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Cytotoxic Effects of Sábila (*Aloe Vera*): Commercial Products as Food Supplement

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Abstract: *Aloe vera* is used intensively in traditional medicine because it's widely number of therapeutic properties, and due to that it is used as food supplement. Although it is well known that the main class of compounds responsible for aloe-induced toxicity is anthraquinones, it seems to be that their concentrations are reduced in commercial products. To our knowledge, few studies concerning to the toxicity of aloe-based product have been documented. This lack of data suggested us to evaluate the effect of two different commercial products on cell viability, which were 200x Dehydrated Inner Leaf Powder (ILP) and 100x Dehydrated Whole Leaf Powder (WLP). The HPLC analysis revealed two peaks in each product; aloin (5.82 ppm for ILP and 4.75 ppm for WLP) and aloe-emodin (5.73 ppm for ILP and 1.68 ppm for WLP) indicating low concentrations compared with other studies. For cytotoxic analysis, both products were dissolved in cell culture medium getting final concentrations of 0.5x, 1x, 5x, and 10x; at pH 7.3 and 50 µg/ml gentamicine. Cells were analyzed by cell membrane integrity (LDH-release), metabolic activity (WST-1), functional lysosomes (NRU) and apoptosis induction (Annexin V/PI). The results indicated that both products are not cytotoxic at the recommended concentration of 1x, which it is used as food supplement; this could be explained due to the low anthraquinones concentration. The cytotoxic effects were observed from 5 up to 10x fold over-concentrated; a possible reason is that the toxic effect might be caused by mechanical stress by the fibres. For another side, no apoptotic effect was detected at concentrations up to 10x with both

products. This work was a short cytotoxicity study in a classical cancer cell line (HeLa), we are considering to do further toxicological evaluations with other commercial products in order to know the risk for to use them as food supplements.

Keywords: *Aloe vera*, cytotoxic assays, commercial products, food supplements.

INTRODUCTION

Aloe vera (*A. vera*) is a plant native from Africa, belonging to the family Asphodelaceae, which can measure 30 to 50 cm tall, their leaves are rosette-shaped, fleshy toothed at the edges, with three layers: an inner layer that is a translucent gel, the middle layer that it is known as latex, and the thick outer layer called whole leaf [1,2].

The whole leaf of *A. vera* contains a complex mixture of compounds with about 98% water and in its dry weight 55% are polysaccharides, 17% sugar, 16% minerals, 7% protein, 4% fats and 1% phenolic compounds. It contains antioxidant and vitamins such as A, C and D, B1, B2 and B12, choline and folic acid. Also carbohydrates such as monosaccharide, and polysaccharides (glucomannans and acemannans), glucose and mannose that form polymannans with β glycosidic bonds (1-4), are present. *A. vera* is characterized by its content of phenolic compounds mainly classified into two groups: anthrones and anthraquinones. The anthraquinones present in aloe latex are: aloin A (barbaloin), aloin B (isobarbaloína), aloe-emodin, emodin and aloesin[3-5].

Different parts of the *A. vera* plant (latex, gel and whole leaf) are used in traditional medicine because of its biological properties as antibacterial [6], anti-inflammatory[7], antiviral, antitumor[8], antiulcer, antioxidant, antidiabetic[9], laxative, gastrointestinal and skin problems[3], among others[10]. Also it is used in the pharmaceutical, food and cosmetic industry[11]. Nearly all scientific studies on raw-extracts of *A. vera* report beneficial effects; albeit a recent technical report stated that an intake of 1.1-1.3 g/kg non-decolorized whole leaf extract in drinking water cause after 2 years a significant increase of adenomas in the large intestine of F344/N rats, although under the same conditions this effect could not be observed in B6C3F1 mice[12]. Due to these contradictory results doubts have arose about the toxic properties of commercial products of *A. vera*.

In order to know cytotoxic effects of the commercial *A. vera* products, *i.e.* dehydrated and pasteurized inner leaf powder (ILP) and whole leaf powder (WLP), we analysed their cellular effects on HeLa cancer cell line.

METHODS

Commercial products of Aloe vera: The included commercial products of *A. vera*, were pasteurized 200x Dehydrated Inner Leaf Powder (ILP) and 100x Dehydrated Whole Leaf Powder (WLP), and were obtained from a local company in the state of Tamaulipas, México. For the cell culture assays both products were dissolved in cell culture medium with final concentrations of 0.5x, 1x, 5x and 10x; pH was adjusted to 7.3 and 50 µg/ml gentamicine (Sigma Aldrich, Steinheim, Germany) was added to avoid bacterial contamination.

Cell line and culturing conditions: HeLa cells (human cervix-uteri) were obtained from the Immunology Laboratory of the Western Biological Research Centre (CIBO) part of the Mexican Institute from Social Security (IMSS). HeLa was grown in DMEM (ATCC, Manassas, USA) supplemented with 5% fetal bovine serum (FBS) (Biowest LLC, Kansas City, USA) at 37 °C, 4 % CO_2 and 95% rH. Assays were carried out in

12-well microtiter plates (MTP) containing 1 ml medium, which was inoculated with 4∗ 10^4 cells. Depending on the assay, the cells were grown on round cover slides. After 24 h incubating for attaching the cells, the medium was changed for medium containing *A. vera* products. In the positive controls necrosis was induced with 30 µl/ml of 1 M H_2O_2 and apoptosis with 5 µl/ml of 2 mM camptothecin (BioVision, Inc. California, USA). Afterwards the cells were incubated for additional 24 h before the analysis.

WST-1 assay: After the additional 24 h of incubation 20 µl of WST-1 (Clontech, Mountain View, USA) were added into each well and incubated for further 4 h. Then 800 µl of the medium were centrifuged for 1 min at 8,000 *g* (5415D, Eppendorf, Germany) to precipitated possible cell debris and before measuring the absorbance (Biopop, Mecasys, South Korea) at λ 440 nm and λ 690 nm (as background) for each sample.

LDH-release assay: The reaction solution, containing INT, lactate, NAD^+ and diaphorase, was prepared according the instructions of the provider (Clontech, Mountain View, USA) just before its usage. Additionally, for this method the FBS used in the culture medium was heat inactivated (15 min, 60 °C) before use. After the additional 24 h of incubation 200 µl of reaction solution were added into each well, incubated for another hour, then 800 µl of the medium were centrifuged for 1 min at 8,000 *g* and the absorbance was measured at λ 500 nm and λ 690 nm (as background) for each sample.

NRU (Neutral Red Uptake) assay: For this assay the cells were seeded over cover slides. After the additional 24 h of incubation 20 µl of 0.33 % neutral red solution (Santa Cruz, USA) were added into each well and incubated for additional 4 h. Afterwards the cover slides were washed with phosphate buffered saline (PBS) and observed under the microscope (Axioskop 40FL, Zeiss, Germany).

Annexin V assay: The assay-buffer was prepared according to the manufactor's manual (Santa Cruz, USA). For this assay the cells were seeded over cover slides, too. After the additional 24 h of incubation the cells were washed with PBS and then once with 400 µl assay-buffer. Afterwards the cover slide was placed upside down onto a 100 µl-drop of assay-buffer containing Annexin V-FITC and PI (propidium iodide), incubated 15 min at room temperature in the dark, then washed briefly in assay-buffer before being analysed by epifluorescence microscopy (green filter: ex. 475/40 & em. 530/50; red filter: ex. 565/30 & em. 620/60).

RESULTS

Composition of commercial A. Vera products: The preliminary chromatography analysis revealed two peaks in each of commercial product: for ILP 5.82 of aloin and 5.73 ppm of aloe-emodin and for WLP 4.75 ppm of aloin and 1.68 ppm of aloe-emodin (more data no shown). Previous studies have shown that the anthraquinones such as aloe-emodin and aloin are responsible for aloe-induced toxicity against rats[5] or cancer cell lines[5, 10, 13.] Those values detected in both products are much lower than those used in many other studies[10, 12, 14.]

On the other side, there are many studies documenting the benefits when it is used as food supplement. The majority of all these studies were done with *A. vera* raw-extracts, made by the researchers themselves and with the objective to obtain as much active compounds as possible. But for studying the safety aspects of aloe-based products for the consumer, it is important to also include assays on the commercial products, because compositions of the active compounds depend highly on the extraction method.

Cytotoxic activity: The metabolic activity (WST-1) of HeLa, exposed for 24 h to ILP at 5x and 10x, was strongly reduced (Fig. **1**); the EC_{50} was calculated to 2.4x. Nevertheless, an increased membrane

permeability (LDH-release) as an indicator for necrosis could not be observed (Fig. **1**). Similar results were found by NRU.

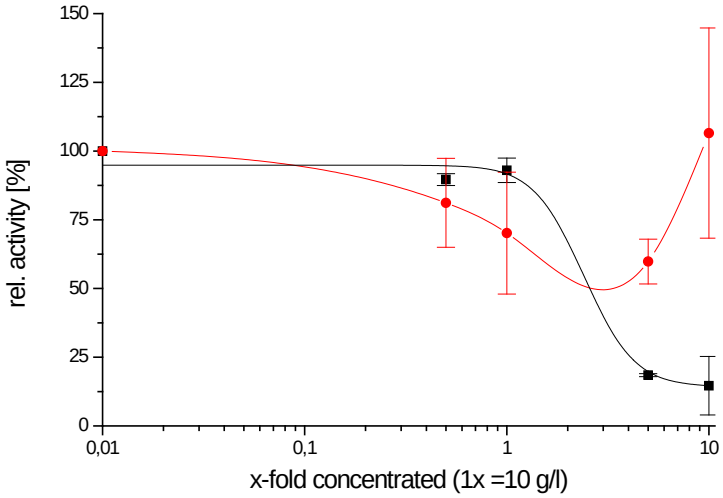

Figure 1: Effects of Inner Leaf Powder on HeLa: the metabolic activity (' WST) was reduced with an EC_{50} = 2.4x, while the membrane permeability (' LDH-release) did not increase.)

At concentrations of 1x or less the cells have red stained lysosomes and normal cell shape (not shown) while 5x is more critical; nearly all cells still have red stained lysosomes, indicating them as functional, but about the half of the cells look deformed (Fig. **2**). At high concentrations of 10x any cell had red stained lysosomes anymore and all of them looked destroyed (not shown). This membrane disintegration could not be documented by the LDH-release test, probably because the extract itself inhibits the test (*e.g.* the required enzyme activity of LDH and/or diaphorase).

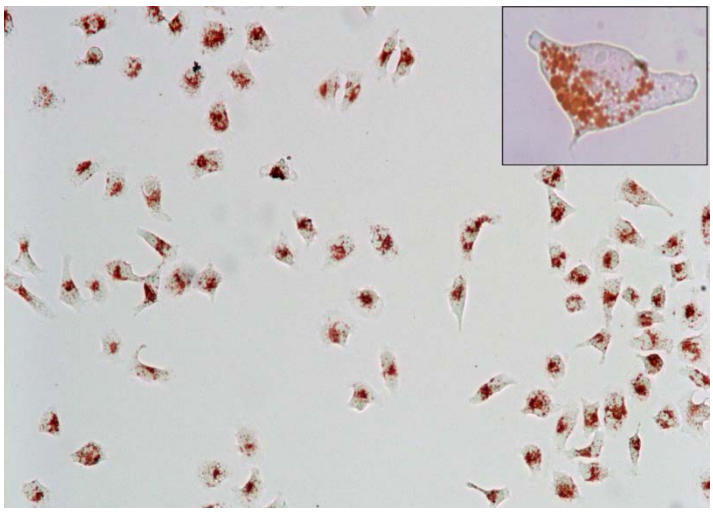

Figure 2: NRU with HeLa at 5x ILP: nearly all cells with red stained lysosomes, indicating metabolic activity; but about the half of the cells have deformed shape. Magnification: 100x, right corner 400x.

For the WLP we found similar results. The metabolic activity (WST) was only decreased by about 50% up to 10x (Fig. **3**) and an increased membrane permeability (LDH-release) was neither measured (Fig. **3**).

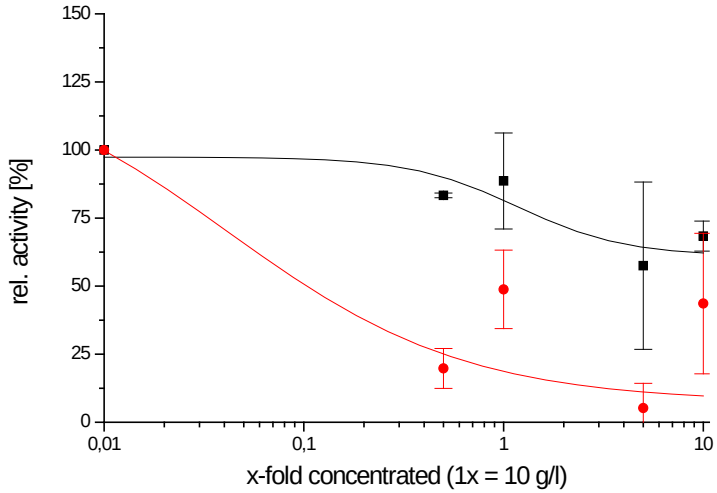

Figure 3: Effects of Whole Leaf Powder on HeLa: Neither the metabolic activity (' WST) was significantly reduced, nor the membrane permeability (' LDH-release) was increased.

But looking at the cells, at 1x HeLa still had red stained lysosomes by NRU-uptake but already at 5x the majority of the cells were deformed and had no functional lysosomes anymore (Fig. **4**). Things worsened at higher concentrations, at 10x any cell had functional lysosomes and all of them were deformed (not shown). This visible effect on the cells could not be demonstrated by the indirect test like WST and LDH-release. Also in this case, probably the high concentrations of substances found in these extracts (and in whole leaf obviously more than in inner leaf) interfere with the test itself. This demonstrates the importance of the direct observation and behaviour of the cells.

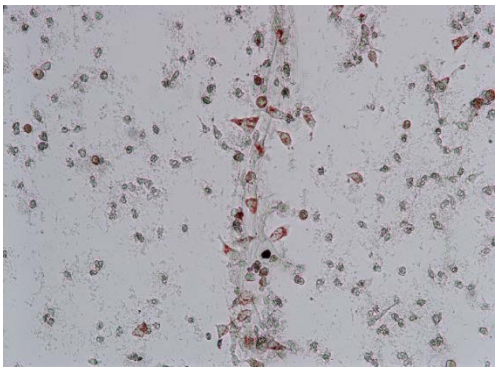

Figure 4: NRU with HeLa and 5x WLP: about 30% of the cells with red stained lysosomes, the rest of the cells look destroyed. Magnification: 100x.

In summary, at the recommended commercial concentration of 1x the retail products did not exhibit cytotoxic effects neither reducing the metabolic activity of the cells (WST, NRU) nor inducing necrosis (LDH-release, PI-staining). Using the products over-concentrated (5x and 10x), we could clearly see cytotoxic effects. The cells had a low metabolic activity and the cells looked necrotic - although this could not be documented by the LDH-release test. Due to the relatively low concentration of aloin and aloin-emodin it is possible that the toxic effect was caused by mechanical stress by the vegetal fibres as have been documented that the *A. vera* leaves are composed mainly of fibres (73.35 %)[15].

Our study about apoptosis induction was done using Annexin V assay. The cells were treated with ILP and WLP and stained with Annexin V-FITC and PI and analysed using a fluorescence microscope. We found that neither 1x ILP (not shown) nor 1x WLP (Fig. **5**) induced apoptosis. By PI-staining even with 1x WLP we found up to 15% of the cells necrotic (red fluorescence; Fig. **5**), while at 1x ILP only 1% of the cells were necrotic using this technique (not shown).

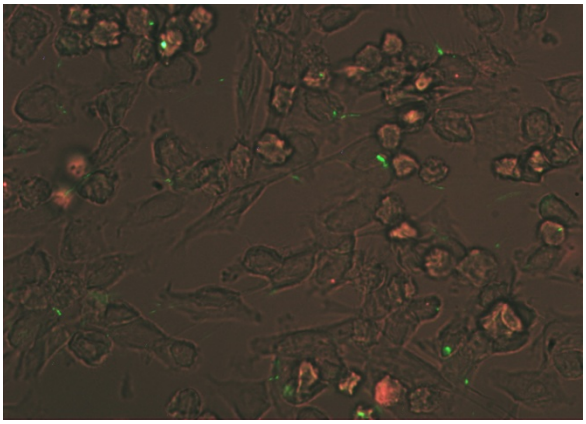

Figure 5: HeLa treated with 1x WLP: Overlay of green (Annexin V-FITC) and red (PI) fluorescence. Any apoptotic effect (green) could be observed, only very few cells were necrotic (red).

Our findings are in accordance with a study done with human Jurkat T lymphocytes cell line 10,[4] where it was shown that *A. vera* supplements, which do not contain aloin, may be safer than those containing aloin. As this study demonstrated an aloin-induced toxicity by an increase of mitochondrial membrane permeability and thus a decrease of mitochondrial membrane potential, we evaluated apoptosis induction with the cancer cell line HeLa. We could not observe any apoptosis inducing effect of the two commercial products at concentrations up to 10x.

CONCLUSIONS

We concluded that the two commercial products, dehydrated inner leaf powder as well as dehydrated whole leaf powder, are not cytotoxic at the recommended use concentration of 1x, which is the retail concentration when used as food supplement. When the products are used 5-fold over-concentrated slight cytotoxic effects could be observed, which changed to strong effects when the products were used 10-fold over-concentrated. On HeLa no apoptotic effect was detected at concentrations up to 10x with both products.

Further cytotoxic analysis will be done, with more commercial products, e.g. decolourised inner leaf and whole leaf, as well as additional human cell lines from colon, liver or kidney, with the objective to evaluate better the risk of its use as food supplement.

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