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Inhibition of Cytotoxicity of Shiga Toxin of *Escherichia coli* O157:H7 on Vero Cells by *Prosopis alba* Griseb (Fabaceae) and *Ziziphus mistol* Griseb (Rhamnaceae) Extracts

M. G. PELLARÍN,[1]* C. ALBRECHT,[1] M. J. ROJAS,[1] J. J. AGUILAR,[2] B. S. KONIGHEIM,[2] M. G. PARAJE,[3] I. ALBESA,[3] AND A. J. ERASO[1]

[1]*Escuela de Nutrición, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Enrique Barros s/n, Ciudad Universitaria, Córdoba, Argentina;* [2]*Instituto de Virología Dr. José María Vanella, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Enfermera Gordillo Gómez s/n, Ciudad Universitaria, Córdoba, Argentina; and* [3]*Departamento de Farmacia, IMBIV-CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Medina Allende s/n, Ciudad Universitaria, Córdoba, Argentina*

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ABSTRACT

The capacity of *Prosopis alba* Griseb. and *Ziziphus mistol* Griseb. fruit extracts to inhibit the toxic action of Shiga toxin (Stx) was investigated. Purification of Stx from *Escherichia coli* O157:H7 was performed by saline precipitation and affinity chromatography using a column with globotriaosylceramide, while the fruits were subjected to ethanolic or aqueous extractions. The protective action of both fruits was determined by pre-, co-, and postincubation of one 50% cytotoxic dose per ml of Stx with different concentrations of ethanolic and aqueous extracts in confluent monolayers of Vero cells for 72 h at 37°C (5% CO_2). The inhibition of the cytotoxic effect of Stx by fruit extracts was determined by the neutral red vital staining technique. The extraction of the polyphenols and flavonoids was effective, and more polyphenols per milligram of dissolved solids were obtained from *P. alba* than from *Z. mistol*. However, there were more flavonoids in *Z. mistol* than in *P. alba*. Components of both fruits increased the viability of cells treated with Stx when the extracts were preincubated with Stx for 1 h before being applied to the cell cultures, with the ethanolic extract of *P. alba* showing 95% cell viability at a concentration of 2.45 mg/ml. The extracts were less effective in protecting cells when Stx, extracts, and cells were coincubated together without a previous incubation of Stx; only the concentrations of 19.46 mg/ml for the *P. alba* aqueous extract and 3.75 mg/ml for the *Z. mistol* ethanolic extract resulted in the inhibition of cytotoxicity, with 52 and 56% cell viability occurring, respectively. Investigation into this difference in the protection of cells indicated that the protein molecule of Stx suffered degradation to advanced oxidative protein products during preincubation with extracts, principally with *P. alba,* which exhibited a greater amount of nonflavonoid polyphenols than *Z. mistol*. The prooxidant action on Stx favored the cells and enhanced the protective action of both fruits.

Shiga toxin (Stx)–producing enterohemorrhagic *Escherichia coli* (EHEC) is the causative agent of hemolytic uremic syndrome (HUS) and is an emerging pathogen in foods. This disease is the leading cause of acute kidney failure in children and the second leading cause of chronic renal failure, being responsible for 20% of kidney transplants in children and adolescents. In Argentina, where HUS is endemic, there are approximately 400 new cases per year *(10)*.

The damage observed in both the intestine and kidneys of infected children is produced by bacterial toxins powered by inflammatory mediators, with type 1 or 2 Stx (Stx1 and Stx2) being necessary for the development of HUS. The Stx family belongs to the AB_5 toxins, which are constituted by one A subunit and five B subunits, thus forming a pentameric structure. This pentamer binds to a receptor, globotriaosylceramide (Gb_3), found in some eukaryotic cells, and then the Stx is endocytosed by following the retrograde transport pathway for protein secretion via the Golgi apparatus, endoplasmic reticulum, and nuclear membrane. The B subunit binding to the Gb_3 receptor triggers intracellular signaling, thereby inducing cell damage by inhibition of protein synthesis *(23)*.

At present, there is no effective therapy to prevent or even limit the microangiopathic process in order to affect the course of this disease. Moreover, the use of antibiotics to treat infection with EHEC has been shown to increase the risk of HUS 17 times, because injury to the bacterial membrane induced by the antibiotic favors the release of large amounts of preformed toxin. Consequently, the use of antibiotics could be a serious disadvantage if EHEC cells are not eliminated quickly from the bowel. Certain antibiotics, particularly quinolone, furazolidone, and trimethoprim, are in fact potent inducers of gene expression of *Stx2* and can increase the level of toxins in the intestine *(36)*.

Given the need to find new therapies for HUS, natural products effective against EHEC have been investigated in

* Author for correspondence. Tel and Fax: (54 0351) 4333024; E-mail: mgpellarin@gmail.com.

recent years. Different studies have appeared in the scientific literature with the purpose of detecting plant foods with antimicrobial activity that are able to modulate infection, Stx production, and translocation of the toxin into the blood *(14, 17, 34, 35)*.

Along these lines, the present investigation was performed by using indigenous plant extracts in a eukaryotic cell line (Vero cells) with the objective of evaluating the inhibition of Stx cytotoxicity by determining the degree of cytotoxic damage.

MATERIALS AND METHODS

Preparation of plant extracts. Fruit extracts were obtained from *Prosopis alba* Griseb (Fabaceae) and *Ziziphus mistol* Griseb (Rhamnaceae) by performing maceration overnight at 4°C with two different solvents, ethanol (Porta Hnos. S.A., Córdoba, Argentina) and sterile distilled water; after filtering, the extracts were concentrated in vacuo in a heat rotary evaporator (Evapomix, Buchler Instruments, Fort Lee, NJ). The resulting solids were solubilized with sterile distilled water and sterilized using a 0.22-μm-pore-size syringe filter (Millipore, Inc., Bedford, MA) before being stored at −20°C until use.

Quantification of DS, total polyphenols, and plant extract flavonoids. Dissolved solids (DS) were quantified by weight on an analytical balance (Ohaus, Parsippany, NJ), and suspensions were prepared at different concentrations (in milligrams per milliliter) with the solvent.

For the determination of total polyphenols, plant extracts were analyzed by a colorimetric method using Folin-Ciocalteu reagent and Na_2CO_3 (Anedra S.A., Buenos Aires, Argentina). The absorbance was measured at 765 nm in a spectrophotometer DU-640 (Beckman Coulter, Inc., Brea, CA), and the readings obtained were compared with a calibration curve obtained by using 1.6 to 250 μg of gallic acid (Anedra) as the standard *(9)*. The results were expressed as micrograms of gallic acid per milligram of DS.

The flavonoid content was determined using the technique of Salamanca Grosso et al. *(28)*. The absorbance was measured at 415 nm, and the readings obtained were compared with a calibration curve using 3.9 to 250 μg of high-performance liquid chromatography–grade quercetin dihydrate (Sigma Aldrich, Buenos Aires, Argentina) as the standard. The results were expressed as micrograms of quercetin equivalents per milligram of DS.

Thin-layer chromatography. The presence of functional groups was confirmed by carrying out thin-layer chromatography on Sil G/UV254 silica gel plates (Merck Chemicals, Buenos Aires, Argentina) with a fluorescent indicator, using chloroform–acetone–formic acid (7.5:1.65:0.85, vol/vol/vol) (Anedra), and the spots were visualized under 254-nm illumination. All organic solvents were distilled prior to use. Quercetin (Sigma Aldrich) and luteolin (Sigma Aldrich) controls were also used *(29)*.

Preparation of Stx. *E. coli* O157:H7 EDL 933, producing both Stx1 and Stx2, and *E. coli* ATCC 25922, as the reference nonpathogenic strain (Stx negative), were provided by the Laboratory of Hygiene and Microbiology, Department of Pharmacy, National University of Córdoba, Córdoba, Argentina. The identity of each strain was confirmed by the National Administration of Laboratories and Institutes of Health (ANLIS). The strains were maintained in culture for 24 h at 37°C in Trypticase soy broth (Britania, Buenos Aires, Argentina) supplemented with 0.3% yeast extract (Britania). Periodically, confirmation of the purity of the strains was performed by growing strains in sorbitol MacConkey agar (Britania) and Chrom ECC agar (CROMagar, Paris, France). The 48-h cultures of both bacterial strains were centrifuged (centrifuge EPP16, Zelian, Buenos Aires, Argentina) for 15 min at 13,000 rpm at 4°C. The cell-free supernatant was precipitated overnight with ammonium sulfate [$(NH_4)_2SO_4$] (Sigma Aldrich) at 60% saturation, and the precipitate was resuspended in phosphate buffer (pH 7) and dialyzed against 0.1 M phosphate-buffered saline (PBS) for 24 h, with three daily changes of buffer. Then, the sample was filtered through a 0.22-μm-pore-size membrane. Confirmation of Stx was performed by affinity chromatography using a column with Gb_3 *(2)*. Briefly, culture supernatants were passed through a small column (1 or 2 ml) of Octyl-Sepharose CL-4B-Gb_3 (Sigma Aldrich) five times. The column was then washed with 10 column volumes of PBS to eliminate unabsorbed proteins, and the Stxs retained were eluted with 4.5 M $MgCl_2$ (Sigma Aldrich) in PBS (10 bed volumes) and dialyzed against PBS.

The presence of Stx was determined by an optical immunoassay (Shiga toxin Biostart kit, Inverness Medical Professional Diagnostics, Inc., Princeton, NJ) and quantified by the Lowry colorimetric technique with Folin-Ciocalteu reagent (Anedra), using a calibration curve with bovine albumin (Merck Chemicals), and the results were expressed as milligrams of protein per milliliter. Samples were stored at −20°C until use.

Vero cell culture. African green monkey (*Cercopithecus aethiops*) kidney cells (Vero 76, ATCC CRL-587) were provided by the Dr. Jose Maria Vanella Institute of Virology, National University of Córdoba. Cells were grown to confluence in minimum essential medium (MEM; Gibco, Invitrogen, Buenos Aires, Argentina) supplemented with 10% fetal bovine serum (Natocor, Villa Carlos Paz, Argentina) and 1% gentamicin (Sigma Aldrich) at 37°C in a controlled atmosphere (5% CO_2).

Cytotoxicity assays and cell viability. Cellular viability was measured by means of the neutral red uptake assay according to the methodology described by Borenfreund and Puerner *(5)*. Suspensions of cells ($2.5 \times 10^5 \pm 0.6 \times 10^5$ cells per ml) were incubated to confluence in supplemented MEM in 96-well tissue culture plates (Millipore) at 37°C under a controlled atmosphere. The 50% cytotoxic dose (CD_{50}) of verotoxin and cycloheximide (Sigma Aldrich) and noncytotoxic doses of *P. alba* and *Z. mistol* extracts were determined in a confluent monolayer of Vero cells after 72 h of incubation at 37°C under a controlled atmosphere. Then, 200 μl of a 50-μg/ml solution of neutral red dye (Sigma Aldrich) was placed in each well, and the cultures incubated for 3 h at 37°C in a controlled atmosphere to determine the viability of Vero cells. The dye was discarded and replaced at 100 μl per well with a decolorizing solution of water, alcohol, and acetic acid (100:99:1) (Anedra). This was stirred for 15 min and read on an enzyme-linked immunosorbent assay reader (BioTek, Winooski, VT) at a wavelength of 540 nm, with the results expressed as optical density (OD). Untreated-cell control experiments were carried out using the OD at 450 nm as 100% viability.

Cytotoxicity inhibition assays. The protective action of both fruits was determined by preincubation of 1 CD_{50}/ml of Stx with different concentrations of ethanolic and aqueous extracts of *Z. mistol* (ethanolic, 0.94 to 3.75 mg/ml, and aqueous, 2.98 to 11.90 mg/ml) or *P. alba* (ethanolic, 0.61 to 2.45 mg/ml, and aqueous, 4.87 to 19.46 mg/ml) for 1 h at 37°C. Then, 96-well plates of confluent monolayers of Vero cells in supplemented MEM were incubated with the samples of Stx plus extracts for 72 h at 37°C (5% CO_2). The inhibition of the cytotoxic effect of Stx by fruit extracts was determined in Vero cells by the neutral red vital staining technique. Coincubation was performed in 96-well plates

TABLE 1. *DS and total polyphenol and flavonoid extracts of* P. alba *and* Z. mistol[a]

Type and amt (mg/ml) of extract (DS)	Polyphenols (μg GAE/mg DS)	Flavonoids (μg QE/mg DS)
P. alba, ethanolic, 24.46 ± 0.15	11.33 ± 0.29	1.07 ± 0.03
P. alba, aqueous, 194.60 ± 0.52	7.82 ± 0.05	0.11 ± 0.005
Z. mistol, ethanolic, 37.53 ± 0.26	20.71 ± 0.44	0.56 ± 0.02
Z. mistol, aqueous, 119 ± 0.42	30.48 ± 0.41	0.12 ± 0.01

[a] Values are means ± standard deviations. DS, dissolved solids; GAE, gallic acid equivalents; QE, quercetin equivalents.

with confluent Vero cells, with 1 CD_{50}/ml of Stx and different concentrations of plant extracts incubated simultaneously in MEM for 72 h at 37°C (5% CO_2), and then the cell viability was determined. Postincubations with different concentrations of ethanolic and aqueous extracts of *Z. mistol* and *P. alba* were performed in confluent Vero cell monolayers in MEM after 1 h of contact at 37°C with 1 CD_{50} Stx. Then, cells were incubated for 72 h at 37°C (5% CO_2) and the cell viability was determined.

In all cytotoxic assays, the Vero cells alone without cytotoxic treatment were used as negative controls. Moreover, negative controls were performed with culture supernatants of *E. coli* ATCC 25922 Stx(−). Two types of positive controls of cytotoxicity were performed, one with Stx at a toxic concentration of 1 CD_{50} and the other with cycloheximide at a concentration of 1 CD_{50}.

The degradation of 10 μg/ml Stx by ethanolic fruit extracts (0.64 mg/ml of *Z. mistol* extract or 0.12 mg/ml of *P. alba* extract) was investigated using 0.1 ml of acetic acid (Anedra) and 50 μl of 1.16 M potassium iodide (Sigma Aldrich) in test tubes to determine the advanced oxidation protein products (AOPP). A standard curve was produced with 1 ml of 0 to 100 μmol of chloramine T (Anedra). The absorbance of the reaction mixture was read at 340 nm to calculate the chloramine T equivalents in micromoles *(8).*

The experiments were performed in triplicate and repeated at least three times. Results were expressed as means ± standard deviations. Statistically significant differences between samples were investigated using analysis of variance and Student's *t* test with significance determined at a *P* value of <0.05.

RESULTS

Quantification of DS, total polyphenols, and flavonoids in plant extracts. The determination of DS was carried out on aqueous and ethanolic extracts of *P. alba* and *Z. mistol,* with the analysis of the components in the extracts obtained from these two species of fruit indicating that the DS content varied depending on the extraction solvent used. The aqueous extracts of *P. alba* and *Z. mistol* revealed greater amounts of DS per milliliter (194.60 ± 0.52 mg DS per ml and 119 ± 0.42 mg DS per ml, respectively) than the ethanolic extracts (Table 1).

The total polyphenol content per milligram of DS was higher than the flavonoid content in all the samples studied, with the aqueous and ethanolic *Z. mistol* extracts exhibiting the highest contents of polyphenols per milligram of DS (Table 1). Additionally, the ethanolic extracts of *P. alba* and *Z. mistol* presented higher contents of flavonoids than the aqueous extracts of these fruits. Thin-layer chromatography with quercetin and luteolin standards confirmed the flavonoid contents of the extracts.

Cytotoxicity assays. The maximum doses of fruit extracts that did not produce microscopic alterations of the Vero cells corresponded to 19.5 mg DS per ml for extracts of *P. alba* and 11.9 mg DS per ml for extracts of *Z. mistol*. Consequently, the assays of inhibition of Stx cytotoxicity were performed with doses of extract below these concentrations. The CD_{50}s determined for Stx and cycloheximide corresponded to concentrations of 3.9 and 10 μg/ml, respectively. Figure 1A and 1B show the percentages of reduction in cell viability with the different concentrations of Stx and the cycloheximide positive control.

Cytotoxicity inhibition assays with vegetable extracts. The cytotoxicity of Stx was inhibited by both fruit extracts. The comparison of the percentages of Vero cell viability with ethanolic and aqueous extracts of *P. alba* showed that the former had a greater protective effect than the latter (Fig. 2), despite the aqueous extract having a greater amount of DS than the ethanolic extract. However, it should be noted that this extract presented more polyphenols and flavonoids than the aqueous extract.

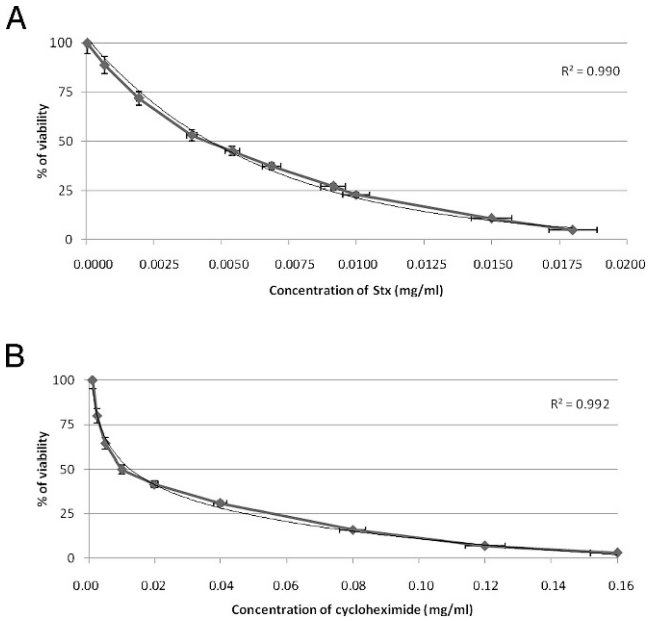

FIGURE 1. *(A) Determination of 50% cytotoxic dose (CD_{50}) of Stx from* Escherichia coli *O157:H7 on Vero cells. Percentages of cell viability after 72 h of incubation at 37°C (5% CO_2/95% O_2) with different concentrations of Stx. Mean values and standard deviations for independent experiments are shown;* $P < 0.05$. *(B) Determination of CD_{50} of cycloheximide on Vero cells. Percentages of cell viability after 72 h of incubation at 37°C (5% CO_2/95% O_2) with different concentrations of cycloheximide. Mean values and standard deviations for independent experiments are shown;* $P < 0.05$.

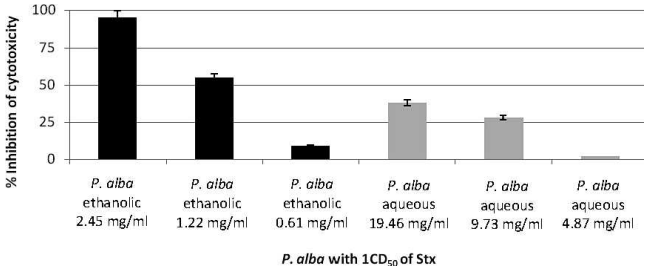

FIGURE 2. *Inhibition of effect of 50% cytotoxic dose (CD_{50}) of Stx on Vero cells by* P. alba *extracts at different concentrations. Black bars, ethanolic extracts; grey bars, aqueous extracts. Mean values and standard deviations for three independent experiments are shown;* $P < 0.05$.

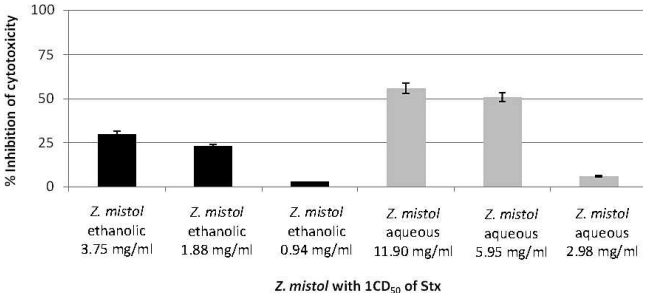

FIGURE 3. *Inhibition of effect of 50% cytotoxic dose (CD_{50}) of Stx on Vero cells by* Z. mistol *extracts at different concentrations. Black bars, ethanolic extracts; grey bars, aqueous extracts. Mean values and standard deviations for three independent experiments are shown;* $P < 0.05$.

With respect to *Z. mistol,* the extracts obtained with both solvents showed inhibition of Stx cytotoxicity, with the percentage of viability of treated cells being higher with the aqueous extract than the ethanolic extract (Fig. 3). In addition, there were more polyphenols in the former than in the latter, despite the flavonoid content being higher in the ethanolic extract than in the aqueous extract.

When the action of fruit extracts against Stx was tested under different conditions of incubation with Vero cells, the extracts did not show any inhibition of the cytotoxic effect when applied for 1 h postincubation with 1 CD_{50} of Stx (data not shown). Nevertheless, preincubation of 1 CD_{50} of Stx for 1 h with ethanolic and aqueous extracts of *P. alba* and *Z. mistol* at the different concentrations assayed revealed inhibition of cytotoxicity. It was also demonstrated that the inhibitory activity varied depending on the concentrations of the extracts used, with the OD readings showing that the extract which demonstrated the highest cell viability was the ethanolic extract of *P. alba* at a concentration of 2.45 mg/ml (OD value for treatment with 1 CD_{50} Stx, 0.705 $\pm$ 0.077; for untreated cells, 1.630 $\pm$ 0.039; and for *P. alba* ethanolic extract, 1.579 $\pm$ 0.047) (Table 2).

In the assays of coincubation of Vero cells with toxins and fruit extracts without previous contact between Stx and extracts, concentrations of 19.46 mg/ml *P. alba* aqueous extract and 3.75 mg/ml *Z. mistol* ethanolic extract resulted in the inhibition of cytotoxicity, with 52 and 56% viability (OD value for treatment with 1 CD_{50} Stx, 0.584 $\pm$ 0.06; for untreated cells, 1.218 $\pm$ 0.03; for *P. alba* aqueous extract, 0.638 $\pm$ 0.03; and for *Z. mistol* ethanolic extract, 0.682 $\pm$ 0.06) (Table 3).

Finally, the reason why the preincubation of Stx with the extracts prior to the inoculation of Vero cells was more effective in reducing the cytotoxicity than coincubation was investigated. Degradation of Stx by the extracts before application to Vero cells was found to be a possible cause of the reduction of cytotoxicity in the preincubation assay. Moreover, inactivation of the protein molecule of Stx by degradation to AOPP was detected during the preincubation of Stx with *Z. mistol* or *P. alba* extracts. These assays indicated that both fruits stimulated the degradation of Stx, with an increase of AOPP from 23.80 to 30.26 μmol of chloramine T equivalents (27% of increment) with *Z. mistol* ethanolic extract and a corresponding rise from 23.80 to 26.48 μmol of chloramine T equivalents (11% of increment) with ethanolic extract of *P. alba* (Fig. 4).

DISCUSSION

The inhibition of Stx has been studied previously by different techniques, including recent assays which used the application of nanoparticles *(20).* An attempt was also made to utilize probiotic bacteria to inhibit Stx cytotoxicity *(18).* Various plant spices have been screened for their ability to repress Stx production, and when the suppressive effect of eugenol was evaluated, the amounts of both intracellular and extracellular Stx were found to decrease, suggesting that eugenol may be useful for reducing the virulence of *E. coli* O157:H7 *(31). Quercus infectoria* nut gall was considered to be an effective medicinal plant against Stx-producing *E. coli* O157:H7. However, its antibacterial mechanisms have not yet been elucidated, although extensive damage to *E. coli* with loss of bacterial integrity through this plant has been demonstrated *(30).* Generally, the inhibition of Stx has been investigated by cell-based assays in the cell line Vero, which was used to screen a panel of plant compounds for antitoxin activities, thus confirming the antitoxin property of the grape extracts *(25).*

In the present investigation, the analyses of the DS contents per milliliter and of the polyphenol and flavonoid components by colorimetric and chromatographic techniques has suggested that these compounds can participate in the inhibition of cytotoxicity provoked by Stx on Vero cells, depending on the solvent used and the concentration of extract used.

The ethanolic extract of *P. alba* had a greater inhibitory effect on cytotoxicity than the aqueous one, despite having a lower amount of DS. These results may have been due to the larger amount of flavonoids in the ethanolic extract than in the aqueous extract, i.e., the greater inhibition of cytotoxicity obtained with the ethanolic extract of *P. alba* than with that of *Z. mistol* was probably due to its higher content of flavonoids, expressed as quercetin equivalents (1.07 $\pm$ 0.03 μg of quercetin eq per mg of DS).

Unlike *P. alba,* the aqueous extract of *Z. mistol* exhibited a higher level of inhibition than the ethanolic extract, probably as a consequence of its larger amount of

TABLE 2. *Optical densities obtained by preincubation of Stx with extracts and subsequent inoculation of Stx plus extracts on Vero cells*

Sample	Neutral red vital stain uptake (mean OD ± SD)
Vero cells	1.630 ± 0.039
Vero cells + 1 CD_{50} Stx + 2.4 mg/ml *P. alba* ethanolic extract	1.579 ± 0.047
Vero cells + 1 CD_{50} Stx + 3.75 mg/ml *Z. mistol* ethanolic extract	0.895 ± 0.032
Vero cells + 1 CD_{50} Stx + 19.46 mg/ml *P. alba* aqueous extract	0.862 ± 0.021
Vero cells + 1 CD_{50} Stx + 11.9 mg/ml *Z. mistol* aqueous extract	0.956 ± 0.024
Vero cells + 1 CD_{50} Stx + 1.22 mg/ml *P. alba* ethanolic extract	0.919 ± 0.048
Vero cells + 1 CD_{50} Stx + 1.88 mg/ml *Z. mistol* ethanolic extract	0.836 ± 0.015
Vero cells + 1 CD_{50} Stx + 9.73 mg/ml *P. alba* aqueous extract	0.823 ± 0.063
Vero cells + 1 CD_{50} Stx + 5.95 mg/ml *Z. mistol* aqueous extract	0.925 ± 0.078
Vero cells + 1 CD_{50} Stx + 0.61 mg/ml *P. alba* ethanolic extract	0.755 ± 0.031
Vero cells + 1 CD_{50} Stx + 0.94 mg/ml *Z. mistol* ethanolic extract	0.730 ± 0.025
Vero cells + 1 CD_{50} Stx + 4.87 mg/ml *P. alba* aqueous extract	0.726 ± 0.048
Vero cells + 1 CD_{50} Stx + 2.98 mg/ml *Z. mistol* aqueous extract	0.741 ± 0.048
Negative control, Vero cells + *E. coli* ATCC Stx(−)	1.580 ± 0.021
Positive control, Vero cells + 0.1 mg/ml cycloheximide	0.925 ± 0.076
Positive control, Vero cells + 1 CD_{50} Stx	0.705 ± 0.077
Cytotoxicity control, Vero cells + 2.45 mg/ml *P. alba* ethanolic extract	1.595 ± 0.029
Cytotoxicity control, Vero cells + 19.46 mg/ml *P. alba* aqueous extract	1.601 ± 0.023
Cytotoxicity control, Vero cells + 3.75 mg/ml *Z. mistol* ethanolic extract	1.590 ± 0.021
Cytotoxicity control, Vero cells + 11.90 mg/ml *Z. mistol* aqueous extract	1.589 ± 0.026

nonflavonoid polyphenols. In general, these compounds are considered to be antioxidants and able to protect cells from damage caused by diverse toxins. However, concerning the inhibition of toxins by natural products, research has shown that a single plant has several compounds with different effects, including antioxidant, prooxidant, and cytotoxic effects *(13)*.

It is necessary to find suitable doses of nutrients in order to obtain antioxidant effects. Nevertheless, antioxidant protective effects are frequently found at certain concentrations, while the opposite effects may occur at other concentrations, as was reported for some dietary nonflavonoid polyphenols from *Oleaeuropaea (7)*. In fact, there are various components of fruits and vegetables which may have an antioxidant (anticytotoxic) or a prooxidant (cytotoxic) effect depending on the concentration *(4, 12)*. Moreover, flavonoids are extensively metabolized, and their metabolites can be cytotoxic, showing that bioconversion can counteract the anticytotoxic (antioxidant) capacity of flavonoids *(3)*.

It should be remembered that Stx is cytotoxic, as it inhibits protein synthesis, and it has also been demonstrated to induce apoptosis with oxidation and fragmentation of DNA *(11)*. Cell death by apoptosis provoked by Stx depends on the cell type and varies with the sensitivity or specificity of the assay used *(6)*. Related to this, the capacity of Stx to induce apoptosis has been reported in different cell types, such as Vero cells *(15),* human renal proximal tubular epithelial cells *(19),* and human renal cortical epithelial cells *(16)*. In addition, there is now abundant evidence that Stx induces apoptosis in epithelial, endothelial, lymphoid, and myeloid cells in vitro. When this toxin has been administered, these cells have been shown to be capable of activating several cell stress response pathways in response to the oxidative stress caused by Stx *(32)*.

Early studies described the inhibition of protein and inactivation of ribosome function by Stx, whereas more recently, investigations have indicated alterations in the redox pathways in the action of Stx on Vero cells, with a reduction of dehydrogenase activity *(26)*. Stx induces the

TABLE 3. *Optical densities obtained in assays with Stx plus fruit extract coincubated in Vero cells without previous contact between toxin and extracts*

Sample	Neutral red vital stain uptake (mean OD ± SD)
Vero cells	1.218 ± 0.035
Vero cells + 1 CD_{50} Stx + 19.46 mg/ml *P. alba* aqueous extract	0.638 ± 0.036
Vero cells + 1 CD_{50} Stx + 3.75 mg/ml *Z. mistol* ethanolic extract	0.682 ± 0.065
Negative control, Vero cells + *E. coli* ATCC Stx(−)	1.193 ± 0.044
Positive control, Vero cells + 0.1 mg/ml cycloheximide	0.621 ± 0.025
Positive control, Vero cells + 1 CD_{50} Stx	0.584 ± 0.061
Cytotoxicity control, Vero cells + 2.45 mg/ml *P. alba* ethanolic extract	1.198 ± 0.025
Cytotoxicity control, Vero cells + 19.46 mg/ml *P. alba* aqueous extract	1.185 ± 0.031
Cytotoxicity control, Vero cells + 3.75 mg/ml *Z. mistol* ethanolic extract	1.203 ± 0.021
Cytotoxicity control, Vero cells + 11.90 mg/ml *Z. mistol* aqueous extract	1.190 ± 0.028

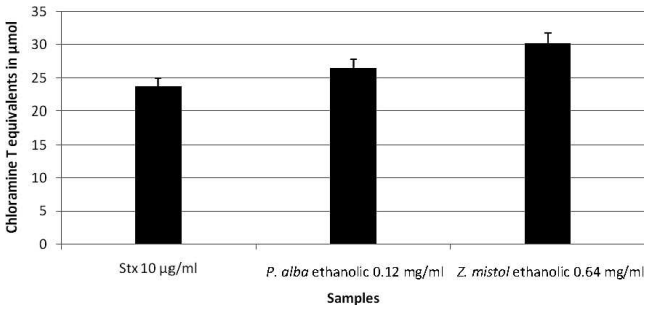

FIGURE 4. *Degradation of Stx to advanced oxidation protein products (AOPP) stimulated by* P. alba *and* Z. mistol *extracts. Mean values and standard deviations for independent experiments are shown;* $P < 0.05$.

endoplasmic reticulum (ER) stress response that leads to apoptosis by the activation of stress sensors, with an alteration in the ER lumen redox state, variations in the rate of flux of newly synthesized proteins, aberrant glycosylation, and anomalous intracellular calcium homeostasis. Thus, the capacity of the ER to correctly fold nascent proteins may become saturated, and this condition is referred to as ER stress *(27)*.

It should be emphasized that Stx is not the only microbial toxin that may induce apoptosis through prolonged activation of the ER stress response *(21)*. In a recent publication, Stx was shown to activate multiple stress-associated signaling pathways in mammalian cells with the activation of a ribotoxic stress response that led to signaling through mitogen-activated protein kinase cascades *(33)*. With respect to the association of Stx with oxidative stress, there is accumulating evidence of disequilibrium of redox status in HUS, since significant decreases in superoxide dismutase activity in erythrocytes from HUS patients have been described *(22)*.

Another cause of oxidative stress in patients with this syndrome was a low plasma vitamin E concentration, subsequently treated with the antioxidant vitamin E. In fact, children with this antioxidant fared considerably better than patients not treated with vitamin E, with an absence of any observed side effects *(24)*. Increased levels of oxidative stress were demonstrated during the acute phase of HUS, with the peak plasma lipid peroxidation values being well above those registered in controls, suggesting a connection between the clinical course of HUS and plasma lipid peroxidation as measured by thiobarbituric acid-reactive substances and total reactive antioxidant potential *(11)*. More recently, important levels of protein oxidation by the action of Stx were found *(1)*.

The results obtained in the present work show the protective effects of *P. alba* and *Z. mistol,* with a reduction in Stx cytotoxicity. However, the percentages of cell viability were higher when Stx was previously in contact with the extracts, suggesting a possible alteration of this toxin. Effectively, the assays of AOPP revealed that both fruits were able to increase the levels of advanced products of oxidative degradation in Stx, and consequently, the extracts reduced the amount of Stx capable of damaging the Vero cells. Curiously, *Z. mistol* exhibited a greater prooxidant effect in Stx than *P. alba,* probably due to the greater number of polyphenols, since there are nonflavonoid polyphenols with important prooxidant capacities. Indeed, a prooxidant effect was reported in nonflavonoid phenols of olive oils (oleuropein and hydroxytyrosol) from *Olea europaea* L., which inhibited low-density lipoprotein oxidation at low concentrations, thus exerting a prooxidant effect. However, dietary supplementation with virgin olive oils was able to increase the total plasma antioxidant status, with the net effect of olive oils being measured by taking into account their pro- and antioxidant capacities *(7)*.

To conclude, the protein molecule of Stx suffered degradation to AOPP during preincubation, principally with the *P. alba* extract, which exhibited a greater amount of nonflavonoid polyphenols than the *Z. mistol* extract. This prooxidant action on Stx favored the cells and enhanced the protective action of the polyphenols and flavonoids, which had antioxidant and prooxidant actions simultaneously.

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