Biosynthesis and characterization of silver nanoparticles produced with aqueous extract of *Pterodon emarginatus* Vogel - Fabaceae seeds associated with gentamicin sulfate and hyaluronic acid with potential antimicrobial activity

Biossíntese e caracterização de nanopartículas de prata produzidas com extrato aquoso de sementes *de Pterodon emarginatus* Vogel - Fabaceae associadas ao sulfato de gentamicina e ao Ácido Hialurônico com potencial atividade antimicrobiana

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ABSTRACT
The synthesis of metallic nanoparticles by biological systems is known as green synthesis or biosynthesis. In this synthesis strategy, instead of using toxic agents to reduce the metal ion, obtaining the metallic nanoparticles (NPs) is mediated by biological components capable of reducing the metal. In this context, the present work uses aqueous extract from the seeds of *Pterodon emarginatus* Vogel to obtain silver nanoparticles (AgNPs-PE). These metallic nanoparticles associated with the aqueous extract of *P. emarginatus* have, among others, antimicrobial property that was enhanced by the

association of 1% gentamicin sulfate (AgNPs-PEG) and hyaluronic acid (AgNPs-PEG-AH2). Therefore, the evaluation of antimicrobial activity was addressed after obtaining the AgNPs-PE and their associations. The characterization of AgNPs-PE was performed by means of UV-Vis spectrophotometry, determination of the average hydrodynamic diameter (AHD), zeta potential (ZP) and polydispersity index (PDI), field emission gun scanning electron microscopy (FEG-SEM), energy dispersive X-ray spectroscopy (EDS) and Fourier transform infrared (FTIR) spectroscopy. Although the green synthesis still does not have its mechanisms clearly elucidated, it is known that the bioreduction of Ag^+ and the stabilization of Ag^0 are carried out by a combination of primary and secondary metabolites present in the extract, such as tannins and flavonoids. After formed, AgNPs-PE, AgNPs-PEG and AgNPs-PEG-AH2 showed, respectively, maximum absorbance around 423 nm, 425 nm and 430 nm. Microscopy (FEG-SEM) showed spherical-shaped nanoparticles, with an average size ranging from 59 nm for AgNPs-PE, 63 nm for AgNPs-PEG and 66 nm for AgNPs-PEG-AH2. The ZP of all AgNPs-PE and their associations remained within the values considered stable for nanoparticles (-27.15 to -39.73 mV). The PDI of the analyzed samples was on average 0.332 to 0.524, median values for the distribution of NPs. AgNPs-PE, AgNPs-PEG and AgNPs-PEG-AH2 showed bactericidal activity against Gram-positive, Gram-negative and yeast bacteria. The present research indicates that AgNPs-PE and their associations have potential as a therapeutic agent against bacterial and fungal infections.

Keywords: green synthesis, *Pterodon emarginatus* Vogel, silver nanoparticles, antimicrobial activity

RESUMO

A síntese de nanopartículas metálicas por sistemas biológicos é conhecida como síntese verde ou biossíntese. Nessa estratégia de síntese, ao invés do uso de agentes tóxicos para a redução do íon metálico, a obtenção das nanopartículas metálicas é mediada por componentes biológicos capazes de reduzir o metal. Nesse contexto, o presente trabalho utiliza extrato aquoso das sementes de *Pterodon emarginatus* Vogel para obter nanopartículas de prata (AgNPs-PE). Essas nanopartículas metálicas associadas com o extrato aquoso de *P. emarginatus* possuem, entre outras, propriedade antimicrobiana que foi potencializada pela associação de sulfato de gentamicina 1% (AgNPs-PEG) e ácido hialurônico (AgNPs-PEG-AH2). Por isso, a avaliação da atividade antimicrobiana foi abordada após a obtenção das AgNPs-PE e suas associações. A caracterização das AgNPs-PE foi realizada por meio de espectrofotometria UV-Vis, determinação do diâmetro médio (DM), potencial zeta (PZ) e índice de polidispersão (IP), microscopia eletrônica de varredura por emissão de campo (MEV-SEM), espectroscopia de raios-X por dispersão de energia (EDS) e espectroscopia no infravermelho por transformada de Fourier (FTIR). Apesar da síntese verde ainda não ter seus mecanismos claramente elucidados, sabe-se que a biorredução da Ag^+ e a estabilização da Ag^0 são realizadas por uma combinação de metabólitos primários e secundários presentes no extrato, como taninos e flavonoides. Após formadas, as AgNPs-PE, as AgNPs-PEG e as AgNPs-PEG-AH2 apresentaram, respectivamente, absorbância máxima em torno de 423 nm, 425 nm e 430 nm. A microscopia (FEG-SEM) apresentou nanopartículas de formato esférico, de tamanho médio variando de 59 nm para as AgNPs-PE, de 63 nm para as AgNPs-PEG e de 66 nm para as AgNPs-PEG-AH2. O PZ de todas as AgNPs-PE e suas associações mantiveram-se dentro dos valores considerados estáveis para nanopartículas (-27,15 a -39,73 mV). O IP das amostras analisadas foi em média 0,332 até 0,524, valores medianos para a distribuição das NPs. As AgNPs-PE, as AgNPs-PEG e as AgNPs-PEG-AH2 apresentaram atividade bactericida contra bactérias Gram-positivas, Gram-negativas e levedura. A presente pesquisa indica que as AgNPs-PE e suas associações possuem potencial como agente terapêutico contra infecções microbianas.

Palavras-chave: síntese verde, *Pterodon emarginatus* Vogel, nanopartículas de prata, atividade antimicrobiana

1 INTRODUCTION

Nanotechnology is a modern field of science that has applications in different fields of science, as in the pharmaceutical area with the objective of increasing the bioavailability of drugs, controlling the release at the site of action, decreasing toxicity, protecting the molecule from extrinsic and / or intrinsic factors and also facilitating the administration of medications (Banu *et al*., 2018; Rosa *et al*., 2020).

Noble metal nanoparticles, such as gold and silver, have attracted much interest due to their versatility, simplicity and physicochemical properties suitable for many biological applications, such as interacting with microorganisms and exhibiting antibacterial and antifungal activity; these properties are mainly dependent on their shape, size and composition. And, there are several strategies described for the synthesis and preparation of these colloidal systems, such as, for example, photochemical, radiolytic and biogenic formations (Melo Júnior *et al*., 2012; Aljabali *et al*., 2018).

Among metallic nanoparticles, AgNPs (Silver Nanoparticles) have won the interest of many researchers. This is due to the characteristics of electrical conductivity, chemical stability and the catalytic and antimicrobial activities of AgNPs. The antimicrobial property of AgNPs makes these nanostructures applicable in different areas of medicine, industry, livestock and other areas where it is necessary to combat the disordered proliferation of microorganisms (Silva, 2014). According to Rai, Prabhuneb, Perry (2010) the advantage of using both antimicrobials and inorganic nanoparticles together is that if the bacteria have resistance against one of the components, a larger component could kill them in a different way. The inappropriate qualitative and / or quantitative use of antimicrobials, such as gentamicin sulfate, associated with an insufficient investment in technology and microbiological investigations, has led to a constant and borderline decline in the effectiveness of current therapies and a shortage of new structural classes, which must be replaced or have a complementary agent in their use (O'Connell *et al*., 2013).

According to Sena *et al*., (2019), green synthesis is gaining ground in research and development today, its use reduces the production of undesirable waste, consequently prevents pollution, and also encourages the use of natural resources. For the synthesis of metal nanoparticles, the use of plant biodiversity has been considered due to the availability of phytochemicals existing in various plant extracts. Biomolecules such as proteins and enzymes, alkaloids, tannins, polyphenols, polysaccharides, saponins, terpenoids and vitamins present in plant extracts (stems, leaves, flowers, roots, fruits and seeds) have medicinal properties, are beneficial to the environment and their chemical structures complexes are used in the synthesis of AgNPs of different shapes and sizes (Lopes, 2017). Tea leaf extracts, rich in polyphenols, such as flavonoids, are powerful reducing agents for the production of AuNPs and AgNPs (Park *et al*., 2011). In nature, polyphenols are the most important compounds in

several biological reduction reactions very common in plants, and flavonoids act as antioxidants because they have the ability to donate hydrogen atoms (Mendes, 2015).

Because hyaluronic acid is a linear anionic polysaccharide that is widely used as a carrier of substances, biocompatible, highly efficient in targeted delivery (Kumar *et al*., 2015) and, because it has hydroxyl groups, a hemiacetal reducing end and other functionalities can play an important role in both the reduction and stabilization of metallic nanoparticles (Kogan *et al*., 2008) and, when associated with phytochemicals present in the green synthesis of gold and silver nanoparticles, it is very useful to provide biocomposites with new uses in nanomedicine (Park *et al*., 2011).

Several plants have already been used in the production of nanoparticles: Skunk vine (*Paederia foetida*) - ornamental plant native to Bangladesh and with a strong odor of sulfur compounds; Papaya - papaya fruit, tree of the species of the genus Carica (*Carica papaya*); Coriander (*Coriandrum sativum*) - plant originating in the Mediterranean and the Middle East, but was already known by the Egyptians who used it as a medicinal plant; the Indian Fig (*Opuntia ficus-indica*) - a species of cactus very common in semi-arid regions; Curry Tree (*Murraya koenigii*) - plant originally from India, from the Rutaceae family (Lopes, 2017). The Geranium plant (*Pelargonium graveolens*) was used in the form of extract as a reducing agent, for the formation of AgNPs (Shankar, Ahmad, Sastry, 2003); Pine (*Pinus desiflora*), Persimmon (*Diopyros kaki*), Ginko (*Ginko biloba*), Magnolia (*Magnolia kobus*) and *Platanus orientalis* leaf extracts have also been used successfully to synthesize stable AgNPs (Song, Kim, 2009).

Pterodon emarginatus Vogel popularly known as “white sucupira”, “faveira”, “faveiro”, “fava-de-Santo-Inácio”, “fava-de-sucupira”, “sucupira”, “sucupira-lisa”, belonging to family Fabaceae (= Leguminosae) and the subfamily Faboideae, is a medium-sized tree that can reach 15 meters, native to the Cerrado areas of Central Brazil, being geographically distributed in the North (Rondônia and Tocantins), Northeast (Bahia, Ceará, Maranhão, Piauí), Midwest (Goiás, Distrito Federal, Mato Grosso and Mato Grosso do Sul) and Southeast (Minas Gerais and São Paulo). With anti-inflammatory, antirheumatic and analgesic properties attributed to it by popular medicine (Dutra, 2008; Lorenzi; Matos, 2008; Miranda *et al*., 2014; Pinto, 2017; Oliveira *et al*., 2019) and, it is cataloged as a medicinal species native to the Midwest region (Fontes, Camillo, Coradin, 2018). All parts of the *P. emarginatus* Vogel plant are used in folk medicine, from the root to the leaves, in the form of infusion and decoction, having as main uses: in the treatment of diabetes, rheumatism, and as an anti-inflammatory (Ferreira, Dantas, Catão, 2014).

Phytochemical analyzes of *P. emarginatus* Vogel showed the presence of alkaloids and terpenoids in the bark, isoflavones and terpenoids in the trunk wood, isoflavones and terpenoids in the sapwood and heartwood, sesquiterpenes, steroids, flavonoids, catechin tannins, flavones and xanthones

in the leaves. In oleoresin obtained from fruits, a wide variety of substances have also been reported, such as furanoditerpene-type terpenoids, sesquiterpenes, vouacapanic skeleton diterpenes, phenolic constituents and flavonoids (Oliveira, 2016).

Considering that the synthesis of nanoparticles by phytochemicals is an area of research with great potential (Wang, Ho, 2009; Mendes, 2015) and that, according to Park *et al*., (2011) currently, sustainability initiatives that use green chemistry to improve and / or protect our global environment are becoming focal issues in many fields of research. Considering the rich Brazilian biodiversity, where some studies in the literature relate the use of endemic plants in the country in the production of AgNPs (Oliveira, 2019) and because there is no published research mentioning the use of aqueous extract of seeds of *Pterodon emarginatus* Vogel (white sucupira) associated with gentamicin sulfate and hyaluronic acid, we opted to use this extract in the synthesis, characterization and research of the activity AgNPs-PE (Nanoparticles of silver associated with the aqueous extract of *P. emarginatus*) and their associations and further investigation of antimicrobial activity.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

The fruits of *P. emarginatus* Vogel were collected by Marcivan Barreto in July 2017, at Serra Boca D 'Água de Uibaí - Irecê Region - Bahia, with the following geographical coordinates: Latitude: 11 ° 20' 24 " South, Longitude: 42 ° 7 '56' 'West. They were identified by Dr. Rosangela Capuano Tardivo from the Department of General Biology - UEPG, Brazil. The fruit and seed samples were deposited at the Carpoteca do Herbário - HUPG of the State University of Ponta Grossa / PR-Brazil under number 22.422.

2.2 OBTAINING THE AQUEOUS EXTRACT OF *P. EMARGINATUS* VOGEL (EAPE)

The aqueous extract was prepared according to the recommendation for popular use (Ferreira, Dantas, Catão, 2014). The sucupira fruits were opened and their seeds were selected and partitioned. 100 mL of ultrapure water was added to a beaker and heated until boiling on a magnetic stirrer with heating, while 2.5 g of seeds were weighed on a semi-analytical balance, when the water boiled the heavy seeds were placed in decoction for 10 minutes. After the time, the decoction was removed from heating and cooled to room temperature. Completely cold, it was filtered with the aid of a glass funnel and qualitative filter paper Whatmann® nº 1 directly into a beaker, after checking the final volume, ultrapure water was added until 100 ml was completed. The extract was then transferred to an identified amber glass bottle and stored under refrigeration, maintained at 4 to 8 ° C, protected from light, and later used as a reducing agent and stabilizer of AgNPs.

2.3 PHYTOCHEMICAL SCREENING

For this analysis, 50 mL of the extract prepared as described above was used and a qualitative research of some secondary metabolites was performed, described in the literature as present in other extracts of *P. emarginatus* Vogel (Matos, 1997; Moreira, 1979).

2.4 PRODUCTION OF AGNPS USING AQUEOUS EXTRACT OF *P. EMARGINATUS* VOGEL (AGNPS-PE)

AgNPs-PE were obtained by adding 9 ml of 2.5% aqueous extract of *P. emarginatus* Vogel seeds, to 1 ml of 1 mM AgNO3. Then the mixture was placed in a water bath (BM) Model Q334 M-24, Quimis, Brazil, at 60-70 ° C, with and without stirring (A). Every 10 minutes, changes in color to brownish yellow were monitored on a UV-Vis spectrophotometer (model NIR VARIAN CARY 50), at wavelengths between 200 and 600 nm, the formation of AgNPs-PE being confirmed at a wavelength of 423 nm.

2.5 PRODUCTION OF AGNPS-PE ASSOCIATED WITH GENTAMICIN SULFATE (AGNPS-PEG)

A 1% gentamicin sulfate stock solution was prepared with ultrapure water in a volumetric flask with a final volume of 25 mL. This mixture was left stirring for 12 h on a magnetic stirrer, protected from light (Model QUIMIS, 235, Diadema, Brazil) without heating and, then stored under refrigeration at 4 to 8 ° C. For the preparation of AgNPs-PEG, 9 ml of synthesized AgNPs-PE were dripped onto the flask containing 1 ml of 1% gentamicin sulfate under agitation and left under agitation, on a magnetic stirrer, protected from light for 18 hours. After the time elapsed, UV-Vis was read to verify the association.

2.6 PRODUCTION OF AGNPS-PEG TO AH2 (AGNPS-PEG-AH2)

A solution of hyaluronic acid (AH2) (15x106 MM), in the concentration of 0.001M was prepared. This solution was sonicated in ultrasound (Sonics-Vibra cell), 530 W, 375 J for 30 min and later stored under refrigeration from 4 to 8° C. To the volume of 5 ml of AgNPs-PEG was added 5 ml of AH2 solution, under magnetic stirring, at 60 ° C for 50 minutes. To the volume of 5 ml of AgNPs-PEG was added 5 ml of AH2 solution, under magnetic stirring, at 60 ° C for 50 minutes.

2.7 UV - VISIBLE SPECTROSCOPY ANALYSIS

The analyzes of the AgNPs-PE and their associations were analyzed using a UV-Vis Nir Varian Cary 50. All samples, without previous dilutions or treatments, were placed in a 1 cm quartz cuvette

with an optical path. All the absorption spectra of the solutions as a function of time were obtained and recorded at wavelengths in the range of 200 to 600 nm.

2.8 DETERMINATION OF THE AVERAGE HYDRODYNAMIC DIAMETER (AHD), ZETA POTENTIAL (ZP) AND POLYDISPERSITY INDEX (PDI)

The size of the nanoparticles, the ZP and the PDI were determined by dynamic light scattering. After preparing a suspension of the nanoparticles (1:20, v / v) in ultrapure water. The measurements, in triplicate, were performed using the Zetasizer Nano series ZS90 equipment (MALVERN INSTRUMENTS, Worcestershire, United Kingdom), with a 90 ° to 25 ° C detection angle.

2.9 FIELD EMISSION GUN SCANNING ELECTRON MICROSCOPY (FEG-SEM) AND ENERGY DISPERSIVE X-RAY SPECTROSCOPY (EDS)

The morphological evaluation and analysis of the surface of the AgNPs-PE and their associations were performed using a field emission scanning electron microscope (TESCAN, model Mira 3, Brno, Czech Republic). The samples were diluted 1:20 v / v and 20 µL of this solution were placed on metallic supports and dried for 24 hours in an oven at 36 ° C. The samples were then metallized with gold in the SC7620 mini sputter Coater; there was no metallization for EDS analysis. To obtain the electromicrographs, an acceleration voltage of 15 kV was used and specific software (Electron Optical Design) was used.

2.10 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

AgNPs-PE and their associations were analyzed by FTIR, using KBr tablets, using 1 mg of each lyophilized sample and 196 mg of KBr spectroscopic grade (2 % m / m), on the IR Prestige 21 equipment (SHIMADZU, Kyoto, Japan), in the range of 4000-400 cm^{-1}, with a resolution of 4 cm^{-1} and 32 $scan.min^{-1}$. The obtained spectra were evaluated to verify the functional groups involved in the formation of the nanoparticles and their associations.

2.11 EVALUATION OF ANTIMICROBIAL ACTIVITY BY THE BROTH MICRODILUTION METHOD

The bacterial suspensions were prepared by adding some colonies of the microorganism to the sterile saline solution, until turbidity equivalent to 0.5 of the McFarland scale (approximately 1.5 x 108 $UFC.mL^{-1}$) was reached. Then the reading was performed on a BTS-330 spectrophotometer (*Byosystems*) at 590 nm to confirm the concentration. All assays were performed in triplicate using relevant health bacteria, strains of *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922,

Escherichia coli GR (Gentamicin Resistant), *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* were tested ATCC 29906, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. The microdilution test was performed in 96-well plates according to the NCCLS (2004) protocol M7-A6 adapted, using 100 µL of BHI broth (brain heart infusion). 100 µL of the AgNPs-PE suspension and its associations were used and the tested concentrations were 5.4; 2.7; 1.35; 0.675 and 0.337 µg/mL, taking into account the silver concentration used in the preparation of the nanoparticles. AgNPs-PEG and AgNPS-PEG-AH2 were tested in the same concentrations above, in relation to the silver mass and in the concentrations: 100; 50; 25; 12.5 and 6.25 µg/ml, referring to the concentration of gentamicin associated with AgNPs-PE. For comparison, a gentamicin solution was tested at concentrations of 1000; 500; 250; 125 and 62.5 µg/ml, a 1mM $AgNO_3$ solution at a concentration of 170 µg/ml was also tested. Then, 10 µL of the microorganism suspension inoculated in saline with a final concentration of approximately $1x10^5$ CFU/mL were added. The plates were incubated at 35 ° C for 24 hours. After 24 hours, to confirm viable microorganisms in non-inhibitory concentrations, 15 µL of the 1% TTC dye (2,3,5 triphenyltetrazolium chloride) was used and waited 30 minutes in an oven at 35 ° C. The minimum bactericidal concentration (CBM) was obtained by sowing the contents of the wells, which did not show color, on Mueller-Hilton agar. After sowing, the plates were incubated at 37 ° C for 24 hours to view growth or not.

3 RESULTS AND DISCUSSION

3.1 PHYTOCHEMICAL SCREENING

The results of the qualitative phytochemical analysis of *P. emarginatus* Vogel are listed in Table 1. It was possible to verify the presence of secondary metabolism compounds found, by other authors, in different types of extracts of *P. emarginatus* Vogel or even of the genus *Pterodon*. Authors such as Rocha and Kaplan (2006), Dutra (2008), Hoscheid and Cardoso (2015), in their research with the genus *Pterodon* demonstrated a composition similar to that found in this work.

Table 1- Identification of groups of secondary metabolism substances present in the 2.5% aqueous extract of *P. emarginatus* Vogel

Classes of substances	Action Pharmacological	Chemical reactions	Results
Tannins	Anti-inflammatory	Basic lead acetate	+
(proanthocyanidins)	Bactericide	Ferric chloride	+
	Fungicide	Lead Acetate	+
		Gelatine	+
Saponins		General reaction	-
Flavonoids	Antitumor	Taubouk reaction	+

(isoflavonoids)	Anti-inflammatory	Pew reaction	+
Fabaceae Family	Antioxidant	1N NaOH	+
	Antiviral	Concentrated HCl	+
Cardiotonic heterosides	Insufficiency	Liebermann-Buchard	+
	Cardiac	Keller-Killiani	+
	Congestive	Kedde	+
		Pesez	+
Alkaloids		Draggendorf, Mayer and Bouchardat	-

Source: The author
Legend: - negative; + positive

The green precipitate in the search for tannins is characteristic of condensed tannins also called proanthocyanidins. Plants rich in tannins are used in traditional medicine to treat various diseases, such as diarrhea, high blood pressure, rheumatism, hemorrhages, wounds, burns, heartburn, nausea, gastritis and gastric ulcer, kidney problems, urinary system, inflammatory processes in general, bactericidal and fungicidal activity. These pharmacological activities are due to the general characteristics common to a greater or lesser degree to the two groups of tannins, condensed and hydrolyzable, they are: 1- complexation with metal ions (iron, manganese, vanadium, copper, aluminum, among others); 2- antioxidant and free radical scavenging activity and; 3- ability to complex with others including macromolecules such as proteins and polysaccharides (Simões *et al*., 2004). Flavonoids have pharmacological importance, for example, antitumor, anti-inflammatory, antioxidant, antiviral, among others. Isoflavonoids, unlike other classes of flavonoids, have their taxonomic distribution restricted to the Fabaceae family, with very few exceptions. The three most important biological properties of isoflavonoids are: estrogenic activity of isoflavones and cumestanes, antifungal and antibacterial activities (phytoalexins) of isoflavonoids and the insecticidal properties of rotenoids. When found in the leaves they can be different from those present in the flowers, in the stems or branches, roots or fruits. (Simões *et al*., 2004). Flavonoids in the form of heterosides are polar constituents, so extraction is more effective with polar solvents (Dutra, 2008).

Cardiotonic heterosides are characterized by their high specificity and powerful action on the heart muscle, it is the class of medication of choice for the treatment of congestive heart failure (Simões *et al*., 2004).

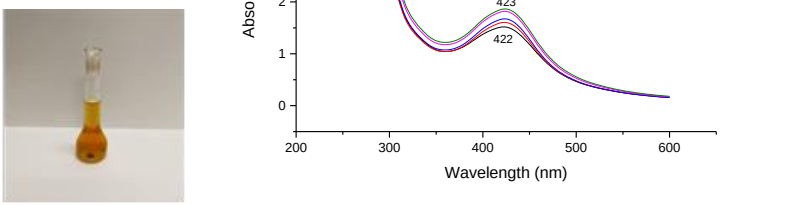
In addition to all the therapeutic activities mentioned, *P. emarginatus* Vogel has great potential as a reducing agent in the green synthesis of AgNPs, due to the wide variety of metabolites with potential redox in its composition. Jafarizad *et al*., (2015) as well as Mendes (2015) in their research with plant extracts and metallic nanoparticles mention that tannins, flavonoids and phenolic acids found in their extracts because they have functional groups such as hydroxyl act as reducing agents and, also

benzyl rings and carboxyl groups act as stabilizing agents. Katas *et al*., (2019) also mentions that mushroom extracts contain constituents such as terpenoids, tannins, polysaccharides, carbohydrates, phenolic compounds and flavonoids can potentially reduce agents in the biosynthesis of metallic nanoparticles.

3.2 PRODUCTION OF AGNPS BY MEANS OF AQUEOUS EXTRACT OF P. EMARGINATUS VOGEL SEEDS (AGNPS-PE)

The formation of AgNPs-PE was observed by changing the color from straw yellow to brown or reddish brown, which is due to the reduction of the metallic salt ($AgNO_3$) by the biomolecules present in the EAPE (aqueous extract of *P. emarginatus*), by the plasmonic surface effect presented by silver nanoparticles when exposed to visible light. The nanoparticles obtained through reduction by plant extracts are mostly spherical, but through other chemical agents they can present varied shapes such as cubes, triangles, sticks and even dendrites (Sena, *et al*., 2019). In the analysis of Uv-Vis at different reading times (10 to 50 minutes) the highest absorption in UV-Vis was at 423 nm as shown in (Fig. 1). These spectral bands are in agreement with Oliveira (2018) who found results between 400-425 AgNPs of aqueous extract of leaves of *P. emarginatus.*

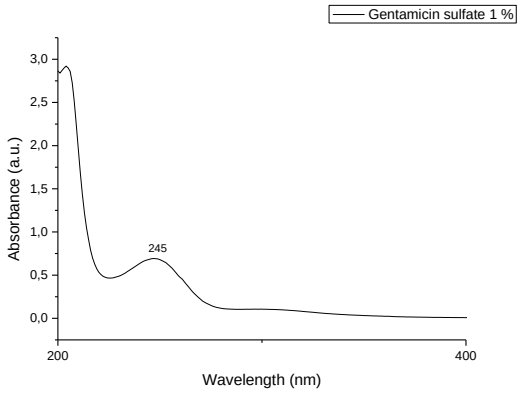
Fig. 1- UV-Vis spectra of AgNPs-PE, with readings every 10 minutes until complete reduction.



According to Solomon *et al*. (2007) spectroscopy at wavelengths from 420 to 435 nm is consistent with AgNPs of size around 35 to 50 nm and at wavelengths from 438 to 450 nm, AgNPs have sizes between 60 to 80nm. It is known that the width of the bands is related to the size distribution of the NPs, while the maximum wavelength of the SPR band is related to the average size of the NPs, and longer wavelengths are indicative of longer NPs (Mendes, 2015). The result obtained in the synthesis of AgNPs-PE indicates the formation of homogeneous sized nanoparticles and, within the wavelengths cited by Ashraf *et al*. (2016) who says that the surface plasmon resonance for silver occurs

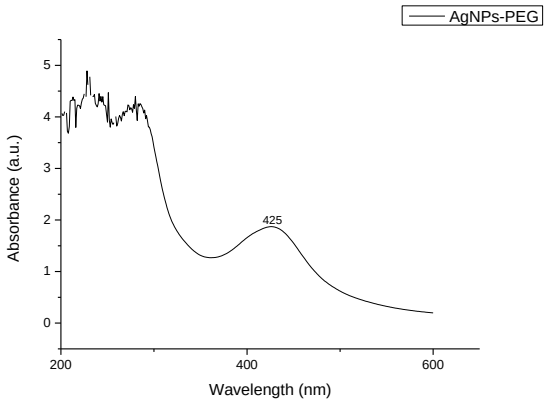
between 400 and 500 nm. And, according to Sena *et al*. (2019) this monomodal curve pattern shows the obtaining of nanoparticles in spherical shape and, according to Mendes (2015) with uniformity in size. AgNPs-PE when combined with gentamicin sulfate and prepared with heating in an AgNPs-PEG water bath, showed an absorption peak at 425, quite close to the absorption peak of AgNPs-PE. A (Fig. 2) shows the UV-Vis spectrum of gentamicin sulfate, with an absorption peak at 245 nm.

Fig. 2- UV-Vis spectrum of 1% gentamicin sulfate



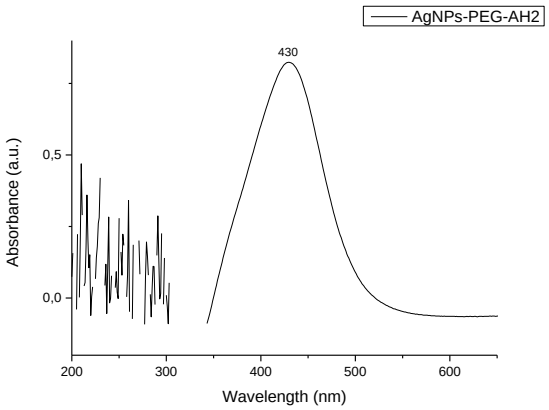
This peak is no longer seen in the spectrum of AgNPs-PEG (Fig. 3), which may be indicative of the association of AgNPs-PE with 1% gentamicin sulfate for the formation of AgNPs-PEG.

Fig. 3- UV-Vis spectrum of AgNPs-PEG



In (Fig. 4) we observe the UV-Vis absorption spectrum at 430 nm of AgNPs-PEG associated with 0.001M hyaluronic acid (AH2). The AgNPs-PEG-AH2 showed a slight shift in the wavelength, with the peak of absorption at 430 nm, we believe that due to the association with 0.001M hyaluronic acid. This association with hyaluronic acid is due to its use in viscosupplementation of synovial fluid in patients with osteoarthritis and gentamicin sulfate to be used together with HA in the treatment of osteomyelitis.

Fig. 4- UV-Vis spectrum of AgNPs-PEG-AH2.



3.3 DETERMINATION OF THE AVERAGE HYDRODYNAMIC DIAMETER (AHD), ZETA POTENTIAL (ZP) AND POLYDISPERSITY INDEX (PDI)

In Table 2 presents the results of the average hydrodynamic diameter (AHD), zeta potential (ZP) and polydispersity index (PDI), as well as the average diameter obtained by the FEG and the absorption peaks of the AgNPs-PE and their associations obtained. A great difference can be observed in the values of the average hydrodynamic diameters (AHD) and those measured in the FEG images; possibly due to different sample conditions. The AHD measurement is performed by light scattering, so that particles that are agglomerated promote greater dispersion. Furthermore, the determination of the AHD of the particles in suspension by scattering light results from the Brownian motion of these particles. When a particle is dispersed in a liquid medium, a solvation layer forms on its surface, which makes this data overestimated when compared, for example, with microscopy techniques (FEG), which measure the diameter of the dry particles individually (Albernaz, 2014; Katas *et al*., 2019). The AHD was affected by the environment that involved the NP and were calculated starting from an isotropic spherical particle. Therefore, these data represent the particle diameter and not the actual particle size, which must be determined by other techniques, for example, Field Emission Scanning Electron Microscopy (FEG-SEM) (Lopes, 2017). AgNPs-PEG-AH2 had the largest diameter, by both techniques employed (AHD and FEG-SEM), probably due to the large association of compounds. These results supported the UV-Vis, where a bathochromic shift in the wavelength was observed, shown in the graph curve, indicating an increase in size, in addition to presenting an average polydispersity index, evidenced by the PDI value. The PDI is a parameter used to assess the size distribution of NPs, this value varies from 0 to 1 and, the smaller, the more monodispersed and, consequently, the more homogeneous will be the nanoparticles (Albernaz, 2014; Sena *et al*., 2019). For Lopes (2017), a value of PDI > 0.7 means a high particle size distribution. According to these references, all samples analyzed are within an IP that is average for the distribution of NPs. The Zeta

Potential (ZP) was used to evaluate the stability of the nanoparticles. Most particles, when in contact with a liquid, acquire an electrical charge on their surface and this potential is called ZP. The "charge" of the colloidal particle is actually an effective charge, resulting from the balance of ion and counter ion charges. ZP correlates with electrostatic repulsion between particles and with the stability of colloidal systems and, the higher the ZP value, the more stable the colloid will be, that is, the speed of formation of the aggregates increases as the ZP is closer to zero. When the value of the Zeta Potential is less than - 30 mV or greater than + 30 mV, the particle will be stable (Lopes, 2017). All NPs obtained showed negative ZP, this can be attributed to the presence of polyphenols, flavonoids and proteins from the plant extract. The values obtained varied between - 27 to - 39 mV, which indicates a good degree of stability. According to Ferreira (2016), a colloidal suspension with a value greater than 25mV (positive or negative) is considered stable. Colloids with low zeta potential tend to flocculate or coagulate, as they are not electrically stabilized. The metallic NPs have a very small electrostatic potential and, according to the Theory of Derjaguin, Landau, Verwey and Overbeek (Theory DLVO), the greater the Zeta Potential, the colloidal dispersion is more likely to be stable, as the charged particles repel each other and this force exceeds the Intermolecular Forces (Van der Waals Forces), which are responsible for aggregation. The addition of AH2 may have acted as a stabilizing agent that protected AgNPs-PE (steric stabilization) and increased its stability in water. The stability of AgNPs-PE in aqueous solution occurs because the macromolecule adsorbs on the surface of the formed nanoparticles and provides steric repulsion (Lopes, 2017).

Table 2- Average values of Hydrodynamic Diameter (AHD), Zeta Potential (ZP), polydispersity index (PDI), diameter by FEG-SEM and UV-Vis wavelength of AgNPs-PE, AgNPs-PEG and AgNPs- PEG-AH2

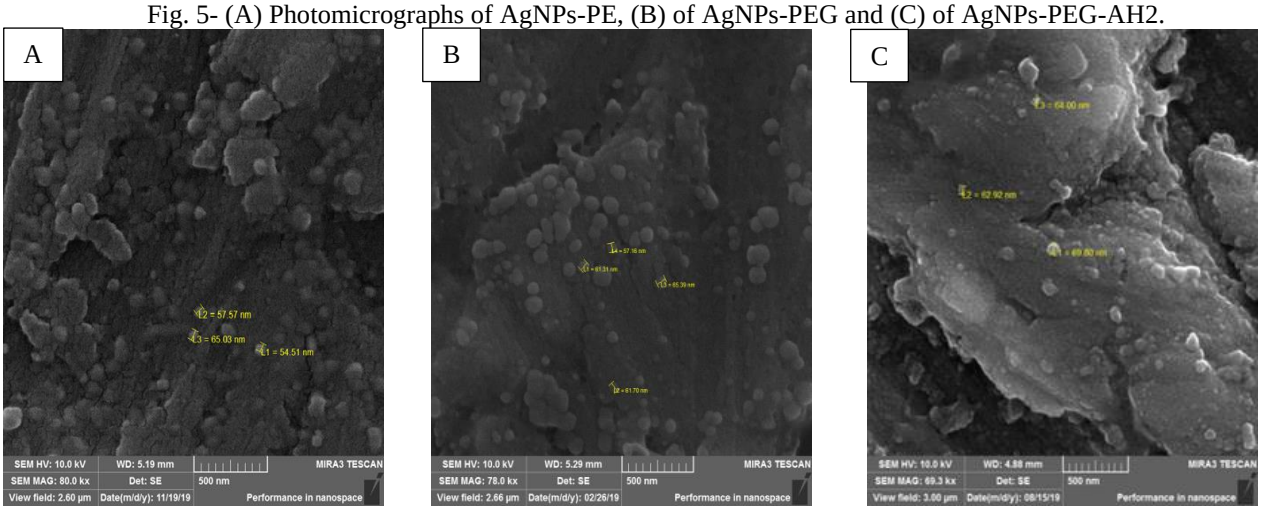
Sample	AHD nm (mean ± SEM)	ZP mV (mean ± SEM)	PDI (mean ± SEM)	FEG-SEM nm (mean ± SEM)	UV-Vis nm (mean ± SEM)
AgNPs-PE	120,37 ± 1,39	- 36,17 ± 1,40	0,332 ± 0,076	59,57 ± 5,41	423 ± 1,53
AgNPs-PEG	132,63 ± 2,25	- 39,73 ± 1,47	0,339 ± 0,070	62,72 ± 2,25	425 ± 1,53
AgNPs-PEG-AH2	134,33 ± 1,85	- 27,15 ± 1,15	0,524 ± 0,091	65,56 ± 3,70	430 ± 1,00

Source: The author

3.4 FIELD EMISSION SCANNING ELECTRON MICROSCOPY (FEG-SEM) OF AGNPS-PE, AGNPS-PEG AND AGNPS-PEG-AH2

AgNPs-PE and their associations were spherical in shape, the average size was around 59 nm for AgNPs-PE, 63 nm for AgNPS-PEG and around 66 nm for AgNPs-PEG-AH2 as shown in (Fig.5).

Fig. 5- (A) Photomicrographs of AgNPs-PE, (B) of AgNPs-PEG and (C) of AgNPs-PEG-AH2.



SOURCE: The author

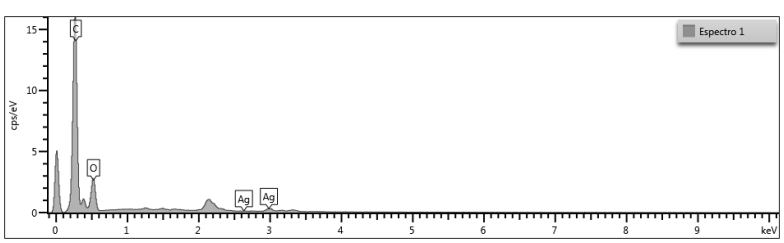
In order to obtain nanoparticles with uniform size distribution, it is necessary that all nuclei be formed at the same time. Once formed under the same conditions, the nuclei are very close in size and grow uniformly (Ferreira, 2016). The absence of agglomerates and dimers was also confirmed by the absorption plasmon band as seen in Figures 1, 2 and 4), because if these phenomena occurred a new plasmon band with a localized surface would be present, the result of the interaction of two or more silver nanoparticles together and in contact. The small particle agglomeration that appears in Fig. 5 (A), (B) and (C) can be attributed to the water drying process in the metallic support and the presence of AH, which provided the coalescence of the nanoparticles (Lopes, 2017).

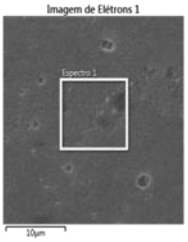
4 ENERGY DISPERSION X-RAY SPECTROSCOPY (EDS)

Energy dispersion X-ray spectroscopy (EDS) is a qualitative and quantitative microanalytical technique that provides information on the chemical composition of the sample. Once the electron beam is focused on the sample, the outermost electrons and ions change their energy level, however, upon returning, the outer electrons and ions change their energy level, however, when they return, there is the release of energy that can be measured through the detector, in the form of spectra (histograms) in which individual elements can be identified. The electrons of each atom have different energies, making it possible to determine which chemical elements are present in the samples, since the EDS spectra correspond to X-ray lines characteristic of a specific element. Thus, the characteristic energies of X-rays allow the identification of the sample elements, while the peak intensity allows the quantification of the concentration of the sample elements (Mendes, 2015). The presence of pure Ag in the sample was confirmed by EDS, at approximately 3 Kev, as can be seen in Fig. 6, as was also demonstrated by Agarwal, Agrawal, Singh (2014); Banala, Nagati, Karnati (2015); Mendes (2015) and thus confirming the biosynthesis of AgNPs. The O, N peaks correspond to the surroundings of the

AgNPs-PE, that is, the components of the plant extract used in the synthesis of the NPs and the C peak of the support used to fix the sample (Mendes, 2015).

Fig. 6- X-ray energy dispersion spectroscopy of AgNPs-PE with histogram and silver quantification





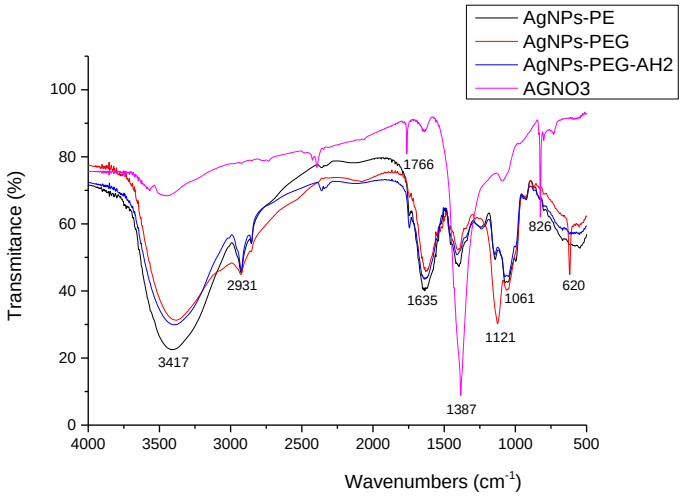
Element	Weight %
C	77,93
O	11,5
N	9,6
Ag	0,97
Total:	100

SOURCE: The author

4.1 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

In (Fig.7) shows Fourier Transform Infrared Spectroscopy (FTIR) of silver nitrate and AgNPs-PE and their associations. The bands at 1380 and 833 cm^{-1} refer to the vibration of the NO3 anion of silver nitrate, with the peak 833 cm^{-1} being suppressed in AgNPs-PE and associations. Such spectral changes with suppression or manifestation of peaks are suggestive of bioreduction of silver. The peak at 1380 cm^{-1} in the silver nitrate solution is very characteristic of nitro groups - NO_2 (1380-1300 cm^{-1}). Its appearance, in less intensity, in the samples of AgNPs-PE and associations indicates that the functional groups of some metabolite of the extracts were associated with silver Ag^+ ions, modifying the connection profile and possibly promoting the reduction in Ag^0 (Oliveira, 2018). We can see broadband in the 3417 cm^{-1} region, this band is characteristic of OH present in alcohols and phenolic compounds or NH_2 group, even from the water itself, which may have a sample origin or from the KBr itself because it is highly hygroscopic (Banala, Nagati, Karnati, 2015; Oliveira, 2018). It is important to remember that as the extracts are composed of several bioactive components, it is not possible to say for sure which active ingredients are directly related to the process of bioreduction and stabilization of AgNPs-PE and associations. This requires further studies to investigate the nature of the extracts molecules involved in these mechanisms (Oliveira, 2018).

Fig. 7- Fourier Transform Infrared Spectroscopy (FTIR) of silver nitrate and AgNPs-PE and their associations.



4.2 EVALUATION OF ANTIMICROBIAL ACTIVITY BY THE BROTH MICRODILUTION METHOD

The results obtained coincided with the literature in the field of nanoparticles and certainly provide proof about the synthesis of AgNPs-PE and its efficiency as an antibacterial agent (Banala, Nagati, Karnati, 2015). The antibacterial nature of silver is enhanced in the form of nanoparticles. AgNPs are cited in several studies as they have antibacterial activity against various species of bacteria, they have the ability to penetrate the bacterial cell wall and damage the cell membrane leading to cell death (Banala, Nagati, Karnati, 2015; Katva *et al*., 2017; Lopes, 2017). According to Fernandes (2010), no significant difference was found in the antimicrobial activity of AgNPs over the different groups tested (Gram-positive versus Gram-negative and resistant versus susceptible antimicrobials), suggesting that AgNPs have a broad spectrum bactericidal effect.

Li *et al*. (2005) and also Lopes (2017) believe that the association of metallic NPs with antibiotics increases their action and reduces the resistance of bacteria to them; they suggest that the change caused in the cell and nuclear wall (DNA / RNA) by NPs is the main cause for the retardation of the growth of bacterial cells. The AgNPs synthesized in the study by Vu *et al*. (2018) exhibited strong antibacterial activity against *E. coli* and *S. aureus*. Katva *et al*. (2017) noted in their research that when AgNPs were used in combination with gentamicin they showed an increased antimicrobial effect. In their study Jamaran and Zarif (2016) found a synergistic antibacterial effect of the combination of AgNPs and gentamicin or neomycin in isolates of *S. aureus* from mastitis. In their research Saeb *et al*., (2014) found that AgNPs associated with Gentamicin demonstrated antimicrobial activity against P. aeruginosa major compared to Gentamicin, demonstrating a clear synergistic effect between AgNPs and Gentamicin. AgNPs associated with hyaluronic acid showed potent antimicrobial activity against *S. aureus* and modest activity against *E. coli* in the study by Kemp *et al*., (2009).

Together, AgNPs synthesized by HA have antibacterial activity and biocompatibility that makes them good candidates for use in medical applications (Park *et al*., 2011).

Several studies on the production of AgNPs, through green synthesis, have shown promising results against microorganisms, to name a few: Sasirekha *et al*., (2018) observed in his study in the synthesis of AgNPs through the aqueous extract of the fruit of *Carica papaya* that it exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*.

The report by Kalpana *et al*., (2018) that describes the synthesis of AgNPs using aqueous extract of *Carica papaya* demonstrated high efficiency against the bacteria *S.* aureus *and P. aeruginosa*. The use of *O. gratissimum* leaf extract by Das *et al*., (2017) where antibacterial activity was tested against resistant *E. coli* strains, AgNPs showed superior efficacy to antimicrobials. The biosynthesized AgNPs, in the research by Anandalakshm, Venugobal and Ramasamy (2016), through the extract of the leaves of *Pedalium murex* revealed a pronounced antibacterial activity against *E. coli*, *K. pneumoniae*, *M. flavus*, *P. aeruginosa*, *B. subtilis*, *B. pumilus* and *S. aureus*. In the green synthesis of AgNPs by the aqueous extract of the leaves of *Arbutus andrachne* in research carried out by Erdoğan *et al*., (2016) these AgNPs associated with the extract exhibited antimicrobial activity against strains of bacteria Gram-negative and Gram-positive *S. aureus*, *S. epidermidis*, *E. coli*, *K. pnemoniae*, *P. aeruginosa* as well as antifungal activity against *C. albicans* and *C. krusei*. The antibacterial activity of AgNPs synthesized from the residues of vegetable peels such as *L. siceraria*, *L. cylindrica*, *S. lycopersicum*, *S. melongena* and *C. sativus* was performed by Sharma, Kaushik and Jyoti (2016), against two Gram-negative bacteria, *E. coli* and *K. pneumoniae* where the results showed a strong antibacterial potential. *Averrhoa carambola* fruit extract was used for AgNPs synthesis by Gavade *et al*., (2015) and showed bactericidal activity against *E. coli* and *P. aeruginosa* by the agar diffusion method. The extracts of *Cassia auriculata* and *Ocimum sanctum* formed AgNPs of small size with significant antimicrobial activity against *E. coli* and *A. niger* according to the report by Choudhary, Bhamare and Mahure (2014).

As can be seen in Table 3, the EAPE did not show antimicrobial action against any of the tested strains, which is in agreement with Oliveira (2018) who used aqueous extract of leaves of *P. emarginatus* against the strains of *S. aureus*, *S. epidermidis* and *E. coli* and also had no antimicrobial action in the tested samples. Compared to the positive control, Gentamicin, AgNPs-PE and their associations showed bactericidal activity at lower concentrations against all tested bacteria, demonstrating a synergistic effect. Both AgNPs-PE, AgNPs-PEG and AgNPs-PEG-AH2 exerted bactericidal activity against the bacterium *E. coli* RG. In addition, no antimicrobial activity was observed in EAPE, which did not have NPs, showing that antimicrobial activity is directly related to AgNPs-PE and their associations. According to Banala, Nagati, karnati, (2015), Lopes (2017) and also Oliveira (2018) the AgNPs for having small particle size can adhere and cross the bacterial cell wall

and then, AgNPs can be oxidized to form metal ions Ag^+, a Lewis "soft" acid ($\eta = 6.8$) that has a natural tendency to react with bases. The cells are composed of sulfur and phosphorus atoms, which are Lewis "soft" bases. Therefore, the interaction between the metal Ag^+ ions and the amino acids present in the bacteria cells was of the Lewis acid / base type. This strong interaction leads to cell damage and cell death. Another factor to be considered is the presence of sulfur and phosphorus in the constitution of DNA; Ag^+ ions may also have interacted with these "soft" Lewis bases and destroyed DNA, which would have caused cell death. The interaction between silver and the sulfur / phosphorus atoms present in the DNA can lead to problems in the replication of the bacteria's DNA and cause the death of these microorganisms. The interaction of NPs with the cell wall of bacteria, DNA and enzymes can cause an imbalance in cell metabolism, causing the induction of a high level of reactive oxygen species (ROS). As well as they can result in damage to the membrane with formation of pores, leakage of intracellular content, loss of viability, lysis and consequently cell death. However, the presence of the extract, gentamicin sulfate, hyaluronic acid associated with AgNPs can influence this process of interaction with the surface of bacteria, so the influence of this bionanointerface on toxicity needs to be elucidated. We observed that there was a greater synergistic effect in the associations of AgNPs-PEG and AgNPs-PEG-AH2 for all bacteria and yeast tested than only with AgNPs-PE. This synergistic effect found in this research, suggests that the combination of AgNPs-PEG and AgNPs-PEG-AH2 may act in the treatment of these microorganisms in a more consistent way than only Gentamicin or AgNPs-PE alone. The exact mechanism of this action is still unclear; however several mechanisms have been proposed. It has been reported that, compared to isolated AgNPs the combination / association of antibiotic + AgNP complexes will release Ag^+ at a higher rate, furthermore, it has also been proposed that the combination of antibiotic with AgNPs through active groups of antibiotics, as hydroxyl group and amine group will result in the conjugation of both molecules, resulting in an increase in the effective concentration of the antibiotic in a specific location (Katva *et al.*, 2017).

Table 3 - Antimicrobial activity of EAPE, AgNPs-PE, AgNPs-PEG, AgNPs-PEG-AH2, gentamicin sulfate 1% and AgNO3 1mM against Gram-positive, Gram-negative bacteria and yeast

Microorganisms	EAPE (µg/mL)	AgNPs-PE (µg/mL)	AgNPs-PEG (µg/mL) Ag + G	AgNPs-PEG-AH2 (µg/mL) Ag + G	Gentamicin sulfate (µg/mL)	$AgNO_3$ 1mM (µg/mL)
E. faecalis	NAA	5,4	1,35 + 25	1,35 + 25	250	170
E. coli	NAA	5,4	0,675 + 12,5	0,675 + 12,5	125	170
E. coli RG	NAA	5,4	2,7 + 50	2,7 + 50	NAA	170
K. pneumoniae	NAA	5,4	0,675 + 12,5	0,675 + 12,5	125	170
P. mirabilis	NAA	5,4	0,675 + 12,5	0,675 + 12,5	125	170
P. aeruginosa	NAA	5,4	1,35 + 25	1,35 + 25	250	170

S. aureus	NAA	5,4	1,35 + 25	1,35 + 25	250	170
C. albicans	NAA	5,4	0,675 + 12,5	0,675 + 12,5	125	170

SOURCE: The author

NAA: there was no action; *E. coli* RG: Gentamicin resistant *E. coli*; EAPE: aqueous extract of *P. emarginatus* Vogel; AgNPs-PE: silver nanoparticles associated with EAPE; AgNPs-PEG: silver nanoparticles associated with EAPE and gentamicin sulfate; AgNPs-PEG-AH2: silver nanoparticles associated with EAPE with gentamicin sulfate and hyaluronic Acid; AgNO3: silver nitrate; Ag + G: silver concentration + gentamicin sulfate concentration

5 CONCLUSION

In this experiment we managed to produce colloidal dispersions of AgNPs by the green synthesis method with aqueous extract of seeds of *P. emarginatus* Vogel, where there was a bioreduction of Ag^{+} to Ag^{0}, demonstrated by the analysis techniques employed. This methodology proved to be simple and fast, easily reproducible and, even allowed to associate compounds such as gentamicin sulfate and hyaluronic acid, enabling microbiological tests against Gram-positive, Gram-negative and yeast bacteria, where we obtained synergistic effects when the associated AgNPs-PE were tested against all tested bacteria and yeast. The exact mode of bactericidal action remains debatable, however, these observations show the potential of the associated AgNPs-PE against the bacteria tested, there is a possibility of pharmaceutical application, after elucidating the chemical reactions involved in the synthesis to optimize the process and seek greater yield and stability.

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