

Cytotoxic activity of *Cardia draba* leaves extracted on some cancer cell line

الفعالية الخلوية السمية لمستخلص نبات الجنجيرا على بعض خطوط الخلايا السرطانية

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Abstract

Cardaria draba is considered as one of the most fundamental plants that have wide diversity in medical uses, other researches had shown that this plant has a strong antioxidant activity for this reason the idea came to look for the cytotoxicity of this plant extract against different carcinogenic cell lines after subsection of this plant to methanolic extraction method. Result declared strong cytotoxicity against those carcinogenic cell line with a variable values of IC_{50} and the best effect was seen against HepG2 cell line, then we looked for the reason behind this reduction in viability of the cells via performing Caspase 9 assay which is used to check the induction of apoptosis and the expression of the TNF- α as a strong transcription factor which is activated in tumor cell lines.

Interestingly, our finding suggested that the plant extract produced a positive effect in the induction of Caspase 9 which led to induce early apoptosis, regarding TNF- α expression; the result explained a significant reduction in expression of those genes which were correlated with induction of tumor. This meant that the reduction in cell viability resulted in the induction of apoptosis and reduction of TNF- α transcription genes.

Key word: *Cardia draba* , tumor cell lines

الملخص:

يعتبر نبات الجنجيرا من النباتات التي تستخدم بشكل واسع في المجال الطبي. اشارت البحوث ان هذا النبات له فعالية قوية مضادة للاكسدة وهذا ما شجع على اختبار فعالية المستخلص الخام للنبات ضد عدد من خطوط الخلايا السرطانية بعد استخلاصه بالميثانول. اوضحت النتائج سمية خلوية عالية للمستخلص النباتي ضد الخلايا السرطانية A375, Hep G2, MCF7 حيث خفضت عدد الخلايا الحية بنسبة تراوحت بين 40-60%, وفضل تأثير ظهر على الخلايا السرطانية للكبد Hep G2 ب IC_{50} 53,2 مايكروغرام/مل. كما وتم دراسة الفعالية المضادة للسرطان من خلال دراسة Caspase9 الذي استخدم لقياس تحفيز الموت المبرمج للخلايا حيث بينت النتائج ان 25 مايكروغرام/مل من المستخلص النباتي لها القدرة على تحفيز فعالية Caspase9. كما تبين ان هنالك انخفاض معنوي في التعبير الجيني ل TNF- α عند معاملة المستخلص مع الخلايا .

الكلمات المفتاحية: جنجيرا ، مضادات السرطان ، الموت المبرمج للخلاية

Introduction

It's well-known that medical plants are considered as one of the most important sources used for the production of drugs and other medical components. The importance of these plants came those active compounds which play substantial role in pharmaceutical production, should undergo certain biological check for other effects when we want to use them on different applications on cell lines like; cytotoxicity and antioxidant to ferret if there is any side effect upon their uses[1].

The focused on *Cardaria Graba*, this plant is about 10- 80 cm length, with white top end with horizontal creeping roots, the stem is branched while leaves are simple, alternating and mostly toothed. The size of leaves of this plant is about 4- 10 cm, with slight stem. While its shape is either lance or egg- shaped. This plant is found normally in southern part of Europe and eastern part of Asian, but it is rarely found in North America, other findings refer to its presence in Australia[2].

For many years, it was well known that medical plants had been used for treating against different types of diseases and cancers that helped in supporting the body to resist any defect that lead to the discovery of new medicine[3,4]. The development of information and knowledge in medicinal plant which came from many experimental trials and that helped in appearance of many traditional, famous therapies like; Chinese medicine, naturopathy, and aromatherapy[5,6,7]

For this reason, the natural drugs are considered as a fundamental source for such treatment and considered as an alternative mean for the treatment of any infection beside the chemical drugs [8,9]. The practical application of herbal medicine came to support other types of medicine due to its importance in treatment of bacterial diseases [10,11,12].

Materials and Methods

Plant collection

The leaves of *Cardaria Graba* were collected in March-April 2016 from Al- Khalidia near Baghdad. The classification of plant based on the morphological characteristics in the department of biology/Baghdad University. Then materials were air-dried at room temperature under shade for 8-10 days. The dried leaves were pulverized to powdered form by mortar and pestle. The powdered form was then stored at room temperature

Plant extraction

Dried plant material (10g) was extracted with 100 ml of methanol using rotary shaker for 24 h. Later on, it was filtered and centrifuged at 5000 g for 15 min. After evaporation of the solvent, the supernatant was collected to make the final volume one-fifth of the original volume. It was stored at 4° in bottles for further experiment[13].

Viability assay

Protocol was performed following the manufacturer instructions. Cells ($1 \times 10^4 - 1 \times 10^6$ cells mL⁻¹) were grown in a 96 flat-well plate, with a final volume of 200 μL per well. The plate was covered by sterilized parafilm, agitated gently, and incubated at 37±°C, 5% CO₂ for 24 h. After incubation, the medium was removed and 200 μL of the two fold serial dilutions of *P. graveolens* crude extract (25, 50, 100, 200, 400 μg mL⁻¹) were added to the wells. Triplicates were performed at each concentration, as well as controls. Plates were incubated at 37±°C, 5% CO₂ for 24 h. After exposure to extract, 10 μL of the MTT solution was added to each well. Plates were further incubated at 37±°C, 5% CO₂ for 4 h. The medium was then carefully aspirated and 100 μL of solubilization solution was added per well and incubated for 5 min. The absorbance was determined with an ELISA reader (Bio-Rad - Germany) at a wavelength of 575 nm. For calculation of IC 50, the optical density readings were subjected to statistical analysis[14].

RNA isolation, cDNA synthesis and rt PCR

RNA isolation from infected cells was implemented using the RNeasy Mini Kit or RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol as mentioned [15]. Concentration of purified total RNA was measured by a Nanodrop ND-1000 spectrophotometer (Peqlab). Then, equal amounts of RNA were used to synthesize cDNA using Thermo Fisher Scientific kit according to the manufacturer's protocol. The quantification of cDNA was performed by quantitative real-time polymerase chain reaction (qRT-PCR) and the following primers: GAPDH_fwd 5'-gcaattccatggcaccgt-3', GAPDH_rev 5'-gccctctgatttggagg-3', TNF_fwd 5'-CTGCTGCACTTTGGAGTGAT-3', TNF_rev 5'-AGATGATCTGACTGCCTGGG-3'. The qRT-PCR reaction mix (Brilliant III SYBR Green QPCR Master Mix) was purchased from Agilent Technologies. Analysis was performed as described earlier, the GAPDH primers used as a control to calculate the CT values [16].

Caspase 9 activity

A time-dependent study of caspase -9 activities was done three times using assay kits Caspase-GloH 9 (Promega, Madison, WI) on our cell lines using a 96-well microplate. A total of 1×10^4 cells/ml was seeded and incubated with 100 ml of methanolic plant extract at different concentration as mentioned in results for 24 hours. Caspase activities were performed according to the manufacture protocol. Briefly, 100 ml caspase-Glo reagent were added and incubated at room temperature for 30 minutes. The activity of caspases from apoptotic cells cleaved the aminoluciferin-labeled synthetic tetrapeptide and led to the release of substrate for the luciferase enzyme. For measurements, we used a Tecan InfiniteH200 Pro (Tecan, Mannedorf, Switzerland) microplate reader [17].

Cell lines

Different cell lines had been used to accomplish this research; Hepatoblastoma (HepG2) cells [18], Human breast adenocarcinoma (MCF-7) cells [19], Human malignant melanoma cell (A375) line [20] and WRL68 cell line as normal cell line were obtained from American Type Culture Collection (ATCC) and cultured in DMEM medium supplemented with 10% Fetal Bovine Serum, 1% sodium bicarbonate, 10^3 IU Penicillin G, and $100 \mu\text{g mL}^{-1}$ Streptomycin. MCF-7 cells were maintained in a CO_2 incubator (5%) at $37 \pm ^\circ\text{C}$.

Statistical Analysis

In this research, one way analysis of variance (ANOVA) was performed to analyze all data. Those were standard deviation (SD) and statistical significances were carried out using a GraphPad \pm expressed as mean Prism version 6.

Results

Cytotoxic Effects of *C.draba* Extracts on different carcinogenic cell lines using the MTT Assay:

MTT assay is considered as fundamental way used to determine the cytotoxic activity of different plant extracts among them; our methanolic extract from *Cardaria draba* plant. Here, we tested the cytotoxic effect of this extract against different carcinogenic cell lines like; Hepatoblastoma (HepG2) cells, Human breast adenocarcinoma (MCF-7) cells, Human malignant melanoma cell (A375) line. While, WRL68 cell line was used as normal cell line. The purpose of this assay is to determine the cell viability and the inhibition rate via using different concentrations of *Cardaria draba* plant extract on these cell lines as mentioned in table 1.

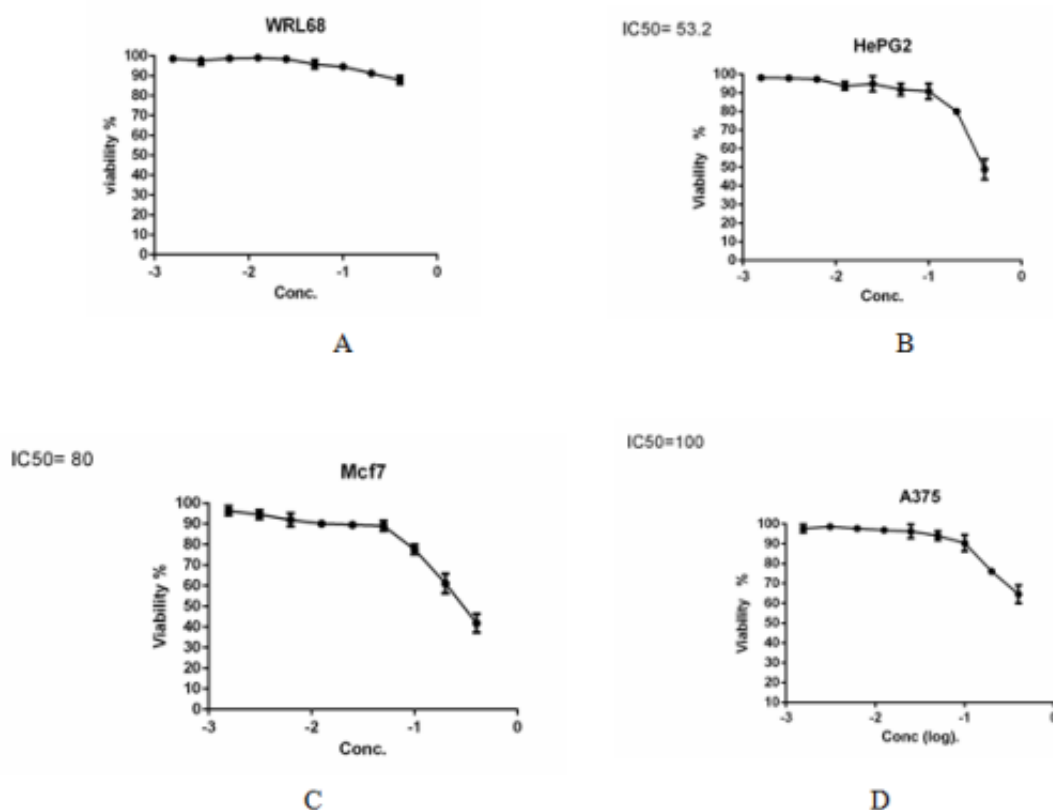
Those results represented the effect of treatment of tumor cell line with different concentrations started with 6.2 and ended with $400 \mu\text{g mL}^{-1}$ and log values of $\mu\text{g mL}^{-1}$ was performed on a Graphpad Prism 6 using log (Inhibitor) versus response curve.

It is well known that the most effective concentrations were selected depending on the most significant and lowest IC_{50} values. Cell viability at each time-point was determined by MTT colorimetric assays.

Figure 1(a) which represents the MTT assay on WRL 68 cell line which is considered as normal cell line and it is considered as a control test while figures 1(b, c & d) showed as different expressions and values from the exposure of HePG2, MCF7 and A375 cell lines respectively to the methanolic extract of *Cardaria Graba* at different concentrations started from 6.2 till $400 \mu\text{g mL}^{-1}$.

In figure 1 (b,c & d); we could see certain differences of vibrations in reduction rate or cell viability upon using our extract on different tumor cell lines 24 hours post the treatment, the cell viability decreases upon increasing the concentrations in those cell lines. The lowest reduction rate between all cell lines ranged from 40- 60 %, while most effective cytotoxic effect which had shown the lowest IC_{50} was 53.2 in HePG2 cell lines.

IN A375 and MCF 7, we got clear reduction in cell viability upon treatment with plant extract, but the IC_{50} that we got were 100 and 80 respectively. This means that the plant extract produced higher cytotoxic effect on other cells. For this reason we selected HepG2 cells for further studies had shown higher values compared with HepG2 cells.



Figure(1): cytotoxic activity of methanolic extract of *Cardaria Graba* on different tumor cell lines like; A735, HePG2, MCF-7 and WRL as normal cell line using different concentrations represented by log values and calculated by ug/ml, while cell viability are calculated by percentage as shown in figure's legends.

Table (1) : represents the count of viable cell no. upon the treatment of *Cardaria Graba* extract on different tumor cell lines.

Treatment ($\mu\text{g/ml}$)	Viable Cell Count HepG2 (Mean \pm SD)	Viable Cell Count MCF7 (Mean \pm SD)	Viable Cell Count A375 (Mean \pm SD)	Viable Cell Count WRL (Mean \pm SD)
400	48.9 \pm 5.4	41.8 \pm 4.5	64.5 \pm 4.5	87.8 \pm 2.1
200	80.1 \pm 1.8	61.1 \pm 4.7	76.0 \pm 1.3	91.0 \pm 0.7
100	90.8 \pm 4.2	77.6 \pm 2.3	90.3 \pm 4.1	94.6 \pm 1.3
50	91.8 \pm 3.0	89.0 \pm 2.2	94.0 \pm 2.3	96.8 \pm 2.1
25	94.9 \pm 4.0	89.5 \pm 1.9	96.2 \pm 3.4	98.4 \pm 0.7
12.5	93.7 \pm 2.2	90.1 \pm 0.4	96.8 \pm 1.4	99.1 \pm 0.9
6.25	97.7 \pm 1.2	91.9 \pm 3.1	97.7 \pm 0.3	98.7 \pm 0.6
3.125	97.9 \pm 1.5	94.5 \pm 2.1	98.6 \pm 0.7	97.8 \pm 2.2
1.5625	98.1 \pm 0.6	96.4 \pm 2.3	97.6 \pm 2.0	98.6 \pm 1.0

Plant extract induces Caspase 9 activity significantly

Its well-known that apoptosis is considered as complicated activity that mobilize no. of molecules, the caspase activity is a good mean to determine such activity. Here we tested the effect of our plant extract on HePG2 cell line because, it had shown the lowest IC_{50} compared with other cell line used in this research. To study the apoptotic activity, we stained the cells with aminoluciferin labeled substrate of caspase to determine the caspase 9 via the estimation of intensities of illumination after 24 hour post labeling. We noticed a graduated increase in caspase activity in HePG2 cells that was labeled with illuminant and treated with our plant extract,

At 25 $\mu\text{g/ml}$, we found significant increase in Caspase 9 compared with control sample and this activity increased more upon increasing the concentration of plant extract with 50 $\mu\text{g/ml}$ The maximum level of caspase had been detected upon the treatment with maximum concentration of our plant extract which was 100 $\mu\text{g/ml}$ indicating that this plant extract is a good or strong activator for caspase activity in tumor cell line as shown in figure 2

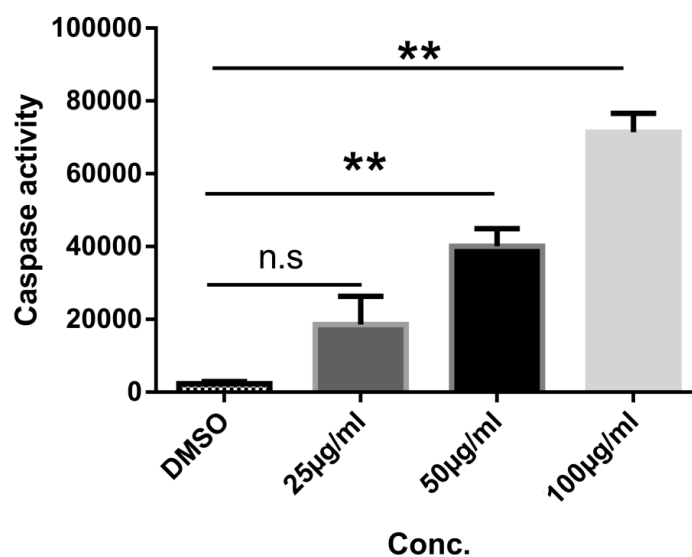


Figure (2): activation of caspase activity by plant extract. Results were normalized with DMSO values as fold of increase compared with control. Data represents mean \pm SD of three independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using DMSO as a control used for statistical analysis of each time point separately (** $p \leq 0.01$).

Plant extract reduces TNF- α activity

To speculate about the role of plant extract on TNF activity, we treated our cell line with different concentration of plant extract (25, 50 and 100 µg/ml) then; we checked the activity of TNF- α by real time PCR after extraction of cDNA.

The results were shown certain reduction in TNF- α activity in treated cells compared with control, TNF- α expression started to decrease when the cell line was treated with 25 µg/ml of the extract. Interestingly, this reduction increased more upon using a concentration of 50 µg/ml till getting a highly significant decrease in expression of TNF- α when we used 100 µg/ml of the plant extract.

Our finding indicates that this extract showed us a positive effect in decreasing the activity of TNF- α which is considered as a strong factor in activation of tumor. That may be considered as a good hint to use this plant extract for medical application as shown in figure 3.

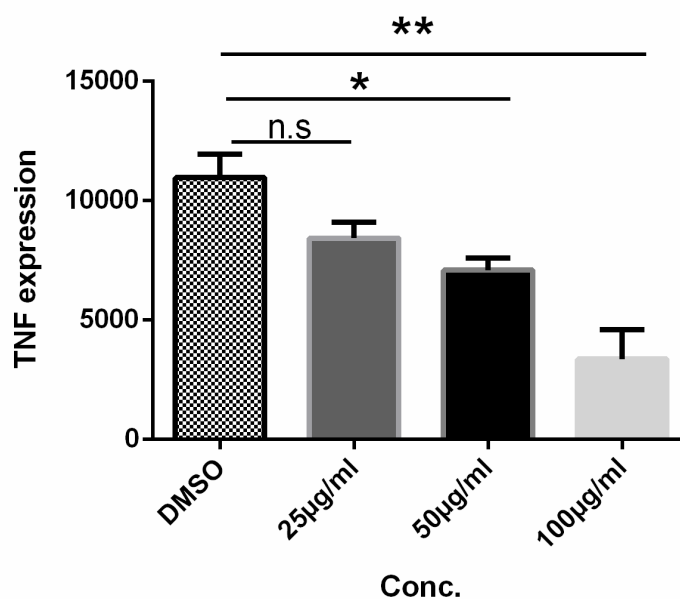


Figure (3): reduction of TNF- α activity due to treatment of tumor cell line with plant extract. Cells were treated with different concentrations of the plant (25,50 and 100 100µg/ml) then, TNF- value was determined by Real time PCR. Data represents mean \pm SD of three independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using DMSO as a control used for statistical analysis of each time point separately (p \leq 0.01).**

Discussion

Cancer is considered as the second-leading cause of mortality and morbidity and that pushed the researcher to discover an urgent way to develop (chemo) therapeutic interventions in order to help in restriction of cancer disease [21], because depending only on excision, surgery to treat cancer cases like breast cancer which is considered as one of the most common types of cancer in women as mentioned by Lopez group [22] is not enough to solve such disastrous problem. For this reason, the need comes to depend on another mean in order to help in surrounding the distribution of cancer.

Here in this study, the methanolic extract of *Cardaria Graba* was used to study the cytotoxic effect against different carcinogenic cell lines like; HepG2, MCF7 and A375 cell line. *Cardaria draba* is considered as one of the most effective medical plant that showed different activities as a strong anti-microbial agent due to its ability to prevent the growth of some gram positive bacteria like *Bacillus Spp.* and gram negative bacteria like *E.coli*. Also, Sharifi- Rad found that this plant is considered as a substantial anti-oxidant agent on synthetic DPPH free radical from its leaf and seed extract and it has a fundamental anti-inflammatory activity[23].

It is well-known that natural plant extract plays important role in decreasing health problems which are associated with chronic diseases due to the presence of anti-oxidant components like; carotenoid, vitamins [24] because, there is an opposite relationship between the antioxidant compounds and human diseases like; cancer and other diseases[25].

The importance of previous finding which confirmed the role of *Cardaria draba* as a strong anti-oxidant pushed us to speculate if there is another biological effect which might help in restricting the growth of tumor. To do so, we checked if this plant has any cytotoxic effect on some type of tumor cell lines and if yes, we looked for the explanation of any mechanism which supports our results.

Recently, studies proved that MTT assay is considered as an important method to check the cell viability, this method can detect survival rate the cells after the treatment with such active substances at specific time point [26]. The studies that talked about the cytotoxic activity of this plant extract is very rare, that pushed us to check cytotoxicity on different tumor cell lines and we got interesting results upon the treatment of those cell lines with our plant extract but with certain variation and the best one which had shown the lowest IC₅₀ was HepG2 cells, for this reason; we chose this cell line to check the mechanism behind this cytotoxicity.

One of the most acceptable theories that give an explanation or correlation with cytotoxic effect on cell lines is apoptosis [27], according to the findings of Looi; it is defined as one of the most complicated actions which triggered by molecules leading to activate caspase pathway in dependent or independent manner and caspase 9 is classified as an extrinsic pathway that trigger apoptosis. Indeed, caspases is related to the cysteine proteases category which is found either as initiator or executioner caspases and caspase 9 is being considered as initiator caspases [28].

Depending on previous finding, we found that treatment with our plant extract induced apoptosis significantly, suggesting that *Cardaria Graba* plant extract has an effective apoptotic activity against cell lines.

Recently, it had been established that TNF- α is considered as an effective activator of the cancer as in breast cancer, this fact pushed us to check if the treatment of tumor cell line with our plant extract has a negative effect on the expression of TNF- α . Our result showed that the incubation of tumor cell lines with *Cardaria Graba* reduced the expression of TNF- α significantly and this result could confirm the correlation between the reduction of TNF and our plant extract which has a strong antioxidant activity toward tumor cell lines as mentioned previously because, the plants that had shown antioxidant activity are able to inhibit TNF- α induced tumor in human cell line by blocking the activation of nuclear factor-kappa B (NF-kB) [29].

Conclusion

In conclusion this study report that the methanolic extract showed a significant antioxidant activity and cytotoxic activity against different cell lines like; A375, HepG2, MCF-7 cell line. The extract induces reduction in the activity of TNF- α which is a causative agent for induction of tumor and at the same time activate Caspase-9 to induce apoptosis.

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