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Chemistry

Antioxidant potential and synthesis of copper oxide nanoparticles from plant extracts of *Piper amalago* L. (Piperaceae)

Potencial antioxidante e síntese de nanopartículas de óxido de cobre a partir de extratos vegetais de *Piper amalago* L. (Piperaceae)

Mayara Cristina Neves Abel[I], Etenaldo Felipe Santiago[I], Ana Francisca Gomes da Silva[II], Jonas da Silva Mota[I], Montcharles da Silva Pontes[I]

[I]Universidade Estadual de Mato Grosso do Sul, Dourados, MS, Brazil
[II]Universidade Estadual de Mato Grosso do Sul, Mundo Novo, MS, Brazil

ABSTRACT

Piper amalago L. popularly known as jaborandi-manso, jaborandi-falso and jaborandinhandi is widely used in the Brazilian folk medicine for treating urinary calculus diseases. The objective of this study was to evaluate the antioxidant activity and determine the levels of phenols, flavonoids and total tannins of the ethanolic and aqueous extracts of *P. amalago* leaves, as well as to synthesize and characterize copper oxide nanoparticles using the aqueous extract, in addition to evaluate its antioxidant activity. Quantification of total phenols, flavonoids and tannins was performed by spectrophotometry in the visible region and the antioxidant activity was evaluated by the DPPH$^{\cdot}$ free radical scavenging method. The synthesis of nanoparticles was prepared with a solution containing aqueous extract of the leaves and copper salt. Subsequently, the characterization was performed by microscopy and FTIR and UV-Vis spectroscopy. Both extracts showed antioxidant activity; the ethanol extract was the most active, reducing the DPPH$^{\cdot}$ at a concentration of 100 μg.mL^{-1} by more than 90%. This extract also presented the highest levels of total phenols (199.17 ± 4.11mg GAE/g extract), total flavonoids (30.84 ± 0.59 mg QE/g extract) and total tannins (158.30 ± 4.83 mg TAE/g extract). The generated nanoparticles showed no great capacity to reduce DPPH$^{\cdot}$. The results suggest that the antioxidant potential evidenced by *P. amalago* extracts is mainly related to the presence of phenolic compounds, such as flavonoids and tannins, recognized as antioxidants and confirmed the green synthesis of copper oxide nanoparticles, but with the lowest antioxidant potential.

Keywords: Phenolic compounds; Free radicals; Green synthesis

RESUMO

Piper amalago L. conhecida popularmente como jaborandi-manso, jaborandi-falso e jaborandinhandi é muito utilizada na medicina popular brasileira no tratamento de doenças do cálculo urinário. Objetivou-se com este trabalho avaliar a atividade antioxidante e determinar os teores de fenóis, flavonoides e taninos totais dos extratos etanólico e aquoso das folhas de *P. amalago,* bem como sintetizar e caracterizar nanopartículas de óxido de cobre utilizando o extrato aquoso, além de avaliar sua atividade antioxidante. A quantificação de fenóis, flavonoides e taninos totais foi realizada por espectrofotometria na região do visível e a atividade antioxidante avaliada pelo método de sequestro do radical livre DPPH$^{\bullet}$. A síntese das nanopartículas foi preparada com uma solução contendo extrato aquoso das folhas e sal de cobre e a caracterização foi realizada por microscopia e espectroscopia de FTIR e UV-Vis. Ambos os extratos apresentaram ação antioxidante, sendo o extrato etanólico o mais ativo reduzindo em mais de 90% o DPPH$^{\bullet}$ na concentração de 100 µg.mL^{-1}. Este extrato foi também o que apresentou os maiores teores de fenóis totais (199,17 ± 4,11mg de EAG/g), flavonoides totais (30,84 ± 0,59 mg de EQ/g) e taninos totais (158,30 ± 4,83 mg de EAT/g). As nanopartículas geradas não apresentaram a menor ação de redução de DPPH$^{\bullet}$. Os resultados sugerem que a potencialidade antioxidante evidenciada pelos extratos de *P. amalago* está relacionada, principalmente à presença de compostos fenólicos, como flavonoides e taninos e confirmaram a síntese verde das nanopartículas de óxido de cobre, porém com o menor potencial antioxidante.

Palavras-chaves: Compostos fenólicos; Radicais livres; Síntese verde

1 INTRODUCTION

Nanoparticles (NPs) can be synthesized by several chemical methods. Traditionally, the vast majority of techniques used for synthesizing metallic nanoparticles (NPMs) involve toxic solvents and the generation of waste that is harmful to health and the environment, limiting their use in various circumstances (SILVA *et al*., 2017).

A promising approach is to explore the wide range of biological resources that are available in nature through the so-called green synthesis, a common name given the synthesis that uses less toxic, biodegradable and low-cost chemicals to synthesize nanomaterials, having as a primary source a biological organism or parts thereof (MITTAL *et al*., 2013; SHUKLA *et al*., 2012). Different sources of plant extracts can be used for green synthesis, however, the synthesis of NPs from plant extracts from species with potential biopesticide use, such as *Piper amalago* L., implies a pioneering study.

Piperaceae is one of the largest families of Angiosperms with about 3,615 species

distributed pantropically, where the genera *Piper* L., *Peperomia* Ruiz & Pav., *Manekia* Trel., *Zippelia* Blume and *Verhuellia* Miq are currently accepted. (CHRIST, 2016). The genera *Peperomia* Ruiz & Pav., *Manekia* Trel., *Piper* L. and *Ottonia* Spreng. are recognized for Brazil, with the latter two continuing to be synonymized (BFG, 2015).

Among the genera belonging to the family Piperaceae, the genus *Piper* has numerous species used in food, as insecticides and also for medicinal purposes, as they present a great diversity of bioactive secondary metabolites (ARAÚJO *et al*., 2014) with chemical properties for insecticide, pesticide (PITON *et al*., 2014; SILVA *et al*., 2018), antioxidant (SILVA *et al*., 2014), antibacterial and antifungal (AVILA *et al*., 2018; MORANDIM-GIANNETTI *et al*., 2010).

Piper amalago L. is popularly known as jaborandi-manso, jaborandi-falso and jaborandinhandi. Traditionally, it is used for the treatment of skin, wounds, burns, as an analgesic, diuretic and in the treatment of urinary calculus (BRATTI *et al*., 2013; NOVAES *et al*., 2014). Several studies on the chemical and biological characterization of *P. amalago* essential oils are described in the literature (SANTOS *et al*., 2016; DOS SANTOS *et al*., 2018), however, there are few describing their extracts and isolated compounds.

Rovani *et al*. (2013) recorded the presence of different organic groups, in addition to vitexin and lupeol substances, and the extract showed antioxidant properties. The obtained extract and amides showed antinociceptive, antihyperalgesic and antiarthritic activities (ARRIGO *et al*., 2016). In another study, two amides isolated from the extract of the species showed antileishmanial activity (CARRARA *et al*., 2013). Novaes *et al*. (2014) achieved positive biological results with the extracts, as they showed diuretic and natriuretic activity, in addition to antilithiasis effects.

Thus, considering the potential as a source of bioactive compounds from the secondary metabolism of *Piper* species, the objective of this study was to evaluate the antioxidant activity and determine the levels of total phenolic compounds in the ethanolic and aqueous leaf extracts of *P. amalago*, as well as synthesize and characterize copper oxide nanoparticles, using the aqueous extract.

2 MATERIAL AND METHODS

2.1 Collection and identification of plant material

The leaves of *P. amalago* were collected in the municipality of Dourados, by the morning. The plant material collected on May 2016 was used for ethanol extraction and the one on Jun 2019 used for aqueous extraction, both located in the following geographic coordinates, S22O12'42.9", W 54O 54'55.6. The species was previously identified (DDMS 4412). Species collection was registered in SisGen (A3C3205). The collected plant material was dried at room temperature for three weeks, and then ground in a knife mill.

2.2 Preparation of extracts

2.2.1 Ethanol extract

Ethanol 700 INPM (4 L) was added to ground leaves (500 g) and the mixture remained at rest for 7 days and then filtered through cotton, the liquid part was concentrated in a rotary evaporator and dried in a hood. This procedure was repeated three times, yielding 50 g ethanol extract.

2.2.2 Aqueous extract

Water at 98°C (2 L) was added to ground leaves (200 g), after 30 minutes of rest, the extract was filtered through cotton. The liquid portion was partially concentrated in a rotary evaporator and subsequently lyophilized, yielding 15 g aqueous extract.

2.3 Synthesis of copper oxide nanoparticles (CuONPs)

Nanoparticles were produced following the methodology of Sankar *et al*. (2014), in which fresh *P. amalago* leaves were dried and ground. Subsequently, 10 g powder was added to 100 mL distilled water in an erlenmeyer flask, and placed in an ultrasonic bath at 60 °C for 10 minutes. After this, at room temperature, the extract was filtered

through filter paper. For the copper oxide nanoparticle synthesis, 90 mL of 5 mM cupric sulfate ($CuSO_4$ $5H_2O$) was mixed with 10 mL leaf extract and allowed to stand at room temperature until color change. Then, obtained the precipitate was purified by repeated centrifugation at 12000 rpm for 20 minutes. Subsequently, it was taken to the electron microscope for characterization.

2.3.1 Characterization of nanoparticles

Scanning electron microscopy (SEM) was used to determine the morphology and size distribution of CuONPs. The nanoparticle suspensions were diluted (1:100), dried on silicone grids, and analyzed using an EVO-LS-15 scanning electron microscope (Carl Zeiss, Germany). SEM images were captured by operating at 15 kV high voltage with a spot size between 3.0-4.0 and a working distance (WD) of 8.5 mm. The CuONPs size distribution histogram was constructed from approximately 100 nanoparticles using ImageJ software. To check the homogeneity or heterogeneity of particle size, the Kernal density map was generated. Particle size distribution (hydrodynamic diameter) and zeta potential were studied using dynamic light scattering (DLS, ZetasizerNano, Malvern Instruments, Inc.). For this, nanoparticles were diluted 10.000 times, and the samples were analyzed in triplicate.

After synthesis, the spectra in the Ultraviolet-Visible (UV-Vis) region of the aqueous plant extract, copper sulfate, and copper oxide nanoparticles were evaluated. Spectra were obtained at room temperature. The scanned spectral region was 200 - 800 nm. Fourier transform infrared spectroscopy (FTIR) was performed using a spectrophotometer Nexus 670 Thermo Nicolet in the spectral range from 400 to 4000 cm^{-1}, with photoacoustic detection mode. FTIR spectrophotometer was purged with dry air to remove water vapor, and the photoacoustic cell was purged with helium gas. The resolution was 8 cm^{-1} and 128 scans were performed, carbon black was used as reference.

2.4 Total Phenols

The content of total phenols was determined by the Folin-Ciocalteau spectrophotometric method (BONOLI *et al*., 2004). Each extract was dissolved in methanol to obtain a 1 $mg.mL^{-1}$ concentration solution. To a 1.0 mL aliquot of this solution was added Folin-Ciocalteu reagent (2.5 mL), then the solution was stirred for 1 minute and made up to 10 mL with 7.5% sodium carbonate. After 1h and 30 minutes, the absorbance of the samples was measured at 750 nm in a Tecnal UV/vis spectrophotometer. The total phenol content was determined by interpolating the absorbance of the samples against a calibration curve constructed with gallic acid standards (15.625 to 250 µg.mL-1; y = 0.0105x + 0.091; R2 = 0.9966). The analyzes were performed in triplicate, with the results expressed as mg of gallic acid equivalent (GAE) per g of sample.

2.5 Total Flavonoids

The determination of the total flavonoid content was performed by the colorimetric method with aluminum chloride (LIN; TANG, 2007). An aliquot of 0.2 mL of 10% aluminum chloride (AlCl3) was mixed with 1 mL of the sample solution (1 $mg.mL^{-1}$) and made up to 10 mL with distilled water. After resting for 30 minutes, the reading was performed in a UV/vis Tecnal spectrophotometer at 420 nm. The content of total flavonoids was determined using a standard curve of quercetin at concentrations from 15.625 to 250 $\mu g.mL^{-1}$ and the equation of the obtained curve was y = 0.007x + 0.0102; R2 = 0.999. Analyzes were performed in triplicate and results expressed in mg of quercetin equivalent (QE) per g of sample.

2.6 Total tannins

The dosage of total tannins was performed by the Folin-Denis spectrophotometric method (PANSERA *et al*., 2003). To a 1.0 mL aliquot of sample solution (1 $mg.mL^{-1}$) was added Folin-Denis reagent (0.5 mL), then the solution was stirred for 1 minute and 0.5

mL of sodium carbonate added 7.5%. Subsequently, the volume was made up to 10 mL with distilled water. After 30 minutes the absorbance of the samples was measured at 725 nm in a Tecnal UV/vis spectrophotometer. The total tannin content was determined using a calibration curve constructed with tannic acid standards (15.625 to 500 $\mu g.mL^{-1}$). The curve equation was y = 0.0054x + 0.1855; R2 = 0.9977. Analyzes were performed in triplicate, with results expressed as mg of tannic acid equivalent (TAE) per g of sample.

2.7 Antioxidant activity

Antioxidant activity was evaluated following the methodology described in the literature (ALVES *et al*., 2010) with modifications, evaluating the consumption of the free radical DPPH· by the samples, through the decrease in absorbance of solutions of different concentrations.

Absorbance measurements of the reaction mixtures (1.0 mL sample solution at concentrations from 6.25 to 100 $\mu g.mL^{-1}$ and 2.0 mL methanolic solution of DPPH· at the concentration 40 $\mu g.mL^{-1}$) were recorded at the end of 60 minutes in a UV/vis Tecnal spectrophotometer at 515 nm, with gallic acid as a positive control. From the absorbance values, the radical oxidation inhibition percentages were calculated according to Equation (1):

$$\% \text{ Reduction (DPPH· consumed} = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100 \quad (1)$$

In where: $A_{control}$ is the initial absorbance of DPPH· at a concentration of 40 $\mu g.mL^{-1}$ and A_{sample} corresponds to the absorbance of DPPH· in the medium, after the reaction with the sample.

The inhibitory concentration (IC_{50}), the amount of antioxidant required to decrease the initial concentration of DPPH· by 50%, was determined from a first-order exponential curve, obtained by plotting the sample concentrations ($\mu g.mL^{-1}$) on the abscissa, and the DPPH reduction percentages on the ordinate axis.

2.8 Statistical analysis

Results were expressed as mean ± standard deviation (n = 3) for each extract. The statistical treatment of data was carried out by analysis of variance (ANOVA) using the computer program Sisvar 5.6. A p value < 0.05 was considered statistically significant.

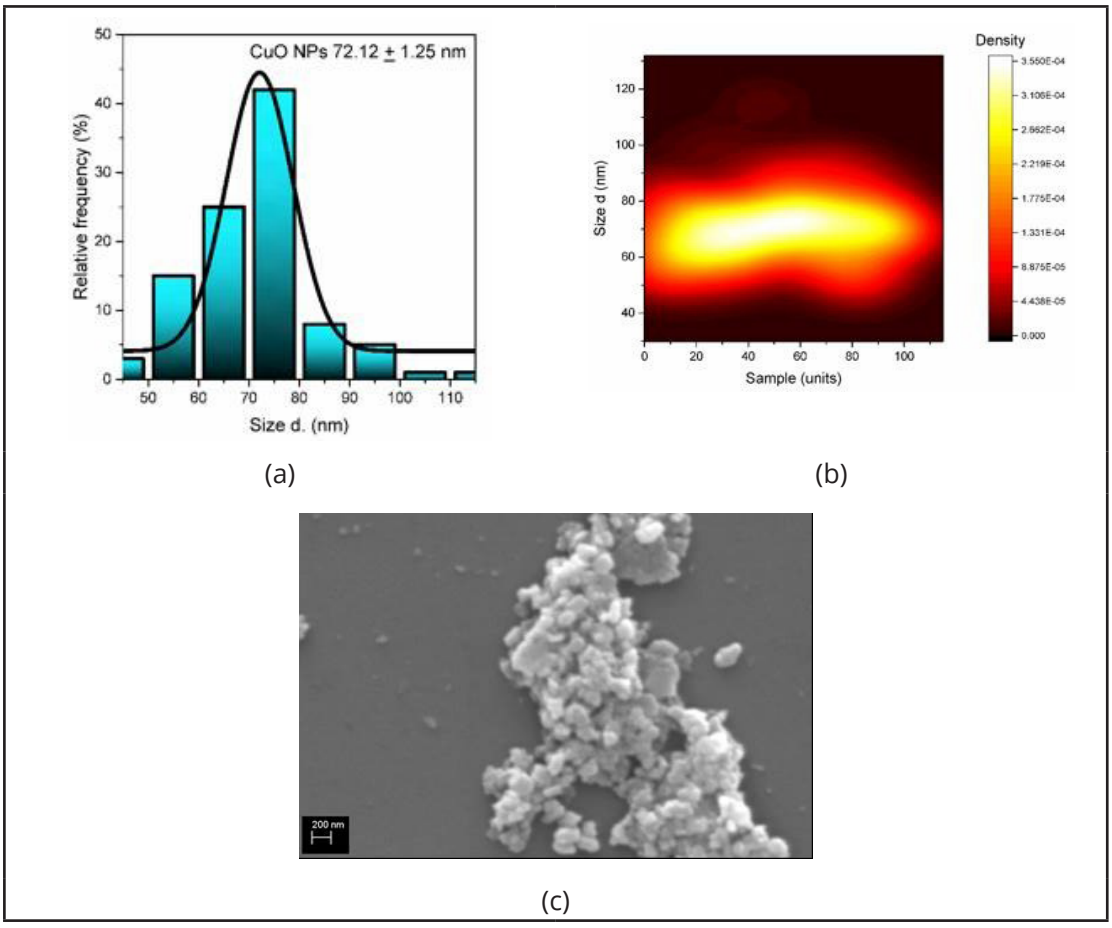
3 RESULTS AND DISCUSSION

The scanning electron microscopy (SEM) analysis showed a frequency distribution of the particle diameter suggesting an average size of approximately 72 nm (Figure 1a). Although the particles occurred in isolation or clusters, they presented a relatively homogeneous distribution, concerning size distribution, as evidenced by the Kernel density map (Figure 1b). The micrograph suggests a spherical shape for the nanoparticles (Figure 1c).

According to the DLS results, the hydrodynamic diameter of CuONP showed a size around c.a. 107.32 nm with a PDI value of 0.07 (Figure 2). They corroborate with the SEM data. The surface charge of nanoparticles evaluated by zeta potential presents negative value (z = -16.9 mV).

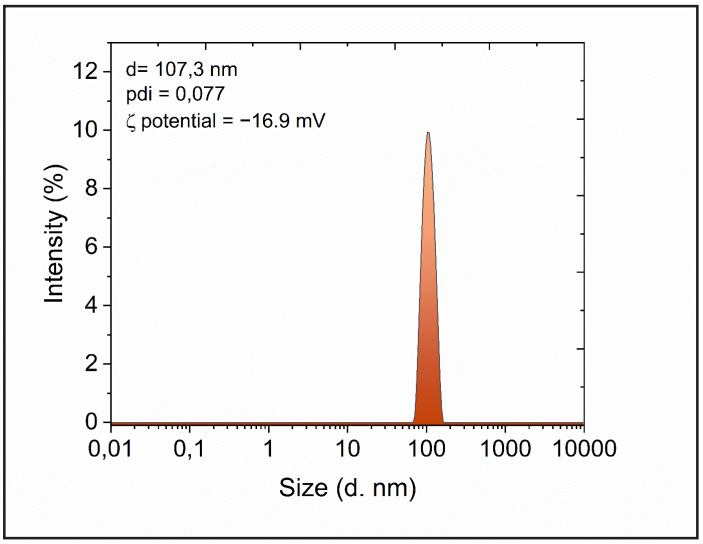
The analysis of UV-Vis spectra confirmed the presence of suspended copper nanoparticles after synthesis. The spectral behavior of the aqueous extract of *P. amalago* L., the copper sulfate, and the synthesized nanoparticles are shown in Figure 3. A shift accompanied by a band broadening from 235 nm to 239 nm was found for the copper sulfate solution and the suspension of nanoparticles after synthesis. The spectral band broadening behavior suggests the role of the plant extract in reducing and folding the synthesized nanoparticles.

Figure 1 – (a) particle diameter frequency distribution, (b) Kernel density, and (c) micrograph of CuO nanoparticles



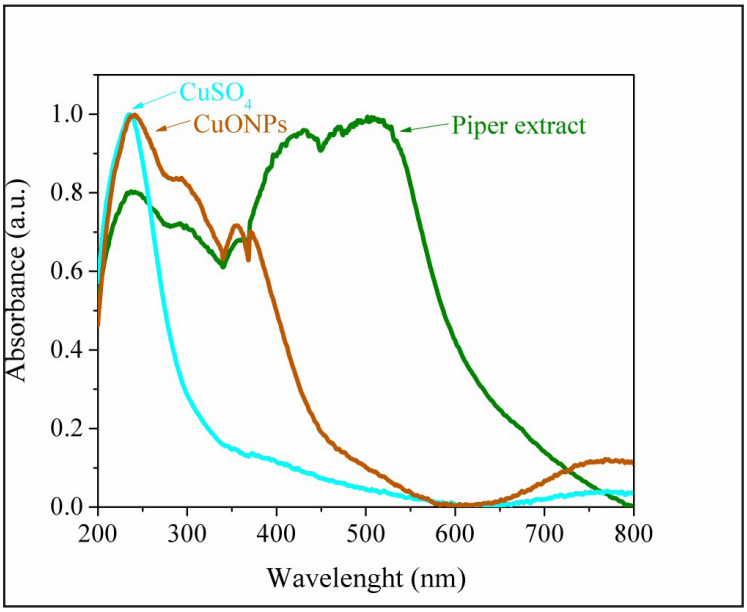
Source: Authors (2022)

Figure 2 – The hydrodynamic diameter of CuO nanoparticles



Source: Authors (2022)

Figure 3 – Spectral behavior of the aqueous extract of *Piper amalago*, copper sulfate and CuO nanoparticles



Source: Authors (2022)

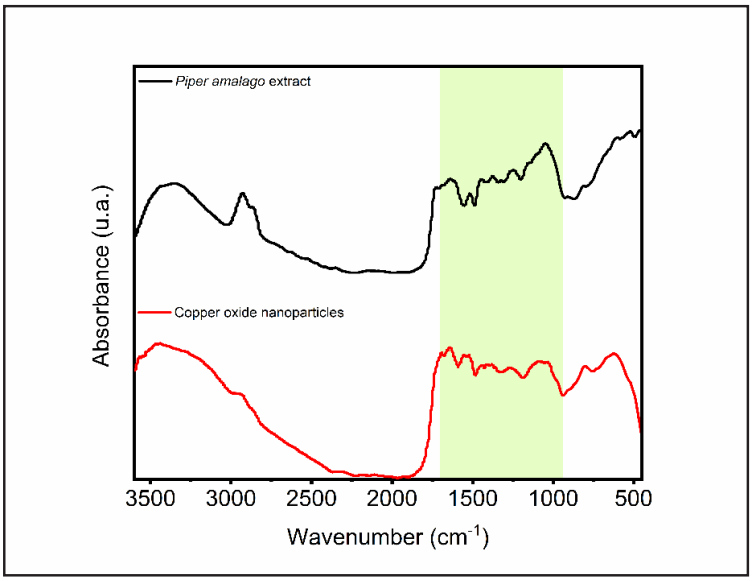
The FTIR results reveal the occurrence of CuONPs formation (Figure 4), in which the absorption band at 625 cm^{-1} was observed due to the stretching vibration mode of Cu_2-O. The band at 716 cm^{-1} also corroborates this result. The fingerprint region of plant extract also is observed from the spectral region of 1750 to 950 cm^{-1}, suggesting the incorporation of plant compounds as reducing agents and capping of nanoparticle surface.

These results show the success of the aqueous extract of *P. amalago* for the green synthesis of CuONPs. The extract showed reducing power for the synthesis of metallic nanoparticles. The reducing activity of plant materials has already been used in several studies involving the synthesis of metallic nanoparticles. The reducing agents involved include several water-soluble plant metabolites, such as alkaloids, phenolic compounds, and terpenoids (MITTAL *et al*., 2013; OLOUMI *et al*., 2015; JADOUN *et al*., 2020; ZHANG *et al*., 2020).

Traditionally, the production of metallic nanoparticles starts with toxic solvents, which end up giving rise to waste that is harmful to health and the environment. Developing synthesis methodologies using natural extracts is simple, fast and highly

relevant, especially concerning environmental issues. In particular, the synthesis of CuONPs with low toxicity can even be a source of copper for the soil (MITTAL *et al*., 2013).

Figure 4 – Fourier transform infrared spectroscopy (FTIR) of CuO nanoparticles



Source: Authors (2022)

Ethanol and aqueous extracts of *P. amalago* leaves and copper oxide nanoparticles (CuONPs) were evaluated through the in vitro antioxidant activity assay using the DPPH$^{\cdot}$ method and the results were expressed as the ability to reduce the DPPH$^{\cdot}$ free radical in percentage (Figure 5) and by the IC_{50} value (Table 1), which is a parameter indicative of the inhibitory concentration required to decrease the radical by 50%. The lower the IC_{50} value, the greater the antioxidant activity (EL; KARAKAYA, 2004; BIGHETTI *et al*., 2018).

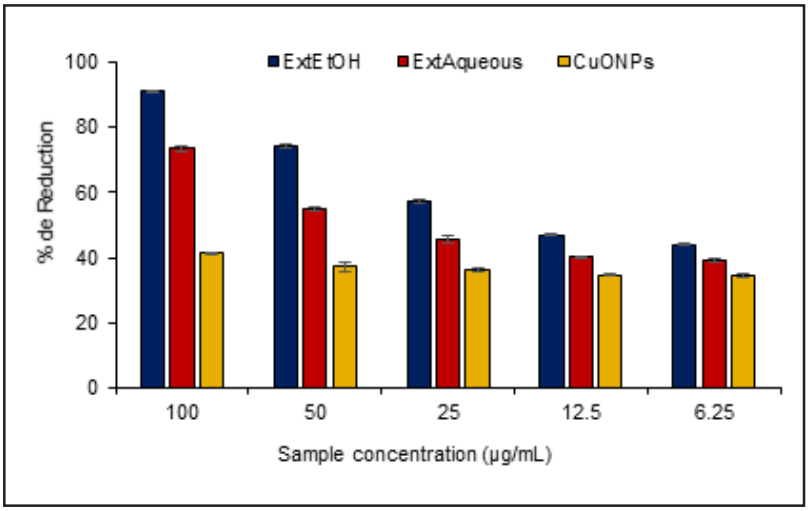
Regarding the ability to reduce or eliminate the DPPH$^{\cdot}$ free radical (% reduction) of the samples analyzed individually, the statistical analysis evidenced that both the ethanolic and aqueous extracts showed differences in the concentrations tested ($p < 0.05$). Only the ethanol extract did not result in differences in radical reduction at concentrations of 12.5 and 6.25 $\mu g.mL^{-1}$ (Figure 5). The CuONPs samples showed a significant difference only at the highest concentrations (100 to 50 $\mu g.mL^{-1}$).

As for the extracts, ethanol was considered the most active at the concentrations tested. At the highest concentration (100 $\mu g.mL^{-1}$), this extract was significantly more

effective, reducing DPPH· by more than 90%. The aqueous extract at this concentration reduced the radical by more than 70%. CuONPs reduced DPPH· the least at the concentrations tested, about 40% at the highest concentration (100 µg.mL^{-1}), therefore with the lowest potential as sources of free radical scavenging substances.

Analyzing the IC_{50} values produced by the extracts and the CuONPs (Table 1), the ethanol extract showed greater effectiveness in the antioxidant capacity (14.34 ± 0.96 µg.mL^{-1}). The aqueous extract produced an IC_{50} of 36.69 ± 1.15 µg.mL^{-1} and the CuONPs, the highest IC_{50} value (213.37 ± 5.29 µg.mL^{-1}). In this study, the lower antioxidant activity presented by CuONPs may suggest an effect of generating reactive oxygen species (ROS) and/or inhibiting the activity of the plant extract, acting as a pro-oxidant (CASTELO-BRANCO; TORRES, 2011).

Figure 5 – Percentage of DPPH reduction of the ethanolic (ExtEtOH) and aqueous (ExtAqueous) extracts of *Piper amalago* leaves and copper oxide nanoparticles (CuONPs)



Source: Authors (2022)

The results obtained in the determination of total phenols indicated that the highest content was found for the ethanol extract (199.17 ± 4.11 mg of GAE/g). This sample also presented a higher content of flavonoids (30.84 ± 0.5 mg of QE/g), in addition to a higher concentration of tannins 158.30 ± 4.83 mg TAE/g, indicating

that this solvent was more efficient for the extraction of these groups of organic compounds. The concentration of total phenols observed for the aqueous extract was 67.33 ± 1.26 mg GAE/g, which registered 24.6 ± 0.62 mg QE/g flavonoids and 64.47 ± 1.12 mg TAE/g total tannins (Table 1).

Table 1 – Contents of total phenols (TF), total flavonoids (TFV) and total tannins (TT) of the ethanolic and aqueous extracts of *Piper amalago* and IC_{50} of the copper oxide extracts and nanoparticles

Samples	TF (mg de GAE/g)	TFV (mg de QE/g)	TT (mg de TAE/g)	IC_{50} (μg.mL^{-1})
ExtEtOH	199.17 ± 4.11a	30,84 ± 0.59a	158.30 ± 4.83a	14.34 ± 0.96c
ExtAqueous	67.33 ± 1.26b	24,6 ± 0.62b	64.47 ± 1.12b	36.69 ± 1.15b
CuONPs				213.37 ± 5.29a
Quercetin				4.07 ± 1.43

Source: Authors (2022)

Values expressed as mean ± standard deviation (n = 3). Means followed by different letters in the column differ statistically after ANOVA, followed by Tukey ($p < 0.05$). Ethanol Extract (ExtEtOH), Aqueous Extract (ExtAqueous) and Copper Oxide Nanoparticles (CuONPs).

Some authors describe that the antioxidant activity of vegetables is related to the presence of phenolic compounds, such as flavonoids and tannins, capable of delaying or inhibiting the oxidation of oxidizable substrates (SOUSA *et al*., 2007; PERES *et al*., 2009).

In the case of *P. amalago*, this approach can be accepted, as the ethanol extract, the most active in terms of antioxidant activity (IC_{50} = 14.34 ± 0.96 μg.mL^{-1}) also contained the highest phenolic content (199.17 ± 4.11 mg GAE/g). The aqueous extract exhibited an IC_{50} of 36.69 ± 1.15 μg/mL, with lower potential for free radical scavenging and lower content of phenolic compounds (67.33 ± 1.26 mg GAE/g). However, this extract showed a reducing power, efficient for the green synthesis of copper oxide

nanoparticles. Probably, in addition to phenolics, other secondary metabolites present in the aqueous extract are responsible for the bioreduction of metal ions, their growth and stabilization.

In view of total phenols, it is possible that the main phenolic constituents of *P. amalago* are flavonoids and mainly tannins, both groups of compounds recognized as antioxidants (SILVA *et al*., 2010; SANTOS; RODRIGUES, 2017).

Rovani *et al*. (2013) evaluated the aerial parts of *P. amalago*, and recorded the presence of flavonoids and tannins, as detected in this study, however, with lower antioxidant potential, as the species was effective at 28.09 $\mu g.mL^{-1}$ to reduce by 50% initial $DPPH^{\cdot}$ concentration.

Previous studies carried out with other species of the genus have shown antioxidant activity. According to Silva *et al*. (2014), the ethanol extract of *Piper divaricatum* leaves, even at the lowest concentration (5 $\mu g.mL^{-1}$) inhibited free radical activity by 70%, in addition to the high activity observed for *Piper arboreum* and *Piper dilatatum*. In a more recent study, the ethyl acetate extract of *Piper betler* showed moderate DPPH radical scavenging activity with an IC_{50} of 100.1 ± 0.32 $\mu g.mL^{-1}$ (SAVSANI *et al*., 2020).

As in this study, Sarma *et al*. (2018) reported that the ethanolic extract of *P. betler* leaves showed greater antioxidant activity (89.46% $DPPH^{\cdot}$ inhibition), while the aqueous extract had lower potential (62.03% inhibition). In the same way, Gülçin (2005) demonstrated that both the ethanolic and aqueous extracts of *Piper nigrum* seeds exhibited strong antioxidant activity. Thus, the potential for pharmacological and biopesticide purposes of this genus is clear.

4 CONCLUSION

In the present study, the ethanolic and aqueous extracts of *P. amalago* leaves exhibited considerable antioxidant activity that may be directly related to phenolic

compounds present in the plant material, such as flavonoids and mainly tannins. The aqueous extract of *P. amalago*, despite its lower antioxidant potential compared to the analyzed extracts, has a reducing power and is efficient for the green synthesis of copper oxide nanoparticles. Due to the fact that *P. amalago* is a species with biopesticide potential, studies on the applicability of the use of metallic nanoparticles from green synthesis from its extracts are fundamental for studies with bionanopesticides.

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Authorship contributions

1 – Mayara Cristina Neves Abel

Universidade Estadual de Mato Grosso do Sul, Master in Natural Resources
https://orcid.org/0000-0002-7314-7877 • mayaranevesouza@gmail.com
Contribution: Conceptualization, Methodology, Software, Formal Analysis, Research, Resources, Data Curation, Writing – original draft, Writing - review & editing, Visualization - Supervision - Project Administration

2 – Etenaldo Felipe Santiago

Professor at the Universidade Estadual de Mato Grosso do Sul, PhD in Biological Sciences
https://orcid.org/0000-0001-6838-1098 • felipe@uems.br
Contribution: Conceptualization, Methodology, Software, Formal Analysis, Research, Resources, Data Curation, Writing – original draft, Visualization, Supervision, Project Administration

3 – Ana Francisca Gomes da Silva

Professor at the Universidade Estadual de Mato Grosso do Sul, PhD in Chemistry
https://orcid.org/0000-0002-7966-1623 • anafranciscagomesdasilva@gmail.com
Contribution: Conceptualization, Methodology, Software, Formal Analysis, Research, Resources, Data Curation, Writing – original draft, Writing - review & editing, Visualization, Supervision, Project Administration

4 – Jonas da Silva Mota

Professor at the Universidade Estadual de Mato Grosso do Sul, PhD in Chemistry
https://orcid.org/0000-0001-5328-8069 • dasilvamotaj@gmail.com
Contribution: Methodology, Formal Analysis, Research, Resources, Writing – original draft, Visualization, Supervision

5 – Montcharles da Silva Pontes

Universidade Estadual de Mato Grosso do Sul, PhD in Natural Resources
https://orcid.org/0000-0002-9844-5838 • montcharles.pontes@gmail.com
Contribution: Conceptualization, Methodology, Software, Formal Analysis, Research, Data Curation, Writing – original draft, Writing - review & editing, Visualization

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