The Synergistic Antioxidant Effect and Antimicrobial Efficacity of Propolis, Myrrh and Chlorhexidine as Beneficial Toothpaste Components

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Toothpastes containing different components which could have beneficial effects on oral health and the identification of better composition are always a research challenge. Our study aims are to evaluate the synergistic effect of propolis, myrrh, and chlorhexidine, separated and combined in some formulated toothpaste. To assess the electrochemical capacity by monitoring of physico-chemical parameters (pH conductivity), the mixture of these components in toothpaste into artificial saliva solution was performed in ambient medium and at 37°C. The antioxidant activity and the antimicrobial potential in vitro of toothpastes formulated against microorganisms that frequently colonize the oral cavity was performed also. It has been found that mixtures of chlorhexidine, propolis, and myrrh exhibit synergistic antioxidant activity with better potential than some commercial toothpaste, also the antibacterial activity shown that this activity is maintained in time against some strains.

Keywords: antioxidant, antimicrobial, chlorhexidine, propolis, myrrh, toothpaste,

The most common oral complaints among the population are gingival inflammation and cavities. This pathology is largely generated by the presence of bacterial plaque that can lead to the appearance of caries, tartar and gingival inflammation [1].

The toothpaste has the role of reducing bacterial plaque, reducing inflammatory conditions and gum bleeding, and incorporating a series of vegetal extracts or volatile oils with various beneficial properties in the composition of such toothpastes, addressing patients suffering from gingivitis or periodontitis [2,3]. The *p*H of the toothpaste varies depending on the ingredients it contains from slightly acidic *p*H to lower alkaline *p*H. Thus, soap based toothpastes have a *p*H of between 9.5 and 10.5. Those based on foaming agents, softener flavors 7.5-9.0 and those with fluoride salts have an acidic pH of between 4.6 and 5.5 [4].

Toothpaste contains a variety of components, the main being abrasives (aluminum hydroxide, calcium carbonate), fluorides (sodium fluoride), detergents (surfactants) and antimicrobial agents (chlorhexidine). In the toothpaste, you can also add natural ingredients of eucalyptus oil, myrrh, propolis tincture and essential oils.

Chlorhexidine (CHX) is the antimicrobial agent most familiar to dental professionals for prevention of dental caries. CHX is a cationic biguanide with a low germicidal efficacy against the bacterial plaque and it has been used for more than twenty years for its action against plaque and gum disease [5]. It exhibits prolonged absorption and maintenance on tooth surfaces, can attach to salivary glycoproteins and thus reduces bacterial plaque formation. Chlorhexidine has duration of action ranging from 8 to 12 h. Bacteriostatically acts at concentrations of 0.1 g/mL and bactericidal in concentration of 100 g//mL on gram-positive germs, aerobic negative gram and optionally anaerobic. It is fungicidal and fungistatic on fungi and yeasts on *Candida Albicans* and virucides including HIV. Chlorhexidine is indicated for: prevention of bacterial plaque deposition, in acute gingivitis; in marginal periodontal abscesses; in chronic gingivitis and chronic marginal periodontitis [3]. It is incorporated into the toothpaste composition at a concentration of 0.04% to inhibit the formation of the bacterial plaque and prevent its consequence, dental caries and periodontitis [3,5,6].

Propolis is a resinous substance collected by *Apis mellifera* from flowers and buds plant, with which they cover holes and cracks in the hive, defend against bacteria and other microorganisms. It is a popular medicine that has a wide range of biological activities because of its chemical components, its strong pharmacological properties and low toxicity. Numerous propolis biological properties have been reported, including cytotoxic, antiherpetic, antioxidant, antimicrobial and anti-HIV [7-9].

Due to the wide range of biological activities, propolis is used in food and beverages and for improving health and preventing disease [8-11]. The medical use of propolis has led to an increased interest in its chemical composition and botanical origin, as flavonoids, polyphenolic compounds, have been identified so far. Polyphenolic compounds vary depending on their structure and concentration, depending on the region of production, availability of sources of vegetable resin collection. This broad spectrum of therapeutic effects makes propolis a potential candidate in several clinical trials. Clinical trials are also ongoing to check the effects of propolis in preventing and treating diseases [10-12].

Myrrh is a resinous substance, obtained by the growth of exotic shrubs: *Styrax Benzoin, Styrax macrothyrsus, Styrax paralleloneurus, Styrax tokinozis, Commiphora Myrrha* from the Styracaceae family, which is used in medicine [13-15]. The word *myrrh* originates in the ancient Hebrew word *myrrh*, which means *bitterness*, myrrh being a bitter substance like most of the resinous ones. Myrrh is a very compact, juicy juice that solidifies in contact with

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the air, becoming a tomato and forming translucent pieces. Slowly, the color becomes golden, because when the resin is hardened to be reddish, gathered in small tears. It has a balsamic taste, burns a pleasant scent and many pharic acid vapors. Dissolve in alcohol at 90° or in ether. The main chemical components of myrrh oil are: a-pinene, cadinene, lindestrene, beta-elemen, gamma-elemen dipentene limonene, cuminaldehyde, eugenol, m-cresol, heerabolen, acetic acid, formic acid and other sesquiterpene and acids [14]. Several myrrh qualities are known after the extraction and preparation mode as well as the age of the trees from which they are extracted [13]. Myrrh is anti-inflammatory of the airways. It is also used as antioxidant, antiseptic, astringent, carminative, deodorant, sedative, expectorant, antifungal, antibacterial and anti-inflammatory. It is recommended for rinsing the mouth by removing bad breath to relieve diseases that appear in the mouth (e.g. gingivitis). Due to its antimicrobial effect, oil is used to fight infections, viral, microbial and fungal infections. It treats oral and gingival problems such as mouth ulcers, pyorrhea, gingivitis and spongy gums. Myrrh as an antimicrobial agent acts in two complementary ways: it stimulates the production of white cells and has an antimicrobial direct role, being used to treat diseases in the oral cavity, especially gingivitis [13-15].

The present study aims to study the efficacy of three active principles (propolis, myrrh and chlorhexidine) will be evaluated separately and combined in formulated toathpastes by monitoring the physico-chemical parameters of pH and conductivity into artificial saliva solution. As well as the evaluation of antioxidant activity and the antimicrobial potential *in vitro* of these toothpastes formulated against microorganisms that frequently colonize the oral cavity will be tested.

Experimental part

Reagents, equipment and methods

The following pharmaceutical preparations were used: toothpaste with CHX, with propolis with myrrh, or mixed toothpaste, tested in artificial saliva solution or in combination with CHX 2%, prepared in laboratory according to good pharmaceutical laboratory practice.

The electrochemical measurements (*p*H, conductivity) were made with the Consort C862 equipped with two electrodes: mixt electrode (glass electrode for *p*H) and a special electrode (platinum electrodes for conductivity). Before measurement, the pH meter was calibrated with buffer solution of known *p*H (*p*H 4.0, 7.0 and 10.0) and the conductometric measurements using solution of KCl 1 n, 10^{-1} n and 10^{-2} n. All measurements were made at medium temperature (24°C) and human body temperature (37°C).

Evaluation of antioxidant activity was performed by redox volummetry (permanganometry), as direct and indirect method. The antioxidant capacity by the action of $KMnO_4$ in the acid medium on most reducing substances in plant products could be evaluated through this qualitative analysis. In direct method, the plant products with a different content of antioxidants are decolorated of $KMnO_4$ in the acidic medium. Standard solutions of $KMnO_4$ of different concentrations (being $2\cdot10^{-2}$N) are reported on reducing primary standard solutions (oxalic acid), of appropriated concentrations. Thus at 2 g of the sample (toothpaste) to distilled water in the presence of H_2SO_4(1:3), heating at 80°C, was titrated with $KMnO_4$ $2\cdot10^{-2}$N dropwise until a pale pink color persists for one minute.

The indirect volumetric method consists in adding excess of $KMnO_4$ $2\cdot10^{-2}$N and titration of the remaining excess unconsumed of the reducing substances with oxalic acid. Thus, at 2 g of the sample (toothpaste) with 50 mL of distilled water was added 15 mL of $KMnO_4$ $2\cdot10^{-2}$N, 5 mL of H_2SO_4 (1: 3) into an Erlenmeyer beaker. After heating, the excess of $KMnO_4$ in the solution was titrated with V mL of oxalic acid $2\cdot10^{-2}$N until the solution was discolored.

The reaction of $KMnO_4$ with e^-(reduction electrons) from sample in the acidic medium is:

$$2KMnO_4 + 3H_2SO_4 + e^- \leftrightarrow K_2SO_4 + 2MnSO_4 + 3H_2O + 5[O]$$

Reaction of $KMnO_4$ with oxalic acid in acidic medium at heating condition is:

$$2KMnO_4 + 5H_2C_2O_4 + 3H_2SO_4 \rightarrow 2MnSO_4 + K_2SO_4 + 10CO_2 + 8H_2O$$

The artificial saliva solution was prepared with NaCl-5% KCl-7.8% $NaH_2PO_3\cdot H_2O$-7.95%, $CaCl_2\cdot H_2O$-0.05%, $Na_2S\cdot 9H_2O$-3%, $(NH_4)_2SO_4$-0.05%, $C_6H_8O_7$-0.05%, $NaHCO_3$-1% and urea 10% added to distilled water to one liter of solution [16]. The solution was stirred until complete dissolution of all substances and complete homogenization.

Formulation and preparation of toothpastes

Recipes (Rp1-Rp4) are composed of base toothpaste and natural or synthetic active principles with antioxidant and antibacterial properties. We prepared the base toothpaste according with rules of good pharmaceutical practice and combined it with CHX, myrrh oil and propolis tincture to get the toothpaste samples (table 1).

The base toothpaste is prepared so that the substances are brought to the mortar in increasing order of mass and decreasing density, calcium carbonate, then sodium bicarbonate and kaolin when forming a composite powder. Substances are dried before use to remove total or partial moisture. When the mass of dust exceeds 20 g, sieving is mandatory, ensuring a homogeneous mixture without agglomeration of particulate matter. The 2.5% tragacanth gum is officially formulated and prepared according to the Romanian Pharmacopoeia, X-th edition [17].

Preparation of working solutions

Diverse types of solutions were prepared from formulated toothpaste and mixtures of components (antibacterial CHX, myrrh and propolis) analyzed in the artificial saliva solution, for constant volume of 10 mL (table 2). The *p*H and conductivity measurements were

Base compounds		Active principles	Rp1	Rp2	Rp3	Rp4
calcium carbonate sodium bicarbonate kaolin mucilage gummi tragacanthae 2.5% glycerin Water added till	40 g 10 g 5 g 10 g 10 g 100 g	Chlorhexidine	3 g	-	-	3 g
		Myrrhoil	-	3 g	-	3 g
		Propolis tincture	-	-	3 g	3 g

Table 1
MAIN AND SPECIFIC ACTIVE COMPONENTS IN THE FORMULATED TOOTHPASTES (g/100 g)

solution A	toothpaste (mg)			
	solution B	solution C	solution D	solution E
CHX 2%	with CHX	mixed (CHX, myrrh oil, propolis tincture)	with propolis tincture	with myrrh iol
between 0.1-1 mL	0.0552; 0.1021; 0.1682; 0.2452; 0.3040.	0.4900; 1.0410; 1.5331; 2.1250; 2.5912.	0.6301; 1.0121; 1.6382; 2.1782; 5.7301.	0.6011; 1.2012; 1.6320; 2.0381; 2.8001.

Table 2
COMPOSITIONS OF THE DIVERSE TYPES OF SOLUTIONS

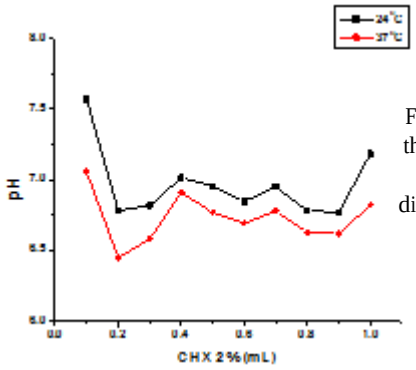


Fig.1a. *p*H evolution of the artificial saliva with added of CHX 2% in different volume, at two temperature

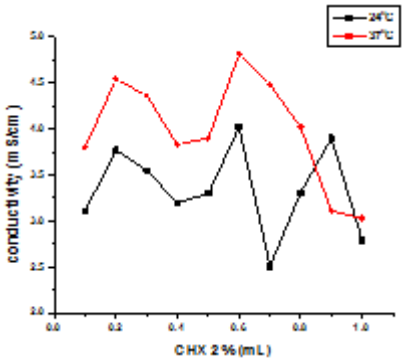


Fig. 1b. Conductivity measurements of the artificial saliva with added of CHX 2% in different volume, at two temperature

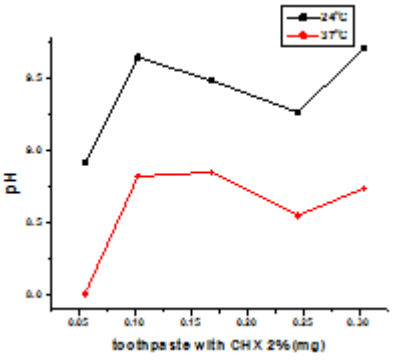


Fig.2a. The pH evolution with temperature on toothpaste with CHX 2% (type B) in artificial saliva solution

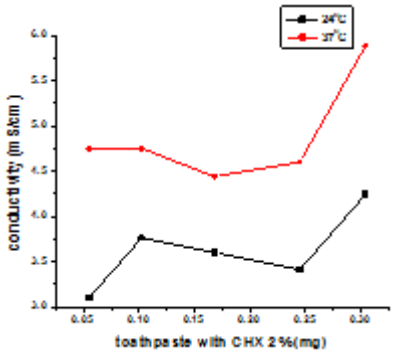
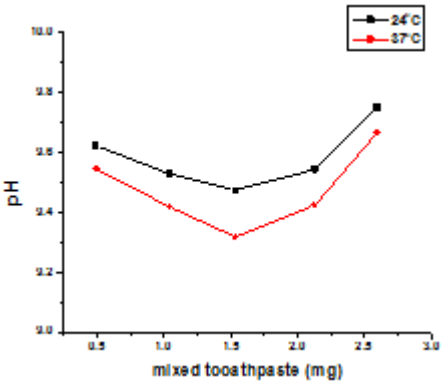


Fig.2b. Conductivity measurements on toothpaste with CHX 2% (type B) in artificial saliva solution



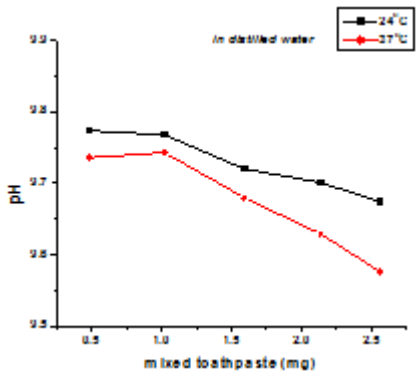
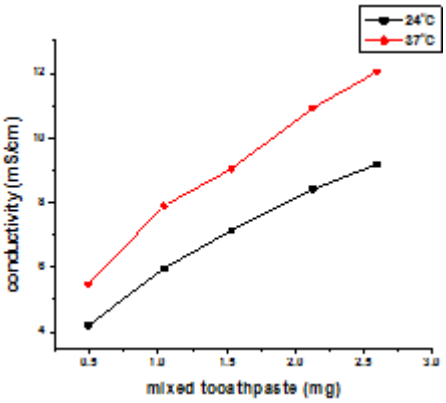


Fig.3a. The pH evolution with temperature on mixed toothpaste (type C) in artificial saliva solution and distilled water

performed (figs.1-6) compared with a solution with different mixture toothpaste amounts (0.482 mg, 1.020 mg, 1.591 mg, 2.139 mg, 2.564 mg) at 10 mL distillated water (fig. 3).

Evaluation of the antimicrobial activity of the formulated toothpastes was performed on culture media: MMA (Agar Malt Extract - for yeast and mold cultivation) and BCA (Agar broth - for bacteria cultivation), respectively. The method for assessing antimicrobial activity *in vitro* was performed as follows: 6 medium tubes (1 BCA and 5 MMA) were heated in a water jar until they became fluids. After fluidization, each medium is placed in a Petri capsule, which solidifies again. Then pour the SFS over the test strains and form suspensions, and with the flame detach the stem from the test tube wall. Add 0.5 mL of each suspension over their respective Petri capsules. With Drigalski spatula, previously flamed and cooled, the suspensions circulate very easily through circular motion across the entire surface of their media. Place 4 wells in each Petri dish over which place 100 µL of each toothpaste (solution: 1 g toothpaste to 10 mL distilled water). It is thermostated for 48 h at 37°C for BCA media and for 4 days at 28°C for MMA environments. The measuring the diameter of the growth inhibition halo (clear area) around the well was evaluated.



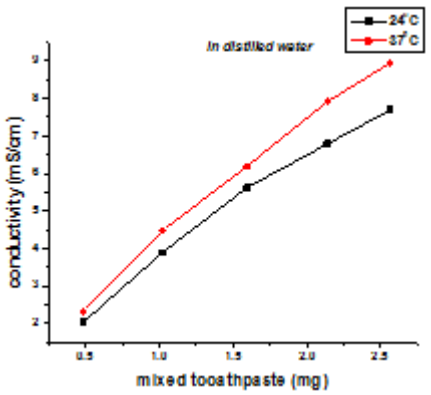


Fig.3b. Conductivity measurements on mixed toothpaste (type C) in artificial saliva solution and distilled water

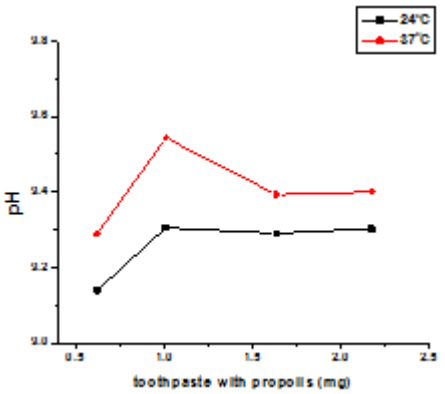


Fig.4a. The pH evolution with temperature on toothpaste with propolis (type D), in artificial saliva solution

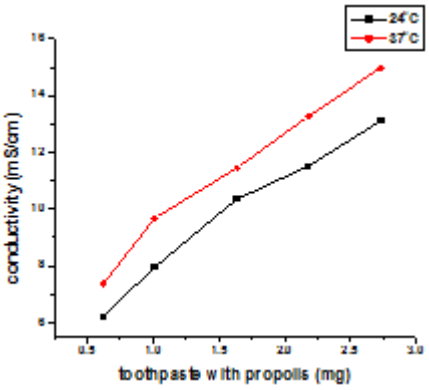


Fig.4b. Conductivity measurements on toothpaste with propolis (type D), in artificial saliva solution

Results and discussions

The role of saliva and characterization of the artificial saliva

Saliva, one of the most complex biological environments, meets implants and prosthetic works. It composition is given by water, mineral salts, organic matter, enzymes, immunological factors, hormones, vitamins. It is an excellent electrolyte with *p*H variations between 4.5–8.0 and with direct action on biomaterials [18]. Salivary secretion fluctuates quantitatively and qualitatively, these fluctuations also defining chlorine and pH concentrations. Some salivary constituents increase or decrease in saliva depending on its flow. The acid-base balance (*p*H) depends on the variety of salivary secretions that is daily, seasonal, and lower at night and in summer depending on diet. The average salivary *p*H is 6.8 and is influenced by diet (the *p*H of an alcoholic is acid), drugs (psychotropic medication leads to an acid salivary *p*H). Changes in *p*H may alter the salivary properties and disturbance of the oral flora ecosystem. The oral bacteria are multiplied to an optimal salivary *p*H and the *p*H of the mouth buccal generates unbalances of the oral flora with the alteration of the enzymes involved in the defense of the periodontal and periimplantation structures. Saliva *p*H influences the electrochemical corrosion and oxidation of biomaterials.

We prepared an *artificial saliva solution* according with reference [16]. Initially, the artificial saliva solution parameters (*p*H and conductivity) at medium temperature (24°C), and human body temperature (37°C) were evaluated. The *p*H is 7.13 (at 24°C) and 6.96 (at 37°C) respectively; and specifically, conductivity is 3.5 mS cm^{-1} (at 24°C) and 4.1 mS cm^{-1} (at 37°C) respectively, according to the literature for ambient temperature. At temperature of 37°C, the *p*H of solution recorded a slight decrease and the conductivity slightly increased by 0.6 $mS \cdot cm^{-1}$. Estimated values correspond to an artificial *p*H neutral sink with dissociation components and a suitable buffer [16,19].

The effect of CHX in contact with artificial saliva solution was tested also. Thus, for *type A solutions* there is a variation of *p*H between 6.4 and 7.0 at 37°C and a slight increase in *p*H values at 24°C, between 6.7 - 7.5, which is not always correlated with the amount of added CHX (table 1). It follows that the *p*H value varies slightly depending on temperature and amount of added CHX, but generally remains constant. Conductivity variations are higher at 37°C than at 24°C, e.g. from 3.8 to 4.8 $mS \cdot cm^{-1}$, higher values for 0.6 mL CHX 2% added in artificial saliva solution (fig. 1b).

Physicochemical evaluation of toothpaste

The pharmaceutical preparations as formulated toothpaste with CHX, myrrh oil, propolis tincture and mixed toothpaste (noted B-E, table 2) were tested for behaviour into artificial saliva solution to observe physicochemical evolution, at medium temperature and at 37°C.

For type B solutions, the *p*H of the solutions registers an increase in *p*H relative to the reference salivary reference parameters, a variation between 8.9 and 9.7 at 24°C and shows a slight decrease at 37°C, around *p*H 8 (fig. 2a). The conductivity values are relatively constant, with variation between 3.10 - 4.24 $mS \cdot cm^{-1}$ at 24°C and respectively of 4.44 - 5.88 $mS \cdot cm^{-1}$ to 37°C (fig. 2b).

The type C solutions showed a lower alkaline *p*H at 24°C and 37°C, around 9.5 (fig. 3 a). Conductivity values shown an increase, at 24°C values varied between 4.2 - 9.18 $mS \cdot cm^{-1}$, and between 5.48 - 12.04 $mS \cdot cm^{-1}$ at 37°C, increase directly proportional to the amount of toothpaste in the prepared solution (fig. 3b).

The *p*H assessment for type D (with propolis tincture) and type E (with myrrh oil) toothpaste solutions indicates stability at temperature variations around of *p*H 9. There are no essential changes for the $[H^+]$ concentration, the *p*H remains weakly alkaline (figs. 4a, 5a), also in conductivity measurements (figs. 4b, 5b).

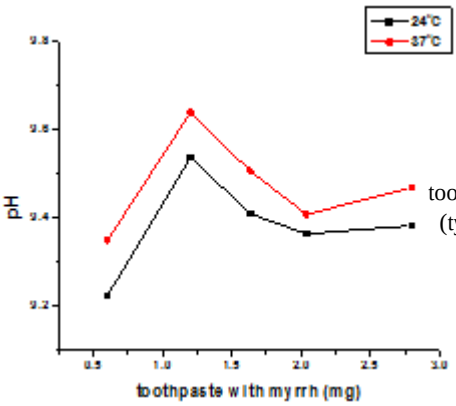


Fig.5a. The pH evolution with temperature on toothpaste with myrrh (type E), in artificial saliva solution

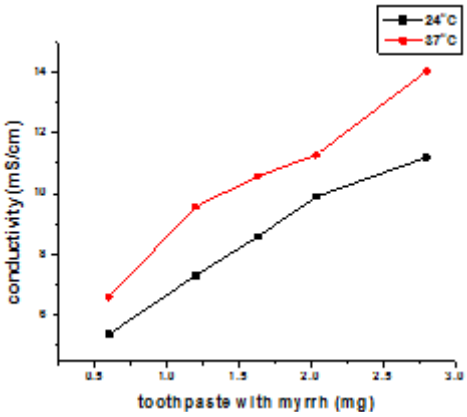


Fig.5b. Conductivity measurements on toothpaste with myrrh (type E), in artificial saliva solution

By comparison, the *p*H obtained for formulated toothpaste, on quantities of approx. 2.50 g, a slightly alkaline pH around 9 is observed. Thus, in toothpaste with CHX, the pH is lower (8.95), and in mixed toothpaste is higher (9.73), but all toothpaste is in according with pH accepted of FRX as alkaline toothpaste [17]. Therefore, it is noted in type C toothpaste solutions that synergistic effects are present between the mixed toothpaste components, that CHX, a chemically interacting antispective and the active principles of myrrh oil, an astringent tonic and the active principles of propolis tincture, a healer and an antimicrobial; and the result is beneficial for the pharmaceutical product.

Evaluation of antioxidant action of formulated toothpastes

From the recorded results, propolis-based toothpaste (Rp 3/table 1) has the most intense antioxidant character, better emphasized by the indirect method. The results obtained by both methods are graphically represented in figure 6. Propolis being a complex compound, and in toothpaste when adding aggressive chemical reagents has induced some changes in structure with reducing potential as it found in our results. By evaluating the antioxidant activity of toothpaste by the indirect method, propolis dentifrice has the highest antioxidant capacity of 3.792 g %, and the other toothpastes indicate lower antioxidant activity. Anyway, the mixed toothpaste (Rp 4/table 1) indicated a higher activity than the other toothpastes, with a insignificant difference for both chemical method (direct and indirect).

Comparison of the antioxidant activity of toothpaste formulated with some commercial toothpaste was

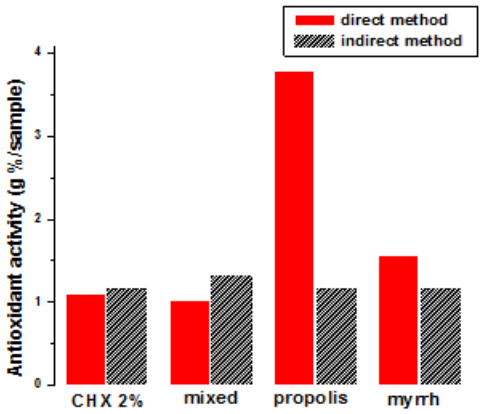


Fig.6. Antioxidant evolution on toothpaste analyzed by direct and indirect permanganometric method

achieved by the direct method because the indirect method resulted in a great deal different for formulated toothpaste due to the chemical reactions of active principles with aggressive reagents used to the analysis method. An antioxidant capacity of about five times greater was found in toothpaste formulated compared to commercial toothpaste tested. Therefore, laboratory toothpastes prepared in the laboratory have relevant antioxidant capacities with higher values than commercial toothpastes and could be a solution to be used with a beneficial potential for some oral affections.

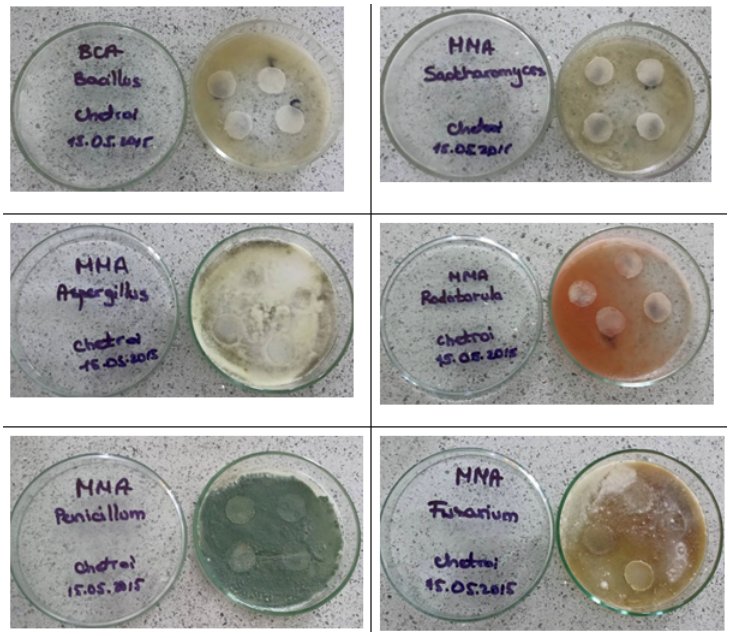


Fig.7. Antimicrobial activity of samples on different culture media after thermostation

Evaluation of antimicrobial activity of formulated toothpastes

The assessment of *in vitro* activity of the specimens aimed at verifying the antimicrobial potential against microorganisms that frequently colonize the oral cavity by measuring the diameter of the growth inhibition halo (the clear area) around the well (fig. 7).

Some areas of inhibition have been observed only when the strain is *Bacillus* sp., *Saccharomyces* sp. and *Rhodotorula* sp., but are very small (2-5 mm). The other fungal strains (*Penicillium* sp., *Fusarium* sp., *Aspergillus* sp.) were not sensitive to the action of the compounds that make up these toothpastes. Unfortunately, repeating the experiment with other diluted solutions was unsuccessful. Considering the age of the preparations, the inappropriate way of preserving the toothpaste, it would be the cause of the observed inactivity for the formulated toothpaste.

Conclusions

There were formulated toothpastes of low alkaline *p*H containing chlorhexidine, propolis tincture and myring oil, simple and combined, which due to sodium bicarbonate in their composition prevents acids from attacking the tooth enamel and producing a cooling effect. Addition of chlorhexidine enhances the synergistic effect due to its dissociation properties in the mixed toothpaste. Mixed composition toothpaste interacts with the artificial saliva solution with a slight variation in *p*H values, so its effects last for a longer period. Due to the synergistic effects of the three analytes (chlorhexidine, propolis and myrrh) created, chlorhexidine based on dissociation is expected to impart an effective therapeutic effect to the studied pharmaceutical products. The *p*H assessment of the elaborate toothpaste indicates stability at temperature variations. There are no essential changes for the concentration of H ions, the *p*H remaining in the predominantly alkaline range 9-10.

The highest conductivity variation is seen in toothpaste with chlorhexidine, ie ionic dissociations with temperature (studies conducted at 24 and 37°C respectively), the conductivity decreased by 4.32 $mS \cdot cm^{-1}$ at 24° C and 5.43 $mS \cdot cm^{-1}$ at 37°C, since the toothpaste solidifies against resistance to the dissociation of some ions.

From the recorded results, propolis teeth paste has the most intense antioxidant character, better evidenced by the indirect method, and the other toothpastes with much less antioxidant activity. Mixed toothpaste exhibits greater activity than other toothpastes, but the difference is insignificant, results obtained by the direct method.

Therefore, laboratory toothpastes prepared have relevant antioxidant capacities with higher values than commercial toothpastes. Toothpastes indicate antioxidant capacity, and the propolis toothpaste in composition is also 10 months old with increased antioxidant capacity compared to commercial toothpastes.

The antimicrobial activity, very small inhibition zones have been observed. However, considering the age of preparations and the inappropriate way of preserving and preserving the toothpaste formulated, it would be the cause of inactivity.

The components in the dentifrice composition impart this beneficial action and correspond to the purpose for which they were manufactured (medical to treat diseases or cosmetics for daily use in oral hygiene). The oral cavity despite having a small percentage in the human body structure is of extraordinary importance in maintaining its health and integrity.

Our study is encouraging and indicates that the chemical interactions that may occur between toothpaste components should be studied more intensely to explore their beneficial potential for the treatment of oral cavity disease.

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