Journal of Applied Pharmaceutical Research
Volume 12 Issue 5, Year of Publication 2024, Page 87 – 101
DOI: https://doi.org/10.69857/joapr.v12i5.679

Check for updates

Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | JOAPR
www.japtronline.com *ISSN: 2348 – 0335*

FORMULATION AND DEVELOPMENT OF COMMIPHORA MYRRHA BASED POLYHERBAL NANOEMULSION MOUTHWASH AND ASSESSMENT OF ITS ANTI-OXIDANT AND CYTOTOXICITY ACTIVITY

Norafiqah Yusof[1], Sheba R David[2], Nuramalina H Mumin[1], Liyana Ahmad[1], Rajan Rajabalaya[1]*

Article Information
Received: 19th May 2024
Revised: 28th August 2024
Accepted: 16th September 2024
Published: 31st October 2024

Keywords
Nanoemulsion, Commiphora myrrha, Polyherbal, Antioxidant, Cytotoxicity

ABSTRACT

Background: A *Commiphora myrrha* (CM)-based polyherbal mouthwash with enhanced stability and oral bioavailability was developed using a high-energy homogenization method. **Methodology:** The formulations primarily consist of herbal extracts from CM, ginger, and white tea, optimized based on various parameters, including organoleptic properties, pH, Dynamic Light Scattering (DLS), and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR- FTIR). Stability studies were also conducted on each formulation. **Results and Discussion:** The particle sizes ranged from 77 to 216 nm, with zeta potential values between -0.92 and -2.09 ± 0.38 mV, indicating stability. ATR-FTIR studies confirmed no interaction between the ingredients. Antioxidant activity was significant, with IC50 values for pure extracts of CM, white tea, and ginger being 0.071 ± 0.003, 0.073 ± 0.004, and 0.066 ± 0.004 mg/ml, respectively. For formulations M1 and M2, IC50 values were 1.030 ± 0.901 and 0.495 ± 0.496 mg/ml, respectively, showing a concentration-dependent increase in antioxidant activity. The MTT cytotoxicity assay showed high cell viability for M1 (96.1%) and M2 (133.3%) at 0.002 mg/ml, suggesting low cytotoxicity, though variability in results indicated further assay optimization. High standard deviations, 0.06 and 0.208, indicated limitations in experimental conditions emphasizing the need for improved assay parameters for accuracy. **Conclusion:** The mouthwash formulations, M1 and M2 Show promise, with future work focusing on increasing CM concentration and refining cytotoxicity testing methods to ensure reliable data for subsequent antibacterial and in vivo studies.

INTRODUCTION

Oral health is integral to overall well-being, whether in health or illness. Periodontal disease is an infectious condition caused by microbial plaque accumulation around the teeth, triggering an inflammatory response in the gingival tissue. While many individuals experience gingival inflammation, only a subset progresses to periodontitis, characterized by attachment loss. Without treatment, periodontitis can lead to tooth decay and, eventually, tooth loss [1]. Severe oral problems are particularly

[1]*PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, BE 1410 Gadong, Bandar Seri Begawan, Brunei Darussalam.*
[2]*School of Pharmacy, University of Wyoming, Laramie, Wyoming, 82071, USA.*

For Correspondence:** **rajan.rajabalaya@ubd.edu.bn

prevalent in individuals with advanced cancer who are admitted to intensive care units [2], highlighting the importance of oral health in overall patient well-being, especially toward the end of life [2,3].

Several studies suggest chlorhexidine (CHX) mouthwash effectively reduces plaque, gingival inflammation, and bleeding [4]. However, prolonged use of CHX and other mouthwashes may lead to side effects, including altered taste perception, tooth decay, and acidic oral conditions that lower saliva pH [5,6]. CHX may also reduce oral antibodies, increasing susceptibility to diseases such as gingivitis [7] and contributing to dental mineralization loss and other dental issues when overused [8]. Elevated concentrations of lactate and glucose in saliva, closely associated with an increased risk of oral diseases such as diabetes mellitus, have also been reported [9]. High saliva lactate and glucose levels, associated with increased oral disease risk, including diabetes mellitus, are also reported [9]. Additionally, CHX may alter oral acidity, impacting bacterial species and nitrite availability and potentially influencing blood pressure regulation. Although CHX helps maintain acid-base balance by modifying the oral bacterial composition, alternative strategies for preventing gingivitis, such as herbal remedies, are desirable [6]. Herbal extracts have long been used to treat various ailments and are increasingly being explored as alternatives for oral health care. Herbal remedies offer a promising alternative, with fewer side effects and bioactive compounds targeting inflammation and microbial growth. However, there is a gap in integrating advanced drug delivery systems, such as nanoemulsions, in oral care products containing herbal extracts[10]. Nanotechnology enhances the delivery of herbal compounds by addressing challenges such as poor solubility and bioavailability, which are common with natural products compared to synthetic compounds. Combining herbal extracts into nanoemulsions improves bioavailability and delivery efficiency [11].

Commiphora myrrha (CM) is a small tree native to the Arabian Peninsula, East Africa, and India, belonging to the Burseraceae family. The resinous exudate of CM, known as myrrh, is obtained from the bark and trunk of the trees [12]. Myrrh is water-soluble and primarily contains 60% frankincense, 40% resin, and volatile oils such as eugenol, sterols, triterpenoids, and furanosesquiterpenes [12–14]. Traditionally, myrrh has been used in perfumery and treating skin diseases and wounds [15]. It is also an effective antimicrobial agent, commonly used in treating oral ulcers, gingivitis, and sinusitis [14]. Furthermore, myrrh oil and crude extracts exhibit various biological activities, including anti-inflammatory, cytotoxic, and antimicrobial effects [16]. Studies have demonstrated myrrh's ability to stimulate plasma cell production, induce angiogenesis, and promote tissue regeneration within seven days [15].

Additionally, myrrh mouthwash has been reported to initiate early rehabilitation, exhibiting superior immunomodulatory, antimicrobial, and antifungal properties [15]. Myrrh has also shown therapeutic potential in inhibiting the growth of various bacteria, fungi, and parasites, such as those causing fascioliasis and schistosomiasis [17]. In diabetic patients, periodontal disease progresses more rapidly, disrupting glycemic control and potentially creating a vicious cycle [18]. Ginger (*Zingiber officinale*) is one of the most widely used medicinal plants [19]. Its active ingredients, such as β-bisabolene, shogaol, gingerol, and paradol, contribute to its anti-glycemic, anti-inflammatory, antioxidant, anti-cancer, and anti-obesity effects [20]. Ginger may also alleviate inflammation by reducing levels of inflammatory markers (TNF-α, IL-1β, IL-6) in diabetic patients, significantly lowering malondialdehyde levels while increasing antioxidant enzyme levels, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione itself [21]. Like green and black tea, white tea is derived from *Camellia sinensis* [22]. Previous studies have highlighted the significant health benefits of white tea, particularly its antimicrobial properties, which surpass those of other tea varieties [23].

White tea contains polyphenolic compounds with many activities, including antioxidant, antiviral, anticancer, antitoxoplasmal, and antihelminthic effects [24]. Nanoemulsions have gained attention for their ability to improve the solubility and bioavailability of poorly soluble herbal extracts in various therapeutic applications, including oral health [25]. Studies have demonstrated that nanoemulsions can effectively deliver active ingredients, such as essential oils and herbal extracts, to the oral mucosa, providing sustained release and enhanced therapeutic effects . For instance, Juniatik et al. developed a nanoemulsion mouthwash containing lemongrass and kaffir lime oils, showing significant antifungal activity against Candida albicans [26]. Similarly, nanoemulsions incorporating eucalyptus oil have been shown to enhance the

formulation's antimicrobial and antioxidant properties. However, using nanoemulsions containing herbal combinations like CM, ginger, and white tea in oral health applications is still underexplored, creating further research and innovation opportunities. Based on the above literature, the primary objective of this study was to develop a polyherbal nanoemulsion mouthwash containing CM, ginger, and white tea. The development process included evaluating the formulation's physicochemical properties, such as organoleptic characteristics, particle size, pH, consistency, and stability. Additionally, the study aimed to investigate the formulated mouthwash's antioxidant properties and in vitro cytotoxicity.

MATERIAL AND METHODS

Materials

The oleo-gum-resin or *Myrrh* of *Commiphora Myrrha* (CM), Virgin Coconut Oil (VCO), and Ginger powder was obtained from a local market in Brunei. White Tea extract (*Camellia Sinesis)* was purchased from (Bareggio, Milan, Italy). Glyceryl Monooleate (GMO) and Tween® 80 were procured from Sigma-Aldrich (Billerica, Massachusetts, USA). The L-Glutamine solution, Penicillin-Streptomycin, Fetal Bovine Serum (FBS), Dimethyl sulfoxide (DMSO), 0.25% Trypsin-EDTA and 0.4% Trypan blue solution were also obtained from Sigma Aldrich (Burlington, MA, USA). Dulbecco's Modified Eagle Medium (DMEM) and Phosphate Buffer Saline (PBS) were obtained from Thermo Fisher Scientific (St. Louis, Missouri, USA). 2,2-diphenyl-1-picrylhydrazyl DPPH solid powder and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma Aldrich (Burlington, MA, USA).

Preparation of Mouthwash

Five different polyherbal mouthwash formulations based on Commiphora myrrha (CM) were prepared, each containing Myrrh along with two secondary herbal components: Ginger extract (GE) and White Tea extract (WTE). Myrrh resin was first rinsed with distilled water, air-dried, and ground into a fine powder to prepare each formulation. Myrrh and Ginger powders were then separately dissolved in Milli-Q water and filtered to obtain aqueous extracts of Myrrh and Ginger. Each formulation was prepared with a constant volume of 100 mL, with a concentration of 2 mg/mL, based on 200 mg of Myrrh in each formulation, as shown in Table 1. The oil phase of the emulsion consisted of Virgin Coconut Oil (VCO) and GMO surfactant, while the aqueous phase contained the Myrrh extract, GE, WTE, and Tween 80 as a co-surfactant. Both phases were heated to 60°C before titration. The aqueous phase was slowly added to the oil phase under continuous stirring at 500 rpm for 10 minutes.

This was followed by homogenization using a high-pressure Silverson Homogenizer at 2500 rpm for 30 minutes to create nano-dispersions for each formulation [27,28]. Each formulation was then observed for one day to check for phase separation and to optimize the concentrations of surfactants and co-surfactants, as described in the references.

Physicochemical Characterization Studies

Organoleptic Properties

The organoleptic properties, including odor and color, were evaluated for the CM-based polyherbal nanoemulsion [29].

pH determination

Exactly 5 mL of the sample formulation was dissolved in 10 mL of distilled water (pH 7.0), and the pH was measured using a calibrated Mettler Toledo pH meter (USA), as shown in Table 2 [30].

Dynamic Light Scattering (DLS) measurements

Dynamic Light Scattering (DLS) was used to characterize the five mouthwash formulations in terms of particle size, zeta potential, and polydispersity index using the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Each formulation was diluted with 4 mL of Milli-Q water and ultrasonicated for 15 minutes to ensure uniform particle dispersion. Then, 2 mL of the solution was placed into sample cuvettes and loaded into the DLS instrument. The analyses were conducted with an average of 30 scans [29].

ATR- FTIR analysis

Fourier Transform Infrared (FTIR) spectroscopy was conducted on all mouthwash formulations and their herbal components to assess compatibility and identify functional groups. The analysis used an IR Spirit (Shimadzu, Kyoto, Japan) equipped with an Attenuated Total Reflectance (ATR) accessory with a diamond crystal. For each measurement, 10 µL of the sample was directly applied to the ATR crystal, ensuring sufficient contact. The spectra were recorded in the 400 to 4000 cm^{-1} range with a resolution of 4 cm^{-1}. The ATR-FTIR method allows direct analysis of the liquid samples without complex sample

preparation like the KBr pellet method. Spectra were collected for each formulation and compared with those of the pure herbal components to confirm the presence and stability of the functional groups [31].

Stability studies

The stability of each mouthwash formulation was evaluated by storing 20 mL of each formulation at 4°C, 25°C, and 40°C for 30 days. The pH of the formulations was measured at 0, 14, and 30 days using the Accumet AE150 pH meter (Thermo Fisher Scientific) [32].

Antioxidant activity of CM-polyherbal nanoemulsion

DPPH Assay

The total antioxidant capacity of the optimized mouthwash formulations was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assay. In this assay, antioxidants reduce the purple DPPH• radicals, producing a pale yellow compound, 1,1-diphenyl-2-picrylhydrazine, which allows for spectrophotometric measurement of antioxidant activity [33]. A 0.2 mM DPPH solution was prepared by dissolving 3.94 mg of DPPH powder in 100 mL of methanol. Various concentrations of each mouthwash formulation, herbal components, and the standard Trolox were prepared by serial dilution in a 96-well plate using methanol, ranging from 2 mg/mL to 0.00625 mg/mL. The volume of each test sample was kept constant at 100 µL per well.

A blank containing 100 µL of methanol was also included. Next, 100 µL of DPPH• solution was added to each well, mixed thoroughly, and incubated at room temperature (25±2°C) for 30 minutes in the dark. Absorbance was measured at 517 nm using the Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). All absorbance values were corrected against the blank, and the ability to scavenge DPPH• free radicals was expressed as the percentage radical scavenging activity, calculated using the following equation [34,35]:

$$\%\ Radical\ Scavenging\ activity = \frac{Abs_{Blank} - Abs_{sample}}{Abs_{Blank}} \times 100$$

Where, Abs = Absorbance.

The results were expressed as the Minimal Inhibitory Concentration (IC_{50}) value, the concentration required to achieve a 50% anti-oxidant effect.

Cell culture

Human Embryonic Kidney 293 (HEK293) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 to maintain optimal conditions for cell growth. These parameters were strictly controlled throughout the experiment to ensure consistency and reproducibility. The cells were cultured in T25 flasks containing 4 mL of DMEM media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, and 1% Penicillin-Streptomycin.

Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were passaged when they reached approximately 90% confluence to maintain optimal density. The old media was aspirated during cell passage, and cells were washed with 4 mL of phosphate-buffered saline (PBS). Cells were then detached by incubating them with 1 mL of 0.25% Trypsin-EDTA for 3–5 minutes at 37°C. Following detachment, 4 mL of fresh media was added to neutralize the Trypsin, and the cell suspension was centrifuged at 300 g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in fresh DMEM media to avoid prolonged Trypsin exposure, which could lead to cell damage. Cell viability and concentration were assessed using a hemocytometer and Trypan Blue exclusion assay to ensure accurate cell counting for subsequent experiments. Residual cells were seeded into new T25 flasks and incubated under the same conditions [36,37].

In-Vitro Cytotoxicity Study

Mouthwash Formulation Treatment

Three concentrations (0.2 mg/mL, 0.02 mg/mL, and 0.002 mg/mL) of the M1 and M2 mouthwash formulations were prepared by serial diluting a 2 mg/mL stock solution in DMEM. Herbal controls of Commiphora myrrha, ginger extract, and white tea extract were ready at a 1:10 dilution to match the highest concentration used in the test samples.

MTT Cytotoxicity Assay

The cytotoxicity of the optimized mouthwash formulations was evaluated using the MTT assay. Before the experiment, cell optimization was conducted to determine the optimal seeding density. HEK293 cells were seeded at densities of 1×10^4, 2×10^4, 3×10^4, and 4×10^4 cells per well in a 96-well plate (Corning, Waltham, Massachusetts, USA) and incubated at

37°C with 5% CO_2 for 24 hours. Based on the optimization results, a seeding density of 2×10^4 cells per well was selected for further experiments. After 24 hours of incubation, the media was replaced with 100 µL of each treatment sample (M1, M2, and controls) and incubated for another 24 hours under the same conditions. Cells treated with 1 mM 5-Fluorouracil (a known cytotoxic agent) served as the positive control, while distilled water was the negative control. Wells containing only fresh DMEM without cells were used as the blank control. After the treatment, the media was removed from each well, and 30 µL of MTT solution (1 mg/mL in PBS) was added. The plates were incubated for 2 hours at 37°C in the dark to allow the formation of formazan crystals by viable cells.

After incubation, 100 µL of isopropanol was added to each well to dissolve the formazan crystals, and the plate was kept in the dark for an additional 2 hours. Absorbance was measured at 570 nm using the Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). Absorbance values were corrected against the blank wells, and cell viability was calculated using the following equation:[38]. This method provides reproducibility by ensuring accurate cell optimization and detailed absorbance measurement procedures.

$$Cell\ Viability\ (\%) = \frac{Abs_{Sample} - Abs_{Blank}}{Abs_{negative\ control} - Abs_{Blank}} \times 100$$

Where, Abs = Absorbance.

Statistical analysis

All experiments were performed in triplicate to ensure reproducibility, and the data are presented as mean ± standard deviation (SD). For statistical analysis, one-way analysis of variance (ANOVA) was used to compare the means across different treatment groups. Tukey's post-hoc test was applied for multiple comparisons to determine the significance between specific groups. Each data point was normalized against the blank control to account for variability in absorbance values, and outliers were identified and excluded using Grubbs' test. Statistical analyses were conducted using GraphPad Prism (version 8.4.3). A p-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Preparation of CM-Based Polyherbal Nanoemulsion

Initially, 12 formulations containing additional secondary herbal components, such as ginger and white tea, were developed. However, only five stable CM-based polyherbal nanoemulsions were successfully prepared. The limitation arose from solubility issues with the ginger extract in the aqueous phase, leading to phase separation. Formulations with lower and higher concentrations of these components led to phase separation, which prompted the selection of five optimized formulations (M1-M5) based on stability.

The chosen concentrations of Virgin Coconut Oil (VCO), Glyceryl Monooleate (GMO), and Tween 80 were optimized to ensure emulsification stability, droplet size uniformity, and formulation integrity [29]. Tween 80 was selected for its ability to reduce interfacial tension and prevent phase separation, enhancing overall stability. GMO and VCO were chosen for their compatibility and capacity to form stable emulsions with small, uniform droplets aided by the medium-chain fatty acids in VCO. These optimized concentrations also ensured long-term stability by maintaining appropriate particle size and zeta potential [39]. The results of the formulations (M1-M5) prepared using Virgin Coconut Oil (VCO), Glyceryl Monooleate (GMO), and Tween 80 with three herbal extracts (Commiphora myrrha (CM), Ginger extract, and White Tea extract) are presented in Figure 1 and Table 1. M3 was excluded from further studies of the five formulations due to phase separation. VCO was dissolved in GMO as the oil phase. GMO has a high solubility in the medium-chain fatty acids (MCFA) found in VCO, promoting maximum stability of the nanoemulsion or liquid crystalline formulations [37]. Despite these efforts, formulation M3 still exhibited phase separation after the stability studies, likely due to insufficient surfactant concentration, and was excluded from further analysis.

Physiochemical Characterization studies

Organoleptic properties

The organoleptic properties were evaluated, and the pH data and particle size values are presented in Tables 1 and 2.

The organoleptic properties of the CM-based nanoemulsion showed a pure white color and an oily odor, as no flavoring agents were added based on preliminary studies. The pH values ranged from 5.02 to 5.37, indicating a slightly acidic formulation. Ideally, mouthwashes should have a pH above 5.5 to prevent enamel erosion in an acidic environment ($pH < 5.5$) [40, 41]. Several factors, such as chemical reactions and acidic esters in VCO, may have contributed to the slight acidity observed in the formulations [42].

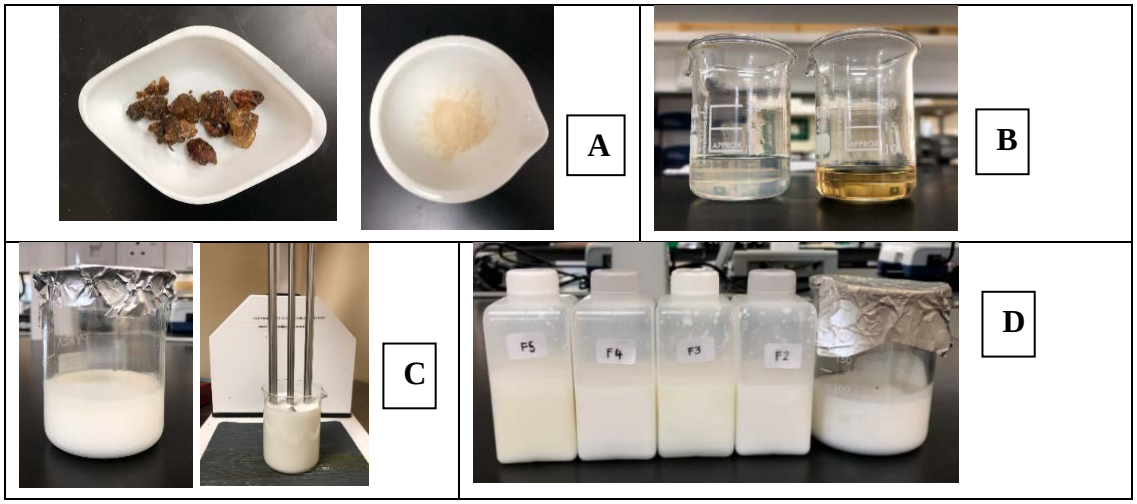

Figure 1: Schematic diagram of the preparation of oil-in-water (o/w) CM based polyherbal nanoemulsion. A- CM & Ginger; B- Aqueous and Oil phase; C- Higher formulation method; D – CM- polyherbal nanoemulsion formulations.

Table 1: Excipients and components of CM-based mouthwash final formulation

Batch code	VCO (g)	Surfactants (%)		CM (mg/mL)	GE (mL)	WTE (mL)	Organoleptic properties		Remarks
		GMO	Tween 80				Colour	Odour	
M 1	10	5	2	2	10	5	White	Oil	Nanoemulsion formed
M 2	10	5	4	2	10	5	White	Oil+	Nanoemulsion
M 3	20	4	2	2	10	5	Off-white	Oil	Phase separation
M 4	20	4	5	2	10	5	White	Oil+	Nanoemulsion formed
M 5	20	4	10	2	10	5	Off-white	Oil++	Nanoemulsion formed

VCO: Virgin Coconut Oil, GMO: Glyceryl Monooleate, CM: *Commiphora Myrrha*, GE: Ginger Extract, WTE: White Tea Extract: + Strong odour, ++ Very strong odour

Table 2. pH values and Particle size of Mouthwash formulations at different intervals

Code	Day	pH value			Particle size	Zeta Potential (mV)	PDI value
		4°C ± 2°C	25°C ± 2°C	40°C ± 2°C			
M 1	0	-	5.37	-	216.3 ± 2.3	-1.18 ± 0.51	0.263 ± 0.01
	14	5.29	5.09	4.75			
	30	5.20	4.90	4.72			
M 2	0	-	5.33	-	135.7 ± 1.6	-1.72 ± 0.26	0.279 ± 0.01
	14	5.16	5.04	4.51			
	30	5.02	4.91	4.73			
M 4	0	-	5.11	-	156.3 ± 2.5	-2.09 ± 0.38	0.230 ± 0.02
	14	5.03	5.10	4.69			
	30	5.01	4.98	4.76			
M 5	0	-	5.02	-	77.7 ± 0.3	-0.92 ± 0.27	0.217 ± 0.06
	14	4.97	4.69	4.44			
	30	4.85	4.88	4.35			

PDI: Polydispersity Index. Values are given as mean ± SD

Dynamic Light Scattering (DLS)

DLS was used to detect fluctuations in scattering intensity due to the Brownian motion of particles in suspension [43]. The droplet sizes of the nanoemulsions with different oil and surfactant ratios (M1-M5) ranged from 77.7 ± 0.3 nm to 216.3 ± 2.3 nm (Table 2). Formulation M5, with the highest Tween 80 concentration, exhibited the smallest particle size (77.7 ± 0.3 nm), while M1, with the lowest Tween 80 concentration, showed the largest particle size (216.3 ± 2.3 nm). Higher surfactant concentrations were associated with smaller droplet sizes, enhancing stability [42]. Polydispersity Index (PDI), another key indicator of stability, ranged from 0.217 ± 0.06 (M5) to 0.279 ± 0.01, indicating mid-range uniformity in particle size distribution [44,45]. Zeta potential values, which measure the surface charge and influence long-term stability, ranged from -0.92 ± 0.27 mV to -2.09 ± 0.38 mV, suggesting relatively low electrostatic stabilization [46]. As shown in Table 1, the formulations with higher concentrations of Tween 80 (e.g., M5) exhibited smaller particle sizes than those with lower concentrations (e.g., M1). This can be attributed to the role of surfactants in reducing interfacial tension, thus leading to smaller, more stable nanoemulsion droplets (Figure 1). Dynamic Light Scattering (DLS) results (Table 2) further confirm that higher surfactant levels lead to smaller particle sizes and a more uniform distribution, as indicated by lower polydispersity index (PDI) values. Although higher zeta potential values typically indicate greater stability, the nanoemulsions in this study maintained stability through careful optimization of formulation parameters, such as surfactant type and stirring speed [47,48]. As presented in Table 2, the zeta potential was also influenced by the concentration of Tween 80. Formulations with higher surfactant levels (e.g., M5) showed more negative zeta potential values, indicating greater stability due to increased surface charge repulsion[49]. This is consistent with the expectation that higher surfactant concentrations enhance the electrostatic stabilization of nanoemulsions. These findings emphasize that stability in nanoemulsions depends on multiple factors, including particle size, PDI, and zeta potential, with surfactant concentration playing a critical role.

Fourier Transform Infrared Spectroscopy

The FTIR spectrum identifies the vibrational characteristics of functional groups in the components and the nanoemulsion. In pure Commiphora myrrha (CM), absorption peaks at 1623 cm^{-1} and 3298 cm^{-1} correspond to (C-H) and (O-H) groups of phenols (Figure 2). For Virgin Coconut Oil (VCO) and Glyceryl Monooleate (GMO), dual peaks at 2925 cm^{-1} and 2851 cm^{-1} represent asymmetric and symmetric (C-H) stretching, characteristic of fatty acids like palmitic, lauric, and stearic acids [47]. Additionally, absorption peaks at 1728 cm^{-1}, 1169 cm^{-1}, and 1103 cm^{-1} indicate carbonyl esters and fatty acid stretching. Tween 80 exhibited characteristic absorption peaks at 2854 cm^{-1}, 1733 cm^{-1}, and 1090 cm^{-1}, corresponding to (C-H), (C=O), and (C-O) stretching, respectively [45]. The FTIR spectra of the CM-nanoemulsion formulations (containing ginger, white tea, Tween 80, VCO, and GMO) showed prominent peaks corresponding to these ingredients, indicating the successful incorporation of active groups into the nanoemulsion particles. The spectra closely matched that of pure myrrh, confirming the stability and integrity of the formulation [48]. After characterization, formulations M1 and M2 were selected to evaluate their antioxidant and cytotoxic properties further.

Stability studies

The pH value is crucial for the stability of nanoemulsions. At Day 0, the pH values of all formulations ranged from 5.02 to 5.37. A gradual decrease in pH was observed from Day 14 to Day 30, with formulations stored at 40°C showing a more significant decline than those stored at 4°C (Table 2). Changes in ionic strength can impact the stability of nanoemulsions by altering electrostatic attraction between particles and lowering interfacial tension [50]. The reduction in pH, especially with coconut oil, may be due to the hydrolysis of fatty acid esters [51]. The observed pH decrease over time may be attributed to the hydrolysis of fatty acids in VCO or the degradation of other components. To address this issue, incorporating buffering agents such as phosphate or citrate buffer could help maintain a stable pH throughout the storage period. Additionally, adjusting the concentration of antioxidants and preservatives may reduce oxidative degradation and improve pH stability[52]. These strategies will be investigated in future formulation optimizations to enhance long-term stability.

Anti-oxidant studies

DPPH Assay

The radical scavenging activity (RSA) of *Commiphora myrrha* (CM), M1, ginger, and white tea extracts was expressed as IC50 values relative to Trolox. The ginger extract exhibited the highest RSA compared to CM and white tea. The RSA for ginger at concentrations of 0.0625 to 2 mg/mL ranged from 77.5% to

96.8%, while for CM, it ranged from 76.9% to 77.7%, and for white tea, from 76.0% to 78.6% (Figure 3a & b). For the formulations, the RSA of M1 ranged from 15.0% to 62.0% and M2 from 4.0% to 49.4% across the same concentration range (Figure 4). Both formulations showed a concentration-dependent increase in RSA. Pure myrrh extract, white tea, and ginger exhibited strong antioxidant activity, with IC50 values of 0.071 ± 0.003, 0.073 ± 0.004, and 0.066 ± 0.004 mg/mL, respectively, compared to Trolox (IC50 = 0.030 ± 0.008 mg/mL). The IC50 values for M1 and M2 were 1.030 ± 0.901 and 0.495 ± 0.496 mg/mL, respectively (Table 3). The DPPH assay results indicated lower antioxidant activity for M1 and M2, likely due to the low encapsulation efficiency of CM [53].

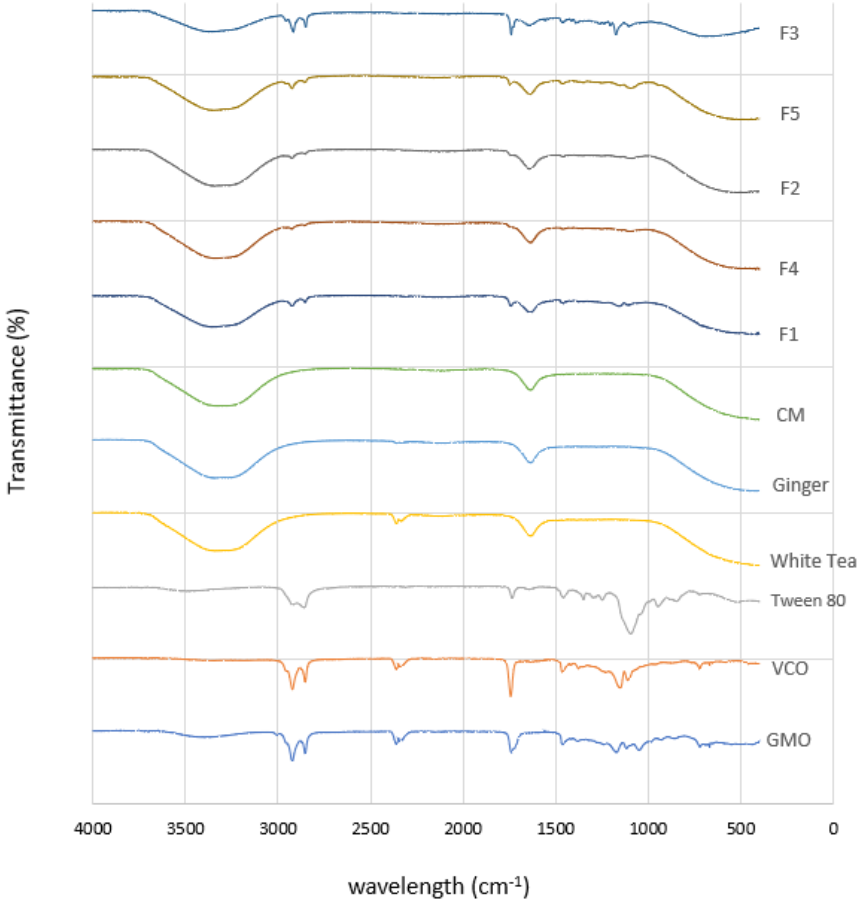

Figure 2: FTIR Spectra obtained for formulation and individual components. GMO: Glyceryl Monooleate, VCO: Virgin Coconut Oil, CM: *Commiphora Myrrha*

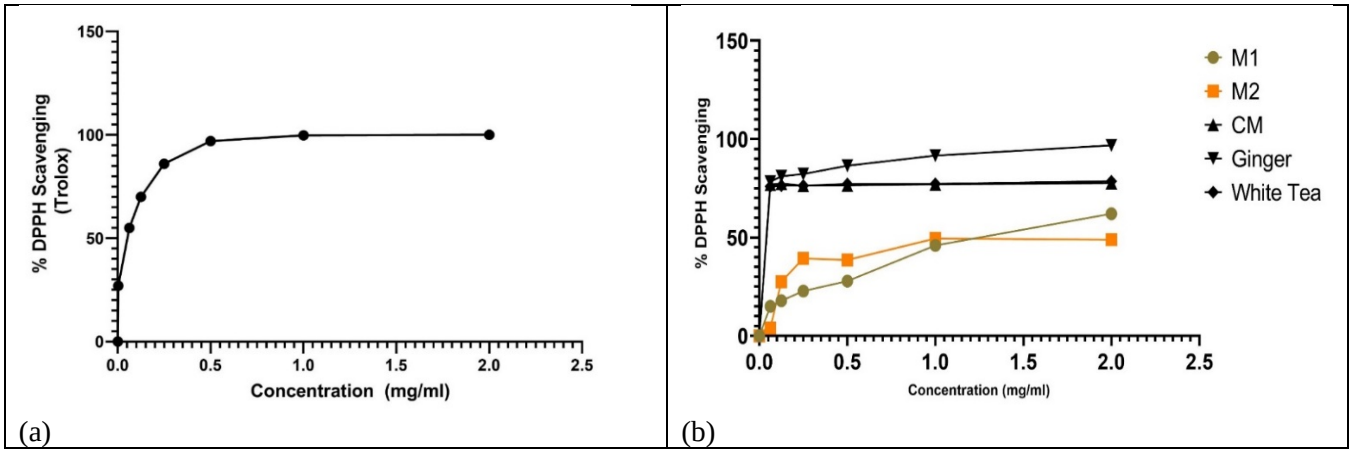

Figure 3: Scavenging Activity of DPPH Radicals by (a) Trolox Standard and (b) Nanoemulsion Mouthwash Containing CM, Ginger, and White Tea. Inhibition of DPPH free radicals by Trolox (standard) at different concentrations

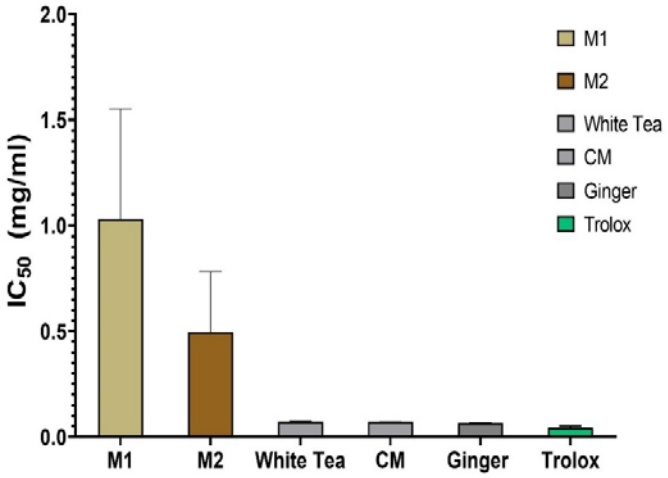

Figure 4: IC_{50} values of Mouthwash formulations, herbal ingredients and control M1 & M2 $p < 0.05$

Table 3: IC_{50} values of Mouthwash Formulations and Herbal components

Formulation code	DPPH Radical Scavenging Activity IC_{50} (mg/ml)
Trolox	0.030 ± 0.008**
M1	1.030 ± 0.901*
M2	0.495 ± 0.496*
CM	0.071 ± 0.003*
Ginger	0.066 ± 0.004*
White Tea	0.073 ± 0.004

CM: *Commiphora* Myrrha. Values are given as mean ± SD; *$p < 0.05$; **$p > 0.05$

The results of our polyherbal mouthwash formulations, particularly in terms of antioxidant activity and cytotoxicity, align with findings from other studies involving herbal ingredients like Commiphora myrrha (CM) and related polyherbal systems. For example, studies have shown that CM possesses significant antioxidant and antimicrobial properties, making it a common ingredient in formulations aimed at oral health care. Like our study, Badran et al. demonstrated that nanoemulsions incorporating herbal components, including CM, exhibited strong antioxidant activity with IC50 values comparable to synthetic antioxidants such as Trolox [54].

This supports our findings where CM-based formulations exhibited good antioxidant potential, although further optimization is needed to enhance encapsulation efficiency and stability. In addition, comparisons can be drawn with studies on other herbal mouthwash formulations. For instance, a study by Anand et al. on polyherbal formulations containing eucalyptus oil and other essential oils demonstrated comparable antioxidant activity and stability when using optimized surfactant concentrations [55]. Like our findings, using surfactants such as Tween 80 was crucial for maintaining stability and ensuring efficient delivery of the active herbal components. These studies reinforce the potential of herbal ingredients like CM in oral formulations and highlight the importance of optimizing formulation parameters to improve efficacy.

The lower encapsulation efficiency of Commiphora myrrha (CM) in formulations M1 and M2 could be attributed to several factors. First, the inherent solubility characteristics of CM resin and its complex chemical composition may have impacted its incorporation into the nanoemulsion system [56]. CM contains a mixture of hydrophobic and hydrophilic components, and the hydrophobic resin may have exhibited partial incompatibility with the surfactant system used (Tween 80 and GMO), leading to reduced encapsulation [57]. Additionally, the high surfactant concentration necessary to stabilize the formulation may have interfered with the efficient loading of CM, as excess surfactant can sometimes hinder the formation of stable drug-surfactant complexes [58].

These factors could explain the lower antioxidant activity (higher IC50 values) observed in M1 and M2. Future studies will explore modifications to the surfactant-to-oil ratio and alternative encapsulation techniques to improve the loading efficiency of CM. To overcome this limitation, one approach would be to optimize further the concentration of surfactants, such as Tween 80 and Glyceryl Monooleate (GMO), to improve the emulsification process and enhance the incorporation of CM into the nanoemulsion system. Increasing the surfactant concentration can reduce interfacial tension and promote better encapsulation of the hydrophobic components[59]. However, care must be taken to avoid excessive surfactant levels, which could lead to destabilization or cytotoxic effects.

In-vitro cytotoxicity study

Cell optimization

The absorbance values for cells with cell densities of 1.0, 2.0, 3.0, and 4.0 × 10^5 cells were 0.382, 1.071, 1.159, and 1.202, respectively. With increasing cell density, an increase in absorbance at 570 nm was observed (Figure 5 & Table 4).

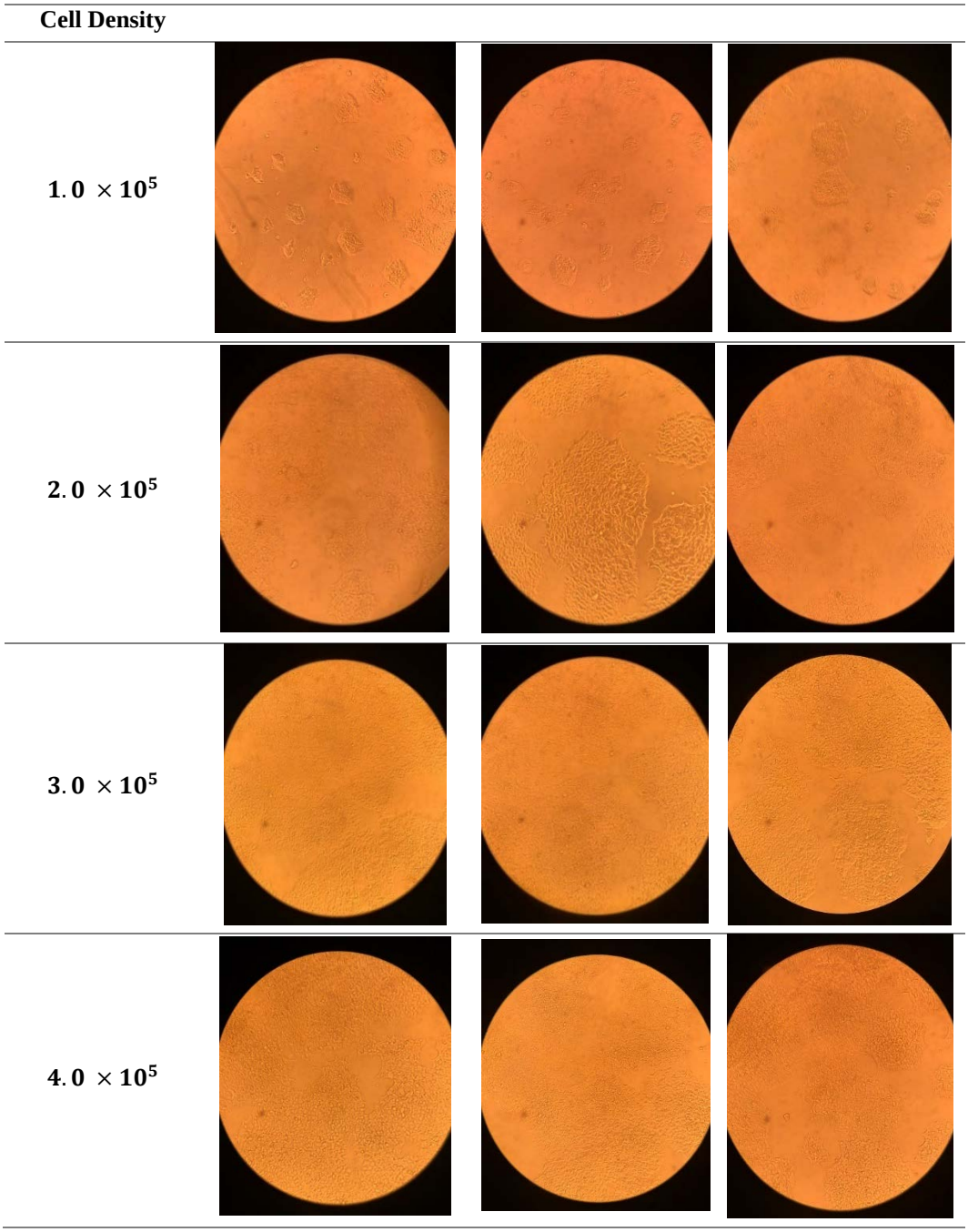

Figure 5: Cell Optimization replicates at different cell densities

Table 4: Absorbance values of different cell density in cell optimization

Cell density	Absorbance at 570nm			
	1	2	3	Mean
1.0×10^5	0.432	0.378	0.335	0.382*
2.0×10^5	1.133	0.855	1.225	1.071*
3.0×10^5	0.983	1.013	1.481	1.159*
4.0×10^5	1.102	1.069	1.436	1.202*

; *$p < 0.05$; **$p > 0.05$

In-Vitro Cytotoxicity Of CM-Polyherbal Nanoemulsion Using MTT Assay

The mean absorbance values for the negative control (distilled water) and positive control (5FU) were 0.992 ± 0.184 and 0.710 ± 0.128, respectively. The absorbance readings for the M1 and M2 formulations at a concentration of 0.002 mg/mL were 1.426 ± 0.06 and 1.88 ± 0.208, respectively. The %CV for M1 at 0.002 mg/mL was calculated as 96.1%, and for M2, it was 133.3% (Figure 6a & b).

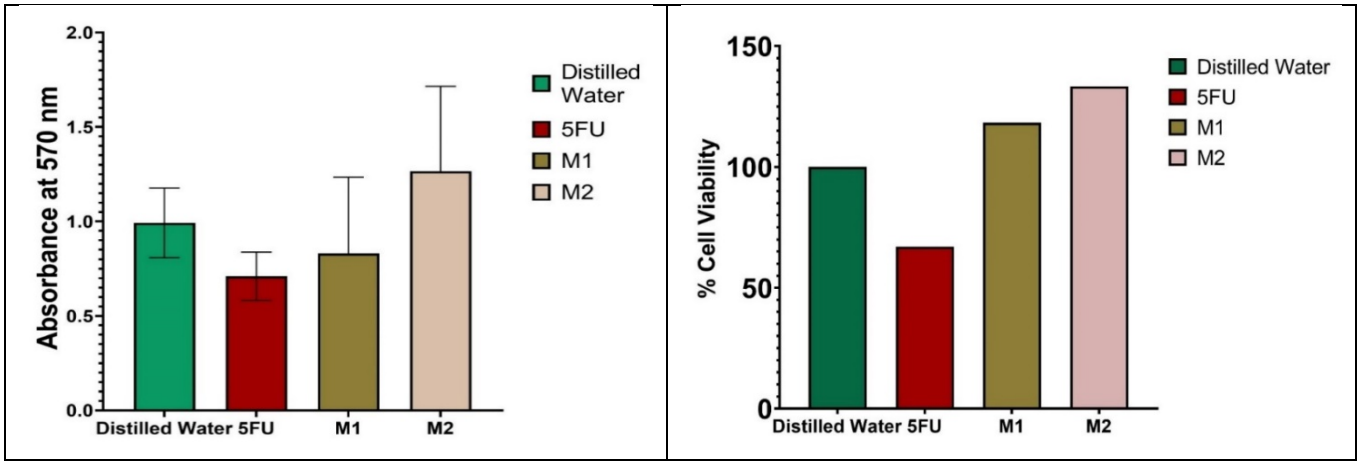

Figure 6: (a) Absorbance values at 570nm of controls and mouthwash formulation (b) Cell Viability (%) of the controls and mouthwash formulations; M1* & M2*- $p < 0.05$.

The optimization of cell number per well in a 96-well plate was performed before the experiment. It was observed that absorbance values increased with higher cell density, ensuring the correct dilution ratio for each cell number. In this study, a cell density of 2.0×10^5 cells per well, which yielded an absorbance reading of 1.071, was selected to evaluate the cytotoxic activity of the mouthwash formulations. This fell within the optimal absorbance range of 0.7 to 1.25, allowing for accurate measurement of both cell proliferation and inhibition of cell death [60]. Distilled water, with an absorbance value of 0.992 and 100% cell viability, was used as the negative control to assess the cytotoxic effects of M1 and M2 formulations. However, both formulations exhibited high standard deviation (SD) values of 0.06 and 0.208, respectively, indicating variability in absorbance within replicates. This inconsistency compromised the accuracy of the cytotoxicity evaluation. Additionally, contrary to expected cytotoxic properties, M1 and M2 formulations at the lowest concentration of 0.002 mg/mL demonstrated an increase in overall cell viability, with 96.1% and 133.3% viable cells, respectively (Figure 3b).

The low absorbance readings and variations in results may be attributed to suboptimal experimental conditions, such as improper cell culture conditions (e.g., temperature, humidity, CO_2 levels, and light exposure) and non-confluent cell suspensions. Uneven cell distribution due to inconsistent mixing before plating could also contribute to low cell counts in certain wells, leading to decreased cell viability. Based on these MTT assay results, it was challenging to accurately assess the cytotoxic effects of both M1 and M2 formulations on HEK293 cells due to ineffective control results. Increasing the number of experimental repeats and improving cell plating techniques in future studies may enhance the accuracy and reliability of the results. Reducing significant variations in %CV between replicates could also be achieved by optimizing cell plating procedures [61,62]. The variability observed in the cytotoxicity assay (Table 3, Figures 5-6) could partially be linked to differences in the surfactant concentration affecting cell interaction with the formulations. Formulations with higher concentrations of Tween 80, such as M5, may have shown less cytotoxicity due to better stabilization of the particles, reducing their potential to interact negatively with cell membranes. This aspect will be further optimized in future studies to reduce variability.

The observed variability in cell viability during the cytotoxicity assays, as indicated by high standard deviations in the MTT assay results, can be attributed to several factors. First, the nanoemulsion formulations might have experienced uneven distribution in the cell culture medium. Due to the hydrophobic nature of some components (such as Virgin Coconut Oil and Commiphora myrrha), non-uniform dispersion could lead to variable exposure of cells to the active ingredients, affecting the reproducibility of results [63]. Surfactants like Tween 80, while necessary for stabilization, may also impact the interaction between the nanoparticles and the cell membranes, influencing cytotoxic responses differently depending on the concentration [64]. Another reason for the variability could be the sensitivity of the Human Embryonic Kidney 293 (HEK293) cells used in the study. These cells may respond differently to slight

nanoparticle size or surfactant concentration variations, leading to inconsistent cell viability results [61]. Factors such as differences in cell confluency at the time of treatment, pipetting errors during the addition of nanoemulsions, and suboptimal culture conditions (e.g., temperature or CO_2 levels) could also introduce variability in cytotoxicity outcomes [60].

CONCLUSION

A *Commiphora myrrha*-based polyherbal nanoemulsion containing ginger and white tea extract was successfully prepared using the inversion emulsion method. The formulations were characterized based on organoleptic properties, particle size, and zeta potential. The particle sizes ranged from 77 to 216 nm, and compatibility studies via FTIR confirmed no interactions between herbal ingredients and surfactants, supporting the stability of the formulations. Antioxidant studies showed relatively low radical scavenging activity for the nanoemulsions, likely due to the lower concentration of *Commiphora myrrha*. The MTT cytotoxicity assay was inconclusive due to unoptimized experimental conditions, which may have reduced cell viability and inconsistent results. Future studies should focus on increasing the concentration of *Commiphora myrrha* to enhance its antioxidant properties. Additionally, optimizing the experimental conditions, particularly for the MTT assay, is critical to evaluate the cytotoxic potential of the formulations accurately. Improvements such as ensuring proper cell culture conditions, adjusting surfactant levels, and refining the concentration of active components will enhance the reliability and efficacy of future studies. Future research should also consider enhancing cytotoxicity assessment techniques by increasing the number of experimental replicates, exploring alternative cell lines, and using complementary cytotoxicity assays to ensure consistent and reproducible results. Moreover, increasing the concentration of *Commiphora myrrha*, exploring the antibacterial properties, and conducting in vivo testing could provide deeper insights into the therapeutic potential of these nanoemulsions for oral health and other therapeutic applications.

FINANCIAL ASSISTANCE

The authors would like to acknowledge Universiti Brunei Darussalam, Brunei Darussalam, for the funding (UBD/RSCH/1.6/FICBF(b)/2021/013).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Rajan R conceptualized and designed the research and experimental work, including the formulation development and optimization of the nanoemulsion. Sheba R. David contributed significantly to supervising the project and provided critical insights into the methodology and data interpretation. Norafiqah Y., Sahira Suhaimin, Nuramalina H. M., and Liyana Ahmad played essential roles in data collection, analysis, and interpretation, ensuring the accuracy and reliability of the experimental results.

Rajan R., Sheba R. David, and Nuramalina H. M. contributed to the manuscript, including drafting and revising the content for intellectual clarity and scientific accuracy. All authors have reviewed and approved the final version of the manuscript, demonstrating their collective effort and expertise in advancing the research on Commiphora myrrha-based polyherbal nanoemulsion mouthwash.

REFERENCES

[1] Könönen E, Gursoy M, Gursoy U. Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues. *Journal of Clinical Medicine*, **8**, 1135 (2019) https://doi.org/10.3390/jcm8081135.

[2] Gustafsson A, Skogsberg J, Rejnö Å. Oral health plays second fiddle in palliative care: an interview study with registered nurses in home healthcare. *BMC Palliative Care*, **20**, 173 (2021) https://doi.org/10.1186/s12904-021-00859-3.

[3] Saini R, Marawar P, Shete S, Saini S, Mani A. Dental expression and role in palliative treatment. *Indian journal of palliative care*, **15**, 26–9 (2009) https://doi.org/10.4103/0973-1075.53508.

[4] James P, Parnell C, Harding M, Whelton H, Worthington H V, Beirne P V. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. *Cochrane Database of Systematic Reviews*, (2010) https://doi.org/10.1002/14651858.cd008676.

[5] Van der Weijden FA, Van der Sluijs E, Ciancio SG, Slot DE. Can Chemical Mouthwash Agents Achieve Plaque/Gingivitis Control? *Dental Clinics of North America*, **59**, 799–829 (2015) https://doi.org/10.1016/j.cden.2015.06.002.

[6] Bescos R, Ashworth A, Cutler C, Brookes ZL, Belfield L, Rodiles A, Casas-Agustench P, Farnham G, Liddle L, Burleigh M, White D, Easton C, Hickson M. Effects of Chlorhexidine mouthwash on the oral microbiome. *Scientific Reports*, **10**, 1–8 (2020) https://doi.org/10.1038/s41598-020-61912-4.

[7] Sanz M, Beighton D, Ma C, Cury J, Dige I, Dommisch H, Ellwood R, Sanz M, Beighton D, Curtis MA, Cury JA, Dige I, Dommisch H, Ellwood R, Giacaman R, Herrera D, Herzberg MC. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases .

Consensus report of group 1 of the Joint EFP / ORCA workshop on the boundaries between caries and periodontal disease. **44**, (2017) https://doi.org/10.1111/jcpe.12682.

[8] Tribble GD, Angelov N, Weltman R, Wang BY, Eswaran S V., Gay IC, Parthasarathy K, Dao DH V., Richardson KN, Ismail NM, Sharina IG, Hyde ER, Ajami NJ, Petrosino JF, Bryan NS. Frequency of tongue cleaning impacts the human tongue microbiome composition and enterosalivary circulation of nitrate. *Frontiers in Cellular and Infection Microbiology*, **9**, (2019) https://doi.org/10.3389/fcimb.2019.00039.

[9] Seethalakshmi C, Jagat Reddy RC, Asifa N, Prabhu S. Correlation of salivary pH, incidence of dental caries and periodontal status in diabetes mellitus patients: A cross-sectional study. *Journal of Clinical and Diagnostic Research*, **10**, ZC12–4 (2016) https://doi.org/10.7860/JCDR/2016/16310.7351

[10] Dakhale R, Paul P, Achanta A, Ahuja KP, Meshram M. Nanotechnology Innovations Transforming Oral Health Care and Dentistry: A Review. *Cureus*, (2023) https://doi.org/10.7759/cureus.46423.

[11] Orabi SH, Al-Sabbagh ES, Khalifa HK, Mohamed MAEG, Elhamouly M, Gad-Allah SM, Abdel-Daim MM, Abd Eldaim MA. Commiphora myrrha Resin alcoholic extract ameliorates high fat diet induced obesity via regulation of UCP1 and adiponectin proteins expression in rats. *Nutrients*, **12**, 1–19 (2020) https://doi.org/10.3390/nu12030803.

[12] Heinz Brieskorn C, Noble P. Furanosesquiterpenes from the essential oil of myrrh. *Phytochemistry*, **22**, 1207–11 (1983) https://doi.org/10.1016/0031-9422(83)80223-4.

[13] Ahmad A, Raish M, Ganaie MA, Ahmad SR, Mohsin K, Al-Jenoobi FI, Al-Mohizea AM, Alkharfy KM. Hepatoprotective effect of Commiphora myrrha against D-GalN/LPS-induced hepatic injury in a rat model through attenuation of pro inflammatory cytokines and related genes. *Pharmaceutical Biology*, **53**, 1759–67 (2015) https://doi.org/10.3109/13880209.2015.1005754.

[14] Abdul-Ghani RA, Loutfy N, Hassan A. Myrrh and trematodoses in Egypt: An overview of safety, efficacy and effectiveness profiles. *Parasitology International*, **58**, 210–4 (2009) https://doi.org/10.1016/j.parint.2009.04.006.

[15] Al-Mobeeriek A. Effects of myrrh on intra-oral mucosal wounds compared with tetracycline- and chlorhexidinebased mouthwashes. *Clinical, Cosmetic and Investigational Dentistry*, **3**, 53–8 (2011) https://doi.org/10.2147/cciden.s24064.

[16] Eid RA Al. Efficacy of Commiphora myrrh mouthwash on early wound healing after tooth extraction: A randomized controlled trial. *The Saudi Dental Journal*, **33**, 44–54 (2021) https://doi.org/10.1016/j.sdentj.2019.11.011.

[17] Batiha GE-S, Wasef L, Teibo JO, Shaheen HM, Zakariya AM, Akinfe OA, Teibo TKA, Al-kuraishy HM, Al-Garbee AI, Alexiou A, Papadakis M. Commiphora myrrh: a phytochemical and pharmacological update. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **396**, 405–20 (2023) https://doi.org/10.1007/s00210-022-02325-0.

[18] Ide R, Hoshuyama T, Wilson D, Takahashi K, Higashi T. Periodontal Disease and Incident Diabetes. *Journal of Dental Research*, **90**, 41–6 (2011) https://doi.org/10.1177/0022034510381902.

[19] Abuajah CI, Ogbonna AC, Osuji CM. Functional components and medicinal properties of food: a review. *Journal of Food Science and Technology*, **52**, 2522–9 (2015) https://doi.org/10.1007/s13197-014-1396-5.

[20] Shidfar F, Rajab A, Rahideh T, Khandouzi N, Hosseini S, Shidfar S. The effect of ginger (Zingiber officinale) on glycemic markers in patients with type 2 diabetes. *Journal of Complementary and Integrative Medicine*, **12**, (2015) https://doi.org/10.1515/jcim-2014-0021.

[21] Mazidi M, Gao H-K, Rezaie P, Ferns GA. The effect of ginger supplementation on serum C-reactive protein, lipid profile and glycaemia: a systematic review and meta-analysis. *Food & Nutrition Research*, **60**, 32613 (2016) https://doi.org/10.3402/fnr.v60.32613.

[22] Hajiaghaalipour F, Kanthimathi MS, Sanusi J, Rajarajeswaran J. White tea (Camellia sinensis) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage. *Food Chemistry*, **169**, 401–10 (2015) https://doi.org/10.1016/j.foodchem.2014.07.005.

[23] Zhao L, La VD, Grenier D. Antibacterial, Antiadherence, Antiprotease, and Anti-Inflammatory Activities of Various Tea Extracts: Potential Benefits for Periodontal Diseases. *Journal of Medicinal Food*, **16**, 428–36 (2013) https://doi.org/10.1089/jmf.2012.0207.

[24] Almajano MP, Carbó R, Jiménez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry*, **108**, 55–63 (2008) https://doi.org/10.1016/j.foodchem.2007.10.040.

[25] Preeti, Sambhakar S, Malik R, Bhatia S, Al Harrasi A, Rani C, Saharan R, Kumar S, Geeta, Sehrawat R. Nanoemulsion: An Emerging Novel Technology for Improving the Bioavailability of Drugs. *Scientifica*, **2023**, 1–25 (2023) https://doi.org/10.1155/2023/6640103.

[26] Abd El-Hack ME, El-Saadony MT, Saad AM, Salem HM, Ashry NM, Abo Ghanima MM, Shukry M, Swelum AA, Taha AE, El-Tahan AM, AbuQamar SF, El-Tarabily KA. Essential oils and their nanoemulsions as green alternatives to antibiotics in poultry nutrition: a comprehensive review. *Poultry Science*, **101**, 101584 (2022) https://doi.org/10.1016/j.psj.2021.101584.

[27] David SR, Rajabalaya R. Development and in vitro Evaluation of Nanoemulsion-based Polyherbal Hair Serum Formulations for Leave-in Hair Treatments. *Current Cosmetic Science*, **03**, 83–97

(2024) https://doi.org/10.2174/0126667797319847240910105239.

[28] Rajabalaya R, Leen G, Chellian J, Chakravarthi S, David S. Tolterodine Tartrate Proniosomal Gel Transdermal Delivery for Overactive Bladder. *Pharmaceutics*, **8**, 27 (2016) https://doi.org/10.3390/pharmaceutics8030027.

[29] Kemel K, Tfaili S, Tfayli A, Baillet-Guffroy A, Faivre V, Laugel C. ATR-FTIR Characterization of Janus Nanoparticles. Part I: Implementation of Spectroscopic Descriptors. *Journal of Pharmaceutical Sciences*, **107**, 2457–64 (2018) https://doi.org/10.1016/j.xphs.2018.04.022.

[30] David SR, Abdullah K, Shanmugam R, Thangavelu L, Das SK, Rajabalaya R. Green Synthesis, Characterization and In Vivo Evaluation of White Tea Silver Nanoparticles with 5-Fluorouracil on Colorectal Cancer. *BioNanoScience*, **11**, 1095–107 (2021) https://doi.org/10.1007/s12668-021-00905-7.

[31] Sheba R David, Nurul Akmar Binti Anwar, Koh Rhun Yian, Chun-Wai Mai, Sanjoy Kumar Das RR. Development and Evaluation of Curcumin Liquid Crystal Systems for Cervical Cancer. *Scientia Pharmaceutica*, **88**, 15 (2020) https://doi.org/10.3390/scipharm88010015.

[32] Seibert JB, Bautista-Silva JP, Amparo TR, Petit A, Pervier P, dos Santos Almeida JC, Azevedo MC, Silveira BM, Brandão GC, de Souza GHB, de Medeiros Teixeira LF, dos Santos ODH. Development of propolis nanoemulsion with antioxidant and antimicrobial activity for use as a potential natural preservative. *Food Chemistry*, **287**, 61–7 (2019) https://doi.org/10.1016/j.foodchem.2019.02.078.

[33] Morales G, Paredes A. Antioxidant activities of Lampaya medicinalis extracts and their main chemical constituents. *BMC Complementary and Alternative Medicine*, **14**, 1–12 (2014) https://doi.org/10.1186/1472-6882-14-259.

[34] Sheba R Nakka David, Miza S Mohammad, Lim Ya Chee RR. Is Sunflower Cooking Oil Beneficial for Colorectal Cancer? In Vivo Studies on Azoxymethane-induced Colon Cancer in Rats. *Current Nutrition & Food Science*, **18**, 329–36 (2022) https://doi.org/10.2174/1573401317666211208144625.

[35] Rajabalaya R, David SR, Chellian J, Xin Yun G, Chakravarthi S. Transdermal delivery of oxybutynin chloride proniosomal gels for the treatment of overactive bladder. *Drug delivery*, **23**, 1578–87 (2016) https://doi.org/10.3109/10717544.2015.1116027.

[36] Keshari R, Tharmatt A, Pillai MM, Chitkara D, Tayalia P, Banerjee R, Sen S, Srivastava R. Eugenol-Loaded Lipid Nanoparticles-Derived Hydrogels Ameliorate Psoriasis-like Skin Lesions by Lowering Oxidative Stress and Modulating Inflammation. *ACS Pharmacology & Translational Science*, **7**, 3592–606 (2024) https://doi.org/10.1021/acsptsci.4c00493.

[37] Yoon HJ, Zhang X, Kang MG, Kim GJ, Shin SY, Baek SH, Lee BN, Hong SJ, Kim JT, Hong K, Bae H. Cytotoxicity evaluation of turmeric extract incorporated oil-in-water nanoemulsion. *International Journal of Molecular Sciences*, **19**, 1–12 (2018) https://doi.org/10.3390/ijms19010280.

[38] David SR, Refai SA, Yian KR, Mai CW, Das SK, Rajabalaya R. Development and evaluation of liquid crystal systems of combination of 5-fluorouracil and curcumin for cervical cancer cell line. *Journal of Pharmacy and Pharmacognosy Research*, **7**, 441–53 (2019).

[39] Rajan Rajabalaya, Muhammad Nuh Musa, Nurolaini Kifli SRD. Oral and transdermal drug delivery systems: Role of lipid-based lyotropic liquid crystals. *Drug Design, Development and Therapy*, **11**, 393–406 (2017) https://doi.org/10.2147/DDDT.S103505.

[40] Musa MN, David SR, Zulkipli IN, Mahadi AH, Chakravarthi S, Rajabalaya R. Development and evaluation of exemestane-loaded lyotropic liquid crystalline gel formulations. *BioImpacts : BI*, **7**, 227–39 (2017) https://doi.org/10.15171/bi.2017.27.

[41] van Swaaij BWM, Slot DE, Van der Weijden GA, Timmerman MF, Ruben J. Fluoride, pH Value, and Titratable Acidity of Commercially Available Mouthwashes. *International Dental Journal*, **74**, 260–7 (2024) https://doi.org/10.1016/j.identj.2023.09.002.

[42] Prabhakar K, Afzal SM, Surender G, Kishan V. Tween 80 containing lipid nanoemulsions for delivery of indinavir to brain. *Acta Pharmaceutica Sinica B*, **3**, 345–53 (2013) https://doi.org/10.1016/j.apsb.2013.08.001.

[43] Osman Mohamed Ali E, Shakil NA, Rana VS, Sarkar DJ, Majumder S, Kaushik P, Singh BB, Kumar J. Antifungal activity of nano emulsions of neem and citronella oils against phytopathogenic fungi, Rhizoctonia solani and Sclerotium rolfsii. *Industrial Crops and Products*, **108**, 379–87 (2017) https://doi.org/10.1016/j.indcrop.2017.06.061.

[44] Clayton KN, Salameh JW, Wereley ST, Kinzer-Ursem TL. Physical characterization of nanoparticle size and surface modification using particle scattering diffusometry. *Biomicrofluidics*, **10**, 054107 (2016) https://doi.org/10.1063/1.4962992.

[45] Yakoubi S, Kobayashi I, Uemura K, Nakajima M, Isoda H, Ksouri R, Saidani-Tounsi M, Neves MA. Essential-Oil-Loaded Nanoemulsion Lipidic-Phase Optimization and Modeling by Response Surface Methodology (RSM): Enhancement of Their Antimicrobial Potential and Bioavailability in Nanoscale Food Delivery System. *Foods*, **10**, 3149 (2021) https://doi.org/10.3390/foods10123149.

[46] Laxmi M, Bhardwaj A, Mehta S, Mehta A. Development and characterization of nanoemulsion as carrier for the enhancement of bioavailability of artemether. *Artificial Cells, Nanomedicine and Biotechnology*, **43**, 334–44 (2015) https://doi.org/10.3109/21691401.2014.887018.

[47] McClements DJ. Emulsion Design to Improve the Delivery of Functional Lipophilic Components. *Annual Review of Food*

Science and Technology, **1**, 241–69 (2010) https://doi.org/10.1146/annurev.food.080708.100722.

[48] Ismail SH, Hamdy A, Ismail TA, Mahboub HH, Mahmoud WH, Daoush WM. Synthesis and Characterization of Antibacterial Carbopol/ZnO Hybrid Nanoparticles Gel. *Crystals*, **11**, 1092 (2021) https://doi.org/10.3390/cryst11091092.

[49] Jusril NA, Bakar SIA, Khalil KA, Saad WMM, Wen NK, Adenan MI. Development and Optimization of Nanoemulsion from Ethanolic Extract of Centella asiatica (NanoSECA) Using D-Optimal Mixture Design to Improve Blood-Brain Barrier Permeability. *Evidence-based Complementary and Alternative Medicine : eCAM*, **2022**, 1–18 (2022) https://doi.org/10.1155/2022/3483511.

[50] Che Marzuki NH, Wahab RA, Abdul Hamid M. An overview of nanoemulsion: concepts of development and cosmeceutical applications. *Biotechnology & Biotechnological Equipment*, **33**, 779–97 (2019) https://doi.org/10.1080/13102818.2019.1620124.

[51] Zhang X, Wei Z, Sun Y, Luo T, Xue C. Preparation of core–shell hordein/pectin nanoparticles as quercetin delivery matrices: Physicochemical properties and colon-specific release analyses. *Food Research International*, **170**, 112971 (2023) https://doi.org/10.1016/j.foodres.2023.112971.

[52] Aslam MS, Ahmad MS, Mamat AS, Ahmad MZ, Salam F. Antioxidant and Wound Healing Activity of Polyherbal Fractions of Clinacanthus nutans and Elephantopus scaber. *Evidence-Based Complementary and Alternative Medicine*, **2016**, 1–14 (2016) https://doi.org/10.1155/2016/4685246.

[53] Chandrasekaran N, Sugumar S, Mukherjee A. Eucalyptus oil nanoemulsion-impregnated chitosan film: antibacterial effects against a clinical pathogen, Staphylococcus aureus, in vitro. *International Journal of Nanomedicine*, 67 (2015) https://doi.org/10.2147/IJN.S79982.

[54] David SR, Lai PPN, Chellian J, Chakravarthi S, Rajabalaya R. Influence of rutin and its combination with metformin on vascular functions in type 1 diabetes. *Scientific Reports*, **13**, 12423 (2023) https://doi.org/10.1038/s41598-023-39442-6.

[55] Khan M, Liu S, Qi L, Ma C, Munir S, Yu L, Hu Q. Liquid crystal-based sensors for the detection of biomarkers at the aqueous/LC interface. *TrAC Trends in Analytical Chemistry*, **144**, 116434 (2021) https://doi.org/10.1016/j.trac.2021.116434.

[56] Pakdel E, Wang J, Kashi S, Sun L, Wang X. Advances in photocatalytic self-cleaning, superhydrophobic and electromagnetic interference shielding textile treatments. *Advances in Colloid and Interface Science*, **277**, 102116 (2020) https://doi.org/10.1016/j.cis.2020.102116.

[57] Adi Idris, Ihsan N Zulkipli, Nurul Ramizah Zulhilmi, Huan F Lee, Rajan Rajabalaya, Lim Y Chee, Mohamed Majid SRD. Melastoma malabathricum ethyl acetate fraction induces secondary necrosis in human breast and lung cancer cell lines. *Pharmacognosy Magazine*, **13**, 688–92 (2017) https://doi.org/10.4103/pm.pm_465_15.

[58] Das SK, Chakraborty S, Roy C, Rajabalaya R, Mohaimin AW, Khanam J, Nanda A, David SR. Ethosomes as Novel Vesicular Carrier: An Overview of the Principle, Preparation and its Applications. *Current Drug Delivery*, **15**, 795–817 (2018) https://doi.org/10.2174/1567201815666180116091604.

[59] Ghasemi M, Turnbull T, Sebastian S, Kempson I. The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International Journal of Molecular Sciences*, **22**, (2021) https://doi.org/10.3390/ijms222312827.

[60] Weyermann J, Lochmann D, Zimmer A. A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics*, **288**, 369–76 (2005) https://doi.org/10.1016/j.ijpharm.2004.09.018.

[61] Fotakis G, Timbrell JA. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, **160**, 171–7 (2006) https://doi.org/10.1016/j.toxlet.2005.07.001.

[62] Gupta A, Eral HB, Hatton TA, Doyle PS. Controlling and predicting droplet size of nanoemulsions: scaling relations with experimental validation. *Soft Matter*, **12**, 1452–8 (2016) https://doi.org/10.1039/C5SM02051D.

[63] Patil S, Sandberg A, Heckert E, Self W, Seal S. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials*, **28**, 4600–7 (2007) https://doi.org/10.1016/j.biomaterials.2007.07.029.

[64] Nielsen CK, Kjems J, Mygind T, Snabe T, Meyer RL. Effects of Tween 80 on Growth and Biofilm Formation in Laboratory Media. *Frontiers in Microbiology*, **7**, (2016) https://doi.org/10.3389/fmicb.2016.01878.