Phytochemical composition and characterization of bioactive compounds of the green seaweed *Ulva lactuca* - a phytotherapeutic approach

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Abstract-

The Moroccan coastline is particularly rich in algae and constitutes a reserve of species with considerable economic, social and ecological potential. This work focuses on research and characterization of algae bioactive compounds that can be used in pharmacology or phytopathology.

The biochemical composition of the green alga *Ulva lactuca* (*Ulvophyceae*) was studied by determining the content of phenols, flavonoids, total tannins and chlorophyll. Seven solvents: distilled water, methanol, ethyl acetate, chloroform, benzene, petroleum ether, and hexane were tested for their effectiveness in recovering chemical compounds. The identification of bioactive chemical compounds was determined by Gas chromatography-mass spectrometry (GC-MS).

Phenol content differed from one solvent studied to another, while chlorophyll *a*, *b* and total chlorophyll were determined at 14%, 9.52% and 26% respectively. Carotenoid was present in considerable amount (8.17%).

The experimental results show that methanol is the most effective solvent for recovering bioactive compounds followed by water. Moreover, the green alga *Ulva lactuca* is characterized by a high level of total polyphenols (45±3.24 mg GAE/gDM), average levels of total tannins and flavonoids (22.52± 8.23 mg CE/gDM, 15.49± 0.064 mg QE/gDM) respectively.

The GC-MS analysis gave precisely the compounds contained in the various extracts such as phenolic compounds, fatty acids, terpenenoids, alcohols, alkanes, hydrocarbons and steroids.

All these results represent only a first step in the search for biologically active natural substances from seaweed. Additional tests are envisaged to confirm the bioactivity of seaweed.

Keywords: Algae, GC-MS, maceration, phenolic compounds, *Ulva lactuca.*

I. INTRODUCTION

Algae are a major concern of economic development. Morocco's seaweed industry is very underdeveloped and mainly depends on the export of raw materials. Investing in the region requires an inventory of our algal resources on both sides of the strait. The involvement of technology should make better use of these natural resources [1].

They are considered a source of bioactive compounds due to their ability to produce a wide variety of secondary metabolites with a wide range of bioactive profiles. They represent a natural source of a variety of drugs for pharmaceutical, food and cosmetic applications, including carotenoids, terpenoids, steroids, amino acids, phenols, haloketones, alkanes and cyclic polysulfides. Thus, known as an excellent source of bioactive compounds with a wide range of biological activities, including antibacterial, antifungal, antiviral, antitumor, antioxidant and anti-inflammatory [2, 3].

Moreover, several authors have shown that the chemical composition of algae varies according to species, habitat, maturity and environmental conditions [4, 5].

In this study, we focus on the phytochemical study and the identification of bioactive compounds contained in *Ulva lactuca* which could provide some solutions in the phytopathological and phytopharmaceutical field.

II. MATERIAL AND METHODS

A. *Harvesting and preparation of the algae*

Ulva lactuca (sea lettuce) was harvested in September 2021 at ambient temperature 22°C, on the Moroccan Atlantic coast, at the beach of Mehdia (34°15'44" N latitude, and 6°40'00" W longitude).

Figure 1: Geographic location of the Ulva lactuca sampling site (KENITRA) (Google Earth, 2021)

The collected sample was washed on site with seawater, to remove external material such as sand, epiphytes and contamination from other algae and then placed in polyethylene plastic bags. In the laboratory, the sample was again washed with tap water and dried for 78 hours at 40°C until the weight became constant. After drying, the sample was collected, ground to a fine powder using a mechanical grinder, stored in tightly sealed jars and placed in the refrigerator at 4°C until use.

B. Preparation of algal extracts

The extraction was done cold by maceration (5%) in seven solvents of different polarities; Distilled water (DW), Methanol (Met.), Ethyl acetate (E.ac.), Chloroform (Chlor.), Benzene (Benz.), Petroleum ether (P.Et) and Hexane (Hex.) under magnetic stirring 6000 rpm for one hour and left for 24 hours at room temperature with occasional manual stirring in order to extract as much as possible the polar compounds such as polyphenols. After filtration on Whatman N°.1 paper, the filtrates obtained are stored in dark glass bottles at 4°C until use [6].

C. Phytochemical screening

The presence of the main families of chemical compounds in the extracts was investigated using the tests described by [7]; [8]: carbohydrates (Fehling test), glycosides (modified Borntraëger test), phenolics (ferric chloride test), flavonoids (alkaline reagent test), tannins (Stiasny reaction followed by ferric chloride reaction), carotenoids (CarrPrice reaction), Anthraquinones (Borntraëger test), anthocyanins (NaOH test), proteins (Biuret test), Phytosterols and Triterpenoids (Salkowski test) and saponosides (foam index).

D. Determination of total pigments

Chlorophyll *a*, chlorophyll *b* and total carotenoids were determined as follows according to [9]: 1 g of algal powder was homogenized manually with a pestle and mortar and the pigments were extracted in 10 ml of 100% acetone, the homogenate is stored at -4°C for 18 hours. The extract is centrifuged at 5000 rpm for 10 minutes, the pellet is discarded and the supernatant containing the pigments is recovered. The absorbance is measured at 662, 645 and 450 nm and the pigment concentrations are calculated according to the formulas below:

C_a (chlorophyll *a*) = 11.75 [A_{662}] − 2.35 [A_{645}];

C_b (chlorophyll *b*) = 18.61 [A_{645}] − 3.96 [A_{662}];

Total carotenoid = (1000 [A_{450}] − 2.270 × C_a − 81.4 × C_b) /227

E. Polyphenol content

The polyphenol contents of the extracts are determined by the folin-Ciocalteu method according to Rebereau-Gayon (1968) modified by [10]. To 0.5 ml of each extract, 2.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) diluted to 1/10 in water is added. After vigorous stirring 1ml of a 7.5% sodium carbonate solution was added. The mixture was homogenized and incubated in the dark at room temperature 22±1°C for 2 hours, and the absorbances were then read with a spectrophotometer (UV-VIS SPECTROPHOTOMETER) at 765 nm, the blanks were prepared by replacing the extract with methanol. Three tests were performed for each concentration of test material. A calibration curve based on a gallic acid dilution series (0.01 to 0.2 mg/ml). The same protocol was used to measure the samples. Results are expressed in milligrams of gallic acid equivalent per gram of dry matter (mg GAE/gDM).

F. Flavonoid content

The aluminum trichloride ($AlCl_3$) method was described by [11], is used to quantify flavonoids. A 1ml sample of each sample was added to 1ml of $AlCl_3$ solution (2% in methanol). After 10 minutes of reaction; the absorbance is read at 430 nm. Blanks were prepared by replacing the extract with methanol. Three trials were performed for each concentration of test material. A calibration curve was established from a dilution series of quercetin (40µg/ml). Then the same protocol was undertaken to assay the samples. The results are expressed in milligram equivalent of quercetin per gram of dry extract (mg QE/gDM).

G. Condensed tannin content

Condensed tannins were determined by the vanillin acid method [12]. 2.5 ml of vanillin reagent (equal volume mixture of 8% HCl in methanol and 1% vanillin in methanol) was added to 0.5 ml of each extract. Blanks were prepared by replacing the reagent with the 4% methanol-acid mixture, the tubes were kept at 30°C for 20 minutes and the absorbance was read at 510 nm. A calibration curve based on a catechin dilution series (0.01 to 0.3 mg/ml). The same protocol was used to measure the samples. Results are expressed in milligrams equivalent of catechin per gram of dry matter (mg CE/gDM).

H. Gas Chromatography- Mass Spectrometry (GC-MS)

The analysis of the green algae extracts was performed by automatic injection of 1µL of each extract using an autoinjector into a BRUKER 456 GC EVOQ gas chromatograph equipped with a RXL-5SIL MS column (30m ×0.25mm× 0.25µm film thickness, BURKER, GERMANY), and coupled to the GC (3Q: Triple Quadrupole) operating in electron impact (EI) at 70 eV, scanning the m/z range of 10-600 atomic mass units. The initial temperature of the column was programmed at 35°C, then increased to 300°C with a speed of 5°C/min to be maintained at this temperature for 10 min. The flow rate of helium (He) as carrier gas was 1.5 ml/ min while the temperature of the injection chamber was 300°C [13].

I. Component identification

The relative percentage quantity of each component was calculated by comparing its mean peak area to the total areas. The name and molecular weight of the components were determined. The identification of biological activities of identified compounds is based on phytochemical and ethnobotanical databases of Duke [14] from Agricultural Research Service/USDA.

III. RESULTS AND DISCUSSION

A. Phytochemical screening

In this study, seven solvents of different polarities were used to perform the various phytochemical tests. Test results are summarized in Table 1.

Table 1: Phytochemical screening of *Ulva lactuca* extracts

Compounds	D.W.	M	E.A	C	B	P.E	H
Carbohydrates	++	++	++	++	+	+	+
Triterpenes	++	+++	+	+	-	-	-
Phytosterols	++	+++	++	+	-	-	-
Phenols	++	+++	++	++	+	-	-
Catechic tannins	++	+++	-	+++	-	-	-
Gallic tannins	-	++	++	++	-	-	-
Proteins	-	++	++	++	-	-	-
Glycosides	+++	+++	+	+	+	+	+
Saponins	+	+	+	+	+	+	+
Anthraquinones	+++	+++	+	+	+	+	+
Anthocyanes	-	++	-	-	+	-	+
Flavonoids	++	++	++	+	+	+	+

(+ + +): Strong positive test**; (+ +):** Positif test**; (+):** Weakly positive test**; (-):** Negatif test

In the preliminary phytochemical screening, 12 compounds were tested for their presence or absence in the various extracts of *Ulva lactuca*.

Flavonoids, anthraquinones, carbohydrates, saponins, and glycosides were present in all extracts while triterpenes, phytosterols, tannins, proteins, were completely absent in hexane, etheric, and benzene extracts of Ulva.

With the exception of petroleum ether and hexane, phenolic compounds were observed in the remaining five solvent extracts. Anthocyanins were only detected in the methanol, benzene, and hexane extracts. The results showed that the phytochemical composition varies with the polarity of the solvent.

B. Chlorophyll contents

The pigment composition of *Ulva lactuca* is shown in the following figure:

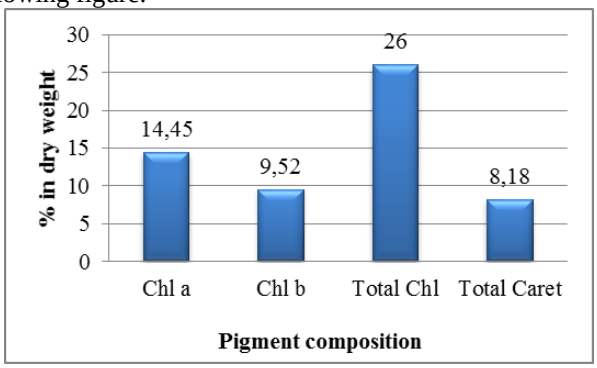

Figure 2: Pigment composition (dry weight percentage) in Ulva lactuca

The results of a study by [15] also show that the green alga *Ulva lactuca* contained high levels of chlorophyll. Thus, the nutrient composition of the growth medium has a significant influence on the production of photosynthetic pigments by *Ulva lactuca*.

C. Determination of the contents of bioactive compounds

The content of total polyphenols is determined from the equation of the linear regression of the calibration curve Y= 11.492X+0.0301 expressed in mg gallic acid equivalent (mg GAE) per g of dry matter (DM).

The analysis of the extracts in phenolic compounds showed that the methanolic extract from the green alga *Ulva lactuca* is rich in these compounds (45± 3.24 mg GAE /gDM) (Figure 3). Our results proved that methanol and water were the best solvents to extract phenolic compounds due to their polarity and the good solubility of these compounds in both solvents, followed by chloroform.

These results, for the green algal extract, are in agreement with the study of [16], which indicates that methanolic algal extracts are the richest in phenolic compounds. This is due to the ability of methanol to inhibit the action of polyphenol oxidase which causes oxidation of polyphenols in plant tissues [17].

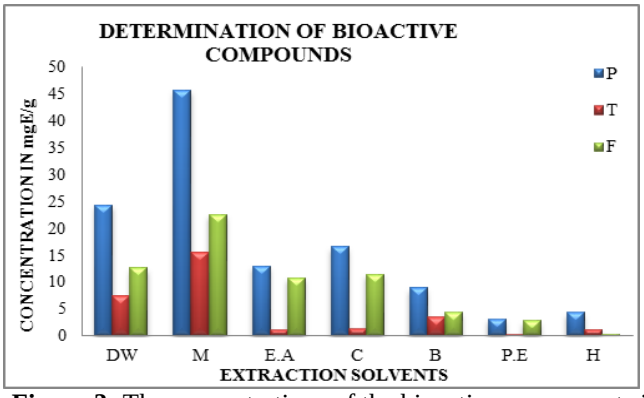

Figure 3: The concentrations of the bioactive components in the different organic solvents

Moreover, the quantitative determination of total flavonoids by the aluminum trichloride method reveals that the methanolic and aqueous extracts are the richest in flavonoids with contents that can reach 15.49±0.064 mg QE/gDM and 7.4±1.102 mgQE/gDM respectively (Figure 3)**.** In accord with that, it is reported that green algae contain flavonoid contents varying between (8.43 and 33.39 mg/gDM) this variation is due to several factors namely species, season and as well as geographical conditions [18].

In the same context, the analysis of algal extracts in total tannins reveals that the methanolic extract has the highest tannin contents with levels that can reach 22.52 ± 8.23 mg CE/gDM.

These results are not confirmed with the data of [19] reporting that the species Ulva lactuca contains contents varying between 40.46 as a maximum value for the chloroform extract and a minimum value of 4.39 mg CE/gDM for the methanol extract. This can be explained by the geographical conditions and the harvest season.

D. Gas Chromatography-Mass Spectrometry

GC-MS analysis of the algal extracts was carried out in order to search for the presence of bioactive substances, which could have therapeutic effects.

The results showed that the methanolic and hexanolic extracts contained important bioactive compounds, mainly steroids, fatty acids and fatty acid esters. In addition, some high molecular weight molecules such as hydrocarbons (tricyclotriacontane, 1, 2-benzenedicarboxylic acid, cyclohexane, undecane) were observed (Tables 2 and 3).

Table 2: Composition of methanolic extract of *Ulva lactuca*

Phytochemical compound	Molecular formula	RT	Area %
D-Limonene	$C_{10}H_{16}$	16.21	2.199%
Nonadecane	$C_{19}H_{40}$	33.1	0.602%
1,1'-bi (cyclohexan)-1'-en-2-	$C_{12}H_{16}O$	33.94	1.416%
Neophytadiene	$C_{20}H_{38}$	42.76	2.151%
Palmitic acid	$C_{16}H_{32}O_2$	46.02	1.689%
2,6-Di-tert-butyl-4-methyl-phenol	$C_{16}H_{26}O_2$	46.59	0.510%
1-Phenanthrenecarboxylic aci	$C_{15}H_{10}O_2$	47.03	0.219%
Phytol	$C_{20}H_{40}O$	49.22	1.679%
Linoleic acid	$C_{18}H_{32}O_2$	50.02	5.819%
9-Octadecenoic acid (Z)	$C_{18}H_{34}O_2$	50.55	2.622%
5,7,9(11)-Androstatriene, 3-	$C_{19}H_{24}O_2$	54.20	0.221%
.gamma.-Sitostenone	$C_{29}H_{48}O$	59.67	31.648%
Z,Z-6,28-Heptatriactontadien	$C_{37}H_{70}O$	65.98	12.466%
Tricyclo[20.8.0.0(7,16)]tria	$C_{30}H_{52}O_2$	73.44	0.244%

The chloroformic extract revealed the presence of phenols, alkanes such as triacontane, tricosane and nonadecane and terpenenoids such as Neophytadiene (Table 4), while the hexanic extract contained palmitic acid, linoleic acid, oleic acid, gamma-sitostenone, terpenoids and some hydrocarbons (Table 3).

Table 3: Composition of hexane extract of *ulva lactuca*

Phytochemical compound	Molecular formula	RT	Area %
D-Limonene	$C_{10}H_{16}$	16.21	2.2%
Nonadecane	$C_{19}H_{40}$	33.1	0.60%
1,1'-bi (cyclohexan)-1'-en-2-	$C_{12}H_{16}O$	33.94	1.42%
Neophytadiene	$C_{20}H_{38}$	42.76	2.15%
Palmitic acid	$C_{16}H_{32}O_2$	46.02	1.69%
2,6-Di-tert-butyl-4-methyl-phenol	$C_{16}H_{26}O_2$	46.59	0.51%
1-Phenanthrenecarboxylic aci	$C_{15}H_{10}O_2$	47.03	0.22%
Phytol	$C_{20}H_{40}O$	49.22	1.68%
Linoleic acid	$C_{18}H_{32}O_2$	50.02	5.82%
9-Octadecenoic acid (Z)	$C_{18}H_{34}O_2$	50.55	2.62%
5,7,9(11)-Androstatriene, 3-	$C_{19}H_{24}O_2$	54.20	0.221%
.gamma.-Sitostenone	$C_{29}H_{48}O$	59.67	31.65%
Z,Z-6,28-Heptatriactontadien	$C_{37}H_{70}O$	65.98	12.47%
Tricyclo[20.8.0.0(7,16)]tria	$C_{30}H_{52}O_2$	73.44	0.24%

Table 4: Composition of chloroform extract from *Ulva lactuca*

Phytochemical ompound	Molecular formula	RT	Area %
Cyclotetradecane	$C_{14}H_{28}$	38.55	15.38%
Nonadecane	$C_{19}H_{40}$	40.28	11.42%
Neophytadiene	$C_{20}0H_{38}$	42.72	22.28%
3,7,11,15-Tetramethyl-2-hexa	$C_{20}H_{40}O$	43.32	12.69%
Tricosane	$C_{23}H_{48}$	45.50	13.86%
2,6-Di-tert-butyl-4-methyl-phenol	$C_{16}H_{26}O_2$	46.52	9.19%
Triacosane	$C_{30}H_{62}$	50.25	13.86%

2, 6-di-tert-butylphenol (antioxidant and UV stabilizer) was detected in ethyl acetate, chloroform, and hexane extracts. Fatty acids such as palmitic acid (saturated fatty acid), linoleic acid (omega 3 fatty acid) were recorded from GC-MS analysis of different extracts; different sterols were also detected, such as stigmasterol, beta-sitosterol and gamma-sitostenone.

Linoleic acid and Neophytadiene were recorded as major compounds in the methanol extract in terms of peak area.

In addition, linoleic acid, palmitic acid, docosahexaenoic acid (omega 3 mono unsaturated fatty acid), phytol, stigmasterol, squalene and beta-sitosterol are the main compounds present in the methanolic extract.

Table 5: Composition of ethyl acetate extract of *Ulva lactuca*

Phytochemical compound	Molecular formula	RT	Area %
9-Acetyl-6-hydroxy-2-methoxy	$C_{34}H_{41}NO_{13}$	4.79	1.98%
p-Cymene	$C_{10}H_{14}$	16.07	0.005%
Neophytadiene	$C_{20}H_{38}$	42.74	0.12%
Heptadecanoic acid, 16-methy	$C_{18}H_{36}O_2$	49.56	0.003%
cis-5,8,11,14,17-Eicosapent	$C_{21}H_{32}O_2$	52.04	0.002%
Z,E-3,13-Octadecadien-1-o	$C_{18}H_{34}O$	58.91	0.001%
Clionasterol -H2O	$C_{29}H_{50}O$	66.80	0.003%

Tableau 6: Composition of benzene extract of *Ulva lactuca*

Phytochemical compound	Molecular formula	RT	Area %
Cyclotetradecane	$C_{14}H_{28}$	38.55	3.62%
Neophytadiene	$C_{20}H_{38}$	42.72	11.15%
Phytol	$C_{20}H_{40}O$	49.15	2.4%

Tableau 7: Composition of petrol ether extract of *Ulva lactuca*

Phytochemical compound	Molecular formula	RT	Area %
2-Butanol, 2,3-dimethyl-	$C_6H_{14}O$	2.98	52.72%
Cyclotetradecane	$C_{14}H_{28}$	38.55	12.44%
Neophytadiene	$C_{20}H_{38}$	43.42	3.62%

1,2-benzene dicarboxylic acid and fatty acids have been evaluated against many microbes as antimicrobial agents [20, 21]**.** Palmitic acid, linolenic acid and oleic acid are known to be potential antibacterial and antifungal agents [22, 23]. Similarly, in the present study, these compounds were found in methanol and ethyl acetate extract of Ulva lactuca. It has been shown that seaweeds are a good source of unsaponifiable sterols, non-toxic and with medicinal value [24, 25].

Based on phytochemical and ethnobotanical data of Duke we have found the biological activities of several chemical compounds identified by GC-MS, we have grouped the data found in the table below:

Table 8: Biological activities based on phytochemical and ethnobotanical databases of Duke, 2009 from the Agricultural Research Service/USDA 2013.

The chemical compound	Nature of the component	Importance/activity
Hexadecanoic acid, ethyl ester	Palmitic acid ester	Antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor
2,4-Difluorobenzoic acid,	Benzoic acid compound	Antimicrobial
Phytol : 2-Hexadecen1-ol, 3,7,11,15-tetramethyl-,[R- [R*,R*-(E)]	Diterpene	Antioxidant, Antimicrobial, Anthelmintic, Anticancer, Anti-inflammatory, Diuretic and Antidiabetic
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Linolenic acid Polyunsaturated omega-6 fatty acid	Antiinflammatory, hypocholesterolemic cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, insectifuge
Squalene	Triterpene lineaire	Antioxidant, antistatic, antibacterial, anticancer, antitumor
Neophytadiene	Sesquiterpene	Antioxidant, Antimicrobial
Nonadecane	Alkane hydrocarbon	Antiinflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge Antihistaminic, Antiarthritic, Anticoronary, Antieczemic Antiacne, 5-Alpha reductase inhibitor Antiandrogenic,
1-Heptatriacotanol		Anti-oxidant, anti-microbial, anti-inflammatory and hormonal secretions
9-Octadecenoic acid (Z)	Fatty acid esters	Antifungal activity
D-Limonene	Terpene derivative	Antimicrobial
p-Cymene	monoterpene	antioxidant, anti-inflammatory, antinociceptive, anxiolytic, anticancer and antimicrobial [26]
Methyl 9-eicosenoate	an omega 9 unsaturated fatty acid	Antioxidant, pesticide, nematicide;
.gamma.-Sitostenone		Antibacterial and antifungal agent. [27]
2,6-Di-tert-butyl-4-methyl-p	compounds are soluble and chiral nature	inhibit bacterial, fungal, protozoan and parasite growth
Methyl 4,7,10,13,16,19-docos	an omega 3 fatty acid	DHA [28]

IV. CONCLUSION

This work aims to identify the natural potentialities of marine green algae that form an Interesting and very promising source of biologically active substances.

The research undertaken includes a phytochemical study and an identification of the substances contained in the various extracts of *Ulva lactuca* based on qualitative and quantitative analyses.

The overall results showed that *Ulva lactuca* methanol extract was the best solvent capable of extracting the maximum amount of bioactive compounds. The GC-MS analysis of Ulva lactuca revealed the presence of many sesquiterpenes and fatty acids, which are known to be a potential source of bioactive compounds and should be investigated for natural insecticides and fungicides.

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