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From synthesis to bioactivity: A comprehensive study of Cu-based biocidal tool

N Anantha Krishna[a,*,†], Y V Mohana Reddy[b,‡] & B Chandra Mohan Reddy[c,#]

[a]Department of Mechanical Engineering, Mohan Babu University erstwhile Sree Vidyanikethan Engineering College, Tirupati 517 102, India

[b]Department of Mechanical Engineering, GPREC, Kurnool 518 001, India

[c]Department of Mechanical Engineering, JNTUA, Ananthapuramu 515 002, India

E-mail: ragakrishnulu@gmail.com[†], yvmr06@gmail.com[‡], cmr_b@yahoo.com[#]

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The present study reports on the biogenic synthesis of a copper-based biocidal material through the fermentation of gruel, a traditional non-alcoholic beverage. This process may involve a bio-beneficiation mechanism, in which the indigenous microorganisms in the ferment interact with the material. X-ray diffraction analysis confirmed the powder's crystalline copper composition. Fourier-transform infrared spectroscopy revealed the crucial role of organic acids in the capping process. Transmission electron microscopy, ultraviolet-visible spectroscopy, and antimicrobial susceptibility tests were conducted to characterize the powder. Furthermore, the biocidal material was combined with the anticancer drug curcumin to explore its additional anti-proliferative effects, including apoptosis, on human hepatocellular carcinoma cell lines *in vitro*. These findings highlight the potential of this biogenic copper material as a promising candidate for biomedical applications.

Keywords: Biocidal, Copper, Fermentation, Metallurgy, Organic, *Rasa shastra*

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Copper ions, both on their own and in copper complexes, have been used to sterilize water, food and even human tissue for ages. Copper is used as an antibacterial, antifouling, algaecide, fungicide, nematicide, molluscicide and water purifier. Investigations revealed that only 1% of the *E. coli* bacteria were alive in the copper loop, while the number of germs stayed the same or increased in other types of plumbing material. Copper sulphate is employed to suppress algae growth in potable water reservoirs; many algae are very vulnerable to it. Also, it has been used to preserve wood, and as a molluscicide[1].

Compared to the modest copper sensitivity of human tissue, bacteria are highly responsive to copper. Copper toxicity in microorganisms may result from the displacement of critical metals from their native binding sites, interference with oxidative phosphorylation and osmotic equilibrium and changes in the conformational structure of nucleic acids, membranes and proteins. Copper is being utilized as a biocide, especially in agriculture. However, there is a vast scope of investigation in other realms of applications.

As mentioned earlier, copper, both in its incinerated and non-incinerated forms, is recommended for therapeutic purposes by *Rasashastra* and Ayurveda. Copper's incinerated form, *Tamrabhasma*, is effective against jaundice, Irritable Bowl Syndrome (IBS) and anemia[2,3]. Accordingly, the green synthesis of copper nanoparticles (CuNPs) h`as also drawn considerable attention from chemists and scientists in recent years. Few researchers have indicated that copper nanoparticles in the form of *Tamrabhasma* can target CYS 145 thiolite in the coronavirus main protease active site[4].

Vacuum vapor deposition[5], thermal reduction[6], chemical reduction[7], etc. have been used to synthesize nanoscale copper recently. By contrast, green synthesis techniques allow more interaction time than chemical reduction procedures[8]. The vast majority involve toxic, labor-intensive and/or energy-intensive procedures[9]. As well as being extremely harmful to life and ecosystems, reducing agents are highly toxic to the human body and other forms of life[10]. The production of nanomaterials in a plant-based

*Corresponding author

environment (also known as "green synthesis") is evidence that plants can serve as "nano factories[9]. The extraction of natural substances and their subsequent synthesis is becoming increasingly crucial in nanotechnology. So far, only a few researchers have successfully synthesized copper nanoparticles using the cementation process[9,11].

Cancer nanotherapeutics is a novel but crucial field of study that looks at how nanomaterials can treat cancer. The harmful consequences of conventional cancer treatments can be avoided with nano-mediated therapeutic delivery devices. Due to their small and variable size, vast surface area, capacity to carry many genes and medicines, and mediation of improved therapeutic payload absorption, nanoparticles (NPs) are viewed as ideal tumor-targeting vehicles. There is a considerable improvement in the NPs' solubility, bioavailability, therapeutic index, and anticancer characteristics once Curcumin is added as a capping agent. From its rhizomes, *Curcuma longa* yields Curcumin, the most well-known curcuminoid. Antioxidant, hypotensive, anti-inflammatory, anticoagulant, antifertility, anti-ulcer, anti-microbial, anti-venom, anti-fibrotic, anti-mutagenic, antidiabetic, anticarcinogenic and most importantly, anticancer activities are just some of the biological and pharmacological properties of this bioactive component. In this work, we aim to study the physical properties of nano copper synthesized from fermented rice gruel using modern scientific techniques[12-15]. Further, the potential and anticancer qualities of curcumin-synthesized and capped Cu nanoparticles (NPs) are attempted. This can be an inexpensive, easy, and environmentally friendly biocidal tool for the localized delivery of therapeutic genes and medicines to the microenvironment of cancer tumors with fewer undesirable tumor effects.

Materials and Methods

Synthesis of Cu biocidal tool

Fermented rice gruel was prepared as per the references of *Rasa shastra* texts[12-15]. A significant quantity of contents, as mentioned in Table 1 were boiled and cooked. The ingredients were transferred to the pot, tightly sealed, and kept for fermentation for a period of 30 days at normal room temperature (as it took 30 days for fermentation) even though classic texts mention seven days for it.

Copper powder extraction was carried out per procedures described in references[9-11,12-15]. For seven days, a large quantity of the gruel was stored in an iron container with 100 g of powdered copper sulphate[12,16-19]. Following the reaction period, the copper was removed from the vessel and post-treated using the methods outlined in *Tamra Shodhana*, involving *Dola Yantra* and liquids such as fermented gruel, buttermilk, and cow's urine, before being washed with fermented gruel extract and distilled water[16]. For further investigation, the powder was then air-dried at room temperature.

Characterization and anti-microbial susceptibility test

Copper nano powder was characterized by X-Ray diffraction using CuKα radiation of wave length λ=0.154051 nm. The X-Ray diffraction pattern was recorded in the 2θ range from 100 and 800 at scanning steps of 0.020. The UV-Visible spectrum of copper nanopowder was recorded using Varian Cary 5000 instrument in the wavelength region 400 to 650 nm operated at a resolution of 1 nm. TEM analysis was carried out in a JEOL/JEM 2100 microscope and is used to estimate the morphology mean size of the copper nanoparticles. Scanning Electron Microscopy analysis of the Copper Nano Powder was performed using JEOL/EO model JSM-6390 cv. Avatar 370 model instrument was used for FTIR analysis which is responsible for reducing copper ions with the spectral range of 400 -4000 cm^{-1}.

S. auresus and *E. coli* bacterial species were obtained and sustained on Luria- Bertani agar. Prior to the investigation with the cu powder, the organisms were cultured overnight within 5ml of Luria – Bertani broth (Fulka) in a certomat BS-T incubation shaker at 370 and 150 rpm till the culture reached on OD600 of

Table 1 — Ingredients used in Kanji

Ingredient	Latin Name	Part Used	Quantity Taken (g)
Rajika	*Brassica juncea* Hook	Seeds	240
Saindhava	Rock Salt	---	480
Kulatha	*Dolichus biflorus* Linn	Seeds	480
Odana	----	Cooked rice	480
Haridra	*Curcuma longa* Linn	Rhizome	120
Vamsha	*Bambusa arundinaceae* Wild	Leaves	120
Shunthi	*Zingiber officinale*	Rhizome	60
Jeera	*Cuminum cyminum* Linn	Fruits	60
Hingu	*Ferula narthax* Bioss	Resin	5
Jala	H_20	Water	4800
Sarshapa Taila	*Brassica campestris* Linn	Seed oil	Q.S
Masha	*Phaseolus mungo* Linn	Seeds	120

1.0, in correspondence to 8 x 108 colony units per ml, as determined by a UV 3000 spectrophotometer

Both species were cultivated on Nutrient agar medium. One hundred milliliters of distilled water was thoroughly combined with 2.8 g of agar powder. The agar medium was powdered in Petri plates, thoroughly sterilized earlier. After that, they were left alone to set. Incubation time was used to dry the surfaces created before any organisms were streaked across them. The organisms were collected from their source (*i.e*., culture tube) with a metallic loop (pre-sterilized) and streaking was carried on the solidified plates. We used a metal borer (sterilized) to make holes in the Petri dishes, and then we put our samples in the cup. For 36 h, they were kept in an incubator.

Synthesis of Curcumin – CuNp's anti-cancer drug

A substantial amount of copper powder was suspended in water and sonicated for fifteen minutes to prevent the formation of aggregates. The solution was continually swirled for five hours after introducing an excess amount of obtained Curcumin (minimum complexation forming time). The mixture was then homogenized at 8000 rpm for 30 min to guarantee uniformity. After this, centrifugation separated the undissolved medication from the remainder. This produced a curcumin-induced copper medication for the apoptosis test, which was freeze-dried. The flow cytometry assay was used to check for apoptosis. Propidium iodide reagent was used to stain the cells after they were fixed in 80% ethanol (50 Ag/mL propidium iodide in 0.1% sodium citrate containing 0.1% Triton X-100). Cell Quest software analyzed the data from ten thousand flow cytometer events (Becton Dickinson, San Jose, CA). Moreover, In vitro studies are being incorporated into the research to estimate better drug release (the time period of 24 h). Triplicate formulations were considered as shown in Table 2.

Results and Discussion

Metal extraction has been linked to specific bacteria in several studies. SEM analysis of the synthesized gruel (559 nm to 1.20 µm) was performed to ascertain the impact of such organisms in copper extraction from copper sulphate (Fig. 1).

Table 2 — Formulation of drug

Formulation	Curcumin (g)	Synthesized CuNp's (g)
F1	0.5	0.5
F2	1	0.5
F3	1.5	0.5

Microorganisms can concentrate metals and Toxic elements can be removed from biomass using a bio-beneficiation process, which relies on microorganisms native to certain media at certain pH levels. Even with *shodhana*, this was a point of emphasis[20]. However, definitive theories on these grounds have not yet been made and support for the argument comes from a relatively small number of academics.

Peaks at 43.150, 50.270 and 73.930 were observed in the XRD of copper nano, which corresponds to the (1 1 1), (2 0 0) and (2 2 0) planes of copper, respectively, and were compared to the standard powder diffraction card and JCPDS, copper file No. 04-0836. The XRD analysis shows that pure (FCC) copper nanopowder is the final product. The Debye-Scherrer Equation. Nanopowder has a surface area to volume ratio of about 10,000 and its crystallite size is estimated to be 15.69 nm using the Debye-Scherrer equation. The XRD pattern of copper nanoparticles is displayed in Figure 2.

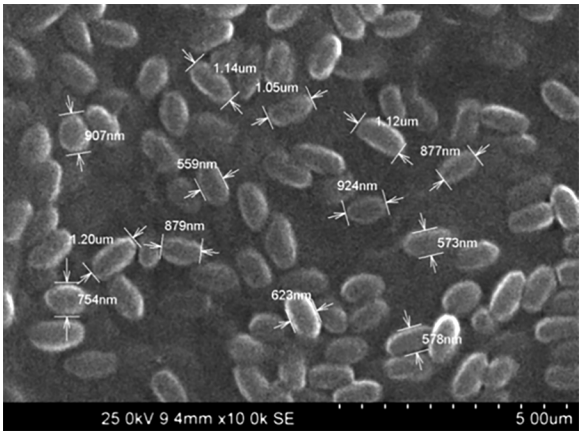

Fig. 1 — Rod-shaped organisms in the gruel

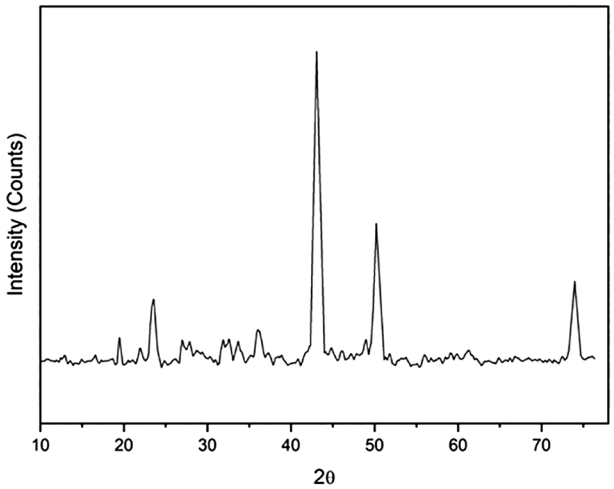

Fig. 2 — XRD of Copper nano particles

The ultraviolet-visible spectrum of the artificial copper powder is shown in Figure 3. The 570 nm wavelength was found to be the location of the characteristic absorption band. The SPR of Cu nanoparticles typically occur between 500 and 600 nm[21]. The 570 nm surface plasmon band provided strong evidence of copper nanoparticles' presence. The wide size distribution of nanoparticles is likely to blame for the broad absorption peak.

The absorption peaks of curcumin's UV spectrum occur at roughly 425 nm and 280 nm as shown in Figure 4. The peak at 425 nm is due to the conjugated pi-electron system of the -diketone moiety in curcumin, whereas the peak at 280 nm is due to the phenolic groups in the molecule. The UV absorption spectrum of curcumin can be utilized to identify and quantitatively analyze the chemical.

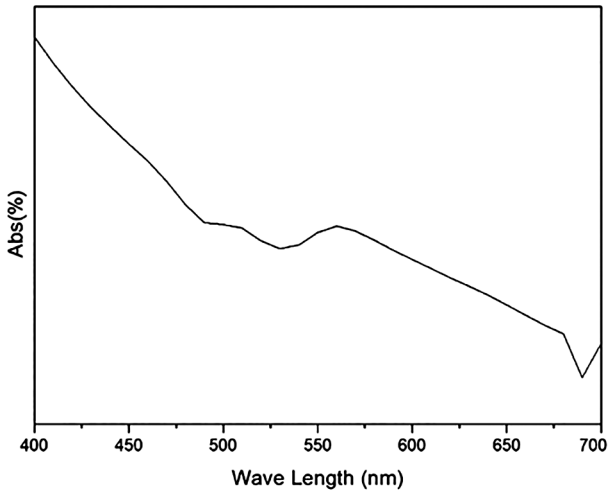

Fig. 3 — Shows UV-Vis sprectra of copper nano particles

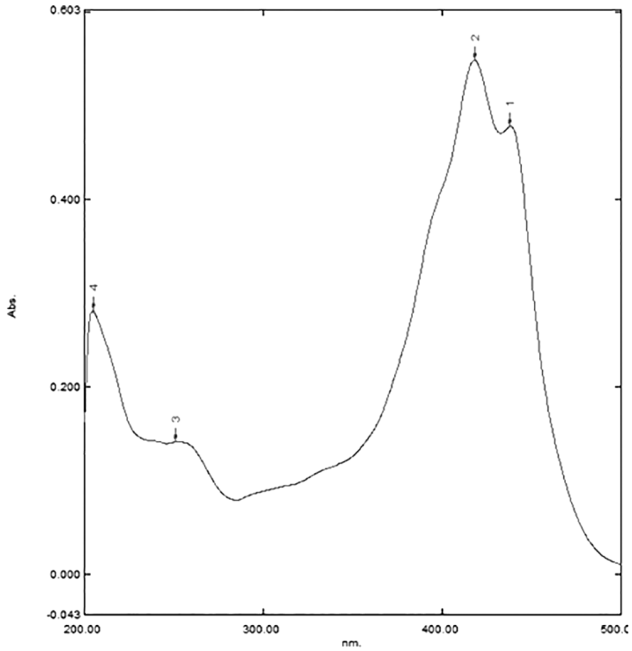

Fig. 4 — Shows UV-Vis spectra of curcumin

FTIR spectroscopy is a helpful tool for studying the functional group of any organic molecule. The FTIR spectrum is displayed in Figure 5. The broad peak represents the presence of aliphatic hydrocarbon chains at 3423 cm^{-1} and 3197 cm^{-1}, which is due to OH stretching; the peaks also indicate the presence of aliphatic chains at 2872 cm^{-1} and 2825 cm^{-1}, which are due to CH_2 stretching vibrations. The distinct peak indicates C=O stretching frequency at 1591 cm^{-1}. Accordingly, the FTIR Spectral data proves that organic acids from natural products were crucial to the capping process.

Copper deposition on iron is a well-known cementation process, as several lines of evidence indicate. Here, a more reactive metal is replacing a less reactive one. It has been shown that the gruel's acidity inhibits copper oxidation.

Figure 6 displays TEM measurements of the synthesized nano particles' size and shape. Micrographs taken with a transmission electron microscope (TEM) confirm the metal nanoparticles' nanoscale size, spherical shape, lack of a limiting shell, and wide size distribution. In most cases, their size was measured between 15 and 30 nm.

These particles were tested further for their anti-microbial studies, where incubation was carried for 36 h. Petri plates were removed and the zone of inhibition formed around the cups was analyzed. It can be seen from Figure 7(a) and (b) that both *E. coli* and *S. aureus* were proliferated thoroughly by copper

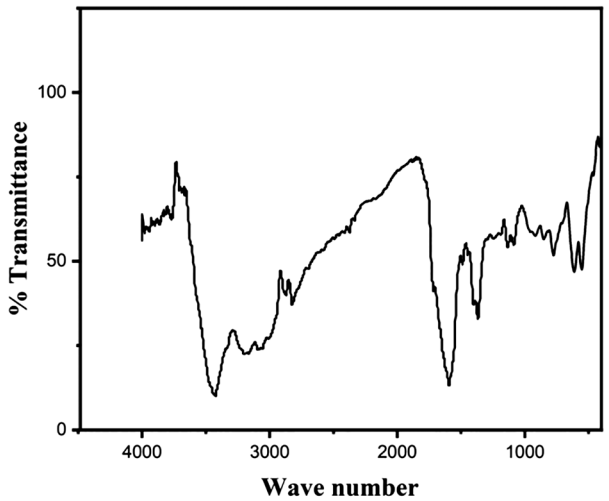

Fig. 5 — Depicts FTIR analysis of copper nanoparticles

powder at their highest concentration. The particles can penetrate the membrane and become embedded in the cell wall. The cytoplasm of the bacteria degrades and eventually disappears as the wall is destroyed by its ions. The potent adsorption of copper ions to cells is mainly responsible for the widespread antibacterial effect, which underpins the substance's antimicrobial propensity.

Cancer continues to be the leading cause of death worldwide, with a bleak prognosis, making it a devastating disease that can take lives. Cancer rates and associated expenditures will eventually rise as the average lifespan of the human population rises. Cancer alters healthy cells through inherited and acquired genetic abnormalities that offer growth and survival benefits, resulting in malignant neoplasms that infiltrate adjacent tissues and spread to distant organs[22,23].

As a result, it is crucial to find ways to reduce the risk of cancer in the population as a whole. Histologically, cancer progresses from average to hyperplasia to mild, moderate and severe dysplasia and carcinoma in situ over several years following the initial carcinogen exposure[24]. Cancer develops over a long period and eclectic preventive measures can be taken at any stage to slow down or stop the disease. Massive research efforts have led to the development several cancer prevention techniques, including behavioral change, vaccinations, surgical manipulation, and chemoprevention[25].

Healthy lifestyle choices, including a balanced diet, frequent exercise, abstinence in smoking and alcohol use, maintaining a healthy weight and managing stress have been shown to reduce cancer risk in several studies[26-30].

"Chemoprevention" is a preventive technique in which either natural or synthetic chemicals are deployed to halt, reverse or prevent cancer progression. Curcumin, prevalent in the Indian spice Haldi, is one such chemical being studied in clinical trials for cancer chemoprevention. Several studies focused on the three polyphenols extracted from *Curcuma longa*[31]. Curcumin triggers programmed cell death (apoptosis) in numerous cancer cells. Also, curcumin activates both intrinsic and extrinsic apoptotic pathways.

The biocidal tool consisting of Curcumin-Cu nanoparticles was investigated for *in vitro* studies as three formulations which are shown in Table 3. The study was carried out from 0 to 24 h and as shown in Figure 8, it can be observed that the drug

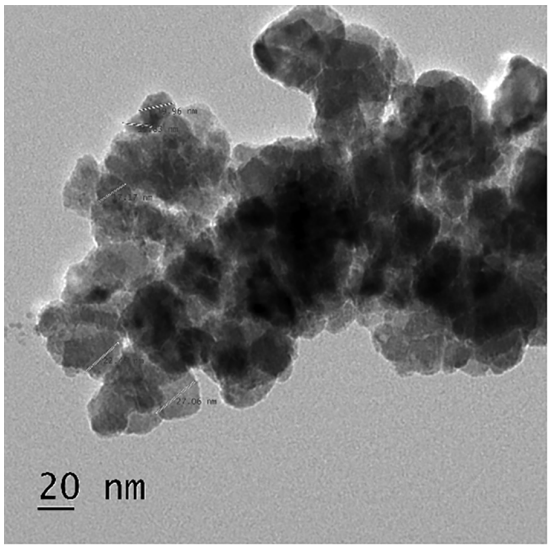

Fig. 6 — Represents TEM image of copper nanoparticles

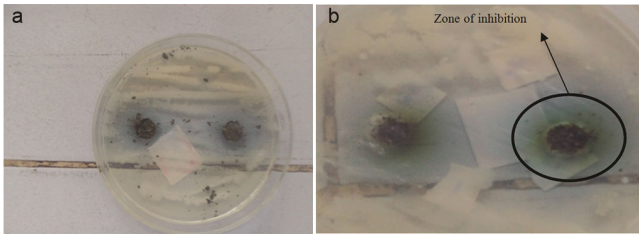

Fig. 7 — (a) & 7 (b) Anti-microbial studies against Gram Positive and Gram Negative bacteria

Table 3 — *In vitro* studies of formulated Curcumin-CuNP's drug

Hours	F_1	F_2	F_3
0	0	0	0
0.5	11	15	0
1	19	30	13
2	48	55	18
4	63	70	37
6	71	78	59
12	82	85	63
24	88	94	78

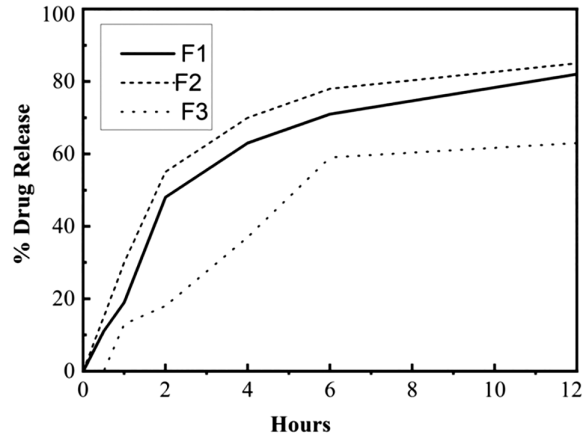

Fig. 8 — *In vitro* studies of formulated Curcumin – CuNp'sdrug

Table 4 — *In vitro* studies of formulated curcumin-CuNP's drug and pure curcumin

Hours	F_2	Curcumin
0	0	0
0.5	15	12
1	30	32
2	60	48
4	70	69
6	80	72
12	85	90
18	90	
24	94	

release percentage for the F2 formulation is relatively high (about 90%) in comparison to other formulations. Further, when F2 was compared with pure curcumin drug as shown in Table 4, the in vitro studies showed a significant percentage of drug release even after 12 h and up to 24 h compared to the pure drug as highlighted in Figure. 9. The comparison included both the study and control groups. An ANOVA is run for F2 and pure drug to ascertain the variations among the tested products. According to an analysis of variance (ANOVA), the *in vitro* findings of the comparison between F2 and drug formulations revealed statistically significant differences ($p< 0.05$). Hence, curcumin-copper nanoparticles impart a favorable circumstance to extend the clinical cluster of this dynamic agent with its influential properties.

Various cell stressors, such as irreversible DNA damage, a faulty cell cycle or the absence of growth hormones, can generate death signals via the intrinsic pathway and finally transmit them to mitochondria. However, in the extrinsic pathway, the cell's exterior environment, tumor necrosis factor and death receptors trigger death signals. In addition, Curcumin's anticancer action is also defined as being opposed to leaky arteries and loss of adhesion, which are strongly linked with cancer development and pervasiveness. Nanotherapeutics are potential prospects to challenge standard cancer treatments because of improvements in therapeutic indices, targeting biodistribution, oral bioavailability and water solubility[32,33]. Hence an attempt was made to evaluate the apoptosis potential of Curcumin- Copper nanoparticles.

Using human hepatocellular carcinoma (HepG2) cells as a model, apoptosis tests with the MTT assay revealed that curcumin-copper nanoparticles possessed anti-cancer capabilities. Figure 10 shows cell viability drops from 30000 cells/cm^2 to 7000

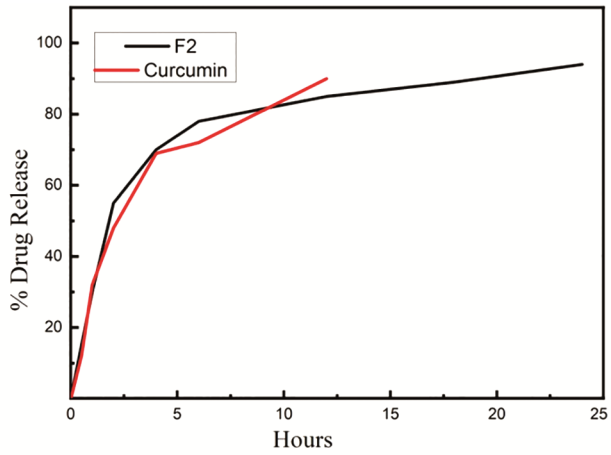

Fig. 9 — *In vitro* studies of formulated Curcumin – CuNp'sdrug and pure curcumin

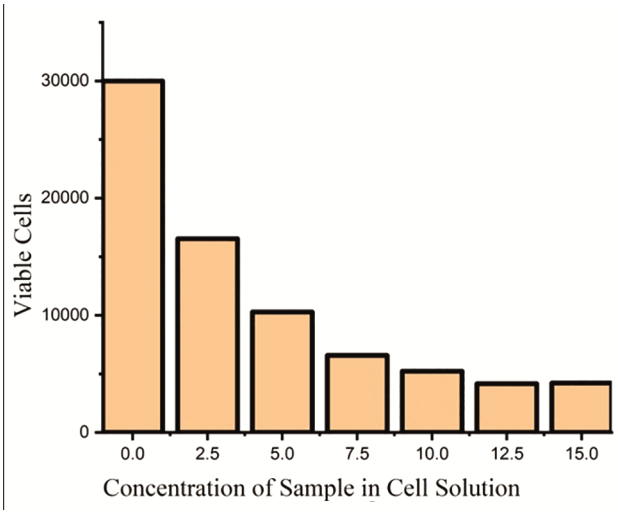

Fig. 10 — Concentration of sample Vs viable cells

cells/cm^2 as the Cu-curcumin nanoparticle concentration in the media increases from 0 to 15 mg/mL. Almost half of the viability is lost at a dosage of 2.5 mg/mL, which is remarkable. Therefore, as there are indications that Curcumin possesses direct and indirect anti-angiogenic activity both in vitro and in vivo, produced Cu- curcumin nanoparticles can potentially suppress Hepatic Carcinoma cell proliferation[34].

Conclusion

The study successfully synthesized a biocidal tool using fermented rice gruel, forming crystalline nano copper. The UV spectrum and TEM image analysis confirmed the presence of spherical copper nanoparticles in the nano range. As indicated by FTIR spectral data, the capping process was facilitated by

organic acids from natural products. The copper powder exhibited potent antimicrobial activity against Gram-positive and Gram-negative bacterial strains. With significant drug release, the Cu-Curcumin drug formulation exhibited remarkable apoptotic effects on human hepatocellular carcinoma cell lines. These findings suggest that the synthesized Cu biocidal tool could be a promising candidate for various biomedical and environmental applications.

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Conflict of Interest

Authors declare that there is no conflict of interest.

Authors' Contributions

NAK: Conceptualization, Data Curation, Investigation, Methodology, Writing-original draft preparation, validation and Reviewing; YVMR: Conceptualization, Methodology, Supervision, Validation, Writing- review and Editing; and BCMR: Conceptualization, Supervision, Validation, Writing-review and Editing.

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