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Green-synthesized copper nanoparticles: A promising molecule for wound healing with antibacterial and angiogenic activities

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Abstract

Wound healing is an active process with intricate and highly programmed phases and any improper occurrence of this sequence will lead to delayed or improper wound healing. Hence, the bioprospecting of potential molecules alone or in combination with antibacterial and angiogenesis activity is a prerequisite for better therapeutic wound healing activity. With this background, the present study was conducted using copper nanoparticles (CuNPs) green synthesized using *Calendula officinalis* L. flowers. The CuNPs were characterized using UV-Vis Spectroscopy, SEM, TEM, EDAX, FTIR and XRD. The UV-Vis analysis revealed the characteristic peak at 342 nm. SEM and TEM analysis showed irregular and elliptical-shaped nanoparticles with mean sizes of 79.30 nm and 41.91 nm, respectively. The functional groups present in *C. officinalis* extract were confirmed using FTIR and copper presence was confirmed using EDAX and the crystallinity was identified using XRD. The antibacterial activity of CuNPs was assessed against *E. coli* and *S. aureus*. The results revealed a better zone of inhibition of 16.61 ± 0.50 mm for *E. coli* and 24.33 ± 0.64 mm for *S. aureus* which was compared with ceftriaxone-sulbactam (24.18 ± 0.21 mm) and enrofloxacin (24.10 ± 0.20 mm), respectively. Further, the angiogenesis activity of CuNPs was assessed through chick chorioallantoic membrane (CAM) assay and the findings showed the CuNPs produced 32.00 ± 0.58 new blood vessels whereas, the control group produced 10.83 ± 0.48 new blood vessels. Hence, it was concluded that the detection of significant antibacterial and angiogenic activities of green synthesized CuNPs might indicate their use as a potential wound healing agent.

1. Introduction

In recent years, metal nanotechnology has attained extensive fields of research in all disciplines because of its varied applications such as lubricant additives, coating for medical devices, optical sensors, antibacterial and antifungal when added to plastics, photocatalysis, electrocatalysis and inkjet printing. Copper nanoparticles, one of the metal nanoparticles, possess vital chemical and physical properties (Dutta *et al.,* 2018) and it has been used in multiple arenas such as the biomedical, food, and healthcare industries. Since ancient times, copper has been known to possess antiseptic properties and acted as cofactor for cytochrome oxidase (Kim *et al.,* 2008), superoxide dismutase (Sheng *et al.,* 2014) and lysyl oxidase enzyme (Rucker *et al.,* 1998) which is necessary for collagen cross linking during healing of wound. Also copper stimulates angiogenesis (Borkow, 2014) which further could benefit the wound healing process. Hence, the antimicrobial and angiogenic activity of copper could be tapped to develop a wound healing nanoformulation as a better alternative to allopathy based medicines.

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The synthesis of nanoparticles involves various methods, *viz.,* physical, chemical and thermal which encompass the utilization of costly equipment and hazardous chemicals (Das *et al.,* 2017). To overcome this, a better alternative will be the green-mediated eco-friendly method of nanoparticle synthesis using plant extracts. The green synthesis method eliminates the use of toxic chemicals and high-cost equipment involved in other methods (Kumar and Yadav, 2009).

With this aspect, *C. officinalis* belonging to the Asteraceae family has been selected through bioprospecting for the green synthesis of copper nanoparticles. *C. officinalis* was extensively used in traditional medicine for wound healing, and inflammation and incorporated in many skin care products (Zitterl-Eglseer *et al.,* 1997).

Hence, the objectives of this study were to synthesize the copper nanoparticles using *C. officinalis* flower extract and to detect the antibacterial and angiogenic activities of green synthesized nanoparticles to authenticate the combination utility as a wound healing agent.

2. Materials and Methods

2.1 Collection of *C. officinalis* flowers

Fresh flowers of *C. officinalis* were procured from the Government Botanical Garden in Ooty, Nilgiris district of Tamil Nadu, India and it was authenticated by a Botanist affiliated with the Survey of Medicinal Plants and Collection Unit at the Central Council for Research in Homeopathy, Department of AYUSH, Government of India.

2.2 Preparation of ethanolic extract of *C. officinalis* flowers

The flowers were shadow-dried and finely ground and stored for the extraction process. A quantity of 20 g of powder was added to 200 ml of ethanol and placed in a rotary shaker for about 72 h. Then, it was filtered using Whatman filter paper No.1 to separate the extract. The filtrate was subjected to evaporation at 35°C by placing it in a hot air oven. The resulting dried extracts were preserved at 4°C for further usage (Vikrama Chakravarthi *et al.,* 2022).

2.3 Qualitative phytochemical analysis

Fifty milliliters of distilled water were added to 5 g of dried ethanolic extracts of *C. officinalis* and it was heated below 50°C for 1-2 min and then utilized for detecting various phytochemicals like alkaloids (Hager's test), flavonoids (Alkaline reagent test), phenols (Ferric chloride test), saponins (Froth test), terpenoids, volatile oil and carbohydrates (Fehling's test), cardiac glycosides, hydrolyzable tannins, phylobotannins and vitamin C (Trease and Evans, 1983; Kokate *et al.,*1990).

2.4 Green synthesis of copper nanoparticles

Twenty ml of *C. officinalis* extract was slowly added to 100 ml of 10 mM copper sulfate placed on a magnetic stirrer and the pH was maintained at 8. The stirring continued for 4 h at room temperature. The solution was centrifuged at 10,000 rpm for 5 min, followed by two washes with distilled water and ethanol to eliminate residual copper and plant debris. After centrifugation, the resulting pellets were collected and dried using a hot air oven, yielding green-synthesized CuNP powders, which were stored for subsequent use (Rajeshkumar *et al.,* 2019).

2.5 Characterization of green synthesized CuNPs

Copper nanoparticles synthesized using *C. officinalis* were characterized using various techniques such as; the surface plasmon resonance of the CuNPs was analyzed by double beam UV-Visible (UV-Vis) spectrophotometer (M/s Systronics 2201) immediately after synthesis and 60 days after synthesis for determining the stability of the synthesized nanoparticles. The morphological characteristics of the CuNPs were examined by scanning electron microscope (SEM Carl Zeiss Evo 18) and Transmission electron microscope (TEM-Tecnai Sprit) (Sandhya and Lankalapalli, 2023), the elemental composition was identified by energy dispersive x-ray spectroscopy (EDAX- Vega Tescan), the structural investigation was performed using powder X-ray diffractometer (XRD- Malvern Panaltical) and the phytocompounds associated in the production of nanoparticles were examined using fourier transform infrared spectroscopy (FT-IR, Bruker Alpha).

2.6 Antibacterial activity of green synthesized CuNPs

The antibacterial activity of green synthesized CuNPs and *C. officinalis* extract was assessed by disc diffusion assay (Wu *et al.,* 2020) against *Escherichia coli* and *Staphylococcus aureus* isolated from the infected wound samples. The overnight cultures were swabbed uniformly onto the Muller Hinton agar plates. The sterile discs were impregnated with *C. officinalis* extract (2 mg/disc), and green synthesized CuNPs (1 mg and 2 mg/disc) were placed over the swabbed agar plates and incubated for 24 h. Dimethyl sulfoxide is used as solvent control. After 24 h the size of the inhibitory zone was measured. Based on the pilot study among different antibiotics, the highest zone size antibiotics, *viz.*, ceftriaxone with sulbactam and enrofloxacin against *E. coli* and *S. aureus*, respectively were taken as standard antibiotics for comparison.

2.7 Angiogenic activity of green synthesized CuNPs

This unique assay allows the observation of blood vessel sprouting in response to angiogenic agents. During this assay, embryo mortality was observed since the openings made in the shell might aid in contamination and thus strict aseptic measures were followed to curtail the chick embryo mortality. Thirty numbers of ten days old embryonated chicken eggs were divided into five groups. Ethical approval is not required for this assay, since pain perception mechanisms will not be developed for the chick embryos before seventeenth day of incubation (Sarogni *et al.,* 2021). The air sac was punctured through an opening in the blunt end to make the CAM drop. Another opening was made on the shell and the test formulations (200 µg of *C. officinalis*, 100 and 200 µg of CuNPs) were instilled over the CAM. DMSO served as solvent control and distilled water was inoculated for the control eggs. Then the eggs were incubated at 37°C with 80% humidity for 72 h. Then the CAMs were excised and placed over a glass slide to capture images using a stereo microscope at 10X magnification attached to a digital camera. The number of newly formed blood vessels was measured to determine the angiogenic effect (Talekar *et al.,* 2017).

2.8 Statistical analysis

Experimental data were analyzed as a completely randomized design (Snedecor and Cochran, 2004) using the one-way ANOVA in SPSS software (version 20). Post-hoc analysis was carried out by Duncan's significant difference test.

3. Results

3.1 Qualitative phytochemical analysis

The qualitative phytochemical analysis of *C. officinalis* flowers showed the existence of phenols, alkaloids, flavonoids, saponins, terpenoids and volatile oil. The carbohydrates, cardiac glycosides, hydrolyzable tannins, phylobotannins and vitamin C could not be detected in the phytochemical analysis.

3.2 Green synthesis of copper nanoparticles

The color of the copper sulfate was initially changed from blue to brownish green color following the addition of the flower extract. Then, the solution gradually changed to a green color upon continuous stirring which confirmed the reduction and synthesis of copper nanoparticles (Figure 1).

3.3 Characterization of green synthesized nanoparticles

3.3.1 UV-Vis spectroscopy

The UV-Vis spectra of the CuNPs were shown in Figure 2**.** The absorption spectra were evaluated at various wavelengths from 200 - 600 nm in the double beam UV-Vis Spectrophotometer. The green synthesized CuNPs exhibited the Surface Plasmon Resonance peak absorption at 342 nm. Also the peak absorption of the CuNPs was 341 nm after 60 days of synthesis.

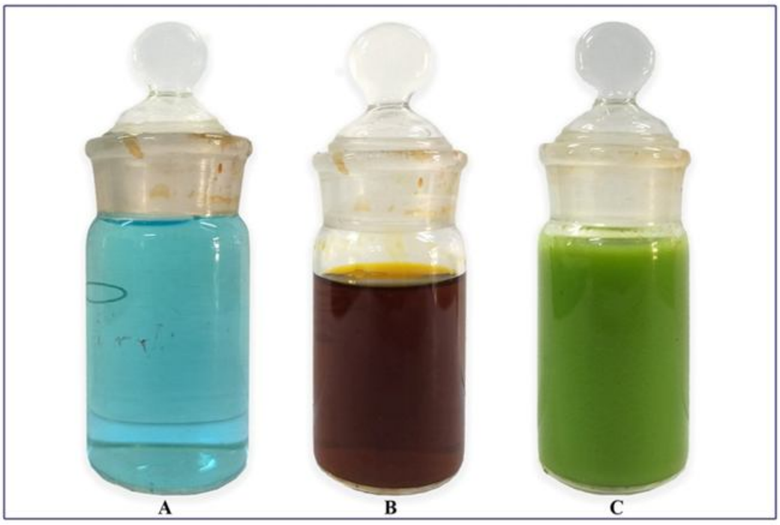

Figure 1: A-$CuSO_4$, B-*C. officinalis* extract and C-CuNPs.

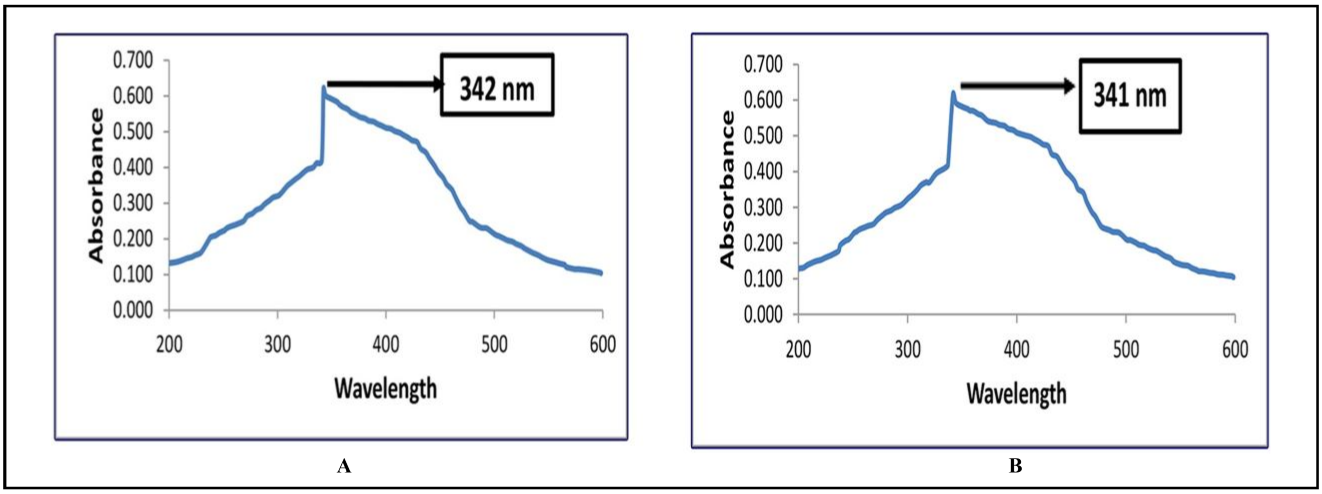

Figure 2: A: UV-Vis spectroscopy of CuNPs; B: UV-Vis spectroscopy of CuNPs-60 days after synthesis.

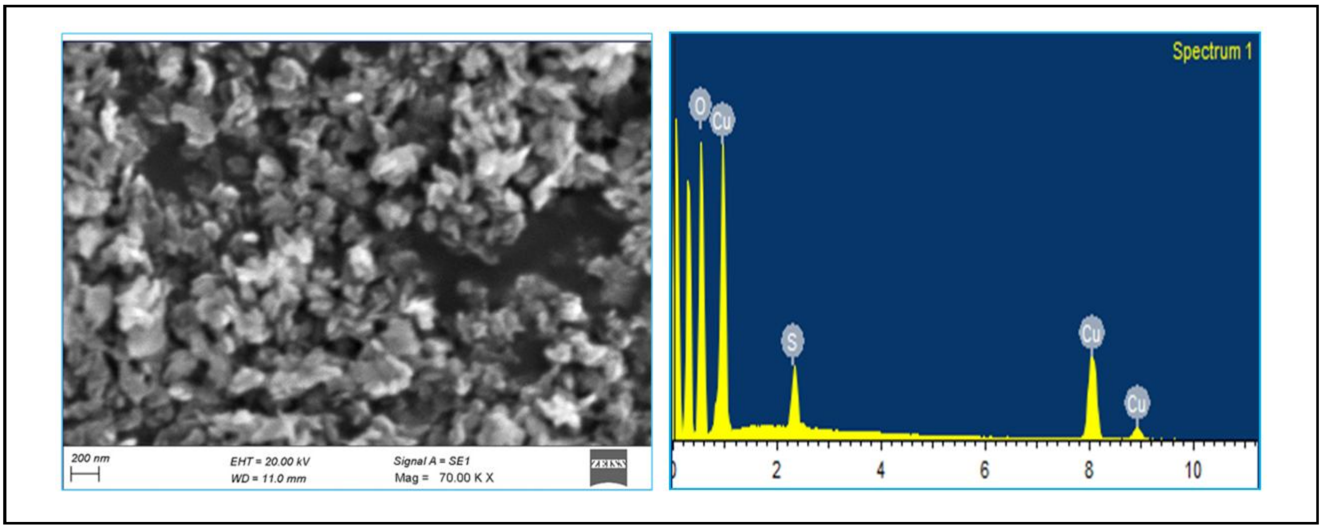

Figure 3: SEM and EDAX of CuNPs.

3.3.2 Scanning electron microscopy with energy dispersive x-ray spectroscopy

The morphology of the green synthesized CuNPs was investigated using SEM and it is shown in Figure 3. The nanoparticles were irregular and elliptical shaped in a few places. The size of the nanoparticles was between 54.4 to 92.3 nm with a mean particle size of 79.3 nm which was determined using Image J software.

The elemental composition of the green synthesized CuNPs was analyzed using EDAX and the result is shown in Figure 3. EDAX analysis indicated the presence of peaks corresponding to elemental copper, oxygen and sulfur. The weight percentages of copper, oxygen and sulfur were 42.66, 52.19 and 5.15, respectively.

3.3.3 Transmission electron microscopy

TEM investigation was conducted to attain deep insight into the morphometry of the green synthesized nanoparticles. TEM images exhibited the synthesized nanoparticles in various shapes such as elliptical, rod and irregular which is depicted in Figure 4. The size of the particles was in the range of 22.8 to 62.8 nm and the mean size was observed as 41.91 nm using Image J software.

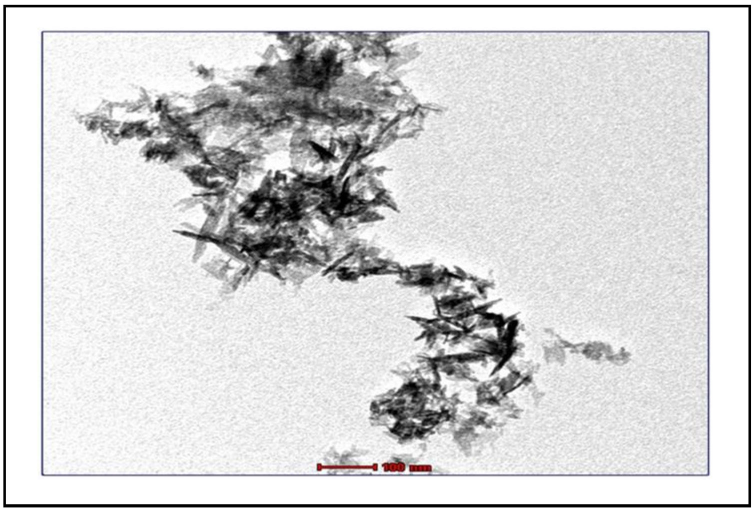

Figure 4: TEM of CuNPs.

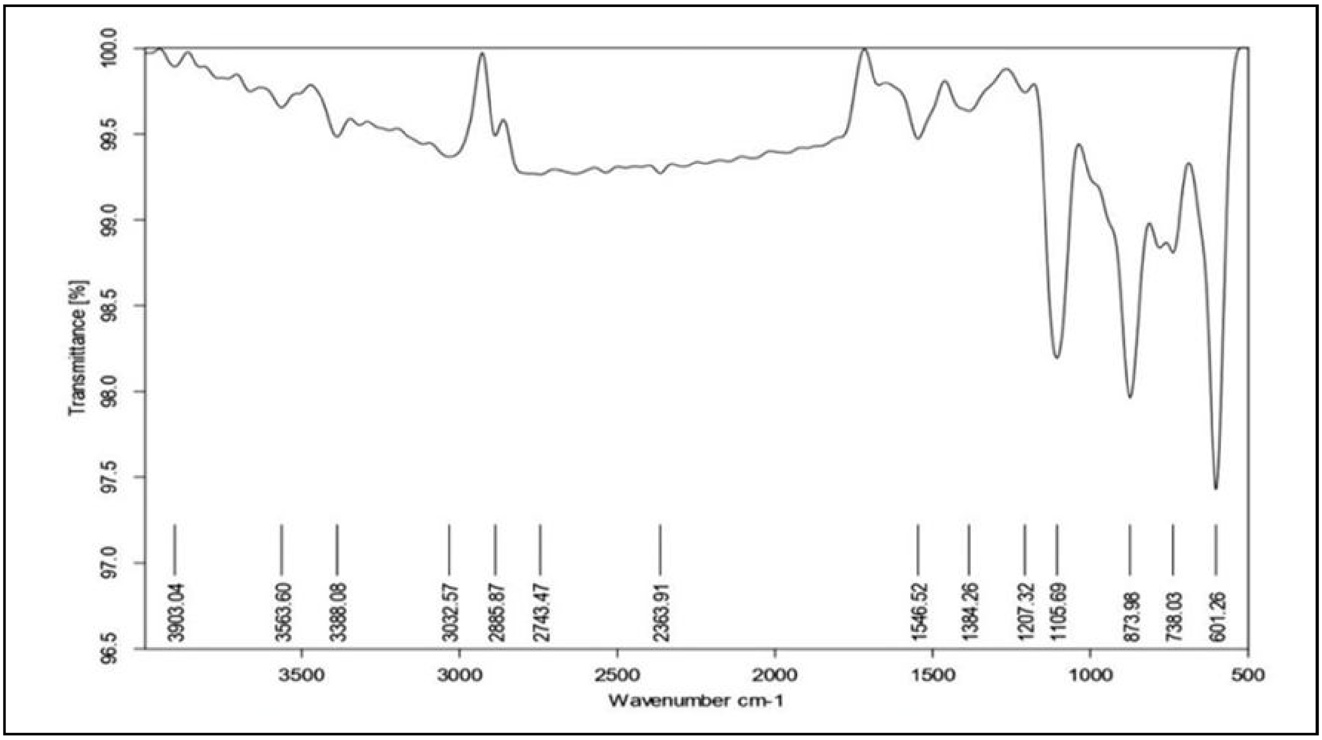

Figure 5: FT-IR of CuNPs.

3.3.4 Fourier transform infrared spectroscopy

FT-IR spectrum was depicted in Figure 5. In the 3500-3000 cm^{-1} region, absorption of hydroxyl group (OH) appeared at 3234 cm^{-1}. The bands at 2000-1500 cm^{-1} were due to the C = C bond. The band at 1654 cm^{-1} was determined to carbonyl and carboxylic (CO) bands of peptide linkages. The band at 1384 cm^{-1} was imposed to the –O–H bend of carboxylates. The red shift at 1593 to 1654 cm^{-1} was due to oxidation of –CO groups of the extract. The band at 601 cm^{-1} was relevant to Cu-O functional group resonance.

3.3.5 X-ray diffraction

The XRD peaks of CuNPs at 35.58°, 52.55° and 61.57° could be allotted to (111), (020) and (-113) planes, respectively which denoted that the synthesized CuNPs have a face-centered cubic structure. The two diffraction peaks at the 2-theta value of 29.1° and 60.5° were indexed to cuprous oxide (Figure 6).

3.4 Antibacterial activity

The zone of inhibition of CuNPs and *C. officinalis* is demonstrated in Figure 7 and Table 1. The results showed that the green synthesized CuNPs @ 2 mg/disc, exhibited an equivalent zone of inhibition (24.33 ± 0.64 mm) as that of enrofloxacin (24.10 ± 0.20) concerning *S. aureus* and a lower but substantial zone of inhibition (16.61± 0.50 mm) for *E. coli* in comparison with ceftriaxone and sulbactam combination (24.18 ± 0.21 mm). The zone of inhibition of *C. officinalis* extract was lower for both organisms than the standard and both concentrations of CuNPs.

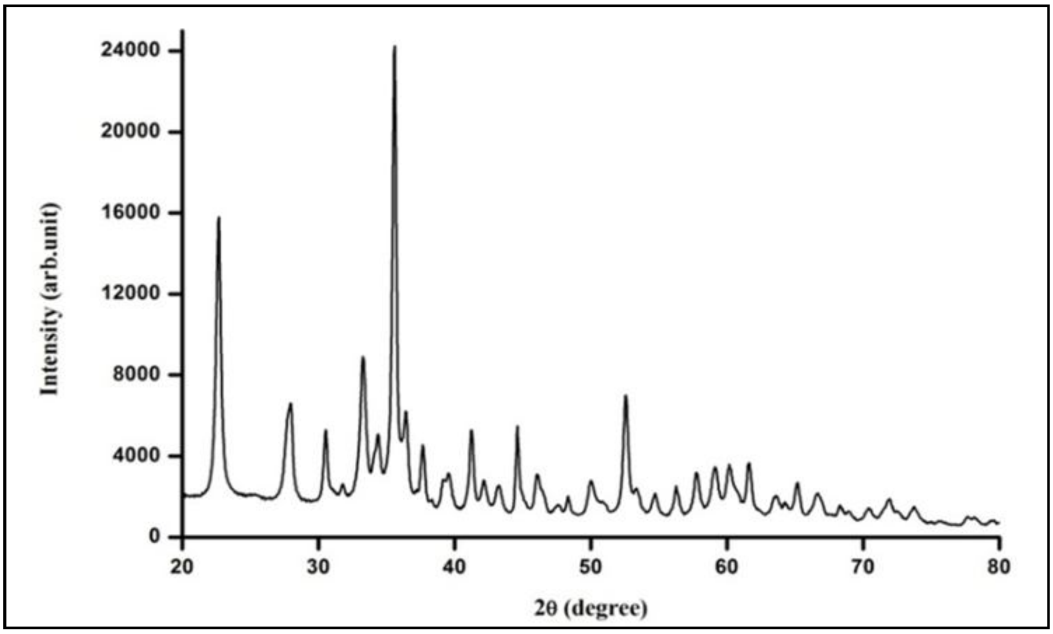

Figure 6: XRD of CuNPs.

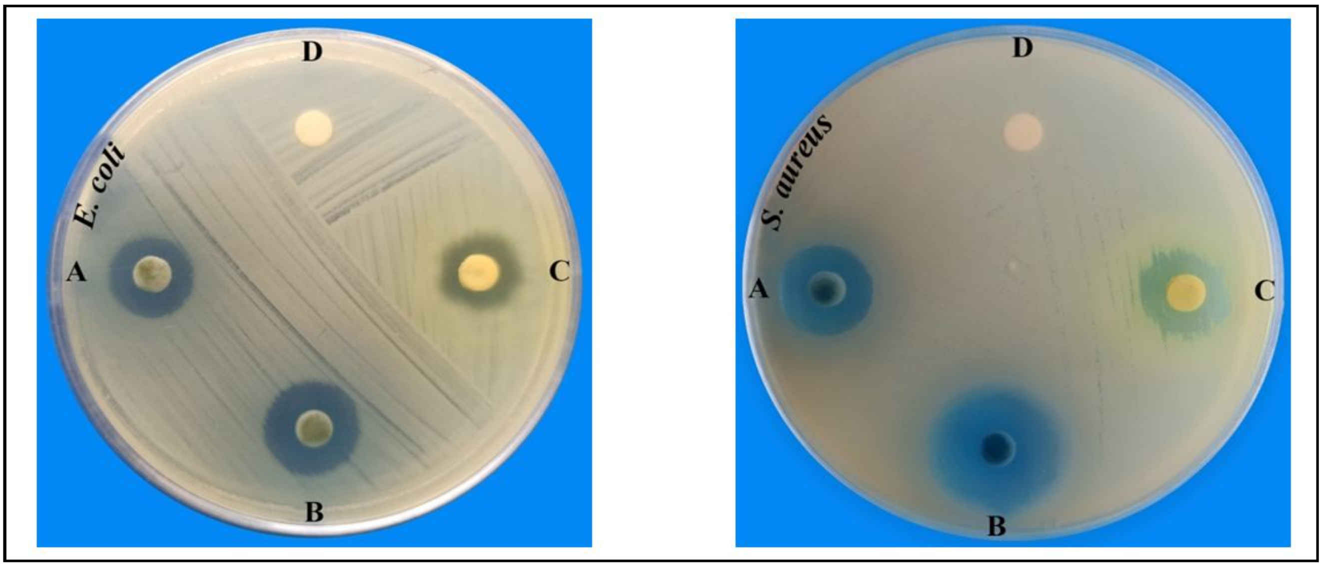

Figure 7: Zone of inhibition of CuNPs: A-1 mg green synthesized CuNPs; B-2 mg green synthesized CuNPs, C-2 mg *C. officinalis* and D-DMSO.

Table 1: Zone of inhibition (mm) of green synthesized CuNPs against *E. coli* and *S. aureus* (n=6) (Mean ± SE)

Organism	T_1 (DMSO)	T_2 (Standard)	T_3 (*C. officinalis* – 2 mg)	T_4 (Green synthesized CuNPs – 1 mg)	T_5 (Green synthesized CuNPs – 2 mg)
E. coli	0.00	$24.18^d \pm 0.21$	$10.80^a \pm 0.33$	$13.40^b \pm 0.66$	$16.61^c \pm 0.50$
S. aureus	0.00	$24.10^c \pm 0.20$	$9.41^a \pm 0.63$	$18.14^b \pm 0.67$	$24.33^c \pm 0.64$

Columns bearing common superscript did not vary significantly at 5% ($p<0.05$) level.

3.5 Angiogenic activity

The *C. officinalis* at 0.2 mg, green synthesized CuNPs at 0.1 mg and 0.2 mg groups showed a markedly increased number of blood vessels than control and solvent control groups. Between the treated groups, green synthesized CuNPs at 0.2 mg showed a significantly higher number of blood vessel formation which was followed by green synthesized CuNPs at 0.1 mg and *C. officinalis* at 0.2 mg (Figure 8 and Table 2). The newly formed blood vessels in the CuNPs treated CAM were much firmer which was an additional finding in this study.

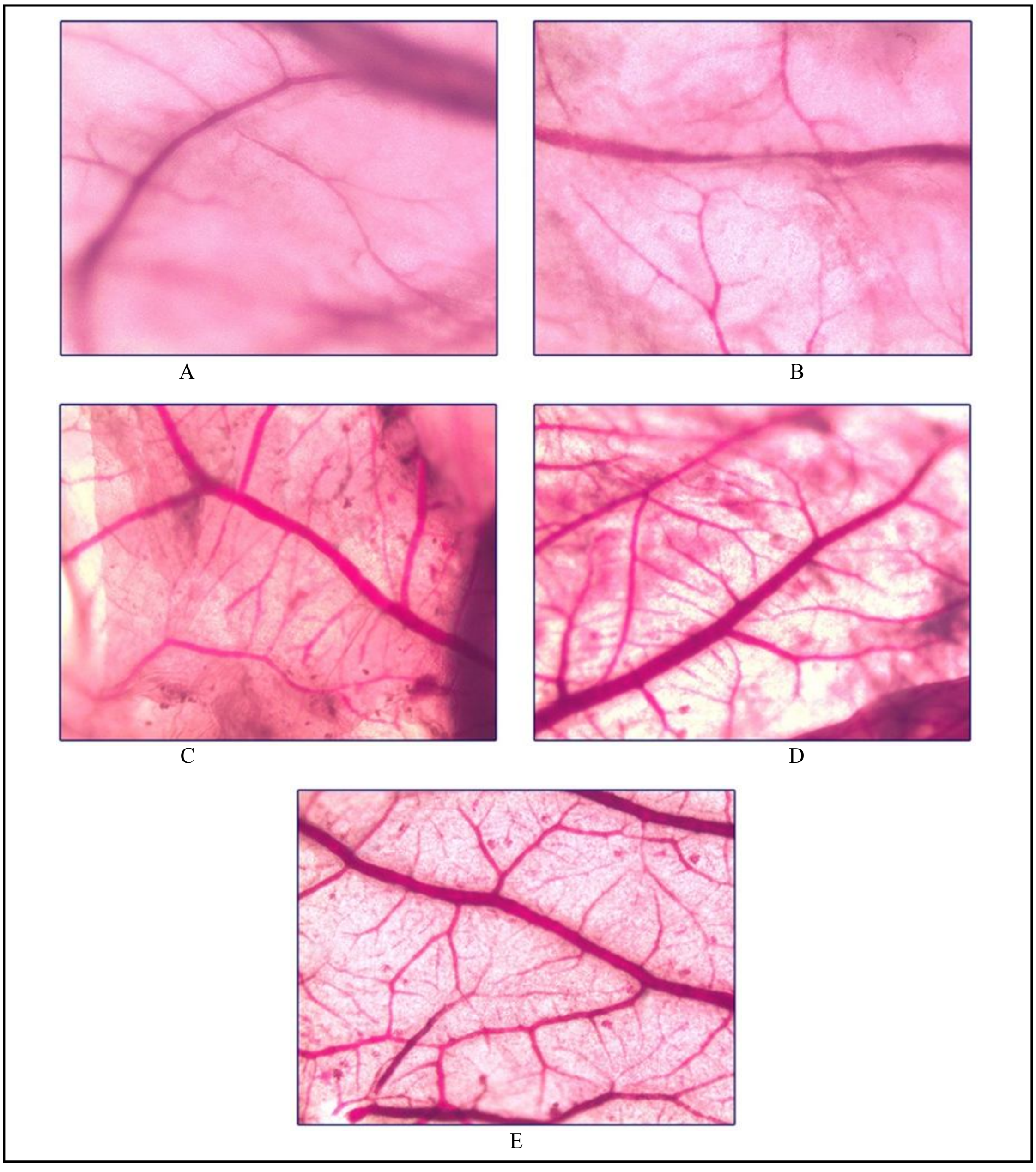

Figure 8: Angiogenic activity: A- Control, B- Solvent control, C- *C. officinalis* (0.2 mg), D- Green synthesized CuNPs (0.1 mg) and E- Green synthesized CuNPs (0.2 mg).

Table 2: Effect of green synthesized CuNPs on angiogenesis in CAM model (n=6) (Mean ± SE)

Treatment groups	Number of blood vessels
Control	$10.83^{a} \pm 0.48$
Solvent control	$10.50^{a} \pm 0.77$
C. officinalis – 0.2 mg	$20.17^{b} \pm 0.80$
Green synthesized CuNPs – 0.1 mg	$26.83^{c} \pm 0.60$
Green synthesized CuNPs – 0.2 mg	$32.00^{d} \pm 0.58$

Columns bearing common superscript did not vary significantly at 5% ($p<0.05$) level.

4. Discussion

4.1 Qualitative phytochemical analysis

Alkaloids, saponins (Chong *et al.,* 2008), flavonoids and terpenoids were detected in the phytochemical analysis of *C. officinalis* and they showed antimicrobial activity in many studies (Subashini *et al.,* 2011). Likewise, saponins and flavonoids were also detected in the phytochemical analysis and they possessed angiogenic property (Parente *et al*., 2011).

4.2 Green synthesis of copper nanoparticles

The phytocompounds were associated with the reduction (Abubucker Peer Mohideen, 2021), synthesis and stabilization of the copper nanoparticles (Iravani, 2011). The color transformation observed during the reaction signified the synthesis of nanoparticles (Haris Variyathody *et al.,* 2023; Sangita *et al.,* 2023).

4.3 Characterization of green synthesized copper nanoparticles

4.3.1 UV- Vis spectroscopy

The formation of CuNPs using *C. officinalis* was confirmed with the gradual color change of solution at different times and it was recorded through the peak of surface plasma resonance (SPR) by UV-Vis Spectroscopy. The green synthesized CuNPs showed the peak absorption at 342 nm in the UV-Vis spectroscopic analysis. The initial confirmation of the formation of nanoparticles can be determined with this peak absorption (Wu *et al.,* 2020). The peak absorption obtained 60 days after synthesis confirmed the stability of the nanoparticles.

4.3.2 Scanning electron microscopy with energy dispersive x-ray spectroscopy

This method of characterization of nanoparticles does surface analysis, which will enable us to know the morphology, shape, size and orientation of materials. In this study, the SEM images of the green synthesized CuNPs revealed heterogeneity in the size and shape of the particles with mild agglomeration. The shapes of the particles were irregular to spherical with an average size of 79.3 nm. The results concur with the findings of Rajeshkumar *et al.* (2019), who obtained irregular and spherical-shaped agglomerated nanoparticles.

The broad size variation of the nanoparticles might be due to the phytocompounds present in the *C. officinalis* extract, particularly the phenolic compounds exhibit strong electrostatic attractive force between the small particles and result in holding the smaller particles together to form aggregates and medium-sized particles (Murthy *et al.,* 2020; Wu *et al*., 2020).

EDAX analysis indicated elemental weight percentages of copper, oxygen and sulfur were 42.66, 52.19 and 5.15, respectively. This finding was in accordance with the results of Amaliyah *et al.* (2020). The synthesized CuNPs were highly pure which can be evident from the presence of only copper, oxygen and sulfur peaks without any additional peaks (Murthy *et al.,* 2020).

4.3.3 Transmission electron microscopy

TEM investigation exhibited the nanoparticles were in elliptical, rod and irregular shapes with an average size of about 41.91 nm. The broad size variation of the nanoparticles might be due to the phytochemicals present in the *C. officinalis* extract particularly the phenolic compounds (Murthy *et al*., 2020).

4.3.4 Fourier transform infrared spectroscopy

The FTIR analysis showed the existence of various functional moieties like Alcohol (OH stretch H-bonded, free), Alkane (C-H stretch, -C-H bending), Alkene (=CH bending, C=C stretch), Amine (C-N, stretch), Nitro compounds (N-O stretch), Acid (OH, stretch) and Ester (CO, stretch). These functional moieties play a crucial part in nanoparticle production. The presence of a peak at 601 $cm^{-}1$ is relevant Cu-O functional group which confirms that copper particles are present in the nanoparticles sample (Ethiraj and Kang, 2012).The biomolecules present in the *C. officinalis* flower extract were confirmed by their corresponding peaks and these biomolecules are responsible for the synthesis and stabilization of the CuNPs (Ahmed *et al.,* 2019; Sneha *et al.,* 2022).

4.3.5 X-ray diffraction

In XRD investigation, the sharp peaks denoted that the CuNPs were crystalline. The results were in agreement with the findings of Kumar *et al.* (2015).

4.4 Antibacterial activity

The observed antibacterial activity of green synthesized CuNPs against *E. coli* was lower than the standard antibiotic (ceftriaxone with sulbactam) whereas the antibacterial effect of green synthesized CuNPs against *S. aureus* was equivalent to that of the standard antibiotic (enrofloxacin). From this finding, it was clear that the CuNPs exhibited better antibacterial activity against Gram-positive organism than the Gram-negative organism. Also, it was observed that the antibacterial activity of the CuNPs was augmented in a dose-dependent manner. A similar outcome was observed when CuNPs were synthesized using Strawberry extract (Hemmati *et al.,* 2020).

The increased effect of CuNPs on gram-positive organism might be due to the presence of a single-layered cell wall made of peptidoglycan and the absence of an outer membrane and lipopolysaccharide layer which is present in the gram-negative bacteria (Hemmati *et al.,* 2020). The mechanism by which the CuNPs produced antibacterial activity was that it might produce and accumulate the reactive oxygen species in the cytoplasm leading to bacterial cell membrane damage, leakage of intracellular components and cell death (Zhang *et al.,* 2010). Further, the CuNPs generate hydroxyl radicals which cause oxidation of protein, separation of DNA and RNA and damage to bacterial cell membrane (Wu *et al.,* 2020). The presence of alkaloids, saponins, flavonoids and terpenoids in *C. officinalis* extract might have contributed to the elaboration of antibacterial activity (Subashini *et al.,* 2011).

4.5 Angiogenic activity

The *C. officinalis* - 200 µg (T_3), green synthesized CuNPs - 100 µg (T_4) and green synthesized CuNPs - 200 µg (T_5) groups showed a significantly increased number of blood vessels than control and solvent control groups. The highest number of blood vessel formation was noted in the green synthesized CuNPs - 200 µg (T_5) group. The result of the angiogenic effects of *C. officinalis* was in concurrence with the findings of Patrick *et al*. (1996) and Mroczek-Sosnowska *et al.* (2015).

The phytocompounds detected in the *C. officinalis* extract such as saponins and flavonoids might have contributed to the elaboration of angiogenic activity (Parente *et al.,* 2011). Also, the possible mechanism behind the stimulation of angiogenesis by CuNPs might be the stimulation of hypoxia-inducible factor-1, which is essential for the expression of vascular endothelial growth factor (Zhang *et al.,* 2014).The newly formed blood vessels in the CuNPs treated CAM were much firmer than the control groups which was an additional finding in this study. Generally in wound healing, angiogenesis is required for the migration of fibroblast for collagen production and proper supply of oxygen and nutrients to the extracellular matrix. It was evident from this CAM assay that the CuNPs stimulated the formation of a greater number of firmer blood vessels which were less prone to damage and might aid in faster wound healing in humans and animals.

Secondary bacterial infection is a major impending factor that delays the wound healing process and most of the wound treatment is aimed at preventing microbial contamination. Fibroblast migration plays a vital role in wound contraction and it depends majorly on the amount of angiogenesis at the wound site. Hence, the detection of significant antibacterial and angiogenic activities together would pave the way for utilizing the green synthesized CuNPs as a better wound healing agent.

5. Conclusion

The novel combination of copper nanoparticles synthesized using ethanolic extract of *C. officinalis* flowers was very well characterized concerning morphology, crystallinity, elemental composition and biomolecules involved in the synthesis. The findings revealed that *C. officinalis* could be utilized as a reducing and capping agent for the synthesis of copper nanoparticles. The discovery of valuable antibacterial and angiogenic activities of green synthesized copper nanoparticles could be tapped to utilize this novel combination for possible wound healing activity.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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