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Effect of the *Thymus vulgaris* essential oil on the growth of *Streptococcus mutans*

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ABSTRACT

The traditional Mediterranean herb thyme (*Thymus vulgaris*) is a source of an essential oil that has been shown to possess antimicrobial activity against many microorganisms. A considerable part of the general population has dental caries and *Streptococcus mutans* is one of the microorganisms responsible. The aim of this study was to assess the effect of the essential oil extracted from thyme on the growth of *S. mutans*, the main bacterium involved in the etiology of dental caries, as well as to incorporate this oil into a toothpaste formulation for preliminary assessment. The broth dilution technique was used in threefold tests for antibacterial activity. The concentrations tested were 1%, 5% and 10% essential oil diluted in ethanol or mineral oil. The controls were triclosan at 0.25% and 0.5%, chlorhexidine digluconate at 0.06% and 0.12%, and ethanol. The 1% solution of thyme essential oil in ethanol proved to be the most efficient against *Streptococcus mutans* and may be considered viable as an ingredient of toothpaste, both with regard to cost and to the sensory profile of the product. Also, analyses of the characteristics of the formulation indicated that the product is stable.

Keywords: Thyme/antimicrobial activity. *Thymus vulgaris*/ antimicrobial activity. Essential oil/pharmaceutical applications. *Streptococcus mutans*. Caries/prevention.

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INTRODUCTION

The history of humanity shows that the use of medicinal plants is very old. The World Health Organization (WHO) reports that a large portion of the population uses plants for therapeutic purposes, owing to poverty or a bad healthcare system (Migliato, 2007). According to Isaac et al., (2008), it is the active plant compounds present in a phytocosmetic product that define the effectiveness of the product.

The family *Lamiaceae* consists of approximately 150 plant genera with roughly 2800 species throughout the world, among which *Thymus vulgaris L.*, popularly known as common thyme, stands out. This culinary and medicinal herb is native to the Mediterranean region (Spain, Italy, France, Greece, Egypt, Lebanon, Turkey) (Carreto et al., 2007; Lorenzi & Matos, 2002). The hybridization that occurs among *Thymus* species that are geographically close and have coincident flowering periods results in great variability, which affects the homogeneity and yield of the essential oil and its chemical composition. Antiseptic, expectorant, carminative and antispasmodic activities are attributed to thyme oil. Such activities are associated with the content of thymol (2-isopropyl-5-methylphenol) and its conformational isomer, carvacrol (5-isopropyl-2-methylphenol), which have greater antibacterial and antifungal activities than phenol and are less toxic (Santurio et al., 2007; Simões et al., 2007). Thymol, a phenolic antioxidant from plants and important ingredient in toothpastes, has demonstrated antibacterial, anthelminthic and antifungal activity, while carvacrol has been investigated for its antibacterial activity (Carreto et al., 2007; Rubin et al., 2007).

The bacteria *Streptococcus mutans* are oval Gram-positive cocci of diameter 0.5 to 0.75 µm, group in pairs or chains and require a nutritionally rich medium and an average temperature of 37°C for optimal growth (Moreira, 2006; Neiva & Vicente, 2007). Like other streptococci,

S. mutans is classified as a facultative anaerobe, that is, an organism that can grow both in aerobic and anaerobic conditions (Fejerskov & Kidd, 2005).

Compared to other human body sites, the oral cavity has the highest numbers and diversity of microorganisms (Marinho & Araújo, 2007; Fejerskov & Kidd, 2005). Some groups of microorganisms can be found in specific oral niches in most individuals. The normal microbiota consists of microorganisms that are best adapted to the environmental characteristics that are specific to each niche (Fejerskov & Kidd, 2005). Dental caries is one of the main oral health problems. It is localized, transmissible and infectious, since it depends on infection by specific cariogenic microorganisms (Fejerskov & Kidd, 2005; Rosell et al., 2004). Dental caries is also considered a multifactorial disease, depending on the association of four primary factors: the host, the cariogenic microbiota, the dietary substrates (that are metabolized by the microorganisms) and time, since the first three factors must be present for a period of time for enamel demineralization to occur. Meanwhile, secondary factors, such as reduced salivary flow rate and tooth mineralization, influence caries progression and severity (Fejerskov & Kidd, 2005).

Caries begins with an imbalance in the native microbiota, favoring the proliferation of some opportunistic pathogenic microorganisms, since, under normal conditions, the levels of these microorganisms are low. *Streptococcus mutans* is considered the main species involved in the development of caries. It is therefore one of the best studied species and its only known natural habitat is the dental surface (Fejerskov & Kidd, 2005; Marinho & Araújo 2007).

The formation of a biofilm by microorganisms attached to a tooth surface, under continuous salivary flow, is the initial stage in the development of dental caries (Neiva & Vicente, 2007). Bacterial plaque is a dense, uncalcified mass formed by microorganisms embedded in a matrix rich in extracellular bacterial polysaccharides and salivary glycoproteins, firmly attached to the tooth. Normally, it develops over the salivary biofilm (acquired film) that lines the entire oral cavity (Marinho & Araújo, 2007; Rahim & Khan, 2006).

The cariogenic microbiota and, consequently, the appearance of new caries, can be controlled by proper oral hygiene allied to fluoride therapy, reduced sugar intake and intake frequency, restorative treatment of the existing caries and use of antimicrobial substances (Marinho & Araújo, 2007; Rosell et al., 2004). According to Fejerskov & Kidd (2005) there is little chance of suppressing the cariogenic microbiota once it is established.

Many essential oils and their constituents have been tested in recent years, in view of their antimicrobial properties, resulting in their use in a range of health-related areas, including food, cosmetics and pharmaceuticals, in which essential oils are used as preservatives or as drugs (Kalemba & Kunicka, 2003; Nascimento et al., 2007).

The main products and cosmetics used for oral hygiene and to maintain the oral cavity in good working condition are toothpaste, tooth brush, dental floss and mouthwash. Chlorhexidine, a cationic antibacterial agent used during dental treatments, is one of the best-studied agents and considered to be the most effective (Marinho & Araújo, 2007; Zanatta & Rösing, 2007). Chlorhexidine inhibits glucose incorporation by *S. mutans* and consequently, its metabolism to lactic acid. Furthermore, it inhibits enzymes that are essential for bacteria to form plaque on the dental surfaces, such as the glycosyltransferase secreted by *S. mutans* (Fejerskov & Kidd, 2005; Torres et al., 2000). Long-term use of chlorhexidine is not recommended because of its adverse side effects, such as tooth staining and changes in taste sensation (Fejerskov & Kidd, 2005; Torres et al., 2000; Zanatta & Rösing, 2007). Chlorhexidine has been used in many formulas and innumerable studies show that a concentration of 0.12% is enough to effectively reduce the oral biofilm (Marinho & Araújo, 2007).

Triclosan is another antimicrobial agent used in toothpastes prophylactically to reduce plaque formation and inhibit or slow the development of gingivitis (Fejerskov & Kidd, 2005).

The objective of the present study was to assess the effect of thyme (*Thymus vulgaris*) essential oil on the growth of *Streptococcus mutans* and to prepare a toothpaste formulation with this essential oil.

MATERIAL AND METHODS

Material

The thyme essential oil was purchased from *Brasil Portrait Cosméticos Ltda* (Sorocaba, SP, Brazil) - lots 4418 and 4419. Chlorhexidine digluconate and triclosan were purchased from *Henrifarma* (São Paulo, SP, Brazil). The culture media were Brain Heart Infusion (**BHI**) broth and Trypticase Soy Agar (TSA) from Difco™.

Samples

The test samples were: thyme essential oil in ethanol (1%, 5% and 10%); thyme essential oil in mineral oil at the same concentrations; triclosan in ethanol (0.25% and 0.5%); chlorhexidine digluconate in ethanol (0.06% and 0.12%); combinations: (A) 5% thyme essential oil in mineral oil and 0.25% triclosan; (B) 5% thyme essential oil in mineral oil, 0.25% triclosan and 0.06% chlorhexidine digluconate; (C) 0.25% triclosan and 0.06% chlorhexidine digluconate, and (D) 5% thyme essential oil in mineral oil and 0.06% chlorhexidine digluconate.

Antimicrobial assay

The essential oil was tested against the *Streptococcus mutans* strain ATCC 25175, by broth dilution assay. The inoculum of *Streptococcus mutans* was prepared by dispersing a colony in a 0.9% saline solution and adjusting cell density to tube 0.5 on the McFarland scale (Erna Cona, 2002; Longhini et al., 2007).

To each test tube (triplicate) containing 5mL of BHI were added 100µL of the bacterial suspension and 250µL of a sample. The tubes were incubated at 37ºC for 72 hours, under low oxygen tension in stainless steel jars. The negative control was culture medium alone and positive

control was culture medium and microorganism. The assay was reproduced threefold.

The presence or absence of microbial growth, which was determined by the turbidity of the culture medium, was recorded. If the sample was already turbid at inoculation (5% and 10% thyme essential oil in ethanol and 0.25 and 0.5% triclosan), impairing the later reading of turbidity, it was incubated as above and, after this period, samples of the homogenized broth were transferred to a dish containing TSA agar and this was incubated, under the same conditions, to determine whether microbial growth had occurred.

Formulation containing thyme essential oil

A toothpaste formulation was prepared for the addition of thyme essential oil (Table 1).

Table I. Formulation of a toothpaste.

Components (INCI*)	Function	% w/w
Calcium carbonate	Abrasive	50.0
Glycerin	Humectant	20.0
Green dye	Coloring agent	0.1
Methylparaben	Preservative	0.10
Peppermint essence	Flavoring	1.50
Sodium carboxymethyl cellulose	Thickener	0.30
Sodium lauryl sulfate	Surfactant	3.00
Sodium saccharin	Sweetener	0.15
Thymus vulgaris essential oil	Active ingredient	1.00
Water	Vehicle	to 100

* International Nomenclature of Cosmetic Ingredients

After a sensory test of the color, odor and appearance of the prepared toothpaste, it was packaged in sealed plastic tubes and stored at room temperature or in a dry Fanem® incubator (315 SE) thermostated at 60°C for 120 days and reassessed at 15-day intervals (Brasil, 2004; Brasil, 2007).

RESULTS

The results of all antimicrobial activity assay of thyme essential oil are described in Table II and the results of the formulation assessment are described in Table III.

Table II. *Streptococcus mutans* growth in the presence of various concentrations of *Thymus vulgaris* essential oil, triclosan or chlorhexidine digluconate, alone or combined.

Sample	Reading (*n*=9)
1% Thyme essential oil in ethanol	Absence of growth
5% Thyme essential oil in ethanol	Absence of growth
10% Thyme essential oil in ethanol	Absence of growth
1% Thyme essential oil in mineral oil	Presence of growth
5% Thyme essential oil in mineral oil	Presence of grouth
10% Thyme essential oil in mineral oil	Absence of growth
Ethanol	Presence of growth
0.5% triclosan	Absence of growth
0.25% triclosan	Absence of growth
0.06% chlorhexidine digluconate	Absence of growth
0.12% chlorhexidine digluconate	Absence of growth
Combination A *	Absence of growth
Combination B **	Absence of growth
Combination C ***	Absence of growth
Combination D ****	Absence of growth

* 5% Thyme essential oil in mineral oil + 0.25% triclosan;
** 5% Thyme essential oil in mineral oil + 0.25% triclosan + 0.06% chlorhexidine digluconate;
*** 0.25% triclosan + 0.06% chlorhexidine digluconate;
**** 5% Thyme essential oil in mineral oil + 0.06 % chlorhexidine digluconate.

Table III. Color, odor and general appearance of the toothpaste containing *Thymus vulgaris* essential oil at various times and two storage temperatures.

Time (days)	Room temperature			60°C		
	Color	Odor	Appearance	Color	Odor	Appearance
0	LG	C	I	LG	C	I
15	LG	C	I	LG	C	I
30	LG	C	I	LG	C	I
45	LG	C	I	LG	C	I
60	LG	C	I	LGY	F	I
75	LG	C	I	LGY	F	D
90	LG	C	I	LGY	F	D
105	LG	C	I	BY	G	D
120	LG	C	I	LB	G	D

BY: Brownish yellow; C: Characteristic; D: Dry; F: Fading; G: gone; I: initial/unmodified ; LB: Light brown; LG: light green; LGY: Light green yellowish

DISCUSSION

The idea for the present study came from the population's tendency to use plants in their search for effective alternative medicines, which are cheaper and possibly less aggressive to the human body. The prophylactic use of personal hygiene products on a daily basis is extremely important. While screening medicinal plants that could have real antimicrobial activity, our group came across the culinary herb, thyme (*Thymus vulgaris*). Thyme is an aromatic plant whose essential oil has been an object of considerable interest to the international scientific community.

After preliminary tests, the concentrations of thyme essential oil tested in the present study were 1%, 5% and 10%, since no published articles were found with suggestions of optimal concentrations.

Thyme essential oil was diluted in ethanol and the results showed that 0.5% triclosan (positive control) and thyme essential oil, at all the above three concentrations, were effective against the microorganism (Table II). When the thyme essential oil was diluted in mineral oil, however, the results were different and growth inhibition of *S. mutans* occurred only at the highest concentration (10%).

A second positive control used in the study was chlorhexidine digluconate, another antimicrobial substance commonly used in oral care formulations (Marinho & Araújo, 2007; Torres et al., 2000). Triclosan and chlorhexidine digluconate were used at their usual concentrations and at half strength (0.5% and 0.25%; 0.12% and 0.06%, respectively). Their concentrations were reduced to 50% to test whether synergy occurred between these substances and thyme essential oil.

The possibility of synergy between thyme essential oil and the other substances was considered because the only concentration of thyme essential oil in mineral oil that seemed effective against the microorganism was 10%. This concentration would be unviable in any type of formulation because of the high cost and especially because of its intense flavor, which would render the product unpalatable.

Since 10% thyme essential oil in mineral oil is an effective but unviable concentration for use in cosmetic formulations, combinations of 5% thyme essential oil and half the usual concentrations of triclosan and chlorhexidine digluconate were tested to see if these substances would show synergy. The concentration of 5% thyme oil was

chosen because it is considered the most viable for use in formulations in terms of cost and interactions with the other components of the formulation. Since this concentration (in mineral oil) gave inadequate results when used alone, this concentration could be used to test for synergy. Half of the usual concentrations of triclosan and chlorhexidine digluconate were used for the same reason: to test for synergy, since these concentrations, alone, would not be effective against the microorganism. Positive results were obtained for all concentrations of these antimicrobial agents when used alone, suggesting that the activity shown by all the combinations was not due to synergism (Table II).

In order to test the effectiveness of thyme essential oil dissolved in ethanol against *S. mutans*, it was necessary to determine how ethanol alone affected *S. mutans* growth. Ethanol did not affect microbial growth in any of the experiments (Table II). This shows that, at all three concentrations, the effectiveness of this solution against bacterial growth was due to thyme essential oil and not to ethanol.

The results obtained show that the sample that prevented microbial growth most efficiently was that containing 1% thyme essential oil in ethanol, since even at this low concentration, thyme essential oil showed activity against *S. mutans*, inhibiting its growth. Obviously, the same happened at concentrations of 5% and 10%.

When thyme essential oil was diluted in mineral oil, it did not afford satisfactory action against *Streptococcus mutans*. It is possible that the lipophilic nature of mineral oil did not allow it to mix well with the BHI broth, so that the two phases (mineral oil and BHI broth) separated. Consequently, the thyme essential oil might not have been in close contact with the microorganism, despite being inside the same test tube. Ethanol promoted homogenization of the mixture, possibly because the mixture of essential oils has both short-chain terpenes and oxygenated compounds.

Thyme essential oil in mineral oil was investigated in association with other commonly used antimicrobial agents, to determine if synergism occurred, but the results showed no improvement. The concentrations used in the study were lower than the usual concentrations in oral care products (mouthwash, for example), to try to avoid microbial inhibition by these substances alone. However, inhibition still occurred at these concentrations, so these results were discarded, since thyme essential oil, the object of the study, was efficient at the lowest concentration (1%) investigated. This concentration can be considered viable both in terms of cost and in terms of palatability.

An important factor to consider when analyzing the results is the use of various thyme essential oil lots because, as Nascimento et al., (2007) point out, lots can be affected by intraspecific genetic variation and differences in the conditions of cultivation of the plant or preparation methods, such as climate, sowing time, soil, use of pesticides, use of fertilizers, state of plant material (dry or fresh) and extraction technique. These variations can affect the chemical composition of the oil and the contents of the active substances significantly, thereby influencing their antimicrobial activity. Furthermore, adverse storage conditions can affect the stability of the oil components and reduce their activity. In the present study, two different lots were mixed.

As a typical vehicle for the thyme essential oil, a toothpaste formula was prepared and assessed, in order to test the performance of this oil in this type of product (Table I).

The results obtained in the present study could not be compared with similar results in the literature because published studies of the effect of thyme essential oil on *Streptococcus mutans* were not found.

However, many investigations of the activity of other essential oils, plant extracts and other plant derivatives against a variety of microorganisms, including *Streptococcus mutans*, were found. For example, Nogueira et al., (2007) assessed the activity of propolis essential oils against the cariogenic bacteria *S. mutans* and *Lactobacillus casei*, using the agar diffusion test. The essential oils showed significant activity against the two microorganisms, and some had an even larger zone of inhibition than the positive control (1% chlorhexidine digluconate).

Vasconcelos et al., (2008) assessed the antimicrobial activity of a dental cement containing *Copaifera multijuga* Hayne oleoresin against *Streptococcus mutans* and *Streptococcus sanguinis* by the dilution test in a liquid medium. Inhibition of microbial growth occurred in all tested groups of both bacterial species, showing that copaiba oil has great potential to be used in dental cement.

Silva et al., (2008) investigated the *in vitro* antimicrobial activity of a hydroalcoholic extract of *Rosmarinus officinalis* Linn. (rosemary) against standard *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus casei* strains. The minimum inhibitory concentration (MIC) was determined by the agar diffusion test, in Petri dishes. Satisfactory results were found for most of these microorganisms, except *Streptococcus mitis*, suggesting the possibility of using rosemary extract as an oral antimicrobial agent. However, the authors proposed to use study models that reproduce the characteristics of the oral cavity more accurately, for a better assessment of the use of this extract to treat and prevent oral infections.

Studies have been published on the antimicrobial activity of thyme essential oil against microorganisms other than *S. mutans*. For example, Santurio et al., (2007) assessed the antimicrobial activity of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and cinnamon (*Cinnamomum zeylanicum*) essential oils against samples of *Salmonella enterica* of varied serovars, all taken from bird carcasses. The results showed that oregano and thyme essential oils are effective against *Salmonella*.

In addition to antibacterial assessments, thyme essential oil has also been tested against viruses. Koch et al., (2008) used a plaque reduction assay to test thyme oil activity against the herpes simplex virus type 2 (HSV-2) in RC-37 cells *in vitro*. This study showed that thyme essential oil has satisfactory activity against the virus.

Given that no studies were found on the activity of thyme essential oil against *Streptococcus mutans*, the present study is of great relevance, since the test substance gave very satisfactory results against this microorganism. Thus, taking our results and the above-mentioned studies together, thyme essential oil can be used as a broad-spectrum prophylactic agent, protecting the oral cavity as

a whole, since the oral cavity is known to be the route of entry for many microorganisms.

Regarding the use of this oil in a formulation, the results obtained for the toothpaste indicate that the formulation is stable, showing no changes when stored at room temperature for the study period of 120 days (Table III). However, the formulation stored at 60ºC showed considerable changes in color, odor and consistency (darkening, fading odor and dryness) after 60 days, which was to be expected, since the active ingredient is a highly volatile essential oil, subject to oxidation when exposed to high temperatures.

The incubator used in this study was a dry incubator, which did not maintain high humidity levels inside its chamber. Hence, any product stored in such an environment at 60ºC tends to dry up and the loss of water raises the concentrations of all the components of the formulation. Since the chemical decomposition kinetics of these compounds is generally of first order, an increase in concentration increases the decomposition rate of the product even more than high temperature alone, further reducing its stability (Prista et al., 1990).

The results, in particular for the formulation stored at room temperature, show that this formulation appears to have good stability. Today, it is common to use active plant ingredients in cosmetic formulations. Therefore, the formulation prepared in this study containing thyme essential oil, with its previously demonstrated activity, is promising for the prophylaxis of oral diseases.

The tests carried out in this study showed that 1% thyme essential oil diluted in ethanol performed best against *Streptococcus mutans* and thus can be considered effective at this concentration in the fight against this organism. In the experimental conditions of this study, this concentration proved viable, both in terms of cost and in terms of sensory characteristics, making it suitable for use in formulations. When the toothpaste containing thyme essential oil was prepared and subjected to a simple stability test based on its organoleptic characteristics, the formulation proved stable, since, when stored at room temperature, its characteristics remained unchanged for 120 days. When stored at 60ºC, its characteristics only started to change after 60 days, indicating that the product was still quite stable when stored under such a severe temperature condition.

In the experimental conditions of the present study, *Thymus vulgaris* essential oil was effective against *Streptococcus mutans* at the smallest concentration tested (1%), which can be considered viable in terms of cost and sensory characteristics. In addition, the toothpaste formulation containing thyme essential oil was stable, so the use of *Thymus vulgaris* essential oil in cosmetic products is promising.

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RESUMO

Óleo essencial de Thymus vulgaris sobre o crescimento de Streptococcus mutans

***Thymus vulgaris* (tomilho) é uma fonte de óleo essencial que tem demonstrado atividade antimicrobiana. Uma parcela considerável da população tem apresentado problemas dentários, tais como a cárie, na qual o *Streptococcus mutans* é um microrganismo de fundamental importância. O objetivo deste trabalho foi avaliar o efeito do óleo essencial de tomilho sobre o crescimento do *Streptococcus mutans*, a principal bactéria relacionada com a etiologia da cárie dentária, bem como veicular este óleo essencial em uma formulação de creme dental para estudo preliminar. O método empregado foi diluição em caldo. As concentrações utilizadas de óleo essencial foram 1%, 5% e 10% de óleo essencial diluído em etanol ou óleo mineral. Os controles foram triclosan a 0,25% e 0,5%, bem como digluconato de clorexidina a 0,06% e 0,12%. A amostra contendo 1% de óleo essencial de tomilho diluída em etanol foi a mais eficaz, sendo efetiva contra *Streptococcus mutans* e, portanto considerada viável em relação ao custo e ao sensorial conferido ao produto, sendo que a formulação avaliada foi considerada estável.**

Palavras-chave: Tomilho/atividade antimicrobiana. *Thymus vulgaris*/atividade antimicrobiana. Óleo essencial/aplicações farmacêuticas. *Streptococcus mutans*. Cárie/prevenção.

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