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Research Article

Antioxidant and Cytotoxicological Effects of *Aloe vera* Food Supplements

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Currently, food industries use supplements from *Aloe vera* as highly concentrated powders (starting products), which are added to the final product at a concentration of 1x, meaning 10 g/L for decolourized and spray-dried whole leaf powder (WLP) or 5 g/L for decolourized and spray-dried inner leaf powder (ILG) and also for nondecolourized and belt-dried inner leaf powder (ILF). Flavonoids, tannins, or saponins could not be detected for any starting product at this concentration and their total phenol concentration of 68–112 μM gallate-eq. was much lower than in fresh extract; however, their antioxidant capacity of 90–123 μM ascorbate-eq. for DPPH was similar to the fresh extract. Starting products, dissolved at 1x, had an aloin concentration of 0.04 to 0.07 ppm, a concentration much lower than the industry standard of 10 ppm for foodstuff. While decolourized starting products (i.e., treated with activated carbon) exhibited low cytotoxicity on HeLa cells (CC_{50} = 15 g/L ILG or 50 g/L WLP), ILF at CC_{50} = 1–5 g/L exhibited cytotoxic effects, that is, at concentrations even below the recommended for human consumption. Probable causes for the cytotoxicity of ILF are the exposure to high temperatures (70–85°C) combined with a high fibre content.

1. Introduction

The *Aloe* genus comprises 581 accepted species [1] that belong to the family Xanthorrhoeaceae which was previously categorized under Liliaceae [2]. *Aloe vera* (L.), a.k.a. *Aloe barbadensis* Miller, is a perennial, leaf-succulent xerophyte. Their leaves consist of an outer waxy cuticle and a single cell layer of epidermis; underneath is an 8–10-cell layer of chlorenchyma cells. The vascular bundles are located between the thick watery inner parenchyma, called *Aloe* fillet, and the thin chlorenchyma [3]; extruded *Aloe* fillet is called *Aloe* gel.

The whole leaf of *A. vera* consists mainly of water, and the residual dry mass of 2.6% is composed of approximately 73.4% carbohydrate fibres, 16.9% ashes (minerals), 6.9% proteins, and 2.9% lipids [4]. Additionally, it contains many secondary metabolites such as complex polyphenols (e.g., tannins and flavonoids), lignins, saponins, anthraquinones, glycoproteins, polysaccharides, and enzymes and also smaller metabolites, like sterols, fatty acids, alcohols, vitamins, amino acids, and saccharides [3, 5, 6]. The *Aloe* gel consists of about 99% water; the residual dry mass is composed of approximately 35.5% crude fibres, 26.8% soluble saccharides, 23.6% ashes (minerals), 8.9% proteins, and 5.1% lipids [7]. More than 95% of the soluble saccharides are glucose; the nonstarch polysaccharides (apart from pectin, cellulose, and hemicellulose) consist mainly of mannose, glucose, and galactose, forming β1,4-linked polymers of 30–40 kDa [7, 8]. The thin intermediate layer, composed of chlorenchyma and vascular bundles, produces a yellow bitter tasting exudate called *Aloe* latex; this liquid contains secondary metabolites such as glycosylated anthrones (up to 35% aloin A and aloin B), glycosylated chromones (aloesin and aloeresin), glycosylated anthraquinones, and polyphenols. Free anthraquinones (aloe-emodin) are only minor components of the exudate and are more abundant in the root [9, 10].

Several positive effects have been attributed to *Aloe* products [11]; in fact, it has been used in traditional medicine for centuries; therefore, the food industry started to use *Aloe* extracts as a supplement for functional food [3, 12]. Typical health claims for this kind of products are to provide relief from rheumatoid arthritis, cancer, diabetes, digestive and intestinal disorders, or ulcers. The related pharmacological actions include anti-inflammatory, antiproliferation, and antimicrobial activity, as well as laxative and blood glucose lowering effects; to prove those claims, *Aloe* gel extracts especially have been analysed by several research groups [8, 13–15].

Aloe latex contains even more secondary metabolites than *Aloe* gel and several effects could be attributed to them, for example, the induction of apoptosis in several cell lines like HL60, HeLa, Jurkat, and MCF-7 [16–19]. Another desired effect is that C_8-hydroxy substituted anthranoids act as laxatives; this effect is caused by changes in the colonic motility and absorption. However, in vitro studies revealed potential mutagenic activity of aloe-emodin in the Ames test and carcinogenic activity of 1-hydroxyanthraquinone in vivo [20]. The safety aspects that have arisen for potential consumers are summarized in the report on the safety assessment of different *Aloe* products: cell culture studies for gel and whole leaf extracts revealed proliferative effects, while the latex fraction had cytotoxic effects [21]. The same report quoted no acute oral toxicity when mice were fed with 3 g/kg of an ethanolic leaf extract; even when rats were fed with 1% or 10% *Aloe* extract in drinking water during a period of 5.5 months, no negative health effects could be observed when the extract was decolourized (i.e., treated with charcoal to eliminate anthranoids); when left untreated, the rats suffered from diarrhoea and a lower growth rate with the 10% extract. Their final conclusion was that *Aloe*-derived products for cosmetic industries (i.e., topical use) are considered safe if the anthraquinone concentration is below 50 ppm [21]. In 2002, the US Food and Drug Administration (FDA) declared that *Aloe* extracts as laxative were not generally recognized as safe and effective and that more evidence was needed [22]. As a consequence, the International Aloe Science Council (IASC) established the industry standard for oral consumption to less than 10 ppm aloin [23]. Moreover, within the National Toxicology Program (NTP), toxicological aspects were reviewed with results pointing to potentially carcinogenic effects: F344/N rats fed during a period of 2 years with 1% (nondecolourized) whole leaf extract in drinking water developed significantly more intestinal adenomas or carcinomas; on the other hand, the same treatment did not significantly increase neoplasms in B6C3F1 mice [24]. This report was heavily criticized, especially by the IASC, because the aloin content with approximately 6500 ppm was more than 650 times higher than the industry standard [25]. Thereafter, Sehgal et al. [26] reported no negative effects (neither on organs nor on blood values) on F344 rats when fed during a period of 13 weeks with charcoal decolourized whole leaf extract, even when the rats consumed 5 to 6 times more *Aloe* extract than the recommended maximum for humans.

Until now, most studies have analysed raw extracts from *A. vera*, but the starting material for food industries is processed in a very different way; thus, the safety aspects for those products have not been fully evaluated, meaning that a risk of adverse outcomes or collateral damage to consumers exists. In order to address this discrepancy, we began to examine cytotoxic effects of starting products produced and commercialized by Mexican facilities of a US enterprise: preliminary studies revealed that those powders were not cytotoxic to the cell line HeLa when up to 12 g/L (2.4x) concentrated inner leaf extract or less than 50 g/L (5x) whole leaf extract was used; both (concentrated) products had only 5-6 ppm of aloin [27]. We then continued by analysing the phytochemical components, antioxidant activity, and their cytotoxic effects in greater detail.

2. Materials and Methods

2.1. Molecular Identification by ITS Sequencing. The genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Briefly, the sample was frozen at −70°C and ground to a fine powder. The nuclei were then lysed (65°C) and the RNA was degraded by adding RNase (37°C). After cooling down to room temperature (RT), the proteins were removed by salt precipitation. After centrifugation, the supernatant was transferred into a new tube to precipitate the genomic DNA with isopropanol; after centrifugation, the pellet was washed with 70% ethanol, air-dried, and finally resuspended in buffer.

The universal primer pair ITS-1 (5′-TCC GTAGGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used in the PCR (Polymerase Chain Reaction) to amplify the polymorphic Internal Transcribed Spacers (ITS1 and ITS2) and the intervening 5.8S rRNA gene region as described by White et al. [28]. The PCR products of approximately 650 bp were purified with the ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, USA) and sequenced by the Sequencing Laboratory of the Center of Genomic Biotechnology of the Instituto Politécnico Nacional (Cd. Reynosa, Mexico). The sequence was compared to reported sequences of the GenBank database using the BLAST (Basic Local Alignment Search Tool) algorithm [29], available at the NCBI (National Center for Biotechnology Information) website. Finally, a neighbour-joining phylogenetic tree was calculated with the program CLC Sequence Viewer 7.6.

2.2. Starting Products of Aloe vera. A US enterprise provided the starting products (powders) from its facilities in Mexico, which are commercialized as food supplements of *A. vera* to food processing industries. The whole leaf was mashed, filtered and pasteurized (HTST-juice), decolourized with activated charcoal, filtered through diatomaceous earth, and dehydrated by spray-drying resulting in 100x decolourized whole leaf powder (WLP). The inner leaf fillet was washed and mashed, and the juice was decolourized, filtered through diatomaceous earth, and dehydrated by spray-drying: 200x inner leaf gel (ILG) powder or the inner leaf fillet was washed, dehydrated by belt-drying, and crushed resulting in

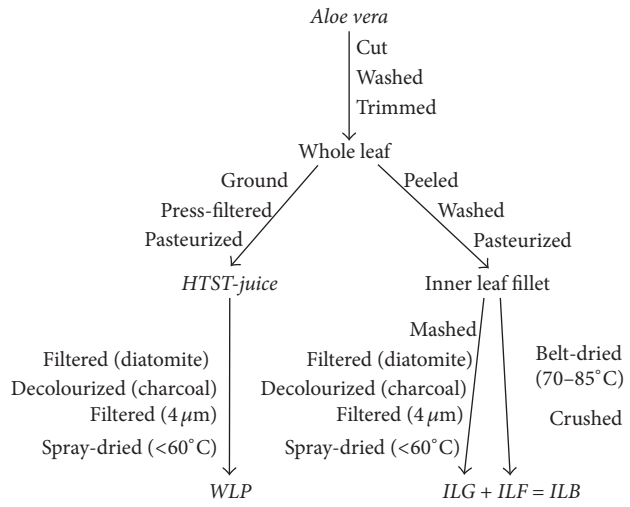

Figure 1: Sample preparation scheme. Leaves from *Aloe vera* are cut, washed, and trimmed to get the whole leaves. For HTST-juice, the whole leaves are ground to a particulate slurry, which is press-filtered, and the resulting juice is pasteurized. Whole leaf powder is produced by filtration of HTST-juice through diatomaceous earth, and the aloin content is then reduced by charcoal treatment, which itself is removed by another filtration; the resulting liquid is spray-dried resulting in a 100x concentrated powder (WLP). The whole leaf can also be peeled, washed, and pasteurized to get the inner leaf fillet. This fillet can be mashed and then treated like HTST-juice to get a 200x concentrated inner leaf gel (ILG) powder, or it can be (probably sliced) belt-dried and crushed to get a 200x concentrated inner leaf gel powder, rich in fibres (ILF). The main difference between WLP/ILG and ILF is that the latter has no charcoal treatment and is exposed to higher temperatures during the drying process. The 1 : 1 mixture of ILG and ILF results in a 200x inner leaf blended (ILB) powder.

a powder with a high fibre content: 200x dehydrated inner leaf powder with fibre (ILF) (Figure 1). The fourth sample, inner leaf powder blended (ILB), is a mixture of ILG and ILF. The powders were dissolved in water or culture medium to different concentrations; the enterprise recommended a concentration of 1x for their products, meaning 5 g/L for the 200x samples (ILG, ILB, and ILF) or 10 g/L for the 100x sample (WLP). When indicated, samples were centrifuged for 20 min at 15,000*g* or filtered through a nylon membrane with 0.20 μm pore size to reduce the fibre content. The liquid HTST-juice is a preproduct and is not commercialized by this enterprise and was used by us as an internal control (nearly a nonprocessed extract, similar to a raw extract).

2.3. Phytochemical Screening. For the qualitative analysis of flavonoids, tannins, terpenoids, and saponins [30], the samples were dissolved in distilled water (0.5 g/mL WLP, 0.25 g/mL ILG, 0.17 g/mL ILB, and 0.1 g/mL ILF; 1 : 1 v/v HTST-juice) and shaken (80 rpm) for 12 h at RT in the dark and subsequently filtered (Whatman No. 4). Only positive tested samples were quantified using concentrations of 10 g/L for WLP and 5 g/L for ILG, ILB, or ILF; HTST-juice was diluted at 1 : 7 (v/v).

Flavonoids. 1 mL of each sample was mixed with 5 mL 1% NH_4Cl, and then 5 drops of conc. H_2SO_4 were added. An unstable yellow colour was formed in the presence of flavonoids.

Tannins. 1 mL of each sample was boiled for 2 min, and then 5 drops of 0.1% $FeCl_3$ were added forming a brownish green or blue-black colour in the presence of tannins.

Terpenoids. 5 mL of each sample was mixed with 2 mL dichloromethane, and then 3 mL of conc. H_2SO_4 was added forming a stable layer. A reddish-brown colour in the interface indicates positive results.

Saponins. 5 mL of each sample was boiled for 2 min, diluted with water, and shaken to form foam; then, 3 drops of olive oil were added and shaken vigorously. The formation of an emulsion indicated the presence of saponins.

Quantification of Total Phenols. 75 μL of each centrifuged sample was mixed with 1.2 mL water and 75 μL water-Folin-Ciocalteu reagent (1 : 1). After 3 min, 150 μL of 1 N Na_2CO_3 was added and then incubated for 2 h at RT in the dark. A light violet colour was quantified with a photometer (Mecasys Optizen-Pop, Daejeon, South Korea) at 725 nm. The standard curve was prepared with up to 1.176 mM gallate (#G7384, Sigma-Aldrich, St. Louis, USA) [31].

Quantification of Flavonoids. 100 μL of each sample was diluted with 500 μL distilled water, and then 30 μL of 5% $NaNO_2$, after 6 min 60 μL of 10% $AlCl_3 \cdot 6\,H_2O$, and after further 5 min 200 μL of 1 M NaOH were added. This mixture was filled up to 1 mL with distilled water before the absorbance at λ = 510 nm was quantified with a photometer (Optizen-Pop). Up to 0.34 mM (+)-catechin (#C1251, Sigma) was used as standard [32].

2.4. Chromatographic Analysis. The analytical standards, aloin (#B6906) and aloe-emodin (#93938), were obtained from Sigma-Aldrich, and the necessary solvents were purchased from J. T. Baker (Center Valley, USA). The standards and samples were analysed by high pressure liquid chromatography (HPLC) using an Agilent 1100 series with diode array detector (Agilent, Santa Clara, USA) and a Kromasil C-18 column (#K08670357, Bohus, Sweden): (1) aloin (acetonitrile-water, 40 : 60 v/v; flow rate 0.6 mL/min; detection at 360 nm; run time of 25 min) and (2) aloe-emodin (methanol-water, 70/30 v/v; flow rate 0.6 mL/min; detection at 254 nm; run time of 25 min). 0.5 g or 0.5 mL of each sample was extracted with 5 mL 70% ethanol for 30 min and filtered (0.45 μm) before HPLC analysis. 20 μL standard or sample was injected with an isocratic elution gradient at RT. A calibration curve was done for each standard at concentrations from 0.5 to 5 ppm.

2.5. Antioxidant Capacity. 10 g/L of WLP and 5 g/L of ILG, ILB, or ILF were dissolved in distilled water, and HTST-juice was diluted at 1 : 7 (v/v). All standard curves were prepared with up to 400 μM ascorbate (#11140, Sigma).

DPPH. For a stock solution, 19.7 mg of 2,2-diphenyl-1-picrylhydrazyl (#D9132, Sigma) was dissolved in 100 mL methanol, which, diluted at 1 : 4.55 to a working solution, had an absorbance of ~1.38 at λ = 515 nm. 100 μL of each sample was mixed with 900 μL DPPH working solution, stored for 24 h at RT in the dark, and centrifuged (1 min at 15,000*g*) and the absorbance was measured at λ = 515 nm.

ABTS. 22.6 mg of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (#11557, Sigma) was dissolved in 10 mL acetate buffer (20 mM, pH 4.5) and then mixed with 10 mL of 1.44 mM $(NH_4)_2S_2O_8$ in acetate buffer. This ABTS-working solution was incubated for 12–16 h at RT in the dark and then diluted at 1 : 25 with acetate buffer; the absorbance was ~1.24 at λ = 734 nm. 100 μL of each sample was mixed with 900 μL ABTS-working solution and incubated for 4 h at 37°C in the dark and the absorbance was measured at λ = 734 nm.

FRAP. The ferric reducing antioxidant power-working solution was made by mixing 2.5 mL of TPTZ (10 mM 2,4,6-tris(2-pyridyl-s-triazine) [#93285, Sigma] in 40 mM HCl) and 2.5 mL of 20 mM $FeCl_3 \cdot 6\ H_2O$ in distilled water with 25 mL of acetate buffer (300 mM, pH 3.6). 100 μL of each sample was mixed with 900 μL FRAP-working solution and incubated for 2 h at 37°C in the dark and the absorbance was measured at λ = 593 nm.

2.6. Cell Culture

Cell Line and Culturing Conditions. The cell line HeLa (human cervix-uteri) was purchased from ATCC (Manassas, USA). HeLa was grown in DMEM (ATCC) supplemented with 10% FBS (Biowest, Kansas City, USA) at 37°C, 4% CO_2, and 95% RH. The samples were dissolved in concentrations up to 100 g/L WLP or 50 g/L ILG, ILB, and ILF (10x) in culture medium; pH was adjusted with NaOH (1 M) to pH 7.3 and 50 μg/mL of gentamycine (Sigma-Aldrich) was added to avoid bacterial growth. HTST-juice was directly added to the medium without using antibiotics. The experiments were previously described [27]; in short, the cells were seeded over cover slides in 12-well microtiter plates (MTP) and incubated for 1 d to let them attach, the samples were added or the medium was changed for sample-containing medium, and then the cells were grown for one more day prior to analysis.

Cell Viability and Necrosis Induction. The metabolic activity was determined by neutral red uptake (NRU): 20 μL/mL of 0.33% neutral red solution (Santa Cruz, USA) was added and incubated for an additional 4 h. Afterwards, the cover slides were washed once with phosphate buffered saline (PBS) and observed under the microscope (Axioskop 40FL, Zeiss, Oberkochen, Germany). For quantification, after adding 30 μL/mL of 0.33% neutral red solution and incubation, the cells were washed once with PBS and the neutral red was extracted with 800 μL of 1% acetic acid in 50% ethanol during a period of 15 min. The supernatant was centrifuged for 3 min at 10,000*g* (Eppendorf 5415D, Hamburg, Germany) and the absorbance was measured at λ = 690 nm. For the WST-quantifying method, the cells were exposed to the sample and the medium was then changed for a fresh culture medium. Then, 20 μL/mL of WST-1 (Clontech, Mountain View, USA) was added and the cells were incubated for an additional 4 h. Finally, 800 μL of the medium was centrifuged (1 min, 8,000*g*) to precipitate possible cell debris before measuring the absorbance at λ = 440 nm and λ = 690 nm (as background). To detect necrosis, 0.05% (final concentration) trypan blue (Biowest) was added and incubated for 5 to 10 min, and the cover slides were washed with PBS and then observed under the microscope.

Table 1: Concentration of aloin and aloe-emodin in different commercial starting products at the concentration 1x compared to the raw extract (HTST-juice). Values are expressed as mean ± 1,96∗SEM (n = 3).

Sample (1x)	Aloin (μg/mL)	Aloe-emodin (μg/mL)
ILG	0.036 ± 0.012	0.003 ± 0.004
ILB	0.069 ± 0.007	0.050 ± 0.008
ILF	0.053 ± 0.002	0.130 ± 0.010
WLP	0.046 ± 0.011	0.009 ± 0.018
HTST-juice	10.04 ± 0.82	7.57 ± 0.47

2.7. Statistical Analysis.

Values are expressed as means ± 1.96 ∗ standard error of the mean (1.96∗SEM). Differences between groups were determined by one-way analysis of variance (ANOVA) and subjected post hoc to Tukey's HSD multiple comparison tests using the program SPSS 19. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Phytochemical Analysis.

Plant material from the enterprise, which also provided the starting products, was identified by ITS sequencing as *Aloe vera* with 98% identity to the sample KC893746 of the NCBI GenBank database.

Most secondary metabolites are found in the *Aloe* latex; representative metabolites were analysed by HPLC to prove whether the decolourization process for the starting products was efficient and whether those products fulfilled the IASC standard of < 10 ppm aloin. The aloin content ranged from 4.6 to 13.8 μg/g dry weight for the 100x or 200x concentrated powders; when those samples were diluted to the recommended concentration of 1x (i.e., 10 g/L for WLP or 5 g/L for ILG, ILB, and ILF), the aloin concentration diminished accordingly to only 0.04–0.07 μg/mL. Those values were considerably lower than the ~10 μg/mL of the HTST-juice (Table 1). The anthraquinone aloe-emodin was found in similar concentrations (0.003–0.13 μg/mL) in 1x solutions (i.e., 10 g/L for WLP or 5 g/L for ILG, ILB, and ILF) and was high only in the HTST-juice (Table 1).

Tannins or saponins were not detected in those starting products and a hint of terpenoids was observable only in HTST-juice. Flavonoids were clearly detectable in HTST-juice, barely detectable in WLP, ILG, and ILB, and even absent in ILF. The quantitative analysis for *Aloe* gel powders revealed that the total phenol concentration decreased in conjunction

Table 2: Concentration of total phenols and flavonoids in different commercial starting products at the concentration 1x compared to the raw extract (HTST-juice). Values are expressed as mean ± 1,96∗SEM (n = 9 for total phenols, n = 3 for flavonoids); ND: not detected.

Sample (1x)	ILG	ILB	ILF	WLP	HTST-juice
Total phenols [μM gallate-eq.]	111.7 ± 15.5	92.3 ± 15.0	57.0 ± 15.8	85.7 ± 14.1	2,712.4 ± 87.7
Flavonoids [μM catechin-eq.]	ND	ND	ND	ND	38.2 ± 4.1

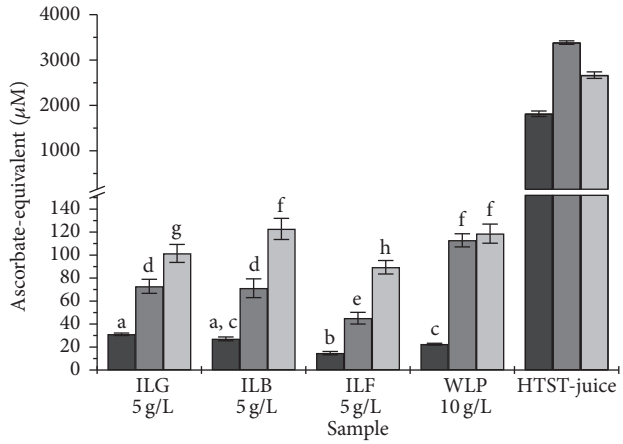

Figure 2: Antioxidant capacity of different starting products at the concentrations of 5 g/L for ILG, ILB, and ILF and 10 g/L for WLP (i.e., 1x) and HTST-juice measured as FRAP (dark grey), ABTS (grey), and DPPH (light grey). Values are expressed as mean ± 1,96∗SEM (n = 7–9). Letters indicate statistically significant differences.

with an increase of the fibre content (Table 2) accordingly. The HTST-juice had 25 times more total phenols than the starting products. Consistent with the qualitative analysis, the quantification of flavonoids resulted to be positive only for the HTST-juice; for all other samples, the concentration was below the detection limit (Table 2).

3.2. Antioxidant Capacity (AOC). AOC was determined by three independent methods: FRAP and ABTS, which measure mainly water soluble antioxidants with Single Electron Transfer (SET), and DPPH, which detects methanol soluble antioxidants with SET and H-Atom-Transfer (HAT) mechanisms. A rather consistent observation for all three methods was found in the *Aloe* gel samples: the more fibre the product had, the lower the AOC; that is, AOC was decreasing from ILG via ILB to ILF (Figure 2). WLP had a similar AOC to ILB; only the HTST-juice exhibited 20 to 30 times higher (DPPH and ABTS, resp.) and even 80 times higher (FRAP) AOC than the starting products (Figure 2).

3.3. Cell Viability and Necrosis. Results for HeLa viability for the WLP and ILG samples have been previously reported by us [27]. ILB inhibited metabolic activity of HeLa (NRU) and induced necrosis (trypan blue) at a concentration of 15 g/L (3x) (Figure 4) and ILF at even 5 g/L (1x) (Figure 3), the recommended concentration for human consumption. Moreover, at 5 g/L ILF, only very few cells were still present and much debris and fibres could be seen (Figure 3). This effect was observed not only after 24 h of incubation, but already after 4 h (data not shown), which means that ILF acts very fast. Only a further reduction of the ILF concentration to 0.5 g/L abolished the cytotoxic effect (Figure 3). In order to narrow the cytotoxic principle, the sample was centrifuged or filtered. The cytotoxicity for ILF decreased slightly: now, at 5 g/L ILF, few cells (and less fibres) could be seen and exhibited metabolic activity visualized as NRU (Figure 3). Centrifugation of ILB hardly reduced its cytotoxicity: compared to the noncentrifuged sample, at 15 g/L of centrifuged ILB (3x), some more cells were visible, yet many of them were necrotic (Figure 4); thus, centrifugation to reduce the fibre content was less effective for the sample, which contained a priori less fibres.

Those effects could not be quantified directly using the WST test; the sample probably interfered with the tetrazolium salt (and for the same reason LDH release as a necrosis indicator could not be determined). To address this problem, the standard protocol was changed slightly: after 4 h of exposure, the ILF-containing medium was changed for fresh medium and then WST was added to determine metabolic activity. The cytotoxicity could be determined like this to $CC_{50} \approx 1$ g/L ILF (0.2x). Centrifugation as well as filtration slightly reduced the cytotoxic effect, for both techniques, to $CC_{50} \approx 2.25$ g/L ILF (Figure 5), which is still below the recommended 5 g/L for human consumption.

30 to 80 μL/mL of HTST-juice reduced the metabolic activity of HeLa to 50% of the control, measured quantitatively as NRU. Observing the cells by the microscope revealed that at 60 μL/mL HeLa was metabolically still active (red stained with neutral red), at 100 μL/mL it was nearly inactive (faint red) yet normally shaped, and at 300 μL/mL only few cells were seen and were not metabolically active (Figure 6). As HTST-juice has ~10 μg/mL aloin, the cells had been exposed to ~1 μg/mL aloin when metabolic activity declined (at 100 μL/mL HTST-juice); but when the cells were exposed directly to aloin, they did not show any signs of cytotoxic effects even at concentrations up to 50 μg/mL (Figure 6).

4. Discussion

Starting products for foodstuff from *Aloe vera* are mostly commercialized as concentrated powders, but the quality among them can vary considerably. The products can be old (<10% of polysaccharide acemannan) and suffer from bacterial fermentation (>10% lactate) or other adulterations (typically with maltodextrin or citrate). Previous analysis

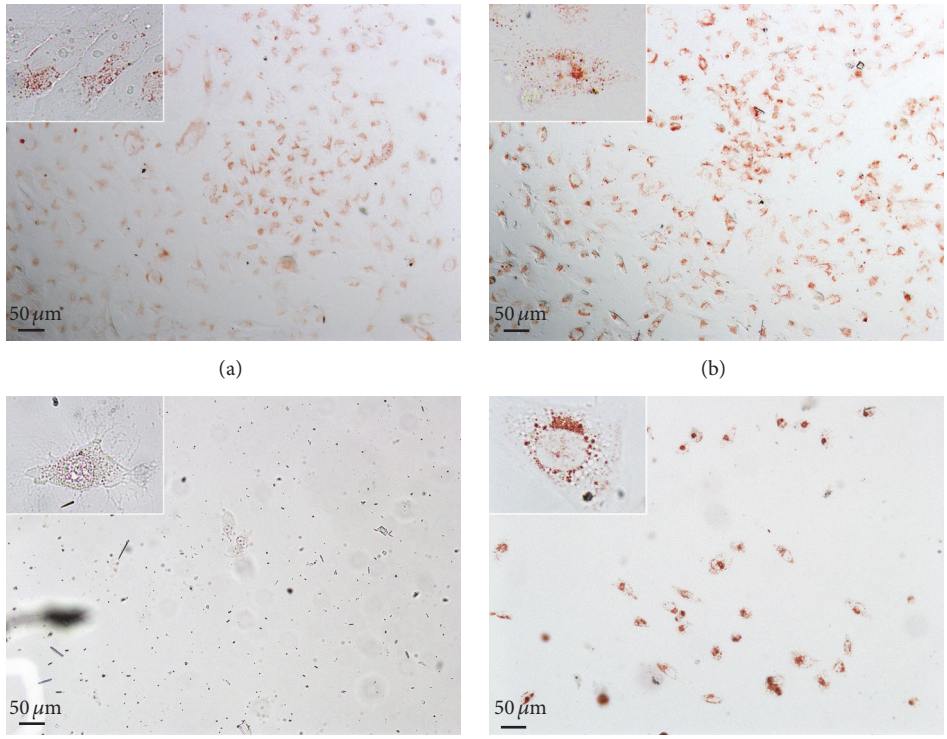

Figure 3: Effect of ILF on HeLa cells measured as NRU; all images at 100x magnification and 400x magnification at the upper left corner. Growth control w/o ILF (a), at 0.5 g/L (b), at 5 g/L (c), and at 5 g/L centrifugation (d). The recommended concentration for human consumption is 5 g/L (1x). Red stained cells indicate metabolic activity and functional lysosomes.

of those products revealed large differences among them, although all have been certified by IASC. For instance, the acemannan content of different 200x inner leaf gel powders ranged between 4 and 9% and the aloin concentration varied between "not detectable" and up to 15 ppm [33]. The aloin concentrations for our samples ranged from 4.6 to 13.8 μg/g dry weight; but as the samples are 100 or 200 times concentrated, the aloin concentration in the final product for the consumer (meaning 10 g/L for WLP or 5 g/L for ILG, ILB, and ILF, i.e., at 1x) ranged between 0.04 and 0.07 μg/mL. A comparison to the aloin content of the HTST-juice (~ 10 μg/mL) demonstrates that not only the decolourization but also the filleting and dehydration process is very effective in reducing the aloin content. It is known that heat treatment (as of 80°C) halves the aloin content of fresh *Aloe* gel juice within 4 h [34]. The biological activity for the highly concentrated powders cannot be estimated according to their chemical constitution as can readily be done for raw extracts [35]. Therefore, the principal constituents of our samples, regarding potentially bioactive molecules, were determined: tannins, saponins, and terpenoids were not detected in any of the highly concentrated powders. For fresh *Aloe* gel, Lucini et al. [5] determined 12.76 mmol/kg gallate-eq. total phenols and 0.16 mmol/kg rutin-eq. flavonoids. For the HTST-juice, we found with 2.7 mM gallate-eq. total phenols and 0.04 mM catechin-eq. flavonoids similar values; but in our powder samples, in 10 g/L WLP and 5 g/L ILG, ILB, or ILF (1x), respectively, we found only 0.07 to 0.11 mM gallate-eq. total phenols and flavonoids could not be detected. This indicates that the treatment for reducing the aloin content also reduces the content of other biologically active compounds. Many studies on the effects of *A. vera* were done with fresh material, but as the constitution of those starting products differs considerably from the fresh material, their properties should be analysed in order to address (or not) effects to end products.

For all four powder samples, when dissolved at 10 g/L WLP or 5 g/L ILG, ILB, and ILF (1x), we found AOC values ranging from 89.4 to 122.8 μM ascorbate-eq. for the DPPH test. Lucini et al. [5], using the same test, measured 108 μM trolox-eq. for fresh whole leaf extract and 54 μM trolox-eq. for *Aloe* gel extract; those values are comparable because ascorbate and trolox have nearly the same redox potential [36]. The decolourization process, used to lower the aloin concentration, also reduced the total phenol and the flavonoid content and, along with this, the AOC. Only the ILF powder is not decolourized but dried by heat; compared to the decolourized sample (ILG), ILF has a higher aloin and aloe-emodin content but less total phenols and thus the lowest AOC of all the samples.

To evaluate the consequences of these constitutional differences among the samples, we performed cytotoxicological studies: we differentiated between a reduction of metabolic activity (WST and NRU) and necrosis induction (trypan

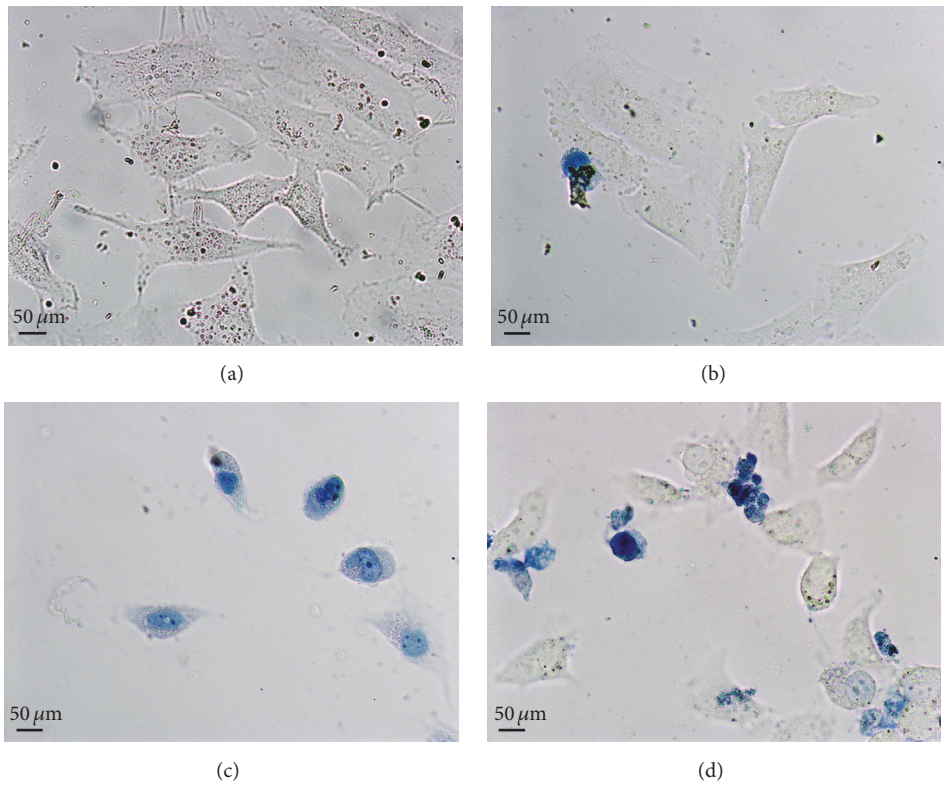

Figure 4: Effect of ILB on HeLa cells measured as trypan blue; all images at 400x magnification. Growth control w/o ILB (a), at 5 g/L (b), at 15 g/L (c), and at 15 g/L centrifugation (d). The recommended concentration for human consumption is 5 g/L (1x). Blue stained cells indicate necrotic cell death.

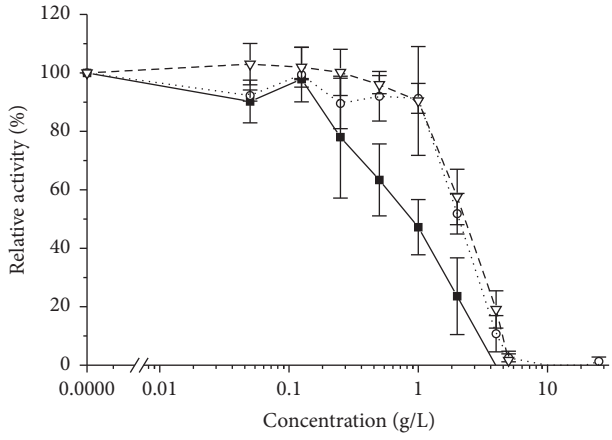

Figure 5: Effects of ILF (square), centrifuged ILF (circle), and filtered ILF (triangle) on the metabolic activity of HeLa quantified as relative WST activity. Growth control activity was set to 100% for each experiment. The recommended concentration for human consumption is 5 g/L (1x). Values are expressed as mean ± 1,96∗SEM (n = 3; for ILF filtered n = 4).

blue). In our case, the lowest cytotoxicity on HeLa was exhibited by both decolourized samples, with 50 g/L WLP (5x) or 25 g/L ILG (5x), followed by approximately 15 g/L ILB (3x); ILF showed cytotoxic effects in a range of 1–5 g/L (0.2–1x). But as HeLa was not inhibited by up to 50 ppm aloin, we conclude that aloin itself is not responsible for cytotoxicity of the samples. Moreover, flavonoids, tannins, and saponins were not detected in the sample powders; therefore, these compounds should not contribute to cytotoxicity either. The difference between the gel fillet samples is that while ILF is a washed, heat-dried, and powdered fillet that still contains all of the *Aloe* gel fibres, ILG is a decolourized and spray-dried fillet extract whose *Aloe* gel fibres are mostly removed when making an extract (Figure 1); samples comparable to ILG have a fibre content ranging from 3.0 to 6.4%, while samples similar to ILF have up to 18.4% fibre content [35]. These fibres contain, among other polysaccharides, acemannans, a polymer of C_2, C_3, or C_6 O-acetylated mannose, interspaced by a few glucose residues and branched by some α-1,6-bond galactose residues [7, 8]. To prove a possible cytotoxic effect by the fibres alone, the ILF sample was centrifuged or filtered and the cytotoxicity reduced slightly from CC_{50} = 1 g/L for the nontreated to about CC_{50} = 2.25 g/L for the centrifuged or filtered sample. Thus, by centrifugation or filtration, the cytotoxic component could not be readily removed. Another major difference between the samples is the heat treatment, which causes changes in the acemannan composition compared to fresh *Aloe* gel. When *Aloe* gel is dehydrated, debranching reactions occur at temperatures starting at 60°C, especially for terminal galactose; at temperatures starting at 70°C, deacetylation reactions increase and the polymers gain molecular weight; this is followed by browning reactions, which start at 80°C [37]. The changes for

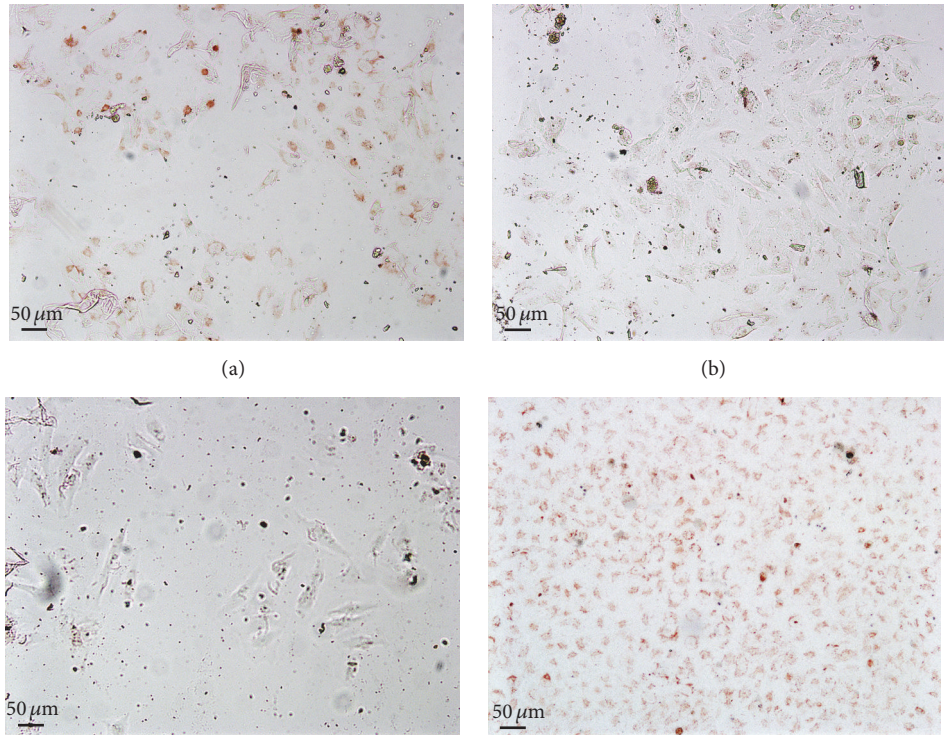

Figure 6: Effects of HTST-juice on HeLa measured as NRU; red stained cells indicate metabolic activity (magnification: 100x): 60 μL/mL (a), 100 μL/mL (b), 300 μL/mL (c), and HTST-juice and 50 μg/mL aloin (d).

pasteurized *Aloe* gel are similar: deacetylation and debranching reactions are observed as well as molecular weight gains [38]. Deacetylation reactions cause major structural changes within acemannans, which affect their biological activity, perhaps because of an altered affinity to members of the mannose receptor family; however, cytotoxic effects were not observed [39]. Thus, it is likely that the fibre itself, and even after being altered by heat treatment, did not induce the cytotoxic effect either. On the other hand, the browning of *Aloe* samples at higher temperatures indicates that complex Maillard reactions occurred, although the precise changes, which introduce those reactions, still remain unclear. The ILF sample received a heat treatment (belt-drying) at higher temperatures (70–85°C) than all other samples, and thus it is reasonable that the heat treatment, possibly combined with a high fibre content, introduces a toxic principle; the exact nature and mode of action remain to be elucidated. We found only one study on starting products, which reported a cytotoxic concentration of CC_{50} = 0.27 g/L^1 for a heat-dried gel powder on HeLa [40], which is in a similar range to what we have found for ILF. On the other hand, the same study reported a cytotoxic concentration of CC_{50} = 0.41 g/L^1 for a whole leaf powder, which would be considerably lower than our result of about 50 g/L WLP (5x).

Finally, we could show that the standard procedures to reduce the aloin content also reduced other biologically active compounds, for instance, tannins, saponins, and terpenoids. Nevertheless, the antioxidant capacity was still comparable to fresh extracts. On the other hand, we detected that the fibre content correlated with a very fast (less than 4 h) cytotoxic effect and could exclude the aloin content being responsible for that. The strong cytotoxic effect is probably not caused exclusively by the high fibre content itself, but by chemical reactions that occur when those fibres are exposed to heat treatment as was done for ILF; however, the nature of the cytotoxic substance has yet to be elucidated. Therefore, more (cyto)toxicological studies with starting products should be done, especially when those products receive a heat treatment and contain a high fibre content.

Abbreviations

ABTS:	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)
AOC:	Antioxidant capacity
ATCC:	American Type Culture Collection
DMEM:	Dulbecco's modified Eagle's medium
DPPH:	2,2-Diphenyl-1-picrylhydrazyl
FBS:	Fetal bovine serum
FDA:	Food and Drug Administration
FRAP:	Ferric reducing antioxidant power
HPLC:	High pressure liquid chromatography
HTST:	High temperature short time
ILB:	*Aloe* inner leaf blended powder
ILF:	*Aloe* inner leaf fibre powder
ILG:	*Aloe* inner leaf gel powder
ISAC:	International Aloe Science Council
NRU:	Neutral red uptake

NTP: National Toxicology Program
PBS: Phosphate buffered saline
RH: Relative humidity
RT: Room temperature
WLP: *Aloe* whole leaf powder
WST: Water soluble tetrazolium.

Disclosure

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Competing Interests

The authors report no competing interests regarding the publication of this paper.

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Endnotes

1. We assume these concentrations. The authors stated in the Materials and Methods that they dissolved 10 g/L = 10,000 mg/L powder in culture medium (resulting for a typical 100x concentrated starting product powder in a final concentration of 1x) and from this solution they made serial dilutions, which (as we suppose) range from 0.01 to 1,000 mg/L. The authors claim that the dilutions range from 0.01 to 1,000 mg/mL = g/L, but then the highest concentration (i.e., 1,000 mg/mL = 1,000 g/L = 1 kg/L) would mean a 100-fold higher concentration than the initial solution (and it is hard to imagine dissolving 1 kg 100x *Aloe* powder in 1 L of water or medium); therefore, we suppose a typo (mL versus L).

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Aloe barbadensis leaf, *Aloe barbadensis* leaf extract, *Aloe barbadensis* leaf juice, *Aloe barbadensis* leaf polysaccharides, *Aloe barbadensis* leaf water, *Aloe ferox* leaf extract, *Aloe ferox* leaf juice and *Aloe ferox* leaf juice extract," *International Journal of Toxicology*, vol. 26, no. 2, pp. 1–50, 2007.

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