Asian Journal of Chemistry; Vol. 34, No. 4 (2022), 926-930

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23661

Green Synthesis and *in vitro* Anti-Tubercular Activity of Monometallic and Bimetallic Oxide Nanoparticles

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Received: 15 November 2021; *Accepted*: 10 January 2022; *Published online*: 10 March 2022; AJC-20735

Tuberculosis (TB) is an infectious disease with limited drugs and currently the research is focused on its prevention, diagnosis and treatment. In recent studies, it is observed that the therapeutics approach of tuberculosis is more convenient to use nanoparticles for the treatment. With this regard, we foccussed present study towards modified green synthesis of monometallic oxide and bimetallic Cu-Zn and Cu-Mn oxide nanoparticles using *Ficus religosa* extract. All the synthesized bimetallic Cu-Zn oxide and Cu-Mn oxide nanoparticles were characterized by powdered XRD, FTIR, FE-SEM with EDS reports and ESR spectra. All the monometallic and bimetallic oxide nanoparticles were tested for *in vitro* anti-tubercular, antibacterial and cytotoxic activity. The Cu-Mn oxide nanoparticles showed promising result with IC_{50} = 6.27 µg/mL, MIC = 53.61 µg/mL and IC_{50} = 8.81 µg/mL, MIC= 54.82 µg/mL against *M. tuberculosis* H37Ra at dormant state and active state, respectively. Whereas Cu-Zn and Cu-Mn oxide nanoparticles showed moderate effect against *M. tuberculosis* H37Ra. On the other hand, all these nanoparticles were inactive against two Gram-positive and two Gram-negative bacterial strains with low cytotoxicity.

Keywords: Bimetallic nanoparticles, *Ficus religosa*, Green synthesis, Anti-mycobacterial activity, Cytotoxicity.

INTRODUCTION

Tuberculosis is one of the major death causing contagious disease among 10 diseases reported by WHO. In 2019, around 1.4 million people were died because of tuberculosis [1,2]. However, tuberculosis is preventable and cured by therapeutic medicines [3]. Various rapid assays are available to diagnose the tuberculosis but it is very complex to diagnose at early stage. So there is extreme need to pay attention on treatment than diagnosis. Moreover it is ultimately effective solution to cure tuberculosis than its expensive preventative measures [4,5]. Currently, a tuberculosis patient treatment includes standard six months course of four drugs of isoniazid, rifampin, streptomycin, ethambutol and pyrazinamide [6]. However, due to consumption of high concentration of these drugs for longer period it gets accumulated in blood cells and thus adverse effect is observed [7].

In this regard, nanotechnology is remedy over such kind of problems caused during the treatment. In many cases nanoparticles are used either as a drug or as drug delivery vehicle for the administration of drug for the treatment. For tuberculosis particularly, Ag, Au and ZnO nanoparticles are reported as a potential anti-tubercular drug [8,9]. Nanoparticles can be synthesized by using physical, chemical and biological methods [10].

Present work aims to synthesize monometallic and bimetallic oxide nanoparticles using extract of *Ficus religiosa* and their *in vitro* anti-tubercular, antibacterial and cytotoxic activities.

EXPERIMENTAL

All the solvents and other chemicals *viz*. $Cu(OAc)_2{\cdot}2H_2O$, $Zn(OAc)_2{\cdot}2H_2O$ and $Mn(OAc)_2{\cdot}2H_2O$ were purchased from Sigma-Aldrich, USA.

Leaves of *Ficus religiosa* L. were collected in large amount from local area of Pune city, India. Leaves were washed with distilled water to remove dirt and dried in shed. Dried leaves were ground to make fine powder and used to prepare plant extract. Redistilled ethanol was used to make plant extract.

The IR spectra were recorded on Nicolleate iD5 instrument while powdered X-ray diffraction pattern for each sample was recorded on JEOL ESR 59 at 77 K. FE-SEM images were recorded on FEI Nova Nano SEM 450 and EDS reports were obtained from Bruker XFlash 6130, ESR spectra were recorded on JEOL 49 at room temperature.

In biological studies, all the chemicals such as sodium salt XTT and MTT, DMSO, ampicillin and rifampicin were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. Synthesized compounds were dispersed in DMSO and used as stock solution (10 mg/ml) for further biological testing.

Preparation of plant extract: A plant extract of *Ficus religiosa* L. was prepared using maceration. A dried leaves powder of *Ficus religiosa* L. (50 g) were soaked in 500 mL distilled ethanol for 48 h at room temperature. After two days, ethanol was filtered and concentrated under reduced pressure at 40 ºC to obtain plant extract.

Synthesis of CuO nanoparticles (sample A): In 250 mL Erlenmeyer flask, 5 mL alcoholic extract of *Ficus religiosa* was added to 200 mL 1mM Cu $(OAc)_2{\cdot}2H_2O$ solution and slightly alkaline pH (7.2-7.4) was maintained. The reaction mixture was sonicated for 0.5 h at room temperature and brown coloured CuO nanoparticles were formed. The nanoparticles were isolated on centrifugation and dried in microwave oven for 0.5 h with 30 s time interval. Finally, obtained product was characterized by UV-Vis, FT-IR and powder XRD techniques.

Similar process was applied for the synthesis ZnO (sample B) and Mn_2O_3 (sample D) nanoparticles from $Zn(OAc)_2{\cdot}2H_2O$ and $Mn(OAc)_2{\cdot}2H_2O$ salts, respectively.

Synthesis of bimetallic Cu-Zn oxide (sample C) and Cu- Mn oxide nanoparticles (sample E): A solution of 100 mL each 1 mM $Cu(OAc)_2{\cdot}2H_2O$ and 1mM $Zn(OAc)_2{\cdot}2H_2O$ was taken in 250 mL Erlenmeyer flask and 10 mL 50% ethanolic plant extract was added. Alkaline pH (7.2-7.4) of reaction mixture was maintained and sonicated for 15 min. A reaction mixture colour changes to black. Finally, the Cu-Zn oxide nanoparticles were isolated on centrifugation and dried in microwave oven with 30 s time interval with slow water evaporation. Finally, a uniform finely powdered product of hybrid Cu-Zn Oxide nanoparticles were obtained.

Similar procedure was used for the synthesis of hybrid Cu-Mn oxide nanoparticles by using 1 mM $Cu(OAc)_2{\cdot}2H_2O$ and 1mM $Mn(OAc)_2{\cdot}2H_2O$. Both the hybrid products were characterized by UV-Vis, FT-IR, pXRD, ESR, EDS and FESEM techniques. All these synthesised nanoparticles were tested for anti-tubercular, antibacterial and cytotoxic activities.

Anti-tubercular assay: The anti-tubercular activity of synthesized compounds was carried out by measuring inhibition of growth against a virulent strain of *M. tuberculosis* H37Ra (ATCC 25177) in liquid medium using an established XTT reduction menadione assay (XRMA) method. The optical density was read on a micro plate reader (Spectramax plus384 plate reader, Molecular Devices Inc) at 470 nm filter against a blank prepared from cell-free wells. Absorbance given by cells treated with the vehicle alone was taken as 100% cell growth. The compounds were tested for their inhibitory activity against active (8 days incubation) and dormant (12 days incubation), initially primary screening was done at 3 µg/mL concentration. Compounds showing 90% inhibition of *bacilli* at 3 µg/mL, which were particularly used for further dose response curve. MIC and IC_{50} values of sample E compound were calculated from their dose response curves (0-10 µg/mL) by using Origin 6 software.

Activity against MTB was determined using XTT reduction menadione assay (XRMA) reading absorbance at 470 nm as per the protocol [11]. Percentage inhibition was calculated using the following formula:

$$\text{Inhibition } (\%) = \frac{\text{Control} - \text{CMPD}}{\text{Control} - \text{Blank}} \times 100$$

where, control is the activity of mycobacteria without compounds, CMPD is the activity of mycobacteria in the presence of compounds and blank is the activity of the culture medium without mycobacteria.

The experiment was performed in triplicate and the quantitative value was expressed as the mean ± standard deviation (S.D.)

***in vitro* assay:** *In vitro* activity against MTB at active (8 days) and dormant (12 days) stages was performed using the XRMA 12 assay.

Antimicrobial activity: Compounds at concentration of 3 µg/mL were tested against four bacterial strains comprising of two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 23857) and two Gram-negative bacteria: *Pseudomonas fluorescens* (ATCC 13525), *Escherichia coli* (ATCC 25292) by antibacterial assay.

To determine specificity, compounds at concentrations of 3 µg/mL were tested against bacteria. Bacterial strains comprised of two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 23857) and two Gram-negative bacteria: *Pseudomonas fluorescens* (ATCC 13525), *Escherichia coli* (ATCC 25292). For the antimicrobial assay, the OD-adjusted ($OD_{620} = 1$) culture was inoculated in LB broth (1% v/v). Then 2.5 µL of compound and 247.5 µL of culture were dispensed in a 96-well microtitre plate and were incubated for 18 h at 37 ºC before reading the absorbance at 620 nm [12-15]. Ampicillin was used as a positive control. Wells for growth and sterility control were also included.

Cytotoxicity assay: *in vitro* potential effects of compounds on cell viability were investigated through reduction of tetrazolium (MTT) dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) as described earlier [16-19], a widely adopted method of measuring cellular proliferation. The effect of compounds on cell growth was determined in a panel of HeLa cell line cervical cancer cells obtained from the European Collection of Cell Cultures (ECCC, Salisbury, UK). Percentage cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity } (\%) = \frac{\text{Control} - \text{CMPD}}{\text{Control} - \text{Blank}} \times 100$$

where, control is the cell growth in medium without compounds, CMPD is the cell growth in the presence of compounds from rhizome and blank is the culture medium without cells.

Paclitaxel was used as positive control and purchased from Sigma which is ≥ 96% pure. The experiment was performed in triplicate and the quantitative value was expressed as the mean ± S.D.

RESULTS AND DISCUSSION

Green synthesis of metal oxide nanoparticles using *Ficus religiosa* L. is rapid, good yield and benign method. A plant extract was preferably prepared in ethyl alcohol so that most of the polar organic compounds get extracted from the plant material. These organic functional groups from alcoholic plant extract of *Ficus religiosa* L .acts as a precursor and capping agent for the synthesis of CuO, ZnO, Mn_2O_3 nanoparticles, Cu-Zn oxide and Cu-Mn oxide nanoparticles.

FTIR studies: The IR spectra of CuO, Cu-ZnO, Mn_2O_3 and Cu-MnO nanoparticles shows peak in finger print region *i.e.* 800-700 cm^{-1} indicates the formation of metal oxide nanoparticles. A weak frequency peak for -C=O observed at 1700-1600 cm^{-1} and -OH at 3300 cm^{-1}. The -OR at 1050 cm^{-1} indicates presence of glycoside linkages from plant extract, which acts as a stabilizing agent for metal oxide nanoparticles.

P-XRD studies: Average particle size of synthesized nanoparticles were calculated by using Debye-Scherrer equation [20]:

$$D = \frac{0.95\lambda}{\beta \cos\theta}$$

Fig. 1a represents the X- ray diffraction of spectra of CuO, ZnO and bimetallic Cu-Zn oxide nanoparticles whereas, Fig. 1b represents X-ray diffraction spectra of CuO, Mn_2O_3 and bimetallic Cu-Mn oxide nanoparitcles. The average particle size of bimetallic Cu-Zn oxide nanoparticles and Cu-Mn oxide nanoparticles were obtained as 82.80 and 66.12 nm, respectively which specifies the formation of bimetallic nanoparticles. The patterns for the CuO, ZnO and Mn_2O_3 monometallic nanoparticles were indexed and resembles with JCPDS No. 80-0076, JCPDS No. 80-0074 and JCPDS No. 76-0636, respectively from observed peaks. After the determination of individual metal oxide structure, the XRD pattern for bimetallic metal oxide nanoparticle was compared with them and confirmed its formation.

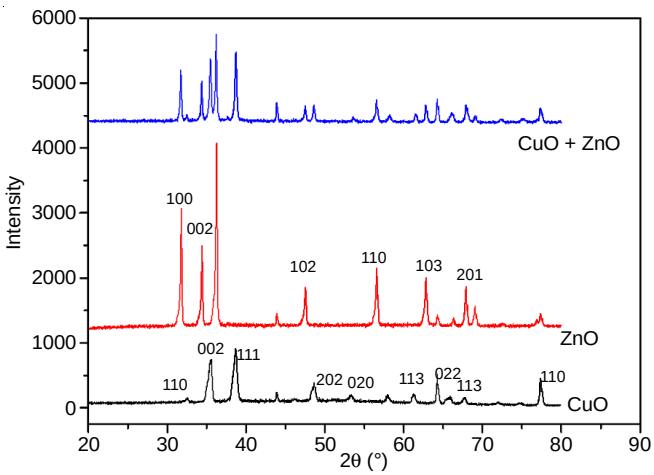

Fig. 1a. Combined powder X-ray diffraction pattern of CuO nanoparticles, ZnO nanoparticles and bimetallic Cu-Zn oxide nanoparticles

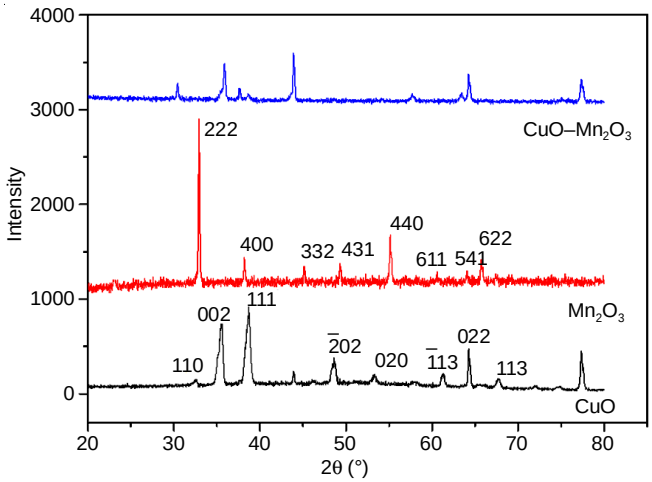

Fig. 1b. Combined powder X-ray diffraction pattern of CuO nanoparticles, MnO nanoparticles and bimetallic Cu-Mn oxide nanoparticles

FE-SEM and EDS studies: FE-SEM images determined the morphology analysis of bimetallic Cu-Zn oxide and Cu-Mn oxide nanoparticles. From Fig. 2a, it was observed that Cu-Zn oxide nanoparticles have spherical shaped morphology. A size of bimetallic Cu-Zn oxide nanoparticles observed between 85-150 nm with development of polymorphs. Whereas, FE-SEM image of bimetallic Cu-Mn oxide from Fig. 2b showed nanoparticles have trigonal shaped morphology of size 55-120 nm. The actual size observed from FE-SEM images is analogous to size calculated from XRD pattern. It is also observed that both metal nanoparticles adopted new morphology from their individual identity. Both the images exhibited lots of void space, which supports catalysis property and ability of binding sites with receptors.

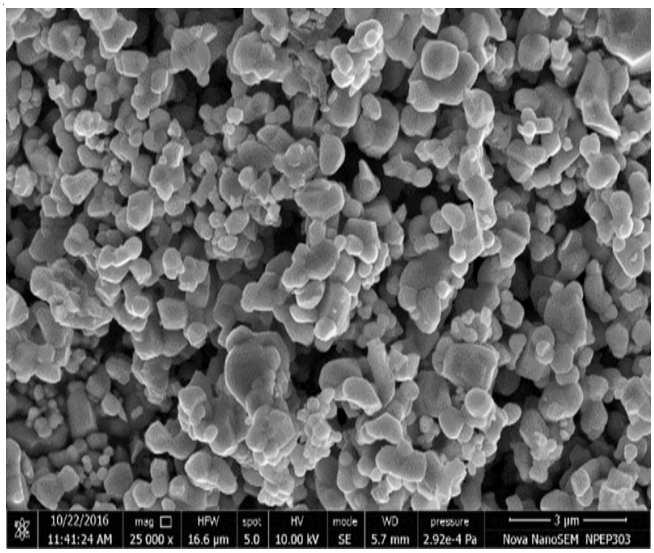

Fig. 2a. FE-SEM image of Cu-Zn oxide nanoparticles

Bimetallic nanoparticle formation was also supported with EDS reports of Cu-Zn oxide and Cu-Mn oxide nanoparticles. It was observed that the Cu, Zn and Cu, Mn metals were present in 1:1 ratio with oxygen. This supports the formation of bimetallic metal oxide nanoparticles formation.

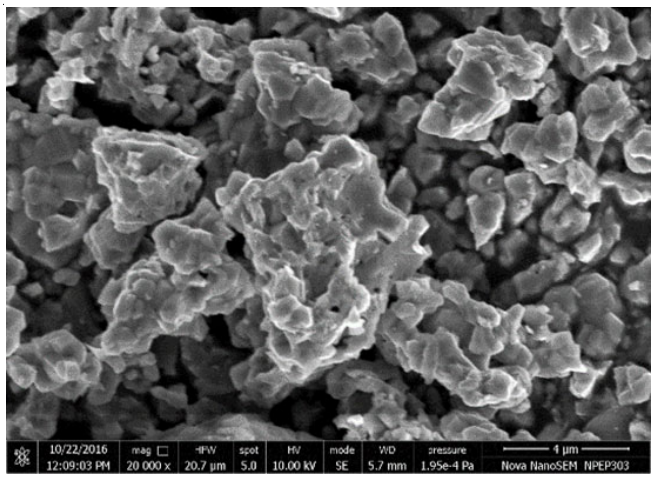

Fig. 2b. FE-SEM image of Cu-Mn oxide nanoparticle

ESR studies: ESR spectra of Cu-Zn oxide nanoparticles and Cu-Mn nanoparticles in Fig. 3a and 3b, respectively shows the formation of bimetallic nanoparticles. In Fig. 3a, the g values at 2.16 shows an intense absorption at high field which is isotropic. This confirms the crystal field is tetragonal at metal centre, most probably at Cu(II) site due to unpaired electron in the ground state is in $d_{x^2-y^2}$ and increase in R value from principal axis indicates bimetallic Cu-Zn nanoparticle formation

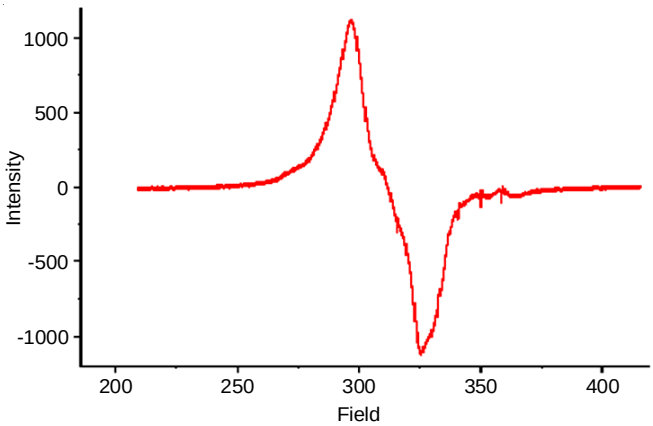

Fig. 3a. ESR spectra of Cu-Zn oxide nanoparticles

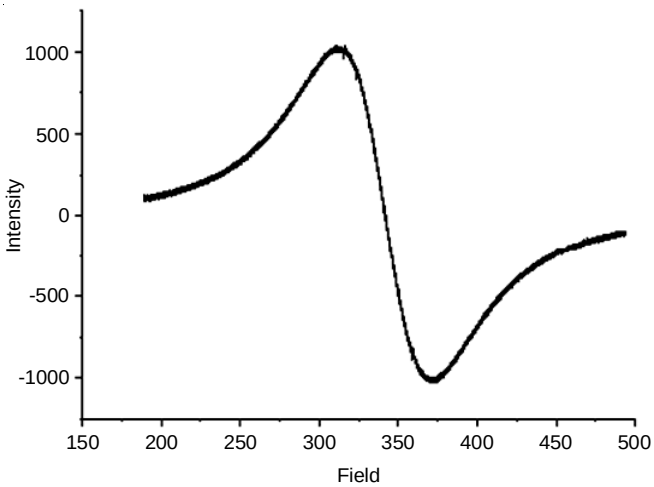

Fig. 3b. ESR spectra of Cu-Mn oxide nanoparticles

[21,22]. In Mn(II) compounds, *d-d* transitions are weak so the transitions from the ground state to the states of four fold multiplicity are also of a very weak intensities. Fig. 3b exchange energy is not matching with hyperfine value therefore we can see hyperfine splitting of Mn(II) with six signals from 2I + 1= 2×5/2 + 1 = 6 signals where I = 5/2 for ^{55}Mn nuclear spin quantum number value. The field strength varies from 319 mT to 364.2 mT with six g values range from 2.11 to 1.85 along with enhance-ment in R value from principal axis due to presence of Cu(II) indicates the formation of bimetallic Cu-Mn oxide nanoparticles.

Biological studies: The synthesized metal oxide nanoparticles and their bimetallic nanoparticles were tested for anti-tuberculosis study. The drug rifampicin was used as reference. The anti-tubercular activity was carried out for both active and dormant states. All the metal oxide nanoparticles are ineffective for active stage but encouraging results were obtained for dormant state.

In vitro anti-tubercular studies, it is observed that at dormant state bimetallic Cu-Mn oxide (sample E) nanoparticles showed high anti-tubercular activity (81.82% for dormant state), Mn oxide (sample D) and bimetallic Cu-Zn oxide nanoparticles (sample C) showed a moderate anti tubercular activity (65% and 56% at 3 µg/mL, respectively) against *M. tuberculosis* H37Ra (ATCC 25177) strain (Fig. 4). Dose response curve showed IC_{50} = 6.27 µg/mL, MIC = 53.61 µg/mL for dormant state and IC_{50} = 8.81 µg/mL, MIC = 54.82 µg/mL for active state respectively.

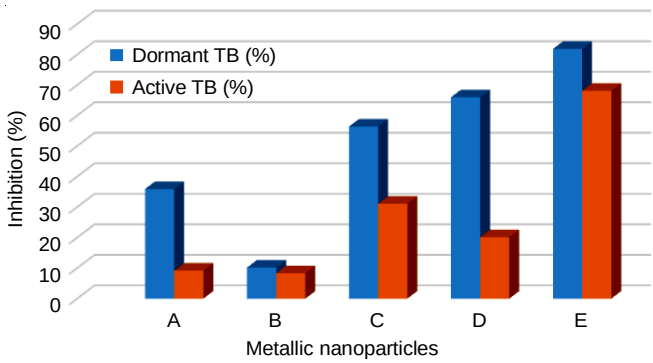

Fig. 4. Antitubercular activity of compounds by *in vitro* assay at 3 µg/mL

All these compounds were inactive against two Gram-positive and two Gram-negative bacteria (Table-1). Moreover, all these monometallic and bimetallic nanoparticles were also showed low cytotoxicity against HeLa cell line (Table-2).

TABLE-1
ANTIBACTERIAL ACTIVITY OF COMPOUNDS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA AT 3 µg/mL

Compd.	Bacterial strains			
	S. aureus	*B. subtilis*	*P. fluorescens*	*E. coli*
A	-12.01±0.4	18.20±0.7	-28.36±0.6	6.44±0.9
B	-6.27±0.3	7.09±0.5	-18.86±0.4	-5.19±0.6
C	-2.32±0.5	22.98±0.4	12.20±0.6	1.32±0.5
D	-3.57±0.3	20.08±0.3	-15.53±0.4	-64.20±0.3
E	-0.13±0.6	54.81±0.8	-12.89±0.9	-22.72±0.2

TABLE-2
CYTOTOXIC ACTIVITY OF COMPOUNDS FOR CERVICAL CANCER CELL LINE AFTER 48 h OF EXPOSURE AT 3 µg/mL

Compound	HeLa cell line	Compound	HeLa cell line
A	23.34693878	**D**	-2.248037677
B	15.21507064	**E**	-16.62127283
C	-1.500784929		

Conclusion

Synthesis of monometallic and bimetallic oxide nanoparticles using *Ficus religiosa* plant extract is a green and cost effective method. The process is benign and with less hazardous chemicals. The Cu-Mn oxide nanoparticles showed promising result whereas Cu-Zn and Mn oxide nanoparticles showed moderate effect against *M. tuberculosis* H37Ra at dormant stage. On the other hand, all these nanoparticles were inactive against two Gram-positive and two Gram-negative bacterial strains with low cytotoxicity.

ACKNOWLEDGEMENTS

The authors are grateful to Principal Prof. P. B. Buchade, MES Abasaheb Garware College, Pune, India for providing all facilities for research work. The authors are also thankful to Dr. Dhiman Sarkar, Scientist, National Chemical Laboratory, Pune, India for biological studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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