Advances in Pure and Applied Chemistry (APAC)
Vol. 1, No. 1, 2013, ISSN 2167-0854

www.worldsciencepublisher.org

From Biochemical Assays in Cells to Zoological Applications: A Herbal Antidotal Treatment of Intoxication by Pussley (*Portulaca oleracea*)

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Abstract – The assessment of the cytotoxicity of pussley (*Portulaca oleracea* L.) enrolls in the first series of studies of *ex vivo* evaluation of toxicity of range plants in Palestine. In parallel, this study looks for an antidotal treatment in Milk Thistle (*Silybum marianum*) which is available in the Palestinian environment. This study has an economical, ecological, agricultural and political importance through preserving livestock, ranges and land.

Starting at 500 µg of *Portulaca oleracea* extract/ml of HepG2 cell culture medium, a clear toxicity for HepG2 cell line as expressed by MTT viability test is demonstrated as directly proportional to the plant dose. *Silybum marianum* shows a similar profile even though with a viability profile weaker than that of *Portulaca oleracea*. This is unexpected for a plant (*Silybum marianum*) used classically as hepatic regenerative and antidote agent, recall that *Portulaca oleracea* is an edible plant.
However, *Silybum marianum* is demonstrated to be efficient (at 1.5 mg of plant extract/ml of HepG2 cell line culture medium) as an antidote against the toxicity of *Portulaca oleracea*. This opens horizons for using *Silybum marianum* as an antidote in case of intoxication of livestock and also humans.

Keywords– Plants toxicity; livestock; pussley (*Portulaca oleracea* L.); antidote effects of milk thistle (*Silybum marianum).*

1. Introduction

In many cases the prevailing knowledge of the toxicity of many range plants in Palestine is popular and unfortunately non-objective. The example of intoxication attributed to *Cichorium pumilum* is demonstrative. Actually the indoor livestock that do not graze in ranges manifest intoxication symptoms still attributed to *Cichorium pumilum*. An objective assessment of intoxication reasons and of toxicity of ranges plants is clearly needed. The objectives of this present study are, therefore, the following:

(a) Production of a credible assessment of plants in the Palestinian ranges and ideally prevention of intoxication. This study is conducted on one the suspected plants (i.e. pussley (*Portulaca oleracea*))
(b) Providing recommendations for farmers to treat intoxication by plants available in the Palestinian environment like Milk Thistle (*Silybum marianum*).
(c) Ultimately, conservation of Palestinian farmers, their livestock and land, an important and essential element for the Palestinian emerging State.

Farmers, indeed, report suffering of their grazing animals from several plants growing in the Palestinian pastures. This plays a negative role in driving them out of production. This kind of studies (assessment of toxicity and antidotal virtues of Palestinian ranges plants) is, therefore, of vital economical, ecological, agricultural and even political importance. Let us recall that 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 are accessible to Palestinians [1].

Furthermore, Palestinian ranges undergo a severe deterioration due to rainfall variation, bad management of grazing that led to vegetation damage, decrease in plant productivity, and an increase in poisonous and unpalatable plants and, consequently, severe soil erosion. All these factors are leading to desertification of the Palestinian land [2]. Of utmost scientific and political importance is an efficient management of the available rudimentary areas which necessitates recognition and characterization of the toxic plants.

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 research is based on a field survey in Jenin district and aims to yield an objective assessment of the hear-say reports of toxicity reports regarding ranges plants, the focus is being made on pussley (*Portulaca oleracea*). This plant (Figure 1) is used in the Palestinian kitchen, however! and known as Farfaheina, Baqla or Rejla.

Pussley (*Portulaca oleracea*) is reported to contain high concentrations of soluble oxalates poisonous to sheep and goats [3]. *P. oleracea* contains many biologically active compounds and is a source of many nutrients. Some of the biologically active (and, in some cases, potentially toxic compounds) include free oxalic acids, alkaloids, omega-3 fatty acids, coumarins, flavonoids, cardiac glycosides, and anthraquinone glycosides. It has high contents of Omega-3 fatty acids and protein, compared to other vegetables [4]. Taken together, *Portulaca oleracea*, however, is traditionally considered as a comestible plant of high gastronomic, nutritive, and medicinal values. The toxicity reports need, therefore, to be verified as being conducted in this research.

Figure 1: Pussley (*Portulaca oleracea*)

2. Materials and methods

Preparation of Plant Extracts

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-July) and are dried in shadow at room temperature and then manually finely ground and semi-powdered. Each 2.5g ground plant material is extracted by refluxing with 25 ml ethanol for 30 min and kept overnight at room temperature before filtration. This method is appropriate in extracting the organic as well as the non organic components. After filtration, ethanol is evaporated until dryness and the crude extracts are weighed. 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 cytotoxicity tests [5]. The concentrations used throughout this manuscript are described as weight of plant dry matter extract (mg or µg) / medium volume unit (ml) where cells are grown (RPMI).

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 [6] and therefore, this cell line permits long-term studies to be performed. The cells are grown in Dulbecco's modified Eagle's medium (RPMI) 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/m1 streptomycin. Cells are maintained in humidified atmosphere with 5% CO2 at 37°C. The medium of cells from both cell lines is changed twice a week. At 70–80% confluence, cells are trypsinized and seeded in 96-well plates in cell density of 1.5x104 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 [7,8]. Therefore, any measured plant toxicity on HepG2 can be expected to appear in the whole organism.

Parallel treatment of HepG2 cells with pussley (*Portulaca oleracea*) and Milk Thistle (*Silybum marianum*) intends the assessment of the latter as an antidotal plant.

MTT Assay

Vol. 1, No. 1, 2013, ISSN 2167-0854

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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 in 1983 [9], 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 are 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 [10].

Statistical Analysis

A series of experiments is conducted using plant extracts of Pussley (*Portulaca oleracea*) 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.

3. Results

A series of concentrations of *Portulaca oleracea* are applied on the Hepatic cell line HepG2 as described in Materials and Methods. The toxicity effects are quantified as described by the viability test (MTT assay) described also in materials and Methods. The viability profile is shown in Figure 2:

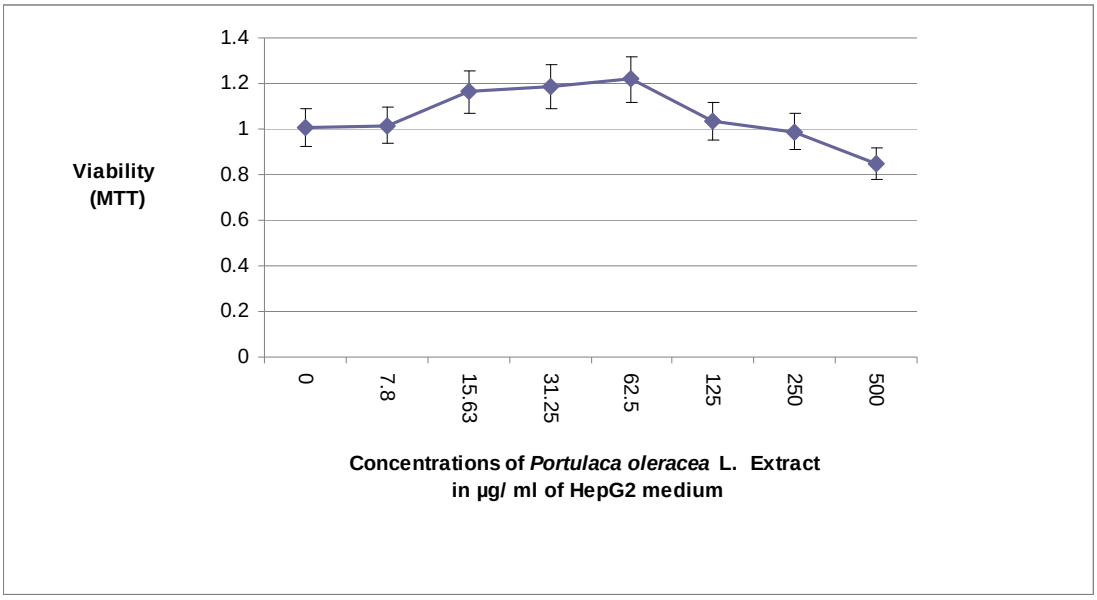

Figure 2: Viability of HepG2 cell line upon applying different pilot concentrations of pussley (*Portulaca oleracea*). Concentrations are expressed in µg of plant extract/ ml of HepG2 culture medium (RPMI).

Up to 250 µg of Portulaca *oleracea* plant extract/ml of HepG2 cell culture medium, no significant toxicity level is clear as shown in Figure 2.

Further concentrations (higher than those employed in Figure 2) of pussley (*Portulaca oleracea)* as well as of Milk Thistle (*Silybum marianum*) are applied on the hepatic cell line HepG2 as described above. Results are shown in Figure 3. Milk thistle (*Silybum marianum*) is shown in Figure 4.

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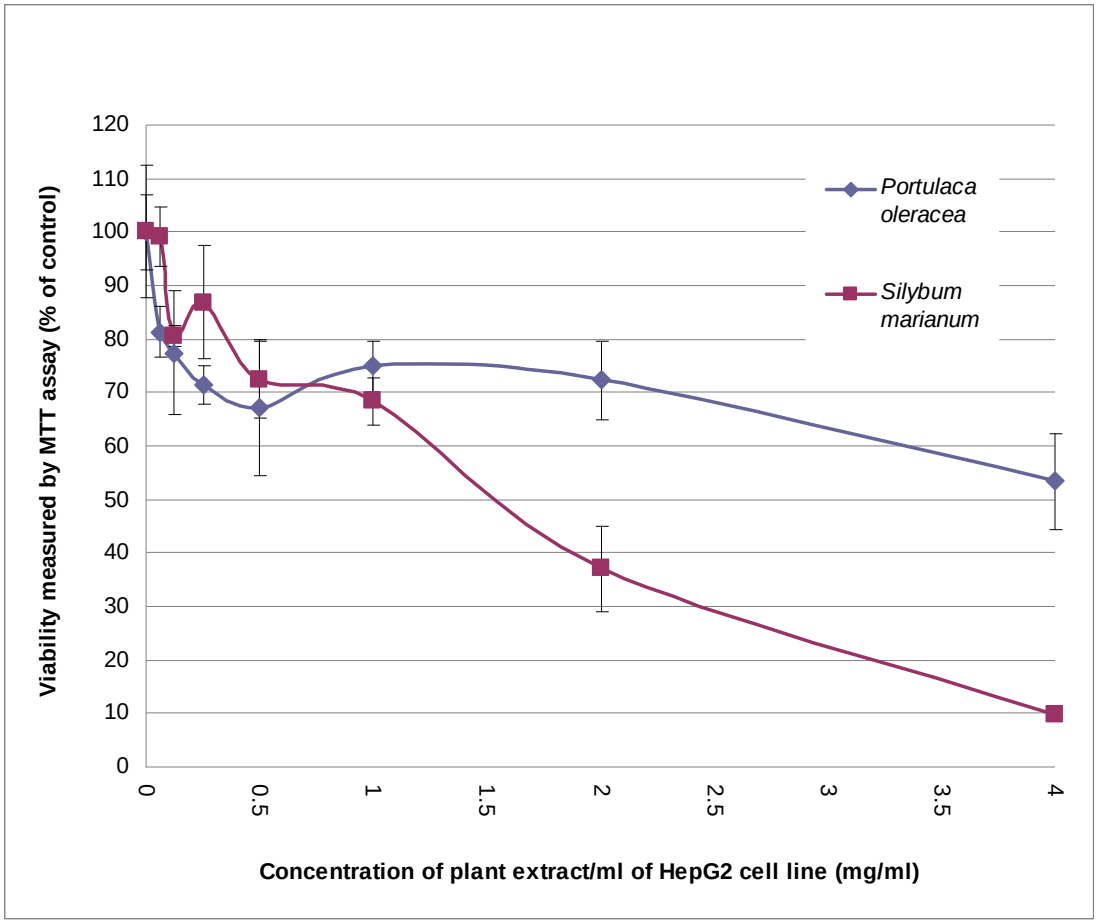

Figure 3: Further concentrations of *Portulaca oleracea* are assayed for possible toxicity effect when applied on HepG2 cell line. *Silybum marianum* is assayed in parallel for possible use as an antidote. Concentrations are expressed in terms of mg of plant extract/ ml of HepG2 cell line culture medium (RPMI).

In this study, hepatic cells treated with toxic plant extract are also treated with *Silybum marianum* extract in order to evaluate its antidotal and hepatic cells regeneration capacities after assaying for determining an appropriate dose to be used possibly as an antidote (Figure 5).

Figure 4: Milk Thistle (*Silybum marianum*)

At approximately 1.5 mg of *Silybum marianum* extract/ml of HepG2 cell culture medium, the viability of HepG2 cell line is 50% (Figure 3). This intermediate dose is useful as an antidote concentration used to obtain further results (Figure 5). This concentration is neither too low to be inefficient, nor too high to be excessively toxic.

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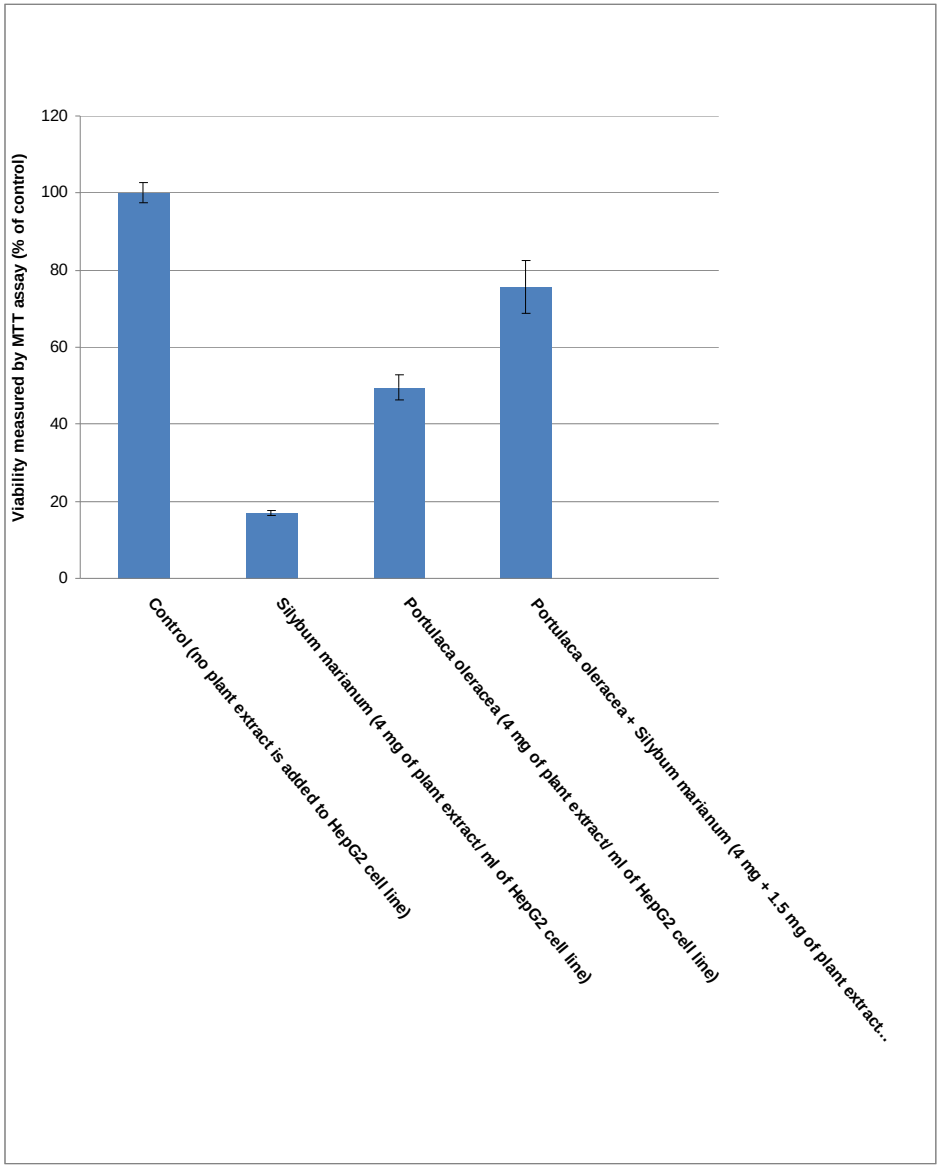

Figure 5: *Portulaca oleracea* without and with *Silybum marianum* are assayed for toxicity and antidote virtues respectively using the viability test, MTT. Besides, *Silybum marianum* and no plant (Control) are also assayed. Concentrations are shown in terms of mg of plant extract/ ml of HepG2 cell line culture medium (RPMI).

4. Discussion

The results depicted from Figure 2 gives an expected profile for a plant commonly used in the kitchen. Furthermore, it is considered as a delicious Palestinian traditional meal! However, in ranges, the picture can be different especially in case of deterioration of Palestinian pastures and poverty of the available grazing plants where animals can be forced to feed excessively on *Portulaca oleracea*.

In general, plants considered poisonous to humans are also considered to be poisonous to animals. However, there have been specific cases where animals have been poisoned by plants considered safe to humans. Also, it should be kept 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 [11].

Coherently with the profile obtained in Figure 2, a clear toxicity is manifested at doses higher that 0.5 mg of plant extract/ml of HepG2 cell culture medium. The effect is directly proportional to the dose. This applies to both Portulaca *oleracea* and to *Silybum marianum* which, shows a viability profile weaker than that of *Portulaca oleracea*. This is striking as *Silybum marianum* is recognized as cell regenerative and antidote agent. It is reported to possess antidote and liver regeneration virtues [12,13,14,15,16,17,18,19,20]. Milk Thistle (*Silybum marianum*), therefore, could be as an antidotal herb for intoxicated animals.

Portulaca oleracea shows a clear toxicity level at 4 mg of plant extract/ ml of HepG2 cell culture medium (Figure 5). This is compatible with Figure 3. Importantly, at that toxic dose, when *Silybum marianum* is applied, a clear antidote result is obtained and the viability of cells rises significantly as can be shown by the right column (Figure 5).

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The toxicity of *Portulaca oleracea* might seem not to be severe in Figure 5. However, toxicity is evident and even the "low toxicity" should never be underestimated. Actually, toxins fortunately cause herbivores to limit their intake of plants and play, therefore, a role in regulating the intake of many "mildly poisonous plants" [21]. 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 an 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 [22]. 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 ingest 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-ingestion consequences that animals experience from nutrients in the plant.

5. Perspectives

Construction of a data base on Palestinian ranges that tries to answer the following questions and elements:
* What are the plants objectively poisonous to livestock and humans? This should be answered by assaying for toxicity *ex vivo* (in cells) and *in vivo* (in animals).

Many of plants are reported to be toxic in the Jordanian and Palestinian environments [22]. Ramram or Ratreet (*Chenopodium spp*.) is reported to cause toxicity to grazing animals due to soluble oxalates [23]. 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 [24]. Harmal (*Peganum harmala* L.) contains alakaloids toxic to sheep, goats and cattle, guinea pigs [25]. Hasak (*Tribulus terrestris* contains steroids and nitrates and propably high toxic selenium content [23]. Areina (*Hypericum perforatum* L.) is reported to be toxic for sheep and cattle. Goats and horses are less sensitive to its toxicity [23].

* If a plant under study is toxic, under what doses? Growth stage? Animal species, Circumstances…
* Antidotal virtues of Milk Thistle (*Silybum marianum*) and other antidotal herbs against toxicity *ex vivo* and *in vivo,* appropriate doses…

In an old literature of 1887, certain plants are described as alexipharmics or snake-bite Antidotes [26]. A long list of plants used as antidotes by the tribes of Bihar are cited. These plants with their used parts and tribal use include *Achyranthes aspera* Linn. (Crushed fresh root and paste of leaves in scorpion stings), *Alangium salviifolium* (Linn. f.) Wang (fresh bark extract in insect bites), *Aristolochia indica* Linn. (Fresh root extract in snake bites), *Azadirachta indica* A. Juss. (Fresh leaf extract in insect bites), *Calotropis procera* (Ait.) R. Br. (latex in insect bites), *Carica papaya* Linn. (Latex in insect bites), *Clerodendrum infortunatum* Linn. (Fresh leaf extract in insect bites), *Clitoria ternatea* Linn. (Root extract in snake bites), *Costus speciosus* (Koenig ex Retz.) Sm. (fresh root extract in snake bites), *Curculigo orchioides* Gaertn. (Crushed fresh root in scorpion stings), *Cyperus kyllingia* Endl. (Fresh tuber as antidote), *Drypetes roxburghii* (Wall.) Hurusawa (crushed seed in insect bites), *Fimbristylis cymosa* R.Br. = F. spathacea Roth (fresh root in snake bites), *Gmelina arborea* Roxb. (crushed bark in insect bites), *Gloriosa superba* Linn. (fresh root in scorpion stings), *Murraya paniculata* (Linn.) Jack. (Root in snake bites), *Tamarindus indica* Linn. (crushed seeds in snake bites), *Typhonium trilobatum* Schott (crushed tuber in snake bites), *Vitex peduncularis* Wall. ex Schauer (fresh leaf extract in snake bites) [27].
* Recommendation for policy makers to safeguard Palestinian farmers and ranges.
* Phyto-chemical analysis to define the toxic ingredients. At the risk of oversimplifying, plants toxic substances can be classified in the following seven categories: Alkaloids, cyanogenic glycosides, cardiac glucosides, saponins, toxic organic acids, selenium (Se) and photosenstizers [22,28]. Others classify toxic plants in the following eight categories: Alkaloids, glycosides, oxalates, resins and resinoids, proteins and polypeptides, nitrates and nitrites, photosensitizers and finally mineral elements [22,29,30]

6. Conclusions

This paper targets reduction of animal suffering from intoxication by range toxic plants. In addition, the methodology is using hepatic cell line HepG2 instead of experimental animals. Actually, in Islam, animals, like other creatures are considered as genera and "nations" like yourselves:
And there is no animal that walks upon the earth nor a bird that flies with its two wings but (they are) genera like yourselves; We have not neglected anything in the Book, then to their Lord shall they be gathered [31].

This paper demonstrates the cytotocity of pussley *(Portulaca oleracea)* to hepatic cell line HepG2 in a dose-dependent directly proportional manner similar to Milk Thistle (*Silybum marianum*), strikingly. Importantly, however, the cytotoxicity of the former can be counteracted by the later plant which opens horizons for curing in case of intoxication of livestock and principally humans.

Evaluation of the cyto-toxicity of the following ranges plants besides the antidotal effects of Milk Thistle *(Silybum marianum)* has already been successfully started in our laboratory of animal cell culture at Arab American University. The already studied plants are *Crozophora tinctoria*, *Cichorium pumilum* and *Nerium oleander*

[32,33,34 respectively]. The mentioned papers as well as the present one are all conducted in the hepatic cell line, HepG2. Hepatic cells are known to represent the detoxification center of animals [7,8]. Therefore, any measured plant toxicity on HepG2 can be expected to appear in the whole organism which should mean that the biological system used in these experiments is appropriate.

Acknowledgments

My deep acknowledgments for Allah first of all "Praise be to Allah, the Cherisher and Sustainer of the worlds" [35]

This research was kindly funded by Arab American University (AAUJ).

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