Lutein encapsulated oleic - linoleic acid nanoemulsion boosts oral bioavailability of retinal protective carotenoid lutein in rat model

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Abstract

There is excessive interest in emerging colloidal delivery systems to enhance the water solubility and oral bioavailability of lutein, which is a hydrophobic carotenoid claimed to possess health benefits. The present study aimed to design lutein-enriched nanoemulsions with improved physicochemical properties and to achieve various health benefits of lutein. The prepared lutein nanoemulsion was characterized, and its bioavailability was examined in vitro (simulated gastrointestinal digestion) and in vivo. The mean size, PDI and zeta potential of the lutein nanoemulsion were 110 ± 8 nm, 0.271 and 36 ± 2 mV, respectively. Furthermore, TEM examination revealed that the particles are nanosized and spherical in shape. Notably, the aqueous solubility of the nanoemulsion was 726-fold higher than that of free lutein. The composite nanoemulsion also showed exceptionally higher (87.4%) in vitro bioaccessibility than that of nonencapsulated or free lutein (15%). The in vivo bioavailability of lutein nanoemulsion (112.6 ng/mL) was much higher than that of nonencapsulated lutein (48.6 ng/ml) and mixed micelles (68.5 ng/mL), and the tissue distribution pattern of lutein nanoemulsion showed higher lutein accumulation in the liver (2.80- and 1.70-fold) and eye (1.91- and 1.48-fold) compared to free lutein and mixed micelle-fed groups. These results suggested that oleic acid-linoleic acid composite nanoemulsions may be a promising delivery system for lutein and may help enhance the solubility, oral bioavailability and bioecacy of lutein and could be used as an ingredient for the formulation of beverages or functional foods.

1. Introduction

Lutein is a hydrophobic non-provitamin A carotenoid, rich in dark green leafy vegetables, fruits and flowers [1]. However, lutein is commercially extracted from marigold flowers (Tagetes erecta). Lutein is an important pigment found in the macular of the retina and possesses a wide range of biological functions, includes scavenging free radicals, improvement of vision, nerve protection, improving brain and cognitive function, prevention of age-related macular degeneration and cataracts, cardiovascular disease, anti-inflammatory, protection retinal cells from photo-oxidation and anti-angiogenic, anti-mutagenicity and anti-cancer [2, 3]. Having such biological functions, lutein is not synthesized de nova in the human body and must be obtained through diet [4]. However, the bioavailability of lutein from food, fruits and raw vegetables is relatively poor (≤ 10–15%) due to its association with a complex matrix [5]. Hence, various commercial formulations have emerged in the market to tackle lutein scarcity (6–10 mg/day). Although studies have reported poor bioavailability, wide inter-individual variation was evidenced due to differences in its solubility [6]. Despite the extensive biological importance of lutein, poor solubility and bioavailability forage its application in the food and pharmaceutical industries.

Furthermore, the poor aqueous solubility of lutein limits its intestinal absorption (passive absorption), thus hinders its bioavailability. In addition, lutein is sensitive to environmental factors, processing and storage conditions such as heat, light, oxygen, intestinal pH, temperature, water activity, water peroxides and lipoxygenase, which leads to poor chemical stability [7]. The hydrophobic nature of lutein due to its C40 isoprenoid structure tends to form aggregation in the intestinal mucosa, hindering bioavailability [8].
cataracts and macular degeneration require strategies to protect their stability, solubility and biological activity.

Nanoencapsulation is one of the modern strategies advocated to effectively deliver and enhance the biological activity of lipophilic molecules [9]. Nanoencapsulation of bioactives is generally applied to improve the solubility, stability and bioavailability of hydrophobic phytochemicals such as lutein due to their smaller size and high stability [10]. Among nanotechniques, nanoemulsion is a simple and effective delivery system because of safety and easy industrial adoption and is widely accepted for food and pharma applications. Thus, nanoemulsion of bioactives like lutein has received increasing attention [11] as an effective approach to improving the bioavailability and bioefficacy [12] apart from protecting against degradation by systemic conditions (acidic pH of the stomach and enzymatic actions in the intestine) and interaction with other ingredients [13]. Further, the slow and controlled release of lutein from the nanocomplex system improves lutein bioavailability. In contrast to native or free lutein, nano-lutein was found to remain in the circulation for a longer duration indicating enhanced stability and bioavailability [14]. Vishwanathan, Wilson and Nicolosi [12] prepared phospholipid nanoemulsions and showed improved bioavailability of lutein in a human clinical trial. do Prado Silva, Geiss, Oliveira, da Silva Brum, Sagae, Becker, Leimann, Ineu, Guerra and Gonçalves [15] prepared lutein nanoemulsions and found improved declarative memory function in mice compared with conventional lutein supplementation.

Further, the nanoencapsulation-based system has been designed to deliver bioactives for the targeted release. The nanoemulsion with the inclusion of medium-chain triglycerides was reported to enhance the bioaccessibility of lipophilic bioactive compounds [16]. Earlier, we have reported that inclusions of phospholipids enhanced intestinal permeation of lutein and a positive correlation with their plasma and tissue lutein levels [17]. Nevertheless, to the best of our knowledge, there are no reported data on the effect of unsaturated fatty acids, such as oleic acid-linoleic acid nanoemulsion-based lutein delivery systems. The knowledge of digestion kinetics in the human GI tract is also an essential component in establishing the processing parameter at manufacturing stages to achieve the desirable release of active compounds.

Based on the literature survey and our previous findings, in the present study, we have focused on the preparation, characterization of lutein-loaded oleic acid-linoleic acid emulsions on bioavailability using a simulated gastrointestinal tract (GIT) simulation process, *in vitro* digestion kinetics and bioavailability of lutein in rats.

### 2. Materials And Methods

#### 2.1. Chemicals

Lutein (≥ 96% purity), butylated hydroxyl toluene (BHT), linoleic acid, oleic acid and LC-MS grade solvents were purchased from Sigma-Aldrich (St Louis, MO). Pepsin, bile salts, bovine pancreatin, sodium taurocholate and monooleoyl glycerol were purchased from Hi-Media (Mumbai, India). HPLC grade
solvents methanol, hexane, acetonitrile, dichloromethane (DCM), acetone and sodium sulfate, potassium hydroxide, ammonium acetate, tween 20 and other chemicals were purchased from Sisco Research Laboratory (Mumbai, India).

2.2.1. Sample for lutein

Marigold flowers were purchased from the local agricultural market (Mysore, Karnataka, India), petals were separated and moisture was removed using a hot air oven (45°C). Dried petals were grounded using a mixer grinder and used for lutein extraction as described by Lakshminarayana, Aruna, Sangeetha, Bhaskar, Divakar and Baskaran [18].

2.2. Preparation of lutein nanoemulsion (lutein-NEL)

Oleic acid and linoleic acid (1 % w/v) were prepared in acetone separately, stirred using a magnetic stirrer for 20 min at 600 rpm. Lutein (0.4 mg/ml) was added to linoleic acid under magnetic stirring for 20 min at 600 rpm. Then, to the mixture of linoleic acid-lutein, oleic acid was added at a ratio of 1:1 under magnetic stirring for 30 min at 700 rpm with 0.1% Tween-20 (surfactant), and the mixture was sonicated for 15 min. The solvent was evaporated under nitrogen flux, and the lutein-NEL was redissolved in PBS followed by sonication for 15 min.

2.3. Preparation of blank nanoemulsion (blank-NEL)

The blank-NEL (NEL with no lutein added) was prepared using linoleic acid and oleic acid (1% w/v) was prepared in acetone separately and stirred for 20 min at 600 rpm. Then, at a ratio of 1:1, oleic acid and linoleic acid were mixed under magnetic stirring for 30 min at 700 rpm, and 0.1% Tween-20 was added. Then, the samples were sonicated for 15 min, the solvent was evaporated under nitrogen flux, and the NEL was redissolved in PBS followed by sonication for 15 min and treated as control.

2.4. Particle size distribution of NEL

The hydrodynamic diameter and particle size distribution of the prepared lutein-NEL and control NEL was determined by dynamic light scattering (Zetasizer Nano, Malvern Instruments, UK). Samples were prepared (n = 3) by diluting the lutein-NEL and control NEL separately with ultrapure water (1:10 ratio) and loaded into a polystyrene cuvette (DTS0012®, Malvern, UK). All measurements were performed in triplicate at 25°C with an angle of detection of 173° and a refractive index of 1.33 for the dispersion medium.

2.4.1. Surface charge of nanoemulsion

The surface charge or zeta potential of the diluted nanoemulsion (1/10 v/v in water) was measured (n = 3) using a Zetasizer instrument (Nano-ZS®, Malvern, UK). The dielectric constant of the dispersion medium was 78.5. A capillary cell (DTS1070®, Malvern, UK) was used for the zeta potential analysis.

2.4.2. Transmission electron microscopy (TEM)
TEM was used to examine the size and shape of the nanoparticles. In brief, diluted samples (1/10 in double-distilled water) were deposited on a carbon-coated copper grid and air-dried for 5 min and the excessive samples were removed with filter paper. A droplet of sample stain, sodium phosphotungstate (2 %, w/v), was placed on a dry copper grid for 3–5 min. The samples were observed by Titan Themis transmission electron microscopy (FEI, ThermoScientific™, Waltham, Massachusetts, USA) at 300 kV randomly scanned, and photomicrographs were taken at different magnifications.

2.5. Fourier transform infrared (FTIR) spectroscopy

Infrared spectra of oleic acid, linoleic acid, lutein and lutein nanoemulsion were recorded using a Fourier transform infrared spectrophotometer (Bruker, Tensor II, Germany). Briefly, samples were taken directly, and spectra were analyzed in the range of 4000–400 cm\(^{-1}\).

2.6. Extraction of lutein from lutein-NEL

Lutein was extracted from lutein-NEL by the procedure described earlier by Toragall, Jayapala and Vallikannan [19]. Briefly, 0.5 ml of lutein-NEL was added to DCM: MeOH (1:2 v/v) vortexed and 1.5 ml of hexane was added, vortexed and the hexane layer was collected. The process was repeated with DCM and hexane until the yellow colour disappeared. The hexane layer was pooled, evaporated under nitrogen flux and redissolved in the mobile phase for HPLC analysis.

2.7. Preparation of mixed micelles

Mixed micelles were prepared in phosphate-buffered saline containing mono-oleoyl-glycerol (2.5 mM), sodium taurocholate (12 mM), oleic acid (7.5 mM) and lutein (600 µM). These chemicals were separately dissolved in chloroform and methanol, and solvents were evaporated to dryness using nitrogen. The micelle was resuspended in phosphate-buffered saline (pH 7.2) with vigorous mixing using a vortexer (REMI CM-101 plus, India) and followed by sonication (PCI, Mumbai, India) for 15 min to obtain a clear solution. Lutein content in the lutein-NEL and mixed micelles was quantified using HPLC before it was used for further studies [10].

2.8. Confirmation of lutein core in NEL

The lutein core in the lutein-NEL was examined by phase-contrast microscopy (Olympus, Tokyo). In brief, lutein-NEL were mixed with ‘oil red O’ stain at a working concentration (0.3 %) followed by stirring and sonication. A drop of stained lutein-NEL was placed on a clean glass slide with a coverslip and visualized under phase contrast microscopy [19].

2.9. Solubility of lutein nanoemulsion

To determine the solubility of lutein, lutein-NEL, lutein mixed micelles and free lutein in oleic acid (1% w/v) (0.2 mg) were dispersed in 10 mL of distilled water and incubated in a shaking water bath at 37°C for 24 h. Samples were filtered through Whatman no. 1 filter paper, and lutein was extracted from the filtrate and quantified by HPLC [19].
The prepared lutein NEL (n = 3) were immediately transferred into screw-capped glass vials and stored at different storage temperatures at 25°C and 4°C in the dark for 30 days. An aliquotes of the sample were periodically drawn (n = 3) for particle size and lutein content analysis. The percent of lutein retention was at each time point with respect to initial concentration was calculated [20].

2.11. In vitro bioaccessibility of lutein from NEL

In vitro bioaccessibility of lutein from lutein NEL in comparison with free lutein in oleic acid and mixed micelles (control) was measured. The bioaccessibility was examined in simulated gastrointestinal digestion as per method Garrett, Failla and Sarama [21], [22] with slight modifications. Lutein in the free form was dissolved with oleic acid, and mixed micelles and nanoemulsions (600 µM) were taken in screw-tightened glass vials and subjected to in vitro (gastric and intestinal phase) digestion. Gastric digestion: In brief, pepsin (porcine gastric mucosa 88–2500 U/mg protein) was dissolved (3 mL) in a phosphate buffer (3.6 mmol/L CaCl\(_2\), 12 mmol/L KCl, 6.4 mmol/L KH\(_2\)PO\(_4\), 1.4 mmol/L MgCl\(_2\).6H\(_2\)O, 49 mmol/L NaCl) was added to the sample containing vial, and the pH was adjusted to 2.0 using 2 M HCl. The vials were filled with nitrogen to prevent the oxidation of lutein, tightly capped and incubated at 37°C in a water bath shaker (Scigenics Orbitek, India) at 120 strokes/min for 1h.

Intestinal phase: After incubation, samples were loaded to room temperature (28°C) and the pH of the digest was raised to 7.4 using 1 M NaHCO\(_3\) followed by 6 mL of pancreatin (porcine pancreas 89 U.S.P) prepared in 0.1 M NaHCO\(_3\) and 25.38 g/L bile extract (porcine) were added. Furthermore, the pH of the digest was adjusted to 7.4 using 1 N NaOH, followed by incubation for 3 h in a shaking water bath after filling extra space with nitrogen. After the incubation period, the digesta (1 mL) was withdrawn from each sample at different time intervals (0–4 h) and ultracentrifuged at 144,000 x g at 4°C for 60 min (Beckman Optima-100 ultracentrifuge, Indianapolis, US). The aqueous phase containing micelles was used for the extraction and quantification of micellized lutein by HPLC [19]. The percent of transformation, bioaccessibility and bioavailability was calculated as per the equations given below [23]. Values represent the amount of lutein present in the small intestine without chemical degradation or alteration in the structural complexity. Perhaps the in vitro gastrointestinal digestion (GIT) simulated was cannot precisely replicate the complex process occurring in the gastrointestinal tract of in vivo. However, this method can be used for rapid screening of samples to identify important physicochemical interactions for drug/nutraceutical delivery.

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\text{Transformation(\%)} = \frac{\text{Concentration of flutein in digesta at ending}}{\text{Initial concentration of flutein}} \times 100
\]

\[
\text{Bioaccessibility(\%)} = \frac{\text{Mass of flutein in micellar}}{\text{Mass of flutein in starting emulsion}} \times 100
\]

\[
\text{In vitro bioavailability} = \text{Bioaccessibility} \times \text{Transformation}
\]
2.12. Lutein bioavailability in rat model

To investigate lutein bioavailability from lutein NEL, the animal experiment was performed after clearance from the CFTRI animal ethics committee (IEAC NO. FT/AHF/Al/108/2019). Weanling Wister rats weighing 50 ± 5 g were housed in polypropylene cages in the institute animal house facility at 28 ± 2°C with a 12 h light:dark cycle and given free access to feed and water. The standard pellet diet (crude protein-18.34%, crude fat-3.28%, crude fiber-5.40%, calcium-1.20%, phosphorus-0.58%, total ash-5.8%, carbohydrate-64% and moisture-7.42%) fed to rats was purchased from M/s Champaka feeds and foods (Bengaluru, India). Rats were acclimatized for one week before treatment started.

A single oral dose of equimolar concentration (600 µM, a physiological dose) of either micellar lutein (control) or lutein NEL and the free lutein-oleic acid mixture (0.2 mL) was gavaged to a group of rats (n = 6) separately. After 8 h of gavage, rats received no sedation before being sacrificed with CO₂ anesthetic. The CO₂ flow rate was monitored with AIMS CO₂ flow meter (AIMS™ SC100) with 25 L/min CO₂ for rapid euthanasia. Blood was collected in heparin-coated tubes for the analysis of lutein in the plasma, liver and eyes. To obtain the plasma, the blood was centrifuged at 1000 x g for 15 min at 4°C. The liver and eyes of rats were homogenized with ice-cold isotonic saline (0.9%). The lutein was extracted from plasma (1 mL), liver and eye homogenate (1 mL) and analyzed by HPLC.

2.13. HPLC and LCMS analysis

The purified lutein from the marigold flower petals extracted lutein from lutein NEL, plasma and tissues were analyzed by HPLC (Alliance 2690, Waters, UK). Standard lutein and lutein extracts were injected (10 µL) into an HPLC system equipped with a C-18 column (250*4.6 mm²; SGE company, Mumbai, India) and PDA detector. The mobile phase (acetonitrile/methanol/DCM; 6:2:2 v/v/v) containing 0.1% ammonium acetate was used for the separation of lutein at a flow rate of 1 mL/min by monitoring at 444 nm. LCMS analysis of lutein was carried out as per the method of Toragall, Jayapala and Vallikannan [19] to validate the mass of the lutein extracted with the lutein standard.

2.13. Statistical data analysis

Experiments were carried out in triplicate (NEL preparation and characterization) or n=6/group (animal experiments), and the results were statistically tested for significance (p ≤ 0.05) for the analysis of variance with one-way and two-way ANOVA. All statistical calculations were performed using GraphPad Prism 5.0 software (GraphPad Prism 5 ®, GraphPad Software, San Diego, CA).

3. Results And Discussion

3.1. Lutein purity
Marigold flower petals were used for lutein extraction. Extracted lutein was purified through open column chromatography. The presence of lutein in the purified extract was confirmed by the spectral maxima at $\lambda_{\text{max}} = 444$ nm recorded using spectrometry. Furthermore, lutein purity ($\geq 96\%$) was authenticated by HPLC and LC-MS results (Fig. 1). Purified lutein was used for lutein nanoemulsion (lutein-NEL) preparation.

3.2. Particle size, polydispersity index (PDI) and zeta potential

Dynamic light scattering (DLS) and transmission electron microscopy were used to measure the particle size, zeta potential and morphology of the lutein-NEL. The average size of the NEL was $110 \pm 8$ nm with a polydispersity index of 0.271 (Fig. 2A and 2C), showing that the monomodal dispersion of particles in the nanoemulsion and zeta potential (Fig. 2B) of lutein-NEL showed a higher positive charge ($36 \pm 2$ mV), indicating higher stability of lutein in NEL compared to free lutein in oleic and micellar lutein. The present results are in agreement with our previous study of a lutein nanocarrier system developed using chitosan and sodium alginate along with oleic acid that showed higher lutein bioavailability of lutein rats due to the smaller particle size (60–140 nm) and higher zeta potential ($+38 \pm 4$ mV) [10, 19]. Furthermore, TEM analysis of the lutein-NEL confirmed that the mean particle size is $<100$ nm and sphericle in shape (Fig. 2D). Moreover, the droplet distributions were uniformly dispersed with no aggregation and aggregation, which may promote higher intestinal absorption without gastric and intestinal degradation as reported by Ranganathan, Manabe, Sugawara, Hirata, Shivanna and Baskaran [24]. However, the average particle size perceived by TEM ($<100$ nm) was significantly smaller than that of DLS detection ($110 \pm 8$ nm), which may be due to the hydration status or hydrodynamic size. At the same time, the TEM samples were dried before the examination. Acevedo-Fani, Silva, Soliva-Fortuny, Martín-Belloso and Vicente [25] also found that droplet sizes examined under TEM were in good agreement with those obtained by DLS. In addition, TEM reflected the local particle size of the nanoemulsions. In contrast, DLS reflected the average particle size of the nanoemulsions. However, the particle size of the DLS (Fig. 2C) has an agreement with the TEM results. The oil red O staining also revealed that the morphology of the particles inside the lutein-NEL were spherical with a lutein core (Fig. 2D), which is in line with other lutein nanocarrier studies [19, 24].

3.3. FT-IR of NEL

The interaction of carrier molecules (oleic acid, linoleic acid) and lutein was determined using FTIR spectroscopy. Figure 3A shows the FTIR spectra of (a) lutein, (b) pure oleic acid, (c) linoleic acid and (d) lutein-NEL. Figure 2A (a) shows that the characteristic lutein peak was observed at 1650 cm$^{-1}$, indicating the C = C stretch (alkene) that usually appears at 1660 – 1600 cm$^{-1}$ and OH bond stretching observed at 1016 represents the functional group of lutein [26]. The FTIR spectrum of pure oleic acid shows two bands at 2853 and 2922 cm$^{-1}$ corresponding to the symmetric -CH$_2$ stretch and the asymmetric -CH$_2$ stretch, respectively. A wide, intense band is seen at 2673 cm$^{-1}$ and 3008 cm$^{-1}$ due to O-H bond stretching, the band observed at 1708 cm$^{-1}$ is attributed to asymmetric -C = O stretching and the band at
1284 cm$^{-1}$ corresponds to C-O stretching [27]. Linoleic acid FT-IR characteristic bands exhibit stretching at 2929 cm$^{-1}$ and 2857 cm$^{-1}$, which may be due to the aliphatic C-H group. C = O and C-O band stretching was observed at 1708 cm$^{-1}$ and 1222 cm$^{-1}$, respectively. A band was observed at 726 cm$^{-1}$ due to aliphatic C-H group vibration. The C-H group bending and scissoring was observed at 1455 cm$^{-1}$ because of the methylene group [28] (Fig. 3A). Figure 2D represents the lutein-NEL, where the peak at 2353 cm$^{-1}$ is due to the C = C group present in lutein, which usually appears at 1160–1600 cm$^{-1}$. There is also a shift of the OH bond in lutein from 1016 to 1634 cm$^{-1}$. The presence of C = C and OH band stretch indicates the presence of lutein and indicates that nanoemulsion of lutein results from weak intermolecular forces such as hydrogen bonds.

### 3.4. Aqueous solubility of the lutein-NEL

The aqueous solubility of lutein is poor due to its hydrophobic nature, which limits its application in the food and pharma industries. As shown in Fig. 3B, the prepared lutein-NEL was (120 µg/mL) 726-fold higher than lutein dissolved in oleic acid (165.3 ng/mL), which might be due to the higher volume to surface ratio of the nanoparticles. The present results are in good agreement with our previous findings that polymer-based lutein nanoencapsulation had a higher volume to surface ratio [19, 26]. The higher volume to surface ratio is also one of the important factors required for favorable intestinal absorption of carotenoids [12, 29]. A recent report on lutein nanoencapsulation using zein/soluble soybean polysaccharide composites has shown that the aqueous solubility of lutein (64.40 µg/mL) was enhanced over 30-fold in comparison to free lutein [30]. In contrast, to their report, the present study showed that the lutein-NEL (120 µg/mL) exhibits 72.6% higher solubility.

### 3.5. Storage stability of lutein-NEL

Figure 4 shows the storage stability of the lutein-NEL at different temperatures. The particle size of the lutein-NEL increased, and lutein retention decreased gradually with prolonged storage time (30 days) at 25°C (Fig. 4A & 4C). However, lutein-NEL stored at 4°C showed no significant variation in the particle size, denoting that the prepared nanoemulsion can be stored at a lower temperature up to 30 days. The particle size of the lutein-NEL reached 350 nm, and the retention of lutein was 65 ± 3% at 25°C after 30 days. Whereas at 4°C, the particle size of the lutein-NEL was 130 nm, and the retention rate of lutein was 94 ± 4%, indicating the possible protection of the stability of lutein against degradation (Fig. 4C). Furthermore, the reduction in the zeta potential over the storage time at 25°C supports the aggregation of nanoparticles in the nanoemulsion, causing a larger particle size and a reduction in the lutein content. The zeta potential at 4°C after 30 days showed no significant changes (+ 30 ± 2 mV) over time, indicating that the stability of the lutein-NEL at lower temperatures is higher (Fig. 4B). This could be the reason for the retention of higher lutein content and smaller particles in the prepared nanoemulsion stored at 4°C. Xu, Ma, Gong, Li, Huang and Zhu [31] reported that carboxymethylcellulose-modified rice protein lutein nanoparticles showed an increase in particle size (350 nm) and less lutein retention (45%) after 35 days of storage at 25°C. In contrast, nanoemulsions prepared in the present study showed almost the same
3.6. In vitro digestion of lutein-NEL (bioaccessibility)

Carotenoids are easily degraded in the GIT, which limits their bioaccessibility and bioavailability. Therefore, it is mandatory to find out the stability of lutein released from the carrier. Hence, a simulated GIT was used to evaluate the fate of digestion on lutein stabilized by oleic and linoleic acid.

Freshly prepared lutein-NEL (stored at 4°C) and micellar lutein were used to investigate the bioavailability of lutein because there was no evident change (non-significant) in droplet size (Fig. 4). In vitro simulated gastrointestinal digestion showed that the percent lutein micellization (available lutein for intestinal absorption) after 4 h digestion of lutein-NEL was significantly higher (5.83-fold) than that of free lutein and micellar lutein (2.25-fold) (Fig. 5A). Samples drawn at different time points revealed that the bioaccessibility of lutein was enhanced gradually with incubation time. However, after 3 h of digestion, there was no change in the enhancement of lutein bioaccessibility was evidenced. Transformation is defined as the amount of lutein remaining after simulated gastrointestinal digestion. Lutein degradation under simulated GIT conditions occurs mainly due to exposure to aqueous neutral or alkaline environments and due to multiple factors such as low pH, enzymatic action [29]. Hence, free lutein transformation was found to be 61.2%, whereas lutein-NEL showed 30.6%. On the other hand, the bioaccessibility of free lutein (15%) was significantly lower than that of lutein-NEL (87.4%) (Fig. 5A). Several physicochemical properties may contribute to the difference in bioaccessibility [32]. First, the aqueous solubility of hydrophobic lutein tends to form aggregates, leading to an increase in the particle size that hinders bioavailability. Second, the higher surface-to-volume ratio leads to a faster dissolution rate of lutein from nanoparticles than larger crystals as in free lutein. The absolute amount of lutein present in mixed micelles after simulated in vitro gastrointestinal digestion is a measure of the amount available for absorption and can be used as a measure of in vitro bioavailability. The amount of bioavailable lutein from the NEL (20.75%) was much higher (2.26- and 1.66-fold) than free lutein (9.18%) and micellar lutein (12.5%), respectively (Fig. 5B). These results clearly demonstrate that oleic acid-linoleic acid nanoemulsions greatly enhance lutein bioaccessibility by protecting lutein from gastrointestinal conditions [33]. Cheng, Ferruzzi and Jones [34] reported that zein nanoparticles (ZNPs) containing lutein showed 40% micellization. The nanoemulsion prepared in the present study showed 87.4% micellization, indicating that oleic acid and linoleic acid are superior vehicles compared to ZNPs for transferring hydrophobic molecules to micelles [35]. Another study showed the micellization of resveratrol was 60–70% using lecithin-conjugated zein nanoparticles, representing the prime role of lipids in enhanced bioaccessibility [36]. Feng, Li, Tan, Fu, Zhang and Huang [37] reported that lutein-loaded emulsions prepared using corn fiber gums showed lower bioaccessibility of 13-32.4%. Additionally, Ma, Yuan, Yang, Wang and Lv [38] reported similar results from lutein-encapsulated zein/tea saponin composite nanoparticles, which displayed 67.17% bioaccessibility of lutein. Furthermore, the bioaccessibility of lutein from vegetables would be 40–60% [39] compared to nanoemulsion prepared in
the present study, showing 2-fold lower bioaccessibility, signifying that fatty acid nanocarrier (oleic acid-linoleic acid) is a promising carrier for the delivery of hydrophobic molecule lutein.

3.7. In vivo bioavailability of lutein

In vitro measurements provide valuable information about the physicochemical factors that influence the bioavailability of lutein. However, they cannot accurately model the complexity of the GIT in vivo. For the same reason, in vivo bioavailability of lutein from NEL compared free lutein and lutein mixed micelles was determined by oral administration to rats at a dose of 600 µM. The impact of the type of delivery system on the bioavailability of lutein was determined by measuring the serum lutein concentration and tissue distribution after administration.

Lutein performs various biological activity, prominently as an eye-protective function against various microvascular complications such as diabetic retinopathy, neuronal injury, age-related macular degeneration (AMD) and cataract [3, 40]. However, there are constraints in its effective biological application in humans due to very active carotenoid cleavage enzymes present in the bloodstream, which decreases the active carotenoid levels in tissues. Hence, the nanocarrier systems are in practice to enhance the plasma and tissue lutein levels [10]. Therefore, in the present study, we have developed lutein-NEL and examined the lutein bioavailability and tissue distribution. Results of the current study showed 2.32- and 1.65-fold higher (112.6 ng/mL) plasma lutein levels in the lutein-NEL fed group compared to free lutein (48.6 ng/mL) and mixed micelles (68.5 ng/mL) groups, respectively (Fig. 6A).

Arunkumar, Prashanth, & Baskaran [8] have also reported that feeding lutein PLGA-PEG nanoparticles (244 pg/ml) enhanced lutein bioavailability (27.7%) compared to free lutein in mice. Interestingly, lutein bioavailability from NEL found in the present study had evidenced higher bioavailability (502-fold) compared to the above literature.

Furthermore, the enhanced bioavailability from the lutein-NEL was attributed to the smaller particle size, slow, controlled lutein release and its prolonged circulation time in the blood [41]. Peng, Li, Zou, Liu, Liu and McClements [23] also reported that curcumin nanoemulsion had an appreciably higher (2.7-3.6-fold) bioavailability than the free curcumin (100 mg/kg) in rats. Furthermore, Vishwanathan, Wilson, and Nicolosi [12] also reported higher bioavailability of lutein from phospholipid nanoemulsion compared to commercial lutein supplements in human trials. Since lutein bioavailability from NEL in vitro and in vivo is found to be higher hence the NEL systems can be considered the potential delivery system.

The liver and eye levels of lutein is shown in Fig. 6B. The results show 2.80- and 1.70-fold higher levels of lutein in the liver of lutein-NEL fed group than the group received free lutein and lutein mixed micelle. A similar pattern was observed in the case of target tissue (eye), in which the nanoemulsion displayed higher (1.91- and 1.48-fold) lutein accumulation than the free lutein and lutein mixed micelles, respectively. The higher levels of lutein in plasma and tissues of NEL group may be due to the nanosize, surface charge, surface-to-volume ratio and lipid surface protecting the lutein from the physiological conditions of the GIT, which improves the stability, solubility, bioaccessibility and intestinal permeability.

The available literature clearly demonstrate that lutein-NEL...
improves the hydrophilicity, stability, bioaccessibility and biological availability of lipid-soluble bioactive lutein and hence can be considered for effective delivery of lutein to subjects with retinopathy, cataract and AMD.

4. Conclusion

Lutein is used as a functional ingredient in food and pharma applications for the treatment of various ocular complications. However, poor water solubility and bioavailability forage its application. Therefore, to address these deficiencies, lutein was nanoemulsified in the lipid carrier system. The particle size, zeta potential, PDI and degradation were used to evaluate the physicochemical stability of the lutein-NEL. The lutein-NEL prepared in this study had greater physicochemical stability. Further, results indicate a slow and controlled release of lutein from NEL under stimulated gastrointestinal digestion and higher bioavailability in rats compared to free and micellar lutein. The findings of the study can have a greater impact on the design of fatty acid-based lutein nanoemulsions for therapeutic applications. It is concluded that the nanoencapsulation of highly hydrophobic bioactive lutein is an efficient podium to enhance hydrophilicity, stability, bioaccessibility and oral bioavailability.

Declarations

Declaration of Competing Interest

The authors declare no conflicts of interest.

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**Figures**
Figure 1

(A) Purified lutein HPLC chromatogram extracted from marigold petals (B) LC-MS profile of lutein extracted demonstrating purity.
Figure 2

Physicochemical characterization of lutein-NEL (A) particle size, (B) zeta potential, (C) size distribution of particles in the nanosuspension and (D) TEM image indicating that the particles are in the nano range ($\leq 200$ nm).
Figure 3

(A) FTIR spectra of (a) lutein, (b) oleic acid, (c) linoleic acid and (d) lutein-NEL and (B) the solubility of lutein and lutein-NEL. Values not sharing similar superscript between the group are significantly different ($P \leq 0.05$).
Figure 4

Changes in the (A) particle size, (B) zeta potential and (C) lutein retention in the nanoemulsion at 4 °C and 25 °C over the period of 30 days. Values not sharing similar superscript between group and time interval are significantly different (P ≤ 0.05).
Figure 5

Percent transformation and bioaccessibility of lutein from lutein in oleic acid, mixed micelles and lutein-NEL after simulated gastrointestinal digestion. Values not sharing similar superscript among the group within time points are significantly different ($P \leq 0.05$).
Figure 6

Lutein concentration in the (A) plasma lutein, (B) lutein tissue distribution (liver and eye) of rats fed with an equimolar concentration of lutein (600 µM) nanoemulsion and mixed micelles. Values not sharing similar superscript among the group are significantly different (P ≤ 0.05).