Assessment of the Cyto-toxicity of Bethlehem Star (*Ornithogalum umbellatum*) to HepG2 Cell Line and Antidotal Virtues of Milk Thistle (*Silybum marianum*)

تقييمُ سُمِيَّةِ نباتِ نجمة بيتَ لحم (*Ornithogalum umbellatum*) للخلايا الكبدية HepG2 وتقييمُ القدرة المضادة للسُّمية في نبات الخرفيش (*Silybum marianum*)

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Received: (31/7/2011), Accepted: (12/2/2012)

Abstract

Intoxication of animals in Palestinian ranges represents a major problem and needs an objective assessment for prevention as well as treatment. Bethlehem Star plant (*Ornithogalum umbellatum*) is assayed in this study for toxicity to HepG2 hepatic cell line using the cellular viability test, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Among the several doses applied, toxicity is demonstrated to start at around 0.125 mg of the plant extract/ml of HepG2 culture medium. Toxicity increases in a directly-proportional manner as the plant dose increases (up to 4 mg/ml). Astonishingly, Milk Thistle (*Silybum marianum*) classically reviewed and used as an antidote plant is demonstrated in this paper to manifest a toxicity profile parallel to that of Bethlehem Star (*Ornithogalum umbellatum*). Milk Thistle shows 50% viability of HepG2 cells at an approximate dose of 1.5 mg of plant extract/ml of HepG2 cell line culture medium. This concentration is applied in parallel to 4 mg of Bethlehem Star (*Ornithogalum umbellatum*)/ml of HepG2 cell line medium in order to look for antidotal virtues of Milk Thistle. At these concentrations, the antidotal virtues of

Milk Thistle cannot be demonstrated. The used plant concentrations of Bethlehem Star (*Ornithogalum umbellatum*) showed a toxicity profile similar to that of Milk Thistle (*Silybum marianum*). The later plant, surprisingly, failed to manifest antidotal virtues. However, this study can be used for future investigations of adjusting the appropriate doses of Milk Thistle to be used in case of intoxication of animals and possibly humans.

Key words: Toxicity, grazing animals, Bethlehem Star *(Ornithogalum umbellatum*), antidote effects of Milk Thistle (*Silybum marianum)*

ملخص

يُشكل تسمم حيوانات الرعي في المراعي الفلسطينيةمشكلة كبيرة مما يتطلب تقييما موضوعيا بهدف الوقاية والعلاج. يتناول هذا البحث تقييمَ سُميَّة نبتة نجمة بيت لحم (*Ornithogalum umbellatum*) للخلايا الكبدية HepG2 باستخدام فحص الMTT -3] (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] الذي يقيس حيوية الخلايا. من بين تراكيز عديدة وُجد أن السُّمية تبدأ على ما يقارب ٠.١٢٥ ملغرام من مستخلص النبات/مللتر من الوسط المغذي للخلايا الكبدية HepG2 وتزداد السمية كلما ازداد تركيز المستخلص النباتي (حتى تصل أقصى حد لها في هذا البحث على تركيز ٤ ملغم/مل). إن الدراسات والاستخدامات التقليدية للخرفيش (*Silybum marianum*) تشير إلى قدرته المضادة للسُّمية، إلا أنه من اللافت للنظر في هذا البحث أن الخرفيش يُظهر نمطاً سُمياً للخلايا الكبدية HepG2 يُشبه النمط الذي يُظهره نباتُ نجمة بيت لحم (*Ornithogalum umbellatum*). كما يُظهر نبات الخرفيش حيوية لخلايا HepG2 بنسبة ٥٠% على تركيز ١.٥ ملغرام تقريباً من مستخلص النبات/مللتر من الوسط المغذي للخلايا الكبدية HepG2 . وقد استخدم هذا التركيز بالتزامن مع نبات نجمة بيت لحم بتركيز ٤ ملغم/ مللتر من الوسط المغذي للخلايا الكبدية HepG2 من أجل الكشف عن مقاومة السّمية لنبات الخرفيش التي لم تظهر باستخدام التراكيز المذكورة. لقد أظهر نباتُ نجمة بيت لحم (*Ornithogalum umbellatum*) على التراكيز المستخدمة في هذا البحث نمطا للسُّمية يشابه نبات الخرفيش (*Silybum marianum*) وقد فشل هذا الأخير في إظهار مُضاديته للسُّمية على التراكيز المستخدمة إلا أنه يمكن الاستفادة من نتائج هذا البحث في أبحاث أخرى من أجل ضبط الجرعة الأمثل من نبات الخرفيش التي يمكن استخدامها في حالات تسمم الحيوان وربما الإنسان.

الكلمات الدالة: السُّمية، حيوانات الرعي، نجمة بيت لحم (*Ornithogalum umbellatum*)، الخرفيش (*Silybum marianum*)

Introduction

This paper is integral in a series of studies, which is the first on evaluation of toxicity of grazing plants in Palestine. This study focuses on Bethlehem Star (*Ornithogalum umbellatum*) shown in Figure 1. It is well known in the Palestinian environment from where its name was derived and it is known in Arabic as "Najmat Beit Lahem". This plant is perennial of the family Liliaceae with bulbs below ground; the bulb is 15-25 mm long and 18-32 mm diameter. It has six to ten leaves, linear with a white line on the upper surface, up to 30 cm long and 8 mm broad, and a scape of 10-30 cm. The flowers group in a corymbose raceme with 6-20 flowers, and are white with a green stripe outside (Abu-Rmeileh, 2000, Blamey and Grey-Wilson, 1989; http://plants.usda.gov/java /profile?symbol=ORUM; Palestinian plant taxonomist Banan Al-Sheikh, personal communication).

Figure (1): Bethlehem Star (*Ornithogalum umbellatum*) http://en.wikipedia.org/wiki/File:Liliaceae_-_Ornithogalum_umbellatum-2.JPG)

In addition, this study assays for the anti-toxicity virtues in Milk Thistle (*Silybum marianum*) shown in Figure 2. This is an annual or biannual plant possessing red to purple flowers found throughout the world and popular in the Palestinian environment where it is known as

"Khurfeish". The Milk Thistle is a thistle of the genus *Silybum* Adans., a flowering plant of the daisy family (Asteraceae), subfamily Carduoideae and tribe Cynareae. They are native to the Mediterranean regions of Europe, North Africa and the Middle East. The achenes are black, with a simple long white pappus, surrounded by a yellow basal ring (Abu-Rmeileh, 2000, Rose, 1981; http://www.websters-online-dictionary.org/definitions/Milk%20Thistle, Banan Al-Sheikh, personal communication). The name "Milk Thistle" derives from two features of the leaves; they are mottled with splashes of white and they contain a milky sap (Hogan *et al.,* 2007).

Figure (2): Milk Thistle (*Silybum marianum*)
http://en.wikipedia.org/wiki/File:Silybum_marianum_2004.jpg.

Milk Thistle (*Silybum marianum*) is reported to manifest antidotal and liver regenerative characteristics (Flora *et al.,* 1998; Luper, 1998; Buzzelli *et al.*, 1993; Vailati *et al.*, 1993; Lirussi and Okolicsanyi, 1992; Wagner, 1981; Magliulo *et al.*, 1978; Bode *et al*., 1977; Desplaces, 1975; Anonymous, 1999). In this research, this plant is assayed in a perspective of using it as an available anti-dote agent in case of intoxication.

Israeli occupation has drastically limited the land available for Palestinians. Dramatic confiscated areas are classically reported. For example, only 70,000 ha were accessible (Braigith, 1998). In a recent report, detailed confiscated surface areas as well displacement of Palestinians are demonstrated (Ma'an, 2011). Occupation, negligence, rainfall variations as well as bad management of ranges led to general deterioration of ranges and a decrease in productivity, severe soil erosion and consequently desertification of the Palestinian land (Mohammad, 2005).

This research is based initially on "toxicity citations" from a field survey among farmers in Jenin and Toubas supported by literature. The toxicity of many Jordanian and Palestinian ranges plants to livestock is reported (Abu Rmeileh, 2000). The whole plant of *Ornithogalum umbellatum* is poisonous, including the flower and bulb. Livestock, especially sheep, are sensitive to the toxins. Bethlehem Star *Ornithogalum spp*. is reported to have poisoned 1000 sheep in one year in Maryland (Abu Remeileh, 2000).

In general, toxic plants cause physiological problems, pains, diseases especially in the 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 (Mohammad, 2005).

Plants toxic substances are classified in the following categories: Alkaloids, Cyanogenic glycosides, Cardiac glucosides, Saponins, Toxic organic acids, Selenium (Se) and Photosenstizers (Sankari, 1978). Another classification puts toxic plants in eight categories: Alkaloids, glycosides, oxalates, resins and resinoids, proteins and polypeptides, nitrates and nitrites, photosensitizers and finally mineral elements (Abu Remeileh, 2000; Jaffe, 1972; Kingsbury, 1964)

Objectives

1. Recognition of toxic plants on an objective assessment basis to help in a better management of grazing and livestock. Prevention of intoxication is better than attempting to cure it.
2. In case of intoxication, it is ideal to look for cures from the local environment. The antidotal virtues of Milk Thistle (*Silybum marianum*) are assessed in this research.
3. In addition of the vital importance for farmers, reduction of suffering of livestock is also an important objective of this study.

Materials and methods:

Preparation of Plant Extracts

Bethlehem Star (*Ornithogalum umbellatum*) and Milk Thistle (*Silybum marianum*) plants are collected from different locations in Jenin area located in the northern Palestinian Territories and are pooled for extraction. Fresh above ground plant parts are harvested in 2010 (March-April) and are dried in shadow at room temperature and then manually and finely ground and semi-powdered. As described previously by Saad *et al*., (2006), 2.5g ground plant material is extracted by adding to 25 ml of distilled water and boiled for 10 min. The boiled water extracts are filtered through filter paper and freeze-dried in a lyophilizer. The freeze-dried extracts are stored at -70°C. 0.1g of the crude extract is dissolved in dimethyl sulphoxide (DMSO) to a final stock concentration of 10 mg/ml. All extracts are kept at -20°C until carrying out assays. The concentrations used throughout this manuscript are described as weight of plant dry matter extract (mg or µg) per medium volume unit (ml) where cells are grown (DMEM).

Cell Culture

The hepatic cell line HepG2 is used in this study. HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes

including the expression of P450 isoenzymes (Medina-Diaz and Elizondo, 2005), and therefore, this cell line permits long-term studies to be performed. The cells are 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 penicillin /ml, and 10 mg streptomycin /m1. Cells are maintained in humidified atmosphere with 5% CO_2 at 37°C. The medium of cells is changed twice a week. At 70–80% confluence, cells are trypsinized and seeded in 96-well plates in cell density of $1.5x10^4$ HepG2 cells. Twenty four hours after cell seeding, cells are exposed to various concentrations of the plant extracts in fresh serum-free medium.

Hepatic cells are known to represent the detoxification center of animals (Behnia *et al.,* 2000; Lerche *et al.,* 1997). Therefore, any measured plant toxicity on HepG2 can be expected to appear in the whole organism.

Parallel treatment of HepG2 cells with Bethlehem Star (*Ornithogalum umbellatum*) and Milk Thistle (*Silybum marianum*) intends the assessment of the latter as an antidotal plant.

MTT Assay

The MTT assay is performed to assess the effect of the plant extracts on the viability and proliferation of cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] standard colorimetric assay, first described by Mosmann (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 is 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 solublized. 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) at 570 nm.

Duplicate samples are run for each concentration of plant extracts. Cells are seeded in 96-well culture plates and treated with different concentrations of plant extracts (µg or mg/ml of cell medium) for 24 hours. Then, 20 µl of MTT (5 mg/ml stock) solution is added to the wells and incubated at 37°C for 5 hours. Thereafter, the medium is gently removed from the wells, and 200 µl of DMSO are added to each well to dissolve the purple formazan crystals. The absorbance at 570 nm is recorded using the Dynatech MR5000 spectrophotometer, Dynatech Laboratories, Inc., Chantilly, VA (Raju *et al.,* 2004).

Statistical Analysis

A series of experiments is conducted using plant extracts of Bethlehem Star (*Ornithogalum umbellatum*) with and without Milk Thistle (*Silybum marianum*). The *ex vivo* experimentation variable tested is the viability of cells (determined by MTT assay) upon application of plant extract(s). Error limits and error bars represent simple standard deviations of the mean. Results are presented as the average and standard deviation of multiple replicates compared to appropriate controls.

Results and Discussion

As demonstrated in Figure 3, no toxic dose of Bethlehem Star (*Ornithogalum umbellatum*) could be detected at the used concentrations (expressed in µg of Bethlehem Star extract/ml of HepG2 cell line medium (DMEM). Unexpectedly, a slight mitogenic effect is shown at the used concentrations as compared to the control where no plant is applied to HepG2 cell line:

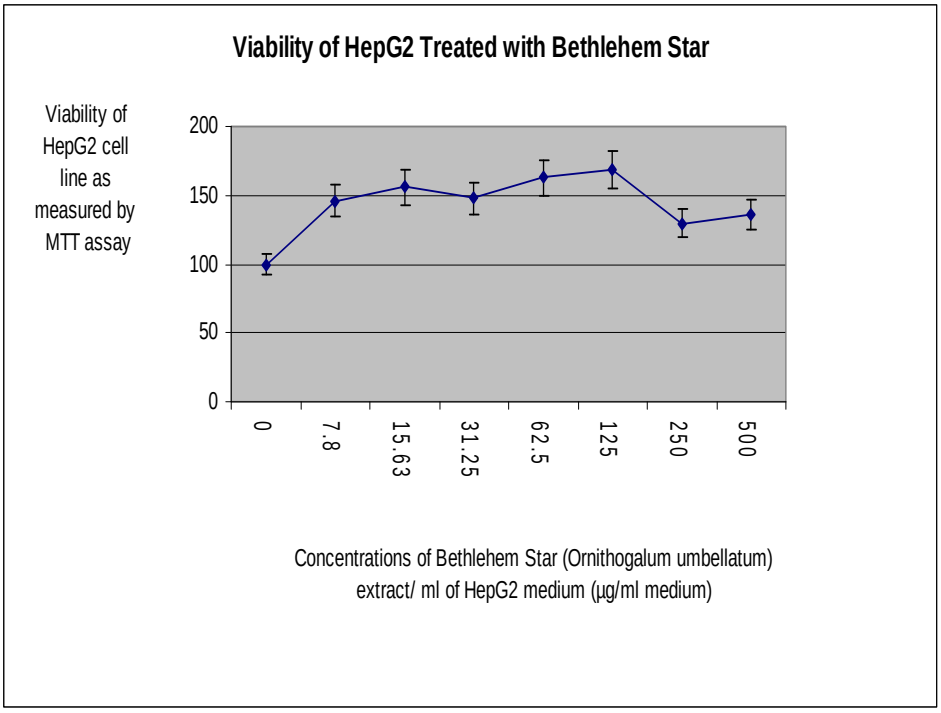

Figure (3): Different concentrations of the plant Bethlehem Star (*Ornithogalum umbellatum*) are applied on the hepatic cell line HepG2. The applied concentrations are expressed in terms of µg of plant extract/ ml of HepG2 culture medium. The effect is measured in terms of MTT that measures the viability of cells.

In the light of the results displayed in Figure 3, higher doses are assayed and later displayed in Figure 4. Bethlehem Star (*Ornithogalum umbellatum*) along with another plant (Milk Thistle, *Silybum marianun*) are assayed for toxicity to HepG2 cell line.

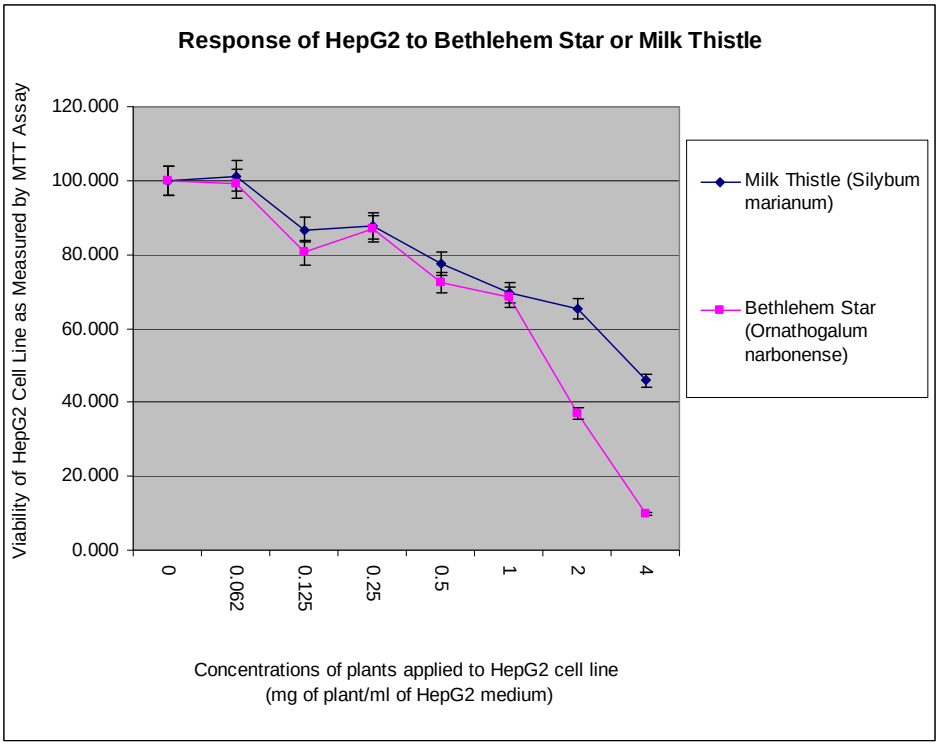

Figure (4): Different concentrations of the plant either Bethlehem Star (*Ornithogalum umbellatum*) or Milk Thistle (*Silybum marianum*) are applied on the hepatic cell line HepG2. The applied concentrations are expressed in terms of mg of plant extract/ ml of HepG2 culture medium. The effect is measured in terms of MTT that measures the viability of cells.

Clearly, Figure 4 demonstrates that at 0.062 mg of plant extract/ml of HepG2 medium, there is still no detectable toxicity. Toxicity of either Bethlehem Star (*Ornithogalum umbellatum*) or Milk Thistle (*Silybum marianum*) starts at 0.125 mg of plant extract/ml of HepG2 medium and continues in a directly proportional manner as the dose increases. At the highest plant extract doses (1-4 mg/ml of HepG2 medium), the decline in the viability of cells is sharper. This can indicate that cellular tolerance mechanisms lose their vigor as toxic doses increase.

In this paper, we apply also a parallel treatment of cells with Milk Thistle (*Silybum marianum*) in order to study and confirm the antidotal and liver regenerative virtues of Milk Thistle that are reported by many authors (Flora *et al.,* 1998; Luper, 1998; Buzzelli *et al.*, 1993; Vailati *et al.*, 1993; Lirussi and Okolicsanyi, 1992; Wagner, 1981; Magliulo *et al.*, 1978; Bode *et al*., 1977; Desplaces, 1975; Anonymous, 1999). Milk Thistle is added to HepG2 in parallel to the toxic plant as displayed in Figure 5:

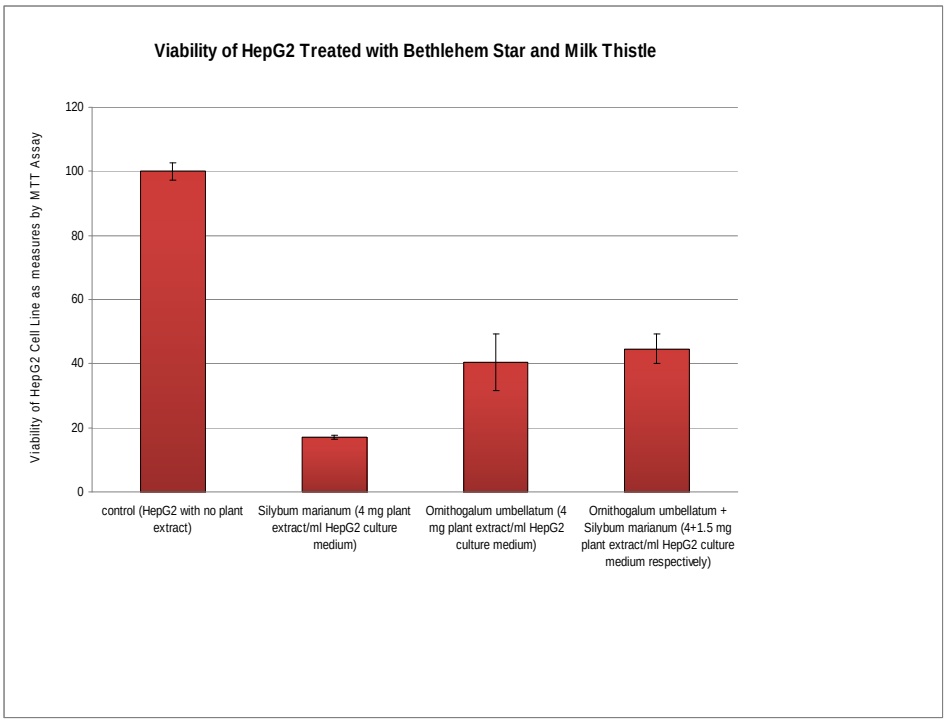

Figure (5): Different concentrations of the plant, Milk Thistle (*Silybum marianum*) or a combination of Bethlehem Star with Milk Thistle are applied on the hepatic cell line HepG2 beside the control where no plant extract is applied to HepG2. The applied concentrations are expressed in terms of mg of plant extract/ ml of HepG2 culture medium. The effect is measured in terms of MTT that measures the viability of cells.

At the used doses, Milk Thistle (*Silybum marianum*) demonstrates a clear toxicity to HepG2 cell line. Controversial to the known literature therefore, Milk Thistle is demonstrated here to be toxic at the above displayed dose (1.5 mg of plant extract/ml of HepG2 medium). Consequently, this classically used antidote (Milk Thistle) fails to neutralize the toxicity of Bethlehem Star (Figure 5). Therefore, the reported antidotal virtues of Milk Thistle (if any) should be used within careful limits! These results contrast with previous studies accomplished on other range plants (i.e. *Crozophora tinctoria*, *Cichorium pumilum* and *Nerium oleander*) (Ghareeb *et al.,* 2007, Ghareeb, 2008 and 2011) respectively.

Actually, the antidotal concentration applied in this paper (1.5 mg of plant extract/ml of HepG2 medium) is considered by extrapolation on the basis of results in Figure 4 where at that concentration, about 50%, viability should be manifested. Therefore, this dose should have represented, a reasonable (intermediate) dose that could have manifested antidotal virtues. A much lower dose could simply be inefficient, and a much higher dose could simply be toxic rather than antidotal (Figure 4).

Perspectives

In the light of results obtained in this paper, further investigations can be followed to find out the appropriate dose at which Milk Thistle (*Silybum marianum*) manifests its antidotal virtues. Such investigations can lead to an antidotal Milk Thistle weight to be given for intoxicated animals as a field prescription in case of intoxication.

Another main research work can be *in vivo* (in animals) validation of the toxicity of the mentioned plants as well the antidotal virtues of Milk Thistle (*Silybum marianum*) using experimental small and large animals.

Assaying more range plants for their toxicity levels. Abu Remeileh, 2000 reviewed many of these plants. Ramram or Ratreet (*Chenopodium spp*.) is reported to cause toxicity to grazing animals due to soluble oxalates (Amole and Izegbu, 2005). Aslaj (*Ankyropetalum gypsophiloides* Fenzl.) contains high levels of githagenin and can cause

death for grazing animals which eat a weight of this plant equivalewnt to 3% of their body weight (Abu Rmeileh, 2000). Harmal (*Peganum harmala* L.) contains alakaloids toxic to sheep, goats and cattle, guinea pigs (Lamchouri et al., 2002). Hasak (*Tribulus terrestris*) contains steroids and nitrates and probably high toxic selenium content (Abu Rmeileh, 2000). It is reported to cause disorders in sheep (Bourke, 1987). Areina (*Hypericum perforatum* L.) is reported to be toxic for sheep and cattle. Goats and horses are less sensitive to its toxicity (Abu Rmeileh, 2000). Its toxic does notably for cattle are assessed (Bourke and White, 2004)

Ultimately, Phyto-Chemical evaluation of the assayed plants can be conducted using TLC and GC in the light of results obtained *ex vivo* (in cells) and *in vivo* (in animals).

Conclusions

The ultimate goal of this research is to come up with a credible assessment of plants in the Palestinian ranges, treatment in case of intoxication and ideally preventing such intoxications and consequently conservations of our farmers, their livestock and land. In Palestine, applied research especially that intending to preserve land, livestock and consequently farmers is of utmost importance for ecological, economical and political evident reasons.

This study demonstrates the doses at which Bethlehem Star plant (*Ornithogalum umbellatum*) is toxic to HepG2 cell line. At the dose used in this study, no antidotal virtue of Milk Thistle (*Silybum marianum*) is obtained. Unexpectedly, Milk Thistle shows the same viability profile as that of Bethlehem Star (Figure 5). Milk Thistle shows a viability value lower than Bethlehem Star at 4 mg of plant extract/ml of HepG2 medium (Figure 5).

Acknowledgments

وَهُوَ اللَّهُ لا إِلَهَ إِلاَّ هُوَ لَهُ الْحَمْدُ فِي الأُولَى وَالآخِرَةِ وَلَهُ الْحُكْمُ وَإِلَيْهِ تُرْجَعُونَ (القصص ٢٨: ٧٠).

And He is Allah. There is no god but He. To Him be praise, at the first and at the last: for Him is the Command, and to Him shall ye (all) be brought back (The Glorious Qu'an Chapter 28, Al-Qasas, The Story, Stories, Verse 70)**.**

This research was kindly funded by Arab American University (AAUJ). Many thanks are addressed to Mr. Banan Al-Sheikh for his kind help in classification, to Dr. Aysar Yaseen and Mr. Osama Jarrar for their kind linguistic revision of English and to Dr. Mohammad Dawabsheh for his kind linguistic revision of Arabic.

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