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BIOSYNTHESIS OF COPPER NANOPARTICLES USING *AEGLE MARMELOS* LINN. LEAF EXTRACT AND RUTIN: CHARACTERIZATION AND THEIR ANTITUBERCULAR EFFICACY

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ABSTRACT

The biological activities of natural products are well-known and contribute to their value. The present situation has seen a substantial expenditure of resources for studying plant leaf extract and synthesizing nanoparticles with their biological activity. Due to its claimed function as an antitubercular agent in the medical area and its lethality, copper is the metal of choice for synthesizing nanoparticles. In this study, we have used *Aegle marmelos* (L.) leaf extract to test the antitubercular properties of copper nanoparticles which have been compared with copper nanoparticles made with rutin (flavonoid common in Rutaceae family). The use of DLS verified the synthesis of CuNPs. Further analysis was carried out using UV-Vis spectrophotometer, FTIR and SEM analysis. The findings (ZOI and MIC investigations) showed that copper nanoparticles (made with *Aegle marmelos* leaf extract) exhibited more potent antitubercular properties in comparison to the copper nanoparticles biosynthesized with rutin when tested against *Mycobacterium tuberculosis*. Based on the results of these investigations, copper nanoparticles show great efficacy as antitubercular agent. In light of these findings, it is reasonable to assume that medical devices and antitubercular systems are two areas where copper nanoparticles can prove to be an invaluable asset as a therapeutic agent.

Keywords: *A. marmelos* (L.), rutin, biosynthesized copper nanoparticles, antitubercular activity, *M. tuberculosis*

INTRODUCTION:

The medium-sized deciduous tree known as *Aegle marmelos* L. (Rutaceae) is often known as "Bael" or "Wood apple" in India. It grows in Indo-China, Thailand, Ceylon, Bangladesh, and India (Parichha, 2004) **[1]**. Utilized parts include fruit, leaves, roots, and bark (Nigam and Nambiar, 2015) **[2]**. As a result of the presence of various coumarins, polyphenols, triterpenes, alkaloids, sterols, marmesolin, psoralen and essential oils in the plant, it has therapeutic qualities (Bhardwaj and Nandal, 2015) **[3]**. Due to its great phytochemical and pharmacological relevance, the Ayurvedic medical system uses this plant's many components at different stages of maturity and ripening to cure a wide range of illnesses (Manandhar *et al*., 2018) **[4]**. Diabetes is often treated with *A. marmelos* (L.) in Bangladesh and India (Lampronti *et al*., 2003) **[5]**. *A. marmelos* (L.) also has a well-documented history of antibacterial, anti-hepatotoxic, anti-inflammatory, antipyretic and anticancer effects (Sharma *et al*., 2011) **[6]**. Hypochondriasis, depression and palpitations are among conditions that it can alleviate (Maity *et al*., 2009) **[7]**.

Since nanoparticles have the potential to replace antibiotics, they are the subject of increasing attention in antimicrobial development studies (Ge *et al*., 2014) **[8]**. Nowadays, plant mediated approaches are employed for nanoparticle biogenesis instead of physical and chemical processes since they are more eco-friendly and cost-effective (Niraimathi *et al*., 2013) **[9]**. Plant extracts and other naturally occurring substances provide endless potential for the development of novel pharmaceuticals (Sasidharan *et al*., 2011) **[10]**. New data from the pharmaceutical industry confirms that it is still a very valued chemical entity with potential applications in treating complicated disorders (Chin *et al*., 2006) **[11]**. Medicinal plants often include a wealth of chemicals that prevent the growth of microbes, including phenolic compounds, steroids, alkaloids and diterpenoids (Ranjitham *et al*., 2013) **[12]**. Furthermore, phytochemicals found in plant extracts have the dual role of reducing metal ions to metal nanoparticles and coating these particles (Swarnalatha *et al*., 2013) **[13]** and are so extensively used in the synthesis of metal nanoparticles, which have applications in drug delivery, biosensing, catalysis, and other fields. In addition to being a helpful and economical alternative to the mass manufacturing of metal nanoparticles, it is also very cost-effective (Sujitha and Kannan, 2013; Geethalakshmi and Sarada, 2012) **[14]**, **[15]**. The size range of nanoparticles is from one hundred nanometers down to the microscopic level. Due to their very high

surface-to-volume ratio and the quantum size effect, these nanoparticles are highly active and have shown surprising characteristics (Luo *et al*., 2005; Monga and pal, 2015) **[16]**, **[17]**. Copper, along with other metals like gold, silver, iron, palladium, zinc, etc., has many uses in electrical, optical, catalytic, biomedical, and antibacterial applications, making it the most utilized substance in the world. Because of its extreme toxicity to bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and relative lack of toxicity to mammalian cells, copper is considered to be an efficient antimicrobial metal (Pawar *et al*., 2014) **[18]**. Many researchers have synthesized copper nanoparticles using extracts from *A. marmelos* (L.), and have demonstrated their antimicrobial action against bacterial strains viz. *Bacillus subtilis*, *Escherichia coli, Streptococcus mutans, Staphylococcus aureus, Salmonella* spp, *Shigella flexneri, etc.* **[19-21]**. But to the best of our knowledge not much work has been reported in relation to antitubercular action of copper nanoparticles using leaf extract from *A. marmelos (L.)* against *Mycobacterium tuberculosis*. And this is where the presented research work holds its significance as *M. tuberculosis* is responsible for the air-borne infectious disease tuberculosis (TB), which for ages has been a threat to public health because of a number of complex reasons **[22]**. Another purpose of this research is to compare the antibacterial action of copper nanoparticles made from (a) leaf extract from *A. marmelos* (L.) and (b) rutin. Rutin is the main flavonoid (glycoside of quercetin) present in *A. marmelos* (L.) **[23, 24]**. Some semi-synthetic flavonoids, particularly derivatives of rutin, are used as therapeutic agents in the treatment of diseases involving free radicals **[25]**.

MATERIALS AND METHODS:

Materials: *A. marmelos* (L.) leaves were collected from local area of Jodhpur in the Indian state of Rajasthan (Figure 1). Copper sulfate and silver nitrate were used individually as precursor for the biosynthesis of two types of metallic nanoparticles with leaf extract of *A. marmelos* (L.). Only analytical-grade chemical reagents were employed in this experiment which were used directly without purification.

Figure 1: ***A. marmelos (L.)*** **plant**

Preparation of leaf extract: The leaves of *A. marmelos (L.)* (10 g) were cleaned well and then chopped before being cooked in 100 mL of de-ionized water for 15 minutes

in a heating pot set at 80^0C. After filtering, the product was stored in the fridge to be used for future studies.

Qualitative tests for flavonoids: The presence of flavonoids in *A. marmelos (L.)* leaf extract was confirmed by lead acetate test **[26]**, Shinado's test **[27]** and alkaline reagent test **[28]**.

Biosynthesis of copper nanoparticles:

(a) Biosynthesis of copper nanoparticles with *A. marmelos (L.)* (CuAM-NPs):

The *A. marmelos (L.)* mediated copper nanoparticles were synthesized using 50 mL of 0.01 M copper sulfate ($CuSO_4.5H_2O$) (stirred magnetically at 45-50 ^{0}C for about 15 minutes). About 5 mL of *A. marmelos* (L.) leaves extract was added to this $CuSO_4.5H_2O$ solution (with continuous stirring). Eventually the solution turned dark green (indicating the formation of copper nanoparticles), which were kept away from light for approximately 12 hours **[29]**.

(b) Biosynthesis of copper nanoparticles with rutin (CuRut-NPs):

10 mL of 0.02 M rutin (molecular weight= 610.52; Sigma-Aldrich Production GmbH, Switzerland) was mixed with 80 mL of 5mM $CuSO_4.5H_2O$ in a 250 mL beaker. On unstopped shaking, the solution changed its color from blue to reddish brown (indicating the synthesis of copper nanoparticles).

Characterization of biosynthesised Cu-NPs:

At 25^0C, a particle size analyzer based on DLS (dynamic light scattering) (Zetasizer Nano ZSP (ZEN 5600), Malvern Instrumnets Ltd. United Kingdom) was used to determine PDI (polydispersity index) and particle size of the prepared Cu-NPs. For monitoring colour of the solution and variations in absorbance brought on by the biosynthesis of Cu-NPs, double beam UV-VIS spectrophotometer: 2202 (Systronics) was used at a scanning range of 200 to 800 nm. Cary 630 FTIR Spectrometer (Agilent Technologies) was employed to obtain Fourier transform-infrared (FT-IR) spectra of Cu-NPs which gave information about the chemical structure of the Cu-NPs. These samples were analyzed in the spectral range of 300–4000 cm^{-1}. FE-SEM Supra 55 (Carl Zeiss, Germany) was used to get information on surface morphology and elemental composition of Cu-NPs.

Antitubercular activity assay (CuNPs vs. *Mycobacterium tuberculosis*):

(a) Antitubercular -Zone Inhibition Test:

The antitubercular activity was checked by following Zone Inhibition Method (Kirby-Bauer method) **[30]**. The MHA plates (A, B, C) were inoculated by spreading with 100 µl of Bacterial culture, *M. tuberculosis* (adjusted to 0.5 McFarland Unit - Approx

cell density (1.5 X 10^8 CFU/mL) and followed by placing the discs containing 10 µl of different concentration (0 to 100 %) of (a) CuAM-NPs and (b) CuRut-NPs. One disc in each plate was loaded with solvent alone which served as vehicle control and Ciprofloxacin disc (10µg) were taken as positive control. The plates of *M. tuberculosis* were incubated (Basil Scientific Corp. India- Incubator) at 37 °C for 24 hrs. A clear zone created around the disc were measured and recorded **[31]**.

Requirements:

- Mueller-Hilton Agar (MHA) Plates
- Bacterial Culture (M. tuberculosis, MTCC 300)
- Whatman No 1 filter paper discs (5mm)
- Solvent (vehicle control)- Dimethyl Sulfoxide- DMSO (SRL Chem 28580)
- Ciprofloxacin - (SRL Chem- 78079)- 2mg/ml
- Amount Loaded – (0 to 100 %/disc)

(b) Minimum Inhibitory Concentration (M.I.C.) Assay:

0.5 Mc farland standard dilution of microbes was used for the study. 500 µl diluted log cultures of bacteria (*M. tuberculosis*, MTCC 300) were added to the micro centrifuge tube and then treated with 10 µl of prepared treatment dilutions of different concentrations of (a) CuAM-NPs and (b) CuRut-NPs **(Tables 2 and 3)** to the defined tubes and incubated for the 15 days. After Incubation all content was transferred to the 96 well plate and added with MTT Solution (a final concentration of 250µg/ml) and incubated for 24 hours. After incubation, reading was taken by Elisa Plate Reader (iMark Biorad) at 490nm and 595 nm. Levofloxacin (10µg) was used as Positive Control **[32]**.

RESULTS AND DISCUSSION:

(a) Qualitative tests for flavonoids:

Flavonoids in *A. marmelos (L.)* leaf extract was confirmed by lead acetate test [yellow color precipitate], Shinado's test [pink color] and alkaline reagent test [yellow color to colorless].

(b) DLS studies:

Biosynthesised CuAM-NPs (dark green) and CuRut-NPs (red-brown) were investigated individually for their particle size distribution. The DLS studies (at 25 ^{0}C) showed that the average diameter (Z avg) of CuAM-NPs and CuRut-NPs was 37.75 nm **(Figure 2)** and 216.1 nm **(Figure 3)**, respectively. For both the nanoparticles the poly dispersity index (PDI) values were between 0.460 and 0.500, indicating that the samples were moderately dispersed **[33]**. The CuAM-NPs showed higher percentage of particles in the nanometer range in comparison to CuRut-NPs **(Table 1)**.

Table 1: DLS of CuRut-NPs vs. CuAM-NPs

	Peak	Size	% Intensity	SD	Z-Avg	Pd I	Intercept
CuRut-NPs	Peak 1	220.6	82.3	116.2	216.1 nm	0.495	0.921
	Peak 2	923.8	10.6	271.7			
	Peak 3	4884	7.1	688.8			
CuAM-NPs	Peak 1	77.76	84.1	46.004	37.75 nm	0.469	0.767
	Peak 2	11.21	15.9	3.621			
	Peak 3	0.00	0.0	0.000			

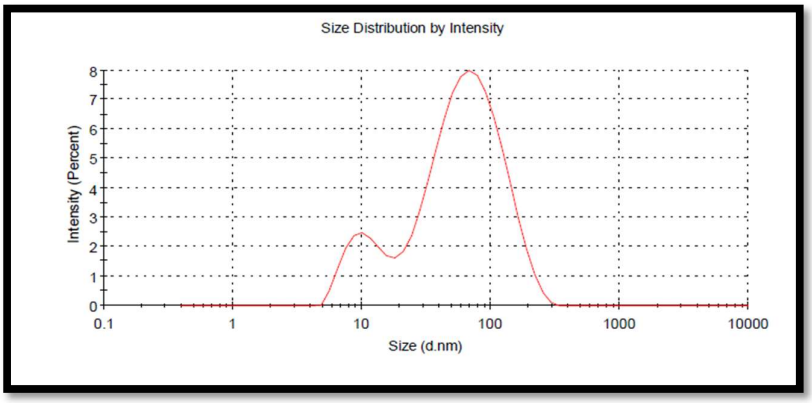

Figure 2: CuAM-NPs Size Distribution Report by Intensity

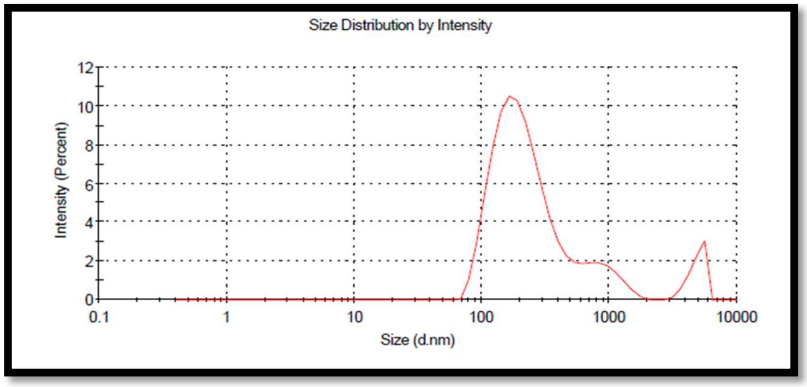

Figure 3: CuRut-NPs Size Distribution Report by Intensity

(c) UV-VIS spectra analysis:

When studying the production of nanoparticles or the stability of metal nanoparticles in aqueous solution, ultraviolet-visible spectroscopy is an important technique. Through the use of UV-Vis spectroscopy, the process of reducing copper sulphate (Cu^{2+}) to copper (Cu^{0}) using an aqueous extract was observed **[34]**. Using a vortex, the purified CuAM-NPs were dissolved in 5 mL of double distilled water before being examined under UV-Vis light. CuAM-NPs formation was ascertained using this method. Using a UV-VIS spectrophotometer, an absorption spectra in the 200–800 nm wavelength region was recorded, with λ max around 450 nm **(Figure 4)**. Since no other detectable peak is present, this spectrum only verifies the existence of Cu **[35]**.

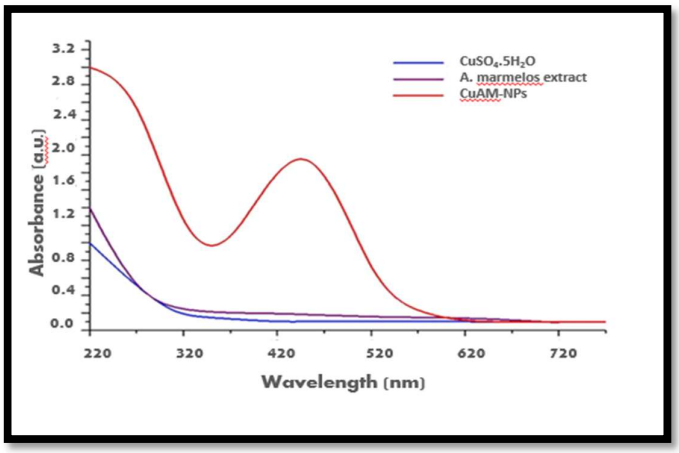

Figure 4: UV-VIS spectra of CuAM-NPs

(d) Fourier-Transform Infrared (FTIR) analysis:

The biomolecules needed for capping and effective stabilisation of the copper nanoparticles produced by *A. marmelos (L.)* leaf extract were identified using FTIR measurements **[36]**. **Figure 5** displays the FTIR spectrum of CuAM-NPs. The assignment of peak positions in the FTIR-spectrum of CuAM-NPs are as follows:

- 3470.8 cm^{-1}: N-H stretching in amines
- 3442.2 cm^{-1}: -OH stretching in phenols
- 3330.1 cm^{-1}: O-H stretching in phenols
- 2935.3 cm^{-1}: C-H stretching in aromatics
- 2222.9 cm^{-1}: -C=N stretching in nitriles
- 1649.8 cm^{-1}: -C=O stretching in flavonoids and terpenoids
- 1631.0 cm^{-1}: -C=C stretching in flavonoids and terpenoids
- 1527.1 cm^{-1}: N-O stretching in nitro groups
- 1399.9 cm^{-1}: N=O stretching in aliphatic nitro compounds
- 1111.9 cm^{-1}: C-O stretching in anhydrides
- 613.1 cm^{-1}: C-I stretching in halo groups

The presence of stretching frequencies in the range of 3550 to 3230 cm^{-1} are suggestive of presence of polyphenols in the CuAM-NPs which presumably act as capping biomolecules for the nanoparticles **[37]** **(Figure 5)**. Flavonoids present in *A. marmelos* (L.) are established through the stretching frequencies of 1649.8 cm^{-1} and 1631.0 cm^{-1}, respectively. The flavonoids are considered to be a good reducing as well as capping agents **[38]**.

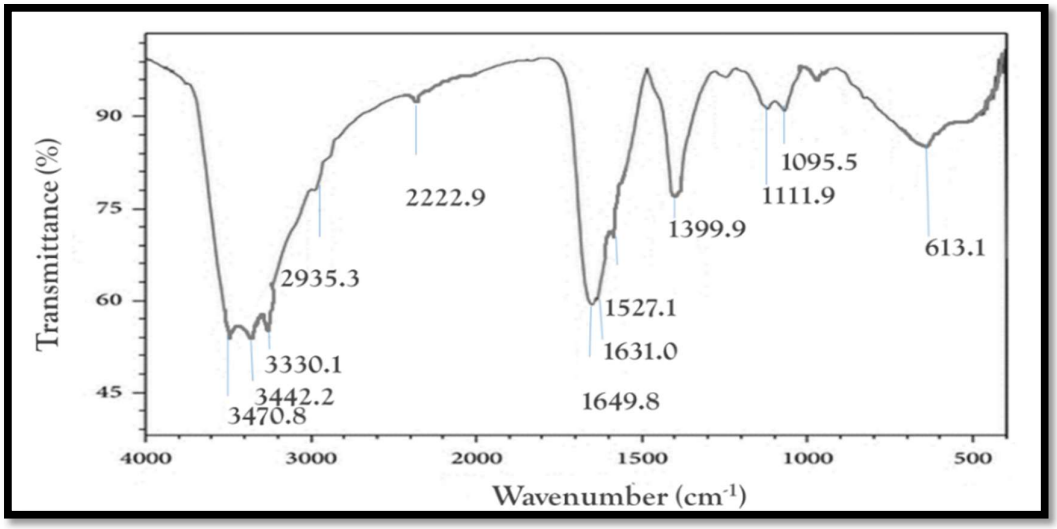

Figure 5: FTIR spectrum of CuAM-NPs

(e) Scanning Electron Microscope (SEM) analysis:

The morphology, size and distribution of differently synthesized nanoparticles can be efficiently studied using SEM **[39]**. The SEM images for as prepared CuAM-NPs at two different magnifications are given in **Figure 6 and 7**, respectively. Particle sizes of Cu nanoparticles ranged from 30 to 65 nm (as calculated manually using the resolution scale). The images depict a nearly spherical morphology of nanoparticles (arranged in flaky or leaf-like form) which also appear to be moderately uniformly distributed.

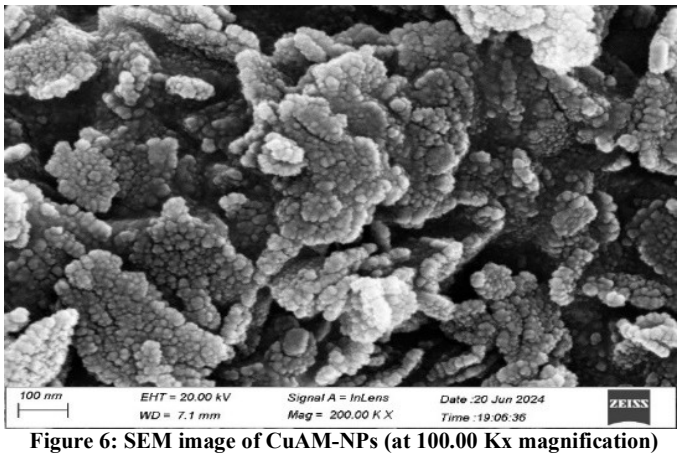

Figure 6: SEM image of CuAM-NPs (at 100.00 Kx magnification)

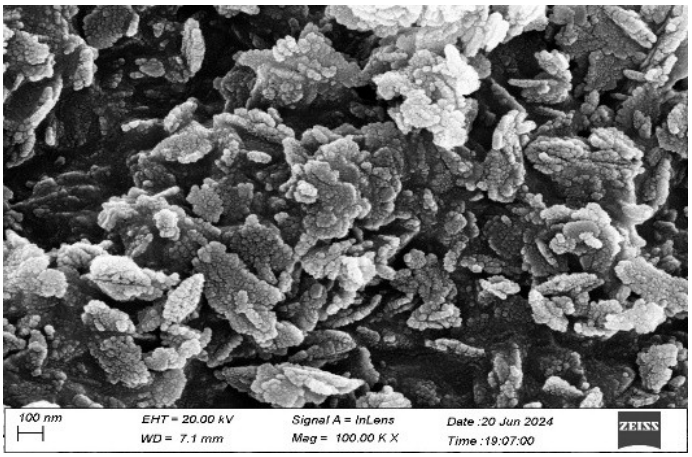

Figure 7: SEM image of CuAM-NPs (at 200.00 Kx magnification)

(f) Antitubercular -Zone Inhibition Test:

Agar plates (A, B, C) for different amount of sample (CuAM-NPs**)** and agar plates (D, E, F) for different amount of sample (CuRut-NPs**)** were used to treat test organism (*M. tuberculosis)*. Results obtained from the study showed that both the nanoparticle samples were active against *M. tuberculosis* as compared to positive control (maximum zone of inhibition 39 mm at 10 µg dose). The maximum zones of inhibition observed for CuAM-NPs and CuRut-NPs were 21 mm and 13 mm at 100% concentration doses, respectively. The results for anti-bacterial-zone inhibition studies are depicted in **Table 2** and **Figures 8, 9**, respectively. In this study, ciprofloxacin which belongs to class of fluoroquinolones was used as the positive control (PC). Ciprofloxacin, like- ofloxacin, and levofloxacin is a promising drug for the treatment of tuberculosis (TB), particularly multi drug resistant (MDR)-TB **[40]**. The zone of inhibition is an area around a disk on an agar plate where no bacterial growth is observed due to the presence of an antimicrobial agent. It is used to determine whether a particular test organism is susceptible to the action of a particular antimicrobial agent or not **[41]**.

The maximum zone of inhibition (ZOI) for CuAM-NPs is more than that for CuRut-NPs at 100% concentration doses **(Table 2)**. This data suggests that along with rutin (flavonoids), there are some other phytoconstituents in *A. marmelos (L.)* leaf extract which are responsible for enhancing the antibacterial activity of CuAM-NPs against *M. tuberculosis*.

Table 2: Antitubercular -Zone Inhibition data for Copper nanoparticles

	Conc. (%/disk)	Plate A Zone size (mm)	Plate B Zone size (mm)	Plate C Zone size (mm)	Average Zone size (mm)	SD (±mm)
CuAM-NPs against *M. tuberculosis*	PC	40	39	40	39.6667	0.57735
	0	0	0	0	0	0
	6.25	0	0	0	0	0
	12.5	0	0	0	0	0
	25	9	10	9	9.33333	0.57735
	50	17	16	17	16.6667	1.1547
	100	22	21	21	21.3333	0.57735
CuRut-NPs against *M. tuberculosis*	PC	40	39	39	39.3333	0.57735
	0	0	0	0	0	0
	6.25	0	0	0	0	0
	12.5	0	0	0	0	0
	25	4	4	3	3.66667	0.57735
	50	8	8	8	8	0
	100	13	14	13	13.3333	0.57735

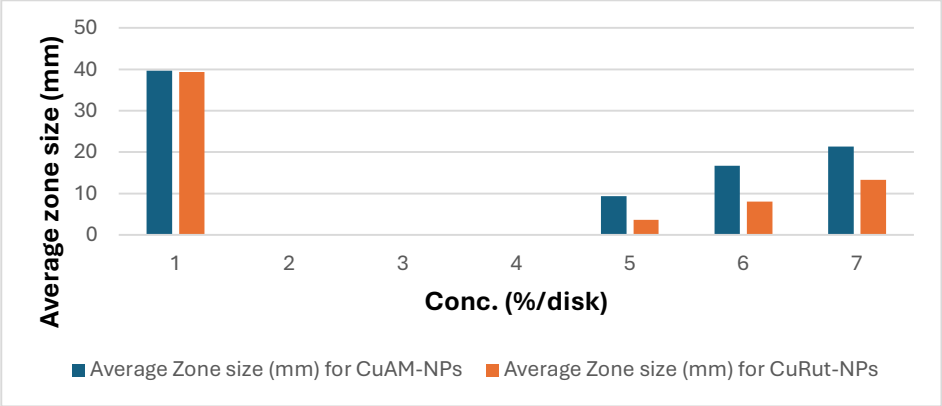

Figure 8: Antitubercular -Zone size vs. Conc. for CuAM-NPs and CuRut-NPs against *M. tuberculosis*

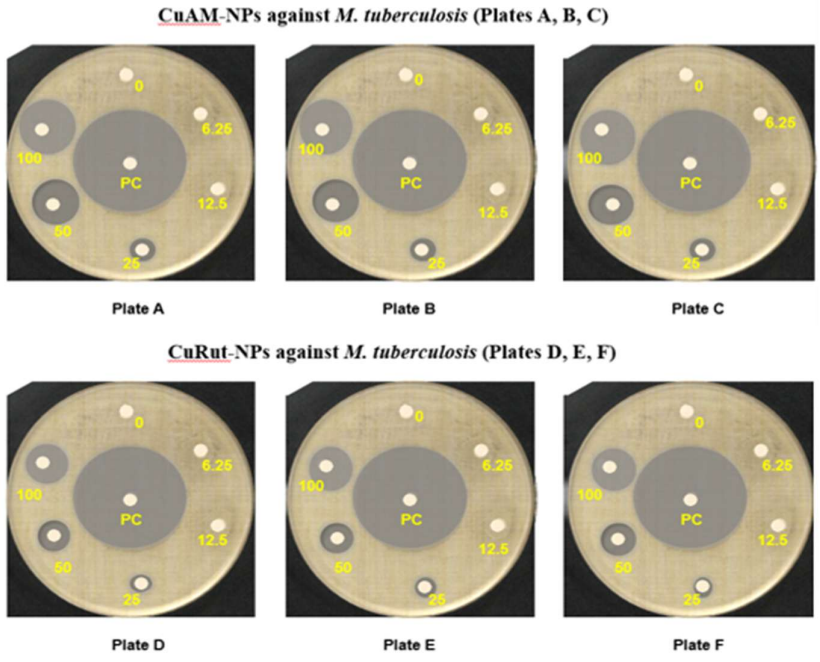

Figure 9: Antitubercular -Zone Inhibition plates for Cu-NPs against *M. tuberculosis*

(g) M.I.C. Assay:

Based on the study, it was observed that when test organism *M. tuberculosis* was exposed with different concentrations of the Cu-NPs samples then Minimum Inhibitory Concentration estimated for **CuAM-NPs was** approx 0.3-0.5 **µg/ml and that for CuRut-NPs was** 1.0-1.3 **µg/ml (Tables 3 and 4).** The MIC results have been generated by taking a mean of 4 replicates for % **MTB** wrt PC for both the types of Cu-NPs. MIC has been most commonly defined as that minimum concentration of an antibacterial agent at which the growth of the test strain of a microbe is inhibited completely when grown under carefully regulated in vitro conditions is measured in micrograms per millilitre, or µg/mL, and is the concentration at which the test strain of an organism cannot grow at all under carefully regulated in vitro conditions **[42]**. For this MIC assay levofloxacin was taken as PC. Like ciprofloxacin it also belongs to the class of fluoroquinolones, and is a 2nd line anti-TB agent **[43]**. In a recent study carried out on *M. tuberculosis* ($H_{37}Rv$), susceptible and resistant clinical isolates by de Souza Santos **[44]**, it was reported that the MIC for levofloxacin fell in the range of 0.12–0.25 µg/mL. This MIC value is slightly less than that found for **CuAM-NPs in the presented work.** In the past few years, studies on the antibacterial activity of green Cu-NPs have been published; nevertheless, the field of Cu-NPs' antitubercular activity has not received as much attention **[45, 46]**. Additionally, CuAM-NPs have a lower MIC value than that for CuRut-NPs, indicating that they have superior antitubercular efficacy (**Table 3 and 4)**.

Table 3: MIC assay (*M. tuberculosis*) vs. CuAM-NPs

CuAM-NPs Conc. (µg/mL)	Replicte-1	Replicte-2	Replicte-3	Replicte-4	Mean (% MTB wrt PC)	SD*	SEM**	N***
0	99.626168	88.03738	101.8692	110.4673	100	9.243049	4.621524	4
0.1	85.962	84.3214	83.23231	83.0222	84.13448	1.344848	0.672424	4
1	66.3697	64.4393	69.4221	68.8097	67.2602	2.296753	1.148377	4
10	60.6689	55.5607	57.9991	56.4535	57.67055	2.238357	1.119178	4
100	49.6321	45.7196	42.6449	43.63632	45.40823	3.09374	1.54687	4
500	33.17757	31.3178	31.1602	28.0302	30.92144	2.134127	1.067064	4
1000	16.3103	14.23141	13.8889	13.27103	14.42541	1.317939	0.658969	4
PC	4.2990654	3.551402	2.056075	-2.42991	1.869159	3.013928	1.506964	4

***= standard deviation, **= Standard error of the mean, ***= Total number of replicates**

Table 4: MIC assay (*M. tuberculosis*) vs. CuRut-NPs

CuRut-NPs Conc. (µg/mL)	Replicte-1	Replicte-2	Replicte-3	Replicte-4	Mean (% MTB wrt PC)	SD*	SEM**	N***
0	99.626168	88.03738	101.8692	110.4673	100	9.243049	4.621524	4
0.1	88.42056	87.1028	90.46729	89.71963	88.92757	1.481544	0.740772	4
1	77.2804	79.4393	81.4221	82.8097	80.23788	2.408392	1.204196	4
10	66.6689	65.5607	67.9991	66.4535	66.67055	1.007294	0.503647	4
100	54.6321	49.7196	50.6449	47.66355	50.66504	2.923512	1.461756	4
500	40.17757	41.3178	39.1602	38.0302	39.67144	1.404956	0.702478	4
1000	26.3103	24.23141	23.1256	20.2632	23.48263	2.519827	1.259914	4
PC	4.2990654	3.551402	2.056075	-2.42991	1.869159	3.013928	1.506964	4

*= standard deviation, **= Standard error of the mean, ***= Total number of replicates

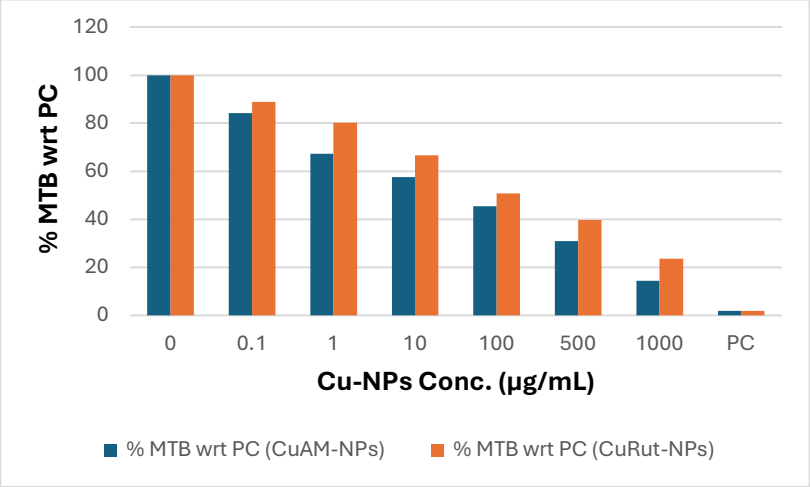

Figure 10: Comparison of %MTB wrt PC and concentration (µg/mL) for CuAM-NPs and Cu-RutNPs

CONCLUSION:

Through a flexible, non-toxic, and bio-safe process, we have achieved the effective biosynthesis of two types of Cu nanoparticles using leaf extract of *A. marmelos (L.)* as well as rutin (flavonoid). UV-VIS spectrophotometry, FT-IR, DLS and SEM confirmed the formation of Cu-NPs in the nanometer range (30-65 nm). The presented research work is unique in a way that the antitubercular activities of both the biosynthesized Cu-NPS were assessed against *Mycobacterium tuberculosis*, and both of them proved to be potent antitubercular agents. Though the CuAM-NPs demonstrated an upper edge than CuRut-NPs in this area of investigation. The strong correlation seen between the minimum inhibitory concentration (MIC) values of CuAM-NPs and data from previous studies implies that doping CuAM-NPs with noble metals such as Au/Pt can open up new avenues for enhanced antitubercular efficacy. Also, it is necessary to assess the CuAM-NPs' antitubercular action *in vivo*.

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