Chemiluminescence determination of antioxidant property of *Zizyphus mistol* and *Prosopis alba* during oxidative stress generated in blood by Hemolytic Uremic Syndrome-producing *Escherichia coli*

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ABSTRACT: This study was undertaken to elucidate the antioxidant effect of *Zizyphus mistol* and *Prosopis alba*, with the hypothesis that these fruits can counteract the induction of reactive oxygen species (ROS) caused by toxins produced by *Escherichia coli*. In the search of nutrients effective against the Hemolytic Uremic Syndrome (HUS), we detected by chemiluminescence a protective role of both plants, due to their natural antioxidants significantly decreasing the levels of ROS induced by toxins from *E. coli* in blood. The ferric reducing antioxidant power (FRAP) was found to be higher in *Z. mistol* than in *P. alba*. The chemical analyses of the phenols and flavonoids present in the fruit extracts indicated that the FRAP correlated with the amount of phenolic compounds, but not with the flavonoids analyzed. Both fruits studied reduce the induction of ROS, and in this way help to prevent the development of complications related to oxidative stress generated in the blood of patients with HUS. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: antioxidants; *Zizyphus mistol*; *Prosopis alba*; Uremic Hemolytic Syndrome; chemiluminescence

Introduction

Diverse food components have been demonstrated to have beneficial effects on human health, since dietary constituents have important antioxidant properties. Natural antioxidants, such as extracts from vegetable species, have been investigated to develop new therapeutic and preventive strategies against different pathologies, since they are considered to be important sources of radical scavenging (1,2).

Polyphenolic compounds, present for example in fruits, contribute in a similar way to the flavonoids by counteracting the noxious effects of oxidative stress induced in an array of diseases (3,4).

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is able to provoke enteric illness, which results in clinical features of variable severity, ranging from uncomplicated diarrhea and hemorrhagic diarrhea to Hemolytic Uremic Syndrome (HUS), with renal and microvascular thrombosis. This latter syndrome is the most common cause of acute renal failure in children below 3 years of age. It presents a hemolytic anemia associated with fragmented erythrocytes, thrombocytopenia and acute renal failure, including activation of the platelet aggregation and local intravascular coagulation, especially in the renal microvasculature. Although Shiga toxin (Stx) or Vero toxin (VT) is the main pathogenic factor in HUS; hemolysin (Hly) also has a distinctive role in this disease (5,6).

The link between HUS and oxidative damage has been demonstrated by different authors. Patients with this syndrome presented alterations in plasma composition, and also in blood cells, including the viability and function of leukocytes. With respect to the plasma composition, an increase in lipid peroxidation to malonic dialdehyde in response to an enhanced ROS production, including the hydroxyl radical, was observed in patients with this syndrome (7). Related to this, Stx has direct effects on polymorphonuclears (PMNs) that can contribute to tissue injury in this disease (8). Moreover, the PMNs of patients with HUS are functionally impaired and degranulated, thus causing endothelial damage, as a consequence of activation with the release of ROS, thus causing endothelial damage (9). In addition, in the acute phase of HUS, the erythrocytes are also exposed to an oxidative imbalance, which is associated with a decrease in

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membrane fluidity and hemolysis (10). A significant decrease in superoxide dismutase (SOD) activity was found in erythrocytes from HUS patients (11).

The above work stimulated us to investigate if a potential dietary treatment could alter the natural course of this disease by avoiding its severe consequences, with the hypothesis that indigenous fruits could be antioxidants able to reduce oxidative stress in HUS. *Zizyphus mistol* and *Prosopis alba* were analyzed with the objective of finding potential nutritional or pharmaceutical treatments to counteract the outcome of oxidative damage in patients with HUS.

Experimental

Culture conditions of STEC and separation of supernatants free of cells

The clinically isolated STEC stain was grown in tryptic soy broth for 48 h at 37°C in an orbital rotator (150 rpm). Bacteria were then pelleted, and the cell-free culture supernatant was obtained by a sterilizing filtration with a membrane of 0.22 µm diameter pores.

Purification of Stx and Hly toxins

Shiga toxin was separated from culture supernatants by precipitation with 60% saturation of ammonium sulfate $(NH_4)_2SO_4$ and dialyzed for 24 h in phosphate buffer (pH 7). Then, Stx was purified using a receptor-mediated affinity chromatography following a method previously described (12).

The supernatant of STEC culture was precipitated with 60% $(NH_4)_2SO_4$ and the Hly was purified by chromatography (13). The toxin purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining.

Detection of ROS by chemiluminescence (CL)

The capability of Stx and Hly to generate ROS was examined in venous whole blood using 3-aminophthalhydrazide 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) sensitized CL. All experiments were carried out following the guidelines of the Ethics Committee of Cordoba University with blood of normal volunteers ($n = 6$). The production of ROS in 10 µL of whole blood was measured with 10 µL of Hanks's balanced salt solution (HBSS) and 600 µL of reagent mixture, composed of 5 mL 0.067% luminol in HBSS, 0.2 mL 5% glucose, 1 mL of Ringer lactate solution and 3.6 mL distilled water. Then, 10 µL of different concentrations of toxins were added (1–40 µg/mL Stx, or 1–40 HU/mL Hly) and the CL was tested in a BioOrbit luminometer. Controls used 10 µL of HBSS instead of toxins. The light emission results were expressed as relative light unities (RLU), with the maximum RLU reached in each assay being named V_{max}. The area under the curve (AUC) for the curves of RLU vs time was calculated using the Origin computer program. 4,5-Dihydroxi-1,3- benzene-disulfonic acid (Tiron, 0.5 mM) was applied in some assays instead the 10 µL HBSS.

Assays of ROS scavenger capacity with fruit extracts by means of CL assays

The antioxidant effect of fruit extracts in blood treated with toxins at concentrations 1–40 µg/mL Stx and 1–40 HU/mL Hly was investigated by the addition of 10 µL of 50 mg/mL extracts (instead of the 10 µL of HBSS in the CL assay described above) to detect the production of ROS in these cultures.

Determination of ferrous reduction antioxidant potential (FRAP)

The ability to reduce Fe^{+3} to Fe^{+2} was tested by using a reaction of 1.12 mL of 300 mM acetate buffer (pH 3.6); 0.14 mL of 20 mM $FeCl_3·6H_2O$; 0.14 mL of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl; and 0.1 mL of 50 mg/mL vegetable extracts. After 20 min, the OD at 593 nm was determined. Standard $FeSO_4$ diluted into different concentrations was employed to express the results in µM $Fe SO_4$.

Preparation of fruits extracts

Z. mistol or *P. alba* fruits were macerated with different solvents for 24 h at 4°C with acetone, hexane, ethyl alcohol and water. Then the fruits were filtered to separate the solids, the different extracts were dried until they reached a constant weight of 0.2–6.7 g in a rotatory extractor with yields of 5–43%. Different factors, such as extraction temperature, solvent concentration and time of maceration, were investigated to optimize the extraction process. Dry extracts were dissolved in water (50 mg/mL), before being conserved at −20°C. The extracts of *Z. mistol* and *P. alba* were evaluated by considering their putative application as an antioxidant.

Phenolic compounds and flavonoids assay

The fruit extracts were analyzed by the assay described previously (14), using the Folin–Ciocalteu reactive and Na_2CO_3. Results were expressed in µg gallic acid/mg dry extract.

The flavonoid content was determined as in previous investigations (15) with $AlCl_3$ and CH_3COOK. These results were expressed in µg quercetin/mg dry extract. The results of the CL assay and FRAP were compared with the amount of phenolic compounds and flavonoids present in the vegetable extracts.

Results

In order to select the most appropriate fruit extract to act as an antioxidant in our model, we used different extraction methods and evaluated the presence of important antioxidant components, such as phenols and flavonoides, in the extracts obtained. The analyses of the phenolic compounds and flavonoids per mL of extracts indicated that the content varied depending on the solvent used. The aqueous and alcoholic procedures of extraction were more efficient than extractions with acetone, with the aqueous procedure being more useful than the alcoholic one to obtain phenolic compounds from *Z. mistol*, although the alcoholic treatment extracted more µg of gallic acid/mg dry extract of *P. alba* than the aqueous one. Hexane was not effective in extracting phenolic compounds from either *Z. mistol* or *P. alba* (data not shown). Therefore, the two procedures selected were the aqueous and alcoholic, based on these extraction results. Moreover, these extracts showed good activities and stabilities over the year of the study.
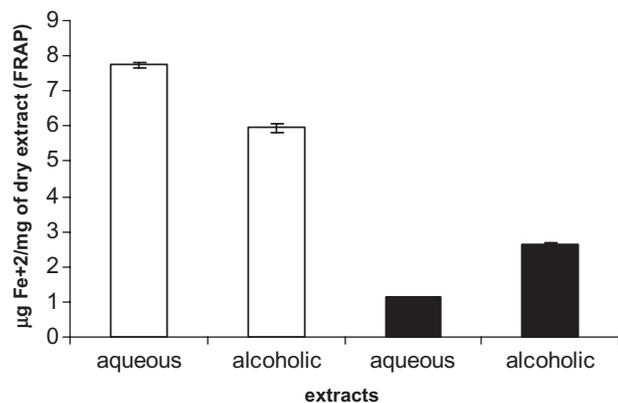
Figure 1. Ferrous reduction antioxidant potential: FRAP/mg dry extracts of *Z. mistol* (□) and *P. alba* (■).
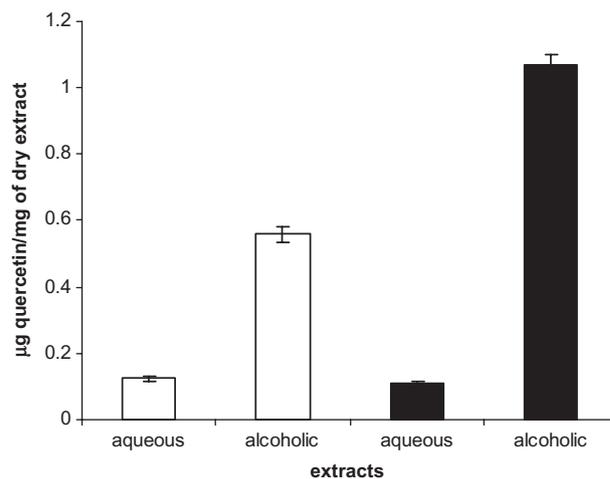

Figure 2. Phenolic compounds in μg gallic acid/mg dry extracts of *Z. mistol* (□) and *P. alba* (■).

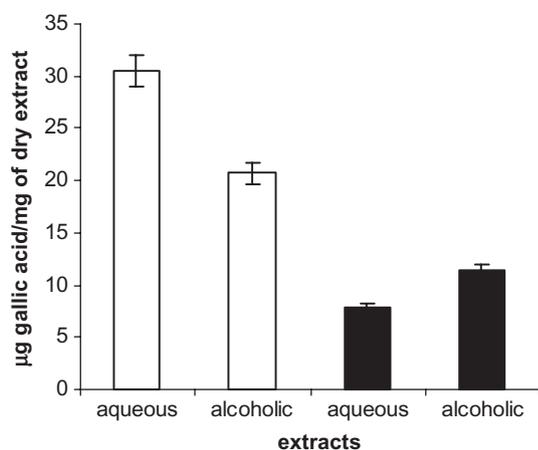**Figure 3.** Flavonoids in μg quercitin/mg dry extracts of *Z. mistol* (□) and *P. alba* (■).

The assays of FRAP demonstrated that extracts of *Z. mistol* and *P. alba* had antioxidant capacity. The μg Fe^{+2}/mg dry extract obtained with each solvent were the highest in aqueous *Z. mistol*, followed by alcoholic *Z. mistol*, alcoholic *P. alba* and aqueous *P. alba* (Fig. 1).

In addition, the analysis of phenolic compounds indicated the following decreasing order of μg galic/mg dry extract found: aqueous *Z. mistol*, alcoholic *Z. mistol*, alcoholic *P. alba* and aqueous *P. alba* (Fig. 2). There was an important correlation between FRAP and the content of these compounds, with a correlation coefficient r^2 of 0.97, adjusted to the equation $y = 0.2929x - 0.7827$.

With respect to the flavonoids, the alcoholic procedures were more effective than the aqueous ones to extract flavonoids per milligram of dry extract, with the following decreasing order of μg quercetin/mg dry extract obtained: alcoholic *Z. mistol*, alcoholic *P. alba*, aqueous *Z. mistol* and aqueous *P. alba* (Fig. 3). Unlike phenolic compounds, it was observed that flavonoid levels showed little correlation with FRAP ($r^2 = 0.22$).

CL was performed to evaluate if blood cells of normal persons suffered oxidative stress in the presence of purified Stx or Hly. This assay detected an increase of ROS in all the samples of blood studied. Blood cells suffered stress in the presence of their own plasma. Figure 4(A) shows the increase in ROS generated by puri-fied Stx (10, 20 and 40 μg/mL) in whole human blood. Purified Hly (10, 20 and 40 HU/mL) were also able to stimulate oxidative stress in blood with increases of ROS similar to or higher than that caused by Stx (Fig. 4B).

The results indicate that the extracts obtained had a scavenger capacity, since they were able to decrease significantly the production of ROS generated by 1 μg/mL Stx or 1 HU/mL Hly, concentrations that toxins present in bacterial cultures. *Z. mistol* and *P. alba* extracts were able to decrease the stimuli of ROS caused by Stx or Hly, at a similar intensity to Tiron, a scavenger of superoxide anion (Fig. 5). Since Stx and Hly were able to stimulate ROS, assays were performed with samples obtained by saline precipitation of both toxins together, in order to study the effects of these two stressing factors which are produced simultaneously by the bacteria. Dialyzed samples of precipitated toxins (Stx + Hly) from supernatants of STEC cultures also generated an oxidative stress that was detected by CL with luminol, with the 50 mg/mL extracts being able to counteract the stimuli of ROS caused simultaneously by both toxins. Table 1 shows the percentage of decrease in AUC and V_{max}, with aqueous and alcoholic extracts of *Z. mistol* and *P. alba*.

Discussion

Chemiluminescence (CL) has been previously applied to investigate oxidative bursts of PMNs in HUS patients (16). However, the assay of CL with luminol has not been used before to study the stress of erythrocytes together with other blood cells in the presence of STEC toxins. Therefore, the results obtained in the present study contribute to understanding the relation between oxidative stress and HUS. In our model, the CL assay was useful to show that the oxidative stress can be generated by the toxins of *E. coli*, isolated in a patient with HUS, with a similar increase in ROS being provoked by Stx and Hly.

Previous results obtained in our laboratory with other stressing substances demonstrated that an increase in their concentration resulted in a rise in the oxidative response to a point at which the exhausted cells decreased the production of ROS (17,18). This behavior was reflected in the results obtained with the HUS toxins, since ROS increased with 10 and 20 μg/mL of Stx, but
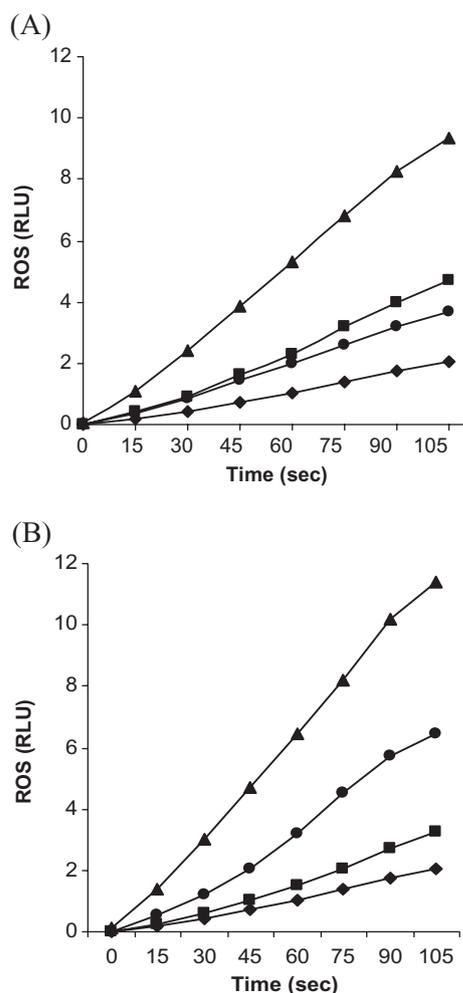
(A)

(B)

Figure 4. Production of reactive oxygen species (ROS) in blood stressed by: (A) purified Stx 10 µg/mL (●), 20 µg/mL (▲), 40 µg/mL (■) and 0 µg/mL (♦); (and B) purified Hly 10 HU/mL (●), 20 HU/mL (▲), 40 HU/mL (■) and 0HU/mL (♦).

(A)

(B)

Figure 5. Generation of reactive oxygen species (ROS) by (A) 1 µg/mL Stx (■), 0 µg/mL Stx (♦),1 µg/mL Stx together with 5 mM Tiron (▲), 1 µg/mL Stx together with 50 mg/mL of aqueous *Z. mistol* (×), alcoholic *Z. mistol* (○) or alcoholic *P. alba* (□); (B) 1 HU/mL Hly (■), 1 HU/mL Hly together with 0.5 mM Tiron (▲) and 1 HU/mL Hly together with 50 mg/mL of alcoholic *P. alba* (□).

decreased with 40 µg/mL Stx. In similar way, a rise in ROS occurred with 10 and 20 HU/mL of Hly, but a reduction was seen with 40 HU/mL of Hly.

The stimuli of ROS detected when blood was incubated with purified Stx or Hly indicated that both toxins were able to cause oxidative stress. Therefore it was decided to investigate the anti-oxidant capacity of fruit against both toxins used together, due to the fact that they are produced simultaneously by the bacteria. In addition, it should be mentioned that the oxidative stress generated by both toxins was manifested even in the presence of the natural antioxidant defenses of plasma, which were not sufficient to counteract the stimuli of ROS caused by Stx and Hly.

Counteraction of the oxidative damage produced by Stx and Hly by means of using the extracts of *Z. mistol* or *P. alba* was achieved. The extractive method employed was useful to obtain antioxidants, which prevented the increase of ROS induced by the toxins from *E. coli*. The aqueous fraction of *Z. mistol* was more effective than the alcoholic one, and conversely, in *P. alba*, the alcoholic extract was more effective than the aqueous extract. The decrease in ROS produced by the incorporation of the Tiron to the CL assay indicated that anion superoxide was involved in the stress generated by both toxins, and also showed that the
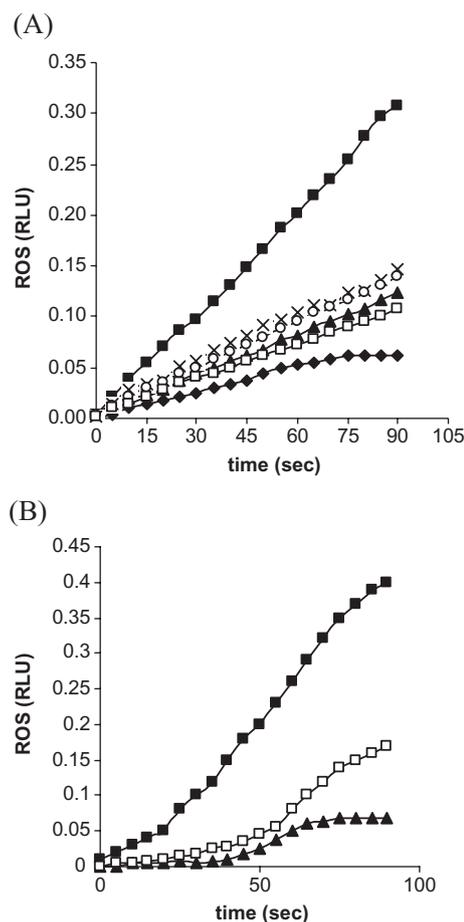
Table 1. Scavenger effect of Tiron and extracts of *Z. mistol* and *P. alba* on reactive oxidant species (ROS) stimulated by 40µg/mL Stx and 40 HU/mL Hly toxins of STEC

	Percentage decrease of	
	AUC	V_{max}
Tiron	55 ± 5	62 ± 6
Aqueous *Z. mistol*	65 ± 7	66 ± 8
Alcoholic *Z. mistol*	55 ± 9	64 ± 7
Aqueous *P. alba*	50 ± 6	51 ± 8
Alcoholic *P. alba*	53 ± 5	56 ± 6

extracts of both fruits studied present a scavenger capacity similar to the universally accepted scavenger, Tiron.

The fact that antioxidants could be derived from the aqueous extract of *Z. mistol* can be considered an advantage as they can be easily obtained and to administered in drinking juices. Moreover, the aqueous extracts and phenolic compounds showed good stabilities, with this being useful for future medicinal preparations. This characteristic was also emphasized as favorable by authors who had investigated the health-beneficial constituents of kiwi fruit (19).

The protective effect of these extracts could be, at least in part, a result of the antioxidant capacity observed in *Z. mistol* or *P. alba*. This study of the natural components of *Z. mistol* and *P. alba* suggests that the phenolic compounds were involved in the antioxidant capacity evaluated by the FRAP assay. On the other hand, the capacity to scavenge ROS seemed to be related to other compounds different from flavonoids, since the FRAP did not correlated with the milligrams of flavonoids detected in both fruits. The reduction of ROS obtained in the present study coincides with results of other investigations that studied the antioxidant activity of selected medicinal plant extracts, and emphasized the importance of phenolic contents and total flavonoids (20). However, despite flavonoids being a subclass of the polyphenols, some flavonoids were found not to be related to the antioxidant capacity (21).

The potential beneficial use of *Z. mistol* or *P. alba* could be an alternative for infections that still do not have an effective cure at present, in spite of diverse techniques being tried. In particular, the application of antioxidants may be useful in order to avoid blood cell damage in patients with HUS. In addition, in the present study the polyphenols observed in the antioxidant extracts showed that *Z. mistol* and *P. alba* are indigenous fruits with a high proportion of effective antioxidants against the oxidative stress generated by toxins of *E. coli* during HUS. The use of these foods or their phenolic components could help to prevent severe renal damage, thus avoiding the need for transplantation in patients with signs of recurrence of HUS.

Summing up, these results indicate that both fruits have a potential application in the long-term diet to reduce the severity of the chronic evolution in patients with HUS, a pathology that has been associated with the oxidative stress of diverse tissues, and principally, with oxidative damage of the blood and kidney.

Acknowledgments

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