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Synthesis and Characterization of Proteolytic Enzyme Loaded on Silver Nanoparticles

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Abstract

Bromelain is a proteolytic enzyme rich in cysteine proteases, extracted from the stem and fruit of pineapple (*Ananas comosus*). There are several therapeutic applications of the bromelain enzyme, where it has anti-inflammatory, anti-cancer, and antimicrobial activity, reduces joint pain, and accelerates wound healing. In the current study, bromelain enzyme was loaded on silver nanoparticles (Br-AgNPs) prepared using the citrate-reduction Turkevich method. Different characterization analyses were performed, including UV-Vis spectrophotometers, FTIR, SEM, and XRD analyses. Moreover, the antioxidant activity of prepared Br-AgNPs was evaluated by DPPH assay. The results of UV-Vis showed a peak at 434 nm, which referred to the AgNPs formation, and FTIR results revealed groups of (C=O, C=C) at 1519.91 and 1539.20, respectively, and the amine group at 1384.89 and the flavonoids group at 1357.89. SEM results exhibit that the synthesized Br-AgNPs were spherical in shape, with average sizes of about 84.73 nm. Also, the AgNPs were crystalline in nature with face-centered cubic symmetry. The synthesized BR-AgNPs showed strong in vitro antioxidant activity in a dose-dependent manner. In conclusion, successfully synthesized silver nanoparticles and bromelain had a potent antioxidant effect and may be a possible therapeutic agent for many diseases in the future.

Keywords: Bromelain, antioxidant, silver nanoparticles, Turkevich method.

1. Introduction

Nanotechnology is a scientific field that aims to produce devices or materials with special functional properties by manipulating and controlling these materials at the nanolevel, i.e., the level of atoms and molecules. Nanotechnology controls materials by changing their internal

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structure via rearranging their atoms and molecules to become nanomaterials with distinctive properties that can be used in various scientific applications [1]. One of the most synthesized nanoparticles is AgNPs, due to their unique properties such as size and shape, which depend on electrical, magnetic, and optical properties [2]. AgNPs have many applications in the industry field, like the manufacture of textiles and cosmetics, and in the medical field, such as using them as antimicrobials, gene therapy, dental fillings, wound and burn dressings, and in drug delivery to target organs [3].

Plants are rich sources of natural products; molecules of primary metabolism like proteins or enzymes appear as therapeutic agents; proteolytic enzymes, or proteases, are the most common species of enzymes used for clinical applications; proteolytic enzymes act as cleave agents for peptide bonds of proteins [4].

Bromelain is a proteolytic enzyme rich in cysteine proteases that is extracted from the stem and fruit of pineapple (*Ananas comosus*). Pineapple extract from the pineapple stem is more economical to produce and is a more commonly available commercial product [5]. Bromelain contains several enzymes, i.e., peroxidases, phosphatases, glucosidases, cellulases, and escharasein, in addition to glycosidase-like components, carbohydrates, glycoproteins, and many protease inhibitors. Bromelain has cysteine proteinases that have proteolytic activity, and crude bromelain consists of proteinases, and the complex of proteolytic enzymes also contains sulfhydryl proteases [6]. There are several therapeutic applications of Bromelan enzyme, where it has anti-inflammatory, anti-cancer, and antimicrobial activity, in addition to other medical inquiries that help to treat digestive disorders, allergies, asthma, reduce joint pain, and accelerate wound healing [7]. Thus, this study was undertaken to provide appropriate information on the synthesis, characterization, and loading of bromelain on silver nanoparticles, along with evaluating their free radicle scavenging activity for future potential as a therapeutic agent.

2. Materials and Methods

2.1. Chemicals

Bromelain (EC: NO. 253-387-5), silver nitrate ($AgNO_3$), and tri-sodium citrate solution were purchased from Sigma-Aldrich, USA. All reagents used are of analytical grade.

2.2. Preparation of bromelain and $AgNO_3$ solutions

$AgNO_3$ solution was prepared by dissolving 0.0618 g of $AgNO_3$ in 100 ml of distilled water at 45 °C to obtain a 1 mM silver nitrate solution, then 2 ml of (1%) trisodium citrate was added. The solution was kept on a stirrer for minutes, and then stored until used at 4°C [8]. Freshly prepared bromelain with a final concentration of 10 mg/mL dissolved in distilled water was added to the AgNPs solution and stirred for 6 hours [9].

2.3. Characterization of biosynthesized AgNPs

2.3.1. UV–VIS spectral analysis

The absorption spectrum was measured by a UV-VIS spectrophotometer (Shimadzu 1900, Japan) with a range from 100 to 1100 nm at room temperature; distilled water was used as a control solution [10].

2.3.2. FT-IR analysis

To identify the functional groups in both bromelain solution and silver nanoparticles, Fourier Transform Infra-Red (FTIR) Spectroscopy (Shimadzu 1800, Japan) was used, at wavelengths ranging from 400 cm^{-1} to 4000 cm^{-1}. The analysis was done using a Kbr disc [11].

2.3.3. SEM analysis

A scanning electron microscope (Tescan Mira, Czech) was used to detect the shape and size of particles of silver nanoparticles and conjugated bromelain using the method of [12]. The samples were spread evenly on the sample holder with the help of carbon tape and sputter-coated with gold. Samples were dried at room temperature and analyzed using different microscope powers.

2.3.4. XRD analysis

The crystalline nature of biosynthesized AgNPs was determined by using the X-ray diffraction Technique. The amount of prepared samples was placed on a glass slide, dried at 100 degrees, and analyzed using XRD (Philips PW1730). The patterns of XRD were measured with Cu Kα radiation (λ=1.54 A) at a 2θ angle in the range of 20 to 80 degrees [13].

2.4. Free radical scavenging activity

The DPPh assay was employed in accordance with the [14] method to assess the capacity of Br-AgNPs to inhibit free radicals. The samples with a range of concentrations (12.25, 25, 50, 100, and 200 μg/ml) were mixed in an equivalent volume with DPPH solution at 60 μM and left in the dark for 1 h at 37°C. Vitamin C (L-ascorbic acid) was used as a positive control. Absorbance was then determined spectrophotometrically at 517 nm, and the scavenging activity was calculated following the equation:

$$\text{Scavenging inhibition activity } \% = (\text{AbC-AbS})/\text{AbS} \times 100$$

3. Results and Discussion

3.1. Silver nanoparticles and bromelain-AgNPs preparation

As shown in Figure 1, adding tri-sodium citrate to tetra-chloroauric acid changed the color mixture to brownish; this change is an initial indication for the synthesis of silver nanoparticles from Ag+. Then bromelain was loaded with AgNPs, and the color changed to dark red. This change may be due to the difference in the electronic density of the particles as a result of the changing nanoparticle sizes [10].

Figure 1. Color change in the silver nanoparticles process. (A) silver nitrate solution, (B) silver nanoparticles solution (silver nitrate and tri-sodium citrate), (C) bromelain solution and (D) bromelain-silver nanoparticles (Br-AgNPs).

3.2. Characterization of biosynthesized AgNPs

3.2.1. UV–VIS spectral analysis

The results of UV-VIS showed an absorbance peak at 434 nm, as shown in Figure 2, which is within the reported absorbance peak of silver nanoparticles ranging from 400 to 450 nm [15].

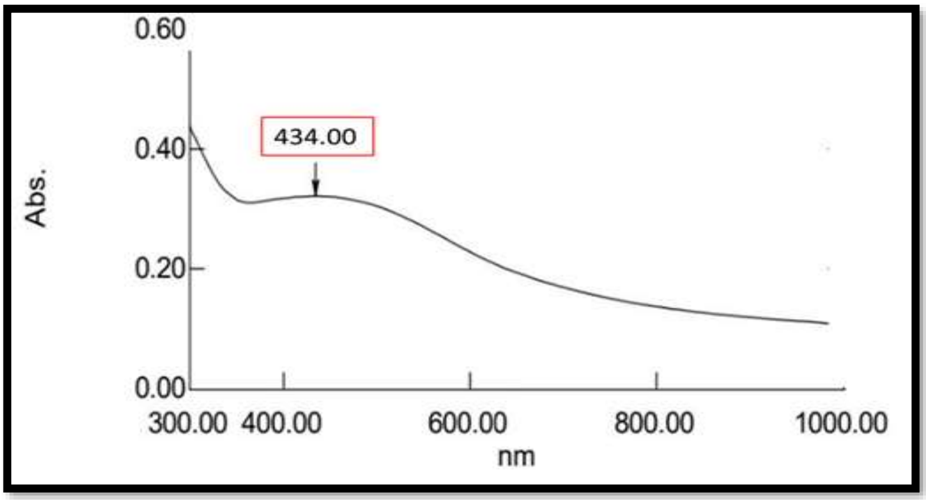

Figure 2. UV-Visible spectra of Br-AgNPs

This peak was recorded at 434 nm due to surface plasmon resonance that appears on the surfaces of some minerals as a result of the collective movement of electrons in the nanoparticle when light falls on it [16]. Moreover, the properties of the plasmon resonance peak depend on the size, shape, and location of the nanoparticles. The region between 400 and 450) nm represents the blue light emission region; these emissions are achieved through silver nanoparticles whose diameters range from 40 to 100 nm [17]. This study agrees with a study [18] where the result of UV analysis of silver nanoparticles prepared from pineapple juice extract and a solution of silver nitrate showed an absorption peak at 430 nm. Also, the results agree with a study by [19], where the result of the

UV analysis of the nanoscale mixture Ag-Au (core-shell) prepared from pineapple leaf extract showed an absorbance peak at 442 nm after 20 minutes of adding silver nitrate solution.

3.2.2. FTIR analysis of bromelain

The bromelain solution was analyzed using FTIR to verify the functional groups. Figures 3 and 1 showed the results of the FTIR analysis of the bromelain solution, where the adsorption band at 3321.42–3545.16 - 3545.16 cm^{-1} could be identified as the OH group of the alcohol and phenol compounds. There are two peaks at 2125.56 cm^{-1} and 1639.49 cm^{-1} that indicate the alkene group and alkyne group, respectively, and the peaks at 1789.94 and 1080.14 cm^{-1} refer to the carbonyl groups of double bonds and single bonds, respectively, and the peaks that appeared at 601.79 and 983.70 cm^{-1} indicate effective groups. The FTIR analysis results of Br-Ag-NPs revealed a peak at 3456.44, which indicates the presence of the OH group of alcohol and phenol; the peak at 1577.77 cm^{-1} refers to the presence of amine and secondary amide groups. The adsorption bands at 1539.20, 1519.91, and 1504.48 cm^{-1} could be identified as C=C cm–1 cm^{-1} stretching of the cyclic aromatic ring, and the peak at 1384.89 reveals the presence of secondary amine and carbonyl groups (Figure 3, 2), amine and carbonyl groups (Figure 3, 2).

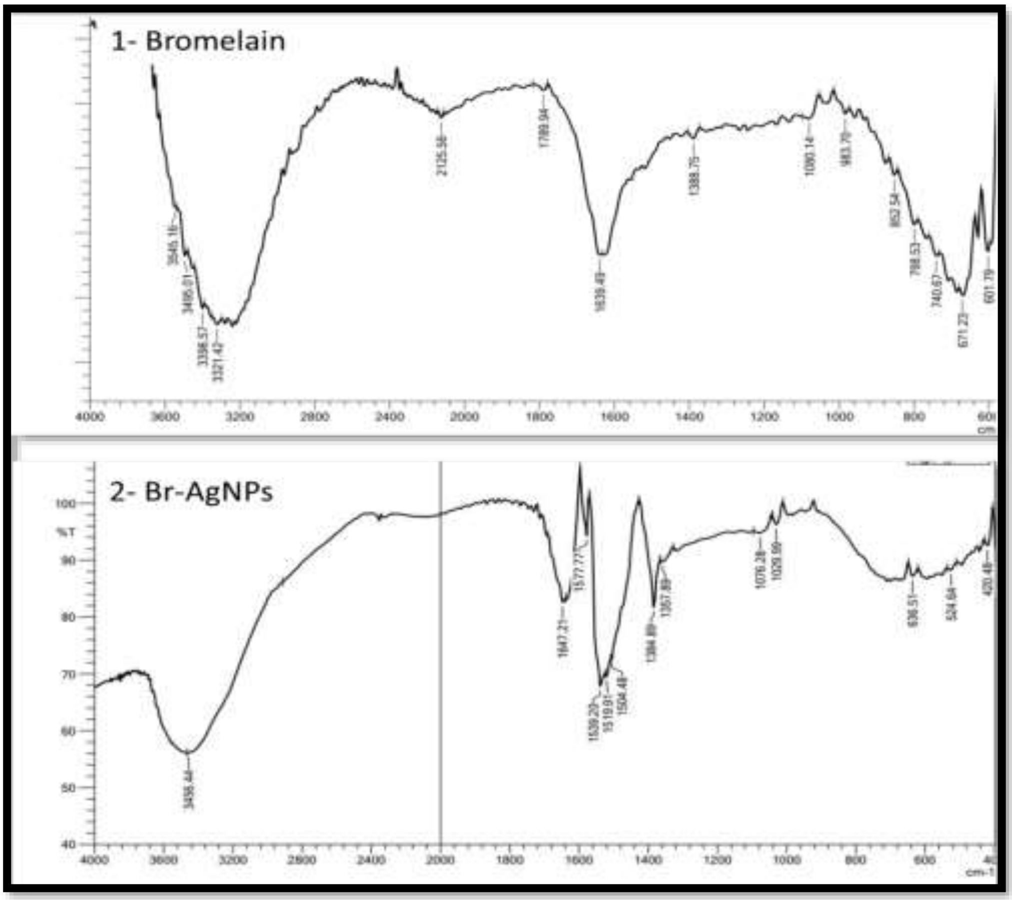

Figure 3. FTIR of 1- bromelain and 2- bromelain loaded on silver nanoparticles (Br-AgNPs)

When comparing the results of FTIR analysis of the bromelain solution and Br-AgNPs, observing the presence of hydroxyl groups of the alcohols and phenol compounds in both spectrums indicates that the synthesized AgNPs contain natural components of the bromelain solution. A study by [20] recorded that surface nanostructure has so many applications in microelectronics, biomaterials, and medicine that altering the surface can influence how it interacts with cells. Studies have shown that the reason behind the use of plants in the synthesis of various nanoparticles is due to the action of enzymes present in biomolecules, especially phenolic compounds in plants, where these enzymes undergo oxidative cleavage during the process of biosynthesis, which leads to the release of free electrons for their use. In reducing the ions of the chemical solution to their metallic state, which leads to the formation of nanoparticles [21], they found that recombinant bromelain was used to create nanoparticles utilizing a green chemical approach. The response of the chemical with recombinant bromelain determined the absorption and weight of the nanoparticles, and according to 3D plots, temperature and time are the two main factors that determine the ideal condition for high responses. The current study corresponds to the study of [22], where the results of the FTIR analysis of the aqueous solution of pineapple leaves and AgNPs synthesized by pineapple leaves showed that natural substances such as sugars (sucrose, glucose) are responsible for the biosynthetic reduction of Ag^+ and the formation of AgNPs. This also corresponds to the study of [23], where the results of the FTIR showed the presence of hydroxyl, carbonyl, and carboxyl groups of the main compounds present in pineapple leaf extract, and these groups are responsible for the Ag^+ reduction and stability of the AgNPs synthesized. Moreover, a study by [24] found that plant-based proteolytic enzymes can be used as agents for the reduction and stability of metal ions for the synthesis of nanoparticles by synthesizing gold nanoparticles using the bromelain enzyme.

3.2.3. SEM analysis

SEM analysis of synthesis particles is shown in Figure 4. A displayed clear homogeneity and an almost single distribution with fewer aggregations or agglomerations. This might be the result of the AgNPs citrate reduction; citrate ions may leave the AgNPs surface with a negative charge. Additionally, most of the AgNPs particles were spherical, with an average size of 55.37 nm. The shape of Br-AgNPs particles was spherical, which is the dominant appearance of the particles with an average size of 84.73 nm (Figure 4, B). The analysis also proved the success of the process of loading bromelain into the AgNPs, and this appears from the preservation of the silver nanoparticles in their dominant spherical shape as well as the slight change in the average size of the nanoparticles.

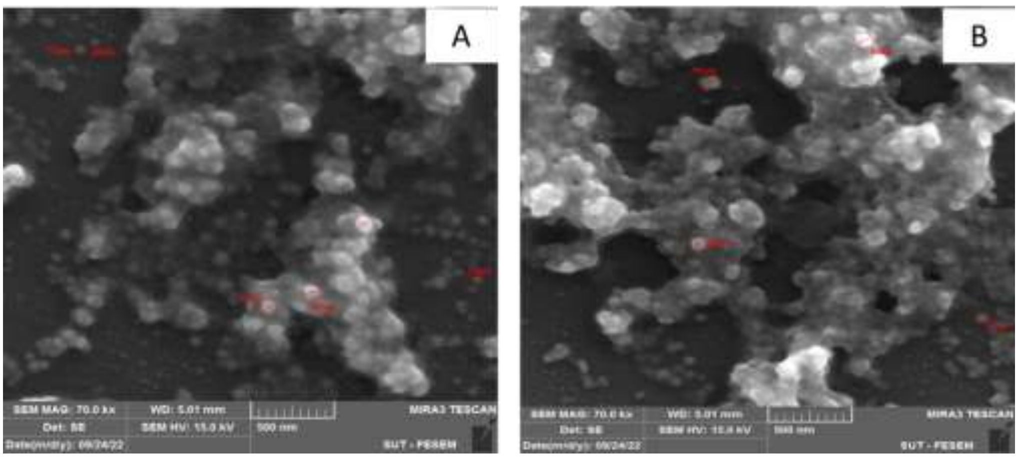

Figure 4. SEM images of synthesized (A) sliver nanoparticles (AgNPS) and (B) bromelain-AgNPs (Br-AgNPs).

The results of this study, in terms of particle shapes, are consistent with the results of the [25] study, where the synthesis of nanoparticles was done using different fruits, i.e., pineapple, lemon, and guava, and the SEM analysis of pineapple-derived silver nanoparticles revealed that the particles were spherical in shape. It is also consistent with the study of [26], where the shape of the particles in a sample of a solution of silver nanoparticles prepared from the crumbs of pineapple peels was spherical, either in terms of size or shape. The results of this study agree with the results of the study [27], where the size of the particles. The chitosan nanoparticles loaded with bromelain are 100 nm. It also agrees with the study of [28], where the particle size ranged from 20 to 60 nm for gold nanoparticles prepared from the extract of pineapple plants containing bromelain.

3.2.4. XRD Analysis

The XRD pattern of Br-AgNPs is shown in Figure 5. The crystallographic results obtained from XRD analysis revealed that the sample was face cubic center (fcc) structured. Four distinct diffraction peaks were recorded that appeared in magnitude 2θ at angles of 38.5, 45.5, 66.5, and 78.5, which correspond to silver nanoparticles (111, 200, 220, 311), by comparing the measurements with the X-ray diffraction database ICDD file 0783-JCPDS04 for silver nanoparticles [29]. According to a study [20], the additional peaks that appear in the result of XRD analysis are due to the presence of biomolecules related to stabilizing factors such as enzymes, proteins, and other phytochemical components on the surface of the nanoparticles. The results of this study are consistent with the results of the study [30], where XRD of AgNPs synthesized by the outer peel extract of pineapple has diffraction peaks at the angles that correspond to X-ray diffraction measurements of AgNPs. These results are also consistent with the results of [31], where papaya peel extract contains many biomolecules such as proteolytic enzymes (papain) and phenolic compounds responsible for the reduction and stability of nanoparticles in the synthesis of AgNPs. The results of the XRD indicated the presence of diffraction peaks that are consistent with the X-ray diffraction measurements of the silver nanoparticles, and the appearance of the additional peaks might be attributed to the coverage and stability factors of the nanoparticles.

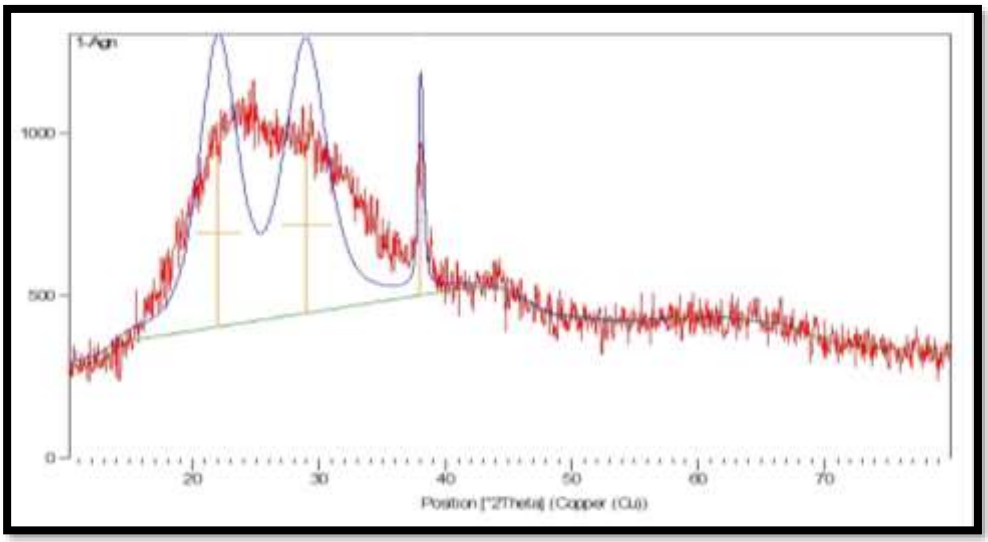

Figure 5. XRD spectra of Br-AgNPs

3.3. Free Radical Scavenging Analysis

The potential of the Br-AgNPs for DPPH radical scavenging activity is represented in Figure 6. The antioxidant activity was increased in a dose-dependent manner, by which it was about 68% at a concentration of 200 µg/ml and decreased to 18% at a concentration of 12.5 µg/ml. When compared to the control (ascorbic acid), the results revealed an effective scavenging activity adjacent to the activity of ascorbic acid. These results indicated that the conjugate is effective in removing unstable electrons from the UPPH and donating hydrogen atoms. The hydroxyl group's ability to operate by giving the hydroxyl radicals a hydrogen atom and an electron may be the cause of the citrate-capped AgNPs ability to scavenge the hydroxyl radical [32]. This study is consistent with the study of [20], where the results of the DPPH test for AgNPs prepared from the external peel of pineapple showed that at concentrations of 25–100 µg/ml, the free radical scavenging capacity ranged from 27.23–43.41%. Also, a study by [33] reported that bromelain enzyme conjugated with chitosan nanoparticles showed highly antioxidant activity against bromelain at 40%.

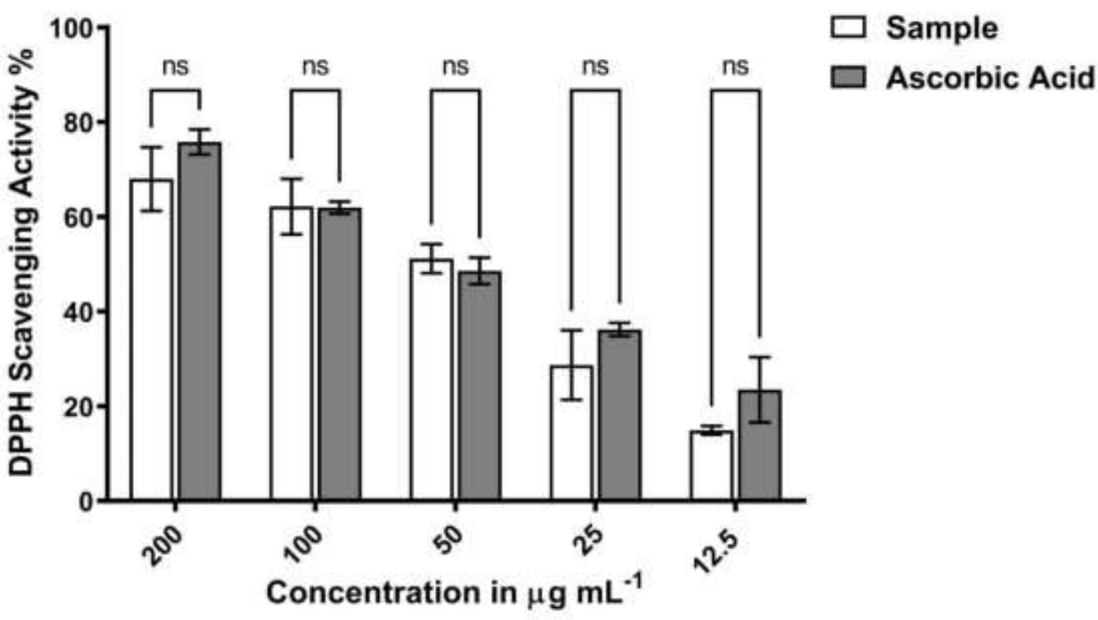

Figure 6. Scavenging free radicals' percentage using DPPH assay of Br-AgNPS and positive group (vitamin C). Data are mean ± SD. Ns = not statically significant.

5. Conclusions

The current study showed that bromelain can be made safely, easily, and cheaply, and that it can be attached to silver nanoparticles to make powerful antioxidants. As a result, it is thought to be a good drug delivery system, and further in vivo and in vitro studies are required.

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Conflict of Interest

There are no conflicts of interest.

Funding

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