Pumpkin Oil–Based Nanostructured Lipid Carrier System for Antiulcer Effect in NSAID-Induced Gastric Ulcer Model in Rats

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Background: Peptic ulcer disease, a painful lesion of the gastric mucosa, is considered one of the most common gastrointestinal disorders. This study aims to investigate the formulation of pumpkin seed oil (PSO)-based nanostructured lipid carriers (NLCs) to utilize PSO as the liquid lipid component of NLCs and to achieve oil dispersion in the nano-range in the stomach.

Methods: Box–Behnken design was utilized to deduce the optimum formula with minimum particle size. The optimized PSO-NLCs formula was investigated for gastric ulcer protective effects in Wistar rats by evaluating ulcer index and determination of gastric mucosa oxidative stress parameters.

Results: PSO was successfully incorporated as the liquid lipid (LL) component of NLCs. The prepared optimum PSO-NLCs formula showed a size of 64.3 nm. Pretreatment of animals using the optimized PSO-NLCs formula showed significantly (p<0.001) lower ulcer index compared to indomethacin alone group and significantly (p<0.05) less mucosal lesions compared to the raw oil.

Conclusion: These results indicated great potential for future application of optimized PSO-NLCs formula for antiulcer effect in non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcer.

Keywords: natural products, gastric ulcer, pumpkin seed oil, nano-lipid carriers, optimization, Box–Behnken experimental design

Introduction

Peptic ulcer disease (PUD) is a common gastrointestinal disorder with 10% prevalence in the human society.1 It is a disease related to damage caused by balance disturbance between aggressive and defense factors in the stomach. The aggressive factors include pepsin and stomach acid secretion, active free radicals and oxidants, leukotrienes, endotoxins, in addition to exogenous factors such as alcohol intake and nonsteroidal anti-inflammatory drugs (NSAIDs). Contrastingly, gastric mucin, prostaglandins (PGs), bicarbonate, nitric oxide (NO), growth factors, and antioxidant enzymes or antioxidant peptides like glutathione (GSH) constitute the defensive factors. Nonetheless, the most commonly affected organs are the lesser curvature in the stomach and proximal duodenum, however, ulceration may also occur anywhere in the gastrointestinal tract (GIT) from pylorus to cardia.2,3

Importantly, the prolonged use of NSAIDs is the second most common cause of PUD.4 NSAIDs used for anti-inflammatory, antipyretic, pain-relief, anti-platelet...
aggregation, and anti-thrombogenesis indications. In particular, Indomethacin, an member of NSAIDs family, is widely used for the management of rheumatoid arthritis, several inflammatory diseases, and for its well-established cardiovascular protection properties; however, its contribution to gastric ulceration has been documented in literature. The induced inhibition of the cyclooxygenase enzyme (COX-2) enzyme is responsible for indomethacin’s anti-inflammatory effect. Nevertheless, when used to alleviate inflammation and pain, it is known to exert a severe damaging effect on epithelial cells of the digestive tract, which constitutes its serious side effect. It is believed that the pathogenesis of indomethacin-induced gastric ulceration occurs via its potential to block the activities of the COX-1 enzyme, the major protective factor of gastrointestinal system, and the subsequent deficiency of protective factors such as prostaglandin E2 (PGE2), the production and secretion of mucus and bicarbonate, decreased mucosal blood flow, platelet aggregation dysfunction, impairment of microvascular structures. In addition, indomethacin increases aggressive factors, eg, acid, and oxidant parameters. On the other hand, indomethacin reduces anti-oxidant parameters; altogether indomethacin previously indicated the effects lead to epithelial damage.

Numerous treatment modalities are presently available to prevent indomethacin-induced peptic ulceration and to promote healing of mucosal damage, for instance, histamine receptor antagonists (H2RAs), proton pump inhibitors, PGs analogues, and cytoprotective agents. A superior drug to prevent and treat gastric-related side effects caused by NSAIDs in general remains somewhat controversial in clinical practice. Besides, most of these drugs have been reported to produce severe adverse reactions and toxicities upon chronic usage. Hence, a search for less toxic drugs is highly required, particularly in cases when they are to be used for an extended period.

Amongst the novel compounds recently researched for alleviation of gastric ulcer is pumpkin seed oil (PSO). Research has been carried out to investigate the potential efficacy of the aforementioned drug as a potent anti-oxidant for management and protection against peptic ulcer; yet data with this regard remain scarce in literature. PSO is rich in mono- and polyunsaturated fatty acids, mainly oleic and linoleic acid (37–41.7%). In addition, PSO contains carotenoids, in high concentration, and sterols as stigmastatrienol, stigmastadienol, and spinasterol. Reports have shown the therapeutic effects of PSO, primarily highlighting the antidiabetic, antibacterial, anti-oxidant and anti-inflammatory properties of the edible oil with the highest contribution to the anti-oxidant capability being related to the polar fraction of the oil, mainly tocopherols. The mechanism underlying the anti-oxidant activity involves the blockage of 5-alpha reductase enzyme action.

Nanostructured lipid carriers (NLCs), second-generation solid lipid nanoparticles (SLNs), are high-performance pharmaceutical nanocarrier systems developed to enhance water solubility, stability as well as oil compounds' bioavailability. Mainly intended for parenteral administration of anti-cancer therapeutics, SLNs introduced in 1991, are nanosized particulate carrier system prepared either with physiological lipids or phospholipids, forming a lipid matrix that is solid at physiological temperature, with a size range of 50 to 1000 nm, dispersed in water vehicle or alternatively, in an aqueous surfactant solution. Unlike most polymeric microspherical and nanoparticulate carrier systems, the production of both lipid-based nano-formulations, SLNs and NLCs, eliminates the employment of potentially toxic organic solvents, which often leads to detrimental effect on certain drugs. Nevertheless, due to their lipophilic nature, they are primarily developed for the purpose of incorporating lipophilic active pharmaceutical ingredients. However, hydrophilic drugs are also incorporated yet to a lesser extent.

In fact, NLCs were developed in order to overcome the formulation-associated pitfalls of the SLNs. They are formulated using physiological, non-irritating lipids, unlike those used in forming polymeric nanoparticles (NPs). With a slight modification to the SLNs, NLCs are prepared by incorporating bioactive liquid oil component into the lipid-based formulation. Particularly enhancing oral delivery of drugs, the notable advantage raised by NLCs is the ability to encapsulate extensive drug quantities by the formation of imperfect, less structured lipid matrices, for better encapsulation efficiency. Importantly, the imperfection is owed to liquid oil incorporation within the core matrix of solid lipid (SL). Further to the remarkable advantages imparted by SLNs and other novel drug delivery systems of nanoparticles, NLCs demonstrated further enhancement of stability, reduced expulsion of the encapsulated drug from the carrier during storage period due to the imperfection of crystalline lattice, properties not possessed by SLNs. Moreover, using SLNs and NCLs has minimized the fairly large number of shortcomings associated with liposomes and NPs, such as difficulty of upscaling, high-cost production process and materials, and potential toxicity.
Lately, the trend towards using NLCs as vehicles for oils is extremely promising. The NLC as a liquid core demonstrated minimal toxicity. In addition, it has enhanced the in vivo performance and potentiated the immunosuppressive effects of the carried drug Tacrolimus, through inhibition of Interleukin-2 (IL-2) cytokine release. Furthermore, Muchow et al have developed a paste-like formulation of omega-3-loaded NLC in order to chemically stabilize the fatty acids.

This study aims to investigate the formulation of PSO-based NLCs in order to utilize PSO as the liquid lipid (LL) component of NLCs and to achieve oil dispersion in the nano-range in the stomach. Box–Behnken design was utilized to deduce the optimum formula. The optimized PSO-NLCs formula was investigated for gastric ulcer protective effects by evaluating ulcer index and determination of gastric mucosa oxidative stress parameters.

**Materials and Methods**

**Materials**

Pumpkin seed oil (PSO), d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MI, USA). Precirol® ATO 5 was obtained as a kind gift from Gattefosse (Saint-Priest, France). Soybean L-α-phosphatidylcholine (soybean lecithin) was purchased from Lipoid (Ludwigshafen, Germany). The PSO oil has been used as supplied.

**Formulation of PSO-NLCs**

Precirol was utilized as the solid lipid (SL), PSO was utilized as the liquid lipid (LL) and Tween 80: TPGS: soybean lecithin (6:2:2 ratio) mixture was used as surfactant in the formulation of the NLCs. PSO-NLCs were formulated by high-shear homogenization and ultrasonication technique as previously reported. Briefly, lipid formula components, precirol (SL) and PSO (LL), were melted at 75 °C. Separately, aqueous surfactant mixture solution (Tween 80: TPGS: soybean lecithin) was heated to 75 °C and mixed with the melted lipids. The mixture was homogenized (T25 Ultra-Turrax (IKA® Werke GmbH & Co. KG, Staufen, Germany)) at 12,000 rpm for 60 seconds, in a water bath at 75 °C. The mixture was then subjected to probe-sonication for 5 minutes. The final volume was adjusted to 20 mL using distilled water. The formed nanoemulsion was then left to cool to form PSO-NLCs and stored at 20 °C.

**Optimization of PSO-NLCs**

The development of the Box–Behnken experimental design for PSO-NLCs formulation components was carried out, based on preliminary investigation, using the Statgraphics plus, version 4 (Statgraphics software) (Manugistic Inc., PA, USA). The selected factors were the concentrations of precirol as SL (X1); the concentration of oil PSO as LL (X2); and the sonication time (ST) (X3). The design response was PSO-NLCs size (nm). The design goal was set to minimize the size of the investigated response (PSO-NLCs). The design generated 15 formulations of various combination of the investigated factors that were prepared as described in the ‘Formulation of PSO-NLCs’ section.

**PSO-NLCs Size Determination**

PSO-NLCs size was determined utilizing Nano-ZS particle size analyzer (Malvern Instrument, Worcestershire, UK). One hundred microliters of each PSO-NLCs formulation was 100-times diluted with distilled water that was passed through a 0.1-µm membrane filter, vortexed for 1 minute and then measured.

**Prediction and Preparation of Optimized PSO-NLCs Formulation**

The data collected from PSO-NLCs formulations, proposed by the experimental design, were statistically analyzed utilizing the software (ANOVA and multiple response optimization). The proposed optimum formulation obtained (predicted formula) was practically prepared and compared to the predicted formula by the design for result validation.

**Fourier-Transform Infrared Physicochemical Characterization of the Optimized PSO-NLCs**

The optimized PSO-NLCs formula and formula components were assessed using Fourier-transform infrared (FTIR) analysis as previously described. Briefly, FTIR spectra of PSO, precirol, TPGS, Tween 80, soybean lecithin and the prepared optimized PSO-NLCs formula were recorded over the wavelength range from 400 to 4000 cm-1 using FTIR spectrophotometer (Nicolet IZ 10, Thermo Fisher Scientific, Waltham, MA, USA). Samples were directly applied to the FTIR spectrophotometer without treatment.
In vivo Evaluation of Optimized PSO-NLCs Formulation

Animals

Adult male Wistar rats (180–200 gm) were obtained from the animal house of King Fahd Medical Research Center, King Abdulaziz University. Animals were acclimatized for 1 week before the experiment. The study protocol was approved by the Faculty of Pharmacy Research Ethics Committee, King Abdulaziz University (Reference #: PH 122–41). Care and use of animals according to the EU Directive 2010/63/EU and DHEW publication NIH 80–23 was ensured. One day prior the induction of gastric ulcers, all rats were fasted in mesh-bottomed cages to minimize coprophagia with free access to water. The rats were then divided to four groups (8 animals each): 1) control group: non-treated rats with no induction of ulcer, 2) indomethacin group: in which the rats received 50 mg/kg of indomethacin, 3) PSO group: in which the rats received pure PSO 30 minutes before injection of indomethacin and finally 4) optimized PSO-NLCs formula group: in which the rats received optimized PSO-NLCs formula 30 minutes before injection of indomethacin. PSO was given at a dose of 100mg/kg and PSO-NLCs optimized formulation was given as an equivalent dose and was administered orally 30 minutes before the induction of ulcer. Gastric ulceration was induced by an intraperitoneal injection of indomethacin (50mg/kg). Four hours later, all rats were sacrificed by decapitation. Their stomachs were removed, opened along the greater curvature, and washed with ice-cold saline. Animal stomachs were scored for macroscopic gross mucosal lesions. Gastric mucosae were collected and stored at −80 °C until used for estimation of oxidative stress parameters. Another set of stomachs from each group was immersed in 10% formalin for histopathological examination.

Gastric Mucosal Lesion Assessment

Mucosal lesions in all animal groups were quantified according to a previously described method by Szabo and Hollander. Briefly, images were captured for pinned stomachs and areas of mucosal damage were measured using ImageJ software and then expressed as % of the total stomach surface area. For each group, mean ulcer score expressed as ulcer index (U.I) and the percentage of inhibition (preventive index) against indomethacin-induced ulcers was determined using the equation:

\[
\text{Ulcer inhibition (\%) = } \left( \frac{\text{U.I. in indomethacin} - \text{U.I. in treated rats}}{\text{U.I. in indomethacin}} \right) \times 100
\]

\[\text{Equation (1)}\]

Determination of Gastric Mucosa Oxidative Stress Parameters

Gastric mucosal tissues were homogenized (0.1 g/mL) using phosphate buffer saline (ice-cold) using then centrifuged for 20 minutes at 4 °C. The following parameters were calculated from the aspirated supernatant:

- Malondialdehyde (MDA), a measure of lipid peroxidation, was determined according to the method of Uchiyama and Mihara. 
- Nitric oxide (NO) was assayed colorimetrically using Griess reagent.
- Catalase activity was determined using a commercially available kit (Biodiagnostic, Egypt), according to the method of Fossati, Prencipe.

Results and Discussion

Formulation and Optimization of PSO-NLCs

NLCs composed of SL and LL. The inclusion of LL in NLCs, different from SL nanoparticles, aims to reduce crystallinity and increase the fluidity of the matrix with reduced lipid packaging density. This leads to improved storage life when compared with SL nanoparticles. The important criteria for efficacy and efficiency of nanoparticles (drug release, biodistribution and cellular uptake) are particle size and size distribution.

Table 1 shows the PSO-NLCs size variabilities for the prepared formulations. The results revealed that the size ranged from 65.0 to 284.0 nm for formulations F13 and F14, respectively. The polydispersity index for the prepared PSO-NLCs formulations was in the range of 0.2–0.5 that shows acceptable unimodal size distribution. Two-way ANOVA analysis showed a significant antagonistic effect of the SL (X1) and ST (X3) percentages on the PSO-NLCs size with p-values of 0.00001 and 0.0001, respectively (Table 2, Figure 1). In addition, the quadratic term of X3 showed a significant synergistic effect on PSO-NLCs size with a p-value of 0.0313. The equation of PSO-NLCs size prediction according to correlation with the factors is shown in Equation (1).

\[\text{PSO} - \text{NLCs size (nm)} = 491.65 - 162.5X_1 - 17.32X_2 - 24.44X_3 - 242.59X_1^2 - 11.11X_2X_3 - 5.83X_1^3 - 20.37X_2^2 + 17.5X_3X_3 + 2.26X_3^3\]
The results indicated that increasing SL (precirol) percent (content) in the formulation showed a reduction in the produced PSO-NLCs size. This has been observed in formulation number, 2, 6, 12 and 13. The reduction in PSO-NLCs size with increased SL content is attributed to the formation of more dense rigid crystalline structure of the formed nanoparticles. In addition, the inverse relationship between ST (X3) and PSO-NLCs size is attributed to the ability of ultrasound sonication force to breakdown the coarse emulsion droplets to smaller nano-range sizes. Consequently, increased ST provides more energy to breakdown emulsion droplets to smaller sizes.

On the other hand, the results of pareto chart, Figure 1, showed a direct relationship, although non-significant at the specified concentration range, between LL and PSO-NLCs size. Previous reports revealed the direct relationship between LL and PSO-NLCs size.

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rational for this relation either unknown or attributed to the inability of surfactant to cover the melted lipid droplets’ surface when the LL-to-SL ratio was more than 50%.\(^{49}\) Pareto chart and response surface plot revealed the relationship between the investigated factors (X1–X3) and PSO-NLCs size (Figures 1 and 2).

### Validation of the PSO-NLCs Optimized Formula

The obtained data from the 15 formulations generated by the experimental design were analyzed with ANOVA. The Box–Behnken design predicted the optimum formulation that was practically prepared and evaluated and compared with the predicted results generated by the design (Table 3). The prepared optimum formula showed a size of 64.3 nm that was compared with the predicted value (62.9 nm) of PSO-NLCs size generated by the design (Table 3). The optimized PSO-NLCs formulation was utilized in the in vivo evaluation

### Table 3 Optimum Levels for PSO-NLCs Factors and Predicted, Actual and Residual Values for PSO-NLCs Size

<table>
<thead>
<tr>
<th>Factor</th>
<th>Optimum Level</th>
<th>Low Level</th>
<th>High Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>0.9</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>X2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>X3</td>
<td>5.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Response</td>
<td>Prediction</td>
<td>Actual</td>
<td>Residual</td>
</tr>
<tr>
<td>PSO-NLCs size</td>
<td>62.9 nm</td>
<td>64.3 nm</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Desirability constraint: Minimize the particle size.

### Abbreviations:

PSO, pumpkin seed oil; NLCs, nanostructured lipid carriers; X1, concentrations of precirol as solid lipid; X2, the concentration of oil PSO as liquid lipid; X3, the sonication time.

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**Figure 3** Size distribution of optimized PSO-NLCs.

**Abbreviation:** PSO-NLCs, pumpkin seed oil nanostructured lipid carriers.

**Figure 4** FTIR spectra of PSO, Tween 80, precirol, TPGS, soybean lecithin and the optimized PSO-NLCs formula.

**Abbreviations:** FTIR, Fourier-transform infra-red; TPGS, d-α-Tocopheryl polyethylene glycol 1000 succinate; PSO-NLCs, pumpkin seed oil nanostructured lipid carriers.
studies. Size distribution of the optimized formula is shown in Figure 3 that revealed unimodal narrow size distribution.

**FTIR Physicochemical Characterization of the Optimized PSO-NLCs**

Figure 4 shows the FTIR spectra of optimized PSO-NLCs and its individual formula components. The main PSO IR peaks were 3300-3500 cm\(^{-1}\) as weak broad peaks that refer to the OH and COOH groups. The indicated PSO OH and COOH did not interfere with the characteristic peak region (around 3000 cm\(^{-1}\)) of other formula components. The results indicated no change in the characteristic functional group peaks of individual components when formulated in the optimized PSO-NLCs. FTIR is a useful tool for the evaluation of possible formula components interaction. Incompatibility among formula components could be predicted by changes in the characteristic peaks of the functional groups of each component of the optimized formula.

**In vivo Evaluation of Optimized PSO-NLCs Formulation**

**Effect of PSO and PSO-NLCs Formula on Indomethacin-Induced Gastric Lesions**

As shown in Figure 5, indomethacin resulted in the development of ulcer lesions, which were quantified as ulcer index of 6.2 ± 0.6 (Figure 5A). Pretreatment using both PSO and
optimized PSO-NLCs formula resulted in a significantly (p<0.01 and 0.001, respectively) lower UI compared to indomethacin (Figure 5B). The effect of the formula was more pronounced showing significantly (p<0.05) less mucosal lesions compared to the raw oil. Representative photos of the stomachs from the four different groups are shown in Figure 5C.

Effect of PSO and PSO-NLCs Formula on Gastric Mucosal Oxidative Stress Parameters

Lipid peroxidation, catalase activity and total nitrite levels were evaluated in the gastric mucosal tissues. As shown in Figure 6, the results for the indomethacin group indicated that gastric MDA and total nitrite levels were elevated compared to control that indicated increased oxidative stress. Similarly, the activity of catalase was higher than the control rat group (p<0.001) reflecting a compensatory increase in antioxidative parameters to counteract the elevated reactive oxygen species generation. PSO and PSO-NLCs formula administration had protective effects against these alterations showing significantly lower MDA, NO and catalase activity (Figure 6A–C).
Histopathological Examination of Stomach Sections (PSO versus PSO-NLCs Formula)

Figure 7 shows the results of histopathological examination of H&E stained stomach sections showing normal structure with no evidence of inflammation or ulceration in control rats (Figure 7A). Sections from indomethacin-treated groups show features of acute gastritis in the form of foveolar hyperplasia, edema, hyperemia and focal necrosis of foveolar cells. The lamina propria shows signs of neutrophilic infiltration (Figure 7B). No pathological lesions could be detected in muscularispropria. Sections from PSO-treated rats showed pits of normal gastric mucosal glands with no ulceration in which one or pit is found to have focal foveolar necrosis, mild edema and hyperemia in the lamina propria with submucosal area of congestion and hyperemia and no abnormalities in muscularispropria (Figure 7C). The stomach of optimized PSO-NLCs formula-treated rats (Figure 7D) shows normal gastric mucosal glands with foveolar arrangement and of normal length. No inflammation or infiltrates in lamina propria could be detected.

Gastric ulcer occurs when there is an imbalance between certain aggressive factors and defensive endogenous factors. There is therefore a great need for healthy, economic and effective antiulcer agents. Natural products have emerged as a source of compounds with potential antiulcer activity. Previous report by our group investigated the PSO solubilizing ability of ibuprofen in self-nanoemulsifying drug delivery system for improved solubility and as protection factor from peptic ulcer induced by the enhanced solubility of ibuprofen. These promising results were taken a step further to prove the efficacy of PSO in ulcer protection through formulation into NLCs with improved stability and efficacy characteristics when compared with the self-nanoemulsifying drug delivery system. Optimized PSO-NLCs showed improved efficacy in protection of anti-inflammatory drug-induced ulcer. The protection is attributed to PSO components (polyunsaturated fatty acids, tocopherol and sterols). In addition, PSO has been reported for wound healing characteristics. The optimized formula could have the ability to re-epithelialize the internal tissues as a result of tocopherol content of formula (from PSO and TPGS) that has scavenger activity of peroxy, hydroxyl, and superoxide radicals with the ability to heal the internal ulceration. In addition, soybean lecithin content of the
surfactant mixture in the optimized PSO-NLCs formulation offers gastric mucosal barrier.  

Conclusion

In this study, PSO was successfully incorporated as the LL component of NLCs. Box–Behnken experimental design for PSO-NLCs formulation components was carried out to achieve oil dispersion in the smallest formulation size in the stomach. Pretreatment using the optimized PSO-NLCs formula showed lower UI compared to indomethacin and less mucosal lesions compared to the raw oil. These results indicated great potential for future application of optimized PSO-NLCs formula for antiulcer effect in NSAID-induced gastric ulcer.

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Disclosure

The authors report no conflicts of interest in this work.

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