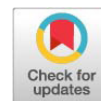


Research Article



Potential of Morrio-NP as a Natural Immunostimulant for Foot and Mouth Disease (FMD) Prevention

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Abstract | In early 2025, Foot and Mouth Disease (FMD) re-emerged in Indonesia, causing significant mortality and economic losses in ruminant livestock. The objective of this research was to develop a natural immunostimulant based on chitosan nanoparticles formulated with a combination of *Moringa oleifera* leaves and *Durio zibethinus* seeds (Morrio-NP). This study aims to evaluate the potential of these bioactive compounds to enhance immune responses and support sustainable livestock health management by introducing plant-derived nanoparticle formulations as an innovative strategy for FMD prevention in ruminants. This study employed Morrio-NP formulation based on chitosan-TPP loaded with ethanolic Moringa-durian seed extract, followed by bioactive compound analysis, particle size and zeta potential characterization, and DPPH-based antioxidant activity assessment across four Morrio-NP concentrations and vitamin C. Morrio-NP was found to contain key bioactive compounds including tannins, flavonoids, alkaloids, saponins, and steroids, with measured levels of 6.9 mg RE/g dw flavonoids, 13.8 mg GAE/g dw total phenols, and 9.2% total tannins. Morrio-NP exhibited a uniform particle size of 111.8 nm and a zeta potential of +32.5 mV, indicating good stability. Antioxidant assays showed that Morrio-NP has strong free radical scavenging activity, with IC₅₀ values ranging from 90.3 to 98.1 ppm, highlighting its potential as a natural antioxidant. The antioxidant properties observed strongly suggest the potential of Morrio-NP as a natural immunostimulant for preventive support against FMD in ruminants. Although these findings are promising, further research including antiviral assays and in vivo studies is urgently required to validate its efficacy in practical livestock applications.

Keywords | Additive, Feed, FMD, Morrio-NP, Preventive

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INTRODUCTION

Foot-and-mouth disease (FMD) has re-emerged as a major threat to Indonesia's livestock sector after more than 30 years of WOAHA-recognized freedom. The 2022–2025 outbreak caused severe economic losses, estimated at IDR 38.67 trillion, with smallholder farmers

losing more than 10% of their annual income (Rohma *et al.*, 2022; Handoko, 2025). Beyond reducing productivity and causing high mortality, FMD restricts trade and jeopardizes national food security (Kargbo *et al.*, 2025). Vaccination remains the primary control strategy but faces limitations such as poor accessibility, waning immunity, and inadequate cross-protection, highlighting the need for

innovative preventive approaches (Hoelzer *et al.*, 2018).

The limitations of vaccines and conventional therapies have driven research into alternative approaches focused on enhancing host immunity. Promising options include probiotics, bacteriocins, plant extracts, and natural immunomodulators (Zubareva *et al.*, 2024). In this context, nanoparticle technology offers significant advantages by improving the bioavailability and efficacy of bioactive compounds. Chitosan-based nanoparticles, in particular, have been reported to enhance antibacterial, anti-inflammatory, and immunostimulatory activities, making them strong candidates as feed additives and livestock health enhancers (Arshad *et al.*, 2021). Thus, nanoparticle-based delivery of these natural immunomodulators represents a logical extension of current preventive strategies by addressing the bioavailability and stability limitations of conventional vaccines and therapies.

Natural sources such as *Moringa oleifera* leaves and *Durio zibethinus* seeds (Morrio-NP) possess well-documented immunomodulatory properties. Their rich content of flavonoids, curcuminoids, vitamins, and minerals supports immune cell proliferation, restores suppressed immunity, and exerts strong antioxidant and anti-inflammatory effects (Hossain *et al.*, 2022; Poluan *et al.*, 2023). Combined extracts have also demonstrated antiviral potential in preclinical studies. Both plants have been proven to restore immune function in cyclophosphamide-induced immunosuppressed rats and to enhance the populations of CD4+, CD8+, and B220+ cells in mice (Mahana *et al.*, 2018). The combination of *Moringa* and curcuma extracts has also (Kharisma *et al.*, 2022). These findings indicate that *Moringa* and turmeric have strong potential as natural immunomodulators and may contribute to overall health maintenance (Jacques *et al.*, 2020). Despite increasing interest in natural immunostimulants, the role of nanoparticle-based natural formulations (e.g., Morrio-NP) in enhancing immune responses against FMD remains insufficiently explored, especially in livestock systems. Therefore, this study aims to evaluate the potential of nanoparticle-based formulations of natural compounds as immunostimulants to enhance bioactivity and support sustainable strategies for the prevention of FMD.

MATERIALS AND METHODS

LOCATION

This study on the analysis of the preventive potential of Morrio-NP (*Moringa oleifera* + *Durio zibethinus* seed formulation) against Foot-and-Mouth Disease (FMD) in ruminants was conducted from July 1 to August 30, 2025, at the KST Soekarno, National Research and Innovation Agency (BRIN), Indonesia. *Moringa oleifera* leaves and *Durio zibethinus* seeds were collected from community

gardens in Kediri Regency, East Java, Indonesia, in June 2025. Plant identification was carried out based on morphological characteristics following standard taxonomic keys. No voucher specimens were deposited in a herbarium.

PREPARATION OF MORRIO-NP

Based on the solubility test, Chitosan (1.5% w/v, medium molecular weight, degree of deacetylation $\geq 85\%$; Sigma-Aldrich, USA, product code 102566488) was dissolved in 1% (v/v) acetic acid under continuous stirring (magnetic stirrer) until a homogeneous, pale-yellow viscous solution was obtained; this solution served as the chitosan base for Morrio-NP synthesis. *Moringa* leaves (*Moringa oleifera*) and durian seeds (*Durio zibethinus*) were dried using a hot-air oven at 40–45 °C until a constant weight was achieved, then ground into a fine powder and passed through a 60-mesh sieve to ensure uniform particle size. The powdered materials were extracted by maceration using 96% ethanol with a solvent-to-material ratio of 10:1 (mL/g) for 48 h at room temperature with occasional stirring. The extracts were filtered using Whatman No. 1 filter paper and subsequently concentrated under reduced pressure using a rotary evaporator at 45 °C to obtain a thick crude extract. The extracts of *Moringa oleifera* leaves and *Durio zibethinus* seeds were mixed at a 1:1 (w/w) ratio and incorporated dropwise into the prepared chitosan solution under continuous magnetic stirring. The total extract volume added was adjusted to obtain a final Morrio-NP solid concentration. Sodium tripolyphosphate (TPP; supplier, catalog number) was then added dropwise as a crosslinking agent at a chitosan:TPP ratio of 2:1 while stirring at 1000 rpm for 30 minutes at room temperature (25 ± 2 °C) to prevent nanoparticle agglomeration. The pH of the suspension was maintained at pH 4.5–5.0 and adjusted when necessary using 0.1 M NaOH. The resulting Morrio-NP suspension was allowed to stabilize before further characterization.

IDENTIFICATION OF BIOACTIVE COMPOUNDS IN MORRIO-NP

The bioactive components of the Morrio-NP were qualitatively identified using the method outlined by Adeyemi *et al.* (2017). Quantitative analysis of total phenolic content (TPC) in the combined extract of *Moringa oleifera* leaves and *Durio zibethinus* seeds (Morrio extract) was performed using the Folin–Ciocalteu method. A gallic acid calibration curve (0–200 µg/mL) was used, and absorbance was measured at 765 nm after 30 min of incubation at room temperature. Results were expressed as mg gallic acid equivalents (GAE) per g extract. Concurrently, total flavonoid content (TFC) of the same extract was determined using a colorimetric aluminum chloride method, employing rutin as the standard (0–100

µg/mL). Absorbance was recorded at 415 nm after 30 min of incubation. Results were expressed as mg rutin equivalents (RE) per g extract. All measurements were conducted in triplicate (n = 3), following the procedure described by Prasetyo *et al.* (2024).

CHARACTERIZATION OF MORRIO-NP

Morrio-NP characterization was carried out by diluting 100 µL of the nanoparticle suspension with distilled water to a final volume of 5 mL (dilution factor 1:50) to avoid multiple scattering effects during measurement, followed by homogenization using a vortex mixer for 30 s. Particle size and size distribution were measured using a Particle Size Analyzer (PSA; Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) based on dynamic light scattering. Zeta potential was determined using the same instrument via electrophoretic light scattering. Measurements were conducted at 25 °C, using distilled water as the dispersion medium with a refractive index of 1.33. Each sample was measured in triplicate, and the results were expressed as mean ± standard deviation (SD), including the polydispersity index (PDI).

ANTIOXIDANT ACTIVITY EVALUATION OF MORRIO-NP

The antioxidant activity of Morrio-NP was evaluated in vitro using the DPPH radical scavenging assay with modifications from Rastuti and Purwati (2012). For the DPPH assay, Morrio-NP samples were prepared at final concentrations of 10, 25, 50, 75, and 100 ppm, which were obtained by diluting the respective Morrio-NP stock formulations. The Morrio-NP formulations at 6%, 9%, and 12% (w/v) represent the original nanoparticle preparations, while the ppm concentrations were used specifically for the in vitro antioxidant assay. The maximum wavelength (λ_{max}) was determined by mixing 4.5 mL of 96% ethanol with 1.5 mL of 0.2 mM DPPH solution and incubating at 37 °C for 10 min. Absorbance was measured in the range of 500–530 nm at 5 nm intervals using a UV-Vis spectrophotometer (Thermo Fisher Scientific, USA).

Antioxidant activity was expressed as percentage inhibition (%) and calculated using the formula described by Molyneux (2003):

$$\% \text{ Inhibition} = [(A_0 - A_s) / A_0] \times 100$$

Where; A₀ is the absorbance of the control and A_s is the absorbance of the sample.

All measurements were performed in triplicate (n = 3), and results are presented as mean ± SD. Ascorbic acid (Vitamin C) was used as a positive control.

STATISTICAL ANALYSIS

Data were analyzed using one-way ANOVA after confirming normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Differences were considered significant at p < 0.05, followed by Tukey HSD post hoc analysis. All experiments were performed in triplicate, and results are expressed as mean ± SD.

RESULTS

The results of qualitative (Table 1) observations of plant bioactivity showed that Morrio-NP proved positive for containing tannins, flavonoids, alkaloids, saponins and steroids. While, the results of quantitative analysis of bioactive compound on this study such as flavonoids, total phenols and tannin are presented in Table 2. The flavonoid content of the Morrio-NP was 6.9 (mg RE/g dw), the total phenolic content obtained was 13.8 (mg GAE/g dw), the total tannin content is 9.2%, with condensed tannins detailed at 6.8% and hydrolysable tannins at 5.5%.

Table 1: Analyses of qualitative phytochemicals of Morrio-NP.

Secondary metabolite	Morrio-NP
Tannin	+
Flavonoid	+
Alkaloid	+
Saponin	+
Steroid	+

Table 2: The quantification of Morrio-NP

Mate- rial	Flavonoids content (mg RE/g dw)	Total phe- nolic (mg GAE/g dw)	Tannin Con- total (%)	den- sified tannin, CT (%)	Hydro- lyzed tannin, HT (%)
Mor- rio-NP	6.9	13.8	9.2	6.8	5.5

Table 3: Results of particle size analyzer (PSA) test.

Sample	Replication 1	Replication 2	Replication 3	Average
Morrio-NP	108.2 nm	115.4 nm	111.9 nm	111.8 nm

Table 3 shows that the average particle size of Morrio-NP obtained from PSA analysis was 111.8 nm, which falls within the nanometer range (<200 nm) suitable for biomedical applications as a natural immunostimulant. The relatively narrow particle size distribution (108.2–115.4 nm) indicates that the formulation process produced homogeneous and reproducible nanoparticles. Meanwhile, the results of the zeta potential test showed an average value of +32.5 mV, indicating a fairly good dispersion stability. A zeta potential value above ±20 mV suggests the presence of electrostatic repulsion forces between particles,

thereby preventing aggregation (Table 4).

Table 4: Results of zeta potential test.

Sample	Replica- tion 1	Replication 2	Replication 3	Average
Morrio-NP	+31.3 mV	+33.3 mV	+32.9 mV	+32.5 mV

Table 5: IC₅₀ values of morrio-NP.

Treatment	IC ₅₀ (ppm)	Category
Ascorbic Acid	2.820 ± 0.050 ^a	Very Strong
Morrio NP 6%	90.327 ± 8.815 ^b	Strong
Morrio NP 9%	98.113 ± 14.954 ^b	Strong
Morrio NP 12%	97.953 ± 15.223 ^b	Strong

^{a,b} Different superscript letters in a column indicate significant difference (p<0.05).

The results of the antioxidant activity tests presented in Table 5 indicate that all treatments fall into the “strong” category for free radical scavenging activity. Ascorbic acid, used as a positive control, showed the lowest IC₅₀ value of 2.820 ± 0.050 ppm (p < 0.05). Meanwhile, Morrio NP at concentrations of 6%, 9%, and 12% exhibited IC₅₀ values of 90.327 ± 8.815 ppm, 98.113 ± 14.954 ppm, and 97.953 ± 15.223 ppm, respectively. Although the IC₅₀ values of Morrio NP are higher than that of ascorbic acid, they still fall within the strong antioxidant category, demonstrating that Morrio NP is effective in inhibiting free radical activity, albeit at higher concentrations than ascorbic acid.

DISCUSSION

The qualitative and quantitative analyses of Morrio-NP revealed the presence of key bioactive compounds, including tannins, flavonoids, alkaloids, saponins, and steroids, with significant levels of flavonoids (6.9 mg RE/g dw), total phenols (13.8 mg GAE/g dw), and total tannins (9.2%). These compounds are well-documented for their antioxidant, antimicrobial, and health-promoting properties (Prasetyo *et al.*, 2024; Baihaqi *et al.*, 2024; Harahap *et al.*, 2024; Rokana *et al.*, 2024; Lisnanti *et al.*, 2024; Sakti *et al.*, 2024; Baihaqi *et al.*, 2024), suggesting that Morrio-NP exerts a multifaceted bioactivity. In particular, the high phenolic and flavonoid content likely underpins its strong free radical scavenging potential, while the combination of condensed and hydrolysable tannins may enhance both antioxidant and antimicrobial effects (Hassanpour *et al.*, 2023). Collectively, these findings indicate that Morrio-NP is a potent natural source of bioactive compounds, supporting its potential application in nutraceuticals, functional foods, and natural therapeutics.

The particle size analysis of Morrio-NP showed a narrow distribution with an average size of 111.8 nm, which falls within the ideal range (<200 nm) for biomedical

applications (Bashir *et al.*, 2022). This size supports efficient cellular penetration and optimal biomolecular interactions, consistent with previous findings indicating that nanoparticle size influences circulation time, targeting ability, and cellular uptake (Hoshyar *et al.*, 2016). The narrow size distribution also indicates that the formulation process produced homogeneous and reproducible nanoparticles, an important factor for consistent bioactivity and delivery efficiency (Masarudin *et al.*, 2025).

The use of chitosan-based nanoparticles as immunostimulant delivery systems has long been reported to enhance the stability, bioavailability, and biological activity of natural compounds (Landriscina *et al.*, 2015). These early studies emphasized the role of chitosan nanoparticles in modulating immune responses through macrophage activation and increased cytokine production (Rajitha *et al.*, 2016). More recent findings have expanded this concept by integrating immunomodulatory phytochemicals into nano-based systems to improve efficacy against viral diseases in livestock (Gourapura and Renu, 2020). The zeta potential measurement of Morrio-NP showed an average value of +32.5 mV, indicating good colloidal stability due to electrostatic repulsion between particles, minimizing aggregation and enhancing shelf-life and functional performance (Calva-Estrada *et al.*, 2021). This value aligns with literature reports stating that zeta potentials above ±30 mV indicate high nanoparticle stability (Kaasalainen *et al.*, 2017). Overall, Morrio-NP demonstrates potential as a candidate for applications in nutraceuticals and natural therapeutics.

The results of the antioxidant activity assay of Morrio-NP are presented in Table 5. Statistical analysis was performed using one-way ANOVA, and the results indicated a statistically significant difference among treatment groups (p < 0.05). These differences are denoted by different superscript letters on the IC₅₀ values. Ascorbic acid exhibited very strong antioxidant activity (Eisvand *et al.*, 2025) with the lowest IC₅₀ value and was significantly different from all Morrio-NP formulations. Meanwhile, no significant differences were observed (p > 0.05) among Morrio-NP at concentrations of 6%, 9%, and 12%, all of which were classified as having strong antioxidant activity. Morrio-NP exhibited strong antioxidant activity, with IC₅₀ values ranging from 90.33 to 98.11 ppm, confirming its effectiveness in scavenging free radicals across various concentrations (Untea *et al.*, 2024). Although these values are higher than ascorbic acid (IC₅₀ = 2.820 ppm) used as a positive control, Morrio-NP remains a strong antioxidant, highlighting its potential as a natural source of bioactive compounds. The high content of flavonoids, phenolic compounds, and tannins likely acts synergistically to enhance immune responses and reduce oxidative stress, which are critical factors in improving livestock resistance

to infections (Piao *et al.*, 2023). Therefore, Morrio-NP may function not only as an antioxidant but also as a natural immunostimulant, supporting the prevention of FMD in ruminants. Integrating Morrio-NP into feed or functional supplements could strengthen animal immunity, decrease susceptibility to pathogens, and potentially reduce FMD prevalence through mechanisms of cellular protection and oxidative stress mitigation.

CONCLUSION

Morrio-NP contains significant bioactive compounds, including flavonoids, phenols, and tannins, and exhibits strong antioxidant activity (IC₅₀ 90.33–98.11 ppm). Its nanoparticle characteristics (111.8 nm, +32.5 mV) ensure stability and bioavailability. Morrio-NP not only neutralizes free radicals but also supports immune function, highlighting its potential as a natural feed additive or nutraceutical to enhance livestock health and aid in the prevention of Foot-and-Mouth Disease (FMD).

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NOVELTY STATEMENT

This study is the first to develop and characterize a chitosan-TPP nanoparticle formulation combining *Moringa oleifera* leaves and *Durio zibethinus* seeds (Morrio-NP) as a natural immunostimulant candidate for FMD prevention in ruminants.

AUTHOR'S CONTRIBUTION

ER, ZAB, FT dan AD designed the study and collected samples and performed examinations. All authors have drafted and revised the manuscript. All authors have read, reviewed, and approved the final manuscript.

ETHICAL APPROVAL

The research obtained approval from the Animal Care and Use Committee at Universitas Brawijaya, Indonesia under the reference number: 208 – KEP – UB – 2023.

GENERATIVE AI AND AI ASSISTED TECHNOLOGY STATEMENT

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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