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Biogenic amines and HL60 citotoxicity of alfalfa and fenugreek sprouts

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Abstract

The use of germinated seeds as food originated in far east countries and has recently spread to the western world where they are seen as fresh and healthy ingredients. While sprouted alfalfa is widely consumed, sprouted fenugreek seeds are not commonly produced, yet could be active ingredients for blood glucose and cholesterol control. As part of a safety evaluation of sprouted alfalfa and fenugreek flours, as novel ingredients for use in functional foods, their contents of biogenic amines and HL60 cytotoxicity were studied. Alfalfa (*Medicago sativa*) and fenugreek (*Trigonella foenum-graecum*) were germinated or 4 days at 20 °C and 30 °C, with and without light. Ungerminated seeds contained putrescine, cadaverine, histamine, tyramine, spermidine and spermine. Bioactive amine levels found in alfalfa sprouts were twice higher than those found in raw seeds and germination at 20 °C without light provided the lowest levels of total biogenic amines. In sprouted fenugreek, only putrescine and cadaverine increased during germination and temperature and light exposure brought about little change. The amount of biogenic amines in sprout seeds was always below acceptable healthy levels. Results obtained in HL60 leukemic cells showed apoptosis, cell proliferation and cell viability values similar to those found for distilled water and no toxic effects were found. The results provide support for the use of germinated alfalfa and fenugreek seeds as ingredients in functional foods.

Keywords: Alfalfa; Fenugreek; Sprouts; Biogenic amines; HL60 citotoxicity

1. Introduction

Seed germination is a traditional practice that has long been carried out in eastern countries. At present, sprouts are becoming a fashionable food in western countries because consumers demand light (low calorie) and healthy safe foods, and these novel products are considered as fresh and dietetically useful (Kuo, Rozan, Lambein, Frías, & Vidal-Valverde, 2004; Rozan, Kuo, & Lambein, 2001, 2000). Seedlings are easily available in European food shops and they provide many legume and cruciferous species, such as soybean, mungbean, garden pea, lentil, alfalfa (lucerne), fenugreek (alhova), cress and radish. Seedlings are often consumed in fresh salads or as decorative appetisers and, despite abundant reports that sprouts improve nutritional value compared with unprocessed seeds, either by increasing digestibility (Kuo et al., 2004; Prodanov, Sierra, & Vidal-Valverde, 1997; Sierra & Vidal-Valverde, 1999; Urbano et al., 1995; Vidal-Valverde & Frias, 1992) or by reducing antinutritional factors (Frias, Díaz-Pollán, Hedley, & Vidal-Valverde, 1995, 1996; Hooda & Jood, 2003), little has been documented on the content of bioactive amines or effects on living cells of these so- called "healthy food".

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Santos, 1996) and, also, as a consequence of microbial

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activity, by enzymatic decarboxylation of corresponding amino acids (Glória, 2005; Halàsz, Baràth, Simon-Sarkadi, & Holzapfel, 1994). They are organic bases of low molecular weight that can be found in plant food since they are required in cellular metabolism and in growing tissues (Matilla, 1996; Santos, 1996) Polyamines, such spermidine, spermine and their diamine obligate precursor putrescine, are ubiquitous in all plant cells. They are formed by *de novo* synthesis during plant growth, as indispensable components of living cells and important in the regulation of nucleic acid function and protein synthesis (Bouchereau, Aziz, Larher, & Martin-Tanguy, 1999; Smith, 1981). They may also have functions in cell differentiation and membrane stabilization and prevent lipid peroxidation by scavenging free radicals (Kebski, Geuns, & De Proft, 1999; Tabor & Tabor, 1984). In bacteria and plants, putrescine is also involved in the control of cell pH (Smith, 1981).

Biogenic amines, such as histamine, tyramine and cadaverine can be naturally present in some plant tissues and their distribution has proved useful in chemotaxonomic studies (Bouchereau et al., 1999). The metabolic function of these amines in plants is not well established, though they are the precursors of a series of alkaloids and they likely benefit the plant by acting as deterrents to predators; therefore, they have agricultural significance. Many factors can alter the levels of biogenic amines, including ripening, storage conditions, variety of seeds and conditions of growth (Bouchereau et al., 1999).

In virtually all foods that contain proteins or free amino acids, and that are subjected to conditions enabling biochemical and/or microbial activity, the presence of biogenic amines can be expected. Therefore, knowledge of the level of biogenic amines in food products is needed. In mature seeds, different authors have reported a large variation in biogenic amines, not only among species (Shalaby, 2000) but also among cultivars and year of cultivation (Glória, Tavares-Neto, Labanca, & Carvalho, 2005). In four commercial mung bean sprouts, putrescine, cadaverine, spermidine and spermine were detected and levels found varied depending on the producer. Some papers have related the biogenic amines to the microbial quality of sprouts (Skowronek, Simon-Sarkadi, & Holzapfel, 1998; Simon-Sarkadi & Holzapfel, 1995). However, the presence of biogenic amines in food does not necessarily indicate the presence of spoilage organisms and vice versa, because they are not all decarboxylase-positive (Santos, 1996; Suzzi & Gardini, 2003).

It is generally accepted that seed sprouts constitute fresh and healthy food. However, excessive consumption of bioactive amines can be a health concern because high consumption of bioactive amines can cause diseases of different degree in humans, determined by their action on nervous, gastric and intestinal systems and blood pressure (Bardócz, 1995). On the other hand, amines have been considered as a possible mutagenic precursor, since may be nitrosylated or act as precursors of other compounds capable of forming nitrosamines, which are a potential health hazard to humans (Shalaby, 1996). Therefore, it is important to evaluate the changes that occur during germination and the effects of these vegetables on cell viability. HL60 leukemia cells have been used for studying apoptosis (Birnie, 1988) induced by external agents (Zhou, Wang, Sun, Liu, & Zhang, 2001) and plant extracts (Pilarski, Poczekaj-Kostrzewska, Ciesiolka, Szyfter, & Gulewicz, 2007) and they could be used for studying sprout extracts. Furthermore, the survival of these cells can easily be monitored by the cleavage of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a blue-coloured product (formazan) by the mitochondrial enzyme, succinate-dehydrogenase (Slater, Sawyer, & Strauli, 1963) and this method is potentially very useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of living cells present and can be spectrophotometrically quantified. It is a rapid and simple assay which has been used in different cell lines (Denizot & Lang, 1986) and HL60 survival has already been tested with food extracts (Pilarski et al., 2007).

The present investigation, carried out on alfalfa and fenugreek seeds, was undertaken to study the effect of different germination conditions (temperature and light exposure for 4 days of germination) on the bioactive amines and to examine their potential effect on HL60 cell survival. Optimising the conditions of germination, in association with toxicological data, makes possible the production of high quality food.

2. Materials and methods

2.1. Plant material

Alfalfa (*Medicago sativa*) and fenugreek (*Trigonella foenum-graecum*) were purchased in an herbalist shop.

2.2. Germination procedure

Different germination conditions were carried out with alfalfa and fenugreek seeds. The procedure was as follows: 10 g of seeds were soaked for 30 min with 50 ml of 0.07% sodium hypochlorite. After draining and washing with distilled water to neutral pH, the seeds were soaked with 50 ml of distilled water for 5.5 h and shaken every 30 min. The imbibed seeds were germinated on a pilot scale by layering them over moist filter paper in a germination tray. The tray was placed in a seed germinator, G-120 model (ASL Snijders International S.L., Holland), where the filter paper was kept moist by capillary action. The germination process was carried out at different temperatures (20 °C and 30 °C) and light conditions (with and without light) for 4 days at a relative humidity of 90%. Fenugreek seeds did not germinate at 30 °C under light conditions. Samples were named as follows: AL, raw alfalfa seeds; AL4D20L, alfalfa seeds germinated for 4 days at 20 °C with light; AL4D20NL, alfalfa seeds germinated for 4 days at 20 °C

without light; AL4D30L, alfalfa seeds germinated for 4 days at 30 °C with light; AL4D30NL, alfalfa seeds germinated for 4 days at 30 °C without light; F, raw fenugreek seeds, F4D20L, fenugreek seeds germinated for 4 days at 20 °C with light; F4D20NL, fenugreek seeds germinated for 4 days at 20 °C without light; F4D30NL, fenugreek seeds germinated for 4 days at 30 °C without light.

Sprouted seeds were freeze-dried and ground to pass through a sieve of 0.5 mm and kept in plastic bags in a vacuum at 4 °C under dark conditions prior to further analysis.

2.3. Analysis of biogenic amines

The extraction and HPLC analyses of biogenic amines were done according to Moret, Smela, Populin, and Conte (2005). Briefly, 0.5 g of raw or germinated flour sample was homogenized with 10 ml of 0.1 M HCl in an Ultra-Turrax T25 homogenizer for 2 min. The resulting homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was collected and the residue was re-extracted under the same conditions. The two combined extracts were filtered through a Whatman no. 1 filter paper and diluted to 100 ml in a volumetric flask. To prepare dansyl-derivates, a 1 ml aliquot of the diluted extract was mixed with 0.5 ml of saturated $NaHCO_3$ and 1 ml of dansyl chloride (Sigma) reagent (20 mg/ml in acetone). The mixture was then kept at 40 °C in darkness under agitation for 60 min. The residual dansyl chloride was removed by adding 200 μl of a proline (Sigma) solution (100 mg/ml), vortexing for 1 min and left to react at room temperature in darkness for 15 min. Finally, the sample was extracted twice with 1 ml of diethyl ether (SDS). The combined extracts were dried under nitrogen flow and the residue was dissolved in 0.5 ml of acetonitrile (HPLC grade, Scharlau), and then filtered through a 0.45 μm PVDF Millipore filter for injection.

Putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tyramine, spermidine trihydrochloride and, spermine tetrahydrochloride were purchased from Fluka (Spain). A stock standard solution of amines was prepared by placing an accurately weighed amount of each standard (ca. 50 mg) in a 25 ml volumetric flask (made up with water) and processed as previously for the samples. Standards were derivatised as described for samples and they were used as external standards.

The chromatographic system consisted of an Alliance Separation Module 2695 (Waters, Milford, USA), a photodiode array detector 996 (Waters) and a personal computer running the Empower II for windows chromatographic software (Waters). The sample (20 μl) was injected onto a C_{18} Kromasil 250 × 4.6 mm i.d., 5 μm size (Symta) column equipped with a C_{18} guard column (Symta), both thermostatted at 30 °C. The mobile phase consisted of bidistilled water (solvent A) and HPLC-grade acetonitrile (solvent B). The elution programme was held at 65% of B for 1 min, ramped at 80% (10 min), 90% (12 min), 100% of B (16 min) and held until the end of the run (23 min) with a flow rate of 0.8 ml/min. Calibration curves were obtained for standard amines and "*r*" was always above 0.990.

2.4. Cytotoxicity evaluation

To obtain the extracts, 50 mg of the seed flour were dissolved in 2.5 ml of deionized water and sonicated for 30 min (Sonorex AK103H). Then, the extracts were centrifuged for 15 min at 12,000 rpm. The supernatant was filtered through 0.22 μm membranes into sterile test tubes. Aliquots (1 ml) of each filtered supernatant were evaporated in tared vessels and the obtained dry masses were weighed after 24 h of desiccation with P_2O_5. The residues were dissolved in sterilized water to a final concentration of 1 mg/ml.

The human leukemic cell line (HL-60), derived from a patient with acute promyelocytic leukemia, was obtained from American Type Culture Collection (ATCC). The culture were maintained on RPMI medium containing 10% fetal calf serum and 1% penicillin G (Sigma) and streptomycin (Sigma), at 37 °C in a humidity-controlled incubator at 90% relative humidity and 5% CO_2. After a few passages, cells were centrifuged, resuspended in fresh medium at the concentration of 0.30×10^6 cell per millilitre and transferred onto several plates in 2 ml volumes. The cells were exposed to 100 μg/ml of the raw and germinated seed extracts and control (sterilized water).

The mortality cell assay was performed in duplicate for 24, 48 and 72 h after starting exposure to seed extracts. Then, trypan blue dye (Sigma) was added to cell cultures in a ratio of 1:1 and left for 10 min. 20 μl aliquots of cell suspension were then loaded, by a micropipette into Bürker chambers. The cells were counted under a microscope at 100 × magnification. The ratios of live to dead cells were determined.

The tetrazolium reduction assay (MTT) was performed in duplicate, following the method of Denizot and Lang (1986). Briefly, 100 μl volumes of the medium with cells were transferred into Eppendorf tubes and centrifuged for 5 min at 1600 rpm. The supernatants were removed and cells resuspended in 100 μl of fresh medium. The cells were exposed to 2, 5, 10, 100, 500 and 1000 μg/ml of the raw and germinated seed extracts and control (sterilized water) for 24 h. After this time, 20 μl of MTT (Sigma) at a concentration of 5 mg/ml in PBS, were added to each sample. All plates were incubated for 4 h at 37 °C in a humidity-controlled incubator at 90% relative humidity; 5% CO_2 and 120 μl of DMSO (Sigma) were added. After 3 h of incubation at 37 °C, samples were centrifuged and 25 μl of Sorensen buffer (0.1 M glycine, 0.1 M NaCl, pH = 10.5) were added to supernatants. Absorbance of the formazan product was measured at 570 nm.

2.5. Statistical analyses

All analyses were performed in triplicate using different test samples, and data expressed as means ± standard

deviation. Data were subjected to multi-factor analysis of variance with the use of the least significant difference test with the Statgraphic 5.0 Program (Statistical Graphics Corporation, Rockville, Md).

3. Results

Tables 1 and 2 show the content of bioactive amines in flours of raw and germinated alfalfa and fenugreek, respectively. Mature legume seeds showed the presence of putrescine, cadaverine, histamine, tyramine, spermidine and spermine. In both seeds, cadaverine was the major amine (25 and 22 mg/kg DM in alfalfa and fenugreek, respectively). The content of histamine was higher in alfalfa (21 mg/kg DM) than in fenugreek (17 mg/kg DM). Levels of spermidine and spermine were quite similar in both seeds (19 and 17 mg/kg DM in alfalfa and 20 and 18 mg/kg DM in fenugreek, respectively) and putrescine and tyramine were the bioactive amines present in the lowest amount (14 and 10 mg/kg DM in alfalfa and 14 and 17 mg/kg DM in fenugreek, respectively). The total content of biogenic amines reached 107 and 108 mg/kg DM, for alfalfa and fenugreek, respectively (Tables 1 and 2).

Germination caused different effects on biogenic amines, depending on the seed and conditions under which the process was carried out. In alfalfa sprouts, the content of amines underwent a sharp increase after 4 days of germination and, in general, the bioactive amines doubled their content in comparison with raw material. Putrescine, cadaverine and histamine were affected by germination temperature and germination at 20 °C brought about lower levels of these amines than did germination at 30 °C. The levels of tyramine, spermidine and spermine were not affected by temperature. Light exposure during germination affected only putrescine and cadaverine when it was carried out at 20 °C, and flour from alfalfa sprouted in the dark at 20 °C provided 34.4 and 53.5 mg/kg DM of putrescine and cadaverine, respectively, compared with values of 37.7 and 57.0 mg/kg DM when seeds were germinated with light. Germination, carried out at 20 °C in darkness for 4 days, provided the lowest total bioactive amine content (220 mg/kg DM), in comparison with the other assayed conditions (Table 1).

Fenugreek seeds were germinated for 4 days at 20 °C with and without light but, when sprouting temperature was 30 °C, these seeds only germinated in darkness. Under the studied conditions, only putrescine and cadaverine showed a rise after germination compared with raw material (from 13.7 to 19–20 mg putrescine/kg DM and from 22 to 42–44.7 mg cadaverine/kg DM). Tyramine showed a slight, but significant ($P \leqslant 0.05$) increase (from 17 to 18.8 mg/kg DM) whilst histamine and spermidine did not change during germination. Light exposure brought about a very small, but significant ($P \leqslant 0.05$) spermine increment when sprouted fenugreek seeds were obtained at 20 °C (from 18.2 to 18.7 mg/kg DM) and temperature did not affect biogenic amine content of fenugreek sprouts. Total biogenic amine content of sprouted fenugreek flours ranged from 137 to 140 mg/kg DM (Table 2).

Table 1
Biogenic amine content of raw and germinated alfalfa (*Medicago sativa*)[A]

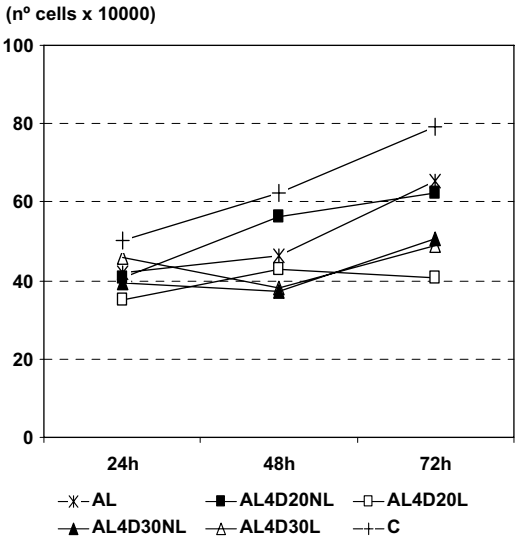
Alfalfa seeds	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	Total biogenic amines
AL	14.49 ± 0.42^{a}	25.04 ± 1.43^{a}	21.33 ± 0.46^{a}	9.53 ± 0.33^{a}	19.15 ± 0.57^{a}	17.65 ± 0.24^{a}	107.18 ± 0.57^{a}
Germinated							
AL4D20L	37.66 ± 1.09^{c}	57.00 ± 2.01^{c}	35.09 ± 1.03^{b}	22.16 ± 1.15^{b}	38.38 ± 1.31^{b}	36.21 ± 1.15^{b}	226.51 ± 1.29^{c}
AL4D20NL	34.39 ± 0.97^{b}	53.47 ± 1.40^{b}	35.68 ± 0.59^{b}	22.03 ± 1.12^{b}	38.00 ± 0.64^{b}	36.74 ± 0.83^{b}	220.30 ± 0.92^{b}
AL4D30L	42.68 ± 0.73^{d}	75.28 ± 1.32^{d}	48.09 ± 1.00^{c}	22.14 ± 1.06^{b}	37.55 ± 1.01^{b}	36.31 ± 0.28^{b}	262.05 ± 0.90^{d}
AL4D30NL	41.73 ± 0.74^{d}	74.80 ± 1.45^{d}	47.51 ± 0.71^{c}	22.22 ± 0.80^{b}	37.77 ± 0.31^{b}	36.21 ± 1.10^{b}	260.24 ± 0.85^{d}

Different superscript in the same column means significant difference ($P \leqslant 0.05$).
AL, raw alfalfa seeds; AL4D20L, alfalfa seeds germinated for 4 days at 20 °C with light; AL4D20NL, alfalfa seeds germinated for 4 days at 20 °C without light; AL4D30L, alfalfa seeds germinated for 4 days at 30 °C with light; AL4D30NL, alfalfa seeds germinated for 4 days at 30 °C without light; and C, control.
[A] Data in mg/Kg dry matter and are the mean of four determinations ± standard deviation.

Table 2
Biogenic amine content of raw and germinated fenugreek (*Trigonella foenum-graecum*)[A]

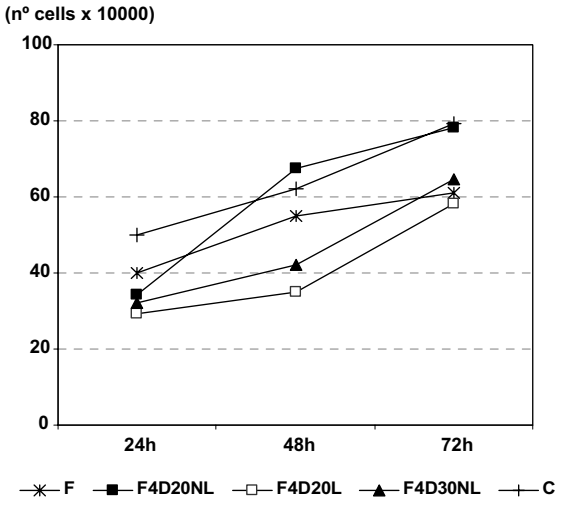
Fenugreek seeds	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	Total biogenic amines
F	13.74 ± 0.07^{a}	22.29 ± 0.19^{a}	17.02 ± 0.49^{a}	16.94 ± 0.36^{a}	20.07 ± 0.16^{a}	18.23 ± 0.05^{a}	108.29 ± 0.22^{a}
Germinated							
F4D20L	19.86 ± 0.76^{b}	44.69 ± 0.67^{d}	17.84 ± 0.43^{a}	18.38 ± 0.38^{c}	19.96 ± 0.08^{a}	18.69 ± 0.09^{b}	139.63 ± 0.34^{d}
F4D20NL	19.56 ± 0.35^{b}	43.48 ± 1.63^{cd}	17.42 ± 0.12^{a}	18.74 ± 0.46^{c}	20.10 ± 0.27^{a}	18.08 ± 0.19^{a}	137.39 ± 0.50^{c}
F4D30NL	19.61 ± 0.80^{b}	42.73 ± 1.02^{c}	17.11 ± 0.32^{a}	18.85 ± 0.56^{c}	20.28 ± 0.50^{a}	18.34 ± 0.34^{a}	136.93 ± 0.59^{c}

Different superscript in the same column means significant difference ($P \leqslant 0.05$).
F, raw fenugreek seeds; F4D20L, fenugreek seeds germinated for 4 days at 20 °C with light; F4D20NL, fenugreek seeds germinated for 4 days at 20 °C without light; F4D30NL, fenugreek seeds germinated for 4 days at 30 °C without light; and C, control.
[A] Data in mg/Kg dry matter and are the mean of four determinations ± standard deviation.



AL = Raw alfalfa seeds
AL4D20NL = Alfalfa seeds germinated for 4 days at 20ºC without light
AL4D20L = Alfalfa seeds germinated for 4 days at 20ºC with light
AL4D30NL = Alfalfa seeds germinated for 4 days at 30ºC without light
AL4D30L = Alfalfa seeds germinated for 4 days at 30ºC with light
C = Control

Fig. 1. Proliferation of HL-60 cells exposed to raw and sprouted alfalfa (*Medicago sativa*) extracts.



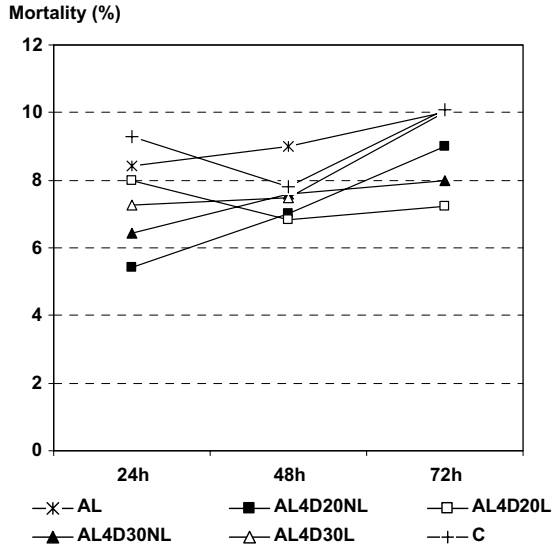
F = Raw fenugreek seeds
F4D20NL = Fenugreek seeds germinated for 4 days at 20ºC without light
F4D20L = Fenugreek seeds germinated for 4 days at 20ºC with light
F4D30NL = Fenugreek seeds germinated for 4 days at 30ºC without light
C = Control

Fig. 2. Proliferation of HL-60 cells exposed to raw and sprouted fenugreek (*Trigonella foenum-graecum*) extracts.

Fig. 1 shows the proliferation of HL60 cells exposed to extracts of alfalfa and control. Cell proliferation reached levels of 35–50 (× 10,000) cells after 24 h of alfalfa extract exposure. After 48 h, the numbers of cells found with alfalfa sprouts obtained with light at 20 °C and 30 °C, and without light at 30 °C for 4 days remained almost constant until 72 h, whilst cells exposed to extracts of raw alfalfa and germinated at 20 °C without light increased after 48 h and, at 72 h, more than 70,000 cells were counted. However, levels of HL60 proliferation were always lower than those found in the control assay with cells exposed to distilled water (almost 80,000 cells) (Fig. 1).

Fig. 2 shows results of the exposure of HL60 cells to fenugreek extracts. Similar behaviour to that of alfalfa seeds after 24 h of exposure was observed. However, cell proliferations increased when they were in contact with fenugreek extracts for 48 h, especially the sprouted seeds obtained at 20 °C in darkness, that reached levels above those of the control experiment (68,000 and 62,000 cells, respectively), levels that were equal after 72 h of exposure (almost 80,000 cells) (Fig. 2).

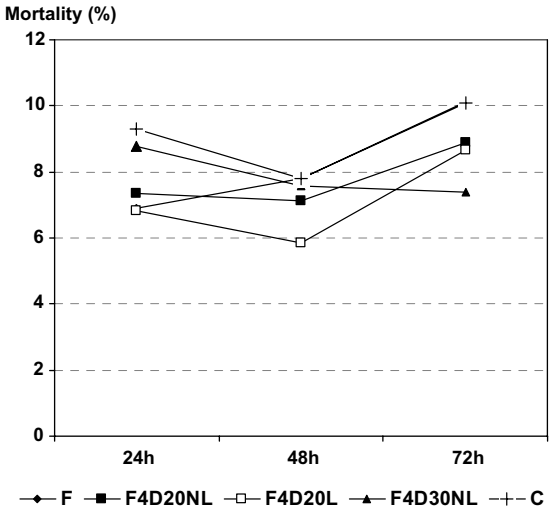
When percent mortality was calculated, no significant differences ($P \leqslant 0.05$) were observed between raw and sprouted seeds (Figs. 3 and 4). Therefore, the studied seed preparations did not enhance mortality of the exposed HL-60 cells, since ratios of live and dead cells in all cell cultures were similar to those found for the control, in which dead cells were up to 10% of the whole population (Figs. 3 and 4).



AL = Raw alfalfa seeds
AL4D20NL = Alfalfa seeds germinated for 4 days at 20ºC without light
AL4D20L = Alfalfa seeds germinated for 4 days at 20ºC with light
AL4D30NL = Alfalfa seeds germinated for 4 days at 30ºC without light
AL4D30L = Alfalfa seeds germinated for 4 days at 30ºC with light
C = Control

Fig. 3. Apoptosis of HL-60 cells exposed to raw and sprouted alfalfa (*Medicago sativa*) extracts.

Figs. 5 and 6 show the results of MTT assay performed on HL60 cells exposed to extracts of raw and sprouted seeds of alfalfa and fenugreek, respectively. The obtained



F = Raw fenugreek seeds

F4D20NL = Fenugreek seeds germinated for 4 days at 20ºC without light

F4D20L = Fenugreek seeds germinated for 4 days at 20ºC with light

F4D30NL = Fenugreek seeds germinated for 4 days at 30ºC without light

C = Control

Fig. 4. Apoptosis of HL-60 cells exposed to raw and sprouted fenugreek (*Trigonella foenum-graecum*) extracts.

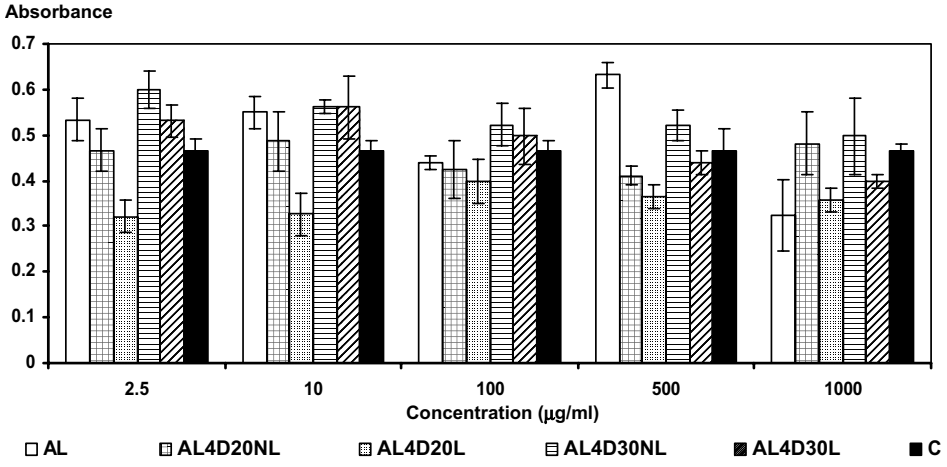
MTT results indicated no difference or minimal differences between the cell viabilities obtained for the studied extracts and the control (distilled water). Biological activities of the studied preparations were evaluated within the ranges of the results obtained for the control (Figs. 5 and 6). Similar results in cell viability were obtained with different extract concentrations (from 2.5 to 1000 μg/ml).

4. Discussion

The extraction and determination of dansyl chloride amines in raw and germinated seeds of alfalfa and fenugreek were carried out using a simple preparation method that does not require complicated procedures, as recently published by Moret et al. (2005). The extraction with 0.1 M hydrochloric acid instead of trichloracetic acid and further derivatization with dansyl chloride, prior to high performance liquid chromatographic analysis, make the analysis of biogenic amines in vegetables simple and rapid compared with other proposed methodologies (Glória et al., 2005; Shalaby, 2000). Furthermore, spermidine and spermine are detected as DCl-derivatives using a UV detector, since they are not detectable as OPA-derivatives with a fluorescence detector (Moret et al., 2005).

The levels of bioactive amines in seeds of alfalfa (*M. sativa*) and fenugreek (*T. foenum-graecum*) show that both raw seeds had quite similar contents, data which are in agreement with previously reported data in broad bean, chick pea, lupin and fenugreek seeds (Martínez-Villaluenga, Gulewicz, Pérez-Romero, Frias, & Vidal-Valverde, 2006; Shalaby, 2000), higher than those in soybean cultivars (Glória et al., 2005), but lower than those in mung bean, lentil and radish (Simon-Sarkadi & Holzapfel, 1995). All these data demonstrate that biogenic amine levels seem to depend on the seed type and variety.

Only few papers have been published about the effect of germination on the biogenic amines of seed. Martínez-Villaluenga et al. (2006) reported an increasing trend, throughout the germination period, for biogenic amines in lupins and fenugreek seeds germinated for 2, 3, 4 and 5



AL = Raw alfalfa seeds

AL4D20NL = Alfalfa seeds germinated for 4 days at 20ºC without light

AL4D20L = Alfalfa seeds germinated for 4 days at 20ºC with light

AL4D30NL = Alfalfa seeds germinated for 4 days at 30ºC without light

AL4D30L = Alfalfa seeds germinated for 4 days at 30ºC with light

C = Control

Fig. 5. MTT assay results performed on HL-60 cells exposed to raw and sprouted alfalfa (*Medicago sativa*) extracts.

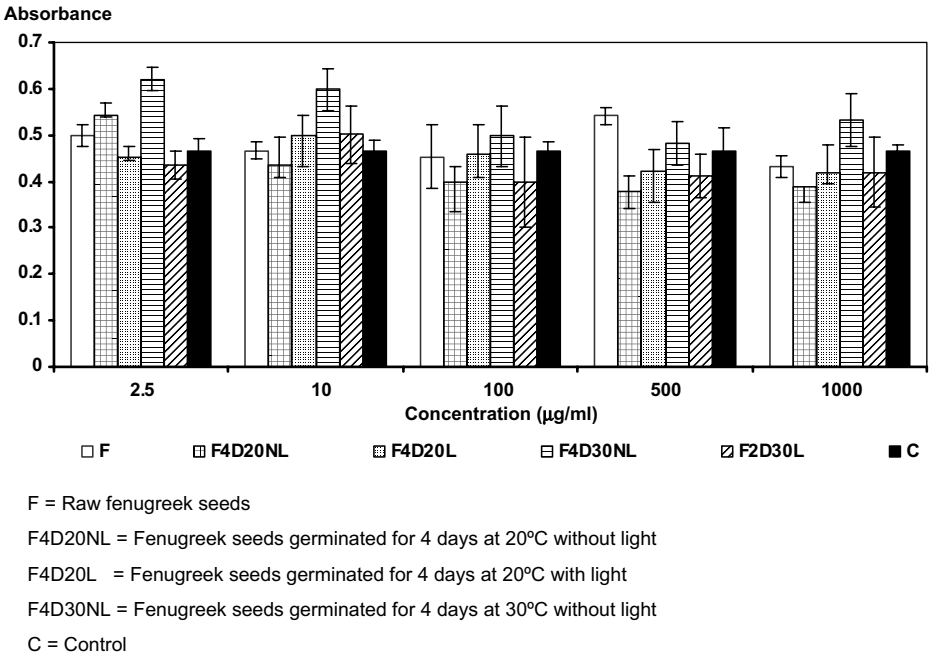


Fig. 6. MTT assay results performed on HL-60 cells exposed to raw and sprouted fenugreek (*Trigonella foenum-graecum*) extracts.

days at 25 °C under dark conditions, reaching levels more than three times higher than those in the raw seed. No data have been found on biogenic amines in alfalfa sprouts. In other legumes, such as commercial mung bean sprouts, Skowronek et al. (1998) found the presence of putrescine, cadaverine, spermidine and spermine, and concentrations ranged from 4 mg/kg for cadaverine to 114 mg/kg for putrescine, whilst spermidine and spermine did not show a great difference among commercial samples. Simon-Sarkadi and Holzapfel (1995) reported a large variation in the individual biogenic amines of mung beans, lentils and radish during germination for 7 days, depending on the process conditions, since mung bean were germinated with light, while lentil and radish were kept first in darkness for 2 days before exposure to light. According to Shalaby (2000) and Glória et al. (2005), amines are endogenously produced during germination and during this biosynthetic process, protein is synthesized rapidly and increasing levels are expected. Glória et al. (2005) attributed the significantly higher levels of polyamines spermidine, spermine and putrescine, during germination, to the greatest cellular multiplication period and growth that have been reported to be related to protein and nucleic acid synthesis (Tabor & Tabor, 1976). However, little changes in the spermidine and spermine concentrations have been observed during the germination period of radish (Simon-Sarkadi & Holzapfel, 1995) and fenugreek (Martínez-Villaluenga et al., 2006). Flores, Protacio, and Signs (1989) associated the presence of cadaverine in sprouts with its role in the elongation of the root and the increase in cell size, and increase of cadaverine has been observed during the present study in alfalfa and fenugreek sprouts. Histamine has been associated with allergic reactions, skin irritations, headache, dizziness, vomiting and diarrhea and FAO (Pan & James, 1985) has set limits for histamine in fish at 100–200 mg/100 g. Results for histamine presented here, for sprouted alfalfa and fenugreek are much lower (17–48 mg/kg). Acceptable healthy levels of tyramine have been proposed for fermented and non-fermented food at 100–800 mg/kg (Nout, 1994) and none of the studied seed sprout flours studied here reached such levels. For putrescine, 11–20 mg/kg has been suggested as acceptable for human health (Tailor, Shulman, Walker, Moss, & Gardner, 1994) and results obtained in germinated alfalfa and fenugreek seeds are below these levels.

An antihypercholesterolaemic effect has been described in rabbits (Malinow, McLaughlin, Stafford, Livingston, & Kohler, 1980b), in rats (Malinow, McLaughlin, & Stafford, 1980a) and in cynomolgus macaques (Malinow, McLaughlin, Bardana, & Craig, 1984) when their diets were supplemented with alfalfa seeds. Fenugreek seeds have been used in traditional medicines because they have hypoglycemic and hypocholesterolemic activities (Khosla, Gupta, & Nagpal, 1995; Neeraja & Rajyalakshmi, 1996); however, current use of fenugreek in foodstuffs has been limited to its role as flavouring agent, and not as a major ingredient. Flammang, Cifone, Erexson, and Stankowski (2004) have determined that fenugreek seed extracts were not genotoxic and these authors suggested the addition of fenugreek extracts to foodstuffs. These results agree with the present work that, in general, shows no cytotoxicity for all tested samples. On the other hand, the same methodological approach for other extracts studied in our laboratory

clearly demonstrated their genotoxic potency. For example, under the same experimental conditions, 100 μg of *Uncaria tomentosa* extract kills over 50% of cells after 24 h (Pilarski et al., 2007).

Other studies have demonstrated that fenugreek may have a beneficial influence on the human organism. Thirunavukkarasu, Anuradha, and Viswanathan (2003) reported that aqueous extract of fenugreek could offer a significant protection against ethanol toxicity, prevent enzymatic leakage and enhance the antioxidant potential. Due to high protein and fibre contents the seeds are a good supplement for cereals (Rao, Sesikeran, & Rao, 1996). In some traditional cooking recipes of North India, e.g. *Laddo*, *Methi*, *Suhali*, wheat-fenugreek flour is used and widely consumed by diabetic and hypercholesterolemic people (Hooda & Jood, 2003) and germinated seeds could be included in this type of foodstuff since antinutritional factors, such as raffinose, stachyose, verbascose phytic acid, tannic acid and trypsin inhibitors, are considerably reduced (Mansour & El-Adawy, 1994). Nevertheless, Randir, Lin, and Shetty (2004) reported that fenugreek sprouts contained phenolic compounds that were related to the antioxidant activity and, at the same time, fenugreek sprouts had a high antimicrobial activity against peptic ulcer-linked *Helicobacter pylori*.

Therefore, the results obtained in the present paper for the studied sprouts together with those found in the literature, indicate that the consumption of these sprouted alfalfa and fenugreek flours, included as ingredients in functional foods (pasta, biscuits, or salsa) is not a risk for healthy consumers.

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