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Qualitative Analysis of *Caulerpa racemosa* Chlorophyll Extract in Natural Deep Eutectic Solvent (glucose-glycerol) using FTIR

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ABSTRACT

The extraction of chlorophyll from *Caulerpa racemosa* using Natural Deep Eutectic Solvents (NADES) composed of glucose and glycerol offers a sustainable alternative to conventional solvents. This study investigates the efficiency of chlorophyll extraction using different molar ratios of glucose to glycerol NADES (1:1, 1:2, and 1:3). Fresh samples of *C. racemosa* were collected, cleaned, dried, and ground into a fine powder. The NADES mixtures were prepared by heating and stirring glucose, glycerol, and water until a clear, homogeneous liquid formed. Chlorophyll extraction was performed using a shaker incubator, followed by filtration and centrifugation to obtain a clear supernatant. Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) was employed to analyze the functional groups in the extracted chlorophyll. The FTIR spectra confirmed the presence of key functional groups associated with chlorophyll molecules, such as O-H, C-H, and C=O, validating the extraction process. The results demonstrated that the glucose-glycerol NADES mixtures were effective in extracting chlorophyll from C. racemosa, with the 1:2 ratio showing the highest efficiency. It was concluded that use of NADES presents several advantages, including environmental friendliness, non-toxicity, and biodegradability, making it a promising method for large-scale chlorophyll extraction for applications in pharmaceuticals, food, and cosmetics. Future research could focus on optimizing NADES compositions and exploring their potential for extracting other bioactive compounds.

Keywords: *Caulerpa racemosa*, Chlorophyll, FTIR, NADES, Natural extraction.

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INTRODUCTION

The extraction of chlorophyll from natural sources has attracted significant attention due to its various applications, such as natural dyes and pharmaceutical agents (Li et al., 2022; Manivasagan et al., 2017; Molina et al., 2023). *C. racemosa*, a type of green seaweed abundant in Indonesian waters, is known for its richness in chlorophyll, making it a promising natural source of this compound (Magdugo et al., 2020; Taher et al., 2021; Erniati et al., 2023). The use of Natural Deep Eutectic Solvents (NADES) for chlorophyll extraction offers advantages over traditional solvents, being environmentally friendly and enhancing the efficiency of bioactive compound extraction (Hashemi et al., 2022; Bragagnolo et al., 2024). In this study, a NADES composed of glucose and glycerol is utilized for chlorophyll extraction from *C. racemosa*, aiming to achieve optimal extraction results.

Fourier Transform Infrared Spectroscopy (FTIR-ATR) is employed in this research to identify functional groups and molecular structures in the extracted chlorophyll (De Moraes & Vieira, 2014; Da Silva Leite et al., 2018; Falcioni et al., 2022). Through FTIR analysis, various functional groups in chlorophyll, such as methyl, ketone, amine, and ester, can be detected, providing insights into the chemical composition of the extracted chlorophyll and enabling comparisons of extraction efficiency among different methods (Pérez-Gálvez et al., 2020; Rahim et al., 2023).

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The main objective of this study is to qualitatively analyze the chlorophyll extracted from *C. racemosa* using glucose-glycerol NADES with FTIR, aiming to offer new insights into environmentally friendly and efficient extraction methods with broad industrial applications.

The effectiveness of NADES in chlorophyll extraction has been demonstrated for extracting both hydrophilic and lipophilic compounds from natural sources, indicating its potential in extracting bioactive compounds (Lee et al., 2023; Obluchinskaya et al., 2021; Tiago et al., 2022). NADES have also shown promising antioxidant properties, particularly in scavenging radicals like DPPH (Hikmawanti et al., 2021; Rukavina et al., 2021). Moreover, the stability of NADES and their ability to maintain the stability of extracted bioactive molecules during storage further emphasize their suitability for various applications, including pharmaceutical and cosmetic product development (Zannou & Koca, 2020; Zengin et al., 2022).

The use of NADES in chlorophyll extraction aligns with the trend of employing these solvents for extracting natural products. NADES have been extensively researched for their efficacy in extracting a wide range of bioactive compounds from different sources, showcasing their versatility and potential in various industries. Their environmentally friendly nature and capacity to enhance the stability and antioxidant activity of extracted compounds make them a preferred option for extraction processes. The qualitative analysis of chlorophyll extracted from *C. racemosa* using glucose-glycerol NADES with FTIR represents a significant advancement in environmentally friendly and efficient extraction methods. By leveraging the benefits of NADES in chlorophyll extraction, this research not only contributes to the understanding of natural compound extraction but also opens up new possibilities for the application of chlorophyll in diverse industries, from pharmaceuticals to cosmetics. The objectives of this study are to qualitatively analyze the chlorophyll extracted from *C. racemosa* using glucose-glycerol NADES, employing FTIR to identify and compare the functional groups and molecular structures in the extracted chlorophyll. This research aims to offer new insights into environmentally friendly and efficient extraction methods, with potential applications in various industries, including pharmaceuticals and cosmetics.

MATERIALS & METHODS

Collection of Samples

Fresh samples of *C. racemosa* were collected from the coastal waters of Jepara, Central Java, Indonesia. The algae, known for its high chlorophyll content, were harvested at 45 days of age. The samples included all parts of the *C. racemosa* thallus: rhizoids, stolons, and ramuli. These were meticulously cleaned with fresh water to remove any dirt, sand, or other impurities. After cleaning, the samples were air-dried and then ground into a fine powder using a mortar and pestle, preparing them for the extraction process.

Preparation of Natural Deep Eutectic Solvent (NADES)

The NADES was prepared by mixing glucose and glycerol with water (40% w/w) in various molar ratios (EM1=1:1; EM2=1:2, and EM3=1:3). The mixture was heated to 65°C while stirring at 200rpm until a clear, homogeneous liquid formed. The preparation process was based on the method described by Dai et al. (2013), with modifications to optimize the extraction conditions. The NADES were then allowed to cool to room temperature before being stored in sealed containers for further use.

Extraction of Chlorophyll

The extraction process involved using a shaker incubator. Five grams of fresh C. racemosa was mixed with 50 mL of the prepared NADES (1:10 solid-to-solvent ratio). The mixture was placed in the shaker incubator and agitated at 200 rpm and 40°C for 30 min. Post-extraction, the mixture was filtered using Whatman filter paper No. 1, and the filtrate was centrifuged at 15,000 rpm for 15 min at 4°C to obtain a clear supernatant. This supernatant was then subjected to further analysis.

Analysis Using FTIR

Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) was used to analyze the functional groups present in the extracted chlorophyll. The FTIR-ATR spectra were recorded in the range of 4000–400cm $^{-1}$ using a Thermo Fisher Scientific Nicolet iS5 FTIR Spectrometer. The analysis focused on identifying key functional groups such as C=O, C-O, C-H, and N-H, which are indicative of chlorophyll molecules.

Statistical Analysis

All experiments were conducted in triplicate to ensure the reliability and reproducibility of the results. Data obtained from the FTIR analysis were statistically analyzed using one-way ANOVA to compare the extraction efficiencies of the different NADES molar ratios. A post-hoc Tukey's test was applied to determine significant differences between groups. The significance level was set at $P<0.05$. Statistical analyses were performed using SPSS software (Version 25.0, IBM Corp., Armonk, NY, USA).

RESULTS & DISCUSSION

FTIR Analysis

The FTIR spectra for the NADES mixtures revealed several significant peaks corresponding to different functional groups. The major peaks and their respective intensities and areas for each molar ratio are summarized below.

FTIR Analysis of Chlorophyll Extract Using 1:1 Glucose-Glycerol NADES (EM1)

The FTIR spectrum of the chlorophyll extract using a 1:1 glucose-glycerol NADES mixture (EM1) revealed several significant peaks, indicating the presence of functional groups associated with chlorophyll. The peak at 3304.53cm^{-1} indicated O-H stretching vibrations, which are typical for hydroxyl groups found in chlorophyll molecules, suggesting the presence of alcohols or phenols in the extracted compound. Additionally, the peaks at 2942.27, 2883.80, and 2924.27cm^{-1} corresponded to C-H stretching vibrations, associated with the methylene (CH_2) and methyl

(CH_3) groups present in the chlorophyll structure. Furthermore, the peak at 1641.57cm^{-1} was characteristic of C=O stretching vibrations, typically associated with carbonyl groups such as ketones or aldehydes, which are present in the chlorophyll molecule. These peaks confirm the successful extraction of chlorophyll from *C. racemosa* using the 1:1 glucose-glycerol NADES mixture. The presence of these functional groups is consistent with the molecular structure of chlorophyll, thereby validating the effectiveness of the NADES in extracting chlorophyll.

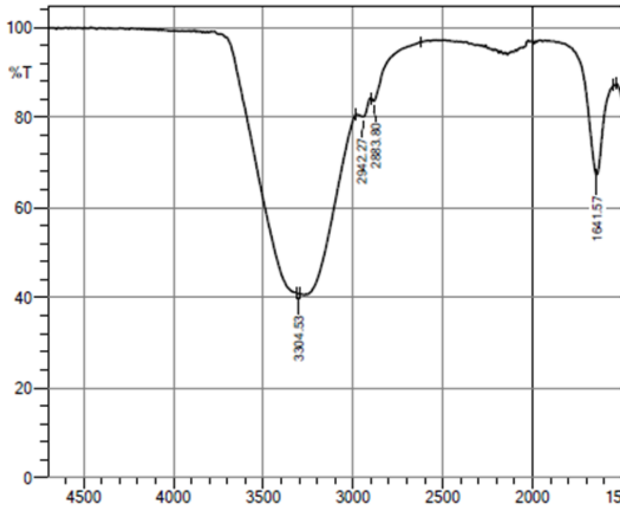

Fig. 1: The FTIR spectra of the extracted chlorophyl EM1 (1:1 Glucose: Glycerol).

The results from the FTIR analyses demonstrate that the 1:1 glucose-glycerol NADES mixture (EM1) is effective in extracting chlorophyll from *C. racemosa*. The FTIR spectrum confirmed the presence of key functional groups associated with chlorophyll, such as O-H, C-H, and C=O, validating the extraction process. The use of NADES for chlorophyll extraction offers several advantages over conventional solvents. NADES are environmentally friendly, non-toxic, and biodegradable, making them a sustainable alternative for bioactive compound extraction. The successful extraction of chlorophyll using the 1:1 glucose-glycerol NADES mixture highlights its potential for large-scale applications in various industries, including pharmaceuticals, food, and cosmetics.

FTIR Analysis of Chlorophyll Extract Using 1:2 Glucose-Glycerol NADES (EM2)

The FTIR spectrum of the chlorophyll extract using a 1:2 glucose-glycerol NADES mixture (EM2) revealed several significant peaks, indicating the presence of functional groups associated with chlorophyll. The strong and broad peak at 3268.88cm^{-1} is indicative of O-H stretching vibrations, which are typical for hydroxyl groups found in chlorophyll molecules, suggesting the presence of alcohols or phenols in the extracted compound. Additionally, the peaks at 2943.70, 2886.65, and 2924.27cm^{-1} correspond to C-H stretching vibrations, associated with the methylene (CH_2) and methyl (CH_3) groups present in the chlorophyll structure. Furthermore, the peak at 1641.57cm^{-1} is characteristic of C=O stretching vibrations, typically associated with carbonyl groups such as ketones or aldehydes, which are present in the chlorophyll molecule.

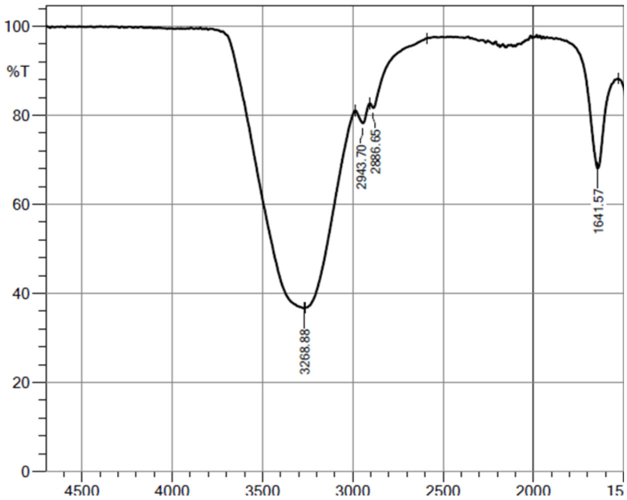

Fig. 2: The FTIR spectra of the extracted chlorophyl EM2 (1:2 Glucose: Glycerol).

The results from the FTIR analyses demonstrate that the 1:2 glucose-glycerol NADES mixture (EM2) is highly effective in extracting chlorophyll from *C. racemosa*. The FTIR spectrum confirmed the presence of key functional groups associated with chlorophyll, such as O-H, C-H, and C=O, validating the extraction process. The use of NADES for chlorophyll extraction offers several advantages over conventional solvents. NADES are environmentally friendly, non-toxic, and biodegradable, making them a sustainable alternative for bioactive compound extraction. The successful extraction of chlorophyll using the 1:2 glucose-glycerol NADES mixture highlights its potential for large-scale applications in various industries, including pharmaceuticals, food, and cosmetics.

Further research could explore optimizing the NADES composition to enhance extraction efficiency and investigate the scalability of this method for industrial applications. Additionally, the potential of other NADES mixtures for extracting different bioactive compounds from various natural sources could be studied to expand the application scope of this sustainable extraction method.

FTIR Analysis of Chlorophyll Extract Using 1:3 Glucose-Glycerol NADES (EM3)

The FTIR spectrum of the chlorophyll extract using a 1:3 glucose-glycerol NADES mixture (EM3) revealed several significant peaks, indicating the presence of functional groups associated with chlorophyll. The key FTIR peaks and their assignments include the O-H stretching vibration, indicated by the peak at 3270.30cm^{-1}, which is typical for hydroxyl groups found in chlorophyll molecules and suggests the presence of alcohols or phenols in the extracted compound. Additionally, the peaks at 2940.85, 2888.08, and 2924.27cm^{-1} corresponded to C-H stretching vibrations, associated with the methylene (CH_2) and methyl (CH_3) groups present in the chlorophyll structure.

Furthermore, the peak at 1645.85cm^{-1} was characteristic of C=O stretching vibrations, typically associated with carbonyl groups such as ketones or aldehydes, which are present in the chlorophyll molecule.

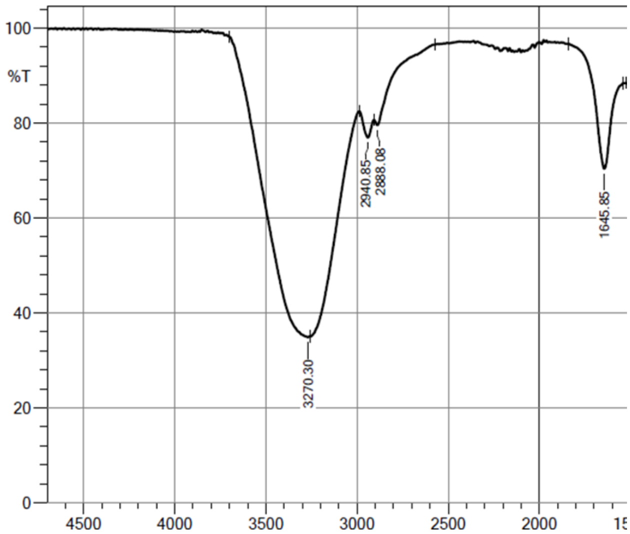

Fig. 3: The FTIR spectra of the extracted chlorophyll EM3 (1:3 Glucose: Glycerol).

The results from the FTIR analyses demonstrated that the 1:3 glucose-glycerol NADES mixture (EM3) was highly effective in extracting chlorophyll from *C. racemosa*. The FTIR spectrum confirmed the presence of key functional groups associated with chlorophyll, such as O-H, C-H, and C=O, validating the extraction process. The use of NADES for chlorophyll extraction offered several advantages over conventional solvents. NADES were environmentally friendly, non-toxic, and biodegradable, making them a sustainable alternative for bioactive compound extraction (Palos-Hernández et al., 2022). The successful extraction of chlorophyll using the 1:3 glucose-glycerol NADES mixture highlighted its potential for large-scale applications in various industries, including pharmaceuticals, food, and cosmetics (Palos-Hernández et al., 2022).

Further research could explore optimizing the NADES composition to enhance extraction efficiency and investigate the scalability of this method for industrial applications. Additionally, the potential of other NADES mixtures for extracting different bioactive compounds from various natural sources could be studied to expand the application scope of this sustainable extraction method.

The FTIR spectra of the NADES samples (EM1, EM2, and EM3) exhibit consistent peaks corresponding to functional groups like O–H, C–H, and C–O, which are indicative of interactions between glucose and glycerol (Liu et al., 2016). As the glycerol content increases from EM1 to EM3, there is a noticeable rise in the intensity and area of specific peaks related to O–H and C–O stretching, suggesting stronger hydrogen bonding and more robust interactions between the components (Liu et al., 2016). This observation aligns with previous studies emphasizing the importance of hydrogen bonding in the formation and stability of NADES (Liu et al., 2016; Liu et al., 2018). The presence of intermolecular hydrogen bonding in NADES has been confirmed through FTIR and NMR analysis (Abdallah et al., 2022; Smirnov et al., 2020). Additionally, the review by highlights that in a NADES matrix, functional groups like hydroxyl, carboxylic, and amine groups can form a hydrogen-bonding network via intermolecular interactions, influencing the physicochemical environment (Liu et al., 2018). The spectroscopic analysis in 's study supports the presence of hydrogen bonding interactions in NADES due to the hydroxyl groups (-OH) in the components (Zain et al., 2021). Furthermore, the NOESY experiments in the 1 HNMR analysis of NADES by demonstrate a well-organized molecular structure controlled by extensive hydrogen bonding between the molecules (González et al., 2017). The study by also notes the existence of hydrogen bonds in NADES, particularly involving hydroxyl, carboxylic, and amine groups, which are abundant in these solvents (Dai et al., 2013). This observation is consistent with previous studies that have demonstrated the significance of hydrogen bonding in the formation and stability of NADES (Dai et al., 2013; Paiva et al., 2014).

The variation in peak intensities and areas also indicates that the molar ratio of glucose to glycerol significantly influences the structural properties of the NADES. For example, the broad and intense peaks in the range of 2883.80cm^{-1} to 3304.53cm^{-1} are indicative of extensive hydrogen bonding networks, which are essential for the solvent's functionality and effectiveness (Smith et al., 2015). Additionally, the strong peaks observed at 1038.28cm^{-1} and 1092.48cm^{-1} for EM1, and similar peaks for EM2 and EM3, further confirm the presence of significant C–O stretching vibrations, which play a crucial role in the solvent's properties (Zhang et al., 2012). The data also aligns with the findings of Abbott et al. (2004), who reported that the interactions within NADES are primarily governed by hydrogen bonding and van der Waals forces. Overall, the FTIR analysis provides valuable insights into the structural characteristics and interactions within the glucose-glycerol-water NADES, highlighting the importance of the molar ratios in determining the properties and potential applications of these solvents.

The study focused on preparing and characterizing Natural Deep Eutectic Solvents (NADES) composed of glucose and glycerol mixed with water (40% w/w) in various molar ratios: EM1 (1:1), EM2 (1:2), and EM3 (1:3). The FTIR analysis of these NADES mixtures revealed significant peaks corresponding to functional groups such as O–H, C–H, and C–O, which are indicative of interactions between glucose and glycerol. The results demonstrate that the molar ratio of glucose to glycerol substantially influences the structural properties and interactions within the NADES. The presence of broad and intense peaks in the O–H stretching region, particularly as the glycerol content increases, suggests enhanced hydrogen bonding networks, which are essential for the solvent's stability and functionality. Peaks associated with C–O stretching further confirm the robust interactions within the mixtures. These findings align with previous studies highlighting the significance of hydrogen bonding and van der Waals forces in NADES.

The insights gained from FTIR analysis underscore the potential of NADES for various applications, particularly in green technology and sustainable chemical processes. NADES have been shown to exhibit clear hydrogen bonding between components (Dai et al., 2013). The ability to tailor the properties of NADES by adjusting the molar ratios of their components opens up opportunities for their use in diverse fields such as biomass valorization, food waste extraction, and bioactive compound recovery (Kalhor & Ghandi, 2019; Ozkan, 2023; González-Laredo et al., 2023). The utilization of NADES in the extraction of phenolic compounds, flavonoids, and bioactives from various sources showcases their versatility and efficiency in extraction processes (Oliva et al., 2024; Vo et al., 2023; Ivanović et al., 2022). Moreover, the formation of NADES through hydrogen bonding interactions between hydrogen bond donors and acceptors highlights their unique properties, including lower melting points and enhanced solubility (Jauregi et al., 2024; Caviglia et al., 2024). Overall, the research on NADES demonstrates their potential as green and sustainable solvents with wide-ranging applications in various industries, highlighting their role in advancing environmentally friendly practices and processes (Wu et al., 2022; Gómez-Urios et al., 2022). The ability to modulate the properties of NADES through FTIR analysis and understand their structural characteristics further enhances their attractiveness for use in green chemistry initiatives and sustainable product development (Dai et al., 2013).

Conclusion

The glucose-glycerol-water NADES exhibit promising characteristics influenced by their molar ratios, which dictate the extent of hydrogen bonding and structural interactions. Future research could explore the practical applications of these solvents in different fields, leveraging their environmentally friendly properties and effectiveness as green solvents.

Author Contributions

SR, HK, and DS conceived and designed the experiment. SR and HN performed the sample collection and preparation. SR and HK prepared the NADES mixtures and conducted the chlorophyll extraction process. DS and HN performed the FTIR-ATR analysis. SR supervised and coordinated the overall experiment and provided expertise in natural deep eutectic solvents. SR, HK, and DS analyzed the data and interpreted the results. SR prepared the draft of the manuscript. All authors critically revised the manuscript and approved the final version.

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