ORIGINAL ARTICLE

Enhancing antioxidant properties of lime juice powder through polyelectrolyte microparticles of chitosan-alginate: Formulation, characterization and stability study

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Lime (*Citrus aurantifolia*) juice was reported to contain ascorbic acid (AA) and flavonoids, which has bioactivity as antioxidants. To develop an antioxidant product, improving its stability is necessary due to the perishable characteristics of compounds in lime. Therefore, the formulation of polyelectrolyte microparticles using chitosan and alginate was conducted to overcome the weaknesses. This study aims to evaluate the effect of various chitosan, alginate, and lime juice powder (LJP) concentrations on the physical characteristics and antioxidant activity of LJP encapsulated in chitosan-alginate microparticles (CALM). Microparticles with various concentrations of chitosan and alginate were prepared by ionic gelation method using $CaCl_2$ as a crosslinker. The microparticles were evaluated for its physical properties and its antioxidant activity using 2-2-diphenyl-1-picrylhydrazyl reagent. A one-way ANOVA test and Tukey's honest significant difference *post hoc* were used to determine the effect of LJP amount on the antioxidant activity. The highest AA content in CALM was 0.14 mg/100 mg, with a % encapsulation efficiency of 18.38% ± 0.02%. Antioxidant activity tests revealed that LJP possessed the strong antioxidant activity with an IC_{50} value of 32.59 μg/mL, whereas IC_{50} values of the microparticles ranged from 24.79 ± 0.03 μg/mL to 39.96 ± 0.07 μg/mL. During storage, the IC_{50} of LJP decreased from 32.59 ± 0.13 μg/mL to 65.53 ± 0.03 μg/mL, whereas the IC_{50} of microparticles remained stable. This study concluded that the chitosan-alginate polyelectrolyte microparticle formulation can improve and protect LJP's antioxidant activity.

Key words: Alginate, antioxidant, chitosan, lime, microparticles

INTRODUCTION

Free radicals can cause various chronic diseases, such as premature aging. However, the effects of free radicals can be prevented by antioxidants, which stabilize free radicals.[1] Antioxidants are divided into two categories: natural and synthetic antioxidants. However, synthetic antioxidants

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Submitted: 31-Dec-2023 **Revised:** 28-May-2024
Accepted: 01-Jul-2024 **Published:** 22-Jul-2024

Access this article online	
Quick Response Code:	Website: https://journals.lww.com/JAPTR
	DOI: 10.4103/JAPTR.JAPTR_556_23

How to cite this article: Rahmiati N, Sari R, Wahyuni TS, Lestari ML. Enhancing antioxidant properties of lime juice powder through polyelectrolyte microparticles of chitosan-alginate: Formulation, characterization and stability study. J Adv Pharm Technol Res 2024;15:231-6.

have been reported to have side effects such as increasing carcinogenesis; hence limiting their use.[2] Therefore, natural antioxidants, which are abundant sources, are expected to be an alternative for preventing free radicals.

Lime (*Citrus aurantifolia*) contains natural antioxidants such as ascorbic acid (AA) and flavonoids. Lime fruit juice is widely used to increase Vitamin C intake. However, lime fruit is difficult to store for a long time because it is easily damaged even at low temperatures, high temperatures, and when exposed to light and oxygen.[3] In addition, AA in lime fruits is not resistant to heating and is easily oxidized, losing its function when exposed to moist air and light,[4] hence becoming an obstacle to their utilization.[3] Microparticle formulation can be used to overcome the issue by protecting the compound with a polymer layer. Alginate, a polysaccharide polymer, can be used as a microparticle matrix due to its anionic functional group. One method for preparing the alginate microparticles is ionic gelation based on the ability to crosslink with divalent ions such as Ca^{2+}, which can produce polymer gelation.[5] This method can provide high encapsulation efficiency (EE)[6] and does not require a heating process, which is advantageous for encapsulating heat-sensitive compounds.[7] However, the gel's macroporous structure can lead to a sudden release of core substances. The addition of chitosan will form a polyelectrolyte complex (PEC) to overcome this issue.

PEC is formed by polyelectrolytes that have opposite charges through electrostatic attraction in aqueous solution.[8] Chitosan-alginate PEC is formed through interactions between the primary amino groups (NH^{3+}) of chitosan (polycationic polymer) and carboxyl groups (COO^{-}) of alginate (polyanionic polymer) resulting in gelation polymer. The formation of PEC can increase the stability of microparticles and reduces the porosity of alginate gel.

Therefore, this study was conducted to formulate lime juice powder (LJP) in PEC microparticles by combining chitosan-alginate to protect the antioxidant compound. The physical characteristics of Chitosan-Alginate-LJP-Microparticle (CALM) were evaluated, as well as the antioxidant activity of CALM. The novelty of this research is the utilization of natural ingredients, lime in freeze-dried powder form as an antioxidant, and improve the stability with the use of polyelectrolyte microparticles chitosan and alginate.

MATERIALS AND METHODS

Materials

Lime fruit (*C. aurantifolia*) harvested from the lime plantation in Ujung Pangkah, Gresik City, Indonesia has been determined at the Herbal Laboratory, Materia Medica, Batu (Certificate number 074/372/102.20-A/2022). Acetic acid (MERCK), $CaCl_2$ dihydrate pro analysis (Merck), chitosan 19 cps (Biotech Surindo), alginate 80–120 cps (Wako), L - AA powder (EMSURE, Merck), and 2-2-diphenyl-1-picrylhydrazyl (DPPH) powder (Chemindo, Analytical reagent).

Methods

Chitosan-alginate-LPJ microparticles (CALM) preparation

CALM was prepared by formulating different compositions of chitosan and alginate [Table 1].

Alginate was dissolved in 45 mL of distilled water with stirring at 500 rpm. LJP was dissolved in 5 mL of distilled water and mixed with alginate.

Chitosan was dissolved in 50 mL of 1% acetic acid, then 50 mL of 4% $CaCl_2$ solution was added to the chitosan solution.

The alginate-LJP solution was dripped into the chitosan-$CaCl_2$ solution using a 22G syringe. After 10 min, the microparticles were filtered and washed with distilled water. The microparticles were then dried at 40°C ± 5°C for 2 h. Dry microparticles were stored in a desiccator for further evaluation.

Macroscopic and microscopic examination

The microparticles were then observed for their color, shape, and odor. Microscopic (KERN) observations were made at ×40.

Fourier transform infrared

Infrared spectral analysis of microparticles from each formula was performed using Fourier transform infrared (FTIR) spectrometer Bruker Eco-ATR (ALPHA II), and OPUS 8.1.29 software was used to analyze the infrared spectrum.

X-ray diffraction

X-ray diffraction (XRD) analysis of microparticles was performed with a diffractometer (Panalytical X'pert Pro) at a short angle with a range of 2Ø 5°–40°.

Particle size

Particle size measurements were made on 100 particles with ×40 using an optical microscope (KERN) and Optics Lab Software.

Moisture content evaluation

The moisture content (MC) of microparticles was determined

Table 1: Formulation of chitosan-alginate microparticles

Materials	Amount (%)			
	F1	F2	F3	F4
LJP	0.4	0.4	0.4	0.4
Chitosan	0.25	0.25	0.5	0.5
Alginate	0.75	1	0.75	1
$CaCl_2$	4	4	4	4

LJP: Lime juice powder

using a moisture analyzer (Mettler Toledo, HC103). 500 mg of microparticles was placed on a plate and then heated. The % MC was recorded.

Encapsulation efficiency

The EE was determined by dissolving 50 mg of CALM in 10 mL methanol p.a (5000 ppm), then filtered and diluted to 2000 ppm. The samples were analyzed spectrophotometrically at a wavelength of 277 nm. The sample concentrations were calculated using a linear regression equation.[9] The EE was calculated by the equation below:

$$\% \text{ EE} = \frac{\text{Actual drug amount}}{\text{Theoretically drug amount}} \times 100\%$$

Antioxidant activity test

Antioxidant activity tests were carried out using the DPPH method. A 50 mg CALM was dissolved in 10 mL of methanol p.a and incubated for 3 h then sonicated for 10 min. Samples were diluted into 15, 20, 25, 30, 35, and 40 µg/mL for each formula. Further, 1 mL sample was added to 3 mL of 0.004% DPPH solution. The mixture was incubated for 30 min. The absorption was measured using a ultraviolet-visible spectrophotometer at 517 nm. The IC_{50} was determined by calculating the value using a calibration curve by plotting the concentration of the solution and % inhibition.

Antioxidant stability during storage

Stability test on antioxidant activity was carried out under accelerated conditions for 21 days. 50 mg of LPJ and microparticles were put into the vial and stored at a temperature of 40°C ± 2°C and RH of 75% ± 5% in a Climatic Chamber (Climacell, MMM-Group). The samples were tested on days 0, 2, 7, 14, and 21. Each sample was analyzed for its antioxidant activity using the DPPH method.[9,10]

Data analysis

The %MC and EE were statistically analyzed using two-way ANOVA, whereas IC_{50} was analyzed using one-way ANOVA, followed by *post hoc* Tukey honest significant difference (HSD) test.

RESULTS AND DISCUSSION

Based on the study by Rahmiati *et al*., LJP was yellow-orange with a specific odor of lime [Figure 1] and contains flavonoid compounds that have antioxidant activities, but LJP is a hygroscopic powder.[11] Thus, entrapment of LJP in biodegradable chitosan-alginate microparticles can extend the shelf life of LPJ, and LPJ encapsulated in chitosan-alginate beads is protected from heat and degradation.

The macroscopic examination showed that the particles were spherical and not easily damaged after drying [Figure 2]. The dried microparticles were in the form of granules with a yellowish color and did not have any odor despite containing LJP [Figure 2]. Microscopic examination [Figure 3] revealed that the microparticles were spherical.

The results of the FTIR examination in Figure 4 show a stretching band around 3600-3100 per cm^{-1}, indicating an increased hydrogen bond between chitosan and alginate. The bending of the NH group at 1581 per cm and the stretching of the-C-O group at 1592 per cm and 1409 per cm in the FTIR spectra of microparticles F1, F2, F3, and F4 appeared to disappear, indicating that the $–NH^{3+}$ group of chitosan reacted with the $–COO^-$ group of alginates.[12] The shift in the absorption of LJP bands at 1133 per cm and the absorption of chitosan bands at 1022 per cm and alginate at 1028 per cm showed the interaction of the three materials. Microparticles F1, F2, F3, and F4 showed similar results, such as the presence of O-H groups at absorption 3395–3377 per cm^{-1} [Figure 4].

The diffractogram in Figure 5 shows that the sharp peaks of LJP were not visible in the microparticle, indicating that the LJP contained in the microparticles had been encapsulated in the CALM.[13] Hence, the results of FTIR and XRD examinations showed that LJP had been entrapped in chitosan-alginate microparticles.

The particle size of CALM with higher alginate concentrations (1%) in F2 and F4 was more extensive compared to formulas with alginate concentrations of 0.75% (F1 and F3) [Table 2]. This follows the theory that microparticles with higher alginate concentrations can increase their particle size[14] due to the alginate's viscosity. In addition, an increase in chitosan concentration from 0.25% to 0.5% also affects the viscosity of the chitosan solution, resulting in larger particle sizes.[13]

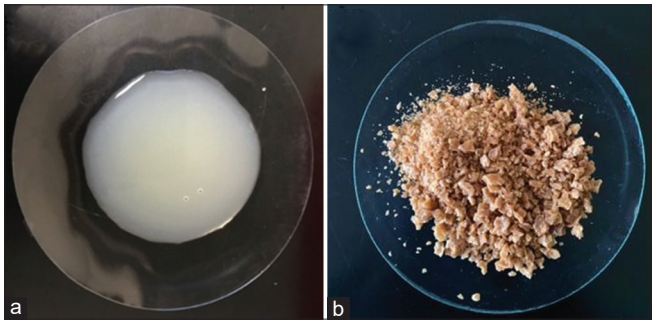

Figure 1: Photograph of (a) Lime juice, (b) Freeze drying powder (lime juice powder)

Table 2: Particle size and moisture content of chitosan-alginate microparticles

Formulas	Particle sizes (µm)	MC (%)±SD
F1	32.34–54.12	4.27±0.04
F2	38.55–52.92	4.50±0.03
F3	47.59–59.92	4.53±0.03
F4	51.45–76.74	4.96±0.02

MC: Moisture content, SD: Standard deviation

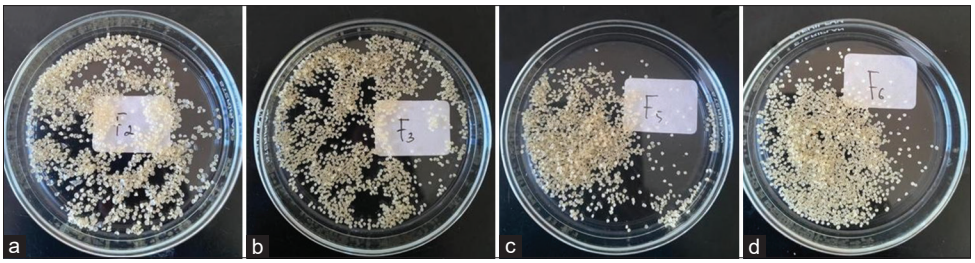

Figure 2: Photograph of CALM with various concentrations of chitosan: Alginate: (a) 0.25:0.75, (b) 0.25:1, (c) 0.5:0.75, (d) 0.5:1.0

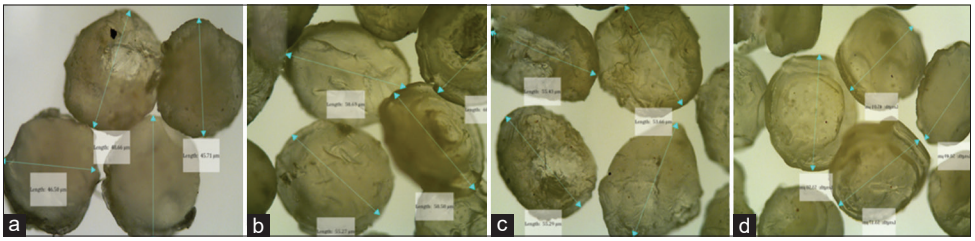

Figure 3: Micrograph of CALM with various concentrations of chitosan: Alginate: (a) 0.25:0.75, (b) 0.25:1, (c) 0.5:0.75, (d) 0.5:1.0

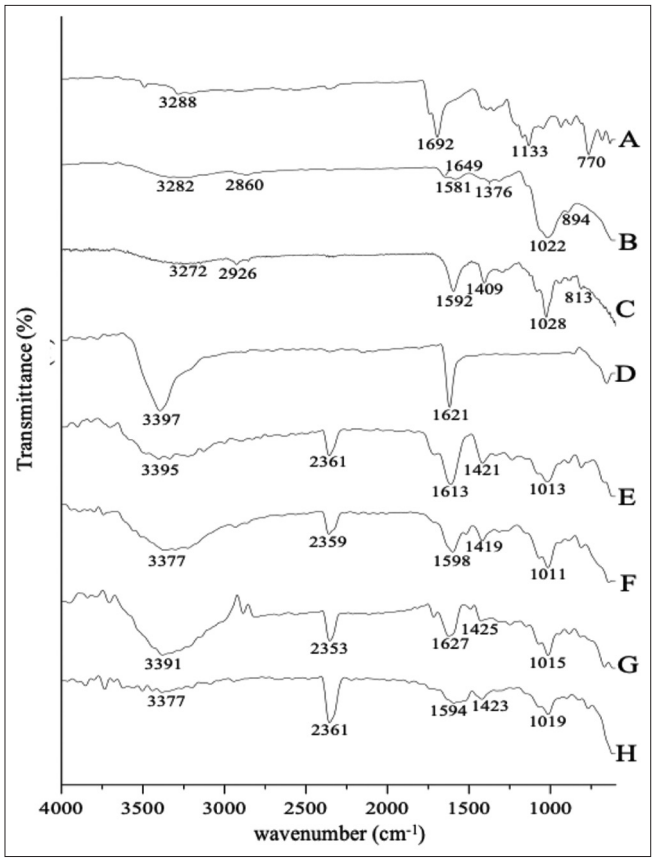

Figure 4: Fourier transform infrared spectra of: A: Lime juice powder, B: Chitosan, C: Alginate, D: $CaCl_2$, and CALM with various concentrations of chitosan: Alginate: E: 0.25:0.75, F: 0.25:1, G: 0.5:0.75, H: 0.5:1.0

Table 2 shows a slight increase of MC caused by increasing both polymer concentrations. Microparticles with 1% alginate and chitosan concentration had the highest % MC. Two-way ANOVA analysis revealed that the concentrations of chitosan and alginate significantly affected % MC ($P = 0.000$, $P < 0.05$), and there was an interaction between chitosan and alginate. Microparticles with low % MC can have high efficiency and good chemical and physical stability,[5] affecting antioxidant activity because more LJP will be encapsulated.

As shown in Table 3, F2 with the lowest chitosan and highest alginate amount had the highest EE compared to the other formulas. This indicated that the interaction of alginate and $CaCl_2$ plays a more significant role in encapsulating LJP because it forms a stronger bond between the COO^- group of alginates and Ca^{2+} than alginate-chitosan. Furthermore, microparticles with various LJP amounts were prepared to determine the loading capacity using the F2 ratio of chitosan and alginate. The EE results in Table 4 indicated that F2-300 had the highest EE compared to the other formulas. It demonstrated that the more LJP added, the lower the strength of the microparticle matrix protecting the encapsulated material. Two-way ANOVA analysis followed by *post hoc* Tukey HSD revealed that the EE values of all formulas were significantly different ($P = 0.000$, $P < 0.05$).

The antioxidant activity test using DPPH is often used to assess the antioxidant activity of various natural ingredients and is stable at room temperature.[15]

According to the previous study, the IC_{50} of AA was 8.57 µg/mL, whereas the IC_{50} of LJP was 32.59 µg/mL.[11] As seen in Table 4, F2-200 exhibited the lowest IC_{50}, indicating that the antioxidant activity of F2-200 is more potent compared to other formulas.

One-way ANOVA analysis followed by *post hoc* Tukey HSD revealed that the IC_{50} values of all formulas were

significantly different (P = 0.000, <0.05). However, the antioxidant activity of microparticles was categorized as strong antioxidants with IC_{50} below 50 µg/mL.[16]

Table 3: The ascorbic acid content and encapsulation efficiency of chitosan-alginate microparticles with different concentrations of chitosan and alginate

Formula	AA content (% b/b)±SD	EE (%)±SD
F1	0.11±0.0001	14.41±0.01
F2	0.14±0.0001	18.38±0.02
F3	0.11±0.0002	14.32±0.02
F4	0.11±0.0002	14.64±0.03

AA: Ascorbic acid, EE: Encapsulation efficiency, SD: Standard deviation

Table 4: Encapsulation efficiency and Inhibitory concentration 50 antioxidant activity of chitosan-alginate microparticles with different lime juice powder amount

Sample	EE (%)±SD	IC_{50}±SD (µg/mL)
F2-200	18.38±0.02	24.79±0.03
F2-300	28.99±0.14	37.84±0.04
F2-400	27.48±0.10	39.96±0.07
F2-500	26.22±0.05	36.50±0.09

EE: Encapsulation efficiency, SD: Standard deviation

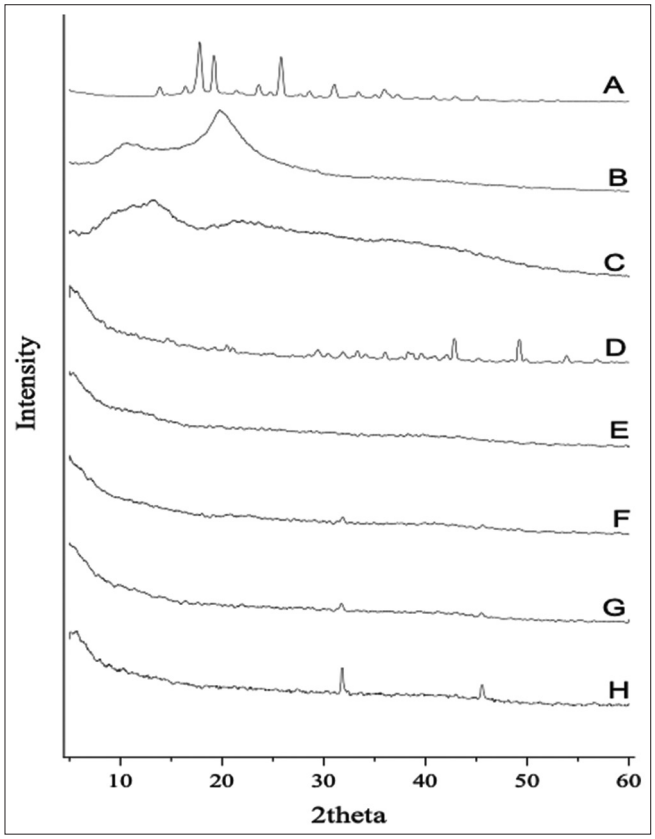

Figure 5: Diffractogram of A: Lime juice powder, B: Chitosan, C: Alginate, D: $CaCl_2$, and CALM with various concentrations of chitosan: alginate: E: 0.25:0.75, F: 0.25:1, G: 0.5:0.75, H: 0.5:1.0

Moreover, the antioxidant activity of LJP decreased during storage, whereas LJP in chitosan-alginate microparticles remained stable [Figure 6]. These results demonstrated that the PEC microparticles of chitosan-alginate can protect the antioxidant activity. These results were in accordance with research conducted by Boškov *et al.*,[17] after encapsulation in alginate-chitosan microparticles, the antioxidant activity of extract flower significantly increased.

CONCLUSION

It was shown that increasing chitosan and alginate concentrations enhanced particle size (40.59 µm–60.25 µm) and CALM MC (4.27%–4.96%). The formula with the least chitosan and highest alginate concentration had the maximum EE. Polyelectrolyte microparticles chitosan-alginate of LJP had strong antioxidant activity (IC_{50} < 50 µg/mL). The antioxidant activity of LJP reduced considerably with storage, whereas the polyelectrolyte microparticles remained stable. Chitosan-alginate polyelectrolyte microparticles are promising for a stable natural and synthetic antioxidant delivery system that will boost application in the health sector.

Acknowledgment

This research was financially supported by Airlangga Research Fund, Penelitian Dasar Unggulan Scheme 2023, Airlangga University, Indonesia, Contract No. 748/UN3.1.5/PT/2023.

Financial support and sponsorship

This research was financially supported by Airlangga Research Fund, Airlangga University, 2023.

Conflicts of interest

There are no conflicts of interest.

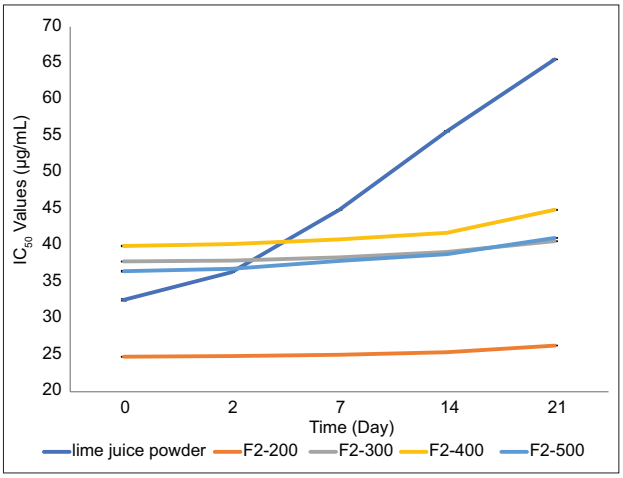

Figure 6: Graph of IC_{50} of stability test of lime juice powder and CALM with different lime juice powder amount

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