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Study Of Stability And Anti-Inflammatory Action Of Naringin Loaded ß-Lactoglobulin Nanoparticles

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Abstract
In this study, the effect of naringin loaded β-lactoglobulin nanoparticles on stability of naringin and its anti-inflammatory action were studied. When exploring the effect of adding β-lactoglobulin nanoparticles, 0.01g β-lactoglobulin nanoparticles was evaluated for stability by total phenolic content and the total antioxidant activity. The total phenolic content in the test samples was determined quantitatively using Folin-Ciocalteu reagent method. The antioxidant action of the synthesized compounds was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The anti-inflammatory activity of the test samples of naringin and naringin β-Lactoglobulin nanoparticles was determined by albumin denaturation assay and the antiprotease activity determination assay. The total phenolic content increased on addition of β-lactoglobulin and preparation of nanoparticles further improved the total phenolic content suggesting higher heat stability of naringin when formulated as β-lactoglobulin nanoparticles. It was also witnessed that the formulation of β-Lg nanoparticles loaded with naringin improved the antioxidant activity of naringin suggesting improved stability of the encapsulated naringin. The naringin loaded β-lg nanoparticles exhibited higher anti-inflammatory action in comparison to pure naringin in both the assay methods. The improved stability of naringin in nanoparticles may be an attributing factor for the improved activity.
Keywords:*Naringin, β-lactoglubulin nanoparticles, anti-inflammatory, stability, phenolic*

INTRODUCTION

Phenolic compounds despite having have emerged as a class of natural products shown to have anti-oxidant, anti-atherogenic, and normolipidemic effects have limited use owing to poor aqueous solubility and low bioavailability. Naringin is a natural antioxidant isolated for Grapefruit and is known to possess a large number of pharmacological actions. The antioxidant potential of the molecule is mainly responsible for almost all of its effect on the human body. The half-life of the molecule is though low (3.5h) which limits the use of the molecule in therapy by lowering its bioavailability. Naringin is hydrolyzed to naringenin by gut flora prior to being absorbed and naringenin has been found to have very low bioavailability of around 5.8% [1]. In our previous study we optimized naringin loaded ß-lactoglobulin nanoparticles with an encapsulation efficiency of 38.6% with particle size 227 nm and PDI 0.213. The optimized conditions were found to be 5:1 molar ratio of drug to ß-Lg, 45 min heating at 75°C and pH of 6.0. The in vitro release exhibited controlled release of naringin from the nanoparticles for 12 h. The results of our previous study led to the conclusion that protein nanoparticles (ß-lacotoglobulin) could be an effective method to improve bioavailability of naringin [2]. In continuation to our study, the present work focused on assessing the stability of naringin in β-lactoglobulin nanoparticle formulation and evaluating the anti-inflammatory activity of the nanoparticles.

MATERIAL AND METHODS

Effect of heating and β-lactoglobulin and β-lactoglobulin nanoparticle on the stability of naringin

Varying concentrations of naringin solutions/suspensions (20-100 µg/mL) were heated at 75°C in a water-bath for 45 minutes and moved to an ice bath for 10 minutes afterwards. Then, samples were taken for analysis. When exploring the effect of adding β-lactoglobulin, 0.01g β-lactoglobulin powder was added to 5mL naringin standard solutions/suspensions of varying concentrations in a 7mL sterile container. Then samples were evaluated for stability by total phenolic content and the total antioxidant activity. When exploring the effect of adding β-lactoglobulin nanoparticles, 0.01g β-lactoglobulin nanoparticles was evaluated for stability by total phenolic content and the total antioxidant activity [3].

Total Phenolic Content

The total phenolic content in the test sampes would be determined quantitatively using Folin-Ciocalteu reagent method, using gallic acid as the reference standard. For total phenolic content determination, 200 µL of sample was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After incubating at room temperature for 15 min, 300 µL of 20% Na_2CO_3 aqueous solution was added and

the mixture was allowed to incubate at room temperature for 2 h. The absorbance of the solution was measured at 760 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample [4].

Antioxidant activity

The antioxidant action of the synthesized compounds was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [5]. The free radical scavenging activity of the synthesized molecules was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The test samples (100 μL, 100-500 µg/mL) were prepared in DMSO and were mixed with 1.0 mL of DPPH solution and filled up with methanol to a final volume of 4 mL. Absorbance of the resulting solution was measured at 517 nm in a visible spectrophotometer. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(\text{Ao} - \text{At})}{\text{Ao}} \text{ X } 100$$

where Ao is the absorbance of the control (blank, without sample) and At is the absorbance in the presence of the test samples. All tests were performed in triplicate and the results were expressed as mean values ± standard deviations.

Anti-inflammatory action

Preparation of test samples

The naringin powder and naringin nanoparticles were individually dissolved in dimethyl sulfoxide (DMSO) and appropriately diluted to prepare solutions of 100, 200, 300, 400 and 500 µg/mL concentration. Briefly, 10 mg of the either naringin powder or naringin-ß-lactoglobulin nanoparticles was dissolved in 10 mL DMSO to obtain stock solution of 1 mg/mL. From the stock solution 0.5, 1.0, 1.5, 2.0 and 2.5 mL were pipetted out in separate volumetric flasks and the volume of each flask was made upto 5 mL using DMSO resulting in solutions of 100, 200, 300, 400 and 500 µg/mL concentration.

Inhibition of albumin denaturation

The technique of inhibition of albumin denaturation reported previously [6,7] was used with slight modifications.

Preparation of albumin solution

A solution of 1% BSA in deionized water was prepared for the test. It was prepared by dissolving 0.1g BSA in 10 mL of deionized water.

Procedure of Assay

The reaction vessel was filled with 200 µL of BSA, 1400 µL of PBS and 1000 µL of the test solutions. Ibuprofen solution (10 µg/mL) was used in the positive control and distilled water was used in the negative control vessels instead of test solution. The reaction mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. The mixtures were then allowed to cool to room temperature and the absorbance of constituent of each vessel were analyzed in UV-Visible spectrophotometer at 660 nm. The inhibition of percent denaturation of albumin was determined using the following formula:

% Denaturation inhibition = (1−D/C) × 100%

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

RESULTS AND DISCUSSION

Effect of heat on stability of naringin

Total Phenolic Content

The effect of heat on stability of naringin was evaluated by quantifying the total phenolic content of the heated samples of naringin, naringin with native ß-Lg and naringin ß-Lg nanopartticles. Standard curve of gallic acid was plotted in distilled water (Figure 1). The result of the total phenolic content of the extract examined using Folin-Ciocalteu method (Table 1).

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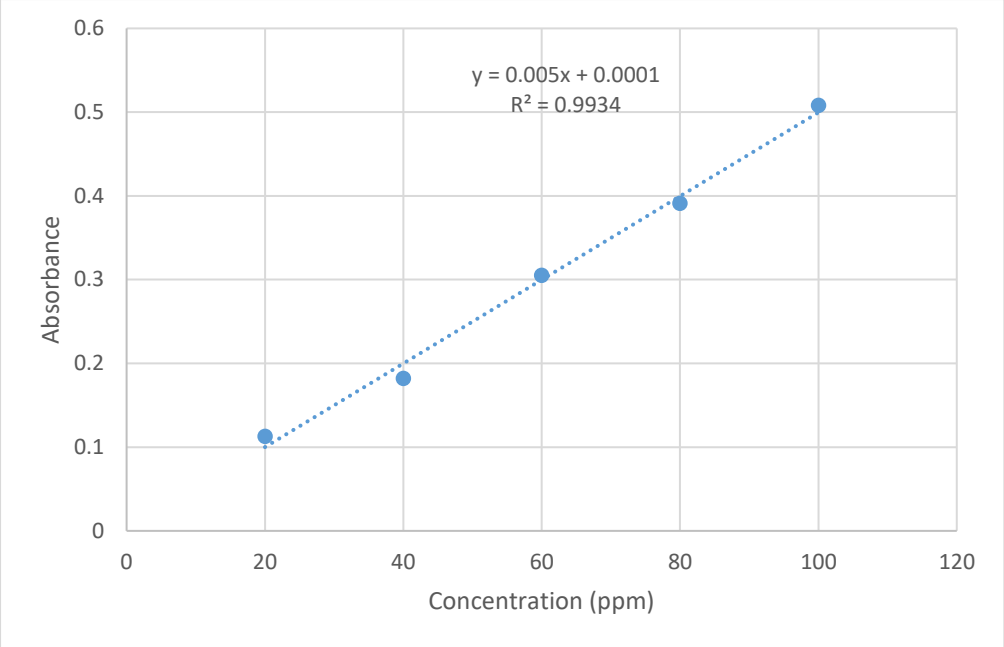

Figure 1 Calibration curve of gallic acid

Table 1 Total Phenolic Content of solutions

Concentration	Total Phenolic Content		
	Naringin	Naringin + ß-Lg	Naringin ß-Lg Nanoparticles
20	31.18	36.78	48.98
40	45.58	51.18	60.18
60	52.78	58.98	71.18
80	62.18	66.58	79.58
100	74.98	80.38	88.18

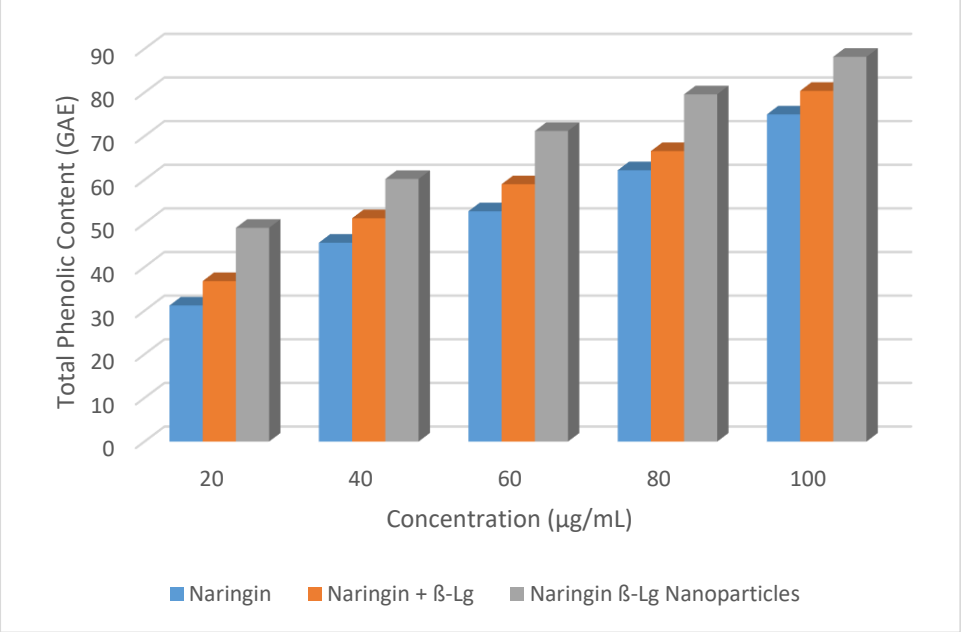

Figure 2 Comparison of TPC of various solution

The TPC increased on addition of ß-lactoglobulin and preparation of nanoparticles further improved the TPC suggesting higher heat stability of naringin when formulated as ß-lactoglobulin nanoparticles.

Antioxidant Action

The absorbance of control (DPPH + methanol) as well as various concentration of the test solution was measured at 517 nm using UV-visible spectrophotometer and the % DPPH inhibition was measured (Table 3).

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Table 3 DPPH radical inhibition by test samples

Concentration	Naringin	Naringin + ß-Lg	Naringin ß-Lg Nanoparticles
10	12.59	24.18	34.38
20	18.83	28.95	40.94
30	24.53	32.16	46.73
40	26.78	38.69	52.08
50	29.26	44.23	57.29

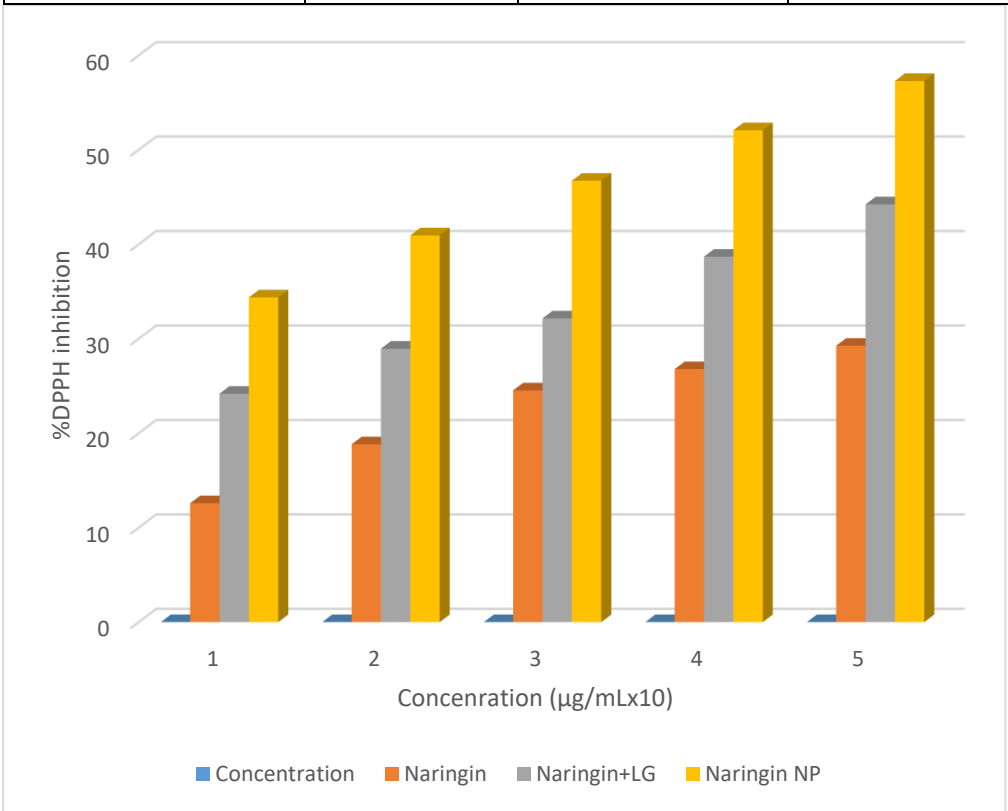

Figure 3 Comparison of antioxidant action of various test samples

It was witnessed that the formulation of ß-Lg nanoparticles loaded with naringin improved the antioxidant activity of naringin suggesting improved stability of the encapsulated naringin.

Anti-inflammatory action

The anti-inflammatory action of the naringin and naringin ß-Lg nanoparticles was evaluated using two of the well established in vitro methods viz., protease inhibition activity and inhibition of albumin denaturation. The results are presented in table 4 and 5 respectively.

Table 4 Inhibition of albumin denaturation by test compounds

Treatment	Inhibition of albumin denaturation (%)					
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	10 µg/mL
Naringin	9.60 ± 1.169	17.58 ± 2.016	30.04 ± 2.032	40.64 ± 2.116	49.76 ± 0.066	ND
Naringin ß-Lg Nanoparticles	15.86 ± 2.105	24.57 ± 2.004	35.28 ± 3.036	56.04 ± 3.101	64.47 ± 1.033	ND
Ibuprofen	ND	ND	ND	ND	ND	55.38 ± 2.516

ND-Not Determined; n=3; Values are Mean ± SD

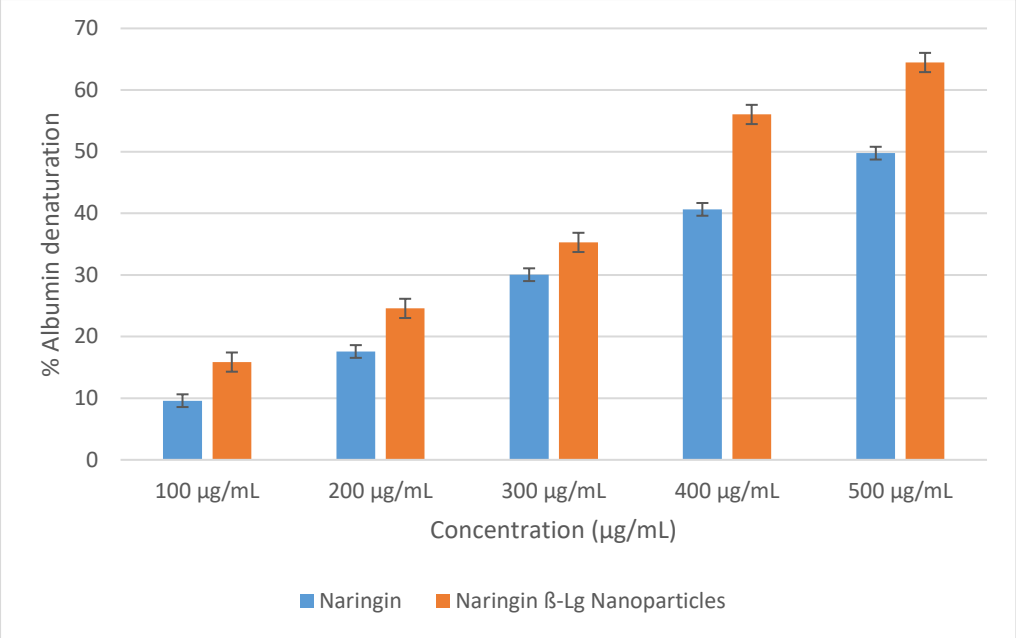

Figure 4 Inhibition of albumin denaturation

Table 5 Percent inhibition of protease action by test compounds

Treatment	Inhibition of Protease Action (%)					
	10 µg/mL	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
Ibuprofen	52.26 ± 1.066	ND	ND	ND	ND	ND
Naringin	ND	8.26 ± 1.039	16.44 ± 0.911	22.36 ± 2.136	25.19 ± 1.299	35.04 ± 3.113
Naringin ß-Lg	ND	11.49 ± 1.066	18.05 ± 1.038	25.84 ± 2.111	36.40 ± 2.036	51.43 ± 3.011

ND-Not Determined; n=3; Values are Mean±SD

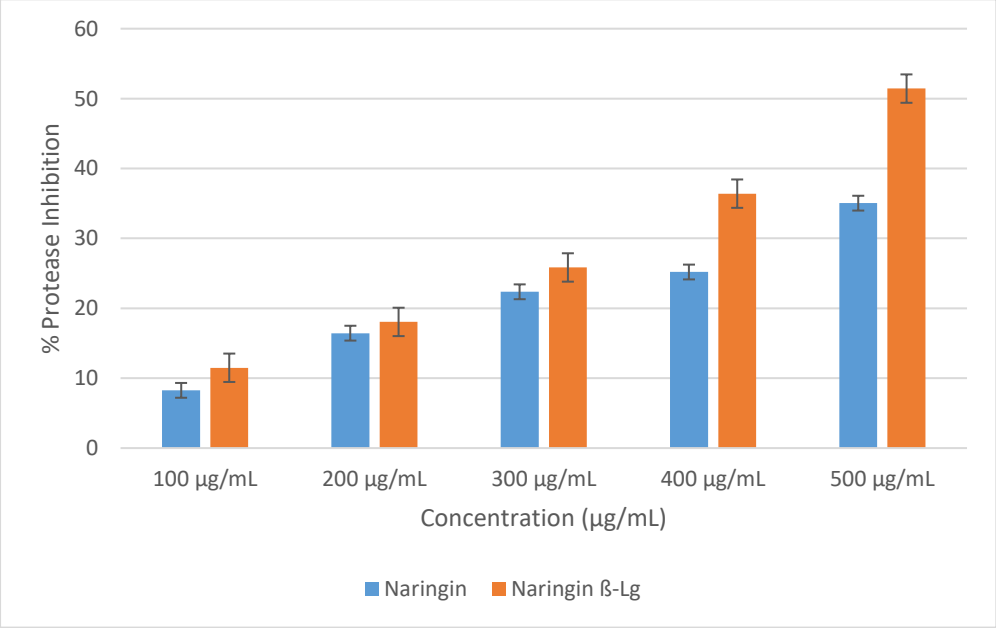

Figure 5 Inhibition of protease action

Protein denaturation has been significantly correlated with the occurrence of the inflammatory response and may lead to various inflammatory diseases including arthritis. It has been said that tissue injury might be due to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of the test compounds to inhibit the denaturation of protein signifies obvious potential for anti-inflammatory activity. It has also been reported that leukocytes protease have an important role in the development of tissue damage during inflammatory reactions and significant level of protection could be

provided by protease inhibitors. Hence the inhibition of protease action by test compounds signifies its role as anti-inflammatory molecules. The naringin loaded ß-lg nanoparticles exhibited higher anti-inflammatory action in comparison to pure naringin in both the assay methods. The improved stability of naringin in nanoparticles may be an attributing factor for the improved activity.

CONCLUSION

The use of naringin as a nutraceutical is greatly limited by its poor water solubility and bioavailability. The water solubility of naringin was measured to be 81.1 μM at 25°C in the pH range of 1-7.6. The poor digestive absorption of the crystalline form in bioactive materials has been reported due to the low solubility and diffusivity. The objective of the study was to prepare ß-lactobulin based nanoparticles of naringin for improving its stability and bioavailability. The results of the study led to the conclusion that the nanoparticle formulation was able to improve the stability of naringin as witnessed from the improved TPC and DPPH radical inhibition. Also the release of naringin was prolonged for more than 12 hours suggesting and improved half-life of the phenolic by formulating as nanoparticles. The anti-inflammatory activity of the phenolic was also improved when formulated as nanoparticles. Hence the objective of the study was concluded to be fulfilled.

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