Fabrication and physicochemical characterization of copper oxide–pyrrhotite nanocomposites for the cytotoxic effects on HepG2 cells and the mechanism

Abstract: Novel CuO–FeS nanocomposites were synthesized to exert anticancer effects on HepG2 cells. The formation was initially demonstrated using UV–Visible spectrophotometry analysis, which indicated two peaks at 335 and 370 nm. Characteristic Fourier transform infrared spectroscopy peaks for Cu–O and Fe–S bonds were observed at 516, 577 and 619 cm$^{-1}$ in addition to other notable peaks. The Miller indices correspond to the lattice spacing of monoclinic CuO and FeS as observed by selected area diffraction rings concurrent with the X-ray diffraction observations. The morphology was interpreted by scanning electron microscopy and transmission electron microscopy, indicating a particle size of 110 nm. As per energy-dispersive X-ray spectroscopy analysis, strong peaks for Cu (0.9, 8 and 9 keV), Fe (6–7 keV), O (0.5 keV) and S (2.5 keV) indicated the formation of CuO–FeS blend with no impurities. A mean particle size of 121.9 nm and polydispersity index of 0.150 were displayed by dynamic light scattering analysis and the zeta potential was $-29.2$ mV. The composites were not toxic to normal 3T3-L1 cells and were not haemolytic even at higher doses. In addition, the stable composites exerted cytotoxic effects on HepG2 cells ($IC_{50} = 250 \pm 5.7 \mu g/mL$) and induced cell death by creating a loss in mitochondrial membrane potential and induction of mitochondrial apoptosis in a ROS-independent manner.

Keywords: nanocomposite, HepG2, apoptosis

1 Introduction

Noncommunicable diseases are known to affect the productive population (ages 30–69) accounting for more than 70% of global deaths each year [1]. Cancer has a noteworthy global noncommunicable disease burden and accounts for approximately 16% of deaths related to this burden [2,3]. Tobacco use, elevated BMI, drug and alcohol abuse, limited intake of dietary fibres and the shortage of physical activity account for more than 30% of the deaths associated with cancer [4]. To address this issue, the World Health Organization has made its strong efforts to reduce the alcohol dependence and provide impartial access to health care for all affected individuals [5].

To progress efficiently with attempts to manage cancer globally, cell lines are primarily used as in vitro models for preclinical cancer research. These cells serve as an unlimited biological resource for drug testing and form a base for the resultant drug discovery [6,7]. As a well-differentiated human hepatoma of a 15-year-old Caucasian with 50–60 chromosomes and a size range of 12–19 µm, HepG2 is an oncogenic model for liver cancer. It is used in studying the metabolism of several anticancer drug candidates. Molecular studies have determined that it is related more to hepatoblastoma, an
early childhood malignancy, than to hepatocellular carcinoma as it lacks the expression of hGSTP1 [8].

Cancer registries form the basis for controlling cancer at the national and international levels. In this regard, publications at the global level from several countries provide updated information on several malignancies every year. This is related to the incidence, chronological aspects and projections of cancer-related incidence and mortality. This can help in exploring the current challenges and future perspectives for managing cancer [9,10]. With this background, the American Cancer Society data provide the information that an estimated 41,260 new cases and 30,520 deaths related to liver cancer are expected to occur in the United States alone in 2022 [11]. Additionally, the United States projections for liver cancer in 2023 are 41,210 new cases and 29,380 deaths with a low survival rate of 21% [12]. For the same year, an astounding 4,820,000 new cancer cases and 3,210,000 deaths are projected to happen in China. As the second-most common cause of death after lung cancer, liver cancer accounts for 431,383 new cases and 412,216 deaths [13].

As an alternative to current therapeutic approaches intended for cancer therapy, nanomedicine is projected to fill a global demand with increases of approximately 13% every year. Subsequently, revenue may rise from 8,500 million USD in 2019 to 30,000 billion USD by 2030. Health care and medicine are considered the key sectors for this growth [14]. In this regard, the worldwide market for cancer medications accounted for nearly one-third of the overall nanomedicine arena in 2014 [15]. Although it is a growing market, nanomedicine is still relatively in its infancy due to insufficient research on safety and other guideline issues [16]. To accomplish the increased application of nanomedicine, nanomaterials are comprehensively used in diagnosing, detecting and managing tumours. This may be attained through drug delivery and therapy at the primary or distant sites after they metastasize [17–19].

To achieve the enhanced use of nanomedicine, novel metallic nanoparticles have been developed for everyday use in onco-nanotherapy. In this regard, bimetallic nanoparticles display enhanced therapeutic effects compared to their metallic counterparts with limited systemic toxicity. Therefore, the beneficial effects of both monometallic nanoparticles can be attained simultaneously when used in combination. Hence, improved therapeutic efficacy can be achieved based on the intrinsic properties of the individual material [20]. Metallic nanoparticles fabricated based on copper oxide (CuO) have been used extensively in preclinical trials for use in cancer therapy [21–24]. Although ferrous sulphide (FeS) based nanoparticles have been synthesized for purposes other than in medicine, they are seldom studied for their anticancer effects [25–27]. Furthermore, CuO and iron-based nanoparticles exhibited improved cytotoxic effects on cancer cell lines when used in combination with several other nanomaterials compared to their use alone [28–30]. This may be because their magnetic and other properties related to synergism can make the bimetallic nanoparticles more efficient in their biological applications than their monometallic counterparts [31].

In general, nanocomposites are prepared by combining organic or inorganic compounds with the intention of being created with smaller sizes and larger surface area. They are frequently loaded with standard anticancer drugs, fluorescent probes and other biological macromolecules by covalent or noncovalent contact and/or binding [32,33]. Among bimetallic nanocomposites, CuO is usually prepared and blended with zinc oxide and this nanocomposite is commonly used for antibacterial effects [34–37]. The combination of CuO with zinc oxide and/or graphene oxide as composites has also been tested for cytotoxic effects on a small number of cancer cell lines [38–40]. However, the copper oxide–pyrrhotite (CuO–FeS) composite is a novel blend and has not been studied for use in nanomedicine, especially for cancer therapy. In this connection, we report the synthesis of CuO–FeS nanocomposites and attempt to determine their cytotoxic effects for the first time on HepG2 cells with the probable mechanism involved.

2 Materials and methods

2.1 Preparation of the CuO–FeS composite

All chemicals used for this study were of analytical grade and bought from Sigma Aldrich. In a simple synthesis through the hydrothermal route, 10 mL of 0.01 M Fe(NO3)2, 10 mL of 0.05 M Na2S, 10 mL of 0.01 M Cu(CH3COO)2 and 10 mL of 1 M NaOH aqueous solutions were added into a Teflon beaker, sealed and kept in an autoclave at 220°C for 12 h. After completion of the reaction, the mixture was cooled to room temperature and washed with ethanol and water. Finally, the obtained product was filtered and dried at 60°C overnight in an oven and used further [41–44].

2.2 Physicochemical characterization of the composite

UV–Visible spectrophotometry (UV–Vis) analysis was performed using Spectramax 190 absorbance reader. The surface properties of the composites were studied using Fourier transform infrared spectroscopy (FTIR) analysis (Perkin
Elmer Spectrum RX1 model). Particle size and polydispersity index (PDI) were measured using Particulate Systems model Nano Plus instrument. Zeta potential was determined using Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). The crystalline nature of the composites was studied using X-ray diffraction (XRD) analysis (D8 ADVANCE, Bruker AXS, Germany). The morphology and diffraction patterns were studied using transmission electron microscopy (TEM) and selected area diffraction (SAED) analysis (JEM-2100 Plus, JEOL Ltd, Japan). Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDAX) analyses were conducted using Thermo Scientific Apreo S instrument (ThermoFisher Scientific, USA).

### 2.3 Cytotoxic effects of the composite

Human hepatocellular carcinoma cell line HepG2 (ATCC® HB8065™) was cultured in Minimum Essential Media at $1 \times 10^5$ cells/well. The media with the cancerous cells was then treated with standard antibiotics (1× antibiotic antimycotic solution) besides 10% fetal bovine serum and incubated at 37°C with 5% CO₂. Furthermore, the cells were washed and treated with various concentrations of the composite (25–500 μg/mL) and incubated for 24 h. Later, the medium was aspirated, treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) and incubated at 37°C for 4 h. At the end of the incubation period, MTT was discarded, and the suspension (0.5 mg/mL) and incubated at 37°C for 4 h. At the end of the incubation period, MTT was discarded, and the suspension was read at 570 nm using a microplate reader. HepG2 cells were replaced with 3T3-L1 fibroblasts (ATCC® CL-173™) and the same methodology was adopted for safety testing of the composites [45]. The cytotoxic effects of the standard doxorubicin were also determined using the same protocol.

### 2.4 Hemocompatibility assay

Sheep blood was collected in heparin or sodium citrate tubes and the erythrocytes were instantaneously separated by centrifugation at 3,000 rpm for 5 min. The supernatant was aspirated, and the erythrocytes were washed using phosphate-buffered saline (PBS) and centrifuged again at 3,000 rpm for 5 min. The supernatant was centrifuged thrice until it turned clear. The erythrocyte-containing pellet was then diluted to 1:100 in PBS. Later, 1% erythrocyte solution was prepared and mixed with equal volume of the composites. 10% Triton X-100 was used as a positive control, while PBS was used as a negative control. The blend was incubated at 37°C for 60 min and centrifuged at 3,000 rpm for 5 min. The final supernatant was collected and the absorbance was read at 450 nm using a microplate reader [46]. The activity was expressed via the following formula:

$$\text{Haemolysis ratio} = \frac{(\text{OD(test)} - \text{OD(negative control)})}{(\text{OD(positive control)} - \text{OD(negative control)})} \times 100\%.$$ 

### 2.5 Mechanistic analysis of the composite–HepG2 interactions

#### 2.5.1 Evaluation of mitochondrial membrane potential (MMP), ROS and acridine orange (AO)-ethidium bromide (EB) staining

The MMP was measured by adhering $2 \times 10^5$ cells/well overnight, treating them with composites and incubating for 8 h. After incubation, the cells were loaded with 1 μg/mL rhodamine 123 for 30 min at 37°C. The cells were later washed and observed using a fluorescent microscope (Nikon ECLIPSE TE2000-U). The average fluorescence was studied using ImageJ software and a histogram was plotted [47,48]. For determining the intracellular reactive oxygen species (ROS), $5 \times 10^5$ cells were allowed to adhere overnight, treated with the composites and incubated for 8 h. Later, the media was removed and 5 μM H2DCFDA was added and left for 30 min at 37°C. The cells were observed as described above for the MMP assay [49]. For determining the induction of apoptosis, HepG2 cells were treated with dual fluorescence staining solution (1 μL) containing 100 μg/mL AO and 100 μg/mL EB and observed after 20 min using a fluorescent microscope [50]. All these experiments were conducted in triplicate.

#### 2.5.2 Quantitative PCR based analysis of gene expressions

Total RNA was extracted with TRIzol reagent (Ambion, USA) at the IC₅₀ dose and PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA BIO INC) was used for cDNA synthesis. Furthermore, the reaction was continued at 42°C for 60 min followed by termination at 95°C for 5 min. SYBR® Select Master Mix (Applied Biosystems) was used in a Rotor-Gene Q 2PLEX HRM Real-Time PCR system (Qiagen) for the quantification of each gene using the $\Delta\Delta$CT method. The conditions applied for enzyme activation were followed as per previous reports with slight modifications. β-actin was used as the internal standard [51–53]. The list of primers used for analysing the expressions of every gene is enlisted in Table 1.
Table 1: List of primers used for qPCR

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Primers</th>
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<tbody>
<tr>
<td>Bax</td>
<td>FP: 5′ CCTGTGCACCAAGTGCCGAAT 3′&lt;br&gt; RP: 5′ CCACCCCTGGTCTTGGATCCAGCCC 3′&lt;br&gt;</td>
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2.6 Statistical analyses

The expression of apoptosis-related genes was analysed in triplicate and is presented as the mean ± SEM (P value < 0.05 was considered significant). Central composite design of response surface methodology (RSM) was employed for the study. The ideal responses for cell viability of HepG2 cells after being treated with the nanocomposites were determined using Design-Expert software Version 12 (State-Ease Inc., Minneapolis, MN, USA).

3 Results and discussion

3.1 Physicochemical characteristics of the nanocomposites

3.1.1 Surface, structure and morphology

UV–Vis spectra of CuO was observed at 305 nm which corresponds well to the peak range of 280–380 nm previously reported for CuO nanoparticles [54–56]. The peaks at 310 and 350 nm correspond well to the peak range reported for FeS nanoparticles [57,58]. Two peaks with very minor shift were observed at 335 and 370 nm for the composites. These two peaks relate to the peaks observed for CuO and FeS supporting the formation of the CuO–FeS blend. The minor shift in absorbance is commonly observed during the formation of nanocomposites [59–62] (Figure 1). The FTIR peaks at 517 and 603 cm⁻¹ are due to the existence of CuO. The peak at 3,432 cm⁻¹ is due to the O–H stretching and the peak at 2,963 cm⁻¹ is due to the C–H bonds. The peaks at 1,628 and 1,455 cm⁻¹ are due to the alkenes and C–H stretching, whereas the peaks at 1,377 and 1,162 cm⁻¹ are due to the pyrrole ring and vibrating C–N bonds [63]. Additionally, the peak at 3,388 cm⁻¹ is due to the n (N–H) vibrations, while the peak at 2,347 cm⁻¹ is possibly due to the sp stretching vibrations. The peaks at 1,095 and 618 cm⁻¹ are due to the S–O and Fe–O stretching vibrations [58,64,65]. A combination of these peaks is observed for the CuO–FeS composite. The FTIR peaks for CuO–FeS composites at 516, 577 and 619 cm⁻¹ are a combination of peaks characteristic for the Cu–O and Fe–S bonds. The multiple peaks at the range of 400–1,500 cm⁻¹ are due to the vibrations of the SO₄ group. Remarkably, the peaks at 1,636 and 3,435 cm⁻¹ signify the presence of O–H vibrations relating to the chemistry of pyrite surfaces at the nanoscale [63,66–68]. The surface properties of the composite displayed the characteristic features of CuO and FeS as exhibited by the FTIR analysis (Figure 2).

The XRD patterns of CuO, FeS and the CuO–FeS composite are depicted in Figure 3. The diffraction peaks at 2θ = 18.2°, 32.07°, 34.82°, 46.9° and 52.6° corresponds to (001), (101), (203), (206) and (220) monoclinic crystal phases of FeS [69–72]. Similarly, the peaks at 2θ = 32.53°, 37.58°, 48.12°, 50.40°, 62.41°, 70.69° and 72.24° correspond to (001), (111), (202), (200), (−113), (310) and (311) phases of CuO [37,73–78]. A combination of these peaks is observed for the composite. The crystal planes of the composite as analysed using SAED pattern is presented in Figure 4. The d-spacing values and Miller indices observed in this study correspond to lattice spacing of monoclinic CuO and FeS, respectively [79–83]. These results are consistent with the XRD observations which indicated a particle size of the composites to be 30.5 nm.

After the structure and surface characteristics were elucidated, the morphology was studied using TEM. Since TEM is generally used for analysing nanomaterials at diverse magnifications, it was adopted for studying the morphology of the prepared composites [84,85]. According to TEM, the materials had an average size of 110 nm. Furthermore, the morphology of the composites as evidenced by TEM is presented in Figure 5. The SEM observations are presented in Figure 6. The EDAX analysis indicated the elemental composition with peaks for Cu, O, Fe and S confirming the synthesis of CuO–FeS composite as elucidated in Figure 7. The strong peaks at 0.9, 8 and 9 keV signifies the presence of Cu, whereas the peak at 0.5 signifies the presence of O [86–88]. The peaks around 6–7 keV signifies the presence of Fe, whereas the peak around 2.5 keV signifies the presence of S [89,90]. This elucidates the formation of CuO–FeS composite with no impurities.
3.1.2 Particle size, PDI and zeta potential

To validate the prepared composite further, dynamic light scattering (DLS) was implemented to determine the particle size and PDI. This method is commonly adopted by a diverse network of interacting scientists as a valid method to determine particle characteristics at the nanoscale. It is key to recognize that these two measures are used to indicate the quality of the materials at the nanoscale [91,92]. As per the analysis, the mean particle size was 121.9 nm and the PDI was 0.150. Depending on the width of a hypothetical Gaussian distribution, nanomaterials in the PDI range of 0.1–0.4 are considered moderately polydisperse [93–95].

Since the composites had a mean size less than 200 nm, they are expected to flow out into the tumour microenvironment efficiently. Thus, the composites can be used better in tumour targeting as the vascular spaces of the tumour microenvironment have gaps of 400–600 nm. This is because nanomaterials of sizes less than 200 nm have been verified and found to permeate and amass into the tumour openings through enhanced permeability and retention effect [96–100]. In addition, the uptake of nanomaterials by cancer cells is dependent on their particle size [101]. In this regard, materials with sizes of approximately 100 nm were found to be more effective in being taken up by cancer cells in comparison to their shorter counterparts by clathrin-mediated endocytosis [102,103]. These results suggest that the prepared composites were predominantly monodisperse and are suitable for targeting cancer cells (Figure 8). As particles less than 150 nm can extravasate into the intratumoural

**Figure 1:** UV–Vis peaks of CuO, FeS and CuO-FeS composites.
environment efficiently, it is interesting to note that the absorption, distribution, metabolism, excretion and biodistribution of nanomaterials with sizes greater than 200 nm can change in vivo. These materials with sizes greater than 200 nm can accumulate systemically in major organs like spleen, lungs, liver and kidney over durations longer than usual. Therefore, particles with sizes around 100 nm are better candidates for drug delivery [104–107]. This is consistent with the particle sizes measured by using DLS (121.9 nm) TEM (110 nm) and XRD (30.5 nm) in this study.

For determination of the particle size of nanomaterials, DLS, XRD and TEM can be used and each technique has its individual advantages and disadvantages [108]. The particle sizes observed using TEM is generally greater than those observed using XRD [109,110]. This is consistent with the observations of our study. There was no considerable difference in the particle size of the composites as measured by DLS and TEM. The outcomes of DLS and TEM analyses can be used to efficiently determine particle aggregation. If the particle sizes of a material calculated using DLS and TEM remain similar or do not have much difference, the suspended particles are deemed to be free of aggregates [111]. The composites were therefore highly monodisperse, unaggregated and possessed sizes optimum for tumour-targeting. These are the properties largely expected of materials intended for use in nanomedicine [104,112]. Determination of particle size by TEM is dependent on the quantity of particles in a specific area as a measurement of individual geometric size; whereas, DLS-based determination focuses on the intensity of light scattered around the area under investigation and provides the average hydrodynamic size of the nanoparticles. In this regard, DLS is considered a more suitable method for materials used in solvents meant for biological assays [113,114].

Zeta potential is used to define the interfaces of the colloids and the electrolytes in addition to flocculation. The basic mechanism underlying this effect is that the counter ions are attracted, while the coions are believed to repel each other [115]. It is the electrokinetic potential of surface charge involving the electrical dual layer of particles with a

Figure 2: FTIR spectra of the CuO, FeS and CuO-FeS composites.
thickness of 0.2 nm and is measured in millivolts (mV) [116]. As per our study, the zeta potential of the composite was −29.2 mV (Figure 9). It is generally accepted that nanomaterials with a zeta potential in the range of −10 and +10 mV are considered neutral, whereas particles of potential less than −30 mV are believed to be strongly anionic and stable [93,117]. Additionally, charged and harder nanoparticles with metallic or magnetic behaviour are internalized and easily taken up into the cells. Once these nanomaterials enter the bloodstream and interact with the biomolecules, they can form an active protein corona based on the size, shape and charge of the materials [118]. Materials with such anionic corona are expected to exhibit greater levels of apoptosis [119].

3.2 Interactions of the composites with cancerous cells and the possible mechanisms

3.2.1 Cytotoxicity of the composites on HepG2 cells

In vitro cell lines have long been used for identifying the clinical outcome of an approach to predict the anticancer effects of a drug under trial. In this process of identifying new drug possibilities, cell lines form the basis for developing a drug by studying the associated mechanisms [120,121]. After exposing the HepG2 cells to the nanocomposites for 24 h, the cytotoxic effects were determined using the MTT assay. The MTT assay is a typical standard and the most common tool
used for recognizing cell viability and in turn the cytotoxic effects of preclinical-level drugs to be studied further [122,123]. As per the assay, the cell killing effects were too low at the initial dose of 25 μg/mL (99% viable) and with increasing concentrations, the effects began to progress. The mean percentage of viability was 12.91 ± 0.869 at the highest dose tested of

**Figure 4:** Diffraction patterns of the composites determined by SAED.

**Figure 5:** Morphological analysis of the composites using TEM.

**Figure 6:** Morphological analysis of the composites using SEM.
500 μg/mL (Figure 10). The cytotoxicity of the composites was therefore prominent with increasing concentrations and remained dose-dependent. In this regard, the dose or concentration that can result in reducing the cell growth to 50% in comparison to the control cells or simply the IC$_{50}$ was calculated to be 250 ± 5.7 μg/mL [124,125]. Although there are no existing reports on the cytotoxic profiling of CuO–FeS nanocomposites, the IC$_{50}$ of composites prepared based on the combination of CuO with other materials ranges between 6.5 and 169 μg/mL [126,127].

In addition, the IC$_{50}$ of the standard doxorubicin was 12.25 μg/mL or 23 μM (Figure 11). The IC$_{50}$ value was similar to those supported by previous studies and the cytotoxic effects of doxorubicin are usually observed to be higher than those of any drug under testing, such as nanomaterials [128]. The cytotoxic activities of other such tumour-targeting compounds have been identified to be 5–50 folds lower than that of doxorubicin on HepG2 cells [129].

RSM as a mathematical and statistical tool for optimizing several experiments including the optimization of cell viability has been previously reported [130]. It has also been reported to optimize the synthesis of nanoparticles with regard to size and other surface parameters [131–134]. The model $f$ value observed in the study (575.70) indicates that the possibility of this as a result of noise is
considerably inadequate (0.01%). The linear model is significant, as the $P$ value is <0.0001. The observed signal-to-noise ratio was 57.989. The predicted $R^2$ and adjusted $R^2$ values were 0.9894 and 0.9919, respectively [135,136]. Representing the outcomes of the RSM technique, the contour and response surface plots are presented in Figure 12.

3.2.2 Nanocomposites decreased MMP and triggered apoptotic cell death in HepG2 cells

MMP is a consistent and dependable indicator of the functioning of mitochondria. An MMP value in the range of −100 to −140 mV is considered optimum for ATP production by living cells. Alternatively, an MMP value of approximately −200 mV is observed in cancer cells owing to their higher metabolic needs. Alterations or decreases in MMP levels can lead to a decrease in cellular ATP levels. Mitochondria are therefore a key target in the induction of apoptosis. Therefore, therapeutic interventions for normalizing MMP levels can help in managing several disorders including cancer [137–140]. Additionally, changes in MMP can cause mitochondrial dysfunction and improper functioning of the cells and eventually lead to a decrease in the synthesis of ATP [141,142]. This reduction in cellular ATP can induce the killing of cancer cells as they display decreased glucose levels due to the Warburg effect [143,144]. Hence, cells with decrease in MMP can eventually undergo apoptosis by releasing cytochrome C from the mitochondria [145]. Accordingly, fluorescent dyes such as rhodamine 123 are used to detect MMP across cells as the rate of reduction in fluorescence is proportionate to MMP [146,147]. The decreased fluorescence after treatment with the composites at a dose of 250 µg/mL indicates that the MMP was decreased considerably in comparison to the control (Figure 13). This decrease in MMP can account for decreased ATP levels and subsequent glucose deprivation in HepG2 cells treated with the composites.

Since endocytosis is a key mechanism for the uptake and trafficking of nanomaterials by cancerous cells, they can enter specific organelles such as mitochondria after lysosomal escape resulting in apoptosis among such cells [148,149]. Since a decrease in MMP was evidenced, AO/EB staining was subsequently used to determine the change in morphology and apoptosis in the cancerous HepG2 cells [150–153]. In this study, the number of viable cells decreased significantly after treatment with the composites and amplification of apoptotic

Figure 11: Cytotoxic effects of doxorubicin on HepG2 cells.

Figure 12: Contour and response surface plots for cell viability.
characteristics was observed. The colour of the HepG2 cells changed from green to yellowish orange after treatment with composites, indicating early apoptosis [154–156]. These observations indicate that apoptosis was induced in HepG2 cells treated with the composites (Figure 14).

3.2.3 Nanocomposites induced ROS-independent apoptosis

The incubation of HepG2 cells with the composite decreased the cellular fluorescence in comparison to the positive control as per DCFDA staining. This indicates decreased production of ROS by the cancer cells after treatment with the composites (Figure 15). In general, nanomaterials produce ROS to promote cancer cell death and oxidative stress is a vital mechanism for this cytotoxic effect of metal-based nanomaterials [157–159]. However, the nanotoxicity profile of materials is significantly dependent on the overproduction of ROS leading to oxidative stress. This can lead to a consequent failure in the regular physiological redox-dependent functioning and progression to diseases like cancer. Therefore, the generation of ROS is considered to be an adverse effect of nanomaterial interaction with cells of multiple origins. Hence, redox imbalance is not preferred for therapeutic interventions of diseases and regulating ROS

Figure 13: Composite-mediated reduction in MMP as elucidated by cell fluorescence and quantitative mean fluorescence (*\(P < 0.01\)).

Figure 14: Decreased number of viable cells and the establishment of apoptotic characteristics in HepG2 cells by the composites as determined using AO-EB staining.
remains key for managing diseases like cancer. The production and after-effects of ROS is dependent on the particle size, shape, surface, dissolution, aggregation and the manner in which they interact with cells [160,161]. Hence, it is important to note that overproduction of ROS can possibly promote the nanotoxic effects of materials being tested for therapeutic benefits. A significant number of studies elucidate that apoptosis can be triggered regardless of the production of ROS as they are not the effectors of an apoptotic trigger. In such cases, loss of MMP and induction of apoptosis was observed although there was a decrease in production of intracellular ROS. In these ROS-independent mechanisms of cell death, ROS is primarily observed as a side-effect of apoptosis. Additionally, the adverse effects on normal cells remain minimal in such modes of cell death [162].

Loss of mitochondria membrane potential and limited production of ROS reveal that the cell death was caused with mitochondria as the organelle being targeted [163–165]. This ROS-independent mechanisms can reduce the unwanted side-effects on normal cells and result in enhanced uptake and targeting of the cancer cells which is usually expected in nano-oncotherapy [166,167]. ROS-independent apoptosis is not so widely studied or observed in cancer cells, especially in nanoparticle-mediated cancer therapy. It is therefore thought of as a highly valued therapeutic approach and the one to be appreciated more for its enhanced and specific targeting of the mitochondria and subsequently the tumour [168,169]. The results of investigating the intracellular ROS determine that the composites decreased the generation of ROS and specifically targeted the mitochondria which could be beneficial in minimizing the side effects of cancer therapy commonly observed while using nanomaterials. This can protect the normal cells from the outcomes of nonspecific targeting, a common consequence of nanomaterial usage.

3.2.4 Quantitative PCR based analysis of gene expressions

Since the cytotoxic effects of the composite were determined, the probable mechanisms of such effects have been elucidated in the following section. Among the methods for determining the mechanisms of cytotoxic effects, quantitative PCR is an easy, sensitive and reliable method to determine the differential expression of genes at the molecular levels [170–172]. In this type of quantification, upregulated expression of a target gene is determined by a positive fold change, whereas the downregulation of a gene expression is determined by a negative fold change [53]. This can help in identifying the biological markers for a disease and improve the therapeutic efficacy of a drug precisely [173]. Considering these advantages, the expression of six different genes related to apoptosis were analysed among the composite-treated and nontreated cells. The genes such as Bax, Bcl-2, CASP8, CASP9, CASP3 and p53 are classic components of apoptosis and were therefore studied [174].
First, caspases are endoproteases that can cause breaks in peptide bonds known to assist aspartic acid in initiation and execution of apoptosis. Even though the “isoleucine–glutamic acid–threonine–aspartic acid” is a target motif for CASP8, it can also cleave the “aspartic acid–glutamic acid–valine–aspartic acid” motif of CASP3 and aid in the suppression of malignant transformations through extrinsic apoptosis [175–178]. This may cause an apoptotic trigger based on ER-stress through the formation of a stressosome [179,180].

Additionally, Bcl-2 is an anti-apoptotic gene upregulated in several cancers which can antagonize the effects of pro-apoptotic Bax, leading to reduced release of cytochrome C. The upregulation of Bcl-2 can promote resistance among cancer cells. Since the Bcl-2 family is known to possess both pro-apoptotic and anti-apoptotic members, the upregulation of Bax, a pro-apoptotic domain and target of p53 can induce apoptosis in harmony. In addition, the increased expressions of CASP9 can cleave Bcl-2 and result in mitochondrial damage by the amplification of the caspase-cascade [181–186]. Since Bcl-2 was downregulated in the study, the role of CASP9 becomes significant.

In this study, the components of initiator caspases such as CASP8 and CASP9 along with the executioner caspase CASP3 were studied. The expressions of the executioner caspase CASP3 and the initiator caspases such as CASP8 and CASP9 were found to be upregulated [187]. Furthermore, the release of cytochrome C could be a critical constituent responsible for activation of an apoptotic trigger [188]. The involvement of caspase-cascade and cytochrome C release along with p53 (known to be a tumour suppressor involved in cell cycle arrest, senescence and apoptosis) being engaged can lead to intrinsic apoptosis. Eventually, the CASP3 release can trigger apoptosis as the final outcome. The upregulation of the caspase-cascade suggests that the probable mechanism of cell death induced by the composite was dependent on both intrinsic (upregulation of CASP9) and extrinsic (upregulation of CASP8) apoptosis. Generally, after being internalized into the cytosol, cytochrome C can combine with apoptosis activating factor-1 (ApaF-1) to form the apoptosome, leading to the activation of pro-caspase-9 and subsequent activation of CASP9. The so-formed CASP9 can activate CASP3 leading to the induction of apoptosis [189–191]. This confirms that the composite can activate ROS-independent mitochondrial apoptosis of HepG2 cells by involving both the extrinsic and intrinsic modes (Figure 16). The mechanism of cytotoxic effects of the composite is pictorially presented in Figure 17.

### 3.3 Safety testing and hemocompatibility of the nanocomposites

To test the safety of nanocomposites, normal 3T3-L1 fibroblasts with the ability to differentiate into white adipocytes were used [192,193]. Specific targeting of any drug under analysis to be used as an anticancer drug, is typically conducted on normal cell lines and compared with the effect on cancer cells [194,195]. It is widely accepted that drugs with the ability to target cancer cells precisely are not toxic.
to normal cells [196]. The outcomes of the study indicate that the composites were competent in targeting cancerous HepG2 cells in comparison to normal 3T3-L1 fibroblasts. This is based on the observation that the IC50 for toxic effects on normal cells was not reached at the maximum concentration tested (500 μg/mL). More than 95% of the cells were viable at the tested dose (Figure 18). Therefore, the outcomes indicate that the composites were cytotoxic to cancerous HepG2 cells and not toxic to normal 3T3-L1 cells. This signifies that the composites are safe for use in cancer therapy.

Measurement of hemocompatibility is an important tool for assessing the credential of an agent to be used in medicine and its application in vivo. The agent to be tested must not create any adverse effect on blood after being treated. No significant toxic effects such as thrombosis, haemolysis, platelet, leukocyte and complement activation or other blood-associated adverse effects should be observed after the composite (the test agent in this study) is being treated. In this regard, the analysis was performed using blood as an in vitro model for interactions with biological materials. Using an in vitro model has several advantages over using in vivo models. Well-controlled settings, such as
blood flow and anticoagulation in vitro, can eradicate factors such as flow obstruction, surgical interventions and effects related to the usage of tissues [197,198].

For systemic administration of drug candidates such as nanomaterials, hemocompatibility tests seem essential for analysing immunological outcomes [199]. This is because nanomaterials can interact directly with blood, cells and tissues and their physicochemical properties determine their hemocompatibility [200]. Relatedly, the composites showed no haemolytic effects even at a dose of 500 µg/mL, the dose at which the haemolysis ratio was only 2.03 ± 0.93 in comparison to the 100% lytic effect of Triton X 100 (10%) (Figure 19). Based on the outcomes of safety testing on normal cells and the hemocompatibility assay, the composites were deemed to possess excellent biocompatibility [201,202].

4 Conclusions

Novel CuO–FeS nanocomposites were prepared through a facile route and physicochemically characterized using standard techniques where the outcomes determine the formation of the CuO–FeS blend. Initially, the UV–Vis and FTIR analyses indicated stable peaks representing the optical and surface characteristics relating to the fabrication of the composite. The observations of SAED rings and XRD patterns were concurrent. The morphology was determined by TEM and SEM; whereas, the elemental composition was determined using EDAX and no impurities other than constituents of CuO–FeS were observed. The composites were stable, free of aggregates and effective in tumour targeting according to the determination of zeta potential and elucidation of particle size using DLS. These stable nanocomposites possessed cytotoxic effects on HepG2 cells by creating a loss in MMP and induction of mitochondrial apoptosis in a ROS-independent manner. The composites were not cytotoxic to normal 3T3-L1 cells and were not haemolytic at higher concentrations; therefore, they are extremely biocompatible. Further in vivo studies are warranted to determine the clinical efficacy of the composite in the management of cancer.

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