Green synthesis and characterization of copper nano-particles with propolis and their potential inhibitory effect on uropathogenic E.coli

Síntesis verde y caracterización de nanopartículas de cobre con propóleo y su potencial efecto inhibidor sobre E. coli uropatógena

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Abstract

A current focus in nanotechnology and nanoscience is the green biosynthesis of nanoparticles (NPs) using biomaterials. Research on green methods for making metal oxide NPs is gaining momentum to safeguard the environment from the potential dangers associated with toxic chemicals. This study aimed to synthesize copper NPs (CuNPs) via propolis extraction, a novel application of nanoscience. The results of our study were characterized by UV-visible absorption spectra, XRD, FTIR, and SEM and then to figure out the antibacterial activity against UPEC strains. According to an FTIR examination, substances present in propolis extract may affect how the surface of the created NPs is modified. The propolis extract spectrum exhibited sharp peaks at 2924.45cm^{-1}, 1624.57cm^{-1}, and 1517.11cm^{-1} to 1056.15cm^{-1} and 3422 cm^{-1}, which might be due to C = O and C = C aromatic stretching frequencies. We found a characteristic peak at 385nm signifying the surface plasmon resonance (SPR) with propolis extract. Our results confirmed of the potential role of antibacterial activity by the green synthesized CuNPs. Significant MIC values were seen at 8.4 ± 0.192 (1mg/ml) (P<0.05). We also found significant biofilm inhibition (84%) and reduction in motility (92%) when compared to a positive control (Ampicillin). The qPCR study found *fimH* and *fliC* gene members to be downregulated to 35% and 66%. However, we could not find an effect on the *BamA* gene member. For clinical uses, such as drug delivery systems, medication formulation, and biomedical applications, the green production of CuNPs from propolis may be a suitable candidate.

Keywords: Propolis, Copper nanoparticles, MIC, UPEC, qPCR.

Resumen

Un enfoque actual en nanotecnología y nanociencia es la biosíntesis verde de nanopartículas (NP) utilizando biomateriales. La investigación sobre métodos ecológicos para fabricar NP de óxido metálico está ganando impulso para proteger el medio ambiente de los peligros potenciales asociados con los productos químicos tóxicos. Este estudio tuvo como objetivo sintetizar NP de cobre (CuNP) mediante la extracción de propóleo, una nueva aplicación de la nanociencia. Los resultados de nuestro estudio se caracterizaron mediante espectros de absorción UV-visible, XRD, FTIR y SEM y luego determinaron la actividad antibacteriana contra las cepas UPEC. Según un examen FTIR, las sustancias presentes en el extracto de propóleo pueden afectar la forma en que se modifica la superficie de las NP creadas. El espectro del extracto de propóleo exhibió picos agudos en 2924,45 cm-1, 1624,57 cm-1 y 1517,11 cm-1 a 1056,15 cm-1 y 3422 cm-1, lo que podría deberse a las frecuencias de estiramiento aromático C = O y C = C. Encontramos un pico característico a 385 nm que significa resonancia de plasmón superficial (SPR) con extracto de propóleo. Nuestros resultados confirmaron el papel potencial de la actividad antibacteriana de las CuNP sintetizadas en verde. Se observaron valores de CIM significativos en 8,4 ± 0,192 (1 mg/ml) (P<0,05). También encontramos una inhibición significativa de la biopelícula (84%) y una reducción de la motilidad (92%) en comparación con un control positivo (ampicilina). El estudio qPCR encontró que los miembros de los genes fimH y fliC estaban regulados negativamente en un 35% y un 66%. Sin embargo, no pudimos encontrar ningún efecto sobre el miembro del gen BamA. Para usos clínicos, como sistemas de administración de fármacos, formulación de medicamentos y aplicaciones biomédicas, la producción verde de CuNP a partir de

propóleos puede ser un candidato adecuado.

Palabras clave: Propóleo, Nanopartículas de cobre, MIC, UPEC, qPCR**.**

Introduction

In recent years, nanoparticles (NP) and nanometric materials have provided special qualities that allow for their usage in a variety of industries, making nanotechnology increasingly prominent[1]. NPs are a great study subject for battling infectious diseases because of their antibacterial and antiviral characteristics, which have recently received significant awareness in the medical community[2]. NPs are capable of resolving several technological and scientific issues. As a result, they make a great subject for research on the prevention and management of infectious diseases[3].

The method utilized to synthesize the NPs has an impact on the morphology, size, and shape of the molecules. This field of research is difficult because of the quick oxidation[4]. Despite substantial research, there has been relatively research on copper despite its cost-effectiveness. Compared to other noble metals, copper (Cu) is abundant, reasonably priced, and antimicrobial, according to the United States Environmental Protection Agency[5]. COVID-19 pandemic, which states that SARS-CoV-2 can break down faster on copper surfaces than on plastic surfaces (4 hours), has recently increased interest in copper[6].

An important topic of nanoscience research in recent decades has been the environmentally friendly synthesis of nanomaterials, which does not include harmful chemicals. This encourages the creation of eco-friendly procedures[7]. Plant extracts and microorganisms can be used to create metallic nanoparticles, with the active biological component acting as a capping and cost-effective reducing agent[8]. Therefore, excessive pressure and energy are not required for experimental studies, leading to procedures that are both energy-efficient and environmentally benign. Optimising reaction conditions is one of the key strategies used in green synthesis to produce stable and well-characterized nanoparticles[9].

Pollen, waxes, phenolic acids, flavonoids, and aromatic balsams make up propolis. Propolis composition differs based on the location and method of production as well as the species of plants used[10]. Propolis' flavonoids make it biologically active, which have special biochemical capabilities that allow them to bind to biological polymers and heavy metal ions as well as scavenge free radicals and catalyse electron transport[11]. Propolis has antibacterial qualities, but it also interferes with the integrity of the membranes to stop bacterial enzyme activity and movement, making propolis stop the development of germs that are resistant to antibiotics[12]. In addition to its numerous uses, remedies for ailments such as urinary tract infections have been discovered. Propolis has a wide range of uses and has been found to be effective in treating a number of ailments, including urinary tract infections, periodontal disease, ear infections, cancer, sinus congestion, Parkinson's disease, gastritis, influenza, intestinal infections, headaches, conjunctivitis, and warts[13].

Currently, drug resistance can occur anywhere in the world. Over the past few decades, medicine and health have advanced significantly as a result of the discovery of antimicrobials. A significant fraction of the human population is afflicted with urinary tract infections (UTIs), which are common. Each year, approximately 150 million people experience UTI, which has significant socioeconomic implications[14]. According to Micali et al., 11% of women over the age of 18 experience a UTI episode each year, and it is predicted that 40% of women will experience at least one UTI at some point in their lives[15]. Very few reports are seen with a study on synthesizing copper nanoparticles with propolis. We report for the first time, that propolis synthesized Copper nanoparticles against UPEC strains. This research aimed to green synthesize the CuNPs using propolis extracts and screen their potent antibacterial activity against UPEC strains.

In this context, the goal of this research was to examine the possibility of synthesizing CuNPs using propolis. In order to assess the synthesis process and characterize the functional elements most likely in charge of producing the CuNPs compounds, the creation of CuNPs was observed using microscopy and spectroscopy. The uropathogenic (UPEC) *Escherichia coli* were tested for antibacterial and anti-biofilm activities using the green synthesized CuNPs.

Propolis sampling and extraction: Propolis was purchased from the local beekeepers in 2022 in Khalkhal (Ardabil province) and Gilan province, Iran. Frozen samples were thawed and about 5g of propolis sample was added to 100 ml of 70% ethanol and incubated in the dark for three days. Following incubation, the mixture was filtered on Whatman no. 1 filter paper and concentrated using a rotating evaporator. The powdered sample obtained was then used for the synthesis of nanoparticles[13].

Green synthesis of Copper nanoparticles (CuNPs): 50mg of propolis extract (PE) was added to 100ml of deionized water and added to an equal volume of 6mM copper sulphide solution with constant stirring (800rpm). The contents are stirred for 24 to 48 hours in the dark at 40°C. The change in color from light-brown to reddish-brown confirms the formation of CuNPs, and the reaction was stopped. The mixture was centrifuged at 13,000rpm for 15 minutes to pellet down the synthesized CuNPs. The pellet obtained was washed twice with deionized water and dried in a hot air oven at 80^{0}C. The CuNPs synthesized were used for characterization tests[16]. Following characterization, the particles were used for antibacterial evaluation against UPEC.

Characterization of synthesized CuNPs: The polydispersity index (PDI) and the size distribution of the synthesized green CuNPs were measured utilizing dispersed light at angles of 90 or 173 degrees (default) using a DLS model SZ-100. To check the dispersion and general stability of the synthesized AgNPs, the zeta potential measurement was carried out at a temperature of 25^{0}C. Using a UV-vis spectrophotometer model (1800, Shimadzu) the absorbance spectra were measured over the wavelength range of 200 to 800nm. The study made use of nanoparticle solutions and control-$CuSO_4$ salt.

The biomolecules present in the plant extracts around the synthesized green CuNPs were subsequently classified using a Fourier Transform Infrared (FTIR) spectrometer at 500–4000cm^{-1} (Shimadzu, Koyoto, Japan) after the green CuNPs were freeze-dried. Using the Nanoparticles Tracking Analysis (NTA) LM-20 (NanoSight Ltd. UK), the synthesized nanoparticles were examined for their particle size and distribution. The brownian motion of the particles is also determined by NTA since it assists in particle separation based on size and intensity.

The chemical and crystalline structure of the substance were determined using XRD analysis. The green synthesized CuNPs underwent crystallographic studies using an X-ray powder diffractometer (XRD D8 Advance, Bruker, Madison, WI, USA) with a Ni filter and a Cu K**α** radiation source (**λ** =1.540 **Å**). At a scan speed of 0.4°/min, the diffraction pattern was seen over the 2**θ** range (10-700). Later, the diffractogram was compared to the ICDD's (International Centre for Diffraction Data) reference database.

The hydrodynamic size (Z average), polydispersity index (PDI), and surface charge (zeta potential) of the greenly synthesised CuNPs were screened using the DLS method using a Horiba SZ-100 analyzer (Kyoto, Japan). At a medium count rate of 210 kCPS and a scattering angle of 90° at about 25°C, particle size analysis was performed. SEM was used for the morphological examination. In order to determine whether any aggregates or agglomerates had formed, the morphology of the green synthetic CuNPs was examined.

Bacterial strains: *E. coli* strain was obtained from Mustansiryah university, College of Pharmacy, Iraq. Ampicillin, Nutrient broth and chemicals were purchased from HiMedia Ltd, India. Bacteria were revived and sub-cultured in Nutrient broth at 37ºC for 12-18hr and maintained as pure stock.

Antibacterial Activity Tests: The agar well diffusion method was used to screen the antimicrobial activity of GCuNPs. A loopful of overnight culture of UPEC was inoculated into a nutrient broth media and incubated at 37°C overnight. Cell suspensions adjusted to approximately were used as inoculum. About 100µl of culture (1 x 10^8 CFU/ml) was used for the pour plate (Mueller-Hinton agar). Wells were cut at equidistant positions and 20µl of GCuNPs along with positive control (Ampicillin 20mg/ml) were added. GCuNps of varying concentrations were used for testing. Sterile de-ionized water was used as a negative control. The plates were incubated at 37°C for 24hr under aerobic conditions. following incubation, the diameter of the zone of inhibition (in millimetres) was recorded.

Minimum Inhibitory Concentration (MIC): MIC of the synthesized NPS was determined as described by Sudhakar Malla *et al.* but with slight modification[17]. Varying concentrations (100, 200,400, 800 and 1000µg/ml) of the GCuNPs were prepared with de-ionized water. About 20µl of culture was added to all the tubes including the control. Tube 1 served as a negative control without treatment, tube 2 served as a positive control (Ampicillin). To the medium 50µl of treatment was added to their respective wells. The contents are mixed thoroughly and incubated at 37°C for 12-18hr. Following incubation, the growth or inhibition was observed by recording the OD at 650nm. MIC was considered the minimum concentration at which the growth was inhibited.

Biofilm inhibition assay: The effectiveness of the green synthesized nanoparticles to inhibit the biofilm formation was done according to Sudhakar Malla *et al.* but with slight modification[17]. In brief, overnight cultures were diluted (1: 100) using nutrient broth containing glucose solution. The wells labelled were loaded with 170µl of nutrient broth and seeded with 10µl of UPEC culture. Well 1 served as negative control (without treatment), well 2 served as positive control and wells from 3-7 were treated with 20µl of the nanoparticle suspension of varying concentrations as performed in the previous

section. The plate was incubated for overnight at 37^{0}C and following incubation, wells were washed thrice with 200µl of PBS (pH7.4). Following washing, the plates were air dried and stained with crystal violet (2%) for 15min. The wells were then added with 200µl of ethanol: acetone (80:20) to solubilise the crystal violet and plates were kept in a plate reader (Genetix, Germany) to record the absorbance at 590nm. Biofilm inhibition is calculated with the formula: %biofilm inhibition = 1 - (OD590nm of treated cells / OD590nm of non-treated cells) * 100. The MIC for anti-biofilm potential was expressed as an IC_{50}.

Motility assay by Capillary method: The assay was done according to the protocol mentioned by Cooper et al. but with modifications[18]. Overnight cultures (OD600 of 0.3 to 0.4) were centrifuged and resuspended in phosphate-buffered saline (PBS (1:10). Bacterial suspensions (500µl) were added to the wells of the chambers. Capillaries of length 32mm with a capacity of 1µl were filled with PBS buffer (0.1M, pH *7.0)* and closed at one end. The open end was gently placed in the bacterial suspension on a glass slide. 100 and 200µg/ml of CuNps were used as treatment. The number of bacteria that moved up the capillary was determined by diluting the contents along with buffer and plating them onto nutrient agar. Following incubation for 24-48 hr, colonies were counted, and their number was multiplied by the dilution factor. Results are presented as the percentage of bacteria present in the capillary tubes.

RNA extraction:

Treatment: A 20µl of bacterial suspension was added to 50ml of media and thoroughly mixed. The tube marked «treatments» received 10µl of the respective fraction. Ampicillin (10mg/ml) was used as a «positive control.» A control tube is one that has not been treated. The mixture is adequately vortexed and incubated for 24-48 hours at 37^{0}C. The cultures were taken out after incubation and used for the RNA extraction process.

RNA extraction: The manual's instructions for the RNA extraction technique were followed. The cultures (1 x 10^8 bacteria) were centrifuged at 5000xg for five minutes at 4^{0}C, and the resultant supernatant was thrown away. 500µl of Buffer RLT were added to the pellet and vortexed forcefully for 5 -10sec. The contents were then centrifuged at full speed for approximately 10 seconds before being collected in a new tube. By pipetting, an equivalent volume of 70% ethanol was added and properly mixed. A 2ml collection tube holding the 700µl of obtained lysate was introduced to the RNeasy spin column. After centrifuging the contents for 15 seconds at a speed of roughly 9000 x g, the flow was discarded. To clean the spin column, 700µl of Buffer RW1 was added to the column and centrifuged for 15 seconds at 9000 x g. The RNeasy spin column was washed with 500µl of Buffer RPE by centrifuging it for 15 seconds at 9000 x g while discarding the flow-through. The membrane was cleaned by repeating the previous step twice. 30–50µl of RNase-free water was added to the column before it was placed in a fresh collection tube. The RNA was extracted from the contents by centrifuging it for 1 minute at 9000rpm, and it was then kept at -20°C. The UV spectrophotometer was used to evaluate the RNA quality before it was utilised to synthesise complementary DNA (cDNA).

Reverse transcription (RT) PCR: cDNA synthesis: The SuperScript TMII Reverse Transcriptase, 200U/l (HiMedia) RT PCR kit was used to create the cDNA. In a nutshell, the beginning reaction was initiated with approximately 2µg of the RNA acquired in the preceding phase. The amount of RNA that was retrieved was 1.98µg/l. Therefore, 1.12µl of total RNA, 1µl of RT enzyme, and random primers were used. The mixture was properly combined before being incubated at 25°C for 10 minutes. The generated cDNA was then kept in storage pending further usage for gene expression after 45 minutes of incubation at 70°C.

Real-time PCR: Primer3 software was used to design the real-time primers (Table 1), which were then obtained from Sigma-Aldrich. The iQ™ SYBR Green Supermix (HiMedia) was then used to conduct the real-time PCR experiment in accordance with Monika Kauna et al. findings[19]. In the PCR experiment, the primers (600nM) and 1µl of the RT products were employed, and a total volume of 12.5µl was used for the reaction. To verify the positive amplification, each reaction was conducted in triplicate and concurrently with its corresponding negative control.

Type 1 fimbriae D-mannose specific adhesin *fimH* (ID: 948847), *fliC* flagellar filament structural protein, *BamA* outer membrane protein assembly factor of *Escherichia coli str. K-12*] were used as drug target gene members.

Table 1: Table showing the primer details of the genes used in the study.

Gene		Sequence	Length	Tm	GC%	Product
fimH (ID: 948847)	FW	GGGCAACTCGATTTTCACCA	20	58.76	50	151
	RV	TGCCGTTAATCCCAGACTCA	20	58.73	50	
fliC (ID: 949101)	FW	TTACGCTGCGGATGTGAATG	20	58.99	50	211
	RV	CTCACCACCAGCAGTCAAAC	20	59.05	55	
bamA (ID: 944870)	FW	CCGACCTGTCCGACTATACC	20	59.05	60	152
	RV	CAGATAACGCCACATCGCAA	20	58.99	50	
recA	FW	ATCTCCGTCAATCTCCGCAC	20	59	55	382
	RV	ACGCGCTGAACAAAAGGTTC	20	59.97	50	

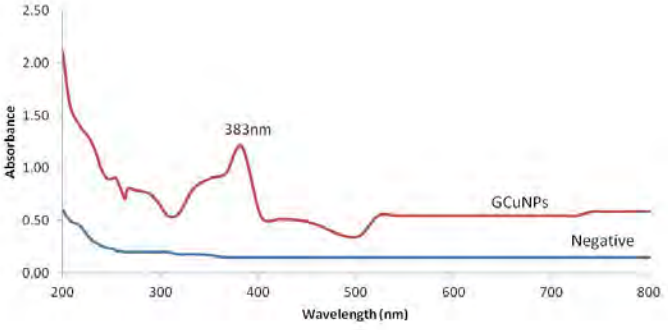
Expression of drug target gene members: The samples (both control and treatment) were quantified in real-time using the Corbett Research cycler (Bio-Rad). The amplification programme utilised fimH, fliC and bamA primers at a concentration of about 600nM. The programme was performed for approximately 40 cycles at 92°C for 60s, 63°C for 55s, and with an elongation at 72°C for 45s using 1.12µl of the RNA products. In order to compare the mRNA expression, the housekeeping gene *recA* was amplified together with the relevant genes of interest. Using the ΔΔCt technique, the relative amounts of mRNA in the test samples (including the control) were compared. The gene of interest's Ct values were acquired and normalised to its housekeeping gene.

Statistical analysis: Triplicates of each experiment were performed. Data was conducted to one-way analysis of variance (ANOVA) when appropriate, and differences between samples were verified by the Tukeys test ($P<0.05$) and were regarded statistically significant. Analyses were conducted using the Microsoft Excel 2010 statistical program throughout the investigation. The faculty version of SPSS was used to conduct the statistical analysis.

Results

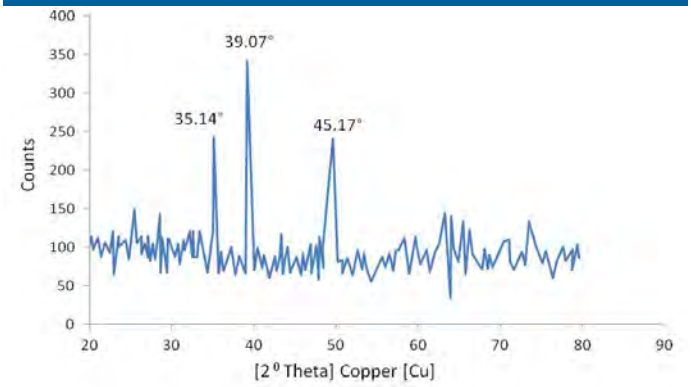
UV spectroscopy: The surface plasmon resonance (SPR) characteristics of the green synthesised CuNPs were evaluated using a UV-visible spectrophotometer. It is the SRP of the electrons on the surface of a nanoparticle that creates a distinctive peak at particular light wavelengths. CuNPs' UV-Vis spectra with propolis extract showed an SPR with a distinctive peak at 385 nm (Figure 1). Similar reports were seen with propolis and Copper nanoparticles, which peaked at 385nm[20].

Figure 1: UV spectra obtained with the biosynthesis of GCuNPs with propolis extract (PE). All the experiments were done in triplicates. The peak at 383nm can be seen in the graph. Deionized water was used as a negative control.



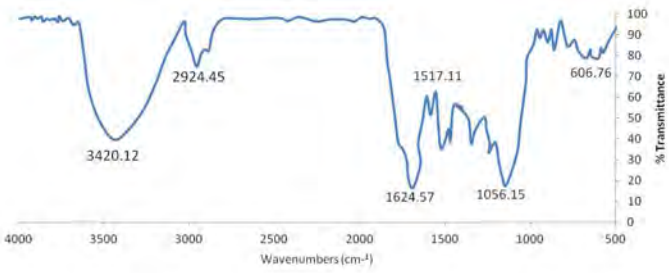
XRD: Propolis extract was used to produce copper nanoparticles with distinct peaks that could be observed through X-ray diffraction. When propolis extract was used to reflect CuNPs, diffraction peaks at 2θ = 35.14°, 39.07°, and 45.17° were observed, which corresponded to the crystallographic planes of face-centred cubic (FCC). Similar reports were reported by Xiang Zhou et al. wherein they observed 5 distinct peaks[21]. Similar to our propolis extract study, YS Hajizadeh *et al.* also observed 3 peaks but at 35, 39 and 49[20].

Figure 2: Image showing the XRD pattern for copper oxide NPs formed from propolis extract (PE).



In FTIR spectroscopy, the Fourier transform is employed to calculate the frequency of molecular vibration. Figure 3, displays the infrared spectra of CuNPs utilizing propolis extract in the frequency range of 4000-400cm^{-1}. The propolis extract spectra showed a strong peak at 3420cm^{-1} caused by free hydroxyl groups and their intra- and intermolecular H-bonds. Sharp peaks at 2924.45, 1624.57, and 1517.11 to 1056.15cm^{-1} were connected to C = O and C = C aromatic stretching frequencies. While, CuNPs monoclinic phase showed a 606.76cm^{-1} absorption band. The peaks which were seen at 2924.45 and 1624.57 and ranged from 1517.11 to 1056.15cm^{-1} seem to be related to saturated hydrocarbons (CSp3-H) and aromatic stretching frequencies of C=O and C=C. NPs in the monoclinic phase of Cu can be seen to have absorption bands 606.76 cm^{-1}. Similar peaks were also reported with propolis[20].

Figure 3: Graph showing the FTIR spectra of GCuNPs for copper oxide NPs formed from propolis extract (PE) from 400 to 4000cm^{-1}.



Particle Size Distribution and Surface Charge Analysis of AgNPs

Prior to evaluating the synthesized CuNPs' antibacterial and anti-biofilm activities, it was critical to establish the particle size and charge of the materials. The main elements that affect nanoparticles' in vitro toxicity are their size, surface charge, shape, and composition[22]. Particle size and charge in an aqueous solution were determined using a dynamic light scattering spectroscopy (DLS) technique. Using this method, it is possible to quickly determine the surface charge and particle size distribution of nanoparticles in solution[23]. The average particle size of the CuNPs produced from the PE was 70.55, according to the results of the DLS study (Figure 4). Because the type of interaction that takes place between nanoparticles and cells is greatly dependent on the size of the nanoparticle, particle sizes <100 nm have higher promise in biomedical applications. Another important characteristic of nanoparticles that influences their capacity to connect with or compound with externally or internally located macromolecules is their surface charge. In order to examine the CuNPs' capability for interacting with biological macromolecules, their charge or Zeta potential was measured. The CuNPs produced by PE had a charge of -21.6 mV, according to the results. Our findings are supported by earlier research on the charge and particle size of CuNPs[24].

One to one hundred nanometer-sized nanoparticles exhibit unusual and novel features[25].

Figure 4: Graph showing the DLS analysis of Green synthesized CuNPs (GCuNPs) using Propolis extract.

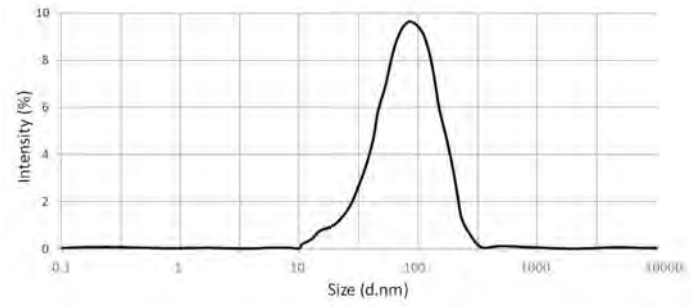
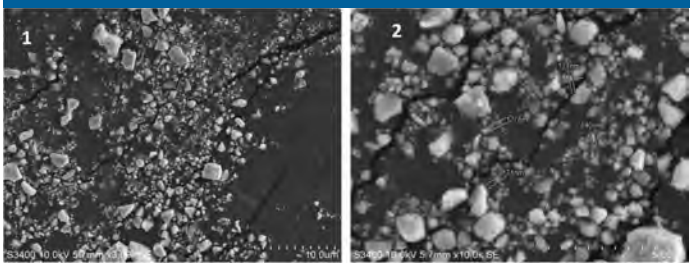


Figure 5: Micrograph images of green synthesized CuNPs. 1: Scale 10.00µm. 2: Scale 5.00µm.



The present study's AgNPs were synthesised using the scanning electronic microscope (SEM), which was used to characterise their size and morphology. SEM is a helpful instrument for learning about the structural characteristics of nanoparticles. The synthesised AgNPs were identified as distinct, uniform-shaped, face-centred, and well-separated nanoparticles based on the inspection of SEM micrographs of the AgNPs. The particles' typical sizes ranged from 48 to 85 nm.

Agar well diffusion assay and MIC: Initial antibacterial activity testing of the extracts was carried out using disc diffusion, in which bacterial activity is determined by the inhibitory zone created by the diffusion of GCuNPs into the agar media. When compared to the positive control and the control, the inhibition zone results indicated that GCuNPs had greater antibacterial activity. The zone of inhibition was found to be 16mm for 1mg/ml when compared to the positive control. Positive control showed 10mm at 0.2mg/ml (Figure 6). The MIC values of GCuNPs obtained from broth dilution assay are presented (Table 2). The MIC values ranged from 77.64 ± 0.113 to 8.4 ± 0.192 for GCuNPs at a concentration range of 0.1 to 1mg/ml. on the other hand, MIC values ranged from 7.2 ± 0.54 to 0.2± 0.82 for ampicillin at the concentration range of 0.1 to 1mg/ml. GCuNPs showed MIC of 8.4 ± 0.192 at 1mg/ml when compared to positive control (7.2 ± 0.54 but at 0.1mg/ml) (Figure 2).

Figure 6: Left: Plate showing the zone of inhibition values in mm as obtained from agar well diffusion method using varying concentrations of GCuNPs. Right: Graph showing the zone of inhibition values in mm as obtained from agar well diffusion method using varying concentrations of GCuNPs. All the values are average of triplicates and expressed as value ± SD.

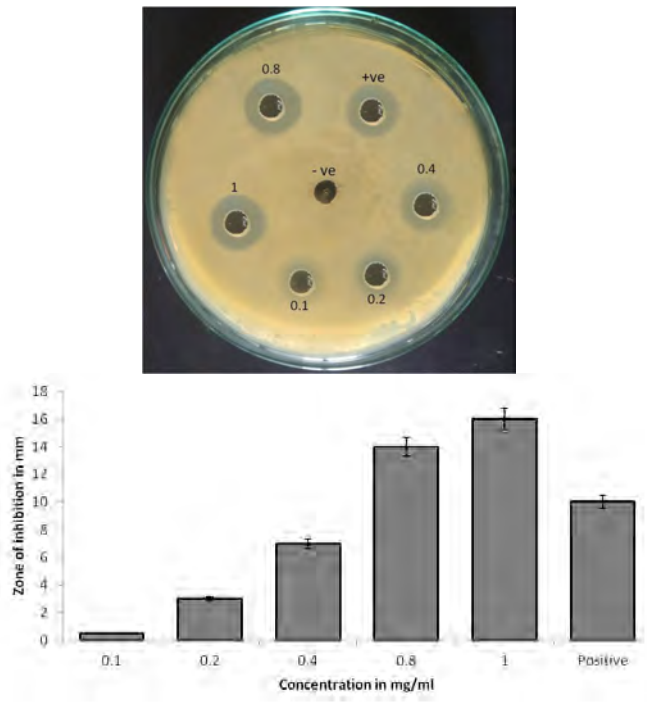
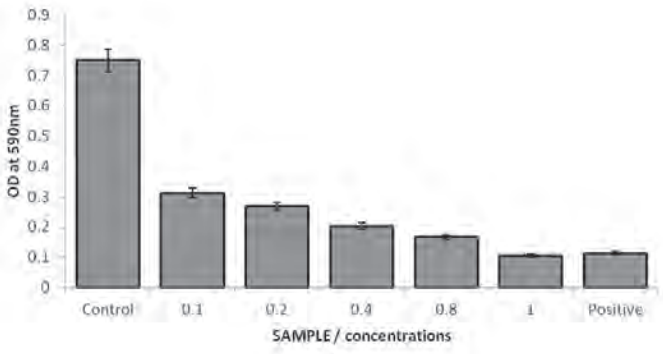


Table 2 shows the MIC values in mg/ml as obtained from the broth dilution method using varying concentrations of GCuNPs. All the values are average of triplicates and expressed as value ± SD.

Conc in mg/ml	0.1	0.2	0.4	0.8	1
GCuNPs	77.64 ± 0.113	51.2 ± 0.71	34.5 ± 0.117	12 ± 0.21	8.4 ± 0.192
Ampicillin (10mg/ml)	7.2 ± 0.54	4 ± 0.31	2 ± 0.31	1 ± 0.62	0.2± 0.82

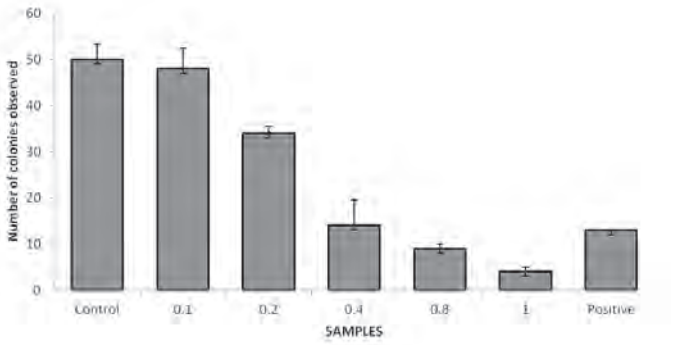
Effect on Biofilm Formation: Green synthesized copper nanoparticles were also assayed for anti-biofilm activity against UPEC strain. In our study, in*vitro* anti-biofilm activity was evaluated in a dose-dependent manner. We found that GCuNPs inhibited biofilm formation by the UPEC species, with respect to the control used in the experiment **(Figure 7**). The MICs of biofilm inhibition were expressed in terms of IC_{50} and all of the GCuNPs, showed an excellent MIC value. Notably, the GCuNPs of all the concentrations exhibited anti-biofilm activity against UPEC in a dose-dependent manner. *E. coli*. concentrations of 80, 50, and 100 µg/ml. AgNPs derived from *G. lanceolarium, S. anacardium*, and *B. retusa*, respectively, exhibited >99% inhibition against biofilm formation by *E. coli*, a Gram-negative bacterium. GCuNPs exhibited antibiofilm activity in the range of 58 to 86%. Positive control showed 81% biofilm inhibition at 0.2mg/ml whereas our GCuNPs at the same concentration showed 64% which is significant (P<0.05). 86% activity was observed at 1mg. ml concentration. Control OD was found to be 0.751±0.32 and its activity was assumed to be 0.

Figure 7: Graph showing the biofilm inhibition of GCuNPs at varying concentrations (0.1,0.2,0.4,0.8 and 1mg/ml). Ampicillin (10mg/ml) was used as positive control. Control is without treatment. All the values are the average of 3 independent experiments (P<0.05).



Motility assay by Capillary method: Our results confirmed the effect of GCuNPS on the motility of the UPEC. The motility behaviour of the UPEC strain was observed to be dose-dependent (p<0.05). The number of colonies was found to be reduced with increasing concentration depicting the antibacterial activity of the GCuNPs. GCuNPs showed a 92% reduction in the number of colonies at 1mg/ml. on the other hand, positive control showed a 74% reduction at 0.2mg/ml. control was assumed to be 0 (Figure 8).

Figure 8: Graph showing the number of colonies formed on treatment with GCuNPs at varying concentrations (0.1,0.2,0.4,0.8 and 1mg/ml). Ampicillin (10mg/ml) was used as positive control. Control is without treatment. All the values are the average of 3 independent experiments (P<0.05).



Gene expression studies: From the qPCR study, we found that overall all of the three gene members were downregulated on treatment with the GCuNPs when compared to positive control (P<0.05). We found a strong correlation between the virulence gene members and the treatments, which was quite obvious from the loads of PCR products (p<0.05). The expression of each gene member was indicated as the ratio of its relative expression to that of control (non-treated). From the real-time expression profile, we found fimH gene member was found to be downregulated by 35% when compared to the positive control (75%). On the other hand, fliC was found to be downregulated by 66% on treatment with GCuNPs when compared to positive control (85%).

Strikingly, there was no significant downregulation seen with GCuNPs when compared to positive control (33%) which is far lower when compared to other gene members' regulation. Even positive control showed a lower effect (p<0.05).

Figure 9: Graph showing the relative gene expression of fimH, fliC and BamA expression on treatment with GCuNPs. Control expression (without treatment) was assumed to be 1 or 100%. Ampicillin was positive control (10mg/ml). All the values are averages of triplicates. ** Significant; * highly significant (P<0.05). A 1.2% agarose gel image showing the amplified bands of the gene members can be seen in the figure (Bottom).**

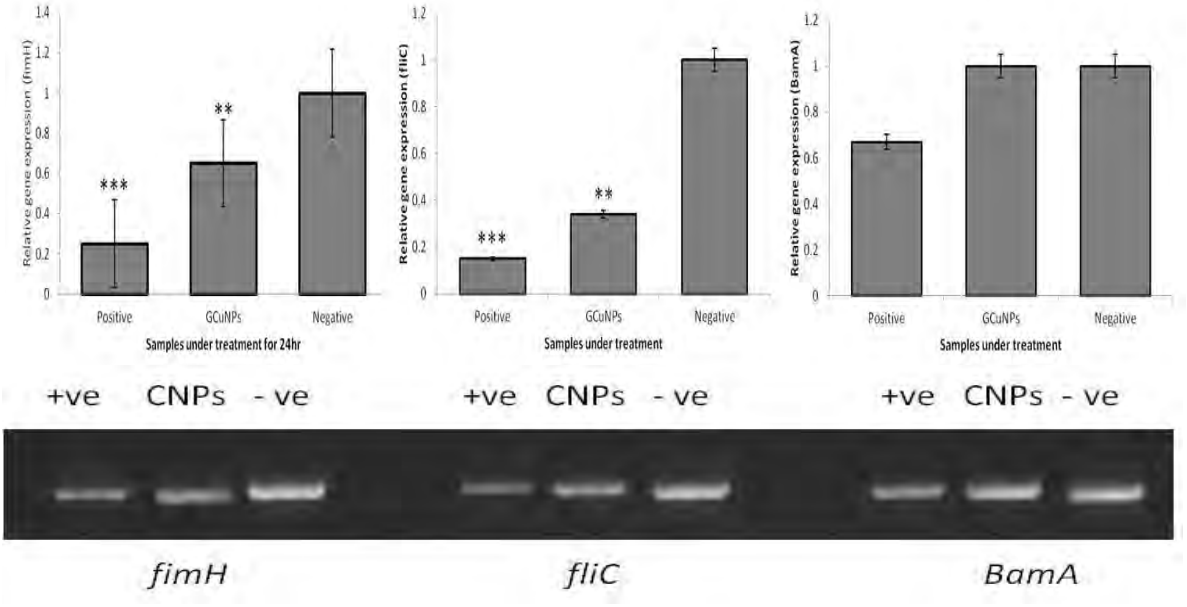
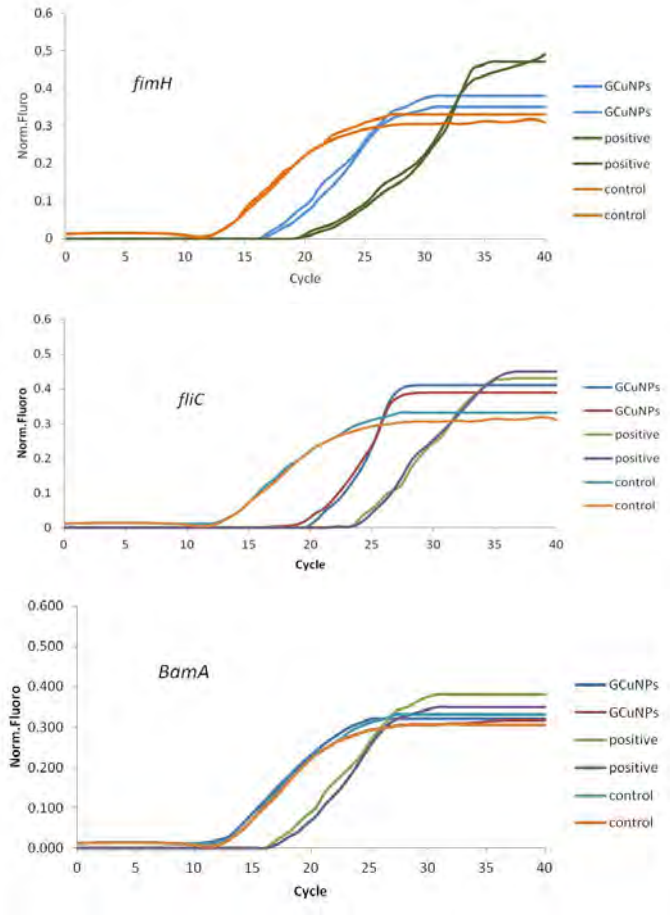


Figure 10: Real-time CT values showing the relative gene expression of fimH, fliC and BamA expression on treatment with GCuNPs. Ampicillin was positive control (10mg/ml). All the values are averages of triplicates. RecA was used as a housekeeping gene (Normalizer).



Discussion

In this study, propolis extract was used to create CuNPs that were then optimised and characterised. Due to their capacity to stabilise AgNPs, inherent antibacterial capabilities, high level of efficacy, and minimal toxicity, plant extracts or green products have an advantage over the chemical or physical manufacture of NPs. Their application represents an environmentally acceptable method of NP synthesis. According to our research, propolis extract has great potential for the industrial synthesis of CuNPs.

The surface plasmon resonance (SPR) characteristics of the green synthesised CuNPs evaluated with a UV-visible spectrophotometer revealed an absorbance band at 385nm (Figure 1). Similar reports were seen with propolis and Copper nanoparticles, which peaked at 385nm[20]. Studies done by Jain S, et al.(2017) reported the UV spectra of CuNPs at about 403nm. This absorption band is basically due to the surface plasmon resonance of g-Cu NPs. A similar result was reported after the analysis of synthesized Cu NPs using the *Ocimum sanctum* leaf extract[26]. The surface plasmon absorbance basically depends on the size of NPs, and hence, every researcher reports a different value for NPs synthesized using different plant extracts.

Studies using FTIR identify potential biomolecules in plant extract that are in charge of CuNP stabilisation and reduction[19][26]. Our FTIR study depicts the peak bands at 2924.45, 1624.57, and 1517.11 to 1056.15cm^{1}.

Similar matching peaks were observed in other studies as well[26].

These distinctive characteristic bands are said to reveal the presence of flavonoids and phenolic compounds[27]. By unleashing a reactive hydrogen atom, the flavonoid biomolecules changed the enol form into the keto form, which could then reduce Cu2+ ions to generate CuNPs. These biomolecules chelate metal ions via their carbonyl groups or π -electrons to stabilise NPs[28]. This leads to the conclusion that the flavonoid and other phenolic compounds in these leaf extracts could stabilise and cap the surface of the synthesised CuNPs[29].

It was crucial to determine the particle size and charge of the materials before testing the antibacterial and anti-biofilm properties of the synthesised CuNPs. Particle size and charge in an aqueous solution were determined using a dynamic light scattering spectroscopy (DLS) technique. We found the average particle size of the GCuNPs to be 70.55 from the DLS study (Figure 4). Jeevanandam *et al*., (2019) stated that particles of size 10-100nm are very much capable of unusual and novel features. To investigate their potential role in interacting with other biological macromolecules, their charge or Zeta potential was also estimated wherein we found a charge of -21.6 mV was produced. Our findings are supported by earlier research on the charge and particle size of CuNPs[24].

Propolis samples from various regions isolated showed moderate to high antibacterial activity or MIC against both gram-positive and gram-negative microorganisms ranging from 0.08 mg/ml to 7.5 mg/ml[30]. From our study, we found the MIC value to be highly significant with other reports. We found a significant MIC at 8.4 ± 0.192 (1mg/ml). Our studies are completely in line with Veiga R.S. et al(2017) and Seidel V et al(2008).[31, 32]. But we report for the first time, the MIC and antibacterial potential of the GCuNPs against UPEC strains.

Although many antibiotics can freely penetrate the EPS, cells within the biofilm are often still protected. Biofilm bacteria show much greater resistance to antibiotics than their free-living counterparts. The creation of starved, stationary phase dormant zones in biofilms seems to be a significant factor in the resistance of biofilm populations to antimicrobials, particularly against antibiotics such as β-lactams, which are effective against rapidly dividing gram-positive bacteria by interruption of cell wall synthesised[33].

We found MBC values to be highly significant in inhibiting the biofilm formations. Our GCuNPs showed significant antibiofilm activity ($P<0.05$) in the range of 58 to 86% when compared to positive control (81%). Our biofilm results are also in accordance with motility studies. GCuNPs showed a 92% reduction in the number of colonies at 1mg/ml when compared to positive control (74%). Similar report was seen wherein Propolis extract derived copper nanoparticles showed potent MBC activity[20]. Study done by Murthy H. C. Ananda et al. also stated the significant role of green synthesized CuNPs on antibiofilm activity[34].

Biofilm and virulence genes were studied in various transcriptomics reports. We investigated the significant role of virulence genes (*fimH, fliC and BamA*) through gene expression studies. We aimed to search the regulation of the three aforementioned genes under the treatment of GCuNPs. We could confidently say we are the first to report such expression studies on these genes under the effect of GCuNPs. Interestingly, we found both fimH and fliC genes were downregulated signifying their role in biofilm and pathogenesis. However, we found there is no significant change in the expression of BamA. Further studies need to be done to validate these results.

Conclusions

Though propolis was previously used to synthesize CuNPs, this study presents a novel method of evaluating antibacterial activity against UPEC strains. Green biosynthesis was effective because it was carried out using a non-toxic, simple-to-use, economical, and environmentally beneficial method. Tools for characterising CuNPs give information about their stability in upcoming uses. Due to their environmentally friendly biosynthetic synthesis from natural materials, CuNPs are anticipated to be utilised in drug delivery systems, drug formulations, and biomedical applications in the future. The precise antibacterial and synergistic effects of combining CuONPs and propolis may be clarified, though. To ascertain the long-term efficacy of this technique and propolis extract, clinical studies must be planned to assess the function of propolis-based green biosynthesized CuONPs.

The biological sciences should investigate this area of study more thoroughly. Due to climate change, there is an increased prevalence of recently evolved, drug-resistant bacteria in semi-tropical habitats, which poses a serious health risk. Current antibiotics and the idea of employing antibiotics to fight diseases are quickly losing their efficacy in the face of emerging pathogen strains and disease-causing organisms. High-throughput nanobiotechnology-tailored antimicrobials provide a fresh method for treating biofilms and microorganisms that are resistant to conventional medical treatments. The current study shows the potential of employing AgNPs produced from plants to prevent biofilm development for therapeutic treatments, which provides a new approach to successfully treating a variety of infectious disorders brought on by pathogenic bacteria.

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Conflicts of Interest: The authors declare no conflicts of interest.

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