

In vitro evaluations of cytotoxicity of *Crozophora tinctoria* (Ghbeira) and antidote effects of *Silybum marianum* (Khurfeish) applied aspects for grazing in Palestine

تقييم سمية نبات الغبيرة للخلايا الكبدية وتقييم قدرة نبات الخرفيش كمضاد للتسمم (بحث تطبيقي لحيوانات الرعي في فلسطين)

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Abstract

This is the first study of evaluation of toxicity of some plants in the Jenin area in Palestine, where farmers report suffering of their grazing animals from some plants. This plays a negative role in driving them out of production. This kind of study is, therefore, of vital economical, ecological and even political importance. Based on a survey in the villages of the Jenin area, we focus our study on a list of suspected plants and started with *Crozophora tinctoria* (Ghbeira). The objective is to assess the toxicity of this plant on grazing animals and to produce recommendations for farmers in case of intoxications. The study model is the hepatic cell line HepG2. *Crozophora tinctoria* plants were extracted using ethanol 50%. A series of concentrations were added to HepG2 and the effect was evaluated using the MTT assay, which assesses the viability of cells. A clear toxic effect was found to be directly

proportional to the concentration of the plant extract in HepG2 medium. *Silybum marianum* (Khurfeish), when added alone to HepG2 cells has a neutral effect on the growth of cells. According to literature, *Silybum marianum* has regenerative effect on cells and consequently antidotal virtues. This can be promising in case of intoxication. But, surprisingly, no such regenerative effect was found when added in combination with *Crozophora tinctoria*.

Key words: Toxicity, grazing animals, *Crozophora tinctoria* (Ghbeira), *Silybum marianum* (Khurfeish), antidote.

ملخص

هذه هي الدراسة الأولى لتقدير سُمية بعض النباتات في فلسطين أجريتها في منطقة جنين، إذ يعاني مربي الماشية من تسمم حيواناتهم، مما يؤدي للأسف إلى إخراجهم من دورهم كمنتجين. وبالتالي هذه الدراسة لها أهمية اقتصادية وبيئية ولا تخفى كذلك الأهمية السياسية في المحافظة على الأرض والإنتاج الحيواني. بناء على استطلاع لمنطقة جنين فقد بدأنا بدراسة سُمية بعض النباتات بالنسبة لحيوانات الرعي مبتدئين بالغبيرة بهدف عمل توصيات للمزارعين لتجنب السُمية وللتعامل مع الحيوانات في حال التسمم. تتم الدراسة بإضافة تراكيز مختلفة من نبات الغبيرة المستخلص بواسطة الكحول والماء إلى سلالة خلايا كبدية، ثم فحص مدى السُمية عن طريق عد الخلايا الحية المتبقية بواسطة فحص MTT. أسفرت التجارب عن أثر واضح لسُمية نبات الغبيرة على الخلايا الحية. وهذا الأثر يتناسب طردياً مع تركيز مستخلص نبات الغبيرة. وفي محاولة للبحث عن نبات مضاد للتسمم، فقد أضفنا مستخلص نبات الخرفيش إلى الخلايا، إذ نُشرت عن هذا النبات خصائص كمجدد لنمو الخلايا. وبالتالي أضفنا مستخلص نبات الخرفيش لسلالة الخلايا الكبدية، فلم يظهر أثر معنوي يذكر تنشيطاً أو تثبيطاً. ولكن وبالعكس ما كان متوقعا، فعند إضافة مستخلص نبات الخرفيش مع مستخلص نبات الغبيرة، لم يتم الكشف عن قدرة تجديدية لنمو الخلايا.

Introduction

The Palestinian Territories suffer severely from lack of natural resources including the most vital of resources: land. Efficient management of the rudimentary areas available is, therefore, of capital importance. In the Palestinian Territories, the total rangeland area is about 218,000 ha, mainly situated in the eastern slopes. However, due to the Israeli occupation, only 70,000 ha is accessible to Palestinians (Braighith A., 1998). At the same time that the vertical agricultural

expansion is important for the local developing agriculture, one has to benefit maximally from the available range areas for more than one reason: the traditional dependence on range grazing in our territories, the weak and non-supported economic situation of our farmers, and the adaptation of our local farm animal varieties to open field grazing rather than closed-door farms. These reasons are added to the political importance of realizing the value of the remaining range areas that will motivate our own as well as international support to keep and protect this land against confiscation and settlement building.

A severe deterioration takes place in Palestinian ranges and is caused by rainfall variation, bad management of grazing and poverty leading to use of trees and shrubs as domestic fuel source. Furthermore, abandoning ranges due to economical and political reasons during the long years of occupation has played a role in the deterioration of ranges. All these factors led to vegetation damage, decrease in plant productivity, and an increase in poisonous and unpalatable plants and, consequently, severe soil erosion. That results in desertification of the Palestinian land (Mohammad A., 2005).

An efficient use of range areas necessitates recognition and characterization of the toxic plants causing physiological problems, pains, diseases especially in digestive and nervous systems, fetus malformation, abortion and even death for farm animals. All these diverse losses caused by toxic plants lead to a decrease in animal production and even the plant production, as the toxic plants present a competition effect on the non-toxic ones. Consequently, an efficient grazing management of ranges containing toxic plants (not to mention elimination of toxic plants for their evident importance in the natural equilibrium of the local flora and fauna) is an important factor in decreasing the evacuation of Palestinian land which has been for the benefit of working in Israeli colonies and factories.

Our present knowledge of this field can be described as popular, based on empirical and, in many cases, non-objective observations. We can cite here the example of *Cichorium pumilum* flowers, which are “accused” by farmers in Jenin region (North of Palestinian Territories) to

be toxic for both goats and sheep. Some farmers, however, report that the so-called “*Cichorium pumilum* flowers syndrome” affects even animals that never graze outdoors (in ranges)! This citation demonstrates the need for objective investigations in this matter.

In addition to plants toxic to grazing animals in ranges, we have to note also the importance of the evaluation of the toxicity of many cultivated plants from which wastes and by-products are used to feed animals at least in certain seasons. Many examples can be given in this respect, such as tomato, cabbage, potato and cauliflower. The problem of toxicity is aggravated during drought due to reduced grazing selectivity.

In general, plants considered poisonous to humans are considered to be poisonous to animals. The degree of toxicity for humans has been classified from 1 (non toxic) to 4 (major toxicity), 5 (dermatitis), 6 (possibly toxic), and 7 (animal toxicity). However, there have been specific cases where animals have been poisoned by plants considered safe to humans. Also, we should keep in mind that animals tend to eat larger amounts of plant materials than humans and that may account for the problems seen in animals. Furthermore, animals are less selective to plant parts than humans, which might expose them more to toxicity (Owen W. R. and Collins A., 2003).

Many people assume all plants that contain toxins cause a higher risk of death or decrease production by impairing an animal’s physiology. In reality, the number of toxic plants eaten by herbivores and causing overt signs of poisoning is limited, fortunately. Rather, toxins cause herbivores to limit their intake of plants and play, therefore, a role in regulating the intake of many “mildly poisonous plants” (Launchbaugh K. (2001). At high concentrations, most toxins cause plants to be unpalatable, fortunately again! But, unfortunately, toxins at low concentrations do not render a plant unpalatable. Whether a herbivore will eat a toxic plant depends on several factors. Herbivores are less likely to eat a toxic plant if it is low in nutrients or contains high levels of acutely toxic compounds. They are more likely to eat toxic plants high in nutrients when they contain low concentrations of compounds that are not acutely toxic. For example, lambs offered unlimited access to alfalfa pellets will

consume grain laced with toxins, because the grain provides needed energy and variety in their diet (Abu Rmeileh B., 2000). However, well-fed lambs will only ingest limited amounts of toxins. On the other hand, when herbivores have no other foods to eat, they may be forced to eat plants high in toxins. Hungry animals will often over-ingest toxic plants and die rather than starve. Intake of nutritious plants high in toxins is typically cyclical. Herbivores gradually increase intake of nutritious toxic plant over several days. When intake exceeds the toxin satiation threshold, intake of food declines for a few days, then gradually increases due to the positive post-ingestive consequences animals experience from nutrients in the plant.

Crozophora tinctoria belongs to the family Euphorbiaceae. Tinctoria means the sap used for dyeing or has a sap which can stain. This plant is doubtfully poisonous. The ash-gray green appearance is caused by the dense cover of white, wood-like (tomentose) hairs. Undoubtedly, the most conspicuous parts of the plant are its fruits. When mature, the fruit darkens to dark green color. It bursts upon twisting its walls, sending the seeds to a considerable distance away.

This plant is mainly found in the Mediterranean region and Central/South Asia. It is said that edible red and blue dyes are obtained from the flowers, fruits and sap. Medical properties are transmitted in the folk medicine such as considering it as a vomiting agent, lowering body temperature to alleviate fever and using it to control and treat warts. The US Food and Drugs Administration reports indicate that the plant has toxic properties to some farm animals and even to man and should be considered as an unpalatable poisonous plant. Toxicity evidence on this plant, however, needs more research work. The objective of this study is, therefore, to evaluate the toxicity of *Crozophora tinctoria* as well as the antidotal virtues of *Silybum marianum* using the hepatic cell line HepG2. This should guide a future research *in vivo* and might provide our farmers with a free antidote in case of intoxication.

Materials and methods

Plants were collected from different locations in the north of Palestinian Territories, especially in Jenin area, and were pooled for extraction.

Preparation of Plant Extracts

The plants were dried in shadow at room temperature and the leaves were taken for extraction. In order to extract the organic as well as the non organic extracts, ethanol (50% water) was added to leaves which were finely pulverized using a kitchen blender. This mixture was then boiled for 15 minutes while stirring. The filtrate was then taken and freeze-dried and diluted in a Phosphate Buffered Saline (PBS) buffer and preserved at -20°C . The concentration is described as weight of plant dry matter (μg) in the medium volume unit (ml), where cells were grown.

Cell Culture

HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes, including the expression of P450 isoenzymes (Medina-Diaz et al., 2006) thus permitting long-term studies to be performed. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose content (4.5 g/L) supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 10 mg/ml streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C . The medium of cells from both cell lines was changed twice a week. At 70–80% confluence, cells were trypsinized and seeded in 96-well plates in cell density of 1.5×10^4 HepG2 cells. Twenty-four hours after cell seeding, cells were exposed to various concentrations of the plant extracts in fresh serum-free medium.

We present in this report the profiles of viability of cells under the effect of plant extracts using suitable controls: HepG2 cultures without any plant treatment for the experiments of toxicity of plants on cells.

Hepatic cells treated with toxic plant extract will also be treated with *Silybum marianum* extract in order to evaluate its antidote and liver regeneration capacities of *Silybum marianum*. As a control, *Silybum marianum* was added alone and in combination with the toxic plants extract.

For the "antidotal" experiments using *Silybum marianum*, this plant was added to HepG2 in parallel to the toxic plant, the controls were HepG2 without any plant extract, HepG2 with *Silybum marianum*, and HepG2 with the plant extract. The concentrations of each plant were 64,000 µg of plant DM/ ml of medium.

Silymarin (SM) is the commercial name for a group of active substances extracted from *Silybum marianum*. SM includes silichristin, silidianin A, silibin B, isosilibin A and isosilibin B. It was purchased from SigmaTM. SM was used as control for *Silybum marianum* extracted in the laboratory.

MTT Assay

MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] standard colorimetric assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue or purple formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multi-well scanning spectrophotometer (ELISA reader)

Statistical Analysis

Error limits cited and error bars plotted represent simple standard deviations of the mean. Usually, numerical results are only accurate

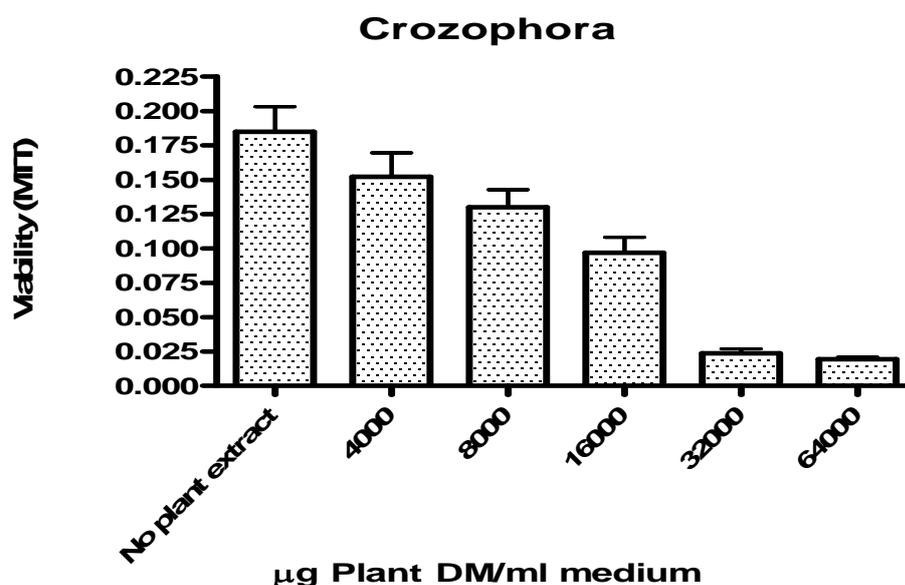


Figure (1): A clear effect of toxicity of *Crozophora tinctoria* is demonstrated on HepG2 cell line. The effect is directly proportional to the concentration of the *Crozophora tinctoria*.

Silybum marianum has antidotal and liver regeneration virtues (Luper S., 1998; Wagner H., 1981; Desplaces A., 1975; Anonymous, 1999) and could, therefore, be as an antidotal herb for intoxicated animals. So hepatic cells treated with toxic plant extract are also treated with *marianum* extract in order to evaluate its antidotal and liver regeneration capacities. As control, *marianum* was added alone and in combination with the toxic plant extracts.

Marianum, when added alone to HepG2 gave a level of viability parallel to that obtained without any plant extract (Figure 2: *Crozophora*+*Silybum*). The commonly known virtues of *marianum* as a regenerative agent could not be demonstrated in HepG2 cells. This can be due to the detoxification virtue of the hepatic cells. Possibly, in this system, no more detoxification effects could be detected by *Silybum*

Conclusion

Despite the existence of many works on toxicity of plants, many local plants suspected to be toxic for animals need to be assessed for their toxicity on grazing animals. This demonstrates the importance of the present proposed research work for the Jenin area in the Palestinian Territories.

Evaluation of toxicity is of vital importance for better grazing range management, for instance, by giving additional rest to plants during drought. Good grazing management helps to maintain an alternative forage choice over toxic plants. A good practice is not to release hungry animals into pastures known to have toxic plants, especially when toxic plants are the only green forage available. Prevention of livestock poisoning on rangelands is easier to accomplish than curing poisoned animals. It is extremely important to know where, when and how to control toxic plants, especially during drought.

This work demonstrates the toxicity of plants for the first time in Palestine. More studies are needed on the cellular level as well as on small animals and then farm animals in the final stage.

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