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Original Research Article

Antioxidant Activity and Stability of Nanocapsules of Red Ginger (*Etlingera flexuosa*) Endemic to Central Sulawesi

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ABSTRACT

Nanoencapsulation is a technique used to maintain the active compounds' stability against environmental stressors. *Etlingera flexuosa*, an endemic ginger found in Central Sulawesi, is being explored for its potential as a herbal medicine by formulating its extract into nanocapsules. The objectives of this research are to evaluate the antioxidant activity, physicochemical characteristics, and stability of *E. flexuosa* ginger nanocapsules under varying conditions of temperature, pH, and sodium chloride (NaCl) concentrations. The rhizome, stem, and leaf of *E. flexuosa* ginger were extracted using ethanol. The nanoencapsulation employed κ-carrageenan as the coating matrix, with extract-to-matrix ratios of 1:1, 1:2, 1:3, 2:1, and 3:1. Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and the nanocapsules' stability was evaluated using UV-Vis spectrophotometry.

The results showed that the best antioxidant activity (IC_{50}) of the nanocapsules was obtained at an extract-to-matrix ratio of 1:2. The IC_{50} values of rhizome, stem, and leaf extract of *E. flexuosa* ginger, respectively, were 53.73 µg/mL, 80.36 µg/mL, and 102.10 µg/mL. After nanoencapsulation at an extract-to-matrix ratio of 1:2, the IC_{50} values of rhizome-NPs, stem-NPs, and leaf-NPs were 43.22 µg/mL, 77.38 µg/mL, and 94.61 µg/mL, respectively. Particle size analysis revealed that the nanocapsules derived from three parts of *E. flexuosa* ginger had sizes below 1000 nm. The zeta potential (ζ) values for rhizome-NPs, stem-NPs, and leaf-NPs were -6.4 mV, -6.9 mV, and -8.1 mV, respectively. The nanoencapsulation effectively preserved the bioactive compounds' stability from the rhizome, stem, and leaf of *E. flexuosa* ginger under varying pH, temperature, and NaCl concentrations.

Keywords: *Etlingera flexuosa*, 2,2-diphenyl-1-picrylhydrazyl, Nanoencapsulation, Particle size, Zeta potential

Introduction

Ginger (*Zingiber officinale*) is a plant species classified under the Zingiberaceae family. It contains antioxidants such as gingerol, shogaol, zingerone, and other volatile compounds.[1,2] These antioxidant compounds are crucial in neutralizing free radicals and reducing the risk of degenerative diseases.[3] However, they have limited stability, particularly when exposed to environmental aspects such as temperature, pH, and light, which can reduce their effectiveness during product storage or application.[33,34,35] One method developed to address the instability of these active compounds is nanoencapsulation. Nanoencapsulation is a promising technique to overcome stability issues.[4] This technology not only enhances the stability of active compounds against environmental stressors but also improves their bioavailability and biological activity in the body.[5] Nanoencapsulation protects the compounds from degradation and provides a longer-lasting protective effect when used in food, cosmetic, or pharmaceutical formulations.[6]

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Improved stability also enables extended product shelf life without losing bioactivity.[7] Central Sulawesi has a species of ginger known as *Etlingera flexuosa*, one of 36 species of the *Etlingera* genus reported as new and endemic to Sulawesi island, and belongs to the *Zingiberaceae* family.[8] Traditionally, *E. flexuosa* is used as a flavor enhancer in food and as a traditional medicine to treat diarrhea. Previous studies demonstrated that *E. flexuosa* can inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. Phytochemical screening using GC-MS revealed that *E. flexuosa* contains phenolic compounds and essential oils distributed throughout the plant and can act as immunomodulators, possessing antifungal, antiviral bioactivities.[8,10,11]

Building upon these known pharmacological properties, this study aims to further explore the potential of *E. flexuosa* —a member of the Zingiberaceae family endemic to Central Sulawesi—as a herbal medicine by formulating its extracts into nanocapsules. Specifically, this study investigates the antioxidant activity and stability of nanocapsules formulated from rhizome, stem, and leaf extracts of *E. flexuosa* at various pH, temperature, and sodium chloride (NaCl) concentrations.

Materials and Methods

Materials

Etlingera flexuosa ginger, ethanol p.a (Merck), pH universal (Merck), distilled water, κ-carrageenan (Sigma Aldrich), sodium chloride (NaCl) (Sigma Aldrich), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich).

Collection and authentication of plant materials

Etlingera flexuosa ginger was collected from Tambing, Lore Lindu National Park, Poso Regency, Central Sulawesi (geographical coordinates: 1°19'31.8"S, 120°18'29.8"E) in November 2023. The plant was identified and confirmed by a Botanist by comparing it with voucher specimen No. **2655** from the Herbarium Celebense (CEB) and the Museum Zoologicum Celebense, UPA Sulawesi Natural Resources, Tadulako University. The voucher specimen has been deposited in the Herbarium Celebense for reference.

Etlingera flexuosa Preparation
The dried simplicia of *E. flexuosa* ginger (rhizome, stem, and leaf) were ground into a powder. A total of 500 g of the powdered rhizome, stem, and leaf was extracted using the maceration method with 1 L of ethanol 96% (24 hours), and this process was repeated three times. The filtrates from each plant part were filtered using a Buchner funnel to obtain ethanol extracts. Subsequently, *E. flexuosa* extracts were concentrated with a rotary vacuum evaporator to obtain thick extracts of the rhizome, stem, and leaf of *E. flexuosa* ginger.

Nanoencapsulation
The extracts of *E. flexuosa* ginger from the rhizome, stem, and leaf were nano-encapsulated using κ-carrageenan as a matrix. Nano-encapsulation and stability were carried out based on previous research with some modifications. *E. flexuosa* extract (1 g/100 mL of water) was mixed with kappa carrageenan (1 g/100 mL of water) at extract-to-matrix ratios of 1:1, 1:2, 1:3, 2:1, and 3:1. The mixtures were centrifuged for 60 minutes and then subjected to ultrasonication for 60 minutes at a power level of 60% and a frequency of 20–25 kHz[5,28]. The nanocapsule solution was freeze-dried to obtain a solid product. The *E. flexuosa* extract and *E. flexuosa* nanocapsules were evaluated for their antioxidant activity and stability through UV-Vis spectral patterns and turbidity grades under varying of temperature, pH, and NaCl conditions.

Antioxidant Activity Test
The concentrated extracts of the rhizome, stem, and leaf, as well as rhizome-NPs, stem-NPs, and leaf-NPs, were evaluated for their antioxidant activity using a UV-Vis spectrophotometric method with the DPPH reagent. The DPPH radical scavenging test was carried out following the procedure described by Noreen *et al*.[12] with some modifications. Each sample was prepared at 20, 40, 60, 80, and 100 µg/mL concentrations. 1 mL of each sample concentration was combined with 3 mL of 50 µM DPPH solution. The mixtures were thoroughly homogenized and left to incubate in the dark at room temperature (34^0C) for 30 minutes. All the samples were prepared in triplicate. Subsequently, the absorbance of the solution was determined at a wavelength of 517 nm. A control test was also conducted using the DPPH solution. Absorbance data obtained from the assay were used to calculate the inhibition percentage based on the equation below:

$$\%\ \boldsymbol{Inhibition} = \frac{\boldsymbol{Abs.\ DPPH\ -\ Abs.Samples}}{\boldsymbol{Abs.\ DPPH}}\ \boldsymbol{X}\ \mathbf{100}\%$$

Determination of IC_{50} is based on the linear regression equation (y = a + bx).

Characterization and stability of rhizome-NPs, stem-NPs, and leaf-NPs
Zeta potential (ζ) and particle size, determined using a Zetasizer Nano ZS (Malvern), were employed to evaluate the physicochemical characteristics of rhizome-NPs, stem-NPs, and leaf-NPs. Further assessment of rhizome-NPs, stem-NPs, and leaf-NPs involved testing their stability under different pH, temperatures, and NaCl concentrations, as indicated by UV-Vis absorption patterns (200–700 nm, Perkin Elmer) and turbidity levels.[1,28]

Stability on temperature
Nanocapsules were heated from 30°C to 100°C, then placed into a cuvette. Their absorbance was recorded using a UV-Vis spectrophotometer at wavelengths of 200-700 nm. Subsequently, the heated nanocapsules were tested for turbidity levels using a turbidimeter.[1,28]

Stability of pH
Nanocapsules with pH variations ranging from 2 to 11 were placed into a cuvette, and their absorbance was determined by a UV-Vis spectrophotometer at a wavelength range of 200-700 nm. Subsequently, the turbidity levels of the nanocapsules were measured using a turbidimeter.[1,28]

Stability on NaCl concentrations
Nanocapsules were added with NaCl solution at various concentrations of 0, 0.1 M, 0.15 M, 0.2 M, 0.25 M, and 0.3 M, placed into cuvettes, and their absorbance was analyzed using a UV-Vis spectrophotometer at wavelength range of 200 nm-700 nm. Subsequently, the turbidity of the nanocapsules with the different NaCl concentrations was tested using a turbidimeter.[1,28]

Results and Discussion

This study formulated nanocapsules from ethanol extracts of the rhizome, stem, and leaf of the *E. flexuosa* plant using the ultrasonication method and κ-carrageenan as the matrix. Carrageenan was selected as the encapsulating material due to its pseudoplastic properties, which promote the formation of spherical microcapsules with smooth surfaces. For this reason, carrageenan is widely used in the food and pharmaceutical industries.[13] Carrageenan also possesses several advantageous properties, including biodegradability, biocompatibility, and non-toxicity.[14] In addition, it exhibits various bioactivities such as antiviral, antibacterial, antioxidant, antitumor, and immunomodulation.[15,16] In the pharmaceutical field, carrageenan is widely used in drug delivery systems to obtain longer drug action.[14]

Antioxidant activity
The antioxidant activity of the rhizome, stem, and leaf parts of *Etlingera flexuosa* has been evaluated. Based on the antioxidant assay using the DPPH method, differences were observed between the crude extracts and the nanocapsule formulations of the three plant parts regarding antioxidant activity, as presented in Table 1. The antioxidant activity (IC_{50}) of the rhizome extract of *E. flexuosa* was the highest compared to that of the stem and leaf extracts, with an IC_{50} value of 53.731 µg/mL. Following nanoencapsulation, the antioxidant activity (IC_{50}) of rhizome-NPs, stem-NPs, and leaf-NPs showed that nanocapsules with a ratio of 1:2 demonstrated the greatest antioxidant. The antioxidant activity (IC_{50}) at a ratio of 1:2 for the rhizome, stem, and leaf parts was 43.221 µg/mL, 77.380 µg/mL, and 94.607 µg/mL, respectively. A sample with an IC_{50} value below 50 µg/mL is considered to have very strong antioxidant activity.[30] The greater antioxidant activity of rhizome extract, as indicated by its ability to scavenge DPPH radicals, is associated with higher concentrations of phenolic derivative compounds whose capacity to donate hydrogen atoms helps stabilize free radicals. The total phenolic content is directly proportional to the high antioxidant activity.[17] Therefore, the rhizome-NPs, stem-NPs, and leaf-NPs at a ratio of 1:2 were further analyzed regarding their characterization and stability. Although several studies have shown that nanocapsules can improve pharmacological activity, encapsulation of plant extracts must be based on an appropriate ratio between the extract and the coating material.

Characterization of Nanocapsules
The physicochemical characterization of nanocapsules involved analyzing particle size and zeta potential (ζ) (Table 2). Nanoparticles are colloidal particles composed of organic or inorganic substances, including polymers, polysaccharides, proteins, and lipids, with particle sizes ranging from 1-1000 nm.[18,19] Respectively, the particle sizes of rhizome-NPs, stem-NPs, and leaf-NPs were 615.8 nm, 707.3 nm, and 768.1 nm. Based on the particle size of the three types of *E. flexuosa* nanocapsules, nanoencapsulation using κ-carrageenan as the matrix meets the criteria for nanoparticles. Some sources mention that nanoparticles can show their typical properties at diameters below 100 nm. However, this limit is difficult to achieve for a nanoparticle system as a drug delivery system. Herbal medicines/drugs in nano form generally must be contained in sufficient quantities in the matrix of each particle, thus requiring a relatively larger particle size compared to non-pharmaceutical nanoparticles.[20]

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Table 1: I_{50} Values of *Etlingera flexuosa* Ethanol Extracts and Nanocapsules in The DPPH Assay

Sample	Antioxidant Activity (IC_{50}) (µg/mL)	Sample	Antioxidant Activity (IC_{50}) (µg/mL)	Sample	Antioxidant Activity (IC_{50}) (µg/mL)
Rhizome Extract	53.73	Stem Extract	80.36	Leaf Extract	102.10
Rhizome-NPs 1:1	51.23	Stem-NPs 1:1	95.72	Leaf -NPs 1:1	111.89
Rhizome-NPs 1:2	43.22	Stem-NPs 1:2	77.38	Leaf -NPs 1:2	94.61
Rhizome-NPs 1:3	96.61	Stem-NPs 1:3	115.48	Leaf -NPs 1:3	136.60
Rhizome-NPs 2:1	84.33	Stem-NPs 2:1	109.18	Leaf -NPs 2:1	122.75
Rhizome-NPs 3:1	113.97	Stem-NPs 3:1	128.45	Leaf -NPs 3:1	148.26

Table 2: Physicochemical Properties of *Etlingera flexuosa* Nanocapsules

Parameters	Rhizome-NPs (1:2)	Stem-NPs (1:2)	Leaves-NPs (1:2)
Size (nm)	615.8	707.3	768.1
ζ (mV)	-6.4	-6.9	-8.1

However, it is commonly agreed that nanoparticles are particles with a size below 1 micron.[21,22] Submicron particles, often referred to as nanoparticles, are generally recognized in the scope of medicine and pharmaceutics.[23]

Zeta potential (ζ) denotes the parameter that characterizes the electric charge on the surface of nanoparticles. The zeta potential value indicates the magnitude of repulsive forces among particles, crucial for maintaining stable sample dispersion. Nanoparticles exhibiting a zeta potential exceeding ±30 mV are classified as strongly cationic and strongly anionic. Generally, a higher zeta potential indicates stronger electrostatic repulsive forces between the particles, stabilizing them and preventing them from aggregating.[24] The zeta potential values of rhizome-NPs, stem-NPs, and leaf-NPs were -6.4 mV, -6.9 mV, and -8.1 mV, respectively (Table 1). The three nanocapsules of *E. flexuosa* were unstable because they had zeta potential values below ± 10 mV, resulting in a tendency to flocculate, agglomerate, or agglomerate after long-term storage.[19]

Stability of Nanocapsules Etlingera flexuosa

Stability on Temperature

The stability of rhizome-NPs, stem-NPs, leaf-NPs, and *E. flexuosa* extract under various temperatures was evaluated by heating the samples between 30°C and 100°C. Additionally, turbidity measurements were conducted to assess their stability. The turbidity values of *E. flexuosa* extract were higher than those of *E. flexuosa* nanocapsules, as the active compounds in the extract were directly exposed to heat, resulting in a greater risk of degradation. In contrast, *E. flexuosa* nanocapsules demonstrated greater stability due to the protective effect of κ-carrageenan polymers, which helped prevent degradation. Turbidity analysis indicated that stem-NPs and leaf-NPs were minimally affected by thermal exposure, with no significant degradation of their bioactive constituents. Except for rhizome-NPs, the nanocapsules maintained thermal stability, as reflected by turbidity values ranging from 3.2 to 5.8 NTU (Table 3).

In addition, the stability of *E. flexuosa* extracts and nanocapsules was observed through their UV spectral patterns. An increase in heating temperature resulted in changes in the UV spectrum, particularly in the stem and leaf extracts. The rhizome extract showed only a slight increase in absorbance intensity at a wavelength of 281 nm as the temperature increased. In contrast to the extracts, the nanocapsules of *E. flexuosa* exhibited no shift in the maximum wavelength at 281 nm or changes in the UV spectral pattern with increasing temperature (Figure 1). These results indicate that the bioactive compounds in the nanocapsule form were highly stable under heat exposure.

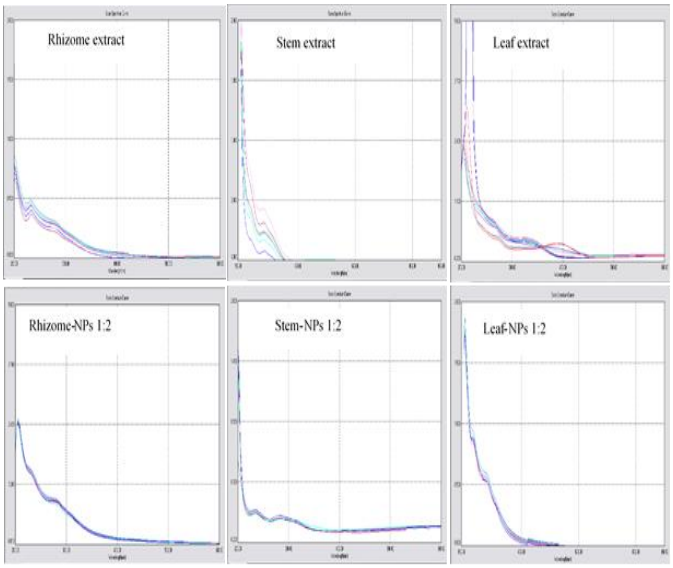

Figure 1: Effect of temperature on the UV-Vis Spectra of *Etlingera flexuosa* ethanol extracts and nanocapsules

Stability of pH

The stability of *E. flexuosa* extract and nanocapsules under varying pH conditions was evaluated based on turbidity levels and UV-Vis spectral patterns. The turbidity values of *E. flexuosa* extract and nanocapsules are presented in Table 4. The turbidity measurements of the rhizome, leaf, and stem extracts indicated instability under pH changes, as evidenced by high turbidity values. In contrast, increasing the pH in *E. flexuosa* nanocapsules generally reduced in turbidity levels, with values below 80 NTU. Rhizome-NPs exhibited high stability against pH changes, with turbidity values ranging from 2.9 to 4.1 NTU. Under alkaline conditions, phenolic compounds formed stable phenoxide ions. The stability of these ions was attributed to resonance, which was initiated by the release of hydrogen atoms from the hydroxyl groups, formating phenoxide salts. These salts exhibited better water solubility

than their phenolic compounds.[28] pH significantly impacts the stability of nanoparticles by influencing their surface charge and electrostatic interactions, ultimately affecting their aggregation behavior.[25] Generally, higher pH values, often in the alkaline range, enhance stability, while lower pH or acidic conditions can promote aggregation and instability[26]. Turbidity refers to the degree of clarity of a liquid, which is affected by suspended particles. However, turbidity is not a direct measurement of the number of suspended particles, but rather an indication of the extent to which those particles scatter and attenuate light. An increase in light scattering or attenuation intensity correlates with elevated turbidity levels. [27]

The stability of the bioactive components in the rhizome extract and rhizome-NPs against pH changes was evaluated based on their UV-Vis spectral patterns. At pH 6, which corresponds to the natural pH of the extract, the rhizome, stem, and leaf extracts exhibited λ-max values ranging from 280 to 281 nm. After the extract was acidified, a decrease in absorption intensity (hypochromicity) was observed. The absorption intensity also decreased at pH 7 and 8, similar to the behavior observed under acidic conditions. However, at pH levels between 9 and 11, an increase in absorption intensity (hyperchromicity) was noted. The UV-Vis spectral patterns of the rhizome, stem, and leaf extracts indicate that *E. flexuosa* extracts are unstable under pH variation. In contrast, the UV spectral patterns of the nanocapsules remaind unchanged across different pH levels, suggesting that the encapsulated compounds did not undergo degradation (Figure 2).

Table 3: The turbidity levels of *Etlingera flexuosa* extracts and nanocapsules at different temperatures

Temperature (^{0}C)	Rhizome Extract (NTU)	Rhizoma-NPs (NTU)	Stem Extract (NTU)	Stem-NPs (NTU)	Leaf Extract (NTU)	Leaf -NPs (NTU)
30	36.3	3.2	262.8	78.1	126.1	105.1
40	48.5	3.5	277.7	82.6	130.7	107.8
50	58.4	3.8	280.8	88.8	135.2	109.3
60	67.9	4.1	284.6	92.6	139.4	111.5
70	65.4	4.5	286.9	98.8	144.4	112.2
80	72.7	4.9	288.6	103.2	138.7	112.7
90	75.4	5.5	289.4	107.8	143.3	113.5
100	86.3	5.8	292.8	114.3	145.3	114.2

Table 4: The turbidity levels of *Etlingera flexuosa* extracts and nanocapsules at different pH levels

pH	Rhizome Extract (NTU)	Rhizoma-NPs (NTU)	Stem Extract (NTU)	Stem-NPs (NTU)	Leaf Extract (NTU)	Leaf-NPs (NTU)
2	145.1	4.1	191.9	65.6	223.6	78.3
3	121.5	3.9	177	62.3	112.2	75.9
4	116.8	3.9	325.7	59.6	293.8	71.8
5	104.2	3.7	310.8	55.1	338.6	68.8
6	99.6	3.5	356.2	52.8	198.5	64.7
7	95.6	3.3	343.6	48.9	307.1	62.6
8	71.3	3.1	258.8	46.4	225.8	59.6
9	82.8	3.1	191.5	43.2	186.8	55.5
10	80.6	2.9	118.1	40.7	77.6	53.8
11	65.6	2.9	109.7	38.9	85.7	51.4

Stability on NaCl

The stability of *E. flexuosa* extracts, rhizoe-NPs, stem-NPs, and leaf-NPs in response to salt was evaluated under NaCl concentrations ranging from 0 to 0.3 M. In the crude extract of *E. flexuosa*, the turbidity value tended to decrease with increasing NaCl concentration (Table 5). This phenomenon was attributed to coagulation, as indicated by forming solid materials (crust) at the container's base, thereby increasing the solution's clarity. Coagulation occurred due to the salting-out effect, which decreased the solubility of the chemical compounds as the NaCl concentration increased.[29] In *E. flexuosa* nanocapsules, increasing the concentration of NaCl led to a rise in turbidity values. This change was attributed to the instability of the coating matrix, specifically κ-carrageenan, within the nanocapsules. The presence of Na^+ cations from NaCl induced gelation in carrageenan, leading to particle aggregation and resulting in increased turbidity.[31] The interaction strength between phenolic compounds and κ-carrageenan can influence physical properties, including hydrophilicity, extensibility, and structural morphology. Hydrogen bonds and ionic interactions between phenolic compounds and κ-carrageenan mainly determine these properties.[32] The high content of phenolic compounds in rhizome extracts contributes to the excellent stability of the rhizome-NPs, as indicated by a significantly lower turbidity value compared to stem-NPs and leaf-NPs.

Table 5: The turbidity levels of *Etlingera flexuosa* ethanol extracts and nanocapsules at different NaCl concentrations

NaCl (Molar)	Rhizome Extract (NTU)	Rhizoma-NPs (NTU)	Stem Extract (NTU)	Stem-NPs (NTU)	Leaf Extract (NTU)	Leaf -NPs (NTU)
0	93.1	7.3	271.3	75.3	222.4	98.3
0.1	83.4	17.8	153.8	88.6	116.8	113.8
0.15	82.3	19.6	146.5	89.2	112.6	115.6
0.2	72.1	21.1	141.9	91.1	109.5	118.2
0.25	61.8	21.9	138.4	92.8	106.6	120.4
0.3	63.5	22.4	129.9	94.1	98.9	121.9

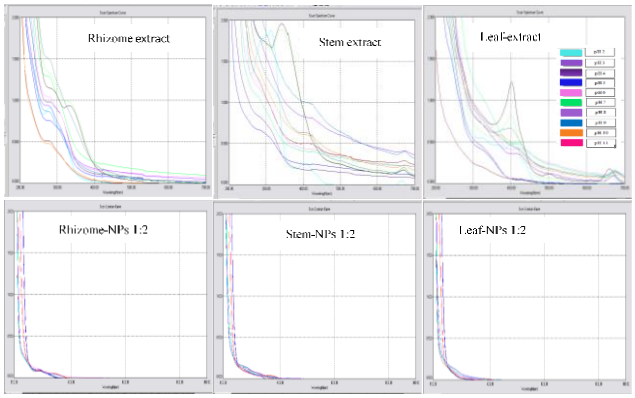

Figure 2: Effect of pH on the UV-Vis spectra of *Etlingera flexuosa* ethanol extracts and nanocapsules

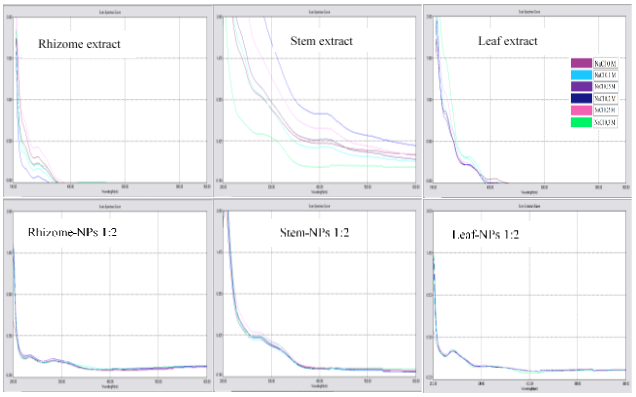

Figure 3: Effect of NaCl concentrations on the UV-Vis Spectra of *Etlingera flexuosa* ethanol extracts and nanocapsules

The addition of NaCl to the *E. flexuosa* extract resulted in significant changes in the UV spectral pattern, indicating that the bioactive components in the extract underwent structural alterations due to increasing NaCl concentrations. In contrast, *E. flexuosa* nanocapsules generally did not exhibit bathochromic or hypsochromic shifts around the wavelength of 280 nm. However, a slight increase in absorption intensity was observed in the UV spectrum of stem-NPs at salt concentrations of 0.25 and 0.3 M (Figure 3). Overall, the addition of NaCl at various concentrations demonstrated that the nanoparticle form of the extract—particularly rhizome-NPs—was more stable, as evidenced by low turbidity values (below 22.4 NTU) and unchanged UV spectra.

Conclusion

Antioxidant test results indicated that the *E. flexuosa* extract in nanoparticle form exhibited enhanced antioxidant activity at an optimal extract-to-matrix ratio. The highest antioxidant activity in rhizome-NPs, stem-NPs, and leaf-NPs was observed at a ratio of 1:2. Among the three types of nanocapsules, rhizome-NPs demonstrated very strong antioxidant activity. The particle sizes of all three nanocapsules were below 1000 nm, with zeta potential values of less than ±30 mV. Nanoencapsulation effectively maintained the stability of the chemical compounds from the rhizome, stem, and leaf parts of *E. flexuosa* ginger under varying pH, temperature, and sodium chloride (NaCl) concentrations. The potent antioxidant activity of the rhizome nanoparticle formulation is considered worthy of further investigation, including toxicity tests against cancer cells and evaluations against other diseases related to antioxidant compounds, both in vitro and in

vivo, to support its potential future development as a standardized herbal medicine.

Conflict of Interest
Authors 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.

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