



Development of chitosan-based *Centella asiatica* nanoparticles and their immunostimulatory activity through macrophage phagocytosis assay

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Abstract

Background *Centella asiatica* (L.) extract (ECA) has demonstrated immunostimulatory effects, with macrophages playing a key role in immune regulation. However, a significant proportion of bioactive compounds derived from natural sources exhibit poor aqueous solubility and may pose toxicity concerns, hindering their absorption and reducing their bioavailability. To address these limitations, nanotechnology-based drug delivery approaches have been explored, including the use of chitosan, a biocompatible polymer, as a carrier material.

Methods *Centella asiatica* extract-loaded chitosan nanoparticles (NECA) were developed and evaluated in this study to investigate their physicochemical properties and immunostimulatory activity. The nanoparticles were formulated using the ionic gelation technique, followed by freeze-drying, with different concentrations of ECA and sodium tripolyphosphate as the crosslinking agents. Comprehensive characterization was conducted, including particle size analysis, morphological observation, Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and X-ray diffraction (XRD).

Results The resulting NECA particles ranged in size from 136.4 to 451.6 nm, with encapsulation efficiencies of 79.47% to 89.27%. XRD analysis indicated a transformation of the material from crystalline to predominantly amorphous, which may enhance solubility. The immunostimulatory activity was assessed in Balb/c mice using the carbon clearance assay. Administration of NECA at doses of 100 and 200 mg/kg significantly increased macrophage phagocytic activity and resulted in higher lymph and liver indices compared with the control group ($p < 0.05$).

Conclusion These results demonstrate that NECA can enhance macrophage-mediated immune responses and may serve as a promising nanoparticle-based immunostimulant delivery system.

Keywords *Centella asiatica* · Chitosan nanoparticles · Phagocytosis index · Immunostimulant · Health outcomes

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1 Introduction

As an important component of the immune response, phagocytosis involves the activity of neutrophils and macrophages that function to engulf and eliminate foreign materials [1]. Immune system dysregulation can negatively impact health and quality of life [2]. The immune system relies on macrophages as key effector cells responsible for phagocytosis, a mechanism that facilitates the elimination of harmful pathogens. Various factors, including aging and disease, can compromise immune performance and subsequently reduce the efficiency of cell-mediated immunity (CMI) [3]. An effective immune system is vital for combating invading pathogens. Immunostimulants contribute to this process by stimulating multiple immune mechanisms, such as phagocytosis, complement activation, antibody generation, and the responses of T and B lymphocytes.

Medicinal plants represent an important source of immunostimulatory compounds, although such agents may also be produced synthetically. One of the most widely studied herbs is *Centella asiatica* (L.), or gotu kola, which is known for its broad spectrum of therapeutic effects. Its biological activities have been evaluated in numerous studies ranging from in vitro experiments to in vivo and clinical investigations. This plant contains several secondary metabolites, including alkaloids, saponins, tannins, triterpenoids, and glycosides. Among these constituents, asiaticoside is the predominant compound and is recognized for its ability to modulate immune responses [4–6]. However, its low water solubility reduces its bioavailability and therapeutic effectiveness. To improve solubility and bioavailability, various strategies, such as nanotechnology-based delivery systems, have been explored [7–9].

The application of nanotechnology in pharmaceutical formulations has been widely explored to improve the delivery efficiency of bioactive substances [10]. By converting compounds into nanoscale particles, their surface area increases, which can enhance dissolution and ultimately improve bioavailability [11]. In addition, nanoparticle-based systems enable more controlled drug release and may help minimize undesirable side effects during treatment. For this reason, the materials used as nanoparticle carriers must exhibit good biocompatibility and minimal toxicity. One polymer that has attracted considerable attention for this purpose is chitosan [3]. This natural polymer is a linear polysaccharide consisting of repeating units of D-glucosamine and N-acetyl-D-glucosamine linked through β -(1 $\rightarrow$ 4) glycosidic bonds. The molecular structure of chitosan contains several reactive groups, including amine, primary hydroxyl, and secondary hydroxyl groups, which contribute to its versatility in

nanoparticle preparation [12]. Duraisamy et al. [13] demonstrated the effectiveness of chitosan as a nanoparticle-forming material, producing spherical particles with an average diameter of approximately 208 nm, a positive zeta potential of + 27 mV, and an encapsulation efficiency of 51% [13].

One of the most widely used techniques for preparing chitosan nanoparticles is ionic gelation. During this process, electrostatic attraction occurs between the positively charged amino groups of chitosan and negatively charged multivalent polyanions, with sodium tripolyphosphate (STPP) commonly utilized as a non-toxic crosslinking compound. This technique results in nanoparticles that are stable and uniform, making them suitable for drug delivery [14]. The properties of the nanoparticles are influenced by various factors, including the concentrations of chitosan and crosslinker, pH, and crosslinking time. [15]. Variations in the amounts of chitosan and TPP used during formulation can markedly influence key nanoparticle characteristics, including particle size, surface morphology, and encapsulation efficiency, which are important determinants for successful drug delivery performance [16–18].

Although chitosan nanoparticles have been widely studied for drug delivery, research on their application with *Centella asiatica* extracts and their impact on immune cell function remains limited. This study aims to develop *Centella asiatica* extract-chitosan nanoparticles (NECA) with varying concentrations of chitosan, characterize their physical and chemical properties, and assess their ability to stimulate immune activity in Balb/c mouse macrophages using the carbon-scavenging method.

2 Materials and methods

2.1 Plant materials

Authentication of the dried *Centella asiatica* (L.) herb used in this study was conducted by the Technical Implementation Unit – Herbal Materia Medica Laboratory, Batu, Indonesia, and documented under certificate number 074/367/102.20-A/2022.

2.2 Chemicals

The reagents utilized in this study consisted of chitosan (Biotech Surindo, Indonesia), glacial acetic acid (Merck, Germany), sodium tripolyphosphate (STPP) (Nacalai Tesque, Japan), sodium carboxymethyl cellulose (CMC-Na) (Sigma-Aldrich, USA), 0.9% sodium chloride solution (Widatra, Indonesia), ethanol 70% (Merck, Germany), methanol (Merck, Germany), distilled water (Merck, Germany), and 4001-Black Ink (Pelikan, Germany).

2.3 Extraction

Extraction was performed by macerating 900 g of dried *Centella asiatica* herb in 70% ethanol at a 1:10 ratio for 24 h at room temperature, with three cycles of extraction. The extract was then concentrated using rotary evaporation and freeze-dried to obtain a dry extract [6].

2.4 Experimental animal

A total of 25 healthy Balb/c mice (*Mus musculus*) aged 6–8 weeks, weighed 25–30 g were used in this research. Before the experiment, all animals were allowed to acclimate to laboratory conditions for at least 7 days, with controlled temperature (22–24 °C), 50–60% humidity, and a 12-h light–dark cycle. Food and water were provided ad libitum during the acclimatization and experimental periods. In this study, five groups were used ($t = 5$), with n representing the number of animals per group, ensuring sufficient sample size for reliable data while minimizing animal use in line with ethical guidelines. The groups include: a negative control group receiving 0.5% CMC-Na solution, an experimental group treated with ECA at a dose of 40 mg/kg BW, and three groups receiving NECA at doses of 50, 100, and 200 mg/kg BW. These groups are designed to evaluate the effects of different doses of ECA and NECA while maintaining an adequate sample size for statistically significant results. The sample size ($n = 5$) was determined based on the following formula (Eq. 1):

$$\begin{aligned} (t-1)(n-1) &\geq 15 \\ (5-1)(n-1) &\geq 15 \\ n-1 &\geq 3.75 \\ n &\geq 4.75 \sim 5 \end{aligned} \quad (1)$$

2.5 Ethical Clearance

This research was approved by the research ethics committee of the Faculty of Veterinary Medicine, Universitas Airlangga, with ethical clearance certificate No. 2.KEH.005.01.2023.

2.6 Preparation of *Centella asiatica* extract – Chitosan Nanoparticles (NECA)

NECA were formulated using the ionic gelation approach by varying the concentration of chitosan to 0.03% (F1), 0.04% (F2), and 0.05% (F3). The extract solution was first prepared by dissolving 40 mg of ECA in 10 mL of ethanol. In a separate preparation, 40 mg of chitosan was solubilized in 0.15% glacial acetic acid to obtain the required polymer

solutions. The ECA solution was then incorporated into the chitosan solution with continuous mixing. For the crosslinking step, sodium tripolyphosphate (STPP) solution was prepared by dissolving 20 mg of STPP in 80 mL of distilled water pH 7. This solution was gradually introduced into the chitosan–extract mixture under magnetic stirring at 500 rpm at room temperature to induce nanoparticle formation. The resulting suspension was subsequently centrifuged at 30,000 rpm for 30 min at 4 °C to separate the formed nanoparticles. The precipitated nanoparticles were collected and subjected to freeze-drying for 24 h to obtain a dry powder. The formation of nanoparticles in this system is driven by ionotropic gelation, which occurs through electrostatic interactions between the protonated amino groups of chitosan and the negatively charged groups of STPP.

2.7 Morphology evaluation

Morphological analysis of the nanoparticles was conducted using Scanning Electron Microscopy (SEM) (Thermo Fisher Phenom™ Pro X), providing high-resolution imaging of the nanoparticle surface. To improve conductivity, a gold–palladium coating was applied for about 120 s. SEM was operated under vacuum at 10 kV, a standard condition for nanoscale imaging. This technique offers important details on particle shape, size distribution, and surface texture, essential for understanding the nanoparticles' physicochemical properties [19].

2.8 Particle Size evaluation

Nanoparticle size distribution was evaluated by Dynamic Light Scattering (DLS) using a Delsa Nano C Particle Analyzer (Beckman Coulter). The analysis was conducted in a glass measurement cell with an integration time set at 30/100. DLS determines particle size by monitoring variations in scattered light intensity that occur as nanoparticles undergo Brownian motion in suspension, allowing estimation of their hydrodynamic diameter. Along with the average particle diameter (ZAve), the polydispersity index (PI) was also calculated. The PI indicates the particle size distribution, with values close to 0.1 indicating a narrow distribution, and values near 1.0 suggesting a wider distribution [20].

2.9 Spectroscopy ATR-FTIR analysis

The Fourier-transform Infrared (FTIR) spectra were obtained using an Alpha II FTIR Spectrometer (Bruker). This technique identifies functional groups in the sample by measuring infrared light absorption at specific wavelengths. The sample was placed on the Attenuated Total Reflectance (ATR) crystal and gently pressed for optimal contact. FTIR

spectra were recorded in the 400–4000 cm^{-1} wavelength range. The software used for analysis was OPUS 8.1.29 (Bruker), enabling accurate peak identification and functional group analysis [21].

2.10 X-ray diffractogram evaluation

The crystalline structure of the prepared nanoparticles was characterized by X-ray diffraction (XRD) using an X'Pert Diffractometer manufactured by Philips (Netherlands). The analysis was carried out at ambient temperature employing Cu $K\alpha$ radiation filtered with Ni, while the diffractometer was operated at 40 kV and 40 mA to generate the diffraction profile. The scan range was $2\theta = 5^\circ - 40^\circ$ to identify the diffraction patterns. XRD is valuable for assessing crystallinity and phase transitions, and it plays a key role in confirming the amorphous or crystalline nature of the nanoparticles [19].

2.11 Differential scanning calorimeter analysis

The melting point and thermal properties of the nanoparticles were analyzed using a Linseis Differential Scanning Calorimetry (DSC) TP-1600. DSC measures heat flow into or out of a sample as it is heated or cooled, providing information on the material's thermal transitions, such as the melting point, glass transition temperature, and crystallization behavior. The analysis was conducted between 50 $^\circ\text{C}$ and 300 $^\circ\text{C}$ at a heating rate of approximately 5 $^\circ\text{C}$ per minute. DSC is particularly useful for evaluating the stability of nanoparticles, as it helps determine if the encapsulated drug or other components undergo phase transitions, which could affect the performance of the drug delivery system [22].

2.12 Encapsulation efficiency and drug loading evaluation

The encapsulation efficiency (EE) and drug loading capacity of NECA nanoparticles were determined through thin-layer chromatography (TLC) based on a previously by Pratiwi et al. [6]. A standard solution was first prepared by dissolving 1 mg of asiaticoside in 1 mL of methanol. For sample preparation, the NECA powder was dispersed in ethanol and subjected to centrifugation to obtain a clear supernatant fraction. A volume of 32 μL from the resulting solution was carefully applied in triplicate onto a GF254 pre-coated TLC plate (20 $\times$ 10 cm). Chromatographic separation was performed using a solvent system consisting of chloroform, glacial acetic acid, methanol, and water in a ratio of 60:32:12:8. After plate development, the separated spots were quantified using a TLC densitometer at a detection wavelength

of 276 nm, and the corresponding peak areas (AUC) were recorded for analysis [6]. The percentage of encapsulation efficiency was subsequently calculated using the following equation:

$$\text{Encapsulation Efficiency} \left(\frac{\% w}{w} \right) = \frac{(\text{Mass of the total drug} - \text{Mass of free drug})}{\text{Mass of total drug}} \times 100 \quad (2)$$

For drug loading evaluation, the same TLC method was used, and the drug loading (DL) was calculated using the equation:

$$\text{Drug Loading} = \frac{\text{Amount of Drug Encapsulated}}{\text{Total Weight of Nanoparticles}} \times 100 \quad (3)$$

2.13 Macrophage activity test and organ weight analysis

The immunostimulatory activity of the samples was evaluated by assessing macrophage phagocytic function using the carbon clearance assay in experimental animals divided into five groups. The negative control group was administered 0.5% CMC-Na at a dose of 10 mL/kg body weight, while the extract-treated group received 200 mg/kg body weight. The remaining three groups were given NECA derived from Formula 1 at doses of 50, 100, and 200 mg/kg body weight, respectively. All treatments were administered orally for seven consecutive days. On the eighth day, each animal was intravenously injected with a 6.4% carbon suspension to initiate the phagocytosis assay. Blood samples were subsequently collected at predetermined time intervals (0, 5, 10, 15, 20, and 25 min), and the absorbance of the samples was measured at 650 nm to monitor the rate of carbon clearance from circulation. Based on these data, the phagocytosis constant and phagocytic index were calculated. Following completion of the assay, the animals were euthanized and their organs were harvested for further analysis. The weights of the liver and lymph nodes were determined to assess immune response and evaluate the safety of the immunostimulant treatment, while changes in lymph and liver mass were used as indicators of immune activation and potential toxicity [42].

$$\text{Phagocytosis constant} = \frac{\text{Log } A(n) - \text{Log } A(n-1)}{t(n-1) - t(n)} \quad (4)$$

In this calculation, the parameter K describes the phagocytosis constant. $A(n)$ is defined as the absorbance value at a specific measurement time, whereas $A(n-1)$ represents the absorbance recorded at the preceding time point. The variable T indicates the sampling times, namely 5, 10, 15, 20, and 25 min, while n designates the corresponding sampling sequence from the first to the fifth interval.

Table 1 Average values of particle size, PDI, encapsulation efficiency, and drug loading of NECA formulations ($n=3$)

Formula	Particle size (nm) $\pm$ SD	PDI $\pm$ SD	Encapsulation Efficiency (%) $\pm$ SD	Drug loading (%) $\pm$ SD
F1	451.6 $\pm$ 1.60	0.266 $\pm$ 0.02	89.28 $\pm$ 1.39	35.71 $\pm$ 0.56
F2	153.2 $\pm$ 1.90	0.264 $\pm$ 0.02	82.25 $\pm$ 2.77	32.90 $\pm$ 1.10
F3	136.4 $\pm$ 1.83	0.295 $\pm$ 0.02	79.47 $\pm$ 1.47	31.79 $\pm$ 0.59

$$\text{Phagocytosis index} = \frac{\text{phagocytosis constant of mice}}{\text{average phagocytosis constant of control mice}} \quad (5)$$

2.14 Statistical analysis

Data on phagocytosis index, liver relative weight, and lymph node relative weight were analyzed using One-Way Analysis of Variance (ANOVA), with Tukey's post hoc test to compare differences between groups. Before performing ANOVA, normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was assessed using Levene's test. A p -value of less than 0.05 was considered significant. All analyses were performed using GraphPad Prism software.

3 Results and discussion

3.1 Characterization of *Centella asiatica* extract – chitosan nanoparticles (NECA)

3.1.1 Particle size and morphology evaluation

NECA formulations were prepared using the ionic gelation method, incorporating freeze-dried ECA into chitosan nanoparticles crosslinked with STPP. The extract was standardized by quantifying the marker compound, asiaticoside [6]. Three formulations (F1, F2, F3) were prepared at different chitosan concentrations to evaluate their effects on particle characteristics.

In this study, variations in chitosan concentration were investigated to assess their effects on nanoparticle characteristics, including particle size, morphology, and encapsulation efficiency. Before freeze-drying, NECA was supplemented with maltodextrin as a lyoprotectant to safeguard the nanoparticles from freezing-induced stresses. Freeze-drying can cause significant stresses that help stabilize nanoparticle colloidal suspensions, especially during freezing. Additionally, ice crystallization can exert pressure on the nanoparticles, potentially destabilizing them [23, 24].

As shown in Table 1, the three formulations exhibited distinct particle sizes, with F1 measuring 451.6 ± 1.60 nm, while F2 and F3 displayed smaller sizes of 153.2 ± 1.90 nm and 136.4 ± 1.83 nm, respectively. Nevertheless, the polydispersity index for all formulations remained within a narrow

range (0.264–0.295), indicating a relatively uniform particle population and stable dispersion after reconstitution. A PDI value below 0.3 generally indicates a consistent particle size distribution. These findings suggest that the freeze-drying process did not substantially alter particle dimensions and that maltodextrin functioned effectively as a stabilizing agent, preserving nanoparticle structure. This aligns with Jiang et al.'s findings, showing that oligosaccharides prevent aggregation and maintain particle size in freeze-dried nanoliposomes. Particle size and PDI are key to assessing nanoparticle stability, especially after freeze-drying [25].

SEM images of the freeze-dried NECA formulations (Fig. 1) revealed an amorphous, porous structure instead of distinct spherical nanoparticles. This is typical when using polysaccharide cryoprotectants like maltodextrin during freeze-drying, as ice formation can compress and rearrange the particles, changing their morphology [24]. Maltodextrin forms an amorphous matrix that traps the nanoparticles, obscuring their individual shapes in SEM. Studies have shown that polysaccharide cryoprotectants create thick layers around particles, making it difficult to observe their original structure. The ice crystal network left after sublimation results in large cavities that reflect the dried matrix's macro-morphology instead of the true nanoscale size. Despite DLS measurements showing particle sizes of 136–451 nm, SEM does not reveal spherical nanoparticles due to their entrapment in the maltodextrin matrix [23, 26, 27].

3.1.2 FTIR spectra evaluation

The FTIR spectrum of chitosan (Fig. 2) displayed characteristic peaks associated with hydroxyl and amine functional groups, which are typical structural features of this polymer. After nanoparticle formation with sodium tripolyphosphate (STPP), several spectral changes were observed in formulations F1–F3, indicating the occurrence of intermolecular interactions. In particular, the disappearance of the amine-related absorption band and the emergence of new signals in the region of 1390 – 1410 cm^{-1} suggested the involvement of these functional groups in the crosslinking process. Additional spectral shifts were also detected, including the displacement of the carbonyl stretching band from 1629 cm^{-1} to approximately 1539 – 1546 cm^{-1} , accompanied by minor changes in the C–H vibration region. The appearance of bands between 1521 and 1591 cm^{-1} was associated with

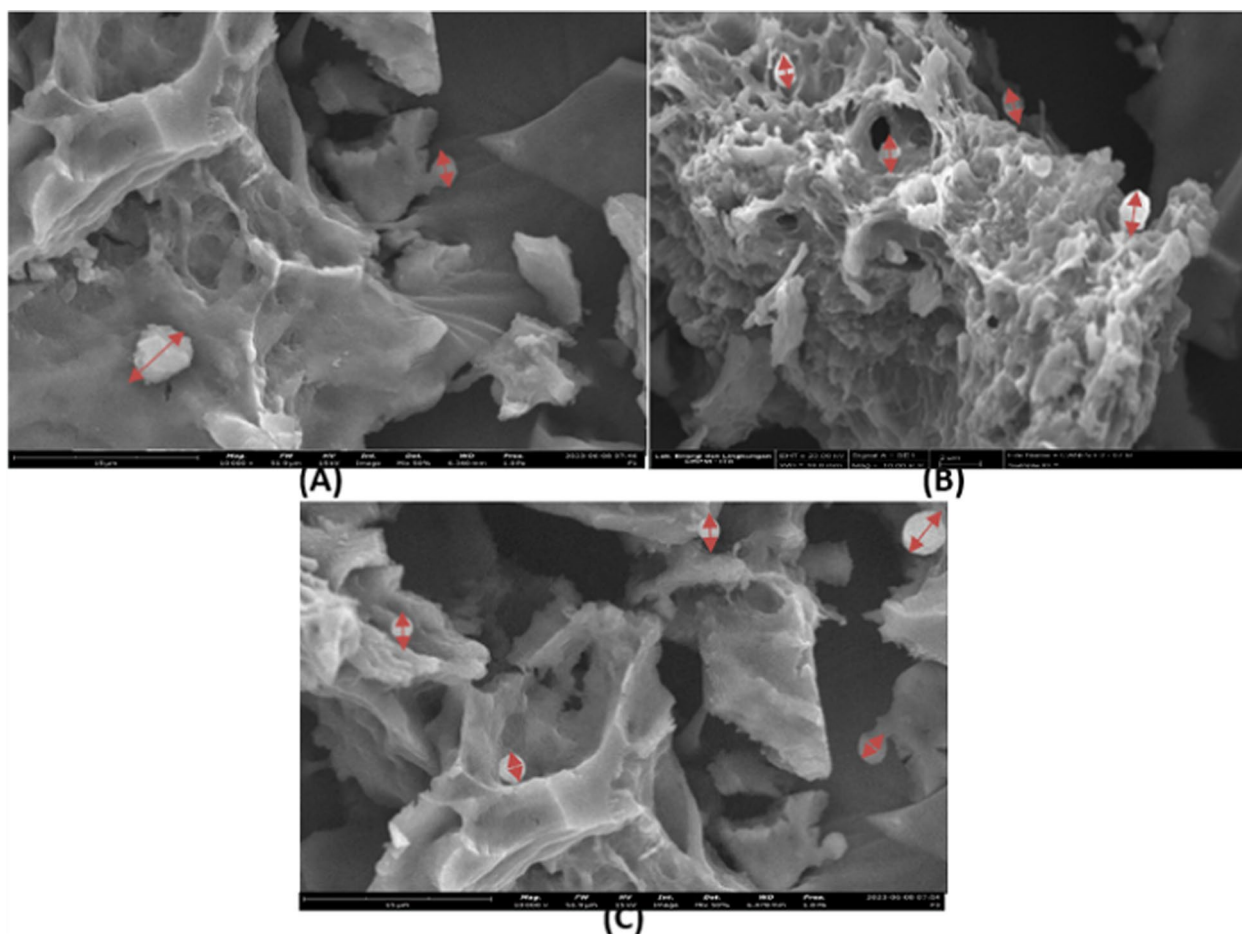
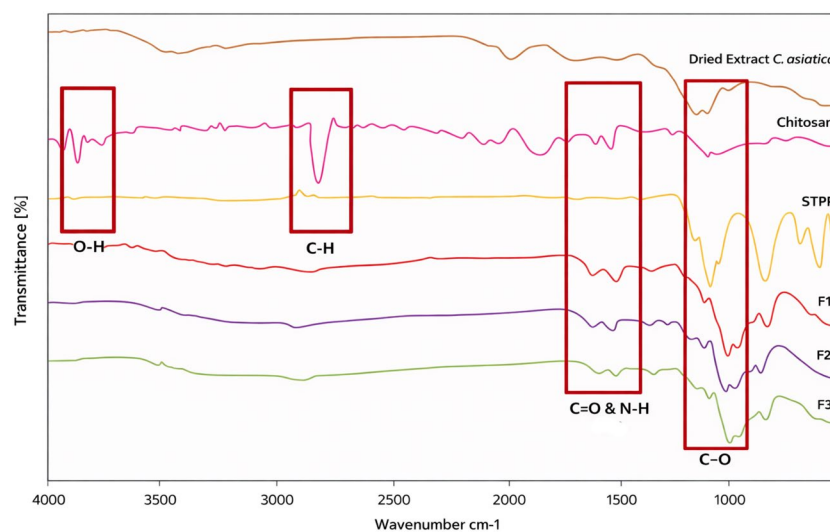


Fig. 1 Morphology observation of NECA with SEM at 10,000× magnification (A) Formula 1 (B) Formula 2 (C) Formula 3

Fig. 2 IR spectra of NECA with different chitosan concentrations: F1 (0.03%), F2 (0.04%), and F3 (0.05%)



carbonyl-containing structures, while absorption signals within the 997–1126 cm^{-1} range indicated the presence of P=O bonds derived from STPP. Furthermore, peaks

detected at 1009–1074 cm^{-1} corresponded to carboxylate-related vibrations.

The attenuation of several absorption bands originating from the extract suggested that the active constituents were successfully incorporated into the chitosan nanoparticle matrix, where their spectral signals became less prominent due to overlapping with the polymer and crosslinking network. Differences in peak intensity and spectral shifts were also influenced by the amount of chitosan used in each formulation. Notably, F1, which contained the lowest concentration of chitosan, exhibited more pronounced spectral alterations, indicating stronger ionic interactions and a higher degree of crosslinking. These observations support previous findings that the concentration of chitosan plays a critical role in modulating the electrostatic interaction between chitosan and STPP during nanoparticle formation, enhancing nanoparticle stability and encapsulation efficiency [28]. Thus, the FTIR results highlight the importance of chitosan concentration in determining the nanoparticle system's properties.

Moreover, several recent studies on chitosan–STPP nanoparticles confirm that during ionic gelation, the characteristic amino/amide and hydroxyl peaks of native chitosan are modified, while new phosphate-associated bands appear, in line with the findings of this study. It has been reported that in chitosan–STPP nanoparticles, the native $\text{-NH}_2/\text{-OH}$ broad band around 3421 cm^{-1} becomes broadened and shifts slightly, while new absorption bands appear around $\sim 1246\text{ cm}^{-1}$ (attributed to asymmetric P=O stretching) and in the $1080\text{--}1030\text{ cm}^{-1}$ region (attributed to P-O-C/P-O-P linkages). These changes indicate that phosphate groups from TPP are incorporated into the polymer matrix through electrostatic cross-linking [29, 30].

These spectral trends suggest that higher chitosan concentrations, as seen in the NECA F1 formulation, facilitate more extensive chitosan–STPP cross-linking, thereby improving the structural integrity and stability of the nanoparticles. Encapsulation studies of bioactive compounds in chitosan–STPP nanoparticles also report that many characteristic absorbances of the free ECA either disappear or are masked after nanoparticle formation, due to the dominant FTIR signals from the polymer-crosslinker matrix [30–32]. This observation supports the interpretation that in the NECA formulations, the absence or attenuation of certain extract-specific bands likely indicates successful encapsulation within the chitosan–STPP matrix.

3.1.3 X-ray diffractogram evaluation

The diffraction profiles obtained from XRD revealed that ECA and STPP possessed well-defined peaks, indicating a highly crystalline structure. Meanwhile, chitosan showed relatively broad diffraction signals, consistent with its semicrystalline nature. Upon nanoparticle formation, the intensity of the crystalline peaks was significantly reduced or

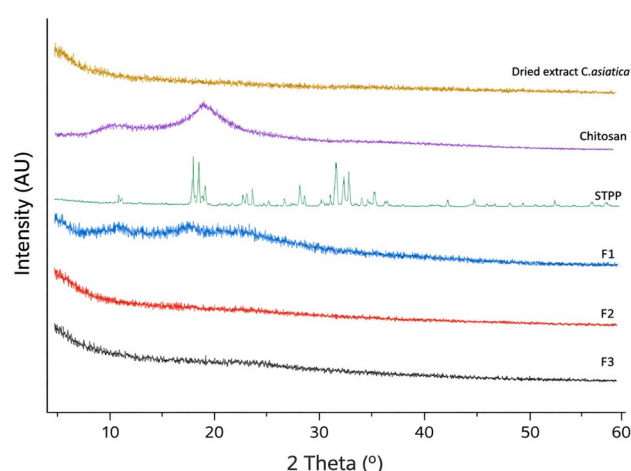


Fig. 3 Diffractogram of NECA with different chitosan concentrations: F1 (0.03%), F2 (0.04%), and F3 (0.05%)

completely absent. Consequently, the diffractogram of the final nanoparticle system, as presented in Fig. 3, exhibited a predominantly amorphous structural pattern..

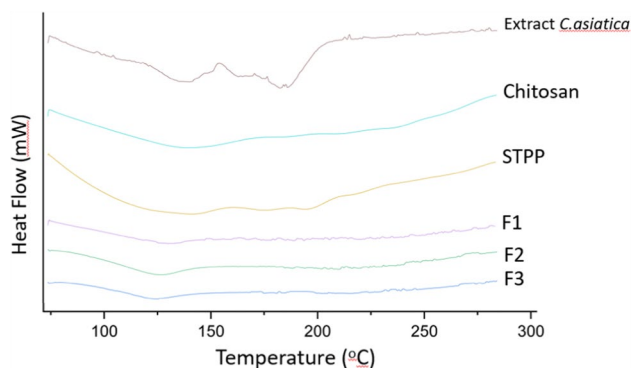
F1 showed minimal changes in crystalline structure, with sharp peaks from both chitosan and the extract still visible. The lower chitosan concentration in F1 led to greater crosslinking with STPP. F2 showed moderate crystallinity changes, with a slight shift towards amorphousness, while F3 had the most significant shift to amorphous patterns, with sharp peaks replaced by broader ones. F1 retained more crystalline characteristics due to limited amorphization, while F3 showed increased amorphization, which improves solubility and bioavailability. F3 also exhibited better structural stability. The observed structural changes were closely related to the concentration of chitosan present in the formulation. A higher chitosan content promoted the transformation toward a more amorphous structure, which is generally associated with better nanoparticle stability. These findings are in agreement with the report of Costa et al. (2023), which indicated that increasing chitosan concentration can lower crystallinity and enhance the functional performance of nanoparticle systems [33].

3.1.4 Differential scanning calorimeter analysis

The differential scanning calorimetry (DSC) results presented in Table 2 and Fig. 4 reveal variations in melting temperature and enthalpy among the analyzed samples, which reflect their distinct structural characteristics and thermal behaviors. The ECA sample exhibited a relatively broad melting interval between 179.3 and $199.1\text{ }^{\circ}\text{C}$ with an enthalpy value of 31.72 J/g , suggesting the presence of thermally stable bioactive constituents and a partially crystalline structure. In contrast, chitosan displayed a wider melting

Table 2 Distance melting point and enthalpy of ECA, chitosan, STPP and NECA

Sample	Melting distance (°C)	Enthalpy (J/g)
ECA	179.3–199.1	31.72
Chitosan	92.8–144.3	85.74
STTP	187.6–203.6	4.13
F1	86.9–123.2	89.56
F2	85.1–115.5	48.46
F3	93.2–122.5	40.33

**Fig. 4** DSC Thermogram of NECA with different chitosan concentrations: F1 (0.03%), F2 (0.04%), and F3 (0.05%)

range from 92.8 to 144.3 °C accompanied by a higher enthalpy value of 85.74 J/g. This behavior may be associated with structural rearrangements of the polymer and the release of bound water during heating. Meanwhile, STPP demonstrated a comparatively high melting temperature ranging from 187.6 to 203.6 °C but exhibited a much lower enthalpy value of 4.13 J/g, which likely reflects its relatively simple crystalline structure and the smaller amount of thermal energy required for its phase transition [34, 35].

Compared with the individual components, the NECA nanoparticle formulations (F1–F3) exhibited noticeably lower melting temperatures, ranging from approximately 85 to 123 °C. This reduction indicates that the interactions among chitosan, STPP, and the plant extract altered the original crystalline arrangement of the materials, resulting in a structure that is more amorphous in nature. Among the formulations, F1 presented the highest enthalpy value (89.56 J/g), which was close to that observed for pure chitosan, suggesting a stronger crosslinking interaction within the polymer network. In contrast, F2 and F3 showed lower enthalpy values of 48.46 and 40.33 J/g, respectively, implying a higher degree of amorphous structure within these formulations. These observations are consistent with previous

studies reporting that ionotropic gelation between chitosan and STPP leads to the formation of a new ionic network within the polymer matrix, thereby modifying its thermal behavior and generally reducing both melting temperature and enthalpy [36–38].

3.1.5 Encapsulation efficiency and drug loading evaluation

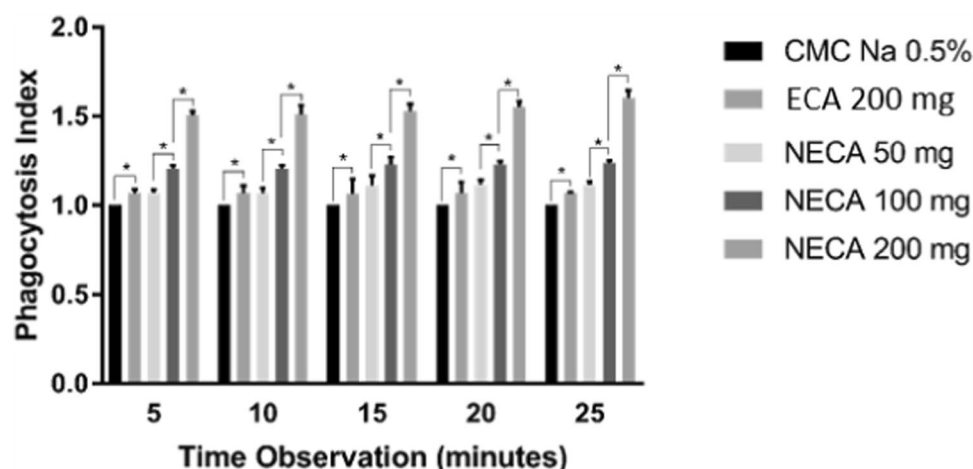
The analytical procedure used to quantify *Centella asiatica* extract (ECA) through its asiaticoside content was previously validated by Pratiwi et al. (2023). In this study, TLC densitometry was utilized for quantitative determination of the analyte, providing improved sensitivity and reproducibility compared with conventional TLC observation. The validated method therefore ensured reliable determination of encapsulation efficiency (EE) in nanoparticles containing *Centella asiatica* extract, supporting the accuracy of the analytical results obtained [6].

The encapsulation efficiency values for the three NECA formulations (F1–F3) are summarized in Table 1, along with their mean and standard deviation. Among the tested formulations, F1 demonstrated the highest encapsulation efficiency, at $89.27 \pm 1.39\%$, indicating effective incorporation of the active compounds into the nanoparticle matrix. Meanwhile, formulations F2 and F3 showed slightly lower EE values of $82.25 \pm 2.77\%$ and $79.47 \pm 1.47\%$. The calculated drug loading percentages were 35.71% for F1, 32.90% for F2, and 31.79% for F3.

Differences in encapsulation performance among the formulations may be attributed to variations in the composition of formulation components, particularly the proportions of chitosan, STPP, and the plant extract, as well as processing parameters such as polymer concentration, pH conditions, and stirring intensity. Similar findings have been reported in recent studies involving chitosan–TPP nanoparticle systems. For instance, quercetin-loaded chitosan–TPP nanoparticles have been reported to achieve an EE of approximately 85.16% under optimized formulation ratios [39]. In another study, curcumin encapsulated in chitosan–TPP microparticles demonstrated EE values exceeding 90%, which were influenced by the concentration and ratio of the crosslinking agent [36].

The relatively high encapsulation efficiency and low variability observed in F1 suggest a strong interaction between the active compound and the polymeric nanoparticle matrix. This may be attributed to a more favorable chitosan-to-TPP ratio and appropriate processing conditions, which promote effective crosslinking within the nanoparticle network. In contrast, the slightly lower EE values observed in F2 and F3 may reflect less optimal polymer network formation, such as reduced crosslinking density or greater heterogeneity in

Fig. 5 Average value of phagocytosis index of ECA and NECA ($n=5$). * significantly different with the value $p < 0.05$



particle size distribution. Achieving a high EE, as observed in F1, is advantageous because it enables a greater proportion of the active compound to be retained within the nanoparticle system, thereby minimizing potential loss during formulation.

3.2 Macrophage activity test and organ weight analysis

The evaluation of macrophage activity presented in Fig. 5 indicates that administration of NECA markedly enhanced the phagocytic index compared with both the negative control group (0.5% Na-CMC) and the group receiving the crude extract (ECA). Among the tested treatments, NECA administered at a dose of 200 mg/kg BW produced the greatest phagocytic response throughout the observation period. These findings suggest that nanoencapsulation of the extract using a chitosan-based carrier enhances innate immune responses, as demonstrated by the increased ability of macrophages to internalize and degrade foreign particles. Enhanced macrophage phagocytosis is widely recognized as a key indicator of immunostimulatory activity [40, 41].

The phagocytic index reflects the functional efficiency of macrophages in capturing and eliminating particulate matter and is commonly used as a parameter of immune activation. In the present study, NECA administered at doses of 50, 100, and 200 mg produced statistically significant differences in phagocytic activity compared with the group treated with ECA at 200 mg. This significance is indicated by the asterisks (*) shown in the figure, representing $p < 0.05$. These results demonstrate that nanoformulated extract produces a stronger stimulatory effect on macrophage function than the extract alone, suggesting improved biological performance of the nanoparticle system.

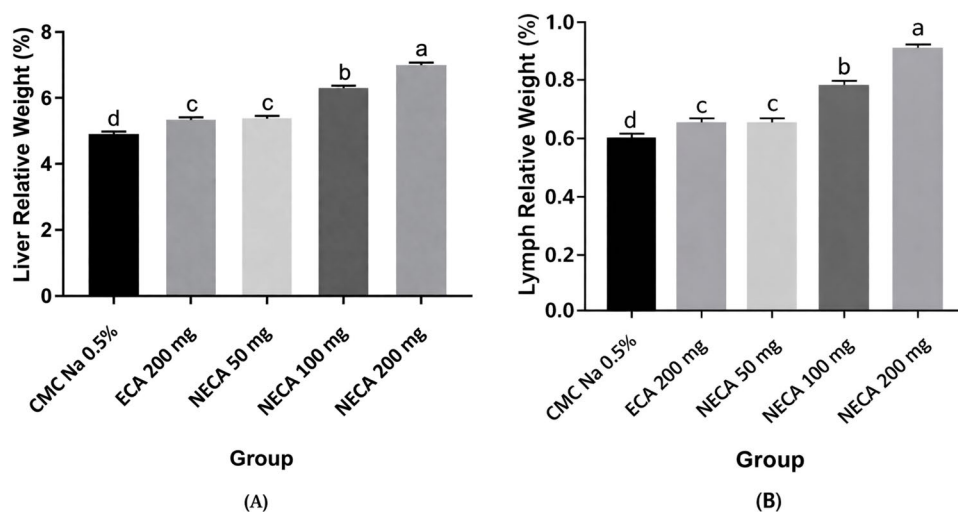
Further evidence of the immunostimulatory properties of NECA was obtained through the carbon clearance assay, which evaluates systemic macrophage activity. This

method assesses the ability of circulating phagocytic cells, particularly Kupffer cells in the liver and macrophages in the lymph, to remove injected carbon particles from the bloodstream. The rate at which these particles are cleared reflects the functional capacity of the reticuloendothelial system [42, 43]. As a well-established technique for evaluating immunomodulatory activity, the carbon clearance assay provides a quantitative measure of macrophage-mediated clearance of foreign materials. By monitoring the reduction of carbon particles in circulation over time, the assay enables assessment of immune cell activation and phagocytic efficiency.

One advantage of this assay is its methodological simplicity and reproducibility, as it does not require complex instrumentation or elaborate experimental procedures. For this reason, it is frequently employed as an initial screening method to investigate the immunological effects of various bioactive compounds, including nanoparticle formulations. Moreover, the *in vivo* nature of the assay allows repeated measurements and dynamic monitoring of immune responses over time. In the present study, the method proved useful for evaluating the immunostimulatory potential of chitosan-based nanoparticles containing *Centella asiatica* extract, providing valuable insights into their ability to enhance macrophage-mediated immune responses [44, 45].

The higher carbon clearance rate observed in animals treated with NECA may be attributed to the nanoencapsulation of asiaticoside within the chitosan matrix. Encapsulation improves the compound's bioavailability and facilitates interaction with immune cells through pattern recognition receptors (PRRs), ultimately promoting macrophage activation [46]. These findings reinforce the immunostimulatory role of NECA and are consistent with the results obtained from macrophage phagocytosis measurements and organ index analysis [47]. Collectively, the *in vitro* and *in vivo* findings indicate that NECA activates the mononuclear phagocyte system, thereby enhancing phagocytic responses and improving the systemic elimination of foreign particles.

Fig. 6 Average (A) liver relative weight and (B) lymph relative weight after macrophage activity test ($n = 5$). The different letters indicate significant value ($p < 0.05$)



This supports the potential of NECA as a plant-derived immunomodulatory system with possible clinical applications [48].

Additional support for the immunostimulatory effect of NECA was obtained from the analysis of relative organ weights, particularly those of the liver and lymph, as shown in Fig. 6. Animals treated with NECA at 200 mg showed significantly higher liver and lymph weights than the control group. The liver relative weight data (Fig. 6A) indicate that the ECA 200 mg group also exhibited a higher liver index than other NECA groups, suggesting involvement of hepatic immune responses. However, the overall increase in liver weight in NECA-treated groups compared with both the control and ECA groups may reflect enhanced immune activity associated with hepatic macrophages.

Similarly, analysis of lymph node relative weight (Fig. 6B) revealed a significant increase in the NECA 200 mg group compared with other treatments. This finding suggests that the nanoparticle formulation promotes lymphatic immune responses, possibly due to increased immune cell activation and proliferation. Encapsulation of the plant extract within nanoparticles appears to enhance its immunostimulatory potential relative to the free extract. This effect may be explained by improved stability, solubility, and bioavailability of the active compounds, particularly asiaticoside, which facilitates sustained release and more efficient interaction with immune cells such as macrophages. As a result, nanoparticle formulations can achieve comparable or even stronger immunological responses at lower doses.

The increase in liver and lymph node weights observed in this study further supports the occurrence of immune activation. Both organs play essential roles in immune regulation: the liver contains Kupffer cells that participate in systemic immune responses, while lymph nodes serve as sites of immune cell activation and antigen presentation. Previous studies have shown that changes in the relative weight

of immune-related organs, including the liver and lymph nodes, can indicate activation of immune mechanisms or inflammatory responses, particularly following exposure to immunomodulatory nanoparticles [49, 50]. Overall, these findings indicate that NECA—especially the optimized F1 formulation—functions as an effective nanocarrier capable of enhancing both the pharmacokinetic properties of asiaticoside and the activation of immune defense mechanisms. The dual role of this formulation suggests its potential for further development as a plant-based immunostimulant, with possible applications in conditions involving compromised immunity or as an adjuvant in vaccine formulations.

4 Conclusions

In this study, NECA were successfully produced via ionic gelation, followed by freeze-drying. The resulting formulations demonstrated favorable physicochemical characteristics, including particle sizes in the nanometer range and low polydispersity index ($PDI < 0.3$), indicating a relatively uniform particle distribution. In addition, the nanoparticles exhibited a high encapsulation efficiency of up to 89.27%. Structural analysis also revealed a transformation from crystalline to predominantly amorphous forms, which may improve dissolution behavior and enhance the bioavailability of the encapsulated compounds. Biological evaluation using an *in vivo* model further showed that administration of NECA significantly stimulated macrophage phagocytic activity in mice. This enhancement of innate immune response was also reflected by increased relative weights of immune-related organs, particularly the lymph and liver, when compared with the control group. These observations suggest that the nanoformulated extract effectively enhances macrophage-mediated immune defense mechanisms. Taken together, the findings highlight the potential of NECA as a

promising plant-derived immunostimulant formulation. The chitosan-based nanoparticle system not only improves the physicochemical properties of the extract but also enhances its immunological activity. Therefore, NECA may have potential applications in the development of pharmaceutical preparations, nutraceutical products, and functional foods to support immune function. Further research is recommended to investigate long-term stability, drug release behavior, and clinical efficacy in order to fully establish its therapeutic applicability.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflicts of interest The authors declare no competing interests.

Ethics statements This research was approved by the research ethics committee of the Faculty of Veterinary Medicine, Airlangga University, with a certificate number of ethical clearance No. 2.KEH.005.01.2023.

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