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Synthesis, Characterization of Biogenic Copper Nanoparticles and Their Therapeutic Activity

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Abstract

Green synthesis of copper nanoparticles (Cu NPs) is an economical, environmentally friendly and non-toxic approach that has been the subject of research in health and industry. Therefore, in this study, Cu NPs were synthesized using Pimpinella anisum (P.anisum) seed extract and their pharmacological activities were evaluated. Characterization of Cu NPs was performed by UV-vis, FT-IR and SEM-EDX analyses. Copper metal was reduced by reacting with the seed extract and reached the maximum peak at 385 nm in the UV-vis spectra, confirming the surface plasmon resonance. FT-IR spectroscopy showed the participation of phytochemical components in P. anisum in the synthesis. SEM analysis determined that the size of the biosynthesized nanoparticles is 10-20 nm in diameter and has a spherical structure. Strong signals of copper metal were confirmed by EDX analysis. The therapeutic effect of Cu NPs was evaluated by antioxidant and antibacterial assays. The DPPH radical scavenging activity IC50 inhibition values of Cu NPs were better than the seed extract and exhibited strong antioxidant activity. Antibacterial activity was performed by the disk diffusion method and Cu NPs were more effective against gram-positive bacteria. It had the highest zone diameter (18.0±2.8 mm), especially on Bacillus subtilis bacteria. These results showed that Cu NPs may have a selective effect against drug-resistant bacteria as an alternative agent to pharmaceutical applications. This study showed that P. anisum seed extract-mediated bioconjugation of Cu NPs can be done simply, quickly and cost-effectively. As a result, Cu NPs should be supported by more detailed in vivo studies to create antioxidant and antibacterial agents.

Keywords: Antibacterial; Antioxidant; Copper nanoparticles; Pimpinella anisum; Green synthesis

1. INTRODUCTION

Antibiotics destroy pathogenic bacteria either by bacteriostatic or bactericidal mechanisms of action. These drugs are used in the treatment of infections in humans and animals. Unfortunately, the irrational use of antibiotics causes bacteria to develop resistance to these drugs. This situation prolongs the treatment period of microorganism-induced diseases and creates a heavy burden on the economies of countries. (Kocak et al., 2022). According to the report of the World Health Organization, they pointed out that antibiotic resistance is spreading and it is an important public health problem of this century (Morrison & Zembower, 2020).

Recently, various methods have been tried to develop new-generation therapeutic agents against antibiotic resistance. One of these methods is the synthesis of nanoparticles (NPs). NPs can be synthesized by physical, chemical and biological methods. However, since chemical and physical methods, high temperature, pressure and toxic chemicals are used, it contains risks in terms of environment, human and animal health. (Abbasi et al., 2016; Meydan and Seçkin, 2021; Kocak et al., 2023). In biological methods, the interest in the use of non-toxic, environmentally friendly and cost-effective materials such as plants and fungi has increased. (Alkhulaifi et al., 2020). The advantage of plant-mediated biosynthesis of NPs over other methods allows their use in various diseases such as cancer treatment and inhibition of infections (Bazancir and Meydan, 2022; Kocak et al., 2022). In

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addition, NPs show that they can be used harmoniously in different areas with their large surface area, stability and durable properties (Thanh and Green, 2010).

Green synthesis of NPs is the reduction of alkaloids, tannins, balsams, phenolic and flavonoid metabolites in the content of plants with noble metal salts such as copper (Cu) (Cho et al., 2005). Cu metal has been the focus of attention due to its low cost, easy availability and similar therapeutic properties as silver and gold. Cu NPs have beneficial effects on living organisms and provide balance and stability in tissues (Ginting et al., 2020). There is also evidence that Cu has antibacterial potential (Mehdizadeh et al., 2020). Many plants such as Diaplazium esculentum (Sinha et al., 2022), Cissus arnotiana (Rajeshkumar et al., 2019), Aloe barbadensis Miller (Gunalan et al., 2012), Diplotaenia turcica (Seckin, 2021) and Persea americana (Rajeshkumar and Rinitha, 2018) have been used for the green synthesis of Cu NPs.

Pimpinella anisum L (Anise) is an essential oil-rich flowering plant belonging to the Apiaceae family. Anise is used in traditional folk medicine for the treatment of various diseases such as cancer, asthma, cholera and cough (Sihoglu Tepe and Tepe, 2015). In addition, its antibacterial, antioxidant, antiseptic, diuretic and anti-depressant effects have been proven. (Sihoglu Tepe and Tepe, 2015). To the best of our knowledge, no study has been found on the green synthesis of Pimpinella anisum (Pa) seed extract with Cu metal. In this study, the biosynthesis and characterization of P. anisum-mediated Cu NPs were designed to evaluate their antibacterial and antioxidant potential.

2. MATERIALS and METHODS

2.1. Preparation of seed extract

P. anisum seeds were obtained from a local market and indentified. P. anisum was rinsed by washing in tap water and then in distilled water to remove dust particles. The seeds were left to dry at room temperature. The dried seeds were ground into a powder with the help of an electric mill. The seed powder (10 g) was transferred to a beaker containing 250 mL of distilled water and heated at 60°C for 10 minutes with stirring. It was then allowed to cool at room temperature and filtered with filter paper (Whatman's). The filtered seed extract was centrifuged (5 min at 5000 rpm) and supernatants were taken and stored at +4 °C for biosynthesis study.

2.2. Synthesis of plant-mediated Cu nanoparticles

10 mL of anise seed extract was added by distillation to 100 mL of 1 mM copper sulfate solution in a 250 mL beaker. It was left to react for 24 hours at room temperature. The solution was placed in Eppendorf tubes and centrifuged at 10000 rpm for 10 minutes. The obtained Cu NPs were washed twice with distilled water. It was then dried in an oven at 50°C for 48 hours. (Keerthika et al., 2021).

2.3. Characterization of Cu NPs

The color change and wavelength of Cu NPs formation were measured by ultraviolet-Visible (UV-Vis) spectroscopy. The morphological structure, size and shape of the NPs were visualized by scanning electron microscopy (SEM). Elemental analysis was performed by energy dispersive X-ray spectroscopy (EDX) analyses. Anise molecules bound to NPs were identified by Fourier transform infrared spectroscopy (FTIR) (Gopalakrishnan and Muniraj, 2021).

2.4. Antibacterial activity of Cu NPs

Antimicrobial activities of Cu NPs biosynthesized with P. anisum were determined using the disc diffusion method (Senthilkumar and Sivakumar, 2014). Pathogens were grown in broth and Mueller-Hinton agar medium was used for disc diffusion. P. anisum seed extract and Pa-Cu NPs were adsorbed into blank discs. Rifampim was used as a reference control. The medium was then incubated at 37oC for 24 hours. The results were evaluated by measuring the zone diameters of its antibacterial activity.

2.5. Antioxidant activity of Cu NPs (DPPH method)

DPPH radical scavenging activity Pyo et al. (2004) was used by modifying the method proposed. This method is based on the spectrophotometric measurement of the reduction in color as a result of the destruction of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, which is a stable purple compound. Properly diluted 0.1 mL of extract was mixed with 3.9 mL of DPPH solution (0.025 g/L methanol) and kept at room temperature for 60 minutes. At the end of the time, the absorbance of the sample was measured at 515 nm, and the inhibition rate of the DPPH radical was calculated. Results are expressed as the IC50 value (Pyo et al., 2004).

2.6. Statistical analysis

The results of the 3-replication analysis of this study were analyzed using the SPSS package (Ver. 22) program values were expressed as mean ± standard deviation. Obtained data were evaluated with one-way analysis of variance (ANOVA) and Duncan's multiple test was used for significant differences ($p < 0.05$).

3. RESULTS and DISCUSSION

3.1. Characterization

Green synthesis of Cu-NPs was performed using copper sulfate and extract from P. anisum seed. During the first 25 minutes of the reaction, the bluish color of the mixture turned green, indicating the formation of nanoparticles (Figure 1A). P. anisum-mediated synthesis of Cu-NPs was confirmed by UV-vis spectrophotometer showing two peaks (Figure 1B).

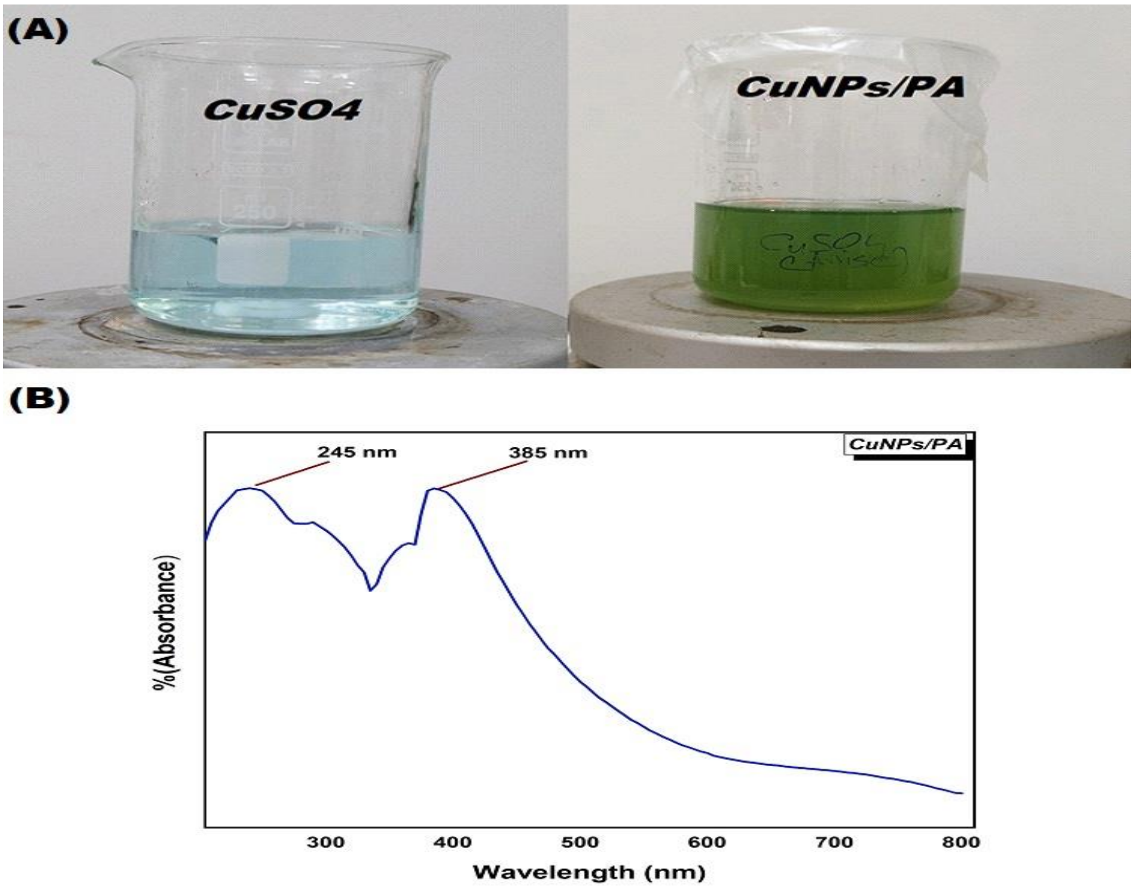

Figure 1. *Color change (A) and UV-vis spectrum (B) in P. anisum-mediated synthesis of Cu nanoparticles.*

The first peak was observed at 245 nm and the second peak at 385 nm. The findings were similar to the two peak values detected in UV-vis of Cu NPs synthesized by Achillea millefolium (Rabiee et al., 2020). Xiong et al. (2011) reported that copper nanoparticles biosynthesized with L-ascorbic acid have two values (Xiong et al., 2011). The data of the presented study show the successful formation of Cu NPs, which is in agreement with the literature (Issaabadi et al., 2017). The changes in the color and surface plasmon resonance in the formation of Cu-NPs confirm that the phytochemicals in the P. anisum content are due.

FT-IR analysis was carried out to identify *P. anisum* phytocomponents responsible for the formation of copper nanoparticles that are reduced in the biosynthesis step. FT-IR spectra of *P. anisum* seed extract and Cu NPs are shown in **Figure 2**. Changes in the peak points are observed as a result of the reaction of copper nanoparticles with the seed extract. The FT-IR spectrum of *P. anisum* mediated Cu NPs corresponds to the hydroxyl groups (-OH) in the phenolic compounds of the broad absorption band at 3394.7 cm^{-1} (Saif et al., 2016). The band at 2983.8 cm^{-1} represents C-H vibrations in aliphatic compounds. The peak at 1654.6 cm-1 can be attributed to alkene (C=C) groups. The 1500-1600 cm^{-1} twists can be attributed to the presence of aromatic rings (Amaliyah et al., 2020; Rajesh et al., 2018). The band gap at 1050-1300 cm-1 can be assigned to the bending vibration of organic carboxylic acids. The signals in the FT-IR spectrum can be concluded that the phenolics and flavonoids in the seed extract act as stabilizing and sealing agents in the formation of nanoparticles.

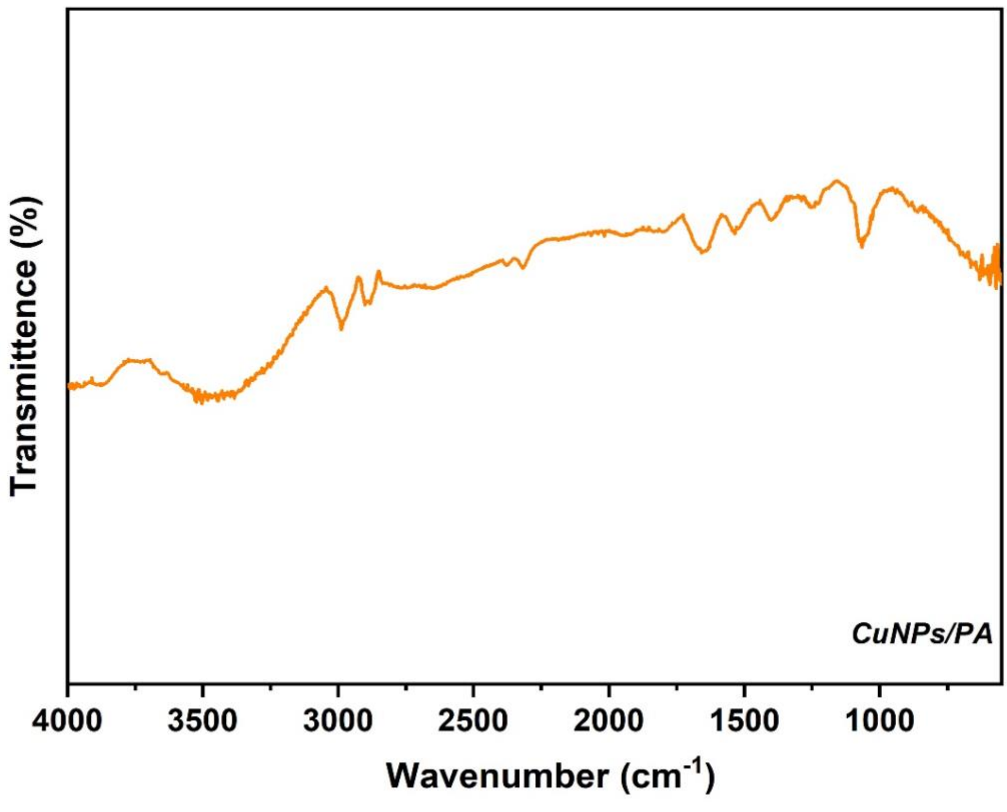

Figure 2. *FT-IR spectra of P. anisum seed extract and synthesized Cu nanoparticles.*

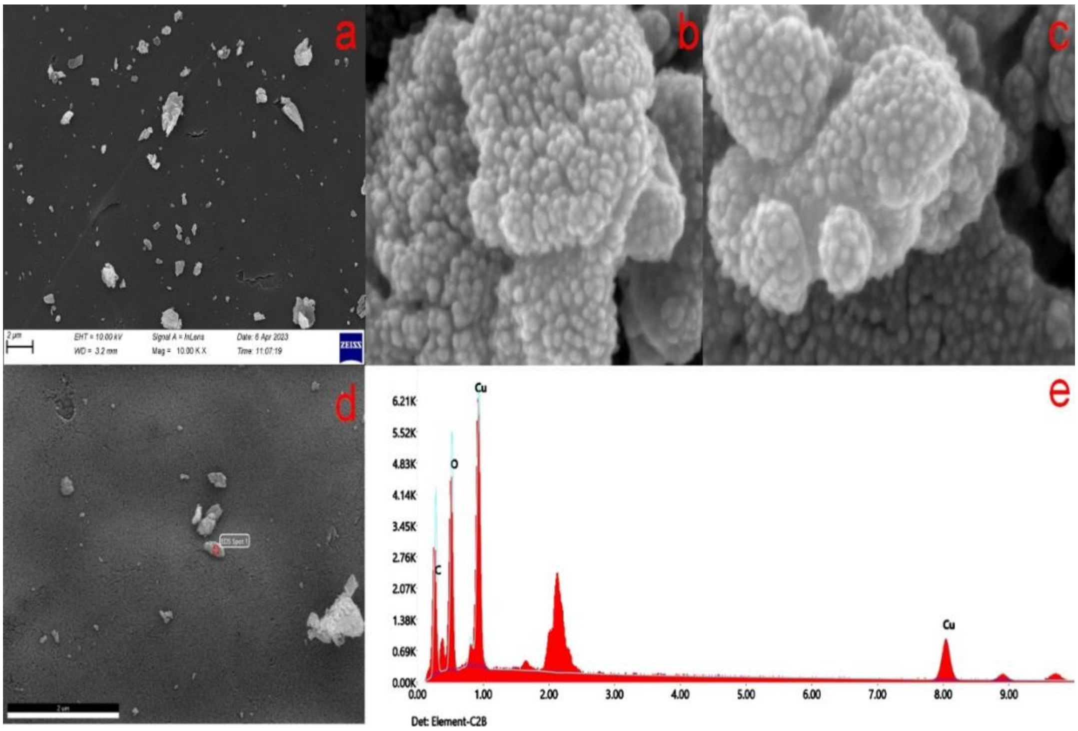

Figure 3. *SEM (a-c) and EDX (d-e) of copper nanoparticles synthesized using P. anisum seed extract.*

The shape and size of Cu NPs/PA were determined by SEM analysis (**Figure 3** (a-c)). The biosynthesized copper nanoparticles had an average of 10 to 20 nm in size (Xu et al., 2022). The shape of the Cu NPs was spherical (Asghar and Asghar, 2020) and biogenic nanoparticles tended to agglomerate slightly. This is known as a general feature in the green synthesis method with noble metals (Liu et al., 2021). Elemental analysis of Cu NPs/PA was performed with EDX (**Figure 3** (d,e)). The presence of Cu metal in EDX confirmed the successful shape fabrication of nanoparticles. The presence of carbon (C) and oxygen (O) elements other than Cu indicates that secondary metabolites in *P. anisum* contribute to the formation of nanoparticles (Liu et al., 2021; Wu et al., 2020). The unidentified peak in EDX belongs to the gold element used in the SEM-EDX analysis acquisition. The results show the purity of Cu NPs in EDX analysis and prove the formation of biogenic nanoparticles.

3.2. Antioxidant potential of *P. anisium*-mediated Cu NPs/PA

The antioxidant activity of *P. anisium* seed extract and Cu NPs was determined by the DPPH method. In this study, the antioxidant capacity of the seed extract and copper nanoparticles at different concentrations was evaluated and compared with the positive control BHA (Butylhydroxyanisole). The findings showed better antioxidant activity than the seed extract of Cu NPs at increasing concentrations. IC_{50} values of BHA, inhibition activity of both seed extract and Cu NPs/PA were more effective. The IC_{50} values of *P. anisum*, Cu NPs and BHA were 41.27, 7.24 and 1.45 µg/mL, respectively (**Figure 4**). The mechanism of antioxidant activity of biogenic copper nanoparticles is attributed to their scavenging activity against reactive oxygen derivatives, decomposition of peroxides and inhibition of various chain reactions, and the high antioxidant potential of Cu NPs has been attributed to the availability of phytochemicals in plant extracts as capping agents (Adewale Akintelu et al., 2021). It has been reported that it increases the antioxidant capacity as a result of the synergistic interaction of phenolics and flavonoids that contribute to the production of nanoparticles (Das et al., 2020; Liu et al., 2021; Subbaiya and Masilamani Selvam, 2015). Phenolic compounds in plant extracts contribute to antioxidant activity. Therefore, it has been reported that the antioxidant potential of *P.anisum* is due to its richness in polyphenolic components. (Zayed et al., 2020). The findings show that P.anisum seed extract can be used in the biosynthesis of potential antioxidant Cu NPs for safe pharmacological applications.

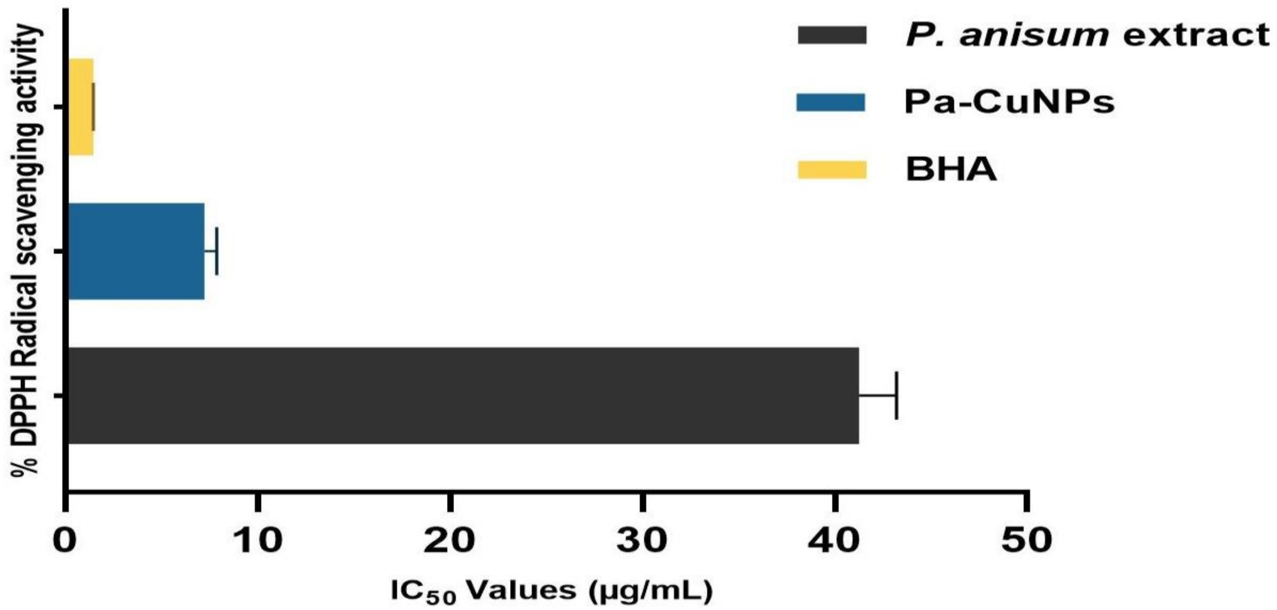

Figure 4. *P. anisum seed extract, DPPH scavenging activity and IC50 values of Cu NPs.*

(BHA; Butylhydroxyanisole), Cu NPs; copper nanoparticles, DPPH; 2,2-diphenyl-1-picrylhydrazyl).

3.3. Antibacterial activity of *P. anisium*-mediated Cu NPs/PA

P. anisum mediated Cu nanoparticles were evaluated against four pathogenic bacteria, *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus and Pseudomonas aeruginosa,* and these pathogens were compared with the antibiotic Rifampin. The obtained results are presented in **Table 1**. The inhibition zone of Cu NPs/PA nanoparticles differed according to the bacterial species. Cu NPs/PA inhibition zone was more effective in gram-positive bacteria than gram-negative bacteria. This may be mainly due to differences in bacterial cell structure (Rastogi & Arunachalam, 2011). In this study, it was observed that nanoparticles formed a zone of maximum inhibition against *Bacillus subtilis* among gram-positive bacteria (**Figure 5**). This may indicate that Cu nanoparticles may be effective in bacterial cell membranes as a result of protein oxidation, DNA cleavage and lipid peroxidation (Wu et al., 2020). Indeed, the findings were consistent with the literature (Keihan et al., 2017; Thiruvengadam et al., 2019). On the other hand, Cu NPs/PA did not form a zone of inhibition against gram-negative *Pseudomonas aeruginosa*. This result confirmed previous studies. (Rastogi and Arunachalam, 2011; Thiruvengadam et al., 2019). *P.anisum* extract was only partially effective against *Bacillus subtilis* pathogen. The results of seed-mediated synthesis of Cu NPs showed that it can exhibit antimicrobial activity against disease-causing bacteria.

Tablo 1. *Inhibition zone diameters (mm) of P. anisum seed extract and Cu NPs/PA against pathogenic bacteria.*

Test Microorganisms	Zone of Inhibition (mm)		
Bacteria	***P.anisum* extract**	**Cu NPs/PA**	**Rifampin**
Staphylococcus aureus ATTC 29213	-	8.1±1.2	8.5±1.8
Bacillus subtilis ATCC 6633	8	18.0±2.8	11.0±1.7
Bacillus cereus ATTC 10876	-	8.0±2.1	9.4±2.6
Pseudomonas aeruginosa ATTC 27853	-	-	-

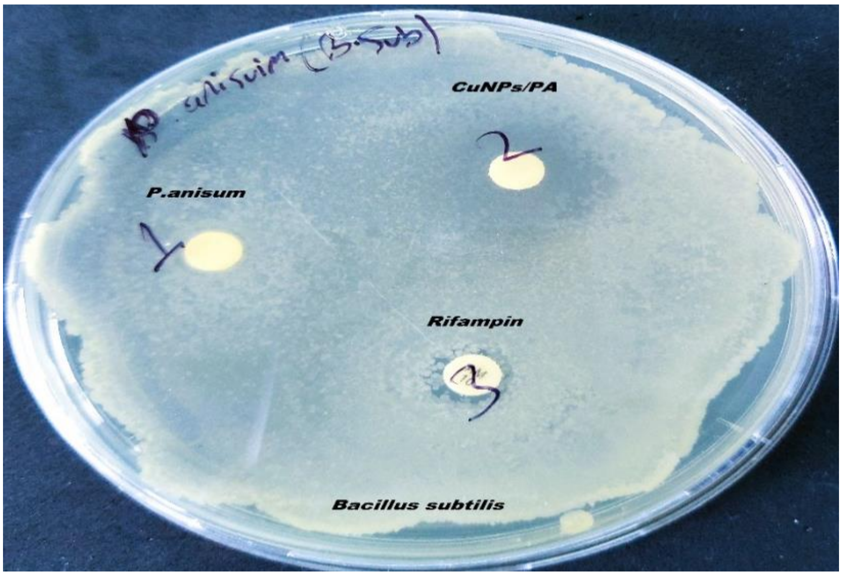

Figure 5. *Inhibition zone diameter of P. anisum seed extract and Cu NPs/PA impregnated on discs against pathogenic bacteria Bacillus subtilis.*

4. CONCLUSION

This research demonstrates the successful rapid synthesis of Cu NPs mediated by *P. anisum* seed extract. It confirmed that Cu nanoparticles exhibit antimicrobial and antioxidant activity against DPPH free radicals and some pathogenic bacteria. The color change in the reaction of copper metal with phytochemicals in the seed content was observed with the location of the surface plasmon resonance band with two peaks at 245 and 385 nm in the UV-vis spectrum. SEM analysis revealed that the nanoparticles had a spherical structure and had an average size of 10-20 nm. It showed that polyphenols or flavonoids in the seed extract could play a role as the sealing agent of nanoparticles in FT-IR. Cu NPs had better biological activity than seed extract. The nanoparticles were more effective against the Bacillus subtilis pathogen. This study is an environmentally friendly and economical approach that demonstrates the formation of Cu NPs without the use of any toxic agents. In conclusion, it shows that nanoparticles can function as a possible therapeutic agent and can be used in pharmacological applications.

5. ACKNOWLEDGEMENTS

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6. CONFLICTS of INTEREST

The authors declare there is no conflict of interest.

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