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RESEARCH ARTICLE

Study of Antioxidant, Antimicrobial and Cytotoxic Activities of Ag-Co Bimetallic Nanoparticles Biosynthesized from Red Alga (*Amphiroa* sp.)

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Abstract

Objectives: To biosynthesize, evaluate, and investigate the green synthesis of silver-cobalt bimetallic nanoparticles using red alga *Amphiroa* sp. and its interactions with human pathogens in a colloidal condition. **Methods:** The marine red algal extract of *Amphiroa* sp. was used to synthesize the bimetallic nanoparticles of Ag and Co. For this 50mL of 10^{-3} aqueous Ag-Co prepared solutions were combined with 50mL of pure algal extract. It was characterized by UV-Vis, FTIR, XRD, and SEM. It was tested for its antioxidant, antibacterial, and cytotoxic activities. The antibacterial activity of bimetallic nanoparticles was tested on five human pathogens *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The MTT assay method was used for cytotoxic activity for MCF-7 Breast cancer cell lines. **Findings:** The green synthesized *Amphiroa* sp. bimetallic nanoparticles showed a UV-Vis spectrum absorption peak at 517nm. Analysis of the FTIR spectra verified the functional groups involved in the production of the Ag-Co nanoparticles. The diffraction pattern of silver-cobalt nanoparticles and the X-ray diffraction pattern of silver nanoparticles showed diffraction angles at 2θ values of 32.5°(15), 46.5°(11) which correspond to (111), (200), and (220). The particle size distribution, which ranges from 56 to 250nm, and the shape was revealed by SEM investigation to be cubic to rhomboidal, thus, they were confirmed to be nanoparticles as well as fine particles/particulate matter. Radical scavenging activity by DPPH, ABTS, and ferrous-reducing power assays were used to investigate the antioxidant potential. The antibacterial activity of bimetallic nanoparticles was tested on five human pathogens and with the zone of inhibition of 23, 18, and 20 mm for *Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis*, respectively. The cytotoxic effect was observed on MCF-7 cancer cell lines were evaluated by MTT assay. The IC_{50} value of our Ag-Co bimetallic sample was found to be 70.90μg/ml at 200μg/mL concentrations.

Novelty: Biosynthesizing and investigating Ag-Co bimetallic nanoparticles using red alga and its characterization, antioxidant, antimicrobial and cytotoxic activity.

Keywords: *Amphiroa* sp.; Ag-Co Bimetallic nanoparticles (BNPs); antioxidant, antibacterial and cytotoxic activity

1 Introduction

One of the scientific fields that are expanding quickly is nanotechnology, a very young field of study. Because of its extensive use in sectors like as food, cosmetics, pharmaceuticals, chemicals, and agriculture, it is a subject of great interest to academicians [(1)].Metallic nanoparticles are extremely small particles, usually between one and one hundred nanometers in size composed of metals such as platinum, gold, and silver [(2)].Due to their unique size-dependent behaviour and characteristics, metallic nanosystems have drawn a lot of attention in this unusual field of study and are highly recommended as robust and appropriate instruments for targeted, regulated, and prolonged drug release [(3)].The benefits of green nanoparticle are reduced costs, environmental friendliness, and the potential for simple and inexpensive industrial production [(4)].It is common for the preparation techniques to use expensive, hazardous, or environmentally carcinogenic substances. Therefore, it is imperative to enhance synthesis processes in order to confront every problem that chemical approaches cannot solve. Thus, green synthesis methods (biological synthesis) were introduced by several researchers using bacteria, fungi, and plant extracts [(5–7)].

Bimetallic nanoparticles (BNPs) are created by combining two distinct metals to give each of the two metals new characteristics. Because they have better particular qualities than their related monometallic nanoparticles (MNPs), they are more significant [(8)].By combining two metals, bimetallic nanoparticles provide scientists with a versatile technique to modify the properties of the particle, improving its uses in biology, imaging, and catalysis [(9)].Because of their great stability, low toxicity, and good biocompatibility, bimetallic nanoparticles are essential. The combination of bimetallic nanoparticles such as Ag/Pd, Ag/Ni, and Au/Pt NPs was reported as more promising photocatalysts than monometallic NPs such as silver, gold, and palladium [(10)]. Plant extracts were frequently used to achieve the desired size and shape of Ag monometallic and Ag/Pd bimetallic nanoparticles. BNPs were synthesized using plant extracts, i.e., *Terminalia chebula, Catharanthus roseus*, and *Cacumen platyclade*, which were used for its antibacterial, anticancer, and hydrogen production [(11–14)].

However, no reports were found, based on the synthesis of Ag/Co BNPs using algae. Algae are proved to have more natural products to synthesize NPs, which serve as capping agents on nanoparticles [(15)].

Cancer, which is a serious health concern for the ageing population, lifestyle choices, environmental changes, genetics, and heredity factors, all contribute to the pronounced prevalence of it [(16)]. In order to address this, nanosized formulations of developed drug treatments may be able to treat cancer by specifically targeting the malignant cells which are proliferating. This would eliminate the need for the currently available therapies, which are often redundant, damaging, and also impair the viability of non-cancerous cells [(17)].

From this perspective, our goal in exploring into this work was to highlight the use of algae in the creation of bimetallic Ag-Co bimetallic nanoparticles as a first report. Thus, our study involved synthesis of a bimetallic nanoparticle containing both silver and cobalt metal using our algal extract of *Amphiroa* sp. was characterized by UV-Vis, FTIR, XRD, and SEM, and studied for its antioxidant potential (DPPH, ABTS, and ferrous-reducing activity) as well as its antibacterial activity relation to five bacterial

pathogens namely *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Additionally, the bioactive potency of the bimetallic nanoparticles against MCF-7 breast cancer cell lines using MTT assay was also evaluated.

2 Methodology

2.1 Chemical and reagents

The chemicals used were Cobalt chloride ($CoCl_2.6H_2O$), silver nitrate ($AgNO_3$), 1,2-diphenyl-2-picryl-hydrazine radical (DPPH), 2,20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Ferrous sulphate, potassium persulfate, Methanol, Ethanol. Dry algal powder of *Amphiroa* sp, was used for extract preparation. All reagent solutions and dilutions were prepared using distilled water and for DPPH, ABTS and Ferrous sulphate were prepared by using methanol.

2.1.1 Collection of sample and extract preparation

The macroalgae samples *Amphiroa* sp. was imported from Rameshwaram coast, Tamil Nadu. Tap water was used to clean the collected samples. Following a sterile distilled water wash, it was left to dry in the shade for 4-5 days at room temperature. Next, a fine powder was made from the algal samples. 1g of algal powder was added to 100mL of glass distilled water and boiled for 15min, and then the extract was cooled, filtered, and used for further studies.

2.1.2 Synthesis Ag-Co BNPs at room temperature

Aqueous solutions of 10^{-3}M $AgNO_3$ and $CoCl_2$ solutions were prepared separately and mixed together. From this bimetallic solution 50mL was added to the 50mL of algal extract for synthesis of Ag-Co bimetallic nanoparticles. At room temperature, the reaction mixture was maintained. The colour variations of the reaction solution mixture changes were visually observed. The solution was stored for 24 hours after which the nanoparticles obtained were evaporated and dried in an oven at 105°C[18].

2.1.3 Characterization of Synthesized Ag/Co BNPs

Ultraviolet-Visible spectroscopy (UV-Vis), Fourier transfer infrared spectroscopy (FTIR), X-ray diffraction (XRD), and Scanning electron microscopy (SEM), were the techniques used to study nanoparticles. The primary use of FTIR was for the analysis of the functional groups involved in the bio-reduction of metal ions into metal atoms. The XRD was used to determine the particle size and crystalline phases. Using an SEM, the morphology and structure of the BNPs were evaluated. To characterize the biosynthesized particle, analysis was conducted in advanced laboratories and across many institutions.

2.1.4 Analysis of the antioxidant activity of Ag-Co BNPs

Three methods were followed for the evaluation of the antioxidant activity of BNPs i.e., DPPH (1,2-diphenyl-2-picryl-hydrazine), $ABTS^+$[2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], Ferrous reducing power assay was carried out according to the method of Kourti[19] with slight modifications.

2.1.5 Antibacterial activity

Antibacterial activity of algae-assisted silver nanoparticles was carried out by well diffusion test technique against gram-positive and gram-negative bacteria. Bacterial cultures such as *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae*, *Staphylococcus aureus, Pseudomonas aeruginosa*, were acquired from Mahathi biotechnologies, Ramapuram, Chennai-89. BNPs were tested for their bactericidal action by dissolving 10 mg of Ag-Co nanoparticles in 1 ml of glass distilled water. Using sterile cotton swabs, McFarland standard (104–106 CFU/mL) was applied to the Muller-Hinton agar plate surface, resulting in homogeneous bacterial lawns. A sterile metallic well borer was used to drill 6mm wells, and various concentrations of dissolved bimetallic nanoparticles were added to each well. The plates were incubated at 37°C for 24hrs after being left in the biosafety cabinet for 10mins to allow for adequate diffusion. Amoxicillin was used as positive control. The diameter in millimetres was utilized to quantify an activity within the zone of inhibition.

2.1.6 Cell Viability Assay

The assay was carried out using (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48hr incubation, the wells were added with MTT (5mg/mL) and left for 2hrs at room temperature. All wells' contents were

removed using the pipette and 100μl DMSO was added to dissolve the formazan crystals; absorbance was read in a Readwell Touch Microplate reader at 570nm [20].

Cell Viability = OD of Sample / OD of Control × 100

3 Results and Discussion

One of the intriguing aspects of this work is the green synthesis of *Amphiroa* sp. (a red alga) mediated Ag-Co bimetallic nanoparticles, as on record no reports are available on synthesis of bimetallic nanoparticles from this alga, and many previous studies concentrated only on single metal nanoparticles. In this research, we have synthesized, characterized, and investigated for possible medical uses. Our findings have demonstrated antioxidant potential along with antibacterial and cytotoxic properties that may help increase the effectiveness of anticancer drugs.

3.1 Synthesis of BNPs

A reduction in metal ions and the production of NPs are indicated by a change in the colour of the reaction media. The colour shift of the reaction mixture indicates that the formation of silver cobalt nanoparticles occurs simultaneously with the instantaneous commencement of green reduction of the silver ions. The colour variation of the algal extract was obvious on visual observation from pinkish to light brown [18,21]. This transition of colour is mainly attributed to the algal active compounds that act as reducing, stabilizing, and capping agents [Figure 1].

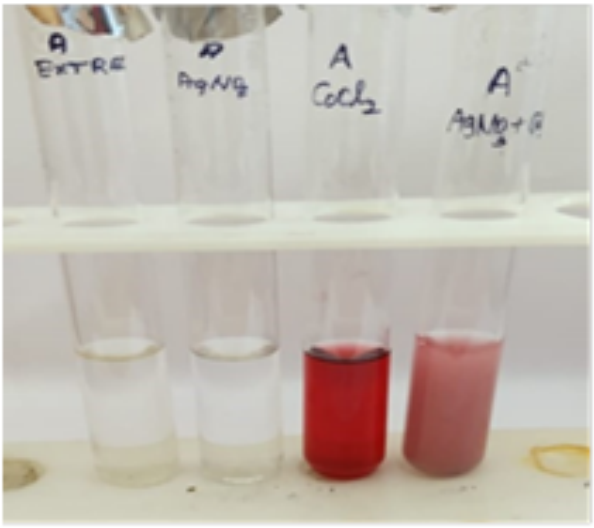

Fig 1. Green Synthesized *Amphiroa*sp. mediated Ag-Co BNPs

3.2 UV-Vis analysis

UV-visible spectra of silver cobalt bimetallic nanoparticles were obtained to assess the formation of nanoparticles. The formation of biosynthesized BNPs was confirmed by the absorption spectrum peak of Ag-Co bimetallic nanoparticles between 400 and 700nm wavelengths. After the reduction of BNPs, the reaction mixture colour also changed, which indicated the confirmation of the synthesis of BNPs. The BNPs showed peak at 517 nm and this characteristic absorbance peak confirmed that BNPs were successfully synthesized [Figure 2]. The intensity of colour increased which was in direct proportion to the incubation period. It may be due to the excitation of surface plasma resonance (SPR) effect and reduction of $AgNO_3$ [22–24].

3.3 FTIR analysis

The FTIR spectrum of BNPs of *Amphiroa* sp. shows strong IR bands characteristic peaks around 447.00, 760.51,818.79, 1045.31, 1330.80,1637.51, 2324.90, 3368.40 and 3485.04cm^{-1},respectively. The observed peaks denote the presence of -C-O-C-, ether

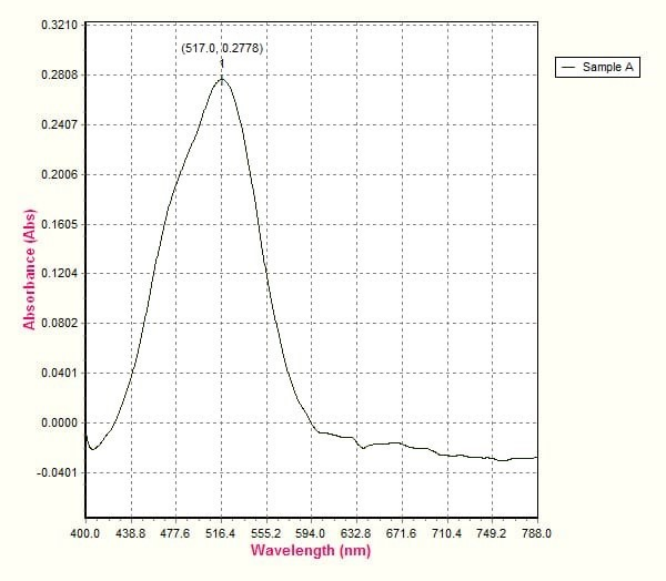

Fig 2. UV-Visible Spectrum of *Amphiroa* sp. mediated Ag-CoBNPs

linkages, -C-N of aromatic amines, -C=C- group aromatic –C=C- stretch, -NH_2 and -OH groups stretch, respectively [Figure 3]. Concurrently, it was observed that absorption peak values differed among others [18,25,26].

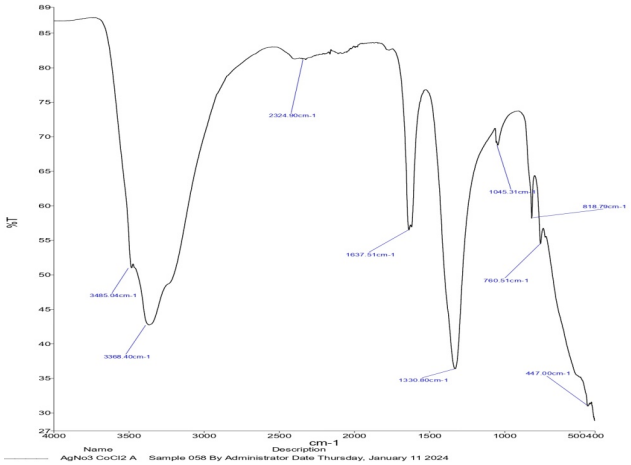

Fig 3. FTIR Spectrum of *Amphiroa* sp. mediated Ag-CoBNPs

3.4 XRD analysis

The bio-synthesized bimetallic nanoparticles powder XRD peaks, which were obtained from *Amphiroa* sp. aqueous extracts, agree well with the JCPDS - International Centre for Diffraction Data Sample Preparation Methods in X-Ray Powder Diffraction. The diffractogram indicates the reflection peaks numbers 2, 7, and 9 at 38.1°, 64.5°, and 77.5°, corresponding to the refection of Ag-Co crystalline planes (111), (200), and (311) diffraction planes, respectively [27,28]. Moreover, the spectrum also shows the diffraction at 2θ values of 32.5^o (15), 46.5^o (11). Based on these results, we conclude that Ag is the core and Co is the shell; that is, Ag atoms form the dark solid core of particles, whereas Ag atoms are responsible for the bigger greyish sphere around Ag atoms [Figure 4]. Antony [29] and coworkers in 2019 worked with Fe_3O_4@CS_AgNi bimetallic NPs in a similar way, and ours peaks are in accordance with their Powdered XRD results.

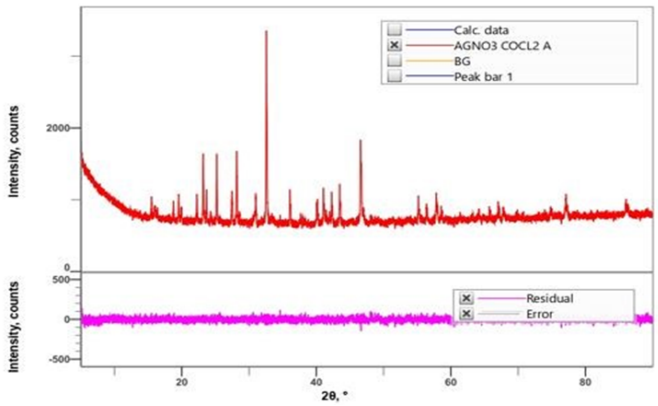

Fig 4. X-RD of *Amphiroa* sp. mediated Ag-CoBNPs

3.5 SEM analysis

Our SEM images remarkably show how the Ag-CO bimetals changed into cubic to rhomboidal, non-aggregating silver nanoparticles with an average diameter of 56–250 nm and thus, our investigations show that it was nanoparticles as well as fine particles/particulate matter. Co-core SEM photos are displayed at various magnifications. It is evident that the core particles varied in size from 200 nm to over 1 μm, exhibiting cubic to rhomboidal forms. The significant size variance may result from agglomeration (caused by the magnetic nature of cobalt) comprising single cobalt particles or parts that combine to form bigger particles [Figure 5 **a, b**]. Our results are in accordance with other reports. The findings of Jamil [30] SEM images of Fe-Ni bimetals revealed that the nanoparticles had merged surfaced and were irregular in form and ranged from 50-100μm and had a compact shape were uncertain and irregular [31].

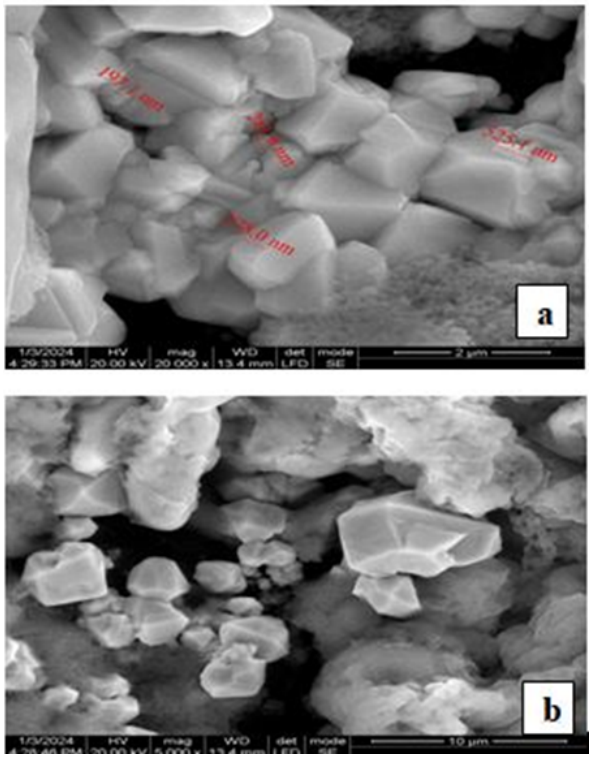

Fig 5. SEM of *Amphiroa* sp. mediated Ag-Co BNPs at (a) at 2 μm (b) at 10 μm

3.6 Antioxidant activity

Three *in vitro* assay techniques were used to assess the antioxidant activity of the algal extracts: the ferrous reducing assay, ABTS+[2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid)], and DPPH (2,2-diphenyl-1-picryl-hydrazine) [32]. Regression equations derived from the extract concentrations and percentage inhibition were created, taking into account 0% inhibition in the assay combination without plant extract. The regression equation was used to determine the IC_{50} values, or the concentration of sample needed to scavenge 50% of the free radical. Figure 6 **a, b** and **c** demonstrate the IC_{50} values, correspondingly, of 66.43, 173.87, and 88.55 respectively in the assays.

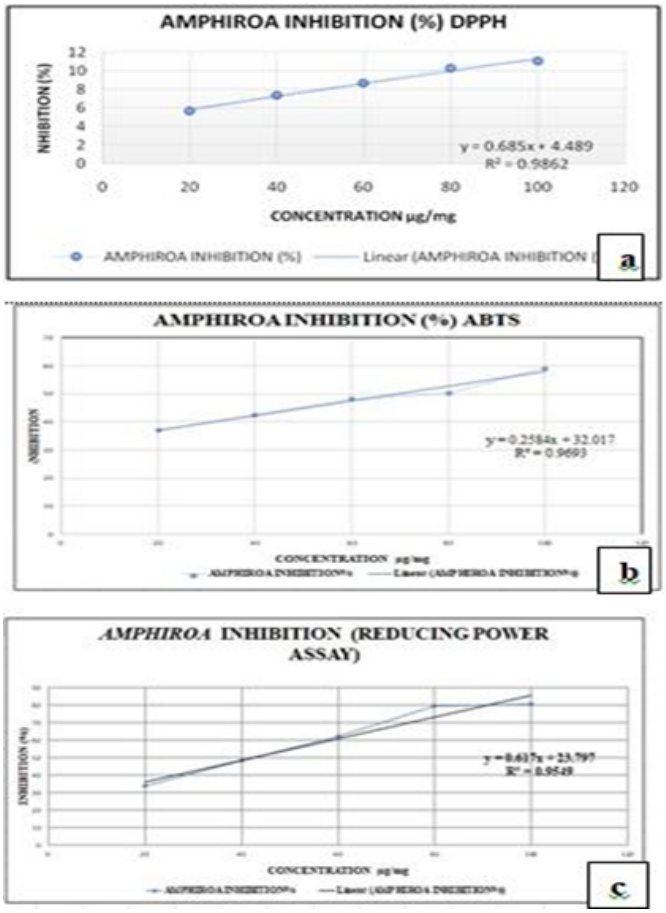

Fig 6. Antioxidant activity of the *Amphiroa* sp mediated Ag-Co BNPs. (a) DPPH, (b) ABTS and (c) Ferrous reducing power assay

3.7 Antibacterial activity

The synthesized BNPs antibacterial activity against both Gram-positive and Gram-negative bacteria was evaluated using the conventional Mueller-Hinton agar well diffusion method. Silver nitrate was used as a negative control and amoxicillin as a positive control. The results in Figure 7 and Table 1 depicted that the inhibition zone for BNPs increased for *Escherichia coli* compared to other bacterium. The maximum antibacterial activity in 50μl concentrations of the synthesized bimetallic nanoparticles were 18, 23 and 17 mm for gram-negative bacteria like *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia* and 20, 18 mm for gram-positive bacteria like *Bacillus subtilis* and *Staphylococcus aureus*, respectively. *Pseudomonas aeruginosa* showed the highest antibacterial activity for synthesized bimetallic nanoparticles than other bacterial strains. This confirms that bimetallic nanoparticles from Ag-Co exhibited good antibacterial potential against gram-negative bacterial strains when compared to gram-positive strains. The reason for the different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these organisms. Gram negative bacteria have outer phospholipidic membrane which carries the structural lipopolysaccharide components. This makes the cell wall

impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da. Hence, the gram-positive bacteria should be more susceptible having only and outer peptidoglycan layer which is not an effective permeability barrier[33,34].

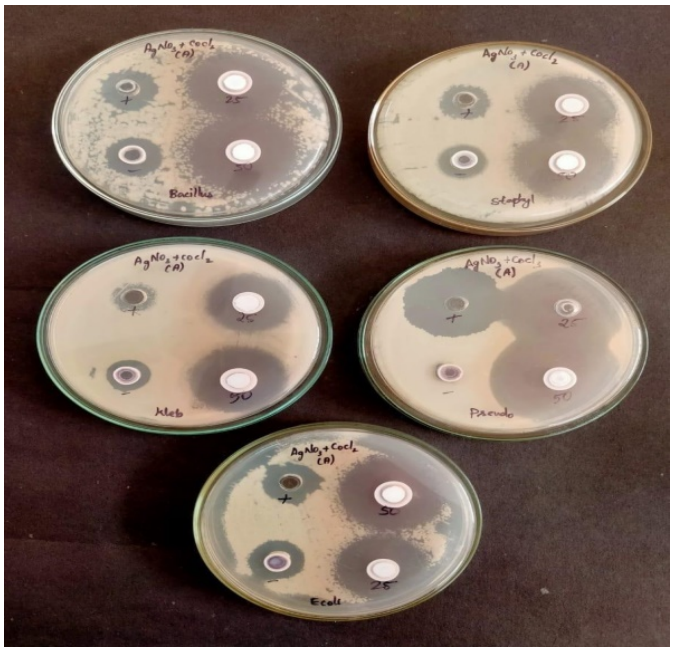

Fig 7. Antibacterial activity of *Amphiroa* sp. mediated Ag-Co BNPs against (1) *Bacillus subtilis* (2) *Escherichia coli* (3) *Pseudomonas aerunginosa* (4) *Staphylococcus aureus* (5) *Klebsiella pneumoniae*

Table 1. Antibacterial activity of *Amphiroa* sp. mediated Ag-Co BNPs against human pathogens

Clinical Pathogens	Positive Control (mm)	Ag-Co BNPs (mm)	*Amphiroa* sp.	
			25μl	50μl
Bacillus subtilis (+ve)	12	10	17	20
Escherichia coli (-ve)	10	9	15	18
Pseudomonas aeruginosa (-ve)	19	5	**22**	**23**
Staphylococcus aureus (+ve)	9	8	18	18
Klebsiella pneumonia (-ve)	7	7	15	17

3.8 Cell Viability Activity

The prepared BNPs was assessed using MTT assay method upon MCF-7 (breast cancer), the bimetallic test samples varied significantly in concentration, which had a significant impact on the *in-vitro* cytotoxicity activities in MCF-7 cell lines [Table 2]. After 48 hours of treatment, the MCF-7 cell line demonstrated substantial cytotoxicity effects in the tested samples at greater concentrations. This observation also indicated that test sample cytotoxicity increased with increasing concentration [Figure 8 **a**]. In comparison to various concentrations, the IC_{50} value of our Ag-Co bimetallic sample was 70.90 μg/ml at 200μg/mL [Figure 8 **b**]. While Copper/Zinc bimetallic nanoparticles on MCF-7 cancer cells was studied in other investigation with an IC_{50}value of roughly 54μg/mL. When these nanoparticles were added to the MCF-7 cell line, they exhibited 100% cytotoxic effects at concentrations greater than 600 μg/mL concentrations[35]. All of these findings suggest that using bimetallic nanoparticles together as a nanoparticle works well against cancer cell lines. Al-Radadi 2021found that Half maximal inhibitory

concentration (IC_{50}) of licorice root extract mediated synthesized AuNPs observed at concentration of 50 μg/ml towards MCF-7 cell line [32,36,37].

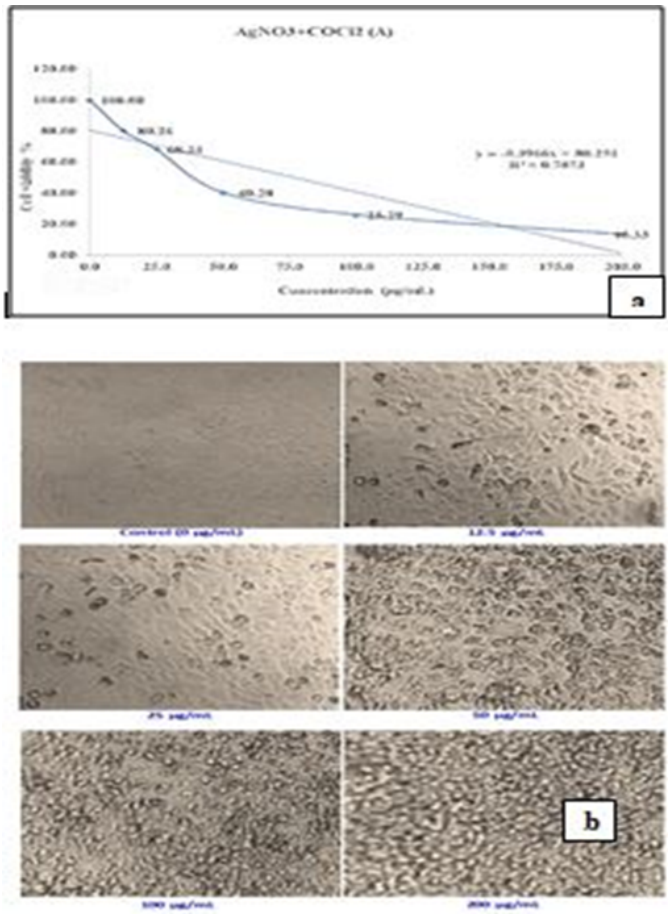

Fig 8. (a) Cell viability and (b) cytotoxicity effect of *Amphiroa* sp. mediated Ag-Co BNPs against MCF-7 Cell lines

Table 2. *In vitro* Cell viability of *Amphiroa* sp. mediated Ag-Co BNPs against MCF-7 Cell lines

Concentration (μg/ml)	Cell Viability (%)
	$AgNo_3$ + $CoCl_2$ (A)
0.0	100.00
12.5	80.26
25.0	68.23
50.0	40.20
100.0	25.79
00.0	13.33

4 Conclusion

This investigation may be the first ever report for using the red alga *Amphiroa* sp. for green synthesizing and testing as an Ag-Co bimetallic agent. We conclude that the extracts of marine alga *Amphiroa* sp. are capable of producing the Ag-Co bimetallic nanoparticles, extracellularly, and they were stable in solutions due to capping likely by proteins. The amines, peptides groups and secondary metabolites, in the extract helped in the bioreduction and stabilization of the bimetals. They were characterized by UV-visible spectra, FTIR and XRD. The UV-visible spectra showed a peak at 517 nm, and the FTIR showed the presence of

-C-O-C-, ether linkages, -C-N of aromatic amines, -C=C- group aromatic –C=C- stretch, -NH_2 and -OH groups stretch, that confirmed the formation of nanoparticles. Based on The XRD results, we conclude that Ag is the core and Co is the shell; that is, Ag atoms form the dark solid core of particles, whereas Ag atoms are responsible for the bigger greyish sphere around Ag atoms. SEM studies revealed that the bimetals had a cubic to rhomboidal, non-aggregating nanoparticles with an average diameter of 56–250 nm, thus, our investigations show that it was nanoparticles as well as fine particles/particulate matter. These bimetals showed very good antioxidant activities and had IC_{50} values, of 66.43, 173.87, and 88.55 for the ferrous reducing power assay, ABTS+, and DPPH activities respectively.

Towards the applications-oriented aspect also, they showed positive and favorable outcomes for both antibacterial and anticancer activities. The bimetals had a very good antibacterial activity, and it was maximum for *P. aeuruginosa* (-ve) bacteria with a zone of inhibition of 23mm at 50 μl concentration, among the tested five pathogens. The cytotoxic studies revealed that the MCF-7 cell line, the IC_{50} concentration of our Ag-Co bimetallic sample had 70.90μg/ml at 200μg/mL suggesting that it works effectively against this cancer cell lines. Thus, the BNP is a straightforward, adaptable, and easy-to-synthesize, a general-purpose vehicle with the ability to facilitate the delivery of a wide-variety of therapeutic agents for antibacterial and anticancer agents. The results obtained are optimistic and promising enough, and we trust they can be used for many applications in nanomedicine, food and other industries.

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