Chemical and biological characterization of lipid profile from *Hydroclathrus clathraus*


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Abstract

This research addresses studying the lipid content of *Hydroclathrus clathraus* brown algae from the Red Sea, Egypt Region and their assess acute oral LD<sub>50</sub> of sterol fraction in albino mice and anticancer activity against different cancer cell lines including; colon cancer cell line (Caco-2) and liver cancer cell line (HuH-7) using MMT, apoptosis analysis, EGFR and genes' expression. Total lipids, glycolipid, sulfolipid, and phospholipid of *Hydroclathrus clathraus* content were 1mg/g, 0.08 mg/g, 0.25±0.01 mg/g and 0.57±0.04 mg/g respectively. The LD<sub>50</sub> of *Hydroclathrus clathraus* sterols was greater than 3g/kg in the mice. The major fatty acid is palmitic acid (67.04%). Polyunsaturated fatty acids content was arachidonic (8.05%), α-Linolenic acid (4.36%) and Linoleic acid (1.69%). The major sterols fractions were Hexacosane (18.54%), Octacosane (14.96%) and Nonadecane (13.31%). Cholesterol (8.32%), β-Sitosterol (2.08%) and Campesterol (1.36%) were presented as minor sterols. However, algal sterol showed the best potential antitumor activity with IC<sub>50</sub> of 0.22 µg/ml and 3.09µg/ml against CaCo2 and HuH7. These sterols fraction reduced tumor genesis by three mechanisms that firstly, the cells' apoptosis which may reduce the regulatory suppressive activity of the epidermal growth factor receptor (EGFR) enzyme, secondly, gene expression of bcl2 was downregulated and thirdly, it induced cell cycle arrest at the G1/S. Thus, algal sterols can be used as medicine to treat disorders liver and colon cancers.

Keywords: Brown algae- lipids- anticancer- cell apoptosis- LD<sub>50</sub>

1. Introduction

Algae are considered a proper source for manufacturing cheaper and safe new pharmaceutical industries. In Egypt, macro-algae grows naturally on the seashores. Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths. Brown algae compounds have many biologically active such as UV skin photo-protection, anti-melanoma [1], ovarian cancer suppression [2], breast and colon cancer antiproliferative activities [3], and anti-pancreatic cancer [4]. It can be seen that not only one component of brown algae was responsible for these biological activities, but also polysaccharides, diterpenoids, fatty acids, and sterols have different and versatile effects against tumor with.

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different modes of action [5]. Hydroclathrus clathratus is a brown marine alga known to have anti-
cancer, anti-herpetic, and anti-coagulant activities [6].

The main lipids content of marine macrophytes
including the fatty acids, waxes, sterols, carotenoids,
mono, di, triacylglycerols (TGs), phospholipids
(PLs), glycolipids (GLs) and betaine lipids [7].
Sterols are a vital family of lipids. Algal sterols are
used as a functional constituent to reduce chronic
diseases in the human body. Macro-
algae lipids and
sterols have antibacterial, antiviral, antitumor, anti-
inflammatory, anti-proliferative and antioxidant
activity [8].

This study aims to isolate and identify the lipid
contents in the Hydroclathrus clathraus and assess the
acute oral LD50 of sterol fraction in mice and
evaluate anticancer activities against liver and colon
cancers by using MTT, EGFR enzyme, gene
expressions, and cell cycle arrest.

2. MATERIALS AND METHODS

2.1. Marine algae material

*Hydroclathrus clathraus* collected (during December
2017) from El Ain El-Sokhna sector of Suez Galf –
Egypt. *Hydroclathrus clathraus* identified by Dr.
Rauhaiya Abdul-Latif, Professor of Botany,
Department of Botany, Faculty of Science, Al-Azhar
University.
The total lipids of *Hydroclathrus clathraus* extracted
according to the method of Roughan and Bartt [9].

2.2. Chemical composition

2.2.1. Determination of *Hydroclathrus clathraus*
sulfolipids

Algal lipids (0.5 ml) hydrolyzed by 0.1N
hydrochloric acid (1.0 N) ethanol absolute (2ml) in
sealed tubes. The hydrolyzed samples clarified by
centrifugation. One ml BaCl2 buffer and 1.5 ml
sodium rhodizonate reagent pipette into each tube
shacked well and allowed to stand for 10 min in the
dark under room temperature. The absorbance was
spectrophotometrically at 520 nm. Standard series
concentrations of sulfate ranges from 2-12 µg /ml
treated as the samples [10].

2.2.2. Determination of *Hydroclathrus clathraus*
Phospholipids

Phosphorus was determined according to the method
of Rouser et al. [11]. Forty µl of algal lipid extract
was transferred into clean glass tubes, after that the
solvent was completely evaporated under N2. Then,
0.65 ml perchloric acid (70%) was added and heated
in boiling water for about 30 min. After cooling, the
reaction mixture containing 3.3 ml water, 0.5 ml of
molybdate solution (2.5 %) and 0.5 ml of ascorbic
acid solution (10 %) added mixed well and heated.
The tubes were placed in a boiling water bath for 5
min. After cooling, the absorbance of each of the
samples was measured at 800 nm. A standard series
concentration of KH2PO4 (5 to 50µg/ml) treated as
the sample.

2.2.3. Determination of *Hydroclathrus clathraus*
glycolipids

Total carbohydrates were determined by the use of
phenol-
sulfuric acid reagent as modified by
Kushwaha and Kates [12]. Lipid (40 µl) was
transferred into tubes and the solvent evaporated
under an N2 stream. Then, 2 ml of water, 1.0 ml of
phenol solution (5%), and 5 ml sulfuric acid were
added and heated for 5 min in a boiling water bath.
After cooling, the samples were measured
spectrophotometrically at 490 nm

2.2.4. GC analysis of the fatty Acids composition of
*Hydroclathrus clathraus* lipids

*Hydroclathrus clathraus* lipids methylated in 1.5%
sulfuric acid – methanol at 95°C for 2 h. The Gc
analysis described by El Gengaihi et al. [13].

2.2.5. GC analysis of the sterols fraction of
*Hydroclathrus clathraus* lipids

*Hydroclathrus clathraus* lipids saponified with KOH
(20ml, 10%) at 80°C for 3h under reflux. Sterols
methyl esters were identified by GC and conditions
of GC were described by El Gengaihi et al [13].

2.3. Biological activity:

2.3.1. Determining the acute oral toxicity dose for
*Hydroclathrus clathraus* sterol fraction: The median
lethal dose (LD50) of sterols fraction was performed
on Swiss albino mice [14]. The experiment was
conducted according to the ethical committee standards and approval of the Egyptian National Research Center. Twenty-five males and females Swiss albino mice were selected randomly homogenous in age, with body weights around 40 g ± 5 g. The groups fasted for 18 h before the beginning of the experiment. This experiment was conducted for 14 days with precise monitoring for any toxicological effects.

2.3.2. Cytotoxic effect of sterol fractions on liver and colon cancer cells.

The Cytotoxicity of sterols fraction was measured against human epithelial colorectal adenocarcinoma (Caco-2) cell line and human hepatocellular carcinoma (HuH-7) cell line by using the MTT assay (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide) [15].

2.3.2.1. EGFR inhibition effect of the sterol fractions on colon and liver cell lines

EGFR enzyme was assayed by using ab193764 – soluble EGFR Human Simple Step ELISA® Kit, USA Abcam [16].

2.3.2.2. Effect of sterol hydrocarbons on apoptotic genes’ expression

Caspase-3 (CASP3), Bcl-2, and P53 Tumor Suppressor genes were evaluated using the q-PCR technique to detect the up-regulation or down regulation effects of the sterol hydrocarbons extracts on different evaluated cancer cells. Strata gene Mx3000P qPCR System, Agilent Technologies (Carpinteria, California, USA) was used. Total RNA was extracted according to the method of RNeasy Mini Kit, Cat No./ID: 74104, USA protocol [17]. Total RNAs were amplified by PCR using the primers: Caspase3 (forward 5′- GCA GCA AAC CTC AGG GAA AC-3′ and reverse 5′- TGT CCG CAT ACT GTT TCA GCA -3′), Bcl2 (forward 5′- TTT GAG TTC GGT GGG GTC AT-3′ and reverse 5′- TGA CTT CAC TTG TGG CCC AG-3′), P53 (forward 5′-TGC GTG TGG AGT ATT TGG ATG-3′ and reverse 5′-TGG TAC ATG AGT CAG CAA CCT C-3′).

2.3.2.3. Effect of sterol fractions on cell cycle and apoptosis

Each cell line was treated with IC50 of sterol fraction for 24 h in the cell culture flasks. Cells were harvested, washed and counted to have not less than 3*105 cells/ each group with at least 90% living cells. Then the cells were stained with EZCellTM Cell Cycle Analysis Kit, BioVision, Inc., San Francisco Bay Area Catalog #K920-100. Another portion of cells was treated with Annexin V-FITC Apoptosis Kit Plus, BioVision Inc., San Francisco Bay Area. Apoptotic divisions were measured according to the Biovision protocol (Biovision.com. Annexin V-FITC Apoptosis Detection). CytoFLEX V0-B3-R1 Flow Cytometer, Beckman Coulter Life Sciences (Indianapolis, United States) was used.

2.3.3. Statistical analysis

The experiments were carried out by the use of three replicates For Cell variability; Graph Pad Prism v5 was used. Data statistically analyzed using t-test, for flow cytometry analysis (FlowJo®• Software (for Mac), Version 10.2, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA,) analysis platform was used.

3. Results and Discussion

3.1. Lipids content of Hydroclathrus clathraus

Total lipids, glycolipid, sulfolipid and phospholipid contents of Hydroclathrus clathraus were 1.5mg/g, 0.08± 00 mg/g, 0.25±0.01 mg/g and 0.57±0.04mg/g, respectively (Table 1). The results of total lipid were in agreement with those obtained by Parekh and Chauhan [18] they found that the total lipid of Hydroclathrus clathraus was 1.7mg/g. In addition, these results are in accordance with those of El Baz [19], who stated that the sulfolipid contents of D. fasciola and Taonia atomaria brown algae ranged from 4.50 to 11.80 % of the total lipids. Abd El Baky [20]found that the phospholipid of D. fasciola and Taonia atomaria brown algae were 7.27% and 3.18%, respectively.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Total lipids content of Hydroclathrus clathraus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid mg/g</td>
<td>Sulfolipid mg/g</td>
</tr>
<tr>
<td>0.57±0.04</td>
<td>0.25±0.01</td>
</tr>
</tbody>
</table>

Mean ± SD using T- test (n=3)

3.2. Fatty acids composition of Hydroclathrus clathraus lipid

The fatty acids composition of Hydroclathrus clathraus is shown in Table (2). The fatty acids of Hydroclathrus clathraus had the highest plamitic...
acid content (67.04%). Moreover, polyunsaturated fatty acids of Hydroclathrus clathraus lipid content were arachidonic (C_{20:4}) (8.05%) and α-Linolenic acid (C_{18:3}) (4.36%). These results are in agreement with those obtained by Bhaskar et al.,[21] he found that the major fatty acid of Sargassum marginatum was palmitic acid (16:0) (43.76%) followed by arachidonic (20:4) (7.76%). El Baz et al.,[22] found that palmitic was the most abundant fatty acids in T. atomaria (11.89%) and D. fasciola (37.49%).

Table 2
Fatty acids composition of lipid Hydroclathrus clathraus

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Common name</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{14:0}</td>
<td>Myristic acid</td>
<td>4.35</td>
</tr>
<tr>
<td>C_{15:0}</td>
<td>Pentadecanoic acid</td>
<td>1.32</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>Palmitic acid</td>
<td>67.04</td>
</tr>
<tr>
<td>C_{16:1}</td>
<td>Palmitoleic acid</td>
<td>2.66</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>Stearic acid</td>
<td>4.87</td>
</tr>
<tr>
<td>C_{18:1}</td>
<td>Oleic acid</td>
<td>4.36</td>
</tr>
<tr>
<td>C_{18:2 (ω 6)}</td>
<td>Linoleic acid</td>
<td>1.69</td>
</tr>
<tr>
<td>C_{18:3 (ω 3)}</td>
<td>α-Linolenic acid</td>
<td>4.36</td>
</tr>
<tr>
<td>C_{20:4}</td>
<td>arachidonic</td>
<td>8.05</td>
</tr>
</tbody>
</table>

3.3. Sterols hydrocarbon composition of Hydroclathrus clathraus lipid

The sterols composition of Hydroclathrus clathraus lipid is illustrated in Table (3). The highest percentage of sterols was found in Hexacosane (18.54%), Octacosane (14.96%) and Nonadecane (13.31%). Tetracosane (C_{24}, 10.8%), Eicosane (7.33%) and Heptacosane (5.75%) were the most abundant sterols, while cholesterol (8.32%), β-Sitostero (2.08%) and Campasterol (1.36%) were presented as a minor sterols. Eman et al. [23] observed that hexacosane (21.78%) was the highest compound in chloroform fraction from Taonia atomaria ethanol extract, followed by campasterol (20.66%) and nonadecane (18.06%). In addition, cholesterol, β-sitosterol, and pentadecane showed 15.92, 14.07, and 9.523%, respectively.

Table 3
Sterols fractions composition of Hydroclathrus clathraus lipid

<table>
<thead>
<tr>
<th>Sterols</th>
<th>lipid extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane(C_{16})</td>
<td>1.36</td>
</tr>
<tr>
<td>Heptadecane(C_{17})</td>
<td>1.12</td>
</tr>
<tr>
<td>Octadecane(C_{18})</td>
<td>3.74</td>
</tr>
<tr>
<td>Nonadecane(C_{19})</td>
<td>13.31</td>
</tr>
<tr>
<td>Eicosane (C_{20})</td>
<td>7.33</td>
</tr>
<tr>
<td>Tricosane (C_{21})</td>
<td>4.15</td>
</tr>
<tr>
<td>Tetracosane (C_{24})</td>
<td>10.8</td>
</tr>
<tr>
<td>Hexacosane (C_{26})</td>
<td>18.54</td>
</tr>
<tr>
<td>Heptacosane (C_{27})</td>
<td>5.75</td>
</tr>
<tr>
<td>Octacosane(C_{24})</td>
<td>14.96</td>
</tr>
<tr>
<td>Nonacosane (C_{29})</td>
<td>5.11</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.32</td>
</tr>
<tr>
<td>Campasterol</td>
<td>1.36</td>
</tr>
<tr>
<td>β-Sitostero</td>
<td>2.08</td>
</tr>
<tr>
<td>α- amyrine</td>
<td>2.07</td>
</tr>
</tbody>
</table>

3.4. Determination of LD_{50} of Hydroclathrus clathraus sterols

Throughout the fourteen days of the experiment, no gross toxicity occurred to any animal member of the administered groups. All animals showed a normal behavior, feeding habits, and no symptoms of toxicity were detected (Table 4). The lethal dose (LD_{50}) of the sterol fraction in our experiment exceeded 3 g/ kg body weight in albino mice. The high LD_{50} indicates the safety of the used component and the wide therapeutic margin of this sterol fraction [24]. This criterion is the aim for all researchers to find a potent anticancer drug from edible, safe and no side effects raw materials. We can see that the resulted LD_{50} exceeded thousands folds of the effective anti-cancer used dose.
CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF LIPID......

Table 4
LD<sub>50</sub> of algal sterols determination in albino mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Number of rats in each group</th>
<th>Number of dead animals</th>
<th>Mortality %</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; mg/Kg b.w</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3000</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2500</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>over 3000 mg/Kg</td>
</tr>
<tr>
<td>3</td>
<td>2083.33</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1736.11</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.5. Cytotoxic effect of algal lipid fractions on colon cancer and liver cancer cell lines

Crude lipid, sterol, and fatty acid fraction were evaluated by Caco-2 and HuH-7 cell lines and determined their respective IC<sub>50</sub> using MTT assay. The IC<sub>50</sub> of sterol fraction was 0.22 and 3.09 µg/ml on Caco-2 and HuH-7 cell lines. IC<sub>50</sub> of the fatty acids was 4.49 and 8.06 µg/ml on Caco-2 and HuH-7 cell lines. While the crude lipid extract was 14.72 µg/ml and 43.69 µg/ml on Caco-2 and HuH-7 cell lines, respectively (Fig. 1). These results are in agreement with those obtained by Eman et al., (2016), they found that the sterol from Taonia atomaria have strong inhibitory activity against different cancer cell lines, HCT116, MCF7, HepG2 and A549 which showed reducing percent 94.20, 100.00, 94.50 and 99.20%, respectively. The sterols from red algae were also of specific interest as anti-cancer against colon cancer cell lines and tumor-implanted mice [25].

![Fig. 1. IC<sub>50</sub> of Hydroclathrus clathraeus lipid fractions on Caco-2 and HuH-7 cell lines.](image1)

3.6. Sterol fractions inhibited EGFR in both Caco-2 and HuH-7 cell lines

Sterol fraction has high inhibitory against EGFR with IC<sub>50</sub> values from 88.3 µg/ml on Caco-2 and 116.8 µg/ml on HuH-7 cells compared to IC<sub>50</sub> of Erlotinib which ranged 35 µg/ml on Caco2 and 67 µg/ml on HuH7 cell lines (Fig. 2). Sterol fraction showed a significant decrease in the level of EGFR in both treated cell lines compared to their respective non-treated controls. EGFR is a well-known tyrosine kinase member expressed in many carcinomas and it plays an important role in pathogenesis and progression of many cancer types [26]. Phosphorylation of EGFR leads to activation of downstream cascade including extracellular signal-regulated kinase (ERK) and, AKT (protein kinase B), and mitogen-activated protein kinase (MAPK). These proteins work activated the pro-apoptotic transcription factor of Bcl-2 family. They also phosphorylate pro-caspase-9, and many other cascades which lead to eventually cell proliferation, angiogenesis, motility, and expression of extracellular proteins [27]. Erlotinib is a potent tyrosine kinase inhibitor in different statuses of EGFR in lung cancers [28].

![Fig. 2. Inhibitory effect of sterol fraction and standard Erlotinib on EGFR enzyme.](image2)

3.7. Sterol fraction up regulated apoptotic genes; P53 and Casp3 and down regulated anti-apoptotic gene; Bcl-2

Both apoptotic genes P53 and Casp3 showed 9 and 11 fold up-regulation respectively. On the other hand, the anti-apoptotic gene Bcl-2 showed down-regulation with only 0.2 and 0.3 fold in both cell lines (Fig. 3). Sterol fraction led to significant up-regulation of both cancer inhibitory genes; p53 and Casp3 at both treated cell lines in comparison with their respective non-treated controls and in the same time. Also, it led to a significant down-regulation of...
the anti-apoptotic gene Bcl-2 at both treated cell lines compared to their respective non-treated controls. The fold change up-regulation of the p53 and Casp3 genes reached up to 10 fold changes in Caco-2 treated cell lines with sterol fraction. While in the HuH-7 treated cell lines the up-regulation fold change was only 4 and 6 folds change in both p53 and Casp3 genes respectively. The P53 gene is one of the powerful tumor suppressors due to its ability to activate apoptosis and regulates cellular senescence and causes cellular aging [29]. In many cancers, the p53 gene loses its function by different mutations either contact or structural ones leading to protein inactivation. Such inactivation leads to increased proliferation, inhibited DNA repair, and diminutive apoptosis [30]. The caspase-3-encoding gene for inactivation and subsequent apoptosis plays an important role in the development of many cancers. Inactive mutated caspase-3 leads to inhibition of DNA degradation, inhibition of the plasma membrane, and inhibition of nuclear condensation [31]. On the other hand, Bcl-2 plays an important role in apoptosis, maintaining cell survival and decrease the proliferation of malignant cells, thus showing its importance in the treatment of cancer cells. [32].

The Annexin V-FITC Apoptosis Kits proved the percentage of early apoptosis, late apoptosis, and necrosis in the treated cells in comparison to their respective controls (Fig.4). Caco-2, treated with the sterol fraction, showed a higher late apoptosis fraction up to 11% of cells in comparison to the non-treated control. Also, hepatocellular carcinoma HuH-7 cell lines showed only about 6% of the cell with late apoptosis. Although late apoptosis was the prevailing fraction of cells. We cannot ignore the fraction of early apoptosis which was about 8% of the cell population in Caco-2 cell line treated with IC_{50} of the extract. While, the same fraction was only half in the hepatocellular carcinoma cells treated with the same concentration of the sterol. In the cell cycle analysis, it was clear that the cells were treated with

### Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caco-2 Apoptosis</th>
<th>HuH-7 Apoptosis</th>
<th>Caco-2 G0-G1</th>
<th>HuH-7 G0-G1</th>
<th>Caco-2 S</th>
<th>HuH-7 S</th>
<th>Caco-2 G2-M</th>
<th>HuH-7 G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.62</td>
<td>3.24</td>
<td>69.32</td>
<td>68.55</td>
<td>24.87</td>
<td>24.23</td>
<td>5.81</td>
<td>7.22</td>
</tr>
<tr>
<td>Sterol (IC_{50})</td>
<td>23.52</td>
<td>11.26</td>
<td>72.36</td>
<td>70.52</td>
<td>26.82</td>
<td>27.09</td>
<td>0.82</td>
<td>2.39</td>
</tr>
</tbody>
</table>

*Effects of Sterol (IC_{50}) on Caco-2 and HuH-7 cell cycle distribution after 24 h exposure. The results are representative of two independent experiments.*

3.8 Sterol fraction caused cell cycle arrest and apoptotic changes in both cell lines

With the use of cell cycle analysis kits, sterol fraction caused cell arrest in G1/G2 phase with a considerable decrease in the G2/M phase in both treated cell lines when compared with their respective controls (Table5). Also, we can see high percentage of apoptotic cells in both treated cell lines compared to respective controls.
sterols due to cell arrest at the G1 phase. Beta-Sitosterol plant stops cell cycle progression such as G0/G1, S or G2/M phases and then stimulates apoptotic cell death [33]. Phytoesterol from Grewia tilaefolia induce cells cycle arrest at G2/M phase in lung cell and promotes the mitochondrial dependent apoptosis in A549 cells [34].

4. Conclusion

This study indicated that the lipid content of Hydroclathrus clathrus enhanced antitumor activities by reducing EGFR enzyme, up-regulation of Caspase 3 and p53, and down-regulation of bc12 against Caco2 and HuH7. Also by investigating cell cycle analysis, sterols arrest cell cycle in G1/S with significant apoptosis rate in Caco2 and HuH7 cells.

5. Acknowledgement

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