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Chemical Characterization and Cytotoxic Potential of a Chloroform Fraction Obtained from Marine Plant *Thalassia testudinum*

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Abstract

Marine plants and algae represent a prolific source of new bioactive metabolites with multiple pharmacological applications. The hydroethanolic extract of marine angiosperm *Thalassia testudinum* is enriched in polyphenols and glucopyranosides and it has demonstrated cytoprotective and antioxidant properties in different models, however, the non-polar components of this extract have not been fully characterized and their cytotoxic capacity has not been evaluated. In this work, it was obtained and characterized a chloroform fraction from *T. testudinum* hydroethanolic extract. By GC-MS analysis 69 compounds were identified, where palmitic acid was the main component. Our study also revealed a cytotoxic potential of this organic fraction in the cell lines A549 (human lung carcinoma) and EA.hy926 (human immortalized endothelial cells).

Keywords: *T. testudinum*; Chloroform fraction; Palmitic acid; Cytotoxicity; Human cancer cells; Human endothelial cells

Abbreviations: HIF-1: Hypoxia inducible factor 1; GC-MS: Gas chromatography-Mass spectrometry; ChFT: Chloroform fraction from *T. testudinum*; MSTFA: N-methyl-N-(trimethylsilyl) trifluoroacetamide; NIST: National Institute of Standards and Technology; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl Sulfoxide; SD: Standard Deviation; ROS: Reactive Oxygen Species.

Introduction

Natural products are getting attention as an attractive alternative in the therapeutics of multiple chronic diseases, due to their variety of pharmacological effects and their relatively low toxicity [1,2]. Marine flora, represents a promising source of new anti-inflammatory and anticancer agents [1,3]. Due to the extreme conditions of pH, salinity, temperature, sunlight exposure and pathogen threat in which these organisms grow, they produce secondary metabolites with a huge chemical variety [4-11]. These substances have been demonstrating a wider spectrum of pharmacological action, including anti-inflammatory, immunomodulatory, neuroprotective antitumor, antioxidant, photoprotective and antimicrobial activities [9-15].

The chemical composition and the biological properties of polar extracts from marine plants and algae have been extensively studied, but less is known about non-polar extracts and lipophilic fractions derived from these organisms. Methanolic and hydroethanolic extracts of different macroalgae such as *Plocamium telfairiae*, *Gracilaria tenuistipitata*, *Ulva lactuca* and marine plants such as *Thalassodendron ciliatum* have demonstrated cytotoxic and anti-proliferative activity in cancer cells with induction of apoptosis and oxidative stress-dependent cell death [16-20]. Other studies have revealed the cytotoxic potential of organic fractions and fatty acids isolated from various algal species [19,21,22]. The hydroethanolic extract obtained from the leaves of marine angiosperm *Thalassia testudinum* has demonstrated antioxidant, regenerative, photoprotective, antinociception, hepatoprotective and neuroprotective properties *in vitro* and *in vivo* [23-26]. A chemical characterization of this polar extract showed the presence of phenolic acids, flavonoids and glucopyranosyl compounds [27]. However, the lipophilic components of this extract have not been completely elucidated and their antitumor potential has not been explored either. In a previous analysis of n-hexane fraction, obtained from a chloroform extract of *T. testudinum* leaves, 43 compounds were identified, mainly hydrocarbons [28]. This fraction only exhibited strong antioxidant and photoprotective effects [28]. Based on this previous background, we decided to obtain and characterize a chloroform fraction from the hydroethanolic extract of *T. testudinum* to study its cytotoxic potential in human lung cancer cells and human immortalized endothelial cells.

Materials and Methods

Chemicals

Analytical-grade reagents and reference patterns for Gas chromatography-Mass spectrometry were obtained from Sigma, USA. Culture media and supplements were purchased from GIBCO (Gibco BRL, Paisley, UK).

Plant material

Thalassia testudinum (Banks and Soland ex. Koenig) was collected on September 2016 in "Guanabo" beach (22° 05' 45" N, 82° 27' 15" W). The specimen was identified by Dr. Areces J.A. (Institute of Oceanology, Havana, Cuba). A voucher sample (No. IdO40) was deposited in the herbarium of the Cuban National Aquarium, Havana, Cuba. Whole dry and ground *T. testudinum* leaves (500 g) were continuously extracted

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by mechanical stirring at 800 rpm with EtOH-H_2O (50:50, v/v) during 1 h at 60°C. The extract was filtered and concentrated under reduced pressure and low temperature (40 °C) to total dryness. Then, 3 g of crude extract were macerated with 300 mL of $CHCl_3$ with mechanical stirring at room temperature for 1 h. The obtained fraction was filtered and dried under reduced pressure, yielding 1.2%.

Cell culture

A549 cells (human lung carcinoma) and EA. hy926 cells (immortalized human umbilical endothelial cells) were obtained from ATCC collection. They were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with glutamine 2 mmol/L, antibiotic and 10% fetal bovine serum, according to the recommendations of the supplier. Cells were maintained in a 5% CO_2 atmosphere at 37°C in incubator.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Samples of chloroform fraction (5.0 mg) from *T. testudinum* (ChFT) were accurately weighed into a 2 mL vial, then 0.15 mL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were added, the vial was tightly capped, heated at 80°C for 1 h and 0.5 µL were analyzed by gas chromatography-mass spectrometry (GC-MS).

The analysis was performed using an Agilent GC 6890N equipped with a mass selective detector 5975 B inert and a split-splitless injector, in splitless mode, was used (Agilent, Palo Alto, CA, USA). Separations were made on a HP-5Ms fused-silica capillary column (30 m × 0.25 mm), with a film thickness of 0.25 µm D_f (Agilent, Avondale, PA).

The GC oven temperature was kept at 60°C for 2 min and programmed to 200°C at a rate of 20°C/min, then from 200°C to 300°C at a rate of 8°C/min and kept constant at 300°C for 5 min. The temperature of the injector was fixed at 320°C and that of the source at 280°C, while MS interface temperature was 250°C. Helium (purity, 99.9995%) was the carrier gas; its flow rate was fixed at 1 mL/min. Ionization of the sample components was performed in electron impact mode (EI, 70 eV). The mass range from 40 to 1000 m/z was scanned at a rate of 3.0 scans/s. One microliter of the organic extract was manually injected into the GC-MS system by using a Hamilton syringe, for total ion chromatographic analysis by splitless injection. The total running time of the GC-MS system was 70 min. The relative percentage of each extract constituent was expressed as percentage with respect to peak area normalization. Peak identification was achieved by computer matching mass spectra against commercial libraries (National Institute of Standards and Technology (NIST) 2011 GC/MS), as well as MS literature data [22,28-30].

Cytotoxic evaluation by MTT reduction assay

Cell viability of A549 and EA. hy926 cells in presence of different concentrations of ChFT (0.01-1000 µg/mL) was evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following a previously described method [31]. The organic fraction was dissolved in culture media with dimethyl sulfoxide (DMSO) 1%. The concentration causing reductions of 50% in cell viability (IC_{50}) was calculated using the GraphPad Prism software. Untreated cells and cells treated with DMSO 1% were used as controls. Cell viability was determined from three independent experiments with three replicas each.

Statistical analysis

The statistical analysis was carried out using the GraphPad Prism software. The values are expressed as the mean ± standard deviation (SD), of at least three independent experiments. For multiple mean comparisons was used a one-way ANOVA with a Tukey post-test ($p<0.05$).

Results

Chemical characterization of ChFT

The GC-MS analysis of ChFT revealed the presence of saturated and unsaturated fatty acids (ω-3 and ω -6), phenolic acids, sterols and glycerides (Figure 1 and Table 1). The chromatogram of the chloroformic fraction after chemical derivatization showed 11 chromatographic peaks between 10 and 30 min of retention time, corresponding with the main components of the organic fraction, as well as other secondary peaks which represent other constituents (Figure 1). The subsequent analysis of these chromatographic products by MS allowed the identification of the chemical structures present in ChFT. For retention times from 10 to 60 min, a total of 69 compounds were detected, where palmitic acid (C16:0) was the most abundant (48.4%), followed by oleic (C18:1), linolenic (C18:3), linoleic (C18:2) and stearic acids (C18:0) (Table 1). The identification of palmitic acid by comparison of its mass spectra with a commercial pattern is shown in Figure 2.

Cytotoxic activity of ChFT in A549 and EA.hy926 cells

The presence of long-chain fatty acids as well glycerides and phenolic acids, suggests possible cytotoxic and antitumor properties for ChFT. According to this, the cytotoxic potential of the organic fraction was evaluated in the human lung carcinoma A549 and in the human immortalized endothelial cell line EA. hy926. As we expected ChFT was able to significantly reduce the viability of treated cells in comparison to control cells (Figure 3). ChFT exhibited a concentration-dependent

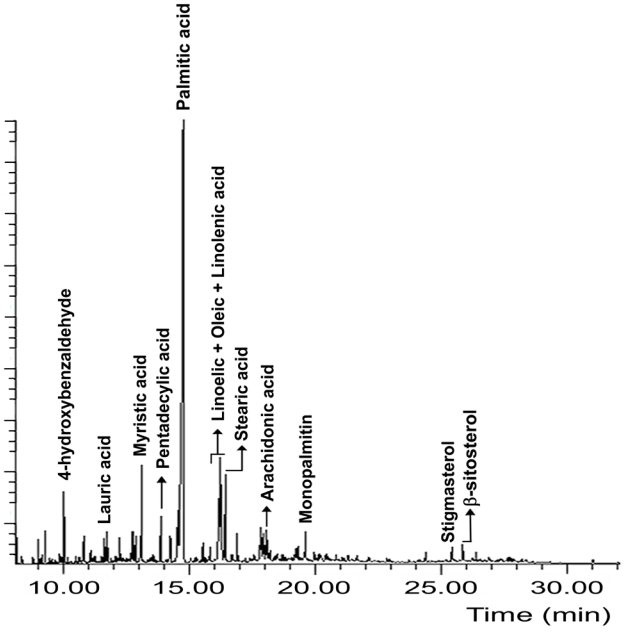

Figure 1: Chromatogram of the chloroform fraction obtained from *T. testudinum* showing its main components. The sample was derivatized with MSTFA and analyzed by GC-MS.

cytotoxicity in A549 and EA. hy926 cells with an IC_{50} of 20.4 and 248.4 µg/mL, respectively (Figure 3; Table 2).

Discussion

Through GC-MS analysis we have identified around 69 compounds in ChFT, a chloroform fraction derived from the hydroethanolic extract of marine angiosperm *Thalassia testudinum*. Most of these compounds were identified for the first time in this specie, representing an important contribution to the chemo-taxonomical characterization of *T. testudinum*. Fatty acids are the main constituents of ChFT, representing around 80% of this organic fraction. These compounds are preferentially extracted due to the polarity of chloroform and they have been reported in other chloroformic extracts from marine plants and algae [29,32,33]. Such compounds were also found as majors in the other specie of the same genus *Thalassia hemprichii* (Ehrenberg) Ascherson and other seagrasses collected in India [34] and Australia [35]. Among detected sterols, β-sitosterol, stigmasterol, cholesterol, hydro cholesterol and 3-methoxy(3B,24S)-stigmastan-4-one, have been identified in different algae and marine plants of Caribbean Sea [36] and Australia [35].

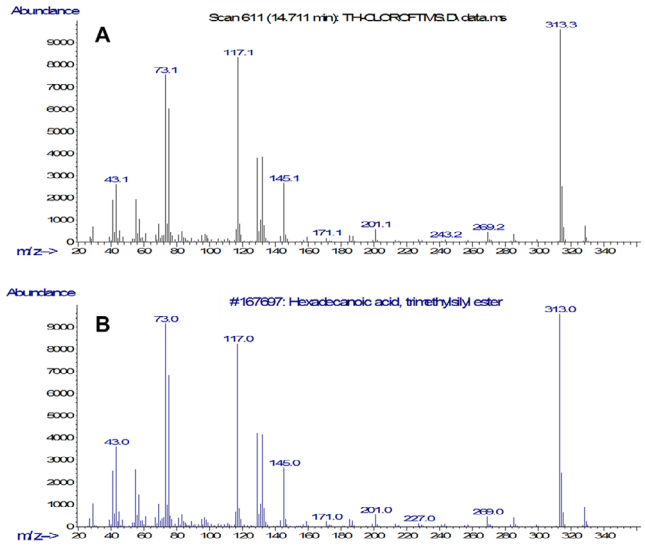

Figure 2: Identification of palmitic acid present in the chloroform fraction of *T. testudinum* by mass spectrometry. The mass spectrum of the main component from the organic fraction with a retention time of 14.71 min, showing identical correspondence with the mass spectrum of the trimethylsilyl derivate of hexadecanoic acid of library NIST. Consequently, this component is identified as hexadecanoic (palmitic) acid.

In a previous chemical characterization, the n-hexane fraction obtained from a chloroform extract of *T. testudinum* leaves was analyzed by GC-MS, but only 43 compounds were identified (a 50% of the total) [28]. Among these constituents (mainly hydrocarbons) the 1,1-bis(p-tolyl) ethane was the most abundant but non-fatty acids were detected. Is important to point out, that *T. testudinum* plant studied by Regalado et al. was collected in other region and time, and the fraction was analyzed without derivatization. Despite that, some of the minor saturated hydrocarbons reported in this paper were also identified by us in ChFT. In addition, the n-hexane fraction obtained by Regalado et al. demonstrated potent antioxidant and photoprotecting capacities; however, the cytotoxic or antitumor potential of this organic fraction was not evaluated.

In our study, palmitic acid was identified as the main component of ChFT. This is in accordance with the chemical characterization of other non-polar extracts and chloroform fractions from different macroalgae such as *Digenia simplex, Colpomenia sinuosa, Halimeda discoidae, Galaxaura oblongata* and *Ulva lactuca*, in which palmitic acid was also the most abundant metabolite and exhibited chemoprotective and antitumor effects [21,22,29]. In one of these studies, Huang et al. revealed that the cytotoxic activity of three organic extracts from marine algae was related with their capacity to induce apoptosis in an oxidative stress-dependent manner. Palmitic acid has also showed cytotoxic effects in the human lung cancer cell line A549 through a mechanism that involves endoplasmic reticulum stress, hypercalcaemia and generation of reactive oxygen species (ROS) [37]. Furthermore, this fatty acid displays a particular mechanism of cell death in EA. hy926 cells, which include mitochondria-dependent necroptosis and autophagy [38]. Interestingly, as occurs in A549 cells, the palmitic acid-induced cytotoxicity in this human transformed endothelial cell line seems to be triggered by intracellular accumulation of Ca^{2+} [38].

Oleic acid has also demonstrated antitumor activity *in vitro*, against murine cancer cells such as TA3 (breast carcinoma) and 6C3HED (lymphosarcoma) [39] as well as in a variety of human tumor cell lines [40]. Likewise, different mixtures of fatty acids showed antileukemic activity in Jurkat cells [41] and increase the cytotoxicity of paclitaxel in human breast carcinoma cell lines such as MDA-MB-231, SK-Br3, T47D and MCF-7 [42]. In addition, other mixtures of polyunsaturated long-chain fatty acids potentiate the antitumor effects of cisplatin in A549 cells, via induction of apoptosis and autophagic cell death [43].

In line with this, ChFT displayed a strong cytotoxicity in A549

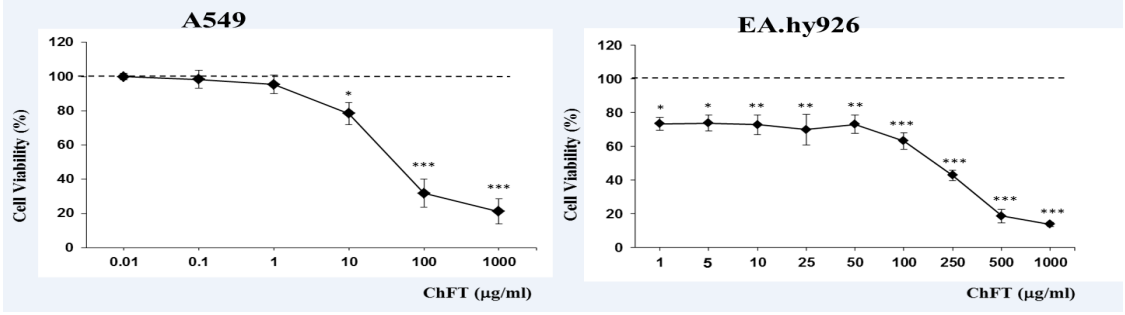

Figure 3: Cytotoxicity of the chloroform fraction of *T. testudinum* in A549 and EA.hy926 cells. Cell viability was evaluated by MTT reduction assay after 48 h of treatment with different concentrations of the fraction (0.01-1000 µg/mL). The values are shown as mean percentages of control ± SD from three independent experiments. *p<0.05, ***p<0.001 vs control (untreated cells).

Compound	Rt (min)	%	Compound	Rt (min)	%
2-pyrrolidone	8.13	0.64	3,7,11,15-tetramethyl-2-hexadecanol	15.82	0.49
Hydroxybutanoic acid	8.28	0.05	Linoleic acid (C18:2)	16.16	3.15
Heptanoic acid (C7:0)	8.32	0.18	Oleic + Linolenic acid (C18:1+C18:3)	16.21	7.73
Diethylene glycol	8.98	0.42	Stearic acid (C18:0)	16.42	3.14
Benzoic acid	9.01	0.14	Unsaturated fatty acid	16.89	0.89
Caprylic acid (C8:0)	9.12	0.18	Sterol	17.79	0.91
Phosphoric acid	9.25	0.71	Unsaturated fatty acid	17.84	1.44
Glyceric acid	9.67	0.04	mono glyceride	17.94	1.32
Nonanoic acid (C9:0)	9.83	0.22	Arachidonic acid (C20:4)	18.04	1.34
4-hydroxybenzaldehyde	9.99	1.55	1-heneicosanol (C21OH)	18.10	0.92
Capric acid (C10:0)	10.48	0.10	Arachidic acid (C20:0)	18.19	0.50
2-2-phenylcyclopropyl-thiophene	10.61	0.15	monopalmitin	19.61	1.31
p-hydroxybenzoic acid methyl ester	10.78	0.58	Behenic acid (C22:0)	19.95	0.38
Unsaturated fatty acid	11.06	0.36	2-hidroxysebasic acid	20.14	0.30
Dihydroactinidiolide	11.14	0.07	Monooleine	21.15	0.12
p-hydroxybenzoic acid	11.64	0.55	Lignoceric acid (C24:0)	21.65	0.25
3-hydroxycapric acid	11.76	0.16	Cerotic acid (C26:0)	23.26	0.04
Lauric acid (C12:0)	11.78	0.72	1-octacosanol (C28OH)	24.08	0.08
p-hydroxybenzoic acid propyl ester	11.82	0.11	α-hydroxycholesterol	24.14	0.09
Salicylic acid	12.19	0.51	Cholesterol	24.39	0.44
p-hydroxy-3-methoxybenzoic acid	12.50	0.05	3-methoxi(3B,24S)-stigmastan-4-one	24.92	0.04
Azelaic acid	12.73	0.96	Campesterol	25.20	0.06
Unsaturated fatty acid	12.85	0.85	Stigmasterol	25.44	0.72
cis-9-Tetradecenoic acid (C14:1)	13.04	0.97	β-sitosterol	25.86	0.83
Myristic acid (C14:0)	13.09	2.62	Lacceroic acid (C32:0)	26.25	0.19
Sebasic acid	13.45	0.07	Lanostan-3-one-18-epoxy	26.40	0.35
Methyltetradecanoate (C14:0met)	13.56	0.27	Sterol	27.65	0.22
Pentadecanoic acid	13.85	1.34	Sterol	27.75	0.25
Ethylhexadecanoate (C16:0et)	14.23	1.19	Sterol	28.22	0.22
Palmitoleic acid (C16:1)	14.53	2.81	Lanosterol (D)	28.37	0.23
Palmitic acid (C16:0)	14.76	50.21	Glyceride	31.04	0.25
Margaric acid (C17:0)	15.52	0.63	Glyceride	34.32	0.59
1-octadecanol (C18OH)	15.62	0.16	Stearinlinolein Glyceride	38.85	0.25
Ethyllinoleate (C18:2et)	15.69	0.07	Glycoside	48.91	0.46
Ethyllinoleate (C18:3et)	15.76	0.26	Glycoside	51.57	0.40

Rt: Retention time; (D): Derivative

Table 1: Chemical composition of the chloroform fraction from *T. testudinum* hydroethanolic extract.

ChFT IC_{50} (µg/mL)[a]	
A549 (ATCC CCL-185)	EA.hy926 (ACTCC CRL-2922)
20.4 ± 10.6	248.4 ± 4.8

[a]IC_{50}: Inhibitory concentration causing reductions in 50% of cell viability

Table 2: Cytotoxic index of the chloroform fraction from *T. testudinum* in A549 and EA.hy926 cells.

cells, in a concentration-dependent manner, suggesting that some of these mechanisms could be involved in a synergistic effect between the fatty acids present in this chloroform fraction. ChFT also inhibited the proliferation of immortalized human endothelial cells EA. hy926, a fact that can be partially attributed to the high content of palmitic acid in the organic fraction. Despite that, ChFT shows lower activity in EA. hy926 cells in comparison to A549 cells. Is important to point out that EA. hy926 cell line is obtained by the fusion of human umbilical vein endothelial cells with the human lung carcinoma A549, and this macro-vascular cell line retains several phenotypic characteristics of umbilical endothelial cells [44]. Thus, the differential cytotoxicity of ChFT in these cell lines could be a result of selectivity toward the more aggressive phenotype of A459 cells.

Other compound isolated from natural sources, the quinone methide triterpene Celasterol can inhibit the proliferation and migration of A549 and EA. hy926 cells, decreasing the expression and activity of the hypoxia inducible factor 1 (HIF-1) [45]. Deeper molecular studies will reveal the mechanisms responsible for the cytotoxic and antiproliferative effects of ChFT, but our results indicate an inhibitory effect of this organic fraction on both tumor and endothelial cells, two important targets in cancer therapy.

On the other hand, the presence of glycerides was also detected in ChFT. This kind of compounds has also demonstrated antitumor and

anti-proliferative capacities. For example, the 1(3)-O-hexadecanoil-2-O-β-D-glucopyranosyl-glycerol and 1(3)-O-oleolil-2-O-β-D-glucopyranosyl-glycerol have been studied in deep, due to their capacity to inhibit the proliferation of malignant cells transformed with Epstein-Barr virus [46].

On the other hand, the glycerol molecule has demonstrated antiproliferative effects, interfering with microtubule assembly and dissociation. In the same way, the 1(3)-O-hexadecanoil-glycerol and 1(3)-O-oleolil-glycerol were identified in the antimitotic fraction isolated from the macroalgae *D. simplex* [22]. These evidences suggest that glycerides could contribute with the cytotoxic activity demonstrated by ChFT. Further research on bio guided fractioning and cytotoxicity is required to define if the observed effects are mediated by an active ingredient or are a result of the synergy between all the compounds present in this chloroform fraction.

Conclusions

It was obtained and characterized by GC-MS a chloroform fraction from the polar extract of marine plant *Thalassia testudinum* grown in Cuban coastal zones. This organic fraction shows, for the first time, a potent cytotoxicity in the human lung carcinoma A549 and antiproliferative effects in the human immortalized endothelial cell line EA. hy926 as well as a new chemical composition for this specie. Thus, our study reveals, at this time, this chloroform extract as a potential source of natural antitumor agents that should be explored deeply in the future.

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Conflict of Interests

The authors declare that there are not conflicts of interest associated with this work.

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