Milk Thistle (*Silybum marianum*) Does not Neutralize the Toxicity of Sorrel (*Rumex obtusifolius*)

Bilal Ahmad Ahmad Ghareeb

(From Assays in HepG2 Cell Line to Applications in the Palestinian Ranges)

Abstract

Palestinian ranges need an objective assessment of the toxicity of some plants that are started to be tested in the first series of research work of its kind in Palestine. In this paper, Sorrel (*Rumex obtusifolius*) is assayed for toxicity to the hepatic cell line HepG2 besides an assessment of Milk Thistle (*Silybum marianum*) for possible antidotal virtues using the cells viability test (MTT).

Up to a given concentration (31.25 µg of plant extract/ml of HepG2 cell culture medium), Sorrel (*Rumex obtusifolius*) has growth stimulation virtues on HepG2 cell line using the viability assay MTT. Afterwards (starting at 125 µg of plant extract/ml of HepG2 cell culture medium), the viability lines decline in a directly proportional manner as the concentration of the plant extract increases/ml of HepG2 medium.

When assayed for its effect on the viability of HepG2 cell line, Milk Thistle (*Silybum marianum*) shows an unexpected low viability profile that seemingly contradicts the classical antidotal uses of this plant. However, it is assayed for such virtues in this paper but unsuccessfully at the concentrations employed tentatively. These results can be used to adjust for finer concentrations of this plant to be used in case of intoxication of animals and humans by Sorrel (*Rumex obtusifolius*) which is consumed by both.

Key words

*T*oxicity of Sorrel (*Rumex obtusifolius*), Antidotal virtues of Milk Thistle (*Silybum marianum*), Grazing Animals, Palestinian Ranges.

Introduction

In the Palestinian Territories acquiring independence, almost every thing has to be built notably in the domain of applied research. This is the first serial study of toxicity of some plants prevailing in Palestine using *ex vivo* assays (e.g. hepatic cell line HepG2 and the *in vitro* cellular viability MTT assay). 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. The plants under study are chosen based on a survey in Jenin and Toubas areas besides literature. Focus is made in this present paper on Sorrel (*Rumex obtusifolius*) shown in Figure 1.

Figure 1: Sorrel (*Rumex obtusifolius*)

A full description of biology and ecology of Sorrel (*Rumex obtusifolius*) is given by Cavers and Harper (1964), Cavers and Harper (1966) and Cottam *et al.,* (1986).

The docks and sorrels belong to the genus *Rumex* L., a genus of about 200 species of annual, biennial and perennial herbs in the buckwheat family (http://en.wikipedia.org/wiki/Rumex). Sorrels (e.g. sheep's sorrel, *Rumex*

acetosella, common sorrel, *Rumex acetosa* and French sorrel, *Rumex scutatus*), have particularly high levels of oxalic acid and some of these are grown as pot herbs or garden herbs for their acidic taste (Łuczaj, Łukasz, 2008)). The soluble oxalates content of *Rumex spp*. L. is reported to be up to 0.27% and thus poisonous and lethal to animals (Hurst, 1942). In Western Europe, dock leaves are a traditional remedy for the sting of nettles Hartfield, 2004).

Substances responsible for toxicity in plants are put in the following eight categories: Alkaloids, glycosides, oxalates, resins and resinoids, proteins and polypeptides, nitrates and nitrites, photosensitizers and finally mineral elements (Jaffe, 1972 and Kingsbury, 1964). Plants toxic substances are, nevertheless, classified by Sankari, 1978 in seven categories: Alkaloids, Cyanogenic glycosides, Cardiac glucosides, Saponins, Toxic organic acids, Selenium (Se) and Photosenstizers.

Despite the existence of many works on toxicity of plants, many local plants suspected to be toxic for animals still need to be assessed for their toxicity on grazing animals. This demonstrates the scientific importance of the present proposed research work which has a particular importance in Palestine. Palestinian farmers report suffering of their grazing animals from some plants available in the Palestinian pastures. This plays a negative role in driving them out of production. This kind of studies is, therefore, of vital economical, ecological and even political importance as it can help in conserving the rudimentary land. Due to the occupation, in 1998 for example, only 70,000 ha were accessible to Palestinians (Braigith, 1998). This limited area suffers from deterioration and desertification (Mohammad, 2005). Large confiscations continue to be committed against Palestinians. This is detailed in terms of surface areas being confiscated from many Palestinian communities as reported recently by Ma'an (2011). This report demonstrates for example that Ein Al-Helweh (Toubas District) suffered from confiscation of 200 Donums on Nov 9, 2010.

In addition this study assays for the anti-toxicity virtues in Milk Thistle (*Silybum marianum*) shown in Figure 2.

Figure 2: Milk Thistle (*Silybum marianum*)

Milk Thistle (*Silybum marianum*) is reported to possess antidote and liver regeneration virtues (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 3, 1999).

This paper is a part of serial studies of plants suspected to be toxic in Palestinian ranges like *Crozophora tinctoria*, *Cichorium pumilum* and *Nerium oleander* (Ghareeb et al., 2007; Ghareeb et al., 2008; Ghareeb, 2011)

A main objective of this study is production of credible and feasible assessment knowledge of toxicity of ranges plants like Sorrel (*Rumex obtusifolius*) and ideally, prevention of intoxications, which is easier than curing of poisoned animals. Treatment in case of intoxication is also an objective of the study, ideally using plants available in the Palestinian environment like Milk Thistle (*Silybum marianum*). Taken together, this paper intends caring for Palestinian farmers, their livestock and the Palestinian land.

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-May) 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 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 for further evaluation as described by Saad *et al.*, (2006). 0.1g of the extract is dissolved in dimethyl sulphoxide (DMSO) to a final stock concentration of 10 mg/ml. 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 (Medina-Diaz *et al.*, 2006) 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 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.

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 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 is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells 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 (Raju *et al.*, 2004).

Statistical Analysis

A series of experiments is conducted using plant extracts of Sorrel (*Rumex obtusifolius*) with and without Milk Thistle (*Silybum marianum*). The *in vitro* 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

When assayed for toxicity to the hepatic cell line HepG2, Sorrel (*Rumex obtusifolius*), shows an interesting profile (Figure 3). A characteristic umbrella shaped curve demonstrates an increase in the viability of cells to reach a kind of plateau at concentrations ranging between 31.5 and 125 µg of plant extract/ml of HepG2 cell culture medium. Consequently, up to a given limit, Sorrel (*Rumex obtusifolius*) has clearly mitogenic virtues to HepG2 cell line. Afterwards, viability of cells starts to decline but does not decrease below the viability at 0 µg of plant extract/ml of HepG2 cell culture medium. The results of this pilot experiment are used to adjust for the doses of Sorrel (*Rumex obtusifolius*) that could be toxic to HepG2 as demonstrated in Figure 4 in the next section.

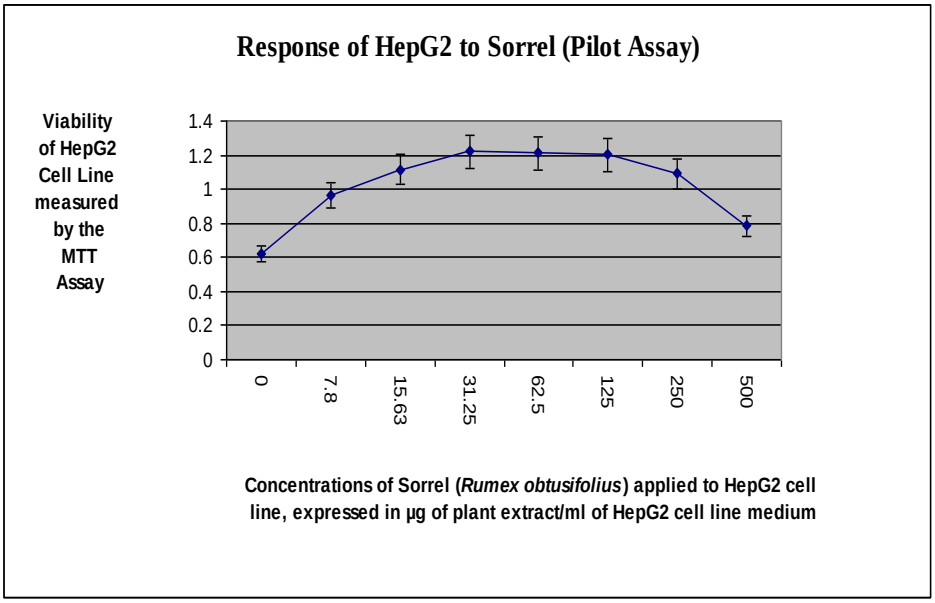

Figure 3: Assaying viability of the hepatic cell line HepG2 (as measured by the MTT viability test against different concentrations of Sorrel (*Rumex obtusifolius*).

In the light of results obtained in Figure 3, more concentrations of Sorrel (*Rumex obtusifolius*) are assayed for possible toxicity to the hepatic cell line HepG2. Expectedly, as a continuity of Figure 3, the viability lines decline in a directly proportional manner as the concentration of the plant extract increases/ml of HepG2 medium.

In parallel to Sorrel (*Rumex obtusifolius*), Milk Thistle (*Silybum marianum*) is assayed for possible toxicity effects. Unexpectedly, Milk Thistle (*Silybum marianum*) shows a viability profile not only resembling that of Sorrel (*Rumex obtusifolius*) at concentrations from 0.5 to 1 mg of plant extract/ml of HepG2 HepG2 medium, but manifests a curve line below that of Sorrel (*Rumex obtusifolius*) at higher concentrations of plant extract. These results are unexpected as Milk Thistle (*Silybum marianum*) is classically reviewed and utilized as antidotal herb. This plant is indeed recognized in the literature as a cell

regenerative agent (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 3, 1999).

However the profile of viability obtained in Figure 4 helps in choosing an appropriate dose of Milk Thistle (*Silybum marianum*) to be used possibly to look for antidotal virtues (if any) that could neutralize the toxicity of Sorrel (*Rumex obtusifolius*) as demonstrated in Figure 5. At 1.5 mg/ml, Milk Thistle (*Silybum marianum*) shows a viability of 50%. It is, therefore, a reasonable plant extract concentration (neither too low to be inefficient nor too high to be excessively toxic).

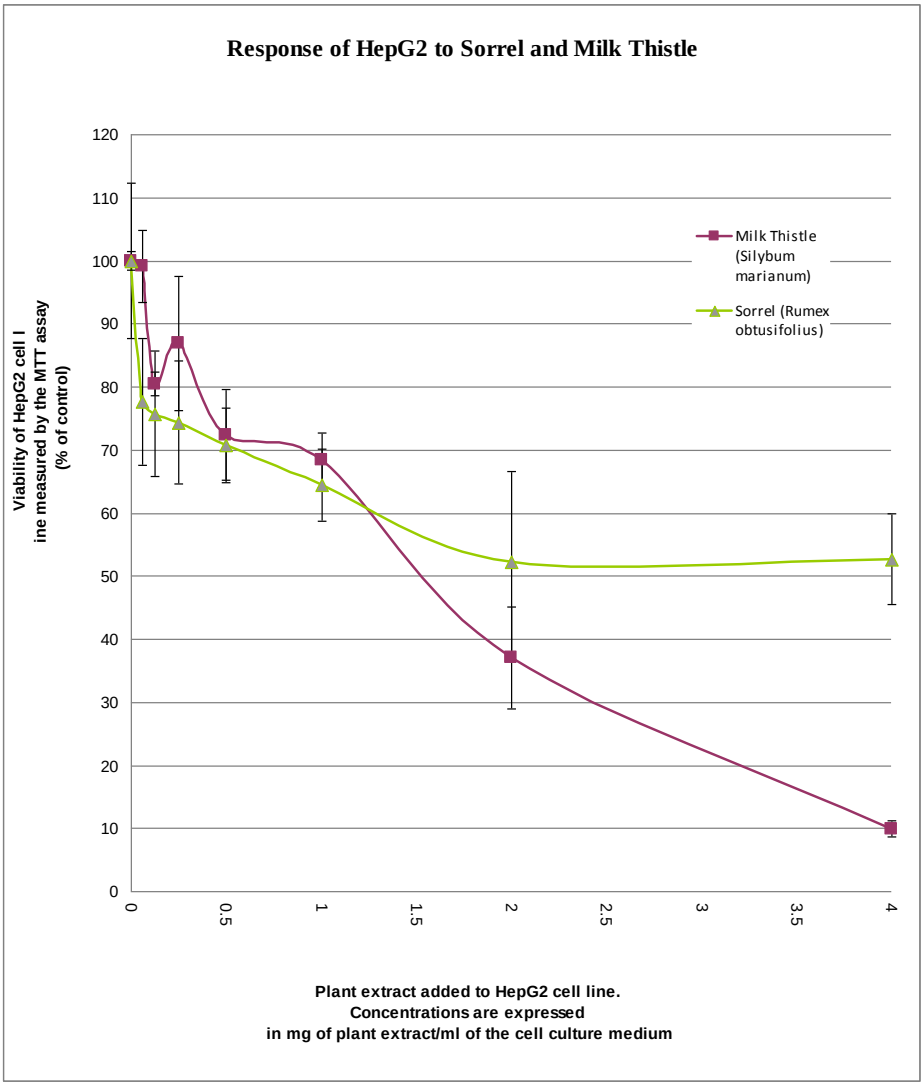

Figure 4: Further concentrations of Sorrel (*Rumex obtusifolius*) are applied on HepG2 cell line to assess for toxicity as measured by the MTT viability test.

What might seem as mildly toxic from the above figure should not be underestimated. Actually, toxins cause herbivores to limit their intake of plants and play, therefore, a role in regulating the intake of many "mildly poisonous plants" (Launchbaugh, 2001). Fortunately again, at high concentrations, most toxins cause

plants to be unpalatable. But unfortunately, toxins at low concentrations do not render a plant unpalatable and therefore, should not be neglected! In addition animals consume relatively huge quantities of plants and with low selectivity under harsh conditions; animals might be exposed intoxication with such plants (Owen and Collins, 2003).

In concordance with the results obtained in Figure 4, Sorrel (*Rumex obtusifolius*) shows a viability level higher than that manifested at the same plant extract concentration (4 mg/ml) for Milk Thistle (*Silybum marianum*). Deceivingly, at the plant extract concentrations used here, this plant fails to neutralize the toxicity caused by Sorrel (*Rumex obtusifolius*). Clearly more assays using finer panoply of concentrations of Milk Thistle (*Silybum marianum*) can demonstrate antidotal virtues of this plant in order to be used in case of intoxication.

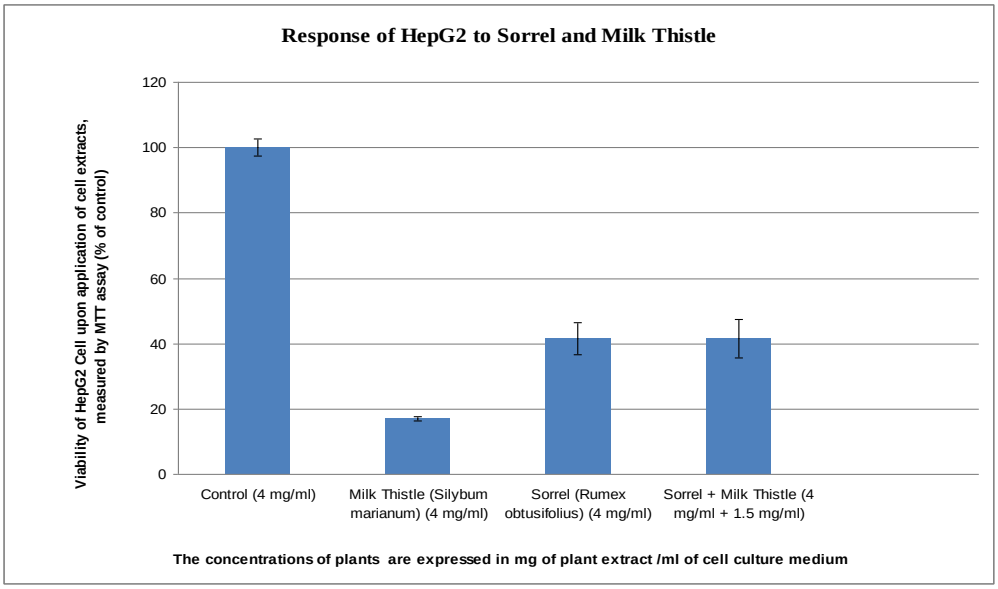

Figure 5: Sorrel (*Rumex obtusifolius*) as well Milk Thistle (*Silybum marianum*) both individually or combined are assayed for their effect on the viability of HepG2 cell line as measured by the viability test MTT.

Conclusions

The Palestinian present knowledge of toxicity of range plants can be described as popular, based on empirical and, in many cases, non-objective observations. An efficient use of the very limited Palestinian range areas necessitates recognition and characterization of the toxic as well as antidotal plants which represent a main goal of this paper. Toxicity of Sorrel (*Rumex obtusifolius*) to the hepatic cell line HepG2 is demonstrated in this paper. Unfortunately, however, at the concentration of Milk Thistle (*Silybum marianum*) employed in this study, no antidotal virtues are demonstrated. This study should, therefore, be followed by assaying more fine concentrations and combinations of Sorrel and Milk Thistle. This opens horizons, however, for further investigations as a perspective of this work. This serial study of toxicity of range plants in Palestine, which is unprecedented, is of vital importance. These studies can help for better grazing range management, for instance by giving additional rest to plants during drought.

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My deep acknowledgments for Allah:

"... رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ" (القرآن الكريم، النمل 27: 19)

…"O my Lord! so order me that I may be grateful for Thy favors, which thou hast bestowed on me and on my parents (The Glorious Qur'an Chapter 27, An-Naml, The Ant, The Ants, Verse 19)

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(القرآن الكريم، النمل 27: 19)

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نباتُ الخرفيش (*Silybum marianum*) لا يعادلُ سُمِّيَّةَ نباتِ الحُميض (*Rumex obtusifolius*)

تلخيص:

تحتاج المراعي الفلسطينية إلى تقييم موضوعي لسُمِّيَّة بعض النباتات، وقد بُدِيء بدراسة هذه النباتات في سلسلة أبحاث، هي الأولى من نوعها في فلسطين. يتناول البحثُ أثرَ نباتِ الحُمّيض (*Rumex obtusifolius*) على الخلايا الكبدية (HepG2) إضافةً لتقدير أثر نبات الخرفيش (*Silybum marianum*) من جهة مقاومته للسُّمِّيَّة باستخدام فحص حيوية الخلايا (MTT).

يُظهرُ نباتُ الحُمّيض بتركيز معين أثراً إيجابياً على تكاثر الخلايا الكبدية يعقبه أثرٌ سُمِّيٌّ (باستخدام فحص حيوية الخلايا (MTT)) ويتناسب طردياً مع تركيز النبات، معبَّراً عنه بتركيز المستخلص النباتي/مل من البيئة المُغذية لخلايا HepG2. عند فحص أثر نبات الخرفيش (*Silybum marianum*) على حيوية خلايا epG2، ان الأثر سلبياً وهذا أثر غير متوقع لهذا النبات يُخالف الاستخدامات والأدبيات المنشورة. ولم تُفلح التراكيز المستخدمة تجريبياً في هذا البحث في إبطال سُمِّيةِ نبات الحميض. إلا أنه من الممكن الاسترشاد بنتائج هذا البحث من أجل الوصول إلى تراكيز من نبات الخرفيش أكثر نجاعة في مقاومة *سُمِّيَّة* نبات الحُميض لدى الإنسان والحيوان وكلاهما يستهلك الحُميض.

الكلمات الدالة

سُمِّيَّة نبات الحُمّيض (*Rumex obtusifolius*)، أثر الخرفيش (*Silybum marianum*) المُقاوِم للسُّمِّيَّة، حيوانات الرعي، المراعي الفلسطينية.