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Anti-carcinogenic and cytotoxic effect of Carthamus *oxycantha Safflower* seed oil extract on RD cell lines

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ABSTRACT

The search for the story of anticancer drugs continues. The Agent that can kill cancer cells should don't kill the normal cells. This study includes the effect of active substances in *Carthamus oxyacantha* on RD malignant cell lines. RD malignant cell lines are Rhabdomyosarcoma cancer cell line which taken from Caucasian girl infected by cancer in pelvic at seven-year-old. Many of solvents used to extraction active compounds from *Carthamus oxycantha*, where prepared oily, aqueous solutions for compartment between them. Cytotoxicity assay, for evaluating the activity of these extracts is done on human cancer cell lines RD, by using eight concentrations (7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000) µg /ml for each extract, and 24, 48, and 72 hours of exposure time. The Results show a gradually cellular toxic effect of extracts on cell lines growth, with dose and time-dependent manner for all types of these cell lines. There are some differences in the effect of these extracts based on the extracts and cancer cell. Through the estimation of cytotoxic concentration that kills 50% of cells (CC50) for each extract and cell type, we found that aqueous extract was the most potent cytotoxic extracts for these cancer cell lines, followed by crude, crude oily extract respectively. RD cells were the most sensitive type of cell lines to these. Active compound Triterpene concreted in the flower and oil extracted oleic acid and linoleic acid will role antitumor activity.

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INTRODUCTION

Over history, there are such attempts to find treatment for cancer. The cancer disease considered the important cause of death; infected people with cancer are increasing during the time. Cancer is usually treated by several methods such as surgery, radiation, and chemotherapy, but all methods have a side effect (Suffines & Pezzuto, 1991). Cancer is a very dangerous disease, and its causes of big numbers of death in all world countries (Canadian Cancer Society, 2014). The deaths will be reached 7.5 billion during next years (Anand *et al.,* 2008).

Alternative or called sometimes the complementary medicine is used plant extracts for treatment all diseases like cancer. The Complementary or plant medicine is used commonly in Europe at 69% in infected patients with cancer (Bock *et al.,* 2004).

Nearly, all the peoples used the plants for the treatment of cancer. And the medicinal plants used for hundreds of years for the cancer treatment. There are more than three hundred of the plant species that used in the treatment of cancer. Well, The Cancer is uncontrolled cell multiplication that results from the over excesses accumulation of the cell (Aberanthy E, 2005).

The Safflower (*Carthamus oxycantha*) is cultivating in native Asia such as Iraq, Iran and Afghanistan. And it's used for the treatment of cancer. This plant is cultivated mainly for production vegetable oil that used to multipurpose for eating and treatment (Chan *et al.,* 1986).

Natural products consist of many compounds that have anticancer effects, and it can use for inhibition and kill cancer cells. Wherever WHO found 80% of the populations used alternative medicine for treating the diseases, some drugs made from medicinal plants (Nabavi *et al.,* 2015; Krishnaiah *et al.,* 2011). The studies in Greek founded the safflower two types: a white and red or called pale and red and it has a great role in treating the cancer diseases (Jhon Chadwick, 1976).

The main aims of our study know effective plant extract of *Carthamus oxyacantha* on RD (Rhabdomyosarcoma cancer cell line). And studying compartment between aqueous extract and oil extract of *Carthamus oxyycantha* and which it has more effective.

MATERIAL AND METHODS

Rhabdomyosarcoma (RD) cell line

Its Rhabdomyosarcoma cell line that taken from Caucasian girl infected with Rhabdomyosarcoma cancer in pelvic at seven-year-old (McAllister *et al.,* 1969)

Plant collection

Plant samples used in this study was collected from the west desert of Iraq in the month Jun to August collected flower of the plant in the morning because consternation of flavonoid and terpenes will be in this time (Al-jumaaly, 2011). It was officially identified. Fresh flower separated and put in the shade inside well ventilation room. Dried flowers were grounded to a fine powder using a coffee grinder.

Extraction (flowers)

Aqueous Extraction

A water extract was prepared as recommended by Harborne (1984) and as described by Al-Aboodi (2001) with some modification imposed by the nature of the extract acquired

Fifty grams of powdered plant flowers were placed in a one-litre flask

- Five hundred millilitres of boiling distilled water (D W) was added to the powder and mixed thoroughly with a glass rod, and the flask was sealed tightly with a cork stopper
- The mixture was lest for approximately 8 hours at room temperature
- The mixture was filtered through muslin and the filtrate centrifuge
- The supernatant was filtered through Whatman No.1filter paper
- The filtrate was gently poured into preweighed glass Petri dishes are placed openly in an electric oven at 37 c for about three days till dryness
- After drying Petri dishes were placed in a desiccator to cool down then they were weight, and the yield of extraction was calculated using the following equation:

The weight of Petri dishes with extract –

$$\frac{\text{The weight of Petri dishes empty}}{50} \times 100$$

- The dried extract was scrubbed off the Petri dishes, placed in labelled and stored in the freezer.

Seed Extraction

The dried seed of *Carthamus oxyacantha* was obtained. The seeds were powdered using an electric blender. 50 gm of the powder was infused in hexane overnight, and then oil by soxhlet apparatus using hexane solvent was then distilled using Whatman no.1 under reduce pressure below 4 c using rotary evaporated. The extract stored in labelled sterile screw-capped bottles until use (Devkota and Dutta, 2001).

Cell Growth Assay

The *vitro* method was used to establish the effect of the aqueous and seed oil extracts of *C. oxyacntha* on (RD tumour cell lines). Solutions were prepared according to ICCMGR standard method.

Antibiotics

a. Benzyl-penicillin vial 1000000IU was dissolved in 5ml of D.W.Then 0.5ml was taken and added to one litre of culture media.
b. Streptomycin vial (1g) was dissolved in 5ml D.W. Then 0.5ml was taken and added to one litre of culture media.

Preparation of the chemical solution

Phosphate buffer saline (PBS) (pH 7.2)

Table 1: substance and its weight that used in the study

NACL	8 gram
kcl	0.2 gram
Na 2 HPO4	0.29 gram
KH2PO4	0.2 gram
D.W.	1000 ml

All these components mixed and sterilisation was performed via autoclave for 10 minutes, and then stored at room temperature.

Table 2: Phytochemical that detected in Aqueous Extract and Hexane Extraction

Hexane Extraction	Aqueous Extract	Phytochemical to be detected
+	++	Alkaloid
++	+	Tannins
+	++	Flavonoids
+	+++	Triterpenoids
+++	+	Saponins
+++	+	Glycoside

Table 3: Effect of concentration and time in % inhibition in RD cell lines (Oil extraction)

Concentration (µg/ ml)	Time (hr.)			LSD value
	24	48	72	
0.00	0.0 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.00 NS
7.81	6.1 ± 0.28	9.3 ± 0.46	10.2 ± 0.53	3.281 *
15.62	9.7 ± 0.43	12.3 ± 0.67	14.9 ± 0.61	4.024 *
31.25	14.1 ± 0.59	16.4 ± 0.54	19.3 ± 1.08	3.983 *
62.5	22.4 ± 0.74	24.5 ± 1.88	27.7 ± 1.30	4.662 *
125	41.9 ± 1.67	44.7 ± 2.09	46.9 ± 2.13	5.823 NS
250	51.7 ± 1.55	56.2 ± 2.53	60.1 ± 2.55	6.552 *
500	70.9 ± 2.83	74.7 ± 3.91	79.5 ± 3.06	6.392 *
1000	82.4 ± 3.78	86.2 ± 3.62	90.5 ± 3.25	6.874 *
LSD value	8.261 *	7.006 *	7.953 *	---

* NS: Non-significant. (P<0.05)

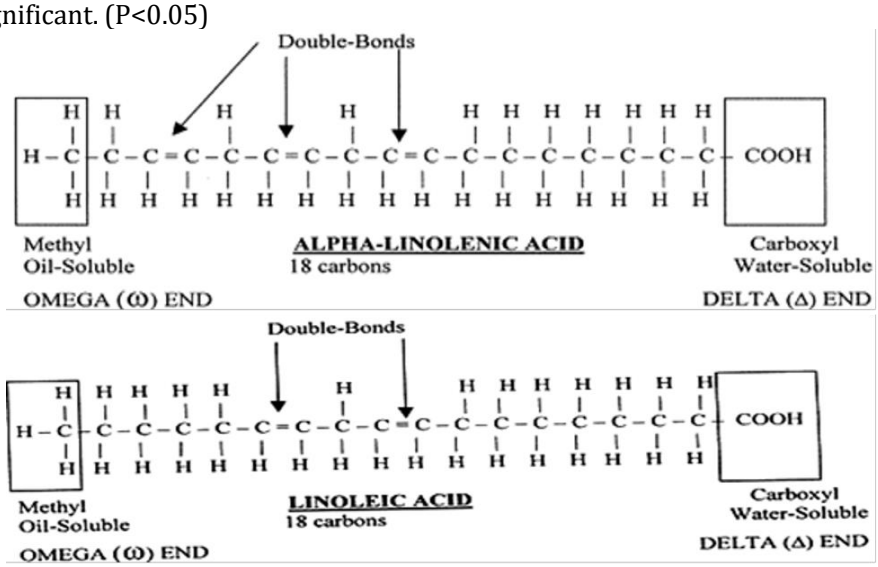

Figure 1: Chemical structure of Linoleic acid And Linolenic acid

Sodium bicarbonate: Sodium bicarbonate was prepared by weighing 4.4g subsequently add to 100 ml D. W., then sterilized by autoclave and stored at 4˚C until use (Jump, 2002).

Trypsin solution: The trypsin powder (1 gram) was added to 100ml of PBS solution and mixed by magnetic stirrer at 25 C. next step the solution sterilized by filtration by using Millipore sterile filters 0.22 µm, stored at -20˚C until use.

Versine solution: It prepared by add 1 gram of (EDTA) material with 100ml of distal water and sterilized for 15 minutes by the autoclave, and stored at 4˚C.

Maintenance of the cell lines: When the cells put in the flask as one layer, apply the following protocols according to (Freshney, 1994)

a. The cell sheet washed with the PBS twice, and the growth medium was decanted off.
b. (2-3) CC of trypsin/versine solutions was added to the flask opposite to the cells. Turning the flask over to flood the monolayer completely, incubated at 37˚C for few minutes, until the cells rounded up and separated into single cells by gentle rocking.
c. Adding to the medium (0.1-0.2ml/cm^2) and dispersing the cells by pipetting in growth medium over the surface bearing the monolayer.
d. Redistribution of the cells at the required concentration into culture flasks and re-incubated at 37˚C.

Table 4: Structure of anti-oxidant that extracted in the study

No	Anti-oxidant structure
1	N-[2-(5-hydroxy-1H-indol-3-yl)ethyl]ferulamide
2	N-[2-(5-hydroxy-1H-indol-3-yl)ethyl]-p-coumaramide
3	, N′-[2, 2′-(5, 5′-dihydroxy-4, 4′-bi-1H-indol-3, 3′-yl)diethyl]- di-p-coumaramide N
4	N-[2-[3′-[2-(p-coumaramido)ethyl]-5, 5′-dihydroxy- 4, 4′ -bi-1H-indol-3-yl]ethyl]ferula-mide
5	, N′-[2, 2′-(5, 5′-dihydroxy-4, 4′-bi-1H-indol-3, 3′-yl)diethyl]- diferulamide
6	N, N-[2-[5-(beta-D-glucosyloxy)-1H-indol-3-yl)ethyl]- p-coumaramide
7	N-[2-[5-(beta-D-glucosyloxy)-1H-indol-3-yl)ethyl]ferulamide

Table 5: Effect of concentration and time in % inhibition in RD cell lines (Aqueous Extraction)

	Time (hr.)			
	24	48	72	
0.00	0.0 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.00 NS
7.81	3.9 ± 0.07	6.5 ± 0.12	8.3 ± 0.32	3.752 *
15.62	6.9 ± 0.12	11.9 ± 0.33	14.9 ± 0.48	5.025 *
31.25	15.8 ± 0.53	20.2 ± 1.42	23.7 ± 1.36	5.279 *
62.5	24.7 ± 1.64	30.7 ± 1.55	40.1 ± 1.75	6.894 *
125	36.6 ± 2.09	40.7 ± 2.07	53.2 ± 2.08	6.433 *
250	50.4 ± 2.31	54.3 ± 2.38	63.9 ± 2.66	7.087 *
500	63.9 ± 2.85	65.6 ± 2.81	71.7 ± 3.49	6.213 *
1000	73.1 ± 2.71	77.4 ± 2.95	82.9 ± 3.28	6.094 *
LSD value	7.258 *	7.849 *	8.141 *	---

* NS: Non-significant. (P<0.05)

Table 6: Growth inhibition of RD cell line by oil extracts of C. Oxycantha. During 24 hrs of exposure

	Time (hr.)			
	24	48	72	
0.00	0.0 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7.81	6.1 ± 0.28	9.3 ± 0.46	10.2 ± 0.53	
15.62	9.7 ± 0.43	12.3 ± 0.67	14.9 ± 0.61	
31.25	14.1 ± 0.59	16.4 ± 0.54	19.3 ± 1.08	
62.5	22.4 ± 0.74	24.5 ± 1.88	27.7 ± 1.30	
125	41.9 ± 1.67	44.7 ± 2.09	46.9 ± 2.13	
250	51.7 ± 1.55	56.2 ± 2.53	60.1 ± 2.55	
500	70.9 ± 2.83	74.7 ± 3.91	79.5 ± 3.06	
1000	82.4 ± 3.78	86.2 ± 3.62	90.5 ± 3.25	

e. The media initially orange once the cells grow, it becomes yellow thereby the reducing media should be changed. The occurrence of turbidity means that the culture is most likely contaminated.

Detection of active compounds

Detection of Tannins

The plant powder (10 gram) and mixed with (50) CC of D.W by using a hot plate magnetic stirrer, it should boil for several minutes, then pour it by filtration, the upper layer treated with the two detection methods of tannins as;

a. According to (Al-Mukter, 1994) prepare watery lead acetate solution 1%, if the gelatin material appears at the bottom that means the presence of tannins.
b. According to (Harborne, 1984) prepare watery ferric chloride solution 1%, if present greenish-blue substances in the bottom that indicate tannins.

Detection of Saponins

a. Making Foam tests for a solution.
b. Preparation mercury chloride solution at concentration 1%. The plant extracts 5 ml mixed with (4 ml) of mercury chloride; the white sediment layer indicates saponin (Al-mukter, 1994).

Detection of Flavonoids

a. The powder explants (10 gram) were added to the ethanol 95% concentration and made filtration.

b. The ethanol 50% (10 ml) was mixed with KOH 50% (10ml) and mix A with B the yellow colour indicted to flavonoids according to (Harborne, 1984).

Detection of Glycosides

a. Same amounts of the watery extract add with Fehling's reagent, then allowed to boil in a water bath for (10) minute in a test tube, present red sediment indicated to glycosides.
b. The Pandect's reagent (1 ml) was mixed with the plant extract (5 ml); if red sediment appears that indicate to glycosides according to (Stahl, 1969).

Detection of Terpenes

The chloride antimony (1 ml) mixed with chloroform 20% concentration and added to plant extract (5 ml), the white sediment material indicated to terpenes present according to (Harborne, 1984).

Detection of Alkaloids

a. The plant powder (10 gram) boiled with mixture consist of distal water (50 ml) with HCL4% then make filtration by filter paper and cooling. (0.5) ml of the solution mix with (0.5) ml as: -
b. Wagner's Reagent: the brown sediment material indicated to alkaloid present according to (Smolensk *et al.,* 1972).
c. Mayer's Reagent: the white sediment material indicates to alkaloid according to (Smolensk *et al.,* 1972).
d. Dragendorff`s Reagent: the orange material sediment at the bottom indicate alkaloids present according to (Harborne, 1973).
e. Tannic Reagent: the tannic acid (1 gram) was mix with distal water (100 ml) and add the reagent (1 ml) with alcoholic extract (1-2 ml), the white sediment in bottom indicate to alkaloids presence according to (Harbone, 1973).

The cytotoxicity and cell viability

The cell killing ability is tested by Crystal violet cell viability technique. It consists of 96-well on the plates (Santa Cruz Biotechnology, USA). Normal Murine Embryonic cells were confluent monolayer achieved or seeded at 7 thousand – 10 thousand cells for each well after one day, the Cells treated with different dilutions from (1/10 v/v), (1/50 v/v) (1/100 v/v) and 1/2000 v/v in culture media. Cell viability measured after 72hours after removing the medium, adding (50 µl) of Crystal violet stain (Sigma Alderch-co) and incubation for two hours at (37°C) after removing the dye, and then washed with PBS. Using microplate reader (Biotek Company, made in the USA) to estimate the absorbency at 492 nanometers. Reagan is assayed used for three times. Endpoint parameters are calculated for the cancer cell lines by apply following formula proliferation rate = (B/A) x100. and inhibiting rate of cell growth (GI) (the percentage of cytotoxicity) was calculated as (A-B)/Ax100, (A) indicates the optical density of untreated wells and (B) indicate to an optical density of treated wells

Statistical Estimation

The Statistical Analysis System (SAS) (2012) software used to study the effect of different factor and parameter (concentration, time and cell lines) in the percentage of inhibition. Also used test of least significant difference (LSD) was used to compare between means in this study. **(**SAS. 2012).

RESULT &DISCUSSION

The affected of aqueous extracted and hexane extraction of flowers and seed of *Carthamus oxyacantha* on RD cell line at eight different concentrations (1000**,** 500**,** 250**,** 125**,** 62.5**,** 31.25**,** 15.62**,** 7.81) µg/mL with a different period (24.48.and 72 hrs) Were tested. The Result was in reducing the optical density of RD at the concentration 1000 ng/ml during 72 hours of exposure.

RD cell line cell line

Result in this type of cell line when treating with seed oil extraction show the effect beginning of 15.62 µg/ml at 72 hours while the effect of concentration 62, 5 µg/ml is beginning from 24 hours Ascension to high concentration.

The effect of treating RD cell line of *C.oxyacantha* seed oil shown in above table dose-dependent was observed in RD seed oil has a highly significant effect ($P<0.05$) at the concentration1000µg/ ml at72, 48, and 24 hrs.(90.5, 86.2, 82.4) respectively. Also, there was a significant effect ($P<0.05$) for the other concentration. Excepted concentration 125 µg/ml there showed no significant effect during the time of period exposer (24.48.72) hrs.

The oil of Safflower demonstrated different cytotoxicity toward RD cell line According to its concentration. Above table the value of inhibition percentage in two cell line and three-period time exposer we can see that inhibition increased to be optimum at 1000 µg. This meaning or indicator That abstract role depending on dose. (Dose-dependent). The result of the study shows the activity of seed oil in the cell line. Caused by linoleic acid, polyunsaturated fatty acid and it sours of Omega -3 that have multi physiology effect on cell live (Carrter, 2004).

The result of this study is compatible with another study. In a vitro study oil of *C.oxyacantha* inhabited

the HT -29malignant human colon cell line (Salerno, 1991). Linoleic acid play role to inhibited three types of brain cancer (Glioma cell lines) through Apoptosis (Leaver *et al.,* 1998). The rat's nutrient on diets rich in n-6 fatty acid (Especially polyunsaturated type) helps cancer lesions to develop in the liver (Ghoshal, 2000). High-fat safflower oil diet revealed a marked induction of hepatic IGFBP-1 and allowed to the protein in the liver cancer development. The reports and the studies found polyunsaturated fatty acids diet are stimuli breast cancer induction, while long-chain n-3 PUFAs compound found in fish oil may inhibit these effects. (Bagga and Glaspy, 2002). There are (7) of antioxidative extracted from the safflower; their structures were confirmed in the table (6):

This Anti-oxidative has strong activities against federals and oxidative compounds, the antioxidant were measured by two specific methods called the alpha, alpha-diphenyl-beta-picrylhydrazyl (DPPH) method and ferric thiocyanate method and according to (Zhang2007). (Turgumbayeva, and Samir, 2014) found oil that extracted from Safflower *plant* was estimate ability of antibacterial activity against *Staphylococcus aureus* isolates and use with fungi *Candida albicans* to studying the activity at details.

Aqueous Extract

The RD cell line was treated with eight different aqueous concentrations for three period's exposer. The viability of cell line decreases gradually with the increase of concentration and time of exposure.

RD Cell line

The toxicity of this extracting increases toward high concentration and period exposure to reach optimum on 1000 µg/ml at 72 hrs. And the effect of aqueous started since 15, 62 µg/ml at 24, 48 hrs respectively. And 7.81 µg/ml at 72 hrs. The value of the inhibition effect of this extraction in tow cell line was increased combined with time exposure period to all cell line RD, the activity of this extraction Dose-dependent**.**

C. oxyacantha have Triterpin compound that found in flower, and it plays as an antitumor activity through toll-like receptor 4/Nf-Kb pathway in the cell assay (Lue, *.et al*, 2009). And Anti-inflammatory activity against 12-O tetraadecanoyphorbol-13 acetate –induce inflammation (Akihisa, 2009). These substances were water soluble.

Active in an Aqueous extract of *C.oxyacantha* return to Carthamin, safflower yellow are the main constituents in the flower of *C. oxyacantha*. Carthamidin, isocarthamidin, hydroxysafflor yellow A, safflor yellow A, safflamin C and luteolin are the main constituents which are reported from this plant. Caryophyllene, p-alkyl toluene, 1-acetoxytetralin and heneicosane were identified as the major components play antithrombotic and Pro-Apoptotic activities in hepatic cell stellate and cytotoxic effect on the rat nervous cells antimycotic properties especially Against *Aspergillus fumigates*.

The neuroprotective properties of Hydroxysafflor yellow an on the neurotoxicity of glutamate in primary cultured rat cortical neurons along with its possible mechanism of action were examined. The excitotoxic neuronal death was attenuated markedly by HSYA treatment. HSYA decreased expression of Bax and rescued the balance of pro and anti-apoptotic proteins. Also, HSYA significantly reversed the upregulation of NR2B-containing NMDA receptors by exposure to NMDA, while it did not affect the expression of NR2A-containing NMDA receptors (Zhaoo, and Guo, 2010). The safflower regulates mechanism of the transporter by using screening cell lines. The Safflower can inhibit serotonin that uptake by Chinese hamster ovary. N (1), N(5)- (Z)-N(10)-(E)-tri-p-coumaroyl-spermidine is active compound was isolated. This compound inhibited serotonin uptake in S6 cells, at IC50 of 0.74±0.15 microM for S6 cells or 1.07±0.23 microM for synaptosomes and with a reversible competitive property for the 5HT-uptake inhibition. The potency of it for 5HT uptake was weaker than that of fluoxetine, whereas efficacy similar for both. The Animals groups treated by these compounds reveal a significant decrease in synaptosomal 5HT uptake capacity (Zahoo *et al.,* 2009).

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