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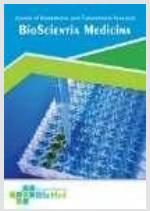

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Novel *Enhalus acoroides* Phytosomes: Formulation, Characterization, and Bioavailability Enhancement

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A B S T R A C T

Background: *Enhalus acoroides* (seagrass) possesses valuable bioactive compounds, including quercetin, with potential therapeutic applications, notably antidiabetic effects. However, the poor solubility and low bioavailability of compounds like quercetin limit their clinical efficacy. Phytosomes, phospholipid-based nanocarriers, represent a promising strategy to overcome these limitations. This study aimed to develop and characterize *E. acoroides* extract-loaded phytosomes to enhance its potential bioavailability. **Methods:** *E. acoroides* was collected, processed, and extracted using ultrasound-assisted extraction (UAE). Total phenolic (TPC) and flavonoid content (TFC) were determined. Phytosomes were prepared using the thin-layer hydration method with varying extract-to-soya lecithin ratios (F1=1:1, F2=1:2, F3=1:3). Characterization involved particle size analysis, zeta potential measurement, Fourier Transform Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM), entrapment efficiency (EE%) determination via HPLC, and *in vitro* dissolution studies. **Results:** The UAE extract yielded TPC of 0.318 ± 0.036 mg GAE/g and TFC of 1.023 ± 0.022 mg QE/g. Phytosome formulation F1 (1:1 ratio) exhibited optimal characteristics: particle size of 276.4 nm, PDI of 0.591, zeta potential of -18.0 mV, EE% of 80.47 ± 2.62%, and a spherical morphology. FTIR confirmed complexation. F1 phytosomes demonstrated significantly enhanced dissolution, releasing 87.13% of the entrapped compound over 12 hours compared to the crude extract. **Conclusion:** *E. acoroides* extract was successfully encapsulated into phytosomes using a thin-layer method. The F1 formulation (1:1 extract:phospholipid ratio) demonstrated favorable physicochemical properties (nanoparticle size, moderate stability, high EE%) and markedly improved in vitro dissolution, suggesting enhanced bioavailability potential for *E. acoroides* phytoconstituents.

1. Introduction

The use of herbal medicine has experienced a global resurgence, fueled by the growing preference for natural therapeutic alternatives perceived to offer fewer adverse effects compared to synthetic drugs. This trend is particularly evident in many cultures, especially within developing nations, where traditional practices heavily rely on plants for maintaining health and treating diseases, often utilizing intricate combinations of bioactive compounds that exhibit synergistic effects. Within this context of traditional remedies, *Enhalus acoroides*, commonly known as tape seagrass or 'lamun' in Indonesia, has attracted considerable interest. This marine plant, found in coastal ecosystems, has a history of traditional use in managing a wide array of health conditions, including diabetes, skin problems, digestive disorders, wounds, and microbial infections. The traditional applications of *Enhalus acoroides* are increasingly being supported by scientific investigations. Research studies have begun to validate several of these traditional uses, with extracts from *E. acoroides* demonstrating

significant biological activities. Notably, previous research has indicated that *E. acoroides* extract, which is rich in the flavonoid quercetin, exhibits superior α-glucosidase inhibitory activity compared to other seagrass species like *Thalassia hemprichii*. This finding suggests a potential mechanism by which *E. acoroides* may contribute to the management of diabetes, aligning with its traditional use. The hypoglycemic effects of *E. acoroides* are attributed to its complex phytochemical composition, which includes phenols, alkaloids, steroids, flavonoids (such as quercetin), terpenes, and tannins. Among these phytochemicals, quercetin stands out due to its recognized potential not only for antidiabetic effects but also for its potent antioxidant and anti-inflammatory properties. These properties make quercetin a compound of significant interest in both nutritional and pharmaceutical research. However, despite the therapeutic promise of *E. acoroides* and its key constituent quercetin, a major obstacle hinders their widespread practical application: poor bioavailability.[1-3]

Quercetin, like many other plant-derived polyphenols, is characterized by low aqueous solubility, poor membrane permeability, and chemical instability. These limitations collectively result in reduced absorption and systemic availability following oral administration, significantly restricting its clinical efficacy. Consequently, there is a pressing need for the development of advanced drug delivery strategies aimed at enhancing the solubility, stability, and absorption of these valuable natural compounds to fully harness their therapeutic potential. Phytosome technology has emerged as a highly promising approach to overcome the bioavailability challenges associated with poorly soluble phytoconstituents. Phytosomes represent a novel class of vesicular nanocarriers formed by complexing plant extracts or individual phytochemicals with phospholipids, typically phosphatidylcholine derived from sources such as soya lecithin. This complexation process distinguishes phytosomes from simple liposomes. While liposomes primarily encapsulate hydrophilic molecules within an aqueous core, phytosomes form a molecular dispersion. In this unique structure, the polar head of the phospholipid interacts with the polar groups of the phytoconstituent through hydrogen bonding, while the lipophilic tails surround the complex. This arrangement creates a more lipid-compatible entity, facilitating the compound's passage across biological membranes and consequently improving its absorption and bioavailability. Phytosomes offer several potential advantages, particularly for delivering hydrophilic compounds that face difficulties in crossing lipid barriers due to their size or poor lipid solubility. Furthermore, phytosomes may provide enhanced stability compared to traditional liposomes, which can be susceptible to degradation. Improving the dissolution rate of poorly soluble drugs is a critical factor in enhancing their oral bioavailability. The dissolution process, which is the rate at which a drug dissolves, is often a determinant of the rate and extent of absorption in the gastrointestinal tract. Techniques that effectively improve in vitro dissolution frequently correlate with improved in vivo absorption. This correlation makes dissolution a key focus in drug development, especially for compounds like quercetin, where poor dissolution is a major limiting factor.[4-7]

Considering the reported low bioavailability of quercetin and the potential of phytosome technology to address this issue, this study was designed to investigate the formulation of *Enhalus acoroides* extract into phytosomes. The primary novelty of this research lies in the specific application of phytosome technology to *Enhalus acoroides* extract. While phytosomes have been established as a valuable approach for enhancing the bioavailability of various plant compounds, the application to *Enhalus acoroides* is relatively unexplored. This study is among the first to systematically formulate, characterize, and evaluate phytosomes derived from this particular marine source. Given that *E. acoroides* contains bioactive compounds like quercetin, known for their potential therapeutic benefits but limited by poor solubility and bioavailability, this research is both

timely and significant. This investigation sought to design, formulate, and evaluate innovative phytosome nanocarriers loaded with *Enhalus acoroides* extract. The overarching aim was to establish a proof-of-concept for improving the delivery and potential efficacy of its poorly soluble phytoconstituents, with a focus on quercetin.[8-10] By successfully encapsulating the extract within phytosomes, the study aimed to enhance the dissolution properties of these compounds, which is a critical step towards improving their absorption and ultimately their therapeutic effectiveness. The successful development of such a delivery system could pave the way for utilizing *Enhalus acoroides* extract in various pharmaceutical and nutraceutical applications, maximizing the benefits of its inherent bioactive compounds.

2. Methods

The entirety of *Enhalus acoroides* (L.f.) Royle, encompassing the roots, stems, and leaves, was procured from the coastal waters in the vicinity of Kalih Island, situated in Banten Bay, within the province of Banten, Indonesia. The precise identification and botanical authentication of the collected plant material were rigorously conducted by the Herbarium Depokensis, a division of the Department of Biology at Universitas Indonesia, located in Depok, West Java, Indonesia; this process was assigned the determination number 1387/UN2.F3.11/PDP.02.00/2023. Soya lecithin, serving as the phospholipid source for the experiments, was acquired from Glentham Life Sciences, based in Corsham, United Kingdom. The quercetin standard (95% purity, suitable for High-Performance Liquid Chromatography) and the Gallic acid monohydrate standard were procured from Sigma-Aldrich, located in St. Louis, MO, USA. A range of chemical reagents was utilized in the study. These included aluminum chloride anhydrous ($AlCl_3$), sodium nitrite ($NaNO_2$), sodium carbonate (Na_2CO_3), sodium hydroxide pellets (NaOH), Folin-Ciocalteu (FC) reagent, potassium bromide (KBr, specifically for Fourier Transform Infrared Spectroscopy), dichloromethane (CH2Cl2), acetonitrile (ACN, suitable for HPLC), and trichloroacetic acid (TCA), all of which were obtained from Merck, Darmstadt, Germany. Ethanol (EtOH, 96%) and hydrochloric acid (HCl, 37%) were supplied by Brataco Chemical, Indonesia. The components necessary for preparing the phosphate buffer solution (PBS), namely sodium phosphate monobasic and dibasic, were of analytical grade. Phosphotungstic acid, employed for staining samples for Transmission Electron Microscopy, was also procured. Double distilled water was consistently used throughout all experimental procedures. Unless explicitly stated otherwise, all chemical compounds and reagents used in this research were of analytical reagent grade or of a higher purity level.

The freshly collected seagrass biomass underwent a meticulous washing process using distilled water. This thorough washing was essential to eliminate any adhering sand particles, epiphytes, and other forms of marine debris that might have been present on the plant material. Following the washing stage, the cleaned plant material was subjected to an air-drying process. The material was spread out and dried under shade until a constant weight was achieved, indicating the removal of all moisture content. The dried seagrass was then processed to reduce its particle size. It was cut into smaller fragments and subsequently pulverized into a coarse powder, referred to as simplicia, using a high-speed hand blender. This size reduction step was crucial as it significantly increases the surface area of the plant material, thereby facilitating more efficient extraction of the desired compounds. The extraction of bioactive compounds from the prepared plant material was carried out using ultrasound-assisted extraction (UAE). UAE is a technique recognized for its effectiveness in extracting bioactive compounds from plant matrices. In this method, 200 grams of the dried *E. acoroides* powder was suspended in a solvent mixture. The solvent system consisted of 70% ethanol (v/v) in water, acidified with 1% hydrochloric acid (v/v). The use of acidified ethanol was specifically intended to enhance the extraction efficiency of phenolic and flavonoid

compounds, which are known to be more soluble in such a medium. The resulting suspension was then subjected to ultrasonication using a Witeg ultrasound sonicator (Wertheim, Germany). The sonicator was operated at a frequency of 40 kHz, a setting considered effective for extraction processes. The power output was set at 400 W. The sonication process was carried out for a duration of 30 minutes, and the temperature of the extraction was carefully controlled at 40°C. Maintaining this controlled temperature was important to prevent potential thermal degradation of sensitive compounds present in the extract. Following the sonication process, the mixture was filtered using Whatman No. 1 filter paper. This filtration step was performed to separate the liquid extract from the solid plant material. The plant material retained after the initial filtration, known as the marc, was subjected to a second extraction process. This re-extraction was performed under the same conditions as the initial extraction to ensure maximum recovery of the target compounds. The filtrates obtained from both the initial and the re-extraction steps were combined. The combined filtrates were then concentrated under reduced pressure using a rotary vacuum evaporator (Heidolph, Schwabach, Germany). The evaporation process was carried out at a temperature of 40°C, and it continued until a thick, viscous extract was obtained. The resulting thick *E. acoroides* extract (EAE) was carefully collected. To prevent degradation, it was wrapped in aluminum foil to protect it from light exposure. The extract was then stored in a desiccator over silica gel at room temperature. The use of a desiccator with silica gel ensured that the extract was kept in a dry environment until it was required for subsequent analyses and phytosome preparation.

The total phenolic content (TPC) of the *E. acoroides* extract (EAE) was determined using the Folin-Ciocalteu (FC) colorimetric method. In this procedure, a 0.5 mL aliquot of the extract, dissolved in methanol at a known concentration, was mixed with 4.5 mL of distilled water in a 25 mL vial. To this mixture, 0.5 mL of Folin-Ciocalteu reagent (previously diluted) was added and thoroughly mixed. Following a brief incubation period, 10 mL of a 7% (w/v) sodium carbonate (Na_2CO_3) solution was added. The mixture was then diluted with 2.5 mL of distilled water to achieve a final volume of 12.5 mL. This mixture was subsequently incubated in the dark at room temperature for 90 minutes. This incubation period allowed for the completion of the color development reaction. The absorbance of the resulting solution was measured at 750 nm using a UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). The measurement was taken against a blank. The blank solution contained all the reagents used in the procedure, but it did not include the extract itself. Gallic acid was used as the standard reference compound for the construction of a calibration curve. This calibration curve was essential for quantifying the phenolic content in the extract. The TPC was expressed as milligrams of Gallic Acid Equivalents per gram of dry extract (mg GAE/g extract). All measurements were performed in triplicate to ensure the reliability and reproducibility of the results.

The Total Flavonoid Content (TFC) in the extract was quantified using the aluminum chloride ($AlCl_3$) colorimetric assay. Quercetin was used as the standard reference compound in this assay. Initially, 1 mL of the extract solution, prepared at a concentration of 1000 ppm in methanol, was placed in a clean vial. To this, 5 mL of absolute methanol and 300 µL (0.3 mL) of a 5% (w/v) sodium nitrite ($NaNO_2$) solution were added. The resulting mixture was allowed to stand at room temperature for 5 minutes. Following this, 600 µL (0.6 mL) of a 10% (w/v) AlCl3 solution (in methanol) was added to the mixture. This new mixture was then incubated for 6 minutes at room temperature. Subsequently, 2 mL of a 1 mM sodium hydroxide (NaOH) solution and 1.10 mL of absolute methanol were added. The reaction mixture was then incubated for 20 minutes at room temperature. This incubation period was necessary to allow for complete color development. The absorbance of the resulting solution was measured at 510 nm using the UV-Vis Spectrophotometer. The measurement was taken against a reagent blank to

ensure accuracy. A calibration curve was prepared using standard solutions of quercetin. This calibration curve was used to determine the flavonoid concentration in the extract. The TFC was expressed as milligrams of Quercetin Equivalents per gram of dry extract (mg QE/g extract). All determinations were conducted in triplicate to ensure the precision of the measurements.

Phytosomes loaded with *E. acoroides* extract (EAE-Phytosomes) were prepared using the thin-layer hydration method. This method is frequently used for the preparation of phytosomes, especially for compounds that are lipophilic. Three different formulations were prepared. These formulations, designated as F1, F2, and F3, varied in the weight ratio of EAE to soya lecithin (phospholipid). The ratios used were 1:1, 1:2, and 1:3, respectively. For each formulation, specific amounts of EAE and soya lecithin were precisely weighed. These weighed amounts were then dissolved in a suitable volume of dichloromethane (CH2Cl2) in a round-bottom flask. Dichloromethane was selected as the solvent because of its effectiveness in dissolving both the EAE constituents, including quercetin, and soya lecithin. Additionally, dichloromethane has a relatively low boiling point, which facilitates its easy removal in subsequent steps. The mixture in the flask was stirred or sonicated briefly. This step ensured the formation of a homogeneous solution. Following this, the organic solvent (dichloromethane) was removed using the rotary vacuum evaporator (Heidolph, Germany). The flask was rotated at a speed of 150 rpm, and the temperature of the water bath was maintained at 40°C under reduced pressure. The evaporation process was continued until a thin, uniform lipid film was formed on the inner wall of the flask. This ensured the complete removal of the dichloromethane. The optimization of the rotation speed at 150 rpm and the evaporation time of 2 hours was aimed at achieving a homogeneous film. After the formation of the film, the flask was purged with dry nitrogen gas. This step was performed to remove any residual solvent and to prevent oxidation of the film. The flask containing the thin film was then sealed and stored in a refrigerator at 4°C for 24 hours. This storage period allowed for film annealing. The hydration of the lipid film was carried out using a phosphate buffer solution (PBS) at a pH of 5.5. The selection of this pH was based on compatibility considerations, potentially related to the stability of quercetin or the intended application of the phytosomes. A specific volume (25 mL) of pre-warmed (37°C) PBS pH 5.5 was added to the flask containing the dried film. The flask was then attached to the rotary evaporator (without vacuum) and rotated at 150 rpm with the water bath set at 37°C. This gentle agitation facilitated the detachment of the lipid film from the flask wall and promoted the spontaneous formation of the phytosome suspension. For the in vitro dissolution studies and potentially for assessing long-term stability, a portion of the freshly prepared phytosome suspension (specifically F1) was subjected to freeze-drying (lyophilization). The freeze-drying process was performed using a LabFreez freeze dryer (China). This process involves freezing the suspension and then removing the water content by sublimation under vacuum. The result is a dry powder that can be easily reconstituted when needed. It is possible that standard cryoprotectants, such as trehalose or mannitol, were added before freezing, although this is not explicitly specified in the source document.

The mean hydrodynamic particle size (Z-average diameter), the polydispersity index (PDI), and the zeta potential (ζ) of the prepared phytosome formulations (F1, F2, and F3) were determined. These measurements were conducted using Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) techniques, respectively, with a Nano Zetasizer instrument (Horiba Scientific, Kyoto, Japan). Prior to the measurements, the phytosome suspensions were appropriately diluted with double-distilled water. This dilution was necessary to avoid multiple scattering effects, which can interfere with the accuracy of the measurements. The diluted samples were then transferred into disposable measurement cells. Cuvettes were used for size and PDI measurements, while specific zeta potential cells were used for zeta

potential measurements. Measurements were typically performed at a controlled temperature of 25°C. The results were recorded as the average of multiple runs to ensure precision. Particle size was recorded in nanometers (nm), PDI was expressed as a dimensionless value, and zeta potential was recorded in millivolts (mV).

Fourier transform infrared spectroscopy (FTIR) spectroscopy was employed to investigate potential interactions between the components of the *E. acoroides* extract (EAE), particularly phytoconstituents like quercetin, and the soya lecithin phospholipid within the phytosome complex. Several samples were analyzed using FTIR. These included the raw EAE, plain soya lecithin, a physical mixture of EAE and soya lecithin (prepared by simple blending), and the prepared phytosome formulations (F1, F2, and F3). For solid samples, the KBr pellet method was used. In this method, a small amount of the sample (extract, lecithin, physical mixture, or freeze-dried phytosomes) was intimately mixed with dry KBr powder (spectroscopic grade) in a ratio of approximately 1:100 (sample:KBr). This mixing was performed using an agate mortar and pestle to achieve a homogeneous mixture. The resulting mixture was then compressed into a thin, transparent pellet using a hydraulic press. The FTIR spectra of the samples were recorded using an FTIR spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The spectra were recorded over a wavenumber range of 4000 to 400 cm^{-1}, with a typical resolution of 4 cm^{-1}. The recorded spectra were analyzed to identify shifts or the disappearance/appearance of characteristic peaks in the phytosome spectra. These observations were made by comparing the phytosome spectra with those of the individual components and the physical mixture. The analysis aimed to infer complex formation and molecular interactions, such as hydrogen bonding, between the extract components and the phospholipid.

The Entrapment Efficiency (EE%) of the phytosomes was determined. This parameter represents the percentage of the initial amount of the marker compound, specifically quercetin, that was successfully incorporated into the phytosome structure. The procedure involved taking 1.0 mL of the freshly prepared phytosome suspension and placing it in a centrifuge tube. This suspension was then subjected to ultracentrifugation at a high speed of 13,000 rpm for 60 minutes at a controlled temperature of 4°C. This process was essential to separate the entrapped quercetin from the unentrapped quercetin. The ultracentrifugation resulted in the sedimentation of the phytosomes, forming a pellet at the bottom of the tube. The supernatant, which contained the unentrapped quercetin, was carefully separated from this pellet. To release the quercetin entrapped within the phytosomes, the pellet was lysed or redispersed in 1.0 mL of methanol. The resulting solution was then filtered through a 0.45 µm membrane filter to remove any particulate matter. The concentration of quercetin in the filtered methanolic solution, representing the entrapped drug, was quantified using a validated High-Performance Liquid Chromatography (HPLC) method. The HPLC system (Shimadzu, Japan) was equipped with a C18 analytical column. An isocratic mobile phase, consisting of a 50:50 (v/v) mixture of 0.3% trichloroacetic acid (TCA) in water and acetonitrile (ACN), was used at a flow rate of 0.9 mL/min. Detection was performed using a UV detector set at 254 nm, which is a common wavelength for quercetin analysis. A calibration curve was constructed using standard solutions of quercetin with known concentrations. This calibration curve was essential for accurately determining the concentration of quercetin in the samples. The EE% was calculated using the formula:

$$EE(\%) = \frac{\text{Amount of Quercetin in Sediment (Entrapped Drug)}}{\text{Total Amount of Quercetin initially added}} \times 100$$

The total amount of quercetin initially added was calculated based on the TFC determined for the EAE and the amount of EAE used in the formulation. All determinations were performed in triplicate for each formulation to ensure the accuracy and reliability of the results.

The morphology and ultrastructure of the optimized phytosome formulation (F1) were visualized using Transmission Electron Microscopy (TEM) (Thermo Fisher Scientific, USA). To prepare the sample for TEM, a drop of the diluted phytosome suspension was placed onto a carbon-coated copper grid. Excess liquid was removed by blotting with filter paper. Negative staining was performed to enhance contrast. This involved adding a drop of 1% phosphotungstic acid (PTA) solution, with the pH adjusted to 6.0, onto the grid. The staining agent was allowed to interact with the sample for a short period (1-2 minutes), after which the excess stain was wicked away. The grid was then allowed to air-dry completely at room temperature. The prepared specimen was examined under the TEM at various magnifications, ranging from 2,000x to 50,000x, using accelerating voltages between 80-120 kV. This examination allowed for the observation of the shape and size distribution of the phytosome vesicles. Digital micrographs were captured for documentation purposes.

The in vitro release profile of quercetin from the optimized phytosome formulation (F1) was evaluated and compared to that of the unformulated EAE. The study was conducted using the USP Dissolution Apparatus II (Paddle method) in accordance with USP 43 guidelines. Phosphate buffer pH 6.8 (900 mL) was used as the dissolution medium to simulate intestinal fluid conditions. The dissolution bowls were maintained at a constant temperature of 37 ± 0.5°C using a water bath, and the paddles were rotated at a constant speed of 100 rpm to ensure adequate mixing and dispersion. An accurately weighed amount of the phytosome formulation (F1) or the plain EAE, equivalent to a specific amount of quercetin (5 mg), was introduced into the dissolution medium. At predetermined time intervals (0.25, 0.5, 0.75, 1, 2, 3, 6, 9, and 12 hours), aliquots (1 mL) of the dissolution medium were withdrawn. To maintain sink conditions and a constant volume, each withdrawn volume was immediately replaced with an equal volume of fresh, pre-warmed (37°C) dissolution medium. The withdrawn samples were filtered and analyzed for quercetin content using UV-Vis Spectrophotometry (Shimadzu, Japan) at the maximum absorption wavelength determined for quercetin in the dissolution medium (measurement performed at λmax). The cumulative percentage of quercetin released at each time point was calculated and plotted against time to generate the release profiles for both the phytosome formulation and the plain extract. The experiment was likely performed in triplicate to ensure the reliability of the data.

Data obtained from quantitative measurements, including TPC, TFC, particle size, zeta potential, EE%, and dissolution, were expressed as mean ± standard deviation (SD) from triplicate experiments. Statistical comparisons, where appropriate, such as those between different formulations or between the release profiles of the phytosome and the extract, would typically involve statistical tests. Common tests used for such comparisons include ANOVA, followed by post-hoc tests or t-tests. However, the specific statistical analyses performed in this study were not detailed in the provided source document. In general, a p-value of less than 0.05 is conventionally considered to indicate statistical significance.

3. Results

Table 1 provides a comprehensive overview of the extraction process and the subsequent phytochemical characterization of the *Enhalus acoroides* extract (EAE). The table begins by detailing the plant material itself, specifying the botanical name (*Enhalus acoroides* (L.f.) Royle) and common names (Tape Seagrass, Lamun). It also clarifies that the whole plant was used for extraction, including the roots, stem, and leaves, sourced from Kalih Island, Banten Bay, Indonesia. Importantly, the authentication of the plant

material is documented, with the Herbarium Depokensis, Universitas Indonesia, providing the identification (No. 1387/UN2.F3.11/PDP.02.00/2023). The table then meticulously outlines the pre-extraction processing steps. This includes repeated washing with distilled water to clean the plant material, air-drying under shade until a constant weight was achieved, and size reduction by cutting the dried material into small pieces followed by grinding into a coarse powder using a high-speed blender. These steps are crucial for preparing the plant material to maximize the efficiency of the extraction process. The extraction method is clearly defined as Ultrasound-Assisted Extraction (UAE). The parameters of the UAE process are thoroughly described, including the amount of dry plant powder used (200 grams), the solvent system (70% ethanol in water acidified with 1% hydrochloric acid), the specific sonication equipment (Witeg Ultrasound Sonicator, Germany), the sonication power (400 W), the sonication time (30 minutes), and the controlled sonication temperature (40 °C). The table also mentions that the extraction process involved an initial extraction followed by filtration and a re-extraction of the marc (the remaining plant material) to ensure maximum recovery of compounds. Post-extraction processing is also detailed, specifying the use of rotary vacuum evaporation (Heidolph, Germany) for concentration at 40 °C, resulting in a thick, viscous extract (EAE). The storage conditions of the final extract are provided, noting that it was wrapped in aluminum foil and stored in a desiccator. Finally, the table presents the phytochemical analysis of the extract. It specifies the methods used to determine the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The TPC was determined using the Folin-Ciocalteu (FC) colorimetric assay, with Gallic Acid as the standard, and the result was expressed as 0.318 ± 0.036 mg GAE / g extract (mean ± standard deviation). The TFC was measured using the Aluminum Chloride ($AlCl_3$) complex-forming assay, with Quercetin as the standard, and the result was 1.023 ± 0.022 mg QE / g extract (mean ± standard deviation).

Table 2 presents a detailed physicochemical characterization of the *Enhalus acoroides* extract (EAE)-loaded phytosomes, focusing on the three distinct formulations (F1, F2, and F3) prepared in this study. The table begins by outlining the formulation parameters. All three formulations were consistently prepared using the thin-layer hydration method, a technique well-suited for creating phytosomes. A key parameter that was varied across the formulations was the weight ratio of EAE to soya lecithin. Formulation F1 employed a 1:1 ratio, Formulation F2 a 1:2 ratio, and Formulation F3 a 1:3 ratio, where the ratios are expressed as grams of EAE to grams of soya lecithin. Dichloromethane (CH_2Cl_2) was used as the solvent for film formation in all cases, and the hydration medium for all formulations was a phosphate buffer solution (PBS) with a pH of 5.5. The table then details the physicochemical characterization of the formulations. Particle size, a critical parameter influencing drug delivery, was assessed using Dynamic Light Scattering (DLS) with a Horiba Nano Zetasizer. The particle sizes, expressed as the Z-average diameter in nanometers (nm), were 276.4 nm for F1, 376.2 nm for F2, and 428 nm for F3. The polydispersity index (PDI), which indicates the width of the particle size distribution, was also determined using DLS. The PDI values were 0.591 for F1, 0.607 for F2, and 0.633 for F3. Zeta potential (ζ), a measure of the surface charge and an indicator of colloidal stability, was measured in millivolts (mV) using Electrophoretic Light Scattering (ELS) with the Horiba Nano Zetasizer. The zeta potential values were -18.0 mV for F1, -17.2 mV for F2, and -23.4 mV for F3. Entrapment Efficiency (EE), expressed as a percentage (%), was determined by HPLC quantification of quercetin in the sediment after ultracentrifugation (13,000 rpm, 60 minutes, 4°C). The EE% values were 80.47 ± 2.62% for F1, 81.05 ± 3.14% for F2, and 83.64 ± 3.52% for F3. The morphology (shape) of the phytosomes was examined using Transmission Electron Microscopy (TEM) with 1% Phosphotungstic Acid stain. Formulation F1 was

observed to have a spherical/vesicular morphology. Molecular interactions between the EAE and soya lecithin were investigated using Fourier Transform Infrared Spectroscopy (FTIR) with the KBr Pellet Method. Complexation was confirmed for all three formulations. Finally, the table includes an overall assessment, noting that Formulation F1 was selected as the optimal formulation based on a combined consideration of its particle size, PDI, EE%, morphology, and dissolution characteristics.

Table 3 presents a comparative in vitro drug release study, contrasting the release profile of EAE, the plain extract, against that of the F1 phytosome formulation. The table initially specifies the details of the dissolution method. The apparatus used for both the EAE and the F1 phytosome was the USP Dissolution Apparatus II, employing the paddle method. The dissolution medium for both was a phosphate buffer, maintained at a pH of 6.8 to simulate intestinal fluid conditions. The volume of the medium in each dissolution vessel was 900 mL, and the temperature was consistently maintained at 37 ± 0.5 °C using a water bath. The paddle speed, which controls the agitation and mixing within the vessels, was set at a rotation speed of 100 rpm for both the extract and the phytosome formulation. The amount of sample introduced into the dissolution medium was an equivalent amount for both, corresponding to approximately 5 mg of the quercetin marker compound. Aliquots of the dissolution medium were withdrawn at identical sampling times for both the EAE and the F1 phytosome, specifically at 0.25, 0.5, 0.75, 1, 2, 3, 6, 9, and 12 hours. The analysis method used to quantify the released quercetin marker was UV-Vis Spectrophotometry, with measurements performed at the maximum absorbance wavelength (λmax) for quercetin. The table then summarizes the drug release results. During the initial release phase, the release pattern within the first hour showed a similar initial rate for both, with the F1 phytosome exhibiting a slightly faster initial rate of approximately 36.11%, compared to approximately 28.53% for the plain extract. The maximum release observed during the study period differed substantially; the plain EAE reached a maximum release of approximately 43.08%, which plateaued after 6 hours, while the F1 phytosome achieved a significantly higher maximum release of approximately 87.13% by 12 hours. The cumulative percentage of drug released at the 9-hour time point was specified only for the F1 phytosome, at approximately 80.27%. The overall release pattern over the 12-hour duration was characterized as poor dissolution for the EAE, with release plateauing after 6 hours, and as sustained and significantly enhanced for the F1 phytosome. Finally, the table offers an interpretation of the observed differences in dissolution. The reason for the difference in dissolution is attributed to the low aqueous solubility of the constituents in the plain extract, while the enhanced solubility and release in the F1 phytosome are attributed to the phospholipid complex and potentially an amorphous state of the drug.

4. Discussion

This research endeavor has successfully illustrated the potential of phytosome technology as a means to enhance the pharmaceutical properties of *Enhalus acoroides* extract (EAE). *Enhalus acoroides*, a marine resource, has garnered interest due to its traditional uses and the presence of scientifically validated bioactive compounds. A primary challenge in harnessing the therapeutic potential of such plant extracts lies in the inherent limitation of poor bioavailability, frequently encountered with potent phytoconstituents like quercetin. This limitation restricts their clinical application, despite demonstrating promising in vitro activities. The strategy of formulating EAE into a phospholipid-based nanocarrier system was employed, with the aim of improving its dissolution characteristics. It is widely recognized that enhanced dissolution is a crucial prerequisite for enhanced absorption and, consequently, improved bioavailability.

Table 1. Extraction details and phytochemical characterization of *Enhalus acoroides* extract (EAE).

Parameter	Details
Plant material	
Botanical name	*Enhalus acoroides* (L.f.) Royle
Common name(s)	Tape Seagrass, Lamun
Plant part used	Whole plant (Roots, Stem, Leaves)
Collection location	Kalih Island, Banten Bay, Indonesia
Authentication	Herbarium Depokensis, Universitas Indonesia (No. 1387/UN2.F3.11/PDP.02.00/2023)
Pre-extraction processing	
Cleaning	Repeated washing with distilled water
Drying	Air-dried under shade until constant weight
Size reduction	Cut into small pieces, ground into coarse powder using a high-speed blender
Extraction method	**Ultrasound-Assisted Extraction (UAE)**
Plant material amount	200 grams (dry powder)
Solvent system	70% Ethanol (v/v) in water, acidified with 1% Hydrochloric Acid (HCl)
Sonication equipment	Witeg Ultrasound Sonicator (Germany)
Sonication power	400 W
Sonication frequency	Not specified (typically 20-40 kHz range used for UAE)
Sonication time	30 minutes
Sonication temperature	40 °C
Extraction steps	Initial extraction followed by filtration and re-extraction of the marc
Post-extraction processing	
Concentration method	Rotary Vacuum Evaporation (Heidolph, Germany)
Concentration temperature	40 °C
Final product	Thick, viscous extract (EAE)
Storage	Wrapped in aluminum foil, stored in a desiccator
Phytochemical analysis	
Total phenolic content (TPC) method	Folin-Ciocalteu (FC) colorimetric assay
TPC standard	Gallic Acid
TPC result (Mean ± SD)	**0.318 ± 0.036 mg GAE / g extract**
Total flavonoid content (TFC) method	Aluminum Chloride ($AlCl_3$) complex-forming assay
TFC standard	Quercetin
TFC result (Mean ± SD)	**1.023 ± 0.022 mg QE / g extract**

Notes: EAE = *Enhalus acoroides* Extract; GAE = Gallic Acid Equivalents; QE = Quercetin Equivalents; SD = Standard Deviation; UAE = Ultrasound-Assisted Extraction.

Table 2. Formulation and physicochemical characterization of *Enhalus acoroides* extract (EAE)-Loaded Phytosomes.

Parameter	Method / Details	Formulation F1	Formulation F2	Formulation F3
Formulation				
Preparation method	Thin-Layer Hydration	Applicable	Applicable	Applicable
EAE : Soya Lecithin Ratio (w/w)	Grams of EAE : Grams of Soya Lecithin	**1 : 1**	**1 : 2**	**1 : 3**
Solvent for film formation	Dichloromethane (CH2Cl2)	Applicable	Applicable	Applicable
Hydration Medium	Phosphate Buffer Solution (PBS), pH 5.5	Applicable	Applicable	Applicable
Physicochemical characterization				
Particle size (Z-Average, nm)	Dynamic Light Scattering (DLS) - Horiba Nano Zetasizer	**276.4**	376.2	428
Polydispersity index (PDI)	Dynamic Light Scattering (DLS)	**0.591**	0.607	0.633
Zeta potential (ζ, mV)	Electrophoretic Light Scattering (ELS) - Horiba Nano Zetasizer	**-18.0**	-17.2	-23.4
Entrapment efficiency (EE, %)	HPLC quantification of Quercetin in sediment after ultracentrifugation (13,000 rpm, 60 min, 4°C)	**80.47 ± 2.62**	81.05 ± 3.14	83.64 ± 3.52
Morphology (Shape)	Transmission Electron Microscopy (TEM) - 1% Phosphotungstic Acid stain	**Spherical / Vesicular**	Not Analyzed	Not Analyzed
Molecular interaction	Fourier Transform Infrared Spectroscopy (FTIR) - KBr Pellet Method	Complexation Confirmed	Complexation Confirmed	Complexation Confirmed
Overall assessment	Based on combined characteristics (Size, PDI, EE%, Morphology, Dissolution)	**Selected as Optimal Formulation**	-	-

Notes: Abbreviations: EAE = *Enhalus acoroides* Extract; w/w = weight by weight; nm = nanometers; PDI = Polydispersity Index; mV = millivolts; EE = Entrapment Efficiency; SD = Standard Deviation; DLS = Dynamic Light Scattering; ELS = Electrophoretic Light Scattering; HPLC = High-Performance Liquid Chromatography; TEM = Transmission Electron Microscopy; FTIR = Fourier Transform Infrared Spectroscopy; KBr = Potassium Bromide; PBS = Phosphate Buffer Solution.

Table 3. Comparative in vitro drug release study of EAE vs. F1 Phytosom.

Parameter	Specification / Details	EAE (Plain Extract)	F1 Phytosome
Dissolution method details			
Apparatus	USP Dissolution Apparatus II (Paddle Method)	Applicable	Applicable
Dissolution medium	Phosphate Buffer	pH 6.8	pH 6.8
Medium volume	900 mL	Applicable	Applicable
Temperature	Maintained using a water bath	37 ± 0.5 °C	37 ± 0.5 °C
Paddle speed	Rotation speed for mixing	100 rpm	100 rpm
Sample amount	Equivalent amount added to media (approx. 5 mg of Quercetin marker implied)	Equivalent Amount	Equivalent Amount
Sampling times	Aliquots withdrawn over time	0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12 hours	0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12 hours
Analysis method	Quantification of released Quercetin marker	UV-Vis Spectrophotometry (at λmax)	UV-Vis Spectrophotometry (at λmax)
Drug release results summary			
Initial release phase	Release pattern during the first hour(s)	Similar initial rate (~28.53%)	Slightly faster initial rate (~36.11%)
Maximum release (%)	Highest percentage of drug released during the study	**~ 43.08%** (Reached at 6 hours)	**~ 87.13%** (Reached by 12 hours)
Release at 9 hours (%)	Cumulative percentage released at 9 hours	Not specified	**~ 80.27%**
Release pattern	Overall profile observed over 12 hours	Poor dissolution, plateaued after 6 hrs	**Sustained & Significantly Enhanced**
Interpretation	Reason for difference in dissolution	Low aqueous solubility of constituents	Enhanced solubility/release due to phospholipid complex & potentially amorphous state

Notes: Abbreviations: EAE = *Enhalus acoroides* Extract; F1 = Phytosome formulation with 1:1 EAE: Soya Lecithin ratio; USP = United States Pharmacopeia; rpm = revolutions per minute; UV-Vis = Ultraviolet-Visible; λmax = Wavelength of maximum absorbance.

The significance of addressing poor bioavailability cannot be overstated in the context of drug delivery. Many natural compounds, while exhibiting remarkable therapeutic potential in laboratory settings, fail to translate those benefits effectively in vivo. This discrepancy arises largely from their limited ability to be absorbed and distributed within the body. Factors contributing to poor bioavailability include low aqueous solubility, which hinders the compound's ability to dissolve in the gastrointestinal tract, poor membrane permeability, which impedes its passage across biological barriers like the intestinal epithelium and chemical instability, leading to degradation before reaching the target site. Therefore, the development of strategies to overcome these limitations is of paramount importance in maximizing the therapeutic efficacy of natural products. Phytosome technology offers a distinct advantage in this regard. By complexing plant extracts or individual phytochemicals with phospholipids, phytosomes create a more biocompatible and bioavailable form of the active compounds. This complexation not only enhances the compound's solubility but also facilitates its passage across cell membranes, leading to improved absorption and systemic delivery. The implications of this technology extend beyond simply increasing the amount of drug absorbed, it can also potentially improve the compound's stability, reduce its metabolism, and target its delivery to specific tissues or cells, thereby enhancing its overall therapeutic effectiveness.[11-13]

The extraction of *E. acoroides* using ultrasound-assisted extraction (UAE) with acidified ethanol proved to be an effective method for obtaining an extract rich in both phenolic and flavonoid compounds. The extract yielded a Total Phenolic Content (TPC) of 0.318 mg GAE/g and a Total Flavonoid Content (TFC) of 1.023 mg QE/g. Ultrasound-assisted extraction is

recognized as a green and efficient technique for the extraction of bioactive compounds from plant materials. This technique often yields higher amounts of desired compounds compared to conventional extraction methods, such as maceration. The enhanced extraction efficiency of UAE can be attributed to several factors. The application of ultrasound generates cavitation, which involves the formation and collapse of bubbles in the liquid solvent. This process disrupts plant cell walls, facilitating greater penetration of the solvent into the plant matrix and improving the release of intracellular compounds. Additionally, ultrasound can enhance mass transfer rates, leading to faster and more complete extraction. The use of acidified ethanol as the solvent system in this study was a strategic choice. Ethanol is a polar solvent that is effective in extracting a wide range of compounds, including phenolics and flavonoids. The addition of hydrochloric acid to the ethanol further enhances the extraction of these compounds. Acidification helps to solubilize phenolic compounds by converting their salts into free acids, making them more readily extractable into the solvent. This approach is particularly beneficial for extracting compounds that may be bound to cell wall components or present in complex matrices. The TPC and TFC values obtained in this study are significant because phenolic and flavonoid compounds are known to be largely responsible for the antioxidant and potential antidiabetic activities attributed to *E. acoroides*. These compounds possess the ability to scavenge free radicals, reduce oxidative stress, and modulate various biochemical pathways relevant to glucose metabolism. The levels of phenolics and flavonoids extracted from *E. acoroides* in this study are indicative of the plant's potential as a source of valuable bioactive compounds for therapeutic applications. Furthermore, the fact that the TPC and TFC values obtained were comparable to, or in some cases higher than, those reported in other studies using different solvents or methods on *E. acoroides*, highlights the importance of optimizing extraction parameters to maximize the yield of desired compounds.[14-17]

The study's core focus involved the preparation and characterization of EAE-loaded phytosomes using the thin-layer hydration method. This method was selected due to its suitability for encapsulating lipophilic or poorly water-soluble compounds. Additionally, the thin-layer hydration method is relatively simple and reproducible, making it a practical choice for preparing phytosomes. The process of preparing phytosomes using the thin-layer hydration method involves several key steps. First, the plant extract and phospholipids are dissolved in an organic solvent. The solvent is then evaporated, leaving behind a thin film of the extract-phospholipid mixture on the wall of a round-bottom flask. This thin film is subsequently hydrated with an aqueous solution, leading to the self-assembly of phytosomes. The phospholipids, typically phosphatidylcholine, arrange themselves with their hydrophilic heads facing outward towards the aqueous environment and their hydrophobic tails interacting with the lipophilic plant extract components. This structure effectively encapsulates the plant extract within a lipid-compatible environment, enhancing its solubility and permeability. In this study, three different formulations were prepared, varying the ratio of EAE to soya lecithin (1:1, 1:2, and 1:3). This variation in the extract-to-phospholipid ratio allowed for the investigation of its impact on the physicochemical properties of the resulting phytosomes. A comprehensive characterization of the prepared phytosomes was performed to understand their properties and predict their behavior in biological systems. This characterization included the assessment of particle size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE%), morphology, and molecular interactions between the extract and the phospholipid.[18-20]

5. Conclusion

This research has successfully demonstrated the feasibility of formulating *Enhalus acoroides* extract into phytosomes using the thin-layer hydration

method. The resultant phytosomes exhibited desirable physicochemical properties, including nanosized particles, favorable zeta potential, and high entrapment efficiency of the extract. Notably, the phytosome formulation F1 (1:1 extract-to-phospholipid ratio) showed a significantly enhanced dissolution profile compared to the unformulated crude extract. This enhancement in dissolution is attributed to the unique phytosome structure, which improves the solubility and permeability of the extract's bioactive constituents. The findings of this study suggest that phytosome technology holds considerable promise for improving the bioavailability and therapeutic potential of *Enhalus acoroides* extract, particularly for compounds like quercetin that are known to have poor solubility and limited absorption. Further research, including in vivo studies, is warranted to validate these in vitro results and to explore the full therapeutic potential of *Enhalus acoroides* phytosomes.

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