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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_{50} 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_{50} 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_{50} 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_{50}

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 (l.0 N)/ ethanol absolute (2ml) in seated tubes. The hydrolyzed samples clarified by centrifugation. One ml $BaCl_2$ 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 N_2. 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 KH_2PO_4 (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 lipid saponified with KOH (20ml, 10%) at 80˚Cfor 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 (LD_{50}) 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 CGG 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 AGT CAG AGC CAA CCT C-3′).

2.3.2.3. Effect of sterol fractions on cell cycle and apoptosis

Each cell line was treated with IC_{50} 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 1
Total lipids content of *Hydroclathrus clathraus*

Phospholipid mg/g	Sulfolipid mg/g	Glycolipid mg/g	Total lipid
0.57±0.04	0.25±0.01	0.08± 00	1.5g/100g

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*

Fatty acid	**Common name**	**%**
$C_{14:0}$	Myristic acid	4.35
$C_{15:0}$	Pentadecanoic acid	1.32
$C_{16:0}$	Palmitic acid	67.04
$C_{16:1}$	Palmitoleic acid	2.66
$C_{18:0}$	Stearic acid	4.87
$C_{18:1}$	Oleic acid	4.36
$C_{18:(2\ ω\ 6)}$	Linoleic acid	1.69
$C_{18:(3\ ω\ 3)}$	α-Linolenic acid	4.36
$C_{20:4}$	arachidonic	8.05

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

Sterols	lipid extract
Hexadecane(C_{16})	1.36
Heptadecane(C_{17})	1.12
Octadecane (C_{18})	3.74
Nonadecane(C_{19})	13.31
Eicosane (C_{20})	7.33
Tricosane (C_{23})	4.15
Tetracosane (C_{24})	10.8
Hexacosane (C_{26})	18.54
Heptacosane (C_{27})	5.75
Octacosane(C_{28})	14.96
Nonacosane (C_{29})	5.11
Cholesterol	8.32
Campasterol	1.36
β-Sitostero	2.08
α- amyrine	2.07

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.

Table 4
LD_{50} of algal sterols determination in albino mice.

Group	Dose (mg/kg)	Number of rats in each group	Number of dead animals Mortality ٪	Mortality ٪	LD_{50} mg/Kg b.w
1	3000	5	0	0	over than 3000 mg /Kg
2	2500	5	0	0	
3	2083.33	5	0	0	
4	1736.11	5	0	0	

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_{50} using MTT assay. The IC_{50} of sterol fraction was 0.22 and 3.09 µg/ml on Caco-2 and HuH-7 cell lines. IC_{50} 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].

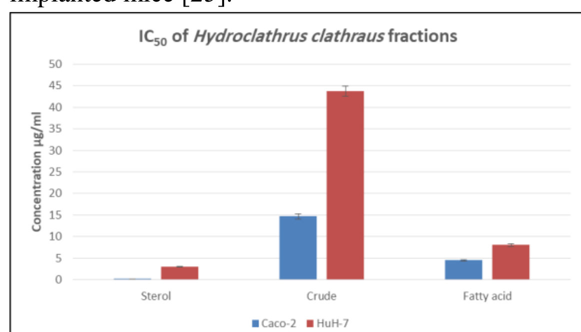

Fig. 1. IC_{50} of *Hydroclathrus clathraus* lipid fractions *on* Caco-2 and HuH-7 cell lines.

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

Sterol fraction has high inhibitory against EGFR with IC_{50} values from 88.3 µg/ml on Caco-2 and 116.8 µg/ml on HuH-7 cells compared to IC_{50} 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].

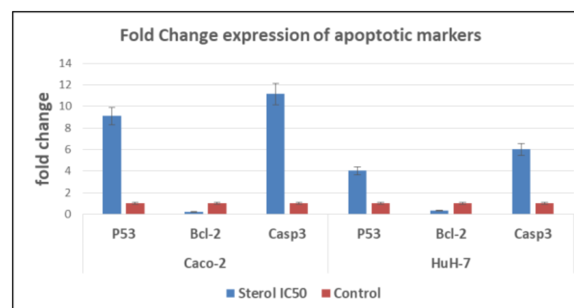

Fig. 2. Inhibitory effect of sterol fraction and standard Erlotinib on EGFR enzyme. Caco-2 and HuH-7 cell lines in comparison to at concentration 20 µg/ml. All samples in comparison to non-treated control cells.

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].

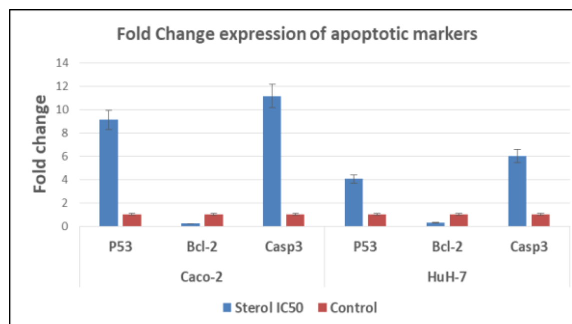

Fig. 3. Fold change expression of apoptotic genes in both Caco-2 and HuH-7 cell lines. Both P53 and Casp3 were up regulated in both cell lines, anti-apoptotic gene Bcl2 was down regulated in both cell lines with $p<0.01$. n=3

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.

Table 5
Effects of Sterol (IC_{50}) on the cell cycle

	Percent cells in							
	Caco-2				HuH-7			
Treatment	Apoptosis	G0-G1	S	G2-M	Apoptosis	G0-G1	S	G2-M
Control	2.62	69.32	24.87	5.81	3.24	68.55	24.23	7.22
Sterol (IC_{50})	23.52	72.36	26.82	0.82	11.26	70.52	27.09	2.39

† 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.

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

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 tiliaefolia* 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 clathraus* enhanced antitumor activities by reducing EGFR enzyme, up-regulation of Caspase 3 and p53, and down-regulation of bcl2 against Caco2 and HuH7. Also by investigating cell cycle analysis, sterols arrest cell cycle in G1/S with significant apoptosis rate in Caco2 and HuH7cells.

5. Acknowledgement

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