Carotenoids derived from mandarin processing wastes as bioengineered nutraceuticals

Mohit Bawa, and Minni Singh

***Abstract*—**Mandarin processing into juice generates a quantum of agrowaste in the form of peels. The bright orange colour of these peels is suggestive of presence of carotenoids, based on which this work was undertaken to extract these bioactives with an objective of developing nutraceuticals from these. For this, supercritical fluid extraction was done for total carotenoids. Mass spectral analysis revealed abundance of xanthophylls, chiefly β-cryptoxanthin in the extract. This provitamin A known for its antioxidative efficacy is limited due to its non-bioavailability attributed to its lipid like structure. Hence, to impart functionality nanotechnological intervention was done. Extract laden solid lipid nanoparticles (SLNs) were fabricated with a particle size of 481.1±14.4 nm and a PdI 0.39±0.07 suggesting monodisperse nature of the bioengineered nanoformulation, which exhibited a promising ζ-potential of -45.5mV. SEM and TEM of nanoparticles reveal spherical morphology and a distinctive core. This work lays the foundation for nanotechnology-driven nutraceutical development for applications in the food industry.

***Keywords*—**β-cryptoxanthin, carotenoids, nutraceuticals, solid l1ipid nanoparticles.

I. Introduction

Mandarin is one of the major citrus fruit crops grown worldwide. It is mainly consumed in the form of juice and thus leaves behind considerable amount of waste in the form of peels [1], which are rich in bioactives such as carotenoids, as suggested by the bright orange color of the peels [2]. These peels can be utilized to extract bioactives, thereby reducing agrowaste which can furthur lead to sustainability. Carotenoids are isoprenoid derivatives which constitute eight isoprene units (5 carbon atoms) joined in head to tail configuration. These are lipid like nutraceuticals which are further classified as carotenes and xanthophylls. Carotenes are purely hydrocarbons, while xanthophylls are oxygenated carotenes. Mandarins are reported to be rich in xanthophylls, mainly β-cryptoxanthin, zeaxanthin, lutein, antheraxanthin [3]-[5]. Carotenoids are provitamin A reservoirs and possess many health benefits such as protection against cancer, cardiovascular diseases, eye related diseases and age related macular degeneration [6]-[8]. The antioxidant potential of carotenoids has remarkable significance in preventing various chronic disorders by their ability to scavenge the reactive oxygen species [9]. Different methods are employed to extract these nutraceuticals such as conventional solvent extraction, enzyme-assisted extraction, ultrasound assisted extraction and supercritical fluid extraction [10]-[13]. Supercritical fluid extraction is a method in which extraction is done using a compound above its supercritical temperature and pressure. Carbon dioxide is the most common supercritical fluid used for the extraction. Above its supercritical conditions CO_2 behaves as a fluid and its extraction power is increased. To change the polarity of the system different concentration of modifiers can be added [14], [15]. Nanotechnology is a multidisciplinary field that has opened up new opportunities of research and development in a number of related sectors such as medicine, cosmetics, agriculture and food [16]. The major limitation of lipophilic bioactive carotenoid is their very low bioavalability [17] which can be enhanced by incorporating them within engineered nanoparticles such as lipid or polymeric nanoparticles, nanoemulsions and nanodispersions. Solid lipid nanoparticles consist of a solid lipid core which contains the dissolved bioactive in it. This lipid core is surrounded by a layer of surfactant which stabilizes it and makes the nanoparticles dispersible in the continuous medium thus enhancing their aqueous dispersibity and therefore lending them boiavailability [18].

In the present study, carotenoids were extracted from mandarin peels by conventional solvent extraction as well as by supercritical CO_2. The extracted carotenoids after quantification were identified by mass spectrum and HPLC. After that, solid lipid nanoparticles were prepared incorporating the carotenoid extract into them and the surfactant concentration and organic to aqueous phase ratio was optimized. Prepared SLNs were characterized by particle size, Polydispersity index (PdI), ζ-potential, SEM and TEM.

II. Materials and Methods

A. Plant material

Mandarin peels were obtained in bulk from Punjab Agro Juices Ltd. (Punjab, India), sun dried, powdered and refrigerated to avoid degradation of thermolabile bioactives.

B. Solvent extraction of carotenoids

To extract carotenoids efficiently, petroleum ether: acetone (1:1, v/v) was used [10]. For this, 30 ml of solvent was added to 2g of mandarin peel powder and incubated for 1 hour at 40°C under shaking conditions (150 rpm). The mixture was then filtered to obtain clear extract.

C. Supercritical fluid extraction of carotenoids

Supercritical fluid extraction was done using CO_2 at pressure

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330 bar and 40°C. Co-solvent concentration was varied as 2.5%, 5%, 10% and total carotenoids were measured.

D. Saponification

Saponification of the carotenoid extract was done to remove esters. It was done by adding 40% methanolic KOH to equal amount of extract and incubated for 16 h in dark. After incubation, carotenoids were partitioned into 10 ml petroleum ether and washed five times with water (20 ml each).

E. Quantification of carotenoids

Total carotenoids were quantified spectrophotometrically at 450 nm using the following expression:

$$Total\ carotenoids\ (mg/g) = \frac{A \times V \times 10}{\varepsilon_{1cm}^{1\%} \times w}$$

where A is Absorbance value of extract at 450 nm, V is volume of extract in ml, $\varepsilon_{1cm}{}^{1\%}$ is percentage extinction coefficient of carotenoids i.e 2500 [19] and w is weight of powder in grams.

F. Mass spectrum

For the identification of major carotenoids present in the extract, positive mode ESI mass spectrometry was done using Thermo LTQ-XL system.

G. High Pressure Liquid Chromatography

For further confirmation of the previously identified carotenoids in mass spectrum, HPLC (model 2650 Waters) was run using C-18 column (250 × 4.6 mm, 5µm). The mobile phase consisted of a mixture of methanol: acetonitrile (9:1) at a flow rate of 1.5 ml/min. The wavelength of detection was 450 nm, column temperature was 25°C, injection volume was 50 µl.

H. Preparation optimization of Solid Lipid Nanoparticles (SLNs)

Solid lipid nanoparticles were prepared using stearic acid as solid lipid carrier, tween 20 as surfactant and glycerol as viscosity enhancer. For the preparation of SLNs organic phase consisting of carotenoid extract in stearic acid was added dropwise to the aqueous phase which consisted of tween 20 in water:glycerol (1:1, v/v) in different ratios i.e. 1:1, 1:4 1:6. Also, concentration of surfactant was varied i.e 1.0 %, 2.5% and 5.0%. This mixture was stirred for 10 min and then ultrasonicated using 6mm probe for 15 min at 35% amplitude and 25on/5off pulse for the preparation of SLNs.

I. Characterization of SLNs

SLNs prepared using optimized parameters were characterized by particle size, ζ-potential, aqueous dispersibility, scanning and transmission electron microscopy and entrapment efficiency. Particle size and ζ-potential were measured using Horiba Nanoparticle analyzer SZ-100. SEM was done using Jeo JSM-6510LV series Scanning Electron Microscope and TEM using Hitachi 7500 Transmission electron microscope.

III. RESULTS

A. Quantification of carotenoids

Total carotenoids were quantified spectrophotometrically at 450 nm and found to be 0.008±0.0006 mg/ml using solvent mixture. Supercritical fluid extraction was done using CO_2 with varied polarity by increasing concentration of co-solvent (ethanol) and total carotenoids were found to be maximum with 10% ethanol as shown in table I. For further work, supercritical fluid extract obtained using 10% ethanol was selected owing to its high extracting ability 0.96±0.03 mg/ml.

TABLE I
TOTAL CAROTENOIDS IN DIFFERENT SUPERCRITICAL FLUID EXTRACTS (SFES)

SFE	Total carotenoids (mg/ml SFE)
2.5% ethanol	0.36±0.01
5.0% ethanol	0.64±0.02
10.0% ethanol	0.96±0.03

B. Mass spectra analysis

Mass spectra of the extract clearly shows the presence of carotenoids particularly xanthophylls, as suggested by the fragmentation pattern shown in Fig. 1. Table II shows the compounds expected to be present in the extract as suggested by mass spectrum.

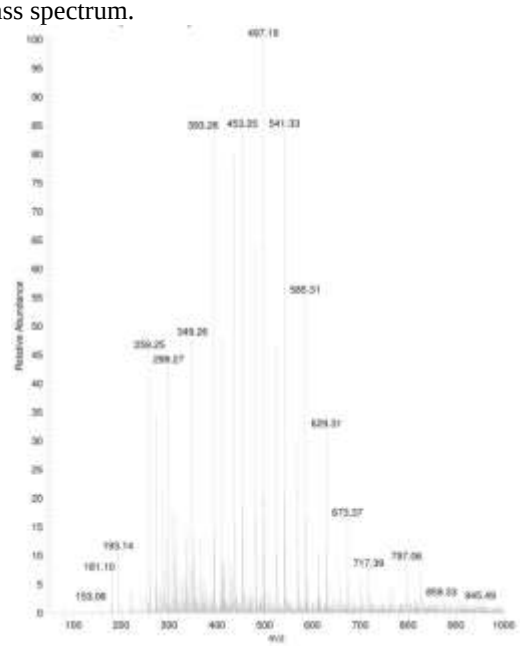

Fig. 1 Mass spectrum of extract

TABLE II
PEAK ASSIGNMENT OF MASS SPECTRUM

% Abundance	m/z ratio	Compound
100	497.19	β-Cryptoxanthin/ Zeaxanthin
85	453.25	β-Cryptoxanthin
85	541.33	ζ-Carotene
85	393.26	Neoxanthin
57	585.31	Antheraxanthin
50	349.26	Neoxanthin

C. HPLC

HPLC chromatogram of saponified extract shows major peak at retention time 14.039 min, which corresponds to β-cryptoxanthin as compared with the standard as shown in Fig. 2.

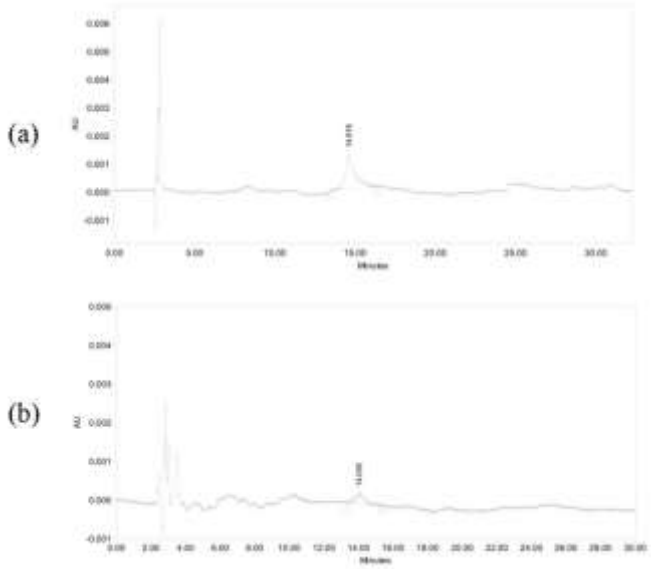

Fig. 2 HPLC chromatogram (a) β-cryptoxanthin and (b) extract.

D. Preparation optimization of SLNs

Different combinations of organic to aqueous phase was used and it was found that minimum particle size and a favorable zeta potential was obtained by the 1:4 ratio as seen from table III. Also, 2.5% surfactant was found to be optimum as shown in table IV.

TABLE III
EFFECT OF ORGANIC:AQUEOUS PHASE RATIO ON PARTICLE SIZE AND PDI

Organic:Aquoeus phase	Particle size (nm)	PdI
1:1	1487.7	0.691
1:4	494.1	0.395
1:6	550.0	0.353

TABLE IV
EFFECT OF SURFACTANT CONCENTRATION ON PARTICLE SIZE AND PDI

Surfactant concentration (%)	Particle size (nm)	PdI
1.0	700.9	0.505
2.5	487.4	0.375
5.0	535.0	0.375

E. Characterization of SLNs

SLNs prepared with the optimized parameters were found to have particle size of 481.1±14.4 nm and a PdI of 0.39±0.07 as shown in Fig. 3. Surface charge of the nanoparticles was found to be -45.5mV.

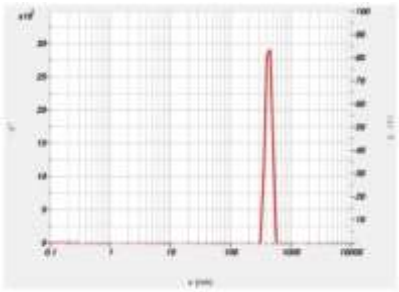

Fig. 3 Particle size of prepared SLNs

SLNs were then imaged under Scanning and transmission electron microscope as shown in Fig. 4.

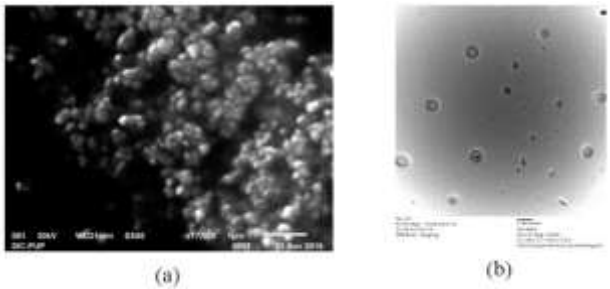

Fig. 4 (a) SEM and (b) TEM micrographs of SLNs

Stability studies of these nanoparticles is underway.

IV. DISCUSSION

Carotenoids possess great potential to be used as a nutraceutical for food fortification. But the use of organic solvents for food applications is unsuitable due to which the replacement of the solvent is important. Further, supercritical fluid extraction was found to be more effective to extract carotenoids than conventional solvent extraction because at supercritical conditions CO_2 behaves as a fluid and has high solvation power. The polarity of the supercritical fluid was increased by adding different concentrations of food grade ethanol. 10% ethanol was optimized to extract total carotenoids efficiently from the mandarin peels. Further, mass spectrum shows the prevalence of xanthophylls over carotenes. HPLC analysis of extract reveals the presence of mainly β-cryptoxanthin as confirmed from standard. SLNs were prepared with an objective to enhance the aqueous dispersiblity of the carotenoids, for which the parameters such as organic to aqueous phase ratio, and surfactant concentration were optimized. When organic: aqueous phase (1:1) ratio was used the size obtained was 1487.7 nm with PdI of 0.691. This can be attributed to insufficient amount of surfactant to coat the particles and the inability to overcome the threshold viscosity of the system imparting due to a high concentration of stearic acid. Thus, ratio 1:4 was used and size obtained was 494 nm with PdI value of 0.395. When 1:6 ratio was used particle size was 550 nm, this may be attributed to the decreased viscosity of the medium as stearic acid concentration was reduced and aqueous phase concentration dominates. Thus, 1:4 (w/w) ratio was used for preparation of SLNs as factors such as viscosity, ratio of surfactant and lipid was optimum. Surfactant tends to reduce the surface tension of a liquid in which it is dissolved. Surfactant contains both a water insoluble component and a water soluble component. Tween 20 is a non ionic surfactant which diffuses in water and adsorbs at the interface between oil and water. With surfactant concentration of 1.0 % particle size obtained was 700.9 nm with PdI value of 0.505, which is due to the lack of sufficient surfactant coating the nanoparticles. Then, concentration of surfactant was increased to 2.5 %, particle size found was 487.4nm with PdI value of 0375. At concentration 5.0 %, particle size increase to 535 nm with PdI value of 0.375, this increase in size is attributed to a thicker coating of the surfactant on the nanoparticle surface, and is assumed to be

over and above the needed concentration to form a stable nanoparticles. Therefore, 2.5% surfactant concentration was opted for further studies. SEM and TEM micrographs of the nanparticles show spherical morphology and distinct surfactant coating over nanoparticles. Stability studies of SLNs is under progress.

V. Conclusion

Carotenoids are sucesssfully extracted from the agrowaste i.e. mandarin peels and converted into a bioengineered product i.e. solid lipid nanoparticles. Supercritical fluid extraction was found to extract caortenoids more efficiently and also it is the solvent free method which can be scaled-up easily. Mass spectra and HPLC both suggested the presence of xanthophylls, particularly β-cryptoxanthin. Electron micrographs confirmed spherical morphology of the nanoparticles. These aqueous dispersible bioengineered nutraceuticals hold promise in the development of functional foods.

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