

Interleukin-6 Secretion in Response to *Onopordum jordanicum* Plant Extracts in Prostate Cancer Cells

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ABSTRACT

Onopordum jordanicum is a Jordanian native medicinal plant with poorly investigated anti-inflammatory properties. The current study aimed to explore the anti-inflammatory effect of *Onopordum jordanicum* in *in vitro* cell models of human lung fibroblast non-cancer MRC-5 and prostate cancer PC3 cells through measuring lipopolysaccharide-induced interleukine-6 secretion. The cells were treated with the plant water and methanol extracts for 72 hrs prior to lipopolysaccharide exposure for 24hrs. The cytotoxicity of the plant extracts was measured using the viability assay water-soluble tetrazolium (WST-1). We observed that the plant water and methanol extracts did not decrease lipopolysaccharide-induced interleukine-6 secretion in fibroblast non-cancer MRC-5 and prostate cancer PC3 cells. However, 15.6µM plant water extract showed an anti-inflammatory trend in both cell lines. The plant water and methanol extracts were not associated with toxic effect in the investigated cells. In conclusion, *Onopordum jordanicum* did not have anti-inflammatory effects in non-cancerous fibroblasts and prostate cancer PC3 cells.

Keywords: Anti-inflammatory, Lipopolysaccharide, PC3, Fibroblast MRC-5, Medicinal Plant.

1. INTRODUCTION

Since the beginning of human history plants have been used for medicinal purposes. A wide range of plants have been investigated for their medicinal properties including their anti-bacterial, antioxidant, anti-cancerous and anti-inflammatory activities. However, nature is full of plants that are still to be investigated. Medicinal plants can be defined as any plant either as a whole or one or more of its parts can be used for therapeutic purposes¹. *Onopordum jordanicum* is a Jordanian local plant from Asteraceae family, commonly called camel thistle. It is found in Jordan eastern desert². The medicinal uses of this plant species were not specifically determined, but

local people in that region have used this plant to feed animals and treat people suffering from kidney problem, thus showing a therapeutic property. However, there are no research studies that have been reported so far on the beneficial health effects of this plant species. There are very limited studies on the beneficial health effects of other species of this plant, in particular its anti-inflammatory properties. A study by Marengo *et al* (2018) showed that the hydro-alcoholic extracts of *Onopordum horridum* Viv. and *Onopordum Illyricum* L. were able to decrease the release of the pro-inflammatory interleukin-8 cytokine and the promoter activity in human gastric epithelial cells AGS³. Whereas, Formisano *et al* (2017) showed that *Onopordum illyricum* contain sesquiterpene lactone that was able to reduce the levels of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) as well as inducing the

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transcription factor Nrf2 that is important for the activation of the cellular antioxidant proteins⁴. Moreover, Lajter *et al* (2015) indicated that in human monocytic (THP-1) cells, *Onopordum acanthium* inhibited the gene expression of both NF- κ B and cyclooxygenase-2, in addition to the inhibition of nitric oxide production as well as the enzymatic activity of 5-lipoxygenase, cyclooxygenase-1 and cyclooxygenase-2⁵. Also, different extracts of the same plant have been shown antiproliferative activities in breast epithelial adenocarcinoma (MCF-7), skin epidermoid carcinoma (A431) and cervix epithelial adenocarcinoma (HeLa) cells^{6,7}. Methanol extract of the *Onopordum acanthium* fruit has been found to decrease the levels of lipopolysaccharide-induced interleukin-8 in endothelial cells (HUVEctert)⁸. Another study by Talhouk and colleagues (2009) on *Onopordum cynarocephalum* showed that the plant water extract decreased the expression of endotoxin-induced inflammatory interleukin-6 in mouse mammary epithelial SCp2 cells at both the protein and gene levels⁹.

Interleukin-6 is a pro-inflammatory cytokine that has been associated with cancer progression, notably prostate cancer¹⁰⁻¹², and is considered a potential target in cancer therapies. To the best of our knowledge, there are no current study that have investigated the effect of this plant extracts on the inflammatory response in prostate and fibroblasts. Fibroblasts has been suggested as one of the strategies for future anti-inflammatory therapies. Therefore, the current study aimed to investigate the effect of water and methanolic extracts of *Onopordum jordanicum* on pro-inflammatory interleukin-6 secretion in human fibroblasts and prostate cancer cells, and will be the first study to investigate the effect of *Onopordum jordanicum* on the secretion of an inflammatory mediator in non-cancerous and cancerous human cells, and provide new understanding of some of the biological profile of this Jordanian medicinal plant.

Materials and Methods**Chemical Reagents and Solutions**

Bacterial lipopolysaccharides were purchased from Sigma-Aldrich (USA) and reconstituted in distilled water to prepare 5mg/ml stock solution. Dimethyl Sulfoxide (DMSO) stock solution was purchased from Duchefa (Netherlands). All stock solutions were diluted to the required final concentrations using media supplemented with 10% heat-inactivated foetal bovine serum (FBS). Water-soluble Tetrazolium (WST-1) proliferation reagent was purchased from Abcam (UK).

Preparation of Medicinal Plant Extracts

Water and methanolic extracts were prepared from the leaves of *Onopordum jordanicum*. The leaves were separated from the plant and washed with distilled water, dried at room temperature and then grind using blender. The resulted plant powder (10g) was extracted by refluxing with 100ml of water or methanol for 72hrs at room temperature with shaking at 150rpm. All samples were filtered through white cheese cloth paper and Whatman filter papers size 11.0cm. Solvents were evaporated by rotary evaporator at room temperature. The crude extracts were dissolved in DMSO to a final stock concentration of 10mg/ml. All extracts were purified by filtration through 0.22 μ m filter units and kept at -20°C.

Cells and Culture Conditions

Human lung fibroblast non-cancer MRC-5 (ATCC # CCL171) and human prostate adenocarcinoma PC3 (CLS # CVCL0035) cells were a gift from Dr. Malek Zihlif, Dr. Mamoun Ahram (School of Medicine, University of Jordan, Jordan) and Ms. Bashaer Abu-Irmaileh (Hamdi Mango Research Centre, Jordan). Cells were routinely cultured in RPMI 1640 medium supplemented with 100mmol/L glutamine, 100units/ml penicillin, 100mg/ml streptomycin and 10% heat-inactivated FBS (Caisson, USA). Both cell lines were grown to 70% confluent at 37°C in a humidified atmosphere of 5% CO₂.

WST-1 Viability Assay

Cell viability in response to water and methanolic extracts of *Onopordum jordanicum* was determined

using water-soluble tetrazolium (WST-1) viability assay. Fibroblast non-cancer MRC-5 and prostate cancer PC3 cells were seeded in 96-well culture plates in a final volume of 100ml/well culture medium and cultured in a humidified atmosphere (37°C, 5% CO₂). Cells were allowed to adhere to the plate surface for 36hrs before being treated with *Onopordum jordanicum* (0–1000µg/ml) or vehicle control (DMSO) for 72hrs. WST-1 reagent (10ml) was added to each well and incubated for 30 min in a humidified atmosphere (37°C, 5% CO₂). Quantification of the formazan dye produced by metabolically active cells was performed by a scanning multiwell spectrophotometer, measuring absorbance at 570 nm with a reference wavelength of 630 nm by microplate reader (TECAN, Austria).

Measuring interleukin-6 Protein Secretion

Fibroblast non-cancer MRC-5 and prostate cancer PC3 cells were allowed to adhere to the plate surface for 36hrs before being treated with plant extracts (15.6 and 125 µg/ml water or methanolic extracts) or vehicle control (DMSO) for 72 hrs in a humidified atmosphere (37°C, 5% CO₂). Cells were then exposed to a pro-inflammatory bacterial agent; lipopolysaccharide, by replacing culture media with fresh media containing lipopolysaccharide at the concentration of 500 mg/ml and plant extracts (15.6 and 125 µg/ml water or methanolic extracts). DMSO was used as vehicle control. Cells were exposed to lipopolysaccharide in the presence of the plant extract for 24 hrs. Supernatants were then collected and appropriately stored at -20°C until analysis. Interleukin-6

levels in supernatants were quantified by using a commercially available ELISA kit (Sigma-Aldrich) following manufacturer's instructions.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Outlier removal and check for normality of residuals were performed before statistical comparisons of the results, made using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The statistical analysis was performed using Graph-Pad Prism Software (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of *Onopordum jordanicum* extracts on the Pro-Inflammatory Cytokine interleukin-6

To assess the anti-inflammatory effects of *Onopordum jordanicum* water and methanolic extracts, lipopolysaccharide-induced interleukin-6 secretion was measured in cells pre-treated with plant extracts at the final concentrations of 15.6 and 125 µg/ml for 72 hrs. The bacterial pro-inflammatory agent lipopolysaccharide was able to significantly induce interleukin-6 production in fibroblast non-cancer MRC-5 cells ($p \leq 0.001$). Pre-treatment with the plant water extract at the tested concentrations did not significantly reduce the secretion of interleukin-6 in response to lipopolysaccharide compared to the lipopolysaccharide-treated cells (Figure 1A).

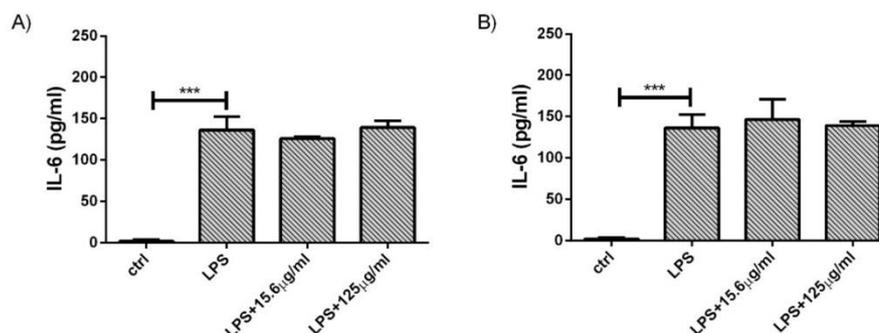


Figure (1): Effect of *Onopordum jordanicum* extracts on interleukin-6 in fibroblast cells

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Figure (1). Effect of *Onopordum jordanicum* extracts on interleukin-6 in fibroblast cells. Interleukin-6 secretion in response to pre-treatment with *Onopordum jordanicum* water (A) and methanolic (B) extracts in lipopolysaccharide (500 ng/ml)-treated fibroblast non-cancer MRC-5 cells. Interleukin-6 was measured in culture medium using a commercially available human interleukin-6 ELISA kit. Results represent means \pm SD of three biological replicates. Statistical analysis was performed using a one-way ANOVA followed by

Tukey's multiple comparison post-test (** $p \leq 0.001$).

A similar trend was observed following treatment with the plant methanolic extract (Figure 1B).

In prostate cancer PC3 cells, lipopolysaccharide was also able to significantly induce interleukin-6 secretion ($p \leq 0.05$). Pre-treatment with 15.6 $\mu\text{g/ml}$ plant water extract decreased the levels of interleukin-6 induced in response to lipopolysaccharide treatment; however, this effect was not statistically significant (Figure 2A).

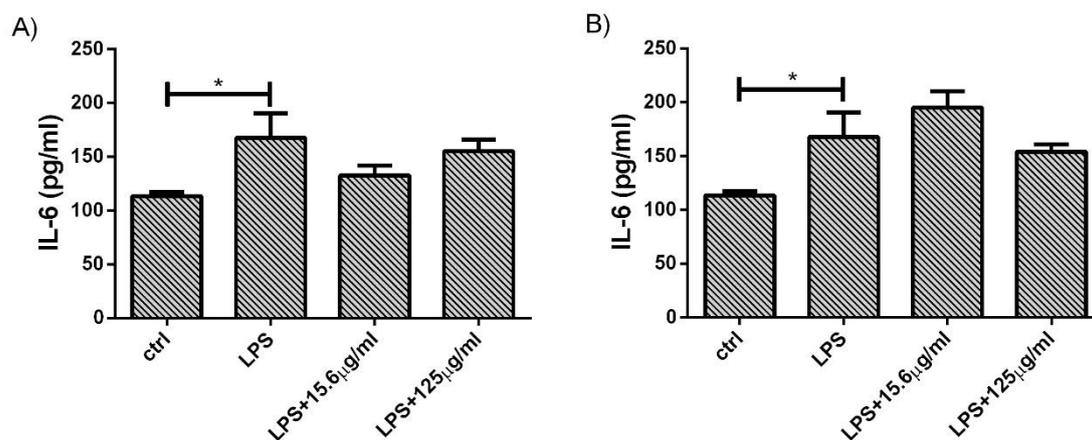


Figure (2): Effect of *Onopordum jordanicum* extracts on interleukin-6 in prostate cancer PC3 cells

Figure (2). Effect of *Onopordum jordanicum* extracts on interleukin-6 in prostate cancer PC3 cells. Interleukin-6 secretion in response to pre-treatment with *Onopordum jordanicum* water (A) and methanolic (B) extracts in lipopolysaccharide (500ng/ml)-treated prostate cancer PC3 cells. Interleukin-6 secretion was measured in culture medium using human interleukin-6 ELISA kit. Results represent mean \pm SD of three biological replicates. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison post-test ($*P \leq 0.05$).

Pre-treatment with the methanolic plant extract did

not reduce the levels of interleukin-6 induced by challenging the cells with lipopolysaccharide (Figure 2B).

Cell Viability in Response to *Onopordum jordanicum* in fibroblast non-cancer MRC-5 and prostate cancer PC3 Cells

Results obtained from the WST-1 assay show that in fibroblast non-cancer MRC-5 cells treatment with *Onopordum jordanicum* (0–1000 $\mu\text{g/ml}$) water and methanolic extracts was not associated with any toxic effect ($p \leq 0.05$) (Figure 3A).

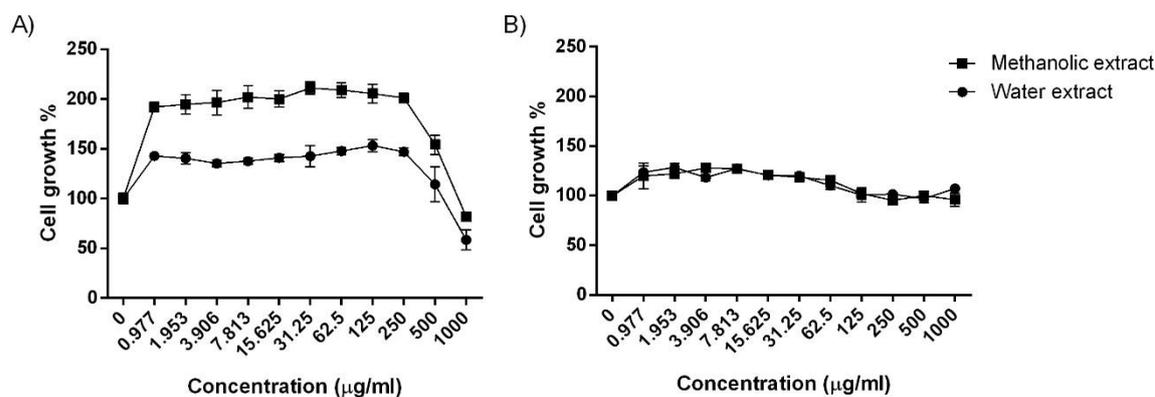


Figure (3): Cell viability in response to *Onopordum jordanicum* water and methanolic extracts

Figure (3). Cell viability in response to *Onopordum jordanicum* water and methanolic extracts. Viability assay performed in fibroblast non-cancer MRC-5 (A) and prostate cancer PC3 (B) cells in response to *Onopordum jordanicum* water (closed circle; ●) and methanolic (closed square; ■) extracts (0–1000 µg/ml) for 72 hrs. Results represent mean±SD of six biological replicates. The treatment with water extract significantly induced cell growth compared to the control ($p \leq 0.01$), except for 500 and 1000 µg/ml in fibroblast non-cancer MRC-5 and 125–1000 µg/ml in prostate cancer PC3 cells. Statistical analysis was performed using a one-way ANOVA followed by Tukey’s multiple comparison post-test).

In contrast, *Onopordum jordanicum* methanolic and water extracts significantly induced cell growth at all concentrations, except for the concentrations of 500 and 1000 µg/ml ($p \leq 0.05$), however, the effect of the plant methanolic extract in increasing the cell growth was more potent compared to the effect of water plant extract. In prostate cancer prostate cancer PC3 cells, exposing the cells to a wide range of concentration of the plant methanolic and water extracts (0 – 1000 µg/ml) induced a significant increase in cell growth, except for the concentrations of 125 – 1000 µg/ml ($p \leq 0.05$) (Figure 3B). This indicates that both aqueous and organic extracts of *Onopordum jordanicum* have no cytotoxic effects at the

concentrations tested in our experimental conditions.

Discussion

Onopordum jordanicum is a Jordanian plant which its beneficial health effects are poorly studied. To the best of our knowledge, this is the first study investigating the effect of this plant on the pro-inflammatory interleukin-6 production in human fibroblasts and prostate cancer as models for non-cancerous and cancerous cells, respectively. Fibroblasts were selected because of their common use in the studies of the inflammatory responses¹³.

It has been shown that the production of the pro-inflammatory interleukin-6 cytokine can be induced by the bacterial endotoxin lipopolysaccharide, and therefore lipopolysaccharide is usually used in *in vitro* models of inflammation¹⁴. lipopolysaccharide induces the production of interleukin-6 through the activation of the transcriptional factor nuclear factor-kappa B (NFκB), which is known to regulate the activity of several pro-inflammatory genes¹⁵. As result of this activation, the levels of pro-inflammatory biomarkers significantly increase in circulation^{10,12}. It is now well known that sustained levels of pro-inflammatory mediators are associated with several pathological conditions including different types of cancer¹⁰. In the current study,

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Onopordum jordanicum show a trend of an anti-inflammatory effect in fibroblast non-cancer MRC-5 cells. The water extract of the plant seems to be able to reduce the increase of interleukin-6 levels in fibroblast non-cancer MRC-5 cells in response to lipopolysaccharide (500 ng/ml) exposure, however, this reduction did not reach a significant level (Figure 1A). The anti-inflammatory effect of this plant has not been investigated in fibroblasts, however, the study by Talhouk and colleagues (2009) on a related species of *Onopordum* showed a decreased endotoxin-induced interleukin-6 levels in the non-cancerous SCp2 cells⁹. The difference in interleukin-6 responses can be attributed to the differences in the cell lines being investigated.

Increasing the concentration of the plant extract did not induce an anti-inflammatory effect. A concentration of 125 μ M did not change the levels of lipopolysaccharide-induced interleukin-6 secretion. A similar response was observed by treating the cells with 15.6 and 125 μ M methanolic plant extract. There was no reduction in the levels of lipopolysaccharide-induced interleukin-6 in fibroblast non-cancer MRC-5 cells (Figure 1B).

The effect of *Onopordum jordanicum* on interleukin-6 production in prostate cancer have not been investigated up to date. In *in vitro* prostate cancer cells, interleukin-6 cytokine production has been induced using lipopolysaccharide¹⁶⁻¹⁸. Since the pro-inflammatory interleukin-6 cytokine has been associated with the progression of prostate cancer¹⁰⁻¹², and that another species of *Onopordum* has been shown to reduce interleukin-6⁹, we tested the hypothesis that *Onopordum jordanicum* water and methanolic extracts can reduce lipopolysaccharide-induced interleukin-6 secretion in prostate cancer PC3 cells. We found that in prostate cancer PC3 cells, both water and methanolic extracts followed the same trend observed in fibroblast non-cancer MRC-5 cells. Water extract at 15.6 μ M showed a potential anti-inflammatory effect in prostate cancer PC3 cells, while 125 μ M water extract did not succeed in

reducing the levels of lipopolysaccharide-induced interleukin-6 (Figure 2A). Methanolic extracts at both 15.6 and 125 μ M did not reduce the levels of interleukin-6 in response to lipopolysaccharide, and therefore did not show an anti-inflammatory effect in prostate cancer PC3 cells (Figure 2B). The differences in the effects of the plant extracts in the two types of the cells can be explained by the differences in the phytochemical composition of the plant water and methanolic extracts, which opens the door for further investigation for the characteristics of both extracts.

Because we decided in our study to investigate whether this medicinal plant could exert a protective effect against the pro-inflammatory stimulus (lipopolysaccharide), we did not measure the levels of interleukin-6 in response to *Onopordum jordanicum* without lipopolysaccharide.

It is important to note that the increase in interleukin-6 levels in response to *Onopordum jordanicum* in both fibroblast non-cancer MRC-5 and prostate cancer PC3 cells was not due the cytotoxic effect of the plant extracts. Data obtained by performing a viability assay have clearly indicated that the plant extract did not reduce cell growth at any concentration tested (Figure 3). In contrast, the concentrations tested were associated with a significant increase in cells growth. On the other hand, the effect of both water and methanolic extracts of *Onopordum jordanicum* in fibroblast non-cancer MRC-5 and prostate cancer PC3 cells cannot be described as a pro-inflammatory effect because treating the cells with plant extracts and lipopolysaccharide did not induce a significant increase in the levels of interleukin-6 compared to the levels of interleukin-6 in response to lipopolysaccharide alone.

Conclusion

This study represents the first *in vitro* report of the effect of *Onopordum jordanicum* in human non-cancer fibroblast non-cancer MRC-5 and prostate cancer PC3 cells. The plant water and methanolic extracts did not show an anti-inflammatory effect in both non-cancer

fibroblast non-cancer MRC-5 and prostate cancer PC3 cells and did not protect the cells from interleukin-6 induced by exposing the cells to lipopolysaccharide. The levels of interleukin-6 induced by lipopolysaccharide in both cell lines followed the same pattern. However, the plant water extract showed a trend of an anti-inflammatory effect in fibroblast non-cancer MRC-5 and prostate cancer PC3 cells. Interestingly, the non-cancerous and cancerous cells respond similarly to the plant methanolic extract in terms of interleukin-6 secretion. Further studies are needed to confirm the results observed in our study and to explore the phytochemical composition of both extracts.

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Declaration of Conflicting Interests

The authors have no conflict of interest related to this work.

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إفراز الإنترليوكين-٦ استجابة لتأثير مستخلصات نبات الأونوبوردوم جورديانيكولوم في خلايا سرطان البروستاتا

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ملخص

الأونوبوردوم جورديانيكولوم (*Onopordum jordanicum*) هو نبات طبي أردني لم يتم دراسته بشكل كافٍ من ناحية خصائصه المضادة للالتهابات. ولذلك يهدف البحث الحالي إلى دراسة التأثير المضاد للالتهابات لنبتة الأونوبوردوم جورديانيكولوم في نماذج من الخلايا البشرية المخبرية المكونة من خلايا الرئة الليفية البشرية غير السرطانية (MRC-5) وخلايا البروستاتا السرطانية (PC3)، وذلك من خلال قياس إفراز الإنترليوكين-٦ الناتج عن ليبوبولي ساكاريد (lipopolysaccharide) البكتيري. في التجربة تم معالجة الخلايا بمستخلص النبات المائي ومستخلص الميثانول لمدة ٧٢ ساعة، ثم تم تعريض الخلايا لمركب البولي ساكاريد (lipopolysaccharide) البكتيري لمدة ٢٤ ساعة. لدراسة التأثير السمي للمستخلصات النباتية على الخلايا تم استخدام فحص التيترازوليوم (WST-1) الذائب بالماء. أظهرت النتائج أن مستخلصات النبات المائية والميثانولية لم تقلل من إفراز الإنترليوكين-٦ الناتج عن تأثير الليبوبولي ساكاريد (lipopolysaccharide) البكتيري في الخلايا الليفية غير السرطانية (MRC-5) وخلايا البروستاتا (PC3) السرطانية، ولكن أظهرت النتائج أن المستخلص المائي للنبات بتركيز ١٥,٦ مايكرومولر كان له تأثير خفيف وغير معنوي على تقليل إفراز الإنترليوكين-٦ في كل الخلايا المستعملة. أيضا أظهرت النتائج أن مستخلصات النبات المائية والميثانولية لم يكن لها أي أثر سمي في الخلايا التي تمت دراستها. ولم يُظهر نبات الأونوبوردوم جورديانيكولوم أية آثار مضادة للالتهابات في كل من الخلايا الليفية (MRC-5) غير السرطانية وخلايا البروستاتا (PC3) السرطانية.

الكلمات الدالة: المضادة للالتهابات، lipopolysaccharide، PC3، الخلايا الليفية MRC-5، نبات طبي.