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MARINE ALGAL STEROL HYDROCARBON WITH ANTI-INFLAMMATORY, ANTICANCER AND ANTI-OXIDANT PROPERTIES

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ABSTRACT

Important ingredients, various bioactivities, biomaterials are derived from marine algae which are used in many products, such as cosmetics and drugs for treating cancer and other diseases. The present research deals with studying the sterol hydrocarbon content of *Taonia atomaria* brown algae and their related biological activities as anti-inflammatory, anticancer activities against different cancer cell lines including; colon cancer cell line (HCT116), breast cancer cell line (MCF7), liver cancer cell line (HePG2) and ovarian cancer cell line (A549). The determination of antioxidant, scavenging activity of *T. atomaria* sterol fraction and Total Antioxidant Capacity (TAC) were also being elucidated. The present results clearly indicated the presence of hexacosane (C26) sterol hydrocarbon with the highest percentage (21.78%), followed by campasterol (C28:20.66%) and nonadecane (C19:18.06%) in addition, cholesterol, β-sitosterol and pentadecane showed 15.92, 14.07 and 9.523%, respectively. Moreover, sterol fraction exhibited anti-inflammatory and anticancer activities in a dose dependent manner as well as it exhibited powerful DPPH free radical scavenging activity due to its high level of total antioxidant capacity. Thus, it could be concluded that, natural products as sterol hydrocarbon fraction derived from *T. atomaria* marine algae protects cells by modulating the effects of oxidative stress. Because oxidative stress plays an important role in inflammatory reactions and in carcinogenesis, marine algal natural products have a potential role for use in anticancer and anti-inflammatory drugs.

KEYWORDS: **Marine algae, Taonia atomaria, Anti-inflammatory, Anticancer, Cancer cell lines, Antioxidant.**

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INTRODUCTION

Marine algae represent the most significant source of bioactive compounds including vitamin A, B, B_{12}, C, D and E besides, the minerals such as Ca, P, Na and K, which attracted the attention of researchers. [1] Phytosterols are mainly found in the cellular membranes of plants and algae. [2] Algal sterols have a unique group that can be used as a chemotaxonomic biomarker to characterize the members of the three main algal divisions: Chlorophyceae (green algae), Rhodophyceae (red algae), and Phaeophycea (brown algae). [3, 4] Generally, C29 sterols, namely fucosterol, and isofucosterol, are the major compounds in brown and green algae. The common Phytosterols are b-sitosterol, campesterol, stigmasterol and ergosterol. [5] Phytosterols constituents of all eukaryotic membranes play important functions in the control of membrane fluidity and permeability and in signal transduction such as hormones or hormonal precursors .[4] Phytosterols are important component of healthy diets to reduce hypercholesterolemia, oxidative stress, inhibitor cancer, and inhibition of inflammatory secretion and control cardiovascular diseases. [2, 5, 6] This study aims to isolate and identify sterols fraction, characterize their chemical constituents in *T. atomaria* algae, and assess their antioxidant, anticancer and anti-inflammatory activities.

MATERIALS AND METHODS

a- Collection of macroalgal sample

T. atomaria alga (brown algae) was collected from the Mediterranean Sea from (Abu-Qir near Alexandria) in August 2014. The algae were identified by Dr. Rauhaiya Abdul-Latif, Professor of Botany Department, Faculty of Science, Al-Azhar University.

b. Preparation of macroalgae sample

Macroalgae were washed several times with tap water, air in shaded area. The dried of algal sample were grinded into fine particle by electric mill and stored in glass containers at room temperature for further experiments.

c. Ethanolic extraction preparation and successive fractionation

Powdered alga was extracted with ethanol (80%, 1L×5) by soaking at room temperature. The combined ethanol extracts were concentrated under reduced pressure at 45 °C. The crude ethanolic extract was dissolved in hot water, left overnight, filtered and was successively partitioned with petroleum ether, chloroform and ethyl acetate, which were further purified.

d. Separation of unsaponifiable matter

The fraction petroleum ether (1 ml) was saponified with methanolic KOH (20 ml, 10%) at 80˚C for 3 hr under reflux. The unsaponifiable matter was extracted with ether (4 x 10 ml), washed several times with distilled water, dried over anhydrous sodium sulphate. Then the solvent was evaporated and the unsaponifiable matter was quantified (g) and kept for further analysis. [7]

e. Identification of the unsaponifiable matter by GLC

The unsaponifiable matter of marine algae *T. atomaria* was identified by using GLC (Central Services Lab. NRC), with the following conditions:

Hewlett Packard HP 6890 apparatus equipped with HP-1 methyl siloxane capillary column (0.25 mm x 30 m), using a flame ionization detector (FID), and nitrogen was used. Carrier gas was Nitrogen; hydrogen and air gases and set at flow rates 30, 30 and 300 ml/min, respectively. The Oven temperature was programmed from 70-280˚C at a rate 8˚C/min. Temperatures of detector and the injectors were 300 and 250˚C, respectively. The hydrocarbon and sterol compounds were identified by comparing the relative retention times of the separated components with those of available standard materials injected under the same conditions. The quantitative estimation of each compound was based on the area of the recorded peak area.

f. Biological evaluation of T. atomaria sterol hydrocarbon

1. ***In vitro anti-inflammatory activity of sterol hydrocarbon of T. atomaria ethanol extracts using bovine albumin serum***:

Anti-inflammatory of different extracts from algal *T. atomaria* ethanol extract were tested using the method of Rahman et al. [8] The different concentration of algal extract or standard drug diclofenac sodium (50, 100, 150, 200 ug/ml) was mixed with 0.45 ml bovine albumin serum. The sample extracts were incubated at 37°C for 20 min and then heated to 57°C for 3 min, after cooling 2.5 ml phosphate buffer pH 6.4 were added to the samples. The absorbance was measured using UV visible spectrophotometer at 255 nm.

2. Cytotoxic effect on human cell lines:

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan. [9] All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium [(for HePG2- MCF7 and HCT116 - DMEM for A549 and PC3)], 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% L-glutamine at 37 ºC under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 ug/ml). After 48 h of incubation, medium was aspirated, 40ul MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO2. To stop the reaction and dissolving the formed crystals, 200µl of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and

incubated overnight at 37ºC. A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions. [10, 11] The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

[(Reading of extract / Reading of negative control) -1] x 100

A probit analysis was carried for IC50 and IC90 determination using SPSS 11 program.

3. Antioxidant activity of sterol hydrocarbon T. atomaria using:

3.1. DPPH free radical-scavenging assay:

The ability of sterol hydrocarbon from *T. atomaria* to scavenge DPPH free radical were determined according to the method of Ye et al. [12] Briefly, a 0.1 mM of ethanolic DPPH solution was prepared, to give the initial absorbance value of 0.993 at 517 nm. The different concentration of samples (in 0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. After incubation for 30 min in the dark, the absorbance was measured at 517nm. The scavenging activity of DPPH was calculated using the following formula:

$$\text{Scavenging activity \%} = [1 - (A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}})] \times 100$$

3.2. ABTS free radical scavenging assay:

The ABTS free radical scavenging capacity assays were carried out according to the method of Arnao et al. [13] Potassium persulfate (2.6 mM) was added to 7.4 mM of ABTS and kept for 12–16 h at room temperature in dark. The ABTS solution (1ml) was diluted with 60ml methanol to an absorbance of 1.1 ± 0.02 at 734 nm before analysis. ABTS solution (2.80 ml) was added to sample fractions (0.150 ml, 50–150 µg/ml). The After incubation for 2h in the dark, the absorbance was measured at 734nm. Trolox was used as the positive control. The ABTS free radical-scavenging capacity (%) was calculated using the following equation:

$$\text{Scavenging activity \%} = [1 - (A_1 - A_2) / A_0] \times 100$$

Where, A_0 was the absorbance of the control (without sample), A_1 was the absorbance in the presence of the sample, and A_2 was the absorbance without ABTS).

3.3. Evaluation of total antioxidant capacity:

The total antioxidant assays were carried out according to the method of Prieto et al. [14] One ml of different extracts from algal *T. atomaria* ethanol extract (100 to 400 µg/ml) was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium moly date). The tubes were capped and incubated at 95°C for 90 min. After cooling, the absorbance of each samples were measured at 695 nm. A standard series concentration of ascorbic acid was treated as the sample.

4. Statistical analysis:

All results are expressed as mean value of three replicate. Data were statistically analyzed through analysis of variance (Anova), Duncans test and Co-Stat Statistics Software, where unshared letters are significant at P≤0.05.

RESULTS

Several sterol hydrocarbon were identified in *T. atomaria* with high percentages for Hexacosane (C26) which represent 21.78 %, followed by Campasterol (C28) 20.66% and Nonadecane (C19) 18.06% (Table 1). In addition, Cholesterol, β-Sitosterol and Pentadecane as they represented 15.92, 14.07 and 9.523% respectively. Sterol fractions of *T. atomaria* exhibited anti-inflammatory effects in a dose dependent manner, showing the highest anti-inflammatory effect at dose 200 ug/ml (73.51± 1.413 %), comparing to diclofenac sodium which demonstrated 88.47± 0.30 % at the same concentration (Table 2).It's clear from Table (3) that, sterol fraction exhibited strong inhibitory activity against different cancer cell lines, HCT116, MCF7, HePG2 and A549 which recorded reducing percent 94.20, 100.00, 94.50 and 99.20%, respectively. In addition, IC_{50} declared significant low value comparing with IC_{90} indicating higher activity of sterol fraction at the different cell line. However, IC_{50} and IC_{90} at hepatocellular carcinoma cell line (HePG2) were not detected. Data tabulated in Table (4) markedly indicated strong sterol fraction DPPH and ABTS inhibitory activities in a dose dependant manner, where it demonstrated strong significant inhibitory activities at the highest concentration 150 ug/ml (43.23 and 73.83%, respectively for DPPH and ABTS) as compared to their relative standard BHT and Trolox at the same concentrations (107.51 and 122.30%, respectively).Table (5) declared sterol fraction of *T. atomaria* exhibited significant total antioxidant inhibitory activity in a dose dependant manner, where the highest statistically significant inhibitory percentage was noticed at 400 ug/ml (244.04±2.38%), as compared to vitamin C (243.46±3.56%).

Table 1
Sterols and hydrocarbon content of T. atomaria fraction

Compound name	Number of carbon atom	Relative percentage (%)
Pentadecane	C_{15}	9.523
Nonadecane	C_{19}	18.06
Hexacosane	C_{26}	21.78
Cholesterol	C_{27}	15.92
Campasterol	C_{28}	20.66
β-Sitosterol	C_{29}	14.07

Table 2
Anti-inflammatory activity of T. atomaria sterol fraction

Concentration (µg/ml)	Inhibition %	
	Sterol fraction	Diclofenac Sodium
50	51.87±4.89^{e}	74.04±0.37^{c}
100	64.96±2.51^{d}	81.74±0.63^{b}
150	66.28±1.81^{d}	85.5±0.21^{ab}
200	73.51±1.413^{c}	88.47±0.30^{a}
LDS	5.99	3.09

Data representing as Mean±SD % of three replicates. Statistical analysis is carried out using Co-state computer program, where unshared letter is significant at p≤0.05.

Table 3
Anticancer activity of sterol fraction of T. atomaria against cancer cell lines (HePG2, A549, HCT116 and MCF7)

	Inhibition activity %			
	HCT116	**MCF7**	**HePG2**	**A549**
Sterol fraction	94.20±4.40	100.00±5.34	94.50±2.87	99.20±5.98
IC_{50}µg/ml	47.20±2.12^{b}	29.60±1.87^{g}	-	63.4±2.43^{a}
IC_{90} µg/ml	77.9±1.78^{c}	50.20±1.90^{h}	-	83.8±2.32^{d}

Data representing as Mean±SD % of three replicates. Statistical analysis is carried out using Co-state computer program, where unshared letter is significant at p≤0.05.

Table 4
Antioxidant activity of sterol fraction of T. atomaria assessed by DPPH and ABTS free radical

		Scavenging activity %	
	Concentrations (µg /ml)	**DPPH**	**ABTS**
Sterol fraction	**50**	38.89±4.88^{a}	29.26±2.55^{f}
	100	41.64±6.88^{c}	54.10±3.06^{d}
	15 0	43.23±3.10^{c}	73.83±5.85^{c}
		BHT	**Trolox**
Control	**50**	46.36±5.06^{c}	40.0.37±2.32^{e}
	100	81.53±4.39^{b}	81.53±0.75^{b}
	150	107.51±10.00^{a}	122.3±1.13^{a}
LSD		13.12	4.99

Data representing as Mean±SD % of three replicates. Statistical analysis is carried out using Co-state computer program, where unshared letter is significant at p≤0.05.

Table 5
Total antioxidant activity of T. atomaria sterol fraction

	Concentration µg/ml			
	100	**200**	**300**	**400**
Sterol fraction	117.45±10.00^{e}	163.09±5.18^{c}	210.70±10.00^{a}	244.04±2.38^{a}
Ascorbic acid	127.97±3.79^{de}	147.37±10.00^{cd}	230.14±6.18^{ab}	243.46±3.56^{a}
LSD	10.89	11.78	8.98	9.65

Data representing as Mean±SD of three replicates. Statistical analysis is carried out using Co-state computer program, where unshared letter is significant at p≤0.05.

DISCUSSION

The present study reveals anti-inflammatory activity of *T. atomaria* sterol fraction in a dose dependent relationship. It's well-known that oxidative stress plays important roles in endothelial dysfunction, lung disease, gastrointestinal dysfunction, atherosclerosis and inflammatory symptoms are involved in these all disorders. [15-18] It was found that, a lots of natural product derived from marine source exhibited anti-inflammatory properties. [19] Phytosterol have been demonstrated to ameliorate coronary heart diseases by reduction in LDL-cholesterol. [20] Direct relation has been established between intake of phytosterol and a

decrease in chronic diseases risk. [21] In concomitant with the present results, Lee et al. [22] declared anti-inflammatory effects of several red, green and brown algae such as phytosterols from *Dunaliella tertiolecta* green algae. [23] *Gracilaria tenuistipitata* aqueous extract from red algae and anti-inflammatory effects of sulfated polysaccharides from galactofucan from *Lobophora variegata* brown algae. [24-25] In addition, Lee et al. [20] reveled brown algae contains high percentages of phlorotannins and its phloroglucinol content was found to have antioxidant effect as well as it suppressed inflammatory markers in cells stimulated by lipopolysaccharide (LPS). [26] The anti-inflammatory effects of sterol fraction of *T. atomira* may be explained on the basis of immunomodulation of an anti-allergic response. [27] Moreover, the suppressive of inflammation may be due to inhibition the production of pro-inflammatory cytokines and inhibition of inducible nitric oxide synthase and cyclooxygeanase-2 expression. [28]In addition, the present research declared that sterol hydrocarbons exhibited anticancer activity against different cancer cell line compared to standard. In this concern, Lopes et al. [2] reported that phytosterols as a sterol fraction appear to exhibit anticancer properties against different cancer cell lines and a protective effect against colon, prostate, and breast cancer. Previously Awad et al. [29] declared that phytosterols-fed animals showing tumor size and metastases reduction by 33% and 20%, respectively as well as breast cancer cells spreading were retarded. This effects of phytosterol may be explained on the basis of phytosterols enter in the cell membrane structure, changing its properties as well as its enzyme activities. Thus, it was suggested that, these products have capability to change signals that initiating growth of tumor and to enhance program cell death of tumor cell line. Also, immune system was stimulated by phytosterols as indicated by *in vitro*. T-cell proliferation enhancement. Moreover, phytosterols have a principle key role in the prevention of colon cancer. [30, 31] Besides, phytosterols enhance switch of sphingomyelin, the consistence of ceramide as well as stimulation of X-receptor of liver. So, these effectiveness lead to weak rate of cell-cycle development, suppression of cell division , and stimulation of cancer cells death by caspase family activation. [31] Several evidences suggested that natural product derived from marine algae like sterol fraction exhibit properties as anticancer through several ways such as retardation in growth of cancer cells, metastasis, as well as initiation of the program cancer cells death. [32] Program cell death including two pathways; mitochondria and death receptor pathways. [33] Caspases stimulation was found to be involved in each of these two pathways leading to apoptosis [34]. Several works investigated brown algae effectiveness on pathways of program cell death. [33-35] In the present results, sterol fraction of *T. atomaria* (brown algae) may inhibit the division of colon cancer cells by stimulation members of the caspase family Fas and Fas receptors leading to activation of apoptosis markers as poly (ADP-ribose) polymerase (PARP). [34,35] Beside, sterol hydrocarbon fraction of *T. atomaria* may regulate ErbB receptors that play the principal adjusting regulation role in cell division, cells life, catabolism and anabolism. While, un-controlled signals pathway of ErbB receptor is connected with many cancer types such as colon cancer. Thus, ErbB receptor is one of the principle cancer causes. [36] Also, sterol hydraocarbone may be diminished Bcl-2 family protein synthesis and suppressed the development of cell cycle by controlling signals pathways of the ErbB. [37] So, the changing in the synthesis of Bcl-2 family protein causing elevation in the membrane permeability of mitochondrial and hence stimulation of caspase 3 and 9 induced apoptosis. [38] It was found that, the combination of β-sitosterol, with campesterol or β-sitosterol alone may protect from different tumor types. [35] So, the anticancer activity of *T. atamaira* may be explained on the basis of the presence of β-sitosterol and campesterol, as these compounds stimulating apoptosis, hence diminished tumor size.In addition, estrogen receptor plays the principle role in breast cancer. It was also found that, high level of estrogen and progesterone receptors are synthesized in tissues of colorectal cancerous and there is a direct relation between estrogen and progesterone receptors level and cancerous tissue. [39] So, Fang et al. [40] declared estrogen receptors controlling cell division, differentiation and growth in normal and cancerous types. Hence, sterol hydrocarbon may exert its anticancer activity by estrogen receptors suppression and /or blocking activity. Thus, sterol hydrocarbon cytotoxic activity in MCF-7 and HT-29 cells in the present results could be attributed to its sterol structure. Beside, hydroperoxy group in sterol hydrocarbon plays critical role determining anticancer activities of this compound, especially previous review indicated that sterol hydrocarbon derived from *Galaxaura marginata* red algae exhibited anticancerous activity against different cancer cell lines; through program cell death induction. [41] Studies of brown algae have shown that glycoproteins from *Laminaria japonica* and fucoidans from *Sargassum horneri*, *Ecklonia cava*, and *Costaria costata* had anticancer effects on human colon cancer cells by activation of caspase-dependent apoptosis. [42,43] Heterofucans from *Sargassum filipendula* exhibited anti-proliferative effects on cervical, prostate, and liver cancer cells. [22] It was found that fucoxanthin which is a carotenoid exhibited anti- prostate cancer cells activity by inhibiting G1 phase of these cells through pathways of GDD45A and SAPK/JNK. [22] Marine algae, including red, green, and brown algae are characterized to have antioxidant activities and in their enzymatic extracts. [44, 45] The present study demonstrated DPPH, ABTS scavenging activity and powerful total antioxidant capacity in a dose dependent relationship. In a parallel results, Lee1 et al. [22] declared that, red algae *Callophyllis japonica* and *G. tenuistipitata*, sesquiterpenoids from *Ulva fasciata*, *Ulva lactuca* green algae as well as methanol extracts of *Fucus vesiculosus* and *Fucus serratus* brown algae exhibited free radical scavenging activities, suppressed H_2O_2-induced cellular apoptosis, DNA damage, counteract cellular proliferation and activated cellular antioxidant enzymes. In addition, marine algae are very rich in flavonoids which have potent antioxidant properties and reduced hepatic oxidative stress[44].

CONCLUSION

Marine algal natural products are rich sources of antioxidants. Sterol fraction bioactive components derived from *T. atomaria* brown algae, showed intensive modifying effects on oxidative stress-related diseases. It has a promising cytotoxic effects on various cell lines particularly human liver, colon, breast and ovarian cells due to its powerful antioxidant activity. Extensive works must be applied for using these active natural products derived from marine algae in drug discovery as nutraceuticals.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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