Spring 4-2021

Effects of Strong Oxidants Present in Acer Spp. on Hemolysis Methemoglobin Production in Equine Erythrocytes

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EFFECTS OF STRONG OXIDANTS PRESENT IN *ACER SPP*. ON HEMOLYSIS AND METHEMOGLOBIN PRODUCTION IN EQUINE ERYTHROCYTES

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March 30, 2021

Submitted in partial fulfillment of the requirements

For graduation with Honors

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Acknowledgements

I would first and foremost like to thank Dr. Jeffrey Lehman for his time and support throughout this project as well as his guidance throughout my time at Otterbein. I would like to thank Dr. Brandon Sinn for acting as my second reader, as well as Louise Captein for serving as my defense advisor. In addition, I would like to thank Dr. Michele Acker and Dr. Karen Steigman for their willingness to answer questions and be a resource throughout the creation of this thesis. I would also like to thank Megan Ray and the Austin E. Knowlton Equine Center for completing early morning blood draws to help the completion of my research.

Abstract

Maple toxicosis is a deadly condition affecting equines after they have ingested wilted leaves of the genus *Acer* (i.e., maple species). It is characterized by hemolytic anemia and methemoglobinemia. Although the toxins responsible for causing toxicosis have not been identified, they are thought to be strong oxidants. The objective of this study is to determine the effect of eight compounds (galloyl derivatives) found in *Acer* spp. on hemolysis and methemoglobin production in bioassays with equine erythrocytes. Seven of the compounds are known constituents of *Acer* spp. that are commercially available. One of the compounds--acertannin--was isolated and characterized from leaf extracts of red maple. Equine erythrocytes were incubated with each compound at concentrations of 0, 0.2, 0.4, 0.8 and 1.6 mg/ml. Toxicity was determined spectrophotometrically based on percentage hemolysis of erythrocytes and on the production of methemoglobin. Experiments were repeated three times. Of the eight compounds, only tannic acid and gallic acid showed a significant effect on hemolysis. Tannic acid caused a higher level of hemolysis than did gallic acid. Concentrations of tannic acid > 0.4 mg/ml caused significantly more hemolysis than the control. The amount of hemolysis caused by the highest concentration (1.6 mg/ml) was roughly 3 times higher than that of the control. For gallic acid, only the most concentrated treatment of 1.6 mg/ml resulted in a significant increase in hemolysis relative to the control. Pyrogallol had the greatest effect on the production of methemoglobin. As little as 0.2 mg/ml of pyrogallol significantly increased methemoglobin production (0.9% versus 56.7% for 0 mg/ml and 0.2 mg/ml, respectively). Quercetin, methyl gallate, gallic acid, and tannic acid significantly affected methemoglobin production, in most cases with as little as 0.2 mg/ml of compound. Acertannin had no effect on hemolysis or on methemoglobin. The results of this study show that maple toxicosis can be largely explained by the presence of tannic acid and pyrogallol which cause hemolysis and the production of methemoglobin, respectively.

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INTRODUCTION

Maple toxicosis is a phenomenon that occurs in equines after the consumption of dried or wilted maple leaves (Boyer *et al*. 2002; Alward, 2019). This condition is characterized by two mechanisms of oxidative damage that work in conjunction with one another: hemolytic anemia and methemoglobinemia (Alward, 2019).

Description of Maple Toxicosis: Clinical symptoms of maple toxicosis include depression, discolored gums, lethargy, fever, tachycardia, abnormal behavior, weakness, dark urine, dark blood and death (Diver *et al*., 1982; Boyer *et al*., 2002; Alward, 2008). The types of symptoms and the severity of these symptoms are reflective of the amount of leaves consumed. Young horses only need to consume 0.22 kg of maple leaves for the leaves to be lethal, while adult horses can consume 0.5-12 kg of leaves before showing symptoms (Adams, 2011). Symptoms normally begin occurring after as little as 18 hours, or as long as five days after consumption (Lenz, 2018). After consumption, equines show large amounts of oxidative damage to their erythrocytes leading to methemoglobinemia and hemolytic anemia. Maple toxicosis is suspected to occur because of strong oxidants found within the dried leaves of *Acer* spp. These strong oxidants are responsible for the methemoglobin production and hemolysis of erythrocytes. This oxidative damage can cause further problems such as clogged kidneys due to the excess waste product, along with severe anemia due to damaged cells not being replaced quickly enough.

Current Preventative Measures: In order to mitigate the risk of red maple toxicosis, equine professionals recommend the removal of red maple trees from pastures and paddocks as a preventative measure. In addition, planting any new maple tree in the area is not recommended. Fallen branches should be quickly removed from the property, and the pasture should be

inspected regularly, especially after large storms (Witonsky *et al*., 2001). During the autumn, it is important that fallen leaves are promptly removed from the area to stop horses from grazing on them. If the pasture does contain maple trees, it is advised that those areas be fenced off, as well as keeping the lower branches pruned. Hungry horses are more likely to eat dried leaves in a pasture, so it is important to keep the horses well fed and monitor their food consumption to ensure they are eating regularly. Inspecting hay before feedings can ensure that no leaves have been incorporated by accident into the existing food.

More studies are being conducted on cures for maple leaf toxicosis, however, preventative measures are currently the best option. If the horse is treated early enough, sometimes large amounts of vitamin C can be administered, or a blood transfusion can be done to clear the toxins from its system. However, both methods are unreliable cures that need to be researched more thoroughly for success rates (Ragone, 2018). Additionally, daily dietary supplements of ascorbic acid (and to a lesser degree tocopherol) can potentially prevent/reduce the effects of erythrocyte oxidation that occurs during maple toxicosis (O'Callaghan et al., 2015).

Description of Hemolytic Anemia and Methemoglobinemia: Hemolytic anemia in maple toxicosis is caused by the denaturation of the hemoglobin. This leads to the formation of Heinz bodies which are cell inclusions indicative of erythrocyte damage (McConnico *et al*., 1992). Heinz bodies form when hemoglobin thiols are oxidized by hydrogen peroxide, which then allows these thiols to form disulfide bonds with other hemoglobin molecules causing inclusions within the blood (Alward, 2008). As a result, Heinz bodies may attach to the cell membrane of erythrocytes, causing loss of ionic composition and ultimately hemolysis, or the deformation of the cell and its subsequent removal from circulation (McConnico *et al*., 1992).

Methemoglobinemia in maple toxicosis is caused by oxidative stress of ferrous hemoglobin (Fe^{+2}) which produces ferric hemoglobin (Fe^{+3} or methemoglobin). This results in an over representation of methemoglobin within the horse. Methemoglobin is normally found in the blood in small amounts (i.e., 1-2%), however, an abnormal increase in methemoglobin can cause irreversible damage to vital organs. Methemoglobin is similar to regular hemoglobin; however, it is unable to carry oxygen due to the increased charge on the iron center of the hemoglobin (Fe^{+3} instead of Fe^{+2}) (Adams, 2011). The conversion between Fe^{+3} and Fe^{+2} forms of hemoglobin occurs naturally, and there is typically a balance between oxidized and reduced states (Alward, 2006). However, when a harmful accumulation of methemoglobin occurs, erythrocytes cannot carry sufficient oxygen. This results in the clinical symptoms of discoloration of mucous membranes, blood and urine (Alward, 2006).

Oxidative Physiology of Equines: When equine erythrocytes encounter oxidative stress, enzymatic antioxidants typically interact with free radicals causing the stress and dispose of them from the body. These enzymes are known as glutathione reductase, glutathione-S-transferase, and glutathione peroxidase. Glutathione reductase normally plays the largest role in protecting erythrocytes against oxidant injury, however, compared to other mammals, equines cannot easily regenerate glutathione reductase (Walter *et al*, 2014). In fact, equines are exceptionally susceptible to oxidative damage because of the lack of biological anti-oxidative mechanisms that normally protect vertebrates (Walter *et al*, 2014). Horses use a lactate-dependent pathway to protect erythrocytes against oxidative damage which is less efficient than the glucose-dependent pathway that is used by other mammalian systems (Robin and Harvey, 1967; Harvey, 1975). Under typical conditions, equines have the sufficient capacity to maintain the production of glutathione reductase and prevent oxidative damage. However, when a toxin is introduced such

as the ones in the leaves of a red maple, the equine can no longer keep up with the needed production of glutathione reductase to combat the oxidative stress, and the equine can no longer reduce methemoglobin levels like normal. This makes them more prone to acquiring hemolytic anemia and methemoglobinemia compared to other animals after ingesting these toxins (George *et al*., 1982; Boyer *et al*., 2002; Walter *et al.*, 2014).

Description of Strong Oxidants: The suspected strong oxidants in *Acer* spp. that are responsible for the production of methemoglobin and hemolysis are of a class of secondary plant metabolites called gallotannins or gallic acid derivatives. In this study, the potential role in maple toxicosis of eight compounds--gallic acid, methyl gallate, lauryl gallate, pyrogallol, quercitin, rutin hydrate, tannic acid, and acertannin—were addressed (Figure 1). Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic acid with the formula $C_6H_3CO_2H$. It is naturally abundant plant phenolic compound and is a well-known component of hydrolysable tannins. It has been reported in higher plants (leaves, shoots, roots, and flowers) in multiple different forms including complex molecules, derivatives, and polymers. In humans, gallic acid is a well-known antioxidant and is absorbed by the body after consumption. It is excreted via the urine as 4-O-methyl gallate (Daglia, 2014). Gallic acid is formed from the hydrolysis of tannic acid through a deglycosylation reaction. Methyl gallate (methyl 3,4,5-trihydroxybenzoate; $C_8H_8O_5$) is a derivative of gallic acid. It is an ester formed from condensation of gallic acid with methanol. It is a plant metabolite and anti-inflammatory (Asnaashari *et al.*, 2019). Lauryl gallate (or dodecyl gallate) is the ester of dodecanol and gallic acid. It is an antioxidant commonly used in the cosmetic and pharmaceutical industry. It is also known for protecting red blood cells and mitochondria from oxidative stress caused by free radicals (Jurak and Miñone, 2016). Pyrogallol ($C_6H_3(OH)_3$) is a strong oxidant derived from the metabolic decarboxylation of gallic acid in the

equine intestine and liver. It is known to release large amounts of free radicals within the body (Upadhyay et al., 2010). Quercetin is a polyphenolic flavonoid found in plants. It has antibacterial and anti-inflammatory properties (Andres *et al.*, 2018). Rutin Hydrate is flavonoid and a well-known neuroprotectant that is normally used to protect against oxidative stress (Mostafa *et al*., 2019). Tannic acid is a common phenolic acid that is structurally related to gallic acid. It is common in many plant species and is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues. It is a common strong oxidant (Chung, 1998). The chemical formula for commercial tannic acid is often given as $C_{76}H_{52}O_{46}$ but it is commonly a mixture of esters of polygalloyl glucoses or polygalloyl quinic acid with the number of galloyl unit per molecule ranging from 2 up to 12 depending on the plant source. The degradation of tannic acid forms gallic acid. Acertannin is a sugar with two gallic acid subunits attached. It can act as an oxidant, however, its effects on oxidative stress have not been as well studied.

Objective: The objective of this study is to determine the effects of eight compounds (galloyl derivatives) found in *Acer* spp. on hemolysis and methemoglobin production in bioassays with equine erythrocytes.

METHODOLOGY

Plant Material: Leaves from species of mature *A. rubrum*, *A. saccharinum*, and *A. freemanii* trees were collected at Dawes Arboretum in Heath, Ohio. For each *Acer* species, 10-20 leaves were collected and placed in an herbarium press that was set in a fume hood for 2 weeks and then into a drying oven set at 40^{o} C for 2 weeks. After the leaves were dried, they were ground with liquid nitrogen and placed in a desiccator until a low humidity of 20% was reached.

The ground tissue was then sealed and stored inside a 4°C refrigerator until analyzed with HLPC.

HPLC Analytical Method Used to Determine Active Fractions of* Acer *Extracts: Plant extracts of *A. rubrum*, *A. saccharinum*, and *A. freemanii* were analyzed with a Restek Ultra C18 column (150×4.6 mm, 5 μm particle size) on an Agilent 1100 HPLC system that included an autosampler and diode array detector. All HPLC procedures were completed at Ashland University under the direction of Jeffrey Weidenhamer. Chromatograms were measured at the absorption maximum for gallic acid (280nm). A linear methanol: water gradient was used, beginning at 2% methanol:98% water (v/v) for the first 8 minutes, increasing to 5% methanol at 12 minutes, 10% methanol at 16 minutes, 25% methanol at 20 minutes, 50% methanol at 30 minutes, 80% methanol at 40 minutes, and 100% methanol at 45-50 minutes. There was a four-minute equilibration time between runs. Sample injection volume was 20μL.

Blood Collection and Erythrocyte Isolation: Blood was collected from the jugular vein of geldings of various ages. All the horses used for this study were housed at the Austin E. Knowlton Equine Center in Westerville, Ohio and were given diets that did not include antioxidant supplements. For every experiment that was run, only the blood of one horse was used to maintain consistency between the samples. The blood was collected in 10 mL lithium heparin-treated tubes between 7:30 am and 8:00 am by a trained faculty member from the Department of Equine Science Otterbein University. Tubes were placed on ice and transported back to the lab. Upon arrival, the samples were centrifuged at 3,400 rpm for 15 minutes. Plasma was discarded and erythrocytes were rinsed twice with a 0.9% saline solution and then suspended in equal amounts of a buffered saline solution (110mM NaCl, 20mM Na_2HPO_4, 4mM KH_2PO_4, water).

Preparation of Oxidants: This study tested the effect of eight chemicals found in *Acer* spp. on equine erythrocytes. These chemicals included commercially prepared tannic acid, methyl gallate, gallic acid, pyrogallol, quercetin, lauryl gallate, and rutin hydrate available from Sigma-Aldrich or Fischer Scientific. In addition, the compound acertannin was isolated and identified from red maple extracts based on HPLC chromatograms. The chemicals were tested individually by dissolving the chemical in dimethyl sulfoxide or water depending on solubility. Each chemical was tested at final concentrations of 0.2 0.4, 0.8, and 1.6 mg/ml. Equine erythrocytes (800 µl) were incubated with chemical/buffer (200 µl) at 37.5° C for 2 hours on a heat block. After incubation, samples were removed from the heat block and vortexed. 25 µl of erythrocyte/chemical sample was added to 1 ml of each of the following NaCl concentrations in 1.5 ml microfuge tubes: 0%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%. These NaCl solutions were centrifuged for 15 min at 2,800 rpm and measured spectrophotometrically to determine toxicity.

Calculation of Relative Area Under Hemolytic Curve (RUHC): The amount of hemolysis or methemoglobin was measured with spectrophotometry in 96 well titer plates. 200 µl of supernatant from each sample in NaCl solution was added to individual wells in a 96-well spectrophotometry plate. A control plate for this test was also made using a 96 well spectrophotometry plate, with each row containing the corresponding saline solutions without any blood added. A Biotrak II plate reader (Biochrom Ltd.,Cambridge, England) was used to read absorbencies at 500, 576, and 630 nm for the blank and experimental plates.

Based on the absorbencies, percentage hemolysis was calculated with the following equation:

$$\text{Percentage Hemolysis} = \frac{(\text{total absorbancy at X})*100}{\text{total absorbancy at 0\% NaCl}}$$

in which X = 0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, or 0% NaCl solution, and total absorbency is the sum of absorbance at 560, 576, and 630 nm at individual salt concentrations. Percentage hemolysis values were plotted against the NaCl concentrations to form the percentage hemolysis curve (Figure 2). Area under the hemolytic curve (AUHC) was calculated from percentage hemolysis data for each level of leaf extract with the following equation:

$$= \sum_{r=1}^{n} \left[\frac{\%Hemolysis_{r+1} + \%Hemolysis_{r}}{2} \right] \times [(salt\ concentration)_{r+1} - (salt\ concentration)_{r}]$$

in which *n* = the concentration of salt from *r* = 1 to *n*. The area was expressed as a percentage of the total possible area under curve and labelled as relative area under hemolytic curve (RUHC)

Calculation of Percentage Methemoglobin: After testing for hemolysis, the samples were incubated for an additional hour at a constant 37.5° C. The tubes were then removed from the heat block and inverted. 20 µl of each blood-extract sample was added to 2.0 ml Triton X buffer (20mM KH_2PO_4, 16mM Na_2HPO_4, 0.05%Triton-X detergent) in 2 ml microfuge tubes. After inversion, the tubes were centrifuged for fifteen minutes at 2,800 rpm. 200 µl of supernatant from each sample were then added to individual wells in a 96-well spectrophotometry plate. A control plate for this test was also made using a 96 well spectrophotometry plate, with each well containing 200µl of Triton X buffer. Absorbencies were read at 500, 576, and 630 nm for the blank and experimental plates. Each plate was read three times. The mean of these three readings were calculated and corrected with an averaged control plate reading.

Absorbencies of the supernatants were used to determine percentage methemoglobin and hemolysis based on the following equations adapted from Wells *et al*. (1997):

$$\text{Oxyhemoglobin (Oxy)} = [1.013\ (A576) - 0.3269\ (A630) - 0.7353\ (A560)\ ] \times 10^{-4}$$

$$\text{Deoxyhemoglobin (Deoxy)} = \lfloor 1.373\ (A560) - 0.747\ (A576) - 0.737\ (A630)\ \rfloor \times 10^{-4}$$

$$\text{Methemoglobin (Met)} = \lfloor 2.985\ (A630) + 0.194\ (A576) - 0.4023\ (A560) \rfloor \times 10^{-4}$$

$$\%\ \text{Methemoglobin} = \lfloor \text{Met}/\ (\text{Oxy} + \text{Deoxy} + \text{Met}) \rfloor \times 100$$

Statistical Analysis: Data for hemolysis and for methemoglobin production were analyzed with ANOVA as a completely random design with 5 treatments (i.e., chemical concentration) and 3 replications. Significant difference among chemical concentration were determined based on Student Newman-Keuls tests (α=0.05).

RESULTS

HPLC analysis: HPLC was used to fractionate extracts of three maple species that had previously been reported to cause maple toxicosis (Weber and Miller, 1997; Boyer *et al*., 2002; Dye, 2015). All samples showed 3-5 major peaks at similar retention times of roughly 21.7, 22.1, 28.5, 30.1 and 30.5. Peaks were larger for *A. rubrum* and *A. freemanii* than for *A saccharinum*. *A. saccharinum* exhibit only 3 major peaks. The largest peak in all chromatograms measured with an absorption maximum of 280 nm had a retention time of 28.5 minutes. Areas under the curve (×1000) for the 28.5-minute peak for *A. rubrum*, *A. saccharinum*, and *A. freemanii* were 151, 96, 223, respectively. The major extract component was isolated in pure form and tentatively identified by nuclear magnetic resonance spectrometry

(NMR) by J. Weidenhamer (Ashland University, Ashland, OH) as a sugar with two gallic acid subunits attached. HPLC-mass spectrometry (LC-MS) was used to further characterize the extracts as 1,5-anhydro-2,6-bis-O-(3,4,5-trihydroxybenzoyl)-D-glucitol, or acertannin.

Analysis of Hemolysis: Typical levels of hemolysis of the control (0 mg/ml) for each compound tested ranged from 11 to 20 except for the control for quercetin which was 26. No treatment caused hemolytic values greater than 32. Of the eight compounds tested, only tannic acid and gallic acid showed a significant effect on hemolysis. Concentrations of tannic acid > 0.4 mg/ml caused significantly more hemolysis than the control. The amount of hemolysis caused by the highest concentration (1.6 mg/ml) was roughly 3 times higher than the control (10.8 versus 31.8). The amount of hemolysis for tannic acid did not differ for the 0.8 and 1.6 mg/ml treatments (31.3 and 31.7, respectively). Gallic acid had less of effect on hemolysis than did tannic acid. For gallic acid, only the most concentrated treatment of 1.6 mg/ml resulted in a significant increase in hemolysis relative to the control (0 mg/ml and 1.6 mg/ml treatments had values of 12 and 22, respectively).

Pyrogallol had the greatest effect on the production of methemoglobin. As little as 0.2 mg/ml of pyrogallol significantly increase methemoglobin production (0.9% versus 56.7% for 0 mg/ml and 0.2 mg/ml, respectively). Quercetin, methyl gallate, gallic acid, and tannic acid significantly affected methemoglobin production, in most cases with as little as 0.2 mg/ml of compound. Acertannin had no effect on hemolysis or on methemoglobin. Values for hemolysis across treatments of acertannin ranged from 20.1 to 24.5

Analysis of Methemoglobin production: The compounds quercetin, methyl gallate, gallic acid, tannic acid and pyrogallol had a significant effect of the production of methemoglobin. Pyrogallol had the greatest effect on the production of methemoglobin across

all treatments. As little as 0.2 mg/ml of pyrogallol significantly increased methemoglobin production (0.9% versus 56.7% for 0 mg/ml and 0.2 mg/ml, respectively). The amount of methemoglobin production for the three highest treatments ranged from 68 to 73% and did not differ significantly from each other. No other compound caused as much methemoglobin production across the range of treatments.

Gallic acid caused a significant amount of methemoglobin production. Methemoglobin values increased dramatically as gallic acid concentration increased. Numerically, gallic acid caused the highest level of methemoglobin production at the 1.6 mg/ml concentration (78%). However, at lower concentration, methemoglobin production was numerically lower than those of pyrogallol. Methyl gallate, quercetin, and tannic acid also significantly affected methemoglobin production, in most cases with as little as 0.2 mg/ml of compound. Maximum levels of methemoglobin production for these compounds were less than those of pyrogallol and gallic acid. Lauryl gallate, and rutin hydrate, and acertannin had no effect on methemoglobin; treatment values were statistically no different than the control.

DISCUSSION

Maple toxicosis is a dangerous condition that is characterized by methemoglobinemia and hemolytic anemia. Methemoglobinemia is caused by the oxidation of the iron core of hemoglobin from the ferrous state (Fe^{+2}) to ferric state (Fe^{+3} or methemoglobin); thereby; limiting the ability of erythrocytes to deliver oxygen to tissues. Hemolytic anemia is caused by the denaturation of hemoglobin; the formation of Heinz bodies, and subsequent removal of the deformed erythrocyte from circulation (McConnico *et al*., 1992).

The initial cases of maple toxicosis were first described in conjunction with *A. rubrum* (red maple) (George *et al*., 1982); hence; the literature often refers to the condition as red maple toxicosis. More recent studies have shown that red maple (*A. rubrum)* may not be the only species of maples able to cause toxicosis. Boyer et al (2002) suggest that *A. saccharinum* and *A. saccharum* can also cause toxicosis. Weber and Miller (1997) report a case of toxicosis involving red × silver maple hybrids. Dye (2015) and Lehman (unpublish) report a number of *Acer* spp. are capable of eliciting toxicity in bioassays of equine erythrocytes. We suggest that the condition be more appropriately name "maple toxicosis", instead of "red maple toxicosis" in veterinary literature and bulletins.

While the toxins in *Acer* spp. that cause hemolytic anemia and methemoglobinanemia have not been identified, galloyl derivatives tannic acid, gallic acid, and pyrogallol have been implicated as possible toxins alone or in conjunction with each other (Boyer *et al*., 2002; Agrawal *et al.*, 2012; Walter *et al.*, 2014). Boyer *et al*. (2002) suggests that in the equine-erythrocyte-*Acer* extract system, gallic acid could be a strong oxidant and partial cause of methemoglobinanemia. They state that in the presence of the Fe^{2+} of the heme group in hemoglobin, gallic acid may generate hydroxyl radicals, resulting in the oxidation of equine hemoglobin. In addition, Boyer et al. (2002) propose that tannic acid may hydrolyze to form greater amount of gallic acid and that gallic acid may be decarboxylated to form pyrogallol which is a stronger oxidant. In *in vitro* assays performed with analytical standards, they showed that pyrogallol has a higher capacity to induce methemoglobin formation in equine erythrocytes than tannic acid and gallic acid. Similarly, Agrawal et al. (2013) report that gallotannins and free gallic acid are present in *A. rubrum* leaves and can be metabolized by the bacteria *Klebsiella pneumoniae* and *Enterobacter cloacae* found in the equine ileum to form pyrogallol, either

directly or through a gallic acid intermediate. Because the main suspects of maple toxicosis--gallic acid, tannic acid, and pyrogallol--are found in many *Acer* spp. in varying concentrations, it is likely that maple toxicosis extends beyond solely red maple (*A. rubrum*) (Walter *et al.*, 2014).

In our study, the chemical that cause the greatest increase of hemolysis (i.e., tannic acid) was not the same as the chemical that caused the greatest increase in methemoglobin production (i.e., pyrogallol). This suggests that in nature, a combination of two or more chemicals likely cause maple toxicity seen in equines. In this study, galloyl derivatives were only tested in bioassays individually. In future studies, we propose testing tannic acid and pyrogallol together to determine whether the two interact synergistically and whether levels of hemolysis increase and more closely resemble levels caused by natural plant extracts.

While tannic acid did affect the amount of hemolysis, mean values for RUHC when treated with 1.6 mg/ml tannic acid were numerically far less that what we have observed in nature. For *A. rubrum* and *A saccharinum*, Dye (2015) reported values for RUHC that were 50 and 70, respectively, in field studies, and 60 and 72, respectively, in greenhouse studies. In our study, values did not exceed 32. Increasing the amount of tannic acid did not corresponding increase levels of hemolysis; treatment concentrations 0.8 and 1.6 mg/ml were not significantly different for the amount of hemolysis. The difference between our RUHC values and those reported using nature plant extracts may be due to the fact that commercially prepared tannic acid is commonly a mixture of esters of polygalloyl glucoses or polygalloyl quinic acid with varying numbers of galloyl unit per molecule depending on the plant source. Hence, the commercially prepare tannic used in this study may be reflective of the natural form of tannic acid present in *Acer* spp. but not be the identical form.

The compounds that caused significant increases in methemoglobin production--tannic acid, quercetin, pyrogallol, methyl gallate and gallic acid--are closely related and are possibly metabolized to the strongest oxidant pyrogallol in the intestine of the horse when consumed (Agrawal *et al*., 2013). Similarly, the conversion of galloyl derivatives to pyrogallol may have occurred during incubation of erythrocytes in bioassays at 37^{o} C for 3 hours. Even with the additional of the lowest concentration of pyrogallol, values for percentage methemoglobin were comparable to values for red maple-equine erythrocyte interactions in field and greenhouse studies (Dye, 2015).

Acertannin was a common constituent of extracts of *A. rubrum*, *A. saccharinum*, and *A. freemanii*. In fact, in HPLC chromatograms measured at the absorption maximum for gallic acid (280nm), acertannin was the most abundant secondary compound. Our initial hypothesize was that acertannin may contribute the hemolysis of equine erythrocyte and production of methemoglobin. Its abundance and chemical nature (i.e., a sugar with two gallic acid subunits attached) were suggestive of a potential role. Based on our bioassays; however, there is little evidence to indicate that acertannin has any effect on maple toxicosis.

In conclusion, this study affirms the importance of galloyl derivative in the phenomenon of maple toxicosis. Our results indicate that maple toxicosis can be largely explained by the presence of both tannic acid and pyrogallol which cause hemolysis and the production of methemoglobin, respectively.

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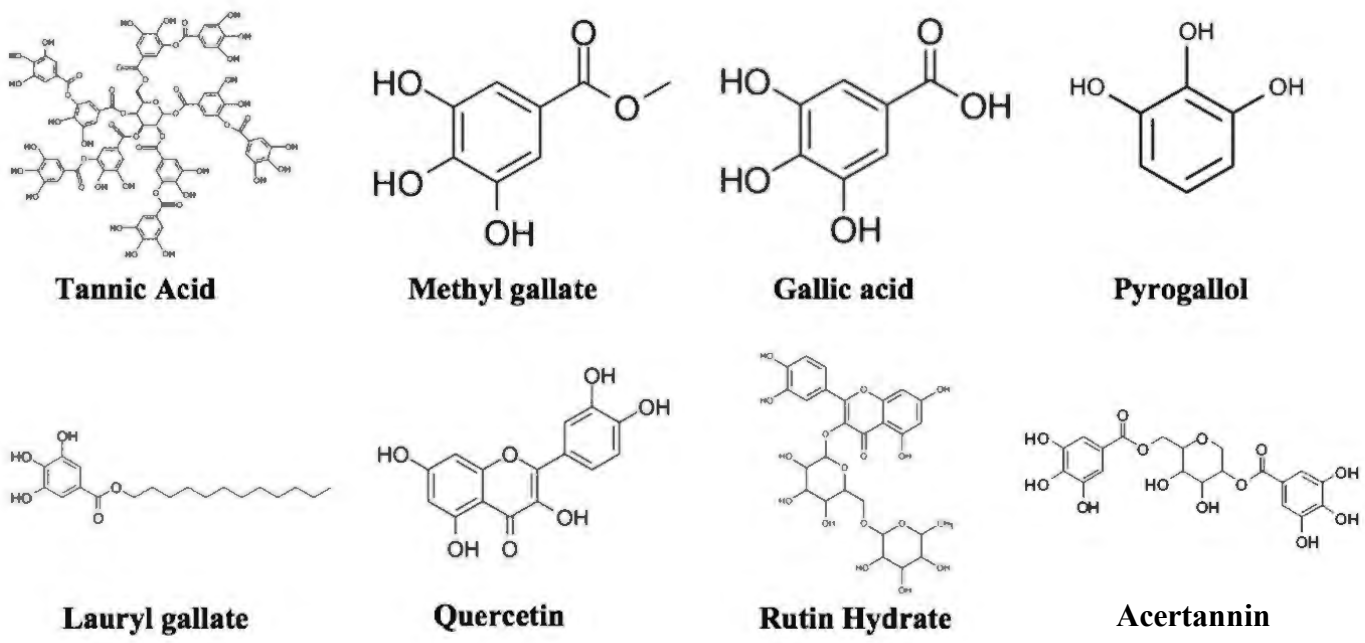

Figure 1: Eight gallotannins or gallic acid deriviative present in *Acer* spp. were tested for toxicity. All compounds except for acertannin were commercially available from Sigma-Aldrich or Fischer Scientific. Acertannin was isolated and identified from red maple extracts based on HPLC chromatograms. Each chemical was tested at concentrations of 0, 0.2 0.4, 0.8, and 1.6 mg/ml with equine erythrocytes in bioassays.

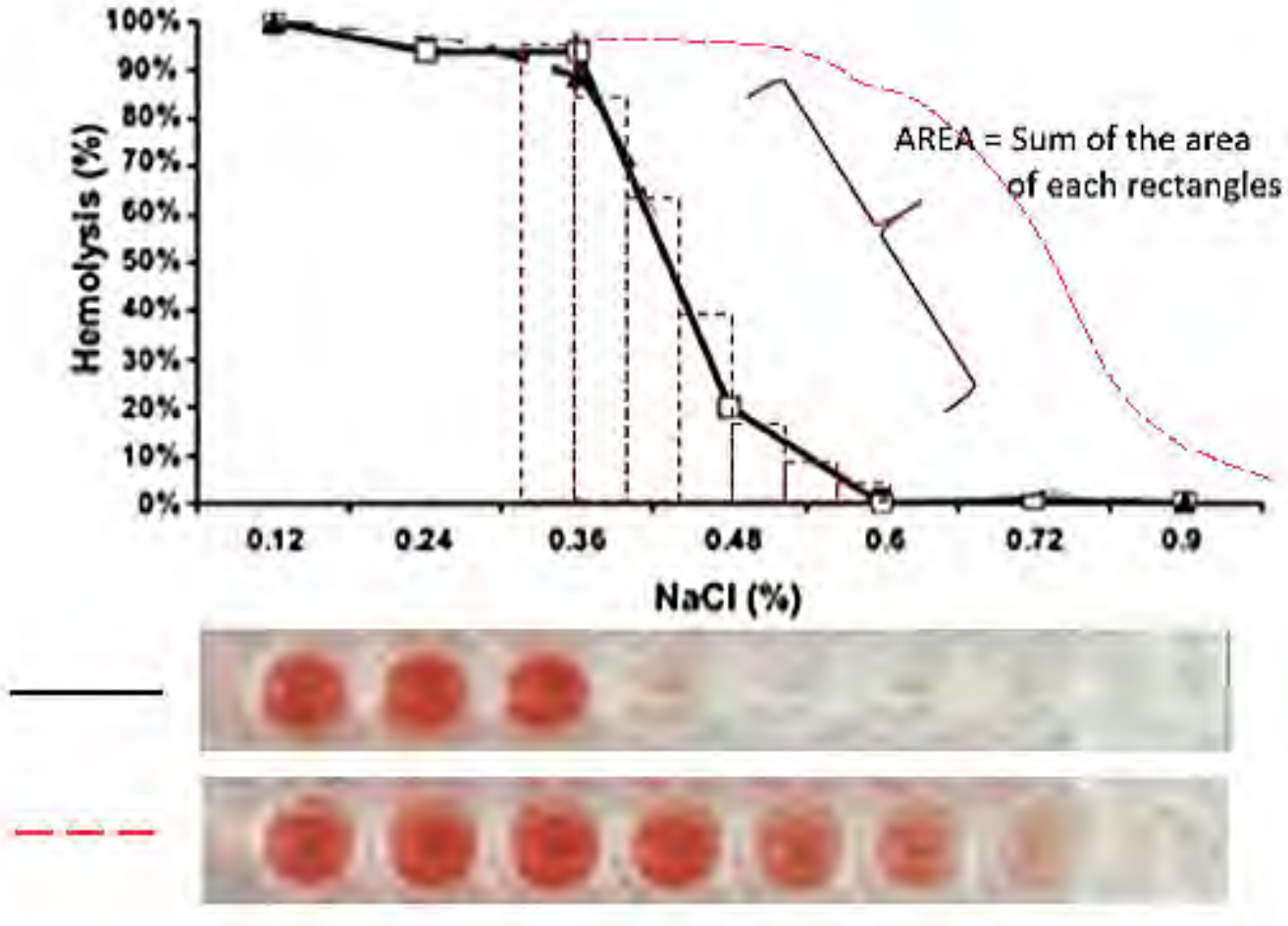

Figure 2: The amount of hemolysis was determined spectrophotometrically in test of cell fragility based on the area under the hemolytic curve. Typical erythrocytes start to lysis in solutions <0.6 NaCl (upper panel; black line). Greater than 90% lysis occurs in solution <0.3 NaCl (upper panel; black line). The amount of lysis can be expressed as the area under the curve (i.e., upper panel; sum of all horizontal rectangles). The addition of *Acer* extracts to erythrocyte solutions can alter the hemolytic curve (upper panel; red dashed line). Visually, these two different responses can be seen colorimetrically across NaCl solutions based on the intensity of red (lower panel; black versus red dashed line).

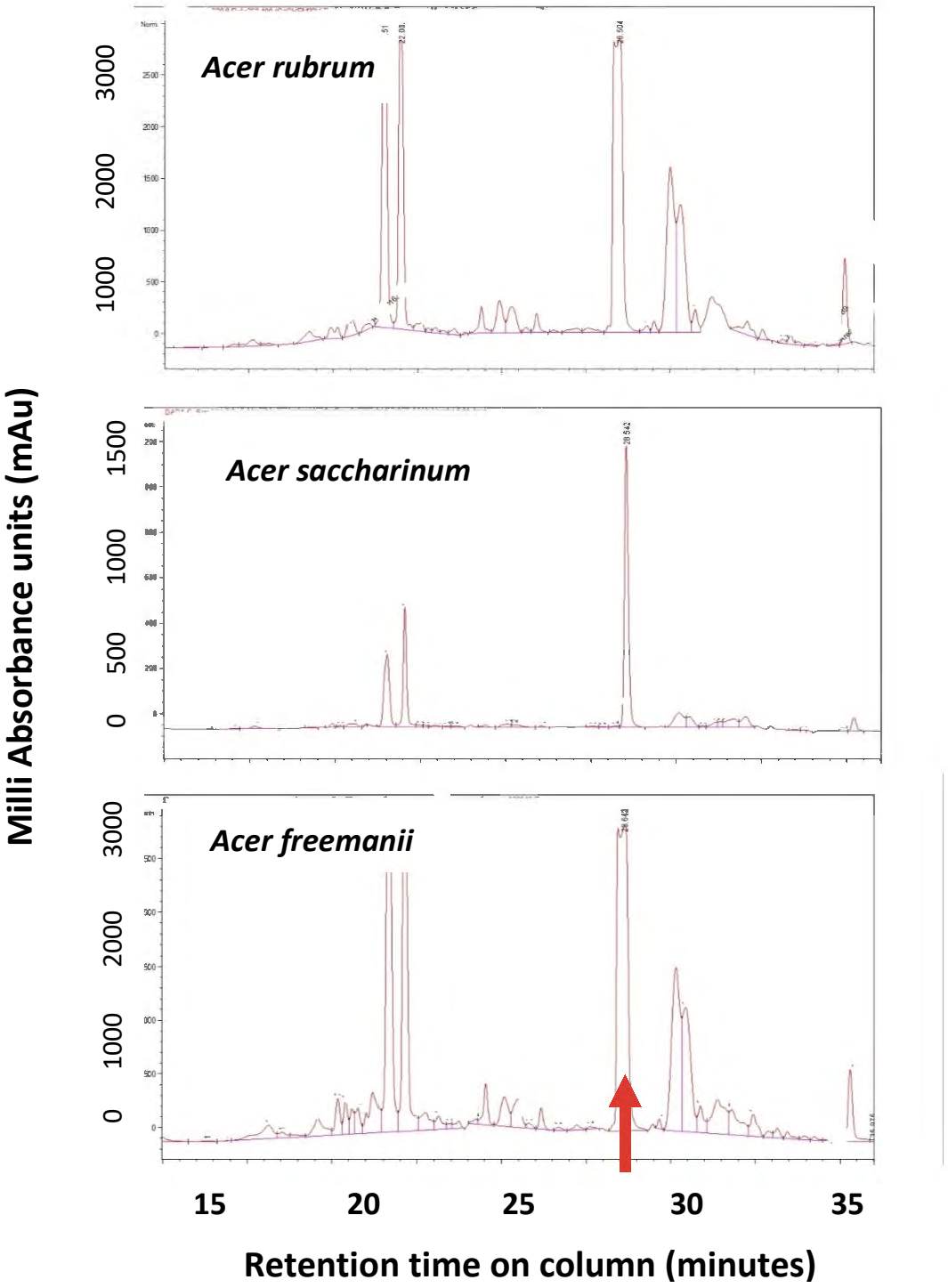

Figure 3: Chromatograms of three *Acer* spp. analyzed with a Restek Ultra C18 column on an Agilent 1100 HPLC system. Chromatograms were measured at the absorption maximum for gallic acid (280nm). All sample showed 3-5 major peaks. The largest peak (red arrow) had a retention time of 28.5 minutes with areas under the curve (×1000) for *A. rubrum*, *A. saccharinum*, and *A. freemanii* were 151, 96, 223, respectively. The major peak was determined to be acertannin in subsequent testing.

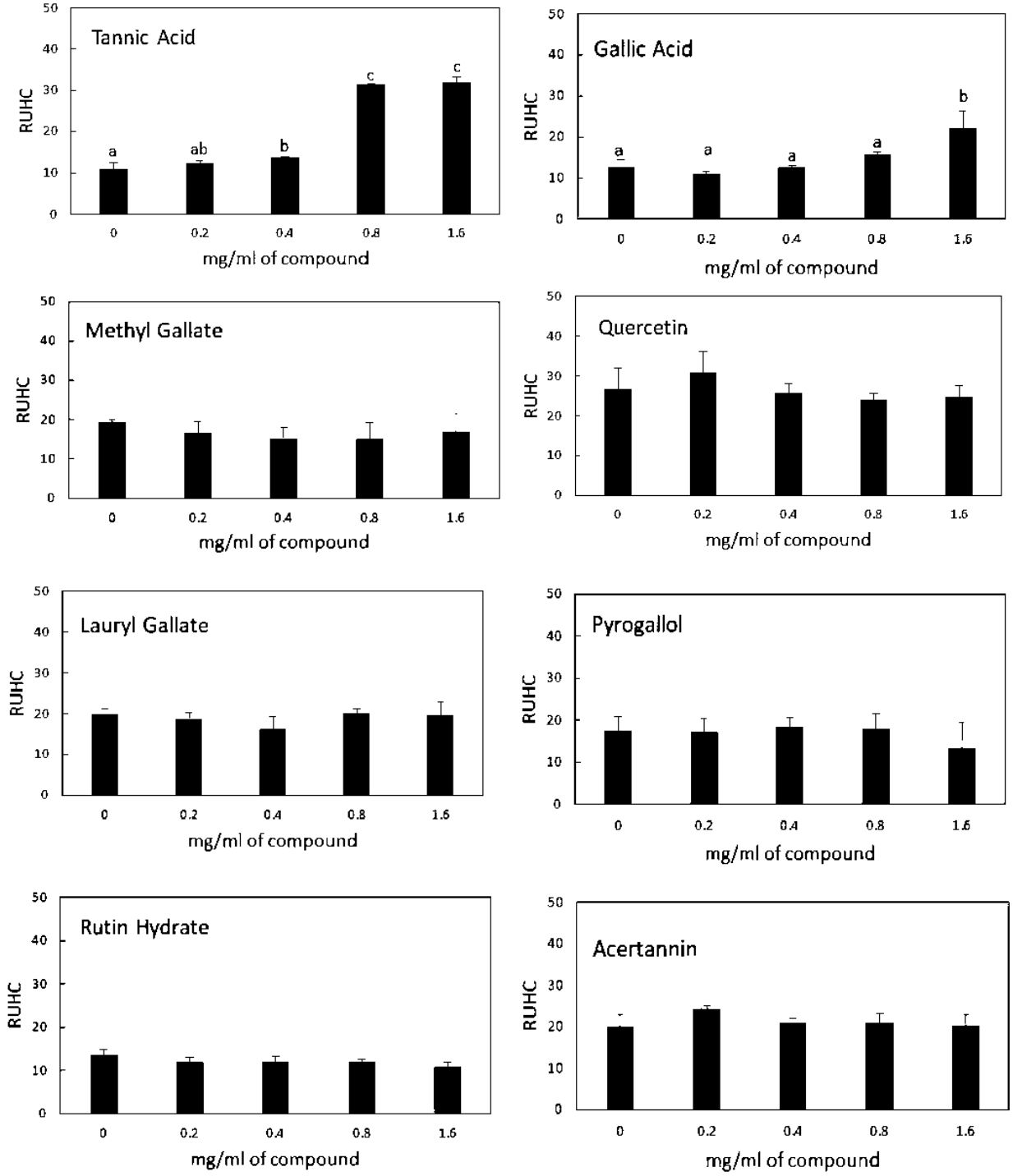

Figure 4: Mean values of relative area under the hemolysis curve (RUHC) for equine erythrocytes suspended in different concentration of gallic acid derivatives present in *Acer* spp. Values are the means of three replications. Columns with a letter in common do not differ significantly according to Student-Newman-Keuls mean separation test (α=0.05).

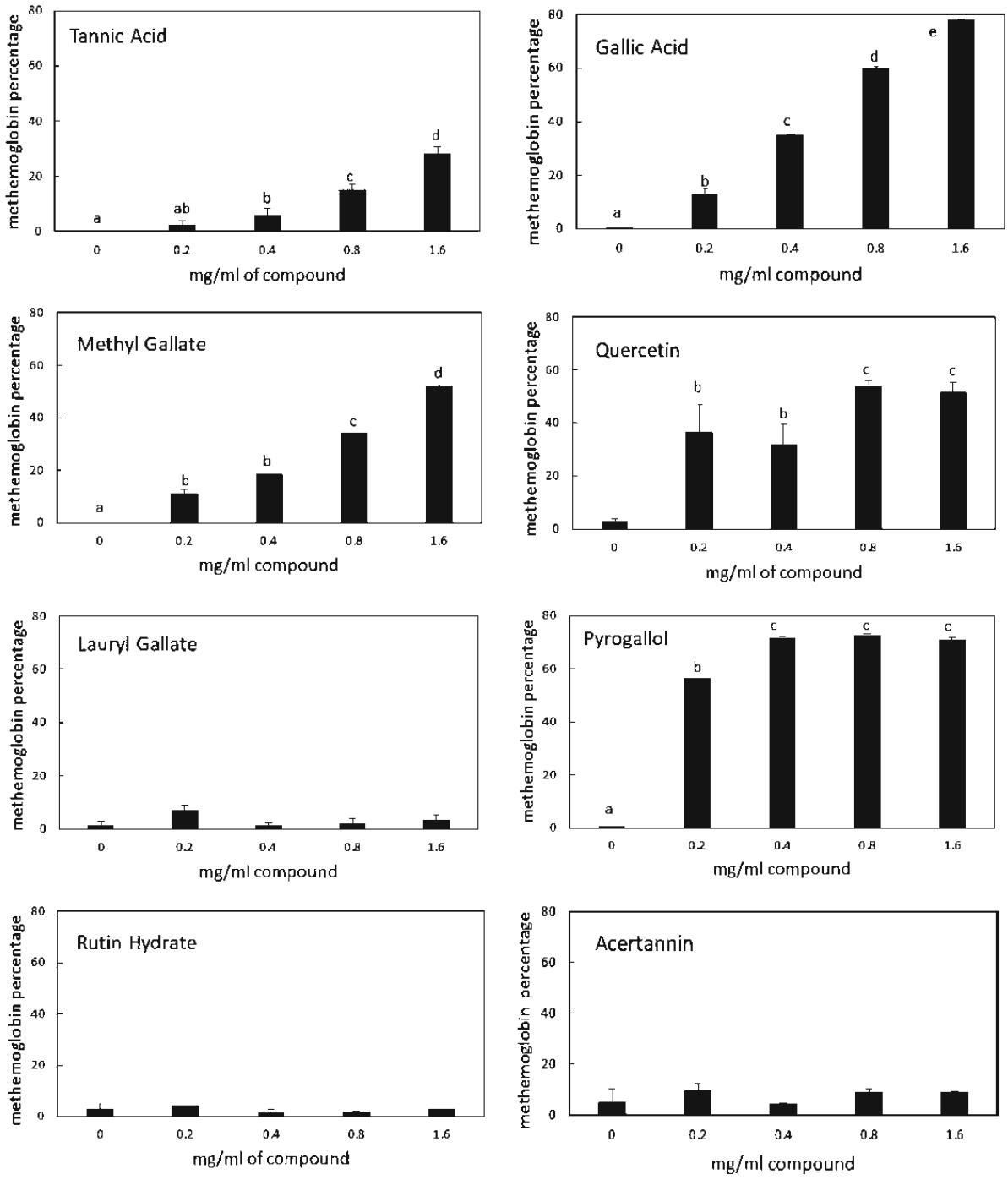

Figure 5: Mean values of percentage methemoglobin for equine erythrocytes suspended in different concentration of gallic acid derivatives present in *Acer* spp. Values are the means of three replications. Columns with a letter in common do not differ significantly according to Student-Newman-Keuls mean separation test (α=0.05).