Câmpus Ipameri

Universidade Estadual de Goiás

PPGPV

Programa de Pós-Graduação em Produção Vegetal

EXTRATO AQUOSO DE FOLHAS DE *Vernonanthura polyanthes:* ANGIOGÊNESE E TOXICIDADE

ANGÉLICA DAIANE LEMOS DO PRADO

MESTRADO

Ipameri-GO
2018

ANGÉLICA DAIANE LEMOS DO PRADO

EXTRATO AQUOSO DE FOLHAS DE *Vernonanthura polyanthes:* ANGIOGÊNESE E TOXICIDADE

Orientadora: Profa. Dra. Luciane Madureira de Almeida

Dissertação apresentada à Universidade Estadual de Goiás – UEG, Unidade Universitária de Ipameri como parte das exigências do Programa de Pós Graduação em Produção Vegetal para obtenção do título de MESTRE.

Ipameri
2018

Prado, Angélica Daiane Lemos
PP896e EXTRATO AQUOSO DE FOLHAS DE Vernonanthura polyanthes: ANGIOGÊNESE E TOXICIDADE / Angélica Daiane Lemos Prado; orientador Luciane Madureira de Almeida; co-orientador Fábio Santos Matos. -- Ipameri, 2018.
68 p.

Dissertação (Mestrado - Programa de Pós-Graduação Mestrado Acadêmico em Produção Vegetal) -- Câmpus-Ipameri, Universidade Estadual de Goiás, 2018.

1. Recursos Genéticos. 2. Biomateriais. 3. Angiogênese. 4. Antiangiogênese. 5. Toxidade. I. Madureira de Almeida, Luciane , orient. II. Santos Matos, Fábio, co-orient. III. Título.

Câmpus Ipameri Universidade Estadual de Goiás

ESTADO DE GOIÁS

CERTIFICADO DE APROVAÇÃO

TÍTULO: "EXTRATO AQUOSO DE FOLHAS DE *Vernonanthura polyanthes*: ANGIOGÊNESE E TOXICIDADE"

AUTORA: Angélica Daiane Lemos do Prado

ORIENTADORA: Luciane Madureira de Almeida

Aprovada como parte das exigências para obtenção do Título de MESTRE EM PRODUÇÃO VEGETAL, pela comissão Examinadora:

Luciane Madureira de Almeida
Profa. Dra. LUCIANE MADUREIRA DE ALMEIDA
Universidade Estadual de Goiás/Câmpus Ipameri-GO

Profa. Dra. ELISA FLÁVIA LUIZ CARDOSO BAILÃO
Universidade Estadual de Goiás/Câmpus Anapólis-GO

Prof. Dr. PAULO ROBERTO DE MELO REIS
Pontifícia Universidade Católica de Goiás/ Goiânia-GO

Data da realização: 23 de fevereiro de 2018

Dedicatória

A Deus por ter me concedido uma vida cercada por pessoas tão especiais.

Agradecimentos

À Deus, por não deixar abalar minha fé e permitir que eu permaneça perseverante nesta caminhada.

À minha orientadora professora Dra. Luciane Madureira de Almeida, que é muito mais que uma orientadora para mim, é um exemplo que eu quero seguir. Obrigada por acreditar em mim desde a graduação e ter contribuído tanto para a minha carreira profissional e também por ter me influenciado tão positivamente na minha vida pessoal. Você tem toda minha admiração e carinho!

À professora Dra. Elisa Flávia Luiz Cardoso Bailão, que muito colaborou para a execução deste trabalho. Muito obrigada pela paciência em transmitir seus conhecimentos, sem dúvida alguma eu aprendi muito neste último ano.

À Joseliana e Adriana, que muitos mais que me auxiliar nas práticas de laboratório foram minhas amigas com quem compartilhei medos durante esse último ano. Obrigada por sempre torcerem por mim, obrigada pela amizade!

Aos funcionários da Universidade Estadual de Goiás, em especial o pessoal da biblioteca que foram minha companhia durante horas de estudos.

Agradeço também aos colegas de laboratório que colaboraram com várias etapas deste trabalho, em especial a Patrícia, Milena, Aparecido e Gleiciane. Com vocês eu apreendi que realmente não se pode fazer nada sozinho!

À professora Dra. Elisa Flávia Luiz Cardoso Bailão e ao professor Dr. Paulo Roberto Melo Reis, por aceitarem o convite para participar da banca.

Aos professores do Programa de Pós-Graduação em Produção Vegetal da Universidade Estadual de Goiás, em especial Nei Peixoto, Fábio Mattos, Fabrício Rodrigues, Ednaldo Rocha Cândido, Adilson Pelá, Flávio Gonçalves de Jesus, Márcio da Silva Araújo. Obrigada por contribuírem com tantos ensinamentos. Vocês também são minhas referências!

À minha família querida que mesmo não entendendo o real significado de tudo isso sempre apoiaram e acreditaram em mim! Vocês me fazem sentir muito amada e com isso eu me sinto mais fortalecida!

À minha segunda família de Paula Silva, obrigada por todo carinho e por sempre me encorajarem a vencer meus desafios!

Ao meu namorado Gabriel que mesmo distante nesse últimos meses me deu força e me fez reconhecer a minha capacidade! Obrigada também por todo amor e paciência nos

meus momentos de desespero nesses últimos dois anos! Com você eu aprendi a ter mais orgulho de tudo que já conquistei até aqui!

Aos meus colegas de turma por todos os conhecimentos compartilhados em aulas e pelo ombro amigo oferecido durante momentos difíceis.

À coordenação do Programa de Pós-Graduação, em especial a Aparecida por sempre me ajudar com a parte burocrática do mestrado e também por escutar meus desabafos.

À Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (Capes) pelo apoio financeiro, sem o qual com certeza teria sido mais difícil a condução dos experimentos.

Enfim extremamente grata por tudo e por todos que contribuíram direta ou indiretamente para a realização de mais um sonho!

SUMÁRIO

RESUMO

A espécie *Vernonanthura polyanthes* conhecida popularmente como assa-peixe, pertence à família Asteraceae e apresenta propriedades farmacológicas em função dos compostos bioativos que possui. Popularmente tem sido utilizada para tratar uma variedade de doenças, incluindo feridas, bronquite, pneumonia, hemoptise, tosse persistente, abscessos internos, dor gástrica e de cálculos renais. Em busca de avaliar o potencial do extrato aquoso do extrato de *V. polyanthes* os objetivos do presente trabalho foram: 1) identificar a atividade angiogênica; 2) realizar análise cienciométrica para identificar as tendências da utilização da técnica da membrana corioalantóide de galinha (CAM) e 3) avaliar o potencial tóxico e citogenotóxico. Para análise da atividade angiogênica foi utilizado o ensaio CAM. Para essa análise foram utilizados os seguintes tratamentos: água esterilizada, Regederm®, dexametasona e o extrato de folhas de *V. polyanthes* nas concentrações de 10, 20 e 40 mg/ml. Como resultado as concentrações de 20 mg/ml e 40 mg/ml apresentam propriedades antiangiogênicas. Esses resultados sugerem o uso dessa planta como possível agente terapêutico que pode ser usado para o tratamento de tumores. A análise cienciométrica foi feita por meio da busca por artigos científicos no ISI – Web of Knowedge utilizando as seguintes combinações de palavras: "chorioallantoic membrane" and CAM, "chorioallantoic membrane" e "chick embryo membrane". Os resultados mostraram que a técnica CAM é um método confiável, que tem sido amplamente utilizado pela comunidade cientifica para responder diferentes processos biológicos. Em relação à avaliação da atividade tóxica e citogenotóxica do extrato aquoso das folhas de *V. polyanthes*, foram usados os métodos de *Artemia salina* e *Allium cepa*. Como resultados, o extrato aquoso de folhas de *V. polyantes* preparados nas concentrações de 20, 40 e 80 mg/ml mostraram-se tóxicos para *Artemia salina*, sendo a dose letal mínima (DL50) igual a 24 mg/ml. Ainda em relação à toxicicidade, o ensaio com *A. cepa* mostrou toxicidade dos extratos de *V. polyanthes* em todas as concentrações testadas (10, 20 e 40 mg/ml). As análises microscópicas do ensaio com *A. cepa* mostrou que os extratos aquosos de folhas de *V. polyanthes* são citotóxicos apenas na concentração de 40 mg/ml. Nenhuma concentração estudada apresentou significante genotoxicidade nas células de *A. cepa.*

Palavras chave: *Allium cepa*, *Artemia salina*, assa-peixe, teste CAM, plantas medicinais

ABSTRACT

The specie *Vernonanthura polyanthes* popularly known as assa-peixe, belongs to the family Asteraceae and presents pharmacological properties in function of the bioactive compounds that it possesses. Popularly it has been used to treat a variety of diseases, including wounds, bronchitis, pneumonia, hemoptysis, persistent cough, internal abscesses, gastric pain and kidney stones. In order to evaluate the potential of the aqueous extract of *V. polyanthes* extract the objectives of the present study were: 1) to identify angiogenic activity; 2) perform a scientiometric analysis to identify trends in the use of chicken chorioallantoic membrane (CAM) technique and 3) evaluate the toxic and cytotoxic potential. For analysis of angiogenic activity the CAM assay was used. For this analysis, the following treatments were used: sterile water, Regederm®, dexamethasone and the leaves extract of *V. polyanthes* at 10, 20 and 40 mg/ml concentrations. As a result, concentrations of 20 mg/ml and 40 mg/ml have antiangiogenic properties. These results suggest the use of this plant as a possible therapeutic agent that can be used for the treatment of tumors. The sciciometric analysis was done by searching for scientific articles in the ISI - Web of Knowedge using the following combinations of words: "chorioallantoic membrane" and CAM, "chorioallantoic membrane" and "chick embryo membrane". The results showed that the CAM technique is a reliable method, which has been widely used by the scientific community to respond different biological processes. In order to evaluate the toxic and cytogenotoxic activity of the aqueous extract of the leafs from *V. polyanthes*, the methods of *Artemia salina* and *Allium cepa* were used. As a result the aqueous extract of leafs from *V. polyanthess* prepared in the concentrations of 20, 40 and 80 mg / ml were toxic to *Artemia salina*, with the LC50 toxicity threshold being 24 mg / ml. Also in relation to toxicity, the *A. salina* showed toxicity of *V. polyanthes* extracts for all concentrations tested (10, 20 and 40 mg/ml). Microscopy analyzes of the *A. salina* showed that aqueous extracts of *V. polyanthes* leaves are cytotoxic in the concentration of 40 mg/ml. No concentration studied showed significant genotoxicity in the cells of *A. salina*.

Keywords: *Allium cepa*, *Artemia salina*, assa-fish, CAM test, medicinal plants

1. INTRODUÇÃO

Ao longo da história da humanidade, o tratamento de diversas doenças tem sido realizado por produtos de origem vegetal, o que torna o uso de plantas medicinais uma prática milenar conservada por meio da transmissão do saber popular às gerações seguintes (TOSCANO RICO, 2011). Plantas medicinais são caracterizadas por possuírem propriedades farmacológicas que exercem efeito curativo ao serem consumidas pelo homem (MOTALEB et al., 2011). Estima-se que cerca de 85% da população mundial utilize plantas medicinais para o tratamento de doenças (Organização Mundial da Saúde, 2017).

As propriedades farmacológicas das plantas medicinais estão geralmente associadas à síntese e acúmulo de diversos compostos bioativos, tais como: taninos, lignanas, cumarinas, quinonas, estilbenos, xantonas, ácidos fenólicos, flavonas, flavonóis, catequinas, antocianinas e proantocianinas (RODRIGUES et al., 2011). Em função desses compostos, as plantas medicinais são usualmente utilizadas como matérias-primas para a manipulação de diversos medicamentos (HASSAN, 2012).

Dentre as diversas plantas utilizadas na medicina popular está a espécie *Vernonanthura polyanthes*, conhecida popularmente como assa-peixe. Essa planta pertence à família Astereceae a qual inclui aproximadamente 24.000 espécies e 1.600-1.700 gêneros encontrados em ambientes diversos (FUNK et al., 2009). A espécie é um arbusto que pode atingir até três metros de altura, composta por folhas do tipo lanceoladas variando entre 5 a 15 cm de comprimento e 1 a 3 cm de largura (VEJA; DEMATTEIS, 2012).

Na medicina popular, os extratos de *V. polyanthes* vêm sendo utilizados para o tratamento de feridas, bronquite, tosse persistente, pneumonia, cálculos renais, distúrbios gástricos, malária, febre, fraturas, entorses, hematomas e luxações (GUERRA-SANTOS et al., 2016). O uso popular de assa-peixe no tratamento de feridas sugere que esta espécie possua biocompostos que estimulem a atividade angiogênica. A angiogênese é uma atividade biológica na qual a partir de vasos sanguíneos pré-existentes há a formação de novos vasos sanguíneos por meio da proliferação, migração, regulação ou diferenciação de células vasculares (FOLKMAN, 2003).

Do ponto de vista das aplicações médicas, biocompostos que induzem a angiogênese são importantes para engenharia de tecidos, para aumentar a proliferação celular ou para promover a cicatrização de feridas (SCHULTZ et al., 2003; SHEN; FALANGA, 2003). Por outro lado, um composto com potencial antiangiogênico é promissor para o tratamento de tumores. Isso porque a ocorrência de angiogênese no tumor estimula o crescimento invasividade e metástase tumoral (WEIS; CHERESH, 2011). Nesse trabalho, foi avaliada a

atividade angiogênica do extrato aquosos das folhas de *V. polyanthes* utilizando o modelo da membrana corioalantóide de galinha (CAM). CAM é a membrana extraembrionária originada pela fusão do córion com o alantóide (RIBATTI, 2010). Essa membrana realiza múltiplas funções durante o desenvolvimento embrionário, como a respiração, o transporte de cálcio a partir da casca do ovo, a homeostase ácido-base e a reabsorção de íons e água do fluído amniótico (YUAN et al., 2014). À medida que a CAM se expande, é gerada uma vasta rede vascular que permite a investigação rápida e de baixo custo de vários processos biológicos, incluindo o crescimento tumoral e a metástase, bem como a análise farmacológica de compostos angiogênicos e antiangiogênicos. Os resultados obtidos em relação ao potencial angiogênico de *V. polyanthes* estão descritos no capítulo 1 da presente dissertação.

Por se tratar de um processo fisiológico fundamental com fortes implicações na homeostase dos tecidos, a angiogênese vem sendo intensamente estudada no meio científico. Existem diferentes modelos experimentais que ajudam a identificar e caracterizar os eventos de angiogênese. Dentre eles, o ensaio CAM é um dos métodos mais utilizados. No presente estudo, foi realizado um levantamento sobre o atual conhecimento científico e utilização experimental do modelo CAM para avaliação da atividade angiogênica. Os dados obtidos na análise cienciométrica da metodologia CAM estão apresentados no capítulo 2.

Independente da atividade angiogênica do extrato de *V. polyanthes*, é fundamental avaliar a toxicidade dessa espécie antes do seu uso empírico. Atualmente, existem poucas informações científicas relacionadas ao uso farmacológico dessa espécie (MARTUCCI et al., 2014). Isso é preocupante, uma vez que as supostas vantagens terapêuticas dessa planta e o seu potencial tóxico não são conhecidos pelo público em geral ou por profissionais da área da saúde. Sendo assim, essa planta é popularmente considerada medicinal, mas pode conter compostos perigosos à saúde humana. Por essa razão faz-se necessários à condução de estudos científicos sobre a toxicidade da espécie para evitar reações adversas. Existe uma grande variedade de testes para se avaliar a toxicidade das plantas medicinais. Um dos mais amplamente utilizados devido sua simplicidade, baixo custo, confiabilidade e concordância com outros testes de genotoxicidade é o teste de *Allium cepa.* Outro teste de toxicidade simples amplamente utilizado é o com *Artemia salina.* Na presente pesquisa, foram avaliadas a toxicidade, citotoxicidade e genotoxicidade de diferentes concentrações do extrato aquoso de *V. polyanthes* e os resultados obtidos estão apresentados no capítulo 3 da presente dissertação.

2. OBJETIVOS

O objetivo desta pesquisa foi avaliar o potencial angiogênico e tóxico do extrato aquoso de folhas de *V. polyanthes.* Além de realizar um levantamento sobre o atual conhecimento científico sobre o uso da metodologia CAM nos artigos científicos publicados nas três últimas décadas.

2.1 OBJETIVOS ESPECÍFICOS

Para atingir esse objetivo geral, foram realizados os seguintes objetivos específicos:

1.) Identificar atividade angiogênica do extrato aquoso de folhas de *V. polyanthes*, utilizando-se o ensaio biológico da membrana corioalantóide (CAM) de ovo fertilizado de galinha;

2.) Análise cienciométrica para identificar as tendências da utilização da técnica da membrana corioalantóide de galinha (CAM).

3.) Avaliar o potencial citotóxico e genotóxico do extrato aquoso de folhas de *V. polyanthes*, por meio do teste de *Allium cepa* e de *Artemia salina*.

Capítulo I

Propriedades antiangiogênicas do extrato aquoso de folhas de *V. polyanthes*

RESUMO

O Cerrado possui uma ampla biodiversidade com diversas plantas medicinais catalogadas e várias outras ainda são pouco estudadas. A espécie *Vernonanthura polyanthes* apresenta propriedades farmacológicas e é frequentemente empregada pela medicina popular para o tratamento de doenças respiratórias, cálculo renal, malária e febre. Além disso outro indicativo, na medicina popular, da utilização dessa espécie é para a cicatrização de feridas de pele, o que sugere a presença de biocompostos que estimulem a angiogênese. Dessa forma o objetivo deste trabalho foi avaliar o potencial angiogênico da espécie utilizando-se diferentes concentrações do extrato das folhas. Para isso foi utilizado o modelo da membrana corioalantóide (CAM) em embriões de galinha. Os tratamentos utilizados foram: água esterilizada, Regederm, dexametasona e o extrato das folhas de *V. polyanthes* nas concentrações de 10, 20 e 40 mg/ml. Como resultado as concentrações de 20 mg/ml e 40 mg/ml apresentam propriedades antiangiogênicas. Esses resultados sugerem que essa planta possa ser utilizada como um agente terapêutico no tratamento contra neoplasias malignas angiogênicas dependentes.

Palavras chave: Teste CAM, assa-peixe, plantas medicinais

ABSTRACT

The Cerrado has a wide biodiversity with several medicinal plants cataloged and several others are still little studied. The species *Vernonanthura polyanthes* exhibits pharmacological properties and is often employed by folk medicine for the treatment of respiratory diseases, renal calculi, malaria and fever. In addition, another indication in popular medicine of the use of this species is for the healing of skin wounds, which suggests the presence of biocomposites that stimulate angiogenesis. Thus, the objective of this work was to evaluate the angiogenic potential of the specie using different concentrations of leaf extract. For this, the chorioallantoic membrane (CAM) model in chicken embryos was used. The treatments used were: sterilized water, Regederm, dexamethasone and the extract of the leaves of *V. polyanthes* at concentrations of 10, 20 and 40 mg / ml. As a result, concentrations of 20 mg / ml and 40 mg / ml have antiangiogenic properties. These results suggest that this plant can be used as a therapeutic agent in the treatment of angiogenic dependent malignant neoplasms.

Keywords: CAM test, assa-peixe, medicinal plants

1. INTRODUÇÃO

O Cerrado cobre aproximadamente 22% do território brasileiro, e é considerado o segundo maior bioma em extensão geográfica da América do Sul (Ministério do Meio Ambiente, MMA, 2017). Apresenta uma elevada biodiversidade, com aproximadamente 11.627 espécies de plantas nativas descritas, das quais 220 espécies possuem uso medicinal comprovado, porém muitas outras ainda não foram avaliadas cientificamente (MMA, 2017). Das 220 espécies com potencial medicinal descrito, 44% são endêmicas do bioma Cerrado (RATTER et al., 2003). Apesar da alta diversidade da flora desse bioma, o Cerrado tem sua biodiversidade ameaçada pela crescente exploração dos recursos naturais e expansão da agricultura, o que tem levado à perda de plantas com potenciais econômicos sem conhecimento científico prévio (VIEIRA; MARTINS, 2000).

Com isso, ressalta-se a importância de estudos relacionados à identificação de plantas do Cerrado com potencial uso farmacológico. Dentre as possíveis utilidades das espécies nativas do Cerrado o uso medicinal é considerado um dos mais representativos (XAVIER, 2005; MOREIRA; GUARIM NETO, 2009; PEREIRA et al., 2012). Neste sentido, a Organização Mundial de Saúde (OMS) apoia a utilização de plantas medicinais e conta com o Programa Nacional de Plantas Medicinais e Fitoterápico, onde procura envolver a produção de fármacos e os conhecimentos tradicionais (OMS, 2017).

Dentre as espécies nativas do Cerrado a família Asteraceae contempla uma grande variedade de espécies em seu estrato herbáceo e arbustivo (BATALHA et al., 2001) e é reconhecida por suas propriedades terapêuticas, aromáticas e cosméticas (FABRI et al., 2011). A espécie *Vernonanthura polyanthes*, conhecida popularmente como assa-peixe, é pertencente à família Asteraceae e apresenta propriedades farmacológicas. Essas propriedades podem ser explicadas pelos diferentes compostos presentes nos extratos da planta, como cumarinas, glicosídeos, esteroides, triterpenos, glicosídeos saponímicos, alcaloides, flavonoides, lactonas sesquiterpênicas e ácidos clorogênicos, presentes no extrato bruto (SOUZA et al., 2008) ou em partes fracionadas (IGUAL et al., 2013).

Na medicina popular, a espécie *V. polyanthes* é frequentemente empregada no tratamento de doenças respiratórias, cálculo renal, malária e febre (JORGETTO et al., 2011). Vários estudos tem atestado o potencial medicinal do extrato para o controle da pressão arterial agindo como um potencial agente de vasodilatação (ROMANEZI DA SILVEIRA et al., 2003), como inibidor do dano da mucosa gástrica conferindo propriedade antiulcerogênica (BARBASTEFANO et al., 2007), assim como atividade antifúngica e leishmanicida (BRAGA et al., 2007), antinoceptiva e anti-inflamatória (TEMPONI, 2012).

Outro indicativo, na medicina popular, do uso de assa-peixe é o de agente cicatrizador para afecções de pele (FERIDOLOGO, 2017). Esse uso sugere que esta espécie possua biocompostos que estimulem a angiogênese. A angiogênese é uma atividade biológica na qual a partir de vasos sanguíneos pré-existentes há a formação de novos vasos sanguíneos por meio da da proliferação, migração, regulação ou diferenciação de células vasculares (FOLKMAN, 2003). Um material que induz a angiogênese é importante não só para cicatrização, mas também na engenharia de tecidos e proliferação celular, representando grande importância para a aplicação medicinal (SHEN; FALANGA, 2003).

É importante salientar que grande parte dos biomateriais usados como cicatrizantes ou regenerativos no Brasil é importada e acaba por gerar custos elevados para pacientes. Dessa forma biocompostos extraídos de plantas podem ser uma alternativa na área de biomateriais como forma de atender às necessidades de melhoria da saúde geral e de redução de custos dos materiais envolvidos. Assim, nas últimas décadas, tem aumentado o interesse por estudos utilizando substâncias derivadas de plantas que mostram sua atividade biológica regenerativa (JONES et al., 2004).

O objetivo deste trabalho foi avaliar o potencial angiogênico de diferentes concentrações do extrato das folhas de *V. polyanthes*. Para isso utilizou-se o modelo *in vivo* da membrana corioalantóide (CAM) em embriões de galinha (TUFAN; SATIROGLU-TUFAN, 2005). Esta metodologia apresenta uma série de vantagens como a facilidade e rapidez de condução do experimento ainda com um menor custo comparando-se com outras metodologias (NOWAK-SLIWINSKA et al., 2014). Além disso, a técnica possui maior aceitabilidade em comparação a outras técnicas (MORENO-JIMÉNEZ et al. 2016) e também apresenta resultados reprodutíveis, semelhantes a outros modelos animais (LOKMAN et al., 2012).

2. MATERIAIS E MÉTODOS

2.1 Coleta material do botânico e obtenção do extrato aquoso bruto

As folhas de *V. polyanthes* foram coletadas em uma área de Cerrado preservada dentro das dependências do Câmpus Anápolis de Ciências Exatas e Tecnológicas da Universidade Estadual de Goiás. Posteriormente, as folhas foram submetidas à secagem à temperatura ambiente e pulverizadas em moinho de facas. O preparo do extrato aquoso foi realizado, conforme utilização popular, por infusão das folhas de *V. polyanthes* na proporção de 0,02 g de material vegetal seco e pulverizado para cada ml de água (BRASIL, 2011).

2.2 Experimento para a avaliação da atividade angiogênica

O potencial angiogênico do extrato de *V. polyanthes* foi avaliado por meio do teste da membrana corioalantóide (CAM) em ovos de galinha fertilizados previamente descrita por MELO-REIS et al. (2010). O ensaio foi conduzido no laboratório de Biotecnologia da UEG, câmpus de Ciências Exatas e Tecnológicas, no município de Anápolis/Goiás.

Foram utilizados 36 ovos de galinha férteis (*Gallus gallus* domesticus) provenientes de produtores do município de Anápolis – Goiás, formando-se cinco grupos experimentais com 6 indivíduos para cada tratamento. Os grupos experimentais utilizados foram: água esterilizada (controle negativo), Regederm® – pomada comercial composta de látex de seringueira (controle positivo), dexametasona (controle inibitório) e o extrato de folhas de *V. polyanthes* preparadas de acordo com o uso popular, formando-se as concentrações de 10, 20 e 40 mg/ml representando o dobro da dose popular, a dose popularmente utilizada e a metade desta dose respectivamente.

Os ovos devidamente higienizados foram incubados a temperatura de 37 °C e umidade variando de 60 a 70% por um período de 5 dias. No quinto dia de incubação, a parte superior do ovo foi removida formando um orifício em formato circular para a exposição da CAM e posteriormente o ovo foi lacrado com fita adesiva e recolocados na incubadora.

No décimo terceiro dia de incubação, os ovos foram submetidos aos diferentes tratamentos por meio de papel filtro contendo as substâncias em teste. Após 72 horas, para avaliar a resposta angiogênica, os embriões de galinha foram eutanasiados por meio de overdose com o analgésico Thiopentax® 325 mg/mL (Cristália) e as CAMs obtidas foram fixadas em soluções de formaldeído (3,7%) durante 5 minutos, removidas do ovo com auxílio de tesouras e mantidas em placa de Petri com solução de formaldeído. Em seguida, as CAMs foram fotografadas por meio de um estereoscópio trinocular com câmera acoplada, da marca Bioptika.

2.3 Análises

As imagens obtidas foram processadas utilizando-se os softwares Gimp e Image J, versão 1.28. O programa Gimp foi utilizado para a edição das imagens quanto à saturação, luz e contraste, a fim de obter uma melhor resolução dos vasos sanguíneos. Já com Image J, determinou-se a área percentual de vascularização de cada membrana, através da quantificação dos pixels correspondentes. Essas porcentagens de vascularização obtidas foram submetidas à Análise de Variância (ANOVA) e as médias de vascularização comparados pelo teste de Tukey, com nível de significância a 5%, através do programa SISVAR (FERREIRA, 2011).

3. RESULTADOS

Imagens representativas da membrana corioalantóide de ovos de galinha tratadas com extrato de folhas de *V. polyanthes* em diferentes concentrações e os respectivos controles são mostrados na Figura 1. Visualmente observa-se uma diferença na rede vascular do controle positivo (Figura 1A) com as diferentes concentrações dos extratos (Figura 1CDE), os quais se assemelham mais ao controle inibidor (Figura 1F), o que evidencia uma menor densidade vascular desses tratamentos.

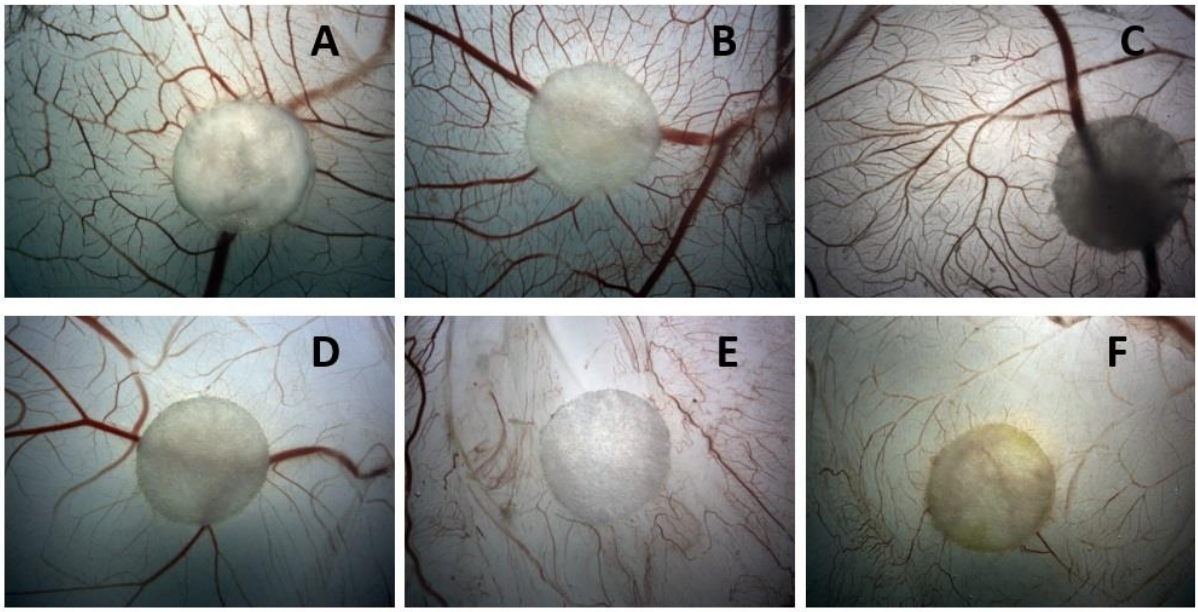

Figura 1. Imagens representativas da CAM submetidas aos diferentes tratamentos: A) Regederm® (controle positivo); B) água para injeção; C) 10 mg/ml do extrato de *V. polyanthes*; D) 20 mg/ml do extrato de *V. polyanthes*; E) 40 mg/ml do extrato de *V. polyanthes*; F) dexametasona (inibidor).

As análises estatísticas realizadas para as porcentagens de vascularização obtidas para os tratamentos: Regederm®, água para injeção, 10, 20 e 40 mg/ml do extrato de *V. polyanthes* e dexametasona encontram-se na tabela 1. Observa-se que os diferentes grupos experimentais apresentaram diferença significativa entre si. A melhor média de vascularização obtida foi para o tratamento Regederm® (14,59%) o qual diferiu estatisticamente dos demais tratamentos. Já as médias para água de injeção (11,22%) e 10 mg/ml do extrato de *V. polyanthes* (9,33 %) foram semelhantes entre si e diferiram das médias encontradas para 20 mg/ml do extrato (5,07%), 40 mg/ml do extrato (4,26%) e para o inibidor dexametasona (4,16 %).

Tabela 1. Porcentagem de vascularização obtida para os diferentes tratamentos submetidas ao teste de Tukey. Médias seguidas das mesmas letras não se diferem estatisticamente.

Tratamentos	Número de amostras	Médias de vascularização (%)
Regederm®	6	14.59 ± 2.01 a
Água para injeção	6	11.22 ± 1.23 b
10 mg/ml do extrato	6	9.37 ± 0.94 b
20 mg/ml do extrato	6	5.07 ± 0.70 c
40 mg/ml do extrato	6	4.26 ± 0.90 c
Dexametasona	6	4.16 ± 0.80c

Médias seguidas das mesmas letras não possuem diferença significativa por meio do teste de Tukey.

4. DISCUSSÃO

A metodologia CAM é amplamente aplicável permitindo a realização de uma diversidade de estudos a fim de investigar o desenvolvimento de células cancerígenas, a cicatrização de feridas, a aplicação de enxertos, o desenvolvimento de novos fármacos, assim como a avaliação de substâncias com potencial atividade angiogênica e antiangiogênica (RIBATTI, 2016).

A busca por biomateriais de origem vegetal com atividade antiangiogênica como tratamentos terapêuticos adicionais tem aumentado nos últimos anos. Em função disso, diversas pesquisas tem sido conduzidas buscando-se avaliar o potencial angiogênico do óleo (ARAUJO et al., 2016), do látex (ALMEIDA et al., 2014), e também do extrato aquoso (KUO et al., 2016) de diferentes plantas.

Os extratos aquosos de folhas de *V. polyanthes* utilizados neste experimento, nas concentrações de 40 mg g/ml e 20 mg/ml, contribuíram para a formação de uma menor rede de vascularização na CAM, caracterizando o processo de antiagiogênese. Assim como a espécie *V. polyanthes*, as espécies *Artemisia sieberi* e *Artemisia arborescens* também pertencem à família Asteraceae, a qual engloba uma diversidade de plantas com potencial anti-angiogênico comprovados. Os extratos aquosos das espécies *Artemisia sieberi* e *Artemisia arborescens*, também inibiram o processo de angiogênese em modelo de membrana corioalantóide de embriões de galinha (COSTA et al., 2016; ABDOLMALEKI et al., 2016).

A atividade antiangiogênica promovida por plantas pode ser em função dos compostos bioativos existentes. O extrato aquoso de folhas de *V. polyanthes* possui diferentes compostos,

tais como: cumarinas, glicosídeos, esteroides, triterpenos, saponinas, alcaloides, flavonoides, lactonas sesquiterpênicas e ácidos clorogênicos, os quais poderiam influenciar essa atividade antiangiogênica observada (SOUZA et al., 2008; IGUAL et al., 2013). Esses compostos são metabólitos secundários sintetizados pelas plantas como mecanismos de defesa contra inimigos naturais ou situação de estresse (SOUZA FILHO; ALVES, 2002). De acordo com VIZZOTO et al. (2010) a produção desses metabólitos está diretamente relacionada com o potencial medicinal das plantas no organismo humano. O composto wogonoside por exemplo, um flavonóide isolado da raiz de *Scutellaria baicalensis*, apresenta alto potencial para inibir a atividade angiogênica, sendo considerado um possível agente terapêutico a ser usado para o tratamento de câncer de mama (HUANG et al., 2015). Outro composto bioativo com atividade antiangiogênica comprovada, são saponinas isoladas da espécie *Rumex hastatus* que mostraram alto efeito antiangiogênico, com potencial aplicação antitumoral (AHMAD, 2016).

5. CONCLUSÕES

Os extratos aquosos de folhas de *V. polyanthes* nas concentrações de 40 mg/ml e 20 mg/ml apresentaram menor rede de vascularização, evidenciando potencial efeito antiangiogênico, o que fornece subsídios para estudos posteriores a fim de investigar essa atividade antiangiogênica.

6. REFERÊNCIAS BIBLIOGRÁFICAS

ABDOLMALEKIA, Z.; ARABA, H.; AMANPOURB , S.; MUHAMMADNEJADB, S. Anti-angiogenic effects of ethanolic extract of Artemisia sieberi compared to its active substance, artemisinin. **Revista Brasileira de Farmacognosia**, v. 26, n. 3, 2016.

ALMEIDA, L. M.; FLORIANO, J. F.; RIBEIRO, T. P., et al. *Hancornia speciosa* latex for biomedical applications: physical and chemical properties, biocompatibility assessment and angiogenic activity. **Journal of Materials Science-Materials in Medicine**, v. 24, p. 2153 – 2162, 2014.

AHMAD, S.; ULLAH, F.; AYAZ, M., et al. Antitumor and anti-angiogenic potentials of isolated crude saponins and various fractions of *Rumex hastatus* D. Don. Biological Research, v. 49, n. 18, 2016.

ARAUJO, L. A.; ARAUJO, R. G. M.; GOMES., et al. Physicochemical/photophysical characterization and angiogenic properties of Curcuma longa essential oil. **Anais da Academia Brasileira de Ciencias**, v. 88, p. 1889 – 1897, 2016.

BARBASTEFANO, V.; COLA, M.; LUIZ-FERREIRA, A.; FARIAS-SILVA, E.; HIRUMA-LIMA, C. A.; RINALDO, D.; VILEGAS, W.; SOUZA-BRITO, A. R. M. *Vernonia polyanthes* as a new source of antiulcer drugs. **Fitoterapia**, v. 78, n. 7, p.545–551, 2007.

BATALHA, M. A.; MANTOVANI, W.; MESQUITA JÚNIOR, H. N. Vegetation structure in cerrado physiognomies in south-eastern Brazil. **Brazilian Journal of Biology**, v. 61, p. 475-483, 2001.

BRAGA, F. G.; BOUZADA, M. L. M.; FABRI, R. L.; MATOS, M. O.; MOREIRA, F. O.; SCIO, E.; COIMBRA, E. S. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. **Journal of Ethnopharmacology**, v. 111, n. 2, p. 396–402, 2007.

BRASIL. Agência Nacional de Vigilância Sanitária. **Formulário de Fitoterápicos da Farmacopeia Brasileira**, Brasília: ANVISA, 2011.

COSTA, R.; RAGUSA, S.; RUSSO, M. et al. J Phytochemical screening of *Artemisia arborescens* L. by means of advanced chromatographic techniques for identification of health-promoting compounds. **Journal of Pharmaceutical and Biomedical Analysis**, doi: 10.1016/j.jpba.2015.10.006, 2016.

EJAZ, S.; ANWAR, K.; TAJ, R.; ASHRAF, M. A novel link between angiogenesis and natural products: Anti-angiogenic effects of *Opuntia dilleni*. Central European Journal of Biology, v. 9, n. 3, p. 298-308, 2014.

FABRI, R. L.; NOGUEIRA, M. S.; DUTRA, L. B.; BOUZADA, M. L. M.; SCIO, E. Potencial antioxidante e antimicrobiano de espécies da família Asteraceae. **Revista Brasileira de Plantas Medicinais**, v.13, n.2, p.183-189, 2011.

FERIDOLOGO – Núcleo interdisciplinar de tratamentos e feridas. Disponível em http://www.feridologo.com.br/fitoterapia.htm. Acesso em 22 de janeiro de 2017.

FOLKMAN, J. A novel anti-vascular therapy for cancer. **Cancer Biology e Therapy**, v. 3, n. 3, p. 338-9, 2004.

FERREIRA, D. F. Sisvar: a computer statistical analysis system. **Ciência e Agrotecnologia**, v. 35, n. 6, p. 1039-1042, 2011.

HUANG, Y.; ZHAO, K.; HU, Y.; et al. Wogonoside inhibits angiogenesis in breast cancer via suppressing Wnt/β-catenin pathway. **Molecular Carcinogenesis**, v. 55, n. 11, 2015.

GUERRA-SANTOS, I. J.; ROCHA, J. D.; VALE, C. R.; SOUSA, W. C.; TELES, A. M.; CHEN-CHEN, L., CARVALHO, S.; BAILÃO, E. F. L. C. *Vernonanthura polyanthes* leaves aqueous extract enhances doxorubicin genotoxicity in somatic cells of *Drosophila melanogaster* and presents no antifungal activity against *Candida* spp. **Brazilian Journal of Biology**, 2016.

IGUAL, M. O.; MARTUCCI, M. E. P.; COSTA, F. B.; GOBBO-NETO, L. Sesquiterpene lactones, chlorogenic acids and flavonoids from leaves of *Vernonia polyanthes* Less (Asteraceae). **Biochemical Systematics and Ecology**, v. 51, p. 94–97, 2013.

JONES, P. H.; CHRISTODOULOS, K.; DOBBS, N., et al. Combination antiangiogenesis therapy with marimastat, captopril and fragmin in patients with advanced cancer. **British Journal of Cancer**, v. 5, n. 1, p. 30-6, 2004.

JORGETTO, G. V.; BORIOLO, M. F. G.; SILVA, L. M.; et al. Ensaios de atividade antimicrobiana *in vitro* e mutagênica *in vivo* com extrato de *Vernonia polyanthes* Less (Assa-peixe). **Revista do Instituto Adolfo Lutz**, v. 70, n. 1, p. 53-61, 2011.

KUO, Z. K.; LIN, M. W.; LU, I. H., et al. Antiangiogenic and antihepatocellular carcinoma activities of the *Juniperus chinensis* extract. BMC complementary and alternative medicine, v.16, p. 277-292, 2016.

LOKMAN, N. A.; ELDER, A. S.; RICCIARDELLI, C.; OEHLER, M. K. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. **International Journal of Molecular Sciences**, v. 13, n. 8, 9959-9970. doi: 10.3390/ijms13089959, 2012.

MINISTÉRIO DO MEIO AMBIENTE (MMA). O Bioma Cerrado. Disponível em: http://www.mma.gov.br/biomas/cerrado. Acesso em 17 de dezembro de 2017.

MOREIRA, D. L.; GUARIM NETO, G. Usos múltiplos de plantas do cerrado: um estudo etnobotânico na comunidade Sítio Pindura, Rosário Oeste, Mato Grosso, Brasil. **Polibotânica**, n. 27, p. 159-190, 2009.

MORENO-JIMÉNEZ I, HULSART-BILLSTROM G, LANHAM SA, JANECZEK AA, KONTOULI N, KANCZLER JM, EVANS ND AND OREFFO RO. The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering, **Scientific Reports**, v. 6, n. 12, doi: 10.1038/srep32168, 2016.

NOWAK-SLIWINSKA, P.; SEGURA, T.; IRUELA-ARISPE, M. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. **Angiogenesis**, v. 17, n. 4, p.779-804, 2014.

PEREIRA, Z. V.; FERNANDES, S. S. L.; SANGALLI, A.; MUSSURY, R. M. Multiple uses of native species of the Cerrado biome of Assentamento Lagoa Grande, Dourados, Mato Grosso do Sul state. **Revista Brasileira de Agroecologia**, v. 7, n. 2, p. 126-136, 2012.

RATTER, J., S.; BRIDGEWATER; RIBEIRO, J. F. Analysis of the floristic composition of the Brazilian Cerrado vegetation. **Edinburgh Journal of Botany**, v. 60, n. 57, p. 109, 2003.

RIBATTI, D. The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. **Mechanisms of Development**, v. 141, p. 70–77, 2016.

ROMANEZI, S. R.; FOGLIO, M. A.; GONTIJO, J. A. R. Effect of the crude extract of *Vernonia polyanthes* Less. on blood pressure and renal sodium excretion in unanesthetized rats. **Phytomedicine**, v. 10, n. 2, p. 127–131, 2003.

SHEN, J. T.; FALANGA, V. Innovative therapies in wound healing. **Journal of Cutaneous Medicine and Surgery**, v.7, n. 3, p. 217-24, 2003.

SOUZA, F. A.; SENA, J.; MARANHO, L. T.; OLIVEIRA, C . M. R.; GUIMAR, A. T. B. Caracterização fitoquímica preliminar de infusões populares obtidas das partes aéreass das espécies *Apium leptophylum* (Pers.) F. Muell. ex Benth. (Apiaceae), *Elvira biflora* L. (DC.) e *Vernonia polyanthes* Less. (Asteraceae). **Revista Brasileira de Farmácia**, v. 89, n. 1, p. 24-27, 2008.

SOUZA FILHO, A. P. S.; ALVES, S. M. **Alelopatia: princípios básicos e aspectos gerais**. Belém: Embrapa Amazônia Oriental, 2002. 260 p.

TEMPONI, V. S.; SILVA, J. B.; ALVES, M. S.; RIBEIRO, A.; PINHO, J. J. R. G.; YAMAMOTO, C. H.; PINTO, M. A. O.; DEL-VECHIO-VIEIRA, G.; SOUSA, O. V. Antinociceptive and Anti-Inflammatory Effects of Ethanol Extract from *Vernonia polyanthes* Less in Robents. **International Journal of Molecular Sciences**, v.13, n.3, p.3887-3899, 2012.

TUFAN, A. C.; SATIROGLU-TUFAN, N. L. The chick embryo chorioallantoic membrane as a model system for the study of tumor angiogenesis, invasion and development of anti-angiogenic agents. **Current Cancer Drug Targets**, v. 5, p. 249–66, 2005.

VIEIRA, R. F.; MARTINS, M. V. M. Recursos genéticos de plantas medicinais do Cerrado: uma compilação de dados. **Revista Brasileira de Plantas Medicinais**, v. 3, n.1, p.13-36, 2000.

VIZZOTO, M.; KROLOW, A. C.; WEBER, G. E. B. Metabólitos secundários encontrados em plantas e sua importância. Embrapa Clima Temperado, 2010, 16 p.

XAVIER, F. F. Conhecimento ecológico tradicional e recursos vegetais em Nossa Senhora da Guia, Cuiabá-Mato Grosso. **Dissertação de Mestrado em Ecologia e Conservação da Biodiversidade submetida à Universidade Federal de Mato Grosso – UFMT**, 89f, 2005.

YANCOPOULOS, G. D.; DAVIS, S.; GALE, N. W., et al. Vascular-specific growth factors and blood vessel formation. **Nature**, v. 407, n. 6801, p. 242-248, 2000.

Capítulo II

Chick embryo choriollantoic membrane assay (CAM) as a model to investigate angiogenesis: A scientometric approach.

Submetido para publicação na revista Anais da Academia Brasileira de Ciências (Qualis B1)

Chick embryo choriollantoic membrane assay (CAM) as a model to investigate angiogenesis: A scientometric approach

ANGÉLICA DAIANE LEMOS DO PRADO[1], ELISA FLÁVIA LUIZ CARDOSO BAILÃO[2], JOÃO CARLOS NABOUT[2], TATIANA RABACHINI[3], PAULO ROBERTO MELO REIS[4], PABLO JOSÉ GONÇALVES[5], LUCIANE MADUREIRA ALMEIDA[1,2]

[1] Universidade Estadual de Goiás, Câmpus Ipameri, Ipameri, GO, Brasil; pradoadl@hotmail.com;

[2] Universidade Estadual de Goiás, Câmpus Henrique Santillo, Anápolis, GO, Brasil; elisaflavia@gmail.com; jcnabout@ gmail.com; almeidalm@hotmail.com;

[3] University of Bern, Hochschulstrasse 3012, Bern, Switzerland; tatianarabachini@hotmail.com;

[4] Pontifícia Universidade Católica de Goiás - PUC, Goiânia, GO, Brasil; melo_reis@yahoo.com.br

[5] Instituto de Física da Universidade Federal de Goiás, UFG, Goiânia, GO, Brazil; pablo@ufg.br

Running Title: Current trends in CAM assay

*__Corresponding author__: Luciane Madureira Almeida, Universidade Estadual de Goiás, Rodovia BR 153, nº 3105, Fazenda Barreiro do Meio – Campus Henrique Santillo-Anápolis, Caixa Postal 459; CEP: 75.132-400, Brasil. Tel: 55 62 3328115; Fax: 55 62 33281177.

E-mail: luciane.almeida@ueg.br or almeidalm@hotmail.com

ABSTRACT

Angiogenesis is a fundamental physiological process with strong implications in tissue homeostasis. Animal models helping to identify how angiogenesis is regulated are fundamental to answer many biological questions. Of note, chick embryo chorioallantoic membrane (CAM) assay is one of the most employed methods to study angiogenesis. In this study we applied a scientometric approach to evaluate the employment of CAM assay in published articles. Our results indicate that CAM assay is widely used and worldwide accepted as a reliable methodology to investigate angiogenesis. Temporal trends indicated that CAM assay was the preferred method to investigate angiogenesis over time. The publications had a significant number of citations and the impact factor of journals publishing articles using this technique. A total of 52 different research areas have articles published using CAM technique. Oncology is the research field in which CAM assay was mostly used. We also identified that 76% of articles published used only CAM assay to answer questions concerning angiogenesis. The results indicated that CAM assay is a robust method to study angiogenesis. Moreover, this data can help researchers who are unfamiliar with the CAM assay to identify if this particular method is suitable for their research.

Keywords: Alternative method, angiogenesis, bibliometric analysis, CAM assay, oncology, tumor vascularization.

1. INTRODUCTION

Angiogenesis is a complex biological process through which new blood vessels form from pre-existing vascular tissue. In general it involves proliferation, migration and differentiation of endothelial progenitor cells (Folkman, 2003). Over the last decades, many efforts have been made to develop therapeutic strategies to promote or to inhibit the angiogenesis (Fisher et al., 2006). Indeed, angiogenesis research is a cutting-edge field with several medical applications. For instance, a compound that is able to induce angiogenesis can be useful for tissue engineering, for boosting cell proliferation and for wound healing (Almeida et al., 2014). On the other hand, a compound that inhibits angiogenesis might be promising to target the abnormal vasculature found in several types of solid tumors (Ribeiro et al., 2012). In fact, millions of patients worldwide are being treated with compounds that are able to regulate angiogenesis.

The use of animal models to investigate angiogenesis is critical, especially when a therapeutic agent seems to be promising in the clinical setting. One of the most widely used methods to investigate angiogenesis is the chick embryo chorioallantoic membrane (CAM) assay (Norrby, 2006). CAM is an extraembryonic membrane, generated by the fusion of the chorion with the vascularized allantoic membrane (Ribatti, 2010). It performs multiple functions during embryonic development, such as respiration, calcium transport from the eggshell, acid-base homeostasis and ion/water reabsorption from the allantoic fluid (Yuan et al., 2014). As it expands, a rich vascular network is generated allowing the quick and low-cost investigation of several biological processes, including tumor growth and metastasis, as well as, pharmacological analysis of angiogenic and anti-angiogenic compounds.

The CAMs can be cultured either *in ovo* or *ex-ovo* as a shell-less culture in Petri dishes or in plastic wrap/cup apparatus (Ribatti, 2010). During the experiment, the chick egg is a self-sufficient chamber that allows a fast animal development, about 21 days, without artificial support media, special culture requirements or storage facilities (Kalirai et al., 2015).

Moreover, the chick eggs are cheap and available during all year. Other advantage is the relative transparency of the internal structures that makes it easy to observe the continued modifications on embryo (Kalirai et al., 2015). It was demonstrated that CAM provides tissue response similar to cell-based and animal based assays (Lokman et al., 2012) with the advantage that CAM is not innervated and thus, no pain is experienced by the chick (Moreno-Jiménez et al., 2016). Moreover, because of its natural immunodeficiency, the CAM accepts transplantation from various tissues and species without immune response. Considering this, CAM assay provides a fast way to investigate angiogenesis in a well-developed vascular tissue (Kue et al., 2015) with the advantages of being a reliable *in vivo* assay that can be readily performed in any laboratory setting with high reproducibility and low-cost (Nowak-Sliwinska et al., 2014).

In order to evaluate the wide use of CAM assay on scientific reports, the present work performed a bibliometric analysis about the employment of CAM assay. Bibliometric analysis is a tool used to evaluate scientific production about certain topic using numerical indicators and statistical analyzes (Razera, 2016; Almeida et al., 2016). In the present work, we identified: trends and biases about the use of CAM assay on scientific research; the quality of the scientific production estimated by number of citations and impact factor of the journals that publish about CAM assay; the main areas and journals interested in this method; the kind of samples usually tested; the exclusivity of CAM assay, as a model to evaluate angiogenesis, on the experimental design of articles; the frequency of CAM assay use in comparison to others *in vivo* angiogenesis models; the variations of CAM assay and the level of cooperation patterns between countries. Our results indicate that CAM assay is widely used and highly accepted as a reliable methodology to investigate angiogenesis.

2. MATERIAL AND METHODS

2.1 Data collection

Data were obtained from Thomson-Reuters database (Web of Science platform) from 1991 to 2016. Initial date was defined based on abstract availability on database. Final data was defined as the last full year with data available since this study began. We divided the production of papers each year by the overall production of papers (obtained in ISI) to eliminate the effect of the temporal increase in papers (Nabout et al., 2012). The impact factor of different journals was collected in February 2017 on the Journal Citation Reports (JCR), published by the Institute for Scientific Information (ISI) and edited by Thomson, exclusively to evaluate indexed papers in the Web of Science platform.

2.2 Search criteria

The combination of the following words were used: (1) "chorioallantoic membrane" and CAM or (2) "chorioallantoic membrane" or (3) "chick embryo membrane", which could be in the title, abstract, or in the list of keywords. The analysis were conducted to obtain the following information from each paper: (1) year of publication; (2) number of citations; (3) name of the Journal and its impact factor; (4) area of study using the CAM assay; (5) if the tested substance was an animal tissue/cell, or a new drug, or a vegetable extract/bioactive plant compound, or a inorganic compound, or nanoparticle, or other; (6) use of other methods to evaluate angiogenesis in combination with CAM assay; (7) temporal trends of others angiogenesis models in comparison to CAM assay; (8) variations of CAM technique; (9) countries of publication and average authorship; and (10) mapping from countries interested in the technique and international research network.

2.3 Data Analysis

Pearson correlation was used to estimate the frequency of papers using CAM assay in relation to the total number of articles published in ISI by year to evaluate the increase of the use of CAM technique over time. The significance value was obtained through the Monte Carlo test with 999 randomizations (Czerwon, 1990). We considered significant p values <

0.05. This analysis was conducted using “R 3.2.2 program” (R CORE TEAM, 2003). Others parameters analyzed were evaluated using basic descriptive statistics.

2.4 Countries Network

To evaluate the networks between countries we used two approaches: i) analysis with all countries (64 countries); ii) Analysis with 31 first countries based in their number of papers. For both approaches, first we determined the country of each author listed in the paper. Thus, we generated the adjacency matrix indicating the number of paper in collaboration for each pair of countries. The importance of each country was estimated by the degree of centrality (DC). The degree of centrality of the country *i* (DCi) is a measure of the number of links (edges) that each country has. The DCi is divided by number of countries minus one to calculate the Relative Degree Centrality (RDCi) of country *i*, which was used to evaluate the importance of countries indicated by different sizes of vertex in network graphs (Koschützki et al., 2005). The figure of all countries was presented just as supplementary material (Fig. S1).

2.5 Temporal trends of CAM assay preference in comparison to other *in vivo* angiogenesis models

To compare the tendencies in CAM assay application in relation to others *in vivo* angiogenic models, we search for the following terms in Web of Science platform: (1) "corneal micropocket"; (2) "sponge implant" or “matrix implant”; (3) "matrigel plug assay"; and (4) “disc angiogenesis assay”. Those words could be in the title, abstract, or in the list of keywords.

3. RESULTS AND DISCUSSION

3.1 Quality indicators of CAM assay-related scientific production

Our analysis identified a total of 2,071 research articles that used CAM assay to address different biological questions. We observed that the number of publications using this method increased constantly and significantly ($r = 0.96$, $p < 0.001$) over the years (Fig. 1a). The publications had a significant number of citations (Fig. 1b) and the impact factor of

journals publishing articles using this technique is relevant for the scientific community (Fig. 1c,d). Our results using different scienciometric approaches indicate a continuous increase in the number and the maintenance of the quality of articles that have used CAM assay to address a particular biological question.

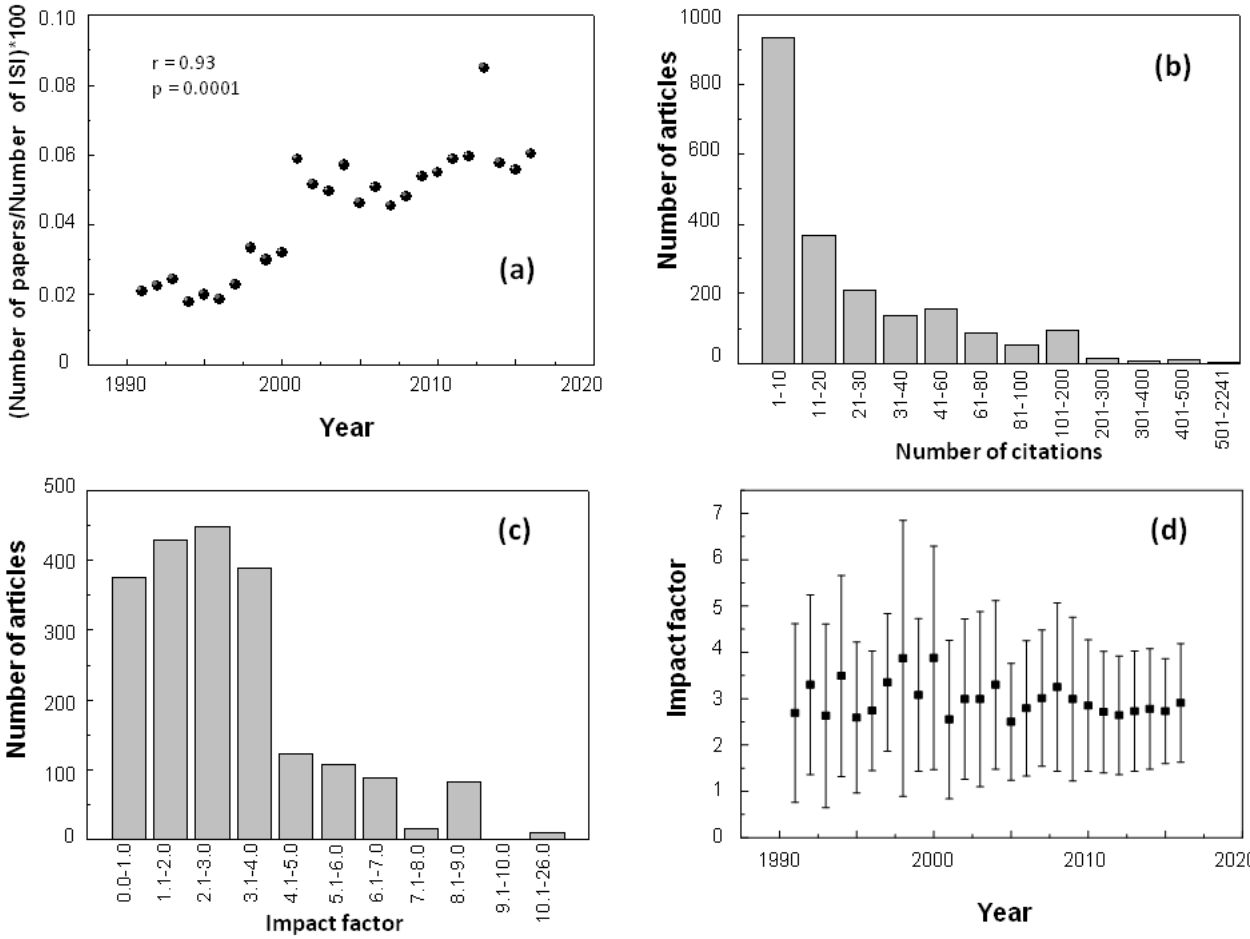

Figure 1. Bibliometric information of the articles using CAM assay: **(a)** number of publications per year; **(b)** frequency of citations per article; **(c)** journal's impact factor; **(d)** mean impact factor (±SD) of journals publishing articles using CAM assay.

Temporal trends indicate that more than a hundred articles using CAM assay were published per year in the last decade. This result may reflect the fact that this particular technique display several advantages such as (a) rapid growth of the embryo choriollantoic membrane, providing faster results; (b) low-cost; (c) simplicity; (d) high reproducibility; (e) easy dynamic observation; (f) immunodeficient environment; (g) high-probability of tumor

cell grafting due to CAM intense vascularization and finally (h) minor ethical concerns (Nowak-Sliwinska et al., 2014; Aleksandrowicz et al., 2015). Still, some disadvantages are also considered and discussed in the literature. Among them we can include (a) difficulty in observing new vessels; (b) difficulty in distinguishing manipulation effects from the effects related to the tested compound; (c) nonspecific inflammatory reaction; (d) presence of perivascular inflammatory infiltrate together with any hyperplastic reaction of the chorionic epithelium; (e) not being a mammalian model; (f) drugs that require metabolic activation can not be assessed; (g) very sensitive to an increase in oxygen tension (Norrby, 2006; Ribatti, 2010; Ribatti, 2014).

It is well established that the number of citations is an important tool to evaluate the impact of a particular work in the scientific community (Carneiro, 2008). In our study, we observed that the number of citations varied from zero to 2,241. However, more than a half of the articles analyzed presented ten to twenty citations (Fig 1b). This observation is in line with common bibliometric patterns (Carneiro, 2008). One article in particular received 2,241 citations and described the application of CAM technique to study the requirement of Vascular Integrin Alpha(V) Beta(3) for angiogenesis (22 Brooks et al. 1994).

The importance of a journal can be measured by traditional Journal Impact Factor (IF) (Nansen et al., 2014). Here, the IF of journals publishing articles using CAM assay ranged from zero to 26, with maximum frequency amid 2.1 to 3.0 (Fig 1c). In general, the mean IF of the journals publishing articles that used CAM assay was close to 3.0 along the years (Fig 1d). This result indicates that CAM assay is used by several applications and there is no biases regarding the use of this particular methodology to address specific biological questions in relation to the relevance of the article published.

3.2 Research areas using CAM assay

Our analysis revealed that CAM assay was used in 52 different areas, as described on ISI database. Considering only the 15 most cited areas (Fig 2a), it is possible to observe that

Oncology is the area with the highest number of articles using CAM assay. A total of 424 articles were published in this field. The reason behind this observation relays on the fact that angiogenesis plays a major role in tumor development and maintenance (Cimpean et al., 2008). Compared to standard mouse models, in which tumor growth takes between 3 to 6 weeks, xenografts tumors generated in chick CAMs are visible between 2 and 5 days after cell transference (Ribatti, 2014). In addition, the natural immunodeficiency of this membrane allows the transplantation from various tissues and species to the CAM. Consequently, different tumor-derived cell lines have been seeded on CAM to study several aspects of tumor biology (Ribatti, 2010). Once tumor cells are seeded on CAM they can provide valuable information regarding tumor cell adherence and invasion of the mesenchyme tissue underneath the blood vessels, tumor cell survival in the circulation, anchorage, penetration and proliferation in distant organs (Ribatti, 2014). For this reason, CAM model has been extensively used in studies aiming to investigate how angiogenesis affects tumor biology and several articles describe the use of this methodology to answer questions about ovarian cancer (Wang et al., 2016), breast cancer (Gautam et al., 2016), cervical cancer (Zhou et al., 2015), glioblastoma (Fornvik et al., 2016), galbladder cancer (Patil et al., 2016), pancreatic cancer (Rovithi et al., 2017), renal cancer (Ferician et al., 2015) and hepatocellular carcinoma (Lv et al., 2016; Han et al., 2016).

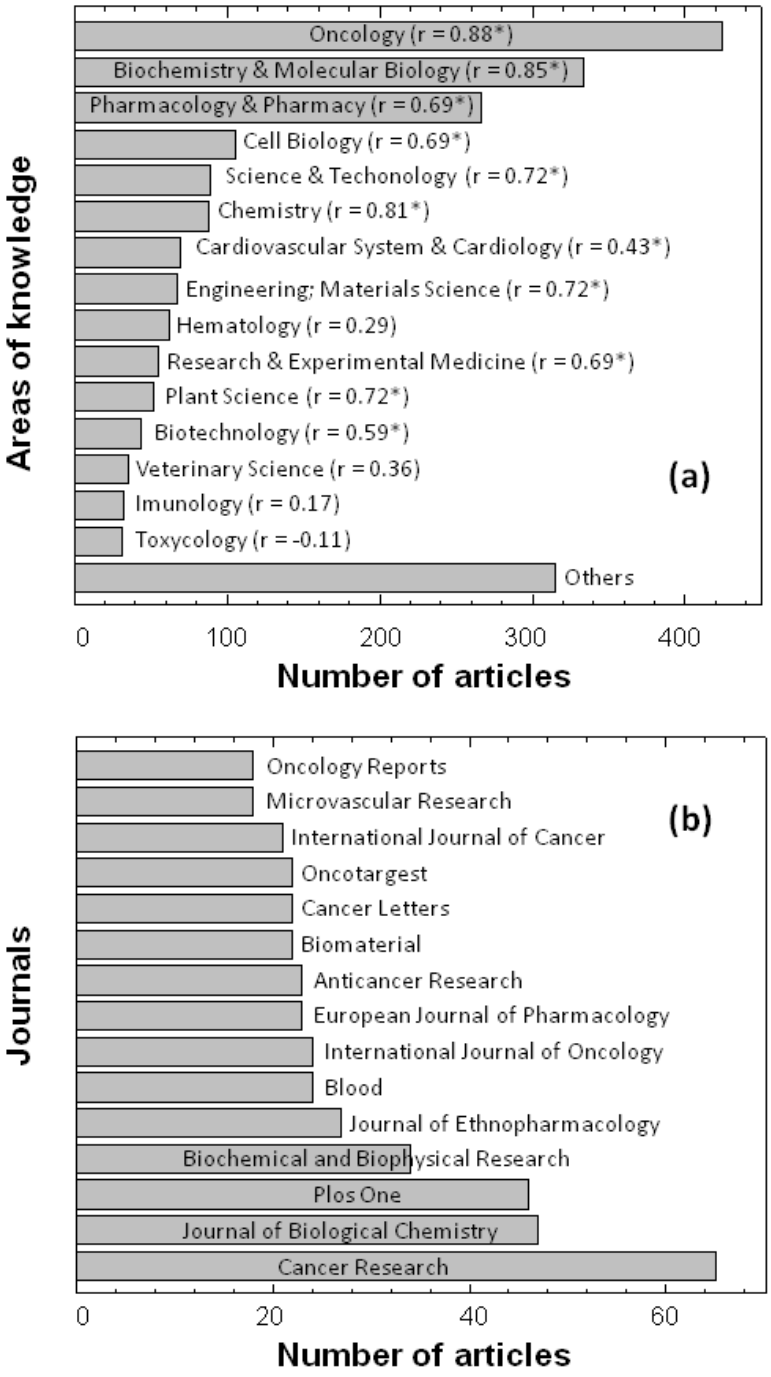

Figure 2. Research areas and journals interested about CAM assay. **(a)** Main areas of knownledge that publish using CAM assay; and **(b)** main journals interested on papers that used CAM assay.

CAM assay has also been used in Pharmacology and Pharmacy to evaluate the angiogenic and anti-angiogenic potential of specific drugs. As an example we can mention the use of CAM to test the angiogenic potential of resveratrol methylated derivatives (Chen et al., 2016)

and the use this technique to test the anti-angiogenic potential of curcumin-capped copper nanoparticles (Kamble et al., 2016).

For Engineering and Material Science, CAM is applied as a method to evaluate angiogenesis in tissue reconstruction (Ishida and Mitsui, 2016). Unlike other *in vivo* models, such as the murine subcutaneous implant, CAM assay is minimally invasive to the chick embryo and hence, considered a refinement model for animal research (Kue et al., 2015). Many studies have used CAM to evaluate tissue repair including studies about osteogenesis (Yan et al., 2016), myocardial regeneration (Fanton et al., 2016) and even induction of angiogenesis using polymer-based constructs (Kanczler et al., 2007). We also analyzed the acceptability of the CAM assay over the time (Fig 2a). For this analysis we verified the use of this methodology regarding research field. Our results indicate that CAM is a reliable method to answer several biological questions, with positive '*r*' values found for all fields, with exception of toxicology. For this particular field, researchers preferred other methods to answer their hypothesis, such as skin and eye irritation assays (Nowak-Sliwinska et al., 2014).

The areas of knowledge identified in the present search also reflected the kind of journals in which the articles were published. Figure 2b shows the journals with more than 20 publications that have used CAM assay. A total of 664 different journals published articles that have used this method. 'Cancer Research' from the 'American Association of Cancer Research' had 65 articles that have mentioned using CAM to address a specific biological question, followed by the 'Journal of Biological Chemistry' from the 'American Society for Biochemistry and Molecular Biology, with 47 published articles and 'Plos One' with 46 articles. Among journals publishing articles using CAM assay, impact factor ranged from 2.8 to 9.1. This result reflects that the assay is used in articles with high acceptability. It also indicates a wide diversity of journals publishing articles using this methodology.

3.3 Applications of CAM assay

CAM assay have been widely used because of its several advantages (Lokman et al., 2012). To elucidate for which particular application CAM method was used, we have stratified articles according to the origin of the main samples tested using CAM. Six categories were created: (a) animal tissues/cells, (b) new drugs, (c) vegetable extracts or bioactive compounds, (d) inorganic compounds, (e) nanoparticles and others. Our results show that a total of 1,162 (56.1%) articles have used CAM to assay animal/tissue samples (Fig 3). The most common samples tested were tumor-derived cell lines, such as neuroblastoma and glioblastoma (Klagsbrun et al., 1976); head and neck squamous cell carcinoma (Gronau et al., 2006); human colorectal cancer (Subauste et al., 2009); osteosarcoma (Balke et al., 2010); human ovarian carcinoma (Adar et al., 2012); and human hepatocellular carcinoma (Lv et al., 2016). A total of 316 (15.3%) used CAM to evaluate the angiogenic and the anti-antiogenic potential of specific drugs (Fig 3). As an example of drugs tested using CAM we can mention agkistin (Yeah et al., 2000); heparin (Casu et al., 2002); curcumin (Hahm et al., 2004); emolin (Kwak et al., 2006); and azaspirine (Asami et al., 2008). The use of CAM to test vegetable extracts/ bioactive plant compounds was observed in 261 (12.6%) articles (Fig 3). Some of these compounds include root methanol extract from *Calliandra portoricensis* (Adaramoye et al., 2015); crude saponis of *Rumex hastatus* (Ahmad et al., 2016); *Hancornia speciosa* latex (Almeida et al., 2014) among others. Finally, we also identified that 159 (7.7%) of articles used CAM method to test inorganic compounds and 61 (3%) used it to test nanoparticles (Fig 3). The main goal of these articles was to evaluate if the tested compound could be used in implants (Gomes et al., 2016); or to verify if the sample tested was toxic (Ahmad et al., 2016); or if the compound could promote tumor angiogenesis (Kamble et al., 2016). Altogether our data indicate that CAM assay is widely used, but seems to be more often seen in articles aiming to investigate biological questions linked to cancer research.

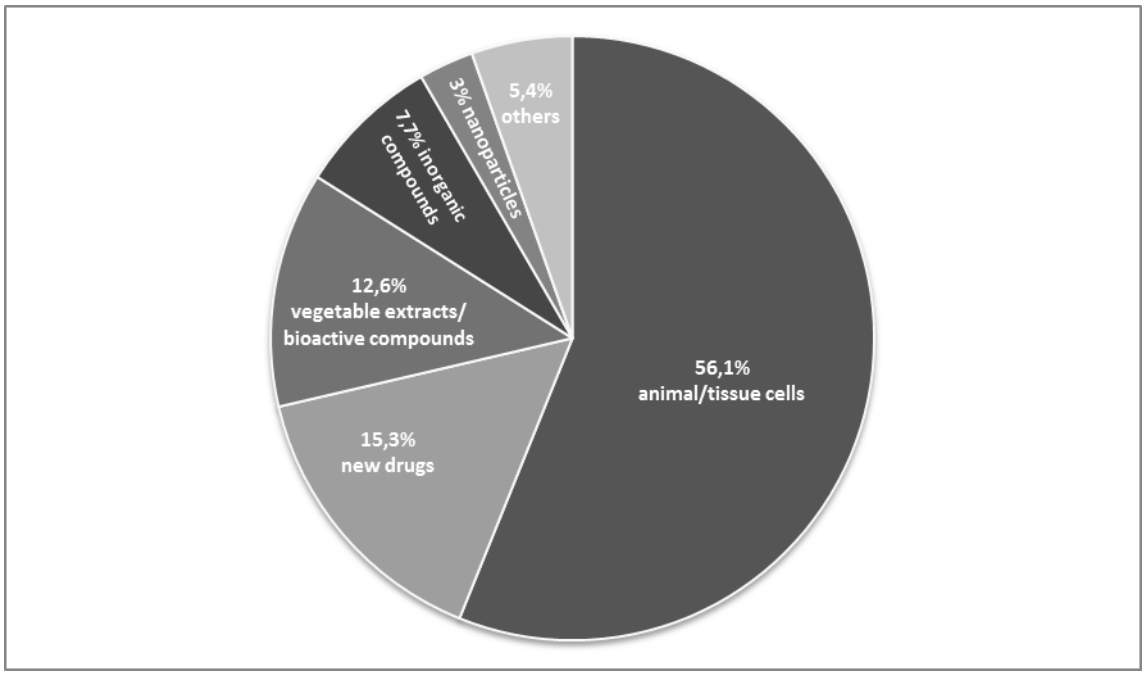

Figure 3. Frequency of articles published using different types of samples in CAM assay.

3.4 Using CAM assay as a single model to investigate angiogenesis

Our data also show that 76% of articles published have used only CAM to investigate angiogenesis. This result indicates that CAM assay is a reliable method to answer a particular biological problem. Articles that have another methodology to further confirm CAM assay results, opted to use xenograft in nude mice (Fornvik et al., 2016; Thanekar et al., 2016). Corneal micropoket assay, sponge implant, disc angiogenesis systems, matrigel plug assay, zebrafish assay, rodent mesentery angiogenesis assay and direct *in vivo* angiogenesis could also be used as angiogenesis models (Norrby, 2006).

3.5 Temporal trends of CAM assay use in comparison to other *in vivo* angiogenesis models

In vivo angiogenesis was evaluated in 2,071 articles using the CAM assay; in 582 articles using matrigel plug assay; in 81 articles using corneal micropocket assay; in 57 articles using sponge matrix implant assay; and in 9 articles using disc angiogenic assay (Table 1). This result indicates that there is a strong preference for choosing CAM assay to test angiogenesis among researchers. Temporal trends also indicate that CAM and matrigel

plug methodologies were constantly and significantly applied over the years to investigate angiogenesis, as indicated by '*r*' values (Table 1). Sponge matrix implant and corneal micropoket also have a positive tendency over the years, but less prominent than CAM or matrigel plug assay. Interestingly, we could observe that the employment of disc angiogenic assay has decreased over the years. This result may reflect the fact that this particular method is not frequently applied to study solid tumors (Norrby, 2006). These results are important to understand that any method used to investigate angiogensesis have advantages and disadvantages. Ideally, the following criteria should be considered before choosing a specific method to answer a particular question in angiogenesis: (a) the assay should provide a quantitative measure of the new vascular network; (b) dose response curves should be created in order to determine concentration, liberation rate and time of treatment of the tested compound; (c) if tumor-derived cell lines are used as a source of angiogenic factors, they should be genetically well defined; (d) there should be a clear distinction between newly formed and pre-existing host vessels; (e) the response seen *in vitro* should be confirmed *in vivo*; (f) tissue damage should be avoided, since it may lead to formation of new vessels; (g) the assay should permit long-term and, if possible, noninvasive monitoring; and (h) it should be cost-effective, rapid, easy to set up, reproducible and reliable (Norrby, 2006; Jain et al., 1997).

Table 1: Bibliometric information of the articles related to different *in vivo* angiogenic models: matrigel plug assay, corneal micropocket, sponge matrix implant, disc angiogenic assay and CAM.

In vivo angiogenesis model	Number of articles	Pearson correlation
Matrigel plug	582	0.90*
Corneal micropocket	81	0.04
Sponge matrix implant	57	0.25
Disc angiogenic assay	9	0.10
Chick embryo chorioallantoic membrane (CAM)	2,071	0.96*

* $P < 0.05$

3.6 Variations of CAM assay

Several protocols are available to perform CAM assay and the most commonly employed are: *in ovo, ex ovo* and Het-CAM. To perform the assay using the *in ovo* protocol is necessary to open a circular hole on the eggshell to expose the CAM. With this protocol it is possible to achieve a more preserved physiological environment, as well as allow the development of the long-term embryo. It is also the protocol of choice found in the vast majority of articles published, with a total of 1,966 studies using this technique. In the *ex ovo* assay the embryo is removed from the egg and transferred to a petri dish. We scored only 42 articles using this method. The main disadvantage of this procedure is related to the low survival rate of the embryo during the process. Most of the time, removing the embryo from the eggshell can damage the yolk membrane (Nowak-Sliwinska et al., 2014). On the other hand, the *ex ovo* method facilitates the testing of a larger number of samples and also improves quantification once a larger area of the CAM can be exposed and quantified (Ribatti, 2016). Alternatively, the Het-CAM is the method of choice when the irritation potential of a substance needs to be addressed. We identified 63 articles using this particular technique. Since the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) considers Het-CAM as an alternative method for conjunctive irritation testing, HET-CAM has been extensively used for this purpose.

3.7 Publications by country, average authorship and collaborative Network

We next sought to investigate if there was any trend of using CAM assay in relation to the country in which the research was conducted. The country of the corresponding author was chose as the identification criteria. A total of 368 articles (17.8%) were published by authors from USA, followed by 318 articles (15.3%) published by authors from China and 167 (8.1%) articles published by authors from Germany (Fig 4A). As expected, this result reflects the fact that the scientific production of nations is related to the socio-economic characteristics of the country (Jaffe et al., 2013). Research cooperation was evaluated by the average of the number of authors (Fig 4B) and the collaboration networks (Fig 5). As

observed, most of the articles had contribution of 4 to 8 authors. Only 6 articles were written by a single author. According to Nabout et al. (2015), there is a natural trend showing a decrease in the number of articles produced by a single author over the years for all subareas of biological sciences. Our results also show that from the 65 countries that published articles using CAM, only 33 established international collaborations (Fig 5). The USA, England and Germany were the countries with the highest number of international collaboration. Although international collaboration network was not highly expressive among groups around the world using CAM assay to assess angiogenesis, it encourages researchers to collaborate in order to increase productivity and to promote better application of financial resources, as assed by Lee and Bozeman (Lee and Bozeman, 2005) and supported by Subramanyam (Subramanyam, 1983) which have shown that international scientific collaboration has increased in volume and importance over the years.

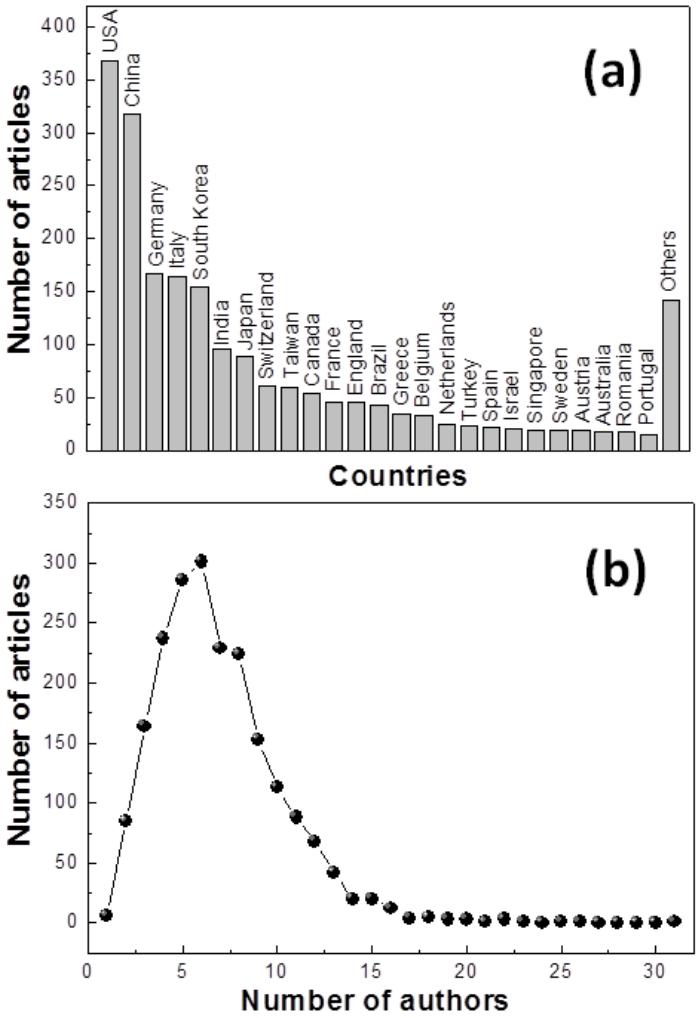

Figure 4: Analysis of author geographic distributions and collaboration network: **A**) shows the number of publish items per country (using data from corresponding author); **B**) shows the average authorship per year.

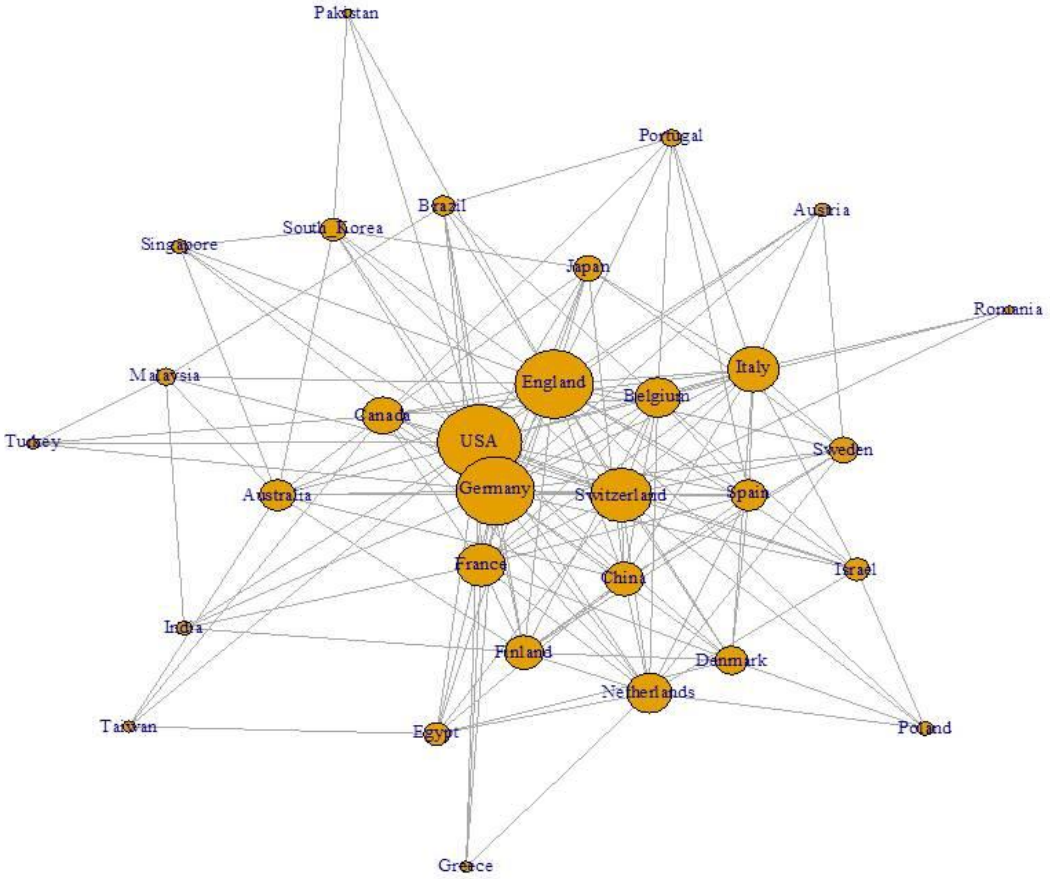

Figure 5: Network of the 31 countries with great number of international collaborations.

4. CONCLUSIONS

This study reported that CAM is a reliable and highly employed method to assess angiogenesis around the world. During 1991 till 2016, a total of 2,071 articles belonging to 52 different areas of knowledge have used CAM assay to address a specific biological question. The field of research in which CAM assay was most used was oncology, especially to evaluate the effect of tumor-derived cell lines influence in angiogenesis. The scienciometric approaches showed a continuous increase of both quantitative (number article per year) and qualitative (IF and article citations) metrics on literature reporting the utilization of the CAM assay. Altogether, the results presented in this article can help researchers who are unfamiliar

with the CAM assay to elucidate how this technique can help to answer questions related to angiogenesis and how is the global acceptability of CAM assay in the research community.

ACKNOWLEDGEMENTS

LMA, EFLCB were supported by Universidade Estadual de Goiás with fellowships at the program PROBIP (Scientific Production Support Program); JCN and PJG were supported by CNPq productivity fellowships and ADLP was supported by CAPES student fellowship.

5. REFERENCES

ADAR Y, STARK M, BRAM EE, NOWAK-SLIWINSKA P, VAN DEN BERGH H, SZEWCZYK G, SARNA T, SKLADANOWSKI A, GRIFFIOEN AW AND ASSARAF YG. 2012. Imidazoacridinone-dependent lysosomal photodestruction: a pharmacological Trojan horse approach to eradicate multidrug-resistant cancers. Cell Death Dis 3:e293. doi: 10.1038/cddis.2012.30

ADARAMOYE O, ERGUEN B, OYEBODE O, NITZSCHE B, HÖPFNER M, JUNG K AND RABIEN A. 2015. Antioxidant, antiangiogenic and antiproliferative activities of root methanol extract of *Calliandra portoricensis* in human prostate cancer cells. J Integr Med 13(3):185-93. doi: 10.1016/S2095-4964(15)60175-3.Adaramoye et al., 2015

AHMAD KA, MOHAMMED AS AND ABAS F. 2016. Chitosan nanoparticles as carriers for the delivery of Phi KAZ14 bacteriophage for oral biological control of colibacillosis in chickens. Molecules 14,21(3):256. doi: 10.3390/molecules21030256.

ALEKSANDROWICZ E AND HERR I. 2015. Ethiccal eutanasia and short-term anestesia of chick embryo. ALTEX 32(2):143-147. doi dx.doi.org/10.14573/altex.1410031.

ALMEIDA LM, FLORIANO JF, RIBEIRO TP, MAGNO LN, da MOTA LS, PEIXOTO N, MRUÉ F, MELO-REIS P, LINO JUNIOR R de S, GRAEFF CF AND GONÇALVES PJ. 2014. *Hancornia speciosa* latex for biomedical applications: physical and chemical properties, biocompatibility assessment and angiogenic activity. J Mater Sci Mater Med 24: 2153-2162. doi: 10.1007/s10856-014-5255-8.

ALMEIDA LM, PRADO ADL, D'ABADIA PL, MACHADO KB, MELO-REIS PR, NABOUT JC AND GONÇALVES PJ. 2016. The state-of-art in angiogenic properties of latex from different plant species. Current Angiogenesis 4: 10-23. doi:10.2174/2211552804011605171645 31

ASAMI Y, KAKEYA H, KOMI Y, KOJIMA S, NISHIKAWA K, BEEBE K, NECKERS L, OSADA H. 2008. Azaspirine, a fungal product, inhibits angiogenesis by blocking Raf-1 activation. Cancer Sci 99: 1853-1858. doi:10.1111/j.1349-7006.2008.00890.x
.
BALKE M, NEUMANN A, KERSTING C, AGELOPOULOS K, GEBERT C, GOSHEGER G, BUEGER H AND HAGEDORN M. 2010. Morphologic characterization of osteosarcoma growth on the chick chorioallantoic membrane. BMC 3:58. doi: 10.1186/1756-0500-3-58.

BROOKS PC, CLARK RAF AND CHERESH DA. 1994. Requirement of vascular integrin alpha(V)beta(3) for angiogenesis. Science 264: 3.

CARNEIRO FM, NABOUT JC, BINI LM. 2008. Trends in the scientific literature on phytoplankton. Limnology 9: 153-158. doi:10.1007/s10201-008-0242-8

CASU B, GUERRINI M, NAGGI A, PEREZ M, TORRI G, RIBATTI D,CARMINATI P, GIANNINI G, PENCO S, PISANO C, BELLERI M, RUSNATI M AND PRESTA M. 2002. Short heparin sequences spaced by glycol-split uronate residues are antagonists of fibroblast growth factor 2 and angiogenesis inhibitors. Biochemistry 41: 10519-10528.

CHEN ZQ, ZHANG L, YU JT, CHEN LK AND ZHOU B. 2016. Identification of resveratrol derivative 3,3 ',4,4 ',5,5 '-hexamethoxy-trans-stilbene as a novel pro-angiogenic small-molecule compound. Eur J Pharmacol 15(791):185-194, doi: 10.1016/j.ejphar.2016.08.034.

CIMPEAN AM, RIBATTI D AND RAICA M. 2008. The chick embryo chorioallantoic membrane as a model to study tumor metastasis. Angiogenesis 11(4): 311-319. doi: 10.1007/s10456-008-9117-1

CZERWON HJ. 1990. Scientometric indicators for a specialty in theoretical high-energy physics: Monte Carlo methods in lattice field theory. Scientometrics18: 5-20. doi.org/10.1007/BF02019159.

FANTON Y, HOUBRECHTS C, WILLEMS L, DANIELS A, LINSEN L, RATAJCZAK J, BRONCKAERS A, LAMBRICHTS I, DECLERCQ J, RUMMENS JL, HENDRIKX M AND HENSEN K. 2016. Cardiac atrial appendage stem cells promote angiogenesis *in vitro* and *in vivo*. J Mol Cell Cardiol 97: 235-244. doi:10.1016/j.yjmcc.2016.06.005

FERICIAN O, CIMPEAN AM, AVRAM S AND RAICA M. 2015. Endostatin effects on tumor cells and vascular network of human renal cell carcinoma implanted on chick embryo chorioallantoic membrane. Anticancer Res 35(12):6521-8.

FISHER C, SCHNEIDER M AND CARMELIET P. 2006 Principles and therapeutic implications of angiogenesis vasculogenesis and arteriogenesis. HEP 176/II. 157-212, Springer-Verlag Berlin Heidelberg, 2006.

FOLKMAN J. 2003. Fundamental concepts of the angiogenic process. Curr Mol Med 3(7): 643-651.

FORNVIK K, ZOLFAGHARI S, SALFORD LG AND REDEBRANDT HN. 2016. ITPP Treatment of RG2 glioblastoma in a rat model. Anticancer Res 36: 5751-5755. doi:10.21873/anticanres.11158

GAUTAM J, BANSKOTA S, REGMI SC, AHN S, JEON YH, JEONG H AND KIM JA. 2016. Tryptophan hydroxylase 1 and 5-HT7 receptor preferentially expressed in triple-negative breast cancer promote cancer progression through autocrine serotonin signaling. Mol Cancer 15 doi:10.1186/s12943-016-0559-6

GOMES PS, ZOMORODIAN A, KWIATKOWSKI L, LUTZE R, BALKOWIEC A, COLACO B, PINHEIRO V, FERNANDES JCS, MONTEMOR MF AND FERNANDES MF. 2016. *In vivo* assessment of a new multifunctional coating architecture for improved Mg alloy biocompatibility. Biomed Mater 10;11(4):045007. doi: 10.1088/1748-6041/11/4/045007.

GRONAU S, THESS B, RIECHELMANN H, FISCHER Y, SCHMITT A AND SCHMITT M. 2006. An autologous system for culturing head and neck squamous cell carcinomas for the assessment of cellular therapies on the chorioallantois membrane. Eur Arch Otorhinolaryngol 263(4): 308-312. doi:10.1007/s00405-005-1008-0

HAHM ER, GHO YS, PARK S, PARK C, KIM KW AND YANG CH. 2004. Synthetic curcumin analogs inhibit activator protein-1 transcription and tumor-induced angiogenesis. Biochem Biophys Res Commun 321: 337-344. doi:10.1016/j.bbrc.2004.06.119

HAN SY, HAN HB, TIAN XY, SUN H, XUE D, ZHAO C, JIANG ST, HE XR, ZHENG WX, WANG J, PANG LN, LI XH AND LI PP. 2016. MicroRNA-33a-3p suppresses cell migration and invasion by directly targeting PBX3 in human hepatocellular carcinoma. Oncotarget 7(27):42461-42473. doi: 10.18632/oncotarget.9886.

ISHIDA K AND MITSUI T. 2016. Generation of bioengineered feather buds on a reconstructed chick skin from dissociated epithelial and mesenchymal cells. Dev Growth Differ 58: 303-314. doi:10.1111/dgd.12275

JAFFE K, CAICEDO M, MANZANARES M, GIL M, RIOS A, FLOREZ A, MONTOREANO C AND DAVILA V. 2013. Productivity in physical and chemical science predicts the future economic growth of developing countries better than other popular indices. PLoS ONE 8(6), e66239. doi:10.1371/journal.pone.0066239.

JAIN RK, SCHLENGER K, HÖCKEL M AND YUAN F. 1997. Quantitative angiogenesis assays: progress and problems. Nat Med 3: 1203-8.doi:10.1038/nm1197-1203

KALIRAI H, SHAHIDIPOUR H, COUPLAND SE AND LUYTEN GPM. 2015. Use of the chick embryo model in uveal melanoma. Ocular Oncology and Pathology 1: 133-140. 10.1159/000370151

KAMBLE S, UTAGE B, MOGLE P, KAMBLE R, HESE S, DAWANE B AND GACCHE R. 2016. Evaluation of curcumin capped copper nanoparticles as possible inhibitors of human breast cancer cells and angiogenesis: a comparative study with native curcumin. AAPS Pharmscitech 17 (5):1030-1041. doi: 10.1208/s12249-015-0435-5.

KANCZLER JM, BARRY J, GINTY P, HOWDLE SM, SHAKESHEFF KM AND OREFFO RO. 2007. Supercritical carbon dioxide generated vascular endothelial growth factor encapsulated poly(dl-lactic acid) scaffolds induce angiogenesis *in vitro*. Biochem Biophys Res Commun 352(1): 135-141. doi:10.1016/j.bbrc.2006.10.187

KLAGSBRUN M, KNIGHTON D AND FOLKMAN J. 1976. Tumor angiogenesis activity in cells grown in tissue culture. Cancer Res 36(1): 110-114.

KOSCHÜTZKI D, LEHMANN KA, PEETERS L, RICHTER S, TENFELDE-PODEHL D AND ZLOTOWSKI O. 2005. Centrality indices in Brandes U., & Erleback T., (Eds.), *Network Analysis*. Springer. doi:10.1007/978-3-540-31955-9_3

KUE CS, TAN KY, LAM ML AND LEE HB. 2015. Chick embryo chorioallantoic membrane (CAM): an alternative predictive model in acute toxicological studies for anti-cancer drugs. Experimental Animals 64: 129-138. doi:10.1538/expanim.14-0059

KWAK HJ, PARK MJ, PARK CM, MOON SI, YOO DH, LEE HC, LEE SH, KIM MS, LEE HW, SHIN WS, PARK IC, RHEE CH AND HONG SI. 2006. Emodin inhibits vascular endothelial growth factor-A-induced angiogenesis by blocking receptor-2 (KDR/Flk-1) phosphorylation. Int J Cancer 118: 2711-2720. doi:10.1002/ijc.21641

LEE S AND BOZEMAN B. 2005. The impact of research collaboration on scientific productivity Social Studies of Science 35:673 doi: 10.1177/0306312705052359

LOKMAN NA, ELDER AS, RICCIARDELLI C AND OEHLER MK. 2012. Chick chorioallantoic membrane (CAM) assay as an *in vivo* model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. Int J Mol Sci 13(8): 9959-9970. doi: 10.3390/ijms13089959.

LV X, LING L, XIA Y, XU YF, HOU FG, REN JL, ZHANG XF AND LI Y. 2016. Establishment of human hepatocellular carcinoma CAM xenograft model and observation of angiogenesis characteristics. Int J Clin Exp Med 9: 3698-3703.

MORENO-JIMÉNEZ I, HULSART-BILLSTROM G, LANHAM SA, JANECZEK AA, KONTOULI N, KANCZLER JM, EVANS ND AND OREFFO RO. 2016. The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering, Scientific Reports 6:32168, doi: 10.1038/srep32168

NABOUT JC, CARVALHO P, UEHARA-PRADO M, BORGES PP, MACHADO KB, HADDAD KB, MICHELAN TS, CUNHA HF, SOARES TN. 2012. Trends and biases in global climate change literature. Natureza & Conservação, 2012; 10(1): 45–51. http://dx.doi.org/10.4322/natcon.2012.008

NABOUT JC, PARREIRA MR, TERESA F, CARNEIRO FM, CUNHA HF, ONDEI LS, CARAMORI SS, SOARES TN. 2015. Publish (in a group) or perish (alone): the trend from single- to multi-authorship in biological papers. Scientometrics 102:357–364. doi 10.1007/s11192-014-1385-5

NANSEN C AND MEIKLE WG. 2014. Journal impact factors and the influence of age and number of citations. Mol Plant Pathol 15(3): 223-225. doi: 10.1111/mpp.12096.

NORRBY K. 2006. *In vivo* models angiogenesis. J Cell Mol Med10(3):588-612.

NOWAK-SLIWINSKA P, SEGURA T AND IRUELA-ARISPE M. 2014. The chicken chorioallantoic membrane model in biology, medicine and bioengineering, Angiogenesis 17(4): 779–804. doi:10.1007/s10456-014-9440-7.

PATIL RS, SHAH SU, SHRIKHANDE SV, GOEL M, DIKSHIT RP AND CHIPLUNKAR SV. 2016. IL17 producing gamma delta T cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients. International Journal of Cancer 139: 869-881. doi: 10.1002/ijc.30134

R CORE TEAM. R. 2013. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/. 2013.

RAZERA JCC. 2016. Contribuições da cienciometria para a área brasileira de Educação em Ciências. Ciência & Educação 22: 557-560. http://dx.doi.org/10.1590/1516-731320160030001

RIBATTI D. 2010. The chick embryo chorioallantoic membrane as an *in vivo a*ssay to study antiangiogenesis. Pharmaceuticals 3: 482-513. doi:10.3390/ph3030482.

RIBATTI, D. 2014. The chick embryo chorioallantoic membrane as a model for tumor biology. Exp Cell Res doi:org/10.1016/j.yexcr.2014.06.010.

RIBATTI D. 2016. The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. Mechanisms of Development141: 70-77. doi: 10.1016/j.mod.2016.05.003.

RIBEIRO SS, JESUS AM, ANJOS CS, SILVA TB, SANTOS ADC, JESUS JR, ANDRADE MS, SAMPAIO TS, GOMES WF, ALVES PB, CARVALHO AA, PESSOA C, de MORAES MO, PINHEIRO ML, PRATA AP, BLANK AF, SILVA-MANN R, MORAES VR, COSTA EV, NOGUEIRA PC AND BEZERRA DP. 2012. Evaluation of the cytotoxic activity of some Brazilian medicinal plants. Planta Med 78: 1601-06. DOI: 10.1055/s-0032-1315043

ROVITHI M, AVAN A, FUNEL N, LEON LG, GOMEZ VE, WURDINGER T, GRIFFIOEN AW, VERHEUL HM AND GIOVANNETTI E. 2017. Development of bioluminescent chick chorioallantoic membrane (CAM) models for primary pancreatic cancer cells: a platform for drug testing. Sci Rep 17(7):44686. doi: 10.1038/srep44686.

SUBAUSTE MC, KUPRIYANOVA TA, CONN EM, ARDI VC, QUIGLEY JP AND DERYUGINA EI. 2009. Evaluation of metastatic and angiogenic potentials of human colon carcinoma cells in chick embryo model systems. Clin Exp Metastas 26(8): 1033-1047. doi: 10.1007/s10585-009-9293-4

SUBRAMANYAM K. 1983. Bibliometric studies of research collaboration: an review. J. Inf. Sci 6:33-38.

THANEKAR D, DHODI J, GAWALI N, RAJU A, DESHPANDE P, DEGANI M AND JUVEKAR A. 2016. Evaluation of antitumor and anti-angiogenic activity of bioactive compounds from Cinnamomum tamala: *In vitro, in vivo* and *in silico* approach. S Afr J Bot 104: 6-14. https://doi.org/10.1016/j.sajb.2015.09.014

WANG C, YAN Q, HU MM, QIN D AND FENG ZQ. 2016. Effect of AURKA Gene expression knockdown on angiogenesis and tumorigenesis of human ovarian cancer cell lines. Targeted Oncol 11, p. 11. doi:10.1007/s11523-016-0436-7

YAN Y, CHONG X, YANG RH, LI H, CHEN JL, MA ZL, WANG G, CHUAI M AND YANG X. 2016. Exposure to excess phenobarbital negatively influences the osteogenesis of chick embryos. Frontiers in Pharmacology 30(7):349. eCollection 2016. doi:10.3389/fphar.2016.00349

YEH CH, WANG WC, HSIEH TT AND HUANG TF. 2000. Agkistin, a snake venom-derived glycoprotein Ib antagonist, disrupts von Willebrand factor-endothelial cell interaction and inhibits angiogenesis. J Biol Chem 275:18615-18618. doi: 10.1074/jbc.C000234200

YUAN YJ, XU K, WU W, LUO Q AND YU JL. 2014. Application of the chick embryo chorioallantoic membrane in neurosurgery disease. Int J Med Sci 11(12): 1275-1281. doi: 10.7150/ijms.10443.

ZHOU X, XU CJ, WANG JX, DAI T, YE YP, CUI YM.; LIAO WT, WU XL AND OU JP. 2015. Metastasis-associated in colon cancer-1 associates with poor prognosis and promotes cell invasion and angiogenesis in human cervical cancer. Nt J Gynecol Cancer 25: 1353-1363. doi:10.1097/IGC.0000000000000524.

Capítulo III

Antiproliferative effect of *Vernonanthura polyanthes* leaves aqueous extracts

ABSTRACT

Vernonanthura polyanthes (Asteraceae), popularly known as assa-peixe, has been widely used in traditional medicine to treat a variety of diseases, including bronchitis, pneumonia, hemoptysis, persistent cough, internal abscesses, gastric and kidney stone pain. The purpose of this study was to evaluate the toxic, cytotoxic and genotoxic activity of *V. polyanthes* leaves aqueous extracts on the brine shrimp and *Allium cepa* assays. As result it was identified a toxic and cytotoxic activity of *V. polyanthes* dependent on the extract concentration. The *A. salina* assay suggests that concentrations greater than 24 mg/ml are toxic for shrimp and kill 50% of naupllis; while the *A. cepa* assay suggests the *V. polyanthes* leaves aqueous extract are toxic in all concentrations tested and cytotoxic only at the 40 mg/ml concentration. This cytotoxic may be assigned to the flavonoids found in the species *V. polyanthes*, since different pharmacologic effects, such as antiproliferative and anticarcinogenic action, are attributed to these compounds. No genotoxic activity was observed in all concentrations of *V. polyantes* extract tested. Our results suggest that *V. polyanthes* could be a good natural source of antitumor compounds and may be has potentially used in human medicine; however more detailed studies need to be performed to confirm its potential.

Key-words: assa-peixe; cancer, cytotoxity, Cerrado, medicinal infusions

1. INTRODUCTION

Medicinal plants have pharmacological properties and can present aromatic and gastronomic uses (VALLEJO et al., 2017; KUSUMA et al., 2014). Their use in medicine, food supplements, cosmetics, and other health related products has increased over the past three decades (JONES et al., 2004). Approximately 50% of all drugs currently in clinical trials are derived from plants (SHAKYA, 2016) and there is speculation that more than two billion people worldwide resort to medicinal plants to treat diseases (SMITH-HALL et al., 2012). The bioactive phytocompounds include an array of compounds, such as tannins, lignans, coumarins, quinones, stilbenes, xanthones, phenolic acids, flavones, flavonols, catechins, anthocyanins, and proanthocyanins, which could cause adverse issues for human health if bad administered. In this way, many plant species commonly considered medicinal can contain potentially dangerous substances (RODRIGUES et al., 2011; MELO-REIS et al., 2011).

The present study is designed to appraise the toxicity and genotoxicity potential of a folklore medicinal plant, *Vernonanthura polyanthes* known commonly as assa-peixe. The *Vernonia* Genus encompasses around 1000 species (KEELEY; JONES, 1979), which it is usually used as food and medicine (TOYANG et al., 2013). Due to the great variability in habit and morphology there was an reclassification in *Vernonia* Genus, that combinate *Vernonathura* and *Vernonia polyanthes* and originated the *Vernonanthura polyanthes* species, the focus of the present work. This shrub presents oval leaves, rough and hairy spear-shaped and occurs primarily in Brazilian Cerrado biome (VEGA; DEMATTEIS, 2010). The white or pink inflorescences are arranged at the apices of the branches in small capitula (ALVES; NEVES, 2003).

In folk medicine, *V. polyanthes* has been used to treat a variety of disturbs, including bronchitis, persistent coughs, pneumonia, kidney stones, gastric disorders, malaria, fever, wounds, fractures, sprains, bruises and dislocations (GUERRA-SANTOS et al., 2016). In addition, the plant is indicated by common sense as diuretic and anti-rheumatic (JORGETTO et al., 2011; OLIVEIRA et al., 2011; SLONGO; HOSCHEID, 2012). It has been demonstrated that *V. polyanthes* is a potential vasodilatation agent, able to manage blood pressure (ROMANEZI et al., 2003).

Previous study identified that the aqueous extract of the *V. polyanthes* showed no toxic, genotoxic and antigenotoxic activity in the experimental conditions tested using the wing somatic mutation and recombination test SMART/wing (GUERRA-SANTOS et al., 2016). However, when the extract was associated with doxorubicin, used as a positive control, the mutagenic potential of doxorubicin was enhanced, increasing the number of mutations in

D. melanogaster somatic cells (GUERRA-SANTOS et al., 2016). The study of toxicity and citogenotoxicity of plants popularly used in traditional medicine are important to avoid adverse reaction (ASARE et al., 2012; CIAPPINA et al., 2017). Toxicological evaluation with several different methodologies is recommended to ensure accurate results.

To evaluate the *V. polyanthes* toxical potential the *Allium cepa* and *Artemia salina* models were selected. Those methods are very useful as a first-tier analysis of toxicity and cytogenotoxicity and they are simple, inexpensive and minimum laboratory facilities are required for its performance (RIBEIRO et al., 2016; NEVES et al., 2014; LEME; MARIN-MORALES, 2009). In addition, both assays have been widely used for evaluation of toxicity of various medicinal herbs (LEITE et al., 2015; BAGATINI et al., 2007). Moreover, the results obtained using those *in vivo* models show a high degree of conformity with the results obtained from mammalian assays (FREIRES et al., 2017; FEDEL-MIYASATO, 2014; LEME; MARIN-MORALES, 2009).

2. MATERIAL AND METHODS

2.1 *Plant and aqueous extract obtaining*

The plant sample was collected in the city of Anápolis, Goiás, in the area of Cerrado within the premises of Universidade Estadual de Goiás, Câmpus Henrique Santillo The collected leaves were dehydrated and pulverized. Then the aqueous infusions were prepared according the popular medicinal use described on Farmacopéia Brasileira (ANVISA, 2011). The *V. polyanthes* leaves aqueous extract concentration commonly used in popular medicine is 20 mg/ml. Then, it was tested three different concentrations: popular dose (20 mg/ml); half from popular dose (10 mg/ml) and the double of popular dose (40 mg/ml).

2.2 *Toxicity assays with Artemia salina*

Eggs (25 mg) from *A. salina* were acquired from local pet shops and hatched at 25-30°C in saline water (pH 8.0). After 24 h, the newly hatched larvae were collected and used in the lethality assay according to procedures described by MEYER et al., (1982). Groups of 10 larvae were exposed to *V. polyanthes* leaves aqueous extract from leaves (5, 10, 20, 40 and 80 mg/ml) diluted in natural seawater and, after 24 h, the survival rates (%) were recorded. The negative control was the saline water used and the positive control was the solution of potassium dichromate. Three independent experiments were performed in triplicate.

2.3 *Toxicity assays with Allium cepa*

The *Allium cepa* test was adapted from the method reported by FISKESJO, (1985). Briefly, *A. cepa* bulbs were growth in water for 48 hours. After that, it was selected for future analysis only the bulbs with approximately 2cm. Next the *A. cepa* bulbs were exposed to three different concentrations of *V. polyanthes* leaves aqueous extract (10, 20 and 40 mg/mL) for 24 hours. Sterile mineral water and sodium azide were used as negative (NC) and positive (PC) controls, respectively. After treatment, the roots were collected for microscopic and macroscopic evaluation to assess toxicity. For macroscopic analysis the following parameters were observed: changes in tissue color, bending or twisting of the roots and presence of tumors. For microscopic analysis, 1.000 cells were evaluated from each bulb, totalizing 5 bulbs per treatment (5.000 cells). The data were compared using t test and $p < 0.05$ was considered statistically significant.

For cytogenotoxic evaluation, the roots were collected, washed with sterile mineral water and placed in Carnoy's fixative solution (3:1 ethanol:glacial acetic acid v/v) refrigerated at -2°C. The roots were subsequently hydrolysed in HCl 5N for 1 min and washed with sterile mineral water. After this, the roots were placed on a microscope slide and one drop of acetic acid 45% was added. Then, the roots were macerated with rusty needles (MONDIN; NETO, 2006). A coverslip were placed on the material and a pressure was made to help separate the cells. The microscope slides were observed under an optical microscope (100x). The following parameters were observed: (a) mitotic index (MI) and the frequency of (b) chromosomal aberrations (CA), (c) micronuclei (MN) and (d) nuclear abnormalities (NA).

The averages for the number of dead individuals of *A. salina* and the mitotic index of the *A. cepa* were compared by Tukey's test, using the statistical program SISVAR (FERREIRA, 2011). In addition, Statistica software was used to calculate the minimum lethal dose.

3. RESULTS

A bioassay that is capable to detecting a wide range of biological properties of crude extracts is the brine shrimp lethality test. This assay is simple, cheap, use a small amount of test material, and it has been used to evaluate plants for potential pharmacological activity (MORALES; PAREDES, 2014). The effects of *V. polyanthes* leaves extracts on rate of mortality of brine shrimps naupillis are presented in Figure 1. The degree of lethality was found to be directly proportional to the concentration of samples. All naupillis of *A. salina* were alive after 24 h of experiment in the negative control and all naupillis in potassium dichromate positive control ($K_2Cr_2O_7$) died at 125 mg/ml. The LC50 obtained using all concentrations tested of *V. polyanthes* leaves aqueous extracts was 24 mg/ml and the LC50 of

$K_2Cr_2O_7$ was 0.19 mg/ml. This means that the medicinal popular dose (20mg/ml) is very close to toxicity limit, but much smaller than the toxicity of $K_2Cr_2O_7$. In addition, statistical analysis showed no significant differences in survival rate between negative control and 5mg/ml of *V. polyanthes* showing that in this concentration the assa-peixe extract is not toxic. On the other hand, no statistical differences were observed between positive control ($K_2Cr_2O_7$) and 40 and 80 mg/ml of *V. polyanthes* which means that in this concentration the *V. polyanthes* extract is highly toxic.

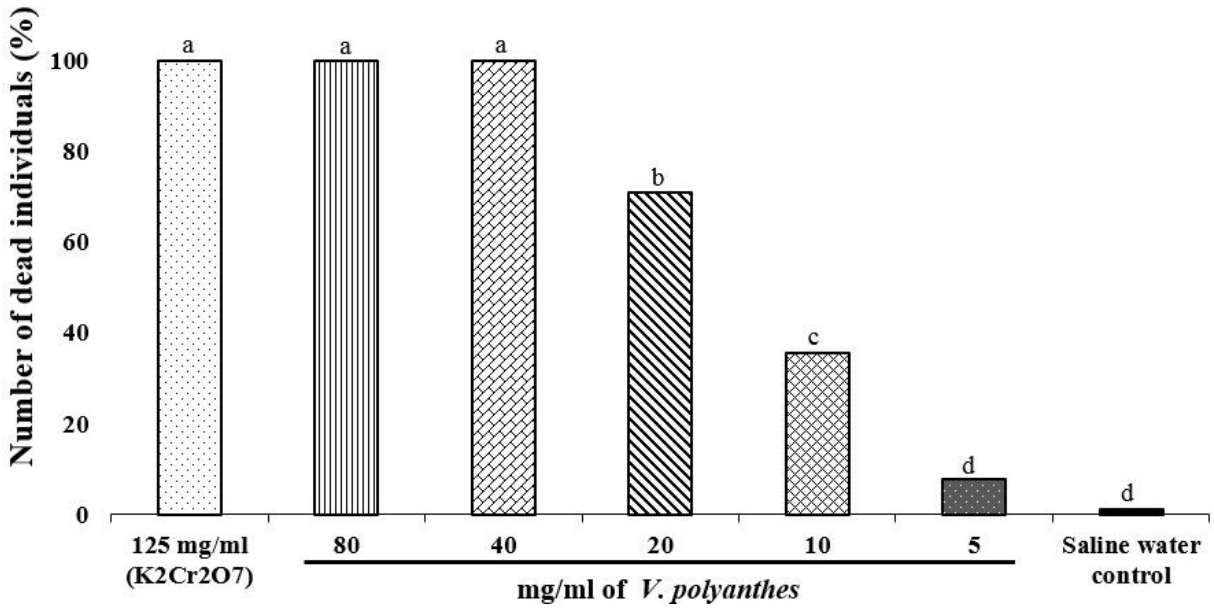

Figure 1. Effects of the *V. polyanthes* leaves aqueous extract on *Artemia salina* assay. Naupliis were treated with 80, 40, 20, 10 and 5 mg/ml of *V. polyanthes* for 24 h. One-way ANOVA followed by Tukey´s test. Averages followed by the same letters do not differ statistically among themselves.

The results of *A. cepa* assay showed that *V. polyanthes* leaves aqueous extracts presented inhibitory effect on *A. cepa* root's growth (Fig. 1A), which can suggest toxicity of *V. polyanthes* extracts. In addition, morphological analysis showed change of coloration in root exposed to *V. polyanthes* leaves aqueous extracts (Figure 2B).

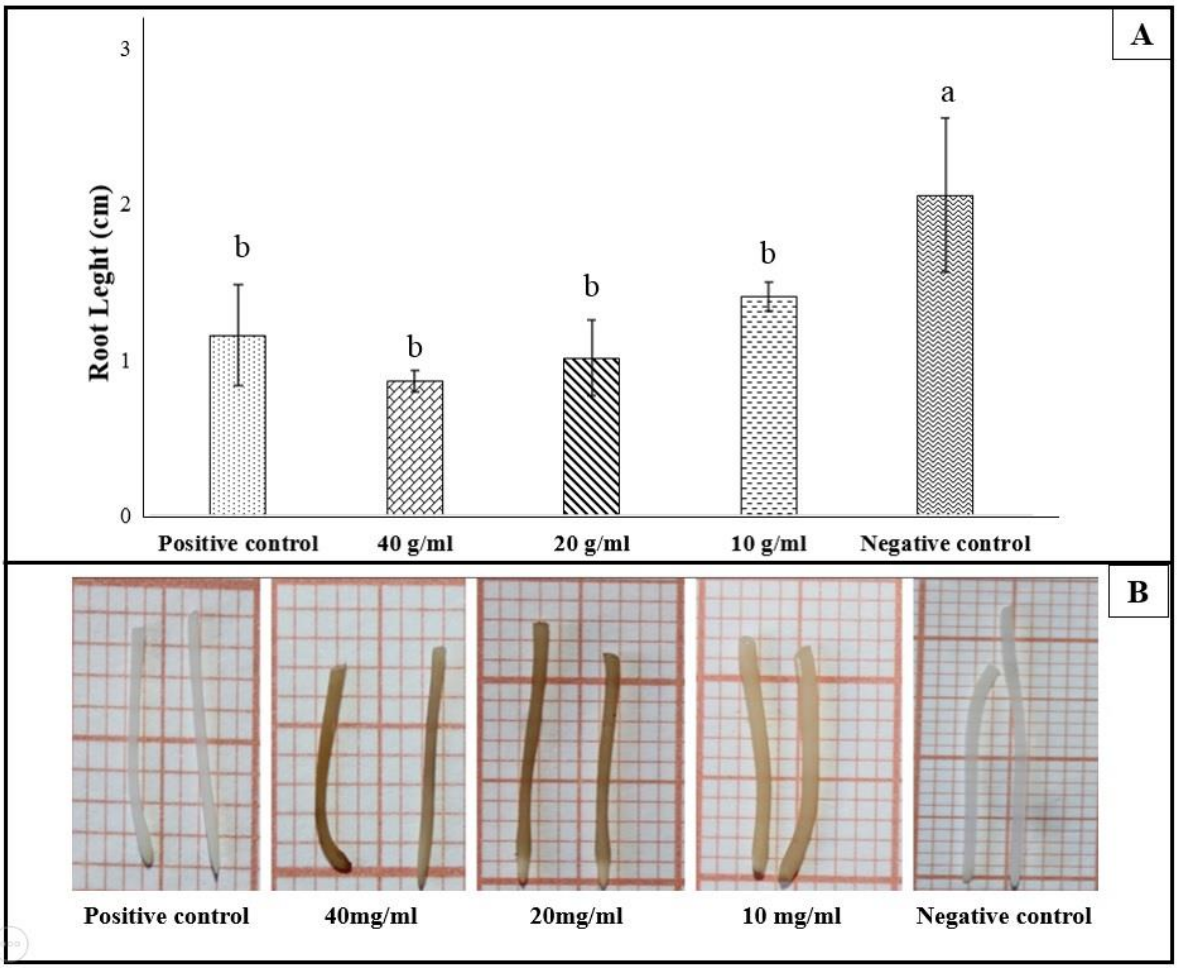

Figure 2. A) Growth analysis of *A. cepa* roots submitted to 40, 20 and 10 mg/ml de *V. polyanthes* for 24 h. One-way ANOVA followed by Tukey´s test. **B)** Root macroscopic analysis of *A. cepa* submitted to the positive control, 40, 20 and 10 mg/ml of *V. polyanthes* and the negative control.

The results obtained for the different concentrations of *V. polyanthes* leaves aqueous extract are shown in Table 1. The results showed no statistical difference between 10 and 20 mg/ml and the negative control, which suggested that on those concentrations the *V. polyanthes* extracts is not cytotoxic. On the other hand, it was observed a decrease in the MI rate in cells subjected to the concentration of 40 mg/ml, which suggest that in highest concentration the *V. polyanthes* inhibit the cell division and is cytotoxic.

Table 1. Cytogenetic analysis of *A. cepa* roots exposed to different concentrations *V. polyanthes* extract.

Treatments	Number of cells	Interphase Cells	Division cells	Cytotoxicity	Genotoxicity	
				Mitotic index (MI)	%Chromosomal aberrations (CA)	% Nuclear abnormalities (NC)
PC (sodium azide)	5000	4730	270	5.2 b	0.30 ± 0.03b	0.28 ± 0.05 b
40 mg/ml	5000	4880	120	2.4 b	0.16 ± 0.006a	0.06 ± 0.01 a
20 mg/ml	5000	4190	810	15.8 a	0,12 ± 0.02 a	0.02 ± 0.006 a
10 mg/mL	5000	4170	830	16.4 a	0,14 ± 0.03a	0.02 ± 0.01a
NC (water)	5000	3865	1135	22.4 a	0.06 ± 0.01a	0.02 ± 0.008 a

Same letters represent no significant difference using Tukey test.

The genotoxicity was characterized by the frequency of chromosomal aberrations and nuclear abnormalities and the results are shown in Table 1. Three main types of chromosome aberrations were recorded: stickiness, bridges and lagging chromosomes. In addition, nuclear abnormalities were also evaluated, such as binucleated cells and micronuclei occurrence. *V. polyanthes* leaves aqueous extracts at all concentrations showed very few chromosomal aberrations and nuclear abnormality frequency, no differing statistically from negative control. This result indicates that *V. polyanthes* leaves aqueous extracts is not genotoxic.

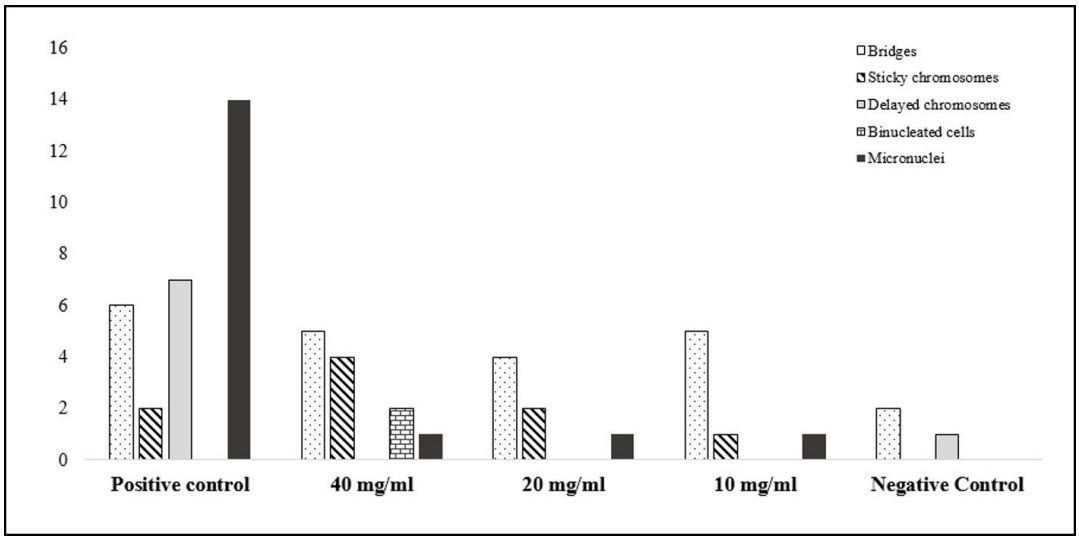

Figure 3. Chromosomal and nuclear alterations in each treatment used.

4. DISCUSSION

In this study, the *in vivo* brine shrimp and *A. cepa* assays were used to assess toxic, cytotoxic and genotoxic potential of leaves aqueous extract of *V. polyanthes*. This is a necessary analysis because the *V. polyanthes* has been empirically used by Brazilian Cerrado population for treatment of various diseases without a detailed evaluation of their effectiveness, toxicity and proper dosage. It is important to mention that others studies yet reveal that plants used in traditional medicine have cytotoxic and/or genotoxic effects, such as rosemary (CARDOSO et al., 2014) and guaco (DALLA NORA et al., 2010).

Previous study using *Drosophila* as a model to evaluate the toxic potential of *V. polyanthes* leaves aqueous extract showed no toxic, genotoxic and antigenotoxic activity in the experimental conditions tested using the wing somatic mutation and recombination test SMART/wing (GUERRA-SANTOS et al., 2016). However, at the present work we identified a toxic and cytotoxic activity of *V. polyanthes* dependent on the extract concentration. The *A. salina* assay suggests that concentrations over 24 mg/ml are toxic for shrimp and kill 50% of naupllis; while the *A. cepa* assay suggests the toxicity of *V. polyanthes* leaves aqueous extract for all concentrations tested (Fig 2A) and cytotoxicity for the 40 mg/ml concentration (Table1). The disagreement with SMART/wing experiment from GUERRA-SANTOS and collaborators (2016) shown how is important evaluate the toxicity of herbal medicinal extracts in different models. According to DEARFIELD et al. (2002), there is no single test to detect the full spectrum of toxicity of the substance.

Similarly to what was observed in this work, the *Costus spiralis* (SOUSA et al., 2017), *Sambucus australis* (TEDESCO et al., 2017), *Luchea divaricata* (FRESCURA et al., 2012) and *Solidago microglossa* (BAGATINI et al., 2009) medicinal extracts, also presented cytotoxic but no genotoxic or mutagenic effect using *A. cepa* test. According to FACHINETTO et al. (2007), a high concentration of some compounds may cause inhibitory or stimulant effect upon the cell cycle and explain the cytotoxic and no genotoxic of some species. According the literature some secondary metabolic was identified in *V. polyanthes* such as: flavones chrysoeriol-7-O-glycuronyl, acacetin-flavones 7-O-glycuronyl, sesquiterpenes, lactones piptocarphin A and piptocarphin B, glaucolide A, chlorogenic acids coumarins, glycosides, steroids, triterpenes, saponin glycosides, alkaloids (MARTUCCI et al., 2014; IGUAL at al., 2013; SOUZA et al., 2008)

The antiproliferative activity of the aqueous extracts analyzed in this study may be assigned to the flavonoids found in the species *V. polyanthes*, since different pharmacologic

effects, such as antiproliferative and anticarcinogenic action, are attributed to these compounds (HOLLMAN et al. 1996, PELZER et al. 1998). In the present research, we can only indirectly assign the antiproliferative and cytotoxic action of the flavonoids present in the extracts of *V. polyanthes*. More details studies need to be performed to understand the toxical potential of this species.

It's important to mention that many of the agents used in cancer therapy are derived from natural compounds extract from plants, such as: vinca alkaloid family isolated from *Catharanthus roseus* (NOBLE 1990; STANTON et al., 2011), the taxanes paclitaxel originally identified from plants of the genus *Taxus* (BAIRD et al., 2010) and others. Then, the identification of cytotoxic and antiproliferative activity of *V. polyanthes* are interesting properties once they could inhibiting cancer cell proliferation. An analysis of the number of chemotherapeutic agents and their sources indicates that over 50% of approved drugs are derived from natural compounds (CRAGG; NEWMAN, 2000) and *V. polyanthes* could be a new candidate.

5. CONCLUSIONS

The aqueous extracts of leafs from *V.* polyanthes with th concentrations: 20, 40 and 80 mg / ml were toxic to *Artemia saline*, with the average lethal concentration (LC50) = 24 mg / ml. Also regarding toxicity, the *Allium cepa* essay showed toxicity of *V. polyanthes* extracts for all concentrations tested (10, 20 and 40 mg / ml). Microscopy analysis of the assay with *A. salina* showed that the aqueous extracts of *V. polyanthes* leaves are cytotoxic at the concentration 40 mg/ml, but it is not genotoxic.

6. REFERENCES

ALVES, V. F. G.; NEVES, L. J. Anatomia Foliar de *Vernonia Polyanthes* Less (Asteraceae). Universidade Rural do Rio de Janeiro. **Revista da Universidade Rural: Série Ciências da Vida**, v. 22, p. 1-8, 2003.

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA – ANVISA, **Formulário de fitoterápicos da farmacopeia brasileira**. Brasília, 2011, 126 p.

ASARE, G. A.; BUGYEI, K.; SITTIE, A., et al. Genotoxicity, cytotoxicity and toxicological evaluation of whole plant extracts of the medicinal plant *Phyllanthus niruri*(Phyllathaceae). **Genetics Molecular Research,** v. 11, n. 1, p. 100-111, 2012.

BAGATINI, M. D.; FACHINETTO, J. M.; SILVA, A. C. F.; TEDESCO, S. B. Cytotoxic effects of infusions (tea) of *Solidago microglossa* DC. (Asteraceae) on the cell cycle of *Allium cepa*. **Revista Brasileira de Farmacognosia**, v. 19, n. 2b, p. 632-636, 2009.

BAGATINI, M. D.; SILVA, A. C. F.; TEDESCO, S. B. Uso de sistema-teste *Allium cepa* como bioindicador de genotoxicidade de infusões de plantas medicinais. **Brazilian Journal of Pharmacognosy, Curitiba**, v. 18, n. 3, p. 509-516, 2007.

BAILÃO, E. F.; DEVILLA, I. A.; DA CONCEIÇÃO, E. C.; BORGES, L. L. Bioactive compounds found in Brazilian Cerrado fruits. **International Journal of Molecular Sciences**. v. 9, n. 16, p. 23760-23783, 2015.

BAIRD, R. D.; TAN, D. S.; KAYE, S. B. Weekly paclitaxel in the treatment of recurrent ovarian cancer. **Nature Reviews Clinical Oncology**, v. 7, p. 575-582, 2010.

CARDOSO, G. H. S.; DANTAS, E. B. S.; SOUSA, F. R. C., et al. Cytotoxicity aqueous extracts of *Rosmarinus officinalis* L (Labitae) in plant test system. **Brazilian Journal Biology**, v. 74, n. 4, p. 886-889, 2014.

CIAPPINA, A. L.; DUTRA, D. M.; DABADIA, P. L.; ALMEIDA, L. M . Phytotoxic Potential of Croton urucurana latex. **Sodebrás**, v. 12, p. 84-89, 2017.

CRAGG, G. M.; NEWMAN, D. J. Antineoplastic agents from natural sources: achievements and future directions. **Expert Opin Investig Drugs**, v. 9, n. 12, p. 2783-2797, 2000.

DALLA-NORA, G.; PASTORI, T.; LAUGHIGHOUSE, H. D., et al. Antiproliferative and genotoxic effects of *Mikania glomerata* (Asteraceae). **Biocell**, v. 34, n. 3, p. 95-101, 2010.

DEARFIELD, K. L.; CIMINO, M. C.; McCARROLL, N. E., et al. Genotoxicity risk assessment: a proposed classification strategy. **Mutation Research**, v. 521, n. 1, p.121-135, 2002.

FACHINETTO, J.M.; BAGATINI, M.D.; DURIGON, J., et al. Efeito anti-proliferativo das infusões de *Achyrocline satureioides* DC (Asteraceae) sobre o ciclo celular de *Allium cepa*. **Brazilian Journal of Pharmacognosy**, v. 17, n. 1, p. 49-54, 2007.

FEDEL-MIYASATO, L. E.; FORMAGIO, A. S.; AUHAREK, S. A., et al. Antigenotoxic and antimutagenic effects of *Schinus terebinthifolius* Raddi in *Allium cepa* and Swiss mice: a comparative study. **Genetics and molecular research**, v. 13 n. 2, p. 3411-3425, 2014.

FERREIRA, D. F. Sisvar: a computer statistical analysis system. **Ciência e Agrotecnologia**, v. 35, n. 6, p. 1039-1042, 2011.

FISKESJO, G. The *Allium* test as a standard in environmental monitoring. **Hereditas**, v.102, p. 99-112, 1985.

FREIRES, I. A.; SARDI, J. C.; DE CASTRO, R. D.; ROSALEN, P. L. Alternative Animal and Non-Animal Models for Drug Discovery and Development: Bonus or Burden? **Pharmaceutical Research**, v. 34, n. 4, p. 681-686, 2017.

FRESCURA, V. D.; LAUGHINGHOUSE, I. V.; TEDESCO, S. B. Antiproliferative effect of the tree and medicinal species *Luehea divaricata* on the Allium cepa cell cycle. **Caryologia**, v. 65, p.27-33, 2012.

GREENWOOD, S. K.; HILL, R. B.; SUN, J. T., et al. Population doubling: a simple and more accurate estimation of cell growth suppression in the in vitro assay for chromosomal aberrations that reduces irrelevant positive results. **Environmental and Molecular Mutagenesis**, v. 43, n. 1, p.36-44, 2004.

GUERRA-SANTOS, I. J.; ROCHA, J. D.; VALE, C. R., et al. *Vernonanthura polyanthes* leaves aqueous extract enhances doxorubicin genotoxicity in somatic cells of *Drosophila melanogaster* and presents no antifungal activity against *Candida* spp. **Brazilian Journal of Biology**, 2016.

HOLLMAN, P. C. H.; VAN TRIP, J. M. P.; BUYSMAN, M. N. C. P. Fluorescence detection of flavonols in HPLC by post-column chelation with aluminium. **Analytical Chemistry**, v. 68, p. 3511-3515, 1996.

IGUAL, M. O.; MARTUCCI, M. E. P.; COSTA, F. B.; GOBBO-NETO, L. Sesquiterpene lactones, chlorogenic acids and flavonoids from leaves of *Vernonia polyanthes* Less (Asteraceae). **Biochemical Systematics and Ecology**, v. 51, p. 94–97, 2013.

JONES, P. H.; CHRISTODOULOS, K.; DOBBS, N.; THAVASU, P.; BALKWILL, F.; BLANN, A. D.; CAINE, G. J.; KUMAR, S.; KAKKAR, A. J.; GOMPERTZ, N.; TALBOT, D. C.; GANESAN, T. S.; HARRIS, A. L. Combination antiangiogenesis therapy with marimastat, captopril and fragmin in patients with advanced cancer. **British Journal of Cancer**, v. 5, n. 1, p. 30-6, 2004.

JORGETTO, G. V.; BORIOLO, M. F. G.; SILVA, L. M.; NOGUEIRA, D. A.; JOSÉ, T. D. S.; RIBEIRO, G. E. Ensaios de atividade antimicrobiana *in vitro* e mutagênica *in vivo* com extrato de *Vernonia polyanthes* Less (Assa-peixe). **Revista do Instituto Adolfo Lutz**, v. 70, n. 1, p. 53-61, 2011.

KEELEY, S. C.; JONES, S. Distribution of pollen types in *Vernonia* (Vernonieae - Compositae). **Systematic Botany**, v. 4, n. 3, p. 195-202, 1979.

KUSUMA, W. I.; MURDIYANTO; ARUNG E. T.; SYAFRIZAL; KIM, Y. Antimicrobial and antioxidant properties of medicinal plants used by the Bentian tribe from Indonesia. **Food Science and Human Wellness**, v. 3, n. 3-4, p. 191-196, 2014.

LEITE, A. S.; DANTAS, A. F.; OLIVEIRA, G. L. S., et al. Evaluation of Toxic, Cytotoxic, Mutagenic, and Antimutagenic Activities of Natural and Technical Cashew Nut Shell Liquids Using the *Allium cepa* and *Artemia salina* Bioassays. **BioMed Research International**, 16 p., 2015.

LEME, D. M.; MARIN-MORALES, M. A. *Allium cepa* test in enverionmental monitoring: A review on its application. **Mutation Research**, v. 682, n. 1, p. 71-81, 2009.

MARTUCCI ME, DE VOS RC, CAROLLO CA, GOBBO-NETO L. Metabolomics as a potential chemotaxonomical tool: application in the genus Vernonia schreb. **Plos One**, v. 15, 9(4): doi: 10.1371/journal.pone.0093149, 2014.

MELO-REIS, P. R.; BEZERRA, L. S. A.; VALE, M. A. A. B., et al. Assessment of the mutagenic and antimutagenic activity of *synadenium umbellatum* Pax latex by micronucleus test in mice. **Bazilian Journal Biology**, v. 71, n. 1, p. 169-174, 2011.

MEYER B. N., FERRIGNI N. R., AND. PUTNAM J. E. Brine shrimp: a convenient general bioassay for active plant constituents, Planta Medica, v. 45, n. 1, p. 31-34, 1982.

MONDIN, M.; NETO, A. D. Citogénetica vegetal enfatizando a família Orchidaceae. **Orchidstudium**, *4*, 24-54, 2006.

MORALES G, PAREDES A. Antioxidant activities of *Lampaya medicinalis* extracts and their main chemical constituents. **BMC Complement Alternative Medicine**, v. 21, n. 14, 2014

NEVES, E. S. B.; FERREIRA, P. M.; LIMA, L. H.; PERON, A. P. Action of aqueous extracts of *Phyllantha niruri L* (Euphorbiaceae) leaves on meristematic root cells of *Allium cepa*. **Annals of the Brazilian Academy of Science**, v. 86, n. 3, p. 1131-1136, 2014.

NOBLE , RL. The discovery of the vinca alkaloids – chemotherapeutic agents against cancer. **Biochemistry and Cell Biology**, v. 68, p. 1344-1351, 1990.

OLIVEIRA, A. K. M.; OLIVEIRA, N. A.; RESENDE, U. M., et al. Ethnobotany and traditional medicine of the inhabitants of the Pantanal Negro sub-region and the raizeiros of Miranda and Aquidauna, Mato Grosso do Sul, Brazil. **Brazilian Journal of Biology**, v. 71, n. 1, p. 283-289, 2011.

PELZER, L.; GUARDIA, T.; JUAREZ, A., et al. Acute and chronic Acute and chronic antiinflammatory effects of plant flavonoids. **Farmaco**, v. 53, n. 6, p. 421-424, 1998.

RIBEIRO, T. P.; SOUSA, T. R.; ARRUDA, A. S.; PEIXOTO, N., et al. Evaluation of cytotoxicity and genotoxicity of *Hancornia speciosa* latex in *Allium cepa* root model. **Brazilian Journal of Biology**, v. 76, p. 245-249, 2016.

RODRIGUES, H. G.; MEIRELES, C. G.; LIMA, J. T. S.; TOLEDO, G. P.; CARDOSO, J. L.; GOMES, S. L.Efeito embriotóxico, teratogênico e abortivo de plantas medicinais. **Revista Brasileira de Plantas Medicinais**, Botucatu, v. 13, n. 3, p. 359-366, 2011.

ROMANEZI, S. R.; FOGLIO, M. A.; GONTIJO, J. A. R. Effect of the crude extract of *Vernonia polyanthes* Less. on blood pressure and renal sodium excretion in unanesthetized rats. **Phytomedicine**, v. 10, n. 2, p. 127-131, 2003.

SHAKYA, A. K. Medicinal plants: future source of new drugs. **International Journal of Herbal Medicine**, v. 4, n. 4, p. 59-64, 2016.

SLONGO, J. M.; HOSCHEID, J. Avaliação fitoquímica preliminar de extratos de folhas e raízes de *Vernonanthura polyanthes* obtidos por maceração, infusão e decocção. **Revista de Fitoterapia**, v. 12, p. 77, 2012.

SMITH-HALL, C.; LARSEN, H. O.; POULIOT, M. People, plants and health: a conceptual framework for plant consumption. **Journal of Ethnobiology and Ethnomedicine**, v. 8, n. 1, p. 43, 2012.

SOUSA, W, V.; PAZ, A. T. S.; CONCEIÇÃO, E. C.; ALMEIDA, L. M., et al. *In vivo* assessment of cito/genotoxic, antigenotoxic and antifungal potential of *Costus spiralis* (Jacq.) roscoe leaves and stems. **Anais da Academia Brasileira de Ciências**, *in press*, v. 89, p. 2043-2051, 2017.

SOUZA, F. A.; SENA, J.; MARANHO, L. T.; OLIVEIRA, C . M. R.; GUIMAR, A. T. B. Caracterização fitoquímica preliminar de infusões populares obtidas das partes aéreass das espécies *Apium leptophylum* (Pers.) F. Muell. ex Benth. (Apiaceae), *Elvira biflora* L. (DC.) e *Vernonia polyanthes* Less. (Asteraceae). **Revista Brasileira de Farmácia**, v. 89, n. 1, p. 24-27, 2008.

STANTON, R. A.; GERNERT, K. M.; NETTLES, J. H.; ANEJA, R. Drugs that target dynamic microtubules: a new molecular perspective. **Medicinal Research Reviews**, v.31, p. 443-481, 2011.

TEDESCO, M.; KUHN, A. W.; FRESCURA, V. D., et al. Assessment of the antiproliferative and antigenotoxic activity and phytochemical screening of aqueous extracts of *Sambucus australis* Cham. & Schltdl. (Adoxaceae). **Anais da Academia Brasileira de Ciências**, v. 89, n. 3, p. 2141-2154, 2017.

TOYANG, N. J.; ATEH, E. N.; KEISER, J. Toxicity, antimicrobial and anthelmintic activities of *Vernonia guineensis* Benth. (Asteraceae) crude extracts. **Journal of Ethnopharmacology**, v. 144, n. 3, p. 700-704, 2012.

VALLEJO, M. J.; SALAZAR, L.; GRIJALVA, M. Oxidative stress modulation and ros-mediated toxicity in cancer: a review on in vitro models for plant-derived compounds. **Oxidative Medicine and Cellular Longevity**, 2017.

VEGA, A. J.; DEMATTEIS, M. The transfer of *Vernonia perangusta* to the genus *Vernonanthura* (Vernonieae, Asteraceae) and the correct name for *Vernonanthura phosphorica*. **Phytotaxa**, v. 8, n. 1, p. 46-50, 2010.

CONCLUSÕES GERAIS

Ao longo dessa pesquisa foi avaliado o potencial angiogênico e tóxico do extrato aquoso de folhas de *V. polyanthes*. Como resultado observou-se que:

1) Extratos aquosos de folhas de *V. polyanthes* preparados nas concentrações de uso popular (20 mg/ml) e no dobro da dose do uso popular (40 mg/ml) apresentam atividade antiangiogênica;
2) Extratos aquosos de folhas de *V. polyanthes* preparados nas concentrações (20, 40 e 80 mg/ml) mostraram-se tóxicos para *Artemia salina*, sendo o limiar de toxicidade LC50 = 24 mg/ml. Ainda em relação à toxicicidade, o ensaio com *A. cepa* mostrou toxicidade dos extratos de *V. polyanthes* em todas as concentrações testadas (10, 20 e 40 mg/ml).
3) Análise microscópica do ensaio com *A. cepa* mostrou que os extratos aquosos de folhas de *V. polyanthes* são citotóxicos apenas na concentração de 40 mg/ml, mas não são genotóxicos.

Os resultados obtidos sugerem que *V. polyanthes* é uma fonte natural de compostos antitumorais e pode ser potencialmente utilizado na medicina humana. No entanto, estudos mais detalhados precisam ser realizados para confirmar seu potencial antiproliferativo.

Em relação à análise cienciométrica da membrana corioalantóide de galinha, este trabalho mostrou que o teste CAM é um método confiável e altamente empregado para avaliar a angiogênese em todo o mundo. O campo de pesquisa em que o ensaio CAM foi mais utilizado foi à oncologia, especialmente para avaliar o efeito da influência das linhas celulares derivadas de tumores na angiogênese. As abordagens cienciométricas mostraram um aumento contínuo de métricas quantitativas (número de artigo por ano) e qualitativas (IF e citações de artigos) em literatura relatando a utilização do ensaio CAM.

REFERÊNCIAS BIBLIOGRÁFICAS

FOLKMAN J. Fundamental concepts of the angiogenic process. **Current Molecular Medicine**, v.3, n.7, p. 643-651, 2003.

FUNK, V. A.; SUSANNA, A.; STUESSY, T.; BAYER, R. J. Systematics, evolution, and biogeography of the Compositae. IAPT, Vienna, 2009.

GUERRA-SANTOS, I. J.; ROCHA, J. D.; VALE, C. R., et al. *Vernonanthura polyanthes* leaves aqueous extract enhances doxorubicin genotoxicity in somatic cells of *Drosophila melanogaster* and presents no antifungal activity against *Candida* spp. **Brazilian Journal of Biology**, 2016.

HASSAN, B. A. R. Medicinal Plants (Importance and Uses). **Anal acta**, v.3, n. 10, p. 1000-1039, 2012.

MARTUCCI ME, DE VOS RC, CAROLLO CA, GOBBO-NETO L. Metabolomics as a potential chemotaxonomical tool: application in the genus Vernonia schreb. **Plos One**, v. 15, 9(4): doi: 10.1371/journal.pone.0093149, 2014.

MOTALEB M. A.; HOSSAIN M. K. SOBHAN, I. ALAN, M. K.; KHAN, N. A. FIROZ, R. Selected medicinal plants of Chittagong Hill Tracts. IUCN (International Union for Conservation of Nature). Bangladesh, 2011, 116 p.

ORGANIZAÇÃO MUNDIAL DA SAÚDE (OMS). Programa Nacional de Plantas Medicinais e Fitoterápicos. Disponível em: http://www.paho.org/. Acesso em 30 de outubro de 2017.

RIBATTI, D. The chick embryo chorioallantoic membrane as an *in vivo a*ssay to study antiangiogenesis. **Pharmaceuticals**, v. 3, p. 482-513, 2010.

RODRIGUES, H. G.; MEIRELES, C. G.; LIMA, J. T. S.; TOLEDO, G. P.; CARDOSO, J. L.; GOMES, S. L. Efeito embriotóxico, teratogênico e abortivo de plantas medicinais. **Revista Brasileira de Plantas Medicinais**, Botucatu, v. 13, n. 3, p. 359-366, 2011.

SCHULTZ, G. S.; SIBBAD, R. G.; FALANGA V., et al. Wound bed preparation: a systematic approach to wound management. **Wound Repair Regen**, v. 1, p. 21-28, 2003.

SHEN, J. T.; FALANGA, V. Innovative therapies in wound healing. **Journal of Cutaneous Medicine and Surgery**, v.7, n. 3, p. 217-24, 2003.

TOSCANO RICO, J. M. Plantas Medicinais. Academia das Ciências de Lisboa, Instituto de Estudos Acadêmicos para Seniores, Lisboa, 2011.

VEJA, A. J.; DEMATTEIS, M. Chromosome studies of some species of Vernonanthura and Vernonia (Asteraceae, Vernonieae). **Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics**, v. 65, n. 4, p. 271–275, 2012.

WEIS, S. M.; CHERESH, D. A. Tumor angiogenesis: Molecular pathways and therapeutic targets. **Nature Medicine**, v. 17, p. 1359-70, 2011.

YUAN, Y. J.; XU, K.; WU, W. et al. Application of the chick embryo chorioallantoic membrane in neurosurgery disease. **International Journal of Medical Sciences**, v. 11, n. 12, p. 1275-1281, 2014.