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The Effect of Citrit Acid-Glucose NADES Ratio on Young *Avicennia marina* Leaf Extract

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ABSTRACT

Mangrove ecosystems are widely acknowledged as valuable natural sources of bioactive compounds with potential applications in functional food and pharmaceutical industries. *Avicennia marina* leaves, in particular, contain diverse secondary metabolites; however, the efficiency of their extraction is strongly influenced by solvent type and polarity. This study aimed to evaluate the effect of different ratios of citric acid–glucose-based natural deep eutectic solvent (NADES) on extraction yield, pH, and phytochemical profile of young *A. marina* leaf extracts. A Completely Randomized Design (CRD) was applied, consisting of four treatments of citric acid:glucose ratios (1:1, 1:2, 1:3, and 1:4) with four replications. Extraction was performed at 50°C for 60 minutes using a material-to-solvent ratio of 1:20 (w/v). The findings indicated that increasing the proportion of citric acid in the NADES formulation significantly enhanced extraction yield, with the highest yield (20.40%) obtained at the 4:1 ratio. The extract pH ranged from 1.79 to 1.88 and decreased as the citric acid proportion increased. Phytochemical screening confirmed the presence of flavonoids, tannins, and alkaloids, while saponins, steroids, and terpenoids were not detected. Overall, these results demonstrate that citric acid–glucose-based NADES serve as an effective green solvent system for extracting polar bioactive compounds from young *A. marina* leaves.

INTRODUCTION

Indonesia's mangrove ecosystems represent a highly diverse tropical bioresource enriched with high-value secondary metabolites, including phenolics, flavonoids, alkaloids, terpenoids, saponins, tannins, and steroids. These compounds have been widely reported to exhibit antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and anticancer activities (Dahibhate *et al.,* 2019; Beniwal *et al.,* 2024; Rahmania *et al.,* 2025; Botosoa & Shahidi, 2025). Numerous Indonesian mangrove species demonstrate substantial phytochemical richness accompanied by strong antioxidant capacity, highlighting their significant potential for applications in functional foods, pharmaceuticals, and cosmeceuticals (Darmadi *et al.,* 2021; Hidayati *et al.,* 2023; Kalor *et al.,* 2025).

Among mangrove species, *Avicennia marina* has been widely reported to exhibit notable biological activities. Its leaves are rich in various bioactive constituents, such as phenolic compounds, flavonoids, alkaloids, terpenoids, and tannins, which contribute to strong antioxidant and antibacterial effects (Al-Mur, 2021; Ibrahim *et al.,* 2021; Mitra *et al.,* 2023; Rajivgandhi *et al.,* 2023; Zhou *et al.,* 2025). Several studies indicate that young leaves demonstrate more active metabolic processes compared to mature leaves, implying a higher concentration of polar phytochemicals. Nevertheless, due to the predominantly hydrophilic characteristics of young leaf tissues, effective extraction requires highly polar solvents capable of forming strong hydrogen-bond interactions to optimize the recovery of these bioactive compounds.

Natural deep eutectic solvents (NADES) have gained increasing attention as environmentally friendly extraction media capable of overcoming many drawbacks associated with conventional organic solvents. Commonly used solvents such as n-hexane, chloroform, and other dipolar aprotic solvents are widely recognized for their toxicity, high volatility, and potential risks to human health and the environment, particularly due to the emission of volatile organic compounds (VOCs) (Joshi & Adhikari, 2019; Płotka-Wasylka *et al*., 2017; Sheldon, 2019; Jordan *et al.,* 2022; Lemos *et al.,* 2024). In addition, traditional extraction methods, including maceration and Soxhlet extraction, generally involve extended processing times and substantial energy consumption, which can promote thermal degradation of heat-sensitive compounds and consequently decrease both extraction efficiency and bioactivity (Ferreira & Sarraguça, 2024; Alam *et al.,* 2024). To address these challenges, NADES formulated from naturally sourced constituents such as organic acids, sugars, polyols, and choline-based compounds have been introduced. These solvent systems exhibit low volatility, biodegradability, and tunable physicochemical properties, allowing adjustment of polarity and hydrogen-bond interactions to improve extraction selectivity and performance (Pena-Pereira *et al.,* 2015; Chemat *et al.,* 2019).

NADES are generally categorized according to the type of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) used in their formulation, as well as their resulting physicochemical characteristics (Paiva et al., 2014; Vanda et al., 2018). Systems composed of organic acids and sugars typically exhibit high polarity, relatively high viscosity, and extensive hydrogen-bonding interactions. These features make them particularly suitable for dissolving polar bioactive compounds, including phenolics, flavonoids, polar alkaloids, and polysaccharides (Fuad & Nadzir, 2021; Li, 2022; Koh *et al*., 2023; Usmani *et al*., 2023). In addition to serving as extraction media, NADES can also contribute to the stabilization of bioactive molecules through specific intermolecular interactions that help preserve their structural integrity.

Among the different NADES formulations, the citric acid–glucose system has received particular attention because of its pronounced polarity, acidic character, and well-developed hydrogen-bonding structure. NADES prepared from organic acids typically display inherently low pH values, which can improve the solubility and stability of phenolic and flavonoid compounds through stronger hydrogen-bond interactions with their hydroxyl groups (Pires *et al*., 2022; Sakurai *et al*., 2024; Esnal-Yeregi *et al*., 2025). In addition, adjusting the proportion of citric acid (acting as the HBD) and glucose (serving as the HBA) is likely to alter important physicochemical parameters of the solvent system, such as polarity and acidity. These changes may ultimately affect extraction efficiency as well as the resulting phytochemical profile.

Although the application of NADES for bioactive compound extraction has been widely reported, systematic investigations evaluating the influence of citric acid–glucose ratio and directly correlating it with extract pH, extraction yield, and phytochemical profile of young *Avicennia marina* leaves remain limited. Therefore, this study aims to evaluate the effect of varying citric acid:glucose NADES ratios on the phytochemical characteristics of young *A. marina* leaf extracts.

METHODS

Place and Time of Research

This research was conducted at the Laboratory of Aquatic Product Technology and Fishery Product Engineering, and the Fishery Product Safety Division Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, from January to June 2024. Sampling of *A. marina* mangrove leaves was carried out in the Wonorejo Mangrove Forest, Rungkut District, Surabaya, located at coordinates 8°41’32.61” S and 113°10’40.98” E.

Materials

The primary raw material used in this study was young leaves of *Avicennia marina*, collected from the Wonorejo Mangrove Forest, Rungkut District, Surabaya, East Java, Indonesia. The materials used for NADES preparation included distilled water (aquades), citric acid, and glucose. The analytical-grade chemicals used for phytochemical analysis were concentrated hydrochloric acid (HCl, Merck), H_2SO_4, (Merck), ferric chloride ($FeCl_3$, Merck), Dragendorff’s reagent (Merck), acetic anhydride (Merck), chloroform (SmartLab), and 70% ethanol (Merck). The laboratory equipment included beakers, test tubes, dark glass vials, serological pipettes, droppers, pipette fillers, graduated cylinders, analytical balance, spatula, glass stirring rods, funnels, Kjeldahl flasks, Soxhlet apparatus, fat extraction flasks, chroma meter, Erlenmeyer flasks, and a texture analyzer.

Experimental Design

The study was conducted using a Completely Randomized Design (CRD). The experimental factor evaluated was the variation in citric acid:glucose ratio in the NADES formulation, consisting of four treatment levels with four replications each. The preparation of the NADES solutions and the determination of treatment ratios were based on previous studies by Yusuf *et al*. (2021), Ibrahim *et al*. (2021), and Meneses *et al*. (2019), with slight modifications. The treatment are P1: Citric acid : Glucose (1:1), P2: Citric acid : Glucose (2:1), P3: Citric acid : Glucose (3:1), P4: Citric acid : Glucose (4:1).

Research Procedure

The research was carried out in two primary phases. The first stage involved drying of plant material and preparation of NADES. The second stage consisted of extraction using

NADES, followed by extract characterization, including yield calculation, moisture content determination, pH measurement, and phytochemical screening.

The initial phase of the research involved the preparation of dried plant material followed by the formulation of the NADES solution. The drying procedure was adapted from the method reported by Imra *et al.* (2022), with minor modifications. Fresh young leaves of *A. marina* were thoroughly rinsed under flowing water to eliminate surface contaminants and then allowed to drain. The cleaned leaves were subsequently dried in an oven at 50°C for 90 minutes to lower their moisture content. Once dried, the leaves were milled using a blender and passed through a 60-mesh sieve to obtain a homogeneous powder. Prior to the extraction process, the moisture content of the powdered sample was measured.

The preparation of the natural deep eutectic solvent (NADES) was carried out following modified procedures described by Yusuf *et al.* (2021), Ibrahim *et al.* (2021), and Meneses *et al.* (2019). Citric acid and glucose were precisely measured based on the established molar proportions and placed into a beaker. The combined components were then heated on a hotplate at 80°C for 60 minutes, allowing the mixture to gradually transform into a uniform and viscous liquid. Subsequently, 35 mL of distilled water was added, and the system was further homogenized using a magnetic stirrer at 200 rpm and 80°C for 60 minutes under closed conditions to ensure complete dissolution and stabilization of the hydrogen-bonding network. The resulting NADES was allowed to cool to room temperature and stored in a sealed container until used for the extraction process.

The second stage involved the extraction of young *A. marina* leaves using NADES. The extraction procedure was adapted from Ahmad and Prabowo (2020), Savi *et al.* (2019), and Rahman *et al.* (2023) with slight modifications. In brief, five grams of powdered young *A. marina* leaves were accurately weighed and combined with NADES in a 1:20 (w/v) ratio within a beaker. The extraction was conducted at 50°C for 60 minutes to facilitate compound release. Upon completion, the mixture was strained through muslin cloth to separate the extract from the solid residue. The collected filtrate was then centrifuged at 5,000 rpm for 15 minutes to obtain a clear supernatant. This supernatant was carefully collected and subsequently subjected to physicochemical and phytochemical analyses.

The parameters evaluated in this study included extraction yield (%), pH value of the extract, and phytochemical constituents. Phytochemical screening was conducted to qualitatively identify the presence of major secondary metabolites, including alkaloids, saponins, tannins, steroids, and terpenoids.

Statistical Analysis

The experimental data were analyzed using Analysis of Variance (ANOVA) with SPSS software. When significant differences were detected ($p < 0.05$), the means were further compared using Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

RESULTS

The Moisture Content of the *A. marina*

Moisture analysis indicated that the *A. marina* leaf powder contained 15.19% water content. This value exceeds the maximum moisture content requirement established by the Departemen Kesehatan Republik Indonesia (2017), which recommends a moisture level below 10% for dried herbal materials.

Extraction Yield of Young *A. marina* Leaves

The extraction yield of young *Avicennia marina* leaf powder is presented in Figure 1 below.

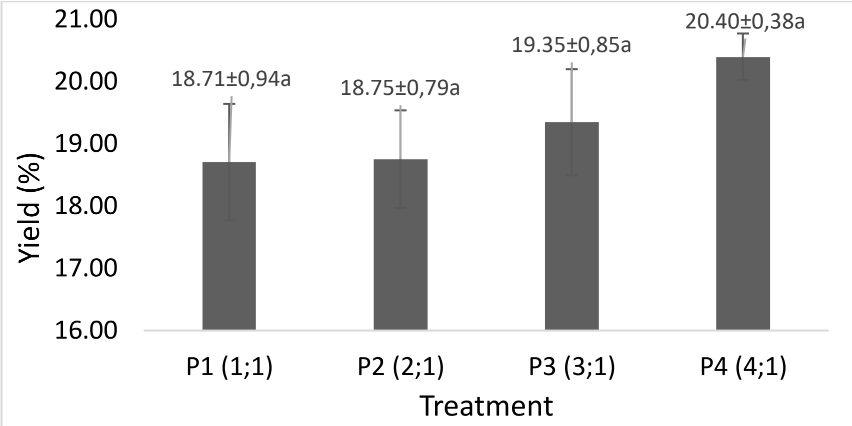

Figure 1. Extraction Yield of Young *Avicennia marina* Leaf Extracts

Figure 1 illustrates the extraction yield of young *Avicennia marina* leaves obtained using different citric acid–glucose NADES ratios. The results indicate that increasing the proportion of citric acid tended to enhance the extraction yield. The highest yield was observed in treatment P4, reaching 20.40%. This value is considerably higher than that reported by Wulandari *et al.* (2022), who extracted *A. marina* leaves using methanol and obtained a yield of 10.93%. Similarly, Rahman & Sasmito (2021) reported lower yields when using conventional organic solvents, with n-hexane, ethyl acetate, and ethanol producing yields of 3.96%, 6.4%, and 14.4%, respectively.

pH of Young *Avicennia marina* Leaf Extracts

The pH values of young *Avicennia marina* leaf extracts obtained using different citric acid–glucose NADES ratios are presented in Figure 2.

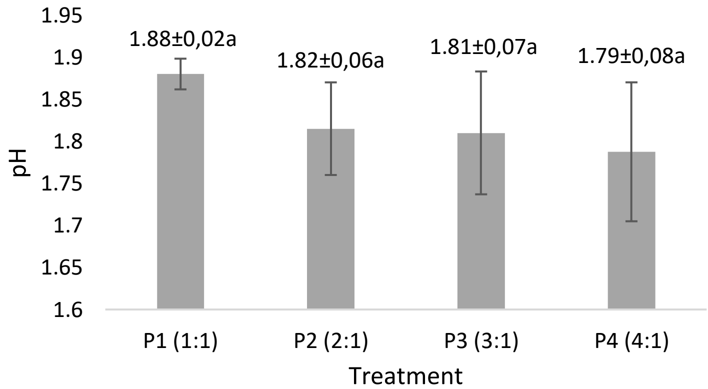

Figure 2. pH Value of Young *Avicennia marina* Leaf Extracts

The pH analysis of young *Avicennia marina* leaf extracts obtained using citric acid–glucose-based NADES revealed strongly acidic conditions, with pH values ranging from 1.79 to 1.88. In general, increasing the NADES ratio from P1 (1:1) to P4 (4:1) resulted in a gradual decrease in extract pH, although some treatments were not statistically different.

Phytochemical Profile of Young *Avicennia marina* Leaf Extracts

The phytochemical screening revealed that extracts obtained from young *Avicennia marina* leaves contained flavonoids, tannins, and alkaloids, showing moderate to strong positive reactions. In contrast, saponins, steroids, and terpenoids were not identified in the extracts. The phytochemical profile of young *Avicennia marina* leaf extracts is presented in Table 1 below.

Table 1. Phytochemical Profile of Young *A. marina* Leaf Extracts

Bioactive Compound	NADES (citric acid:glucose)	Replicate				Positive Indicator
		1	2	3	4	
Flavonoid	1:1	+	++	++	+	Orange to red coloration
	2:1	++	+	+++	+++	
	3:1	++	+	++	+++	
	4:1	++	+++	+	+	
Tannin	1:1	++	+	+	+	Green, red, purple, blue or black coloration
	2:1	+	++	++	+	
	3:1	++	+	+++	++	
	4:1	+++	++	+++	+++	
Alkaloid	1:1	+	+	+	+	Orange precipitate
	2:1	++	+	+	++	
	3:1	++	+++	+++	+++	
	4:1	+++	++	+	+++	
Saponin	1:1	-	-	-	-	Stable foam formation
	2:1	-	-	-	-	
	3:1	-	-	-	-	
	4:1	-	-	-	-	
Steroid	1:1	-	-	-	-	Blue
	2:1	-	-	-	-	
	3:1	-	-	-	-	
	4:1	-	-	-	-	
Terpenoid	1:1	-	-	-	-	Reddish-orange coloration
	2:1	-	-	-	-	
	3:1	-	-	-	-	
	4:1	-	-	-	-	

DISCUSSION

The Moisture Content of the *A. marina*

The elevated moisture content recorded in this study is likely associated with the physiological traits of young mangrove leaves, which are characterized by abundant cellular cytoplasm containing hydrophilic proteins (Widarta & Wiadnyani, 2019).

Leaf maturity and harvesting conditions also influence moisture levels. According to Hanum *et al.* (2018), apical (young) leaves generally exhibit higher moisture content compared to basal (mature) leaves. Additionally, leaves harvested in the morning tend to retain higher water content than those collected in the afternoon or evening due to reduced transpiration rates earlier in the day.

Another contributing factor may be the drying method employed. Wijaya & Noviana (2022) reported that oven drying at elevated temperatures can accelerate moisture removal compared to sun drying; however, insufficient drying time or uneven heat distribution may result in residual moisture. High moisture content in plant materials may promote enzymatic reactions and microbial growth, ultimately leading to deterioration and reduced stability of the dried material (Dharma *et al.,* 2020).

Extraction Yield of Young *A. marina* Leaves

Evidence from previous research indicates that NADES outperform conventional solvents in terms of extraction efficiency. Hikmawanti *et al.* (2021) reported that NADES produced higher extraction yields than 80% methanol. The superior extraction performance of NADES can be attributed to its adjustable physicochemical characteristics, such as polarity, molarity, viscosity, and hydrogen-bonding capability. These tunable properties facilitate stronger and more specific solute–solvent interactions, thereby enhancing solubilization and mass transfer during the extraction process. Consequently, the ability of NADES to optimize intermolecular interactions plays a pivotal role in improving extraction efficiency compared to conventional solvents (Latifah & Nuh, 2024). Based on their composition and chemical characteristics, NADES can be classified into several categories, including ionic liquid-type NADES, neutral NADES, neutral NADES combined with acids or bases, and amino acid-based NADES (Hikmawanti *et al.,* 2021).

pH of Young *Avicennia marina* Leaf Extracts

The highest pH was observed in treatment P1 (1:1), with a value of 1.88 ± 0.02, while the lowest pH occurred in P4 (4:1), reaching 1.79 ± 0.08. This pattern suggests that elevating the proportion of citric acid as the hydrogen bond donor (HBD) within the NADES formulation intensifies the acidity of the resulting extract. The increase in acid concentration likely enhances proton availability in the system, thereby lowering the pH. This finding aligns with previous studies reporting that NADES composed of organic acids particularly those based on citric acid or malic acid naturally exhibit acidic characteristics, often presenting pH values in the range of 2.0–2.5 or even lower, depending on the molar composition and water content of the system (Sakurai *et al.,* 2024; Jauregi *et al.,* 2024).

Moreover, the highly acidic environment of the extracts may be advantageous for the extraction of phenolic and flavonoid compounds, which are generally more stable and exhibit greater solubility under acidic conditions. Esnal-Yeregi *et al.* (2025) and Pires *et al.* (2022) reported that acid-based NADES can enhance hydrogen-bond interactions with phenolic hydroxyl groups, thereby improving extraction efficiency and stabilizing bioactive compounds. Therefore, the low pH values observed in the *A. marina* extracts in this study may represent favorable conditions for the recovery of antioxidant phytochemicals.

Phytochemical Profile of Young *Avicennia marina* Leaf Extracts

These results corroborate previous studies reporting the effectiveness of citric acid–glucose-based NADES in enhancing the extraction of phenolic-rich compounds. Bat Ozmatara (2021) demonstrated that cardamom (*Elettaria cardamomum*) seeds extracted using glucose : citric acid NADES yielded 198.25 µg GAE/g total phenolics and 132.09 mg GAE/g total tannins. Likewise, Ahmad *et al.* (2025) reported that extraction of *Peperomia pellucida* using citric acid–glucose NADES combined with microwave-assisted extraction produced 138.29 mg GAE/g total phenolics. The improved extraction capacity of NADES is associated with their unique composition commonly comprising organic acids, sugars, polyols, or choline derivatives which enables the formation of extensive hydrogen-bonding networks and adaptable intermolecular interactions. These characteristics modulate solvent polarity and enhance the solubility and recovery of phenolic compounds compared to conventional organic solvents (Vidic *et al.,* 2025).

The absence of detectable saponins, steroids, and terpenoids in the young *A. marina* leaf extracts is likely associated with the high polarity of the NADES employed. Steroids and terpenoids are generally nonpolar to semi-polar compounds (Harborne, 1998), making them less efficiently extracted by highly polar solvent systems. Furthermore, the concentrations of these compounds in young leaves may fall below the detection limit of the qualitative phytochemical screening method used. Therefore, the “not detected” result does not necessarily indicate the complete absence of these metabolites but may reflect methodological limitations and solvent selectivity.

CONCLUSION

Extraction of young *Avicennia marina* leaves using citric acid : glucose based natural deep eutectic solvents (NADES) demonstrated that increasing the proportion of citric acid enhanced the extraction yield, with the highest value observed at the 4:1 ratio. This increase in citric acid content also resulted in a progressive decrease in pH, leading to strongly acidic extract conditions. Phytochemical screening confirmed the presence of flavonoids, tannins, and alkaloids, while saponins, steroids, and terpenoids were not detected. These results suggest that citric acid–glucose-based NADES act as effective green solvent systems for selectively extracting polar bioactive compounds from young *A. marina* leaves.

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