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(RESEARCH ARTICLE)

Green synthesis and characterization of bimetallic nanoparticles (Cu-Zn) FROM *Gracilaria edulis* and evaluation of their biological activities

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Abstract

Gracilaria edulis belonged to the Gracilariaceae family. Gracilaria edulis was a type of red algae species. It was an edible seaweed present on the southeast coast of India. It was used as an alternative food supplement to fulfil the basic nutritional needs of humans. It acted against bacteria. It was analysed using the agar-well diffusion technique. In this work bimetallic (Cu-Zn) nanoparticles were synthesized. This aqueous extract acted as both a surfactant and a reducing agent. Then the nanoparticles were characterized by FTIR spectroscopy, UV-visible spectroscopy, SEM, DLS, and XRD to identify the physicochemical properties of the nanoparticles. Each characterization technique was used for various analyses of nanoparticles. The size, shape, and nature of the crystal were identified by SEM, DLS, and XRD techniques. The Fourier transform infrared (FT-IR) spectroscopy technique was used to identify the functional group of the bimetallic nanoparticles. The UV-Vis spectroscopy technique was used to show the optical and chemical properties and formation of colloidal plasmon bands of the bimetallic nanoparticles. Then the bimetallic nanoparticles and aqueous extract were used to evaluate their antimicrobial activity. From there, we confirmed the bimetallic nanoparticles and aqueous extract are acted against bacteria and the bimetallic nanoparticles (Cu-Zn) had more properties compared to monometallic nanoparticles (Cu, Zn).

Keywords: Green synthesis; *Gracilaria edulis*; Phytochemical; Bimetallic nanoparticles; Antimicrobial

1. Introduction

Nanotechnology combines the sciences of biology, chemistry, and physics to create nanoparticles with specific roles. Nanoparticles (NPs) have varying sizes and forms. However, sizes usually range between 1 and 100 nm [1].

Nanoparticles distinctive features are caused by a rise in surface-to-volume ratio, which modifies chemical catalytic properties. Metal nanoparticles are known for their anticancer, antioxidant, and antimicrobial properties, as well as their ability to aid in wound healing. It is essential to note that nanoparticles have numerous uses in organic and applied chemistry [2].

Biologically synthesized nanoparticles, particularly those produced using "green" methods, have gained attention for their unique particle size and shape-dependent physical, chemical, and biological properties. Biological synthesis of nanoparticles is superior to chemical and physical synthesis because it is less expensive, environmentally benign, and a viable alternative process that does not require the use of hazardous chemicals, high pressure, energy, or temperature [3]. Bimetallic nanoparticles (BNPs) are an original form of nanomaterial composed of two distinct metallic components. Although their synthesis is comparable to that of their monometallic counterparts in certain aspects, the synergetic action between the two components allows them to demonstrate a variety of distinctive characteristics and applications [4].

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The synthesis of nanoparticles using algae can be divided into three steps: heating or boiling algal extract in water or an organic solvent for a set period of time, preparing molar solutions of ionic metallic compounds; and incubating algal solutions and molar solutions of ionic metallic compounds for a set period of time under controlled conditions. The creation of nanoparticles is dose-dependent and also influenced by the type of algae employed. Metal reduction is facilitated by a range of biomolecules, including polysaccharides, peptides, and pigments. Proteins stabilise bind the metal nanoparticles in aqueous solutions via amino groups or cysteine residues [5].

Marine macroalgae, often known as seaweeds, are the fundamental components of the marine ecosystem. Seaweeds are a rich source of bioactive chemicals with various properties and have enormous biological activities [6].

The advantage compared to terrestrial plants in producing bimetallic NPs is due to their absence of competition for agricultural land and freshwater supplies. As a consequence, they are a sustainable source for large-scale manufacturing of green nanoparticles [7].

Macroalgae are a major natural resource in the ocean, accounting for 27% of the worldwide production of aquaculture. They are commonly used for human consumption, hydrocolloid production, fertilisation, and animal feed [8]. *G. edulis* is a common red seaweed found along the sea shore. This is a major source for agar extraction and associated goods. It has several biological qualities, including antiviral, antifungal, anti-inflammatory, antioxidant, and cytotoxic activities [9].

Antimicrobials are commonly used to treat diseases in people and promote growth in animals such as fish [10]. Over past years, there has been an enormous increase in research on marine algae to discover new and effective natural medications. Several chemicals seaweeds, which produce proteins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids, cardiac glycosides, phenol and saponin have the possibility of industrial and biotechnological applications [11].

Algal-synthesised NPs are becoming increasingly essential in biomedicine due to their antimicrobial, antioxidant and wound healing properties [5]. In this study Cu, Zn bimetallic NPs have been synthesized using *Gracilaria edulis* extract via a green method. The synthesized bimetallic NPs have been characterized by UV-visible spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, Dynamic light scattering particle size analyser (DLS). Additionally, the antimicrobial activity of these NPs has been evaluated

2. Material and methods

2.1. Collection of Gracilaria edulis and preparation of the extract

Gracilaria edulis was commercially collected from the TSC Purple Turtle Private Limited, Thoothukudi, Tamil Nadu, India. The *Gracilaria edulis* was collected. The collected plant materials were washed thoroughly with running tap water (2–3 times) and distilled water to remove salts and unwanted content. They were dried in a separate room to avoid the sunlight, which led to degradation. Then, the dried algae are chopped using a mixer grinder to produce a fine powder. The powdered algae were stored in an airtight container. Then 10 g of algae powder was mixed with 200 ml of sterile distilled water. The mixture was then stirred and boiled at 60˚C using a magnetic stirrer with a hot plate for 1 hour 30 minutes. Then, extraction was allowed to cool at room temperature for 10 minutes. The mixture was then centrifuged for 10 minutes. Then, this mixture was filtered through the Whatman No. 1 filter paper. The extract was stored at 4 ˚C for further experiments.

2.2. Phytochemical screening of an aqueous extract

Medicinal plants or algae contain bioactive substances like proteins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids, cardiac glycosides, phenol and saponin that provide physiological action in the human body. These compounds are synthesized through the primary or secondary metabolism of living organisms and are widely used in human therapy, veterinary medicine, agriculture, and scientific research. Phytochemicals from various chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro.

2.3. Preparation of Nanoparticles

Prepare a 0.01 M, 0.1 M, 0.2 M solution of zinc acetate and 0.01 M, 0.01 M, 0.1 M, 0.2 M solution of copper sulphate. Combine the two solutions 0.01 M zinc acetate and 0.01 M copper sulphate, 0.1 M zinc acetate and 0.1 M copper sulphate, 0.2 M zinc acetate and 0.2 M copper sulphate and stir each combination separately at 80°C for 30 minutes. To adjust the pH, add 10 ml of NaOH. Continue stirring at 80°C for 1 hour and 30 minutes. The change in colour of the solution

indicates that nanoparticles have formed. Allow the solution to cool to room temperature and then seal it. The next day, centrifuge the nanoparticle sample. Then wash the sample with water two times and ethanol once. Remove the supernatant and transfer the pellet to a plate. Incubate in a hot air oven at 120°C for 1 hour and 30 minutes. After that, grind the nanoparticles using a mortar and pestle. Finally, measure the weight of the ground nanoparticles using a scale.

2.4. Bio synthesis of Bimetallic (Cu-Zn) nanoparticles

Prepare a 0.1M solution of zinc acetate and a 0.1M solution of copper sulphate. Combine the two solutions and stir at 80°C for 30 minutes. To adjust the pH, add 10 ml of NaOH. Then, add 50 mL of algal extract drop by drop continue stirring at 80°C for 1 hour and 30 minutes. The change in color of the solution indicates that nanoparticles have formed. Allow the solution to cool to room temperature and then seal it. The next day, centrifuge the nanoparticle sample. Then wash the sample with water two times and ethanol once. Remove the supernatant and transfer the pellet to a plate. Incubate in a hot air oven at 120°C for 1 hour and 30 minutes. After that, grind the nanoparticles using a mortar and pestle. Finally, measure the weight of the ground nanoparticles using a scale.

2.5. Biological Application

2.5.1. Estimation of Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method with gallic acid as the positive control. Initially, a calibration curve was created using a range of gallic acid solutions with concentrations from 0.01 to 0.16 mg/ml. Subsequently, different volumes of the 10-fold diluted Folin-Ciocalteu (RF-C) reagent were mixed with 1.5 mL of the gallic acid solution. To neutralize any remaining reagent, 1.2 milliliters of 7.5% (w/v) Na_2CO_3 solution were added to the mixture after leaving it at room temperature for five minutes. Absorbances were measured at 760 nm using a SHIMADZU UVmin-1240 UV-VIS spectrophotometer after two hours of incubation at 25 ◦ C. Additionally, oil was produced using 0.3 mL of each solution. The measurements were carried out in three repetitions.

2.5.2. Estimation of Total flavonoid content

Total flavonoid concentration (TFC) of *Gracilaria edulis* extracts was measured using the aluminum chloride ($AlCl_3$) colorimetric technique, as reported by. To summarize, the following mixture was used: 2.4 mL of distilled water, 0.3 mL of a 5% $NaNO_2$ solution, and 1 mL of extract (1 mg/mL). After six minutes, 0.3 mL of a 10% $AlCl_3$ solution was added. One milliliter of a 1.0 M NaOH solution was added to the previous mixture after five minutes of inactivity. The liquid was completely mixed with a vortex before the absorbance at 510 nm was measured. The linear equation of the calibration curve was used to calculate the TFC of the extractions, and the result was expressed in terms of catechin equivalents (CAE) (average ± SD for triplicates).

2.6. Antimicrobial Activity

Two bacterial pathogens used for their antibacterial properties include *Staphylococcus aureus* and *Bacillus subtilis.* The in vitro antibacterial effectiveness of Zn-Cu bimetallic nanoparticles and algal extract against the pathogenic bacteria was evaluated using the agar well diffusion technique. The pure culture was subculture in nutritional broth for a whole night at 37°C. Pathogens were seeded on nutrient agar media for bacteria using sterilized cotton brushes. On agar plates, 6 mm diameter wells were made using sterile micropipette tips. Using a micropipette 50 µl of the algal extract and nanoparticle were applied to each well on all the plates. The bacteria were treated with the medication erythromycin. Multiple sample concentrations (200, 400, 600, 800, and 1000 µg/ml) were used for both bacteria. The plates were incubated at 37°C for 24 hours. Following incubation, the sample-filled well was found to have an inhibitory zone, and the diameters of this zone (measured in millimetres) were determined using a measuring scale.

2.7. Characterization of Nanoparticles

The identification of the nanoparticles was carried out using characterization techniques such as FTIR spectroscopy, UV-visible spectrum, SEM, DLS, and XRD. Each characterization technique used for various purposes. The functional group presentation on nanoparticles was identified using FTIR. The formation of a colloidal plasmon band was identified using UV. The size and shape of the nanoparticles were identified using SEM. The size, crystal structure, and chemical composition of the nanoparticles were identified using XRD. The size and dispersity of nanoparticles were identified using DLS.

3. Results and discussion

3.1. Phytochemical screening of Gracilaria edulis

In the extract of *Gracilaria edulis,* the various phytochemical constituents were present and tested using various quality tests, which revealed the presence of alkaloids, carbohydrates, terpenoids, proteins, phenols, cardiac glycosides, and flavonoids.

3.2. Total Phenolic Content

The standard solution was analysed for linearity. A linear response was identified (r^2= 0.9568), and the equation was Y = 0.3333X - 0.1387. the extract was analysed using UV visible spectrophotometer. The highest phenolic content is 255.4375mg/g.

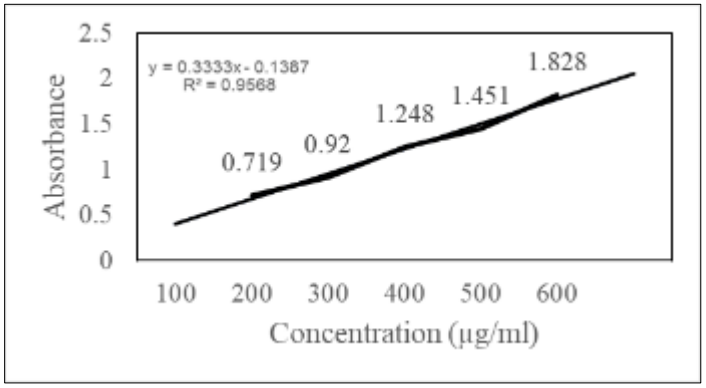

Figure 1 Total Phenolic content of Cu-Zn BMNPs

3.3. Total Flavonoid Content

The standard solution was analysed for linearity. A linear response was identified (r^2 = 0.9808) and the equation was y = 0.2492x – 0.2221. The highest flavonoid content is 364.9166 mg/g.

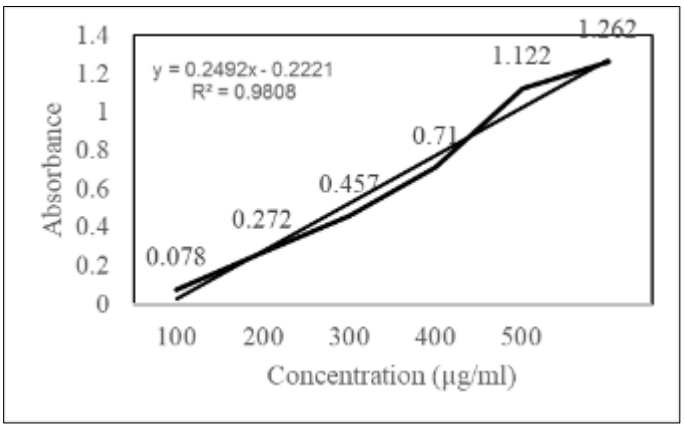

Figure 2 Total Flavonoid content of Cu-Zn BMNPs

3.4. UV Visible Spectroscopy

The UV visible spectrum of the isolated compounds was recorded using a Shimadzu 160A UV visible spectrophotometer. The UV spectrum ranges from 200-1100nm and the UV result of Zn-Cu bimetallic nanoparticles showed the characteristics UV-Vis Spectrum with the peak at 281nm and 386nm. The absorption spectrum resembles that nanoparticle was observed, this suggests the formation of zinc –copper core shell bimetallic nanoparticles

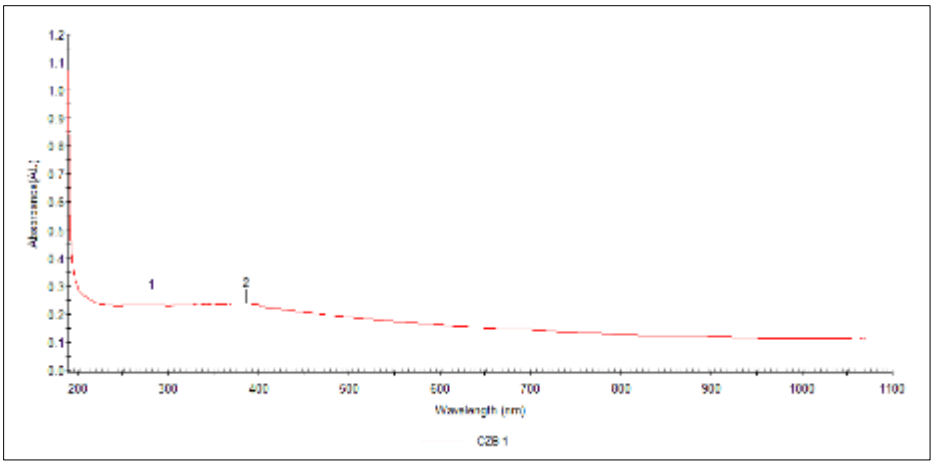

Figure 3 UV Visible Spectra of *G. edulis* mediated bimetallic nanoparticles

3.5. FT-IR Spectroscopy

FT-IR analysis was conducted to identify the biomolecules contained in the extract, as these are responsible for the reduction of Zn^{+} and Cu^{+} ions in the synthesis of nanoparticles. 481.09 cm^{-1}, 682.58 cm^{-1}, 1634.54 cm^{-1}, 2075.13 cm^{-1}, 3436.04 cm^{-1}were identified as the FTIR bands. The band at the 3500–3000cm^{-1}region corresponds to hydroxyl group stretching (OH). The band at 3436.04cm^{-1}, may be attributed to the stretching of aliphatic hydrocarbon (C–H). The bands at the 1634.54 and 2075.13cm^{-1}region can be assigned to C=C and C=O stretching, respectively. The band at 682.58 cm^{-1} may be assigned to the stretching of C-O. The result of the FT-IR analysis investigated in this research, corresponds to that of other researchers.

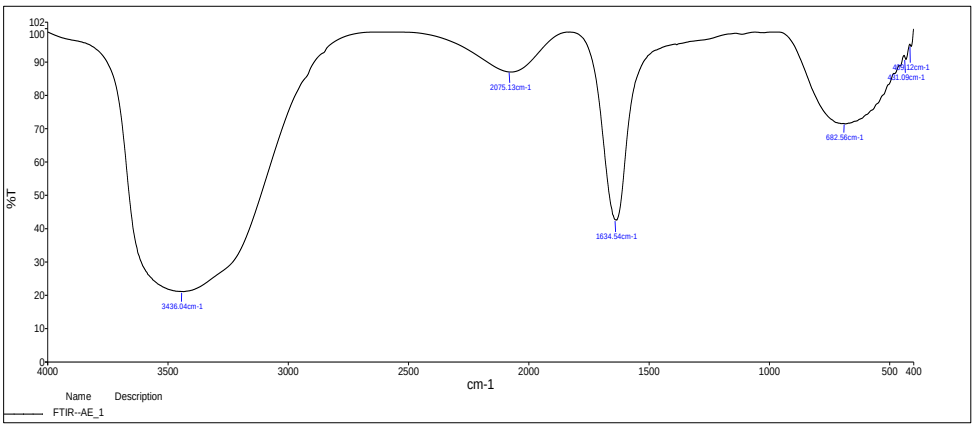

Figure 4 FTIR Spectrum of *G. edulis* mediated bimetallic nanoparticles

3.6. Scanning Electron Microscope

The SEM image is employed to predict the size and morphology of resultant bimetallic nanoparticles using sample. The size of particle ranges from 100.0nm – 128.2nm in diameter and size was about 200nm.

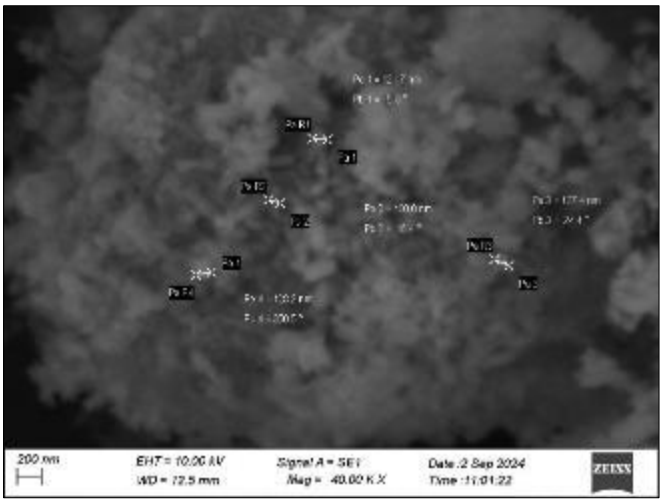

Figure 5 Scanning electron microscope imaging of *G. edulis* mediated bimetallic nanoparticles

3.7. Dynamic Light Scattering particle size analyser

By analysing data, it was found that nanoparticle size was in the range of 30-900nm, however, beyond 100 nm range the percentage of nanoparticles present is very less. The highest fraction of bimetallic nanoparticle presents in the range of 100nm. From the plot it was evident that the nanoparticles having various sizes which are indeed in agreement of the result obtained by SEM analysis.

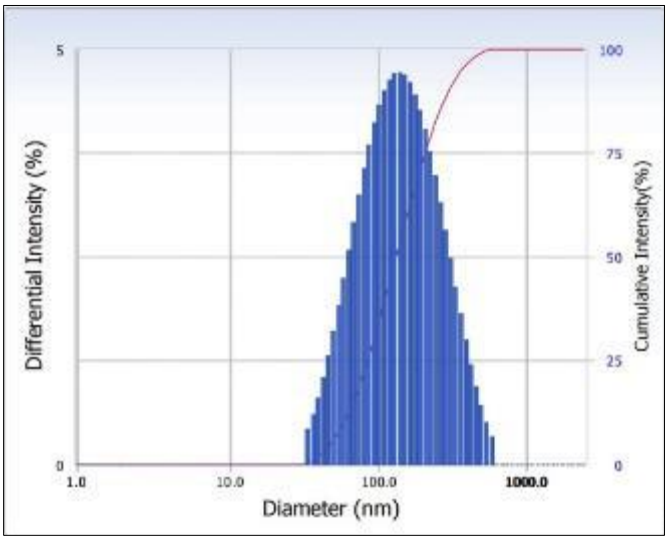

Figure 6 DLS analysis of *G. edulis* mediated bimetallic nanoparticles

3.8. XRD Analysis

The extract-mediated synthesized the nanostructure was confirmed by the characteristic peaks observed in the XRD image. All diffraction peaks correspond to the characteristic face centered cubic lines. These diffraction lines observed at 2θ angle 32.27, 34.93, 38.80° and 57.31 respectively. XRD patterns were analyzed to determine peak intensity, position and width.

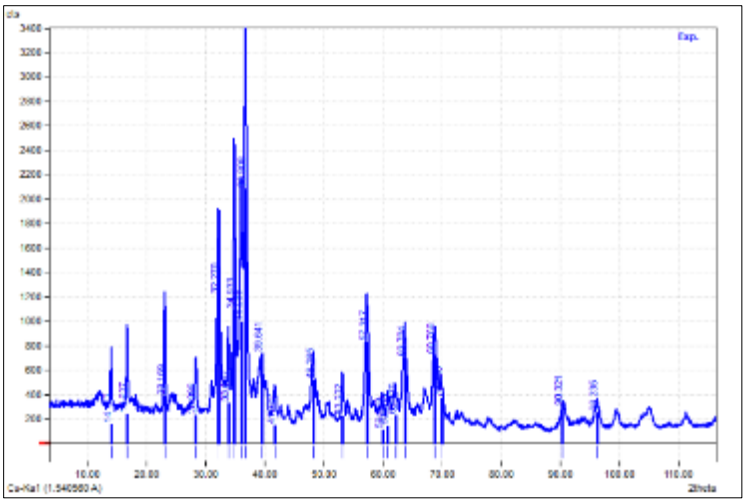

Figure 7 XRD pattern of *G. edulis* mediated bimetallic nanoparticles

3.9. Antimicrobial activity

The zone of inhibition value obtained at 1000µl concentration of bimetallic nanoparticles against *Staphylococcus aureus and Bacillus subtilis* was 8 mm and 2 mm. The zone of inhibition value obtained at 1000µl concentration of algal extract against *Staphylococcus aureus and Bacillus subtilis* was 9 mm and 2 mm. Maximum zone of inhibition was found the concentration 1000µl of bimetallic and algal extract against all the bacteria. When the concentration of bimetallic nanoparticle increases zone of inhibition value also increases.

Table 1 Zone of inhibition of bimetallic nanoparticle against different bacteria

Organisms	Concentration (µg/ml)	Zone of Inhibition (mm)
Staphylococcus aureus	Control (Erythromycin)	±14
	600	±4
	800	±8
	1000	±9
Bacillus subtilis	Control (Erythromycin)	±5
	1000	±2

Table 2 Zone of inhibition of algal extract against different bacteria

Organisms	Concentration (µg/ml)	Zone of Inhibition (mm)
Staphylococcus aureus	Control (Erythromycin)	±13
	200	±4
	400	±6
	600	±6.5
	800	±7.5
	1000	±8
Bacillus subtilis	Control (Erythromycin)	±6
	800	±0.5
	1000	±2

Figure 8 Antimicrobial activity of Cu-Zn BMNPs against *Staphylococcus aureus* and *Bacillus subtilis*

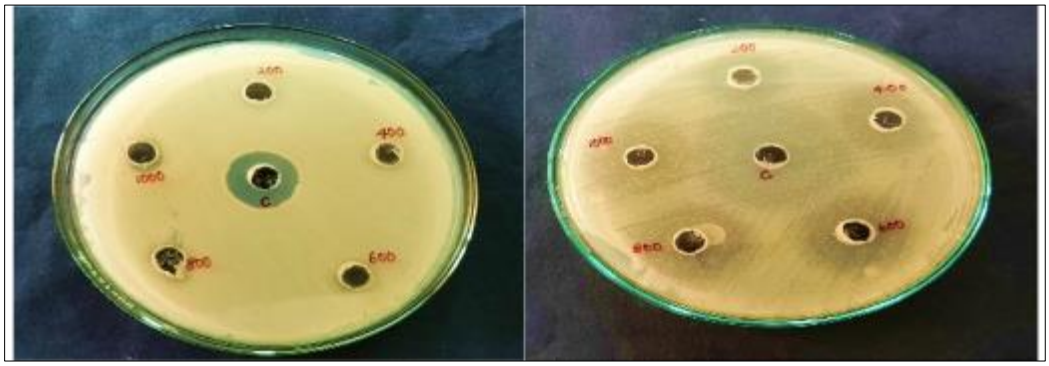

Figure 9 Antimicrobial activity of *G. edulis* extract against *Bacillus subtilis* and *Staphylococcus aureus*

4. Conclusion

In conclusion, the present investigation was a very economical and eco-friendly method to biosynthesize bimetallic (Cu-Zn) nanoparticles using aqueous extracts of *Gracilaria edulis*. In this method, toxic and hazardous chemicals were not used. This aqueous extract acted as both a surfactant and a reducing agent. Then, phytochemical analysis of the aqueous extract of *Gracilaria edulis* was done to analyse the properties of the extract. Then, the quantitative analysis techniques such as TPC, TFC were completed. Then, the characterization of the nanoparticles was completed by FTIR spectroscopy, UV-visible spectrum, SEM, DLS, and XRD techniques to identify the physicochemical properties of the nanoparticles. Then, the bimetallic nanoparticles and aqueous extract were used to evaluate their antimicrobial activity. It was analysed using the agar-well diffusion technique. And this bimetallic nanoparticle and aqueous extract exhibited good antimicrobial properties. When the concentration of bimetallic nanoparticles and aqueous extracts increased, the zone of inhibition values against the bacteria also increased. From there, we confirmed that the bimetallic nanoparticles and aqueous extract acted against bacteria.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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