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The Effect of Crude Extracts of *Sonchus oleraceus* on Cancer Cell Growth *(In vitro)*

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Summary

This study was designed to evaluate the anticancer, effects of the ethanolic (EE), cold aqueous (CAE), and hot aqueous (HAE) extracts of *Sonchus oleraceus* on cancer cell lines (*in vitro*). *In vitro* study was performed on three cancer cell lines (murine mammary adenocarcinoma AMN-3 cell line, laryngeal carcinoma Hep-2 cell line) and rat embryogenic fibroblast (REF) as normal cell line. Periods of exposure of cell lines were measured at 24, 48, and 72-hr in a microtitration plate under complete sterile conditions. Different concentrations starting from (78.125 to 10000) µg/ml of two fold dilution for each extract were prepared and tested on each cell line, with three replicates for each concentration. The three extracts showed concentration and time dependence with growth inhibitory effects, and the highest effect was obtained from ethanolic extract at higher concentrations after 48 hr. of exposures on both AMN3 and Hep-2 cell lines, while the cytotoxic effect of both cold aqueous and hot aqueous extracts on AMN-3 and Hep-2 cell lines exhibited that the higher concentrations gave a significantly ($P<0.05$) and the higher inhibition growth rate of cells were increased at 24 hrs.**Conclusion:** These results suggest that the cytotoxic concentrations of *Sonchus oleraceus* extracts showed variation in values among cell lines according to cell types *in vitro*.

Key words: cancer, cancer cell lines, *Sonchus oleraceus*, extraction.
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تأثيرا لمستخلصات الخام لنبات المرّير (*Sonchus oleraceus*) على نمو الخلايا السرطانية في الزجاج

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الخلاصة

صممت هذه الدراسة للتحري عن التأثير السمي للمستخلصات الكحولي و المائي (البارد و الحار) لنبات المرّير ***Sonchus oleraceus*** في الخطوط الخلوية السرطانية والخلايا الطبيعية خارج الجسم الحي. شمل الفحص ألمختبري استخدام اثنين من الخطوط الخلوية السرطانية المزروعة في الزجاج(وهي خط سرطان الغدة اللبنية الفأري و خط سرطان الحنجرة البشري) و خط الخلايا الجنينية الليفيه الفأري الطبيعي, وقد تم معاملة الخطوط الخلوية خلال 24 ساعة ، 48 ساعة و 72 ساعة وبظروف التعقيم التامة في أطباق المعايره الخاصة بالزراعة النسجية. تم تحضير تراكيز مختلفة ثنائية التخفيف من المستخلصات الخام الثلاث ابتدائا من التركيز 78.125 مايكروغرام\ملليتر وإنتهاءً بالتركيز 10000 مايكروغرام\ملليتر واختبارها لكل من خطوط الخلايا السرطانية بمعدل ثلاث مكررات لكل تركيز. أظهرت المستخلصات الثلاثة تأثيرات تثبيطية في النمو متعلقة .

48 بمقدار تراكيزها ومدة تعريضها وإن أعلى تأثير لوحظ عند التراكيز العالية للمستخلص الكحولي بعد مرور ساعة من التعريض لكل من خطوط الخلايا السرطانية (الغدة اللبنية الفأري والحنجرة البشري), بينما التأثير السمي لكلا المستخلصين المائي (البارد و الحار) على خطوط الخلايا السرطانية (الغدة اللبنية الفأري والحنجرة البشري) أظهرت بأن التراكيز العالية أعطت فرقآ معنويا ($P<0.05$) وأن أعلى معدل لتثبيط نمو الخلايا ازداد خلال الفترة 24ساعة.

Introduction

Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age and causes about 13% of all deaths, 7.6 million people died from cancer in the world during 2007 (1). The current medicinal methods to deal with cancer include surgery, radiation and chemotherapy. Radiation and chemotherapy have the disadvantage of destroying healthy as well as malignant cells and thus can cause severe side-effects (2). A safe and effective cancer treatment has been the goal of scientists for many decades. Such a technique must be selective in destroying the cancer cells without irreversibly damaging normal cells (3). Nature has been a source of medical treatments for thousands of years and even today plant based systems continue to play an essential role in the primary health care of 80% of the world's population. There are at least 250,000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties. Nature has provided many effective anticancer agents in current use, which include drugs of microbial origin, and a number of plant-derived drugs (4). *Sonchus oleraceus* (Common sowthistle) is quite common in Iraq; this plant is a winter to spring annual adaptable and found in full or partial sun, moist to slightly dry sites, and soil that is fertile, so it is a weed. The seeds are used for medicine and young leaves are edible. It was suggested that the antioxidant activity in plants depend on environment factors such as growing season (5) and location (6). Extracts of *Sonchus oleraceus* aim to study the effect of cold and, hot aqueous and ethanolic extracts of *Sonchus oleraceus* on the growth of several cancer cell lines (Hep-2, AMN-3) andon normal cell line (Ref) *in vitro*.

Metraials and methods

The cell lines used in this study were supplied by tissue culture unit/ Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR) maintained in RPMI- 1640.

Human Larynx Epidermoid (Hep-2 cell line)

The passage number was 229 and 231. The origin and the cell line were mentioned by (7. It was a human laryngeal carcinoma excised from 57 years old man, then transplanted in immune suppressed rat by cortisone. It was kept at -169˚C (in liquid nitrogen). In preparation to any *in vitro* assay, the frozen cell line was withdrawn and maintained in RPMI-1640 containing 10% bovine calf serum, when the *in vitro* cells culture forms a monolayer. These cells were treated with trypsin/ versine mixture in order to pursue subculture process (8).

Ahmed-Mohammed-Nahi-2003 (AMN-3 cell line)

The cell line was supplied by tissue culture unit / ICCMGR, Baghdad, Iraq (passage number 122). The origin and description of this cell line was first mentioned by (9). The specimen was taken from murine mammary adenocarcinoma. The procedure of manipulation of this cell line is similar to that mentioned in Hep-2 cell line.

Rat Embryo Fibroblast (REF)

The normal culture of the rat embryo is the most important source for the undifferentiated fibroblastic culture. This cell line was supplied by tissue culture unit / ICCMGR, Baghdad, Iraq (passage 48). The specimen was taken from rat embryo then killed and analyzed by using Trypsin, then maintained in RPMI-1640 medium with 20% bovine calf serum, when it becomes confluent monolayer, the cells treated with Trypsin-Versine mixture in order to pursue subculture process .

Preparation of ethanolic, hot and cold aqueous extracts of *Sonchus oleraceus*

According to (10) ethanolic and cold aqueous extract of the plant were prepared.

Hot aqueous extract- This extract was prepared according to (11).

Cytotoxicity Assay: Cell cultures in microtitration plate (96wells) were exposed to range of plant extract concentrations during the log phase of growth and the effect determined after recovery time. (12) was performed on ethanolic,hot and cold aqueous extracts of *Sonchus oleraceus*.

Results

Cytotoxic effect of ethanolic, cold aqueous and hot aqueous extracts on (AMN3), (Hep-2) and (REF) cell lines: Three cell lines were used (AMN-3, Hep-2 and Ref cell lines) at three times of exposure (24, 48 and 72 hours). Two fold dilution was made to get concentrations from 78.125 µg/ml to 10000 µg/ml of ethanolic, cold and hot aqueous extracts of the plant. In table (1) the results revealed significant cytotoxic effect of ehanolic extract at levels ($P<0.05$) for all concentrations except (2500µg/ml) on AMN3 cell line had non significant effect. All extracts inhibited cell growth at highest concentrations and reduced at the lower concentrations. The ethanolic extract had highest growth inhibition on AMN3 and Hep-2 cell lines at the concentrations (5000 and 10000 µg/ml) for the period of 48 hrs. AMN3 and Hep-2 cell lines revealed variable inhibitory growth when treated with different concentrations of plant extracts of *Sonchus oleraceus*, and some concentrations inhibited the growth of cell lines like (5000 µg/ml, 625 µg/ml, 1250 µg/ml) for the periods (24, 48 and 72 hrs) respectively of AMN3 cell line , and (5000 µg/ml, 1250 µg/ml and 5000 µg/ml) for the periods (24, 48 and 72 hrs) respectively of Hep-2 cell line, more than the other concentrations, on the same cell lines, and in same periods.

Table (2) shows the effect of the cold aqueous extract. Cold aqueous extract had highest inhibitory effect on growth of AMN3 and Hep-2 cell lines at the concentration (5000µg/ml) for the periods of 24 hrs and 72 hrs respectively. The cytotoxic effect on AMN-3 cell line revealed that the concentrations gave a significantly ($P<0.05$) and cold extract had highest growth inhibition on AMN3 cell line at the concentrations (5000 & 625) µg/ml for the period of 24 hrs and 48 hrs respectively. The cytotoxic effect on Hep–2 cell line showed significant effect at levels ($P<0.05$) except the concentration 2500 µg/ml and the highest inhibitory effect after (24, 48, and72) hrs at the concentration 5000 µg/ml. Cold aqueous extract had inhibitory effect on growth of Ref cell lines at the concentration (2500, 2500, 10000µg/ml) for the periods of (24, 48, 72 hrs) respectively.

Table (1): Mean values of inhibition rate (IR%) of (AMN3, Hep.2 and REF)cell lines after treatment with different concentrations of ethanolic extract of *Sonchus oleraceus* for (24, 48 & 72) hours.

Concentration µg/ml	IR% AMN3				IR% Hep.2				IR% REF			
	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD
78. 125	16	17	11.6	*2.6	3.1	35	56.4	*4.2	6	3.2	2.5	*1.3
156. 25	13.6	32.8	18.9	*3.3	20	32	46	*3.9	5.6	8.3	10.3	*1.6
312. 5	26	21.4	33.3	* 5	21	48	42.5	*4.8	7.3	121.9	11.9	*1.2
625	33	35.4	50.7	*7.6	22	45	30	*5.4	3	9.5	24.7	*2.4
1250	32	44.3	61	* 6.9	40.3	52	36.4	*6.8	4.9	18.5	17.7	*2.6
2500	40.5	44.4	45.4	n.s7.3	34	44	36.6	*5.8	8.4	10.2	17.3	*1.2
5000	60	79.7	53	*8.1	48.5	87	56	*6.2	11	6.4	15.4	*3.3
10000	51	84.7	56.3	*7.4	45.6	87	55	*4.8	12.8	20.5	11.5	*2.8
LSD	*5.2	*5.5	*5.7		*4.7	*5.2	*3.9		*1.6	*1.9	*2.1	

(*) Significant difference between means (comparison between columns and comparison with rows at levels (P<0.05).(n.s) Non-significant difference between means.

Table (2): Mean values of inhibition rate (IR%) of (AMN3, Hep.2 and REF)cell lines after treatment with different concentrations of cold aqueous extract of *Sonchus oleraceus* for (24, 48 and 72) hours.

Concentration µg/ml	IR% AMN3				IR% Hep.2				IR% REF			
	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD
78. 125	11.5	12.3	10.4	n.s2.6	13.7	13.2	22.6	*3.5	3.1	9.2	1.7	* 1.6
156. 25	19.2	15.2	17.5	n.s3.7	15.6	19.6	39.8	*4.6	5.2	7.5	2.6	* 0.2
312. 5	28.4	20.6	21.4	* 7.7	13	28.9	33	* 2.3	10.7	6	8.5	* 2.3
625	32.6	31	28.3	* 0	29	35.3	18.5	*3.5	8.6	8.3	14	* 2.3
1250	46.3	27.4	18.4	* 0.4	32.4	48.6	24.5	* 2	9.6	11	19.4	* 1.1
2500	67.5	37.7	25.4	* 5.3	40.8	38	38.3	n.s3	19.3	19.6	27	* 0.2
5000	65.9	43.2	46	* 2.5	43.5	48.7	61	*5.7	10.3	18.9	17.8	* 0.6
10000	50.8	47.3	48.9	* 3.5	41.5	44.2	52	*5.7	13.4	11.6	20.4	* 2.3
LSD	*1.8	*2.3	*0.9		*3.3	*3.6	*3		*1.4	*1.5	*1.2	

(*) Significant difference between means (comparison between columns and comparison with rows at levels (P<0.05). (n.s) Non-significant difference between means.

Table (3) shows the inhibitory effect of hot aqueous extract of *Sonchus oleraceus* on the growth of cell lines (AMN3, Hep-2 and REF). The highest inhibitory growth of AMN3 and Hep-2 cell lines at concentrations (2500 and 10000) µg/ml after 72 hrs and after 48 hours respectively. Hot aqueous extract had the highest inhibitory effect on the growth of Ref. cell line at the concentration 5000 µg/ml after 72 hours of exposure and at the concentration 10000 µg/ml after 24 hours.

Table (3): Mean values of inhibition rate (IR%) of (AMN3,Hep.2 and REF)cell lines after treatment with different concentrations of hot aqueous extract of *Sonchus oleraceus* for (24, 48 and 72) hours.

Concentration µg/ml	IR% AMN3				IR% Hep.2				IR% REF			
	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD	24 hrs	48 hrs	72 hrs	LSD
78. 125	13.4	6.9	12.8	* 1.2	21.4	23	30.3	*1.3	2.4	9.2	20.5	*1.7
156. 25	13.3	18.3	25	* 3.5	18.5	26	16.8	*2.8	4.3	15.7	17.3	*2.3
312. 5	13.6	18.9	42.4	* 3.5	36	37.4	28.3	*5.9	7.3	8.7	25	*3.6
625	25	25.8	49.4	* 4.4	48.5	35.6	35.7	*6.2	6.9	11.9	21.6	*1.6
1250	29	37.6	51.1	* 5.8	49	43	30.6	*4.7	9.8	7.4	23.5	*2.6
2500	25	38.9	65.2	* 7.3	40.7	39.3	42.9	n.s5.2	12.9	9.4	21.2	*2.6
5000	42	30.2	40	* 5.4	56.2	50	38.4	*5.3	6.8	14.2	25.9	*3.5
10000	56	38.4	50.2	* 5.9	62.4	75.2	40.6	*7.1	6.4	18.5	21.6	*2.6
LSD	*3.4	*3.8	*5.4		*3.8	*5	*4.4		*1.8	*2.3	*2.7	

(*) Significant difference between means (comparison between columns and comparison with rows at levels (P<0.05). (n.s) Non-significant difference between means.

Discussion

After treatment with different concentrations of the extracts of *S. oleraceus* during 24, 48 and 72 hours the optical densities (OD) for the stained cell lines plate, revealed differences of (OD) between concentrations, that the high concentration gave low value of OD, indicating maximum response because the affected (dead) cells are removed by washing during staining procedure leaving a light colour represented the attached viable cells. The low concentration gave high value of OD, which indicates minimum response in proportion to high percentage of viable cells. Since *S. oleraceus* extracts are relatively acidic (11), it may alter the PH of the solution in which the cells were suspended particularly at its highest concentration, while this effect was diminished as concentration dropped, or this selective effect of extractions may appears on the cell adhesion. These results indicate that *S. oleraceus* extracts have one or more constituents capable to interfere with the adhesion process of cells leading to detachment from plate and not involved in the measurement of O/D. Most cells in culture need a period of lag phase to attach on the substratum and adapt to medium conditions before they will start to proliferate (12). The other factor may be osmolality effect, in which extracts of *Sonchus oleraceus* rich in proteins, carbohydrates, minerals and other constituents that make it hypertonic solution (13) and may cause dose-dependent osmotic shock to cell-lines.

Three cell lines of mammary gland adenocarcinoma (AMN3), human epidermoid carcinoma (Hep-2), and normal rat embryo fibroblast (REF) were grown *in vitro* to study the cytotoxic effect of *S. oleraceus* extracts, at different concentrations and times of exposures. The results showed that the three types of *S. oleraceus* extracts had significant cytotoxic effect (P<0.05) on AMN-3 and less on Hep-2 cell lines, at

different concentrations and times of exposures. During comparison of effect among the *Sonchus oleraceus* extracts on AMN3 and Hep-2 cell lines, AMN3 cell line was more sensitive than Hep-2 when treated with extracts.(14) in studying the effect of *Cyperus rotundus* crude extracts on cancer cell lines, explained the highest sensitivity of AMN-3 cell line among other types by the activation of some glutathione-S-transferase enzymes (GSTs) via several compounds in plants extract, especially the polyphenolic compounds, (15), also mentioned that during his study, for the effect of Polyphenols and Terpenoids isolated from green tea on some cancer cell lines. The (GSTs) acted as an anti oxidant causing cellular detoxification by enhancing their combination with reduced glutathione leading the cancer cell toward programmed cell death, apoptosis. Also cultures will vary significantly in many of their properties between exponential growth and stationary phase (12).

The differences in Hep-2 response toward different treatments might indicate a presence or absence of specific cellular receptors in each type of cell lines; making the cells interact at same concentration in different manners. Moreover, the metabolic pathways in response to each treatment differed from one line to another. This fact was mentioned in different studies which investigated different plant extracts in treating several types of cell lines (16). On the other hand it can be suggested that extracts of *S. oleraceus* may early block the expression of gene responsible for the milk-like secretion from AMN-3 cell-lines, which is the most characteristic feature of this cell-line (9).

The cytotoxic effect of the plant extracts may be due to the presence of flavonoid, which has been proved to have anticancer action. This study was consolidated by some researchers emphasized that plants and herbs have many active compounds. The active compounds may have cytotoxic character against many important diseases, including cancer cells *in vitro* and *in vivo*.

The inhibitory effect of plant extracts of *Olea europea* (17) and *Urtica dioica* (18) on (AMN3 and Hep-2) cancer cell lines growth may be attributed with their content due to contain of many active compounds which have anti cancer effect, the compounds like flavonoids, phenols, tannins, terpenes and vitamins. Some of these compounds are present in *Sonchus oleraceus* extracts. There were no differences between the results of this study compared with the results of (19) proved that crude extracts from *Salix acmophylla* had inhibitory effect on (AMN3 and Hep-2) cancer cell lines. (4) mentioned that certain flavonoids are known to possess antitumor activity, and the high free radical-scavenging activity and total antioxidant activity result from the existence of both phenolic and flavonoid-type compounds (20). The higher content of total phenolic and flavonoid compounds in *S. oleraceus* may account for the antioxidant effect (21).Chang (22) Reported that the flavonoids profile of *Sonchus oleraceus* particularly their water soluble fractions are (luteolin 7-0-glycoside), (apigenin 7-0-glycoside), (3t, 4, 7-trihydroxyflavone) having the pharmacological activities and antihepatotoxic properties.

Cytotoxic inhibitory effect was found to be different between concentrations of *Sonchus oleraceus* extractions and this may be due to the phenomenon, Hormetic effect, in this type of effect and contributed to all types of cell lines, stimulation in growth was at low concentrations while the growth was inhibited at higher concentrations. It was a biological effect in toxicology representing a contrast effect between stimulation of growth in regard to the control cell growth while the higher concentrations showed a growth inhibition, partially or completely, and according (23) the phenomenon Hormetic effect appeared in the *Plantago major* on AMN-3and Hep-2 cell lines.

Tables (1, 2 and 3) revealed comparisons between the three cell lines (AMN-3, Hep-2 and REF) exposure to (ethanolic, cold aqueous and hot aqueous) extracts of *S. oleraceus* respectively in different concentrations during 24, 48 and 72 hours of exposure on cell lines. The result exhibited the variation in cytotoxic effects of the extract depending on the type, concentration, and duration of exposure.Itharat (24) reported *in vitro* studies about the cytotoxicity activity of different plant extracts against various cancer and normal cell lines that using (ethanolic and water extracts). The ethanolic extract of *S. oleraceus* extracts had the greatest effect on the growth inhibition of both AMN-3 and Hep-2 cell lines than the hot aqueous extract and cold aqueous extract after the three periods of exposure. According to (25) the ethanolic extracts of different plants showed cytotoxic activity against numerous cancer cell lines.

The cold aqueous extract had lower effect than the ethanolic and hot aqueous extracts on AMN-3 and Hep-2 cell lines (Tables 1, 2 and 3). This result may be due to the solubility of both organic and non organic substances (phenols, glycosides, flavonoids, saponine, and tanine) in hydroalcoholic solvent, while in aqueous solution just the substances which dissolved in water will be extracted according to (26). The results showed the hot aqueous extract had cytotoxic effect against cell lines better than the cold aqueous extract.Yoo (27) Suggested that the heat processing of medicinal plants represents a possible route to the development of antitumour agents.

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