Research, Society and Development, v. 11, n. 12, e367111234804, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i11.34804

Biomonitorated study of *Euphorbia umbellata* (Pax) Bruyns extracts to be used to prepare silver nanoparticles with antimicrobial and cytotoxic potential

Estudo biomonitorado de extratos de *Euphorbia umbellata* (Pax) Bruyns para ser utilizado na preparação de nanopartículas de prata com potencial antimicrobiano e citotóxico

Estudio biomonitorizado de extractos de *Euphorbia umbellata* (Pax) Bruyns para preparer nanopartículas de plata con potencial antimicrobiano y citotóxico

Received: 08/31/2022 | Reviewed: 09/07/2022 | Accept: 09/09/2022 | Published: 09/17/2022

Rosana Letícia da Rosa
ORCID: https://orcid.org/0000-0003-2409-514X
Universidade Estadual de Ponta Grossa, Brazil
E-mail: rosanaleticia@hotmail.com
Patricia Mazureki Campos
ORCID: https://orcid.org/0000-0003-2659-8023
Universidade Estadual de Ponta Grossa, Brazil
E-mail: patimazureki@hotmail.com
Luiza Stolz Cruz
ORCID: https://orcid.org/0000-0001-6705-0967
Universidade Estadual de Ponta Grossa, Brazil
E-mail: luizastolz@gmail.com
Robson Schimandeiro Novak
ORCID: https://orcid.org/0000-0002-6997-3260
Universidade Estadual de Ponta Grossa, Brazil
E-mail: robsonnovak@hotmail.com
Carla Cristine Kanunfre
ORCID: https://orcid.org/0000-0002-2865-3084
Universidade Estadual de Ponta Grossa, Brazil
E-mail: cckanunfre@gmail.com
Luis Antônio Esmerino
ORCID: https://orcid.org/0000-0003-2495-3490
Universidade Estadual de Ponta Grossa, Brazil
E-mail: esmerino@uepg.br
Diego José Schebelski
ORCID: https://orcid.org/0000-0001-8647-6760
Universidade Estadual de Ponta Grossa, Brazil
E-mail: diego.ski@hotmail.com
Josiane de Fátima Padilha de Paula
ORCID: https://orcid.org/0000-0002-2717-8867
Universidade Estadual de Ponta Grossa, Brazil
E-mail: jopadilha@uepg.br
Flavio Luís Beltrame
ORCID: https://orcid.org/0000-0001-7067-5802
Universidade Estadual de Ponta Grossa, Brazil
E-mail: flaviobeltra@gmail.com

Abstract

This study proposes to develop a biomonitorated study of the *Euphorbia umbellata* leaves extracts to identify those that could be used to prepare, by green synthesis, silver nanoparticles (AgNPs), with cytotoxic and antimicrobial potential. Hexane, dichloromethane, acetone, methanol, and aqueous extracts were evaluated to measure the terpenes amounts, antimicrobial activity (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), and the cytotoxic potential (A549, H292 cell lines). Characterization of AgNPs was performed by determination of zeta potential, polydispersity index, mean diameter, UV/Vis spectrometry, and scanning electron microscope (SEM), and evaluated against cytotoxic and antimicrobial models. Hexane and dichloromethane extracts presented the higher amounts of terpenes, exhibited microbiological inhibition against *K. pneumoniae* and *S. aureus*, and cytotoxic potential against both cell lines. AgNPs prepared with hexane and dichloromethane extracts were determined after 35 minutes of artificial light exposure and presented spherical form with aggregates, mean diameter of 177.9 ± 1.3 nm, 288.8 ± 1.4 nm, polydispersity index of 0.416, 0.496, and zeta potential -35.7, -30.5 mV, respectively. AgNPs obtained from dichloromethane extract incremented in the biological activities, with IC_{50} of 1.56 µg/mL and 0.25

µg/mL against *K. pneumoniae* and *S. aureus*, respectively, and IC_{50} of 0.28 µg/mL for A549 and 0.18 µg/mL for H292 cell lines. The development of AgNPs with dichloromethane extract of *E. umbellata* using green synthesis demonstrated high potential to be used as cytotoxic and antimicrobial product, associating the phytochemical composition and the presence of AgNPs in a synergic effect.
Keywords: Terpenes; Silver nanoparticles; Green synthesis; Lung cancer; Antimicrobial action.

Resumo
Este trabalho propõe desenvolver um estudo biomonitorado de extratos de folhas de *Euphorbia umbellata* para identificar aqueles a serem usados no preparo de nanopartículas de prata (AgNPs), por síntese verde, com potencial citotóxico e antimicrobiano. Extratos hexano, diclorometano, acetona, metanol e aquoso foram avaliados para quantificar terpenos, determinar a atividade antimicrobiana (*Klebisiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*), e o potencial citotóxico (linhagens A549 e H292). A caracterização das AgNPs com extratos de *E. umbelatta* foi realizada pela determinação do potencial zeta, índice de polidispersidade, diâmetro médio, espectrometria UV/Vis e microscopia eletrônica de varredura (MEV); as AgNPs foram avaliadas frente a modelos citotóxicos e antimicrobianos. Extratos hexano e diclorometano apresentaram maiores quantidades de terpenos, inibição microbiológica contra *K. pneumoniae* e *S. aureus* e potencial citotóxico. AgNPs preparadas com extratos hexano e diclorometanos foram determinadas após 35 minutos de exposição a luz artificial, e apresentaram formato esférico com agregados, diâmetro médio de 177,9 ± 1,3 e 288,8 ± 1,4 nm, índice de polidispersidade de 0,416 e 0,496 e potencial zeta -35,7 e -30,5 mV, respectivamente; AgNPs preparadas com extrato diclorometano apresentaram aumento nas atividades antimicrobiana (*K. pneumoniae* e *S. aureus*, CI_{50} de 1.56 µg/mL, 0.25 µg/mL, respectivamente), e citotóxica (linhagens A549 e H292, CI_{50} de 0,28 µg/mL e 0,18 µg/mL, respectivamente). O desenvolvimento de AgNPs com extrato diclorometano de *E. umbellata,* utilizando síntese verde, demonstrou potencial citotóxico e antimicrobiano, devido ao efeito sinérgico entre os compostos fitoquímicos e a AgNPs.
Palavras-chave: Terpenos; Nanopartícula de prata; Síntese verde; Câncer de pulmão; Ação antimicrobiana.

Resumen
Este estudio propone desarrollar un estudio biomonitorizado de los extractos de hojas de *Euphorbia umbellata* para identificar aquellos a ser utilizados para preparar nanopartículas de plata (AgNPs), por síntesis verde, con potencial citotóxico y antimicrobiano. Se evaluaron hexano, diclorometano, acetona, metanol y extractos acuosos para cuantificar terpenos, determinar actividad antimicrobiana (*Klebisiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*) y el potencial citotóxico (linajes A549 y H292). La caracterización de AgNPs con extractos de *E. umbelatta* se realizó mediante la determinación del potencial zeta, índice de polidispersidad, diámetro medio, espectrometría UV/Vis y microscopía electrónica de barrido (SEM); Las AgNPs se evaluaron frente a modelos citotóxicos y antimicrobianos. Los extractos de hexano y diclorometano presentaron mayores cantidades de terpenos, inhibición microbiológica frente a *K. pneumoniae* y *S. aureus* y potencial citotóxico. Las AgNPs preparadas con extractos de hexano y diclorometano se determinaron después de 35 minutos de exposición a luz artificial y presentaron forma esférica con agregados, diámetro medio de 177,9 ± 1,3 y 288,8 ± 1,4 nm, índice de polidispersidad de 0,416 y 0,496 y potencial zeta -35,7 y -30,5 mV, respectivamente. Las AgNPs presentaron un aumento en las actividades biológicas evaluadas contra *K. pneumoniae* y *S. aureus* (CI_{50} de 1,56 µg/mL, 0,25 µg/mL, respectivamente), y CI_{50} de 0,28 µg/mL y 0,18 µg/mL contra los linajes A549 y H292. El desarrollo de AgNPs con extracto diclorometano de *E. umbellata*, utilizando síntesis verde, demostró potencial citotóxico y antimicrobiano, debido al efecto sinérgico entre compuestos fitoquímicos y AgNPs.
Palabras clave: Terpenos; Nanopartículas de plata; Síntesis verde; Cancer de pulmon; Acción antimicrobiana.

1. Introduction

The genus *Euphorbia* seems to be native from Africa and expanded to America nearly 30 million years ago. It is a rich source of chemical compounds, being the polycyclic diterpenes described as chemotaxonomic makers for this genus (Ernst et al., 2019). Several species are widely used in folk medicine to treat different kind of pathologies such as allergies, Chagas disease, cramp, pain, flu, internal bleeding, tinea, leprosy, and antimicrobial activity, however, it is mostly used to treat ulcers and cancer (Ahmad et al, 2019; Kemboi et al, 2020). Specifically, *Euphorbia umbellata* is used in folk medicine to treat health problems, mainly, neoplastic diseases.

To correlate the popular uses with scientific data, several studies were accomplished in the antimicrobial area, showing that *Euphorbia helioscopia* presents potential activity against different pathogenic microorganisms (El-Mahmood, 2009); and *Euphorbia hirta* has antibacterial activity against gram negative ICU pathogens (Patel & Patel, 2014).

Previous ethnopharmacological studies, for *E. umbellata,* have demonstrated the cytotoxic and anticancer activities of compounds present in this specie (Kanunfre et al, 2017). Oliveira et al. (2013) demonstrated that nonpolar compounds [as terpenes (euphol) and steroids (citrostadienol)] of *E. umbellata* latex promoted reduction in the volume of the tumor of melanoma in mice. Luz et al. (2016) proved that nonpolar extracts of the same vegetal matrix have cytotoxic effects against different cancer cells lines and Cruz et al. (2020) demonstrated that enriched terpene extracts of the latex promoted apoptosis in leukemic cells.

Related to anti-inflammatory and antioxidant properties and modulation of the immune system, Oliveira et al. (2021) studied the action of the phytocomplex compounds (terpenes) of *E. umbellata* latex, which modulated the functions of the immune system (able to activate the complement pathways). Further, the flavonoids and terpenes of the bark extracts of this specie demonstrated high anti-inflammatory and antioxidant activities (Minozzo et al., 2016, Munhoz et al., 2014).

Nanosized materials are widely used due to their applications in biological and biomedical areas. They can be used for the treatment or diagnosis of many diseases because, their nanoscale dimension offers unique physic-chemical features compared to their bulk counterpart (Abbasi et al., 2014, Lee & Jun, 2019; Bamal et al., 2021). Among the metal nanoparticles, the silver nanoparticles (AgNPs) stand out for the size, shape, surface property and structure, which impose distinguished antibacterial, anti-viral and anticancer applications (Yadi et al., 2018; Lee & Jun, 2019). AgNPs have been used in hospital devices coatings and wound dressings for acne, ulcers, and dermatitis (Abbasi et al., 2014). Inside of the synthesis categories to the AgNPs, the green synthesis is the most acceptable and promising for several reasons such as easily, non-toxicity, hydro solubility, stability and high yield of production besides well-defined morphology and size, being interesting for translational research (Zhang et al., 2016).

The green synthesis through plant extracts is a chemical method performed with a short reaction time; and reduced compounds that generate monodisperse nanoparticles. It is advantageous by being environmental-friendly with no use of toxic materials and by providing stabilizing agents to the synthesized metal nanoparticles (Yadi et al., 2018; Bamal et al., 2021). Furthermore, the natural composition of secondary metabolites is beneficial and promotes a single step of biosynthesis with no harm to humans and is safe for nature (Bamal et al., 2021).

The AgNPs synthesis with a plant extract with prominent biological activities as mentioned before for the genus *Euphorbia* and specifically *E. umbellata* is few named (Kumar et al, 2019). Thereby, the AgNPs produced by and associated to *E. umbellata* extracts may bring promising and synergic therapeutical effects, mostly for cytotoxic and antibacterial activities. Thus, this study proposes to develop a biomonitorated study of the *E. umbellata* leaves extracts aiming to identify those with cytotoxic and antimicrobial potential, as well as prepare the AgNPs using the active extracts, evaluate their physicochemical characteristics, cytotoxic potential, and microbiological activity as a pharmaceutical product for medical application.

2. Methodology

In this research, it was developed qualitative and quantitative studies. For the terpene's measurement, antimicrobial and cytotoxic analyses were applied quantitative analysis to measure the amounts of chemical compounds in the extracts, as well as the biological potential of these extracts. Related to the silver nanoparticles, the physic-chemical characterization was based on qualitative (macroscopic evaluation and scanning electronic microscopy) and quantitative (size, polydispersity index, zeta potential and UV-Vis spectrophotometry measurements) methods, since the goals were to identify the all characteristics of the silver nanoparticles obtained.

2.1 Plant material and obtaining the extracts

The plant material (leaves) was collected in December of 2019 in the region of Ponta Grossa (Brazil, altitude: 975 meters, latitude: 25º05´38´´S, longitude: 50º09´30´´W). A voucher was stored at *Maria Eneida P. Kauffamnn Fidalgo Herbarium* (#453920). The leaves were dried in a greenhouse (37 °C) for 7 days. The dry and milled material (35 g) was added into a small paper bag and placed in Soxhlet equipment. The vegetal matrix was subsequently extracted for 6 hours with 750 mL of each solvent (hexane, dichloromethane, acetone, and methanol). The aqueous extract also was obtained using turbo extraction. The solvents were removed under vacuum at 40 °C and lyophilized. The dried samples were stored under refrigeration (5 °C) until the moment of use. The yield was calculated for all the extracts.

2.2 Terpenes quantification

The total of terpenes content was determined according presented by Cruz et al. (2020). Each extract sample (10 mg) was resuspended individually in 1 mL of methanol; then, 100 µL of each solution was mixed with 150 µL of vanillin solution in glacial acetic acid (5 %, w/v) and 500 µL of perchloric acid solution. Samples were heated for 45 minutes at 60 °C and after cooled in an ice water bath to room temperature. In sequence, it was added 2.25 mL of glacial acetic acid. The content of terpenes was measured at 548 nm using a UV/Vis spectrophotometer (Thermo Scientific Orion-AquaMate 8000). A calibration curve of euphol was performed (0.05–1 mg/mL, r^2=0.9921). The results were expressed in mg of euphol per g of extract.

2.3 Microbial activity determination

Three microbial species (*Klebsiella pneumoniae* (ATCC® 13883), *Pseudomonas aeruginosa* (ATCC® 27853), and *Staphylococcus aureus* (ATCC® 25923)) were taken from the Clinical Analyses Department from State University of Ponta Grossa (UEPG). Before the tests, the cultures of microorganisms were prepared in physiological serum and the turbidity was standardized with $BaSO_4$ (control), equivalent to the scale 0.5 of McFarland (1.5 10^8 x UCF/mL), and after that it was inoculated for 24 hours.

2.4 Minimum inhibitory concentration (MIC)

The MIC method was carried out according described by Karaman et al. (2003) with adaptation. A stock solution of each extract (40 mg/mL) was prepared using DMSO (dimethyl sulphoxide). An aliquot of 20 µL of each extract with concentrations of 1.562; 3.125; 6.25; 12.5; 25; 50, 100 e 200 mg/mL were deposited in each tube containing Müller-Hinton broth with a suspension of microorganisms (50 µL) for a final volume of 1000 µL. Controls of the extract were Müller-Hinton broth (negative control), and microorganism suspensions (positive control). After a 24-hour incubation period at 35 °C, the MIC of each sample was measured by the optical density in the reader of ELISA (630 nm) and compared with the negative control. The minor concentration of the extract capable of inhibiting microbial growth was considered as MIC. All determinations were executed in triplicate.

2.5 Minimum bactericidal concentration (MBC)

The samples obtained in the MIC were inoculated in a Sensitive Plate Microtiter with 96 wells containing Müller-Hinton broth, for 24 hours at 35 °C. The lowest dichloromethane silver nanoparticles and extract concentration capable to inhibit the microorganism growth was considered the MBC. All determination were executed in triplicate.

2.6 Cell cultures

Lung carcinoma cells (A549 and H292 – ATCC CCL-185 and CRL-1848) were defrosted and transferred to appropriated culture bottles with Dulbecco's modified eagle medium (DMEM) and Roswell Park Memorial Institute medium

(RPMI 1640) supplemented with 10% (v/v) fetal bovine serum, 24 mmol/L of sodium bicarbonate, 2 mmol/L of glutamine and 1% (v/v) penicillin and streptomycin and maintained at 37 °C with 5% CO_2. The cells cultures were discarded after 30 passages.

2.7 MTT reduction assay

The cells (A549 or H292 cell line, 1×10^5 cell/mL), in log phase, were seeded in a 96-well plate under culture conditions and treated with extracts at different concentrations (up to 500 µg/mL, solubilized in DMSO 2 %), and dichloromethane silver nanoparticles (0; 0.1; 0.25; 0.5; 0.75; 1 µg/mL, solubilized in medium). After 72 hours, the medium was discarded and replaced with 100 µL of MTT solution (500 µg/mL). The cells were incubated at 37 °C for 60 minutes, and the medium discarded. The formazan crystals were solubilized with DMSO (100 µL) and the optical density read at 570 nm. The IC_{50} (concentration that inhibit cellular proliferation by 50%) value was calculated. The negative control was the cells incubated with medium/DMSO. DMSO at the concentration used was not cytotoxic. All the experiments were performed in quadruplicate and repeated 3 folds.

2.8 Protein staining assay (Sulforhodamine B – SRB)

The cells (A549 or H292 cell line, 1×10^5 cell/mL), in log phase, were seeded in a 96-well plate under culture conditions and treated with extracts at different concentrations (up to 500 µg/mL, solubilized in DMSO 2 %) and dichloromethane silver nanoparticles (0; 0,1; 0,25; 0,5; 0,75; 1 µg/mL, solubilized in medium). After the incubation time (72 hours), the medium was removed, and the 96-well plate was washed using phosphate buffer solution (PBS, pH 7.4). After, 100 µL of 10% trichloroacetic acid were added into each well and the plates were kept under refrigeration for 30 minutes. Then, the 96-well plate was washed again and dried at room temperature. Later, into each well, it was added 100 µL of Sulforhodamine B (SRB) 0.4% solution, and left for 30 minutes, at room temperature. Afterwards the wells were washed with acetic acid (1%, v/v) and dried at room temperature. For the last step, 150 µL of TrisBase 10 mM were added (Papazisis et al., 1997). The optical densities were read at 520 nm and 620 nm. DMSO at the concentration used was not cytotoxic. All the experiments were done in quadruplicate and repeated 3 folds.

2.9 Synthesis of silver nanoparticles

For the development of the silver nanoparticles synthesis (AgNPs) it was prepared several solutions such as: $AgNO_3$ aqueous solution (10^{-4} M), stock solution (10 mg/mL) for the dichloromethane extract and stock solution (50 mg/mL) for the hexane extract. A 30 µL of each aqueous solution of the dichloromethane and hexane extracts were solubilized under mild heating with distilled water (1 mL). 500 µL of these solutions were mixed with $AgNO_3$ (1 mL) and exposed to artificial light (JBL lamp, Tropic Ultra T5 24W 55CM 4000K model). The exposure periods were 10, 15, 20, 25, 30, 35, 40 and 45 minutes, to determine the best reaction time from Ag^+ ($AgNO_3$) to Ag^0 (AgNPs) with color change from colorless/light yellow to brownish, respectively, and confirmed by UV-Vis spectrophotometer analysis. The AgNPs solutions were stored at 4–8 °C for further analysis.

2.10 The physic-chemical characterization of AgNPs

2.10.1 Size, polydispersity index and zeta potential

Analysis of size, polydispersity index and zeta potential were determined using dynamic light scattering (DLS) by intensity with a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). The measurement of hydrodynamic diameter and size distribution were performed at 25 °C and a scattering angle of 90 °. The zeta potential was measured with the

standard capillary electrophoresis cell at 25 °C. AgNPs samples were diluted (1:100, v/v) and analyzed in triplicate. The data represent the average values from three separate measurements.

2.10.2 UV-Vis spectrophotometer analysis

The reduction of Ag^+ to the AgNPs formation was observed through the UV-Vis spectrum of the reaction solutions of AgNPs by UV-Vis/NIR Varian® Cary 50 (Agilent Technologies, USA) in the wavelength range of 190–500 nm. The blank was distilled water and the samples were analyzed without dilutions.

2.10.3 Field emission gun – scanning electron microscopy (FEG-SEM)

The morphology and surface of AgNPs were evaluated by field emission gun – scanning electron microscopy Myra 3 LMH (Tescan, Czech Republic). AgNPs samples were added onto the holder and oven dried at 36 °C for 24 hours. Following, it was metalized on the SC7620 mini sputter Coater. The photomicrographs were visualized using acceleration voltages between 10 e 25 kV and images recorded with the equipment software.

2.10.4 Antimicrobial and cytotoxic activity of the AgNPsD

The antimicrobial and cytotoxic evaluation of the silver nanoparticles were executed as described for the extract's evaluation, using 0; 0.1; 0.25; 0.5; 0.75 and 1 µg/mL of the dichloromethane silver nanoparticles.

3. Results and Discussion

3.1 Extracts yields and terpenes quantification

The yield of the extraction after the dry process was as follows: 21.33% for the hexane extract; 19.95% for the dichloromethane extract; 19.83% for the acetone extract; 20.91% for the methanolic extract; and 18.21% for the aqueous extract. The data obtained showed that the hexane fraction presented the highest total terpene content, followed by the dichloromethane fraction. This result was expected, once it has already been identified terpenes at different parts of *E. umbellata* (Andrade, 2020; Cruz et al., 2020). The extracts were obtained aiming to separate the compounds according to their chemical affinity with such solvent (Vasas & Hohmann, 2008), so the extraction process allowed to obtain the increased concentration of terpene compounds of the plant phytocomplex at nonpolar extracts (Azevedo, 2014), as presented in Table 1.

Table 1 - Total terpenes content from *E. umbellata* leaves extracts.

Extract	mg euphol/g of extract
Hexane	7.24 ± 0.81
Dichloromethane	6.12 ± 2.44
Acetone	3.86 ± 1.06
Methanol	1.03 ± 0.30
Aqueous	0.20 ± 0.02

Source: Authors.

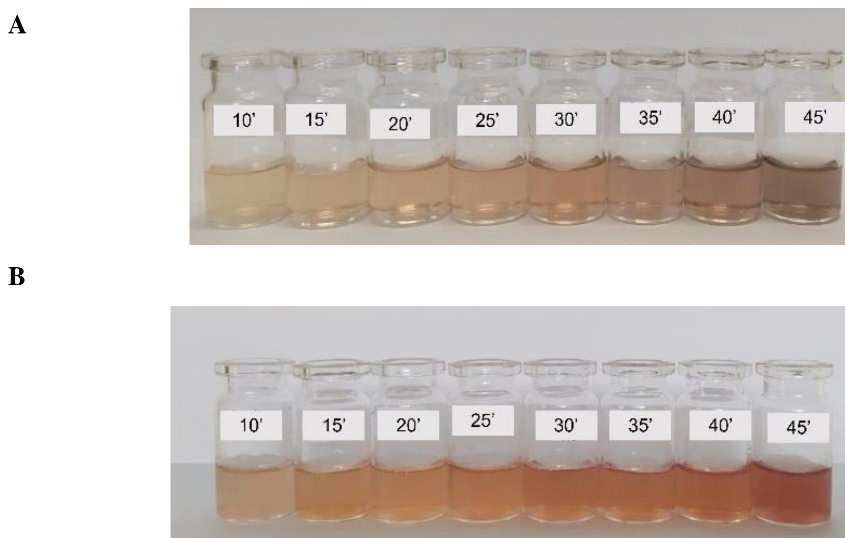
3.2 Macroscopic analysis of silver nanoparticles (AgNPs)

The solution of silver nitrate mixed with hexane or dichloromethane extracts of *E. umbellata* were exposed to artificial light to produce the silver nanoparticles (AgNPsH and AgNPsD, respectively). The green synthesis of the AgNPs was

(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i11.34804

confirmed by the color change visualization from colorless/yellowish to brown for dichloromethane and hexane extracts, as shown in Figure 1. The big number of terpenes present in these extracts (Table 1) could be responsible for reducing the silver ions (Ag^{+}) to Ag^{0} in AgNPs. Further, at the same time as the production of AgNPs, there is nanoparticle stabilization by the plant biomolecules, being the process divided into three steps: ion reduction; nucleation/growing/aggregation/capping; stabilization (Salayová et al., 2021).

Figure 1 - AgNPs suspensions exposed to artificial light in times of 10, 15, 20, 25, 30, 35, 40 and 45 minutes. (**A**) AgNPsD; (**B**) AgNPsH.



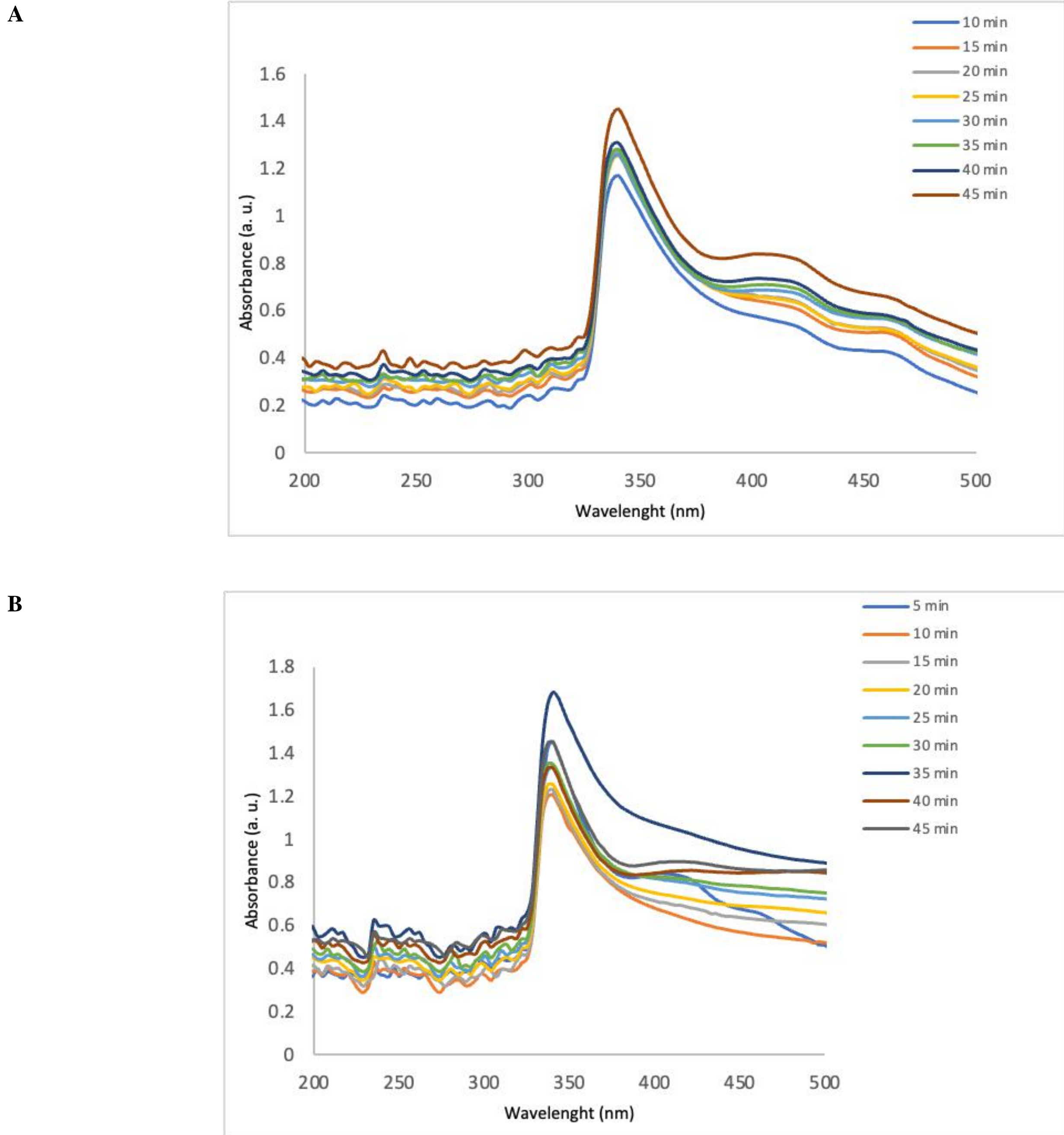
Source: Authors.

The nanoparticles size is influenced by exposure time to artificial light as studied here. AgNPsD suspension had color change over time, most evident at 40 minutes. For the AgNPsH suspension, occurred the same, however, at 45 minutes. Reaction conditions should be controlled to assure uniform morphology and size, preventing aggregation (Alayande et al., 2021).

3.3 Ultraviolet spectroscopy

From 10 to 30 minutes there was little formation of nanoparticles; 35 minutes being the ideal exposure time for both extracts; from 40 minutes of artificial light exposure, AgNPs tend to increase in size as evidenced by peak displacement towards higher absorbances. Nasrollahzadeh, Atarod, & Sajadi (2016) utilized the *Euphorbia heterophylla* leaf extract for AgNPs formation, performing the more adequate UV-Vis wavelength range between 250 and 350 nm, and have developed metallic nanoparticles through aqueous leaf extract of *Euphorbia granulate* with 5 minutes of exposure time at wavelengths of 260 – 320 nm (Nasrollahzadeh & Sajadi, 2016). The surface plasmon resonance band at which occurred high absorption was 340 nm for both extracts, as can be observed in Figure 2.

Figure 2 - Specters over time reaction from 10 to 45 minutes. (**A**) AgNPsD; (**B**) AgNPsH



Source: Authors.

3.4 Size, polydispersity index and zeta potential

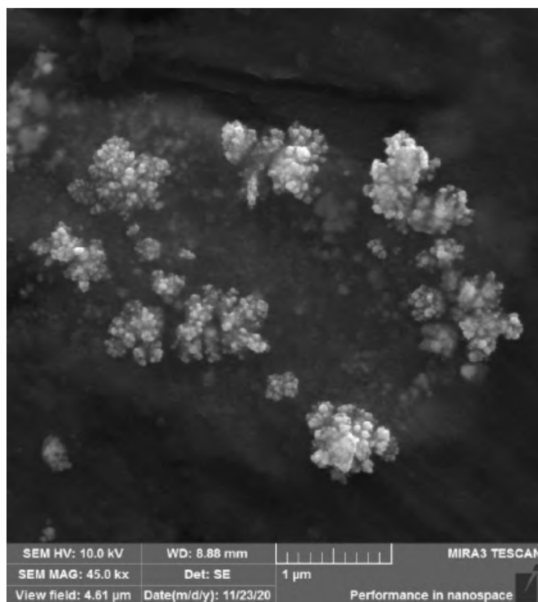
The AgNPsH had an average size of 177.9 nm (with 100% intensity), on the other hand, the AgNPsD presented an average size of 288.8 nm, being size of 191 nm, (89.7% intensity) plus size of 76.61 nm (10.3% intensity). With the latex of *Synadenium grantii* Hook, the size range of AgNPs was 106 – 147 nm (Durgawale et al., 2015). The polydispersity indexes were 0.416 and 0.496 for the AgNPsH and AgNPsD, respectively, which are considered as homogenous size populations. Zeta potential values were -35,7 ± 9.16 mV and -30,5 ± 11.7 mV for the AgNPsH and AgNPsD, respectively, higher than |25 – 30| mV, thus, with electrical stability and minimal flocculation due to the negative charge on the nanoparticle's surfaces (Espinoza, 2021).

3.5 Scanning electron microscopy (SEM)

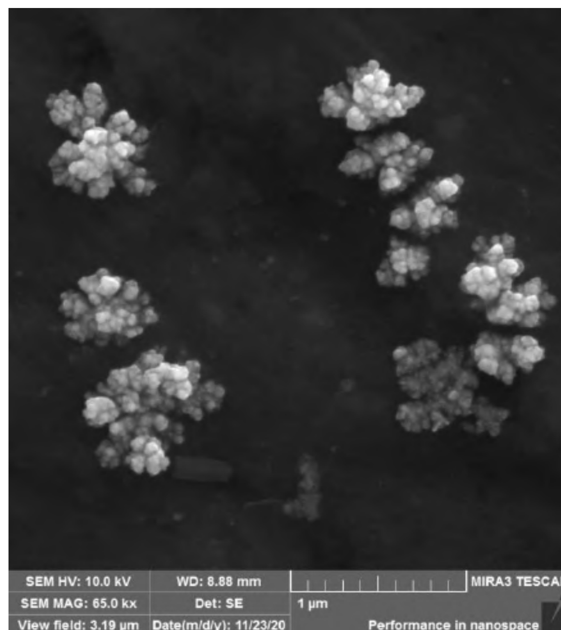
According to Figure 3, both AgNPsH and AgNPsD had presented spherical format and aggregates, which can be related to the presence of extract in excess. They presented average sizes ranging between 86.87 ± 9.11nm and 42.78 ± 17.22 nm, respectively. The same profile was observed for the development of copper nanoparticles with the aqueous extract of *Euphorbia prolifera* leaves (Momeni et al., 2016). The agglomeration formation can be attributed to the plant residual matrix which keeps the nanoparticles connected after the biosynthesis (Salayová et al., 2021).

Figure 3- Micrographs of AgNPs obtained by green synthesis. (**A**) AgNPsH; (**B**) AgNPsD. Images with 145 Kx magnification.

A



B



Source: Authors.

3.6 Evaluation of the antimicrobial activity of the *Euphorbia umbellata* extracts and AgNPsD

For *K. pneumoniae* and *S. aureus* was observed growing inhibition, mainly, for the hexane and dichloromethane extracts (IC_{50}: 3.12 mg/mL). High concentrations of the *E. umbellata* extracts present a greater number of secondary metabolites responsible for the antibacterial activity, mainly terpenes. Terpenes had already been associated with this prominent activity for plant extracts (Espadero et al., 2019). The essential oil and crude extract of *Euphorbia macrorrhiza* also presented antimicrobial effect on gram-positive and gram-negative bacteria (Lin et al., 2012).

Acetone extract showed growing inhibition for *K. pneumoniae* and *P. aeruginosa*, which did not occur for *S. aureus* due to the low minimum inhibitory concentration. Methanol and aqueous extracts did not display growing inhibition for all bacterial strains tested, even in their high concentrations.

As the dichloromethane extract presented better results than hexane extract in microbiological and cytotoxic experiments, and the AgNPsD demonstrated better physic-chemical characteristics, this material (AgNPsD) was selected to evaluate the biological potential.

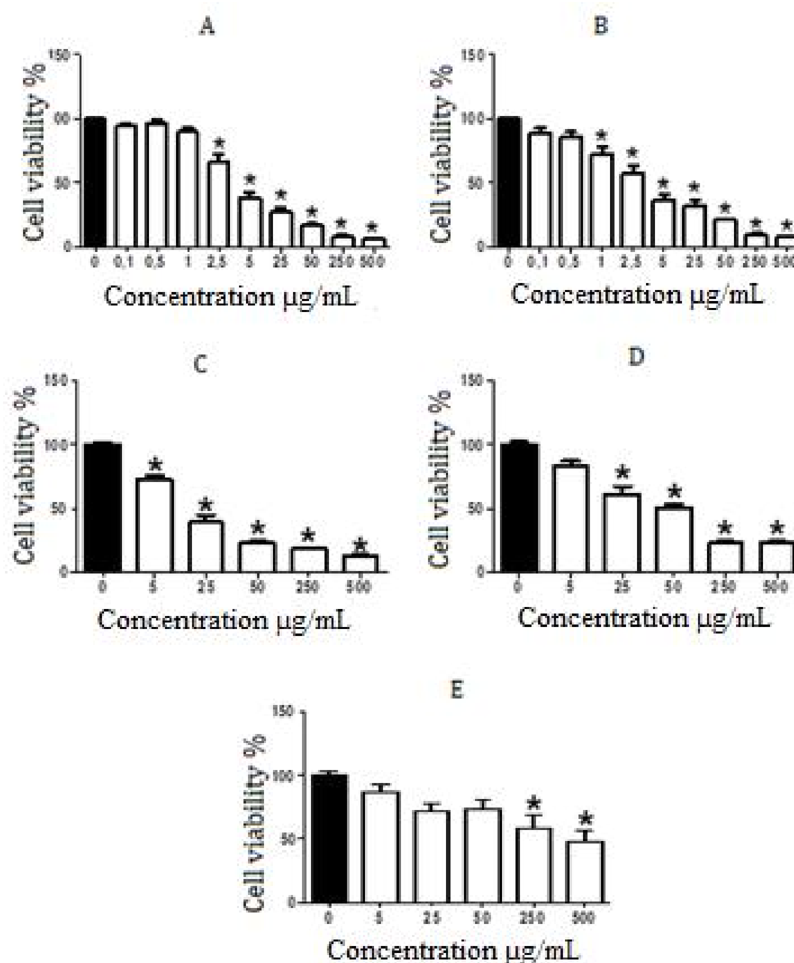
The AgNPsD presented increment in the biological activities evaluated, with IC_{50} of 1.56 µg/mL and 0.25 µg/mL against *K. pneumoniae* and *S. aureus*. According to Banala, Nagati & Karnati (2015), the AgNPs can adhere to and cross the bacterial cell wall due to their small size. They can be oxidized to form metal ions that could react with amino acids present in bacteria, thus, resulting in cell damage and death.

No extract and no AgNPsD showed minimal bactericidal concentration, that is, they only inhibited the growth, without killing the bacteria.

3.7 Evaluation of cytotoxicity of the *Euphorbia umbellata* extracts and AgNPsD

The A549 cell line, cells from lung cancer, treated for 72 hours with different extracts (hexane, dichloromethane, acetone and aqueous) of *E. umbellata* had their cell viabilities decreased in a dose-dependent manner as displayed in Figure 4.

Figure 4 - Cell viability of A549 cell line after 72 hours of treatment with extracts *Euphorbia umbellata* by MTT assay. The results are shown as the mean of three independent experiments ± standard deviation (n=4). Data were analyzed using a one-way analysis of variance (ANOVA) complemented with a post hoc Tukey test * p< 0.001 versus negative control (cells treated with DMSO 2%). (**A**) Hexane; (**B**) Dichloromethane; (**C**)**:** Acetone; (**D**) Methanol; (**E**) Aqueous.



Source: Authors.

For the hexane and dichloromethane extracts were observed a significant decreased in the cell viability compared with negative control (cells without treatment with *E. umbellata* extracts). Hexane extract decreased significantly the A549 cell viability from 2,5 µg/mL while the same effect was observed at 1 µg/mL for dichloromethane extract. The other extracts were able to decrease the cell viability in higher concentrations as 5, 25 and 250 µg/mL for the acetone, methanol, and aqueous extracts, respectively.

For the H292 cell line (cells derived from a lymph node metastasis of the pulmonary mucoepidermoid carcinoma), after treatment with six concentrations, was observed most significant reduction of cell viability for 5 µg/mL for the hexane ($p<0.01$) and dichloromethane ($p<0.001$) extracts, which can be related with the high concentration of terpenes (data not shown).

The SRB assay exhibited higher cytotoxicity for smaller concentrations compared with the MTT assay for both cell lines. These results are due to the SRB technique, which stain only the protein constituents present in the cells adhered to the

well of the culture plate. Based on the values of IC_{50} for dichloromethane, hexane, and acetone extracts, in ascending order, were able to decrease the cell viability at both lineages. On the other hand, methanol and aqueous extracts demonstrated higher IC_{50} values. Nonpolar molecules such as terpenes found in *Euphorbia* genus had already demonstrated cytotoxic effects (Jadranin et al., 2013). Being the nonpolar extract of *E. umbellata* specie was related to the cytotoxicity effects against HRT-18 (adenocarcinoma cells from the large intestine), HeLa (cervical cancer cells) and Jurkat cells (T lymphocyte cells) after treatment of 48 hours (Luz et al., 2016). The IC_{50} values obtained by MTT reduction and SRB assays, for all extracts of *E. umbellata* are shown in Table 2.

Table 2 - Values of IC_{50} (µg/mL) obtained by MTT and SRB method for the A549 and H292 cell lines after 72-hours treatment with *Euphorbia umbellata* extracts. Data were expressed as IC_{50} ± mean standard error and were calculated by Probit analysis and Finney method.

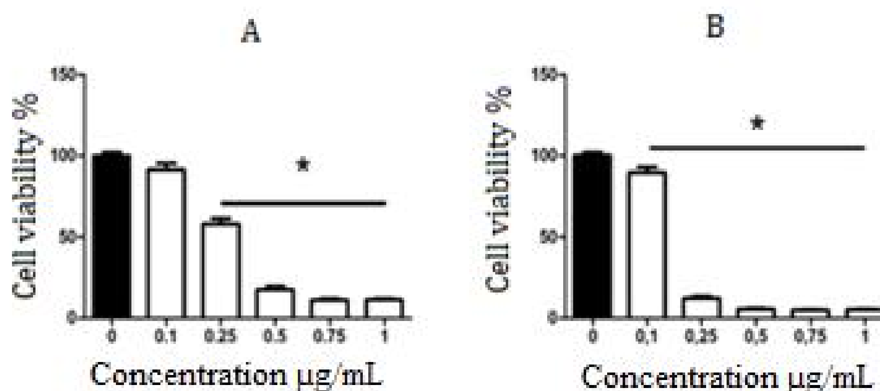
Extract	MTT assay		SRB assay	
	A549	H292	A549	H292
Hexane	5.28 ± 1.40	53.75 ± 19.69	2.30 ± 0.68	5.28 ± 5.98
Dichloromethane	2.74 ± 0.89	27.52 ± 7.43	0.84 ± 0.32	33.83 ± 14.05
Acetone	13.16 ± 45.93	190.15 ± 75.38	2.54 ± 1.11	44.50 ± 29.70
Methanol	61.80 ± 8.82	>500	19.54 ± 3.25	301.72 ± 196.09
Aqueous	157.94 ± 27.60	>500	49.80 ± 21.44	>500

Source: Authors.

All extracts were able to reduce the cell viability, however, when compared to A549 cell line it is possible to notice that H292 required higher concentrations to promote cell death.

For the cell viability evaluation of the AgNPsD, the same A549 and H292 cell lines were treated for 72 hours, both demonstrated cell viability decrease with the increase of the AgNPsD concentrations as displayed in Figure 5.

Figure 5 - Cell viability of A549 (**A**) and H292 (**B**) lineages after 72 hours of treatment with AgNPsD obtained from dichloromethane extract of *Euphorbia umbellata* by MTT assay. The results are shown as the mean of three independent experiments ± standard deviation (n=4). Data were analyzed using a one-way analysis of variance (ANOVA) complemented with a post hoc Tukey test * $p < 0.001$ versus negative control (cells treated with medium).

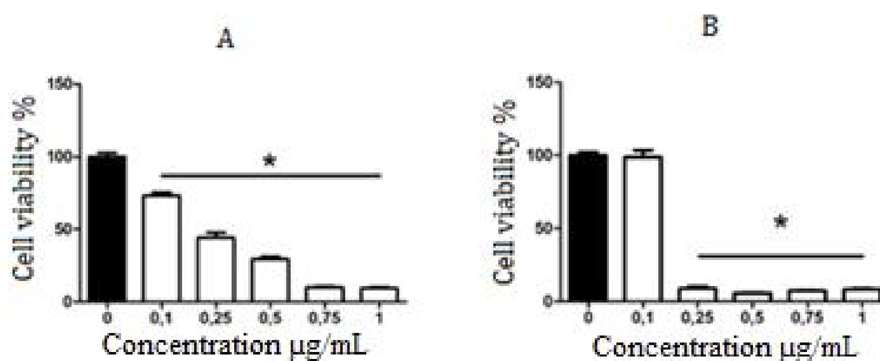


Source: Authors.

The treatment with AgNPsD concentrations were from 0.1 to 1 µg/mL, for the A549 cell line there was a significant cell viability reduction from 0.25 µg/mL ($p<0.001$), which is 4-fold lower in comparison with the treatment of 1 µg/mL dichloromethane extract only. This find can be related to AgNPs ability to disrupt the mitochondrial respiratory chain of cancer cells, which generates reactive oxygen species and, consequently, interrupts ATP synthesis and promotes cell death (Guo et al., 2015). H292 cells were more sensitive to AgNPsD treatment, which can be observed by the significant cell viability reduction from the concentration of 0.1 µg/mL, a value 10-folder lower when compared to the dichloromethane extract concentration for this same cell. The AgNPs cytotoxicity depends on several factors such as small sizes, on average of 288 nm have easily penetrated. Furthermore, surface area and reducing agents for AgNPs synthesis should be taken into consideration (Barabadi et al.,2017; Zhang, Shen & Gurunathan, 2016).

The SRB assay for the AgNPsD treatment of the A546 cell line evidenced a significant reduction of cell viability from 0.1 µg/mL ($p<0.001$), a smaller concentration compared with the value obtained by the MTT assay. On the other hand, when the H292 cells were treated with the AgNPsD this significant reduction appeared from 0.25 µg/mL, a higher value compared with that of the MTT assay. This difference may be related to SRB technique peculiarities. The cell viability of both cells' lineages after treatment with AgNPsD by SRB assay was presented in Figure 6.

Figure 6 - Cell viability of A549 (**A**) and H292 (**B**) lineages after 72 hours of treatment with AgNPsD obtained from dichloromethane extract of *Euphorbia umbellata* by SRB assay. The results are shown as the mean of three independent experiments ± standard deviation (n=4). Data were analyzed using a one-way analysis of variance (ANOVA) complemented with a post hoc Tukey test* $p < 0.001$ versus negative control (cells treated with medium).



Source: Authors.

IC_{50} values of AgNPsD were very low for both A549 and H292 cell lineages (Table 3) when compared with the values of IC_{50} of dichloromethane extract for the same cell lines (Table 2), being 11-fold and 145-fold lower, respectively, which clearly disclosure the great potential of the AgNPsD in decrease the cell viabilities.

Table 3 - Values of IC_{50} (μg/mL) obtained by MTT and SRB methods for the A549 and H292 cell lines after 72-hours treatment with AgNPsD obtained from the dichloromethane extract of *E. umbellata*. Data were expressed as IC_{50} ± mean standard error and were calculated by Probit analysis and Finney method.

	A549	**H292**
MTT	0.28 ± 0.01	0.18 ± 0.05
SRB	0.22 ± 0.02	0.19 ± 0.07

Source: Authors.

4. Conclusion

It was observed that the nonpolar extracts (hexane and dichloromethane) presented better microbiological activity, mainly against *K. pneumoniae* and *S. aureus* and a dose-dependent cytotoxicity against A549 and H292 cell lines. These biological effects could be related with the amounts of terpenes present in these two extracts from *E. umbellata* leaves.

The AgNPs formed using the dichloromethane extract demonstrated a higher increase in microbiological and cytotoxic activities. These data suggest that the nanoparticles developed by green synthesis present great potential to be evaluated as a medicinal product, despite more studies are necessary, mainly through *in vivo* research.

References

Abbasi, A., Emam-Djomeh, Z., Mousavi, M. A. E., & Davoodi, D. (2014). Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate. *Food chemistry*, *143*, 379-383.

Ahmad, S., Perveen, S., Arshad, M. A., & Rehman, T. (2019). Pharmacological and nutritive potential of *Euphorbia granulata*. *Journal of Complementary and Integrative Medicine*, *16*(2).

Alayande, S. O., Akinsiku, A. A., Akinsipo, O. B., Ogunjinmi, E. O., & Dare, E. O. (2021). Green synthesized silver nanoparticles and their therapeutic applications. *Comprehensive Analytical Chemistry*, *94*, 585-611.

Andrade, E. A. D. (2020). Efeito citotóxico do látex de *Euphorbia umbellata* (Pax) Bruyns (Euphorbiaceae) para modelo de melanoma murino.

Azevedo, L. F. P., Faria, T. S. A., Pessanha, F. F., Araujo, M. F., & Lemos, G. C. S. (2014). Triagem fitoquímica e atividade antioxidante de *Costus spicatus* (Jacq.) Sw. *Revista Brasileira de Plantas Medicinais*, *16*, 209-215.

Bamal, D., Singh, A., Chaudhary, G., Kumar, M., Singh, M., Rani, N., ... & Sehrawat, A. R. (2021). Silver nanoparticles biosynthesis, characterization, antimicrobial activities, applications, cytotoxicity and safety issues: an updated review. *Nanomaterials*, *11*(8), 2086.

Banala, R. R., Nagati, V. B., & Karnati, P. R. (2015). Green synthesis and characterization of Carica papaya leaf extract coated silver nanoparticles through X-ray difextract, electron microscopy and evaluation of bactericidal properties. *Saudi Journal of Biological Sciences*, *22*(5), 637-644.

Barabadi, H., Honary, S., Ali Mohammadi, M., Ahmadpour, E., Rahimi, M. T., Alizadeh, A., ... & Saravanan, M. (2017). Green chemical synthesis of gold nanoparticles by using Penicillium aculeatum and their scolicidal activity against hydatid cyst protoscolices *of Echinococcus granulosus*. *Environmental Science and Pollution Research*, *24*(6), 5800-5810.

Cruz, L. S., Kanunfre, C. C., de Andrade, E. A., de Oliveira, A. A., Cruz, L. S., de Faria Moss, M., ... & Beltrame, F. L. (2020). Enriched terpenes extracts of the latex of *euphorbia umbellata* promote apoptosis in leukemic cells. *Chemistry & Biodiversity*, *17*(9), e2000369.

Durgawale, P. P., Phatak, R. S., & Hendre, A. S. (2015). Biosynthesis of silver nanoparticles using latex of *Syandenium grantii* Hook f and its assessment of antibacterial activities. *Digest Journal of Nanomaterials and Biostructures*, *10*(3), 847.

El-Mahmood, M. A. (2009). Antibacterial activity of crude extracts of *Euphorbia hirta* against some bacteria associated with enteric infections. *Journal of Medicinal Plants Research*, *3*(7), 498-505.

Ernst, M., Nothias, L. F., Van der Hooft, J. J., Silva, R. R., Saslis-Lagoudakis, C. H., Grace, O. M., ... & Rønsted, N. (2019). Assessing specialized metabolite diversity in the cosmopolitan plant genus *Euphorbia L. Frontiers in Plant Science*, *10*, 846.

Espadero, M., Avilés, H., Armijos, L., Ávila, L., Idrovo, L., Idrovo, M., & Oyola, C. (2019). Evaluación microbiológica y composición química de extractos orgánicos de *Euphorbia aff. viridis* (Klotzsch & Garcke) Boiss sobre *Staphylococcus Aureus, Klebsiella Pneumoniae* y *Escherichia Coli*. *LA GRANJA. Revista de Ciencias de la Vida*, *29*(1), 119-129.

Espinoza, J. T. (2021). Desenvolvimento, caracterização e avaliação de lipossomas contendo β-sitosterol em um modelo experimental ex vivo de cataratogênese.

Guo, Y., Zhang, L., Zhang, S., Yang, Y., Chen, X., & Zhang, M. (2015). Fluorescent carbon nanoparticles for the fluorescent detection of metal ions. *Biosensors and bioelectronics*, *63*, 61-71.

Jadranin, M., Pešić, M., Aljančić, I. S., Milosavljević, S. M., Todorović, N. M., Podolski-Renić, A., ... & Tešević, V. V. (2013). Jatrophane diterpenoids from the latex of *Euphorbia dendroides* and their anti-P-glycoprotein activity in human multi-drug resistant cancer cell lines. *Phytochemistry*, *86*, 208-217.

Kanunfre, C. C., Leffers, T., Cruz, L. S., Luz, L. E., Crisma, A. R., Wang, M., ... & Beltrame, F. L. (2017). *Euphorbia umbellata* bark extracts–an in vitro cytotoxic study. *Revista Brasileira de Farmacognosia*, *27*, 206-213.

Karaman, I., Şahin, F., Güllüce, M., Öğütçü, H., Şengül, M., & Adıgüzel, A. (2003). Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *Journal of ethnopharmacology*, *85*(2-3), 231-235.

Kemboi, D., Peter, X., Langat, M., & Tembu, J. (2020). A review of the ethnomedicinal uses, biological activities, and triterpenoids of *Euphorbia* species. *Molecules*, *25*(17), 4019.

Kumar, S. A., Priyadarshini, H. S., Paramita, A. P., Pinki, S., Sakshi, G., Kar, B. R., & Kiran, B. K. (2019). Green synthesis and biochemical characterization of silver nanoparticles by using *Euphorbia umbellata* leaf extract and analysis of antimicrobial activity against plant pathogens. *GSC Biological and Pharmaceutical Sciences*, *8*(2).

Lee, S. H., & Jun, B. H. (2019). Silver nanoparticles: synthesis and application for nanomedicine. *International journal of molecular sciences*, *20*(4), 865.

Lin, J., Dou, J., Xu, J., & Aisa, H. A. (2012). Chemical composition, antimicrobial and antitumor activities of the essential oils and crude extracts of Euphorbia

Luz, L. E. C., Kanunfre, C. C., Paludo, K. S., da Silva Justo, A., Petry, V. K., Lemes, B. M., ... & Beltrame, F. L. (2016). Cytotoxic biomonitored study of *Euphorbia umbellata* (Pax) Bruyns. *Journal of ethnopharmacology*, *183*, 29-37.

Minozzo, B. R., Lemes, B. M., da Silva Justo, A., Lara, J. E., Petry, V. E. K., Fernandes, D., ... & Beltrame, F. L. (2016). Anti-ulcer mechanisms of polyphenols extract of *Euphorbia umbellata* (Pax) Bruyns (Euphorbiaceae). *Journal of ethnopharmacology*, *191*, 29-40.

Momeni, S. S., Nasrollahzadeh, M., & Rustaiyan, A. (2016). Green synthesis of the Cu/ZnO nanoparticles mediated by *Euphorbia prolifera* leaf extract and investigation of their catalytic activity. *Journal of colloid and interface science*, *472*, 173-179.

Munhoz, A. C., Minozzo, B. R., Cruz, L. S., Oliveira, T. L., Machado, W. M., Pereira, A. V., ... & Beltrame, F. L. (2014). Chemical and pharmacological investigation of the stem bark of *Synadenium grantii*. *Planta Medica*, *80*(06), 458-464.

Nasrollahzadeh, M., Atarod, M., & Sajadi, S. M. (2016). Green synthesis of the Cu/Fe3O4 nanoparticles using Morinda morindoides leaf aqueous extract: a highly efficient magnetically separable catalyst for the reduction of organic dyes in aqueous medium at room temperature. *Applied Surface Science*, *364*, 636-644.Nasrollahzadeh, M., Sajadi, S. M. (2016). Pd nanoparticles synthesized in situ with the use of *Euphorbia granulate* leaf extract: Catalytic properties of the resulting particles. *Journal of Colloid and Interface Science*, *462*(15), 243-251.

Oliveira Junior, L. F. G., Santos, R. B., Reis, F. O., Matsumoto, S. T., Bispo, W. M. S., Machado, L. P., & Oliveira, L. F. M. (2013). Efeito fungitóxico do óleo essencial de aroeira da praia (*Schinus terebinthifolius* RADDI) sobre *Colletotrichum gloeosporioides*. *Revista Brasileira de Plantas Medicinais*, *15*, 150-157.

Oliveira, T. L., Fontana, P. D., Bavia, L., Cruz, L. S., Crisma, A. R., Sassaki, G. L., ... & Messias-Reason, I. J. (2021). Effects of *Euphorbia umbellata* extracts on complement activation and chemotaxis of neutrophils. *Journal of Ethnopharmacology*, *265*, 113348.

Papazisis, K. T., Geromichalos, G. D., Dimitriadis, K. A., & Kortsaris, A. H. (1997). Optimization of the sulforhodamine B colorimetric assay. *Journal of immunological methods*, *208*(2), 151-158.

Patel, N. B., & Patel, K. C. (2014). Antibacterial activity of *Euphorbia hirta* L. ethanomedicinal plant against gram negative UTI pathogens. *International Journal*, *3*(2), 24-29.

Salayová, A., Bedlovičová, Z., Daneu, N., Baláž, M., Lukáčová Bujňáková, Z., Balážová, Ľ., & Tkáčiková, Ľ. (2021). Green synthesis of silver nanoparticles with antibacterial activity using various medicinal plant extracts: Morphology and antibacterial efficacy. *Nanomaterials*, *11*(4), 1005.

Vasas, A. & Hohmann, J. (2014). *Euphorbia* diterpenes: isolation, structure, biological activity, and synthesis (2008). *Chemical Review*, 114, 8579–8612

Yadi, M., Mostafavi, E., Saleh, B., Davaran, S., Aliyeva, I., Khalilov, R., ... & Milani, M. (2018). Current developments in green synthesis of metallic nanoparticles using plant extracts: a review. *Artificial cells, nanomedicine, and biotechnology*, *46*(sup3), S336-S343.

Zhang, X. F., Liu, Z. G., Shen, W., & Gurunathan, S. (2016). Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. *International journal of molecular sciences*, *17*(9), 1534.