Biogenic Copper Nanoparticles from Rosemary Leaf Extract of Antibacterial Impact

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DOI: 10.62579/JAGC0005

ABSTRACT

Rosemary leaf extract (**RMLE**) was employed for the eco-friendly biogenic synthesis of copper nanoparticles (**CuNPs@RMLE**). The copper nanoparticles **CuNPs@RMLE** were characterized by X-ray fluorescence (XRF) and Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy in solid state. The antibacterial effectiveness of **CuNPs@RMLE** was assessed against Gram-negative strains, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), as well as Gram-positive strains as *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus aureus* (*S. aureus*). It is compared with the corresponding effectiveness exhibited by **RMLE** and copper(II).

KEYWORDS:

Biological Inorganic Chemistry; Copper Nanoparticles; Rosemary Extract Leaves; Antimicrobial Activity

INTRODUCTION

Bacterial infections are projected to remain a leading cause of mortality in the coming decades [1]. This is due to the bacteria resistance to antibiotics as a significant challenge impacting global health since the early 21st century [2]. The drug resistance has reached epidemic levels, particularly in developing countries [2]. One strategy suggested to tackle antibiotic resistance involves utilizing metal and metal oxide nanoparticles (MetNP's, MetONP's). Among the transition elements, copper is being considered as the preferred element for creating these nanoparticles (MetNP's and MetONP's) [2]. Copper plays a role in various essential biological functions within the body [3] and demonstrates antibacterial properties against both Gram-positive or negative bacteria [4].

Biogenic synthesis, on the other hand, is valuable due to its lower environmental impact in contrast to certain physicochemical production methods [5-6], but also because it enables the production of substantial quantities of nanoparticles [7]. Different synthesis methods involve the use of different types of biological agents to yield nanoparticles of different sizes and shape [8].

Rosemary, known as *Rosmarinus officinalis L.*, is a evergreen shrub naturally found across most Mediterranean countries. Belonging to the Lamiaceae family, this plant extract, as per traditional medicine, is believed to aid in various health aspects. Rosemary contains several substances that hold promise for biological activity [9]. In addition to double distilled water and other solvents such as ethanol are used for the rosemary's active components extracting [9]. Rosemary leaves extracts bioactive components include caffeolyl derivatives (rosmarinic acid), diterpenes (carnosic acid, carnosol), phenolic mono-terpenes (Scheme 1) [9]. Rosemary has shown inhibitory effects against various bacteria, such as *S. aureus*, *L. monocytogenes* and *E. coli* [10-11]. By disrupting and reducing the impermeability of these bacteria's membranes, rosemary exhibits the potential to combat drug resistance in specific bacterial strains [11].

Scheme 1

In the course of our research towards the development of new efficient multi targeted antimicrobial agent which therefore prevent the bacterial resistance [12-13] we report here the biogenic synthesis of copper nanoparticles **CuNPs@RMLE** using rosemary (*Rosmarinus officinalis L.*) plant extract.

Results and Discussion

General Aspects: The rosemary leaf extract (RMLE) was employed for the biogenic synthesis of copper nanoparticles (**CuNPs@RMLE**). RMLE extract serves as capping agent in the chemical synthesis process of nanomaterials. The synthetic route of nanoparticles involves diluting copper nitrate in the water extract of rosemary under stirring, followed by microwave irradiation of the nanoparticles, sonication, and centrifugation. After removal of the supernatant to dryness, using a rotary evaporator, the **CuNPs@RMLE** obtained and subsequently dried in oven at 50 ^{o}C for 24 h (Scheme 2).

Scheme 2

X-ray Fluorescence Spectroscopy: Dry solid **CuNPs@RMLE** was ground to powder. The XRF spectrum of **CuNPs@RMLE** confirmed the presence of copper in the samples (Figure 1). Thus, the presence of the photon energies, of principal K-, L-, and M-shell emission lines for copper CuK*a* and CuK*β* at 8.05 and 8.91 KeV in the XRF spectrum of the **CuNPs@RMLE** (Figure 1) confirms the presence of copper [14]. X-ray emission spectroscopies, particularly K*β* mainlines, have been demonstrated to diagnose the metal oxidation state and spin state in 3d transition metals. However, since the K*β* mainlines of copper show very similar $K\beta_{1,3}$ energies (8904.6–8904.7 eV) with no significant shifts between the various copper oxidation states, it is impossible to determine the oxidation state of the metal ion [14].

The content of copper in **CuNPs@RMLE** was determined to (41.62±0.07)% w/w.

Figure 1

Vibrational Spectroscopy: In order to confirm the formation of **CuNPs@RMLE** nanoparticles, FTIR spectra were obtained for both RMLE and **CuNPs@RMLE** (Figure 2). The FTIR spectrum of rosemary displays distinctive absorption bands. These include a broad vibrational band at 3224 cm^{-1}, which corresponds to *ν*(O-H) stretching bands of alcoholic groups. The band at 2932 cm^{-1} is consistent with the vibration of the $\nu(C_{conjugate}\text{-H})$ bonds. Furthermore, bands observed in the spectrum at 1586, 1386, 1360, 1254, 1067, 1020 and 807 cm^{-1}, are consistent with the ν_{as}(COO) and ν_s(COO) of the carboxylic acid groups, ν(C=O), ν(C-O), ν(C-C) of phenyl groups, and ν(C-O) vibrations, respectively (Scheme 1, Figure 2). These bands emerge at 3127, 1604, 1390, 1290, 1021, and 806 cm^{-1} with the formation of **CuNPs@RMLE** nanoparticles (Figure 2). The most notable alteration is observed in the ν_s(COO) band at 1290 cm^{-1} in **CuNPs@RMLE**, which is initially appeared at 1254 cm^{-1} in RMLE. This is consistent with the interaction between copper ions and the carboxylic groups of RMLE ingredients.

Figure 2

Antibacterial activity of **CuNPs@RMLE**

Determination of the Inhibition Zone (IZ) through agar disk-diffusion method: The agar disk-diffusion technique was utilized to assess the microorganism's sensitivity to the antibacterial agent [15]. Figure 3 shows the diameter of the inhibition zones formed when *P. aeruginosa, E. coli, S. epidermidis,* and S. *aureus* are treated with **CuNPs@RMLE** after incubation for 20 hours. The biological experiments evaluating the antimicrobial activity of the material were performed using a consistent amount of material administered to the microbes (100 mg/mL), which corresponds to a copper contented of 41.6% w/w. Each experiment was repeated at least three times to ensure statistical significance in the results. The inhibition zones caused by **CuNPs@RMLE** are 18.3±1.7 mm, 23.5±1.0 mm, 31.0±2.0 mm, and 21.5±1.0 mm against *P. aeruginosa, E. coli, S. epidermidis,* and *S. aureus,* respectively (Figure 3). Copper nanoparticles inhibit both Gram negative (*P. aeruginosa, E. coli)* and positive bacteria (*S. epidermidis* and *S. aureus)* effectively (Figure 3). However, more pronounced activity is noted against Gram-positive bacteria. Copper nanoparticles exhibited the strongest activity against *S. epidermidis,*, being twice as effective as against *P. aeruginosa* or *S. aureus*.

Additionally, the antibacterial activity of **RMLE** at 100 mg/mL and a Cu(II) solution (in the form of $Cu(NO_3)_2 \cdot 3H_2O$) at 41.6% w/w were also assessed (Figure 3). The inhibition zones (IZ) created by **RLME** against *P. aeruginosa, E. coli, S. epidermidis,* and *S. aureus,* were measured at 9.0±0.0, 11.5±0.9, 13.8±0.5, and 11.0±0.0 mm, respectively. In comparison, the IZ of the Cu(II) solution were 26.7±1.7, 26.3±0.7, 32.7±2.8, and 27.8±2.6 mm, respectively. Therefore the antibacterial activity of **CuNPs@RMLE** is comparable to that of Cu(II) ions but superior to that exhibited by **RMLE** alone.

Figure 3

Usually, microbial strains are classified into three groups based on the size of the inhibition zone (IZ) produced by a specific amount of an antimicrobial agent in their agar dilution culture: (i) strains showing an IZ ≥17 mm are labeled as susceptible, (ii) those with an IZ between 13 to 16 mm (13 ≤ IZ ≤ 16 mm) are classified as intermediate, and (iii) strains with an IZ ≤ 12 mm are deemed resistant [16]. The classification of the activity of the **CuNPs@RMLE** towards the microbe strains studied here was based on the criterion mentioned above rather the comparison of the inhibition zones developed by microbe cultures that exposed to a specific amount of the agent **CuNPs@RMLE** and its precursors, (copper salt and solidified extract of rosemary). Therefore, the antibacterial activity of **CuNPs@RMLE** is superior to that exhibited by **RMLE** alone. In comparison to CuNPs, **CuNPs@RMLE** demonstrates similar antimicrobial activity against *P. aeruginosa, E. coli, S. epidermidis,* and slightly superioractivity against *S. aureus*.

This suggests that additive benefits or synergies may be realized with **CuNPs@RMLE** although this appears to be bacteria specific. Further work is underway to identify cases where such enhanced antimicrobial activity by CuNPs@RMLE is achieved.

Conclusion

Biogenic nanoparticles are essential due to their unique properties and environmentally friendly production techniques. Their biocompatibility makes them promising candidates for treating various diseases. As part of our ongoing investigations in this domain, we conducted research into the development of copper nanoparticles (**CuNPs@RMLE**) employing leaf extract (RMLE) and explored their antimicrobial efficacy.

The antibacterial activity of copper nanoparticles synthesized with rosemary leaf extract, has been demonstrated to be highly effective against bacterial strains including *P. aeruginosa, E. coli, S. epidermidis,* and *S. aureus*. With a substantial copper content of 40% in **CuNPs@RMLE** and its notable solubility in water, this material demonstrates exceptional efficacy against both Gram-negative and Gram-

positive strains. The antibacterial effectiveness of **CuNPs@RMLE** surpasses that of **RMLE** alone. When compared to **Cu(II), CuNPs@RMLE** shows comparable antimicrobial activity *against P. aeruginosa, E. coli, S. epidermidis*, and slightly enhanced activity against *S. aureus*. This implies that there may be additional benefits or synergies with **CuNPs@RMLE**, although this seems to vary depending on the bacteria. However, further research into the mechanisms underlying the antibacterial activity of copper nanoparticles, as well as their safety and efficacy in various applications, is warranted to fully harness their therapeutic potential in combating bacterial infections.

Experimental

Materials and instruments: Double distilled water was obtained using the BIDY WATER BI-DISTILLER B.E. 115 apparatus manufactured by BICASA, MILANO, ITALY. Copper nitrate trihydrate ($Cu(NO_3)_2$ $3H_2O$) was purchased from Merk. Tryptone tryptophan medium, beef extract powder, peptone bacteriological, soy peptone was purchased from Biolife. Agar and yeast extract were purchased from Fluka Analytical. D(+)-glucose, di-potassium hydrogen phosphate trihydrate were purchased from Merck. Melting points were measured in open tubes with a Stuart Scientific apparatus and are uncorrected. ATR-FT-IR spectra in the region of 4000-370cm^{-1} were obtained with a Cary 670 FTIR spectrometer, Agilent Tech-nologies. XRF measurements were carried out with a Rigaku NEX QC EDXRF analyzer (Austin, TX, USA).

*Preparation of **RMLE** and **CuNPs@RMLE**:* To prepare the rosemary extract, 2 g of commercially dried rosemary leaves were ground until a fine powder and it placed in a Soxhlet extraction apparatus. A spherical flask containing 40 ml of distilled water was assembled into the apparatus. After 12 hours under reflux, a brownish-clear solution of rosemary extract was obtained.

0.241 g (1 mmol) of copper nitrate trihydrate ($Cu(NO_3)_2$ $3H_2O$) was added into 10 ml of the extract and stirred for 3 h. Then, the suspension, was irradiated under microwave radiation (700 W microwave

oven) for 1 minute, followed by ultrasonic treatment for 10 minutes. The solution is centrifugated at 4000 rpm for 20 minute afterwards. The supernatant was isolated and concentrated to dryness using a rotary evaporator. Green powder, highly soluble in water, was collected and stored in darkness.

CuNPs@RMLE: yield 150 mg, green powder; melting point >250 ^{o}C, ATR-FTIR (cm^{-1}): 3127br, 2372m, 1289vs, 1020s, 806s, 423m

Biological tests:

Bacterial Strains: The bacterial strains of *P. aeruginosa*, *E. coli*, *S. epidermidis* (ATCC® 14990™) and *S. aureus* (ATCC® 25923™) were used in the experiments [12-13]. The bacterial strains *P. aeruginosa and E. coli* were kindly offered from Medical School of the University of Ioannina [12-13]. The biological experiments were performed in triplicates. The statistical analysis software package included in the MS Office excel was used for the data processing.

Determination of the inhibition zone (IZ),: The procedure was performed as previously reported [12-13]. A standardized inoculum (10^8 cfu/mL) of the microorganisms was incubated in agar plates. A cotton swab is dipped in the inoculum (10^8 cfu/mL) of the microorganisms. The excess fluid is removed by turning the swab against the inside of the tube. The inoculum is spread evenly over the entire of the agar petri dishes in three directions. Disks with 10 mm diameter were soaked in solutions of **CuNPs@RMLE** at the concentration of 100 mg/mL and placed on the agar surface and the Petri plates were incubated for 20 h at 37^{o}C.

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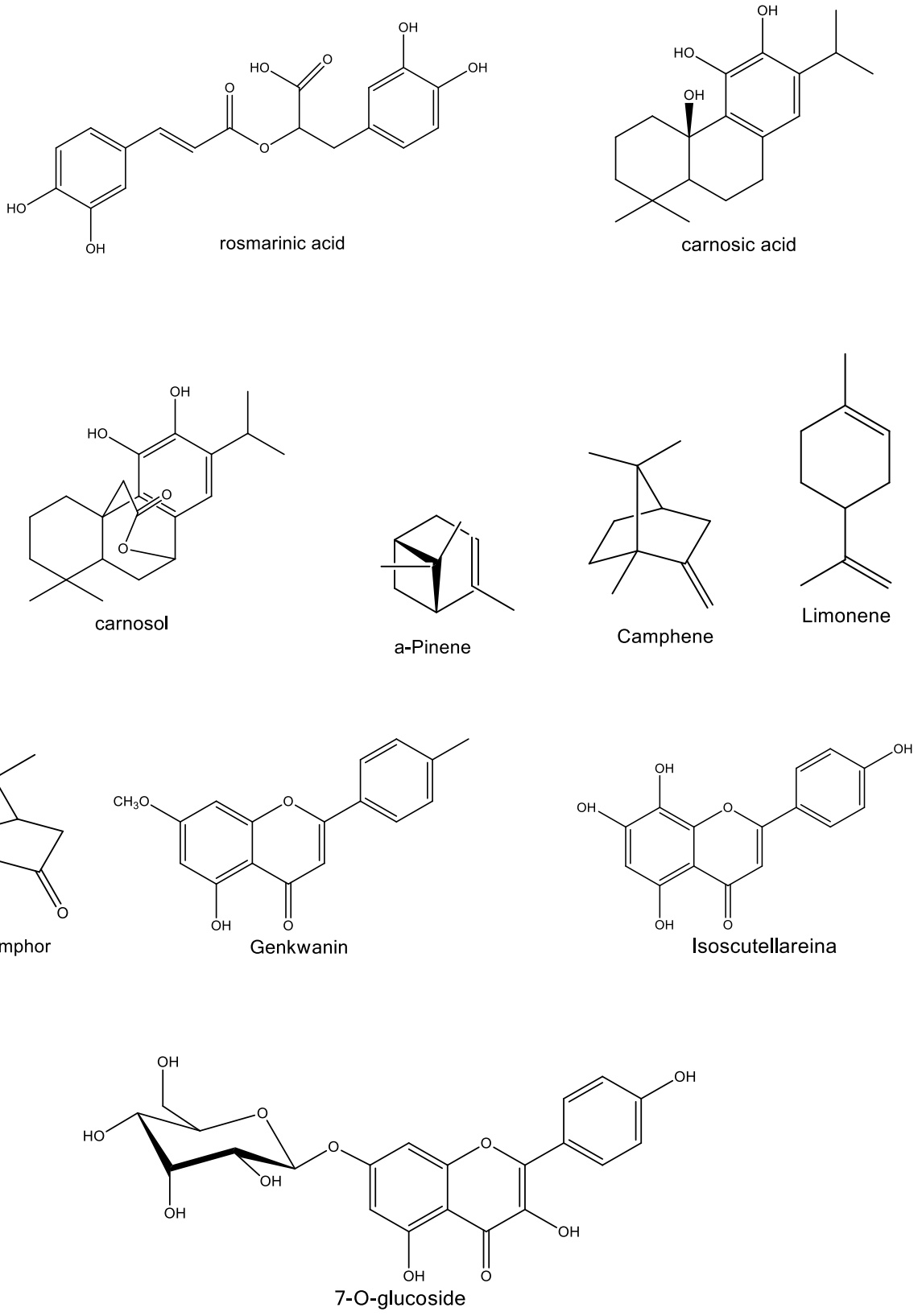
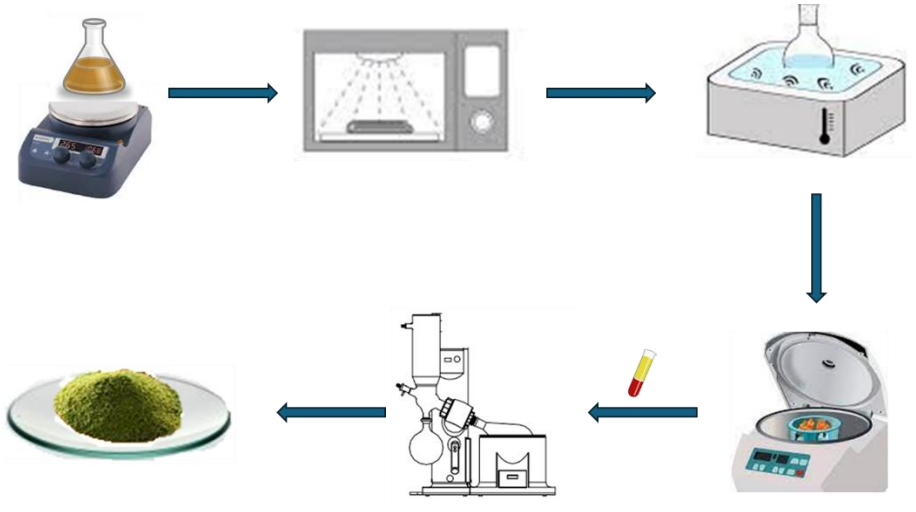


Scheme 1. Rosemary main ingredients



Scheme 2. The synthetic route for **CuNPs@RMLE** preparation

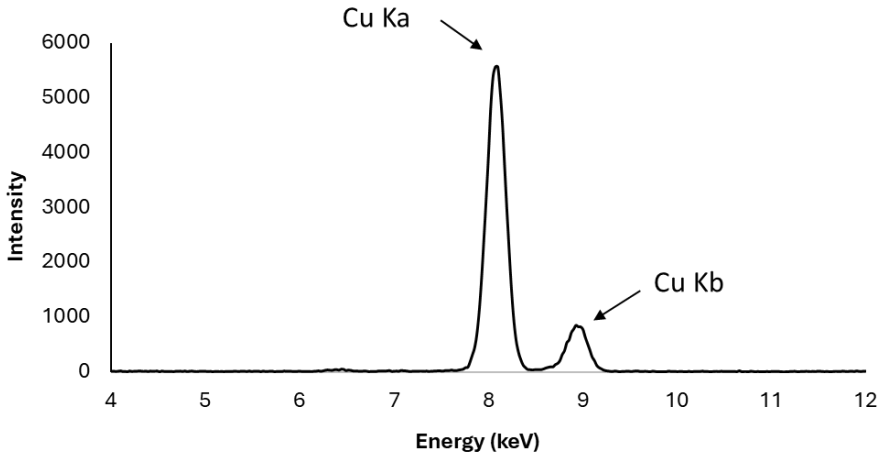


Figure 1. XRF spectrum of **CuNPs@RMLE**

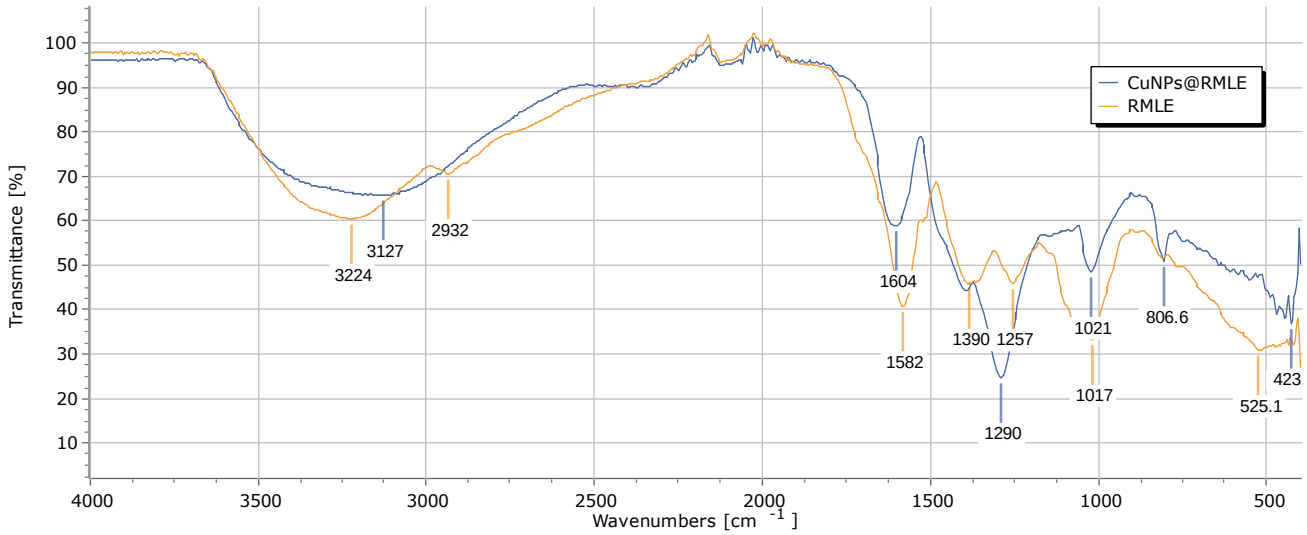


Figure 2. ATR-FTIR of RMLE and **CuNPs@RMLE**.

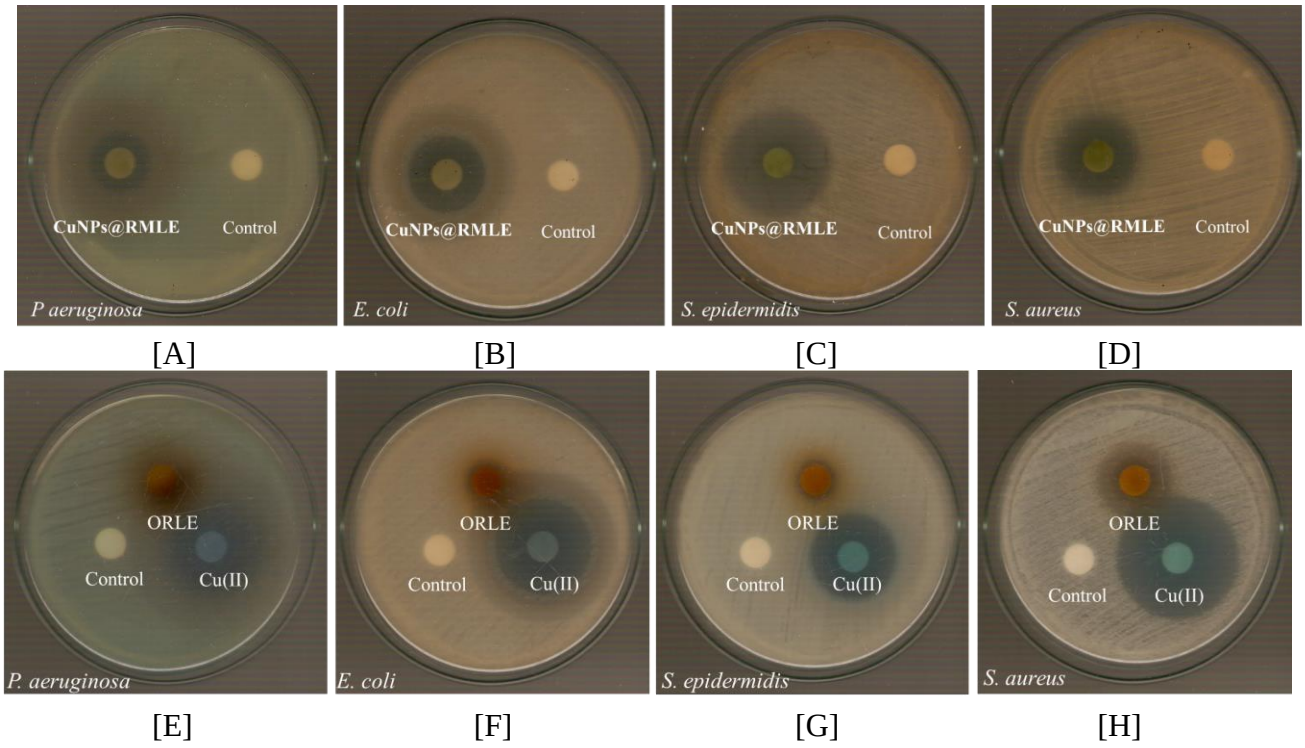


Figure 3. Inhibition zones of microbial strains treated with **CuNPs@RMLE** ([A] *P. aeruginosa* (18.3±1.7 mm); [B] *E. coli* (23.5±1.0 mm); [C] *S. epidermidis* (32.0±2.0 mm); and [D] *S. aureus* (21±1.0 mm)); **RMLE** ([E] *P aeruginosa* (9.0±0.0 mm); [F] *E. coli* (11.5±0.9 mm) [G]; *S. epidermidis* (13.8±0.5 mm) and [H] *S. aureus* (11.0±0.0 mm); Cu(II) ([E] *P aeruginosa* (26.7±1.7 mm); [F] *E. coli* (26.3±0.7 mm) [G]; *S. epidermidis* (32.7±2.8 mm) and [H] *S. aureus* (27.8±2.6 mm).