboring pathogenic bacteria are the major contributing virulence factors associated with these diseases (2). The human oral cavity is inhabited by more than 500 species of bacteria at 10^8-10^9 bacateria per mL saliva or mg dental plaque (3). Caries is a disease caused by plaque bacteria such as *Streptococcus mutans* or *Streptococcus sobrinus* (4).

An apparent connection between the proportions of certain bacterial species in the oral biofilm and the absence or presence of tooth decay (caries) was established (5). Most strikingly, mutans streptococci including *Streptococcus mutans*, are clearly correlated with caries (6), whereas *S. pyogenes* is associated with health. In general, mouthwashes should be recommended after the patient has brushed and cleaned interdentally. However, with chlorhexidine, as many dentifrice ingredients can reduce its antibacterial efficacy, the manufacturer recommends that the patient should be instructed to completely rinse all traces of toothpaste from the mouth or wait 0.5 h between the tooth cleaning and rinsing (7). Patients using the essential oil mouthwash do not need

to take these precautions. In microbiological terms, long-term use of essential oil mouthwashes has been shown to be safe. After 6 months of daily, continued use, essential oil mouthwashes cause no change in the bacterial composition of supragingival plaque although they do produce a decrease in total microbial flora. Specifically, there is no evidence of increased putative and/or opportunistic oral pathogens (8). Similar observations were made for chlorhexidine mouthwash (9). Selected natural products that originate in plants can influence microbial biofilm formation. For example, halogenated furanones, a class of compounds that inhibit biofilm formation by interfering with bacterial quorum sensing, were identified in a marine alga and are thought to have evolved to reduce biofouling (10). Other plant-derived compounds inhibit peptidoglycan synthesis (11), damage microbial membrane structures (12), modify bacterial membrane surface hydrophobicity (13), and modulate quorum sensing (11), all of which could influence biofilm formation. In this study we compared the antibacterial activities of phytochemical essential oils from *Rosemarinus officinalis* and *Thymus eriocalyx* against two oral bacteria namely *S.mutans* and *S.pyogenes*.

MATERIALS AND METHODS

Strains and culture conditions

The primary causative agent of dental caries i.e. *Streptococcus mutans* PTCC 1601 was our main target in this study. A clinical isolate of *Streptococcus pyogenes* was taken as a model oral pathogen to find out possible side effects of anti *Streptococcus mutans* agents. All strains were cultured on blood agar (Merck-Germany).

Oil extraction and analysis

The plant materials were steam distilled for 90 minutes in full glass apparatus. The oils were isolated using a Clevenger-type apparatus. The extraction was carried out after a four-hour maceration in 500 mL of water. The oils were stored in dark glass bottles in a freezer until they were used. GC analyses were performed using a Shimadzu-9A gas chromaph equipped with a flame ionization detector, and quantitation was carried out on Euro Chrom 2000 from Knauer by the area normalization method neglecting response factors. The analysis was carried out using a DB-5 fused-silica column (30m × 0.25 mm, film thickness 0.25 µm, J & W Scientific Inc., Rancho Cordova, CA, USA). The operating conditions were as follows: injector and detector temperature, 250°C and 265°C, respectively; carrier gas, Helium. Oven temperature programme was 40°C-250°C at the rate of 4°C/min.

Gas Chromatography-Mass Spectrometry

The GC/MS unit consisted of a Varian Model 3400 gas chromatograph coupled to a Saturn II ion trap detector was used. The column was same as GC, and the GC conditions were as above. Mass spectrometer conditions were: ionization potential 70 eV; electron multiplier energy 2000 V. The identities of the oil components were established from their GC retention indices, relative to C7- C25 n-alkanes, by comparison of their MS spectra with those reported in the literature (14, 15), and by computer matching with the Wiley 5 mass spectra library, whenever possible, by co-injection with standards available in the laboratories.

Oil dilution solvent

The oils were diluted with dimethylsulphoxide (DMSO) (Merck-802912). These dilutions were used in antibacterial analysis. Bacterial strains were streaked on Mueller Hinton agar (Merck-Germany) plates using sterile cotton swabs. 5µl of dimethylsulphoxide (DMSO) loaded on sterile blank disks were placed on the agar plates and were incubated at 37^{O}C for 24 hours. There was no antibacterial activity on the plates and hence DMSO was selected as a safe diluting agent for the oil. 5µl from each oil dilution followed by sterilization using a 0.45 µm membrane filter was added to sterile blank discs. The solvent also served as control.

Preparation of essential oil blended toothpastes

Ten, 20, 30, and 40 µl of each essential oil was mix blended with 50ml of toothpastes aseptically in order to obtain 200, 400, 600 and 800ppm oil in each toothpaste. The pastes were then reintroduced into the tubes and capped tightly.

Disc diffusion method

The agar disc diffusion method (16) was employed for the determination of antimicrobial activities of the essential oils in question. Briefly, 0.1 ml from 10^8 CFU/mL bacterial suspension was spread on the Mueller Hinton Agar (MHA) plates. Sterile blank filter paper discs (6 mm in diameter) were impregnated with 5µl of the oil and were placed on the inoculated plates. These plates, after remaining at 4^{o}C for 2h, were incubated at 37^{o} C for 24 h. The diameters of the inhibition zones were measured in millimeters. All tests were performed in triplicate.

Determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations

All tests were performed in Brain Heart Infusion (BHI) (Merck-Germany) broth supplemented with Tween 80 (Merck-Germany) detergent (final concentration of 0.5% (v/v). Test strains were suspended in BHI broth to give a final density of 10^7 cfu/ml and these were

confirmed by viable counts. Geometric dilutions, ranging from 0.036 to 72.0 mg/ml of the essential oil, were prepared in a 96-well microtitre plate (Lab Logistic Group-Denmark), including one growth control (BHI+Tween 80) and one sterility control (BHI+Tween 80+test oil). Plates were incubated under normal atmospheric conditions, at 37^{o}C for 24 h. The bacterial growth was indicated by the presence of a white ''pellet'' on the well bottom. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were assessed according to our modified procedure (16). MIC was determined by a broth dilution method in test tubes as follows: 40µl from each of various dilutions of the oils was added to 5mL of Brain Heart Infusion (BHI) tubes containing 10^7 CFU/mL of live bacterial cells. The tubes were then incubated on an incubator shaker as to evenly disperse the oil throughout the broth in tubes. The highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. One hundred micro liters corresponding to 0.1 mL of the cell suspensions from the tubes showing no growth were subcultured on BHI agar plates in triplicate to determine if the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Bactericidal kinetics of the oils

Forty µl of the each oil at the dilution determined by MBC was added to each 5 ml of Brain Heart Infusion (BHI) broth tubes containing bacterial suspension of 10^7 cfu/ml and were then incubated at 37^{O} C in an incubator shaker. 0.1 ml samples were taken after 5, 10, 15, 20, 25, 30, 45, 90, 120, 150, 180, 210, 240, 270 and 300 minutes. The samples were immediately washed with 1ml sterile phosphate buffer pH-7.0, centrifuged at 10000 rpm/1 minute, resuspended in the buffer and were then spread cultured on BHI agar for 24 h at 37^{O}C. Phosphate buffer was used as diluent when needed. Bactericidal experiments were performed three times. Microbial colonies were counted from triplicates after incubation period and the mean total number of viable cells per ml was calculated.

Determination of Decimal Reduction Time (D value)

The mean total number of viable bacteria from bactericidal kinetics experiments at each time interval was converted to $\log_{10}$ viable cells using routine mathematical formulae. The time required to decrease the microbial population in a sample by 90% on the basis of logarithmic value of one was regarded as D value.

Specific biofilm formation (SBF) assay

Due to its biofilm forming properties *Streptococcus mutans* alone was employed in this part of the study. Bacteria were grown in 14-ml polystyrene culture tubes containing 2 ml of LB medium (Merck-Germany) and various concentrations of essential oils or individual chemicals. The total volume of the test compounds added never exceeded 1.5% of the culture volume. Nine identically prepared tubes were used for each concentration. Three of the nine tubes were used to measure growth in suspended culture (G tubes), three tubes were used to measure biofilm growth (B tubes), and three tubes served as controls for abiotic factors (NC tubes). Inocula were grown to the late log phase. Subsequently, the B and G tubes received 20 µl of inoculum, and all tubes (B, G, and NC tubes) were incubated in an orbital cabinet shaker for 17 ± 1 h. Following incubation, cells in the G tubes were mixed well, and the optical densities at 600 nm (OD_{600}) of the cultures were measured. The B and NC tubes each received 125 µl of a 0.3% solution of crystal violet (CV) (Merck-Germany). After 15 min, the suspended culture was poured out, and the tubes were rinsed well with distilled deionized water (six rinses, approximately 4 ml per rinse). Any remaining crystal violet was dissolved in 2 ml of an ethanolacetone (80:20) solution, and the absorbance at 570 nm of each resultant solution was measured spectrophotometrically. Biofilm accumulation was normalized with respect to growth, which yielded the SBF. SBF was determined by using the following formula: SBF = $(B - NC)/G$, where B is the amount of biofilm formed, NC is the amount of CV that adhered to the polystyrene tubes due to abiotic factors, and G is the optical density of cells grown in suspended culture (17). At least two replicate experiments were performed for each concentration of chemical that was tested.

In vivo studies

This part of the research has been conducted with the understanding and written consent of each subject and in full accordance with ethical principles. The study has been independently reviewed and approved by an ethical board. Equal number of male and female dentistry students of Shahed university, Tehran volunteered to participate in this study. Their age range was 20–30 years, they all showed an overall healthy mouth status. Three major groups were designed in the present study. Group one brushed using standard toothpaste. This group served as control. Group two and its subgroups brushed using the same

type of toothpastes as in control group that were blended with different concentrations of essential oils. Group three brushed using toothpastes blended with 0.2% chlorhexidine. After the baseline examinations, we randomly assigned subjects to one of three groups. Each subject then received a complete dental prophylaxis to remove plaque, stain and calculus, which was confirmed by the use of disclosing solution. The groups were instructed to brush twice daily with an ADA-Accepted toothbrush (Oral-B 35). We instructed all subjects to brush thoroughly twice daily and gave them toothbrushes and dentifrice as needed. We allowed the subjects to follow their usual dietary habits, but we instructed them to refrain from using any oral care products other than what we provided to them for the study. We permitted limited interdental cleaning in all groups in instances of considerable food entrapment. All the subject groups were examined once a week for four weeks. On each examination, after using a disclosing solution on the teeth, we scored the plaque area using the Turesky modification of the Quigley-Hein Plaque Index (18) on six surfaces of all scorable teeth as follows: no plaque (0); separate flecks or discontinuous band of plaque at the gingival (cervical) margin (1); thin (up to 1 mm), continuous band of plaque at the gingival margin (2); band of plaque wider than 1 mm but less than one-third of surface (3); plaque covering one-third or more, but less than two-thirds, of surface (4); plaque covering two-thirds or more of surface (5).

Statistical analysis

The values were calculated as the mean percentage of bacterial adherence, compared with control groups. Differences between two means were evaluated by the Student's paired *t*-test was used to compare each test with the control. The level of significance was determined at $p < 0.05$. A one-way ANOVA was performed for comparison of multiple means.

RESULTS

GC and GC–MS analysis of the essential oils of *Rosemarinus officinalis* and *Thymus eriocalyx* led to identification of twenty and eighteen compounds respectively (Tables 1 & 2). *Rosemarinus officinalis* oil was characterized with prominent (>7%) concentrations of Piperitone (23.65%), α-pinene (14.94%), Linalol (14.89%), Camphor (4.97%), 1,8-Cineole (7.43%) as the major compounds. *Thymus eriocalyx* oil was also distinctive in its high concentrations of Thymol (64.33%), β-phellandrene (13.22%) and Cis Sabinene hydroxide (8.38%). The results of antimicrobial tests are summarized in Table 3. The essential oils exerted variable antimicrobial effects on different microorganisms taken under study with greater antibacterial effect than chlorhexidine (Table 3). Minimal bactericidal concentrations (MBC) of the *Rosemarinus officinalis* and *Thymus eriocalyx* oils were found to be 2000 and 4000ppm for *S.mutans* and *S.pyogenes* respectively. *S.pyogenes* was killed at 4000ppm of both oils. MBC values of chlorhexidine (0.2%) were 8000 and 1000 ppm with respect to *S.mutans* and *S.pyogenes* respectively. Using these values, kinetics of bacterial death was studied in order to determine the decimal reduction time (D value) (Figures 1,2). Decimal reduction time of *S.mutans* brought about by each of the essential oils from *R. officinalis* and *T.eriocalyx* at their MBC levels was 2.8 minutes (Figure 1) while cholrhexidine showed longer time to completely kill *S. mutans*. D value of *S.pyogenes* on exposure to the MBC levels of *R. officinalis* and *T.eriocalyx* oils and of chlorhexidine were 4.28, 3.6 and 2.8 minutes indicating higher efficacy of chlorhexidine (Figure 2). Lower amount of Rosemary oil could completely kill *S.mutans* within 20 minutes of exposure which is comparable to the same killing time with double amount of Thyme oil while exhibiting smaller zone of growth inhibition than thyme oil (Figure 3). *S.pyogenes* was affected by both oils and by lower amount of chlorhexidine (Figure 4). In vitro inhibition of biofilm formation by *S.mutans* was studied at four concentrations of 1000, 2000, 4000 and 8000ppm of essential oils and chlorhexidine (Figure 5). Antibiofilm properties of all the concentrations of Rosemary oil were significantly ($p < 0.001$) higher than Thyme oil and chlorhexidine (Figure 6).

DISCUSSION

Many studies have shown that the essential oils of thyme and rosemary are among the most active against a number of pathogen microorganisms (19). The compositions of essential oils from a particular species of plant can differ between harvesting seasons (20) and between geographical locations (21); hence the principal constituents of these oils have to be chemically characterized. Atti-Santos et al. (22) reported that, while rosemary oils contained large quantities of camphor and 1,8-cineole, the main component was α-pinene. In the present study α-pinene was the second most abundant constituent of the rosemary oil confirming the report by Atti-Santos et al. (22). Our results are in agreement with another study that confirmed 1,8-cineole, camphor and α-pinene as the main constituents of rosemary oil (23). *Rosemarinus officialis* has been reported to have the

same composition as of this study but at different proportions of Piperitone (6.68%), α-pinene (4.21%), Linalol (3.37%), Camphor (10.08%), 1,8-Cineole (52.20%) (24). Our *T.eriocalyx* is rich in thymol content than *T.vulgaris* which was reported to contain 17.37% thymol (24). Commercial forms of chlorhexidine and essential oil mouthrinses are now available. Peridex is a 0.12% solution of chlorhexidine, a bisbiguanide antiseptic (7), while the active ingredients in Listerine are four essential oils: thymol 0.064%, eucalyptol 0.092%, methyl salicylate 0.060% and menthol 0.042%. Statistical analysis of data obtained from disc diffusion experiments revealed significant ($p < 0.05$) antibacterial effect of the oils as compared to chlorhexidine. There was no significant difference between anti *S.mutans* effects of both oils. The oils however showed significant ($p < 0.05$) difference when applied to *S.pyogenes* (Table 3). This indicates statistically equal effect of the oils on *S.mutans* and different activities against *S.pyogenes*. These differences may be attributable to the major chemical components of essential oils that play the prime role in the antibacterial activities. Chlorhexidine (0.2%) could inhibit both bacterial growth with significant difference ($p < 0.05$) (Table 3). Due to the variation of diffusion and solubility properties of the different oils, the results obtained by the disk diffusion method are not directly comparable to those obtained with the microdilution broth assay (25). Thyme oil that exhibited larger inhibition zones for both bacteria was confirmed as the antistreptococcal agent with higher (4000 ppm) MBC values (Figures 1 &2). The essential oil of *T.eriocalyx* is characterized by the presence of high concentrations of thymol with well documented antimicrobial activity. An essential oil from *Lippia sidoides* Cham., rich in thymol (66.67%), presented antimicrobial activity (26). A mouth-rinse prepared with this oil reduces plaque-bacteria growth in humans (27). It has been reported that there is a relationship between the chemical composition of essential oils and the antimicrobial activity. The phenolic compounds are widely reported to possess high levels of antimicrobial activity (28). The antimicrobial natures of the thyme essential oil are apparently related to its high phenolic content, particularly thymol. The oils were still stronger *S.mutans* killer than chlorhexidine (Figure 3). Higher susceptibility of *S.pyogenes* to chlorhexidine compared to *S.mutans* (Figure 4) suggests additional application of chlorhexidine in the control of oral infections such as sore throat caused by *S.pyogenes*. The aim of this study was first to assess the antimicrobial effects of two essential oils and chlorhexidine against an important cariogenic bacterium *Streptococcus mutans,* and an oral bacterium *Streptococcus pyogenes* in particular against a biofilm growth. Secondly, we sought to evaluate the effect of these essential oils with chlorhexidine with a view to reducing the formation of biofilm in vitro (Figure 5) and in vivo (Figure 6). The results from in vitro studies show higher efficacy of chlorhexidine at 1000 ppm while *R. officinalis* oil was the most effective agent inhibiting biofilm formation at concentrations over 2000 ppm (Figure 5). Cinnamon essential oil has been reported to exhibit the greatest antimicrobial potency at 1.25–2.5 mg/ml concentration against planktonic and biofilm cultures of *Streptococcus mutans* (29). Our results show effective doses of 2 and 8 mg/ml corresponding to 2000ppm and 8000ppm for *R. officinalis* and *T.eriocalyx* oils in planktonic cultures (Figure 5). In vivo experiments (Figure 6) indicated that lower concentrations of the oils, in particular the rosemary oil, were high significantly ($p< 0.001$) effective during the course of the study as compared to chlorhexidine. Minor changes in plaque scores were recorded in a 1-year study in 10 patients who had been treated non-surgically for periodontal disease in which the subjects were instructed to use chlorhexidine (0.2%) twice daily as a rinse but were not given any other formal oral hygiene instructions (30). Similarly designed studies for Listerine showed plaque reductions ranging from 22 to 36%. (31). These clinical studies also clearly demonstrate that essential oil mouthwashes have excellent safety and tolerability profiles. They showed no evidence of extrinsic tooth stain compared with controls, and intraoral soft-tissue examinations showed no aberrations of any kind (31, 32). In addition, the users reported no changes in taste perception and showed no increase in calculus formation (31, 32). However, there are side effects to chlorhexidine treatment such as an objectionable taste, tooth discoloration, and desquamation and soreness of the oral mucosa (33). Recent reports have suggested that chlorhexidine was ineffective against dental caries in clinical trials (34), and it has been implicated as the potential cause of the selection and persistence of bacteria with low level antibiotic resistance (35). The present study shows that essential oils are capable of affecting biofilm formation. They significantly decreased bacterial adhesion and affected bacterial viability in biofilms. Lin et al. (36) postulated that phenolic-substances may create a low pH microenvir

Figure 1- Decimal reduction time (D value) of *S.mutans* exposed to the MBC levels of essential oils and chlorhexidine

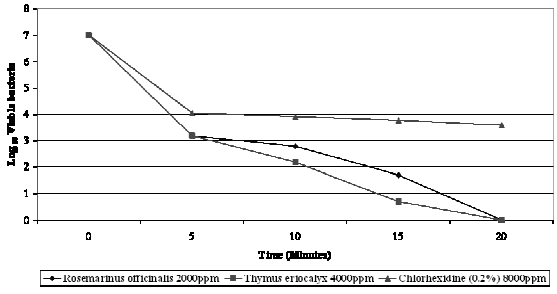


Figure 2- Decimal reduction time (D value) of *S.pyogenes* exposed to the MBC levels of essential oils and chlorhexidine

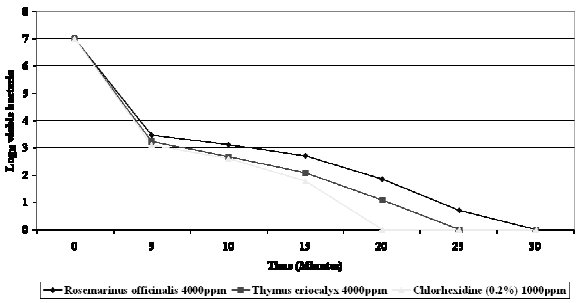


Figure 3- Effect of MBC levels of the essential oils on the zone of inhibition and the time required for complete elimination of *S.mutans*

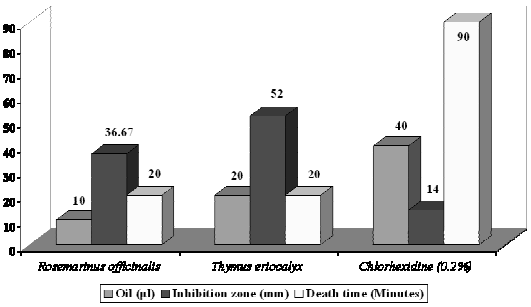


Figure 4- Effect of MBC levels of the essential oils on the zone of inhibition and the time required for complete elimination of *S.pyogenes*

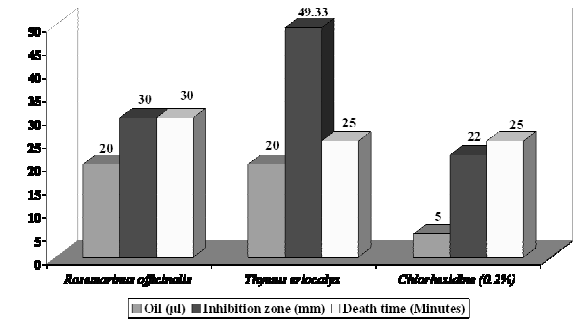


Figure 5- In vitro biofilm formation by S.mutans exposed to various levels of different essential oils and chlorhexidine

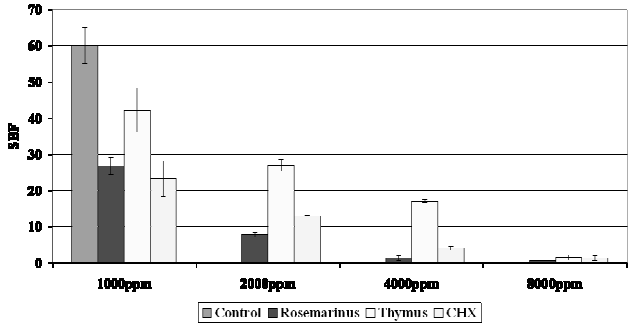
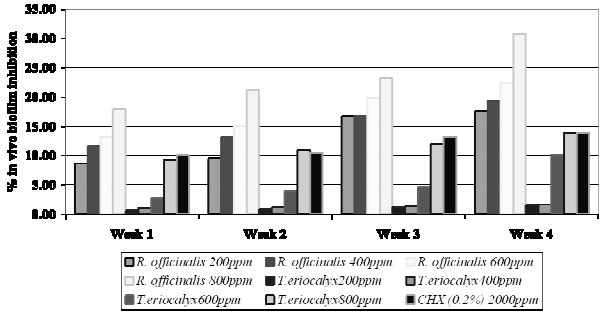


Figure 6- Percent in vivo inhibition of biofilm formation compared to the control groups



-onment due to proton donation and cell membrane disruption, due to stacking. Mourey and Canillac (37) found that the constituents of essential oils, such as monoterpenes (pinene, limonene, and cineole), contribute to the antimicrobial effect. In addition to these, rosemary essential oil contains abundant oxides and monoterpenes, and has the main action of stimulating the nervous system under sympathetic control, leading to increases in memorizing and concentrating abilities (38). These compounds are revealed in high content in the essential oil of rosemary. Most of the studies on the mechanism of phenolic compounds focused on their effects on the cellular membrane, altering its function and in some instances structure, causing swelling and increasing its permeability. These could justify higher antistreptococcal activities of *Rosemarinus officinalis* and *Thymus eriocalyx* as novel agents in combatting biofilm formation.

CONCLUSION

We conclude that there may be a potential role for essential oils in the development of novel anticaries treatments.

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