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BIOSYNTHESIZED GOLD NANOPARTICLES FROM RED SEAWEED AMPHIROA FRAGILISSIMA THROUGH ANTIOXIDANT AND ANTICANCER ACTIVITY AGAINST OSTEOSARCOMA CANCER CELLS

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Abstract

The red seaweed Amphiroa fragilissima was used for the ecologically friendly and efficient green manufacture of stable gold nanoparticles (AuNPs) with potential efficiency against osteosarcoma cancer cell lines. The gold nanoparticles synthesised via the use of Amphiroa fragilissima were examined utilising a range of analytical methods. The use of Ultra Violet spectroscopy (UV) revealed that the Surface Plasmon Resonance (SPR) of A. fragilissima-AuNP occurs at a wavelength of 540 nm. The A. fragilissima-AuNPs were analysed using Fourier Transform Infra-red spectroscopy (FTIR) to identify the biological components present. The Af-AuNPs were observed to possess a spherical and irregular morphology and measured 25 nm in size using scanning electron microscopy (SEM). Af-AuNPs have significant capabilities in scavenging DPPH (71.88%) and hydrogen peroxide (65.97%), indicating their potential as antioxidants. The percentage inhibition of protein denaturation of Af-AuNPs was 67.78%. Furthermore, these nanoparticles demonstrate potent anticancer activity against osteosarcoma (MG-63) cancer cells, with an IC50 value of 23.92 µg/mL. The findings of the study suggest that synthesised A. fragilissima-AuNPs have significant promise as an anti-cancer treatment.

Keywords: Gold Nanoparticles, Amphiroa Fragilissima, Osteosarcoma (MG-63), Antioxidant, Anti-inflammatory, Anticancer

1. Introduction

Cancer is a pathological condition characterised by uncontrolled proliferation and infiltration of certain cells, leading to its invasion of adjacent tissues and organs. These cancer cells originate from normal bodily cells (Brown et al., 2023). Immature or osteoid bone, formed by primitive mesenchymal cells originating in bone, may proliferate and lead to osteosarcoma. The prevalence of osteosarcoma is 2.3-3 million instances per year in the general population, with a higher incidence among teenagers aged 15-19 (Ritter et al., 2010).

Nanotechnology, a technology-driven scientific field, has garnered significant interest due to its ability to create functional systems at the nanoscale, enabling applications such as medication delivery and diagnostic instruments (Alangari et al., 2022). A nanoparticle is a solid particle or a dispersion of particles with a size between 1-100 nanometers. A nanoparticle matrix either encapsulates or attaches the medication or is dissolved inside it (Mohanraj et al., 2006). It has many possible medical uses, including antimicrobial coatings for medical equipment, better medication administration, less inflammation, faster healing after surgery, and the ability to identify cancer cells in the blood (Gajanan et al., 2018). Numerous methods like physical, chemical, and mechanical methods exist for the synthesis of nanoparticles. These methods are not only costly and detrimental to the environment, but they also have health hazards such as cytotoxicity, genotoxicity, and carcinogenicity (Alangari et al., 2022).

Nanoparticles made of metals have several applications in fields as diverse as electronics, chemistry, healthcare, and pharmaceuticals (Tao 2018). Gold nanoparticles (AuNPs) are unique among nanoparticles due to their high biocompatibility, low toxicity, and exceptional stability. AuNPs have distinct advantages in the biomedical domain, including their many functionalities, biodegradability, and non-toxic nature, which have captured the interest of the scientific research community (Rajeshkumar et al., 2021). The large surface area-to-volume ratio and varying sizes of gold nanoparticles (AuNPs) make them very effective in penetrating biological membranes (Al Saqr et al., 2021).

The marine algae known as seaweed are said to contain a wide variety of bioactive compounds. The bioactive substances are amines, sulphates, carboxyl and hydroxyl groups, providing various commercial and medicinal uses (Ponnuchamy et al., 2016, Liu et al., 2019). Rhodophytes are one of the three main groups of algae and are

sometimes called red seaweed. It is estimated that this group has over 7,000 distinct species. Their diverse biomolecules make them ideal candidates for the development of novel nutraceutical, cosmetic, and medicinal products, as well as for the synthesis of new biologically active chemicals (Aziz et al., 2021). It is readily scalable for large-scale synthesis since it is eco-friendly, easy, and cost-effective (Pandimurugan R et al., 2016). Secondary metabolites of *Amphiroa fragilissima* have a wide range of therapeutic applications, including antibacterial, antioxidant, antifungal, anticancer, and antiviral effects, are abundant (Algotiml et al., 2022).

There is currently no published information on the production of AuNPs using *Amphiroa fragilissima* extract. The purpose of this study was to investigate the synthesis of gold nanoparticles via the biological process employing *Amphiroa fragilissima*, as well as to evaluate their potential as antioxidants and anti-cancer agents against osteosarcoma (MG-63) cancer cells.

2. Materials and Methods

2.1 Chemicals used

The chemicals used in the experiment include chloroauric acid ($HAuCl_4$), methanol, dimethyl sulfoxide (DMSO), crystal violet, and trypan blue (purchased from HiMedia, Mumbai, India). Foetal bovine serum (FBS), Phosphate buffer solution (PBS), Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, Bovine serum albumin (BSA), antimycotic solution, and antibiotic solution were purchased from Gibco, Thermo Fisher Scientific, and India, respectively. The compound 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was acquired from Sigma Aldrich in Mumbai, India.

2.2 Seaweed collection

Amphiroa Fragilissima (Af), a red seaweed species, was gathered from the shore of Mandapam in the Ramanathapuram district of Tamil Nadu, India ($9^o16'51"$ N $79^o10'37"$ E). The seaweed species was verified by the Botanical Survey of India (BSI) in Coimbatore, India. The seaweed was washed and rinsed with distilled water to remove any undesired material. The cleaned algae were then dried in the shade and blended into a powder. The seaweed powder will be saved for future studies.

2.3 Preparation of Amphiroa fragilissima extract

One gram of powdered algae was combined with 100 mL of distilled water and subjected to heating at a temperature of 60^o C for a duration of 20 minutes. Subsequently, the extract was cooled to the ambient temperature and filtered using Whatmann No.1 filter paper. The resulting filtrate was then used for the synthesis of gold nanoparticles (Viswanathan et al., 2022).

2.4 Biosynthesis of gold nanoparticles from Amphiroa fragilissima (Af-AuNPs)

A. fragilissima-AuNPs were synthesized by combining 30 mL of seaweed extract with 70 mL of a 1 mM solution of chloroauric acid (HAuCl4). The reaction mixture was then heated to 60 degrees Celsius in an aqueous solution and agitated for 20 minutes using a magnetic stirrer. The rapid change in the solution's colour to a deep purple verified the biosynthesis of gold nanoparticles. The reaction solution was stirred for 48 hours at ambient temperature to achieve full reduction. The colloidal solution was subjected to UV vis-spectroscopy at regular intervals, spanning a wavelength range of 250 to 750 nm. Ultimately, the colloidal solution containing gold nanoparticles underwent centrifugation at a speed of 15,000 revolutions per minute in order to extract pellets for the purpose of conducting a characterization investigation (Baskar et al., 2023).

2.5 Characterization of biosynthesised A. fragilissima-AuNPs

The absorbance spectrum of gold nanoparticles synthesized from A. fragilissima was verified using Ultraviolet-visible spectroscopy, Perkin- Elmer MA, USA. The bioactive components in the AuNP were assessed using FTIR spectroscopy (SHIMADZU, IRTRACER 100). The morphology of A. fragilissima was examined by Scanning

Electron Microscopy (FEI, Quanta 200). The crystalline structure of the AuNPs mediated by A. fragilissima was assessed by X-ray Diffraction (PANalytical, Netherlands).

2.6 Antioxidant activity

2.6.1 DPPH scavenging activity

The capacity of the samples and ascorbic acid to scavenge DPPH radicals was evaluated using the antioxidant activity. The ascorbic acid standard and test samples, ranging in concentrations from 10 to 50 µg/mL, were mixed with 1 mL of 0.1 mM DPPH and then diluted in 50% ethanol. The reaction mixture was thereafter incubated in the absence of light for a duration of 30 minutes. Following this, the absorbance of both the control and samples was measured using spectrophotometry at a wavelength of 517 nm (Baskar et al., 2024).

2.6.2 H_2O_2 scavenging activity

For the H_2O_2 scavenging test, 40 mM of hydrogen peroxide was prepared using phosphate buffer (pH 7.4). Afterwards, 0.6 mL of H_2O_2 was added to the Hv-AuNPs and standard ascorbic acid in separate experiments, with concentrations ranging from 10 to 50 $\mu g/mL$. Each combination was then left to incubate at room temperature for 10 minutes. A control sample was prepared using a hydrogen peroxide solution. The next step was to analyse the absorbance of the sample at 230 nm using spectrophotometry (Baskar et al., 2024).

2.7 Anti-inflammatory activity

The anti-inflammatory effect of synthesized *A. fragilissima*-AuNPs was tested using the BSA denaturation assay, using a modified methodology from (Gunathilake et al., 2018). In brief, 400 µL of bovine serum albumin (BSA, Sigma Aldrich, USA) were supplemented with 40 µL of Af-AuNPs at varying doses (15.6-250 µg/mL). Phosphate buffer saline (PBS, pH 6.4) was added to the mixture and left to incubate in a water bath at 37 °C for 20 minutes before being heated to 72 °C for 10 minutes. After letting the solution cool to ambient temperature, its turbidity was measured at 660 nm. Various quantities of diclofenac sodium salt (Sigma Aldrich, USA) (1 mg/mL stock, produced in methanol) ranging from 15.6-250 µg/mL were used as standards and evaluated comparably. The following formula was used to compute the percentage inhibition of protein denaturation:

% inhibition = $[(Ac-As)/(Ac)] \times 100$, where Ac absorbance of control; As absorbance of the sample.

2.8 Cell culture

The MG-63 cancer cell lines were used for the current study. The National Centre for Cell Science (NCCS) in Pune, India, is where these cell lines were obtained. In an incubator with a humidity level of 37°C and a 5% CO_2 environment, the cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum, 1% antibiotic and antimitotic solution. T-25 tissue culture flasks were used to maintain the stocks. Cells were collected by trypsinization and their numbers were counted.

2.9 In vitro cytotoxicity of biosynthesised A. fragilissima-AuNPs

The MTT test was used to evaluate the cytotoxicity of the synthesised gold nanoparticles in a cell culture medium at doses varying from 3.125 to 50 µg/mL. After that, the necessary concentration of nanoparticles was applied to every well, and the plates were kept at 37◦C for 24 hours to incubate. After incubation, the wells were washed with phosphate buffer saline (PBS). Following this, MTT solution was added to each well, and the wells were incubated in a CO_2 incubator for 4 hours. Once the crystals had formed, 50 µL of dimethyl sulphoxide (DMSO) was poured into each 96-well plate. The plates were then left in darkness for 10-15 minutes to let the crystals dissolve entirely. The optical density of the solutions was measured at 450 nm to ascertain cell survival, with the formula OD of nanoparticles/OD of Control ×100 being used. The findings could be accurately understood since the experiment was conducted in triplicates (Viswanathan et al., 2023).

2.10 Statistical analysis

The mean value was accompanied by the inclusion of the standard deviation (SD) in all reported measurement data. To compare the outcomes of the control and test samples, we used Microsoft Excel 2021. Results were

regarded statistically significant if the p-value was below 0.05.

3. Results and Discussion

3.1 Synthesis of gold nanoparticles using Amphiroa fragilissima

In order to synthesis colloidal AuNPs, the chloroauric acid ($HAuCl_4$) solution was added to the aqueous extract of *A. fragilissima*, causing a colour change from light yellow to purple (Figure. 1A&B). After 2 hours of incubation, the gold nanoparticles were completely reduced. Nanoparticle production resulted in the observed colour variations via surface plasmon resonance (SPR), which is induced by coherent oscillation of electron gas at the nanoparticles' surfaces (Sanchez-Ramirez et al., 2008).

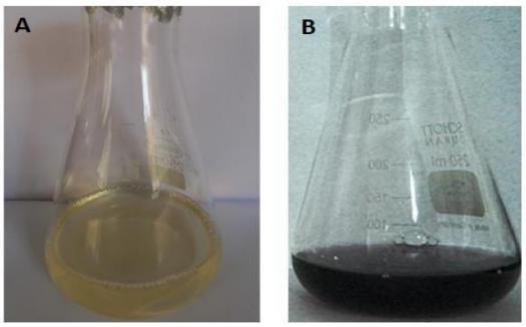

Fig 1. Synthesis of gold nanoparticles *Amphiroa fragilissima* A) Before synthesis B) After synthesis

3.2. Characterization of biosynthesized A. fragilissima-AuNPs

3.2.1. UV–Visible spectroscopy analysis of A. fragilissima-AuNPs

The reduction rates of biosynthesized gold nanoparticles are often evaluated by means of UV-visible spectroscopy, the most fundamental technique for characterising nanomaterials. The ultraviolet (UV) spectra of A. fragilissima-AuNPs were examined within the 400–700 nm range, with the absorbance peaking at 540 nm (Figure. 2). Gold nanoparticle stability was first verified by UV analysis. Results demonstrated a clear correlation between nanoparticle formation and absorbance intensity as well as the incubation period. A peak at 520 nm was detected for the Gracilaria verrucosa synthesised gold nanoparticles, suggesting that the colour change of the aqueous colloidal solution upon heating is caused by the activation of localised surface plasmon resonance (LSPR) (Chellapandian et al., 2019).

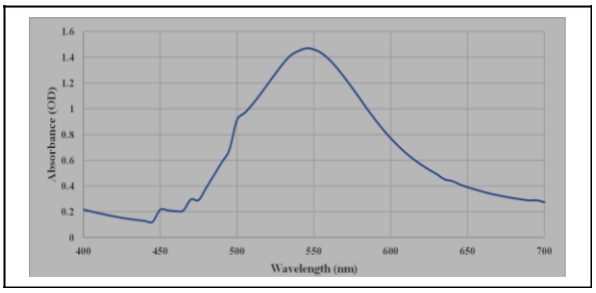

Fig 2. UV analysis of Af-AuNPs

3.2.2. Fourier Transform Infrared spectroscopy (FTIR) analysis of A. fragilissima-AuNPs

Green synthesised gold nanoparticles showed a strong peak at 2402.96 cm^{-1} and 3302 cm^{-1} belonging to O=C=O stretching (carbon dioxide) and O-H stretching (phenolic compounds). The vibrations of the C=C stretching in conjugated alkene compounds were detected at the mean peak of 1637 cm^{-1}. The weak peak at 2110 cm−1, the (C=C stretching) corresponds to alkyne groups was shown Figure. 3. This functional group is very likely to assist in the reduction and stabilisation of iron nanoparticles (Yadav, E et al., 2021). According to the reviewed literature, red seaweeds are efficient in producing phenolic chemicals, particularly bromophenols, in large quantities (Singh

et al., 2019, Gholami et al., 2022).

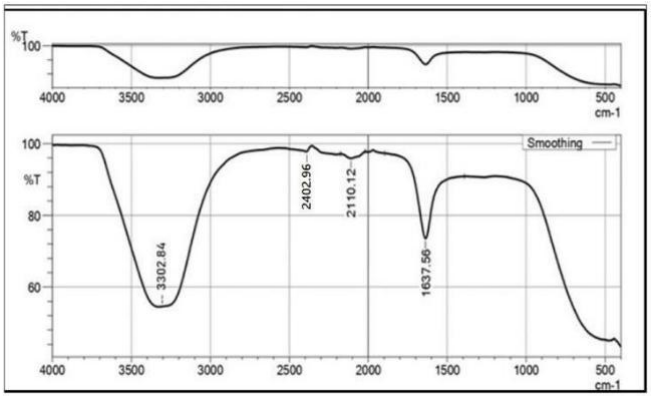

Fig 3. FTIR analysis of Af-AuNPs

3.2.3 Scanning electron microscope (SEM) analysis of A. fragilissima-AuNPs

The morphological properties of *A. fragilissima*-AuNPs were examined using scanning electron microscopy. The SEM picture indicated the Af-AuNPs in their aggregated state. Figure 4 shows that the majority of the synthesised gold nanoparticles were spherical and irregular in form and 25 nm in size. Particles of varying sizes formed spherical and circular patterns as a result of the interaction between the temperature, pH, and other biomolecules in the reaction mixture that was involved in the reduction of nanoparticles (Chen et al., 2021).

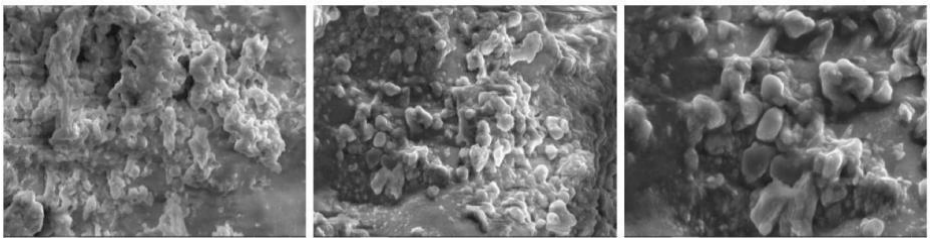

Fig 4. SEM images of Af-AuNPs

3.3 Antioxidant activity of synthesized Af-AuNPS

Cellular metabolism generates free radicals, which are very reactive and contain unpaired electrons; these molecules may cause harm to cells. Antioxidant capabilities are well-known to be present in seaweeds and their byproducts. To reduce the effects of oxidative stress, antioxidants are stable molecules that provide electrons to neutralise free radicals. The secondary metabolites known as phenolic compounds are abundant in seaweeds and are essential for the body to have enough antioxidants (Palanisamy et al., 2017). After evaluating these free radicals with Af-AuNPs and standard vitamin C (10-50 µg/mL), the results showed that they were effectively inhibited. An increase in the percentage inhibition of free radicals occurred when the sample concentration was raised. Figure 5 shows that at a dose of 50 µg /mL, Af-AuNPs exhibited the greatest inhibitory effect of 71.88% in the DPPH scavenging experiment. At a concentration of 50 µg/mL, Af-AuNPs showed a 65.97% suppression of free radicals in a hydrogen peroxide test Figure. 6. Minimising the effect of oxidative stress, the hydroxyl group of phenolic compounds capped gold nanoparticles scavenge free radicals by transferring hydrogen and electron molecules to hydroxyl radicals (Badeggi et al., 2020). The results demonstrated that lower quantities of Af-AuNPs had a stronger DPPH scavenging activity than before (Babu et al., 2020).

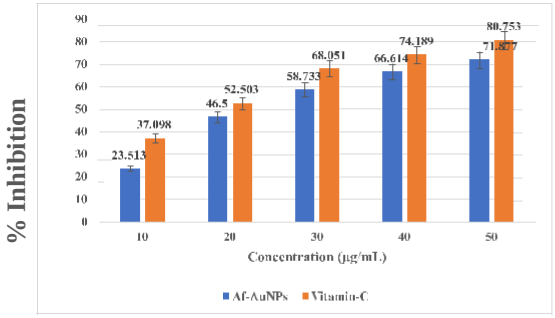

Fig. 5 DPPH radical scavenging activity activity of Af-AuNPs.

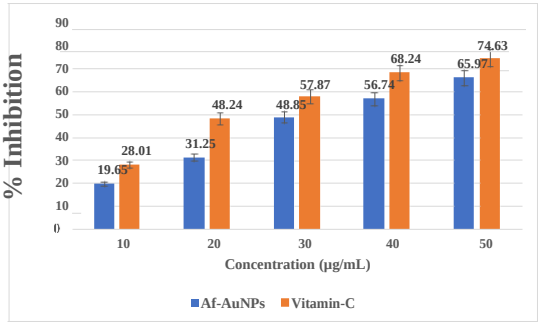

Fig. 6 H_2O_2 radical scavenging of Af-AuNPs

3.4 Anti-inflammatory activity of A. fragilissima -AuNPs

The inhibitory impact of synthesised Af-AuNPs was assessed against the denaturation of proteins/albumin. The Af-AuNps demonstrated a substantial reduction of albumin denaturation, even at low doses, in comparison to the conventional medication, Diclofenac sodium salt. An increase in inflammation was seen when the concentration of the test sample and standard Diclofenac sodium salt (10-50µg/mL) increased. A level of 67.78% inflammation was detected at 50 µg/mL Figure 7. The findings indicate that synthesised Af-AuNPs may regulate the formation of auto-antigens, which lead to protein denaturation under inflammatory conditions. Based on the aforementioned findings, it can be concluded that synthesised AuNP have the ability to regulate the formation of auto-antigens that lead to protein denaturation in the context of inflammation (Rajput et al., 2020).

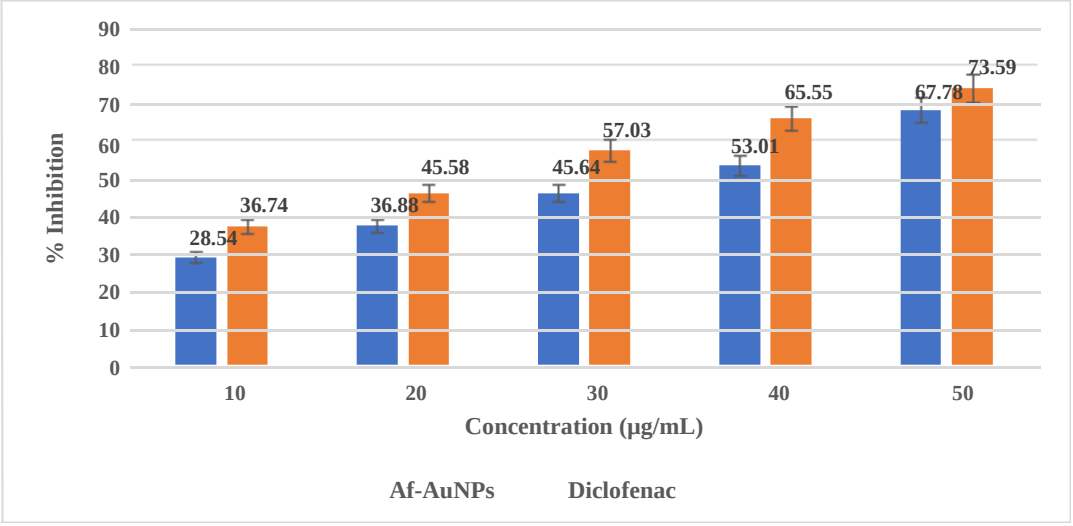

Fig 7. Anti-inflammatory activity of Af-AuNPs

3.5. In vitro anti-cancer activity of A. fragilissima -AuNPs

The MTT assay was used to determine the in vitro anticancer effectiveness of Af-AuNPs against the MG-63 osteosarcoma cell lines. *A. fragilissima* -AuNPs were added to the cells at concentrations varying from 3 to 50 µg/mL. After 24 hours, cells of osteosarcoma cancer treated with Af-AuNP showed signs of growth suppression Figure 8. As concentrations increased, the fraction of viable cells decreased linearly Figure 9. For biosynthesized gold nanoparticles, the IC50 in MG-63 cells was found to be 23.92 µg/mL. Out of all the metal nanoparticles, AuNPs are commonly considered to be the least harmful to normal cells and the most appropriate for distributing nanoparticles in living organisms (Hatipoğlu et al., 2023).

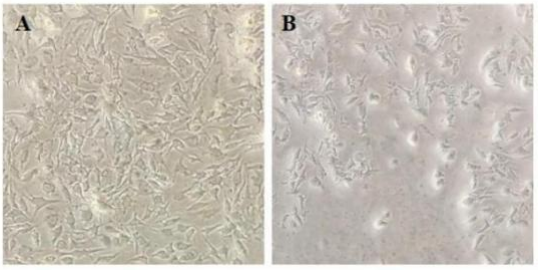

Fig 8. Anticancer activity of Af-AuNPs A) Control- Lung cancer cells B) Af-AuNPs treated cells

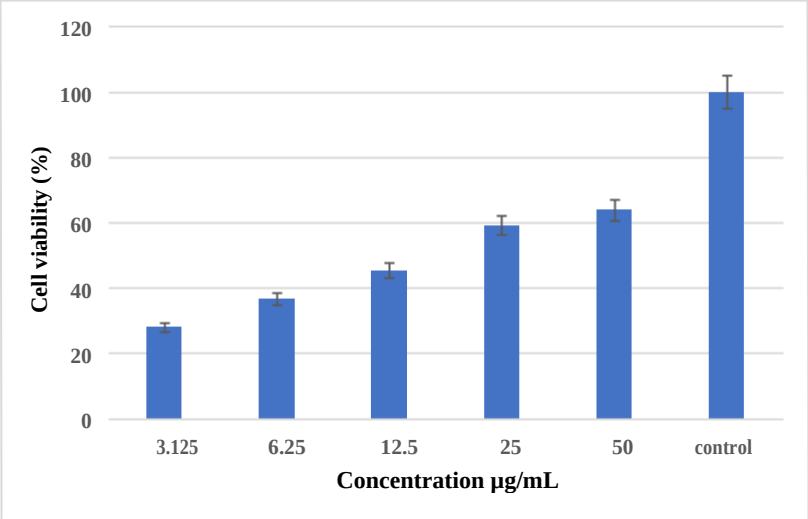

Fig 9. % inhibition of Af-AuNPs

4. Conclusion

In this study, the synthesis of gold nanoparticles from the red seaweed *Amphiroa fragilissima* is first documented. Biosynthesis is a safe and environmentally beneficial process. The synthesised *A. fragilissima*-AuNPs were validated by UV-visible spectroscopy, and the morphology was shown by SEM. The presence of functional groups was determined by FTIR analysis. The antioxidant activity property of Af-AuNPs was comparable to that of ascorbic acid, suggesting that they may be useful as an antioxidant. Producing gold nanoparticles from Amphiroa fragilissima may have anti-inflammatory properties. Furthermore, they showed promising anticancer effects by decreasing cell viability and inducing cell death in human osteosarcoma (MG-63) cancer cells. The results show that *A. fragilissima* -AuNPs might be very effective against osteosarcoma cancer cells.

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