


 Cite this: *RSC Adv.*, 2021, 11, 38161

Surface-engineered liposomal particles of calcium ascorbate with fenugreek galactomannan enhanced the oral bioavailability of ascorbic acid: a randomized, double-blinded, 3-sequence, crossover study

 Ashil Joseph, Dinesh Kumar, Abhilash Balakrishnan, Prasanth Shanmughan, Balu Maliakel and Krishnakumar IM *

The antioxidant, anti-inflammatory, immunomodulating, anti-thrombotic, and antiviral effects along with its protective effects against respiratory infections have generated a great interest in vitamin C (vitC) as an attractive functional/nutraceutical ingredient for the management of COVID-19. However, the oral bioavailability and pharmacokinetics of vitC have been shown to be complex and exhibit dose-dependent non-linear kinetics. Though sustained-release forms and liquid liposomal formulations have been developed, only marginal enhancement was observed in bioavailability. Here we report a novel surface-engineered liposomal formulation of calcium ascorbate (CAAS), using fenugreek galactomannan hydrogel in powder form, and its pharmacokinetics following a randomized, double-blinded, single-dose, 3-way crossover study on healthy human volunteers ($n = 14$). The physicochemical characterization and *in vitro* release studies revealed the uniform impregnation of CAAS liposomes within the pockets created by the sterically hindered galactomannan network as multilaminar liposomal vesicles with good encapsulation efficiency (>90%) and their stability and sustained-release under gastrointestinal pH conditions. Further human studies demonstrated >7-fold enhancement in the oral bioavailability of ascorbate with a significant improvement in pharmacokinetic properties (C_{max} , T_{max} , $T_{1/2}$, and AUC), compared to the unformulated counterpart (UF-CAAS) when supplemented at an equivalent dose of 400 mg of CAAS as tablets and capsules.

 Received 27th August 2021
 Accepted 12th November 2021

DOI: 10.1039/d1ra06483e

rsc.li/rsc-advances

Introduction

Vitamins and minerals, collectively known as micronutrients, are essential elements for the survival of organisms owing to their vital role in the maintenance of the body's homeostasis. L-Ascorbic acid (AA), commonly known as vitamin C (vitC), is an important water-soluble antioxidant micronutrient involved in the regulation of a number of gene expressions and is an essential cofactor for various classes of enzymes involved in the biosynthesis of amino acids, neurotransmitters and hormones.^{1,2} Supplementation of vitC is very often recommended (up to 2000 mg per day) to eliminate its deficiency and hence for efficient cellular processes since a mutation which encodes L-gulonolactone oxidase does not allow its synthesis in humans.^{3,4} Large population studies have reported that vitC deficiency is common even in developed countries.⁵ A significant association has also been demonstrated between vitC

deficiency, balanced immune functions and morbidity/mortality.^{1,5,6} Low levels of plasma vitC ($\sim 20 \mu\text{M}$) has also been shown to correlate with the severity of illness among pneumonia, sepsis and COVID-19 patients.⁷

Vitamin C has been shown to degrade quickly depending on the environmental factors such as pH, temperature, light and oxygen.⁸ Under *in vivo* conditions, it is mainly found in the reduced or anionic ascorbate (ASC) form which may reversibly be oxidized to dehydroascorbate (DHAA) and further to inactive 2,3-diketogulonic acid.^{2,9} In humans, body concentration of vitC depends on its intake, intestinal absorption, tissue distribution, renal re-absorption and excretion.^{10,11} The pharmacokinetics of vitC has been shown to be complex due to a saturable active transport mechanism mediated by sodium-dependent vitC transporters (SVCT) and exhibited dose-dependent non-linear kinetics.¹¹⁻¹³ Though regular uptake of 200 to 400 mg dose has been shown to achieve a steady state plasma concentration of 50 to 70 μM , either multiple gram dosage or infusion is suggested to overcome its tight intestinal absorption control and hence to exceed the homeostatic saturation level.^{11,14} Though 100% absorption was reported for 200 mg single dose, the absorption

R&D Centre, Akay Natural Ingredients, Ambunad, Malaidamthuruth P. O., Cochin, 683561, India. E-mail: krishnakumar.im@akay-group.com; Fax: +91 484 2680891; Tel: +91 484 2686111



was reported to reduce to 33% when the dose was increased to 1250 mg.¹⁵ Therefore, there exist tremendous interest in food-grade delivery forms that can enhance the bioavailability and hence the pharmacokinetic properties of vitC.

Considering the high water solubility and transporter proteins-mediated absorption, multiple and smaller doses per day was hypothesised to be the best mode of dosage for vitC.¹¹ But, no significant differences in pharmacokinetic variables has been observed between the plain and slow release formulations even after 4 weeks of supplementation.¹⁶ Another major approach was based on liposomes. But, supplementation of doses ranging from 4 to 36 g of liposomal vitC was found to offer only marginal enhancement in the oral bioavailability, probably due to its limitations.^{17–20} Liposomal forms are susceptible to chemical changes (hydrolysis of phospholipid ester bonds and oxidation of unsaturated acyl chains) and exhibit poor stability under stomach and storage conditions.²¹ The liquid state of liposomes, usage of various chemical stabilizers during its preparation, relatively low drug loading ability and hence the high dosage are the common issues associated with liposomes in addition to opsonisation (strong interaction with the blood proteins leading to rapid elimination from systemic circulation).²²

We hypothesised that surface modification of liposomal CAAS using suitable methods and novel food-grade biopolymers would enhance the *in vivo* stability and hence oral bioavailability of vitC avoiding the use of synthetic excipients and chemicals. Surface modification of liposomes with synthetic hydrophilic polymers such as polyethylene glycol, polyvinyl alcohol, and chemically modified chitosan have already been reported.^{23,24} Here we report the development of a stable powder formulation of liposomal calcium ascorbate (CAAS) employing a novel surface modification of liposomes with fenugreek (*Trigonella foenum-gracum*) galactomannans (FG) for the first time. The process involved the gel-phase dispersion of a liposomal CAAS into FG-hydrogel network to provide a “capping effect” whereby to stabilize the liposomes from the harsh environmental conditions. Previously, we had reported that FG can form mucoadhesive and amphiphilic gels containing lipophilic molecules as water dispersible powders capable of re-swelling in the gastrointestinal tract to release self-emulsified colloidal particles for better absorption.^{25–27}

Materials and methods

General

All solvents used for analysis were of high performance liquid chromatography (HPLC) grade and those used for the extraction were reagent grade, procured from Merck life sciences Private Limited, Bengaluru, India. MilliQ plus (Millipore India Private Limited, Bengaluru, India) purified water was used for the experiments. The analytical standards of CAAS (CAS no: 5743-28-2 Sigma-Aldrich, Bangalore, India) and ascorbic acid (CAS no: 50-81-7) were procured from Sigma Aldrich, Bangalore, India. Food-grade calcium ascorbate 98% suitable for human supplementation (DSM Nutritional Products Limited, Heanor, UK) and the surface-modified liposomal particles of CAAS with fenugreek galactomannan (FC+) was received from Akay Natural Ingredients, Cochin, India along

with a detailed certificate of analysis indicating food safety parameters. Ascorbic acid content was estimated using a HPLC (Shimadzu SPD20AT) coupled with SPD-M20A prominence photodiode array detector (PDA) (Shimadzu India Private Limited, Mumbai, India) and a Phenomenex reverse phase C18 column (150 × 4.6 mm; 3 μm, 100 Å) following a previously reported validated procedure by Robitaille and Hoffer.²⁸

Preparation and characterisation

FC+ prepared by a gel-phase dispersion process of lipidated CAAS into FG-hydrogel matrix to form a hybrid-hydrogel (hybrid-FENUMAT™). Briefly, about 4.2 g of CAAS was dissolved in water, mixed with an ethanolic solution of lecithin (2.1 g), and homogenized. The homogeneous solution was then mixed uniformly with fenugreek galactomannan solution and the resulting mass was evaporated to dry powder form.

The physicochemical properties including particle size, zeta potential, Fourier-transform infrared spectroscopy (FTIR) and morphology of the formulation FC+ was carried out using high resolution transmission electron microscope (HR-TEM) (JEOL JEM-2100 LaB6, Jeol Co Limited, Japan), field emission scanning electron microscope (FE-SEM) (ZEISS Sigma 500 VP, ZEISS microscopy, Oberkochen, Germany), dynamic light scattering (DLS) (Horiba SZ-100 particle size analyser, Horiba India Private Limited, Bengaluru, India) and FTIR spectra from PerkinElmer Spectrum 400 spectrometer.

Encapsulation efficiency. To evaluate the encapsulation efficiency (EE) of CAAS in liposomes, 100 mg of liposomal CAAS was dissolved in 100 mL water. The solution was then passed through a 0.2 μm filter and analysed by HPLC method. The column temperature was kept at 25 °C and 20 μL sample was injected and chromatogram was monitored at 245 nm.²⁹ The EE of the liposomes was calculated using the following equation:

$$\text{Encapsulation efficiency (EE\%)} = \frac{W_t}{W_i} \times 100\%$$

where W_t is the total amount of drug (CAAS) in the liposomes suspension and W_i is the total quantity of drug added initially during preparation.

The concentration of drug loaded was determined using a HPLC method. The following equation was used to analyse the efficiency of drug loading

$$\text{Drug load (\%)} = \frac{(\text{total drug} - \text{free drug})}{\text{total drug}} \times 100$$

***In vitro* release kinetics.** *In vitro* release of ascorbic acid from FC+ granular powder was determined in water containing 1.6 mmol of disodium salt of EDTA (ethylenediamine tetra acetic acid) at pH 2.0 and 6.8 using 0.1 M HCl and phosphate buffer respectively. A known amount of FC+ (500 mg) was weighed into the dissolution flask and 500 mL of EDTA solution was poured into it. The pH of the solution was adjusted and agitated at 100 rpm. About 1 mL of the solution was carefully withdrawn at regular intervals of 0, 1, 2, 4, 6 and 8 h and made up to 100 mL with water. Ascorbate content was then analysed by HPLC method.²⁸ Analysis was performed in triplicate and



mean cumulative percentage of released ascorbate was calculated and plotted against time to study the release kinetics of CAAS from the fenugreek galactomannan matrix.

Stability study

Accelerated stability study was performed as per the guidelines of International Conference on Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use.³⁰ Briefly, about 10 g sample packets of FC+ were packed in polyethylene kit and kept in air-tight high density polypropylene (HDPE) bottles and incubated at $40 \pm 2^\circ\text{C}$ and $70 \pm 5\%$ relative humidity for a period of six months in a stability chamber (Remi, Mumbai, India). Samples were retrieved at 0, 1, 2, 3 and 6 months' intervals and subjected to analysis for various parameters including colour and appearance, ascorbic acid content, moisture content, and microbial parameters (total plate counts, yeast and mould, *Coliforms*, *Escherichia coli* and *Salmonella*).

Bioavailability studies

Relative bioavailability of the formulation FC+ with respect to the unformulated CAAS powder with >98% purity (UF-CAAS) was investigated in healthy volunteers.

Study design and participants. The study was designed as a randomized, double-blinded, single dose study on healthy volunteers. The protocol was approved by a registered institutional ethical committee (Registration No: ECR/321/INDT/TN2020, Central Drugs Standard Control Organization, Government of India) and was performed in accordance with the guidelines for Good Clinical Practices for Clinical Research in India established by the Government of India (CTRI/2021/01/030450), following the Helsinki declaration. Participants were detailed with the objective and procedure of the study and received a signed informed consent to participate in the study.

Healthy volunteers (males and females) ($n = 14$) aged 18 to 65 years having BMI between $18\text{--}25\text{ kg m}^{-2}$ were enrolled for the study. Those who were having a history of gut disorders such as irritable bowel syndrome, ulcerative colitis, gastro-oesophageal reflux

disease, duodenal or gastric ulcers, gastritis, kidney stone or those who are using proton pump inhibitors were excluded from the study. Smokers, chronic alcoholics, and those who are under any dietary supplements or medication were also excluded along with others having any symptoms of viral infection. In addition, participants with any condition that in opinion of the investigator does not justify the participation in the study were also excluded.

Selected participants were randomized into one of the three treatment arms consisting of three single dose treatments, with either formulated (tablets and capsules) or unformulated (UF-CAAS) tablets. All the doses were provided after an overnight fasting of $10 \pm 1\text{ h}$ as depicted in Fig. 1. The participants were refrained from consuming diets rich in/or supplemented with any form of vitC for five days prior to the study date. A washout period of $10 \pm 2\text{ days}$ was provided between supplementation.

Intervention and dosage. Tablets and hard-shell two-piece hypromellose capsules of both FC+ and UF-CAAS were prepared in such a way that each tablet weigh about $1000 \pm 25\text{ mg}$ and each capsule contains $500 \pm 25\text{ mg}$ with similar content of CAAS. Microcrystalline cellulose (MCC) and maltodextrin were used as binder and filler for both tablets and capsules. To investigate the bioavailability, volunteers were orally administered with single dose of 1000 mg tablet or capsule as per the study protocol, along with $200 \pm 10\text{ mL}$ of normal drinking water.

Blood sampling. About 2 mL venous blood was collected in K_2EDTA tubes, mixed and kept on ice bath for an hour. Plasma was separated in a cooling centrifuge at $2740g$ for about 10 minutes at 4°C . About 0.2 to 0.4 mL of supernatant was taken and immediately added with equal volume of 10% (w/v) metaphosphoric acid (MPA) in 2 mmol L^{-1} disodium EDTA, kept on ice for 5 min, and again centrifuged at $16\text{ 000}g$ for 10 minutes at 4°C . The protein free acid supernatant was immediately frozen and stored at -80°C for analysis.³¹

Quantification of vitamin C in plasma. Quantification of vitC in plasma was carried out by a validated HPLC method following an earlier report by Robitaille and Hoffer.²⁸ Briefly, a solution of 1.8 mmol L^{-1} sulphuric acid at pH 2.7 was used as the mobile phase and a flow rate of 0.8 mL min^{-1} was

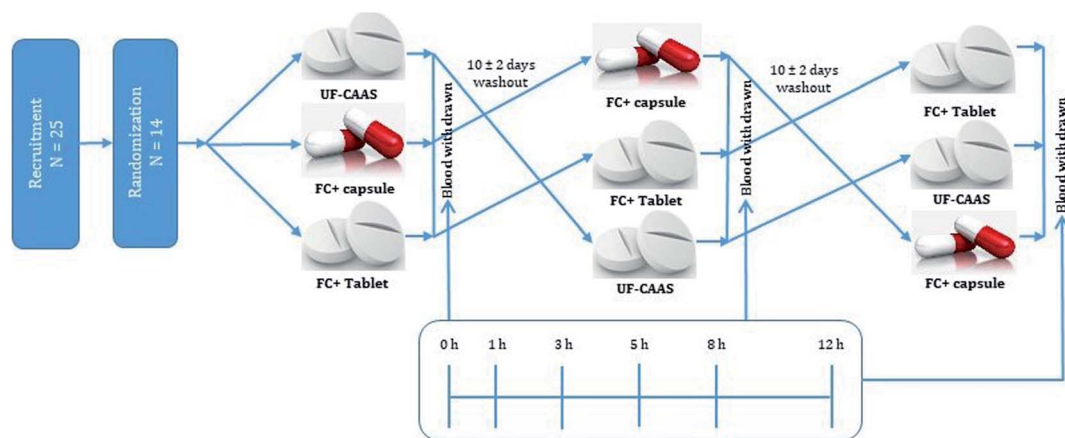


Fig. 1 Schematic representation of the protocol adopted for the randomized, double-blinded, 3-way crossover, 3-sequence comparative pharmacokinetic study. UF-CAAS, unformulated plane release CAAS tablets; FC+, surface-modified liposomal particles of CAAS using fenugreek galactomannan.

Table 1 Properties of formulated and unformulated tablets and capsules used in the present study

Properties	Tablets (FC+)	Capsules (FC+)	Tablets-unformulated (UF-CAAS)
Weight (mg)	1000 ± 25	500 ± 25	1000 ± 25
Calcium ascorbate content (mg)	397.2 ± 10	202.1 ± 5	394.5 ± 10
Friability (%)	0.92 ± 0.2	—	1.95 ± 0.4
Thickness (cm)	0.62 ± 2	—	0.65 ± 2
Hardness (kg cm ⁻²)	5.7 ± 0.3	—	4.0 ± 0.5

maintained. The column temperature was kept at 25 °C, 20 μL sample was injected, and chromatogram was monitored at 245 nm. A standard curve was obtained using peak area linear regression equation from six ascorbic acid standard solutions prepared in MPA/EDTA, ranging from 50–1000 μmol L⁻¹.

Injection solution of plasma was prepared from thawed samples by dividing them into two parts, each containing 30 μL, in separate Eppendorf tubes and mixing with an equal volume of 5 mmol L⁻¹ tris(2-carboxy ethyl) phosphine hydrochloride (TCEP) at pH 2 in water. The mixture was allowed to react with the sample for about 20 min at room temperature under dark conditions. An equal volume of water was then added and the samples were kept on ice bath and centrifuged for 5 min at 4 °C at 16 000g. The supernatant was stored in cold conditions (4 ± 1 °C) and transferred to auto injection vials for analysis.

Statistical analysis

Statistical analysis was performed using SPSS version 27. All data were expressed as mean ± SD and all analyses were performed using analysis of variance (ANOVA). The differences observed were evaluated using a *t*-test and *P* < 0.05 were considered as statistically significant. The pharmacokinetic parameters were deduced from plasma concentration *verses* time plot using GraphPad Prism 5.0.

Results

Food-grade CAAS with not less than 98% purity was used as the vitC supplement in the present study. The formulation (FC+) was a free flowing granular powder with a particle size of 20 to 100 mesh size with a calcium ascorbate content of 40.3% and tapped

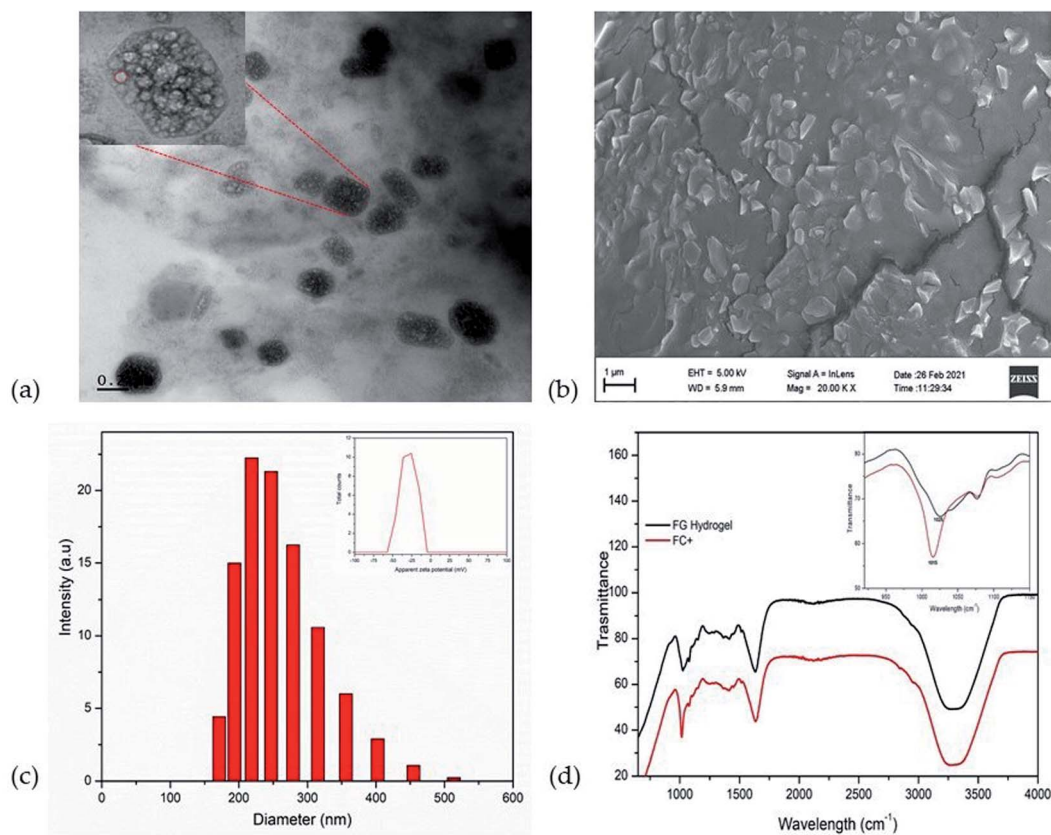


Fig. 2 Characterisation of FC+. (a) HR-TEM image of FC+ at 200 nm. The multilamellar vesicle structure is shown in inset, (b) FE-SEM image of multilamellar vesicles engulfed in the galactomannan hydrogel matrix, (c) the hydrodynamic size distribution of FC+ by DLS. Zeta potential (−29.3 mV) of the particles in solution is shown in inset, and (d) FTIR spectra of FG and FC+.



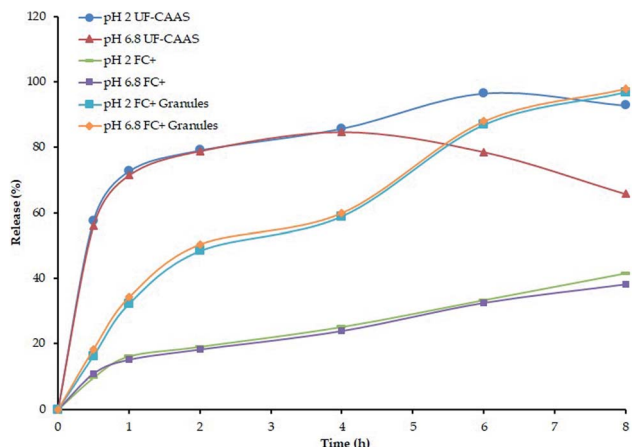


Fig. 3 *In vitro* release of calcium ascorbate from FC+ tablet and granular powder form used for capsules and tablets used in the present study.

density 0.8 g mL^{-1} . Technical specification sheet, certificate of analysis and material safety data sheet of the investigational products showed that they are produced under good manufacturing practices (GMP) and possess all necessary documentations and safety data required for human consumption. The properties of both the formulated and unformulated tablets and capsules used in the study are shown in Table 1.

Characterisation

Electron microscopic studies (HR-TEM and FE-SEM) showed that the formulation of CAAS as FC+ is an encapsulated form of liposomal CAAS in the hydrogel matrix of fenugreek galactomannans to form a typical hybrid-hydrogel. The encapsulation was evident from HR-TEM image as non-spherical liposomes with a surrounding membrane structure held in the hydrophobic pockets created by the galactomannan chains in the gel phase (Fig. 2a). The FE-SEM image also showed the uniform impregnation of non-spherical particles of about $\sim 200 \text{ nm}$ diameter, identified as liposomes, in the gel matrix of fibre network (Fig. 2b). The mean particle size of encapsulated liposomal particles was observed as $212.9 \pm 5.5 \text{ nm}$ with a zeta potential of -29.3 mV (Fig. 2c), while the mean particle size of

uncoated liposomes was $89 \pm 3.9 \text{ nm}$ with a zeta potential of -3.2 mV . CAAS was encapsulated into the liposomes with a high encapsulation efficiency of $95.3 \pm 0.55\%$ as determined by HPLC and the drug loading efficiency was found to be $4.65 \pm 0.18\%$.

The FTIR spectra of FC+ in comparison with the FG matrix when recorded at in the frequency range 4000 to 800 cm^{-1} revealed the nature of entrapment of liposomes in the FG hydrogel matrix (Fig. 2d). The C–O stretching vibrations of alcohol group at 1025 cm^{-1} , the C–O–C vibration at 2920 cm^{-1} and the O–H stretching vibration at 3296 cm^{-1} were identified as the characteristic frequencies of the carbohydrate moieties in the biopolymer galactomannan.³² In FC+, the peak corresponding to the C–O stretching vibration was found to be shifted from 1025 cm^{-1} to 1015 cm^{-1} due to the strong molecular interaction of liposomes with the FG hydrogel network.³³

In vitro release studies of FC+ granular powder and the tablet indicated sustained release of ascorbate under both stomach and intestinal pH conditions (Fig. 3). While the granular powder form of FC+ released almost 85% of ascorbate in 4 h, the compression into tablets further delayed the release in such a way that only 25% release was observed in 4 h, at pH 2 and 6.8 respectively.

Accelerated stability studies revealed no significant changes in the physicochemical properties including colour, appearance, bulk density, moisture content, microbial load and ascorbic acid content. All tested parameters prevailed within $\pm 2\%$ of the initial value, indicating sufficient stability of FC+ for a storage of 2 years when kept in air-tight closed containers under ambient conditions of less than $30 \text{ }^\circ\text{C}$ in dark, without moisture or direct sunlight (Table 2).

Bioavailability study

A total of 25 healthy volunteers were screened; out of which 14 (10 males and 4 females) eligible participants were randomized and completed the study as per the double-blinded crossover design depicted in Fig. 1. The demographics and baseline characteristics of the participants is given in Table 3. The validated HPLC-PDA method of Robitaille and Hoffer was adopted for the quantification of ascorbate content in blood plasma.²⁸ The method showed linearity over wide range (50 to $1000 \text{ } \mu\text{mol L}^{-1}$) of vitC concentration with an r^2 value of 0.99981 (Fig. 4). Matrix-matched calibration was performed in

Table 2 Accelerated stability data of FC+ powder incubated at $40 \pm 2 \text{ }^\circ\text{C}$ and relative humidity of $70 \pm 5\%$ ^a

Parameter	Specification	Initial	1 st month	2 nd month	3 rd month	6 th month
Appearance	Free flowing granular powder	Complies	Complies	Complies	Complies	Complies
Colour	Off white	Complies	Complies	Complies	Complies	Complies
Calcium ascorbate content	NLT* 40%	40.8%	39.9%	39.6%	40.1%	38.4%
Microbiology – USFDA (FDA)						
Total plate count [#]	$<10000 \text{ cfu g}^{-1}$	100 cfu g^{-1}	130 cfu g^{-1}	120 cfu g^{-1}	130 cfu g^{-1}	100 cfu g^{-1}
Yeast & mould [#]	$<200 \text{ cfu g}^{-1}$	$<10 \text{ cfu g}^{-1}$	$<10 \text{ cfu g}^{-1}$	$<10 \text{ cfu g}^{-1}$	$<10 \text{ cfu g}^{-1}$	$<10 \text{ cfu g}^{-1}$
Coliforms [#]	$<3 \text{ MPN g}^{-1}$	$<3 \text{ MPN g}^{-1}$	$<3 \text{ MPN g}^{-1}$	$<3 \text{ MPN g}^{-1}$	$<3 \text{ MPN g}^{-1}$	$<3 \text{ MPN g}^{-1}$
<i>E. coli</i> [#]	Absent per g	Absent per g	Absent per g	Absent per g	Absent per g	Absent per g
<i>Salmonella</i> [#]	Absent per 25 g	Absent per 25 g	Absent per 25 g	Absent per 25 g	Absent per 25 g	Absent per 25 g

^a *NLT denotes 'not less than'; [#] each value was presented as an average of three measurements.



Table 3 Demography and blood routine values of the participants enrolled in the present study^a

Parameter	Male	Female
No. of volunteers	10	4
Age (years)	31.67 ± 3.73	32.33 ± 4.15
Weight (kg)	65.37 ± 9.65	57.3 ± 3.37
BMI	23.18 ± 2.66	24.43 ± 1.20
Hematology		
Hemoglobin (g dL ⁻¹)	16.28 ± 0.88	14.03 ± 1.26
Total leukocyte count (cells per cumm)	7677.78 ± 1208.07	6966.67 ± 585.95
Total RBC count (million per cumm)	5.49 ± 0.62	6.15 ± 0.50
Platelet (lakhs per cumm)	2.04 ± 0.47	2.00 ± 0.63
Lymphocytes (%)	42.89 ± 5.40	38.33 ± 3.51
Eosinophil's (%)	2.56 ± 0.73	3.0 ± 1.00
Neutrophils (%)	53.2 ± 4.38	48.0 ± 2.65
Monocytes (%)	1.78 ± 0.67	1.33 ± 1.15
Biochemical		
ALT (U L ⁻¹)	38.3 ± 15.76	31.3 ± 4.16
AST (U L ⁻¹)	25.3 ± 6.32	16.6 ± 6.43
ALP (U L ⁻¹)	59.0 ± 17.2	82.3 ± 11.9
Total protein (g dL ⁻¹)	7.56 ± 0.26	7.43 ± 0.60
Bilirubin (mg dL ⁻¹)	0.47 ± 0.18	0.58 ± 0.14
Albumin (g dL ⁻¹)	4.60 ± 0.43	4.56 ± 0.26
Globulin (g dL ⁻¹)	2.74 ± 0.31	2.60 ± 0.62
A/G ratio	1.6 : 1	1.6 : 1
Cholesterol (mg dL ⁻¹)	178.1 ± 21.89	178.3 ± 12.1
Triglycerides (mg dL ⁻¹)	114.1 ± 31.56	83.3 ± 15.7
HDL (mg dL ⁻¹)	57.6 ± 7.48	52.6 ± 4.04
LDL (mg dL ⁻¹)	94.6 ± 7.38	16.6 ± 3.14
VLDL (mg dL ⁻¹)	22.8 ± 6.31	30.5 ± 2.50
Creatinine (mg dL ⁻¹)	0.79 ± 0.12	0.78 ± 0.21

^a HDL – high-density lipoprotein, LDL – low-density lipoprotein, VLDL – very low density lipoprotein, ALT – alanine aminotransferase, AST – aspartate aminotransferase, ALP – alkaline phosphatase, RBC – red blood cell, BMI – body mass index.

the present study. Under the specified conditions, ascorbate peak was found at a retention time (Rt) of 3.2 min with a relative standard deviation within the accepted levels of ± 5%. MPA/FEDTA sample preparation method used in the present study showed a recovery of 87.8% in the extraction of vitC from plasma.

Administration of 1000 mg single dose of FC+ in both capsule and tablet form resulted in significantly ($P < 0.05$) higher concentration of plasma ascorbate concentration as

compared to the equivalent dose of UF-CAAS over 1 to 12 h of post-administration time period (Fig. 5). Each 1000 mg of FC+ was found to contain 400 ± 10 mg of UF-CAAS. The pharmacokinetic properties of the formulations are provided in Table 4. It was observed that the area under curve over 12 h of post-administration time period ($AUC_{0-12\text{ h}}$) for both tablets and capsules of FC+ were 2232 and 2119 respectively, which was about 7.2 and 6.8 times higher than the $AUC_{0-12\text{ h}}$ of the

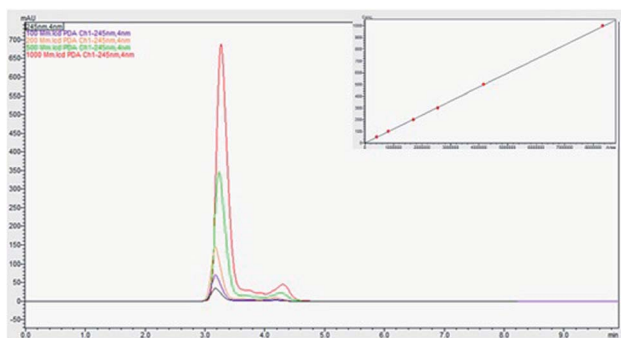


Fig. 4 Panel shows HPLC chromatogram for a series of calibration samples and plasma samples. Calibration curve for ascorbate is shown in inset.

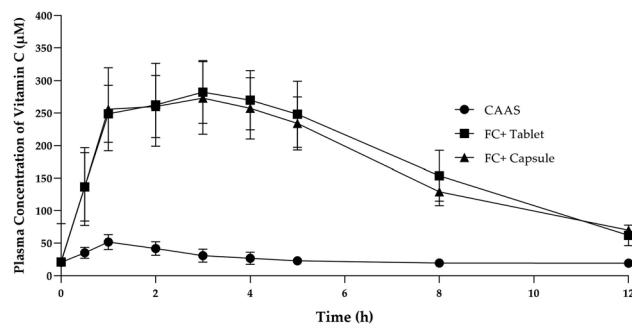


Fig. 5 Pharmacokinetic study: plasma ascorbate concentration versus time plot for unformulated plane release CAAS tablets (UF-CAAS), FC+ (capsule) and FC+ (tablet) followed by single oral dose of 1000 mg.



Table 4 Pharmacokinetic parameters of unformulated plane release CAAS tablets (UF-CAAS), FC+ (capsule) and FC+ (tablet)

Sample	Dose (mg)	C_{\max} (μM)	T_{\max} (h)	$T_{1/2}$ (h)	AUC ($\mu\text{M h mL}^{-1}$)	MRT (h)	F_{rel}
UF-CAAS	1000 \pm 25	51.7 \pm 21	1	3.6	311.4 \pm 63.87	22.67 \pm 2.02	—
FC+ (tablets)	1000 \pm 25	282.4 \pm 43.86	3	8.5	2232 \pm 447.50	22.21 \pm 1.7	716.76 \pm 291.01
FC+ (capsules)	500 \pm 25 \times 2	273 \pm 59.88	3	7.6	2119 \pm 465.19	22.33 \pm 2.2	680.47 \pm 288.95

corresponding forms of UF-CAAS, indicating \sim 7-fold enhancement in the bioavailability of ascorbate from FC+. The maximum observed concentration of ascorbate in plasma (C_{\max}) was 282.4 and 273 μM for the FC+ tablets and capsules respectively, as compared to 51.7 μM for UF-CAAS tablet. In the case of FC+, maximum plasma concentration of about 230 μM was found to be achieved in 1 h time post-dose and remained in almost 240 \pm 20 μM level for more than 5 h ($P > 0.05$) in both capsules and tablets. Therefore, the time at which the maximum plasma concentration was observed (T_{\max}) for FC+ can be considered as 1 to 5 h, precisely about 3 h, *verses* 1 h for T_{\max} UF-CAAS. The elimination half-life ($T_{1/2}$) for FC+ tablet was found to be 8.5 h *verses* 7.6 h for capsules which were significantly higher as compared to UF-CAAS tablets (3.6 h) (Fig. 5 & Table 4). The relative bioavailability (F_{rel}) for the tablet was found to be 716.76 \pm 291.01 and that for capsule was 680.47 \pm 288.95.

Administration of FC+ did not cause any adverse effects, and none of the participants reported any discomfort or tolerability issues. The haematological and biochemical assessment showed no significant ($P > 0.05$) differences in the baseline values (Table 3).

Discussion

The present study was aimed at the characterisation and determination of bioavailability and pharmacokinetic properties of a novel, food-grade, and green formulation of CAAS as surface-engineered liposomal microparticles using the galactomannan from fenugreek seeds for the first time. The formulation (FC+) was achieved by a water-based gel-phase dispersion process of lipidated CAAS in FG matrix to form a hybrid-hydrogel where the water-soluble CAAS is first encapsulated in phospholipids and further engulfed within the pockets created by the conformationally restricted highly sterically hindered three-dimensional galactomannan network as shown in Fig. 2 and 6. Electron microscopic images (FE-SEM and HR-TEM) could also reveal the entrapment of liposomal CAAS with a surface coating by galactomannans in an obloid form. The images were in agreement with the earlier reports on the surface modified liposomes.^{34,35} Particle size analysis and zeta potential measurements could also confirm the surface coating and improved stability of FC+, as evident from the increased mean particle size (212.9 \pm 5.5 nm *verses* 89 \pm 3.9 nm) and zeta potential (−29.3 mV *verses* −3.2 mV) of the formulation as compared to the uncoated liposomes. The non-spherical

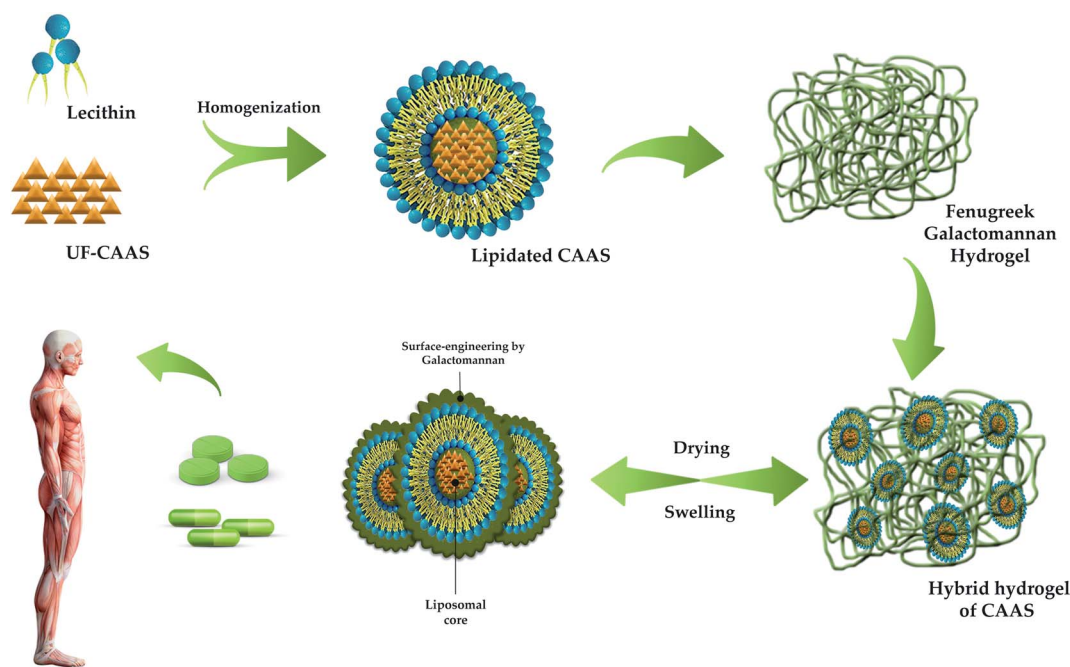


Fig. 6 Schematic representation of FC+ formulation using hybrid-FENUMAT technology employed for the surface modification of liposomal CAAS.



Table 5 Pharmacokinetic properties of various formulations of vitC reported in the literature^a

Formulation	Dose	Dose pattern (oral)	Design	Number of subjects (<i>n</i>)	Analysis method	Pharmacokinetic properties				Number of folds of enhancement in bioavailability	Reference
						<i>C</i> _{max} (μM)	<i>T</i> _{max} (h)	<i>T</i> _{1/2} (h)	AUC (μg h mL ⁻¹)		
Slow-release ascorbic acid	250 mg × 2/day 4 weeks	Repeated	SB, RCT, PC, SS	48 (F19, F19, P10) [#]	HPLC-UV*	91.6	2.4	34.2	1010	1.33	16
Ester-C® (calcium ascorbate)	500 mg × 2	Single	DB, PC, CT, SS, RCT	30 (F15, P15) [#]	HPLC-UV*	43.89	4.0	—	483	1.16	38
PureWay-C®	1000 mg	Single	RCT, DB, PT	10	HPLC-ECD**	123.21	2	—	—	—	39
Nutra-C® (calcium ascorbate)	500 mg × 1	Single	SD, OP, RCT, PG	20 (F10, P10) [#]	HPLC-UV*	102.49	3.3	10.5	822	1.09	40
Liposomal sodium ascorbate	4 g	Single	PC, RCT	11	HPLC-ECD**	187	3	—	584	1.35	19
Liposomal sodium ascorbate	10 g	Single	—	20	HPLC-UV*	303	3	6	1359	1.79	18
Liposomal ascorbic acid	1000 mg/5 mL	Single	OP, RCT, PC, SD, TT, TS, TP, TWC	24 (P12, F12) [#]	HPLC-UV*	296.9	3.5	12.3	3171	1.77	20
Liposomal sodium ascorbate	5 g	Single	SB	2	HPLC-PDA***	230	3.5	—	—	—	17
Liposomal ascorbic acid -powder	150 mg	Single	DB, RCT, CT	8 (F4, P4) [#]	HPLC-UV*	379.8	3.06	3.27	3135	5.9 [§]	41
Hybrid-FENUMAT (FC+) (tablets)	1000 mg	Single	DB, RCT, CT	14	HPLC-UV*	282.4	3	8.5	2232	7.17	Present study
Hybrid-FENUMAT (FC+) (capsules)	500 × 2 mg	Single	DB, RCT, CT	14	HPLC-UV*	273	3	7.6	2119	6.81	Present study

^a Abbreviations: DB – double-blind, PC – placebo-controlled, OP – open label, SB – single-blinded, CT – crossover trial, PG – parallel group, RCT – randomized clinical trial, TC – trail controlled, SS – single site, PT – prospective trail, SD – single dose, TT – two-treatment, TS – two-sequences, TP – two-period, TWC – two-way crossover. *HPLC-UV – denote HPLC estimation using ultraviolet spectrophotometric detector; **HPLC-ECD – denotes HPLC estimation using electrochemical detector; ***HPLC-PDA – denotes HPLC estimation using photodiode array detector; [#]P denotes unformulated ascorbic acid and F represents formulation; [§]the pharmacokinetics study reported as 2-fold higher *C*_{max} value than the administrated dose.

shape shows the multi-lamellar liposomal structure, probably due to its non-covalent interactions of the vesicles with the galactomannans, as evident from FTIR data. Non-spherical multi-lamellar liposomes are of great interest to drug delivery, owing to their controlled and sustained drug release profile, resulting in enhanced bioavailability and efficacy.³⁶ The non-spherical liposomes have longer physiological lifetime when compared to their spherical counterparts. Further, the drug release profile of non-spherical liposomes has been seen to be different from conventional unilamellar spherical liposomes due to the inhomogeneous thickness and larger surface area of the non-spherical particles giving rise to a larger drug flux per unit volume.³⁵

The density, particle size and flow ability of FC+ powder was found to be suitable for a wide range of pharma and food delivery forms. It was found to swell extensively under gastrointestinal tract conditions and further to release stable and stabilized liposomal forms of CAAS indicating its behaviour as a reversible hydrogel for drug delivery (Fig. 6). Accelerated stability data demonstrated a shelf life of minimum 2 years if kept under ambient conditions, away from sunlight and moisture. Direct compressibility of FC+ powder and its high drug loading ability (around 40% w/w) further implies the versatility of the present surface modified liposomal hydrogel

technology for the delivery of water-soluble and labile bioactive molecules for dietary applications.

Calcium ascorbate is a widely recommended vitC supplement due to its compatibility under gastrointestinal conditions, compared to the ascorbic acid which may result in elevated gastric reflux leading to gastrointestinal disorders.³⁷ Intestinal absorption of ascorbate has been found to be tightly controlled and follow nonlinear pharmacokinetics, with a steady state plasma concentration level attained through a membrane transport mechanism unlike the diffusion-mediated absorption and distribution process of the lipophilic small molecule drugs.¹¹ Table 5 describes the various approaches that have been reported for the oral delivery of vitC to overcome the tight control observed on intestinal absorption and hence to provide pharmacologically relevant plasma concentrations with improved pharmacokinetic properties. Though extended-release forms were initially hypothesised to be better for ascorbate delivery due to its slow elimination by delaying the gastric emptying, no significant enhancement in the bioavailability or plasma concentration of slow release formulations was observed by Viscovich *et al.*¹⁶ A randomised controlled trial on Ester-C® (a formulation of CAAS as a mixture of dehydroascorbate, calcium threonate, and 4-hydroxy-5-methyl-3(2H)-furanone) at a dose of 1000 mg vitC reported no significant change in AUC_{0–24 h} of plasma concentration *versus* time plot;



but showed a significant leukocyte retention of ascorbate.³⁸ In another comparative randomised controlled trial using 1000 mg dose of ascorbic acid, CAAS, Ester-C® and Pureway-C® (a proprietary formulation of vitC-lipid metabolites), both Ester-C® and Pureway-C® showed almost similar enhancement in bioavailability with maximum plasma concentration (C_{\max}) as: Pureway-C® ($2.17 \pm 0.19 \text{ mg dL}^{-1}$) > Ester-C® ($1.69 \pm 0.27 \text{ mg dL}^{-1}$) > ascorbic acid ($1.64 \pm 0.18 \text{ mg dL}^{-1}$) > CAAS ($1.12 \pm 0.17 \text{ mg dL}^{-1}$).³⁹ Yet another proprietary form of CAAS as Nutra-C® showed 1.28-fold enhancement in bioavailability and 1.34 times enhancement in C_{\max} ($102.5 \mu\text{M}$) followed by a 500 mg single dose when compared with the corresponding dose of UF-CAAS.⁴⁰ Recent approaches in vitC delivery were based on liposomal technology. Davis *et al.*, has reported about 1.3-fold enhancement in bioavailability when 4.25 g of sodium ascorbate was delivered as liposomes.¹⁹ In another study, supplementation of 10 g of liposomal sodium ascorbate was shown to enhance ascorbate bioavailability by 1.79 times with a C_{\max} of only $300 \mu\text{M}$ as compared to $180 \mu\text{M}$ for the equivalent dose of unformulated one.¹⁸ Another liposomal formulation of ascorbic acid showed 1.77-fold enhancement in bioavailability when supplemented at a dose of 1000 mg vitC/5 mL.²⁰ Supplementation of very high doses of liposomal vitamin C, as high as 25 and 36 g, was also attempted and observed C_{\max} in the range of 350–400 μM only.¹⁶ The most recent report on liposomal formulation was a powder form with 6-fold enhancement in bioavailability from a single dose of 150 mg of ascorbic acid.⁴⁰ But, the pharmacokinetic study of this paper faces a serious issue that the reported C_{\max} of 6.69 mg dL^{-1} is 2-fold higher than the administered amount of ascorbic acid, warranting further studies on its pharmacokinetic properties.⁴¹

It is observed from Table 5 that the pharmacokinetic parameters for FC+ in the present study is significantly higher than the human bioavailability reports on any kind of vitamin C formulation reported so far. FC+ formulation is based on the hypothesis that the protection of vitamin C from its degradation in the gastrointestinal tract and its conversion to mucoadhesive colloidal particles can enhance its absorption and hence the plasma concentration at low dosage. Fenugreek galactomannan-based hybrid-hydrogel technology (hybrid-FENUMAT) provided a novel 100% natural and food-grade powder form for the sustained-release of self-emulsified multi-laminar liposomal particles of CAAS ($200 \pm 20 \text{ nm}$) encapsulated in mucoadhesive galactomannan networks as evident from the *in vitro* release, particle size and electron microscopy studies. Enhanced bioavailability (>7-fold) of FC+ with improved pharmacokinetic properties (C_{\max} , T_{\max} , $T_{1/2}$, AUC) and a plasma concentration of around >230 μM for 5 h following the oral administration of a single dose of 1000 mg FC+ containing about 400 mg of CAAS points towards the efficiency of FC+ to deliver stable CAAS particles in the intestine overcoming the acidic stomach conditions. *In vitro* studies have shown >280 $\mu\text{M L}^{-1}$ as an optimised dosage for antimicrobial and antiproliferation effects of vitC.¹⁷ So, the reported enhancement in ascorbate bioavailability from FC+ is highly significant, since a dosage as high as 2000 mg of CAAS \times 3 per day has shown to provide only 250 μM plasma concentration.⁴²

Liposomal formulation of CAAS was achieved with significant encapsulation efficiency of $95.3 \pm 0.55\%$.²⁹ *In vitro* release studies showed that the powder form of FC+ allowed the sustained release of CAAS by controlling the diffusion process through the galactomannan network, as described by Nguyen *et al.*, for surface modified liposomes.²³ The tablets possess relatively more delayed release kinetics as compared to the granular powder form of FC+ used in capsules. However, FC+ provided similar bioavailability and pharmacokinetic properties following the administration of both capsules and tablets. Therefore, it is the *in vivo* stability, particle size and nature of liposomes released from the fibre matrix, which play critical role in ascorbate pharmacokinetics from FC+ than the extended release profile. It is generally known that the tablets and capsules form can be bioequivalent and provide similar bioavailability.⁴³

Hydrogels are three-dimensional cross-linked polymeric porous structures that absorb and retain huge amount of water.⁴⁴ The nature of hydrophilic polymer chains and the extent of crosslinking determine the porosity and stability of a hydrogel.⁴⁵ Swelling in water and shrinkage upon dehydration is a critical property of hydrogel for its drug loading and sustained-release properties through diffusion or environmental stimuli such as pH.^{44,45} The synthetic hydrogels are widely suggested for the delivery of lipophilic bioactive molecules.⁴⁶ The novelty of the present study lies in the fact that this accounts the first report on the enhanced bioavailability of water-soluble salts like CAAS using non-chemically altered biopolymer hydrogel derived from a food component source such as fenugreek. It has already been shown that under conditions of optimised swelling, the degree of crosslinking resulting from the interpenetration of sterically hindered galactomannan chains can be tuned to have hydrophobic pockets suitable for the loading of lipophilic molecules and further to convert them as water soluble colloidal particles with enhanced absorption.²⁵ Yet another very important feature is the functionality of fenugreek galactomannan as a clean label (non-genetically modified, allergen-free, vegetarian, non-toxic) soluble dietary fiber with biocompatibility and prebiotic potential.⁴⁷ Hypoglycaemic, hypolipidemic, hypotriglyceridaemic and gastroprotective effects of fenugreek dietary fibre has already been reported, in addition to its safety and tolerance.⁴⁸

Conclusion

The present study demonstrated the development of a natural hybrid-hydrogel system for the self-emulsified oral delivery of a water-soluble molecule like CAAS with enhanced bioavailability and pharmacokinetic properties at relatively low dosage of about 400 mg per day of CAAS to provide physiologically significant levels of vitC in the systemic circulation for longer duration as compared to the unformulated counterpart. The methodology used fenugreek galactomannan as a biopolymer matrix for the uniform impregnation of lipidated CAAS and thereby to protect the liposomal forms from its inherent problems of poor *in vivo* stability and rapid elimination for the first



time. Electron microscopy and particle size analysis revealed the surface modification with galactomannan to form the multilaminar vesicles of CAAS engulfed into the galactomannan network. The sustained release of stable nano forms (~200 nm) was achieved by controlled diffusion process through galactomannan hydrogel network for better absorption. The formulation showed >7-fold enhancement in the bioavailability of vitC, when supplemented as both tablets, and capsules, possessed good stability under accelerated testing conditions. Natural status, biocompatibility, safety, functional benefits, easy availability and water-based process makes hybrid-FENUMAT as a green and food-grade technology for the oral delivery of water-soluble micronutrients.

Author contributions

B. M. & K. I. M. – conceptualisation, writing – review & editing, resources, supervision; A. J. – methodology, formal analysis; P. S. – formal analysis, characterisation; D. K. – methodology; A. B. – methodology, software, data curation. All authors have read and agreed to the published version of the manuscript.

Data availability

The dataset analysed in the present study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors disclose the conflict of interest. Hybrid-FENUMAT is a patented technology of Akay Natural Ingredients, Cochin, India.

Acknowledgements

Authors thank Akay Natural Ingredients, Cochin, India, for providing the study samples. Authors are grateful to Dr P. Prathibha, for her support in the preparation of the manuscript.

References

- 1 A. C. Carr and S. Maggini, *Nutrients*, 2017, **9**, 1211, <https://pubmed.ncbi.nlm.nih.gov/29099763/>.
- 2 S. Englard and S. Seifter, *Annu. Rev. Nutr.*, 1986, 365–406, <https://pubmed.ncbi.nlm.nih.gov/3015170/>.
- 3 A. C. Carr and M. C. M. Vissers, *Nutr*, 2013, **5**, 4284–4304, <https://pubmed.ncbi.nlm.nih.gov/24169506/>.
- 4 C. S. Tsao, An overview of ascorbic acid chemistry and biochemistry, in, *Vitamin C in health and disease*, ed. Packer L. and Fuchs J., Marcel Dekker Inc., New York, 1997, pp. 25–58.
- 5 M. Lindblad, P. Tveden-Nyborg and J. Lykkesfeldt, *Nutr*, 2013, **5**, 2860–2879, <https://pubmed.ncbi.nlm.nih.gov/23892714/>.
- 6 S. Rowe and A. C. Carr, *Nutr*, 2020, **12**, 1–20, [/pmc/articles/PMC7400810/](https://pubmed.ncbi.nlm.nih.gov/PMC7400810/).
- 7 P. Holford, A. C. Carr, T. H. Jovic, S. R. Ali, I. S. Whitaker, P. E. Marik, *et al.*, *Nutr*, 2020, **12**(12), 1–17, <https://pubmed.ncbi.nlm.nih.gov/33297491/>.
- 8 A. L. Herbig and C. M. G. C. Renard, *Food Chem.*, 2017, **220**, 444–451, <https://pubmed.ncbi.nlm.nih.gov/27855924/>.
- 9 S. Padayatty and M. Levine, *Oral Dis.*, 2016, **22**(6), 463–493, DOI: 10.1111/odi.12446.
- 10 A. J. Michels, T. M. Hagen and B. Frei, *Annu. Rev. Nutr.*, 2013, **33**, 45–70, <https://pubmed.ncbi.nlm.nih.gov/23642198/>.
- 11 J. Lykkesfeldt and P. Tveden-Nyborg, *Nutrients*, 2019, **11**, 2412, <https://pubmed.ncbi.nlm.nih.gov/31601028/>.
- 12 I. Savini, A. Rossi, C. Pierro, L. Avigliano and M. V. Catani, *Amino Acids*, 2008, **34**, 347–355, <https://pubmed.ncbi.nlm.nih.gov/17541511/>.
- 13 S. Hasselholt, P. Tveden-Nyborg and J. Lykkesfeldt, *Br. J. Nutr.*, 2015, **113**(10), 1539–1549, <https://pubmed.ncbi.nlm.nih.gov/25865869/>.
- 14 M. Levine, S. J. Padayatty and M. G. Espey, *Adv. Nutr.*, 2011, **2**, 78–88, <https://pubmed.ncbi.nlm.nih.gov/22332036/>.
- 15 M. Levine, C. Conry-Cantilena, Y. Wang, R. W. Welch, P. W. Washko, K. R. Dhariwal, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1996, **93**(8), 3704–3709, <https://pubmed.ncbi.nlm.nih.gov/8623000/>.
- 16 M. Viscovich, J. Lykkesfeldt and H. E. Poulsen, *Clin. Nutr.*, 2004, **23**(5), 1043–1050, <https://pubmed.ncbi.nlm.nih.gov/15380894/>.
- 17 S. Hickey, H. J. Roberts and N. J. Miller, *J. Nutr. Environ. Med.*, 2008, **17**(3), 169–177, DOI: 10.1080/13590840802305423.
- 18 M. Łukawski, P. Dalek, T. Borowik, A. Foryś, M. Langner, W. Witkiewicz, *et al.*, *J. Liposome Res.*, 2020, **30**(3), 227–234, <https://pubmed.ncbi.nlm.nih.gov/31264495/>.
- 19 J. L. Davis, H. L. Paris, J. W. Beals, S. E. Binns, G. R. Giordano, R. L. Scalzo, *et al.*, *Nutr. Metab. Insights*, 2016, **9**, NMI.S39764, <https://pubmed.ncbi.nlm.nih.gov/27375360/>.
- 20 S. Gopi and P. Balakrishnan, *J. Liposome Res.*, 2020, 356–364, <https://pubmed.ncbi.nlm.nih.gov/32901526/>.
- 21 A. Gabizon, H. Shmeeda and Y. Barenholz, *Clin. Pharmacokinet.*, 2003, **42**, 419–436, <https://pubmed.ncbi.nlm.nih.gov/12739982/>.
- 22 G. Bozzuto and A. Molinari, *Int. J. Nanomed.*, 2015, **10**, 975–999, <https://pubmed.ncbi.nlm.nih.gov/25678787/>.
- 23 T. X. Nguyen, L. Huang, M. Gauthier, G. Yang and Q. Wang, *Nanomedicine*, 2016, **11**(9), 1169–1185, <https://pubmed.ncbi.nlm.nih.gov/27074098/>.
- 24 H. He, Y. Lu, J. Qi, Q. Zhu, Z. Chen and W. Wu, *Acta Pharm. Sin. B*, 2019, **9**(1), 36–48.
- 25 M. B. Abhilash, D. Kumar, A. Deepti, A. Nair, V. Greet, V. An-Katrien, *et al.*, *J. Funct. Foods*, 2021, **79**, 104405.
- 26 L. Vijayasteltar, I. J. Jismy, A. Joseph, B. Maliakel, R. Kuttan and I. M. Krishnakumar, *Toxicol. Rep.*, 2017, **4**, 382–390, <https://pubmed.ncbi.nlm.nih.gov/28959663/>.
- 27 I. M. Krishnakumar, A. Ravi, D. Kumar, R. Kuttan and B. Maliakel, *J. Funct. Foods*, 2012, **4**(1), 348–357.
- 28 L. Robitaille and L. J. Hoffer, *Nutr. J.*, 2016, **15**(1), 40, <https://pubmed.ncbi.nlm.nih.gov/27102999/>.



- 29 N. Joshi, A. Kaviratna and R. Banerjee, *Integr. Biol.*, 2013, **5**(1), 239–248, <https://pubmed.ncbi.nlm.nih.gov/22911380/>.
- 30 ICH. ICH Topic Q 1 A (R2) Stability Testing of new Drug Substances and Products Step 5 Note For Guidance On Stability Testing: Stability Testing Of New Drug Substances And Products 2003, <http://www.emea.eu.int>.
- 31 A. Karlsen, R. Blomhoff and T. E. Gundersen, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2005, **824**(1–2), 132–138, <https://pubmed.ncbi.nlm.nih.gov/16046288/>.
- 32 Y. L. López-Franco, C. I. Cervantes-Montañó, K. G. Martínez-Robinson, J. Lizardi-Mendoza and L. E. Robles-Ozuna, *Food Hydrocolloids*, 2013, **30**(2), 656–660.
- 33 F. Rashid, S. Hussain and Z. Ahmed, *Carbohydr. Polym.*, 2018, **180**, 88–95, <https://pubmed.ncbi.nlm.nih.gov/29103525/>.
- 34 U. Baxa, Imaging of liposomes by transmission electron microscopy, in *Methods in Molecular Biology*, Humana Press Inc., 2018, 73–88, <https://pubmed.ncbi.nlm.nih.gov/29039095/>.
- 35 J. Chen, N. E. Clay, N. hyung Park and H. Kong, *Chem. Eng. Sci.*, 2015, **125**, 20–24, <https://pubmed.ncbi.nlm.nih.gov/25838583/>.
- 36 B. S. Pattni, V. V. Chupin and V. P. Torchilin, *Drug Delivery*, 2015, **115**, 10938–10966, <https://pubmed.ncbi.nlm.nih.gov/26010257/>.
- 37 J. K. Lee, S. H. Jung, S. E. Lee, J. H. Han, E. Jo, H. S. Park, *et al.*, *Korean J. Physiol. Pharmacol.*, 2018, **22**(1), 35–42, <https://pubmed.ncbi.nlm.nih.gov/29302210/>.
- 38 S. H. Mitmesser, Q. Ye, M. Evans and M. Combs, *Springerplus*, 2016, **5**(1), 1161, <https://pubmed.ncbi.nlm.nih.gov/27512620/>.
- 39 D. Pancorbo, C. Vazquez and M. A. Fletcher, *Med. Sci. Monit.*, 2008, **14**(11), CR547–CR551.
- 40 K.-M. Choi, K. M. Hoon, H. T. Won, J.-D. Kim, K. duck Park, M.-Y. Kim, *et al.*, *Anal. Sci. Technol.*, 2016, **29**(4), 162–169.
- 41 J. Jacob, N. P. Sukumaran and S. Jude, *ACS Omega*, 2021, **6**, 5560–5568.
- 42 T. K. Nielsen, M. Højgaard, J. T. Andersen, H. E. Poulsen, J. Lykkesfeldt and K. J. Mikines, *Basic Clin. Pharmacol. Toxicol.*, 2015, **116**(4), 343–348, <https://pubmed.ncbi.nlm.nih.gov/25220574/>.
- 43 S. Kim, J. W. Ko and J. R. Kim, *Int. J. Clin. Pharmacol. Ther.*, 2018, **56**(9), 443–450, <https://pubmed.ncbi.nlm.nih.gov/30021691/>.
- 44 R. Narayanaswamy and V. P. Torchilin, *Molecules*, 2019, **24**, <https://pubmed.ncbi.nlm.nih.gov/30744011/>.
- 45 A. Onaciu, R. A. Munteanu, A. I. Moldovan, C. S. Moldovan and I. Berindan-Neagoe, *Pharmaceutics*, 2019, **11**(9), <https://pubmed.ncbi.nlm.nih.gov/31450869/>.
- 46 S. Grijalvo, J. Mayr, R. Eritja and D. D. Díaz, *Biomater. Sci.*, 2016, **4**(4), 555–574, <https://pubmed.ncbi.nlm.nih.gov/26818789/>.
- 47 M. Majeed, S. Majeed, K. Nagabhusanam, S. Arumugam, S. Natarajan, K. Beede, *et al.*, *Food Sci. Nutr.*, 2018, **6**(3), 666–673, <https://pubmed.ncbi.nlm.nih.gov/29876118/>.
- 48 K. Srinivasan, *Food Rev. Int.*, 2006, **22**(2), 203–224, DOI: 10.1080/87559120600586315.

