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Oxidant-induced damage to equine erythrocytes from exposure to *Pistacia atlantica*, *Pistacia terebinthus*, and *Pistacia chinensis*

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Abstract

Two horses were referred for methemoglobinemia and hemolytic anemia following 5 acute deaths in their herd from an unidentified toxin source. Horses have a greater risk than other mammalian species of developing methemoglobinemia and hemolytic anemia following ingestion of oxidizing toxins, due to deficiencies in the mechanisms that protect against oxidative damage in erythrocytes. Their susceptibility to oxidative erythrocyte damage is evident in the numerous cases of red maple (*Acer rubrum*) toxicosis. The suspected toxins causing *A. rubrum* toxicosis are tannic acid, gallic acid, and a metabolite of gallic acid, pyrogallol. These compounds can be found in a variety of plants, posing a risk to equine health. In order to quickly identify toxin sources, 2 rapid in vitro assays were developed to screen plant extracts for the ability to induce methemoglobin formation or cause hemolysis in healthy equine donor erythrocytes. The plant extract screening focused on 3 species of the genus *Pistacia: P. atlantica*, *P. terebinthus*, and *P. chinensis*, which were located in the horse pasture. Extracts of the seeds and leaves of each species induced methemoglobin formation and resulted in hemolysis, with seed extracts having greater potency. The in vitro assays used in the current study provide a useful diagnostic method for the rapid identification of oxidizing agents from unidentified sources. There is no effective treatment for oxidative erythrocyte damage in horses, making rapid identification and removal of the source essential for the prevention of poisoning.

Keywords

Equine; hemolysis; methemoglobin; oxidants; *Pistacia* spp; poisoning

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The susceptibility of horses to oxidant-induced erythrocyte damage is clearly demonstrated in the numerous cases of red maple (*Acer rubrum*) toxicosis, which often results in acute methemoglobinemia, hemolytic anemia, and in severe cases, death.[5] Gallic acid and tannic

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acid have been proposed as the primary oxidants in *A. rubrum*; however, it has been suggested that a metabolite of gallic acid, pyrogallol, may contribute to oxidant-induced damage following ingestion.[3] Pyrogallol is a decarboxylated metabolite of gallic acid and is generated in the equine intestine and liver. An in vitro assay performed with analytical standards suggests that pyrogallol has a higher capacity to induce methemoglobin formation in equine erythrocytes than tannic acid and gallic acid.[3] Gallic and tannic acids have also been identified in *Acer saccharum* (sugar maple) and *Acer saccharinum* (silver maple),[7] as well as a variety of other plant species, which may present a health threat to horses. In cases of exposure to oxidants, treatment relies heavily on supportive care,[16] as there is no reliable treatment. The lack of effective treatment options makes it imperative to quickly identify and remove the source of the toxin to prevent additional poisonings. The current study describes the use of rapid in vitro equine met-hemoglobin and hemolysis assays to identify the plant source responsible for the poisoning of 7 horses, 2 of which were treated at the Veterinary Medicine Teaching Hospital (VMTH) at the University of California, Davis (UC Davis).

Equine erythrocytes are uniquely susceptible to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage.[6,7,12] Oxidants typically damage erythrocytes by oxidizing the heme iron in hemoglobin, reactive sulfhydryls, or unsaturated lipids in the membranes. The oxidation of the heme iron in hemoglobin to the ferric (Fe^{3+}) state generates methemoglobin, which is incapable of transporting oxygen. Methemoglobin can be enzymatically reduced back to the functional ferrous (Fe^{2+}) state, primarily by nicotinamide adenine dinucleotide (NADH)-dependent methemoglobin reductase.[19] Horses utilize a lactate-dependent pathway of methemoglobin reduction, which is less efficient than the glucose-dependent pathway of methemoglobin reduction utilized by most mammalian erythrocytes,[7,15,17] making horses more prone to the accumulation of methemoglobin. Oxidation of sulfhydryl groups in the globin portion of hemoglobin can induce protein denaturation and the formation of Heinz body aggregates.[12] The oxidation of sulfhydryl groups and unsaturated lipids can also compromise the erythrocyte membrane integrity.[11] Reduced glutathione (GSH) can protect erythrocytes against oxidant injury, being oxidized itself to a disulfide[11]; however, horses have a reduced ability to regenerate reduced GSH, compared with other mammals, likely due to the decreased activity of glutathione reductase in equine erythrocytes.[2,14,18] Under normal conditions, equine erythrocytes have sufficient capability to prevent oxidative damage. However, increased levels of catalyzing oxidants in circulation, as may be seen following ingestion of an oxidizing toxin, may overwhelm the horse's ability to reduce methemoglobin and regenerate reduced GSH to prevent oxidative damage to erythrocytes, making horses more likely to develop hemolytic anemia and methemoglobinemia following ingestion of oxidizing toxins.[6,7,12,17]

In October 2013, 2 horses were referred to UC Davis VMTH following 5 acute deaths of unknown cause. Previous cases exhibited signs of anemia from intravascular hemolysis, hemoglobinuria, and renal failure. Both horses treated at UC Davis were severely anemic and diagnosed with intra-vascular hemolysis and methemoglobinemia (R Bozorgmanesh, personal communication, 2013). Eccentrocytes were present in blood smears from both horses, suggesting oxidative damage to erythrocytes, potentially from toxin ingestion. The clippings of 3 species in the genus *Pistacia* were present on the property and identified as

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potential toxin sources: *Pistacia atlantica* (Mt. Atlas mastic tree), *Pistacia terebinthus* (turpentine tree), and *Pistacia chinensis* (Chinese pistache). These are ornamental trees found in California, as well as a number of other states ranging from the Southwest to the Southeast of the United States including Arizona, New Mexico, Texas, Alabama, Georgia, North Carolina, and Texas. The approximately 20 species in the genus *Pistacia* are native to Central Asia, the Mediterranean region of southern Europe, North Africa, and the Middle East,[4] and grow favorably in warm, arid climates. Of the approximately 20 *Pistacia* species, *Pistacia vera*, the pistachio, is the only species cultivated for commercial use. However, many *Pistacia* spp. have been used in traditional medicine and investigated for pharmacological activity as antioxidants, antimicrobials, antivirals, anthelmintics, and antimutagens.[8,13] Limited efforts have been made to evaluate the potential of toxicity following ingestion of *Pistacia* spp. in human beings or animals. A variety of chemical compounds from different phytochemical groups have been identified in *Pistacia* species, including terpenoids, phenolic compounds such as gallic acid, fatty acids, and sterols,[8] few of which have been thoroughly evaluated for toxicological action.

To evaluate *P. atlantica*, *P. terebinthus*, and *P. chinensis* as sources of oxidizing toxins to equine erythrocytes, 2 rapid diagnostic assays were utilized to determine if plant extracts could cause methemoglobin formation and/or hemolysis in healthy equine donor erythrocytes. Extracts of both the seeds and leaves of *P. atlantica*, *P. terebinthus*, and *P. chinensis* were evaluated, along with an additional leaf sample suspected to have contributed to another 2013 case of equine toxicosis. This leaf sample was later identified as *P. atlantica* (referred to as *P. atlantica* sample 2 in figures and table). In addition, extracts of the seeds and stalks of the hay being fed to the herd at the time of poisoning were also evaluated.

To prepare the extracts, the seeds and leaves of *P. atlantica*, *P. terebinthus*, and *P. chinensis* as well as the seeds and stalks of the hay sample (1.0 ± 0.01 g) were homogenized in 15 ml of 100% methanol[a] and ultrasonicated[b] for 25 min. Methanol extracts were evaporated to dryness under gentle nitrogen stream in a water bath (40°C).[c] The residues were reconstituted in 5.0 ml of ultrapure water[d] and vortexed[e] for 2 min to complete resuspension. Samples were filtered through 0.22-μm sterile acetate filters.[f]

Assays were performed using heparinized equine donor blood from the UC Davis VMTH. Four samples of whole blood were combined to form a composite equine blood sample (40 ml) and centrifuged[g] at 500 × *g* for 5 min. Plasma was discarded and replaced with 9% NaCl to the original volume and inverted to wash. Centrifugation and washing steps were repeated 3 times, and the erythrocytes were brought back to the original volume with 9% NaCl.

To evaluate the extracts' potential to induce methemoglobin formation, a qualitative assessment of erythrocyte color change from red to dark brown, characteristic of

[a]Methanol (ACS certified reagent grade), Fisher Scientific, Pittsburgh, PA.
[b]1510 ultrasonic cleaner, Branson Ultrasonics, Danbury, CT.
[c]24 position N-EVAP nitrogen evaporator, Organomation Associates Inc., Berlin, MA.
[d]EMD Millipore Milli-Q academic bench-integrated water purification system, Millipore Corp., Billerica, MA.
[e]Mini vortex, Fisher Scientific, Pittsburgh, PA.
[f]Cameo 25AS 0.22 μm sterile acetate filter, Micron Separations Inc., Westborough, MA.
[g]IEC centra CL4, Thermo Corp., San Jose, CA.

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methemoglobin formation, was conducted. Clean equine donor erythrocytes were diluted 1:1 (v/v) with 9% NaCl, and 1.0-ml aliquots were added to a 24-well clear polystyrene flat-bottom plate.[h] Extracts of *P. atlantica*, *P. terebinthus*, and *P. chinensis* leaf and seed and hay seed and stalks were added to erythrocytes (200 μl), in duplicate, along with 3 controls: ultrapure water, pyrogallol[i] (0.17 mg/ml), which causes extensive methemoglobin formation in equine erythrocytes in vitro at 0.1–0.2 mg/ml,[3] and Triton X-100[j] (1%), a known hemolytic agent. Plates were mixed on an orbital shaker[k] for 1 min at low speed to ensure complete mixing and incubated for 2 hr at 37°C.[l] Following incubation, samples were qualitatively evaluated for the formation of methemoglobin (Fig. 1). The extent of color change observed was classified into 4 categories: none, minimal, moderate, and severe, which is indicative of the formation of methemoglobin (Table 1). Equine erythrocytes treated with the positive control pyrogallol (0.17 mg/ml) exhibited a color change to dark brown, suggesting severe methemoglobin formation. Erythrocytes treated with *P. atlantica*, and *P. chinensis* seed extracts also exhibited color change indicative of severe methemoglobin formation, and those treated with *P. terebinthus* seed extract exhibited moderate methemoglobin formation. Minimal color change was observed in the erythrocytes treated with *P. atlantica*, *P. terebinthus*, and *P. chinensis* leaf extracts. Erythrocytes treated with extracts of the hay seeds and stalks exhibited no color change (data not shown), similar to the ultrapure water. Triton X-100 (1%) did not induce color change; however, the erythrocyte sample became translucent due to the extensive hemolysis. These results suggest that incubation with the seed extracts, especially *P. atlantica* and *P. chinensis*, caused more extensive formation of methemoglobin than leaf extracts, indicating that leaf extracts were not nearly as potent as the seed extracts.

To evaluate the extracts' potential to cause hemolysis in equine erythrocytes, a hemolysis assay based on the spectro-photometric measurement of hemoglobin in the cellular supernatant was performed. Clean equine donor erythrocytes were diluted 1:50 (v/v) with 9% NaCl. Diluted erythrocytes (190 μl) were aliquoted into a 96-well clear bottom plate[m] in triplicate and spiked with *Pistacia* spp. leaf and seed extracts (10 μl) or controls. In addition, extract-specific color blanks were used to account for differences in the color of each extract (190 μl of ultrapure water with 10 μl of each *Pistacia* spp. extract). The plate was mixed on an orbital shaker for 1 min at low speed and incubated at 37°C for 1 hr. The plate was centrifuged[n] for 5 min at 500 × *g* to pellet intact erythrocytes. The supernatant (100 μl) from each well was transferred into a clear, flat-bottomed 96-well plate, and absorbance at 440 nm was measured using a multimode microplate reader.[o] The absorbance of the extract-specific color blanks was subtracted from erythrocyte supernatant absorbance readings. Percent hemolysis was determined relative to the absorbance measured in the supernatant of erythrocytes treated with 1% Triton X-100, which should cause complete hemolysis (Fig. 2).

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[h] 24-well tissue culture plate with low evaporation lid, BD Biosciences, Bedford, MA.
[i] Pyrogallol, Aldrich Chemical Company, Milwaukee, WI.
[j] Triton X-100, Fisher Scientific, Pittsburgh, PA.
[k] Innova 2000 orbital shaker, New Brunswick Scientific Co., Enfield, CT.
[l] Forma steri cycle CO_2 incubator, Thermo Corp., San Jose, CA.
[m] 96-well ELISA microplate, Greiner Bio-One, Monroe, NC.
[n] IEC centra CL3R centrifuge, Thermo Corp., San Jose, CA.
[o] Spectramax M3, Molecular Devices, Sunnyvale, CA.

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These steps were repeated with *Pistacia* spp. leaf and seed extracts diluted to 50% of the original concentration.

The rapid hemolysis assay demonstrated that the extracts of *P. atlantica*, *P. terebinthus*, and *P. chinensis* seeds induced a greater percent hemolysis of the equine erythrocytes, 52.2%, 55.4%, and 67.4%, respectively, than the leaf extracts, 27.8%, 6.2%, and 18.0%, respectively. The extracts at 50% of the original concentration exhibited the same pattern, and induced hemolysis at approximately half the extent of the 100% *Pistacia* spp. extracts. The most potent effect was demonstrated by the *P. chinensis* seed extract, which caused 67.4% erythrocyte hemolysis. The extracts of the hay seeds and stalk caused minimal hemolysis, only slightly elevated from the ultrapure water control. Interestingly, the control pyrogallol (0.17 mg/ml) did not induce hemolysis at a level much greater than the water control, suggesting that this toxin may oxidize hemoglobin to methemoglobin but not cause sufficient cellular damage to cause hemolysis.

The results of the 2 biological assays demonstrated that compounds present in *P. atlantica*, *P. terebinthus*, and *P. chinensis*, especially seeds, can cause the formation of methemoglobin and hemolysis in healthy equine donor blood, strong evidence that 1 or multiple of these plants contributed to the poisoning of the 7 horses. The use of these rapid in vitro tests was central to the quick identification of *Pistacia* spp. as the source of oxidizing toxins likely responsible for the methemoglobinemia and intravascular hemolysis of the horses treated at the UC Davis VMTH. Rapid identification of the toxin source quickly minimized the risk to the rest of the herd, and additional poisonings were prevented. Given the high susceptibility of equine erythrocytes to oxidant-induced damage, these assays may be very useful as a rapid screening tool to identify potential sources of oxidizing toxins in future cases. One advantage of the assays is that it is not essential to know the identity of the ultimate toxin. If no known toxins are identified in patient blood or urine samples, these biological assays can still identify the source of the toxin without the need for specific standards. In addition, both assays can be completed within a few hours and with limited laboratory equipment, making them accessible to a broad range of veterinarians and diagnosticians.

In each of the assays used in the current study, the outputs used to identify plant sources of oxidative toxins that may have contributed to methemoglobinemia and hemolysis in the equine patients were based on the quickest evaluation method. For the methemoglobin assay, a qualitative evaluation of color change to dark brown, characteristic of methemoglobin formation, was used; however, this could be expanded in future cases or studies to determine the percent methemoglobin formed.[7] For the hemolysis assay, the percent hemolysis was based on normalization to 1% Triton X-100 and the spectrophotometric absorbance at 440 nm. Additional methods can be used to determine percent hemolysis, such as the Harboe method,[10] which uses a hemoglobin standard curve and multiwavelength spectrophotometric evaluation or the Drabkin cyanomethemoglobin method.[1] While neither of these methods is substantially more time consuming, they each require reference standards and instrumentation that may not be immediately available. For the purpose of a rapid diagnostic assay, the methodology outlined in the current study is appropriate for the rapid determination of the source of a hemolytic agent.

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The 2 assays described in the present study provide a rapid and repeatable method for identification of plant extracts capable of inducing methemoglobin formation and hemolysis. However, the assays do not provide information regarding the ultimate toxic agent, the concentration of the toxin or amount of the plant necessary to cause toxicity, or the concentration of toxin in the plant sample. In addition, the assays will not reflect any biochemical modification of toxins that may have occurred in the intestines or liver, thus they may not be completely representative of in vivo toxicity. While these specific assays do not provide this information, positive results could be evaluated further to identify potential toxins and their concentrations in the plant samples.

The positive results of the rapid methemoglobin and hemolysis assays, coupled with the clinical evaluation of the affected horses treated at the UC Davis VMTH provided strong evidence that ingestion of *Pistacia* spp. clippings caused oxidant-induced damage to equine erythrocytes, resulting in death or serious illness. Further evaluation of *P. atlantica*, *P. terebinthus*, and *P. chinensis* may be necessary to definitively conclude that ingestion was the cause of toxicosis and to identify the ultimate toxin(s) responsible. Additional toxicology testing confirmed the presence of pyrogallol in urine of both horses treated at the VMTH, suggesting that the compounds in *Pistacia* spp. may be similar to those in red maple (*A. rubrum*). The suggested oxidants responsible for *A. rubrum* toxicosis are tannic acid, gallic acid, and pyrogallol, all of which induce methemoglobin formation. Pyrogallol is not detectable by gas chromatography–mass spectrometry analysis of *A. rubrum* leaves; however, it is rapidly produced by decarboxylation of gallic acid, or indirectly by the metabolism of tannic acid to gallic acid in equine intestine and liver. Pyrogallol is a more potent oxidizer than both gallic acid and tannic acid.[3] The assays used in the current study do not account for in vivo digestion or metabolism of the plant samples; therefore, more thorough analysis is necessary to determine the relative contributions of tannic acid, gallic acid, and pyrogallol to *Pistacia* spp. toxicity. The pyrogallol standard used in these assays did not cause hemolysis, but the *Pistacia* spp. extracts, especially from seeds, did. This suggests the presence of another hemolytic agent in the plant extracts that contributes to toxicity. An additional hemolytic compound of unknown identity was determined to contribute to toxicity in *A. rubrum* also.[7] This hemolytic factor is believed to act in addition to the methemoglobin-forming toxins to cause a greater level of oxidative damage. In clinical cases of *A. rubrum* toxicosis, horses typically die because of methemoglobinemia and/or hemolytic anemia; the presence of 2 ultimate toxins with different mechanisms may contribute to the 2 patterns of toxicosis.[9] The toxicity of *A. rubrum* varies seasonally, with the highest toxicity observed in late summer and early fall.[9] If *Pistacia* spp. toxicity is caused by the same toxins as *A. rubrum*, there may also be seasonal variation in toxic potential following ingestion. In order to fully assess the risk that ingestion of *Pistacia* spp. pose to equine health and safety, it is essential to develop a better understanding of the ultimate toxins responsible for the clinical outcome, the in vivo action of these toxins, and the potential seasonal variation of toxicity. A thorough understanding of these factors could be important for the diagnoses of future cases and development of effective treatment options.

Pistacia atlantica, *P. terebinthus*, and *P. chinensis* grow favorably in warm arid climates and in a range of soil types. The plants are native to China, the Mediterranean, the Canary

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Islands, and the Middle East, and are now widely distributed in North America. All 3 species can be found in Mexico and a range of states from the Southwest to the Southeast regions of the United States. Of the 3 species, *P. chinensis* is the most abundant and is promoted as an ornamental because of its hardiness, drought tolerance, and vibrant fall colors. However, in Texas, *P. chinensis* is considered an invasive species. The widespread distribution of *Pistacia* spp. makes them a global concern for the veterinary community. These plants are likely to be found near horse and livestock pastures, and based on this data, pose a significant risk to horses, if ingested. The findings of the current study suggest that *P. atlantica*, *P. terebinthus*, and *P. chinensis* can cause acute poisoning in horses, and should be monitored for and eliminated from locations where animals, particularly horses, have access to leaves or seeds.

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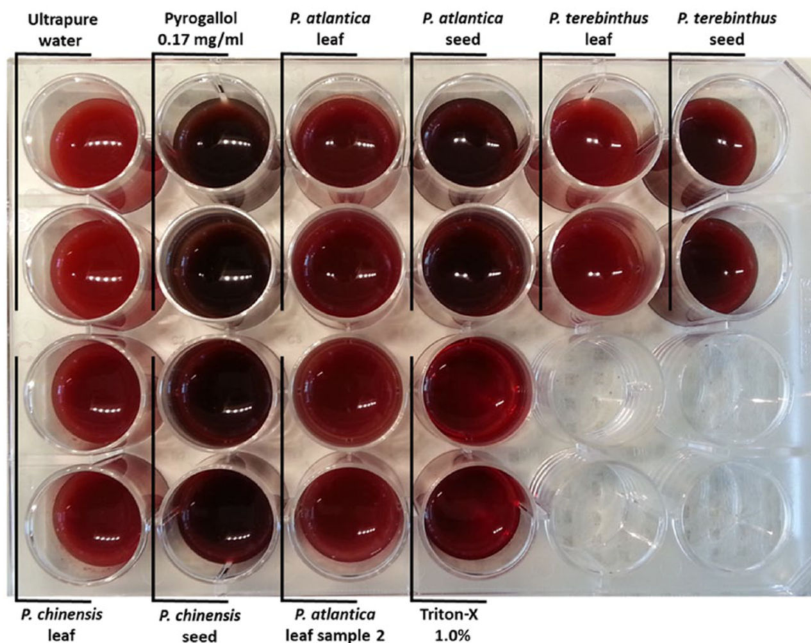

Figure 1.

Representative image depicting results of the rapid in vitro methemoglobin assay. Healthy equine donor erythrocytes were incubated for 2 hr with ultrapure water (control), pyrogallol (0.17 mg/ml), extract of *Pistacia atlantica*, *Pistacia terebinthus*, or *Pistacia chinensis* leaf or seed, or Triton X-100 [j] (1%). The color change of treated erythrocytes to dark brown indicates the formation of methemoglobin. The extent of methemoglobin formation varied between treatments with pyrogallol, *P. atlantica* seed, and *P. chinensis* seed treatments exhibiting severe methemoglobin formation; *P. terebinthus* seed treatment exhibiting moderate methemoglobin formation; *P. atlantica*, *P. terebinthus*, and *P. chinensis* leaf treatments exhibiting minimal methemoglobin formation; and ultrapure water and Triton X-100 (1%) treatments no methemoglobin formation.

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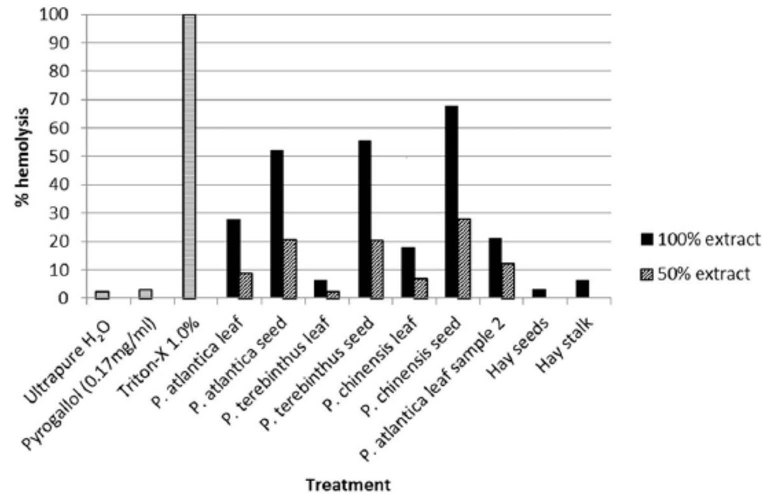

Figure 2.

Percentage hemolysis of equine donor erythrocytes induced by treatment with ultrapure water (control), pyrogallol (0.17 mg/ml) or Triton X-100 j (1%) compared to treatment with extracts of *Pistacia atlantica*, *Pistacia terebinthus*, and *Pistacia chinensis* leaf and seed, and hay seed and stalk at 100% and 50% dilution. The supernatant of treated erythrocytes was evaluated by spectrophotometric absorbance at 440 nm, which varies with concentration of hemoglobin in solution. Percent hemolysis was determined by normalizing absorbance values to those obtained from erythrocytes treated with Triton X-100 (1%), which causes 100% hemolysis.

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Table 1

Evaluation of methemoglobin formation based on the extent of color change of equine erythrocytes.

None	Minimal	Moderate	Severe
Ultrapure water	*Pistacia atlantica* leaf	*P. terebinthus* seed	*P. atlantica* seed
Triton X-100 j 1%	*Pistacia terebinthus* leaf		*P. chinensis* seed
	Pistacia chinensis leaf		
	P. atlantica leaf sample 2		