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IN VITRO CHECKERBOARD ASSAY OF A GREEN NANODRUG SYNTHESIZED FROM *CINNAMON VERUM* AGAINST 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, *in vitro* checkboard assay was done to evaluate the effect of the test drug (nano-conjugate) when used in combination with a standard antituberculosis drug such as rifampicin. **Results:** The initial Minimum Inhibitory Concentration of the nanoconjugate was found to be 250 µg/ml against the *M. tuberculosis* strain H37Rv from a previous study. When the nanoconjugate was used in combination with rifampicin, the Minimum Inhibitory Concentration further reduced to 62.5µg/ml. The Fractional Inhibitory Concentration was found to be 0.347 upon calculation and this indicated that there was a synergistic relationship between the nanodrug and rifampicin. **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, checkboard assay, Minimum Inhibitory Concentration

BACKGROUND

The checkerboard test is an experimental method that is used to assess the interaction of two test drugs (Martinez-Irujo *et al*., 1996). They are tested in serial dilutions, and the concentration of each drug is tested both alone and in combination (Bellio *et al*., 2021). Therefore, it is not only possible to determine the effect of the individual drug, but also, it is a useful method to ascertain the effects generated when two drugs are used in combination. The different effects that are produced are synergism, additivity, antagonism and indifference (Roell *et al*., 2017). Drugs that are synergistic and additive may have the potential to tackle and combat antibiotic resistance as combining different compounds can prove to be the key in creating novel formulations that don't always necessitate the discovery of new compounds (Lehár *et al*., 2009).

METHODS

Collection of the plant material and biosynthesis of copper nanoconjugate

The dried bark of *Cinnamomum verum* was collected from Chennai. It was authenticated by Dr G Jeya Jothi, Assistant Professor, Dept of Plant Biology and Biotechnology, Loyola College, Chennai. It was then powdered and extracted in distilled water using a soxhlet apparatus for five hours. The copper nanoconjugates (CuNC) were synthesized with the prepared plant extract using 0.1 M $CuSO_4.5H_20$ as the precursor. 60 ml of the plant aqueous extract was taken and added to 40 ml of Copper sulphate penta hydrate and 10 ml of chitosan solution was added to it. This was prepared by taking 0.02 g in 2 ml of 5% acetic acid and kept aside for 24 hours. The nanoconjugates were centrifuged at 5000 rpm for 20 mins and the pellet was dried and used for characterization.

Characterization of nanoconjugates

The nanoparticles (NP) were characterized by UV-Visible spectroscopy to find its wavelength and the range was recorded between 200 to 800 nm, Fourier Transformation Infra-Red Spectroscopy (FTIR) was done to find out the biomolecules present in the extract that aided in the synthesis of the NPs and the spectrum was recorded from 4000 cm^{-1} to 500 cm^{-1}, Scanning Electron Microscopy (SEM)- Energy Dispersive Spectroscopy (EDX) was done to study the surface morphology and the elemental composition of the NPs and Zeta Potential was done to understand the stability of the nanoparticles.

In vitro checkerboard assay

100 µl of the mycobacterial strain H37Rv in 7H9 enriched medium was added to the wells of a 96 well plate. The nanodrug was serially diluted starting from its minimum inhibitory concentration (MIC) against the *Mycobacterium tuberculosis* strain H37Rv which was done previously and found to be 250 µg/ml. The dilutions were made along the abscissa of the well plate at the following concentrations: 250, 125, 62.5, 31.25, 15.625, 7.815, 3.9 and 1.95 µg/ml. The standard anti tuberculosis drug Rifampicin (0.5µg/ml) was serially diluted along the ordinate of the well plate in the following concentrations: 0.5, 0.25, 0.125, 0.0625, 0.031, 0.015, 0.007, 0.003, 0.0015, 0.0007 µg/ml. The total volume of the combination was 100 µl per well. Then 100 µl of each bacterial suspension (1×10^6 Colony Forming Units (CFU)/mL) was added to each well of a 96-well microplate Two sets of columns in the well plate were maintained as positive controls. The well plate was sealed and incubated for 10 days. Results were interpreted as synergistic interaction - Fractional Inhibitory Concentration (FIC ≤ 0.5), partial synergy (FIC- 0.5- 0.75), additive interaction (FIC- 0.75 - ≤ 1.0), indifferent (FIC- 1.0 - 4.0) and antagonistic interaction (FIC > 4.0) (Jeong *et al*., 2023).

RESULTS

Biosynthesis of copper nanoconjugate

The copper nanodrug (Fig 1) appeared to be dark brown in colour (Kazemi *et al*., 2023).

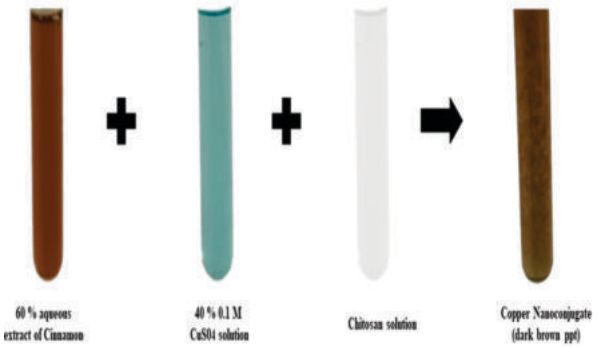

Fig 1- Green synthesis of copper nanodrug

Characterization of nanoconjugates

UV- Visible Spectroscopy

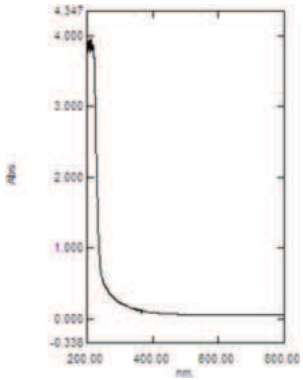

Fig 2- UV –Vis spectra of copper nanodrug from Cinnamon

The spectrum exhibited a distinguishable peak at λ max 340 nm (Fig 2) which corresponded to $CuS0_4$ from previous literature (Venkata et al., 2019).

FTIR

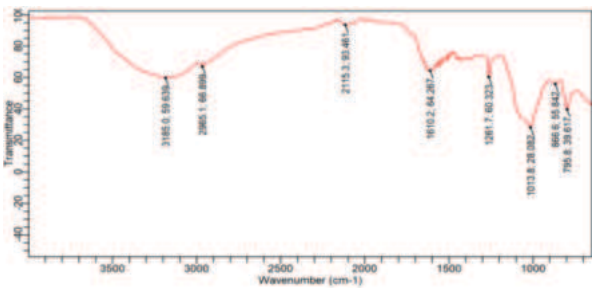

Fig 3- FTIR spectra of copper nanodrug from Cinnamon

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 (Mao *et al*., 2016). 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 (Amjad *et al*., 2021).

SEM- EDX

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

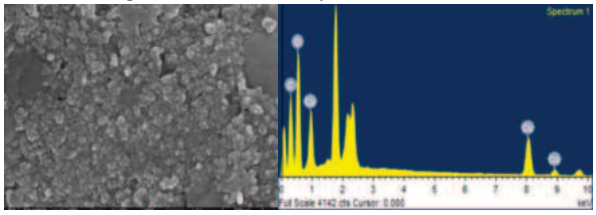

Fig. 4 - SEM-EDX images of copper nano drug from Cinnamon

SEM analysis indicated that the average particle size of the nanoparticles varied from 25-45 nm and the particles were mostly spherical in shape (Fig 4). The EDX spectrum indicated that the particles had peaks of Copper, Carbon and Oxygen indicating the purity of the nanoparticles.

Element	Weight%	Atomic%
C K	37.59	51.24
O K	42.69	43.68
Cu K	19.72	5.08
Totals	100.00	

Zeta Potential

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

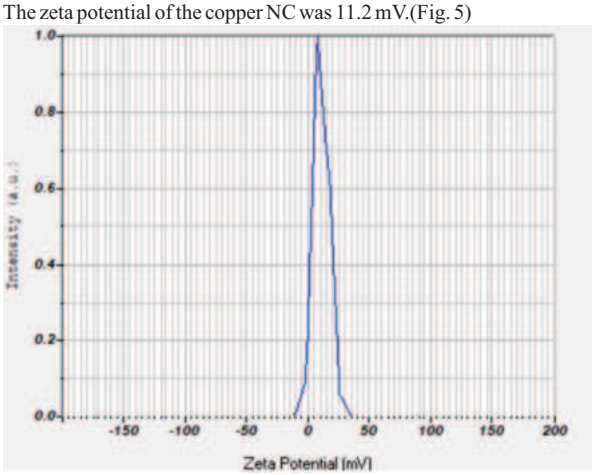

Fig. 5 –Zeta potential graphs of copper nanodrug from Cinnamon

***In Vitro* Checkboard Assay**

The columns 1 and 2 were control wells. "G" indicated Growth and "N" indicated No growth of *Mycobacterium tuberculosis* (Fig 6). The lowest concentration of the nanodrug and rifampicin when used in combination that could inhibit the mycobacterial growth was found in Row C Column 9 of the 96 well plate. The concentration of the nanodrug used was 62.5 µg/ml and rifampicin was 0.0625 µg/ml.

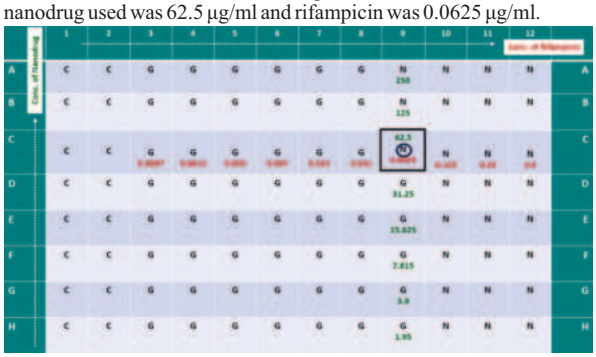

Fig 6- *In vitro* checkboard assay to find out the combinational effect between the nanodrug and rifampicin

The Fractional Inhibitory Concentration (FIC) (Meletiadis et al., 2010) index range between 0.5 and 4 was calculated using the following formula

$$FIC = \frac{MIC\ A}{MIC\ a} + \frac{MIC\ B}{MIC\ b}$$

$$FIC = \frac{62.5}{250} + \frac{0.062}{0.5}$$

$$= 0.25 + 0.124$$

$$= 0.374$$

Where A and B is the MIC of the checkboard assay results of the nanodrug (62.5 µg/ml) and rifampicin (0.062 µg/ml) and a and b is the original MIC of the nanodrug (250 µg/ml) and rifampicin (0.5 µg/ml). The FIC was found to be 0.374 indicating a synergistic association between the nanodrug and rifampicin.

DISCUSSION

Tuberculosis is a highly contagious disease caused by *M. tuberculosis*. The rise of multidrug resistant and sensitive forms of tuberculosis makes the treatment more challenging (Zhou *et al*., 2021). The WHO suggested that combining innovative treatments with existing tuberculosis therapies would be an effective strategy to treat tuberculosis (Ignatius & Dooley, 2019). In this context, the investigation of old and well-known medications in combination with traditional anti-TB drugs is a promising alternative to the existing drugs that are available because it helps to reduce the time period for treatment, is effective at a lower dose, works well against drug-resistant and sensitive strains of tuberculosis, and has fewer harmful side effects (Xu *et al*., 2017) (Kim *et al*., 2022).

Previously, in a study made by (Intorasoot *et al*., 2022) checkerboard

assay was carried out with *M. tuberculosis* H37Rv and the results indicated that human lactoferrin peptide DhLF 1-11 displayed an additive effect when combined with isoniazid and a synergistic effect when combined with rifampicin, with fractional inhibitory concentration indices of 0.730 and 0.312 respectively. A subsequent study was done by (Zhou *et al*., 2021) where orbifloxacin was tested along with rifampicin and the FIC was found to be 0.2 indicating a synergistic effect. A study carried out by (Mahmud *et al*., 2021) concluded that benzonaphthofurandione (BNF15) showed an additive effect when used in combination with isoniazid (FIC 0.75) and rifampicin (FIC 1). Another study by (Rao *et al*., 2021) NZX which was a broad spectrum anti-bacterial peptide had an additive effect when combined with Ethambutol (FIC 0.75) and Isoniazid (FIC 1) and an effect of indifference against rifampicin (FIC 2.1) and amikacin (FIC 1.1). Q203 (Nguyen *et al*., 2022) a first class drug candidate against TB was tested in combination with isoniazid, rifampicin and streptomycin using the checkerboard and it showed an additive effect with a FIC of 0.75.

Nanoparticle-based ideology has demonstrated effective treatment and promising results for chronic infectious illnesses (Nasiruddin *et al*., 2017). Various types of nanocarriers have been investigated as potential drug delivery systems because of their controlled and prolonged drug release over free pharmaceuticals (Edis *et al*., 2021) (Kumar *et al*., 2023). It also minimises dosing frequency and eliminates the issue of low or poor compliance (Baryakova *et al*., 2023).

The current study proves to be extremely useful because the green nanodrug synthesized is effective against the H37Rv strain even when used separately with a MIC of 250 µg/ml and the MIC was further reduced to 62.5 µg/ml when used in combination with rifampicin thereby showing a strong synergistic effect as the FIC was found to be 0.37. Combination therapy not only reduces the required dose and frequency of drug administration, but can also help shorten the period of treatment, lowers the risk of adverse effects and also will avoid development of drug resistance (Goossens *et al*., 2020) . Therefore, the nanodrug based combination therapy can be investigated further in the treatment of tuberculosis for humans.

CONCLUSION

The study uncovered a promising anti-tuberculosis drug candidate, which could aid in the rational development of a more robust tuberculosis combination therapy.

List Of Abbreviations

µl- Microliter
CFU- Colony Forming Units
Cu – Copper
CuNC- Copper nanoconjugate
EDX- Energy Dispersive X-ray Spectroscopy
FIC- Fractional Inhibitory Concentration
FTIR – Fourier Transform Infra-Red Spectroscopy
M. tuberculosis- Mycobacterium tuberculosis
MIC- Minimum Inhibitory Concentration
ml- Milliliter
mm- Millimeter
Nm – Nanometer
NP- Nanoparticle
SEM – Scanning Electron Microscopy
TB- Tuberculosis
UV Vis – Ultra- Violet Visible
µg – Micogram

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