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Formulation of Curcumin-based Polyherbal Nanoemulsion Mouthwash and Assessment of its Antioxidant and *in-vitro* Cytotoxic Properties

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Abstract

Curcumin is a hydrophobic compound with excellent antibacterial and antioxidative properties but it exhibits poor bioavailability which limits its therapeutic value. Nano emulsion (NE) is a modern drug delivery system that can accommodate the poor bioavailability and solubility of hydrophobic curcumin. The objective of this study was to formulate curcumin-based polyherbal NE mouthwash (Cur-polyherbal NE) and evaluate the antioxidant and cytotoxic properties. The NE formulations were prepared using glyceryl monooleate (GMO), Pluronic® F-127, virgin coconut oil (VCO) and distilled water. Physiochemical characterisation of Cur-polyherbal NE was performed based on FTIR and dynamic light scattering analyses, and their stability at different temperatures was also determined. The antioxidant activity of formulations was evaluated using DPPH scavenging assay, and their in-vitro cytotoxicity was investigated on HEK293 cells. This study demonstrates that the antioxidant activity of Cur-polyherbal NE formulation was superior compared to corresponding non-NE formulation ($p < 0.05$). In-vitro cytotoxicity performed on HEK293 cells revealed better % cell viability in dose-dependent manner. The promising antioxidant activity and non-toxicity on HEK293 cells of Cur-polyherbal NE may suggest its potential use as safer mouthwash alternative.

Keywords: Nano emulsion, curcumin, polyherbal, antioxidant, cytotoxicity

Introduction

Gingival and periodontal disease are commonly affiliated with the chronic accumulation of bacterial plaque due to poor oral hygiene (1). The management of plaque-induced gingivitis focused on regular and appropriate dental hygiene practices including the use of chemical mouthwashes such as chlorhexidine as useful adjuncts for mechanical plaque control measures (2). However, prolonged use of chemical antibacterial mouthwashes are associated with several adverse events (3). As an alternative, plant-derived mouthwash had received substantial importance due to the numerous therapeutic benefits of herbal extracts (4).

Curcumin is a natural polyphenolic compound derived from the rhizomatous herbaceous perennial plant, Curcuma longa Linnaeus (C. longa L.). Curcumin is widely recognised for its wide range of therapeutic properties including antioxidant, antimicrobial and anti-inflammatory properties (5). The therapeutic properties of curcumin are associated with its potent free radical scavenging activity that are linked to various signalling pathways (6). Traditionally, curcumin has been used as an herbal remedy to treat various ailments such as inflammatory diseas-

es, wounds, and ulcers (7). In modern practice, several narratives had reported the utilisation of curcumin for various diseases (8–10). Previous studies demonstrated the topical application of curcumin oral gel had significantly reduced inflammation in severe gingivitis and periodontal infections (8,11). However, several reports had addressed the limitations of native curcumin as a therapeutic agent due to its low bioavailability and poor solubility which greatly hindered its therapeutic efficacy (12). Recently, the emergence of nanotechnology received recognition for its ability to solubilise lipophilic drug and ever since, had drastically improved the efficacious delivery and bioavailability of hydrophobic compounds, such as curcumin (13).

Various nanoparticle-based drug delivery systems (DDS) had been pursued including nanoliposomes, solid-lipid nanoparticle, and nano emulsion to enhance the bioavailability of curcumin (14). Amongst these, nano emulsion had been extensively formulated for the enhancement of solubility and absorption of curcumin (15). Nano emulsion (NE) is a lipid-based colloidal system composed of dispersed oil and aqueous phases stabilised by means of surfactants (16). The organisation of NE system allows the solubilisation of hydrophobic compound and thus, providing better uptake of the compound for therapeutic application (17). Several studies had discussed the promising alternative of NE system in restoring therapeutic properties of curcumin by reducing rapid clearance and promoting controlled-release to minimise toxicity (13,15).

Improved understanding of the pleiotropic effects of herbal plants resulted in the appreciation of polyherbal formulation in achieving greater therapeutic effects that may be unattainable from individual plant (18). Herbal plants generally have high antioxidant content attributed from their secondary metabolites, polyphenols (19). The antimicrobial effect of antioxidants has become considerably recognised as plants' antimicrobial defence mechanism (20). White tea (Camellia sinensis L.) is a renowned therapeutic agent associated with its antioxidative, antimicrobial, and anti-inflammatory properties. The antioxidative properties of white tea are attributed to the high content of catechins and polyphenolic compounds (21). It was previously reported that white tea contains the highest concentration of antioxidants compared to the other tea variants (22). Previous studies had demonstrated the bactericidal properties of white tea against several oral pathogens associated with its polyphenolic compounds (23).

Ginger (Zingiber officinale) is another notable rhizomatous plant possessing a wide range of therapeutic properties including antioxidant, anti-inflammatory, and antimicrobial attributed to several phenolic compounds such as 6-gingerol and shogaols (24). Several narratives had reported the antibacterial activity of ginger against several periodontal and plaque-inducing bacteria strains related to phenolic compounds in ginger that caused destruction of bacterial membrane integrity (25,26). Therefore, the objective of this study was to develop and characterise oil-in-water (o/w) polyherbal nano emulsion mouthwash composed of curcumin, white tea, and ginger extracts, and assessed the antioxidant and in-vitro cytotoxic properties.

Materials and methods

Chemicals

Curcumin and ginger powder extract were Pi Chemicals Ltd. (Shanghai, China). Virgin coconut oil (VCO) was procured from local supermarket, Brunei. White tea (*Camellia Sinensis L.*) extract with product code FSS10580 was purchased from Formulator Sample Shop (FSS®, Bareggio, Italy). Glyceryl monooleate (GMO) was purchased from Croda™ International (East Yorkshire, UK). Pluronic® F-127 (P-F127) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The human embryonic kidney cells (HEK293) cell line was purchased from American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle's Medium (DMEM)

was purchased from Gibco™ (Thermo Fisher Scientific Inc., Waltham, US). 1% penicillin with streptomycin, 10% (v/v) fetal bovine serum (FBS), 3-((4,5-Dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide (MTT), and 0.25% trypsin-EDTA, were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6-hydroxy-2, 5, 7, 8-tetramethychroman-2 carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of curcumin-based polyherbal nano emulsion

Oil-in-water (o/w) curcumin-based polyherbal nano emulsion (Cur-polyherbal NEs) were prepared by performing a two-step low-energy Emulsion Inversion Point (EIP) method (27). Different concentrations of GMO (1%, 2% and 4%) and P-F127 (1%, 2%, 4% and 8%) were used as surfactants, respectively. As seen from the composition of Cur-polyherbal NE formulations on Table 1, the white tea liquid extract (WTE) and filtered ginger liquid extract (GE) contents of Cur-polyherbal NEs were kept at 5% and 20% and curcumin content was either 25 and 50 mg. The aqueous phase solution composed of WTE (5%, w/w), filtered GE (20%, w/w), and surfactant P-F127 was added dropwise to the oil phase mixture composed of VCO, surfactant GMO, and curcumin powder extract (Figure 2). Finally, distilled water was gradually added to mark up the total volume of mixture to 100 mL. The mixture was mixed under continuous magnetic stirring at 400 rpm, 45 – 50°C for 30 minutes to create spontaneous o/w emulsion. The formulation of o/w nano emulsion was achieved by further stirring the emulsion at a higher shear speed of 3000 rpm for another 30 minutes using a L5M-A Laboratory Mixer homogeniser (Silverson® Ltd., Chesham, UK). Obtained nano emulsion formulations were contained in sealed bottles and stored inside a cabinet at room temperature (25 ± 2°C) throughout analysis period.

Physiochemical characterisation of curcumin-based polyherbal nano emulsion

Organoleptic properties

Formulations were evaluated for colour, odour and density. A 100 mL pycnometer was used to determine the density in g/mL of the formulations. Whereas, the colour and odour of the formulations were evaluated based on its intensity(28)50cPs.

pH determination

AE150 pH meter (Fisher Scientific) was used to determine the pH of Cur-Polyherbal NEs. 5 mL of each formulation was diluted with 10 mL of tap water before measuring the pH at room temperature (29).

Dynamic light scattering (DLS) measurements

A Zetasizer Nano ZS (ZEN3600) analyser (Malvern Instruments Ltd., Malvern, UK) was used to measure the particle size, polydispersity index (PdI) and ζ-potential measurements of the o/w nano emulsions. Prior to the experiment, 100 µL of each formulation was diluted with 4 mL of distilled water and sonicated in 37°C water bath for 30 seconds to ensure homogenous scattering of particles. Analysis of formulations was measured at scattering angle of 173° and temperature of 25°C. All measurements were performed in triplicate and results are expressed as mean ± SD (29,30).

Stability study

The stability of formulations was assessed by evaluating the change in organoleptic properties, phase separation, pH, and density at three different temperatures of 4 ± 2°C, 25 ± 2°C, and 40°C ± 2°C with 5% humidity. Measurements were carried out on the day of storage (day 0) and at predetermined time intervals of 14 and 30 days. All formulations were kept in sealed vials (31).

FTIR analysis

Optimised Cur-polyherbal NE formulations were scanned using a Fourier transform infrared spectrometer (FTIR LabSolutions IR, Shimadzu, Columbia, Maryland, USA) at wavelength of 400 – 4000cm^{-1} and resolution of 4 cm^{-1} (32,33)"ISSN":"18469558","abstract":"© 2017 Rajan Rajabalaya et al., published by De Gruyter Open 2017. The purpose of the study was to develop a transdermal tolterodine tartrate (TT.

Antioxidant activity of Cur-polyherbal NE

DPPH assay

In order to analyse the antioxidant activity of Cur-polyherbal NE, DPPH scavenging activity assay was performed based on the capability of the herbal extracts of the formulations in scavenging the DPPH• free radical. A 0.1 mM stock solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) in methanol was prepared. All tested sample including free curcumin and Cur-Polyherbal NE formulations (C1-NE and C3-NE) were dissolved in methanol. The non-nanosized formulations (C1-E and C3-E) were also prepared to investigate the effect NE system on the antioxidant capacity of the formulations. The DPPH assay was performed in a 96-well microplate.

The tested compounds containing DPPH free radicals were incubated in the dark for 30 minutes at room temperature to allow free-radical scavenging activity to occur. The antioxidant compounds present in the test formulations reduced DPPH free radicals (purple) to produce 1-Diphenyl-2-picryl hydrazine (yellow). After 30 minutes, the amount of DPPH free radicals remaining was measured using a spectrophotometer microplate reader (EPOCH 2, BioTek Instruments) at 517 nm. The DPPH assay was performed triplicate for each tested compounds and formulations at three independent experiments.

The percentage of radical scavenging activity was calculated according to Equation (1):

$$\% \text{ DPPH scavenging activity } (\%) = \frac{(As - Ai)}{Ac} \times 100\% \quad (1$$

Where, A_s represented the absorbance of sample and DPPH-MetOH solution, A_i represented the absorbance of sample in methanol, and A_c represented the absorbance of DPPH-MetOH solution.

The calculated percentage of DPPH scavenging activity was plotted against sample concentration and IC_{50} values were determined by nonlinear regression analysis.

A powerful antioxidant, Trolox, was used as a reference positive control. A Trolox calibration curve was acquired by measuring the absorbance of 0.1mM DPPH methanolic solution at 517 nm after addition with increasing concentration of Trolox (0 – 30 mM). The absorbance readings were obtained after the DPPH reduction reaction of Trolox at 30 minutes after Trolox addition. Finally, the DPPH radical scavenging capacity were expressed as Trolox equivalent antioxidant capacity (TEAC) in µg/mL of tested compounds (34).

Trolox Equivalent Antioxidant Capacity (TEAC) values were calculated as:

$$\text{TEAC} = \frac{(\text{IC50 of Trolox } (\mu g/mL))}{(\text{IC50 of sample } (\mu g/mL))} \quad (2$$

In-vitro cytotoxicity study

HEK293 cell line

Human embryonic kidney (HEK293) cells were grown in 25 cm^2 culture flasks with complete culture medium containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, USA) supplemented with 4mM L-glutamine (Gibco, CA, USA), 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 µm/mL streptomycin. The cells were maintained under a controlled humidified atmosphere of 5% CO_2 and 95% humidity at 37°C. Cells were regularly sub-cultured using 0.25% Trypsin-EDTA when 80 – 90% cell confluent was reached. Trypan-blue solution (ThermoFisher) and haemocytometer were used for

cell counting (35)anti-oxidative, and anti-cancer properties but has poor bioavailability. Liquid crystals (LC.

HEK293 cell density optimisation for MTT Assay

Cell density optimisation for MTT assay was performed prior to MTT cytotoxic experiments. Different ranges of cell seeding density (1 x 10^4, 2 x 10^4, 3 x 10^4, and 4 x 10^4 cells/well) were seeded in triplicates on 96-well plate. Each cell density was suspended in complete culture medium.

In-vitro cytotoxicity study of Cur-polyherbal NE using MTT Assay

To evaluate the cytotoxicity of Cur-polyherbal NE formulations, *in-vitro* cytotoxicity using MTT assay was performed on HEK293 cells. HEK293 cells were resuspended in 100 µL complete culture medium, seeded in 96-well culture plate at a density of 2 x 10^4 cells/well and incubated overnight at 37°C, 5% CO_2 and 95% humidity to allow cell attachment. Following overnight incubation, culture medium was removed and cells were treated with 100 µL of culture medium containing different dilutions (1:1000, 1:100, and 1:10) of C1-NE, C3-NE, non-nano-sized formulations (C1-E and C3-E) and empty NE system without herbal extracts (Empty-C1 and -C3) for 24 hours. The HEK293 cells were also treated with 1% of free herbal extracts (curcumin, GE, and WTE) used in the formulation of C1-NE and C3-NE. As negative control, cells were treated with 1% distilled water. The viability of cells was determined after 24-hour of incubation. After 24-hour exposure, 30 µL of MTT (1 mg/mL in PBS) was added to respective wells and incubated at 37°C for 2 hours until purple formazan crystals were formed. The formazan crystals were dissolved in 100 µL of isopropanol and left overnight in the dark before reading the absorbance at 570 nm using spectrophotometer plate reader (EPOCH 2, BioTek Instruments). The measured absorbance is identified as the number of viable cells in respective well. The cell viability was expressed as the percentage of formazan absorbance at 570 nm (36).

Statistical analysis

All tests were performed in triplicates and data are presented as mean ± standard deviation (SD). All statistical analyses were analysed by one-way analysis of variance (one-ANOVA) and multiple comparison by Tukey's test using GraphPad Prism (version 8.4.3). A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Formulation & preparation of curcumin-based polyherbal nano emulsion (Cur-Polyherbal NE)

Six formulations were prepared in this study by investigating the effect of different concentrations of surfactants (GMO and P-F127)

Table 1. The different formulations prepared with varying concentration of VCO, GMO and P-F127

Formulation code	Oil-phase			Aqueous phase			Phase separation
	VCO (g)	GMO (%)	Cur (mg)	GE (%)	WTE (%)	P-F127 (%)	
F1	5	1	25	20	5	1	No
F2	5	.2	50	20	5	2	No
F3	10	1	25	20	5	1	Yes
F4	10	2	50	20	5	.2	No
F5	5	2	25	20	5	4	Yes
F6	10	4	50	20	5	8	No

and VCO on the stability of the nano emulsion formulations. Amongst these, four formulations (F1, F2, F4, and F6) were successfully produced stabilised nano emulsion with no visible phase separation after 24-hour post-production (Table 1). Therefore, these optimised formulations (C1-NE - C4-NE) were chosen to be further tested for subsequent physiochemical characterisation and stability studies. The optimised formulations with no phase separation are shown at Table 2, and the visualisation for the optimised formulations is shown on Figure 1.

Table 3. The organoleptic properties, pH values and density of the optimised Cur-polyherbal NE

Formulation code	Oil-phase				Aqueous phase		
	VCO (g)	GMO (%)	Cur (mg)	GE (%)	WTE (%)	P-F127 (%)	Distilled water (mL)
C1-NE	5	1	25	20	5	1	70
C2-NE	5	2	50	20	5	2	70
C3-NE	10	2	50	20	5	2	65
C4-NE	10	4	50	20	5	8	65
F5	5	2	25	20	5	4	Yes
F6	10	4	50	20	5	8	No

Abbreviations: VCO, virgin coconut oil; Cur, curcumin; GE, ginger extract; WTE; white tea extract; GMO, glyceryl monooleate.

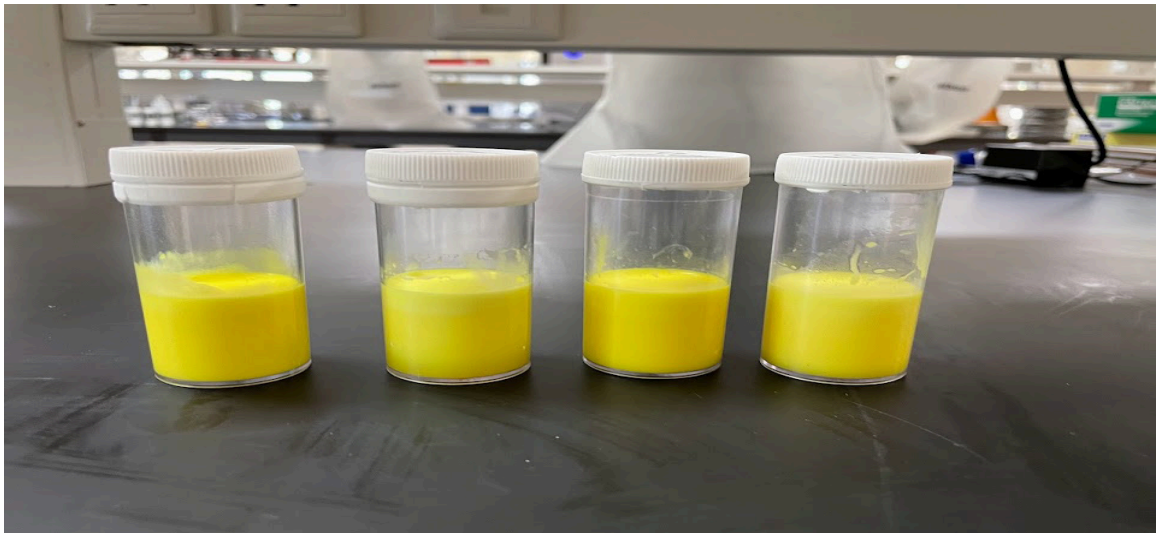

Figure 1. Physical appearance of the optimised oil-in-water curcumin-based polyherbal nano emulsion. From left to right: C1-NE, C2-NE, C3-NE, and C4-NE.

Physiochemical evaluation of optimised Cur-Polyherbal NE

The organoleptic properties, pH, and density of Cur-Polyherbal NE formulations were evaluated. All of the Cur-Polyherbal NE formulations were opaque with pale yellow colour. The measured pH values, density, and odour of the formulations are presented in Table 3.

Table 3. The organoleptic properties, pH values and density of the optimised Cur-polyherbal NE formulations.

Characteristics	Formulation code			
	C1-NE	C2-NE	C3-NE	C4-NE
Colour	Yellow	Yellow	Yellow	Yellow
Odour	Oil-like	Oil-like	Oil-like	Oil-like
(intensity)	(+)	(+)	(+++)	(++++)
pH	5.43	5.40	5.62	6.05
Density (g/mL)	0.977	0.975	0.957	0.994

Note: '+' mild; '+++' moderate; '++++' strong odour.

Stability study

The stability of Cur-Polyherbal NE formulations was assessed based on the measured pH values, density and physical appearance after 30 days storage. At day 30, the Cur-Polyherbal NE formulations presented with acidic pH values ranging from 4.44 – 6.15 (Table 4). All formulations stored at 4 ± 2°C and 40 ± 2°C remained visually stable with no phase separation, although, an increase in density by 0.002 – 0.012 g/mL was observed on most of the formulations (Table 4). Furthermore, reduction in the density of formulation C1-NE and C2-NE was observed by 0.011 g/mL and 0.002 g/mL from its initial density, respectively, when stored at 40 ± 2°C after 30 days. Overall, based on the findings, all formulations presented with relatively acceptable pH values ranging from 5.43 – 6.02 when stored at room temperature (25 ± 2°C) after 30 days. On the other hand, it was shown that formulation C3-NE had the lowest pH values of 4.44 and 4.88 when stored at 4 ± 2°C and 40 ± 2°C, respectively (Table 4).

Table 4. The measured pH values of the Cur-Polyherbal NE formulations at three different storage temperature.

Formulation code	Day	pH			Density (g/mL)		
		4 ± 2°C	25 ± 2°C	40°C ± 2°C	4 ± 2°C	25 ± 2°C	40°C ± 2°C
C1	0	5.43	5.43	5.43	0.977	0.977	0.977
	14	5.35	5.97	4.98	0.977	0.965	0.970
	30	5.61	5.43	4.96	0.978	0.965	0.959
C2	0	5.40	5.40	5.40	0.975	0.975	0.975
	14	5.38	5.52	5.11	0.971	0.963	0.970
	30	5.50	5.50	5.10	0.980	0.963	0.968
C3	0	5.62	5.62	5.62	0.957	0.957	0.957
	.14	4.78	5.60	4.59	0.966	0.956	0.971
	30	4.88	5.79	4.44	0.968	0.962	0.973
C4	0	6.05	6.05	6.05	0.994	0.994	0.994
	14	5.77	5.77	5.78	0.923	0.930	0.931
	30	6.15	6.02	5.58	0.935	0.930	0.931

Particle size, polydispersity index, and zeta potential

The mean particle size, polydispersity index (PdI), and ζ-potential of all four formulations were determined based on Dynamic Light Scattering (DLS) measurements are presented in Table 6. All Cur-polyherbal NEs presented with narrow size distribution (PdI <0.3) and negative ζ-potential values ranging from -0.418 ± 0.373 to -1.12 ± 0.695 (Table 5). It was shown that formulation C3-NE presented the best NE characteristic with relatively smaller mean particle size of 179.8 ± 2.15 nm, PDI of 0.157 ± 0.012, and ζ-potential of -1.12 ± 0.695 mV. In the contrary, formulation C1 presented with relatively larger particle size of 268.3 ± 2.88 nm, PDI of 0.275 ± 0.013, and low ζ-potential of -0.418 ± 0.373 mV. Therefore, due to the distinctive characteristics of C1-NE and C3-NE, these formulations were selected for further antioxidant and cytotoxic studies.

Table 5. Mean particle size, polydispersity index (PdI), and ζ-potential of Cur-polyherbal NEs.

Formulation code	Mean particle size (nm)	Polydispersity Index (PdI)	Zeta (ζ)-potential (mV)
C1-NE	268.3 ± 2.88	0.275 ± 0.013	-0.418 ± 0.373
C2-NE	135.7 ± 2.15	0.169 ± 0.016	-1.027 ± 0.120
C3-NE	179.8 ± 2.15	0.157 ± 0.012	-1.12 ± 0.695
C4-NE	208.1 ± 1.71	0.292 ± 0.004	-0.183 ± 0.352

Data are presented as mean ± SD (n = 3).

FTIR Analysis

The successful incorporation of curcumin, WTE and GE in the Cur-polyherbal NE formulations were analysed using FTIR analysis. The FTIR spectra of formulation C1-NE, C3-NE and individual components of the Cur-polyherbal NE formulations were compared for changes in spectra shifts in the formulations and are depicted in Figure 2. The intense peak at 2926.00 cm^{-1} was revealed to be attributed from the asymmetrical methylene $–CH_2$ (aliphatic) group stretching, whereas, the alkyne –C≡C group stretching resulted in the characteristic dual peaks at 2359.91and 2342.79 cm^{-1} (37). Meanwhile, the disulfide –C-S group stretching resulted in the peak at 668.76 cm^{-1}. Furthermore, these characteristic peaks in FTIR spectra of formulation C1-NE (2926.00, 2359.91, 2342.79 and 668.76 cm^{-1}) were also observed at VCO, GE, GMO, WTE, and curcumin with slight minor shift. Conversely, the characteristic dual peaks observed in formulation C3-NE at 2924.57 and 2854.70 cm^{-1} corresponded to the asymmetrical and symmetrical methylene $–CH_2$ (aliphatic) group stretching, respectively (38)which are sensitive to minute structural changes. The lack of specificity of this technique, on the one hand, permits us to probe directly the vibrational properties of almost all the cofactors, amino acid side chains, and of water molecules. On the other hand, we can use reaction-induced FTIR difference spectroscopy to select vibrations corresponding to single chemical groups involved in a specific reaction. Various strategies are used to identify the IR signatures of each residue of interest in the resulting reaction-induced FTIR difference spectra. (Specific. Meanwhile, the corresponding alkyl carbonate –C=O and nitrate –C=N stretching resulted in strong peak at 1743.91 and 1636.96 cm^{-1}, respectively. Furthermore, the characteristic peaks in formulation C3-NE (2924.57, 2854.70, 1743.91 and 1636.96 cm^{-1}) were also observed at VCO, GMO, P-F127.

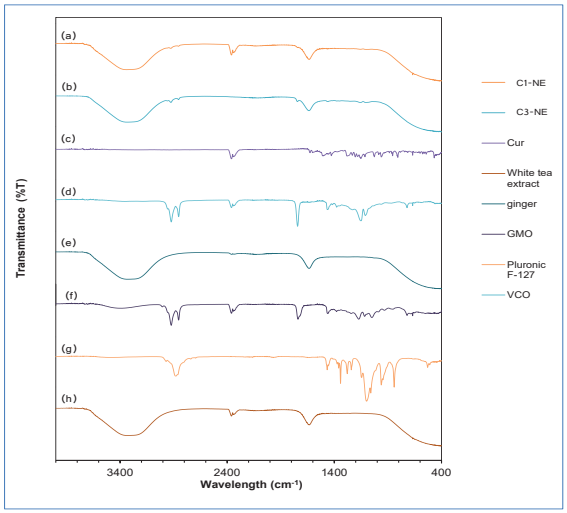

Figure 2. FTIR spectra of (a) C1-NE, (b) C3-NE, (c) curcumin powder extract, (d) VCO, (e) Ginger extract (GE), (f) Glyceryl monooleate (GMO), (g) Pluronic® F-127, and (h) WTE.

Antioxidant activity of Cur-Polyherbal NE

DPPH assay

All tested samples presented with DPPH scavenging activity in a dose-dependent manner (Figure 3). At an equivalent curcumin concentration of 250 µg/mL, C1-NE and C3-NE showed higher DPPH scavenging activity of 96.3 ± 0.577% and 97.7 ± 2.155%, respectively, compared to free curcumin (89.7 ± 1.229%) at the same concentration (Figure 5(a)), although the differences was not significant. When compared between NE and non-NE formulations, the DPPH scavenging activities were significantly reduced ($p < 0.05$) in C1-E (78.6 ± 5.154%) compared to C1-NE (96.3 ± 0.577%) at the same curcumin concentration of 250 µg/mL. Conversely, the reduction in DPPH scavenging activities of C3-NE compared to C3-E was not statistically significant ($p > 0.05$). The DPPH scavenging activities of the three herbal extracts are presented on Table 7. It was revealed that free curcumin and GE presented with excellent antioxidant activities of more than 80%. The IC_{50} values of free curcumin, C1-NE, C3-NE, C1-E and C3-E, from the DPPH scavenging assay, were 3.70, 8.55, 7.74, 16.8, and 11.1µg/mL, respectively (Table 8). The corresponding TEAC values of free curcumin, C1-NE, C3-NE, C1-E and C3-E, are presented in Table 8.

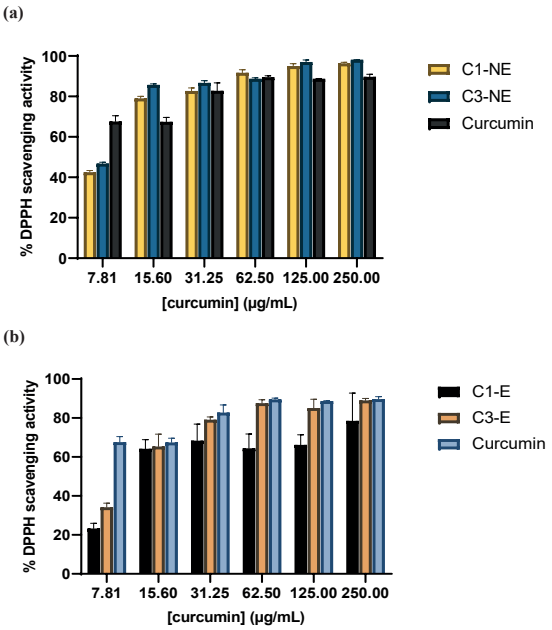

Figure 3. The % DPPH scavenging activity of **(a)** C1-NE, C3-NE, **(b)** C1-E, C3-E at different equivalent curcumin concentrations (7.81 – 250 µg/mL).

Table 6. DPPH radical scavenging antioxidant activity of against the highest concentration of individual herbal extracts used in the development of Cur-polyherbal NE formulations

Herbal extracts	Concentration (µg/mL)	% DPPH scavenging activity
WTE	50	27.0 ± 0.223
GE	200	85.4 ± 0.031
Curcumin	500	90.7 ± 1.15

Table 7. DPPH radical scavenging antioxidant activity (IC_{50}) performed by DPPH assay.

Sample	**IC_{50} (µg/mL) from DPPH assay**	**TEAC (IC_{50}/ IC_{50})**
C1-NE	8.55	0.375

C3-NE	7.74	0.415
C1-E	16.8	0.191
C3-E	11.1	0.289
Curcumin	3.70	0.868
Trolox	3.21	1.000

In-vitro cytotoxicity study

Cell optimisation

Prior to cytotoxic study by MTT assay, determination of the optimal cell density was performed on four different cell seeding density of HEK293 cells. The optimal cell density was selected according to the ability of the cells to reach 80 – 90% confluency after 48 hours on the 96-well plate. This was taken into consideration that HEK293 cells was treated with test samples after overnight incubation and thus, 100% cell confluence should be avoided prior to MTT assay. It was stated that the absorbance for optimum number of cells for MTT assay should be within the range of 0.75 – 1.25 (39). Out of the four cell seeding densities, 2 x 10^4 and 3 x 10^4 cells/well had an appropriate absorbance of 1.07 and 1.16 (Figure 4 (a)). Whereas, 4 x 10^4 cells/well displayed absorbance of 1.80, indicating 100% cell confluence (Figure 4 (b)). Taking into account of the 80 – 90 % cell confluency, 2 x 10^4 cells/well were selected for the MTT assay.

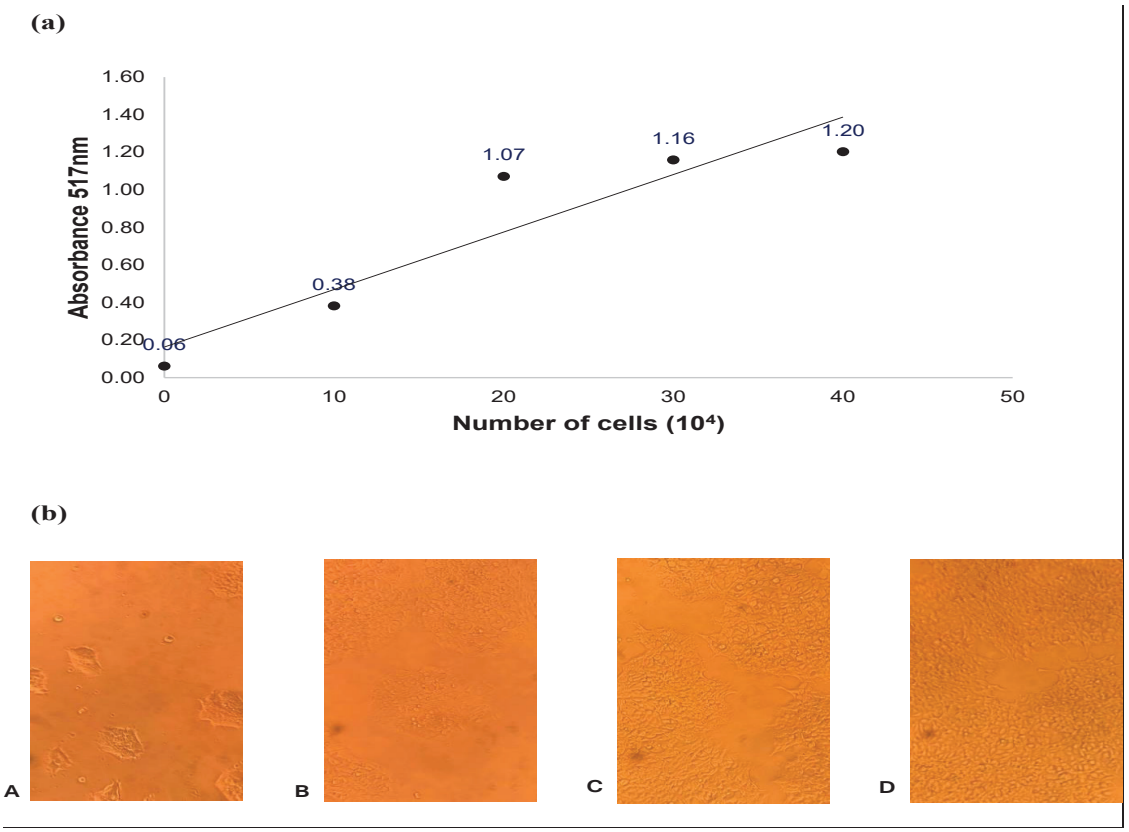

Figure 4 (a) The absorbance (517 nm) of different number of HEK293 cells after 48-hour incubation for cell density optimisation. Figure 4 (b) HEK293 cells viewed under inverted microscope (10x) after 48-hour incubation for cell density optimisation. Note: (A) 1 x 10^4 cells, (B) 2 x 10^4 cells, (C) 3 x 10^4 cells, (D) 4 x 10^4 cells.

In-vitro cytotoxicity of Cur-Polyherbal NE using MTT Assay

In order to determine whether the selected NE formulations (C1-NE and C3-NE) were safe and not toxic to normal cells, different dilutions of the formulations were tested on HEK293 cells and the effect on cell viability was determined by MTT assay. MTT assay was performed on the three herbal extracts used to prepare the NE formulation (curcumin, WTE and GE). It was revealed that free curcumin presented lowest cell viability of 21.3 ± 3.06% followed by WTE with 46.7 ± 2.08%, respectively, after 24-hour incubation (Figure 5). In comparison, GE expressed negligible cytotoxicity with 107.3 ± 4.73% cell viability. Since these NE formulations were prepared in distilled water, the effect of 1% distilled water on cell viability was also tested, and a decrease in cell viability (84.7 ± 4.16%) was observed (Figure 5).

When performed on the NE formulations, the MTT assay revealed a dose-dependent cytotoxicity of C1-NE and C3-NE (Figure 6). Evident cytotoxicity of C1-NE and C3-NE were observed when cells were treated with the formulations diluted at 1:10 which induced a reduction of cell viability of 49.0 ± 2.83 and 44.0 ± 6.66%, respectively. On the other hand, no evident cytotoxicity was observed for C1-NE and C3-NE when diluted at 1:1000 with cell viability above 80%. It was also demonstrated that empty-C1, -C3 and non-nanosized formulations presented a dose-dependent cytotoxicity on HEK293 cells. When compared to NE formulations (C1-NE and C3-NE), the corresponding non-nanosized formulations (C1-E and C3-E) demonstrated a relatively lower cell viability of 36.3 ± 5.34 and 43.6 ± 4.04 % when diluted at 1:10, respectively (Figure 6 (a) and (b)). Conversely, empty-C1 and -C3 did not present with substantial cytotoxicity when diluted at 1:1000 with cell viability above 80%.

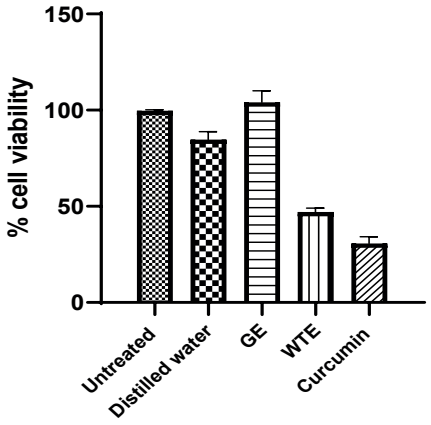

Figure 5. The cytotoxic effects of individual herbal components (GE, WTE and curcumin) on HEK293 cells after 24-hour treatment by MTT assay. Error bars represent standard deviation of triplicate, where n=1.

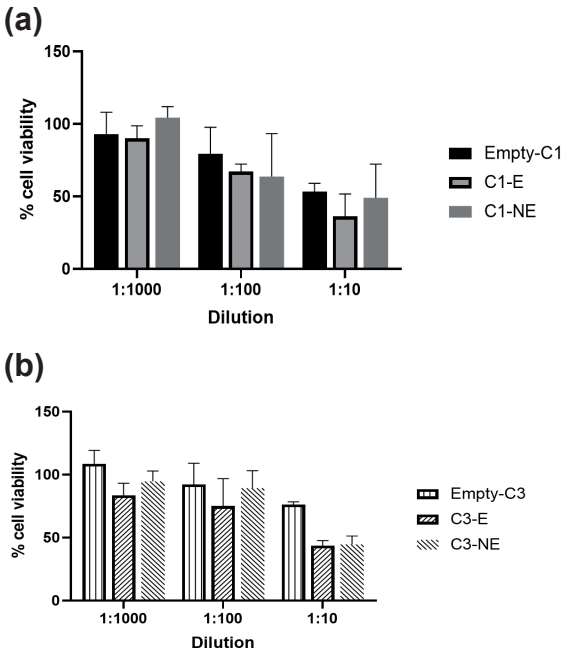

Figure 6. The cytotoxic effect at different dilutions of **(a)** C1-NE, and **(b)** C3-NE on HEK293 cells after 24-hour treatment by MTT assay. Error bars indicate the standard deviation of triplicates, where n=1.

Discussion

Formulation and preparation of Cur-polyherbal NE

Curcumin was able to completely dissolve within oil phase composed of VCO and surfactant GMO. VCO was selected due to its excellent ability to dissolve curcumin attributed to the saturated medium-chain fatty acids (MCFA) contents of VCO that promotes maximum curcumin loading into the NE system (40,41). This is in agreement with previous study whereby VCO demonstrated as a superior plant-based carrier oil for development of NE system for hydrophobic polyphenols such as curcumin (42). Another important consideration during the formulation of NE was to select components that are pharmaceutically acceptable. In this study, non-ionic surfactants (GMO and P-F127) were chosen as they are less toxic compared to the conventional ionic surfactants (43). Previous studies had also described the use of GMO and P-F127 in the development of NE (44), (45). Surfactant is required to create a stabilised interfacial tension present between oil and aqueous phase of NE by ensuring two immiscible phases are sufficiently adsorbed and thereby, providing homogenous NE system (46). Furthermore, it has been reported that the oil-aqueous interfacial tension can be further reduced with increasing surfactant-to-cosurfactant content (47). In this study, the NE system was prepared at varying GMO to P-FF127 ratio contents. Results showed that higher GMO to P-FF127 ratio in combination with higher VCO content exhibited mechanically stable NE system. This is consistent with previous study that reported the incorporation of cosurfactant promoted better interpenetration between surfactants, thereby producing compact interfacial tension that are resistant to particle flocculation (48).

Physiochemical characterisation of Cur-polyherbal NE

Organoleptic properties, pH and density

In this study, the pH values of the optimised formulations were found to be on a slightly acidic pH between 5.40 – 6.04, on average. It has been recommended that the acceptable pH value for mouthwashes should be more than 5.5 to prevent enamel erosion in acidic environment (pH < 5.5) (49). Several factors including chemical reactions and acidic ester contents of VCO might cause the formulations to be slightly acidic (50).

Particle size, PdI and zeta potential

The particle size of the Cur-polyherbal NE formulation served a critical role in providing enhanced curcumin bioavailability (16). The small particle size of NE systems provide greater surface area that enables better drug permeability (51). It was described that the conventional particle size for nano emulsion ranges between 10 – 200 nm in diameter (52). In this study, C1-NE and C4-NE presented with mean particle sizes of more than 200 nm. It was expected that increasing the VCO content with limited concentration of surfactants to compensate for the excessive oil droplet may contribute to the increment of particle size (53). On the other hand, all optimised formulations presented with PdI values less than 0.30 indicating a monodisperse distribution of the particles in the NE system and better stability (54)or lipidic carriers, are being extensively employed to enhance the bioavailability of poorly-soluble drugs. They have the ability to incorporate both lipophilic and hydrophilic molecules and protecting them against degradation in vitro and in vivo. There is a number of physical attributes of lipid-based nanocarriers that determine their safety, stability, efficacy, as well as their in vitro and in vivo behaviour. These include average particle size/ diameter and the polydispersity index (PDI.

The value of zeta potential provides an indication of the physical stability of an NE system and its resistance to particle flocculation based on electrostatic repulsion between the particles (55). It has been reported that an electrostatically stabilised NE system should present with zeta potential values around ± 30mV (56). In

this study, only formulation C2-NE and C3-NE presented with a more negative zeta potential values of -1.027 ± 0.120 mV and -1.12 ± 0.695 mV, relatively, which inherently suggest higher tendency to agglomerate, eventually. The negative zeta potential of the formulations may be due to the dispersion of the anionic fatty acids and glycerol present on GMO and VCO (57). It has been described that the physical stability of NE system is not exclusive to zeta potential values but also on the concentration of surfactant used during the formulation (45). The addition of sufficient surfactant contents may have provided a rigid oil-aqueous interfacial tension which may be sufficient to prevent destabilisation of the NE system.

Stability study

It has been reported that pH alterations due to chemical interactions greatly impact the stability and quality of NE systems due to the electrostatic repulsive interaction that disturb the interfacial tension (58)a major obstacle that remains to be overcome is the capacity of the active molecules in C. asiatica to cross the blood-brain barrier (BBB. Several authors had discussed the influence of pH alteration due to internal chemical reactions which eventually compromise the quality of emulsions. (59). It was also reported that NEs produced using vegetable oil, in this case was VCO, may observe a reduction in pH attributed to the hydrolysis of fatty acid esters (60).

FTIR analysis

The FTIR spectra of Cur-polyherbal NE formulations in comparison to their individual components showed no absence in any functional peaks in any of the spectra. Furthermore, no significant peak shifts or new functional group stretching were observed in the Cur-polyherbal NE formulations (C1-NE and C3-NE). However, some minor shifts in wavelength peaks were observed which may suggest that some interactions occurred between the components of the Cur-polyherbal NE formulations resulting in the broadening of the peaks.

Antioxidant activity

In this study, the selected NE formulations (C1-NE and C3-NE) were investigated for their antioxidant activity using DPPH assay based on the capability of curcumin to scavenge DPPH free radicals when loaded inside the NE system. The increased in DPPH scavenging activity of the NE formulations may be attributed from the well-known antioxidant properties of the polyherbal extracts (WTE, GE and curcumin) present in the NE system which might work synergistically to produce excellent antioxidant activity (61). This observation was expected as both curcumin and GE are renowned for their potent antioxidative properties (62). The increase in antioxidant activity of renowned antioxidant compounds after nanoencapsulation has been reported by some authors (63,64). It has been reported from a previous study that curcumin and resveratrol extracts displayed significant improvement in their antioxidant profile when associated into lipid-nanoencapsulation (65,66). The authors suggested that this was influenced by the kinetic release of antioxidants from the oil core. In-vitro cytotoxicity of Cur-Polyherbal NE using MTT Assay

Cytotoxicity of Cur-Polyherbal NE

In this study, it should be noted that the absolute concentration of the NEs were unable to be deduced as additional assay needs to be performed (67). Thus, in order to assess the cytotoxicity of the formulations, several dilutions of stock NE formulations were made in order to prepare different concentrations of C1-NE and C3-NE, accordingly. However, it was known that C1-NE and C3-NE stock solutions carried curcumin concentration of 250 µg/mL and 500 µg/mL, respectively. Thus, the cytotoxicity of C1-NE and C3-NE towards HEK293 cells were described by taking into account the concentration of curcumin (µg/mL) present at different dilution factors.

In this study, cytotoxicity of C1-NE and C3-NE were in a dose-dependent manner whereby reduction in percentage of cell viability

was observed with increasing concentration of the NE formulations. It can be deduced that C1-NE and C3-NE were evidently cytotoxic towards HEK293 cells when treated with both formulations at a lower dilution of 1:10, whereby, C1-NE and C3-NE had equivalent curcumin concentrations of 25 µg/mL and 50 µg/mL, respectively. This observation is in agreement with previous study that reported higher toxicity of curcumin-loaded NE (< 40% cell viability) at 40 µM on HaCaT cell line (68). Furthermore, the differences in particle sizes between C1-NE (268.3 ± 2.88 nm) and C3-NE (179.8 ± 2.15 nm) may influence the different cytotoxicity observed in this case. It has been reported that NE system with smaller particle size had an increased ability to efficiently deliver loaded drug, in this case was curcumin, across cellular compartments leading more exposure of the drug leading to cytotoxicity especially at a higher dose (69). On the other hand, both empty-C1 and -C3, which comprised of VCO, GMO and P-F127, did not exhibit any inherent cytotoxicity at 1:100 dilution which are in agreement with previous studies that regarded GMO and P-F127 as cytocompatible (70,71).

This study also demonstrated that both C1-NE and C3-NE generally showed higher cell viability when compared with free curcumin. Zheng et al. (72)which was then investigated against human embryonic kidney cells (HEK 293 reported that free curcumin exhibited 50% cytotoxicity at 10 µg/mL compared to curcumin nanoparticle (CNP) with less than 40% cytotoxicity at same concentration after 24 h incubation on HEK293 cells. Several narratives suggested that the reduction in cytotoxicity was accounted from the controlled and sustained release of curcumin when loaded inside NE system (68,73). This suggests that the incorporation of nanotechnology potentially promotes greater bioavailability of curcumin with less toxicity.

One of the limitations of this cytotoxicity study by MTT assay was the lack of independent experimental repeats and statistical analysis was unable to be performed due to the nature of the replicates. Thus, this study cannot conclude whether the cytotoxicity observed in dose-dependent manner between C1-NE and C3-NE was altogether significant. Furthermore, the unknown concentration of NE present on the formulations limits the understanding of the toxicity of formulations associated with NE system. Thus, additional assay should be performed in future studies to determine the concentration of NE present on the formulations. However, as this was only a preliminary study, results obtained from this cytotoxicity study can provide as guidelines for future studies to be performed using a lower concentration of curcumin loaded on NE system and assess its safety profile.

Conclusion

The selected Cur-polyherbal NE formulation (C1-NE and C3-NE) presented with mean particle sizes within 100 to 300 nm, low polydispersity index (PdI) (< 0.3), and acceptable stability when stored at room temperature. This study had shown that the NE formulation (C1-NE and C3-NE) had retained the renowned antioxidant properties of curcumin, WTE and GE, with DPPH scavenging activities over 90%. Moreover, when investigated with the corresponding non-nanosized formulation (C1-E and C3-E), a significant reduction in DPPH scavenging activities were observed ($p < 0.05$) which suggests the importance of nanotechnology in providing better therapeutic effects of the formulation. Whereas, cytotoxicity study of the formulations on HEK293 cells using MTT assay exhibited dose-dependent toxicity. This preliminary study serves as a baseline for further exploration of polyherbal NE formulations as mouthwash alternative. Studies such as antimicrobial study on plaque-inducing pathogens should be evaluated in the future to investigate the efficacy of the Cur-polyherbal NE mouthwash to control gingival and periodontal diseases.

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Competing interests

The authors declare that they have no competing interests.

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