Enhanced Anticancer Performance of Eco-Friendly-Prepared Mo-ZnO/RGO Nanocomposites: Role of Oxidative Stress and Apoptosis

Maqusood Ahamed, Mohd Javed Akhtar, M.A. Majeed Khan, and Hisham A. Alhadlaq

ABSTRACT: ZnO nanoparticles (NPs) have attracted great attention in cancer therapy because of their novel and tailorable physicochemical features. Pure ZnO NPs, molybdenum (Mo)-doped ZnO NPs, and Mo-ZnO/reduced graphene oxide nanocomposites (Mo-ZnO/RGO NCs) were prepared using a facile, inexpensive, and eco-friendly approach using date palm (Phoenix dactylifera L.) fruit extract. Anticancer efficacy of green synthesized NPs/NCs was examined in two different cancer cells. The potential mechanism of the anticancer activity of green synthesized NPs/NCs was explored through oxidative stress and apoptosis. The syntheses of pure ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were confirmed by X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and photoluminescence (PL). Dynamic light scattering (DLS) study indicated the excellent colloidal stability of green prepared samples. Mo-ZnO/RGO NCs exhibited threefold higher anticancer activity in human colon (HCT116) and breast (MCF7) cancer cells as compared to pure ZnO NPs. The anticancer activity of Mo-ZnO/RGO NCs was mediated through reactive oxygen species, p53, and the caspase-3 pathway. Moreover, cytocompatibility of Mo-ZnO/RGO NCs in human normal colon epithelial (NCM460) and normal breast epithelial cells (MCF10A) was much better than those of pure ZnO NPs. Altogether, green stabilized Mo-ZnO/RGO NCs exhibited enhanced anticancer performance and improved cytocompatibility because of green mediated good synergism between ZnO, Mo, and RGO. This study suggested the high nutritional value fruit-based facile preparation of ZnO-based nanocomposites for cancer therapy.

1. INTRODUCTION

Cancer is among the leading causes of death globally, with more than 10 million new cancer cases each year. Current chemotherapy has several limitations including multiple drug resistance, nonspecific toxicity, and requirement of high doses. Hence, current research is now focused on a more effective and safe treatment of cancer. Nanostructured materials offer a potential alternative mode of cancer therapy. Currently, ZnO nanoparticles (NPs) have attracted great attention in cancer therapy because of their novel and tailorable physicochemical features. ZnO NPs exhibit intrinsic selective cytotoxicity toward cancerous cells with minimum effects on noncancerous normal cells. Ostrovsky observed that ZnO NPs had a cytotoxic effect on several human glioma cells and spare normal human astrocytes. Earlier, we also observed that ZnO NPs selectively kill human lung (A549) and liver (HepG2) cancer cells while little effects on primary rat astrocytes and hepatocytes. Antiproliferative activity of biosynthesized ZnO NPs against several murine cancer cells (WEHI-3B, CT-26, and CRL-1451) with no effects on normal mouse fibroblasts (3T3) was also observed by Namvar and co-workers. A recent study observed the anticancer effects of ZnO NPs against human small-cell lung cancer in an orthotopic mouse model.

However, there are some limitations that restrict the development of ZnO-based anticancer agents such as poor selectivity toward cancer cells. Earlier studies also reported the toxic potential of ZnO NPs in a number of organisms including bacteria, yeast marine organisms, zebra fish, and mice. Hence, further improving the anticancer performance and the biocompatibility of ZnO NPs is one of the current research efforts.

Graphene is a two-dimensional sheet of sp²-hybridized carbon atoms (hexagon honeycomb lattice) with exceptional mechanical, electrical, and optical features. Graphene derivatives, especially reduced graphene oxide (RGO) has been considered a revolutionary nanomaterial and shows crucial application prospects in energy, environment, and biomedicine. Hence, NCs of ZnO/RGO are expected to...
achieve enhanced anticancer performance. Additionally, noble-metal NPs are usually added into the ZnO/RGO NCs to further improve their anticancer efficacy. Molybdenum (Mo) is an essential trace metal that serves as a cofactor for several enzymes. Studies reported that a low soil content of Mo metal in a geographical region from northern China to Iran was associated with an increased rate of esophageal cancer. Anticancer activity of Mo has also been observed in recent studies. The motivation for opting Mo metal doping in ZnO/RGO NCs was due to its tunable properties, anticancer potential, and high biocompatibility.

Studies demonstrated that the anticancer efficacy of ZnO NPs could be enhanced by metal-ion doping and integration of graphene derivatives. Recently, Nagajyothi et al. observed that biosynthesized ZnO/Ag NCs exhibit superior anticancer activity in cervical (HeLa) and ovarian (SKOV-3) cancer cells. Our recent study also reported that ZnO/RGO NCs show greater anticancer efficacy against cancer cells. The anticancer activity of ZnO-based NCs was found to be mediated through oxidative stress and apoptosis pathways.

ZnO-based NCs can be developed by applying several physical and chemical methods such as ultrasonic spray pyrolysis, electrochemical, hydrothermal, magnetron sputtering, coprecipitation, and sol–gel methods. Physical methods are expensive and require high pressure, temperature, and energy. Chemical routes release toxic chemicals to the environment. Moreover, NPs/NCs synthesized using these physical and chemical methods are not appropriate for biomedical applications. New routes for efficient preparation of NPs/NCs by plant extracts are rapid, facile, cost-effective, and eco-friendly, as well as appropriate for biomedical applications.

In this study, Mo-ZnO/RGO NCs were prepared via green route utilizing date palm (Phoenix dactylifera L.) fruit extract. The objective was to integrate the beneficial properties of ZnO, Mo, and RGO in a composite form to enhanced anticancer performance along with improved biocompatibility. Saudi Arabia, one of the most important countries in date palm cultivation, has approximately 14% share of global date fruit production. Date palm fruit is a rich source of nutrients, bioactive phytochemicals, and fibers. Date palm fruits may provide health benefits when taken as a medicine or as a diet. In the present study, phenolic compounds and flavonoids of date fruit extract act as reducing and stabilizing agents for the green synthesis of Mo-ZnO/RGO NCs from zinc nitrate, sodium molybdate, and graphene oxide (GO).

Green synthesized ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were characterized by X-ray diffraction (XRD), field emission-transmission electron microscopy (FE-TEM), field emission-scanning electron microscopy (FE-SEM), energy-dispersive X-ray spectroscopy (EDS), photoluminescence (PL), and dynamic light scattering (DLS). Anticancer performance of green synthesized NPs/NCs was examined in human colorectal cancer (HCT116) and breast cancer (MCF7) cells. Possible mechanisms of the anticancer activity of pure ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were explored through oxidative stress and apoptosis. Additionally, cytocompatibility of these nanostructured materials was assessed in human normal colon epithelial (NCM460) normal breast epithelial (MCF10A) cells. These cell lines were selected in the present study because colon and breast cancers are the leading cause of cancer-related death worldwide.

2. RESULTS AND DISCUSSION

2.1. Mechanism of Green Synthesis of Mo-ZnO/RGO NCs. The bioactive phytochemicals (e.g., phenolic acids and flavonoids) present in the date fruit extract behave as ligands, and their specific potential to chelate metal ions increases the reduction and subsequent stabilization of the metal ions into NPs/NCs. The phenolic compounds readily chelate metal ions to form stable complexes because of their ability to donate electrons and hydrogen atoms. Zn(NO₃)₂ and Na₂MoO₄ dissolved in date fruit extract release free-moving Zn²⁺ and Co³⁺ ions that attack the active sites of bioactive phenolic compounds present in the extract to gain stability, hence, reducing into nano complexes. The heat treatment of nano complexes releases the Mo-ZnO/RGO NCs. The abundant number of -OH functional groups of phenolic compounds plays a major role in the reduction process. GO also reduced to Mo-ZnO/RGO NCs. The schematic of the green preparation of Mo-ZnO/RGO NCs (Figure 1A) and the possible mechanism of green synthesis (Figure 1B) are provided.

Figure 1. (A) Schematic of the green preparation of Mo-ZnO/RGO NCs. (B) Possible mechanism of the green synthesis of Mo-ZnO/RGO NCs.
2.2. XRD Study. The XRD spectra of green prepared pure ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs are presented in Figure 2A. The sharp diffraction peaks corresponding to the crystal planes (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202) of Mo-ZnO/RGO NCs indicate the formation of the single-phase hexagonal wurtzite structure of ZnO according to JCPDS card number 36-1451. The addition of Mo and RGO did not modify the original crystal structure of ZnO. The absence of the RGO peak in Mo-ZnO/RGO NCs could be due to the low intensity of RGO peaks as well as the uniform distribution of ZnO NPs on RGO sheets. No diffraction peaks of Mo in Mo-ZnO NPs and Mo-ZnO/RGO NCs suggested homogeneous mixing of Mo throughout the ZnO lattice. In this case, the ionic radius of Mo\(^{6+}\) ions (0.062 nm) is smaller than the ionic radius of Zn\(^{2+}\) ions (0.074 nm). Hence, Mo\(^{6+}\) ions can readily penetrate the ZnO crystal lattice without affecting the crystal structure of ZnO.\(^{31,43}\) In addition, sharp diffraction peaks of XRD spectra indicate the high crystallinity of the prepared NPs and NCs. Slight shifting of the XRD peaks (100, 002, and 101), in comparison to pure ZnO, indicated the successful incorporation of Mo\(^{6+}\) ions in the ZnO crystal lattice in Mo-ZnO NPs and Mo-ZnO/RGO NCs (Figure 2B). The crystallite sizes of the green prepared samples were estimated corresponding to the prominent peak (101) by Scherrer’s equation.\(^{44}\) The average particle sizes of pure ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were 23, 19, and 14 nm, respectively. The reduction in the particle size after metal-ion doping and RGO integration was also reported by other investigators.\(^{22,45}\)

2.3. TEM Study. Structural characterization of the green prepared nanoscale materials was further carried out by field emission-transmission electron microscopy (FETEM). Figure 3A–C represents the low-magnification images of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. The shape of ZnO NPs was nearly spherical with a size of 22 nm. It was observed that the shape of ZnO NPs remains the same, but the particle size decreases, following Mo-doping (17 nm) and anchoring on RGO (13 nm). Moreover, Mo-doped ZnO NPs were strongly anchored on RGO sheets and act as spacers to avoid restacking of RGO sheets and increase the surface area. The lower particle size and the higher surface area of NPs/NCs are associated with increased biological activity.\(^{46,47}\) Figure 3D–F shows the high-resolution TEM images of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs, respectively. These pictures depict that particles were highly crystalline and confirm the synergism of ZnO, Mo, and RGO with good-quality lattice fringes without defects, which is important in composite materials for potential biomedical applications. The measured interplanar spacings of adjacent lattice fringes of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were 0.273, 0.267, and 0.258 nm, respectively, that corresponds to the (002) plane of the hexagonal wurtzite structure of ZnO.\(^{48}\) The lattice fringes were according to the XRD results.

2.4. SEM Study. FESEM images displayed the smooth surface morphology of ZnO NPs, and Mo-doping and RGO integration did not change its morphology (Figures 4A–C).
Mo-ZnO/RGO NC micrograph (Figure 4C) indicated that Mo-doped ZnO NPs were strongly anchored on RGO sheets, supporting FETEM results. EDS analysis showed the presence of Zn, Mo, O, and C elements in Mo-ZnO/RGO NCs (Figure 4D). Elemental mapping of Mo-ZnO/RGO NCs further supports the homogeneous distribution of Zn, Mo, O, and C elements (Figure 5).

2.5. PL Study. Figure 6 exhibits the PL spectra of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs obtained with an excitation wavelength of 300 nm. The PL spectra of the prepared samples show three main peaks at 340, 380, and 466 nm.49,50 We observed that the PL emission intensity of pure ZnO NPs was higher than those of Mo-ZnO NPs and Mo-ZnO/RGO NCs. A decrease in the emission intensity is allied with the hindrance of the recombination of charge carriers (e−/h+), caused geometric distortions, and created more oxygen vacancies. The lowest PL intensity was found in Mo-ZnO/RGO NCs, which was expected, and it defines the reduction of the recombination of charge carriers. The low PL intensity suggests the high separation rate of charges, which might induce intracellular ROS generation. Increased ROS generation favors the enhanced anticancer activity.51

2.6. DLS and Zeta Potential Measurement. Measurements of particle size distribution, surface charge, and colloidal stability of the aqueous suspension of nanoscale materials are indispensable to understand their interactions with biological/
cellular systems. In this study, the hydrodynamic size and zeta potential of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were examined in deionized water and different culture media. Results showed that the particle size in aqueous suspension (hydrodynamic size) in deionized water and culture media were 3–5 times higher as compared to the particle size of nanopowders calculated from XRD and TEM (Table 1). This could be due to the agglomeration of nanoscale materials in the aqueous state and also reported in earlier studies. Zeta potential results showed that the surface charge of particles in the aqueous suspension of deionized water and different culture media ranged from 21 to 29 mV. These values suggested that the aqueous suspension of green prepared ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs was fairly stable. Previous studies suggested that a higher value (±30 mV) of zeta potential (either positive or negative) was associated with the greater stability of aqueous suspension. In addition, the positive surface charge of NPs and NCs in deionized water and culture media favors their interaction with negatively charged cancer cells.

The anticancer performance study revealed that ZnO NPs have inherent potential of anticancer activity that can be further improved by tailoring their physicochemical properties. In the present study, we attempted to improve the anticancer performance of ZnO NPs by Mo-doping and RGO integration via a facile green method. The anticancer activity in colorectal cancer (HCT116) cells was examined, following exposure for 24 h to different concentrations (1–200 μg/mL) of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. MTT cell viability results indicated that all three samples induce dose-dependent cytotoxicity in the dosage range of 5–200 μg/mL (Figure 7A). Moreover, the anticancer performance of Mo-ZnO/RGO NCs was three times higher than that of pure ZnO NPs. The IC50 values for ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were 45, 30, and 14 μg/mL, respectively (Table 2). The neutral red uptake (NRU) results on the cytotoxicity of these samples in HCT116 cells were in agreement with the 3-(4,5-

Table 1. DLS Study of Green Prepared Nanoscale Materials (n = 3)

| parameters        | pure ZnO NPs | Mo-ZnO NPs | Mo-ZnO/RGO NCs |
|-------------------|--------------|------------|----------------|
| hydrodynamic size (nm) |              |            |                |
| deionized water   | 66.4 ± 3.5   | 53.6 ± 4.6 | 37.3 ± 2.3     |
| DMEM              | 75.5 ± 4.2   | 64.1 ± 5.3 | 51.4 ± 3.7     |
| McCoy’s 5A growth medium | 73.6 ± 3.9 | 62.3 ± 2.9 | 50.9 ± 5.5     |
| M3 medium         | 76.6 ± 5.7   | 59.7 ± 3.2 | 48.4 ± 2.1     |
| MEGM              | 71.8 ± 3.7   | 55.4 ± 3.5 | 47.4 ± 2.5     |
| zeta potential (mV) |              |            |                |
| deionized water   | 25.5 ± 1.8   | 24.4 ± 1.3 | 29.3 ± 1.5     |
| DMEM              | 23.5 ± 2.1   | 25.6 ± 1.6 | 27.5 ± 1.8     |
| McCoy’s 5A growth medium | 22.5 ± 1.2 | 24.4 ± 1.8 | 25.1 ± 1.7     |
| M3 medium         | 21.3 ± 1.7   | 23.7 ± 1.6 | 24.5 ± 2.5     |
| MEGM              | 24.6 ± 1.4   | 25.3 ± 2.3 | 27.3 ± 2.5     |

Figure 7. Anticancer activity of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs in human colorectal cancer (HCT116) cells. (A) MTT assay. (B) NRU assay. Quantitative data were presented as mean ± SD of three independent experiments (n = 3). *p < 0.05 NPs/NCs vs control. #p < 0.05 ZnO NPs vs Mo-ZnO/RGO NCs. (C) Cell morphology following exposure for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. Cell images were captured at 10× magnification.
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) data (Figure 7B). The cell morphology following exposure for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs is presented in Figure 7C. This picture depicted a significant number of cell death, following the treatment of prepared nanoscale samples. In agreement with MTT and NRU results, cell death caused by Mo-ZnO/RGO NCs was higher than that caused by pure ZnO NPs. In this study, higher anticancer performance of Mo-ZnO/RGO NCs could be due to green mediated (bioactive phytochemicals) good synergism between three functional materials, ZnO, Mo, and RGO. Additionally, glucans such as (1−3)-β-D-glucans found in date fruits are potent anticancer agents.57,58 Eid et al. reported that date fruit extracts inhibit the proliferation of colon cancer (Caco-2) cells.59 Hence, date fruit extract-mediated synthesis of Mo-ZnO/RGO NCs suggested their potential to act as chemotherapeutic drugs.

2.8. Apoptosis Response. ZnO NPs are known to induce apoptosis by the activation of several genes along with mitochondrial membrane potential (MMP) loss.60,61 In this study, effects of green prepared nanoscale materials were examined on the regulation of several apoptotic genes (p53, bax, bcl-2, and casp-3) and the MMP level in HCT116 cells. Results showed that ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs altered the expression of the mRNA level of these apoptotic genes. Tumor suppressor gene p53 and proapoptotic gene bax were upregulated, while the antiapoptotic gene bcl-2 was downregulated after exposure to green prepared samples. Apoptotic gene casp-3 was also upregulated upon exposure to ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs (Figure 8A). Moreover, the effect of Mo-ZnO/RGO NCs on apoptotic genes was significantly greater than that of pure ZnO NPs. To support mRNA data, the activity of caspase-3 enzymes was also examined in HCT116 cells, following exposure for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. The results showed that all three types of nanoscale materials induced the activity of caspase-3 enzymes, and the effect of Mo-ZnO/RGO NCs was significantly higher than that of pure ZnO NPs (Figure 8B).

MMP loss is a vital and an initial indicator of apoptosis.58 A recent study demonstrated that ZnO NPs induced apoptosis in melanoma cells (A375) through caspase activation and MMP depletion.62 Wang et al. also observed that ZnO NP-induced apoptosis in gingival squamous cell carcinoma was mediated...
through the mitochondrial pathway. In this study, both quantitative (Figure 8C) and fluorescence microscopy (Figure 8D) studies suggested that ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs depleted the MMP level of HCT116 cells. Moreover, MMP depletion caused by Mo-ZnO/RGO NCs was significantly higher as compared to pure ZnO NPs.

2.9. Oxidative Stress Response. Pro-oxidant generation and antioxidant diminution are considered as one of the mechanistic approaches of ZnO NP-mediated anticancer activity. Higher production of intracellular ROS leads to the reduction of antioxidant molecules (e.g., GSH) and enzymes that ultimately cause oxidative damage of cell macromolecules. In the present study, colorectal cancer HCT116 cells were exposed for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs, and oxidative stress markers were assessed by measuring the levels of ROS, H$_2$O$_2$, GSH, and GPx. Figure 9 showed that pro-oxidant levels (ROS and H$_2$O$_2$) were significantly higher while antioxidants

Figure 9. Oxidative stress response of human colorectal cancer (HCT116) cells, following exposure for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. (A) Intracellular ROS level. (B) Intracellular H$_2$O$_2$ level. (C) GSH level. (D) GPx enzyme activity. Data are presented as mean ± SD of three independent experiments (n = 3). *p < 0.05 NPs/NCs vs control. †p < 0.05 ZnO NPs vs Mo-ZnO/RGO NCs.

Figure 10. Role of oxidative stress in the anticancer activity of green prepared nanoscale materials. HCT116 cells were exposed for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs in the presence or absence of NAC (ROS scavenger). (A) ROS level in the presence or absence of NAC. (B) Cytotoxicity in the presence or absence of NAC. Data were presented as mean ± SD of three independent experiments (n = 3). *p < 0.05 NPs/NCs vs control. †p < 0.05 preventive effects of NAC against the ROS generation and cytotoxicity of NPs/NCs.
(GSH level and GPx enzyme activity) were significantly lower in ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NC-treated cells as compared to the control. Moreover, effects of Mo-ZnO/RGO NCs on oxidative stress markers were significantly higher than that of pure ZnO NPs.

To further confirm the role of ROS in anticancer activity, HCT116 cancer cells were exposed for 24 h to 25 μg/mL of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs in the presence or absence of N-acetyl-cysteine (NACs) (ROS scavenger). Results indicated that NAC significantly abrogated the ROS induction and cytotoxicity caused by ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs (Figure 10A, B). These results further supported that the anticancer activity of green prepared ZnO-based NCs was mediated through oxidative stress.

**2.10. Cytocompatibility.** It is crucial for an anticancer agent to have minimum or no toxicity toward normal cells. Hence, the toxicity of green prepared ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs was further examined in human normal colon epithelial cells (NCM460). Cells were exposed for 24 h to various concentrations (1–200 μg/mL) of green synthesized ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs, and cytotoxicity was examined by the MTT assay. Figure 11A indicates that ZnO NPs and Mo-ZnO NPs were cytocompatible up to 50 μg/mL. However, they were cytotoxic at 100 and 200 μg/mL. However, Mo-ZnO/RGO NCs were cytocompatible even at higher concentrations (up to 200 μg/mL). Figure 11B further indicates that ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs at a concentration of 25 μg/mL for 24 h did induce ROS generation in NCM460 cells. These data suggested that green prepared Mo-ZnO/RGO NCs display enhanced anticancer activity against colorectal cancer (HCT116) cells along with improved cytocompatibility in its normal counterparts (NCM460).

**2.11. Anticancer Performance of Green Prepared Samples in Human Breast Cancer Cells and Their Normal Counterparts.** To avoid cell-type specific response, the anticancer efficacy of ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs was further examined in breast cancer cells (MCF7) and their normal counterparts (MCF10A). Figure 12A demonstrates that all three samples induce dose-dependent cytotoxicity to MCF7 cancer cells. The IC$_{50}$ values for ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs were 45, 33, and 15 μg/mL, respectively (Table 2). Moreover, the anticancer performance of Mo-ZnO/RGO NCs in MCF7 cells was three times higher than that of ZnO NPs. Further study demonstrated that ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs induced ROS generation (Figure 12B), suggesting ROS-mediated process in anticancer activity in MCF7 cells. Altogether, the anticancer performance of green prepared ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs in MCF7 cancer cells was in agreement with HCT116 cancer cell data.

Effects of green prepared samples were also studied in normal breast epithelial cells (MCF10A). Figure 12C shows that ZnO NPs and Mo-ZnO NPs were cytocompatible up to 50 μg/mL. However, ZnO NPs and Mo-ZnO NPs generated slight toxicity at 100 and 200 μg/mL. Nonetheless, Mo-ZnO/RGO NCs did not induce cytotoxicity in all selected concentrations (1–200 μg/mL). Figure 12D further confirms that ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs at a concentration of 25 μg/mL for 24 h did induce ROS generation in MCF10A cells. Altogether, Mo-ZnO/RGO NCs selectively induce cytotoxicity in cancer cells via ROS generation while sparing the normal counterparts. The possible mechanism of the anticancer activity of Mo-ZnO/RGONCs is depicted in Figure 13.

**3. CONCLUSIONS**

*Phoenix dactylifera* L. fruits are readily available, and their fruit extract was applied for simple, cost-effective, and eco-friendly preparation of Mo-ZnO/RGO NCs. The XRD, TEM, SEM, and EDS studies confirmed the formation of Mo-ZnO/RGO NCs. Bioactivity study demonstrated that Mo-ZnO/RGO NCs exhibit threefold higher anticancer efficacy toward human colorectal cancer (HCT116) and breast cancer (MCF7) cells in comparison to pure ZnO NPs. Greater anticancer performance of Mo-ZnO/RGO NCs could be due to green mediated good synergism between three functional materials, M, ZnO, and RGO. The anticancer activity of Mo-ZnO/RGO NCs was found to be mediated through oxidative stress via the p53 and caspase-3 pathway. Additionally, Mo-ZnO/RGO NCs displayed greater cytocompatibility in human normal colon epithelial (NCM460) and breast epithelial (MCF10A) cells than those of pure ZnO NPs. Altogether, *Phoenix dactylifera* L. fruit extract-stabilized Mo-ZnO/RGO NCs could be a potential candidate in cancer therapy. This novel approach warranted further study on the antitumor activity of green prepared Mo-ZnO/RGO in suitable animal models.
4. MATERIALS AND METHODS

4.1. Preparation of Date Palm Fruit Extract. Ripe, soft, and fleshy ajwa date fruits that have basal white lines on black exocarp were purchased from a local market of Riyadh, Saudi Arabia. After multiple washing with deionized water, the edible part of date fruits was manually removed, dried in a food drier, and ground into powder using a grinder. Extraction was performed using the maceration method. Briefly, 10 g of date fruit powder was placed in a glass container with 500 mL of deionized water and magnetically stirred for 48 h. Once macerated, each mixture was maintained at 60 °C for 2 h in a water bath (Cole-Parmer, Vernon Hills, IL, USA). Lastly, the mixture was filtered (0.2 μm pore size filter paper), and the extract was stored at 4 °C for later application.

4.2. Synthesis of Pure ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs. Date fruit extract, zinc nitrate hexahydrate (Zn(NO₃)₂·6H₂O) (Millipore-Sigma, St Louis, MO, Figure 12. Anticancer performance of green prepared ZnO NPs, Mo-ZnO NPs, and Mo-ZnO/RGO NCs in breast cancer (MCF7) cells and its normal counterparts (MCF10A). (A) MTT cytotoxicity assay in MCF7 cells following exposure for 24 h to different concentrations of the green prepared samples. (B) ROS generation in MCF7 cells following exposure for 24 h to 25 μg/mL of green prepared samples. (C) MTT cytotoxicity assay in MCF10A cells following exposure for 24 h to different concentrations of the prepared samples. (D) ROS generation in MCF10A cells following exposure for 24 h to 25 μg/mL of the prepared samples. Data were presented as mean ± SD of three independent experiments (n = 3). *p < 0.05 NPs/NCs vs control. #p < 0.05 ZnO NPs vs Mo-ZnO/RGO NCs.

Figure 13. Possible mechanisms of the anticancer activity of Mo-ZnO/RGO NCs.

Green Prepared Mo-ZnO/RGO NCs

Exposure to cells

Cancer cells

MMP

Apoptotic genes

Pro-oxidants

Antioxidants

Cell death via apoptosis

Normal cells

Oxidative stress

Genes expressions

MMP level

Higher or upregulation

Lower or downregulation

Balanced level

No cell death

https://doi.org/10.1021/acsomega.1c06789
ACS Omega 2022, 7, 7103−7115
USA), sodium molybdate dehydrate (Na₂MoO₄·2H₂O) (Millipore-Sigma), and GO (Millipore-Sigma) were utilized as starting materials for the green synthesis of pure ZnO NPs, Mo–ZnO NPs, and Mo–ZnO/RGO NCs. The synthesis medium was deionized water. Zinc nitrate (2.5 g), 0.1 g of sodium molybdate, and 0.1 g of GO were added into 50 mL of date fruit extract. The mixture was stirred for 2 h at room temperature and then placed in a water bath at 60 °C for 2 h. The mixture was now dried at 120 °C for 2 h to obtain the precipitate of the nanocomplex. The precipitate was washed multiple times with deionized water and heat-treated at 500 °C for 1 h in air. Finally, the precipitate was ground to fine powder of NCs. Mo-doped ZnO NPs were synthesized using a similar method without mixing GO. Pure ZnO NPs were also prepared using the same protocol without the addition of sodium molybdate and GO in the reaction mixture. Figure 1A represents the schematic diagram of Mo–ZnO/RGO NC green preparation.

4.3. Characterization. The XRD (PANalytic X’Pert Pro, Malvern Instruments, UK) instrument equipped with Cu-Kα radiation (λ = 0.15405 nm, at 45 kV and 40 mA) was applied to analyze the phase purity and the crystallinity of green synthesized NPs and NCs. Shape, size, and structural characterization was further examined by FETEM (JEM-2100, JEOL, Inc., Tokyo, Japan). The surface morphology, elemental composition, and mapping were performed by field emission-scanning electron microscopy (FESEM, JSM-7600F, JEOL, Inc.). PL spectra were recorded using a fluorescence spectrophotometer (Hitachi F-4600, Tokyo, Japan). DLS (ZetaSizer Nano-ZS, Malvern Panalytical, UK) was carried out to examine the aqueous properties (zeta potential and hydrodynamic size) of NPs and NCs.

4.4. Cell Lines and Their Culture. Human colorectal cancer (HCT116), breast cancer (MCF7), and normal breast epithelial (MCF10A) cell lines were bought from American Type Culture Collection (ATCC, Virginia USA). The human normal colon epithelial (NCM460) cell line was purchased from INCELL Corporation LLC (San Antonio, Texas, USA). HCT116 cells were grown in McCoy’s 5A growth medium with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin). NCM460 cells were grown in INCELL’s enriched M3 medium with 10% FBS and antibiotics. MCF7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS and antibiotics. MCF10A cells were grown in a mammary epithelial cell growth medium (MEGM) kit (Lonza Group Ltd) and 10 ng/mL cholaer toxin (Millipore-Sigma). Cells were maintained at 37 °C with 5% CO₂ supply in a humidified CO₂ incubator (Heracell 150i, Thermo Fisher Scientific, Waltham, MA, USA).

4.5. Preparation of Stock Suspension and Treatment to Cells. The stock suspension (1 mg/mL) of ZnO NPs, Mo–ZnO NPs, and Mo–ZnO/RGO NCs was prepared in deionized water. Working concentrations (1–200 μg/mL) were diluted in a culture medium. First, cells were treated for 24 h with various concentrations (1–200 μg/mL) of NPs and NCs for a dose-dependent anticancer performance assay. Based on these results, we have chosen one moderate concentration (25 μg/mL) of each nanoscale material to delineate potential mechanisms of anticancer activity. Some experiments were performed in the presence or absence of NAC (2 mM), following the exposure of NPs and NCs. Cells devoid of nanoscale materials were designated as the control in each experiment.

4.6. Biochemical Parameters. The anticancer activity of green prepared ZnO NPs, Mo–ZnO NPs, and Mo–ZnO/RGO NCs was examined by MTT and NRU assays with a few specific changes. The cellular morphology of the control and exposed cells was examined using an inverted phase-contrast microscope (Leica Microsystems, GmbH, Germany). The expression of several of apoptotic genes (p53, bax, bcl-2, and casp-3) at the transcriptional level was assessed by real-time polymerase chain reaction (PCR) (ABI PRISM, 7900HT Sequence Detection System) (Applied Biosystems, Foster city, CA, USA) as per the protocol described previously. Fluorometric assay of the caspase-3 enzyme was determined utilizing 7-amido-4-trifluoromethylcoumarin (AFC) standard. Red–orange cationic fluorescent dye tetramethylrhodamine methyl ester, perchlorate (TMRM) (Thermo Fisher Scientific), is rapidly taken up by the mitochondria in a potential-dependent manner. The TMRM probe was applied to assess the MMP level in control and treated cells. In brief, 20,000 cells/well were seeded in a 96-well plate and allowed for 24 h to attach to the surface. Then, cells were treated for 24 h to NPs and NCs. After the completion of exposure time, cells were washed twice with phosphate buffer saline (PBS). Cells were further exposed with 100 μM of the TMRM dye for 30 min at 37 °C in the dark. Cells were washed with PBS, and the fluorescent intensity of TMRM was quantified using a microplate reader (excitation/emission wavelength: 548/574 nm) (Synergy-HT, BioTek, Winooski, VT, USA). A parallel set of experiments in the 24-well plate (1 × 10⁵ cells/well) was also prepared, as reported above. Then, the intracellular brightness of TMRM was captured using a DMi8 fluorescence microscope (Leica Microsystems) using a green excitation filter (detecting red–orange TMRM emission). Intracellular generation of ROS in control and treated cells was examined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Millipore-Sigma). The intracellular level of hydrogen peroxide (H₂O₂) was determined using a fluorometric assay kit (Millipore-Sigma). Ellman’s protocol was applied to measure the intracellular level of glutathione (GSH). The assay of the glutathione peroxidase (GPx) enzyme was performed using the protocol of Rotruck and co-workers. The protein level was measured by applying the method of Bradford.

4.7. Statistical Analysis. Biological activity data were assessed by one-way analysis of variance (ANOVA) followed by Dennett’s multiple comparison tests. p < 0.05 was ascribed as statistically significant.

■ AUTHOR INFORMATION

Corresponding Author

Maqsood Ahamed — King Abdullah Institute for Nanotechnology, King Saud University, Riyadh 11451, Saudi Arabia; orcid.org/0000-0001-6025-1950; Email: mahamed@ksu.edu.sa

Authors

Mohd Javed Akhtar — King Abdullah Institute for Nanotechnology, King Saud University, Riyadh 11451, Saudi Arabia; orcid.org/0000-0002-9596-7745

M.A. Majeed Khan — King Abdullah Institute for Nanotechnology, King Saud University, Riyadh 11451, Saudi Arabia; orcid.org/0000-0003-1287-8155

Hisham A. Alhaddaq — King Abdullah Institute for Nanotechnology and Department of Physics and Astronomy,
Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c06789

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This work was supported by the National Plan for Science, Technology, and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, under Award 13-NAN908-02.

REFERENCES

(1) Wiesmann, N.; Tremel, W.; Brieger, J. Zinc Oxide Nanoparticles for Therapeutic Purposes in Cancer Medicine. J. Mater. Chem. B 2020, 8, 4973–4989.

(2) Yang, Z.; Ma, Y.; Zhao, H.; Yuan, Y.; Kim, B. Y. S. Nanotechnology Platforms for Cancer Immunotherapy. Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol. 2020, 12, e1590.

(3) Yan, L.; Shen, J.; Wang, J.; Yang, X.; Dong, S.; Lu, S. Nanoparticle-Based Drug Delivery System: A Patient-Friendly Chemotherapy for Oncology. Dose-Response 2020, 18, No. 155932582093616.

(4) Wicki, A.; Witzigmann, D.; Balasubramanian, V.; Huwyler, J. Nanomedicine in Cancer Therapy: Challenges, Opportunities, and Clinical Applications. J. Controlled Release 2015, 200, 138–157.

(5) Ostrovsky, S.; Kazimirsky, G.; Gedanken, A.; Brodie, C. Selective Cytotoxic Effect of ZnO Nanoparticles on Glioma Cells. Nano Res. 2009, 2, 882–890.

(6) Akhtar, M. M.; Akhtar, M. J.; Khan, M. M.; Ahmad, J.; Alrokayan, S. A. Zinc Oxide Nanoparticles Selectively Induce Apoptosis in Human Cancer Cells through Reactive Oxygen Species. Int. J. Nanomed. 2012, 7, 845–857.

(7) Namvar, F.; Rahman, H. S.; Mohamad, R.; Azizi, S.; Tahir, P. M.; Charlton, M. S.; Yeap, S. K. Cytotoxic Effects of Biosynthesized Zinc Oxide Nanoparticles on Murine Cell Lines. eCAM 2015, 2015, 1.

(8) Tanino, R.; Amano, Y.; Tog, X.; Sun, R.; Tsubata, Y.; Harada, M.; Fujita, Y.; Isobe, T. Anticancer Activity of ZnO Nanoparticles against Human Small-Cell Lung Cancer in an Orthotopic Mouse Model. Mol. Cancer Ther. 2020, 19, 502–512.

(9) Wang, D.; Li, H.; Liu, Z.; Zhou, J.; Zhang, T. Acute Toxicological Effects of Zinc Oxide Nanoparticles in Mice after Intratracheal Instillation. Int. J. Occup. Environ. Health 2017, 23, 11–19.

(10) Verma, S. K.; Panda, P. K.; Jha, E.; Suar, M.; Parshar, S. K. S. Altered Physicochemical Properties in Industrially Synthesized ZnO Nanoparticles Regulate Oxidative Stress; Induce in Vivo Cytotoxicity in Embryonic Zebrafish by Apoptosis. Sci. Rep. 2017, 7, 13909.

(11) Vimercat, L.; Cavone, D.; Caputi, A.; De Maria, L.; Tria, M.; Prato, E.; Ferri, G. M. Nanoparticles: An Experimental Study of Zinc Nanoparticles Toxicity on Marine Crustaceans. General Overview on the Health Implications in Humans. Front. Public Health 2020, 8, 192.

(12) Sruhti, S.; Ashanti, J.; Mohanan, P. V. Biomedical Application and Hidden Toxicity of Zinc Oxide Nanoparticles. Mater. Today Chem. 2018, 10, 175–186.

(13) Novoselov, K. S.; Fal’Ko, V. I.; Colombo, L.; Gellert, P. R.; Schwab, M. G.; Kim, K. A Roadmap for Graphene. Nature 2012, 490, 192–200.

(14) Garaj, S.; Hubbard, W.; Reina, A.; Kong, J.; Branton, D.; Golovchenko, J. A. Graphene as a Subnanometre Trans-Electrode Membrane. Nature 2010, 467, 190–193.

(15) Yang, K.; Feng, L.; Shi, X.; Liu, Z. Nano-Graphene in Biomedicine: Theranostic Applications. Chem. Soc. Rev. 2013, 42, 530–547.

(16) Campbell, E.; Hasan, M. T.; Pho, C.; Callaghan, K.; Akkaraju, G. R.; Naumov, A. V. Graphene Oxide as a Multifunctional Platform for Intracellular Delivery, Imaging, and Cancer Sensing. Sci. Rep. 2019, 9, 416.

(17) Indrakumar, J.; Korrapati, P. S. Steering Efficacy of Nano Molybdenum Towards Cancer: Mechanism of Action. Biol. Trace Elem. Res. 2019, 194, 121–134.

(18) Odularu, A. T.; Ajibade, P. A.; Mbese, J. Z. Impact of Molybdenum Compounds as Anticancer Agents. Bioinorg. Chem. Appl. 2019, No. 6416198.

(19) Dar, N. A.; Mir, M. M.; Salam, I.; Malik, M. A.; Gullar, G. M.; Yato, G. N.; Ahmad, A.; Shah, A. Association between Copper Excess, Zinc Deficiency, and TP53 Mutations in Eosinophilic Squamous Cell Carcinoma from Kashmir Valley, India – A High Risk Area. Nutr. Cancer 2008, 60, 585–591.

(20) Liu, Y.; Peng, J.; Wang, S.; Xu, M.; Gao, M.; Xia, T.; Weng, J.; Xu, A.; Liu, S. Molybdenum Disulphide/Graphene Oxide Nanocomposites Show Favorable Lung Targeting and Enhanced Drug Loading/Tumor-Killing Efficacy with Improved Biocompatibility. NPG Asia Mater. 2018, 10, e458–e458.

(21) Nagajothy, P. C.; Muthuraman, P.; Tettey, C. O.; Yoo, K.; Shim, J. In Vitro Anticancer Activity of Eco-Friendly Synthesized ZnO/Ag Nanocomposites. Ceram. Int. 2021, 47, 34930–34948.

(22) Ahamed, M.; Javed Akhtar, M.; Majeed Khan, M. M.; Alhadaq, H. A. Facile Green Synthesis of ZnO-RGO Nanocomposites with Enhanced Anticancer Efficacy. Methods 2021, DOI: 10.1016/j.jchem.2021.04.020.

(23) Wang, S.-W.; Lee, C.-H.; Lin, M.-S.; Chi, C.-W.; Chen, Y.-J.; Wang, G.-S.; Liao, K.-W.; Chu, L.-P.; Wu, S.-H.; Huang, D.-M.; Chen, L.; Shen, Y.-S. ZnO Nanoparticles Induced Caspase-Dependent Apoptosis in Gingival Squamous Cell Carcinoma through Mitochondrial Dysfunction and P70S6K Signaling Pathway. Int. J. Mol. Sci. 2020, 21, 1612.

(24) Yousefi, A.-M.; Safarogli-Azar, A.; Fakhroueian, Z.; Bashash, D. ZnO/CNT@Fe3O4 Induces ROS-Mediated Apoptosis in Chronic Myeloid Leukemia (CML) Cells: An Emerging Prospective for Nanoparticles in Leukemia Treatment. Artif. Cells, Nanomed., Biotechnol. 2020, 48, 735–745.

(25) Ahamed, M.; Akhtar, M. J.; Khan, M. M.; Alhadaq, H. A. SnO2-Doped ZnO/Reduced Graphene Oxide Nanocomposites: Synthesis, Characterization, and Improved Anticancer Activity via Oxidative Stress Pathway. JIN 2021, 86, 19–104.

(26) Rahemi Ardekan, S.; Sabour Rouhaghdam, A.; Nazari, M. N-Doped ZnO-CuO Nanocomposite Prepared by One-Step Ultrasonic Spray Pyrolysis and Its Photocatalytic Activity. Chem. Phys. Lett. 2018, 705, 19–22.

(27) Miao, Y.; Wang, X.; Wang, W.; Zhou, C.; Feng, G.; Cai, J.; Zhang, R. Synthesis of Cobalt-Doped ZnO/RGO Nanoparticles with Visible-Light Photocatalytic Activity through a Cobalt-Induced Electrochemical Method. J. Energy Chem. 2017, 26, 549.

(28) Vanitha, M.; Joni, I. M.; Camellia, P.; Balasubramanian, N. Tailoring the Properties of Cerium Doped Zinc Oxide/Reduced Graphene Oxide Composite: Characterization, Photoluminescence Study, Antibacterial Activity. Ceram. Int. 2018, 44, 19725–19734.

(29) Shewale, P. S.; Yun, K. S. Synthesis and Characterization of Cu-Doped ZnO/RGO Nanocomposites for Room-Temperature H2S Gas Sensor. J. Alloys Compd. 2020, 837, No. 155527.

(30) Labhane, P. K.; Sonawane, S. H.; Sonawane, G. H.; Patil, S. P.; Huse, V. R. Influence of Mg Doping on ZnO Nanoparticles Decorated on Graphene Oxide (GO) Crumpled Paper like Sheet and Its High Photo Catalytic Performance under Sunlight. J. Phys. Chem. Solids 2018, 114, 71–82.

(31) Umar, K.; Aris, A.; Parveen, T.; Jaafar, J.; Abdul Majid, Z.; Vijaya Bhaskar Reddy, A.; Talib, J. Synthesis, Characterization of Mo and Mn Doped ZnO and Their Photocatalytic Activity for the Decolorization of Two Different Chromophoric Dyes. Appl. Catal., A 2015, 505, 507–514.

(32) Pal, G.; Rai, P.; Pandey, A. Green Synthesis of Nanoparticles: A Greener Approach for a Cleaner Future. In Green Synthesis,
(65) Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. J. Immunol. Methods 1983, 65, 55–63.

(66) Borenfreund, E.; Puerner, J. A Simple Quantitative Procedure Using Monolayer Cultures for Cytotoxicity Assays (HTD/NR-90). J. Tissue Cult. Methods 1985, 9, 7–9.

(67) Ahamed, M.; Akhtar, M. J.; Siddiqui, M. A.; Ahmad, J.; Musarrat, J.; Al-Khedhairy, A. A.; AlSalhi, M. S.; Alrokayan, S. A. Oxidative Stress Mediated Apoptosis Induced by Nickel Ferrite Nanoparticles in Cultured A549 Cells. Toxicology 2011, 283, 101–108.

(68) Akhtar, M. J.; Ahamed, M.; Alhadlaq, H. Gadolinium Oxide Nanoparticles Induce Toxicity in Human Endothelial Huvecs via Lipid Peroxidation, Mitochondrial Dysfunction and Autophagy Modulation. Nanomaterials 2020, 10, 1–18.

(69) Siddiqui, M. A.; Alhadlaq, H. A.; Ahmad, J.; Al-Khedhairy, A. A.; Musarrat, J.; Ahamed, M. Copper Oxide Nanoparticles Induced Mitochondria Mediated Apoptosis in Human Hepatocarcinoma Cells. PLoS One 2013, 8, e69534.

(70) Ellman, G. L. Tissue Sulphhydril Groups. Arch. Biochem. Biophys. 1959, 82, 70–77.

(71) Rotruck, J. T.; Pope, A. L.; Ganther, H. E.; Swanson, A. B.; Hafeman, D. G.; Hoekstra, W. G. Selenium: Biochemical Role as a Component of Glutathione Peroxidase. Science 1973, 179, 588–590.

(72) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analzyt. Biochem. 1976, 72, 248–254.