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Formulation and Antimicrobial Activity of Toothpastes from Four Citrus Peel Extracts Combined with Surfactants

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Abstract: Many oral infections like dental caries and the well-known periodontal diseases come from bacterial imbalances in the oral microbiome. While conventional antimicrobials risk resistance and adverse effects, citrus-peel extracts, rich in bioactive compounds, exhibit antibacterial properties. This study mainly aimed to formulate and evaluate citrus-peel toothpaste containing commonly available anionic and non-ionic surfactants. Four citrus peel extracts (*C. limonum*, *C. reticulata*, *C. aurantium*, and *C. sinensis*) and three different surfactants (sodium lauryl sulphate [SLS], Poloxamer-407, and Poloxamer-188) were incorporated into six toothpaste formulations (TP1–TP6). These formulations were evaluated for physical properties; including pH, foaming ability, cleaning ability, moisture content, abrasiveness, spreadability, extrudability, and stability. Additionally, their antibacterial activity was evaluated against various bacterial species, along with antifungal activity against a single fungal strain. The formulations met standard parameters, ensuring quality and physicochemical properties. Two formulations with SLS surfactants (TP1 and TP2) demonstrated strong antimicrobial effects. *Staphylococcus aureus* was the most sensitive to TP1 and TP2 (inhibition zones of 25 and 27 mm, respectively), followed by *Proteus vulgaris* (21 and 22 mm for TP1 and TP2, respectively). However, both formulations had no effect on *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. On the other hand, both TP1 and TP2 exhibited a high effect against *Candida albicans* when compared to voriconazole as a positive control. The citrus-peel toothpaste, combined with surfactants, offers a refreshing aroma and antimicrobial effects against Gram-positive and Gram-negative microorganisms. It could be a safer alternative to synthetic toothpaste, but further studies are needed to confirm its safety and *in vivo* efficacy.

Keywords: Citrus peel, Oily extracts, Poloxamer, Surfactant, Toothpaste.

Introduction

Infections of the oral cavity is one of the most prevalent types of infections. Caries in teeth and periodontal diseases are the most common wide spread multi-factorial infectious diseases, which result in damage and infection of enamel, dentine, and gum [1]. The normal flora in humans comprises over 200 species of opportunistic bacteria, most of which are non-pathogenic bacteria. However, the imbalance of this ratio between the pathogenic and non-pathogenic bacteria creates infection and tooth decay [1,2]. According to the World Health Organization (WHO), 80% of the global population in developing countries relies on folk medicine in their healthcare. This high percentage emphasizes the significant role that traditional medicine plays in these communities and the importance of understanding and integrating these practices with contemporary healthcare approaches. Bacterial infections, which are recognized as a major cause of illness in both developed and developing countries, are typically treated with conventional therapies, such as antibiotics [3]. Poor oral hygiene, a high-sugar diet, and infrequent dental check-ups are major causes of these infections, as bacteria create biofilms on teeth and gums, causing inflammation and infection. The typical symptoms include pain, swelling, redness, and bleeding gums, with severe cases potentially resulting in tooth loss and broader systemic health problems. Remarkably, there is a well-documented link between periodontal disease and systemic conditions like heart disease and diabetes, and this highlights the crucial role of maintaining good oral health for overall well-being [4]. However, the utilization of synthetic antimicrobials has been associated with adverse effects, like hypersensitivity, asthma, gastrointestinal diseases, and cancer. Moreover, the overuse of synthetic antimicrobials may contribute to antimicrobial resistance and diminish the population of beneficial bacteria in the human gut. The challenge of multidrug resistance has augmented the severity of bacterial infections, which continue to account for a significant proportion of deaths in human. Multidrug-resistant bacteria alone are responsible for about 700,000 fatalities annually. Toothpaste (TP) is a semi-solid pharmaceutical dosage form that promotes oral health through its cleaning action and provides therapeutic benefits such as remineralization, whitening, and antimicrobial effects [2]. TPs are either synthetic or natural. Essential ingredients include abrasive agents, surfactants, binders, humectants, buffering agents, flavouring agents, and the vehicle. Some may also contain therapeutic components like fluoride, antiplaque, or antigingivitis agents [5].

Modern TP formulations use surfactants to remove debris, biofilms, and plaque, and to micelize flavouring agents and hydrophobic molecules for thorough mouth coverage. Proper surfactant levels are crucial for optimal cleaning and irritation prevention. One of the most common surfactants used is sodium lauryl sulphate (SLS) [6]. Given the potential

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side effects of antibiotics and other antimicrobial substances, researchers are exploring natural alternatives.

Citrus peel extracts show promise in combating oral germs due to their antibacterial and antifungal properties. In addition, they exhibited anti-inflammatory and analgesic benefits. The antibacterial activity of citrus peels might be due to components such as alkaloids, phenolics, flavonoids, and rutin [7,8,9,10]. Citrus oily extracts (COE) in toothpaste provide therapeutic and flavouring benefits. These aromatic compounds, consisting of approximately 400 components, are abundantly present in the oil glands of fruit peels. The COE can be made from lemon (*Citrus limonum*), tangerine (*Citrus reticulate*), bitter orange (*Citrus aurantium*), and sweet orange (*Citrus sinensis*) peels.

Citrus limonum, lemon, is an evergreen tree or shrub in the Rutaceae family. This fruit is a bright yellow, oval-shaped lemon, known for its sour taste and high acidity. Traditionally, digestive diseases were treated by lemon, as well as other diseases like scurvy. This miracle fruit (lemon) shows anticancer, anti-inflammatory, antioxidant, and antiseptic properties. The main constituents that contribute to its health benefits include citric acid, flavonoids, and essential oils [9,11].

Citrus reticulata, known as mandarin orange, a small tree belonging to the family Rutaceae. This tree aspects glossy leaves and fresh-smelling white flowers. This fruit is small, easy to peel with lovely sweet, with a bright orange color. Traditionally, mandarin oranges have been used in medicine to assist digestive health and relieve flu, coughs, and colds. Modern researches suggest that mandarin type oranges have antioxidant properties and may assist to reducing cholesterol levels. The health-caring effects are attributed to their important content of flavonoids, carotenoids, and essential oils [11].

Citrus aurantium, commonly called (bitter orange), a member of the Rutaceae family. This tree is famous for its thorny branches and odorous white flowers. The fruit is small, round with a thick, bumpy rind. Traditionally, bitter orange has been utilized to address digestive problems, anxiety, and as a sedative. Most modern research has demonstrated that bitter orange may assist in weight loss and exhibit antimicrobial properties. The medicinal effects of bitter orange are attributed to its chemical contents, which include synephrine, flavonoids, and essential oils [12,13].

Citrus sinensis, commonly known as (sweet orange), belongs to the Rutaceae family. This tree is characterized by its glossy leaves and redolent flowers. The fruit of the sweet orange is large, round, and sweet, with a bright orange color with a juicy interior. Traditionally, sweet oranges have been used to improve immunity, treat colds, and improve digestion. Many modern studies indicate that sweet oranges are rich in vitamin C and antioxidants, which can enhance immune function and reduce inflammation. The health benefits of sweet oranges are due to their rich content of vitamin C, flavonoids, and essential oils [11,14].

The antimicrobial activity of a toothpaste containing citrus essential oils might be attributed to the mixed action of bioactive compounds such as limonene, flavonoids, and phenolics. Limonene may disrupt bacterial cell membranes, leading to lysis of cell and death, and intervene with bacterial metabolic processes inhibiting its growth. Flavonoids may have very strong antioxidant activities, neutralizing free radicals and boosting the immune response, while also obstructing bacterial DNA, RNA, and protein synthesis. Phenolic compounds destroy bacterial cell walls and membranes, enhancing permeability and causing cellular leakage, and hinder bacterial enzyme activity, interfering with metabolic pathways. Together, these mechanisms could make citrus essential oils remarkably effective antimicrobial agents in herbal toothpaste, promoting oral health [11,15].

This research aimed to formulate, then evaluate a herbal toothpaste containing citrus peel extract with commonly used surfactants. The physical properties and potential antimicrobial effect of these formulations were also investigated.

Chemicals and Reagents

Calcium carbonate was purchased from Readial Deahein, Germany. Sorbitol was procured from Sigma Chemical Co., USA. Carboxymethylcellulose (CMC) was purchased from SHARKCMC®L, China. Sodium bicarbonate was bought from a local market in Mosul, Iraq. Titanium dioxide (TiO_2) and SLS were purchased from BDH, England. Poloxamer-188 and -407 were gifted from Ludwigshafen, Germany. Propylene glycol was obtained from THOMAS BAKER Co., India. All oily extracts of citrus fruit peels were prepared from *Citrus limonum*, *Citrus reticulate*, *Citrus aurantium*, and *Citrus sinensis,* as detailed in previous studies [16,17].

Surfactants used in the study

In the current study, three different types of surfactants were used: Sodium Lauryl Sulphate (SLS or SDS) and two types of poloxamers, poloxamer-188 (Kolliphor®P188) and poloxamer-407 (Kolliphor®P407). SLS is an anionic surfactant used as a detergent, emulsifying agent, tablet and capsule lubricant, skin penetrant, solubilizer, and wetting agent [18]. Poloxamers, non-ionic EO/PO block copolymers, include bio-compatible poloxamer-188 and poloxamer-407. These were used in pharmaceuticals as surfactants, emulsifying, solubilizing, fluidizing, and dispersing agents, making them valuable for biomedical applications [19].

Collection of fruit peels

All fruits were purchased from local farmers and fruit vendors in Mosul, Iraq from November to March, 2023. The fruits were identified by Dr. May Taha Hamid Alwattar, a botanist and plant taxonomist at the College of Science, Mosul University. The samples were matched with the herbarium specimen at the herbarium of the Department of Biology, College of Science, University of Mosul with the code numbers 810, 811, 812, and 813 for *Citrus limonum*, *Citrus reticulate*, *Citrus aurantium*, and *Citrus sinensis*, respectively. Before the extraction, fruits were taken to the laboratory at

the College of Pharmacy, Mosul University, where they were washed with water, exfoliated, and diced into tiny pieces.

Hydrodistillation for oil extraction

Fresh peels were processed using the Clevenger apparatus to extract the COE following the method described recently by Abid and Yahya [16]. The peels of each species were ground in a blender, and 100 g of each was mixed with 500 mL of distilled water in a Clevenger apparatus. The temperature was set at 70–80˚C for five hours while the Clevenger apparatus was installed on a thermostatic heating mantle. Water runoff was used to separate the oil-water mixture, while the calibrated tube provided the oil measurement. The proportion of oil extracts was determined using the following formula:

$$\textbf{Percentage of oil} = \frac{\textbf{Volume of collected oil (mL)}}{\textbf{Weight of peels (g)}} \textbf{ x 100\%}$$

Within one hour, the oily extract accumulated in the collecting funnel and was easily separated. The process was repeated several times until the required quantity was obtained. The oil peel extracts were used for further biochemical and microbiological tests [16,20].

Formulation and preparation of toothpaste

Different formulations of the toothpaste were prepared, including TP0 to TP6 (Table 1). The formulations contained the following ingredients: calcium carbonate used as an abrasive material, sorbitol as a humectant, sweetening agents and binders. Carboxymethylcellulose and starch were used as binders while citric acid was used as a buffering agent. Sodium lauryl sulphate, Poloxamer-407, and Poloxamer-188 were used as surfactants while titanium dioxide was used as an opacifier. Solid ingredients were weighed and ground using a mortar and pestle before being sieved through a #60 (250 micron) sieve. Oily extracts, propylene glycol, and distilled water were manually mixed in a beaker prior the addition to the above solid mixture in the mortar. After 5 minutes of trituration of the solid ingredients with liquids, a paste was formed. The finished paste was retained in a nylon-sealed container and placed in a refrigerator [21]. To find a formula with the best antimcrobial activity, a blank TP formulation was prepared as a negative control, named TP0, and its composition is shown in Table 1. This enabled the comparison of the antimicrobial effects of the different formulations.

Table (1): Composition of toothpaste formulations.

Ingredient	Toothpaste (TP) formulation						
	TP0	TP1	TP2	TP3	TP4	TP5	TP6
Calcium Carbonate (g)	38	38	38	38	38	38	38
Sorbitol (g)	21	21	21	21	21	21	21
CMC (g)	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Starch (g)	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Citric acid (g)	1.8	1.8	1.8	1.8	1.8	1.8	1.8
TiO_2 (g)	0.04	0.04	0.04	0.04	0.04	0.04	0.04
SLS (g)	-	1.4	1.4	-	-	-	-
Poloxamer-407 (g)	-	-	-	-	2.85	2.85	-
Poloxamer-188 (g)	-	-	-	2.85	-	-	2.85
Bitter orange peel extract (mL)	-	14	-	14	-	14	-
Tangerine peel extract (mL)	-	14	-	14	-	14	-
Orange peel extract (mL)	-	-	14	-	14	-	14
Lemon peel extract (mL)	-	-	14	-	14	-	14
Propylene glycol (mL)	10	10	10	10	10	10	10
Distilled Water (mL) up to 100 mL	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.

Total amount for each formulation is 100 mL; CMC: Carboxymethyl cellulose; SLS: Sodium lauryl sulphate; TiO_2: Titanium dioxide; TP0: Negative control.

Determination of physicochemical characteristics

pH

A sample (2 g) of each TP was placed in a 50 mL beaker, and 10 mL of distilled water were added. The dispersions were stirred vigorously to make a uniform suspension for each formula. The pH of the prepared TP was determined by using a pH meter (Peak Tech 5307, Bulgaria) [22].

Foaming ability

To prepare the dispersion, 5 g of the prepared TP was added to 10 mL of distilled water in a 50 mL beaker and left to stand for approximately one hour to ensure thorough mixing. The contents of the beaker were then stirred (110 rpm, 40˚C) using a hotplate stirrer (AccuPlate PC420D Hotplate, Labnet International, Mexico) for 5 min and were left overnight to allow complete mixing of the TP with water. The next day, the diluted slurry was rinsed and transferred from the beaker to a 100 mL plastic graduated cylinder with further addition of 8-9 mL of water [23]. The cylinder was sealed with a stopper and shaken vigorously 30 times to ensure thorough mixing. The foaming ability was calculated as follows:

$$\textbf{Foaming ability} = \textbf{V1} - \textbf{V2}$$

where V1 is the volume in mL of foam with diluted slurry and V2 is the volume in mL of diluted slurry only.

Cleaning ability

A natural eggshell, which is high in calcium and maybe similar to tooth enamel, were focusly used to test the cleaning ability of toothpaste (TP). Each TP formula was tested on a hard-boiled egg stained with a bright orange food coloring solution. The eggshell was divided in half, with one side brushed using an electric toothbrush soaked in distilled water and the other side brushed with the TP formulation. The process was repeated three times for each TP formula, and the eggs were examined for colour removal after rinsing [24].

Moisture content

A 5 g sample of each prepared TP was heated in an oven at 100°C for approximately 24 hours. After cooling, the sample was reweighed. The heating and reweighing process was repeated until a constant mass was achieved in

consecutive measurements [25]. The mass loss was used for calculating moisture content using the following equation:

$$\mathbf{MC}\% = \frac{(\mathbf{Original\ sample\ mass} - \mathbf{Dry\ sample\ mass})}{\mathbf{Original\ sample\ mass}}\ x\ \mathbf{100}\%$$

Where MC is the moisture content.

Viscosity measurement

Viscosity and torque were measured for all formulations using a viscometer (Drawell Scientific Instrument Co., Ltd, Shanghai) at 25°C with spindle number L4. The selected spindle was inserted into the holder, immersed in a 30 mL sample, and rotated at 60.0 rpm. Viscosity and torque measurements were recorded in triplicate [25].

Spreadability

A handmade apparatus was used, where about 1 g of formulated toothpaste (TP1-TP6) was placed on the center of a glass plate, and then a second glass plate was placed over it. A 1 kg object was carefully placed on top of the glass plate and left to stand for 10 minutes. After removing the weight, a ruler was used to measure the diameter of the paste (in centimeters). The larger the diameter, the more the spreadability [26].

Abrasiveness measurement

About 0.2 g of each formula (TP1-TP6) was placed on a plastic microscopic slide with a drop of distilled water. Using a clean cotton swab, a short stroke was applied, and rub back and forth on the slide 30 times. The slide was carefully cleaned and then dried with a piece of soft tissue. The slide was examined under a dissecting microscope (OPTIKA, Italy) to assess surface scratches, which were rated on a scale of 0 to 5, where 0 indicated no scratches and 5 represented a high degree of scratching.

Measurement of gritty texture

A small amount of each formula was rubbed on a butter paper. The intensity and number of scratches that appeared on the piece of butter paper were noted as absence or presence of gritty matter [24].

Homogeneity and extrudability test

In this method, the formulated toothpaste was filled into a capped collapsible aluminum tube and sealed with metal clips. The filled tube's weight was recorded. The tube was placed on a hard surface, the cap was removed, and a 500 g object was applied to extrude the paste onto aluminum foil for homogeneity assessment. The extruded toothpaste was weighed by subtracting the tube's post-extrusion weight from its initial weight. This process was repeated for all formulations, and the percentage of extruded toothpaste was calculated [26].

$$\mathbf{Ex}\% = \frac{(\mathbf{weight\ before\ extraction} - \mathbf{weight\ after\ extraction})}{\mathbf{weight\ before\ extraction}}\ x\ \mathbf{100}\%$$

where Ex is the extrudibility.

Evaluation of antimicrobial activity

Source of isolates

All isolates were obtained as pure strains from pathogenic sources. Their identities were confirmed through biochemical and molecular tests, including the VITEK-2 compact system and polymerase chain reaction (PCR). These strains were sourced from the strain bank at the Department of Biology, College of Science, University of Mosul.

Antimicrobial susceptibility testing

The antimicrobial activity of all the extracted citrus oils and formulated toothpastes were assessed using the agar well diffusion method at the College of Pharmacy, Mosul University. Six Gram-positive and four Gram-negative bacterial strains, along with *Candida albicans* (*C. albicans*), were tested. Sterile Mueller-Hinton or Sabouraud Dextrose Agar was used, and wells were created in the solidified medium using a 7 mm cork borer. Bacterial and yeast cultures were prepared, adjusted to 0.5 McFarland turbidity (1.5×10^8 CFU/mL for bacteria, 1.4×10^6 CFU/mL for yeast), and spread on the medium. Initially, the four different extracted oil were poured into each well seperatly, and the outcomes were recorded. After that, toothpaste was used to repeat the experiment. In addition, a comparison was made with a toothpaste formulation prepared with all the ingredients without citrus oils and surfactants (negative control, TP0).

Each well was filled with 100 µL of toothpaste (TP1-TP6) using a sterile syringe. Ciprofloxacin, gentamicin, and voriconazole were used as positive controls. After incubation (37˚C for 24-48 hours for bacteria, 28˚C for 48 hours for *C. albicans*), the clear zone diameters were measured and compared to the positive controls [8,16].

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of TP1 and TP2 was determined using the brain heart infusion broth assay (Lab M Limited, Topley House, UK) and the macro-dilution broth method. Serial dilutions (100, 50, 25, 12.5, 6.25, and 3.125 mg/mL) were prepared following the guidelines of the National Committee for Clinical Laboratory Standards [26]. Considering the standard turbidity of the 0.5 McFarland solution, each tube received 200 µL. After a 24-hour incubation at 37°C, turbidity was observed.

Stability

About 1 g of each formula of toothpastes (TP1 to TP6) was transferred into a 10-mL glass test tube and stoppered. The test tubes were heated at 45˚C for 72 hours, allowed to cool and the contents were examined visually for homogeneity or any sign of fermentation and deterioration manifestation. Other samples were tested after five months of storage in a refrigerator. Assessments of homogeneity, fermentation or deterioration, pH, spreadability, and foaming ability were repeated and compared with the initial results obtained after toothpaste preparation.

Statistical analysis

Statistical analysis of the data were made by Student's t-test (independent samples) and one-way analysis of variance (ANOVA) with post-hoc analysis using GraphPad Prism (version 5.01) software. P value of less than 0.05 was considered statistically significant.

Results

Oil extracts of citrus fruit peels

Different quantities of oil extract (EO) were obtained by hydrodistillation extraction from 100 g of different types of citrus fruit peels. The highest yield of phytochemical oil extract was obtained from *C. reticulata* (2.5%), followed by *C. aurantium* (2%), *C. sinensis* (1.5%), and *C. limonum* (1%), respectively. Sensory examination revealed that they had a watery consistency, were colorless, and possessed a

characteristic fragrance, which was utilized for further biochemical and antibacterial studies.

Physicochemical characteristics

All of prapared formulations showed white colour and mild bitter taste. The refreshing aromatic odors were distictive. Additional physicochemical properties are presented in details in Table 2. The *p*H of all formulations is regarded as acceptable and within the most specified range (7 to 10). The foaming ability, which frequently depends on surfactant type and concentration, affects the mouth-cleaning effect. TP2 and TP6, with SLS and Poloxamer-188, respectively, showed high amount. Surfactant type (SLS) produced better foam amount at lower concentrations than poloxamers. There is no impaction of extracts on foaming but they may enhance cleaning through their natural ingredients. The primary power of cleaning of toothpaste comes from its abrasive particles combined with the brushing mechanical action. Cleaning effectiveness of (TP1–TP6) which is demonstrated in Table 2. This might be attributed to the mechanical brushing of eggshells in the applied test, and the presence of synthetic surfactants at specific concentrations of (SLS, poloxamer-188, and poloxamer-407), which act as surfactant/detergent/foaming always with stabilizing agents in the prepared formulations. In general, toothpaste seek a moisture content that balances spreadability, microbiological safety, freshness and customer satisfaction. A range of 45-50 % (w/w) moisture content is commonly desirable, even though, no universally defined optimum percentage is found. The percent moisture content in this study presented only slight variations, Table 2. Among all the tested samples, TP1 recorded the highest moisture content at 51.46%, which mildly exceeds the typical range. This puts forward that TP1 may be too moist. Inversly, TP2 had the lowest moisture content: 46.54%, which lies within the acceptable range. This points to that TP2 keeps a balanced moisture level. Other formulations (TP3, TP4, TP5, and TP6) showed moisture contents which are within the acceptable range. The viscosity of (TP1-TP6) is acceptable and affected significantly by polymer types like (CMC and starch), which have complex rheological properties. Toothpaste viscosity ranges from 70,000 to 150,000 cP, together with variable individual preferences. The prepared formulations showed significant differences in viscosity. Specifically, the formulations containing (orange and lemon oil extracts): TP2, TP4, and TP6 manifested distinct viscosity characteristics if compared to the other TP formulations. This is probably attributed to aromatic compounds such as limonene. These extracts may enhance thickness and texture. TP5 had the highest viscosity at 106180 cP. The surfactant (SLS and poloxamers) impact the desired viscosity, with poloxamers enhancing texture and stabilizing the formulation, though their impaction on overall viscosity is minimal. Regarding the spreadability, all formulations exhibited very acceptable spreadability with no significant differences among them.

Toothpaste abrasiveness was measured by a light microscope. On a scale of (1 – 5), a recommended value of around (2 – 3) corresponds to mild to moderate abrasiveness, which is generally so safer for enamel. The abrasiveness is considered relatively high due to the presence of calcium carbonate, a good abrasive agent. This high abrasiveness is vital for cleansing and removing stains, particularly for smokers. However, high abrasiveness may negatively affect the enamel, making these formulations recommended primarily for smokers. It is evident that all the produced formulations (TP1-TP6) exhibit acceptable consistency and are free from gritty texture. This aligns with the ideal characteristics of toothpaste being silky-smooth without any perceptible grit. All formulations (TP1-TP6) exhibited good homogeneity when extruded from commercially collapsible aluminum tubes. The extrudability results are presented in Table 2, influenced by factors such as moisture content and volatile ingredients. After heating the test tubes to 45°C for 72 hours and allowing them to cool, the contents were visually examined for homogeneity and the presence or absence of fermentation or deterioration. Additionally, another set of samples stored in a refrigerator for five months was analyzed. The results for pH, spreadability, and foaming ability were compared with the initial values obtained after TP preparation. No significant changes were observed after storage, as shown in Table 3.

Antimicrobial susceptibility of toothpaste formulations

A preliminary work conducted on citrus oils alone. The extracted oils showed a significant effect on most of the bacteria and fungi used as shown in Table 4. Generally, it looks like citrus oils extract are more efficient on Gram-positive than Gram-negative bacteria. As expected, the TP0 toothpaste formulation exhibited no antimicrobial activity as it does not contain oils or surfactants. Formulations TP3 to TP6 demonstrated no significant antimicrobial effect, while TP1 and TP2 were effective against most of the investigated microorganisms (Table 5).

Table (2): Characteristics of the formulated toothpastes.

Parameter	Toothpaste formulation						
	TP0	**TP1**	**TP2**	**TP3**	**TP4**	**TP5**	**TP6**
Color	White	White	White	White	White	White	White
Odor	None	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic
Taste	Slight sweet	Mild bitter	Mild bitter	Mild bitter	Mild bitter	Mild bitter	Mild bitter
pH	7.6±0.10	7.6±0.01	7.78±0.01	7.86±0.11	7.86±0.18	7.43±0.44	7.71±0.07
Foaming ability (mL)	None	5±0.11	26±0.10	11±0.10	10±0.10	7±0.10	15±0.10
Cleaning ability	Not detected	+	+	+	+	+	+
Moisture content (%)	32±0.20	51.46±0.19	46.54±0.15	49.12±0.17	49.5±0.19	46.96±0.18	48.32±0.16
Viscosity (mPa.s)	106638±0.1	5507.0±0.1	6147.0±0.2	9307.0±0.2	8837.0±0.1	10618.0±0.1	10607.0±0.1
Torque (%)	100	53.5	50.7	50.7	90	106	106
Abrasiveness	Not detected	4±1	4±1	4±1	4±1	4±1	4±1
Gritty matter	Not detected	Absent	Absent	Absence	Absent	Absent	Absent
Homogeneity	Good	Good	Good	Good	Good	Good	Good

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Spreadability (cm)	Not detected	6.73±0.54	6.13±0.29	5.46±0.24	6.26±0.20	6.00±0.21	5.53±0.20
Extrudability	Not detected	61.84±0.23	59.67±0.11	65.95±0.19	53.22±0.25	57.94±0.20	60.25±0.14
Stability	Not detected	Pass	Pass	Pass	Pass	Pass	Pass

All values are expressed as mean ± SD, n = 3

Table (3): Stability of the toothpaste formulations (TP1 and TP2) before and after storage.

Parameter	Before		After	
	TP1	TP2	TP1	TP2
Homogeneity	Good	Good	Good	Good
Presence of fermentation	No	No	No	No
pH	7.6	7.78	7.32	7.75
Spreadability (cm)	6.73±0.54	6.13±0.29	5.55	6.13
Foaming ability (mL)	5	26	5	25

Table (4): Citrus oils activity against several microorganisms using agar well diffusion method.

Microorganisms		ZOI (mean ± SD) (mm)			
		C. limonum	*C. reticulata*	*C. aurantium*	*C. sinensis*
Gram +ve bacteria	*Staph. aureus*	30±0.2	26±0.0	28±0.0	19±0.01
	Staph. epidermidis	20±0.34	18±0.0	20±0.53	19±0.3
	Strept. mutans	20±0.36	0±0.0	0±0.0	0±0.0
	Strept. pyrogenes	21±04	18±0.17	20±1.0	22±0.2
	L. liquificans	20±0.0	15±0.3	22±0.26	17±0.17
	E. faecalis	14±0.9	20±0.0	18±0.45	0±0.0
Gram -ve bacteria	*E. coli*	14±0.0	6±0.0	9±0.26	3±0..5
	P. aeruginosa	5±0.53	2±0.0	3±0.0	0±0.0
	P. vulgaris	22±0.32	20±0.2	22±0.56	21±0.6
	K. pneumonia	7±0.46	2±0.0	7±0.42	3±0.0
Fungi	*C. albicans*	28±0.26	30±0.1	29±1.0	28±0.56

ZOI: Zone of inhibition; SD: standard deviation

Table (5): Antimicrobial activity of formulated toothpastes against some selected microorganisms.

Microorganism		ZOI (mean ± SD) (mm)							
		TP0	TP1	TP2	TP3	TP4	TP5	TP6	Positive Control
Gram +ve bacteria	*Staph. aureus*	0	25±0.00dA	27±0.45Db	0	0	0	0	30±0.2C
	Staph. epidermidis	0	18±0.2bA	20±0.0bB	0	0	0	0	20±0.0B
	Strept. mutans	0	0±0.0a	0±0.0a	0	0	0	0	26±0.33
	Strept. pyrogenes	0	18±0.86bA	21±0.0cB	0	0	0	0	28.9±0.1C
	L. liquificans	0	18±0.0bB	20±0.0bC	0	0	0	14±0.0A	20±0.0C
	E. faecalis	0	0±0.0aA	20±0.5bB	0	0	0	0	19.9±0.2B
Gram -ve bacteria	*E. coli*	0	0±0.0a	0±0.0a	0	0	0	0	20±0.1
	P. aeruginosa	0	0±0.0a	0±0.0a	0	0	0	0	20±0.0
	P. vulgaris	0	21±0.23cB	22±0.0cC	0	0	0	0	20±0.3A
	K. pneumonia	0	0±0.0a	0±0.0a	0	0	0	0	20±0.33
Fungi	*C. albicans*	0	26±0.28eA	28±0.0dC	0	0	0	0	25±0.13B

ZOI: Zone of inhibition; SD: standard deviation; Positive control: Ciprofloxacin for Gram+ve, Gentamicin for Gram–ve, and vericonazol for *C. albicans*; TP0: Negative control; Different small letters arranged vertically indicate significant difference in mean between different groups at a given time interval at P ≤ 0.05; Different capital letters arranged horizontally indicate significantly difference in mean between each group at different time interval at P ≤ 0.05.

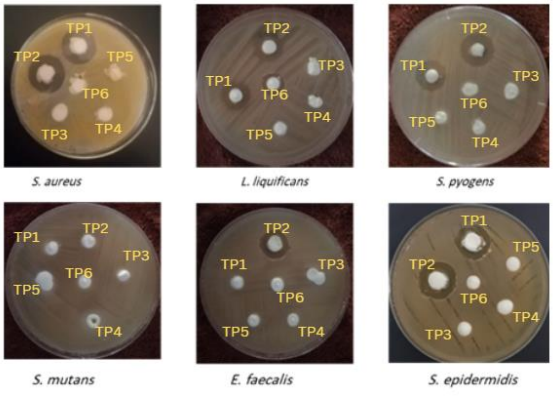
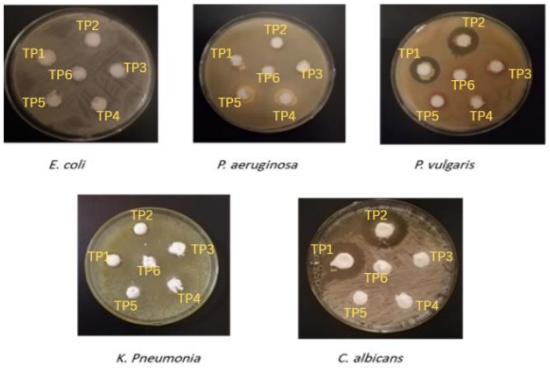


Figure (**1**)**:** Antibacterial activity of formulated toothpastes against Gram-positive bacteria.

The formulated toothpastes (TP1 and TP2) demonstrated greater activity against *Staphylococcus aureus* (*Staph. aureus*) but remained less effective than ciprofloxacin. Compared to their positive control, both TP1 and TP2 exhibited stronger antibacterial effects against *Proteus vulgaris* (*P. vulgaris*) and *Candida albicans* (*C. albicans*). Meanwhile, *Staphylococcus epidermidis* (*Staph. epidermidis*), *Lactobacillus liquefaciens* (*L. liquefaciens*), and *Enterococcus faecalis* (*E. faecalis*) exhibited equal or greater sensitivity to the TP2 formulation compared to ciprofloxacin, as shown in Figures 1 and 2.



Concerning the antibacterial action against Gram-negative bacteria, TP1 and TP2 showed no effect against

Escherichia coli (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Klebsiella pneumoniae* (*K. pneumoniae*). However, both exhibited strong activity against *P.vulgaris*, with an effect equivalent to the antimicrobial effect of gentamicin.

Figure (**2**)**:** Antimicrobial activity of formulated toothpastes against Gram-negative bacteria and oral yeast, *Candida* species.

Minimum inhibitory concentrations of formulated toothpastes

Regarding the MIC, the results (Table 6) indicate that TP2 exhibited an MIC equal to or lower than that of TP1 against the tested bacteria and fungi, except for *Enterococcus faecalis*, which was not affected by TP1.

Table (6): Minimum inhibitory concentration of TP1 and TP2 against the tested microorganisms.

Microorganism	MIC of the two formulations of toothpaste (µg/mL)	
	TP1	TP2
Staph. aureus	25	25
Staph. epidermidis	25	12.5
E. faecalis	0	25
L. liquificans	50	25
P. vulgaris	50	50
Strept. pyogens	100	50
C. albicans	12.5	12.5

MIC: Minimum inhibitory concentration

Discussion

It is obvious that formulations TP3 and TP4 exhibited noticeably higher pH than other formulations and this is due to the presence of Poloxamer-188 and Poloxamer-407, respectively. SLS is anionic alkaline surfactant, while poloxamer, which is present in all other formulations, is a non-ionic surfactant. However, there are some differences among the remaining formulations (TP1, TP2, TP5, and TP6), and this might be due to the different essential oils used in them [27]. The high foaming abilities observed in TP2 and TP6 could be attributed to the presence of SLS and Poloxamer-188. SLS, in particular, is known to produce higher foam at lower concentrations compared to poloxamers, which may encounter to the enhanced cleaning effect of these formulations [28]. While the extracts do not directly influence foaming ability, their natural ingredients play a role in the overall cleaning process. The cleaning efficiency of all formulations (TP1-TP6) is likely due to the combination of abrasive particles and the mechanical action of brushing, supported by synthetic surfactants [28]. The variations in moisture content among the toothpaste formulations suggest that ingredient composition and extracts are impact the moisture content, significantly. The slightly higher moisture content in TP1 indicates potential issues with spreadability and microbiological safety, whereas TP2's balanced moisture content falls within the desirable range, providing a favorable balance between spreadability and freshness [29]. The differences in viscosity between formulations can be attributed to the presence of polymers like CMC and starch, as well as the additional effect of some compounds from extracts like limonene. The higher viscosity observed in TP5 is a result of a well-planned formulation strategy, ensuring that spreadability is not compromised despite its high viscosity. These findings highlight the importance of carefully selecting and balancing the ingredients to achieve the desired properties in toothpaste formulations. The acceptable spreadability of all formulations aligns with existing research, emphasizing the role of solid content in determining this characteristic [30,31]. Regarding the abrasiveness, the high abrasiveness observed in these formulations could be attributed to the presence of calcium carbonate, which is effective for cleansing and stain removal, particularly for smokers. However, it is important to balance abrasiveness to avoid potential negative effects on enamel. The formulations' lack of gritty texture indicates a successful achievement of a silky-smooth consistency, which is desirable for consumer comfort.

The results of stability tests indicated that the toothpaste formulations are robust under varying storage conditions. The absence of fermentation or deterioration after heating and cooling, as well as after five months of refrigeration, suggests that the formulations maintain their homogeneity and quality over time. The stability of pH, spreadability, and foaming ability further supports the reliability of these formulations, making them suitable for long-term use and storage. Concerning the antimicrobial effect, the significant antimicrobial effect observed in TP1 and TP2 may be attributed to the presence of SLS surfactant in these formulations, unlike the poloxamers in the others (Table 1). Surfactants are essential for foaming and cleaning properties in toothpaste. SLS is widely used in various applications, and citrus essential oils have microbicidal action through cell membrane disruption and protein denaturation [32–34]. It is obvious that both TP1 and TP2 have antimicrobial effects against all of the investigated Gram-positive bacteria except *Streptococcus mutans* (*Strept. mutans*). Both TP1 and TP2 demonstrated significant antimicrobial effects ($p < 0.05$), exhibiting greater efficacy against certain organisms compared to their respective positive controls. The antimicrobial effect of these two formulations might be due to the essential oil content, which have an inhibitory effect on the growth of some bacterial species, and this is consistent with the findings of Al-Snafi (2016) [33].

The possible explanation for the antimicrobial effect is the presence of bioactive substances with antibacterial qualities, including limonene, flavonoids, and phenolic compounds, which are abundant in citrus oil extracts. The main monoterpene in citrus peels, limonene, has been shown to damage bacterial cell membranes, increasing permeability and causing the leakage of intracellular contents. The bacterial cell's integrity is jeopardized by this disruption, which eventually results in cell demise [21]. Flavonoids and phenolic compounds inhibit the growth and spread of bacteriomes through various mechanisms, including the inhibition of bacterial enzymes, disruption of quorum sensing, a microbial communication system crucial for biofilm formation, and the generation of reactive oxygen species (ROS) [35]. The results of this study are consistent with previous research, showing that chemicals derived from citrus are efficient against Gram-positive bacteria, including *Enterococcus faecalis* and *Staphylococcus aureus*. The increased vulnerability of Gram-positive bacteria is attributed to their more porous cell walls, as they lack the outer membrane that characterizes Gram-negative bacteria. Gram-negative bacteria have an outer membrane that acts as a barrier, making hydrophobic substances like essential oils less effective [36]. Certain chemicals in the citrus extracts may be able to efficiently target *Proteus vulgaris*, as evidenced by the strong antibacterial activity of TP1 and TP2 against this species, which is similar to that of gentamicin that was used as a positive control. According to previous studies, certain citrus phenolics, such as naringenin and hesperidin, can interfere with DNA replication and damage bacterial cell walls, potentially explaining their observed antimicrobial effects. [21]. The primary active ingredients, phenolics, flavonoids, and limonene, have strong antibacterial

properties but are less effective against Gram-negative organisms. In order to increase activity, future developments might involve the inclusion of permeabilizing agents or synergistic antimicrobial molecules [21].

On the other hand, both TP1 and TP2 exhibited a stronger effect against *Candida albicans* compared to voriconazole. There was a significant difference ($p < 0.05$) between the two formulations (TP1 and TP2) and the positive control. Additionally, a significant difference ($p < 0.05$) was observed between TP1 and TP2 when compared with each other (Table 5). The stronger effect of TP2 on the investigated microorganisms ($p < 0.05$) may be attributed to its specific ingredients. When combined with SLS as a surfactant, the oily extracts from *C. sinensis* and *C. limonum* appear to form a more effective TP formulation than the others. Concerning the inhibitory zone of the formulated toothpaste (Figures 1 and 2), the statistical analysis revealed significant differences in the antimicrobial activity of the formulated toothpastes. TP1 and TP2 consistently demonstrated higher ZOIs compared to TP3–TP6 ($p \leq 0.05$). The positive control exhibited the highest ZOI value in most cases, except for *Candida albicans*, where TP2 demonstrated a slightly higher ZOI (28 ± 0.0 mm) compared to the positive control (25 ± 0.13 mm). Post-hoc analysis (Tukey's HSD) confirmed that the differences between TP1, TP2, and the positive control were statistically significant for most of the microorganisms, while TP3–TP6 showed no significant activity. The difference in MIC between TP1 and TP2 against various bacteriomes may be attributed to the distinct peel extracts incorporated in each formulation. This finding aligns with previous studies confirming that oils extracted from citrus peels exhibit inhibitory effects on the growth of various bacterial species and fungi [17].

Conclusion

This study succeeded in formulating toothpastes with citrus peel oil extracts combined with surfactants. The preparation was straightforward and successful, with all formulations exhibiting satisfactory physicochemical characteristics, including a white color and a pleasant aroma. Bitter taste from citrus peel extract can be mitigated with sweeteners. TP2 stood out for its foaming ability and stability. The antimicrobial activity of the citrus peel toothpaste formulations (TP1 and TP2) can be directly correlated to the presence of bioactive compounds such as limonene, citral, hesperidin, and caffeic acid. These compounds act through multiple mechanisms, including membrane disruption, enzyme inhibition, and ROS generation. The use of SLS as a surfactant could enhance the delivery and efficacy of these compounds, resulting in significant synergistic action of citrus oils and SLS. Moreover, further studies are recommended to investigate the potential whitening effects of these formulations.

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