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Cytotoxic Activity of Enteromorpha intestinalis Extracts against Tumor Cell-Line HeLa

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Abstract
Two extracts (methanol and hexan) were extracted from green algae *Enteromorpha intestinalis* which is isolated from Basra / Iraq, the cytotoxic activity of these extracts were evaluated by using MTT assay on HeLa cells in *vitro* , according to the results both extracts are effectiveness on cancer cells with the superiority of methanol extract , the IC_{50} of methanol extract was 79.08µg/ml and 156.3µg/ml for hexan extract . Also during the current study a number of cytotoxic compounds were isolated from both algal extracts according to the chemical analysis results by using Gc mass technique , these compounds are loliolide , palmitic acid , ethyl palmitate , phytol and squalene from methanol extract while the hexane extract contains palmitic acid , ethyl palmitate and dibutyl phthalate.
Keywords : *Enteromorpha intestinalis* , cytotoxic activity, HeLa cells

1. Introduction

Algae have a great importance in different fields particularly in the medical aspect. As a nature source for production of bioactive metabolic compounds. Recently, an increase research interest focused on the activity of algal metabolic compounds and their applications in medical therapy as antitumor and antioxidant agents (Shalaby 2011).

The alga *E. intestinalis* (chlorophyta) or seaweeds known as intestinal herb with length of (10-15cm) and width of (1-8 mm) and characterized by smooth tubular shape similar to the intestine and usually unbranched .This algae is floating in aquatic habitats and appears as green to dark green when becoming aggregated near the shore . Is consists of semi-circular cells of (10-22µm) diameter, with vegetative cells containing laminated chloroplast (Ruangchuay *et al.* 2007; Josh & Krishnamurthy 1972; Ruangchuay *et al*. 2012) .

This alga grows through the year, which is widely distributed, especially in the Asian countries and grows in salt water habitats and brackish waters and fresh water (Prud'homme van Reine & Trono 2001; Bellinger & Sigee 2015).

Now a days there is an interest of using *E. intestinalis* as anti-tumors as pointed out by researchers . A study of Jiao *et al*. (2009) on Polysaccharides compounds has been isolated from *E. intestinalis* and tested its effectiveness in *vitro* and in *vivo* against tumor cell type Sarcoma, the study has shown these compounds has been effective against the tumor .Another study of Paul & Kundu (2013) examined the activity of the methanol extract of *E. intestinalis* against HeLa cancer cells in *vivo* , and showed that extract was effective against cancer cells , Polysaccharides compounds have been also isolated from *E. intestinalis* by Jiao *et al*. (2010) and proved their effectiveness as anti-tumor and immunomodulating in *vivo* by using laboratory rats. Wang *et al.* (2014) reported that Sulfated Polysaccharides compounds isolated from *E. intestinalis* was active against Hepatoma HepG2 , Acetone extract of *E. intestinalis* revealed a highly effective on Human colon carcinoma LS174 cells and Human lung carcinoma A549 cells and Malignant melanoma Fem-x and Chronic myelogeneous leukemia K562 (Kosanic *et al.* 2014).

The present aimed to examine activity of *E. intestinalis* extracts as anti- tumors using type HeLa cancer cells in *vitro* and chemical analysis of the extracts was made .

2. Materials and methods

2.1. Algal collection: Algal samples were collected in February 2014 from the Abu Sokhir area in Basra / Iraq by using clean sample containers , and transferred directly to the lab . Algal samples were washed thoroughly in distilled water and dried at room temperature away from direct sunlight. Dried samples were grinded and kept at 18 ˚C for further use.

2.2. Algal extracts preparation

2.2.1. Hexane extract preparation **:** 50 g of dried alga are extracted with 500 ml of n-Hexan for 24 hours by a continuous extraction via soxhlet apparatus and then concentrated by rotary evaporator (Harborn 1984).

2.2.2. Methanol extract preparation **:** According to Rios *et al.* (1987) 250 ml of absolute methanol added to 50 g of dried alga in flask then the mixture shaken for 24 h by using the magnetic stirrer apparatus then filtrated by Whatman filter paper No. 1, and the filtrated was concentrated by rotary evaporator to obtain the extract.

2.3. Chemical analysis **:** The chemical compounds are identified via Gas chromatography - mass spectroscopy

technology (GC-mass) by using Agilent Technologies device GC7890A GC, the test was carried out in Tarbiat Modares University / Tehran - Iran.

2.4. MTT assay: The cytotoxicity of algal extracts was tested using cancer cells type HeLa (Human cervical carcinoma cells) via MTT assay, the cells were obtained from Iraqi center for cancer and medical genetics research, Al-Mustansiriya University, Baghdad, Iraq and grown in tissue culture lab at the Bbiology Department / Education for Pure Sciences College - Basrah University, the MTT assay method according to Carmicheal *et al.* (1987) as a following steps:

- 1×10^4 cells of HeLa cells were seeded in 96 well flat bottom culture plates by using the EME medium and incubated at 37˚C , 5% Co_2 for 24 hr. period.
- After incubation, cells were treated with different concentrations of algal extracts (75,150,300,600µg/ml) of five replications along with control (growth media only), incubated for 48 hr. at the same conditions of the previous step .
- After incubation, the medium with treatments were removed and replaced with fresh medium along with 100 µg of MTT (3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl-tetrazolium bromide) in each well then incubated at the same conditions for 4 hr .
- Dimethyl sulfoxide (DMSO) was added for each well and left for five minutes.
- The absorbance was determined at 490 nm by using Microplate reader.
- The value of Half maximal inhibitory concentration (IC_{50}) for each extract was determined by using the Graph Pad Prism software 6.04, also the percentage of inhibition for each concentration was determined according to following equation:
  Inhibition % = [(absorbance of control - absorbance of treated) \ absorbance of control]× 100.

2.5. Statistical analysis: The data were analyzed via IBM SPSS Statistics ver. 19 software by using One way analysis of variance (ANOVA) ,and $P \leq 0.05$ was considered to be statistically significance .

3. Results and discussion

3.1. MTT Assay: According to the results of cytotoxicity of algal extracts against cancer HeLa cells , the IC_{50} of methanol extract was 79.08µg / ml versus 156.3µg / ml of hexan extract , and the inhibition percentages of both extracts concentrations are shown in Figure 1 , the concentrations of methanol extract showed significant differences with the exception of 300 and 600 concentrations that did not show significant difference between them. However, the result of hexane extract concentrations were similar to the concentrations of methanol extract and significant (Table 1).

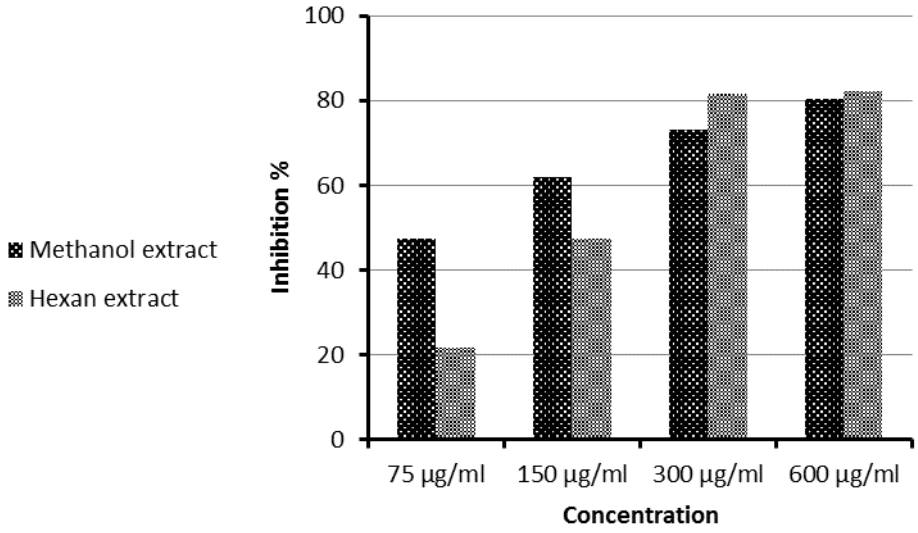

Figure 1. Anti proliferativ effects of *E. intestinalis* extracts on HeLa cells

Table 1. Cell viability of treated and control cells in MTT assay

Extracts	Concentration µg/ml				Control	R.L.S.D.
	600	300	150	75		
Methanol	0.331[d]	0.423[d]	0.601[c]	0.829 [b]	1.58[a]	0.149
Hexan	0.318 [d]	0.33[d]	0.936 [c]	1.394 [b]	1.74[a]	0.140

The data were compared against control and P≤0.05 was considered to be statistically significance

From the present study it appeared that the algal extracts exhibit a high cytotoxicity against cancer cells , and the value of the IC_{50} of methanol extract was 79.08µg / ml versus 156.3µg / ml of hexan extract which suggests that the methanol extract is more cytotoxic than hexan extract against cancer cells. This can be attributed to the quality and quantity of active compounds extracted by methanol as compared with hexan extract.

3.2. The chemical analysis :

According to GC mass analysis of methanol extract and hexan extract, contained different compounds (Table 2,3).

Table 2. Chemical analysis of methanol extract

Compounds	Molecular Formula	M.W. g/mole	% of total
Loliolide	$C_{11}H_{16}O_3$	196.24294	3.296
Ethyl stearate	$C_{20}H_{40}O_2$	312.53	1.07
Palmitic Acid	$C_{16}H_{32}O_2$	256.42408	3.538
Ethyl palmitate	$C_{18}H_{36}O_2$	284.47724	23.744
Phytol	$C_{20}H_{40}O$	296.531	2.8413
Ethyl oleate	$C_{20}H_{38}O_2$	310.51452	4.916
Squalene	$C_{30}H_{50}$	410.718	4.91
Ethyl hexyl adipate	$C_{14}H_{26}O_4$	258.35384	21.855

Table 3. Chemical analysis of hexan extract

Compounds	Molecular Formula	M.W. g/mole	% of total
N,N-Dimethyltetradecylamine	$C_{16}H_{35}N$	241.4558	0.41
Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34348	1.79
Palmitic Acid	$C_{16}H_{32}O_2$	256.42408	2.15
Ethyl palmitate	$C_{18}H_{36}O_2$	284.47724	1.341
Butyl stearate	$C_{22}H_{44}O_2$	340.58356	0.294
Ethyl hexyl adipate	$C_{14}H_{26}O_4$	258.35384	12.171

The activity of both extracts against cancer cells derived from the active compounds as confirmed by the chemical analysis of GC mass , which that both extracts contain a number of active compounds (Table 2,3).

Monoterpene Loliolide was found in the methanol extract and it was isolated from *E. intestinalis* in a previous study by Güven *et al.* (2015) , it was also isolated from marine algae and plants and animals (especially insects), it has antioxidant activity and cytotoxic activity against tumors as well as antifungal and antibacterial activity (Grabarczyk *et al.* 2015).The cytotoxicity of Loliolide was tested against Nasopharyngeal carcinoma and Murine lymphocytic leukemia (Pettit *et al.,* 1980). Another study has shown that the Loliolide from *Sargassum ringgoldianum* marine brown algae exhibits an antioxidant activity (Yang *et al.* 2011).

The results shown that both extracts contained Palmitic acid compound, which is saturated fatty acids and found in plants, animals and microbes. It has different activities but the most important as a anti tumors. Palmitic acid was isolated from marine red algae by Harada *et al.* (2002) and its cytotoxic activity against leukemia was tested and revealed high activity against cancer cells and didn't show any toxicity on normal cells (Human dermal fibroblast) .

Also both extracts contained Ethyl palmitate compound at a higher rate in the methanol extract. Ethyl palmitate compound is a fatty acid that did not prove to be effective as anti tumors but it has an activity as an anti-inflammatory on laboratory rats as reported by Saeed *et al.* (2012).

The methanol extract contain Phytol compound which is dihterpene that has different biological activities as antitumor and antioxidant (Pejin *et al.* 2014). The cytotoxic activity of Phytol against seven types of tumors in *vitro* has been investigated by Santos *et al.*(2013) has confirmed that the Phytol is an important as a antioxidant compound .

The methanol extract also contained Squalene compound which is a triterpenes and has different uses in cosmetics and numerous benefits for the skin due to its antioxidant and anti-cancer activities (Huang *et al.* 2009). Squalene is a highly effective antitumor and antioxidant, Desai *et al.*(1996) was confirmed the activity of

Squalene in the treatment of skin cancer by using laboratory rats, Smith (2000) explained that the Squalene has a role in inhibition of Skin Cancer in laboratory rats. Also the activity of Squalene as a antioxidant has been examined by many studies (Amorowicz, 2009; Saint-Leger *et al*. 1986; Kohno *et al.* 1995; Aioi *et al.* 1995).

This study also indicated that hexan extract contain Dibutyl phthalate compound which is considered as a secondary metabolic products found in plants and animals and it has the different biological activities including an antitumor, as reported by Chu-tse *et al.*(1993) this compound has shown cytotoxicity against leukemia cells.

4. Conclusions

We concluded that the two extracts (methanol and hexan) of *Enteromorpha intestinalis* algae have cytotoxic activity on cancer cells (HeLa) with the superiority of methanol extract due to a number of cytotoxic compounds were isolated from both algal extracts which have the biological activity according to the previous studies .

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