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Necrotic and Apoptotic Activity of the Protein Extract from *Mangifera indica* Mesocarp in Human Lymphocytes in Culture

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Abstract

Mangifera indica, a species of mango in the Anacardiaceae family, in some cases may cause dermatitis and anaphylaxis, in some people who touch the tree or intake the fruit due to its content of allergenic compounds such as polyphenols and proteins with lectin activity. In aim of this study, was to analyze if the protein extract of the *Mangifera indica (mango manila=mango)* mesocarp with lectin activity that recognizes glucose, galactose and glucosamine, has necrotic and apoptotic effect in human lymphocytes in culture by identified proliferation by MTT assay and cytotoxicity by the presence of enzymes involved in necrosis and apoptosis such as, acid sulphatase, phosphatase and caspase-3 and apoptotic bodies in the nuclei by staining with iodide propidium, as well as the quantification of extracellular calcium. Results: Protein extracts of mango at low concentrations showed an increase in proliferation, while at high concentrations was a cytotoxic (necrotic-apoptotic) depending on the time of exposure. At low exposure times increased the activity of sulfatase and acid phosphatase and caspase-3, whereas high exposure times increased caspase-3, the amount of extracellular calcium and the formation of apoptotic nuclear bodies, therefore the results suggest that the protein extract of mango with lectin activity, depending of the concentration and exposure time, have effect on lymphocyte proliferation and cell death by necrosis and apoptosis.

Proteic extracts, lectins, necrosis, apoptosis, acid sulphatase, acid phosphatase, caspase-3

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Introduction

Mangifera indica covers 69 species, which are spread over different geographical areas, determining its genetic variability. It is a species in the Anacardiaceae family, including some poisonous species [1, 2]. The consumption of the fruits of these species causes allergic responses in certain population groups [3, 4], some anaphylactic. One case describes the symptoms of edema, tongue swelling and rash on arms and sides exhibited by a fruit vendor after eating a mango [3, 5]. Cases were children develop dermatitis after climbing mango trees, as well as rashes on the lips and face after eating the fruit have been also reported [3]. On the bark of *Mangifera. indica* the presence of different secondary metabolites, including benzoic acid, methyl gallate, propyl gallate, mangiferin, mangiferol, catechin, epicatechin and propylbenzoate, which are used to prepare medicinal products because their antioxidant, anti-inflammatory and analgesic capacity has also been detected [6, 7]. Furthermore, it has been reported that on leaves, peel and fruit there are proteins possessing lectin activity [8, 9].

Lectins are mainly present in the cotyledons and endosperm of plants; they are widely distributed from bacteria to vertebrates. They are non-immune origin proteins that recognize 20mM of specific free or present carbohydrates on the cell surface and they have the ability to agglutinate cells [8, 9, 10]. Therefore, in order to identify these proteins haemagglutination tests (HA) of erythrocytes are used, among others [9, 10, 11, 12]. The biological activity of the lectins is wide and they play an important role in proliferation and cell adhesion, essential for some viruses and bacteria to develop pathogenicity during the infectious process. They are also involved in cellular transformation, citotoxicity and cellular hypersensitivity [8, 10, 12, 13, 14, 15].

Hypersensitivity to species of the Anacardiaceae family has been investigated in people with a history of sensitivity to pistachio by immunoglobulin-E detection (IgE), type 1hypersensitivity to five proteins 9, 41, 43, 70 and 80kDa (kD) in *Mangifera indica* pulp, identified as allergens was found [1]. The presence of two proteins of 24 and 28 kD lectin activity by an immune blot test of extracts from *Mangifera indica* has also been reported [5].

In leaves, bark and peel of *Mangifera indica*, the presence of three bands of 30kD and 50kD, 15 kD has been reported, as well as an additional 27kD in a graft, in which the 30kD band showed lectin activity and specificity for glucose and N-acetyl glucosamine and N-acetyl galactosamine, sugars present within chitin derivates [9].

In a preliminary study that used type O male blood, lectin activity in various fruits with gastrointestinal activity was found, particularly the protein extract from guava, whereas for mango extract nothing was found [8]. However, in another study with the mature mesocarp of mango, a different human erythrocyte hemagglutination activity depending on sex gender was found [16].

The lectin specificity, in the hemagglutination test, in the case of male gender only recognized the type A blood with 1.24 hemagglutinating units per microgram of protein (UHA/µg protein), whereas in the female case it only recognized the type O with 9.92 UHA/µg. In the same study, a similar recognition for fucose, glucosamine and galactosamine sugars for both female Type O and male Type A was determined.

The molecular weights of the proteins found in the mango extract were 250, 149, 54, 24 and 18 kD [16].

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Moreover, it is widely known the mitogenic effect on human lymphocytes, most lectins such as phytohemagglutinin [9, 12] as well as the cytotoxic effect of RIP type lectins have the ability to inactivate the ribosome [17]. There are also reports on lytic type cytotoxic effects on neutrophils after being exposed to this type of protein [13]. Other studies have shown that some lectins, like those of the mistletoe (ML-I), with recognition for d-galactose enables a signaling caspase pathway involved in the apoptotic pathway [18]. It is also known that lectins isolated from *Boleptosis Leucomelas* with a molecular weight of 15 kD and recognition for diacetil chitiobiosa a chitin derivates also induce apoptosis [18, 19].

Cytotoxicity by apoptosis.

Apoptosis plays a key role in the survival of organisms and for proper functioning of the immune system, among others [20, 21]. In lymphocytes apoptosis can be induced by two or more different ways. the first one associated with the mitochondrial permeability (intrinsic pathway), which causes the output of various proteins into the cytoplasm, including C cytochrome, which acts as a cofactor of a protein called "apoptosis 1 activating factor" (Apaf 1), involved in the activation of caspase-9, initiating the apoptotic effector pathway comprising degranulation of the core with participation of caspase-3.

The second apoptotic pathway (extrinsic pathway) is triggered by the binding of death receptors such as Fas/CD95, between the target cell and effector cell that induces activation of proteolytic enzymes, caspases [20, 21, 22].

In apoptosis activation via receptor serine/threonine phosphatases or PP1 type phosphomono esterases are involved.

They are transmembrane esterases also known as acid phosphatase or alkaline phosphatase, because of their broad range of pH activity ranging from 5 to 9.5 and they have two binding ions. Thus, they are considered as metalloprotease, which is involved in signal transduction, activated by receptors associated with growth, differentiation and apoptosis [23, 24, 25].

It is noteworthy that these phosphomono esterases are indicators for soil quality and have roles in many organisms from bacteria to plants. In some poisonous species, such as crotalid and elapidae, they are associated with their toxin activity and produce more alkaline phosphatase, whereas in ophidian they produce both enzymes, and in some aphidae they produce acid phosphatase, which is a potent allergen. [23, 25, 26].

Cytotoxicity by necrosis

Necrosis is an acute and massive process comprising the loss of cell viability as a result of pathological conditions due to acute exposure to chemical and physical compounds, microbes and toxins. Necrosis is characterized by alterations in the integrity of the membrane, cell lysis and rupture of cellular organelles, releasing their contents which triggers the immune response and induces inflammatory processes [21, 22]. In necrotic cytotoxicity mediated by lymphocytes the target cell undergoes osmotic imbalance because the input and output of ions, resulting from the formation of pores on the membrane, lead to cell lysis due to the releasing of cytotoxic factors, immersed in granules with acid pH containing *perforin* (proteins that interact with phospholipid membranes, causing pores) and *granzimez* (serine-esterases, carboxypeptidases, cathepsin D, aryl-sulfatases and beta-glucuronidase) [21, 23].

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The *granzyme* are enzymes located within cytoplasmic granules of T cytotoxic-lymphocytes and natural killer cells (NK cells), which are released as a response upon interaction with a target cell. This family of proteases is involved in the activation of caspases by expression of the Fas/FasL receptor, and also in the formation of pores, a process performed in the necrotic cytotoxic response [21, 23].

The aim of this work is to identify the type of cytotoxic effect of mango protein extracts with lectin activity in lymphocytes culture through the enzymatic activity involved in necrosis and apoptosis death, and calcium realese.

Material and methods

Extraction of total proteins from mango

Four ripe of mangos were macerated in mortar with buffer of 10 Mm pH 7.4 phosphates. The mash was filtered with gauze and stored at 4 ºC. In order to remove the lipid content acetone was added in a 1 to 4 proportion overnight. The acetone extract was centrifuged at 7,000 rpm. for 15 minutes (min.) in a Rotina 35R Hettich centrifuge, while the precipitate was resuspended in 150 mL of phosphate buffer solution that was called protein extract from mango and used to determine protein quantifications, lectin activity by haemagglutination tests [8], identification of bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), evaluate cytotoxic activity in cell culture, or enzymatic activity of enzymes as acid phosphatase, alkaline phosphatase, acid sulfatase, esterase and caspase-3.

Quantification and Visualitation of proteins from mango

Protein quantification

The method of Bradford 1976 [27] was used for protein quantification. The analysis was performed on a 96-well micro plate. Readings were obtained using a Spectramax-190 spectrophotometer with a wavelength of 593 nm. A standard curve of bovine albumin was used.

Electrophoresis analysis

Both proteins and their molecular weights were determined by SDS- PAGE at 10% using a Molecular marker (Dual Color cat.161-307 4BIO-RAD). Two gels were run simultaneously, one to visualize the proteins and the other for carbohydrates. The gels were stained with Coomassie blue and Schiff's reagent respectively.

Lectin activity identification in protein extracts from mango

Lectin activity was qualitatively determined by the ability to agglutinate erythrocytes and lymphocytes. The degree of agglutination was classified as: high (+++), moderate (++), light (+). We also confirmed the specificity of lectin activity by agglutination inhibition using competition of the extract with standard sugars: glucose, glucosamine and galactosa (1mM), which have been reported to recognize m*ango* lectins in Type O erythrocytes [16].

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Lymphocyte culture

Lymphocytes were extracted by centrifugation at 1,500 rpm, from 10mL of peripheral blood from a male healthy donor of the Rh O^+ type and cultured in 100 mL of McCoy 5A medium with phytohemagglutinin (2 µg/mL) at 37 ºC. After 48 hours of incubation, the medium was distributed into four Falcon type tubes, which were treated with the protein extract from mango at different concentrations: 0.076, 0.771 and 3.804 µg/mL using the phosphate buffer solution as control (500 µL). After aliquot sampling at 30 minutes, 2 and 24 hours, the culture was centrifuged at 1,500 rpm/10 min., and the cell pellet was washed with saline solution and re-suspended in 500 mL of buffer solution for the several tests.

Cell proliferation

To determine the cell proliferation induced by the mango protein extract, in lymphocytes. The Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (cat. 465002 Roche 1465526) cell proliferation kit was used on a 96-well micro plate, where each well contained 25 µL of the treated culture; 25 µL of saline solution (0.9 %) and 10 µL of MTT reagent. After four hours of incubation at temperature with or without stirring, 500 mL of solubilizing solution was added. According to provider, abs at 500 and 690 nm (OD) was determined on a Spectra Max 190.

Enzymatic analysis

For determination of activity enzymatic 10 µL of the sample from the treated lymphocyte culture were placed in micro plates, as well as 10 µL of substrate and 130 µL of the appropriate buffer solution for each enzyme and their respective reaction controls (Table 1).

This mixture was incubated for 30 at 37ºC min. Then the absorbance ratio was determined at 410 nm for substrates coupled with p-nitrophenol chromophore and 415 nm for p-nitroaniline. Enzymatic activity was determined using the extinction coefficient for each substrate used according to the following equation:

$$U = Do / Ɛ * T * V \quad (1)$$

Units (U) = µM of the substrate converted by min^{-1} = specific activity per µg of $protein^{-1}$

Where: Ɛ = extinction coefficient, T = time and V =volume. Ɛ of p-nitrophenyl=10.8. Ɛ of p-nitroaniline = 18

Enzyme	Buffer	Substrate
Acid phosphatase	Acetate buffer 20 mM pH5	p-Nitrophenyl-phosphate 20 mM
Alkaline phosphatase	Sodium bicarbonate buffer 20 mM pH 8	p-Nitrophenyl-phosphate 20 mM
Sulfatase	Acetate buffer 20 mM. pH 5	p-Nitrophenyl-sulphate 20 mM
Caspase-3	Assay buffer20 mM HEPES, CHAPS 0.1%, DTT 5 mM y EDTA 2 mM pH 7.4	N-acetyl-asp-glu-val-asp-p-nitroanilide 15 mM
Esterase	Phosphate buffer 20mM + 0.1% triton pH 7.4	p-Nitrophenyl-acetate 20 mM

Table 1 Substrate and buffer solution used for determining the activity of every enzyme studied

The enzymatic activity was also conducted in the extract from mango to rule out they were involved in lymphocyte response.

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Quantifying extracellular calcium

The Calcium Arsenazo (ELITECH 08-3327) kit was used to determine the extracellular calcium concentration of lymphocytes exposed to the extract from mango . The assay was performed on an ELISA micro plate, where 10 µL of the supernatant of the cell sample of the exposed lymphocytes (previously centrifuged at 2,000 rpm) and 150 µL of Arsenazo were added. The plate was read at a wavelength of 650 nm after 75 seconds of incubation. A target with 10 µL of water and 150 µL of Arsenazo, and a standard with 10 µL of Ca^{+2} with a concentration of 100 mg/L or 100 µg/µL and 150 µL of Arsenazo was placed as controls. The Ca^{+2} concentration was quantified using a standard curve according to provider.

The calcium concentration was identified in the protein extract from the mango mesocarp.

Nuclei staining

The lymphocytes treated with the extract from mango were fixed on a slide and permeabilized for 5 minutes using cold acetone. Then they were stained for 10 min., with propidium iodide solution (1µg/mL). The morphology of nuclei was observed using a NIKON H55OL fluorescence microscopy at 100x and 40x.

Statistical analysis

For analysis of the results the T-test was conducted to compare groups regarding the control values. A Pearson correlation was also performed, for normally distributed variables, using the SPSS statistical software version 18. The values with a $P \leq 0.05$ were considered as significant.

Results

Characterization of the protein extract from mango

The protein extract from mango was characterized regarding the basal content of proteins, and calcium (Table 2) and the activity of the enzymes studied in order to rule out they were involved in the response of the exposed lymphocytes (Table 3).

Extract	Protein	Calcium
Mango	188.2 µg/mL	5.6 µg/mL

Table 2 Basal calcium and protein concentration in the mango extract

In 1 mL of protein extract from mango there is 188.2µg of protein and in this one 5.6 µg of calcium based on protein concentrations used: 0.076, 0.71 and 3.6 µg/mL. The amount of added calcium does not interfere in the results.

The values of enzyme activity in the protein extract from the mesocarp of mango are below the values of the growth medium used to seed lymphocytes.

Units $x10^{-3}$	ACP	ALP	ACS	CAS-3	ES
Medium	2.03	41	4.97	0.66	59
*9.41 µg/mL	0.47	0.40	0.55	0.24	0.25

Table 3 Basal enzymatic activity in the medium and within the protein extract from mango, Acid phosphatase (ACP), Alkaline phosphatase (ALP) Acid sulphatase (ACS), Caspase-3 (CAS-3), Estarease (ES). *p-ext Mango

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Determining the presence of lectins in the protein extract from mango

In the following microscopic photographs the presence of protein with lectin activity in the protein extract of mango is corroborated. It was an increased agglutination in lymphocyte test in the extract with a concentration of 0.771µg (Figure 1) was observed.

Figure 1. A). Lymphocytes in the absence extract from mango. B). Cells agglutinated in the presence of extract from mango. Last seen at 100X and stained with propidium iodide 40X in presence of mango extract.

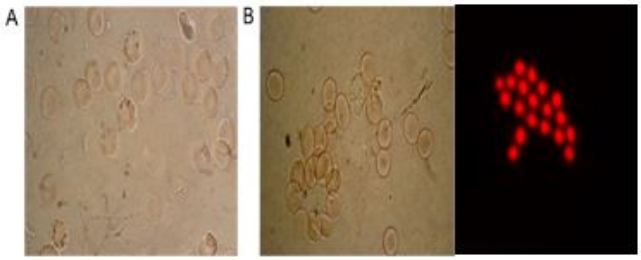

Figure 1

When the protein extract competes with different sugars, a greater specificity for galactose was observed; as the agglutination of both erythrocytes and lymphocytes decreased compared with other sugars (Table 4).

cells	Saline solution	Extract	Glucose	Glucosamine	Galactose
LYM	-	xxxx	xx	xx	x
ERY	-	xxx	xx	x	x

Table 4 Degree of agglutination for both erythrocytes and lymphocytes in the presence of protein extract from mango competing with different sugars. A greater number of "X" determines a greater agglutination, High (+++), moderate (++), light (+) agglutination. Lymphocytes (LYM), Erythrocytes (ERY)

Detecting protein bands by electrophoresis

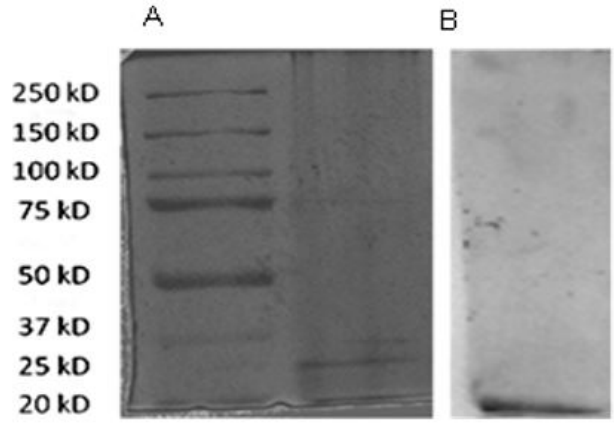

Figure 2 Polyacrylamide gel at 10% stained with Coomasin and Schiff´s reagent´s A). With Coomassie blue. Rail1: Dual Color (cat.161-3074) BIO-RAD molecular weight marker. Rail2: 50µg of protein from the protein extract from mango. B). With the Schiff's reagent

In the protein detection by Coomassie blue, we found the most representative two proteins with molecular weights of 20 and 25 KD and two less sharp bands with molecular weights of 75 and 34 kD. (Figure 2A). In the Schiff's reagent staining, we observed a glycosylated band with a molecular weight about 20 Kd (Figure 2B).

Effect of protein extracts from mango on lymphocyte proliferation

Cell proliferation

The effect caused by the protein extract in lymphocytes, with respect to the response in the proliferation and/or mitochondrial metabolic activity using the MTT technique, showed after 30 min. and 2 horas (hrs.) a significant linear response for all concentrations.

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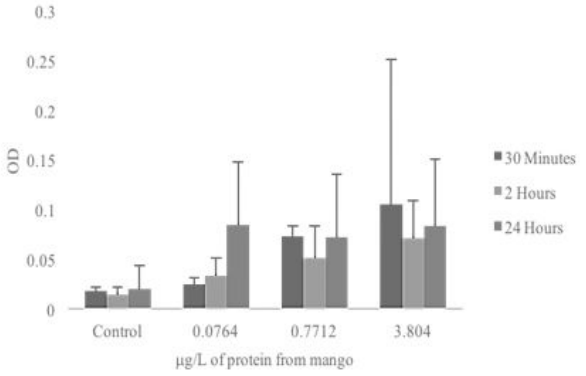
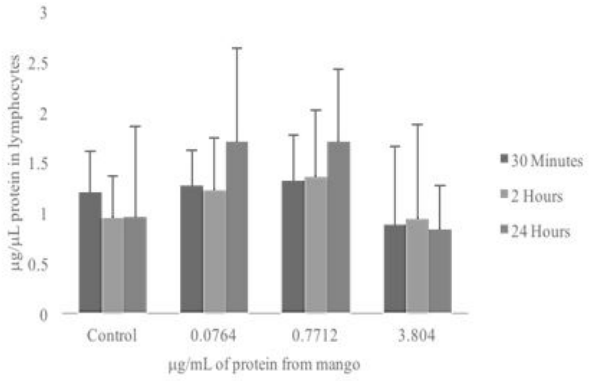
However, after 2 hrs., the response was less than that observed at 30 min; and after 24 hrs., only the lowest concentration of 0.076 µg/mL showed a linear increase overtime, whereas both concentrations of 0.771 µg/mL and 3.804 µg/mL despite they increased regarding control, not show significant changes with respect to grow that 2hours, what is more, after 30min. they decreased (Graphic 1). This suggests that at 30 min., a change in mitochondrial activity in most concentrations does not correspond to the proliferation. activity detected at 24 hrs., only for the lowest concentration of 0.076 µg/mL.



Graphic 1 Effect of protein extract from mango on lymphocytes treated at different concentrations and exposure times in the MTT test used for proliferation, viability and/or mitochondrial activity

Regarding the protein concentrations, in the first two concentrations (0.0764, 0.7712 µg/mL) we found an increased protein concentration in lymphocytes exposed for 2 and 24 hrs., according to the control, whereas at the dose of 3,804 µg/mL are markable decrease, below the control from 30 min., to 24 hrs., was observed probably by the proliferative and cytotoxic effect at the concentrations of 0.0764 and 3.804 µg/mL (Graph 2).



Graphic 2 Quantification of protein for lymphocytes exposed to various concentrations and exposure times compared with the protein extract from mango

After analyzing statistically, the data using the T-test, it showed that both the exposure time and the concentration of protein extract are crucial to the effect on proliferation or mitochondrial activity of lymphocytes with the MTT test. At 24 hrs., the highest concentration of 3,804 µg/mL was induced a significant decrease compared to the control, P=0.048. The increase in other concentrations were so close to be marginal with P=0.056; while at 2 hrs., all concentrations showed a significant increase with P=0.008, for the concentration of 3.804 µg/mL; P=0.01, for the concentration of 0.771 µg/mL; and finally P=0.032, for the lowest one 0.076 µg/mL, suggesting that at 2 hrs., all concentrations had an effect on mitochondrial metabolic activity measured by the MTT test to determine activity for the proliferation or cell viability at 24 hrs., with concentrations of 0.0764 and 3.804 µg/mL, respectively.

Regarding protein concentrations, at 24 hrs., the concentration of 0.771 µg/mL showed a significant increase, P=0.03, while 0.076 µg/mL had a marginal tendency P=0.09; 0.771 µg/mL from 30 min., showed a slight significant increase P=0.01.

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However, the highest concentration of 3,804 µg/mL showed in all exposure times are markable but not significant (Graph 2) decrease, a contradictory result to that observed with the MTT test that showed a significant increase with P=0.048 (Graph 1), which is not reflected in the protein concentration, possibly due to the effect on mitochondrial activity and viability (cytotoxicity) rather than proliferation level as would be the case of the results with concentration of 0.771 µg/mL, 0.076 µg/mL in which the value of the MTT and the protein concentration at 24 hrs., were increased.

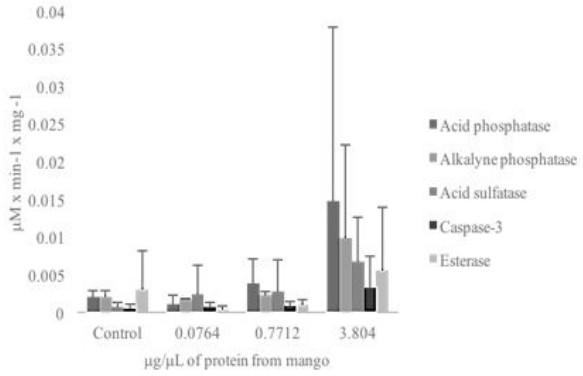
Effect of protein extracts from mango on the enzymatic activity of lymphocytes culture

Analysis of enzymatic activity

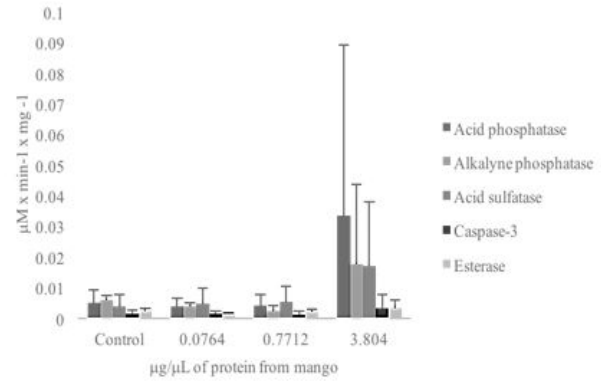
Determining the enzyme activity studied was evaluated for each of the concentrations and exposure times and expressed by protein concentrations.

At the highest concentration of 3,804 mg/mL after 30 min., we observed a high activity of all enzymes, especially acid phosphatase and alkaline phosphatase and caspase-3. Only the marker enzymes for necrosis and apoptosis were shown significant; acid phosphatase and sulfatase acid with P=0.013 and P=0.028, respectively (Graphic 3), as well as in caspase-3 activity with P= 0.05; at 2 hrs., except for a decrease in both esterase and caspase-3, the other ones increased almost twice their activity (Graphic 4), in contrast at 24 hrs., caspase-3 increased P=0.002 and sulfatase acid marginally P=0.12, contradictorily acid phosphatase decreased significantly P= 0.05, as well as alkaline phosphatase with a marginal P=0.08 (Graphic 5), possibly due to the cytotoxic apoptotic/necrotic response.

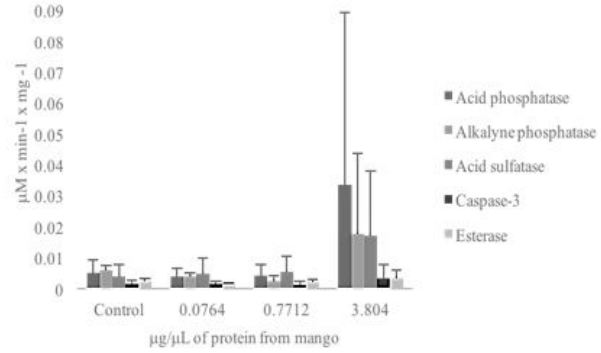
Both concentrations of 0.076 µg/mL and 0.771 µg/mL, after 30 minutes, showed no significant changes in the enzymatic activity (Graph 3 and 4), there was only an insignificant increase in esterase activity, while at 2 hrs., a significant decrease was observed in esterase activity in the concentration of 0.076 µg/mL with P=0.025 and a marginal decrease in alkaline phosphatase activity with P=0.08, for both concentrations, whereas at 24 hrs. In the concentration of 0.076 µg/mL, sulphatase and caspase-3 activity increased and decreased with a value of P=0.03 and P=0.05 respectively (Graphic 5). The results suggest a necrotic/apoptotic cytotoxic effect in the highest concentration at 30 min., and apoptotic at 24 hrs., while the lowest concentration produced necrotic cytotoxicity at times longer than 24 hrs.



Graphic 3 Activity specific of the acid phosphatase, alkaline phosphatase, acid sulfatase, and esterase and caspase-3 enzymes in lymphocytes exposed to protein extract from mango for 30 min



Graphic 4 Activity specific of the acid phosphatase, alkaline phosphatase, acid sulfatase, and esterase and caspase-3 enzymes in lymphocytes exposed to protein extract from mango for 2 hrs



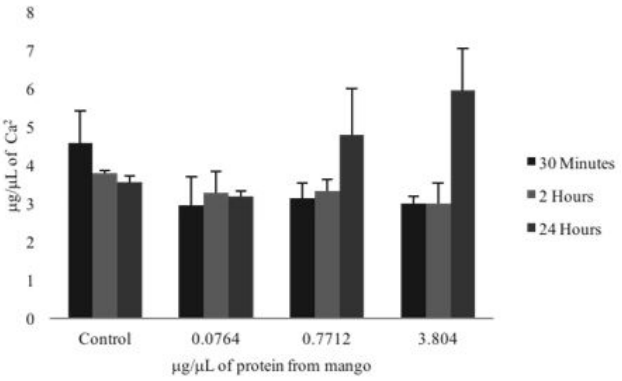
Graphic 5 Activity specific of the acid phosphatase, alkaline phosphatase, acid sulfatase, and esterase and caspase-3 enzymes in lymphocytes exposed to protein extract from mango for 24 hrs

Effect of mango protein extract on the amount of extracellular calcium in the lymphocytes cultures

Quantifying extracelular calcium

Calcium quantification in the supernatants of control samples after centrifugation showed that the amount of Ca^{+2} is slightly higher at 30 min., unlike the decrease at 2 hrs., and 24 hrs.

The amount of external calcium in the supernatants of control groups was not significant in the different times. The amount of extracellular Ca^{+2} decreases in all concentrations after 30 minutes with a marginal tendency P=0.06 compared to the control, suggesting they move inside the cell, which was maintained at 2 hours with the concentrations of 0.076 µg/mL and 0.771 µg/mL, the latter being significant with P=0.044. Contradictorily, at 24 hours an increase was observed in the extracellular calcium of concentrations 0.771 µg/mL and 3.8 µg/mL significant with the highest concentration P=0.033, suggesting they move towards the outer part of the cell. The effect of calcium according to time was marginal with P=0.075 (Graphic 6).



Graphic 6 Extracellular calcium quantification in lymphocytes exposed to different concentrations and times of the protein extract from mango

Effect of mango protein extract on the morphology of the nucleus

Nuclei staining

Figure 3 shows that in the A1, B1 and C1 photographs the nuclei remain intact and are not agglutinated, while in A2, B2 and C2 nuclear bodies close to agglutinated cells are observed, while in A3, B3, C3, A4, B4 and C4, apoptotic bodies are observed.

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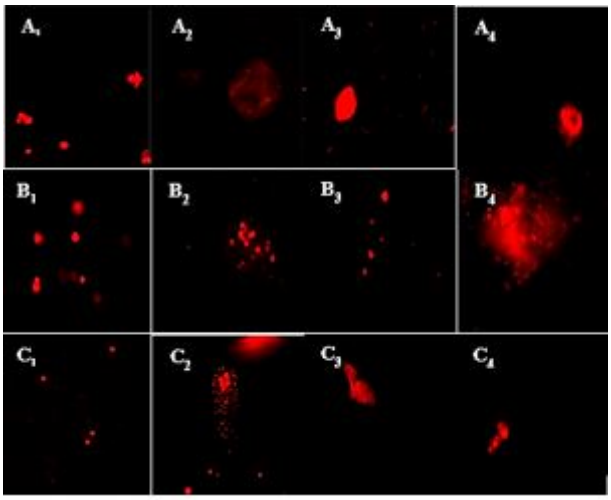

Figure 3 Nuclei of lymphocytes exposed to different doses and times in the extract protein from mango stained with propidium iodide. A (30 minutes), B (2 hours), C (24 hours); 1 (control), 2 (10 L), 3 (100 L) 4 (500 uL). A_1-A_4; B_1-B_4 photographs were taken at 100x. C1-C4 photographs were taken at 40x

Correlations						
		Dose	Time	Prot	ACP	ACS
Dose	Pearson Correlation				.292*	.352*
	Sig. (bilateral)				0.044	0.014
Time	Pearson Correlation					
	Sig. (bilateral)					
Prot*	Pearson Correlation					
	Sig. (bilateral)					
ACP*	Pearson Correlation	.292*				.750***
	Sig. (bilateral)	0.044				0.0001
ACS*	Pearson Correlation	.352*			.750***	
	Sig. (bilateral)	0.02			0.0001	
ES*	Pearson Correlation			-.328*	.282	
	Sig. (bilateral)			0.023	0.053	
ALP*	Pearson Correlation	.279			.954***	.745***
	Sig. (bilateral)	0.06			0.0001	0.0001
CASP-3*	Pearson Correlation	.379**	.287*	-.295*	.660***	.520***
	Sig. (bilateral)	0.018	0.048	0.042	0.0001	0.0001
Ca*	Pearson Correlation				-.261	-.328*
	Sig. (bilateral)				.074	.023
MTT	Pearson Correlation	.505***			.321*	.364*
	Sig. (bilateral)	0.001			0.026	0.011

Correlations						
		ES	ALP	CASP-3	Ca	MTT
Dose	Pearson Correlation		.279	.379**		.505***
	Sig. (bilateral)		0.055	0.008		0.0001
Time	Pearson Correlation			.287*		
	Sig. (bilateral)			0.048		
Prot*	Pearson Correlation	-.328*		-.295*		
	Sig. (bilateral)	0.023		0.042		
ACP*	Pearson Correlation	.282	.954***	.660***		.321*
	Sig. (bilateral)	0.053	0.0001	0.0001		0.026
ACS*	Pearson Correlation		.745***	.520***	-.328*	.364*
	Sig. (bilateral)		0.0001	0.0001	0.023	0.011
ES*	Pearson Correlation		.290*	.649***		.301*
	Sig. (bilateral)		0.045	0.0001		0.038
ALP*	Pearson Correlation	.290*		.626***		.294*
	Sig. (bilateral)	0.045		0.0001		0.043
CASP-3*	Pearson Correlation	.649***	.626***			.552***
	Sig. (bilateral)	0.0001	0.0001			0.0001
Ca*	Pearson Correlation					
	Sig. (bilateral)					
MTT	Pearson Correlation	.301*	.294*	.552***		
	Sig. (bilateral)	0.038	0.043	0.0001		
N = 48						
*. The correlation is significant at level 0. 05 (bilateral).						
**. The correlation is significant at level 0. 01 (bilateral).						
***The correlation is significant at level 0.001 (bilateral).						

Table 5 Statistical analysis using SPSS version 18. A Pearson correlation for normally distributed data was performed.
*Abreviatures: Protein (Prot), Alkaline phosphatase (ALP) Acid phosphatase (ACP), Estarease (ES), Acid sulphatase (ACS), Caspase-3 (CAS-3), Calcium (Ca), Methylthiazolyldiphenyl-tetrazolium bromide Test (MTT)

Discussion

It was confirmed that the protein extract from mango pulp, as expected by the background, possesses lectin activity [16] and recognizes both erythrocytes and lymphocytes and shows specificity for galactose, as well as for glucosamine, fucose and glucose previously detected with the hemagglutination inhibition test [16].

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The electrophoretic pattern for the most proteins we found (25 and 20 kD) and those lacking visibility (34, 75, 150kD) is close to those found in previous studies on mango pulp (250, 149, 75, 54, 24, 18 and 15 kD) and 80, 70, 43, 41 and 9 kD [1, 16]. Some authors report the presence of bands around 15, 30 and 50 kD and the protein band around 30kD with lectin activity and glucose and *N*-acetylglucosamine and *N*-acetylgalactosamine recognition, as well as galactose recognition [5] on peel and leaves of mango.

While other studies on mango pulp have reported the presence of two bands with lectin activity of 25 and 28 kD [5]. These different proteins patterns, may be due to its glycosylation states.

The carbohydrate content of the electrophoretic pattern of proteins showed a single band of approximately 20 kD highly glycosylated, perhaps a proteoglycan, whose presence has not been reported by other authors in extracts from mango.

However, could be the case that the 18 or 20 kD protein band is highly glycosylated and perhaps is responsible for the necrotic response to high and low concentrations observed in this study, as it is one of the principal proteins and it might be involved in hypersensitivity reactions that have been reported.

Nevertheless, reports on pistachio, which also shows cross reaction for mango allergies, suggest the presence of 5 protein bands 9, 41, 43, 70 and 80 kD with allergenic activity and they might be involved in hypersensitivity [3, 5].

The proliferation results at 24 hours with MTT test and the amount of protein concentrations with 0.076 and 0.77 µg/mL suggest an increase in the number of lymphocytes regarding control, indicator of mitogenic effect(s) of lectin(s). The marginal increase in the MTT test with P=0.06 and in the protein concentration P=0.09 at low concentration 0.076 µg/mL corroborate the proliferative effect of lectins and, hence, perhaps that is why the significant increase in the protein concentration of P=0.038, with that of 0.77 µg/mL. However, contradictorily to these concentrations a significant increase for acid sulfatase P=0.03 and decreased activity of acid phosphatase and basic, as well as caspase-3 both with P=0.05, are also detected. It may be the case that increased acid sulfatase besides being involved in necrosis; it might be involved in proliferation, as well as decreased caspase-3 related to MTT. Regarding the higher concentration of 3.804 µg/mL, the significant results observed with the MTT test in all of the exposure times are due to the cytotoxic effect resulting from the mitochondrial activity because of the increased activity of caspase-3 since 30 min., with P=0.05 and marginal P=0.06 at 24 hrs., and not by an increase in proliferation by the fact that was not any significant increase in the activity of MTT at 24 and 2 hours of exposure P=0.048 and P=0.01, respectively, also it is not related to an increase in the amount of protein as from 30 min., to 24 hours a decrease was observed. Besides, a decrease in lymphocytes and lysate lymphocytes was detected with a microscope and by nuclei staining (Figure 3), which indicates loss of lymphocytes since 30 min., due to the necrotic cytotoxic effect, as well as apoptotic, corroborated by the significant increase at 30 min., in both necrosis indicator enzymes: acid sulfatase and acid phosphatase with P=0.013 and P=0.028, respectively (Graph 3), as well as the increase in caspase-3 with P=0.05.

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While at 24 hours it was more apoptotic because an increased caspase-3 with P=0.002. Interestingly enough, we detected a marginal decrease activity in alkaline phosphatase with P=0.08 and significance decrease activity in acid phosphatase with P=0.05 at 2 and 24 hours respectively, as well as the non-significant increase in esterase at 30 min., perhaps as a response to the loss of membrane integrity leads to cell lysis and, thus, a loss of alkaline and acid phosphatase.

It may be the case that the observed decrease is due to the loss by lysis and secretion, where there is evidence that the alkaline phosphatase is secreted and its secretion is involved in gene expression [28]. The results of lymphocytes exposed to protein extract from mango showed a proliferative and necrotic/apoptotic effect depending on concentration and time.

There are reports in the literature indicating that the proliferative and apoptotic dual effect is due to the presence of reactive oxygen species [29].

No oxidative stress was measured. Nevertheless, there are evidences that it is related to both proliferation and cell death (necrosis/apoptosis). Reactive oxygen species such as superoxide's and hydroxyl groups are involved in gene expression with dual effects both toxic and proliferative [29]. On the other hand, the secreted alkaline phosphatase is also considered a marker enzyme for gene expression [28, 29]; and in the urine, is an early marker of renal tubular injury [28, 30]. A marginal decline at 2 and 24 hrs., in all concentrations was observed, meaning a loss primarily with the highest concentration, perhaps at the lowest concentration is only secreted, and we do not know this because we did not measure it.

An association of the caspase-3 with the results of MTT Test (r=0.552, P=0.0001) was found, suggesting a relationship between mitochondrial activity and caspase-3 activity, related to the integrity of the mitochondrial membranes, as a slight association between MTT with acid sulfatase and acid phosphatase and esterase, r =0.364 P≤0.011, r=0.321 P≤0.026P and r=0.301 P≤0.038, respectively, was found.

However, the caspase-3 was the enzyme associated, except calcium, with all parameters that showed a high association with acid and alkaline phosphatase and esterase with r=0.660, r=0.626 and r=0.649 respectively and P=0.0001 in almost all of them, as well as sulfatase with r=0.520 P=0.0001, perhaps because it belongs to the esterase group. Moreover, showed a negative association with the protein concentrations r=-0.295 P=0.042 perhaps related to cellular death.

Esterases comprise a wide range of enzymes that according to the functional they break include acetylases, carboxylases sulfatases and phosphatases, which may explain why we found that association among the most studied enzymes. Remarkably, results shows the high association between acid phosphatase and alkaline phosphatase r=0.954, P=0.0001 could be the same enzyme and correspond to the phosphomono esterase, which are nonspecific enzymes by the broad pH range in which acts ranging from 5 to 7.5, and that both the acid phosphatase and alkaline decrease at 24 hrs. Furthermore, these phospho monoesterases are related to death and the toxic effects of some poisons from crotalids, elapides, ophiodes and aphidoidea [26] Similarly both phosphatases (acid and alkaline) with similar values are highly associated with acid sulfatase and caspase-3 with r=0.750 r=0.745 and r=0.660, r=0.626 respectively, and with P=0.0001 in both, suggest strongly that them are phosphomonoesterase.

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The protein concentration was slightly negative associated with esterase, and caspase-3 r=0.328 P=0.023 and r=-0.295 P=0.042, further the association between esterase and caspase-3 r=0.649 and P ≤0.0001, and MTT with caspase-3 r=0.552 P=0.0001 is of great interest. It could be the case that esterease–which could comprehend phosphomonoesterase (acid and basic phosphatases)-is related with the caspase-3 coming probably from the two induction paths; the apoptosis death receptor and mitochondrial inner origin.

Esterases are also lipases such as phospholipase-C (PLC) and phospholipase-A (PLA).

Phospholipase-C (PLC) is involved in the inner cell signaling mechanisms and, when activated via receptors, it produces both diacyl diglycerides and inositol triphosphate and mobilizes the internal Ca^{+2} from endoplasmic reticulum (RE) [22, 31], while the PLA is activated by calcium and produces arachidonic acid, which is involved in the production of potent inflammation mediators. In insects two kinds of esterases, A and B that differ in their inhibitors have been described. However, lymphocytes are characterized by having nonspecific esterases (serine-esterase), due to their activity on the synthetic substrate of p-acetate nitrophenyl, principally found in T cells and involved in necrosis.

The results from the esterase activity, compared to control, showed an increase and a decrease and different exposure times in most concentrations and exposure times.

Regarding the lowest concentration of 0.076 μg/mL, the increase at 30 min., was no significant, whereas at 2 hrs., the decrease was significant with P=0.025, which could be due to the loss of membrane integrity when releasing during necrosis event, suggesting they are involved in membrane permeability, at the first moments when lymphocytes make contact with the protein extract the membrane is depolarized and there might be movement of calcium that activates inespecific and specifc esterases such as phosphomonoesterase, PLA and PLC, where PLA is capable of inducing the inflammation and proliferation mediators, whereas the PLC could also be activated via receptor and may mobilize the calcium from the ER [22, 31, 32, 33].

Arylsulfatases, is another esterase focuses on removing sulfates from sulfolipids [34], which could be destabilizing plasma and organelle membranes such as lysosomes and mitochondria involved in proliferation and necrotic/apoptotic cytotoxicity effect, since Ca^{++} also activates caspases and endonucleases. However, the data suggest that is very likely that phosphomono esterases and a transmembrane esterases [25] are common because of the decrease in esterase activity, as well as the loss in the activity of acid phosphatase and alkaline phosphatase at 24 hrs., and for it is association with those phosphatase r=0.29 p=0.045; r=289 p=053 respectively. On the other hand, the association of esterase with MTT r=0.301 p=0.038 and caspase-3 r=0.649, p=0001 suggest that is involved in proliferation but principally in apoptosis.

Since we wanted to work in conditions closer to human consumption, we worked with the crude extract and also did not work with inhibitors for each enzyme therefore, we do not know which of the proteins is responsible for the activation of the necrotic o apoptotic effect.

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Though the necrotic effect may be due to the 20KD major protein, a component that because of its degree of glycosidation is capable of forming a transmembrane pore, as well as inducing cell injury and causing necrotic cell death related to the movement of calcium. Hence, in the highest concentration at 30 min., the acid phosphatase and sulfatase activity was increased, as well as caspase-3.

Therefore, it may be the case that the effect of mango on the cytotoxic apoptotic response is due to the activation of a death receptor (Fas) or other receptor associated with phosphomonoesterase dependent on calcium and an apoptosis inducer [25] Such effect is probably due to a lectin similar to that of mistletoe lectin (ML-1), which recognizes galactose, a galectin that, has two subunits and one of the subunits has a molecular weight of 34 kD [18] with a molecular structure similar to that of RIP-2 type lectin and is involved in activating the death receptor, since our protein extract also recognizes galactose and has an discreet band of 34 kD and is similar to that reported by other authors that indicate the presence of lectins recognizing n-acetyl-galactosamine, n-acetyl glucosamine and glucose in both leaves and bark from mango, this activity is probably due to a 30 kD protein [5], as well as by the increase detected in caspase-3 with the high concentration of 3.8 µg/mL from 30 min., to 24 hrs., whereas the necrotic effect was detected only after 30 min., by the increased acid phosphatase and sulfatase activity at this concentration, while at 24 hrs., only acid sulfatase and caspase-3 significantly increased while both acid phosphatase and alkaline phosphatase decreased, possibly because it is the phosphomonoesterase.

It is noteworthy that lectins recognizing galactose are known as galectins, which are not only involved in apoptosis, but also, they are versatile cell adhesion modulators, cell proliferation and cell death as they regulate inflammatory immune responses. [14, 35, 36]. Galectins are also useful cancer markers [15] and are considered as a potentiall cancer therapy.

Furthermore, lectins with low molecular weight from 15 to 18 kD recognizing chitin derivatives (n-acetyl glucosamine) Induce cell death [19]. The protein extract from mango also recognizes n acetyl glucosamine and induces both apoptosis and necrosis, effect that might be also due to granzymes activation [20] where the pore formation would be the trigger through of the effect of chitin type lectins.

Extracellular calcium levels decreased at 30 min., and at 2 hrs., for all of the extract concentrations from mango, indicating entry to lymphocyte. Calcium mobilization could be involved in the binding of the effect or with galectin type death FAS receptor dependending on calcium and associated with an independent caspase-3 of mitochondria inducing apoptosis in lymphocytes.

Mitogens such as lectins induce changes in the polarity and permeability of the cell membrane by altering calcium mobilization and activating esterase type enzymes depending on calcium such as phospholipases, and MAKS or MAPS type phosphorylases kinases associated with proliferation. Additionally, calcium activates the phosphomonoesterase involved in both proliferation and apoptosis and mobilization of internal calcium stimulates enzymes such as endonucleases and exonucleases, nitrate synthase and apoptotic pathways dependent on mitochondrial caspase-3 [22, 31, 33].

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With respect to calcium results, in all extract concentrations from mango pulp, at 30 min., a marginal decrease P=0.06 extracellular calcium was detected, the same as at 2 hrs., where only the decrease was significant in the concentration of 0.771 µg/mL P=0.044, while at 24 hrs., we observed an increased extracellular calcium at all concentrations, but the highest concentration was the most significant P=0.033 was observed, meaning calcium entrance at short times mainly at low concentrations, possibly by membrane depolarization where calcium mobilizes into and at long times the calcium internal concentrations it may destabilize cell membranes such as the endoplasmic reticulum, mitochondria and lysosomes by causing cell lysis and, thus, releasing calcium and increasing its extracellular amount [22, 33 37].

High levels of internal calcium are responsible for the loss of mitochondrial membrane potential and the nucleus, and plays a very important role in the structural and functional changes in them like the lysosome during necrotic apoptotic cell death [20, 22, 37].

The level of extracellular calcium showed a low negative association only with acid sulfatase r=-0.328 and P =0.023 and marginal with acid phosphatese r=-0.267 and P=0.074. Since external calcium was measured, it would correspond to increase or decrease the internal calcium, hence, the lysosomal enzyme activity such as acid sulfatase, positively associated with calcium.

Since calcium modifies the membrane integrity of different organelles such as the lysosomes. No association with caspase-3 was detected.

Probably, the instability of mitochondria and lysosomes are not due to calcium, but with esterase activity, which it was associated with caspase-3 r=0.649 P=0.00012 and with the MTT test r=0.301, P=0.038. Esterase and caspase-3 were negatively associated with protein concentration with r=-0.328 P=0.23, r=-0.295 P=0.042, respectively.

Most mitogens, lectins in this case, as well as depolarizing liposoluble, activate the signaling pathway of MAPKS kinases and also generate ROS, including OH, perhaps involved in the activation and alkaline phosphatase secretion. Both hydroxyl ions and secreted alkaline phosphatase are involved in gene expression, in the case of secreted alkaline phosphatase it is a gene expression marker [28]. In lymphocytes, we detected a decrease and a marginal increase P=0.06 in concentrations of 0.076 and 3.8 µg/mL respectively. Regarding alkaline phosphatase activity at 2 hrs., depending on activity and production of ROS, gene expression associated with proliferation or apoptosis [29] will be inducted. At 30 min., a marginal increase P=0.06 was detected in all of the protein extracts from mango, while at 2 hrs., in all of the concentrations a significant increase was observed in the protein concentration (P=0.008 for the concentration of 3.804 µg/mL; P=0.01 for the concentration of 0.771 µg/mL and finally P=0.032 for the concentration of 0.076 µg/mL).

Similarly, at 24 hrs., both concentrations of 0.076 and 0.771 µg/mL had a significant increase. Contrarily, the concentration of 3.8 µg/mL showed a protein decrease because of the proliferative and cytotoxic effect respectively, and given the protein concentration was found to be negatively associated with esterase and caspase-3 r = -0.328. p=023 and r = -0. 294 p=0.042 respectively.

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Thus, means that the higher the caspase-3 and esterase activity's, the lower is the protein concentration, perhaps because esterase and caspase-3 are involved in the cytotoxic effect. This suggests that the protein extract from mango contains components that act directly on the membrane and also lectins or proteins that recognizes the death factor may actually be involved, as well as the glycosylated 20 kD component.

Calcium levels, along with activity of phosphatase and esterase, etc., play an important role in contrary and dual events such as proliferation and cell death, as well as in the duality of the apoptotic or necrotic cytotoxicity and depending on the cytotoxic effect a response immune dual will occur the anti-inflammatory or pro inflammatory, that could be exacerbated or inhibited by the hypersensitivity to mango that affects some people. Thus, it is important to determine precisely the ingredient(s) from mango responsible for the necrotic/apoptotic effect, but more important to determine why some people have very severe hypersensitivity to mango, since this sensitivity is related to other foods or allergens such as pistachio, strawberry and latex. Much of this sensitivity is associated with the presence of Chi or chitin type lectins.

Furthermore, it is known that atopic subjects with no allergy to any food are hypersensitive to them because Chi type lectins could be involved. For example, these types of lectins from potato activate mast cells and basophils that interact with a chitiobiose nucleus from cells bounded to nonspecific E immunoglobulin [38]. Therefore, foods containing Chi type lectins that recognize polymeric compounds from n acetylglucosamine may trigger allergic reactions to them.

The fact that the protein extract from mango recognizes n-acetyl glucosamine and also the molecular weight of one of the bands is 18 kD, suggest that it also possesses Chi type lectins.

Working with raw extract from Mango opens up a wide range of opportunities for further research to determine one of the possible causes experienced by people allergic to this fruit caused by the presence of proteins, including lectins, that can cause allergic disorders, as well as conducting research to identify the protein or lectin responsible for causing apoptotic cell death to use it for therapeutic purposes and also for diagnostic purposes.

Conclusions

The protein extract from *mango* with lectin activity that recognizes galactose, glucosamine and fucose shows a protein pattern bands similar to what other authors have reported. However, a highly-glycosylated band between 18 and 20 kD was detected.

These protein extract shows a dual proliferative and cytotoxic effect Proliferation is stimulated at low concentrations, while at high concentrations with short exposure times we detected an apoptotic/necrotic cytotoxic effect and apoptotic for long exposure times.

Those effects are associated with acid sulphates and phosphates, as well as alkaline phosphates and caspase 3 and calcium release; probably a protein extract stimulated a membrane phospho-monoesterase which may be involved to shut both cytotoxic and proliferation effects.

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