Trop J Nat Prod Res, September 2025; 9(9): 4273 - 4278 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Potential of Nano *Phyllanthus niruri* L. as Anti-DENV Candidate

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ARTICLE INFO

Article history:
Received 07 April 2025
Revised 06 July 2025
Accepted 30 July 2025
Published online 01 September 2025

ABSTRACT

Dengue fever infection, spread by the *Aedes aegypti* mosquitos, is cause of dengue fever. One of the home grown plants with guarantee as an antiviral operator against dengue is *Phyllanthus niruri* L. Be that as it may, since of the destitute steadiness and bioavailability of the dynamic fixings, its adequacy is still limited. This consider pointed to extend the dengue antiviral movement of *P. niruri* L. by applying a nanoencapsulation technique using κ-carrageenan matrix. *P. niruri* L. was nanoencapsulated using the ultrasonication technique, and its bioactive constituents were subsequently characterized. Nano *P. niruri* L. (Pn-NPs) is a significant advance that aims to overcome the limitations of herbal-based therapies. Pn-NPs had a particle size of 115.42 ± 23.72 nm with a loading efficiency of 99.99 ± 0.06%. The stability of Pn-NPs was better than the extract against changes in NaCl concentration, temperature and pH. *P. niruri* L. extract was able to inhibit dengue virus with an EC_{50} of 1.73 ± 0.35 µg/mL, while Pn-NPs had an EC_{50} degree of 0.84 ± 0.01 µg/mL. Nanoencapsulation of *P. niruri* L. was proven to increase the anti-DENV activity with high SI value and low cytotoxicity (CC_{50}) compared to *P. niruri* L. extract.

Keywords: Anti-DENV, κ-Carrageenan, Dengue, Nanoencapsulation, *Phyllanthus niruri* L.

Introduction

Dengue Hemorrhagic Fever (DHF) is respected as a noteworthy around the world open wellbeing issue, especially nations in warm and humid climate zones, agreeing to the World Health Organization (WHO). DHF infection has four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4, which are dispersed by the *Aedes aegypti* mosquito.[1] It was reported that in 2022 there were 2,597,067 cases of DHF with 2,065 deaths worldwide.[2] Meanwhile, in Indonesia, in 2020, DHF cases reached 71,633, making DHF one of the diseases that need special handling.[3] Indonesia is one of the countries with abundant biodiversity, and particularly rich in medicinal plants. Since ancient times, Indonesian ancestors have used medicinal plants as raw materials for traditional medicine *(Jamu)*. Herbal plants that are believed to have properties against dengue fever include *Syzygium samarangense*,[4] *Phyllanthus niruri* L.,[5] *Cymbopogon citratus*,[6] *Carica papaya*, *Curcuma longa*, and *Andrographis paniculata*.[7] *Phyllanthus niruri* L., a widely distributed species throughout Indonesia a diverse range of phytochemicals, including nitrogenous compounds (alkaloids), polyphenolic substances (such as flavonoid derivatives like quercetin and quercitrin), astringent agents (tannins), triterpenes (lupeol), and other terpenoid constituents.[8] In addition, it contains sterols, glycosides, resins, carbohydrates, balsam, saponins, and phlobatanin. One of the benefits of the active compounds in *P. niruri* L. is as an antiviral.[9] It is believed that *P. niruri* L. can be used as an anti-malarial, antipyretic, and anti-dengue fever.[5]

One of the main concerns with the use of herbs is the effectiveness and safety of herbal remedies. Since not all home grown items are secure and viable, and attention has been drawn to their the toxicity.[10] One of the ways to minimize the challenges with herbal medicines is the design of Drug Delivery System (DDS) that has the ability to increase the effectiveness and control the rate of drug release.[11] Nanoencapsulation is one advancement in DDS. By expanding the surface area at the nanoscale, a technique known as nanoencapsulation may improve the stability and bioavailability of bioactive substances.[12,13] Using nanocapsules, which are built on a κ-carrageenan matrix, is a potential way to increase *P. niruri* L.'s antiviral effectiveness against the dengue virus. Because it may preserve active chemicals from degradation, boost their stability and bioavailability, and provide a targeted and regulated delivery system, the use of nanocapsules is regarded as an effective technique.[14] It is anticipated that this nanoencapsulation would improve the anti-dengue hemorrhagic fever (DHF) properties of *P. niruri* L. extract.

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Citation: Maharani MP, Tsalsabila M, Fajriyah DR, Sucipto TH, Tukiran, Wardana AP. Potential of nano *Phyllanthus niruri* L. as anti-DENV candidate. Trop J Nat Prod Res. 2025; 9(9): 4273 – 4278 https://doi.org/10.26538/tjnpr/v9i9.24

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Materials and Methods

Materials

Methanol, κ-carrageenan, deionized water, 2,2-diphenyl-1-picrylhydrazyl (TCI), Folin reagent (Merck), buffer solutions pH 2, 7, and 8,5; NaCl (Merck), NaOH (Merck), HCl (Merck), Na_2CO_3 (Merck), $AlCl_3$ (Merck), CH_3COONa (Merck), fetal bovine serum (Merck), Vero cell line, fungizone (Sigma), Viral ToxGloTM assay reagent (Promega), CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega), Eagle's Minimum Essential Medium (MEM), 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), Trypan-EDTA, Trypan blue, DENV-2 (NCBI No.KT012513, Dengue Fever Study Group, Institute of Tropical Disease, Universitas Airlangga.

Plant collection and identification

The leaves of *P. niruri L.* were gathered in April 2024 from Sidoarjo, located in East Java, Indonesia, at the geographic coordinates of 7° 36.48826" S and 112° 76.50096" E. The plant specimen was authenticated by the Indonesian Biology Generation Foundation and was cataloged under the voucher code BT-0784.

Total phenolic content

The measurement of total phenolic content (TPC) in *P. niruri L.* extract was carried out using a spectrophotometric method with Folin-

Ciocalteu reagent.[15] In this procedure, A volume of 1 mL from the test solution was mixed with 0.4 mL of Folin–Ciocalteu reagent and left to react at room temperature for 3 minutes. Following this, 5 mL of distilled water and 4 mL of 7% sodium carbonate (Na_2CO_3) solution were incorporated into the mixture, which was then incubated for 90 minutes. The optical density of the resulting solution was measured at 750 nm using a spectrophotometer. Gallic acid was utilized as the comparison standard, and the total phenolic content (TPC) was derived using the standard curve regression formula. The results were reported as milligrams of gallic acid equivalent per gram of sample (mg GAE/g).

Total flavonoid content (TFC)

The quantification of total flavonoid content (TFC) was carried out using a modified protocol adapted from Rosyantari *et al*. (2021)[15] Briefly, the sample solution was combined with 3 mL of methanol, 0.2 mL of 10% aluminum chloride ($AlCl_3$), 0.2 mL of sodium acetate (CH_3COONa), and 6 mL of purified water. The mixture was then incubated in a dark setting for 30 minutes to facilitate the reaction. After incubation, the absorbance was measured at 430 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800). Quercetin served as the reference compound for calibration. The TFC was calculated based on the quercetin standard curve, and results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

Determination of quercetin content

Standard solutions of quercetin at concentrations of 0.5, 1, 2, 4, and 8 ppm were analyzed using a UV-Vis spectrophotometer (Shimadzu UV-1800), with absorbance readings taken at a wavelength of 371 nm.[16]

Antioxidant assay

The free radical scavenging capacity of *Phyllanthus niruri* L. extract was assessed through the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.[17] stock solution of 0.004% DPPH was obtained by dissolving 4 mg of the compound in 100 mL of methanol. The optimal absorbance wavelength of this solution was established using a UV-Visible spectrophotometer. Subsequently, 3 mL of the DPPH solution was combined with varying **dosages** of the extract (1000, 500, 250, 125, 62.5, and 31.25 ppm). The mixtures were vigorously agitated and incubated for 30 minutes at ambient conditions. Absorbance readings were then taken at 516 nm. The antioxidant effectiveness was represented as the percentage of radical inhibition, which was calculated using a standard inhibition formula:

$$\% \text{ Inhibition} = \frac{\text{A Blank} - \text{A Sample}}{\text{A Blank}} \times 100 \quad ...(1)$$

Where;

A Blank is the absorbance of DPPH + methanol without the test sample
A Sample is the absorbance of DPPH + methanol + the test sample

Nanoencapsulation of P. niruri L.

Nano *P. niruri* L. (Pn-NPs) was processed by combining *P. niruri* L. extract with κ-carrageenan matrix using the ultrasonication method.[18] A 0.625 g sample of *P. niruri* L. extract was dissolved in 5 mL of methanol. Concurrently, 2.5 g of κ-carrageenan was dissolved in demineralized water and heated at 80 - 100°C until a homogeneous solution was obtained. The two solutions were combined and subjected to ultrasonication for 30 minutes. Physicochemical properties of Pn-NPs were determined by Dynamic Light Scattering (DLS) (Zetasizer nano ZS, Malvern); The identification of chemical functional moieties was carried out using a (FTIR) spectroscopy technique (Shimadzu IRTracer-100); and topology was analyzed using Atomic Force Microscopy (AFM) (Bruker, nanoscan).

Determination of loading and release profile of Pn-NPs

An osmotic semipermeable membrane was used to determine the loading amount and loading efficiency. Pn-NPs (10 mL) were added to a dialysis membrane. thereafter, the mixture was agitated in demineralized water at 400 rpm. Equations (2) and (3) were used to compute the loading efficiency and loading amount of bioactive components, respectively, while equation (4) was used to calculate the release at pH 2, 7, and 8.5.[13]

$$\%LE = \frac{\text{Mass of Samples on Pn-NPs}}{\text{Mass of Samples in feed}} \times 100\% \quad ...(2)$$

$$\%LA = \frac{\text{Mass of Samples on Pn-NPs}}{\text{Massa of Pn-NPs}} \times 100\% \quad ...(3)$$

$$Ct' = Ct + \frac{v}{V}\sum_0^{i-t} Ct \quad ...(4)$$

Description:

Ct' = corrected concentration at time t
Ct = measured concentration at time t
ν = volume of aliquots
V = total PBS volume

Determination of Pn-NPs stability

The durability of Pn-NPs was assessed by monitoring alterations in their UV-Visible absorption profiles and clarity levels when exposed to different environmental factors, including variations in temperature, acidity (pH), and salt (NaCl).[18]

Anti-DENV assay

DENV-2 (KT012509) with a concentration of 2×10^3 FFU/mL was incubated alongside *Phyllanthus niruri* L. extract (0.001 mg) or Pn-NPs (0.006 mg) for a period ranging from 48 to 144 hours at 37.5°C in a 5% CO_2 Surroundings.Once the incubation period, 100 µL of the Viral ToxGlo assay Compound was introduced into mixture and allowed to react for an additional 10 minutes. The resulting absorbance values for each sample were recorded using the GloMax microplate reader. From the data obtained, the EC_{50} and CC_{50} values were determined, and the selectivity index (SI) was calculated based on the appropriate formula (5).

$$SI = \frac{EC_{50}}{CC_{50}} \quad ...(5)$$

Where:

SI = *Selectivity Index*
EC_{50} = *Effective Concentration*
CC_{50} = *Cytotoxicity Concentration*

Results and Discussion

Antioxidant activity

The extraction process of *P. niruri* L. leaves by maceration in methanol produced an extract yield of 12.98%. from the results of the bioactive compound content determination, *P. niruri* L. extract was found to contain total phenols, total flavonoid, and quercetion at concentrations of 0.68 ± 0.07 mg GAE/g, 0.01 ± 0.02 mg QE/g, and 0.02 ± 0.02 mg/g, Correspondingly (Table 1).

The methanolic extract derived from *Phyllanthus niruri* L. leaves demonstrated strong antioxidant potential, as indicated by an IC_{50} value of 39.08 ± 0.44 µg/ml based on the DPPH free radical scavenging method. this notable antioxidant activity is in line with the extract's elevated total flavonoid concentration. an IC_{50} below 50 µg/ml suggests that the appearance of flavonoid compounds serves a significant contribution to enhancing extract's antioxidant properties. The interaction between phenolic constituents and DPPH occurs primarily through the donation of hydrogen atoms. The hydroxyl group on the phenolic molecule donates one hydrogen atom to DPPH in the reaction, creating DPPH hydrazine, a more stable DPPH radical.[19]

Table 1: Bioactive component level of *P. niruri* L.

Parameters	Result
TPC (mg GAE/g)	0.68 ± 0.07
TFC (mg QE/g)	0.01 ± 0.02
Quercetin Contents (mg/g)	0.02 ± 0.02

Physicochemical properties of Pn-NPs

Pn-NPs are nanocapsules of *P. niruri* L. extract encapsulated with κ-carrageenan matrix. In general, nanoparticle sizes of 100 - 500 nm can be used as a drug delivery system.[18,20] Pn-NPs have a particle size of 115.42 ± 23.72 nm, which is a hydrodynamic size. The Polydispersity Index (PDI) indicates the consistency of particle size distribution in a sample, reflecting the extent of size variation caused by distribution, clumping, and particle clustering.[21] Analysis results showed that Pn-NPs had an heterogeneous size variation within the sample is represented through the PDI value of 0.89 ± 0.08.[18] The AFM analysis results (Figure 1) confirm these findings, showing that the Pn-NPs have

an average size of about 65 nm.

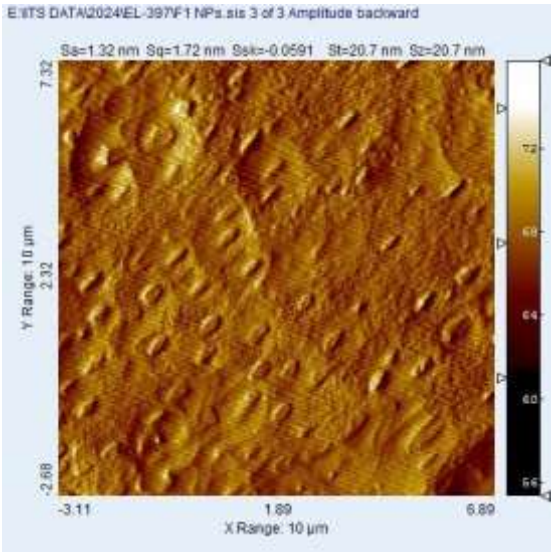

Figure 1: 2D Pn-NPs atomic force microscopy (AFM) Topology

FTIR characterization of Pn-NPs confirmed the successful combination of *P. niruri* L. extract with κ-carrageenan, as illustrated in (Figure 2). This was confirmed by the presence of key absorption bands in the

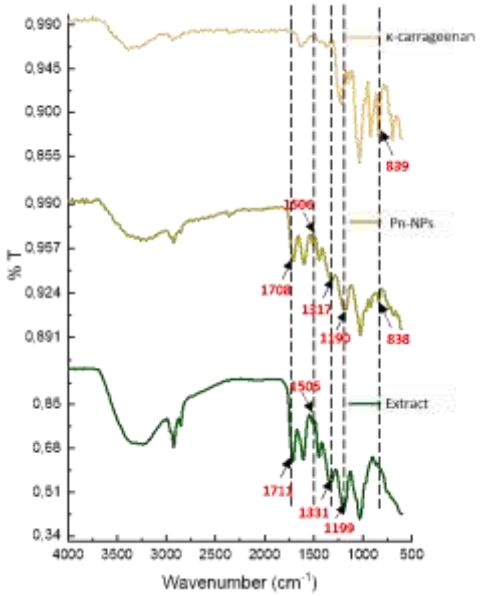

Figure 2: FTIR Spectrum of *P. niruri* L. extract, Pn-NPs, and Matrix κ-carrageenan

FTIR spectrum corresponding to both components. The absorption band located at 1708 cm^{-1} is linked attributed to carbonyl group stretching (C=O) groups, whereas the signal near 1506 cm^{-1} is indicative of the stretching of aromatic carbon-carbon double bonds (C=C). Meanwhile, the peak at 1317 cm^{-1} is connected to the bending of C–H bonds, and the band around 1190 cm^{-1} signifies the stretching of carbon–oxygen (C–O) bonds. These spectral features are typical for functional groups identified in *Phyllanthus niruri* L., the absorption band at 838 cm^{-1}, which is characteristic of galactose 4-sulfate, indicates the presence of κ-carrageenan. The coexistence of these absorption features in the Pn-NPs spectrum suggests that the structural integrity of both components is maintained, confirming the formation of a combined nanoparticle system.[22]

Loading and release profile of Pn-NPs

LE% of Pn-NPs was 99.99 ± 0.06%, and the loading amount (%) of Pn-NPs was 19.99 ± 0.01%. These results demonstrate that nanoencapsulation of *P. niruri* L. extract with κ-carrageenan successfully encapsulates bioactive components from herbal extracts.

The rate at which active phytochemicals are liberated from Pn-NPs is presented in (Figure 3). The results showed that the most optimal release of Pn-NPs occurred at pH 7, reaching a percentage of 62.45%. It is suspected that at pH 7, the sulfate group on κ-carrageenan is deprotonated to produce OSO_3^- ions. This condition causes electrostatic repulsion between ions so that the distance between chains widens and facilitates the release of more bioactive components.[23] At pH 2, Pn-NPs showed the smallest release at 15.83%. In an acidic environment, there is an attraction between particles caused by hydrogen bonds, resulting in smaller particle pores and a slight release of bioactive components.[22]

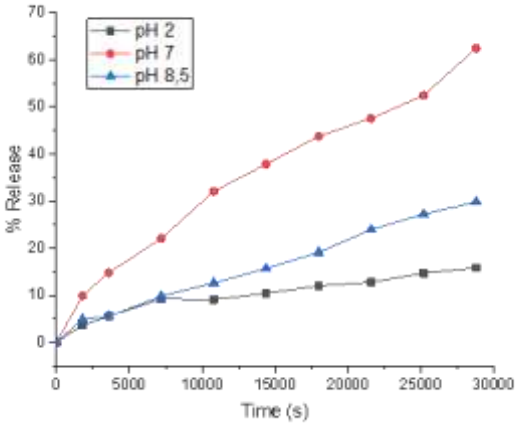

Figure 3: Percent Release of Pn-NPs

Stability of Pn-NPs against NaCl

Increasing the NaCl content from 0 to 0.3 M allowed for the determination of the stability of *P. niruri* L. extract and Pn-NPs. Due to salt-induced precipitation, adding NaCl to the *P. niruri* L. extract reduced turbidity and formed sediment at the bottom. Turbidity increases at a concentration of 0.15 M, suggesting that *P. niruri* L. extract is generally less stable against NaCl than Pn-NPs. This is evidenced by the UV-Vis spectra of *P. niruri* L. extract, which shows a significant hypochromic absorption band shift at a concentration of 0.15–0.3 M NaCl, while Pn-NPs remained relatively stable, as indicated by the absence of fluctuating absorption band shifts (Figure 4).

Stability of Pn-NPs against temperature

The stability of the extract and Pn-NPs was evaluated by raising the temperature within the range of 30°C to 100°C. Between 50°C and 70°C, the *P. niruri* L. extract's turbidity level increased, presumably as a result of the depolymerization-induced synthesis of κ-carrageenan gel.[22] The κ-carrageenan matrix protects the active compounds of *P. niruri* L., so the turbidity level of the Pn-NPs mixture exhibits greater stability and lower susceptibility to decomposition. In this case, the encapsulation process of *P. niruri* L. effectively maintains the stability of its bioactive components, likely due to the effects of heating. Furthermore, the UV-Vis absorption bands of both the extracts and Pn-NPs remained relatively stable, showing no significant shifts (Figure 5).

Stability of Pn-NPs against pH

The stability of *P. niruri* L. extract and Pn-NPs measured at pH 2-10 showed that *P. niruri* L. extract had two absorption bands at an acidic pH of 5. Under pH 2-8 conditions, there was a hypsochromic shift in the 278 nm absorption band. While in Pn-NPs, which have a slightly acidic pH of 6, when acidified (pH 2), there was a hypsochromic shift in the 267 nm band. The turbidity level of the *P. niruri* L. extract showed a significant decrease, which was influenced by the conversion

of keto-enol species on the carbonyl groups of phenolic compounds during the oxidative polymerization process. In contrast, Pn-NPs showed a relatively stable turbidity level. Thus, *P. niruri* L. nanoencapsulation showed superior potential in enhancing the stability of bioactive components against pH variation compared to *P. niruri* L. extract (Figure 6).

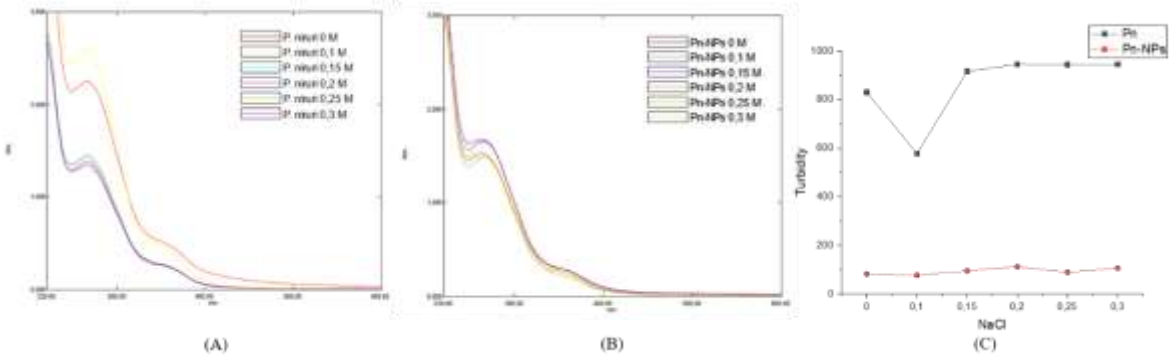

Figure 4: Stability of nano *P. niruri* L. against NaCl concentration **(A)** Extract of *P. niruri* L. **(B)** Pn-NPs **(C)** Turbidity level of extract and Pn-NPs

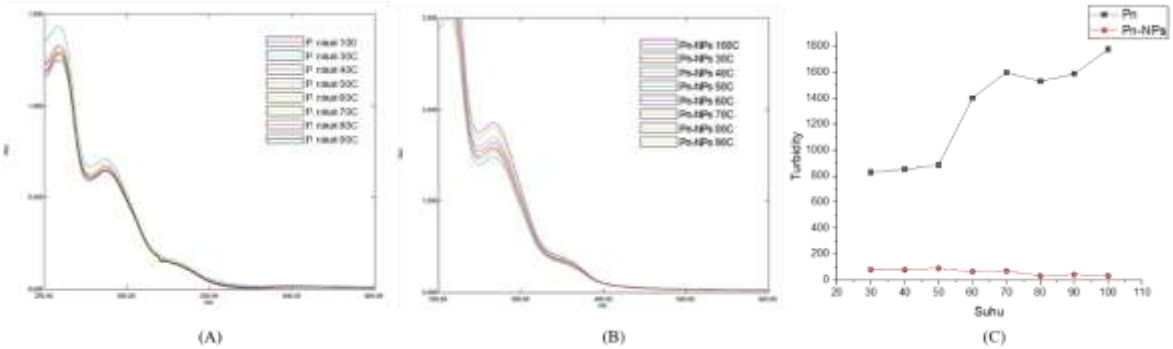

Figure 5: Stability of Nano *P. niruri* L. against temperature change **(A)** Extract of *P. niruri* L. **(B)** Pn-NPs **(C)** Turbidity level of extract and Pn-NPs

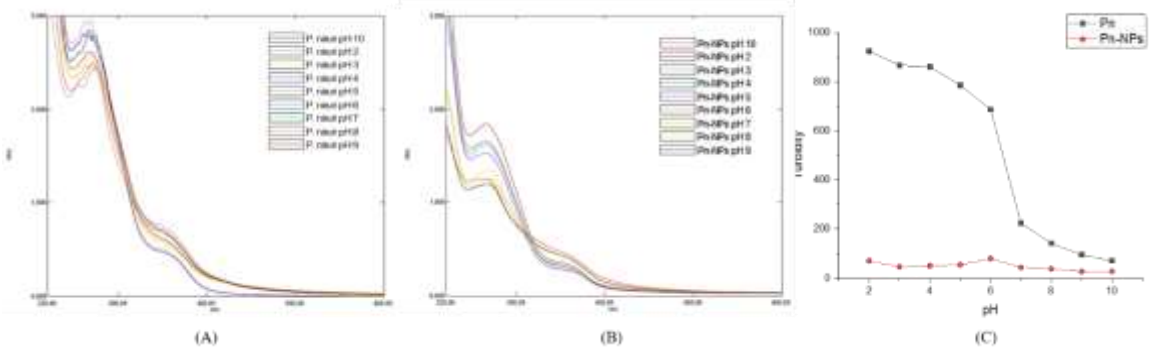

Figure 6: Stability of nano *P. niruri* L. against pH changes **(A)** extract of *P. niruri* L. **(B)** Pn-NPs **(C)** Turbidity level of extract and Pn-NPs

Anti-DENV activity

The anti-dengue virus type 2 activity of Pn-NPs is presented at Table 2. CC_{50} levels of *P. niruri* L. *isolate and* Pn-NPs **managed** 48.21 ± 0.12 and 60.20 ± 4.81 µg/mL, respectively. These results indicate that Pn-NPs were able to reduce the toxicity of *P. niruri* L. while maintaining comparable anti-dengue activity, as demonstrated by the higher CC_{50} value of Pn-NPs relative to the extract. Pn-NPs has higher anti-DENV activity than *P. niruri* L. extract with EC_{50} of 0.84 ± 0.01 and 1.73 ± 0.35 µg/mL, in sequence. This indicates that the nanoencapsulation process of *P. niruri* L. can reduce cytotoxicity and increase anti-DENV activity of Pn-NPs. The minimum SI limit for potential drug candidates is 10.[13,24] Based on the SI results, Pn-NPs has more potential as a specific anti-DENV candidate owing to its greater SI value of 71.10 relative to the extract of only 27.18. This shows that Pn-NPs can inhibit the proliferation of the dengue virus without damaging host cells. They

also provide adequate safety between hazardous dosages against vero cells and effective inhibitory concentrations against dengue virus serotype 2.[25] Based on the ANOVA analysis, a notable statistical variation was detected *P. niruri* L. extract and Pn-NPs in their activity against dengue virus. This is indicated by a level of p-value of 0.010 ($p = 0.010$) lower than the threshold of $\alpha = 0.05$. These findings confirm that both *P. niruri* L. extract and its κ-carrageenan-encapsulated form exhibit significant inhibitory effect against dengue virus, thereby supporting the potential of nanoencapsulation as an advantageous strategy to enhance the potency of bioactive agents.

Table 2: Anti-DENV Activity of *P. niruri* L. extract and Pn-NPs

	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
P. niruri L. Extract	48.21 ± 0.12	1.73 ± 0.35	27.18
Pn-NPs	60.20 ± 4.81	0.84 ± 0.01	71.10

Conclusion

Nanoencapsulated *Phyllanthus niruri* L. (Pn-NPs) exhibited superior anti-DENV activity compared to its extract. This was confirmed by the EC_{50} value of *P. niruri* L. extract (1.73 ± 0.35 µg/mL), that showed a notable higher than that of Pn-NPs (0.84 ± 0.01 µg/mL). In addition, Pn-NPs showed a lower toxicity level with a high selectivity index of 71.10 compared to the *P. niruri* L. extract, which was only 27.18. The promising anti-DENV activity of Pn-NPs is consistent with their stability under varying temperatures and NaCl concentrations, although they exhibit less stability at different pH levels. Moreover, Pn-NPs are capable of releasing bioactive components up to 62.45%. This study successfully showed that *P. niruri* L. extract nanoencapsulated with κ-carrageenan efficiently increased the anti-DenV activity, producing Pn-NPs that were less toxic and had stronger antiviral activity than *P. niruri* L. extract. These results offer a solid foundation for Pn-NPs continued research as possible anti-dengue viral medication options.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

Funding for this analysis was provided by the Indonesian Ministry of Education, Culture, Research, and Technology through Universitas Negeri Surabaya, both of which also supported the implementation of the 2024 Student Creativity Program.

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