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In vitro efficacy of cinnamon derived copper nanoconjugates against drug-resistant and sensitive strains of *Mycobacterium tuberculosis*

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Abstract

Background: Tuberculosis is a chronic infectious disease that is caused by *Mycobacterium tuberculosis*. In the treatment forefront there are drugs like rifampicin, pyrazinamide, isoniazid that are used. However, long-term use of these medications causes multidrug-resistant bacteria and drug-induced liver damage. In the present study, the effect of the test drug (nano-conjugate) synthesized from Cinnamon was tested for its anti-tuberculosis activity by broth microdilution method. In addition, its efficacy was further tested against multi drug resistant and sensitive strains of tuberculosis.

Results: The initial Minimum Inhibitory Concentration of the nanoconjugate was found to be 250 µg/ml against the *M. tuberculosis* strain H37Rv. Furthermore, it was effective against the sensitive and resistant strains of tuberculosis with a MIC of 250 and 500 µg/ml respectively.

Conclusion: The current investigation found a prospective anti-tuberculosis drug candidate which could aid in the rational creation of more potent medications against tuberculosis.

Keywords: Tuberculosis, nanodrug, Minimum Inhibitory Concentration

1.Introduction

Tuberculosis (TB) is a disease that typically affects the lungs and it is caused by *Mycobacterium tuberculosis* (Ravimohan et al., 2018). If left untreated, it may prove to be fatal. The present treatment plan calls for the use of medications such as pyrazinamide, rifampicin, ethambutol, and isoniazid. However extended use of these might cause liver toxicity (Tweed et al., 2019). Moreover, the medications could result in drug resistant and sensitive strains of *Mycobacterium*, which would further reduce its effectiveness (Seung et al., 2015). Since ancient times, drugs originating from natural sources have been vital in the prevention and treatment of diseases (Chaachouay & Zidane, 2024)**.** Modern technology has made it possible to modify traditional sciences such that they are effective even at low dosages. Because of their tiny size, nanoparticles (NP) have a high surface to volume ratio and are being used to treat a wide range of illnesses (Altammar, 2023).

2. Methods

2.1 Collection of the plant material and biosynthesis of chitosan mediated copper nanoconjugates

The bark of *Cinnamomum verum* was collected from Chennai and it was authenticated by Dr. G Jeya Jothi, Assistant Professor in the Plant Biology and Biotechnology department at Loyola College in Chennai. After that, it was pulverised and extracted for five hours in double distilled water using a Soxhlet device. After that, the extract was chilled and kept for later use at 4ºC. Copper sulphate pentahydrate was used to produce the nanoparticles. It was obtained from Emplura and utilised directly without being purified. A solution of 0.1 M $CuSO_4$. $5H_20$ was made in double-distilled water. 60 ml of the plant aqueous extract was added to 40 ml of $CuSO_4$ solution and a dark brown precipitate was observed.

0.02 g of chitosan was weighed out and added to 5 ml of 2% acetic acid. The prepared copper nanoparticles were taken and added to chitosan and stirred for 30 mins. Finally, a few drops of

0.6 M NaOH was added and the pH was adjusted to 10. The mixture was centrifuged for 20 minutes at 5000 rpm and the pellet was dried and used for characterisation.

2.2 Characterization of copper nanoconjugates

The absorption peak, functional groups, and size of the nanoparticles were determined using UV-Vis spectroscopy, Fourier Transformation Infra-Red Spectroscopy (FTIR), scanning electron microscopy-energy dispersive spectroscopy (SEM-EDX), and zeta potential.

2.2.1 Ultra Violet- Visible Spectroscopy (UV-Vis)

The sample was diluted with 10 parts of double distilled water. A Shimadzu UV 1800 - UV Visible spectrophotometer that operates between the wavelengths of 200 and 800 nm was used to record the UV-Vis spectrum.

2.2.2 Fourier-Transform Infrared Spectroscopy (FTIR)

Agilent FTIR spectrophotometer, which is fully computerised, was used for the structural characterization. A spectrum recording was made between 4000 and 500 cm^{-1}.

2.2.3 Scanning Electron Microscopy- Energy Dispersive Spectroscopy (SEM-EDX)

The surface morphology of the synthesized $CuSO_4$ nanoparticles was studied by FE-SEM, CARL Zeiis, Germany model Ultra 55 FESEM with 1 nm resolution and detector was INLENS. The surface elemental composition was studied using Energy Dispersive Spectroscopy (EDS), Oxford instruments, model 20 nm X-Max.

2.2.4 Zeta Potential

The stability of the synthesized NPs was elucidated on reviewing its zeta potential. It is the charge that develops at the interface between the solid surface and the liquid medium that prevents it from aggregation. This was done at Translational Research Platform for veterinary Biologicals (TRPVB) using Horiba Scientific SZ-100 Zeta Potential Analyser.

2.3 Screening for antimycobacterial activity of copper nanoconjugates synthesized against the tuberculosis strain H37Rv by broth microdilution method

The antimycobacterial activity of the copper NC produced was tested against the tuberculosis strain H37Rv. A loopful of the H37Rv culture was scraped out from Lowenstein Jensen medium and dissolved in 7H9 enriched medium. It was then vortexed and the supernatant was adjusted to 0.5 Mc Farland. 1:10 and 1:100 dilutions of the culture were prepared and used as positive controls. 0.1 ml of 1 μg/ml of rifampicin was taken as the negative control. 0.1 ml of

the enriched 7H9 medium was added to the wells of a well plate. Finally, 0.1 ml of the test drug (the copper NP) were added in four concentrations- 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 31.25 µg/ml and its minimum inhibitory concentration (MIC) was checked. 0.1 ml of 0.1 M $CuS0_4$ was also added to a well to check if it could inhibit the growth of MTB when used separately. The experiment was carried out in duplicates. The plates were sealed and incubated for 14 days. The results were read under an inverted microscope and the inhibition of mycobacterium was determined by the absence of cord formation in the wells (Makane et al., 2019).

2.4 Screening for antimycobacterial activity of copper nanoconjugates synthesized against sensitive and multi drug resistant (MDR) strains of *M. tuberculosis* from patient isolates by broth microdilution method

The same method was used to test the efficacy of the synthesized copper nanoconjugates against sensitive and resistant strains of tuberculosis. The sensitive strains were sensitive to all the first line anti tuberculosis drugs such as rifampicin, isoniazid, ethambutol and pyrazinamide. The MDR strains were resistant against rifampicin and isoniazid. The results were read after 14 days under an inverted microscope.

3.Results:

3.1 Biosynthesis of copper nanoparticles

The copper nanoparticles synthesized from the extract of *Cinnamomum verum* turned brown indicating the completion of the reaction (Kothari, 2023). Upon the addition of chitosan, there was no colour change to the brown that was obtained previously (Gowda & Sriram, 2023) (Fig. 1).

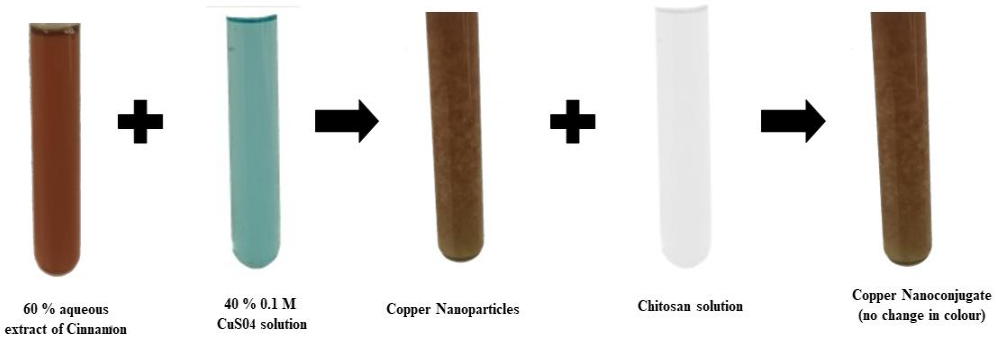

Fig. 1 – Green synthesis of copper nanoconjugates from *Cinnamomum verum*

3.2 Characterization of copper nanoconjugates

3.2.1 UV- Visible Spectroscopy

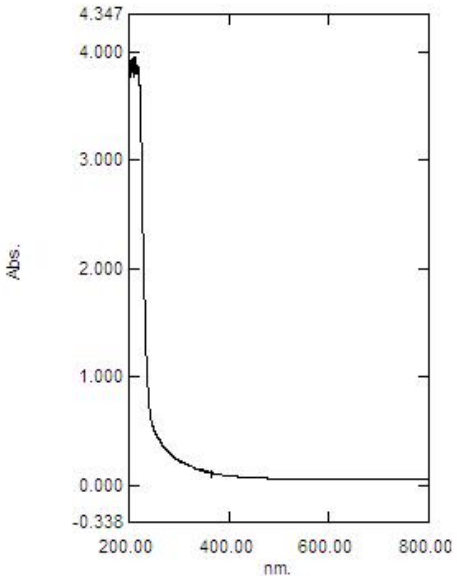

Fig. 2- UV spectrum of chitosan mediated copper nanoconjugates from Cinnamon

The spectrum exhibited a distinguishable peak at λ max 340 nm (Fig. 2) which corresponded to $CuS0_4$ from previous literature (Gawande et al., 2016).

3.2.2 FTIR

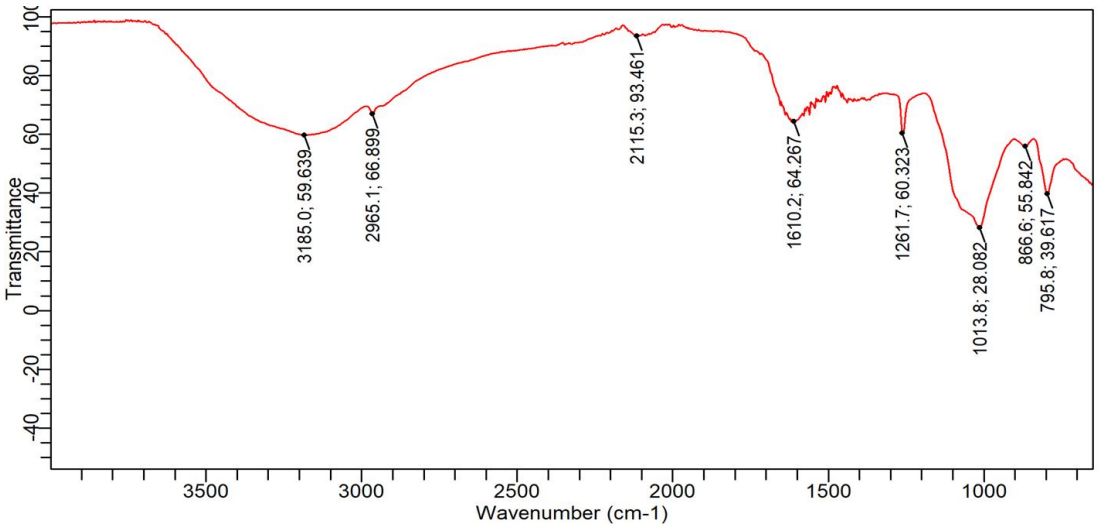

Fig. 3- FTIR spectrum of chitosan mediated copper nanoconjugates from Cinnamon

FTIR analysis of the nanoconjugate revealed the presence of eight peaks (Fig. 3) at 3185.01 cm^{-1}, 2965.09 cm^{-1}, 2115.26 cm^{-1}, 1610.20 cm^{-1}, 1261.70 cm^{-1}, 1013.83 cm^{-1}, 866.60 cm^{-1} and 795.78 cm^{-1}. The peak at 3185 represented a weak broad 0-H stretching alcohol. 2965 was a strong broad N-H stretching amine. 2115 was a weak C ≡ C stretching alkyne. 1610

was medium N-H bending amine.1261 was a strong S = O sulphate stretching band. 1013 was a strong S=O stretching sulphoxide. 866 and 795 showed a strong and medium C=C bending alkene respectively.

The reduction of copper ions into copper nanoparticles was achieved under the effect of hydroxyl and carbonyl linkages in the extract's constituents (Mohamed, 2020). The phytochemicals present act as capping agents thus providing the nanoparticles with more stability (Restrepo & Villa, 2021). This indicates that functional groups play a major role in the synthesis of copper nanoparticle as they have reducing groups which help in the synthesis of nanoparticles (Adewale Akintelu et al., 2021).

3.2.3 SEM- EDX

The morphological observation of CuNC was done by SEM and its elemental composition was checked by EDX (Fig. 4).

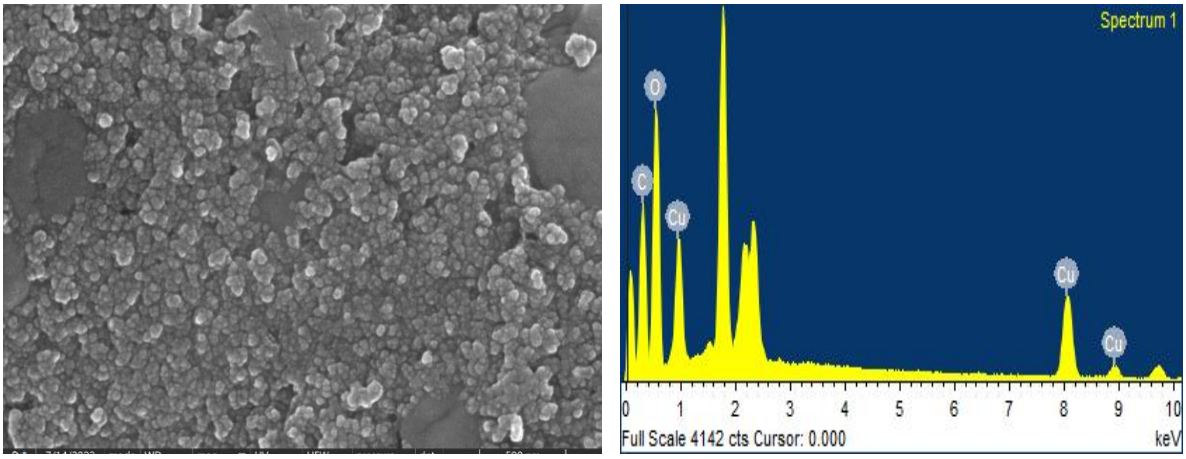

Fig. 4- SEM EDX spectrum of chitosan mediated copper nanoconjugates from Cinnamon

SEM analysis indicated that the average particle size of the nanoparticles varied from 25-45 nm and the particles were mostly spherical in shape. The EDX spectrum indicated that the particles had peaks of Copper, Carbon and Oxygen indicating the purity of the nanoparticles (Chand Mali et al., 2019).

3.2.4 Zeta Potential

The zeta potential of the copper NC was 11.2 mV (Fig. 5).

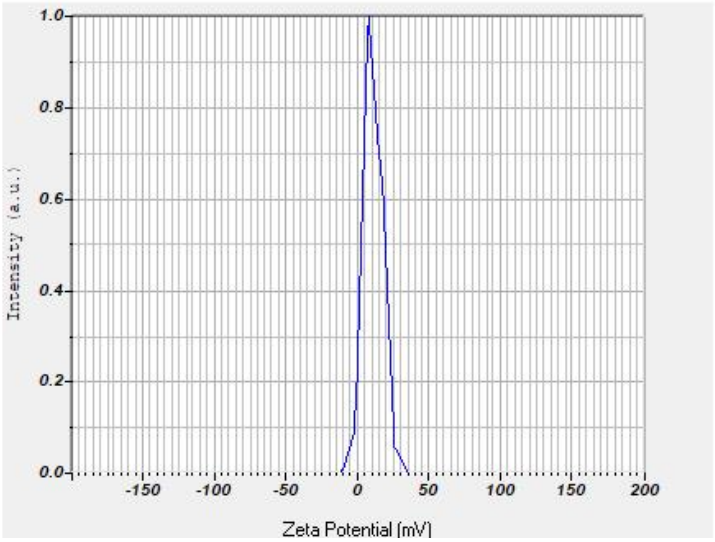

Fig. 5- Zeta potential analysis of chitosan mediated copper nanoconjugates from Cinnamon

3.3 *In vitro* screening for antimycobacterial activity of copper nanoparticles against the tuberculosis strain H37Rv by broth microdilution method

Table 1: *In vitro* screening for antimycobacterial activity of copper NC

Sample	Mycobacterial growth (H37RV Standard Strain)
Control (1:10 dilution, Drug free)	Positive
Control (1:100 dilution, Drug free)	Positive
Rifampicin (1µg/ml)	Negative
0.1 M CuSO4	Positive
CuNC (31.25 µg /ml))	Positive
CuNC (125 µg /ml)	Positive
CuNC (250 µg /ml)	Negative
CuNC (500 µg /ml)	Negative
CuNC (1000 µg /ml)	Negative

From the results (Table 1) it was observed that the positive controls were not able to inhibit mycobacterial growth and the negative control rifampicin was able to inhibit mycobacterial growth. Amongst the test samples the copper nanoconjugate at 31.25 µg/ml and 125 µg/ml concentrations were not able to inhibit the growth of mycobacterium whereas the copper nanoconjugates at 250, 500 and 1000 µg/ml were able to successfully inhibit its growth. 0.1

M $CuS0_4$ when used separately was not able to inhibit mycobacterial growth. These results indicated that the MIC of the CuNC was 250 µg /ml (Fig. 6).

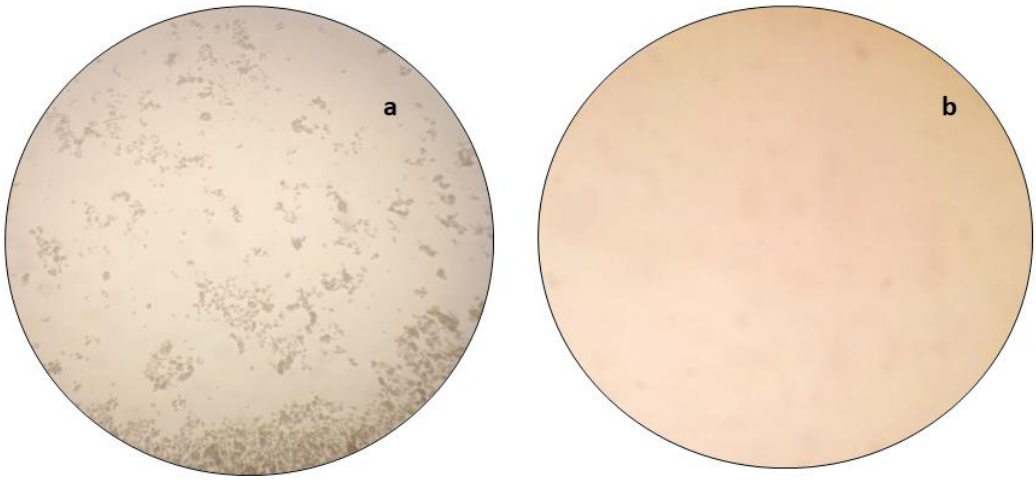

Fig. 6- (a) Control: Mycobacterial growth observed as cord formation under an inverted microscope, (b) Test: Chitosan mediated copper nanoconjugates from *C. verum* that could inhibit Mycobacterial growth with a MIC of 250 µg/ml as observed under an inverted microscope

3.4 *In vitro* screening for antimycobacterial activity of copper nanoconjugates against the sensitive and resistant strains of *Mycobacterium tuberculosis* by broth microdilution method

The nanoconjugate that was synthesized showed good results against drug resistant and sensitive strains of tuberculosis (Table 2). It had a MIC of 250 µg/ml against the sensitive strains and a MIC of 500 µg/ml against the drug resistant strains of *Mycobacterium tuberculosis* from patient isolates.

Table 2: *In vitro* screening copper NC against sensitive and resistant strains of *M. tuberculosis*

Sample	**MIC in Sensitive Strain (µg/ml)**		**MIC in Resistant Strain (µg/ml)**	
	Strain 1	**Strain 2**	**Strain 1**	**Strain 2**
CuNC	250	250	500	500

4. Discussion:

Tuberculosis (TB) is a bacterial infectious illness caused by Mycobacterium tuberculosis. TB continues to negatively impact and pose severe health, social, and economic challenges at a global scale (Gupta et al., 2020). Ancient Indian literature and traditional knowledge both promote the use of indigenous botanicals to treat TB (Y. Xu et al., 2021). Plants produce several antimycobacterial chemical compounds, providing numerous possibilities for efficient disease therapy to lessen the worldwide burden of TB and drug-resistant M. tuberculosis strains (Chinsembu, 2016). Numerous types of nanoparticles, such as those made of copper, iron, gold, and silver, when paired with polysaccharides like chitosan, have inherent antibacterial properties (Manivasagan et al., 2019). *Cinnamomum verum*, is a common spice that contains secondary metabolites such as quercetin, gallic acid, catechin, salicylic acid, cinnamic acid, and cinnamaldehyde (Sarangi et al., 2021). These can be employed in treating several respiratory disorders including tuberculosis (National Toxicology Program, 2004). Cinnamaldehyde is the primary bioactive molecule that is responsible for mycobacterial growth suppression and bactericidal actions.

(Sawicki et al., 2018).

Previous research concluded that agar dilution method showed a minimum inhibitory concentration (MIC) of 500µg/ml for both aqueous and methanolic extracts of cinnamon (Vaidya et al., 2016). Cinnamaldehyde essential oil has a MIC of 100 µg/ml and is effective against tuberculosis (TB)

(Sergio et al., 2013). In another study the MIC of *C. verum* against *M. tuberculosis* was found to be 10mg/ml (Vaidya et al., 2020). The MIC of Cinnamaldehyde from *C. cassia* was 640 µg/ml (Wan et al., 2022). The MIC of Zinc oxide nanoparticles (NP) from *C. verum* was determined by the broth microdilution method and it was found to be 125 µg/ml (Ansari et al., 2020)

The antimycobacterial mechanism of cinnamic aldehyde is currently not fully understood. Some authors have proposed that cinnamic aldehyde possibly alters the cell membrane stability of *M. tuberculosis* (Sawicki et al., 2018). The postulated mechanism is that it promotes gene expression in the redox process, resulting in the death of bacteria. The nano size, surface area, and photothermal nature of the NPs directly influence the activity (J.W. Xu et al., 2019).

Cu NPs are mainly exploited for their antimicrobial properties (Bhavyasree & Xavier, 2022). Several potential mechanisms for antimicrobial activity have been hypothesised by researchers, including oxidative stress injury, gene toxicity, and mechanical damage (Antonio-Pérez et al., 2023). Cu based nanomaterials exhibit antibacterial properties due to microbial adsorption and non-electrostatic forces, including Vander Vaals force and hydrogen bonding (G & S, 2021). Copper primarily enters microbial cells in the form of ions (Sarkar et al., 2020). The process of oxidation can lead to irreversible damage to the cell membrane, including protein oxidation, molecule rupture, and membrane destruction due to free-radical lipid peroxidation (Ivanova et al., 2024).

Multi Drug Resistant (MDR) and sensitive strains of *Mycobacterium* are urgent and difficult provocation in TB treatment (Maher & Raviglione, 2005). Like other bacterial illnesses, the ultimate goal of the TB drug research endeavour is to eradicate both active and latent disease, potentially in a matter of weeks (Koul et al., 2011). A short-term tuberculosis treatment regimen may enhance rates of adherence, reduce side effects, and minimise expenses (Gillespie et al., 2014). This study gives as a facile method to tackle even the multi drug resistant and sensitive strains of TB at a very low MIC which has not been established very well from previous studies thus proving to be extremely effective.

5. Conclusion

Tuberculosis, a lung disease caused by *Mycobacterium tuberculosis*, can be fatal if left untreated. Current treatment involves medications like pyrazinamide, rifampicin, ethambutol, and isoniazid, but prolonged use may cause liver toxicity and multi drug resistant and sensitive strains of TB which further reduces its effectiveness. Natural drugs have been vital since ancient times, but modern technology has modified traditional methods for low dosages. Nanoparticles (NPs) have a high surface to volume ratio and are being used to treat various illnesses due to their small size. This study provides a simple and alternative approach to tackle tuberculosis using the synthesized nanoconjugates.

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