Ferulic acid loaded pickering emulsions stabilized by resistant starch nanoparticles using ultrasonication: Characterization, in vitro release and nutraceutical potential

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ABSTRACT
The use of starch based nanoparticles have gained momentum in stabilizing pickering emulsions for it’s numerous advantages. In present study resistant starch (RS) was isolated from lotus stem using enzymatic digestion and subjected to nanoprecipitation and ultrasonication to yield resistant starch nanoparticles (RSN). RSN of varying concentrations (2%, 10% and 20%) were used to stabilize the flax seed-oil water mixture to form pickering emulsions. The emulsions were used to nanoencapsulate ferulic acid (FA) – a well known bioactive via ultrasonication. The emulsions were lyophilized to form FA loaded lyophilized pickering emulsion (FA-LPE). The FA-LPE (2%, 10 % and 20%) were characterized using dynamic light scattering (DLS), light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) and attenuated total reflectance fourier transform infra-spectroscopy (ATR-FTIR). AFM showed FA-LPE as spherical droplets embedded in the matrix with maximum peak height of 8.47 nm and maximum pit height of 1.69 nm. SEM presented FA-LPE as an irregular and continuous surface having multiple folds and holes. The ATR-FTIR spectra of all the samples displayed peaks of C = C aromatic rings of FA at 1600 cm⁻¹ and 1439 cm⁻¹, signifying successful encapsulation. In vitro release assay displayed more controlled release of FA from FA-LPE (20%). Bioactivity of FA-LPE was evaluated in terms of anti-cancer, anti-diabetic, angiotensin converting enzyme (ACE) inhibition and prevention against oxidative damage under simulated gastro-intestinal conditions (SGID). The bioactivity of FA-LPE (20%) was significantly higher than FA-LPE (2%) and FA-LPE (10%). Key findings reveal that pickering emulsions can prevent FA under harsh SGID conditions and provide an approach to facilitate the design of pickering emulsions with high stability for nutraceutical delivery in food and supplement products.

1. Introduction

Pickering emulsions stabilized by food grade particles have gained a lot of attention in recent times because of their biodegradability, low toxic nature and excellent biocompatibility. In comparison to conventional emulsions, particle stabilized pickering emulsions display excellent stability against lipid oxidation and can prove as an effective delivery vehicle for various bioactives [1]. The solid particles used for stabilizing the oil water interface provide a physical barrier which prevents the degradation of bioactive compounds. Ovotransferrin combined with anti-oxidants like gallic acid was used to stabilize pickering emulsion which led to a significant reduction in degradation of curcumin against UV light [2]. The solid particles can also provide thermal resistance to the bioactives. 3. Pickering emulsions stabilized by wheat gluten nanoparticle–xanthan gum complex particles improved the stability and enhanced the bioavailability of β-carotene in SGID [3]. Research based on developing the particles derived from natural polymers like chitosan [4], starch [5] and cellulose [6] has been conducted to form pickering emulsions. Starch has been the most attractive and promising polysaccharide used to develop particle stabilizers owing to their inherent biodegradability and overwhelming abundance [7]. Various studies have been conducted, where native starch granules showed ability to stabilize pickering emulsions [8–10]. However the frequent intake of foods with highly digestible starch has been associated with hyperglycemia and hyperinsulinemia that can in turn result in insulin resistance and metabolic syndrome such as diabetes, obesity and
cardiovascular disease [11,12]. So, an alternative can be used to replace digestible starch with a starch that cannot be hydrolyzed by enzymes in the digestive tract i.e., resistant starch (RS). RS lowers the insulin responses and postprandial plasma-glucose, thus combating insulin resistance and metabolic syndrome [13,14]. Also, the microflora present in the gut causes fermentation of RS producing short chain fatty acids that can prevent colon cancer [15]. Besides, RS can act as a prebiotic and an encapsulating agent for loading of various bioactives and probiotics [16,17].

Lotus (Nelumbo nucifera) is an important fresh water aquatic plant belonging to family Nelumboaceae [18]. The edible parts of the plant including buds, rhizome, stem, seeds, flowers and leaves have been used in Asian traditional medicines to treat various disorders like hypertension, depression, vomiting, heart problems, diarrhoea, insomnia and cancer [19]. In the present study, RS isolated from lotus stem has been chosen to act as a stabilizer for stabilizing the bioactive loaded pickering emulsion. RS obtained from lotus stem was subjected to nanoreduction. The use of starch based nanoparticles have gained momentum in stabilizing pickering emulsions for it’s numerous advantages over macro starch such as, high absorption capacity, minimal diffusional limitations and high surface area per unit mass. Nanoprecipitation is the technique that has been chosen to obtain starch nanoparticles. It involves successive addition of non solvent into polymer solution or gradual addition of a dilute polymers solution to a non solvent which ultimately forms nanosized polymer particles. The process basically involves deposition of biopolymer on interface and displacement of semi-polar solvent miscible with water from a lipophilic solution. The process has been used to produce starch nanoparticles from waxy corn, potato, high amylose corn, sweet potato and pea [20]. Chang et al. used the combined technique of ultrasonication and nanoprecipitation for producing starch nanoparticles with high efficiency and lower cost [21].

Although, the characterization of particle based pickering emulsions have been carried out extensively, there is a limited literature available regarding the encapsulation of bioactive compounds, their release profile in SGID, retention of bioactivity and stability after storage. So, the bioactive chosen for this study is Ferulic acid (FA). FA is a hydroxy-cinnamic acid having strong anti-oxidant, anti-inflammatory, anti-cancer and anti-viral properties [22]. It has also been used as a promising candidate for treatment of disorders like diabetes mellitus, cancer, Alzheimer’s disease, skin diseases, and cardiovascular diseases. FA is approved by United States Food and Drug Administration and is used as a preservative to prevent auto-oxidation of oils [22]. However, it is susceptible to photo-degradation and degrades upon exposure to oxygen. So using RNS stabilized pickering emulsion as a delivery vehicle for FA could be a good area of research. To increase the shelf life, improve usage and facilitate transportation, dehydration of pickering emulsions is important [23]. One such approach can be freeze drying. The process involves crystallization of water at low temperature which is then directly sublimated into vapour on decreasing the pressure around the product. The process is suitable for preservation of heat labile food materials and causes less damage to delicate structures [24].The size of the stabilizing agents governs the rate of coalescence during freeze drying and this goes well for starch stabilized pickering emulsions. It has been reported that oil droplets surrounded by thick films protect the penetration of ice crystals and partial coalescence [25].

The objective of the study was to produce freeze dried pickering emulsions loaded with FA stabilized by RSN. The freeze dried pickering emulsion powder was characterized in terms of particle size, zeta potential and morphology. Furthermore, the retention of anti-oxidant, ACE inhibition, anti-cancer and anti-diabetic activity of freeze dried emulsions upon SGID were also studied. The proposed work could be extrapolated and used for encapsulation of sensitive nutraceuticals in food and pharmaceutical applications.

2. Materials and methods

2.1. Materials

The indigenous variety of lotus (Nelumbo Nucifera) was procured from the market around the banks of Dal Lake, Srinagar, Jammu and Kashmir, India. The stems were scraped to remove the adhering dirt and then washed with cold water. All the chemicals used in this study were procured from Sigma Aldrich (USA). The most important chemicals used in this study were FA, amyloglucosidase from Aspergillus niger, protease from Bacillus lichenformis, α-amyrase from porcine pancreas, bile salts, pepsin, pancreatic lipase, MT (3,4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) dye, DMEM (dulbecco’s modified eagle medium) and PBS (fetal bovine serum). Cancerous cells including HELA (cervical cancer cell line), HEK (human embryonic kidney cells) and U2OS (human bone osteosarcoma epithelial cells) were purchased from NCS, Pune, India.

2.2. Extraction of RS through enzymatic digestion method

Lotus stems were cut into small pieces and pulverized with water using a domestic blender for 5 min. The native starch (NS) was extracted using the method given by Noor et al. [26]. RS was then extracted from NS using the method given by Noor et al. [26]. 5 g of NS was added with 10 mL of phosphate buffer (0.2, pH 5.2) and vortexed for 5 min. 5 mg of protease was added to digest any residual protein. The solution was vortexed for 2 min and kept on water bath at 37 °C for 1 h. After incubation, the solution was centrifuged for 10 min at 2850 rpm. The pellet recovered was washed with ethanol (99%) for inactivation of residual enzyme. The pellet was treated with pancreatic lipase to digest the traces of lipids which may form amylose–lipid complex (RS type 5) during interaction with amylose. The enzyme solution was made by dissolving 5 mg of porcine pancreatic lipase in 10 mL of buffer [100 mM PBS and NaCl (0.5%), pH 7.2]. This solution was incubated for 1 h, centrifuged at 2850 rpm for 10 min and washed with 99% ethanol. The recovered pellet was then treated with pancreatic α-amylase enzyme solution (10 mg/mL with activity of 25 U/mL) and 3.2 mL of amyloglucosidase (3U/ml) prepared in 250 mL of sodium acetate buffer (0.1 M, pH 5.2) to hydrolyse digestible starch. The solution was kept on a shaking water bath at 37 °C for 16 h. The starch suspensions were then centrifuged for 10 min at 3000 rpm. The recovered residues were subjected to repeat washing using ethanol (99%), followed by washing with 50% ethanol. The resulting RS precipitates were dried at 45 °C and stored in air tight containers for further analysis.

2.3. Nanoprecipitation-ultrasonication of RS

RSN were obtained using the method given by Tan et al. [27]. RS weighing 500 mg were mixed with 25 mL of acetone followed by dropwise addition of 100 mL of distilled water. The suspension was ultrasonicated using an ultrasonic probe (Cole-Parmer, 04711–35) at an amplitude of 60%, frequency of 20 kHz and power of 150 W for 10 min and then placed on magnetic stirrer till acetone was completely vaporized. The suspension was freeze dried (Buchi-Iyovap-L- 200) for 12 h and the prepared nanoparticles were stored in air tight containers for further use.

2.4. Characterisation of RSN

2.4.1. Particle size, polydispersity index and zeta potential

The average particle diameters, polydispersity index and zeta potential of RSN were analyzed using zeta sizer. For measuring zeta potential, 0.01gm of sample was dispersed in distilled water and placed on a sonicator bath at 40 kHz for 15 min. 3 mL of the prepared dispersion was filled in a cuvette and monitored on zeta sizer. For measuring zeta potential, 0.01gm of sample was dispersed in...
KCl (0.1 mM), maintaining neutral pH. The samples were left for equilibration overnight before measurement. The measurements were carried out at neutral pH at 25 °C.

2.4.2. Measurement of RS content

The RS content of starch was estimated using the Megazyme Assay Kit (Megazyme International, Wicklow, Ireland), following the approved AACC 2000 method [28].

2.5. Fabrication of FA loaded pickering emulsions

FA was dissolved in flax seed oil at the concentration of 10 mg/mL by heating at 45 °C for 30 min to form a transparent solution. FA-FO solution and sodium acetate buffer (92%, 5 mM, pH 7, 0.2 M NaCl) were mixed in the ratio of 2: 25. Three equal portions were mixed with three concentrations of RSN (2%, 10% and 20%). RS stabilized pickering emulsions were then formed in a two step process; first by emulsification using a vortex mixer (2 min) and second by subjecting the emulsion to ultrasonic probe sonicator (Cole-Parmer, 04711-35) having a probe diameter of 12 mm. The ultrasonic treatment was carried out in an ice bath with pulse mode of 1 s on/1 s off for total processing time of 20 min. The frequency of the instrument was 20 KHz. The non-lyophilized FA loaded pickering emulsion stabilized with 2%, 10% and 20% RSN immediately after ultrasonication were designated as FA-PE (2%), FA-PE (10%) and FA-PE (20%), respectively. The samples ([FA-PE (2%), FA-PE (10%) and FA-PE (20%)] were then freeze dried (Buchi-Iyovapor L-200) for 12 h. The lyophilized FA loaded pickering emulsions stabilized with 2%, 10% and 20% RSN were designated as FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%), respectively. The lyophilized samples were stored around 2–8 °C in air tight vials.

2.6. Light and confocal microscopy of FA-PE

The microstructure of FA-PE were examined under light microscope at 40x magnification (LMI, Leedz Micro Imaging Ltd, UK) and Confocal laser scanning microscope (FV3000, Olympus, Tokyo, Japan). For light microscopy, one drop of FA-PE was diluted with 5 drops of glycerol/ water mixture (1, 1 V/V) and placed on a glass slide. For confocal microscopy, FA-PE droplets were dyed with Nile red (0.01%) to stain oil phase. The emulsion droplet was placed on a microscope slide and covered with a cover slip. The fluorescent dye was excited by using an argon laser at 488 nm for Nile Red dye.

2.7. Comparison between particle size, polydispersity index (PDI) and zeta potential of FA-PE and FA-LPE

The methodology for analyzing the hydrodynamic diameters, PDI and zeta potential of FA-PE and FA-LPE (2%, 10% and 20%) is same as provided in section 2.4.1.

2.8. Characterization of FA-LPE

2.8.1. Scanning electron microscopy

The surface morphology of FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%) was observed under scanning electron microscope (Hitachi Se 3600 N-Tokyo, Japan). The samples were loaded onto a circular aluminum stub attached with a double sided adhesive tape and coated with a layer of gold under vacuum. The samples were then analyzed at an accelerating voltage of 10 kV.

2.8.2. Atomic force microscopy

AFM images of FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%) were collected using AGILENT-5500 AFM according to the method of Zhu et al. [29]. 1 mg of samples was diluted in 10 mL of distilled water and vortexed for 10 min. The samples were spread onto the surface of freshly cleaved mica for 24 h of drying at room temperature prior to imaging.

2.8.3. Transmission electron microscopy

TEM (EDAX-JEOLJSM-IT200) was used to analyze the structure of samples. A drop of FA-LPE samples was placed on a carbon-coated grid and stained negatively. The samples were analyzed under the accelerating voltage of 60 kV. Finally, the high resolution images were taken using the Gatan camera.

2.8.4. ATR – FTIR spectroscopy

The ATR-FTIR spectra of FA, RSN, FO, FA-LPE (2%, 10% and 20%) were recorded using FTIR spectrometer system (Cary 630 FTIR, Agilent Technologies, USA). The samples were analyzed in the range from 600 to 3700 cm⁻¹.

2.9. Encapsulation efficiency (EE)

EE of FA-LPE (2%, 10% and 20%) was estimated according to the method used by Noor et al. [30] with modifications. In this method, the amount of FA added in the solution before ultrasonication and the amount of non-encapsulated FA remaining in the supernatant gives the measurement of encapsulation efficiency (EE). 100 mg of sample was washed with 4 mL ethanol (20%) to remove the FA adhered to the external surface. The solution was centrifuged at 3500 rpm for 10 min. The supernatant was discarded and the pellets were dispersed in 4 mL ethanol and kept on magnetic stirrer at the speed of 250 rpm for 24 h. This was again followed by centrifugation at 3500 rpm for 10 min. Finally the supernatant was collected and its absorbance was measured at 321 nm (Eppendorf BioSpectrometer ® basic). Encapsulation efficiency was estimated using the given formula:

\[ EE(\%) = \frac{FA_{\text{released}}}{FA_{\text{added}}} \times 100 \]

Where, FA_{\text{released}} is the content of FA released from particles and FA_{\text{added}} is the content of FA added during dissolution.

2.10. In vitro release of FA

The release of FA from FA-LPE was analyzed under SGID using the method described by Noor et al. [30]. 10 mg of FA-LPE (2%, 10% and 20%) samples were added with 10 mL of α-amylase, prepared in phosphate buffered saline (PBS, pH 7.2). The reaction mixture was kept on a shaking water bath at 37 °C for 5 min. The solution was centrifuged for 10 min at 5500 rpm and absorbance was noted at 321 nm. The recovered pellet was treated with 10 mL simulated gastric juice made by dissolving pepsin (3g/L) in sterile NaCl solution (8.5g/L) and pH adjusted to 3.0 using 1.0 mol/L HCl. The reaction mixture was incubated at 37 °C, centrifuged at 5500 rpm for 10 min and noted for absorbance at 321 nm after the time interval of 30 and 60 min. This will give the amount of FA released after simulated gastric conditions (SGD). In the next stage the pellet was treated with 10 mL of simulated intestinal juice, made by dissolving 3 g/L of bile salts, 10 (g/L) pancreatin in phosphate buffered saline (PBS) at pH 7.5. The absorbance was observed after the interval of 90, 120 and 180 min at 321 nm to estimate the amount of FA released after simulated intestinal digestion (SID).

2.11. Bioactivity of FA-LPE under SGID

2.11.1. Sample preparation

The samples were analyzed for their bioactivity in SGID in terms of anti-cancer, ACE inhibiting, anti-diabetic activity and DNA damage inhibiting potential. For this, 50 mg of FA-LPE (2%, 10% and 20%) was dissolved in 5 mL of simulated gastric enzyme mixture (3 g/L of pepsin in sterile NaCl solution (8.5 g/L) and pH adjusted to 3.0 with 1.0 mol/L HCl). The samples were vortexed for 1–3 sec and incubated at 37°C for 60 min. The enzyme-substrate solution was then added with 5 mL of intestinal juice (3 g/L bile salts and 10 g/L pancreatin dissolved in PBS with pH 7.5) and incubated for 3 h. Finally the solution was
centrifuged for 10 min at 4500 rpm and the supernatant was analyzed for various bioactivities.

2.11.2. Anti-cancer activity

The anti-cancer activities of samples were evaluated using the method given by Ashraf et al. [31]. Cancer cell lines including HELA, HEK and U2OS were grown in 200 μL of DMEM media. The media comprised of 45 mL DMEM + 5 mL of foetal bovine serum + 5 mL of antibiotic, streptomycin. The cells were grown for 24 h in 96 well plate. After incubation, cells were replaced with fresh media and added with 200 μL of sample solutions of FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%), and again incubated for 24 h. After incubation, the reaction mixture was added with 20 μL of tetrazolium dye (MTT dye) and incubated for 3 h. Next, 150 μL of DMSO was added to dissolve the precipitates by shaking the reaction mixture for 15 min. Finally, the absorbance was measured at 590 nm.

2.11.3. Angiotensin converting enzyme (ACE) inhibition activity

ACE inhibition assay was carried out using the method of Ashraf et al. [31]. The reaction mixture consisted of incubating 50 μL of sample and 50 μL of ACE solution (50 μU/mL) for 10 min at 37 °C. Following this, 150 μL of HHL (Hippuryl- histidyl-leucine) was added and incubated for 30 min. 500 μL of 1 M HCl was added to cease the reaction. Then hippuric acid was extracted by evaporation in rotary vacuum evaporator (at 95 °C) using ethyl acetate. The residue obtained was diluted in 2 mL distilled water and the absorbance was measured at 228 nm. The ACE enzyme inhibition activity (%) was calculated using the following formula:

$$ACE\text{Inhibition(\%)} = \left(\frac{A_o - (A_s - A_o)}{A_o - A_s}\right) \times 100$$

Where, $A_s$ denotes absorbance of sample, $A_o$ represents absorbance of positive control, $A_b$ represents absorbance of blank, and $A_n$ denotes absorbance of negative control.

2.11.4. Anti-diabetic activity

Anti-diabetic activity of the samples was evaluated in terms α-amylase inhibition (%). The assay was performed using the method of Joseph-Leenose-Helen et al. [32]. The assay included 50 μL of sample solution, 50 μL of α-amylase solution (40 U/mL in distilled water), and sodium phosphate buffer (0.02 M, pH 6.9) incubated at 37 °C for 10 min. Next, 40 μL of starch solution acting as substrate was added and allowed to undergo incubation for 15 min at 37 °C. Finally, 40 μL of HCl was added to cease the reaction. The change in colour was analyzed by adding 5 mL of iodine reagent and absorbance was read at 620 nm. Acarbose was used as a standard. The α-amylase inhibition (%) was calculated using the formula:

$$\alpha - \text{amylase inhibition(\%)} = \left(\frac{A_o - A_s}{A_o - A_b}\right) \times 100$$

Where, $A_s$ represents absorbance of sample, $A_o$ denotes absorbance of negative control and $A_b$ denotes absorbance of positive control.

2.11.5. Anti-oxidant activity against oxidative damage of DNA

The antioxidant activity of samples against the hydroxyl radical-mediated DNA damage was analyzed using the procedure of Jhan et al. [33]. The sample extract of 5 μL and 2.5 μL of calf thymus DNA (0.25 μg/50 μL) was incubated at 25 °C for 15 min. This was followed by addition of 10 μL of Fentons reagent (30 mM H2O2, 800 μM FeCl2 and 500 μM ascorbic acid). After incubating at 37 °C for 1 h, the reaction was terminated using 2.5 μL of loading buffer (30% glycerol and 0.25% bromophenol blue). The reaction mixtures were then loaded on agarose gel (1%) stained with 4 μL ethidium bromide. Finally, the electrophoresis was carried out using horizontal slab apparatus with Tris/boric/Ethylendiaminetetraacetic acid gel as buffer. The gels were observed using Gel Doc (BIO RAD, Gel DOC™ EZ Imager).

2.12. Statistical analysis

Statistical analysis was performed using commercial statistical package SPSS 25.0 (USA) and the data were analyzed by analysis of variance (ANOVA) using Duncan’s multiple range test at 5% significance level. The results are displayed as means ± standard deviation (n = 3). All experiments were performed in triplicate.

3. Results and discussions

3.1. Characterisation of RSN

RSN obtained after nanoprecipitation-ultrasonication method were characterized for RS content, particle size, zeta potential and polydispersity index. The results are presented in Table 1. RSN had significantly higher (p < 0.05) value of RS content (96.24 ± 0.96 %) as compared to the RS. The increase may be attributed to the molecular disintegration of amylpectin. The amylpectin is easily destroyed by ultrasonication as compared to amylose, and increased apparent amylose content may be responsible for increased RS content [18]. The concentration of starch solution used during nanoprecipitation also plays an important role in determining the particle size of the nanoparticles [27]. The high viscosity of starch solution hampers the dispersion of starch solution towards non-solvent, resulting in the formation of large particles. The concentration of the starch solution used for the present study was 2 g, which can result in formation of larger particles. However, the synergistic action of ultrasound can reduce the size of RS particles owing to the mechanism of cavitation which results in collapse of microbubbles [34]. The covalent bonds present in the starch are broken down by the shear forces generated by the collapsing of bubbles [35]. Zeta potential values determine the surface charge of sample. All the samples displayed negative values of zeta potential which could be attributed to the naturally occurring phosphate groups in amylpectin molecules of RS [18]. However, the zeta value of RSN (-25.14 ± 0.36 mV) was significantly higher (p < 0.05) than RS (-12.12 ± 0.01 mV) and NS 12.13 ± 0.02 (mV). This might be due to the increase in the number of hydroxyl groups on the surface as a result of surface degradation of polymer matrix caused by ultrasonication [36]. Similar results of increased charged groups on the surface of starch nanoparticles due to the conformational changes brought up by sonication were also reported by Agi et al. [37]. The higher value of zeta potential indicates high electrostatic repulsion and less Van Der Waals force. The agglomeration of particles is caused by Van der waals forces, so a decrease in the magnitude of these forces is favourable for the stability of the nanoparticles. Therefore, higher value of zeta potential of suspension dispersion implies that agglomeration will decrease and stability of emulsion/dispersion will increase [38]. The zeta potential value of – 20

| Sample | RS content (%) | Hydrodynamic diameter (μm) | Zeta potential (mV) | Polydispersity index |
|--------|----------------|-----------------------------|---------------------|---------------------|
| NS     | 28.19 ± 0.23a  | 12.13 ± 0.022μm             | -12.12 ± 0.01c      | 1.34 ± 0.09b        |
| RS     | 94.33 ± 1.12b  | 7.86 ± 0.333μm              | -18.98 ± 0.31b      | 2.38 ± 1.07b        |
| RSN    | 96.24 ± 0.96c  | 143.63 ± 0.211μm            | -25.14 ± 0.36d      | 0.158 ± 0.42a       |

NS, RS, and RSN represent lotus stem native starch, lotus stem resistant starch and nano-reduced resistant starch obtained from nanoprecipitation-ultrasonication method. Values expressed are mean ± standard deviation. Mean in the same columns with different superscripts are significantly different at p ≤ 0.05.
mV for biopolymers is sufficient to provide them stability due to the stabilization of steric parameters [39]. Polydispersity index (PDI) gives an idea about the particle size distribution and samples with large PDI have wide particle size distribution which includes large particles or aggregates. The lower values of PDI (i.e. \( i \leq 0.4 \)) indicate homogeneity of particles and narrow particle size distribution. RSN displayed significantly low PDI (0.158 ± 0.42), these can be assumed to have homogeneity and narrow particle size distribution. Thus nanoprecipitation-ultrasonication resulted in RSN narrow particle size distribution and higher surface charge.

3.2. Light and confocal microscopy of FA-PE

The effect of increasing RSN concentration on the stabilization of emulsions was observed using light microscopy. FA-PE stabilized using RSN (2%) (Fig. 1A) displayed unstable droplets. This indicated that the surface coating provided by the solid particles was not enough to form a coating around oil–water interface resulting coalescence. Pickering emulsions formed at low starch concentration (<10%) are unstable [40]. As the concentration of RSN increased from 10% to 20% as shown in Fig. 1B, an effective stabilization was achieved, with RSN surrounding the oil droplets, which form the core. As the concentration of RSN increased to 20%, (Fig. 1c), a black layer surrounding the oil droplet shows that the droplet interface was more densely coated with RSN. Our results are in line with those reported by Leal-Castañeda et al. where starch concentration of 20% and 30% provided effective stabilization to the emulsions [40]. Confocal laser microscopy revealed that FA-PE droplets stabilized by RSN (10%) had high coverage of the oil indicating a stable emulsion. FA-PE formed using RSN (2%) had uneven oil coverage.

3.3. Comparison between particle size, polydispersity index (PDI) and zeta potential of FA-PE and FA-LPE

The results of particle size, zeta potential and PDI of FA-PE and FA-LPE are presented in Table 2. The hydrodynamic diameters of FA-PE (2%), FA-PE (10%) and FA-PE (20%) were observed to be 0.756 ± 0.09 µm, 0.543 ± 0.05 µm and 1.582 ± 0.75 µm, respectively whereas...
The PDI of FA-PE and FA-LPE is presented in Table 2. The values of PDI for FA-PE and FA-LPE (2%, 10% and 20%) increased with the increase in concentration. Overall PDI values were ≤ 0.4 which indicate homogeneity of particles and narrow particle size distribution. The zeta potential values for FA-PE (10%) and FA-LPE (10%) were observed to be −25.89 ± 0.23 mV and −24.97 ± 0.34 mV which makes it moderately stable [26]. The higher the amount of RSN (i.e., 20%) higher could be the agglomeration which is evident from the results of zeta potential of FA-PE (-13.98 ± 0.76 mV) and FA-LPE (-12.09 ± 0.09 mV). Zeta values less negative than − 15 mV indicate the starting point of agglomeration [26]. Particle size, PDI and zeta potential results reveals stability of FA-PE and FA-LPE stabilized using RSN at a concentration of 10%.

Where FA-PE and FA-LPE represent non-lyophilized and lyophilized ferulic acid loaded pickering emulsion. Values expressed are mean ± standard deviation. Means in the same columns with different superscripts are significantly different at (p ≤ 0.05).

### 3.4. Characterisation of FA-LPE

#### 3.4.1. Morphology of FA-LPE

The morphologies of FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%) were observed using the techniques of SEM, AFM, and TEM. The SEM images of pickering emulsions are given in Fig. 2 (A, A, B, B, C & C). The external structure of all the samples presented an irregular and continuous surface having multiple folds which could be related to the oily consistency of the pickering emulsions (Fig. 2: A, B & C) whereas Fig. 2: A, B & C gives a closer look of the surface topography for FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%), respectively. FA-LPE (2%) and FA-LPE (10%) displayed numerous hole like structure on the surface which could be probably caused due to formation of ice crystals that may penetrate into oil droplets disrupting their interfacial membranes in the aqueous phase of emulsions during freezing at ~40°C (Fig. 2 A and B). Fioramonti et al. also reported pore formation in freeze dried microcapsules of flax seed oil formulated with maltodextrin [44].

FA-LPE (20%) is selected as the representative image and its TEM image is given in Fig. 3. TEM image displayed spherical particles clustered around each other confirming the agglomeration of emulsion

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Table 2

| Sample | Hydrodynamic diameter (µm) | Polydispersity Index | Zeta Potential (mV) |
|--------|-----------------------------|----------------------|---------------------|
| FA-PE  |                             |                      |                     |
| 2%     | 0.756 ± 0.09                 | 0.189 ± 0.07         | -16.16 ± 0.24       |
| 10%    | 0.543 ± 0.05                 | 0.210 ± 0.06         | -25.89 ± 0.23       |
| 20%    | 1.582 ± 0.75                 | 0.278 ± 0.10         | -13.98 ± 0.76       |
| FA-LPE |                             |                      |                     |
| 2%     | 0.850 ± 0.06                 | 0.345 ± 0.01         | -18.45 ± 0.11       |
| 10%    | 1.009 ± 0.02                 | 0.367 ± 0.19         | -24.97 ± 0.34       |
| 20%    | 1.956 ± 0.04                 | 0.400 ± 0.03         | -12.09 ± 0.09       |

Where FA-PE and FA-LPE represent non-lyophilized and lyophilized ferulic acid loaded pickering emulsion. Values expressed are mean ± standard deviation. Means in the same columns with different superscripts are significantly different at (p ≤ 0.05).
droplets when the RSN concentration is high. Similarly FA-LPE (20%) was also selected as the representative image of AFM (presented in Fig. 4). The AFM image shows that FA-LPE (20%) appeared like spherical droplets embedded in the matrix. Spherical structures with maximum peak height of 8.47 nm and maximum pit height of 1.69 nm for each spherical particle were observed. The internal structure of the colloidal particles displayed intricate triple scale architecture with small spherical particles found clustered to each other and the results are consistent with TEM observations.

3.4.2. ATR – FTIR

The FTIR spectra of FA-LPE (2%, 10% and 20%) are presented in Fig. 5. The characteristic peak of FA in section A i.e. 3499 cm \(^{-1}\) corresponds to the \(-\text{OH}\) stretching vibration, whereas, the peaks at 1600 cm \(^{-1}\) and 1439 cm \(^{-1}\) in section C are assigned to the presence of \(\text{C} = \text{C}\) aromatic ring. The asymmetric stretching vibration of peaks at 1277 cm \(^{-1}\) indicates the presence of \(\text{C} = \text{O}\)-C bond. In the fingerprint region of FA, the characteristics peaks at 863 cm \(^{-1}\) and 804 cm \(^{-1}\) correspond to the two atoms adjacent to the phenyl ring of FA [22]. For RSN, the peaks at 3300 cm \(^{-1}\) and 1695 cm \(^{-1}\) represent \(-\text{OH}\) and \(\text{C}-\text{O}\) stretching vibration which might be due to the assembly of amylose helices during the enzymatic treatment. In the fingerprint region, RSN displayed the band at 1022 cm \(^{-1}\) which may be assigned to the disordered and amorphous structure of RSN. Also, the peaks at 995 cm \(^{-1}\) and 1047 cm \(^{-1}\) indicate the ordered and crystalline structure of RSN [26]. FTIR spectra of FO was similar to that observed by Comin [45]. The strong and sharp peaks observed in the section C near 1745 cm \(^{-1}\) correspond to the stretching vibrations \(\text{C} = \text{O}\) of ester linkages whereas the bands in the region B from 2996 to 2750 cm \(^{-1}\) indicate \(\text{C}-\text{H}\) vibrations [45]. The ATR-FTIR spectra of FA-LPE (2%, 10% and 20 %) displayed peaks of \(\text{C} = \text{C}\) aromatic rings of FA, signifying successful encapsulation. Furthermore, the fingerprint region of all the FA-LPE samples displayed stretching of the characteristics peaks of RSN at 995 cm \(^{-1}\) and 1047 cm \(^{-1}\) indicating the increase in crystallinity and ordered arrangement of FA-LPE samples. The increase in crystallinity may be attributed to the increased number of hydroxyl groups due to the encapsulation of FA in the samples. Similar phenomenon was also observed for FA encapsulated in maltodextrin [22]. The phenyl rings corresponding to the bands at 863 cm \(^{-1}\) and 804 cm \(^{-1}\) and aromatic ring of FA at 1600 cm \(^{-1}\) and 1439 cm \(^{-1}\) interact with RSN and result in stretching of peaks in RSN. The results are similar to those reported by Garnero et al. [46]. Conversely the peaks of FA at 3436, 1589, 1510 and 863 cm \(^{-1}\) decreased in intensity. This may be attributed to the \(-\text{OH}\) bond that shifts to lower frequency as a result of stretching vibration [47]. The presence of FO in FA-LPE samples (2, 10 and 20 %) was confirmed by the presence of twin peaks from 2996 to 2750 cm \(^{-1}\) shoulders \(\text{C}-\text{H}\) vibrations and strong peaks of \(\text{C} = \text{O}\) ester linkage at 1745 cm \(^{-1}\). In conclusion, based on the ATR-FTIR results, it can be affirmed that FA was successfully encapsulated in FA-LPE.

3.5. Encapsulation efficiency and in vitro release

The results of EE and in vitro release of FA-LPE (2%, 10% and 20%) are shown in Table 3. The EE of FA-LPE (10%) was significantly high (p ≤ 0.05) among the samples (63.98 ± 0.34 %), but as the concentration of RSN increased to 20 % EE declined to 52.23 ± 0.76%, even though the latter provided slow and sustained release. This phenomenon could be due to the amount of stabilizer (RSN) and the encapsulate present in equal weights of FA-LPE (20%) and FA-LPE (10%) samples, taken for the study of EE and the release. EE is calculated based on the amount of FA present in the samples of equal weight. The bulky layer of protection provided by FA-LPE (20%) might give better stability during release, but the amount of FA-FO mixture present is lower compared to that of equal weight of FA-LPE (10%). According to this logic alone FA-LPE (2%) might look like an ideal candidate for transporting more amount of encapsulate, but that is proved otherwise from Table 3 (30.09 ± 0.02%). The loss of encapsulate in FA-LPE (2%) could be due to insufficient amount of stabilizer, which in turn means less protection, and more FA-FO mixture could have been lost during the ethanolic washing, carried out before measuring the EE. Determination of the amount of stabiliser, i.e., RSN should be based on the desired release profile and on weight to encapsulate ratio.

The aim of encapsulation is to safeguard FA from harsh conditions of gastro-intestinal tract until it reaches to target site. Under SGD, FA displayed a rapid release within 1 h of digestion. Next, the release of FA from FA-LPE (2%) during first 30 min was observed to be 11.98 ± 0.04
% which was significantly high (p ≤ 0.05) than FA-LPE (10%) and FA-LPE (20%), with the values corresponding to 8.78 ± 0.33% and 6.24 ± 1.08 %. In the next 30 min, the release of FA from FA-LPE (2%) was observed to be 50.67 ± 0.43 % and within next 30 min whole of the FA was released from FA-LPE. This could be due to insufficient RSN covering the FA-FO mixture which does not resist the harsh SGD conditions. For FA-LPE (10% and 20%), a sustained release was observed for 180 min with FA-LPE (20%) showing significantly (p ≤ 0.05) low values as compared to FA-LPE (10%). Increasing RSN concentration could provide a physical barrier around the emulsion droplets which prevent the diffusion of FA and enables its sustained release. RSN could hamper the diffusion of FA through the pores as RSN is less susceptible to the enzymatic hydrolysis, thus contributing to its slow release.

A similar behaviour of pickering emulsion stabilized by polycationic chitosan nanoparticles in polyanionic alginate hydrogel beads also acted as a barrier for the release of emulsion in gastric medium whereas released the contents in a medium at near-neutral pH [48].

3.6. Bioactivity of FA-LPE under SGID

3.6.1. Anti-cancer activity

The results of MTT assay on three cell lines, HELA, HEKT and U2OS are shown in Fig. 6A. The percent viability of all the three cell lines after the treatment with FA-LPE demonstrated a significant decrease (p ≤ 0.05) with the increase in RSN concentration from 2 to 20%. The percent viability of HELA cells, after 24 h of incubation with FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%) was observed to be 52.89 ± 0.73%, 35.64 ± 0.11% and 15.43 ± 0.02%, respectively. The significantly (p ≤ 0.05) high anti-cancer activity of FA-LPE (20%) could be due to the protective effect offered to FA by RSN in SGID. Although FA-LPE (10%) demonstrated highest EE, which could mean higher amount of FA available for anti-cancer activity, but the protective effect offered by RSN (20%) in SGID outweighed. The higher concentration of RSN might not only control the release of bioactive but also enhances the permeation of bioactives into the cells thereby increasing the bioavailability [49]. The results on other two cell lines, HEK and U2OS also displayed a significant decrease (p ≤ 0.05) in cell viability with the increase in amount of RSN. Pickering emulsion stabilized by CS-GA nanoparticles at the higher concentration significantly improved the bioavailability of curcumin in comparison to the CS-GA nanoparticles at low concentration [50]. FA’s anti-carcinogenic activity is related to its ability to scavenge free radicals and to stimulate cytoprotective enzymes [51]. This scavenging ability in turn reduces lipid peroxidation, inactivation of proteins, disruption of biological membranes and rupturing of single strand DNA [52]. Studies have reported the inhibitory activity of FA on growth of colon cancer cells and suppression of tumor in breast cancer cell lines [53,54].

3.6.2. Angiotensin converting enzyme inhibition activity

The ACE inhibition activity of FA-LPE (2%), FA-LPE (10%) and FA-
LPE (20%) under SGID was found to be 15.07 ± 0.9 %, 45.29 ± 0.01 %, and 25.67 ± 0.3 %, respectively as shown in Fig. 6B. The results of the ACE-inhibition activity of FA-LPE show that the emulsions possess antihypertensive properties. FA may potentially inhibit the ACE, an enzyme that facilitates the conversion of angiotensin I (Ang-I) to angiotensin II (ANG-II) [55]. It has been reported earlier that intake of FA (1–100 mg/kg body weight) decreased the blood pressure with a maximum effect of 34 mm Hg in both spontaneously and stroke-prone spontaneously hypertensive rats after 2 h of oral intake [56]. Furthermore, FA has been shown to inhibit platelet aggregation, reduce serum lipids and prevent thombus formation [57]. The significantly higher ACE inhibiting activity of LPE (10%) can be associated with the high loading of FA in RSN. As shown in encapsulation efficiency studies, FA-LPE (10%) had higher loading efficiency, which means more the concentration of FA, more will be ACE inhibiting activity. The emulsions may act as a good carrier of FA for delivering their ACE inhibition activity.

3.6.3. Anti-diabetic activity (α-amylase activity)
Anti-diabetic activity of the FA-LPE (2%, 10% and 20%) under SGID was evaluated in terms of α-amylase inhibition potential and the results are presented in Fig. 6C. α-amylase acts on the dietary starch and converts the starch into maltose and dextrin which may subsequently be converted into glucose by the action of α-glucosidase [58]. Hence, to relieve postprandial glycaemia, an inhibitor of the intestinal α-amylase enzyme could be an effective treatment. The α-amylase enzyme inhibition activity of FA-LPE samples increased significantly (p ≤ 0.05) with the increase in RSN concentration under SGID. The inhibition values were observed to be 22.09 ± 0.01%, 53.11 ± 0.06% and 38.09 ± 0.4% for FA-LPE (2%), FA-LPE (10%) and FA-LPE (20%), respectively. The mechanism of α-amylase inhibition action of FA can be explained that FA not only competes with α-amylase for active starch-binding sites but also with α-amylase–starch inclusion complex in a non-competitive mode [59]. Molecular docking studies have revealed that FA docked into the active site of α-amylase and the binding strengthened the FA-α-amylase complex. This binding hampers the access of the substrate or occupies the binding site of starch, which may in turn suppress the activity of the hydrogous groups of FA at C3 and C4 via interaction with Asp197 and Asp300 of α-amylase [60]. Therefore, the increase in the FA molecules will compete for more sites resulting in enhanced α-amylase inhibition activity of FA. FA encapsulated in chitosan nanoparticles were observed to reduce blood glucose levels in animals [61].

3.6.4. Antioxidant activity against oxidative damage to DNA
FA-LPE samples (2%, 10% and 20%) were assessed for their anti-oxidant activity against damage to DNA. The results are presented in Fig. 7. The damage to the DNA strand is induced by Fenton reaction (FeSO₄ + C₆H₅O₆ + H₂O₂). Fenton’s reaction generates free hydroxyl radicals which can cause the breakage of DNA strand, modification of base, rearrangement of chromosomes and cross linking with proteins [62,63]. The reaction involves abstraction of deoxyribose hydrogen which unwinds the DNA strand and makes it to run fast. The runoff is presented in lane 2. The native calf thymus DNA is represented by Lane 1. The lanes numbered from 5 to 7 reveal that FA-LPE samples were able to protect the DNA strand, although the highest protection was offered by FA-LPE (20%) as seen by the clarity of band in Lane 7. Additionally, Lane 4 displays the incubation of RSN with calf thymus DNA, providing an idea that RSN had no toxic effect on calf thymus DNA. The bands were similar to those obtained in case of Lane 1. The enhanced visible protective effect of LPE-FA (20%) may be attributed to the strong antioxidant properties of the FA. The strong electron accepting ability of the FA is due to its phenolic ring that offers strong resonance stability and allows it to readily accept the electron from free radical, thus acting as the direct scavenger of free radicals. Earlier FA at a concentration of 250 mg/mL has been reported to scavenge hydroxyl radicals completely in fentons reaction system [64].

4. Conclusion
The key findings in this study reveal that FA-LPE (10%) were stabilized more effectively whereas RSN (20%) led to the agglomeration signifying that the amount of stabilizer plays an important role in stabilization of pickering emulsions. FA-LPE (10%) also displayed high encapsulation efficiency thus providing better option to use it as wall material for loading of FA. FA-LPE (20%) displayed significantly higher retention of anti-cancer and prevention of oxidative DNA damage

| Table 3 |
| --- |
| Encapsulation efficiency and in vitro release of FA-LPE (2%, 10% and 20%). |
| &nbsp; | Encapsulation efficiency (%) | 30 min | Percentage release of FA |
| &nbsp; | &nbsp; | &nbsp; | 60 min | 90 min | 120 min | 180 min |
| FA-LPE (2%) | 30.09 ± 0.02^c | 11.98 ± 0.04^c | 50.67 ± 0.43^c | 35.43 ± 1.1^c | 28.09 ± 0.16^c | 17.43 ± 0.22^c |
| FA-LPE (10%) | 63.98 ± 0.34^d | 8.78 ± 0.33^b | 14.89 ± 0.13^b | 22.56 ± 0.18^b | 28.09 ± 0.16^c | 17.43 ± 0.22^c |
| FA-LPE (20%) | 52.23 ± 0.76^c | 6.24 ± 1.08^a | 10.09 ± 0.16^a | 13.87 ± 0.09^a | 17.08 ± 0.32^a | 22.23 ± 0.14^a |
| FA | – | 35.98 ± 0.11^d | 54.35 ± 1.09^d | – | – | – |

Values expressed are mean ± standard deviation. Means in the same columns with different superscripts are significantly different at p ≤ 0.05.
potential under SGID whereas FA-LPE (10%) displayed higher ACE inhibiting and α-amylase inhibiting activity. The results display that the properties of the pickering emulsions were dependent on RSN concentration. Furthermore, the retention of bioactivities of FA-LPE (2%, 10% and 20%) shows that the pickering emulsions can prevent FA under harsh SGID conditions, thus providing a new opportunity to deliver the nutraceuticals in functional foods and pharmaceuticals.

CRediT authorship contribution statement

Nairah Noor: Investigation, Writing – original draft. Adil Gani: Supervision, Resources, Conceptualization. Faiza Jhan: Formal analysis. Mohammad Ashraf Shah: Writing – review & editing. Zanoor ul Ashraf: Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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