

# Copper oxide nanoparticles induce anticancer activity in A549 lung cancer cells by inhibition of histone deacetylase

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## Abstract

**Objectives** Copper oxide nanoparticles (CuO NPs) promoting anticancer activity may be due to the regulation of various classes of histone deacetylases (HDACs).

**Results** Green-synthesized CuO NPs significantly arrested total HDAC level and also suppressed class I, II and IV HDACs mRNA expression in A549 cells. A549 cells treated with CuO NPs downregulated oncogenes and upregulated tumor suppressor protein expression. CuO NPs positively regulated both mitochondrial and death receptor-mediated apoptosis caspase cascade pathway in A549 cells.

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**Conclusion** Green-synthesized CuO NPs inhibited HDAC and therefore shown apoptosis mediated anticancer activity in A549 lung cancer cell line.

**Keywords** Anticancer activity · Apoptosis · A549 cells · Copper oxide nanoparticles · Epigenetic · Histone deacetylase

## Introduction

The equilibrium of histone acetylation and deacetylation is an epigenetic layer with a key role in the regulation of gene expression. Histone acetylation promoted by histone acetyl transferases (HATs) is linked with gene transcription, whereas histone hyperacetylation, induced by histone deacetylases (HDACs) activity, is connected with gene silencing (Sankar et al. 2015). Differential expression of genes that encode

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HDACs have been linked to cancer development, possibly by altering the transcription of key genes that are involved in the regulation of various cellular functions including cell proliferation and apoptosis (Ropero and Esteller 2007). Inhibition of HDACs may control transcription and induce apoptosis in cancer cells. HDAC inhibitors have strong anticancer activities in several cancer cells through initiation of apoptosis, differentiation, and inhibition of tumor angiogenesis (Qian et al. 2006). Numerous HDAC inhibitors have been developed but each has its own drawback that including solubility, bioavailability and toxicity (Sankar and Ravikumar 2014). Hence, we are in the need of developing novel agents to actively target HDACs.

Metal nanoparticles may target cancer cells (Sankar et al. 2014). Among different metal nanoparticles, copper oxide nanoparticles (CuO NPs) are widely used to attack cancer cells (Sun et al. 2012). Hanagata et al. (2011) showed the copper ions released from CuO NPs arrest the cell cycle, induced DNA damage and downregulated the proliferating cell nuclear antigen in lung epithelial A549 cells. Moreover, we have recently reported that *Ficus religiosa* leaf extract mediated CuO NPs potent anticancer activity in A549 lung cancer cells using cell proliferation, intracellular reactive oxygen species and mitochondrial membrane potential assay (Sankar et al. 2014).

In the present study, we hypothesize that the CuO NPs-induced anticancer activity in A549 lung cancer cells could be due to the regulation of various classes of HDACs. To prove this, CuO NPs were used to check the regulation of various classes of HDACs using a total HDAC assay and the mRNA expression profile of different classes of HDACs in A549 cells. Furthermore, anticancer activity of CuO NPs was also demonstrated.

## Materials and methods

### Synthesis and characterization of CuO NPs

The synthesis of *F. religiosa* leaf extract-mediated CuO NPs was achieved using our protocol (Sankar et al. 2014). Briefly, 10 g *F. religiosa* leaves powder was dissolved in 100 ml deionized water and boiled at 60 °C for 10 min. The filtered extract (10 ml) was added to 90 ml 5 mM CuSO<sub>4</sub> and incubated at room

temperature. The formed CuO NPs were collected by centrifugation. The heat dried CuO NPs powder was prepared on aluminum stub and coated with gold prior to field emission-scanning electron microscope (FE-SEM) with energy dispersive X-ray spectroscopy (EDAX) (VEGA3 TESCAN, 30 kV) examination.

### Cell culture

A549 human lung cancer cell line was obtained from the National Center for Cell Science, Pune, India. It was cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (10,000 U/ml) in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C.

### Reverse transcription PCR (RT-PCR)

A549 cells were either left alone or treated with CuO NPs or the known HDAC inhibitor trichostatin A (TSA) for 36 h; total RNA was extracted by Trizol. Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. RNA (2 µg) was used for the cDNA synthesis. cDNA pool was used for RT-PCR reaction. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. 10 µl of RT-PCR product was analyzed by 1% agarose gel electrophoresis. Sequence of the primers is given in Supplementary Table 1.

### Western blotting

A549 cells were either left alone or treated with CuO NPs or TSA for 36 h; cells were harvested and whole-cell lysates were prepared with dignal buffer. Cellular protein was quantified by the Bradford method. 50 µg of each protein was exposed to SDS-PAGE. Protein was transferred to nitrocellulose membrane, blocked by Tris-buffered saline containing 0.1% (w/v) Tween 20 and 5% (w/v) non-fat milk powder for 1 h. The blot was probed with the appropriate dilution of primary antibodies [Bax, Bcl<sub>2</sub>, caspase 3, 8, 9, cytochrome C, p21 and p53 and matrix metalloproteinases (MMP)-2, and 9 (1:1000 dilution)] for 12 h at 4 °C. The membrane was washed and incubated (1 h) with a secondary antibody containing horseradish peroxidase-conjugated with anti-rabbit or anti-mouse IgG antibody 1 h at 4 °C. The blot was developed using 3,3'-diaminebenzidine.

## Histone deacetylase (HDAC) activity assay

Assay was achieved using a colorimetric HDAC activity assay kit according to the manufacturer guidelines. Lethal dose concentration of CuO NPs (200 µg) treated A549 cell lysate was used to check the total activity. Untreated cell lysate and TSA-treated cell lysate was used as a control. Samples were added in 96 well plate and final volume was made up to 85 µl using deionized water. 10 µl 10× HDAC assay buffer and 5 µl HDAC colorimetric substrates were added, after 1 h incubation at 37 °C reaction was stopped by adding 10 µl lysine developer for 30 min at 37 °C. The absorbance was measured by an ELISA plate reader at 400 nm. HDAC activity was expressed as relative optical density values per µg of protein.

## Statistical analysis

Statistical significance was investigated using SPSS software version (SPSS Inc., Chicago, IL, USA). One-way ANOVA and student's *t* test was done for the expressing significance. A value of  $P < 0.05$  was considered to indicate statistical significance. All the results were expressed as mean  $\pm$  S.D.

## Results and discussion

### Characterization of CuO NPs

We have reported *F. religiosa* leaf extract mediates CuO NPs preparation and physicochemical characterization (Sankar et al. 2014). Here, green-synthesized CuO NPs was confirmed by FE-SEM analysis. The synthesized CuO NPs are spherical and evenly distributed throughout the colloidal solution (Supplementary Fig. 1A). The elemental copper and oxide peaks were confirmed by EDAX spectrum (Supplementary Fig. 1B). The morphological and elemental analysis results were comparable with our previous study (Sankar et al. 2014).

### In vitro HDAC inhibition assay

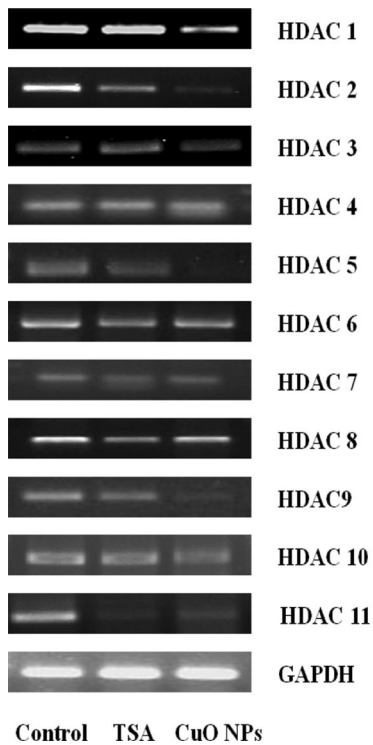
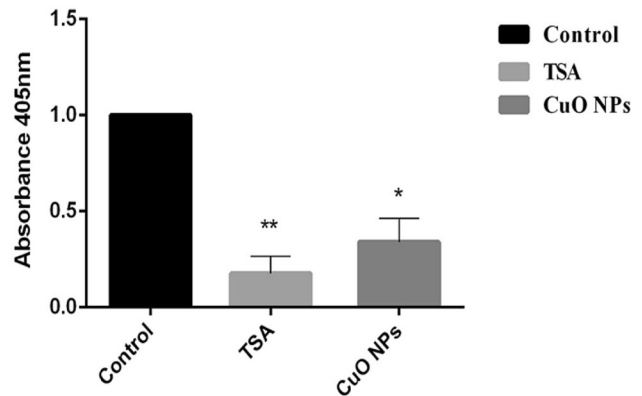
Epigenetic modifications in over-expression of HDACs play a crucial role in carcinogenesis (Ropero and Esteller 2007; Qian et al. 2006). We checked the

effect of CuO NPs on the regulation of HDAC. A549 cells were treated with CuO NPs and the total HDAC level was calculated using the colorimetric assay kit. The known HDAC inhibitor (TSA) was used as a positive control. As shown in Fig. 1, CuO NPs significantly arrested the total HDAC level in A549 cells compared to control. The CuO NPs total HDAC arrest result was comparable with the trichostatin (TSA)-treated A549 cells. The CuO NPs inhibit total HDAC and modulate transcription thereby shown differentiation and apoptosis in lung cancer cells (Sun et al. 2012). The assay result clearly suggested that CuO NPs could be able to act as a HDAC inhibitor.

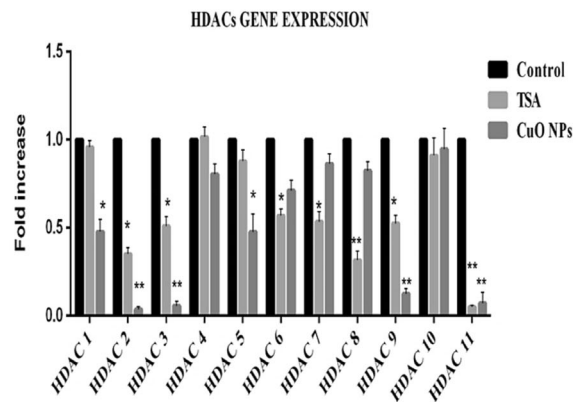
### CuO NPs regulated various classes of HDACs mRNA expression

Different classes of HDACs inhibition have been implicated to modulate transcription and induced apoptosis or differentiation in cancer cells (Ropero and Esteller 2007). The HDAC assay result given the insight that the CuO NPs actively downregulated the total HDAC compared to control cells. Furthermore, we tried to find out the CuO NPs role in regulation of individual HDACs-classes. As shown in Fig. 2, all the HDACs-classes (Class I- 1, 2, 3, and 8; class II- 4,5,6,7,9,10; class IV- 11) were up regulated in A549 lung cancer cells but were, however, downregulated in CuO NPs treatment. The mRNA expression results suggest that green-synthesized CuO NPs could be a novel agent to arrest the HDACs. Suppression of HDACs activity is one of the extensively used mechanism for regulating cancer cells. The high expression of HDACs is seen in several human cancer cell lines and also tissues including cancer of the lung (Xu et al. 2007). Most of human cancer tissues and their corresponding non-cancerous epithelium showed high expressions of class-I HDACs, considered as a potential therapeutic target (Wang et al. 2012). The HDAC inhibitors regulated gene expression by transcription and altering chromatin structure by histone acetylation; play a significant role in regulation of HDAC gene expression (Xu et al. 2007).

**Fig. 1** In vitro inhibition of HDAC activity by TSA and CuO NPs in A549 cells. \* $P < 0.05$  and \*\* $P < 0.01$  compared to the control group (HDAC Histone deacetylase, TSA Trichostatin A, CuO NPs Copper oxide nanoparticles)



**Fig. 2** The effect of TSA and CuO NPs on regulation of class-I histone deacetylase (1, 2, 3, and 8), class-II (4, 5, 6, 7, 9 and 10) and class-IV (11) mRNA expression in A549 cells. GAPDH was used as an internal control. \* $P < 0.05$  and \*\* $P < 0.01$



compared to the control group (TSA Trichostatin A, CuO NPs Copper oxide nanoparticles, GAPDH Glyceraldehyde 3-phosphate dehydrogenase)

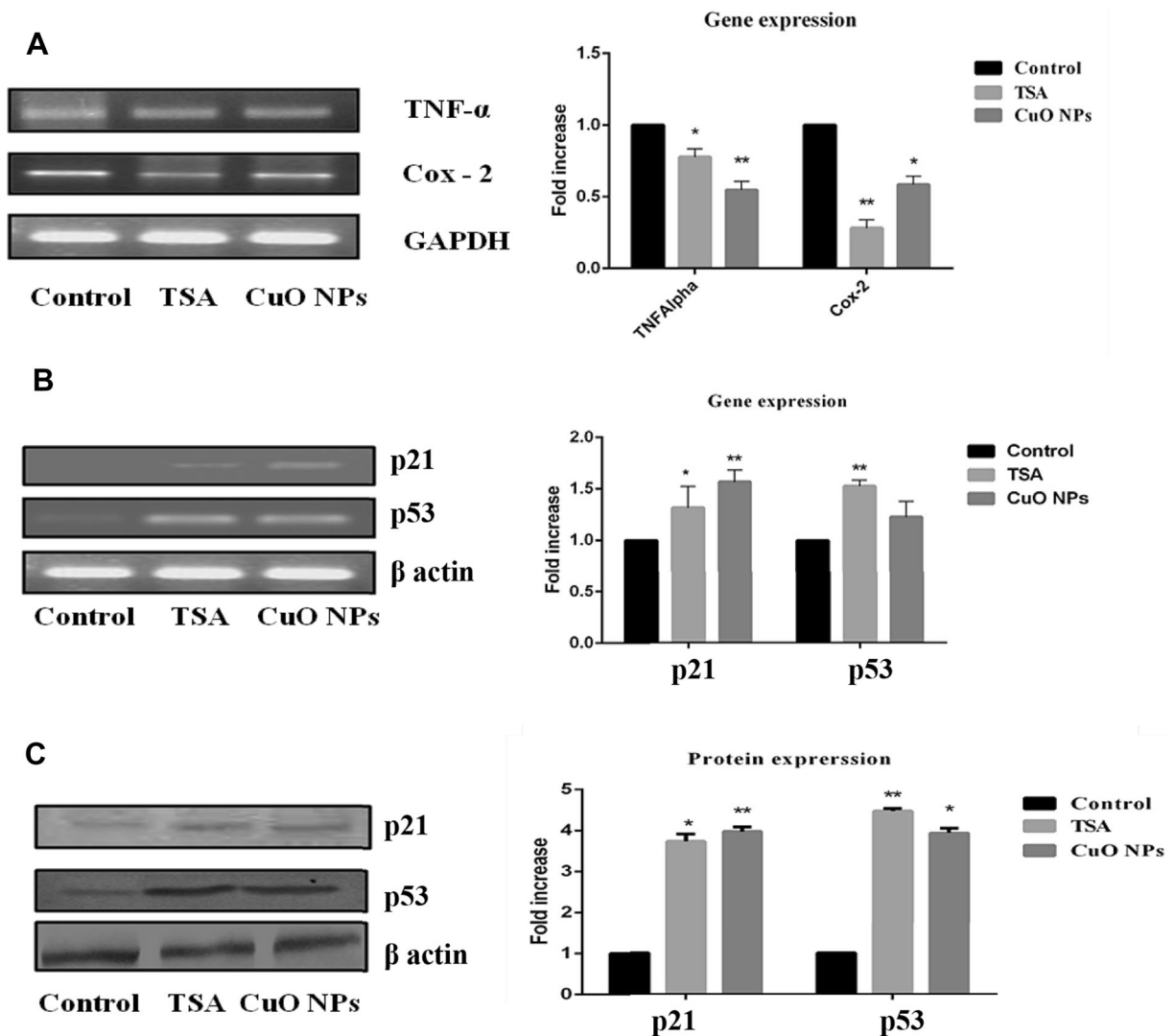
CuO NPs regulated inflammatory, oncogenes and tumor suppressor genes mRNA and protein expression

To test whether CuO NPs produce significant regulation of HDACs and thus may help to induce anticancer activity, we checked different oncogenes and tumor suppressor genes mRNA and protein expression in

A549 cells after the treatment with CuO NPs. The tumor necrosis factor alpha (TNF- $\alpha$ ) made by the immune system and its dysregulation have a significant role in tumor necrosis. TNF- $\alpha$  is also produced by tumors and can act as an endogenous tumor promoter. COX-2 is thought to play a main role in tumor growth because they decrease apoptotic cell death, stimulate angiogenesis and invasiveness. The inflammatory

responsible genes, such as TNF- $\alpha$  and COX-2, were highly expressed in the A549 cells, whereas after the treatment with CuO NPs actively downregulated the mRNA expression (Fig. 3a). The results show that CuO NPs also have a potential to regulate the inflammatory response gene expression. The down-regulation of TNF- $\alpha$  and COX-2 expression indicated the arrest of cancer cell proliferation and angiogenesis (Gupta et al. 2010).

We checked CuO NPs positive regulation of tumor suppressor genes (p53 and p21) mRNA and protein expression in A549 cells. The result showed CuO NPs significantly upregulated both mRNA and protein expression of p53 and p21 in A549 cells compared to control (Fig. 3b, c). Thus, activation of p21 and p53 after the treatment with CuO NPs suggested their ability to act as a potent anticancer agent. Similarly, Siddiqui et al. (2013) reported chemically synthesized CuO NPs significantly upregulated both mRNA and



**Fig. 3** TSA and CuO NPs on regulation of various inflammatory and tumor suppressors mRNA and protein expression in A549 cells. **a** mRNA expression ratio of TNF- $\alpha$  and COX-2 **b** p21 and p53 analyzed by RT-PCR **c** tumor suppressor p21 and p53 protein expression profile analyzed by western blotting.

GAPDH and  $\beta$ -actin was used as an internal control. \*P < 0.05 and \*\*P < 0.01 compared to the control group (TSA- Trichostatin A; CuO NPs- Copper oxide nanoparticles; TNF- $\alpha$ - Tumor necrosis factor alpha; COX-2- Cyclo-oxygenase-2; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase)

protein expression of p53 in human hepatocarcinoma cells. MMP is a vital regulator of many biological processes such as angiogenesis, inflammation and cancer. The key effect of MMP in angiogenesis, tumor growth and metastasis is degradation of extracellular matrix and thereby activation of growth factors, results in cancer development (Shuman Moss et al. 2012).

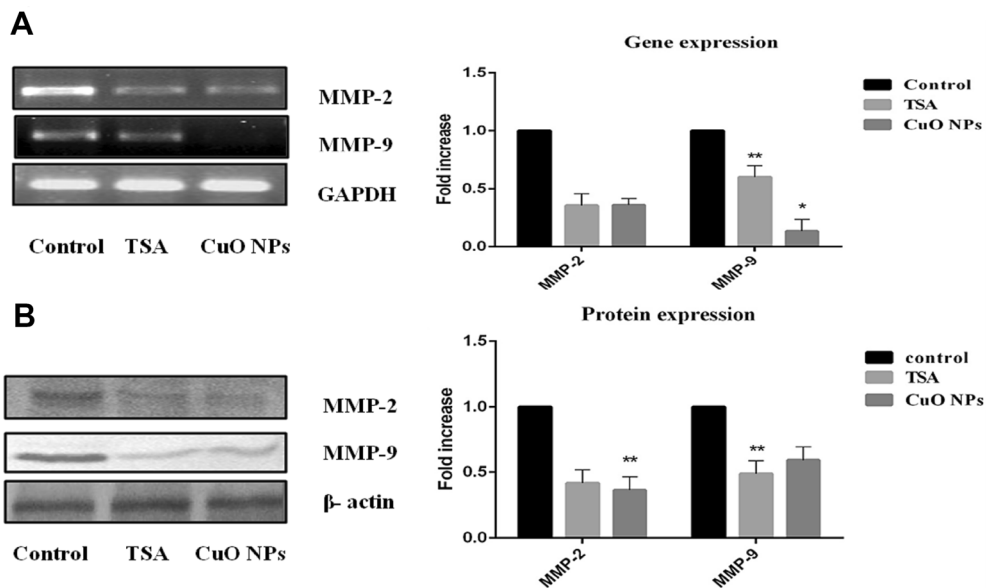
We studied the effect of CuO NPs on regulation of oncogenes (MMP-2 and 9) mRNA and protein expression in A549 cells. MMP-2, 9 were highly expressed in both mRNA and protein level in A549 cells, however, treatment with CuO NPs significantly arrested the expression (Fig. 4a, b). Shafagh et al. (2015) established the CuO NPs showed anticancer activity in human K562 cancer cell line through the regulation of mitochondrial pathway, tumor suppressor genes and reactive oxygen species.

#### CuO NPs regulated apoptosis associated caspase pathway

We checked different apoptosis-associated caspase cascade pathways. Caspase are proteases that have a vital role in triggering and executing apoptosis. We examined the mRNA expression of caspase 3, 8 and 9 after the 8 treatment with CuO NPs in A549 cells. The

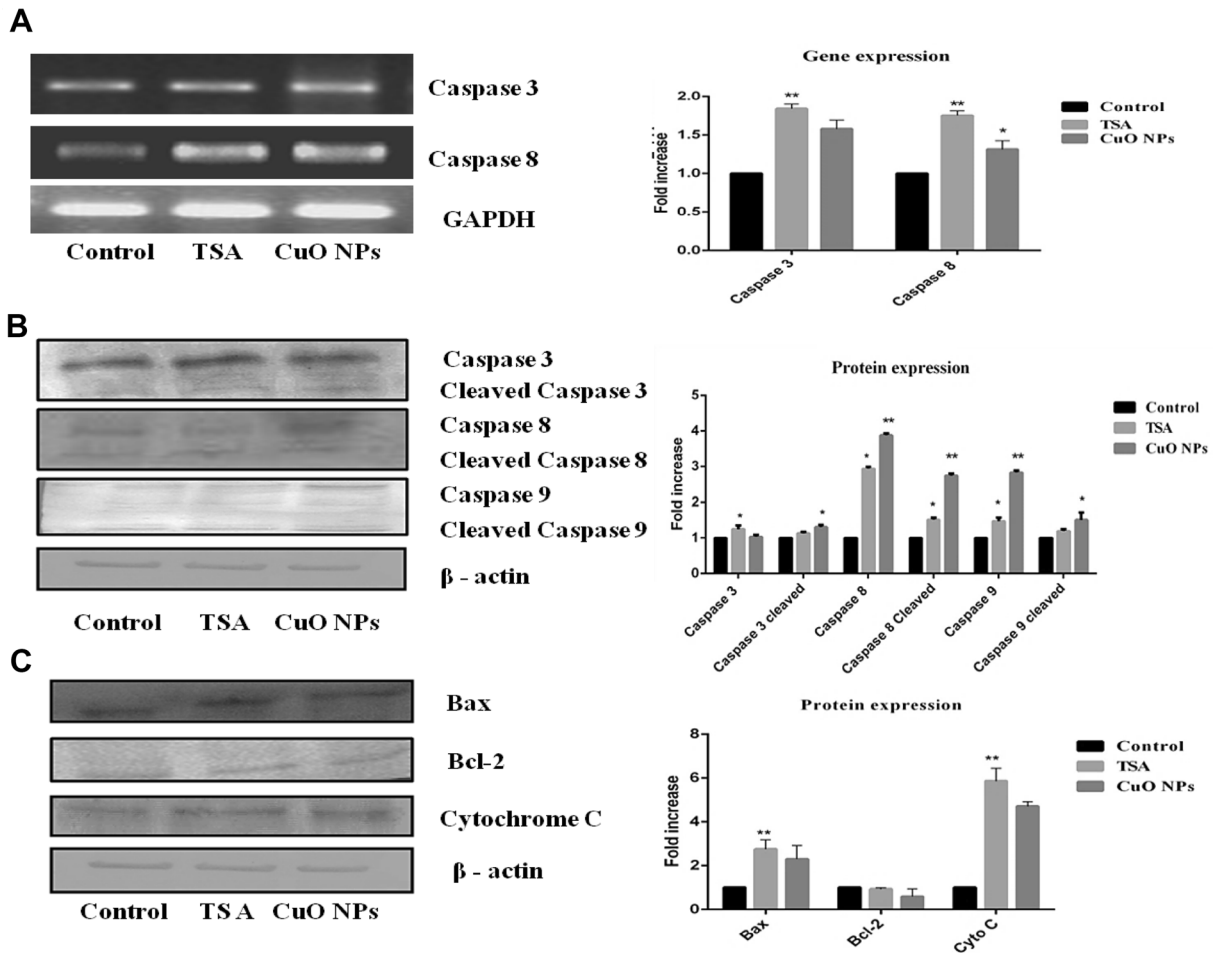
result showed CuO NPs significantly activated the caspase cascade pathway in A549 cells compared to control (Fig. 5a). We also examined protein expression of caspase 3, 8 and 9 after the treatment with CuO NPs. Treatment resulted in clear cleavage of caspase 8, 9 and 3 (Fig. 5b). The result is comparable with TSA-treated A549 cells. Caspase-9 is one of the main initiator for caspase pathway. The result indicated CuO NPs initiate caspase-9 and thereby activated apoptosis pathway in A549 cells. Jänicke et al. (1998) reported that the caspase pathway is stimulated during apoptosis in many cancer cells and play a key role in the initiation and implementation of apoptosis and also in cellular DNA damage.

We also checked pro-apoptotic (Bax), anti-apoptotic (Bcl2) protein and cytochrome c expression in A549 cells after the treatment with CuO NPs. The result clearly exhibited CuO NPs treated cells decreased Bcl2, increased Bax and cytochrome c expression compared with untreated cells (Fig. 5c). Increase in Bax/Bcl2 ratio followed by the initiation of p53 stimulated mitochondria to release cytochrome c and activates apoptosis caspase cascade pathway (Siddiqui et al. 2013). The Bcl2 family protein activated by compounds, Bax is inserted into the mitochondrial membrane and increased membrane



**Fig. 4** The effect of TSA and CuO NPs on regulation of MMP-2 and 9 expression in A549 cells. **a** MMP-2 and 9 mRNA expression analyzed by RT-PCR. **b** MMP-2 and 9 protein expression analyzed by western blotting. GAPDH and  $\beta$ -actin

was used as an internal control. \* $P < 0.05$  and \*\* $P < 0.01$  compared to the control group (TSA Trichostatin A, CuO NPs Copper oxide nanoparticles, MMP Matrix Metalloproteinase, GAPDH Glyceraldehyde-3-phosphate dehydrogenase)



**Fig. 5** Effect of TSA and CuO NPs on the regulation of apoptosis mRNA and protein expression in A549 cells. **a** Caspase 3 and 8 mRNA expression analyzed using RT-PCR. **b** Caspase 3, 8 and 9 pre and cleaved protein **c** pro-apoptotic (Bax), anti-apoptotic (Bcl2) and cytochrome c protein

expression analyzed by western blotting. GAPDH and  $\beta$ -actin was used as an internal control. \* $P < 0.05$  and \*\* $P < 0.01$  compared to the control group (TSA Trichostatin A, CuO NPs Copper oxide nanoparticles, GAPDH Glyceraldehyde-3-phosphate dehydrogenase)

permeability to release cytochrome c and thereby promoting apoptosis (Appaix et al. 2000). The results showed CuO NPs activated both intrinsic (mitochondrial mediated) and extrinsic (death receptor mediated) apoptotic pathway, arrest A549 cancer cell proliferation.

**Conclusion**

Green-synthesized CuO NPs has strong inhibition against total HDAC and different classes of HDACs. They also have a potential to regulate the oncogenes and tumor suppressor genes mRNA and protein

expression. CuO NPs activated both intrinsic and extrinsic caspase cascade pathway against A549 cells. Overall, our previous and present study results revealed CuO NPs induced anticancer activity in A549 lung cancer cells might be due to the regulation of various classes of HDACs. Further studies are required to prove its effect on different cancer cells.

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**Supporting information** Supplementary Fig. 1—Field emission-scanning electron microscopic analysis of **a** CuO NPs **b** EDAX spectrum (CuO NPs- Copper oxide nanoparticles; EDAX- Energy dispersive X-ray spectroscopy).

Supplementary Table 1—The sequence of the primers used for the RT-PCR (RT-PCR- Reverse transcription PCR).

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest concerning this article.

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