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Cytotoxic Effect of *Datura stramonium* Extract on Cancer Cell lines

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Summary

In order to investigate the *in vitro* antitumor activity of *Datura stramonium* seeds on cancer cell lines. Extract of this plant was prepared by using different concentrations of seed extract 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75 and 5 mg/ ml. Cytotoxicity was estimated on mammary adenocarcinoma (AMN3), brain cancer, and normal rat embryonic fibroblast (Ref3) cell lines. The results exhibited that the extract has cytotoxic effect by decreasing the viability of AMN3 (42.91%) and brain cell lines (32.79%). However, it produced little effect on viability of normal cell line Ref3, indicating the specificity of this extract against malignant cells.

تأثير خلوي سمي لنبات الداتورة ستراموينيوم في الخلايا السرطانية

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***** المركز العراقي لبحوث السرطان والوراثة الطبيةالجامعة المستنصرية**

الخلاصة

لغرض الكشف عن فعالية بذور نبات الداتورة ستراموينيوم ضد الخلايا السرطانية, حضر مستخلص من هذا النبات.استعملت تراكيز مختلفة من المستخلص هي3, 3,25, 3.5, 3.75 ,4, 4.25 4.5, 4,75, 5 مكروكرام\مل ثم حسبت سمية المستخلص في الخلايا السرطانية (المتمثلة بسرطانة الغدة اللبنية AMN3 وسرطان الدماغ) وفي الخلايا الطبيعية (المتمثلة بخلايا الارومة الليفية لجنين الجرذ Ref3). . سبب المستخلص الاسيتوني انخفاضا في حيوية خلايا الغدةاللبنية بنسبة 42.9% وبنسبة 32% في خلايا سرطان الدماغ. اظهر المستخلص تاثيرا محدودا في الخلايا الطبيعية و مما يدل على الخاص في الخلايا السرطانية

Introduction

Cancer is a worldwide disease associated with high mortality rate. It is anticipated that in the next 5-10 years death from cancer will overtake other diseases (1). The World Health Organization estimated if unchecked, that cancer death could be rise to 15 million by 2020 (2). Today, cancer is considered the second cause of death after cardiovascular diseases in the world (1). Recent research have directed toward phytochemicals for cancer therapy (3, 4, 5). Plants are rich in variety of secondary metabolites as tannins, terpenoids, alkaloids, flavonoids, steroids, glycosides, volatile oils and lectins. It is necessary to identify the phytochemical components of local medicinal plants usually employed by herbalists in the treatment of diseases (6).

Datura stramonium is an erect branched shrub with long white flower and spiny spherical fruits, the use of the leaves and seeds locally in the treatment of ailments like common cold, headache and asthma. Recent research reported *D. stramonium* has antimicrobial activity against *Pseudomonous aurginosa, Klebsiella Pneumonia and E. coli* (6). The anti-tumor activity of *D. stramonium* agglutinin has been reported by (7). Therefore, because the presence of suitable condition for doing such studies and in order to screen the presence of anti tumor compounds in Iraqi plants, this project was designed to perform the following aim: Detect the activity of *D. stramonium* extracts on cell lines; mammary adenocarcinoma (AMN3), brain cancer, and rat embryonic fibroblast (Ref3) cell lines.

Materials and Methods

Cell Lines

AMN3 Cell Line

This cell line is a murine mammary adenocarcinoma cell line. It was provided by Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), and used at 110-115 passages.

Brain Cancer Cell Line

This cell line is brain tumor cell line; it was isolated by Dr. Ahmed Al-Shamery/ ICCMG, and kindly provided by him. It was used at passage 40-45.

Rat Embryo Fibroblast (REF) Cell Line

It is a normal cell line. It was prepared by Dr. Ahmad Al-Shamery/ ICCMG, and kindly supplied by him. The passage used in this study was 13.

Cell Line Handling

Maintenace of Cell Lines

Following the protocol was carried out (8), cell lines used in this study were sub-cultured when monolayer of each cell line was confluent. The growth medium was decanted off and the cell sheet washed twice with sterile PBS. Two to three ml of trypsin-versene was added to the side of the falcon opposite to the cells and the falcon rocked gently. Incubated for few (2-3) minutes at 37^{o}C until they had dettached from the falcon. Growth medium (15) ml was added and dispersed cell by pippetting with growth medium. Approximately half of this growth medium containing cell suspension was poured off to other falcon and re-incubated at 37^{o}C.

The color of the growth medium is pale orange in color and when the cells grow, the color will change to yellow. If there, is any turbidity in the media that means there is contamination and should be disposed off.

Cell line Preparation for Cytotoxicity Study

When cells in the incubated falcon became monolayer, the confluent monolayer was treated as in subculture described above. After trypsinization, twenty ml of growth medium was added and dispensed cells by pippetting in growth medium. Afterwards, the cell suspension (200µl) was seeded in each well of flat button 96 well micro titer plates. The plate was covered by its coverlid, sealed well by using parafilm, and incubated at 37C° for 24 hours. All steps were carried out under sterile conditions and at sterile place.

Preparation of Seed Extract Concentrations:-

Extract was prepared by using acetone for precipitation (9). Then a stock was prepared by dissolving 0.2 g of prepared extract powder in 10 ml PBS, and filtering with sterile (0.22µm) Millipore filter.

By using sterile serum free medium, different concentrations (dilutions) were prepared, calculating from the following equation [Concentration 1(C1) X Volume1 (V1) = Concentration 2 (C2) X Volume 2 (V2)].

Cell Lines Treated With Extracts

When the cells of micro- titer plates are in log phase, the medium was removed, and the cells were exposed to a range of seed extract concentrations. A pilot study was done to determine the most effective concentrations (the data not shawing). The concentrations used for treatment of tissue cultures were (3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, and 5) mg/ ml. Four replicates were used for each concentration. The middle and last columns were used as control (treated with Serum free media "SFM" only). Afterwards, the plates were incubated at 37^{o}C for the selected exposure times (24, 48 or 72) hours.

The AMN_3 and brain cancer cell lines were exposed for three different durations. However, Ref3 was exposed for only to 72 hours. At the end of recovery time, 150µl of crystal violate were add to each cultured well, the plates incubated in an incubator at 37^{o}C for 25 minutes. After incubation, the plates were washed with water and left to dry.

The optical density of each well was read by using a micro – ELISA reader at a transmitting wavelength of 492 nm (8).

The percentage of cell viability was estimated according the following equation produced by (10):

Percentage of Cell Viability =

*Optic Density of Treated Well*s */ Optic Density of Control Wells* $\times 100$

Results

Effect of Crude Extracts on Mammary Adenocarcinoma (AMN3) Cell line:

The effect of acetone extract of *D. stramonium* on mammary adenocarcinoma was time dependent, and it was not dose dependent (Table 1). The low and medium concentrations of this extract showed high viability of cells at 24 hours, these values were 89.75 $\pm$4.43, 84.9$\pm$24.25, 85.07$\pm$ 4.3, 85.9 $\pm$4.00, 86.88$\pm$ 4.34, and 95.8 $\pm$ 3.00% at 3, 3.25, 3.5, 3.75, 4, and 4.25 mg/ml, respectively. Then the viability of the cells decreased at 48 hours and the values were 84.39 $\pm$4.48, 57.46$\pm$ 3.6, 59.44 $\pm$3.8, 60$\pm$3.01, 61.53$\pm$3.4, and 65.01$\pm$3.20 % at 3, 3.25, 3.5, 3.75, 4, and 4.25 mg/ml, respectively. While the viability reached its lowest value at 72 hours and these values were 64.23 $\pm$3.38, 42.91$\pm$ 3.24, 54.24$\pm$ 3.3, 49.3$\pm$ 3.03, 43.89$\pm$ 3.46, and 51.0$\pm$2.00% at 3000, 3250, 3500, 3750, 4000, and 4250 µg/ml, respectively. Figure (1) illustrated the cytotoxic effect of this extract at 72 hours.

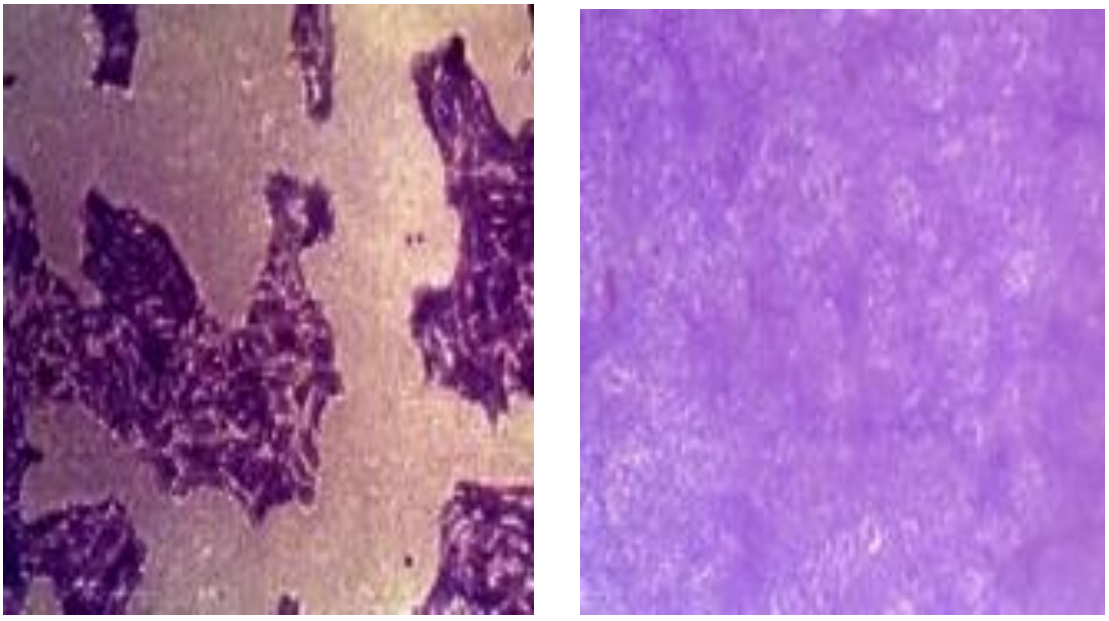
The highest concentrations (4.5, 4.75, and 5 mg/ml) showed a proliferative effect at 24 hours; 104.83 $\pm$ 4.48, 112.0$\pm$3.02 and 125.19$\pm$ 4.76 %, respectively. However, this effect regressed obviously at 48 hours; 69.16$\pm$3.9, 78.0$\pm$2.2 and 89.51 $\pm$ 4.44 %, respectively, and at 72 hours reached; 57.07$\pm$ 3.48, 63.0 $\pm$3.4 and 68.02$\pm$3.46 % at 4.5, 4.75 and 5 µg/ml, respectively.

The statistical analysis showed that there was significant difference at level P ≤ 0.01 among periods (24, 48, and 72 hours) and at level P ≤ 0.05 among concentrations at the same period.

Table (1): Cell viability (%) of AMN3 Cell Line after Exposure to the Acetone Extract of *D. stramonium* for 24, 48, and 72 Hours.

Capital letters mean there are significant differences among periods.
Small letters mean there are significant differences among concentrations

Concentration mg/ml	24 Hours	48 Hours	72 Hours	P- Value
3	^{a} 89.75±4.43 A	^{a} 84.39±4.45 A	^{b} 64.23±3.38 B	≤ 0.01
3.25	^{a} 84.92±4.25 A	^{a} 57.46±3.6 B	^{a} 42.91±3.24 C	≤ 0.01
3.5	^{a} 85.07±4.3 A	^{a} 59.44±3.8 B	^{ab} 54.24±3.3 B	≤ 0.01
3.75	^{a} 85.9±4.00 A	^{a} 60.0±3.01 B	^{a} 49.3±3.03 C	≤ 0.01
4	^{a} 86.88±4.34 A	^{a} 61.53±3.4 B	^{a} 43.89±3.46 C	≤ 0.01
4.25	^{a} 95.8±3.00 A	^{a} 65.01±3.20 B	^{a} 51.0±2.0	≤ 0.01
4.5	^{b}104.83±4.48 A	^{a} 69.16±3.9 B	^{ab} 57.07±3.48 C	≤ 0.01
4.75	^{bc} 112.0±3.02 A	^{b} 78.0±2.2 B	^{b} 63.0±3.4	≤ 0.01
5	^{c}125.19±4.76 A	^{b} 89.51±4.44 B	^{b} 68.02±3.46 C	≤ 0.01
P-Value	≤ 0.05	≤ 0.05	≤ 0.05	



(2) (1)

(Figure1) Effect of *D. stramonium* extract on AMN3 cell line1). Confluent monolayer (control) 2). After exposure to the acetone extract (4 mg/ml) for 72 hours. 100X.

Effect on Brain Cancer Cell Line (Giloma)

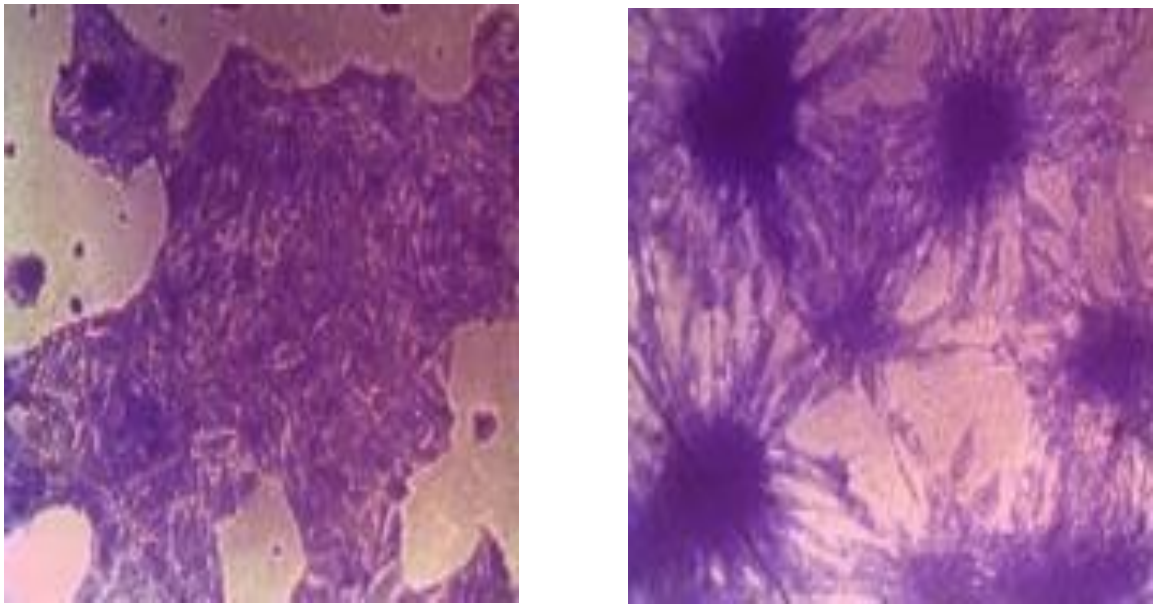
Table (2) showed low cytotoxic effect of the acetone extract at 24 hours in most concentrations used in the study. The values of cell viability which reflect the cytotoxic effect of extract were 80± 4.2, 82.33± 4.82, 88.67±4.17, 90.1± 3.0, 90.74 ± 4.2 and 91.67± 4.77% at 3.5, 3.75, 4, 4.25, 4.5, and 5 mg /ml. This effect progressed at 48 hours in all concentrations used, while at 72 hours exposure the cytotoxic effect regressed at low concentrations 3.5 and 3.75 mg/ml, but progressed at the rest concentrations 4, 4.25, 4.5, 4.75, and 5mg/ml. The values of viability at 72 hours exposure were; 82.76± 4.8, 90.91±5.1, 51.54± 4.6, 48.3 ± 1.3, 47.29±4.77, 32.79± 4.84, 41.93±3.98%, respectively. Figure (2) showed the reduction in cell growth due to exposure to this extract.

Statistical analysis showed that there were significant differences among periods at the level $P < 0.01$, while there were no significant differences among concentrations at 24 hours except at 4750 µg /ml, but at 48 and 72 hours there were significant differences among concentration at level $P \leq 0.05$.

Table (2): Cell Viability (%) of brain cancer Cell line After Exposure to the Acetone Extract of *D. stramonium* for 24, 48, and 72 Hours.

Concentration mg/ml	24 Hours	48 Hours	72 Hours	P- Value
3.5	^{b} 80±4.2 B	^{b} 64.011±4.8 A	^{a} 82.76±4.8 B	≤ 0.01
3.75	^{b} 82.33±4.82 B	^{b} 6493±4.76 A	^{a} 90.91±5.1 B	≤ 0.01
4	^{b} 88.67±4.17 B	^{a} 84.89±5.03 B	^{b} 51.54±4.6 A	≤ 0.01
4.25	^{b} 90.1±3.01 B	^{a} 70.0±2.00 B	^{bc} 48.3±1.3 A	≤ 0.01
4.5	^{b} 90.74±4.2 B	^{c} 54.67±4.78 A	^{bc} 47.29±4.76 A	≤ 0.01
4.75	^{a} 112.53±4.65 B	^{c} 53.84±4.65 B	^{d} 32.79±4.84 A	≤ 0.01
5	^{b} 9167±4.77 B	^{c} 46.42±4.55 A	^{Cd} 41.93±3.98 A	≤ 0.01
P-Value	≤ 0.05	≤ 0.05	≤ 0.05	

Capital letters mean there are significant differences among periods.
Small letters mean there are significant differences among concentrations



(2) (1)

Figure (2): Effect of *D. stramonium* extracts on brain cancer cell line.
1).Confluent monolayer (control) 2). After exposure to the acetone extract (4.75 mg/ml) for 72 hours. 100X.

Effect on Ref Cell Line:

This extract illustrated a proliferative effect at 3.25 and 3.5 mg /ml, while at the other concentrations (3, 3.75, 4, 4.25, 4.5, 4.75, 5 mg/ ml) showed low cytotoxic effect (77.8 ±4.3, 89.34 ± 5.52, 84.89 ±4.86, 71.87 ± 4.2, 72.07 ± 4.85, 56.56 ± 4.67, 80.33± 4.63% respectively) as shown in Table (3).

Statistical analysis showed there were significant differences among concentrations at level P ≤

Table (3): Cell Viability (%) of Ref Cell Line
After Exposure to Extract of *D.Stramonium* for 72 Hours

Concentration µg/ml	72 Hours
3	[bc] 77.88±4.3
3.25	[e] 110.11±5.2
3.5	[e] 110.11±5.22
3.75	[d] 89.34±5.52
4.	[cd] 84.89±4.86
4.25	[b] 71.87±4.2
4.5	[b] 72.07±4.85
4.75	[a] 56.56±4.67
5	[cd] 80.33±4.63
P-Value	≤ 0.05

Small letters mean there are significant differences among concentrations

Discussion:

The results showed that the acetone extract had cytotoxic effect above 50% on tumor cell lines; mammary adenocarcinoma (AMN3) and brain tumor (Table 1 and 2) and beyond 50% on normal cell line represented by Ref (Table 3). This means that this extract was more toxic on tumor cells than normal cells or may be *D. stramonium* lectin had selective action. Lectins have capacity to distinguish between different cells types, including malignant and normal cells (10). This action was probably resulted from affinity of lectin towards glycosyl group in the membranes of cells (11). This can be explained the fact that transformed cells had more glycosyl receptors in plasmatic membrane in comparison to normal cells (12), so that the transformed cells were frequently much more sensitive to cytotoxic effect of lectins than normal cells. On the other hand, lectin may act mainly on highly proliferative cells as differentiating agents do (13). Natural compounds and certain drugs can induce differentiation in cancer cells by acting on actively dividing cells but not on cells in the G_0 resting phase (13,14). This action of pure *D. Stramonium* lectin was reported by Sasaki *et al.* (7) on glioma, but this effect was not well demonstrated in this study, probably attributed to the type of cancer because some cancer cells are more easily induced to differentiate than others (13), and probably the pure lectin is more effective than crude.

At 24 hours, the higher concentrations induced cell proliferation, this phenomenon lead to suggest that presence of trace amounts of other phytochemicals and when these phytochemical reached certain concentrations, they may stimulate cell growth. Cytotoxicity of this extract appeared time dependent.

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