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INSTITUTO DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA - PATOLOGIA E CIÊNCIAS CLÍNICAS

TESE

DOENÇA CARDÍACA FIBRÓTICA INDUZIDA POR ACETOGENINA NA INTOXICAÇÃO POR ABACATEIRO (*Persea americana,* Lauraceae) EM EQUINOS

Marina Sereno de Freitas

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UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
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MARINA SERENO DE FREITAS

Sob a Orientação do Professor
Daniel Guimarães Ubiali

Tese submetido como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária,** no Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Patologia Animal.

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RESUMO

FREITAS, Marina Sereno de. **Doença cardíaca fibrótica induzida por acetogenina na intoxicação por abacateiro (*Persea americana, Lauraceae*) em equinos.** 2024, 33p. Tese (Doutorado em Medicina Veterinária). Instituto de Veterinária, Programa de Pós-Graduação em Medicina Veterinária – Patologia e Ciências Clínicas, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2024.

A intoxicação por folhas e frutos de abacate (*Persea americana*) foi relatada em ovinos, caprinos, cães, coelhos e avestruzes. Os sinais clínicos e lesões são atribuídos à acetogenina, persina. Porém pouco se sabe sobre a epidemiologia, sinais clínicos, lesões e terapia causada por lesão cardíaca induzida por acetogeninas. Durante o estudo de dois anos, foi realizada a investigação de uma fazenda com seis cavalos que frequentemente se alimentavam com folhas de *P. americana* ou polpa e casca de frutas maduras no chão. Dois cavalos morreram e um foi submetido à necropsia, histopatologia e imuno-histoquímica com anticorpo anti-troponina C (cTnC). Macroscópica e histologicamente havia fibroplasia cardíaca grave. Na avaliação imuno-histoquímica houve diminuição multifocal ou expressão negativa de cTnC no citoplasma dos cardiomiócitos. Folhas de *Persea americana* foram confirmadas no trato alimentar utilizando anatomia botânica e técnicas moleculares. A investigação química por (LC-ESI-MS) revelou a presença de acetogeninas, persina e avocadene 1-acetato de *P. americana*. A persina esteve presente nas folhas e frutos (semente e polpa), enquanto o avocadene 1-acetato foi encontrado nas folhas e frutos (semente, casca e polpa) com maior concentração na polpa. Outros quatro cavalos foram examinados por eletrocardiograma, ecocardiograma e troponina 1 sérica (cTnI). Os resultados deste estudo sugerem que a intoxicação crônica por abacate deve ser incluída como causa de necrose miocárdica e fibroplasia de miocárdio em cavalos.

Palavra-chave: Intoxicação, *Persea americana*, equinos.

ABSTRACT

FREITAS, Marina Sereno de. **Fibrotic heart disease caused by acetogenin in avocado (*Persea americana*, *Lauraceae*) poisoning in horses.** 2024, 33p. Thesis (Doutorado em Medicina Veterinária) Instituto de Veterinária, Programa de Pós-Graduação em Medicina Veterinária – Patologia e Ciências Clínicas, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2024.

Poisoning by avocado (*Persea americana*) has been confirmed in sheep, goats, dogs, rabbits, and ostriches. The clinical signs and lesions are attributed to the acetogenin, persin. Little is known regarding the epidemiology, clinical signs, lesions, and therapy caused by acetogenin–induced heart damage. During the two–year study, we investigated a horse farm with six horses that often fed themselves with *P. americana* leaves or mature fruit pulp and skin on the ground. Two horses died, and one underwent necropsy, histopathology, and immunohistochemistry using the anti–cardiac troponin C (cTnC). Grossly and histopathologically, there was severe cardiac fibroplasia. Immunohistochemically, there was a multifocal decrease or negative expression in the cTnC cardiomyocytes' cytoplasm. *Persea americana* leaves were confirmed in the alimentary tract using botanical anatomy and molecular techniques. The chemical investigation by (LC–ESI–MS) revealed the presence of the acetogenins, persin and avocadene 1–acetate from *P. americana*. Persin was present in leaves and fruits (seed and pulp), while avocadene 1–acetate was found in leaves and fruits (seed, peel, and pulp) with a higher concentration in the pulp. Four other horses have been examined by electrocardiogram, echocardiogram, and serum Troponin 1 (cTnI). The results of this study suggest that avocado poisoning chronic avocado poisoning should be included as a cause of myocardial necrosis and fibroplasia in horses.

Keywords: Poisoning, *Persea americana,* horses.

LISTA DE ABREVIATURAS

AV	Nodo atrioventricular
cTnC	Anticorpo Anti-troponina C
ECG	Eletrocardiograma
MFA	Monofluoracetato

SUMÁRIO

1 INTRODUÇÃO

O abacateiro, gênero *Persea*, pertence à família Lauraceae tem origem no México e América Central. As três principais espécies conhecidas de abacate são mexicano (*P. americana* var. drymifolia), Índico Ocidental (*P. americana* var. americano), e guatemalteco *(P. nubigena* var. guatemalensis). O Brasil produziu mais de 338 mil toneladas de abacate em 2022 (IBGE, 2023), sendo um grande produtor mundial de abacate.

Apesar de ser um alimento consumido por humanos, existem relatos de intoxicação em espécies animais como caninos, bovinos, equinos, caprinos, camelídeos, roedores e aves (Zoltani, 2018; Aguirre et al., 2019; Buoro et al., 1994; McKenzie e Brown et al., 1991; Grant et al., 1991; Burger et al., 1994). O quadro clínico varia com a espécie acometida e quantidade consumida, podendo se manifestar somente como agalactia até insuficiência cardíaca aguda e morte (Stadler et al., 1991; Oelrichs, et al., 1995). Apesar de serem descritas 25 espécies de plantas tóxicas para cavalos no Brasil, não há relatos anteriores de intoxicação por *P. americana* em equinos no país (Rocha et al., 2022)

A substância responsável pela intoxicação é principalmente a persina, porém também há ação de outras acetogeninas encontradas no abacate. Seu mecanismo de ação ainda não foi completamente elucidado, mas estudos mostram que persina pode provocar a parada da fase G2-M do ciclo celular e induzir apoptose dependente de caspase (Butt et al., 2006; Roberts et al., 2007).

Pouco se sabe sobre a epidemiologia, sinais clínicos, lesões e terapêutica envolvidos na cardiomiopatia fibrótica induzida por acetogeninas em equinos. Os relatos na espécie são escassos, antigos e não abordam métodos diagnósticos ou lesões histológicas relacionadas ao quadro de intoxicação.

O objetivo desta tese é descrever o quadro clínico e lesões encontradas na intoxicação natural por *Persea americana* em cavalos. Além disso, confirmar o consumo da planta tóxica através de estudo botânico anatômico e molecular do conteúdo gastrointestinal de equinos; realizar estudo imuno-histoquímico com anticorpo anti-troponina cardíaca C para confirmação de lesões em cardiomiócitos e mensuração da troponina sérica para avaliar lesão cardíaca crônica em. Esta tese de doutorado sobre intoxicação por abacateiro em equinos está organizada em (1) Revisão de literatura e (2) Artigo científico publicado na revista Toxicon, em 2022 “Freitas MS, Pereira AHB, Pereira GO, Menezes IS, Lucena AR, Almeida CRF, Pereira EG, Santos LA, Tozin LRS, Alves FM, Macedo AL, Silva DB & Ubiali DG. Acetogenin-induced fibrotic heart disease from avocado (*Persea americana*, Lauraceae) poisoning in horses. doi: 10.1016/j.toxicon.2022.09.007.

2 REVISÃO DE LITERATURA

2.1 Plantas Tóxicas No Brasil

Existem 162 espécies plantas tóxicas que causam surtos em rebanhos de ruminantes e equídeos no Brasil e 219 na América do Sul (Riet-Correa et al., 2023). Para equinos as principais plantas tóxicas no Brasil são *Nerium oleander* (Tokarnia et al., 2012), *Crotalaria retusa, C. juncea, C. spectabilis* (Nobre et al., 2004; Câmara et al., 2022), *Coffea arabica* (Delfiol et al., 2012), *Megathyrsus maximus* (sin. *Panicum maximum*) (França et al., 2021), *Brachiaria humidicola* (Tokarnia et al., 2012), *Bambusa vulgaris*

(Barbosa et al., 2006), *Baccharis coridifolia* (Alda et al. 2009), *Chamaecrista serpens* (Mendonça et al., 2021), *Enterolobium contortisiliquum* (Machado et al., 2019), Froelichia humboldtiana (Pimentel et al., 2007*), Indigofera lespedezioides* (Lima et al., 2012), *Leucaena leucocephala* (Porto et al., 2017)*, Senecio brasiliensis* (Panziera et al., 2017), *Sida carpinifolia* (Bassuino et al., 2017) e *Trema micranta* (Lorenzett et al., 2018).

O Brasil tem mais de 5 milhões e 800 mil cabeças de equinos (IBGE, 2022), estima-se 5% de mortalidade anual e 14 % de causa de morte por intoxicação por plantas (Riet-Correa, 2001; Assis et al., 2010), sendo assim seriam aproximadamente 39.000 equinos por ano mortos pelo consumo de plantas tóxicas (Pessoa et al., 2013; Rocha et al., 2022).

2.2 Plantas Cardiotóxicas No Brasil

2.2.1 Plantas que contém monofluoroacetato

São cerca de 12 espécies de plantas tóxicas que contém monofluoroacetato (MFA) capazes de causar morte súbita, sendo a principal espécie envolvida os bovinos (Riet-Correa et al., 2023; Tokarnia et al., 2002; 2000; Carvalho et al., 2009). As espécies que causam esse quadro são *Palicourea marcgravii, P. grandiflora, P. juruana, P. aeneofusca, Amorimia pubiflora, A. exotropica* e *A. septentrionalis, A. rigida, A. amazonica, Niedenzuella stannea, Tanaecium bilabiatum* (Riet-Correa et al., 2023; Ubiali et al., 2020). É estimado que 50% das mortes por plantas tóxicas no Brasil sejam causadas por plantas que contém MFA (Pessoa et al., 2013; Tokarnia et al., 2002).

Os animais acometidos pela intoxicação por monofluoroacetato apresentam sinais clínicos superagudos como colapso, tremores, dispneia e falência cardíaca que leva a morte em poucos minutos (Cunha et al., 2022). Quando intoxicados por essas espécies não há alterações macroscópicas significativas e na microscopia a lesão frequentemente encontrada é a degeneração hidrópicovacuolar das células epiteliais dos túbulos contornados distais (Tokarnia; Peixoto; Döbereiner, 2000).

2.2.2 Plantas que contém glicosídeos cardiotóxicos

No Brasil existem duas espécies de plantas que contém glicosídeos cardiotóxicos sendo *Nerium oleander* e *Kalanchoe blossfeldiana* (Riet-Correa et al., 2023). Estas espécies são ornamentais e as intoxicações relatadas são relacionadas a erros de manejo, onde há exposição dos animais as plantas (Riet-Correa; Méndez, 2007).

Os glicosídeos cardiotóxicos inibem a bomba de sódio e potássio, causando distúrbio eletrolítico que afeta a condutividade elétrica do coração (Aslani et al., 2004). Experimentalmente ovelhas intoxicadas por *N. oleander* apresentaram sinais clínicos gastrointestinais como dor abdominal, atonia ruminal e timpanismo, também apresentaram poliúria e fraqueza (Aslani et al., 2004).

As lesões encontradas na intoxicação por *N. oleander* são principalmente renais, como degeneração e necrose dos túbulos renais e poucas alterações em cardiomiócitos, como alterações na coloração sugerindo uma degeneração em fase inicial (Barbosa et al., 2008).

2.2.3 Outras plantas cardiotóxicas com compostos tóxicos desconhecidos

As espécies de plantas *Ateleia glazioviana, Niedenzuella acutifolia* e *Nidenzuella multiglandulosa* são capazes de causar três quadros clínicos em ruminantes, podendo

ocorrer de forma singular ou em conjunto, sendo: a forma cardíaca, ocorrendo fibrose em coração causando morte súbita ou falência cardíaca congestiva; forma perinatal causando aborto ou morte neonatal e; a forma nervosa causando espongiose do sistema nervoso (Tokarnia et al, 2012; Gava et al., 2021).

A intoxicação por *A. glazioviana* é caracterizada por sinais clínicos associados a falência cardíaca crônica como perda de peso, fraqueza, edema submandibular e facial, podendo ocorrer morte súbita após a indução de exercício (Gava et al., 2021). Também pode ocorrer sinais neurológicos como letargia e cegueira devido as lesões encontradas na substância branca do encéfalo, principalmente em medular do cerebelo, pedúnculos cerebelares e tronco encefálico (Gava et al., 2001).

2.3 Abacateiro

O abacateiro pertence à família Lauraceae, gênero *Persea*, e compreende dois subgêneros: Perseguir e Eriodaphne. As três principais espécies conhecidas de abacate são mexicanas (*P. americana* var. drymifolia), Índico Ocidental (*P. americana* var. americano), e guatemalteco (*P. nubigena* var. guatemalensis). (Koller, 1992). Existem também variedades híbridas resultantes de cruzamento entre raças como Breda, Fortuna, Geada, Margarida, Ouro Verde e Quintal (híbridos das raças Antilhana e Guatemalense), e Hass e Fuerte (híbridos das raças Mexicana e Guatemalense) (Francisco; Baptistella, 2005).

O abacate é utilizado mundialmente na alimentação e na extração de óleos da semente, principalmente para cosméticos (Rohwer et al., 1993). O abacate é uma espécie cosmopolita arbórea polimórfica originada em ampla área geográfica que se estende desde as terras altas do México central e oriental, passando pela Guatemala até a costa do Oceano Pacífico na América Central (Schaffer et al., 2013).

O Brasil é um dos maiores produtores de abacate do mundo. Em 2022, foram produzidas mais de 338 mil toneladas (IBGE, 2023).

2.3.1 Componentes bioativos do abacate

Os principais componentes bioativos presentes na polpa, casca, semente e folhas do abacate são os polifenóis, seguidos de carotenoides, tocoferóis, esteróis e alcanos (Jimenez et al., 2020; Ferreira da Vinha, 2020). Além de suas propriedades nutricionais, também possui componentes com propriedades terapêuticas como efeito anti-inflamatório, antioxidante e antimicrobiano em humanos (Gupta et al., 2018).

O extrato aquoso de *P. americana* foi capaz de produzir efeito anticonvulsivante em ratos, devido ao aumento da neurotransmissão GABAérgica (Ojewole; Amabeoku, 2006).

As acetogeninas do abacate, como a persina, são derivadas da biossíntese de ácidos graxos de cadeia longa homologia estrutural próxima ao monoglicerídeo do ácido linoleico e pode ser encontrada nas folhas, frutos e semente de *P. americana* (Butt et al., 2006). A persina foi estudada para tratamento para câncer de mama devido ao seu efeito necrótico em células da glândula mamária, sendo um dos seus efeitos celulares é a indução da apoptose através da parada da fase G2-M do ciclo celular (Butt et al., 2006; Roberts et al., 2007).

O extrato de frutos e folhas de abacate foi capaz de gerar instabilidade genômica e dano genético em linfócitos de humanos. Houve diferença entre os grupos expostos a extratos de folhas ou frutos, sendo maior a instabilidade a exposição do extrato de folhas, possivelmente pela maior concentração de bioativos nas folhas (Kulkarni, 2010).

2.3.2 Intoxicação por *Persea americana*

Apesar do considerável consumo de abacate pela espécie humana, há relato de intoxicação em diversas espécies animais, como caninos, equinos, bovinos, caprinos, camelídeos, roedores e aves (Zoltani, 2018; Aguirre et al., 2019; Buoro et al., 1994; McKenzie e Brown et al., 1991; Grant et al., 1991; Burger et al., 1994).

Devido ao potencial tóxico em várias espécies animais, foram feitos estudos para avaliar a toxicidade de extratos do abacate. Ozolua e colaboradores (2009) avaliariam extrato aquoso da semente de *P. americana* em ratos em doses variadas (de 2,5g/kg a 10g/kg) e não houve manifestação de intoxicação ou morte durante o experimento. Padilla-Camberos (2013) relatou mortalidade de ratos consumindo extrato etanólico da semente de *P. americana* nas doses de 500mg/kg a 2000mg/kg, determinando que há diferenças entre a forma de extração e toxicidade. Os sinais clínicos podem variar entre a quantidade consumida e tempo de consumo, sendo as altas doses responsáveis por causar insuficiência cardíaca (Forero, 2010).

Em bovinos (Coit, 1943), ovinos e caprinos (Stadler et al., 1991) e equinos (McKenzie et al, 1991) há relatos de intoxicação por *P. americana* associado com mastite necrohemorrágica. Em ratos experimentalmente intoxicados por *P. americana*, houve necrose do epitélio tubular mamário, edema intersticial e hemorragia nas doses 60-100 mg/kg e necrose de miocárdio assim como fibrose de miocárdio nas doses acima de 100mg/kg (Oelrichs et al., 1995).

As principais lesões em cães (Buoro et al., 1994), equinos (McKenzie e Brown et al., 1991), coelhos (Aguirre et al., 2019), cabras (Grant et al., 1991) e avestruzes (Burger et al., 1994) são cardíacas. Buoro e colaboradores (1994) obtiveram resultados clínicos eletrocardiográficos, radiográficos e clínico-patológicos de dois cães com acesso a abacateiro e quadro clínico-patológico de cardiopatia congestiva.

Em Salta, Argentina, um estudo com coelhos intoxicados espontaneamente após serem suplementados com folhas de *P. americana,* apresentavam na histopatologia cardíaca, degeneração e necrose miocárdica (Aguirre et al., 2019). Experimentalmente, para investigar a causa de morte de avestruzes após serem introduzidos em pomares com abacateiro, as folhas e os frutos do cultivar Hass e Fuerte, foram acrescentados na dieta. As aves morreram após 3 a 5 dias de experimento. Na histopatologia havia necrose de cardiomiócitos (Burger et al., 1994).

Também foi feito um experimento como prova diagnóstica de cabras que morreram após serem alimentadas com folhas de abacate. O experimento foi feito em carneiros que foram alimentados com folhas em doses diferentes. Os carneiros devolveram sinais clínicos compatíveis com insuficiência cardíaca e na histopatologia havia degeneração e necrose de miocárdio (Grant et al., 1991)

Apenas um relato de equinos com acesso a abacateiros do cultivar Hass associa intoxicação por abacateiro com sinais clínicos de insuficiência cardíaca congestiva como abafamento dos sons cardíacos na ausculta, edema em cabeça e presença de líquido no tórax. Houve aumento sérico de CK e AST. Os equinos foram tratados conforme a sintomatologia e os autores não mencionaram mortes (McKenzie e Brown et al., 1991).

2.4 Identificação Microhistológica De Plantas Tóxicas, Incluindo *Persea americana*

A identificação botânica é primordial para gerar dados seguros de taxonomia de plantas tóxicas (Kingsbury et al., 1964, Tokarnia et al., 2012). Para identificação da espécie da planta pode ser coletado material como ramos de florescência, frutos e folhas para análise (Martins-da-Silva, 2014).

No entanto, poucos trabalhos apresentam folhas e frutos no conteúdo gastrointestinal como possível auxiliar na interpretação de quadros-clínico-patológicos. Para a avaliação de ingestão de planta tóxica em animais é possível avaliar o conteúdo gastrointestinal morfologicamente e comparar com amostras da planta tóxica suspeita. Torna-se necessário realizar a correta fixação das amostras e a avaliação de um botânico especialista. Aguirre e colaboradores (2019) determinaram a microhistologia como a principal prova de um caso de intoxicação espontânea por *P. americana* em coelhos. Foram compatíveis as características anatômicas entre o material presente no estômago e as folhas dos abacateiros que os coelhos tiveram acesso

A avaliação morfológica de folhas de *P. americana* já foi realizada para objetivos de saúde pública na Turquia, devido a comercialização de folhas da espécie para fins medicinais (Kendir; Koroglu, 2018).

2.5 Identificação Molecular De Plantas Tóxicas, Incluindo *Persea americana*

A identificação da espécie botânica em casos de intoxicação é crucial para o tratamento adequado. Uma abordagem altamente eficaz para essa identificação é o uso da análise molecular da planta tóxica, a qual pode ser realizada por meio da investigação do vômito ou da diarreia apresentados pela vítima intoxicada (Nithaniyal et al., 2021).

Na medicina forense, a detecção de intoxicação por plantas é realizada por meio da técnica de PCR, que permite a identificação molecular da espécie envolvida. Para esse fim foi criada uma biblioteca de códigos de DNA abrangendo 100 espécies de plantas, facilitando a identificação a partir de amostras do conteúdo gastrointestinal (Nithaniyal et al., 2021).

2.6 Diagnósticos Diferenciais De Fibrose Cardíaca Em Cavalos

Em decorrência de injúria cardíaca que resulta na necrose de cardiomiócitos, ocorre a ativação de miofibroblastos, os quais desempenham um papel na preservação da integridade estrutural do órgão. Em conjunto, a ação das células inflamatórias os miofibroblastos também respondem com produção de colágeno resultando na formação de tecido cicatricial fibroso (Weber et al., 2013; Gyöngyösi1 et al., 2017).

As lesões em miocárdio podem resultar de causas tóxicas, virais, bacterianas, traumáticas, isquêmicas, hipóxias ou metabólicas, de endocardite ou pericardite ou migração parasitária (Coundry et al., 2007). A fibrose cardíaca está relacionada a injúrias crônicas (Schiff; Knottenbelt, 1990).

Em cavalos a fibrose de miocárdio pode estar relacionada a migração da larva de *Strongylus vulgaris,* sendo que as lesões não são causadas diretamente pelo contato com a larva, mas pela microembolização de lesões arteriais, produzindo lesões arterioscleróticas obstrutivas nas arteríolas miocárdicas (Cranley; McCullagh, 1981).

2.7 Avaliação Cardiológica Em Equinos

2.7.1 Eletrocardiograma

O eletrocardiograma (ECG) registra a diferença potencial entre dois pontos na superfície corporal, permitindo a detecção de mudanças no campo elétrico ao redor do coração durante a despolarização e repolarização (Van Loon; Patteson, 2010).

As alterações em frequência e ritmo cardíaco podem ser avaliadas durante a ausculta cardíaca, porém somente o eletrocardiograma consegue fornecer o diagnóstico definitivo de arritmias cardíacas (Mitchell, 2019).

A propagação do sinal elétrico no coração inicia-se no nodo sinoatrial, localizado no átrio direito. Este sinal se difunde pelos átrios, culminando no nodo atrioventricular (AV). A despolarização dos átrios é refletida pela onda P no ECG. Subsequentemente, ocorre um intervalo conhecido como intervalo PR, durante o qual ocorre um retardo na condução para o AV, influenciado pelo sistema nervoso autônomo. Em cavalos, observa-se uma condução elétrica no septo interventricular, propagando-se para as paredes externas e resultando na deflexão QRS. A repolarização dos ventrículos é representada pela onda T que pode sofrer alterações de traçado em cavalos. (Morgan, 2012).

2.7.2 Ecocardiograma

O ecocardiograma é utilizado para a avaliação das estruturas cardíacas através da ultrassonografia, podendo ser realizada a avaliação de lesões morfológicas, anormalidades de contração, avaliação das câmaras, função das válvulas, distúrbios do fluxo sanguíneo, função ventricular sistólica, diastólica e variáveis hemodinâmicas (Schwarzwald, 2019).

O modo B do ecocardiograma utiliza a abordagem convencional da ultrassonografia em duas dimensões para exibir detalhadamente as estruturas internas do coração, abrangendo as câmaras cardíacas, válvulas, miocárdio, pericárdio e grandes vasos (Marr, 1994).

O modo M do ecocardiograma apresenta imagens unidimensionais das estruturas cardíacas ao longo do tempo, sendo empregado para aferir os movimentos dessas estruturas. Realizado simultaneamente ao eletrocardiograma, permite a avaliação desses movimentos em pontos específicos do ciclo cardíaco (Marr, 1994).

O modo Doppler utiliza o princípio Doppler para quantificar a direção e velocidade das hemácias em movimento pelo coração. Essas informações são representadas em um traçado espectral, no qual o eixo horizontal indica a direção do fluxo, enquanto o eixo vertical representa a velocidade do fluxo (Schwarzwald, 2019).

2.7.3 Troponinas cardíacas

As troponinas são proteínas regulatórias que fazem parte do aparato contrátil do tecido muscular cardíaco e esquelético. O complexo das troponinas consiste em três proteínas distintas, sendo troponina I, T e C (Wells; Sleeper, 2008).

A troponina C se liga ao cálcio e tem duas isoformas, sendo uma presente em músculos de contração rápida e outra presente na musculatura cardíaca e de contração lenta. A troponina I e a troponina T são utilizadas como marcadores bioquímicos de lesão cardíaca (Adams et al., 1993; Ohman et al., 1996).

O uso de anticorpos anti-Troponina-C e anti-troponina-T podem ser utilizados na imunohistoquímica para marcar lesões em miocárdio, com potencial de interpretar lesões discretas, paralelamente à morfologia tradicional utilizando-se lâminas coradas per Hematoxilina e Eosina (Fishbein et al., 2003; Martínez Díaz et al., 2005). A utilização deste recurso foi eficiente para detecção de lesões cardíacas em gatos com doença renal crônica (Cid et al., 2021). Em bovinos expostos a *Amorimia exotropica* a utilização de análise imunohistoquímica com anticorpo anti-Troponina-C foi capaz de identificar lesão cardíaca causadas pela intoxicação pela planta (Pavarini et al., 2012).

3 RESULTADOS

Os resultados serão apresentados na forma de artigo científico em anexo.

4 CONCLUSÃO

Os resultados obtidos através das análises macroscópica, histológica, imuno-histoquímica confirmam que houve necrose e fibroplasia em miocárdio, e em conjunto com os resultados dos estudos botânicos anatômico e molecular do conteúdo gastrointestinal do equino, assim como a presença de acetogeninas nas amostras botânicas sugerem fortemente o consumo de abacate como principal causador da lesões cardíacas encontradas.

A intoxicação crônica por abacate deve ser considerada como diagnóstico diferencial de necrose e fibrose miocárdica em cavalos. O diagnóstico deve ser baseado na epidemiologia, clínica, patologia e exames auxiliares.

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ANEXOS

Anexo A. Artigo Publicado na revista Toxicon

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Acetogenin-induced fibrotic heart disease from avocado (*Persea americana*, Lauraceae) poisoning in horses

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ABSTRACT

Poisoning by avocado (*Persea americana*) has been confirmed in sheep, goats, dogs, rabbits and ostriches. The clinical signs and lesions are attributed to the acetogenin, persin. Little is known regarding the epidemiology, clinical signs, lesions and therapy caused by acetogenin–induced heart damage. During the two–year study, we investigated a horse farm with six horses that often fed themselves with *P. americana* leaves or mature fruit pulp and skin on the ground. Two horses died, and one underwent necropsy, histopathology, and immunohistochemistry using the anti–cardiac troponin C (cTnC). Grossly and histopathologically, there was severe cardiac fibroplasia. Immunohistochemically, there was a multifocal decrease or negative expression in the cTnC cardiomyocytes' cytoplasm. *Persea americana* leaves were confirmed in the alimentary tract using botanical anatomy and molecular techniques. The chemical investigation by (LC–ESI–MS) revealed the presence of the acetogenins, persin and avocadene 1–acetate from *P. americana*. Persin was present in leaves and fruits (seed and pulp), while avocadene 1–acetate was found in leaves and fruits (seed, peel, and pulp) with a higher concentration in the pulp. Four other horses have been examined by electrocardiogram, echocardiogram and serum Troponin 1 (cTnI). To establish a causal effect of consumption of *P. Americana* and heart fibroplasia in horses, long-time experiments must be carried out.

1. Introduction

Plants have been employed since immemorial times for different purposes by humans and animals, but the ingestion of specific substances can pose health risks due to the bioactive compounds and their toxic potential (Mensah et al., 2019). Plant toxicity is a significant challenge for Brazilian livestock, as poisoning causes great economic losses (Pessoa et al., 2013). Poisoning horses by plants is a relatively common veterinary problem, and naturally occurring cases may occur when the fresh plant is ingested in pasture or when the poisonous plant contaminates the feed (Tokarnia et al., 2012; Caloni and Cortinovis, 2015).

Several species of plant have been reported as poisonous to horses under Brazilian field conditions, such as *Nerium oleander* (Tokarnia et al., 2012), *Crotalaria retusa, C. juncea, C. spectabilis* (Nobre et al., 2004; Câmara et al., 2022), *Coffea arabica* (Delfiol et al., 2012), *Megathyrsus maximus* (sin. *Panicum maximum*) (França et al., 2021), *Brachiaria humidicola* (Tokarnia et al., 2012), *Bambusa vulgaris* (Barbosa et al., 2006), *Baccharis coridifolia* (Alda et al., 2009), *Chamaecrista serpens* (Mendonça et al., 2021), *Enterolobium contortisiliquum* (Machado et al., 2019), *Froelichia humboldtiana* (Pimentel et al., 2007), *Indigofera lespedezioides* (Lima et al., 2012), *Leucaena leucocephala* (Porto et al.,

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2017), *Senecio brasiliensis* (Panziera et al., 2017), *Sida carpinifolia* (Bassuino et al., 2017) and, *Trema micranta* (Lorenzett et al., 2018). There are no reports in Brazil of spontaneous or experimental *Persea americana* poisoning in horses (Pierezan et al., 2009; Pessoa et al., 2013; Tokarnia et al., 2012; Marcolongo–Pereira et al., 2014; Gava, 2022 personal communication; Riet–Correa, 2022 personal communication).

Avocado (*Persea americana* Mill.), an angiosperm belonging to the family Lauraceae, presents their fruit as the most important product of this botanical family. It is employed worldwide in healthy eating and oil extraction, mainly for cosmetics, from the mesocarp of the seed (Rohwer, 1993). Avocado is a cosmopolitan cultivated, polymorphic arboreal species that originated in a wide geographical area extending from the highlands of central and eastern México, passing through Guatemala to the Pacific Ocean coast in Central America (Schaffer et al., 2013). The avocado includes three botanical races: Guatemalan avocado (*P. americana* var. *guatemalensis*), the West Indian (*P. americana* var. *americana*), and the Mexican (*P. americana* var. *drymifolia*) (Scora and Bergh, 1989; Ochoa–Zarzosa et al., 2021). Hybridization, which occurs freely between these races, has given rise to numerous varieties and cultivars (Pliego-Alfaro et al., 2013). Margarida and Dourado cultivars originated in Brazil and play an important role in Brazilian avocado production (Carvalho et al., 1983). Brazil is an important producer of avocados. In 2018, more than 235 thousand tons were produced, and national production is expanding (Pio, 2020).

Avocado trees' leaves, fruits, and seeds could be toxic to susceptible animals (Kingsbury et al., 1964). Avocado poisoning in animals only occurs by chance and at a high dosage (Burger et al., 1994). The leaves would be toxic in farm animals at a relatively high dose, but the ingestion quantity to produce clinical signs is still unknown (Stadler and Van RensburgNaude, 1991; Aguirre et al., 2019). The toxic effects of avocado are attributed to persin, which can be found in the leaves, fruits, and seeds of *P. americana* (Butt et al., 2006). Persin is an acetogenin derived from the biosynthesis of long-chain fatty acids with close structural homology to the essential n–6 polyunsaturated fatty acid, linoleic acid (Rodriguez-Sanchez et al., 2015).

Avocado poisoning has been described in several animal species, such as sheep (Grant et al., 1988), goats (Craigmill et al., 1989), dogs (Buoro et al., 1994), rabbits (Aguirre et al., 2019), and ostriches (Burger et al., 1994). Avocado toxicity in horses was only reported in an outbreak that caused clinical disease in Australia (McKenzie and Brown, 1991), but the authors did not describe the pathological findings.

This study describes the clinical–pathological, and ancillary findings of a naturally occurring *P. americana* poisoning in horses from Brazil. The toxicological results of *P. americana* consumption and the cardiologic parameters in field horses are presented together with the chemical analyses of leaves and fruits.

2. Material and methods

The Ethical Committee has approved this clinical toxicology field study of Animals Use (CEUA) by the Federal Rural University of Rio de Janeiro (UFRuralRJ) with a number protocol 2824200521/2021.

2.1. Animals and clinical–epidemiological data

Six trips were conducted for the clinical–pathological and toxicological study (Timeline, Fig. 1). The veterinary practitioner (Ms. DVM Marina S. Freitas) and the farmer obtained the clinical and epidemiological data. The small horse breeding farm used for subsistence agriculture and equestrian purposes is located in the Municipality of Queimados, Rio de Janeiro, Brazil (coordinates E 645899.277 N 7485670.493) (Fig. 2).

2.2. Botanical examination of P. americana trees and anatomy of leaves

Botanical samples (leaves, flowers and fruits) of all avocado varieties present on the property were collected and sent to the UFRuralRJ (RBR Herbarium). The varieties' morphological aspects of the plant were identified by Prof. Dr. Flávio Macedo Alves from the Federal University of Mato Grosso do Sul (CGMS herbarium).

A pool of leaves and fruits from the avocado trees (*P. americana*) growing in the field area. We fixed all samples using formalin – acetic acid – 50% ethanol, 5:5:90, v/v/v, (FAA 50) (Johansen 1940). For anatomical studies, hand-cut cross-sections from the leaves samples (10 μm thick) were stained with Safrablau (Bukatsch, 1972). According to De Strittmatter (1973), we clarified the samples and stained them with

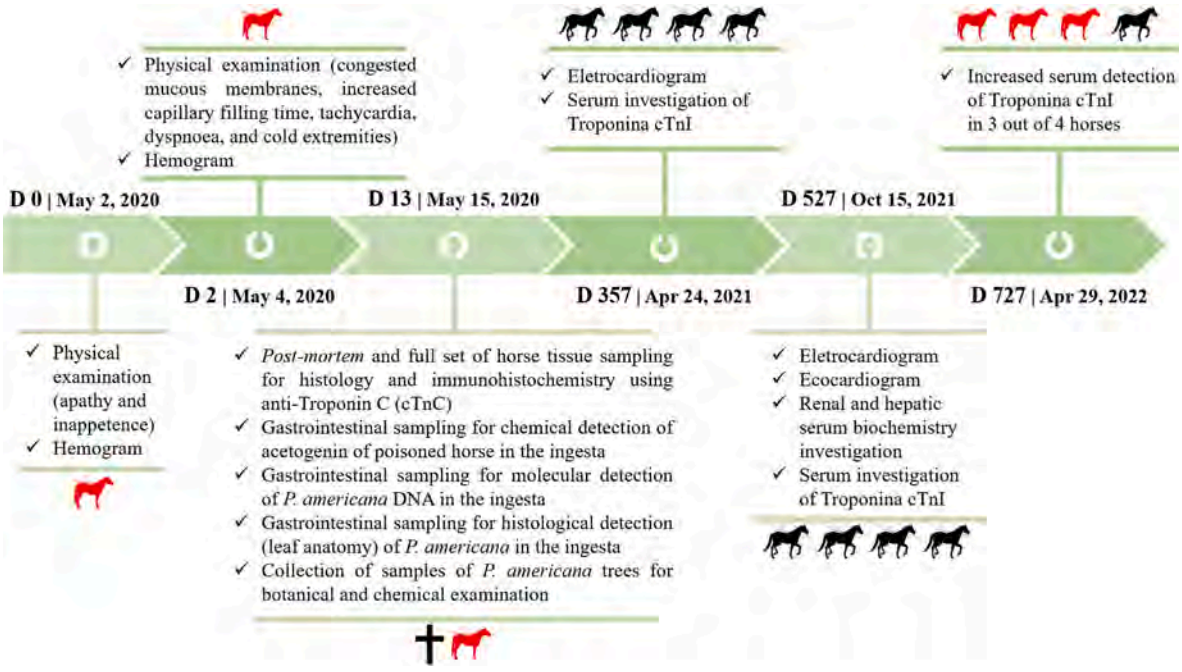

Fig. 1. Flowchart of clinicopathological and ancillary tests on avocado poisoning in horses. Clinical approaches were realized twice in a horse, and the postmortem and histological examinations were done on this horse after natural death. Furthermore, the other four horses were submitted to clinical and ancillary examination.

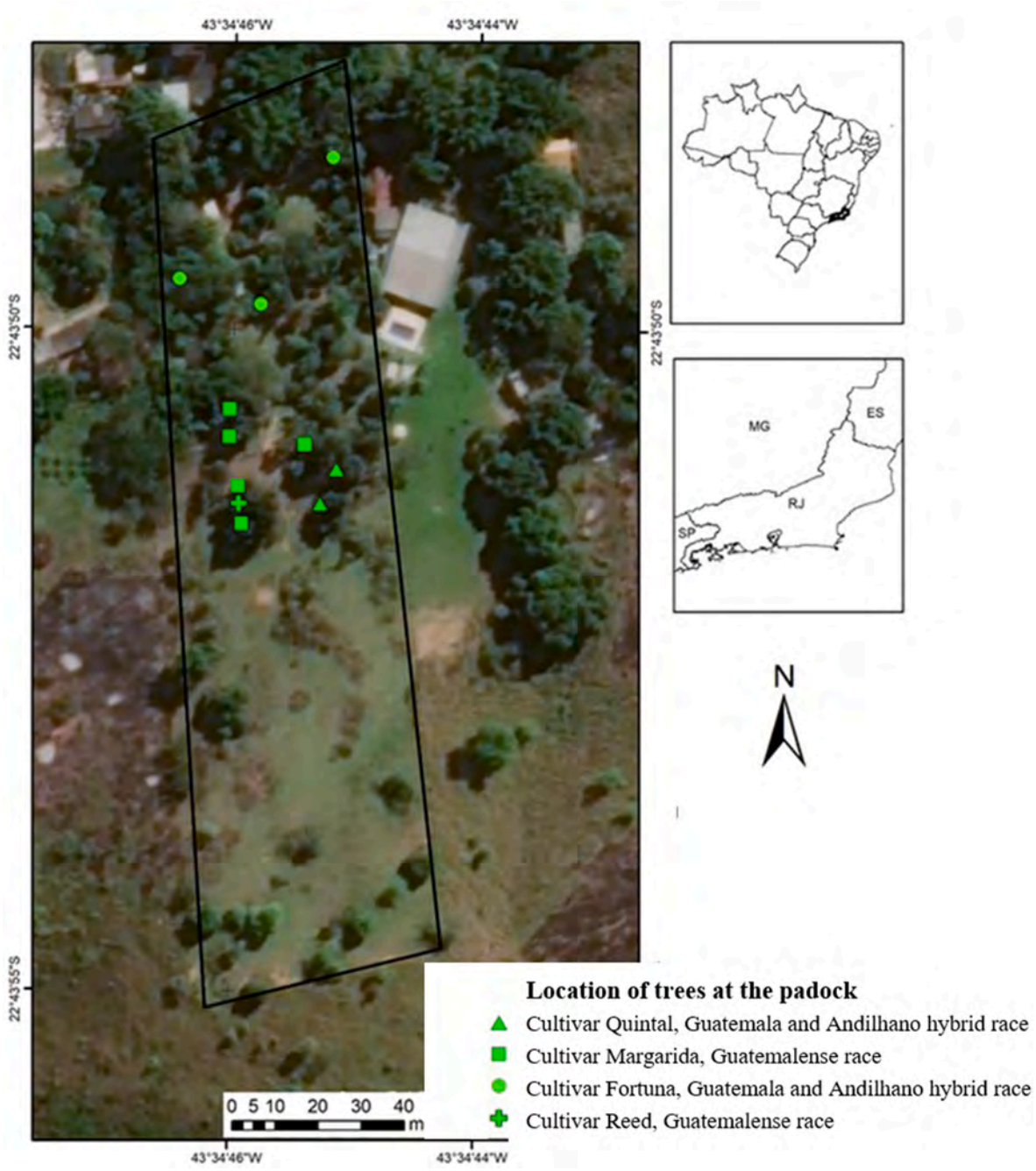

Fig. 2. Paddock for horse pasture with 1,12 ha, where the avocado poisoning occurred, containing eleven avocado trees. The black lines represent the fence, and the green symbols represent the respective avocado cultivar. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

1% safranine in 80% ethanol to better analyze epidermal cells and their appendages. Semi-permanent slides were mounted with glycerin jelly. The slides were evaluated under an optical microscope.

2.3. Necropsy, histopathology, immunohistochemistry, and gastrointestinal ingesta evaluation

One of the two horses that died underwent a *postmortem* examination. The necropsy was conducted by the pathologists of the *Setor de Anatomia Patológica* (SAP) from *Universidade Federal Rural do Rio de Janeiro* (UFRuralRJ). The gastrointestinal ingesta, liver, and kidneys were collected and frozen at −20 °C for further ancillary investigation. The whole heart and the whole brain were collected, as well as fragments of skin, skeletal muscle, trachea, esophagus, lungs, lymph nodes, spleen, kidneys, adrenal, liver, stomach, small and large intestines and pancreas. These organs were fixed by immersion in 10% buffered formalin, processed by routine methods, embedded in paraffin wax, sectioned at 3 μm, and stained with hematoxylin and eosin. The heart was cut into 14 distinct anatomical sections, adapted from racehorse protocol for heart examination (Diab et al., 2017). These heart sections were also submitted to the histochemical Masson's Trichrome technique.

Anti-human troponin C antibody (a-cTnC) immunohistochemistry was performed on the right ventricle, sinoatrial node and atrioventricular node sections (Pavarini et al., 2012a, Cid et al., 2021). The histological sections were incubated for 20 min in 3% hydrogen peroxide.

Antigen retrieval was performed with citrate buffer (pH 6.0) at 98 °C for 20 min. Blocking of nonspecific reactions was achieved with 5% skimmed milk (Molico®, Brazil) for 30 min. The sections were incubated for 2 h at 37 °C with primary anti-human troponin C monoclonal antibody (Novocastra®, clone 1A2) diluted 1:100 in ADS-125 antibody diluent. The biotin-free polymer-HRP detection system (Spring) was revealed with 3,3′-diaminobenzidine (DAB). All sections were counterstained with Harris' hematoxylin and evaluated under an optical microscope.

To demonstrate the ingestion of avocado leaves by the poisoned horse, we collected samples from the gastric and intestinal ingesta during the necropsy. We manually processed the gastric and intestinal ingesta of the horse necropsied and separated all samples similar to avocado leaves. We fixed (Johansen, 1940), hand cut, stained (Bukatsch, 1972), and clarified all samples for evaluation in optical microscopy.

2.4. *Molecular investigation of* P. americana *DNA in the gastrointestinal ingesta*

New and healthy leaves were collected from four avocado varieties (Quintal, Reed, Fortuna, and Margarida) from the horses' paddock. Four species occurring in the equine access area (*Bambusa vulgaris*, *Cynodon dactylon*, *Pennisetum purpureum*) and *Medicago sativa* hay were collected from the smallholding as negative controls. The samples were stored in a thermal box with ice and transported to the Laboratory of Mineral Nutrition of Plants at UFRuralRJ, where they were stored in a freezer (−80 °C). Samples of gastric (CG) and intestinal (CI) ingesta were collected during the necropsy of the horse and stored according to the description above (Fig. 3).

DNA was isolated using the CTAB method (cetyl-trimethyl ammonium bromide) with modifications (Areias et al., 2006). Quantification and analysis of the isolated DNA quality were performed with the Qubit 2.0 fluorimeter (Invitrogen) aid in 1% agarose gel. Microsatellite region (SSR) PCRs were performed for each sample using five pairs of primers (Table 1). Two PCR reactions were performed to improve the identification of DNA in an agarose gel.

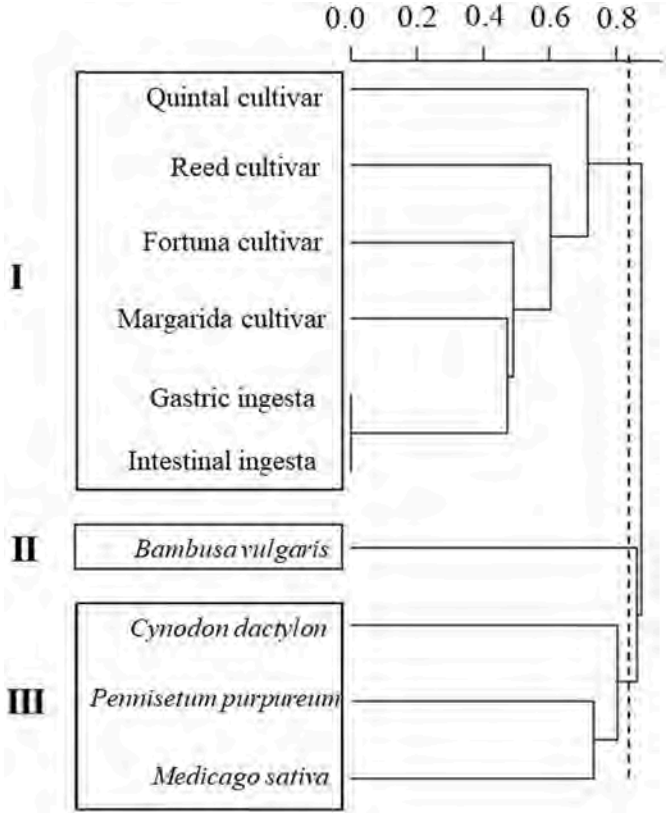

Fig. 3. Dendrogram of genetic dissimilarity, obtained from Jaccard distance and the UPGMA grouping method, as follows: Four varieties of avocado (*Quintal, Reed, Fortuna,* and *Margarida*), four negative controls (*Bambusa vulgaris*, *Cynodon dactylon*, *Pennisetum purpureum*, and *Medicago sativa*), and samples of gastric and intestinal ingesta. Cophenetic correlation coefficient = 0.983.

The first amplification reaction, in 10 μl volume, consisted of PCR buffer (1X), 0.1 mM of each dNTP, 2 mM MgCl, 1 μM of forwarding and Reverse primer, 2 units of Platinum Taq DNA polymerase (Invitrogen, BR), 20 ng of genomic DNA. In the second reaction, 2 μl of the product from the first PCR was used, and the other components were maintained with their respective concentrations, except genomic DNA, which was not added. The conditions for thermal cycling were: 3 min at 94 °C (denaturation), then 35 cycles from 30 s to 94 °C, 30 s to 50 °C (annealing), 15 s to 72 °C (extension), 10 min to 72 °C (final extension).

The amplification products were separated on 2.5% agarose gels, running with TAE (1X), for 3 h at a constant voltage of 100 V. To estimate the size of the amplified fragments (alleles), they were used in the same run electrophoretic two standards of known molecular size, one of 25 bp and the other of 50 bp (DNA Step Ladder, Promega, Madison, USA).

From the analysis of the patterns formed, the binary matrix was assembled by assigning scores based on fragments' presence (1) and absence (0). The genetic distance matrix was calculated using the dissimilarity index of Jaccard (1908). For clustering analysis, the UPGMA algorithm (Unweighted clustering method based on the Arithmetic Mean) was used (Sokal, 1958), and the validation of the graphical representation was determined by the cophenetic correlation coefficient (CCC). The optimal number of groups in the dissimilarity dendrogram was estimated according to Kelley et al., (1996). The consistency of the clustering pattern was verified through the degree of fit between phenetic and cophenetic matrix (Tables 2 and 3) according to Mantel (1967). The software R (R Core Team 2019) was used for these analyses.

2.5. *Chemical investigations from* P. americana *and the gastrointestinal ingesta*

2.5.1. *Sample preparation and extraction*

Leaves of four cultivars (Quintal, Margarida, Fortuna, and Reed), fruits of two cultivars (Margarida and Fortuna) and the gastric ingesta were frozen until the analyses. The fruit samples were submitted for manual separation of pulp, peel and seed. All samples were cut into small pieces using a stainless-steel knife, frozen with liquid N_2 and crushed with mortar and pestle. After that, all samples were lyophilized.

About 2 g of dried samples were weighed and homogenized with acetone (1:5, w/v), sonicated for 5 min and centrifuged at 3000 rpm for 10 min. The supernatant was collected and evaporated under reduced pressure at 30 °C, yielding an acetone crude extract.

2.5.2. *LC–ESI–MS analysis*

All extracts were solubilized in methanol and water (7:3, v/v) at a concentration of 5 mg/mL, then filtered on a 0.22 μm PTFE syringe filter. They were analyzed by an LC-20AD (Shimadzu, Kyoto, Japan)

Table 1
Sequences and annealing temperature of 5 pairs of Avocado SSR primers with their locus names.

Locus[a]	Forward (5′–3′)	Reverse (5′–3′)	T (°C)[b]
AVAG06	CGACCTCTTCTTATACTC	GTACCTCTGATAATGAGCAT	50
AVAG10	GAATTACAAAGCACTAGAG	GTAGAAAGTGGGCACACAT	50
AVAG11	AGCGATGAACATTACCA	ATTTCTTCAACCCATCTGTC	50
AVAG13	TGCGATAACAACTGGAC	AACTAGGACCTGAAACCG	50
AVAG25	ATGGTTTTTTCCTGCCCTTT	AACAAGCCCCCTAAAAGAA	50

[a] Sharon et al. (1997).
[b] Temperature.

Table 2
Phenetic matrix, obtained from dissimilarity index of Jaccard, showing the original genetic distance between the samples.

	Gastric ingesta	Intestinal ingesta	Quintal	Margarida	Fortuna	Reed	*Bambusa vulgaris*	*Pennisetum purpureum*	*Cynodon dactylon*
Intestinal ingesta	0.00								
P. americana Quintal cultivar	0.734	0.734							
P. americana Margarida cultivar	0.471	0.471	0.620						
P. americana Fortuna cultivar	0.500	0.500	0.734	0.471					
P. americana Reed cultivar	0.577	0.577	0.756	0.548	0.707				
Bambusa vulgaris	0.905	0.905	0.894	0.920	0.905	0.913			
Pennisetum purpureum	0.877	0.877	0.829	0.845	0.877	0.886	0.866		
Cynodon dactylon	0.856	0.856	0.894	0.874	0.856	0.866	0.845	0.775	
Medicago sativa	0.886	0.886	0.791	0.856	0.886	0.894	0.877	0.734	0.840

Table 3
Cophenetic matrix, obtained from the clustering methodology via UPGMA, showing the genetic distance obtained between the samples.

	Gastric ingesta	Intestinal ingesta	Quintal	Margarida	Fortuna	Reed	*Bambusa vulgaris*	*Pennisetum purpureum*	*Cynodon dactylon*
Intestinal ingesta	0.00								
P. americana Quintal cultivar	0.716	0.716							
P. americana Margarida cultivar	0.471	0.471	0.716						
P. americana Fortuna cultivar	0.490	0.490	0.716	0.490					
P. americana Reed cultivar	0.602	0.602	0.716	0.602	0.602				
Bambusa vulgaris	0.877	0.877	0.877	0.877	0.877	0.877			
Pennisetum purpureum	0.877	0.877	0.877	0.877	0.877	0.877	0.863		
Cynodon dactylon	0.877	0.877	0.877	0.877	0.877	0.877	0.863	0.807	
Medicago sativa	0.877	0.877	0.877	0.877	0.877	0.877	0.863	0.733	0.807

coupled with a diode array detector and ESI-qTOF mass spectrometer (MicrOTOF-Q III, Bruker Daltonics, Billerica, USA). The chromatographic column used was a Kinetex® C-18 (2.6 μm, 150 × 2.2 mm, Phenomenex®) protected by a precolumn packed with the same material, which was in an oven with the temperature set at 50 °C. The mobile phase was ultrapure water (solvent A) and acetonitrile (solvent B), both mixed with 0.1% formic acid (v/v), and the gradient elution profile was as follows: 0–2 min, 3% of B; 2–25 min, 3–25% of B; and 25–40 min, 25–80% of B, followed by washing and reconditioning of the column (8 min). The flow rate was 0.3 mL min^{-1}, and the injection volume was 1 μl. The analysis was performed by monitoring the ultraviolet (UV) wavelength between 240 and 800 nm and a mass spectrometer operating in negative and positive ion modes (*m/z* 120–1200). The capillary voltage applied was 4500 Kv, and nitrogen was employed as the nebulizer gas (4 Bar) and drying gas (9 L min^{-1}). Raw data were processed in DataAnalysis 4.2 (Bruker Daltonics, Billerica, USA).

Peaks with *m/z* 351.250 (avocadene 1–acetate – $[C_{19}H_{36}O_4Na]^+$) and 403.281 (persin – $[C_{23}H_{40}O_4Na]^+$) were integrated, and the peak area was adjusted to obtain a value that expressed area/g of dry material. A heatmap was plotted in Metaboanalyst 5.0.

2.6. Electrocardiogram and echocardiogram of four horses that consume P. americana

Eleven to 17 months after the postmortem diagnosis of avocado poisoning in a herd-mate, 4 other horses kept in the paddock with 11 *P americana* trees underwent electrocardiographic evaluation. For 15 min, an InCardio® digital electrocardiograph (Florianópolis, Santa Catarina, Brazil) was used for examination. The electrodes were fixed to the skin by "alligator" clips, with alcohol applied to the fixation points. The tracing was recorded and standardized with a sensitivity of 1 mV at a speed of 50 mm/s for all leads. To record the frontal plane leads, DI, DII, DIII (bipolar leads), AVR, AVF and AVL (enlarged unipolar leads), the electrodes were attached to the animal's skin in the region of the olecranon in the thoracic limbs, and of the patella, in the pelvic limbs. To record the pre-cordial leads, the electrodes were fixed in the sixth lower right intercostal space (at the area of the costochondral junction, V1); sixth high right intercostal space (at the area of the scapulohumeral joint, V3); sixth low left intercostal space (V2); sixth high left intercostal space (V4) and in the spinous process of the seventh thoracic vertebra (V10) (Fregin, 1982). The results obtained were evaluated according to the reference values for the species (Van Loon and Patterson, 2010).

The same horses underwent echocardiography examination 17 months after the death and postmortem diagnosis of avocado poisoning in the herd-mate. The equipment was Vivid IQ 4D by GE Heathcare® (São Paulo, São Paulo state, Brazil) with a GE 3 S–RS probe sector. B–mode, Doppler, and M–mode echocardiograms were performed systematically for a complete echocardiographic examination (Schwarzwald, 2019).

2.7. Serological biochemistry of four horses that consume P. americana

Serum samples were collected from the surviving herd-mates at one, eleven, and twenty-three months after the deaths occurred. The twelve serum samples were examined by chemiluminescence to detect Tropin–1 cTnI (Rossi et al., 2014). To assess kidney functions, serum urea and creatinine were analyzed (Câmara et al., 2022). Liver function was evaluated by measuring the serum enzymes aspartate aminotransferase and creatine kinase.

3. Results

3.1. Animals and clinical–epidemiological data

It was common to observe horses consuming the leaves and fruits of *P. americana*, all six horses in the 2-ha smallholding. Two horses died, and four other horses were still held in the 1.12-ha paddock containing eleven avocado trees. The two horses that died had been on the property for 3 and 20 years. The four remaining horses had been on the farm for 4–20 years. These often fed on the aerial leaves of the trees (Supplementary Material 1) (Fig. 4), as well as mature fruit pulp and peel on the ground. The diet of these horses was composed of two meals of 1.5 kg of

Fig. 4. Fruits of one avocado tree (Fortuna Cultivar, Guatemalan and Andean hybrid race) producing avocado poisoning in horses.

concentrate per day and forage *ad libtum*, composed of *Paspalum notatum* grass, *Cynodon dactylon* hay and *Medicago sativa* hay.

Two 15-year male horses, Brazilian Marchador breed, died after a clinical evolution of about 15 days with a similar clinical picture of apathy and inappetence. The horse's clinical evaluation demonstrated congested mucous membranes, increased capillary filling time, tachycardia, dyspnoea, and cold extremities. During pulmonary auscultation, thick crackles and areas of auscultatory silence were found. Treatment for possible pleuropneumonia was established using antibiotic therapy with penicillin (Agrovet; Novartis; Brazil, 20,000UI kg/IM) and gentamicin (Gentatec; Chemitec, Brazil; 6,6 mg kg/IV). Supportive treatment also was applied with fluid therapy (Ringer's Lactate; JP Farma; Brazil; 40 mL/kg IV), mucolytic bromhexine hydrochloride (Aliv V; Agener; Brazil; 70mg/animal/IM), dimethylsulfoxide (DMSO; Fort Dodge Animal Health; 500 mg kg/IV), and flunixin (Banamine; Schering–Plough Animal Health Canada, QC, Canada; 1.1 mg kg/IV). The horse was treated for one week and showed improvement in the general clinical picture. Thirteen days after the beginning of the treatment, the horse usually fed with concentrate and was released with other animals and died during the morning.

3.2. Botanical identification

All eleven trees were identified as *Persea americana*, (Lauraceae, Mill.). The samples sent to the RBR Herbarium from UFRuralRJ were numbered 46,861 to 46,865.

The cultivars and race of samples sent to the CGMS herbarium were identified: Two of eleven trees were of the cultivar Quintal, Guatemalan and Andilhano hybrid race; five were the cultivar Margarida, Guatemalan race; three were the cultivar Fortuna, Guatemalan and Andilhano hybrid race, and one was the cultivar Reed, Guatemalan race.

All horses of this farm had free access to leaves and fruits and fed naturally on the four different cultivars and races of *P. americana*.

3.3. Necropsy, histopathology and immunohistochemistry

The gross evaluation of the horse showed notable morphologic alterations in the heart (Table 4). Multifocal to coalescing white fibroplasia areas were seen in the epicardium (Fig. 5A–C), myocardium (Fig. 5DE) and endocardium (Fig. 5F). In the epicardium and myocardium, multifocal mottled darkened hemorrhage areas interspersed with white fibrosis areas were seen. Changes in the kidney were also found, with red multifocal punctiform areas, varying between 0.1 and 0.3 cm (petechiae). At the cut surface, multifocal petechiae were found in the cortical region. Grossly, no significant alterations were observed in the other organs of this horse.

Multifocally, in the histological horse heart sections, a severe number of necrotic cardiomyocytes with pycnotic nuclei and hypereosinophilic sarcoplasm were associated with marked proliferation of fibroblasts and abundant collagen deposition (Fig. 6A). The proliferated fibroblasts and collagen deposits were interspersed with cardiomyocytes and the extracellular space of epimysium and perimysium. A moderate multifocal lymphohistiocytic infiltrate interspersed with muscle fibers with rare multinucleated giant cells was noted. In histology sections of the sinoatrial node, atrioventricular node, and the interventricular septum at the level of the His bundles, there was a severe number of necrotic and degenerate Purkinje cells, often interspersed by a large number of fibroblasts (Fig. 6BCE).

In the lung sections, there was marked interstitial fibroplasia. There was multifocal congestion and significant extravascular red blood cells (hemorrhage). The alveolar septa were multifocally distended by a moderate number of lymphocytes, plasma cells, and histiocytes. The kidney has moderate multifocal interstitial fibroplasia and multiple foci, sometimes coalescing, with a marked lymphohistiocytic infiltrate. No

Table 4
Mapping of histological lesions in the heart.

	Fibrosis			Necrosis	Edema	Fibroplasia and Collagen	Inflammation			
	Endocardium	Myocardium	Epicardium				Lymphocytes	Histiocytes	Giant cells	Plasma cells
Interventricular septum (LV)	++	+++	–	+++	++	+++	+	++	+	+
Apex	–	+	++	+	+++	++	++	++	–	–
Left papillary muscle (LV)	++	+++	–	++	+++	+++	++	++	–	–
Right papillary muscle (LV)	+++	+++	–	+++	+++	+++	+++	++	–	+
Coronary artery	–	–	–	–	++	+	–	+	–	–
Left atrium	+++	+++	+++	+++	+++	+++	+++	+++	–	+
Left ventricle	–	+	–	–	+	+	+	+	–	–
Left auricle	+++	++	+	++	+	++	++	++	–	+
Bicuspid	+++	++	–	+	++	+++	+++	+++	–	+
Right ventricle	–	+++	–	+++	++	+++	+++	+++	+	+
Interventricular septum	–	–	–	++	++	++	+++	+++	+	+
Sinoatrial node	++	+++	+	++	++	+++	+++	+++	+	+
Atrioventricular node	+++	+++	–	+++	+	+++	+	+	–	–
Right atrioventricular valve	+	+++	–	+++	++	+++	+++	++	–	–

*Anatomical sections evaluated, adapted from racehorse protocol for heart examination (Diab et al., 2017). (–) Absent; (+) Mild; (++) Moderate; (+++) Severe.

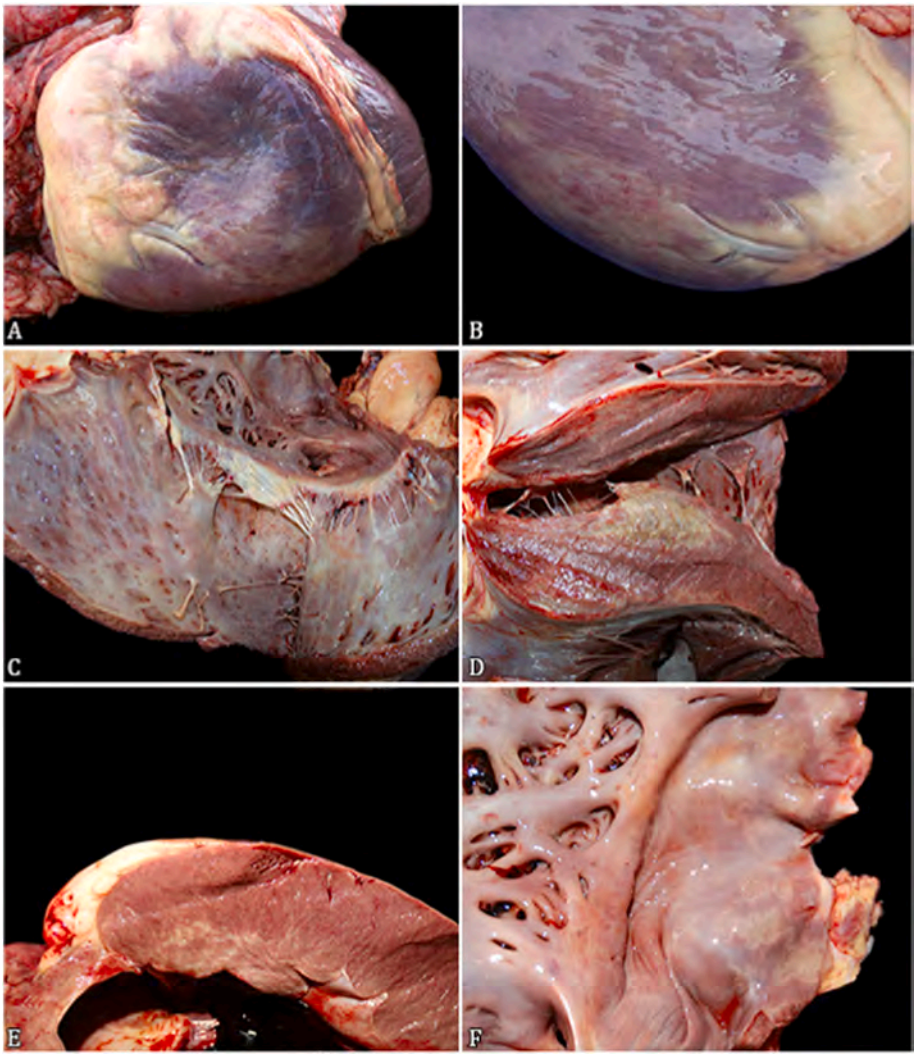

Fig. 5. Gross findings in the heart of a horse poisoned by avocado. (**A**) Locally extensive white stretch marks are shown in the epicardium of the right ventricle (fibroplasia). (**B**) Locally extensive fibroplasia and multifocal hemorrhage in the epicardium of the left ventricle. (**C**) Multifocal to coalescing white moted endocardium in the right ventricle (fibroplasia). (**D**) Focal myocardial white area fibroplasia of papillary muscle of left ventricle. (**E**) Multifocal to coalescing myocardial fibroplasia in the left ventricle wall. (**F**) Multifocal white areas in the endocardium of the left atrium (fibroplasia).

significant changes were seen in the other organs at microscopic evaluation.

The immunohistochemistry showed a decrease or the negative expression for the anti-Troponin C human antibody (a–cTnC) in the sarcoplasm of the cardiomyocytes in all sections of the right ventricle, sinoatrial node and atrioventricular node. The immunoexpression varied in levels of decreased and moderate. The sections of the sinoatrial node and right ventricle showed an immunoexpression total loss of about 70% (Fig. 6D). In other areas of the right ventricle and atrioventricular node, almost 40% of total loss immunoexpression of anti–cTnC was seen.

3.4. *Anatomy of leaves from gastrointestinal ingesta*

The gastric and intestinal ingesta showed that leaf samples collected from both intestinal and gastric ingesta match the anatomical features present in the avocado leaves (Fig. 7A–H). All analyzed leaves were hipostomatic with normocytic stomata (Fig. 7A–D). The epidermis was uniseriate (Fig. 7E), formed by common cells with sinuous shape (Fig. 7A–D) and unicellular non-glandular trichomes only on the abaxial surface. The mesophyll was dorsiventral (Fig. 7E and F) with several oil cells (idioblasts) widely spread in the spongy (Fig. 7G) and palisade (Fig. 7H) parenchyma. The vascular bundles were collateral (Fig. 7F).

3.5. *Molecular investigation of* P. americana *DNA in the gastrointestinal ingesta*

The cophenetic correlation coefficient, obtained between the phenetic and cophenetic matrices, was equal to 0.98, significant at 1% probability by the Mantel test. The high correlation observed indicated a good fit between the matrices and, consequently, a low distortion between the values of the initial dissimilarity matrix and the derived dendrogram. The estimate of the cophenetic correlation varies from zero to one, and the closer to one, the more representative the dendrogram is concerning the matrix of genetic distances (Cruz et al., 2011). Cophenetic correlation values above 0.8 indicate a good fit between the original distance matrices and the resulting cluster analysis.

The formation of three optimal groups was observed in the genetic dissimilarity dendrogram (Fig. 3), according to the Kelley–Gardner–Sutcliffe algorithm (Kelley et al., 1996). Group I, the largest group, was formed by six of the ten samples analyzed (60%), group II by one sample (10%) and group III by three samples (30%). It is important to note that the gastric and intestinal ingesta samples were grouped into a separate group (Group I) along with the four varieties of avocado present on the property. The negative control samples were distributed in the other two groups (Groups II and III).

The genetic dissimilarities among the ten analyzed samples ranged between 0.471 and 0.877, with 0.764 being the estimated value of the mean genetic distance. No genetic distinction was observed between

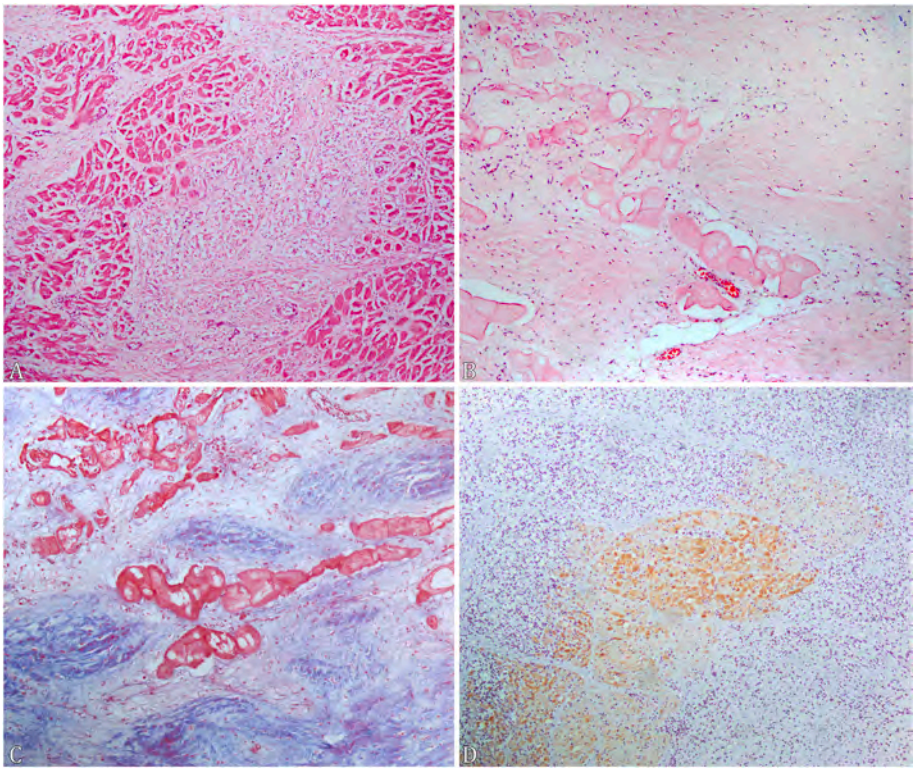

Fig. 6. Histopathology and immunohistochemistry of the heart. (**A**) Focally extensive myocardial fibroplasia that dissects the epimysium and perimysium. (**B**) Sinoatrial node with Purkinje fibers surrounded by fibroplasia and collagen deposition. HE, obj. 20x. (**C**) Sinoatrial node with Purkinje fibers surrounded by fibroplasia and collagen deposition. Masson Trichrome, obj. 20x. (**D**) Right ventricle myocardium with negative or decreased expression of anti–Troponin C antibody (a–cTnC). The negative or decreased expression indicates cardiomyocyte lesion. Immunohistochemistry using primary a–cTnC, obj 40x.

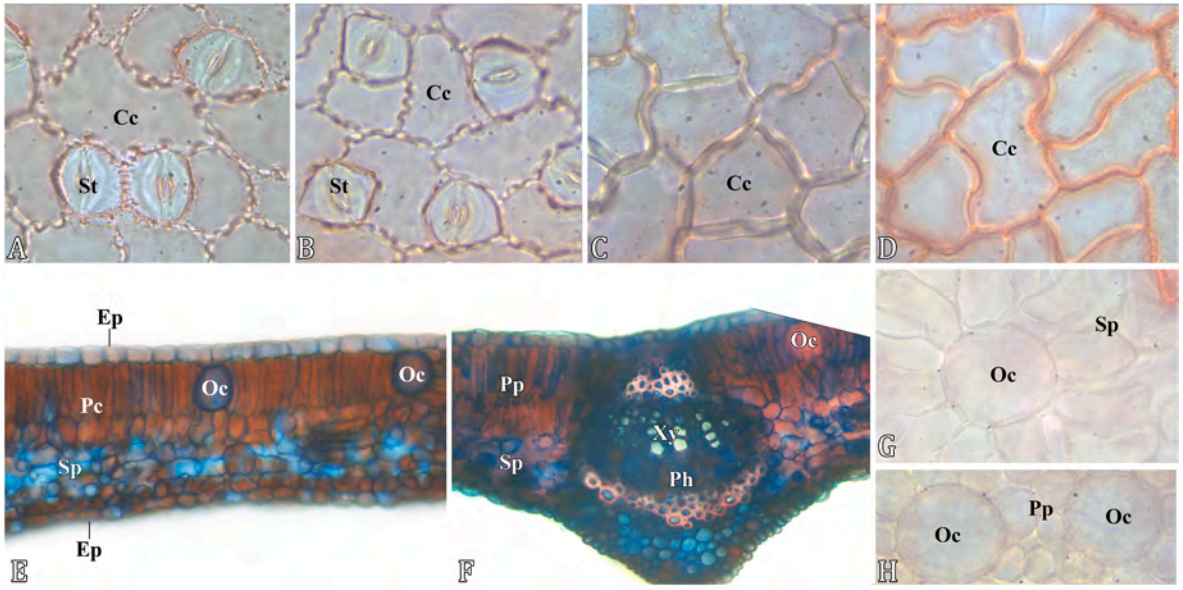

Fig. 7. Photomicrographs of *Persea americana* Mill. Leaf blades collected from tree (**A, C**), gastric ingesta (**B, D, F, H**) and intestinal ingesta (**G**). Frontal view of common epidermical cell and stomata under abaxial (**A, B**) and adaxial (**C, D**) leaf surfaces. Cross–section of the leaf blade showing uniseriate epidermis, palisade and spongy parenchyma, and oil cell (**E**). Detail showing palisade, spongy parenchyma, oil cell, and a collateral vascular bundle (**F**). Detail showing oil cell in the spongy (**G**) and palisade (**H**) parenchyma. Cc: common cell; Ep: epidermis; Oc: oil cell; Ph: phloem; Pp: palisade parenchyma; Sp: spongy parenchyma; St: stomata; Xy: xylem.

gastric and intestinal ingesta samples, showing a genetic similarity of 100% between the alleles identified in these two samples. It was observed that, compared to the other samples analyzed, the samples of gastric and intestinal ingesta were less dissimilar to the variety Margarida (0.471), showing more significant similarity between the DNA found in the gastric and intestinal ingesta with the DNA of the avocado variety Margarida.

3.6. *Chemical investigation from* P. americana *and the gastrointestinal ingesta*

The LC–ESI–MS analysis of the extracts from the avocado plant parts (fruits (seed, peel, and pulp) and leaves) and gastric ingesta of the horse revealed two annotated acetogenins avocadene 1–acetate and persin. These substances showed sodiated ion at *m/z* 351.2506 and 403.2819, which are compatible with molecular formulas $C_{19}H_{36}O_4$ and $C_{23}H_{40}O_4$

Table 5
Annotation of acetogenins present in the extracts of leaves, pulp, peel and seed of avocado and the gastric ingesta of the dead horse.

Compound	Molecular formula	Measured (*m/z*) $[M+H]^+$	Calculated (*m/z*) $[M+H]^+$	MS/MS (*m/z*)
Avocadene 1–acetate	$C_{19}H_{36}O_4$	329.2682	329.2686	269, 251, 233, 191, 177, 163, 149
Persin	$C_{23}H_{40}O_4$	381.3007 403.2819 $[M+Na]^+$	381.2999 403.2818 $[M+Na]^+$	– 363, 321, 303

(Table 5). They revealed a similar fragmentation profile in the MS/MS, exhibiting losses of water (18 *u*) and ketene molecules (42 *u*), suggesting the presence of aliphatic hydroxyl and acetyl groups in the structure, respectively. For example, the acetogenin avocadene 1–acetate showed fragment ions at *m/z* 269 $[M + H–H_2O–CH_2CO]^+$ and 251 $[M + H–2xH_2O–CH_2CO]^+$. The annotation was done based on the molecular formula obtained from the accurate MS data and the comparison with the ion fragmentation pattern reported by Rodríguez-Sánchez et al., (2013). The compound avocadene 1–acetate was found in all samples, and higher intensities were observed from fruit pulp extracts. On the other hand, the acetogenin persin was found in leaves, seeds and pulp, but it was marked with higher intensity in leaves of the variety Fortuna and Reed, in seeds of varieties Margarida and Fortuna and the pulp of variety Fortuna (Fig. 8). This compound was absent in fruit peel and gastric ingesta.

3.7. *Electrocardiogram and echocardiogram of four horses that consume* P. americana

Studies made at 11 months *after the first diagnosis of the poisoned horse by P. americana.* One 5-year–mare (Horse 4) presented mild alterations, standard T and P wave morphology, but elevated ST segment in DIII and concave wave. The electrocardiogram of the other three horses had no physiological abnormalities.

Studies made at 17 months *after the first diagnosis of the poisoned horse by P. americana.* The electrocardiograph of four horses had no physiological abnormalities. One 23-year-horse (Horse 3) presented mild echocardiographic alterations, compatible with systolic function with a contractile force at the lower limits of normal (26%), that is, mild systolic dysfunction in the subjective assessment. The doppler echocardiographic assessment reveals mild echocardiographic alterations, compatible with slight impairment of systolic function. The echocardiography of the other three horses had no abnormalities.

3.8. *Serological biochemistry of four horses that consume* P. americana

All eight serum samples collected from the four horses eleven and seventeen months after the first death due to *P. americana* poisoning resulted in the normal rates of Troponin 1 (cTnI). Three out of four serum samples collected from the four horses twenty-three months after the first death resulted in elevated Troponin 1 (cTnI). The results ranged from 0.2 to 0.3 ng/mL (Table 6). The reference value is < 0.04 ng/mL (Rossi et al., 2014).

Serum chemistry to evaluate liver and kidney function revealed normal values of aspartate aminotransferase, creatine kinase, urea and creatinine.

4. Discussion

We investigated a chronic clinical picture in horses with severe necrosis and fibroplasia of the myocardium associated with a long-term *P. americana* consumption, presumably at low doses. Our results are highly suggestive, of avocado toxicity as a cause of chronic heart disease in horses. The cardiotoxicity of avocado has been reported as the cause of acute cardiac damage in mammals (Sani et al., 1991; Grant et al., 1991; Buoro et al., 1994; Craigmill et al., 1989; Aguirre et al., 2019). The acquired myocardial injuries in horses often lead to degenerative and necrotic changes in the cardiomyocytes (Buergelt, 2003).

Susceptible animal species may die suddenly after ingesting a few leaves, whereas others may show few ill effects (Robison and Robison 2016). The botanical varieties can influence the toxicity to animals and the signs and clinical manifestation (Buoro et al., 1994). We have confirmed by LC–ESI–MS analysis that the acetogenins, avocadene 1–acetate and persin, from all avocado investigated leaves and fruits (seed, peel, and pulp) from all four cultivars. We have highlighted that the cultivar Reed has higher levels of the acetogenin persin from leaves. In comparison, the acetogenin avocadene 1–acetate was annotated in higher levels from fruit pulp extracts from Margarida and Fortuna. For instance, the cultivar Fuerte of *P. americana* has been associated with cardiac failure syndrome in goats and sheep (Sani et al., 1991; Grant et al., 1991), while mastitis was associated with poisoning by the

Table 6
Serum evaluation of Troponin– 1 cTnI from four horses that had access to the avocado trees.

Horse	Troponin 1 (cTnI) ng/ml April 30, 2021[a]	Troponin 1 (cTnI) ng/ml November 19, 2021	Troponin 1 (cTnI) ng/ml April 22, 2022
1	<0,001	0007	0,01
2	<0,001	0004	**0,3**
3	0,003	0011	**0,2**
4	<0,001	<0,001	**0,2**

[a] Reference value is < 0.04 ng/mL.

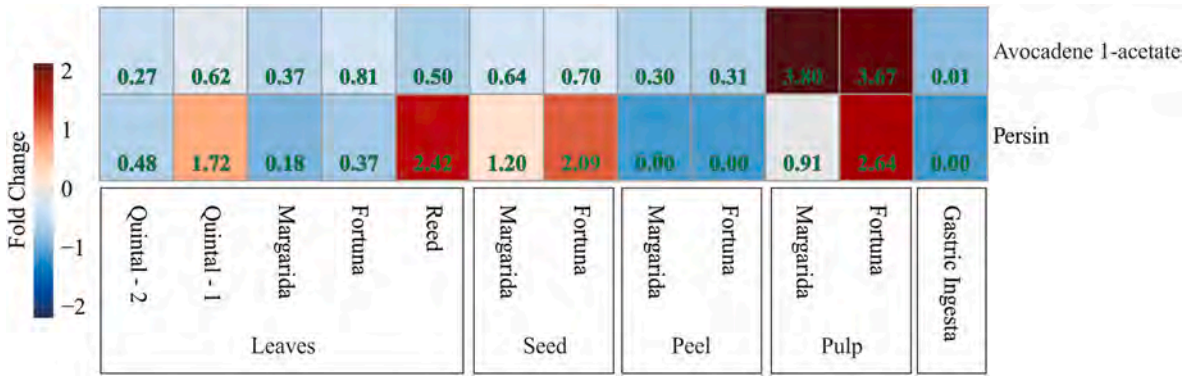

Fig. 8. Heatmap with the distribution pattern of acetogenins present in the leaves, pulp, peel and avocado seed extracts and the dead horse's gastric ingesta. The values in the cells represent the fraction: individual peak area/mean peak area for each compound.

Guatemalan race (Craigmill et al., 1984; Robison and Robison, 2016).

In sheep and goats (Stadler and Van RensburgNaude, 1991), and horses (McKenzie and Brown 1991), there are reports of *P. americana* poisoning associated with necrohemorrhagic mastitis. Furthermore, myocardial necrosis has been seen in cases of avocado poisoning in dogs (Buoro et al., 1994), rabbits (Aguirre et al., 2019), goats (Grant et al., 1991), and ostriches (Burger et al., 1994). The clinical signs reported of avocado poisoning in horses include colic, non-infectious mastitis, varying degrees of dyspnoea, and edematous swelling of the head and pharynx (McKenzie e Brown, 1991). In our case, the absence of edema in soft tissues at horse necropsy can be attributed to previous clinical treatment with dimethylsulfoxide and flunixin.

In general, lesions of avocado poisoning in animals consist of variable severity endocardial hemorrhage, especially of the papillary muscles, and cardiomyocyte necrosis (Robison and Robison, 2016). The exact mechanism of heart failure due to *P. americana* poisoning remains unsolved (Oelrichs et al., 1995). Acetogenins are natural secondary metabolites derived from fatty acids like avocadene 1–acetate and persin in avocado (Rodriguez–Saona et al., 1998). An experimental study in adult rats demonstrated that the cardiotoxicity of *P. americana* enriched extract (means acetogenin) occurs due to an inhibition of complex I and opening of the permeability transition pore of the mitochondria, which leads to the leaking of important metabolic and proapoptotic components (Silva–Platas et al., 2012). The authors describe the loss of mitochondrial membrane potential, inhibiting fatty acid oxidation, and reactive oxygen species production in the tissues. Presumably, these mitochondrial effects play an essential role in cardiotoxicity in horses and need to be better elucidated. Considering all evidence raised by these studies, we agree that acetogenin poisoning in horses is an example of toxicity by secondary plant metabolite (Pavarini et al., 2012b).

The acetogenin compound avocadene 1–acetate was also found in the gastric ingesta but in lower levels compared to fresh leaves. We did not find persin in the gastric ingesta of the dead horse, but it was found in the leaves and fruit pulp of the cultivars Margarida and Fortuna, which showed the presence of acetogenin persin in the injested avocado. Thus, persin was probably absorbed or chemically transformed due to gastric pH or diluted (below the detection analytical capacity) by the ingesta.

Although we have found eleven trees from four *P. americana* cultivars at the farm, it is impossible to track horse ingestion predominance of any single cultivar based on the leaf anatomy data from gastric and intestinal ingesta. On the one hand, the anatomical characteristics certainly do not vary between cultivars, and on the other hand, this examination is conclusive and means ingestion of *P. americana* in cases of rabbit poisoning (Aguirre et al., 2019).

As Aguirre et al. (2019) suggested, in this case, the microhistological examination of ingesta was considered a conclusive means of proving *P americana* exposure in rabbits. In addition, we performed the molecular amplification of *P. americana* DNA in the gastrointestinal ingesta as an auxiliary method to determine the cause of the poisoning. In this study, the presence of avocado DNA was verified both in the gastric and intestinal ingesta samples. Furthermore, according to the UPGMA grouping, the Margarida variety showed more remarkable similarity with the gastric and intestinal ingesta samples, suggesting that, although the animal had access to all avocado varieties present on the property, the Margarida variety was overrepresented in the gastric and enteric contents of the horse examined. Molecular tools have been recently used to investigate animal and human-plant poisoning (Nithaniyal et al., 2021).

Despite the lack of consistent alteration in the electrocardiogram and echocardiogram exams, three out of four horses demonstrated elevated serum troponin cTnI 24 months after the first *P. americana* poisoning case. Troponin cTnI is a highly sensitive biomarker of injury of the myocardium in dogs and humans (Mair et al., 1996; Ricchiuti et al., 1998). In race-training thoroughbred horses, the reference range of troponin 1 (cTnI) is 0.11 ng/mL (Phillips et al., 2003). An increase in serum troponin is a consequence of cardiomyocyte injury. We hypothesized that this increase in three horses after long-term *P. americana* ingesting means an initial clinical picture of acetogenin poisoning. These horses don't have any cardiac clinical signs, despite the elevated serum troponin 1 (cTnI). Human serum troponin testing is highly sensitive to myocardial injury (Hodgson et al., 2022). Increased serum cTnI concentration in a horse with multifocal areas of acute myocardial necrosis was reported (Cornelisse et al., 2000). Few studies have reported increased cTnI serum concentration in horses with cardiac lesions.

Furthermore, some studies showed that leaf extracts of *P. americana* have potent hypotensive and antihypertensive pharmacological activities (Owolabi et al., 2005; Ojewole et al., 2007). Ischemic cardiomyopathy develops secondary to the impedance of blood flow to the energy-dependent cardiomyocytes, with heart failure developing in the setting of significant or persistent interruption or decreased perfusion (Sekulic et al., 2019). The cardiomyocyte necrosis in this horse may be due to prolonged exposure to active hypotensive biomolecules that can lead to a hypoxia microenvironment. The same systemic hypotensive mechanism may be related to kidney injuries in this case. A progressive decrease in blood flow contributes to hypotension and renal failure (Robison and Robison 2016).

Marcus and Ross (1967) described progressive changes in hearts of aged horses and myocardial noninflammatory fibrosis in 18 horses. However, we discard this hypothesis due to marked myocarditis in the horse naturally poisoned by avocado since inflammation did not appear in age-related fibrosis cases by these authors. Plants that contain cardiac glycosides of both the bufadienolide and cardenolide types are well known. Poisoning by cardiac glycoside-containing plants may be acute or chronic in many animal species (Robison and Robison, 2016). *Nerium oleander* is a worldwide plant reported to cause sudden death by cardiac failure in horses (Hughes et al., 2002; Renier et al., 2013). Plants containing cardiac glycosides most commonly associated with poisoning in livestock are oleander (*Nerium* spp.), milkweed (*Asclepias* spp.), and foxglove (*Digitalis* spp.) (Galey, et al., 1998; Woods et al., 2007). There was no evidence of the presence or consumption of those plants by the horses in the present study.

Furthermore, degenerative and necrotizing lesions in the myocardium are associated with ionophores poisoning, mainly monensin, lasalocid, narasin and salinomycin (Nogueira et al., 2009). There are variations in the susceptibility to the effect of ionophores in different animal species (equids, rabbits, sheep, swine, horses, dogs and poultry). For horses, ionophores are highly toxic (Nogueira et al., 2009; Robison and Robison, 2016). No ionophores were labeled in the specific ratio for horses on this farm.

Avocados are often grown on farms along with animal husbandry throughout Brazil (Carvalho et al., 1983; Pio, 2020). So it is uncertain how many animals are exposed to the possible poisoning by avocado. The results presented here can help practitioners and pathologists diagnose poisoning by *P. americana* in horses. Chronic avocado poisoning should be included as a cause of myocardial necrosis and fibroplasia in horses. The diagnosis of avocado poisoning in horses should be based on epidemiological, clinical, pathological, and ancillary methods. To avoid spontaneous cases of avocado poisoning in horses, we suggest limiting access to this plant and not providing *P. americana* in the diet. We also encourage the use of auxiliary methods to determine the diagnosis of poisoning in horses, such as botanical leaf microhistology of gastrointestinal ingesta, molecular analyses of gastrointestinal ingesta and chemical analyses to detect acetogenin compounds.

Credit author statement

Marina S. Freitas, Asheley H.B. Pereira, Daniel G. Ubiali: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing–reviewing and editing.; **Asheley H.B. Pereira:** Writing–original draft preparation.; **Gabriela O. Pereira, Islaine S.**

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Ethical statement

The submission of this manuscript is ethically coherent and respected all aspects of network of knowledge. Thank you very much for considering this manuscript for publication.

Ethics approval

The Ethical Committee of Animals Use (CEUA) by the Federal Rural University of Rio de Janeiro (UFRuralRJ) has approved this clinical toxicology field study with a number protocol 2824200521/2021. The authors were responsible for the welfare of horses. All animals' procedures have complied with the Ethical Principles in Animal Research adopted by the College of Animal Experimentation (CONCEA).

Consent for publication

The horse breeder authorized this clinical toxicology field study.

Data availability. The datasets used and analyzed during the current study are available from the corresponding author upon request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2022.09.007.

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