Development of a nanoemulsion loaded with naringenin**

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to deterioration of a patient's memory, cognition and communication abilities. Numerous studies have indicated that oxidative stress is also a major reason behind the pathophysiology of AD. Oxidative stress can cause neuronal damage and modulate intracellular signaling, ultimately leading to neuronal death by apoptosis or necrosis. Hence, antioxidants have been studied for their effectiveness in reducing these deleterious effects. Naringenin, a polyphenolic compound obtained from grapefruit and sour oranges helps lessen oxidative stress and has been found to be effective for the treatment of AD. In the present study, an o/w nanoemulsion (NE) loaded with naringenin was prepared using labrasol as oil, igepal as surfactant and glycerol as co-surfactant, and characterized (particle size, polydispersity and zeta potential) by *in vitro* methods. The NE was clear (100% optical transmittance) with spheres of mean diameter 74.8 nm, monodisperse (polydispersity index PDI = 0.229) and a zeta potential of –29.6 mV. *In vitro* release into phosphate buffer saline (pH 6.8), artificial cerebrospinal fluid and simulated nasal fluid was studied up to 24 h. The encapsulation of naringenin in a NE sustained its antioxidative potential and stability compared to an aqueous solution. It was concluded that the NE could be used as an effective carrier of naringenin for the management of AD.

Keywords: Alzheimer's disease, *in vitro* release, oxidative stress

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder associated with dementia in the elderly brain [1]. It is characterized by progressive and considerable loss of cognitive functions due to dysfunctions in specific neurotransmitter systems like the cholinergic system, which is well

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known to play a prominent role in learning and memory [2]. It has become a major problem worldwide and affected millions of people [1, 3]. The main cause behind the progress of this disorder is oxidative stress, which results in imbalance of pro-oxidant/antioxidant homeostasis in the brain and leads to the overproduction of toxic, reactive oxygen species (ROS) [4]. Due to the brain's high consumption of oxygen and high content of polyunsaturated fatty acids, it is more prone to oxidative damage than other tissues of body [5]. Various metabolic processes form ROS, which in turn cause oxidative damage of proteins (→ protein carbonyl), lipids (peroxidation), DNA (→ 8-hydroxy-2-deoxyguanosine) etc. [6]. This oxidative damage leads to modification of the structural and functional integrity of the cell membrane, enzyme inactivation and ultimately cell death. Markers of free radical damage have also been seen in AD patients (increased protein and DNA oxidation, enhanced lipid peroxidation, glycation end products, carbonyls etc.) [7, 8]. Furthermore, extracellular plaque formation also occurs in the brains of AD patients due to aggregation of amyloid beta peptides. These amyloid plaques are another cause of oxidative stress in the patients of AD due to the concomitant release of superoxide and nitric oxide [9].

Currently very few preventive and therapeutic approaches are available for AD. Most of the available agents have failed clinical trials due to therapeutic restrictions such as providing only symptomatic relief from cognitive impairment, and adverse reactions. There is an urgent need of drugs that prevent neuronal death and improve cognitive function. Antioxidant agents from food can have a significant, beneficial effect on various neurodegenerative disorders associated with oxidative stress [10].

Polyphenols have been reported to have potential therapeutic benefits in age-related disorders due to their potent free-radical scavenging and antioxidant abilities. Polyphenols are secondary plant metabolites involved in plant defence against pathogens and ultraviolet damage [11]. They are found in many fruits, herbs and vegetables and thus serve as important dietary micronutrients. Chemically, polyphenols comprise a wide variety of biomolecules, which have several hydroxyl groups on one or more aromatic rings. They can be divided into various groups according to chemical structure, such as flavonoids, stilbenes and lignans. One of the flavonoids, naringenin (4, 5, 7-trihydroxyflavanone) is found in grapefruit juice and many other citrus fruits [12], tomatoes [13], cherries [14] and cocoa [15]. It has been reported to have various biological effects on human health, acting as an antioxidant, free radical scavenger, immunomodulator and memory enhancer [16]. The hydroxyl groups present in the structure of naringenin can donate hydrogen to ROS and scavenge the free radicals [17].

In addition, naringenin has also been extensively investigated for its pharmacological activities, including antitumour, anti-inflammatory [18], and hepatoprotective effects. But clinical studies exploring different schedules of administration of this drug have been hindered by its extreme water insolubility. Hence, unsurprisingly, the absolute bioavailability of naringenin was reported as only 4% in rabbits after oral administration [19]. Hence only tiny amounts can be expected to reached the brain for treatment of AD. In the present study, an oil-in-water (o/w) nanoemulsion *loaded with naringenin* was developed by homogenization and ultrasonication methods and further characterized on the basis of particle size, zeta potential etc. The nanoemulsion was further optimized and explored for *in vitro* release of naringenin in different simulated biofluids. The aim is to enhance the bioavailability of naringenin in the brain, using the NE as a suitable delivery system for the treatment of AD.

2. Experimental

2.1 Materials

Labrasol was a gift sample from Gattefosse, Mumbai, Maharashtra, India. Naringenin, Tween 20, soybean oil, propylene glycol and cetyl pyridinium chloride were purchased from Sigma–Aldrich (Bangalore, India). Glycerol was from CDH (P) Ltd, India. Milli-Q (Millipore, USA) highly purified water was used throughout. All other solvents were of HPLC grade.

2.2 Spectrophotometric analysis of naringenin

A stock solution of naringenin was prepared in *methanol* (50 μg/mL) and dilutions prepared in the range 0–25 μg/mL [20]. The wavelength of maximum absorbance (λ_{max}) was observed at 288 nm for the stock solution using UV/Vis spectrophotometry (Shimadzu). Then a standard plot was prepared by measuring the absorbance of a series of diluted samples (Fig. 1).[1]

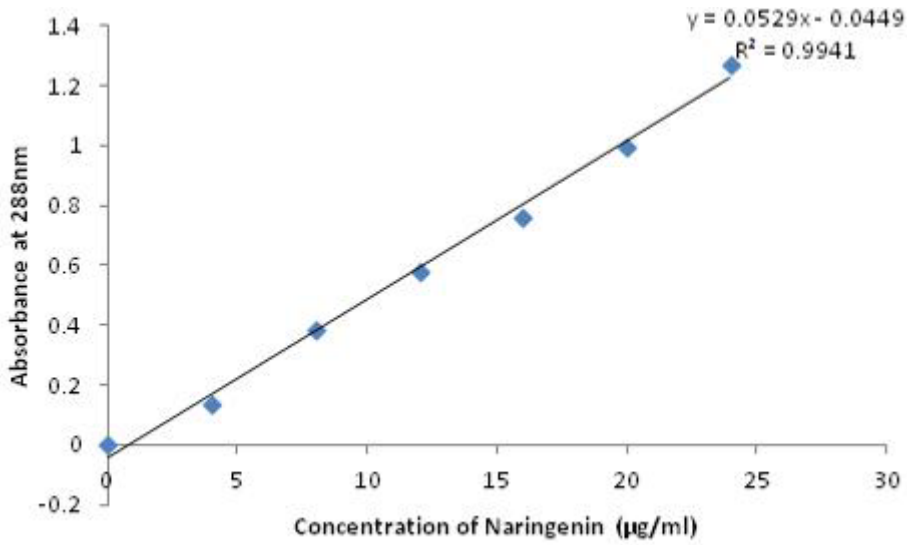

Figure 1. Absorbance of naringenin at 288 nm determined by UV spectrophotometry.

2.3 Development of the nanoemulsion

The solubility of naringenin was assessed in different oils, surfactants and cosurfactants (Table 1). The excipients in which the drug showed maximum solubility were selected (oils: oleic acid, soybean oil and labrasol; surfactants: tween 80, tween 20, cetylpyridinium chloride and igepal; cosurfactants: glycerol, propylene glycol and ethanol). Naringenin was dissolved in the selected oils, vortexing continuously during addition of the surfactants, cosurfactants and water, and kept overnight to check for any creaming or flocculation. Only homogenous and transparent combinations (nos 1, 2 and 4) were selected (Table 2). Transparency was achieved

[1] Cf. the results of Sahu et al. [20], who investigated naringenin encapsulated in solid lipid nanoparticles and found maximum absorbance at 283 nm.

by lowering the particle size: the preemulsions were subjected to high-shear homogenization using a Tissue Master 125 homogenizer (Omni International, Georgia, USA) in an ice bath and further subjected to high-energy ultrasonication in a Model UP400S, 24 kHz, 400 W benchtop ultrasonicator (Hielscher Ultrasound Technology, Germany), using an amplitude of 40% for 200 s to avoid overprocessing, cycling between 0.3 s on and 0.7 s off [21].

Table 1: Solubility of naringenin in various oils, surfactants and cosurfactants.

Excipient	Dissolved amount/mg mL^{-1}
Olive oil	2.0±2.4
Coconut oil	5.0±2.0
Liquid paraffin	2.0±3.2
Canola oil	2.5±9.0
Labrasol	30±7.0
Oleic acid	28.5±7.7
Soybean oil	25.5±4.4
Igepal co630	38.2±2.9
Tween20	25.0±4.7
Tween80	28.0±6.7
Span20	11.0±12.3
Span80	12.6±1.2
Cremophor EL	9.0±3.0
Transcutol HP	13.0±5.0
Propylene glycol	22.34±2.2
Glycerol	31.5±8.1
Diethylene glycol monohexyl ether	13.5±3.2
Ethylene glycol	21.0±4.1

Table 2: Selected combinations of excipients and their transmittances at 650 nm.

№	Oil	Surfactant	Cosurfactant	Aqueous phase vol.	Drug conc.	Appear-ance	Trans-mittance
1	Labrasol (10%)	Cetylpyridinium chloride (1%)	Glycerol (10.00%)	80.00%	5 mg/mL	Clear	90.8%
2	Labrasol (10%)	Igepal (10%)	Glycerol (8.52%)	71.48%	6 mg/mL	Clear	100%
3	Soybean oil (10%)	Tween 20 (10%)	Propylene glycol (8.52%)	71.48%	4 mg/mL	Opaque	72.0%
4	Oleic acid (10%)	Tween 80 (10%)	Glycerol (8.52%)	71.48%	5 mg/ mL	Clear	92.5%
5	Oliec acid (10%)	Tween 20 (10%)	Ethanol (8.52%)	71.48%	5 mg/mL	Opaque	70.4%

2.4 Measurement of transmittance

The developed NE was checked for transmittance at 650 nm using a UV/Vis spectrophotometer (Shimadzu), with water as the blank. Measurements were made in triplicate [22].

2.5 Further characterization of the developed nanoemulsion

Average particle size, polydispersity index (PDI) and ζ-potential of the NE were determined using a Zetasizer 100 HS (Malvern Instruments, Worcestershire, UK). The naringenin-loaded and unloaded (placebo) NEs were diluted 1/50 v/v in water [21] for the measurements, made in triplicate.

2.6 Stability studies

The naringenin-loaded NE containing 6 mg/mL of naringenin (no 2 in Table 2) was stored at 4 °C and 25 °C (in dark-coloured 10 mL bottles to afford light protection) for one month. The stability of the nanoemulsion was evaluated by determining mean particle size and polydispersity index (§2.5) every 6 days during the month [21].

2.7 *In vitro* release studies

The dialysis bag diffusion technique at 100 rpm was employed. Phosphate buffer saline (PBS), pH 7.4,[2] artificial cerebrospinal fluid (ACSF—two solutions were prepared separately and then mixed in equal proportion: Solution A, 8.66 g NaCl, 0.224 g KCl , 0.206 g $CaCl_2 \cdot H_2O$ and 0.163 g $MgCl_2 \cdot H_2O$ were added to 400 mL water and the volume made up to 500 mL; Solution B, 0.214 g $Na_2HPO_4 \cdot 7H_2O$ and 0.027 g $NaH_2PO_4 \cdot H_2O$ were added to 400 mL water and the volume made up to 500 mL) [23], simulated nasal fluid. pH 5.0–5.5 (7.45 g NaCl, 1.29 g KCl and 0.32 g $CaCl_2 \cdot H_2O$ were added to 800 mL water and the volume made up to 1L) [23] were used to maintain the sink conditions. 2 mL of the NE sample was enclosed in each dialysis bag (cellulose membrane, M_r cutoff 12 400, Sigma) and incubated in 500 mL of each medium at 37 °C with agitation in a USP II dissolution test apparatus. Samples of the medium were collected at predetermined time intervals and analysed for donepezil content after suitable dilution by UV spectrophotometry (absorbance at 288 nm); at each sampling, the volume of fluid removed was replaced by fresh medium to maintain sink conditions.

2.8 Determination of the antioxidant potential of the nanoemulsion

The free radical scavenging activity of the NE was compared with that of the aqueous drug via a 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assay. DPPH in methanol was freshly prepared from a 5 mM stock solution by adjusting the absorbance at 517 nm to 0.7. Reaction mixtures containing the sample and DPPH (1:1) were incubated for 30 min at room temperature away from light, after which the absorbance at 490 nm was recorded.

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[2] To 800 mL of water 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were added, followed by adjusting the pH to 7.4 using HCl, and finally making up the volume to 1000 mL with water [22].

In order to to estimate the antioxidant activity of the NE with respect to ascorbic acid a standard graph (straight line) was plotted for increasing concentrations of ascorbic acid (0, 20, 40, 60, 80, 100, 120 μg/mL) diluted in methanol. The plot was collinear with $R^2 = 0.973$.

DPPH inhibition (%) and DPPH radical scavenging activity (%) were calculated using the following equations:

$$\text{DPPH inhibition (\%)} = \{(\text{O.D. of control} - \text{O.D. of sample})/(\text{O.D. of control})\} \times 100 \quad (1)$$

and

$$\text{DPPH radical scavenging activity (\%)} = 100 - \text{DPPH inhibition (\%)}. \quad (2)$$

The NE, the placebo (unloaded NE) and the aqueous drug solution were taken in concentration multiples of C_0 (20 ng/mL of the active ingredient, naringenin): $C_{max}/4$, $C_0/2$, C_0, $2C_0$, $4C_0$, $6C_0$, $8C_0$ and $10C_0$. Graphs of percentage activity versus concentration were plotted and IC_{50}, namely the concentration reducing DPPH absorbance by 50%, was determined from the graphs [25].

3. Results and discussion

3.1 Final formulation of the naringenin nanoemulsion

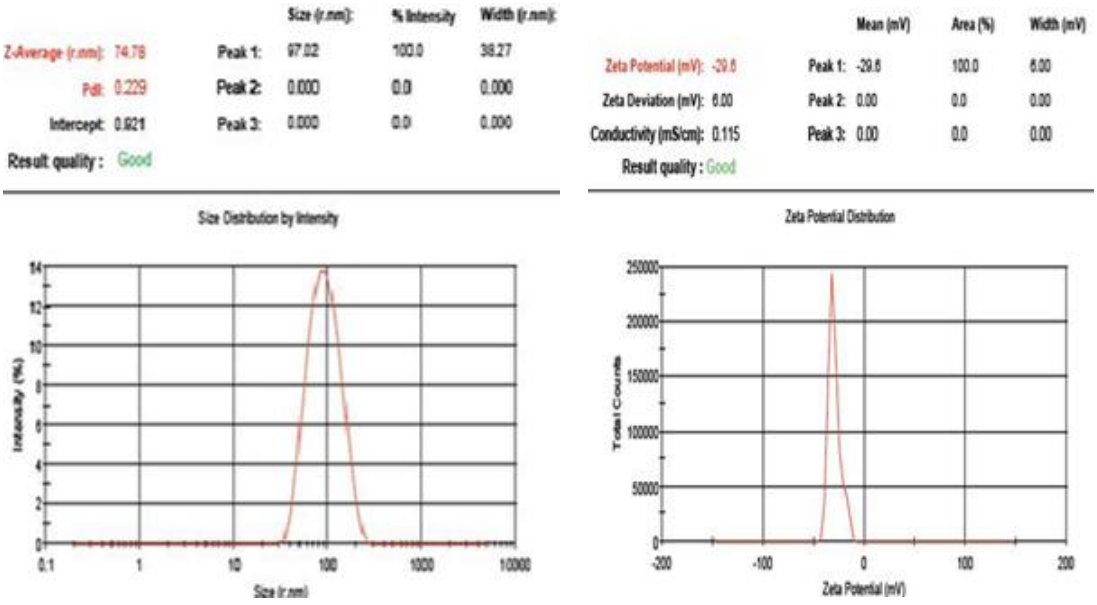
On the basis of transparency and clarity of the nanoemulsions, combination no 2 (Table 2) was selected as the final formulation (Table 3 summarizes the parameters), with a nominal content of 6 mg/mL of naringenin.

Table 3. Preparation parameters of the final naringenin nanoemulsion.

FORMULATION		
Drug	Naringenin	6 mg/mL
Oil	Labrasol	10.00%
Surfactant	Igepal	10.00%
Cosurfactant	Glycerol	8.52%
Aqueous phase	Milli Q water	71.48%
FORMULATION PARAMETERS		
Homogenization speed	10 000 rpm	
Homogenization time	20 min	
Ultrasonication time	150 s	
Ultrasonication duty cycle	3 s on / 7 s off	
Ultrasonication amplitude	40% of max.	

3.2 Nanocharacterization of the nanoemulsion

Mean particle size was 75 nm and the PDI of 0.23 indicated that the developed formulation was practically monodisperse (Fig. 2a), consistent with the clear and transparent appearance. The nanoscale particle size (< 100 nm) made the formulation suitable for brain drug delivery [22]; it assures rapid and transport across barriers like the mucosal epithelial surfaces of the nasal cavity [26]; moreover, the rate of *in vivo* drug release is dependent on particle size [27]. The ζ-potential was –29.6 mV (Fig. 2b), which is very close to the value ±30 mV that assures adequate interparticle repulsion to prevent aggregation [27].



(a) (b)

Figure 2. Determination of (a) particle size and polydispersity index and (b) zeta potential and its distribution of the selected naringenin nanoemulsion formulation.

3.3 *In vitro* release studies

In PBS as the sink (external) medium, maximum release (85±0.15%) of the drug was observed at 6 h and thereafter release remained practically constant for almost 24 h (Fig. 3). In SNF, maximum release was 88±0.22% after 4 h; similarly in ACSF maximum release (90±028%) was observed at 4 h. Hence, the stability- and clarity-optimized NE gave fast release in all simulated media.

Presumably the small particle (droplet) size of the NE contributes to the fast release [21]. This is encouraging for achieving the aim of increased solubilization of the drug by its encapsulation and it can be hypothesized that this enhances the efficacy of the drug by promoting release at the target site.

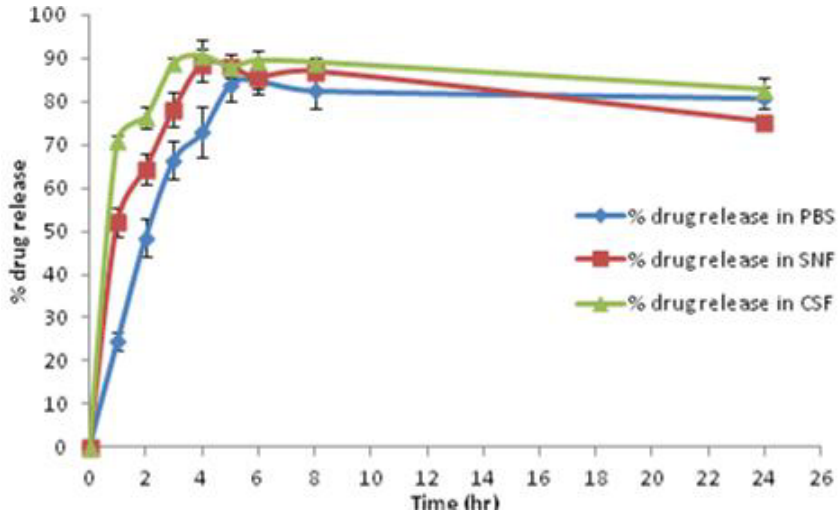
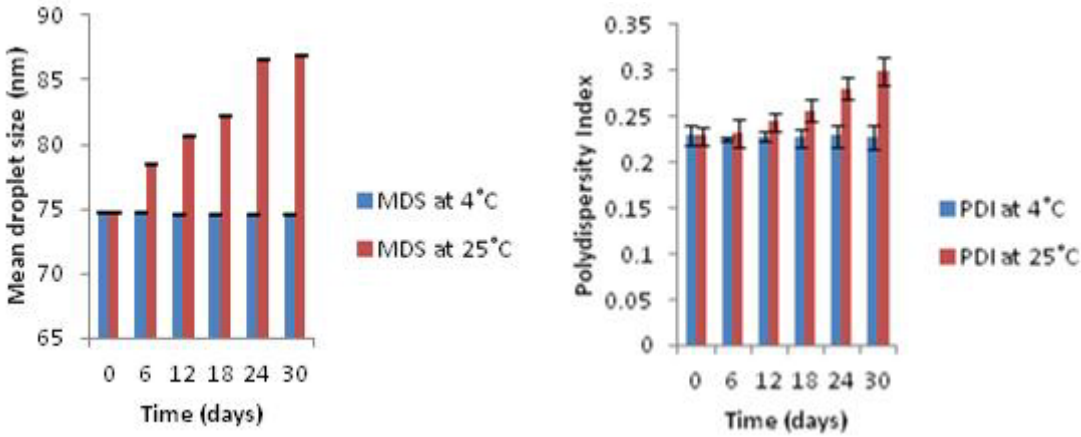


Figure 3. *In vitro* release of the drug from the nanoemulsion in different artificial media (PBS: phosphate buffer saline; SNF: simulated nasal fluid; CSF: simulated cerebrospinal fluid).

3.4 Stability

There were no changes in particle size and PDI of the nanoemulsion stored at 4 °C (Fig. 4), whereas significant changes in particle size and PDI were observed in the nanoemulsion stored at 25 °C. It can therefore be inferred that the developed nanoemulsion was stable at 4 °C.



(a) (b)

Figure 4. Variation of naringenin-loaded nanoemulsion gel during storage at 4 °C and 25 °C: (a) mean droplet size (MDS); (b) polydispersity index (PDI).

3.5 Determination of antioxidant potential

Narengenin in the NE showed higher antioxidant potential compared to the aqueous narengenin (Fig. 5). The calculated IC_{50} value for the NE was about $8C_{max}$, whereas for aqueous narengenin

it was >10C_{max}—the lower the IC_{50}, the better the antioxidant potential [25]. That it was higher for the narengenin-loaded NE compared to aqueous narengenin could, however, be at least partly due to excipients in the formulation (i.e., the oil, suractant and cosurfactant) contributing to the antioxidant potential of the NE; one notes that at some concentrations the unloaded ("placebo") NE had—within experimental uncertainty—the same antioxidant activity as the narenginin-loaded NE.

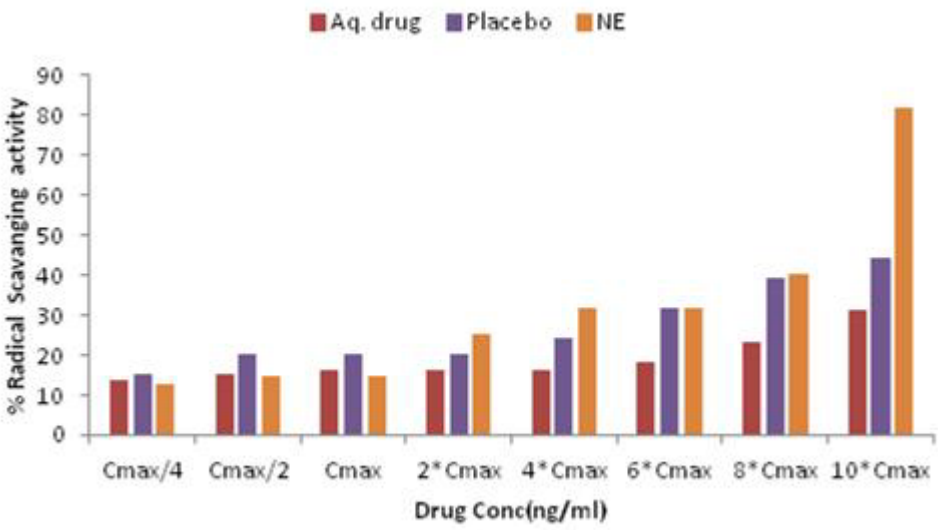


Figure 5. Determination of free radical scavenging activity (indicating antioxidant potential) of the aqueous drug, placebo (unloaded nanoemulsion) and naringenin-loaded nanoemulsion using the DPPH assay.

4. Conclusions

A nanoemulsion formulation for encapsulating naringenin was developed on the basis of preliminary solubility, transparency and clarity studies. The developed NE had particle sizes in the nano range and zeta potential in the range required to prevent coagulation of the particles. *In vitro* release studies showed that release of the drug from the NE was good, and best into artificial cerebrospinal fluid. Stability studies showed that the NE was stable at 4 °C. Taking due account of experimental uncertainties, the naringenin nanoemulsion gave better antioxidant activity than an aqueous drug solution. Hence, it can be proposed that loading naringenin into a nanoemulsion enhances its absorption in the brain and therefore its efficacy as an anti-Alzheimer's drug.

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