Metabolomic Profiling, *In Vitro* Antioxidant and Cytotoxicity Properties of *Caulerpa racemosa* : Functional Food of the Future from Algae

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Research Article

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Abstract

Marine macroalgae are nutraceuticals rich in nutritional profile and secondary bioactive metabolites. However, they have varied nutritional and biochemical qualities due to a variety of reasons. This study aimed to determine the phytochemical profile and biological activities of *Caulerpa racemosa*, edible green algae also known as Sea grapes. The study successfully identified secondary metabolites through metabolomic profiling untargeted by LC-HRMS as well as a bioactive peptide. In addition, antioxidant activity and cytotoxicity of extracts and compounds were determined. A total of 103 metabolites were identified in *C. racemosa* extract obtained by the maceration, while 48 were detected in the soxhlet extract. A peptide with the sequence ELWKTF (Glu-Leu-Trp-Lys-Thr-Phe; $C_{41}H_{58}N_8O$) and its abundance was identified in the α-chymotrypsin hydrolysate of *C. racemosa*. In the antioxidant activity test, Soxhletated-extract (ES) and purified fraction 1 (PF1) had half-maximal effective concentration (EC_{50}) < EC_{50} of control/GSH (DPPH inhibition) and PF1 had EC_{50} < EC_{50} of control/Trolox (ABTS inhibition). The cytotoxicity results showed that macerated-extract (EM), ES, and PF1 as antioxidant agents in the observed EC_{50} were safe. In general, *C. racemosa* contains antioxidant nutrients, metabolites, and bioactive peptides, a factor that makes it a promising functional food and pharmaceutical.

1 Introduction

Marine life is a rich natural source of numerous bioactive compounds. Marine organisms exist in various complex habitats with extreme circumstances, and these biochemically and ecologically significant differences provide a wide variety of specific, potent, and novel compounds that are not yet extensively explored [1,2]. Among these organisms, marine macroalgae are currently recognized as 'superfoods' because of their superior nutritional profile and abundance of bioactive secondary metabolites. They are rich in carbohydrates, proteins, unsaturated fatty acids, a complete set of vitamins, and estimated minerals 10 – 100 higher than terrestrial vegetables due to their bioabsorption and bioaccumulative properties [2,3]. The global harvest of macroalgae in 2013 was estimated at $ 6.7 billion, with more than 95% produced in mariculture countries, making Indonesia one of the top producers [4]. Indonesia has around of its territory as sea and is well known as one of the mega-diversity areas in the world, with more than 555 macroalgal species reported from its waters [5]. Furthermore, most of the islands of Indonesia are located within the Coral Triangle, which has been identified as an area with a high diversity of *Caulerpa*, a genus of green algae [4].

Caulerpa racemosa or Sea grapes is one of the green seaweeds that grow naturally in the waters of Indonesia, found in coral reef areas or sand-rubble substrates. It is traditionally used as a fresh vegetable; however, its consumption range is still limited to fishermen or communities in the coastal region [6]. *C. racemosa* is famous for its high nutritional content, which includes minerals, dietary fibers, rich polyunsaturated fatty acids, secondary metabolites such as phenolics, alkaloids, polysaccharides, flavonoids that act as bioactive compounds, and many more [7,8]. Studies have shown that these bioactive molecules are behind a diverse range of health benefits, including antioxidant, anticancer, antibacterial, antiobesity, and antidyslipidemic properties [9,10]. In addition, *C. racemosa* is also rich in macro and micro minerals, including Mg, Ca, K, Na, Fe, Cu, and Zn, which are needed to sustain metabolic processes. Due to these beneficial findings, *C. racemosa* has been considered a potentially valuable functional food, with tremendous development prospects due to its distinctive taste and color [11].

To further incorporate these bioactive molecules into ideal formulations with enormous health and economic potential, these compounds must first be separated by extraction, analysis, and identification [12]. Classical maceration and Soxhlet extraction are the most popular techniques among conventional extraction methods. Maceration is an easy and low-cost method of extracting bioactive compounds because it uses non-complicated utensils with barely any operator skills [13–15]. The plant source is ground to increase the surface area and then mixed with chosen solvents, followed by periodic shaking to increase diffusion. This method is suitable for thermolabile plant materials, water as a solvent, extended exposure to the menstruum, and the need for large final volume products [12,16]. Meanwhile, Soxhlet extraction, known as continuous hot extraction, is carried out by repeatedly washing the matrix with a warm solvent, allowing higher possible solubilization of the compounds [17,18]. Advantages of Soxhlet extraction include that large amounts of drugs can be extracted with a lower amount of solvent than maceration, no filtration is required, and a high amount of heat can be applied. However, this method is labor-intensive and unsuitable for thermolabile sources [19]. Furthermore, hydrolysis extraction methods use protease enzymes (such as the enzyme α-chymotrypsin) which are usually used to extract a bioactive peptide content in foodstuffs [20].

However, it should also be noted that natural populations of *C. racemosa* tend to have varying nutritional and biochemical properties due to several environmental factors such as sedimentation, salinity, temperature, pollution, and nutrients; therefore, different geographical growing fields can contribute to varying levels of nutrients and secondary metabolites [9]. Despite its abundance, the exploration, identification, and isolation of Indonesia's *C. racemosa*-specific bioactive molecules profiling, bioactive peptides, and their direct activities are still minimal. A compelling approach to conduct this metabolomics identification is liquid chromatography coupled with high-resolution mass spectrometry (HPLC-ESI-HRMS/MS), which is increasingly used in metabolomics, allowing comprehensive analysis of phytochemicals and semiautomatic collection of study samples [21]. Therefore, this research aims to identify bioactive molecules of *C. racemosa* by metabolomic profiling, bioactive peptides by proteomics, and examine its antioxidant potentials and cytotoxicity *in vitro* MTT assays on normal cell lines to ensure its safety. This research is part of foodomics (a comprehensive study involving genomics, proteomics, metabolomics, nutrigenomics, and chemogenomics of food) and their interactions with humans, which is currently a trend in food and health research [22,23].

2 Materials And Methods

The sample collection has been approved by the local authorities and the owner of the Sea grapes pond. Fresh Sea grapes (*Caulerpa racemosa*) were collected from the Sea grapes cultivation pond in Jepara Regency, Central Java Province, Indonesia (6°35'12.5"S latitude 110°38'36.0"E longitude). Botanical identification and authentication were confirmed in the Integrated Laboratory of the Faculty of Sciences and Technology (Herbarium Laboratory), UIN Sunan

Kalijaga, Yogyakarta-55281, Indonesia, conducted by Dian Aruni Kumalawati, M.Sc and then followed by confirmation by biologist Prof. Dr. Trina Ekawati Tallei (Expert and Professor of Biology), and has complied with National Center for Biotechnology Information (NCBI) Taxonomy ID 76317 (Eukaryota/Viridiplantae/Chlorophyta/Ulvophyceae/Bryopsidales/Caulerpaceae/Caulerpa). Specimens were collected for future reference. Researchers (authors) state and confirm that all methods carried out in this study are in line or in accordance with relevant guidelines and regulations of *in vitro* and algae study.

2.1. Sea Grapes Extract Preparations

Sea grapes *(C. racemosa)* were thoroughly washed so that the dirt attached to the sea grapes becomes lost and clean. Washed Sea grapes were then twisted and dried in an oven (Memmert Incubator IN55) at a temperature of 60 °C for 3 x 24 hours. Sea grapes (whole-body) were dried, cut into small pieces, and then mashed with a blender to obtain Sea grapes simplicia powder (*C. racemosa*). Dried simplicia was mashed and then extracted using two methods: the hot and cold ways. Maceration represents the cold way, while Soxhlet extraction represents the way of heat.

2.1.1. Maceration Extraction Method

A total of 1,000 g of simplicia powder Sea grapes *(C. racemosa)* were put in a dark bottle, then 96% ethanol solvent (C H OH; Merck) as much as 2 L with a ratio of 1:2 between simplicia and solvent were mixed and soaked for 3 x 24 hours. Every 1 x 24 hours, the acquired filtrate was occasionally stirred, then filtered with Whatman 41 paper, and the residue was re-macerated with a new 96% ethanol solvent. The extracted sample was concentrated using a rotary evaporator (RV 8 IKA) under low pressure (100 millibars) for 90 minutes and re-evaporated in the oven (Memmert Incubator IN55) at a temperature of 40 °C so that a thick extract of Sea grapes were obtained. The extract was stored in the refrigerator at a temperature of 10 °C until used in research.

2.1.2. Soxhlet Extraction Method

Fifty grams of Sea grapes simplicia powder *(C. racemosa)* was wrapped in filter paper and inserted into a Soxhlet tube (thimble) on installed Soxhlet tools (PYREX® Soxhlet extractor). 96% ethanol solvent (C_2H_5OH; Merck) along with 250 ml of the solution were divided into two parts; 150 ml was inserted into the Soxhlet gourd (pumpkin round base), and 100 ml was inserted into the Soxhlet tube to moisten the simplicia. The ratio between simplicia and solvent was 1:5. The Soxhlet extraction process was carried out at a temperature of 70 – 80 °C, and the extraction was carried out for up to 3 repeat cycles. The extract was stored in the refrigerator at a temperature of 10 °C until used in research.

2.1.3. Hydrolysis Extraction Method by α-Chymotrypsin for Bioactive Peptide Measurements

C. racemosa simplicia was dissolved in a 1.0 mM phosphate buffer and then hydrolyzed with the α-chymotrypsin enzyme under its optimal conditions, referring to [20], which utilizes 1,200 U/mg enzyme activity, 37 °C temperature, at 8.0 pH with digestion time of 2 hours, substrate concentration of 20 mg/mL, 4.0 E/S (w/w) (%). The reaction was stopped by heating at 95 °C for 15 minutes, and the hydrolysate protein was centrifuged at 16,000 rpm for 10 minutes at 4 °C. The supernatant protein (PS) was lyophilized and stored at a temperature of -20 °C for further use in the analysis of the bioactive profiling of peptides.

2.2. Metabolomic Profiling Extract from Maceration and Soxhlet Methods

The untargeted metabolomics profiling test on Sea grapes extract samples (Maceration; Soxhlet Method) was carried out using the Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS) method at the Laboratorium Sentral Ilmu Hayati (LSIH; ISO 9001:2008 and ISO 17025:2005; Central Laboratory of Life Sciences; Brawijaya University, Malang-65145, Indonesia) testing services, with the test number 041/LSIH-UB/LK/II/2022.

2.2.1. Analysis of the Maceration and Soxhlet samples by HPLC-ESI-HRMS/MS

Fifty (50) µl of extract samples (Maceration; Soxhlet Method) were diluted using 96% ethanol up to a final volume of 1,500 µl. The solutions were vortexed at 2,000 rpm for 2 minutes and then span-down at 6,000 rpm for 2 minutes. The supernatant was taken and then filtered using a 0.22µm syringe filter and injected into the vial. The sample in the vial was ready to be inserted into an autosampler and then injected into LC-HRMS (Liquid Chromatography High-Resolution Mass Spectrometry). LC-HRMS uses High-Performance Liquid Chromatography (HPLC) Thermo Scientific Dionex Ultimate 3000 RSLC nano with microflow meter. Solvents A and B consist of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The analytical column uses Hypersil GOLD aQ 50 x 1 mm x 1.9 µ particle size with a flow rate of 40 uL/min, a flow gradient run time of 30 minutes, and a column oven with a temperature of 30 °C. High-Resolution Mass Spectrometer using Thermo Scientific Q Exactive with a full scan at 70,000 resolution, data-dependent MS/MS at 17,500 resolution, and run time of 30 minutes, with both positive and negative mode.

2.2.2. Processing Data Software

Annotated or detected compounds were automatically identified via mzCloud MS/MS Library (Thermo Scientific Q Exactive Software), which were performed by Midia Lestari Wahyu Handayani, S.TP, M.Sc., MP., Ph.D., a certified laboratory technician at the Sentral Ilmu Hayati Laboratorium (LSIH; ISO 9001:2008 and ISO 17025:2005; Central Laboratory of Life Sciences; Brawijaya University, Malang-65145, Indonesia).

2.3. Proteomics Assay of Sequence and Molecular Weight of Amino Acids

2.3.1. Ultrafiltration and Reversed-Phase HPLC (RP-HPLC)

The hydrolyzed protein (PS) of *C. racemosa* was divided into 3 peptide fractions based on molecular weight ((F1; < 3 kDa), (F2; 3–10 kDa), and (F3; > 10 kDa)) using ultrafiltration membrane of 10 kDa and 3 kDa. This approach referred to [20], which showed that peptide fraction of F1 (MW < 3 kDa) at 4 mg/mL

had better antioxidant activity than F2 (3 – 10 kDa) dan F3 (MW > 3 kDa). Peptides with lower molecular weight are more active than those with a high molecular weight [20].

2.3.2. Separation of fraction F1 by RP-HPLC

Fraction 1 (F1; < 3 kDa) was purified using RP-HPLC. 40 mg of peptide fraction was dissolved into 1 mL 0.05% TFA (v/v) and then was filtered using micropores membrane (0.22 m) before being inserted into Agilent ZORBAX SB-C18 (5 m, 9.4 × 150 mm). A binary moving phase system was used in this study along with eluent A (0,1% TFA (v/v)) and moving phase B (ACN, 0.05% (v/v) TFA). The solution was eluted with a linear gradient of 0 – 40% moving phase B from 0 – 40 minutes dan 40% moving phase B from 40 – 55 minutes. All fractions were collected and lyophilized for further activity assays. The purification level of fractions with the highest activity was further analyzed using the Sunfire C18 column (5 m, 4,6 mm × 150 mm; Waters, USA). The column was eluted using a linear gradient of 0 – 20% moving phase B from 0 – 20 minutes and 20% moving phase B from 20 – 25 minutes, resulting in a purified fraction 1 (PF1).

2.3.3. Analysis of Amino Acid Sequence and MW

The ELWKTF (Glu-Leu-Trp-Lys-Thr-Phe) peptide was synthesized in the Laboratory of Biochemistry and Biomolecular Medicine, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. A protein sequencer from Applied Biosystems 494 (ProciseTM 494 N-terminal sequencer; Applied Biosystems Inc, Foster City, CA, USA) was used to examine the amino acid sequence of the purified fraction (PF1) based on time-of-flight quadrupole mass spectrometer (MS/MS) paired with electrospray ionization (ESI) source to determines the molecular weight of the ELWKTF (Glu-Leu-Trp-Lys-Thr-Phe). Analysis of the sequence and molecular weight of ELWKTF was performed according to the method described by Zhang *et al.* (2019) [24]. The results of research by Xiaoqian Zhang et al. 2019 showed good activity of ELWKTF (Glu-Leu-Trp-Lys-Thr-Phe) against radical scavenging activity (DPPH and ABTS) [20].

2.4. DPPH Antioxidant Radical Scavenging Activity Assay

The percentage (%) of the inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured using a method referring to Kaur *et al.*, (2021) [25] and Permatasari *et al.*, (2022) [26], on all samples which include a macerated extract (EM), soxhlet extract (ES), and purified fraction 1 (PF1), while glutathione (GSH; 354102, Sigma-Aldrich) was used as a positive control. In the testing vial (at a concentration of 1, 2, 3, 4, 5 µg, an aliquot (100 µL) of samples and control was added, followed by a DPPH reagent addition (3 mL). The DPPH-extract combination that resulted was then left undisturbed (30 min; dark cycle). The samples were read at 517 nm absorbance with a UV-Vis Shimadzu 80 spectrophotometer. To ensure the validity of the data results, each sample was checked three times (n = 3). Inhibition of DPPH was expressed as a percentage and is determined according to the formula below:

$$\%\ \textbf{DPPH Inhibition} = \frac{\mathbf{A0-A1}}{\mathbf{A0}}\ \mathbf{x}\ 100\%$$

A0 = Absorbance of blank; A1 = Absorbance of standard or sample.

The half-elimination ratio (EC_{50}) was used to express the radical scavenging capacity of EM, ES, PF1, and GSH and defined as the concentration of a sample that caused a 50% decrease in the initial radical concentration.

2.5. ABTS Radical Scavenging Activity assay

For testing the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) or diammonium salt radical cation (ABTS+; Sigma-Aldrich), the procedure follows the method introduced by Arnao et al. (2010) with some modifications [27]. The stock solution includes 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and letting them react for 14 hours at room temperature under dark conditions. The solution was then diluted by mixing 1 mL of ABTS solution with 60 mL of ethanol to set absorbance to 0.706 ± 0.01 units at 734 nm using a spectrophotometer (Thermo Scientific™ GENESYS™) is obtained. A fresh or new ABTS fresh/new solution was prepared for each test. Samples (at a concentration of 1, 2, 3, 4, and 5 µg) were allowed to react with 1 ml of ABTS solution, and the absorbance was taken at 734 nm after 7 minutes using a spectrophotometer. Treatment was carried out in the same way for all samples, including extract maceration (EM), extract-soxhletation (ES), purified fraction 1 (PF1), and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) was used as a positive control. All determinations were performed in three replication (n = 3).

$$\%\ \textbf{ABTS radical scavenging activity} = \frac{\mathbf{A0-A1}}{\mathbf{A0}}\ \mathbf{x}\ 100\%$$

A0 = Absorbance of blank; A1 = Absorbance of standard or sample.

The half-elimination ratio (EC_{50}) was used to express the radical scavenging capacity of EM, ES, PF1, and GSH and defined as the concentration of a sample that caused a 50% decrease in the initial radical concentration.

2.6. Cytotoxicity Evaluation using MTT Assay

Cell viability was assessed on the Human Caucasian skin fibroblast cell line (Normal cell; Bud-8) [28]. The proliferation rate of the Bud-8 cell line after sample treatment was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. A mitochondrial dehydrogenase reduces MTT to a purple compound formazan that is insoluble in water, depending on the viability of the cell. Cells were preserved in Dulbecco's Modified Essential Medium

(DMEM), which was supplemented with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin-Neomycin (PSN). One hundred microliters of cells (4 × 10^4 cells/mL) were seeded in a 96-well plate and incubated at 37°C, 5% carbon dioxide for 24 hours. After 24 hours of incubation, the cells were treated with 100 µL of 100, 200, 300, 400, and 500 µg/mL of samples (EM, ES, PF1). The plate was incubated at 37°C, with 5% CO_2 for 24 and 48 hours. After incubation, the morphology of the cells was examined under a microscope. Twenty microliters of MTT (5 mg/mL) (Sigma) solution was added to each well plate. The plate was further incubated for 2 to 4 hours, and the medium was removed. Formazan crystals dissolved with 100 µL dimethyl sulfoxide (DMSO; Sigma). Absorbance was measured at 560 nm, and the percentage of cell viability and LC_{50} cells is calculated by:

$$\% \textbf{ Cell Viability} = \frac{\textbf{A1}}{\textbf{A0}} \textbf{ x } 100\%$$

Where A0 is absorbance control in cells given 1%, DMSO and A1 are cells' absorbance samples given the test sample.

Lethal concentration (LC_{50}) is the lowest concentration of samples that inhibits 50% of cells. In general, a low LC_{50} value indicates high toxicity. Extracts with high LC_{50} are preferred for use due to their low toxicity effect on host cells [28].

2.7. Data Management and Analysis

Data from *in vitro* tests (DPPH antioxidants, ABTS antioxidants, and Cytotoxicity) were analyzed for significance or not between groups (EM, ES, PF1, or control) using two-way ANOVA CI 95% (0.05) with the MacBook version of GraphPad Prism 9.0.0 premium software. All data were presented in the form of average ± SEM. Graphic visualizations were presented using the MacBook version of GraphPad Prism 9.0.0 premium software. The graphical abstract was designed using the author's licensed BioRender Premium (Fahrul Nurkolis).

3 Results

3.1 Caulerpa racemosa metabolite profiles by non-targeted metabolomic profile

Figure 1 indicated the LC-MS/MS total ion chromatogram and mass spectrum of the macerated Sea grapes (*C. racemosa*) extract used to determine the peaks representing the number of annotated ions, the retention time, and the relative abundance of the ions. Annotated compounds were automatically identified via mzCloud MS/MS Library (Thermo Scientific Q Exactive Software). Based on non-targeted metabolomic profiling results with Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS), within a retention time of 40 minutes, macerated Sea grapes (*C. racemosa*) Extract (which represents cold extraction) contained a total of 103 compounds that were eluted between 0.00-30.0 minutes (Figure 1A). One hundred and three metabolite derivatives were successfully identified in Sea grapes (*C. racemosa*) from macerated extracts and were presented in Table 1. Figure 1B FTMS + p ESI Full ms. [50.0000-750.0000] is a spectrum with a base peak intensity of 2.90 x 10^6 counts (combination of electrospray ionization (ESI) with Fourier transform mass spectrometry (FTMS)). This FTMS was in positive mode and with electrospray ionization (ESI) continuous measurements from m/z 50 to m/z 600 (NL = normalization rate).

The data shown in Table 1 were based on matches with libraries or mzCloud Best Match (>90%) and in order according to their abundance. This was what causes the difference between the calculated exact mass (*) observed in the LC-HRMS results and the predicted-calculated molecular mass (**) in both PubChem and ChemDraw databases. Therefore, we presented molecular mass data by juxtaposing Calculated Exact Mass (*) with Predicted-calculated molecular mass (**) from ChemDraw in Table 1.

Table 1. 103 Compounds Observed from HPLC-ESI-HRMS/MS Analysis of Macerated Sea grapes (*C. racemosa*) Extract.

No.	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
1.	15.508	2,607,709,506.66	366.0987	344.1100	$C_{15}H_{20}O_9$	3-[3-(beta-D-Glucopyranosyloxy)-2-hydroxyphenyl]propanoic acid	Carboxylic Acids
2.	0.948	1,702,868,432.32	103.0996	104.1100	$C_5H_{13}NO$	Choline	Amines (Quartenary Ammonium Compounds)
3.	0.952	815,230,823.19	117.0787	117.0800	$C_5H_{11}NO_2$	Betaine	Amines (Quartenary Ammonium Compounds)
4.	18.019	530,234,031.11	278.1504	278.1500	$C_{16}H_{22}O_4$	Dibutyl phthalate	Carboxylic Acids (Phthalic Acids)
5.	10.763	484,746,646.13	312.0885	312.0900	$C_{18}H_{11}F_3N_2$	2-(1H-indol-3-yl)-3-[4-(trifluoromethyl)phenyl]acrylonitrile	Organofluorine compounds
6.	0.931	464,700,110.40	189.0429	167.0600	$C_8H_9NO_3$	2-(3,4-dihydroxyphenyl)acetamide	Organonitrogen compound (carboxamide)
7.	12.259	312,358,366.23	157.146	156.1400	$C_9H_{19}NO$	2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)	Heterocyclic compounds (piperidines)
8.	21.93	275,377,138.03	255.2549	255.2600	$C_{16}H_{33}NO$	Hexadecanamide	Fatty Acids (Palmitic Acids)
9.	17.905	273,288,427.76	278.1504	276.1400	$C_{16}H_{22}O_4$	Diisobutylphthalate	Carboxylic Acids
10.	0.927	209,833,574.73	205.0169	205.0200	$C_{10}H_7NO_2S$	5-(2-Thienyl)nicotinic acid	Carboxylic acid (aromatic carboxylic acid)
11.	0.916	204,192,224.93	87.10487	87.1000	$C_5H_{13}N$	Isoamylamine	Amines
12.	18.231	188,856,575.32	296.2341	Cannot be generated	$C_{18}H_{34}O_4$	NP-008993	unknown
13.	15.894	188,602,459.59	276.2077	254.2200	$C_{16}H_{30}O_2$	Palmitoleic Acid	Fatty Acids
14.	17.615	128,180,581.48	294.2184	294.2200	$C_{18}H_{30}O_3$	9-Oxo-10(E),12(E)-octadecadienoic acid	Fatty Acids
15.	17.427	123,110,274.85	294.2183	312.2300	$C_{18}H_{32}O_4$	(±)13-hydroperoxy-9Z,11E-octadecadienoic acid	Fatty Acids
16.	13.158	120,142,739.65	294.1819	294.1800	$C_{17}H_{26}O_4$	6-Gingerol	Alcohol (Fatty Alcohols)
17.	1.001	115,965,792.41	270.108	135.0500	$C_5H_5N_5$	Adenine	Heterocyclic compounds (purines)
18.	21.564	113,705,808.63	281.2706	281.2700	$C_{18}H_{35}NO$	Oleamide	Fatty Amides
19.	16.815	101,832,983.44	278.2234	278.2200	$C_{18}H_{30}O_2$	α-Eleostearic acid	Fatty Acids
20.	16.451	95,661,314.03	268.2027	Cannot be generated	$C_{16}H_{30}O_4$	NP-001596	unknown
21.	11.582	85,055,308.35	326.1044	326.1000	$C_{16}H_{14}N_4O_4$	4-{[(4,6-Dimethoxypyrimidin-2-yl)amino]methylidene}-2-phenyl-4,5-dihydro-1,3-oxazol-5-one	Organofluorine compounds
22.	14.177	82,274,117.71	148.0883	148.0900	$C_{10}H_{12}O$	Cuminaldehyde	Hydrocarbons (Terpenes)
23.	15.669	79,749,057.14	326.2445	326.2500	$C_{19}H_{34}O_4$	1,2-dihydroxyheptadec-16-yn-4-yl acetate	Organic Hydroxy compound (alcohol)
24.	1.024	79,366,759.62	267.0956	267.1000	$C_{10}H_{13}N_5O_4$	Adenosine	Carbohydrates (Purine Nucleosides)

No.	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
25.	20.046	75,757,786.32	278.2234	278.4400	$C_{18}H_{30}O_2$	α-Linolenic acid	Essential fatty acid
26.	17.351	73,810,505.50	342.0991	320.1200	$C_{19}H_{16}N_2O_3$	ethyl 3-oxo-5,6-diphenyl-2,3-dihydropyridazine-4-carboxylate	Fatty acid transporters
27.	16.633	70,444,302.73	292.2026	322.2500	$C_{18}H_{28}O_3$	12-Oxo phytodienoic acid	Fatty Acids (Octadecanoids)
28.	1.044	70,264,037.81	122.0476	122.0500	$C_6H_6N_2O$	Nicotinamide	Heterocyclic compounds (Pyridinecarboxylic acids)
29.	23.794	66,634,752.38	283.2858	283.2900	$C_{18}H_{37}NO$	Stearamide	Amides
30.	21.936	62,781,318.81	307.2861	325.3000	$C_{20}H_{39}NO_2$	Oleoyl ethanolamide	Amines (Amino alcohols)
31.	13.43	61,670,246.92	276.1714	Cannot be generated	$C_{15}H_{26}O_3$	NP-020014	unknown
32.	17.56	58,853,299.74	302.2233	320.2400	$C_{20}H_{32}O_3$	11,12-epoxy-5,8,14-eicosatrienoic acid	Eicosanoids
33.	20.518	57,317,714.92	622.2406	584.6600	$C_{29}H_{44}O_{12}$	Ouabain	Autacoids (Eicosanoids)
34.	12.847	56,359,590.26	164.0831	182.0900	$C_{10}H_{14}O_3$	1-(4-methoxyphenyl)propane-1,2-diol	Hydrocarbon (cyclic hydrocarbon)
35.	0.873	53,487,771.60	235.1412	235.1400	$C_{14}H_{18}FNO$	4-Fluoro-α-pyrrolidinobutiophenone	Butyrophenones
36.	1.305	53,461,055.41	167.0611	167.1600	$C_8H_9NO_3$	Pyridoxal	Heterocyclic compounds (Pyridine carboxaldehydes)
37.	14.862	53,396,851.21	214.135	232.1500	$C_{15}H_{20}O_2$	(4aR,5R,6R)-6-hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl)-2,4a,5,6,7,8-hexahydronaphthalen-2-one	Terpenoid
38.	14.874	51,133,618.50	356.1586	334.1800	$C_{19}H_{26}O_5$	(3S,3aR,4S,4aR,7aR,8R,9aR)-3,4a,8-trimethyl-2,5-dioxo-2H,3H,3aH,4H,4aH,5H,7aH,8H,9H,9aHazuleno[6,5-b]furan-4-yl 2-methylpropanoate	Heterocyclic Compounds
39.	15.089	47,187,926.45	328.0835	328.0800	$C_{18}H_{17}ClN_2S$	1-(4-chlorobenzyl)-2-{[(4-methylphenyl)thio]methyl}-1H-imidazole	Protein Enzyme
40.	16.835	44,926,299.27	328.2602	328.2600	$C_{19}H_{36}O_4$	1,4-dihydroxyheptadec-16-en-2-yl acetate	Organic Hydroxy compound (alcohol)
41.	22.189	43,991,117.28	500.2191	250.1100	$C_{16}H_{14}N_2O$	Methaqualone	Heterocyclic compounds (Quinazolines)
42.	16.48	41,454,327.51	250.1923	426.3000	$C_{16}H_{26}O_2$	Octylphenol Ethoxylates (OPEO)	alkylphenols
43.	14.714	38,806,980.21	273.2655	256.2400	$C_{16}H_{32}O_2$	Palmitic Acid	Fatty acids
44.	16.334	37,032,281.51	300.2076	318.4600	$C_{20}H_{30}O_3$	8-Hydroxyeicosapentaenoic acid ((±)8-HEPE)	Hydroxy fatty acid
45.	20.987	35,975,153.82	282.2548	282.2600	$C_{18}H_{34}O_2$	Ethyl palmitoleate	Fatty acid ester (Fatty acid ethyl ester)
46.	21.041	34,105,859.15	280.239	Cannot be generated	$C_{18}H_{34}O_3$	NP-011548	unknown

No.	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
47.	11.577	33,493,328.71	164.0831	164.0800	$C_{10}H_{12}O_2$	4-Phenylbutyric acid	Carboxylic Acids (Phenylbutyrates)
48.	14.077	32,107,702.51	248.1766	266.1900	$C_{16}H_{26}O_3$	Tetranor-12R-HETE	Organy Hydroxy compound (Hydroxy carboxylic acid)
49.	16.588	31,279,593.58	314.1846	292.2000	$C_{18}H_{28}O_3$	4-hydroxy-6-[2-(2-methyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethyl]oxan-2-one	Heterocyclic compound (oxacycle)
50.	17.366	31,243,191.82	354.2756	354.2800	$C_{21}H_{38}O_4$	1-Linoleoyl glycerol	Glycerides
51.	18.021	31,197,110.75	323.2083	306.1800	$C_{18}H_{26}O_4$	n-Pentyl isopentyl phthalate	Phthalates
52.	16.258	30,927,401.68	268.2028	Cannot be generated	$C_{16}H_{30}O_4$	NP-001596	unknown
53.	7.466	30,636,932.36	197.1198	197.1200	$C_{14}H_{15}N$	Dibenzylamine	Amines
54.	18.515	30,124,986.19	318.216	Cannot be generated	$C_{18}H_{32}O_3$	NP-014287	unknown
55.	12.819	29,968,764.20	232.1455	Cannot be generated	$C_{15}H_{22}O_3$	(1aR,1bR,2R,3R,7R,7aS)-1b,2-dimethyl-7a-(prop-1-en-2-yl)-1aH,1bH,2H,3H,4H,5H,7H,7aH-naphtho[1,2-b]oxirene-3,7-diol	unknown
56.	15.64	28,287,214.04	196.0881	196.0900	$C_{14}H_{12}O$	4-Methylbenzophenone	Ketones (Benzophenones)
57.	14.449	27,409,945.13	214.135	232.1500	$C_{15}H_{20}O_2$	(4aR,5R,6R)-6-hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl)-2,4a,5,6,7,8-hexahydronaphthalen-2-one	Terpenoid
58.	16.302	27,286,206.71	302.2444	302.2500	$C_{17}H_{34}O_4$	2,3-dihydroxypropyl 12-methyltridecanoate	Glyceride
59.	15.864	25,328,627.10	316.2003	316.2000	$C_{20}H_{28}O_3$	Cafestol	Hydrocarbons (Terpenes)
60.	14.695	24,803,082.67	508.263	526.2700	$C_{27}H_{37}F_3N_2O_5$	[6-Hydroxy-1-(hydroxymethyl)-1,4a-dimethyl-5-(2-oxo-2-pyrrolidin-1-ylethyl)-2,3,4,5,6,7,8,8a-octahydronaphthalen-2-yl] N-[3-(trifluoromethyl)phenyl]carbamate	Organofluorine compounds
61.	20.606	24,591,569.01	308.2339	302.2200	$C_{20}H_{30}O_2$	Eicosapentaenoic acid	Fatty Acids
62.	21.592	23,630,078.82	313.2967	313.3000	$C_{19}H_{39}NO_2$	R-Palmitoyl-(2-methyl) ethanolamide	Lipid (Fatty Amide)
63.	12.163	23,533,439.36	370.094	Cannot be generated	$C_{21}H_{20}Cl_2N_2$	2-(2,4-dichlorophenyl)-4,4,7,9-tetramethyl-4,5-dihydro-3H-naphtho[1,2-d]imidazole	unknown
64.	12.76	23,504,570.02	234.1611	252.1700	$C_{15}H_{24}O_3$	Ageratriol	Terpenoids
65.	14.233	23,426,676.24	244.1091	262.1200	$C_{15}H_{18}O_4$	(3aR,4aS,5R,8S,9aR)-5-hydroxy-4a,8-dimethyl-3-methylidene-2H,3H,3aH,4H,4aH,5H,6H,8H,9H,9aH-azuleno[6,5-b]furan-2,6-dione	Terpenes
66.	13.406	23,234,665.52	232.1455	Cannot be generated	$C_{15}H_{22}O_3$	(1aR,1bR,2R,3R,7R,7aS)-1b,2-dimethyl-7a-(prop-1-en-2-yl)-1aH,1bH,2H,3H,4H,5H,7H,7aH-naphtho[1,2-b]oxirene-3,7-diol	Nuclear receptor
67.	12.099	22,988,983.05	384.1096	Cannot be generated	$C_{18}H_{19}F_3N_2O_2S$	ethyl 2-(methylthio)-4-tetrahydro-1H-pyrrol-1-yl-8-(trifluoromethyl)quinoline-3-carboxylate	unknown
68.	18.446	22,839,809.05	218.1662	Cannot be generated	$C_{15}H_{24}O_2$	NP-004713	unknown
69.	20.376	22,099,138.42	330.2546	330.2600	$C_{22}H_{34}O_2$	Eicosapentaenoic acid ethyl ester	Fatty Acids
70.	17.161	21,808,214.15	270.2183	Cannot be generated	$C_{16}H_{32}O_4$	NP-020214	unknown

No.	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
71.	13.431	20,606,367.65	261.1354	239.1500	$C_{13}H_{21}NO_3$	Levalbuterol	Amines (Phenethylamines)
72.	20.074	20,450,089.14	306.2545	306.2600	$C_{20}H_{34}O_2$	Linolenic acid ethyl ester	Fatty Acids
73.	21.707	19,935,241.14	310.2857	310.2900	$C_{20}H_{38}O_2$	Ethyl oleate	Fatty Acids
74.	14.41	19,870,753.40	182.0725	182.0700	$C_{13}H_{10}O$	Benzophenone	Ketones (Benzophenones)
75.	17.367	18,964,856.34	262.2285	280.4500	$C_{18}H_{32}O_2$	Octadec-9-ynoic acid	Hydrocarbons (Alkynes)
76.	15.997	18,954,242.24	228.1141	246.1300	$C_{15}H_{18}O_3$	(3aS,5aS,9bR)-5a,9-dimethyl-3-methylidene-2H,3H,3aH,4H,5H,5aH,6H,7H,8H,9bH-naphtho[1,2-b]furan-2,5-dione	Terpenes
77.	19.433	18,184,819.14	328.2388	328.2400	$C_{22}H_{32}O_2$	Docosahexaenoic acid	Fatty Acids
78.	26.392	18,033,386.90	283.3227	284.3300	$C_{19}H_{41}N$	Cetrimonium	Amines (Quartenary Ammonium Compounds)
79.	17.063	17,943,409.82	398.169	398.1700	$C_{22}H_{26}N_2O_3S$	3-(3,4-dimethoxyphenethyl)-2-[(4-isopropylphenyl)imino]-1,3-thiazolan-4-one	Organic Chemicals
80.	9.957	17,802,168.49	236.1402	254.1500	$C_{14}H_{22}O_4$	(±)-C75	Organic heterocyclic compound
81.	16.951	17,509,512.15	234.1611	234.1600	$C_{15}H_{22}O_2$	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	Aldehydes (Benzaldehydes)
82.	20.317	17,492,831.93	299.2812	299.2800	$C_{18}H_{37}NO_2$	Palmitoyl ethanolamide	Amines (Amino Alcohols)
83.	15.118	16,405,749.44	467.3231	467.3100	$C_{28}H_{41}N_3O_3$	Oxethazaine	Amines (Amino Alcohols)
84.	19.858	16,337,402.48	305.2705	Cannot be generated	$C_{20}H_{35}NO$	NP-016582	unknown
85.	20.015	16,138,319.09	321.2654	299.5000	$C_{18}H_{37}NO_2$	Sphingosine (d18:1)	Amines
86.	16.742	15,941,236.73	692.3268	Cannot be generated	$C_{22}H_{22}N_2O_2$	4-(3-methoxy-5,6-dihydrobenzo[c]acridin-7-yl)morpholine	unknown
87.	13.152	15,540,877.24	334.1743	334.1700	$C_{21}H_{22}N_2O_2$	(-)-Strychnine	Alkaloids (Indole Alkaloids)
88.	20.726	15,378,473.45	304.2391	304.2400	$C_{20}H_{32}O_2$	Arachidonic acid	Autacoids (Eicosanoids)
89.	16.279	15,308,423.13	356.0783	356.0700	$C_{20}H_{12}N_4OS$	3,4-Diphenylpyrimido[4',5':4,5]thieno[2,3-c]pyridazin-8(7H)-one	Thienopyridazine derivatives
90.	17.305	14,960,482.55	378.2755	378.2800	$C_{23}H_{38}O_4$	2-Arachidonoyl glycerol	Glycerolipid
91.	19.83	14,601,900.81	442.3432	442.3400	$C_{29}H_{46}O_3$	Testosterone decanoate	Gonadal Steroid Hormones (Testosterone congeners)
92.	14.886	14,357,946.57	274.1558	252.1700	$C_{15}H_{24}O_3$	(5E)-7-methylidene-10-oxo-4-(propan-2-yl)undec-5-enoic acid	Terpenes
93.	17.72	13,897,675.48	312.2651	306.2600	$C_{20}H_{34}O_2$	γ-Linolenic acid ethyl ester	Fatty acid derivative
94.	15.669	12,500,838.24	510.2786	Cannot be generated	$C_{27}H_{40}N_2O_6$	6-Hydroxy-1-(hydroxymethyl)-5-{2-[2-(hydroxymethyl)-1-pyrrolidinyl]-2-oxoethyl}-1,4a-dimethyldecahydro-2-naphthalenyl phenylcarbamate	unknown
95.	19.475	12,426,002.76	344.195	344.2000	$C_{21}H_{28}O_4$	Nor-9-carboxy-δ9- tetrahydrocannabinol	Terpenes

No.	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
96.	26.449	12,331,286.20	131.0942	131.0900	$C_6H_{13}NO_2$	6-Aminocaproic acid	Carboxyclic Acid (Caproates)
97.	17.805	9,806,912.19	340.1637	340.1600	$C_{20}H_{24}N_2OS$	Propionylpromazine	Sulfur compounds (Phenothiazines)
98.	23.138	9,589,300.00	390.2753	390.2800	$C_{24}H_{38}O_4$	Di(2-ethylhexyl) phthalate	Carboxylic acid (Phthalic acid)
99.	20.548	9,339,555.94	176.1194	194.1300	$C_{12}H_{18}O_2$	Sedanolide	Heterocyclic Compound (Benzofurans)
100.	19.703	9,113,695.23	303.2549	303.2600	$C_{20}H_{33}NO$	Arachidonoyl amide	Fatty acid derivative (fatty amide)
101.	16.962	8,865,034.10	248.1766	266.1900	$C_{16}H_{26}O_3$	Tetranor-12(S)-HETE	Organic Hydroxy compound (hydroxy carboxylic acid)
102.	18.548	8,618,378.15	306.2545	306.2600	$C_{20}H_{34}O_2$	8Z,11Z,14Z-Eicosatrienoic acid	Autacoids (Eicosanoids)
103.	21.448	8,239,070.91	363.3121	357.3000	$C_{24}H_{39}NO$	Oleyl anilide	Amines (Anilides)

RT = Retention time (minutes); * = Calculated exact mass observed from LC-HRMS; Predicted-calculated molecular mass from ChemDraw.

Figure 2 indicated the LC-MS/MS total ion chromatogram and mass spectrum of the soxhlet extraction of Sea grapes (*C. racemosa*) used to determine the peaks representing the number of annotated ions, the retention time, and the relative abundance of the ions. Just like in the maceration extract, Annotated compounds were automatically identified via mzCloud MS/MS Library (Thermo Scientific Q Exactive Software). Based on non-targeted metabolomic profiling results with Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS), within a retention time of 40 minutes, soxhlet of Sea grapes (*C. racemosa*) extract (which represents heat extraction) contained a total of 48 compounds that were diluted between 0.00-30.0 minutes (Figure 2A). Forty-eight (48) metabolite derivatives were successfully identified in Sea grapes (*C. racemosa*) from soxhlet extraction extracts then presented in Table 2. Figure 2B FTMS + p ESI Full ms. [50.0000-750.0000] was a spectrum with a base peak intensity of 2.52 x 10^6 counts (combination of electrospray ionization (ESI) with Fourier transform mass spectrometry (FTMS)). This FTMS was in positive mode and by electrospray ionization (ESI) continuous measurements from m/z 50 to m/z 670 (NL = normalization rate).

Table 2. Forty-eight Compounds Observed from HPLC-ESI-HRMS/MS Analysis of Soxhlet Extraction of Sea grapes (*C. racemosa*).

No	RT (min)	Abundance (Area Max.)	Observed HR-ESIMS m/z*	Calculated HR-ESIMS m/z**	Molecular Formula	Tentatively Identified Compound	Category
1.	0.902	615,708,699.02	117.0787	117.0800	$C_5H_{11}NO_2$	Betaine	Amines (Quartenary Ammonium Compounds)
2.	21.916	353,793,967.91	255.25505	255.2600	$C_{16}H_{33}NO$	Hexadecanamide	Fatty Acids (Palmitic Acids)
3.	17.877	344,623,223.38	278.15076	276.1400	$C_{16}H_{22}O_4$	Diisobutylphthalate	Carboxylic Acids
4.	12.294	274,478,961.83	157.14607	156.1400	$C_9H_{19}NO$	2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)	Heterocyclic compounds (piperidines)
5.	18.209	195,885,637.15	296.23405	Cannot be generated	$C_{18}H_{34}O_4$	NP-008993	unknown
6.	16.424	119,602,748.90	268.20276	Cannot be generated	$C_{16}H_{30}O_4$	NP-001596	unknown
7.	21.547	104,636,468.21	281.27056	281.2700	$C_{18}H_{35}NO$	Oleamide	Fatty Amides
8.	0.901	104,452,391.68	131.09424	131.0900	$C_6H_{13}NO_2$	DL-β-Leucine	Amino Acids
9.	16.928	82,954,091.16	278.22365	278.2200	$C_{18}H_{30}O_2$	α-Eleostearic acid	Fatty Acids
10.	23.776	82,837,237.90	283.28606	283.2900	$C_{18}H_{37}NO$	Stearamide	Amides
11.	13.397	79,829,227.71	276.17153	Cannot be generated	$C_{15}H_{26}O_3$	NP-020014	unknown
12.	18.681	75,543,295.89	282.25472	282.2600	$C_{18}H_{34}O_2$	Ethyl palmitoleate	Fatty acid ester (Fatty acid ethyl ester)
13.	17.587	75,423,822.44	294.21851	312.2300	$C_{18}H_{32}O_4$	(±)13-HpODE	Fatty acid
14.	0.911	70,567,131.89	87.10485	87.1000	$C_5H_{13}N$	Isoamylamine	Amines
15.	21.92	67,501,000.61	307.2863	325.3000	$C_{20}H_{39}NO_2$	Oleoyl ethanolamide	Amines (Amino alcohols)
16.	0.906	62,987,796.05	103.0996	104.1100	$C_5H_{13}NO$	Choline	Amines (Quartenary Ammonium Compounds)
17.	0.835	60,193,728.47	228.97644	228.9800	$C_9H_5ClFNOS$	2-(3-Chloro-2-fluorophenyl)-2,3-dihydroisothiazol-3-one	Enzyme
18.	18.938	42,693,613.10	308.19524	Cannot be generated	$C_{16}H_{30}O_4$	NP-001596	unknown
19.	17.881	42,504,568.52	323.20854	306.1800	$C_{18}H_{26}O_4$	n-Pentyl isopentyl phthalate	Phthalates
20.	16.455	39,764,527.54	250.19238	426.3000	$C_{16}H_{26}O_2$	Octylphenol Ethoxylates (OPEO)	alkylphenols
21.	17.434	39,577,349.70	316.20043	316.2000	$C_{20}H_{28}O_3$	Cafestol	Hydrocarbons (Terpenes)
22.	16.654	37,079,182.87	254.22351	254.2200	$C_{16}H_{30}O_2$	Palmitoleic acid	Fatty Acids
23.	22.8	33,398,733.18	309.30147		$C_{20}H_{41}NO_2$	Stearoyl Ethanolamide	Fatty Acids
24.	20.591	32,595,365.73	308.23406	302.2200	$C_{20}H_{30}O_2$	Eicosapentaenoic acid	Fatty Acids
25.	17.87	31,871,173.23	294.21853	294.2200	$C_{18}H_{30}O_3$	9-Oxo-10(E),12(E)-octadecadienoic acid	Fatty Acids
26.	14.577	31,454,460.53	273.26564	256.2400	$C_{16}H_{32}O_2$	Palmitic Acid	Fatty acids
27.	7.411	31,100,701.46	197.11981	197.1200	$C_{14}H_{15}N$	Dibenzylamine	Amines
28.	17.367	30,483,737.39	280.2392	Cannot be	$C_{18}H_{34}O_3$	NP-011548	unknown

				generated			
29.	26.447	29,296,796.45	131.09427	131.0900	$C_6H_{13}NO_2$	6-Aminocaproic acid	Carboxyclic Acid (Caproates)
30.	19.991	29,136,008.89	278.22365	278.4400	$C_{18}H_{30}O_2$	α-Linolenic acid	Essential fatty acid
31.	13.398	25,493,790.28	261.13552	239.1500	$C_{13}H_{21}NO_3$	Levalbuterol	Amines (Phenethylamines)
32.	22.054	19,542,351.23	361.2968	361.3000	$C_{23}H_{39}NO_2$	Methanandamide	Fatty acid derivative
33.	16.914	17,243,572.51	234.16117	234.1600	$C_{15}H_{22}O_2$	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	Aldehydes (Benzaldehydes)
34.	13.132	16,983,601.06	276.17153	276.1700	$C_{17}H_{24}O_3$	Shogaol	Phenols
35.	20.295	16,462,133.15	299.28127	299.2800	$C_{18}H_{37}NO_2$	Palmitoyl ethanolamide	Amines (Amino Alcohols)
36.	14.576	14,874,891.90	414.20268	414.2000	$C_{24}H_{30}O_6$	Bis(4-ethylbenzylidene)sorbitol	Sugar alcohol
37.	19.986	14,176,669.67	321.26558	299.5000	$C_{18}H_{37}NO_2$	Sphingosine (d18:1)	Amines
38.	19.715	13,852,334.54	336.20778	336.2100	$C_{20}H_{29}FO_3$	Fluoxymesterone	Hormones
39.	17.688	13,159,097.66	350.24193	328.2600	$C_{19}H_{36}O_4$	2,4-dihydroxyheptadec-16-en-1-yl acetate	Unsaturated Fatty Acids
40.	26.392	12,768,022.53	283.32268	284.3300	$C_{19}H_{41}N$	Cetrimonium	Amines (Quartenary Ammonium Compounds)
41.	23.12	12,428,205.36	390.2755	390.2800	$C_{24}H_{38}O_4$	Bis(2-ethylhexyl) phthalate	Carboxylic Acids
42.	16.474	11,621,213.91	266.16368	266.1600	$C_{12}H_{27}O_4P$	Tributyl phosphate	Organophosphorus Compounds
43.	16.926	10,593,568.28	248.17679	266.1900	$C_{16}H_{26}O_3$	Tetranor-12(S)-HETE	Organic Hydroxy compound (hydroxy carboxylic acid)
44.	20.537	9,753,652.00	176.11943	194.1300	$C_{12}H_{18}O_2$	Sedanolide	Heterocyclic Compound (Benzofurans)
45.	21.427	8,882,476.13	363.31224	357.3000	$C_{24}H_{39}NO$	Oleyl anilide	Amines (Anilides)
46.	11.188	8,843,434.72	294.21855	294.2200	$C_{18}H_{30}O_3$	13(S)-HOTrE	Unsaturated Fatty Acids
47.	11.662	8,423,640.09	191.13033	191.11300	$C_{12}H_{17}NO$	Diethyltoluamide or DEET	Carboxylic Acids
48.	21.688	7,026,193.73	310.28584	310.2900	$C_{20}H_{38}O_2$	Ethyl oleate	Fatty Acids

RT = Retention time (minutes); * = Calculated exact mass observed from LC-HRMS; Predicted-calculated molecular mass from ChemDraw.

3.2. Identification of the peptide in purified fraction 1 of C. racemosa

PF1 of *C. racemosa* found a bioactive peptide named (2S,5S,8S,11S,14S,17S)-11-((1H-indol-3-yl)methyl)-17-amino-8-(4-aminobutyl)-2-benzyl-5-((R)-1-hydroxyethyl)-14-isobutyl-4,7,10,13,16-pentaoxo-3,6,9,12,15-pentaazaicosanedioic acid or ELWKTF (Glu-Leu-Trp-Lys-Thr-Phe) which was eluted at a retention time of 30.9 minutes with a peak area of 815,602,581.03 (Table 3). The structural visualization of ELWKTF is shown in Figure 3.

Table 3. Amino Acids (ELWKTF) Analysis.

Peptide Sequence	Average Local Confidence (ALC, %)	Length*	m/*z*	Retention Time (RT)	Theoretical Mass/Observed Mass (Da)	Peak Area (max)
ELWKTF	97	6	412.2320	30.9	822.43/822.428	815,602,581.03

***Chemical Formula**: C41H58N8O10. **Exact Mass**: 822.43. **Molecular Weight**: 822.96. **m/z**: 822.43 (100.0%), 823.43 (45.4%), 824.43 (13.0%), 823.42 (3.0%), 825.44 (2.4%). **Elemental Analysis**: C, 59.84; H, 7.10; N, 13.62; O, 19.44. *, The number of amino acids on the peptide.*

3.3. The DPPH Radical Scavenging Activity of C. racemosa

Figure 4 showed the results of an in vitro study inhibiting DPPH radical scavenging activity. The inhibitory activity of DPPH was compared among *C. racemosa* macerated extract (EM), Soxhlet extract (ES), purified fraction 1 (PF1), and glutathione (GSH). The results showed lesser DPPH inhibition activities than GSH or control at 1 µg/mL, 2 µg/mL, 3 µg/mL, and 4 µg/mL of EM; and 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL of ES and PF1($p < 0.0001$). The DPPH inhibition of EM was to close with GSH at a dose of 5 µg/mL with a percentage of 87.43 ± 0.67% and 86.73 ± 0.61%, respectively (Figure 4).

As shown in Figure 5, the EC_{50} yields of EM, ES, PF1, and GSH were 2.945 µg/mL, 2.297 µg/mL, 2.302 µg/mL, and 2.691 µg/mL, respectively. ES and PF1 show good potential effectiveness in DPPH radical elimination activity because the EC_{50} values are lower than the control or GSH.

3.4. ABTS Radical Scavenging Activity of C. racemosa

Figure 6 showed the results of an *in vitro* study inhibiting the radical scavenging activity of ABTS. The inhibitory activity of ABTS was compared among the macerated extract of *C. rasemosa* (EM), the soxhletated extract (ES), the purified fraction 1 (PF1), and the Trolox or control. The results showed lesser of ABTS inhibition activities at doses 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL and 5 µg/mL for ES and PF1, compared to Trolox or control ($p < 0.0001$).

As shown in Figure 7, the EM, ES, PF1, and Trolox yield was EC_{50} of 3.306 µg/mL, 3.244 µg/mL, 2.508 µg/mL, and 2.547 µg/mL, respectively. PF1 showed good potential effectiveness in the radical elimination activity of ABTS because it has a lower EC_{50} value than the control or Trolox.

3.5. Cytotoxicity Evaluation of C. racemosa using MTT Assay

Figure 8 showed the difference in the viability percentage of normal cells or fibroblasts from the human Caucasian cell line. There was a significant difference ($p<0.05$) between each concentration between the groups during 24 hours of incubation and 48 hours. The sequence of LC_{50} samples that are lowest or show the highest cytotoxicity to the lowest cytotoxicity is EM 914.78 µg/mL, PF1 2069.21 µg/mL, and ES 2227.85 µg/mL at 24 hours; and, ES 1816.17 µg/mL, PF1 2173.02 µg/mL, and EM 2971.15 µg/mL at 48 hours (Table 4). This result suggested EM, ES, and PF1 as antioxidant agents in the observed EC_{50} were safe. Furthermore, in terms of cytotoxicity, it was observed that *C. racemosa* was safe to be potentially developed into various products. In addition, the value of LC_{50} is presented in Table 4.

Table 4. LC_{50} Value of *C. racemosa* on Cytotoxicity Test in BUD-8 Cell Lines

Hours of incubation	LC_{50} (µg/ml)		
	EM	ES	PF1
24 hours	914.78	2227.85	2069.21
48 hours	2971.15	1816.17	2173.02

4 Discussions

Seaweed is traditionally used as a sea vegetable in Asian countries, especially Indonesia, but its consumption is still minimal. *C. racemosa* is one of the green seaweeds whose metabolite profiles, health properties, and potential use as functional ingredients in food, supplements, and pharmaceuticals should be further explored. This will be a new opportunity to introduce *C. racemosa* indirectly into the human food chain in western countries, especially in Europe.

Foodomics is a discipline that studies the domain of food and nutrition through the application and integration of advanced technology "-omics" to improve the well-being, health, and knowledge of consumers [22,23]. One part of Foodomics is the metabolomic study [29] which was applied in this study and successfully profiled the secondary metabolites of *C. racemosa* with different extraction methods. On the other hand, the bioactive peptides were also successfully identified using proteomics approaches. Secondary metabolites and bioactive peptides are expected to be a data challenge for other researchers or follow-up research to find their continued effects on health and product development based on *C. racemosa*. This untargeted metabolomic profiling study that we conducted has succeeded in profiling secondary metabolites of *C. racemosa*, which was previously a challenge from the research of Pangestuti *et al*. (2021) [30]. Study by Pangestuti *et al*. (2021) only observed at phytochemicals of *C. racemosa* in terms of total phenolic, saponins, and flavonoid contents without the metabolite compounds [30].

There was a difference in the number of compounds identified in the maceration (cold) and soxhletation (heat) methods of extraction of sea grapes Sea grapes (*C. racemosa*) extract through the extraction-maceration method had higher bioactive compound than the Sea grapes extracted using a Soxhlet. The soxhlet extraction method integrates the advantages of reflux and percolation extraction, which utilizes the principle of reflux and siphon to continuously extract the herb with fresh solvent [13]. Extraction using the soxhlet method has the advantage of an automatic continuous extraction method with less extraction time and less solvent use than maceration or percolation [13]. However, high temperatures and long extraction times in soxhlet extraction will increase the likelihood of thermal degradation. This is what is strongly suspected to cause differences in the results of compounds from the two extracts in this *C. racemosa* study. In line with the results of our study, other studies showed that the degradation of catechins in tea was also observed in soxhlet extraction due to the high extraction temperature [31]. The total polyphenol and total alkaloid concentrations of the soxhlet extraction method decreased compared to the maceration method [31,32].

The bioactive compounds found in this study showed both health benefits and toxic effects based on other literature (see Supplementary). However, not all bioactive compounds will have a significant effect since the dose of each metabolite should be considered. For example, an *in vivo* intervention study using 450 mg/kgBW of sea grapes extract on rats showed no adverse effects [10].

Proteins in foods sourced from marine resources and their by-products have high structural diversity and are a considerable resource for exploring bioactive peptides [33]. Previous literature suggests that the types of amino acids in Bioactive Peptides (BPs) are considered a critical factor in their activity [34]. Residual hydrophobic groups from hydrophobic amino acids such as Pro, Met, Ala, Leu, and Ile, can strongly react with hydrophobic polyunsaturated fatty acids (PUFAs) to inhibit lipid peroxidation in lipid-rich foods [35,36]. The EC_{50} of PF1 which was allegedly derived from ELWKTF activity in this study was more potential than Trolox (in ABTS inhibition assay) and GSH (DPPH inhibition Assay) as a control based on the EC_{50} value. Carboxyl and amino groups in polar amino acid residues are essential to capture hydroxyl radicals and the metal-ion chelating capacity of BPs [36,37]. In addition, Glu and Leu residues can maintain the high flexibility of the polypeptide skeleton, and its single hydrogen atom can be donated to neutralize Reactive Oxygen Species (ROS) [34,38]. Therefore, polar amino acids, including -Glu and -Leu residues in ELWKTF, may have played an essential role in hydroxyl radical capture activities. *C. racemosa,* which has an abundance of ELWKTF, can be a source of free radical inhibition activity through the mechanism presented in the previous sentence. Recent scientific evidence suggested that dietary proteins may have function as nutrients and can also modulate the body's physiological functions [39]. This physiological function is mainly regulated by several encrypted peptides in the original protein sequence. This bioactive peptide can provide beneficial properties for health and is therefore considered a significant compound for developing nutraceuticals or functional foods to fight metabolic syndrome, obesity, cancer, diabetes, and aging, which are associated with cardiovascular disease. This study that we conducted showed novelty on the measurement of bioactive peptides and their antioxidant activity, which in previous studies had never been reported or carried out [30,40].

Lethal concentration (LC_{50}) is the lowest concentration of samples that inhibits 50% of cells. In general, a low LC_{50} value indicates high toxicity. Extracts with high LC_{50} are preferred for use due to their lower toxicity effect on host cells [28]. The interpretation of the LC_{50} value was based on the National Cancer Institute [41], LC_{50} value of 20 µg/mL indicates strong cytotoxic properties, 21-200 µg/mL indicates moderate cytotoxicity, 201-500 µg/mL exhibits weak cytotoxicity, and > 500 µg/mL indicates no cytotoxic properties. *C. racemosa* has a potential antioxidant activity of EC_{50} value accompanied by an LC_{50} value which was in a safe category. This is in line with similar studies that observed at the cytotoxicity of *C. racemosa* in subcritical water extraction, which did not show significant cytotoxicity activity [30]. Furthermore, a review study by Aroyehun *et al.* (2020) stated that in addition to minimal toxicity, *C. racemosa* has bioprospecting as a promising nutraceutical due to its nutritional values [9].

4.1 The Potential and Prospects of Caulerpa racemosa as Nutraceuticals and Pharmaceuticals in Terms of Cultivation and Use in Commercial Industrial Production Applications.

Fewer incidences of diet-related diseases, especially non-communicable diseases and including cancer and cardiovascular disease have been observed in countries that consume high amounts of seaweed as a supplement or food [42,43]. Seaweed consumption has been shown to reduce the prevalence of different non-communicable diseases due to the metabolites and other bioactive compounds working as a defense mechanism [43]. Studies carried out with seaweed extracts or their specific metabolites proved that they have cytotoxins, which prevent the proliferation of cancer cells [44]. In this study, the results showed that. ES and PF1 show good potential effectiveness in DPPH radical elimination activity because the EC_{50} values are lower than the control or GSH. This was in line with a review study by Collins *et al.,* 2016, which focused on the potential use of bioactive seaweed derivatives, including polysaccharides, antioxidants, and fatty acids, among others, to treat chronic non-communicable diseases [42]. Furthermore, the main compounds resulting from the identification of *C. racemosa* metabolomic profiling, such as choline[45], betaine[45], oleamide[46], hexadecanamide[47], palmitoleic acid[48], and α-eleostearic acid[49] may become therapeutic drugs for non-communicable diseases. Furthermore, we also explored the antioxidant activity of each secondary metabolite of metabolomic profiling results by a review approach, which can be seen in **Supplementary Table 1**.

The therapeutic activity of food-derived bioactive proteins and peptides – such as from medicinal plants – is attracting increasing attention in the research community [39]. Bioactive peptides offer promising potential as antiviral drugs, and therapeutic peptides are an exciting alternative to be developed into anti-Dengue virus drugs due to their safety and diverse biological and chemical properties[50]. In addition, many potential compounds were identified from *C. racemosa* metabolites profiles, such as choline[51], betaine[52], oleamide[53], hexadecanamide[54], palmitoleic acid[55], and α-eleostearic acid[56]. More research is needed to examine the clinical effects of *C. racemosa* as a nutraceutical.

4.2 Potential for Future Research.

Many strategies are needed to further develop *C. racemosa* products to explore their secondary metabolites and health benefits. In addition, environmental factors play an essential role in influencing the composition of secondary metabolites in *C. racemosa* [57,58], which is important to be considered. To ensure the validity and safety of Sea grapes research development, the drug discovery process should be carried out through various steps as recommended by the Food and Drug Administration (FDA, US), which can be seen in Figure 10.

The process initiated by metabolomic profiling identifies what processes/pathways can be targeted to affect the disease/condition. Compound screening can also be done to identify compounds that can be promising candidates for further development. Next, an *in silico* study is needed for target validation, determining whether the drug can provide therapeutic benefit to the target, and lead discovery to identify the lead compound to be observed for further studies. Subsequently, preclinical trials are needed to evaluate therapeutic index and dose using *in vitro* and *in vivo* approaches and evaluate the aspects of pharmacokinetics and pharmacodynamics in experimental animals (Figure 10). Finally, to ensure efficacy and safety, three phases of clinical trials need to be conducted, first on healthy patients and then on obese or patients with other cardiovascular diseases.

4.3 Strengths and Limitations of Study

The results of this study complemented the published data related to secondary metabolites from *Caulerpa racemosa* found and grown in Asia, especially in Indonesia waters. Furthermore, this study succeeded in profiling the bioactive peptide and their bioactivity properties from the purified fraction 1 of *Caulerpa*

racemosa (Graphical Abstract). In addition, the synthesis and purification of their respective compounds (metabolites and bioactive peptides) for the development of food and drug products and the exploration of bioactive carbohydrates and other bioactive peptides is a limitation of this research. It is hoped that more research will be carried out in the future. Seeing the metabolomic results which show indications of several compounds that are thought to be anti-nutrients, further studies are needed to confirm whether they are naturally present in Sea grapes or as contaminants. References indicating the activity of the health benefits of each metabolite that have been successfully identified are still very few and limited, so computational molecular docking or *in silico* studies are needed for various disease-causing receptors.

5 Conclusions

The abundance of secondary metabolites and bioactive compounds in Sea grapes *(Caulerpa racemosa)* was obtained from the maceration method's extraction process. The bioactive peptide purified fraction 1 (PF1), as shown in ELWKTF, was also analyzed for its antioxidant and cytotoxicity activity. These metabolites were responsible for high biochemical activity (antioxidants, scavenging, and reducing) and have good prospects of cytotoxicity. The study revealed that *C. racemosa* contained antioxidant nutrients, metabolites, and bioactive peptides (Graphical Abstract); these factors make it a promising functional food and pharmaceutical. From a future perspective, *C. racemosa* is a potential candidate for development as a functional food and other nutraceutical applications, including pharmaceuticals.

6 Patents

Patent Number S00202107179 (Fahrul Nurkolis is a patent holder, https://pdki-indonesia.dgip.go.id/detail/S00202107179?type=patent&keyword=Formula+Anggur+Laut).

Abbreviations

ABTS = 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **BPs** = Bioactive Peptides; **DENV** = Dengue virus; **DMEM** = Dulbecco's Modified Essential Medium; **DMSO** = Dimethyl sulfoxide; **DPPH** = 2,2-diphenyl-1-picrylhydrazyl; **ELWKTF**: Glu-Leu-Trp-Lys-Thr-Phe; **EM** = Macerated Extract (Extract Maceration); **ES** = Soxhletated Extract (Extract-Soxhletation); **ESI** = Electrospray ionization; **FBS** = Fetal bovine serum; **GSH** = Glutathione; **HPLC** = High Performance Liquid Chromatography; **HRMS** = High Resolution Mass Spectrometry; **IUPAC** = International Union of Pure and Applied Chemistry; **MTT** = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PS** = Protein Supernatant; **PSN** = Penicillin-Streptomycin-Neomycin; **PUFAs** = Polyunsaturated fatty acids; **ROS** = Reactive Oxygen Species.

Declarations

Author Contributions or Authors CRediT (Contributor Roles Taxonomy): FN and HH: conduct experiments, analyzed data, write the manuscript, design research, and conceptualize ideas; while HH, VMY, MY, RJK: contribute to data analysis, critiquing manuscript, interpret manuscript results, assisting in the processing of data, as well as helping to revise and graphical abstract editing. IWH, WBG, SR, NAT, NM, NS, AT, RK: critiquing, writing – review & editing manuscript. TET, RK, SR, EI, and CFT reviewed and edited the final manuscript text. All authors have read and also approved this final manuscript.

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Figures

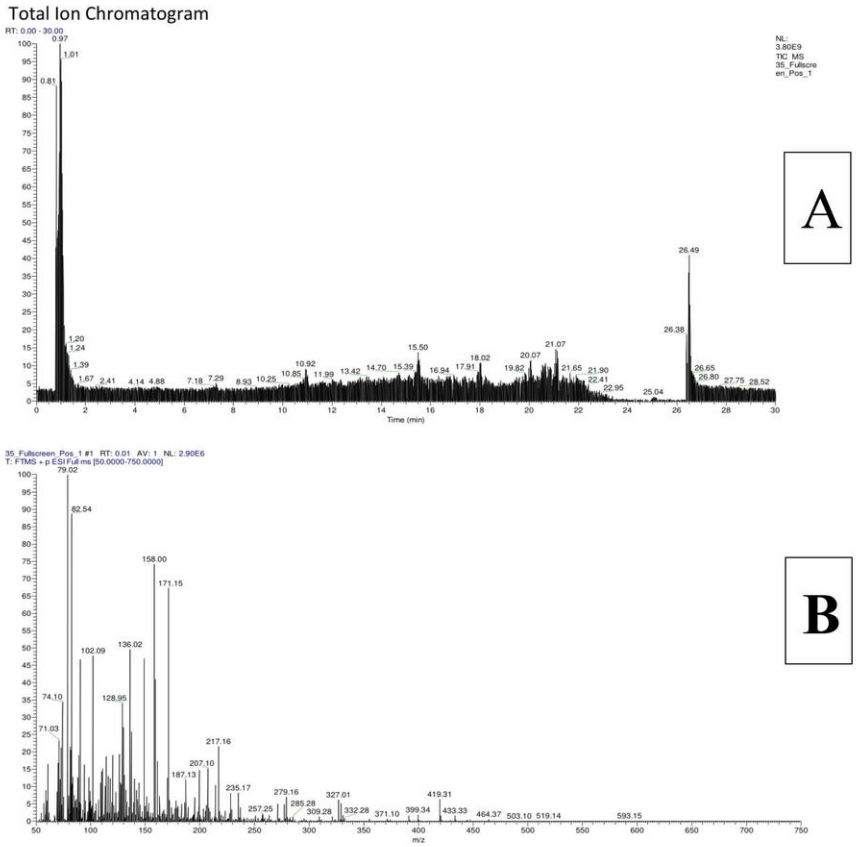

Figure 1

1A. Total Ion Chromatogram (TIC) of Macerated Sea grapes (*C. racemosa*) Extract by HPLC; **1B.** Mass spectrum of Macerated Sea grapes (*C. racemosa*) Extract by MS/MS. S#: Scan number; RT: Retention time; AV: Averaged (followed by the number of averaged scans); SB: Subtracted (followed by subtraction information); NL: Neutral loss; T: Scan type; F: Scan filter.

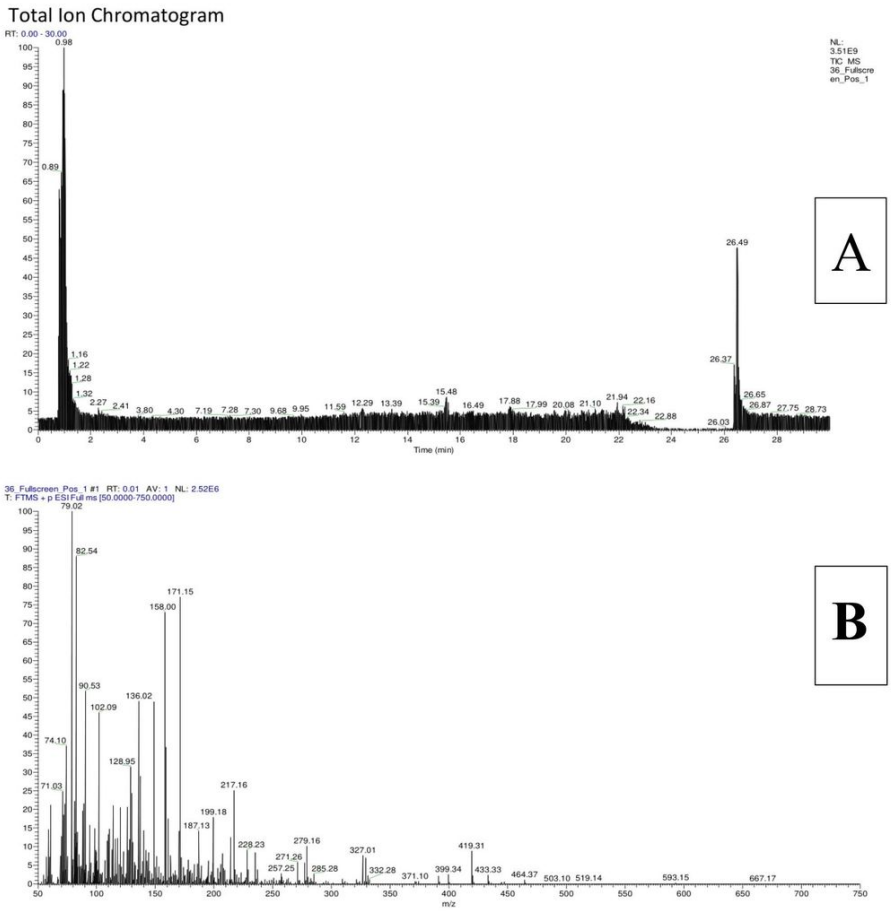

Figure 2

2A. Total Ion Chromatogram (TIC) of Soxhlet Extraction of Sea grapes (*C. racemosa*) Extract by HPLC; **2B.** Mass spectrum of Soxhlet Extraction of Sea grapes (*C. racemosa*) Extract by MS/MS . S#: Scan number; RT: Retention time; AV: Averaged (followed by the number of averaged scans); SB: Subtracted (followed by subtraction information); NL: Neutral loss; T: Scan type; F: Scan filter.

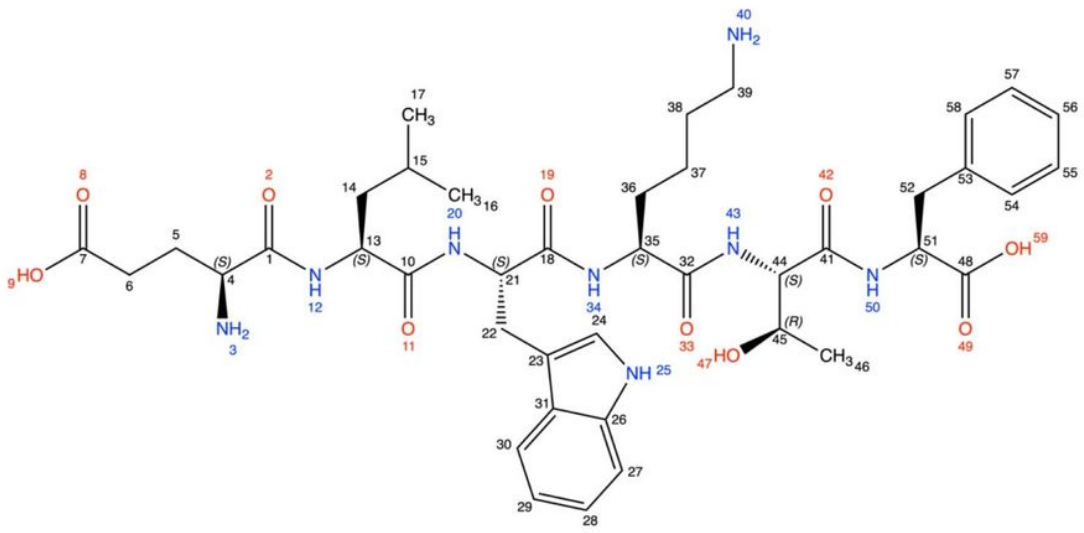

Figure 3

ELWKTF Analysis and Visualization Results Using ChemDraw 21.0.0 Macbook Version.

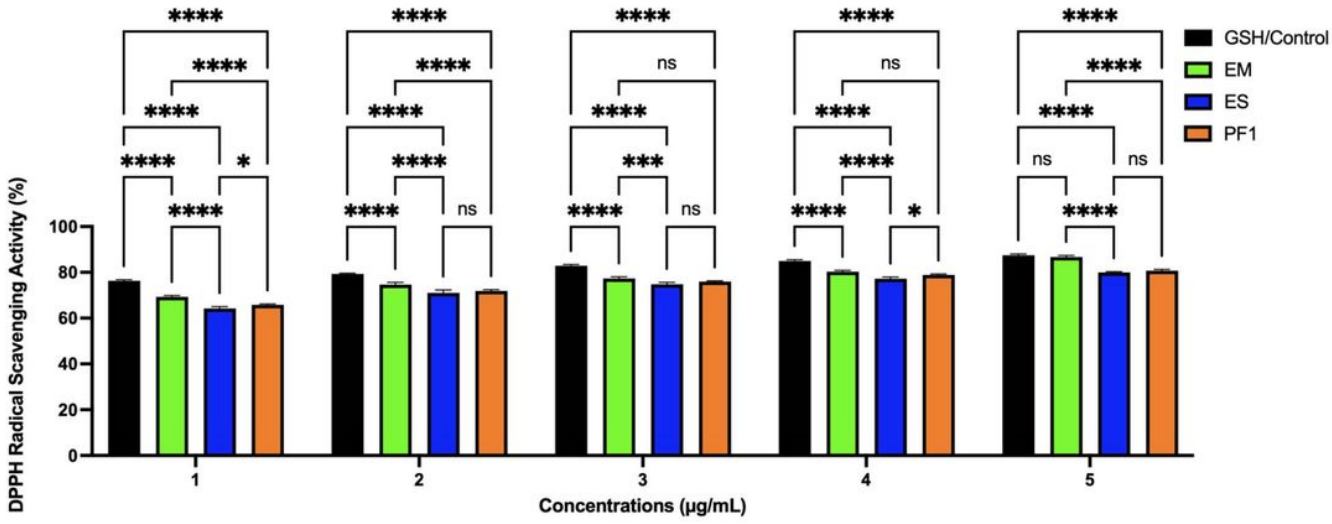

Figure 4

Comparison of DPPH Radical Scavenging Activity. * = p 0.0374; *** = p 0.0002; **** = p < 0.0001; ns = Not significant (p > 0.05).

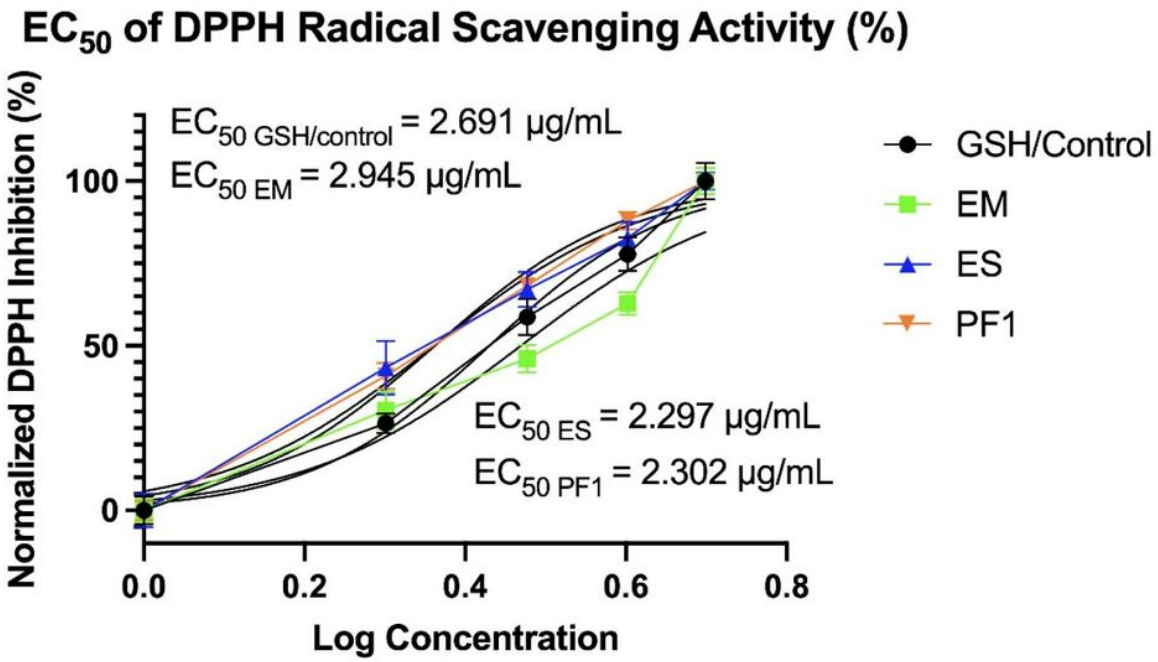

Figure 5

Dose-response Curve of EM, ES, PF1, and GSH Regarding DPPH Radical Scavenging Activity. EC_{50} = Half-maximal effective concentration. EM = Macerated extract (Extract maceration); ES = Soxhletated extract (Extract-soxhletation); PF1= Purified fraction 1.

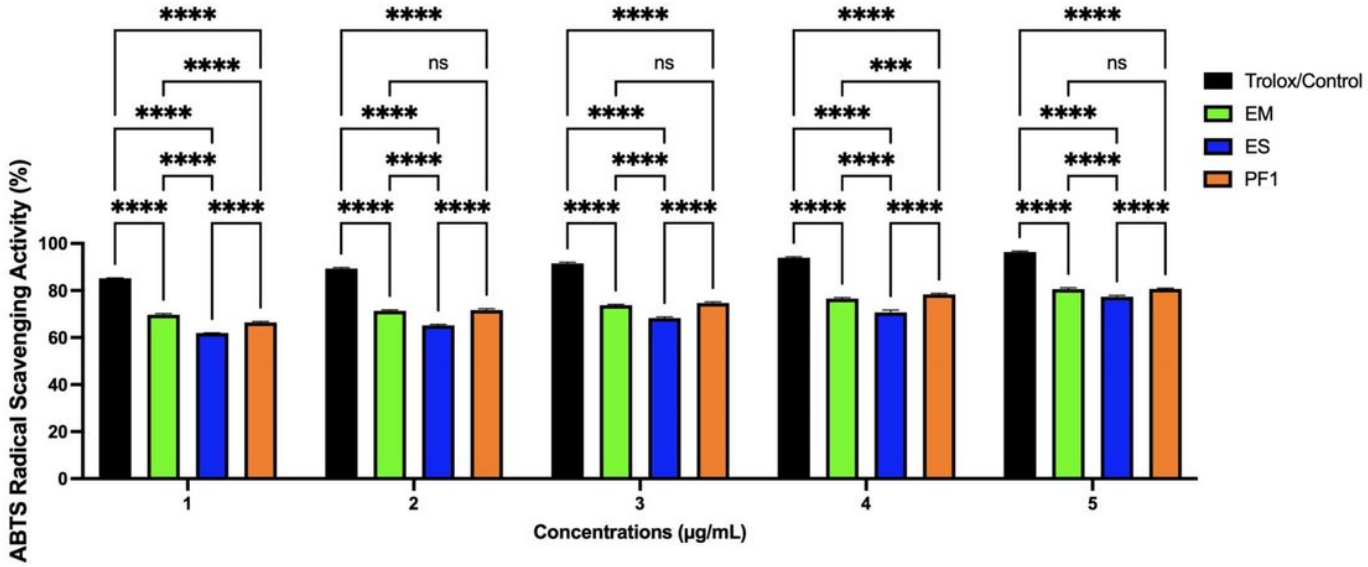

Figure 6

Comparison of ABTS Radical Scavenging Activity. *** = p 0.0004; **** = p < 0.0001; ns = Not significant (p > 0.05).

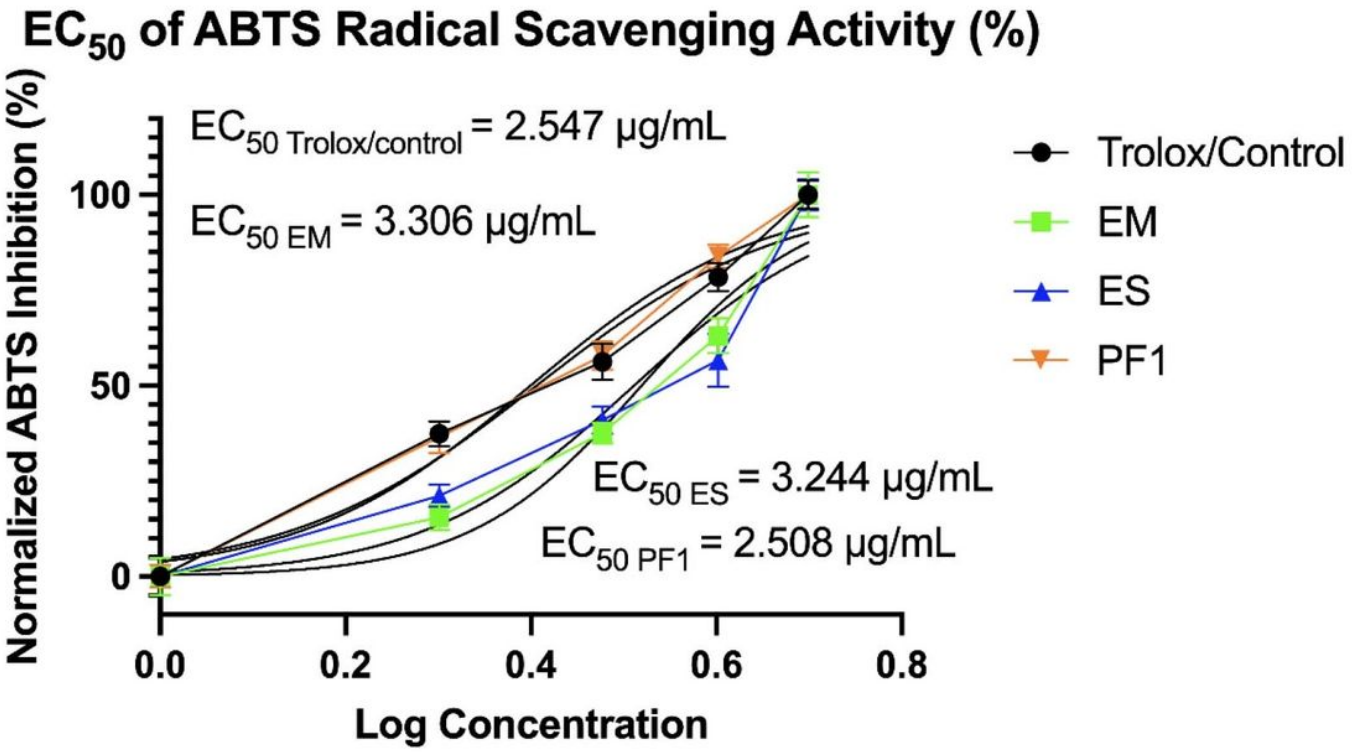

Figure 7

Dose-response Curve of EM, ES, PF1, and Trolox Regarding ABTS Radical Scavenging Activity. EC_{50} = Half-maximal effective concentration. EM = Macerated extract (Extract maceration); ES = Soxhletated extract (Extract-soxhletation); PF1= Purified fraction 1.

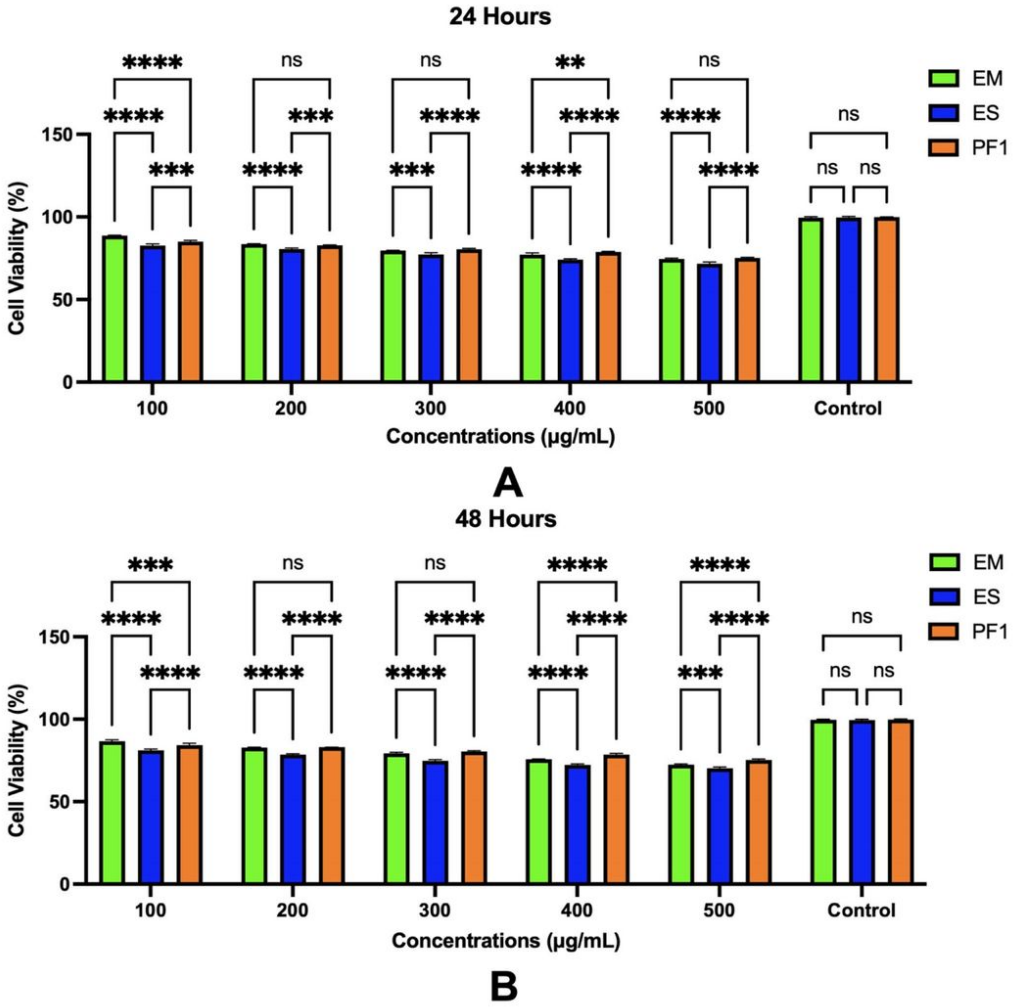

Figure 8

Percentage of cell viability evaluation of *C. racemosa* cytotoxicity using MTT assay on the human Caucasian skin fibroblast cell line. **A=** Cytotoxicity Evaluation using MTT Assay at 24 Hours. **B**= Cytotoxicity Evaluation using MTT Assay at 48 Hours. ** = p 0.0096; *** = p 0.0002; **** = p <0.0001 ; ns = not significant (p = 0.2745). EM = Macerated extract (Extract maceration); ES = Soxhletated extract (Extract-soxhletation); PF1= Purified fraction 1.

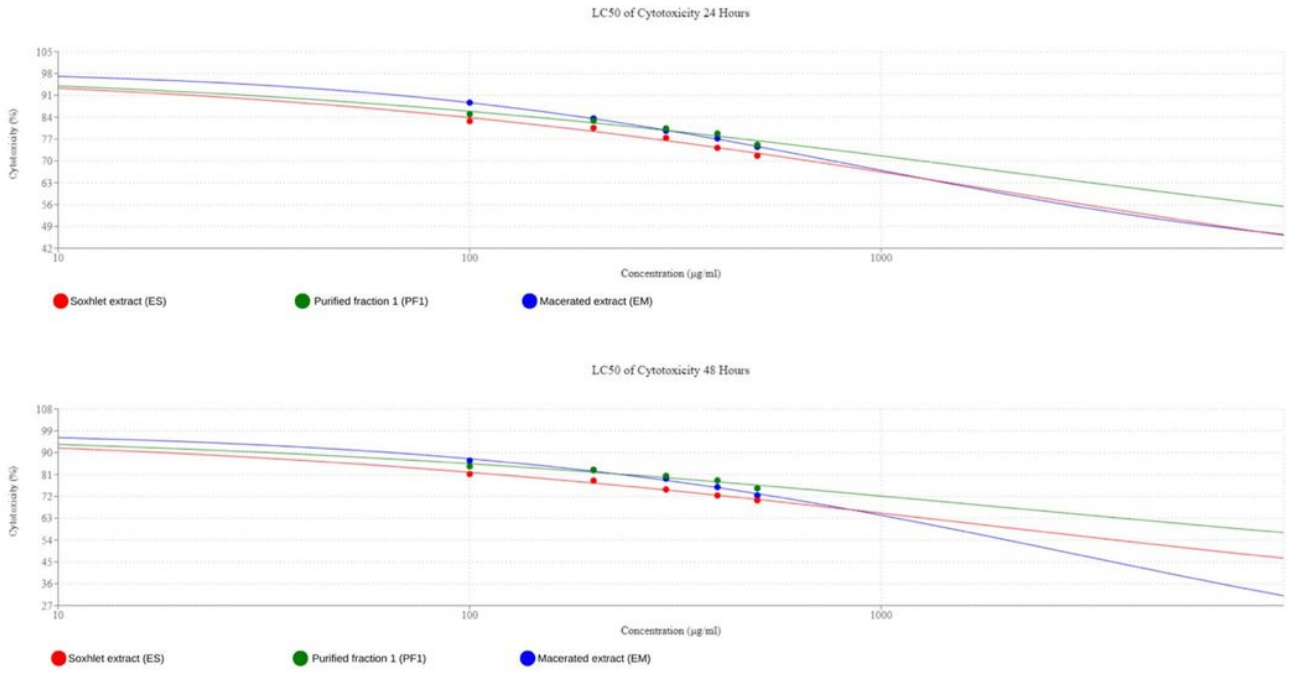

Figure 9

Log Concentrations of *C. racemosa* Cytotoxicity Assay. Macerated Extract (EM), Soxhletated Extract (ES), Purified Fraction 1 (PF1). LC_{50} = half-maximal lethal concentration (lowest concentration of samples that inhibit 50% of cells).

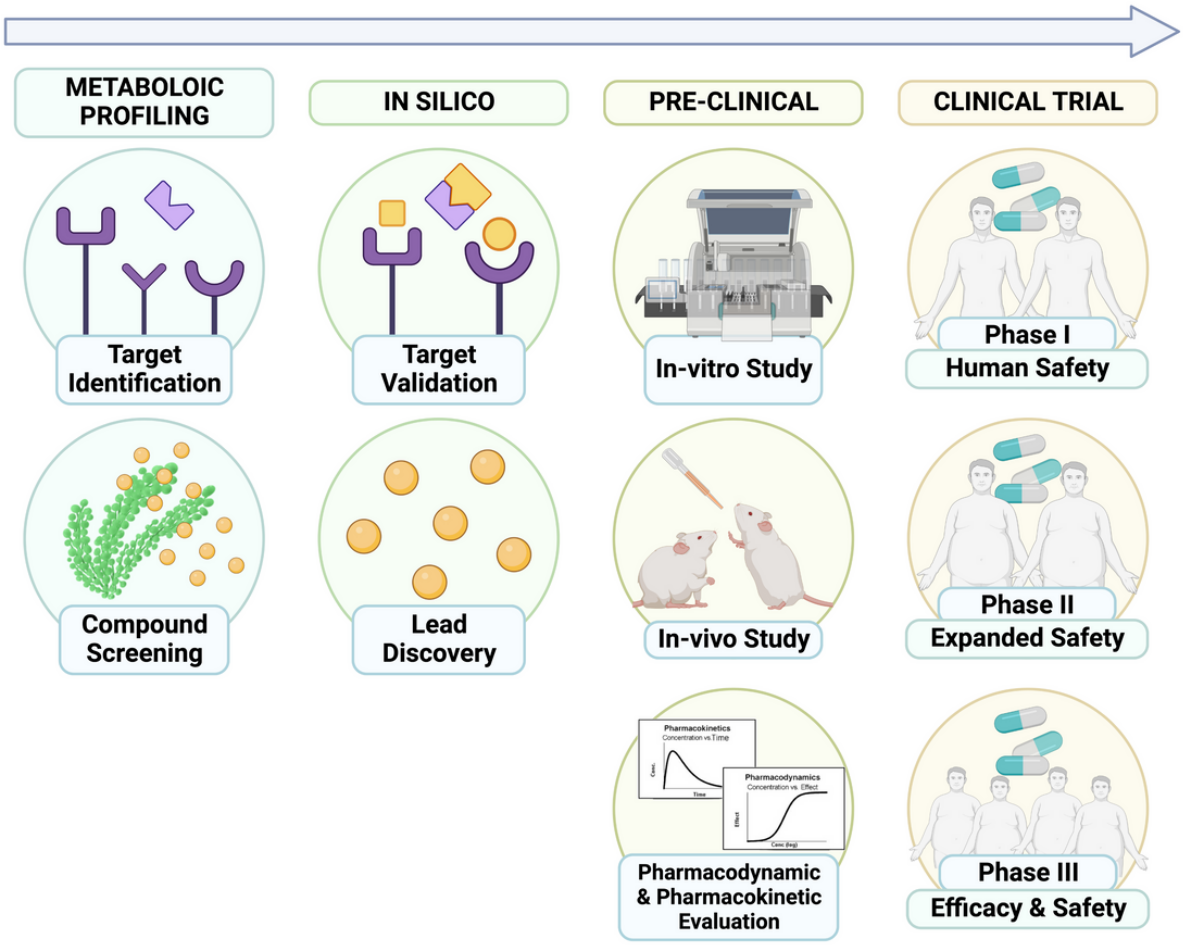

Figure 10

The Flow of Research and Product Development of *C. racemosa* in the Future.

Supplementary Files

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- TableSupplementary1.AntioxidantReviewofMetabolitesCaulerparacemosa.docx
- GraphicalAbstractMetabProfiling2.png