EVALUATION OF MINIMUM INHIBITORY CONCENTRATION AND ANTIMICROBIAL ACTIVITY OF BASIL EXTRACT (Ocimum basilicum L.) INCORPORATED TO THE MOUTHWASH

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ABSTRACT

AIM: This study has as aim to evaluate the minimum inhibitory concentration and the antimicrobial activity of basil extract incorporated to the mouthwash against the bacteria S.mutans. **MATERIAL AND METHODS:** For the study, the hydro alcoholic basil extract (Ocimum basilicum L.) incorporated to the mouthwash was used in order to evaluate the effect of this formulation on bacteria and its Minimum Inhibitory Concentration (MIC). As positive control, TBS + S. mutans was used; as negative control, only the TBS; the fluid hydro alcoholic basil extract 20% and the concentrated basil extract 12%, both incorporated to the mouthwash, were also evaluated. As MIC verification method and antimicrobial activity, the micro dilution was used in the concentrations: pure, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128; and carried out in triplicate. The microtiter plates were incubated and evaluated after 24 and 72hs. **RESULTS:** The results showed there was no antimicrobial activity of mouthwash associated to the fluid and concentrated basil extract. However, the mouthwash insulated showed antimicrobial activity only as pure; other dilutions did not presented the same result. **CONCLUSION:** Before the findings in this study, it is possible conclude that hydro alcoholic basil extract incorporated to the mouthwash did not present antimicrobial activity against the bacteria S. mutans.

KEYWORDS

Ocimum basilicum. Mouthwash. Streptococcus mutans.

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INTRODUCTION

Within the context lived by developing countries where several diseases occur due to the lack of sanitation, malnutrition and the lack of free access to medicine[1], the WHO – World Health Organization stimulates the use of regional herbal as medicine as a way to decrease the costs of Public Health Programs. The use of vegetal species as drug result in lower cost products and lower production time, consequently they are more accessible to the population[1].

Antioxidant, antimicrobial and antifungal activity of basil (*Ocimum basilicum L.*) has been reported in the literature in alimentary[2], microbiological[3], Medical Dentistry[4-5] and in agriculture[6], demonstrating effective antimicrobial results.

According to Ostrosky *et al*. (2008)[7], the evaluation of antimicrobial activity of vegetal extracts must be defined through the obtainment of minimum inhibitory concentration (MIC), that is the smaller quantity of substance necessary to inhibit the growth of test-organism. For the same authors, determination of MIC can suffer variations depending on the organism and the strain used in the text; in this way, it has to be applied in accordance with the primary etiological agent and the type of pathology in which the medicine will be proposed as therapy.

Caries is an infectious, transmissible dental disease, with multifactorial etiology, like diet, oral microbiota, hygiene habits, and salivary characteristics, representing one of the most frequent oral problems in Public Health[8-10]. Caries is directly related to the presence of several microorganisms involved on dental biofilm, among them the *Streptococcus mutans*, gram-positive cocci microorganisms, and they have been related with this pathogenesis main due to the good adhesion to the dental surface and several cariogenic properties[8].

Caries prevention is directly related to the effectiveness of oral hygiene[11]. Oral hygiene is performed through mechanic and chemical means. The mechanic control is carried out using toothbrushes and dental floss/tape, further auxiliary ones. However, the use of mechanic means is performed in a deficient way by most people, leading to the use of chemical control of bacterial film, an important oral hygiene factor. Chemical agents are widely proposed as an auxiliary mean on the prevention of the bacterial biofilm formation[12], used as mouthwashes, varnish or gel.

The literature demonstrates that the use of mouthwash as an accessorial to the mechanical techniques for oral hygiene is very common on the plaque and gegivitis[13]. Mouthwashes based on chemicals, like chlorhexidine and triclosan are effective against pathogenic periodontal microorganisms[14]. Among the agents used as

chemical control of bacterial biofilm, Chlorhexidine digluconate is considered the Golden standard due to the good antimicrobial effects and its substantiality[15]. However, despite its beneficial effects, it has been verified that prolonged use of Chlorhexidine digluconate may bring side effects like stains on teeth, restorations, prosthesis and tongue, sloughing of oral mucosa, reduced sensitivity of taste and formation of supragingival calculus[16]. It leads to detach the importance of possibility to use a product with similar action, but presenting lower aggressive power to the oral cavity.

Several agents are available in the market, although these chemical products can change the oral microbiota and have side effects, like vomit, diarrhea and tooth staining[17]. Because of these reasons, the search by alternative medicines based on natural products has been a research target all over the world. The use of standardized preparations based on natural products is safe and less toxic than some synthetic drugs[18].

We are aware of antimicrobial effect of basil described on the literature and its importance on the control of oral microbiota on the prevention of caries. This work has as aim to verify the antimicrobial action and the Minimum Inhibitory Concentration (MIC) of hydro alcoholic basil extract *(Ocimum basilicum L.)* incorporated to the mouthwash against the bacteria of *S. mutans* lineage.

MATERIAL AND METHODS

To perform this study, the following solutions are used: (1) Fluid extract of *Ocimum basilicum L*. (basil) was obtained from the laboratory Bio Tae (Tatuí – São Paulo - Brazil) with certificate of analysis (attached file, figure 1); (2) Concentrated extract of *Ocimum basilicum L*. (basil) obtained from the fluid extract previously described, on the laboratory of Microbiology of the Center University of Araraquara – UNIARA – São Paulo - Brazil; (3) Mouthwash manipulated in the laboratory of Pharmacy of the Center University of Araraquara – UNIARA, with the following formulation: 2.5g of glycerin, 0.1g of tween 20, 0.13g de saccharin, 0.05g of sodium benzoate, water *q.s.p*. for 50ml (figure 2); (4) Fluid extract of *Ocimum basilicum L*. incorporated on the mouthwash in 20% concentration; (5) Concentrated extract of *Ocimum basilicum L*. (concentrated) incorporated to the mouthwash 12% concentration; (6) Culture medium Trypitone soya agar (TSA) and Trypitone soya broth (TSB) (figure 2).

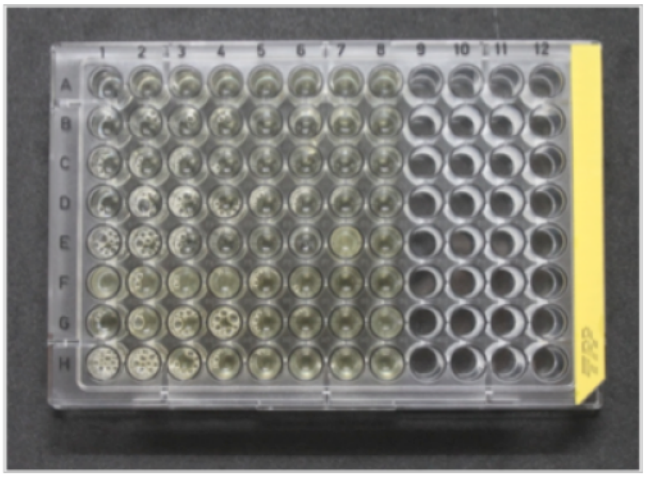
In this study, standard strains of S. *mutans* (ATCC 25175) ceded by School of Dentistry of Araraquara – Unesp – São Paulo – Brazil, were used. The strains were refrigerated and conserved in cryoval, with nutrient broth of glycerin 40%, and they were reactivated in the moment of use.

The culture medium used, Tryptic Soy Agar (TSA) and Tryptic Broth (TSB), were

prepared according to the supplier's instructions, added 0.25 % glycose. From the cryovals (micro tubes for conservation), a sprinkle was carried out on plates TSA with a handle 10µl. The plates TSA were incubated by 24 horas at 37°C. After the incubation period, using a bacteriological handle, about 3 to 4 colonies on the plates TSA were transferred to Falcon tubes (15ml) containing 5 – 10 ml TSB.

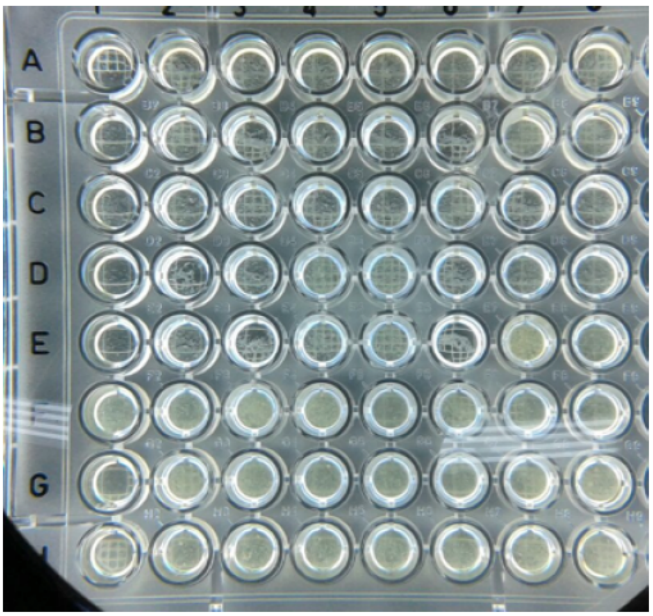
The tubes were incubated by 16/18 hours (one night) at 37 °C. After the incubation period, the tubes were submitted and centrifuged by 5 minutes at 2500 rpm. The supernatant was discarded and the biomass obtained was re-suspended in 10 ml of TSB, in order to obtain a turbidity correspondent to 0.5 on the Mac Farland scale, which corresponds to $1.5x10^8$.

Figure 1. Microtiter plate of 96 wells with the solutions inserted.



The culture mediums used were according to the following formulation: (1) TSA: 6g TSA + 15g glucose + 200ml of distilled water; (2) TSB: 6g TSB + 15g glucose + 200ml of distilled water.

Figure 2. Microtiter plate demonstrating presence of bacteria (by turbidity) after 24 hours.



The solutions were placed in a micro titer plate of 96 wells, according to described below (figure 1): (1) Column 1, line A, B and C: 200 **µ**l pure mouthwash; (2) Column 1, line D, E and F: 200 **µ**l basil mouthwash (concentrated) 12%; (3) Column 1, line F, G, H: 200 **µ**l basil mouthwash (fluid) 20%; (4) Columns 2 until 8, lines A until H: 100 **µ**l of culture medium (TSB).

The controls used were: (1) Positive control: TSB (column 10, line H); (2) Negative control: pure mouthwash (column11, line H).

All the experiments were carried out in triplicate.

Then, the serial dilution of all the wells from the column 1 until 8 was performed,

transferring 100 ml from one well to another, discarding the last 100ml remaining.

Aseptically in a biological cabin, after serial dilution, the *S. mutans* was added in an aliquot of 100µL of bacterial suspension (Streptococcus *mutans*) in all the wells. The plates were incubated at 37°C for 48 and 72 hours. The Minimum Inhibitory Concentration was considered the lower concentration of mouthwash able to inhibit the bacterial growth. The visualization was performed in naked eye by a trained and calibrated person.

RESULTS

Antibacterial activity of *Ocimum basilicum L*. extract (basil) fluid and concentrated incorporated to the mouthwash against *S. muttans* bacterial strains was conducted evaluating the determination of Minimum Inhibitory Concentration (MIC) of concentrated and fluid mouthwash.

It was observed that both mouthwashes with basil incorporated (concentrated and fluid) did not present antimicrobial activity on the early 24 hours, maintained the same result for 72 hours. The pure mouthwash formulation, anterior to the dilution, was the only solution that resulted in inhibition of bacterial growth (figures 2 and 3, table 1).

DISCUSSION

This study proved that hydro alcoholic extract of *Ocimun basilucum* incorporated to the mouthwash on concentration 12% (concentrated) and 20% (fluid) did not present antibacterial action. Most studies, regarding to the antimicrobial activity of basil, used the essential oil of *Ocimun basilucum* for analysis[3,5,19-28]. However, other extracts were also found[29], extraction with acetone, chloroform and methanol. Silva (2001)[19] compared the antimicrobial action of the essential oil and of hydro alcoholic extract of basil, and verified that only the essential oil demonstrated antibacterial activity. Nevertheless, the bacteria used were *Salmonella enteritidis and Staphylococcus aureus,* corroborating with the results of this study, using the same methodology, despite the different bacterial lineage.

The studies that used the essential oil as extractor method obtained positive antibacterial action by Silva, 2001[19]; Opalchenova & Obreshkova, 2003[3]; Hussain *et al.* 2008[20]; Ahonkhai, 2009[21]; Runyoro *et al.*, 2010[22]; Bassolé *et al.*, 2010[23]; Aquino *et al.* 2010[24]; Almeida *et al.* 2012[26]; Cavalcanti *et al.* 2012[5]; Sienkiewicz *et al.* 2013[27]; but the studies performed by Pozzo *et al.* 2011[25]; Freire *et al.,* 2014[28] did not show antibacterial action.

Still on the studies with essential oils, there was great diversity regarding to the methodologies employed. Hussain*et al.* (2008)[20] and Sienkiewicz *et al.* (2013)[27] used the disk diffusion method; Silva (2001)[19],

Ahonkhai (2009)[21], Runyoro *et al.* (2010)[22], and Cavalcanti *et al.(* 2012)[5], the diffusion method (or dilution) in agar; and the microdilution broth method was used by Opalchenova & Obreshkova (2003)[3]; Aquino *et al.* (2010)[24]; by Pozzo *et al.* (2011)[25]; and Freire *et al.*,(2014)[28].

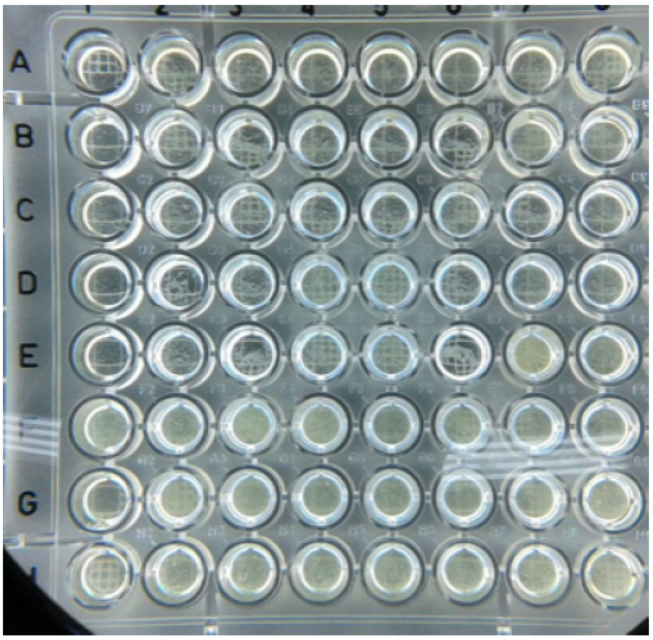
Table 1. Result of inhibition (+) and non-inhibition (-) of solutions in different times 24 and 72 hours.

Dilution	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128
200 µl Mouthwash	Soft inhibition	-	-	-	-	-	-	-
200 µl pure mouthwash	Soft inhibition	-	-	-	-	-	-	-
200 µl pure mouthwash	Soft inhibition	-	-	-	-	-	-	-
200 µl mouthwash + basil 12% concentration	-	-	-	-	-	-	-	-
200 µl mouthwash + basil 12% concentration	-	-	-	-	-	-	-	-
200 µl mouthwash + basil 12% concentration	-	-	-	-	-	-	-	-
200 µl mouthwash + basil 20% fluid	-	-	-	-	-	-	-	-
200 µl mouthwash + basil 20% fluid	-	-	-	-	-	-	-	-

A study about the hydro alcoholic extract about the possible toxicity in mice confirmed that this plant might affect the hematological system of the mouse; in this work the antimicrobial action was not evaluated. The scarcity of works regarding to the antimicrobial action of the basil hydro alcoholic extract and its easiness of incorporation stimulated, therefore, performing this study.

Regarding to the antifungal action of Basil, Almeida *et al.* (2012)[26] did not found antifungal action for Candida albicans insulated of HIV patients; the same result was found by Runyoro *et al.* (2010)[22] against three species of Candida: C. albicans, C. tropicalis e C. glabrata. However, Cavalcanti *et al.* (2012)[5] proved an effective antifungal action of basil against Candida albicans tropicalis C. krusei. The method of these studies consisted in micro dilution in broth for Almeida *et al.* (2012)[26], and diffusion in agar for the others.

Figure 3. Microtiter plate demonstrating presence of bacteria (by turbidity) after 72 hours.



The negative result of antibacterial action for mouthwashes with basil incorporated - in fluid or concentrated - could be justified by the extraction method, by seasonal harvest time and by the plants used. Regarding to the extraction method, the hydro alcoholic one presents lower antimicrobial activity compared to the essential oil, what maintain more the medicinal properties of the plant[19]. About the harvest times, in the winter and autumn, the antimicrobial potential is higher when compared to the summer and the spring harvests. However, this information is hardly provided by the manipulation laboratories[20].

Figure 4. Titer plate showing the positive control solution (+) TSB + bacteria and the negative control (-) TSB pure.

When other basil species are verified (O. basilicum, O. kilimandscharicum, O. lamiifolium, O. soft), Runyoro *et al.* (2010)[22] confirmed the O. soft is stronger, and O. basilicum is weaker, regarding to the bacterial action. Another species, O. sanctum was evaluated when incorporated to the mouthwash in a random clinical trial (RCT) on the control of bacterial plaque and gingival inflammation, and they verified an equally effective to the mouthwash containing Chlorhexidine.

The antimicrobial action found in mouthwash without basil can be due to the sodium benzoate present in the formulation, which has both bacteriostatic and antifungal properties[30]. The hydro alcoholic extract of basil used in this work, associated to the mouthwash formulation, possibly interacted with some component, resulting in inhibition of antibacterial effect of the formulation used.

CONCLUSION

Before the limitations of this study, it is possible conclude that the hydro alcoholic extract of basil incorporated to the mouthwash did not present antimicrobial activity against the bacteria *S mutans*. The need of new studies is evident verifying other concentrations, extraction methods, besides an *in vivo* analysis.

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