MATERIALS AND METHODS

3.1 Materials

All the chemicals and reagents used in this study were of analytical grade with high purity. The reagents acetonitrile, methanol, ethanol, sodium hydroxide, sodium carbonate, sodium nitrate, methyl blue, congo red and malachite green were obtained from CDH Pvt. Ltd., India. Methylene blue (S.D. Fine, Pvt. Ltd., India), guar gum and potassium persulphate were obtained from Loba Chemie. Pvt. Ltd., India. Microbial strains (Escherichia coli, Staphylococcus aureus, Aspergillum nigres and Candida albicans) were obtained from Bio technology research laboratory of Shoolini University, Solan. DMEM, Ham’s F-12, heat inactivated fetal bovine serum and antimycotic-antibiotic solution were procured from Gibco, Invitrogen. Sulforhodamine B (SRB) was obtained from Sigma Aldrich, Trichloroacetic acid (TCA) from Merck and well flat bottom plates were from corning. All the reagents were used as received without further purification. Double distilled water was used for each preparation and dilutions.

3.2. Instruments

X-ray diffraction (Panalytical.s X.Pert Pro), scanning electron microscopy (SEM) (S-3400N, Hitachi, Japan), transmission electron microscopy (TEM) (FEI Tecnai F 20), thermal analysis (TGA/DTA) (Perkin Elmer, model. STA 6000), Fourier transformation infrared spectroscopy (FTIR)(Perkin Elmer - Spectrum RX-IFTIR), ultraviolet-visible spectroscopy (UV-vis) (Systronics 2202 double beam spectrophotometer), hot air oven, muffle furnace (Shree Sati scientific Industries, Ambala, India). Magnetic stirrer, centrifuge electrophoresis power supply digital (INCO Pvt. Limit., India), copper plate, aluminum plate (procured from the local market), platinum electrode (Phillips), were used in this study.

3.3. Methods

3.3.1. Electrochemical Synthesis of Copper oxide Nanoparticles (CuO NPs)

Electrochemical deposition method has been used for the synthesis of CuO NPs under different reaction conditions. 1.25 mM of supporting electrolyte (sodium hydroxide,
sodium carbonate and sodium nitrate) was dissolved in 200 mL solvent (water, water-acetonitrile (12:1) and water-methanol (12:1). The copper plate (3×2 cm$^2$) and inert platinum (1×1 cm$^2$) were used as sacrificial anode and cathode, respectively. Before electrochemical deposition, both electrodes were cleaned with hydrochloric acid and double distilled water. The distance between both the electrodes was fixed at 1 cm for all the experiments. The electrolysis reaction was carried out in an undivided electrochemical cell at 50 V - 100 V potential with vigorous stirring at room temperature. The electrolysis was performed at currents of 20 mA, 50 mA, 100 mA and at time of 30 min, 60 min, 120 min. After the electrolysis, the dark brown precipitates were centrifuged for 15 minutes at 4000 rpm, washed with ethanol and finally with double distilled water several times. The products were then dried at 60 °C in hot air oven for 6 h. The materials obtained were calcined at 300°C, 600°C and 900°C for 1 h to study the effect of temperature on particle size. The particles so obtained were analyzed for spectral analysis such as Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX). The electrolytic cell is represented in Scheme 3.1.

### 3.3.2. Electrochemical Synthesis of Aluminum oxide Nanoparticles (Al$_2$O$_3$ NPs)

In this procedure, 1.25 mM of supporting electrolyte (sodium hydroxide, sodium carbonate and sodium nitrate) was dissolved in 200 mL of solvents (water, water-ACN and water-methanol (12:1)). The electrolysis was performed at different current (20mA, 50mA and 100mA) and electrolysis time (10 min, 30 min, 60 min). The electrolysis has been conducted at sacrificial aluminum (2×5 cm$^2$) anode and inert platinum (1×1 cm$^2$) cathode in a beaker. Aluminum plate was used as anode and platinum plate as cathode. Both electrodes were connected to the source of direct current power supply. Before electrochemical deposition, both electrodes were cleaned with hydrochloric acid and distilled water.
The distance between both the electrodes was fixed at 1 cm for all the experiments. The electrolysis reaction was carried out in an undivided electrochemical cell with vigorous stirring at room temperature. The electrolysis reaction was performed at 20 mA, 50 mA and 100 mA. After the electrolysis, the white precipitates were centrifuged at 4000 rpm for 15 minutes, washed with ethanol and finally with distilled water. The products were then dried at 60 °C in a hot air oven for 6 h. The materials obtained in different conditions were calcined at 900°C, 1100°C and 1200°C for 1 h to evaluate the effect of temperature. The Al$_2$O$_3$ NPs were analyzed Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX). The electrolytic cell is presented in Scheme 3.2.
3.3.3. Preparation of Guar gum/CuO Nanocomposite (GG/CuO)

GG/CuO nanocomposite was prepared by simple sol-gel method. In this method, gel of guar gum was prepared by mixing 150 mg of guar gum into 50 mL of deionized water with continuous stirring for 1-2 h at 50 °C. Then, the suspension of CuO NPs (prepared in section 3.3.1) was added into this solution with continuous shaking. It was followed by the addition of potassium persulphate solution drop wise with continuous shaking. The resultant mixture was stirred for 3 h on magnetic stirrer at 50 °C. The brown colored precipitates obtained were kept for 24 h for digestion. Then supernatant liquid was decanted and precipitates were filtered under suction and washed with double distilled water to remove the impurities. Finally, the composite material was dried in a hot air oven at 50 °C.
3.3.4. Preparation of Guar gum/\( \text{Al}_2\text{O}_3 \) NPs nanocomposite (GG/\( \text{Al}_2\text{O}_3 \))

GG/\( \text{Al}_2\text{O}_3 \) nanocomposite was prepared by simple and ambient sol-gel method. In typical procedure, 150 mg of guar gum was dissolved in 50 mL of deionized water and stirred for 2 h at 50 °C. The suspension of \( \text{Al}_2\text{O}_3 \) NPs (as prepared in section 3.3.2) was added into above solution with continuous shaking. It was followed by the addition of potassium persulphate solution drop wise with shaking. The resultant mixture was stirred for 3 h on magnetic stirrer at 50 °C. The white colored precipitates obtained were kept for digestion for 24 h. The supernatant liquid was decanted and precipitates were filtered under suction and washed with double distilled water to remove the excess of impurities. The material thus obtained was dried in hot air oven at 50 °C.

3.4. Details of Various Characterization Techniques

3.4.1. X-rays Diffraction (XRD)

X-rays diffraction is a powerful structural technique used for studying crystalline structure of the material. It is a non-destructive analytical technique that provides detailed information of the crystal structures, grain size, orientations, phases and chemical composition of materials. The characteristic spectra consists of discrete energy, which occurs due to the X-rays emitted by transition of electrons from K shell into L shell, which gives rise to copper K\( \alpha \) peaks. The transition of electrons from M shell gives K\( \beta \) peaks and the electrons from the N shell give K\( \alpha \) peaks. K\( \alpha \) and K\( \beta \) peaks are the most prominent in the peaks characteristic spectrum. The X-rays get scattered from a crystalline solid interfere constructively and produce a diffracted beam of light. The d spacing between diffraction planes is calculated using Bragg’s law as:

\[ n\lambda = 2dsin\theta \]

where, \( d \) = inter planar spacing, \( \theta \) = diffraction angle and \( \lambda \) = wavelength and \( n = 0, 1, 2, 3 \) etc.

XRD of nanomaterials were performed using (X’pert PANaytical) equipped with Cu K\( \alpha 1 \) (\( \lambda =1.5406 \ \text{Å} \)). The instrument was equipped with graphite monochromator and operated
at 40 kV and 30 mA. Average particle size has been estimated by using Debye-Scherrer formula:

\[ D = \frac{0.9}{\beta \cos \theta} \]

where, \( \lambda \) is wavelength of X-ray (0.1541 nm), \( \beta \) is FWHM (full width at half maximum), \( \theta \) is the diffraction angle and D is particle diameter size.

3.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a powerful technique used to determine the function group in the material. Infrared spectroscopy is the study of interaction between matter and electromagnetic fields in IR region, which reveal the properties of the matter. In this spectral region, the electromagnetic wave mainly couple with the molecular vibrations. In other words, a molecule can be excited to a higher vibration state by absorbing IR radiation. The probability of a particular IR frequency and the molecule being absorbed depends on the actual interaction between the frequency and the molecule. In generally, a frequency will be strongly absorbed if its photon energy coincides with the vibrational energy levels of the molecule. IR spectroscopy is a very powerful technique which provides fingerprint information on the chemical composition of the sample. FTIR spectra of samples were recorded using Perkin Elmer spectrophotometer. In this, 10 mg of material was thoroughly mixed with 100 mg of KBr. Then an appropriate pressure was exerted to from a transparent pellet. FTIR spectra of the materials were recorded between the ranges from 4000 to 400 cm\(^{-1}\).

3.4.3. Transmission Electron Microscopy (TEM)

Transmission electron microscopy has been used for the production of images at higher resolutions compared to other conventional light microscopes. TEM provides detailed information of the crystal structure, interface structure, particle size, crystal defects and single crystal diffraction. TEM analysis of different samples was performed using FEI Tecnai F 20. In this, 0.01 mg of material was added to minimum quantity of ethanol to form fine suspension. The suspension was sonicated in ultrasonic cleaner for 30 minutes. Then drop of suspension was placed onto a carbon coated copper grid. High energetic
beam of electrons first generated by an electron gun (field emission) are focused by a series of magnetic lenses. This beam is then transmitted through the ultra-thin sample on TEM grid and interacting with the atoms of the specimen. The transmitted electrons through the specimen create an image as the result of the interaction of the electrons and the specimen. This image is magnified and focused onto a fluorescent screen or camera. Thus, the working principle of the TEM is quite identical to the optical microscope. The combination of high-resolution imaging, bright field, dark field imaging, electron diffraction and detailed micro analysis are the important features of TEM. The elemental composition of the samples was determined by energy dispersive X-ray coupled with transmission electron microscopy.

3.4.4. Scanning Electron Microscopy (SEM)

The morphology of the materials were studied by scanning electron microscope. SEM is one of the most versatile instruments widely used to study the surface microstructure. SEM images the sample surface of a solid specimen using a focused beam of high-energy electrons. The signal contains information about surface topography, external morphology, chemical composition, crystallographic information, and electrical conductivity. The image formation in SEM depends on signals produced from elastic and inelastic interactions between the high energy electron beam and the specimen surface. The working principle of SEM is similar to the TEM, where a high energy electron beam (few keV) is focused by condenser magnetic lenses and passes through a scanning coil inside the electron column. The scanning coil deflects the electron beam in the x and y axes. The interaction of the incident beam with the specimen involves random electron scattering, absorption, penetration, and backscattering. These interactions result in various emitted signals that are detected by different types of electron/photon detectors. SEM analysis of nanomaterials was performed on a S-3400N, Hitachi, Japan. The samples were stocked over a holder and gold-sputtered before investigation.

3.4.5. UV-visible Spectroscopy

The absorption spectra or optical density and band gap studies of nanoparticles and nanocomposite were attempted by UV-visible spectrophotometer (Perkin-Elmer spectrometer). In this method, 5 mg of sample was dispersed in distilled water. The
suspension was ultrasonicated for 1 h. UV-visible spectrum was recorded using double beam spectrophotometer. If suppose \( I_i \) and \( I_o \) are the intensities of incident and transmitted rays of light. Then the absorbance ‘A’ is defined as

\[
A = \log \frac{I_i}{I_o}
\]

Or,

\[
A = \varepsilon cx
\]

Where, \( x \) = sample path length (cm), \( c \) = concentration (mol L\(^{-1}\)). \( \varepsilon \) = molar extinction coefficient (mol\(^{-1}\)cm\(^{-1}\))

This technique usually gives the preliminary concept of particle size and size distribution such as mono or polydispersity. Usually a blue shift (decrease in wavelength) is associated with a decrease in particles size and vice-versa. Light with wavelength from 400 nm to 1100 nm is generated by a halogen lamp and a deuterium lamp. Then the light is divided into two equal-intensity beams and passes through a prepared sample and a reference sample, respectively. A silicon photodiode is used to record the intensity of the transmitted light. The optical band gap of nanomartials was determined from Tauc relation as follow:

\[
\alpha h\nu = A(h\nu-E_g)^n
\]

Where, \( \alpha \) is the absorption coefficient, \( h\nu \) represents the photon energy, \( E_g \) is the energy band-gap, \( A \) is a constant and \( n \) depends on the nature of the band gap (\( n = 1/3 \) for indirect forbidden transition, \( n = 1/2 \) for indirect allowed transition, \( n = 2/3 \) for direct forbidden transition, and \( n = 2 \) for direct allowed transition).

### 3.4.6. Thermo Analysis (TGA/DTA)

Thermogravimetric analysis (Shimadzu thermal analyzer TGA50 model-Japan) was used to determine the physical property of a substance as a function of temperature while the substance is subjected to a controlled temperature program. Thermal analysis has been used for the determination of thermal stability, material characterization, compositional analysis, simulation of industrial processes, kinetic studies and corrosion studies etc. In the analysis, powdered sample was heated in a nitrogen atmosphere in the temperature range of 50 °C to 750 °C. The thermal stability and percentage weight loss was analyzed through TGA/DTA instrument.
DTA, give the information about the sample behavior such as exothermic (heat emission during the reaction) or endothermic (heat absorption during the reaction). An exothermic process is plotted with upward deflection while an endothermic process is plotted with a downward deflection in the DTA curve.

3.5. Applications of Nanomaterial

3.5.1. Photocatalytic Activity

In the typical procedure, (2 x 10^-5 mol/L solution of malachite dye and 1.5 x 10^-5 solution of methylene blue, methyl red, congo red and malachite green dye were prepared in double distilled water (Liu et al., 2013). In this method, 0.1 g of sample was added into 100 mL solution of dye in pyrex beaker with continuous stirring. In adsorption experiments, the suspension was stirred magnetically and placed in dark to establish the adsorption/desorption equilibrium. Then for photocatalytic studies the suspension was exposed to sun light irradiation. 3 mL solution was withdrawn at different intervals of time, centrifuged to remove particles. The absorbance was recorded at wavelengths of 664, 497, 410 and 662 nm for MB, CR, MR and MG dye. The percent degradation of all dyes were calculated using formula as:

\[
\% \text{ Degradation} = \frac{C_e - C_t}{C_e} \times 100
\]

where \( C_e \) and \( C_t \) are the concentrations of dye at equilibrium and at time t.

The rate of photocatalytic degradation of dyes was determined using pseudo-first-order kinetic model as follow.

\[
r = -\frac{dc}{dt} = k_{app}t
\]

On integrating the above equation, we get

\[
\ln \frac{C_o}{C_t} = k_{app} t
\]

Where \( k_{app} \) is the apparent rate constant, \( C_o \) is the concentrations of dye before illumination and \( C_t \) is the concentration of dye at time t.
3.5.2. Antimicrobial Activity

Antimicrobial activities of nanomaterials were investigated under two methods: growth curve method and colony forming unit (CFU).

3.5.2.1. Growth Curve Method

Antimicrobial activity of NPs was carried out by growth curve method against bacterial strains (*Escherichia coli* and *Staphylococcus aureus*) and fungal strains (*Aspergillum nigres* and *Candida albicans*). The bacteria were cultured overnight in nutrient broth and fungus is cultured in potato dextrose broth (PDB) solution at 37 °C in incubator shaker. The microbial culture was exposed to NPs of different concentration (25 μg/mL and 50 μg/mL) and microbial cell concentration was adjusted to approximately 10^7 CFU/mL. The control was used without the nanoparticles. The cultures were shaken at 150 rpm at 37 °C. After 1 hr, 3 mL of suspension was taken out to determine the microbial growth rates by measuring optical density at 600 nm (OD_{600}) using a UV-visible spectrophotometer. The efficiency of nanoparticles for inhibiting the growth of microorganisms was determined by differences in percentage of microbes before and after treatment as:

\[
\text{Inhibition rate} = \frac{1 - OD_{sample}}{OD_{control}} \times 100
\]

3.5.2.2. Colony Forming Unit (CFU)

Antimicrobial activity of nanocomposite was analyzed using colony forming unit (CFU) methods (Gupta et al., 2015). To examine the susceptibility of bacteria to different samples, nutrient agar plates were prepared. Three tubes of bacterial suspension 5mL (with a concentration of 10^7 CFU/ml of *Staphylococcus aureus*) cultured in NB were prepared. Out of three tubes of bacterial suspension, two were supplemented with different amounts of composite material (with a concentration of 0.8 μg/mL and 0.4 μg/mL of nanoparticles). A 100 μL sample of bacterial suspension from each tube was then plated in three different nutrient agar plates. Nanoparticle-free bacterial suspension incubated under the same conditions was used as controls. The plates were then incubated at 37°C. The tubes of bacterial suspension were incubated at 37°C on a rotary shaker at
200 rpm for 24 h. The numbers of resultant colonies of *S. aureus* were counted after 24 h of incubation. The counts from three independent experiments (plated at 0 hour, 24 h & 48 h) corresponding to a particular sample were averaged.

### 3.5.3. Cytotoxicity Studies of Nanocomposite

In vitro cytotoxicity studies of nanocomposite using Sulforhodamine B dye: The CHO-K1 cells (CCL-61) were cultured in Ham’s F-12 media, oral cancer cells KB (ATCC No. CCL-17) and rat glioma C6 cells (ATCC No. CCL-107) were cultured in DMEM supplemented with 10% heat inactivated FBS and 1% antimycotic-antibiotic solution. The cells were grown until 70-80% confluency achieved and maintained for cytotoxicity testing. The cells were seeded in 96-well plate with the cell density of 20,000 cells per well (100µl) to evaluate the toxicity of the drug, and incubated for 12 h in CO₂ incubator, maintained at 5% CO₂ and 95% humidity, for cell adherence. Further, treated with 100 µl of 4 different concentrations of the nanocomposite (10, 25, 50 and 100μg/ml), in triplicates and were incubated for 24, 48 and 72 hrs. After the completion of treatment, SRB assay was performed and fraction of viable cells was counted.