

Green Nanotechnology and Phytochemical Mediated Production of Ketone Encapsulated Protein Nanoparticles—in vitro and in vivo Bioavailability Investigations

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Aim: Low carbohydrate, ketogenic foods have shown convincing evidence for their metabolic role in mitigating severe adversities due to obesity and other chronic diseases. They induce systemic ketosis: a process where ketone bodies, namely β -hydroxybutyrate, acetoacetate and acetone are produced in vivo. Beyond serving as an alternative source of energy besides glucose, various analogs of ketones present unprecedented opportunities for therapeutic interventions in the management of numerous chronic diseases and neurological disorders. The profound benefits of ketone bodies to human health, unquestionably, demand exogenous administration of ketone molecules in doses that promote and enhance energy levels in the human body. Hence, it is of paramount importance to develop sophisticated delivery vehicles wherein ketones are made bioavailable in a sustainable fashion in vivo. Engineering nano-formulations of ketone molecules allow efficient cellular penetration of ketones, thus presenting prospects for enhanced bioavailability of energy molecules in vivo. In this article, we report nanoencapsulation of (R)-3-hydroxybutyrate monoglyceride, a Ketone Molecule (KM) within biocompatible pea protein nano-framework utilizing natural phytochemical crosslinking.

Purpose: The goal was to develop a sophisticated delivery vehicle wherein ketones are made bioavailable in a sustainable and biocompatible fashion.

Methods: We present full details on the production of well-defined Ketone Molecule (KM) encapsulated nanoparticles of pea protein using naturally available crosslinking agents such as mangiferin, epigallocatechin 3-O-gallate (EGCG) and quercetin from their respective plant extracts. The Ketone Molecule (KM) encapsulated Pea Protein Nanoparticles by phytochemical crosslinking was fully characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) size and zeta potential (ZP) measurements. The KM concentration was estimated using gas chromatography–mass spectrometry (GC-MS). Phytochemical and water-soluble pea protein interaction was comprehensively studied using nuclear magnetic resonance (NMR) spectroscopy.

Results: Green nanotechnology offers the most effective means to encapsulate and transform small molecules into pea protein nanoparticles with optimum size for effective cell-specific delivery, thus offering an attractive delivery vehicle to enhance bioavailability. The Ketone Molecule (KM) encapsulated Pea Protein Nanoparticles, by phytochemical crosslinking importantly, demonstrated the most favorable in vivo pharmacokinetics with sustained (R)-3- hydroxybutyrate (BHB) levels and higher area under the curve (AUC) relative to free KM.

Conclusion: Novel pathways toward the design and development of protein nanoparticle-encapsulated ketone molecules were explored utilizing plant-based proteins from a biocompatibility, biodegradability, and biosafety perspective.

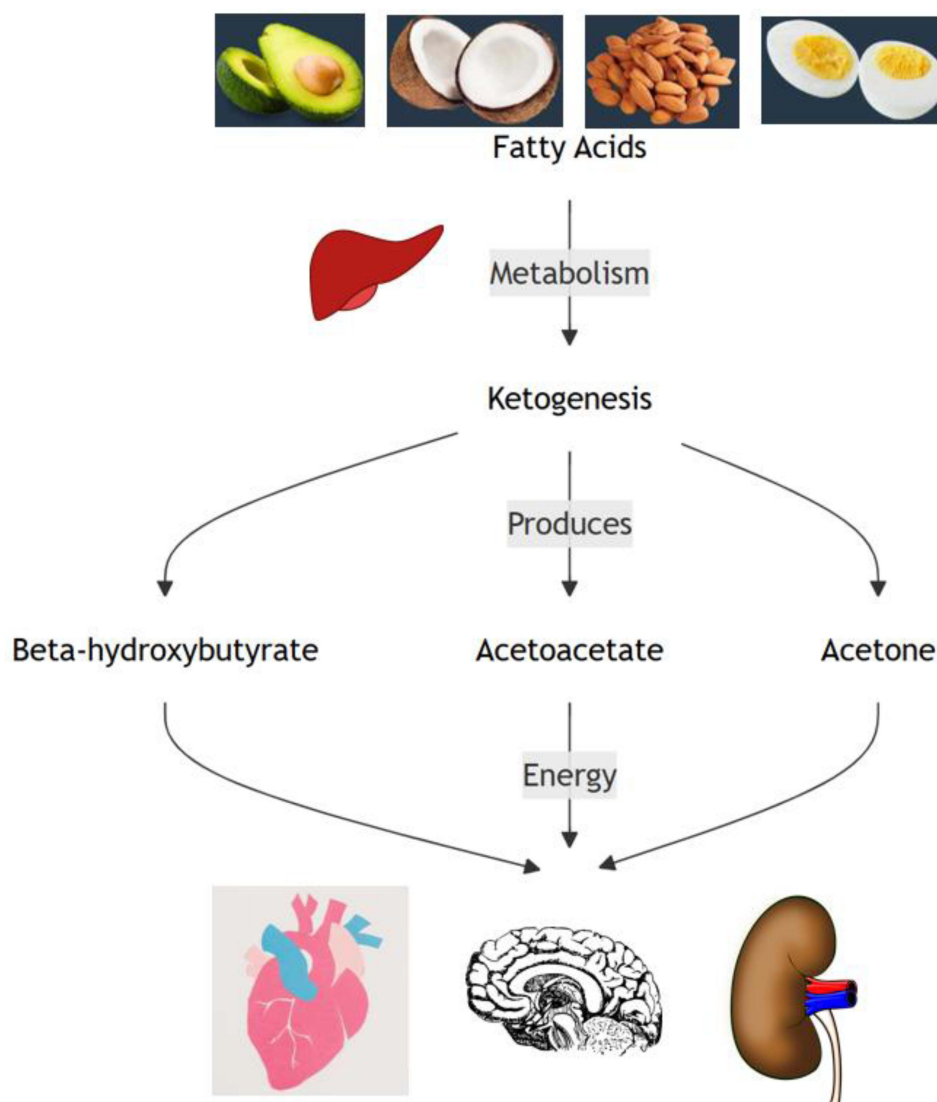
Keywords: green nanotechnology, nanoparticle delivery, ketone molecule, nutraceutical applications

Introduction

There is burgeoning interest in the development of healthy foods, from the available food supplies, or diets, that translate efficiently into sustainable good health with nutrient retention and enhancement of energy.^{1–6} Improving access to carbohydrates, fats, proteins, vitamins, and allied energy-rich food components, with carefully balanced key nutrients, offers the potential for transforming lives of global population by maintaining nutritional energy as the focus.^{7,8} It is well-known that cellular transformation of energy in the human body is mediated through upstream oxidative processes (which include glycolysis, beta-oxidation, and citric acid cycle) to mitochondrial oxidative phosphorylation.⁹ Cellular energy cycle primarily involves oxidation of energy-rich molecules such as carbohydrates, lipids, and proteins. Such oxidation processes continuously provide free energy for storage in phosphoanhydride bonds through intermediacy of a myriad number of macromolecules including adenosine diphosphate (ADP), adenosine triphosphate (ATP), and a host of other biomolecules including phosphoenolpyruvate, carbamoyl phosphate, 2,3-bisphosphoglycerate, phosphagens, phosphoarginine phosphatase, and phosphocreatine.¹⁰ Although glucose molecules are reliable sources of energy to the body, fatty diets continue to attract significant attention because of their superiority as energy reservoirs.¹¹ Indeed, fats translate into more energy which can be stored per weight and volume, without need for additional water for maintaining solubility and conformation. For example, glycogen, a glucose polysaccharide occurring in most mammalian and nonmammalian cells, comprises a glucose to water ratio of 1:2 (weight/weight) and therefore provides about seven times less calories per weight than fat.^{12,13} Therefore, keto diet, which promotes metabolic ketosis, provides a reliable and alternative source of energy especially when the body does not have enough glucose to provide energy.

Rollin Woodyatt's landmark discovery that when liver is starved of carbohydrates, it releases a myriad of ketone molecules (3-hydroxybutyrate/beta-hydroxybutyrate, acetoacetate, and acetone as shown in [Scheme 1](#))—opened up unprecedented opportunities in the development of ketogenic diet for human consumption.¹⁴ The proven importance of ketone molecules (often called as ketone bodies) as a source of energy to human body has spawned rapid development of ketogenic foods or diets comprising food supplements for regular use that are low in carbohydrates, with measured protein content, and with high amount of fat.^{1,2} This combination of food is expected to induce systemic ketosis—a process where ketone bodies are produced in vivo, serving as an alternate energy source, for neurons and various cellular pathways.^{15,16} While a plethora of ketone-rich dietary supplements is available through various outlets, there is burgeoning interest toward the development of ketone body products that offer reliability in ketosis effects systemically. To develop products that offer quantitative measure on ketone intake in the human body, there is extensive ongoing research in developing analogs of beta-hydroxybutyrate, a monomer of microbially produced natural biopolymer, polyhydroxybutyrate (PHB).^{17,18} It is important to recognize that beta-hydroxybutyrate is a naturally available bio-ketone that is produced in the human body for providing energy to cells, heart, and brain—through metabolic means to counter situations of carbohydrate deprivation.^{19–24} Compelling clinical evidence suggests that the monomeric beta-hydroxybutyrate, is directly associated with amplification of pleiotropic effects with measurable benefits on the human body for improving metabolic and physical health.^{2,25–27} It may be noted that although consumption of various forms of ketogenic diets, as well as exogenous ketone derivatives including ketone salts or ketone esters, can increase serum beta-hydroxybutyrate levels, due caution is imperative because of gastrointestinal distress and related severe complications of excessive use of these ketogenic analogs.^{5,25,26,28}

The mechanism through which ketogenic diets act on our body involves production of acetone, acetoacetate, and beta-hydroxybutyrate through downstream metabolic transformation of fatty acids.¹⁴ The high water-solubility of ketone molecules, due to ketogenesis, allows effective transport without the aid of lipoproteins. Systemic presence of ketone molecules is known to promote resistance to oxidative and inflammatory stress, and that many of the beneficial effects of non-metabolic actions of ketone analogs, on enhanced motor skills and various organ functions, are associated with their ability to act as a ligand to several innate cellular targets. In addition to their proven utility as alternative sources of energy, optimum level of ketones in the body, either achieved through consumption of ketogenic diet or exogenous ketone products, has beneficial effects on brain function.^{3,27} Increase in plasma ketone levels correlates with brain ketone metabolism with consequent source of energy supply to the brain resulting in mitigating cognitive impairment in Alzheimer's disease.^{3,27,29,30} Additionally, optimum blood ketone levels achievable through intravenous infusion, has



Scheme 1 Ketone bodies - energy source to human body alternative to glucose.

shown reduction in myocardial glucose utilization without affecting myocardial free fatty acids (FFA) metabolism.³¹ Low carbohydrate, ketogenic foods have also shown convincing evidence for their metabolic role in mitigating severe adversities of obesity, and associated diseases.^{1–6} It is, therefore, vitally important to recognize that beyond serving as an alternative to glucose, as a source of energy to humans, various analogs of ketones continue to present unprecedented opportunities for therapeutic interventions in the management of numerous diseases including cardiac, chronic kidney and age-related diseases and disorders.^{3,27,29,31–33}

The profound benefits of ketone bodies to human health, unquestionably, demand exogenous administration of ketone molecules in doses that promote and enhance energy levels in the human body. This means it is of paramount importance to develop sophisticated delivery vehicles wherein ketones are made bioavailable in a sustainable fashion in vivo. An attractive solution involves the development of biocompatible nanoparticles of ketone molecules in sizes smaller than normal cells. Engineering nanoparticles of ketone molecules allow efficient cellular penetration of ketones, thus presenting prospects for enhanced bioavailability of energy molecules in vivo.

As part of our longstanding interest in achieving bioavailability of various drug molecules, Katti et al have developed innovative green nanotechnology pathways for delivering therapeutic molecules through biocompatible nanoparticles.^{34–38} As the work advanced to targeted radio-nanotherapeutics,^{36,39,40} and green-synthesis platforms,^{41–43} our laboratory also

explored plant-protein stabilization strategies,^{44,45} that demonstrated biocompatibility and environmental safety.^{46–48} Herein, we present novel pathways toward the design and development of ketone molecule encapsulated-protein nanoparticles. Specifically, we have chosen plant-based proteins from a biocompatibility, biodegradability, and biosafety perspective. Plant-based proteins are considered “Generally Recognized As Safe” (GRAS) by the United States Food and Drug Administration (FDA). Plant-based proteins are also beneficial for human health from a nutritional standpoint. They reduce inflammation and possess inherent antibacterial and chemo-preventive properties as well. In this paper, we report nanoencapsulation of (R)-3-hydroxybutyrate monoglyceride [referred to as ketone molecule (KM)] within biocompatible nanoparticles derived from pea proteins. Our choice of plant-based protein stems from our rationale that such proteins are highly sustainable when compared to proteins derived from animal sources: which have huge carbon and water footprints. The choice of pea protein is further justified because it is naturally gluten free and contains all nine amino acids our body needs.^{49,50} Furthermore, pea protein is rich in lysine for strengthening cartilage, bones, as well as aiding optimum calcium absorption. It is also noteworthy that pea protein has a lower glycemic index, and thus helps to maintain a healthy body weight, while controlling blood sugar levels steady throughout the day. Although plant-protein and other protein-based nanoparticle systems have been shown to protect bioactives and deliver lipophilic compounds in vitro,^{51,52} and while exogenous ketone supplements have demonstrated in vivo bioavailability and physiological effects,⁵³ to date, there is no published work demonstrating effective in vivo delivery of exogenous ketones via fully plant-based nanoparticle carriers. This constitutes an important gap that our current study seeks to address.

Herein, we present full details on (i) the production of well-defined nanoparticles of pea proteins using naturally available crosslinking agents such as mangiferin (from mango peel), epigallocatechin 3-O-gallate (EGCG, from tea leaves) quercetin (from blackberry) and (ii) encapsulation of Ketone Molecule (KM), within these biocompatible nanoparticles derived from pea proteins.

Materials and Methods

Materials

Ketone molecule (KM) was obtained from Tecton Group, LLC, Alexandria, LA. Batch numbers 20201202, 20210702 and 20221104 were used for encapsulation. KM is composed of $\geq 80\%$ (R)-3-hydroxybutyrate monoglyceride, $\leq 15\%$ glycerol, and $\leq 8\%$ (R)-3-hydroxybutyrate as confirmed by gas chromatography-mass spectrometry (GC-MS). Pea protein was bought from Water Soluble Protein, China. Mango (*Mangifera indica*) peel powder was purchased from Jamadar Hosur farms, Vidyanagar, located in Belgaum, India. Wagh Bakri black premium loose tea and Jungle Powders blackberry powder were purchased directly from Amazon. Mangiferin, EGCG, quercetin and MTT (Thiazolyl blue formazan) dye were obtained from Sigma (St. Louis, MO, USA). Human aortic endothelial cells (HAEC, Manassas, VA, USA) were purchased from the American Type Culture Collection (ATCC) and cultured at the University of Missouri, Cell and Immunobiology Core facility according to ATCC recommendations. RPMI 1640, DMEM, fetal calf serum and TrypLE, Trypan blue, and DAPI (4',6-diamidino-2-phenylindole) were obtained from Thermo Fisher Scientific, USA. Double-distilled water was used throughout the experiments.

Synthesis of KM Encapsulated Pea Protein Nanoparticles by Mango Peel Crosslinking

KM encapsulated Pea Protein Nanoparticles were synthesized by Mango Peel crosslinking (KM-PP-MP-NP). Fifteen-gram water-soluble Pea Protein (PP), 15 g Mango Peel (MP) powder and 6 g Ketone Molecule (KM) were added to a 2 L conical flask with a stir bar. 1 L distilled water was added to the flask, and the reaction mixture was stirred overnight. Then, the solution was centrifuged at 4,000 rpm for 15 minutes and the supernatant collected, and the residue discarded. 625 mL of the supernatant was transferred to a 2 L round bottom flask with a stir bar and 210 mL of absolute ethanol was added to the flask contents whilst vigorous stirring. The reaction mixture was stirred overnight. The resulting KM encapsulated Pea Protein Nanoparticles by Mango Peel crosslinking (KM-PP-MP-NP) were fully characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) size and zeta potential (ZP) measurements. The KM-PP-MP-NP solution is refrigerated ($5^{\circ}\text{C} \pm 3^{\circ}\text{C}$) for extending shelf life. Blank Pea Protein Nanoparticles by Mango Peel crosslinking (PP-MP-NP) were synthesized following the same procedure without the addition of KM.

Formulations with any concentration variation of pea protein, KM and extract were also synthesized following the same procedure. Six hundred milligrams per liter of sodium benzoate and 350 mg/L of potassium sorbate, food grade preservatives, were added to the nano-formulations to extend shelf-life.

Synthesis of KM Encapsulated Pea Protein Nanoparticles by Tea Extract Crosslinking

KM encapsulated Pea Protein Nanoparticles were synthesized by Tea Extract crosslinking (KM-PP-TE-NP). Tea Extract (TE) solution was first prepared for nanoparticle synthesis. Six grams of Darjeeling loose-leaf tea was weighed and transferred to a 1 L conical flask. Three hundred-milliliter of distilled water was added to the flask and the contents boiled for 10 minutes at 100°C. Then heating was switched off to allow the tea extract solution to cool down to room temperature. Then the solution was centrifuged at 4,000 rpm for 15 minutes and supernatant collected, and the residue discarded. The clear, deep-brown TE supernatant solution was utilized for nanoparticle synthesis. Weigh 8 g water soluble Pea Protein (PP) and transfer to a 2 L conical flask with a stir bar. Add 500 mL distilled water to the flask and let the pea protein solution stir for 30 minutes. Then weigh 3 g Ketone Molecule (KM) and transfer to the flask and continue stirring for 30 more minutes. Further, add 100 mL of the TE solution and continue stirring the reaction mixture for another 2 hours. Then, 170 mL of absolute ethanol was added to the flask contents whilst vigorously stirring and left to stir overnight. The resulting KM-encapsulated Pea Protein Nanoparticles by Tea Extract crosslinking (KM-PP-TE-NP) was then characterized by TEM, DLS size and ZP measurements. The KM-PP-TE-NP solution was refrigerated ($5^{\circ}\text{C} \pm 3^{\circ}\text{C}$) to extend the shelf life. Blank Pea Protein Nanoparticles by Tea Extract crosslinking (PP-TE-NP) were synthesized following the same procedure without addition of KM. Formulations with any concentration variation of pea protein, KM and extract were also synthesized following the same procedure. Six hundred-milligram/L of sodium benzoate and 350 mg/L of potassium sorbate, food grade preservatives were added to the nano-formulations to extend shelf-life.

Synthesis of KM Encapsulated Pea Protein Nanoparticles by Berry Extract Crosslinking

KM encapsulated Pea Protein Nanoparticles were synthesized by Berry Extract crosslinking (KM-PP-BE-NP). Begin by preparing an aqueous solution of Blackberry Extract (BE). One hundred grams of blackberry powder was weighed and transferred to a 1 L conical flask. One thousand milliliters of distilled water was added to the flask and the contents boiled for 15 minutes at 100°C. Then turn off the heat and let the solution cool down to room temperature, allowing any remaining insoluble blackberry fruit powder residue to settle down. Then centrifuge the solution at 4,000 rpm for 15 minutes and collect the supernatant solution and discard the residue. For the nanoparticle synthesis, the clear purple BE supernatant solution was used. Fifty grams of water-soluble Pea Protein (PP) and 300.0 g Ketone Molecule (KM) were weighed and transferred to a 2 L conical flask with a stir bar. Then, 500 mL of the BE extract and 500 mL distilled water were added to the flask and the contents boiled for 15 minutes at 100°C. Three hundred and thirty-three milliliters of absolute ethanol was then added to the flask and the reaction mixture left to stir overnight at room temperature. The resulting KM encapsulated Pea Protein Nanoparticles by Berry Extract crosslinking (KM-PP-BE-NP) was characterized by TEM, DLS size and ZP measurements. The KM-PP-BE-NP solution was refrigerated ($5 \pm 3^{\circ}\text{C}$) for extending shelf life. Blank Pea Protein Nanoparticles by Berry Extract crosslinking (PP-BE-NP) were synthesized following the same procedure without addition of KM. Formulations with any concentration variation of pea protein, KM and extract were also synthesized following the same procedure. Six hundred-milligram/L of sodium benzoate and 350 mg/L of potassium sorbate, food-grade preservatives were added to the nano-formulations to extend shelf-life.

Characterization of Nanoparticles

KM encapsulated pea protein nanoparticles were centrifuged before measurements to remove any residue. The supernatant was diluted 10-fold in water to determine hydrodynamic size and zeta potential using a Malvern Nano ZS90 Zetasizer (Malvern Instruments Ltd. USA). Each sample was measured five times, and at least 10 runs were performed per measurement.

Transmission electron microscopy (TEM) images were obtained on a JEOL 1400 TEM (JEOL, LTE, Tokyo, Japan). The samples were stained using negative stain (methylamine tungstate, Nano-W Negative Stain) to visualize the core size

of the nanoparticles. The absorption measurements were obtained by UV-visible spectrophotometer (Varian Cary 50 conc, USA).

Nuclear Magnetic Resonance Spectroscopy

Proton (^1H) nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz on a Bruker 600 MHz spectrometer. Carbon (^{13}C) NMR spectra were recorded at 151 MHz on a Bruker 600 MHz spectrometer. NMR spectra were recorded in deuterated water (D_2O) or deuterated methanol (CD_3OD) or a combination of both. The ^1H chemical shifts are reported relative to internal D_2O or CD_3OD . The ^{13}C NMR chemical shifts are reported relative to an external tetramethylsilane (TMS) standard. NMRs of KM, water-soluble pea protein and phytochemicals were recorded.

Fluorescence Measurements

The intrinsic tryptophan fluorescence spectrum at 25 °C was recorded using a Varian Eclipse spectrofluorometer (Varian Inc). The fluorescence emission spectrum of KM encapsulated pea protein nanoparticles was recorded between 300 and 400 nm using 295 nm as the excitation wavelength. The excitation and emission band passes were 5 nm each.

Gas Chromatography–Mass Spectrometry (GC-MS)

The KM concentration was estimated using gas chromatography–mass spectrometry (GC-MS) analysis and a neat KM standard curve. One microliter of KM was diluted 1000 times by adding 1000 μL of pyridine. Twenty-five microliter of this solution was trimethylsilylated with 25 μL MSTFA [N-methyl-N-(trimethyl-silyl)trifluoroacetamide] + 1% TMCS (chlorotrimethylsilane) reagent by incubating at 50 °C for an hour. The derivatized samples were then analyzed using an Agilent 6890 GC coupled to a 5973N MSD mass spectrometer with a scan range from 50 to 650 m/z (Agilent Technologies, Inc., Santa Clara, CA). One microliter of sample was injected into the GC column with a split ratio of 1:1 with a 60-minute run time. Separation was achieved with a temperature program of 80 °C for 2 minutes, then ramped at 5 °C/min to 315 °C and held at 315 °C for 12 minutes, on a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 μm film thickness) and a constant flow of 1.0 mL/minute of helium gas. A standard alkane mix was used for GC-MS quality control and retention index calculations. The chromatographic peaks in sample were deconvoluted using Automated Mass Spectral Deconvolution and Identification System (AMDIS) and annotated through mass spectral and retention index matching to an in-house constructed spectral library and commercial National Institute of Standards and Technology 17 (NIST 17) mass spectral library. Neat KM standard, with five different concentrations prepared in pyridine, was similarly analyzed. The Peak area was calculated using Agilent, Enhanced Data Analysis (EDA) software to generate a calibration curve. Using the calibration curve and the peak area of the GC peak of the sample solution at retention time (RT) ~30 min, the concentration of the KM monoglyceride was accurately calculated.

Estimation of KM Encapsulation Efficiency

The amount of KM in the nanoparticle formulation was measured to calculate the encapsulation efficiency of the framework. A high KM encapsulation efficiency is important for better bio-delivery of KM. After synthesis of the KM plant protein nanoparticles, it was filtered using an Amicon Ultra Centrifugal Filter (3 kDa MWCO) to remove any unincorporated KM in the formulation. Then, GC-MS analysis of the filtered KM nano-formulation was carried out for estimation of the encapsulation efficiency. Quantification was performed using a neat KM standard calibration curve. About 50% encapsulation efficiency was observed for KM plant protein nanoparticles crosslinked with phytochemicals. Considering the green nanotechnology approach (no harsh chemicals, organic solvents or rigorous experimental conditions) and biocompatibility of the materials, this is very significant.

Stability Study

The stability of KM encapsulated pea protein nanoparticles was monitored over a six-month period by tracking the hydrodynamic size and zeta potential of the nanoparticles ([Supplementary Material](#), Stability Study). The nanoparticles were refrigerated ($5^\circ\text{C} \pm 3^\circ\text{C}$) in glass or plastic containers with a tight seal. Negligible change in size and zeta potential confirmed the stability of nanoparticles for long-term storage ([Supplementary Table 1](#)). An example Zetasizer dynamic

light scattering (DLS) size measurement of KM encapsulated Pea Protein Nanoparticles by Berry Extract crosslinking (KM-PP-BE-NP) is shown in [Supplementary Figure 1](#). An example Zetasizer zeta potential (ZP) measurement of KM-PP-BE-NP is shown in [Supplementary Figure 2](#). Moreover, GC-MS analysis was performed to quantify the amount of KM and monitor for changes during the stability study. In the present study, stability of KM-encapsulated pea protein nanoparticles was assessed over a six-month period using both dynamic light scattering (DLS) and gas chromatography–mass spectrometry (GC-MS). This sampling schedule enabled evaluation of temporal consistency in hydrodynamic size, zeta potential, and KM content throughout storage.

Cell Viability Assay

The in vitro cytotoxicity of ketone molecule and ketone molecule encapsulated pea protein nanoparticles was investigated against human aortic endothelial cells (HAEC). HAEC cells (4×10^4 cells/mL) were seeded into 96-well tissue culture plates. The cells were incubated in a CO₂ incubator (37°C, 5% CO₂, 90% RH) for 24 hours. After incubation, the medium was replaced with the medium containing different dilution of Ketone Molecule and Ketone Molecule encapsulated pea protein nanoparticles. Treatment was evaluated at three incubation time-points 24, 48 and 72 hours. After incubation, MTT dye (10 µL/well) was added in the cell suspension and incubated for 4 hours in the CO₂ incubator. Formazan crystals formed after 4 hours were dissolved with DMSO buffer (100 µL) in each well. The plates measured using a microplate reader (Spectra Max plate reader, USA) operated at 570 nm wavelength. Percent of cell viability was calculated by using the formula:

$$\text{Cellviability}(\%) = \frac{(T_{Ab} - B_{Ab})}{(C_{Ab} - B_{Ab})} \times 100$$

where the absorbance of the treatment (T_{Ab}), blank (B_{Ab} , representing the medium), and control (C_{Ab} , representing untreated cells) were measured.

Statistical Analysis

All experimental data are expressed as average $\pm$ SD. Statistical analysis was carried out using the one-way analysis of variances (ANOVA) using Graph Pad Prism software. $P < 0.05$ was considered significant.

KM and KM-PP-BE-NP in vivo Study

Experimental Animals

Male adult New Zealand White rabbits (~3kg) were used for the study. Animals were maintained on a Laboratory Rabbit Diet HF (High Fiber) throughout the experimental period. The animals were administered KM and KM-PP-BE-NP orally (PO gavage) at 60 g human equivalent dose (HED). Blood sample will be collected at pre-dose, 15 minutes, 30 minutes, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours and 24 hours post dosing (11 time-points).

Overview and Compliance

This study was performed at Vipragen Biosciences Private Limited. The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were followed at the laboratory (Registration number 1683/PO/RcBiBt/S/13/CPCSEA) following all ethical practices as described in the guidelines for animal care and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, USA. This study was approved by the Institutional Animals Ethics Committee (IAEC) of the test facility – VIP/IAEC/350/2022.

Acclimatization

The animals were acclimatized for at least five days to laboratory conditions and were observed for clinical signs once daily. Veterinary examinations of all the animals were performed on the day of receipt and prior to the start of the treatment.

Environmental Conditions

Animals were housed in an environmentally monitored air-conditioned room maintained at a temperature of $20 \pm 3^\circ\text{C}$, at a relative humidity of 30% to 70%. Air changes of 10–15 per hour were maintained in the experimental room with 12 hours dark and 12 hours light cycle. Temperature and humidity were recorded once daily.

Housing

The rabbits were housed individually in conventional laboratory cages with floor area of $5,600\text{ cm}^2$ and a height of 60 cm. Cage interiors were smooth surfaces without any projections or breaks in the slats or wire of the floor. Waste was removed from cage trays frequently. It was cleaned and sanitized regularly.

Food and Water

Rabbits were fed a complete and nutritionally balanced pelleted diet (Rabbit Diet HF) limited to 4–6 ounces of pellets daily. They had free access to clean, potable water via a Lixit or water bottle.

KM and KM-PP-BE-NP Treatment and Randomization

Healthy male rabbits were grouped and allocated to their respective treatment groups using a body weight-based stratified randomization. The *in vivo* study was conducted as a pilot experiment with six animals per group, a sample size commonly used in exploratory nutraceutical delivery studies.⁵⁴ While no formal *a priori* power calculation was performed, this group size has been shown to provide sufficient sensitivity to detect moderate differences in pharmacokinetic parameters under comparable conditions. Animals were randomly assigned. We also note that large-scale studies have already been reported by our group, including detailed statistical analyses on ketone-induced glucose modulation and on KM toxicology, further supporting the translational reliability of our findings in rats.^{55,56}

The details of study design and animal allocation are given below:

The following test groups were used: $n=6/\text{group}$

Group 1 – KM dose level at 60 g HED

Group 2 – KM-PP-BE-NP dose level at 60 g HED

Groups of 6 male adult New Zealand White rabbits (average weight: $3.0 \pm 0.3\text{ Kg}$) were fasted overnight (about 16 hours) and the test article was administered orally (PO gavage). Ketone was administered on a per unit body weight (kg) basis, where the exact amount of ketone for each animal was calculated based on the individual respective body weight at the time of dosing. The animal equivalent dose (amount/kg) was scaled from the specified human equivalent dose via allometric scaling using the species (rabbit) specific body surface area-based conversion factors. Based on average adult body weight the hourly caloric requirement of rabbit is 11 kcal/h. Based on the nutritional facts of the test item (KM 5 kcal/g), equivalent quantity was reconstituted in the required dose volume. All calculations considered the individual body weight of each animal ahead of dosing. Specified HED to be tested in this study were 60 g KM per adult (average body weight of 70 kg), as these dosages are commonly used by humans in a free-living environment.

Blood Collection and Analysis

Blood was collected from the marginal ear vein and the blood (R)-3- hydroxybutyrate (BHB) was measured using beta-hydroxybutyrate assay kit (Catalog Number Ab83390) purchased from Abcam, MA, USA. Blood BHB levels were measured at pre-dose, 15 minutes, 30 minutes, 1.5 hours, 2 hours, 3 hours, 4 hours, 8 hours and 24 hours post dosing (11 time-points).

Results

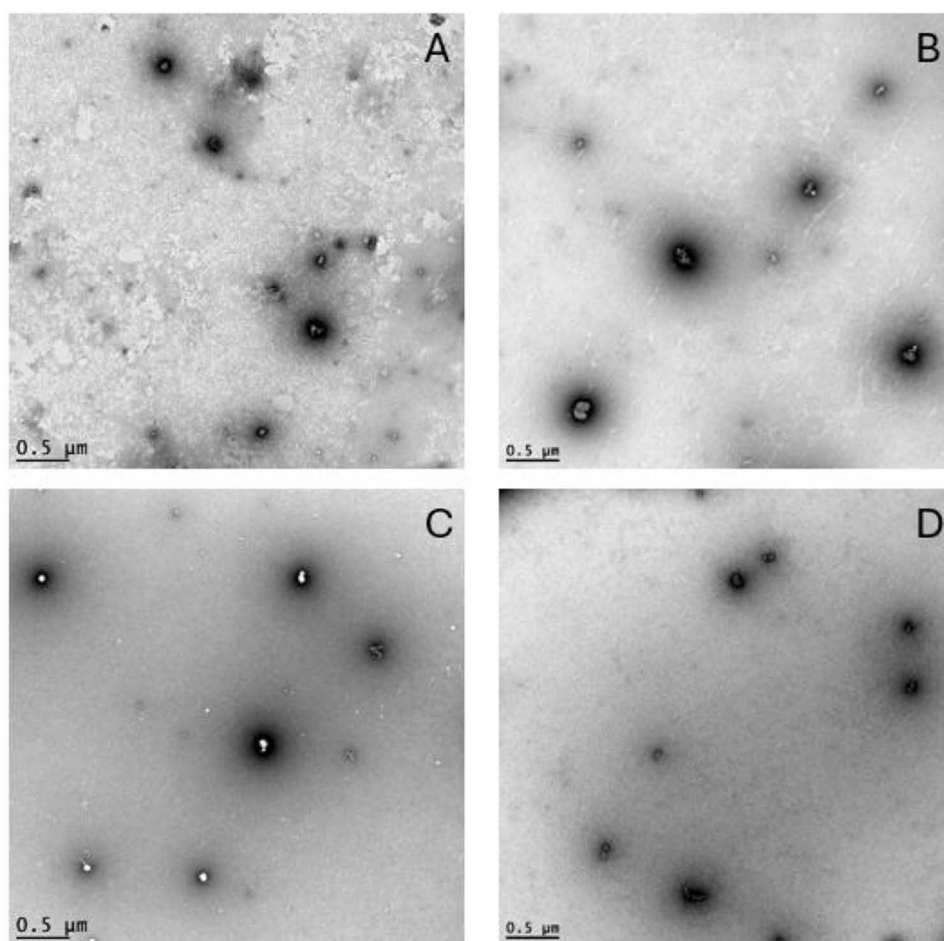
KM Encapsulated Pea Protein Nanoparticles by Mango Peel Crosslinking

Naturally, available plant-based materials were explored for crosslinking. Mangiferin (MGF), a phytochemical is present in mango peel. The multiple hydroxyl groups in MGF are effective for protein crosslinking. KM encapsulated water-soluble Pea Protein Nanoparticles using Mango Peel crosslinking (KM-PP-MP-NP) was attempted by ethanol desolvation method. After synthesis, ethanol can be removed from the formulation by rotatory evaporation at 37°C . KM-PP-MP-NP hydrodynamic size was between 336–422 nm, with an average of 367 nm using DLS and showed an average zeta

Table 1 KM Encapsulated Pea Protein Nanoparticles by Mango Peel Crosslinking (KM-PP-MP-NP)

Sample ID	Pea Protein (PP) (mg/mL)	Ketone Molecule (KM) (mg/mL)	Mango Peel (MP) (mg/mL)	Water (mL)	Ethanol (mL)	PP:KM:MP Ratio	Water: Ethanol Ratio	DLS Size (nm)	PDI	ZP (mV)
A	15	5	17	47	16	3:1:3	3:1	343	0.2	-18
B	15	6	15	625	210	3:1:3	3:1	336	0.2	-17
C	15	0	17	42	14	3:0:3	3:1	369	0.2	-19
D	100	300	15	1000	350	0.3:1:0.1	3:1	422	0.2	-9
E	100	0	17	5	2	6:0:1	3:1	486	0.3	-12

potential of -15 mV (Table 1) indicating reasonable stability. Polydispersity index (PDI) of 0.2 indicated that KM-PP-MP-NP of uniform size distribution. Using negative staining technique nanoparticle core size was explored by TEM (Figure 1). The surface morphology of the protein nanoparticle reveals spherical dense particles with size in the ~300-500 nm range.

**Figure 1** TEM images (A–D) of various KM encapsulated Pea Protein Nanoparticles by Mango Peel crosslinking (KM-PP-MP-NP).

KM Encapsulated Pea Protein Nanoparticles by Tea Extract Crosslinking

Tea leaves extract contains a variety of polyphenols including flavonoids, epigallocatechin gallate (EGCG) and other catechins. Hence, tea extract can be effective in crosslinking protein nanoparticles. KM-encapsulated water-soluble Pea Protein Nanoparticles using Tea Extract Crosslinking (KM-PP-TE-NP) was carried out by ethanol desolvation method. After synthesis, any insoluble residue was removed by centrifugation/filtration and the ethanol can be removed from the formulation by rotatory evaporation at 37 °C. KM-PP-TE-NP hydrodynamic size was between 316–373 nm, with an average size of 338 nm using DLS and showed an average zeta potential of −13 mV (Table 2), indicating reasonable stability. The PDI of ~0.2 indicated nanoparticles of uniform size distribution. Using negative staining technique, nanoparticle core size was estimated by TEM (Figure 2) and revealed stable particles with size in the ~300-500 nm range.

KM Encapsulated Pea Protein Nanoparticles by Berry Extract Crosslinking

Crosslinking was investigated using naturally occurring plant-based materials from blackberries. Quercetin (QUE), a phytochemical is present in berry extracts. The hydroxyl groups in QUE are effective at crosslinking proteins. The ethanol desolvation method was used to prepare KM encapsulated water-soluble Pea Protein Nanoparticles with Berry extract crosslinking (KM-PP-BE-NP). Centrifugation/filtration was used to eliminate any insoluble residues following nanoparticle production. Ethanol can be removed from the formulation by rotatory evaporation at 37 °C. KM-PP-BE-NP hydrodynamic size was between 354 and 474 nm, with an average size of 407 nm determined by DLS and showed an average zeta potential of −10 mV (Table 3). PDI of ~0.3 suggests mostly uniform sized nanoparticle distribution. TEM was used to investigate the size of nanoparticles using the negative staining technique as shown in Figure 3 and revealed size in the range ~500 nm.

KM Encapsulated Pea Protein Nanoparticles Fluorescence Spectroscopy

The fluorescence emission spectra of KM-PP-MP-NP and PP-MP-NP were recorded by excitation at 285 nm corresponding to the tryptophan amino acid. A broad emission peak at 351 nm with fluorescence intensity of ~40 was seen in the emission spectra of (Figure 4). Changes in the tryptophan microenvironment can be monitored through fluorescence spectroscopy. KM-PP-MP-NP and PP-MP-NP fluorescence maximum peak and intensity are comparable, indicating not much change in the tryptophan region of the pea protein nanoparticles. Similarly, the fluorescence emission spectra of KM-PP-TE-NP and PP-TE-NP were recorded by excitation at 285 nm corresponding to the tryptophan amino acid. A broad emission peak at 353 nm with fluorescence intensity of ~30 is seen in the emission spectra of (Figure 5). KM-PP-TE-NP and PP-TE-NP fluorescence maximum peak and intensity are comparable, indicating not much change in the tryptophan region of the pea protein nanoparticles. Since KM is water soluble and hydrophilic, it does not interact with the hydrophobic tryptophan microenvironment of the pea protein. The fluorescence measurements give insights into pea protein interaction with KM.

Table 2 KM Encapsulated Pea Protein Nanoparticles by Tea Extract Crosslinking (KM-PP-TE-NP)

Sample ID	Pea Protein (PP) (mg/mL)	Ketone Molecule (KM) (mg/mL)	Tea Extract ^a (TE) (mL)	Water (mL)	Ethanol (mL)	PP:KM Ratio	Water: Ethanol Ratio	DLS Size (nm)	PDI	ZP (mV)
A	15	5	10	50	17	3:1	3:1	320	0.2	−15
B	15	5	100	500	170	3:1	3:1	342	0.2	−14
C	15	0	5	25	9	3:0	3:1	362	0.1	−14
D	50	300	2,000	8,000	2,000	1:6	4:1	373	0.2	−6
E	15	5	2	10	3	3:1	3:1	316	0.3	−17

Notes: a = Tea leaves (20 mg/mL) boiled at 100 degrees for tea extract (TE).

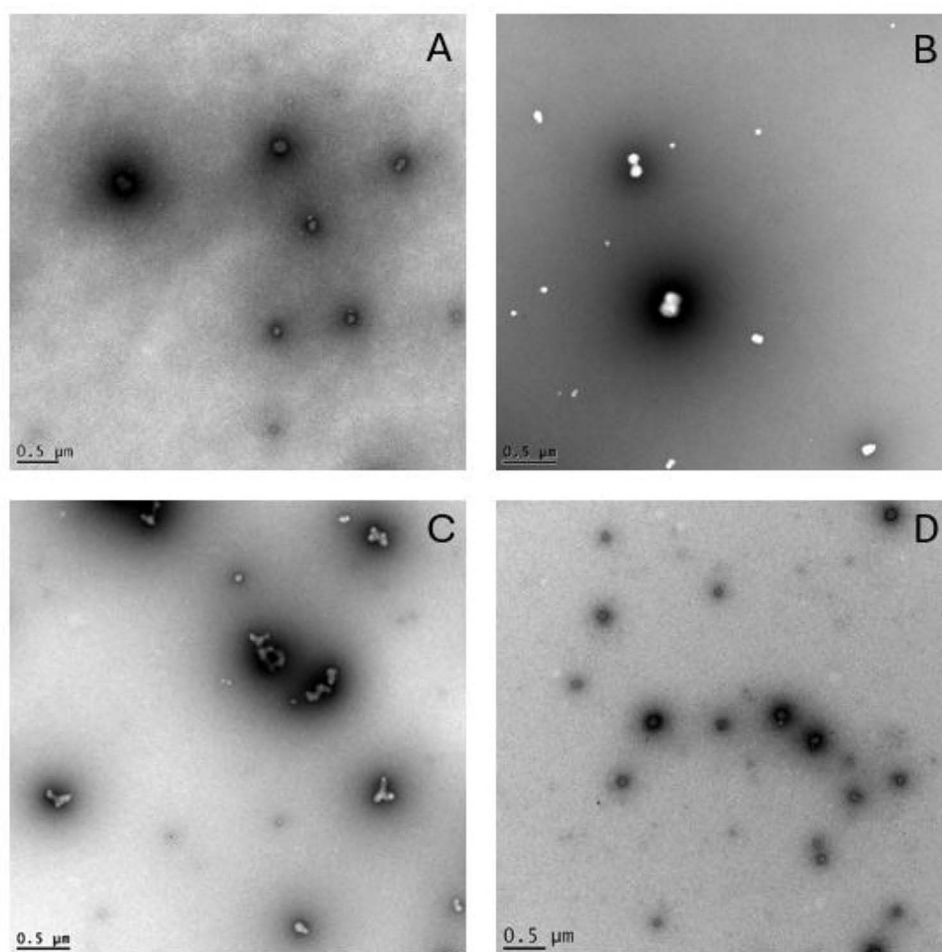


Figure 2 TEM images (A–D) of various KM encapsulated Pea Protein Nanoparticles by Tea Extract crosslinking (KM-PP-TE-NP).

GC-MS Analysis of KM Encapsulated Pea Protein Nanoparticles

The amount of KM in the nanoparticle formulation was established using GC-MS analysis. It was found to be ~50% KM encapsulation. A high KM encapsulation efficiency is important for bio-delivery of KM. The GC-MS column provides good resolution of the starting materials and various glycerides. Quantification of KM can be carried out by using neat KM to obtain a calibration curve. The curve should have good linearity, and the analysis concentration should fall within the calibration range. GC-MS can be used for quality control of the KM encapsulation process.

Table 3 KM Encapsulated Pea Protein Nanoparticles by Berry Extract Crosslinking (KM-PP-BE-NP)

Sample ID	Pea Protein (PP) (mg/mL)	Ketone Molecule (KM) (mg/mL)	Blackberry Extract (BE) (mL)	Water (mL)	Ethanol (mL)	PP:KM Ratio	BE: Ethanol Ratio	DLS Size (nm)	PDI	ZP (mV)
A	50	300	10 ^a	0	2	1:6	5:1	392	0.3	−10
B	50	300	10 ^b	0	2	1:6	5:1	474	0.3	−9
C	50	0	10 ^a	10	2	1:0	5:1	415	0.2	−10
D	50	300	500 ^c	500	333	1:6	2:1	354	0.2	−10

Notes: a = 25 mg/mL, b = 50 mg/mL and c = 100 mg/mL berry powder boiled at 100 degrees for berry extract (BE).

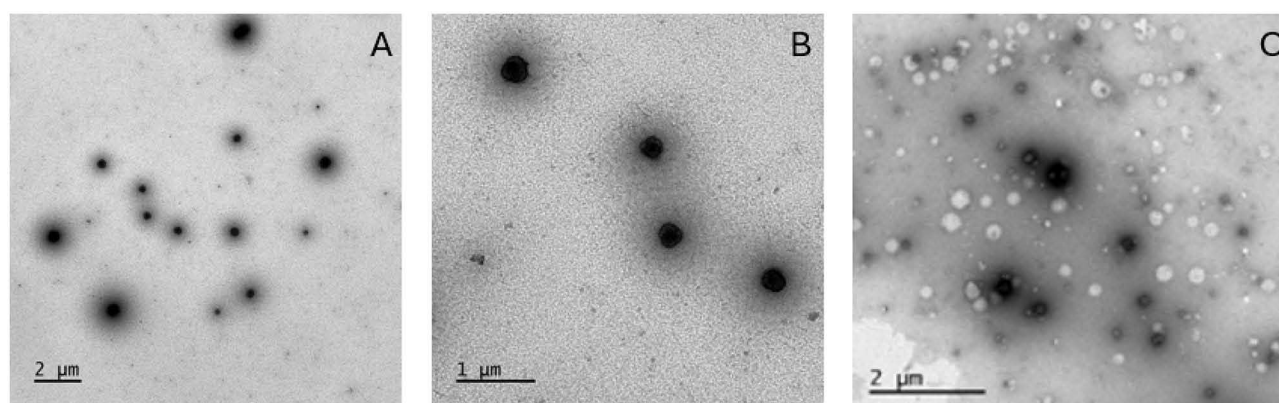


Figure 3 TEM images (A–C) of various KM encapsulated Pea Protein Nanoparticles by Berry Extract crosslinking (KM-PP-BE-NP).

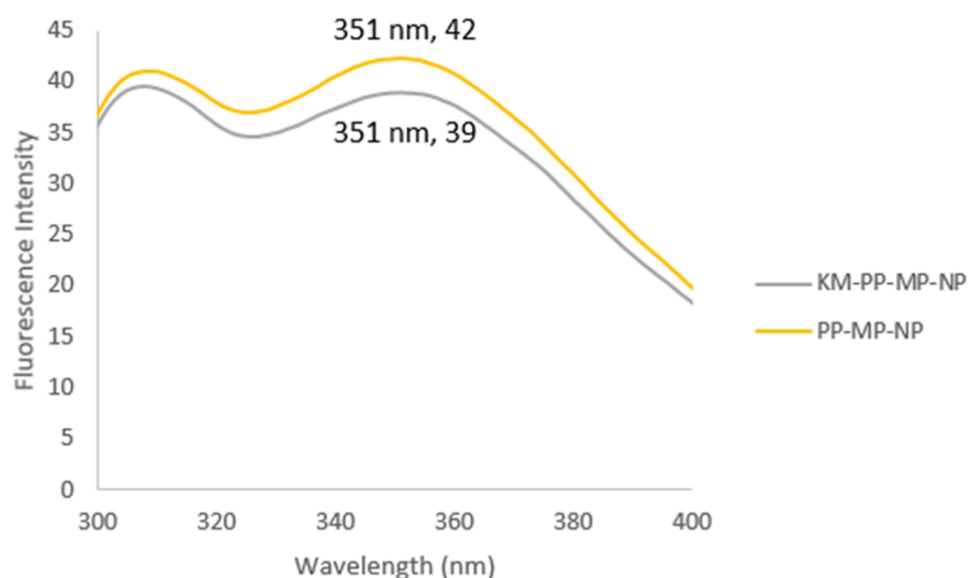


Figure 4 Fluorescence emission spectra of KM encapsulated Pea Protein Nanoparticles by Mango Peel crosslinking (KM-PP-MP-NP) and blank Nanoparticles (PP-MP-NP).

Cellular Assay Toxicity Studies for KM and KM Encapsulated Pea Protein Nanoparticles by Mango Peel Crosslinking

Cellular assay toxicity studies for KM and KM encapsulated pea protein nanoparticles by Mango Peel crosslinking (KM-PP-MP-NP) were carried out with HAEC cell line. This step was critical in determining KM and KM encapsulated nanoparticles toxicity to normal cells. This will determine the amount of KM utilized in formulations designed for in vivo investigations. The cell proliferation assay kit uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a tetrazolium dye for measurement of the cell viability. MTT assay revealed that the cell viability for KM and KM encapsulated nanoparticles against HAEC cell line after 24-, 48- and 72-hours post incubation showed no toxicity over the concentration range 9–142 $\mu\text{g/mL}$ for KM (Figure 6). KM encapsulated pea protein isolate nanoparticles cross-linked with mango peel (KM-PP-MP-NP) were also tested. HAEC after 24-, 48- and 72-hours post incubation with KM-PP-MP-

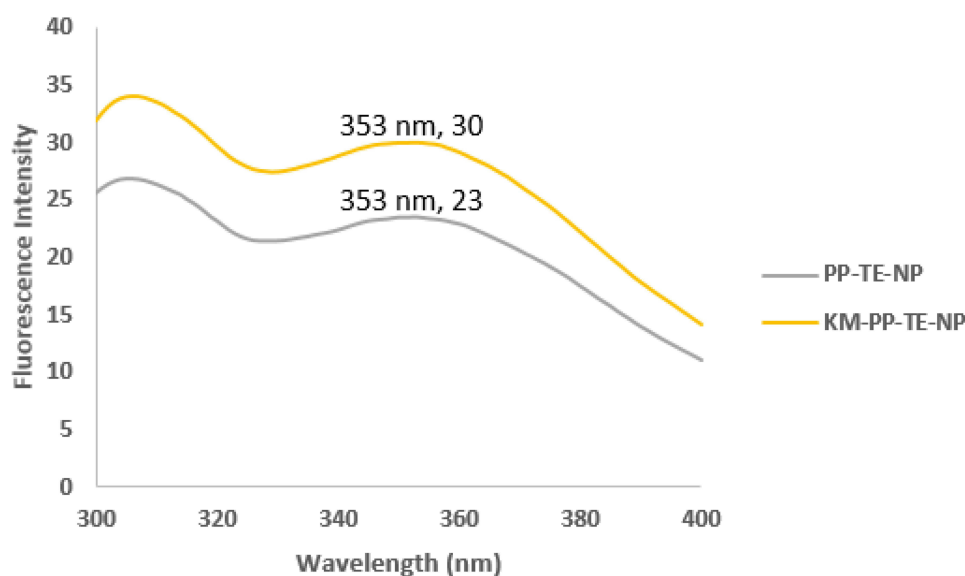


Figure 5 Fluorescence emission spectra of KM encapsulated Pea Protein Nanoparticles by Tea Extract crosslinking (KM-PP-TE-NP) and blank Nanoparticles (PP-TE-NP).

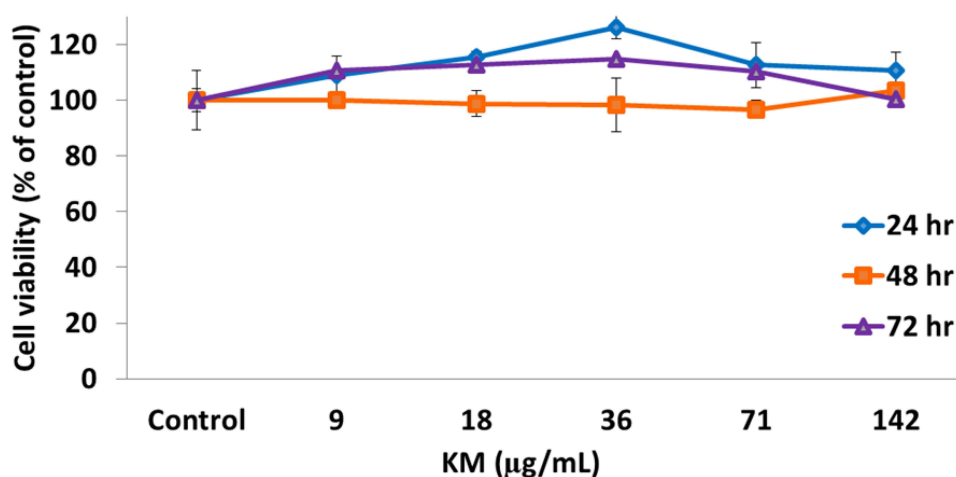


Figure 6 Cell viability of human aortic endothelial cells (HAEC) after 24-, 48- and 72-hours post incubation with KM (9-142 µg/ml). Control is untreated cells.

NP shows good cell viability over the concentration range 9–142 µg/mL (Figure 7). KM concentration in KM-PP-MP-NP was used for plotting the concentration values.

Cellular Assay Toxicity Studies for KM and KM Encapsulated Pea Protein Nanoparticles by Tea Extract Crosslinking

Cellular assay toxicity studies for KM and KM-encapsulated pea protein nanoparticles by Tea Extract crosslinking (KM-PP-TE-NP) were carried out with HAEC cell line. Using the MTT assay, cell viability was measured for both KM and KM-encapsulated nanoparticles. HAEC after 24-, 48- and 72-hours post incubation with KM showed no toxicity over the concentration range 81–1300 µg/mL (Figure 8). KM-encapsulated pea protein nanoparticles cross-linked with tea extract (KM-PP-TE-NP) were also tested. HAEC after 24-, 48- and 72-hours post incubation with KM-PP-TE-NP shows no toxicity over the concentration range 81–650 µg/mL (Figure 9). Compared to the free KM, the toxicity concentration for

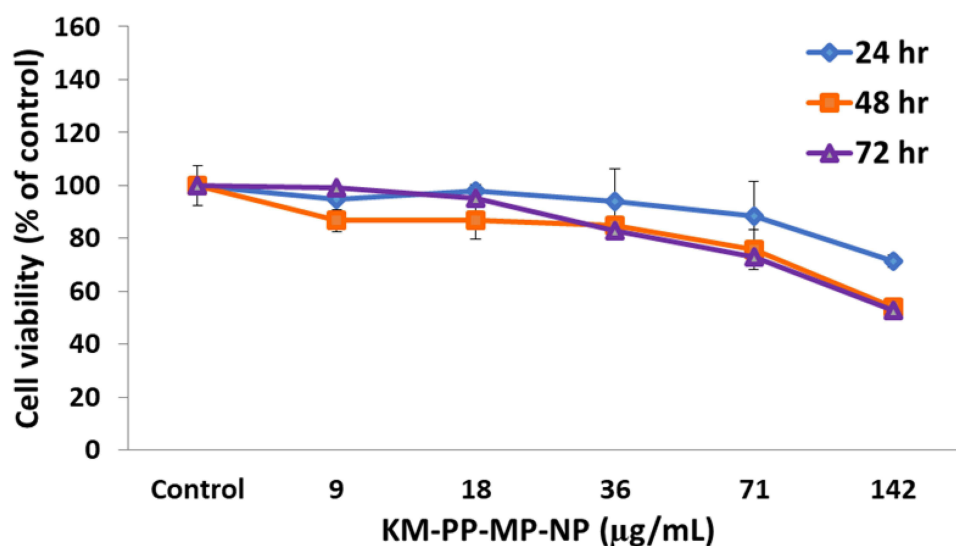


Figure 7 Cell viability of human aortic endothelial cells (HAEC) after 24-, 48- and 72-hours post incubation with KM encapsulated pea protein nanoparticles with Mango peel crosslinking (KM-PP-MP-NP). KM concentration in KM-PP-MP-NP is used for plotting. Control is untreated cells.

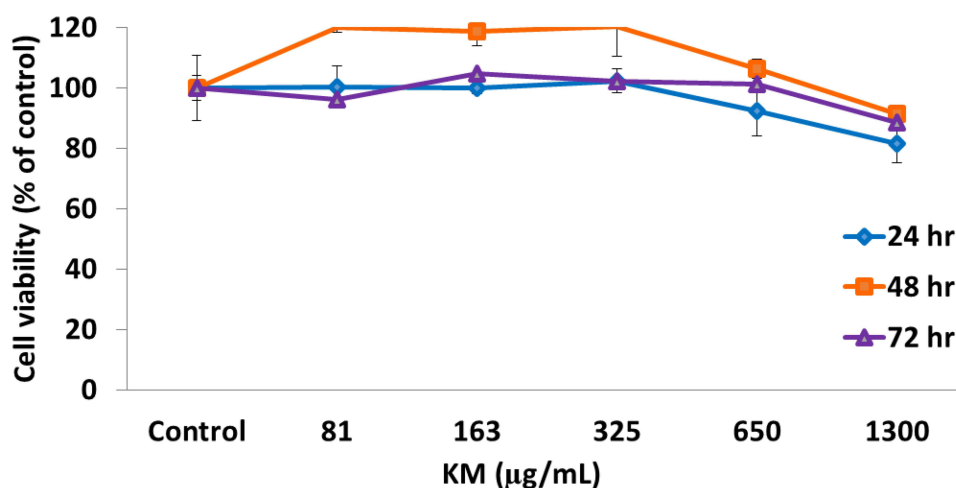


Figure 8 Cell viability of human aortic endothelial cells (HAEC) after 24-, 48- and 72-hours post incubation with KM (81-1300 µg/ml). Control is untreated cells.

KM encapsulated nanoparticles is slightly lower. This observation infers enhanced bioavailability of KM in KM-PP-TE-NP. KM concentration in KM-PP-TE-NP was used for plotting the concentration values.

KM and KM-PP-BE-NP in vivo Study

Cellular dose dependent increase in (R)-3- hydroxybutyrate (BHB) was seen with exogenous KM gavage infusion over time in Male adult New Zealand White rabbit in vivo study (Figure 10). The endogenous (BHB) level increase is seen by increased area under the curve (AUC) for the KM-PP-BE-NP (dose level at 60 g HED) in comparison to the neat KM (dose level at 60 g HED). Also, administration of nano-formulated ketone KM-PP-BE-NP versus neat ketone KM resulted in a shift in T_{max} (time to maximum concentration), suggesting a sustained release kind of profile with the nano-formulation (Table 4).

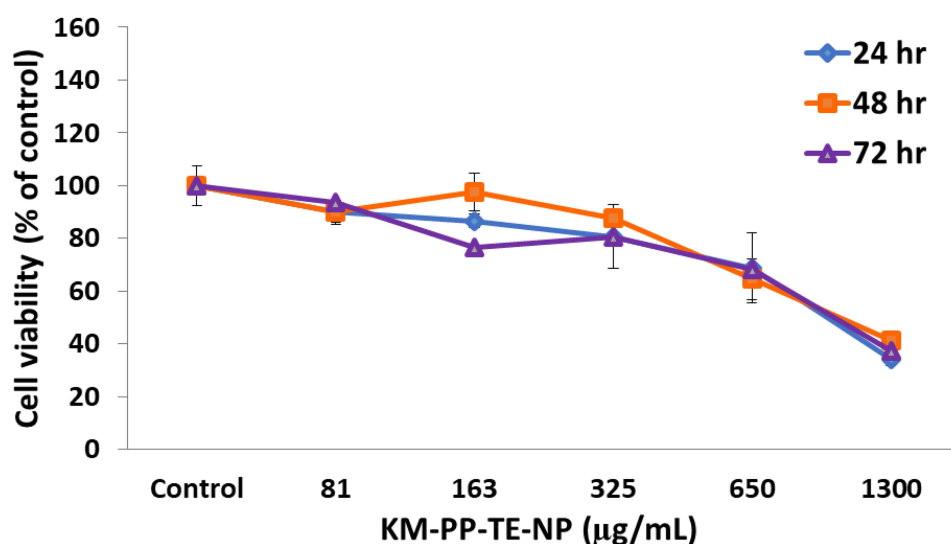


Figure 9 Cell viability of human aortic endothelial cells (HAEC) after 24-, 48- and 72-hours post incubation with KM encapsulated pea protein nanoparticles with tea extract crosslinking (KM-PP-TE-NP). KM concentration in KM-PP-TE-NP is used for plotting. Control is untreated cells.

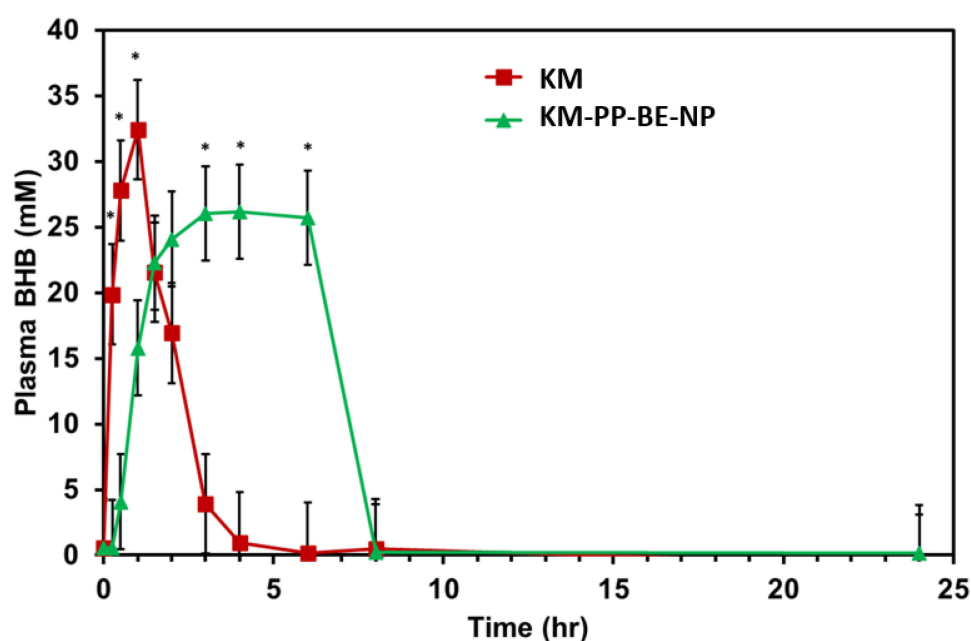


Figure 10 KM and KM encapsulated Pea Protein Nanoparticles by Berry Extract crosslinking (KM-PP-BE-NP) in vitro study in male adult New Zealand White rabbits. Statistical significance was assessed using independent two-sample t-tests at each timepoint; asterisks (*) indicate $p < 0.05$.

Discussion

Advantages Offered Through Encapsulation of Ketone Molecule (KM) into Pea Protein Nanoparticles Through Phytochemical Crosslinking

Green nanotechnology offers the most effective means to encapsulate and transform small molecules such as the Ketone Molecule (KM) into pea protein encapsulated nanoparticles with optimum sizes for effective cellular specific delivery, thus offering an attractive delivery vehicle to enhance bioavailability. Nanoparticulate KM, delivered directly into cells, can achieve optimum bioavailability. This allows various plant-based polyphenols as crosslinking agents to create pea-

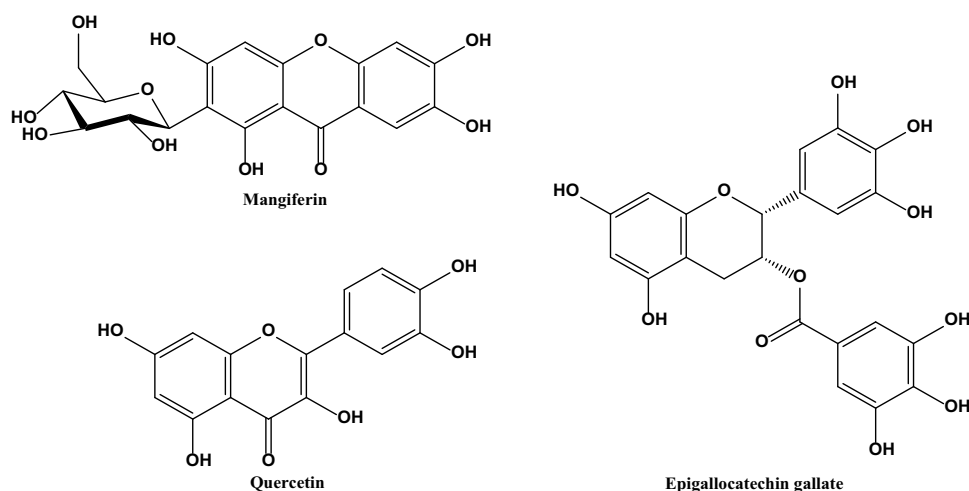
Table 4 Pharmacokinetic Parameters (PK) for KM and KM-PP-BE-NP (Nanoparticles) in vitro Study in Male Adult New Zealand White Rabbits

Pharmacokinetic Parameters (PK)	Ketone Molecule (KM)	KM-PP-BE-NP (Nanoparticles)
[BHB] _{max} (mmol/L)	1.33	1.17
T _{max} (hours)	0.75	2.67
AUC _{inf} (hours.mmol/L)	7.74	8.08
AUC _{last} (hours.mmol/L)	4.22	7.46
MRT (hours)	6.60	6.42

based nanoparticles and encapsulation of KM into such nanoparticles. Overall, this eliminates the use of the industrial chemical glutaraldehyde in the crosslinking process while demonstrating the use of non-toxic plant-based polyphenols to crosslink proteins in the overall green nanotechnology strategy as outlined in the sections below.

Relative to conventional liposomes like polymeric nanoparticles (eg, PLGA), and nanoemulsions, our fully plant-based pea-protein/polyphenol nanocarriers target a different balance of food-grade composition, gastrointestinal (GI) robustness, and practical stability. Oral liposomes often suffer bile-salt and lipase-mediated destabilization that compromises payload retention in the small intestine, despite many adaptations for oral use.^{57–59} Poly(lactic-co-glycolic) acid (PLGA) nanoparticles offer controlled release and regulatory maturity in parenteral products, but they can exhibit burst release, scale-up complexity, and higher cost footprints for food/nutraceutical contexts. Food-grade nano-emulsions are excellent for solubilizing lipophilic actives yet can be limited by Ostwald ripening and long-term physical stability without careful formulation.^{60,61} In contrast, plant-protein nanocarriers leverage protein–polyphenol crosslinking to create colloids with high nutritional value and inherent antioxidant interfaces while avoiding synthetic crosslinkers/surfactants typical of many alternatives, aligning with GRAS-leaning materials and sustainability goals.⁶² Within this framework, our pea-protein nanoparticles maintain size/zeta stability under refrigeration and show sustained in vivo exposure compared with neat ketone, supporting their suitability as a food-compatible delivery platform for exogenous ketones.

We recognized the huge commercial importance of plant-based proteins and phytochemicals from biocompatibility, bioavailability and biosafety perspectives. These fall under the “Generally Recognized As Safe” (GRAS) classification by the FDA. Plant-based proteins are biodegradable, abundantly available, and economically viable. Biocompatible plant-based materials can also be effective as natural and non-toxic cross-linking agents for protein nanoparticle

**Figure 11** Plant phenolic compounds – biocompatible crosslinking agents.

formulations. Our synthesis entails the application of naturally available crosslinkers from plants, fruits and herbs. We have discovered that plant-based compounds like mangiferin, EGCG and quercetin (Figure 11) effectively crosslinks and encapsulates KM into pea protein nanoparticles.^{45,63–65} Naturally available phytochemicals in mango peel extract (mangiferin and other polyphenols), tea leaves extract (EGCG, catechins and other polyphenols) and blackberry extract (quercetin and phenolic acids) are effective crosslinkers and nanoparticle initiators offering excellent biocompatibility through our innovative green nanotechnology approaches (Figure 11).^{45,63,66}

Plant-based proteins are beneficial for human health from a nutritional standpoint. They reduce inflammation and have inherent antibacterial and chemo-preventive properties as well. Plant-based polyphenols have important role as micronutrients and offer great health benefits because they are highly effective in mitigating oxidative stress.^{63,67} Their antioxidant properties are beneficial for use as prophylactics in boosting immune system of healthy human beings and in treating cancer, cardiovascular diseases and numerous age-related diseases and disorders. Radical scavenging properties, pharmacological activity and affinity for proteins are attractive attributes for using polyphenols in food, beverages and in nutraceuticals.^{50,62,68,69} Mango peel extract contains significant amounts of mangiferin, a glucose-functionalized xantho-noid and various other quercetin and alkaloid derivatives. The amount of mangiferin is 40–60 µg/g of dried mango peel powder.^{63,70} Tea extract contains a variety of polyphenols like catechins, theaflavins and thearibigins. The amount of EGCG is 9.36 mg per g black tea.^{45,71} A variety of polyphenols like anthocyanins (cyanidin 3-glucoside), flavonoids (quercetin) and various other polyphenols are present in blackberry extract. Quercetin content in blackberry is 0.5–3.5 mg/100 g fresh weight.⁶⁶

Pea protein-derived nanoparticles offer unprecedented opportunities for encapsulating ketone energy molecules for use in next-generation energy drinks. The unique combination of plant-based polyphenols with plant-based proteins opens new opportunities in the design and development of novel pharmaceuticals, nutraceuticals, food ingredients, beverages, flavor encapsulants and allied significant applications in human food, health and hygiene sectors.

Nanoparticles of pea protein, created through plant-derived cross linkers, engulf Ketone Molecule (KM). This approach of nano-encapsulation of KM using naturally available polyphenols, apart from being effective crosslinkers and nanoparticle initiators for KM, the encapsulated products offer effective cellular specific delivery—thus enhancing bioavailability (Figure 12).

Plant-based proteins and phytochemicals are considered generally safe pharmaceuticals.^{44,46,48,50,68} However, technologies to exploit their commercial potential as crosslinkers in the creation of plant-derived protein nanoparticles have

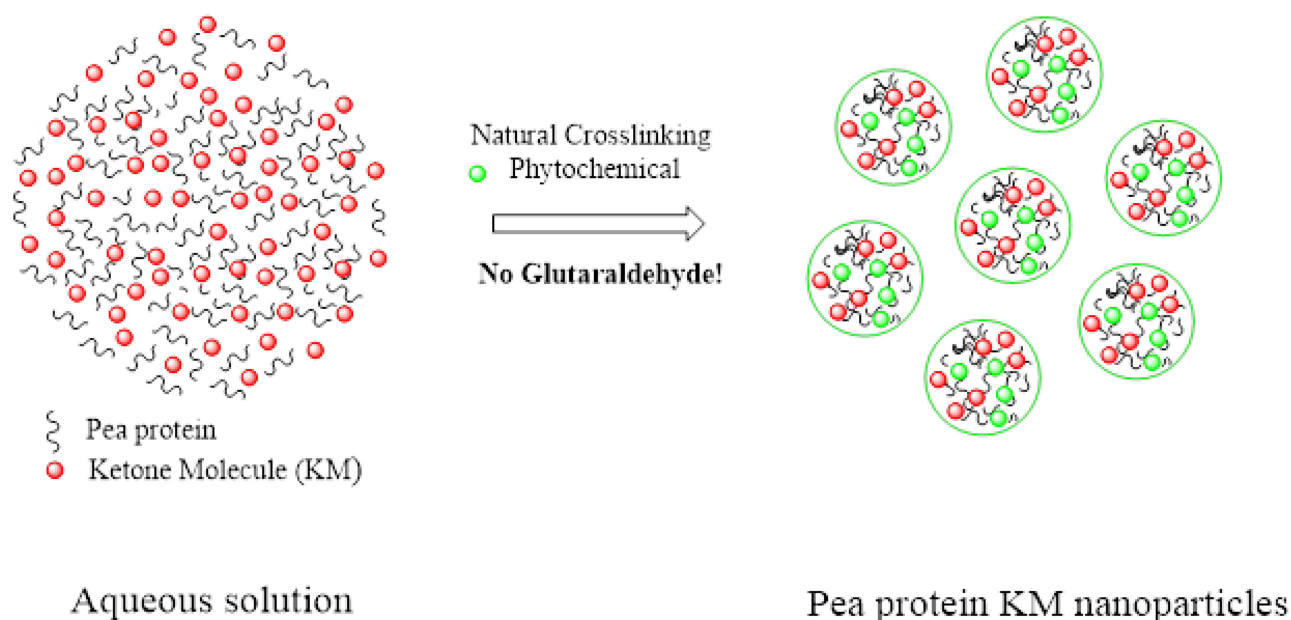


Figure 12 Pea protein KM nanoparticle synthesis utilizing biocompatible plant-based crosslinking agents.

lagged due to scientific and technical challenges. This is where we have achieved groundbreaking discoveries through our green nanotechnology approaches—which have allowed us to exploit the innate biocompatible and nutraceutical benefits of plant-based nanoparticles.

Stability Considerations

In the present study, stability testing was performed under refrigerated conditions ($5 \pm 3^\circ\text{C}$), reflecting practical storage environments for nutraceutical formulations and consistent with the pilot-study scope. Six hundred-milligram/L of sodium benzoate and 350 mg/L of potassium sorbate, food grade preservatives, were also added to the nano-formulations to extend shelf-life. Under these conditions, nanoparticles retained hydrodynamic size, zeta potential, and KM content for ~six months, indicating excellent stability ([Supplementary Table 1](#)). While encouraging, we acknowledge that comprehensive International Council for Harmonization (ICH)-compliant stability studies: including multiple temperature and humidity conditions as well as accelerated stress testing will be required to fully establish commercial readiness. This limitation will be addressed in future investigations, particularly as KM encapsulation formulations advance toward large-scale deployment. Beyond demonstrating encapsulation efficiency and stability, it was equally important to evaluate the cytotoxicity of KM-based nanoparticles, since cellular safety is critical for translational relevance.

Cytotoxicity Considerations

Evaluation of cytotoxicity was essential to complement encapsulation and stability studies. Human aortic endothelial cell (HAEC) assays demonstrated that KM-encapsulated nanoparticles were largely biocompatible at physiologically relevant concentrations. At lower doses ($\leq 71\ \mu\text{g/mL}$ for KM-PP-MP-NP; $\leq 325\ \mu\text{g/mL}$ for KM-PP-TE-NP), cell viability remained ~80–90% even after 72 hours, consistent with accepted thresholds for nanoparticle safety.^{72,73} Higher concentrations ($\geq 142\ \mu\text{g/mL}$ and $\geq 650\ \mu\text{g/mL}$, respectively, for KM-PP-MP-NP and KM-PP-TE-NP) produced a dose-related reduction in viability, though acute toxicity was limited within ranges relevant for translational use. These findings align with previous reports of plant-derived biopolymeric nanoparticles, which typically preserve viability at moderate exposures but show stress responses under supra-physiological conditions.^{74–76}

We recently published important toxicological aspects of the ketone molecule.^{55,56} It is crucial to recognize that parent ketone molecule, used in our investigations, exhibited no systemic toxicity when evaluated *in vivo* even at concentrations ranging from 14 to 21 g/kg bw/day for rats. Ketone Molecule (KM) at 20 g human equivalent dose (HED) was utilized for attenuation of blood glucose study in Healthy Male Sprague Dawley Rats.

The Ketone Molecule (KM) has demonstrated no systemic toxicity *in vivo* rats.^{55,56} Our phytochemical crosslinkers, mangiferin, EGCG, and quercetin, are derived from widely consumed dietary sources, reinforcing biosafety. Moreover, nano-encapsulated ketone formulations are non-toxic at clinically relevant exposures of 60 g HED in male adult New Zealand White rabbit *in vitro* study. Collectively, these results from cell-based, animal, and early human studies support the preliminary biocompatibility of KM nanoparticles and provide a foundation for further mechanistic safety investigations.

Crosslinker Comparison

To clarify the relative performance of the phytochemical crosslinkers, we have described the comparative data across mango peel, tea extract, and berry extract systems. Mango peel–derived nanoparticles were moderately sized (~336–422 nm) with favorable zeta potential (–15 mV) and good cell viability over the concentration range 9–71 $\mu\text{g/mL}$ ([Figure 7](#)). Tea extract–based formulations yielded particles of comparable size between 316 and 373 nm and comparable zeta potential of ~–15 mV. KM-PP-TE-NP nanoparticles showed high HAEC cell viability across a broad concentration range of 81–325 $\mu\text{g/mL}$ ([Figure 9](#)). Berry extract–crosslinked nanoparticles were also comparable in size (~354–474 nm) and surface charge of (~–10 mV) and showed ~50% KM encapsulation efficiency. Importantly, demonstrated favorable *in vivo* pharmacokinetics, with sustained BHB levels and higher AUC relative to free KM. All three systems were biocompatible and comparable and provided promising translational potential for systemic ketone delivery.

Evidence for Protein Phytochemical Crosslinking Interaction

Crosslinking agents are required for the synthesis of Ketone molecule (KM) encapsulated protein nanoparticles. Crosslinking agents crosslink the protein amino groups to form denser particles. The common chemical crosslinker used for protein nanoparticle synthesis is glutaraldehyde. It is an effective crosslinking agent imparting nanoparticle stability and sustained drug release. Although effective, it may exert measurable systemic toxicity on humans, especially when used for extended periods. As there are no alternative nontoxic crosslinking agents, food, beverage, and pharmaceutical industries have continued to use glutaraldehyde in lower concentrations albeit the fear of long-term systemic toxicities with irreversible adverse toxic effects on human health.

In this context, our application of biocompatible and non-toxic plant-based materials as effective and natural crosslinking agents for protein nanoparticle formulations are unprecedented (Figure 13). We hypothesized that under oxidizing conditions, polyphenols like mangiferin, EGCG and quercetin interact with amino groups of pea proteins to produce covalently bound crosslinks thus resulting in bioconjugations of various polyphenols with protein networks. In this invention, we have experimentally validated this hypothesis as outlined in Figures 13 and 14.

In our efforts toward creating biocompatible protein nanoparticles for encapsulating KM, we have employed naturally available plant-based materials for effective crosslinking and encapsulation. Naturally, available plant-based polyphenol like mangiferin was utilized for effective protein crosslinking and encapsulation of KM. Mangiferin, a xanthonoid, is the glucoside of norathyriol and a natural phenolic compound. From the leaves and bark of *Mangifera indica* (mango), it was first isolated. It can also be found in mango peel (MP) and the seed kernel. Catechin, a natural phenol belongs to the flavonoid family. It is found in tea and fruits and is an antioxidant. EGCG is a catechin formed by the esterification of gallic acid and epigallocatechin. Polyphenols like EGCG and catechin are found abundantly in tea leaves. Quercetin, a polyphenol belonging to the flavonoid group, is widely found in fruits and vegetables. Blackberry extract contains quercetin and related polyphenols.

We have discovered that the acidic phenolic functional groups found in plant-derived phytochemicals are effective for crosslinking with the amino groups in proteins. Creation of possible plant protein-polyphenol platforms, through interactions of polyphenolic compounds with protein amino side chains, are shown in Figures 13 and 14. Through extensive fundamental research in green nanotechnology of proteins and polyphenols, we have developed this state-of-the-art understanding on interactions of various plant-derived proteins-polyphenols platforms. Depending on physiochemical factors and nature of plant-derived protein and polyphenols, well-defined protein-polyphenol nanoparticles can be formulated. The reactive phenolic hydroxyl functionalities on the polyphenols enable crosslinking of proteins.

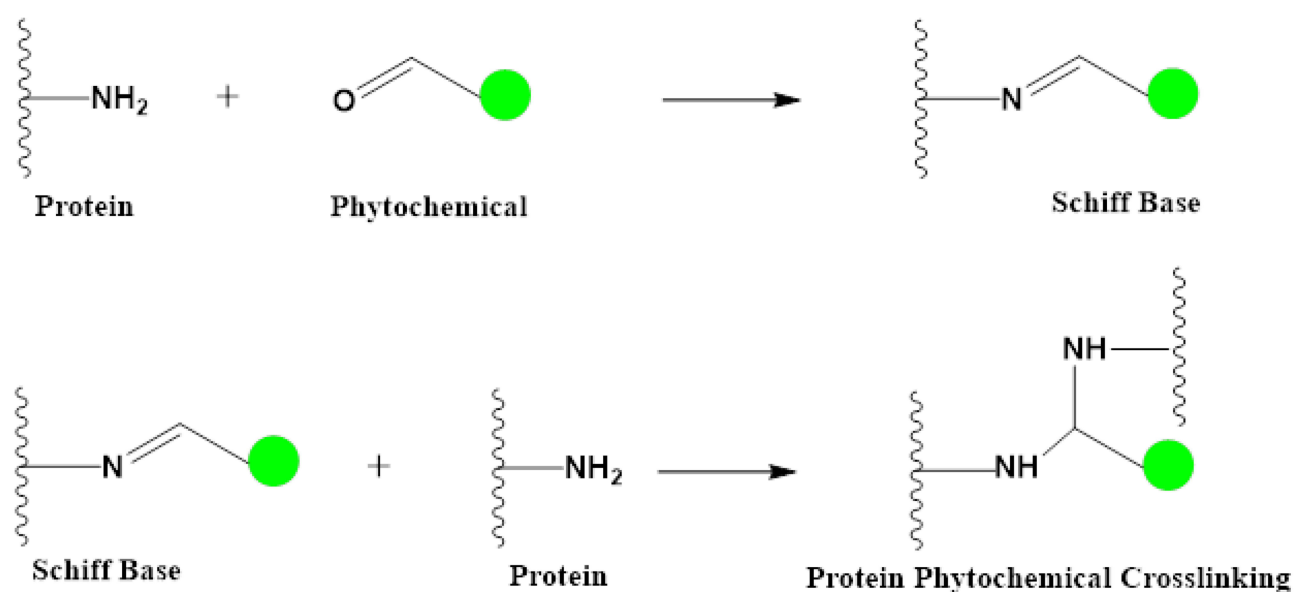


Figure 13 Creation of plant protein-polyphenol platform through interactions of polyphenolic compounds with protein amino side chains.

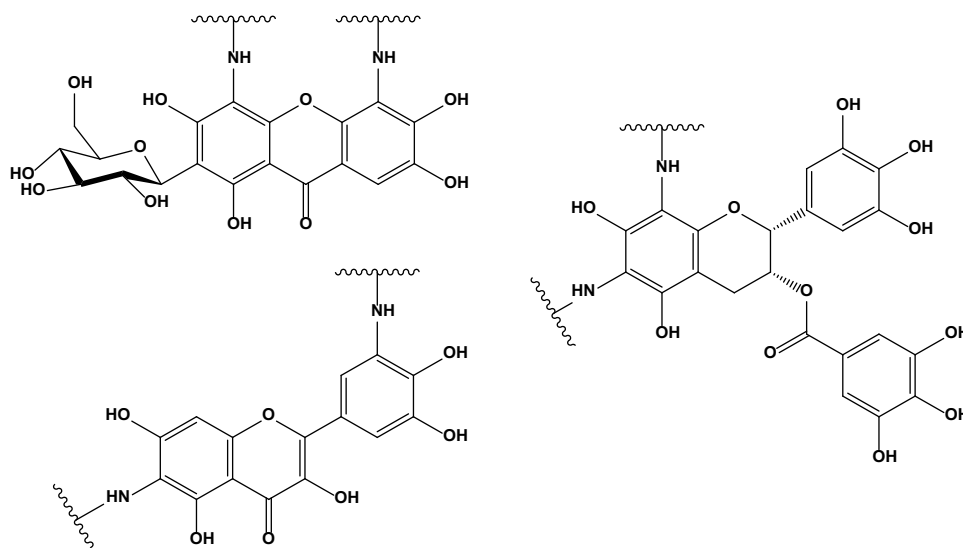


Figure 14 Mangiferin, EGCG and quercetin crosslinking products with pea protein.

Phytochemical and water-soluble pea protein interaction was comprehensively studied using nuclear magnetic resonance (NMR) spectroscopy. Mangiferin, EGCG and quercetin reactions were carried out individually with the pea protein to monitor changes in the phytochemical and pea protein. NMR analysis clearly shows distinct changes in the phytochemical indicating covalent interaction with the protein ([Supplementary Figures 3–13](#)). Then KM was added to the phytochemical and pea protein reaction mixture and further NMR analysis done. In the presence of KM there was no change in the phytochemical or protein, indicating the stability of KM as well. While using the mango peel/tea/blackberry extracts for the KM nanoparticle synthesis, it should be noted that the respective phytoextract is a phytochemical cocktail containing various other active ingredients, which can also play a role in the crosslinking and encapsulation process whilst adding nutraceutical value to the formulations. The aromatic ring protons of the phytochemical show distinct changes indicating covalent interaction with the protein. These changes enable crosslinking with protein amino functional groups. The mangiferin proton NMR showed equivalent peaks at 7.5, 6.8 and 6.4 ppm. But after reaction with the pea protein there is significant change in the 6.8 and 6.4 ppm peaks ([Supplementary Figure 6](#)). In the EGCG proton NMR the doublet peak at 6.1 ppm disappeared after reacting with the pea protein ([Supplementary Figure 9](#)). The quercetin proton NMR showed equivalent peaks at 7.7, 7.6, 6.9, 6.4 and 6.2 ppm. But after reaction with the pea protein, the peak at 6.4 ppm decreased significantly ([Supplementary Figure 12](#)). The carbon NMRs show corresponding changes as well.

Under oxidizing conditions, polyphenols react with protein amino groups (forms crosslinks and creates network). The reaction of ortho-quinones with proteins to form C-N or C-S bonds enable in the crosslinking process ([Figure 13](#)). Plant proteins like pea and soy have plenty of lysine amino acid residues. These contain primary amino groups for crosslinking with phytochemicals. Plant based compounds conjugate to the lysine residue's primary (ϵ -) amino group to generate a protein-polyphenol crosslinking platform. Sulphur containing amino acid cysteine is present in small amounts in pea and soy protein. Phytochemicals crosslink proteins by conjugating to the thiol group as well. Possible pea protein-mangiferin, EGCG and quercetin crosslinking products are shown in [Figure 14](#).

Extensive hydrogen bonding is a key feature of KM, protein, and phytochemical structures in an aqueous environment. This is not surprising considering the ester, hydroxyl, amine and oxo functional groups in their structures. Both intermolecular and intramolecular hydrogen bonding opportunities are extensive. Hydrogen bonding will be very conducive for KM interaction with protein and phytochemical for nanoparticles synthesis and encapsulations purposes ([Supplementary Figure 14](#)). Extensive hydrogen bonding feature facilitates KM nanoencapsulation and interaction of KM with protein and phytochemical. It is also favorable for interaction of protein and phytochemical as well.

Compelling Synergy of Pea Proteins with Ketones

The rationale for selecting pea protein is because of its food-grade (GRAS) status, high nutritional value, and ability to form stable nanoparticles via protein–polyphenol interactions. These attributes, along with its favorable environmental footprint, make pea protein a particularly suitable carrier for nutraceutical applications. Plant proteins, in dietary supplements, have gained significant scientific interest due to several advantages, including the amino acids in certain plant-derived proteins stimulate insulin release, both directly as well as through an increased production of glucagon-like peptide-1 (GLP-1) hormones. The choice of pea protein to encapsulate our ketone molecule is rationalized based on a lower glycemic index for this protein.⁶⁸ Pea protein is a rich resource for naturally available plant-derived essential amino acids, including leucine, isoleucine, and arginine.⁶⁹ It is well-known that leucine, isoleucine, and arginine, in combination, stimulate insulin secretion. In fact, recently, three GLP-1-secreting peptides have been discovered from pea protein hydrolysate (PPH) through calcium-sensing receptor (CaSR) activation-based molecular docking.⁷⁷ Indeed, it is now known that PPH-triggered GLP-1 secretion is presumably mediated by CaSR activation. In the context of glucose mitigation, GLP-1, exerts several physiological actions related to digestion, and is also associated with the modulation of glycemic response. The proven glucose mitigation efficacy of ketones made the choice of “GLP-1-secreting peptides-rich” pea protein compellingly synergistic. In addition, our continued quest to reduce carbon footprint in drug design, as well as in functional foods, prompted us to utilize plant-based protein as an encapsulating agent^{42,47} for the development of protein functionalized ketone diet.^{78–84}

These findings establish phytochemical-based protein KM nanoparticles as the most promising formulation for sustained ketone delivery and underscore their translational potential for nutraceutical and functional food applications.⁸² Practical opportunities include incorporation into energy drinks to support athletic performance and recovery,⁸³ clinical nutrition supplements to aid patients with metabolic or neurological disorders, and oral ketone therapies as safer alternatives to high-dose ketone esters or salts.⁸⁴

The study was designed as a pilot with a modest sample size.⁵⁴ Stability testing was restricted to refrigerated conditions, and resilience under commercial manufacturing (pasteurization, high shear mixing etc.) has not been established.⁶⁰ Looking ahead, future investigations should prioritize comprehensive stability evaluations under ICH-compliant and processing conditions to confirm robustness during manufacturing and storage. Further optimization of formulation scalability, sensory and consumer acceptance, and chronic dosing regimens in human subjects will be necessary to translate these plant protein–polyphenol nanocarriers from pilot studies into commercial nutraceutical and clinical applications.

Conclusion

This study demonstrated that fully plant-based phytochemical crosslinkers (mangiferin, EGCG, quercetin) can be effectively used to stabilize pea protein nanoparticles for ketone molecule delivery. KM encapsulated Pea Protein Nanoparticles by Berry Extract crosslinking (KM-PP-BE-NP) provided highly favorable outcome, yielding very high plasma BHB levels, a sustained release profile, and superior AUC compared with free KM. These findings highlight the novelty of our approach and establish phytochemical crosslinked protein nanoparticles as a promising food-compatible delivery platform for exogenous ketones with clear translational potential.

At the same time, several limitations like the modest sample size typical of pilot studies, stability testing restricted to refrigerated conditions, and the lack of validation under industrial processing settings must be acknowledged. Continued efforts to validate long-term stability, scalability, and clinical performance will determine the full translational potential of these biocompatible nanocarriers.

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Disclosure

Mr Alton Chesne reports a patent PCT/US2023/021578 pending to NA; and Alton Chesne is an unpaid advisor to Tecton Group, LLC. Professor Kattesh Katti reports a patent. We have filed a patent regarding the work described in this manuscript, pending to None. The author(s) report no conflicts of interest in this work.

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