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Evaluation of the Physicochemical and Antimicrobial Properties of Nanoemulsion-Based Polyherbal Mouthwash

Nazrul Haq, Mudassar Shahid, Ahmed L. Alaofi, Zeeshan Heera Ahmad, Yasser F. Alrayyes, Ibrahim A. Alsarra, and Faiyaz Shakeel*

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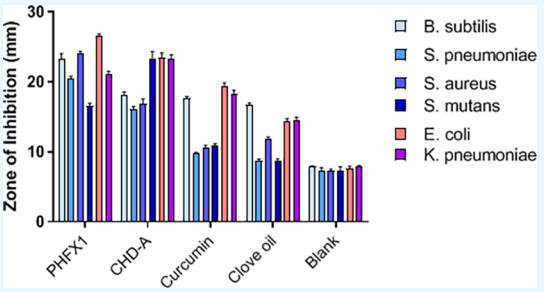
ABSTRACT: A nanoemulsion-based polyherbal mouthwash (PHFX) of *Curcuma longa* hydroalcoholic extract was developed and evaluated for its antibacterial effects against a variety of Gram-positive and Gram-negative oral pathogens in comparison to standard chlorhexidine acetate (CHD-A) (positive control). Various nanoemulsion-based mouthwashes of *C. longa* extract were produced using an aqueous phase titration approach via construction of pseudoternary phase diagrams. The developed nanoemulsion-based PHFX was studied for thermodynamic stability tests. Selected formulations (PHFX1−PHFX5) were characterized physicochemically for droplet diameter, polydispersity index (PDI), refractive index (RI), transmittance, and pH. The drug release studies were performed using the dialysis method. Based on the minimum droplet diameter (26.34 nm), least PDI (0.132), optimal RI (1.337), maximum %*T* (99.13), optimal pH (6.45), and maximum cumulative drug release (98.2%), formulation PHFX1 (containing 0.5% w/w of *C. longa* extract, 1.5% w/w of clove oil, 7.0% w/w of Tween-80, 7.0% w/w of Transcutol-HP, and 84.0% w/w of water) was selected for antimicrobial studies in comparison to standard CHD-A. The antibacterial effects and minimum inhibitory concentration were studied against various Gram-positive oral pathogens such as *Streptococcus mutans*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Bacillus subtilis* and Gram-negative oral pathogens such as *Escherichia coli* and *Klebsiella pneumoniae*. The antibacterial effects of PHFX1 were found to be significant over standard CHD-A against most Gram-positive and Gram-negative oral pathogens. The antimicrobial studies showed that the formulation PHFX1 was effective against all oral pathogens even at 3- to 4-fold lower working concentrations. These findings indicated the potential of nanoemulsion-based mouthwash in the treatment of a variety of oral pathogen infections.



1. INTRODUCTION

Gingivitis and periodontitis are the two main oral diseases.[1] The sixth most common disease in the world, severe periodontitis, had an age-standardized prevalence of 11.2% according to the Global Burden of Disease [GBD] (2010) survey.[2] The GBD (2017) study estimates that 796 million people worldwide are affected by periodontal disorders, primarily gingivitis and periodontitis.[3] Their most prevalent symptoms, which can be stopped by preventing plaque accumulation and routinely removing it, are gingival irritation and bleeding, foul breath, gingival recession, and teeth loosening.[4] Antiplaque agents often stop the development of plaque by their antimicrobial characteristics.[5] A broad-spectrum antibacterial agent with long-lasting effects, chlorhexidine acetate (CHD-A), adheres to oral substances.[6,7] Tooth discoloration, an increase in calculus development, taste disruption, and carcinogenic consequences are some of the drawbacks of CHD-A.[8]

Due to their effectiveness and lack of negative side effects, the usage of herbal medications has dramatically expanded recently. Numerous researchers from throughout the world have evaluated and endorsed the antibacterial and anti-inflammatory properties of various herbal medications.[9] Turmeric, known as "Indian saffron," has been in use for thousands of years in Indian medicine and cuisine as well as for religious purposes. The bright yellow-colored rhizome is a product of *Curcuma longa* from ginger (family: Zingiberaceae), native to tropical South Asia.[10] Together with essential oils and other curcuminoids, curcumin (CCM) is a principal bioactive compound of turmeric powder—an oriental spice commonly obtained from this plant.[10,11] CCM (CCM I, diferuloylmethane) is a dimeric derivative of ferulic acid, composed of two *o*-methoxyphenol rings connected by a heptadienedione

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chain (Figure 1). It has a chemical formula of $C_{21}H_{20}O_6$ and a molecular weight of 368.38 g/mol.

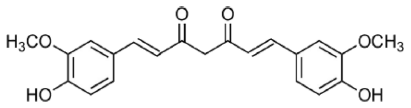

Figure 1. Chemical formula and structure of curcumin (CCM).

This naturally occurring yellow-orange pigment is a lipophilic polyphenol.[10] CCM has a wide range of beneficial features that can be used to treat a wide range of ailments, including analgesic, anti-inflammatory, antioxidant, antiseptic, and antibacterial.[12−17] In the treatment of gingivitis, the anti-inflammatory impact has been investigated, both as a topical application in the form of a gel or as a mouthwash.[12,13] The health-promoting effects of CCM, which are principally attributed to its potent antioxidant and anti-inflammatory properties, have been proven in numerous *in vitro* and *in vivo* investigations.[14−16] In patients with inflammatory diseases (arthritis, inflammatory bowel disease, peptic ulcer, and *Helicobacter pylori* infection), metabolic syndrome, neurodegenerative diseases, and cancer, including colorectal, pancreatic, and breast cancers, clinical trials have shown the therapeutic benefits of CCM supplementation.[17−20] Researchers have been very interested in CCM because of its diverse spectrum of biological activities and its pleiotropic therapeutic potential. Due to its weak water solubility and low bioavailability, CCM has only found limited use in formulation development.[21−23]

Clove (*Syzygium aromaticum*) is a herb whose essential oil is most widely used in food seasoning.[24] Its antimicrobial capacity was identified when many Gram-positive and Gram-negative species including certain fungi were killed by its essential oil extracts.[24,25] Clove essential oil's (CEO) potent biological and antibacterial properties are due to the high amounts of eugenol it contains.[24] It is widely known that phenolic compounds like eugenol and CEO may denature proteins, interact with phospholipids in cell membranes to change how permeable they are, and inhibit a wide range of Gram-negative and Gram-positive bacteria as well as many yeast species.[24−26]

Different colloidal systems have been developed to address the issues of poor solubility.[27] To increase the bioavailability and solubility of bioactive chemicals, numerous researchers have investigated drug delivery systems based on nanoemulsions and nanomedicine.[28] Nanoemulsions are thermodynamically stable, transparent/translucent, and isotropic dispersions of oil and water stabilized by an interfacial film of surfactant/cosurfactant molecules having a droplet size a less than 100 nm.[29,30] Due to their improved physical stability against droplet aggregation and phase separation, enhanced optical clarity, and unique rheological features, nanoemulsions have been proposed to offer a number of advantages over conventional emulsions.[31−33] The potential of nanoemulsions in enhancing therapeutic effects, solubility/dissolution, and bioavailability of numerous essential oils and herbal medicines such as CEO, *Eucalyptus* oil, *Piper cubeba* oil, niaouli essential oil, sinapic acid, vanillin, thymoquinone, apigenin, and luteolin has been studied well in the literature.[34−43] Recently, numerous nanomedicine-based drug delivery systems of CCM such as nanomicelles,[44,45] nanocellulose-based drug delivery systems,[46] niosomes,[47] nanoliposomes,[48] folic acid receptor-targeted nanoparticles (NPs),[49] chitosan-ZnO NPs,[50] mesoporous silica NPs,[51] PEGylated mesoporous silica NPs,[52] and self-nanoemulsifying drug delivery systems (SNEDDS)[53] have been reported to overcome the CCM solubility problems

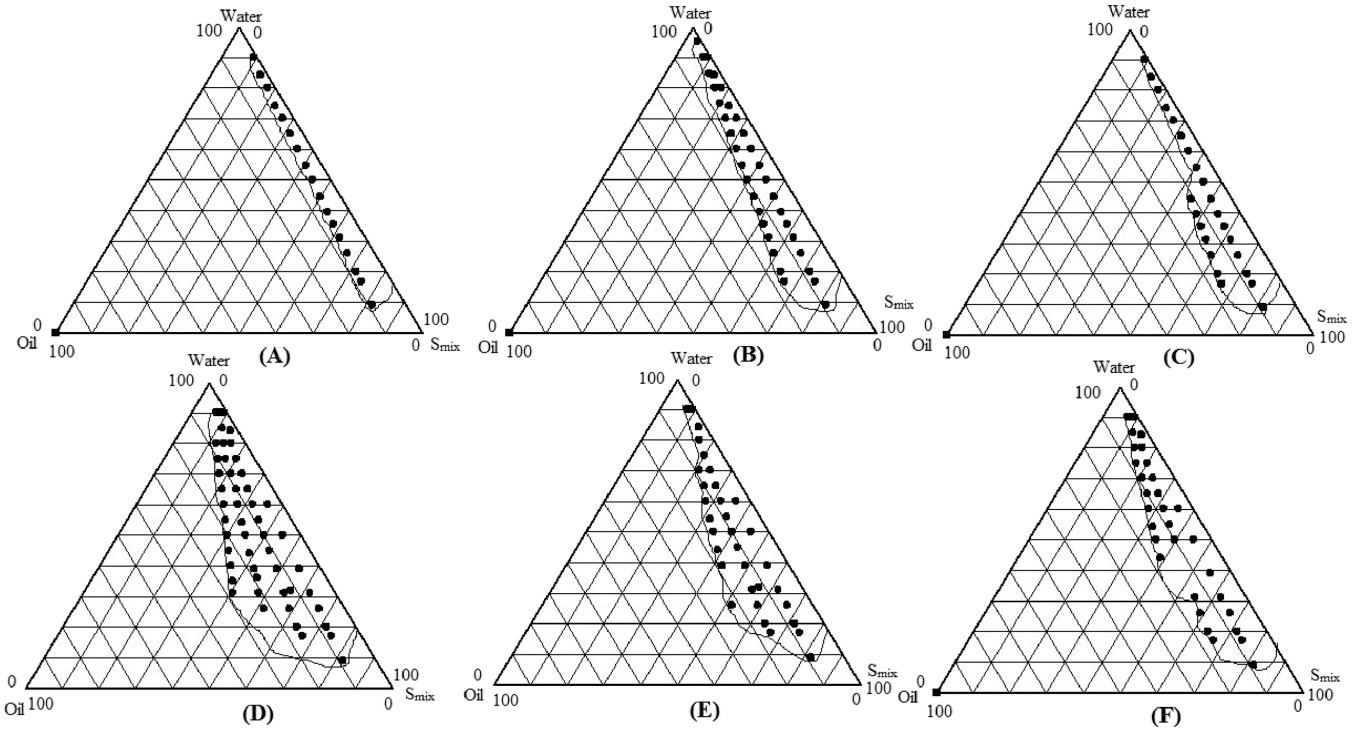

Figure 2. pseudoternary phase diagrams illustrating the nanoemulsion zones for the CEO as the oil phase, water as the aqueous phase, Tween-80 as the surfactant, and Transcutol-HP as the cosurfactant for S_{mix} ratios of (A) 1:0, (B) 1:2, (C) 1:3, (D) 1:1, (E) 2:1, and (F) 3:1.

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and to enhance its drug targeting efficiency and therapeutic effects.

So far, nanoemulsion-based mouth rinse (PHFX) containing *C. longa* extract and CEO has not been explored as an effective measure against the microbes of the oral cavity. In addition, the amount of *C. longa* extract dissolved in this formulation without the use of alcohol has not been achieved so far. The demand for new and safe substitutes has increased as a result of drug resistance and the negative effects associated with the usage of synthetic medications. The purpose of this study was to assess the effectiveness of *C. longa* extract in the suppression of oral microbiota growth utilizing nanoemulsion-based PHFX, taking into account the need for new therapeutic mouthwashes and the advantages of natural care products for both oral and general health. In this way, there is a chance to discover a brand-new material for use in dental hygiene that could help prevent oral bacteria.

2. RESULTS AND DISCUSSION

2.1. Preparation of *C. longa* Extract and Determination of CCM. The hydroalcoholic extract of *C. longa* was successfully produced. CCM, a significant biomarker, was quantified in *C. longa* extract using a published high-performance liquid chromatography (HPLC) method at 425 nm.[11] The hydroalcoholic extract of *C. longa* contained 81.15 ± 0.64% CCM, according to the research. According to recognized literature,[54] the percentage of CCM in six different commercially available *C. longa* extracts ranged from 69.82 to 86.79%. The quantity of CCM in *C. longa* hydroalcoholic extract reported in this study was according to the literature.[54]

2.2. Preparation of Nanoemulsion-Based PHFX Mouthwash and Construction of Pseudoternary Phase Diagrams. A total of six phase diagrams (Figure 2A−F) was created, and each one featured various ratios of the aqueous phase, oil phase, and surfactant and cosurfactant (S_{mix}). The initial phase diagram (Figure 2A) with an S_{mix} ratio of 1:0 showed the least amount of nanoemulsion zones. Phase diagrams B (Figure 2B) and C (Figure 2C), which had S_{mix} ratios of 1:2 and 1:3, respectively, were next to it and similarly had small nanoemulsion zones. For phase diagrams D (Figure 2D), E (Figure 2E), and F (Figure 2F) with 1:1, 2:1, and 3:1 S_{mix} ratios, respectively, the enhanced nanoemulsion zones were recorded, but phase diagram D was superior to phase diagrams E and F with higher nanoemulsion zones. From these results, phase diagram D (Figure 2D) with a 1:1 S_{mix} ratio and the maximum nanoemulsion zones was chosen for the preparation of nanoemulsion-based PHFX mouthwash. After selecting the 1:1 S_{mix} ratio (Figure 2D), which gave the maximum nanoemulsion zones, five nanoemulsion-based PHFX mouthwashes (PHFX1−PHFX5) were produced. The formulations were created with a fixed minimum possible S_{mix} concentration of 14.0% w/w, a range of CEO (oil phase) concentrations of 1.5, 3.0, 4.5, 6.0, and 7.5% w/w, and 84.0, 82.5, 81.0, 79.5, and 78.0% w/w concentrations of deionized water (aqueous phase). The 0.5% (w/w) of *C. longa* hydroalcoholic extract was dissolved completely in CEO before the addition of Tween-80 and Transcutol-HP followed by titration with water. The composition of the nanoemulsion-based mouthwashes (PHFX1−PHFX5) is tabulated in Table 1.

2.3. Thermodynamic Stability Testing. The qualitative thermodynamic stability testing was performed. The tests revealed no phase separation (flocculation, coalescence, and phase inversion), drug precipitation, or loss of stability for any of the nanoemulsion-based PHFX mouthwashes (PHFX1−PHFX5). These results suggested that the produced formulations were thermodynamically stable.

Table 1. Composition of Nanoemulsion-Based PHFX Mouthwashes

	formulation composition (%w/w)				
code	C. longa extract	oil	S_{mix}	water	S_{mix} ratio
PHFX1	0.5	1.5	14.0	84.0	1:1
PHFX2	0.5	3.0	14.0	82.5	1:1
PHFX3	0.5	4.5	14.0	81.0	1:1
PHFX4	0.5	6.0	14.0	79.5	1:1
PHFX5	0.5	7.5	14.0	78.0	1:1

2.4. Physicochemical Characterization. Numerous physicochemical parameters of nanoemulsion-based mouthwashes (PHFX1−PHFX5) were determined, which includes the droplet diameter, polydispersity index (PDI), refractive index (RI), percent transmittance (%*T*), pH, and surface morphology. The findings for the physicochemical characterization are listed in Table 2. The droplet diameter of PHFX1−PHFX5 was ranged from 26.34 to 65.52 nm. All the formulations showed the droplet diameter in the nanometer range. As the percentage of CEO (oil phase) decreased, it was discovered that the droplet diameter of formulations decreased. The smallest mean droplet diameter was seen in formulation PHFX1 (26.34 ± 0.18 nm), which may have been caused by the formulation's low oil phase concentration but high solubilizing potential. The largest droplet diameter (65.52 ± 0.54 nm) was observed in the formulation PHFX5, which may have been caused by the presence of the high amount of oil phase.[32] The PDI of PHFX1−PHFX5 ranged from 0.132 to 0.181. The least PDI was 0.132 ± 0.021 in mouthwash PHFX1, and the maximum PDI was 0.181 ± 0.014 in mouthwash PHFX5. All formulations showed low values of PDI, suggesting uniformity in droplet diameter.[34] The RI of PHFX1−PHFX5 ranged from 1.337 to 1.344. The lowest RI was recorded in mouthwash PHFX1 (1.337 ± 0.001), and the highest one was found in mouthwash PHFX5 (1.344 ± 0.002). The RIs for all mouthwashes were relatively close to that of water (1.33), indicating the transparent nature and o/w type of nanoemulsions.[39] The %*T* of mouthwashes made with nanoemulsions was chosen to assess how well their clarity and transparency translated into their capacity to transmit light as opposed to absorb or block it.[38] The range of the PHFX1−PHFX5 %*T* was 95.89−99.13%. The lowest %*T* (95.89%) and highest %*T* (99.13%) were found in mouthwashes PHFX5 and PHFX1, respectively. These findings indicated the transparent behavior of nanoemulsion-based mouthwashes.[38] The pH range of PHFX1−PHFX5 was 6.45−6.60. The pH of each formulation was acidic and close to the pH of oral mucosa (pH = 6.30). The acidic pH range of each formulation was suitable for its delivery to oral mucosa.

The surface morphology and size of optimized formulation PHFX1 were evaluated by transmission electron microscopy (TEM). Figure 3 presents the TEM image of PHFX1. The droplets of PHFX1 were spherical in shape and distributed within a nanometer range. The droplet size of PHX1 measured by TEM was in accordance with those measured using the DLS scattering technique. The spherical shape of droplets was most probably due to the presence of Tween-80 and Transcutol-HP in PHFX1.

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Table 2. Physicochemical Parameters of Nanoemulsion-Based PHFX Mouthwashes (Mean ± SD, n = 3)[a]

	characterization parameter ± SD				
code	droplet diameter (nm)	PDI	RI	%T	pH
PHFX1	26.34 ± 0.18	0.132 ± 0.021	1.337 ± 0.001	99.13 ± 0.12	6.45 ± 0.06
PHFX2	35.28 ± 0.29	0.142 ± 0.024	1.339 ± 0.002	98.41 ± 0.13	6.50 ± 0.07
PHFX3	44.74 ± 0.35	0.147 ± 0.028	1.342 ± 0.002	97.03 ± 0.15	6.52 ± 0.08
PHFX4	52.13 ± 0.42	0.153 ± 0.034	1.343 ± 0.001	96.35 ± 0.14	6.55 ± 0.10
PHFX5	65.52 ± 0.54	0.181 ± 0.014	1.344 ± 0.002	95.89 ± 0.13	6.60 ± 0.11

[a]PDI: polydispersity index, RI: refractive index; %T: percentage of transmittance.

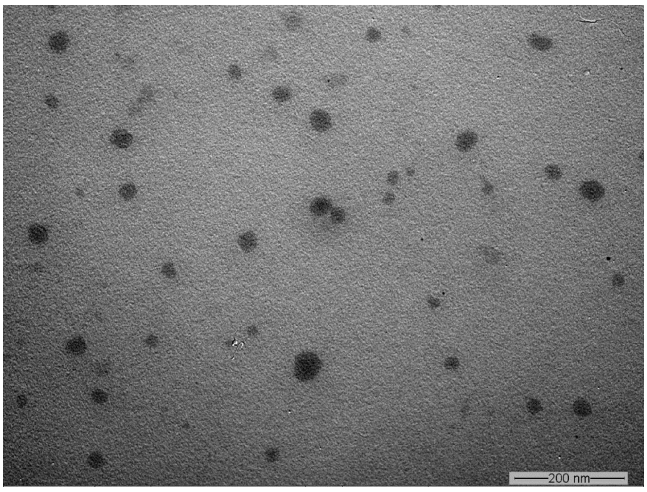

Figure 3. TEM image of optimized formulation PHFX1 presenting spherical-shaped droplets within the nanometer size range.

2.5. Assessment of Drug Release. Figure 4 displays the results of the drug release trials. The drug release profile from mouthwashes (PHFX1−PHFX5) based on nanoemulsions was a two-step release profile, with rapid release occurring in the first step and sustained release occurring in the second. However, the release profile of CCM from the *C. longa* extract was a slow release profile throughout the study. For the first 8 h of the study, all mouthwashes showed immediate drug release profiles. However, after 8 h of the study, all mouthwashes presented sustained release type profiles. After 8 h of the study, the cumulative drug releases for formulations PHFX1, PHFX2, PHFX3, PHFX4, and PHFX5 were 85.4, 82.1, 77.4, 71.3, and 65.2%, respectively. However, the release of CCM from *C. longa* extract was 13.4% only after 8 h of study. The cumulative drug release from mouthwashes based on nanoemulsions increased significantly throughout the trial up until its conclusion (after 24 h) compared to *C. longa* extract. At 24 h, the cumulative drug releases from PHFX1, PHFX2, PHFX3, PHFX4, and PHFX5 were 98.2, 93.4, 87.8, 81.2, and 76.3%, respectively. However, the cumulative drug release from *C. longa* extract was 23.2% only after 24 h. Formulation PHFX1 showed the maximum percentage of cumulative drug release (98.2%). Meanwhile, formulation PHFX5 had the least drug release compared to the other formulations reaching 76.3% cumulative drug release after 24 h of the study. The findings of the drug release profile were in accordance with their recorded physicochemical parameters. The enhanced drug release profile of nanoemulsion-based mouthwashes was probably due to the presence of Tween-80 and Transcutol-HP compared to *C. longa* extract. Overall, the two-step drug release profile from all nanoemulsion-based mouthwashes was similar to those reported from nanoemulsion formulations of other herbal drugs such as sinapic acid, vanillin, thymoquinone, and luteolin.[35,36,38,42] From these

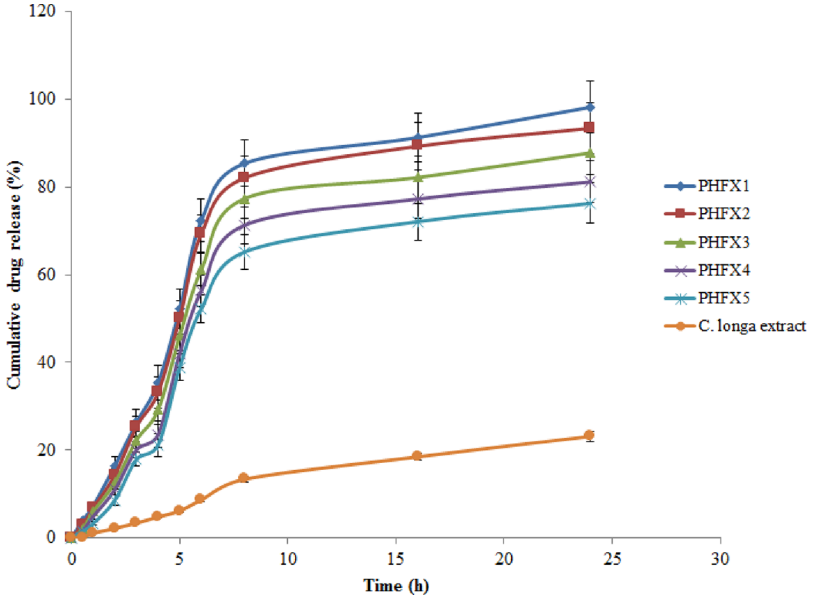

Figure 4. Cumulative *in vitro* release of CCM from nanoemulsion-based mouthwashes (PHFX1−PHFX5) and *C. longa* extract via dialysis bags over 24 h (mean ± SD, n = 3).

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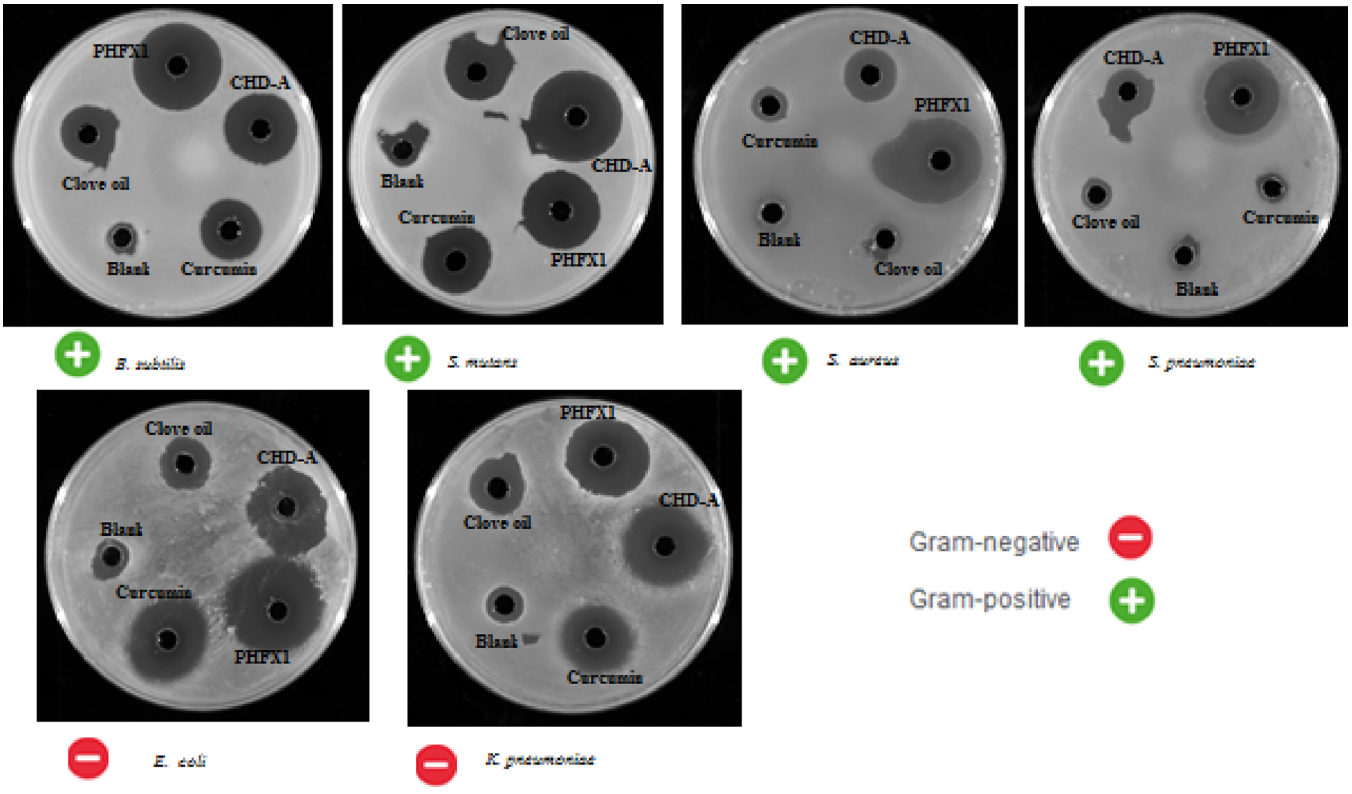

Figure 5. Zone of inhibition (mm) of nanoemulsion-based mouthwash PHFX1 (test), standard CHD-A, free CCM, and pure CEO against a variety of Gram-positive and Gram-negative oral pathogens.

findings, formulation PHFX1 was selected as the best nanoemulsion-based mouthwash based on its minimum droplet diameter (26.34 ± 0.18), least PDI (0.132 ± 0.021), optimal RI (1.337 ± 0.001), optimal pH (6.45), maximum %*T* (99.13 ± 0.12), and maximum cumulative drug release (98.2%). As a result, the formulation PHFX1 was selected for further antimicrobial studies against a variety of oral pathogens.

2.6. Antimicrobial Analysis by the Disc Diffusion Assay. Based on the above findings, nanoemulsion-based mouthwash PHFX1 was chosen for antimicrobial evaluation against a variety of oral pathogens. As compared to the aqueous phase, the concentration of S_{mix} is too low in formulation PHFX1. We selected the lowest possible concentration of S_{mix} in each formulation. In formulation PHFX1, the amount of S_{mix} is 14% w/w, and the amount of the aqueous phase is 84% w/w. For 2 g of formulation, it will have 10 mg of *C. longa* extract, 30 mg of CEO, 280 mg of S_{mix}, and 1680 mg of the aqueous phase. So, the concentration of S_{mix} is low. In addition, the nanoemulsion will not form without CEO as it was used as the oil phase in the preparation of nanoemulsion. Therefore, antimicrobial activity of formulation without *C. longa* extract and CEO was not performed. Antimicrobial susceptibility was evaluated by using the agar diffusion method. The pictures for the zone of inhibition (ZOI) for nanoemulsion-based mouthwash PHFX1, standard CHD-A, free CCM, pure CEO, and control are presented in Figure 5.

The quantitative values of ZOI for tested mouthwash PHFX1 and standard CHD-A against a variety of Gram-positive and Gram-negative oral pathogens are displayed in Figure 6. Antimicrobial assessment showed significantly high

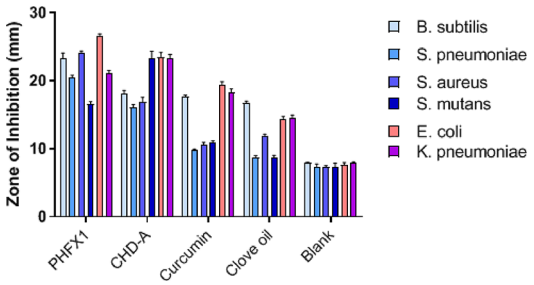

Figure 6. Quantitative values of the zone of inhibition (mm) of PHFX1 (test), standard CHD-A, free CCM, and pure CEO against a variety of Gram-positive and Gram-negative oral pathogens (mean ± SD, $n = 3$).

ZOI for PHFX1 against selected bacteria. Gram-positive bacteria, such as *Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus mutans*, showed susceptibility toward PHFX1 with ZOIs of 23.37 ± 1.20, 20.43 ± 0.71, 24.03 ± 0.60, and 16.53 ± 0.71 mm compared to the standard CHD-A ZOIs of 18.07 ± 0.91, 16.13 ± 0.67, 16.87 ± 1.20, and 23.27 ± 1.90 mm, respectively ($p \leq 0.05$). Moreover, Gram-negative bacteria, such as *Escherichia coli* and *K. pneumoniae*, also showed susceptibility toward PHFX1 with ZOIs of 26.53 ± 0.60 and 21.17 ± 0.61 mm, which were similar to the standard CHD-A ZOIs of 26.53 ± 0.60 and 23.27 ± 1.60 mm, respectively. In addition, the ZOI values for PHFX1 were significant compared to free CCM and pure CEO ($p \leq 0.05$). PHFX1 inhibited the growth of *B. subtilis*, *S. pneumoniae*, *S. aureus*, *S. mutans*, and *Klebsiella pneumoniae* even at one-third of the concentration of formulation and was most active against *E. coli* where it was a potent inhibitor, inhibiting the growth at one-fourth of the concentration.

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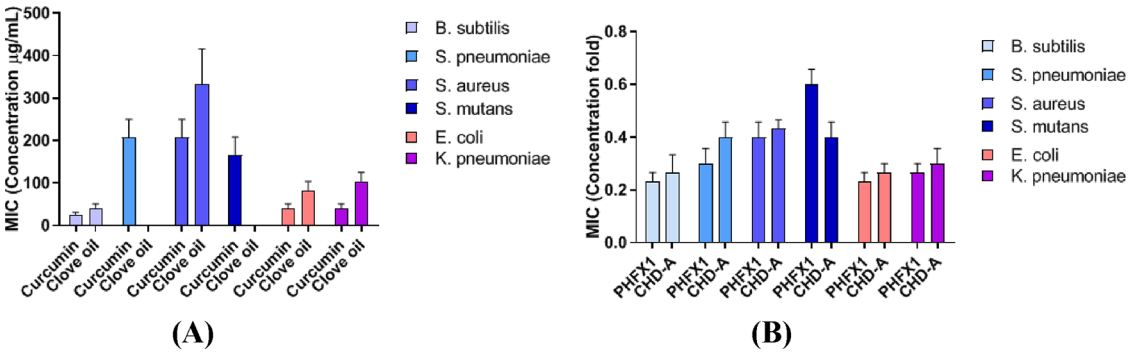

Figure 7. MIC (μg/mL) values of (A) CCM and standard CEO and (B) PHFX1 (test) and standard CHD-A against a variety of Gram-positive and Gram-negative oral pathogens (mean ± SD, n = 3).

Standard CHD-A on the other hand inhibited bacteria at 42% of the working concentration. The enhanced antimicrobial effects of PHFX1 compared to standard CHD-A, free CCM, and pure CEO were possible due to the presence of Tween-80, Transcutol-HP, and synergistic effects of CCM and CEO in a nanoemulsion-based mouthwash.

2.7. Determination of Minimum Inhibitory Concentration (MIC) Values. The MIC values for tested mouthwash PHFX1, standard CHD-A, free CCM, and pure CEO against a variety of Gram-positive and Gram-negative oral pathogens are displayed in Figure 7A,B.

The MIC values for tested mouthwash PHFX1 and standard CHD-A against a variety of Gram-positive and Gram-negative oral pathogens were assessed as a fold change in the concentration. The MIC values of PHFX1 against all Gram-positive bacteria studied, such as *B. subtilis*, *S. pneumoniae*, *S. aureus*, and *S. mutans* measured at 0.33-fold, were found to be 0.23 ± 0.06, 0.30 ± 0.10, 0.40 ± 0.10, and 0.60 ± 0.10, respectively. However, the MIC values of standard CHD-A against *B. subtilis*, *S. pneumoniae*, and *S. aureus* determined at 0.42-fold were 0.27 ± 0.11, 0.40 ± 0.10, and 0.43 ± 0.06, respectively, and 0.50-fold against *S. mutans* (0.40 ± 0.10). The MIC values of PHFX1 against Gram-negative bacteria, such as *E. coli* at 0.25-fold and *K. pneumoniae* at 0.33-fold, were found to be 0.23 ± 0.06 and 0.27 ± 0.06, respectively. Meanwhile, the MIC values of standard CHD-A against both Gram-negative bacteria, such as *E. coli* and *K. pneumoniae* determined at 0.42-fold, were 0.27 ± 0.06 and 0.30 ± 0.10, respectively. Overall, the MIC values of PHFX1 against all oral pathogens were significantly lower than standard CHD-A ($p \leq 0.05$). The enhanced antibacterial effects of PHFX1 compared to standard CHD-A were possible due to the presence of CCM and CEO in the formulation PHFX1. When the main components of the mouth rinse were analyzed individually for their antimicrobial effect, both CEO and CCM showed good antimicrobial properties and the results were found to be in consonance with the previous reports.[12,13,24] Therefore, it was expected that the enhanced antibacterial effects of formulation PHFX1 were possible due to the synergistic effects of CCM and CEO.[13,24] These findings indicated the great potential of the proposed nanoemulsion-based mouthwash for the treatment of a variety of oral pathogen infections.

3. CONCLUSIONS

In this work, a nanoemulsion-based PHFX of *C. longa* hydroalcoholic extract was produced and evaluated for its antibacterial effects against a variety of Gram-positive and Gram-negative oral pathogens. The aqueous phase titration was used to create various formulations, which were then physicochemically characterized and tested for drug release profiles. Based on the lowest droplet size, least PDI, maximum %T, optimal values of RI and pH, and maximum drug release profile, formulation PHFX1 (containing 0.5% w/w of *C. longa* extract, 1.5% w/w of CEO, 14.0% w/w of S_{mix}, and 84.0% w/w of the aqueous phase) was chosen for antimicrobial evaluation against a variety of Gram-positive and Gram-negative oral pathogens. Overall, Gram-positive and Gram-negative oral pathogens were found to be much more sensitive to PHFX1's antibacterial activities than to conventional CHD-A. The MIC value of PHFX1 was significant over standard CHD-A against most of the oral pathogens. These findings suggested that nanoemulsion-based mouthwash may have application in the management of a range of oral pathogen infections. To fully investigate the potential of nanoemulsion-based polyherbal mouthwash, additional preclinical and clinical investigations are needed.

4. MATERIALS AND METHODS

4.1. Materials. Pure CCM was obtained from E-Merck (Darmstadt, Germany). The fresh *C. longa* rhizomes were procured from a hypermarket in Riyadh, Saudi Arabia. High-pure diethylene glycol monoethyl ether (Transcutol-HP) was procured from Gattefossé (Lyon, France). Ethanol and polyoxyethylene (20) sorbitan monooleate (Tween-80) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CEO was procured from Loba Chemie Pvt., Ltd. (Mumbai, India). Ultrapure deionized water was obtained from the ELGA water purification unit (Wycombe, UK). CHD-A was obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as a positive control. A treated dialysis bag (MWCO: 12−14 kDa) was obtained from Spectrum Medical Industries (Mumbai, India). The clinical isolates of *S. mutans* (ATCC 25175), *S. aureus* (ATCC 29213), *S. pneumoniae* (ATCC 3300), *B. subtilis* (ATCC 10400), *E. coli* (ATCC 25922), and *K. pneumoniae* (ATCC 13883) were obtained from King Saud University Medical City, King Saud University (Riyadh, Saudi Arabia). All other chemicals used in this study were of analytical/pharmaceutical grade.

4.2. Preparation of *C. longa* Extract and Determination of Its Biomarker Compound, CCM. The hydroalcoholic extract of *C. longa* was obtained using the procedure presented in our recent publication.[11] The amount of biomarker compound of *C. longa*, i.e., CCM, was determined using a reported HPLC method. The measurement of CCM

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was carried out at 25 ± 1 °C. CCM was quantified using a Nucleodur (150 mm × 4.6 mm) reversed-phase C_{18} column with a particle size of 5 μm. The binary combination of ethanol and ethyl acetate (83:17% v/v) was used as the mobile phase, which was delivered with a flow speed of 1.0 mL/min. At a 425 nm wavelength, CCM was quantified. The HPLC method development and validation details are also included in our recent publication.[11]

4.3. Preparation of Nanoemulsion-Based PHFX and Construction of Pseudoternary Phase Diagrams. The solubility studies are required when a pure compound is selected for the preparation of nanoemulsion. We have used the hydroalcoholic extract of *C. longa*, so solubility studies were not performed in this study. The solubility measurements can be applied in pure compounds only. For the creation of nanoemulsion systems in this work, CEO, Tween-80, Transcutol-HP, and deionized water were used as the oil phase, surfactant, cosurfactant, and aqueous phase, respectively. Through the creation of pseudoternary phase diagrams, oil-in-water (o/w)-type nanoemulsions were created using a spontaneous emulsification process.[34−36] The weight ratios of the surfactant (Tween-80) and cosurfactant (Transcutol-HP) were varied and included 3:1, 2:1, 1:1, 1:2, 1:3, and 1:0. These Tween-80 and Transcutol-HP combinations were chosen based on the ratio of rising Tween-80 content to Transcutol-HP concentration and vice versa.[34] Varied S_{mix} ratios were taken to identify the maximum number of nanoemulsion zones in the phase diagrams. To clearly define the phase boundaries in the phase diagrams, CEO (oil phase) and a particular combination of S_{mix} were mixed in a range of weight ratios (from 1:9 to 9:1). By slowly adding deionized water (the aqueous phase) to an oil phase and specific S_{mix} combination while titrating the mixture, pseudoternary phase diagrams were created. The deionized water was added at around 5.0% w/w interval, and all titrations were performed at 25 °C. Visual observations were taken after each addition of water. Upon visual observation, the clear transparent solutions upon each addition of water were considered as nanoemulsions, and turbid formulations were considered as emulsions. Based on visual observations, the pseudoternary phase diagram's nanoemulsion zones were found to be the area where clear, transparent, and easily flowable mixes were produced.[34,35] Different nanoemulsions were selected from pseudoternary phase diagrams utilizing the minimum possible S_{mix} concentration to avoid toxicity and irritation effects of S_{mix}. The 0.5% w/w *C. longa* extract was loaded into each formulation to obtain nanoemulsion-based PHFX. Table 1 provides a summary of the ingredients in each nanoemulsion-based PHFX formulation.

4.4. Thermodynamic Stability Tests. Thermodynamic stability tests on prepared nanoemulsion-based PHFX formulations were carried out to exclude metastable or unstable formulations.[36] Three distinct tests were carried out, including centrifugation, heating and cooling cycles, and freeze−pump−thaw cycles. Centrifugation of the produced formulations at 5000 rpm for 30 min was done while checking for any physical alterations.[35−37] For the heating and cooling cycles, PHFX formulations that remained stable at centrifugation were chosen. At each storage temperature, four heating and cooling cycles between 4 and 45 °C were carried out for 48 h while being watched for any physical changes. For freeze−pump−thaw cycles, PHFX formulations that remained stable during the heating and cooling cycles were used. Between −21 and 25 °C, four freeze−pump−thaw cycles were carried out for 24 h.[36] For physicochemical characterization, formulations that remained stable under all stress conditions in thermodynamic stability tests were chosen.

4.5. Physicochemical Characterization of Nanoemulsion-Based PHFX Formulations. In nanoemulsion-based PHFX formulations, the droplet diameter, PDI, RI, %*T*, pH, and surface morphology were all determined. The droplet diameter and PDI of the nanoemulsion-based PHFX formulations were measured by using a Malvern Zetasizer (Nano ZS90, Malvern Instruments Ltd., Holtsville, NY, USA). At a temperature of 25 °C and a scattering angle of 90°, the measurements were made. To evaluate the droplet diameter and PDI, 3 mL of each PHFX formulation was placed to an acrylic plastic cuvette after being diluted approximately 1 mL of each formulation with water (1:100).[37]

The RI of each PHFX formulation was determined using an Abbes-type refractometer (Precision Standard Testing Equipment Corporation, Darmstadt, Germany). RI measurements were performed on undiluted samples using castor oil as the standard.[38]

According to the literature,[34] a UV-visible spectrophotometer (SP1900, Axiom, Germany) was used to quantify the turbidity/ %*T* of each PHFX formulation at 550 nm.

The pH of each PHFX formulation was determined using a digital pH meter (Mettler Toledo, Greifensee, Switzerland).

TEM was used to evaluate the surface morphology and size of optimized formulation PHFX1. A JEOL TEM (JEOL JEM 1010, USA) instrument was used to operate TEM on PHFX1. Central Laboratory, Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia conducted the TEM measurements. Optimized formulation PHFX1 was diluted according to the procedure included for droplet size and PDI measurements. On a carbon-coated grid, a drop of diluted formulation PHFX1 was applied and left to dry for about 30 s. The operation was performed using TEM at 80 kV.

4.6. *In Vitro* Drug Release Studies. The biomarker compound of *C. longa* is CCM, and hence, CCM was determined using a reported HPLC method in *in vitro* drug release samples.[11] Using a dialysis method, *in vitro* drug release studies of CCM from different nanoemulsion-based PHFX formulations and *C. longa* extract were performed.[42] Phosphate buffer, which has a pH of 6.8, was utilized as the drug release/dissolution medium in these trials and was 200 mL. The appropriate glass beakers were filled with the drug release medium. Each nanoemulsion-based PHFX formulation included approximately 1.0 mL, which was transferred to a dialysis bag and secured with plastic clips. The glass beakers containing the clamped dialysis bags and 200 mL of the drug release/dissolution liquid were submerged. The entire system was put into a WiseBath WSB shaking water bath (Model WSB-18/30/-45, Daihan Scientific Co. Ltd., Seoul, Korea) set to shake at 100 rpm at 37 ± 0.5 °C. Samples (1.0 mL) from each formulation were carefully taken out at various time intervals and replaced with the same volume of freshly made drug release media. The amount of CCM in each nanoemulsion-based PHFX formulation and *C. longa* extract was estimated utilizing a reported HPLC method at 425 nm at each time point.[11]

4.7. Antimicrobial Analysis by the Disc Diffusion Assay. The nanoemulsion-based PHFX1 was optimized and chosen as a test formulation for antimicrobial analysis based on the smallest droplet diameter, least PDI, the highest percentage

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of *T*, and, ultimately, the least amount of oil phase (CEO). Using the agar diffusion method, PHFX1 mouthwash was evaluated for its antibacterial properties.[55,56] The microbe cultures were produced in the microbiology department of the College of Pharmacy at King Saud University in Riyadh, Saudi Arabia, and the microbial strains were selected from the list of global priority pathogens (GPP). *S. mutans*, *S. aureus*, *S. pneumoniae*, *B. subtilis*, *E. coli*, and *K. pneumoniae* were tested for antibiotic susceptibility against the PHFX1 mouthwash. Each microbial strain's pure colonies were selected and cultured on Mullar Hilton broth for 18−24 h at 37 °C. On the Mullar Hilton agar plates, 0.5 McFarland standard cultures were prepared. Freshly prepared PHFX1 mouthwash, standard CHD-A mouthwash (containing 2% CHD-A) (positive control), free CCM, and free CEO were loaded on a disc and place on an agar plate. One blank disc well served as the negative control. After incubation for 24 h, the zone of inhibition was measured. The experiments were carried out in triplicates.

4.8. Determination of MIC. To demonstrate the inhibitory effects of the PHFX1 mouthwash against each microbial strain, the turbidometric (TB) assay was used. A positive control was utilized, which was the standard mouthwash of CHD-A. The Clinical and Laboratory Standards Institute (CLSI) methodology for broth microdilutions was exactly followed.[56] A sterile 96 microtiter plate, for each bacteria culture, was used for the assay with positive control CHD-A, and the negative control contained no bacteria. PHFX1 mouthwash, CHD-A, free CCM, and free CEO were loaded onto the first well and 2-fold diluted thereafter until the seventh well; the last well contained no drug and no bacteria (negative control). Then, all wells (except from the negative control) received 5 μL of diluted bacterial suspension (1.5×10^6 cells/mL) and were carefully mixed. For each type of bacterial species, microdilution was carried out in triplicates. Growth was observed and noted after an overnight incubation at 37 °C. The MIC was defined as the lowest concentration before turbidity.

4.9. Statistical Analysis. The results of three independent experiments were used to express all the data as mean ± SD. Using the GraphPad Instat software (San Diego, CA, USA), all the physicochemical parameters were statistically evaluated using one-way analysis of variance (ANOVA) and Dennett's test. A *t*-test was used to compare the results of antimicrobial activity of nanoemulsion-based PHFX1 mouthwash and standard CHD-A. The $p < 0.05$ was considered a significant result.

AUTHOR INFORMATION

Corresponding Author

Faiyaz Shakeel − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia;* orcid.org/0000-0002-6109-0885; Email: fsahmad@ksu.edu.sa

Authors

Nazrul Haq − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Mudassar Shahid − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Ahmed L. Alaofi − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia;* orcid.org/0000-0001-8967-173X

Zeeshan Heera Ahmad − *Dental University Hospital, King Saud University Medical City, Riyadh 11545, Saudi Arabia*

Yasser F. Alrayyes − *Dental University Hospital, King Saud University Medical City, Riyadh 11545, Saudi Arabia*

Ibrahim A. Alsarra − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06176

Notes

The authors declare no competing financial interest.

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