https://doi.org/10.31925/farmacia.2026.5.9 **ORIGINAL ARTICLE**

ANTIOXIDANT AND ANTIHYPERTENSIVE EFFECTS OF *ELEUTHERINE BULBOSA* (MILL.) CHITOSAN NANOPARTICLES IN NACL-INDUCED HYPERTENSIVE RATS

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Abstract

Eleutherine bulbosa contains flavonoids and phenolic compounds with antioxidant properties and has potential as a natural antihypertensive agent. This study evaluated the antioxidant and antihypertensive effects of *Eleutherine bulbosa* extract and its chitosan nanoparticles (EB-CNPs) in NaCl-induced hypertensive rats. The extract (EB-e, 60 mg/kg B.W.) and three EB-CNP formulations (100, 200, and 400 mg/kg B.W.) were prepared and characterized based on pH, particle size, zeta potential, and morphology using scanning electron microscopy (SEM). Hypertension was induced in male Wistar rats by oral administration of NaCl (3.75 g/kg B.W.) for 21 days, followed by treatment for 7 days. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and nitric oxide (NO) levels were measured. Among the formulations, F2 containing 0.75% chitosan exhibited the most favorable physicochemical characteristics, with a particle size of 291.17 nm. Treatment with EB-CNPs significantly reduced SBP and DBP and increased NO levels compared with the negative control group ($p < 0.05$). The highest dose (400 mg/kg B.W.) reduced SBP from 156.87 ± 16.17 to 115.40 ± 17.89 mmHg and DBP from 124.13 ± 20.27 to 92.20 ± 16.50 mmHg, while increasing NO levels from 16.43 ± 2.67 to 28.72 ± 2.86 µmol/L ($p < 0.05$). The antihypertensive effect of EB-CNPs at 400 mg/kg B.W. was comparable to that of captopril and greater than that of the crude extract. In conclusion, EB-CNPs enhance the biological activity of *Eleutherine bulbosa*, improving its antihypertensive potential and supporting its development as a promising natural therapeutic agent.

Rezumat

Eleutherine bulbosa conține flavonoide și compuși fenolici cu proprietăți antioxidante, prezentând potențial ca agent antihipertensiv natural. Studiul a urmărit evaluarea efectelor antioxidante și antihipertensive ale extractului de *Eleutherine bulbosa* și ale nanoparticulelor de chitosan conținând acest extract (EB-CNPs). Au fost preparate și caracterizate extractul vegetal (EB-e, 60 mg/kg corp) și trei formulări de nanoparticule (100, 200 și 400 mg/kg corp), prin determinarea pH-ului, dimensiunii particulelor, potențialului zeta și morfologiei prin microscopie electronică de baleiaj (SEM). Hipertensiunea a fost indusă la șobolani masculi Wistar prin administrarea orală de NaCl (3,75 g/kg corp), timp de 21 de zile, urmată de tratament timp de 7 zile. Au fost evaluate tensiunea arterială sistolică (TAS), tensiunea arterială diastolică (TAD) și nivelurile de oxid nitric (NO). Dintre formulările investigate, F2, cu un conținut de 0,75% chitosan, a prezentat cele mai favorabile caracteristici fizico-chimice, având o dimensiune a particulelor de 291,17 nm. Tratamentul cu EB-CNPs a determinat reducerea semnificativă a TAS și TAD și creșterea nivelurilor de NO, comparativ cu lotul martor negativ ($p < 0,05$). La doza maximă de 400 mg/kg corp, TAS a scăzut de la 156,87 ± 16,17 la 115,40 ± 17,89 mmHg, iar TAD de la 124,13 ± 20,27 la 92,20 ± 16,50 mmHg, în timp ce nivelul NO a crescut de la 16,43 ± 2,67 la 28,72 ± 2,86 µmol/L ($p < 0,05$). Efectul antihipertensiv al EB-CNPs la această doză a fost comparabil cu cel al captoprilului și superior celui al extractului vegetal neformulat. Rezultatele sugerează că formularea extractului de *Eleutherine bulbosa* sub formă de nanoparticule de chitosan îi îmbunătățește activitatea biologică și potențialul antihipertensiv, susținând dezvoltarea sa ca posibil agent terapeutic de origine naturală.

Keywords: antihypertensive activity, chitosan nanoparticles, *Eleutherine bulbosa*, hypertension, nitric oxide

Introduction

Hypertension is a global health problem that remains a major challenge because it plays a significant role in increasing morbidity and mortality caused by cardiovascular disease, stroke, and kidney disorders (1). The pathogenesis of hypertension is not only related to genetic and environmental factors but is also strongly influenced by increased oxidative stress (2). An imbalance between the production of free radicals and

the capacity of endogenous antioxidant systems leads to cellular damage, endothelial dysfunction, and reduced nitric oxide (NO) bioavailability, which ultimately triggers elevated blood pressure (3).

Oxidative stress can impair endothelial function by increasing the production of reactive oxygen species (ROS), particularly superoxide. Superoxide reacts with nitric oxide (NO), reducing its availability and limiting its vasodilatory effect. As a result, vascular resistance increases, which may contribute to the development of hypertension. Oxidative stress can also disrupt endothelial nitric oxide synthase (eNOS) activity, leading to lower NO production and further impairment of vascular function. For this reason, therapies that reduce oxidative stress while maintaining NO availability may help improve blood pressure control (4).

Natural products have increasingly been explored as complementary approaches for hypertension management because they contain bioactive compounds with pharmacological activity and are generally associated with fewer adverse effects than synthetic drugs. Among the medicinal plants found in Indonesia, *Eleutherine bulbosa* (Mill.) Urb., commonly known as bawang dayak, has attracted interest due to its potential antihypertensive properties (5). This plant contains several bioactive compounds, including flavonoids, phenolics, naphthoquinones, and eleutherin, which have been reported to exert antihypertensive, anti-inflammatory, and cardioprotective effects (6).

The antihypertensive activity of these phytochemicals may be explained by their antioxidant properties. Flavonoids and phenolic compounds help neutralize free radicals, reducing oxidative stress and protecting endothelial function. This can preserve nitric oxide (NO) availability, allowing blood vessels to relax more effectively and improving blood flow. Naphthoquinones, together with other phenolic constituents, have also been reported to inhibit angiotensin-converting enzyme (ACE), reduce inflammatory responses in blood vessels, and protect endothelial cells from oxidative damage. These actions may work together to lower vascular resistance, resulting in a reduction in blood pressure (7).

Previous studies have demonstrated that *Eleutherine bulbosa* extract may contribute to blood pressure reduction and improvement of vascular function (8). Previous investigations have also demonstrated that *Eleutherine bulbosa* possesses strong antioxidant activity owing to its high flavonoid and phenolic contents, which may contribute to its cardiovascular protective effects (9). However, these studies have primarily evaluated *Eleutherine bulbosa* in the form of conventional crude extracts, while investigations employing advanced drug delivery systems to enhance its therapeutic efficacy remain limited.

However, despite its pharmacological potential, the application of *Eleutherine bulbosa* extract is limited by its relatively low bioavailability, which is mainly associated with poor solubility and limited stability of its active compounds (6). These limitations may reduce its therapeutic effectiveness when administered in conventional extract form. Therefore, strategies to enhance the delivery and bioavailability of its active constituents are required.

Nanoparticle-based delivery systems have attracted attention as a way to improve the delivery of plant-derived compounds. Chitosan is often used to prepare these systems because it is biocompatible, biodegradable, and readily forms nanoparticles with sodium tripolyphosphate (NaTPP). This type of formulation helps protect bioactive compounds, improves their absorption, and allows a more gradual release, potentially leading to better therapeutic effects (10). Numerous studies have demonstrated that nanoparticle formulations improve the oral bioavailability of herbal phytochemicals by increasing aqueous solubility, protecting bioactive compounds against chemical and enzymatic degradation, enhancing intestinal absorption, and providing sustained release. Chitosan is commonly used in nanoparticle formulations because of its good biocompatibility, biodegradability, and mucoadhesive properties. Its ability to adhere to mucosal surfaces may prolong residence time and improve the absorption of encapsulated compounds (11). Evidence from previous studies suggests that chitosan-based nanoparticles provide greater stability and biological activity than conventional herbal extract formulations (12). For several medicinal plants, nanoparticle encapsulation has successfully enhanced antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and antihypertensive activities compared with crude extracts. Nevertheless, studies specifically investigating chitosan nanoparticle formulations containing *Eleutherine bulbosa* remain scarce, and evidence of their antihypertensive efficacy remains limited. The nanoparticle formulation is expected to enhance the pharmacological effects of *Eleutherine bulbosa* extract by increasing its bioavailability and facilitating improved interactions with biological systems (13).

Previous studies have confirmed the antioxidant and antihypertensive potential of *Eleutherine bulbosa* crude extract. In contrast, separate investigations have demonstrated that chitosan nanoparticle formulations improve the stability, bioavailability, and biological activity of numerous herbal medicines. However, to the best of our knowledge, no previous study has directly compared the antihypertensive efficacy of *Eleutherine bulbosa* crude extract and *Eleutherine bulbosa*-loaded chitosan nanoparticles in NaCl-induced hypertensive rats while simultaneously evaluating nitric oxide modulation as an indicator of endothelial function. Therefore, this study was designed to address this research gap and determine whether the nano-

particle formulation provides additional therapeutic benefits compared with the conventional extract.

Materials and Methods

Materials

The tools used in this study included a water bath (Memmert, Germany), rotary evaporator (Heidolph, Germany), UV–Vis spectrophotometer (Shimadzu, Japan), ELISA reader (Bio-Rad, USA), micropipettes (Eppendorf, Germany), analytical balance (Ohaus, USA), pH meter (Hanna Instruments, Romania), centrifuge (Hettich, Germany), and a CODA device for blood pressure measurement (Kent Scientific, USA), as well as scanning electron microscopy (Vega3 Tescan®) and a particle size analyser (VASCO FlexTM®) for nanoparticle characterization.

The materials used in this study included fresh bulbs of *Eleutherine bulbosa* obtained from Banjarmasin, South Borneo (3°18′51.78″S 114°35′33.05″E). The plant material had been previously authenticated, and a voucher specimen was deposited at Balai Materia Medica East Java (No. 000.9.3/2154/102.20/2025). Other materials included DPPH solution (Merck, Germany), methanol (Merck, Germany), vitamin C (Sigma-Aldrich, USA), chitosan solution (Sigma-Aldrich, USA), sodium tripolyphosphate (NaTPP) (Merck, Germany), polysorbate 80 (Merck, Germany), Wistar rats (obtained from the Laboratory of Pharmacology, Universitas Ahmad Dahlan, Indonesia), sodium carboxymethyl cellulose (NaCMC) (Merck, Germany), sodium chloride (NaCl) (Merck, Germany), captopril (Kimia Farma, Indonesia), and Griess reagent for nitric oxide (NO) analysis.

Preparation and etraction of Eleutherine bulbosa bulbs

Fresh bulbs of *Eleutherine bulbosa* were collected from Banjarmasin, South Borneo. Fresh plant material was washed thoroughly, cut into small pieces, dried in the shade, then ground and passed through a 40-mesh sieve to obtain a uniform powder for extraction. The simplicia powder was extracted by maceration using 96% ethanol at a 1:10 (w/v) ratio for three days. The filtrate was separated, and the residue was re-macerated using the same solvent. All filtrates were combined and concentrated on a rotary evaporator, then further concentrated in a water bath to obtain a thick extract (14).

Phytochemical screening of Eleutherine bulbosa extract

Phytochemical screening of *Eleutherine bulbosa* extract was conducted to identify major secondary metabolites, including alkaloids, flavonoids, saponins, tannins, and terpenoids/steroids, using standard qualitative methods (15). Alkaloids were detected by Mayer's reagent, flavonoids by the magnesium-hydrochloric acid reaction, saponins by foam formation, tannins by the $FeCl_3$ test, and terpenoids/steroids by the Liebermann Burchard reaction.

Total flavonoid and phenolic content of Eleutherine bulbosa

Total flavonoid and phenolic contents were quantified using a UV–Vis spectrophotometer. Flavonoid content was measured by the aluminum chloride ($AlCl_3$) colorimetric assay with quercetin as the reference standard. Briefly, the extract was mixed with $AlCl_3$ solution, incubated under the specified conditions, and its absorbance was recorded at approximately 415 nm. The results were expressed as mg quercetin equivalents (QE)/g extract. Phenolic content was evaluated using the Folin–Ciocâlteu assay with gallic acid as the standard. The extract was mixed with Folin–Ciocâlteu reagent followed by sodium carbonate, incubated in the dark, and the absorbance was recorded at 760 - 765 nm. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g extract (16).

Antioxidant activity test of Eleutherine bulbosa extract using the DPPH method

The antioxidant activity of *Eleutherine bulbosa* extract was evaluated using the DPPH (*1,1-diphenyl-2-picrylhydrazyl*) method. All measurements were performed in triplicate (n = 3). A 0.1 mM DPPH solution was prepared in methanol and stored in the dark. The extract and vitamin C (positive control) were prepared at concentrations of 50 - 90 ppm and 2 - 10 ppm, respectively. A 1 mL sample and 1 mL DPPH solution were incubated for 30 minutes in the dark at room temperature, and absorbance was measured at 517 nm using a UV-Vis spectrophotometer (17). The percentage of inhibition was calculated using the formula:

$$(\%)\ \text{inhibition} = \frac{\text{absorbance of blank - absorbance of sample}}{\text{absorbance of blank}} \times 100.$$

The percentage of inhibition at each concentration was used to construct a calibration curve. Linear regression ($y = ax + b$) was then applied to estimate the concentration required to achieve 50% inhibition (IC_{50}) (18).

Preparation of Eleutherine bulbosa extract nanoparticles (EB-CNPs)

The preparation of *Eleutherine bulbosa* extract nanoparticles was carried out based on the method developed by (12), with several modifications, including the preparation of the chitosan and NaTPP solutions and the nanoparticle formation process for the *Eleutherine bulbosa* extract. The complete nanoparticle formula used in this study is presented in Table I.

Table I

Eleutherine bulbosa extract nanoparticles (EB-CNPs)

Material	F1	F2	F3
Eleutherine bulbosa extract	500 mg	500 mg	500 mg
0.5% chitosan solution	100 mL	-	-
0.75% chitosan solution	-	100 mL	-
1% chitosan solution	-	-	100 mL
NaTPP solution	50 mL	50 mL	50 mL
Polysorbate 80	1 mL	1 mL	1 mL

Evaluation of Eleutherine bulbosa extract nanoparticles (EB-CNPs)

The preparation of *Eleutherine bulbosa* chitosan nanoparticles (EB-CNPs) was carried out according to the method reported by (19) with slight modifications. Three formulations were prepared using 100 mL of chitosan solutions at concentrations of 0.5% (F1), 0.75% (F2), and 1.0% (F3, w/v)**,** each containing 500 mg of *Eleutherine bulbosa* extract**.** Subsequently, 50 mL of sodium tripolyphosphate (NaTPP) solution (0.2%, w/v; prepared by dissolving 0.1 g NaTPP in 50 mL distilled water) was added dropwise under magnetic stirring at 1,500 rpm for 4 h to induce ionic gelation and nanoparticle formation. The pH of each formulation was monitored using a calibrated pH meter to ensure formulation stability.

The morphology of nanoparticles was observed using Scanning Electron Microscopy (Vega3 Tescan®), while particle size, polydispersity index (PDI), zeta potential, and stability were analysed using a Particle Size Analyzer (VASCO FlexTM®). Encapsulation efficiency (%EE) and loading capacity (%LC) were determined using UV-Vis spectrophotometry (20). The values of %EE and %LC were calculated according to the following equations:

$$(\%)\ EE = \frac{\text{weight of loaded extract}}{\text{weight of intial extract}} \times 100,$$

$$(\%)\ LC = \frac{\text{weight of loaded extract}}{\text{weight of sample}} \times 100.$$

Animal preparation

This study was approved by the Research Ethics Committee of Ahmad Dahlan University (Approval No. REC-UAD/02/01/06-2025/071). Thirty-five male Wistar rats (200 - 250 g) were acclimatized for two weeks under standard laboratory conditions and randomly divided into seven groups (n = 5) (21). Group I served as the normal control, Group II received NaCl (3.75 g/kg B.W.), Group III received captopril (4.5 mg/kg B.W.), Group IV received *Eleutherine bulbosa* extract (60 mg/kg B.W.), Group V received *Eleutherine bulbosa* chitosan nanoparticles (EB-CNPs) at 100 mg/kg B.W., and Group VI received EB-CNPs at 200 mg/kg B.W., Group VII received EB-CNPs at 400 mg/kg B.W. All treatments were administered orally throughout the experimental period (22).

Antihypertensive activity test

Hypertension was induced by oral administration of NaCl (3.75 g/kg B.W.) once daily for 21 consecutive days. After the induction period, the rats were treated orally with *Eleutherine bulbosa* chitosan nanoparticles (EB-CNPs) for 7 days. Systolic and diastolic blood pressure were recorded on Days 0, 21, and 28 using the CODA® non-invasive tail-cuff system. Before each measurement, the animals were allowed to acclimatize to the restrainer and warming platform for 10 - 15 min to reduce stress during the procedure. Blood pressure was measured using the volume-pressure recording (VPR) sensor incorporated into the CODA® system, which monitors changes in tail blood volume. Each rat underwent 15 measurement cycles, and the average of 10 valid readings was used for data analysis (23).

Measurement of nitric oxide levels in hypertensive rats

Nitric oxide levels were measured using an ELISA reader (Asys) based on a modified Griess method (24). Blood samples from male Wistar rats were collected on days 21 and 28, then centrifuged at 3,000 rpm for 15 minutes to obtain serum (25). Nitrite levels were determined by mixing 100 µL of serum or nitrite standard solution with 100 µL of Griess A and Griess B reagents in a 96-well microtiter plate. The mixture was allowed to stand until a colour change occurred, after which absorbance was measured at a wavelength of 550 nm using an ELISA reader (26).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Systolic and diastolic blood pressure measured repeatedly on Days 0, 21, and 28 were analysed using a two-way repeated-measures analysis of variance (ANOVA), with treatment group as the between-subjects factor and time as the within-subjects factor. When significant differences were detected, Tukey's post hoc test was applied for multiple pairwise comparisons. Nitric oxide (NO) levels were analysed using one-way ANOVA, followed by Tukey's post hoc test to compare differences among treatment groups. Statistical analyses were performed using IBM SPSS Statistics version 25, and differences were considered statistically significant at $p < 0.05$ (27).

Results and Discussion

Characterization of Eleutherine bulbosa extract

The extraction of *Eleutherine bulbosa* using 96% ethanol yielded 142.59 g of thick extract from 2,400 g of dried simplicia powder, corresponding to an extraction yield of 5.94%. The screening of secondary metabolites of *Eleutherine bulbosa* extract showed that this extract contains alkaloids, flavonoids, saponins, tannins, and terpenoids/steroids (Table II).

Table II

Phytochemical screening results

Compound	Result
Alkaloid	+
Flavonoid	+
Saponin	+
Tannin	+
Terpenoids/steroids	+

(+) indicates a positive result for the presence of the tested phytochemical compounds, whereas (−) indicates a negative result

The phytochemical screening showed that the extraction process retained a broad range of secondary metabolites. This is important because these compounds are known to contribute to various biological

activities. Flavonoids are recognized for their antioxidant activity and may be involved in blood pressure regulation (28), while alkaloids have various physiological effects (29). Saponins are known for their immunomodulatory activity (30), tannins possess antimicrobial and astringent properties (31), and terpenoids and steroids have been reported to exhibit anti-inflammatory and anticancer activities (32).

Total flavonoid and phenolic content of Eleutherine bulbosa

The quantitative analysis of total flavonoid and phenolic contents in *Eleutherine bulbosa* bulbs is summarized in Table III.

The results of the analysis showed that the total flavonoid content of *Eleutherine bulbosa* bulbs was 12.451 ± 0.038 mg QE/g extract, while the total phenolic content was 43.322 ± 0.049 mg GAE/g extract (Table III). The replicate measurements for both parameters demonstrated consistent values, indicating good reproducibility and reliability of the analytical methods used.

The analysis showed that *Eleutherine bulbosa* extract contained substantial amounts of flavonoids and phenolic compounds, indicating that the bulbs are a rich source of bioactive phytochemicals (33). These compounds are well known for their antioxidant activity. Flavonoids act by donating hydrogen atoms or electrons to neutralize free radicals and limit oxidative chain reactions (34). Their glycosylated forms are generally more soluble in polar solvents, which may have contributed to the extraction efficiency observed in this study. Phenolic compounds also exhibit antioxidant activity through their hydroxyl groups, enabling them to donate hydrogen atoms or electrons and stabilize reactive oxygen species (35).

Table III

Total flavonoid and phenolic content of *Eleutherine bulbosa*

Sample	Flavonoid (mg QE/g)	Phenolic (mg GAE/g)
Eleutherine bulbosa Extract	12.451 ± 0.038	43.322 ± 0.049

The extract contained more total phenolics than total flavonoids, indicating that phenolic compounds are the predominant antioxidant constituents (36). Flavonoids are part of the larger phenolic group, so both are expected to contribute to the extract's antioxidant activity. It is common to find differences in flavonoid and phenolic contents between studies, as these values can be influenced by factors such as the plant's growing environment, harvest stage, geographical origin, and extraction procedure (37).

Antioxidant activity test of Eleutherine bulbosa extract using the DPPH method

Antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. This method relies on the reduction of the stable DPPH radical by antioxidant compounds. As the radicals are neutralized through the donation of hydrogen atoms or electrons, the solution gradually changes from purple to yellow. The change in absorbance at 517 nm was then used to estimate the extract's free radical-scavenging activity (38). The antioxidant activity results are presented in Table IV.

Based on the DPPH assay, *Eleutherine bulbosa* extract demonstrated strong antioxidant activity. At a concentration of 90 ppm, the extract inhibited 65.994% of DPPH free radicals, with an IC_{50} of 63.734 ppm, indicating strong antioxidant activity. This activity is attributed to the presence of flavonoids and phenolic compounds, which can donate hydrogen atoms or electrons to stabilize free radicals. The hydroxyl groups attached to their aromatic rings contribute to their ability to terminate radical chain reactions and protect cells against oxidative damage (40).

Table IV

Antioxidant activity of *Eleutherine bulbosa* extract

Samples	Antioxidant activity (IC_{50}) (ppm)
Eleutherine bulbosa extract	63.734 ± 0.514
Vitamin C	1.283 ± 0.086

Vitamin C was used as a reference antioxidant because of its well-established radical scavenging activity. The IC_{50} value of Vitamin C (1.283 ppm) indicated very strong antioxidant activity and was substantially lower than that of *Eleutherine bulbosa* extract. Previous studies have similarly reported strong antioxidant activity for *Eleutherine bulbosa* ethanol extract, with IC_{50} values ranging from 26.98 to 41.46 mg/L (41). These findings confirm that *Eleutherine bulbosa* extract possesses considerable antioxidant potential, although its activity remains lower than that of the standard antioxidant, Vitamin C.

The DPPH assay in this study was performed only on the crude *Eleutherine bulbosa* extract, so the antioxidant activity of EB-CNPs remains unknown. This makes it difficult to determine whether nanoencapsulation altered the extract's antioxidant potential. Although nanoparticle formulation may affect the stability and release of bioactive compounds, these aspects were not investigated in the current study. Further evaluation of EB-CNPs using the same antioxidant assay would provide a clearer understanding of the effect of nanoencapsulation.

Evaluation of Eleutherine bulbosa extract nanoparticles (EB-CNPs)

In this study, three nanoparticle formulations were developed by varying the concentrations of chitosan and tripolyphosphate (TPP). The interaction between the positively charged amino groups of chitosan and the negatively charged TPP produces ionic gelation, which determines the physical characteristics of the nanoparticles. Three formulations were prepared using 0.5% chitosan (F1), 0.75% chitosan (F2), and 1% chitosan (F3). A chitosan-to-TPP ratio of 5:1 was used because it has been shown to yield smaller, more compact nanoparticles *via* stronger ionic cross-linking (42). The chitosan nanoparticles showed an encapsulation efficiency (%EE) of 96.08% and a loading capacity (%LC) of 13.80%. The high encapsulation efficiency suggests that most of the *Eleutherine bulbosa* extract was successfully incorporated into the chitosan nanoparticles. The loading capacity indicates the proportion of extract carried by the nanoparticle system. Encapsulation may help protect the bioactive compounds and support their delivery after administration. This may partly explain why EB-CNPs exhibited stronger antihypertensive effects than the crude extract in the present study (43).

Characterization of the *Eleutherine bulbosa* nanoparticles was carried out using a Particle Size Analyzer (PSA) to determine particle size and Scanning Electron Microscopy (SEM) to observe particle morphology. The measured sizes of F1, F2, and F3 were 325.67 nm, 291.17 nm, and 441.70 nm, respectively. All particle sizes fell within the general nanoparticle range of 10 - 1000 nm. The ionic gelation method typically produces particles within 200 - 500 nm, and the results obtained in this study align with that expected size range. The evaluation results are shown in Table V.

Table V

Evaluation of *Eleutherine bulbosa* extract nanoparticles (EB-CNPs)

Formula	pH	Size (nm)	Zeta (mV)	Polydispersity index (PI)	Conductivity (mS/cm)
F1	5.14 ± 0.00	325.67 ± 71.74	19.69 ± 0.58	0.86 ± 0.21	0.42 ± 0.00
F2	5.19 ± 0.00	291.17 ± 48.43	25.52 ± 1.43	0.78 ± 0.19	0.33 ± 0.02
F3	5.19 ± 0.00	441.70 ± 13.51	24.35 ± 0.22	0.57 ± 0.03	0.34 ± 0.00

F1: EB-CNPs (chitosan 0.5% with TPP 0.2%); F2: EB-CNPs (chitosan 0.75% with TPP 0.2%); and F3: EB-CNPs (chitosan 1% with TPP 0.2%)

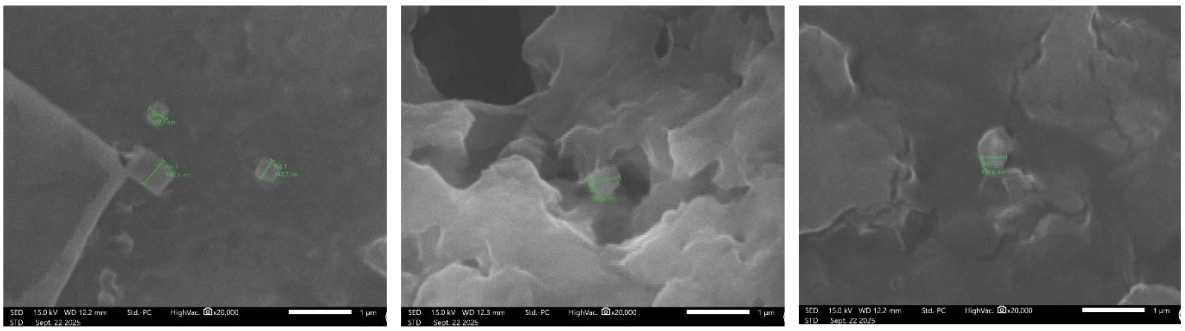
Among the three formulations, F2 had the smallest particle size. This result may be related to the longer stirring time used during its preparation, which likely helped break up larger particle aggregates and produced a more homogeneous nanoparticle dispersion. Since F2 showed the most desirable physicochemical properties, especially its smaller particle size, it was selected for the *in vivo* antihypertensive study.

Ideally, increasing the concentration of chitosan tends to produce larger particles; however, the particle size obtained is also influenced by mechanical processing conditions, such as the intensity and duration of stirring (44). The polydispersity index (PI) reflects the variation in particle size within a nanoparticle system. A lower PI value indicates a more uniform particle population, while a higher value suggests greater differences in particle size and a higher possibility of aggregation. Generally, PI values above 0.7 are considered to represent a relatively broad size distribution and reduced colloidal stability. In this study, the formulations showed PI values ranging from 0.57 to 0.86, suggesting that the particles were not completely uniform. Among the tested formulations, F3 showed the lowest PI value, indicating a more homogeneous particle distribution compared with F1 and F2 (45).

Similarly, zeta potential measurements yielded + 19.69 mV for F1, + 25.52 mV for F2, and + 24.35 mV for F3, all of which were below the ± 30 mV threshold typically associated with good electrostatic stability. These findings indicate that the prepared EB-CNPs exhibited only moderate physicochemical stability and remained susceptible to particle aggregation. Therefore, although F2 demonstrated the most favourable physicochemical characteristics among the tested formulations, particularly the smallest particle size, the relatively high PI values and moderate zeta potential indicate that further formulation optimization is required to improve particle homogeneity and colloidal stability before enhanced stability or delivery performance can be claimed (46).

Another factor influencing nanoparticle size is pH. Variations in pH affect the degree of amine group protonation in chitosan, which in turn affects the ionic gelation process. In this study, increasing the chitosan concentration resulted in slightly lower pH values. The observed pH range around 5 is favourable for nanoparticle formation, and particle size tends to decrease as the pH approaches the optimal range for chitosan–TPP crosslinking. This suggests that pH plays a critical role in determining the final characteristics of the nanoparticles (47).

Furthermore, the morphological analysis using Scanning Electron Microscopy (SEM) showed that the nanoparticles from all three formulations exhibited rough surfaces. This roughness is likely attributed to suboptimal gold coating during sample preparation, which can produce surface artifacts and reduce image clarity. The SEM results for formulations F1, F2, and F3 are presented in Figure 1.



(a) **(b)** **(c)**

Figure 1.
SEM images of nanoparticle morphology for F1 (a), F2 (b), and F3 (c) at 20,000× magnification

Evaluation of antihypertensive activity via systolic blood pressure measurement

In this study, systolic blood pressure in male Wistar rats was measured with the CODA® non-invasive blood pressure system. One limitation of the present work is the use of a non-invasive tail-cuff system to measure blood pressure. Compared with direct arterial methods, this technique is more prone to measurement variability. Although the animals were acclimatized before each measurement, this source of variation may still have influenced the results. The mean systolic blood pressure values are presented in Table VI. On Day 0, systolic blood pressure did not differ significantly among the experimental groups ($p > 0.05$), indicating that all groups had similar baseline blood pressure before hypertension induction. On Day 21, the negative control group, captopril (4.5 mg/kg B.W.), *Eleutherine bulbosa* extract (60 mg/kg B.W.), and EB-CNPs at doses of 100, 200, and 400 mg/kg B.W. showed systolic blood pressure values of 147.73, 151.27, 161.07, 166.67, 150.73, and 156.87 mmHg, respectively. All treatment groups exhibited significantly higher systolic blood pressure than the normal group ($p < 0.05$), confirming successful induction of hypertension following NaCl administration (3.75 g/kg B.W.) for 21 days. These findings indicate that prolonged high-salt administration effectively induced sustained hypertension, consistent with previous reports (48).

Table VI
Mean systolic blood pressure

Groups	Dose (mg/kg b.w.)	Systolic Blood Pressure (mmHg) ± SD			Difference (mmHg)
		Day 0	Day 21	Day 28	
Normal	-	130.33 ± 11.38^{a}	132.33 ± 9.33^{a}	121.80 ± 14.27^{a}	10.53
Control	-	115.80 ± 17.08^{a}	147.73 ± 27.48^{b}	151.47 ± 12.70^{c}	3.74
Captopril	4.5	120.07 ± 9.04^{a}	151.27 ± 10.14^{b}	106.93 ± 22.35^{a}	44.34
EB-e	60	121.67 ± 8.13^{a}	161.07 ± 8.16^{b}	142.17 ± 8.33^{c}	18.9
EB-CNPs	100	130.47 ± 11.05^{a}	166.67 ± 17.85^{b}	140.27 ± 7.22^{c}	26.4
EB-CNPs	200	125.80 ± 12.37^{a}	150.73 ± 11.30^{b}	117.07 ± 12.92^{b}	33.66
EB-CNPs	400	117.00 ± 10.73^{a}	156.87 ± 16.17^{b}	115.40 ± 17.89^{b}	41.47

Values are expressed as mean ± SD. Within the same row, values with different superscript letters **(**a, b, c**)** indicate statistically significant differences ($p < 0.05$, Tukey's post hoc test). In contrast, values with the same superscript letter are not significantly different

On day 28, a reduction in systolic blood pressure was observed in the captopril group (106.93 mmHg), *Eleutherine bulbosa* extract group (142.17 mmHg), EB-CNPs 100 mg/kg B.W. (140.27 mmHg), EB-CNPs 200 mg/kg B.W. (117.07 mmHg), and EB-CNPs 400 mg/kg B.W. (115.4 mmHg), with reductions of 44.34, 18.90, 26.40, 33.66, and 41.47 mmHg, respectively. EB-CNPs at 400 mg/kg B.W. exhibited a blood pressure-lowering effect comparable to captopril, indicating a strong antihypertensive response at this dose.

Analysis using two-way repeated-measures ANOVA showed that treatment, time, and their interaction significantly affected systolic blood pressure ($p < 0.05$). These results suggest that the reduction in blood pressure was related to the treatment and its duration. The hypertensive control group exhibited a progressive increase in systolic blood pressure throughout the study, whereas captopril and EB-CNPs significantly reduced systolic blood pressure by Day 28. The antihypertensive effect of EB-CNPs was dose-dependent, with the 400 mg/kg B.W. dose showing the greatest reduction and efficacy approaching that of captopril, suggesting that the nanoparticles enhanced the therapeutic activity of the extract (49).

The reduction in systolic blood pressure observed in the *Eleutherine bulbosa*–treated groups may be attributed to the presence of bioactive compounds, particularly flavonoids and phenolics, which are known to improve vascular function. A high-salt diet promotes

oxidative stress, which can reduce nitric oxide (NO) availability and disturb normal vascular function. The bioactive compounds present in *Eleutherine bulbosa* may counteract these effects by maintaining NO availability and supporting vascular relaxation, which could contribute to the decrease in blood pressure observed in this study (50). In addition, the nanoparticle formulation improves the bioavailability of the extract, resulting in more effective pharmacological activity compared with the crude extract (51). The nanoparticle formulation enhances the bioavailability observed in the EB-CNP groups, particularly at doses of 200 and 400 mg/kg B.W..

On Day 28, the captopril group had a lower systolic blood pressure than the normal control group. Although captopril is widely used as a positive control in experimental hypertension studies, the greater reduction observed in this study may reflect the response to the 4.5 mg/kg B.W. dose under the present experimental conditions. Variation among animals and the use of a non-invasive tail-cuff system may also have contributed to this result. For this reason, the antihypertensive effects of EB-CNPs and captopril should be compared with caution.

Evaluation of antihypertensive activity via diastolic blood pressure measurement

In this study, the diastolic blood pressure of male Wistar rats was measured using the CODA® non-invasive blood pressure system. The mean diastolic blood pressure values are presented in Table VII. The diastolic blood pressure measurements on day 21 showed that the negative control group, captopril (4.5 mg/kg B.W.), *Eleutherine bulbosa* extract (60 mg/kg B.W.), and EB-CNPs at doses of 100, 200, and 400 mg/kg B.W. exhibited diastolic values of 111.20, 128.53, 124.53, 125.13, 125.87, and 125.27 mmHg, respectively. All treatment groups showed significantly higher values compared with the normal group ($p < 0.05$), indicating successful induction of diastolic hypertension following NaCl administration (3.75 g/kg B.W.) for 21 days. This finding is consistent with salt-sensitive hypertension, in which increased extracellular fluid volume and vascular dysfunction contribute to elevated diastolic pressure (52).

Table VII

Mean diastolic blood pressure values

Groups	Dose (mg/kg b.w.)	Diastolic Blood Pressure (mmHg) ± SD			Difference (mmHg)
		Day 0	Day 21	Day 28	
Normal	-	$102.00 \pm 10.77^{a,b}$	102.00 ± 12.57^{a}	99.20 ± 9.81^{a}	2.8
Control	-	$98.53 \pm 12.82^{a,b}$	111.20 ± 21.85^{b}	124.13 ± 20.27^{c}	22.93
Captopril	4.5	$94.13 \pm 12.17^{a,b}$	128.53 ± 7.42^{b}	88.80 ± 13.17^{a}	39.73
EB-e	60	$100.07 \pm 9.56^{a,b}$	124.53 ± 9.10^{b}	114.47 ± 8.72^{b}	10.73
EB-CNPs	100	105.93 ± 5.62^{b}	125.13 ± 19.33^{b}	114.00 ± 5.51^{b}	11.13
EB-CNPs	200	86.27 ± 5.19^{a}	125.87 ± 12.08^{b}	93.80 ± 16.32^{a}	32.07
EB-CNPs	400	84.4 ± 10.30^{a}	125.27 ± 23.11^{b}	92.20 ± 16.50^{a}	33.07

Values are expressed as mean ± SD. Within the same row, values with different superscript letters **(**a, b, c**)** indicate statistically significant differences ($p < 0.05$, Tukey's post hoc test), whereas values with the same superscript letter are not significantly different

On day 28, reductions in diastolic blood pressure were observed in the captopril group (88.8 mmHg), *Eleutherine bulbosa* extract group (114.47 mmHg), EB-CNPs 100 mg/kg B.W. (114.0 mmHg), EB-CNPs 200 mg/kg B.W. (93.8 mmHg), and EB-CNPs 400 mg/kg B.W. (92.2 mmHg), with reductions of 39.73, 10.73, 11.13, 32.07, and 33.07 mmHg, respectively. EB-CNPs at 200 and 400 mg/kg B.W. demonstrated stronger diastolic-lowering effects, with the 400 mg/kg B.W. dose showing an effect comparable to captopril, suggesting improved vascular function and reduced peripheral resistance (52).

Diastolic blood pressure was significantly affected by treatment, time, and their interaction, as indicated by the two-way repeated-measures ANOVA ($p < 0.05$). These results suggest that the effect of each treatment varied throughout the experimental period. The hypertensive control group maintained elevated diastolic blood pressure throughout the experimental period, whereas captopril and EB-CNPs significantly reduced diastolic blood pressure by Day 28. The higher doses of EB-CNPs (200 and 400 mg/kg B.W.) produced greater reductions than the crude extract, indicating that the nanoformulation improved antihypertensive efficacy, possibly through enhanced bioavailability of the active phytochemicals (11).

The greater increase in diastolic blood pressure than systolic blood pressure observed in the negative control group may reflect biological variability and the inherent limitations of the non-invasive tail-cuff method. These findings should be considered with caution. However, treatment with *Eleutherine bulbosa* extract and EB-CNPs reduced diastolic blood pressure, suggesting a possible antihypertensive effect of the bioactive compounds in the plant. Diastolic blood pressure is closely associated with vascular tone and arterial elasticity, both of which are strongly influenced by endothelial function. Flavonoids and phenolic compounds in *Eleutherine bulbosa* may support endothelial function by maintaining nitric oxide (NO) availability, enhancing vasodilation, and reducing peripheral vascular resistance. These effects may contribute to the lower diastolic blood pressure observed in the treated groups. In addition, a high-salt

diet promotes oxidative stress, which can impair vascular function, reduce arterial flexibility, and interfere with normal vascular relaxation (53). The improved activity of EB-CNPs may be related to better delivery of the bioactive compounds from *Eleutherine bulbosa*, although further studies are needed to confirm the effect of nanoencapsulation on vascular protection and arterial function (54, 55).

Evaluation of nitric oxide levels measurement

This study evaluated the effect of administering ethanol extract of *Eleutherine bulbosa* Extract Nanoparticles (EB-CNPs) on nitric oxide (NO) levels in hypertensive rats using the Griess reaction method. NO levels were determined based on the formation of a pink azo compound produced when nitrite reacts with sulfanilamide under acidic conditions, followed by coupling with N-napthylethylenediamine. Absorbance was measured using an ELISA Reader, which utilizes spectrophotometric detection and enables simultaneous analysis of multiple samples and standards in a 96-well microplate with a minimum volume of 100 µL. The measurement results are presented in Table VIII.

Table VIII

Nitric oxide level measurement results

Groups	Dose (mg/kg b.w.)	Nitric Oxide (µmol/L)		Difference (µmol/L)
		Day 21	Day 28	
Normal	-	29.52 ± 2.16^{a}	29.33 ± 2.11^{a}	0.20
Control	-	16.47 ± 2.60^{b}	16.43 ± 2.67^{c}	0.03
Captopril	4.5	16.52 ± 1.51^{b}	30.03 ± 2.30^{a}	13.51
EB-e	60	17.05 ± 0.78^{b}	20.23 ± 0.46^{b}	3.18
EB-CNPs	100	16.69 ± 2.02^{b}	21.20 ± 2.23^{b}	4.51
EB-CNPs	200	16.28 ± 1.48^{b}	24.56 ± 2.20^{b}	8.28
EB-CNPs	400	17.60 ± 1.80^{b}	28.72 ± 2.86^{a}	11.13

Values are expressed as mean ± SD. Within the same row, values with different superscript letters (a, b, c) indicate statistically significant differences ($p < 0.05$, Tukey's post hoc test), whereas values with the same superscript letter are not significantly different

The NO level measurements on day 21 showed a significant decrease in all hypertensive groups compared with the normal group ($p < 0.05$), indicating that NaCl administration (3.75 g/kg B.W.) for 21 days effectively impaired nitric oxide production. Reduced NO levels reflect endothelial dysfunction, commonly associated with hypertension, due to decreased endothelial nitric oxide synthase (eNOS) activity and increased oxidative stress, which together reduce NO bioavailability and increase vascular resistance (56). The analysis using two-way repeated-measures ANOVA showed significant effects of treatment, time, and their interaction on nitric oxide levels ($p < 0.05$). Nitric oxide levels changed over the treatment period and differed among the experimental groups. The hypertensive control group exhibited persistently low nitric oxide concentrations, whereas treatment with captopril and EB-CNPs significantly restored nitric oxide levels over time. The increase was dose-dependent, with EB-CNPs at 400 mg/kg B.W. producing nitric oxide levels comparable to those of the captopril group, suggesting improved endothelial function through enhanced nitric oxide bioavailability following nanoparticle administration of the extract (44).

On day 28, an increase in NO levels was observed in the captopril group (30.03 µmol/L), *Eleutherine bulbosa* extract group (20.23 µmol/L), EB-CNPs 100 mg/kg B.W. (21.20 µmol/L), EB-CNPs 200 mg/kg B.W. (24.56 µmol/L), and EB-CNPs 400 mg/kg B.W. (28.72 µmol/L). The greatest improvement was observed in the captopril group (13.51 µmol/L increase), followed by EB-CNPs 400 mg/kg B.W. (11.13 µmol/L), EB-CNPs 200 mg/kg B.W. (8.28 µmol/L), EB-CNPs 100 mg/kg B.W. (4.51 µmol/L), and *Eleutherine bulbosa* extract (3.18 µmol/L).

In this study, nitric oxide levels were estimated indirectly through serum nitrite concentrations rather than direct measurement of nitric oxide. Since baseline (Day 0) nitrite levels were not assessed, the extent to which treatment restored nitric oxide levels could not be confirmed. Serum nitrite concentrations may also be influenced by other factors, including dietary nitrate intake and physiological variation, particularly under high-salt dietary conditions (56). These factors should be taken into account when interpreting the results. Future work would benefit from measuring nitric oxide at baseline and including additional markers of endothelial function to better explain the antihypertensive mechanism of EB-CNPs.

The nanoparticle formulations increased nitric oxide levels more effectively than the crude extract, with this effect becoming more pronounced at higher doses. One possible explanation is the antioxidant activity of flavonoids and phenolic compounds in *Eleutherine bulbosa*. Flavonoids and phenolic compounds may reduce oxidative stress by lowering reactive oxygen species (ROS) levels. This effect can help maintain nitric oxide availability, support normal endothelial function, and promote vascular relaxation, which may contribute to the reduction in blood pressure observed in this study (57).

The stronger effect observed with EB-CNPs than with the crude extract suggests that the nanoparticle formulation enhances the bioavailability of bioactive

compounds, thereby allowing more effective protection of NO from oxidative degradation. This improved pharmacological response, particularly at higher doses, indicates that EB-CNPs may enhance NO-mediated vasodilation and contribute significantly to antihypertensive activity (58).

The enhanced antihypertensive activity observed in the EB-CNPs formulations may be related to the effect of chitosan nanoparticles on the absorption, distribution, metabolism, and excretion of the bioactive compounds in *Eleutherine bulbosa*, where chitosan nanoparticles improve intestinal absorption and protect active compounds from degradation, resulting in higher bioavailability compared with the crude extract (59). In addition, the smaller particle size facilitates better distribution into vascular tissues and helps maintain more stable concentrations of active compounds in circulation, thereby enhancing nitric oxide (NO) bioavailability, improving endothelial function, and contributing to the stronger antihypertensive activity observed in EB-CNPs, particularly at doses of 200 and 400 mg/kg B.W. (60).

Eleutherine bulbosa extract and its chitosan nanoparticle formulation demonstrated antihypertensive activity in NaCl-induced hypertensive rats, as shown by reductions in systolic and diastolic blood pressure and improvements in nitric oxide (NO) levels. EB-CNPs, particularly at doses of 200 and 400 mg/kg B.W., exhibited antihypertensive effects comparable to captopril, suggesting enhanced efficacy through improved bioavailability and vascular function.

The enhanced effect of EB-CNPs may not only result from the bioactive compounds of *Eleutherine bulbosa*, but also from the properties of chitosan as the nanoparticle matrix. Chitosan has been reported to possess antioxidant activity and may support endothelial function by maintaining nitric oxide availability. These characteristics may have contributed to the improved antihypertensive response observed with EB-CNPs (61).

The greater antihypertensive activity of EB-CNPs may be associated with their smaller particle size. A smaller particle size increases the available surface area, which may facilitate interaction with biological membranes. As a result, the encapsulated phytochemicals may be absorbed more effectively and achieve higher bioavailability than the crude extract (62).

Increasing the dose of EB-CNPs resulted in a greater reduction in blood pressure and higher nitric oxide levels. This dose-related response suggests that the amount of formulation administered may influence the antihypertensive effect, although other factors should also be considered. Compared with the crude extract, EB-CNPs exhibited greater activity, which may be attributed to improved delivery of *Eleutherine bulbosa* bioactive compounds *via* the nanoparticle system and the supportive role of chitosan.

However, the present study did not include pharmacokinetic or bioavailability evaluations. Therefore, the enhanced therapeutic efficacy was inferred from the observed pharmacological responses rather than directly confirmed. In addition, oxidative stress biomarkers and histopathological examinations were not performed, limiting further elucidation of the antioxidant mechanisms and structural tissue protection associated with EB-CNP treatment.

Future studies integrating pharmacokinetic analysis, oxidative stress biomarkers, and histopathological evaluation are warranted to provide a more comprehensive understanding of the antihypertensive mechanisms of EB-CNPs and to further validate their therapeutic potential.

Conclusions

Eleutherine bulbosa chitosan nanoparticles (EB-CNPs) effectively lowered blood pressure in NaCl-induced hypertensive rats. Doses of 200 and 400 mg/kg B.W. significantly reduced both systolic and diastolic blood pressure while increasing nitric oxide (NO) levels compared with the negative control group ($p < 0.05$). Overall, the nanoparticle formulation produced a stronger antihypertensive response than the crude extract. At 400 mg/kg B.W., the antihypertensive effect was comparable to that of captopril, suggesting that the nanoparticle formulation improved the therapeutic performance of the extract.

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Availability of data and materials

The data supporting the findings of this study are included in this article. Additional information may be obtained from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All experimental procedures involving animals, including housing, treatment administration, and euthanasia, were conducted in accordance with ethical

guidelines and were approved by the Research Ethics Committee, Universitas Ahmad Dahlan (Approval No. REC-UAD/02/01/06-2025/071).

AI use disclosure

During the preparation of this manuscript, the authors used Grammarly to improve spelling, grammar, word choice, readability, and clarity.

Conflict of interest

The authors declare no conflict of interest.

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