

Mouthwash Formulas for Xerostomia and Malodour

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Research Article

Keywords: Vitis vinifera L. seed oil, Corylus avellana L. seed oil, Citrus × bergamia Risso et Poiteau. fruit peel oil, Cinnamomum zeylanicum Blume seed essential oil, dry mouth, mouthwash

Posted Date: January 12th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3821986/v1>

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Abstract

Xerostomia also known as dry mouth is often associated with bacterial malodour and oral-dental disorders. A successful treatment must provide oral hygiene and moisten the mouth. In this study, it was aimed to prepare mouthwash formulas using essential oils (1–6%), fixed oils and carboxymethyl cellulose that can meet both requirements. *Citrus bergamia* fruit peel oil, *Cinnamomum zeylanicum* leaf oil, *Vitis vinifera* L. and *Corylus avellana* L. seed oils was used as essential and fixed oils, which were analyzed by using GC-FD/MS. The organoleptic, pH, density, viscosity, antimicrobial activity, and cytotoxicity controls were conducted on the formulas. Stability studies were conducted in a stabilization cabinet at 25°C, 60%RH for 90 days, revealing that all formulations remained stable based on organoleptic assessments, pH, and density controls. Formulations containing 2% or more cinnamon oil exhibited antimicrobial activity against all investigated mouth pathogens (*S. pyogenes*, *S. mutans*, *S. mitis*, *S. aureus*, *K. pneumonia*, *C. albicans*) while formulations with 1–6% bergamot fruit peel oil displayed limited activity, proving ineffective against *S. mitis*. The most and the lowest cytotoxic formulations were respectively including 6% and 1% cinnamon oil (47,25% and 7,82%) while all the others were in acceptable range against gingival fibroblast cells. In conclusion, the formulation, comprising 2% cinnamon leaf oil, 2–4% hazelnut oil, and a 0.5% CMC solution, demonstrated successful physicochemical characterization with antimicrobial efficacy against all investigated mouth pathogens and exhibited acceptable safety in terms of cytotoxicity. This formulation holds promise for local treatment of xerostomia, malodor, and orodental infections.

Introduction

Xerostomia, commonly known as dry mouth, arises due to reduced saliva production or alterations in its composition (Furness et al. 2013; Kapourani et al. 2022). It is frequently observed in cancer patients undergoing radiation therapy targeting the head, face, or neck, and can emerge as a side effect of certain chemotherapeutic agents (Scully 2008). Additionally, polypharmacy—especially in the treatment of mood and anxiety disorders—systemic diseases like diabetes mellitus, thyroid disorders, Sjögren's syndrome, and rheumatoid arthritis, as well as lifestyle factors such as alcohol, caffeine, and tobacco consumption, can contribute to xerostomia (Kapourani et al. 2022; Ristevska et al. 2015).

While the condition is prevalent across the population, its incidence is notably higher among postmenopausal women and the elderly (Kapourani et al. 2022; Ristevska et al. 2015). Dry mouth symptoms include oral mucosa sores, dysphagia, dysgeusia, and difficulties in speaking, chewing, and swallowing, all of which significantly impact the oral health-related quality of life. Moreover, there is an increased risk of dental caries, infections in the oral salivary glands, and oral candidiasis. These symptoms also adversely affect sleep quality, as well as the social and emotional well-being of affected individuals (Furness et al. 2013; Kapourani et al. 2022). The primary objective in treating xerostomia is to alleviate oral discomfort by restoring adequate mouth moisture levels (Furness et al. 2013; Ristevska et al. 2015). To achieve this, topical and systemic saliva substitutes or saliva stimulants are commonly employed in treatment (Furness et al. 2013).

Dryness in the mouth often coincides with bacterial malodor and oral-dental disorders. Hence, it becomes imperative to address both moisture and hygiene for effectively treating xerostomia (Furness et al. 2013; Kapourani et al. 2022). Herbal essential oils are exceptional resources in managing dry mouth due to their delightful aromas and inherent antimicrobial properties. These oils contain secondary metabolites responsible for their biological activities (Blowman et al. 2018; Tambur et al. 2021). Notably, terpenes and terpenoids present in essential oils show promising potential in preventing and treating various diseases (Noriega 2000). Cell and animal models have demonstrated the antioxidant, antifungal, antidepressant, anxiolytic, analgesic, anti-convulsant, anti-inflammatory, antimutagenic, antibacterial, and antiproliferative activities of terpenes and terpenoids (Blowman et al. 2018; Lizarraga-Valderrama 2021). Essential oils derived from medicinal plants serve as excellent bases for mouthwash formulations due to their robust antifungal, antibacterial, anti-inflammatory, and antioxidant properties. For example, Mulyanti et al. conducted an *in vitro* study revealing the organoleptic properties and inhibitory efficacy of a mouth rinse formulated with lime essential oil [*Citrus aurantifolia* (Christm.) Swingle] against the growth of *Streptococcus mutans* (2020). Alipour et al. prepared a herbal mouthwash formulation containing *Zataria multiflora* Boiss. leaf essential oil, demonstrating its antimicrobial activity against *Lactobacillus acidophilus*, *Staphylococcus aureus*, *S. mutans*, and *Candida albicans* (2018). Additionally, an *in-vivo* study by Patil et al. showcased the reduction effect of the essential oil mouthwash—comprising eucalyptol 0.092%, menthol 0.042%, methyl salicylate 0.060%, and thymol 0.064% as active ingredients—on plaque formation and gingivitis (2023).

Citrus × bergamia Risso et Poiteau, commonly known as “bergamot” belongs to the Rutaceae family and is extensively cultivated in southern Italy (Calabria), East Africa, Argentina, Brazil, Türkiye, and various other Mediterranean countries (Navarra et al. 2015; Marzocchi et al. 2019). This hybrid species originated from a cross between the sour orange (*Citrus aurantium* L.) and lemon (*Citrus limon* L. Burm. f.) species (Navarra et al. 2015). In Italian folk medicine, *C. bergamia* has been traditionally used to alleviate fever, sore throat, and mouth, as well as to treat skin and respiratory system infections (Nauman and Johnson 2019). The essential oil extracted from the plant finds widespread application in the perfume, cosmetics, and food industries owing to its valuable biological properties (Navarra et al. 2015). Bergamot essential oil is also extensively employed in aromatherapy to alleviate symptoms associated with stress-induced disorders, depression, and anxiety (Lizarraga-Valderrama 2021). Various studies have confirmed the antimicrobial, anti-inflammatory, antiproliferative, antidepressant, anxiolytic, analgesic, neuropsychopharmacological, and neuroprotective activities of *C. bergamia* fruit peel essential oil (Lizarraga-Valderrama 2021; Navarra et al. 2015). The main components identified in the essential oil from the fruit peel of *C. bergamia* include limonene, linalyl acetate, linalool, and -terpinene (Navarra et al. 2015; Marzocchi et al. 2019). Research by Filipović et al. demonstrated the potent inhibitory effects of *C. bergamia* essential oils against *Parvimonas micra* and other anaerobic bacteria, with non-toxic effects on gingival cells at concentrations exhibiting antimicrobial activity (2020).

Cinnamomum zeylanicum Blume, commonly known as cinnamon, belongs to the Lauraceae family and is found across southeast Asia, China, and Australia (Cardoso-Ugarte et al. 2016). In Indian folk medicine, the bark and leaf essential oil of *C. zeylanicum* has been historically used to alleviate aching joints and numb pain (Han and Parker 2017; Wang et al. 2020). The essential oils extracted from the bark and leaves of these species are highly valued in the pharmaceutical, confectionary, perfumery, and food industries due to their chemical components (Cardoso-Ugarte et al. 2016). Cinnamaldehyde is the predominant component in *C. zeylanicum* bark essential oil, while eugenol predominates in the leaf essential oil (Paranagama et al. 2001). This divergence in

chemical composition leads to distinct activities between these essential oils. Although the literature predominantly focuses on cinnamon bark essential oil, there is relatively limited research on leaf essential oil (Wang et al. 2020; Yanakiev 2020). Studies have demonstrated the antioxidant and antifungal activities of *C. zeylanicum* essential oil, as well as the antimicrobial, anti-cancer, analgesic, and antioxidant properties of its major compound, eugenol (Schmidt et al. 2006; Ulanowska and Olas 2021). Rangel et al. conducted an *in vitro* study showcasing the potential use of *C. zeylanicum* leaf essential oil in combating oral infectious diseases. They determined its antifungal activity against *Candida albicans* and *Candida tropicalis* and observed its non-cytotoxic effects on human cells (2018). Oliveira et al. further supported the antifungal activity of *C. zeylanicum* leaf essential oil against *Candida* species, demonstrating its safety and tolerability in a phase I clinical trial involving users of removable dentures (2014).

Oil pulling is an old Ayurvedic technique that is becoming more popular for its oral health benefits. In traditional Indian medicine, it was believed that the “ama” (toxin) would be removed from the mouth by shaking and spitting the fixed oil. (Singh and Purohit 2011; Jong et al. 2023). Thus, this technique was used for tooth decay, gum bleeding, toothache and dry mouth in Indian folk medicine. Organic oils such as sesame oil, sunflower oil and coconut oil are the most popular oils for oil pulling. It is very beneficial to use fixed oils obtained by the cold-pressing method for oil pulling, especially since the amount of trans fats in cold-pressed oils is much lower than in commercial oils extracted with petroleum-based solvents (Jong et al. 2023; Shanbhag 2016). When the studies in the literature were examined, it was understood that regular oil pulling reduced the number of bacteria counts such as *Streptococcus mutans* and *Lactobacillus* and reduced the gingival index (Jong et al. 2023). For instance, Asokan et al. revealed oil pulling with sesame oil had a significantly decreasing effect on the number of *S. mutans* in a 2-week intervention clinical study (2008). Sezgin et al. showed that oil pulling with coconut oil has the same plaque inhibition activity as 0.2% chlorhexidine gluconate in a 4-day plaque regrowth study model (2019). The medium-chain fatty acid constituents and the phenolic compounds of the fixed oils could show antimicrobial activities against oral bacteria and fungi (Jong et al. 2023). In addition to all these properties, fixed oils also moisturize the oral flora. Although there are very few studies conducted for this purpose, Nardi et al. prepared a mouthwash formulation containing ozonated olive oil and demonstrated its *in vitro* antibacterial and antibiofilm activities (2020).

Vitis vinifera L. (grape) seed oil is rich in phenolic compounds, carotenoids, unsaturated fatty acids -mainly linoleic (65–75%) and oleic (15–40%) fatty acids- and tocopherols. Several studies have revealed the *in-vitro* antioxidant, anti-inflammatory, antimicrobial, anticancer and cardioprotective effects of *Vitis vinifera* L. seed oil. These activities that grape seed oil has been due to the rich chemical compounds it contains. Grape seed oil, which is very popular in the pharmaceutical and cosmetic industries, is also very famous in the food industry especially in European cuisines such as Germany, France, and Italy because of its lovely aroma and taste (Di Pietro Fernandes et al. 2023; Đorđevski et al. 2022). *Corylus avellana* L. (hazelnut) seed oil is very popular because of its unique taste and health-beneficial properties. It contains phenolic compounds, fatty acids -primarily consisting of oleic acid (73.6–82.6%), linoleic acid (9.8–16.6%) and palmitic acid (4.1–6.8%)-, α -tocopherol and β -sitosterol majorly. The antioxidant, antimicrobial, anti-aging and hypolipidemic activities of hazelnut oil have been proven (Sun et al. 2022; Selli et al. 2022). *Corylus avellana* L. seed oil and *Vitis vinifera* L. seed oil have different tastes and they are suitable for oral use. Both fixed oils obtained by the cold pressing method were chosen for the formulations because of their bioactive chemical components with their antioxidant and antimicrobial activities. These fixed oils moisturize the oral flora and they are compatible with the essential oils that will be included in the formulation. As far as we know, this study is the first to use *Corylus avellana* L. seed oil and *Vitis vinifera* L. seed oil in a mouthwash formulation.

In our study, we aimed to develop a mouthwash formulation with antimicrobial effects targeting dry mouth and the elimination of malodor. Dryness in the mouth is associated with bacterial malodor and oral-dental disorders therefore, a successful mouthwash formulation should not only provide moisturize of the mouth but also ensure proper oral hygiene (List et al. 1999; Munot and Gujar 2013). For this purpose, carboxymethyl cellulose (CMC) which is the most abundant used polymer in the saliva substitutes’ development combined with the essential and fixed oils for preparing mouthwash formulations (Kapourani et al. 2022). Additionally, glycerin and propylene glycol were added to the formulations due to their lubricant and moisturizing effects (Kapourani et al. 2022; Sinjari et al. 2020). Two groups of formulations were developed. The first group formulations (F1B, F3B, F5B and F7B) included *Citrus × bergamia* Risso et Poiteau fruit peel oil (bergamot oil) with *Vitis vinifera* L. seed oil (grape oil) and the second group formulations (F2C, F4C, F6C and F8C) contained *Cinnamomum zeylanicum* Blume leaf oil (cinnamon oil) with *Corylus avellana* L. seed oil (hazelnut oil). Each group of formulations was combined with carboxymethyl cellulose, glycerin, propylene glycol, polysorbate 80 (Tween 80), sodium benzoate and deionized water. The amount of essential oil varied from 1–6% and fixed oil varied from 2–4% in these formulations. They were subjected to evaluation through organoleptic, pH, density, viscosity, antimicrobial activity, and cytotoxicity tests. The chemical components of the essential oils and the fixed oils were analyzed using GC-FD/MS. Stability assessments were conducted in a stabilization cabinet at 25°C, 60%RH for 90 days.

Materials and Methods

Chemicals and instruments

Chemical compositions of essential oils were determined by using GC-MS (Thermo, Bremen, Germany). *Cinnamomum zeylanicum* Blume leaf essential oils, *Citrus × bergamia* Risso et Poiteau fruit peel essential oil, *Vitis vinifera* L. seed oil and *Corylus avellana* L. seed oil were purchased from FitoBio (Istanbul, Türkiye). Glycerin, propylene glycol, polysorbate 80 and sodium benzoate were obtained from Merck (Darmstadt, Germany). Carboxymethyl cellulose was purchased from Sigma Aldrich (St. Louis, Missouri, USA), as certified reference materials.

Gas Chromatography/ Mass Spectrometry (GC/MS) Analysis of Essential Oils

Cinnamomum zeylanicum Blume leaf and *Citrus × bergamia* Risso et Poiteau fruit peel essential oils were analyzed by using GC-FID/MS (Thermo, Bremen, Germany). The qualitative and quantitative analyses of the essential oils were detected by using Agilent 7890B GC-FID (Santa Clara, CA, USA) connected to Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA) through a two-way capillary splitter. An Agilent G4513A (Santa Clara, CA, USA) auto-injector was used to inject 1 μ L of samples. HP-INNOWAX column (60m, 0.25mm, 0.25 μ m) was used with the following temperature program; 70 °C for 15 minutes and raised to 180 °C at a rate of 2 °C/min. After maintaining an isothermal temperature of 180°C for 5 minutes, the column temperature was

increased to 230°C at a rate of 5°C/min. Then, the isothermal column temperatures of 230°C for 15 minutes were set. 100 minutes were assigned for the analysis. Injector port, quadrupole, MSD transfer line, ion source, and FID temperatures were, in order, 220°C, 150°C, 250°C, 230°C, and 220°C. Mass spectra were recorded at 70 eV. Mass range was 45–450 m/z . As a carrier gas, helium was employed (constant flow rate of 1.5 mL/min). A split ratio of 1:50 was chosen. H₂ flow was arranged to 30 mL/min while FID air flow was 400 mL/min (İnal et al. 2023). The chemical components of the essential oils were identified by comparing the mass spectra of compounds obtained from the Wiley Registry of Mass Spectral Data, 9th edition (April 2011) with the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH). Relative retention indices (RRI) of the components on the DB-WAX column were calculated from the co-injected saturated n-alkane series (C₇-C₄₀), and the outcomes were appraised with previous studies and the NIST online webbook. Relative percentage amounts of the separated compounds were calculated from the FID chromatograms. Three simultaneous auto-injections were conducted to provide the same operational conditions on GC-FID/MS.

Fatty acid analysis of fixed oils

Saponification and methylation of the oils

The two seed oils were weighed independently in 50 ml volumetric flasks and 12 ml 0.5 N methanolic NaOH was added to each seed oil for saponification. Then, they were heated in a steam bath for 5 minutes until the fat globules disappeared. 20 ml of BF₃/MeOH (Sigma Co.) was added to each flask and the mixtures were boiled for 2 minutes more. After cooling down at room temperature, they were made up to 50 ml with saturated NaCl solution. Then they were transferred to a separatory funnel, and extracted with 30 ml petroleum ether. The ether phases were taken and evaporated in a water bath at 60°C. The obtained methyl esters of fatty acids were dissolved in 5 ml of hexane and 1 µl of samples were injected and analyzed by GC-MS (Sener et al. 2007).

Gas Chromatography/ Mass Spectrometry (GC/MS) Analysis of fixed oils

Gas Chromatography-Mass Spectrometry analysis of the fixed oils was carried out on an Agilent 7890B GC-FID (Santa Clara, CA, USA) system connected to an Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA). The capillary column used was an Agilent DB-Wax (60m x 0,25mm x 0,25 µm). Helium was used as carrier gas at a flow rate of 2 ml/min with 5 µl injection volume. Samples were analyzed with the column held initially at 50°C after injection with 1 min hold time, then increased to 200°C with 25°C/min heating ramp. The final temperature was increased to 230°C with a 3°C/min heating ramp and kept at 230°C for 38 min. The injection was performed in split mode (50:1). Detector and injector temperatures were 220°C. The run time was 55 min. MS scan range was (m/z): 35–450 atomic mass units (AMU) under electron impact (EI) ionization (70 eV). (Sener et al. 2007).

The peaks were identified by using the Wiley 9-NIST 11 Mass Spectral Database, comparison of retention time (Rt) and comparison of standard compounds with mass spectrums. The relative content of % fatty acids was determined with the area under peaks using Agilent software. Three simultaneous auto-injections were conducted to provide the same operational conditions on GC-FID/MS.

Preparation of Mouthwash Formulations

0.5% w/w Carboxymethyl cellulose solution was prepared with distilled water by using magnetic stirring. Ingredients except oils were added and mixed. Finally fixed oil and essential oil were added respectively and stirred until a homogeneous solution was obtained.

Physicochemical Characterization Studies

Organoleptic Controls

Appearance, colour, odour, taste, and consistency were evaluated by organoleptic controls.

pH

The pH of herbal gel formulations was determined by using digital pH meter (ST 300, Ohaus Corporation USA). The measurement of pH of formulation was carried out in three times and the average values are reported.

Density

The density of the mouthwashes was determined using the dry pycnometer method (Venâncio et al. 2015). The densities were defined in the 50 mL pycnometer (ISOLAB 023.02.100, Germany) (empty, with distilled water and with the mouthwash), in triplicate, using an analytical balance (Shimadzu AUW220D/China).

Viscosity

The viscosity measurements of the mouthwashes were determined at room temperature 25°C between the plates of a controlled shear rate rheometer (Physica MCR 302 Anton Paar, Germany). The upper plate (cone plate) was set at a distance of 0.001 mm before the onset of the reactions. Measurements were drawn in the shear rate range 1-100 s⁻¹ at 293 K (AOAC 2019; TS 3036 2010).

Stability Studies

Stability studies were carried out at 25°C, 60%RH for 90 days, in a stabilization cabinet (TK 252. 000 Nuve Test Cabinet, Türkiye).

Antimicrobial Activity Test

For determining the in vitro antibacterial and antifungal activities of the formulations, minimum inhibitory concentrations (MICs) were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI 2000; CLSI 2004). For these experiments, standard strains

of *Streptococcus pyogenes* ATCC 19615 (Group A beta haemolytic Streptococci/GABHS), *S. mutans* ATCC 25175, *Streptococcus mitis* ATCC 49456, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 5352, and the yeast *Candida albicans* ATCC 10231, were used. Serial two-fold dilutions of all 8 mouthwash formulations were prepared in Brain heart infusion broth (BHIB, Difco Laboratories) for bacteria, and Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) buffered to pH 7.0 with morpholine propane sulfonic acid (MOPS, Sigma) for fungi in 96 well, U shaped, microtiter plates (Greiner). Each well was inoculated with 10 µL of fresh broth cultures that gave a final concentration of 5×10^5 cfu/mL for bacteria, and 5×10^3 cfu/mL for fungi in the test trays. The trays were covered and placed in plastic bags to prevent evaporation and incubated for 18–24 hours at 37°C. GABHS, *S. mutans*, and *S. mitis* were cultured under 5% CO₂ atmosphere. The MIC values were defined as the lowest concentrations that producing complete inhibition of visible growth. Also, rezasurin was used for indicating the growth of microorganisms as coloured pink. Ampicillin and fluconazole were used as reference antibiotic and antifungal, for bacteria and fungi, respectively.

Cytotoxicity Test

Eight mouthwash formulations were tested for in vitro cytotoxicity against the human gingival fibroblat (HGF) ATCC CRL-2014 cell lines (Oyardi et al. 2022). Cells were grown in Dulbecco's Minimum Essential Medium (DMEM) (Gibco), supplemented with 10% FBS (Gibco) and 100 units per mL penicillin G and streptomycin under the humidified, 5% CO₂ atmosphere at 37°C. The cells were seeded as 1×10^4 cells per well in 96 well plates and allowed 24 h to ensure the attachment, then they were incubated in the absence or presence of mouthwash formulations for 24 h. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in accordance with the manufacturer's protocol. After incubation, MTT solution was removed from the wells, DMSO solution (Sigma, 100 µl) was added, and the plates were placed on a shaker for 15 minutes. The optical density of the cells was measured at 570 nm in the microplate reader (EON-BioTek Instruments, Winooski, VT, USA). In each assay, two replicates were used for each formulation, and the process was repeated two times. The % cytotoxic effects of each formulation were calculated by comparing the untreated control cells.

Results and Discussion

During the selection of essential oil combinations for the formulated mouthwashes, consideration was given to antimicrobial activity, low cytotoxicity, and the development of formulas with two different aromas. The fixed oils in the formulations were chosen for their antioxidant properties, ability to form a thin protective film in the mouth, and contribution to alleviating dry mouth.

Qualitative analysis of essential oils by GC-MS

A total of 21 compounds in *Citrus × bergamia* Risso et Poiteau fruit peel essential oil were quantified. The characterized components indicate 99.891% of the essential oil. Table 2 demonstrates the constituents in terms of the retention indices order. Monoterpene hydrocarbons (57.427%) and oxygenated monoterpenes (41.806%) were found to be the predominant component class of the *C. bergamia* essential oil. The main components of *C. bergamia* fruit peel essential oil were detected as limonene ($39.497 \pm 0.006\%$), linalyl acetate ($28.374 \pm 0.019\%$), linalool ($10.666 \pm 0.011\%$), -Terpinene ($6.822 \pm 0.007\%$) and β -pinene ($6.379 \pm 0.015\%$). Our results were similar to studies from the literature. According to the literature, limonene (25–53%), linalool (2–20%), linalyl acetate (15–40%), -terpinene, and β -pinene are the major components of the *C. bergamia* fruit peel essential oil (Navarra et al. 2015).

Table 1
Ingredients of F1-F8 formulations.

Ingredient %	F1B	F3B	F5B	F7B	F2C	F4C	F6C	F8C
<i>C. bergamia</i> eo ¹	2	6	1	3	-			
<i>C. zeylanicum</i> eo	-				2	6	1	3
<i>V. vinifera</i> so ²	2–4				-			
<i>C. avellana</i> so	-				2–4			
%0.5 CMC sol ³	qs							
Glycerine	5–10							
Propylene glycol	2.5-5							
Polysorbate 80	2–4							
Sodium benzoate	0.1–0.2							
Distilled water	0.5-1							

¹essential oil: eo, ²seed oil: so, ³solution: sol

Table 2
Composition of *Citrus × bergamia* Risso et Poiteau fruit peel essential oil determined by mass spectroscopy.

No	Components	KI	RRI	Relative %
1	α -Pinene	1018-1032 ^a	1025	1.408 ± 0.007
2	β -Pinene	1102-1118 ^a	1112	6.379 ± 0.015
3	Sabinene	1115-1132 ^a	1122	0.988 ± 0.013
4	Myrcene	1155-1169 ^a	1159	0.804 ± 0.005
5	Limonene	1190-1204 ^a	1202	39.497 ± 0.006
6	<i>B</i> -Phellandrene	1202-1218 ^a	1212	0.244 ± 0.001
7	-Terpinene	1238-1255 ^a	1248	6.822 ± 0.007
8	p-cymene	1264-1280 ^a	1272	0.992 ± 0.001
9	Terpinolene	1277-1290 ^a	1284	0.293 ± 0.002
10	cis-Linalool oxide	1410-1478 ^a	1469	0.084 ± 0.001
11	Linalool	1537-1553 ^a	1549	10.666 ± 0.011
12	Linalyl acetate	1548-1565 ^a	1561	28.374 ± 0.019
13	cis- α -Bergamotene	1545-1577 ^a	1572	0.092 ± 0.002
14	trans- α -Bergamotene	1560-1590 ^a	1587	0.277 ± 0.009
15	β -Caryophyllene	1585-1612 ^a	1595	0.289 ± 0.003
16	Neral	1668-1693 ^a	1679	0.231 ± 0.003
17	α -Terpineol	1682-1706 ^a	1694	0.217 ± 0.016
18	Neryl acetate	1706-1726 ^a	1725	0.824 ± 0.003
19	Geranyl acetate	1728-1772 ^a	1729	0.802 ± 0.008
20	Nerol	1752-1832 ^a	1756	0.541 ± 0.006
21	Geraniol	1830-1857 ^a	1843	0.067 ± 0.002
Total identified				99.891
Monoterpene hydrocarbons (1–9)				57.427
Oxygenated monoterpenes (10–12, 16–21)				41.806
Sesquiterpene hydrocarbons (13–15)				0.658
RRI: Relative retention indices calculated against n-alkanes; % calculated from the FID data.				
KI (Kovats Indices) were given from literature mostly of RI data ranges for each compound (Babushok et al. 2011a).				

A total of 22 compounds were quantified in *Cinnamomum zeylanicum* Blume leaf essential oil. The characterized components indicate 99.989% of the essential oil and Table 3 shows the components in the order of the retention indices. The main class detected for *C. zeylanicum* leaf essential oil was phenylpropanoids (89.983%). Eugenol (80.831 ± 0.001%) was detected as the major component of the *C. zeylanicum* leaf essential oil. The trans-cinnamaldehyde (4.028 ± 0.008%), β -caryophyllene (3.238 ± 0.001%) and linalool (2.066 ± 0.005%) compounds were also found in the *C. zeylanicum* leaf essential oil respectively. Our results were confirmed by the literature. For instance, Paranagama et al. detected the chemical components of the *C. zeylanicum* leaf essential oil. The total phenyl propanoid content was determined as 85.4% and eugenol (76.74%) was detected as the major of them (2001).

Table 3
Composition of *Cinnamomum zeylanicum* Blume leaf essential oil determined by mass spectroscopy.

No	Components	KI	RRI	Relative %
1	α -Pinene	1018-1032 ^a	1025	0.637 $\pm$ 0.014
2	Camphene	1060-1076 ^a	1070	0.102 $\pm$ 0.001
3	β -Pinene	1102-1118 ^a	1112	0.191 $\pm$ 0.008
4	α -phellandrene	1160-1176 ^a	1163	0.399 $\pm$ 0.003
5	Limonene	1190-1204 ^a	1202	0.230 $\pm$ 0.009
6	1,8-cineole	1203-1220 ^a	1211	0.417 $\pm$ 0.013
7	p-cymene	1264-1280 ^a	1271	0.702 $\pm$ 0.006
8	α -Copaene	1481-1497 ^a	1484	0.517 $\pm$ 0.007
9	Benzaldehyde	1504-1533 ^a	1520	0.104 $\pm$ 0.004
10	Linalool	1537-1553 ^a	1549	2.066 $\pm$ 0.005
11	β -Caryophyllene	1585-1612 ^a	1595	3.238 $\pm$ 0.001
12	α -Humulene	1655-1682 ^a	1667	0.561 $\pm$ 0.004
13	α -Terpineol	1682-1706 ^a	1694	0.380 $\pm$ 0.005
14	Safrole	1853-1876 ^a	1869	0.849 $\pm$ 0.005
15	Benzenepropyl acetate	1919-1971 ^b	1938	0.790 $\pm$ 0.001
16	Caryophyllene oxide	1970-2008 ^a	1979	0.462 $\pm$ 0.007
17	trans-Cinnamaldehyde	2015-2044 ^a	2035	4.028 $\pm$ 0.008
18	Cinnamyl acetate	2125-2153 ^c	2144	1.613 $\pm$ 0.009
19	Eugenol	2151-2185 ^a	2157	80.831 $\pm$ 0.001
20	Eugenol acetate	2252-2277 ^a	2253	1.646 $\pm$ 0.002
21	trans-Cinnamyl alcohol	2257-2289 ^d	2274	0.127 $\pm$ 0.001
22	Chavicol	2320-2358 ^a	2328	0.099 $\pm$ 0.001
Total identified				99.989
Monoterpene hydrocarbons (1–5, 7)				2.261
Oxygenated monoterpenes (6, 10, 13)				2.863
Sesquiterpene hydrocarbons (8, 11, 12)				4.316
Oxygenated sesquiterpenes (16)				0.462
Phenylpropanoids (14, 15, 17–22)				89.983
Others (9)				0.104
RRI: Relative retention indices calculated against n-alkanes; % calculated from the FID data.				
KI (Kovats Indices) were given from literature mostly of RI data ranges for each compound (Babushok et al. 2011a; NISTWebbook, 3-Phenyl-1-propanol, acetate 2023b, NISTWebbook, Acetic acid, cinnamyl ester 2023c, NISTWebbook, 2-Propen-1-ol, 3-phenyl-, (E) 2023d)				

Fatty acid analysis of fixed oils by GC-MS

Fatty acids analysis of the *V. vinifera* and *C. avellana* flaxseed oils are demonstrated in Table 4. Consistent with the literature, linoleic acid was detected as the predominated in *V. vinifera* seed oil (67.667%) and the oleic acid is the major fatty acid of *C. avellana* seed oil (81.936%). Unsaturated fatty acids were measured highly in both *V. vinifera* (83.940%) and *C. avellana* (91.096%) seed oils. Monounsaturated fatty acids were detected as more constituted in *C. avellana* seed oil (79.809%) while the polyunsaturated fatty acids were in a higher quantity in *V. vinifera* (66.197%) seed oil. According to our results, both *V. vinifera* and *C. avellana* seed oils' fatty acids and their content are in accordance with the literature.

Table 4
Fatty acid composition of the flaxseed oils.

No	Fatty acids	Relative %	
		<i>C. avellana</i> seed oil	<i>V. vinifera</i> seed oil
1	Myristic acid (C _{14:0})	0.048 ± 0.001	-
2	Palmitic acid (C _{16:0})	6.523 ± 0.009	12.500 ± 0.016
3	Palmitoleic acid (C _{16:1})	0.139 ± 0.002	-
4	Stearic acid (C _{18:0})	2.332 ± 0.001	3.560 ± 0.005
5	Oleic acid (C _{18:1})	79.670 ± 0.014	17.743 ± 0.010
6	Linoleic acid (C _{18:2})	11.287 ± 0.019	66.197 ± 0.017
	SFA (1, 2, 4)	8.903	16.060
	UFA (3, 5, 6)	91.096	83.940
	MUFA (3, 5)	79.809	17.743
	PUFA (6)	11.287	66.197
	SFA: Saturated fatty acid		
	UFA: Unsaturated fatty acids		
	MUFA: Monounsaturated fatty acid		
	PUFA: Polyunsaturated fatty acid		

Physicochemical Characterization Studies

Organoleptic Controls

The organoleptic properties of the mouthwash formulations are given in Table 5 and Table 6. Also, the mouthwash formulations (F1, F3, F5, F7 and F2, F4, F6, F8) can be seen in Fig. 1. While the pH of the prepared formulations varied between 5.47 ± 0.19–6.01 ± 0.01, their density was determined to be close to that of water (1 g/mL). The viscosity of the formulas in which the amount of glycerin and propylene glycol is half (F5B, F7B, F6C and F8C) was found to be higher than the viscosity determined in the formulations F1B, F3B, F2C and F4C. This result is consistent with the finding that the viscosity of the CMC solutions decreased at higher concentrations of glycerol (Wagner et al. 2023). The results of the physicochemical characterization studies indicated that the formulated mouthwashes are suitable for oral rinsing, and the pH is compatible with the physiological pH value in the oral cavity.

Table 5
Organoleptic controls of mouthwash formulations.

Controls	Formulations							
	F1B	F3B	F5B	F7B	F2C	F4C	F6C	F8C
Apperance	Opaque liquid, no sediment	Opaque liquid, no sediment	Transparent liquid, no sediment	Opaque – transparent liquid, no sediment	Opaque liquid, no sediment	Opaque liquid, no sediment	Transparent liquid, no sediment	Opaque – transparent liquid, no sediment
Colour	Light Yellowish white	Yellowish white	White	Very Light Yellowish white	Light Browish white	Brownish white	White	Very light brownish white
Odour	Bergamot	Pungent Bergamot	Fresh scented light bergamot	Fresh scented bergamot	Cinnamon	Pungent Cinnamon	Fresh scented light cinnamon	Fresh scented cinnamon
Taste	Aromatic bergamot	Pungent aromatic bergamot	Fresh aromatic bergamot	Aromatic bergamot	Aromatic cinnamon	Pungent aromatic cinnamon	Fresh aromatic cinnamon	Aromatic cinnamon
Consistency	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky	Homogeneous not sticky

Table 6
Viscosity, density and pH measurements of mouthwash formulations.

Controls	Formulations							
	F1B	F3B	F5B	F7B	F2C	F4C	F6C	F8C
pH	5.54 ± 0.07	5.47 ± 0.19	5.83 ± 0.02	5.96 ± 0.12	5.76 ± 0.04	5.62 ± 0.01	6.01 ± 0.01	5.94 ± 0.03
Density (g/mL)	1.05 ± 0.11	1.05 ± 0.05	1.061 ± 0.01	1.06 ± 0.08	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.01	1.01 ± 0.02
Viscosity (cP)	15.19 ± 0.82	13.21 ± 0.75	47.81 ± 0.48	44.28 ± 1.22	7.48 ± 0.68	7.03 ± 0.47	34.38 ± 2.72	24.69 ± 1.73

Result of stability evaluation

The formulations were stayed in a stabilization cabinet at 25°C, 60%RH for 90 days days, after which they underwent examination for organoleptic properties, pH levels, and density. At the conclusion of this period, no changes were observed, indicating the stability of the mouthwash formulations.

Antimicrobial activities of formulations

According to the MIC results, as shown in Table 7, F2C, F4C and F8C were detected to be effective against almost all tested mouth-throat and upper respiratory tract pathogens and potential pathogens. The most active essential oil and fixed oil combination was the group that included *C. zeylanicum* and *Corylus avellana* seed oil formulations. They were effective against all tested pathogens, especially at 0.375% and 0.750% concentrations of F4C against *K. pneumoniae* or *C. albicans*, and *St. aureus* or *S. pyogenes* (GABHS) respectively. However, *C. bergamia* essential oil and *V. vinifera* oil combination was not active against *S. mitis*, it has antibacterial activity at 0.750% or 1.500% concentrations of F7B or F3B against *St aureus* or *K. pneumonia*, and antifungal activity against *C. albicans* at 0.375% concentration of F3B. The MIC values of the standard antibiotic and antifungal against standard strains were within the accuracy range of CLSI, throughout the study (CLSI 2022).

Table 7
Minimum inhibitory concentrations (MICs) of the formulations.

Formulations	Strains					
	<i>S. pyogenes</i>	<i>S. mutans</i> ATCC 25175	<i>S. mitis</i>	<i>St. aureus</i>	<i>K. pneumoniae</i>	<i>C. albicans</i>
	ATCC 19615		ATCC 49456	ATCC 29213	ATCC 4352	ATCC 10231
F1B	2	-	-	1	1	0.250
F2C	2	2	2	0.500	0.500	0.250
F3B	3	6	-	1.500	1.500	0.375
F4C	0.750	1.500	1.500	0.750	0.375	0.375
F5B	-	-	-	1	0.500	0.120
F6C	1	-	-	0.500	0.250	0.120
F7B	3	3	-	0.750	0.750	0.375
F8C	1.500	1.500	1.500	0.750	0.375	0.375
Results were given as dilutions of formulations, -: no any antimicrobial effect						

Cytotoxicity test

As depicted in Table 8 and Fig. 2 the cytotoxicity test results against healthy human gingival fibroblast cells (HGF ATCC CRL-2014) revealed that the most cytotoxic formulation was F4C, exhibiting 47.25% cytotoxicity, followed by F8C at 16.48%. Conversely, formulations F6C and F5B demonstrated the lowest cytotoxic effects, measured at 7.82% and 8.57%, respectively, against gingival fibroblast cells.

Table 8
% Cytotoxicities of the
formulations.

Formulations	Cytotoxicity %
F1B	9,89
F2C	10
F3B	11,5
F4C	47,25
F5B	8,57
F6C	7,82
F7B	10,21
F8C	16,48

Research on the *in vitro* cytotoxicity of cinnamon leaf essential oil on gingival fibroblast cells suggests various outcomes depending on the concentration used and the specific experimental conditions. The cytotoxic effects of cinnamon leaf oil on gingival fibroblast cells appear to be dose-dependent. Lower concentrations might exhibit minimal or no cytotoxicity, while higher concentrations could lead to more pronounced cytotoxic effects. However, despite its potential cytotoxicity at higher concentrations, cinnamon leaf oil has also been reported to possess anti-inflammatory properties that might benefit gingival tissues. It may modulate inflammatory responses and reduce inflammation in gingival cells under certain conditions (Marcoux et al. 2020; Veilleux and Grenier 2019). The cytotoxic and antimicrobial effects of cinnamon leaf essential oil at high concentrations are probably due to its high content of eugenol. In our study, the cinnamon leaf essential oil that we used in preparing F4C and F8 formulations contains $80.831 \pm 0.001\%$ eugenol. Eugenol has been used in dental practice for a long time because of its anti-inflammatory, anesthetic, and antimicrobial properties. In particular, the antimicrobial activities of eugenol against oral pathogens such as *Porphyromonas gingivalis*, *S. sobrinus*, *S. sanguinis*, *S. anginosus* and *Aggregatibacter actinomycetemcomitans* have been proven (Marchese et al. 2017). Gerosa et al. revealed that the eugenol in the alcohol solution had a cytotoxic effect at concentrations of above $1.9 \mu\text{M}$ against gingival fibroblasts (1996). For this reason, it is very important to evaluate the cytotoxicity of eugenol, which is widely used in oral health, at different concentrations. In this study, it was observed that formulations F4C and F8C, which contained the highest concentrations of cinnamon leaf oil, exhibited the highest levels of cytotoxicity. However, it is noteworthy that these formulations, F4C and F8C, which demonstrated the highest cytotoxicity, also exhibited the highest and broadest antimicrobial activity among all formulations.

F6C and F2C, formulations containing the same essential oil in slightly lower amounts, were effective against all tested microorganisms, although weaker than F4C, and had acceptable cytotoxic effects. On the other hand, formulations containing bergamot fruit peel oil, which has limited antimicrobial activities, showed much lower cytotoxicity against gingival cells.

Conclusion

The studied mouthwash formulations demonstrated appropriate physicochemical characteristics and exhibited dose-dependent antimicrobial activities against nearly all studied oral pathogens. Particularly, formulations containing *C. zeylanicum* oil displayed significant effectiveness against all tested microorganisms at notably lower doses. However, the F4C formulation, despite showing the most potent antimicrobial activity and favorable properties in other tests, was evaluated as unsuitable for oral tissue application due to its high cytotoxicity to gingival cells. Formulations excluding F4C but containing cinnamon leaf oil proved valuable as mouthwashes, showcasing acceptable cytotoxicity alongside notable antimicrobial activities and other tested properties. The F2C formulation, comprising 2% cinnamon leaf oil, 2–4% hazelnut oil, and a 0.5% CMC solution, demonstrated successful antimicrobial efficacy against all investigated mouth pathogens and exhibited acceptable safety in terms of cytotoxicity. This formulation holds promise for local treatment of xerostomia, malodor, and orodental infections.

Additionally, formulations containing bergamot fruit peel oil, characterized by low cytotoxicity, favorable properties in other tests, and relatively lower antimicrobial activities, are also beneficial as mouthwashes. The F7B formulation, consisting of 3% bergamot fruit peel oil, 2–4% grape oil, and a 0.5% CMC solution, exhibited successful antimicrobial efficacy against all investigated mouth pathogens except *S. mitis* and displayed acceptable safety in terms of cytotoxicity, making it a viable option for local treatment of xerostomia, malodor, and orodental infections. On the other hand, formulations F3B, F1B, F5B, and F6C displayed good safety profiles with acceptable cytotoxicity and mild antimicrobial efficacy, suitable for local treatment of xerostomia, malodor, and oral hygiene.

Declarations

Conflict of interest

The authors declare no competing interests.

Funding

Not applicable.

Data Availability

The data used in this study are available in the supplementary material.

Author contributions

Study design, EAY; experimental studies, Eİ, FNY, CÖ, EAY; writing—original draft preparation, EAY, SD, MSE, Eİ; writing—review and editing, MSE, BAK; supervision M.K.; All the authors have read the final manuscript and approved the submission.

Ethics Approval

Not applicable.

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Figures



Figure 1

Appearance of the formulas. Left to right F1B, F3B, F5B, F7B and F2C, F4C, F6C, F8C respectively.

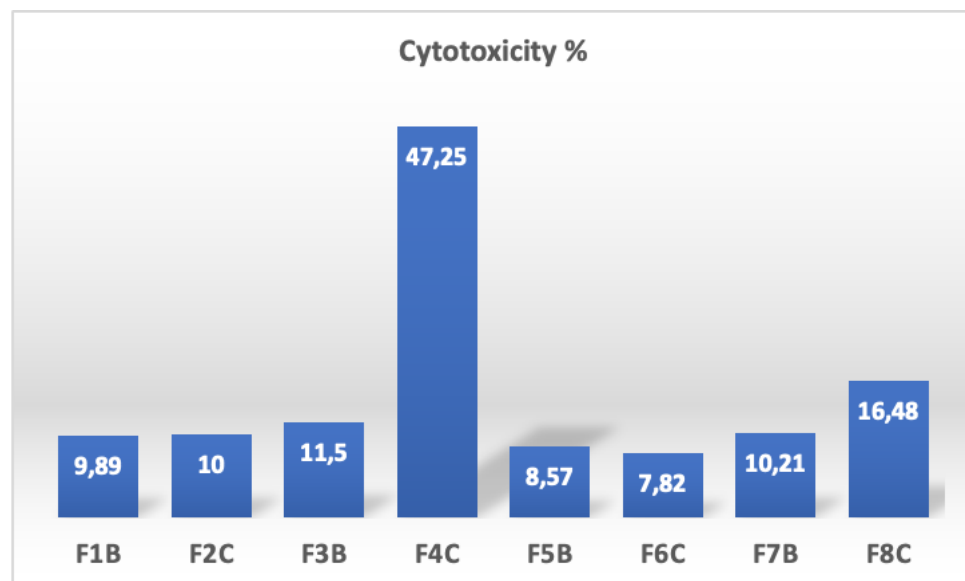


Figure 2

% Cytotoxicities of the formulations

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