ANA CAROLINA SILVEIRA RABELO

Evaluation of the antitumor effect of *Calotropis procera* (Aiton) Dryand (Apocynaceae) *in vitro* and *in vivo*

São Paulo

2020

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Thesis presented to the Graduate Program in Anatomy of Domestic and Wild Animals at Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo to obtain the title of PhD in Science

Department:

Surgery

Concentration area:

Anatomy of Domestic and Wild Animals

Advisor:

Prof[a]. Dr[a]. Maria Angélica Miglino

São Paulo

2020

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CERTIFICADO

Certificamos que a proposta intitulada "Avaliação do efeito antitumoral e antioxidante de espécies vegetais da Região do Baixo Munim, Maranhão, em células tumorais caninas", protocolada sob o CEUA nº 1100210217 (ID 003591), sob a responsabilidade de **Maria Angélica Miglino** *e equipe; Ana Carolina Silveira Rabelo; Rodrigo da Silva Nunes Barreto; Fernando José da Costa Carneiro* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 10/05/2017.

We certify that the proposal "Evaluation of the antitumor and antioxidant effect of plant species from the Baixo Munim Region, Maranhão, on canine tumor cells", utilizing 1 Dogs (males and females), protocol number CEUA 1100210217 (ID 003591), under the responsibility of **Maria Angélica Miglino** *and team; Ana Carolina Silveira Rabelo; Rodrigo da Silva Nunes Barreto; Fernando José da Costa Carneiro* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/10/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 06/2017 a 06/2019 Área: Anatomia dos Animais Domésticos E Silvestres

Origem:	Amostras biológicas estocadas						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	1 a 10 anos	N:	1
Linhagem:	não se aplica			Peso:	5 a 20 kg		

Local do experimento: Laboratório de Produtos Naturais do Campus São Luis Monte Castelo, do Instituto Federal do Maranhão e Laboratório de Cultivo Celular do Setor de Anatomia dos Animais Domésticos e Silvestres da FMVZ-USP

São Paulo, 20 de agosto de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 20 de agosto de 2020
CEUA N 1100210217

Ilmo(a). Sr(a).
Responsável: Maria Angélica Miglino
Área: Anatomia Dos Animais Domésticos E Silvestres

Título da proposta: "Avaliação do efeito antitumoral de Calotropis procera (Aiton) Dryand (Apocynaceae) in vitro e in vivo".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 006821)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 19/agosto/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Olá! Gostaríamos de solicitar alteração do título da proposta. O primeiro título era denominado: "Avaliação do efeito antitumoral e antioxidante de espécies vegetais da Região do Baixo Munim, Maranhão, em células tumorais caninas. No entanto, de todas as espécies que testamos no início do projeto, apenas a Calotropis procera foi promissora e, portanto, seguimos apenas com ela. Devido a isso, gostaríamos de alterar o título para: "Avaliação do efeito antitumoral de Calotropis procera (Aiton) Dryand (Apocynaceae) in vitro e in vivo". "Evaluation of the antitumor effect of Calotropis procera (Aiton) Dryand (Apocynaceae) in vitro and in vivo"".

Comentário da CEUA: "Título da proposta alterado atendendo solicitação do responsável".

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação do efeito antitumoral de Calotropis procera em modelo xenográfico de câncer de mama", protocolada sob o CEUA nº 2378110219 (ID 007704), sob a responsabilidade de **Maria Angélica Miglino** *e equipe; Ana Carolina Silveira Rabelo* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 08/04/2020.

We certify that the proposal "Evaluation of the antitumor effect of Calotropis procera in a breast cancer xenographic model", utilizing 40 Isogenics mice (40 females), protocol number CEUA 2378110219 (ID 007704), under the responsibility of **Maria Angélica Miglino** *and team; Ana Carolina Silveira Rabelo* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/08/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 12/2019 a 12/2020 Área: Oncologia

Origem:	Biotério Central do Instituto de Ciências Biomédicas da USP						
Espécie:	Camundongos isogênicos	sexo:	Fêmeas	idade:	3 a 5 semanas	N:	40
Linhagem:	Balb/c nude			Peso:	20 a 30 g		

Local do experimento: Biotério do NUCEL (Núcleo de Terapia Celular e Molecular) da USP

São Paulo, 08 de abril de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

DIVISION OF RESEARCH

Research Compliance and Biosafety

January 23, 2018

MEMORANDUM

TO: Dr. Susanne Talcott
ALRSRCH - Agrilife Research - Nutrition And Food Science

FROM: Dr. John N. Stallone, Chair
Dr. Michael S. Smotherman, Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: **Approval of AUP IACUC 2017-0378**
Title: Beneficial Effects of Botanical extracts in Breast Cancer Chemotherapy
Reference Number: 066824
Funding Source: Washington State Fruit Commission
AUP Approval Date: 01/23/2018
AUP Expiration Date: 01/22/2021
Species: Mice, athymic nude, Mus musculus

The above referenced AUP has been approved by the IACUC for a period of 3 years. It is the responsibility of the principal investigator to assure all animal work is conducted in accordance with this AUP.

If you have indicated that you will be performing post procedural monitoring of animals at specific intervals, please provide documentation of your observations in the medical record or by using Animal Observation cards that are available through the Comparative Medicine Program.

A copy of this approval will be sent to the housing facility. ***You must consult with the housing facility manager prior to ordering animals to ensure that space is available.***

Pc: Comparative Medicine Program
Housing Facility Manager

750 Agronomy Road, Suite 2701
1186 TAMU
College Station, TX 77843-1186

Tel. 979.458.1467 Fax. 979.862.3176
http://rcb.tamu.edu

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A94401C

A atividade de acesso ao Conhecimento Tradicional Associado, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A94401C**
Usuário:	**fernando josé costa carneiro**
CPF/CNPJ:	**466.665.753-34**
Objeto do Acesso:	**Conhecimento Tradicional Associado**
Finalidade do Acesso:	**Pesquisa**

Espécie

Calotropis procera (ASCLEPIADACEAE)

Fonte do CTA

CTA de origem não identificável

Título da Atividade:	**ESTUDO QUÍMICO E ATIVIDADE ANTITUMORAL DE Calotropis procera (Asclepiadaceae)**

Equipe

fernando josé costa carneiro	**instituto federal do maranhão**
Antonio José Cantanhede Filho	**ifma**
Ana Carolina Silveira Rabelo	**usp**
Rodrigo da Silva Nunes Barreto	**usp**
Adriano Souza Fonseca	**ifma**

Parceiras Nacionais

63.025.530/0019-33 / Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia

Data do Cadastro:	**16/05/2019 16:55:11**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **16:55** de **16/05/2019**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

EVALUATION SHEET

Author: RABELO, Ana Carolina Silveira

Title: **Evaluation of the antitumor effect of *Calotropis procera* (Aiton) Dryand (Apocynaceae) *in vitro* and *in vivo***

Thesis presented to the Graduate Program in Anatomy of Domestic and Wild Animals at the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo to obtain the title of Doctor of Science

Date: _____/_____/_____

Examining Committee

Prof. Dr._____

Institution:_____ Judgment:_____

Prof. Dr._____

Institution:_____ Judgment:_____

Prof. Dr._____

Institution:_____ Judgment:_____

Prof. Dr._____

Institution:_____ Judgment:_____

Prof. Dr._____

Institution:_____ Judgment:_____

ACKNOWLEDGMENT

I thank **God**, for always guiding my steps and allowing all achievements to be possible.

To my parents, **Carlos Henrique Rabelo** and **Maria Ambrosina da S. Rabelo**, for being my examples of love, dedication, respect and affection, I owe you everything that I know and I am. Thank you for being such wonderful parentes and for building our Family wich I am so proud.

To my brothers **Carlos Henrique S. Rabelo** and **Flávio Henrique S. Rabelo**, for being my examples of wisdom, achievements and brotherly love. Words could not describe the admiration and love I have for you, I am very grateful to have you as brothers.

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I am grateful to all the **animals** who donated their freedom, their life and their body for this project to be carried out.

I am grateful to everyone who contributed to this work in some way. To all of you, thank you very much!

“O que vale na vida não é o ponto de partida e sim a caminhada.

Caminhando e semeando, no fim terás o que colher”

Cora Coralina

RESUMO

RABELO, ACS. **Avaliação do efeito antitumoral de *Calotropis procera* (Aiton) Dryand (Apocynaceae) *in vitro* e *in vivo*. 2020**. 133 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

O câncer é o principal problema de saúde pública no mundo e embora avanços terapêuticos tenham sido feitos, ainda existem muitas lacunas. Nesse sentido, o objetivo do trabalho foi avaliar o efeito antitumoral de *Calotropis procera*, uma planta popularmente utilizada para tratar diversas doenças, especialmente relacionadas às desordens gástricas e inflamatórias. Para tal, dividimos a tese em três capítulos, sendo o primeiro uma revisão geral dos principais fitoquímicos e efeitos biológicos descritos para essa espécie vegetal. No segundo capítulo, realizamos a caracterização do extrato etanólico bruto das folhas de *C. procera* (CE), onde foram encontrados principalmente flavonoides, glicosídeos e cardenolídeos. Também avaliamos o potencial antitumoral de CE e das frações metanólica (MF) e acetato de etila (EAF) em células de tumor canino mamário (CMT) e osteossarcoma canino (OST). Como controle positivo foi utilizado o quimioterápico doxorrubicina. Os resultados demonstraram que o tratamento com os extratos de *C. procera* reduziram a viabilidade celular e proliferação de CMT e OST, refletindo no aprisionamento das células na fase G0/G1. *C. procera* também alterou a morfologia das células tumorais, deixando-as mais arredondadas e menores, o que é sugestivo de apoptose. De fato, mediante a análise por citometria de fluxo, foi comprovado que *C. procera* aumentou caspase-3, reduziu angiogênese, o processo de transição epitélio mesenquimal, e aumentou p53 nas células OST; além de reduzir PCNA nas células CMT. Por fim, no terceiro capítulo avaliamos o potencial antimetastático do extrato fenólico bruto de *C. procera* (CphE) em células de câncer de mama 4T1 e em tumores mamários de camundongos num modelo xenográfico. A quercetina, um dos flavonoides presentes na *C. procera*, foi utilizada como controle positivo. Os resultados *in vitro* demonstraram que CphE reduziu a viabilidade das células 4T1; os níveis de ROS; induziu apoptose; modulou as vias de crescimento celular Akt/mTOR e MAPK; reduziu migração celular; e amenizou a transição epitélio-mesenquimal. *In vivo*, *C. procera* reduziu ERK1/2 nos tumores mamários, e reduziu metástase hepática e pulmonar, através da redução de Cenpf e Twist. Todos esses resultados analisados em conjunto sugerem fortemente que *C. procera* apresenta um grande potencial como agente antitumoral.

Palavras-chave: câncer de mama; osteossarcoma; metástase; fitoterapia; flor-de-seda.

ABSTRACT

RABELO, ACS. **Evaluation of the antitumor effect of *Calotropis procera* (Aiton) Dryand (Apocynaceae) *in vitro* and *in vivo*.** 2020. 133 f. Thesis (pHD in Sciences) – Faculty of Veterinary Medicine and Zootechnics, University of São Paulo, São Paulo, 2020.

Cancer is the main public health problem worldwide and although advances have been made in anticancer therapy, there are still many gaps. In this sense, our goal was to evaluate the antitumor effect of *Calotropis procera*, a plant popularly used to treat several diseases, especially related to gastric and inflammatory disorders. For this, we divided the thesis into three chapters. First, it was demonstrated a general review of the main phytochemicals and biological effects described for this plant. In the second chapter, it was carried out the characterization of the crude ethanolic extract of *C. procera* leaves (CE), where mainly flavonoids, glycosides and cardenolides were found. We also evaluated the antitumor effect of CE, methanolic (MF) and ethyl acetate (EAF) fractions of *C. procera* in canine mammary tumor (CMT) and canine osteosarcoma (OST) cells. As a positive control, it was used the chemotherapy drug-doxorubicin. Results demonstrated that *C. procera* treatment reduced the cell viability and proliferation of CMT and OST, reflecting the cell arrest in the G0/G1 phase. *C. procera* also altered the morphology of tumor cells, making them more round and smaller, which is suggestive of apoptosis. In fact, through flow cytometry analysis, we found that *C. procera* increased caspase-3, reduced angiogenesis and the mesenchymal epithelial transition, and increased p53 in OST cells; in addition to reducing PCNA in CMT cells. Finally, in the third chapter, the antimetastatic effect of crude extract phenolics from *C. procera* (CphE) was evaluated in 4T1 breast cancer cells and in mammary tumors of nude mice xenograft model. Quercetin - a flavonoids present in *C. procera*- was used as positive control. The *in vitro* results demonstrated that CphE reduced 4T1 cells viability and the levels of ROS, induced apoptosis, modulated Akt/mTOR, MAPK pathways, reduced cell migration, and epithelial-mesenchymal transition. *In vivo*, CphE reduced ERK1/2 in breast tumors, and reduced liver and lung metastasis, by reducing Cenpf and Twist. All of these results analyzed together strongly suggest that *C. procera* has great potential as an antitumor agent.

Keywords: breast cancer; osteosarcoma; metastasis; phytotherapy; milkweed.

SUMÁRIO

1. INTRODUCTION

Cancer is a generic term for a large group of diseases that can affect any part of the body and is the leading cause of death worldwide (World Health Organization-WHO, 2020). In 2018, it was estimated more than 18 million new cancer cases and 9.6 million deaths due to cancer (Bray et al., 2018). According to WHO (2020), about a third of cancer deaths are due to behavioral and dietary risks, such as high body mass index, low fruits and vegetables intake, lack of physical activity, use of tobacco and alcohol.

The development of cancer involves successive genetic and epigenetic alterations that allow cells to escape from homeostatic controls that ordinarily suppress inappropriate proliferation and inhibit the survival of aberrantly proliferating cells outside their normal niches. In addition to increased cell proliferation, there are also resistance to apoptosis, metabolic changes, genetic instability, induction of angiogenesis, and increase in migratory capacity (Sever and Brugge, 2015). Furthermore, progression of cancer is associated with a complex interplay between the tumor cells and surrounding non-neoplastic cells and the extracellular matrix (ECM) (Sever and Brugge, 2015).

At the molecular level, oncogenic mutations can cause the affected genes to be overexpressed or produce mutated proteins whose activity is dysregulated. Examples include proteins involved in signaling pathways that are commonly activated in many physiological responses, such as growth factor receptor tyrosine kinases (RTKs) [e.g., the epidermal growth factor receptor (EGFR)]; small GTPases [e.g., Ras], serine/threonine kinases [e.g., Raf and Akt], cytoplasmic tyrosine kinases [e.g., steroid receptor coactivator (Src)], lipid kinases [e.g., phosphoinositide 3-kinases (PI3Ks)], as well as nuclear receptors [e.g., estrogen receptor (ER)] (Sever and Brugge, 2015).

Surgery, radiotherapy and chemotherapy are now the main currents of cancer therapy coupled with the emerging targeted and cancer immunotherapies (Liu et al., 2019). To a certain extent, the growth and spread of cancer were controlled and the patients' survival time was prolonged by these methods, but the overall effectiveness and many of the associated side effects have caused more and more concerns (Yan et al., 2017). In some cases, these treatments are effective, but the therapeutic effects did not last long, or the cancer cells gained resistance to chemical drugs, limiting their further

use. Therefore, it is necessary to develop a more ideal drug therapy or adjuvant treatment strategy for cancer (Yan et al., 2017; Liu et al., 2019).

In this context, natural molecules, which are thought to have remarkable physiological efects, low toxicity and non-mutagenic properties in the human body when consumed properly, have gained more interest in anti-cancer agent development (Yan et al., 2017; Liu et al., 2019). Among natural products, plants have played a key role in treating a number of diseases, including cancer. The sheer variety and number of plants with medicinal properties around the world is quite astonishing. It is estimated that around 70,000 plant species have been used at one time or another for medicinal purposes (Kuruppu et al., 2019).

Plant-derived agents have played a vital role in the treatment of cancer with more than 3,000 species reported to have anti-cancer properties. Over the years medicinal plants have been exploited as an initial point for the synthesis of new compounds for cancer with different structural parameters in the synthetic, combinatorial and biotechnological sciences. Over 60% of currently used anti-cancer agents are derived from natural sources such as plants (Kuruppu et al., 2019).

The curative properties originate in different parts of plants, due to the presence of an array of low-molecular-mass substances known as secondary metabolites. Examples of secondary metabolites in plants are flavonoids, phenolics, terpenoids, alkaloids and sulphur-containing compounds (Kuruppu et al., 2019). These compounds exert their antitumor effect through inhibition of cell growth, reduction of metastasis and invasion, promotion of cancer cell apoptosis, and enhancement of immunity (Abu-Darwish and Efferth, 2018; Liu et al., 2019).

Calotropis procera belongs to the family Apocynaceae and is well known for its various medicinal properties. Leaves, roots, flowers and latex from this plant are used in several systems of medicinal preparations (Samy et al., 2012). The leaves of *C. procera* are effective in treating migraines (Prasad, 1985). The root extract of *C. procera* has been found to produce cytotoxic effect on COLO 320 tumor cells (Smit et al., 1995). Moreover, a cardenolide isolated from the root barks of *C. procera* showed a strong cytotoxic effect on several human cancer lines, a high *in vivo* tolerance to tumor growth and prolonged

survival in the human xenograft models of nude mice (Van Quaquebeke et al., 2005). The chloroform-soluble fraction of its roots, ethanolic extract of its flowers and aqueous and organic extracts of its dried latex also exhibit a strong anti-inflammatory activity in animal models of acute and chronic inflammation (Arya and Kumar, 2005). However, even more studies with *C. procera* regarding its antitumor potential should be carried out to elucidate its mechanism of action. In view of this, this work proposed to carry out a literary review on the main biological and pharmacological properties of *C. procera*; evaluate the cytotoxic and antitumor activities of *C. procera* in mammary and osteosarcoma canine cells; and elucidate the main metastatic pathways modulated by *C. procera* in 4T1 breast cancer cells and in a xenograft mice model.

2. OBJECTIVES

2.1. Main objectives

To evaluate the antitumor effect of *Calotropis procera* in canine mammary cancer and osteosarcoma canine cells, and also to elucidate the metastatic pathways modulated by *C. procera* in breast cancer.

2.2. Specific objectives

ARTICLE I

- Review current scientific evidence on the pharmacological effects of *C. procera* and its phytochemicals, and evaluate possible research opportunities using *C. procera* as complementary and alternative medicine.

ARTICLE II

- Characterize the crude extract of *C. procera* (CE);

- Evaluate the cytotoxicity of methanolic fraction (MF), ethyl acetate fractions (EAF) and CE of *C. procera* in osteosarcoma canine tumor cells (OST), canine mammary tumor (CMT), and canine fibroblast (non-tumor cells);

- Evaluate the growth curve, morphology, cell cycle and proliferation of OST and CMT after treatment with MF, EAF and CE;

- Evaluate markers of apoptosis (Bax, Bcl-2 and caspase-3), angiogenesis (CD31 and VEGF) and mesenchymal-epithelium transition (TGF-β) in OST cells after treatment with MF, EAF and CE. And proliferation (PCNA) on CMT cells after treatment with MF, EAF and CE.

ARTICLE III

- Evaluate the cytotoxicity of crude extract phenolics from *C. procera* (CphE) in 4T1 breast cancer cells;

- Evaluate the production of reactive oxygen species (ROS) and the migration of 4T1 breast cancer cells after CphE treatment;

- Evaluate the expression of proteins related to tumor growth and migration (p-Akt/Akt, p-mTOR, p-p38/p38, p-ERK1/2, and p-CREB); epithelial-mesenchymal transition (FAK and Src); and apoptosis (caspase-3 and PARP) in 4T1 breast cancer cells and in tumors from a xenograft mice model of breast cancer after CphE treatment;

- Quantify the proteins involved in the metastatic process (IR, IRS-1, IGF-1, PTEN, Akt, mTOR, P70S6k, RPS6, GSK3-a, GSK3-b, and TSC2) in 4T1 breast cancer cells and in tumors from a xenograft mice model of breast cancer after CphE treatment;

- Evaluate the expression of genes involved in the metastatic process (EGFR, p38, mTOR, and Akt); and in the apoptotic process (caspase-8, caspase-3, cytochrome C and Bax) in 4T1 breast cancer cells and in tumors from a xenograft mice model of breast cancer after CphE treatment;

- Evaluate the gene expression of Twist and Cenpf in the lungs and liver from a xenograft mice model of breast cancer after CphE treatment;

3. ARTICLE I - C*alotropis procera* (Aiton) Dryand (Apocynaceae): a review on phytochemistry and pharmacological properties

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Abstract: *Calotropis procera* (Aiton) Dryand (Apocynaceae) popularly known as milkweed, has been traditionally used to treat diseases, particularly associated with gastric disorders and inflammatory processes. The present investigation aimed to review the current scientific evidence regarding pharmacological effects of *C. procera* extracted phytochemicals, and possible research opportunities as complementary and alternative medicine. Scientific publications were searched in various electronic databases using the following search terms: *Calotropis procera*, medicinal plants, toxicity, phytochemical characterization, and biological effects. Collected data showed that cardenolides and flavonoids were the main classes of phytochemical identified in *C. procera* latex and leaves, which were correlated with biological activities including mainly antitumoral, antioxidant, anti-inflammatory, hypoglycemic, gastric protective, anti-microbial, insecticide, anti-fungal, and anti-parasitic. However, some of the studies were carried out with only a single dose, or with a high dose not achievable under physiological conditions. Therefore, the validity of *C. procera* biological activity may be questionable. Not less important to highlight are the risks associated with its use and the absence of clinical studies. In conclusion, the need of bioassay-guided isolation of bioactive compounds, bioavailability and efficacy, as well as pharmacological and toxicity studies are needed using *in vivo* animal models in order to support the traditionally claimed human health benefits.

Keywords: *Calotropis procera*, milkweed, medicinal plants, phytochemical characterization, biological effects.

3.1. Introduction

Calotropis procera (Aiton) Dryand (Apocynaceae) is a native plant from Africa, and Asia, introduced in America during the middle of the 19th (Gracia et al., 2019). Several parts of the plant (e.g. leaves, roots, flowers and latex) are used in different systems of medicinal preparations to treat a large range of diseases, especially related to gastric, skin disease and inflammatory disorders (Prasad, 1985; Samy et al., 2012). In the Middle East, for example, the leaves are widely used in the treatment of jaundice (Murti et al., 2010); while the latex is used for treating intermittent fevers, and rheumatoid swellings (Hassan et al., 2015).

C. procera is a wild shrub that can vary from 2.5 to 10 m in height (Aslam et al., 2013). The leaves are opposite oblong-ovate to nearly orbicular (Sharma et al., 2010). The flowers are umbelliform cymes, while the fruits are inflated. Both flowering and fruiting occur throughout the year (Sharma et al., 2010). However, *C. procera* may show some morphological and physiological adaptations, due to its original occurrence in regions with water deficit and poor nutrient soils (Gracia et al., 2019). Details of *C. procera* shrub, flowers, leaves and fruits can be seen in figure 1.

3.2. Database search method

All information on *C. procera* was collected from various electronic databases, named PubMed, Scopus, Web of Science, Google Scholar, Springer, Wiley, and Mendeley. The following search terms were selected: *Calotropis procera*, medicinal plants, toxicity, phytochemical characterization, and biological effects.

3.3. Phytochemistry

Several parts of *C. procera* have been investigated for phytochemical composition, however, most studies reported the metabolites found in leaves and latex. The main solvents used for extraction and isolation of these phytochemicals were ethanol and methanol. In general, the presence of cardenolides, steroid glycoside and flavonoids were the most commonly described secondary metabolites (Jucá et al., 2013; Kazeem et al., 2013; Moustafa et al., 2010; Sweidan and Abu Zarga, 2015), although lignans, terpenes, coumarins, and phenolic acids have already been found (Verma et al., 2013; Mohamed et al., 2015; Abdel-Mageed et al., 2016). The main parts of the plant, solvent, compounds and classes of metabolites are summarized in Table 1.

3.4. Pharmacological and biological activities

Several *in vitro* and pre-clinical animal studies have investigated the biological and pharmacological activities of *C. procera* extracts and fractions from different parts of the plant within the context of their potential to fight human diseases.

3.4.1. Antitumor activity

Ethanolic extract of *C. procera* leaf, flower, and fruit has been tested on MCF-7 (breast cancer), HCT-116 (colon carcinoma), HepG-2 (hepatocellular carcinoma) and A-549 (human lung carcinoma) tumor cells. Overall, the extracts of flower showed the highest cytotoxic activities, being more potent against HCT-116 and HepG-2 cells (IC_{50}: 28.60 and 24.50 µg/ml, respectively) (Al-Taweel et al., 2017). However, even the extract that had the best response still showed a cytotoxicity of 4 times lower than vinblastine sulfate, used as a positive control in this study (IC_{50}: 5.39 and 3.48 µg/ml, respectively) (Al-Taweel et al., 2017).

Crude methanolic extract of aerial parts of *C. procera* inhibited the growth of MCF-7, HepG-2, MDBK (Madin-Darby bovine kidney) and A-549 cells with different potencies (IC_{50}: 6.05, 11.64, 12.16, and 1.9 µg/ml, respectively). However, the petroleum ether, chloroform, and methanol fractions did not present cytotoxicity for most of the

tested cells, presenting IC > 50 μg/ml (Esmaeili et al., 2014). Other investigation showed that the ethyl acetate and acetone crude extract from *C. procera* stem was more cytotoxic to colon (HCT-8) and melanoma (B-16) cells (IC_{50}: 0.8 and 4.4 μg/ml, respectively). However, the hexane and dichloromethane fraction did not show cytotoxicity for any cell line (IC_{50} > 25) (Magalhães et al., 2010). Although the different extracts of *C. procera* had a promising effect in some strains, especially the crude extract, the potency of cytotoxicity was much lower than the positive controls used in these studies (Tamoxifen and Doxorubicin, respectively). Furthermore, a non-tumor cell control was not used to assess the selectivity of *C. procera*, thus impaired the validity of the findings.

The treatment with *C. procera* latex for 72 h reduced viability of HL-60 (promyelocytic leukemia), HCT-8 (colon carcinoma), MDA-MB-435 (breast cancer) and SF295 (brain tumor) cells, presenting the highest potency against SF295 cells (IC_{50}: 0.42 μg/ml). The underlying mechanisms were linked to reduction of DNA synthesis, inhibition of topoisomerase I and apoptosis induction at similar levels as those induced by the chemotherapeutic doxorubicin (10 or 25 μg/ml) (Soares de Oliveira et al., 2007). Similarly, there was no comparison with a non-tumor cell to validate the findings.

Treatment with *C. procera* protein (CP-P) isolated from root bark (12.5 and 50 μg/ml) alone or in combination with the drug cyclophosphamide (0.2 mg/kg) induced apoptosis of human MCF-7 and MDA-MB-231 breast cancer cells (Samy et al., 2012). Activation of apoptosis also appears to be an important mechanism for reducing the viability of human skin melanoma cells (SK-MEL-2) treated with *C. procera* methanolic extract (20 and 40 μg/ml) for 24 hours (Joshi et al., 2015). Other manuscript showed a reduction of hepatoma (Hub-7) cell viability, mediated by a marked increase in DNA cellular fragmentation, which occurred at a concentration and time dependent fashion (0.1 – 10 mg/l). However this occurred without changes in Bcl-2 or caspases-3 levels, suggesting caspase-independent apoptosis (Choedon, 2006). Conversely, a reduction in the percentage of apoptosis was observed in erythrocytes after exposure of fish to *C. procera* latex (Sayed et al., 2016).

Some authors have investigated the potential compounds associated with the anti-tumor effect of *C. procera*. The cytotoxic effects of *C. procera* against tumor cells have

been associated with the calotroposide isolated from the butanolic fraction of the root bark. This compound (0.5 to 0.7 μM) inhibited U373 (glioblastoma) and PC-3 (prostate cancer) cell viability (Ibrahim et al., 2015). Another study reported that chitinases isolated from the latex was cytotoxic against HCT-116 (colon carcinoma), OVCAR-8 (ovarian carcinoma), and SF-295 (glioblastoma) tumor cell lines (IC_{50}: 1.2 to 2.9 μg/ml) (Viana et al., 2017). Moreover, cardenolides isolated from the latex (calactin and calotoxin) exhibited higher inhibitory activity against A549 and Hela (cervix) tumor cells compared to the non-glycosidic derivatives (12β-hydroxycoroglaucigenin; afrogenin; procegenin A; and procegenin B). A preliminary structure-activity relationship among these cardenolids suggests that the double linked six-membered ring sugar moiety gives an additional cytotoxic effect (Ibrahim et al., 2015). Furthermore, the cancer pro-apoptotic effect of *C. procera* was attributed to the cardenolides, like 2"-oxovoruscharin, that showed potential in inhibiting Na^+/K^+ ATPase (Juncker et al., 2009; Van Quaquebeke et al., 2005).

In addition, treatment with a new protein from *C. procera*, matched with apolipoprotein A-I protein, can suppress tumor growth in breast cancer (MCF-7 and MDA-MB-231 cells) by suppressing the NF-kB pathway (Samy et al., 2012). NFκB is an important transcription-factor of five subunits, in which its dimers are inactive in the cytoplasm. When cells are stimulated by external signals (e.g. TNFα, IL-1, lipopolysaccharide, and radiation), NFκB is activated and enters in the nucleus to bind to target genes, such as products involved in cell proliferation (e.g. cyclin D1), and anti-apoptosis (e.g. Bcl-2) (Xia et al., 2018). Thus, inhibition of NF-kB activation by *C. procera* may account for its antiproliferative, pro-apoptotic and chemosensitizing properties (Samy et al., 2012).

In an *in vivo* study, proteins extracted from *C. procera* latex reduced tumor weight in mice bearing sarcoma 180 tumors after 7 days treatment with 2 or 5 mg/kg in a dose-dependent manner; these effects were similar to those exerted by the chemotherapeutic drug-5-fluorouracil (5-FU) (10 mg/kg). Moreover, the anti-tumor activity of *C. procera* were maintained after heat treatment, but were lost after proteolysis, acidic treatment, or iodoacetamide treatment (Oliveira et al., 2010).

In another study, the aqueous suspension of *C. procera* latex fed to X15-myc transgenic mice with hepatocellular carcinoma for 15 weeks (400 mg/kg) protected animals from the malignant changes occurring in the liver, and decreased serum vascular endothelial growth factor (VEGF) levels (Choedon, 2006). Furthermore, a significant tumor weight reduction was observed in mice bearing sarcoma 180 tumor after 7 days of treatment with *C. procera* stem ethyl acetate and acetone extracts (250 mg/kg/day). These effects were similar to those exerted by 5-FU (50 mg/kg/day). In contrast, methanolic extract obtained from same *C. procera* stem and used under same experimental conditions, was not effective in delaying tumor growth (Magalhães et al., 2010).

Although the *in vivo* results supported the *in vitro* findings, and we assume that the extracts will present the same activity in humans as in mice, the result obtained is therapeutically irrelevant anyway. Since for an extract to be considered potential for pharmacological studies *in vivo*, the dose range between 100-200 mg/kg should be considered as being the upper limit (Heinrich et al., 2020; Izzo et al., 2020). Thus, further investigation is necessary to establish a complete pharmacological profile, in order to verify the exact mechanisms involved in the antitumor effect, and the establishment of lower therapeutic doses.

3.4.2. Antioxidant activity

The antioxidant activities of *C. procera* extracts were based on assays assessing the radical scavenging abilities of phytochemicals such as the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), superoxide radicals, hydroxyl radicals, lipid peroxidation, metal chelating assays etc. However, the results obtained from these studies present several pitfalls (Tan et al., 2020). For example, the *in vitro* studies revealed that aqueous extract from *C. procera* roots showed substantial and dose-dependent free radical scavenging activity and metal ion chelating activity (Kumar et al., 2013). As the study used high doses ranging from 1000 to 20000 μg/ml, data collected from their studies can't be translated into physiological conditions, and they were no relevant for further investigation.

An investigation showed the ethanolic extract of *C. procera* leaves, flowers, and fruits (200 and 400 mg/kg/bw) containing phenolics compounds (e.g. lignin and flavonoids) reduced thiobarbituric acid reactive substances (TBARS) and upregulated glutathione (GSH) levels in gastric tissues of albino rats after ethanol-induced gastric ulcer (Al-Taweel et al., 2017). Although the results are promising they have not been compared to the reference drug-ranitidine (50 mg/kg/bw) used in this study. In contrast to the phytochemicals extracted from *C. procera* roots, the methanolic extract of *C. procera* latex (50 and 150 mg/kg/bw) containing asclepin, calactinic acid, calotoxin, calotropagenin, calotropin, coroglaucigenin, procesterol, uscharidin and uscharin (Table 1), was effective as the drug mesalazine (300 mg/kg/bw), reducing TBARS and increasing GSH levels and superoxide dismutase (SOD) activity in colitis induced rats with acetic acid (Kumar et al., 2019).

Other study refers to a carrageenan-induced paw inflammation model, were it was found that the high molecular weight protein fraction of *C. procera* latex (5 and 25 mg/kg/bw) normalized the levels of oxidative stress markers (GSH and TBARS) and myeloperoxidase (MPO), similarly to diclofenac (5 mg/kg/bw) (Chaudhary et al., 2015). However, *C. procera* was administered intravenously, while diclofenac was orally, which does not make treatments fully comparable.

Other research model to assess the antioxidant enzymes modulatory properties of *C. procera*, was the Freund's complete adjuvant induced monoarthritis reported by Kumar and Roy (2007). In this study, *C. procera* methanolic extract of latex (50 and 500 mg/kg/bw) produced a dose-dependent increase in the level of GSH, SOD, glutathione peroxidase (GPx), and catalase (CAT); and decrease in TBARS levels. Although *C. procera* offered general protection, the anti-inflammatory drug rofecoxib (20 and 100 mg/kg/bw) was more pronounced than the extract (Kumar and Roy, 2007). Otherwise, in alloxan-induced diabetes in rats, *C. procera* latex (100 and 400 mg/kg/bw) produced an increase in the hepatic levels of endogenous antioxidants (SOD, CAT and GSH), comparable to the standard drug-glibenclamide (10 mg/kg/bw) (Roy et al., 2005). Possibly, the antioxidant effect attributed to *C. procera* latex was due to the presence of cardinolides, lignans and flavanol glycosides (Roy et al., 2005).

It is quite possible that the aerial parts of *C. procera* exhibit the antioxidant protective effect due to the presence of abundance of latex and its phytoconstituents, including asclepin, calactinic acid, calotoxin, calotropagenin, calotropin, coroglaucigenin, procesterol, uscharidin and uscharin (Awaad et al., 2018; Kumar et al., 2019). Furthermore, compounds found in *C. procera*, such as quercetin, luteolin and kaempferol, have demonstrated a potential to activate nuclear erythroid 2-related factor 2 (Nrf2) (Pallauf et al., 2017). This transcription factor is normally present in the cytoplasm, associated with Kelch-like ECH-associated protein 1 (Keap1) and is rapidly degraded by the ubiquitin-proteasome pathway. However, under oxidative stress, phosphorylation of Nrf2 leads to its dissociation from Keap1 and subsequent translocation to the nucleus, where it binds to the antioxidant response element (ARE) and improves the transcription of genes that mount strong antioxidant and cytoprotective responses, including GPx and others antioxidant enzymes (Jadeja et al., 2016). Although the activity of Nrf2 is increased in the presence of these compounds, there is no obvious indication between chemical structure and induction of Nrf2 (Pallauf et al., 2017).

Although *C. procera* extracts appear to be counteracted oxidative stress and lipid peroxidation, especially due to improved antioxidant defense, reliability of the results, the therapeutic benefits and the mechanism of this assay is still unknown.

3.4.3. Anti-Inflammatory

Previous studies have described pro- and anti-inflammatory activities displayed by the latex from *C. procera* (Abdel-Mageed et al., 2016; Alencar et al., 2004; Sehgal and Kumar, 2005; Shivkar and Kumar, 2003). However, these antagonistic activities were reported for the whole latex and varied according to the solvent used to extract the bioactive compounds and the route of administration in experimental animals. Scientific evidence indicates that *C. procera* latex contains molecules of different molecular weight, which can be fractionated by a simple protocol based on centrifugation and dialysis (Alencar et al., 2006). The low molecular weight molecules have been involved in pro-inflammatory effects (Kumar, 2006; Kumar and Sehgal, 2007; Ramos et al., 2007; Seddek

et al., 2009); while those with a greater molecular weight have been associated with anti-inflammatory effects (Freitas et al., 2012).

A study demonstrated that the crude and methanolic extracts from *C. procera* latex (50 and 500 mg/kg/bw) decreased the levels of histamine, serotonin, prostaglandin E2 (PGE2), and bradykinin, similarly to standard drugs-phenylbutazone (100 mg/kg/bw), chlorpheniramine (3 mg/kg/bw), cyproheptadine (3 mg/kg/bw), and bradyzide (0.5 and 1.0 µmol/kg/bw), in a model of carrageenan induced-paw oedema (Arya and Kumar, 2005). The histological analysis revealed a significant decrease in the number of cellular infiltrates in the paw tissues, especially in animals treated with crude extract (Arya and Kumar, 2005). However, these findings were found just for the highest concentration of extract (500 mg/kg/bw). Furthermore, no phytochemical analysis was carried out in order to correlate the chemical-biological effects.

The latex proteins extracted from *C. procera* by dialysis also exerted anti-inflammatory activities as demonstrated when inhibited the carrageenin-induced neutrophil migration *in vivo* (Alencar et al., 2004). Hence, paw edema in Wistar rats exacerbated by carrageenin, was severely inhibited (80%) when *C. procera* (100 mg/kg/bw) was administrated 30 min before inflammation-induction, and was almost completely suppressed after 4 hours (Alencar et al., 2004). However, the same latex fraction was unable to control the paw edema induced with dextran stimulation, and the study was done with one single dose experiment in comparison with drug-mesna (40 mg/kg/bw).

In the study conducted by Lima-Filho et al. (2010), Swiss mice received a single dose of *C. procera* latex proteins (30 or 60 mg/kg/bw) by the intraperitoneal (i.p.) route, 24 h before or after lethal challenge containing 10^6 CFU/ml of *Salmonella enterica*. *C. procera* (60 mg/kg/bw) promoted a discretely reduction in IL-12 serum levels, but there was no difference in TNF-α, when administered before inoculation of S. *Typhimurium*. Histopathological analysis of the spleen and liver that were given *C. procera* revealed tissue necrosis and inflammatory infiltrates on the same way as the control group, not being effective in reducing *S. enterica*. However, bacteria population was drastically reduced in blood. In addition, animals receiving the latex 24 h after bacteria inoculums

did not resist and mortality rate was documented to be similar to the control group (Lima-Filho et al., 2010). Other study with very similar experimental conditions (Swiss mice; 10^6 CFU/ml of *Salmonella enterica*; single dose of *C. procera* 24 h before the challenge; i.p. route) showed a recruitment of neutrophils into the peritoneal cavity after treatment with *C. procera* latex proteins (60 mg/kg/bw). Besides that, early tumor necrosis factor alpha (TNF-α) mRNA expression was observed at 4 h post-infection in the *C. procera* group, but interferon gamma (IFN-ϒ) was not observed at this early time point. In addition, animals that received *C. procera* maintained 100% survival, in which it was accompanied by high bacterial clearance from the bloodstream (Oliveira et al., 2012). Despite the experimental conditions of the two studies being very close, the *C. procera* latex proteins induced different mechanisms. This difference can probably be attributed to the difference during protein preparation, since Oliveira et al. (2012) performed chromatography and isolated only one of the protein peaks to perform the treatment, whereas Lima-Filho et al. (2010) used all the soluble proteins.

Some authors have sought a better understanding of the anti-inflammatory mechanism of *C. procera*. According to Kumar and Roy (2007) the mechanisms exerted by compounds extracted from *C. procera* latex with methanol (50 and 500 mg/kg/bw) are mediated, at least in part, by a decrease in prostaglandin E2 (PGE_2), TNF, MPO, and nitric oxide (NO) in a dose-dependent manner. These changes improved the joint arthritic in a Freund's complete adjuvant-induced monoarthritis rat, similarly to standard anti-inflammatory drug rofecoxib (20 and 100 mg/kg/bw) (Kumar and Roy, 2007).

Likewise, soluble proteins obtained from *C. procera* latex (5 mg/kg/bw, i.p.) decreased levels of TNF-α, interleukin 1 beta (IL-1β), nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) on inflamed conjunctive and epithelial tissue in a mucositis-induced mucositis hamsters model (Freitas et al., 2012). However, the study was performed only with a single dose experiment, and lacking a positive control which is unable to analyze the dose-effects relationship of the plant extract.

Other report suggested that anti-inflammatory activities of compounds extracted from *C. procera* are not related to their polarity. Thus, anti-inflammatory effect in carrageenan-induced peritonitis of fractions extracted from *C. procera* crude latex

partitioned within 49.4% hexane, 5.2% dichloromethane, 2.0% ethyl acetate, 2.1% n-butanol, and 41.1% aqueous. Results showed that activity in reducing neutrophil migration in rats was in the following order of potency: aqueous fractions (72%) > dichloromethane soluble fraction (67%) > ethyl acetate soluble fraction (56%), whereas both hexane and n-butanol fractions did not inhibit neutrophil migration. Triterpenes were detected in both hexane and dichloromethane fractions, while flavonoids were detected in the dichloromethane and ethyl acetate fractions (Jucá et al., 2013). Although this study was one of the only ones to correlate the phytochemical composition with the anti-inflammatory activity, there is still a lack of positive control for comparison.

Notwithstanding the anti-inflammatory activity of *C. procera* supports its use in traditional medicine, there is still a lack of understanding of the mechanisms involved in this process and, mainly, which are the compounds responsible for this activity.

3.4.4. Hypoglycemic activity

In *in vitro* assay, the ethanolic and aqueous extracts of *C. procera* showed to act as inhibitors of α-amylase (IC_{50}: 7.80 mg/mL) and α-glucosidase (IC_{50}: 3.25 mg/mL), which was attributed to the presence of some phytochemicals such as flavonoids, tannins and saponins (Kazeem et al., 2014). However, in this research there was no comparison with a standard antidiabetic drug, and the authors did not make it clear how many replicates were performed, leaving the reproducibility of the experiment questionable.

In alloxan-induced diabetic rats, treatment with *C. procera* latex (100 and 400 mg/kg/bw), for 31 days, reduced glycemic and increased the hepatic glycogen, in a dose-dependent manner, similarly to standard drug glibenclamide (10 mg/kg) (Roy et al., 2005). In the study conducted by Yadav et al. (2014), *C. procera* roots, stem and leaves (100 and 250 mg/kg) sequentially extracted with petroleum ether, chloroform, ethyl acetate, and methanol were tested in streptozotocin-induced diabetic rats for 31 days. Results showed that the root methanolic extract at 100 mg/kg/bw/day was the best treatment for promoting β-cell regeneration in pancreas, and improving plasma insulin levels (Yadav et al., 2014).

The intravenous (i.v.) administration of *C. procera* latex proteins (5 mg/kg/bw) also promoted reduction in glucose, and these effects seems to be related to decreased glucose synthesis from pyruvate, since there was a decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels (de Oliveira et al., 2019). *C. procera* also promoted the activation of 5'AMP-activated protein kinase (AMPK), which induces suppression of glucose-6-phosfatase and PEPCK gene expression, both of which are target genes for gluconeogenesis (de Oliveira et al., 2019).

In contrast, *C. procera* leaves methanolic extract (100 and 250 mg/kg/bw), did not exert hypoglycemic effect after 2h of treatment (Rahmatullah et al., 2010). Moreover, intravenous administration of *C. procera* latex proteins (5 mg/kg/bw) did not change insulin secretion (de Oliveira et al., 2019). The inconsistencies regarding the hypoglycemic effects of extracts obtained from *C. procera* may be related to the length of intake and the part of the plant used to extract the bioactive components (e.g. leaves vs roots). Therefore, further studies should be conducted to better understand and validate the hypoglycemic effect. In addition, further studies on the phytochemical characterization of the possible compounds related to this effect should be carrying out.

3.4.5. Gastric protector

C. procera leaves, flowers, and fruits (200 and 400 mg/ml) were administrated in Sprague-Dawley rats with ulcers. *C. procera* leaf was the most efficient in reducing gastric secretion, titratable acidity, gastric lesion index in pylorus, intraluminal bleeding score, and gastric lesion ulcer index after 6 h treatment (Al-Taweel et al., 2017). However, only the highest concentration (400 mg/ml) showed promising results, yet it does not match the standard drug-ranitidine (50 mg/ml).

The *C. procera* stem bark chloroform and hydroalcoholic extracts (200 and 400 mg/kg/bw/day) showed potent anti-inflammatory and gastric protector activities in albino rats when administered 30 min before aspirin and ethanol induced ulcer (Tour and Talele, 2011). Moreover, the aqueous suspension of *C. procera* latex (20 and 100 mg/kg) was associated with marked reduction in gastric hemorrhage, maintenance of tissue integrity

and normalization of levels of oxidative stress markers in rats induced ulcer with ethanol, pyloric ligation and aspirin (Bharti et al., 2010).

Another study reported that alcoholic, polar and non-polar fractions of *C. procera* aerial parts (200 and 400 mg/kg/bw/day) exerted a dose-dependent anti-ulcerative colitis activity in Wistar rats induced colitis with acetic acid after 5 days of treatment. The anti-ulcerative colitis activity was characterized by improved inflammation, decreased lesion score and ulcer area (Awaad et al., 2018). Similarly, the *C. procera* root extract (5, 15 and 25 mg/kg) significantly inhibit arachidonic acid metabolism, through the inhibition of 5-lipoxygenase, consequently decreasing inflammation and improving gastric parameters (Sen et al., 1998). However, there is a lack of a standard drug for comparison of results and better investigation of the mechanisms involved.

Although the gastroprotective effect of *C. procera* has been attributed to extract active principles, such as flavonoids, triterpenoides, tannins, coumarins (Tour and Talele, 2011; Awaad et al., 2018), steroids, and saponins content (Tour and Talele, 2011), there is still a need for further studies to investigate the structure-function relationship. In addition, there are still many gaps in the gastric protection mechanisms carried out by *C. procera*.

3.4.6. Antimocrobial

C. procera extracts have been tested as anti-microbial compounds since late 1980s through the measurement growth inhibition zone diameters (Mascolo et al., 1988). Different solvents were used to obtain the corresponding extracts from the distinct plant parts. Overall, results indicated that *C. procera* extracts have potential in inhibiting the growth of several bacterial strains, especially *Staphylococcus aureus* (Kareem et al., 2008; Mascolo et al., 1988; Samy et al., 2012), *S. epidermidis, Pseudomonas aeruginosal*, *Salmonella typhi* H. (Mascolo et al., 1988; Perumal Samy and Chow, 2012), and *Escherichia coli* (Akhtar et al., 1992; Mohamed et al., 2014; Perumal Samy and Chow, 2012). Even more, these extracts also showed antifungal activity against *Candida albicans* (Kareem et al., 2008; Mohamed et al., 2014), *Trichophyton* spp.(Aliyu et al., 2015; Mohamed et al., 2014), *Aspergillus terreus* (Mohamed et al., 2014), *Macrophomina*

phaseolina (Waheed et al., 2016), *Microsporum* spp. (Aliyu et al., 2015), *Fusarium oxysporum* and *Colletotrichum gloeosporioides* (Freitas et al., 2016a).

It should be highlighted that the antimicrobial effect of *C. procera* extracts results from a mixture of coumpounds, including flavonoids (like quercetin, kaempferol and isorhamnetin) (Nenaah, 2013), fatty acid ethyl ester, palmitic acid ester, linoleic acid and amino acid (Pattnaik et al., 2017). Some proteins are also involved in the role of *C. procera* against microorganisms, such as osmotin (De Freitas et al., 2011) and chitinase (Freitas et al., 2016b).

Although *in vitro* studies have demonstrated the great potential of *C. procera* crude extracts and some secondary metabolites as antimicrobials, *in vivo* studies are scarce, and therefore, necessary to confirm these findings.

3.4.7. Insecticide

It has been estimated that approximately $38 billion are spent on pesticides per year, and only about 0.1% of pesticides reach their targets. The remaining pesticides contaminate the surrounding environment, contributing to acute and chronic human diseases (Kaur and Garg, 2014). This situation has led to the search for eco-friendly pesticides (Al-Sarar et al., 2012). *In vitro* investigation of the insecticidal effect of benzenic and methanolic extract from *C. procera* latex showed that benzene extract exerted a higher toxicity against *Monacha cantiana* land mollusks. In this case, the dose range required to achieve a mortality of 13.3 to 96.7% was from 10 to 20 mg/kg, with a median lethal dose (LD_{50}) value of 12.62 mg/kg (Al-Sarar et al., 2012).

C. procera leaves and fruits aqueous solution (concentration of 1:1, 1:2, 1:3 w/v of solvent) exerted a higher toxicity against *Coptotermes heimi* compared to *Heterotermes indicates* after 10 days of feeding. Also, *C. procera* flower extracts caused more mortality than the leaves extracts for *H. indicates* (21.3% of leaves and 45.3% for flowers), suggesting that toxic compounds are more concentrated in the flowers (Badshah et al., 2004).

The aqueous extract of *C. procera* leaves (100-1000 ppm) showed high level of toxicity against 2nd, 3rd and 4th instar larvae of mosquitoes *Anopheles arabiensis* and *Culex quinquefasciatus* 24 h post-treatment (Elimam et al., 2009). Moreover, the methanolic extract of *C. procera* leaves showed to be less toxic than its latex when tested in both *An. arabiensis* and *Cx. quinquefasciatus* species (LC_{50}: 109.71 and 387.93 mg/L, respectively) (Shahi et al., 2010). In addition, *C. procera* latex collected into distilled water 1:1 (v/v) induced 100% mortality of 3rd instar larvae of *Aedes aegypti* within five minutes, and most of individual growing under experimental conditions died before reaching 2nd instars or stayed in 1st instars (Ramos et al., 2006).

Polyherbal spray containing a mix of *C. procera* (1%) and other plant species [*Andropogon citrates* (3%), *Cymbopogon citratus* (3%), *Ocimum sanctum* (2%), *Pinus longifoia* (10%) and 1% each of *Datura stramonium*, *Aegle marmelos*, *Ricinus communis*, *A. indica*, *Allium sativum*, *Carica papaya*, *A. squamosa* and *Pongamia glabra*] exerted a potent acaricide effect in tick-infested cattle. Single application of the spray decreased by over 70% the average tick count from 3 to 21 days after treatment (Bhikane et al., 2018). It was also reported that *C. procera* whole-plant extracts reduced the egg laying index by over 95% and inhibited adult female tick oviposition in 35% when assessed by the adult immersion test at 40 mg/ml (Khan et al., 2019).

In general, *C. procera* has shown good potential as an insecticide. However, studies that evaluate the possible secondary metabolites related to this activity are still scarce, and the mechanisms behind it are still not well understood.

3.4.8. Potential application in the food industry

C. procera latex has been described as rich in proteolytic enzymes and with a higher potency than chymosin and that j-casein, which are already widely used in the food industry (Rayanatou et al., 2017). The latex fractions of *C. procera* exhibited dose-dependent milk-clotting activity up to 50–60 μg to 2 ml of milk, similar to *Carica papaya* latex fraction and papain (cysteine peptidases used as controls). However, doses higher than 60 μg were not effective in improving milk-clotting activity (Freitas et al., 2016a).

The proteolytic activity of *C. procera* latex was assessed with 9 and 40 mg of latex peptidases were added to 500 ml of milk and coagulation after 7 and 17 min at 25°C. Results showed that yield, dry mass, and soluble proteins were similar to those obtained with commercial chemosin (Freitas et al., 2016a). Moreover, *C. procera* latex peptidases hydrolyzed the caseins extensively, even at the lowest concentration evaluated (20 µg of latex per 4.5 mg casein; 1:225 ratio), and at similar levels than the peptidase bromelain, used as the positive control (Oliveira et al., 2019).

The proteolytic activity of *C. procera* latex extracted proteins were shared by at least four distinct cysteine proteinases. However, aspartic proteinase activities were barely detected, while serine and metalloproteinases were not (Soares de Oliveira et al., 2007). Another study indicates that the proteolytic effect of *C. procera* latex can be attributed to the presence of procerain and procerain B, as well as to 3 new other peptidases isolated, named CpCP-1, CpCP-2 and CpCP-3, which all shared common catalytic properties with cysteine peptidases (Ramos et al., 2013).

In addition to its application for curd production (Issa Ado et al., 2018; Rayanatou et al., 2017), *C. procera* latex proteins have also shown potential as meat tenderizing (Rawdkuen et al., 2013). Finally, the use of *C. procera* latex peptidases in food industry seems to be a safe alternative because of their low immunogenicity as demonstrated in *in vitro* and *in vivo* studies (Oliveira et al., 2019).

3.4.9. Other activities

Other activities have already been described for *C. procera*, and they are summarized in table 2. Briefly, *C. procera* latex has been widely studied and has shown several promising activities *in vivo*, such as myocardial protection (Mueen Ahmed et al., 2004), analgesic (Dewan et al., 2000), anticonvulsant (Lima et al., 2012), antinociceptive (Soares et al., 2005), healing (Aderounmu et al., 2013; Rasik et al., 1999), hepatoprotective (Padhy et al., 2007) and anti-diarrhea (Kumar et al., 2001). The leaves and flowers of *C. procera* have also exhibited anthelmintic effects (Aggarwal et al., 2016;

Chauhan et al., 2017; Seif el-Din et al., 2014), anti-depressant (Garabadu et al., 2019) and hepatoprotective (Ramachandra Setty et al., 2007).

3.5. Toxicity of *Calotropis procera*

Several studies have investigated about the safety of *C. procera* extracts. It was demonstrated that the chloroformic extract of *C. procera* wax up to 3 μg/ml was not toxic to mouse skin fibroblast (M5S) cells. Similarly, the wax extracted with acetone was not toxic to M5S cells when used at 1 μg/ml based on lactato desidrogenase (LDH) assay. However, the cytotoxicity increased to 57.2% when cells were treated with 3 μg/ml (Sharma et al., 2019).

Other research reported that *C. procera* latex (66 and 132 μl/kg) and ethanolic extract from leaves (4.78 and 9.56 mg/kg) fed to male albino rats for 4 and 8 weeks increased creatinine kinase-MB (CK-MB) and LDH activities in serum and reduced hormonal parameters [testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH)] (Ahmed et al., 2016). Based on this study and the fact that elevated CK-MB activity may be related to cardiac injuries (Maghamiour and Safaie, 2014), whereas hormonal changes may compromise fertility (Rehman et al., 2018), it is evident that *C. procera* may have toxic effects.

The exposure to the single dose of *C. procera* leaves (30 and 60 g/kg) was responsible for tachycardia and transient cardiac arrhythmias in sheep. While the treatment with 60 g/kg/day for 10 days promoted mild ascites, exudates on the trachea, pulmonary edema, mild hemorrhage in the liver, hydropericardium, flaccid heart, ulcers on the abomasum and kidneys presenting pale juxtamedullary cortex (de Lima et al., 2011). Moreover, healthy rats treated with *C. procera* aqueous extract (25, 50 and 75 mg/kg daily) promoted an increase in the erythrocyte parameters, and a disruption in blood electrolytes (Ajagbonna et al., 1999).

Studies evaluating the safety of ethanolic extracts from *C. procera* leaves revealed that no visible toxic symptoms or mortality were observed with a single high dose of up to 3 g/kg. However, upon chronic treatment (100 mg/kg) for 90 days, an animal mortality

rate of 50% was recorded (Mossa et al., 1991). Toxicity of *C. procera* latex was also assessed in Swiss mice via oral (5000 and 2000 mg/kg/bw) and i.p (300, 150, 75 and 15 mg/kg/bw) route. Results showed that the orally treated animals presented light behavioral changes within the first 24 h (e.g. hypothermia and abdominal contortions), but all animals appeared as healthy as the control group day 14 after treatment. In contrast, the i.p. administration of latex caused animal death in a dose-dependent manner, of which 300 mg/kg/bw provoked 100% death within 30 min (Bezerra et al., 2017).

Furthermore, *C. procera* latex administered at 0.3 ml/kg/day intra vaginally in pregnant and non-pregnant goats for 14 days induced the production of mucus at vulva and plugs, pain, and maternal- and fetal-toxicity (El Badwi and Bakhiet, 2010). The toxicity was correlated with higher AST and alkaline phosphatase activities, as well as higher concentrations of creatinine, globulin, urea and progesterone. In non-pregnant goats, *C. procera* treatment promoted vaginitis and anorexia (El Badwi and Bakhiet, 2010). A potent anti-implantation activity was demonstrated in proven fertile female Wistar rats treated with the ethanolic extract of *C. procera* roots (250 mg/kg) for 7 consecutive days (Kamath and Rana, 2002). These studies demonstrated that *C. procera* latex can promote infertility.

Treatment of Nubian goats with *C. procera* latex (0.2, 0.4 and 0.8 ml/day) for 7 days did not promote any clinical signs of intoxication or changes in plasma protein levels. However, on day 7 there was a significant increase in AST activity, liver fat accumulation, and kidney congestion at the glomerular junction (El Sheikh et al., 1991). Toxicity seems to be exercised by some organic solvents since the aqueous suspension of *C. procera* latex did not produce any toxicity when fed to rats for 45 days (10, 100 and 400 mg/kg/day) (Singhal and Kumar, 2009). *C. procera* latex proteins obtained by dialysis did not exert any toxicity or death to Wistar rats when administered for 35 days as daily doses of 1.5 mg/ml (Ramos et al., 2006).

Human eye exposure to *C. procera* latex promoted transient corneal edema, but completely disappeared within 2 weeks, despite a reduction in endothelial cell count and abnormal morphology. These clinical manifestations suggest that latex is relatively non-toxic to the corneal epithelium and highly toxic to the corneal endothelium (Al-Mezaine

et al., 2008). Another research reported that *C. procera* latex caused sudden vision darkness with photophobia, conjunctival congestion, and mild to severe corneal edema. Some of them had epithelial defect, iridocyclitis and associated secondary glaucoma. However, eyes recovered completely within 3-14 days treatment with topical corticosteroids or similar medications (Basak et al., 2009; Waikar and Srivastava, 2015).

It has been shown that some heavy metals, such as lead (Pb), can accumulate in the leaves of *C. procera*, and this concentration can vary throughout the year (Khalid et al., 2018). Since it has already been demonstrated that Pb presents toxicity to the environment and to living organisms (Wani et al., 2015), it is suggested that the accumulation of Pb in *C. procera* may be involved in the cytotoxic effects already reported.

Overall, these studies strongly indicate that toxicity of *C. procera* phytochemicals extracted with different solvents is specific to the extraction method and to the receiver organism/body tissue or organ where it is applied.

3.6. Conclusions and future perspective

Calotropis procera is an important species in traditional medicine and, consequently, a number of pharmacological studies have been performed. However, there are many gaps to be resolved, specially related to the phytochemical profile and the secondary metabolites responsible for given biological effect. In addition, the mechanisms of action of *C. procera* demonstrated *in vitro* and *in vivo* are not yet fully understood. Furthermore, no clinical trial has been conducted with this plant, requiring an understanding of the mechanisms of absorption, distribution, metabolism and excretion. Finally, it is worth highlighting the toxic potential of the plant and the need for the development of extraction methods using environmentally friendly solvents to optimize the isolation of bioactive compounds. This should be accompanied by studies aimed to provide dose-safety recommendations to ensure the health benefits without toxicity to the target tissues or organisms.

Figure

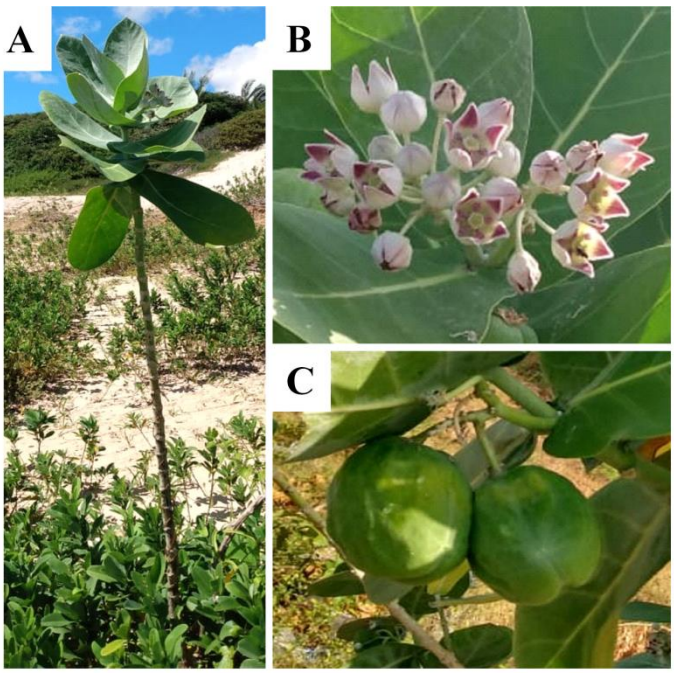

Figure 1: Details of *Calotropis procera*. **A:** the plant is found in a shrub form; **B:** the flowers are fleshy and variable in color from white to pink, often spotted or tinged with purple. **C:** fruits with inflated, obliquely ovoid follicles.

Tables

Part Used	Extract	Compound	Compound class	References
Flowers	Ethanolic	7'-methoxy-3'-*O*-demethyl-tanegool-9-*O*-β-D-glucopyranoside	Lignan	Al-Taweel et al., 2017
		Methyl ferulate	Hydroxycinnamic acid	
		Rosmarinic acid	Caffeic acid ester	
		Methyl rosmarinate	Coumaric acids	
		Methyl 4-β-D-glucopyranosyl-ferulate	Ferulic acid ester	
		Pinoresinol 4-*O*-glucoside	Lignan	
Fruits	Ethanolic	Favonoid glycoside kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	Flavonoid glycoside	Al-Taweel et al., 2017
		Syringaresinol 4-*O*-glucoside	Lignan	
Aerial parts	Ethanolic	Ischarin	Cardenolide	Sweidan and Zarga, 2015
		Ischaridin	Cardenolide	
		Uzarigenin	Cardenolide	
		Calotropin	Cardenolide	
		Calactin	Steroid glycoside	
		Calotoxin	Steroid glycoside	
		β-sitosterol glucoside	Phytosterol	
		19-dihydrocalotropagenin	Cardenolide	

		3'-*O*-methyl quercetin-3-*O*-rutinoside	Flavonoid glycoside	
		Calotropenyl acetate	Triterpenoid	
		β-sitosterol	Phytosterol	
		Uscharin	Cardenolide	
Aerial parts	Methanolic	Proceragenin	Cardenolide	Akhtar et al., 1992
Leaves	Ethanolic	β-sitosterol	Phytosterol	Al-Taweel et al., 2017
		Lupeol	Triterpenoid	
		Lupeol-3-*O*-acetate	Triterpenoid	
		Calotropagenin	Steroid glycoside	
Leaves	Not described	2-propenyl-2'-hydroxyethyl carbonate	Organic carbonate	Olea et al., 2002
		Stigmasterol	Phytosterol	
		β-sitosterol	Phytosterol	
Leaves	Hydroethanolic	Calotropagenin	Steroid glycoside	Kanojiya and Madhusudanan, 2011
		Calotoxin	Steroid glycoside	
		Calactin	Steroid glycoside	
		Uscharidin	Steroid glycoside	
		Asclepin	Steroid glycoside	
		Isomers of calactinic acid methyl ester	Steroid glycoside	
Leaves	Ethanolic	L-glutamic acid	Amino acid	

Butane, 2,2-dimethyl	Saturated hydrocarbon	
1,2-Benzenedicarboxylic acid, dimethyl ester	Phthalate	
1-Dodecene	Alkene	
Heptane, 3,3-dimethyl	Alkane	
6-Octen-1-Ol, 3,7-dimethyl acetate	Monoterpenoid	
(+)-(1s,2's)-3-(2'-isopropylcyclopropyl)-1-propanol	Alcohol	
Per(trimethylsilyl)-D-fructose	Fructose derivative	
Hexadecanoic acid, methyl ester	Saturated fatty acid	
1,2,3,4-Tetrakis-O-(trimethylsilyl)pentopyranose	D-xylopyranose derivative	
Hexadecanoic acid, ethyl ester	Fatty acid ethyl esther	Verma et al., 2012
1-[(T-Butyl)dimethylsilylthio)butane	Hydrocarbon derivative	
1-Octanol	Alcohol	
9-Octadecenoic acid (Z)- methyl ester	Fatty acid methyl esther	
2-Hydroxyhexadecyl butanoate	Fatty acid butane esther	
9,12-Octadecadienoic acid (Z,Z)-	Unsaturated fatty acid	
Ethyl (9Z,12Z)-9,12-octadecadienoate	Unsaturated fatty esther	
Alpha-D-glucopyranoside, methyl 2,3,4-Tris-O- (trimethylsilyl)-, hexadecanoate	D-glucopyranoside derivative	
1,2,3,4-Tetrakis-O-(trimethylsilyl)pentopyranose	D-xylopyranose derivative	
Ethanamine, 2,2'-oxybis[N,N-dimethyl	Amino ether	

		1,3-Hexanediol, 2-ethyl	Aliphatic diol	
		2-Dodecanol, 1,1-dichloro	Halogenated fatty alcohol	
		1,2-Benzenedicarboxylic acid, diisooctyl	Phthalate	
		1-Octanol, 3,7-dimethyl	Aliphatic alcohol	
		Cyclohexanol, 3-methyl	Ciclic alcohol	
		(E,E)-4,8,12-Trimethyl-3,7,11-tridecatriene-1-Ol	fatty alcohol	
Leaves	Chloroformic	2-Tert-butyl-4-(1,1,3,3-tetramethylbutyl) phenol	Phenolic	Verma et al., 2012
		2-Methylene-1,5-pentanediol	Aliphatic diol	
		2,6,10-Trimethyl,14-ethylene-14-pentadecene	Neophytadiene	
		Bicyclo[4.1.0]heptane, 7-butyl	Terpenoid	
		3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Fatty alcohol	
		Nonanoic acid, 7-methyl methyl ester	Fatty acid metyl esther	
		Bis-(3,5,5-trimethylhexyl) ether	Aliphatic ether	
		Methyl 9-octadecenoate	Unsaturated esther	
		6-Octen-1-ol, 3,7-dimethyl	Monoterpenoid	
		(+)-(1r,2r)-2,7,7-Trimethyl-3-oxabicyclo[4.1.1.]Octan-4-one	Unsaturated lactone	
		6(e),9(z),13(e)-Pendectriene	Unsaturated hydrocarbon	
		Beta-L-galactopyranose, 6-deoxy-1,2,3,4-tetrakis-O- (trimethylsilyl)	L-galactopyranose derivative	
		D-xylopyranose, 1,2,3,4-tetrakis-o-(trimethylsilyl)	D-xylopyranose derivative	

		Bis-(3,5,5-trimethylhexyl) ether	Phthalate	
		1,2-Benzenedicarboxylic acid, diisooctyl ester	Phthalate	
		1-(2-Hydroxyethoxy)-pentadecane	Aliphatic hydrocarbon	
		2,6,10-Dodecatrienoic acid, 7,11-dimethyl-3- (trifluorpmethyl)-, methyl ester, (Z,Z)	Fatty acid mehyl esther	
Leaves	Methanolic	Quercetagetin-6-methyl ether 3-*O*-*β*-D-4C1-galacturonopyranoside	Flavonoid glycoside	Mohamed et al., 2011
		(*E*)-3-(4-methoxyphenyl-2-*O*-*β*-D-4C1-glucopyranoside)-methyl propenoate	Flavonoid glycoside	
		Isorhamnetin 3-*O*-*β*-D-rutinoside	Flavonoid glycoside	
		Isorhamnetin 3-*O*-*β*-D-robinoside	Flavonoid glycoside	
		Methyl caffeate	Caffeic acid esther	
		Caffeic acid	Phenolic acid	
		Isoquercitrin	Flavonoid	
		Quercetin	Flavonoid	
		Isorhamnetin	Flavonoid	
		Azaleatin	Methylated flavonol	
		3,3’-dimethoxy quercetin	Flavonoid	
		3,6,3’,4’-tetramethoxy quercetin	Flavonoid	
		3,6,7,3’,4’-pentamethoxy quercetin	Flavonoid	
Leaves	Methanolic	Quercetin	Flavonoid	Oraibi and Hamad, 2018
		Rutin	Flavonoid	

		Kaemprferol	Flavonoid	
Latex	Crude	Asclepin	Steroid glycoside	Kumar et al., 2019
		Calactinic acid	Cardenolide	
		Calotoxin	Steroid glycoside	
		Calotropagenin	Steroid glycoside	
		Calotropin	Cardenolide	
		Coroglaucigenin	Steroid glycoside	
		Procesterol	Steroid	
		Uscharidin	Steroid glycoside	
		Uscharin	Cardenolide	
Latex	Crude	Calotropin	Cardenolide	Seiber et al., 1982
		Calactin	Steroid glycoside	
		Uscharidin	Steroid glycoside	
		Uscharin calotoxin	Cardenolide glycoside	
		Voruscharin	Cardenolide glycoside	
Látex	Chloroformic	Calactin	Steroid glycoside	Mohamed et al., 2015
		15-β-hydroxy calactin	Steroid glycoside	
		Afroside	Steroid glycoside	
		Uscharin	Steroid glycoside	
		15β-hydroxy uscharin	Steroid glycoside	

		Calotoxin	Cardenolide	
		12β-hydroxycoroglaucigenin	Cardenolide glycoside	
		Afrogenin	Cardenolide	
		Calactoprocin	Cardenolide	
		Procegenin A	Cardenolide	
		Procegenin B	Cardenolide	
Latex	Ethyl acetate	urs-19(29)-en-3-yl acetate	Terpenoid	Cavalcante et al., 2016
		(3β)-Urs-19(29)-en-3-ol	Terpenoid	
		1-(2',5'-dimethoxyphenyl)-glycerol	Phenolic triol	
Latex	Ethanolic	(+)-pinoresinol 4-O-b-D-glucopyranoside	Lignan glycoside	Abdel-Mageed et al., 2016
		(+)-medioresinol 4-Ob-D-glucopyranoside (Eucommin A)	Lignan glycoside	
		(+)pinoresinol 4-O-[6"-O-vanillyl]-β-D-glucopyranoside	Lignan glycoside	
		pinoresinol-4'-O-[6"-O-(E)-feruloyl]-β-D-glucopyranoside	Lignan glycoside	
		(+)-pinoresinol 4-O-[6"-O-protocatechuoyl]-βD-glucopyranoside	Lignan glycoside	
Stems	Ethanolic	5-hydroxy-3,7-dimethoxyflavone-4'-*O*-β-glucopyranoside	Flavonoid glycoside	Shaker et al., 2010
		β-anhydroepidigitoxigenin	Cardenolide	
		Uzarigenine	Cardenolide	
		2β,19-epoxy-3b,14β-dihydroxy-19-methoxy-5α-card-20(22) enolide	Cardenolide	
		β-anhydroepidigitoxigenin-3β-*O*-glucopyranoside	Cardenolide glycoside	
Stems	Ethanolic	Uzarigenone	Cardenolide	

		Uzarigenine	Cardenolide	Elgamal et al., 1999
		Deglucouzarin	Cardenolide	
		Frugoside	Cardenolide	
Root barks	Methanolic	12-*O*-benzoylisolineolon	Steroid glycoside	Ibrahim et al., 2015
Root barks	Methanolic	2''-oxovoruscharin	Cardenolide	Quaquebeke et al., 2005
Roots	Methanolic	Calotropin	Cardenolide	Van Quaquebeke et al., 2005
		Calactin	Steroid glycoside	
		Uscharin	Steroid glycoside	
		Voruscharin	Steroid glycoside	
		2″-oxovoruscharin	Cardenolide	

Table 1: Main constituents found in different parts of *Calotropis procera.*

Activity	Plant parts	Extract type	Dose range	Model/Assay	References
Anthelmintic	Leaves	Crude	0.4 and 0.6%	*in vivo Eimeria tenella, E. necatrix, E. acervulina, E. maxima, and E. mitis*	Chauhan et al., 2017
Anti-depressant	Leaves	Ethanolic	50, 100 and 200 mg/kg	*in vivo* chronic unpredictable mild stress	Garabadu et al., 2019
Hepatoprotective	Flowers	Hydroethanolic	200 and 400 mg/kg	*in vivo* paracetamol-induced hepatic damage	Ramachandra Setty et al., 2007
Anthelmintic	Flowers	Ethanolic and aqueous	12.05 and 23.52 mg/ml	*in vitro Gastrothylax indicus*	Aggarwal et al., 2016
Anthelmintic	Latex and flowers	Aqueous	250 mg/kg/bw	*in vivo Schistosoma mansoni*	Seif el-Din et al., 2014
Myocardial protection	Latex	Ethanolic	300 mg/kg/bw	*in vivo* isoproterenol -induced heart damage	Mueen Ahmed et al., 2004
Analgesic	Latex	Crude	165 to 830 mg:kg	*in vivo* acetic acid-induced writhings	Dewan et al., 2000
Anticonvulsant	Latex	Crude	50 and 100 mg/kg	*in vivo* pentylenetetrazol-, pilocarpine-, and strychnine-induced convulsions	Lima et al., 2012
Antinociceptive	Latex	Crude	12.5, 25 and 50 mg/kg	*in vivo* acetic acid-induced writhings	Soares et al., 2005
Healing and Anti-keloid	Latex	Crude	2 ml	*in vivo* excisional wounds	Aderounmu et al., 2013
Healing	Latex	Crude	20 µl	*in vivo* excisional wounds	Rasik et al., 1999
Hepatoprotective	Latex	Crude	5, 50 and 100 mg/kg	*in vivo* tetrachloromethane-induced hepatitis	Padhy et al., 2007
Anti-diarrhoeal	Latex	Crude	1, 5, and 50 mg/kg	*in vivo* irinotecan-induced diarrhoea	de Alencar et al., 2017
Anti-diarrhoeal	Latex	Crude	500 mg/kg	*in vivo* castor oil induced diarrhoea	Kumar et al., 2001

Table 2: Pharmacological activities of *C. procera* extracts.

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4. ARTICLE II - *Calotropis procera* as an anti-cancer agent against osteosarcoma and mammary carcinoma canine cells

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Abstract: Cancer is a disease with high social impact, and although great strides have been made in the treatment and control of disease progression, many points still require further studies and assessments. Efforts are being made to identify natural anticarcinogens that prevent, delay and/or reverse cancer induction and subsequent developments. The anticancer properties of plants have been recognized for centuries, and a variety of bioactive compounds and their derivatives have shown inhibition of carcinogenesis in various experimental systems. Our goal was to carry out phytochemical characterization and evaluate the antitumor potential of *Calotropis procera.* The phytochemical constitution of the crude extract (CE) was performed by mass spectrometry, revealing the presence of flavonoids, glycosides and cardenolide. The MTT assay was used to evaluate the cytotoxicity of CE, methanolic (MF) and ethyl acetate fractions (EAF) of *C. procera* in osteosarcoma canine cells (OST), canine mammary tumor (CMT), and canine fibroblasts (non-tumor cell). In addition to the extracts, doxorubicin was also used as a positive control. Our results showed that the CE, MF and EAF promoted a decrease in the viability of OST and CMT cells and did not alter the viability of the fibroblasts, showing cellular selectivity. There was also a decrease in the number of cells after the treatments, corroborating to the decrease in proliferation and the cell cycle arrest in the G0/G1 phase. It was also evaluated the cell morphology by light and fluorescence microscopy, where it was verified a change in the shape and size, being characterized by cytoplasmic decrease and

cell rounding. Moreover, flow cytometry data demonstrated that CE treatment promoted increase of caspase-3 and p53, showing that the apoptotic process was activated in OST cells. In addition, there was a decrease in CD31, VEGF, osteopontin and TGF-β after CE treatment, suggesting that angiogenesis and epithelial-mesenchymal transition process were softened in OST cells. Moreover, CMT cells showed a reduction in PCNA after treatment with MF and CE. Analyzing the data together, we demonstrated that *C. procera* has an antitumor potential in both OST and CMT cells, encouraging us to continue investigating its use in cancer therapy.

Keywords: osteosarcoma, mammary cancer, medicinal plants, leaves, ethanolic extract.

4.1. Introduction

Cancer is characterized by cells in continuous multiplication, followed by the inability to be controlled or stopped, forming tumors of malignant cells with the potential to be metastatic (Greenwell and Rahman, 2015). Cancer is responsible for one in eight deaths worldwide, it kills more than AIDS, tuberculosis, and malaria together (Moten et al., 2014). In addition to affecting human health, cancer has been increasingly diagnosed in dogs (Baioni et al., 2017). Among the neoplasias common to humans and dogs stands out the mammary cancer, which affects 21 million women every year (WHO, 2018) and even worse in bitches, since its incidence is three times higher than in women (Dutra et al., 2004). Osteosarcoma is also prominent, accounting for 20% of bone cancers in humans (Kundu, 2014), and a frequency 10 times higher in dogs (Feng et al., 2014). The similarities between human and canine cancer with regard to histology, biological behavior, and molecular genetic alterations, suggest that the dog provides an excellent model for preclinical oncological studies of novel therapies (Schiffman and Breen, 2015). Current treatments for both mammary cancer and osteosarcoma include chemotherapy, radiotherapy and chemically derived drugs. Treatments such as chemotherapy may not be effective in some tumor types and can put patients under a lot of strain and further damage their health. Therefore, there is a focus on using alternative treatments and therapies against cancer (Greenwell and Rahman, 2015).

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment (Greenwell and Rahman, 2015). *Calotropis* is a

widespread genus of the family Apocynaceae were many plants are included for their known medicinal value. *Calotropis* species, particularly *Calotropis procera*, have been used in traditional medicine as the therapy for prevention and treatment of various diseases, consumed as tea, tincture and in many other ways (Chaudhary et al., 2015; Kumar et al., 2019). The use of this plant is based on its confirmed antifungal (Aliyu et al., 2015), healing (Patil e Makwana, 2015), anti-ulcerogenic (Al-Taweel et al., 2017), anti-inflammatory (Chaudhary et al., 2015; Kumar et al., 2019), and antioxidant effects (Qureshi et al., 2007; Chaudhary et al., 2015). Regarding its effects on cancer, a cardenolide called 2-oxovoruscharin 02 has already been isolated and, from synthetic derivation, has given rise to the cardiotonic steroid UNBS1450 (Van-Quaquebeke et al., 2005). This cardiotonic steroid showed a potent anticancer effect, through the inhibition of the Na^+/K^+ ATPase and the NF-kB signaling pathway (Juncker et al., 2009). The antiproliferative activity was also verified in sarcoma 180 rats (Magalhães et al., 2010) and in tumor cells H1975, HT29, MDA-BM-231, PANC1, PC3 (Kwon et al., 2018).

The cytotoxic activity of different *Calotropis* species was investigated by numerous authors (de Oliveira et al., 2007; Ravi et al., 2011; Habib et al., 2016). *C. procera* showed cytotoxic activity against colon and liver carcinomas (Al-Taweel et al., 2017), besides oral and central nervous system cell lines (Bhagat et al., 2010). However, little is known about the mechanism of its action, active constituents and effects on the other cancers. The necessity to investigate effects of *C. procera* on osteosarcoma and canine mammary cancer emerged due to the lack of new strategies for the treatment of these frequent diseases with high mortality rate, both in humans and dogs (Feng et al., 2014; Kundu, 2014; Vascellari et al., 2016; WHO, 2018). This seemed to be a rational approach once high daily intake of fruit, vegetables and plants protects against various cancers (Key et al., 2011; Zheng et al., 2018). The anticancer activity of natural products includes many different mechanisms of action, such as inhibition of cancer cell proliferation, suppression of migration and metastasis, modulation of redox status, induction of apoptosis, among others (Greenwell e Rahman, 2015; Thapliyal et al., 2018). In this sense, the aim of this study was to examine the antitumor effect of *C. procera* on osteosarcoma (OST) and canine mammary tumor (CMT).

4.2. Materials and methods

4.2.1. Chemicals, antibodies, and reagents

Dimetilsulfoxido (DMSO); (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT); and RNAse were purchased from Sigma-Aldrich (San Louis, MO, USA). The DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) vectashield; phalloidin; propidium iodide and CellTrace Blue Cell Proliferation® kit were purchased from Fisher Scientific (Pittsburgh, PA USA). Antibodies against caspase-3 (ab2171), Bax (ab32503), p53 (ab26), TGF-B (ab215715), and VEGF (ab2349) were purchased from Abcam (Cambridge, UK). Bcl-2 (100/D5), CD31 (MA3100), and osteopontin (MA5-17180), were purchased from ThermoFischer Scientific (Grand Island, NY, USA). PCNA (sc-46) was purchased from Biotecnologia Santa Cruz (Inc, Europe).

4.2.2. Plant material

The aerial parts of *C. procera* were collected in São Luís city, in Maranhão state, Brazil. The specimens were authenticated and deposited at the Herbarium of Maranhão voucher number MAR 9481. After identification, the leaves were dried at room temperature, pulverized (Wallita, Power 2i) and stored in plastic bottles. To obtain the crude extract (CE), approximately 19 g of leaves was extracted with 3 liters of ethanol, by maceration for 48 hours. The CE obtained was filtered (paper filter: Whatman qualitative filter paper, Sigma, pore size 4-12Âµm, filtration speed 20-25s) and concentrated in a rotary evaporator (IKA, RV 3V, Landsberger, Berlin). The extract was solubilized with 10% methanol/water and then partitioned with the organic solvents to obtain methanolic (MF) and ethyl acetate (EAF) fractions.

For cell culture experiments MF, EAF and CE extracts (~0.1 g/ml) were dissolved with 0.6% dimethylsulfoxide (DMSO) and supplemented with culture medium to obtain a 2200 µg/ml stock solution.

4.2.3. Phytochemical analyzes

The phytochemical characterization of CE was performed in LC- QqToF. Briefly, 1 mg of sample was dissolved in 1 mL of methanol/water (1:1, v/v). Samples were filtered and 20 µl aliquots were injected into the apparatus. The analyzes were performed using Luna-C18-HST column (10 x 0.2 mm, 2.5 µm). The mobile phases used were: water and acetonitrile, both acidified with 0.1% formic acid. Data acquisition and processing were performed using Data Analysis® software (Bruker Daltonics GmbH, Bremen, Germany).

4.2.4. Cell culture

This study was reviewed and approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Veterinary Medicine and Animal Science in the University of São Paulo (USP), Brazil (protocol no. 1100210217).

Primary culture was performed to obtain the OST, CMT and fibroblast cells. Thus, a small fragment of the osteosarcoma and mammary tumors, as well as a healthy dog skin fragment was washed with phosphate solution (PBS) and antibiotic (Penicillin-Streptomycin 1%) (Invitrogen, Carlsbad, CA, EUA). The fragment was transferred to a petri dish (Corning, EUA) and punctured with a scalpel blade. The contents were transferred to a falcon-type tube containing collagenase type I (1:10 ratio) (Invitrogen, Carlsbad, CA, EUA) and incubated for 6 hours. After this, the high glucose DMEM medium (LGC Biotecnologia, Cotia, São Paulo) was add and the tube was centrifuged at 1500 rpm for 5 minutes, and the supernatant discarded. The pellet was resuspended in 1 ml of DMEM medium supplemented with 10% fetal bovine serum (FBS) and placed in the cell culture dish. Then, the plates were incubated at 37°C humidified with 5% carbon dioxide (CO_2), and the medium being replaced every two days, according to the confluence of the cell monolayer. Subcultures were performed when the cells had 80% confluence.

4.2.5. Cell viability

Cell viability was determined using colorimetric MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay as described previously by Fotakis and Timbrell (2005). In brief, OST, CMT and fibroblast cells (1×10^3) were cultured in 96 well-plates with or without EAF, MF and CE (10–1000 μg/ml), and the positive control-doxorrubicin (1–15 μg/ml) for 24, 48 and 96 h. We also performed an incubation with 0.6% DMSO, mimicking the conditions that the extract was diluted. After incubation, medium was removed and 100 μl of 5 mg/ml MTT solution was added and incubated at 37°C for further 1 h. Subsequently, 100 μl of DMSO was added to dissolve formazan crystals and the absorbance was measured at 570 nm using microplate reader (MQuant– Bio Tek Instruments, VT, USA). The viabillity percentage was calculated based in the formula: (absorbance of treated cells/absorbance control) × 100. The control was assigned 100% of viability.

4.2.6. Selectivity index

From the MTT results, the cytotoxic concentration to 50% of cells (CC_{50}) and half maximal inhibitory concentration (IC_{50}) were calculated. Then, the selectivity index (SI) was calculated according to the following equation:

$$SI= CC_{50} \text{ (no cancer cells)}/ IC_{50} \text{ (cancer cells)}$$

where a SI ≥10 was considered to belong to a selective compound, according to Orme et al. (1999).

4.2.7. Growth curve and morphology

The OST and CMT cells ($7x10^4$) were plated in six-well plates and incubated for 24 hours for adhesion. After this, the supernatant was removed and 250 μg/ml of EAF, MF and CE were added, as well as 10 μg/ml of doxorubicin. The plates were incubated for 48 hours at 37° C humidified with 5% CO_2. After this, the plates were photographed with inverted light microscope (Nikon Eclipse TS-100) to observe the cellular morphology. Thereafter, cells were trypsinized and counted in a neubauer chamber with trypan blue dye.

4.2.8. Immunofluorescence

For the immunofluorescence assay a 24-well plate was prepared with coverslips. Soon after, OST and CMT cells were plated in each well. After 24 hours, 250 μg/ml of EAF, MF and CE, as well as 10 μg/ml of doxorubicin, were added. A further incubation of 48 hours was performed and the cells were fixed in 4% paraformol for 2 hours. Then, phalloidin (1:2000 dilution in PBS) was added for 1 hour. After the time, 3 washes of 10 minutes were performed with 1x PBS, and the coverslips were removed from the culture dish and placed on slides containing DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) vectashield. The photos were taken at the fluorescence light microscope (Nikon Eclipse 80i).

4.2.9. Cell cycle

The OST and CMT cells ($5x10^4$) were plated in 9 mm^2 dishes with culture medium supplemented with 10% FBS. After 24 hours of incubation, the supernatant was removed and 5 ml of culture medium supplemented with 0.2% FBS was added. The plates were incubated for a further 24 hours and then EAF, MF and CE were added at the concentration of 250 μg/ml and the doxorubicin at 10 μg/ml. After 48 hours, the cells were trypsinized and counted with trypan blue dye in the neubauer chamber. The cell contents were transferred to a polystyrene tube and 50 μl of RNAse (10 μg/ml) was added. A new incubation of 15 minutes was performed. Then, 1 μl of propidium iodide (100 μg/ml) and 200 μl PBSA were added. The reading was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA). For each sample 10,000 events were counted. The Modfit program 2.9. was used to obtain the graphs and percentage of the phases of the cell cycle.

4.2.10. Cell proliferation

CellTrace Blue Cell Proliferation® kit was used to evaluate cell proliferation. Basically, the kit has a non-fluorescent ester that enters the cell and it is converted into fluorescent derivatives. The active ester binds to amine groups present in proteins, retaining the dye. Thus, it is possible to visualize five or more generations of proliferating cells before the signal is dominated by intrinsic cellular autofluorescence.

The OST and CMT cells ($2x10^5$) were incubated for 20 minutes with 1 µl of the CellTrace dye. Then, they were plated in 9 mm^2 plates with culture medium for cells adhesion. After 24 hours, the supernatant was removed and the cells were collected at time zero (T_0). Subsequently, 250 µg/ml of EAF, MF and CE, or 10 µg/ml of doxorubicin were added, and a new incubation of 24 hours was performed. After time, the cells were collected and counted in neubauer chamber. This process was repeated through 72 hours. At the end, a tube was obtained for each treatment at T_0, T_{24}, T_{48}, T_{72}. The reading was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA). The data were analyzed in the FCS Express program (5.0).

4.2.11. Flow cytometry

The OST and CMT cells were treated with EAF, MF and CE at 250 µg/ml, or doxorubicin at 10 µg/ml. After 48 hours, the cells were fixed in paraformaldehyde (4%) for 2 hours. Then, the cells were centrifuged at 1500 rpm for 5 minutes. After the time, a total of $5x10^4$ cells were resuspended in 100 µl of PBSA + BSA (2%), and 2 µl of triton (0.1%) was added for 15 minutes. After this, the primary antibody (1:100 v/v) was added in OST cells for apoptosis (caspase-3, Bax and Bcl-2); p53; angiosenesis (VEGF and CD31); TGF-β; and osteopontin. For CMT cells, the primary antibody (1:100 v/v) for cell proliferation (PCNA) was added. The tubes were incubated in the refrigerator for 30 minutes. The secondary antibody was added (1:250 v/v), and incubated for another 30 minutes. After this, the tubes were centrifuged at 1500 rpm for 3 minutes. The supernatant was discarded and 250 µl of PBSA was added. The reading was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif., USA), which was calibrated using unlabelled cells and cells labeled with only the non-specific secondary antibody. For each sample, 10.000 events were counted. The data were analyzed using Flowjo software version vX-0.7 and were plotted in% expression.

4.2.12. Statistical analysis

The data were submitted to the Shapiro-Wilk normality test, followed by the ANOVA variance test, with Tukey post-test, using R software version 3.5.1. All values are expressed as

the mean ± standard error of the mean (SEM). Differences were considered significant when $p<0.05$.

4. 3. Results

4.3.1. LC-QqToF analysis of *Calotropis procera* extract

Four flavonoids (Isorhamnetin-3-O-hexose-deoxyhexoside; Kaempferol 3-O-hexoside; Kaempferol-3-O-hexose-deoxyhexoside; Quercetin-3-O-hexoside); six glycosides (Isorhamnetin; Coroglaucigenin; Calotropagenin; Reseoside I e II; Adynerin; Roseoside); and onde cardenolide (Calotropin) were detected in CE. The LC-QqToF fingerprint is shown in Fig 1 and compounds in Table 1.

4.3.2. Cell viability and selectivity index

The first step was to evaluate the viability of OST, CMT and fibroblast cells after incubation with different concentrations of EAF, MF, CE, and DOXO. DMSO was also tested at 0.6%, mimicking the same dilution conditions as the extract. Results showed that DMSO did not significantly ($p > 0.05$) alter cell viability, showing it to be a safe diluent (Fig 2A-I).

MF treatment for 24 h reduced the viability of OST and CMT cells from 10 μg/ml. However, the cell viability increased in 33.3 and 54% in CMT cells treated with 750 and 1000 μg/ml compared to those cells treated with 500 μg/ml (Fig 2A), suggesting that the most effective concentration was 500 μg/ml. MF treatment for 48 h showed a similar profile of 24 h, but with 250 μg/ml being the most effective concentration in reducing the viability of CMT cells (Fig 2B). In 96 h, there was a reduction of OST cells viability from 250 μg/ml. Regarding CMT cells, only the treatment with 1000 μg/ml was effective in reducing cell viability, and although there was a reduction of 13 to 30% when CMT cells were treated with MF in a concentration range of 10 to 750 μg/ml, there was no statistical difference (Fig 2C). Regarding fibroblasts, there was a significant increase in cell viability after treatment with MF in the highest concentrations (from 750 μg/ml in 24, and 500 μg/ml in 48 and 96) (Fig 2A-C).

EAF treatment for 24 and 48 h reduced cell viability in both OST and CMT in the range of 100 to 1000 μg/ml in a dose-indepenent manner (Fig 2D, E). In 96 h, treatment with EAF was more effective in CMT cells, since from 10 μg/ml there was a reduction in cell viability, while in OST cells it was from 500 μg/ml (Fig 2C). Regarding fibroblasts, although there was an increase in viability in some concentrations over time, there was no statistical difference ($p > 0.05$) compared to the control (Fig 2D-F).

CE treatment for 24 h was effective only in reducing CMT cell's viability from 500 μg/ml (Fig 2G). In 48h, CE promoted a reduction in the viability of both OST and CMT, from 10 and 100, respectively (Fig 2H). At 96 h, there was also a reduction in the viability of OST and CMT cells from 250 and 10 μg/ml, respectively (Fig 2I). Regarding fibroblasts, there was no statistical difference in any of the tested concentrations and times (Fig 2G-I).

Doxorubicin treatment did not promote any change in OST viability in 24 h, but promoted a decrease in cell viability from 5 and 1 μg/ml in 48 and 96 h, respectively. On the other hand, there was a decrease in CMT viability from 1 μg/ml at all times tested (Fig 2J-L). Regarding fibroblast, there was a reduction in viability when cells were treated from 15, 10 and 1 μg/ml at 24, 48, and 96 h, respectively (Fig 2J-L).

The selectivity index (SI) was calculated through the MTT, and CE presented the best SI in 48h (SI = 31.90 μg/ml for OST cells, and SI = 17.32 μg/ml for CMT cells) (Table 2). The EAF presented SI = 10.17 μg/ml, whereas doxorubicin showed an SI = 14.77 μg/ml, both for CMT cells in 48h. Based on this, 48 hours were chosen to be the treatment time and the concentration of 250 μg/ml to the extracts and 10 μg/ml to doxorubicin.

4.3.3. Treatment with *Calotropis procera* decreased cell proliferation and promoted cell cycle arrest in G_0/G_1

To confirm the reduction in cell viability promoted by extracts and doxorubicin, cell counting, proliferation assay and cell cycle analysis were performed. After 48 h, it was possible to observe a reduction in the number of cells (OST and CMT) incubated with MF, EAF and CE, when compared to their respective control group (C), similar to the positive control-doxorubicin (Fig 3A and B).

The proliferation of OST and CMT cells was analyzed at 24, 48 and 72h. We observed that the OST cells of the C group showed a proliferation over time, especially from 24 to 48 hours (Fig 3C). Treatment with MF showed an increase in proliferation over time, but lower than group C (Fig 3C). EAF and CE treatments remained constant over time, however with a much lower proliferation than C group (Fig 3C). Treatment with doxorrubicin promoted a reduction in proliferation in 72h and when compared to the C group (Fig 3C). Regarding CMT cells, it was also observed that C group showed a cell proliferation over time, while the cells treated with MF, CE and doxorubicin remained constant. Although the cells of the EAF group showed increase in proliferation in 72h, this increase was lower than the C group at all times evaluated (Fig 3D).

The OST and CMT cells were also evaluated for cell cycle. In Fig 3E and F we observed that both cells of C group are mainly in the S phase. The OST cells in MF and EAF groups also remained in S and G_2/M. However, treatment with CE arrested the OST cells to G_0/G_1 and G_2/M phases. The CMT cells of the CE group remained mainly in the G_0/G_1 phase, similar to doxorubicin. Although the CMT cells in MF and EAF groups also maintained mainly in G_0/G_1, there was no significant difference when compared to the C group (Fig 3F).

4.3.4. Tumor cell morphology was altered after *Calotropis procera* treatment

The OST and CMT cells of the C group were fibroblast-like. After treatment with MF and EAF, the cells presented a fusiform shape. The CE treatment changed the cells morphology, leaving them with rounded shape, similar to doxorubicin treatment. These morphological changes were visualized by light microscopy and confirmed by phalloidin and DAPI labeling, analyzed by fluorescence microscopy (Fig 4).

4.3.5. *Calotropis procera* modulated apoptosis, angiogenesis, and mesenchymal-epithelium transition in OST cells and decreased proliferation in CMT cells

Many medicinal plants have antitumor effect through their ability to promote apoptosis, inhibit angiogenesis and epithelial-mesenchymal transition. We evaluated the apoptotic process after MF, EAF and CE treatment in OST cells, through the markers caspase-3, Bax and Bcl-2

(Fig 5A-C). We can observe that treatment with MF and EAF did not promoted any change in apoptotic markers (Fig 5A-C). On the other hand, CE treatment promoted an increase in caspase-3 similarly to doxorubicin, but there was no change in Bax and Bcl-2. In addition, CE was able to increase p53 levels (Fig 5D). The angiogenic process was evaluated by VEGF and CD31 markers, where CE treatment promoted a decrease in both parameters more efficiently than doxorubicin (Fig 5E and F). To analyze the role of *C. procera* in the epithelium-mesenchymal transition, the TGF-β marker was used. Figure 5G shows a decrease in TGF-β after CE and doxorubicin treatment. Osteopontin has also been evaluated and it is possible to observe a reduction in its levels after CE and doxorubicin treatment (Fig 5H).

Regarding CMT cells, we observed a reduction in proliferation marker-PCNA after incubation with MF, CE and DOXO (Fig 5I).

4.4. Discussion

Natural products mainly isolated from plants or plant-derived extracts, used in traditional medicine, continue to be an important source of novel therapeutic agents. Although they are consumed as food, tea, supplement or in other ways for prevention and treatment of various diseases, including cancer, many are not adequately investigated. This study provided original scientific data about chemical contents and anticancer properties of *C. procera* extracts. We showed that *C. procera* contains numerous compounds responsible for its biological activities, including flavonoids, cardenolides and glycosides, similar to other studies (Pandey et al., 2016; Al-Taweel et al., 2017; Kumar et al., 2019). It is known that environmental conditions, geographic location, and many other factors can influence the contents of secondary metabolites in the *C. procera* (Jucá et al., 2013; Aliyu et al., 2015), thus influencing various biological activities of this plant.

The crude (CE) and fractions (MF and EAF) extracts of *C. procera* investigated for cytotoxic activity on osteosarcoma (OST), canine mammary tumor (CMT) and normal fibroblast, showed selective effects through significant cytotoxicity on OST and CMT cells, and non-cytotoxicity on normal fibroblast. The cytotoxic activity of *C. procera* was confirmed in earlier study on colon and liver carcinomas, oral and central nervous system cancer cells (Bhagat et al., 2010; Al-Taweel et al., 2017), making this plant suitable for further anticancer

studies. This cytotoxic effect can be attributed, at least in parts, to calotropin, one of the cardenolide found in the crude extract, since it has already been demonstrated that this compound had cytotoxicity on multiresistant cells (A549/CDDP) (Mo et al., 2016). In addition to the *C. procera* extracts, the doxorubicin cytotoxicity was also evaluated. This drug belongs to the anthracyclines class and has been widely used as a chemotherapeutic agent since the 1960s (Renu et al., 2018). Our results demonstrated that doxorubicin promoted cytotoxicity in all cells evaluated, especially after 48 hours, being in agreement with the cytotoxicity in breast cancer cells found by Oliveira et al. (2014). According to selectivity index (SI) values, it was evident that the cancer cells were more sensitive to CE extract than to the other two fractions (SI= 31.9 for OST, and 17.32 for CMT), which was correlated with secondary metabolites accumulation. It is worth mentioning that the CE presented a greater selectivity than the positive control-doxorubicin (SI= 0.40 for OST, and 14.77 for CMT). The cytotoxicity found for extracts and doxorubicin was confirmed by the growth curve, which showed reduced number of OST and MCT cells, when compared to the control group, and decreased proliferation of tumor cells.

The cell cycle was also evaluated, and analysis showed that the OST and CMT cells of the control group were mainly in the S phase and G2M, showing that the cells are synthesizing DNA and allowing the cell division. It is known that cells normally depend on the growth signaling of a tightly regulated cell cycle to proliferate and maintain tissue homeostasis. Cyclin-dependent kinases (CDKs), cyclins, CDK inhibitors and phosphorylation events contribute greatly to the regulation of the cell cycle (Vermeulen et al., 2003). However, this process is discontinued in the case of tumor cells (Fouad and AanEI, 2017). In mammary cancer, for example, the increase of cyclin D1, with consequent formation of cyclin D-CDK4/6 complexes, has been shown to allow the promotion and progression of the cell cycle and cell division (Thu et al., 2018). It has also been reported that deregulation of CDK4/cyclin D; cyclin E; CDK11 cells play an important role in cell cycle progression in osteosarcoma cells (Peyressatre et al., 2015). In mammalian cells, p53 also provides important cell-cycle checkpoint functions (Chen, 2016). Our data showed that there is a low expression of p53 in the OST cells of the control group. The p53 gene is mutated in >50% of all types of malignant tumor and its mutations have been demonstrated to be involved in osteosarcoma tumorigenesis (Chen, 2016). Based on this

results, seems that OST and CMT cells lost the mechanisms of cell cycle regulation, consisting of the increased proliferation found in the control group.

When the OST cells were treated with CE there was a predominance in G0G1 and G2M phase, whereas the CMT cells remained in G0G1. However, fractions of *C. procera* do not alter the cell cycle profile. In human melanoma cells (SK-MEL-2) treated with *C. procera* crude methanolic extract was a predominance of G2M phase, which was accompanied by more than 60% of cells in apoptosis (Joshi et al., 2015). Yan et al. (2008) demonstrated that calotropin, found in *C. procera*, promoted a decrease in cyclins, stopping the cell cycle in the G2/M phase. Another report showed that cardenolides are able to block the cell cycle in the G2/M phase and induce apoptosis in human leukemia cells (HL-60) (Huang et al., 2013). These findings are in agreement with the decrease in proliferation found in our study after *C. procera* treatment. Doxorubicin-treated CMT cells also showed a predominance in G0G1 phase, followed by the S phase. Other researches have also demonstrated that MCF7 cells after doxorubicin treatment remained mainly in the G1 phase (Guo et al., 2003; Meiyanto et al., 2011). This effect can be attributed to its mechanism of action, which induces apoptosis and prevents cell division (Renu et al., 2018).

Regarding cell morphology, it was observed that treatment with CE and its fractions promoted morphological alterations in OST and CMT cells, being characterized by reduction in cell volume and formation of fusiform and rounded cells. These characteristics are usually associated with apoptosis induction (Vermes et al., 2000). Although no difference was found in Bax and Bcl-2 levels after treatment with CE, there was a significant increase in caspase-3 levels in OST cells. Several mechanisms are known to regulate apoptosis, including Bax, Bcl-2 and p53 (Aubrey et al., 2018). In this way, CE appeared to activate apoptosis mainly by p53, which reflected the increase of caspase-3. Joshi et al. (2015) also demonstrated that the methanolic extract of *C. procera* induced apoptosis in human skin melanoma cells (SK-MEL-2). In addition, dried latex of *C. procera* induced apoptosis in hepatoma cells (Hub-7) (Choedon et al., 2006). However, in both studies apoptosis was caspase independent way.

Epithelial-Mesenchymal Transition (EMT) is a process involved in tumor progression, with metastatic expansion and generation of tumor cells with stem cell properties, which plays an important role in cancer treatment resistance (Roche, 2018). Studies indicate that

transforming growth factor (TGF)-β stimulates EMT, playing an important role in the metastasis process (XU et al., 2009; XIE et al., 2018). In our study, we observed a decrease in TGF-β in OST cells after CE treatment. Flavonoids are known to alter the expression and functionality of important proteins in signaling pathways that control cellular mesenchymal characteristics (Amawi et al., 2017). Some of the pathways inhibited by these compounds are: TGF-β; epidermal growth factor (EGF); hepatocyte growth factor (HGF); fibroblast growth factor (FGF); among others (Amawi et al., 2017). As flavonoids were found in CE extract (e.g.: kaempferol and quercetin), they are possibly acting to decrease TGF-β, softening the EMT process. Moreover, TGF-β promotes the expression of osteopontin, increasing the malignancy of OST cells (Han et al., 2019). Probably, the increase in TGF-β found in the control group of our study contributed to the increase in osteopontin. But the treatment with MF and CE was able to reverse this situation.

Another important mechanism in tumor growth, progression and metastasis is angiogenesis, which is characterized by new blood vessels (Deshpande et al., 2011). Some angiogenic molecular markers are overexpressed in tumors and can be used as targets for early cancer detection. These include vascular endothelial growth factor (VEGF) and CD31 (Schlüter et al., 2018). In our study we found a decrease in VEGF and CD31 after CE treatment. Reactive oxygen species (ROS) are known to stimulate angiogenesis (Kim and Byzova, 2019), while flavonoids neutralize ROS by avoiding this process (Quideau et al., 2011). In our study, we found flavonoids such as kaempferol and quercetin in CE, what has been correlated to reduction in VEGF (Batra and Sharma, 2013).

4.5. Conclusion

By assessing the key parameters for the protective effects against cancer cells, treatment with *Calotropis procera* revealed favorable antitumor effect by reducing OST and CMT viability, whereas did not alter the viability of fibroblasts, showing even more selectivity than the positive control doxorubicin. *C. procera* also demonstrated its antitumor effect by reducing proliferation and stopping the cycle in G0/G1 in both OST and CMT cells. CE proved to be the most effective treatment among the extracts, since it reduced angiogenesis, mesenchymal-epithelium transition, and induced apoptosis in OST cells. These effects were probably because

CE accumulated more secondary metabolites than the fractions (MF and EAF). All these effects encourage us to continue researching this specie plant as anticancer therapy.

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Figures

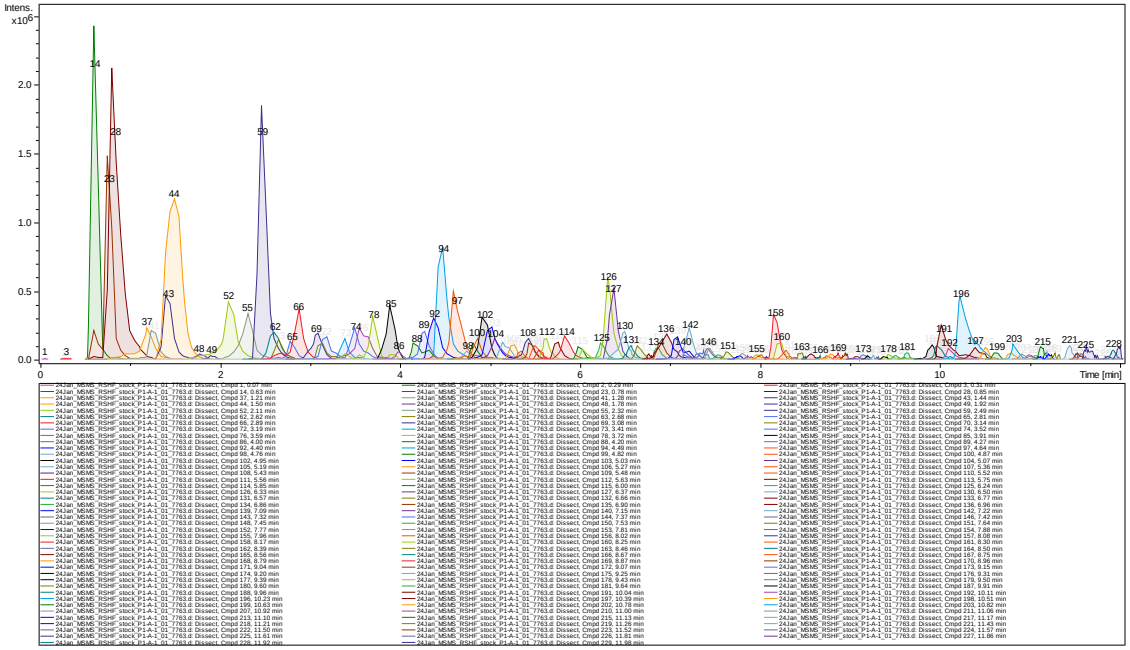

Figure 1: The LC-QqToF fingerprint of crude extract of *Calotropis procera*. Five flavonoids (Isorhamnetin-3-O-hexose-deoxyhexoside; Kaempferol 3-O-hexoside; Kaempferol-3-O-hexose-deoxyhexoside; Quercetin-3-O-hexoside) and seven glycosides (Eriojaposide A; Isorhamnetin; Calotropin; Coroglaucigenin; Calotropagenin; Reseoside I e II; Adynerin; Roseoside) were detected in crude extract of *C. procera*.

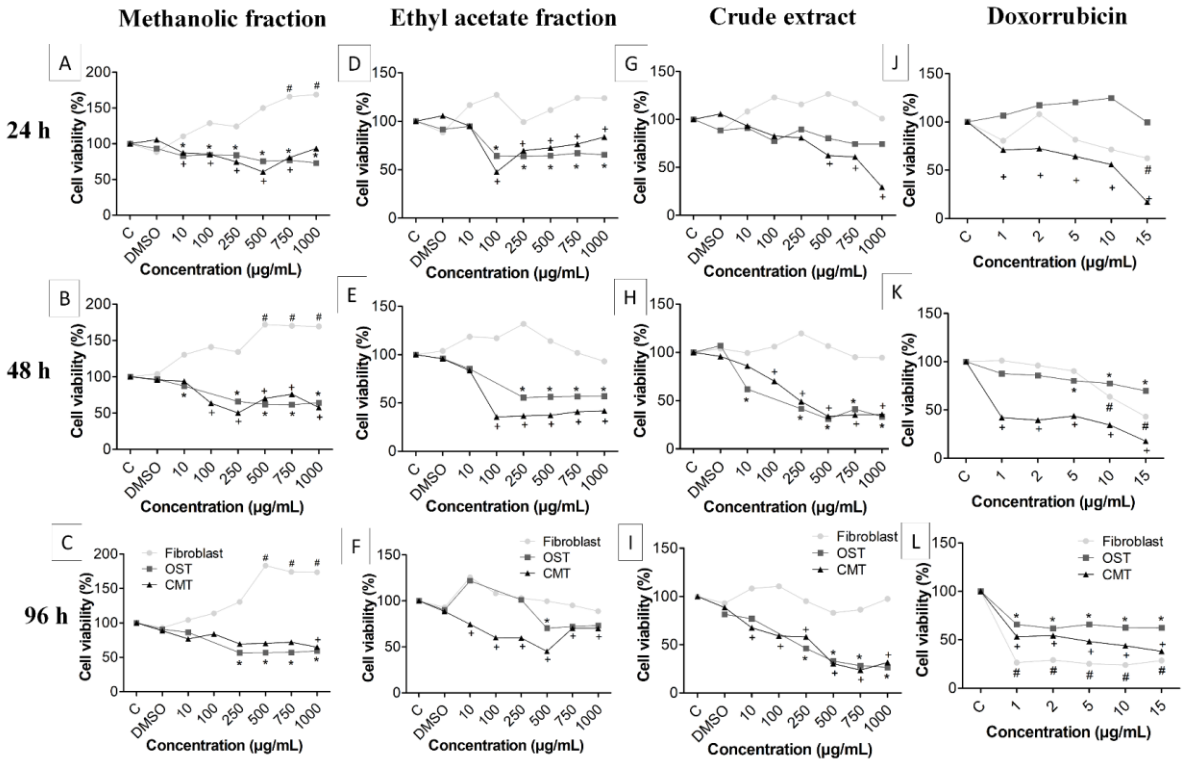

Figure 2: Evaluation of cellular viability by MTT assay. Canine osteosarcoma (OST), canine mammary tumor (CMT) and fibroblast cells were incubated with different concentrations of ethyl acetate fraction (A-C); methanolic fraction (D-F); and the crude extract of *C. procera* (10-1000 µg/ml) (G-I) for 24 h, 48 h and 96 h. The cells were also incubated with doxorubicin (1-15 µg/ml) (J-L), used as positive control. DMSO: dimethylsulfoxide, used as solvent for the extracts. The experimental groups were compared with the control (C) and statistical difference was considered when $p < 0.05$. Asterisk (*) shows the statistical difference between the extract concentrations and the control group of the OST cells. Plus (+) signal shows the statistical difference between extract concentrations and the control group of CMT cells. Hashtag (#) shows the statistical difference between extract concentrations and the control group of fibroblasts.

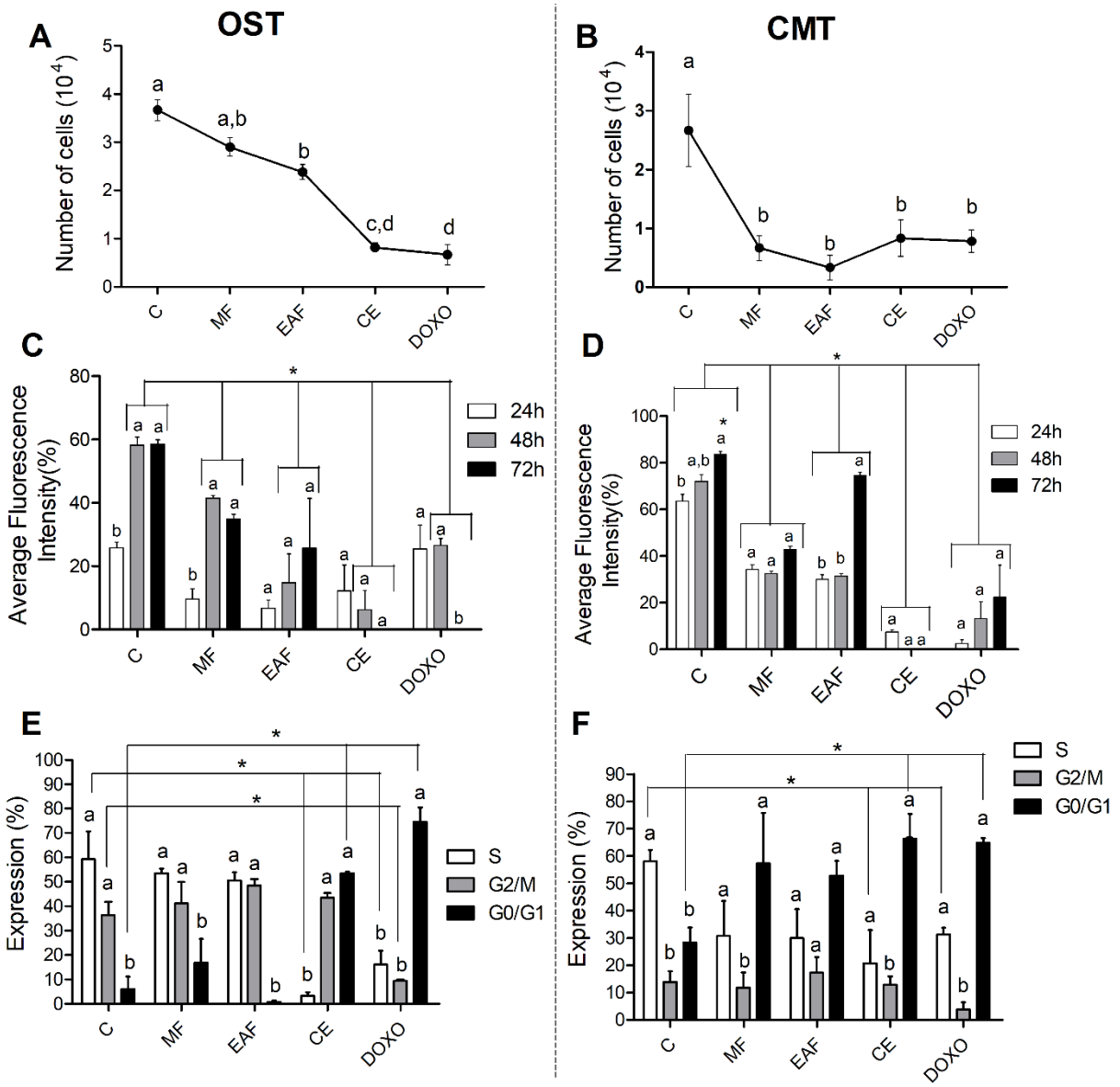

Figure 3: Growth curve, proliferation and cell cycle analysis of osteosarcoma (OST) and canine mammary tumor (CMT) cells, after incubation with *C. procera* extracts and positive control-doxorubicin. A-B: growth curve; C-D: proliferation assay; E-F: cell cycle analysis. OST and CMT cells were incubated with 250 µg/ml of methanolic (MF), ethyl acetate fraction (EAF) and crude extract (CE) of *C. procera* or 10 µg/ml of doxorubicin (DOXO), for 48 h. The experimental groups were compared with the control (C) and statistical difference was considered when $p < 0.05$. Different letters mean statistical difference. Asterisk (*) represents statistical difference between different groups.

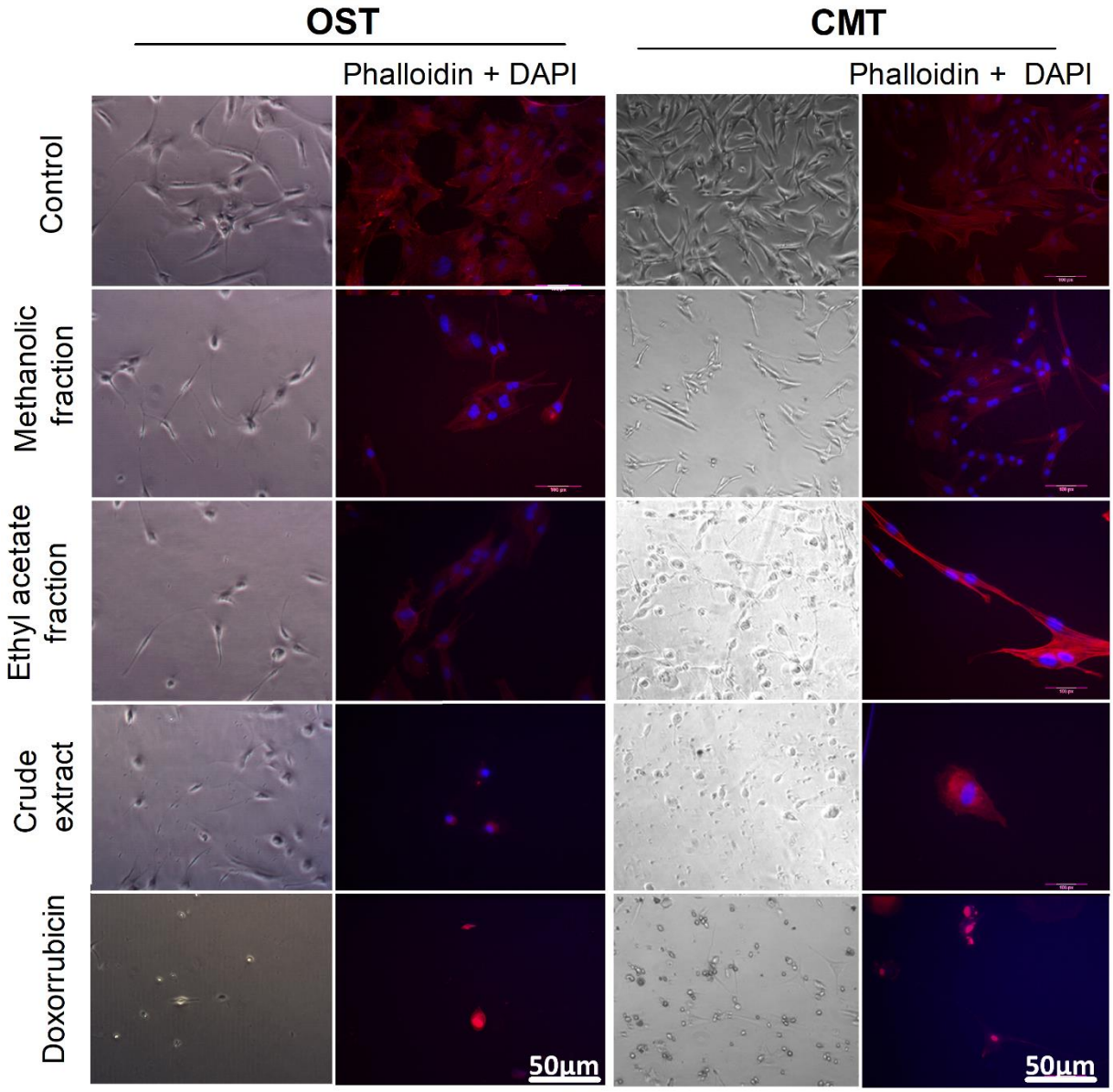

Figure 4: Cell morphology after incubation with *C. procera* extracts and positive control-doxorubicin. Canine osteosarcoma (OST) and canine mammary tumor (CMT) cells were incubated with 250 µg/ml of methanolic, ethyl acetate fraction, and crude extract of *C. procera* or 10 µg/ml of doxorrubicin, for 48 h. Then, the cells were photographed by light microscopy or incubated with phalloidin and DAPI for imaging by fluorescence microscopy.

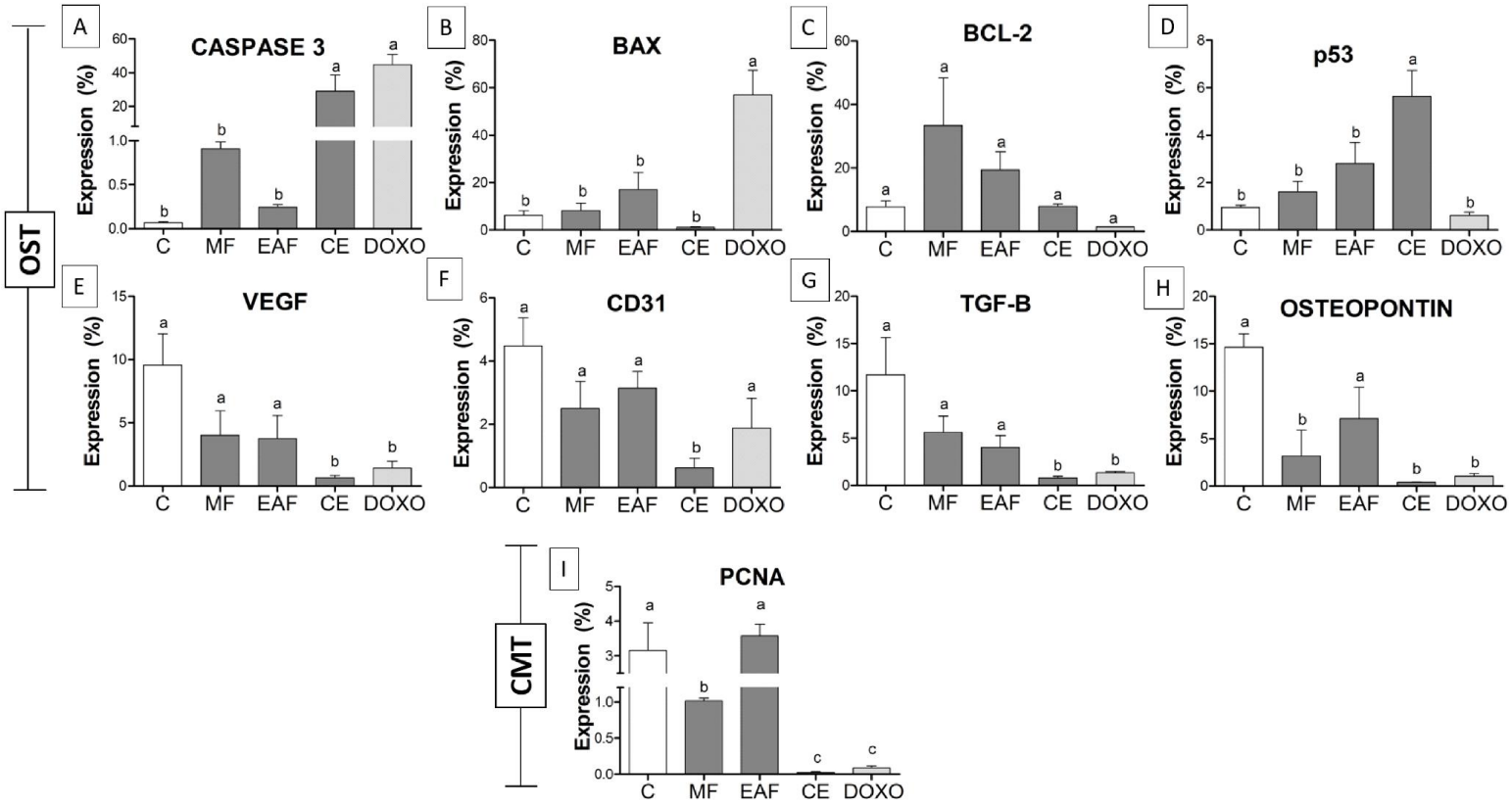

Figure 5: Evaluation of apoptosis, angiogenesis, mesenchymal epithelium transition and proliferation markers by flow cytometry. Canine osteosarcoma cells (OST) were incubated with 250 µg/ml of methanolic fraction (MF), ethyl acetate fraction (EAF) and crude extract (CE), of *C. procera* or 10 µg/ml of doxorubicin (DOXO), for 48 h. Then, the cells were analyzed by flow cytometry for the markers caspases-3 (A), Bax (B), Bcl-2 (C), p53 (D), VEGF (E), CD31 (F), TGF-β (G), and osteopontin (H). Canine mammary tumor (CMT) cells received the same treatments and were evaluated for PCNA (I). The experimental groups were compared with the control (C). Statistical difference was considered when $p < 0.05$. Different letters mean statistical difference.

Tables

Number	$[M- H]^-$(m/z)	Fragments (ms^n)	Compounds
1	625.1771	479.1176; 317.0659; 153.0179	Isorhamnetin-3-O-hexose-deoxyhexoside
2	449.1076	287.0548; 153.0179	Kaempferol 3-O-hexoside
3	595.1660	449.1071, 287.0551, 153.0506	Kaempferol-3-O-hexose-deoxyhexoside
4	465.1020	303.0495; 153.0177	Quercetin-3-O-hexoside
5	503.1915	371.2047; 209.1536; 133.1007	Eriojaposide A
6	317.0655	302.0410; 153.0179	Isorhamnetin
7	533.2741	515.2626; 497.2505; 479.2416	Calotropin
8	391,2466	375.2304; 343.2384	Coroglaucigenin
9	405.2287	ms/ms*	Calotropagenin
10	387.2015	225.1482; 133.1010	Reseoside I
11	317.3155	499.3052; 481.2935; 325.2275	Adynerin

Table 1: Compounds identified in the crude extract of *Calotropis procera*. *Did not reach the minimum for fragmentation (intensity of 10^3).

Treatment/Time		Fibroblast	OST	CMT	Selectivity index	
		CC_{50}	IC_{50}	IC_{50}	OST	CMT
Crude extract	24h	5680.3	4361.8	755.7	1.30	7.51
	48h	4645.7	145.6	268.2	31.90	17.32
	96h	2566.2	413.7	319.7	6.20	8.02
Methanolic fraction	24h	1112.2	3004.9	1962.01	0.37	0.56
	48h	11537.4	1405.8	1938.1	8.20	5.95
	96h	811.2	1268.7	3704.0	0.63	0.21
Ethyl acetate fraction	24h	12173.0	1750.8	5178.5	6.95	2.35
	48h	1425.0	1062.4	140.0	1.34	10.17
	96h	2252.0	1246.9	402.9	1.80	5.58
Doxorrubicin	24h	20.6	35.55	8.2	0.57	2.51
	48h	13.3	33.14	0.9	0.40	14.77
	96h	0.9	83.13	7.1	0.01	0.12

Table 2: Index of selectivity of *Calotropis procera* and positive control-doxorubicin. Canine osteosarcoma (OST), canine mammary tumor (CMT) and fibroblast cells were incubated with 250 μg/ml of the crude and fractions extract of *Calotropis procera* or 15 μg/ml of doxorrubicin, for 48 h. The selectivity index was calculated from the division of CC_{50} by IC_{50}.

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5. ARTICLE III: C*alotropis procera* selectively impaired the 4T1 breast cancer cells growth by preferentially blocking Akt/mTOR signaling and reduced lung and liver metastasis through twist and cenpf dowregulation *in vivo*

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Abstract: *Calotropis procera* is a commonly used herb in folk medicine to treat various diseases. In this study, we aimed to investigate the biological effects of crude extract phenolics from *C. procera* (CphE) and its mechanism on 4T1 breast cancer cells and on xenograft tumors in nude mice. As a positive control we chose quercetin, one of the flavonoids present in *C. procera*. To prepare the extract, leaves were ground and extracted with ethanol and water, by maceration. Cells were treated with CphE and quercetin at concentrations that inhibited cell growth by 50%. *In vitro* results showed that CphE supressed cell viability through apoptosis activation via caspase-3 cleavage and decrease in total PARP. CphE also scavenger ROS and supressed Akt, mTOR, ERK1/2, CREB, and Src activation, that contributed to the cell motility inhibition. Moreover, CphE modulated the levels of IR, PTEN, p70s6, rps6, GSK3a and TSC2. For the *in vivo* study, 4T1 cells were injected into the mammary chain of nude mice. After 9 days of tumor growth, the animals were treated with 150 mg/kg of CphE for one week, by gavage. *In vivo* results showed dowregulation of ERK1/2 expression and reduction in mTOR mRNA levels. An increase in mRNA levels of caspase 8 in tumors was also found after CphE treatment. In addition, CphE dowregulated Twist mRNA levels in lung, and Cenpf in the liver and lung. Taken together, the inhibited cell proliferation and induced cell apoptosis effect promoted by CphE extract via Akt/mTOR provided a new insight into anti-cancer effect of *C. procera* as a promising agent in breast cancer treatment.

Keywords: 4T1 cells; milkweed (*Calotropis procera*); Aakt/mTOR pathway; metastasis

5.1. Introduction

Breast cancer is the most commonly occurring cancer in women and the second most common cancer overall (World Health Organization [WHO], 2020). Worldwide, about 2.1 million new cases of female breast cancer were diagnosed in 2018, representing almost 1 in 4 cancer cases among women (Bray et al., 2018). Breast cancer still has a high mortality rate, accounting for approximately 600,000 deaths in 2018 (WHO, 2020). Over 90% of these patients die of metastasis, also known as stage IV, which is when cancer cells move away from their original tumors, spread systemically and invade distant organs (Jin and Mu, 2015).

Conventionally, breast cancer is classified into four subtypes based on the receptor status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), termed luminal A, luminal B, HER2 positive, and triple negative (TNBC) (Dai et al., 2017). Among them, TNBC subtype features low or no expression of all three markers (ER-PR-HER2-), and share the worst prognosis (Dai et al., 2017). Moreover, TNBC accounts for 10–20% of invasive breast cancers and represents a consistent subgroup of breast cancers with heterogeneous clinical presentation, behavior, pathology and response to treatment (Kumar and Aggarwal, 2016). The standard of care for many TNBC patients is neoadjuvant chemotherapy, which includes a combination of taxanes and anthracyclines (Gadi and Davidson, 2017). However, about 50% of patients develop drug resistance, leading to poor overall survival (Kim et al., 2018). Because of this, several studies have focused on the search for new therapeutic possibilities and models that clinically mimic TNBC (Khan et al., 2019). In this sense, 4T1 cells has been widely applied for this purpose, since its classification proved to be triple-negative in stage IV, and showed high potential for metastasis in the lungs, liver, brain and bones (Bailey-Downs et al., 2014). In addition, the 4T1 orthotopic model closely mimics the progressive forms of human metastatic breast cancer (Kim et al., 2011).

The phosphatidylinositol-3-kinase (PI3K) - protein kinase B (Akt) - mammalian target of rapamycin (mTOR) pathway is one of the most important and active pathways involved in chemoresistance and survival of TNBC (Khan et al., 2019). The

phosphatidylinositol-3,4,5-triphosphate messenger (PIP3) activates the downstream PI3K pathway and Akt, which leads to protein synthesis, cell growth, angiogenesis and drug resistance by activating the downstream effector mTOR through tuberous sclerosis complex 1 and 2 (TSC1/2) (Khan et al., 2019; Hosonaga et al., 2020). In addition, the mitogen-activated protein kinase (MAPK) signal transduction pathway has shown to play key roles in cell growth, differentiation, inflammation, and responses to environmental stresses (Kaboli et al., 2020). Typically, there are 4 major cascades in the MAPK pathway: the extracellular-signal regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), Big MAP kinase (BMK, also known as ERK5), and p38 MAPK (Koul et al., 2013; Kaboli et al., 2020). The function of MAPKs in breast cancer is complex due to different responses they modulate and their interaction with different pathways (Ahmad et al., 2016). Moreover, it is known that the crosstalk between MAPK and PI3K/Akt occurs frequently, leading to aberrant signaling activation and poor prognosis (Cao et al., 2019).

Furthermore, one of the known characteristics of stage IV breast cancer is the predominance of mesenchymal attributes; the cells have undergone epithelial-mesenchymal transition (EMT), in which they go through changes in adhesion ability, in polarity, and in differentiating characteristics, making them more migratory and invasive (Vijay et al., 2019). Plenty of reports have suggested a central role for focal adhesion kinase (FAK) and steroid receptor coactivator (Src) in cancer through its ability to promote EMT, proliferation and anoikis resistance in tumor cells (Bolós et al., 2010).

A large body of evidence suggests that intake of plant phytochemicals is a relatively easy and practical strategy for significantly reducing the incidence of cancer (Russo, 2007; Kapinova et al., 2017; Mitra and Dash, 2018). Many of them interfere in the regulation of signal transduction at different levels, such as the modulation of hormones/growth factors, inhibition of oncogenes and activation of tumor suppressor genes, induction of apoptosis, restoration of the immune response, inhibition of angiogenesis, and decrease of inflammation (Russo, 2007). In breast cancer, for example, herbs, mixture of green tea catechins, and phytochemical-rich foods have shown potential in reducing invasion, proliferation, angiogenesis, oxidative damage and increase apoptosis rate (Kapinova et al., 2017; Mitra and Dash, 2018).

Calotropis procera is a wild and perennial shrub used in folk medicine to treat several diseases, particularly associated with the gastric disorders and inflammatory processes (Kumar, 2006; Al-Taweel et al., 2017). Until now, the major phytochemicals found in CP were cardenolides, steroid glycoside and flavonoids (Jucá et al., 2013; Kazeem et al., 2013; Moustafa et al., 2010; Sweidan and Abu Zarga, 2015). Quercetin has been reported as one of the most present flavonoid in leaves of *C. procera* (Mohamed et al., 2011; Sweidan and Zarga, 2015; Oraibi and Hamad, 2018).

The potential of *C. procera* phytochemicals in promoting cytotoxicity against the breast cancer cell lines MCF-7 and MDA-MB-435 *in vitro* was reported (Soares de Oliveira et al., 2007; Samy et al., 2012; Al-Taweel et al., 2017). However, the mechanisms involved in its anticancer activity have not been explored nor the potential *in vivo* anticancer activity. Our goal was to investigate the anticancer activity and underlying mechanisms of *C. procera* phenolic extracts (CphE) *in vitro* using the highly invasive mouse 4T1 breast cancer cell line and the potential activity *in vivo*.

5.2. Materials and Methods

5.2.1. *C. procera* extract and quercetin preparation

The aerial parts of *C. procera* were collected in São Luís city, in the State of Maranhão, Brazil. The specimens were deposited at the Herbarium of Maranhão voucher number MAR 9481. After identification, the leaves were dried at room temperature and pulverized in microprocessor (Wallita, Power 2i). The dried leaves were homogenized with ethanol (~1g:150 mL), left for 48 h at room temperature, followed by filtration with paper filter (Whatman qualitative filter paper, Sigma, pore size 4-12Âµm, filtration speed 20-25s) and evaporation using a rotary evaporator (IKA, RV 3V, Landsberger, Berlin). Dried *C. procera* crude extract was kept at -20°C for further use in cell culture and animal experiments.

5.2.2 Chemicals, antibodies, and reagents

Quercetin standard was purchased from Sigma-Aldrich (San Louis, MO, USA). The Folin-Ciocalteu reagent, and solvents were purchased from Fisher Scientific

(Pittsburgh, PA USA). Antibodies against phospho-ERK1/2, phospho-Akt, phospho-CREB, phospho-Src, phospho-mTOR, phospho-p38, phospho-FAK were purchased from Cell Signaling Technology (Danvers, MA USA). Western blotting chemiluminescence luminol reagent was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA USA). Direct-zol™ RNA MiniPrep Plus mRNA extraction kit was purchased from Zymo Research (Irvine, CA USA), iScript™ reverse transcription supermix and SsoAdvanced™ Universal SYBR® Green Supermix were purchased from Bio-Rad Laboratories (Hercules, CA USA). Primers for human caspase 3 and 8, cytochrome *c*, human Bax, EGFR, MAPK, p38, and mTOR were purchased from Integrated DNA Technologies (Coralville, IA USA); while primers for Akt were purchased from Sigma-Aldrich (St. Louis, MO, USA). MILLIPLEX MAP Akt/mTOR Phosphoprotein Magnetic Bead 11-Plex Kit - Cell Signaling Multiplex Assay was purchased from Millipore (Billerica, MA, USA).

5.2.2. In vitro *study*

5.2.2.1. Cell line

The 4T1 breast cell line derived from mouse metastatic site was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI medium (with L-glutamine and sodium bicarbonate) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin antibiotic mix (ThermoFisher Scientific, Grand Island, NY, USA) and maintained at 37ºC with a humidified 5% CO_2 atmosphere. For cell culture experiments, a stock solution of *C. procera* crude phenolic extract (~0.1 g/mL) dissolved with 0.6% dimethylsulfoxide (DMSO) in phosphate buffer solution (PBS) was used. Total phenolics in *C. procera* crude extract stock solution were quantified as gallic acid equivalent (GAE) using the Folin–Ciocalteu method (Kaur and Kapoor, 2002). Cells were treated with *C. procera* crude phenolic extracts (CphE) at 31-500 µg/ml GAE/mL dose-range. The flavonoid quercetin (Sigma-Aldrich, San Louis, MO, USA) was dissolved with 0.6% DMSO in PBS to obtain a 100 µM stock solution to be used in cell culture as positive control.

5.2.2.2. Cell viability

To evaluate the effect of CphE on cell viability, the *in vitro* toxicology resazurin based assay kit was used (Sigma-Aldrich, San Louis, MO, USA). Briefly, 4T1 cells seeded in a 96-well plate were allowed to reach approximately 80% confluence before treatment with CphE (31 to 500 µg GAE/mL) or quercetin (2-10 µM) for 24 and 48h according to the manufacturer's protocol. Relative fluorescence units (RFU) were measured at 560 nm and 590 nm excitation and emission wavelength, respectively using the FLUOstar Omega plate reader (BMG Labtech, NC, USA). Cell viability (% of control) was calculated as: [RFU sample/ RFU control] × 100. The dose needed to inhibit cell viability by 50% (IC_{50}) was calculated to be used in following experiments.

5.2.2.3. Evaluation of reactive oxygen species (ROS) production

The 4T1 cells seeded in a 96-well plate were allowed to reach approximately 100% confluence before treatment with CphE (31 to 250 µg GAE/mL) or quercetin (4 to 8 µM) for 24 and 48 h. Production of ROS was assessed with the Carboxy-H2DFFDA probe (10 µM) (Fisher Scientific, Pittsburgh, PA, USA) following the manufacturer's protocol. RFU were measured at 480 nm excitation and 520 nm emission using FLUOstar Omega plate reader (BMG Labtech, NC, USA). RFU were normalized to cell density assessed using the Janus green reagent (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells fixed with 100% methanol (100 µL) for 3 min were air-dried and stained with 100 µL of Janus green (1 mg/mL) for 3 min. Stained cells were washed 2X with PBS and Janus green crystals were dissolved with 100 µl of 50% methanol. Cell density was proportional to absorbance at 654 nm.

5.2.2.4. Wound healing assay

Cells seeded on 24-well plates were allowed to reach 100% confluence. Well surfaces were scratched with 200 µL sterile tip to form a wound. Debris was removed by washing twice with PBS followed by CphE or quercetin treatments at dose shown to inhibit cell viability by 50% (IC_{50}) in 10% FBS-supplemented culture medium. Cell migration to wounded area was assessed after 24 and 48 h of treatment through

microphotographs from random fields ($n \geq 3$) taken with Keyence BZ-X710 Microscope (40X). The number of cells that migrated towards the wounded area were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). Data from at least three independent experiments was analyzed with Graph Prism 5.0 (San Diego, CA, USA).

5.2.2.5. Multiplex magnetic bead-based immunoassay

Cell and tissue lysates were analyzed using the MILLIPLEX Akt/mTOR Phosphoprotein Magnetic Bead (Millipore, Billerica, MA, USA) to quantify total proteins involved in tumor growth and metastasis like insulin receptor (IR); insulin receptor substrate 1 (IRS-1); insulin-like growth factor-1 (IGF-1); phosphatase and tensin homolog deleted on chromosome 10 (PTEN); Akt, mTOR, P70S6k, RPS6, glycogen synthase kinase 3 α (GSK3-α) and b (GSK3-b); and tuberous Sclerosis Complex 2 (TSC2) following the manufacturer's protocol on a Luminex system. Data were analyzed using xPonent 3.1 software (Austin, TX, USA).

5.2.3. *In vivo* study

In order to investigate the anti-invasive and anti-metastasis effects of *C. procera* phenolic extracts, orthotopically injection of 4T1 cells was performed into mammary pads ($1x10^6$ cells/100 uL growth factor reduced matrigel, BD Bioscience, San Jose, CA). The mice were allowed to adjust to their environment for one week and anesthetized using isoflurane before 4T1 cells injection into the right and left targeted mammary fat pad. Mice were allowed to recover from anesthesia and placed back into it's home cage for 9 days for tumor growth. After this time, animals were fed with CphE resuspended in PBS (150 mg GAE/kg body weight/day, n = 2) or PBS (control, n = 2) by gavage for one week. Body weight and tumor volumes were measured every other day. Tumor volume was calculated as a^2 x b/2, were "a" was the shortest and "b" was the longest diameters of tumors.

Animals were terminated by CO_2 asphyxiation followed by cervical dislocation. Tumors and major organs (lungs, liver, kidneys, heart, and spleen) were harvested, flash-

frozen with liquid nitrogen and stored at -80ºC for furture analyses. This study was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (IACUC 2017-0378).

5.2.4. Protein expression analyses

4T1 cells seeded onto 10 cm culture plate were allowed to reach 90% confluence before overnight serum starvation in FBS-free medium followed by treatment with CphE or quercetin in culture medium supplemented with 3% FBS. Cell lysates were obtained after 24h or 48h treatments using xTractor Buffer (Takara Bio Company, Mountain View, CA, USA) suplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA) following the manufacturer's protocol.

Tumor tissues were grinded with liquid nitrogen and lysed with xTractor buffer as above.

Cell and tissue lysates were analyzed for protein concentration using Bradford reagent (BioRad, Hercules, CA, USA) according to manufacturer's protocol. Cell and tissue lysates (60 μg) were subjected to electrophoresis and wet blotting transfer onto 0.46 μm nitrocellulose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes blocked with 5% skim milk dissolved in 0.1% tween in PBS (PBS-T) for 60 min were incubated with primary and secondary antibodies as detailed by Noratto et al. (2011). Protein bands were detected with luminal reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) after 1 min of reaction. The band intensities were quantified using the Image-J software.

5.2.5. Gene expression analyses

4T1 cells starved overnight and treated with CphE or quercetin for 24 h at IC_{50} doses were subjected to mRNA extraction using the the Quick-RNA-MicroPrep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's protocol.

Tumors, liver and lung tissues grinded with liquid nitrogen were subjected to mRNA extraction using Direct-zol-RNA-MiniPrep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's protocol.

The reverse transcription supermix iScript™ (Bio-Rad, Hercules, CA, USA) was used to synthesize cDNA from mRNA followed by amplification using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) using specific pairs of primers to perform real-time polymerase chain reaction (RT-PCR). Relative mRNA levels were calculated as reported (Schmittgen and Livak, 2008). Primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA, USA), except for Twist, Cenpf that were purchased from Sigma-Aldrich (St. Louis, MO, USA). GAPDH was used as housekeeping gene. Primer sequences are presented in supplementary Table 1.

5.2.6. Statistical analysis

Quantitative data represent mean values with standard error of the mean (SEM) corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post test, where treatments were compared to controls. Data were analyzed using GraphPad Prism 5.0 (San Diego, CA, USA).

5.3. Results

5.3.1. CphE reduced 4T1 breast cancer cell viability and modulated ROS production

Results showed that CphE treatments for 48 h reduced cell viability in a dose-depenent manner up to 125 µg/mL (IC_{50} = 49.6 µg/mL) (Fig. 1A). The dose- response cell viability inhibition was consistent with reduction of ROS production down to 70% of control within same dose-range (Fig. 1B). However, the ROS levels increased in 21% in cells treated with 250 µg/mL CphE compared to those cells treated with 125 µg/mL. Cancer cells use ROS signaling to stimulate proliferation and other events necessary for tumor progression (Schumacker, 2006); therefore, inhibition of ROS generation exerted by CphE within 31-125 µg/mL may contribute to slow down the rate of tumor progression. However, CphE treatment at 250 µg/mL reversed the trend in ROS

generation suggesting that 125 µg/mL is the threshold for ROS-mediated tumor growth downregulation.

Quercetin also reduced cell viability at 48 h within a dose-range of 2-8 µM (IC_{50} = 5.79 µM) (Fig. 1C). However, in contrast to the CphE, quercetin induced ROS generation within same dose-range. These results suggest a contrasting response from 4T1 breast cancer cells to individual phenolics present in CphE regarding ROS generation. The quercetin-induced ROS production seemed to be counteracted by the other phenolics in CphE when total phenolics are below 125 µg/mL. This may explain that above 125 µg/mL ROS levels increased (Fig. 1D).

Based on these results, the IC_{50} concentrations for CphE and quercetin were used for future experiments to investigate the underlying mechanisms involved in cell viability inhibition.

5.3.2. CphE induced caspase-dependent apoptosis in 4T1 breast cancer cells

In order to investigate whether the apoptotic pathway could contribute to the reduction of cell viability, it was investigated the role of CphE in apoptosis. Results from gene expression showed no changes in mRNA levels of biomarkers of the intrinsic mitocondrial apoptotic pathway Bax, cytochrome C, as well as the executioner caspase-3 in cells treated with CphE or quercetin (data not shown). However, these molecules undergo significant post- translational regulation, especially through phosphorylation and proteolytic processing (Subasic et al., 2016; Cui et al., 2018). Results from protein expression analysis showed that CphE promoted a significant increase in cleaved caspase-3/caspase 3 ratio in 48 hours, similarly to quercetin (Fig. 2A, B). The activation of effector caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates including PARP. After treatment with CphE for 48 h, a reduction in total PARP was observed (Fig. 2A, C). Quercetin also decreased total PARP levels (Fig. 2A, C), indicating that quercetin contributes to caspase-dependent apoptosis.

5.3.3. CphE dowregulated Akt/mTOR pathway in 4T1 breast cancer cells

The Akt/mTOR pathway play a major role in regulating cell growth, survival and differentiation (Kilbas et al., 2019). Results from mRNA analyses showed mTOR upregulation induced by CphE and quercetin at similar levels after 24 h treatment (Fig. 3A), while the Akt mRNA levels showed no difference compared to control (data not shown).

Protein phosphorylation is one of the most important post-translational modifications that plays a key role in cell signaling and gene expression pathways. In addition, an upregulation of protein phosphorylation (e.g. Akt and mTOR) have been reported in different types of cancer and are related to a poor cancer prognosis (Ardito et al., 2017). Accordignly, results showed that CphE and quercetin dowregulated the phosphorylation of Akt and its downstream mTOR at similar levels after 48 h treatment (Fig. 3B, C).

Because phosphorylated Akt is known to activated transcription factors, such as response element-binding protein (CREB), which regulates the transcription of genes involved in cell metabolism, growth and migration, we next examined the effects of CphE on CREB phosphorylation. Our results demonstrated a dowregulation in CREB phosphorylation after treatment with CphE and quercetin for 48 hours (Fig 3D), suggesting that quercetin contributes to the effect of CphE in reducing p-CREB.

It is known that Akt is a serine/threonine kinase that phosphorylates over 100 protein substrates and is often itself hyperphosphorylated in various tumor types (Xie et al., 2016). Among the Akt/mTOR pathway proteins analyzed by multiplex immunoassays, only the upstream regulators of Akt IR and PTEN were modulated by CphE and/or quercetin treatments between 24-48 h (Figure 3E); as well as the downstream of Akt GSK3-α, GSK3-b, TSC2, mTOR, p70S6K, and RPS6 (Figure 4A-F).

Results showed that CphE downregulated the oncogens IR and Akt down to 2.4 and 1.5-fold of control, respectively. Quercetin treatment failed to modulate levels of total IR and total Akt (Fig 3E). The IGF-1 levels were also downregulated by CphE treatment to 1,2-fold of control, but did not reach significance ($p > 0.05$) (data not shown). However,

both CphE and quercetin downregulated total PTEN at similar levels (down to 1.3- and 1.37-fold of control, respectively). PTEN is known to be modified by phosphorylation, acetylation and oxidation to act as a tumor suppressor (Chen et al., 2018); therefore, the reduction in total PTEN levels is probably occurring to promote its active form. But more studies would have to be carried out to confirm this hypothesis.

Downstream of Akt, the GSK3α plays a paradoxical role as a tumor suppressor or a tumor promoter. However, evidence suggests that GSK3 inhibits p53, PTEN and other molecules responsible for controlling tumor growth, while is stimulated by Akt (Hermida et al., 2017). Results showed that ChpE promoted a dowregulation in GSK3α levels to 2.4-fold of control, while quercetin did not modulate this parameter (Fig 4A). On the other hands, only quercetin dowregulated GSK3-b in 1.4-fold of control (Fig 4B). Likewise, the TSC2 complexes with TSC1 to inhibit the protein G Rheb thereby inhibiting mTOR (Hua et al., 2019). Akt also plays a role on TSC2, but in an inhibitory way through phosphorylation (Huang and Manning, 2008). Results showed that TSC2 was downregulated by CphE to 2.2-fold of control and quercetin did not modulate this parameter (Fig 4C).

Modulation of the Akt target mTOR complex has been identified as a relevant target for cancer treatment due to its role as regulator of growth and metabolism. Moreover, mTOR drives multiple anabolic pathways and is closed related to proliferation and metastasis. Due that, several anticancer therapies have focused on inhibiting mTOR (e.g. rapamycin) (Xie et al., 2016). Results showed that treatment with CphE dowregulated mTOR to 5.8-fold of control, which was consistent with the reduction of mTOR targets p70S6K and RPS6 (1.5- and 3.1-fold of control, respectively) (Fig. 4D-F).

5.3.5. CphE modulated MAPK signaling pathway in 4T1 breast cancer cells

MAPKs signaling pathway regulate a variety of biological processes in a dual way since they can act as an activator or inhibitor, depending on the cell type and the stimulus. For example, MAPKs may act as oncogene when is activated by growth factors or as pro-apoptotic when is activated by cellular stress (Yue and López, 2020). Because of this we

evaluated the mRNA levels of MAPK-ERK1/2, epidermal growth factor receptor (EGFR), and p38 after treatment with CphE and quercetin throughout 24h. With the exception of quercetin which reduced MAPK levels, the other parameters were not modulated (data not shown).

We also analyzed p38 and ERK1/2 protein expression. Results showed that p38 phosphorylation was upregulated only by quercetin treatment (Fig 5A, B). These results suggest that p38 may be involved in apoptosis activation by quercetin, since p38 is able to promote dissociation of mitochondrial permeability transition pore (mPTP) and activate cell death (Gräb and Rybniker, 2019).

ERK1/2 has generally been associated with cell survival and proliferation, however, activation of ERK can also mediate cell death through ROS and oxidative stress (Mebratu and Tesfaigzi, 2009). Results showed that ERK1/2 phosphorylation was down and up regulated by CphE and quercetin, respectively (Fig. 5A, C). Results seem to have a rational approach since quercetin induced ROS, which would increase oxidative stress and ERK1/2 phosphorylation to mediate mitocondrial permeabilization. However, CphE has shown an oposite effect on ROS generation, therefore as a whole mixture of phenolics the overall effect seems to be the decrease in oxidative stress - cell growth signaling cascades such as p-ERK1/2.

5.3.6. CphE lessened epithelial-mesenchymal transition in 4T1 breast cancer cells

Src and FAK are closely related to the epithelial-mesenchymal transition and play a central role in promoting cell proliferation and anoikis resistance that allows survival after detachment from the primary sites and travel through the circulatory and lymphatic systems (Bolós et al., 2010). Results demonstrated that CphE dowregulated Src phosphorylation at higher levels than quercetin (Fig 6A, B). Src regulates several downstream signaling pathways including the STAT3 pathway, the PI3K pathway and the MAPK pathways (Bjorge, 2011) as well as the FAK phosphorylation that lead to invasion, migration, and adhesion (Mayer, 2010). Unexpectedly, the Scr modulation by CphE was not consistent with changes in p-FAK levels, as it was in cells treated with quercetin (Fig. 6A, C). This suggests that CphE Scr-induced downregulation and its

dowwnseam targets Akt and MAPK/ERK1/2 pathways may be the primary cell signaling pathways modulated by CphE to inhibit cell growth and invasion; while quercetin alone may target also FAK pathway to contribute to its anti-invasive and antiproliferative activity on 4T1 cells.

The anti-invasive potential of CphE and quercetin was furhter confirmed by the cell wounding assay (Fig. 6D, E). Wound healing was suppressed by CphE and quercetin, in which treatments decreased the number of cells that invaded the wounded area down to 60 and 78%, in 24h, and by 6% and 10% in 48 hours, respectively.

5.3.7. CphE inhibited breast tumor growth *in vivo* cancer dowregulated mTOR and ERK1/2 in xenograft tumors in nude mice

Results from pilot trial confirmed the anticancer activity of CphE as shown in the reduced volume and weight of tumors. However, there was not significance due to the lack of power of this pilot test (Fig 7A, B).

Furthermore, results from levels of mRNA in tumor tissues supported the *in vitro* findings regarding the modulation of tumor growth related signaling molecule mTOR that decreased down to 1.8-fold of control (Fig 7C) and the pro-apoptotic biomarker caspase-8 that was upregulated to 5-fold of control (Fig 7C). Likewise, phosphorylation levels of ERK1/2 in tumor tissues supported the CphE *in vitro* activity in inhibing tumor growth (Fig. 7D).

5.3.8. CphE modulated pro-invasive Twist and Cenpf mRNA levels *in vivo*

The assessment of expression levels of genes that in distant organs is a common technique to assess the anti-invasive potential of anticancer plant extracts. Evidence has increasingly shown that Twist and Cenpf are proteins that act as stimulators of EMT process contributing to metastasis (Wang et al., 2018; Sun et al., 2019). Results showed that Twist mRNA levels in lungs were significantly downregulated to 2.8-fold of control, while in liver decreased but did not reach statistical difference (Fig. 7E). When assessing

the Cenpf, we found a significant reduction in the liver and lung (3.5 and 2.2-fold of control, respectively) (Fig 7F).

5.4. Discussion

Calotropis procera has been studied extensively within the last years because of its comprehensive medicinal value. During our investigation it was possible to observed that CphE can directly inhibit 4T1 cell growth *in vitro*, indicating that the cytotoxic effect may be beneficial to breast cancer patients. The *C. procera* cytotoxicity has also been demonstrated in MCF-7 and MDA-MB-435 breast cancer cell lines (Samy et al., 2012; Al-Taweel et al., 2017). However, in these studies the breast cancer cells were treated with a protein named CP-P isolated from root bark, and the whole flowers extracts, respectively, instead of phenolic compounds. Much of the cytotoxic effect promoted by *C. procera* can be attributed to the set of phenolic compounds such as flavonoids, lignans, and triterpenoid (Mohamed et al., 2011; Sweidan and Zarga, 2015; Al-Taweel et al., 2017); but also to cardenolides (e.g. calotropin, and 2"-oxovoruscharin) that have potential in inhibiting Na^+/K^+ATPase contributing to cell death (Juncker et al., 2009; Van Quaquebeke et al., 2005). Quercetin treatments also reduced 4T1 cell viability (Fig 1C), suggesting that it contributed to the anticancer effect of the CphE. Consistently with previous reports, quercetin has shown to inhibit the growth of 4T1 and MCF-7 cells within dose range 5-40 μM and 10-120 μM, respectively (Kim et al., 2013; Niazvand et al., 2019).

ROS is a generic term for unstable, reactive and partially reduced oxygen derivatives from normal and/or pathological processes (Yang et al., 2018). The literature demonstrates that there is an over-expression of ROS in breast cancer cell lines in relation to non-tumoral breast cells and mitochondria is the main source of ROS in TNBC cell lines (Yang et al., 2018; Sarmiento-Salinas et al., 2019). In this study, CphE treatment reduced ROS levels, while quercetin induced their production (Fig. 1B, D). This ability to reduce ROS has been reported for the flavonoids present in CphE (e.g. kaempferol and rutin) (Mohamed et al., 2011; Oraibi and Hamad, 2018). These flavonoids may induce the production of antioxidant enzymes and modulation of oxidative stress (Roy et al.,

2005; Chaudhary et al., 2015). Furthermore, kaempferol has been reported to activate the nuclear erythroid 2-related factor 2 (Nrf2); a transcription factor that play a key role on the expression of genes that mount strong antioxidant responses (Pallauf et al., 2017). Quercetin, in turn, despite being a flavonoid, promoted an increase in ROS over time in 4T1 cells. The increase in ROS promoted by quercetin may occur due to its potential to donate electrons for scavenging reactive peroxyl radicals. The resultant quercetin radicals enter the redox cycle to potentially increase ROS (Jeong et al., 2009). However, this increase in ROS levels promoted by quercetin appears to be beneficial, since its is closely related to induction of apoptosis in different types of cancer (e.g. breast and cervical carcinoma) (Bishayee et al., 2013; Niazvand et al., 2019), and inhibition of cell cycle progression of human breast carcinoma (Jeong et al., 2009). Thus, it appears that the other phenolic compounds present in CphE are overcoming the pro-oxidative effect of quercetin.

Apoptosis is one of the most potent mechanisms against cancer and appears to be induced by many chemopreventive agents, including plant extract (Kuno et al., 2012). Caspase-3 is considered to be the most important of the executioner caspases (Elmore et al., 2007). CphE induced apoptosis via upregulation of cleaved caspase-3, reflecting the reduction in total PARP (Fig 2A-C). PARP cleavage by caspase 3 is considered a hallmark of apoptosis (Elmore et al., 2007); CphE promoted the reduction of total PARP, suggesting that it is being cleaved by caspase-3, thus completing programmed cell death. Our findings confirmed a high pro-apoptotic potential of *C. procera* by targeting apoptotic mechanisms, and demonstrated for the first time, to our knowledge, the potential to cleave caspase-3. This result is supported by other studies. For example, treatment with methanolic extract of *C. procera* activated apoptosis in human skin melanoma cells (SK-MEL-2), mostly caspase independently way (Joshi et al., 2015). In another study, *C. procera* reduced hepatoma cell viability (Hub-7) through an increase in cell DNA fragmentation, but without changes in Bcl-2 or caspase-3 levels; suggesting apoptosis independent of caspase (Choedon, 2006). Quercetin also induced apoptosis in 4T1 cells (Fig. 2A-C), being consistent with its potential for activating apoptosis in human breast cancer cell line MDA-MB-231 (20 μM) and cervical carcinoma (HeLa) cells (90 μM) (Bishayee et al., 2013; Nguyen et al., 2017). Interestingly, quercetin needed a much

lower dose (5.79 μM) to activate apoptosis in 4T1 cells, compared to MDA-MB-231 (20 μM) and HeLa cells (90 μM) (Bishayee et al., 2013; Nguyen et al., 2017). With these results in mind, it appears that the activation of apoptosis exercised by ChpE is due to the set of phenolic compounds present in its leaves, including quercetin; however independently of ROS.

Aberrations in the PI3K/Akt/mTOR pathway are among the most common genomic abnormalities in breast cancer, and is also involved in drug resistance (Costa et al., 2018; Kilbas et al., 2019). In breast cancer, Akt is mutated by approximately 2.5%, contributing to tumor growth and anti-apoptotic activity (Pascual and Turner, 2019). The phosphorylation of Akt is required for its full activation (Kilbas et al., 2019). Results showed that Akt phosphorylation's were very potently suppressed by CphE and quercetin, suggesting that Akt is one of the targets of phenolic compounds present in *C. procera* (including quercetin) to reduce cell proliferation. This fact is totally unprecedented for the species *C. procera*, since until now only coroglaucigenin, the steroid glycoside isolated from *C. gigantean*, had demonstrated a potential to modulate Akt and reduce proliferation in colorectal cancer cells (Huang et al., 2008). On the other hands, quercetin had already demonstrated a potential to modulate Akt in other breast cancer cells (MDA-MB-231 and MCF-7, respectively) (Rivera et al., 2016; Cao et al., 2018). Similarly to Akt, mTOR is also activated by phosphorylation to promote the growth and spread of TNBC cells (Guerrab et al., 2020). Results also showed a dowregulation in mTOR expression promoted by CphE and quercetin, while mRNA levels was upregulated. These results suggest that the mTOR pathway is a target of CphE and quercetin as underlying anti-proliferative mechanism, which contributed to reducing the viability of 4T1 cells.

Akt/mTOR pathway can still modulate the activity of several transcriptional factors, such as CREB. When CREB is activated, expression of antiapoptotic genes such as Bcl-2 and mcl-1 occurs allowing tumor progression (Ortega et al., 2020). In our study, both CphE and quercetin promoted dowregulation of CREB phosphorylation, which must have occurred due to the downregulation of Akt/mTOR. This is the first time, to our knowledge, that CphE is being related to p-CREB dowregulation. This is especially relevant considering that CREB is related to the progression of breast cancer and its

signaling serves as a therapeutic strategy for triple-negative breast cancer (Qin et al., 2020).

Akt activation is complex and controlled by several processes, with the PI3K-dependent pathway being the most common. When PI3K is activated there is phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2), generating PIP3. PIP3 levels are strongly regulated by the action of phosphatases such as PTEN, which act as an inhibitor of Akt activation (Nicholson and Anderson, 2002; Kilbas et al.,2019). In our study, CphE promoted dowregulation in total PTEN, suggesting that PTEN may be being converted to phosphorylated form, which means its active form. However, the regulation of total protein levels is not always correlated with changes in phosphorylation pathways. Therefore, changes in PTEN phosphorylation remain to be investigated to confirm whether treatment with CphE induced the corresponding signal transduction pathway.

The insulin and the insulin-like growth factors I and II (IGF-I and IGF-II) signaling is mediated by hormone interaction with cognate tyrosine kinase receptors, IR and IGF-IR. They can still catalyze the phosphorylation of specific substrates, such as IRS-1 to IRS-4. Following tyrosine phosphorylation, IRS proteins interact with growth factor receptor binding protein 2 (GRB2) and with the p85 regulatory subunit of PI3K, activating Akt (Malaguarnera and Belfiore, 2011). In breast cancer, IR/IGF-IR signaling is often deregulated with consequent loss of signaling specificity and overlap between IR and IGF-IR actions (Malaguarnera and Belfiore, 2011). Herein, a reduction in IR was found after CphE treatment, contributing to Akt dowregulation.

Of the many pathways and processes downstream of Akt, we focus here on multi-functional Ser and Thr protein kinase glycogen synthase kinase 3 (GSK3); TSC2 and mTOR. There are two highly homologous forms of GSK-3 in mammals, GSK-3α and GSK-3β, both can be inactivated through phosphorylation promoted by Akt (Ougolkov and Billadeau, 2006; Malaguarnera and Belfiore, 2011). In human breast carcinoma, GSK-3β overexpression has been shown to be associated with several indicators of poor prognosis. Moreover, breast cancer patients with GSK-3β expression in the highest quartile had an increased risk of distant relapse (Ugolkov et al., 2016). In our study, CphE reduced GSK-3α, whereas quercetin reduced GSK-3β. These results suggest that the

amount of quercetin in CphE seems to not be sufficient to reduce GSK-3β. However, GSK-3α reduction promoted by CphE must be related to other phenolic compounds (e.g. lupeol and kaempferol) that have already demonstrated the potential to modulate this protein (Harish et al., 2008; Ren et al., 2019).

Active Akt phosphorylates TSC2 inhibiting the formation of the TSC complex (Mehta et al., 2011). The TSC complex inhibits the protein G Rheb thereby inhibiting mTOR (Hua et al., 2019). When TSC2 complex is not functional, there is uncontrolled mTOR activity leading to cell growth and tumor formation (Mehta et al., 2011). In our study, CphE and quercetin promoted a reduction in total TSC2 protein levels. This data suggest two points: First, the reduction in TSC2 promoted by CphE and quercetin is probably independent of Akt, since this protein was dowregulated and could not then inhibit TSC2. Second, the reduction of mTOR by CphE appears to be independent of TSC2.

The 70 kDa ribosomal protein S6 kinase (p70S6K) has contributed to the development of breast cancer (Miller et al., 2011). For instance, a study conducted with breast tissue biopsy from women with high grade of Tamoxifen resistance revealed high levels of Akt/p70S6K activation and confirmed that Akt/p70S6K signaling pathway was activated in early stage breast disease (Wang et al., 2017). In our study, the mixture of phenolics in CphE reduced p70S6K levels in 4T1 cells more effectively than quercetin alone. In addition, CphE also reduced ribosomal protein S6 (rpS6) levels, a protein that has been linked to the development of HER-2+ cancers (Yang-Kolodji et al., 2015). The downregulation of these proteins confirm that mTOR is one of the pathways targeted by CphE that contributed to inhibit cell growth.

The stimulation of PI3K/Akt and MAPK cascades may also become unbalanced with the consequent amplification of mitogenic signals (Malaguarnera and Belfiore, 2011). MAPK pathway is one of the most important regulatory mechanisms in eukaryotic cells. MAPKs mainly regulate the functions of other proteins through phosphorylation and play an important role in the occurrence, development and metastasis of multiple tumors (Jiang et al., 2020). In TNBC cell lines, for example, MAPK plays a central role in the expression of ER, PR and HER-2, being closely related to metastasis and poor

prognosis (Zhao et al., 2017). In addition, MAPK can activate ER in response to estrogen deprivation and thus facilitate the interaction of ER and coactivators, leading to resistance from endocrine therapy (Peng et al., 2017). EGFR is one of the receptor tyrosine kinases that can activate the MAPK pathway (Rachakhom et al., 2019). CphE treatments did not modulate ERK or EGFR at gene expression levels, in contrast to quercetin which decreased MAPK-ERK mRNA levels at significant levels ($p < 0.05$). But interestedly, CphE and quercetin modulate ERK1/2 phosphorylation in oposite directions. Downregulation of phospho-ERK1/2 is a well known mechanism to reduce cell proliferation and metastasis as it blocks interaction with the extracellular matrix and reduces the crosstalk PI3K/Akt pathway (Mebratu and Tesfaigzi, 2009; Salaroglio et al., 2019; Wang et al., 2020) and seems to be the CphE underlying antiproliferative mechanism. However, quercetin seems to induce ERK1/2 phosphorylation which has been reported as stress response that leads to mitochondria permeabilization and caspase-dependent apoptosis (Mebratu and Tesfaigzi, 2009; Cook et al., 2017), as shown in Fig.1D and Fig. 2B. p38 MAPK participates in a broad range of cellular activities, being able to act as a tumor-suppressor kinase, but can also function as a tumor promoter, as demonstrated by extensive experimental data (Grossi et al., 2014; Martínez-Limón et al., 2020). In our *in vitro* study, treatment with quercetin increased the phosphorylated p38/total p38 ratio. Modulation of p38 in cancer cells by quercetin proved to be a critical step in proliferation controlling and apoptosis induction (Reyes-Farias and Carrasco-Pozo, 2019). All of these results together suggest that quercetin does induce cell apoptosis in response to stress.

Epithelial mesenchymal transition (EMT) is a complex cellular process by which epithelial cells acquire mesenchymal, migratory and aggressive properties. FAK and Src have been associated with solid tumor metastasis through their ability to promote EMT (Bolós et al., 2010). CphE and quercetin effectively reduced the expression of Src, which is responsible for FAK activation. However, only quercetin was able to reduce FAK. Studies have shown that crosstalk PI3K/Akt/mTOR/Src/FAK is essential for metastasis in breast cancer and the downregulation of this pathway can serves as a potential therapeutic target (Paul et al., 2020). In fact, quercetin contributed to reducing metastasis of epidermoid carcinoma cells through the downregulation of Akt, Src and FAK (Chen et

al., 2018). Thus, at least in part, Akt/mTOR downregulation promoted by CphE and quercetin is contributing to modulation of Src and EMT easing.

In our *in vivo* study, using 4T1 cells xenograf model, tumor diameter and weight were reduced compared to controls after CphE treatment (150 mg GAE/kg/bw), but did not reached significant difference ($p>0.05$). It has been reported that administration of organic extracts (ethyl acetate and acetone) obtained from *C. procera* (400 mg/kg) stem reduce *in vivo* tumor growth of Sarcoma 180 transplanted mice (Magalhães et al., 2010). Moreover, the aqueous suspension of *C. procera* latex (250 mg/kg/day) fed to X15-myc transgenic mice with hepatocellular carcinoma also promoted a reduction in tumor size (Choedon, 2006). Even with a lower dose than reported in other studies and a small number of animals, CphE showed a potential to reduce the tumor and be beneficial for the treatment of breast cancer.

Twist-1 protein (Twist) is a transcription factor, which is one of the master regulators of EMT process. An aberrant Twist expression is often found in different types of cancer, and is related to apoptosis inhibition, generation of drug resistance, increase in angiogenesis, and support of cancer stem cells, evidencing its role in cancer metastasis (Khan et al., 2013). Breast cancer mainly metastasizes to the bony skeleton, lungs, liver, and brain via the circulation (Ma et al., 2015). Moreover, if breast cancer liver metastasis is left untreated, the survival time is only 4–8 months (Adam et al., 2006). CphE treatment reduced significantly ($p < 0.05$) the mRNA levels of Twist in lungs, but not in the liver. In a study where MCF-7/Twist cell line were injected into the mammary fat pad of female mice evidenced the functional role of Twist in promoting breast cancer metastasis with preference to lung (Vesuna et al., 2017). Thus, it appears that CphE was able to act in the early stages of metastasis targeting the pulmonary Twist. Consistent with Twist's role in treatment resistance in breast cancer, centromere protein F (Cenpf) positively associated with poor prognosis in endocrine resistant breast cancer (Browne et al., 2018). CphE also reduced the mRNA levels of Cenpf in the lung and liver showing to be a beneficial candidate in metastatic breast cancer control.

These results are, to our knowledge, reported for the first time and strongly suggest the potential of phenolics extracted from *C. procera* as breast cancer tumor growth and

invsasive suppressors. In general, the underlying mechanisms modulated by CphE is illustrated in Fig. 8 and demonstrate that quercetin contributed to downregulate PI3K/Akt/mTOR, CREB, Src, and induce apoptosis, while antagonized to the CphE in modulation of ERK1/2 and upregulation of ROS.

5.5. Conclusion

In conclusion, we demonstrated that phenolics extracted from *C. procera* inhibited 4T1 breast cancer cell viability, in part due to the reduction of oxidative stress, and downregulation of Akt/mTOR and MAPK/ERK1/2 cell signaling pathways involved in cell growth. One of the phenolics present in *C. procera* leaves quercetin was found to contribute to the Akt/mTOR pathway downregulation, while induced ERK1/2 phosphorylation, thus antagonizing with the CphE action and likely contributing to stress mediated apoptosis induction. Quercetin still upregulated p38 contributing to cell stress and induction of apoptosis. Both CphE and quercetin induced targeted caspase-3 cleavage, which suggest a caspase dependent apoptotic pathway induced by the mixture of phenolics in CphE. Furthermore, both CphE and quercetin reduced the phosphorylation of CREB and Src, contributing to the reduction of cell migration.

The acute mice treatment of 150 mg GAE *C. procera* phenolics/kg/day for one week, even though did not reach significance due to the high variability, reduced tumor volume and tumor weigh. In addition, downregulated mRNA levels of Twist and Cenpf in lungs, and Cenpf in liver. This preliminary results are relevant considering that liver and lungs are the organs most frequently affected by metastasis from breast cancer. These results suggest that *C. procera* could provide an alternative strategy for breast cancer patients, however, further studies are needed to better evaluate its therapeutic potential.

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Figures

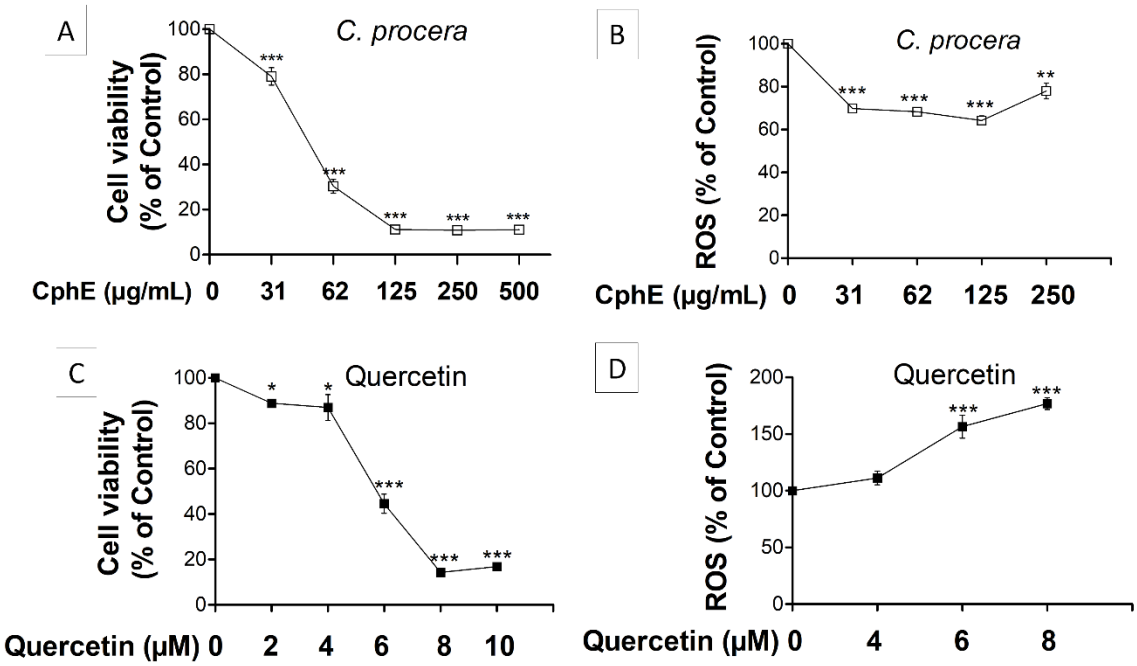

Figure 1: CphE and quercetin reduced 4T1 cell viability in part through modulating the generation of ROS in oposite directions. 4T1 cell viability (A) and ROS levels (B) after 48 h *C. procera* phenolics extract (CphE) treatment. 4T1 cell viability (C) and ROS levels (D) after 48 h quercetin treatment. Cell viability was assessed by resazurin and ROS levels were determined by carboxy-H2DFFDA probe as detailed in materials and methods. Data are average ($n \geq 6 \pm$ SEM). Comparisons against control were performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

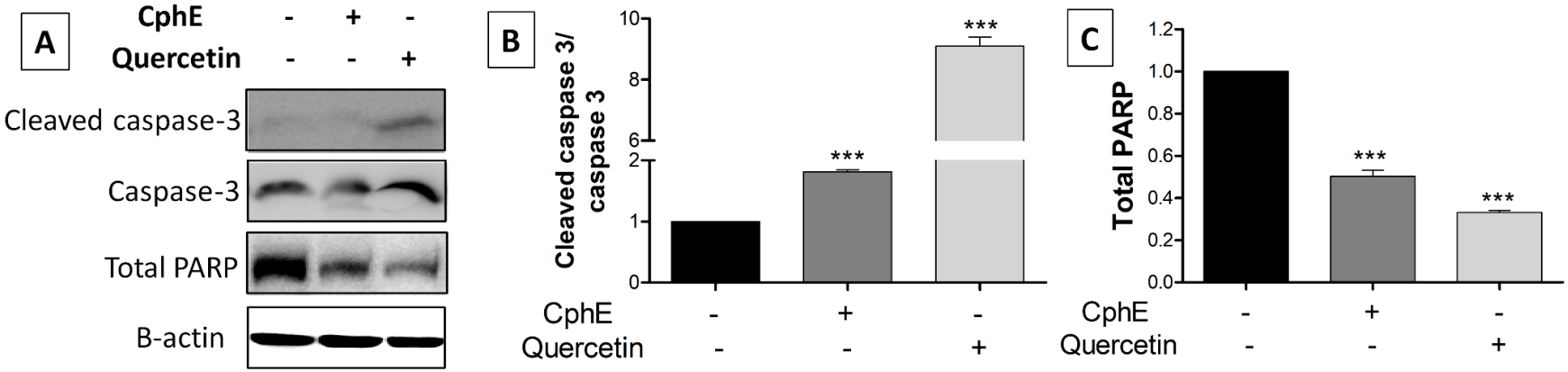

Figure 2: CphE and quercetin activate caspase-dependent apoptosis in 4T1 breast cancer cells. (A): protein band of cleaved caspase-3, total caspase-3 and total PARP after 48h of *C. procera* phenolics extract (CphE) and quercetin treatment. (B): protein levels of total and cleaved caspase-3. (C) protein levels of total PARP. Band intensities of target proteins divided by β-actin were normalized against control. Data are average ($n \geq 3$ ± SEM), performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, ***$p < 0.001$.

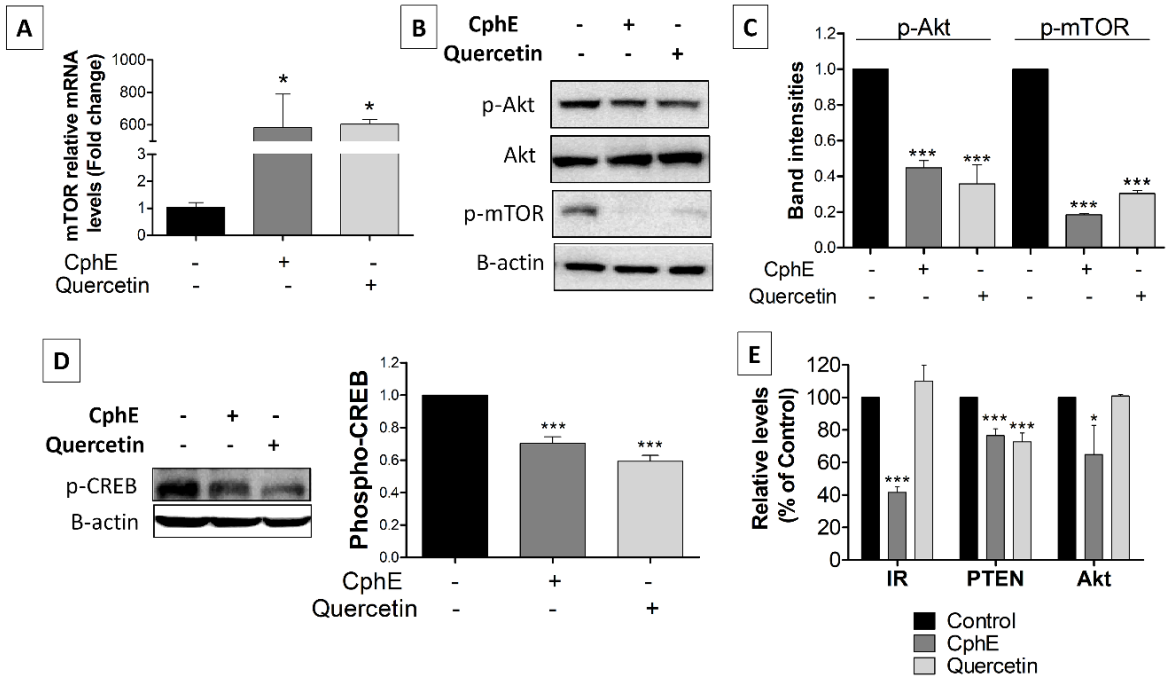

Figure 3: CphE and quercetin downregulated Akt/mTOR pathway in 4T1 breast cancer cells. (A): mRNA levels of mTOR after 24h of *C. procera* phenolics extract (CphE) and quercetin treatment. GAPDH was used as the loading control for mRNA expression analyzes. (B), (C) and (D): protein band and band intensities of total and phospho-Akt, total Akt and phospho-mTOR, and phospho-CREB after 48h of CphE and quercetin treatment. Band intensities of target proteins divided by β-actin were normalized against control. (E): Protein levels Insulin receptor (IR); Phosphatase and tensin homolog deleted on chromosome 10 (PTEN); and of Protein quinase B (Akt). Data are average ($n \geq 3 \pm$ SEM), performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, $*p < 0.05$, $***p < 0.001$.

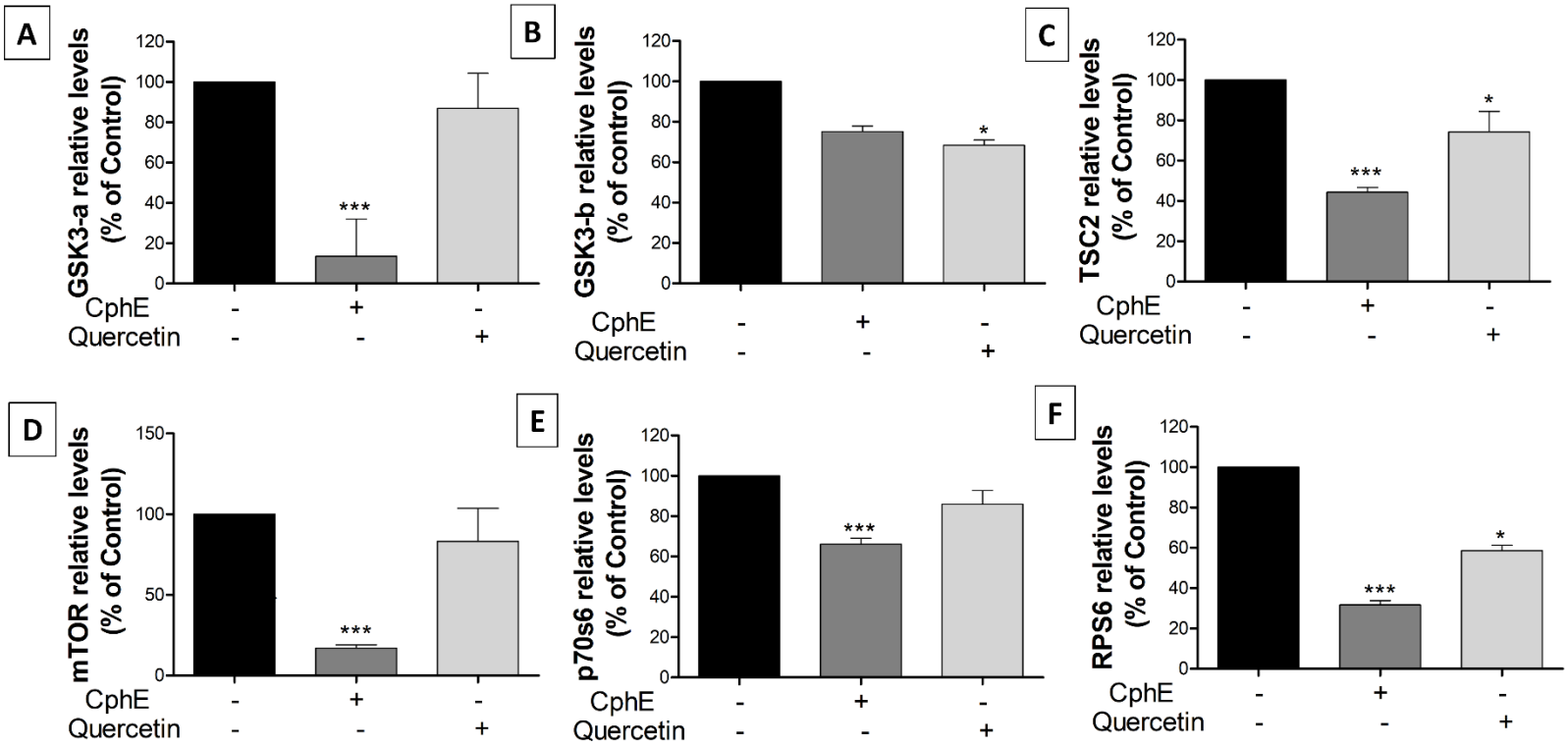

Figure 4: CphE and quercetin modulated PI3K signaling pathway in 4T1 breast cancer cells. Proteins levels of (A): Glycogen synthase kinase 3 α (GSK3-α); (B): Glycogen synthase kinase 3-b (GSK3-b); (C): Tuberous Sclerosis Complex 2 (TSC2); (D): mammalian target of rapamycin (mTOR); (E): p70 ribosomal S6 kinase (p70S6K); (F): S6 ribosomal protein (RPS6). GSK3-α and RPS6 were measured after 24 h of *C. procera* phenolics extract (CphE) and quercetin treatment; while mTOR, p70s6, and TSC2 were measured after 48 h of both treatments. Data are average ($n \geq 3 \pm$ SEM), performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, $^{**}p < 0.01$, $^{***}p < 0.001$.

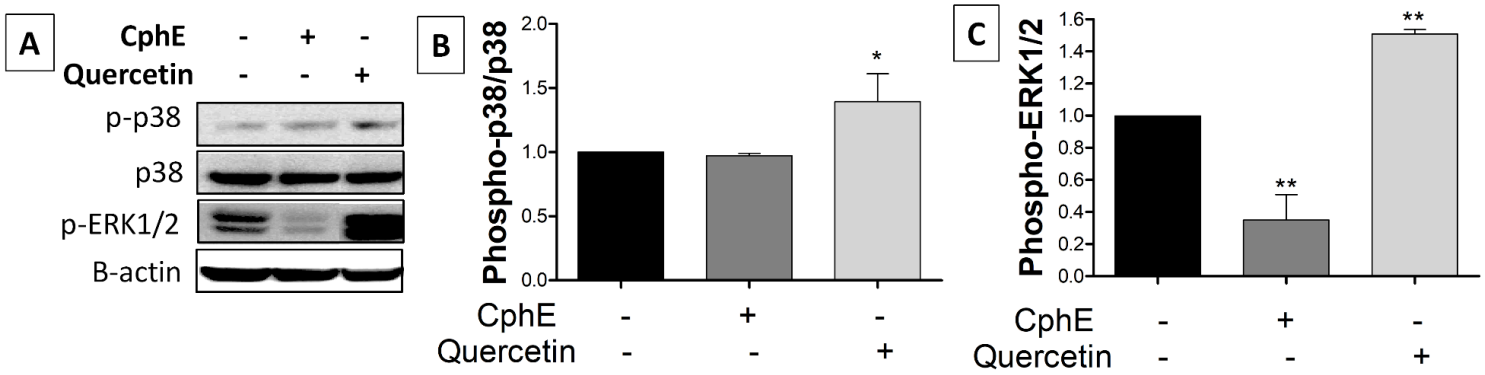

Figure 5: CphE and quercetin modulate the MAPK pathway in opposite directions in 4T1 breast cancer cells. (A): protein band of total and phospho-p38 and phospho-ERK1/2 after *C. procera* and quercetin treatment over 24h. (B) and (C): protein expression of total and phospho-p38, and phospho-ERK1/2, respectively. Band intensities of target proteins divided by β-actin were normalized against control. Data are average ($n \geq 3$ ± SEM), performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, $^{**}p < 0.01$, $^{***}p < 0.001$.

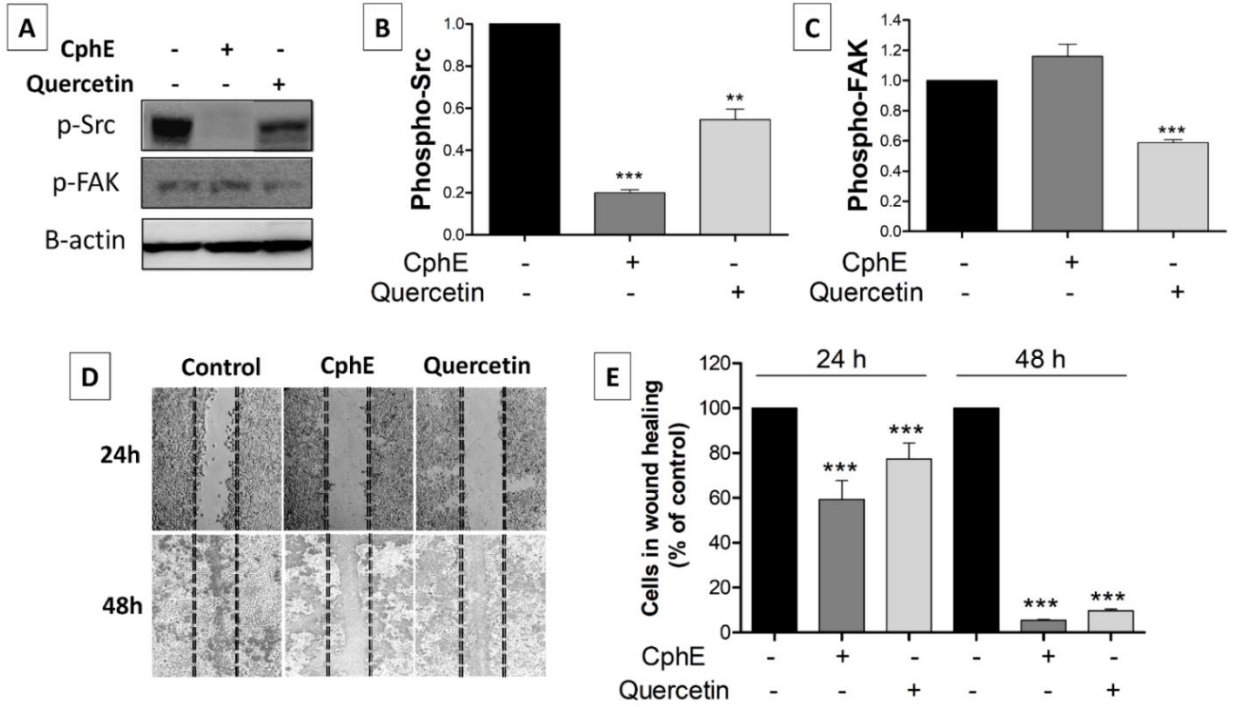

Figure 6: CphE and quercetin soften the epithelial-mesenchymal transition and reduce the migration of 4T1 breast cancer cells. (A): protein band of phospho-Src and phospho-FAK after *C. procera* and quercetin treatment throughout 24 h. (B) and (C): protein levels of phospho-Src and phospho-FAK, respectively. Band intensities of target proteins divided by β-actin were normalized against control. (D) and (E): microphotographs (40X) and quantification of migrating cells to wounded area after 24 and 48 h treatment with CphE and quercetin. Data are average ($n \geq 3 \pm SEM$). Comparisions against control groups were performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

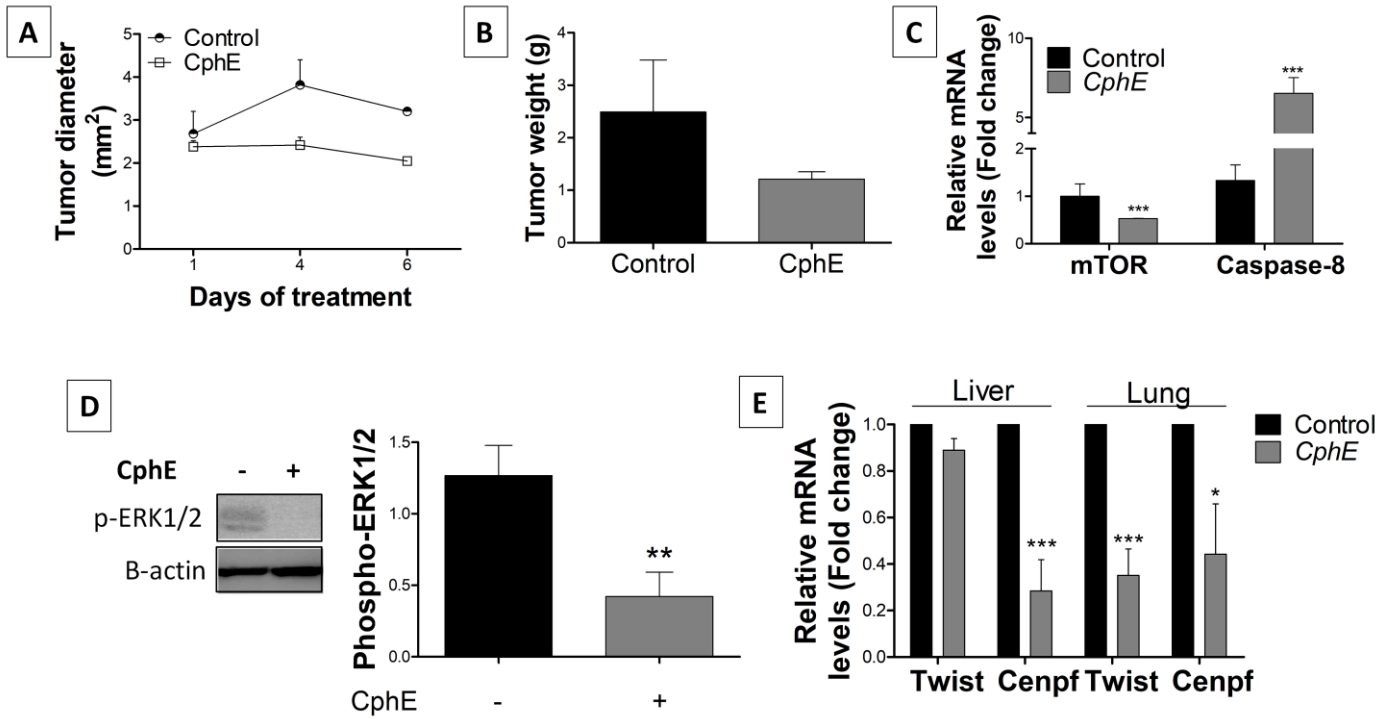

Figure 7: CphE inhibite breast tumor growth *in vivo*, dowregulated mTOR and ERK1/2, as well as modulate pro-invasive Twist and Cenpf in xenograft tumors in nude mice. (A): tumors diameter measured over a week of treatment with *C. procera* compared to the control. (B): tumors weight measured after the euthanasia. (C): mRNA levels of mTOR and caspase-8 on tumors. (D) and (E): protein band and protein expression of phospho-ERK1/2 and phospho-CREB on tumors. Band intensities of target proteins divided by β-actin were normalized against control. (F): mRNA levels of twist and cenpf on liver and lung. GAPDH was used as the loading control for mRNA expression analyzes. Data are average ($n \geq 3 \pm$ SEM), performed with one-way analysis of variance (ANOVA) as a Bonferroni post test, ***$p < 0.001$.

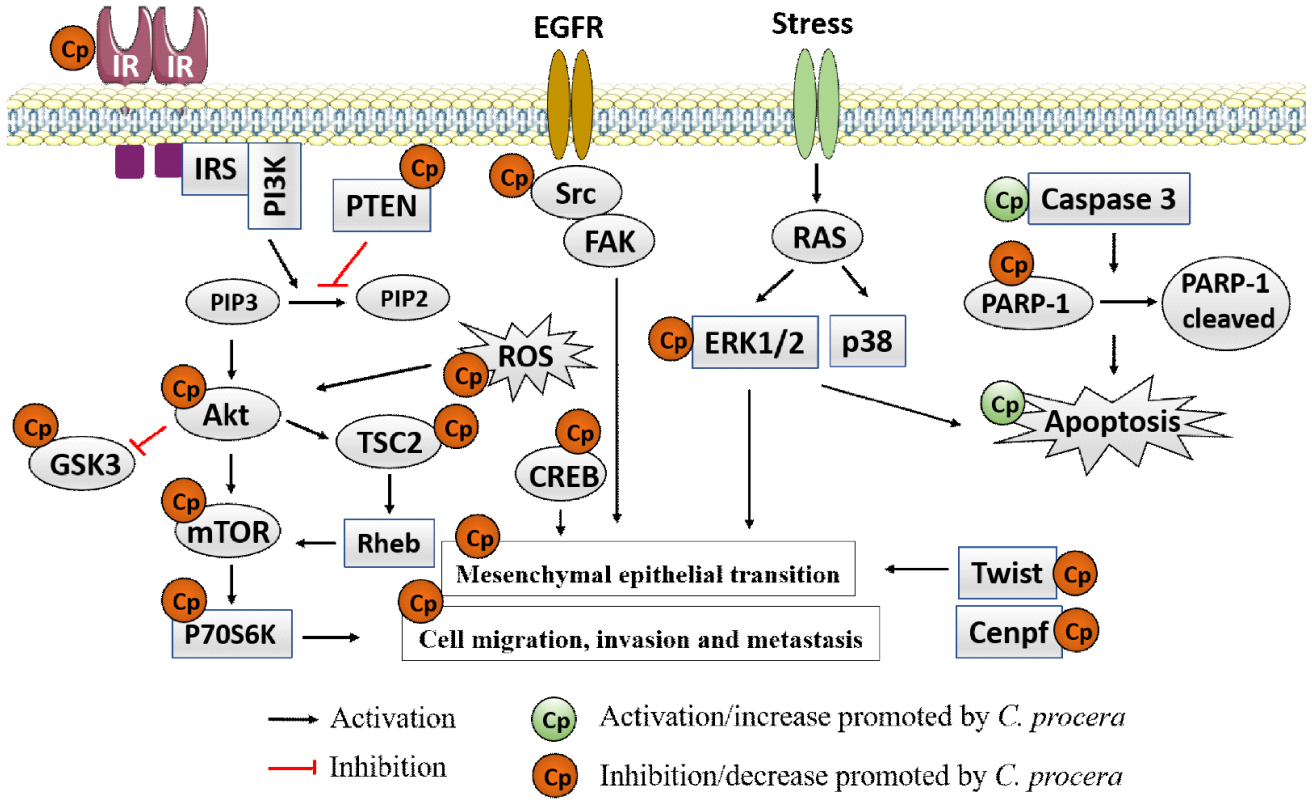

Figure 8: Schematic summary of the main pathways modulated by *C. procera*. *C. procera* reduced the migration and invasion of 4T1 breast cancer cells by dowregulating the Akt/mTOR pathway and reduced the mesenchymal epithelial transition (EMT) by reducing Src. In addition, *C. procera* modulated the MAPK pathway through ERK1/2 and p38. In addition, there was stimulation of apoptosis via increased cleaved caspase-3 and decrease in total PARP. *In vivo*, *C. procera* also reduced Twist and Cenpf levels, contributing to a reduction in EMT.

Complementary table

Table 1: Primer sequences.

Gene	Primer sequence	
	Forward	Reverse
MAPK-ERK1/2	GAAGCATTATCTTGACCAGC	TCCATGGCACCTTATTTTTG
EGFR	GCGTCTCTTGCCGGAATG	GGCTCACCCTCCAGAAGGTT
p38	GCCCGAGCGTTACCAGAAC	TGTCAAAAGCAGCACACACAGA
mTOR	CCAACAGTTCACCCTCAGGT	GCTGCCACTCTCCAAGTTTC
Akt	TCCCGAGGCCAAGTCCTT	CCGCCAAGCCTCTGCTT
Caspase-3	CTGGACTGTGGCATTGAGACA	CGGCCTCCACTGGTATTTTATG
Caspase-8	GGCTCCCCCTGCATCAC	CCTGCTAGATAAGGGCATGAATCT
Cytochrome c	TCTTGGACTCCTGACCTCGT	AAACCACCAGAAGTGCTTGG
Bax	CCAAGGTGCCGGAACTGA	CCCGGAGGAAGTCCAATGT
GAPDH	CCTCCCGCTTCGCTCTCT	TGGCGACGCAAAAGAAGAT
Twist	GAGACCTAGATGTCATTGTTTC	GAATTTGGTCTCTGCTCTTC
Cenpf	GACTTACCCAGGAGTTACAG	CTTCATTTCCTCACTGCTTC

Primer sequence used in PCR analysis. MAPK-ERK1/2: mitogen-activated protein kinase; EGFR: endothelial growth factor receptor; mTOR: mammalian target of rapamycin; Akt: protein kinase B; Bax: Bcl-2-associated protein X; Twist: twist-1 protein; Cenpf: centromere protein F; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

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5. FINAL CONCLUSION

In conclusion, *C. procera* revealed favorable antitumor effect by reducing the viability of canine tumor cells (OST and CMT) and mice cells (4T1) *in vitro* and *in vivo*. The antitumor effect seems to be related to the set of secondary metabolites [e.g. flavonoids (quercetin), glycosides, and cardenolides] found in the crude extract of the leaves of *C. procera*. At the molecular level, *C. procera* proved to be effective in reducing the proliferation of tumor cells, through the modulation of the cell cycle, the epithelium-mesenchymal transition and the main cell growth pathways (Akt/mTOR and MAPK). In addition, *C. procera* induced caspase-3 dependent apoptosis, which was particularly surprising, since this effect has not been described for the species *C. procera*. All of these results together suggest that *C. procera* could provide an alternative strategy for cancer patients, especially breast cancer. However, further *in vivo* studies with a larger sample and clinical studies are needed to better evaluate its therapeutic potential.

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