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Comparative analysis of fatty acid profiles through transesterification in macroalgae from Gulf of Mannar reveal Ulva lactuca for potent edible oil synthesis

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***Abstract*---**Selective macroalgae comprising green algae (*Ulva lactuca),* brown algae (*Padina gymnosphora, Sargassam wightii, Turbinaria ornate)* and red algae (*Gracilaria corticata, Halimeda macrolopa, Halymenia dilatata, Gracilaria crassa)* were collected from Gulf of Mannar, India. Taxonomic and molecular identification affirmed the identity of the microalgal species. Free fatty acids and Fatty Acid Methyl Ester (FAME) synthesis by transesterification was assessed for cataloguing the edible oil source from the various macroalgae collected. Lipid components were extracted employing various solvents namely, Hexane, Chloroform: methanol (2:1), Chloroform: hexane (1:1), Chloroform: hexane (1:2) and Dichloromethane: methanol (2:1). The effects of different solvents based on Soxhlet extraction were preceded and GC-MS was utilized for cataloguing the various lipid components in the macroalgae. Furthermore, *in vitro* anticancer activity against the hepatocellular carcinoma cell line, HepG2 indicate 15.6µg/ml concentration showed significant cytotoxicity compared to control. Thus, the present report emphasizes the optimal role of *Ulva lactuca* as a potential edible oil source supplement, along with pharmaceutical and neutraceutical benefits.

***Keywords*---**Ulva lactuca, fatty acid profile, edible oil, nutraceuticals, transesterification.

Introduction

Ulva lactuca belongs to the green macroalgae family, Chlorophyceae that are responsible for destructive green tides, ubiquitously. Upon anthropogenic activities, algal blooms pose serious threat by producing vapours causing deleterious consequences. The macroalgae belongs to sea lettuce group and accounts for green tides implying environmental limitations. However, the microalgal species have been documented for the presence of potent bioactive compounds, food source, biomass, bioenergy and biofuel prospects. Multi-omics analysis, big data analytics and machine learning approaches have been postulated as an effective means in deciphering the effective usage of *Ulva* sp (Dominguez and Loret, 2019). *Ulva lactuca* has also been proved for phosphorous recovery enabling sustainable agriculture. Thus the macroalgae proves as an organic fertilizer in augmenting yield and productivity (Breure, 2014). Moreover, *Ulva lactuca* has been established as edible seaweed (Sinurat and Fransiska, 2021). Food and food products derived from *Ulva lactuca* has been demonstrated for large-scale cultivation in supply of rich dietary nutrients and healthy alternative (Roleda et al., 2021). Thus the energy efficiency, food supplementation, biomass, biofuel and oil compositions render the macroalgae as an effective alternate in multiude benefits surpassing limitations. The present study aims to catalogue the total fatty acids, FAMEs (Fatty Acid Methyl Esters), various soxhlet extractions for solvent optimization, GC-MS profiling in deciphering the edible oil characteristics of selected macroalgae in the Mandapam coastal regions, Gulf of Mannar. Further, *Ulva lactuca* as a potent edible oil source are critically summarized, along with their anticancer cytotoxicity against Hela cells indicating pharmaceutical and nutraceutical benefits.

Materials and Methods

Collection of algae

Different species of seaweeds (Chlorophyceae, Phaeophyceae and Rhodophyceae) was collected from Mandapam coastal waters in the Gulf of Mannar on India's southeast coast. Algal thalli were separated, put in plastic bags, kept in an icebox and transported to the laboratory. They were properly cleansed with tap water to get rid of any impurities. The algae were spread out on filter paper after the water had been drained out in order to remove the extra moisture. The samples were weighed and dried. Using a home mixer grinder, the dry biomass is pulverised to obtain fine particles. The original weight was reduced by about a factor of 10. Then 1 kg of wet seaweed was weighed to 100 g (10 to 1 wet to dry ratio). For further oil extraction and analysis, 100 g of this fine particle were obtained. The cylindrical cellulose holder was filled with algae and removed during the batch run. Distillation was done to separate the oil solvent combination in the end. Using the following equation, the yield percentage was obtained (Subramanian et al. 2015).

$$\% \text{ oil yield} = W1 \div W2 \times 100$$

To determine the lipid content (%) in dry biomass, the lipid residue was dried in an oven at 60°C and weighed. (Satpati et al., 2015).

Estimation of free fatty acid

Free fatty acids were estimated using the protocol of Sadhasivam and Manickam, 1996. Briefly, 1g of oil or melted fat was dissolved in 50mL of the ethyl acetate solvent in a 250mL Erlenmeyer flask. A few drips of phenolphthalein was added and titrated against 0.1N potassium hydroxide, constantly mixed until a pink color which persists for fifteen seconds was obtained. Following formula was used to calculate acid values.

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titer value x Normality of KOH x 56.1}}{\text{Weight of the sample (g)}}$$

Fatty Acid Methyl Ester (FAME) Production by Transesterification

FAMEs production was estimated by the transesterification method. After extraction, the lipid samples were placed in a 10 mL glass screw-cap tube (BOROSIL, Mumbai, India) and the transesterification reagents methanolic hydrochloric acid (1: 4 v/v) was then added. The tube was placed in a glass beaker filled with double-distilled water and heated in a hot air oven for 6 to 8 hours at 70°C. To remove particles, the solution was centrifuged for 10 minutes at 10,000 rpm after being allowed to cool. The GC-MS auto sample vials were then filled with the FAME extract to begin the analysis (Satpati et al., 2015).

Thin layer chromatography

The modified thin-layer chromatography (TLC) method was used to separate the extracted lipids. The merck readymade thin layer sheets were used (50mm X10mm). The lipid content was dissolved in chloroform or methanol and 50µL of lipid was taken and loaded on TLC plate. The plate was placed in the chamber to develop using solvent system (chloroform: acetone: methanol: acetic acid: water (50:20:10:10:5 v/v/v/v/v). The chamber was covered and left running for around an hour, until the solvent front reached the upper line. The plate was then taken out and placed under the fume hood to dry. A brief exposure to iodine vapor in the fume hood allowed for the visualization of the lipids after complete drying. The TLC plates were placed in the TLC chamber containing iodine. Lipid patches that change colour to a yellow-brown were considered to indicate the point of separation. Carbon pencil marks were made around the spots' edges (Arjun, 2011).

Measuring R_f values

The distance travelled by the solute divided by the distance travelled by the solvent is known as the retention factor (or R_f). For instance, the R_f is 0.75 if a compound moves 2.1 cm and the solvent front moves 2.8 cm. The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant: The formula used for lipid separation was

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

Effect of different solvents on Soxhlet extraction and GC-MS

Five different solvents were used for lipid extraction (1) Hexane, (2) Chloroform: methanol (2:1), (3) Chloroform: hexane (1:1), (4) Chloroform: hexane (1:2), (5) Dichloromethane: methanol (2:1) (Byreddy et al., 2015). Further the presence of constituent components in the aqueous extracts was compared to NIST library database in profiling fatty acids (Arjun et al., 2011).

In vitro anticancer activity (Hela cell line)

The MTT assay was used to evaluate the cancer activity of sample on HELA cells (Mosmann, 1983). In 96-well plates, cells (1×10^5/well) were plated in 0.2 ml of medium/well. The cells were then kept in an incubator with 5% CO2 for 72 hours. Various concentrations of the samples were added with 0.1% Dimethyl Sulfoxide for 24 hrs at 5 % CO_2 incubator. Photos were obtained after the images were seen under a 40X inverted microscope. After removing the sample solution, 20µl of MTT reagent was added to each well. By measuring the absorbance at 540 nm, viable cells were identified. The IC50 value, or 50% inhibition of cell viability, was graphically determined. The effect of the samples on the proliferation of HELA cells was expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = A540 \text{ of treated cells} / A540 \text{ of control cells} \times 100\%$$

Result and Discussion

Different compositions of oil contents are present in algal species. Some species of algae are studied in this current research to identify their fatty acid composition. Algae can grow in different conditions even in the presence of low nutrient contents. The representative macroalgae have been taxonomically characterized as green algae (*Ulva lactuca),* brown algae (*Padina gymnosphora, Sargassam wightii, Turbinaria ornate)* and red algae (*Gracilaria corticata, Halimeda macrolopa, Halymenia dilatata, Gracilaria crassa).* Free fatty acids and FAME production by transesterification reactions showed the respective inherent lipid components. Of which, *Ulva lactuca* revealed the presence of Octadecane ($C_{18}H_{38}$), Hexadecane ($C_{16}H_{34}$), 1-Nonadecene ($C_{19}H_{38}$), 1-Pentadecene ($C_{15}H_{30}$) and 4-Trifluoroacetoxypentadecane ($C_{17}H_{31}F_3O_2$). The above listed lipid precursors have been amply reported as edible oil precursors for eicosapentanoic acid (EPA) showing the potential edible oil synthesis mechanisms. The identified algal samples were then subjected for the lipid content estimation. This was carried out by the extraction method in soxhlet apparatus. The oven dried lipid content was weighed and the percentage of lipid in the dry biomass was estimated and recorded. Followed by this the presence of free fatty acid was determined. The appearance of pink colour is confirmed as the presence of free fatty acid in the tested algal sample. Successively Fatty acid methyl ester (FAME) production test was carried out by transesterification method and this FAME extract was then subjected to GCMS test. The highest fatty acid was seen in Green Algae, *Ulva lactuca* (1.68%) and Brown Algae, *Turbinaria ornata* .Very low fatty acid was present in Red algae, *Halimeda macrolopa* (0.20%). Table 1 enlists the fatty acid values for the selected macroalgae. For cost-effective oil extraction, solvent selection plays a major role (Fig. 1).

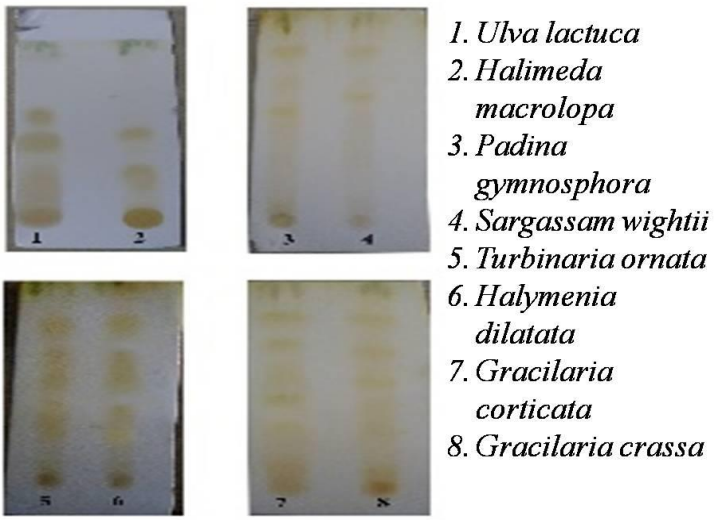

Fig. 1. Types of algae and compound profiling by Thin Layer Chromatography techniques

Table 1: Determination of Fatty Acid Value for the algal samples

S. No	Sample name	Fatty acid Value (%)
1.	Green Algae: *Ulva fascita*	1.68
2.	Green Algae: *Ulva lactuca*	1.38
3.	Brown Algae: *Padina gymnosphora*	1.09
4.	Brown Algae: *Sargassam wightii*	1.27
5.	Brown Algae: *Turbinaria ornata*	1.63
6.	Red Algae: *Gracilaria corticata*	1.28
7.	Red Algae: *Halimeda macrolopa*	0.20
8.	Red Algae: *Halymenia dilatata*	0.40
9.	Red Algae: *Gracilaria crassa*	0.21

Hexane is a non-polar solvent that, in general, has been used frequently for extracting vegetable oils due to its high stability, minimal greasy residue effects, boiling point, and low corrosiveness. A greater extraction yield of algal oil was made possible by solvent extraction employing chloroform-methanol (4.21%). A low extraction yield is obtained when the same method is repeated in a hexane solvent while depending solely on the diffusion of lipids through the cell membrane. This is because the polarity of different lipids varies. Polar lipids in the cellular membrane have strong hydrogenic or electrostatic bonds with protein molecules. Adding polar solvent (alcohol) breaks the link between lipid and protein before extraction. The impact of various solvents on the separation of the components of fatty acids is shown in Table 2. Hexane is thus able to interact with neutral lipid. As a result, the yield of oil extraction was 2.91% (w/w). The current findings are in line with a prior publication by Hidalgo et al (2016).

Table 2: Effect of different solvents for separation of fatty acid

S. No	Sample name	Fatty acid Value (%)
1.	Hexane	2.91
2.	Chloroform: methanol(2:1)	4.21
3.	Chloroform: hexane(1:1)	0.82
4.	Chloroform: hexane(1:2)	1.74
5.	Dichloromethane: methanol (2:1)	1.1

GCMS was used to determine the fatty acid composition of the algal oil. Some of the identified fatty acid components from the selected 8 algal samples are pictorially depicted in Figure 2.

Green Algae: *Ulva lactuca* – Octadecane, Hexadecane, 1-Nonadecene, 1-Pentadecene, 4-Trifluoroacetoxypentadecane (Table 3). Pourkarimi et al. (2021) evaluated the impact of key pyrolysis factors, such as pyrolysis temperature, carrier gas flow rate, and heating rate, on bio-oil yields. Their study provided evidence in support of our findings. Accordingly, the pyrolysis produced the maximum bio-oil yields (34.29%) under the optimum operating conditions (at 500 °C, 0.2 L/min nitrogen flow rate, and 10 °C/min heating rate).
Brown Algae: *Padina gymnosphora* - Hexadecane, 2,6,10,14-tetramethyl-, Hexadecane, 2-methyl-, 2-Trifluoro acetoxypentadecane, Octadecane, 9-ethyl-9-heptyl-, 1-Nonadecene (Table 4).

Table 3: Fatty acid profile of Green Algae, *Ulva lactuca*

S.no.	Retention time	Compound Name	Molecular formula	M. Wt. $gmol^{-1}$	Area %
1	14.197	Octadecane	$C_{18}H_{38}$	254.494	0.26
2	14.374	Hexadecane	$C_{16}H_{34}$	226.448	0.29
3	16.574	1-Nonadecene	$C_{19}H_{38}$	266.5	0.20
4	16.763	1-Pentadecene	$C_{15}H_{30}$	210.3987	2.77
5	18.096	4-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324.42205	0.23

Similarly, Bhuyar *et al.*, (2021) showed that the presence of few polysaccharide components from the brown seaweed *Padina gymnosphora* in which n-Hexadecanoic acid is high in content at about 26.31% of area.

Brown Algae: *Sargassam wightii* - Octadecane, 5,14-dibutyl-, Dodecane, 1,1`-oxybis-, n-Hexadecanoic acid, Oleic acid, Octadecane, 1-chlolo-; Brown Algae: *Turbinaria ornate* -Heptacosane, 1-chloro-, 9-Octadecenoic acid, Hexadecane, 1-iodo-, Nonadecane, 9-methyl-, Tridecane (Table 5).

Table 4: Fatty acid profile of Brown Algae, Padina gymnosphora

S.no.	Retention time	Compound Name	Molecular formula	M. Wt. $gmol^{-1}$	Area %
1	14.363	Hexadecane, 2,6,10,14-tetramethyl-	$C_{20}H_{42}$	282.5475	0.40

2	19.341	Hexadecane, 2-methyl-	$C_{17}H_{36}$	240.4677	0.37
3	19.441	2-Trifluoro acetoxypentadecane	$C_{17}H_{31}F_3O_2$	324.42205	0.60
4	21.201	Octadecane, 9-ethyl-9-heptyl-	$C_{27}H_{56}$	380.7335	1.10
5	22.662	1-Nonadecene	$C_{19}H_{38}$	266.5	1.13

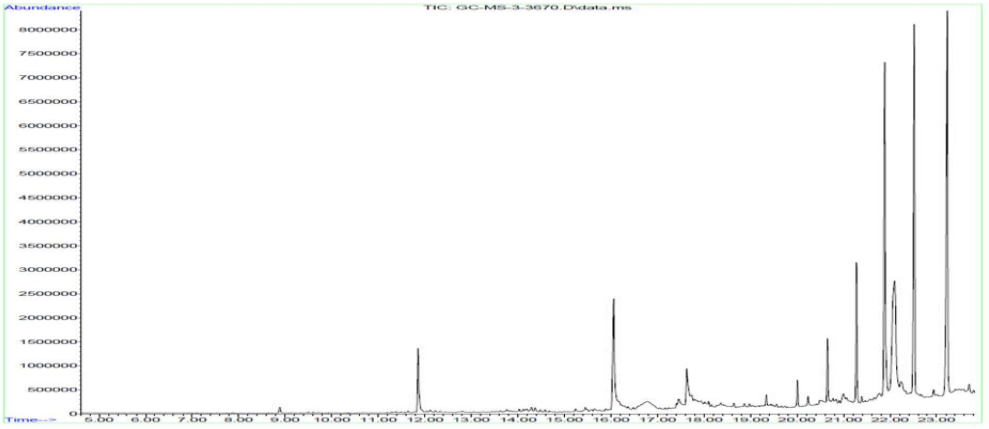

Fig. 2. Fatty acid profile of the algal oil was determined by GC-MS.

Similar presence of various compounds in the bio-oil have been reported (Lee et al., 2021): 4-hexenoic acid, 2-amino-6- hydroxy-4-methyl, heptamethylene diacetate, cyclohexanone, 2–((dimethyl amino) methyl), 9-hexadecenoic acid, 9-octadecenyl ester, 2,2,5-

Table 5: Fatty acid profile of Brown Algae, Sargassam wightii

S.no.	Retention time	Compound Name	Molecular formula	M. Wt. $gmol^{-1}$	Area %
1	14.363	Octadecane, 5,14-dibutyl-	$C_{26}H_{54}$	366.7070	0.56
2	15.885	Dodecane, 1,1`-oxybis-	$C_{24}H_{50}O$	354.6532	0.38
3	16.052	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	19.34
4	17.474	Oleic acid	$C_{18}H_{34}O_2$	282.47	1.51
5	19.152	Octadecane, 1-chlolo-	$C_{18}H_{37}Cl$	288.94	0.52

trimethylcyclohexane 1,4-diol, 9,12,15–octadecatrienoic acid, 2–((trimethylsilyl)oxy– 1– (trimethylsilyloxy) methyl)ethyl ester (Z,Z,Z), 2-ethoxycarbonyl-3-methyl-4-azafluorenone-2-flourenylimine, 1,2,3,4—cyclopentanetrol, and piperidine, 2,3-dimethyl.

Table 6: Fatty acid profile of Red Algae, *Gracilaria corticate*

S.no.	Retention time	Name of the Compound	Molecular formula	M. Wt. $gmol^{-1}$	Area %
1	14.363	Tetradecane	$C_{14}H_{30}$	198.39	0.50
2	16.085	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	35.38
3	17.463	Oleic acid	$C_{18}H_{34}O_2$	282.47	1.92
4	17.641	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	6.92

5	18.641	Nonadecane, 9-methyl-	$C_{20}H_{42}$	282.5475	0.43

Red Algae: *Gracilaria corticata* – Tetradecane, n-Hexadecanoic acid, Oleic acid, Octadecanoic acid, Nonadecane, 9-methyl (Table 6). Gaubert *et al.*, (2019) showed the presence of main saturated fatty acids (SFAs) in *G. corticata* were palmitic acid (C16:0), margaric acid (C17:0) and stearic acid (C18:0).

Red Algae: *Halimeda macrolopa* – Cyclopentadecane, Heptadecane, Dodecanoic acid, hexadecyl ester, Hexadecane, 3-methyl-, Dodecanoic acid, tetradecyl ester; *Halymenia dilatata* - Hexadecanoic acid, Dodecane, Tetradecane, Heptadecane, 9-octyl, Nonadecane; *Gracilaria crassa* – Tridecane, Hexadecane, Heptadecane, 9-octyl, Nonadecane, Cyclododecane (Table 7).

Table 7: Fatty acid profile of Red Algae, *Halimeda macrolopa*

S.no.	Retention time	Name of the Compound	Molecular formula	M. Wt. $gmol^{-1}$	Area %
1	8.320	Cyclopentadecane	$C_{15}H_{26}O_2$	238.37	1.28
2	10.886	Heptadecane	$C_{17}H_{36}$	240.471	0.83
3	20.651	Dodecanoic acid, hexadecyl ester	$C_{28}H_{56}O_2$	424.7	1.56
4	20.851	Hexadecane, 3-methyl-	$C_{17}H_{36}$	240.5	1.06
5	20.651	Dodecanoic acid, tetradecyl ester	$C_{26}H_{52}O_2$	396.6899	1.56

Fatty acid dysregulation is linked to a number of disorders, because fatty acids are essential components of numerous biological processes. The total amount of fatty acids in macroalgal biomass can be calculated using the method that has been presented. Several studies with cross-disciplinary attributes for augmenting the utility of *Ulva lactuca* has been reported in establishing the potential multitude benefits. TiO_2-ZnO blended nanocomposite materials with silver nanoparticles enhanced the biodiesel yield along with antimicrobial properties (Gurusamy et al., 2019). Thus it is evident that *Ulva lactuca* possess pharmaceutical and nutraceutical potentials. The purpose of the current study was to decipher the dosage compensation of aqueous extracts of the macroalgae against the hepatocellular cancer cell line, HepG2.

Figure 3 shows the comparative cytotoxicity profiles and clearly show the increased concentration of 500 µg/ml revealed 5.1% of cell viability. Similar studies with *U. pinnatififida* have proved the nutraceutical efficacy and positive health potentials as a food supplement and balanced diet of necessary fatty acids (Rocha et al., 2021). Nutritional benefits of *Ulva lactuca* corresponds to the presence of ulvans, too. Hence edible oil synthesis from the macroalgae could impose further research in enhancing the utilization for indigenous usage and circular bionomy approaches. Moreover, it was evident that the macroalgae, *A. taxiformis* showed potential antitumour activities against A549 cell line showing anti-Alzheimer's potentials (Nunes et al., 2020).

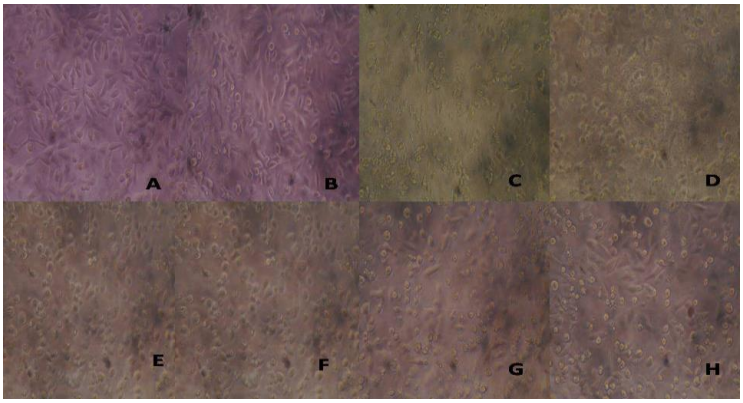

Fig. 3. Comparative cytotoxicity profiles shows (500 µg/ml) revealed of cell viability (5.1%)

Future research perspectives in *Ulva lactuca* nutraceutical benefits have been correlated to in-depth assessment of phenolic constituents, polysaccharides, proteins, amino acids, sulfated compounds, pigments, minerals and lipids (Garcia-poza et al., 2022). The solvent extraction of fatty acids has been followed in earlier studies for Galician macroalgae, including *Ulva lactuca* revealed the effectiveness in antioxidant and antimicrobial activities (Otero et al., 2018). Thus the significance of the present study can be emphasized for rationalizing the approach for other cancer studies, together with edible oil properties. Further, the antioxidant and radical scavenging activities of this edible seaweed have also been postulated for cosmeceuticals, together with nutraceutical potentials (Pangestuti et al., 2021). The lipid components present in *Ulva lactuca* has the significant polar lipids for developing functional foods and nutritional dietary incorporations for better health (Uribe et al., 2019; Moriera et al., 2021).

Conclusion

Green algae, brown algae and red algae were collected from the Mandapam coastal waters, Gulf of Mannar region. Morphological and molecular identification revealed the presence of ten distinct macroalgae. The microalgal samples were then analyzed for total fatty acids, FAME production by transesterification employing various solvents using soxhlet extraction. Thin layer chromatography and GC-MS analysis revealed the presence of principal eicosapentanoic acid (EPA) precursors establishing the edible oil synthesis from the edible sea lettuce, *Ulva lactuca*. Further nutraceutical and pharmaceutical benefits of the macroalgae was coerced with *in vitro* anticancer activity against hepatocellular carcinoma cell line, HepG2. Thus the present assessment emphasizes the multiple benefits of *Ulva lactuca*, as edible oil, biodiesel, bioenergy, food supplement, functional foods, Organic agriculture and mass cultivation, biofuel, pharmaceutical and nutraceutical potentials. Future research is necessitated for the delineating the limitations of the microalgal cultivation in arresting green tides.

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