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RESEARCH ARTICLE

Potential transformation of seagrass (*Syringodium isoetifolium*) into a bioactive food ingredient using different extraction techniques

[version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: *Syringodium isoetifolium* is a seagrass that grows abundantly in Indonesian territorial waters and has been known to be of high significance not only for the seawater ecosystem, but also for human beings (as food, nutritional and pharmaceutical products). In this study, the bioactive constituent of *Syringodium isoetifolium* was extracted using several different techniques to recover a maximum yield.

Methods: Extraction was carried out by conventional and non-conventional (Microwave-assisted extraction, ultrasound-assisted extraction-bath system, and ultrasound-assisted extraction- (UAE) probe system) techniques with green solvents (water, 50% ethanol, and 100% ethanol).

Results: As a result, 50% ethanol and water extracts exhibited a significantly higher yield. Total phenol content was significantly higher for 50% ethanol extract. Different extraction techniques (using 50% ethanol solvent) showed that the UAE-probe was the best technique since it yielded the highest total phenol (17.37 ± 2.16 mg GAE/g) and the richest bioactive compounds (Choline, betaine, 3,5-di-tert-butyl-4-hydroxybenzoic acid, 7-Hydroxycoumarine, 4-Methoxycinnamic acid, Zearalenone, Caffeic acid, Levalbuterol, Phloretin, Dihydrocaffeic acid, Quercetin-3β-D-glucoside and Quercetin). Interestingly, choline was the most abundant compound in the extract obtained with different extraction techniques. In this *in silico* assay, choline from seagrass extract was shown as an anti-inflammatory. The interaction pathway of the choline compound with receptors (TNF-α, IL-1β, and IL6) had a higher binding affinity value than the inhibitor-receptor interaction

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(i.e. -3.4, -3.0, and -2.8 kcal/mol). The cytotoxicity test on TIG-1 cells showed that the extract did not have a toxic effect on them.
Conclusions: These findings support the potential use of *Syringodium isoetifolium* as a bioactive food ingredient.

Keywords
Extraction technique, Phenolic compounds, Phytochemical, Syringodium isoetifolium, Ultrasound

This article is included in the Agriculture, Food and Nutrition gateway.

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Introduction

Indonesia's vast coastline is a habitat for seagrass, which grows to form seagrass meadows. *Syringodium isoetifolium, Thalassia hemprichii, Cymodocea rotundata, Halophila ovalis, Enhalus acoroides, Cymodocea serrulata, Halodule pinifolia,* and *Halodule uninervis* are species that are often found in seagrass beds in Indonesian waters (Kiswara *et al.*, 2009; van Katwijk *et al.*, 2011). The species *S. isoetifolium, Cymodocea rotundata, and Cymodocea serrulata* have a growth period that tends to be faster (Kiswara *et al.*, 2009). Recently, Li *et al.* (2021) succeeded in seeding *E. acoroides* for the restoration of seagrass beds in the tropics. Therefore, seagrass is a marine plant that has the potential to be produced on a large scale. However, the high production potential of seagrass has not been followed by its postharvest utilization to produce high economic value products. So far, seagrass beds have been allowed to grow wild and play an important role not only in coral reef ecosystem and maintain the sustainability of the marine biota, but also in maintaining other marine ecosystem's balance (van Katwijk *et al.*, 2011).

Only the leaves of seagrass that has optimal growth can be harvested so that it does not cause the plant's death. In addition, there is no need to replant after harvesting seagrass leaves. Seagrass leaves have a dark green color and a strong aroma, and are rich phytochemical compounds such as phenolics. Phenolic compounds are molecules with high diversity formed by the attachment of one or more –OH groups to the phenyl ring. Phenolics are divided into several classes including flavonoids and other benzopyran derivatives, phenolic acids and ester derivatives, quinones, lignins, lignans, tannins, stilbenes, curcuminoids, chalcones, and essential oils (Chiorcea-Paquim *et al.*, 2020).

As seagrass has a deep green color and a quite strong aroma, it is predicted that it is rich in phytochemical compounds such as phenolics. A previous study by Rengasamy *et al.*, 2019 reported that *S. isoetifolium* has a potential as a natural antioxidant and clinical enzyme inhibitor that can be applied in pharmaceutical cosmeceutical industries. Moreover, *S. isoetifolium* could also be a good candidate as a food complement, feed, and biomedical field due to its rich biochemical profile (Bharatharathna & Santhanam, 2019).

The extraction technique plays an important role in extracting nutrient content and phytochemical constituents. Efficient extraction techniques that are fast, inexpensive, economical in materials and solvents, high-yield, environmentally friendly, and do not damage the structure and functional properties of the target compounds, were chosen. Conventional extraction is usually carried out by maceration, which involves immersing the material with a solvent at a certain temperature for a certain time with agitation assistance (Abd Aziz *et al.*, 2021); meanwhile, modern extraction techniques are carried out using microwave-assisted extraction (MAE) and ultrasonics. The principle of MAE is to use energy from microwaves to heat a mixture of sample material and extraction solvent, which is induced by microwave irradiation (Lefebvre *et al.*, 2021). Microwave power is strongly absorbed by the interior of plant cells due to their water content. The pressure on the cell wall is generated by swelling of the plant cell when water evaporates, which pushes the cell wall from within to stretch and eventually rupture. This event can release metabolites from the broken cells into the surrounding solvent (Bachtler & Bart, 2021). On the other hand, the principle of UAE is to use energy from ultrasonic waves. At a certain frequency and amplitude, it creates cavitation bubbles, which when they reach an unstable point, then release high temperatures and pressures by the blasting process. This phenomenon can damage cell walls and promote the release of metabolites from the plant (Lefebvre *et al.*, 2021). These different extraction techniques affect the quantity and bioactivity of the target compounds that are crucial to study.

To our knowledge, a recent study by Kalaivani *et al.* (2021) has reported the biological activities such as antibacterial, antifungal, antimicrobial, antifouling, and anticancer properties of *S. isoetifolium.* This research was conducted to develop the potential use of *S. isoetifolium* as a food bioactive contituents. To get the best phytochemical and nutrient compounds from *S. isoetifolium*, four different techniques, namely maceration, MAE, and UAE-bath (UAE with bath) and UAE-probe system were applied. Meanwhile, in this study, the phenol extract was analyzed and phytochemical compounds were screened by liquid chromatography–high resolution mass spectrometry (LC-HRMS). While the potential development is proven by *in silico* study and cell toxicity.

Methods

Materials

All experiments were carried out at Universitas Brawijaya, Indonesia. Fresh *S. isoetifolium* was harvested from Kondang Merak Beach, East Java, Indonesia (-8.3964160388196N, 112.51923979683157E) and then directly washed with fresh water to remove attached dirt and salt particles. Afterward, the seagrass was dried using an oven at 40°C to a constant weight (13.87 $\pm$ 0.20% moisture) and then with a blender. The dried powder (297.00 μm) was used for extraction. Chemicals used for extraction and analysis comprised 2,2,1-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3), gallic acid, Aquadest, and absolute ethanol, from Meck, Singapore. All chemicals used were analytical grade except water and ethanol for LC-HRMS analysis, which were HPLC-grade.

Production of *Syringodium isoetifolium* extracts

Preparation

Water, ethanol 50%, and ethanol 100% were applied for extraction with different techniques. So far, they have been considered green solvents because they have been proven safe (non-toxic) for either phytochemical extraction or food usage (Ahmad *et al.*, 2021; Molino *et al.*, 2018). In addition, green solvents have a positive environmental impact due to their lower eco-toxicity and are biodegradable due to a strong solvency (Ahmad *et al.*, 2021). Also, these solvents are highly efficient to completely extract the molecules in various polarities (Farooq *et al.*, 2021; Harscoat-Schiavo *et al.*, 2021; Lao *et al.*, 2017).

Extraction process

Seagrass powder in each extraction solvent (*viz.* water, ethanol 50%, and ethanol 100%;1:20 w/v) was extracted with different techniques, *i.e.* MAE (Anton Paar - Multiwave Pro), UAE-Probe (Branson 250/450), UAE-Bath (Soltec - Sonica 2400 EP Ultrasonic cleaner) and conventional maceration (Waterbath shaker – Memmert WNB 45). MAE followed the Albuquerque *et al.* (2017) method with a slight modification in extraction time. MAE settings were: 50°C, stirrer at high speed, a 5 min duration, and factory set (Maximum microwave power 700 W; Maximum pressure 18.0 bar; Maximum pressure increase rate 0.5 bar/s). UAE-Bath followed the optimum method by Albuquerque *et al.*, 2017) with the following equipment settings: off-temperature control, 60 min duration, and factory set (40 kHz frequency, 260 W peak ultrasonic power). UAE-Probe followed (Albuquerque *et al.*, 2017) method by modifying the maximum time. UAE-Probe settings were: 70% amplitude, off-temperature control, 5 min duration, continuous duty cycle, and factory set (19-20 kHz horn frequency, 400 W kinetic energy). Conventional based on the optimum method by (Albuquerque *et al.*, 2017), extraction was processed in a water bath shaker, agitated at medium speed, at 30°C for 1440 min. Each treatment was done in triplicate.

Dry extract generated

The extracted mixture was filtered with filter paper and then centrifugated at 3000 rpm, 25°C, for 5 minutes to separate the solute and the material residue. The extract solution was dried with a vacuum oven at 40°C to achieve constant weight. The water, 50% ethanol, and 100% ethanol dry extracts were yielded for each extraction technique. The dry extracts were then stored at -20°C for the next analysis. The yield of dry extracts was calculated using Equation ((1) (Aboulghazi *et al.*, 2022).

$$\text{Yield}\,(\%) = \frac{\text{Weight of dry extract}\,(\text{g})}{\text{Weight of initial dried sample}\,(\text{g})} \times 100 \quad (1)$$

Total phenolic content

Analysis of total phenolic content followed a Folin-Ciocalteau method (Lim *et al.*, 2019). Briefly, 30 µl of Folin-Ciocalteu reagent (1.0 N) and 60 µL of diluted samples were mixed in a 96-well microplate, then incubated for 5 min. Afterward, 150 µL of 20% sodium carbonate solution was added and incubated in a dark place at room temperature for 40 min. The absorbance of the supernatant was measured at 730 nm using Microplate Reader after centrifugation for 8 min (1600×*g*). The total phenolic content of the samples was calculated with a standard curve using gallic acid (mg GAE/g w.b.).

Identification of bioactive compounds by LC-HRMS

Identification of phenolic compounds contained in 50% ethanol extract of *S. isoetifolium* using different extraction techniques was carried out using a non-targeted screening method (Susilo *et al.*, 2020) by dissolving 100 µL of seagrass extract in 1400 µLof water and ethanol depending on the solvent used for extraction. Samples were filtered with a 0.22 m minisart RC and then injected into the LC-HRMS apparatus. The 10 µL samples were processed automatically by using a hypersil gold aQ 50 mm × 1 mm × 1.9 µm (length x diameter x particle size) column with positive polarity conditions, flow rate 40 L/min, oven column temperature 30°C with elution gradient as follows: 0–2 min 5% B, 15–22 min 60% - 95% B, stabilized at 95% B for 3 min then dropped to 5% B for 5 min. The chromatogram data generated from the injection process was analyzed using Compound Discoverer 3.2 software based on the Mzcloud online library.

In silico assay of choline bioactivity

Ligand and protein preparation

The ligands used in this study consisted of choline compounds and inhibitors of each protein. All ligands were obtained from the PubChem web server in Sybil Data Files (SDF) format. The ligands were converted into a 3D structure in Protein

Data Bank (PDB) format with BIOVIA Discovery Studio 2019. Choline is known to have anti-inflammatory activity, and in this study, it was hoped that it could bind to TNF-α, IL-1β, and IL6, which function as pro-inflammatory agents. The 3D structures of TNF-α (2AZ5), IL-1β (1ITB), and IL6 (1P9M) were obtained from the RCSB PDB web server. The receptors were then prepared to remove water molecules and ligands with BIOVIA Discovery Studio 2019.

Molecular docking analysis and ligand-receptor interactions

Ligand-receptor interactions were analyzed by molecular docking using AutoDock Vina integrated in PyRx version 0.9.5. The molecular docking process was carried out using a specific docking method based on the active site of each receptor (Boulanger *et al.*, 2003; Halim *et al.*, 2015; He *et al.*, 2005; Zia *et al.*, 2020). Docking results, bond positions, and amino acid residues formed between the ligand-receptors were analyzed using the 2019 BIOVIA Discovery Studio.

Molecular dynamic simulation

Molecular dynamic (MD) was used as a simulation method to analyze the physical movements of atoms and molecules. The Yet Another Scientific Artificial Reality Application (YASARA) has been used for MD simulations. This simulation was carried out to compare the interaction of ligands and inhibitors in binding to receptors. The parameters in the simulation correspond to the physiological conditions of the cell at 37°C, 1 atm, pH 7.4, and 0.9% salt content for 50 ns with autosave every 25 ps. The simulation was run by the md_run macro program, and the results were displayed by the md_analyze and md_analyzebindenergy programs (Bagheri & Fatemi, 2018; Deeba *et al.*, 2017; Krieger & Vriend, 2015).

Cytotoxicity test with MTT method

In this study, the MTT assay based on Miasih *et al.* (2022) was applied with slight modification. The extract of *S. isoetifolium* was treated in TIG-1 cells. Finally, the absorbance was measured using a microplate reader at a wavelength of 595 nm. The results were used to construct a graph of cell viability percentage against extract concentrations.

Statistical analysis

The quantitative data served as average values ± standard deviation. Analysis of variance (ANOVA) used a Nested design with Minitab 18 software. Furthermore, the Tukey test with $p < 0.05$ revealed a significant difference.

Results and discussion

Yield

Using 100% ethanol, 50% ethanol, and water as solvents aims to extract phytochemical compounds with high polarity to low polarity. The extraction results are presented in Table 1, the water and 50% ethanol solvent produced the highest yield

Table 1. Effect of different extraction techniques with green solvents on the yield and total phenolic content of *Syringodium isoetifolium* extract.

Technique	Green solvent	Yield (%)	Total phenolic (mg GAE/g w.b.)
Conventional	Water	7.55 ± 0.15 a	1.97 ± 0.11 e
	Ethanol 50%	8.40 ± 0.70 a	5.39 ± 0.67 cd
	Ethanol 100%	2.55 ± 0.45 b	-
MAE	Water	7.47 ± 0.70 a	2.62 ± 0.07 e
	Ethanol 50%	8.20 ± 0.60 a	11.29 ± 1.18 b
	Ethanol 100%	1.60 ± 0.26 b	-
UAE-Bath	Water	7.27 ± 0.75 a	2.59 ± 0.23 e
	Ethanol 50%	8.53 ± 0.15 a	7.49 ± 0.43 c
	Ethanol 100%	2.63 ± 0.31 b	-
UAE-Probe	Water	8.00 ± 0.62 a	2.92 ± 0.12 de
	Ethanol 50%	7.50 ± 0.52 a	17.37 ± 2.16 a
	Ethanol 100%	1.30 ± 0.26 b	-

Note: (-) = not detected, and average values are followed by different letters in each column indicating a significant difference ($p < 0.05$).

while 100% ethanol solvent produced a significant yield for each extraction technique. It was expected, due to the phytochemical compounds (such as dihydrocaffeic acid, quercetin, caffeic acid, levalbuterol, phloretin, and zearalenone; see in Table 3) of *S. isoetifolium* having high polarity. In other plants (*Blepharis linariifolia Pers., Guiera senegalensis JF Gmel.* and *Limnophila aromatica*), 50% ethanol solvent also showed a higher yield than 100% ethanol, 75% ethanol, water, acetone, and dichloromethane (Dirar *et al.*, 2019; Do *et al.*, 2014). Many studies have stated the advantages of non-conventional extraction methods compared to conventional ones, namely high yields (Ameer *et al.*, 2017). However, the yield of *S. isoetifolium* extraction with various non-conventional and conventional techniques showed no significant difference. The extraction yields depends on the extraction time (Đurović *et al.*, 2018).

Total phenolic content

Phenolics are molecules with high diversity defined by the attachment of one or more –OH groups to the phenyl ring. Phenolics are divided into several classes including flavonoids and other benzopyran derivatives, phenolic acids and ester derivatives, quinones, lignins, lignans, tannins, stilbenes, curcuminoids, chalcones, and essential oils (Chiorcea-Paquim *et al.*, 2020). For each extraction technique, extraction of *S. isoetifolium* with 50% ethanol as a solvent produced the highest total phenol, meanwhile, with 100% ethanol solvent, phenol could not be obtained (Table 1). The polarity of the phenolic compounds is medium so extraction with 50% ethanol in many plants (*Moringa oleifera* L. leaves, rambutan peel, *Cyperus rotundus* L., and pomegranate) yielded the highest total phenol compared to extraction with 100% ethanol, 80% ethanol, 70% ethanol , water, acetone, and dichloromethane (Dirar *et al.*, 2019; Harscoat-Schiavo *et al.*, 2021; Phuong *et al.*, 2020; Wu *et al.*, 2020). The total phenol content in *S. isoetifolium* extract was influenced by the extraction technique used. UAE-probe yielded a significantly higher total phenol (17.37 ± 2.16 mg GAE/g) compared to UAE-bath, MAE, and conventional (Table 1). Đurović *et al.* (2018) reported that the total phenol content of yellow soybean seeds extracts, which was extracted using ultrasound-probe, was not different from that of ultrasound-bath and MAE. This is due to the different samples extracted, the structure of yellow soybean seeds being harder than *S. isoetifolium* leaves, as well as the different processing conditions of the ultrasound-probe (20 kHz, 30% of maximum amplitude, 10 min duration, the temperature was held constant at 25 ± 1°C) and UAE-probe (50/60 kHz, 70% amplitude, 5 min, at room temperature and the temperature was increased but not held).

Identification of bioactive compounds

Phenolic compounds are non-polymeric phytochemical compounds that present bioactivity as antioxidants. Their main structure is an aromatic ring or phenol unit containing at least one hydroxyl group (Akyol *et al.*, 2016; Lao *et al.*, 2017; Yang *et al.*, 2018). The complex phenolic structure causes a wide variety of molecular weights, and there are about 10,000 different phenolic derivatives (Chiorcea-Paquim *et al.*, 2020). Caffeic acid, quercetin, coumarin, vanillic acid, benzoic acid, cinnamic acids, phloretin, vanillin, and benzopyran derivatives are examples of phenolic compounds (Akyol *et al.*, 2016; Chiorcea-Paquim *et al.*, 2020; Xiong *et al.*, 2019). In this study, the extraction of *S. isoetifolium* with 50% ethanol produced the highest total phenol; therefore the use 50% ethanol for each extraction technique identified its phenolic compounds content. As can be seen in Table 2, UAE-probe yielded the most phenolic compounds (10 types), including 3,5-di-tert-Butyl-4-hydroxybenzoic acid, 7-hydroxycoumarine, 4-methoxycinnamic acid, zearalenone, caffeic acid, levalbuterol, phloretin, dihydrocaffeic acid, quercetin-3β-D-glucoside, and quercetin. Meanwhile UAE-bath, MAE, and conventional extraction yielded fewer phenolic compounds: 5, 5, and 6 types, respectively. Specifically, zearalenone, caffeic acid, phloretin, dihydrocaffeic acid, and quercetin were detected only by UAE-probe. The advantages of the UAE for the extraction of phenolic compounds have also been proven in other plants. The yield of ellagic acid and quercetin extraction from rambutan peel with UAE was significantly higher than conventional extraction (Phuong *et al.*, 2020). Extraction yield of quercetin, gallic acid, vicenin-2, p-Hydroxybenzoic acid, orientin, rutin, hyperoside, kaempferol-3-O-rutinoside, isorhamnetin 3-O-glucoside, rosmarinic acid, apigenin, and kaempferol on *Moringa oleifera* L. Leaves with UAE was higher than with conventional extaction (maceration and stirring-assisted extraction) (Wu *et al.*, 2020). Interestingly, choline was abundantly present in all extraction techniques, especially with UAE-probe. Choline is an essential nutrient that plays an important role for neurotransmitter synthesis (acetylcholine), cell-membrane signaling (phospholipids), lipid transport (lipoproteins), and methyl-group metabolism (homocysteine reduction). Moreover, choline is also required to make the phospholipids phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, and sphingomyelin – essential components for all membranes (Zeisel & da Costa, 2009). Betaine also presents in all extraction techniques, except MAE. According to findings from a previous study by Cho *et al.* (2006) and Detopoulou *et al.* (2008), a combination of dietary intake rich in choline and betaine are associated with lower concentrations of all of the inflammatory markers, including C-reactive protein (CRP), homocysteine, interleukin-6, and tumor necrosis factor.

In silico assay of choline bioactivity

Ligand-receptor interactions

Molecular docking is used to determine the interaction between the receptor and the ligand, indicated by the binding affinity value and the amino acid residues involved (Figure 1). Since the presence of choline is abundant, this study used a

Table 2. Identification of bioactive compounds on *Syringodium isoetifolium* extracts from the different extraction techniques.

Bioactive compounds	Formula	Molecular weight (g/mol)	Retention time (min)	Area (Max.)			
				Conventional	MAE	UAE-Bath	UAE-Probe
Choline	$C_5H_{13}NO$	103.10	0.97	5,681,769,957.18	1,993,336,001.52	5,722,177,008.90	3,994,678,105.68
Betaine	$C_5H_{11}NO_2$	85.05	0.88	344,214,182.61	-	3,663,629,437.98	1,327,942,848.69
3,5-di-tert-butyl-4-hydroxybenzoic acid	$C_{15}H_{22}O_3$	250.16	15.58	19,435,054.29	192,482,978.00	192,406,362.92	203,105,033.75
7-Hydroxycoumarine	$C_9H_6O_3$	162.03	15.53	50,589,614.18	51,295,922.08	67 966 766.40	26,516,672.75
4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	178.06	21.19	18,070,168.60	27,356,643.60	19,611,271.36	17,730,545.83
Zearalenone	$C_{18}H_{22}O_5$	300.13	18.75	-	-	-	165,649,696.48
Caffeic acid	$C_9H_8O_4$	180.04	7.29	-	-	-	156,428,174.34
Cirsimaritin	$C_{17}H_{14}O_6$	314.08	13.32	-	-	41,062,662.46	-
Vanillin	$C_8H_8O_3$	152.05	7.61	30,078,430.10	8,901,899.48	-	-
Levalbuterol	$C_{13}H_{21}NO_3$	261.14	18.21	-	22,208,426.74	-	28,041,597.92
Phloretin	$C_{15}H_{14}O_5$	274.08	8.81	-	-	-	18,247,889.49
Dihydrocaffeic acid	$C_9H_{10}O_4$	164.05	14.29	-	-	-	17,703,918.51
Shogaol	$C_{17}H_{24}O_3$	276.17	13.82	16,843,826.02	-	10,665,694.43	-
Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.09	7.61	13,153,256.73	-	-	15,943,406.62
Quercetin	$C_{15}H_{10}O_7$	302.04	7.61	-	-	-	7,730,951.51

Note: (-) = not detected.

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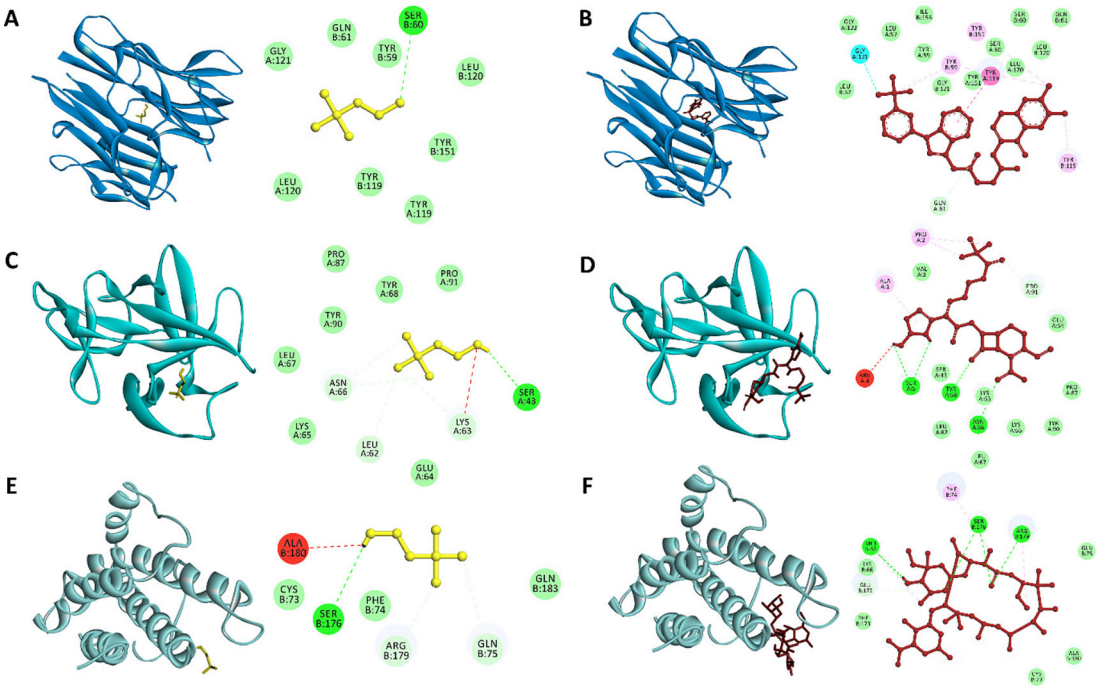


Figure 1. Ligand-receptor interaction and amino acid residues involved. (A) Choline-TNF-α, (B) Inhibitor-TNF-α, (C) Choline-IL-1β, (D) Inhibitor-IL-1β, (E) Choline-IL6, and (F) Inhibitor-IL6. Choline and inhibitors are presented as yellow and red sticks, respectively. TNF-α, IL-1β, and IL6 are presented as ribbon structures with the different blue color.

ligand consisting of choline compounds and inhibitors from each protein. The inhibitor of each protein is a compound used as a control, to compare the strength of the interaction between choline-receptors and inhibitor-receptors. Molecular docking results show that the interaction of choline compounds with receptors (TNF-α, IL-1β, and IL6) had a higher binding affinity value than the inhibitor-receptor interaction, namely -3.4, -3.0, and -2.8 kcal/mol. The binding affinity between the inhibitor and TNF-α was the highest compared to the other inhibitor-receptors, with a value of -9.1 kcal/mol. The higher the binding affinity value, the weaker the bond between the ligand-receptor (Table 3).

Table 3. Molecular docking and amino acid residues on ligand-receptor interactions.

Interaction			Binding affinity (kcal/mol)	Amino acid residues	
Ligand	**CID**	**Receptor**		**Hydrogen interaction**	**Hydrophobic interaction**
Choline	305	TNF-α	-3.4	**Ser60**	**Tyr59**, **Gln61**, **Tyr119**, **Leu120**, **Gly121**, **Tyr151**
		IL-1β	-3.0	Ser43	**Leu62**, **Lys63**, **Glu64**, **Lys65**, **Asn66**, **Leu67**, **Tyr68**, **Pro87**, **Tyr90**, **Pro91**
		IL6	-2.8	**Ser176**	**Cys73**, **Phe74**, **Gln75**, **Arg179**, **Ala180**, Gln183
Inhibitor	5327044	TNF-α	-9.1		Leu57, **Tyr59**, **Ser60**, **Gln61**, **Tyr119**, **Leu120**, **Gly121**, Gly122, **Tyr151**, Ile155
	139595263	IL-1β	-6.3	Ser5, **Asn66**, **Tyr68**	Ala1, Pro2, Val3, Arg4, Ser43, **Leu62**, **Lys63**, **Glu64**, **Lys65**, **Leu67**, **Pro87**, **Tyr90**, **Pro91**
	447043	IL6	-5.6	Met67, **Ser176**, **Arg179**	Lys66, **Cys73**, **Phe74**, **Gln75**, Glu172, Phe173, **Ala180**

Note: The bolded amino acid residues show equal interaction between the ligand and the control at the receptor.

Molecular docking results were then visualized to determine the binding site of the ligand and receptor. Visualization with Discovery Studio showed that the choline compound had the same binding site as the inhibitor on each protein. The presence of the same binding site indicates that the choline compound can have the same function as the control, even though it has a relatively high binding affinity value. The same binding site between choline compounds and inhibitors in each protein was indicated by several amino acid residues that interact through hydrogen and hydrophobic interactions. Tyr59, Ser60, Gln61, Tyr119, Leu120, Gly121, and Tyr151 are seven amino acid residues involved in the interaction of choline-TNF-α and inhibitor-TNF-α. However, there was a slight difference: Ser60 in the choline-TNF-α reaction interacts through hydrogen bonds, whereas in the inhibitor-TNF-α reaction, it interacts through hydrophobic bonds. Tyr119 is an amino acid residue that plays an essential role in ligand-receptor interactions (Zia *et al.*, 2020).

Leu62, Lys63, Glu64, Lys65, Asn66, Leu67, Tyr68, Pro87, Tyr90, and Pro91 are 10 amino acid residues involved in the interaction between choline-IL-1β and inhibitor-IL-1β. Asn66 and Tyr68 are two amino acid residues that interact through hydrogen bonds in the inhibitor-IL-1β interaction while interacting through hydrophobic bonds in the choline-IL-1β interaction. Ser176 is an amino acid residue that interacts via hydrogen bonds in the reaction of choline-IL6 and inhibitor-IL6. Four other amino acid residues involved in the reaction of the two ligands with IL6 interact via hydrophobic bonds, namely Cys73, Phe74, Gln75, and Ala180. Amino acid residues involved in the ligand-receptor interaction complex through hydrogen bonds and hydrophobic bonds can increase the affinity of the ligand bonds in proteins (Chen *et al.*, 2016). It is known that the formed hydrophobic interactions in ligand-receptor complex play an important role in stabilizing the ligand-protein bond and helping to increase the affinity of the ligand bond with protein (Varma *et al.*, 2010).

Molecular dynamic simulation results

The stability of the ligand complex with each pro-inflammatory protein is known based on molecular dynamic (MD) simulations. As seen in Figure 2, the parameters used in this simulation were the RMSD of the ligand-receptor complex, RMSF, and the number of hydrogen bonds of the ligand-receptor complex. MD analysis showed that the choline-TNF-α complex and the inhibitor-TNF-α had the same stability up to 20 ns simulation, indicated by an RMSD value of 3 Å. The stability of the choline-TNF-α complex fluctuated with RMSD values, reaching 3.5 Å at several simulation times. Unlike the inhibitor-TNF-α complex, which experienced relatively high fluctuations during the simulation time of around 29 ns with an RMSD value of 4 Å, the simulation stabilized again after 30 ns with an RMSD value of less than 3 Å. The simulation of the choline-IL-1β complex was very unstable, as indicated by very high fluctuations and an average RMSD value of more than 4 Å. These results are inversely proportional to the stable inhibitor-IL-1β complex from start to end of the simulation, indicated by the average RMSD value <3 Å. The choline-IL6 and

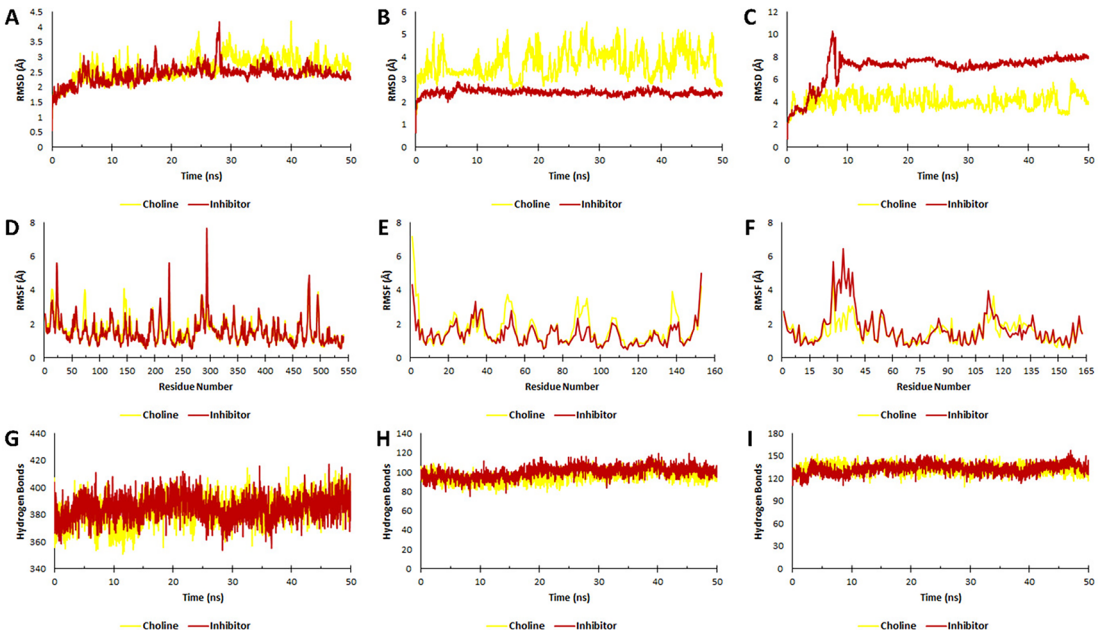


Figure 2. Molecular dynamic simulation. During simulation, the stability of the ligand-protein interaction complex is indicated by the RMSD value. RMSF value is based on fluctuations in the amino acid residues and the hydrogen bond numbers. A, D, G. Ligands-TNF-α complex. B, E, H. Ligands-IL-1β complex. C, F, I. Ligands-IL6 complex.

inhibitor-IL6 complex was unstable compared to other interactions, indicated by an RMSD value of >3 Å. However, when paying attention to the choline-IL6 complex, it had a higher level of stability than the IL6-inhibitor, indicated by a lower RMSD value. Previous studies have shown that the ligand-receptor complex, which is stable during simulation, has an RMSD value of 3 Å (Martínez, 2015; Wargasetia *et al.*, 2021).

The fluctuations of the amino acid residues formed can also determine the stability of the ligand-receptor complex. The choline-TNF-α and inhibitor-TNF-α complexes are the most stable compared to other ligand-receptor complexes. This is indicated by fluctuations in amino acid residues that are not too high compared to the others. It is known that the more amino acid residues that experience fluctuation, the more unstable the ligand-receptor complex (Azminah *et al.*, 2019). The number of hydrogen bonds in the choline-TNF-α and inhibitor-TNF-α complexes were the highest compared to the other complexes. A large number of hydrogen bonds makes the two ligand-receptor complexes the most stable compared to other ligand-receptor complexes.

Cytotoxicity test on TIG-1 cells

A toxicity test was conducted to evaluate the effect of the extract on the viability of TIG-1 cells. TIG-1 cell viability was determined by the MTT-assay method, using mitochondrial dehydrogenase activity in living cells against 3[4,5-dimethylthiazole-2-yl]-2,5-diphenyltertrazolium bromide (MTT) into insoluble purple formazan crystals. The MTT test requires the addition of a solvent (DMSO) to dissolve the formed insoluble formazan product.

The data obtained were the absorbance values of purple formazan crystals measured at a wavelength of 595 nm, which were then converted to determine the percentage of living cells. The number of viable cells was measured by colorimetry and the quantity of formazan product was directly proportional to the number of live cells in the culture (McCauley *et al.*, 2013).

The results of the toxicity test (Figure 3) showed a decrease in the viability of TIG-1 cells in line with the increase in the concentration of the extract, but not significant. Exposing the lowest extract concentration of 12.5 µg/mL showed no decrease in TIG-1 cell viability compared to control cells, which was 96.1%. Meanwhile, exposing the highest extract concentration of 800 µg/mL showed a decrease in cell viability of 78.4%. TIG-1 cell viability in the range of extract concentrations of 25-400 µg/mL did not show a significant difference in the range of 84.5-92.5%. The results of the extract toxicity test on TIG-1 cells showed that the extract did not have a toxic effect on TIG-1 cells.

Referring to international guidelines such as those of the International Organization for Standardization (ISO 10993-5:2009) it has been shown that if the viability is reduced to <70% of the control, then the test substance has the potential for cytotoxicity. Based on these guidelines, giving the extract to TIG-1 cells with the highest dose has a viability value above 70% which indicates that the extract has no cytotoxic potential on normal cells.

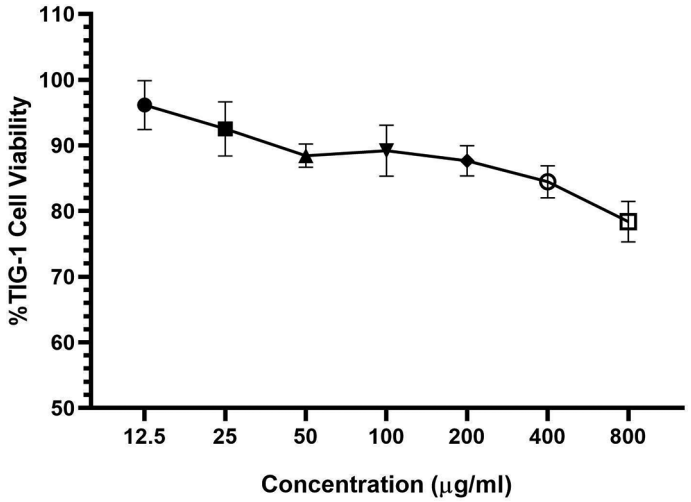


Figure 3. Effect of *Syringodium isoetifolium* extract on the profile of TIG-1 cell viability.

An *in vitro* cytotoxicity test was used to evaluate the toxicity of extracts following international guidelines such as those by the International Organization for Standardization (ISO 10993-5:2009). This test can be used to determine the structure–activity relationship of the extract used, as well as determine the initial concentration for further research. Inhibition of cell death, cell growth, or cell proliferation are important parameters used to evaluate the cytotoxicity of an extract (Aydin *et al.*, 2016).

Conclusions

Bioactive constituents of *Syringodium isoetifolium* can be extracted efficiently using the UAE-probe method with 50% ethanol; in this study, it resulted in both the highest yields and total phenol, as well as the richest bioactive constituents. Moreover, choline was the most dominant compound in extracts yielded by different extraction techniques. Since choline was an abundant compound, the *in silico* assay was done using this compound, and resulted in choline having the ability to be anti-inflammatory. Furthermore, based on a cytotoxicity test, the *S. isoetifolium* extract by UAE-Probe with 50% ethanol did not have a toxic effect on TIG-1 cells. Hence, *Syringodium isoetifolium* extract had a high potential as a bioactive food ingredient.

Data availability

Underlying data

Figshare: Experimental Data: Potency Development of Seagrass (Syringodium isoetifolium) as Food Bioactive Constituents by Different Extraction Techniques, https://doi.org/10.6084/m9.figshare.21780674.v7 (Susilo *et al.*, 2022).

This project contains the following underlying data:

- Experimental Data (yield, total phenolic, bioactive compounds & cytotoxicity).xlsx
- RCSB and Protein DataBank accession numbers of molecular docking.docx

Extended data

Figshare: Experimental Data: Potency Development of Seagrass (Syringodium isoetifolium) as Food Bioactive Constituents by Different Extraction Techniques, https://doi.org/10.6084/m9.figshare.21780674.v7 (Susilo *et al.*, 2022).

This project contains the following extended data:

- Instruments.xlsx
- Minitab Project - ANOVA OF YIELD DATA.MPJ.BAK
- Minitab Project - ANOVA of Yield Data.MPJ
- Minitab Project - ANOVA OF TOTAL PHENOLIC DATA.MPJ.BAK
- Minitab Project - ANOVA of Total Phenolic Data.MPJ
- GraphPad Prism - Graphic of TIG-1 cell viability profile.pzfx
- Table. Molecular docking and amino acid residues on ligand-receptor interactions.docx
- Figure. Molecular dynamic simulation.png
- Figure. Ligand-receptor interaction and amino acid residues involved.png

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

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✓ **Larry Chañi**
Universidad Nacional Autonoma Altoandina de Tarma (Ringgold ID: 486233), Tarma, Junin, Peru

The manuscript "Potential transformation of seagrass (*Syringodium isoetifolium*) into a bioactive food ingredient using different extraction techniques" presents interesting and updated scientific information about seagrass, it is a promising source of bioactive with potential application for the industry. The manuscript adequately describes the methods and results of the application of emerging technologies for the extraction of bioactive compounds from seagrass. It also details the quantification of total phenolic compounds and the identification of phenolics by LC-HRMS. The most abundant component was choline, this compound shows anti-inflammatory properties with potential for the pharmaceutical industry.

Minor comments:

The manuscript requires minor corrections, which are detailed below:

Improve the keywords, do not use very general words or words used in the title.

I recommend changing “Extraction technique” and “Phytochemical” for the word’s “Green extraction methods” and “Marine plant phytochemicals”, respectively. Use the scientific name as a keyword as long as it is not used in the title. Other keyword alternatives are to include the name of the extraction methods.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Extraction of bioactives by emerging green methods, especially by supercritical fluids.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 18 May 2024

Bambang Susilo

Dear Reviewer,
Thank you kindly for your minor comments. Here with Author(s) response addressed for your comments and recommendations:
1. Keywords: The keywords are improved into Choline., Cytotoxicity., Marine plant., Microwave., Ultrasound.
2. Changing “Extraction technique” and “Phytochemical” words: Author(s) approve changing the “Extraction technique” and “Phytochemical” words into the “Green extraction methods” and “Marine plant phytochemicals” words, respectively, for changes the appropriate words in the entire manuscript.

Competing Interests: No potential competing interest was disclosed by the author(s).

Reviewer Report 07 February 2024

https://doi.org/10.5256/f1000research.141336.r238828

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?

Carlos Eduardo Barão
Federal Institute of Paraná (IFPR), Paranavaí, Brazil

Dear Authors

The article is interesting, but some points need to be improved
Abstract: Name the bioactive compound of interest.

Keywords: Use words other than the title.

Extraction process: The conditions, even based on the work of Albuquerque (2017), seem random and do not offer similar conditions in each treatment to compare the results and define which would be better.

Identification of bioactive compounds by LC-HRMS: the type of solvent and concentration used in extraction can offer different profiles of phenolic compounds. It would be interesting to identify all extracts and not just the one that provides the highest yield.

The authors state that the extraction time influences the yield, so the same time should have been used in all conditions.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Emerging technologies Extraction Processes

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Feb 2024

Bambang Susilo

Dear Reviewer,

Thank you very much for your comments and suggestions. Here Author would like to respond to your comments.

1. Abstract: Name the bioactive compound of interest. --> Choline and phenolic are the bioactive compounds of interest.
2. Keywords: Use words other than the title. --> Thank you. Other relevant keywords i.e. extract yield, bioactive compounds, and solvents are added as replacements.
3. Extraction process: The conditions, even based on the work of Albuquerque (2017), seem random and do not offer similar conditions in each treatment to compare the results and define which would be better. --> Our research does not compare Albuquerque's (2017) method with our method so we do not present data comparisons. However, we apply Albuquerque's (2017) method by making some modifications have need to be constructed, based on the conditions of the tools we used in the research laboratory.
4. Identification of bioactive compounds by LC-HRMS: the type of solvent and concentration used in extraction can offer different profiles of phenolic compounds. It would be interesting to identify all extracts and not just the one that provides the highest yield. --> We do agree. However, based on the LC-HRMS results the dominant compounds detected are very good and relevant bioactive compounds as mentioned in Table 2. In addition, the highest yield extract is needed as the bioactive food ingredient, since even though it contains bioactive compounds, if the yield is low, it is less efficient as the bioactive food ingredient candidate. Accordingly, other extracts (by different solvents and concentrations) whose yield is not the highest do not need to be identified by LC-HRMS.
5. The authors state that the extraction time influences the yield, so the same time should have been used in all conditions. --> Although the extraction time is different. However, all extraction techniques have used the optimum time based on citations from the previous research.

Competing Interests: There are no competing interests in this research

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