BIODIVERSITAS
Volume 27, Number 2, February 2026
Pages: 11

ISSN: 1412-033X
E-ISSN: 2085-4722
https://doi.org/10.13057/biodiv/d270225

Nepenthesin activity of three *Nepenthes* species from Lore Lindu National Park, Central Sulawesi, Indonesia

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Manuscript received: 18 August 2025. Revision accepted: 21 February 2026.

Abstract. *Rahmadina, Tarigan MRM, Panggabean NH, Daulay A, Fadillah MA, Faisal M, Hidayat, MI, Sudarman S. 2026. Nepenthesin activity of three* Nepenthes *species from Lore Lindu National Park, Central Sulawesi, Indonesia. Biodiversitas 27 (2): d270225. https://doi.org/10.13057/biodiv/d270225.* Carnivorous pitcher plants of the genus *Nepenthes* rely on proteolytic enzymes to supplement nutrient uptake in nutrient-poor habitats. Among these enzymes, nepenthesins are acid-stable aspartic proteases found in the digestive fluid. This study compared the morphology and enzymatic activity of three *Nepenthes* species (*Nepenthes maxima*, *Nepenthes nigra*, and *Nepenthes tentaculata*) from Lore Lindu National Park, Central Sulawesi, Indonesia. Morphological traits were measured in four 10×10 m plots, while nepenthesin activity was tested at four concentrations (50-200 µg/mL) using casein as substrate. Lower pitchers were generally larger and more robust, whereas upper pitchers were slender and showed interspecific variation. Enzymatic activity increased with concentration and ranged from 0.20 to 0.58 U, with *N. tentaculata* exhibiting the highest proteolytic activity. These results indicate species-specific digestive strategies shaped by habitat conditions and prey availability. Morphological traits showed weak correlations with enzymatic activity, suggesting that biochemical mechanisms play a more decisive role than pitcher structure. Overall, the findings enhance understanding of the adaptive ecology of *Nepenthes* and suggest the potential relevance of nepenthesins as acid-stable proteases for future biotechnological exploration.

Keywords: Carnivorous plant, digestion, Lore Lindu National Park, morphology, nepenthesin, *Nepenthes,* protease

INTRODUCTION

Nepenthes is a carnivorous plant characterized by the modification of its leaves into pitcher-shaped structures that function simultaneously as prey traps and digestive chambers, exhibiting extensive morphological diversity across species in size, shape, peristome architecture, coloration, and fluid composition as adaptations to distinct ecological niches (Buch et al. 2015; Tarigan et al. 2024). The acidic pitcher fluid contains a dynamic biochemical milieu enriched with hydrolytic enzymes, diverse microbial consortia, secondary metabolites, and various bioactive compounds that work synergistically to decompose captured organisms, with its viscosity, enzymatic profile, and antimicrobial properties capable of adjusting in response to prey input or environmental changes (Takeuchi et al. 2015). Among these biochemical components, nepenthesin stands out as a highly stable aspartic protease that remains active under extremely acidic conditions and plays a central role in protein hydrolysis, amino acid release, and nutrient mineralization. Its activity, supported by complementary enzymes such as amylases and lipases as well as microbial contributions, enables efficient nitrogen and phosphorus acquisition, allowing *Nepenthes* to persist and flourish in nutrient-poor habitats while exemplifying sophisticated adaptations in plant carnivory and symbiotic digestion (Ravee et al. 2018; Dkhar et al. 2020; Mansur et al. 2021).

Nepenthes species from Lore Lindu are particularly compelling to study because this region lies within the Wallacea biogeographic zone, known for its high endemism, heterogeneous ecological conditions, and environmental gradients that may influence plant physiology, including the composition and enzymatic activity of pitcher fluid. Each species exhibits variations in pitcher morphology, fluid volume, and trapping type, ranging from lower pitchers that primarily capture ground-dwelling insects to upper pitchers that are more adapted to flying prey. These differences are suspected to be associated with variation in nepenthesin activity, although this relationship has not yet been systematically investigated. Furthermore, the adaptive strategies of *Nepenthes* in response to nutrient scarcity such as efficient prey capture, the ability to inhibit competitive microbes, and the optimization of enzymatic activity result in a complex digestive system that remains incompletely understood. Although previous studies have reported the presence of nepenthesin in several species from Sumatra and Kalimantan, comprehensive investigations of this enzyme's activity in Sulawesi's distinct species, evolutionarily isolated and potentially physiologically divergent, are still lacking.

Nepenthes is widely studied in plant physiology because its pitcher-based digestive system is a remarkable adaptation to nutrient-poor habitats. Unlike most plants that depend mainly on soil nutrients, *Nepenthes* supplements its nutrition by trapping and digesting animal prey in modified leaves called pitchers. This carnivorous habit helps the plant acquire key nutrients, especially nitrogen and phosphorus, which are limited in the acidic, oligotrophic environments where it often grows (Buch et al. 2015; Chan et al. 2016). Digestion in the pitcher involves not only plant-produced hydrolytic enzymes but also interactions among plant tissues, microbial communities, and environmental factors. Thus, pitcher fluid acts as a dynamic medium in which enzymatic processes, microbial activity, and physicochemical conditions jointly control prey breakdown and nutrient release (Buch et al. 2013). Because pitcher shape, prey type, and habitat conditions can affect this process, understanding enzyme regulation is essential for explaining nutrient acquisition and adaptation in *Nepenthes*.

Despite *Nepenthes* being recognized for its unique physiological attributes among carnivorous plants, knowledge of interspecific variation in nepenthesin activity remains limited, particularly for species originating from biodiversity-rich regions such as Lore Lindu National Park in Central Sulawesi. To date, most research has emphasized pitcher morphology, species diversity, or surface-level ecology, whereas comparative studies on digestive enzyme dynamics, especially nepenthesin as the primary protease, are rare. This research gap is critical because nepenthesin activity holds the key to explaining mechanisms of protein digestion, nutrient-capture efficiency, and species-specific adaptive strategies. Therefore, examining nepenthesin activity in three *Nepenthes* species from Lore Lindu National Park is essential to fill the existing knowledge gap regarding interspecific enzymatic variation, relate these patterns to the biological and ecological traits of each species, and clarify how habitat factors contribute to the digestive strategies of these carnivorous plants. Such research not only strengthens the literature on carnivorous plant physiology but also opens possibilities for the application of extremophilic enzymes in various biotechnological fields.

MATERIALS AND METHODS

Study area

This study combined qualitative morphological observations with quantitative laboratory assays to compare nepenthesin activity in three *Nepenthes* species (*Nepenthes maxima* Reinw., *Nepenthes nigra* Nerz, Wistuba, Chi.C.Lee, Bourke, U.Zimm. & S.McPherson, and *Nepenthes tentaculata* Hook.f.) from Lore Lindu National Park, Central Sulawesi, Indonesia. Sampling was conducted purposively between August and December 2024 across four 10×10 m plots representing distinct microhabitats where *Nepenthes* naturally occur (forest edges, understory, and swampy areas) (Gay et al. 2012). The purposive sampling approach was chosen to ensure that all species and pitcher types (lower and upper) were adequately represented across ecologically distinct sites, despite their uneven natural distribution. Lore Lindu National Park, part of the UNESCO-designated Lore Lindu Biosphere Reserve, covers an area of 229,117.5 ha and is characterized by high endemism and heterogeneous microhabitats favorable for *Nepenthes* growth. The study sites were distributed across Mount Rore Kautimbu (Helipad I and Puncak Dingin) and Padang Padeha, where the target species naturally occur (Figure 1).

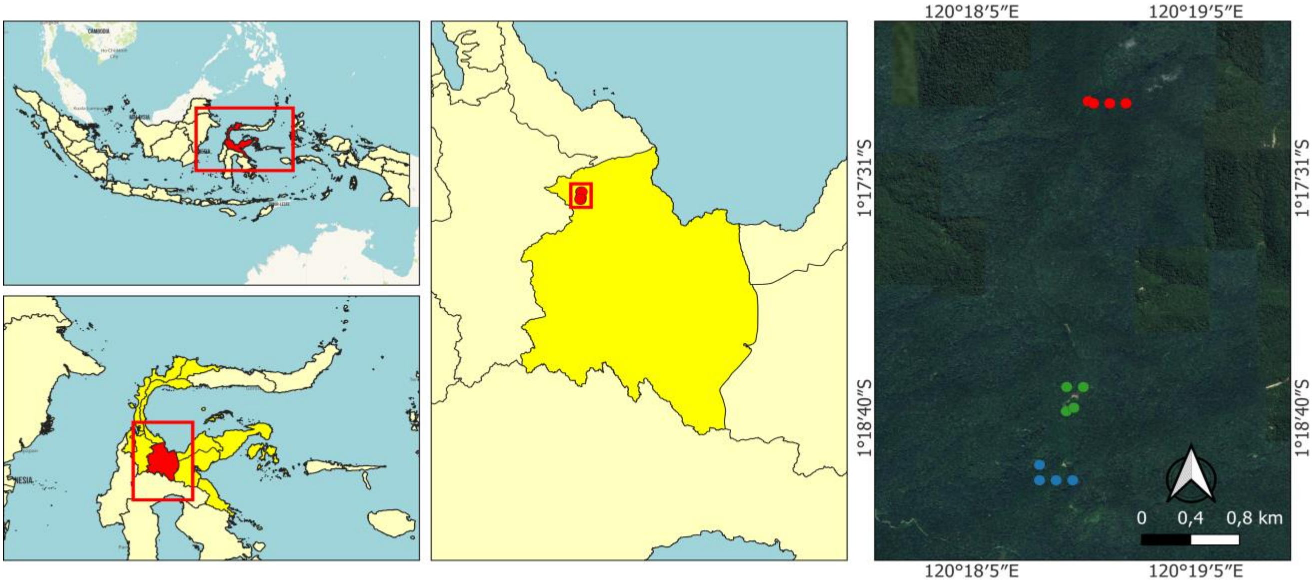


Figure 1. Map of study area showing collection sites for *Nepenthes* in Lore Lindu National Park, Poso, Central Sulawesi, Indonesia. ●: *Nepenthes maxima*, *Nepenthes tentaculata* (Padang Padeha), ●: *Nepenthes maxima*, *Nepenthes tentaculata* (Mount Rore Kautimbu, Helipad I), ●: *Nepenthes maxima*, *Nepenthes nigra* (Mount Rore Kautimbu, Puncak Dingin)

Field procedures

A purposive sampling design was employed to represent the natural variation of *N. maxima*, *N. tentaculata*, and *N. nigra* across distinct ecological gradients within Lore Lindu National Park, Central Sulawesi, Indonesia. Sampling was conducted in four 10 × 10 m plots located at three main sites (Mount Rore Kautimbu with Helipad I and Puncak Dingin sublocations, and Padang Padeha) that were selected purposively to capture differences in altitude, microhabitat, and canopy exposure known to influence pitcher morphology and physiology. In each plot, three healthy individuals per species (n = 3 biological replicates) were selected to provide biological replication that captures inter-individual variation while accommodating logistical and accessibility constraints in remote montane habitats, consistent with minimum biological replication commonly applied in ecological and functional plant studies. Each biological individual bore both lower and upper pitchers, from which two mature, newly opened pitchers of each type (m = 2 technical-level pitcher replicates) were sampled to represent within-individual structural variability while maintaining the plant individual as the biological unit of inference. This design yielded twelve pitchers per species, with pitcher-level measurements averaged at the individual level for comparative analyses (Hurlbert 1984; Legendre and Legendre 2012).

Morphological documentation followed standardized morphometric criteria commonly applied in *Nepenthes* taxonomy (e.g., Clarke 2001; Clarke 2006; Cheek and Jebb 2013), including tendril length and coiling, pitcher body dimensions, peristome width and coloration, lid morphology, and spur architecture. Quantitative measurements were obtained using digital calipers. These characters represent the core diagnostic traits consistently used in species delimitation and intraspecific comparison within the genus *Nepenthes*. Environmental parameters such as air temperature, relative humidity, and soil pH were measured in situ using portable instruments, with air temperature and relative humidity recorded using a factory-calibrated digital thermo-hygrometer (e.g., Lutron HT-3017) that had been verified against a reference thermometer prior to field deployment, and soil pH measured using a portable pH meter (e.g., Hanna HI-99121) calibrated daily with pH 4.01 and 7.00 buffer solutions following the manufacturer's protocol. Pitcher fluids were sampled aseptically by wiping the outer surface with 70% ethanol, discarding the initial fraction, and aspirating 2 mL of fluid into sterile, pre-chilled microtubes, followed by storage at 4°C during transport and at -80 °C for long-term preservation to avoid freeze–thaw degradation. The purposive selection of functionally active pitchers was further supported by ecological observations indicating that upper pitchers more frequently exhibit active prey-capture functions than lower pitchers, ensuring that sampled pitchers represented comparable digestive phases across species (Moran 1996).

Laboratory procedures

All laboratory analyses were conducted at the Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Indonesia, between February and May 2025. Digestive fluids were diluted in 50 mM sodium acetate buffer (pH 3.5) prepared from sodium acetate trihydrate (Merck, Darmstadt, Germany) and adjusted using glacial acetic acid (Merck, Germany) to obtain final enzyme concentrations of 50, 100, 150, and 200 µg/mL. To maintain enzyme stability, the pH of all working buffers was verified before each assay session using a calibrated digital pH meter (Hanna Instruments, USA), and samples were kept on ice to minimize proteolytic self-degradation. No exogenous protease inhibitors were added to avoid interfering with endogenous nepenthesin activity; therefore, all steps from dilution to reaction setup were performed rapidly at low temperature to reduce potential enzyme denaturation. Nepenthesin activity was assayed using 1% casein from bovine milk (Sigma-Aldrich, St. Louis, MO, USA as substrate in the same buffer, and reaction mixtures were incubated at 37°C for 30 minutes in a temperature-controlled water bath (Memmert, Germany) before termination with 10% trichloroacetic acid (TCA) (Merck, Germany) to precipitate undegraded proteins. Following centrifugation at 12,000×g for 10 minutes at 4°C using a refrigerated centrifuge (Eppendorf 5424 R, Germany), absorbance of the supernatant was measured at 280 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). One unit (U) of nepenthesin activity was defined as the amount of enzyme releasing 1 µmol of tyrosine per minute per mg of protein under assay conditions. Tyrosine concentrations were determined in µmol/mL using a calibration curve fitted with the equation $y = 0.6934x + 0.0071$ ($R^2 = 0.998$) using tyrosine standard (Sigma-Aldrich, USA) (Alakbaree et al. 2022). To maintain standardization and prevent redundancy, results were expressed in µmol/mL without conversion to µg/mL (Figure 2). A negative control consisting of buffer and substrate without enzyme extract was included in each assay to detect non-enzymatic casein degradation and to ensure that all measured activity originated solely from enzymatic reactions. All assays were performed in triplicate technical replicates to ensure analytical accuracy and reproducibility.

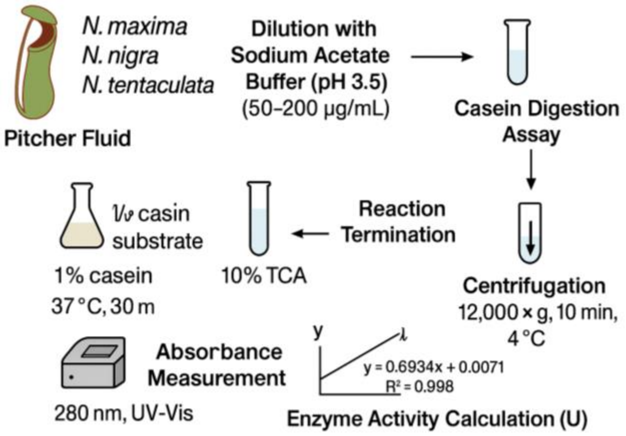


Figure 2. Workflow of nepenthesin enzyme activity assay from *Nepenthes* pitcher fluids

Statistical analysis

All statistical analyses were performed using Minitab® version 19.1. The distribution of upper and lower pitchers among *N. maxima, N. tentaculata*, and *N. nigra* was assessed using a chi-square test of independence to examine the relationship between pitcher type and species identity. Enzyme activity data were expressed as mean ± standard deviation and analyzed using two-way ANOVA with species (three levels) and enzyme concentration (four levels: 50-200 µg/mL) as fixed factors. Prior to conducting ANOVA, data were evaluated for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to ensure that model assumptions were met. When significant effects were detected ($p<0.05$), Tukey's HSD post-hoc test was applied to determine specific pairwise differences. Additionally, Pearson correlation analyses were conducted to evaluate the relationships between pitcher morphological traits (e.g., peristome width, pitcher volume, and lid size) and nepenthesin activity, with scatterplots and residual checks used to confirm linearity and the absence of major outliers, providing an integrated quantitative understanding of how morphological features influence digestive enzyme performance across species.

RESULTS AND DISCUSSION

Species occurrence and pitcher type distribution

All three species, i.e., *N. maxima*, *N. nigra*, and *N. tentaculata*, were documented across the four sampling plots in Lore Lindu National Park (Figures 3, 4, 5). Both upper and lower pitchers were recorded in each species, with differences in abundance across plots (Table 1). *N. maxima* was the most widespread, occurring in all three sites, whereas *N. tentaculata* was present at two sites, and *N. nigra* was restricted to Mount Rorekatimbu (Puncak Dingin). Across all species and sites, a total of 334 pitchers were documented, consisting of 120 lower pitchers and 214 upper pitchers. On average, each site yielded 20±8.51 lower pitchers and 35.67±12.41 upper pitchers. Upper pitchers were consistently more abundant than lower pitchers across species, particularly in *N. maxima* and *N. tentaculata*. The opposite trend was observed in *N. nigra*, which produced more lower pitchers than upper pitchers at its restricted site. A chi-square test comparing pooled counts of pitcher types confirmed that upper pitchers were significantly more abundant than lower pitchers ($\chi^2 = 28.05$, $df = 1$, $p<0.05$), and the exact p-value was reported to improve reproducibility ($p = 1.17 \times 10^{-7}$).

Environmental characteristics of sampling sites

Environmental conditions across the habitats occupied by the recorded *Nepenthes* species were characterized through systematic measurements conducted in four sampling plots at each site. The assessed parameters included air temperature, soil pH, relative humidity, elevation, and geographic coordinates. Mean values and standard deviations were compiled to capture the extent of abiotic heterogeneity among sites. A comprehensive summary of these environmental characteristics for all study locations is provided in Table 2.

Pitcher morphometrics among *Nepenthes* species

A comprehensive assessment of pitcher morphology across the three *Nepenthes* species is summarized in Table 3. The table documents a range of morphometric attributes—including pitcher height, tendril length, upper and lower pitcher diameters, mouth and lid dimensions, peristome width, spur length, and wing development—for both lower and upper pitcher types. These measurements allow for a clearer comparison of structural variation within and among species, providing essential context for interpreting functional and ecological. Additionally, peristome width for upper pitchers in *N. maxima*, *N. tentaculata*, and *N. nigra* was not numerically quantified due to the high curvature and irregular morphology of the peristome in fully mature upper pitchers, which prevented consistent caliper placement across field conditions. Measurements varied substantially depending on orientation and compression angle, resulting in low repeatability.

Table 1. Number of lower and upper pitchers of *Nepenthes* species observed across four 10 × 10 m plots per site in Lore Lindu National Park, Central Sulawesi, Indonesia

Sites	Species	Pitcher type	Plot 1	Plot 2	Plot 3	Plot 4	Total
Mount Rorekatimbu (Helipad I)	*Nepenthes maxima* Reinw.	Lower	4	5	4	5	18
		Upper	10	11	10	11	42
	Nepenthes tentaculata Hook.f.	Lower	4	5	4	4	17
		Upper	13	14	13	14	54
Mount Rorekautimbu (Puncak Dingin)	*Nepenthes maxima* Reinw.	Lower	3	3	3	3	12
		Upper	7	7	7	7	28
	Nepenthes nigra Nerz, Wistuba, Chi.C.Lee, Bourke, U.Zimm. & S.McPherson	Lower	9	9	9	9	36
		Upper	4	4	4	5	17
Padang Padeha	*Nepenthes maxima* Reinw.	Lower	4	4	3	4	15
		Upper	8	8	9	8	33
	Nepenthes tentaculata Hook.f.	Lower	5	6	5	6	22
		Upper	10	10	10	10	40
Total							334

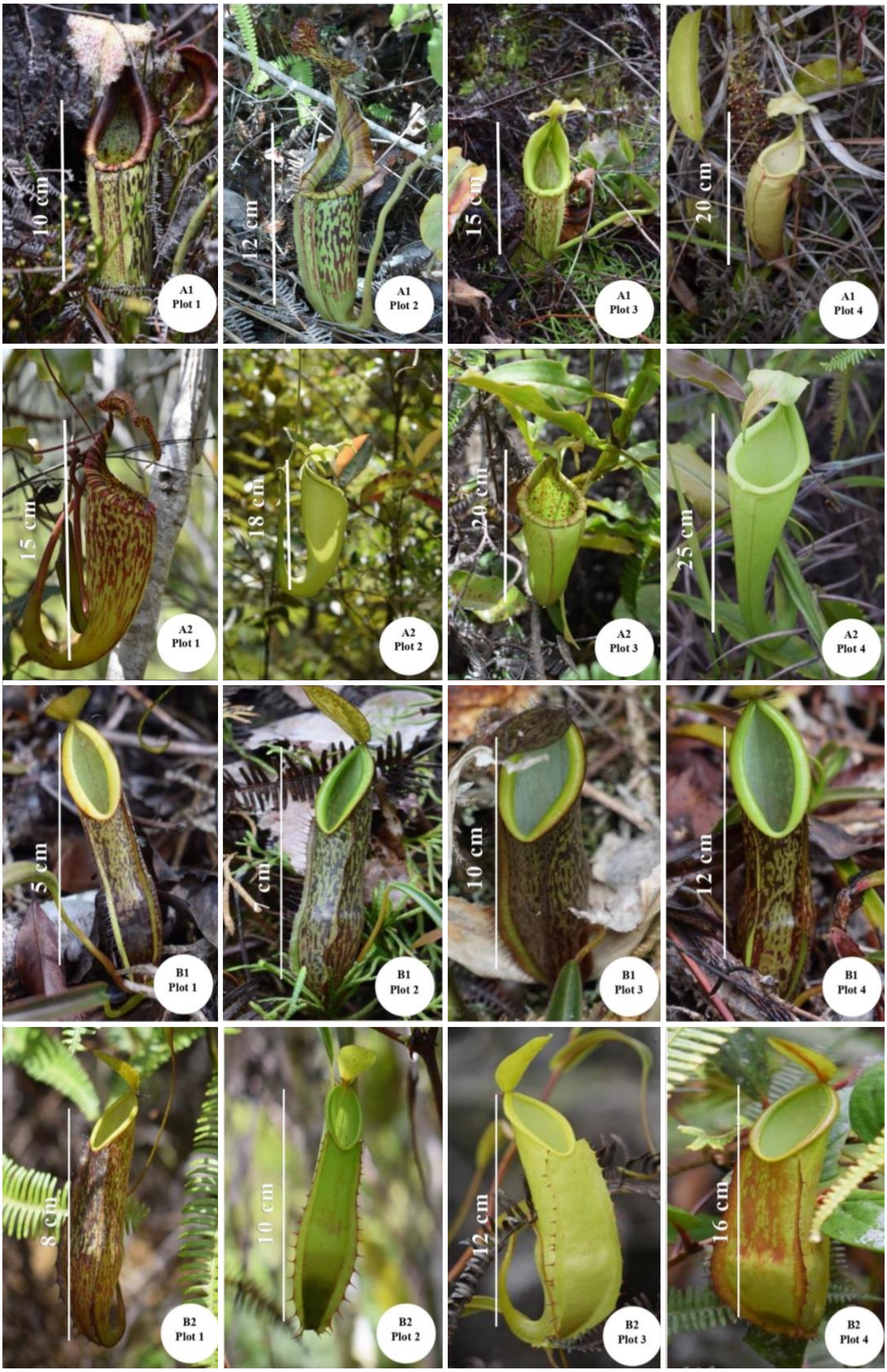


Figure 3. *Nepenthes* species collected from four 10 × 10 m research plots established within Lore Lindu National Park, Central Sulawesi, Indonesia. The plots were distributed across three main sites: Mount Rore Kautimbu (Helipad I and Puncak Dingin) and Padang Padeha. A. *Nepenthes maxima* found across Plots 1-4 in Helipad I showing (1) lower and (2) upper pitchers, B. *Nepenthes tentaculata* found across Plots 1-4 in Helipad I showing (1) lower and (2) upper pitchers. Photographs by Mhd. Rafi'i Ma'arif Tarigan.

Figure 4. *Nepenthes* species collected from four 10 × 10 m research plots established within Lore Lindu National Park, Central Sulawesi, Indonesia. The plots were distributed across three main sites: Mount Rore Kautimbu (Helipad I and Puncak Dingin) and Padang Padeha. C. *Nepenthes maxima* found across Plots 1-4 in Puncak Dingin showing (1) lower and (2) upper pitchers; D. *Nepenthes nigra* found across Plots 1-4 in Puncak Dingin showing (1) lower and (2) upper pitchers. Photographs by Mhd. Rafi'i Ma'arif Tarigan

Figure 5. *Nepenthes* species collected from four 10 × 10 m research plots established within Lore Lindu National Park, Central Sulawesi, Indonesia. The plots were distributed across three main sites: Mount Rore Kautimbu (Helipad I and Puncak Dingin) and Padang Padeha. (E) *Nepenthes maxima* found across Plots 1-4 in Padang Padeha showing (1) lower and (2) upper pitchers; (F) *Nepenthes tentaculata* found across Plots 1-4 in Padang Padeha showing (1) lower and (2) upper pitchers. Photographs by Mhd. Rafi'i Ma'arif Tarigan

Table 2. Environmental characteristics of *Nepenthes* habitats in Lore Lindu National Park. Values represent mean ± SD across four sampling plots per site

Site: Species	Parameters	Plot 1	Plot 2	Plot 3	Plot 4	Mean±SD
Mount Rorekatimbu (Helipad I) *N. maxima*, *N. tentaculata*	Air temperature (°C)	15	17	18	15	16.5±1.3
	Soil pH	6.4	6.5	6.7	6.4	6.5±0.1
	Humidity (%)	77	78	78	77	77.5±0.6
	Altitude (m asl)	2007	2011	2012	2006	2009±3
	Coordinate	01°18'30.6" N, 120°18'30.6" E	01°18'37.5" N, 120°18'30.5" E	01°18'36.5" N, 120°18'32.5" E	01°18'30.6" N, 120°18'35.0" E	-
Mount Rorekatimbu (Puncak Dingin) *N. maxima*, *N. nigra*	Air temperature (°C)	13	14	14	15	14.0±0.8
	Soil pH	8.2	8.4	8.3	8.5	8.4±0.1
	Humidity (%)	82	82	84	83	82.8±0.8
	Altitude (m asl)	2311	2011	2008	2007	2084±133
	Coordinate	01°17'09.1" N, 120°18'36.3" E	01°17'09.7" N, 120°18'37.7" E	01°17'09.7" N, 120°18'42.0" E	01°17'09.7" N, 120°18'46.3" E	-
Padang Padeha *N. maxima*, *N. tentaculata*	Air temperature (°C)	18	16	17	18	17.8±0.8
	Soil pH	6.5	6.4	6.3	6.5	6.4±0.1
	Humidity (%)	82	80	82	80	81.0±1.2
	Altitude (m asl)	1651	1650	1652	1648	1650±2
	Coordinate	01°18'57.2" N, 120°18'27.8" E	01°18'57.2" N, 120°18'32.2" E	01°18'57.2" N, 120°18'23.4" E	01°18'52.8" N, 120°18'23.4" E	-

Table 3. Morphometric characteristics of lower and upper pitchers in three *Nepenthes* species from Lore Lindu National Park

Species	Pitcher type	Height (cm)	Tendril length (cm)	Diameter (upper) (cm)	Diameter (lower) (cm)	Mouth (L×W, cm)	Peristome width (cm)	Lid (L×W, cm)	Spur length (cm)	Wings
N. maxima	Lower	10-20	2-10	4-8	3-6	2-4 × 2.5-5	≤1.5	3-6 × 2-5	0.3-1.5	0.4-1.2 cm
	Upper	15-25	10-30	3-7	2-5	2-5 × 2.5-6	Broad (not quantified)	4-7 × 3-6	0.5-2	Reduced to ridges
N. tentaculata	Lower	5-12	1-8	2-4	1.5-3.5	1-2.5 × 1-3	0.2-0.6	1-2.5 × 1-2.2	0.2-0.6	0.2-0.5 cm
	Upper	8-16	5-15	2-4	1.5-3	1.5-3 × 2-4	Narrow (not quantified)	1.5-3 × 1-2.5	0.3-0.8	Reduced to ridges
N. nigra	Lower	8-15	2-10	3-5	2-4	1.5-3.5 × 24.5	≤1.0	2-4 × 1.5-3.5	0.3-1.0	0.3-0.8 cm
	Upper	12-20	10-25	2-4.5	1.5-3	2-4 × 2.5-5	Narrow to moderate	2-4.5 × 2-4	0.3-0.8	Reduced to ridges

Table 4. Nepenthesin activity and corresponding tyrosine concentrations released from casein substrate at four nepenthesin concentrations (50-200 µg/mL) for three *Nepenthes* species. Values represent means ± standard error ($n = 3$)

Species	50 µg/mL		100 µg/mL		150 µg/mL		200 µg/mL	
	Activity unit (U)	Tyrosine (µmol/mL)	Activity unit (U)	Tyrosine (µmol/mL)	Activity unit (U)	Tyrosine (µmol/mL)	Activity unit (U)	Tyrosine (µmol/mL)
N. maxima	0.21±0.008	0.30±0.011	0.32±0.007	0.45±0.010	0.46±0.015	0.66±0.021	0.52±0.013	0.74±0.018
N. nigra	0.20±0.008	0.27±0.012	0.29±0.014	0.41±0.020	0.42±0.010	0.59±0.014	0.49±0.009	0.70±0.012
N. tentaculata	0.23±0.005	0.33±0.007	0.36±0.007	0.50±0.009	0.51±0.011	0.73±0.015	0.58±0.017	0.82±0.024

Nepenthesin activities among *Nepenthes* species

Nepenthesin assays showed a consistent increase in enzymatic activity and tyrosine release with increasing enzyme concentrations across all three *Nepenthes* species (Table 4). At the lowest concentration (50 µg/mL), activities ranged from 0.20 U in *N. nigra* to 0.23 U in *N. tentaculata*, corresponding to tyrosine releases of 0.27-0.33 µmol/mL. At 100 µg/mL, activities increased to 0.29-0.36 U, with tyrosine concentrations between 0.41 and 0.50 µmol/mL. A further increase was observed at 150 µg/mL, where activities reached 0.42-0.51 U, producing 0.59-0.73 µmol/mL tyrosine. The highest enzyme concentration tested (200 µg/mL) yielded the highest activities, ranging from 0.49 U in *N. nigra* to 0.58 U in *N. tentaculata*, with corresponding tyrosine values of 0.70-0.82 µmol/mL. Among species, *N. tentaculata* consistently displayed the prominent activity and tyrosine release at all enzyme concentrations, while *N. nigra* showed slightly lower values compared to *N. maxima*. Statistical analysis (Table 5) using two-way ANOVA confirmed highly significant effects of both enzyme concentration (p<0.001) and species (p<0.001) on tyrosine release. Moreover, a significant interaction between species and enzyme concentration (p = 0.002) indicated that the rate of increase in tyrosine release differed among species. Post hoc comparisons (Figures 3, 4, 5) further showed distinct groupings, with *N. tentaculata* outperforming the other two species at higher enzyme concentrations. These findings indicate that *N. tentaculata* possesses stronger proteolytic potential than the other species, suggesting biochemical adaptation to compensate for its smaller pitcher volume and limited prey size.

Figure 6 shows that tyrosine release (µg/mL) in *N. maxima*, *N. nigra*, and *N. tentaculata* increases with rising nepenthesin concentrations, indicating a positive correlation between enzyme concentration and proteolytic activity. At all concentration levels, *N. tentaculata* exhibits the highest tyrosine release, followed by *N. maxima*, while *N. nigra* consistently shows the lowest values. The different letters above the bars indicate statistically significant differences among treatments based on Tukey's post hoc test (p<0.05). Physiologically, the increased tyrosine release reflects enhanced protein hydrolysis by nepenthesin in the pitcher fluid, which is essential for nitrogen and amino acid acquisition in these carnivorous plants and the higher activity observed in *N. tentaculata* suggests greater enzymatic efficiency and digestive capacity, supporting its adaptive strategy in nutrient-poor environments.

The heatmap in Figure 7 illustrates the pairwise Pearson's correlation coefficients (r) among seven measured variables—pitcher height, diameter, volume, mouth, lid, nepenthesin, and tyrosine—where color intensity and numerical values indicate the strength of the relationships. Strong positive correlations were found among all structural traits (r = 0.86–1.00), showing that height, diameter, volume, mouth, and lid are closely associated and tend to increase together. This pattern of extremely high intercorrelation also suggests potential multicollinearity, indicating that several structural variables may be capturing overlapping information; therefore, future studies may consider dimensionality-reduction approaches such as Principal Component Analysis (PCA) to identify key morphological axes and reduce redundancy. In contrast, enzymatic traits (nepenthesin and tyrosine release) displayed weak to moderate correlations with structural parameters (–0.04 < r < 0.48), reflecting that enzyme activities were only partially linked to pitcher morphology and were more independent relative to the tightly correlated structural traits.

Table 5. Two-way ANOVA for the effects of species, enzyme concentration, and their interaction on tyrosine release. Both main effects and their interaction were statistically significant

Source	*df*	Adjusted SS	Adjusted MS	*F*-value	*p*-value
Species	2	2144.5	1072.3	137.05	0.000
Concentration(s)	3	37002.8	12334.3	1576.49	0.000
Species×Concentration(s)	6	224.3	37.4	4.78	0.002
Error	24	187.8			
Total	35	39559.4			

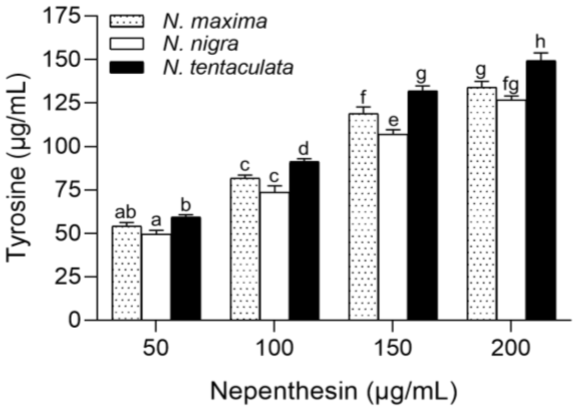
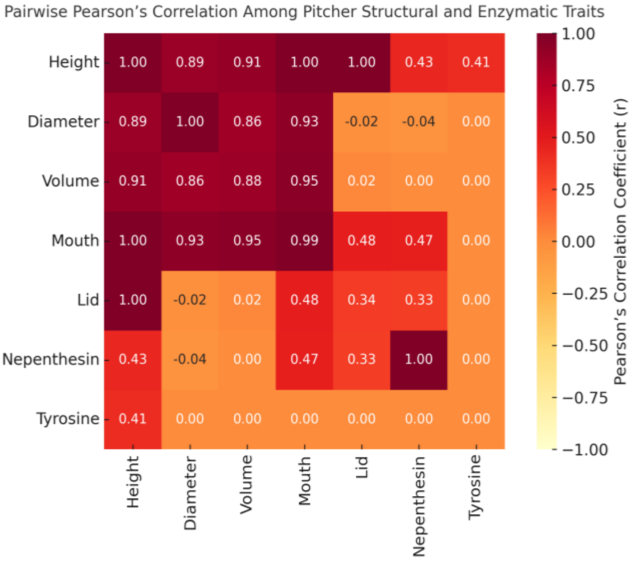


Figure 6. Tyrosine release (µg/mL) in three *Nepenthes* species (*Nepenthes maxima*, *Nepenthes nigra*, and *Nepenthes tentaculata*) at four nepenthesin concentrations. Bars represent means ± SD (n = 3). Different letters above bars indicate significant differences among treatments based on Tukey's post hoc comparisons (p<0.05)



	Height	Diameter	Volume	Mouth	Lid	Nepenthesin	Tyrosine
Height	1.00	0.89	0.91	1.00	1.00	0.43	0.41
Diameter	0.89	1.00	0.86	0.93	-0.02	-0.04	0.00
Volume	0.91	0.86	0.88	0.95	0.02	0.00	0.00
Mouth	1.00	0.93	0.95	0.99	0.48	0.47	0.00
Lid	1.00	-0.02	0.02	0.48	0.34	0.33	0.00
Nepenthesin	0.43	-0.04	0.00	0.47	0.33	1.00	0.00
Tyrosine	0.41	0.00	0.00	0.00	0.00	0.00	0.00

Figure 7. Heatmap of pairwise Pearson correlation coefficients (r) among morphological and enzymatic activities of *Nepenthes*

Discussion

The findings of this study demonstrate that nepenthesin activity across *N. maxima*, *N. tentaculata*, and *N. nigra* follows a consistent enzymatic response to increasing concentrations, reflecting the typical behavior of plant-derived aspartic proteases. The progressive rise in proteolytic activity and tyrosine release from 50 to 200 µg/mL is indicative of a classical Michaelis-Menten-type kinetic pattern, where enzyme activity increases proportionally to concentration until approaching saturation. This saturation trend is expected in systems where the number of available substrates or active catalytic sites becomes limiting, particularly in acidic digestive environments characteristic of *Nepenthes* pitcher fluid. Such a profile is consistent with observations reported by Rottloff et al. (2016) and Zulkapli et al. (2021), who noted that nepenthesin constitutes a central catalytic component of the *Nepenthes* digestive system and exhibits high stability and catalytic efficiency under low pH conditions (pH 2-4). Pavlovič (2025) and Wal et al. (2025) likewise noted that digestive proteases in carnivorous plants often display rapid catalytic turnover but plateau when substrate availability becomes a limiting factor. The data from this study therefore reinforce the notion that *Nepenthes* digestion is governed by controlled physiological regulation aligned with classical enzyme kinetic principles.

The interspecific variation in enzymatic performance provides additional insights into species-specific digestive strategies. *N. tentaculata*, the species with consistently highest nepenthesin activity, appears to invest heavily in biochemical digestion, suggesting that enzymatic efficiency compensates for potentially smaller pitcher sizes or reduced prey capture rates in montane habitats. Brearley (2021) and Gilbert et al. (2020) have shown that montane carnivorous plants often experience significant nutrient scarcity and therefore evolve heightened enzymatic capacity or increased digestive efficiency as a compensatory mechanism. In contrast, *N. maxima*, despite possessing larger pitchers, exhibited only moderate enzymatic activity. This disparity suggests a divergent resource-acquisition strategy where physical traits—such as wide peristomes, larger pitcher volumes, and improved mechanical capture—play a more dominant role in nutrient acquisition. Bazile et al. (2015) proposed that species with larger pitchers benefit from passive nutrient influx, reducing the selective pressure to intensify enzymatic output. *N. nigra,* which showed the lowest enzymatic activity, highlights yet another adaptive strategy. Rather than investing heavily in biochemical digestion, the species appears to rely on morphological or visual lures, including dark pigmentation, conspicuous peristomes, or funnel-shaped geometries that enhance prey attraction and retention. Tarigan et al. (2023) noted similar patterns in other dark-pigmented *Nepenthes* species, where prey capture is optimized through increased visibility or contrast against forest backgrounds. This reinforces that digestive function in *Nepenthes* is multifaceted and governed by an interplay of morphological, ecological, and biochemical adaptations rather than a uniform pattern across species.

Despite significant morphological variability among the species studied, the correlation between pitcher structure and enzymatic activity was unexpectedly weak. Although pitcher height, diameter, and volume are highly correlated with each other, they do not reliably predict digestive capacity. This supports conclusions by Andrew et al. (2025) and Saganová et al. (2018), who demonstrated that enzyme production in *Nepenthes* is influenced more strongly by environmental stimuli—such as prey availability, rainfall patterns, and pH fluctuations—than by pitcher morphology. Rottloff et al. (2016) and Freund et al. (2022) also show that the digestive fluid of *Nepenthes* contains a diverse suite of enzymes beyond nepenthesin, including chitinases, β-1,3-glucanases, nucleases, and phosphatases. Variation in the presence, abundance, or activation states of these enzymes likely contributes to the interspecific differences observed here, suggesting that pitcher morphology is not a reliable proxy for biochemical function.

Environmental conditions at the sampling sites further contextualize the digestive variation among species. The habitats of *N. maxima* and *N. tentaculat*a—Helipad I and Padang Padeha—are characterized by acidic soils, moderate thermal conditions, and relatively stable humidity. These factors are favorable for protease stability and may enhance enzymatic function by reducing protein denaturation. In contrast, *N. nigra*, found exclusively in Puncak Dingin, experiences lower temperatures, higher humidity, and more alkaline substrates. Alkaline seepage is known to suppress the activity of aspartic proteases, which function optimally at acidic pH. Thus, the low enzymatic activity of *N. nigra* may reflect ecological constraints rather than intrinsic biochemical limitations. Studies by Bittleston et al. (2023) and Chan et al. (2016) have shown that microbial communities in pitchers are highly sensitive to shifts in temperature and pH, affecting the abundance of bacteria that contribute auxiliary digestive roles. Since this study did not characterize microbial assemblages, the observed enzymatic patterns may represent combined plant-microbe interactions rather than plant enzyme activity alone.

From an evolutionary perspective, the data support the model of modular adaptation within *Nepenthes*—where morphological, physiological, and biochemical traits evolve semi-independently in response to unique environmental pressures. Pavlovič (2025) and Freund et al. (2022) argue that carnivorous plants frequently evolve multiple, parallel strategies to optimize nutrient acquisition, including enhancements in enzyme production, changes in pitcher chemistry, prey specialization, and microbial symbiosis. The digestive profiles of the studied species differ, indicating variation in digestive strategies. In nutrient-poor ecosystems such as Wallacea, this variation may support adaptation to differing ecological conditions.

In conclusion, the proteolytic activity of nepenthesin varies among species and is influenced by enzyme concentration, pitcher morphology, and habitat conditions, indicating that digestive performance is not driven by a single structural factor but by an interplay of ecological, physiological, and biochemical elements. The weak correlation observed between structure and function suggests that morphological traits and enzymatic activity can shift relatively independently, with each species exhibiting distinct strategies for nutrient acquisition in nitrogen-poor environments. However, several limitations should be acknowledged, including single-season sampling, small sample size, the absence of microbial analyses, and differences between laboratory conditions and natural microhabitats, all of which require cautious interpretation of the results. Despite these constraints, the study provides empirical evidence for meaningful interspecific variation in nepenthesin activity and highlights the need for further research using metagenomic and proteomic approaches, multi-season sampling, broader site coverage, and detailed assessments of nutrient profiles and microbial communities to more comprehensively understand the digestive system of *Nepenthes*.

ACKNOWLEDGEMENTS

The authors are grateful to Universitas Islam Negeri Sumatera Utara, Universitas Negeri Medan and National Research and Innovation Agency (BRIN), Indonesia, for their assistance in supporting the study and publication. Moreover, the authors anticipate that this study will contribute to the institutions and further investigations in the future.

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