Tropical Journal of Pharmaceutical Research October 2018; 17 (10): 1977-1982
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i10.12

Original Research Article

Fatty acid constituents and anticancer activity of *Cladophora fracta* (OF Müller ex Vahl) Kützing

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Sent for review: 31 March 2018 *Revised accepted: 22 September 2018*

Abstract

Purpose: *To determine the fatty acid constituents and anticancer effect of Cladohora fracta*
Methods: *Cladophora fracta (O.F. Müller ex Vahl) Kützing was collected from natural ponds in Tokat, Turkey. Antiproliferative and cytotoxic effects of methanol and hexane extracts of C. fracta were investigated on human colon carcinoma (HT29) and non-tumorigenic African green monkey kidney (Vero) cell lines using BrdU cell proliferation enzyme-linked Immunosorbent assay (ELISA) and lactate dehydrogenase (LDH) test, respectively. The fatty acid composition of hexane extract was analyzed by gas chromatography-mass spectrometry (GC-MS).*
Results: *Oleic acid, palmitic acid, gamma-linoleic acid and linoleic acid were the main constituents of C. fracta. The methanol extract exhibited strong antiproliferative activity on HT29 and Vero cell lines ($p < 0.05$). The hexane extract revealed its good antiproliferative activity at high concentrations on both cell lines. Cytotoxicity results showed that both methanol and hexane extract had low effect on HT29 cell at low concentrations.*
Conclusion: *Due to the strong antiproliferative effect of C. fracta methanol extract on HT29 and Vero cell lines, it has potential anticancer properties and recommended for further development as such.*

Keywords: *Cladophora fracta, Antiproliferative activity, Anti-cancer, Cytotoxic effect, Fatty acid*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Natural products play a considerable role in drug development processes, due to bioactive secondary metabolites in their compositions [1-7]. Bioactive compounds from algae with efficient medicinal potential attract a significant amount of attention in cancer research. Algae have been known as one of the most effective groups of organisms to isolate bioactive natural compounds. Phytochemical research on algae leads to isolation of fascinating secondary metabolites with various biological effects such as antitumor, antifungal, antioxidant, antibiotic, anti-HIV, antimicrobial, anti-inflammatory, herbicidal, immune-suppressive activities [8].

Cancer is a major public health problem and is the leading cause of human death all over the world. Cancer is uncontrolled cell growth which can kill normal cells or spread quickly to all body parts [9]. Taking into account the increasing

cancer incidence rates, safe and effective treatment is crucially needed to inhibit tumor cell proliferation. Nowadays, chemotherapeutic medicines are being used effectively in cancer therapy. However, chemotherapeutic medicines have heavy toxicity and side effects. Therefore, there is an interest in natural agents for cancer treatment [10].

Algae have been extensively used as a food additive and for medicinal purposes. Since some algae have been used in treatment of cancers, many crude extracts and isolated compounds from algae have been investigated for their anticancer effects and these studies revealed that algae contain some novel compounds with therapeutic properties for human diseases [11].

In a previous work, the good inhibitory activities of *Mougeotia nummuloides* and *Spirulina major* on different cancer cell lines were demonstrated [12]. In the present study, the anticancer and cytotoxic activities of *Cladophora fracta* extracts have been investigated along with the fatty acid constituents of the hexane extract.

EXPERIMENTAL

Collection and identification

Cladophara fracta was collected from Tokat Gaziosmanpasa University Campus during the spring of 2015. The alga material was washed with water to get rid of epiphytes, invertebrates, extraneous material. After the addition of 5 % formalin to the alga specimen, identification was executed with a light microscope [13].

Extraction of *C. fracta*

The air dried C. *fracta* (10 g) was powdered and extracted with 100 ml of hexane for 24 h and the filtration was executed by a Buchner funnel with a filter paper. Then, *C. fracta* hexane extract was re-extracted with methanol (100 mL). After filtration, the solvents were removed by a rotary evaporator to yield the hexane extract and methanol extract which were stored (+4 °C) for further analysis.

Esterification of hexane extract

The hexane extract (35 mg) was dissolved in hexane (5.0 mL) and centrifuged at 3500 rpm for 3 min. The supernatant (3 mL) was poured to a glass tube. 3 mL of KOH (2 M in methanol) was added and vigorously shaken. The upper layer was directly injected to Gas chromatography-flame ionization detector (GC-FID).

GC-FID analysis

Fatty acid analysis was carried out on an instrument (Perkin Elmer Clarus 500) with RTX-2330 (Restek) capillary column (30 m × 0.25 mm id., × 0.20 µm film thickness). Helium was used as beared gas at a flow rate of 1 mL/min with 50/1 split ratio. The detector and injection temperatures were 250 and 37 °C, respectively. Fatty acid methyl ester mixtures were used as standards to identify fatty acids.

Preparation of cell culture

Anticancer effects of *C. fracta* extracts were examined on various cell lines such as cancerous HT29 (ATCCR HTB-38™) and non-cancerous Vero cells (ATCCR CCL-81™). The cultivation was executed in Dulbecco's modified eagle's cell medium (DMEM) supplemented with fetal bovine serum (10%, v/v) and Penicillin-Streptomycin solution (2%, v/v). Old medium was removed from plates when cells achieved a congestion of 85%. Then, cells were ripped from flask using trypsin-EDTA (4.0 mL) (Sigma, Germany). Finally, the cell pellet was resuspended using DMEM (4.0 mL) and counted to achieve a concentration of 5×10^4 cell/mL and inoculation was executed in wells [4].

BrdU proliferation assay

A cell suspension of roughly 5×10^3 cells in 100 µL was inoculated into the wells of culture plates. *C. fracta* extracts and standard (5 fluorouracil, 5FU) were dissolved in DMSO (0.5 %), and then were treated with cells at the concentrations of 5, 10, 20, 30, 40, 50, 75, and 100 mg/mL at 37 °C overnight. DMEM was applied to adjust the final volume to 200 µL. The measurement of cell proliferation was executed by ELISA Kit (Roche, USA). Microplate reader was employed to evaluate the BrdU incorporation at 450-600 nm and each trial was carried out three times [5].

Calculation of IC_{50} and % inhibition

XLfit5 software was carried out to calculate the half maximal inhibitory concentration (IC_{50}). Inhibition was calculated as in Eq 1.

$$\text{Inhibition (\%)} = \{(A - B)/B\}100 \quad \ldots\ldots\ldots (1)$$

where A is the absorbance of the extracts and B the absorbance of DMSO.

Evaluation of cytotoxic activity

The cytotoxicity of hexane extract and methanol extract as well as fluorouracil were carried out on

HT29 and Vero cells using a Lactate Dehydrogenase Assay Kit (Roche, USA). Nearly 5×10^3 cells in 100 µL were placed into microtiter plates and reacted with diverse concentrations of *C. fracta* extracts. LDH activity was detected at 492 - 630 nm using a microplate reader [14]. The cytotoxicity results were calculated by Eq 2.

Cytotoxicity (%) = {(EV - LC/HC - LCo) × 100} ..... (2)

where EV= experimental value, LC = low control, and HC = high control.

Assessment of cell morphology

Cells were set up in plates at a density of 5.000 cells per well and kept for 24 h. Morphological changes of the cells were observed by a phase microscope (phase contrast) in every 6 hours for 24 h. The sights of extracts and standard treated with the cells were photographed by a digital camera combined with an inverted microscope.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (Windows version 21.0, SPSS). The data were reported as mean ± standard deviation. Differences between groups were considered significant at the 5% probability level.

RESULTS

Fatty acid content

After esterification of hexane extract, fatty acid constituents were determined using GC-MS analysis (Table 1). Oleic acid (46.0%), palmitic acid (15.6%), gamma linoleic acid (10.6%), linoleic acid (7.5%) were found as main constituents. In addition, lauric acid, myristic acid, pentadecanic acid, palmitoleic acid were detected in hexane extract of *Cladophora fracta*.

Antiproliferative activity

Antiproliferative effects of *C. fracta* hexane and methanol extracts were carried out on Vero and HT29 cell lines using BrdU Cell Proliferation ELISA kit, with 5FU as a positive control for anticancer activity (Figure 1). XLfit5 software was applied for determination of IC_{50} values (Table 2).

C. fracta methanol extract showed higher activity than 5 FU (Table 2). On the other hand, activity of hexane extract was not more effective than 5FU on cell lines. Methanol extract displayed higher tumor specificity on HT29 cell lines than hexane extract.

Table 1: Fatty acid constituents of *Cladophora fracta*

RT (min)	Fatty acid	Area (%)
7.149	C12:0 (Lauric acid)	0.42
11.419	C 14:0 (Myristic acid)	4.46
14.047	C15:0 (Pentadecanoic acid)	0.29
16.923	C16:0 (Palmitic acid)	15.56
18.163	C16:1 (Palmitoleic acid)	1.54
21.487	C17:1 (cis-10-Heptadecenoic acid)	0.70
22.875	C18:0 (Stearic acid)	2.21
24.299	C18:1n9c (Oleic acid)	45.99
25.900	C18:2n6t (Linolelaid acid)	0.54
26.536	C18:2n6c ((Linoleic acid)	7.53
28.865	C20:0 (Arachidic acid)	0.25
29.292	C18:3n6 (gama Linoleic acid)	10.66
34.750	C20:3n3 (Eicosatrienoic acid methylester)	1.02
35.390	C22:1n9 (Erucic acid)	0.22
36.886	C20:5n3 (Eicosatrienoic acid methylester)	8.37

Table 2: Half Maximal Inhibitory Concentration (IC_{50}) values for *C. fracta* extracts and control

	IC_{50} ± SD (µg/mL)		
Sample	*HT29*	*Vero*	*Tumor specificity*
Hexane	35.04±1.3	27.28±1.0	0.78
MeOH	14.84±0.9	14.16±0.8	0.95
5FU	21.58±1.1	20.55±1.0	0.95

SD: standard deviation, 5FU: 5-Florouracil, Tumor-specificity for HT29 = {IC_{50} (Vero)}/{IC_{50} (HT29)}

Both extracts were found to have significant effects on HT29 cell lines to some degree. Activity of methanol extract was higher than 5FU at all concentrations. Nevertheless, hexane extract displayed lower activity up to 50 µg/mL. In Vero Cell lines, methanol extract showed the same activity as 5FU up to 20 µg/mL. Its inhibition increased after 20 µg/mL. In regard to hexane extract, it displayed good activity at high concentrations as usual (Figure 1).

Cytotoxicity

Cytotoxicity of *C. fracta* hexane and methanol extracts were tested on cell lines via LDH cytotoxicity assay kit. Cytotoxicity was evaluated by treating cells with various concentrations of *C. fracta* extracts (5, 10, 20, 30, 40, 50, 75, 100 µg/mL) that displayed breakdown of cell membrane with the concentration dependence. The cytotoxicity tests revealed that reaction of cells with higher concentrations of hexane and methanol extracts of *C. fracta* brought about cell membrane damage but at low concentration

significant cytotoxicity was not detected. Interestingly, both hexane and methanol extracts of *C. fracta* were safest for HT29 cell line at 40 µg/mL and below dose while were highly toxic after 30 µg/mL for other cell lines. *C. fracta* extracts exhibited cytotoxic effect at high concentrations. So *C. fracta* has therapeutic properties (Figure 2).

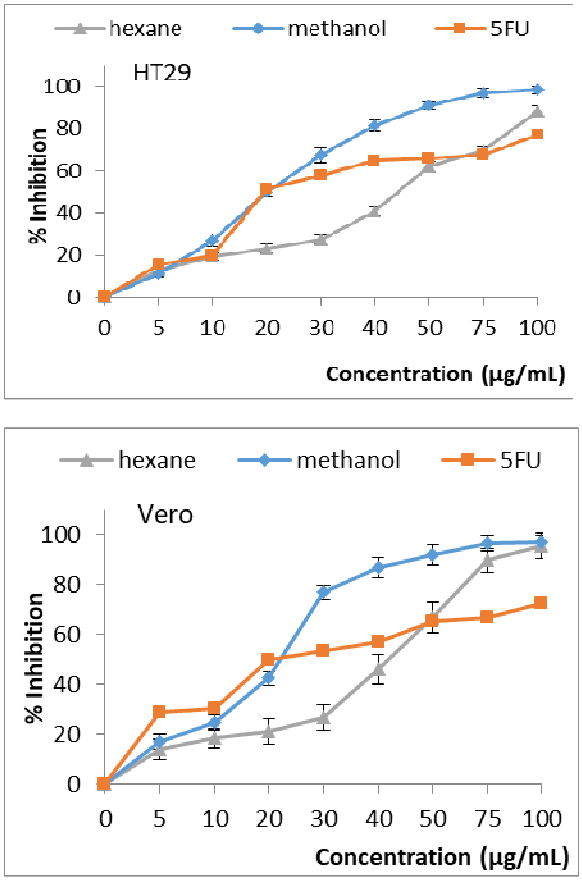

Figure 1: Antiproliferative effect of hexane, methanol extract and 5FU on HT29 and Vero cell lines

Morphological features

Morphological assessment of cytotoxic activity of *C. fracta* hexane and methanol extracts (Figure 3) were executed with a digital camera combined with an inverted microscope (Germany, Leice IL10). Treatment of cells with extracts changed the shape of cells from round to floating form, indicating the death of cells at high concentration. At low concentrations, these extracts did not affect the growth of cells. Activity of methanol extract was better than that of the hexane extract.

DISCUSSION

Freshwater environment has a great potential for discovery of bioactive compounds that could be used in pharmaceutical industries [15]. Especially, green algae harbor considerable amount of bioactive compounds which display a great deal of biological activity [16]. In addition, *Cladophora* species consist of the free sterols, their esters and glycosides [17].

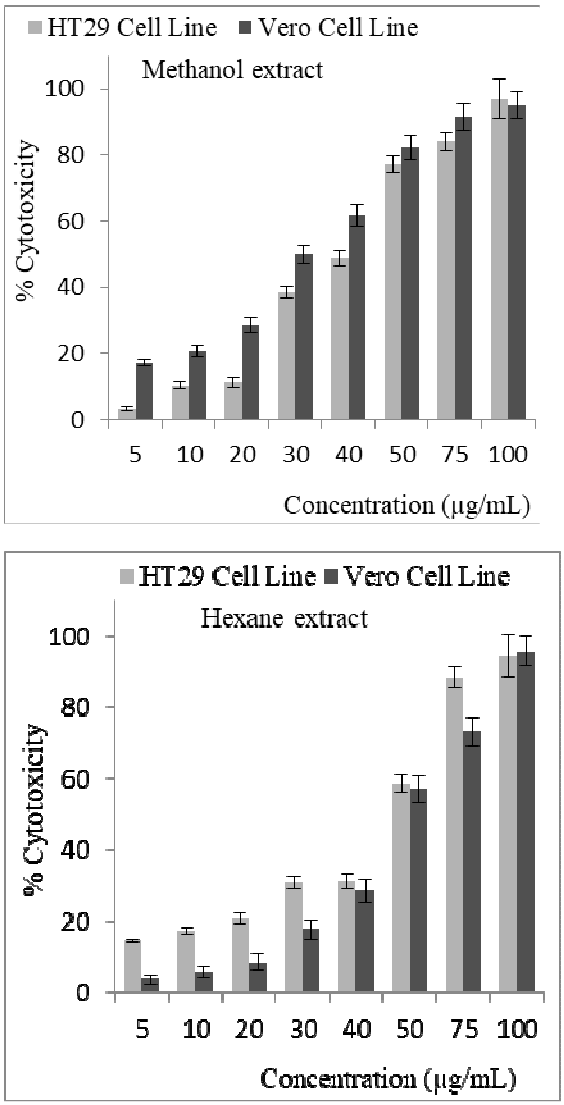

Figure 2: Cytotoxic activity of extracts on HT29 and Vero cell lines.

In the present study, *Cladophora fracta* methanol extract could be promising source of drug development process due to the fact that it displays higher antiproliferative effects of methanol extract than standard 5FU on Vero and HT29 cell lines. In addition; hexane extract also displayed as good an activity as standard on all cell lines. This has to do with fatty acid contents. Fatty acids are abundant in algae species and have significant biological activities such as antiviral, anti-inflammatory [18], anticancer [19] and antimicrobial [20] activities. Two fatty acids,18:4n-3 and 16:4n-3, were isolated from marine algae *Ulva pertusa* and *Ulva pinnatifida* and found to have strong inhibitory effects on

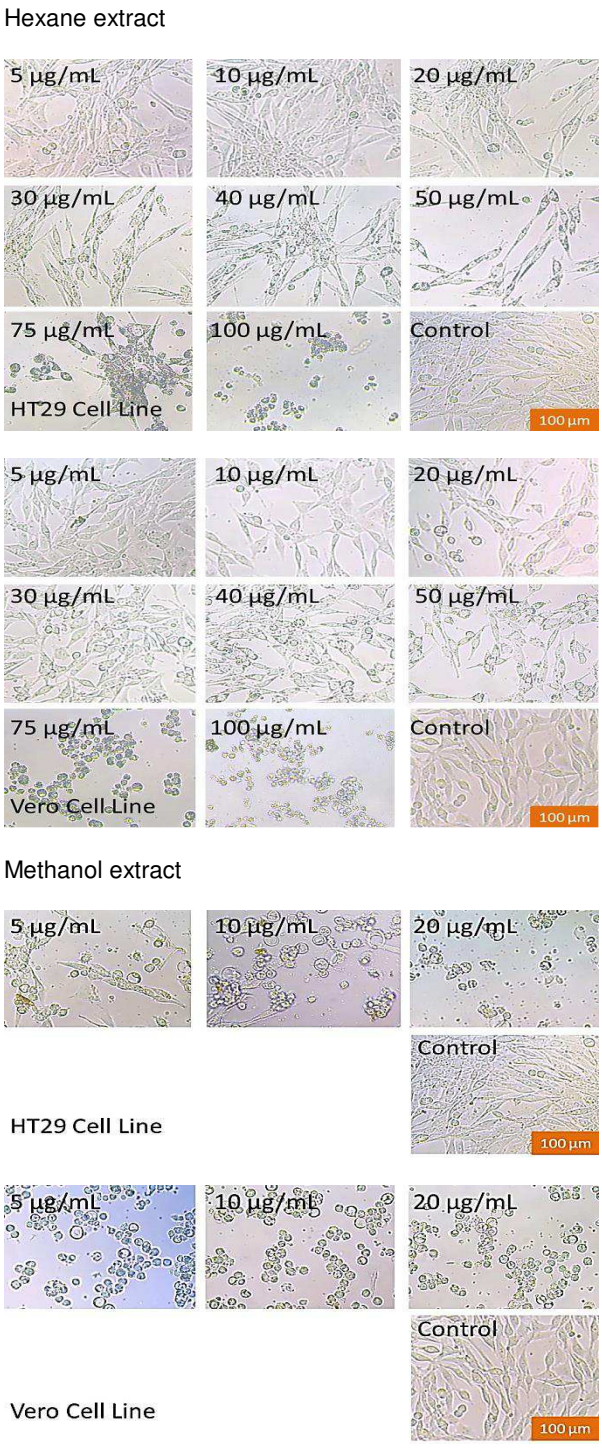

Figure 3: Effect of *C. fracta* extracts on the morphology of HT29 and Vero cells. DMSO treated cells as controls

Lekotrien B4, 5-hydroxyeicsatetraenoic acid and leukotriene C4 in MC/9 mice mast cells [21]. Fatty acids have inhibitory effects on tumor growth and antimetastatic property. They are able to change malignant cell membranes to serve chemotherapy effects [22]. Oleic acid plays a significant role in activation of various intracellular pathways such as carcinoma cell development. Palmitic acid also showed considerable antitumor effect in mice [23]. γ-linolenic acid suppressed cell growth of neuroblastoma cell lines [24]. Linolenic acid gained great attention as a chemotherapeutic agent after being displayed to have inhibitory effects on carcinogenesis [25].

CONCLUSION

C. fracta exhibits antiproliferative effect on HT29 and Vero cell lines. There is a need, however, to isolate and identify the active chemical compounds of *C. fracta*. The fatty acid contents also displayed strong antiproliferative activity on HT29 and Vero cell lines as well. Further studies are required to determine the potentials of both the extract and its fatty acid for anti-cancer therapy.

DECLARATIONS

Acknowledgement

The authors are grateful to Dr. Ali Aydin for executing the antiproliferative activity tests and Dr. Nejdet Kandemir for his critical review of the manuscript.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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