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RESEARCH ARTICLE

Formulation and characterization of 6,6′-dihydroxythiobinupharidine and phloroglucinol nanoemulsion for intranasal approach to neurodegenerative disorders

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Abstract

Neurodegenerative diseases involve complex pathological mechanisms, including oxidative stress and neuroinflammation, which are not adequately addressed by single-target therapies. Therefore, a dual-targeted strategy is to be employed by combining phloroglucinol, a potent antioxidant, with 6,6′-dihydroxythiobinupharidine, a selective protein kinase C inhibitor. Thus, the present study aimed to formulate and evaluate phloroglucinol and 6,6′-dihydroxythiobinupharidine nanoemulsion (NE) for intranasal administration and a dual-targeted approach. NE was formulated using coconut oil, Span80, Tween80 and PEG400 in the ratio of 4.5:4.5:5.5:5.5. After formulation, it was evaluated for size, shape, drug release and stability. NE was homogenously opaque white and clear i.e., without any visible drug particles. It was an oil-in-water type of NE with a circular shape and size of <150 nm. It possessed a polydispersity index of 0.238, a zeta potential of -26.2 mV and was stable for more than 90 days. It was readily dispersible upon addition of water and showed no separation of oil and water. There was no sign of precipitation or cracking and exhibited a percentage transmittance of 93.4 ± 0.67. Nanoemulsified phloroglucinol and 6,6′-dihydroxythiobinupharidine had a cumulative release rate of 88.79 ± 1.92 and 90.39 ± 1.26, respectively, after 2 hr and 1 hr. Over 90 days, an increase in the particle size and a faint odor were observed. Therefore, these results indicate that the NE of phloroglucinol and 6,6′-dihydroxythiobinupharidine enables efficient and targeted delivery of drugs directly to the brain and improves treatment effectiveness for neurodegenerative disorders.

Keywords: Alzheimer's disease; 6,6′-dihydroxythiobinupharidine; intranasal; nanoemulsion; phloroglucinol

Introduction

Neurological disorders, including Alzheimer's disease (AD), Parkinson's disease and other forms of dementia, represent a major and growing global health burden. However, effective pharmacological treatment remains elusive due to significant challenges in delivering drugs to the central nervous system (CNS). A primary unmet need is the effective targeting of therapeutics to the brain with conventional oral medications due to the hindrances posed by the hematoencephalic barrier (1). Thus, to bypass the hematoencephalic barrier, intranasal administration of drugs was demonstrated by William Frey in 1989 (2). The primary benefit of this method is that it can deliver small or large molecules through the olfactory, trigeminal or systemic pathways (2, 3). Additionally, the intranasal method is non-invasive, feasible for self-administration and offers rapid absorption (1, 4, 5). In efforts to manage neurological disorders, clinicians have employed various intranasal medications, each with distinct formulations. However, these treatments have been associated with adverse reactions, including polyuria, cardiomyopathy, nasal irritation and several other side effects (2).

Phloroglucinol acts as a spasmolytic and has antioxidant and antimicrobial properties (6, 7). It also crosses the hematoencephalic barrier, degrades amyloid aggregates and decreases neurotoxicity, thus showing neuroprotective activity (8). In 5XFAD mice, oral administration of phloroglucinol improved cognitive function, reduced amyloid plaque accumulation, restored dendritic spine density and decreased pro-inflammatory cytokine levels (9). Phloroglucinol also hindered the initiation of aggregation and blocked the clumping of $A\beta_{1-42}$ with homocysteine aggregates (8). Increased phloroglucinol in the bloodstream can cause adverse effects such as palpitations, nausea, vomiting, etc (10). Therefore, oral and intravenous administration of phloroglucinol can be excluded and alternative methods like intranasal drug delivery can be considered.

Complementing the actions of phloroglucinol, 6,6′-dihydroxythiobinupharidine (DTBN) was discovered in the late 80s and is now attracting renewed interest due to its promising applications and advances in research. It is a sesquiterpene alkaloid that can be isolated from *Nuphar* species (11). Recent literature shows that DTBN enhances the activity of topoisomerase IIα and IIβ (human) in DNA cleavage roughly 8 times and 3 times, respectively (12). It exhibits anti-inflammatory effects primarily by inhibiting NF-κB. Moreover, it alleviated kidney failure by decreasing inflammatory markers in chronic kidney disease C57BL/6J mice (13). DTBN has been shown to inhibit the catalytic activity of protein kinase C (PKC)

(14). This essential enzyme plays a role in signal transmission pathways, including cancer. PKC has multiple brain functions such as synaptic plasticity, learning, memory and the survival of neurons. Its dysfunction is of particular concern in AD, where irregular PKC signaling has been associated with the onset of AD and other neurological conditions (15). Given that DTBN increases the activity of human topoisomerases, it is advisable to use it in smaller amounts. Therefore, administering DTBN via the intranasal route is preferred over oral or other methods.

Despite advances in neuropharmacology, current therapies for neurodegenerative disorders predominantly target single molecular pathways, which have proven insufficient due to the multifactorial and interconnected nature of these diseases. A growing body of recent reviews highlights that this “one target-one drug” paradigm is unable to halt or reverse disease, prompting a shift toward dual- or multi-target strategies that can simultaneously address multiple pathogenic processes and provide more comprehensive neuroprotection (16). For example, combining phloroglucinol, a potent antioxidant modulating oxidative damage, with DTBN, a selective PKC inhibitor that mitigates neuroinflammation, offers a rational dual-targeted approach that exploits synergistic mechanisms to disrupt the oxidative stress-inflammation cycle central to neurodegeneration.

Moreover, nanoemulsion (NE)-based drug delivery systems offer transformative advantages in this context. NE is a stable two-phase system in which oil is dispersed in water as nano-sized droplets (17). These are easy to administer, highly convenient for usage and economical (18). These render a higher advantage over suspension and emulsion in bioavailability, stability and solubility parameters (19, 20). A NE is a combination of oil, surfactant, cosurfactant and water. Surfactants and cosurfactants assist in tapering the surface tension between water and oil, with solubilization and uniform dispersion of the drug molecule (19, 21).

Intranasal administration of drugs via NE efficiently delivers the drug to the brain compared to any other route of administration (1). These NEs must have a particle size <200 nm for enhanced nasal permeation and brain absorption (2). The previous study investigated the pathways involved in the nasal delivery of huperzine A. They reported that NE can be transported via nerve and systemic pathways, potentially elevating the drug concentration in the brain by inhibiting efflux pumps (22). To further improve brain targeting, vinpocetine NE was combined with borneol, a known brain-targeting molecule. At a borneol concentration of 1 mg/kg, vinpocetine NE achieved a drug-targeting index of 596.1 % and a direct transport percentage of 83.1 % to the brain (23). Recent advancements in managing neurological disorders include dual or multi-target approaches. In a murine model with scopolamine-induced cognitive impairment, simultaneous administration of donepezil and memantine. They reported successful formulation, administration and distribution of the NE through the nanocolloidal carriers (24). However, only a few studies have been reported on dual or multi-target approaches.

Thus, this study hypothesizes that the dual-targeted NE formulation of phloroglucinol and DTBN in coconut oil, administered intranasally, enables efficient, stable and synergistic delivery of antioxidant and anti-inflammatory agents directly to the brain, thereby improving therapeutic efficacy against neurodegenerative disorders by simultaneously disrupting oxidative stress and neuroinflammation. To the best of our knowledge, this study reports the first successful formulation and characterization of a dual-drug NE containing phloroglucinol and DTBN utilizing coconut oil as the oil phase for intranasal administration.

Materials and Methods

Materials

Pure coconut oil was obtained from a regional oil processing facility in Puttur (Karnataka, India). The chemicals purchased from Sigma Aldrich (St. Louis, MO, USA) were DTBN, surfactants and cosurfactants such as Span80, Tween80 and PEG400 and were of analytical grade. Distilled water was utilized for the preparation of NE. The remaining solvents and reagents utilized were of high purity and suitable for analytical applications.

Evaluating the ingredients of NE

In a series of stoppered glass vials, 0.03 mg of DTBN and 300 mg of phloroglucinol were added to 5 mL of surfactants, oils and cosurfactants. The heat was supplied to these vials from a shaking water bath (REMI, India) set at 25 ± 1 °C and equilibrated for 72 hr under dark conditions to ensure that the system reaches thermodynamic equilibrium, allowing for complete solubilization of components, uniform distribution of drug in all phases and reliable phase separation or stability mapping. Later, the mixtures within these vials were centrifuged (Weswox, WT-60) at 7000 rpm for 900 sec. The supernatant was assessed for concentrations of DTBN and phloroglucinol using UV spectrophotometry (SHIMADZU, UV-16100, Japan; range: 190 to 1100 nm). For this, supernatant (10 µL) was diluted with absolute ethanol (1 mL) and absorbance at 331 nm for DTBN and 269 nm for phloroglucinol was recorded, where the corresponding media was used as a blank (25).

Assessment of surfactant choice

A 4 µL of the coconut oil was gradually mixed into 2.5 mL of Span80/Tween80 solution (15 % w/w) with vigorous agitation. The process was continued by adding oil in increments until the solution turned opaque (25). This procedure was performed in triplicate (n = 3) to ensure reproducibility of the observations. No turbidimeter or instrumental turbidity measurement was used at this stage.

Verification of cosurfactant proportions

To verify the composition of the cosurfactant, pseudoternary phase diagrams were created with a constant surfactant:cosurfactant (1:1; Smix) ratio. Various Smix ratios were used to map these diagrams. Each formulation point within these diagrams was assessed visually to confirm the formation of a single, transparent phase (25).

Pseudoternary phase diagram construction

Initially, the surfactant and cosurfactant were blended in a range of specific weight ratios (1:0, 1:1, 1:2, 1:3, 1:4, 2:1, 3:1 and 4:1) to create the surfactant-cosurfactant mixture (Smix). This Smix was combined with the chosen oil in various proportions (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1). Water was gradually added until the clear mixture became opaque. The procedure was performed at controlled room temperature of 25 ± 1 °C. The volumes of oil, Smix and water were converted into percentages and plotted using OriginPro 8.5.1.315 software. In the resulting pseudoternary phase diagrams, the areas were measured and the diagram with the largest NE region was selected for further analysis (25). The data points are provided in Supplementary Material 1.

Formulation of NE

DTBN and phloroglucinol NE were prepared employing magnetic stirring (REMI, 2MLH) and probe sonication (ultrasonic homogenizer; PKS-750FL) method. In this experiment, 100 mg of phloroglucinol and 0.02 mg of DTBN were added into 4.5 mL of coconut oil in a beaker kept on a magnetic stirrer with continuous stirring at 2000 rpm for 30 min maintained at 40 °C. Later, 5.5 mL, 4.5 mL and 5.5 mL of Tween80, Span80 and PEG400 were added. Finally, this emulsion was made up to 50 mL with water and stirred continuously till the system attained homogeneity. Later, the emulsion was subjected to probe sonication for 300 sec with sonication time for 30 sec with a gap of 5 sec, power 70 % and temperature maintained at 22 °C using an ice bath and these parameters were selected based on widely accepted NE optimization studies.

NE characterization

Emulsion type determination

To identify the emulsion type, methylene blue, a water emulsion dye (0.1 % w/v in water) or Sudan III, an oil emulsion dye (0.1 % w/v in oil) was added to the mixture. The emulsions were then examined under a microscope (AmScope, M150C-I) for confirmation (26).

Dispersibility and percentage transmittance measurements

To assess dispersibility, 200 mL of distilled water and 1 mL of the NE were incorporated and stirred at 50 rpm with a magnetic stirrer at 34 °C (25). Particle size analyzer such as Litesizer-500 equipped with Litesizer software version 3.02.02 (Anton Paar GmbH), measured transmittance percentages of the formulations.

Zeta potential, globule size and polydispersity index (PDI)

Dilution of sample and distilled water (for multiple scattering prevention) in the ratio of 1:100 was conducted and then transferred to a polystyrene cuvette. The cuvette was positioned in the particle size analyzer for accurate measurement of globule size, PDI and zeta potential (27).

Stability test

For all stability and thermal stability tests, the samples were transferred into tightly sealed glass vials immediately after preparation.

NE stability was evaluated by exposing it to 40 °C temperature over three months. At four distinct time points (0-90 days; 0, 30, 60 and 90 days) the extracted samples were examined for an increase/decrease in droplet size (28).

Thermal stability

To understand the effect of thermal changes on sample stability, the samples were subjected to 6 cycles of temperature changes between 45 °C and 4 °C, with at least 48 hr at that temperature (29).

Centrifugation

The samples underwent centrifugation for 1800 sec at 2000 rpm. Samples that showed signs of phase separation, creaming or cracking were eliminated (29).

Viscosity and pH viscosity measurements

The sample viscosity was assessed using a Brookfield viscometer (Brookfield, DV1 M), comprising spindle No. 6 at a rotation speed of 100 rpm. Additionally, a digital pH meter (MK VI) measured the pH at 25 °C (25). Both instruments were calibrated according to the manufacturer's instructions prior to measurements.

Measurement of drug content

The prepared NE were quantitatively evaluated for the presence of phloroglucinol and DTBN. The lambda max (λ_{max}) of the individual drugs was assessed using absolute ethanol as a solvent. Phloroglucinol and DTBN standard calibration curves were prepared using serial dilutions of the drugs in absolute ethanol. The calibration curves for phloroglucinol (R^2 = 0.9988) and DTBN (R^2 = 0.9989) were linear over the tested concentration ranges. The absorbances were observed at determined λ_{max}. The obtained absorbance was plotted against the respective concentration, followed by scatter plotting the graph. The resulting calibration curve was analyzed and the regression factor (R^2) and equation of the slope were determined.

The concentration of the drug (phloroglucinol and DTBN) present in 5-50 µL of the NE was calculated and is regarded as theoretical drug content. Based on the equation of the slope for phloroglucinol and DTBN determined, the unknown concentration of phloroglucinol and DTBN at different volumes of the sample (5-50 µL) were calculated by considering the absorbance at 269 nm for phloroglucinol and 331 nm for DTBN and this value was regarded as analyzed drug content. Percentage of drug content = Analyzed/ theoretical drug content multiplied by 100.

Drug release study (*in vitro*)

Phosphate buffer saline (PBS) was prepared and the calibration curves for phloroglucinol and DTBN were plotted. The dialysis bags were thoroughly rinsed with deionized water and soaked overnight in PBS of 6.4 pH. A 1 mL sample of the NE, comprising 2 mg of phloroglucinol and 0.4 mg of DTBN were loaded into a dialysis bag and immersed in 100 mL of PBS (pH 6.4) as the release medium. The bags containing the receptor solution were then placed on a thermal magnetic stirrer set at 100 rpm and maintained at 34 ± 0.5 °C. A milliliter of the PBS was extracted from the system and was substituted with the fresh PBS at pre-determined time intervals (0-24 hr), thus maintaining sink conditions by ensuring the drug concentration in the release medium remained below saturation and preserving a constant concentration gradient. The extracted solution was shifted to a new covered test tube and made up to 2 mL using PBS and stored at 4 °C in the dark until absorbance was measured. The values obtained were converted into 10 folds and cumulative drug release was calculated. All the samples were evaluated in triplicates. The release profiles of phloroglucinol and DTBN from the prepared NE were represented as the cumulative percentage of drug released plotted against time (25).

Organoleptic assessment

The NE samples were stored in tightly closed containers at ambient room temperature (25 ± 2 °C) and protected from direct light throughout the 4-week organoleptic evaluation period. Every week for four weeks, a physical examination was conducted on all NE samples. The evaluated parameters included color, odor and any visible phase separation (30).

Transmission electron microscopy (TEM) analysis

In brief, 20µL of the NE was placed onto a carbon-coated copper specimen grid (200 mesh size), where it was allowed to absorb for 10 min. Excess liquid was gently removed using filter paper to avoid damaging the grid. The grid was subsequently stained by adding a single drop of 3 % w/v phosphotungstic acid and left to air dry for 3 min in a dust-free environment. This negative staining technique enhanced contrast, facilitating clear visualization of the NE droplets. After drying, the prepared grid was examined using a Hitachi TEM HT -7700, which operated at a 60kV accelerating voltage to assess the droplet morphology and size (31).

Results

NE components screening

DTBN and phloroglucinol were dissolved in various surfactants, cosurfactants and oils. Tween80, PEG400 and coconut oil showed the highest absorbance values. Absorbance measurements were performed to quantitatively assess the solubility of both phloroglucinol and DTBN in each tested component (oil, surfactant and cosurfactant) after the equilibration period. Samples with the highest absorbance indicated superior solubilization capacity for the respective drug, allowing optimal component selection for NE formulation. Visual inspection was used to detect any signs of incompatibility or precipitation among components under equilibrium conditions, but no incompatibility or precipitation was observed. Absorbance values of DTBN and phloroglucinol in different surfactants, cosurfactants and oils are provided in Table 1.

Validation of surfactant

In the present study, Tween80 was prioritized for further formulation development because it exhibited superior compatibility and the highest miscibility with coconut oil among the surfactants tested, allowing greater volumes of oil to be solubilized before the onset of turbidity. Addition of Tween80 was 8 additions × 4 µL coconut oil, whereas Span80 and Tween20 had 3 additions × 4 µL oil and 5 additions × 4 µL oil, respectively, for the solution to get visibly opaque white.

Validation of cosurfactant ratio

Regarding cosurfactant ratio determination, PEG400 with Tween80 and coconut oil showed the highest area in the 1:1 ratio of Smix:coconut oil when compared with other ratios. This NE region indicates enhanced compositional flexibility and a broader range of stable formulations. The balanced proportion of surfactant and cosurfactant at 1:1 optimizes interfacial fluidity and reduces tension, facilitating efficient emulsification and improving NE stability. This approach follows established NE formulation strategies where the Smix ratio yielding the widest isotropic region is prioritized to maximize the robustness and reproducibility of the final system. The pseudoternary diagram of the ratio 1:0 of Smix was not drawn as the addition of water caused direct solidification of the system. Solidification was attributed to compositional instability, where the absence of a cosurfactant led to the formation of viscous or gel-like phases upon water addition, rather than the formation of a stable NE. The inclusion of a cosurfactant at appropriate ratios disrupted these ordered phases and enabled the formation of fluid, stable systems. The area for rest of the Smix were 1:1 = 30.06 %, 1:2 = 28.90 %, 1:3 = 29.57 %, 1:4 = 28.71 %, 2:1 = 28.52 %, 3:1 = 27.40 % and 4:1 = 27.12 %. The phase diagram corresponding to the 1:1 Smix ratio is presented in Fig. 1, clearly demonstrating the superior NE formation compared to the other ratios tested.

Pseudoternary phase diagrams

In the pseudoternary phase diagram, the 'area' represents the compositional domain in which the formulated mixtures produced stable and transparent NEs without evidence of phase separation or turbidity.

The lower concentration of Smix gave a milky appearance, which was kinetically stable, with little amounts of water in the NE formulation, whereas, as the concentration increased, it would consume more water and result in the formation of a gel. The Smix weight ratio (1:1) showed the highest area of 30.06 %. There was a slight reduction in the area when the Tween80 concentration was increased. There was also a slight decrease in area when PEG400 concentration was increased. Thus, Smix:coconut oil ratio was 1:1 which was used for further experimentation. This area is considered substantial compared to commonly reported values in literature. NE phase diagrams with areas above 20-25 % are regarded as offering robust formulation flexibility; thus, the 30.06 % area reflects favorable compositional tolerance and indicates a wide range of successful, stable NE formulations in this system.

From the literature, the concentration of effective surfactant blend was found to be 5.5:4.5 of Tween80 and Span80, respectively. Since PEG400 is also to be taken in as same amount as per the pseudoternary diagram, it was taken in the amount of 5.5 mL.

To determine the concentration of coconut oil to be used, the Smix was mixed with different concentrations of coconut oil and was observed for emulsion formation. Coconut oil at a concentration of 4.5 mL was observed with a visibly opaque white stable emulsion (Table 2).

To prepare the NE with the lowest possible particle size, the emulsion was subjected to ultrasonication at different time intervals. The NE with the lowest particle size was successfully created at the end of 300 sec of ultrasonication (Table 3). As the sonication time increased, the mean droplet diameter decreased significantly, indicating improved emulsification and enhanced kinetic stability of the system. Specifically, particle size reduced from 386.2 nm at 180 sec to 73.8 nm at 300 sec, after which no substantial decrease was observed. This plateau suggests that additional sonication beyond 300 sec offers no further advantage in size reduction and may risk droplet coalescence due to over-processing.

Table 1. Absorbance values of DTBN and phloroglucinol in various oils, surfactants and cosurfactants

	Value of DTBN (331 nm)	Value of phloroglucinol (269 nm)
Oils		
Arachis oil	0.021	0.016
Coconut oil	0.042	0.035
Castor oil	0.009	0.013
Surfactants		
Tween80	0.028	0.025
Tween20	0.013	0.018
Span80	0.015	0.021
Cosurfactants		
PEG400	0.033	0.037
Ethanol	0.026	0.029
Glycerol	0.021	0.025

Values are presented as mean values. DTBN: 6,6'-dihydroxythiobinupharidine.

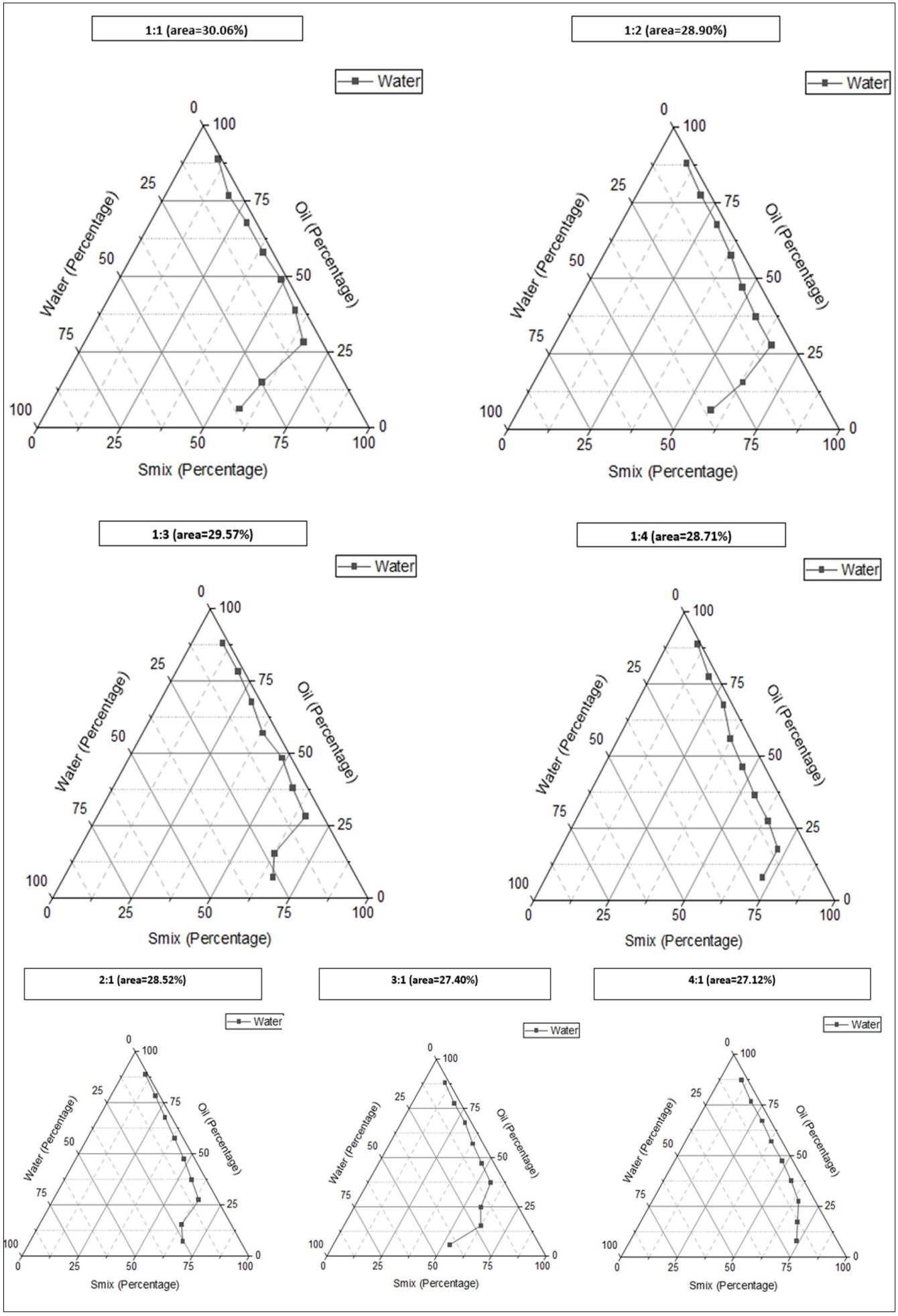

Fig. 1. Pseudo ternary phase diagrams of Tween80, PEG400 and coconut oil at various ratios. PEG: polyethylene glycol.

Table 2. Particle size distribution of the DTBN and phloroglucinol NE

S.No.	Coconut oil concentration	Inference
1	4 mL	Off-white emulsion formed
2	4.5 mL	Visibly opaque white stable emulsion formed
3	5 mL	Separation
4	5.5 mL	Separation
5	6 mL	Separation

DTBN: 6,6′-dihydroxythiobinupharidine; NE: nanoemulsion.

Table 3. Particle size of the NE at various ultrasonication time intervals

S.No.	Sonication time (min)	Particle size (nm)
1	3.0	386.2 ± 3.5
2	4.0	167.7 ± 2.8
3	5.0	73.8 ± 7.1
4	6.0	211.3 ± 5.5
5	7.0	434.6 ± 1.2

Values are expressed as mean ± SEM.

Characterization of NE

Determination of emulsion type

According to accepted classification, emulsions with droplet sizes in the range of 20-200 nm are categorized as NE. The droplet size observed in this study (103.10 nm) thus confirms its designation as an NE. The NE formed was visibly opaque white, clear and homogenous without any visible drug particles. Water-soluble dye (methylene blue) was used to detect the type of emulsion. It diffused fast in water and oil bubbles could be seen with a blue background, indicating o/w NE.

Dispersibility and % transmittance measurements

The NE dispersed rapidly with the addition of water and remained clear. No precipitation or cracking was observed upon visual inspection at the time of preparation at room temperature. The emulsion had a percentage transmittance of 93.4 ± 0.67. NEs with a percentage transmittance closer to 100 % are considered ideal. However, values above 90 % still indicate a clear, transparent and well-dispersed system with nanometric droplet size.

Determination of PDI, globule size and zeta potential of NEs

PDI of <0.1 indicates highly monodisperse (very uniform droplet sizes), whereas, 0.1-0.3 indicates moderately narrow or uniform distribution, typical of well-optimized NEs and >0.3 indicates increasingly broad/dispersed droplet sizes, suggesting less physical uniformity and potential stability concerns. The PDI in this study was measured to be 0.238 ± 0.008. Thus, this confirms the emulsion has a moderately narrow, acceptable size distribution, which is a characteristic of a well-formed and stable NE.

As shown in Fig. 2, the NE exhibited a hydrodynamic diameter of 104.84 ± 1.75 nm, which falls within the typical size range for NEs (20-200 nm), confirming successful formulation. A zeta potential value greater than ±20 mV is generally considered the threshold for acceptable stability in NEs, as it indicates sufficient electrostatic repulsion to prevent droplet aggregation and ensure physical stability of the formulation. Fig. 3 illustrates the zeta potential of the NE was determined to be −26.2 ± 2.4 mV.

Stability studies

The physical stability of the NE was evaluated by monitoring particle size changes over 90 days while storing samples at a controlled temperature of 40 °C (Table 4). The study was conducted in closed containers under ambient laboratory humidity (approximately 50-60 % relative humidity) without direct exposure to light. The NE exhibited an initial particle size of 119 ± 1.2 nm, which gradually increased to 133 ± 1.7 nm at 30 days, 167 ± 0.9 nm at 60 days and 184 ± 2.6 nm at 90 days. Throughout the study, the NE remained below 200 nm, retaining its classification as a NE. Importantly, during the entire storage period at elevated temperature, no visible phase separation, cracking, or creaming was observed.

Table 4. Particle size of the NE over 90 days

S.No.	No. of days	Particle size (nm)
1	0	119 ± 1.2
2	30	133 ± 1.7
3	60	167 ± 0.9
4	90	184 ± 2.6

Values are expressed as mean ± SEM.

Measurement of drug content

All formulations exhibited a drug content exceeding 95 %, validating the preparation method's effectiveness and the accuracy of the dosing in each formulation (Table 5 and 6). This high drug content indicates that most of the initially introduced drug was successfully incorporated within the NE droplets, with minimal loss during

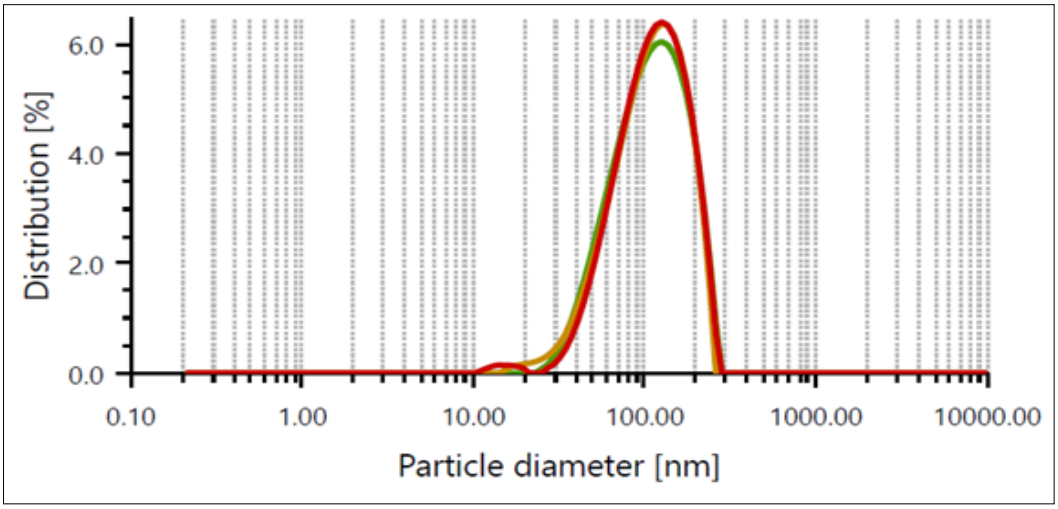

Fig. 2. Particle size distribution of the co-loaded DTBN and phloroglucinol NE. The multiple peaks, Green = 104.84 nm; Yellow = 103.10 nm; and Red = 106.6 nm, are the particle sizes of the same formulation, confirming consistent particle size characteristics. All curves represent the same co-loaded DTBN and phloroglucinol NE sample. Particle size distributions were measured by DLS and are presented as intensity-based distribution (%) as calculated by the instrument software. The mean particle size was 104.84 nm with a PDI of 0.238 ± 0.008 as calculated from three technical replicates. DTBN: 6,6′-dihydroxythiobinupharidine; NE: nanoemulsion.

Table 5. Drug content analysis of phloroglucinol at varying volumes

NE volume (µl)	Absorbance recorded	Drug content (µg)	Drug content (%)
10	0.098	19.17	95.83
	0.097	18.96	94.79
	0.095	18.54	92.71
			94.44 ± 1.59[a]
20	0.186	37.50	93.75
	0.181	36.46	91.15
	0.191	38.54	96.35
			93.75 ± 2.60[a]
30	30	0.279	56.88
	0.282	57.50	95.83
	0.275	56.04	93.40
			94.67 ± 1.22[a]
40	40	0.379	77.71
	0.383	78.54	98.18
	0.374	76.67	95.83
			97.04 ± 1.17[a]
50	50	0.471	96.88
	0.469	96.46	96.46
	0.481	98.96	98.96
			97.43 ± 1.34[a]

[a]Mean ± SEM from three replicate measurements.

Table 6. Drug content analysis of DTBN at varying volumes

NE volume (µl)	Absorbance recorded	Drug content (µg)	Drug content (%)
10	0.111	3.76	93.92
	0.109	3.69	92.19
	0.113	3.83	95.66
			93.92 ± 1.74[a]
20	0.227	7.78	97.31
	0.225	7.72	96.44
	0.221	7.58	94.70
			96.15 ± 1.33[a]
30	0.341	11.74	97.86
	0.338	11.64	96.99
	0.335	11.53	96.12
			96.99 ± 0.87[a]
40	0.454	15.67	97.92
	0.457	15.77	98.57
	0.455	15.70	98.13
			98.21 ± 0.33[a]
50	0.576	19.90	99.51
	0.565	19.52	97.60
	0.571	19.73	98.65
			98.59 ± 0.96[a]

[a]Mean ± SEM from three replicate measurements.

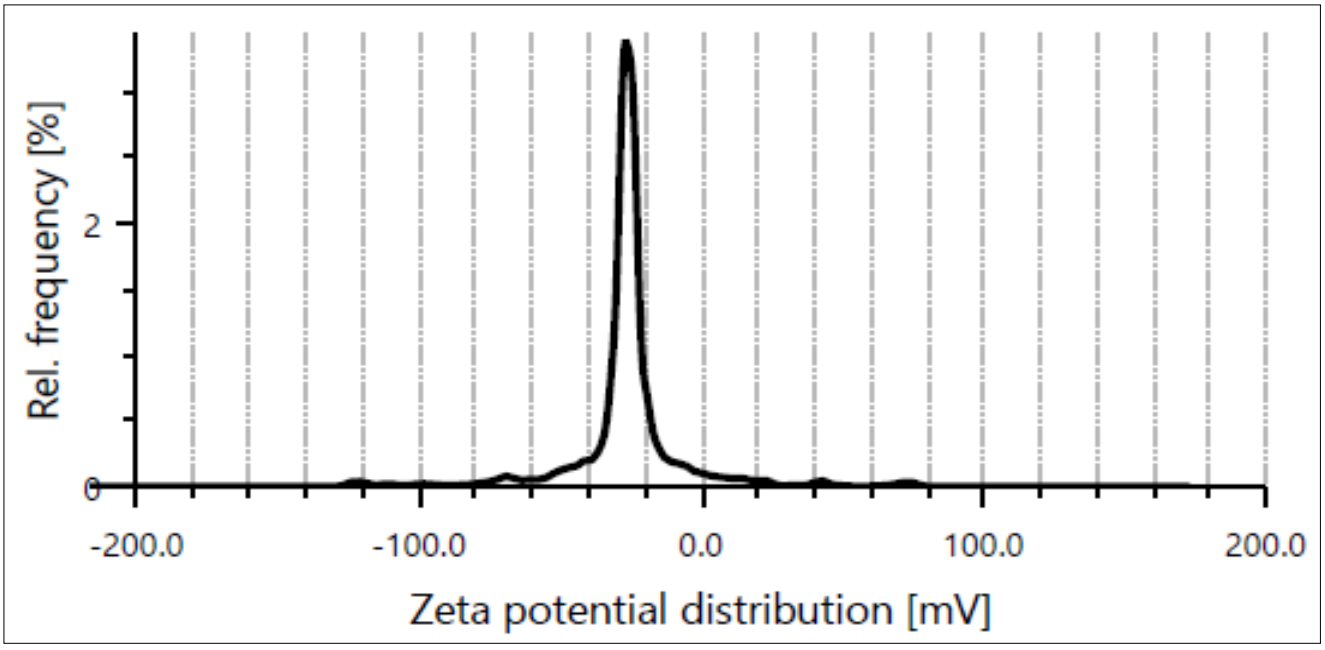

Fig. 3. Zeta potential of the DTBN and phloroglucinol NE. DTBN: 6,6′-dihydroxythiobinupharidine; NE: nanoemulsion.

preparation. Drug loading efficiency above 95 % is widely recognized in the literature as evidence of efficient encapsulation, demonstrating that the formulation process was effective and there was negligible drug partitioning into the external phase or precipitation. Such high encapsulation efficiency ensures consistent dosing, optimal therapeutic effect and minimal wastage, which are critical factors in the quality and reliability of NE-based drug delivery systems.

To ensure the consistency and reliability of the NE formulation, multiple batches were prepared following the same optimized procedure. Key parameters such as particle size, PDI, zeta potential and drug content were measured for each batch. Results showed minimal variation between batches, with particle size and zeta potential values consistently within the reported ranges and drug content exceeding 95 %.

***In vitro* release**

The cumulative drug release profiles of phloroglucinol and DTBN in the PBS system revealed a clear distinction between conventional and NE formulations (Fig. 4). At 1 hr, phloroglucinol NE achieved a cumulative release of 78.58 ± 3.25 %, compared to 28.94 ± 2.13 % from the conventional formulation representing approximately a 2.7 -fold increase in release rate for the NE at this time point. Similarly, DTBN NE showed 90.39 ± 1.00 % release at 1 hr, versus 38.89 ± 1.74 % for conventional DTBN, corresponding to a 2.3-fold faster release. Conventional phloroglucinol and DTBN exhibited a steady release, reaching peak concentrations at 4 and 5 hr, respectively. This accelerated release from NEs can be attributed to their smaller droplet sizes, which increase the interfacial surface area and facilitate enhanced drug diffusion into the PBS medium. Consequently, the NEs provide improved drug release kinetics compared to their conventional counterparts, which is advantageous for rapid onset of action and potentially improved bioavailability. The data points are provided in Supplementary Material 2.

Throughout the *in vitro* release study, sink conditions were maintained by ensuring the drug concentration in the PBS release medium remained below 10-30 % of drug saturation solubility, preventing drug precipitation and ensuring continuous diffusion-driven release. The PBS volume and sampling intervals were chosen accordingly to sustain sink conditions, enabling reliable measurement of cumulative drug release over time.

Organoleptic evaluation

Organoleptic testing was conducted with 10 participants at the beginning (week 0) and again after four weeks of storage. The participants aged 23-24 years, without any acute or chronic illness and with no known hypersensitivity to any component of the formulation were included. Organoleptic testing parameters included color, odor and phase separation. The color of the formulation was visually examined to monitor any changes in opacity or hue over time. Odor was assessed for any change in intensity or development of undesired smells, indicating possible ingredient degradation. Phase separation was checked visually to ensure the formulation remained physically homogeneous without stratification or creaming. The observations are summarized in Table 7. From these observations, it can be considered that the prepared NE is stable with a faint odor, which may be because of Tween80.

TEM analysis

TEM analysis (Fig. 5) revealed dark, circular spots corresponding to NE globules against a lighter background. The images were captured at a magnification of 75000× and the scale bar represents 500 nm as indicated in the figure. The globules were uniformly distributed, discrete and showed no evidence of aggregation. The droplets appeared predominantly spherical in shape and this morphology was consistent across all observed fields, indicating a homogeneous and stable NE system.

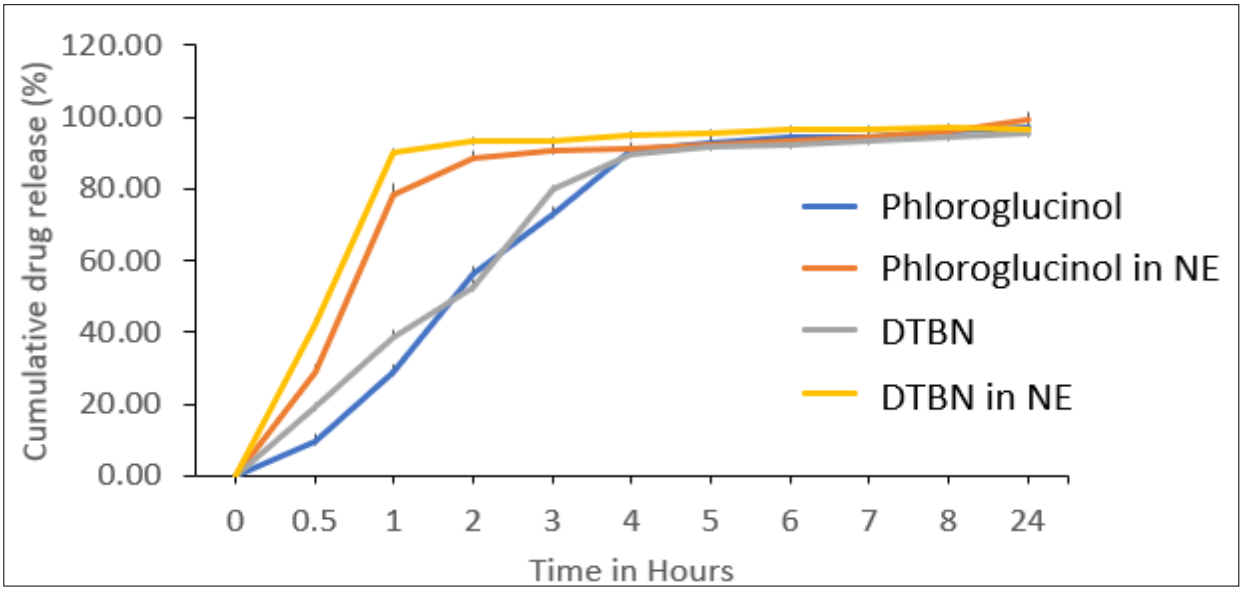

Fig. 4. *In vitro* release of NE loaded with phloroglucinol and DTBN in comparison with pure phloroglucinol and DTBN. DTBN: 6,6'-dihydroxythiobinupharidine; NE: nanoemulsion.

Table 7. Organoleptic evaluation of the DTBN and phloroglucinol NE

S. No.	Test	Week 0	Week 4
1	Color	Visibly opaque white	Off white
2	Odour	Slightly	Moderate
3	Phase separation	No	No

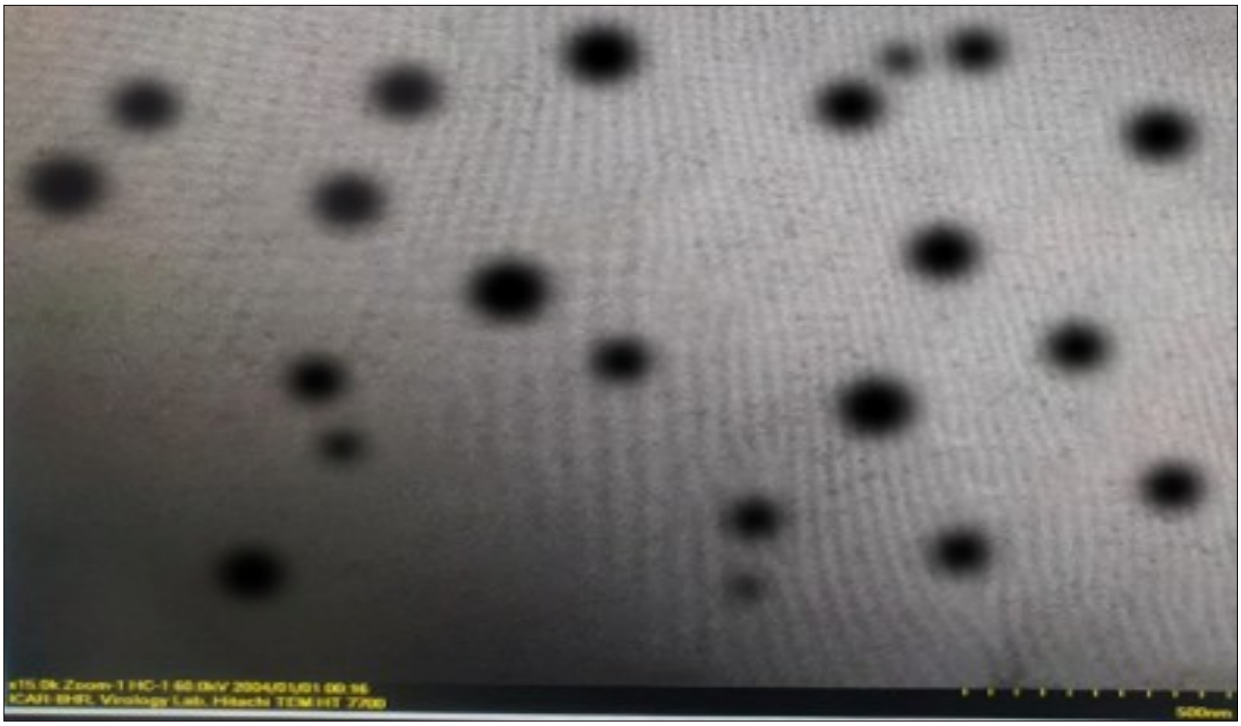

Fig. 5. TEM image of the DTBN and phloroglucinol NE. TEM image was observed at 15× magnification. DTBN: 6,6′-dihydroxythiobinupharidine; NE: nanoemulsion; TEM: transmission electron microscopy.

Discussion

Surfactants play a crucial role in the formulation and stabilization of NE due to their dual nature. They function as intermediaries between the oil and water phases, decreasing interfacial tension and minimizing the energy necessary for the formation of the NE (32). Additionally, surfactants can hinder the merging of globules by creating a layer and affect the lipid bilayer, thereby enhancing the permeability into the membrane (33). Tween80, classified as a nonionic surfactant, possesses a hydrophilic-lipophilic balance (HLB) value of 15 (25). It has been proved that it could create oil-water NE with small and evenly distributed droplets. Furthermore, the selection of Tween80 as the preferred surfactant was influenced by its critical micelle concentration and its superior stability profiles *in vivo*. This choice was made due to its effect and negligible impact on pH levels (34). In the present study, Tween80 has shown more absorbance than the rest of the surfactants. This explains the good solubility and is in accordance with previously published literature.

The addition of a cosurfactant helps to decrease the quantity of surfactant required. This cosurfactant aids in reducing the tension on the surface by allowing the film between each globule to be more flexible enabling it to adapt and conform (33, 35). Furthermore, the cosurfactants addition enhances the fluidity of the film leading to changes in its curvature and resulting in the formation of NE across compositions (25). Cosurfactants enhance the mobility of the hydrocarbon tails thereby facilitating oil penetration in the NE region (36). In the present study, PEG400 has shown good solubilization of drugs and it has been reported in the recent literature that it increases solubility and stability (37), which is in line with the present study.

A literature survey has shown that the inclusion of Span80 in the preparation of NE has increased the stability of the system. This is because Tween80 has a larger head group and Span80 has a smaller head group. These smaller head groups can effectively pack alongside larger head groups at the oil-water interface (38). Thus, in the present study both Tween80 and Span80 have been used.

The solubility of the drug in surfactants and cosurfactants should not be the sole criterion considered when selecting components for NE, thus miscibility of coconut oil in different surfactants was also employed. In the recent past it has been reported that the solubility of the oil was found to decrease when there was an imbalance between the oil and surfactant HLB values (25). HLB of coconut oil is 8, whereas, the HLB of Tween80, Tween20 and Span80 are 15, 16.7 and 4.3, respectively.

In the present study, for the cosurfactant selection glycerol was not used as it is published in the recent literature that it increases globule size (39). Similarly, ethanol was also not used as it may cause creaming and phase separation above 40 % concentration (40). Thus, PEG400 was the right choice and there is literature confirming the stable NE using PEG400 and Tween80 (41).

Raising the Tween80 concentration decreased the area of NE which can be attributed to increasing its concentration resulting in a more stable NE with reduced globule size but a further increase in Tween80 concentration may cause Brownian motion and initiation of Ostwald ripening (42, 43). Furthermore, there is a possibility of the interface being disrupted due to water infiltrating the oil droplets. This could lead to the expulsion of oil droplets, into the phases. Based on the outcomes observed in pseudoternary phase diagrams we can conclude that the formation of NE is affected by the ability of the Smix ratio to reduce surface tension at the oil-water interface. As a result, we observe a decrease in energy and favorable changes in entropy due to a reduction in surface tension and an increase in the interfacial region producing thermodynamically stable NE spontaneously (43, 44).

In this study, the Smix concentration was maintained at a 1:1 ratio as per the ternary phase diagram and Span80 was

added in the ratio of 0.45:0.55 with Tween80 for the formation of NE. The addition of Span80 with Tween80 at this concentration makes the HLB of surfactant near the HLB of coconut oil. Thus, it explains the formation of stable NE (45).

During the determination of the type of NE, methylene blue was used which highlighted only the water region leaving the oil globules. Thus, it was confirmed that the NE formed was oil-water in nature. These results were in line with the preceding published literature which also reported faster diffusion of methylene blue in the water phase (46).

The dispersibility test assesses the ability of NE to form and maintain a dispersed state for effective drug delivery. In the present study, NE dispersed rapidly with the addition of water and remained clear with no precipitation or cracking. The transmittance was above 90 %. The results obtained are similar to the previous published literature (25).

The size of the globules and the PDI are important factors that affect the formulation stability and influence both the degree and rate of drug release and drug absorption (47). It is also reported that globule size is proportional to oil concentration and leads to coalescence and phase separation taking place at low temperatures (25, 48). The droplet size is likely related to the structure of the surfactant used. Tween80 possesses optimum curvatures and packing parameters that enable the formation of micelles. Due to the hydrophobic segments of Tween80, when the organic and aqueous phases are combined, the surfactant can arrange itself more compactly at the interface. This facilitates the formation of micelles (49). In the present study, optimum globule size has been formed and it is well under the size of 200 nm which is required for nasal drug delivery.

The PDI determines the size uniformity of the globules in a formulation. As there is increase in PDI value (0.5-1), the size of the globule in the NE decreases (37). In this study, the PDI was found to be decreased which is an indication of uniformity in the globule size. These results can also be confirmed by the TEM images which showed uniform distribution of the circular NE droplets.

Zeta potential serves as an indicator for anticipating the enduring stability of NE. The electrical charges present in ions of excipients represent their viscoelasticity, which differs significantly from the surrounding solution, near the drug particles (50). Various factors including the type of surfactant, particle size, ionic strength, hydration, morphology and solution pH significantly influence it. In the present study, the zeta potential was found to be above -10, this may be attributed to the hydrophilic groups of Tween80 that can draw water molecules towards them.

Measurement of pH is highly important because the ingredients used in the formulation can impact the pH of the product which in turn determines how it will be administered. Moreover, any alterations, in pH could also affect the zeta potential of the formulation ultimately influencing its stability. In this study, the pH was found to be in the range of nasal pH (5.83-6.47). This confirms that the NE is non-irritating, making it suitable for nasal administration.

The viscosity of an NE is important for making sure that intranasal formulations work effectively and are suitable, for use. In the nasal cavity, it influences how long the formulation stays and how well the drug is absorbed (37). The obtained viscosity in the present experiment is in the range of the required viscosity ensuring easy handling, packing and administration of NE formulation without any complications (25).

In the current experiment, the NE demonstrated stability for 90 days, without adversely affecting the quality of the formulation. This stability may be attributed to the use of two non-ionic surfactants, Tween80 and Span80, which help create a viscoelastic film at the oil-water interface, thereby obstructing droplet coalescence (25).

Drug concentration in an NE is a significant factor to determine. In the present experiment, the drug was maximally available in the NE and is in accordance with the published literature (25).

Evaluation of organoleptic properties reported only slight changes in NE when compared with the initial observation and as shown in the previously published studies (30). Surfactants like Span80 and Tween80, commonly used to stabilize NEs, can undergo slow chemical degradation over time due to factors such as exposure to oxygen, light, temperature variations, or pH changes. This degradation may lead to the formation of oxidation products or breakdown compounds that modify the odor perceptibly, even if the NE remains physically stable overall.

Conclusion

Intranasal NEs represent a significant advancement in drug delivery systems aimed at targeting the CNS. These formulations improve the absorption and bioavailability of therapeutic agents by utilizing their nanoscale characteristics. The present study formulated the NE of phloroglucinol and DTBN with particle size permeable for intranasal administration. The coconut oil provided solubility for therapeutic agents whereas, Tween80 and Span80 provided stability for more than three months. Additionally, the NE had higher *in vitro* release rates than the individual drugs. Thus, these results suggest that the NE of phloroglucinol and DTBN holds significant possibilities as a therapeutic intervention for treating neurodegenerative disorders.

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Authors' contributions

PN carried out the formulation of nanoemulsion, evaluated the parameters and drafted the manuscript. MA reviewed the manuscript. BDR participated in the design of the study and reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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