Manuscript Details

Manuscript number	JDDST_2019_1287_R1
Title	Preparation, characterization and in-vitro efficacy of quercetin loaded liquid crystalline nanoparticles for the treatment of asthma
Short title	Quercetin loaded liquid crystalline nanoparticles for the treatment of asthma
Article type	Research Paper

Abstract

The present study aims to formulate quercetin loaded liquid crystalline nanoparticles (LCN) and surface modified liquid crystalline nanoparticles (sm-LCN) as well as investigate their anti-inflammatory activity in human primary bronchial epithelial cell line (BCi-NS1.1) induced with lipopolysaccharide (LPS). Quercetin LCN were prepared using ultrasonication method. The formulated LCNs and sm-LCNs were characterised in terms of particle size, zeta potential as well as the drug encapsulation efficiency. Furthermore, their morphology and in vitro release profile were also studied. In addition, the anti-inflammatory activity of quercetin LCN and sm-LCNs were evaluated by measuring the concentration of pro-inflammatory markers namely interleukin (IL)-1β, IL-6 and IL-8 in BCI-NS1.1 cell lines via cytometric bead array. The molecular mechanism inherent to the inclusion of quercetin into monoolein nanosystem and surface modification of the nanosystem with chitosan was elucidated via molecular mechanics simulations. Quercetin LCN and sm-LCN significantly ($p < 0.05$) decreased the production of IL-1β, IL-6 and IL-8 compared to LPS only group. Encapsulation of quercetin into LCN and sm-LCN further enhanced its anti-inflammatory activity compared to quercetin in dimethyl sulfoxide (DMSO). In addition to that, quercetin LCN and sm-LCN also exhibited comparable activity to fluticasone in terms of significantly ($p < 0.05$) reducing the production of IL-1β and IL-6. Quercetin loaded LCN and sm-LCN could be a potential therapeutic intervention for asthma as they are efficacious in suppressing the production of key pro-inflammatory cytokines associated with the development of asthma.

Keywords	Quercetin; Liquid crystalline nanoparticles; Asthma; Inflammation
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Suggested reviewers	Pawan Maurya, Srinivas Nammi, Madhur Shastri, Shakti Shukla

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No data was used for the research described in the article

9th September 2019

The Editor
Journal of Drug Delivery Science & Technology

Subject: Regarding submission of an original research article for publication in Journal of Drug delivery Science and Technology entitled: Preparation, characterization and *in-vitro* efficacy of quercetin loaded liquid crystalline nanoparticles for the treatment of asthma.

Dear Editor,

Please find attached our original manuscript entitled "Preparation, characterization and *in-vitro* efficacy of quercetin loaded liquid crystalline nanoparticles for the treatment of asthma" for your kind consideration for publication in the Journal of Drug Delivery Science and Technology. The present research work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

The manuscript discusses about bronchial asthma, being a chronic inflammatory disease of airways, has numerous treatment options, none of which has disease-modifying properties. Quercetin, a dietary flavonoid commonly found in fruits and vegetables exhibits a wide spectrum of pharmacological activities; prominent among which is its anti-inflammatory activity, which has been proved, as a potential therapeutic intervention in the treatment of bronchial asthma, by various *in vitro* and *in vivo* animal studies.

Targeted and controlled delivery of quercetin directly to the lungs is extremely difficult. The use of liquid crystalline nanoparticle as an intervention in asthma is still not fully explored and only minimal attempts have been made. This directs an utmost need and attention, to identify a potential drug delivery platform that have minimum side effects and maximum patient compliance. Keeping all these facts, we have designed and conducted our present unique and a significant study, where, we have developed quercetin-loaded liquid crystalline nanoparticles as a means of delivery. In addition, quercetin is well known for its poor bioavailability and warrants the use of nanoparticles to overcome those limitations along with providing a prolonged release of quercetin.

The prepared liquid crystalline nanoparticles were characterized for their physicochemical properties, *in-vitro* release study and *in-vitro* anti-inflammatory studies focussed on various pro-inflammatory markers using immortalized human airway basal cell line (BCi-NS1.1). The prepared liquid crystalline nanoparticles were found to be stable and exhibited a controlled release pattern along with the suppression of various anti-inflammatory genes clearly demonstrating the potential of quercetin nano-formulations in asthma. Moreover, we have also studied the molecular mechanisms showing the molecular encapsulation pattern of quercetin in liquid crystalline nanoparticles.

Our study, emphasizing the application of nano-quercetin in the intervention of asthma as a promising drug delivery system, would be of great interest to the formulation, biological and respiratory scientists and clinicians. Our study can form the basis for further detailed research in this field, which can help in targeting a better treatment option for asthma. We hope that you find our manuscript acceptable for publication in the Journal of Drug Delivery Science and Technology.

Kind regards,
Dr Kamal Dua

24th September 2019

The Editor
Journal of Drug Delivery Science & Technology

Sub: Revision of manuscript for publication in Journal of Drug Delivery Science & Technology

Dear Editor,

Please find enclosed our revised manuscript "Preparation, Characterization and *In-vitro* Efficacy of Quercetin Loaded Liquid Crystalline Nanoparticles for the Treatment of Asthma" which we would like to re-submit for consideration for publication. We are grateful to you, and that to your reviewers for providing us valuable feedback/suggestions, which we have addressed that has helped improve our manuscript. We am submitting herewith the revised manuscript which incorporates all the suggestions of the reviewers. Changes made in the revised manuscript are done using the red color mode in MS Word file along with below listed point to point clarifications to the reviewer's comments.

Best Regards

Kamal Dua/ Dinesh Kumar Chellappan

Point wise changes have been made in the revised manuscript.

We thank the Editor, Editorial team and the Reviewers for their consideration of our manuscript, and for providing valuable suggestions. We have now addressed all the comments and incorporated changes into a revised version as described in the point-by-point response below.

Reviewer 1
The manuscript entitled by Yong et al. showed the anti-inflammatory potential of the quercetin loaded liquid crystalline nanoparticles (LCN) and surface-modified liquid crystalline nanoparticles (sm-LCN) in asthma where the authors have carried out a blend of physicochemical, molecular modeling characterization and biological studies. The concept of liquid crystalline nanoparticles (LCN) and surface-modified liquid crystalline nanoparticles is novel and emerging in the area of translational research in respiratory diseases. I recommend the manuscript to be considered for publication considering the authors can incorporate and answer the below suggestions.

1. **Comment 1 (C1):** There are no recent statistics provided for asthma as a disease burden. Please include in the introduction section.
   Response (R1): We appreciate the reviewer comment. The statistics for disease burden for Asthma has been included in the introduction. (Page:3; line 4 to 7).
2. **C2:** Is there any particular pathway authors envisages in relation to their biological activity results, if so, please include in the discussion.
   R2: We appreciate reviewer's comment. The NFκB pathway has been discussed specifically in context to our biological activity. (Page:10; line 16).
3. **C3:** Why the authors have selected IL-6, IL-8 and IL-1β as the potential inflammatory markers to examine in the study. Please justify.
   R3: We appreciate the comment made by the reviewer. IL-6, IL-8 and IL-1β has been extensively involved in pathogenesis of Asthma. The authors emphasized these inflammatory markers based on previous studies as follows: https://www.ncbi.nlm.nih.gov/pubmed/20816188, https://www.ncbi.nlm.nih.gov/pubmed/29781141

4. **C4:** The authors need to check the manuscript for language consistency and grammatical errors.
    R4: We appreciate reviewer's concern. The authors have checked the manuscript for grammatical errors and language consistency.
5. **C5:** Please ensure that all the references are correctly cited and are in accordance with the journal guidelines.
    R5: The manuscript has been referenced by Endnote software as per the journal guidelines and specifications.

Reviewer 2

The manuscript entitled "Preparation, Characterization and Comparative *In-vitro* Efficacy of Quercetin Loaded Liquid Crystalline for the Treatment of Asthma" demonstrates the development of quercetin loaded liquid crystalline nanoparticles and surface modified liquid crystalline nanoparticles as formulations having anti-inflammatory activity in human primary bronchial epithelial cell line induced with lipopolysaccharide. The authors have covered all the important components needed for the formulation preparation and testing. However, I have a few below mentioned suggestions which authors can attend and include necessary details in the current version of their research manuscript.

1. **C1:** Is there any particular phenotype of asthma being targeted in the study? If so, mention the details.
    R1: The study was considered to target overall population with asthma pathophysiological features but did not take into account the specific consideration for the phenotype which forms the part of our future studies.
2. **C2:** Introduction lacks a description of the inflammatory markers involved in the pathophysiology of asthma. The authors must include a description with the figure if possible, to provide an overview to the readers considering the authors have done the inflammatory markers assessment in their study.
    R2: We appreciate reviewer's suggestion. We have included the role of inflammatory markers in the introduction section. (Page: 3; Line 7 to 11)
3. **C3:** How CHI solution has been prepared, which solvent has been used please mention?
    R3: The authors thank the reviewers for the feedback. The preparation of CHI solution has been included. (Page: 5; line: 9 & 10)
4. **C4:** Please change morphology to surface morphology.
    R4: The authors have rectified the sub-heading.
5. **C5:** Why authors have selected 0.5, 1, 2, 3, 6, 9, 12& 24 hrs as the time point for in vitro release study? Please justify.
    R5: The following time-points (0.5, 1, 2, 3, 6, 9,12 & 24 h) has been selected considering the intent to look for the prolonged/controlled release of Quercetin in the biological fluids which is the main rationale behind designing the liquid crystalline nanoparticles.
6. **C6:** Please be consistent with using short forms such as "h" for hours and ml or mL etc throughout the manuscript.
    R6: The authors have made the abbreviations consistent in the manuscript.
7. **C7:** Fig 4C, please change IL-b which should be represented as IL-1β.
    R7: Fig 4C: IL-b has been replaced with IL-1β.
8. **C8:** Authors need to do a careful reading in order to fix typographical and grammatical errors.
    R8: The authors thank the review for the suggestion. Authors have read and corrected the manuscript.

Preparation, Characterization and *In-vitro* Efficacy of Quercetin Loaded Liquid Crystalline Nanoparticles for the Treatment of Asthma

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1   **Abstract**

2   The present study aims to formulate quercetin loaded liquid crystalline nanoparticles (LCN) and

3   surface modified liquid crystalline nanoparticles (sm-LCN) as well as investigate their anti-

4   inflammatory activity in human primary bronchial epithelial cell line (BCi-NS1.1) induced with

5   lipopolysaccharide (LPS). Quercetin LCN were prepared using ultrasonication method. The

6   formulated LCNs and sm-LCNs were characterised in terms of particle size, zeta potential as well

7   as the drug encapsulation efficiency. Furthermore, their morphology and in vitro release profile

8   were also studied. In addition, the anti-inflammatory activity of quercetin LCN and sm-LCNs were

9   evaluated by measuring the concentration of pro-inflammatory markers namely interleukin (IL)-

10   1β, IL-6 and IL-8 in BCI-NS1.1 cell lines via cytometric bead array. The molecular mechanism

11   inherent to the inclusion of quercetin into monoolein nanosystem and surface modification of the

12   nanosystem with chitosan was elucidated via molecular mechanics simulations. Quercetin LCN

13   and sm-LCN significantly ($p < 0.05$) decreased the production of IL-1β, IL-6 and IL-8 compared

14   to LPS only group. Encapsulation of quercetin into LCN and sm-LCN further enhanced its anti-

15   inflammatory activity compared to quercetin in dimethyl sulfoxide (DMSO). In addition to that,

16   quercetin LCN and sm-LCN also exhibited comparable activity to fluticasone in terms of

17   significantly ($p < 0.05$) reducing the production of IL-1β and IL-6. Quercetin loaded LCN and sm-

18   LCN could be a potential therapeutic intervention for asthma as they are efficacious in suppressing

19   the production of key pro-inflammatory cytokines associated with the development of asthma.

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21   **Keywords:** Quercetin; Liquid crystalline nanoparticles; Asthma; Inflammation; BCi-NS1.1

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1. Introduction

Asthma is a chronic inflammatory disease involving the airways, which is often associated with narrowing of the airway structure and eventually leading to airway tissue remodeling [1]. Clinical symptoms of asthma include wheezing, coughing as well as shortness of breath. Based on the current estimates provided by the World Health Organization (WHO), around 235 million individuals suffer from asthma, thus, emerging to be a public health concern globally [2]. More than 80% of asthma deaths occur in low and lower middle-income countries. Innate immune cells, dendritic cells and epithelial cells produce various cytokines including interleukin-4 (IL-4), IL-8, IL-1β, IL-25, IL-33, thymic stromal lymphopoietin (TSLP) resulting in Th2 differentiation, recruitment and activation, thus promote IgE production, eosinophil and mast cell recruitment (Figure 1). These are the important factors contributing to the pathology of asthma [3, 4]. Common treatment modalities available to relieve asthmatic symptoms and manage its exacerbations include beta-adrenergic agonist, inhaled and systemic corticosteroids. Despite corticosteroids remarkably inhibiting the cascade of inflammatory reactions pertinent in asthma, the serious long-term side effects [5] associated with its use warrant the need for a potent and safer anti-inflammatory compound [6, 7].

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) (Figure 2) is a dietary flavonoid commonly found in vegetable and fruits, including onions, apples, grapes and nuts [8]. The growing interest of researchers towards this particular flavonoid could be attributed to its various pharmacological properties such as anti-oxidant, anti-inflammatory, anti-bacterial, anti-diabetic and anti-cancer [9]. Numerous studies have also elucidated on the potential therapeutic use of quercetin in the treatment of asthma owing to its potent anti-inflammatory property. Quercetin was found to ameliorate allergic airway inflammation and hyper-responsiveness mediated via attenuation of the NF-κB signalling pathway [10, 11]. Moreover, it also inhibits degranulation of mast cells [10], reduced the recruitment of eosinophils [12] and the levels of pro-inflammatory markers evident in allergic inflammatory diseases [13].

Although quercetin exhibits diverse pharmacological actions, its application in clinical practice is still limited. The main factor could be the poor bioavailability of this compound primarily due to low solubility in aqueous medium as well as poor intestinal absorption [14]. In order to overcome these drawbacks, quercetin can be encapsulated into a novel nanocarrier system

1   such as liquid crystalline nanoparticles (LCNs). Monoolein (MO), a non-toxic amphiphilic lipid is

2   normally used to construct these systems [15]. LCNs are formed by the dispersion of monoolein

3   in water which self-assembles into a well-ordered bicontinuous cubic structure [16]. Surfactants

4   such as poloxamer 407 (P 407) need to be added into the LCN system so that it ensures the

5   formation of a kinetically stable dispersion [17].

6   LCNs shows a great promise as an emerging drug delivery system due to their unique

7   properties such as bioadhesive nature, higher hydrophobic drug loading capacity, sustained-release

8   behaviour as well as their ability to improve physicochemical stability of encapsulated drugs [18,

9   19]. Studies have also proven that the surface modification of LCN with hydrophilic polymers

10 such as chitosan may allow longer residence time, increased mucoadhesiveness and enhanced

11 cellular uptake therefore, improving drug bioavailability. The rationale behind this approach is that

12 the outer shell layer was claimed to be more important than those of the core because the surface

13 is directly contact with body cells and fluids [3, 20-24].

14 Therefore, we believed that quercetin loaded LCNs and sm-LCNs were effective in treating

15 inflammatory respiratory ailments such as asthma as well as to further enhance the anti-

16 inflammatory activity of quercetin. The main aim of this research was to formulate quercetin

17 loaded LCNs and sm-LCNs followed by characterising them and evaluating their in vitro release

18 profile. Finally, the effects of quercetin loaded LCNs and sm-LCNs on the secretion of pro-

19 inflammatory cytokines were evaluated in immortalised human bronchial epithelial cell line (BCi-

20 NS1.1) induced with lipopolysaccharide (LPS).

21 **2. Materials**

22 Quercetin was purchased from Sisco Drug Laboratories, India; whereas, monoolein was obtained

23 from PI Chemicals, China. Poloxamer 407, Spectra Por® dialysis membrane and fluticasone

24 propionate were purchased from Sigma-Aldrich, USA. Immortalized human bronchial epithelial

25 cell line, BCi-NS1.1 was acquired from R. G. Crystal (Weill Cornell Medical College, New York,

26 USA). Bronchial Epithelial Growth Media (BEGM) and Bronchial Epithelial Basal Media

27 (BEBM) were purchased from Lonza, USA. All other solvents and chemicals used were of

28 analytical grade.

29 **2.1.   Preparation of quercetin loaded LCN**

Ultrasonication method was used to prepare quercetin loaded LCNs as described previously [15]. The composition of quercetin loaded LCN formulation is shown in Table 1. Briefly, appropriate amounts of monoolein and quercetin were melted together in a glass vial at 45°C. Distilled water heated to the same temperature was added into another glass vial containing P 407 and subsequently vortexed for one minute. Then, P 407 solution was transferred to the melted MO and quercetin mixture followed by vortexing for another minute. The liquid crystalline cubic phase formed in the mixture was then subjected to ultrasonication for 10 min using probe type sonicator (Sartorius Labsonic P) at 60 amplitudes and 0.5 cycle). Blank LCN without quercetin was also prepared using the same method mentioned above for comparison.

2.2. Preparation of quercetin loaded sm-LCN

0.1% Q sm-LCN was prepared by mixing 5mL 0.1% CHI solution and 5mL Q LCN. CHI solution was prepared in 50mM acetate buffer having 0.02% sodium azide. After that, it was subjected to magnetic stirring for 30 min. The same process was repeated for 0.2% Q sm-LCN except that it was mixed with 0.2% CHI solution [15].

2.3. Characterization of LCN & sm-LCN
2.3.1. Particle Size and Zeta Potential

The particle size and zeta potential of the nanoparticles was evaluated using Zetasizer Nano ZS (Malvern, UK) at 25°C. 0.1mL from blank LCN, Q LCN, 0.1% Q sm-LCN and 0.2% Q sm-LCN were diluted and equilibrated for 120 seconds before analysis [25-31].

2.3.2. Surface Morphology

The morphology of nanoparticles was studied using transmission electron microscopy (TEM). Nanoparticles was diluted suitably to 50-fold and allowed to drop on a carbon-coated copper grid and fixed by vacuum drying. The anchored droplets were then exposed to TEM observation at an acceleration voltage of 100kV [15, 28, 32].

2.3.3. Entrapment Efficiency

The EE of the nanoformulation was evaluated by separating free drug from the nanoparticles system via centrifugation method. 1.9mL of distilled water was added to dilute the 0.1mL of Q LCN and Q sm-LCN. UV-vis was then performed to analyze their concentration via Shimadzu

spectrophotometer at 369nm. 2mL of Q LCN and Q sm-LCN were centrifuged using a benchtop centrifuge (Eppendorf, Germany) at 2.5 Rcf for 15 min. The supernatants were withdrawn cautiously to remove the free quercetin and diluted with distilled water to 2mL. Their absorbance was detected 369nm using UV-Vis spectrophotometer, and the percentage of entrapment efficiency was calculated according to the following equation:

$$\% \ EE = (D_{total} - D_{free}) / D_{total} * 100$$

Where D_{free} and D_{total} are the amounts of free and total quercetin in the nanoparticles.

2.4. In vitro release study

In vitro drug release from the nanoformulation was carried out by using Spectra/Por dialysis membrane bag. 2mL of Q LCN, 0.1% Q sm-LCN and 0.2% Q sm-LCN was placed into separate dialysis bags (MWCO: 3500 g/mole) and immersed into 25mL of release medium (0.01-M phosphate buffer, pH 7.4) in falcon tube. The falcon tube was shaken at 39 rpm at 37°C temperature using water bath. At 0.5, 1, 2, 3, 6, 9, 12 and 24 hrs of the time points, 2mL from the falcon tube was removed for analysis and same amount of fresh dialysis medium was added to maintain the conditions. The amount of quercetin released was measured using UV−vis spectrophotometer at 369nm [27, 33].

2.5. In vitro cell culture study

Human minimally immortalized bronchial epithelial cell line BCi-NS1.1 was obtained from R. G. Crystal (Weill Cornell Medical College, New York, USA), and was cultured in BEGM growth media supplemented with (Lonza). Cells were then treated with Q LCN, 0.2% Q sm-LCN, blank LCN, blank sm-LCN at 25uM for 24hrs, and were then stimulated with LPS (100ng/mL) for 24hrs. 10nM Fluticasone was used as a standard treatment. The supernatants and RNAs were then harvested for further studies [25].

2.6. Cytometric bead array

The concentration of IL-1β , IL-6 and IL-8 in the supernatants collected from the treated BCi-NS1.1 cells was measured using cytometric bead array (BD Biosciences) and flow cytometry (FACSCANTO II, BD Biosciences) as documented in the manufacturer's instructions [34, 35].

2.7. Mechanistic profiling via molecular mechanics simulations

Molecular mechanics simulations were carried out employing using HyperChem™ 8.0.8 Molecular Modelling Software (Hypercube Inc., Gainesville, FL, USA). The molecular structures of chitosan (Cht; 4 glucosamine units) and quercetin (Quer) were generated employing saccharide building tool and natural bond angles, respectively. For monoolein (MO), the molecular segment with functional groups was generated using natural bond angles. The bimolecular structures – MO-Quer and MO-Cht – were developed by parallel disposition of constituent molecules and then AMBER 3 (Assisted Model Building and Energy Refinements) Force Field was applied for energy minimization. Full geometrical optimization was conducted in vacuum employing the Polak–Ribiere Conjugate Gradient method until an RMS gradient of 0.001 kcal/mol was reached [36].

2.8. Statistical analysis

All the values obtained were studied and performed using Graphpad Prisms 8.0. Data shown as mean ± standard deviation. One-way ANOVA tool was used to analyze the significant difference between groups and a value of $p < 0.05$ was considered statistically significant.

3. Results

The mean particle size, polydispersity index (PDI) and zeta potential of the quercetin formulation were measured and summarized in table 1. The mean particle size of the LCN formulations ranged from 210.0 to 268.7 nm with PDI less than 0.4 for all preparations. The zeta potentials for LCN formulations ranged from -14.6 to -15.6 mV whereas the sm-LCN ranged from 15.8 to 22.3 mV. The encapsulation efficiency of quercetin loaded LCN was 99.4% which was slightly higher compared to the surface modified LCNs.

The TEM images (Figure 3) show that both LCN and sm-LCN were spherical in shape. The particle size of LCNs ranged from 100nm to 300nm which was consistent with the dynamic light scattering.

The release profile of quercetin LCN over 24 hours. Release profile of quercetin from LCN exhibits a prolonged manner over the longer duration of time (Figure 4).

The BCi-NS1.1 cells were treated simultaneously with either 100ng/mL of LPS alone or with quercetin loaded LCNs and free quercetin in DMSO solution to investigate their respective effects on the production of LPS induced pro-inflammatory markers in these cell lines. Quercetin loaded LCNs and sm-LCNs with the dose of 25µM showed a consistent trend of significantly ($p < 0.05$) decreasing the concentration of pro-inflammatory cytokines namely IL-1β, IL-6 and IL-8 compared to the positive control group (Figure 5). It is also worth noting that quercetin LCNs as well as sm-LCNs remarkably downregulated the production of IL-6, IL-8 and IL-1β compared to free quercetin dissolved in DMSO, therefore, exhibiting superior anti-inflammatory activity.

4. Discussion

4.1. Characterization of LCN and sm-LCN

The significant increase in particle size indicates the encapsulation of quercetin into the blank LCN. Drug encapsulation into the nanoparticles may be a result of chemical conjugation or physical entrapment [37]. Polydispersity index (PDI) represents the size distribution and uniformity of the nanoparticles formed [38]. PDI values greater than 0.3 indicates a broad size distribution. Ideally, a narrow size distribution identified with PDI values lesser than 0.3 is preferred [38]. The slightly higher polydispersity index of quercetin loaded LCNs may be attributed to the coexistence of cubic liquid crystalline nanoparticles with other types of vesicles in the dispersion as previously reported [39, 40]. It was observed that the mean particle size for sm-LCN was significantly larger than LCN with regards to the amount of CHI used to modify the surface. When 0.1% CHI was used, the particle size observed was 236.1 nm whereas 0.2% CHI used has shown to increase the particle size to 268.7nm. The size increased in proportion to the quantity of CHI used proves that the increment was attributed to the surface coating of CHI on LCN [15].

The positive reading in zeta potential further indicates that the surface of LCN (negatively charged) have been successfully coated by the protonated amino group from CHI (13). Generally, zeta potential values above ±25 mV are required to form a stable dispersion between particles and reduce their aggregation [41]. P 407 aided in stabilising the LCN dispersion as demonstrated

previously [42]. It is presumed that the hydrophobic part of the polypropylene oxide chain adheres to the surface of the cubic nanoparticles whereas the hydrophilic portion of the chain extends out to the surrounding environment to provide steric shielding, therefore, avoiding agglomeration [43]. The negatively charged zeta potential could be ascribed to the subtle amount of oleic acid released by monoolein resulting in anionic behavior of the dispersion caused by adsorption of hydroxyl ions on the surface of the LCNs [41, 43].

TEM images further confirmed the results from the Zetasizer, showing that the particles were spherical in shape and size less than 300nm, which is quite similar to other reported studies [15, 18, 42, 44]. The dense black core surrounding the particle was due to the existence of CHI coating indicating that the surface of LCN has been modified.

Both LCN and sm-LCN have exhibited a high entrapment efficiency whereby >97% of drug was successfully entrapped. This indicates that most of the drug has been encapsulated regardless the surface modification of the nanoparticle. However other studies have successfully encapsulated their drug as high as 99% in LCN [15, 18, 41]. Nevertheless, the entrapment efficiency is still considered high when compared to other nanocarriers such as poly lactide-co-glycolic acid (PLGA) polymer-based nanoparticles where Anwar et al reported that only 86% of quercetin was encapsulated [45].

4.2. In vitro release study

The result has shown that quercetin drug was able to release in a sustained release manner when incorporated into the LCN and by modifying the surface with CHI further prolonged the drug release. This sustained release of quercetin could be attributed to the structure of the LCN. The higher proportion of lipids in the LCN not only allows hydrophobic drug to bind strongly to its inner structure, but also provides a larger surface area for the drugs to be incorporated. As a result, high entrapment efficiency of the drug can be achieved. Yoo et al., also showed that the release of the drug from LCN has sustained as long as 15 days [18].

4.3. *In vitro* evaluation anti-inflammatory activity of quercetin loaded LCN and sm-LCN on immortalised human bronchial epithelial cell line (BCi-NS1.1)

Evaluation of in vitro anti-inflammatory activity of quercetin loaded LCN and sm-LCN in comparison to free quercetin in DMSO solution

In the present study, quercetin loaded LCNs effectively decreased the concentration of pro-inflammatory cytokines namely IL-1β, IL-6 and IL-8. These effects exhibited by quercetin LCN was in consistent with previous studies evaluating the anti-inflammatory activity of quercetin on different cell lines [11, 13, 46-48]. Zhang et al., proved that quercetin remarkably suppressed the secretion of IL-6 and IL-1β in LPS-stimulated human peripheral blood mononuclear cells (PBMCs) [47]. Another research demonstrated that the treatment of quercetin notably reduced the gene expression and release of IL-8, IL-6 as well as IL-1β in human mast cells (HMC-1) induced by phorbol 12-myristate 13-acetate and calcium ionophore A23187 [11]. Although the underlying mechanism was not investigated in this present study, it is well-established from previous studies that quercetin attenuates the activation of NF-κB hence, downregulating the production of pro-inflammatory cytokines [11, 46, 47]. NF-κB signaling pathway plays a crucial role in the pathogenesis of asthma as it modulates the gene expression of pro-inflammatory mediators including chemokines, cytokines and cell adhesion molecules that lead to the infiltration of immune cells such as neutrophils and eosinophils in the airway [49, 50].

Encapsulation of quercetin into liquid crystalline nanoparticles was successful in further improving its anti-inflammatory activity. The enhanced anti-inflammatory effects of quercetin LCNs could be primarily attributed to the characteristics of the LCN itself, whereby it is bioadhesive in nature and improves membrane permeation leading to an increased interaction with the bronchial epithelial cells [41, 51]. The lipid bilayer of LCNs is akin to the epithelial cell membrane hence could be transported easily across the membrane resulting in greater drug absorption [51, 52]. Furthermore, findings from numerous studies also elucidated that LCNs promote greater cellular uptake and internalisation into cells via endocytosis enabling them to be diffused throughout the cytoplasm [18, 41, 52, 53]. On the other hand, quercetin itself could have only remained in the cell membrane due to its high lipophilicity allowing it to be easily incorporated into the cell membrane.

The findings observed in our study has demonstrated that quercetin loaded sm-LCN with the dose of 25μM significantly (p<0.05) decreased the pro-inflammatory markers when compared

to the free quercetin drug in DMSO. This indicates that sm-LCN is able to enhance the anti-inflammatory effect of quercetin when entrapped in the nanocarrier.

There is also a noticeable reduction in the expression of all the pro-inflammatory cytokines which was observed on the blank LCN & sm-LCN. This suggests that the empty vehicle itself can slightly reduce the inflammation. We suspect that one of the components in the LCN formulation might contributed and it was monoolein. However, only one study has confirmed this and Ali et al reported that monoolein possess anti-inflammatory property that inhibits the production of pro-inflammatory markers in LPS stimulated bone marrow-derived dendritic cell (BMDC) via NF-κB and MAPK pathways [54].

Evaluation of in vitro anti-inflammatory activity of quercetin loaded LCN and sm-LCN in comparison to fluticasone

Fluticasone, an FDA approved drug to treat asthma was used as a standard to further establish the anti-inflammatory activity of quercetin LCNs. Quercetin LCNs were comparable to fluticasone in terms of reducing the levels of IL-1β and IL-6 however, fluticasone was much more effective in decreasing the production of IL-8. In this existing study, the BCi-NS1.1 cell lines were only treated with quercetin LCNs at the dose of 25μM. Quercetin was able to reduce the levels of pro-inflammatory cytokines in a dose-dependent manner up to a 100μM as described in the literature [47]. Therefore, the finding suggests the potential of quercetin LCNs to decrease the levels pro-inflammatory cytokines to a greater extent comparable to fluticasone at a higher dose.

4.4.   Molecular mechanics simulations

The molecular simulations performed in this research focused on two aspects: a) encapsulation or incorporation of quercetin within a monoolein matrix, and b) surface modification of the monoolein matrix with chitosan. The geometrical iterations representing the above bimolecular complexes are shown in Figure 5 while the related energetic molecular attributes are depicted in Tables 2 and 3, respectively. In case of MO-Quer; the formation of molecular complex was accompanied by a total energy stabilization of 15.894kcal/mol. Interestingly, the total energy of the MO-Quer complex was less than the individual constituent molecules. The incorporation of Quer in MO was evident from the fact that all bonding (bond, angle, and dihedral) and non-bonding (van der waals forces and H-bonding) energy terms contributed to the geometrical stabilization of

the complex. The major stabilization was provided by the van der Waals function which brought the molecules in close vicinity and hence led to the formation of –OH…HO– bonds between the molecules (Table 2, Figure 5). For the MO-Cht molecular complex; an energy stabilization of 10.336kcal/mol was observed. In line with MO-Quer, the total energy of the MO-Cht complex was less than the individual molecular components. The energy stabilization in this case was mainly due to non-bonding interactions (van der Waals forces and electrostatic interactions) while the bonding interactions (bond and dihedral energies) destabilized the complex. Such destabilization is due to torsional strain experienced by the molecules while interacting within the van der Waals space. Additionally, both inter- and intra-molecular H-bonding was observed in the MO-Cht complex further confirming its favourable formation and conformation. The above discussion justifies the selection and successful incorporation of quercetin in and chitosan surface modification of the monoolein nanosystem.

5. Conclusions

Quercetin was successfully encapsulated into liquid crystalline nanoparticles revealing their sustained release behaviour. Moreover, quercetin LCN and sm-LCN were effective in reducing the levels of pro-inflammatory mediators such as IL-1β, IL-6 and IL-8 whereby encapsulating quercetin into LCN further enhanced its anti-inflammatory activity. This demonstrates that the quercetin LCN could be employed as a potential novel drug delivery for the treatment of asthma. This mode will overcome the issues associated with quercetin such as the low solubility, poor bioavailability and short half-life. The findings of this present study advocate the translation into other associated pulmonary inflammatory diseases such as chronic obstructive pulmonary disorder (COPD) as well as lung cancer which will help in providing a new direction to the pulmonary clinics.
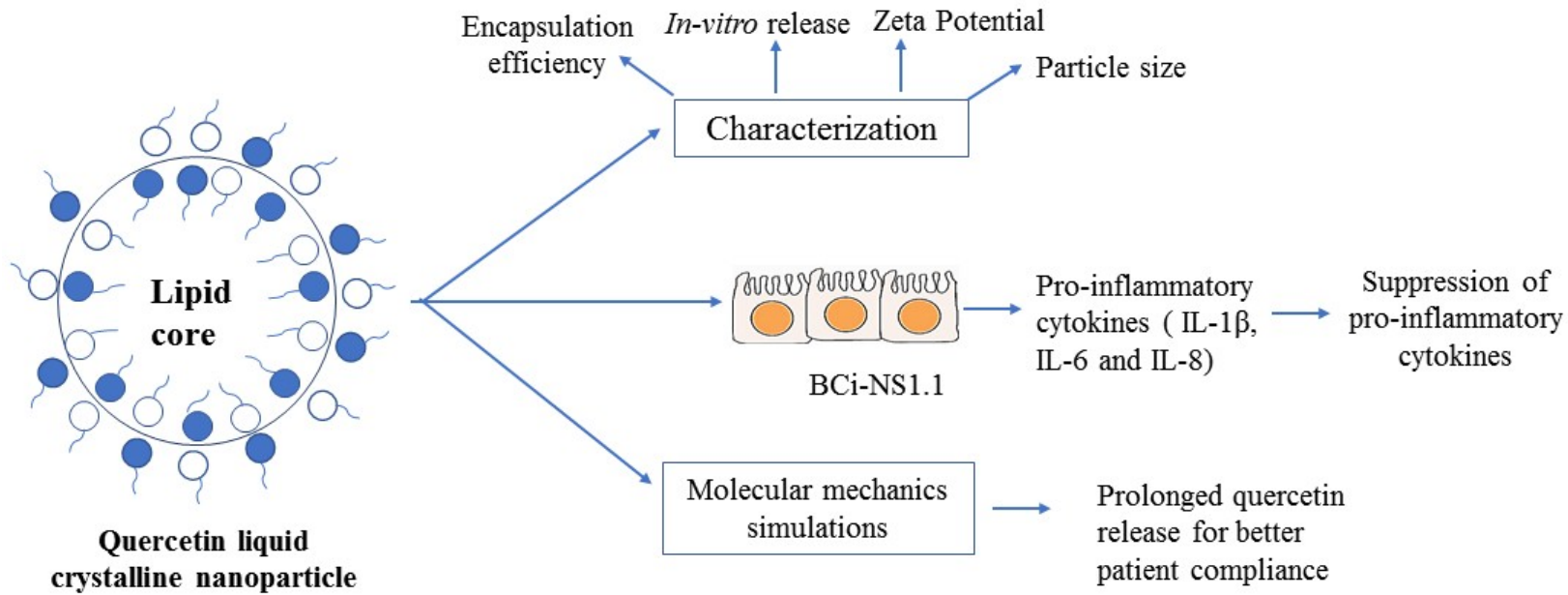
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Encapsulation efficiency   *In-vitro* release   Zeta Potential

Particle size

Characterization

Pro-inflammatory cytokines (IL-1β, IL-6 and IL-8)

Suppression of pro-inflammatory cytokines

BCi-NS1.1

Molecular mechanics simulations

Prolonged quercetin release for better patient compliance

Lipid core

Quercetin liquid crystalline nanoparticle

Preparation, Characterization and *In-vitro* Efficacy of Quercetin Loaded Liquid Crystalline Nanoparticles for the Treatment of Asthma

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Abstract

The present study aims to formulate quercetin loaded liquid crystalline nanoparticles (LCN) and surface modified liquid crystalline nanoparticles (sm-LCN) as well as investigate their anti-inflammatory activity in human primary bronchial epithelial cell line (BCi-NS1.1) induced with lipopolysaccharide (LPS). Quercetin LCN were prepared using ultrasonication method. The formulated LCNs and sm-LCNs were characterised in terms of particle size, zeta potential as well as the drug encapsulation efficiency. Furthermore, their morphology and in vitro release profile were also studied. In addition, the anti-inflammatory activity of quercetin LCN and sm-LCNs were evaluated by measuring the concentration of pro-inflammatory markers namely interleukin (IL)-1β, IL-6 and IL-8 in BCI-NS1.1 cell lines via cytometric bead array. The molecular mechanism inherent to the inclusion of quercetin into monoolein nanosystem and surface modification of the nanosystem with chitosan was elucidated via molecular mechanics simulations. Quercetin LCN and sm-LCN significantly ($p < 0.05$) decreased the production of IL-1β, IL-6 and IL-8 compared to LPS only group. Encapsulation of quercetin into LCN and sm-LCN further enhanced its anti-inflammatory activity compared to quercetin in dimethyl sulfoxide (DMSO). In addition to that, quercetin LCN and sm-LCN also exhibited comparable activity to fluticasone in terms of significantly ($p < 0.05$) reducing the production of IL-1β and IL-6. Quercetin loaded LCN and sm-LCN could be a potential therapeutic intervention for asthma as they are efficacious in suppressing the production of key pro-inflammatory cytokines associated with the development of asthma.

Keywords: Quercetin; Liquid crystalline nanoparticles; Asthma; Inflammation; BCi-NS1.1

1. Introduction

Asthma is a chronic inflammatory disease involving the airways, which is often associated with narrowing of the airway structure and eventually leading to airway tissue remodelling [1]. Clinical symptoms of asthma include wheezing, coughing as well as shortness of breath. Based on the current estimates provided by the World Health Organization (WHO), around 235 million individuals suffer from asthma, thus, emerging to be a public health concern globally [2]. Common treatment modalities available to relieve asthmatic symptoms and manage its exacerbations include beta-adrenergic agonist, inhaled and systemic corticosteroids. Despite corticosteroids remarkably inhibiting the cascade of inflammatory reactions pertinent in asthma, the serious long-term side effects [3] associated with its use warrant the need for a potent and safer anti-inflammatory compound [4, 5].

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) (Figure 1) is a dietary flavonoid commonly found in vegetable and fruits, including onions, apples, grapes and nuts [6]. The growing interest of researchers towards this particular flavonoid could be attributed to its various pharmacological properties such as anti-oxidant, anti-inflammatory, anti-bacterial, anti-diabetic and anti-cancer [7]. Numerous studies have also elucidated on the potential therapeutic use of quercetin in the treatment of asthma owing to its potent anti-inflammatory property. Quercetin was found to ameliorate allergic airway inflammation and hyper-responsiveness mediated via attenuation of the NF-κB signalling pathway [8, 9]. Moreover, it also inhibits degranulation of mast cells [8], reduced the recruitment of eosinophils [10] and the levels of pro-inflammatory markers evident in allergic inflammatory diseases [11].

Although quercetin exhibits diverse pharmacological actions, its application in clinical practice is still limited. The main factor could be the poor bioavailability of this compound primarily due to low solubility in aqueous medium as well as poor intestinal absorption [12]. In order to overcome these drawbacks, quercetin can be encapsulated into a novel nanocarrier system such as liquid crystalline nanoparticles (LCNs). Monoolein (MO), a non-toxic amphiphilic lipid is normally used to construct these systems [13]. LCNs are formed by the dispersion of monoolein in water which self-assembles into a well-ordered bicontinuous cubic structure [14]. Surfactants such as poloxamer 407 (P 407) need to be added into the LCN system so that it ensures the formation of a kinetically stable dispersion [15].

LCNs shows a great promise as an emerging drug delivery system due to their unique properties such as bioadhesive nature, higher hydrophobic drug loading capacity, sustained-release behaviour as well as their ability to improve physicochemical stability of encapsulated drugs [16, 17]. Studies have also proven that the surface modification of LCN with hydrophilic polymers such as chitosan may allow longer residence time, increased mucoadhesiveness and enhanced cellular uptake therefore, improving drug bioavailability. The rationale behind this approach is that the outer shell layer was claimed to be more important than those of the core because the surface is directly contact with body cells and fluids [18-23].

Therefore, we believed that quercetin loaded LCNs and sm-LCNs were effective in treating inflammatory respiratory ailments such as asthma as well as to further enhance the anti-inflammatory activity of quercetin. The main aim of this research was to formulate quercetin loaded LCNs and sm-LCNs followed by characterising them and evaluating their in vitro release profile. Finally, the effects of quercetin loaded LCNs and sm-LCNs on the secretion of pro-inflammatory cytokines were evaluated in immortalised human bronchial epithelial cell line (BCi-NS1.1) induced with lipopolysaccharide (LPS).

2. Materials

Quercetin was purchased from Sisco Drug Laboratories, India; whereas, monoolein was obtained from PI Chemicals, China. Poloxamer 407, Spectra Por® dialysis membrane and fluticasone propionate were purchased from Sigma-Aldrich, USA. Immortalized human bronchial epithelial cell line, BCi-NS1.1 was acquired from R. G. Crystal (Weill Cornell Medical College, New York, USA). Bronchial Epithelial Growth Media (BEGM) and Bronchial Epithelial Basal Media (BEBM) were purchased from Lonza, USA. All other solvents and chemicals used were of analytical grade.

2.1. Preparation of quercetin loaded LCN

Ultrasonication method was used to prepare quercetin loaded LCNs as described previously [13]. The composition of quercetin loaded LCN formulation is shown in Table 1. Briefly, appropriate amounts of monoolein and quercetin were melted together in a glass vial at 45°C. Distilled water heated to the same temperature was added into another glass vial containing P 407 and subsequently vortexed for one minute. Then, P 407 solution was transferred to the melted MO

and quercetin mixture followed by vortexing for another minute. The liquid crystalline cubic phase formed in the mixture was then subjected to ultrasonication for 10 minutes using probe type sonicator (Sartorius Labsonic P) at 60 amplitudes and 0.5 cycle. Blank LCN without quercetin was also prepared using the same method mentioned above for comparison.

2.2. Preparation of quercetin loaded sm-LCN

0.1% Q sm-LCN was prepared by mixing 5ml 0.1% CHI solution and 5ml Q LCN. After that, it was subjected to magnetic stirring for 30 min. The same process was repeated for 0.2% Q sm-LCN except that it was mixed with 0.2% CHI solution [13].

2.3. Characterization of LCN & sm-LCN
2.3.1. Particle Size and Zeta Potential

The particle size and zeta potential of the nanoparticles was evaluated using Zetasizer Nano ZS (Malvern, UK) at 25°C. 0.1mL from blank LCN, Q LCN, 0.1% Q sm-LCN and 0.2% Q sm-LCN were diluted and equilibrated for 120 seconds before analysis [24-30].

2.3.2. Morphology

The morphology of nanoparticles was studied using transmission electron microscopy (TEM). Nanoparticles was diluted suitably to 50-fold and allowed to drop on a carbon-coated copper grid and fixed by vacuum drying. The anchored droplets were then exposed to TEM observation at an acceleration voltage of 100kV [13, 27, 31].

2.3.3. Entrapment Efficiency

The EE of the nanoformulation was evaluated by separating free drug from the nanoparticles system via centrifugation method. 1.9mL of distilled water was added to dilute the 0.1mL of Q LCN and Q sm-LCN. UV-vis was then performed to analyze their concentration via Shimadzu spectrophotometer at 369nm. 2mL of Q LCN and Q sm-LCN were centrifuged using a benchtop centrifuge (Eppendorf, Germany) at 2.5 Rcf for 15 minutes. The supernatants were withdrawn cautiously to remove the free quercetin and diluted with distilled water to 2mL. Their absorbance was detected 369nm using UV-Vis spectrophotometer, and the percentage of entrapment efficiency was calculated according to the following equation:

% EE=(D_total-D_free)/D_total *100

Where Dfree and Dtotal are the amounts of free and total quercetin in the nanoparticles.

2.4. In vitro release study

In vitro drug release from the nanoformulation was carried out by using Spectra/Por dialysis membrane bag. 2ml of Q LCN, 0.1% Q sm-LCN and 0.2% Q sm-LCN was placed into separate dialysis bags (MWCO: 3500 g/mole) and immersed into 25ml of release medium (0.01-M phosphate buffer, pH 7.4) in falcon tube. The falcon tube was shaken at 39 rpm at 37oC temperature using water bath. At 0.5, 1, 2, 3, 6, 9, 12 and 24 hours of the time points, 2ml from the falcon tube was removed for analysis and same amount of fresh dialysis medium was added to maintain the conditions. The amount of quercetin released was measured using UV–vis spectrophotometer at 369nm [26, 32].

2.5. In vitro cell culture study

Human minimally immortalized bronchial epithelial cell line BCi-NS1.1 was obtained from R. G. Crystal (Weill Cornell Medical College, New York, USA), and was cultured in BEGM growth media supplemented with (Lonza). Cells were then treated with Q LCN, 0.2% Q sm-LCN, blank LCN, blank sm-LCN at 25uM for 24hrs, and were then stimulated with LPS (100ng/mL) for 24hr. 10nM Fluticasone was used as a standard treatment. The supernatants and RNAs were then harvested for further studies [24].

2.6. Cytometric bead array

The concentration of IL-1β , IL-6 and IL-8 in the supernatants collected from the treated BCi-NS1.1 cells was measured using cytometric bead array (BD Biosciences) and flow cytometry (FACSCANTO II, BD Biosciences) as documented in the manufacturer's instructions [33, 34].

2.7. Mechanistic profiling via molecular mechanics simulations

Molecular mechanics simulations were carried out employing using HyperChem™ 8.0.8 Molecular Modelling Software (Hypercube Inc., Gainesville, FL, USA). The molecular structures of chitosan (Cht; 4 glucosamine units) and quercetin (Quer) were generated employing

saccharide building tool and natural bond angles, respectively. For monoolein (MO), the molecular segment with functional groups was generated using natural bond angles. The bimolecular structures – MO-Quer and MO-Cht – were developed by parallel disposition of constituent molecules and then AMBER 3 (Assisted Model Building and Energy Refinements) Force Field was applied for energy minimization. Full geometrical optimization was conducted in vacuum employing the Polak–Ribiere Conjugate Gradient method until an RMS gradient of 0.001 kcal/mol was reached [35].

2.8.   Statistical analysis

All the values obtained were studied and performed using Graphpad Prisms 8.0. Data shown as mean ± standard deviation. One-way ANOVA tool was used to analyze the significant difference between groups and a value of $p<0.05$ was considered statistically significant.

3. Results

The mean particle size, polydispersity index (PDI) and zeta potential of the quercetin formulation were measured and summarized in table 1. The mean particle size of the LCN formulations ranged from 210.0 to 268.7 nm with PDI less than 0.4 for all preparations. The zeta potentials for LCN formulations ranged from -14.6 to -15.6 mV whereas the sm-LCN ranged from 15.8 to 22.3 mV. The encapsulation efficiency of quercetin loaded LCN was 99.4% which was slightly higher compared to the surface modified LCNs.

The TEM images (Figure 2) show that both LCN and sm-LCN were spherical in shape. The particle size of LCNs ranged from 100nm to 300nm which was consistent with the dynamic light scattering.

Media only condition represented as negative control while LPS only condition represented as positive control. The significant difference at p<0.05 of pro-inflammatory cytokines were indicated with the '*' sign above the bar when compared to negative control whereas the "#" sign for positive control.

The BCi-NS1.1 cells were treated simultaneously with either 100ng/mL of LPS alone or with quercetin loaded LCNs and free quercetin in DMSO solution to investigate their respective effects on the production of LPS induced pro-inflammatory markers in these cell lines. Quercetin loaded LCNs and sm-LCNs with the dose of 25μM showed a consistent trend of significantly (p < 0.05) decreasing the concentration of pro-inflammatory cytokines namely IL-1β, IL-6 and IL-8 compared to the positive control group (Figure 4). It is also worth noting that quercetin LCNs as well as sm-LCNs remarkably downregulated the production of IL-6, IL-8 and IL-1β compared to free quercetin dissolved in DMSO, therefore, exhibiting superior anti-inflammatory activity.

4. Discussion

4.1.   Characterization of LCN and sm-LCN

The significant increase in particle size indicates the encapsulation of quercetin into the blank LCN. Drug encapsulation into the nanoparticles may be a result of chemical conjugation or physical entrapment [36]. Polydispersity index (PDI) represents the size distribution and uniformity of the nanoparticles formed [37]. PDI values greater than 0.3 indicates a broad size distribution. Ideally, a narrow size distribution identified with PDI values lesser than 0.3 is preferred [37]. The slightly higher polydispersity index of quercetin loaded LCNs may be attributed to the coexistence of cubic liquid crystalline nanoparticles with other types of vesicles in the dispersion as previously reported [38, 39]. It was observed that the mean particle size for sm-LCN was significantly larger than LCN with regards to the amount of CHI used to modify the surface. When 0.1% CHI was used, the particle size observed was 236.1 nm whereas 0.2% CHI used has shown to increase the particle size to 268.7nm. The size increased in proportion to the quantity of CHI used proves that the increment was attributed to the surface coating of CHI on LCN [13].

The positive reading in zeta potential further indicates that the surface of LCN (negatively charged) have been successfully coated by the protonated amino group from CHI (13). Generally, zeta potential values above ±25 mV are required to form a stable dispersion between particles and reduce their aggregation [40]. P 407 aided in stabilising the LCN dispersion as demonstrated previously [41]. It is presumed that the hydrophobic part of the polypropylene oxide chain adheres to the surface of the cubic nanoparticles whereas the hydrophilic portion of the chain extends out to the surrounding environment to provide steric shielding, therefore, avoiding agglomeration [42]. The negatively charged zeta potential could be ascribed to the subtle amount of oleic acid released by monoolein resulting in anionic behavior of the dispersion caused by adsorption of hydroxyl ions on the surface of the LCNs [40, 42].

TEM images further confirmed the results from the Zetasizer, showing that the particles were spherical in shape and size less than 300nm, which is quite similar to other reported studies [13, 16, 41, 43]. The dense black core surrounding the particle was due to the existence of CHI coating indicating that the surface of LCN has been modified.

Both LCN and sm-LCN have exhibited a high entrapment efficiency whereby >97% of drug was successfully entrapped. This indicates that most of the drug has been encapsulated regardless the surface modification of the nanoparticle. However other studies have successfully encapsulated their drug as high as 99% in LCN [13, 16, 40]. Nevertheless, the entrapment efficiency is still considered high when compared to other nanocarriers such as poly lactide-co-glycolic acid (PLGA) polymer-based nanoparticles where Anwar et al reported that only 86% of quercetin was encapsulated [44].

4.2.   In vitro release study

The result has shown that quercetin drug was able to release in a sustained release manner when incorporated into the LCN and by modifying the surface with CHI further prolonged the drug release. This sustained release of quercetin could be attributed to the structure of the LCN. The higher proportion of lipids in the LCN not only allows hydrophobic drug to bind strongly to its inner structure, but also provides a larger surface area for the drugs to be incorporated. As a result, high entrapment efficiency of the drug can be achieved. Yoo et al., also showed that the release of the drug from LCN has sustained as long as 15 days [16].

4.3. *In vitro* evaluation anti-inflammatory activity of quercetin loaded LCN and sm-LCN on immortalised human bronchial epithelial cell line (BCi-NS1.1)

Evaluation of in vitro anti-inflammatory activity of quercetin loaded LCN and sm-LCN in comparison to free quercetin in DMSO solution

In the present study, quercetin loaded LCNs effectively decreased the concentration of pro-inflammatory cytokines namely IL-1β, IL-6 and IL-8. These effects exhibited by quercetin LCN was in consistent with previous studies evaluating the anti-inflammatory activity of quercetin on different cell lines [9, 11, 45-47]. Zhang et al., proved that quercetin remarkably suppressed the secretion of IL-6 and IL-1β in LPS-stimulated human peripheral blood mononuclear cells (PBMCs) [46]. Another research demonstrated that the treatment of quercetin notably reduced the gene expression and release of IL-8, IL-6 as well as IL-1β in human mast cells (HMC-1) induced by phorbol 12-myristate 13-acetate and calcium ionophore A23187 [9]. Although the underlying mechanism was not investigated in this present study, it is well-established from previous studies that quercetin attenuates the activation of NF-κB hence, downregulating the production of pro-inflammatory cytokines [9, 45, 46]. NF-κB signaling pathway plays a crucial role in the pathogenesis of asthma as it modulates the gene expression of pro-inflammatory mediators including chemokines, cytokines and cell adhesion molecules that lead to the infiltration of immune cells such as neutrophils and eosinophils in the airway [48, 49].

Encapsulation of quercetin into liquid crystalline nanoparticles was successful in further improving its anti-inflammatory activity. The enhanced anti-inflammatory effects of quercetin LCNs could be primarily attributed to the characteristics of the LCN itself, whereby it is bioadhesive in nature and improves membrane permeation leading to an increased interaction with the bronchial epithelial cells [40, 50]. The lipid bilayer of LCNs is akin to the epithelial cell membrane hence could be transported easily across the membrane resulting in greater drug absorption [50, 51]. Furthermore, findings from numerous studies also elucidated that LCNs promote greater cellular uptake and internalisation into cells via endocytosis enabling them to be diffused throughout the cytoplasm [16, 40, 51, 52]. On the other hand, quercetin itself could have only remained in the cell membrane due to its high lipophilicity allowing it to be easily incorporated into the cell membrane.

The findings observed in our study has demonstrated that quercetin loaded sm-LCN with the dose of 25µM significantly (p<0.05) decreased the pro-inflammatory markers when compared to the free quercetin drug in DMSO. This indicates that sm-LCN is able to enhance the anti-inflammatory effect of quercetin when entrapped in the nanocarrier.

There is also a noticeable reduction in the expression of all the pro-inflammatory cytokines which was observed on the blank LCN & sm-LCN. This suggests that the empty vehicle itself can slightly reduce the inflammation. We suspect that one of the components in the LCN formulation might contributed and it was monoolein. However, only one study has confirmed this and Ali et al reported that monoolein possess anti-inflammatory property that inhibits the production of pro-inflammatory markers in LPS stimulated bone marrow-derived dendritic cell (BMDC) via NF-κB and MAPK pathways [53].

Evaluation of in vitro anti-inflammatory activity of quercetin loaded LCN and sm-LCN in comparison to fluticasone

Fluticasone, an FDA approved drug to treat asthma was used as a standard to further establish the anti-inflammatory activity of quercetin LCNs. Quercetin LCNs were comparable to fluticasone in terms of reducing the levels of IL-1β and IL-6 however, fluticasone was much more effective in decreasing the production of IL-8. In this existing study, the BCi-NS1.1 cell lines were only treated with quercetin LCNs at the dose of 25µM. Quercetin was able to reduce the levels of pro-inflammatory cytokines in a dose-dependent manner up to a 100µM as described in the literature [46]. Therefore, the finding suggests the potential of quercetin LCNs to decrease the levels pro-inflammatory cytokines to a greater extent comparable to fluticasone at a higher dose.

4.4. Molecular mechanics simulations

The molecular simulations performed in this research focused on two aspects: a) encapsulation or incorporation of quercetin within a monoolein matrix, and b) surface modification of the monoolein matrix with chitosan. The geometrical iterations representing the above bimolecular complexes are shown in Figure 5 while the related energetic molecular attributes are depicted in Tables 2 and 3, respectively. In case of MO-Quer; the formation of molecular complex was accompanied by a total energy stabilization of 15.894kcal/mol. Interestingly, the total energy of

the MO-Quer complex was less than the individual constituent molecules. The incorporation of Quer in MO was evident from the fact that all bonding (bond, angle, and dihedral) and non-bonding (van der waals forces and H-bonding) energy terms contributed to the geometrical stabilization of the complex. The major stabilization was provided by the van der Waals function which brought the molecules in close vicinity and hence led to the formation of –OH…HO– bonds between the molecules (Table 2, Figure 5). For the MO-Cht molecular complex; an energy stabilization of 10.336kcal/mol was observed. In line with MO-Quer, the total energy of the MO-Cht complex was less than the individual molecular components. The energy stabilization in this case was mainly due to non-bonding interactions (van der Waals forces and electrostatic interactions) while the bonding interactions (bond and dihedral energies) destabilized the complex. Such destabilization is due to torsional strain experienced by the molecules while interacting within the van der Waals space. Additionally, both inter- and intra-molecular H-bonding was observed in the MO-Cht complex further confirming its favourable formation and conformation. The above discussion justifies the selection and successful incorporation of quercetin in and chitosan surface modification of the monoolein nanosystem.

5. Conclusions

Quercetin was successfully encapsulated into liquid crystalline nanoparticles revealing their sustained release behaviour. Moreover, quercetin LCN and sm-LCN were effective in reducing the levels of pro-inflammatory mediators such as IL-1β, IL-6 and IL-8 whereby encapsulating quercetin into LCN further enhanced its anti-inflammatory activity. This demonstrates that the quercetin LCN could be employed as a potential novel drug delivery for the treatment of asthma. This mode will overcome the issues associated with quercetin such as the low solubility, poor bioavailability and short half-life. The findings of this present study advocate the translation into other associated pulmonary inflammatory diseases such as chronic obstructive pulmonary disorder (COPD) as well as lung cancer which will help in providing a new direction to the pulmonary clinics.

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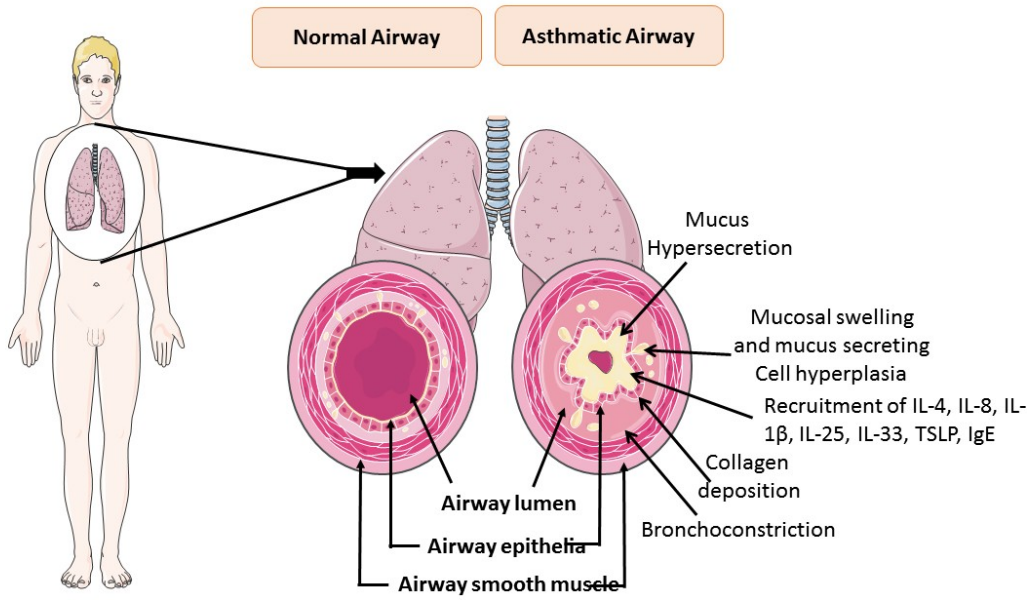attenuating mitogen-activated protein kinase and NF-κB pathways. Food science and biotechnology. 26 (2017) 507-11.
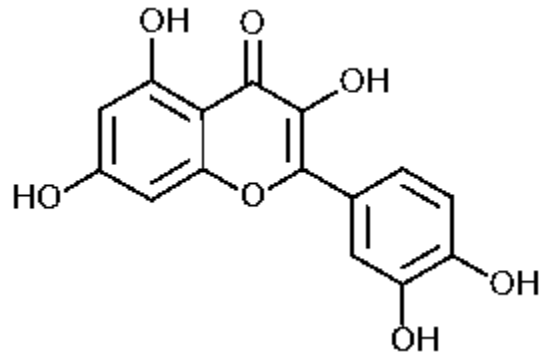
Figure 1: Pathology of Asthma

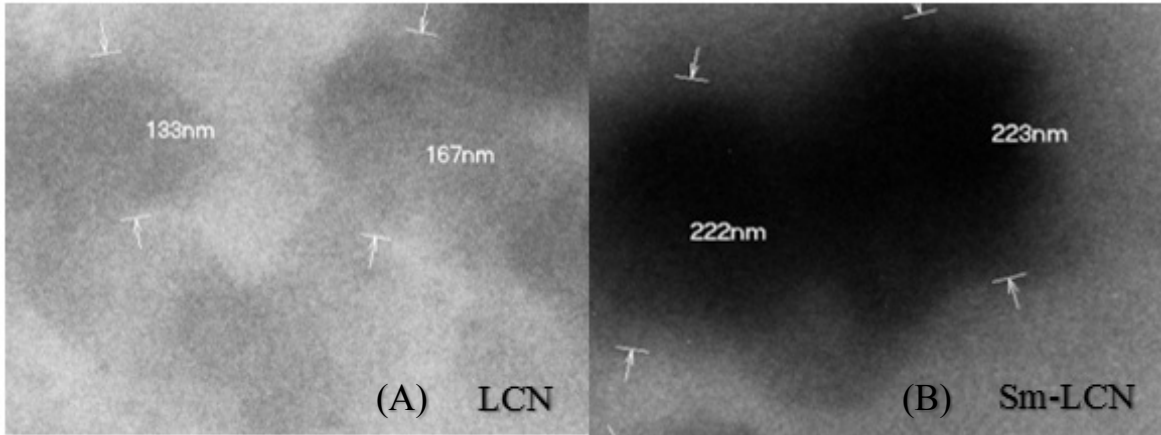**Figure. 2.** Chemical structure of Quercetin

Figure 3. TEM images of LCN and sm-LCN (bar 500 nm). (A) LCN liquid crystalline nanoparticle and (B) sm-LCN Surface-modified liquid crystalline nanoparticle.
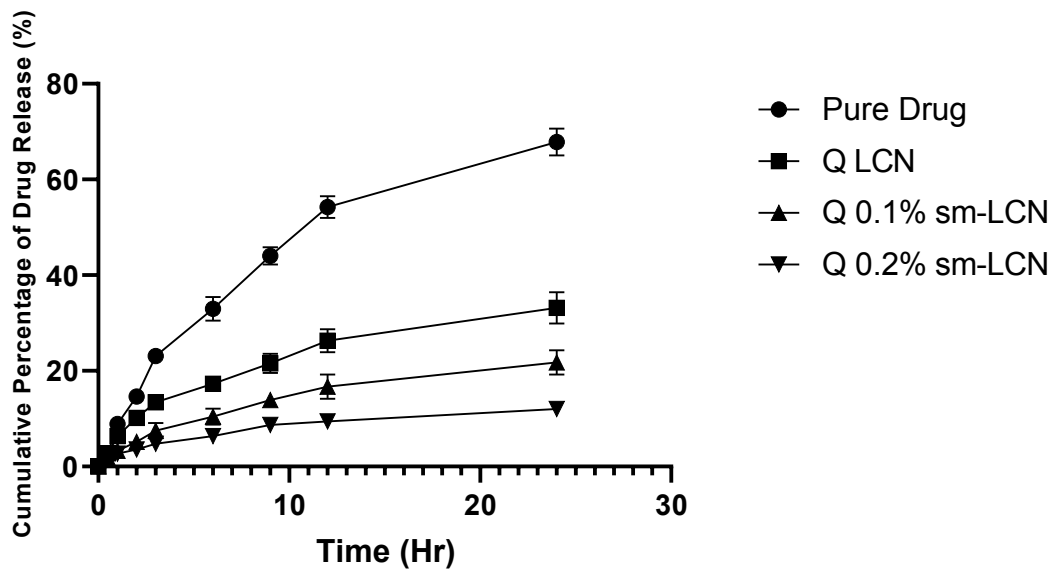

Figure 4. Cumulative Percentage release of Quercetin Vs. Time. The release profile of quercetin LCN over 24 hours. Release profile of quercetin from LCN exhibits a prolonged manner over the longer duration of time.
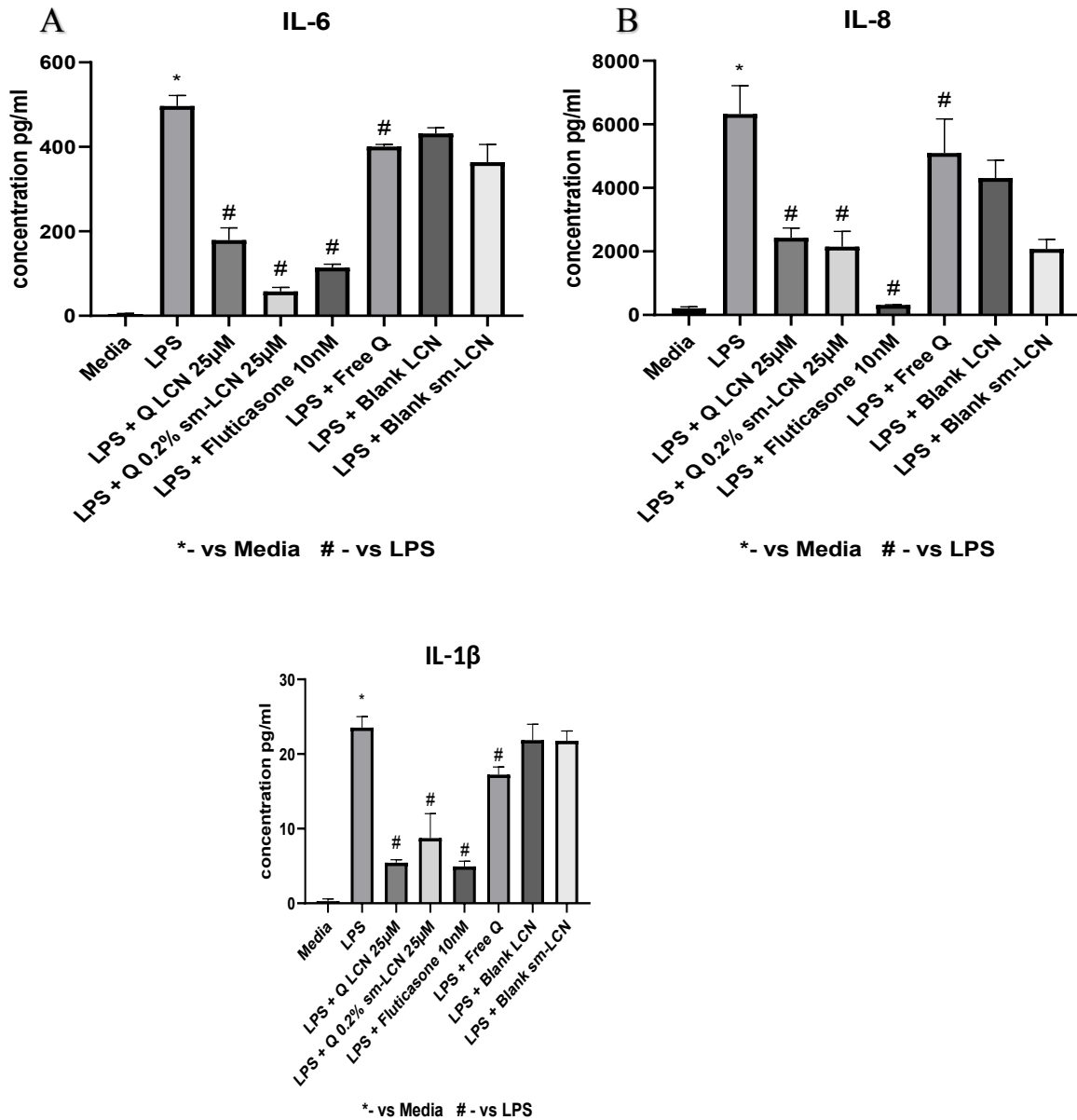
Figure 5A, 5B and 5C. Effects of quercetin LCN treatment on BCi-NS1.1 cell line. Cells were treated with Q LCN, Q 0.2% sm-LCN at 25nM, fluticasone in DMSO at 10nM, Free Q in DMSO, Blank LCN and sm-LCN for 24h (n=3)
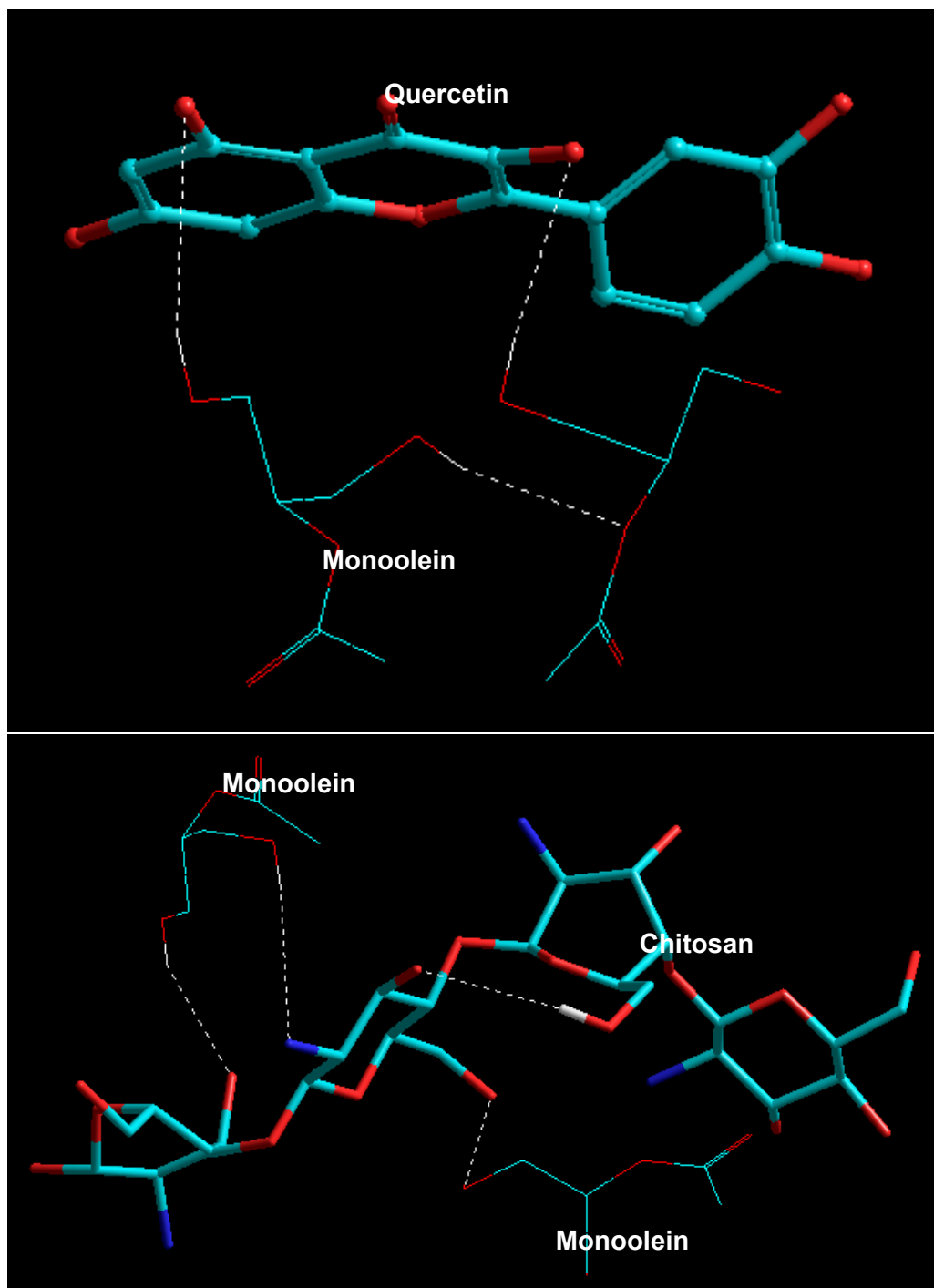
Figure 6: Representation of the geometrical preferences of the two monoolein functional moieties (stick rendering) in complexation with a) quercetin molecule (ball-and-tube rendering); and b) chitosan chain (tube rendering) after molecular mechanics simulations [Colour code for elements: C = cyan; H = white; O = red]. Hydrogen atoms not shown for clarity.
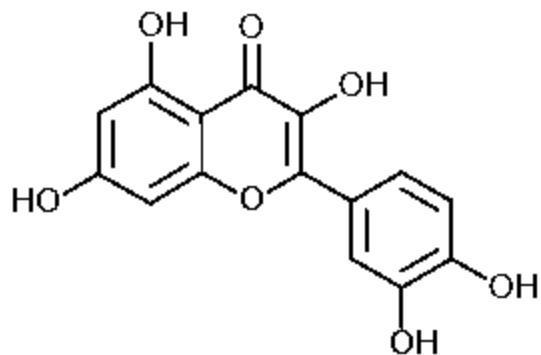
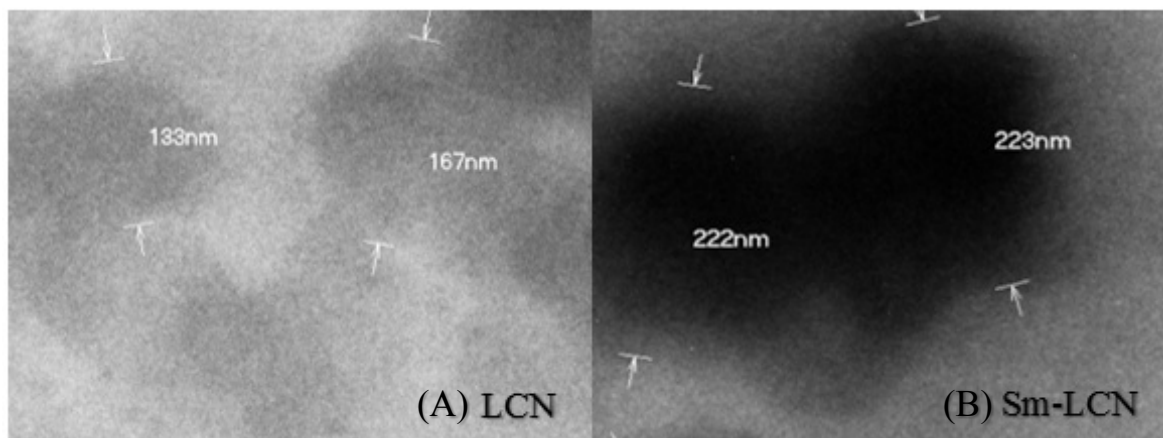**Figure. 1.** Chemical structure of Quercetin

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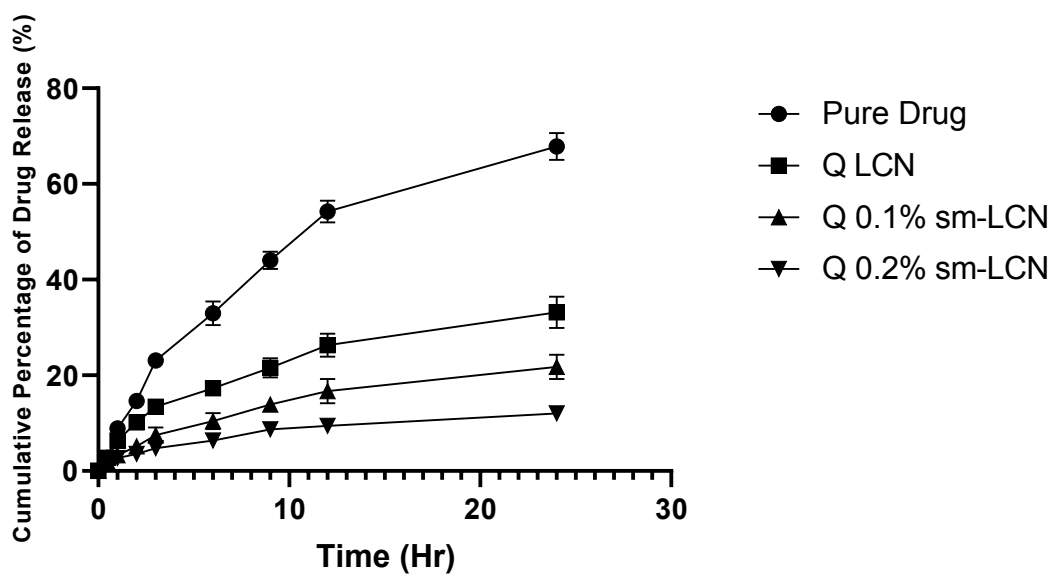

Figure 3. Cumulative Percentage release of Quercetin Vs. Time

Figure 3 The release profile of quercetin LCN over 24 hours. Release profile of quercetin from LCN exhibits a prolonged manner over the longer duration of time.
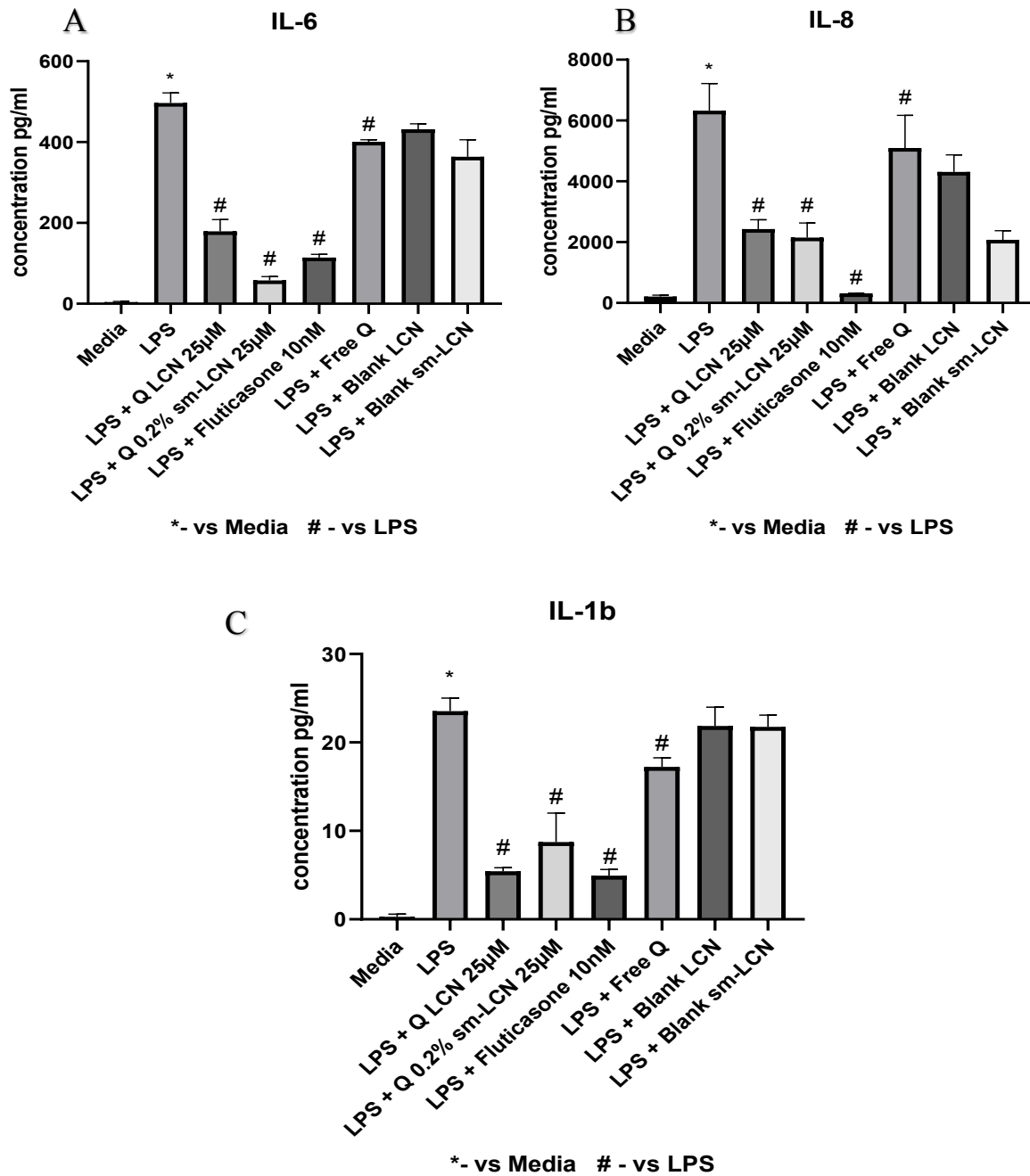

A   IL-6

B   IL-8

C   IL-1b

Figure 4A, 4B and 4C. Effects of quercetin LCN treatment on BCi-NS1.1 cell line. Cells were treated with Q LCN, Q 0.2% sm-LCN at 25nM, fluticasone in DMSO at 10nM, Free Q in DMSO, Blank LCN and sm-LCN for 24h (n=3)
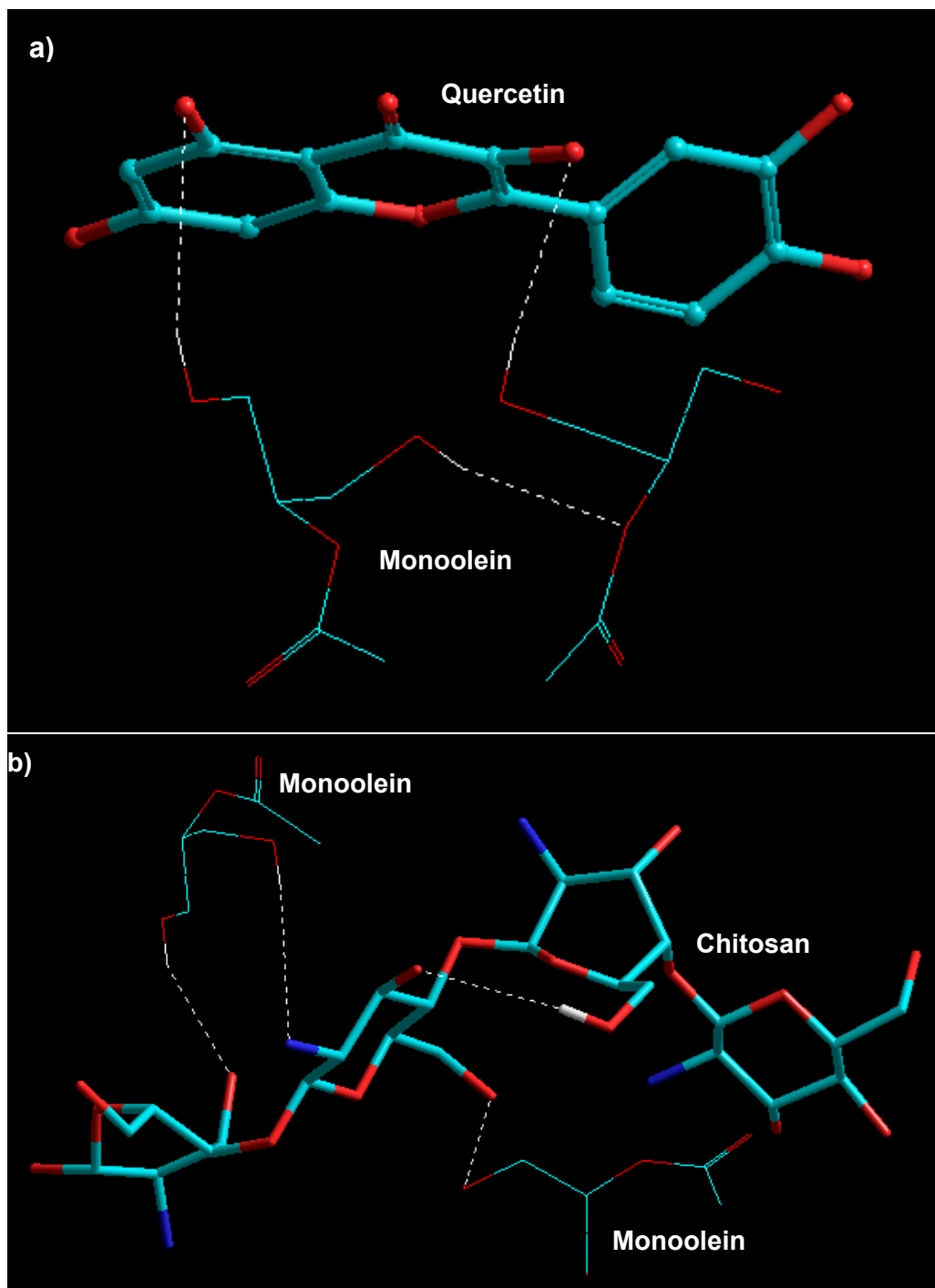
Figure 5: Representation of the geometrical preferences of the two monoolein functional moieties (stick rendering) in complexation with a) quercetin molecule (ball-and-tube rendering); and b) chitosan chain (tube rendering) after molecular mechanics simulations [Colour code for elements: C = cyan; H = white; O = red]. Hydrogen atoms not shown for clarity.

Table 1. Characterization parameter of the nanoformulations.

Formulations	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Encapsulation efficiency (EE) (%)
Blank LCN	210.0 ± 2.77	0.162 ± 0.02	-14.6 ± 0.25	-
Q LCN	223.9 ± 1.83	0.339 ± 0.008	-15.6 ± 0.17	99.4 ± 0.37
0.1% Q sm-LCN	236.1 ± 2.12	0.384 ± 0.03	15.8 ± 0.29	97.6 ± 0.30
0.2% Q sm-LCN	268.7 ± 0.55	0.419 ± 0.03	22.3 ± 0.96	98.5 ± 0.46

Each value is presented as mean ± standard deviation (SD), N=3

Table 2. Inherent energy attributes of monoolein-quercetin complex calculated using static lattice atomistic simulations in vacuum.

Energy component	$(MO)_2$-Quer	$(MO)_2$	Quer	ΔE [a]
Total [b]	3.761	6.580	13.075	-15.894
Bond [c]	1.038	0.428	0.792	-0.182
Angle [d]	3.669	3.592	1.369	-1.292
Dihed [e]	4.590	3.002	3.458	-1.87
vdW [f]	-4.792	-0.442	8.183	-12.533
H-bond [g]	-0.743	0.00	-0.728	-0.015

[a]$\Delta E_{(A/B)} = E_{(A/B)} - [E_{(A)} + E_{(B)}]$; [b]total steric energy for an optimized structure; [c]bond stretching contributions; [d]bond angle contributions; [e]torsional contribution arising from deviations from optimum dihedral angles; [f]van der Waals interactions; [g]hydrogen-bond energy function; values in green represent the structure stabilizing contribution; and values in red represent the structure destabilizing contribution. All values in kcal/mol.

Table 3. Inherent energy attributes of monoolein-chitosan complex calculated using static lattice atomistic simulations in vacuum.

Energy component	$(MO)_2$-Cht	$(MO)_2$	Cht	ΔE [a]
Total [b]	2.701	6.58	6.457	-10.336
Bond [c]	1.517	0.428	1.064	0.025

Angle [d]	9.028	3.592	5.782	-0.346
Dihed [e]	15.563	3.002	8.567	3.994
vdW [f]	-4.388	-0.442	6.189	-10.135
Elec [g]	-19.021	0.00	-15.145	-3.876

[a]$\Delta E_{(A/B)} = E_{(A/B)} - [E_{(A)} + E_{(B)}]$; [b]total steric energy for an optimized structure; [c]bond stretching contributions; [d]bond angle contributions; [e]torsional contribution arising from deviations from optimum dihedral angles; [f]van der Waals interactions; [g]electrostatic interactions; values in green represent the structure stabilizing contribution; and values in red represent the structure destabilizing contribution. All values in kcal/mol.

Conflict of interests

The authors declare that there is no conflict of interests.