Ultrasonics Sonochemistry 86 (2022) 106006

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Nano reduction coupled with encapsulation as a novel technique for utilising millet proteins as future foods

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ARTICLE INFO

Keywords:
Nano protein
Ultrasonication
Crocin
Anti-microbial
Anti-cancer

ABSTRACT

Crocin (saffron bioactive) loaded protein nanoparticles were prepared from three underutilised cereal varieties viz., sorghum (SPCN), foxtail millet (FPCN) and pearl millet (PPCN) using ultrasonication technique. The particle size of crocin loaded protein complex was attained in the nano range with reduced polydispersity index and negative zeta potential. The encapsulation efficiency of crocin in protein nanoparticles was found to be 83.78% (FPCN), 78.74 % (SPCN) and 70.01% (PPCN). The topographical images of crocin loaded protein nano complex was revealed using field emission-scanning electron microscopy (FE-SEM). The attenuated total reflectance fourier transform infra-spectroscopy (ATR-FTIR) analysis showed the characteristic peaks of crocin at 956, 1700 and 3350 cm^{-1} in protein-crocin nanocomplex as a confirmatory test for nanoencapsulation. The antimicrobial activity of crocin loaded protein nanocomplex against three strains *(Escherichia coli, Staphylococcus aureus and Fusarium oxysporium*) were also evaluated. *In vitro* release studies showed higher content of crocin released in simulated intestinal conditions ensuring its controlled release at target site. Bioactivity (anti-cancerous and anti-hypertensive) of crocin upon *in-vitro* digestion were well retained indicating that protein nanoparticles can act as an effective wall material. Our results suggest that protein nanoparticles prepared in this study can act as an effective oral delivery vehicle for crocin that could be used for development of functional foods.

1. Introduction

Crocin, a hydrophilic carotenoid is a glyosidic derivative of crocetin which constitutes the colouring part of saffron [1]. It is considered as one of the most important carotenoid found in nature with its high nutraceutical potential. It acts as a potent antidepressant, antioxidant, antiapoptotic, anticancerous and therapeutic agent imparting beneficial effects on gastric, cardiovascular, nervous and immune systems. Despite of such advantages major limitation for its usage is due to its high susceptibility to pH, oxygen, light and temperature hindering its food system application. When administrated orally the hydrolysis of crocin to crocetin results in the sugar moieties elimination during its absorption in intestines reducing its bioavailability [2,3]. Natural biodegradable polymer, protein is considered now a days a suitable option for the safeguard of bioactives and their target delivery in human gastro intestinal (GI) tract compared to synthetic biodegradable polymers [4]. Moreover, food proteins are considered as vital macronutrient possessing unique functional attributes which include their gelling, emulsion and film forming ability making it suitable as a delivery vehicle for both lipophilic and hydrophilic bioactives [5]. However, the use of micro-protein particles for encapsulation do not enhance the bio accessibility of bioactives in the human GI tract during digestion. Therefore use of nanoreduced protein particles for the purpose of encapsulation could be an effective approach increasing the stability, solubility and cell permeability of the target compound protecting it from degradation by human digestive enzymes [6]. The nanoencapsulation approach also protects the bioactivity of the compound as well as make it bio-accessible during the process of digestion helping in its proper absorption at its target site [7]. Thus, utilisation of nano polymers offer the suitable option for oral delivery of various bioactive compounds since they can easily get adhered to the intestinal membrane thereby increasing the residence time of bioactives in the gastric conditions. Also the M−cells present in the Peyer's patches easily absorb nano polymeric particles by receptor-mediated endocytosis, transcellular and paracellular pathways to directly deliver bioactives into the blood circulation [5]. Subirade and Chen [8] reported that previously casein, gelatin,

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https://doi.org/10.1016/j.ultsonch.2022.106006
Received 11 February 2022; Received in revised form 1 April 2022; Accepted 9 April 2022
Available online 12 April 2022

soy protein and whey protein nanoparticles have been successfully used for encapsulating vitamins, probiotics, unsaturated fatty acids and bioactive peptides, etc. In order to achieve the nanoencapsulation of crocin in protein nanoparticles the effective method of ultrasonication was used for the purpose which has been previously carried out for nano sizing the protein particles in our previous study [9]. The present study aims to use three underutilised sources for extracting the protein viz sorghum, pearl millet and foxtail millet. Millets are an affordable and cheap source of protein which are gluten free in nature [10]. These millets grow abundantly in adverse agricultural conditions with sustainable yields in which major cereal varieties like rice and wheat fail to grow [11]. Thus, present study was aimed to increase the utilisation of such millets by extracting the protein and utilising it as a nano delivery vehicle of crocin in human gastro intestinal (GI) conditions. The *in vitro* release behaviour and bioactivity of crocin from nanoprotein particles in simulated gastric conditions were also investigated. The current study may be useful for future functional industry for the development of protein rich foods with enriched nutraceutical properties especially for people with celiac disorder.

2. Materials and methods

2.1. Materials

Crocin and all other chemicals used were of analytical grade and purchased from Sigma Aldrich (USA). Foxtail millet, sorghum and pearl millet were acquired from local markets of Srinagar, Kashmir, India. Protein extraction and all other experimentations were carried out in laboratory of functional foods and nutraceuticals , food science and technology department, University of Kashmir.

2.2. Protein extraction

Seeds from all the three different varieties were first manually cleaned for removing unwanted foreign constituents. Milling of grains was done using a grinder (Phillips, Mixer Grinder, New Delhi, India) at ambient temperature to obtain fine flour. The flour samples were then sieved using a sieve (60 mesh-British standards). The flour was further defatted and decolorized using methanol:chloroform (60:30). The slurry was then centrifuged (Eppendorf, 5810R, Hamburg, Germany) at 3000 × g for 10 min and the obtained pellet was air dried. The defatted and decolorized flour was used for the extraction of protein using the previously followed method of Jhan et al., 2021 [9]. Briefly, slurry of flour was prepared at the ratio of 1(flour): 4 (double distilled water) w/v. The pH of slurry was maintained at 9.5 using sodium hydroxide (1 N) and kept at stirring for one hour at room temperature which enhanced the protein solubility. The slurry was then centrifuged at 4000 × g for 15 min. The pH of supernatant obtained was then adjusted to 4 using hydrochloric acid (1 N) and kept undisturbed for about one hour. The centrifugation was again carried out for 15 min at 4000 × g and pellet recovered was required protein concentrate. The purity of the extracted protein was found to be 76.9%, 82.87% and 86.65% using kjeldhal method.

2.3. Crocin loaded protein particles

The crocin loaded protein particles were prepared following the protocol of Liang et al. [12] with certain changes. Protein (5 g) was dissolved in borate buffer (pH 8) and total volume was made around 250 mL. The solution was left on magnetic stirrer for about 2 h and then subjected to centrifugation (Eppendorf, 5810R, Hamburg, Germany) for 10 min at 5000 × g. The supernatant was collected and kept in refrigeration for 24 h. Next day, centrifugation under same conditions was carried out to remove any unwanted material and collect precipitated protein particles. Crocin (0.5g) was dissolved in distilled water and total volume was made upto 50 mL. The final solutions were then obtained by mixing crocin and protein solution together at the ratio of 1:5 (v/v).

2.4. Formation of crocin- protein nanocomplex using ultrasonication

The prepared solutions containing crocin and protein solution at the ratio of 1:5 (v/v) were subjected to sonication treatment using probe sonicator (Cole-Parmer, 04711–35, Mumbai, India; probe diameter 12 mm) for about one hour at frequency of 40 kHz. The beaker containing solution was placed in ice cold water and sonication was carried at intervals of 5 min to avoid excess heating which can cause damage to the samples. The probe with diameter 12 mm was dipped into the solution at 10 mm depth with output power of 100 W. The sonicated samples were then freeze dried using lypholizer (Buchi lyovapor L-200, Switzerland) and stored at 4°C in air tight plastic containers for future use.

2.5. Particle size analysis, zeta potential and polydispersity index.

The samples were prepared at dilution of 1:10 w/v for protein powder in distilled water at room temperature. Mean particle size, polydispersity index (PDI) and zeta potential measurements were carried out using malvern nanosizer ZS (Malvern Inc., Malvern, UK).

2.6. Infrared spectroscopy

The spectral analysis of crocin- protein nanocomplex samples were recorded using attenuated total reflectance fourier transform infrared equipment (ATR-FTIR, Agilent -Cary 630, USA) between the wavelength range of 4000–400 cm^{-1}.

2.7. Morphological analysis

Microstructural analysis of the crocin- protein nanocomplex were examined using FE-SEM (GeminiSEM 500 8203017193, UK/GB). The dried samples were first placed on the aluminium stub with the help of adhesive carbon tape, and coated with gold (15 nm thickness). Topography of samples were examined and images were recorded.

2.8. Encapsulation efficiency

The encapsulation efficiency of protein nanoparticles for crocin was calculated by following the method of Jhan et al. [13]. Briefly, nanoencapsulated samples (20 mg) were washed with double distilled water (10%) and subjected to centrifugation at 3000 × g for 5 min. The pellet recovered is free from surface adhered crocin. The pellet was resuspended in double distilled water (2.5 mL) followed by sonication in bath sonicator for 20 min to extract the entrapped crocin from nanoecapsulated samples and then again centrifuged. The supernatant obtained was filtered using whatman No 1 and its absorbance was measured using UV–Vis spectrophotometer (Model U2900 2JI-0003, Hitachi, Japan) at 440 nm. The crocin content was obtained by using the equation based on crocin standard curve by measuring the absorbance at 440 nm of a known concentration of crocin. The encapsulation efficiency of protein nanoparticles was then calculated using following formulae;

EE (%) of protein nanoparticles = Crocin released from wall material / Total crocin added initially ×100

2.9. In vitro release behaviour of crocin

The *in vitro* release behaviour of crocin from protein nanoparticles under simulated GI conditions was studied. Firstly, simulated mouth conditions (SMC) were prepared by dissolving 0.2% alpha amylase in phosphate buffer saline (pH 7.2). 10 mg of nano encapsulated samples were suspended in 10 mL of SMC. The mixture was then stirred and kept

Table 1
Particle size, zeta potential, polydispersity index and encapsulation efficiency of crocin protein nanocomplex.

Sample	Particle size (nm)	Zeta potential	Polydispersity index	E.E (%)
FPCN	386.87	−19.98	0.37	83.78 ± 0.02^{c}
SPCN	486.98	−28.01	0.40	78.74 ± 0.13^{b}
PPCN	664.96	−27.04	0.30	70.01 ± 0.32^{a}

The presented values are average ± standard deviation (n = 3). Different superscripts of averages in the same row are significantly different at $p \leq 0.05$. FPCN, SPCN and PPCN represent crocin encapsulated in protein nanoparticles of foxtail millet, sorghum and pearl millet, respectively.

in a shaking water bath at 37 ℃ for 5 min and then centrifuged for 10 min at 5000 × g. The pellet was recovered and absorbance of supernatant was measured at 440 nm using UV-spectrophotometer. The pellet was then subjected to simulated gastric conditions (SGC) which was prepared by dissolving 3 g/L pepsin in 9 g/L NacL solution (pH 3). The prepared solution was incubated at 37 ℃ and then centrifuged for 5 min at 5000 × g at the time intervals of 30 and 60 min. The absorbance of the supernatant was measured at 440 nm. The recovered pellet was then exposed to 10 mL simulated intestinal juice (SIC), which was prepared by dissolving 3 g/L bile salts and 10 g/L pancreatin in phosphate buffer saline (PBS) at 7.5 pH. The prepared solution was incubated at 37 ℃, centrifuged for 5 min at 5000 × g on time interval of 30, 60 and 120 min and absorbance was taken at 440 nm. Percent crocin released was calculated by the formulae:

Released crocin (%) = Weight of crocin released/Total weight of encapsulated powder added × 100

2.10. Anti-microbial assay

The antimicrobial activity of nano encapsulated samples was evaluated by agar disc diffusion method using three different strains viz. *Escherichia coli, Staphylococcus aureus* and *Fusarium oxysporium.* Briefly, cell suspension (0.1 mL) having density 100–110 CFU/Ml were cultured. For growing *E.coli and S. aureus* nutrient agar was used while as for *F. oxysporium* rose Bengal agar was used. Two sterile discs and one antibiotic disc (positive control) was placed on the agar. 50 µL of sample extract was impregnated on one sterile disc while the other was kept as such. The plates were then incubated for 24 h at 37 ℃. Next day, the zone of inhibition formed by the samples and antibiotic disc was measured using a clipper and expressed in millimetres (mm).

2.11. Bioactivity retention of crocin under simulated gastrointestinal conditions

To analyse the bioactivity retainment of crocin in three different nano protein samples, the samples were subjected to simulated human GI conditions. Nano encapsulated samples (200 mg) were first treated with 10 mL of simulated gastric juice (3 g/L pepsin in 9 g/L NacL solution; pH 3). The solution was then centrifuged for 5 min at 3000 × g. The supernatant was collected and refrigerated for future use. The pellet recovered was further subjected to 10 mL of simulated intestinal juice (3 g/L bile salts and 10 g/L pancreatin in phosphate buffer saline; 7.5 pH). Centrifugation of solution was again carried to obtain supernatant which was collected and stored under refrigerated conditions.

2.11.1. Anti-cancerous activity

The *in vitro* anti-cancerous activity of the nano encapsulated samples was done on three cell lines *viz.*, HELA (cervical cancer cell line) HEK (Human embryonic kidney) and U2OS (Human Bone Osteosarcoma Epithelial Cells) which were bought from National Centre for Cell Science (NCCS), Pune, India following the method of Wani et al. [14]. Media was prepared by mixing Dulbecco's Modified Eagle Medium (DMEM) (45 mL), Foetal bovine serum (5 mL) and streptomycin antibiotic (5 mL). The cells were grown in freshly prepared media overnight in 96 well plate under incubation temperature of 37 ℃ and carbon dioxide 5 %. Next day, the media was replaced with freshly prepared media. 20 µL of nanoencapsulated samples (1 mg/mL) were added and kept at incubation for 24 h. After this, 20 µL of tetrazolium dye [MTT 3-(4,5-dimethylthiazol- 2–144 yl) − 2,5-diphenyltetrazolium bromide] was added in each well and incubated for 3 h. Dimethyl sulfoxide (150 µL) was added in dark for dissolving the precipitates. The absorbance was measured at absorbance of 590 nm on microplate reader (Elisa Biotek, synergy HT, US). The cell viability (%) was calculated by using the following equation:

Cell viability (%) = S–B/ C–B × 100.

Where, S denotes absorbance of sample, B denotes absorbance of blank and C denotes absorbance of control at absorbance of 590 nm.

2.11.2. Anti-hypertensive activity

The *in vitro* anti-hypertensive activity of nanoencapsulated samples was evaluated by following the method of Noor et al.[15]. Briefly, sample solution (8 mg/mL) and 50 mU/mL angiotensin converting enzyme (ACE) solution (50 µL) was mixed together and incubated at 37 °C for half an hour. After this, 150 µL of HHL (Hippuryl-histidyl-leucine) was added to the mixture and again incubated for half an hour to terminate the reaction. The hippuric acid was extracted by ethyl acetate followed by evaporation in rotatory vacuum evaporator at 95 ℃. The residue left was diluted using double distilled water (2mL). The absorbance was taken at 228 nm. The percent inhibition of ACE activity was measured using formula:

Inhibition (%) = $[A_1 - (A_2 - A_3)/ (A_1 - A_4) \times 100$.

Where, A_2 denotes sample/standard absorbance, A_1 denotes positive control absorbance, A_3 denotes absorbance of blank, and A_4 represents negative control absorbance.

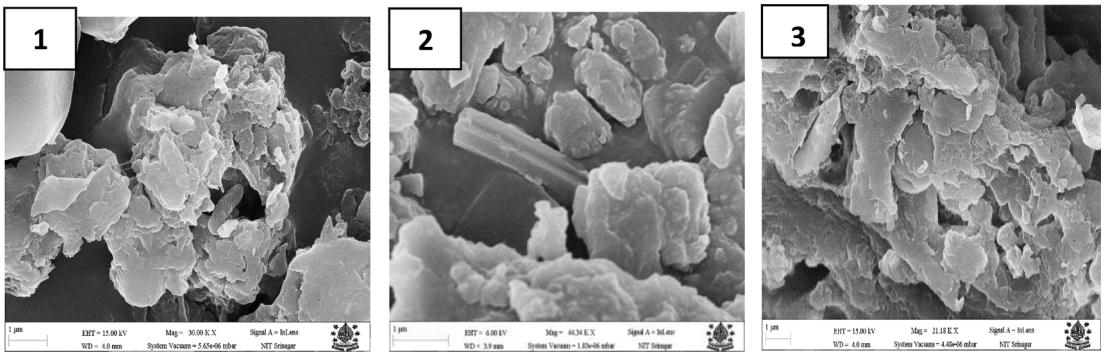

Fig. 1. Surface topographic images of crocin protein nanocomplex of foxtail millet (1) sorghum (2) and pearl millet (3).

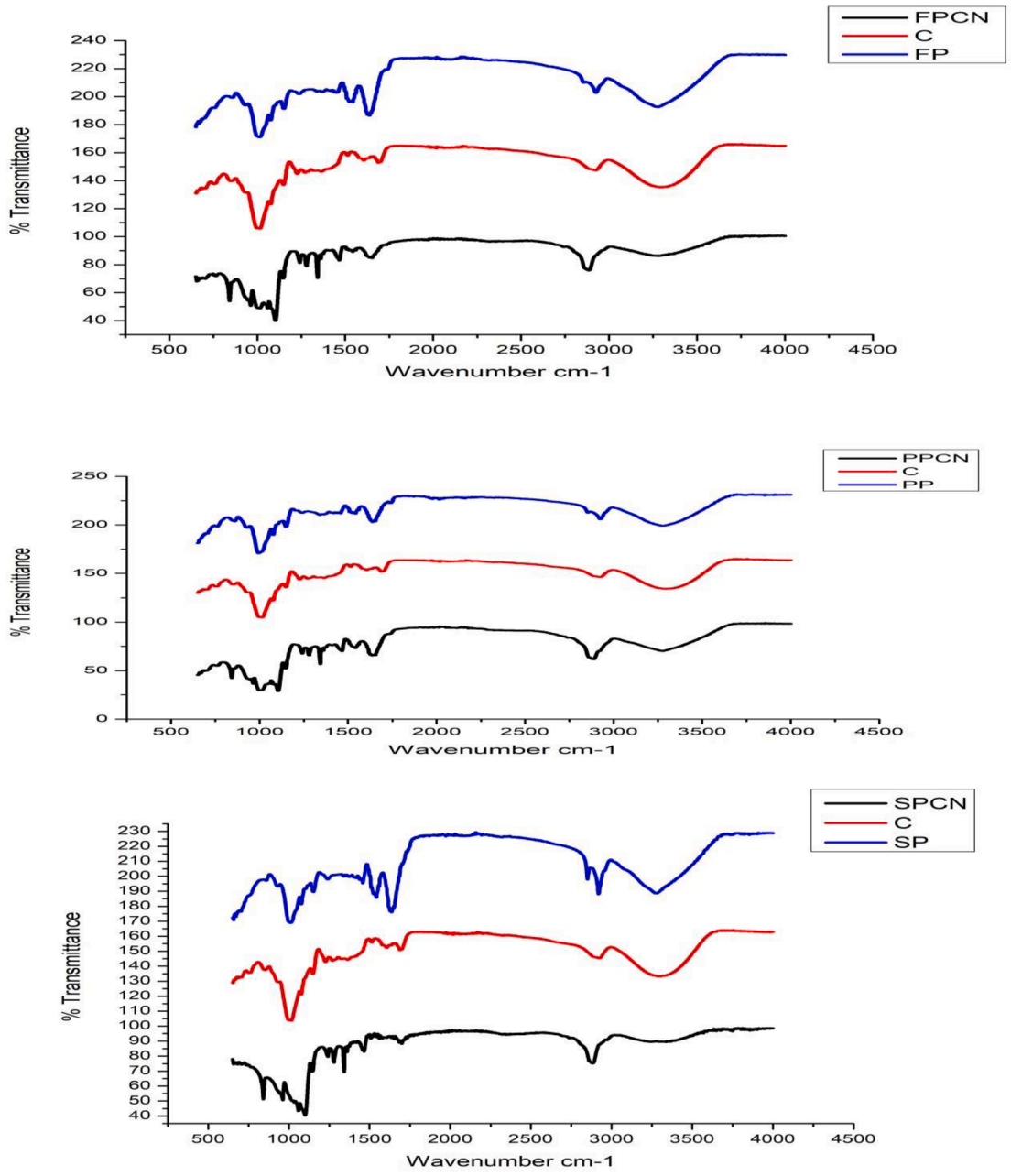

Fig. 2. Structural elucidation of pure crocin (C), pearl millet protein (PP), foxtail millet protein (FP), sorghum protein (SP), crocin protein nanocomplex of foxtail millet (FPCN), sorghum (SPCN) and pearl millet (PPCN), respectively.

Table 2
Anti-microbial activity presented as zone of inhibition (mm) of crocin protein nanocomplex.

Inhibition zone (mm) Anti microbial	PPCN	SPCN	FPCN	Standard	Sterile disc
E. coli	8.7 ± 0.04^{a}	11.87 ± 0.01^{b}	19.87 ± 0.12^{c}	23.75 ± 0.01^{d}	NS
S. aureus	9.78 ± 0.23^{b}	7.09 ± 0.02^{a}	9.0 ± 0.03^{b}	25.04 ± 0.00^{c}	NS
F. oxysporium	15.09 ± 0.22^{c}	10.87 ± 0.12^{a}	12.67 ± 0.23^{b}	20.98 ± 0.03^{d}	NS

The presented values are average ± standard deviation (n = 3). Different superscripts of averages in the same row are significantly different at $p \leq 0.05$. FPCN, SPCN and PPCN represent crocin encapsulated in protein nanoparticles of foxtail millet, sorghum and pearl millet, respectively.

2.12. Statistical analysis

IBM SPSS statistical package was used to check analysis of variance between the means. Statistical significant difference for P-values was considered at less than or equal to 0.05 using Duncan's test. Results were reported as mean ± standard error done in triplicate (n = 3).

3. Results and discussion

3.1. Particle size, zeta potential and polydispersity index

The DLS measurements viz. particle size, zeta potential and polydispersity index of nano encapsulated crocin is presented in Table 1. These parameters are most vital for the formation of stable nano encapsulation system for targeted delivery of bioactives. The average hydrodynamic particle diameter of encapsulated nanoparticles was

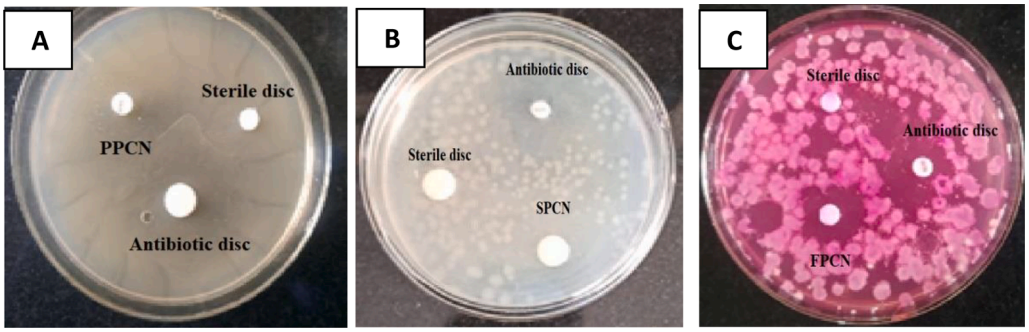

Fig. 3. Representative images of zone of inhibition for antimicrobial activity of crocin loaded pearl millet protein nanocomplex against *E.coli* (A) Crocin loaded sorghum protein nanocomplex against *S. aureus* (B) Crocin loaded foxtail millet protein nanocomplex against *F. oxysporium* (C).

found to be 386.87, 486.98 and 664.96 nm with a relatively narrow polydispersity index (PDI) below 0.4 for FPCN, SPCN and PPCN, respectively. The nano metric particle size was attained for all encapsulated particles attributed to the effects of ultrasonic waves which induces the cavitation process causing the bursting of microbubbles resulting in the degradation, breakage and molecular rearrangement of particles [9]. The reduction of particle size due to ultrasonic waves enhance the coagulation of crocin on protein surfaces, its rate of transport and collision frequency [16]. Moreover, the lower polydispersity values indicate homogenous and narrow size distribution of particles [17]. The zeta potential of all nanoencapsulated particles was found above −30 mV which indicated their good stability. The highest stability was found for SPNC which means the particles will not aggregate due to presence of high surface repulsive charges present [18]. The augmented negative zeta potential values might be caused due to molecular rearrangement during the ultrasonication process [19].

3.2. Surface topography using FE-SEM

The internal microstructure of crocin protein nanocomplex is presented in Fig. 1.The images clearly reveal the disrupted protein matrix with encapsulated crocin molecules. The micrographs show irregular, rough, inhomogeneous and porous structural appearance with some channels and holes present which are holding crocin particles in it. The disruption of the particles was caused due to the turbulence, shock and shear forces of ultrasonic cavitation process resulting into the particle size reduction and exposure of cleave bonds that helps to hold the bioactive more efficiently [20]. Jin et al. [21] reported similar morphological results for corn gluten meal, due to ultrasound waves inducing many holes and even a hollow structure resulting in a curly surface. The micrographs of all three samples also showed cracks and aggregation on the surface of matrix which might have been caused due to protein hydrophobic interactions during freeze drying process of the encapsulated polymer network [22].

3.3. Structural elucidation using fouier transform infrared spectroscopy

The spectral analysis of pure crocin, native protein and crocin protein nanocomplex are shown in Fig. 2. which explains possible intermolecular interaction between crocin and protein particles. The characteristic peaks for pure crocin was found at around 900–1000 cm^{-1} (C–O stretching, sugar groups), 1500–2000 cm^{-1} (CH_2 scissors vibration, C–C stretching), 2800–2900 cm^{-1} (CH stretching) and 3300–3500 cm^{-1} (OH groups) [23]. The FTIR spectral analysis of native protein revealed main functional groups at around 3400, 1550, 1200 and 900 cm^{-1} representing amide A (N–H stretching), amide I band (C-O and C-N stretching), and amide II band (N–H deformation and C-N stretching) positions, respectively [24]. The crocin protein nanocomplex showed the characteristic peaks of native proteins and crocin indicating the intermolecular interaction and encapsulation of crocin in protein molecules with lowered intensity which is due the sonication process. After nanoencapsulation all the peaks were shown but with a slight change in hydrogen bonds which showed a stretch depicting a strong and stable hydrogen bond formation and electrostatic interaction between protein and crocin. Moreover, sharpening and widening of peaks were also observed around amide I and II which indicate the alteration in secondary structure of protein leading to improved interaction and encapsulation of crocin in nanoprotein matrix [24].

3.4. Encapsulation efficiency of crocin in protein nanocomplex

Crocin is highly hydrophilic and sensitive bioactive in nature. To increase its bioavailability and stability the nanoencapsulation in protein nanomatrix was carried out. The percent encapsulation efficiency of crocin in protein nano matrix is presented in Table 1. The encapsulation efficiency (EE) was found significantly ($p \leq 0.05$) different for each sample with highest value for FPCN (83.78%). The difference in EE of three protein matrixes might be due to their different particle size, composition, hydrophobicity and structural differences [18]. The results are in alignment to DLS data. The process of ultrasonication results in the exposure of cleavage bonds, loosening of protein structure as well as size reduction of particles due to the process of cavitation Thus, resulting in the increased surface area of protein molecules enhancing the contact between crocin and protein particles thereby increasing the encapsulation efficiency of protein particles [25]. Patel et al., [26] also stated the similar EE (71–87%) of curcumin in zein-based nanoparticles using anti-solvent precipitation method.

3.5. Anti-microbial activity of crocin in protein nanocomplex

The antimicrobial activity of the crocin encapsulated in three nanoprotein particles has been assessed by determining their potential to inhibit the growth of selective pathogenic microorganisms (*Escherchia coli, Staphylococcus aureus and Fusarium oxysporium*). The results recorded are presented in Table 2 and the representative images are shown in Fig. 3. The anti-microbial activity of crocin against different strains varied significantly ($p \leq 0.05$) with a zone of inhibition ranging from 7.09 −19.87 mm. The highest zone of inhibition was found against *E.coli* by FPCN. This might be corelated to the DLS results which revealed the lowest particle size for FPCN. Theoretically, the modulation effects could be due to the large surface area of nanoparticles, morphology, surface charge and nanostructure-cell interaction [27]. The protein nanoparticles were found to release the encapsulated crocin quickly in agar mediums. The rapid release of bioactive is probably due to the digestion of the wall material i.e., protein nanoparticles by pathogenic strain [28]. The inhibition activity of crocin bioactive is attributed to the presence of alcoholic groups (–OH) in its structure. The alcoholic compounds are regarded as antiseptic agents [29], which results in changed nature of cell proteins and also increases the cell membrane permeability [30]. Reports indicate that nanoformulations prepared using zein

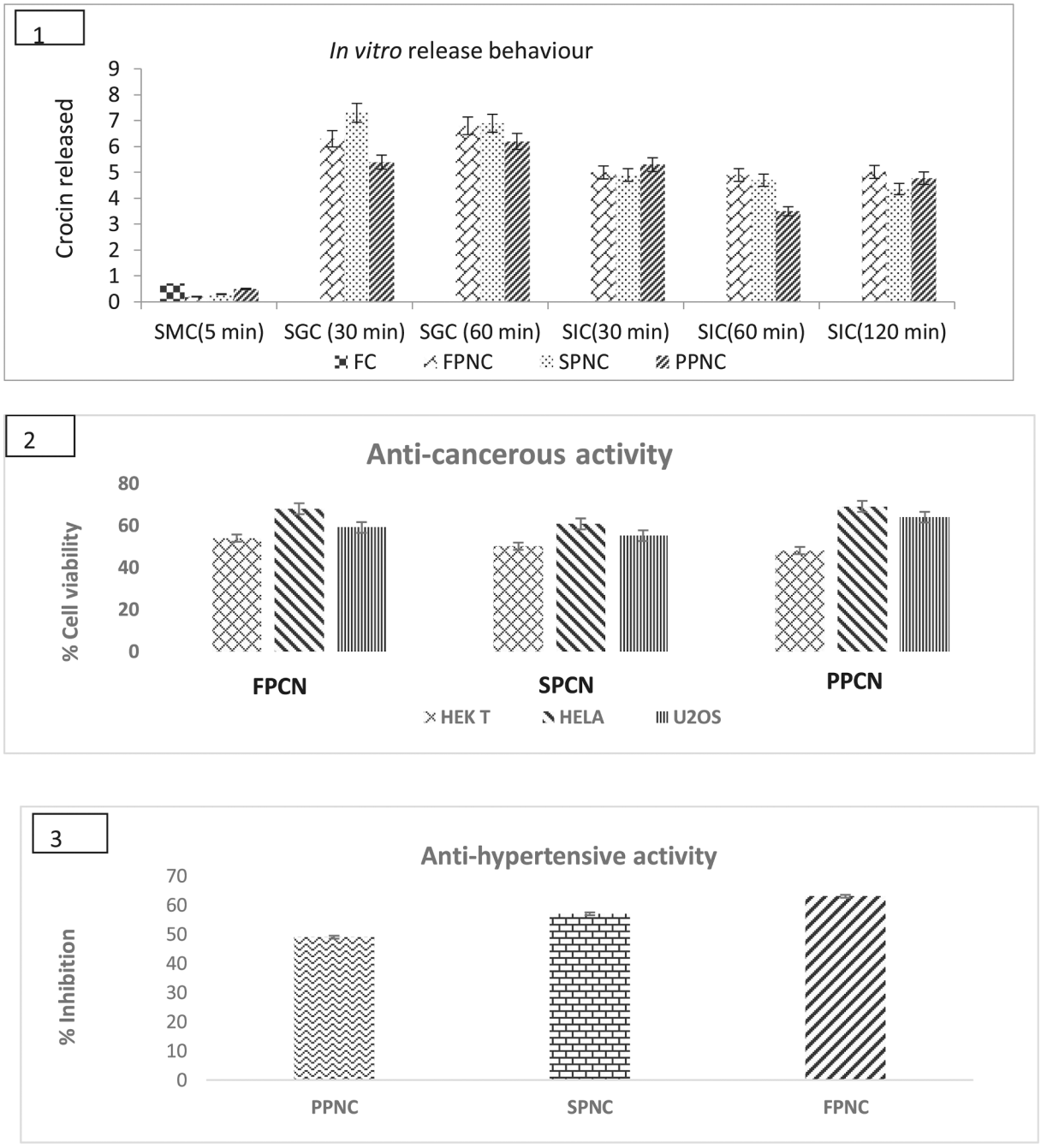

Fig. 4. **(1)** *In vitro* release profile of free crocin (FC), crocin encapsulated in protein nanocomplex of foxtail millet (FPCN), sorghum (SPCN) and pearl millet (PPCN) in simulated mouth conditions (SMC) for 5 min, simulated gastric conditions (SGC) for 30 and 60 min and simulated intestinal conditions (SIC) for 30, 60 and 120 min. **(2)** *In vitro* anti-cancerous activity of crocin encapsulated in protein nanocomplex of foxtail millet (FPCN), sorghum (SPCN) and pearl millet (PPCN). **(3)** *In vitro* anti-hypertensive activity of crocin encapsulated in protein nanocomplex of foxtail millet (FPCN), sorghum (SPCN) and pearl millet (PPCN).

nanoparticles for the encapsulation of thymol and carvacrol showed water dispersibility and enhanced antimicrobial activity against food borne pathogen [31].

3.6. *In vitro release behaviour*

Different food formulations when administrated orally pass through different pH ranges and various digestive enzymes. The nano formulatios should be able to pass through harsh gastric conditions effectively without any aggregation of the core wall material system and degradation of enclosed bioactives [26]. Therefore, in present study, stability of colloidal formulation was evaluated in simulated mouth, gastric and intestinal conditions by monitoring the crocin concentration over a period of 5 h until it gets absorbed at its target site. Therefore *in vitro* release behaviour of the crocin from nano protein matrix encapsulates was evaluated under the simulated gastrointestinal conditions and values as presented in Fig. 4(1). In simulated mouth conditions for 5 min, the release was found very less which might be the free crocin adhered to protein matrix [13]. During the transit through simulated gastric conditions, in first 30 min, the nanoformulation is mediated by the pepsin which creates pores and causes the cracks in the wall material thus facilitating the crocin release by diffusion process [32]. During next 60 min, the sustained release of crocin was observed. In simulated intestinal conditions, gradual release was observed due to the pancreatin

and higher pH present in intestinal fluid causing more relaxation and digestion of nano protein matrix due to their high surface area [33], thereby releasing the crocin at the intestinal site increasing its bio accessibility with minimal degradation of bioactive which remained constant for next 60 and 120 min. Rodríguez-Félix. et al,[33] also reported quercetin encapsulated in zein nanoparticles showed better encapsulation efficiency, enhanced gastrointestinal stability and bioaccessibility than free quercetin.

3.7. *In vitro* bioactivity of nano encapsulated crocin

3.7.1. *Anti cancerous activity*

The bioactivity retention of crocin in nanoencapsulated form in simulated gastric and intestinal conditions is summarised in Fig. 4(2, 3). The results indicate that during *in vitro* digestion in simulated gastric conditions (SGC), bioactive properties of crocin were retained in nano encapsulated forms. The anticancer properties of crocin encapsulated in nanoprotein was evaluated against three cell lines by MTT assay and presented in Fig. 4(2). The cell viability (%) of HELA, HEK T and U20S after incubation for 24 h with FPCN, SPCN and PPCN was found to be in the range of 60.78–69.12 %, 48.09–53.98 % and 55.17–63.98 %, respectively in simulated gastric conditions. Thus, crocin upon nanoencapsulation could exhibit marked inhibition of *in vitro* cancerous activity as depicted by the significant reduction ($p \leq 0.05$) in cell viability. The chemo preventive mechanism of carotenoids include carcinogen metabolism modulation, cell proliferation inhibition, cell cycle progression and growth regulation, immune system modulation, cell differentiation enhancement and apoptosis [34]. The bioactivity retainment of encapsulated crocin is due to the slow effect of crocin loaded nanoparticles on cancer cell line, its sustained release due to the different pathway in comparison with spiked crocin.

3.7.2. *Anti hypertensivity*

The *in vitro* anti-hypertensive activity of crocin encapsulated in protein nanocomplex was found to be 49.09, 57.87 and 63.21% for FPCN, SPCN and PPCN, respectively as presented in Fig. 4(3). Various studies have reported significant effect of crocin which is one of the main constituent of saffron on the hypertension problem. Different mechanisms that contribute to the anti-hypertensive properties of crocin include calcium channels blockage, interference with nitric oxide of endothelium, antioxidative properties and no release of sarcoplasmic reticulum Ca21 into cystol [35]. The results indicate that the ACE inhibiting activity of crocin was successfully retained by the encapsulating nanoprotein particles upto the target site. Imenshahidi et al. [36] also reported that crocin significantly ($p \leq 0.05$) reduced hypertension in anaesthetized rats. The significantly higher anti hypersensitive activity of FPCN can be corelated to its high encapsulation efficiency of crocin (Table 1) which implies that that high concentration of crocin effectively induced ACE inhibiting activity.

4. Conclusion

The world of food is reaching new heights with the development of functional foods and nutraceuticals. Bioactives despite of having numerous health benefits is limited in use due to their easy degradation and unavailability at their target absorption site. The incorporation of biaoctives like crocin by using nanoencapsulation technique provides a suitable option for increasing their stability and bio accessibility. This study concluded that protein nanoparticles showed good encapsulation efficiency for crocin using ultrasonication technique and acted as an effective carrier/ wall material for bioactive protecting them from degradation during their transit through gastric conditions retaining its bioactivity. The nanocomplex can be used to fortify foods for the development of effective functional foods in future.

Funding

Adil Gani is thankful to Indian Council of Medical Research 544 (ICMR), Government of India for the award of Senior 545 Research Fellowship in favor of Faiza Jhan (3/1/2/130/2019-Nut).

CRediT authorship contribution statement

Faiza Jhan: Experimentation, Writing – original draft, Review & editing. **Adil Gani:** Supervision, Resources, Conceptualization. **Nairah Noor:** Formal analysis. **Bashir Ahmad Malla:** Experimentation. **Bilal Ahmad Ashwar:** 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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