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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

MYLENA ANDRÉA OLIVEIRA TORRES

Eficácia da *Morinda citrifolia* (noni) no tratamento de cães com sintomatologia neurológica infectados pela *Ehrlichia canis* e pelo vírus da cinomose

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia, com sede na Universidade Federal Rural de Pernambuco e ponto focal na Universidade Federal do Maranhão, como requisito para obtenção do Título de Doutor em Biotecnologia.

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Orientadora: Profa. Dra. Ana Lúcia Abreu Silva

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“O impossível reside nas mãos inertes daqueles que não tentam”.

(Epicuro)

"Aos meus amados filhos: **Bruna**, **Natália** *e* **Vinícius**, *pelo amor incondicional e por serem a razão da minha existência! Meu amor por vocês é imensurável!*
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LISTA DE ABREVIATURAS E SÍMBOLOS

% - por cento

µg/mL - micrograma por mililitro

ALT - alanine transaminase

ANOVA -analise de variância

AST - aspartato aminotransferase

BBB - barreira hematoencefálica

BHK - células de rim de hamster

BID - duas vezes ao dia

CB_1 - canabinóide 1

CB_2 - canabinóide 2

CD8 + - grupamento de diferenciação

CDV - vírus da cinomose canina

CDV/DV_2-12 - Anticorpo do CDV

COX_2- ciclooxigenase 2

E_2- prostaglandina E_2

EDTA- ácido etilenodiamino tetra-acético

EGFR- fator de crescimento epidérmico

ELISA- ensaio de imunoabsorção enzimática

FOXO1- fator de transição

g/kg- grama por quilograma

GABAa- ácido gama-aminobutírico

HCT-116 - Linhagem celular do carcinoma colorretal humano

Hela - carcinoma cervical humano

$Hepg_2$ - carcinoma hepatocelular humano

Her_2 - receptor do fator de crescimento epidérmico

HIF-1α- fator induzido por hipóxia 1

HIV- vírus da imunodeficiência humana

HT-29 - adenocarcinoma no cólon humano

IFAT - imunofluorescência indireta

IFI - imunofluorescência indireta

IFN-ɤ- interferon ɤ

IGg - imunoglobulina

IHQ - imunohistoquimica

IL - interleucina

KB - carcinoma epidermoide humano

KG- kilogramas

LAN5- células de melanoma B16 – F10

LDL- colesterol

LLC- carcinomatose peritoneal pulmonar de Lewis

LOOH- hidroperóxido lipídico

LPS- lipopolissacarídeo

MCF7- adenocarcinoma mamário

MDA-MB-231- carcinoma mamário

Mg/ks- miligramas por quilogramas

MMP-9- metaloproteinase de matriz 9

MMTV- células de câncer de mama

NF-KB- fator nuclear kappa B

Nm- nanômetro

PBS- tampão fosfato-salino

PC-3- câncer de próstata

PCR- proteína C reativa

PO- posologia oral

PPT- suco do noni rico em polissacarídeos

RAS- células precussoras de tumores malígnos

RNA- ácido ribonucléico

RT-PCR- reação da transcriptase reversa

SAR- ácido superóxido

SID- uma vez ao dia

SLAM CD+150- signaling lymphocyte activation molecule

SNC- sistema nervoso central

Th1- célula T auxiliar tipo 1

TNFα- fator de necrose tumoral - α

TNJ- suco do noni comercial

UV- ultra violeta

UVA- ultra violeta A

UVB- ultra violeta B

RESUMO

A cinomose e erliquiose são duas doenças que acometem cães e que possuem sintomatologia neurológica. A primeira é uma doença viral contagiosa, de alta incidência e mortalidade, que acomete cães domésticos e várias espécies de carnívoros; já a segunda é de origem bacteriana, que apresenta alta frequência em cães. O tratamento de doenças neurológicas é baseado na administração de fármacos, que podem atuar tanto no agente etiológico como na diminuição da progressão da doença; ou na terapia de suporte, no caso da cinomose. Visando tratamentos alternativos para as doenças com quadros neurológicos, que sejam de baixo custo, fácil administração e com efeitos satisfatórios na remissão dos sinais clínicos, esta pesquisa teve como objetivo avaliar a eficácia de *Morinda citrifolia* (noni) no tratamento de cães com sintomatologia neurológica causada pelo vírus da cinomose e pela *Ehrlichia canis*. Para avaliação do efeito da droga vegetal de *M. citrifolia* em cães com erliquiose, foram utilizados 10 cães, divididos em dois grupos, um tratado com a droga padrão doxiciclina (10 mg/kg, BID/PO) e complexo de vitaminas B (0,2 mg/kg, BID/PO), e outro tratado com o protocolo experimental (500 mg da droga vegetal de *M. citrifolia*, SID/PO). Após trinta dias, foi realizada coleta de sangue para avaliação dos parâmetros hematológicos. Foi observado que, embora o noni tenha levado à melhora dos parâmetros hematimétricos, principalmente das plaquetas, os animais não apresentaram cura clínica da doença, o que demonstrou a sua ineficácia no tratamento da erliquiose. Para a avaliação da droga vegetal de *M. citrifolia* em animais com cinomose, foram utilizados 15 animais, divididos em dois grupos, um tratado com as medicações convencionais e outro com o protocolo experimental. Após trinta dias, foi observado que os animais tratados com noni apresentaram remissão dos sinais clínicos, demonstrando sua eficácia no combate aos sinais neurológicos da cinomose. Como última etapa deste trabalho, buscou-se avaliar as lesões no sistema nervoso de cães com estado avançado de cinomose, que foram submetidos à eutanásia. Foram coletadas várias áreas do encéfalo de 7 cães (cerebelo, colículo rostral, colículo caudal, obex, pedúnculo cerebelar e telencéfalo) para análises histopatológicas e imunohistoquímicas. Após avaliação histopatológica, foi observado que a área mais afetada foi o pedúnculo cerebelar, e os principais achados foram processo inflamatório e desmielinização. A imunohistoquímica confirmou os resultados histopatológicos, com marcações em todas as áreas do encéfalo e, contrariando a literatura os neurônios foram as células que apresentaram mais marcações virais.

Palavras-chaves: Cinomose; Erlichiose; Tratamento; Sinais Neurológicos*; Morinda citrifolia*.

ABSTRACT

Canine distemper and ehrlichiosis are two diseases affecting dogs, which have neurological symptomatology. The former is a contagious viral disease, with a high incidence and mortality that affects dogs and other canids; the latter is a bacterial disease highly frequent. The treatment of neurological diseases is based on drug administration, which may act both on etiologic agent as reducing the progression of the disease; or on supportive therapy, in cases of canine distemper. Looking for an alternative treatment for diseases with neurological conditions, low-cost, easy to administrate and with satisfactory effects in the remission of clinical signs, this study aimed to evaluate the effectiveness of *Morinda citrifolia* (noni) in the treatment of dogs with neurological symptoms caused by canine distemper virus and *Ehrlichia canis*. To evaluate the effect of *M. citrifolia* vegetable drug in animals with ehrlichiosis, ten animals were used, divided into two groups, one treated with the standard drug, doxycycline (10 mg/kg, BID, orally) and complex B vitamins (0.2 mg/kg, BID, orally), and the other treated with the experimental protocol (500 mg of *M. citrifolia* vegetable drug, SID, orally). Thirty days post-treatment, blood collection was made to evaluate hematological parameters. Although noni has led to the improvement of these parameters, especially platelets, animals did not show clinical cure, which demonstrated its inefficacy in the treatment of ehrlichiosis. To evaluate the effect of the vegetable drug in animals with canine distemper, fifteen animals were used, divided into two groups, one treated with the conventional drugs and the other with the experimental protocol. After thirty days, was observed that the animals treated with noni showed remission of clinical signs, demonstrating its efficacy against distemper neurological signs. As the last step of this work, were assessed the lesions in the central nervous system of dogs in an advanced stage of distemper, which were euthanized. Several brain areas of seven dogs were collected (cerebellum, rostral colliculus, caudal colliculus, obex, cerebellar peduncle and telencephalon), to perform histopathological and immunohistochemical analyses. In histopathological analysis was observed that the cerebellar peduncle was the most affected area and that inflammation and demyelination were the major findings. Immunohistochemistry confirmed histopathological results, with markings in all areas of the brain and, contrary to the literature; neurons were the cells that presented more viral particles.

Keywords: Canine Distemper Virus; Erlichiose; Treatment; Neurological Signs; *Morinda citrifolia.*

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

O trabalho está estruturado da seguinte forma:

Parte I - Compreende a explanação da temática: Introdução; Revisão de Literatura; Justificativa; Hipótese e Objetivos.

Parte II - Composta de quatro capítulos referentes aos artigos:

- Capítulo I – Evaluation of *Morinda citrifolia* effectiveness in the treatment of canine ehrlichiosis.
- Capítulo II – The higher tropism of CDV for neuron of dogs in severe phase of distemper.
- Capítulo III - A plant, many uses: A review on the pharmacological applications of *Morinda citrifolia.*The higher tropism of CDV for neuron of dogs in severe phase of distemper.
- Capítulo IV – Avaliação da eficácia da *Morinda citrifolia* em cães naturalmente infectados pela cinomose canina.

Os periódicos utilizados para submissão desta pesquisa foram: *Annals of Microbiology* (INSS 1590-4261), cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) é B2, com fator de impacto 1.232. *Archives of Virology* (ISSN: 0304-8608 (Print) 1432-8798 (Online)) conforme o Sistema WebQualis é A2, com fator de impacto é 2.255. *Phytotherapy Research* (ISSN 1099-1573), conforme o Sistema WebQualis é A2, com fator de impacto 2.694. *Viruses* (ISSN 1999-4915), cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é A1, fator de impacto 3.042.

Parte III - Conclusões, Referências e Anexos.

Parte I

Explanação da temática

2 INTRODUÇÃO

As enfermidades infecciosas do sistema nervoso central (SNC) representam um grupo importante de doenças nos cães. Sinais clínicos graves, muitas vezes incompatíveis com a vida do animal, podem ser determinados por diferentes etiologias (ZUBRIGGEN et al., 1995). O aumento da casuística de atendimentos neurológicos na clínica amplia a necessidade de diagnósticos e tratamentos mais específicos (SILVA et al., 2007).

Alterações neurológicas em cães são comuns na rotina da clínica médica veterinária e podem ter origens congênitas, tóxicas, nutricionais, metabólicas, traumáticas, vasculares, degenerativas, neoplásica, idiopáticas e inflamatório-infecciosas (CHRISMAN, 1991). A sintomatologia neurológica está associada principalmente a doenças infecciosas tais como toxoplasmose, neosporose, erliquiose, cinomose, raiva, meningoencefalocele congênita, neospororose, encefalomielite canina por herpesvírus, dentre outras (NEVES, 2010).

A cinomose canina é uma doença viral altamente contagiosa causada por um RNA-vírus, um morbillivirus da família *Paramyxoviridae* (MARTELLA et. al., 2008). Embora seja uma doença de alta incidência e mortalidade, perdendo somente para a raiva canina, não há notificação dos casos, pois não é considerada uma zoonose.

Em regiões endêmicas, como no Brasil, é crescente o número de mortes de cães causadas por vírus da cinomose canina (CDV). Além disso tem sido considerada uma doença reemergente em países onde já esteve controlada (SILVA et al., 2007). No Brasil, especificamente no Maranhão, apesar da cinomose ser uma doença que ocorre o ano inteiro, ela apresenta uma maior ocorrência no período chuvoso, onde a disseminação do vírus é maior (BRITO et al., 2016). CDV causa imunossupressão grave e multissistêmica, estando geralmente associado a disseminação do vírus para o sistema nervoso central, resultando em uma leucoencefalopatia multifocal desmielinizante progressiva (BEINEKE et al., 2009).

Outra doença de cães de grande ocorrência que cursa com sinais neurológicos é a erlichiose canina, que é uma doença infecciosa de alta prevalência em várias regiões do Brasil, correspondendo grande parte aos atendimentos nas clínicas de animais de pequeno porte. É uma doença que se caracteriza por imunossupressão tanto em cães e canídeos silvestres. O agente etiológico é a bactéria do gênero *Ehrlichia*, que pertence a um grupo de bactérias conhecidas como ricketsias, da ordem Rickettsiales, família Anaplasmataceae (VIEIRA et al., 2011).

Para maioria das doenças que comprometem o sistema nervoso não há tratamentos devido ao grau de comprometimento. O tratamento das doenças neurológicas é baseado na administração de fármacos que atuem diretamente no agente etiológico e proporcionem a cura da doença, como por exemplo, antibióticos; ou em fármacos que atuem na diminuição da progressão da doença como nas neoplasias, ou baseado no tratamento suporte para fortalecimento de reativação do sistema imune quando se trata de doenças virais como a cinomose (NEVES, 2010).

A utilização de plantas medicinais e produtos naturais tem sido um aliado no tratamento de doenças, inicialmente de forma empírica e, posteriormente pela validação de pesquisas científicas. Cada vez mais tem aumentado a demanda global pelo uso de fitoterápicos no tratamento de inúmeras doenças (DENG et al., 2015).

Devido à fonte ilimitada de compostos químicos, a busca de novos princípios ativos em produtos naturais tem despertado grande interesse dos profissionais da saúde, indústrias farmacêuticas e comunidades científicas em todo o mundo. Por isso, novas técnicas de isolamento e purificação de princípios bioativos dos produtos naturais tornou-se alvo para descoberta de novos fármacos (NEWMAN & CRAGG, 1997; HEINRICH & GIBBONS, 2001).

Experimentos de avaliação do potencial biológico in vitro e in vivo tem sido realizados com inúmeras espécies vegetais, dentre elas, ressalta-se *Morinda citrifolia*, popularmente conhecida como noni, que é originária do Sul da Ásia e Austrália e recentemente introduzida nos trópicos. Pertence à família *Rubiaceae*, subfamília *Rubioideae*, do gênero *Morinda*. A planta produz flores e frutos o ano todo, o que favorece o uso medicinal e terapêutico (SOLOMON, 1999).

Os princípios biológicos ativos estão presentes em várias partes de *M. Citrifolia*, sendo o sumo do fruto maduro o mais utilizado, para o qual se atribui as seguintes atividades: antifúngica (BANERJEE, 2006)**,** antibacteriana (LEACH et al., 1988; LOCHER et al., 1995), anti-inflamatóra (MCKOY et al., 2002; KIM et al., 2004), imunomoduladora (HOKAMA et al., 1993; HIRAZUMI et al., 1996), analgésica (BASAR et al., 2010) e antiviral (RATNOGLIK et al., 2014) e neuroprotetora (HARADA et al., 2010; MURALIDHARAN et al., 2010).

No Brasil ainda é necessário mais pesquisas sobre doenças neurológicas em cães e,

principalmente sobre a distribuição de casos por regiões geográficas do país (FIGHERA et al., 2008). Estudos epidemiológicos realizados no Rio Grande do Sul (HEADLEY & GRAÇA, 2000; SILVA et al., 2007; FIGHERA et al., 2008), estimaram que 12% de todos os cães da rotina no Hospital Veterinário Universitário encaminhados à necropsia são acometidos por doenças infecciosas com quadro clínico neurológico compatível a cinomose (CHAVES, 2014).

Estudos realizados na Universidade Estadual do Maranhão, com dados obtidos de 481 animais atendidos no Hospital Veterinário no período de 2010 a 2013, baseado em sinais clínicos neurológicos sugestivos da cinomose e avaliados hematologicamente para a pesquisa do corpúsculo de Lentz, constatou a presença da inclusão em 39 animais (DOMINICI & SANTOS, 2014).

No intuito de um tratamento alternativo de baixo custo, fácil administração e com efeitos satisfatórios para as doenças com quadros neurológicos como a cinomose e ehrlichiose, é que se propôs investigar a ação da *M. citrifolia*, nessas enfermidades, visto que estudos já comprovaram importantes efeitos imunomodulador, antiviral, antibacteriano e neuroprotetor desta planta.

3. REVISÃO DE LITERATURA

3.1 Erlichiose canina

Ehrlichia foi identificada na Argélia em 1935 no Instituto Pasteur pelos pesquisadores Donatien e Lestoquard, que observaram estruturas intracelulares em leucócitos de cães infestados por carrapatos. Os microrganismos observados foram denominados *Rickettsia canis* e, posteriormente, em 1945, foram reclassificados como *Ehrlichia canis* por Mashkovski em homenagem a Paul Ehrlich, devido aos seus trabalhos desenvolvidos nas áreas de hematologia, imunologia e quimioterapia que contribuíram para o avanço no diagnóstico de inúmeras doenças infecciosas (VIEIRA et al., 2011).

O primeiro relato descrito da erliquiose canina nas Américas ocorreu em 1957, na região das Antilhas Holandesas, em um cão com coinfeção por *Babesia canis* (ALMOSNY, 2002). Essa é uma doença que causa imunossupressão tanto em cães domésticos quanto em canídeos silvestres. Há ainda evidências sorológicas que sugerem a ocorrência de erliquiose humana no Brasil, entretanto, o agente etiológico ainda não foi identificado (VIEIRA et al., 2011).

A erliquiose canina é uma doença mundialmente distribuída em várias regiões geográficas, as quais incluem sudeste da Ásia, África, Europa, Índia, América Central e América do Norte. Isso tudo coincide com a prevalência nessas áreas do vetor (WOLDEHIWET; RISTIC, 1993). A erliquiose monocítica canina causada *por E. canis* parece ser altamente endêmica em muitas regiões do Brasil embora dados de prevalência não estejam disponíveis em muitas (VIEIRA et al., 2011) (Figura1).

Figura 1. Distribuição geográfica dos casos de infecção por *E. cannis* em cães no Brasil Fonte: Adaptado de Vieira et al. (2011).

Nos estados brasileiros, os níveis de prevalência da doença podem variar de acordo com a distribuição do vetor, as condições climáticas, a população sob estudo, o comportamento animal e seu habitat e até mesmo com a metodologia empregada na investigação do agente. Nos estados do Nordeste e do Sul, diferenças das taxas de prevalência podem ser atrubuídas em parte a melhor adaptação do carrapato ao clima quente e úmido do que ao clima temperado (SILVA et al., 2010).

Em hospitais e clínicas veterinárias das regiões Nordeste, Sudeste, Sul e Centro-Oeste do Brasil foram relatada que aproximadamente 20% dos cães atendidos em hospitais e clínicas veterinárias são acometidos pela erliquiose (LABARTHE et al., 2003; MOREIRA et al., 2003). Na região Nordeste do Brasil, no município de Chapadinha, Estado do Maranhão, um estudo com métodos sorológicos e moleculares, objetivou revelar a exposição e infecção de agentes transmitidos por carrapatos, comprovaram que 14,6% dos cães foram soros reagentes para *E. canis* (COSTA et al., 2015). Em relação aos dois tipos de habitats distintos (zonas urbanas e rurais) amostrados no estado do Maranhão, foi demostrado que a sororreatividade canina a *E.*

canis foi semelhante em áreas urbanas e rurais (COSTA et al., 2015).

O agente etiológico responsável pela erliquiose são rickettsias pertencentes ao gênero *Ehrlichia*, microrganismo intracelular obrigatório com tropismo por células hematopoiéticas (SKOTARCZAK, 2003). Geralmente infecta leucócitos ou trombócitos levando a um quadro de trombocitopenia no hospedeiro (OLICHESKI, 2003; CHAVES et al., 2007). As espécies envolvidas na infecção em canídeos são: *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Anaplasma platys* e *Anaplasma phagocytophilum* (ALMEIDA, 2012).

Em países de clima temperado, tropical e subtropical a erliquiose é causada pela *E. canis*, coincidindo com a ocorrência do seu vetor, *Rhipicephalus sanguineus (*ALMOSNY, 2002). *E. canis* é a principal espécie em cães no Brasil, embora a infecção por *Ehrlichia ewingii* tenha, recentemente, tenha despertado suspeita em cães (VIEIRA et al., 2011).

R. sanguineus é um carrapato da família Ixodidae que se disseminou amplamente no mundo provavelmente através da introdução de cães infestados. Os cães são seus hospedeiros preferenciais e neles é capaz de provocar efeitos debilitantes decorrentes da perda de sangue durante o repasto sanguíneo, e também pela transmissão de agentes infecciosos como *E. cannis*. É um dos carrapatos de maior importância médico-veterinária do mundo e é considerado, juntamente com as pulgas, como os principais ectoparasitas de cães em todo o Brasil. É encontrado parasitando cães de forma habitual em diversos estados brasileiros (LABRUNA, 2004).

O carrapato adquire o agente durante o repasto sanguíneo em um cão infectado e o transmite a outro cão em novo repasto. *E. cannis* é inoculado através da picada do carrapato e das secreções salivares contaminadas cuja secreção contém grande concentração de bactérias, e posteriormente estas serão fagocitadas por células mononucleares (DAGNONE et al., 2001).

Os três estágios de desenvolvimento do ciclo de vida de *E. cannis* são: corpúsculo elementar, corpúsculo inicial e mórula. A forma de mórula é uma característica de todas as espécies de *Ehrlichia* e são estruturas intracitoplasmáticas, que podem ser arredondadas, ovóides ou alongadas, circundadas por dupla membrana limitada por membrana trilaminar, que envolve numerosos corpúsculos elementares (DAVOUST, 1993). As mórulas medem cerca de 1.0 a 6.0 µm de largura e contém de 1 a 40 organismos no seu interior e parasitam principalmente monócitos e neutrófilos. Os corpúsculos do interior da mórula irão infectar

novas células assim que a mórula deixa a célula o que não necessariamente causa o rompimento celular (ANDEREG & PASSOS, 1999).

Após um período de incubação, que dura de 8 a 20 dias, o agente se multiplica nos macrófagos e disseminam-se pelo organismo (THOMAS et al., 2010). Por ser um parasito intracelular, consegue escapar de mecanismos de defesa do sistema imunológico, como por exemplo de anticorpos que não conseguem entrar na célula hospedeira (PEREZ; RIKIHISA; WEN, 1996).

E. canis multiplica-se em células mononucleares circulantes e dos tecidos fagocitários mononucleares do fígado, baço e linfonodos, o que causa linfadenomegalia e hiperplasia linforreticular do fígado e do baço. Ocorre a replicação da bactéria originando mórulas em torno de 7 a 12 dias após a infecção e no endotélio a adesão destas bactérias ocasiona vasculite e infecção subendotelial. Uma vez na corrente sanguínea atingirão diversos órgãos como rins, pulmões, olhos e meninges (HARRUS et al., 1997).

Os sinais clínicos de infecção por *E. canis* podem variar de acordo com a estirpe, a resposta imunitária do cão e a presença de infecções concomitantes com outros agentes patogênicos. Alguns cães podem não apresentar sinais clínicos e laboratoriais relacionados com a infecção e outros podem mostrar sinais graves (LITTLE, 2010). Clinicamente a erliquiose apresenta três fases caracterizadas como aguda, subclínica e crônica.

A fase aguda pode durar de 1 a 4 semanas e a maioria dos cães submetidos a tratamentos específicos se recuperam da enfermidade. Nesta fase o animal apresenta dispnéia, febre, depressão, anorexia e tem anormalidades hematológicas como trombocitopenia, leucopenia, leve anemia e hipergamaglobulinemia (SKOTARCZAK, 2003). O animal também pode apresentar membranas mucosas pálidas, devido à anemia, epistaxe, petéquias, equimoses, sangramento prolongado durante o estro, hematúria ou melena associadas a vasculite, trombocitopatia ou trombocitopenia (SAINZ et al., 2015).

A trombocitopenia é o mais proeminente e consistente achado hematológico que ocorre na erlichiose canina (HARRUS et al., 1997). A destruição e sequestro das plaquetas são as principais causas da trombocitopenia, porém há outros mecanismos que ainda não estão bem elucidados quanto à diminuição da quantidade de plaquetas (PANTANOWITZ, 2002). Alterações no número de leucócitos são variáveis e a anemia ocorre de forma progressiva

devido à supressão da produção de eritrócitos e à destruição acelerada destas células (WANER, 2008). A anemia e trombocitopenia são responsáveis pelos sinais clínicos mais comuns como inapetência, letargia, depressão, vômitos, febre, palidez e epistaxe (YBAÑEZ, 2016).

Durante a fase subclínica que se manifesta em torno de seis a nove semanas após a infecção, em geral não são observados sinais clínicos, mas pode ser evidenciada a presença de trombocitopenia, leucopenia e anemia em hemograma de rotina (SKOTARCZAK, 2003). Mas, mesmo nesse período pode haver persistência de sinais como hemorragias, perda de apetite, depressão, edema de membros e palidez de mucosas (ROSEZ et al., 2001).

Na fase crônica pode ocorrer pancitopenia grave, em decorrência da hipoplasia da medula óssea (SHIPOV et al., 2008) além de supressão medular, sangramentos por mucosas e conjuntivas e alta letalidade (HARRUS et al., 1997). Geralmente nesta fase o animal tem os mesmos sinais da fase aguda porém atenuados, tornando-se apático, caquético e com susceptibilidade aumentada a infecções secundárias, havendo características de doença auto imune (SILVA et al., 2015). Os sinais oculares também são comuns dentre eles a opacidade da córnea, hifema, uveíte anterior, tortuosidade dos vasos da retina, lesões coriorretinianas, hemorragia sub-retiniana, descolamento de retina e cegueira (SAINZ et al., 2015).

Os sinais clínicos e severidade da doença estão associados a produção de citocinas em células esplênicas e leucócitos sanguíneos que ativam células inflamatórias, dentre elas interleucina-1β (IL- 1β), interleucina-6 (IL-6), interleucina-12 (IL-12) e fator de necrose tumoral-α (TNF-α) (FARIA et al., 2011).

Sinais neurológicos decorrentes da erliquiose canina são menos comumente descritos porém podem estar presentes em até 24,2% dos cães acometidos pela doença (UENO et al., 2009). São consequência da doença crônica e severa e são geralmente secundários à meningite, sendo atribuidos a hemorragia, plasmocitose e infiltrados perivasculares nas meninges (MCDADE, 1990). Estes sinais incluem ataxia, disfunção neuromotora, disfunção vestibular central ou periférica, anisocoria, tremor intencional e hiperestesia localizada ou generalizada (GREENE, 2015). Os sinais são um reflexo da meningoencefalite que acomete os animais, os quais demonstram dores severas no pescoço, paraparesia ou tetraparesia, ataxia, déficits dos nervos cranianos e convulsões (MCDADE, 1990).

O diagnóstico laboratorial mais comum é realizado por meio da pesquisa de mórulas em

esfregaços sanguíneos. A imunofluorescência indireta (IFI) detecta a presença de IgG contra *E. canis* no soro, sendo um método mais sensível, porém pode apresentar reação cruzada com outras rickettsias (O'CONNOR, 2006). Outro teste bastante simples e disponível é o teste de ELISA, que se baseia na detecção de anticorpos IgG contra *E. canis* no soro. Este teste é muito útil no monitoramento dos níveis de anticorpos, principalmente nas fases subclínica e crônica, onde é muito difícil encontrar a *E. canis* em esfregaço sanguíneo (BABO-TERRA, 2004). A técnica de PCR permite um diagnóstico preciso, podendo ser usada para detectar o DNA específico do microrganismo em leucócitos de sangue periférico (ALVES et al., 2004).

O tratamento para erliquiose canina instituído em clínicas e hospitais veterinários do Brasil é feito principalmente a base de tetraciclina, oxitetraciclina, doxiciclina e dipropionato de imidocarb. Entretanto, as tetraciclinas e seus derivados, como a doxiciclina, são as que apresentam melhor eficácia (SHIPOV, 2008). A doxiciclina é considerada o fármaco de eleição. Em estudo feito por Garcia Filho et al. (2010) foi observada eficácia após 15 dias de tratamento e melhora significativa nos padrões hematológicos e remissão completa dos sinais clínicos.

A rifampicina foi introduzida no tratamento da erliquiose canina e os resultados mostraram que a droga pode ser uma alternativa no controle da infecção (SCHAEFER et al. 2008). Devido a pouca opção de fármacos para o tratamento dessa enfermidade e a frequente resistência apresentada pela maioria das bactérias aos antibióticos, estudos com outras drogas como a azitromicina foram realizados, todavia, esse antibiótico não levou à recuperação clínica e a normalidade dos parâmetros hematimétricos dos animais infectados (CANTADORI et al., 2014).

Considerando a alta morbidade da erliquiose e a inexistência de vacina, a prevenção é uma ferramenta de suma importância para controle da infecção, principalmente nos locais de grande concentração de animais. As principais medidas profiláticas são: o controle de carrapatos, o uso de coleiras ectoparasiticidas e limpeza dos ambientes onde os animais vivem. Além disso, Koh et al. (2016) alertam que a erlichiose é uma zoonose em potencial, por isso, se faz necessária a adoção de medidas de controle para prevenção das doenças transmitidas por carrapatos.

3.2 Cinomose canina

A cinomose canina é uma doença viral, infecciosa, causada por um *Morbilivirus* da

família *Paramoxiviridae,* da ordem *Mononegavirales*, e é considerada um dos mais importantes patógenos de cães domésticos (SILVA et al., 2009).

Os primeiros dados de cinomose canina foram registrados em 1746 na América do Sul. Em meados de 1760 a doença foi descrita na Espanha, seguida de Inglaterra, Itália e Rússia. Em Madri, aproximadamente 900 cães morreram em um único dia no ano de 1763. A entrada da doença em cães da Europa foi atribuída a migração de colonizadores espanhóis no século XVII vindos do Peru para a Europa (BLANCOU, 2004).

Em 1905, Carré isolou pela primeira vez o vírus causador da doença o que contrariou trabalhos anteriores que atribuíam a doença a bactérias como pasteurelas, brucelas e salmonelas. Foi Koprowski, em 1959 e Imagawa em 1960, que incluíram o vírus da cinomose no mesmo gênero do vírus do sarampo (MENLEN & HALL, 2016).

Na primeira metade do século XX, a cinomose foi uma das doenças fatais mais comuns em cães em todo o mundo. Com o surgimento de vacinas específicas, a partir dos anos 60 a mortalidade em cães diminuiu, no entanto, a mortalidade ainda é comum em animais não vacinados ou naqueles com problemas de falha vacinal (SILVA, 2004).

O vírus da cinomose canina (CDV) afetou populações de cães domésticos (*Canis familiaris*) durante séculos e atualmente causa doença também em animais silvestres, como raposas, furões, leões, leopardos, guepardos e tigres (NORRIS et al., 2006). Alguns desses animais podem ser extintos em decorrência do vírus (BUCZKOWSKI et al., 2014).

No Brasil a doença é endêmica em diversos estados e a prevalência da infecção pelo vírus varia de 10,6% a 65,7% de soropositividade (LÚCIO et al., 2014). Milhares de cães morrem de cinomose anualmente, chegando a corresponder a morte de 11,7% dos cães acometidos (HEADLEY, 2000).

O gênero *Morbillivirus* tem causado grande impacto em populações humanas e animais, e é responsável por diversas doenças como sarampo, peste bovina, peste dos pequenos ruminantes e cinomose canina, relacionando os agentes dessas doenças intimamente, antigenicamente e morfologicamente. Os *Morbillivirus* são conhecidos por causar imunossupressão, problemas cutâneos, respiratórios, gastrointestinais e neurológicos (BEINEKE et al., 2009).

A transmissão ocorre principalmente por meio de aerossóis e gotículas que contêm as partículas virais, secreções respiratórias, fezes e urina (MARTELLA et al., 2008). Durante a exposição natural, o CDV se propaga e entra em contato com o epitélio do trato respiratório superior onde são ativadas as CD150/SLAM, moléculas sinalizadoras de ativação dos linfócitos (TATSUO et al., 2001). No período de 24 horas, as partículas virais se replicam nos macrófagos e se disseminam pela via linfática local para as tonsilas e linfonodos bronquiais (KAPIL & YEARY., 2011).

O vírus é eliminado principalmente na fase aguda que ocorre de 7 a 15 dias, porém a sua eliminação pode ocorrer até até 60-90 dias após a infecção. As fontes de infecção mais comuns os fômites, ar, água e alimentos contaminados. O CDV se propaga mais facilmente em lugares aglomerados, como lojas de animais, abrigos, canis e clínicas (DEL PUERTO et al., 2010). As pulgas podem ser vetores importantes na transmissão horizontal da cinomose em espécies de mamíferos mustelídeos, o que aponta para a importância da biossegurança em torno de fazendas com criatório dessas espécies, a fim de evitar a introdução desse vírus e de novos patógenos que circulam no ambiente silvestre (TREBBIEN et al., 2014).

Em carnívoros selvagens a transmissão pode ocorrer através de urina, fezes ou ingestão de carnes infectadas, sendo estas consideradas importantes vias de infecção da doença (LUDLOW, 2013). Avendano et al. (2016) demonstraram que em carnívoros selvagens na Costa Rica a transmissão se deu por meio das fezes.

O CDV possui um virion relativamente grande (150-250 nm) com uma fita simples de RNA e é recoberto pelo envelope viral que deriva de glicoproteínas da membrana celular da célula hospedeira. Possui 6 genes que codificam 8 proteínas virais, duas não estruturais (C e V) e 6 proteínas estruturais: a proteína do Nucleocapsídeo (NP), a fosfoproteína (P), a proteína da matriz (M), a proteína de fusão (F), a hemaglutinina (H) e a grande proteína (L) (WIENER et al., 2007).

O vírus apresenta uma molécula específica chamada SLAM (molécula de ativação e sinalização de linfócitos) ou CD 150, uma glicoproteína da membrana celular,que funciona como receptor celular para o vírus mais especificamente um receptor para a hemaglutinina presente no envelope viral. A sua expressão ocorre nas superfícies das células do sistema imune como linfócitos T e B, e as células apresentadoras de antígeno é um dos principais determinantes do tropismo viral o qual determina seu efeito imunodepressor (RUDD et al.,

2006).

Há sensibilidade viral ao calor e ao ressecamento, não resistindo a temperaturas de 50°C a 60°C por 30 minutos. Sobrevive 1 hora a 37ºC e por 3 horas a 20°C. Sobrevive por semanas em baixas temperaturas e permanece estável por até 7 anos na temperatura de -65ºC (GREENE, 2015).

O CDV apresenta apenas um único sorotipo do vírus, porém existem inúmeras cepas virais biologicamente diferentes. As cepas de baixa virulência causam infeções inaparentes e as viscerotrópicas de alta virulência promovem depleção do sistema imunológico, ocasionando infeções agudas. O efeito imunossupressor do vírus depende de vários fatores como idade do animal, estado nutricional e a virulência da cepa viral (GREENE, 2015).

Já foram descritas várias cepas do CDV que apresentam inúmeras linhagens, dentre elas estão a América 1, América 2, América 3, Europeia, Ártica, Ásia 1 e Ásia 2. E suas várias estirpes, como a A75/17, 5804P, Onderstepoort, Snyder Hill, Rockborn (MCVEY et al., 2008). Na América do Sul uma nova cepa foi encontrada na Argentina e provavelmente circula apenas em animais silvestres (PANZERA et al., 2012). Na China também foi identificado um novo isolado denominado de XJ12 (QIAO et al., 2011).

Uma nova cepa de CDV foi descrita no Sudoeste dos Estados Unidos, a América 4. Esta cepa apresenta poucas diferenças de genótipo quando comparada a América 3. Esse fato é contraditório pois essas cepas vieram de diferentes espécies de animais e foram coletadas em momentos e locais diferentes, mas a via transmissão não foi elucidada (RYLEY e WILKES, 2015). Na Suíça provavelmente existe uma nova estirpe selvagem, uma vez que testes moleculares não foram capazes de identificar a cepa circulante nos cães (WILLI et al., 2015).

O período de incubação para o surgimento dos sinais clínicos do CDV é de 14 a 18 dias. Após a exposição e infecção os cães apresentam febre não muito elevada, entre o 4º e o 7º dias, sem sintomas evidentes da doença (MATTHIESEN, 2004). A manifestação clínica da infecção depende da cepa viral infectante, da idade e do perfil imunológico do animal. Sinais epiteliais da doença são frequentes e geralmente precedem ou ocorrem simultaneamente aos sinais neurológicos, sendo que estes últimos podem ocorrer sem sinais sistêmicos associados (SANTOS, 2006).

Em cães com cinomose são observadas alterações respiratórias (tosse, dispnéia,

corrimento nasal) gastrointestinais (diarreia, vômito), oculares, dermatológicas (pústulas e hiperqueratose) e neurológicas. Dentre os sinais oftalmológicos estão a conjuntivite purulenta, ceratoconjuntivite seca, secreções oculares serosas ou seromucosas, vermelhidão, lesões na retina e até cegueira (MARTINS et al., 2009).

A infecção no sistema nervoso central (SNC) é a mais grave complicação da cinomose canina, pois causa uma variedade de distúrbios neurológicos, muitas vezes com um prognóstico desfavorável. (VANDEVELDE & ZURBRIGGEN, 2005). Em alguns cães as lesões no SNC podem ocorrer como a única manifestação aparente da infecção, sendo observados sinais como mioclonias, ataxia, paresia, paralisia, incoordenação e, algumas vezes, alterações comportamentais como vocalização intensa e hiperexcitabilidade (MARTELLA et al., 2008).

Após a redução do antígeno viral no SNC, pode haver complicações devido a um aumento na expressão do complexo de histocompatibilidade, principalmente nas células da micróglia, por serem responsáveis pelo processo de desmielinização contínua e pela infiltração mononuclear perivascular disseminada (MANGIA, et al., 2012).

No cérebro, foi observado o aumento das células T CD8+ e a produção precoce de algumas citocinas como a IL-8, IL-6, IL-1 e TNFα, que são mediadas pela ação do vírus, sendo que a indução dessas citocinas está relacionada com o grau de produção do vírus nas células caninas do SNC em experimentos in vitro *(*PARDO, 2005).

Sinais neurológicos geralmente se instauram após a doença sistêmica ou até mesmo sem a presença de sinais clínicos sistêmicos (VANDEVELDE & ZURBRIGGEN, 2005). Estes sinais decorrem da degeneração neural, desmielinização, infiltrado linfoplasmocitário perivascular ocorrendo principalmente no cerebelo, tálamo e medula espinhal (RAW, 1992).

A fase aguda da cinomose é caracterizada por uma encefalomielite aguda que destrói os neurônios localizados na substância cinzenta e a substância branca é afetada por uma encefalomielite não-supurativa subaguda ou crônica causando desmielinização. Animais jovens e cães com imunodeficiência desenvolvem com frequência necrose neuronal (WYSS-FLUEHMANN et al., 2010).

O CDV produz lesões nas células do SNC na fase aguda da doença e afeta diretamente e de forma grave as células produtoras de mielina devido a predileção que algumas cepas virais possuem por esse tipo celular (ORSINI et al., 2008). Já na fase crônica, a desmielinização ocorre

através dos processos inflamatórios ou não, visto que há acumulo de células fagocitárias e anticorpos que causam a destruição da bainha de mielina (LITFALLA et al., 2008).

Em relação à sensação da dor, Aguiar (2015) observou que cães com cinomose apresentaram dor profunda pela pressão vigorosa sobre o periósteo da região interdigital e dor superficial com e sem estímulo tátil a nível cutâneo como resposta a sensibilidade elevada ao estimulo. Neves (2010) também relatou a presença da dor espinhal ou neuropática nos cães acometidos por cinomose, pois houve o envolvimento das meninges, raízes nervosas e dos nervos periféricos, sendo indicado o uso de analgésicos.

Dependendo do nervo lesionado o CDV pode causar perda dos reflexos pupilares e muitas vezes perda da visão. Se atingir os nervos trigêmeo e facial pode provocar alterações na face do animal como lábio mais caído de um lado e salivação excessiva. No nervo vestibulococlear pode provocar ataxias, surdez, dismetria e danos nas vias de propriocepção. Os cães sobreviventes podem ter sequelas neurológicas permanentes, envolvendo espasmo dos flexores e disfunções visuais e olfatórias (FEITOSA, 2014).

Em pesquisa desenvolvida por Aguiar (2015) os animais foram avaliados clinicamente de maneira a identificar possíveis lesões nos nervos cranianos e estimou-se que os mais afetados pelo vírus da cinomose canina foram os nervos trigêmio, oculomotor e acessório.

O diagnóstico da cinomose baseia-se nos sinais clínicos, idade dos animais, histórico de vacinações inadequadas e possibilidades de exposição ao vírus. O diagnóstico clínico em cães sem sinais sistêmicos precedentes ou concomitantes é difícil (SANTOS, 2006).

Pesquisa de corpúsculos de Lentz, que estão presentes em células como hemácias e leucócitos, são observadas nos esfregaços sanguíneos durante a realização do hemograma (SILVA, 2005). A partir do exame sanguíneo pode ser observado em cães com cinomose sistêmica a presença de leucocitose, neutrofilia ou em alguns casos neutropenia, linfopenia, monocitopenia e trombocitopenia (MEINKOTH et al., 2000). Durante a replicação viral no organismo, um excesso de proteínas não utilizadas pelo vírus se organizam sob forma de corpúsculo de inclusão e formação de sincício (JONES, 2000). Os corpúsculos virais de inclusão podem ser observados em amostras de bexiga, estômago, SNC, coxins digitais, no epitélio conjuntival, no epitélio dos brônquios e bronquíolos (TOVAR, 2007).

Na fase neurológica não são observadas alterações hematológicas significativas, porém

há uma expressiva diminuição dos linfócitos (SILVA, 2005). No exame do líquido encefaloraquidiano é observada uma elevação das proteínas e dos leucócitos com predomínio de linfócitos, sugerindo o diagnóstico de doenças de etiologia viral (AMUDE, 2006).

O diagnóstico do CDV pode ser estabelecido por técnicas como a imunofluorescência, RT-PCR, microscopia eletrônica, imunohistoquímica e exames histopatológicos (SCHUMAKER et al., 2014). Várias técnicas têm sido sugeridas para o diagnóstico definitivo da cinomose. Os testes tipo ELISA detectam a presença do antígeno da cinomose por meio de amostras de secreções nasais, oculares e da urina, ou através do soro ou plasma sanguíneo, sendo úteis para os animais não vacinados ou que já tenham tido declínio dos títulos maternos (BRANDÃO, 2009). O isolamento viral em cultivo celular é um teste específico, porém pode ser de difícil realização (QUINN, 2005).

Exames histopatológicos podem ser realizados post-mortem, verificando-se as cinco estruturas no encéfalo de maior predileção pelo vírus: o lobo frontal, o mesencéfalo, o diencéfalo, a ponte e o cerebelo. Griffin et al. (2008) encontraram em cães com cinomose submetidos a ressonância magnética focos hiperintensos e perda de contraste entre as substâncias branca e cinzenta.

Não há tratamento especifico para cinomose, por isso o tratamento de suporte a base de líquidos, eletrólitos, vitaminas do complexo B, vitamina A, complementos nutricionais e antibióticos de amplo espectro para o controle das infecções bacterianas secundárias são indicados (GREENE, 2015).

Quando há comprometimento do sistema respiratório, os animais devem ser tratados com broncodilatadores e expectorantes, a fim de reduzir a tosse e evitar infecções secundárias como pneumonia bacteriana e insuficiência respiratória. Animais que apresentam quadro neurológico podem receber anticonvulsivantes e relaxantes musculares como o diazepam (SIGWALT, 2009).

Em casos de convulsões, devem ser utilizadas medicações como diazepam ou fenobarbital, pelas vias intravenosa, intramuscular ou oral, a cada 12 horas. Para redução de edema cerebral deve-se utilizar terapias a base de corticosteroides, como a dexametasona na dose de 2,2 mg/Kg, por via intravenosa, reduzindo a dose progressivamente. Nos casos severos de encefalite multifocal progressiva, com lesões neurológicas como a tetraplegia, semi coma e

incapacitação, a eutanásia é recomendada (GREENE, 2015).

A ribavirina em culturas de células infectadas pelo CDV mostrou-se eficaz (ELIA et al., 2008). Em cães a ribavirina demostrou ser eficaz contra a infecção do vírus e dimetil-sulfóxido (DMSO) mostrou-se capaz de potencializar a sua ação (MANGIA & PAES, 2008). A terapia antiviral com ribavirina foi capaz de evidenciar a eficácia frente ao vírus da cinomose, pois os animais tratados aumentaram a sobrevida em 70% devido ao tratamento a que foram submetidos (MANGIA et al., 2012).

Carvalho et al. (2012) testaram in vitro a ação antiviral de alguns flavonoides: quercetina, rutina, morina e hesperidina. Todos os flavonoides apresentaram atividade antiviral significativa contra o CDV, sendo estes compostos químicos promissores para formulação de drogas contra o CDV.

Estudos in vitro utilizando o fucoidano, que é um polissacarídeo extraído de uma alga marinha *Cladosiphon okamuranus,* comprovaram atividade antiviral significativa em relação ao CDV. O fucoidano inibiu as fases iniciais do ciclo da infecção viral, provavelmente por ação direta sobre as proteínas (F ou H) responsáveis pela fusão do CDV ou através da inibição da expressão do vírus (TREJO-AVILA et al., 2014).

A melhor maneira de prevenção contra cinomose canina é a vacinação. Existem várias vacinas que foram produzidas com amostras do vírus isoladas de cães naturalmente infectados e atenuadas em culturas de células, como as Snyder Hill, Rockborn e Onderstepoort (GREENE, 2015). Entretando, existem vários fatores que podem diminuir a eficácia das vacinas ocasionando uma baixa imunidade, tais como as diferenças genéticas, a interferência dos anticorpos maternos que podem resultar em uma proteção incompleta e o manuseio inadequado da vacina. No entanto, foram relatadas recentemente alterações na virulência, na antigenicidade e o aparecimento de novas estirpes com uma grande diversidade genética quando comparados com as cepas das vacinas disponíveis já existentes (RYLEY & WILKES, 2015).

3.3 Fitoterapia

As plantas medicinais são utilizadas com finalidades terapêuticas há milhares de anos com seu uso popular sendo propagado de geração em geração e descrito nas farmacopéias. Atualmente tornou-se possível obter substâncias puras através do isolamento de princípios ativos de plantas (TUROLLA & NASCIMENTO, 2006).

A fitoterapia é fundamental na medicina moderna pois pode fornecer fármacos que dificilmente seriam obtidos via síntese química, sendo uma fonte natural que fornece compostos que podem ser modificados de forma a diminuir sua toxicidade e aumentar sua eficácia e que podem ser utilizados como protótipos para obtenção de fármacos com atividades terapêuticas semelhantes a dos compostos originais (ROBBERS et al., 1996).

Devido a fácil obtenção das plantas, o baixo custo, a eficiência na prevenção e no tratamento de doenças, a utilização de plantas medicinais é vista como uma forma de busca por novos produtos fitoterápicos na medicina veterinária, tornando-se uma alternativa viável para a saúde animal, além de proporcionar melhoria na sua qualidade de vida. Os produtos fitoterápicos podem fornecer um tratamento melhor em comparação a outros fármacos já disponíveis no mercado e também podem ser usados de forma auxiliar. Hoje, os fitoterápicos já representam 6,7% do mercado total de medicamentos veterinários (OZAKI & DUARTE, 2006).

No que discerne a fitoterapia e o tratamento de doenças neurológicas em animais, as plantas são usadas devido a sua ação calmante, sedativa e relaxante para cães (OZAKI & DUARTE 2006) (Tabela 1).

Tabela 1. Plantas usadas para o tratamento de doenças que afetam o sistema nervoso em cães

Nome Científico	Família Botânica	Nome popular	Parte da planta	Referência
Valeriana officinalis L	Valerianaceae	Valeriana	Raiz	Viegi et al 2003 Alonso, 1998
Nepeta cataria L.	Lamiaceae	Mentrasto	Partes aéreas da flor	Berschneidr, 2002 Cunha et al 2003
Passiflora alata	Passifloráceas	Maracujá	Toda planta	Cavalcanti, 1997 alonso, 1998
Humulus Lúpulus	Canabináceas	Lúpulo	Cone ou estróbilo	Cavalcanti, 1997 Cunha et al 2003
Ginseng	Araliáceas	Panax ginseng	Raiz	Cavalcanti, 1997 alonso, 1998
Fumaria Officinalis	Fumariaceas	Fumária	Flores	Cavalcanti, 1997 alonso, 1998

Adaptado de OZAKI & DUARTE (2006).

3.4.Legislação em Fitoterapia

No Brasil, a legislação para medicamentos fitoterápicos é regulada e fiscalizada pela Agência Nacional de Vigilância Sanitária (ANVISA) a qual avalia aspectos importantes como a eficácia e segurança do uso destes medicamentos. A Anvisa deve evitar que intoxicações,

fracassos terapêuticos, agravamento de enfermidades, ou até mesmo a morte de pacientes ocorram pela circulação de medicamentos nocivos e de má qualidade, buscando com isso conquistar a confiança da população e a credibilidade dos profissionais de saúde, estimulando a prescrição e o uso racional de medicamentos fitoterápicos (CARVALHO et al., 2007).

Para a Anvisa, planta medicinal pode ser definida como espécie vegetal utilizada para fins terapêuticos e a partir desta planta pode-se obter uma droga vegetal, após esta passar por processos de coleta, estabilização e secagem. De acordo com a Resolução da Diretoria Colegiada (RDC) nº 14 de 2010 são considerados medicamentos fitoterápicos os obtidos com emprego exclusivo de matérias-primas ativas vegetais, cuja eficácia e segurança são validadas por meio de levantamentos etnofarmacológicos, da sua utilização, documentações tecnocientíficas ou evidência clínicas (BRASIL, 2010).

A Portaria n.6 de 1995 estabeleceu prazos para que as indústrias farmacêuticas apresentassem dados de eficácia e segurança dos medicamentos fitoterápicos e a RDC n. 17 de 2000 e a RDC n. 48 de 16 de março de 2004, atualmente em vigor dispõem sobre o registro de medicamentos fitoterápicos (OZAKI & DUARTE 2006).

Em 2006, foi estabelecida no Brasil a Política Nacional de Plantas Medicinais e Fitoterápicos, publicada através do Decreto nº 5.813. Essa política busca o incentivo à pesquisa e ao desenvolvimento do uso de plantas medicinais e fitoterápicos que possam ser disponibilizados com qualidade, segurança e eficácia à população, priorizando a biodiversidade do país (CARVALHO et al., 2007).

3.5 Morinda citrifolia

3.5.1 Caracteristicas e distribuição geográfica

Morinda citrifolia, ou noni, é uma planta nativa do sudoeste da Ásia. A árvore atinge até 6 m de altura, com folhas verde-brilhantes e ovaladas, que chegam a medir de 10 a 30 cm. O fruto tem um formato ovóide e, quando maduro, possui um odor desagradável de ácido butírico e sabor adstringente (Figura 2). As sementes possuem um saco aéreo em uma das extremidades e são flutuantes, o que explica em partes a ampla distribuição das árvores de noni nas ilhas do Indo-Pacífico (POTTERAT & HAMBURGER, 2007).

Figura 2- Fruto do noni. Fonte: Arquivo Pessoal.

Segundo Morton (1992) *M. citrifolia* pertence ao:

- ✓ Reino Plantae
- ✓ Filo Magnoliophyta
- ✓ Classe Magnoliopsida
- ✓ Ordem Rubiales
- ✓ Família Rubiaceae

O gênero Morinda compreende mais de 80 espécies, todas elas presentes exclusivamente em regiões de clima tropical (MORTON, 1992). Há duas variedades distintas de *M. citrifolia* (*M. citrifolia var. Morinda* e *M. citrifolia var. bracteata*) e há uma variedade tipo cultivar (*M. citrifolia* cultivar Potteri) com diferentes morfologias e distribuições geográficas. *M. citrifolia* var. *Morinda* é a variedade mais encontrada e de maior importância econômica (PAWLUS & KINGHORN, 2007).

Noni tem uma longa história de uso como uma planta medicinal na Polinésia, Sul e Sudeste da Ásia, nordeste da Austrália e no Caribe. *M. citrifolia* tem sido usada como uma planta medicinal há séculos e seu uso tem continuado a crescer; foi disseminada ao longo dos trópicos como uma colheita de canoa, interposto pelos primeiros colonos polinésios, e agora se espalhou para o mundo desenvolvido como um suplemento dietético popular (PAWLUS & KINGHORN, 2007).

O advento do suco de noni (Tahitian noni juice) deu-se em 1996 e, devido a sua grande procura, culminou no aumento das pesquisas acerca dos possíveis efeitos que esse material poderia promover com relação aos seus benefícios à saúde (PALU et al., 2008). O emprego tradicional pelos polinésios é devido aos efeitos medicinais atribuídos ao noni relacionados com atividade antibacteriana, antiviral, antifúngica, antitumoral, anti-helmíntica, analgésica, antiinflamatória, hipotensora e imunoestimulante, sendo usado há mais de 2000 anos (VASCONCELOS et al., 2014).

O fruto do noni chegou ao Brasil devido as ações benéficas associadas ao seu consumo e consequente apelo comercial. E apesar da demanda pelos produtos oriundos do noni, a cultura foi introduzida há poucos anos e ainda não há cultivo em escala comercial (SILVA, 2010). O cultivo do noni já foi relatado nos Estados de Minas Gerais, Acre, Sergipe, São Paulo, Pará e Ceará, entre outros (CORREIA et al., 2011).

A região Nordeste do Brasil abriga diversas espécies vegetais nativas e exóticas com potencial fitoterápico, socioeconômico e elevado valor no mercado dentre as quais *M. citrifolia* (CARVALHO et al., 2011). No Estado do Maranhão a espécie é de recente introdução. As primeiras mudas plantadas em São Luís-MA datam de 2005, e nesta região por fatores como localização geográfica, clima, solo e amplitude favoráveis houve propensão para o desenvolvimento dessa espécie. Atualmente é amplamente comercializado em diversos mercados e feiras livres, na capital e no interior do estado (SILVA, 2010). Em Zé Doca-MA, o cultivo de noni também demonstrou bons resultados como elevado índice de sobrevivência da planta, ausência de ataque de pragas ou doenças ao cultivo e produtividade precoce (CARVALHO et al., 2011).

Apesar da grande procura por parte da população para ultização da planta, segundo Anvisa (2007) é proibido o comércio de produtos contendo noni como alimento no Brasil, até que os requisitos legais que exigem a comprovação de sua segurança de uso sejam atendidos. A medida tem como finalidade proteger e promover a saúde da população já que poucas informações e os estudos toxicológicos disponíveis até o momento são insuficientes para um consumo seguro. A Gerência-Geral de Alimentos da Anvisa avaliou por diversas ocasiões produtos contendo *M. citrifolia*, incluindo o suco de noni, desaprovando o consumo do produto no país em todas as avaliações realizadas (ANVISA, 2007).

3.5.2 Composição química

As pesquisas sobre os componentes fitoquímicos de noni tem chamado a atenção dos pesquisadores devido ao seus registros como planta medicinal e crescente uso como suplemento dietético botânico (PAWLUS & KINGHORN, 2007). Os principais compostos isolados de noni podem ser visualizados na tabela 2.

Tabela 2. Compostos identificados dos frutos, folhas e raízes de *M. citrifolia*

Parte da planta	Composto	Classificação química	Referência
Folha	Americanin A	Ligana	Kovendan et al. (2012) e Kovendana et al. (2014)
	Prolina Leucina Cisteína Metionina Glycine Histidina Isolucine Ácido glutâmico Fenilalanina Serina Treonina Triptofano Tirosina Arginina Valina	Aminoácido	Shovic e Whistler (2001), Elkins (1998) e Sang et al. (2001)
	Quercetina 3-O-β-D-glucopiranoside Quercetina 3-O-a-lrhamnopiranosil-(1-6)-β-digropiranoside	Flavonóides	Chan-Blanco et al. (2006) e Sang et al. (2001)
	Ácido ursólico	Triterpenóides	Elkins (1998), Sang et al. (2001) e Dittmar (1993)
	β-sitosterol	Esteróis	Shovic e Whistler (2001) e Wang et al. (2002)
	Citrifolinoside B	Iridóides	Sang et al. (2001)
	Kaempferolm 3-O-β-Dglucopiranosil-(1-2)-a-Lrhamnopiranosil-(1-6)-βD-galactopiranoside	Derivados de clorofila	Sang et al. (2001)
	Escopoletina	Derivado de cumarina	Kamiya et al. (2010) and Mohd Zin et al. (2007)
Fruto	Ácido octanóico (caprílico) Ácido hexanóico Ácido capróico	Ácido graxo	Elkins (1998), Dittmar (1993), Wang et al. (1999, 2002), Mohd Zin et al. (2007), Jayaraman et al.

			(2008), Liu et al. (2001) e Zhang et al. (2014)
	Vitamina C Vitamina E Niancina	Vitaminas	Zin et al. (2002) e West et al. (2011)
	Manganês, Selênio	Elementos químicos	Zin et al. (2002) e West et al. (2011)
	Ácido Asperulosídico	Iridóide	West et al. (2012)
	Quercetina	Flavonóides	Elkins (1998), Sang et al. (2001), Liu et al. (2001), Morton (1992), Yu (2004) e Deng et al. (2007b)
	2,6-di-O-(β-Dglucopiranosil-1-Ooctanoil-b-D glucopiranose	Éster de ácidos graxos	Nelson (2006), Kamiya et al. (2010), Jayaraman et al. (2008) e Lin et al. (2013)
	6-O-(β-D-glucopiranosil-1-O-octanil-β-D glucopiranose		Liu et al. (2001), Wang et al. (1999), Zhang et al. (2014) and Akihisa et al. (2012)
	Damnacanthal	Antraquinonas	Chan-Blanco et al. (2006) e Kamata et al. (2006)
	Americanin A	Ligana	Su et al. (2005)
Raiz	8-hidroxi-8-metoxi-2-metil-antraquinona, rubiadina 1,3-di-hidroxi-6-metil antraquinona	Antraquinonas	Elkins (1998), Morton (1992) e Inoue et al. (1981)
	Morenone 1 Morenone 2		Elkins (1998) e Solomon (1999)
	Asperuloside	Iridóide	Bushnell et al. (1950)
	Quercetina	Flavonóides	Mohd Zin et al. (2007) e Mandukhail et al. (2010)
	Escopoletina	Derivado de cumarina	Sang et al. (2001) e Jensen et al. (2005)
	Damnacanthal	Antraquinonas	Nualsanit et al. (2012)

Adaptado de Assi et al. (2015).

Diversos metabólitos já foram isolados dos frutos de noni, dentre eles os compostos fenólicos, ácidos orgânicos e alcaloides e dentre os compostos ativos encontrados estão cumarinas, ácidos graxos, flavonoides, lignanas, polissacarídeos, irridoides, antraquinonas,

terpenoides e esteróis (DENG et al., 2007). No fruto as vitaminas em maior quantidade são ácido ascórbico e provitamina A (CHAN-BLANCO et al., 2006).

Grandes diferenças regionais são encontradas quando na análise dos frutos de noni, decorrentes de fatores ambientais locais, como solo, radiação, temperatura, umidade, corrente de ar, e também das características do fruto, como ponto de maturação e condições de armazenamento o que reafirma a importância do estudo da composição do frutos nas diferentes regiões do Brasil (PALIOTO et al., 2015).

Em São Luís-MA, em solo deficiente em matéria orgânica e em micro e macronutrientes e teores elevados de ferro, manganês e zinco, os nutrientes encontrados no frutos de noni foram principalmente potássio e manganês, também foram encontrados nitrogênio, enxofre, fósforo, cálcio, magnésio, ferro, sódio, bário e zinco em menores quantidades. Os teores de macronutrientes, micronutrientes e sódio diminuiram com a idade dos frutos até a maturação (SILVA et al., 2011). Correia et al. (2011) caracterizaram o fruto de noni cultivado no estado do Ceará e encontrou alto teor de compostos fenólicos e vitamina C e baixos teores de proteínas e lipídeos. O fruto possuiu em sua composição o predominio de carboidratos e dentre as proteínas identificadas, a pectina foi a que obteve maior quantidade. Frutos maduros de noni cultivados em Maringá-PR apresentaram 10% de proteínas, 22,19% de lipídios, 5,81% de carboidratos, 89,16% de umidade e 0,75% de cinzas (PALIOTO et al., 2015).

3.5.3 Atividades biológicas do noni e de seus compostos isolados

Cada parte da planta, desde suas raízes até as sementes, é amplamente utilizada na medicina popular, com vários efeitos terapêuticos já relatados (WANG et al., 2002). Esta pesquisa foi feita na base de dados do PubMed, SciELO and Periódicos CAPES, no período de abril a maio de 2016, onde foram encontrados 505 artigos sobre *M. citrifolia*, 92 deles estão relacionados com os princípios biológicos. Dentre eles, destacam-se o papel antibacteriano, antiviral, antifúngico, antitumoral, analgésico, anti-inflamatório, imunomodulador e neuroprotetor (HIRAZUMI & FURUSAWA, 1999; ZAIDAN et al., 2005; AKIHISA et al., 2007; PALU et al., 2008; JAINKITTIVONG et al., 2009; BASAR et al., 2010; HARADA et al., 2009; PACHAURI et al., 2013). Nos últimos anos, vários trabalhos in vitro e in vivo foram realizados com o intuito de comprovar as atividades biológicas do noni e de seus compostos

isolados, e os resultados obtidos têm sido promissores. Esta revisão pontua as atividades farmacológicas de *M. citrifolia* já descritas na literatura.

3.5.3.1 Atividade imunomoduladora

Algumas plantas apresentam compostos que têm atividade imunomoduladora, ou seja, possuem substâncias capazes de promover ou suprimir a resposta imunológica do organismo, como por exemplo, podem influenciar no mecanismo de produção de citocinas. Dentre as plantas, *M. citrifolia* destaca-se por sua atividade imunoestimulante, tanto relacionadas à resposta celular como humoral.

De acordo, com Hirazumi & Furusawa (1999), a administração concomitante do suco do noni com drogas imunossupressoras reduziu o efeito imunoestimulante deste, o que confirma sua ação como agente imunomodulador, sendo, portanto, capaz de interferir na resposta imunológica, em diferentes estados patológicos.

Tanto o extrato hidroalcoólico quanto o aquoso dos frutos de noni aumentaram a proliferação de esplenócitos in vitro e estimularam a atividade de linfócitos B e T (NAYAK & MENGI, 2010). Já em esplenócitos isolados de ratos isogênicos F344 de meia idade, quando tratados com o suco do fruto de noni sem sementes, houve diminuição da produção de IL-2 e INF-γ, além da diminuição da atividade linfoproliferativa. No entanto, o suco com sementes apresentou efeitos contrários, com aumento da linfoproliferação em ratos jovens e velhos e aumento da produção das citocinas (PRATAP et al., 2016).

Palu et al. (2008) relataram o efeito imunomodulador de *M. citrofolia* in vitro, o suco comercial (Tahitian Noni Juice-TNJ) e o sumo concentrado dos frutos ativaram os receptores de canabinóide 2 (CB2), mas inibem os receptores de canabinóides 1 (CB1), de maneira dose-dependente. Também observaram que em estudo in vivo, a administração oral do TNJ *ad libitum* durante 16 dias reduziu a produção de IL-4 e aumentou a produção de IFN-γ. Estes resultados sugerem que o noni é capaz de modular o sistema imune, exercendo efeitos benéficos em condições que envolvem respostas imunes inadequadas.

In vivo, o noni induziu a um estado imunitário predominantemente Th1, com efeito promotor desta resposta quando combinado com a citocina INF-γ , mas com essa atividade suprimida quando combinado com IL-4 e IL-10, citocinas do perfil Th2 (FURUSAWA et al., 2003). Também foi demonstrado que o extrato do fruto de *M. citrifolia* suprimiu a resposta

celular em tumor ascítico que havia sido tratado previamente com substâncias imunossupressoras. Além disso, inibiu a produção de IL-2 nesses animais e reduziu a quantidade de células natural killer em camundongos normais (MURATA et al., 2014).

Em animais com tumor de Lewis e tratados com substância rica em polissacarídeos (noni-ppt) isolada do suco de fruta de noni verificou-se que essa substância foi capaz de estimular a liberação de várias citocinas, incluindo TNF-α, IL-1β, IL-10, IL-12p70 e IFN-γ, além de óxido nítrico. Por outro lado, diminuiu a liberação de IL-4 e não teve nenhum efeito na produção de IL-2 (HIRAZUMI & FURUSAWA, 1999).

3.5.3.2 Atividade antitumoral

M. citrifolia é usada na medicina popular como suplemento alimentar em pacientes com diferentes tipos de câncer. Diversos trabalhos têm demonstrado a ação desta planta sobre células tumorais, sobre vias envolvidas na resposta imunológica, como a supressão de ciclooxigenase 2 (COX2), marcadores inflamatórios e aumento do gene supressor de tumor (LIM et al., 2016). A interferência do noni também se estende à inibição de mutações gênicas: compostos carcinogênicos ligam-se de forma covalente ao DNA do indivíduo, formando estruturas denominadas adutos que, se não reparados, provocam mutações. O noni pode ser usado para prevenir a formação dessas estruturas (WANG & SU, 2001).

O efeito antiproliferativo do noni já foi descrito em várias linhagens celulares, como em células de rim de hamster (BHK), células Vero, Hep2, MCF7, neuroblastoma e células de melanoma B16-F10 (LAN5) (ARPORNSUWAN & PUNJANON, 2006; CANDIDA et al., 2014).

O suco do fruto de noni tem a capacidade de regular negativamente, e de maneira acentuada, a expressão da proteína HIF-1α induzida por manganês, em células da linhagem A549 de câncer de pulmão humano. Isso sugere os efeitos benéficos de noni sobre neoplasias pulmonares em que o manganês e a superexpressão de HIF-1α desempenham papéis fundamentais, já que a exposição ao manganês é um fator de risco para muitas doenças pulmonares (JANG, 2012).

O suco de noni também reduziu a expressão do receptor do fator de crescimento epidérmico (EGFR), um biomarcador de adenocarcinoma de pulmão (LIM et al., 2016), além de ter ação no carcinoma pulmonar de Lewis em camundongos singenéticos (HIRAZUMI et

al., 1994). Na composição química do suco encontra-se uma substância rica em polissacarídeos, o noni-ppt, que possui atividade antitumoral frente à carcinomatose peritoneal pulmonar de Lewis (LLC). A administração terapêutica do noni-ppt aumentou significativamente a sobrevivência de camundongos portadores deste tumor (HIRAZUMI & FURUSAWA, 1999).

Em ensaios in vitro, o extrato da folha fresca de *M. citrifolia* apresentou efeito inibitório sobre células KB (carcinoma epidermoide humano), HeLa (carcinoma cervical humano), MCF-7 (adenocarcinoma mamário) e HepG2 (carcinoma hepatocelular humano), bem como em linhagem de células Vero (rim de macaco-verde africano), podendo agir como um suplemento alimentar na quimioprevenção contra câncer epidermóide e cervical (THANI et al., 2010). Já a fração acetato de etila do extrato inibiu a proliferação de células MCF-7 e MDA-MB-231, ambas associadas ao adenocarcinoma mamário. Esse efeito é atribuído ao aumento do número de células em apoptose e à estagnação das fases do ciclo celular (SHARMA et al., 2016).

Em cultivo de células de adenocarcinoma de cólon humano (Caco-2), o tratamento com as frações etanólica e acetato de etila do extrato do fruto de noni reduziram a oxidação intracelular e a inflamação, através da redução de espécies reativas de oxigênio intracelulares, e suprimiu significativamente a produção de COX-2, IL-8 e da prostaglandina E2 (HUANG et al., 2015).

O suco de noni em camundongos transgênicos MMTV-Neu reduziu significativamente o peso e o volume do tumor de mama HER2-positivo. No entanto, a latência desse tumor e a incidência de metástases não foram afetadas pelo tratamento. Esses resultados, ao serem extrapolados para seres humanos, sugerem que mulheres que consomem o suco de noni com outros propósitos medicinais não teriam risco maior ou menor de desenvolver a doença, mas caso apresentassem tal neoplasia mamária, o suco de noni seria capaz de melhorar a resposta ao tratamento (CLAFSHENKEL et al., 2012).

M. citrifolia apresentou potencial citotóxico em células do tumor de Ehrlich em camundongos BALB/c, demonstrando ser útil no tratamento de carcinoma mamário, seja utilizado isoladamente ou em conjunto com a droga doxorrubicina, um potente agente anticâncer. Seu efeito é resultante da indução da apoptose (TASKIN et al., 2009).

Quando utilizado no tratamento do sarcoma 180 (S-180) ou tumor de Crocker em camundongos, o noni apresentou atividade antitumoral com taxa de cura de entre 25 a 45%, e

mostrou efeito sinérgico ou aditivo quando combinado com um largo espectro de fármacos quimioterapêuticos (FURUSAWA et al., 2003).

O damnacanthal isolado a partir da fração clorofórmica do extrato da raiz de *M. citrifolia* inibe significativamente a reprodução de células RAS, precursoras de diversos tumores malignos (HIRAMATSU et al., 1993). Em células MCF-7, esse composto apresentou atividade antiproliferativa após 72 horas de tratamento, em uma concentração de 8,2µg/ml, e induziu a interrupção do ciclo celular na fase G1, além do processo de apoptose, através da ativação do gene p21 e caspase-7 (AZIZ et al., 2014). Também inibiu o crescimento de várias linhas celulares de câncer: câncer colorretal (HCT-116), adenocarcinoma de cólon humano (HT-29), câncer de mama (MCF-7) e câncer de prostáta (PC-3), de forma dose-dependente e tempo-dependente (NUALSANIT et al., 2012; SUKAMPORN et al., 2016).

Um estudo clínico de fase I, envolvendo pacientes em estágio avançado de câncer, buscou avaliar qual a dose máxima diária do damnacanthal recomendada a esses pacientes. A dose máxima tolerada foi de 3 gramas (seis cápsulas de 500 mg do extrato seco do fruto, quatro vezes ao dia), embora não tenha sido relatado nenhum caso de toxicidade da dose. A ingestão de três ou quatro cápsulas, quatro vezes ao dia, é recomendada no controle da fadiga, dor e manutenção da função física. A escopoletina, um componente bioativo do extrato do fruto do noni, é mensurável no sangue e na urina após sua ingestão, e pode ser utilizado no estudo da farmacocinética do noni em pacientes com câncer (ISSELL et al., 2009).

Também se discute o papel dos fungos presentes na planta na inibição de células tumorais, conforme observado por Wu et al. (2015), que demonstraram que endófitos presentes nas folhas de *M. citrifolia* inibiram o crescimento de linhagens de células de carcinoma em humanos.

3.5.3.3 Atividade antidiabética

Cada vez mais a medicina alternativa ganha adeptos entre os indivíduos diabéticos e, isso tem estimulado um número crescente de pesquisas partindo deste conhecimento popular. A ingestão de determinados tipos de alimentos e medicamentos tradicionais como o noni pode diminuir o risco do indivíduo desenvolver diabetes mellitus tipo 2 (OWEN et al., 2008). Além do efeito preventivo, o uso do noni como fonte alimentícia pode ser uma forma de tratamento nestes indivíduos (LEE et al., 2012).

Nerurkar et al. (2012) investigaram os efeitos antidiabéticos de *M. citrifolia* em camundongos C57BL/6 tratados com suco fermentado de noni, que demonstrou melhorar o metabolismo da glicose, através da interferência em FOXO1, um fator de transcrição da família forkhead BOX O (FOXO1). FOXO1 estimula a gliconeogênese no fígado no estado de jejum e durante a alimentação a insulina irá fosforilar e inativar a FOXO1, noni agiu sobre ele através do estímulo a essa via de fosforilação.

Hosfall et al. (2008) observaram que o uso do suco de noni no tratamento de diabetes induzida em ratos levou à redução dos níveis de glicose no sangue, e que o noni possui ação sinérgica quando utilizado em terapia combinada com a insulina.

Os efeitos hipoglicemiantes dos constituintes químicos das raízes de *M. citrifolia* foram avaliados em casos de diabetes induzida por estreptozotocina em ratos. A fração butanólica do extrato das raízes reduziu os níveis de glicose no sangue, sendo esse efeito atribuído aos compostos damnacanthal-3-O-beta-D-primeverosida e lucidina 3-O-beta-D-primeverosida (KAMIYA et al., 2008). Efeito semelhante foi atribuído ao sumo da fruta fermentado, que demonstrou, além de propriedade hipoglicemiante, efeito hepatoprotetor em ratos diabéticos (NAYAK et al., 2011).

3.5.3.4 Atividade antiobesidade

Um estudo in vitro demonstrou que tanto o extrato das folhas como dos frutos de noni podem ser utilizados como agentes no controle do peso corporal, pois ambos inibiram a lipoproteína lipase (PAK-DEK et al., 2008). Também foi avaliado o efeito do extrato das folhas de noni no tratamento de ratos da linhagem Sprague-Dawley com obesidade induzida por dieta rica em gordura. Após 9 semanas de tratamento, foram observados efeitos positivos sobre a adiposidade, teor de gordura fecal, níveis séricos de triglicerídeos, insulina e leptina, demonstrando que o noni melhorou significativamente os distúrbios metabólicos causados pela obesidade (GOODA SAHIB JAMBOCUS et al., 2016).

Em outro estudo, a administração dos extratos das folhas, raiz ou frutos de noni (1000 ou 500 mg/kg), em ratos com hiperlipidemia induzida por triton ou por dieta rica em gordura, causaram redução nos níveis de colesterol total e triglicerídeos. Os dados indicam que o efeito antidislipidêmico dos extratos foi meditado através da inibição da biossíntese, secreção e

absorção de lipídios. Isto pode ocorrer possivelmente devido à presença de constituintes antioxidantes presentes na planta (MANDUKHAIL et al., 2010). O óleo da semente de noni também reduziu o colesterol total e triglicerídeos em ratos normolipidêmicos e hiperlipidêmicos (PAZOS et al., 2011).

Em hamsters submetidos à dieta rica em gordura e colesterol, a ingestão do suco de noni diminuiu os níveis de triacilglicerol e colesterol no soro, a deposição de lipídios no fígado e o índice aterogênico (LIN et al., 2012). Em adultos fumantes, o consumo do suco de noni reduziu os níveis de colesterol, com diminuição do LDL e de triglicérides, além dos níveis plasmáticos de proteína C reativa de alta sensibilidade (hsPCR), um importante parâmetro para avaliação de risco cardíaco e ferramenta de prognóstico em doenças cardíacas. O suco foi, portanto, capaz de suavizar a dislipidemia induzida pelo fumo (WANG et al., 2012).

3.5.3.5 Atividade antibacteriana e antisséptica

Hidrocolóides irreversíveis à base de alginato de cálcio são utilizados na odontologia como material de moldagem na confecção de restaurações dentárias. O uso do extrato de *M. citrifolia* com o pó hidrocolóide no preparo de moldes dentários diminuiu a contaminação por microrganismos, sem prejudicar a qualidade do material (AHMED et al. 2015).

Na fruticultura, observou-se que a imersão de cubos de manga em suco de noni teve efeito antimicrobiano sobre bactérias mesófilas, bolores e leveduras, mostrando que essa pode ser uma tecnologia potencialmente valiosa para a descontaminação de superfícies de frutas frescas de corte (ULLOA et al., 2015).

Na produção animal, foi avaliado o efeito antibacteriano do extrato das folhas de noni sobre *Salmonella typhimurium* na criação de codornas japonesas. Sua ação contra esse agente patogênico foi observada através da redução da mortalidade de codornas e do aumento da produção de ovos (RETNANI et al., 2014).

Em bezerros recém-nascidos alimentados com purê de noni, foi evidenciado papel antibacteriano contra *Escherichia coli*, revelando sua utilidade em sistemas de produção em que o uso de antibióticos é mais restrito (SCHÄFER et al., 2008). O extrato etanólico do fruto também possui atividade contra *E. coli*, além de inibir o crescimento de *Staphylococcus aureus* (CANDIDA et al., 2014).

Estudos in vitro evidenciaram o potencial antibacteriano de *M. citrifolia* sobre *S. aureus* mesmo quando estes apresentam resistência à meticilina (ZAIDAN et al., 2005). O extrato aquoso das folhas apresentou atividade antibacteriana moderada (SERAFINI et al., 2011). Já o extrato etanólico bruto de noni e sua fração hexânica possuem atividade antituberculose (SALUDES et al., 2002).

3.5.3.6 Atividade antifúngica

O extrato do fruto de *M. citrifolia* contra *Candida albicans* mostrou efeito inibitório, que variou conforme a concentração e tempo de contato (JAINKITTIVONG et al., 2009).

3.5.3.7 Atividade antiviral

O desenvolvimento de medicamentos complementares ou alternativos para o tratamento de enfermidades causadas por vírus ainda é uma grande necessidade, por isso, a presença de compostos com atividade antiviral em plantas medicinais constituem potenciais alvos terapêuticos. Em estudo com cultura de células do vírus da hepatite C foi demonstrado que o extrato metanólico das folhas de noni e suas frações hexânica e acetato de etila possuem atividade antiviral (RATNOGLIK et al., 2014).

Compostos isolados a partir do extrato metanólico dos frutos exibiram efeitos inibitórios moderados contra o vírus Epstein-Barr (AKIHISA et al., 2007). Já em células MT-4 infectadas com o vírus HIV-1 e tratadas com extratos metanólico e etanólico dos frutos (suco e extrato seco) de *M. citrifolia*, não foi observada nenhuma atividade anti viral (SELVAM et al., 2009).

3.5.3.8 Atividade leishmanicida

A busca por novos medicamentos para o tratamento da leishmaniose tem aumentado devido à elevada frequência de casos de resistência aos medicamentos utilizados em áreas endêmicas, além dos seus efeitos colaterais e as complicações decorrentes do tratamento (SERENO et al., 2007).

Vários trabalhos in vitro e in vivo têm demonstrado o efeito leishmanicida de *M. citrofolia*. Almeida-Souza et al. (2016), ao avaliarem a ação do extrato dos frutos de noni sobre formas promastigotas de *Leishmania infantum*, verificaram que este não era tóxico em concentrações até 2 µg/mL, com IC_{50} de 260.5 µg/mL. Além disso, foi evidenciado que a

administração do extrato levou a alterações ultraestruturais intensas nas formas promastigotas, acarretando na destruição do parasito.

O potencial leishmanicida do noni também foi avaliado na infecção experimental em modelo murino. Em camundongos BALB/c infectados com *L. amazonensis* e tratados com extrato bruto dos frutos de noni foi observada intensa produção de matriz extracelular na área da lesão, caracterizada pelo predomínio de colágeno maduro; além da ausência de alterações histológicas significativas nos demais órgãos analisados. Em contraste, animais infectados e não tratados apresentaram destruição da matriz extracelular, hiperplasia da polpa branca do baço e infiltrado inflamatório no fígado. Os resultados evidenciam que *M. citrifolia* proporciona um controle eficaz das lesões causadas pela infecção (MONDEGO-OLIVEIRA et al., 2015).

Dois estudos clínicos em pacientes humanos com leishmaniose cutânea foram realizados, para avaliar a eficácia de extratos metanólicos do caule, folhas e frutos de *M. citrifolia*. Foi feita uma preparação tópica a 1% à base dos extratos, aplicada nas lesões cutâneas dos pacientes. Foi observada melhora acentuada e regressão das lesões em 50% dos pacientes tratados com extrato do caule, 30% dos pacientes apresentaram melhora moderada e 20% melhora significativa. As folhas e os extratos de fruta não foram muito eficazes no tratamento (SATTAR et al., 2012; SIDDIQUI et al., 2014).

3.5.3.9 Atividade anti-inflamatória

A ação anti-inflamatória de *M. citrifolia* foi demonstrada em modelos in vitro e in vivo em várias patologias associadas à inflamação. Os efeitos anti-inflamatórios de suco de noni foram investigados in vitro através da mensuração da produção de óxido nítrico e de prostaglandina E2 por macrófagos ativados, e da inibição da COX 1 e 2; e in vivo sobre o modelo de edema de pata induzido por carragenina em ratos. A administração do suco reduziu o edema da pata, inibiu diretamente a ação de COX 1 e 2 e a produção de óxido nítrico e prostaglandina E2 em células J774.G8 de forma dose-dependente, demonstrando a ação anti-inflamatória do noni que também pode ser reforçada por efeitos antioxidantes (DUSSOSSOY et al., 2011).

Nualsanit et al. (2011) também demonstraram o efeito anti-inflamatório in vitro e in vivo do extrato de noni e do seu composto isolado damnacanthal, que reduziu edema de pata e ratos e camundongos, e regulou negativamente a atividade do fator nuclear kappa B (NF-kB)

induzida por lipopolissacarídeos. Foi observado também que a produção de citocinas pró-inflamatórias, COX2 e da enzima óxido nítrico sintase indizível foram suprimidas pelo damnacanthal.

O extrato aquoso das folhas de noni reduziu significativamente a migração de leucócitos e devido a essa ação anti-inflamatória pode ser utilizado como alternativa para situações de dor e inflamação, incluindo as relacionadas com estados de oxidação (SERAFINI et al., 2011). Já o extrato alcoólico dos frutos inibe a produção da metaloproteinase de matriz 9 (MMP-9) por monócitos humanos após estímulo com LPS, efeito semelhante ao da hidrocortisona, medicamento utilizado no tratamento de artrite. Esse resultado aponta a eficácia do noni na diminuição do processo inflamatório em casos de artrite (BASAR et al., 2010).

O extrato etanólico das raízes de *M. citrifolia* pode proteger contra as doenças inflamatórias do cólon (HUANG et al., 2015). Além disso, as frações etanólica e acetato de etila do extrato do fruto de noni regularam negativamente as respostas inflamatórias durante a infecção por *Helicobacter pylori* (HUANG et al., 2014).

A fração clorofórmica do extrato da raiz possui efeitos anti-inflamatórios, reduzindo significativamente o edema da pata induzida por histamina, na concentração de 3 g/kg. Já o damnacanthal, utilizado isoladamente, reduz o edema nas concentrações de 10 a 100 mg/kg (OKUSADA et al., 2011). Da mesma forma, outras antraquinonas isoladas a partir da fração metanólica do extrato dos frutos de noni exibiram potente atividade anti-inflamatória, em modelo de inflamação induzida em camundongos (AKIHISA et al., 2007).

3.5.3.10 Atividade antinociceptiva e analgésica

O extrato aquoso liofilizado das raízes de *M. citrifolia* demonstrou efeitos analgésicos e alterações comportamentais significativos e dose-dependentes em camundongos (YOUNOS et al., 1990). O extrato aquoso da folha de noni mostrou ação antinociceptiva no teste de contorção induzida por ácido acético (SERAFINI et al., 2011).

A fração clorofórmica do extrato das raízes e o damnacanthal isolado da raiz têm efeitos antinociceptivos, reduzindo o comportamento relacionado à dor no teste da formalina. Seus efeitos são mediados em parte pela interferência em receptores de histamina (OKUSADA et al., 2011).

Uma solução a 10% de purê do frutos de noni concentrado adicionada à agua de beber de ratos com artrite, reduziu a sensibilidade à dor de forma comparável ao tramadol, droga analgésica central, mostrando que o suco de noni é eficaz na diminuição da dor causada pela artrite (BASAR et al., 2010).

3.5.3.11 Atividade antioxidante

O noni pode ser utilizado como uma fonte valiosa de antioxidantes naturais (THOO et al. 2013). O sumo dos frutos de noni, com ou sem sementes, é capaz de modular a resposta imunológica mediada por células e a atividade de enzimas antioxidantes (PRATAP et al., 2016). Em modelo com células somáticas de *Drosophila melanogaster*, os efeitos protetores do suco de noni são dependentes da concentração, indicando uma correlação dose-resposta que pode ser atribuída ou a uma poderosa ação antioxidante ou à capacidade de captar radicais livres (FRANCHI et al., 2013).

O extrato hidroalcoólico de *M. citrifolia* demonstrou atividade antioxidante com grande eliminação de superóxido de oxigênio (CALZUOLA et al., 2006). O tratamento oral com 50 mg/kg do extrato metanólico bruto das folhas de noni durante 14 dias aumentou a atividade de enzimas antioxidantes, tais como a catalase, glutationa peroxidase e superóxido dismutase em camundongos portadores de linfoma (ANITHA & MOHANDASS, 2006).

As propriedades antioxidantes do suco dos frutos de noni em humanos envolvem a diminuição da acidose tecidual, reequilíbrio acidobásico, melhora da atividade redox mitocondrial, maior oxigenação dos tecidos e melhoria do metabolismo corporal como um todo (CARAMEL et al., 2015). Em fumantes, a ingestão de noni teve efeitos antioxidantes, com redução dos níveis do ânion superóxido (SAR) e hidroperóxido lipídico (LOOH) do plasma. SAR é uma espécie reativa de oxigênio capaz de danificar a estrutura celular, já LOOH está associado à peroxidação lipídica (WANG et al., 2009). Apesar do potencial antioxidante do noni ter sido comprovado, em alguns estudos, como os de Thani et al. (2010), o efeito antioxidante não foi evidenciado.

3.5.3.12 Atividade neuroprotetora

A ação neuroprotetora do noni foi testada em coelhos, utilizando o modelo de doenças degenerativas induzidas por hidrocefalia, e seu efeito foi comparado com a droga nemantina. Para detectar a degeneração neuronal e apoptose no tecido periventricular do quarto ventrículo

de coelhos, foi realizada imunohistoquímica para marcação da proteína-2 associada à microtúbulos e da caspase-3. Foi observada que a intensidade da marcação na imuno-histoquímica foi significativamente maior no grupo tratado com noni, e que o tratamento reduziu o número de células de caspase-3-positivo em coelhos com hidrocefalia, enquanto a memantina não teve nenhum efeito. Estes achados sugerem que o noni possui efeitos inibitórios mais pronunciados sobre doenças neurodegenerativas induzida por hidrocefalia quando comparados à memantina (KÖKTÜRK et al., 2013).

Os efeitos da proteção neuronal do noni contra o estresse isquêmico foi avaliado em dois experimentos. No primeiro deles, o suco de noni teve um efeito preventivo contra o estresse isquêmico cerebral, demonstrado através da redução da área de infarto e menor déficit neurológico em camundongos (HARADA et al., 2009). No segundo, o tratamento com o suco aumentou os níveis de insulina no soro, facilitando a secreção da mesma após o stress isquêmico e possivelmente atenuando o desenvolvimento de intolerância à glicose (HARADA et al., 2010).

O efeito neuroprotetor da fração acetato de etila do extrato dos frutos de noni também foi demonstrado. A administração da fração em camundongos com disfunção cognitiva induzida por beta-amilóide, na dose de 400 mg/kg, aumentou os níveis de serotonina, dopamina e enzimas antioxidantes (MURALIDHARAN et al., 2010).

A administração do extrato etanólico dos frutos de noni e suas frações clorofórmica e acetato de etila melhoraram a memória e o fluxo sanguíneo cerebral, atenuaram o estresse oxidativo e a atividade da acetilcolinesterase após estímulo com escopolamina, demonstrando a utilidade do uso do noni em problemas de perda de memória (PACHAURI et al., 2012). Em camundongos com Doença de Alzheimer induzida por injeção intracerebral de estreptozotocina, a fração acetato de etila preveniu a perda de memória, melhorou o metabolismo energético no cérebro e a neurotransmissão colinérgica, além de apresentar ação antioxidante (PACHAURI et al., 2013).

A ingestão do suco de noni teve ação protetora no cérebro de camundongos contra a diminuição da função cognitiva induzida por estresse, principalmente por sua influência no giro denteado do hipocampo, melhorando a redução da densidade dos vasos sanguíneos que ocorre por estresse neste local do cérebro (MUTO et al., 2010).

3.5.3.13 Atividade cicatrizante

A atividade cicatrizante de *M. citrifolia* foi avaliada tanto com a aplicação tópica de pomadas à base do extrato das folhas de noni bem como com a administração oral de extrato etanólico das folhas e sumo dos frutos. Em todos os casos, foram observadas evidências de um processo cicatricial mais acelerado, como a diminuição no tempo de contração das feridas e do tempo de reepitelização, que demonstram os efeitos terapêuticos do noni na cicatrização (NAYAK et al., 2007; NAYAK et al., 2009; PALU et al., 2010).

3.5.3.14 Atividade antialérgica

O potencial antialérgico de *M. citrifolia* foi avaliado em camundongos, através da inoculação de substâncias alérgenas (dinitrofluorobenzeno e cloreto de picrila) na orelha dos animais. Foi avaliado o efeito dos extratos etanólico das folhas e frutos, administrados via oral, em reações de hipersensibilidade imediata e tardia. Nos animais tratados com noni foi observada diminuição do edema causado pelas substâncias estranhas, tanto imediatamente após o estímulo como na fase tardia, demonstrando que o extrato diminui a degranulação de mastócitos e a liberação de histamina ou outras substâncias inflamatórias. O noni pode ser um potencial antialérgico, contra reações de sensibilidade tardia ou dermatite atópica (MURATA et al., 2014).

3.5.3.15 Atividade antiangiogênica

Um dos fatores que determina a ação de noni em neoplasias é o seu potencial antiangiogênico. O extrato metanólico das folhas e frutos de *M. citrifolia*, bem como sua fração clorofórmica do extrato metanólico do fruto, têm atividade antiangiogênica in vivo, sendo esse efeito atribuído em partes à escopoletina, presente no noni (BEH et al., 2012).

Hornick et al. (2003), no modelo de matriz tridimensional do coágulo de fibrina, utilizando veias placentárias humanas e tumor de mama como fontes para o desenvolvimento de vasos, avaliaram o potencial antiangiogênico do suco de noni, que inibiu o início da angiogênese e levou à ruptura das redes vasculares recém formadas. Já Piaru et al. (2012), observou pouca atividade antiangiogênica em anel aórtico de rato, com diferentes concentrações do óleo essencial de *M. citrofolia*.

3.5.3.16 Atividade antiemética e antináusea

A eficácia do noni na prevenção de náuseas e vômitos foi demonstrada em pacientes com alto risco para desenvolver esses sintomas após vários tipos de cirurgia. O extrato de noni na dose de 600 mg teve ação anti-emética profilática ao reduzir a incidência de náuseas no pós-operatório imediato (PRAPAITRAKOOL & ITHARAT, 2010).

3.5.3.17 Atividade anti-úlcera gástrica e esofagite

Mahattanadul et al. (2011) avaliaram o efeito do extrato aquoso dos frutos de noni e do seu isolado escopoletina em modelos de inflamação gastro-esofágicos em ratos (esofagite por refluxo ácido, gastrite aguda induzida por etanol e serotonina e úlcera gástrica crônica induzida pelo ácido acético). As concentrações de 0,63 a 2,50 g/kg do extrato inibiram de forma significativa o quadro de esofagite por refluxo ácido, reduziram a formação de lesões gástricas induzidas por álcool e pela serotonina e aceleraram a cicatrização de úlceras gástricas induzidas por ácido acético de modo semelhante à dos medicamentos convencionais (ranitidina e lansoprazole). A escopoletina também produziu resultados semelhantes, demonstrando que tanto o extrato como a substância isolada podem ser benéficas como um potencial agente preventivo e terapêutico para doenças inflamatórias gastro-esofágicas.

3.5.3.18 Atividade antihelmíntica

Os extratos aquosos e etanólico do fruto de *M. citrifolia* auxiliaram no combate a *Ascaridia galli* in vitro e em frangos naturalmente infectados (BRITO et al., 2009).

3.5.3.19 Atividade antimutagênica

Foi verificada ação antimutagênica e antirecombinagênica do suco dos frutos de noni em células somáticas de *D. melanogaster* (FRANCHI et al., 2013).

3.5.3.20 Atividade antipsicótica

Pandy et al. (2012) demonstraram o efeito antidopaminérgico do extrato metanólico dos frutos de noni em camundongos Swiss administrado via oral por 21 dias juntamente com apomorfina e a metanfetamina, sugerindo que o noni possui atividade anti-psicótica e que pode ser utilizado no tratamento de distúrbios psiquiátricos. Esse mesmo extrato também inibe o efeito compensador da heroína (NARASINGAM et al., 2016).

3.5 .3.21 Atividade ansiolítica

O extrato do fruto pode ser visto como alvo para desordens de ansiedade. Em estudo in vitro realizados por Deng et al. (2007), o extrato metanólico bruto dos frutos de noni mostrou afinidade significativa para os receptores do neurotransmissor ácido gama-aminobutírico A (GABAA). Os resultados demonstram a presença de um ligador competitivo, que pode ligar-se ao receptor GABAa como um agonista e assim causar efeitos ansiolíticos e sedativos.

O efeito do extrato do fruto como ansiolítico foi comprovado quando utilizado como suplemento alimentar em modelo animal (KANNAN et al., 2014). Porém ressalta-se a necessidade de considerar também os efeitos tóxicos que o fruto pode ter no organismo (TIN & WIWANITKIT, 2014).

3.5.3.22 Atividade fotoprotetora

A exposição à radiação solar, particularmente a radiação ultravioleta (UV), tem uma variedade de efeitos nocivos na saúde humana. Por isso, o efeito fotoprotetor do noni também passou a ser alvo de pesquisas. Compostos iridoides isolados da folha de noni demonstraram ação inibidora sobre a proteína AP-1 induzida por radiação UVB (SANG et al., 2003). Essa proteína, quando ativada, estimula a transcrição de genes de enzimas desintegradoras da matriz, como as metaloproteínas, que degradam estruturas como o colágeno (ANGEL et al., 2001).

O efeito fotoprotetor de formulações tópicas com o extrato de *M. citrifolia* foi avaliado por Serafini et al. (2014) em ratos expostos à radiação UVA-UVB. Após 7 dias de tratamento com noni e 20 h após a exposição aos raios ultravioleta (UV), foram avaliados os danos na barreira da pele, formação de eritema e alterações histológicas. Os resultados mostraram que as formulações que contêm o extrato protegeram a pele dos animais contra os danos induzidos pela radiação UV.

Em um ensaio clínico com 25 voluntários, submetidos à indução de eritema por radiação UVB e tratados com formulação tópica contendo extrato etanólico das folhas de noni, foi observado efeito protetor, que se mostrou seguro para utilização tópica e para tratamento de injúrias causadas por UVB na pele (WEST et al., 2009).

3.5.3.23 Atividade antirrugas

A antraquinona 1,4-antraquinona-di-hidroxi 2-metoxi-7-metil, derivada do extrato dos frutos de noni, pode ser usada como um novo agente antirrugas, devido à sua capacidade de

induzir a atividade biossintética de componentes da matriz extracelular conforme demonstrado em pesquisas realizadas por Kim et al. (2005), onde verificaram que esse composto aumentou a produção de pro-colágeno tipo I e glicosaminoglicanos e reduziu a expressão de metaloproteinase da matriz (colagenase-1) em fibroblastos dérmicos humanos. Além disso, a nanoemulsão elaborada a partir desse composto aumentou a síntese de pró-colágeno tipo I na pele de ratos.

3.5.3.24 Regeneração do tecido periodontal

Boonanantanasarn et al. (2014) avaliaram a ação regenerativa do extrato liofilizado das folhas de *M. citrifolia* no ligamento periodontal de dentes pré-molares e molares dos quais foram retiradas as raízes. O material, após higienização, foi colocado em cultivo celular em meio completo, meio suplementado com ácido ascórbico e meio suplementado com o extrato de noni para avaliação da proliferação celular, mineralização e síntese de proteínas. Os resultados mostraram que o extrato aquoso da folha de noni foi eficaz na indução de proliferação celular, síntese de proteínas, atividade da fosfatase alcalina e mineralização da matriz in vitro, tendo portanto, efeito osteoindutor para ossos e regeneração dos tecidos periodontais.

4 JUSTIFICATIVA

De um modo geral, as doenças com sinais neurológicos são um grande desafio na rotina médico veterinária e, dentre aquelas que mais comumente acometem os cães, destacam-se a cinomose e ehrliquiose. Em animais na fase neurológica da cinomose, os protocolos terapêuticos são insatisfatórios e normalmente a eutanásia do animal é recomendada. Na erliquiose, a fase neurológica, mais rara, não custuma ser muito estudada, o que também gera desafios no seu tratamento. Desta forma, são necessários estudos objetivando propor tratamentos alternativos para essas doenças, que sejam seguros, com baixo custo e fácil obtenção.

Na busca por tratamentos mais eficazes, a população utiliza plantas medicinais de forma empírica e a partir desse conhecimento popular muitas pesquisas encontraram novos fármacos. Uma vez que o Brasil possui grande biodiversidade vegetal, o uso de produtos naturais é amplamente difundido como suporte na terapêutica de inúmeras doenças ao agir diretamente contra agentes patogênicos, ou até mesmo ao melhorar a ação sistema imunológico de forma mais ampla. Desta forma, drogas vegetais que contenham substâncias com comprovada ação terapêutica são colocadas em evidência como possível solução para o tratamento de doenças.

A escassez de pesquisas que visem o tratamento de doenças neurológicas em cães resulta na indicação da eutanásia em muitos casos, o que causa grande sofrimento ao proprietário que considera o animal como um membro da família. Aliado a essa humanização e a validação científica dos efeitos imunomodulador, antiviral, antibacteriano e neuroprotetor com ação eficaz sobre doenças neurodegenerativas atribuídos ao noni (*M. citrifolia*) justifica-se a realização desta pesquisa no sentido de propor tratamento da cinomose e erlichiose.

5 HIPÓTESE

Morinda citrifolia é eficaz na remissão de sinais neurológicos em cães naturalmente infectados pela *Ehrlichia canis* e/ou pelo vírus da cinomose canina?

6 OBJETIVOS

6.1 Geral

- Avaliar a eficácia do noni (*Morinda citrifolia*) no tratamento de cães com sintomatologia neurológica infectados por *Ehrlichia canis* e pelo vírus da cinomose canina.

6.2 Específicos

- Avaliar parâmetros hematológicos e bioquímicos de cães tratados com noni e drogas convencionais para o tratamento de erlichia e cinomose;
- Identificar alterações histopatológicas e determinar por imunohistoquimica as regiões mais acometidas pelo vírus da cinomose canina;

Parte II

Capítulo I

Artigo "Evaluation of *Morinda citrifolia* effectiveness in the treatment of canine ehrlichiosis"

Submetido ao periódico *Annals of Microbiology* (INSS *1590-4261*), cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é B2, fator de impacto 1,232. O artigo foi elaborado e formatado conforme as normas do periódico supracitado, que seguem anexas ao trabalho.

Evaluation of *Morinda citrifolia* effectiveness in the treatment of canine ehrlichiosis

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Abstract

Canine ehrlichiosis is an infectious disease caused by Ehrlichia canis, which affects dogs in Brazil and around the world. It is transmitted by Rhipicephalus sanguineus, which transports the pathogenic bacteria to the host during blood meal digestion. Animals can present mild to severe clinical signs, depending on the stage of the disease. Tetracyclines are the preferred drugs used in treatment. *Morinda citrifolia* (noni) has bioactive compounds with antibacterial, antifungal and anti-inflammatory activities. The aim of the present study was to evaluate the effect of treatment with noni on dogs infected with *E. canis*. A study was carried out involving ten animals with clinical signs suggestive of ehrlichiosis, and which were parasitized by ticks. After clinical evaluation and laboratory exams, the animals were divided into two groups: Group 1 (G1: six dogs positive for E. canis infection and treated with the dry vegetable drug of

M. citrifolia, encapsulated in a compounding pharmacy) and Group 2 (G2: four dogs positive for E. canis infection and treated with the conventional drug doxycyclin and B complex vitamins). In G1, while there was an increase of platelet count levels in the treated dogs, there was no reduction in clinical signs. There were no significant changes in biochemical evaluation following treatment, revealing that noni did not cause liver or kidney damage. Although noni vegetable drug was not effective in treating canine ehrlichiosis, the treated animals showed a significant increase in platelet count levels. Noni has no therapeutic effect but could be useful as an adjuvant in the treatment of diseases that cause thrombocytopenia.

Key words: Diseases; Dogs; Treatment; Canine ehrlichiosis; Platelet.

Introduction

Canine ehrlichiosis is one of the main infectious diseases affecting dogs, and is highly prevalent in Brazil. It is caused by *Ehrlichia canis*, which causes immunosuppression in dogs and wild canids (Vieira et al. 2011). *E. canis* is an obligate intracellular microorganism that infects leukocytes and thrombocytes, leading to thrombocytopenia in hosts (Silva 2011) (Almosny 2002).

The most frequent clinical signs of ehrlichiosis are fever, appetite loss, dyspnea, petechiae and ecchymosis on the surface of the body, ophthalmic signs (uveitis), polyarthritis and neurological signs as convulsions, lack of motor coordination and paresis(Silva, 2015). Several drugs are used in its chemotherapy, however tetracyclines, particularly doxycycline, are the elected drugs for the treatment of this infection (Aiello 2001).

Natural products have traditionally been used as medicines and have attracted theattention of health care professionals, the pharmaceutical industry and scientific communities around the world. The use of new isolation and purification techniques has made bioactive compounds derived from natural products a target for the drug discovery process, as they represent an

unlimited source of chemical compounds for the treatment of both human and animal diseases (Cragg 1997; Heinrich and Guibbons 2001).

In this context, several experiments evaluating the in vitro and in vivo biological potential of many plants have been conducted. Among these *Morinda citrifolia* is worthy of note. Popularly known as noni, it is a plant native to South Asia and Australia, and was recently introduced in the tropics (Banerjee 2006).

Studies show that *M. citrifolia* has biologically activ compounds with antifungal (Banerjee et al. 2006), antibacterial (Leach et al. 1988, 1995), anti-inflammatory (Mckoy el al. 1995; Kim et al., 2004), immunomodulatory (Hirazumi et al.; 1996, Hokama 1993), analgesic (Basar, 2010) and antiviral (Ratnoglik et al. 2014) effects. All parts of the plant are used in traditional medicine, with the juice of the ripe fruit being the most used form. Due to the antibacterial properties of *M. citrifoli*a, an experiment was performed to evaluate the efficacy of the vegetable drug of this plant in the treatment of canine ehrlichiosis.

Materials and methods

The experiment was approved by the Animal Experimentation Ethics Committee of the Universidade Estadual do Maranhão, Brazil (protocol 17/2010). The animal owners were informed about the therapeutic protocols and, after agreeing to participate, signed a free and informed consent form according to the treatment chosen.

Collection and preparation of the vegetable drug of *Morinda citrifolia*

For the preparation of the dry extracts of *M. citrifolia*, fruit were 71 collected at the Fazenda Escola of Universidade Estadual do Maranhão (the Farm School of Maranhão State University), located in southern São Luís, Maranhão, Brazil (02 ° 35 023 'S, 44 ° 12,551 'W).

The city has a hot and humid equatorial climate, with two well-defined seasons: a rainy season from January to June, and a dry season from July to December. Rainfalls vary between 1700-2300 mm annually. Mean temperature is around 26°C, with a maximum temperature ranging

from 28 to 37°C and a minimum temperature between 20 and 23°C (Silva et al. 2010). Fruit collection was carried out in the morning, from June to November 2014, prioritizing fruit with a greenish color, soft pulp and bark integrity, and which were pest and insect free.

After collection, the fruit were washed with distilled water and dried in a hot chambre 37°C for 72 hours, before being grinded, weighed and packaged in sterile plastic bags. The vegetable drug was sterilized for 30 minutes and sent to a compounding pharmacy for encapsulation. Each capsule contained 500 mg of the vegetable drug.

To perform the therapeutic protocols ten animals with suggestive clinical signs of ehrlichiosis, which was subsequently confirmed by laboratory tests, IFAT were used. The clinical signs evaluated were fever, depression, anorexia, pale mucous membranes, uveitis, petechiae and ecchymosis in the ventral abdomen, and hind limb paresis. Further laboratory tests, such as complete blood count analysis, were also carried out to obtain the hematological profile of the animals before and after treatment with the vegetable drug.

Detailed symptomatic evaluation was performed according to the protocol described by (Feitosa, 2014). The selected animals were divided into two groups:

Group 1 (G1): six dogs, positive for *E. canis* infection, treated with *M. citrifolia* vegetable drug (500 mg, once a day, orally) for 30 days;

Group 2 (G2): four dogs, positive for *E. canis* infection, treated with conventional drugs - doxycycline (5-10 mg/kg, twice a day, orally) and B complex vitamins (0,1-0,2 mg/kg, twice a day, orally) for 30 days.

Clinical follow up of the animals was performed daily for one year, in order to determine the recovery percentage. Body condition score, temperature, mucous membranes, abdominal and the absence of nervous symptoms were evaluated.

Hematological, biochemical and indirect immunofluorescence analysis

For hematological evaluation, blood samples were processed in a Bio 1800 Vet (Bioeasy) hematology analyzer, according to the manufacturer's instructions, to obtain the erythrocyte and leukocyte count. A blood smear was performed for leucocyte (lymphocytes, eosinophils, monocytes, segmented), differentiated cells and *E. canis* morulae identification. Total plasma proteins were measured in a Coleman 295 spectrophotometer.

For the biochemical tests, serum samples were processed in a semi-automatic analyzer (Celm SBA 200) using commercial kits (Labtest), following the manufacturer's recommendations. For the determination of creatinine values, the colorimetric method through kinetic reaction with alkaline picrate was used, while for urea values thenzymatic colorimetric method was applied. Aspartate aminotransferase (AST) analanine transaminase (ALT) were measured by the kinetic method UV optimized at 37ºC.

For the indirect immunofluorescence assay, serum samples 115 and the Imunotest Ehrlichia (IFAT) diagnostic kit were used, in accordance with the manufacturer's instructions.

Statistical analysis

Serum variable fluctuation was analyzed as repeated measures over time. The model included the effects of treatment, time and the treatment/time interaction.

Data was plotted, summarized and presented in the form of graphs and tables, according to the observed variable. Analysis of variance (ANOVA) was performed, by comparing the average values of the hematological and biochemical variables of the two groups, depending on the treatment, using the paired t-test. Analysis was performed using the GraphPad INSTAT version 3.05 software, considering a significance level of 5% ($p < 0.05$).

Results

There was no remission of clinical signs in the group treated with *M. citrifolia* vegetable drug. One dog displayed severe neurological signs such as hind limbs paresis and convulsions and died 15 days after administration of the drug due to canine distemper virus coinfection. The

dogs in the other group showed no signs of clinical remission even after 40 days of treatment and so were submitted to conventional treatment for ethical reasons. As previously reported all the animals presented changes in hematimetric parameters before treatment. Although the treatment increased the platelet levels, it was not observed a statistical significance (Table 01). Three animals treated with the conventional drug showed a remission 136 of clinical signs and an increased platelet count 30 days after treatment. In addition to the treatment with doxycycline, all animals received B vitamin complex as a support therapy.

Biochemical analysis did not reveal significant changes in Biochemical parameters, suggesting that the vegetable drug is safe for use, as the animals did not present hepatotoxic and nephrotoxic effects (Table 2).

Discussion

Science today is marked by the optimization and refinement of the results of applicability searches. The significance and prevalence of canine ehrlichiosis in veterinary medicine and the applicability of alternative therapy models such as medicinal plants and natural products, have resulted in great improvements in the area of animal health. As a result, an increased global demand for herbal products has been observed in recent years (Deng et al. 2015).

Treatment with *M. citrifolia* vegetable drug was not effective in terms of the remission of clinical signs. Although the treatment did not produce satisfactory results, it did induce increased production of platelets. These results suggest that the noni vegetable drug can be an excellent option for the treatment of canine ehrlichiosis as a supplementary medication to conventional therapies. In this work was not possible to identify the compound responsible for the increase in platelets. This effect is probably due to presence flavonoid known by its antioxidant properties as previously described by (Almeida et al. 2016).

Studies conducted by (Tintino et al. 2013) have also reported that the ethanol extract of *M. citrifolia* showed no clinically relevant antimicrobial activity against *Escherichia coli* and

Pseudomonas aeruginosa. In contrast, (Silveira et al. 2011) reported that the hydroalcoholic extract of noni fruit displayed antibacterial activity against standard strain *Staphylococcus aureus*.

Natural plant products are useful due to their antibacterial activity and the fact that they enhance antibiotic activity (Gibbons 2004; Tintino 2013). The antimicrobial function of natural extracts is a result of the ability of such products to cause reduced microbial resistance, as they are composed of complex mixtures, which makes microorganism adaptation more difficult (Daferera et al. 2003). Noni antibacterial activity in vivo was not observed in the present study at the established dose and during the treatment period, as shown in post-treatment laboratory results. It is worth noting that while these in vitro studies were carried out with an extract of *M. citrifolia*, an in vivo study used the vegetable-drug chiefly due to the bitter taste of the noni fruit. For human use noni juice is usually mixed with natural grape juice due to its improved taste. If untreated, ehrlichiosis can trigger a severe neurological condition, leading to animal death. The most used and effective drugs in the treatment of ehrlichiosis are tetracyclines and their derivatives, including doxycycline, which is considered the most effective drug against the causative agent (Amyx et al. 1971; Breitchwerdt 1995).

The treatment of ehrlichiosis consists of doxycycline administration. Studies by (Garcia et al. 2010) demonstrated the efficacy of the drug after 15 days of treatment and found significant hematological improvement and complete remission of clinical signs. In the presente study, the remission of the clinical signs of the animals treated with doxycycline was only observed after 30 days of treatment. Even after extended treatment, the animals presented relapses and hematological changes. These findings indicate that conventional drugs did not display the expected efficacy, suggesting bacterial resistance. Studies performed by (Garcia et al. 2010, Schaefer et al. 2007) reported the persistence of *E. canis* infection in dogs treated with

doxycycline for 14 days. Similar results showed the persistence of rickettsia infection 182 even after long-term treatment with doxycycline (Mcclure et al. 2010).

Rifampicin has also been successfully used in the treatment of ehrlichiosis. However, azithromycin (20 mg/kg, once a day, orally, for 7 days) did not lead to clinical recovery and improvement in the hematimetric parameters of infected animals (Cantadori et al. 2014). Therefore, it is extremely important that new drugs are tested and to find out bioactive compounds identified which are useful for new bactericidal pharmaceutical formulations, or function as adjuvants in the treatment against pathogens. Although noni vegetable drugs did not exert action against *Ehrlichia,* they contributed to the improvement of platelet count.

Conclusion

Noni vegetable-drug did not demonstrate efficacy in the treatment of canine ehrlichiosis, as it did not lead to a remission in clinical signs. However, it did increase platelet production and did not present hepatotoxicity and nephrotoxicity, suggesting it may be a potential adjuvant for the treatment of this or other diseases that cause thrombocytopenia.

°**C -** Celsius degrees; **ALT**- alanine aminotransferase; **ANOVA** - analysis of variance; **AST** - aspartate aminotransferase; **IFAT** - indirect fluorescent antibody test; **kg** - Kilograms; **mg** - milligrams; **mm** - milliliters; **UV** - ultraviolet.

Ethical Approval and Consent to participate

The experiment was approved by the Ethics Committee on Animal Experimentation of the Universidade Estadual do Maranhão, Brazil (protocol 17/2010).

Consent for publication

Not applicable

Availability of supporting data

All the results are included within the manuscript.

Open access

Competing interests

The authors declare no conflicts of interest exist, either financially or with the policy of the journal.

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Contribution of authors

Study concept: ALAS, MAOT. Data acquisition: MAOT, RAS, ACCC, JCS, ZSB, NSM. Statistical analysis: NSM. Manuscript writing: ALAS, MAOT, RAS, RMO, IFBM. All the authors read and approved the final manuscript.

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Table 1. Mean and standard deviation of hematimetric parameters of dogs infected with *Ehrlichia canis*, before and after treatment with doxycycline and *M. citrifolia*

Hematological parameters	Doxycycline			Noni			Reference values
	Before	After	*P*	Before	After	*P*	
GV (%)	33.50[a]±13.92	32.00[a]±6.58	0.82	34.17[a]±9.33	40.67[a]±9.67	0.01	37-55
Erythrocytes (x10⁶/µL)	507.75[a]±210.79	476.00[a]±94.44	0.77	447.83[a]±273.87	616.17[a]±146.1	0.12	5.5-8.5
Hemoglobin (g/dL)	1015.00[a]±421.73	700.00[a]±493.10	0.52	895.67[a]±547.94	814.00[a]±539.60	0.83	12-18
HGM	20.00[a]±0.00	20.00[a]±0.00	-	20.0[a]±0.0	20.0[a]±0.0	-	-
VGM	66.00[a]±0.00	66.00[a]±0.00	-	66.0[a]±0,0	66.0[a]±0.0	-	-
CHGM	303.00[a]±0.00	303.00[a]±0.00	-	303.0[a]±0,0	303.0[a]±0.0	-	-
Leukocytes	15150.00[a]±5612.19	15150,00[a]±7009.28	1.00	11575.00[a]±7325.69	13041.67[a]±7531.76	0.65	6.000-17.000
Segmented leukocytes	57.75[a]±9.81	63.00[a]±10.23	0.28	71.67[a]±13.25	71.67[a]±15.00	1.00	3.000-11.500
Lymphocytes	29.50[a]±12.23	20.75[a]±14.34	0.25	16.17[a]±12.29	15.50[a]±11.84	0.85	1.000-4.800
Monocytes	1.00[a]±1.15	1.50[a]±1.73	0.73	2.83[a]±1.47	2.50[a]±1.87	0.61	150-1.350
Eosinophils	10.00[a]±10.45	5.25[a]±3.20	0.35	7.67[a]±7.81	9.50[a]±5.86	0.65	100-1.250
Rod cells	1.25[a]±0.95	2.75[a]±2.75	0.34	1.50[a]±1.38	0.50[a]±0.84	0.25	00-540
STP (g/dL)	7.00[a]±1.15	7.50[a]±1.00	0.39	6.33[a]±1.97	6.33[a]±1.51	1.00	5.5-8.0
Platelets	106043.50[a]±60338.59	345717.50[a]±75893.08	0.16	171046.67[a]±67485.14	268333.33[a]±89090.2	0.15	200.000-900.000

a – same letters in the same line indicate no statistical differences between means ($p > 0.05$). 1 - ANOVA with means comparison by Mann-Whitney parametric test. GV= globular volume; STP= Serum total protein.

Table 2. Mean and standard deviation of biochemical parameters of dogs infected with *Ehrlichia canis*, before and after treatment with doxycycline and *M. citrifolia*

Biochemical parameters	Doxycycline			Noni			Reference values
	Before	After	*P*	Before	After	*P*	
Ureia (mg/dl)	36.00[a]±21.42	51.30[a]≠37.73	0.1075	33.80[a]≠12.85	43.66[a]≠12.62	0.0713	10-60
Creatinina (mg/dl)	0.58[a]≠0.20	0.79[a]≠0.41	0.697	0.71[a]≠0.24	0.73[a]≠0.13	0.8327	0.5-1.5
AST (UI/L)	43.40[a]≠18.27	47.40[a]≠29.14	0.6520	63.66[a]≠17.61	50.50[a]≠25.332	0.2488	10-88
ALT (UI/L)	43.40[a]≠18.27	37.90[a]≠26.18	0.5433	42.60[a]≠27.41	34.20[a]≠20.04	0.1792	10-88

a – same letters in the same line indicate no statistical differences between means ($p > 0,05$). 1 - ANOVA with means comparison by Mann-Whitney parametric test. AST - Aspartate aminotransferase. ALT- Alanine aminotransferase.

Parte II

Capítulo II

Artigo "Histological and immunohistochemistry CDV through encephalon parenchyma"

Submetido ao periódico *Archives of Virology* (ISSN: 0304-8608 (print) 1432-8798 (Online) cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é A2, fator de impacto 2.255. O artigo foi elaborado e formatado conforme as normas do periódico supracitado, que seguem anexas ao trabalho juntamente com o comprovante de submissão.

Histological and immunohistochemistry CDV through encephalon parenchyma

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Abstract

Although canine distemper virus (CDV) is primarily described as a virus that causes disease in domestic canines, wild animals such as tigers, panthers, lions, jaguars, and nonprimates such as felids and ferrets can be affected. A histological and immunocytochemical study was performed to evaluate the distribution of CDV within the encephalic parenchyma, and its correlation with neurological profile. The results showed that although the virus causes lesions throughout the encephalon, the cerebellar peduncle region was most affected and the cerebellum presented severe damage. Virus antigen detection was observed in all the regions studied and the neurons showed strong immunohistochemical labelling, including in the body and branches of the

neuronal cells. In conclusion, the virus is widespread in the brain with most neurological signs due to neuronal damage and demyelization.

Key-words: CDV, neuron, histopathological and Immunohistochemistry.

Introduction

Canine distemper is a highly contagious viral disease, caused by a morbillivirus (Paramyxoviridae family, Mononegavirales order). Canine distemper virus (CDV) is considered one of the most significant pathogens of domestic dogs [1] and is distributed worldwide. Besides canids, CDV infects animals of the Mustelidae (ferrets), Procyonidae (raccoons), Viverridae (mongoose), Felidae, and Ursidae family and aquatic mammals [2] [3]. In Brazil, the disease is endemic, with high morbidity and mortality rates, especially in immunosuppressed dogs under 5 years old [4] [5]. In addition, it is a reemerging disease in countries where it was once controlled [4].

CDV presents only one serotype, but there are many different biological viral strains. Low virulence strains cause inapparent infections while high virulence strains cause depletion of the immune system, causing acute infection [6] [7]. The immunosuppressive effect of the virus depends on many factors, such as age, nutritional state and strain virulence [8].

The disease is characterized by dermatological, digestive, respiratory and neurological manifestations [9]. The last maned complication is the most severe: in this phase, the animal displays a considerable number of neurological signs, some of which present no clinical systemic signs [10].

Although the histopathology of canine distemper is well known and widely described in literature, there is no study that shows viral distribution in the cerebral parenchyma. In the present study we demonstrated that while the virus is disseminated throughout the encephalon, the most affected area is the cerebellar peduncle region, and severe damage was observed in the cerebellum.

Materials and methods

This study was approved by the Ethical Committee on Animal Experimentation of the Universidade Estadual do Maranhão (Protocol number 17/2010).

The animals used in this study were from the Universidade Estadual do Maranhão Veterinary Hospital. They were aged from 3 to 24 months, of both genders and presented specific signs of disease such as ataxia, tremors, seizures, paraplegia and myoclonus, which was the most common condition They were subsequently submitted to a rapid screening test (CDV Ac test kit" - Alere®), which detects specific antigens of CDV.

The dogs were euthanized with a lethal dose of thiopental (0.3 mg/kg) and the encephalon was removed and fixed in 10% phosphate buffered formalin. After fixation, the encephalon of each animal was sectioned and fragments of the telencephalon, cerebellum, obex, caudal cerebellar peduncles, caudal colliculi, and rostral colliculi were routinely processed for embedding in paraffin wax before 5µm was stained with hematoxylin-eosin [11].

Briefly, for immunohistochemistry analysis, 5 µm sections were deparaffinized and antigen retrieval was performed by heating to 80-85°C in EDTA for 20 min, and then left to steep for 20 min. The protocol of [12] was adapted. A total of 1/100 dilution of CDV antibody (DV2-12/SC-57660/ monoclonal IgG_{2b} Santa Cruz Biotechnology/USA) and an ExtrAvidin Peroxidase Staining Kit consisting of ExtrAvidin Peroxidase and Biotinylated Monoclonal Anti-Rabbit Immunoglobulins (Clone RG-16/Sigma-Aldrich/USA) were used. After antibody incubation and the enzyme substrate reactions, all sections were counterstained with Harris hematoxylin and mounted with Entellan (Merk/ Alemanha). Positive and negative controls consisted of the brain sample of a dog that died due to CDV and a healthy dog, respectively.

Results

Histological and immunohistochemistry analysis showed that the cerebellar peduncle was the most affected area (Table 1).

Table 1: Histopathological and immunohistochemical findings and its score in the central nervous system of animals naturally infected with canine distemper virus

ORGANS	INJURIES	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7
CEREBELLUM	Inflammation	++	++	++	+	+	+	+
	Gliosis	-	-	-	-	-	-	-
	Chromatolysis	-	+	-	-	-	-	-
CAUDAL COLICOLI	Inflammation	++	++	+	-	++	+	+
	Gliosis	-	-	-	+	-	-	-
	Chromatolysis	-	+	-	+	-	-	-
ROSTRAL COLICOLI	Inflammation	+++	+	+	-	+	+	+
	Gliosis	-	-	-	-	-	-	-
	Chromatolysis	-	-	-	-	-	-	-
OBEX	Inflammation	+	-	++	++	+	-	-
	Gliosis	-	-	-	+	-	-	-
	Chromatolysis	-	-	-	-	-	-	-
CEREBELLAR PENDUNCLE	Inflammation	+++	-	+++	++	++	-	+
	Gliosis	-	-	-	+	-	-	-
	Chromatolysis	-	-	+	+	-	-	-
TELENCEPHALON	Inflammation	+	+	+++	+	-	+	-
	Gliosis	-	-	+	+	-	-	-
	Chromatolysis	-	-	-	-	-	-	-

(+ mild; ++ moderate; +++ intense; - no alteration)

Microscopic analysis showed that all dogs presented perivascular cuffing, gliosis, chromatolysis, and demyelination in at least one region (Figure 1 A/B). Additionally, inflammatory mononuclear reaction was observed in the subarachnoid space and pia-mater. Immunohistochemistry confirmed the histopathology results and highlighted significant immunolabeling of the neuron cells, where it was possible to observe intense viral particles within these cells (Fig. 1/C).

Discussion

CDV is a pantropic virus that leads to an inflammatory reaction and demyelination in the central nervous system. It was observed in the present study that all brain regions were affected, but that lesions were more frequent in the cerebellar peduncles, which explains the clinical signs presented by the animals.

Although the blood-brain barrier (BBB) is efficient barrier, CDV is able to cross it and cause damage. Two hypotheses explain the entrance of CDV in the cerebral parenchyma. One is a Trojan horse-like theory, in which infected mononuclear cells reach the brain. Virus replication

causes cell disruption, and releases viral particles that infect the endothelial cells (Figure 2) [13]. Another theory advocates that the virus enters the central nervous system (CNS) via the olfactory bulb. As the respiratory tract is the main infection route, the virus can spread to the brain via the olfactory nerve, and then replicate, as described in ferrets (Figure 3) [14].

The brain lesion is responsible for the most severe neurologic complications of distemper, and depending on the virulence and infection route the animal may present neurological signs instead of system manifestation [15, 16, 10]. CDV virus has a single serotype, but the strains presented a different virulence grade, which explains how infection can range from inapparent to severe, depending on the strain [17].

It was noted that most of the neurons showed a remarkable intracytoplasmic labeling, especially the neurons cells of the cerebellar peduncle. Literature reports that these cells are not primarily affected, but that CDV tropism affects the astrocytes, microglia and ependymal lining cells [15, 18, 14].

The CDV-specific neuron marking found in the present study is probably related to the high percentage of unvaccinated dogs in the city of São Luís, Brazil. A study [1] pointed out that, in Brazil, the high prevalence of distemper occurs due to inadequate vaccination. Additionally, there is a lack of studies about canine distemper epidemiology and strain characterization in this city, which could identify if a more virulent strain circulates among dogs.

According to several authors CDV is primarily found in the ependymal cells, choroid plexus cells and cerebral vessels, supporting the theory that the virus spreads by a hematogenic route or through cerebrospinal fluid, since in subarachnoid space the virus reaches the pia-mater meninges and subsequently the gray matter, causing severe damage due to inflammation and demyelination [19, 20, 21]. In the present study an intense inflammatory response in the subarachnoid space and the occurrence of perivascular cuffs with cerebral parenchyma was observed, reinforcing the idea that dissemination occurs through the blood stream.

An injury frequently observed in the present study was demyelination in both the white and gray matter. The pathogenesis of demyelization is controversial, presumably as it occurs due to direct damage to the oligodendrocytes, impairing functioning; another mechanism that could result in an immune response triggered by stimulation of the microglia [18,22]. Activated microglia promotes the release of ROS and the proteolytic enzymes that increase phagocytic activity 18, 22, 23].

The neurological signs presented by the animals are related to the region of the CNS involved, the CDV strain and the age and immunological status of the animal [2]. Cerebellar lesions lead to a lack of coordination, ataxia, hypermetria and a loss of muscle tone due to disruption of the pathways to the vestibular nuclei [8].

In conclusion, CDV caused severe lesions, mainly in the cerebellar peduncles and infected neurons.

Abbreviation

BBB - Blood-brain barrier; CDV - canine distemper virus; CNS - central nervous system; EDTA - Ethylenediaminetetraacetic acid; ROS - reactive oxygen species.

Ethical Approval

The experiment was approved by the Ethics Committee on Animal Experimentation of the Universidade Estadual do Maranhão, Brazil (protocol 17/2010).

Conflict of interests

The authors declare that they have no competing interests.

Contribution of authors

Study concept: ALAS, MAOT. Data acquisition: MAOT, ACCC, TST, NSM, RMO, APCS, JJSA. Manuscript writing: ALAS, MAOT, JCS, RMO, JJSA, FAM. All authors read and approved the final manuscript.

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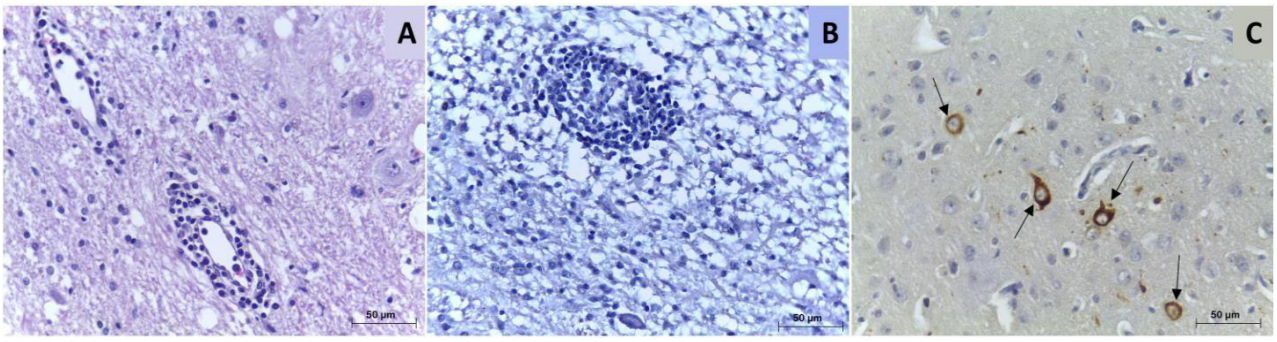

Figure 1: A. Rostral colliculus where was observed perivascular cuffing, composed chiefly by

mononuclear cells(bar=50µm) H&E. B. Spinal cord: white matter shows perivascular cuffing (bar=50µm) H&E. C. Caudal colliculus. Gray matter. Antigenic marking of neurons (arrow) bar=50µm counterstained by Mayer Hematoxylin.

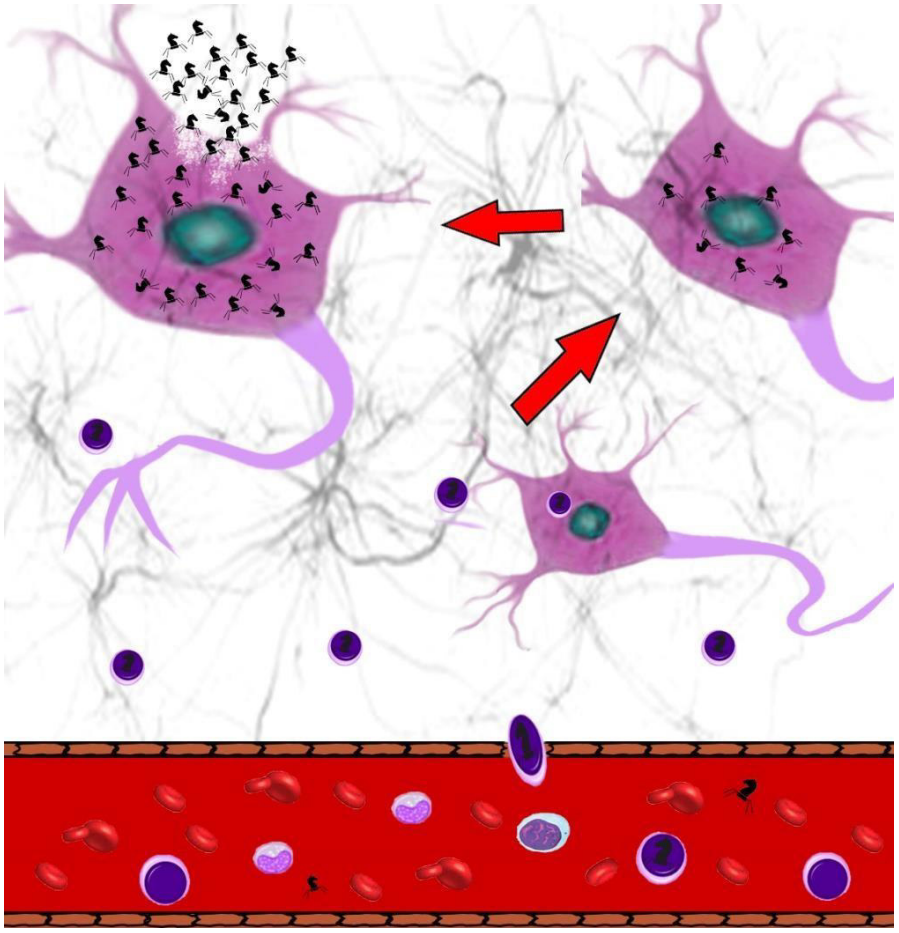

Figure 2: Schematic design that illustrates the hypothesis Trojan horse route as described by Ludlow et al (2013)

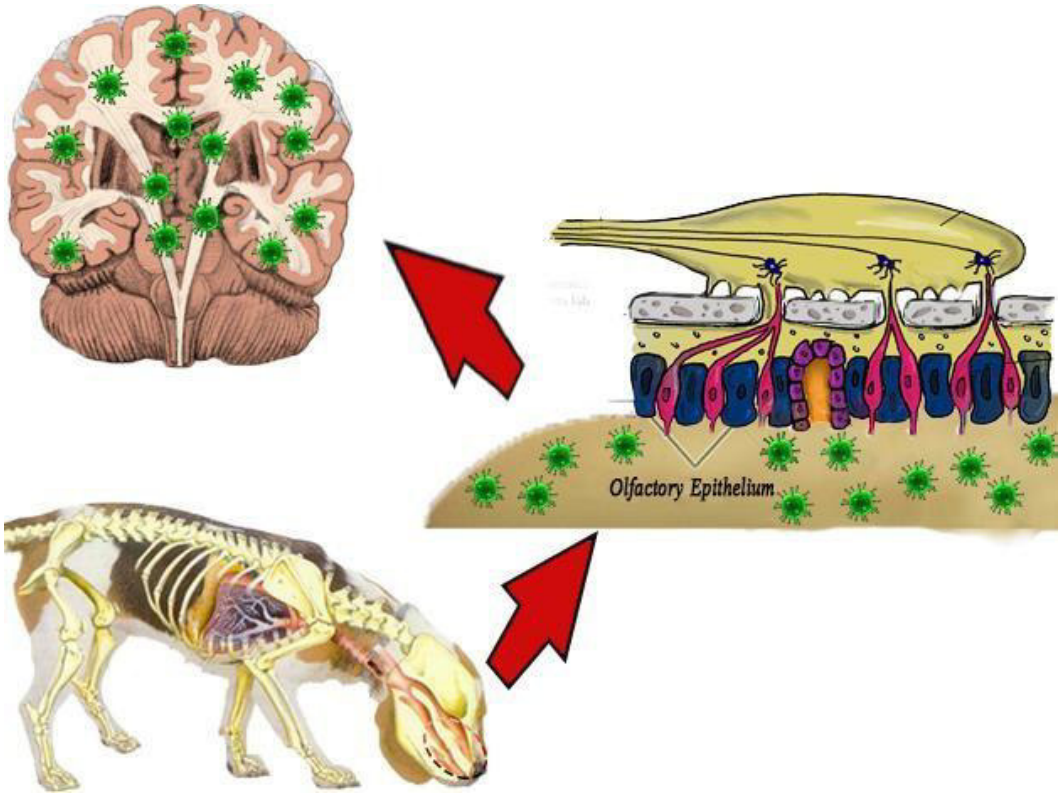

Figure 3: Schematic design hypothesizing the olfactory route described by Rudd et al (2006)

Parte II

Capítulo III

Artigo "A plant, many uses: A review on the pharmacological applications of *Morinda citrifolia***"**

Submetido ao periódico *Phytotherapy Research* (ISSN 1099-1573), cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é B1, fator de impacto 2.694. O artigo foi elaborado e formatado conforme as normas do periódico supracitado, que seguem anexas ao trabalho.

One plant, many uses: A review of the pharmacological applications of *Morinda citrifolia*

Pharmacological uses of *Morinda citrifolia*

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Morinda citrifolia, also known as noni, is commonly used in popular medicine in Brazil. Many parts of the noni tree are utilized in such practices, including the roots, leaves and seeds. Through a search of online databases, the present article reviews 92 research studies on the biological actions of *M. citrifolia*. The paper will discuss the therapeutic effects of noni and its compounds in a variety of forms of presentation, focusing on studies that support its traditional use. A large and diverse number of properties were identified, which were divided into immunostimulatory, antitumor, antidiabetic, anti-obesity, antibacterial and anti-septic, antifungal, antiviral, leishmanicidal, anti-inflammatory, antinociceptive and analgesic, antioxidant, neuroprotective, wound healing, antiallergic, antiangiogenic, antiemetic and anti-nausea, anti-gastric ulcer and oesophagitis, anthelmintic, antimutagenic, antipsychotic, anxiolytic, photoprotective, anti-wrinkle and periodontal tissue regeneration activities. While it was concluded that although *M. citrifolia* is widely and successfully used for the treatment or prevention of various diseases, it should be consumed carefully, and only after exhaustive studies into its chemical constituents and mechanisms of action, both in *in vitro* and *in vivo* models, as well as clinical trials.

Keywords: biological activity; noni; phytotherapy.

INTRODUCTION

Morinda citrifolia, or noni, is a plant native to Southeast Asia. The tree can grow as tall as 6 m and has bright green, oval shaped leaves, which measure from 10 to 30 cm. Its fruit is ovoid in shape and when mature has an unpleasant butyric acid odor and astringent taste. Its seeds have an air sac at one end which allows them to float, partly explaining the wide distribution of noni trees in the Indo-Pacific islands (Potterat and Hamburger, 2007). Each part of the plant, from its roots to its seeds, is widely used in folk medicine, and several therapeutic effects have already been reported (Wang *et al*., 2002). Several *in vitro* and *in vivo* studies have been performed in recent years, seeking to demonstrate the biological potential of *M. citrifolia* and/or its isolated compounds, and the results have been promising. In a search of several databases, including PubMed, SciELO and Periódicos CAPES (CAPES Journals), conducted from April 2016 to May 2016, 505 results were found for *Morinda citrifolia*, 92 of which were related to its biological actions. Of these, 35 were *invitro* studies, 51 were *in vivo* studies, 2 were *ex vivo* studies and 5 were clinical trials involving humans. These studies highlighted the following biological actions of *M. citrifolia*: immunostimulatory, antitumor, antidiabetic, anti-obesity, antibacterial and anti-septic, antifungal, antiviral, leishmanicidal ,anti-inflammatory ,antinociceptive and analgesic, antioxidant, neuroprotective, wound healing, antiallergic, antiangiogenic, antiemetic and anti-nausea, anti-gastric ulcer and oesophagitis, anthelmintic, antimutagenic, antipsychotic, anxiolytic, photoprotective, anti-wrinkle and periodontal tissue regeneration activities, which are described in this review.

IMMUNOSTIMULATORY ACTIVITY

Some plants have compounds that present immunomodulatory activity, or in other words contain substances that can promote or suppress the immunological responses of the body, such as cytokine production. *M. citrifolia* stands out among plants for its immunomodulatory activities, which are related to both cellular and humoral responses.

According to Hirazumi and Furusawa (1999), the coadministration of noni juice and immunosuppressant drugs reduced the immunostimulatory effect in mice, which confirms the action of noni as an immunomodulating agent which can interfere in immune response under different pathological conditions.

Both the hydroalcoholic and aqueous extract of noni fruit increased *in vitro* splenocyte proliferation and stimulated B and T lymphocyte activity (Nayak and Mengi, 2010). In splenocytes isolated from middle-aged F344 inbred rats treated with seedless noni fruit juice, there was a decrease in *in vitro* INF-γ and IL-2 production and a reduction in lymphoproliferative activity. However, juice with seeds had the opposite effect, increasing lymphocyte proliferation in young and old mice and augmenting the production of cytokines (Pratap *et al*., 2016).

Paul *et al*. (2008) reported the immunomodulatory effect of *M. citrifolia in vitro*. The commercial (TNJ) and concentrated fruit juice activated the cannabinoid receptor 2 (CB2), but inhibited the cannabinoid receptor 1 (CB1) in a dose-dependent manner. It was also noted in *in vivo* studies that the ad libitum oral administration of TNJ for 16 days decreased IL-4 production and increased INF-γ. These results suggest that noni can modulate the immune system by exerting beneficial effects in conditions involving inappropriate immune responses.

The beneficial effects of noni fruit juice when combined with IFN-γ *in vivo* have also been demonstrated, although its activity was suppressed when it was combined with IL-4 or IL-10, suggesting that it induces a predominantly Th1 immune status (Furusawa *et al*, 2003). It was also demonstrated that *M. citrifolia* fruit extract suppressed cellular response in an ascitic tumor previously treated with immunosuppressant substances. Furthermore, it inhibited IL-2 production in animals and reduced the amount of natural killer cells in normal mice (Murata *et al*., 2014).

In animals with Lewis tumors treated with noni ppt, an isolated substance, it was found that

noni stimulated the release of several cytokines, including TNF-α, IL-1β, IL-10, IL-12 and IFN-γ, as well as nitric oxide. However, it decreased IL-4 release and had no effect on IL-2 production (Hirazumi and Furusawa, 1999).

ANTITUMOR ACTIVITY

In folk medicine *M. citrifolia* is used as a food supplement in patients with different types of cancer. *In vivo* studies have demonstrated the action of the ethanolic extract of noni leaves on tumor cells and on the pathways involved in immunological response, such as cyclooxygenase 2 (COX2) suppression, inflammatory markers and the increase of the tumor suppressor gene (Lim *et al*., 2016). The administration of 10% TNJ in laboratory animals also extends to the inhibition of gene mutations: carcinogenic compounds bind covalently to the DNA, forming structures known as adducts that, if not repaired, cause mutations. Noni can be used to prevent the formation of these structures (Wang and Su, 2001).

The antiproliferative effect of the methanolic extract of noni fruit has been described in a number of cell lines, such as baby hamster kidney cells (BHK), African green monkey kidney cells (Vero), human epithelial type 2 cells (Hep2), human breast adenocarcinoma cells (MCF7) and neuroblastoma. The ethanolic extract was tested in B16-F10 melanoma cells (LAN5) and also exhibited antiproliferative action (Arpornsuwan and Punjanon, 2006; Candida *et al*., 2014). Noni fruit juice can markedly downregulate the expression of HIF-1α protein induced by manganese *in vitro*, in the A549 cell line of human lung cancer. This suggests that it has potential beneficial effects on lung cancers in which manganese and the overexpression of HIF-1α play key roles, as manganese exposure is a risk factor for many pulmonary diseases (Jang, 2012).

The ethanolic extract of noni leaves also reduced the expression of epidermal growth factor receptor (EGFR), a lung adenocarcinoma biomarker, in mice (Lim *et al*., 2016), as well as acting on Lewis lung carcinoma in syngenetic mice (Hirazumi *et al*., 1994). The chemical composition

of the juice shows the presence of noni-ppt, a substance rich in polysaccharides, which has exhibited antitumor activity against Lewis lung peritoneal carcinomatosis (CLL). The therapeutic administration of noni-ppt significantly increased the survival of tumor-bearing mice (Hirazumi and Furusawa, 1999).

M. citrifolia fresh leaf extract exhibited an *in vitro* inhibitory effect on the lineage cells of human epidermoid carcinoma (KB), human cervical carcinoma (HeLa), MCF-7, human hepatocellular carcinoma (HepG2) and Vero cells, and can act as a food supplement in the chemoprevention of cervical and epidermoid cancer (Thani *et al*., 2010). Its ethyl acetate fraction extract inhibited MCF-7 and MDA-MB-23 cell proliferation, both of which are associated with mammary adenocarcinoma (Sharma *et al*., 2016).

In human colon adenocarcinoma cell (Caco-2) culture, the ethanolic extract of noni fruit and its fractions reduced intracellular oxidation and inflammation through the reduction of reactive oxygen species, and decreased COX-2, IL-8 and PGE2 production *in vitro* (Huang et al., 2015).

TNJ significantly reduced the tumor weight and volume of HER2-positive breast cancer in MMTV-neu transgenic mice. However, tumor latency and metastasis incidence were not affected by the treatment (Clafshenkel *et al*., 2012).

A commercial noni juice (Alnoni®) exhibited cytotoxic potential on Ehrlich tumor cells in BALB/c mice, proving to be useful in the treatment of breast carcinoma, whether alone or in combination with doxorubicin, a potent anticancer agent. The effects of noni are the result of apoptosis induction (Taskin *et al*., 2009).

When used in the treatment of sarcoma 180 (S-180) or Crocker tumor in allogeneic mice, noni-ppt, isolated from ripe noni juice, demonstrated antitumor activity, with a remission rate of 25 to 45% and a synergistic or additive effect when combined with a broad spectrum of chemotherapeutic drugs (Furusawa *et al*., 2003).

In *in vitro* studies, damnacanthal, a bioactive compound isolated from the chloroform fraction

of *M. citrifolia* root extract, significantly inhibits the reproduction of RAS cells, precursors of many malignant tumors (Hiramatsu *et al*., 1993). In MCF-7 cells, this compound exhibited antiproliferative activity after 72 hours of treatment, at a concentration of 8.2µg/ml, and induced cell cycle arrest and apoptosis in the G1 phase, through the activation of the p21 gene and caspase-7 (Aziz *et al*., 2014). It also inhibited the growth of several cancer cell lines: colorectal cancer (HCT-116), human colon adenocarcinoma (HT-29), MCF-7, prostate cancer (PC-3), in a dosage and type-dependent form (Nualsanit *et al*., 2012; Sukamporn *et al*., 2016).

A phase I clinical trial aimed to evaluate the effect of the ingestion of the dry fruit extract on patients with stage 4 cancer. The maximum daily dose the patients tolerated was 3 grams (six capsules, each of which contained 500 mg of the dry fruit extract, four times a day). The ingestion of three or four capsules four times a day is recommended to prevent fatigue and pain and to maintain physical functioning. However, some patients demonstrated side effects associated with the ingestion of noni capsules, such as nausea and abdominal discomfort (Issell *et al*., 2009). This demonstrates the need for further studies to evaluate the toxicity of the extract to ensure the safety of those who use it. This study is of great importance, however, as it is one of very few human clinical trials.

The role of the fungi found in noni leaves and fruit in inhibiting tumor cells *in vitro* was studied by Wu *et al*. (2015), who demonstrated that the endophytes present in *M. citrifolia* leaves inhibited the growth of human cancer cell lines.

ANTIDIABETIC ACTIVITY

The consumption of certain types of traditional foods and medicines, such as noni, can decrease the risk of developing type 2 diabetes mellitus (TIIDM) (Owen *et al*., 2008). Besides its preventive effects, the use of noni as a food source can be a form of treatment for patients with TIIDM (Lee *et al*., 2012).

The increasing number of diabetic patients who support alternative medicine has encouraged a

growing number of studies into popular knowledge. Nerurkar *et al*. (2012) investigated the antidiabetic effects of *M. citrifolia* in C57BL/6 mice with a high-fat diet, who were submitted to treatment with fermented noni juice. The improvement of the glucose metabolism, via phosphorylation of the transcription factor FOXO1, was observed. Similarly, Horsfall et al. (2008) observed that the use of noni juice on the treatment of induced diabetes in rats led to reduced blood glucose levels, and that noni possesses a synergistic action when used in combined therapy with insulin.

The hypoglycemic effects of the chemical constituents of *M. citrifolia* roots were evaluated in streptozotocin-induced diabetes in rats. The butanol fraction of the root methanolic extract reduced blood glucose levels, with this effect attributed to the compounds damnacanthal-3-O-beta-D-lucidinmprimeveroside and 3-O-beta-D-primeveroside (Kamiya *et al*., 2008). A similar effect was attributed to fermented fruit juice, which showed, in addition to hypoglycemic properties, a hepatoprotective effect on diabetic rats (Nayak *et al*., 2011).

ANTIOBESITY ACTIVITY

An *in vitro* study demonstrated that the ethanolic extract of noni leaves and fruit can be used as body weight control agents, as both inhibit lipoprotein lipase (Pak-Dek *et al*., 2008).

The effect of the ethanolic extract of noni leaves on the treatment of obesity induced by a high-fat diet in the Sprague-Dawley rat strain was also assessed. After nine weeks, positive effects were observed on body fat, fat content, triglyceride, insulin and leptin serum levels, which demonstrate that noni significantly improved metabolic disorders caused by obesity (Gooda Sahib Jambocus *et al*., 2016).

In another study, the administration of leaf, root and fruit hydro alcoholic extracts (1000 or 500 mg/kg) in rats with Triton-induced or high-fat diet hyperlipidemia caused a reduction in total cholesterol and triglyceride levels. This data indicates that the anti-dyslipidemic effect of the extracts was meditated through the inhibition of the biosynthesis, secretion and absorption of

lipids. This may be due to the presence of antioxidant constituents in the plant (Mandukhail *et al*., 2010). Noni seed oil also reduced total cholesterol and triglycerides in hyperlipidemic and normolipidemic mice (Pazos *et al*., 2011).

In hamsters submitted to a high-fat and cholesterol diet, the ingestion of noni juice decreased triacylglycerol and cholesterol serum levels, lipid deposition in liver and the atherogenic index (Lin *et al*., 2012). In adult smokers, the consumption of noni juice reduces cholesterol levels, by decreasing LDL, triglycerides and high sensitivity C-reactive protein (hsPCR) plasma levels, the last of which is an important parameter for cardiac risk evaluation and an aprognostic tool of heart disease. The juice was therefore able to soften smoking-induced hyperlipidemia (Wang *et al*., 2012).

ANTIBACTERIAL AND ANTI-SEPTIC ACTIVITIES

Irreversible hydrocolloids are used in dentistry as a molding material in the confection of dental restorations. The use of *M. citrifolia* extract with hydrocolloid powder in the preparation of dental impressions decreased contamination by microorganisms, without harming the quality of the material (Ahmed *et al*. 2015).

In fruit farming, it was observed that the immersion of mango cubes in noni juice had an antimicrobial effect on mesophilic bacteria, molds and yeasts, demonstrating that this can be a potentially valuable technology for the decontamination of sliced fresh fruit surfaces (Ulloa *et al*., 2015).

In animal production, the antibacterial effect of noni leaf extract on *Salmonella typhimurium* was evaluated in Japanese quail breeding. Its action against this pathogen resulted in reduced quail mortality and increased egg production (Retnani *et al*., 2014).

In newborn calves fed with noni fruit puree, antibacterial activity against *Escherichia coli* was observed¸ revealing the usefulness of the fruit in production systems where antibiotic use is more restricted (Schäfer *et al*., 2008). The ethanolic fruit extract also acts against *E. coli* and

inhibits *Staphylococcus aureus* growth *in vitro* (Candida *et al*., 2014).

In vitro studies have demonstrated the antimicrobial activity of *M. citrifolia* against both *S. aureus* and methicillin-resistant *S. aureus* (Zaidan *et al*., 2005). The aqueous extract of leaves exhibited moderate antibacterial activity *in vitro* and *in vivo* (Serafini *et al*., 2011), while ethanolic crude extract of noni and its hexane fraction demonstrated antituberculosis activity *in vitro* (Saludes *et al*., 2002).

ANTIFUNGAL ACTIVITY

Lyophilized fruit extract of *M. citrifolia* demonstrated an inhibitory effect against *Candida albicans in vitro*, which varied according to concentration and contact time (Jainkittivong *et al*., 2009).

ANTIVIRAL ACTIVITY

There remains a major need for the development of complementary or alternative medicines for the treatment of diseases caused by viruses. Therefore, the presence of compounds with antiviral activity in medicinal plants constitutes a potential therapeutic target. In a study involving hepatitis C cell culture, it was shown that the methanol extract of leaves and its hexane and ethyl acetate fractions possess antiviral activity (Ratnoglik *et al*., 2014).

Isolated compounds from the fruit methanol extract exhibited moderate inhibitory effects against Epstein-Barr virus *in vitro* (Akihisa *et al*., 2007). However, *in vitro* anti-HIV activity was not found in HIV-1-infected MT-4 cells treated with *M. citrifolia* fruit (juice and dry extract) methanolic and ethanolic extracts (Selvam *et al*., 2009).

LEISHMANICIDAL ACTIVITY

The search for new drugs to treat leishmaniasis has increased due to the elevated frequency of drug resistance cases in endemic areas, in addition to the side effects and treatment complications of the disease (Sereno *et al*., 2007).

Several *in vitro* and *in vivo* studies have demonstrated the leishmanicidal effect of *M. citrifolia*.

Almeida-Souza *et al*. (2016) evaluated the action of noni fruit extract on *Leishmania infantum* promastigotes, and found the extract was not toxic at concentrations of up to 2 µg/ml, with an IC_{50} of 260.5 µg/mL. Moreover, it was shown that administration of the extract led to intense ultrastructural changes in promastigotes, resulting in parasite destruction.

The leishmanicidal potential of noni has also been evaluated in experimental infection in mice. In BALB/c mice infected with *L. amazonensis* and treated with crude noni fruit extract intense extracellular matrix production was observed at the lesion site, characterized by the predominance of mature collagen and the absence of significant histological alterations in other analyzed organs. In contrast, infected and untreated animals exhibited extracellular matrix destruction, hyperplasia of the white pulp of the spleen and inflammatory infiltrates in the liver. Results show that *M. citrifolia* provides effective control of lesions caused by infection (Mondego-Oliveira *et al*., 2015).

Two clinical trials in human patients with cutaneous leishmaniasis were performed to evaluate the efficacy of *M. citrifolia* stem, leaf and fruit methanolic extracts. A 1% topical preparation was prepared and applied to the skin lesions. A marked improvement and regression of lesions was observed in 50% of patients, while there was a moderate improvement in 30% of patients and a significant improvement in 20%. The leaf and fruit extracts have been found not to be very effective in treatment (Sattar *et al*., 2012; Siddiqui *et al*., 2014).

ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory action of *M. citrifolia* has been demonstrated in *in vitro* and *in vivo* models in a number of pathological conditions associated with inflammation. The anti-inflammatory effects of noni juice were investigated *in vitro* by measuring the production of nitric oxide and prostaglandin E2 in activated macrophages, and the inhibition of COX 1 and 2; and *in vivo* on carrageenan-induced paw edema in rats. The juice reduced paw edema, directly inhibited the action of COX 1 and 2, and reduced nitric oxide and prostaglandin E2 production

in J774.G8 cells, in a dose-dependent manner, demonstrating the anti-inflammatory action of noni, which is also enhanced by its antioxidant effect (Dussossoy *et al*., 2011).

Nualsanit *et al*. (2011) also demonstrated the *in vitro* and *in vivo* anti-inflammatory effects of noni extract and its isolated compound, damnacanthal, which reduced paw edema in rats and mice and down-regulated nuclear factor kappa B activity (NF- kB) induced by lipopolysaccharides. The production of proinflammatory cytokines, COX2 and inducible nitric oxide synthase was also suppressed by damnacanthal.

The aqueous extract of noni leaves has anti-inflammatory potential, significantly reducing leukocyte migration, and can be used as an alternative to pain and inflammatory situations, including those related to oxidation (Serafini *et al*., 2011). The ethanolic extract of noni fruit inhibits the production of matrix metalloproteinase 9 (MMP-9) by human monocytes after stimulation with LPS *in vitro*, with a similar effect to hydrocortisone, a drug used for the treatment of arthritis. This result shows the effectiveness of noni in reducing inflammation in arthritis (Basar *et al*., 2010).

The ethanol and ethyl acetate fractions of noni fruit extract negatively regulated inflammatory responses during *Helicobacter pylori* infection *in vitro* (Huang *et al*., 2014).

The chloroform fraction of the root extract has an anti-inflammatory effect, significantly reducing histamine-induced paw edema at a concentration of 3 g/kg. Damnacanthal, used alone, meanwhile, reduces edema at concentrations of 10-100 mg/kg (Okusada *et al*., 2011). Likewise, other anthraquinones isolated from the methanolic fraction of noni fruit extract exhibited potent anti-inflammatory activity in an induced inflammation model in mice (Akihisa *et al*., 2007).

ANTINOCICEPTIVE AND ANALGESIC ACTIVITY

The lyophilized aqueous extract of *M. citrifolia* roots demonstrated significant and dose-dependent analgesic effects and behavioral changes in mice (Younos *et al*., 1990). Noni leaf aqueous extract exhibited antinociceptive action in a writhing test induced by acetic acid

(Serafini *et al*., 2011).

The chloroform fraction of the root extract and damnacanthal isolated from the root have antinociceptive effects, reducing the pain related to formalin test behavior. Its effects are mediated in part by interference with histamine receptors (Okusada *et al*., 2011).

A 10% solution of concentrated noni fruit puree added to the drinking water of mice with arthritis reduced sensitivity to pain in comparison with tramadol, a central analgesic drug, showing that noni juice is effective at reducing the pain caused by arthritis (Basar *et al*., 2010).

ANTIOXIDANT ACTIVITY

Noni can be used as a valuable source of natural antioxidants (Thoo *et al*. 2013). Noni fruit juice, with or without seeds, is capable of modulating cell-mediated immune response and antioxidant enzyme activity *in vitro* (Pratap *et al*., 2016). In a *Drosophila melanogaster* somatic cell model, the protective effects of TNJ depended on its concentration, indicating a dose-response correlation, which can be attributed to either a powerful antioxidant action or the ability to capture free radicals (Franchi *et al*., 2013).

M. citrifolia hydroalcoholic extract exhibited antioxidant activity, with significant elimination of oxygen superoxide *in vitro* (Calzuola *et al*., 2006). Oral treatment for 14 days with 50 mg/kg of noni leaf ethanol extract increased the activity of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase on mice with lymphoma (Anitha and Mohandass, 2006).

The antioxidant properties of noni fruit juice in humans involve decreased tissue acidosis, acid-basic balance, improvement of mitochondrial redox activity, increased tissue oxygenation and improvement of the body metabolism as a whole (Caramel *et al*., 2015). In smokers, TNJ ingestion had antioxidant effects, reducing the plasma levels of superoxide anion (SAR) and lipid hydroperoxide (LOOH). SAR is a reactive oxygen species capable of damaging cell structure, as LOOH is associated with lipid peroxidation (Wang *et al*., 2009).

Despite the fact that the antioxidant potential of noni has been proven, in some studies, such as that by Thani *et al*. (2010), this effect was not observed. In this case, the dichloromethane extract of the fresh leaves did not present antioxidant activity *in vitro*.

NEUROPROTECTIVE ACTIVITY

The neuroprotective action of a 100% concentrated noni juice (Tahitian Noni Original Concentrate) was tested in rabbits using a hydrocephalus-induced degenerative disorders model, and its effect was compared with that of memantine. To detect apoptosis and neuronal degeneration in the periventricular tissue of the fourth ventricle of rabbits, microtubule-associated protein-2 and caspase-3 immunohistochemistry were performed. The intensity of immunohistochemical staining was observed to be significantly higher in the noni group and the treatment reduced the number of caspase-3-positive cells, while the memantine had no effect. These findings suggest that noni has more obvious inhibitory effects on hydrocephalus-induced degenerative disorders than memantine (Köktürk *et al*., 2013).

The effects of noni neuronal protection against ischemic stress were evaluated in two experiments. In the first, noni juice (Okinawa Noni juice) had a preventive effect against cerebral ischemic stress, demonstrated by a reduction of the infarcted area and fewer neurological deficits in mice (Harada *et al*., 2009). In the second experiment, the treatment increased insulin serum levels *in vitro*, favoring secretion after ischemic stress and possibly attenuating the development of glucose intolerance (Harada *et al*., 2010).

The neuroprotective effect of the noni fruit ethyl acetate fraction was also observed. Its administration in mice with beta-amyloid induced cognitive dysfunction, at a dose of 400 mg/kg, and increased serotonin, dopamine and antioxidant enzyme serum levels (Muralidharan *et al*., 2010).

The administration of noni fruit ethanol extract and its chloroform and ethyl acetate fractions improve memory and brain blood flow and attenuate oxidative stress and acetylcholinesterase

activity after scopolamine stimuli, demonstrating the usefulness of noni in the treatment of memory loss problems (Pachauri *et al*., 2012). In mice with streptozotocin-induced Alzheimer's disease, the ethyl acetate fraction prevented memory loss, improved energy metabolism in the brain and cholinergic neurotransmission, and exhibited antioxidant action (Pachauri *et al*., 2013).

The ingestion of noni juice has a protective action on mice brains, acting against the decline of stress-induced cognitive dysfunction, mainly due to its influence on hippocampus dentate gyrus, improving the reduction of blood vessel density caused by stress (Muto *et al*., 2010).

WOUND HEALING ACTIVITY

The healing activity of *M. citrifolia* was evaluated with topical ointments based on noni leaf extract and through the oral administration of leaf ethanol extract and fruit juice. In all cases, evidence of a faster healing process was observed, such as decreased wound contraction and reduced re-epithelialization time, demonstrating the therapeutic effects of noni on healing (Nayak et al., 2007; Nayak et al., 2009; Palu *et al*., 2010).

ANTIALLERGIC ACTIVITY

The anti-allergic potential of *M. citrifolia* in mice was evaluated by the inoculation of allergens (dinitrofluorobenzene and picryl chloride) in the ears of animals. The effect of the leaf and fruit ethanol extracts, administered orally, was evaluated in immediate and late-phase hypersensitivity reactions. A reduction of edema caused by foreign substances was observed in animals treated with noni, both immediately after the stimulus and in the delayed phase, indicating that the extract reduces mast cell degranulation and the release of histamine and other inflammatory substances. Noni can be a potential antiallergic against delayed hypersensitivity reactions or atopic dermatitis (Murata *et al*., 2014).

ANTIANGIOGENIC ACTIVITY

One factor that determines the action of noni on cancer is its anti-angiogenic potential. *M.*

citrifolia leaf and fruit methanol extract, as well as the chloroform fraction of fruit extract, exhibit anti-angiogenic activity *in vivo*, and this effect is in part attributed to scopoletin, a chemical compound found in the plant (Beh *et al*., 2012).

Hornick *et al*. (2003), in a three-dimensional fibrin clot matrix model using human placental vein and breast tumor explants as sources for vessel development, evaluated the antiangiogenic potential of noni juice, which inhibited early angiogenesis and led to the rupture of newly formed vascular networks. Piaru *et al*. (2012), however, observed low antiangiogenic activity *in vitro* on a rat aortic ring assay, with different concentrations of *M. citrifolia* essential oil.

ANTIEMETIC AND ANTINAUSEA ACTIVITY

The effectiveness of noni in the prevention of nausea and vomiting was demonstrated in patients with a high risk of developing these symptoms after different types of surgery. Noni extract at a concentration of 600mg had prophylactic antiemetic action, reducing the incidence of nausea in the immediate postoperative period (Prapaitrakool and Itharat, 2010).

ANTI-GASTRIC ULCER AND OESOPHAGITIS

Mahattanadul *et al*. (2011) evaluated the effect of noni fruit aqueous extract and its isolated compound, scopoletin, in models of gastro-esophageal inflammation in rats (acid reflux esophagitis, acute gastritis induced by ethanol and serotonin and chronic gastric ulcer induced by acetic acid). Extract concentrations from 0.63 to 2.50 g/kg significantly inhibited acid reflux esophagitis, reduced the formation of gastric lesions induced by alcohol and serotonin and accelerated the healing of gastric ulcers induced by acetic acid, similar to conventional drugs (ranitidine and lansoprazole). Scopoletin also produced similar results, demonstrating that both the extract and the isolated substance may be beneficial as a potential preventive and therapeutic agent for gastro-esophageal inflammatory diseases.

ANTIHELMINTIC ACTIVITY

The aqueous and ethanolic extracts of *M. citrifolia* were effective in controlling *Ascaridia galli in vitro* and in naturally infected chickens (Brito *et al*., 2009).

ANTIMUTAGENIC ACTIVITY

The antimutagenic and anti-recombinogenic action of TNJ in *D. melanogaster* somatic cells has been verified (Franchi *et al*., 2013).

ANTIPSYCHOTIC ACTIVITY

Pandey *et al*. (2012) demonstrated the antidopaminergic effect of noni fruit methanol extract in Swiss mice. The extract was administered orally for 21 days along with apomorphine and methamphetamine, suggesting that noni has antipsychotic activity. The same extract also inhibits the heroin compensatory effect (Narasingam *et al*., 2016).

ANXIOLYTIC ACTIVITY

In vitro studies performed by Deng *et al*. (2007) showed that crude noni fruit methanol extract had significant affinity for gamma-aminobutyric acid A (GabaA) receptors. The results demonstrate the presence of a competitive binder, which can bind to GabaA receptor as an agonist. While this may exert anxiolytic and sedative effects *in vivo*, there is a need to prove this finding in the murine model and in clinical trials with human patients.

The fruit extract can be seen as a target for anxiety disorders, as it has a proven effect when provided as a food supplement in an animal model (Kannan *et al*., 2014). But the need to also consider the toxic effects that the fruit may have in the body should be noted (Tin and Wiwanitkit, 2014).

PHOTOPROTECTIVE ACTIVITY

Exposure to sunlight, particularly ultraviolet radiation (UV), has a variety of adverse effects on human health. Therefore, the photoprotective effect of noni has also become a research target. Iridoid compounds isolated from the ethanolic extract of noni leaves exhibited an inhibitory action on the AP-1 protein, induced by UVB radiation (Sang *et al*., 2003). This protein, when

activated, stimulates the transcription of disintegrative matrix enzymes genes, such as metalloproteins, which degrade structures such as collagen (Angel *et al*., 2001).

The photoprotective effect of topical formulations with *M. citrifolia* lyophilized aqueous extract was evaluated by Serafini *et al*. (2014) in rats exposed to UVA-UVB light. Seven days after treatment and 20 hours after exposure to UV light, the damage to the skin barrier, formation of erythema and histological alterations were evaluated. The results showed that the formulations containing the extract protected the animal skin from damage induced by UV radiation.

In a clinical trial with 25 volunteers submitted to erythema induction by UVB radiation and treated with topical formulation containing noni leaf ethanolic extract, a protective effect against injuries caused by radiation in the skin was observed (West *et al*., 2009).

ANTI-WRINKLE ACTIVITY

The anthraquinone-1,4-dihydroxy-anthraquinone-2-methoxy-7-methyl, derived from noni fruit ethanolic extract, can be used as a new anti-wrinkle agent due to its ability to induce the biosynthetic activity of extracellular matrix components (Kim *et al*., 2005). In the same study, the authors found that this compound increases the production of pro-collagen type I and glycosaminoglycans and reduced the expression of matrix metalloproteinase (collagenase-1) in human dermal fibroblasts. In addition, the nanoemulsion prepared from this compound increased type I procollagen synthesis in the skin of rats.

PERIODONTAL TISSUE REGENERATION

Boonanantanasarn *et al*. (2014) evaluated the regenerative action of *M. citrifolia* lyophilized leaf extract in the periodontal ligament of premolars and molars. After cleaning the material, cell culture was performed using complete medium, ascorbic acid supplemented medium and noni extract supplemented medium, for the evaluation of cell proliferation, mineralization and protein synthesis. The results show that noni leaf aqueous extract was effective at inducing cellular proliferation, protein synthesis, alkaline phosphatase activity and *in vitro* matrix

mineralization, thus having an osteoinductive effect and an effect on periodontal tissue regeneration.

SUMMARY AND DISCUSSION

M. citrifolia is widely used for the treatment or prevention of various diseases. It must be understood, however, that even when using a natural product, consumption should be undertaken with care, and only after exhaustive studies on the chemical constituents and mechanisms of action of the plant, both in *in vitro* and *in vivo* models, as well as clinical trials.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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Parte II

Capítulo IV

Artigo "Avaliação da eficácia da *Morinda citrifolia* em cães infectados naturalmente pela cinomose canina"

O artigo foi submetido ao periódico *viroses (ISSN 1999-4915)*, cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é A1, fator de impacto 3.042.

Eficácia da *Morinda citrifolia* no tratamento de cães infectados naturalmente pelo vírus da cinomose

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RESUMO

O objetivo deste estudo foi avaliar a resposta clínica em cães com cinomose tratados com a droga vegetal de *M. citrifolia*. Foram utilizados quinze cães que foram submetidos ao teste de triagem rápido para detecção de antígenos da classe IgG específicos para o vírus da cinomose

canina. Os animais positivos foram submetidos a exames laboratoriais, como hemograma completo e análises bioquímicas, e divididos em dois grupos: (G1) – sete cães, tratados com a droga vegetal de *M. citrifolia* 500mg/SID e (G2) – oito cães, submetidos ao tratamento convencional com antibiótico e vitaminas do complexo A e B. A avaliação clínica dos animais com cinomose revelou que aos 30 dias pós-tratamento com noni, quatro animais apresentaram remissão dos sinais clínicos. Somente um animal sobreviveu ao grupo do tratamento convencional e teve remissão dos sinais clínicos. A droga vegetal do noni mostrou-se eficaz na remissão dos sinais clínicos neurológicos de cães portadores da cinomose.

Palavras-chave: Cinomose, Sinais neurológicos, *Morinda citrifolia*.

1 INTRODUÇÃO

A cinomose canina é uma doença viral, infecciosa, causada por um *Morbilivirus* da família *Paramoxiviridae,* da ordem *Mononegavirales*, e é considerado um dos mais importantes patógenos de cães domésticos (SILVA et al., 2009). Os *Morbillivirus* são conhecidos por causar imunossupressão, alterações cutâneas, respiratórias, gastrointestinais e neurológicas (BEINEKE et al., 2009).

A transmissão ocorre principalmente por meio de aerossóis e gotículas que contêm as partículas virais, secreções respiratórias, fezes e urina (MARTELLA et al., 2008). No período de 24 horas, as partículas virais se replicam nos macrófagos e se disseminam pela via linfática local para as tonsilas e linfonodos bronqueais (KAPIL et al., 2011).

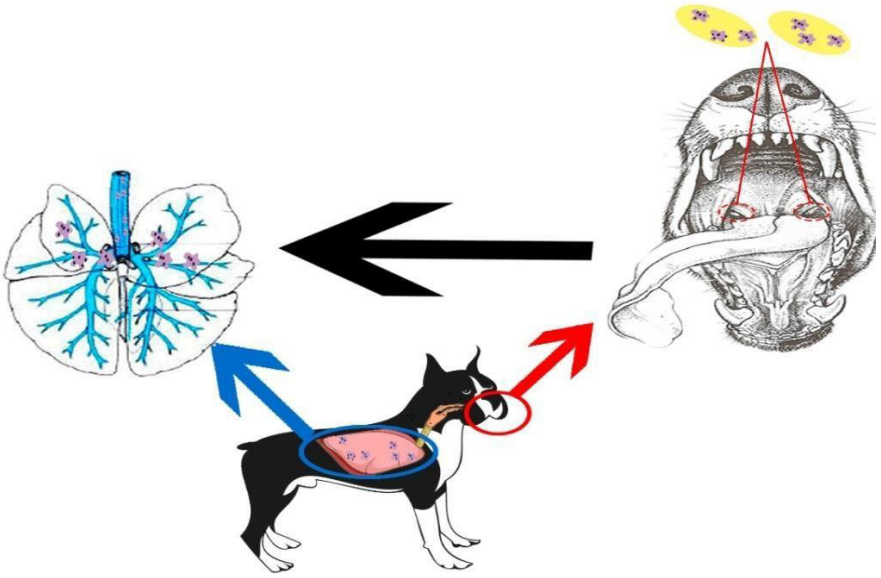

Figura 1: Patogenia da cinomose (KAPIL et al., 2011).

Entretanto, a infecção no SNC é a mais grave complicação dessa doença, que induz a distúrbios neurológicos severos e ao um prognóstico quase sempre desfavorável (VANDEVELDE & ZURBRIGGEN, 2005). Alguns cães apresentam apenas sinais neurológicos tais como: mioclonias, ataxia, paresia, paralisia, falta de coordenação motora e, às vezes alterações comportamentais como vocalização intensa e hiperexcitabilidade (MARTELLA et al., 2008).

À semelhança de todas as viroses, não há tratamento específico para cinomose, sendo na maioria dos casos utilizado um protocolo terapêutico sintomático (GREENE, 2015). Devido as plantas apresentarem metabólitos secundários, podem ser utilizadas no tratamento de muitas doenças e cresce o número de pesquisadores que com base no conhecimento tradicional investigam o potencial terapeuticos das plantas e outros produtos naturais (DENG et al., 2015). Noni (*Morinda citrifolia*) é uma planta originária do Sul da Ásia e Austrália e recentemente introduzida nos trópicos tem sido objeto de estudo de vários pesquisadores. O fato de produzir flores e frutos o ano todo, favorece o desenvolvimento de pesquisas com objetivo a avaliar o uso terapêutico (SOLOMON, 1999).

Os estudos mostram que todas as partes de *M. citrifolia* possuem pelo menos um metabólito com potencial terapêutico, no entanto, o sumo do fruto maduro é o mais utilizado principalmete como imunomodulador (HIRAZUMI et al., 1993; HOKAMA et al., 1993), antiviral (ALI et al., 2000; RATNOGLIK et al., 2014) e neuroprotetor (MURALIDHARAN et al., 2010; PACHAURI et al., 2013; MUTO et al., 2010).

Desta forma, devido a ineficácia do tratamento da cinomose e as propriedades terapêuticas atribuidas a planta é que se propôs investigar a eficácia da droga vegetal oriunda da *M. citrifolia* em cães infectados naturalmente pelo CDV, com a pretensão de desenvolver um tratamento alternativo e/ou coadjuvante de baixo custo e fácil obtenção.

2 MATERIAL E MÉTODOS

O projeto foi aprovado pelo Comitê de Ética em Pesquisa Animal da Universidade Estadual do Maranhão – UEMA, sob protocolo nº 17/2010.

2.1 Plantas

Foram coletados frutos de *M. citrifolia* (noni) para o preparo da droga vegetal na Fazenda Escola da Universidade Estadual do Maranhão (UEMA) no Campus de São Luís – MA, situada ao Sul de São Luís, Maranhão, Brasil, a 02°35.023' S, 44°12.551' W. A temperatura média desse período varia de 23°C a 31°C e a pluviosidade média é de 381 mm (SILVA et al., 2011). As coletas dos frutos foram feitas no período da manhã, nos meses de junho a novembro de 2014.

Os frutos apresentavam cor esverdeada, polpa macia, casca íntegra e estavam livres de pragas e insetos, foram lavados em água corrente e após, desidratados em estufa de secagem por 72 horas a 37°C. Posteriormente foram moídos, pesados, acondicionados em sacos plásticos esterelizados e submetidos a luz ultravioleta por 30 minutos. Em seguida, esse pó obtido foi encaminhado a um laboratório de manipulação para o encapsulamento na concentração de 500 mg de droga vegetal/cápsula.

Para a análise dos componentes do fruto de *M. citrifolia*, utilizou-se 1g do extrato metanólico e diluiu-se em 5 ml de metanol e posteriormente foi separada por cromatografia em coluna utilizando gel de Sephadex LH20 como fase estacionária e o metanol como fase móvel.

As frações foram utilizadas para obter o espectro de massa e foram injetados por inserção directa no espectrómetro de massa. O tempo de aquisição total para cada espectro foi de 1 min.

Os espectros foram extraídos pela polaridade de íons negativos com equipamentos Q - TOF Bruker Compass. Os parâmetros de aquisição foram : Varredura inicial a 50 m / z ; varredura final 1500 m / z ; conjunto capilar 4500 V; set placa de deslocamento final -500 V;

definição da tensão de carga 2000 V ; definição do nebulizador 0,4 bar; Temperatura da estufa de secagem a 200 ° C ; definição do gás a seco 4.0 L / min.

2.2 Animais

Foram utilizados quinze cães não vacinados, ambos os sexos, com idade que variou de 3 meses a 13 anos , provenientes da casuística de atendimento do Hospital Veterinário "Francisco Edilberto Uchôa Lopes", da Universidade Estadual do Maranhão (HOVET-UEMA), que apresentaram sintomatologia clínica específica da doença, em especial as alterações neurológicas como fasciculação, mioclonias, paresia, ataxia, paralisia, sensibilidade na região dorsal, rigidez dos membros torácicos e convulsão além de outras sintomatologias específicas, tais como secreções oculares e nasal.

Após essa análise semiológica detalhada, segundo Feitosa (1997) (2014), os animais foram submetidos ao teste rápido imunoenzimático Alere® Ac para cinomose, sendo utilizado somente aqueles cujo resultado foi positivo. Todos os cães ficaram no canil experimental da UEMA até o final do tratamento e as medicações foram administradas na mesma hora em todos os dias. Foi feito uma coleta de sangue para análise hematológicas e provas bioquímicas, antes e após o tratamento.

Para análise hematológica, foi utilizada máquina Bio 1800 vet (Bioeasy), de acordo com as recomendações do fabricante. Para identificação dos leucócitos e das células diferenciadas (linfócitos, eosinófilos, monócitos e segmentados), utilizou-se o procedimento manual do esfregaço sanguíneo. As proteínas plasmáticas totais (PPT) foram quantificadas em espectrophotometro Coleman 295.

Para as provas bioquímicas de creatinina, ureia, Aspartato amino transferase (AST) e Alanina amino transferase (ALT), utilizou-se soro e as análises foram realizadas em analisador semi-automático (Celm SBA 200). Nestas, foram utilizados kits comerciais (Labtest), seguindo as recomendações do fabricante, para determinação por métodos cinéticos.

Os proprietários dos animais utilizados nesse estudo foram orientados quanto a proposta da pesquisa e, após concordarem, assinaram um termo de consentimento livre e esclarecido conforme o tratamento escolhido.

Os tratamentos utilizados foram :

Grupo 1 (G1): Sete cães submetidos ao tratamento com a droga vegetal de *M. citrifolia*, 500 mg/SID/VO durante 30 dias.

Grupo 2 (G2): Oito cães, submetidos ao tratamento convencional com o antibiótico enrofloxacino 50 mg/kg/BID/VO , durante 15 dias. Foram também utilizados um estimulador neurológico a base de vitaminas A e do complexo B (15 dias) e corticoide, dexametasona, na dose de 1mg/5kg/SID/VO (5 dias).

3 RESULTADOS E DISCUSSÃO

O extrato metanólico foi dividido em nove frações que foram identificados íons moleculares correspondentes a escopoletina, damnacanthal, ácido ursólico, noniosídeo C, ácido asperuloside e ácido deacetil asperulosidic.

Sete animais com sinais neurológicos avançados que variavam de ataxia até paralisia dos membros, foram submetidos ao tratamento com *M. citrifolia.* Todos os animais foram avaliados diariamente verificando a remissão dos sinais clínicos neurológicos. Observou-se que após cinco dias de tratamento os animais já apresentavam uma melhora clínica. Um animal apresentava paresia dos membros pélvicos, voltou a andar com dez dias de tratamento e aos vinte e um dias observou-se uma remissão significativa dos sinais clínicos, porém o animal continuou apresentando fasciculação no membro torácico direito. Segundo Silva (2009), a cinomose na fase neurológica é progressiva, raramente estacionando, levando à morte em curso agudo ou crônico, e sempre deixando sequelas irreversíveis, quando eventualmente o animal sobreviva.

A remissão dos sinais neurológicos foi o aspecto mais importante nesta pesquisa, o que gera perspectivas para o tratamento de doenças que afetam o tecido nervoso de animais e pessoas. No entanto, não foi possível determinar qual o mecanismo que contribui para a melhora dos sinais neurológicos. Um grupo de pesquisadores japoneses descreve que *M. citrifolia* em condições experimentais teve efeito protetor para os neurônios, por facilitar a secreção de insulina e por atenuar os efeitos da intolerância destas células à diminuição de glicose nos casos de isquemia (HARADA et al., 2009).

Comparando-se a resposta clínica frente ao tratamento com o fitoterápico, observou-se que o noni proporcionou uma melhora em cinco dias. Enquanto que a utilização de drogas

experimentais, como ribavirina, os animais só começaram a apresentar uma melhora no quadro clínico a partir do sétimo dia pós-tratamento (MANGIA & PAES, 2008). A eficácia do tratamento do noni em um intervalo de tempo menor pode ser um fator extremamente importante para a minimização dos danos aos neurônios, o que o tornaria muito mais eficaz na terapêutica desta doença. Em estudos desenvolvidos por Akihisa et al. (2007) foi observado que compostos isolados a partir do extrato metanólico dos frutos de *M. citrifolia* exibiram efeitos inibitórios moderados contra o vírus Epstein-Barr.

Um animal também estava positivo para babesia e além do tratamento com a droga vegetal, foi administrado uma aplicação de imizol após dez dias de tratamento. Apresentava paresia dos membros pélvicos, fasciculação do membro pélvico direito, mioclonia e sensibilidade na região dorsal com presença de dor. Após 13 dias de tratamento, o animal voltou a andar. Younos et al. (1990) comprovaram que o extrato aquoso liofilizado das raízes de *M. citrifolia* demonstrou efeitos analgésicos e alterações comportamentais significativos e dose-dependentes em camundongos. Podemos associar a diminuição da dor na região dorsal do animal ao tratamento com a droga vegetal de *M. citrifolia*. Aos 21 dias observou-se a diminuição da fasciculação. Porém, após uma notável melhora clínica, o animal começou a apresentar crises de convulsão, que progrediram rapidamente e veio a óbito após 60 dias ao início do tratamento.

Em casos de convulsões, devem ser utilizados medicações como diazepam ou fenobarbital, pelas vias intravenosa, intramuscular ou oral, a cada 12 horas. Nos casos severos de encefalite multifocal progressiva, com lesões neurológicas como a tetraplegia, semi coma e incapacitação, a eutanásia é recomendada (GREENE, 2015). Verificou-se nesse estudo que a droga vegetal na concentraração testada não tem ação anticonvulsivante em cães.

Na droga vegetal utilizada nesse estudo foi identificada uma substância química, uma cumarina, a escopoletina, que apresenta efeitos analgésicos, antinociceptivo e anti-inflamatória. É conhecido que o CDV causa sérios danos neuronais e leva a desmielinização. Considerando que o suco do noni possui propriedades imunomoduladora, anti-inflamatória e analgésica (BASAR et al., 2010), presumimos que os vários componentes existentes no fruto têm ação conjunta na remissão dos danos causados no SNC e na resposta imunológica.

Dentre as substâncias identificadas no noni destaca-se o dannacanthal, uma antraquinona natural que demonstrou ter atividade no controle da infecção por HIV por induzir

a morte de células infectadas pelo vírus (ALI et al., 2000). Com base na análise fitoquímica, o dannacanthal foi uma das substâncias identificadas na droga vegetal utilizada nesse estudo, o que podemos relacionar a remissão clínica dos sinais neurológicos dos animais que não apresentavam lesões muitos graves.

Após análise estatística dos resultados das coletas feitas no dia 0 e depois de 21 dias de tratamento com a droga vegetal, observou-se que dos parâmetros hematimétricos analisados, somente as plaquetas apresentaram um grau de significância estatística (Tabela 1). Não foi observada alteração dos parâmetros bioquímicos (Tabela 2) após o tratamento com a droga vegetal, sugerindo que o noni não exerceu efeitos hepatotóxicos e nefrotóxicos. Em pesquisas com ratos foi demonstrado que o suco de noni fermentado quando administrado tem um efeito hepatoprotetor e hipoglicemiante (WEST et al., 2009; NAYAK et al., 2011).

No grupo dois, oito animais foram tratados com medicações convencionais. Somente um animal sobreviveu após o tratamento e também foi submetido ao tratamento com cinoglobulim. Mioclonia também foi o sinal clínico mais predominante nesse grupo. Entretanto, não foi possível fazer a coleta após o tratamento de seis animais do grupo dois, pois vieram a óbito no período de 4 a 8 dias após o início do tratamento.

Na atualidade o tratamento existente para a cinomose é paliativo e, normalmente quando é diagnosticada a forma neurológica, a recomendação é a eutanásia dos animais. Com base na inexistência de uma terapêutica adequada utilizamos o noni na perspectiva de obtenção de um produto eficaz no tratamento desta importante doença de canídeos, que acomete milhares de cães, principalmente em países em desenvolvimento.

Conclusão

A droga vegetal do noni mostrou-se eficaz na remissão dos sinais neurológicos de cães portadores da cinomose, porém há necessidade de elucidar o mecanismo de ação dos compostos bioativos e se o aumento da concentração produz efeitos mais satisfatório.

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Tabela 01- Parâmetros hematimétricros das amostras de cães com cinomose, provenientes do município

de São Luís – MA, antes e após o tratamento com a droga vegetal de *M. citrifolia* (noni)

Parâmetros hematológicos	G1[1]		G1[2]		Valores de referência	Valor p
	Mean	± SD	Média	±DP		
VG (%)	34.429[a]	±6.579	37.429[a]	±5.855	37-55	0.3089
Hemácias (x10^6/µL)	5.671[a]	±0.884	5.214[a]	±0.998	5,5-8,5	0.3056
Hemoglobina (g/dL)	10.431[a]	±1.992	11.340[a]	±1.774	12-18	0.3090
Leucócitos	11.064[a]	±5.956	11.650[a]	±4.965	6.000-17.000	0.8652
Segmentados	74.143[a]	±18.197	69.857[a]	±14.622	3.000-11.500	0.3945
Linfócitos	16.429[a]	±17.605	20.000[a]	±11.776	1.000-4800	0.3751
Monócitos	1.143[a]	±1.464	2.571[a]	±3.259	150-1.350	0.3661
Eosinófilos	5.143[a]	±4.413	7.143[a]	±6.176	100-1.250	0.5644
Bastonetes	1.714[a]	±1.113	0.4286[a]	±1.134	00-540	0.0633
PPT (g/dL)	6.286[a]	±1.799	6.857[a]	±1.069	5,5-8,0	0.5686
Plaquetas	271.82[a]	±117.26	443.29[b]	±150.82	200.000-900.000	0.0005

a – letras iguais na mesma linha indicam não haver diferenças estatísticas significativas entre as médias (p > 0,05).
b- letras diferentes na mesma linha indicam haver diferenças estatísticas significativas entre as médias (p < 0,05).
1 - ANOVA com comparação das médias pelo teste não paramétrico de Mann-Whitney.
VG= volume globular; PPT= proteínas plasmáticas totais.
G1[1]- grupo cinomose antes do tratamento com a droga vegetal. G1[2]- grupo cinomose depois do tratamento com a droga vegetal.

Tabela 02- Parâmetros bioquímicos das amostras de cães com cinomose, provenientes do município de São Luís – MA, antes e após o tratamento com *Morinda citrifolia*

Parâmetros bioquímicos	G1[1]		G1[2]		Valores de referência	Valor p
	Média	±DP	Média	±DP		
Uréia (mg/dl)	36,00[a]	±21,42	51,30[a]	±35,73	10-60	0,1075
Creatinina (mg/dl)	0,58[a]	±0,20	0,79[a]	±0,41	0,5-1,5	0,2697
AST (UI/L)	43,40[a]	±18,27	47,40[a]	±29,14	10-88	0,6520
ALT (UI/L)	43,40[a]	±18,27	37,90[a]	±26,18	10-88	0,5433

a – letras iguais na mesma linha indicam não haver diferenças estatísticas significativas entre as médias (p > 0,05).
1 - ANOVA com comparação das médias pelo teste não paramétrico de Mann-Whitney.
G1[1]- grupo cinomose antes do tratamento com noni. G1[2]- grupo cinomose depois do tratamento com noni.
TGO (AST- - Aspartato amino transferase); TGP (ALT- Alanina amino transferase).

Parte III

Conclusões e Referências

9 CONCLUSÕES

- A droga vegetal de *M. citrifolia* (noni) não foi eficiente no tratamento de *Ehrlichia canis*, pois não apresentou cura clínica da doença, porém mostrou-se eficaz na remissão dos sinais neurológicos de cães portadores da cinomose, quando não estavam em fase neurológica muito avançada;

- Nos animais com cinomose e tratados droga vegetal de *M. citrifolia* apresentaram melhora dos parâmetros dos hematológicos e induziu aumento do número de plaquetas;

- Os tratamentos com o noni não interferiram nas análises das funções hepáticas e renais;

- Os neurônios apresentaram intensamente marcados para o vírus CDV e a região anatômica mais afetada foi o pendunculo cerebelar;

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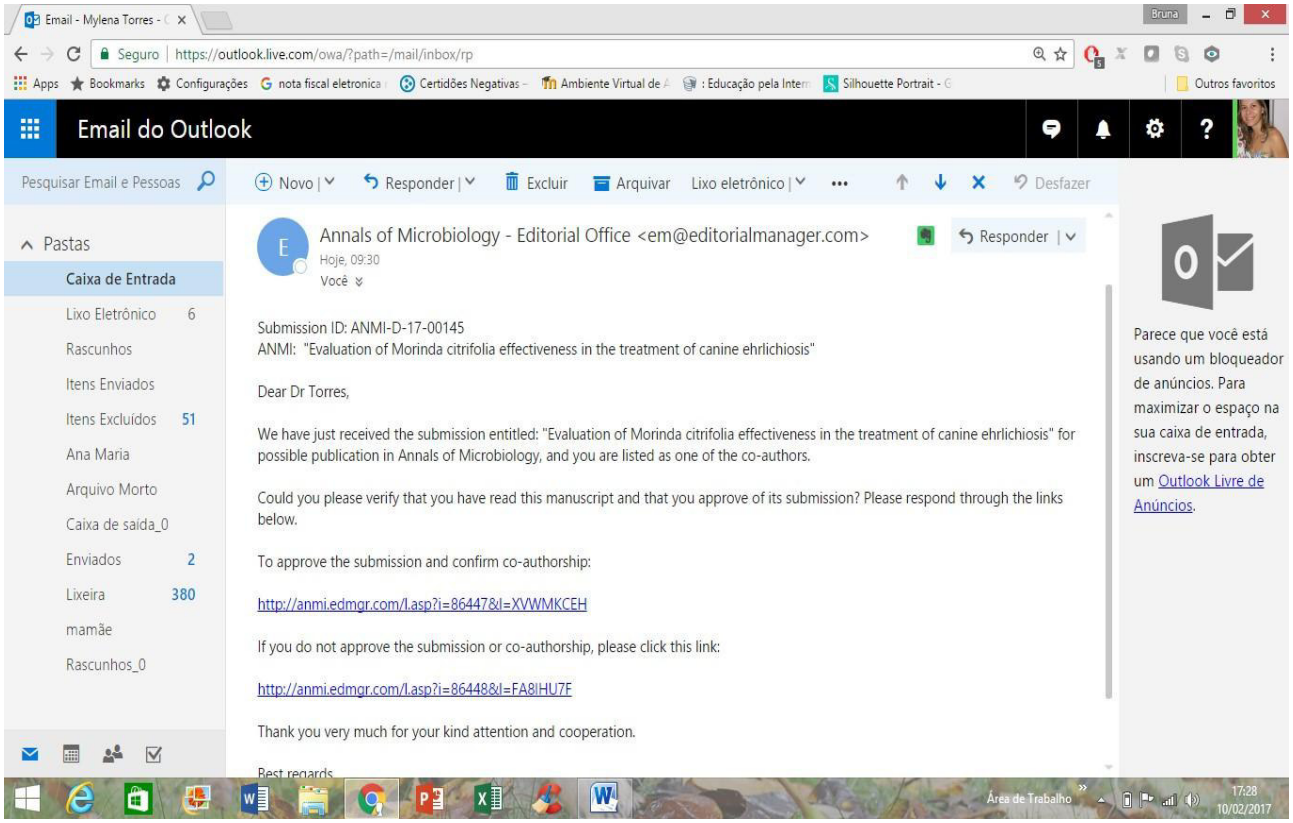
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Dear Editor-in-Chief
Francesca Cappitelli

We are sending the manuscript "*Evaluation of Morinda citrifolia effectiveness in the treatment of canine ehrlichiosis*" which authors are Mylena Andréa Oliveira Torres, Raquel Albuquerque Silva, Anderson Cássio Campelo Costa, Joicy Cortêz de Sá, Zulmira da Silva Batista, Nathálya dos Santos Martins, Renata Mondêgo-Oliveira, Isadora de Fátima Braga Magalhães, Rafael Cardoso Carvalho and Ana Lúcia Abreu-Silva for submission to the *Annals of Microbiology*. We inform that this manuscript has been read and approved by all named authors. Besides that, we confirm that the order of authors listed in the manuscript has been approved by all of us.

Sincerely Yours,

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

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Reference citations in the text should be identified by numbers in square brackets. Some examples:
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- Book

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- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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São Luís, 24 de jan 2017

Dear Editor-in-Chief
Marc van Regenmortel

We are sending the manuscript "*Histological and immunohistochemistry CDV through encephalon parenchyma"* which authors are Mylena Andréa Oliveira Torres, Anderson Cássio Campelo Costa, Tiago da Silva Teófilo, Nathálya dos Santos Martins, Ana Patrícia de Carvalho da Silva, Joicy Cortêz de Sá, Renata Mondêgo-Oliveira, Joanna Jéssica Sousa Albuquerque, Ferdinan Almeida Melo and Ana Lúcia Abreu-Silva for submission to the Journal of Veterinary Research. We inform that this manuscript has been read and approved by all named authors. Besides that, we confirm that the order of authors listed in the manuscript has been approved by all of us.

Sincerely Yours,

Dra. Ana Lucia Abreu-Silva
Departamento de Patologia
Universidade Estadual do Maranhão.

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Anderson Cássio Campelo Costa

Tiago da Silva Teófilo

Nathálya dos Santos Martins

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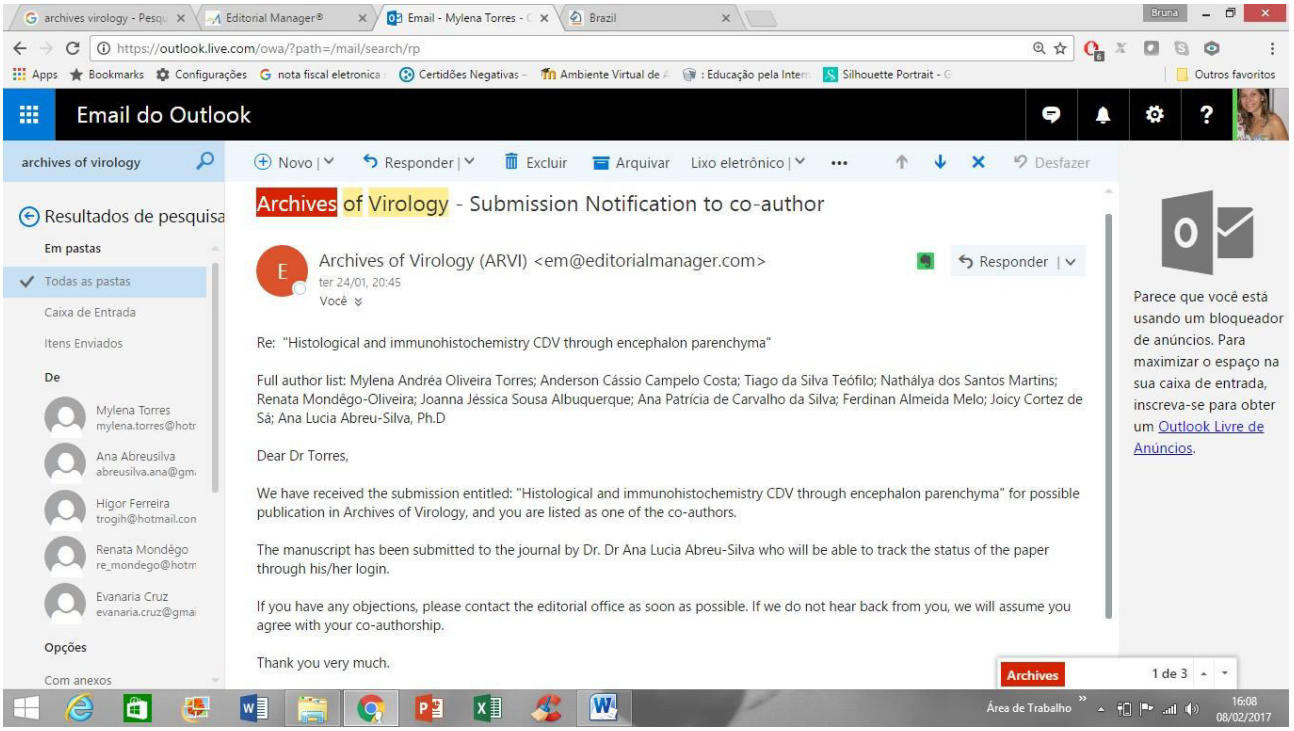

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São Luís,, 16 Jan 2016

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Elizabeth M. Williamson

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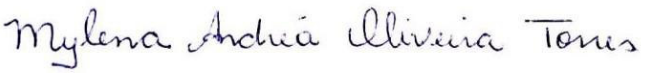

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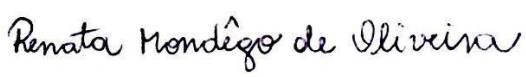

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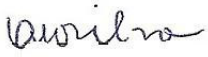

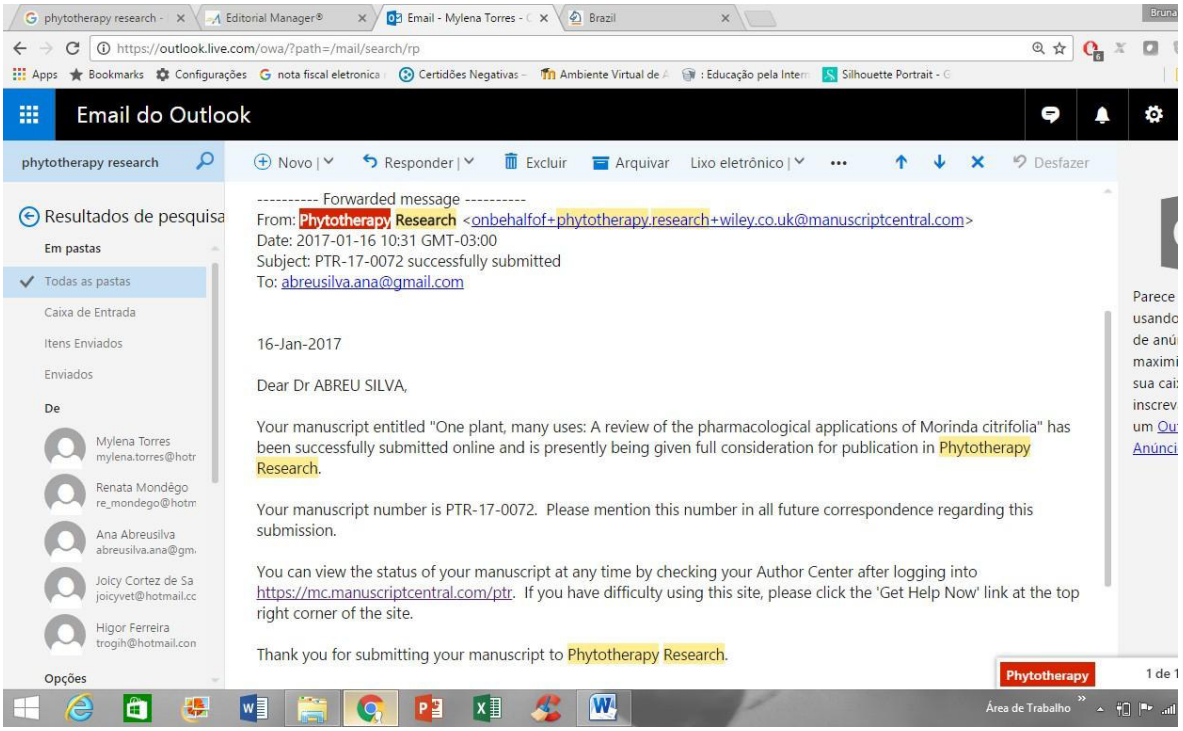
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Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.

- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (http://www.wma.net/en/30publications/10policies/b3/), revised in 2008. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. As a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). A written informed consent for publication must be obtained from participating patients in this case.

Editors reserve the rights to reject any submission that does not meet these requirements.

Example of Ethical Statements:

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code).

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and

- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Viruses endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from ‘Crown Mix’ (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx) were kindly provided by Dr. XXX , institute, city, country).