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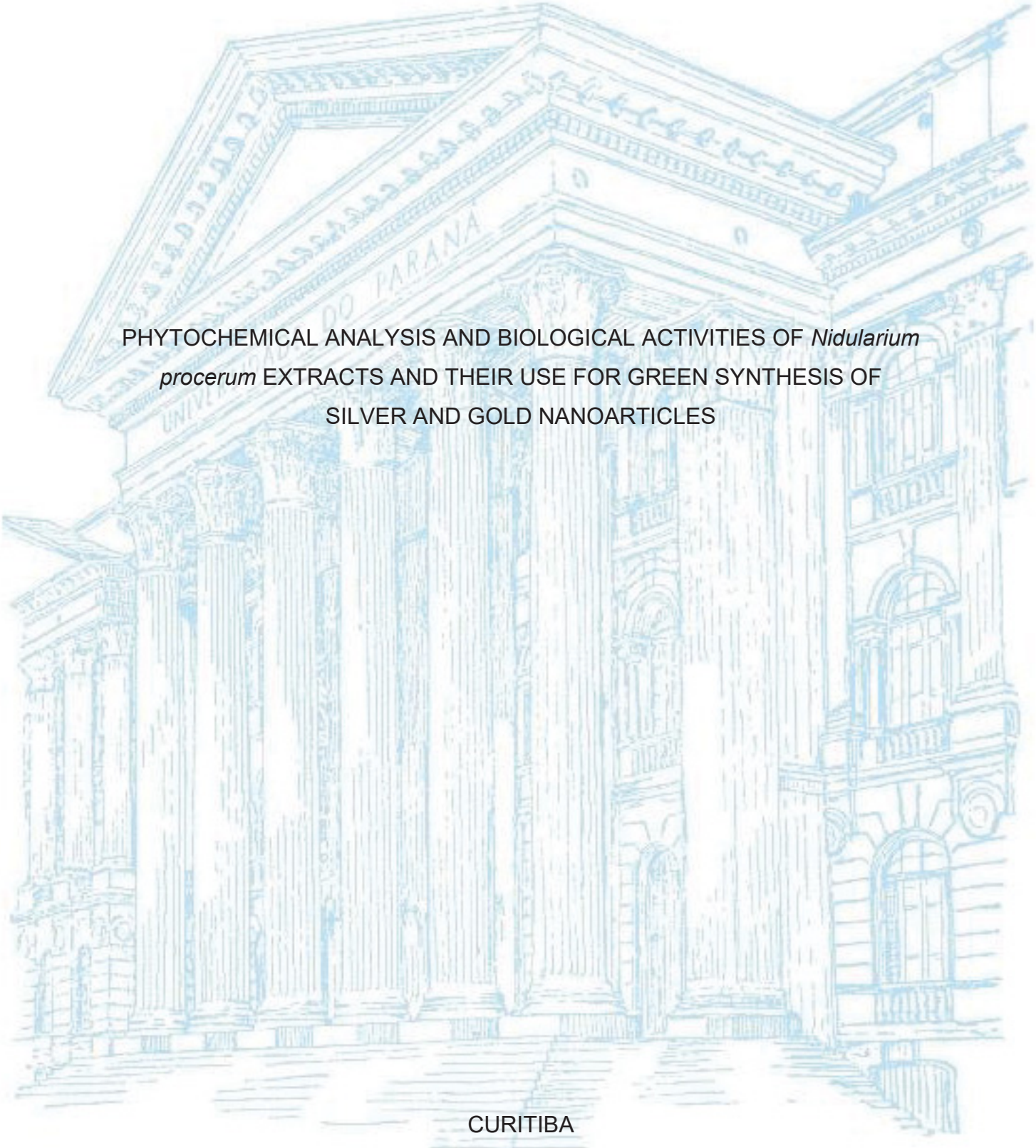

PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF *Nidularium procerum* EXTRACTS AND THEIR USE FOR GREEN SYNTHESIS OF SILVER AND GOLD NANOARTICLES

CURITIBA

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol.

Co-orientadores: Dra. Valcineide Oliveira de Andrade Tanobe.
Prof. Dr. André Luís Lopes da Silva.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANDRE LUIZ GOLLO** intitulada: **PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF NIDULARIUM PROCERUM EXTRACTS AND THEIR USE FOR GREEN SYNTHESIS OF SILVER AND GOLD NANOPARTICLES**, sob orientação do Prof. Dr. CARLOS RICARDO SOCCOL, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 24 de Abril de 2020.

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DEDICATÓRIA

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“Agradeço todas as dificuldades que enfrentei, não fosse por elas, eu não teria saído do lugar. As felicidades nos impedem de caminhar. Mesmo as críticas nos auxiliam muito”.

Francisco Cândido Xavier

(Médium e Filantropo)

1910-2002

“O que somos é consequência do que pensamos”

Buda

“Não ganhe o mundo e perca sua alma, sabedoria é melhor do que prata e ouro”

Bob Marley

(Cantor e Compositor)

1945-1981

RESUMO

A prospecção biotecnológica de compostos bioativos da biodiversidade brasileira é um desafio, pois muitas espécies estão sob vulnerabilidade de extinção, como o *Nidularium procerum*. O objetivo deste estudo foi caracterizaro quimicamente extratos aquosos de mudas de *N. procerum* cultivadas *in vitro*, uma espécie endêmica da Mata Atlântica brasileira, para a subsequente síntese verde de nanopartículas de prata (AgNPs) e ouro (AuNPs). As análises químicas de Cromatografia Líquida de Alto Desempenho (HPLC), Cromatografia a Gás (GC), Cromatografia a Gás acoplada a Espectrofotometria de Massa (GC-MS) mostraram pela primeira vez a composição química parcial das folhas e uma variedade de compostos presentes nos extratos, principalmente flavonoides, esteróides, lipídios e vitaminas, entre outros antioxidantes e antitumorais. Os biocompostos presentes nos extratos aquosos (quente e frio) foram utilizados como agentes redutores para a sínteses das nanopartículas. Os extratos aquosos apresentaram respostas significativas em todos os parâmetros imunomodulatórios, bem como atividade citotóxica contra a linhagem celular de carcinoma adrenocortical (H295R) em ambos os tratamentos, resultando em ~35% de inibição nos extratos brutos e ~80% nos extratos conjugados com nanoparticulas metálicas. Os resultados revelaram que os extratos são fontes promissoras e potenciais candidatos no desenvolvimento de medicamentos e terapias alternativas contra o carcinoma adrenocortical. Os extratos obtidos nesse estudo podem ser utilizados como agentes de redução não tóxicos e eficazes para a síntese verde de AgNPs e AuNPs com efeito antitumoral potencial em aplicações biomédicas.

Palavras-chave: antitumoral, nanopartículas, síntese verde, *Nidularium procerum,* extratos de plantas, micropropagação *in vitro.*

ABSTRACT

The biotechnological prospection for bioactive compounds from Brazilian biodiversity is a challenge since so many species are vulnerable to extinction, such as *Nidularium procerum.* The aim of this study was to characterize chemically aqueous extracts of *in vitro* cultured *N. procerum* seedlings*,* an endemic species of the Brazilian Atlantic forest, for the subsequent green synthesis and characterization of silver (AgNPs) and gold (AuNPs) nanoparticles. The chemical analysis by High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Gas Chromatography-Mass Spectrophotometry (GC-MS) showed for the first time the partial chemical composition of leaves and a range of compounds present in extracts, mainly flavonoids, steroids, lipids, and vitamins, among others considered antioxidants and antitumor. The biocompounds present in aqueous extracts (hot and cold water) were used as reducing agents for the synthesized nanoparticles. The aqueous extracts showed significative responses in all immunomodulatory parameters, as well as cytotoxic activity in both crude (~35%) and nanoparticles-conjugated (~80%) against adrenocortical carcinoma cell line (H295R). The results revealed that N. procerum are promised sources to be used as potential candidates in the development of alternative drugs and therapies against adrenocortical carcinoma. These extracts may be utilized as an effective non-toxic reducing and capping agent for AgNPs and AuNPs green synthesis with a potential antitumoral effect be used in biomedical applications.

Key Words: Antitumor, nanoparticles, green synthesis, *Nidularium procerum*, plant extracts, *in vitro* micropropagation.

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LISTA DE ABREVIATURAS OU SIGLAS

µg – Micrograma
µL - Microlitro
µm – Micrômetro
µM - Micromolar
ABS - Absorbância
ABTS – 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AgNO3 - Nitrato de prata
AgNPs - Silver Nanoparticles
AOCS - American Oil Chemist's Society
AuNPs - Gold nanoparticles
BAP - 6-benzylaminopurine
BDL – Below detection limit
BSE - Back-Scattered Electrons
C - Control
C16H32O2 – Ácido Palmítico
CA - Cold Aqueous
CG - Gas Cromatography
MS – Mass Spectroscopy
CHL – Chloroform Leaf Extract
CO2 – Gás carbônico
Cu/Zn-SOD – Superóxido dismutase
DAD - Diode Array Detector
DLS - Dynamic Light Scattering
DMEM - Dulbecco's Modified Eagle's Medium
DNA - Àcido desoxirribonucleico
DPPH - 2,2-diphenyl-1-picryl-hydrazyl-hydrate
EDX - Energy-dispersive X-ray
ET - Ethanolic
EthD-1 – Ethidium Homodimer-1
FAMEs - Fatty Acid Methyl Esters
FBS - Fetal Bovine Serum

FCC - Face-centered cubic

FDA – Food and Drug Administration

FID - Flame Ionization Detector

g - Grama

GAE - Gallic Acid Equivalent

GP - Glutathione Peroxidase

h - Hora

H2O2 - Peróxido de hidrogênio

HA – Hot Aqueous

HAuCL4 – Ácido Cloráurico

HCl – Ácido clorídrico

HE – Hexanoic Extract

HPLC - High Performance Liquid Chromatography

IC – Inhibition

IUNC – International Union for Conservation of Nature

kDa - Kilodalton

kg - Quilograma

kV – Kilovat

L - Litro

ME – Methanol

mg - Miligrama

min – Minuto

mL - Mililitro

mol.L – Molaridade

MS – Mass Spectrometry

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

mV – Milivolt

NAA – Naphthalene acetic Acid

NADPH-oxidase – nicotinamide adenine dinucleotide phosphate oxidase

NaOH - Hidróxido de sódio

nm - Nanômetro

NPs – Nanoparticles

O2 - Oxigênio

OH - Hydroxyl

P.A. – Alphatec

PAF – Platelet Activating Factor

PDMS – Polydimethylsiloxane

PdNPs – Palladium nanoparticles

PGE2 – Prostaglandin E

pH - Potencial Hidrogeniônico

PKC - Protein Kinase C

PTFE – Politetrafluoretileno

PVDF - Polyvinylidene Difluoride

RAW – Murine Macrophages Cell Line

ROS – Reactive Oxygen species

Rpm – Rotação por minuto

SAED - Selected Area Electron Diffraction

SEM - Scanning Electron Microscopy

SPR - Surface Plasmon Resonance

TEM - Transmission Electron Microscopy

UFPR - Universidade Federal do Paraná

UV-Vis – Ultravioleta visível

LISTA DE SÍMBOLOS

% - porcentagem

© - marca registrada

ºC - graus Celsius

± - mais ou menos

α - alfa

β – beta

γ - gamma

SUMÁRIO

1. INTRODUÇÃO

O uso de plantas como recurso terapêutico é um fato bastante difundido no mundo inteiro (Ministério da Saúde 2006). As plantas têm sido utilizadas como possível cura de doenças há muito tempo. Ainda hoje nas regiões mais remotas do planeta e até mesmo nas grandes cidades, plantas medicinais são comercializadas, em feiras livres, mercados populares e muitas vezes em quintais residenciais.

As observações populares sobre o uso e a eficácia de plantas medicinais contribuem de forma relevante para a divulgação das virtudes terapêuticas dos vegetais, pelos efeitos medicinais que produzem, apesar de não terem todos os seus constituintes químicos conhecidos (Maciel et al. 2002). Por isso, o conhecimento sobre a composição química e a atividade biológicas das plantas frente a enfermidades é muito importante e nos últimos anos as pesquisas desenvolvidas nessa área têm ganhado muita importância. Além de auxiliar na descoberta de novos agentes terapêuticos, elas permitem a descoberta de novas substâncias de interesse, como taninos, óleos, gomas e outras substâncias amplamente presentes nos vegetais (Farnsworth 1966).

Diferentemente de outros organismos, as plantas utilizam uma quantidade significativa do carbono e energia assimilada na síntese de uma variedade de moléculas orgânicas, que não estão diretamente envolvidas nos processos fisiologicos basais, como fotossintético, respiratório, transporte de solutos e síntese de proteínas, carboidrato ou lipídeos. Essas moléculas são chamadas metabólitos secundários (Ávalos, A. y Pérez 2009).

Os metabólitos secundários são compostos estocados em células ou órgãos específicos das plantas e apresentam uma frequência inferior a 1% do carbono total presente em sua estrutura (Harborne 1998). Distintos métodos de caracterização, tais como espectrometrias e cromatografias têm permitido o isolamento de muitas destas moléculas, contribuindo para elucidação estrutural destes compostos.

Biologicamente, os produtos secundários formados estão envolvidos na adaptação das plantas aos seus ambientes. Essas moléculas atuam no estabelecimento das espécies vegetais nos mais diversos ecossistemas (Aerts et al. 1991). São responsáveis por diversas atividades biológicas (antibióticos,

antifúngicos e antivirais) aumentando a probabilidade de sobrevivência das espécies, afastando possíveis patógenos e também podem apresentar atividades antigerminativas ou tóxicas para outras plantas concorrentes. Além disso, também estão relacionadas com a absorção da luz ultravioleta, evitando ou amenizando os danos causados em folhas e demais tecidos vegetais (Li et al. 1993). Eles são usualmente classificados de acordo com a sua rota biossintética de formação (Jeffrey B 1999), sendo as três principais famílias classificadas em compostos fenólicos, esteróides e alcaloides.

Os compostos fenólicos estão envolvidos na síntese de lignina, contribuindo na atração de alguns animais para polinização e dispersão de sementes. Além disso, tem efeito direto na proteção das plantas contra raios ultravioletas, insetos, fungos, vírus e bactérias além de inibirem o crescimento de outras plantas competidoras (Croteau; 2000).

Os flavonoides apresentam, entre outras atividades estudadas, importante potencial antibiótico, relacionado à capacidade destes compostos em complexarem-se com proteínas solúveis e extracelulares, permitindo a formação de ligações com as paredes celulares bacterianas (Osonga et al. 2019). Alguns compostos desse grupo, como os da classe dos flavonoides lipofílicos e isoflavonoides, podem chegar a romper as membranas bacterianas, além de também apresentarem elevadas atividades antifúngicas (Coelho; et al. 2003). Entre outras propriedades, os flavonoides são amplamente conhecidos por apresentarem atividades antioxidantes, anti-inflamatórias, antitumorais e elevado potencial quimiotaxonômico (Manetti; et al 2009). Ou seja, sendo constituintes presentes especificamente em alguns grupos de plantas, como os flavonoides com hidroxilação ou metoxilação na posição C-6, que dentro do grupo das monocotiledôneas são exclusivos para a família das Bromeliaceas (Cruz 2017).

Os esterois são classificados como sendo subclasse dos terpenoides, e apresentam uma estrutura básica formada por quatro anéis ligados entre si, compostos por átomos de carbono. Os esteróis são moléculas hidrofóbicas e apresentam estrutura similar ao do colesterol; entretanto, esses constituintes são sintetizados por plantas ao invés de mamíferos. O anel de esterol presente nesses compostos é comum a todos os esteróis, diferenciando-se apenas através de suas cadeias laterais (Simões; et al 2010). Os esteroides fazem parte

de um grupo fitoquímico oriundo da via do mevalonato, metabólitos dos triterpenos. Dentro deste grupo estão compostos com elevada importância comercial, como as saponinas (Simões; et al 2010). Essas são componentes essenciais da membrana celular de plantas, sendo encontrados em óleos vegetais, sementes e frutas. Em sua forma livre apresentam funções na estabilização da bicamada lipídica de fosfolipídios, além de apresentar ação como reguladores de crescimento ou então na formação de seus precussores (Law 2000). Muitos compostos dessa classe de metabólitos já foram identificados em plantas, sendo o beta-sitosterol, campesterol e estigmasterol os mais abundantes (Yankah 2006).

Por fim destacam-se os alcaloides, compostos farmacologicamente ativos, constituídos quimicamente por um átomo de nitrogênio além de derivados de aminoácidos (Cordell 1981). O papel dos alcaloides nas plantas ainda não foi completamente elucidado, mas algumas pesquisas são focadas nas funções eco-químicas destes compostos (Croteau; 2000), demonstrando que os alcaloides exercem uma grande variedade de efeitos fisiológicos sobre patógenos e concorrentes, ajudando especialmente na defesa química das plantas frente aos herbívoros devido à sua alta toxicidade (Cogni and Trigo 2016).

A produção de metabólitos secundários não só pode ser desencadeada em resposta a diferentes formas de estresse biótico, como também faz parte do metabolismo basal da planta na atração de polinizadores, estabelecimento de simbiose e fornecimento de componentes estruturais à paredes celulares (Ncube and Van Staden 2015). Devido a grande variedade de vias de ativação a qual está atrelada a produção de metabólitos secundários, a padronização desta produção se torna necessária para estudos mais aprofudados. Somado ao fato de que a pesquisa geralmente está atrelada a uma metodologia destrutiva, há também a necessidade de ela ser realizada da maneira mais sustentável possível. Nesse sentido o cultivo de tecidos e explantes *in vitro* é uma excelente alternativa para a propagação de espécimes, podendo ainda ser extendida a plantas sob algum tipo de ameaça ambiental.

A cultura de tecidos é um processo por meio do qual fragmentos vegetais ou suas partes (explantes) são isolados a partir de organismos matrizes, sendo assepticamente cultivados em meio de cultura apropriado sob condições

adequadas e totalmente controladas, como luminosidade, temperatura, fotoperíodo e nutrição (Amaral; and Silva 2003). Entre as vantagens da cultura de tecidos, se destaca a multiplicação de plantas de propagação vegetativa lenta ou de difícil propagação em um reduzido período de tempo, podendo ser realizada em qualquer época do ano. Além de possibilitar a produção de grandes quantidades de plantas em uma área reduzida, auxilia também no melhoramento vegetal, na manutenção e multiplicação de plantas híbridas, mantendo a sua combinação genética (Pasqual and Ferreira 2007).

Atualmente, há uma grande busca por novos produtos de origem natural visando a promoção da saúde e longevidade. Novas tecnologias estão surgindo de forma a intensificar o efeito dos compostos bioativos presente nos extratos, sendo uma delas a nanotecnologia verde. Os compostos orgânicos presentes nos extratos de plantas têm a capacidade de reduzir quimicamente íons metálicos, como íons de prata e ouro (empregados neste trabalho) presentes em soluções, formando nanopartículas naturais, não tóxicas e potencializando a capacidade farmacológica do extrato (Kavitha et al. 2013). Uma forma de melhorar explorar, elucidar e intensificar a importância de alguns espécimes vegetais pouco conhecidos e ameaçados é, portanto, o estudo *in vitro* de suas potencialidades atrelados à novas tecnologias, como a produção de nanopartículas a partir de síntese verde. E inserindo-se nesta situação está a bromélia *Nidularium procerum*, utlilizada no presente estudo.

Espécie endêmica, *Nidularium procerum* está presente apenas no litoral brasleiro onde ainda ocorrem remanescentes da Mata Atlântica, estandodistribuída naturalmente da Bahia ao norte do Rio Grande do Sul (Fontoura; Costa; and Wendt 1991). Pouco estudada, já foi previamente reportada por seus extratos apresentarem importantes efeitos analgésicos, anti-inflamatórios e antialérgicos em modelos animais (Amendoeira, et al. 2005a; Amendoeira et al. 2005b; Vieira-de-Abreu et al. 2005).

Atualmente existem poucos trabalhos publicados na literatura sobre nanopartículas reduzidas a partir de extratos vegetais focando em suas atividades medicinais. Especificamente quando se trata de extratos de bromélias. Muitos são os estudos desenvolvidos a partir de nanoparticulas de prata focando em seu efeito antimicrobiano, bem como de síntese química e/ou

física de nanoparticulas de ouro visando seu efeito farmacológico com fármacos sintéticos.

Dessa forma, torna-se interessante o emprego de biomoléculas de plantas produzidas em ambientes controlados para redução de íons metálicos e formação de nanopartículas mais sustentáveis com possíveis utilidades no campo medicinal.

1.1. JUSTIFICATIVAS

Diante da grande diversidade de moléculas presentes nos vegetais, o estudo e investimento na área fitoquímica é de suma importância. Atualmente, há, muitas vezes, o uso indiscriminado de plantas medicinais pela população na forma de infusões e chás e sem o devido conhecimento da possibilidade de presença de compostos citotóxicos, os quais podem causar efeitos adversos e consequentes danos à saúde às pessoas (Bagatini, Carlos, and Tedesco 2007).

Por outro lado, muitas espécies que possuem atividades farmacológicas de interesse, acabam não sendo exploradas pela falta de estudos no campo de pesquisa aplicada. Como acontece com a *Nidularium procerum*, que apesar de ter sido previamente reportada como potencial no campo da medicina, teve sua investigação limitada a poucos trabalhos realizados há um bom tempo.

Por ser uma espécie endêmica, estando presente apenas no Brasil, e fazer parte de um grupo em risco de extinção devido à perda de seu habitat natural, deve-se ter um cuidado especial ao desenvolver estudos de sua composição fitoquímica. Por isso, a produção *in vitro* de mudas se torna uma alternativa não só sustentável, como também auxilia na padronização da produção de metabólitos secundários naturais da planta quando crescidas em ambiente não hostil.

Além do mais, a síntese verde de nanopartículas é um campo relativamente novo, envolvendo rotas menos tóxicas e mais seguras para aplicação medicinal. Extratos de plantas possuem muitos metabólitos secundários, dentre eles os compostos fenólicos, flavonoides, proteínas e lipídeos que são capazes de reduzir íons metálicos como de prata e ouro, agindo na produção de nanopartículas (LATIF MS et al. 2019).

Até o momento não foram reportadas aplicações focadas no estudo imunomodulador e antitumoral de nanoparticulas oriundas de síntese verde, a

partir de extratos de bromélias. Assim sendo, extratos de *Nidularium procerum* produzidas *in vitro* sob condições controladas poderiam ser agentes verdes mais seguros para produção de nanopartículas metálicas. Visto as atividades medicinais previamente reportadas da espécie, os extratos podem estar relacionados ao tratamento de doenças desenvolvidas a partir da desbalanço do mecanismo imunomodulatório, além de câncer e tumores.

2. Objetivo Geral

Caracterizar parcialmente extratos aquosos de folhas da espécie *Nidularium procerum* e sintetizar nanoparticulas através de síntese verde, avaliando os seus potenciais terapêuticos.

2.1. Objetivos específicos

- Caracterizar os extratos através de *screening* fitoquímico e técnicas cromatográficas de alta resolução.
- Avaliar a atividade antioxidante dos extratos aquosos por técnicas *in vitro*.
- Avaliar o potencial imunomodulatório dos extratos aquosos em macrófagos da linhagem RAW 264.7 e a atividade citotóxica *in vitro* em células de tumor adrenocortical de linhagem H295R.
- Investigar o potencial dos extratos aquosos na redução de íons metálicos para produção de nanopartículas de prata e ouro através de síntese verde.
- Avaliar o potencial citotóxico das nanopartículas sintetizadas na linhagem H295R de carcinoma adrenocortical e sua toxicidade frente a células não tumorais.

CHAPTER I. PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF *IN VITRO* CULTURED *NIDULARIUM PROCERUM*, A BROMELIAD VULNERABLE TO EXTINCTION.

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Introduction

Bromeliaceae is a morphologically distinctive and ecologically diverse family, divided into eight subfamilies (Brochinioideae, Lindmanioideae, Tillandsioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puvoideae and Bromeliodeae) based on morphological and molecular DNA data (Versieux et al. 2018). Almost the entire family is native to the American continent, with the exception of *Pitcairnia feliciana* (A.Chev), an endemic specie of West Africa[2]. Due to their wide distribution and abundance in tropical habitats, bromeliads represent a very important ecological component in many communities, with a direct impact on richness and diversity of fauna and flora (Smith and Till 2013).

Bromeliads are also worldwide recognized for their ornamental value. In the past decades, it has become very popular as a garden plant, which increased the extraction pressures from natural populations. Brazil is the diversity center of Bromeliaceae, with 1,246 species cataloged to date, in which, 1,067 are endemic to the country(Forzza et al. 2012). Among these, six species are classified as vulnerable, three endangered and seven critically endangered, indicating threatened ecosystems according IUNC criteria (Martinelli and Moraes 2013). *Nidularium* is a genus with high vulnerability to extinction and *Nidularium procerum* Lindm is one of the most prevalent bromeliads found in the Atlantic Rain Forest[6]. It is a polymorphic specie, with varying appearance in response to the environment, especially the coloration of the leaves and bracts involved. The populations are mainly concentrated on the coast, where they develop in isolation or in groups of 2-10 individuals (Tardivo and Cervi 1997).

The bromeliad family has been used for centuries in Native American medicine[8]. More recent research has confirmed the beneficial effects of bromeliads supported by traditional medicine, such as improvement of digestive, diuretic and respiratory processes (Duke et al. 2002; Rodrigues and Carvalho

2007). Other biological actions include relief of fever symptoms and diabetes mellitus (Whiterup et al. 1995), as well as anti-inflammatory and anti-allergic proprieties, being able to inhibit the influx of pleural neutrophils and mononuclear cells in allergy-induced mice and, also, decrease the number of eosinophils by inhibiting PAF and eotaxin-induced eosinophil chemotaxis (Amendoeira, Fabio et al. 2005; Vieira-de-Abreu et al. 2005). In addition, bromeliad extracts are reported and used as antitumoral agents. Bromelain and fastuosain — a complex natural mixture of proteolytic enzymes described in the group — was demonstrated to induce the apoptosis pathway of human epidermoid carcinoma and melanoma cells (Guimarães-Ferreira et al. 2007; Rathnavelu et al. 2016). Phytochemical compounds present in bromeliads family were also shown to affect cell adhesion molecules involved in other pathways of carcinoma cells growth (Juhasz et al. 2008).

In the current scenario of vulnerability caused by human exploitation, it is necessary to use alternative methods that allow cultivation of plant species. Thus, *in vitro* plant tissue culture represents an ecological alternative to obtain competent explants (plant parts) under controlled conditions. This cultivation system allows the obtaining of several compounds with pharmacological interest without affecting natural population levels. In addition, micropropagated plants produce secondary metabolites at an early stage of growth (Ncube et al. 2011), which can be a way to provide rapid propagation of a large number of uniform plants, without being affected by adverse natural factors, such as climate, season, diseases and slow plant growth(Arikat et al. 2004). This allows for a technology alternative for rapid production of pharmacological compounds that can be utilized for medicinal purpose.

Recent studies have revealed the pharmacological properties of *N. procerum*. Amendoeira et al., (2005a) (Amendoeira, Fabio et al. 2005), Amendoeira et al., (2005b) (F. C. Amendoeira et al. 2005) and Vieira-de-Abreu et al., (2005) (Vieira-de-Abreu et al. 2005) reported that extracts of *N. procerum* have analgesic, anti-inflammatory and antiallergic properties with nontoxic activities, making it an attractive candidate for future drug development. However, the phytochemical composition of *N. procerum* remains poorly studied. Only a study conducted by Williams (1978) (Williams 1978) reported the flavonoid composition of leaves of *Bromelia* spp. including *N. procerum*, which observed

the presence of quercetin. Here, crude extracts from the leaves of *in vitro* cultivated *N. procerum* were analyzed by diverse chemical analysis, including phytochemical screening by colorimetric tests, target compounds by gas chromatography assays (GC), chlorophyll quantification, total phenolic compounds and individual phenols by high performance liquid chromatography (HPLC), and antioxidant activity. In addition, for the first time, the immunomodulatory and antitumoral activities of aqueous extract of *N. procerum* leaves were assessed using specific tests of murine macrophages modulation and MTT assay against adrenocortical carcinoma, a cancer with rare treatment.

Material and Methods.

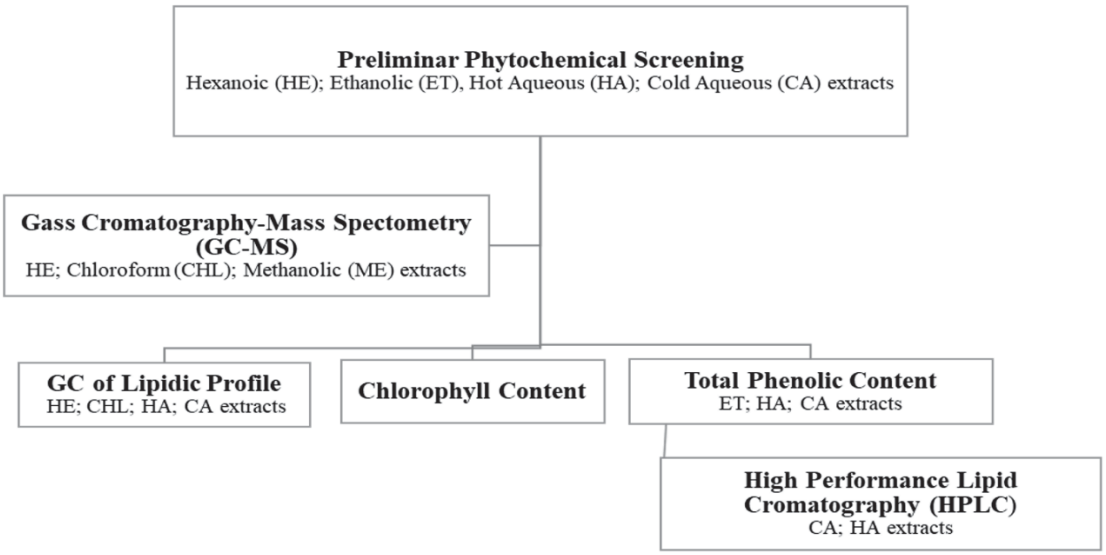
Plant material and extraction.

Plants were stablished and multiplicated *in vitro* according Lopes da Silva *et al* (2012) (Lopes da Silva, André et al. 2012). Shoots (2 cm height) from clusters previously micropropagated *in vitro* were used as explants and subcultured *in vitro* to elongation and rooting for 90 days, free of plant growth regulators, up to the formation of a complete explant (basal and aerial part)- becoming available for extraction of leaves compounds, protocol adapted from Kim *et al* (2019)[80]. Plantlets were removed from culture chambers and the leaves were cut into small pieces and 1 g of fresh leaf mass was macerated and extracted in 10 mL of specific solvent, over 24 hours, under 80 rpm agitation and 25 °C in a dark room. The extracts were filtered with Whatman n° 1 filter paper, lyophilized and stored at -20° C for further characterizations and applications.

Chemical characterization of *Nidularium procerum* extracts.

The chemical investigation of *N. procerum* compounds were carried out in different solvents, in order to detect the maximum range of substances extracted as summarized in the flowchart below (Figure 1**)**. The analyses were performed based on the results obtained in preliminary phytochemical screening and finally focused on aqueous extracts used in biological tests. The antioxidant, immunomodulatory and antitumoral potential of the aqueous fractions were explored due to their lack of toxicity and the low-cost of the process.

FIGURE 1: FLOWCHART OF METHODOLOGIES USED IN CHEMICAL CHARACTERIZATION OF DIFFERENT EXTRACTS OF N. PROCUM LINDM.



Phytochemical Screening.

The phytochemical tests were carried out in four different extracts from *N. procerum*: hexane (Analytical standard – VETEC), ethanolic (Analytical standard – VETEC), hot aqueous (100°C) and cold aqueous (25°C). The screening was performed according to Iqbal *et al* (2015) (Iqbal, Salim, and Lim 2015). Identification of alkaloids was determined using Dragendorff, Mayer and Wagner´s test, reducing sugars using Fehling´s reagent, quinones by Bornträger´s test, saponins by permanent foam appearance, mucilage by gelatinous consistency after cooled, coumarins using Bajlet´s test, steroids/triterpenoids using Liebermann-Buchard´s test, resins by precipitation test, flavonoid by Shinoda´s test and tannins/phenols using Ferric Chloride´s test.

Gas Chromatography–Mass Spectrometry.

The GCMS profiles of solvents with different polarity ranges (HE, CHL and ME) were obtained by electron impact with GC-MS-TQ Series 8040-2010 Plus (Shimadzu-Japan) equipped with a 95% PDMS and 5% Phenyl capillary column (model SH-Rtx-5MS; 30 m × 0.25 mm × 0.25 µm). The temperature program started at 50 °C, maintained for 2 minutes and raised at a flow of 7 °C.min^{-1} up to 280 °C, which remained constant for 15 minutes, for a total of 47 min of

analysis. Helium was the carrier gas used, at a 1mL.min^{-1}, 88.3kPa column press and split ratio of 1:40. The solvent cut off was 2.5 min. The mass spectrometry range was 30–500 (m/z), at an ion source temperature of 250 °C. The chemical compounds were identified by comparison of the mass spectra present in NIST98/2014 and Wiley 7 data library.

Lipid Profile – Gas Chromatography.

Fatty acid profile of CHL, HE, HA and CA extracts were analyzed using a Shimadzu chromatograph (GC 2010 *Plus*), a capillary column (SH-Rtx-Wax - Shimadzu: 30 m x 0.32 mm x 0.25 □m), flame ionization detector (FID) and split injection mode (1:10). The injector and detector temperatures were 240 °C and 250 °C, respectively. The oven temperature was programmed to start at 100 °C during 5 min, followed by an increase up to 240 °C at a rate of 4 °C.min^{-1} and maintained at this temperature for 5 min. The carrier gas was Helium at 32.5 cm^3.min^{-1}. The samples were prepared according to the official method (Ce 2-66) of the American Oil Chemist's Society (AOCS, 1998) to convert triacylglycerol and free fatty acid of samples into fatty acid methyl esters (FAMEs). FAMEs were identified by comparison with retention times of the standard mixture FAMEs (Supelco, MIX FAME 37, St. Louis, MO 63103, USA). The quantification of fatty acids was conducted by area normalization procedure. Results were expressed as percentage of each individual fatty acid present in the sample.

Chlorophyll Quantification.

Fresh leaves (5g) were macerated in 10 mL acetone (P.A. VETEC). The solution was filtered with Whatman n° 1 filter paper and stored at -6 °C for five minutes. The absorbance was measured with spectrophotometry at 470, 662, 645, and 652 nm to chlorophyll a, b, relation between and chlorophyll a/b, respectively(Wellburn and Lichtenthaler 1983). The assays were carried out in triplicate and the results were expressed in μg.g^{-1} of fresh weight.

Total Phenol Content.

Total phenolic contents of ET, HA and CA extracts were determined using Folin-Ciocalteu method(Singleton, Rossi Jr., and Rossi J A Jr. 1965) and the standard curve was performed using 0.39, 3.9, 7.8, 15.6, 31.2, 62.5 and 125

μg.mL^{-1} of gallic acid. The results were expressed in mg of gallic acid equivalent (GAE) in 100g of fresh weight.

Phenolic Content - High Performance Liquid Chromatography.

The phenolic content of aqueous extracts was separated in HPLC using an Agilent Technology 1200 Series system, coupled to a diode array detector (DAD) at wavelengths 235, 260, 275, 280, 290, 311, 357, 370 nm and a scanning from 190 nm to 600 nm. A ZorbaxElipse XDB – C18 (4,6 x 150 mm, 5 – micron) column was used at 0,7 mL min^{-1} flow. The mobile phase was 2,5% acetic acid (solvent A) and methanol (solvent B). The elution gradient was carried out as follows: 90% A/10% B, 0-13 min; 75% A/25% B, 13-28 min; 15% A/85% B, 28-32 min; 10% A/90% B, 32-36 min. Chlorogenic acid, caffeic acid, ferulic acid, tocopherol, genistein, transcinnamic acid, catechin, rutin, p-coumaric acid, gallic acid, resveratrol and epicatechin (SIGMA) were used as standards. To obtain the calibration curve, all standard reagents were solved in mobile phase and used at 1, 2, 5, 8 and 10 ppm. The samples were microfiltered trough a hydrophilic membrane GV (Durapore) made of polyvinylidene difluoride (PVDF), with a pore size of 0,22 μm. The resulting chromatogram values were plotted and a linear equation was generated by calculating the average of triplicate runs for each compound. The equations were used to quantify the phenolic compound contents of the samples. The injection volume was 10 μL. All the assays were also performed in triplicate.

***In vitro* antioxidant activity**

Scavenging ability on DPPH

The antioxidant potential of the aqueous extracts was determined by their ability of quenching the free radical DPPH (Tan et al. 2016). A Trolox (Sigma) standard solution was diluted from 0.25 to 25 mg/ml and used as positive control to the assay, mixing 200 μL of each concentration in 800 μl of 0.004 % methanol solution of DPPH. After 30 min of incubation in absence of light at room temperature, the absorbances were read against blank at 517 nm using a SP-2000 spectrophotometer. The same protocol was used for the HA and CA treatments. DPPH solution was used as negative control with the solvent

extraction. Tests were carried out in triplicate and the percentage of free radical inhibition was calculated by the following equation (1):

$$\%I = (A_{blank} – A_{sample}/ A_{blank}) \times 100 \quad \text{Eq. (1)}$$

Where A_{blank} is the negative control and A_{sample} is the absorbance of extracts. The results were expressed in extract concentration producing 50 % inhibition (IC 50%), calculated from the graph of the DPPH scavenging effect against the extract concentration.

ABTS assay

The ABTS assay was carried out using a radical cation decolorization protocol(Re et al. 1999). The ABTS radical had to be pre-formed by the reaction between 5 mL ABTS 7 mM (Sigma) with 88 µL of 140 mM potassium persulfate, stored in the dark at room temperature for 16 hours. The ABTS solution (1 mL) was previously diluted in 50 mL of ethanol P.A. (Alphatec) to obtain an absorbance of 0.700 at 734 nm. In absence of light, 10 µL of each aqueous plant extract were added to 500 µL ABTS solution. After 6 minutes, the absorbance was read in the spectrophotometer (SP 2000) at 734 nm. Distilled water was used as blank and as negative control. All measurements were carried out in triplicates. The scavenging capability of tests compounds was calculated using the following equation (2):

$$\text{ABTS Scavenging activity (\%)} = (1-(\lambda_{734\text{-Sample}}/ \lambda_{734\text{-Control}})) \times 100 \quad \text{Eq. (2)}$$

Where $\lambda_{734\text{-Sample}}$ is the absorbance of control without radical scavenger and $\lambda_{734\text{-Control}}$ the remaining ABTS in the presence of scavenger. Trolox was used as standard.

Immunomodulatory Activity

Macrophage activity was assessed by its reactive oxygen species production - superoxide anion and hydrogen peroxide, cell adhesion, phagocytic efficiency and phagolysosomal formation (Bonatto et al. 2004). Murine macrophages cells were cultured in Dulbecco's Modified Eagle's medium (DMEM – Sigma Aldrich) supplemented with 10 % fetal bovine serum (FBS - Gibco and 1% antibiotic solution (10.000 $U.mL^{-1}$ penicilin and 10 $mg.mL^{-1}$ streptomycin - Gibco®), maintained in a humidified atmosphere with 5 % CO_2 at 37 °C until 80-90 % confluence was reached. The cells were divided at 10^5 cells/well in 96-well

plate (Biofil) and exposed into the following experimental groups: cells without treatment (C), Hot Aqueous (HA) and Cold Aqueous Extract (CA), both at concentrations 2, 10, 20, 100 and 1000 µg.mL^{-1} for 24h at same conditions of growing. The analyses were performed in 12 repetitions.

Antitumoral Activity

H295R and VERO cells were cultured in DMEM F-12 (Sigma Aldrich) medium supplemented with 10 % fetal bovine serum (Gibco) and 1 % antibiotic solution (Gibco). They were incubated in a CO_2 incubator at 37 °C, with humidified air (95%) and CO_2 (5 %) until 80-90 % confluence was reached. The cells were divided in 10^5 cells/wells in a 96-well plate (Biofil) and exposed into the following experimental groups: cells without treatment (C), Hot Aqueous and Cold Aqueous Extract, both at concentrations 2, 10, 20, 100 250, 500, 750 and 1000 µg.mL^{-1} for 24h at the same growing conditions. The analyses were performed in 12 repetitions(Mosmann 1983).

Statistical analysis

The data were expressed as mean ± standard error and analyzed by One Way Anova followed by Tukey´s test using Graph Pad Prism 6 software. Differences between means at the 5 % of confidence interval ($p<0.05$) were considered significant.

Results and Discussion

Phytochemical Screening

The phytochemical screening of crude extracts from the leaves of *in vitro* cultivated *N. procerum* revealed the presence of some secondary metabolites, according to the solvent used (Table 1). The Ethanolic (ET), Hot Aqueous (HA-100°C) and Cold Aqueous (CA-25 °C) extracts showed positive results for alkaloids, with the appearance of red orange precipitated complexes on Dragendorff test. Two qualitative tests (i.e., Wagner and Mayer) were also carried out for this chemical group, confirming the positive results. Furthermore, the aqueous extracts showed defined turbidity (HA) and precipitate (CA), whereas ET showed only opalescence, indicating that aqueous extracts could also be effective for alkaloid extraction.

The Liberman-Buchard test revealed the presence of steroids/triterpenoids in Hexanoic (HE) and ET extracts. HE showed the formation of yellow color, indicating the possible presence of a methyl group on carbon 14(Cook 1961). In the ET, a green color was observed, related with a carbonyl function in carbon 3 and double bound between carbons 5 and 6 or 7 and 8 in the extracted compounds(Xiong, Wilson, and Pang 2007).

The flavonoid group was observed in ET, HA and CA extracts, with the appearance of yellowish green and intense yellow, respectively, by Shinoda test. In addition, the ferric chloride assay confirmed the presence of tannins in these extracts. The ET showed a blueish black color, indicating the presence of pyrogallol tannins or hydrolysable tannin, which generates gallic acid or ellagic acid when hydrolyzed by acids, bases or appropriated enzymes(Elgailani and Ishak 2016). Finally, both aqueous extracts showed intense green color, referring to the presence of pyrocatecholic tannins (condensed tannins), formed by condensation of two or more flavanols, which are not hydrolyzed by acids, bases or specific enzymes(Elgailani and Ishak 2016).

These secondary metabolites found in the *N. procerum* extracts are important due to their biological activities. Alkaloids, flavonoids, tannins and other biomolecules are known for their antioxidant, antifungal, anticancer, antiviral, anti-inflammatory and antiophidic activities(Cimmino et al. 2017; Goietsenoven et al. 2010; Schrader et al. 2010). Moreover, previous studies reported the presence of sterols, di and triterpenes, phenolic compounds, flavonoids, lignin, saponins, coumarins and cinnamic acids derivatives in other species grown under natural conditions of the Bromeliaceae family(Andrighetti-Frohner et al. 2005; Manetti et al. 2009).

TABLE 1. PRELIMINARY SCREENING OF HEXANE, ETHANOLIC AND AQUEOUS EXTRACTS OF NIDULARIUM PROCERUM LINDM SHOOTS.

Metabolites	Hexane Extract	Ethanolic Extract	Hot Aqueous Extract	Cold Aqueous Extract
Alkaloids Dragendorff	-	+	++	+++
Alkaloids Wagner	-	+	++	+++
Alkaloids Mayer	-	+	++	+++

Reducing Sugars	-	-	-	-
Quinones	-	-	-	-
Saponins	-	-	-	-
Mucilages		-	-	-
Coumarins	-	-	-	-
Steroids/Triterpenoids	++	+	-	-
Resins	-	-	-	-
Flavonoids		+	++	++
Tannins	-	++	+	+

Blank spaces mean that the test was not performed on the extract.
(+) small quantity positive response was obtained for the chemical group in the extract.
(++) medium quantity positive response was obtained for the chemical group in the extract.
(+++) positive response of greater quantity was obtained for the chemical group in the extract.
(-) negative response was obtained for that chemical group in the extract.

Gas Chromatography – Mass Spectroscopy (CG-MS)

GC-MS was carried out in order to describe and quantify the compounds found in a solvent polarity gradient, chosen according to permission of method. A total of 43 phytocompounds were found (Table 2). These compounds belong to different chemical classes, including hydrocarbons, esters of fatty acids, steroids/triterpenes, aldehydes, amides, vitamins and flavones. The highest number of compounds (28) was evidenced in chloroform leaf extract (CHL), followed by methanol (ME) (11) and hexane (4). The major compounds found were hydrocarbons (32,5%), esters of fatty acids (21%) and steroids/triterpenes (9,3%). Some of these compounds, such as tetrapentacontane, tetradecane, stigmasterol, neoftadiene (7,11,15-trimethyl-3-methylidenehexadec-1-ene), n-hexadecanoic acid, oleic acid and octadecanoic acid, have already been reported from leaves extracts of other plant species(Swamy et al. 2017). Steroids (β-cytosterol and stigmasterol) and lipids (palmitic acid, oleic acid, γ-tocopherol, α-tocopherol) were described in *Bromelia Laciniosa* Mart. ex Shult. & Schult.f, *Neoglaziovia variegata* Mez and *Encholirium spectabile* Schult. & Schult(Juvik, Ole et al. 2017), while cyclolaudenol triterpene was reported in *Tillandsia fasciculata* Sw. hexanoic extract(Cantillo-Ciau, Brito-Loeza, and Quijano 2001).

The most commonly found sterols in plants include campesterol, sitosterol and stigmasterol(Tao, Bernie 2007). Stigmasterol, in particular, has been investigated for its pharmacological potential, including cytotoxic, antioxidant,

antitumoral, antimutagenic, among other herbal approaches to pathological states in principles and practice of phytotherapies (Chaudhary et al. 2011). Furthermore, to the best of our knowledge, it is the first time that some important compounds, already described in the literature in other plant species, were reported in the *Nidularium* group. These include α-Tocopherol (vitamin E) and the 5,6,7-trimethoxy-2-(4-methoxyphenyl)chromen-4-one flavone, also known as scuttelarein. Previously, α-Tocopherol was reported in four species of the family *B. laciniosa* (1.8% content in hexane leaves extract), *N. variegate* (1.5% content in hexane leaves extract), *E. spectabile* (0,9% content in hexane leaves extract) and *Ananas erectifolius* (31.4mg/Kg of fiber)(Juvik, Ole et al. 2017; Marques, Gutiérrez, and Del Río 2007). The presence of vitamin E in *N. procerum* crude extracts increase the antioxidant and nutritional importance of the specie. In addition, scutellarein, previously found in *Pitcairnia darblayana* Sallier, *Pitcairnia poortmanii* André, *Pitcairnia xanthocalyx* Mart., *Pitcairnia corallina* Linden at André, *Pitcairnia punicea* Schiedw(Williams 1978) and *Bromelia pinguin* L. bromeliads(Raffauf et al. 1981), were also related as anticancer agent against fibrosarcoma cells, by induction of cells apoptosis pathway(Shi et al. 2015).

TABLE 2. MAIN COMPOUNDS FOUND IN DIFFERENT EXTRACTS OF N. PROCERUM LINDM BY GC-MS.

Coumpounds	Chemical Class	Area	*m/z*	Retencion time
Chloroform				
7,11,15-trimethyl-3-methylidenehexadec-1-ene.	Hydrocarbon	5329	68.0	19.813
Methyl hexadecanoate.	Fatty Acid Ester	10533	74.0	21.079
Tetradecan-2-ylbenzene.	Hydrocarbon	59459	105.0	22.299
Methyl (9*E*,12*E*)-octadeca-9,12-dienoate.	Fatty Acid Ester	2038	67.0	23.357
1-hexadecanoyloxy-3-hydroxypropan-2-yl)hexadecanoate.	Fatty Acid Ester	24428	57.0	25.871
(*Z*)-octadec-9-enamide.	Amide	35867	59.0	26.662
1-iodotriacontane.	Hydrocarbon	38790	57.0	27.020
(2-hydroxy-3-octadecanoyloxypropyl) octadecanoate.	Fatty Acid Ester	9716	57.0	28.218
Tetracontane ..	Hydrocarbon	56949	57.0	29.220
(Z)-9-Octadecenoic acid 1,2,3-propanetriyl ester	Fatty Acid Ester	14734	55.0	29.954
E)-octadec-9-enal.	Aldheyde	14614	55.0	30.112
(1*R*,4*R*)-3,3,4-trimethyl-4-(4-methylphenyl)cyclopentan-1-ol.	Hydrocarbon	3318	147.0	30.717

(*Z*)-docos-13-enamide.	Amide	56502	59.0	31.153
3-[(*E*)-dodec-2-enyl]oxolane-2,5-dione.	NC*	10118	67.0	31.158
2-methyl-3-(4-propan-2-ylphenyl)propanal.	Aldheyde	39439	133.0	31.253
1-chloroheptacosane.	Hydrocarbon	51728	57.0	31.267
2-octyl-3-pentadecyloxirane.	Hydrocarbon	9077	55.0	31.270
3,4-dihexyl-7,7-dimethylcyclohepta-1,3,5-triene.	Hydrocarbon	18042	119.0	31.398
12-[(2*S*,3*R*)-3-octyloxiran-2-yl]dodecanoic acid.	Hydrocarbon	6008	67.0	31.655
4-methyl-2-[(2,4,6-trimethylphenyl)methylsulfanyl]-1*H*-pyrimidin-6-one.	NC	67968	133.0	31.944
Tetrapentacontane.	Hydrocarbon	88322	57.0	32.247
Dotriacontane .	Hydrocarbon	14947	57.0	34.587
4-*O*-(2,2-dichloroethyl) 1-*O*-undecyl (*E*)-but-2-enedioate.	Fatty Acid Ester	26373	69.0	34.595
α-Tocopherol-β-D-mannoside.	Vitamin	10086	165.0	35.329
5,6,7-trimethoxy-2-(4-methoxyphenyl)chromen-4-one.	Flavone	52630	327.0	36.140
β sitoesterol.	Steroid/Triterpene	2386	135.0	38.729
Cyclolaudenol.	Steroid/Triterpene	7665	207.0	41.783
Stigmasterol.	Steroid/Triterpene	34250	95.0	43.329
Methanol				
1-(4-hydroxy-2-methylphenyl)ethanone.	Acetophenone	66807	150.0	13.982
2-(hydroxymethyl)-2-nitropropane-1,3-diol.	Alcohol	25556	57.00	16.115
Trehalose .	Saccharide	56984	73.00	19.635
Methyl hexadecanoate.	Fatty Acid Ester	13085	74.00	23.918
Methyl (9*E*,12*E*)-octadeca-9,12-dienoate.	Fatty Acid Ester	3758	67.00	26.202
Methyl octadeca-9,12,15-trienoate.	Fatty Acid Ester	7985	79.00	26.292
(4*R*)-2-methylpentane-2,4-diol.	Alcohol	10890	59.00	5.676
1-(3-methoxyphenyl)ethanone.	Acetophenone	1754	44.00	14.113
2-chloro-4-methylpentan-3-ol.	Alcohol	7720	57.00	16.126
β-D-galactopyranosyl-(1→4)-D-glucose.	Saccharide	11882	73.00	19.655
[(E)-henicos-10-en-11-yl]benzene.	Hydrocarbon	6891	118.0	24.983
Hexan				
Tetradecan-2-ylbenzene.	Hydrocarbon	12199	105.0	20.874
Tetrapentacontane.	Hydrocarbon	42750	57.0	34.579
α-Tocopherol-β-D-mannoside .	Vitamin	42982	165.0	35.317
Stigmasterol.	Steroid/Triterpene	77970	95.0	43.328

*NC – Not Classified.

Lipid Profile – Gas Chromatography (GC-MS)

The lipidic profile of *N. procerum* was investigated in the same apolar extractors used in GC-MS assay, whereas in the polar phase, it was analyzed in aqueous solvents, used in biological tests. The major identified constituents were Linolelaidic acid methyl ester (C18:2 – Omega 6), Methyl palmitate (C16:0 - Palmitic Acid), Cis-9-Oleic acid methyl ester (C18:1 – Oleic acid) and ɣ-Linolenic acid methyl ester (C18:3 – Gamma linolenic acid/Omega 6) (Table 3). Omega-6 Linolelaidic acid, an isomer of Linoleic acid found in spinach, broccoli, potatoes soya bean, cotton seed oil and sunflower oil (Talapatra, Talapatra, and Nicolaou 2015), was the major component found in the extracts. Furthermore, palmitic, oleic and ɣ-Linolenic acids are lipidic compounds commonly detected in Bromeliads(Payrol, Martinez, Migdalia, and García, Janet 2001)

Other minor constituents detected were stearic and palmitoleic acids, previously reported in *A. erctifolius* L.B.Sm.(Marques et al. 2007)*, B. pinguin* L(Payrol et al. 2001), *B. laciniosa* Mart. ex Shult. & Schult.f*, N. variegata* Mez and *E. spectabile* Schult. & Schult. Bromeliads (Juvik, Ole et al. 2017). Moreover, eicosanoic and tricosanoic acids were also described (Table 3) and, to our knowledge, it was the first time these compounds are found in bromeliads leaves extracts. Eicoisanoic acid was already described in *A. erectifolius* bromeliad (24,2 mk/Kg in Fibers)(Marques et al. 2007) and reported for having anticancer and anti-inflammatory potential(Arora, Kumar, and Meena 2017); while tricosanoic acid was present in hexane extract of leaves from *Ananas cosmosus* bromeliad, which demonstrated potential cytotoxic against tumoral cell lines (Arruda et al. 2018).

Fatty acids play an important role in biological functions of living organisms, contributing to the prevention and treatment of some diseases. Diets with oleic, linolenic, linoleic and linoleic conjugates have been shown to reduce plasma cholesterol levels, in addition to affecting some physiological reactions, such as immune response and inhibition of tumor growth (Oomah and Mazza 1999), decreased risk of coronary heart disease, and protective action against stroke, age-related cognitive decline and Alzheimer disease (Arsic, Stojanovic, and Mikic 2017; Macdonald 2013). Moreover, Omega-6 fatty acids has gained attention in medicine studies for showing potential in preventing sarcopenia, modulate cancer, atherosclerosis, obesity, immune function and diabetes (Kim et al. 2016;

Macdonald 2013). This is the first time the lipid profile of *N. procerum* was described, improving the knowledge of its chemical compounds.

TABLE 3. PERCENTAGE OF FATTY ACID IN RELATION TO THE TOTAL FATTY ACIDS PRESENT IN THE HEXANE, CHLOROFORM, HOT AQUEOUS AND COLD AQUEOUS EXTRACTS OF FRESH N. PROCERUM LINDM PLANTS MULTIPLIED ON MS AFTER 90 DAYS OF IN VITRO CULTURE.

Esters obtained from fatty acids	% of Fatty Acids			
	Hexane	Chloroform	HA	CA
Methyl palmitate (C16:0) (*Palmitic Acid*)	19.77	24.08	23.25	20.78
Methyl palmitoleate (C16:1) (*Palmitoleic Acid*)	5.87	2.86	5.36	6.66
Methyl heptadecanoate (C17:0) (*Margaric Acid*)	ND	ND	4.16	ND
Methyl octadecanoate (C18:0) (*Stearic Acid*)	4.62	9.10	ND	8.20
cis-9-Oleic acid methyl ester (C18:1_cis9) (*Oleic Acid*)	15.81	10.25	34.86	24.12
Linolelaidic acid methyl ester (C18:2) (*Omega-6*)	28.65	21.95	27.72	21.19
Methyl Arachidate (C20:0) (*Eicosanoic Acid*)	8.78	8.56	0	10.53
ɣ -Linolenic acid methyl ester (C18:3 n- 6) (*Omega-6*)	14.71	15.54	4.64	8.52
Methyl tricosanoate (C23:0) (*Tricosanoic Acid*)	1.79	7.66	ND	ND

Chlorophyll quantification

Leaves of *in vitro* cultivated *N. procerum* showed a greater concentration of chlorophyll b (215.06 ±14.8 $\mu g.g^{-1}$ of fresh mass) than chlorophyll a (170.75 ±18.5 $\mu g.g^{-1}$ of fresh leaves). In comparison with *Nidularium campo–alegrense* Lem (56.4 ±11.2 $\mu g.g^{-1}$ fresh mass) and *Aechmea ornate* Baker (97.1±11.2 $\mu g.g^{-1}$ fresh mass) wild-type bromeliads(Prado, Jenny, Paola et al. 2014)*, N. procerum* presented higher amounts of both chlorophylls. The photosynthetic mechanism of plants grown during *in vitro* culture is not completely active and the leaves have a reduced capacity to synthesize organic compounds(Cassana et al. 2010). Then, plants can compensate this failure with greater amounts of photosynthetic

pigments, as they tend to increase its concentration, with reduced light intensity. Furthermore, bromeliads usually grow under the canopy and the leaves of shade plants often have higher content of chlorophylls than sun species(Goodchild, Bjorkman, and Pyliotis 1972).

Despite the low ratio between chlorophylls a/b (0.79 ± 0.1 $\mu g.g^{-1}$), when compared to *N. campo – alegrense* Lem (3.07 $\mu g.g^{-1}$) and *A. ornate* Baker (2.94 $\mu g.g^{-1}$) grown in normal conditions(Prado, Jenny, Paola et al. 2014), the higher amount of pigments can be related to the improvement of biological activity of the extracts. Chlorophyll compounds have been described as potential antioxidants with effective activity against lipidic peroxidation, DNA degradation and some cases of anemia(Durga and Banu 2015; Marková et al. 2018). Furthermore, recent works showed that chlorophyll derivatives, such as chlorophyllide, are also closely correlated to enhanced selectivity and improved cytotoxic activity against a range of carcinoma cells(Wang et al. 2019).

Total phenolic content

The ET extract presented the highest concentration (107.27 mg of gallic acid/100g), followed by CA (96.82 mg of GAE/100 g) and HA (78.57 mg of GAE/100 g). Similar results (70.73 mg of GAE/100 g) were reported in fresh fruit extracts of wild-type *Bromelia anticantha* Berto (Krumreich, Fernanda et al. 2015).

In general, phenolics have gained attention due to their antioxidant, antimutagenic, anticancer and anti-inflammatory capacities(Rondón et al. 2018). The aromatic benzene rings with substituted hydroxyl groups are responsible for their biological activity through the capacity to eliminate or absorb free radicals, and to chelate reactive oxygen species molecules formators. Furthermore, the effectiveness is generally proportional to the number of hydroxyl (OH) groups present in their aromatic rings (Macêdo, Larissa, Alves, Ribeiro et al. 2018).

Phenols Content

The phenolic profile of aqueous extracts was evaluated in HPLC, in order to identify some antioxidants, present in the solvents applied in the biological tests. The compounds were identified and quantified by comparing their retention times and absorption spectrum data in ultraviolet, which presented UV-band

characteristic for gallic acid, p-coumaric acid, rutin, daidzein, quercetin, trans-cinnamic acid and genistein (Table 4).

The main component found was the isoflavone genistein (19.4 $\mu g.g^{-1}$)—a compound belonging to the flavonoids class of phenols. Other flavonoids were also detected, such as tannin gallic acid (2.6 $\mu g.g^{-1}$), flavone rutin (3.4 $\mu g.g^{-1}$) and flavonol quercetin (0.7 $\mu g.g^{-1}$). With the exception of genistein, that is usually found in leguminous(Graham 1995), flavonoids are characteristic of the Bromeliaceae family, having been reported in *Bromelia balansae* Mez, *E. spectabile* Mart. ex Schult. & Schult.f among others (Coelho et al. 2010; Santana, Clara et al. 2012).

Flavonoids and phenolic acids, such the p-coumaric and trans-cinnamic found in this study, are known by their antioxidant, antibacterial, antiviral, anti-inflammatory, cardio and hepatoprotective effects (Kumar and Pandey 2013; Wang, Li, and Bi 2018). Genistein, in particular, have already being described for having chemotherapeutic potential against some tumor lines, such as prostate and gastric cancer (Kumi-Diaka et al. 2004;, Matsukawa et al. 1993).

TABLE 4. RETENTION TIMES OF PHENOLIC COMPOUNDS PRESENT IN AQUEOUS EXTRACTS OF N. PROCERUM LINDM.

	Hot Aqueous extract of *Nidularium procerum*				Cold Aqueous extract of *Nidularium procerum*		
Phenolic Compounds	Wavelength (nm)	Ret. Time	Area	Concentration ($\mu g.g^{-1}$)	Ret. Time	Area	Concentration ($\mu g.g^{-1}$)
Galic Acid *3,4,5-trihydroxybenzoic acid*	275	3.39	252476	2.35±0.27	3.39	279619.3	2.63±0.45
p-Coumaric Acid *3-(4-Hydroxyphenyl)-2-propenoic acid*	311	18.6	991292	4.39±1.98	18.56	1117747.3	4.96±0.22
Rutin *Quercetin-3-Rutoside*	357	21.7	129072	2.99±0.33	21.72	143847	3.41±0.23
Daidzein *Dihydroxyisoflavone*	260	23.4	161855	BDL*	23.1	170331	BDL
Quercetin 3',4',5',7– *Tetrahydrixyflavon-3-ol*	370	25	30647	0.67±0.09	25	33351.6	0.71±0.04

Trans-Cinnamic Acid *Phenylacrylic acid*	275	25.3	322897	1.15±0.09	24.87	272689.6	0.9±0.2
Genistein *5,7,4 Trihydroxyisoflavone*	325	25.7	244231	18.75±3.36	25.9	252668.3	19.44±4.41

*BDL – Below detection limit.

Antioxidant activity of aqueous extracts

DPPH scavenging activity of hot aqueous (IC_{50}: 0.18 ± 0.01 $g.g^{-1}$ dry weight) and cold aqueous extract (IC_{50}: 0.29 ± 0.01 $g.g^{-1}$ dry weight) was dose-dependent, inhibiting up to 90% of free radicals and lower than the standard Trolox (IC 50: 18.30 ± 0.1 $g.g^{-1}$ dry weight). However, HA extract also showed the highest value of Trolox equivalent (21.4 mg of Trolox.g^{-1} dry wheight), than CA extracts (18.83 mg of Trolox.g^{-1} dry weight) in 2,2 – azinobis – 3 – ethyl – benzoatiazolin – 6 – sulfonic acid (ABTS) assay. According to total phenolic content of *N. procerum*, HA showed a significant difference to CA ($p<0,05$), which is closely related to its higher antioxidant potential between the extracts.

In the human body, antioxidants are efficient against some metabolic disorders that compromise the corporal homeostasis, such as lipidic and protein peroxidation, DNA degradation and cell membrane alteration (Miron et al. 2019). The antioxidant power of *N. procerum* extracts can be attributed to the molecular structure of compounds, in particular polyphenolics. They can inhibit free radicals and chelate metals, acting in entire oxidative process. Gallic acid has three hydroxyl radicals attached in its aromatic ring, which are considered to be closely related to its antioxidant, cytotoxic and antiproliferative potential (Gomes et al. 2003). In comparison to other phenolic acids, trihydroxilated derivatives displays grater biological activities than phenolic acids with fewer hydroxyl radicals in their molecular structure, such as p-coumaric, trans-ferulic and trans-caffeic acids (Silva, Borges, and Guimara 2000). Furthermore, compounds such as chlorophylls, alkaloids and some fatty acids, appear to contribute to the antioxidant activity, due to their ability in delocalize the unpaired electrons of free radicals (Gan et al. 2017).

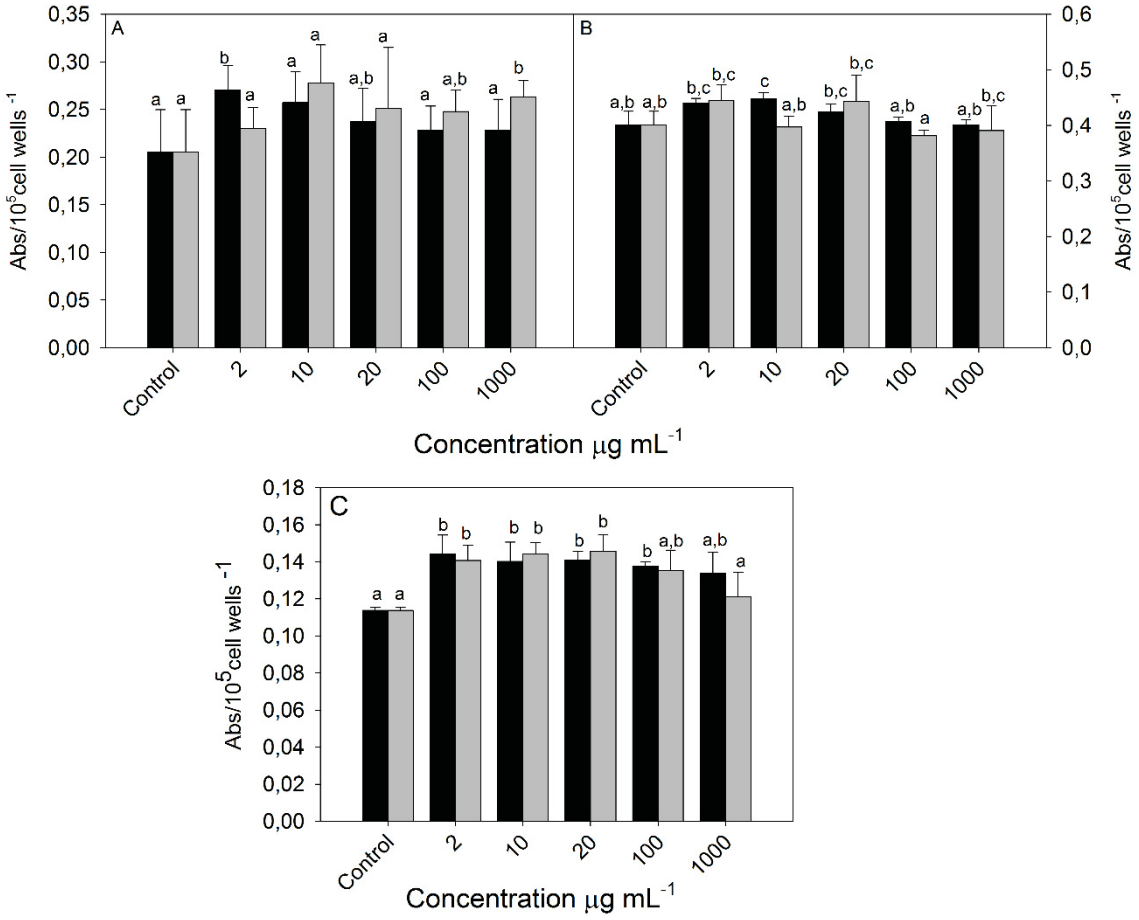
Immunomodulatory Activity

Many plant extracts have long been described as possessing anti-inflammatory and immunomodulatory actions. The first line of human body defense against invading pathogens is the innate immune system, through macrophage cells. In the present study, murine macrophages were assessed *in vitro* by morphological indicators, such lysosomal volume, adhesion and phagocytic capacity, as well as metabolic activities of hydrogen peroxide and superoxide anion (Figure 2). The macrophage adhesion response showed that the lowest concentration (2 $\mu g.mL^{-1}$) of HA extract elicited a significant raise ($p<0.05$) of about 32 % of its activity (Figure 2A**)**. On the other hand, CA extract showed no significant impact at 2 $\mu g.mL^{-1}$, but increased the adhesion capacity at a higher concentration tested (1000 $\mu g.mL^{-1}$) in over 28% of isolated macrophages. The adhesion alteration capacity of cells may be linked to the biological compounds present in HA extract. Fatty acids and polyphenols, especially flavonoids and tannins, can change properties of the plasma membrane, altering its fluidity capacity, as well as the distribution of adhesion molecules within the plasma membrane, such β1 (CD29) and β2 (CD18/11a, b, c) integrins (Kim et al. 2011). β1 (CD29) and β2 (CD18/11a, b, c), molecules involved in mediate the adherence of phagocyte cells to endothelium cell receptors.

Cells treated with 10 $\mu g.mL^{-1}$ HA elicited a significant increase in the phagocytic capacity of macrophages ($p<0.05$), while in CA extract, no significant effect was observed at all concentrations tested (Figure 2B). Similar results were reported in cells treated with methanolic extracts of *Garcinia mangostana* L. and *Annona muricate* L (Harun, Nurul, Septama, and Jantan 2015). Triterpenes, isocoumarins, steroids and flavones described in this study (*e.g.,* stigmasterol, rutin, daidzein and genistein) can induce activities in phagocytic cells, by activating surface receptors, such Fc gamma (FcɣRI, FcɣRIIA and FcɣRIIB), starting the “zippering” phagocytosis (Underhill and Goodridge 2012) and also increasing the expression of complement receptors – CR1, CR3 and CR4 – promoting the “sinking” phagocytosis (Campagne, Wiesmann, and Brown 2007). Furthermore, unsaturated fatty acids have previously shown to improve phagocytosis ability (Calder et al. 1990), and according to Table 3, HA presented higher content of unsaturated fatty acids (73 %) than CA (60 %).

The phagolysosomal formation of macrophages was stimulated when the cells were treated with lower concentrations of extracts ($p<0,05$), raising ~25% of neutral red uptake in both HA and CA, at 20 $\mu g.mL^{-1}$ (Figure 2C). This held the dose-dependent response and the proportional increase rate observed previously in phagocytosis activity. Lysosomes play the role of digesting intracellular components and also break down phagocytosed material, through the fusion of phagosome to hydrolase-containing lysosomal vesicles (Da Rosa et al. 2014), improving the defense cell mechanism.

FIGURE 2: MACROPHAGES ADHESION (A), PHAGOCYTOSIS ACTIVITY (B) AND PHAGOLYSOSOMAL FORMATION (C) OF MACROPHAGES (CELL LINE RAW 264.7) TREATED WITH HOT AQUEOUS EXTRACT (DARK BARS) AND COLD AQUEOUS EXTRACT (GRAY BARS) OF *NIDULARIUM PROCERUM* LINDM. VALUES ARE MEAN ± SE (N=12). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST ($P < 0,05$).



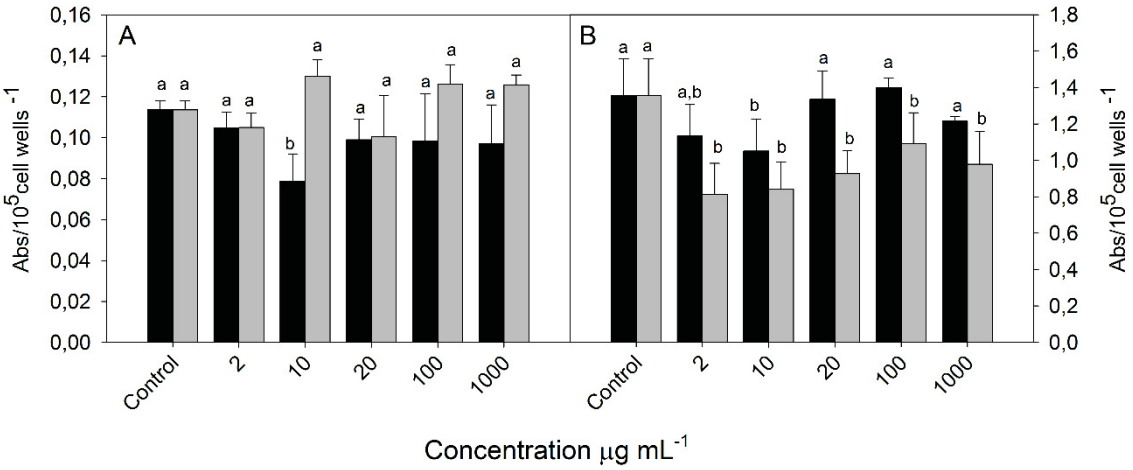
Reactive oxygen species, such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are generated in the first minutes of macrophage stimulation, during

the so-called “respiratory burst” (Tan et al. 2016). They are involved in the inflammation process, acting as efficient protectors; however, uncontrolled or excessive ROS production can further promote oxidative stress —a disruption in the redox balance system that contributes to damaging the body´s own cells and tissues (Castaneda et al. 2017).

HA extract (10 μg.mL^{-1}) was capable to inhibit 36% the production of hydrogen peroxide (H_2O_2) in macrophage cells (Figure 3A), while CA did not differ in comparison to the control at any concentration tested ($p<0.05$). In the same way, HA (10 μg.mL^{-1}) significantly reduced 38% of the production of superoxide anion (Figure 3B), and different from that described in the H_2O_2 assay, CA was capable of inhibiting the production of O_2^- in all concentrations ($p<0,05$), with maximum reduction (40%) of activity at 2 μg.mL^{-1}. This may be related to the potential of some flavonoids, such as rutin and quercetin, present in higher concentrations in CA extracts (3.41 and 0.71 μg.g^{-1} respectively) than HA, in the inhibition of xanthine oxidase and phosphoinositide 3 – Kinase γ enzymes (Selloum et al. 2001). Furthermore, the higher concentration of H_2O_2 in relation to superoxide anion radical could be involved through the formation of enzyme-flavonoids hydrogen bonds, inhibiting the antioxidant activity of some peroxidase enzymes, such as catalase (Majumder, Das, and Saha 2017).

FIGURE 3: HYDROGEN PEROXIDE PRODUCTION (A) AND SUPEROXIDE ANION PRODUCTION (B) OF MACROPHAGES (CELL LINE RAW 267.4) TREATED WITH HOT AQUEOUS EXTRACT (DARK ARS) AND COLD AQUEOUS EXTRACT (GRAY BARS) OF

NIDULARIUM PROCERUM LINDM. VALUES ARE MEAN ± SE (N=12). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST (P < 0,05).



Some molecules described in *N. procerum* extracts, especially flavonoids and derivatives, can act in different mechanisms of enzymes responsible for the oxidative burst in cells. The inhibition of ROS is related to their structure, the number and orientation of the hydroxyl group and the antioxidant potential of each compound; employing in its ability to permeate cell membrane and modulate the pathway signaling of NADPH-oxidase, phospholipase D, protein kinase C-(PKC) alpha, among others (Ciz et al. 2012). Plant extract compounds can also increase the expression of genes associated with the antioxidative system, such Cu/Zn-SOD, Mn-SOD, catalase, and GPx genes, suppressing oxidative stress by increasing antioxidant activity of enzymes (Hwang, Hwang, and Song 2016).

The ability to modulate all macrophage parameters found in *N. procerum* extract can be promising to help fight inflammation and even maintain cell homeostasis under different conditions. Leaf aqueous extract of *N. procerum* was also shown to interfere in different functions of host response capacity against injuries, such the inhibition of lipid body formation, PGE2 and cytokine production of *in vivo* pleural leukocytes (Amendoeira, Fabio et al. 2005). Taken together, these data indicate that the substances described in the leaves of *N. procerum* proved to be efficient in modulating significant responses mediated by macrophages. This can be a potential alternative as a therapeutic agent applied in the prevention and treatment of pathologies related to the immune system. In

addition, previous studies demonstrated that plant-derived compounds are able to alter the immunosuppressive status of patients, increasing antitumor immunity, promoting the proliferation of immune cells and accelerating macrophage phagocytosis (Matsushita and Kawaguchi 2018). To the best of our knowledge, there is no study on the anti-tumor activity of *N. procerum* extract. Studies were also carried out to evaluate the cytotoxic activity of *in vitro* cultured *N. procerum* Lindm against H295R cell line, a carcinoma with rare, heterogeneous malignancy and a very poor prognosis (Kou et al. 2018).

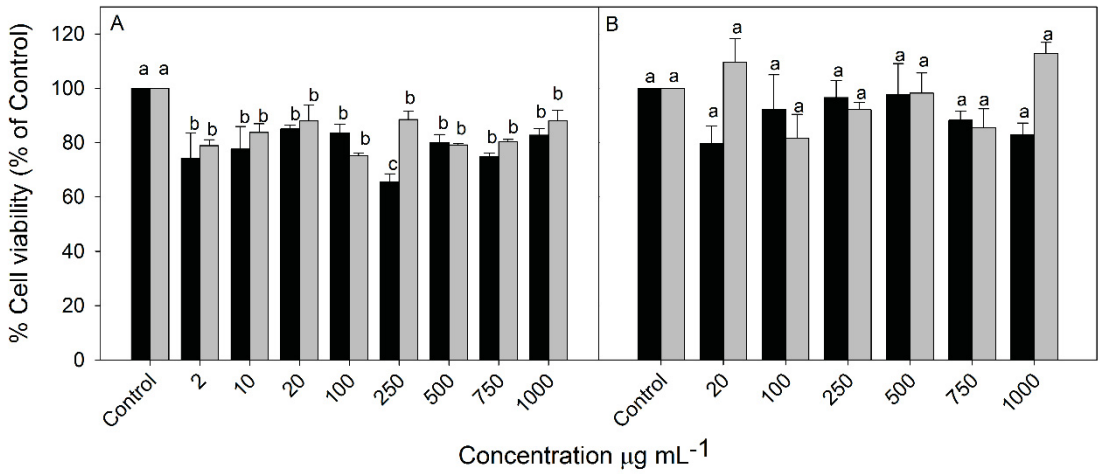
Antitumoral Activity

The key results obtained by MTT assay in H295R and the non-tumoral African green monkey kidney (VERO) cell lines exposed from 2 $\mu g.mL^{-1}$ to 1000 $\mu g.mL^{-1}$ for 24 h are summarized in Figure 4**.** Both HA and CA showed significant decrease in tumor cell viability at all concentrations tested (Figure 4A). The maximum mortality rate was 24,7% (CA at 100 $\mu g.mL^{-1}$) and 34,4 % - (HA at 250 $\mu g.mL^{-1}$). On the other hand, there was no statistical difference among extracts and control in the viability of non-tumor cells (VERO) (Figure 4B). The levels of extracts also showed no statistical differences, with no interaction among them. Molecules, such as phenolics described in this study, can either inhibit or stimulate the oxidative damage process, depending on the dose, structure, target molecule and environment (Khan, Ahmad, and Hadi 2000). In the present work, both HA and CA showed no cytotoxicity against normal cells, which makes these extracts promising as sources for the development of alternative drugs.

Antitumoral activity has already been found in some species or Bromeliaceae, such as *A. comosus* L. (Báez et al. 2007), *Tillandsia recurvata* Baker (Lowe et al. 2017) and *Bromelia fastuosa* Lindl. (Guimarães-Ferreira et al. 2007). The antitumoral activity was attributed to cysteine proteinases (*e.g.,* bromelain and fastuosain) as well as flavonoids, including penduletin, cirsimaritin and HLBT-100 (Raffauf et al. 1998; Lowe et al. 2017). Biological compounds are related to the suppression of some metastatic markers, resulting in regulation of mitogen activated protein kinase and protein kinase B (Kim et al. 2019). Genistein, present in higher amounts in both HA and CA (Table 4), is also related to the inhibition of protein tyrosine kinase and topoisomerase II, and elimination of oxygen free radicals (Uckun et al. 1995; Barnes 1995), inhibiting the bioavailability of sex

hormones, platelet aggregation, angiogenesis, as well as modulating the apoptosis of malignant cell lines (Ravindranath, M. H.; Muthugounder, S.; Presser, N.; Viswanathan 2004). The biological activities of genistein are also related to the intramolecular hydrogen bonding formed by 5-hydroxyl and 4-ketonic oxygen (Hiroshi Ogawara, Testu Akiyama, Shun-Ichi Watanabe, Norik Ito, Masato Kobori 1989; Polkowski and Mazurek 2000). These characteristics may be related to the cytotoxic potential of HA and CA extracts.

FIGURE 4: VIABILITY PERCENTAGE OF TUMOR CELLS (H295R) (A) AND NON-TUMOR CELLS (VERO) (B) TREATED WITH DIFFERENT CONCENTRATIONS OF N. PROCERUM LINDM AQUEOUS EXTRACTS WITHIN 24H. VALUES ARE MEAN ± SE (N=12). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST ($P < 0.05$). BLACK BARS: HOT AQUEOUS EXTRACT (HA). GREY BARS: COLD AQUEOUS EXTRACT (CA).



Furthermore, some tannins, alkaloids, saccharides and fatty acids, especially polyunsaturated, have proved to be efficient as antitumoral agents, inducing autophagy of cells and other pathways (Lee, Yoon, and Lee 2018; Yildirim and Kutlu 2015). Until now, there have been only a few studies in the biological activity of *N. procerum* and none of them included the chemical compounds related to it, nor their potential as antitumor agents. Adrenocortical carcinoma is a rare and aggressive neoplasm with pour prognosis (Parikh et al. 2018; Tripathy et al. 2020), in which most patients diagnosed with advanced disease had a median survival time of less than 12 months and a 5-year survival

rate of less than 15% among patients with metastatic disease (Tran et al. 2016). Brazil has a 15 to 18-fold higher incidence of pediatric adrenocortical carcinoma (ACC), than other countries (CUSTÓDIO et al. 2013). In this scenario, the biological activity reported for *N. procerum* shows potential for the development of alternative treatments against adrenocortical carcinoma, which needs to be further explored through isolation and/or microencapsulation of bioactive compounds.

Conclusions

The extracts obtained from the leaves of *in vitro* grown *N. procerum* were chemically characterized for the first time, showing the presence of phenolic compounds, steroids, fatty acids, polysaccharides, α-Tocopherol and scutellarein. These compounds showed good antioxidant activity and promoted the immunomodulation of murine macrophages. The crude extracts also showed potential against adrenocortical carcinoma cells, without cytotoxicity to non-tumoral cells, making it a potential candidate for alternative therapies against this tumoral line. However, further studies should be carried out to isolate and characterize *N.* procerum-derived compounds to improve cytotoxic activity as well as to prevent other human diseases caused by free radicals and other pathways.

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CHAPTER II. GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *NIDULARIUM PROCERUM* EXTRACTS AND ITS BIOLOGICAL ACTIVITY.

Introduction

Silver nanoparticles (AgNPs), silver structures with nanometric scale, have attracted great attention due to their physical, chemical and therapeutic properties, which make them one of the most commercialized nano-material with five hundred tons of AgNPs per year (Shakeel et al., 2016; Larue et al., 2014). Moreover, it is expected an annual increase of 25% and an increase in the nanotechnology industry value from $ 91 billion by 2009 to $1 trillion by 2015, and $3 trillion by 2020 (Tran, Nguyen, and Le 2018). Such growth is related to AgNPs biological properties, such as antibacterial (Shakeel et al., 2016), anti-fungal (Kim et al. 2008), anti-inflammatory (Wong et al. 2009) and anticancer activities (Guo et al. 2013), increasing the interest of the plastics, soaps, pastes, food, drugs and textiles industries (Dallas, et al., 2011; Fabrega, et al., 2011; García-Barrasa, et al., 2011; Tran et al., 2018).

Different methods are reported for the synthesis of nanoparticles. Chemical and physical methods are currently employed. However, these methods involve the use of hazardous chemicals, high energy requirements and many purification steps, which become quite expensive techniques and harmful to the environment (Shakeel et al., 2016). Based on this, biological synthesis can be considered as an attractive alternative for nanoparticles production due to use naturally occurring reagents, such as sugars, biodegradable polymers, plant extracts and microorganism as reductants and capping agents (S Ahmed, et al., 2014; Shakeel Ahmed & Ikram, 2015; Shakeel et al., 2016; Kharissova et al., 2013). This method provides advantages over physical/chemical synthesis, as it is environmentally friendly, simple and low-cost technique, relatively reproducible and results in more stable particles (Shakeel et al., 2016; Tran et al., 2018).

The synthesis of silver nanoparticles through plant extract has gained attention to be an alternative process for AgNP synthesis and green technology.

The use of plant extract as reducing agent eliminates the problem related to the toxicity caused by using hazardous chemicals, making the nanoparticles more bio-compatible for medical purposes. Moreover, plant extract could be advantageous over other biological processes by eliminating cell cultures (Bar et al. 2009). Recent articles report the production of AgNPs using seed extract of *Jatropha curcas* (Bar et al. 2009) and *Coffea arabica* (Dhand et al. 2016), *Ixora coccinea* leaves extract (Karuppiah and Rajmohan 2013), *Capsicum annuum* L. extract (Li et al. 2007), eucalyptus leaf extract (Mo et al. 2015) and *Cassia angustifolia* leaf extract (Amaladhas et al., 2012). However, the potential of use plant extract as source for nanoparticle synthesis related to its biological effects is still unexplored (Shakeel et al., 2016). The focus of this present study was to investigate the green synthesis of AgNP using extract of the *Nidularium procerum* as reducing agent. *N. procerum* is a member of the Bromeliaceae family, found throughout the Brazilian costal rain forest. It has been reported that the extracts of *N. procerum* are nontoxic and have analgesic, anti-inflammatory and anti-allergic properties, making it a potential bioreductant for AgNP synthesis, and promising alternative in the search for new drugs development against diseases without effective treatment, especially cancer (Fabio Coelho Amendoeira et al. 2005; Vieira-de-Abreu et al. 2005).

AgNPs have proven promising antitumor effects. It was reported that a low concentration of AgNPs can cause DNA damage, inhibit cell viability and cell growth, and genotoxicity against different types of tumor cells, such as angiogenesis, leukemia, breast, lung, and skin/oral cancer (Wei et al. 2015).

The present study report for the first time, the biosynthesis of silver nanoparticles via a single-step reduction of silver ions using *N. procerum* aqueous extract, without the use of any reducing or capping agents. In addition, this study examines the biological effects of AgNP synthesized against adrenocortical cancer, a rare tumor with limited medical treatment.

Material and Methods

Preparation of plant extract

Plants were *in vitro* established according to Lopes da Silva et al (2012). After 90 days of *in vitro* culture on MS multiplication media (Murashige and Skoog 1962), supplemented with 30 $g.L^{-1}$ sucrose, 2 μM NAA (naphthalene acetic acid)

and 4 µM BAP (6-benzylaminopurine), all seedlings were well washed with tap water to remove the residual impurities. They were dried with a paper towel, cut into small pieces and 1 g of fresh leaf mass was macerated and extracted in 10 mL of aqueous solvent – boiling water (HA) or 25 °C water (CA) - over 24 hours, under 80 rpm agitation in a 25 °C – dark room.

Silver Nanoparticles Synthesis

The nanoparticles synthesis was carried out by adding 1 mL of *N. procerum* extract (HA or CA) to 9 mL of $AgNO_3$ aqueous solution (1 mM). The solution was well mixed and stored in the dark at room temperature, until the change of the solution color, from the transparent color to the dark brown. It was continuously observed every 10 minutes via UV-Vis spectrophotometer until the stop of the reaction, which is characterized by the overlapping of the peaks in the scanning diagram. After the reaction took place, the solutions (HA-AgNP and CA-AgNP) were stored at 4 °C until be submitted to characterization analysis. To the biological assays, aliquots of nanoparticles solutions were previously lyophilized and resuspended in distilled water at specific concentrations.

Characterization of Silver Nanoparticles

The resulting solutions with the nanoparticles (extracts/AgNPs, as well unreacted bulk reagents that were not removed) were submitted to UV-visible spectroscopy, Dynamic Light Scattering (DLS) and Zeta Potential analysis, and also to visual evaluation such scanning electron microscopy (SEM) / Electrons (BSE) and transmission electron microscopy (TEM).

Ultraviolet-visible spectrophotometry analysis

The bioreduction of Ag^+ ions with different *N. procerum* extract solutions was monitored by absorbance measurement using an ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu, UV-1601 PC). The spectra of the surface plasmon resonance (SPR) of AgNPs in the samples were measured in 200-800 nm ranges with a resolution of 1 nm. The UV-vis spectra were recorded using a quartz cuvette and deionized water was used as reference.

Transmission Electron Microscopy analysis of nanoparticles

The nanoparticle size and morphology were analyzed by using a transmission electron microscope (JEOL, JEM 1200EX-II) operating at 120 kV. TEM samples were prepared by placing a drop of the suspension of *N. procerum* extract/AgNPs on carbon-coated copper grids and allowing water to evaporate at room temperature. The selected area electron diffraction (SAED) patterns were analyzed using the CrysTBox software in order to identify the silver crystal planes.

Scanning Electron Microscopy analysis of nanoparticles

The nanoparticle size and morphology were analyzed by using Scanning Electron Microscopy with Energy Dispersive X-Ray Analyzer (SEM/EDX) to provide elemental identification and quantitative compositional information of samples. The equipment was VEGAN TESCAN, BSE (Back-scattered Electron Detector) mode and operating at 10 kV.

Dynamic light scattering (DLS) and Zeta potential of nanoparticles

The average sizes of AgNPs in aqueous medium were determined by Dynamic Light Scattering (DLS) (Brookhaven, NanoDLS). For the Zeta potential analyses, 2 mL of *N. procerum* extract/AgNPs resulting solutions were diluted in 8 mL ultrapure water or DMEM medium culture (Sigma®). The analyses were carried out using a Zeta Potential analyzer (Stabino) with a 10 mL PTFE measurement cell and a 400 µm piston. The measurement of Zeta potential was done in a pH-titration mode using 0.05 mol.L^{-1} HCl and 0.05 mol.L^{-1} NaOH as titrants.

Antitumoral Activity

The antitumoral activity of the *N. procerum* extract/AgNPs was tested on adrenocortical carcinoma human cell line H295R acquired from the collection of *Instituto de Pesquisa Pelé Pequeno Príncipe* – Paraná – Brazil, by the protocol adapted from Mosmann (1983). It was measured the ability of viable tumor cells to metabolize the yellow water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in blue-violet insoluble formazan, after being treated with the aqueous extracts. After thawing, the cells were transferred to a

cell culture bottle with nutrient mixture culture medium (DMEM – Sigma Aldrich) supplemented with 10% fetal bovine serum (Gibco®) and 1% antibiotic solution (Gibco®). They were incubated in a CO_2 incubator at 37 °C, with humidified air (95%) and CO_2 (5%) until presented 100% confluence. Subsequently, the cells were treated by adding 4 mL of trypsin (Sigma Aldrich) for 5 min, followed by the addition of 4 mL of supplemented culture medium, to neutralize the trypsin activity and form the cell suspension. The standardization of cancer cells in the 96-well plate (Biofil®) was checked by counting in a Neubauer's chamber using 100 µL of the culture medium and 1 x 10^6 cancer cells per well. A column was reserved for the blank containing only the culture medium and another column was reserved for the negative control (cancer cells without addition of compounds). After incubating the ELISA plate for 24 hours to the cell fixation, 20 µL of the treatments with different concentrations were added in 180 µL of fresh culture medium for 24h. The tumor cells were exposed and divided into the following experimental groups: Control (C), representing cells without treatment, the crude Hot Aqueous Extract (HA) and Cold Aqueous Extract (CA) and the synthetized nanoparticles HA-AgNP and CA-AgNP, at concentrations 20, 100, 250, 500 and 750 µg.mL^{-1}. In the last day of experiment, the treatments were removed and 100 µL MTT (3.33 g.ml^{-1}) was added in each well for 3 hours. After the MTT removal, 100 µL dimethyl sulfoxide was added to formazan solubilization. The viable cell number/well is directly proportional to the production of formazan, which solubilized, was measured at 550 nm on microplate reader. The results were expressed as the mean percent inhibition of tumor cell proliferation, calculated by the formula:

$$\text{Cell inhibitory rate (\%)} = (1 - ABS_{\text{experimental group}}/ABS_{\text{control group}}) \times 100 \text{ - Eq (3).}$$

In addition, it was also evaluated de live / dead viability / cytotoxicity of treatments against tumor cells by microscopy images. A fluorescent microscopy protocol was realized by InCell – Analyzer 1000 Workstation equipment, following the protocol proposed by (Zeiss & Gmbh, 2018). All cells were treated by the same treatment protocols described previously.

Statistical Analysis

All analyses were performed in triplicates. The data were expressed as mean ± standard error and analyzed by One Way ANOVA followed by Tukey's

test using Graph Pad Prism 6. Differences between means at the 5% of confidence interval ($p<0.05$) were considered significant.

Results and Discussion

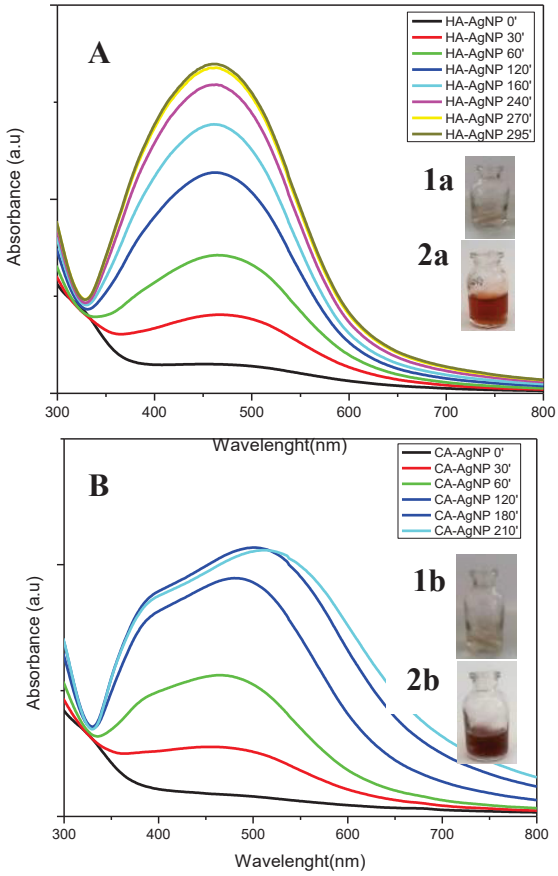
Ultraviolet-visible spectrophotometry analysis

In this study it was investigated the formation of silver nanoparticles (AgNPs) using different solvent temperatures in aqueous extraction of *N. procerum* leaves (HA-AgNP and CA-AgNP). Figure 5 shows that process of silver nanoparticles formation with both extracts was confirmed by the formation of a well-defined absorption peaks exhibited by the nanometallic Ag particles and by a change in color of the reaction solution from almost colorless to yellowish brown (Aziz et al., 2017). This observed color phenomena occurs due to the excitation of surface plasmon vibrations in silver nanoparticles, which is dependent on the size of nanoparticles (Mukunthan et al., 2011).

The kinetic profile of AgNPs formation was monitored for 310 minutes, but trough 270 to 310 min of HA-AgNPs reaction, the process was established in the absorbance intensity. While the maximum reduction and formation of CA-AgNPs was observed at 180 minutes, with no difference in reading at the following readings until the final time.

The UV-Vis spectra of the synthesized AgNPs (Figure 5) were very different from the spectra of the corresponding extracts HA and $AgNO_3$ used as initial reagents. The synthesis of both extracts was similar until 60 minutes of reaction, however after this period there was a formation of two plasmon bands evidenced around 420 nm and in 550nm of CA-AgNP solution, which probably occurs by the complex chemical constitution between the extracts. As previously described in first chapter, hot and cold water are different extractors capable to remove plant compounds with chemical characteristics that act differently in Ag^+ reduction. According to Sorescu et al., (2016), flavones and terpenoid components from leaf broth are able to stabilize the formation of AgNPs, while the heterocyclic components soluble in water are responsible for the reduction of silver ions. Furthermore, the SPR bands are influenced by size, shape, morphology, composition and dielectric environment of the prepared nanoparticles (Kelly et al., 2003).

FIGURE 5. UV-VIS SPECTRA OF SURFACE PLASMON RESONANCE FOR AGNPS. **A** – UV-VIS FORMATION SPECTRA AT DIFFERENT TIMES OF HA-AGNP. **1A** – SOLUTION COLOR BEFORE DE REDUCTION PROCESS AT 0 MINUTE. **2A** – SOLUTION COLOR AT 295 MINUTES AFTER THE REDUCTION PROCESS. **B** – UV-VIS FORMATION SPECTRA AT DIFFERENT TIMES OF CA-AGNP. **1B** - SOLUTION COLOR BEFORE DE REDUCTION PROCESS AT 0 MINUTE. **2B** – SOLUTION COLOR AT 295 MINUTES AFTER THE REDUCTION PROCESS.



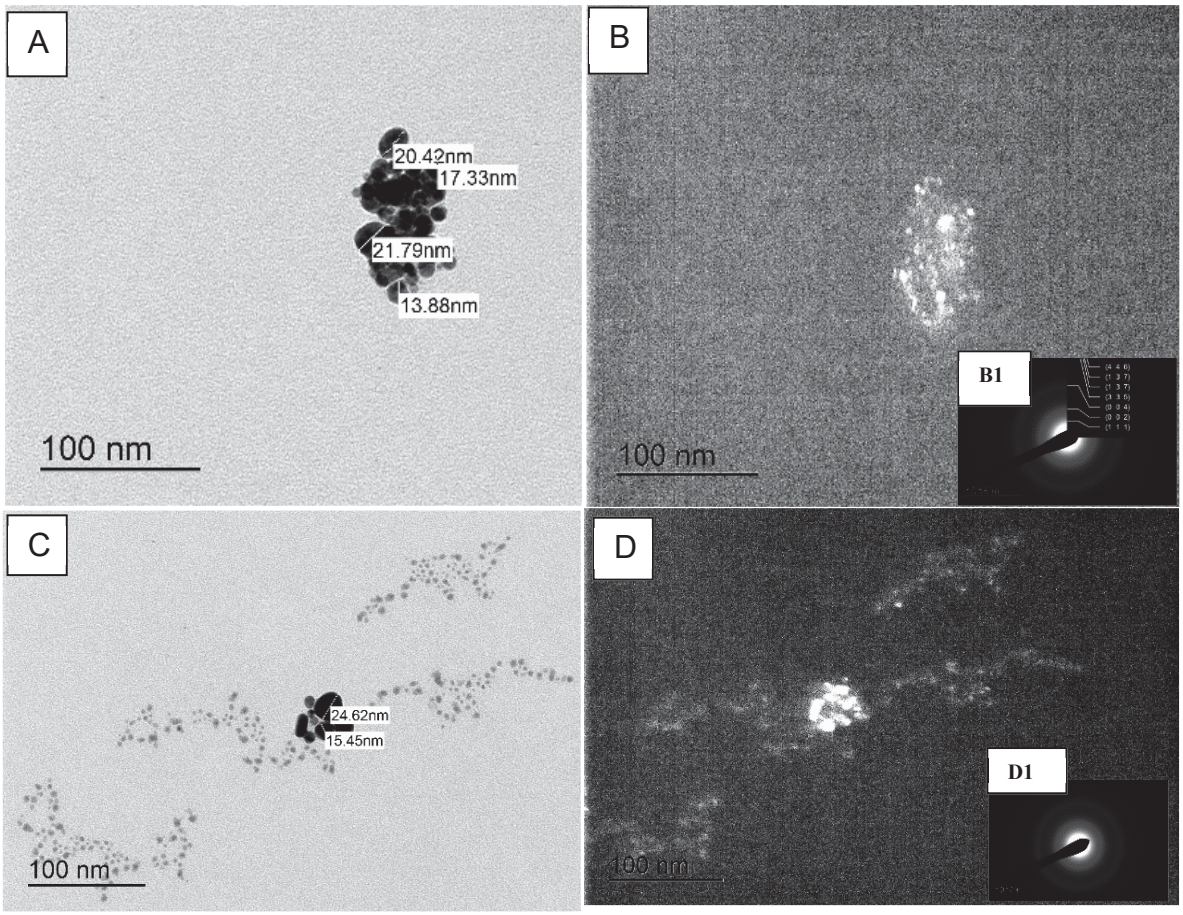
The final scanning evaluated of HA-AgNPs resulting solutions presented a strong, broad SPR peak located between 350 and 600 nm, with maximum absorbance at 460 nm. Whereas CA-AgNPs showed slightly shifted peaks to the right, between 350 and 700 nm, with maximum absorbance at 500-510 wavelength. However, both solutions showed almost the same absorbance values, which is related to similar concentration of AgNP synthesized (Dong et al., 2016).

Transmission Electron Microscopy analysis of nanoparticles diluted in water

Figure 6 shows the TEM mode of both synthesized nanoparticles. In the bright field of HA-AgNPs (Figure 6A), the dark spots revealed that the synthesized AgNPs possess almost spherical morphology with dimensions ranging from 13 to 21 nm, with agglomeration of the particles. The shinning spots on the dark field TEM mode showed metallic silver in the particles, confirming that most of them are real AgNPs (Figure 6B). In Figure 6C, it is possible to verify the bright field of CA-AgNPs that also shows spherical morphology with dimensions ranging from 15 to 25 nm, with a noticeable tendency to aggregate formation. This is explained by the fact that no stabilizing adjuvant was used, with only the plant extract acting as reducer and stabilizer of the nanoparticles. According to Vanaja et al., (2013), the aggregation can occur due to the presence of cell components on the surface of nanoparticles, that acts as capping agents. In addition, many authors also reported this size diversity and aggregation tendency in utilization of plant extract as reducing agents (Heydari & Rashidipour, 2015; Kumar, Smita, Cumbal, & Debut, 2017; Praveena & Kumar, 2014).

The selected area electron diffraction to (SAED) pattern of both extracts revealed the crystalline nature of the nanoparticles. The diffraction rings, from inner to outer, corresponded to (644), (640), (622), (600), (533), (444), (400), (200) and (111) to HA=AgNPs (Figure 6-B1) and (644), (731), (533), (400), (200), (111), (200) to CA-AgNPs (Figure 2 – D1).

FIGURE 6. MORPHOLOGY, DIMENSIONS AND SAED PATTERN OF SILVER NANOPARTICLES SYNTHESIZED FROM N. PROCERUM EXTRACTS DILUTED IN WATER. A-B) HA-AGNPS, BRIGHT AND DARK FIELDS. C-D) CA-AGNPS, BRIGHT AND DARK FIELDS.

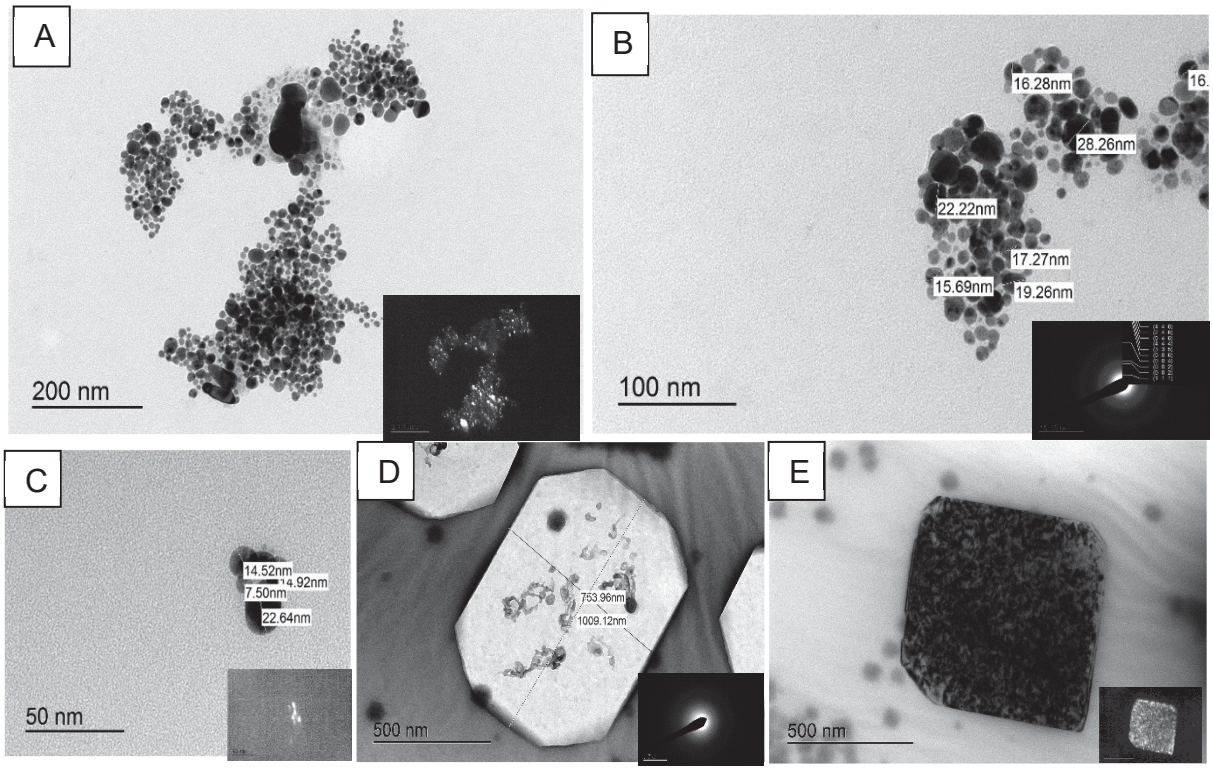


Transmission Electron Microscopy analysis of nanoparticles HA-AgNP diluted in DMEM

Figure 7 shows the TEM mode of both synthesized nanoparticles diluted in DMEM medium culture, nutritional solution utilized in growth cells in biologic tests. In the bright field of HA-AgNPs (Figure 7-A and B), it´s possible to see that HA-AgNPs maintained the tendency to spherical shape and size with more propensity to agglomeration. Besides CA-AgNPs also be present in spherical formats as saw in water dilution (Figure 7C), the microscopy reveals that these nanoparticles, when diluted in DMEM medium, presented a morphological diversity, such spheres, squares, lozenges. Moreover, patterns with nanoparticles aggregates were formed (greater than 500nm to micrometers), which can be attributed to the diversity of the medium culture composition, once it’s constituted by glucose, vitamins, amino acids, inorganic salts (calcium and

magnesium chlorides, magnesium sulphates and sodium bicarbonate) and organics, such sodium pyruvate, phenol red among others, causing the nanoparticles destabilization very quickly (Zook, et al., 2011).

FIGURE 7. MORPHOLOGY, DIMENSIONS AND SAED PATTERN OF SILVER NANOPARTICLES SYNTHESIZED FROM N. PROCERUM EXTRACTS DILUTED IN DMEM MEDIUM CULTURE. A-B) HA-AGNPS, BRIGHT AND DARK FIELDS. C-D-E) CA-AGNPS, BRIGHT AND DARK FIELDS.



Scanning Electron Microscopy analysis of nanoparticles.

The size, shape and distribution of green synthesized silver nanoparticles from both extracts characterized by Scanning Electron Microscopy analysis are showed in Figure 8 and Figure 9. In Figure 8A - 8B, it is possible to observe the spherical formats with non-homogeneous sizes, of HA-AgNPs nanoparticles. While the Back-Scattered Electrons (BSE), presented in quadrant B, shows the aggregates formed by the nanoparticles, with dimensions between 1 and 10 micrometers. The Energy-dispersive X-ray (EDX) (Figure 8-C) is also used to provide elemental identification and quantitative compositional information of solutions, reporting that the structures analyzed were priory constituted by 64.13% carbon, 25.05% oxygen and 7.53% silver, with potassium residual.

The SEM of CA-AgNPs showed that synthesized nanoparticles also present globular forms with undefined size (Figure 9A). The formed aggregates with dimensions between 1 to 20 micrometers are showed in Figure 9B. According to Fatimah (2016), the aggregate formation in SEM analysis can be related to the sample preparation procedure, since it need to be filtered and dried before measurement. The Figure 9C obtained by EDX provide that structures are basically constituted by 63.38% carbon, 25.03% oxygen and 7.4% silver, as well previously reported to HA nanoparticles. CA-AgNPs presented a dimension somewhat larger that HA-AgNPs, which can be due to the different compounds extracted in different temperatures of solvent utilized.

FIGURE 8. SEM MICROGRAPHY OF HA-AGNPS. **A -** IMAGE INCREASE BY 10,000X. **B –** IMAGE INCREASE BY 50,000X. C - ENERGY-DISPERSIVE X-RAY.

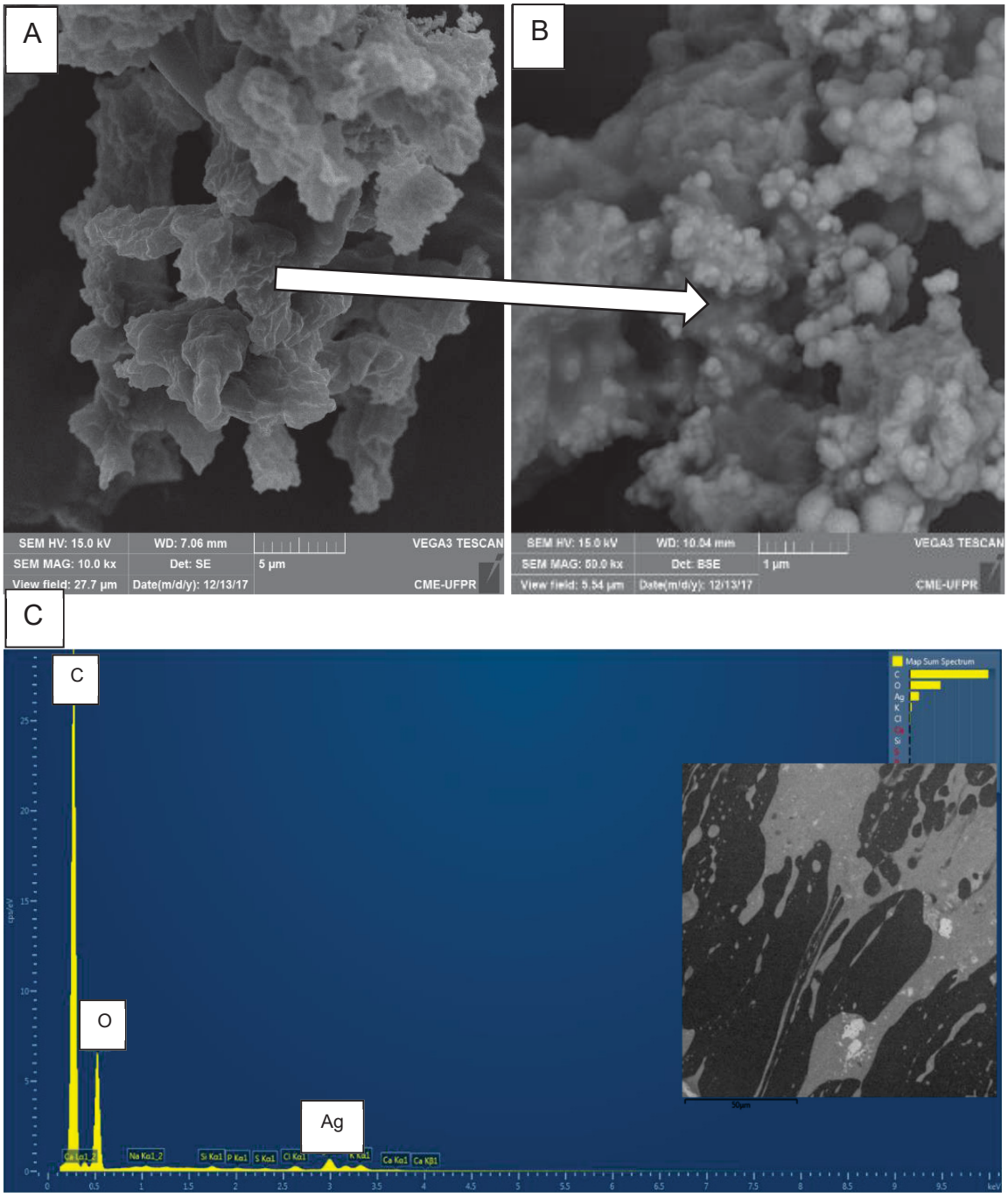
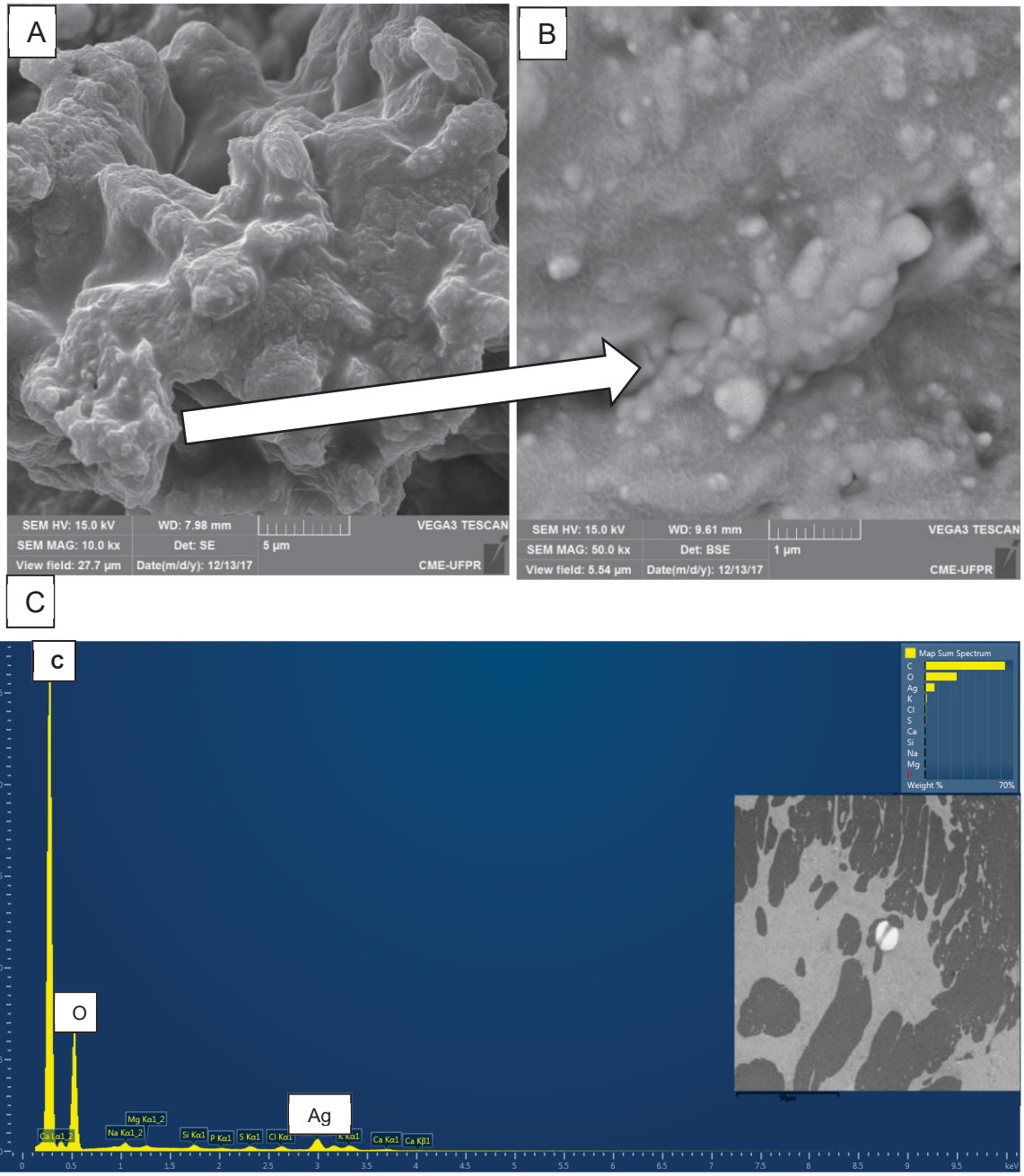


FIGURE 9. SEM MICROGRAPHY OF CA-AGNPS. **A -** IMAGE INCREASE BY 10,000X. **B –** IMAGE INCREASE BY 50,000X. C - ENERGY-DISPERSIVE X-RAY.

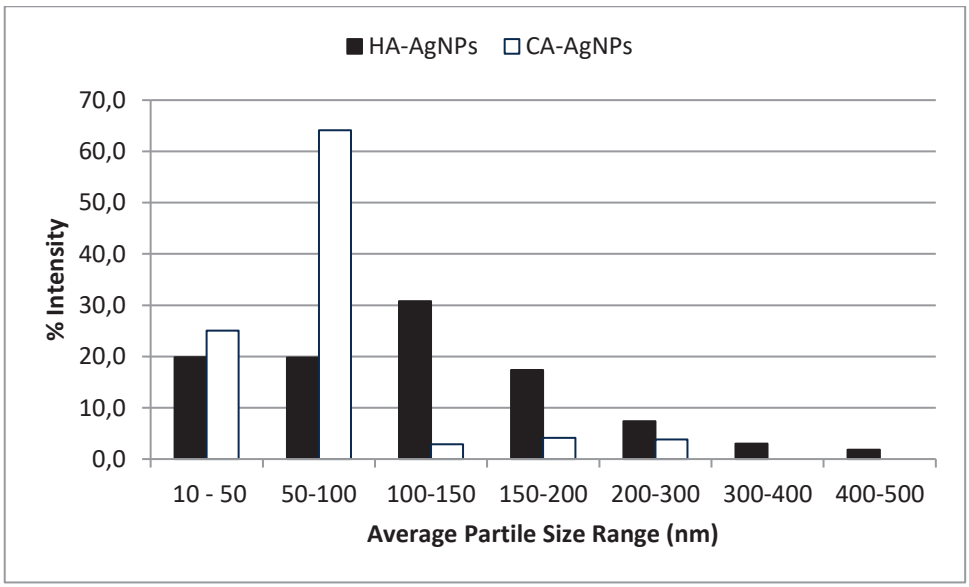


Dynamic light scattering (DLS).

In order to verify the size of the HA-AgNPs and CW-AgNPs, they were analyzed by dynamic light scattering (DLS). Figure 10 shows a broadening size range exhibiting a polydisperse mixture (around 10 nm to 500 nm) that was obtained with a main diameter from 100 to 150 nm for HA-AgNPs, while CA-AgNPs possess most of its particles in the range between 50 – 100 nm. These distribution in size profile can be related due to the detection of minor amounts of

large size particles generates by agglomeration, since there was no adjuvant for this purpose, only the extracts acting with their functional groups in the synthesis. Producing silver nanoparticles by green synthesis of *Lippia nodiflora* aerial extracts, Sudha et al., (2017) also found the average particle size around 145 nm. Although the size from TEM method is within the wide range measured with DLS, CA and HA-AgNPs evaluated by DLS assay presented larger particles size values. The differences in both analyses can be due TEM technique measures only the core size of particles and does not consists of any capping agent, showing smaller diameters that obtained by DLS for coated particles (Tejamaya et al., 2012). Whereas DLS measures the hydrodynamic diameter along ions and associated molecules that are attached to the surface of nanoparticles (Erjaee, et al., 2017). Furthermore, is well-know that methodology and conditions (chemical, physical and biological) used in the synthesis are intrinsically related to size and shape of the nanoparticles (Dhand, et al., 2015; Syafiuddin et al., 2017).

FIGURE 10. PARTICLE SIZE DISTRIBUTION OF HA-AGNPS AND CA-AGNPS (GREEN SYNTHESIS CONDITIONS: 25 °C, pH 5, 310 MINUTES OF SYNTHESIS).



Zeta Potential response of nanoparticles.

The AgNPs colloidal stability characterized by Zeta potential profile is showed in Figure 11. HA-AgNPs exhibited a -20 mV zeta potential between pH 6-10, while CA-AgNP exhibited a zeta potential value of -40 mV at 4-10 pH range when diluted in ultrapure water, which can be consider stable in this region for

both, but higher to CA-AgNP. Generally, nanoparticle suspensions that exhibits an absolute zeta potential less than 20 mV is considered unstable, resulting in the particle precipitation (Sun et al. 2014). On the other hand, high absolute zeta potential values designates high electrical charges on the nanoparticles surface, causing a strong repulsive force among the particles for withholding agglomeration (Guo et al., 2015). Furthermore, the negative values of zeta potential found in *N. procerum* nanoparticles can be related to some factors such, pH, types of functional groups present in solution and molecular weight of the material (Ostolska and Wisniewska 2014). In addition, It can be closely associated due the unbalance of charge on AgNPs surface, which is related to adsorbed polyphenolic compounds and dissociated functional groups present in structures of plant extract (Lopes et al., 2018).

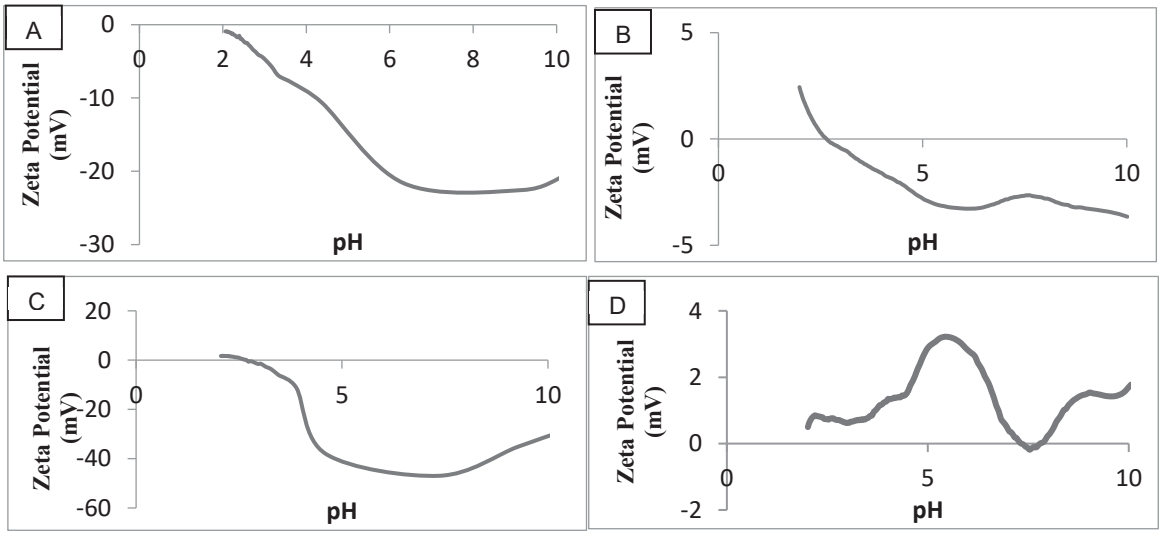
As described in UV-Vis formation profile, the CA-AgNPs showed to have different response that nanoparticles synthesized from HA extract, which can probably be due by the different compounds extracted in each solvent. Furthermore, the results found in zeta potential are in accordance with those described by DLS, since in the natural pH of solution (pH 5), the particles show to be instable to HA-AgNPs, with tendency to aggregate, while in CA-AgNPs the solution are stable and presented minor particles size.

Comparing with other works, green synthesis of AgNPs produced from *Calliandra haematocephala* leaf extract presented a zeta potential value of -17.2 mV (Raja, et al., 2017). Additionally, AgNPs synthesized from leaf extract of *Lawsonia inermis* presented -28.3 mV values of zeta potential, also indicating the production of stable silver nanoparticles (Kiruba, et al., 2013).

The AgNPs were intended to be used in a much more complex solution (culture media) to evaluate its biological activity, so the zeta profiles of AgNPs diluted in DMEM medium culture were also examined. Figure 11 showed that both extract-mediated AgNPs presented instability in colloidal suspension when diluted in this nutritional solution, since the zeta potential changed to -4 mV at pH ranging from 6 to 10 to HA-AgNPs (Figure 11B), and to distinct values close to 0 mV at 2-10 pH ranging to CA-AgNPs (Figure 11D). These anomalous behavior of AgNPs can be related to the agglomeration of nanoparticles but does not necessarily imply that silver ions would not be released from the agglomerate.

According to Albanese et al., (2012), nanoparticles can exhibits distinct biological behavior depending in shape, size and surface chemistry.

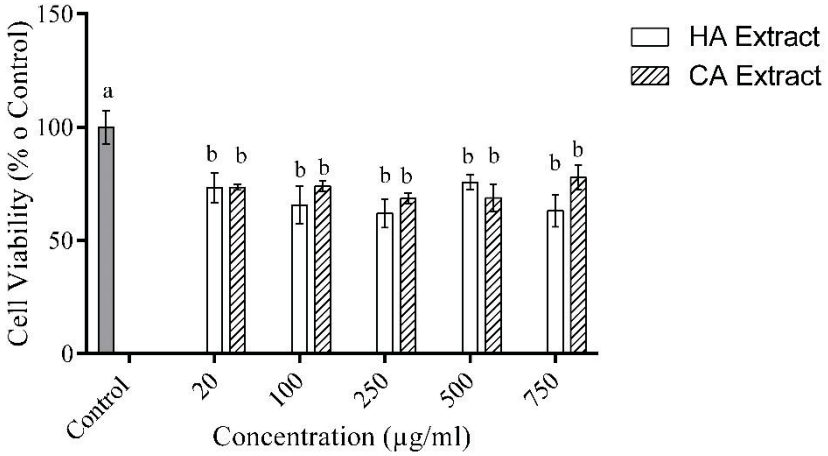
FIGURE 11. ZETA POTENTIAL PROFILES OF SILVER EXTRACT-MEDIATED NANOPARTICLES AT 2-10 PH RANGE. A - HA-AGNPS IN DISTILLED WATER. B - HA-AGNPS IN DMEM CULTURE MEDIA. C - CA-AGNPS IN DISTILLED WATER. D - CA-AGNPS IN DMEM CULTURE MEDIA.



Antitumoral Activity.

The *in vitro* cytotoxicity of *N. procerum* extracts and synthesized nanoparticles were evaluated against human adrenocortical tumor cell line H295R at different concentrations by MTT assay. The cell inhibitory rate of HA and CA leaf extracts (Figure 12) showed no statistical difference between them, but all concentrations evaluated significatively reduced the ability of viable cells in reducing MTT into a blue colored formazan, when compared to control group ($p<0,05$). The maximum inhibition potential observed for HA was 38%, whereas to CA was 31.4%, both at 250 µg/ml. However, it is notable that the increase in concentration of the extracts did not affect the cell proliferation.

FIGURE 12 VIABILITY PERCENTAGE OF ADRENOCORTICAL TUMOR CELLS (H295R) TREATED WITH DIFFERENT CONCENTRATIONS OF N. PROCERUM AQUEOUS EXTRACTS WITHIN 24 H. VALUES ARE MEAN ± SE (N=3). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST (P < 0.05).



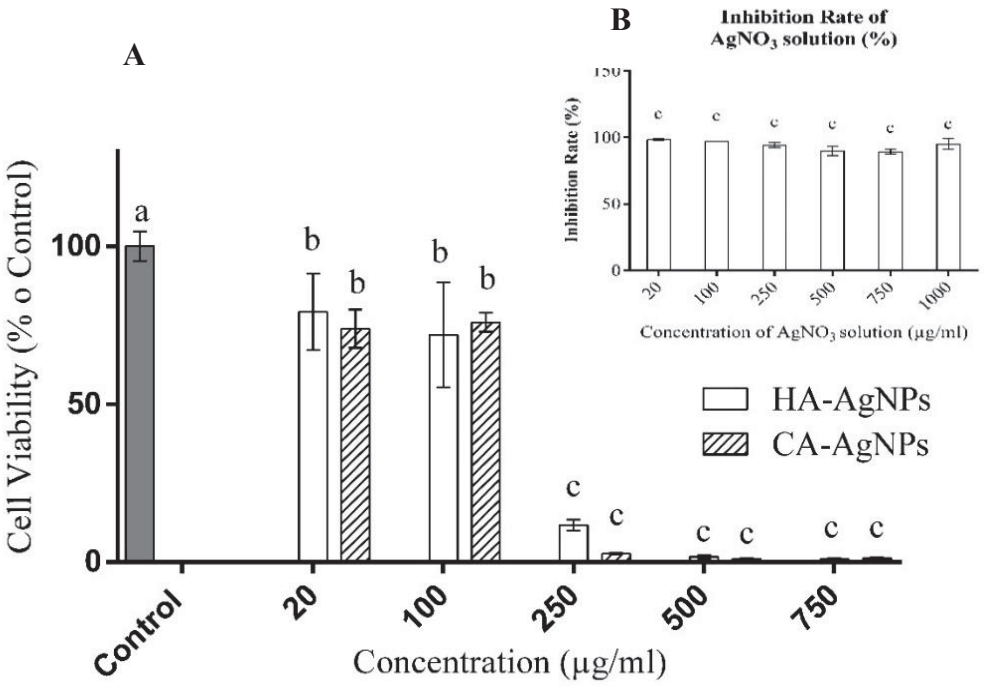
In Figure 13-A it is possible to see the activity of synthesized AgNPs, with no significant difference between both groups. But the results demonstrate inhibitory effect in all concentrations, with a dose dependent reduction of cell viability in the concentrations tested. This dose dependent cytotoxicity was also reported by Wang et al., (2016), in which green silver synthesized from *Dendropanax morbifera* leaf extracts, inhibited A549 human lung cancer cell growth by more than 70% at a concentration of 100 µg/mL. The anticancer potential of AgNPs are involve with modulation of several biological mechanisms in cells, such production of reactive oxygen species, damaging DNA and mitochondria of tumors, activating immune system and inducing cell cycle arrest, apoptosis and necrosis (Asharani, et al., 2009; Miethling-Graff et al., 2014).

Assuming that both treatments were constituted by AgNPs plus reminiscent silver ions that was not bioreduced, it was also investigated the action of pure $AgNO_3$ salt solution at same concentrations (Figure 13-B). It is possible to observe that all concentration tested, even the smallest presented high cytotoxicity against H295R cells, with cell viability reduced in around 100% after 24 h of treatment, when compared to cells in standard situation of proliferation. In the same way, Greulich et al., (2012) reported the cytotoxic action of silver ions in cell cultures, in which concentrations of 2,5 µg/mL showed more than 90% of inhibition of mesenchymal stem cells. However, although the formed

nanoparticles presented similar effects as silver solution at the highest concentrations, they showed distinct profiles in 20 and 100 µg/ml, reducing the tumor cells proliferation in 18.5% HA-AgNPs (20 µg/mL); 20.3% HA-AgNPs (100 µg/mL); 21 % CA-AgNPs (20 µg/mL and 24% CA-AgNPs (100 µg/mL).

Furthermore, the high cytotoxic potential observed in HA-AgNPs and CA-AgNPs solution at 250, 500 and 750 µg/ml cannot be precisely related to the formed nanoparticles, since it was also showed similar results to pure silver solution against the same tumor cell line. However, the lower responses in 20 and 100 µg/ml in comparison with another studies (Al-Sheddi et al. 2018; He et al. 2016) can be related with the morphology of HA-AgNPs and CA-AgNPs synthesized. Particle shape, size and surface chemistry influenced the cytotoxicity of AgNPs (Sayeed and Swamy, Mallappa 2018). These particles showed a clear ability of size-dependence in the solubilization / release of metal ions in the cell culture medium, in which larger nanoparticles presents slower rate of dissolution of ions, than the small nanoparticles (Karlsson et al. 2014). Moreover, Shi et al., (2018) reported that spherical AgNPs can show different cellular uptake results and toxicity in colon carcinoma cells, according with its size. Nanoparticles of 20 nm mainly existed in cytosol, whereas AgNPs around 100 nm nearly exists in cellular phases, which support our findings.

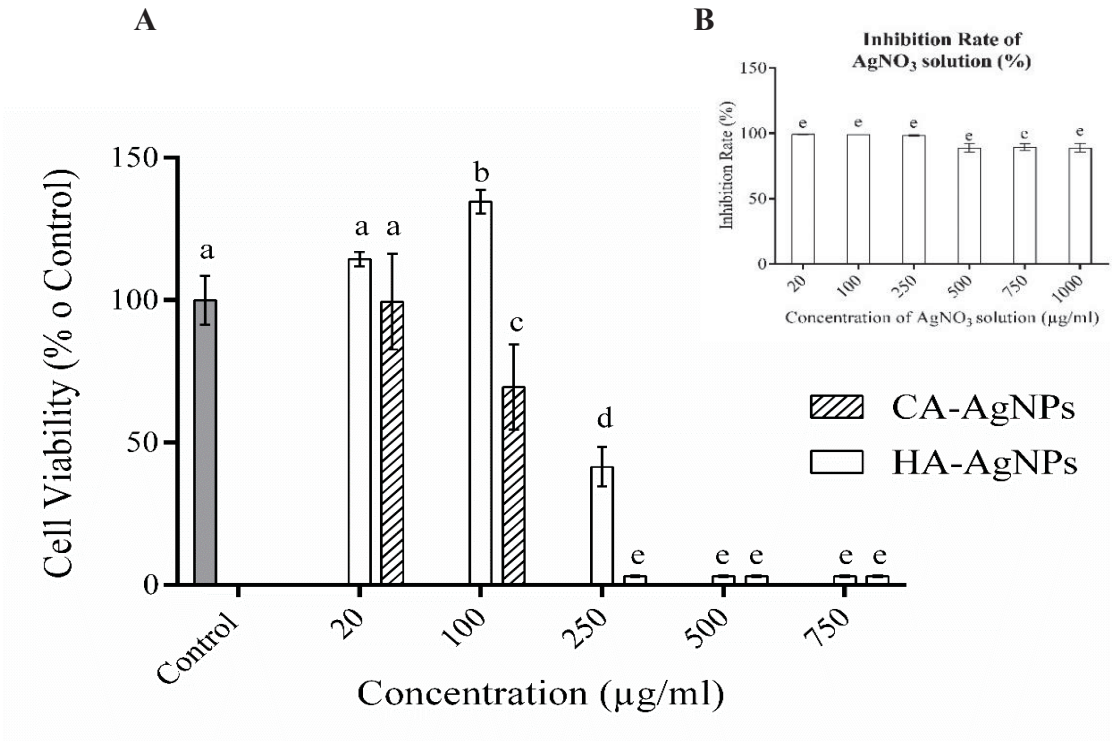
FIGURE 13. A- VIABILITY PERCENTAGE OF H295R CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF EXTRACTS CONJUGATED TO AGNPS WITHIN 24H. B- UNVIABLE PERCENTAGE OF CONTROL OF H295R CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF $AGNO_3$ SALT SOLUTION. VALUES ARE MEAN ± SE (N=3). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST ($P < 0,05$).



In order to analyze the viability of these formed AgNPs in future medicinal uses, it was also investigated their cytotoxic potential against VERO non-tumor cell line (Figure 14-A), as well as the activity of silver salt solution against this cellular linage, expressed by percentage of inhibition rate of control (Figure 14-B). HA-AgNP in special, in addition to inhibiting 28% of tumor cells activity, significantly increased 34.6% the viability of the non-tumor cells at 100 µg/mL. Rosas-Hernández et al., (2009) previously reported that AgNPs had a dual effect regards to non-tumoral cell proliferation by MTT assay, taking coronary endothelial cell as model of study. While low concentrations showed to inhibit the cell proliferation, higher concentrations (100 µg/mL) showed to stimulated. It could be related by the action of AgNPs on specific plasma membrane receptors or sharer receptors active in, such BK-induced proliferation.

FIGURE 14. **A**- VIABILITY PERCENTAGE OF NON-TUMOR CELLS (VERO) TREATED WITH DIFFERENT CONCENTRATIONS OF EXTRACTS CONJUGATED TO AGNPS WITHIN 24H. **B-**

UNVIABLE PERCENTAGE OF CONTROL OF VERO CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF $AGNO_3$ SALT SOLUTION VALUES ARE MEAN ± SE (N=3). DIFFERENT LETTERS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST ($P < 0,05$).



To improve our results, it was also carried out images of fluorescent cell viability, based on the simultaneous determination of live and dead cells that measure recognize parameters of cell viability – intracellular esterase activity and plasma membrane integrity (Figure 15). It is possible to confirm the control group did not present cell death (green points), while both AgNPs showed their inhibitory effect against tumor cells (red and blue points). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permanent calcein to the intensely fluorescent calcein. The dye calcein is retained within live cells, producing an intense uniform green fluorescence in live cells. While the EthD-1 entered in cells with damaged membranes and undergoes the fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescent in dead cells. Blue points were referent to the cell nucleus(Zeiss and Gmbh 2018).

HA and CA leaf extracts showed higher potential of inhibiting the proliferation of tumor cells, without significative cytotoxicity against VERO cell line. This plant is composed by various defined biochemical compounds, according to previous characterization, that were already been reported by their important biological activities in medicine. Among them, the polyphenolics, commonly found in Bromeliaceous family (Magalhães, et al., 2012; Oliveira-Junior, et al., 2015; Vieira, et al., 2017). Several studies have proven that these class of compounds are related with anti-proliferative effect of tumor cells. Their structural diversity influences a role of components in cell cycle regulation, forming active metabolites (Jafari, et al., 2014), sensitizing cancerous cells to chemotherapeutics (Madunić,et al., 2018) and activating different signaling pathways, which results in reduction of cells viability (HE, et al., 2014).

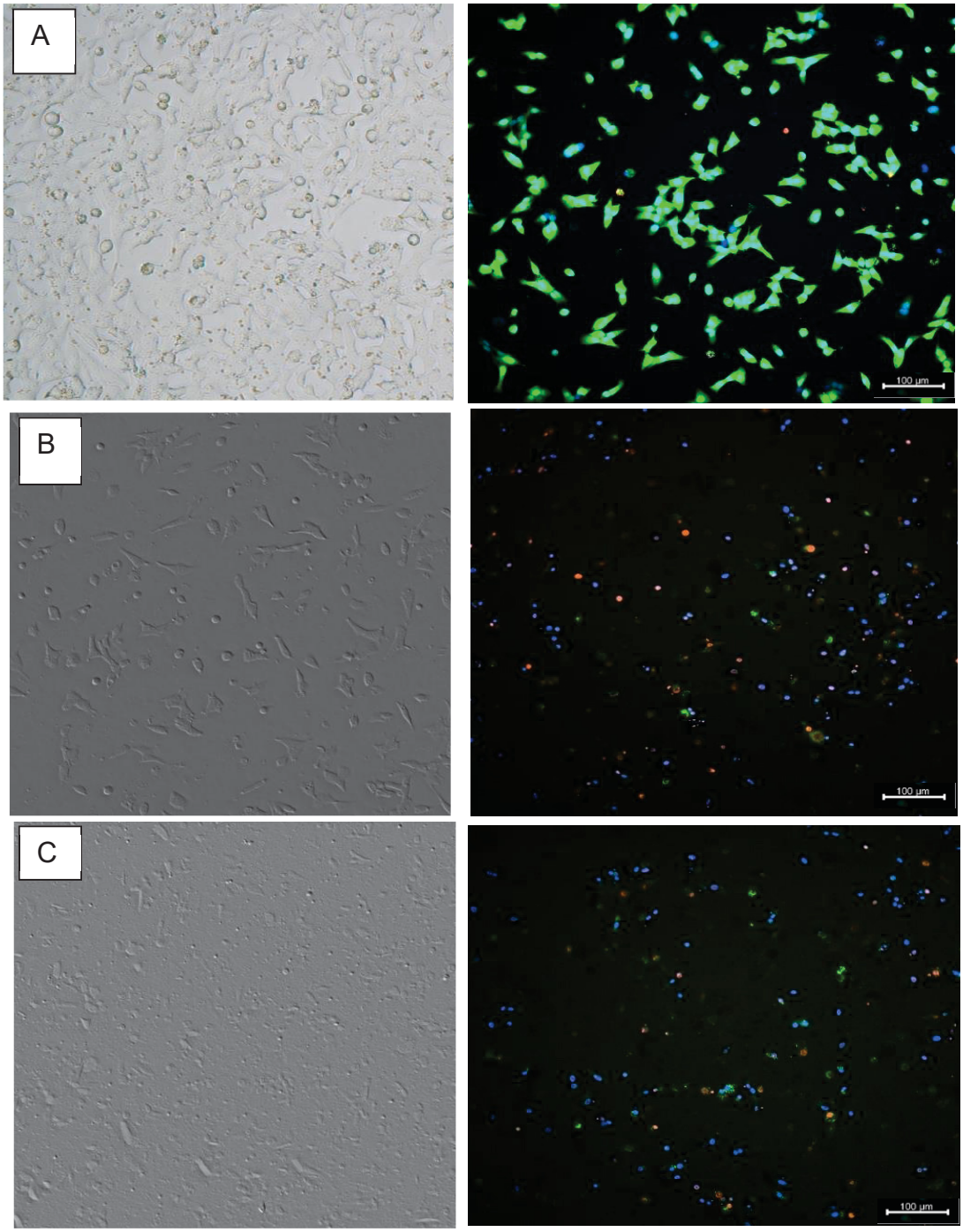
The majoring class of phenolics found in our extracts were flavonoids, and the antiproliferative potential of extracts may also be related with them. It can mediate the regulation of several extracellular and intracellular mechanism via kinase signaling, such cell growth, proliferation and apoptosis (Hsieh, et al., 2012). The apoptosis induction of cancer cells by flavonoids, can be related by their ability to inhibits fatty acids synthase activity (Brusselmans, et al., 2005), induces phosphorylation of γ-H2AX and DSB and p53 expression (Lam et al., 2012), and also acting through a complex intracellular modulation, that can involving intrinsic or extrinsic pathways, according to the flavonoids in question (George, et al., 2017).

To our knowledge, no work has been published, on the use of bromeliad leaf extracts in green synthesis of silver nanoparticles and its cytotoxic effects against adrenocortical tumor models. Moreover, a green synthesis process may produce low-cost biocompatible AgNPs, which may be used in future cancer treatments.

Furthermore, a green synthesis process may produce biocompatible AgNPs, environmentally friendly, non-toxic, and low-cost process that may be useful to improve the activity and promote new combinations to treat cancer and reduce the side effects of chemotherapy.

FIGURE 15. IMAGES OF FLUORESCENCE MYCROSCOPY OF LIVE-DEAD CELLS MADE BY INCELL ANALYZER 1000 WORSKSTATION. **A)** CONTROL (WITHOUT TREATMENT) LEFT SHOWED BRIGHT FIELD, WHILE RIGHT DARK FIELD. **B)** HA – AGNPS, BRIGHT AND DARK

FIELDS (250 µG/ML). C) CA – AGNPS, BRIGHT AND DARK FIELDS (250 µG/ML). GREEN POINT: LIVE CELLS. RED POINTS: DEAD CELLS. BLUE POINTS: CELL NUCLEI.



Conclusions

The present study introduced for the first time a simple and eco-friendly method for silver nanoparticle synthesized using organic compounds from *N. procerum* leaves aqueous extracts.

The characterization methodologies showed that was produced stable silver nanoparticles, which presented mainly spherical shapes with stability at pH

5 to 10 range in water dilution. Showing the possibility in use *N. procerum* extracts as bioreductant agents.

The synergy of compounds presents in the extracts plus green synthesized nanoparticles showed a high inhibition rate against adrenocortical carcinoma cells at 250 µg/mL, with cytotoxicity in non-tumoral cells, showed to begin from 100 µg/mL. These results reveal the *N. procerum* nanoparticles to be promised candidates in the development of the new products in phytomedicine.

More studies need to be carried out to elucidate the pathways of *N. procerum* extracts and AgNPs cytotoxicity and to improve their potential against tumoral cells.

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CHAPTER III. GREEN SYNTHESIS OF GOLD NANOPARTICLES FROM *NIDULARIUM PROCERUM* EXTRACTS AND ITS POTENTIAL AGAINST ADRENOCORTICAL CARCINOMA CELLS.

Introduction

Nanotechnology has gained great attention in recent years, especially focused on biomedicine applications. The synthesis of nanoparticles emerged as a remarkable solution for the control of target drug release. Among the various metallic nanoparticles that have been studied, the gold stands out due to their various nanotechnology-related biomedical applications. Gold nanoparticles (AuNPs) are known as promising agents for cancer therapy, due to its unique optical, physicochemical and biological properties, and thus, have been used for various applications such as biosensor, bioimaging, photothermal therapy and targeted drug delivery (Ahmed, et al., 2016; Murugan, et al., 2013; Sengani, et al., 2017).

The synthesis of nanoparticles can be performed by physical, chemical, biological or green methods. Physical and chemical methods are limited by operational costs and the use of potentially toxic compounds (Santhoshkumar, et al., 2017). The green synthesis is a rapid, clean and eco-friendly approach, in which nanoparticles are obtained using plant tissues (leaf, stem, fruit, bark, root, peel and flower) or microorganism (bacteria, yeast, actinomycetes, and fungi) and their derivatives as reducing and capping agents (Ahmed, et al., 2016). Plant mediated synthesis of nanoparticles has various advantages over microbial synthesis, including the time reduction related to the maintenance of cell cultures and the opportunity to produce high amounts of nanoparticles at a large industrial scale (Kumar and Yadav 2009; Sengani et al. 2017).

Different shapes and sizes of AuNP are generated based on the synthesis procedure and experimental conditions performed. Spherical, triangular and hexagons shapes, ranging from 10 to 100 nm are reported in the biosynthesis of AuNPs (Elahi, et al., 2018). Spherical gold nanoparticles with antibacterial and antifungal activity were obtained using *Plumbago zeylanica* and *Abelmoschus esculentu* extracts, respectively (Jayaseelan, et al., 2013; Priya et al., 2016). High potential AuNPs against tumor cells were obtained using *Hibiscus sabdariffa*

and *Morinda citrifolia* (P. Mishra et al., 2016; Suman, et al., 2013). Fruit extracts of *Couroupita guianensis* were used as bioreductant to synthesize AuNP, formatting safe and stable nanoparticles with a strong antioxidant property (Sathishkumar et al. 2016).

Therefore, in this paper, it was reported the first time the green synthesis and complete characterization of AuNP using the extract of *Nidularium procerum* as reducing agent, a specie of the Bromeliaceae family, vulnerable to extinction found throughout the Brazilian costal rain forest. Their extracts are non-toxic and were reported to present medicinal properties such as analgesic, anti-inflammatory and anti-allergic, making it a valuable alternative for the treatment of some diseases (Amendoeira et al., 2005; Vieira-de-Abreu et al., 2005). The present study also examined the biological effects of AuNPs against adrenocortical cancer cell line H295R, a rare tumor with limited medical treatment.

Material and Methods

Plant material and extraction

Plants were *in vitro* established according to Lopes da Silva et al., (2012). After 90 days of *in vitro* culture, on MS multiplication media (Murashige and Skoog 1962) supplemented with 30g.L^{-1} sucrose, 2 µM naphthalene acetic acid (NAA) and 4 µM 6-benzylaminopurine (BAP), all seedlings were well washed with tap water to remove the residual impurities. They were dried with a paper towel, cut into small pieces and 1g of fresh leaf mass was macerated and extracted in 10 mL of aqueous solvent – boiling water (HA) and 25 °C “cold” water (CA) – over 24 hours, under 80 rpm agitation in a 25 °C – dark room.

Synthesis of Gold Nano particles

In order to synthesize gold nanoparticles (AuNPs) from *N. procerum* extracts, 1 mL of HA or CA was mixed to 9 mL of $HAuCl_4$ (1mM). The solution was hand-shacked and stored in a water bath at 30 °C in the dark until the change of color solution that goes from the light-yellow to a dep-purple color. The pH of crude extracts was 4 with no modification. It was continuously observed every 30 minutes via UV-Vis spectrophotometer until the stop of the reaction, which is characterized by the overlapping of the peaks in the scanning diagram. After the

finish of the reaction, the solutions (HA-AuNP and CA-AuNP) were stored at 4 °C until be submitted to physical and chemical characterization analysis. To the biological assays, aliquots of nanoparticle solutions were previously lyophilized and resuspended in distilled water at specific concentrations.

Characterization of Gold Nanoparticles

The resulting solutions with the nanoparticles synthesized (extracts/AgNPs + unreacted bulk reagents that were not removed) were submitted to UV-visible spectroscopy, Dynamic Light Scattering (DLS) and Zeta Potential analysis, and also to visual evaluation such scanning electron microscopy (SEM) / Back-Scattered Electrons (BSE) and transmission electron microscopy (TEM).

Ultraviolet-visible spectrophotometry analysis

The bioreduction of the Au^{+} ions with different *N. procerum* extract solutions was monitored by absorbance measurement using an ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu, UV-1601 PC) every 30 minutes from the initial time (0 minutes of reaction) to final time (600 minutes of reaction). The spectra of the surface plasmon resonance (SPR) of AuNPs in the samples were measured in 200-800 nm ranges with a resolution of 1 nm. The UV-vis spectra were recorded using a quartz cuvette and deionized water was used as reference.

Transmission Electron Microscopy analysis of nanoparticles

The nanoparticle size and morphology were analyzed by using a transmission electron microscope (JEOL, JEM 1200EX-II using CCD Gatan – Bioscan and Orius SC1000B camera) operating at 120 kV. TEM samples were prepared by placing a drop of the suspension of *N. procerum* extract/AuNPs on carbon-coated copper grids and allowing water to evaporate at room temperature. The analyses of the selected area electron diffraction (SAED) patterns were analyzed using the CrysTBox software in order to identify the gold crystal planes.

Scanning Electron Microscopy analysis of nanoparticle

The nanoparticle size and morphology were analyzed by using Scanning Electron Microscopy with Energy Dispersive X-Ray Analyzer (SEM/EDX) to provide elemental identification and quantitative compositional information of samples. It was used VEGAN TESCAN equipment, BSE (Back-scattered Electron *Detector*) mode, and operating at 10 kV.

Dynamic light scattering (DLS) and Zeta potential of nanoparticles

The average sizes of AuNPs in aqueous medium was carried out by Dynamic Light Scattering (DLS) (Brookhaven, NanoDLS). For the Zeta potential analyses, 1 mL of *N.procerum* extract/AuNPs resulting solutions were diluted in 9 mL of ultrapure water or DMEM medium culture (Sigma®). The analyses were carried out using a Zeta Potential analyzer (Stabino) with a 10 mL PTFE measurement cell and a 400 µm piston. The measurement of Zeta potential was done in a pH-titration mode using 0.05 mol.L^{-1} HCl and 0.05 mol.L^{-1} NaOH as titrants.

Antitumoral Activity

The antitumoral activity of the *N. procerum* extract/AuNPs was tested on adrenocortical carcinoma human cell line H295R acquired from the collection of *Instituto de Pesquisa Pelé Pequeno Príncipe* – Paraná – Brazil, by the methodology adapted from Mosmann, (1983). It was measured the ability of viable tumor cells to metabolize the yellow water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in blue-violet insoluble formazan, after being treated with the aqueous extracts. After thawing, the cells were transferred to a cell culture bottle with nutrient mixture culture medium (DMEM – Sigma Aldrich) supplemented with 10% fetal bovine serum (Gibco®) and 1% antibiotic solution (Gibco®). They were incubated in a CO_2 incubator at 37 °C, with humidified air (95%) and CO_2 (5%) until presented 100% confluence. Subsequently, the cells were treated by adding 4 mL of trypsin (Sigma®) for 5 min, followed by the addition of 4 mL of supplemented culture medium, to neutralize the trypsin activity and form the cell suspension. The standardization of cancer cells in the 96-well plate (Biofil®) was checked by counting in a Neubauer chamber using 100 µL of the culture medium and 1 x 10^6 cancer cells

per well. A column was reserved for the blank containing only the culture medium and another column was reserved for the negative control (cancer cells without addition of compounds). After incubating the ELISA plate for 24 hours to the cell fixation, 20 µL of the treatments with different concentrations were added in 180 µL of fresh culture medium for 24 h. The tumor cells were exposed and divided into the following experimental groups: Control (C), representing cells without treatment, the crude Hot Aqueous Extract (HA) and Cold Aqueous Extract (CA) and the synthetized nanoparticles HA-AgNP and CA-AgNP, all at concentrations 20, 100, 250, 500 and 750 $\mu g.mL^{-1}$. In the last day of experiment, the treatments were removed and 100 µL MTT (3.33 $g.ml^{-1}$) was added in each well for 3 hours. After the MTT removal, 100 µL dimethyl sulfoxide was added to formazan solubilization. The viable cell number/well is directly proportional to the production of formazan, which solubilized, was measured at 550 nm on microplate reader. The results were expressed as the mean percent inhibition of tumor cell proliferation, calculated by the formula:

$$\text{Cell inhibitory rate (\%)} = (1 - ABS_{\text{experimental group}}/ABS_{\text{control group}}) \times 100 \quad \text{Eq. (3).}$$

In addition, it was also evaluated de live / dead viability / cytotoxicity of treatments against tumor cells by microscopy images. A fluorescent microscopy protocol was realized by InCell – Analyzer 1000 Workstation equipment, following the protocol proposed by (Zeiss & Gmbh, 2018). All cells were treated by the same treatment protocols described previously.

Statistical Analysis

All analyses were performed in triplicates. The data were expressed as mean ± standard error and analyzed by One Way ANOVA followed by Tukey test using Graph Pad Prism 6. Differences between means at the 5% of confidence interval ($p<0.05$) were considered significant.

Results and Discussion

Ultraviolet-visible spectrophotometry analysis

The UV-Vis spectra of gold nanoparticles (AuNPs) formed from the bioreduction of Au^{+} ions by two different *N. procerum* leaf extracts (HA-AuNP and CA-AuNP) are showed in Figure 16. The scan images confirmed the AuNPs generation by the formation of strong and well-defined absorption peaks locates

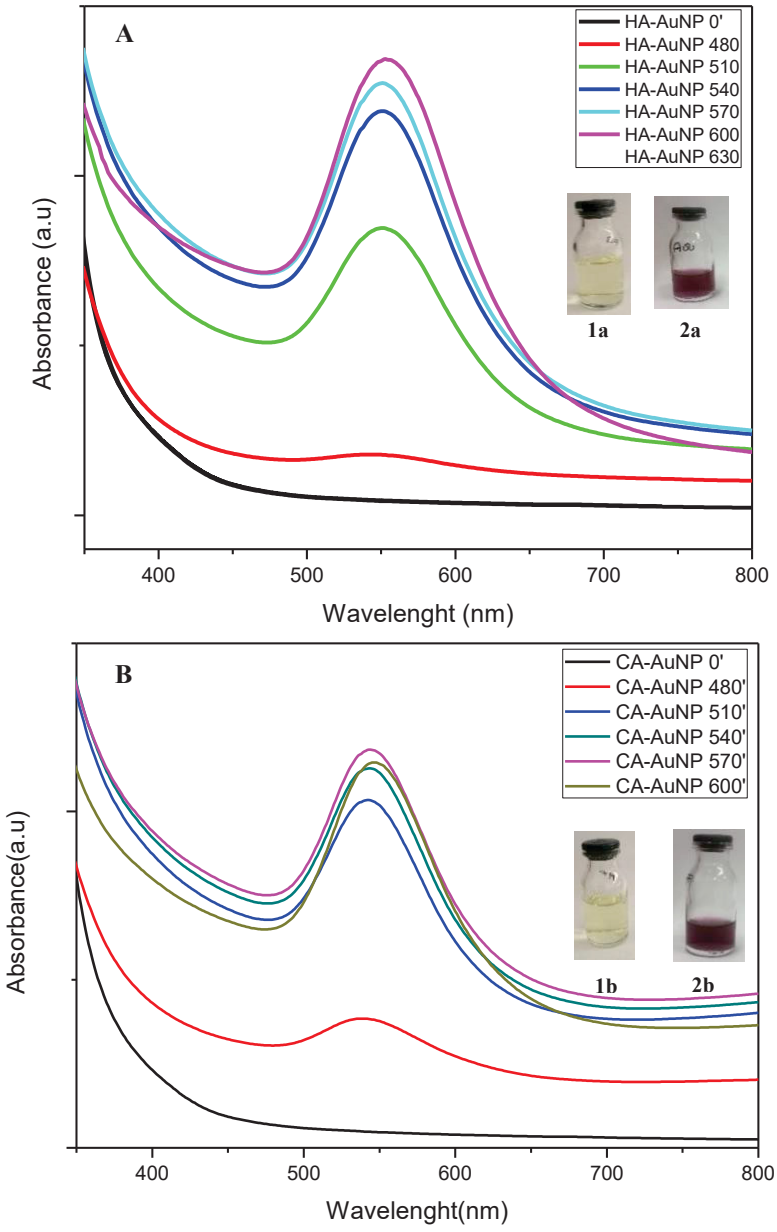
around 550 nm over the reaction time. These picks are assigned to the surface plasmon resonance (SPR) band of the gold nanoparticles formed (Pourmortazavi et al., 2017) and are generally detected by pick range between 500-580 nm (Mishra, et al., 2012). Similar results of green synthesis were also reported by Singh et al., (2013) and Rabadia et al., (2014) with gold nanoparticles peaks observed at 550 nm by UV-Vis scanning.

It was also observed the change in the solution color, in which went through from light-yellow to deep purple color, since transition arises due to excitation of surface plasmon vibration of AuNPs by the interaction of electromagnetic waves and the electrons in the conduction band around the nanoparticles (Ghosh and Pal 2007).

The Kinect profile of AuNPs formation was monitored for 660 minutes for both extracts and the process of HA-AuNPs reaction was established in 600 minutes. While CA-AuNPs showed the maximum reduction and nanoparticle formation at 570 minutes, with no variation in the scanning until the final readings. Bindhani & Panigrahi, (2014) reported that green synthesis of gold nanoparticles using *Azadirachta indica* leaf extracts, took around 24 h to complete gold ion reduction at room temperature. On the other hand, using *Trigonela foenum-graecum* extracts, Younis, et al (2018) showed a few minutes were necessary to complete the synthesis at 100 °C, reporting that the time of reaction are closely related with the temperature used in the process – the hotter the solution, the faster the reaction will end.

The phytochemical analysis of *N. procerum* leaves extracts showed the presence of flavones, isoflavones, terpenoids, carotenoids, fatty acids, phenolic acids, tannins and alkaloids. These compounds present in the leaves extracts are powerful reducing agents, which may be responsible for the reduction of the gold ions due its water soluble heterocyclic structures (Khalil and Ismail 2012; Sarwar et al. 2017).

FIGURE 16. UV-VIS SPECTRA OF SURFACE PLASMON RESONANCE FOR AUNPS. A – UV-VIS FORMATION SPECTRA AT DIFFERENT TIMES OF HA-AUNP. 1A – SOLUTION COLOR BEFORE DE REDUCTION PROCESS AT 0 MINUTE. 1B – SOLUTION COLOR AT 630 MINUTES AFTER THE REDUCTION PROCESS. B – UV-VIS FORMATION SPECTRA AT DIFFERENT TIMES OF CA-AUNP. 2A - SOLUTION COLOR BEFORE DE REDUCTION PROCESS AT 0 MINUTE. 1B – SOLUTION COLOR AT 600 MINUTES AFTER THE REDUCTION PROCESS.



Transmission Electron Microscopy analysis of nanoparticles.

Figure 17 shows the Transmission electron microscopy (TEM) used to identify the size, shape, and morphology of both groups of synthesized nanoparticles. The bright field of HA-AuNPs (Figure 17A and 17C) reveals the spherical and hexagonal formats of nanoparticles (dark spots). While in dark field of TEM mode (Figure 17B), the shining spots are referent to metallic gold portion

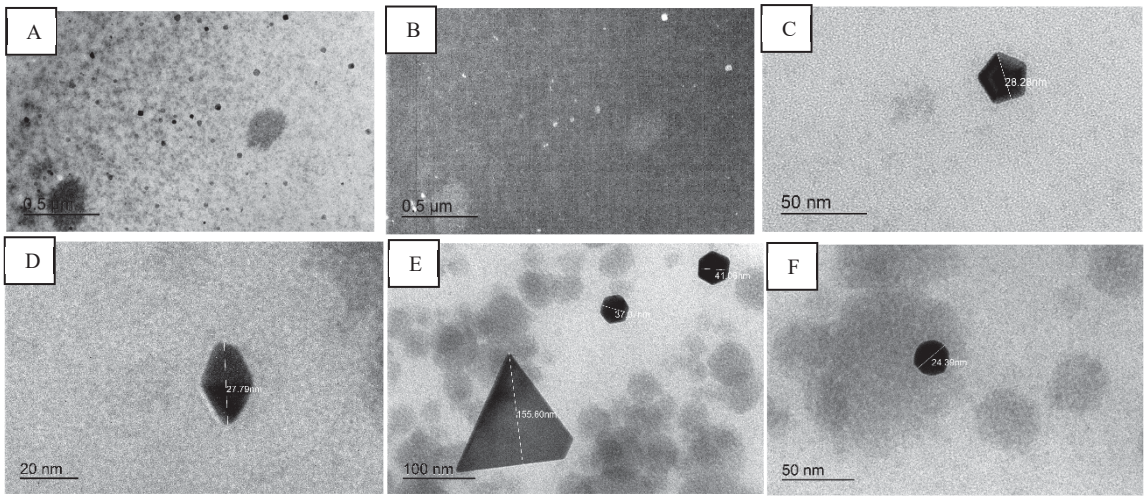
of the nanoparticles, confirming that most of them are AuNPs formed, with no agglomeration of particles and 28 nm of dimension (Figure 17C). In the same figure, the bright field of CA-AuNPs presented in Figure 17D, 17E and 17F indicates that they were well dispersed, without any visible agglomeration and ranging from 24 to 156 nm. As described previously, both *N. procerum* extracts presented a range of chemical compounds that were related to the role process of gold nanoparticles synthesis and stabilization. Among them, palmitic acid ($C_{16}H_{32}O_2$) is known to act as stabilizing agent, preventing the aggregation of gold nanoparticles and have been used as protector of the formed AuNPs (Abdel-Raouf, et al., 2017).

In addition, it was possible to identify a polydisperse population of nanoparticles, with spherical, triangular, hexagonal and diamond shapes. This variation in its morphology can be associated with the temperature of reaction and concentration of extracts used in synthesis. Lower concentrations of leaf broth offer the aid of electron-donating group containing extract, which leads to slow reduction of $HAuCL_4$ ions, facilitating the generation of multiple polygons (Elizondo et al. 2012). According to Teimuri-mofrad et al., (2017), lower temperatures of synthesis are also related to the production of gold nanoparticles with multiple formats, while higher temperatures and extract concentrations are associated with formation of spherical shapes.

Triangular, spherical, hexagonal among other nanostructures were found by Ahmad, et al., (2016) in the bioreduction of gold ions by *Ealise guineensis* aqueous leaf extract at 30 °C of synthesis reaction. As well as Song, et al., (2009), who controlled the shape and size of gold nanoparticles synthesized from *Magnolia kobus* and *Dioperys kaki* leaf extracts, by changing the reaction temperature and leaf broth concentration. In addition, it has been reported that flavonoids present in aqueous extracts, may also be responsible for the formation of irregular-shaped gold nanoparticles (Deshpande et al. 2010).

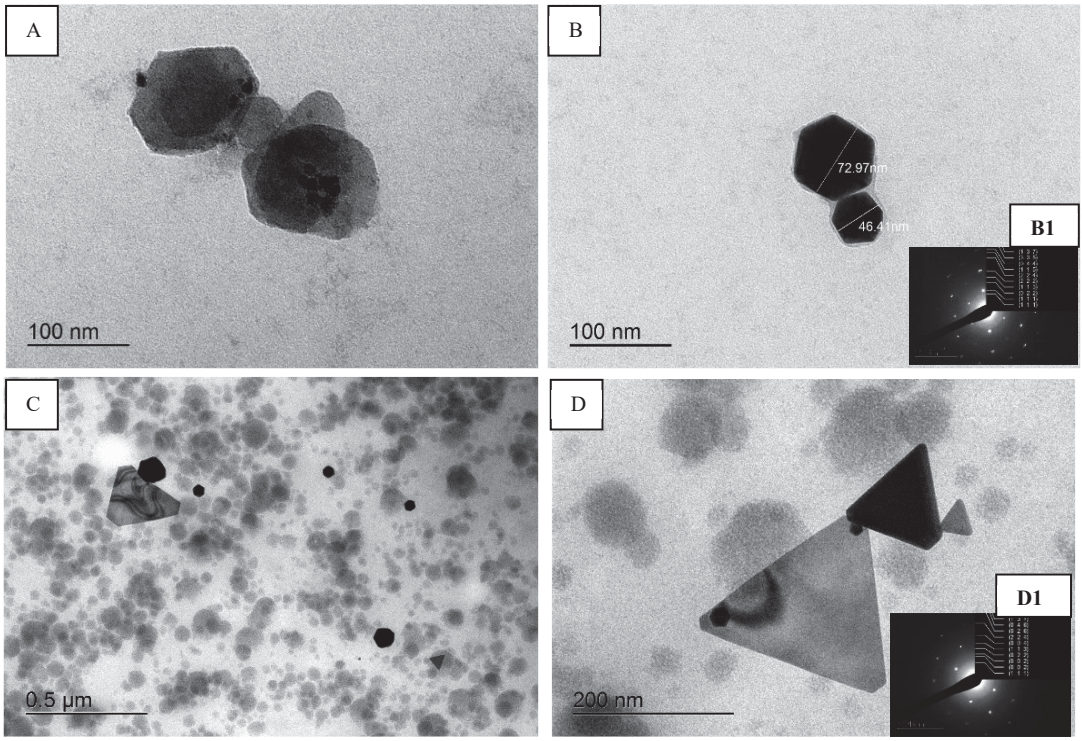
FIGURE 17. MORPHOLOGY AND DIMENSIONS OF GOLD NANOPARTICLES SYNTHESIZED FROM N. PROCERUM EXTRACTS DILUTED IN WATER. A) BRIGHT FIELD OF HA-AUNPS.

B) DARK FIELD OF HA-AUNPS. C) HEXAGONAL SHAPE OF HA-AUNP. D-E-F) MULTIPLE FORMATS OF CA-AUNPS.



The TEM comportment of both synthesized nanoparticles diluted in DMEM medium was also evaluated, in order to understand their behavior when diluted in the cell culture media that was used in subsequent biological assays. Figure 18 (A-B) showed that HA-AuNPs maintained their polygon shape and presented propensity to agglomeration, as well as it possible to see in Figure 18 (C-D) to the CA-AuNPs group, where nanoparticles also demonstrated aggregates tendency but maintaining their morphological diversity. The grouping of gold nanoparticles can be attributed to the high ionic strength of the medium culture, which is constituted by glucose, vitamins, amino acids, inorganic and organic salts among other chemical and biological compounds, causing the nanoparticle destabilization (Grassian 2009).

FIGURE 18. MORPHOLOGY AND DIMENSIONS AND SAED PATTERN OF GOLD NANOPARTICLES SYNTHESIZED FROM N. PROCERUM EXTRACTS DILUTED IN DMEM MEDIUM CULTURE. A-B) AGGREGATES FORMED IN HA-AUNPS. B1) SAED OF HA-AUNPS. C-D) AGGREGATES FORMED IN CA-AUNPS. D1) SAED OF CA-AUNPS.



The selected area electron diffraction to (SAED) pattern of both extracts revealed the crystalline nature of the nanoparticles. The diffraction rings, from inner to outer, corresponded to (731), (640), (422), (400), (311), (220), (200) and (111) for both NPs synthesized with aqueous extracts (Figures 18-B1 and D1). These planes (731), (640), (422), (400) do not show correspondence in the literature of gold nanoparticles but can be explained by the complex matrix of extracts, in which are mentioned as triangles, diamond-shapes, rods among others and we hypothesize they may be related to these forms. However, the planes (111), (200) (220) and (311) have been reported as face-centered cubic structures (FCC), which also have reported by H. Singh et al., (2017). According to Bar et al., (2012) the (111) crystal planes reveal that triangular prismatic geometries are grown through the preferential adsorption of gold atom on the (111) crystal plane.

Scanning Electron Microscopy analysis of nanoparticles (SEM).

The size, shape, and distribution of green synthesized gold nanoparticles from both extracts characterized are shown in Figure 19 and Figure 20. Figure 19A-19B shows the HA-AuNPs images, where it is possible to verifymainly nanoparticles with spherical shapes and non-homogeneous sizes. In addition, in

Figure B is also possible to observe nanoparticle aggregates, with dimensions between 1 and 10 micrometers. The nanoparticle aggregation in SEM analysis can be related to sample preparation procedure since it needs to be dried before measurement (Fatimah 2016). The elemental identification and quantitative compositional information of HA-AuNPs solution (Figure 19C), reported that the field analyzed were priory constituted by 69.98% carbon, 16.31% oxygen, 4.95% gold, 4.77% copper and 2.39% chlorine. Whereas copper value is related to the matrix grid of the samples prepared for analysis and chlorine is referenced to the gold salt source used in the solution.

The electrons scanning of CA–AuNPs (Figure 20) showed the poly-disperse shapes of formed nanoparticles, in accordance with the result found previously in TEM analysis. It´s possible to identify tuber, wires, triangles, hexagons, and cubes, with a wide diversity of sizes. According to Chandran, (2006), nanotriangles, in special, are formed only from the reduction of gold ions by molecules with molecular weights less than 3 kDa. Furthermore, authors also reported that all of these shapes can be regularly synthesized by chemical, biological and physical methods (El-sayed, 2001; Lim, et al., 2008).

FIGURE 19. SEM MICROGRAPHY OF HA-AUNPS. A - IMAGE INCREASE BY 20.000X. B – IMAGE INCREASE BY 50.000X. C - ENERGY-DISPERSIVE X-RAY OF HA-AUNPS.

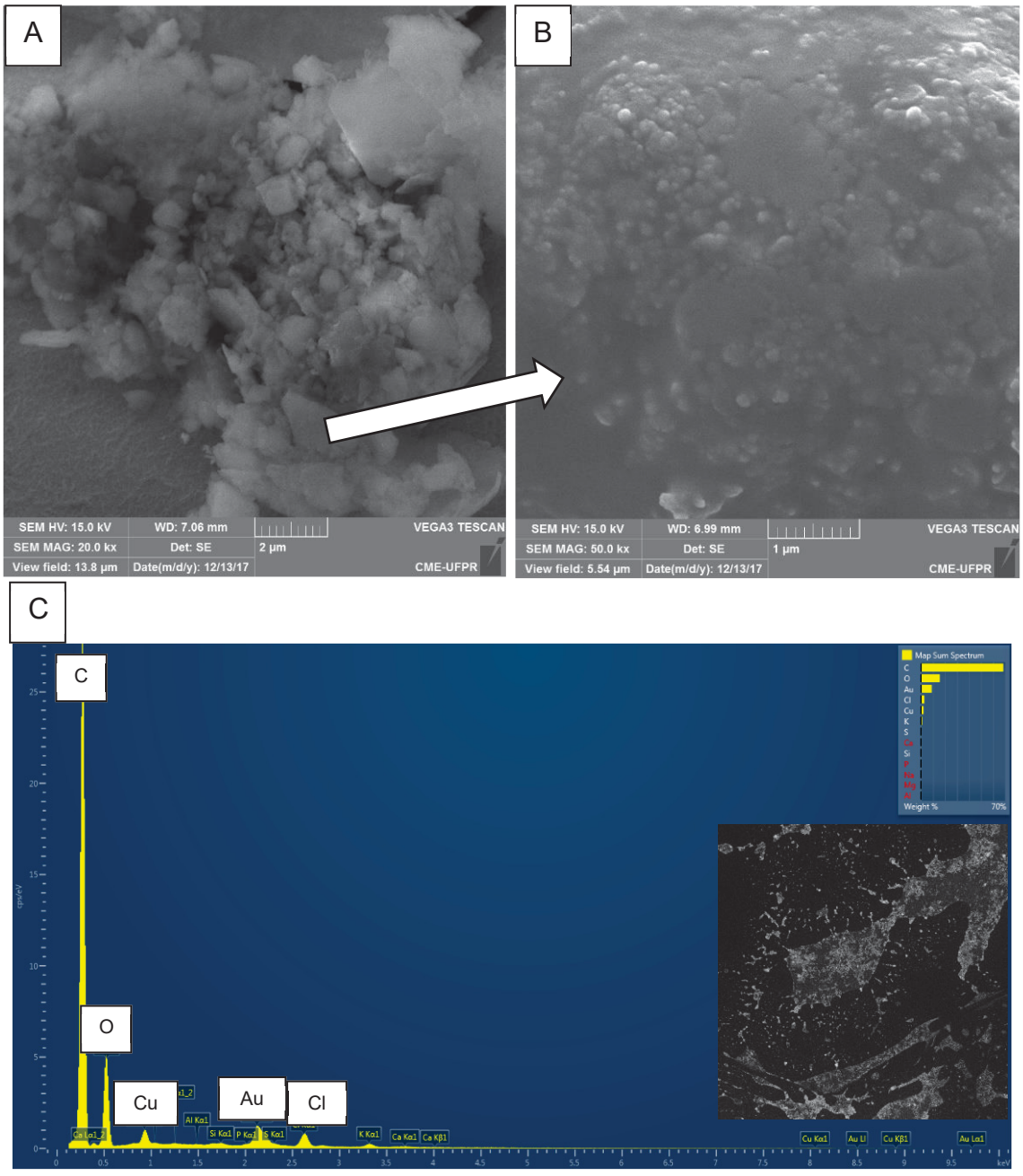
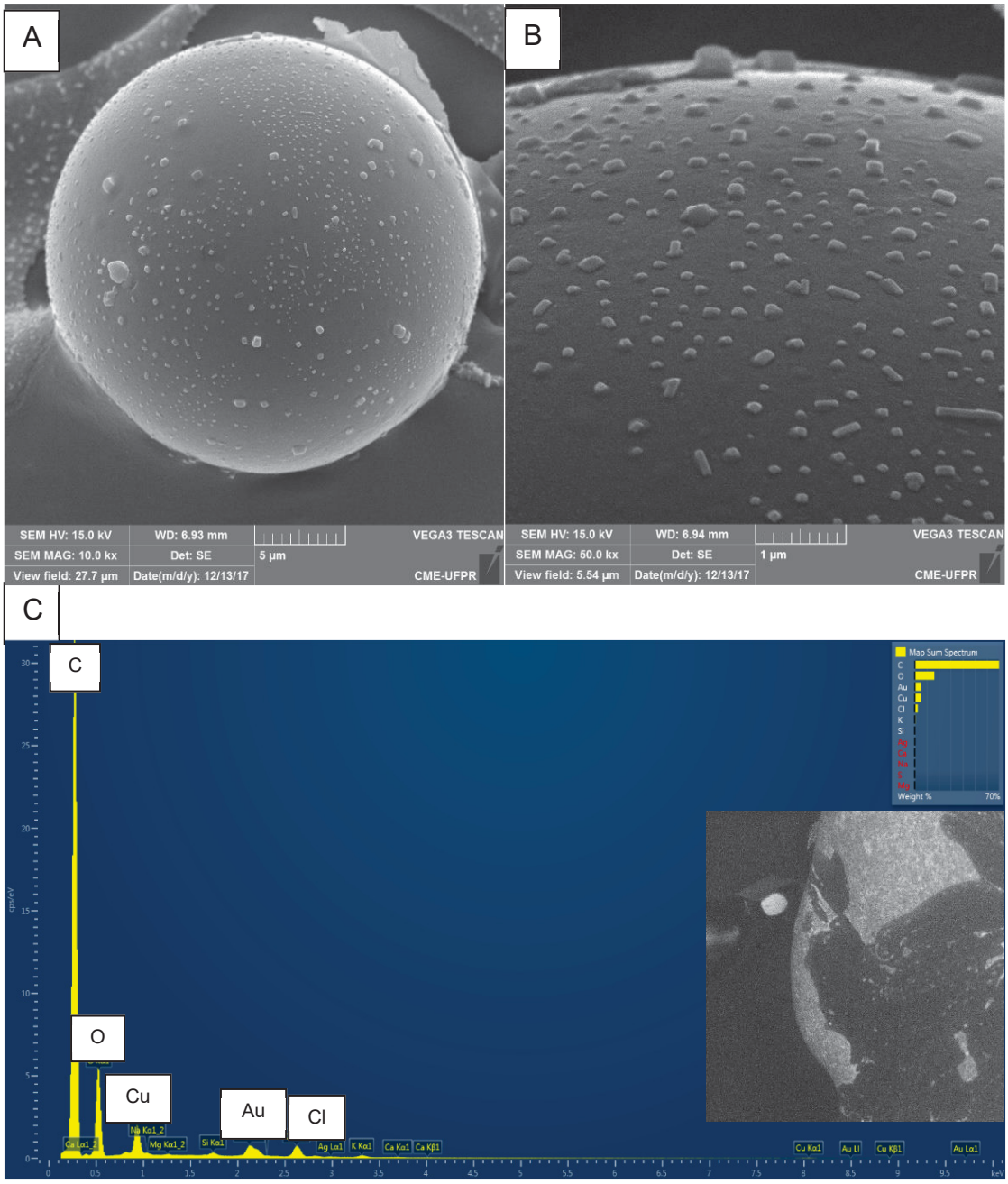


FIGURE 20. SEM MICROGRAPHY OF CA-AGNPS. A - IMAGE INCREASE BY 10.000X. B – IMAGE INCREASE BY 50.000X. C - ENERGY-DISPERSIVE X-RAY OF CA-AUNPS.



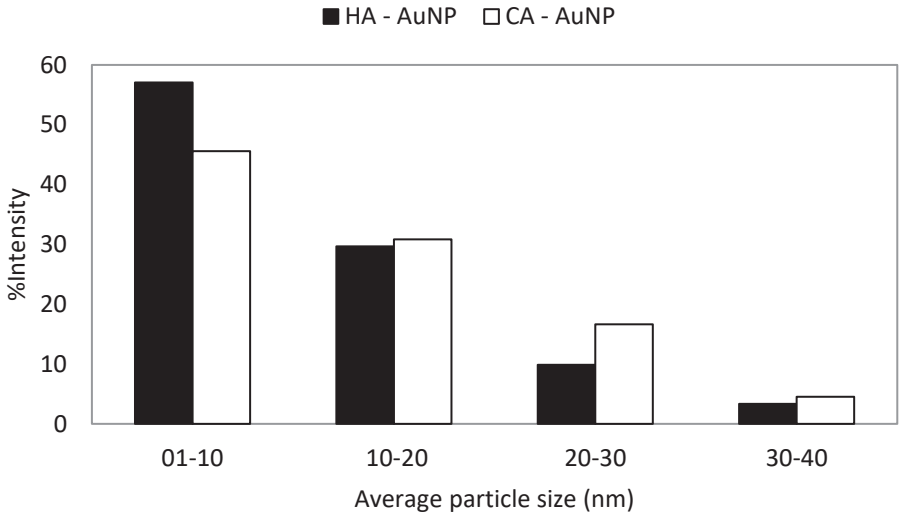
Dynamic light scattering (DLS).

Particle size and dispersity of HA-AuNPs and CA-AuNPs determined by DLS are shown in Figure 21. They showed a polydisperse hydrodynamic diameter ranging from 1 to 40 nm. The main diameters found in both groups of synthesized nanoparticles are distributed around 1-10nm, but HA showed to have more concentrations of lower AuNPs, while CA presented a higher number of nanoparticles distributed in other average sizes. This can be related due to the higher morphological diversity of shapes found in CA-AuNPs, which show to be

bigger than those found in the HA-AuNPs solution, as demonstrated in TEM and SEM analysis.

Similar results were reported in green synthesis studies of bioreduction of golf ion by *Azadirachta indica* extracts (Bindhani and Panigrahi 2014), that produced nanoparticles with 18.4 nm of effective diameter. Moreover, in the synthesis of gold nanoparticles with *Avena sativa*, Armendariz et al., (2004) reports that rather than on their shape, size of AuNPs can be regulated by changing the pH of the reaction.Similarly for the present study, Armendariz et al., (2004) demonstrated the formation of nanoparticles with diameters ranging from 5-20 nm, with most of them having average size of 10 nm at pH 4. The size of nanoparticles also showed to be related to other parameters, such as concentration of leaf broth and temperatures of synthesis. Taking into account that the size of AuNPs decreases upon increasing the extract concentration and temperature (Fayaz, et al. 2009; Khalil & Ismail, 2012).

FIGURE 21. PARTICLE SIZE DISTRIBUTION OF HA-AUNPS AND CA-AUNPS (GREEN SYNTHESIS CONDITIONS: 25°C, PH 4, AFTER 660 MINUTES OF SYNTHESIS).



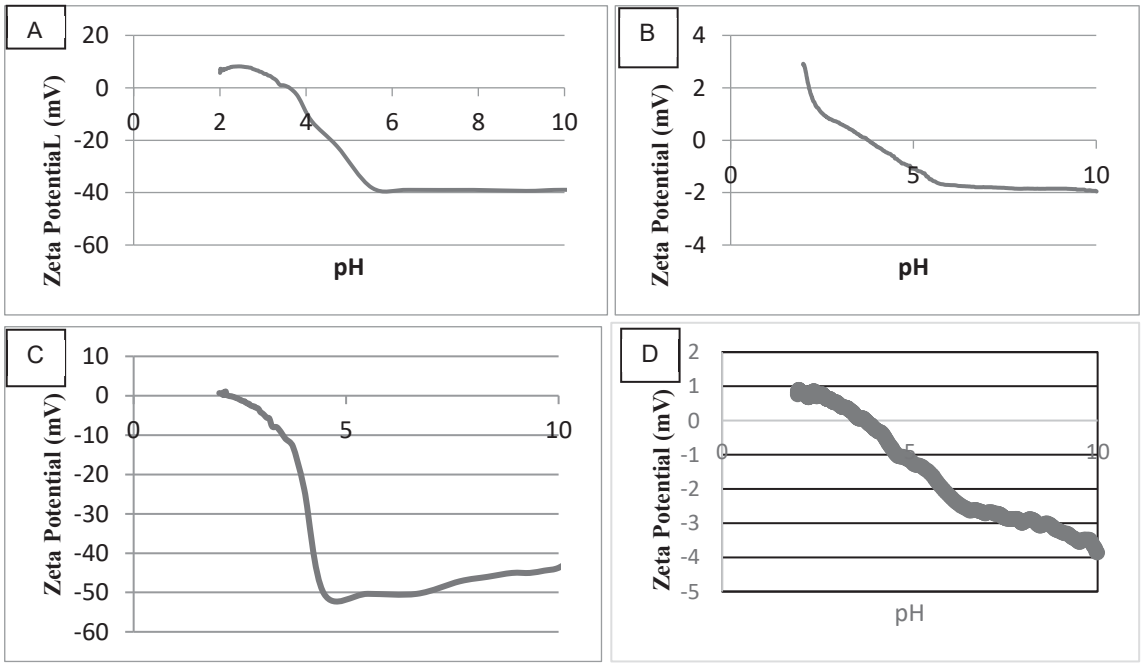
Zeta potential of nanoparticles.

The AuNPs surface charge and colloidal stability characterized by Zeta potential are presented in Figure 22**.** HA-AuNPs showed a -40 mV zeta potential between pH 5-10, while CA-AuNPs exhibited a zeta potential value of -50 mV at pH 5, decreasing gradually until reaching pH 10, both diluted in ultrapure water. These results confirmed the TEM images, where it did not saw visible aggregates

in water dilutions. The absolute values obtained in zeta potential assay replicates the net electrical charge on the particles' external surface that arises from the surface functional groups (Aljabali et al. 2018). Nanoparticles with zeta potentials less than -40 mV are considered to have good stability (Fathiah and Edyvean 2015), since high absolute values mean high electrical charges on the surface of nanoparticles, producing a strong repulsive force among the particles for withholding agglomeration (Guo et al. 2015).

High negative values to zeta potential (-34,7mV) were also reports by Park et al., (2017) using *Garcinia mangostana* extract as the bioreduction agent of gold ions. In addition to the results found by Gopinath, et al., (2014), who described the formation of stable gold nanoparticles from *Terminalia arjuna* extract reduction, with zeta potential value of 21,9 mV.

FIGURE 22. ZETA POTENTIAL PROFILES OF SILVER EXTRACT-MEDIATED NANOPARTICLES AT 2-10 PH RANGE. A - HA-AUNPS IN DISTILLED WATER. B - HA-AUNPS IN DMEM CULTURE MEDIA. C - CA-AUNPS IN DISTILLED WATER. D - CA-AUNPS IN DMEM CULTURE MEDIA.



The medicinal properties of AuNPs synthetized were further *in vitro* explored in the present work, so the zeta potential response of the nanoparticles diluted in DMEM culture was also evaluated. Figure 22 (B-D) showed that both extracts mediated AuNPs presented lower values of zeta potential in this nutritional solution. They changed to -2 mV to HA-AuNPs solution at pH ranging

from 5 to 10, and near to zero to CA-AuNPs at the same pH range. According to Moore et al., (2015), medium culture is a buffered solution comprised of many compounds, such as proteins (serum albumin, globulins), amino acids, and ionic salts. It can interfere with the particle of AuNPs in their first contact with the solution, by the formation of aggregates, as reported in TEM images. It can not only influence in vitro behavior but also change the nanoparticles mobility.

Foregoing facts disclose that phytochemicals present in crude aqueous extracts showed promising results against adrenocortical carcinoma cells, as well as the formed AgNPs. In order to investigate different metallic nanoparticles which could improve this potential, AuNPs formed were also evaluated against the H295R tumoral cell line.

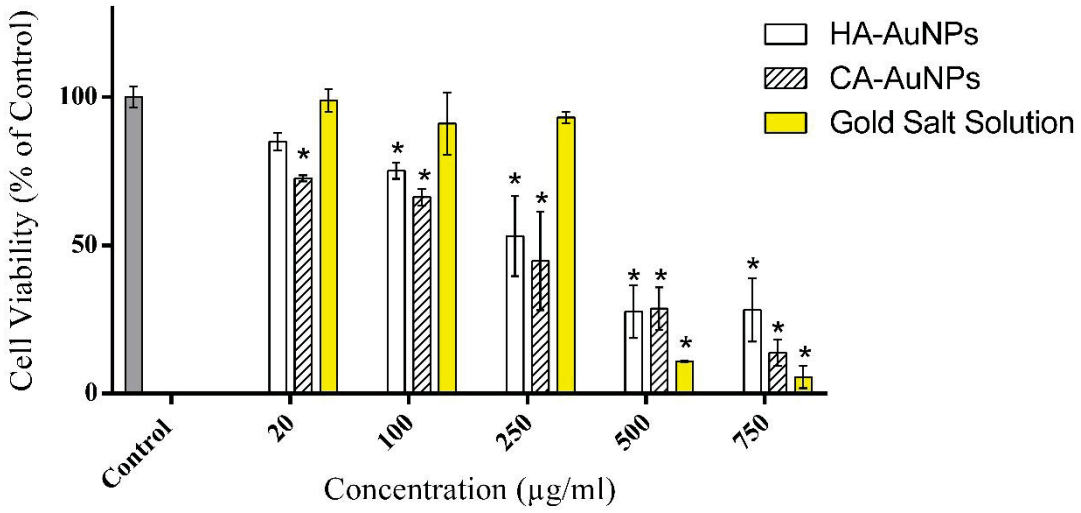
Antitumoral Activity

The *in vitro* viability of adrenocortical tumor cells (H295R) treated with different concentrations of AuNPs synthesized from *N. procerum* extracts during 24h, was evaluated by their ability in reducing MTT into a blue-colored formazan (Figure 23). The lowest concentration that significative inhibited the viability of tumor cells for HA-AuNPs was 100 µg/mL (25%), while to CA-AuNPs was 27% at 20 µg/mL. On the other hand, the highest inhibition rate for HA (38%) and CA (31,4%) crude extracts were found at concentration of 250 µg/mL (Figure 24). Whereas, the gold nanoparticles conjugated with extracts increased the inhibition of tumor cell growth from 38% to 72% in HA-AuNPs and from 31.4% to 86% in CA-AuNPs (250 µg/mL). The inhibition potential of AuNPs formed were dose-depend, in which was observed the increase in their capacity, according to the increase in their concentration, in agreement with previously reported works (Parveen & Rao, 2014; Priya & Iyer, 2015; Vijayan, et al., 2018).

Different results were reported about the cytotoxic capacity of gold nanoparticles. Connor, (2005), showed that gold nanoparticles did not present acute toxicity in human leukemia cells when treated for 24h. As well as Oueslati, et al., (2018), which showed that nanoparticles synthesized from processed extracts of *Lotus leguminosae* leaves, presented a higher rate of proliferation inhibition of 46% in highest concentration tested (1000 µg/mL), using breast cancer cell line as model. On the other hand, similar results were also reported by Geetha & Tamilselvan, (2013), which applied AuNPs from *Couroupita*

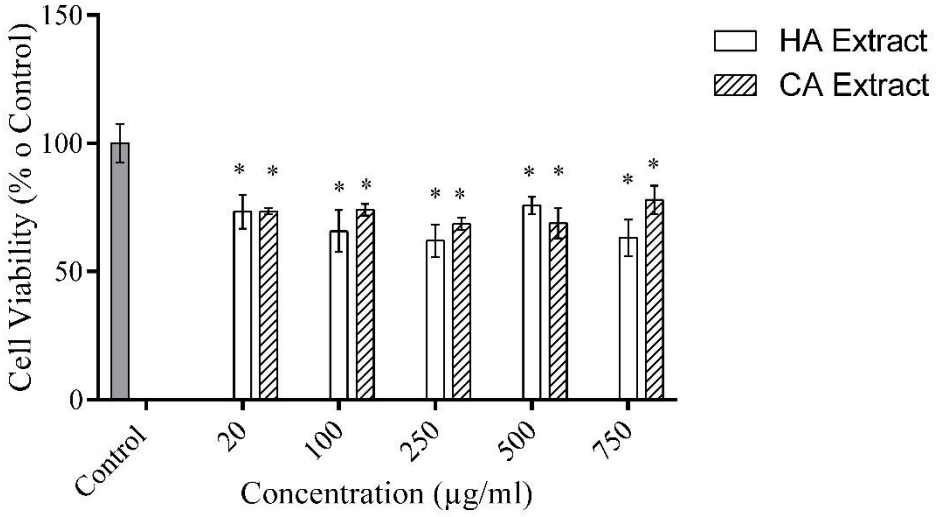
guianensis extracts in leukemia cells and showed around 90% inhibition with 150 µg/mL treatment after 5 days. In addition, Patil, et al., (2016) reported 80% of gastric cancer cells death using 200 µg/mL of AuNPs synthesized from *Rhus chinensis* for 24h. According to Hanan et al., (2018), the different shapes found in our HA-AuNPs and CA-AuNPs are less common and usually present less toxicity than spherical and quasi-spherical shaped nanoparticles.

FIGURE 23. VIABILITY PERCENTAGE OF ADRENOCORTICAL TUMOR CELLS (H295R) TREATED WITH DIFFERENT CONCENTRATIONS OF GOLD SYNTHESIZED NANOPARTICLES FROM N. PROCERUM AQUEOUS EXTRACTS AND GOLD SALT SOLUTION ($HAUCL_4$) WITHIN 24 H. VALUES ARE MEAN ± SE (N=3). ASTERISKS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST ($P < 0.05$).



The toxicity effect of gold nanoparticles is closely related to their high attraction towards the biological macromolecules and easily permeability to the cellular barriers due to their large surface/volume ratio (Khorrami, et al., 2018). Furthermore, according to with its size and shape and surface chemistry, they may enter cells more likely by the non-specific receptor mediated endocytosis mechanism (Jain, et al., 2012), and also have the ability to enhance permeability and retention, passively accumulating at tumor site on *in vivo* models (Toy, et al., 2015). Once present in the tumoral cell, AuNPs can interact with its constituents and disrupt cell signaling pathways, which may result in cytotoxic mediated by different tools, such DNA damage, cytokine release, mitochondrial toxicity, GADPH enzyme degradation, arresting cell cycle and apoptosis (Kang, et al., 2010).

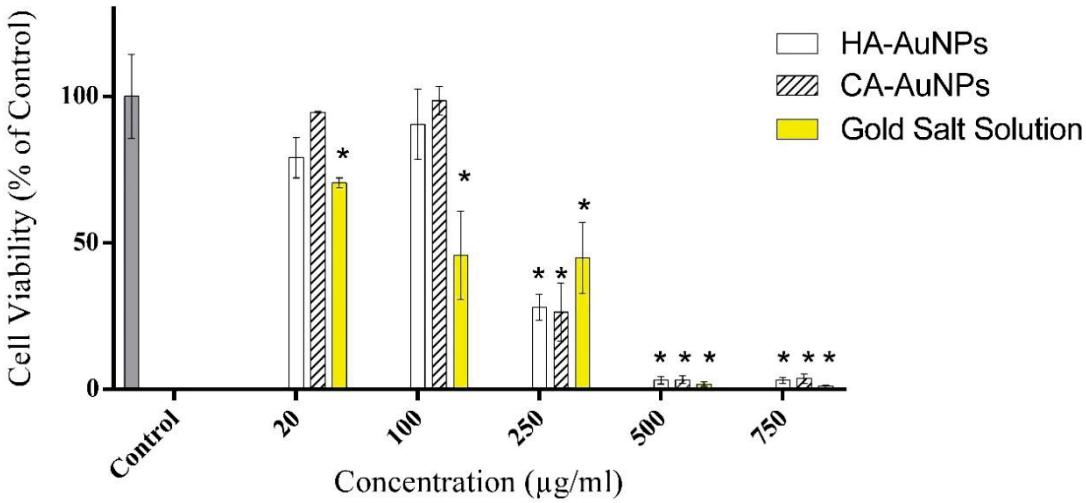
FIGURE 24. VIABILITY PERCENTAGE OF ADRENOCORTICAL TUMOR CELLS (H295R) TREATED WITH DIFFERENT CONCENTRATIONS OF N. PROCERUM AQUEOUS EXTRACTS WITHIN 24H. VALUES ARE MEAN ± SE (N=3). ASTERISKS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST (P < 0.05).



Considering that both treatments were constituted by AuNPs plus reminiscent gold ions that were not bioreduced, it was also investigated the action of the pure gold salt solution against H295R tumor cells at the same concentrations, represented by yellow bars (Figure 25). It is possible to see that it did not present significant toxicity against H295R cells until 250 µg/mL, which proved that the cytotoxic activity is related to both HA-AuNPs and CA-AuNPs.

In order to analyze the viability of these formed AgNPs in future medicinal uses, it was also investigated their cytotoxic potential against VERO, a monkey kidney non-tumor cell line (Figure 25), as well as the activity of gold salt solution against this cellular linage, represented by yellow bars.

FIGURE 25. VIABILITY PERCENTAGE OF NON-TUMORAL CELL LINE (VERO) TREATED WITH DIFFERENT CONCENTRATIONS OF GOLD SYNTHESIZED NANOPARTICLES FROM N. PROCERUM AQUEOUS EXTRACTS AND GOLD SALT SOLUTION ($HAUCL_4$) WITHIN 24 H. VALUES ARE MEAN ± SE (N=3). ASTERISKS ON BARS INDICATE SIGNIFICANT DIFFERENCES BY TUKEY TEST (P < 0.05).



, To prove our findings, it was also carried out images of fluorescent cell viability, based on the simultaneous determination of live and dead cells that measure recognize parameters of cell viability – intracellular esterase activity and plasma membrane integrity (Figure 26). It is possible to confirm that the control group did not present cell death (green points), while both AuNPs showed their inhibitory effect against tumor cells (red points). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permanent calcein to the intensely fluorescent calcein. The dye calcein was retained within live cells, producing an intense green fluorescence in live cells. While the EthD-1 enters cell with damaged membranes and undergoes the fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescent in dead cells. Blue cells were unclassified in live or dead according to program but represents the marking of nuclei cells(Zeiss and Gmbh 2018).

In the same way, the gold salt solution presented a significative cytotoxic effect even at the smallest concentration tested in the VERO cell line (Figure 25). Unlike the AuNPs, which begin to present cytotoxic effect from the concentration of 250 µg/mL, what suggests that cytotoxic effect of HA-AuNPs and CA-AuNPs in higher concentrations may be related to the free gold ions present in solution, and also demonstrates the selective toxicity of HA-AuNPs and CA-AuNPs on adrenocortical carcinoma cells. Yang, et al., (2014) also reported that 160 µg/mL treatment of AuNPs from *Magnifera indica* did not present more than 20% of monkey kidney fibroblast cell death. On the other hand, Balasubramani &

Ramkumar, (2016) showed that AuNPs synthesized from *Albizia amara* leaf extracts presented dose-dependent cytotoxicity at 72.28 µg/mL against VERO cells treated for 48 h.

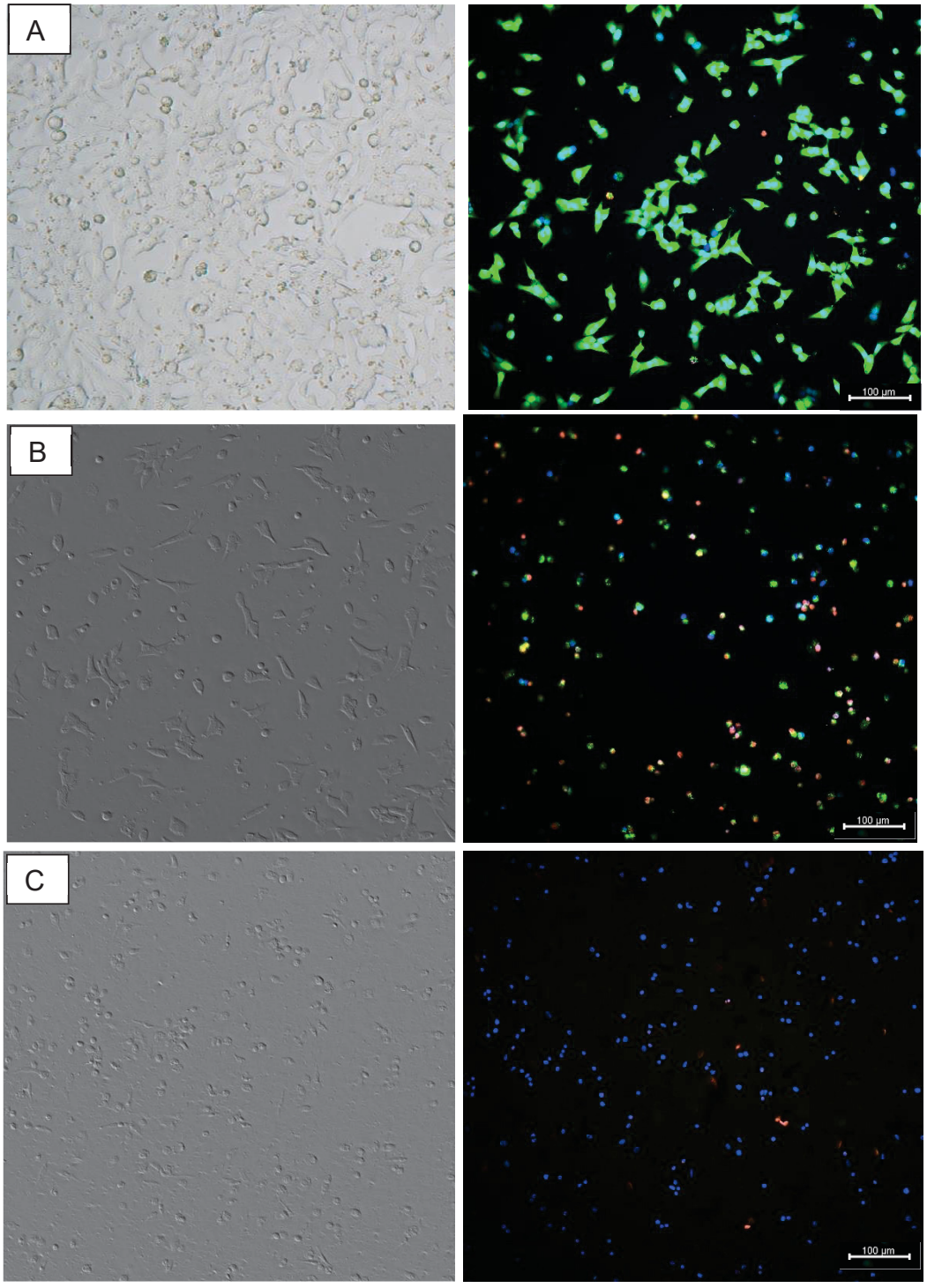
HA-AuNPs and CA-AuNPs presented high potential of inhibiting H295R tumoral cells, and it is important to emphasize that gold nanoparticles are photosensitizing material (Yao et al. 2016). Therefore, the green synthesized AuNPs may also act in the improvement of cancer cells inhibition if combined with conventional treatments, such as photothermal therapy (Roa et al. 2009). Since gold nanoparticles are photothermal agents that can enhance the hyperthermia in cells by light induction (Mendes, et al., 2017), photodynamic therapy (Dolmans, et al., (2015), with the ability of AuNPs in modulation ROS production by generation of singlet oxygen (Tada and Baptista 2015), and chemical therapy, enhancing radiosensitivity of cancer cells, which relates to size, concentration, used energy at cell line (Babaei and Ganjalikhani 2014).

Taking into account the previous chemical characterization of extracts, It is possible to relate the powerful of crude extracts and AuNPs against tumoral cells, to the presence of several components such polyphenolics, flavonoids, vitamins, fatty acids among others (Chen, et al., 2014; George, et al., 2017; Vieira, et al., 2017).

Subramanian et al., (2017) reported that the use of chemical nanoparticles against the H295R cell line may be related to targeting of the steroidogenic pathway, potentializing the apoptosis process. While Kim et al., 2016, showed to downregulate the CYP19 and 17β-HSD1 genes expression toward the treatment of H295R cells with *Gingko biloba* ethanolic extract.

According to our knowledge, there is no study reporting on green synthesized AuNPs against adrenocortical carcinoma cells, much less using extracts from notorious bromeliad *N. procerum* as bioreductant agents. In addition, the only current FDA approved therapeutic is the adrenolytic agent Mitotane, with response rates of 20-30% in advanced disease clinical cases (Subramanian et al. 2017).

FIGURE 26. IMAGES OF FLUORESCENCE MICROSCOPY OF LIVE-DEAD CELLS MADE BY INCELL ANALYZER 1000 WORSKSTATION. **A)** CONTROL (WITHOUT TREATMENT) LEFT SHOWED BRIGHT FIELD, WHILE RIGHT DARK FIELD. **B)** HA – AUNPS, BRIGHT AND DARK FIELDS (250 µG/ML). C) CA – AUNPS, BRIGHT AND DARK FIELDS (250 µG/ML). GREEN POINT: LIVE CELLS. RED: DEAD CELLS. BLUE POINT: CELL NUCLEI.



Conclusions

The present study intrdyced for the first time a simple and eco-friendly method for gold nanoparticle synthesized using organic compounds of *N. procerum* leaves aqueous extracts.

The stable gold colloids synthesized showed mainly spherical NPs in addition to variable morphologies, such as triangles, diamond-shape, hexagons, rods, and square plates, due to the complex nature of leaf extracts and the methodology used. AuNPs also showed stability at pH 6 to 10 range in water dilution.

The synergy of compounds presents in the extracts plus green synthesized nanoparticles showed a high inhibition rate against adrenocortical carcinoma cells, at 1000 µg/mL, with cytotoxicity in non-tumoral cells showed to begin from 100 µg/mL. Revealing to be promised candidates in the development of the new product in phytomedicine.

More studies need to be carried out in order to elucidate the pathways of *N. procerum* extracts and AuNPs cytotoxicity and to the improvement of their potential against tumoral cells.

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3. CONCLUSÕES

Foi possível caracterizar parcialmente extratos de diferentes polaridades de folhas de *Nidularium procerum* cultivadas *in vitro*, a partir de análises qualitativas de *screening* fitoquímico e através de análises quantitativas por cromatografia de alta resolução.

Extratos aquosos de *Nidularium procerum* demonstraram possuir atividade antioxidante frente a testes *in vitro* de neutralização de radicais livres.

Extratos aquosos de *Nidularium procerum* apresentaram efeito potencial na atividade imunomoduladora de macrófagos da linhagem RAW 264.7, observando-se efeitos significativos ($p<0,05$) nos estímulos das atividades de adesão celular, fagocítica e formação fagolisossomal. Enquanto demonstraram efeitos significativos na redução dos radicais livres peróxido de hidrogênio e ânion superóxido.

Extratos aquosos brutos de *Nidularium procerum* inibiram em torno de 35% a capacidade de proliferação *in vitro* de células adrenocortical tumorais (H295R), enquanto não apresentaram citotoxicidade frente às células não tumorais (VERO).

Foi possível sintetizar e caracterizar nanopartículas de prata e ouro utilizando compostos bioativos presentes nos extratos de *Nidularium procerum*, como agentes redutores e estabilizantes das nanopartículas formadas.

Nanopartículas de prata conjugadas com compostos orgânicos dos extratos aquosos de *Nidularium procerum* apresentaram elevada capacidade de inibição da proliferação das células tumorais H295R, como também das células não tumorais VERO.

Nanopartículas de ouro conjugadas com extratos aquosos quentes de *N. procerum* potencializaram a inibição do crescimento de células tumorais de 38% para 72% comparando-se à sua fração bruta (HA). Enquanto a inibição do crescimento das células tumorais passou de 31,4% em extratos aquosos frios brutos, para 86% em nanopartículas conjugadas com o mesmo extrato.

3.1. PERSPECTIVAS FUTURAS

No desenvolvimento deste trabalho pretendeu-se fazer a caracterização química parcial dos extratos de folhas de *N. procerum* cultivadas *in vitro,* e avaliar seus potenciais biológicos.

Entretanto, devido à complexidade envolvida nestes ensaios, não foi possível realizar a caracterização dos extratos em demais solventes, tais como: éter de petróleo, acetato de etila, DMSO entre outros. Os quais podem ser futuramente explorados, bem como realizar *screening* completos de outras estruturas vegetais, como as raízes.

Considerando ainda realizar uma completa caracterização desta planta, seria necessário além da extração, efetuar também o fracionamento, o isolamento e a purificação dos compostos através de técnicas espectrométricas, tal como Ressonância Magnética Nuclear. Sendo necessário também realizar trabalhos comparativos com espécimes selvagens, crescidas em seu habitat natural.

Além do mais, aproveitando a facilidade de manipulação das plantas por meio da cultura vegetal *in vitro,* a atividade biológica poderia também ser incrementada através da maior indução de metabólitos secundários específicos. Utilizando-se de alternativas como crescimento dos explantes em meios de cultura seletivos na micropropagação *in vitro*, cultivos celulares em biorreatores e também indução de calos friáveis por vias hormonais.

Tendo em vista os promissores resultados encontrados nos estudos em modelos celulares *in vitro*, são necessários futuros estudos voltados ao melhor entendimento das vias metabólicas relacionadas tanto aos estímulos imunomoduladores reportados em macrófagos, quanto aos responsáveis pela citotoxidade encontrada em células tumorais.Além de também ser necessário explorar modelos de estudo *in vivo*.

No que diz respeito ao emprego dos extratos como agentes bioredutores na síntese verde de nanopartículas, e considerando que se obteve distintas morfologias nas nanopartículas. Deveriam também ser realizados estudos voltados ao desenvolvimento de diferentes protocolos envolvendo a variação dos parâmetros de síntese, obtendo-se assim um melhor controle das formas estruturais e tamanhos das nanopartículas.

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