Trop J Nat Prod Res, September 2025; 9(9): 4343 - 4352 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Metabolite Profiling of the Ethanol and Ethyl Acetate Extracts of *Ulva lactuca* using UPLC-QToF-MS/MS

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ARTICLE INFO

Article history:
Received 07 February 2025
Revised 28 July 2025
Accepted 06 August 2025
Published online 01 October 2025

ABSTRACT

Ulva lactuca, commonly known as sea lettuce, is a marine green algae known for its diverse pharmacological activities such as antioxidant, antimicrobial, antidiabetic, wound healing, and anti-inflammatory activities. This algae can be found in intertidal zones, where it is attached to rocks. Its growth is quite fast due to its rapid absorption of nutrients from seawater. This study aimed to determine the metabolite profile of *Ulva lactuca* originating from Rembang, Central Java, Indonesia, a region where *Ulva lactuca* has not been explored. *Ulva lactuca* was extracted with 96% ethanol and ethyl acetate using Ultrasound-Assisted Extraction (UAE) method. The extracts obtained were analyzed for their metabolites profile using the Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight-Tandem Mass Spectrometry (UPLC-QToF-MS/MS). The resulting data were processed with MassLynx 4.1 software and further identified using the ChemSpider and MassBank databases. A total of 66 compounds were detected, comprising 43 known compounds and 23 unidentified compounds. The predominant compound in both the 96% ethanol and ethyl acetate extracts was 1-carboxy-3-hydroxyadamantane, with a percentage peak area of 21.71% and 19.87%, respectively.

Keywords: Metabolite profiling, Sea lettuce, *Ulva Lactuca*, UPLC-QToF-MS/MS.

Introduction

Indonesian marine waters are a habitat for various algae, such as green algae, which are the most diverse group with 7000 species. Currently, green algae are used as food ingredients, such as gelling agents, animal feed, fertilizers, and applications in the biomedical, chemical, and agricultural sectors.[1] The exploration of algae remains an essential area of research due to its vast potential as a raw material in both industrial and agricultural sectors. Whether in fresh or dried form, algae offers numerous applications, including bioactive compounds for pharmaceuticals, natural fertilizers, biodegradable packaging, and food additives.[2] Algae produces numerous secondary metabolites with antioxidants,[3-5] antimicrobial,[6-9] antiproliferative and antihyperlipidemics,[10] antidiabetic,[11-14] anticancer,[15-17] anti-inflammatory,[18,19] antiaging,[20] and wound healing properties.[21] Algae also serve as a prebiotic for the colon and contains many polyunsaturated fatty acids, vitamins, and minerals.[22] Indonesia, as an archipelagic nation with vast marine resources, presents significant opportunities for the advancement of pharmaceutical science. The rich biodiversity of its coastal and marine ecosystems, including algae and other marine organisms, offers a valuable source of bioactive compounds with potential applications in pharmaceutical, nutraceutical, and medical research. Algae have several advantages; including high productivity, absence of seasonal variations, ease of extraction, thereby having potential value as raw materials and nutraceuticals for food and pharmaceutical industry.[23]

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Citation: Prasetyanti IK, Damayanti A. Metabolite profiling of the ethanol and ethyl acetate extracts of *Ulva lactuca* using UPLC-QToF-MS/MS. Trop J Nat Prod Res. 2025; 9(9): 4343 – 4352 https://doi.org/10.26538/tjnpr/v9i9.33

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Ulva lactuca (sea lettuce) is one of the most abundant and widely distributed green algae in several regions. In Indonesia, coastal communities commonly use this algae as a source of food. This algae contains the metabolites ulvan which is a sulfate polysaccharide,[24] and contains many nutrients such as iron, protein (15%), iodine, vitamins (A, B1, and C). Phytochemicals that are also found include fatty acids[25], minerals, and fiber,[26] carbohydrates,[27] alkaloids, flavonoids, phenolics, tannins, quinones, mono and sesquiterpenoids,[28] rhamnose, glucuronic acid, xylose, sulfate, glucose, and iduronic acid.[29] Phenolic and flavonoid compounds present in *Ulva lactuca* include catechin, chlorogenic acid, caffeic acid, routine, ellagic acid, quercetin, and camphepherol.[30]

Secondary metabolites are chemical compounds derived from plant metabolic processes that, while not essential for growth or reproduction, serve to enhance the plant's adaptability and survival by providing selective advantages such as defense against herbivores, pathogens, or environmental stress. For example, these metabolites can be used to inhibit the growth of other competing plants, and as self-protection.[31] Currently, more than 100,000 secondary metabolites have been identified, and they are classified based on their chemical structure and functional characteristics such as terpenes, flavonoids, alkaloids, saponins, polyphenols, tannins, anthraquinones, cyanohydrin glycosides, and essential oils.[32]

The selection of ethanol as a solvent for extraction is based on its universal and selective nature in dissolving the desired compounds, as well as its ability to degrade non-polar plant cell walls and cause polyphenol compounds to exit the cell walls.[33] On the other hand, the selection of ethyl acetate as a solvent is based on its semi-polar nature, which is expected to be able to extract non-polar compounds found in *Ulva lactuca*.

Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) is an advanced evolution of the LC-MS technique, designed for high-resolution metabolite profiling. This method separates compounds based on differences in polarity while simultaneously fragmenting them for detailed structural identification, enabling precise and efficient chemical characterization.[34] This instrument has many advantages, including increased efficiency of compound separation, accelerated analysis time, ability to separate

smaller compounds, small amount of samples needed, more accurate monoisotopic mass measurements, high-resolution spectra for confirmation of target compounds and unknown compounds, and faster acquisition of results without reducing the quality of high mass resolution.[35]

Research on the secondary metabolites profile of *Ulva lactuca* aims to provide scientific insights into the chemical composition of its 96% ethanol and ethyl acetate extracts, specifically from samples collected in Rembang Central Java, Indonesia. Metabolite profiling is essential to determine the entire content of secondary metabolites contained in 96% ethanol extract and ethyl acetate extract of *Ulva lactuca*. This metabolite profiling will serve as a foundation for further research by identifying the compounds present in *Ulva lactuca* extracts, and help to determine which bioactive components are associated with each solvent, enabling the selection of the most suitable extraction method for future research. By understanding the solvent-specific distribution of bioactive compounds, researchers can optimize extraction processes for targeted applications in pharmaceuticals, biotechnology, and other industries.

Materials and Methods

Collection and identification of Ulva lactuca

Ulva lactuca was sourced from Rembang Beach, Central Java, Indinesia. The plant sample was identified at the Fish Health and Aquatic Environment Management Service Unit, Faculty of Fisheries and Marine Sciences, University of Airlangga, Indonesia where the voucher number 67/ULMKILP/UA.FPK/12/2023 was assigned.

Extraction of Ulva lactuca

The plant material was sorted, air-dried, and ground into a fine powder. The powdered plant sample (100 g each) was mixed separately with 96% ethanol (1 L) and ethyl acetate (1 L) in an Erlenmeyer flask and subjected to Ultrasonic-Assisted Extraction (UAE) (Soltec Sonica 5300EP S3, Italy) in three cycles of 10 minutes each, with 5-minute breaks between cycles. The resulting liquid extract was filtered, and the filtrate was evaporated using a rotary evaporator (Hei-VAP Core motor lift model, Heidolph G3, Germany) at 50°C with a rotation speed of 70 rpm until a concentrated extract was obtained. The concentrated extract was weighed and the percentage yield calculated.

Metabolite profiling of the extracts

Metabolite profiling of the 96% ethanol and ethyl acetate extracts was carried iut using the UPLC-QToF-MS/MS ACQUITY UPLC® H-Class System with an MS Xevo G2-S QToF detector (Waters, USA) at the Indonesia Police Forensic Laboratory Center. Sample preparation was done by solid phase extraction (SPE) technique with dichloromethane and methanol. The prepared extract (100 ppm) was injected into the UPLC-MS instrument in a 5 µL volume using a micro syringe. Samples were separated on an ACQUITY BEH C18 column (1.7 µm; 2.1 x 50 mm). The mobile phase consisted of acetonitrile + 0.05 % formic acid and water + 0.05 % formic acid at a flow rate of 0.2 mL/min. The sample analysis were in the form of total ion chromatogram (TIC) obtained from electrospray ionization in a posite mode (ESI+). The TIC was analyzed using MassLynx 4.1 software. The analysis started by selecting the BPI chromatogram menu in the display toolbar. On the displayed dialog box, the process was selected and then integrated. After the completion of the process, the detected peaks were selected in the edit toolbar to obtain the retention time (RT), peak height, and peak area. The analysis was continued by entering RT data from the TIC through the spectrum toolbar to obtain the molecular formula of each compound. The results obtained were in the form of measured mass (50 - 1500 amu) from the TIC of each extract. The value was then entered into the toolbar tools, and the calculated mass was immediately selected within a range of + 0.0005 from the measured mass $[M + H]^+$, allowing the molecular formula of the compound to be determined. Finally, the molecular formula obtained was confirmed by matching molecular masses, MS/MS fragmentation patterns, and chemical structures to existing data in the ChemSpider and MassBank online databases.

Results and Discussion

Extraction and yield of Ulva lactuca

The extraction process in this study was carried out using ultrasonic wave-assisted extraction (UAE) (ultrasonication) technique. Ultrasonication is one of the green technologies widely used today in the extraction process. This method enhances efficiency by facilitating the breakdown of cell structures, and improving compound extraction while reducing solvent usage and processing time. Generally, ultrasonic waves can cause cavitation phenomena that produce mechanical deformation force that breakdown cell walls.[36-38] This process reduces temperature, solvent concentration, the use of organic solvent, and the time of the extraction process. UAE uses a smaller amount of solvent compared to traditional extraction methods such as maceration, and the extraction process requires a shorter time. The extract yield is influenced by the extraction method, selection of extracting solvent, temperature, and duration of extraction. From the results of the extraction process, a thick ethanol extract was obtained with a yield of 1.75%, and a thick ethyl acetate extract with a yield of 1.81%.

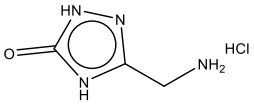
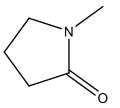
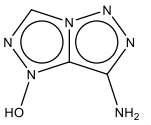
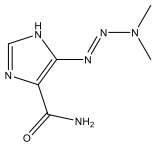
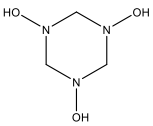
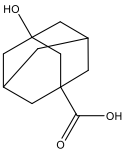
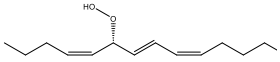
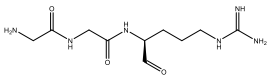
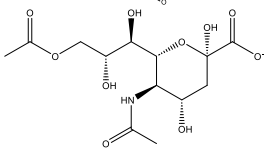
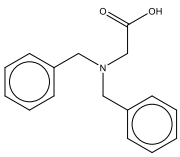
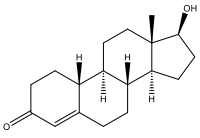
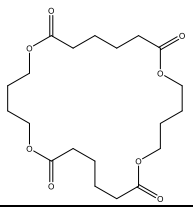
Metabolite profile of Ulva lactuca

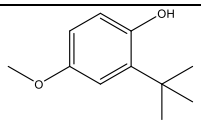
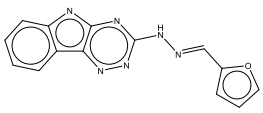
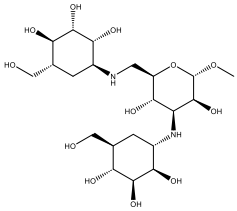
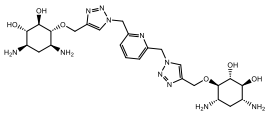
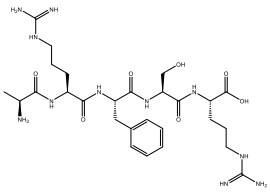
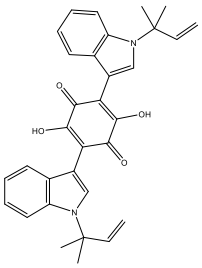
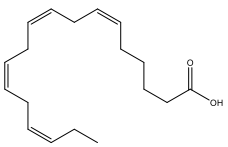
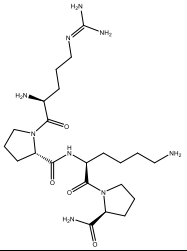
The chromatogram obtained from LC-MS/MS Q-ToF was interpreted using MassLynx 4.1 software to obtain the m/z spectrum of the compound from each detected peak. After obtaining the molecular formula of the compound, confirmation was done using the ChemSpider and MassBank online databases. The chromatogram obtained from LC-MS/MS Q-ToF analysis of *Ulva lactuca* 96% ethanol and ethyl acetate extracts are presented in Figures 1 and 2, respectively. The LC-MS/MS Q-ToF analysis of *Ulva lactuca* yielded 32 components in the 96% ethanol extract, of which 23 were of known structures and 9 of unknown structures (Table 1). Some of the known compounds have been reported to have pharmacological activity. These compounds include 1-Methyl-2-pyrrolidinone, reported as an anti-inflammatory agent,[39] Dacarbazine shown to inhibit metastatic malignant melanoma,[40,41] Nandrolone which is a class of steroids reported as an anabolic androgen steroid,[42] and Stearidonic acid in the carboxylic acid group reported to have anti-inflammatory, cancer preventive, hepatoprotective, and antihistaminic activities.[43]From the LC-MS/MS Q-ToF analysis of *Ulva lactuca* ethyl acetate extract, 34 components were obtained, of which 20 were of known structures, and 14 were of unknown structures (Table 2). Some of the known compounds have been shown to possess pharmacological activities, including antileukemic activity reported for Piperidone,[44] anti-depressant and muscle relaxant reported for Mephenesin,[45] anti-Alzheimer's activity for β-Estradiol, a steroidal compound, [46] and an androgenic effect for testosterone decanoate.[47] Two compounds, namely Butylated hydroxyanisole (BHA) or 2-(1,1-dimethyl ethyl)-4-(methyloxy)phenol a known antioxidant,[48] and 1-carboxy-3-hydroxyadamantane were identified in both extracts. The major compound in the two extracts was 1-carboxy-3-hydroxyadamantane with a percentage peak area of 21.71% and 19.87% in the 96% ethanol and ethyl acetate extracts, respectively.

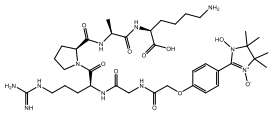
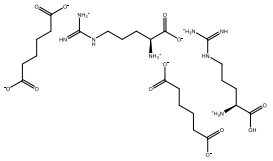
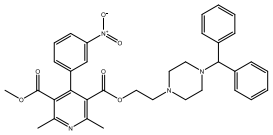
Previous studies on the metabolite profile of *Ulva lactuca* extract using Ultra High-Performance Liquid Chromatography High-Resolution Mass Spectrometry (UHPLC-ESI-HRMS) reported the presence of fatty acids, phenolic acids, pigments, flavonoids, and steroids in *Ulva lactuca* extract.[49,50] this aligns with the results of the present study which also identified fatty acids such as propanoic acid and α-linolenic acid in the ethyl acetate extract of *Ulva lactuca*.

Out of the total 66 compounds detected, 43 of these were identified, while 23 could not be identified, and were indicated as unknown compounds in each of the extracts. Unknown compounds are those that cannot be identified in existing databases. These compounds may be impurities or degradation products, or they could be novel compounds that have not been recorded. These unknown compounds could become particularly relevant when present in high concentrations.[51]

Table 1: Compounds identified in the 96% ethanol extract of *Ulva lactuca*

No.	Retention Time (min)	% Area	Measured m/z	Molecular Formula	IUPAC Name	Chemical Structure
1	1.15	3.22	151.0352	$C_3H_7N_4OCl$	5-(Aminomethyl)-1,2-dihydro-3*H*-1,2,4-triazol-3-one hydrochloride	
2	2.69	0.34	100.0760	C_5H_9NO	1-Methyl-2-pyrrolidinone	
3	3.83	0.15	141.0560	$C_3H_4N_6O$	7-Amino-1*H*-[1,2,4]triazolo[4,3-c][1,2,3]triazol-1-ol	
4	4.20	0.61	183.1027	$C_6H_{10}N_6O$	Dacarbazine	
5	4.57	0.16	136.0765	$C_3H_9N_3O_3$	1,3,5-Triazinane-1,3,5-triol	
6	5.37 and 5.80	5.54 and 16.17	197.1186	$C_{11}H_{16}O$	1-carboxy-3-hydroxyadamantane	
7	6.55	0.07	309.2039	$C_{18}H_{29}O_4$	(9Z,11E,13S,14Z)-13-Hydroperoxy-9,11,14-octadecatrienoate	
8	7.01	1.16	273.1720	$C_{10}H_{20}N_6O_3$	Glycylglycyl-L-argininal	
9	7.61	0.68	369.1519	$C_{13}H_{24}N_2O_{10}$	Ammonium (6R)-5-acetamido-6-[(1R,2R)-3-acetoxy-1,2-dihydroxypropyl]-3,5-dideoxy-β-L-threo-hex-2-ulopyranosonate	
10	7.87	0.98	258.1348	$C_{16}H_{17}NO_2$	N,N-Dibenzylglycine	
11	8.20	0.32	275.2019	$C_{18}H_{26}O_2$	Nandrolone	
12	8.75 and 11.01	2.57 and 2.40	401.2175	$C_{20}H_{32}O_8$	1,6,13,18-Tetraoxacyclotetracosane-7,12,19,24-tetrone	

13	9.06	6.05	181.1236	$C_{11}H_{16}O_2$	3-BHA or 2-(1,1-dimethylethyl)-4-(methyloxy)phenol	
14	9.45	3.13	279.0945	$C_{14}H_{10}N_6O$	3-[(2E)-2-(2-Furylmethylene)hydrazino]-5H-[1,2,4]triazino[5,6-b]indole	
15	9.76	1.95	513.2679	$C_{21}H_{40}N_2O_{12}$	Methyl 3,6-dideoxy-3,6-bis{[(1S,2R,3S,4R,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)cyclohexyl]amino}-α-D-mannopyranoside	
16	10.35	0.09	590.3167	$C_{25}H_{39}N_{11}O_6$	(1S,2R,3R,4S,6R,1'S,2'R,3'R,4'S,6'R)-3,3'-[2,6-Pyridinediylbis(methylene-1H-1,2,3-triazole-1,4-diylmethyleneoxy)]bis(4,6-diamino-1,2-cyclohexanediol)	
17	10.64	1.76	636.3583	$C_{27}H_{45}N_{11}O_7$	L-Alanyl-L-arginyl-L-phenylalanyl-L-seryl-L-arginine	
18	11.32	1.44	708.4155	$C_{31}H_{53}N_{11}O_8$	Unknown	-
19	11.91	6.30	507.2281	$C_{32}H_{30}N_2O_4$	Asterriquinone / Asperphenamate	
20	12.22	0.84	327.0789	$C_{16}H_6N_8O$	Unknown	-
21	12.46	0.24	908.5192	$C40H73N7O_{16}$	Unknown	-
22	12.77	1.20	277.2176	$C_{18}H_{28}O_2$	Stearidonic acid	
23	13.06	0.08	496.3395	$C_{22}H_{41}N_9O_4$	N~5~-(Diaminomethylene)-L-ornithyl-L-prolyl-L-lysyl-L-prolinamide	

24	13.49	2.99	482.3603	$C_{22}H_{43}N_9O_3$	Unknown	-
25	13.78	0.32	818.4513	$C_{37}H_{59}N_{11}O_{10}$	N-{[4-(1-Hydroxy-4,4,5,5-tetramethyl-3-oxido-4,5-dihydro-1H-imidazol-2-yl)phenoxy]acetyl}glycyl-L-arginyl-L-prolyl-L-alanyl-L-lysine	
26	14.13	0.78	495.2924	$C_{18}H_{38}N_8O_8$	Bis[(2S)-2-ammonio-5-{[ammonio(imino)methyl]amino}pentanoate] adipate	
27	15.05	7.69	609.2708	$C_{35}H_{36}N_4O_6$	2-[4-(Diphenylmethyl)-1-piperazinyl]ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate	
28	15.45	6.67	609.2714	$C_{32}H_{28}N_{14}$	Unknown	-
29	15.91	2.83	962.6052	$C_{49}H_{87}NO_{17}$	Unknown	-
30	16.22	8.74	1162.7095	$C_{59}H_{104}NO_{21}$	Unknown	-
31	17.16	5.45	413.2662	$C_{22}H_{32}N_6O_2$	Unknown	-
32	18.22	7.08	813.5833	$C_{44}H_{81}NO_{12}$	Unknown	-

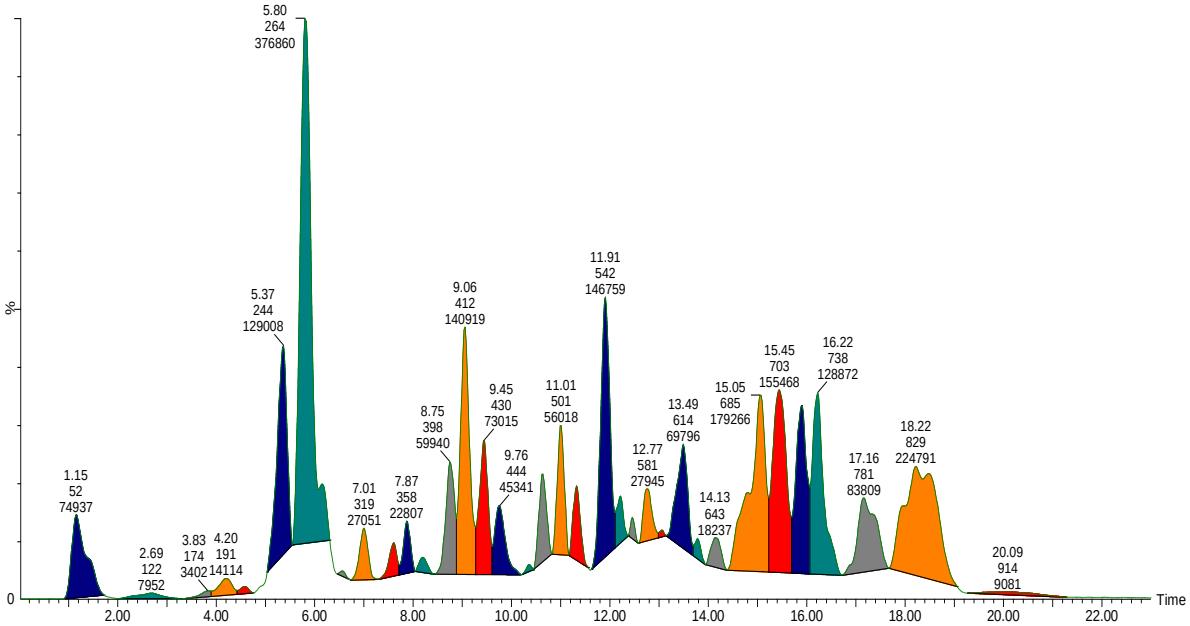


Figure 1: Total ion chromatogram (TIC) of 96% ethanol extract of *Ulva lactuca*

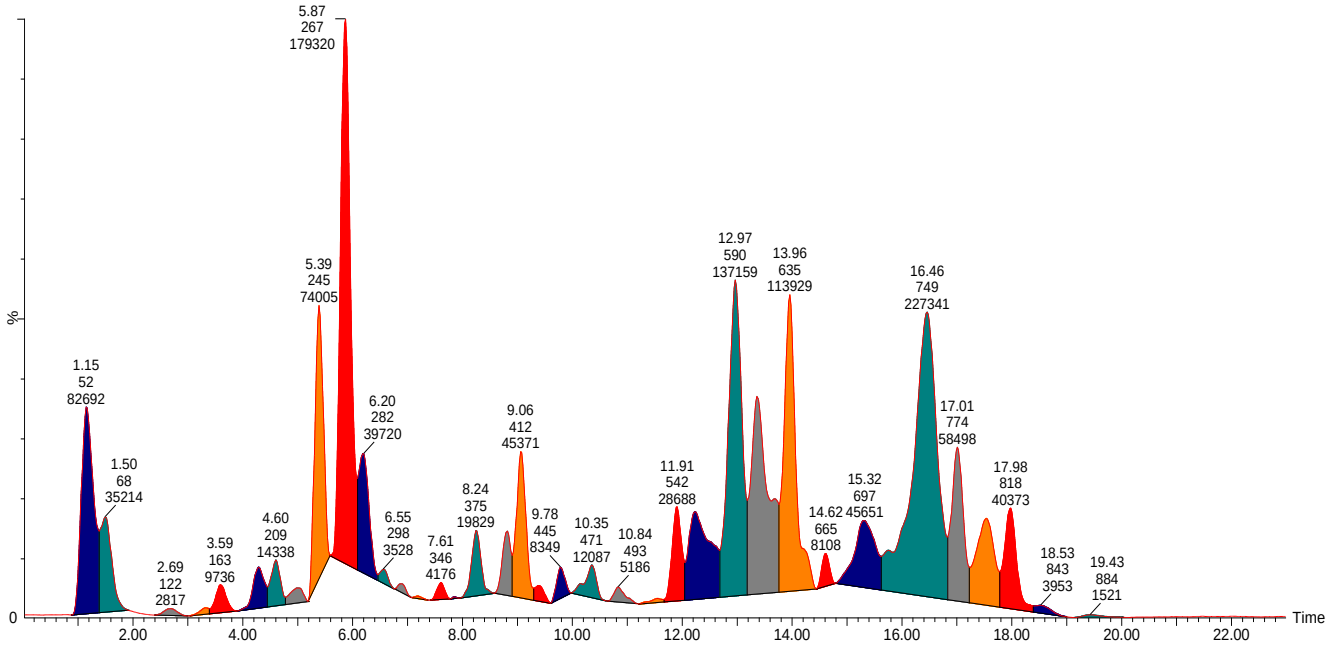
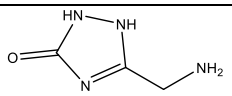
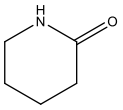
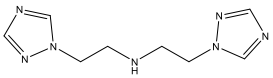
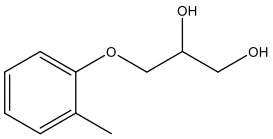
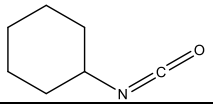
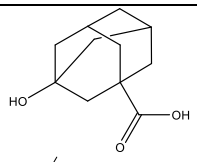
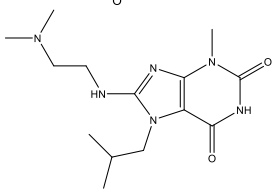
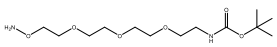
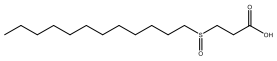
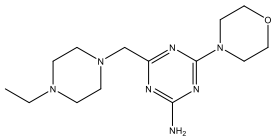
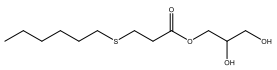
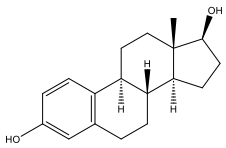
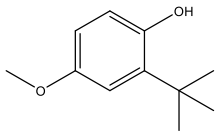
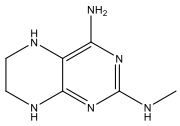
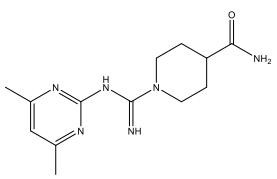
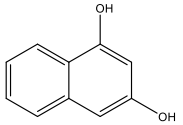
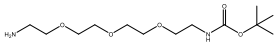
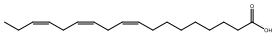
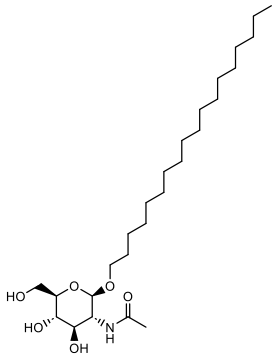
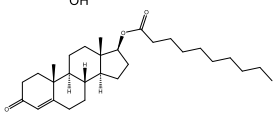


Figure 2: Total ion chromatogram (TIC) of ethyl acetate extract of *Ulva lactuca*

Table 2: Compounds identified in the ethyl acetate extract of *Ulva lactuca*

No.	Retention Time (min)	% Area	Measured m/z	Molecular Formula	IUPAC Name	Chemical Structure
1	1.51	5.61	151.0350	$C_3H_7ClN_4O$	5-(aminomethyl)-1,2-dihydro-1,2,4-triazol-3-one	
2	1.50	2.39	236.1485	$C_6H_{17}N_7O_3$	Unknown	-
3	2.69	0.19	100.0758	C_5H_9NO	Piperidone	
4	3.32	0.12	208.1336	$C_8H_{13}N_7$	2-(1*H*-1,2,4-Triazol-1-yl)-N-[2-(1H-1,2,4-triazol-1-yl)ethyl]ethanamine	
5	3.59	0.66	831.3460	$C_6H_{25}N_{11}O_7$	Unknown	-
6	4.29	1.01	183.1031	$C_{10}H_{14}O_3$	Mephenesin	
7	4.60	0.97	389.9391	$C_7H_8N_5O_6S_3Cl$	Unknown	-
8	4.99	0.43	126.0925	$C_7H_{11}NO$	cyclohexylisocyanate	

9	5.39, 5.87 and 6.20	5.02; 12.16 and 2.69	197.1185	$C_{11}H_{16}O_3$	1-carboxy-3-hydroxyadamantane	
10	6.55	0.24	309.2069	$C_{14}H_{24}N_6O_2$	8-{[2-(Dimethylamino)ethyl]amino}-7-isobutyl-3-methyl-3,7-dihydro-1H-purine-2,6-dione	
11	6.89	0.14	309.2065	$C_{13}H_{28}N_2O_6$	2-Methyl-2-propanyl [2-(2-{2-[2-(aminooxy)ethoxy]ethoxy}ethoxy)ethyl]carbamate	
12	7.19	0.04	291.1994	$C_{15}H_{30}O_3S$	3-(Dodecylsulfinyl)propanoic acid	
13	7.61	0.28	308.2224	$C_{14}H_{25}N_7O$	4-[(4-Ethyl-1-piperazinyl)methyl]-6-(4-morpholinyl)-1,3,5-triazin-2-amine	
14	7.85	0.01	265.1445	$C_{12}H_{24}O_4S$	2,3-dihydroxypropyl 3-(hexylthio)propionate	
15	8.24	1.34	530.3334	$C_{24}H_{39}N_{11}O_3$	Unknown	-
16	8.82	1.16	273.1859	$C_{18}H_{24}O_2$	*β*-Estradiol	
17	9.06	3.08	181.1236	$C_{11}H_{16}O_2$	3-BHA or 2-(1,1-dimethylethyl)-4-(methyloxy)phenol	
18	9.39	0.31	181.1202	$C_7H_{12}N_6$	N~2~-methyl-5,6,7,8-tetrahydropteridine-2,4-diamine	
19	9.78	0.57	277.1777	$C_{13}H_{20}N_6O$	1-[N-(4,6-dimethylpyrimidin-2-yl)carbamimidoyl]piperidine-4-carboxamide	

20	10.35	0.82	590.3182	$C_{28}H_{47}NO_{12}$	Unknown	-
21	10.84	0.35	161.0612	$C_{10}H_8O_2$	Naphthoresorcinol	
22	11.54	0.11	293.2118	$C_{13}H_{28}N_2O_5$	tert-Butyl (2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamate	
23	11.91	1.94	507.2286	$C_{21}H_{47}N_7O_9S$	Unknown	-
24	12.24	4.18	574.3257	$C_{21}H_{47}N_7O_9S$	Unknown	-
25	12.97	9.30	672.4156	$C_{31}H_{61}NO_{14}$	Unknown	-
26	13.36	7.56	279.2331	$C_{18}H_{30}O_2$	Alpha-Linolenic acid	
27	13.96	7.72	474.3786	$C_{26}H_{51}NO_6$	Octadecyl 2-acetamido-2-deoxy-β-D-glucopyranoside	
28	14.62	0.55	443.3517	$C_{29}H_{46}O_3$	Testosterone decanoate	
29	15.32	3.09	1016.6880	$C_{48}H_{81}N_{21}O_4$	Unknown	-
30	16.46	15.41	663.4540	$C_{40}H_{54}N_8O$	Unknown	-
31	17.01	3.97	806.5684	$C_{44}H_{71}N_9O_5$	Unknown	-
32	17.54	3.46	572.5248	$C_{30}H_{66}N_7O_3$	Unknown	-
33	17.98	8.10	792.5891	$C_{44}H_{73}N_9O_4$	Unknown	-
34	18.53	9.87	763,6735	$C_{42}H_{90}N_4O_5S$	Unknown	-

Conclusion

The present study identified several metabolites in *Ulva lactuca* extracts. There were marked differences in the metabolite profile in the 96% ethanol extract and ethyl acetate extract, which indicated that solvent polarity played a significant role in the type of metabolites extracted. Of the total 43 compounds identified, only two compounds, namely butylated hydroxyanisole and 1-carboxy-3-hydroxyadamantane were present in both extracts. In addition, 1-carboxy-3-hydroxyadamantane was found to be the major compound in both extracts, with percentage peak area of 21.71% and 19.87% in the 96% ethanol and ethyl acetate extracts, respectively. Some of the compounds identified have been shown to have promising pharmacological activities such as anti-inflammatory and antioxidant activities. Further studies are therefore needed to isolate these compounds and determine the biological activities of *Ulva lactuca* extract in greater details.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors express profound gratitude to Hang Tuah University for its support in conducting research both in terms of funds and facilities.

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