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Datura Aqueous Leaf Extract Enhances Cytotoxicity via Metabolic Oxidative Stress on Different Human Cancer Cells

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Abstract

This study was designed to evaluate the cytotoxic effect of aqueous *Datura stramonium* leaf extract on different human cancer cell lines *in vitro*. Breast (MDA-MB231), head, neck (FaDu), and lung (A549) cancer cell lines were treated with 1 mg/ mL of *Datura* aqueous extract for 24 and 48 hours. Exposure of MDA-MB231 and FaDu cells to the extract for 24 hours resulted in a significant decrease in cell survival. Same effect was seen with all cell lines exposed to the *Datura* aqueous extract for 48 hours. Treatment with *Datura* aqueous extract also caused perturbations in parameters indicative of oxidative stress, including increased glutathione disulfide (GSSG) in FaDu cells treated for 48 hours. Additionally, an increase on the redox sensitive enzymes was seen in MnSOD and HO-1 on A549 cells, treated with *Datura* aqueous extract for 24 and 48 hours. The results may suggest therapeutic potential of *Datura* aqueous leaf extract for the treatment of different types of cancer. Further investigations are needed to verify whether this cytotoxic effect occurs *in vivo*.

الملخص

صممت هذه الدراسة لتقيم مدى سمية المستخلص المائي لأوراق نبتة الداتورة استرامونيوم (*Datura stramonium*) على العديد من سلالات خلايا الإنسان السرطانية , لتحقيق هذا الهدف قمنا بتعريض خلايا سرطان الثدي (MDA-MB231), الرأس والرقبة (FaDu) وسرطان الرئة (A549) في المختبر لتركيز (1 مغم\مل) من مستخلص هذه النبتة لمدة 24 و48 ساعة.

اتضح من النتائج انه بعد تعرض خلايا MDA-MB231 وFaDu للمدة 24 ساعة انخفاض ملحوظ في عدد الخلايا الحية , وقد تم ملاحظة نفس التأثير على جميع أنواع الخلايا بعد تعرضها للمستخلص لمدة 48 ساعة.

وأسفر معالجة الخلايا السرطانية بهذا المستخلص باضطرابات في معايير جهد الأكسدة بما في ذلك زيادة نسبة الجلوتاثيون المؤكسد (glutathione disulfide (GSSG)) , بالإضافة إلى زيادة إنزيمات جهد الأكسدة مثل MnSOD, HO-1 .

تشير هذه النتائج إلى أنه يمكن إستخدام المستخلص المائي لأوراق نبتة الداتورة استرامونيوم (*stramonium Datura*) لعلاج أنواع مختلفة من السرطان في الإنسان بعد إجراء المزيد من التجارب لمعرفة حيثية عمل هذا المركب في الإنسان .

Keywords: Datura Stramonium; Glutathione; Mnsod; HO-1.

1. Introduction

1.1. Plant

Datura stramonium, more commonly known as jimson weed or thorn apple, is a wild-growing flowering plant belonging to the family Solanaceae and is a medicinal plant with antinociceptive (Abdollahi et al., 2003) antioxidant (Couladis et al., 2003), hypolipidemic (Rasekh et al., 2001), anti-inflammatory, anti-rheumatoid (Tariq et al., 1989), and hypoglycemic (Gharaibeh et al., 1988) properties. Therefore, this study was carried out to evaluate the therapeutic potential of the aqueous *Datura stramonium* leaf extract in the treatment of different types of cancer.

1.2. Oxidative Stress

Mammalian cells continuously produce reactive oxygen species (ROS) through various metabolic pathways. Reactive oxygen species are molecules that contain oxygen and have higher reactivity than ground-state molecular oxygen. These species include not only the oxygen radicals (like $O_2^{\bullet-}$, $^{\bullet}OH$, and peroxyl radicals), but also non-radical molecules such as H_2O_2 and $^1O_2$. Superoxide is formed during the reduction of O_2 by the mitochondrial electron transport system (Boveris and Cadenas, 1982). Eukaryotic cells are equipped with an antioxidant system capable of converting ROS to H_2O *via* different cytosolic enzymes. Oxidative stress results when the balance between the production of ROS exceeds the antioxidant capability of the target cell. It is generally thought that low levels of ROS are not harmful to cells, and indeed even perform useful signaling functions, whereas high levels of ROS are detrimental through covalent reactions with cellular proteins, lipids, and DNA that results in altered target molecule function. The accumulation of oxidative damage has been implicated in

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both acute and chronic cell injury, including possible participation in the formation of cancer. Acute oxidative injury may produce selective cell death or sublethal injury, such as mutations, chromosomal aberrations or carcinogenesis (McCord *et al*., 1971; Klaunig *et al*., 1998). In contrast, chronic oxidative injury may lead to a non-lethal modification of normal cellular growth control mechanisms. Cellular oxidative stress may modify intracellular communication, protein kinase activity, membrane structure and function, and gene expression, and it may result in modulation of cell growth (Klaunig *et al*., 1998).

Cells are protected against oxidative stress by different intracellular antioxidant compounds, mainly Glutathione (GSH) and thioredoxin, and by other antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1) (Tsan, 1989; Guo *et al*., 2001).These antioxidant enzymes were shown to be up-regulated by various physical, chemical, and biological agents and oxidative stress (Tsan, 1989, Wong *et al*., 1989; Bianchi *et al*., 2002).

Little information is available on the antioxidant or pro-oxidant properties of the herbal preparations of *Datura stramonium.* The purpose of this study was to evaluate the therapeutic potential of aqueous leaf extract of this plant in the treatment of cancer *in vitro.*

2. Material and Methods

2.1. Cell Culture

Breast (MDA-MB231) cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal bovine serum, and head, neck (FaDu), and lung (A549) cancer cell lines were routinely kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. All cells were obtained from the European Collection of Cell Cultures (ECACC) and were kept at 37 °C in a humidified 5% CO_2 incubator.

2.2. Preparations of Extract

Datura stramonium, a wild-growing flowering plant belongs to the family Solanaceae, was collected during the flowering period in August 2005 in Jordan. The leaves were separated and dried in the shade in green house for several days; and was deposited in the Herbarium of the Department of Biology at the Hashemite University. The procedure was as follows**:** Leaf part of *Datura stramonium* (150g) of dried plant was ground and the obtained powder was mixed with 1 L of boiling distilled water for 1 hour. The obtained mixture was filtered twice through a funnel by using suction pump. Water was concentrated under vacuum by using a rotary evaporator at a temperature of 50°C. The extract was evaporated under a reduced pressure till it dried by using a lyophilizer (or by using fume hood). The extract was stored in glass flasks to protect them from humidity and light. 1 mg/ mL of the extract was prepared by dilution of the stock with sterile phosphate-buffered saline (PBS) solution.

2.3. Cell Survival Experiments

Cells were plated in 60 mm tissue culture dishes at low density (300 per dish) and grown for 3 days in the presence of antibiotics (Gentamycin). At the beginning of each experiment, the cells were placed in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum. Control cultures were treated identically. Cells were then treated with *Datura* aqueous extract (1 mg/mL). Cultures were then placed in an incubator. At each time point (24 and 48 hours), cells were trypsinized, counted, diluted, and plated at low density (300-1000 per plate) for clonogenic cell survival assay as previously described (Spitz *et al*., 1990). Surviving colonies were fixed and stained with Coomassie Blue stain after 14 days of incubation; and were counted under a dissecting microscope. Colonies containing 50 cells or more were scored.

2.4. Measurement of Glutathione Levels

The intracellular levels of reduced glutathione (GSH) and GSSG in cancer cells were measured. Total glutathione content was determined according to (Anderson, 1985). The total intracellular GSH was determined by the colorimetric reaction of DTNB (5, 5-dithio-*bis*- (2-nitrobenzoic acid)) with GSH to form TNB (5-thio-2-nitrobenzoic acid). The rate of formation of TNB, which is proportional to the total GSH concentration (GSH + GSSG), was measured spectrophotometrically at 412 nm. Cellular GSSG is reduced to GSH by glutathione reductase (GR), using NADPH as a cofactor. Briefly, cell pellet was lysed in 5 % 5-sulfosalicylic acid (SSA); the total GSH was measured by mixing 50 µL sample with 100 µL water, 700 µL working buffer [0.298 mM NADPH in stock solution (0.143 M sodium phosphate, 6.3 mM EDTA)], and 100 µL DTNB (6 mM DTNB in stock solution). The assay was initiated by the addition of 50 µL GR (266 U/mL), and the rate of TNB formation was followed spectrophotometrically at 412 nm, every 15 seconds for 2.5 min. The total GSH of a sample was extrapolated from a standard curve of glutathione concentration as a function of the change in absorbance over time. The cellular GSSG level was determined using the same DTNB assay when the reduced GSH is masked by 2-vinylpyridine (2-VP) (Griffith, 1980). 2-VP (2 µL of a 50% solution in EtOH per 50 µL aliquot of media) was added to 30 µL of sample for 1.5 h to block all reduced GSH, and then 30 µL SSA were added, and this was subjected to DTNB assay as described for total GSH. GSH was determined by subtracting the GSSG content from the total GSH content. All biochemical determinations were normalized to the protein content using the Bradford method (Bradford, 1976).

2.5. Western Blotting Analysis

Cell lines were grown to near confluence, washed with ice-cold PBS, and then collected by scraping and centrifugation. Cells were lysed by sonication in 10 mM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride (PMSF). The cell lysates were then mixed with one volume of 2X sample buffer containing 6% SDS and 10% mercaptoethanol, denatured by heating to 95ºC for 5 min, and separated on 3% acrylamide stacking, and 12% Laemmli running gels for SDS polyacrylamide gel electrophoresis. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (Bio Rad, Hercules, CA). The membrane was blocked with 5% skim milk in TBST (Tris

buffered saline with 0.1% Tween) for 1 hr, and then incubated with the primary antibody for 1–2 hr. The blot was washed with TBST and incubated with secondary antibody (horseradish peroxidase-conjugated anti-IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The immuno-reactive protein was detected using an enhanced chemiluminescence (ELC) detection kit (Amersham Pharmacia Biotech, USA). Primary antibodies were anti-Heme Oxygenase-1 (Stressgen Biotech Serologies, USA), and Rabbit anti-MnSOD (kind gifts from Dr. Larry Oberley, University of Iowa, USA).

2.6. Statistical Analysis

All results are expressed as mean ± 1 standard deviation (S.D). Student's t test was employed ($p<0.05$) for two groups analysis.

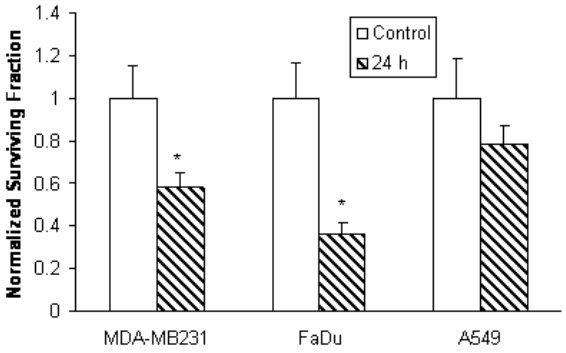


Figure 1. Clonogenic cell survival showed increased susceptibility of human cancer cells (MDA-MB231 and FaDu) to *Datura* aqueous leaf extract -induced cytotoxicity.
Cells were grown for 24 hours in RPMI (MDA-MB231) and DMEM (FaDu and A549) medium in the presence of 1mg/mL *Datura* aqueous leaf extract. Error bars represent ±1 SD of the mean of N=3 experiments performed with at least three cloning dishes taken from one treatment dish. Asterisks indicate significant differences between treated group and their prospective control ($p<0.05$, t-test, n = 3). Data were normalized to sham-treated cultures from each cell line.

3. Results

3.1. Datura Aqueous Leaf Extract -Induced Cytotoxicity and Oxidative Stress in Human Cancer Cell Lines

A variety of human cancer cells derived from breast (MDA-MB231), head and neck (FaDu), and lung (A549) human cancer cell lines were exposed for 24 and 48 hours to *Datura* aqueous leaf extract and clonogenic cell survival as well as parameters indicative of oxidative stress were assayed. MDA-MB231 and FaDu cells significantly enhanced ($p<0.05$) clonogenic cell killing following 24 hours exposure to the extract, relative to each respective control (Figure 1). However, A549 cells were found to be resistant to cell killing induced by exposure to the extract for 24 hours. Some variability in responses between the cell lines was also noted with MDA-MB231 showing 40% killing, and FaDu showing 65% cell killing during 24 hours of *Datura* aqueous leaf extract exposure (Figure 1). Exposure of these cells to the extract for 48 hours showed that all cancer cell lines were sensitive to cell killing induced by *Datura* aqueous leaf extract exposure with some variability ($p<0.05$) (Figure 2). MDA-MB231 showing 61% killing, FaDu showing 63% cell killing, and A549 showing 22% cell killing during 48 hours of *Datura* aqueous leaf extract exposure. Figure 3 shows the results of the glutathione analysis done on co-cultures obtained from the same experiments, shown in Figure 1 and 2. Glutathione is a major intracellular redox buffer such that the ratio of GSH to GSSG can be used as a reflection of intracellular redox status (Schafer *et al*., 2001). Exposure of FaDu cells for 24 and 48 hours to *Datura* aqueous leaf extract caused a ~2-fold increase in total GSH and GSSG as well, whereas a minimal change was seen in MDA-MB231 exposed to *Datura* aqueous leaf extract for 24 hours. However, A549 cells exposure to *Datura* aqueous extract for 24 or 48 hours did not seem to significantly alter GSSG level (Figure 3 A, B).

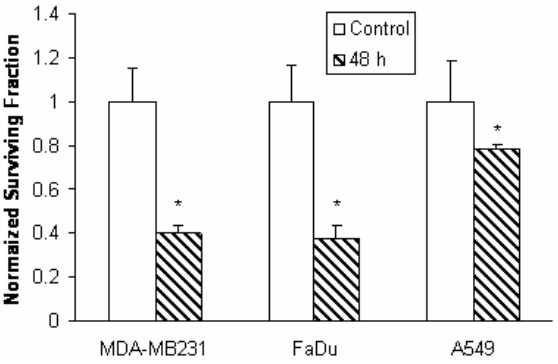


Figure 2. Clonogenic cell survival showed increased susceptibility of human cancer cells (MDA-MB231, FaDu and A549) to *Datura* aqueous leaf extract -induced cytotoxicity.
Cells were grown for 48 hours in medium in the presence of 1mg/mL *Datura* aqueous leaf extract. For more details, see legend of Figure 1.

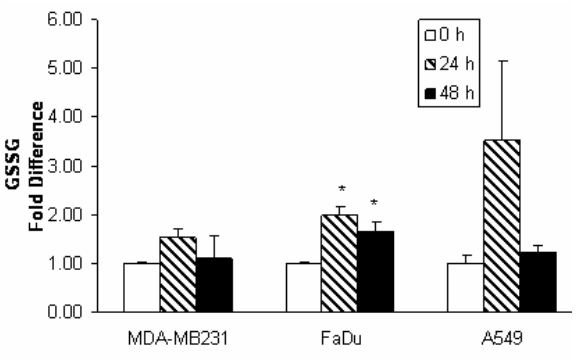
3.2. Datura Aqueous Leaf Extracts Activity and Antioxidants Levels

The protein level of antioxidant enzymes MnSOD and HO-1 was determined by immunoblotting. As shown in Figure 4, there was a significant ($P<0.05$) increase in the protein expression of MnSOD and HO-1 in A549 cells exposed to 1mg/mL of *Datura* aqueous leaf extract for 24 & 48 hours, while no changes were seen in MDA-MB231 and FaDu cells. These results explain the resistance of A549 cells to cell killing–induced by the *Datura* aqueous leaf extract for 24 hours and the minimal toxicity seen for 48 hours.

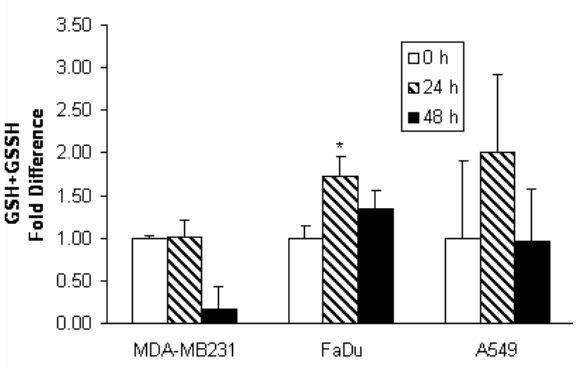
4. Discussion

It has been reported that all parts of the plant *Datura stramonium* are poisonous if ingested by humans or livestock (Radford et al., 1964). However, it could be used for medicinal purposes (King 1984, Mann 1992). The effect of *Datura* extracts on the oxidative stress has not been studied well. In this study, we investigated the toxic effect of *Datura stramonium* aqueous leaf extract on different cancer cells and how this exposure might affect the oxidant/antioxidant status of the cells.

Using clonogenic assay, and after incubation for 24 hours, both MDA-MB231 and FaDu cells showed significantly reduced growth ($P<0.05$) when compared to



(a)



(b)

Figure 3. Effect of *Datura* aqueous leaf extract on total GSH (a) and GSSG levels (b) in human cancer cells (MDA-MB231, FaDu, and A549). Cells were treated with 1 mg/mL *Datura* aqueous leaf extract for 24 and 48 hours. Cells were then harvested for glutathione analysis, using the spectrophotometric recycling assay. For more details, see legend of Figure 1.

control. The situation was different with A549 cells (Figure 1). The killing ability of the *Datura* aqueous leaf extract could be attributed to the imbalance of the internal oxidant/antioxidant capability of the cells caused by this exposure. This can be seen clearly by the level of GSSG changes in FaDu cells. Taken together, the data in Figure 1 and 2 suggest that the cytotoxic effect of the extract in FaDu cells was mediated by disruptions in thiol metabolism consistent with oxidative stress. Although, the extract exposure in MDA-MB231 induced significant cytotoxicity, the level of GSSG was not highly up-regulated. This indicates that the extract might have induced different mechanism of cytotoxicity (pro-apoptotic characteristics). Further studies are still to be done. The higher levels of GSH in A549 cells could be protecting cells from oxidative stress induced by *Datura* aqueous leaf extract exposure for 24 hours (Figure 3).

Upon 48 hours exposure, the picture was different. The level of oxidative stress induced by exposure conditions was causing more killing in the three cell lines. Again looking at the GSH levels, it is clear that exposure to the extract induced GSH response to lesser extent this time. The inability of cells to induce more GSH production could be due to the toxic effect of the *Datura* aqueous leaf extract.

GSH and GSSG are the major redox pair involved in cellular redox homeostasis. A change in the cellular GSH or GSSG is regarded as a representative marker for oxidative stress; and is directly responsible for the

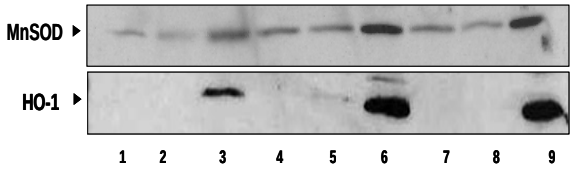


Figure 4. *Datura* aqueous extract produced an increase in MnSOD and HO-1 protein in A549 cells. The effect of *Datura* aqueous leaf extract on the redox sensitive enzymes MnSOD and HO-1 for 24 h and 48 hours, using immunoblot determination technique in MDA-MB231, FaDu and A549 cells. Lane 1, MDA-MB231 control; Lane 2, FaDu cells control; Lane 3, A549 cells control; Lane 4, 5, and 6, MDA-MB231, FaDu and A549 cells treated for 24 hours with the extract respectively; Lane 7, 8, and 9, MDA-MB231, FaDu and A549 cells treated for 48 hours with the extract, respectively.

perturbation of cellular function (Schafer *et al*., 2001). This includes activation of antioxidant defense pathways, as well as induction of cytotoxic responses.

Our results above motivsted us to study the expression of certain antioxidant enzymes such as: MnSOD and HO-1. As we can see the levels of MnSOD or HO-1 were not changed upon exposure to the *Datura* aqueous leaf extract in both MDA-MB231 and FaDu cells, whereas A549 cells showed clear up-regulation on both 24 and 48 hours exposure (Figure 4). This interesting result shows that different cancer cells have different inherent response to oxidative stress. This response will affect the ability of different cancer cells to respond to compounds and chemicals that can induce oxidative stress. Studying signal pathways, involved in different activation processes, could evolve and explain the different responses seen.

In this study, we have demonstrated that *Datura stramonium* aqueous leaf extract induced oxidative stress in different human cancer cell lines. In response, these cells exhibit up-regulating the expression of certain antioxidant compounds and enzymes such as: GSH, HO-1 and SOD. Further studies are still needed to explore the effect of *Datura* aqueous leaf extract on the signaling pathways involved.

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