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Review Article

Synthesis of CuO Nanoparticles Using Aloe Vera Leaf Extract and its Cytotoxic Effects on Human Carcinoma Cells

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ABSTRACT

Copper oxide nano-particles can be manufactured by several methods chemical, physical or biological methods. Copper oxide nano-particles promoting anticancer effect.

In the present study green chemistry methods are used to prepare pure copper oxide by using Aloe-vera. Aloe-vera is a natural plant that has many benefits anti inflammatory, anti viral, antitumor, antibacterial and antiseptic effects. Characterization of the composition of CuO Aloe-vera was done by x-ray diffraction to determine the structure and crystalline size estimation that confirm the presence of the peaks of CuO nano-particles. By using UV-visible spectral analysis energy gap is determined. TEM is used to study nano-particles shape and size and zeta potential for measuring the net charge and stability. The obtained results indicated that addition of the capping molecule Aloe-vera with CuO composition controls the size and distribution of the nano-particles. Anti tumor effect was studied by using MTT assay and RT-PCR analysis on hepatocarcinoma cell culture to confirm the ability of CuO nano-particles to stimulate cancer cell apoptosis. All the previous analysis confirms the presence of CuO nano-particles and their antitumor effect.

INTRODUCTION

Green production of chemicals materials have a role for the wellbeing of humanness and for the preserving of the environment. 'Green Chemistry' considers safe and alternative method of fabrication of materials in contrast to other chemical methods to design nanoscale products [1].

Green synthesis of nanoparticles NPs using plant extracts is an interested research area of synthesizing nanosized particles of desired size and shape [2] in addition to its advantageous over chemical and physical health [3]. Copper oxide (CuO) is a type of the transition metal oxides, that has received considerable attention due to its wide important applications such as p-type semiconductor material, also advanced materials for catalysts, adsorbents, gas and electrochemical sensors, superconductors, lithium-ion batteries, super capacitors and antimicrobial materials [4].

It can be used to prepare copper oxide nanoparticles (CuO NP'S) , including thermal reduction, the capping agent method, sonochemical reduction , vapor deposition

method and induced radiation [5, 6]. The biosynthesis of CuO NPs was developed to prepare nanomaterials, in which plant extract acts as a capping agent [7]. A simple, low cost, stable CuO NPs has been Green synthesized using plant extracts such as aleo-vera. Aloe-Vera belongs to family liliaceae and homegrown belong to several nations with a dry subtropical climate [8,9].

Aloe-vera has many uses like purgative, alexipharmic anthelmintic, analgesic, anticonvulsant, anxiolytic, sedative and antipyretic effect [10,11] . Aloe–vera plant can inhibit the aggregation and it has a wide range of anti-tumor effect. In the present research, antitumor effect of the CuO nanoparticles application has been studied briefly using Hepato-carcinoma (HCC) cell line. The key of cancer progression process and its development is unusual apoptotic mechanism. Cancer cells are able to avoid apoptosis and several genes have significant role in apoptotic cell death. Protein p53 is detected as master guardian of the cell which stimulates the cell cycle arrest.

The bcl-2 protein has an anti-apoptotic effect, whereas the bax is known for pro-apoptotic activity. The ratio of bax/bcl-2 proteins indicates the ability of the cell to complete life or to make self mediated apoptosis. Hepatocellular carcinoma (HCC) is the third most lethal cancer and it is considered the most frequent tumor in the world [12]. Also oxidative stress represents a major risk factor in pathogenesis of liver diseases leading to HCC[13]. Because of the absence of knowledge about the mechanisms of CuO NPs toxicity, this study was done to examine the possible mechanisms of apoptosis caused by CuO NPs in human hepatocellular carcinoma (HepG2 cell line).

The available mechanisms for studying the cytotoxicity were cell viability test, oxidative stress and measuring the mRNA levels of the apoptotic genes as bax, caspase3, p53 and anti apoptotic gene bcl-2 by quantitative RT-PCR analysis.

MATERIAL AND METHODS

Biosynthesis of aloe-vera gel extract was carried out by soaking 50gm of washed and shopped Aloe-Vera in 100ml of warm water for one day. Extracted gel was filtered and added dropwize to copper nitrate. The synthesis of CuO-NPs has been done using three different methods .In the First method (Chemical method), CuO-NPs were synthesized by adding 50 ml of 1 M NaOH into 100mM aqueous solution of copper (II) nitrate with constant stirring at 80°C Within 30 min; the deep blue solution became dark green and then turned to dark black precipitate. CuO-NPs produced is denoted as CuO-C. In the second method (Biosynthesis), NaOH was completely replaced with Aloe-vera gel extract.

This extract was added drop wise into 50 ml of 100 mM aqueous solution of copper (II) nitrate with constant stirring at 80°C. Within few hours, the deep blue solution gradually became dark green and then turned slowly to dark black precipitate. CuO-NPs by this method is denoted by CuO-B. In the two methods, the prepared black precipitate is filtrated via decantation then washed several times by deionized water. Finally,the washed black precipitate is dried over night at 80°C in oven [14]. In the Third method (Chemical and Biosynthesis), 1 M of NaOH was added dropwise to 50ml of 0.24M $CuNO_3$ then after the black precipitate of copper oxide is formed, 50ml of aloe vera filtered gel is added dropwise on Copper Oxide solution under stirring at 80°C for 2 hours. CuO-NPs by this method is denoted as CuO-C+B. (Figure 1) Human liver cancer cell line, HePG2, obtained from Cell Culture Laboratory, National Research Centre, Egypt was used in this work.

All the mentioned procedures were prepared in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, and Stanford, ME, USA). HepG2 cell line was used to determine the cell viability against CuO NPs exposure.

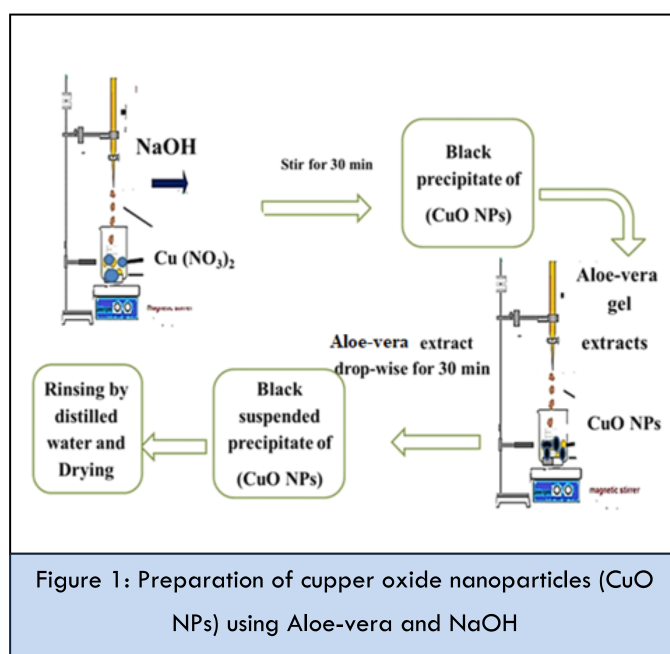

Figure 1: Preparation of cupper oxide nanoparticles (CuO NPs) using Aloe-vera and NaOH

CHARACTERIZATION OF CUO NANO-PARTICLES

Structural assessment

The functional and characteristic spectra of the synthesized CuO-NPs were examined via Fourier Transform Infrared (FTIR) spectra using Perkin-Elmer1650 spectrophotometer. The spectra were taken at 4.0 cm-1 resolution. 64 scans were accumulated to obtain a reasonable signal to noise ration. The dried extract powders were pressed with KBr and tested in parallel [15].

The purity and crystallinity nature were investigated using Analytical-x' Pertpro XRD X-ray powder diffractometer with Cu K radiation (? = 1.5418 ?) at a scanning speed of 0.2 s to detect the specific peaks of the prepared CuO-NPs. The spectra of CuO nanoparticles were done using XRD for 2θ values ranging from 10 to 70°. The spectrophotometric measurements in solutions were made using Optizen recording Spectrophotometer, UV-V in the range from 200-800 nm model 5u470\pop 127022-00 and quartz cell of 1 cm optical length.

The zeta potential surface charge of CuO has been measured to determine the stability of CuO using Zetasizer (ZEN 3690, Malvern Instrumentations Ltd, Worcestershire, UK) at 3.8nm to 100?m and 175° backscattering angle at a fixed refractive index of the respective formulations to assess Nanoparticles metal oxides stability. Three milli liter of the synthesized CuO was dispersed in water and placed in a quartz cuvette, and the measurements were taken by intensity and by volume.Due to the relative small size of nanoparticles they are energetically very unstable.

Therefore, as the particles undergo Brownian motion, colloids tend to balance between the attractive van der Waals' forces and the electrical repulsion due to surface charge. If the zeta potential falls below a certain level, the colloids tend to agglomerate/aggregate due to the attractive force.

Morphological characterizations

In this study, the morphology and arrangement of the synthesized CuO-NPs were performed. For TEM, the synthesized samples were dispersed in ethanol, sonicated for 5 min and deposited onto TEM grids having carbon support film. Using TEM, JEOL (JEM-2100) 200 kV.

Cytotoxic effect of CuO nanoparticles on human cell lines

Apoptotic mechanism has an important role in cancer development and progression. One of the basic characteristics mystery of cancer is the ability of cancer cells to discard apoptosis and persist to propagate [16]. There are several genes that have an important role in apoptotic cell death. The p53 protein is regarded as the master guardian of the cell that stimulates the cell cycle arrest to give the cell its time for repairing damage in DNA or self-mediated apoptosis.

The bcl-2 protein has an anti-apoptotic effect, whereas the bax is known for pro-apoptotic activity [17]. The ratio of bax/bcl-2 protein indicates the ability of the cell to complete life or to make self mediated apoptosis. Because of the abscence of knowledge about the mechanisms of CuO NPs toxicity, this study was done to investigate the possible mechanisms of apoptosis induced by CuO NPs in human hepatocellular carcinoma (HepG2) cells[18]. The available mechanisms for studying the cytotoxicity were by using cell viability, oxidative stress and measuring the mRNA levels of the apoptotic genes as bax, caspase3, p53 and anti apoptotic gene bcl-2 by RT-PCR analysis.

Cell viability assay of cancer and normal cells against CuO-NPs exposure was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide) to purple formazan [19]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. MTT-assay was widely used to assess cell viability. Gene expression by quantitative real time polymerase chain reaction Gene expression of p53, bax, bcl-2, caspase3 and Caspase-9 were determined in HepG2 cell line using Real Time – PCR according to Mitupatum et al., 2016 [2].

The total RNA of treated and untreated cells was extracted using RNA Mini kit. Real were quantified using the formula 2-??CT. Expression levels of mRNA were determined by Stratagene, time PCR amplification was performed using SYPER Green kit. The amount of target gene expression levels MX3000P quantitative PCR System (Agilent technologies) using Maxima SYBR Green qPCR Master Mix (2X) kit purchased from Thermo scientific, cataloug #K0251 and analyzed by MxPro QPCR Software (Agilent technologies). The sequences of the

specific sets of primer for p53, bax, bcl-2, caspase-3 and caspase 9 are mentioned in table 1.

Table 1: The sequences of the specific sets of primer for p53, bax, bcl-2, caspase-3 and caspase 9.

Gene	Primers	Annealing Temp. °C	Ref.
p53	5- ACTTGTCGCTCTTGAAGCTAC-3	51.3	(Wang *et al.*, 2016)
	5 -GATGCGGAGAATCTTTGGAACA-3		
bax	5-CCTGTGCACCAAGGTGCCGGAACT-3	63.4	(Khodapasand *et al.*, 2015)
	5-CCACCCTGGTCTTGGATCCAGCCC-3		
bcl-2	5-TTGTGGCCTTCTTTGAGTTCGGTG-3	56.6	(Khodapasand *et al.*, 2015)
	5--GGTGCCGGTTCAGGTACTCAGTCA-3		
Caspase-9	5 -CACTTCCCCTGAAGACGAGTC-3	52.7	(Barathan *et al.*, 2013)
	5- GTGGGCAAACTAGATATGGCG-3		
Caspase-3	5-TGGCCCTGAAATACGA-AGTC-3	50	(p.kermer et al., 1998)
	5-GGCAGTAGTCGACTCTGAAG-3		

Statistical analysis a statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

The percentage of change in viability =[(Reading of extract / Reading of negative control) -1)] x 100

A probitanalysis was carried for IC50 and IC90 determination using SPSS 11 program.

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy (FT-IR)

The FTIR spectra of Aloe-Vera extract and synthesized CuO NPs [Chemically, Biosynthesized, Chemically and Biosynthesized] are shown in Figure 2. The characteristic peak of the pure Aleo-vera at 870 cm-1 is referring to the C-H out-of-plane deformation of carbohydrate monomers. The absorption peak at 1256 cm^{-1} is corresponding to the C-O-C stretching of $CO\text{-}CH_3$ groups.

The peaks at 1634 and 1418 cm^{-1} are associated with the asymmetrical and symmetrical -COO of carboxylate compounds in the pure Aloe-vera [20]. The broad and strong peak at around 3450 cm^{-1}, observed in both Biosynthesized CuO NPs, can be attributed the O-H groups of alcohols and phenols from Aloe-Vera [2].This peak diminished in the chemically prepared CuO NPs. The bands at region from 2500-2300 cm^{-1} and at 840 cm^{-1} were assigned to C-H stretching and bending. The peaks observed in the range of 1600 and 1060 cm^{-1} had been assigned to C=O and S=O groups attributed to alcohols and phenolic groups [21].

The major peaks were observed in all synthesized CuO NPs to be 510-600 cm^{-1} should be a stretching of Cu-O. Similar results have been reported in literature where CuO NPs was synthesized using different leaves extracts [22].

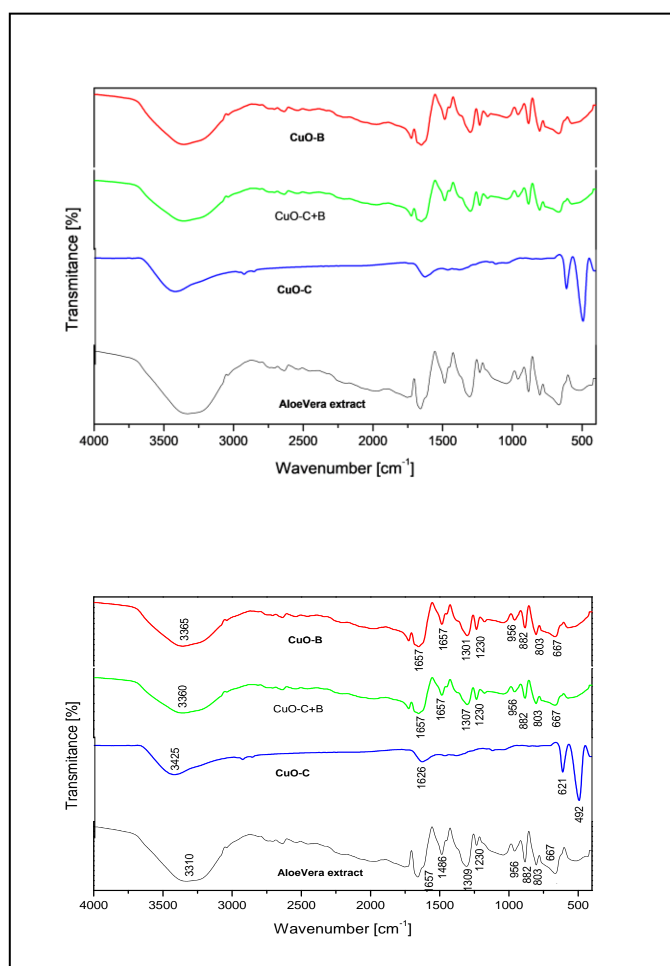

Figure 2: Fourier Transform Infrared Spectroscopy of Aloe vera extract and synthesized CuO nanoparticles with different method (a) NaOH only,(CuO-c) (b), extract and NaOH(CuO-c+b) (c)extract only(CuO-.b).

X-ray diffraction (XRD)

The crystal structure and phase purity of CuO NPs were characterized by XRD. Figure 3 shows the XRD pattern of the CuO prepared using different methods. All preparation method confirmed the synthesis of crystal structure CuO NPs with different puirity, size and crystallinity. XRD patterns at 2θ values 35.17, 38.37, 48.6 which assigned to (002), (111), (202) planes, respectively of monoclinic CuO NPs were observed in all synthesized NPs. The sharp peaks obtained from XRD patterns of chemically synthesized CuO NPs were in corresponds with JCPDS 05-661, which indicates no impurity peaks were observed in these XRD patterns. The average crystallite size calculated from the XRD pattern was 21.10 nm. While other patterns were observed in both Biosynthesized CuO NPs confirms that the Aloe-vera was a strong reducing agent, another characteristic peak for Aloe-vera, were confirmed by XRD at 2θ 38.2, 44 [23]. XRD pattern showed the pure crystalline nature of the as-prepared CuO NPs.

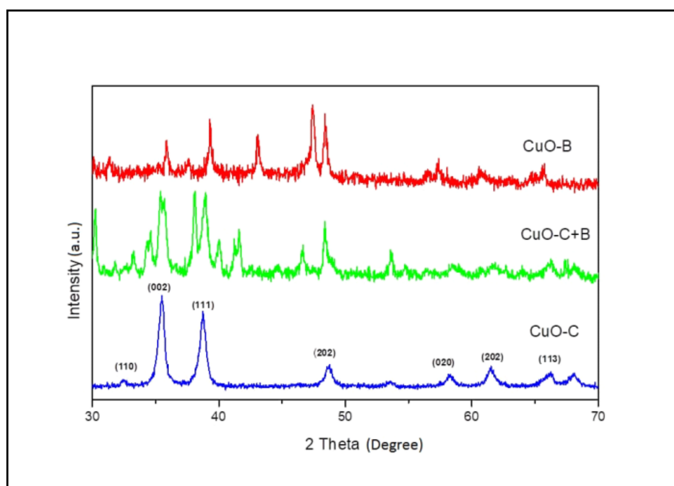

Figure 3: The X-ray diffraction (XRD) of Synthesized CuO nanoparticles with different method (a)NaOH only (CuO-C) (b)Extract only (CuO-B) (c)Extract and NaOH (CuO-C+B).

UV-Vis spectroscopy is a very useful technique for analyzing nanoparticle formation and the stability of metal nanoparticles in aqueous solution [24].

No optical absorption peak for CuO NPs was observed in the UV-Vis spectra of the plant only (extract of aloe vera plant without any addition) . The UV-visible absorption spectrum of synthesized CuO NPs and Aloe-vera extract were shown in Figure 4. The prepared CuO-NPs (CuO-B) had displayed an absorption peak at 285 nm which is assigned to the absorption of CuO NPs.The sample prepared by using NaOH only (CuO-C) gives the same peak as (CuO-B). This spectrum confirms the presence of CuO. The presence of other measurable peak at 550 nm observed in extract and biosynthesized CuO NPs confirm the CuO coating with Aloe-vera residue.

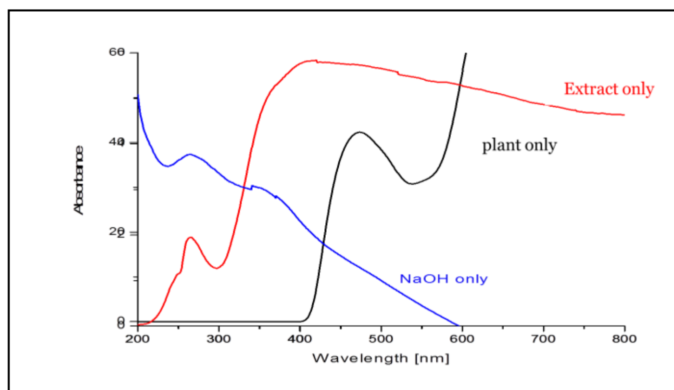

Figure 4: (UV-VIS) spectra of Synthesied CuO nanoparticles with different method (a) NaOH only(b) Extract only (c) plant only.

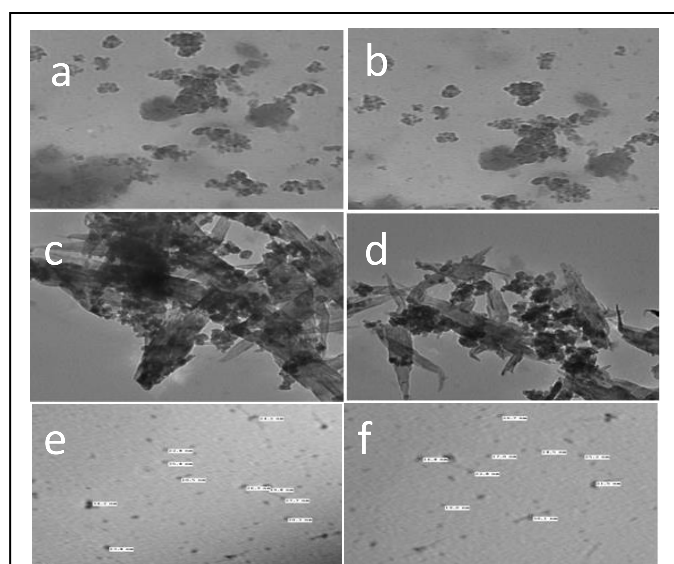

Figure 5: TEM images. a&b are related to CuO nanoparticles using NaOH only. C&d show the fibers of aloe vera in the sample of(CuO-C+B). e&f show the size of the nanoparticles of the sample of (CuO-C+B).

High resolution transmission electron microscope (HRTEM)

Figure 5 a & b reveal rounded but aggregated CuO nano-particles using NaOH (CuO-C) only. The average particle size as indicated by TEM was 23.17 nm, which is in corresponds with the XRD results. This is very similar to those described in

the previous reports Nasrollahzadeh et al., 2015 [1]. Figure 5 c&d show the green biosynthesized of the CuO NPs using extract and NaOH (CuO-C+B). The fibers of the aloe-vera can be clearly seen in the figure. The average particles size is considered lesser on addition of aloe-vera, which considers a good capping reagent. Figure 5 (e&f) showing the synthesized CuO nano-particles using Aloe-vera extract and NaOH; the spherical morphology of the agglomerated CuO with the aloe-vera fibers appears in the figure. The average particles size ranges from 23-34 nm.

Zeta-potential

The potential charges on the surface of the particles reflect their stability. The electric potential at the boundary of the double layer is known as the Zeta potential, was illustrated in Table 2. The zeta Potential was found to be -3.12 and 3.65 mV for the (CuO-C+B) and (CuO-B) respectively while it was found to be 0.19 mv for(CuO-C). The values obtained in this research does fall within the range of +25 or -25 mV, thus according to Malvern (2011) it is stable [25].

Table 2: The average zeta potential for the used different methods.

Sample	Average zeta-potential
Aleo-vera	• 3.12 mv
With NaOH	• 0.19 mv
Aleo-vera and NaOH	• 3.65 mv

Cell viability

In this table 3 the sample was tested against normal human epithelial cell line BJ1 (Normal Skin fibroblast) and HePG2. This table 3 shows the lethal concentration of copper oxide nanoparticles (prepared by using NaOH and aloe vera extract (CuO-C+B)) that cause death of 50% and 90% of HePG2 while the same concentration used to treat normal human epithelial and show no effect on normal cells.

Table 3: The lethal concentration of sample (CuO-C+B) that causes the death of 50% of the cells in 48 hours.

Sample Code	LC_{50} (µg/ml)	LC_{90} (µg/ml)	Remarks
BJ1	--------	--------	23.8% at 100ppm
HEPG2	21.6	37.8	

LC_{50}: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs

LC_{90}: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs

Gene expression by quantitative real time PCR

The action mechanism of the sample in HePG2 cells is due to the examination expression of pro-apoptotic bax.

That was done by using real time PCR in the presence of different concentrations (presented by groupI, II, III,VI,V,I) of the sample (CuO-C+B) in HePG2 cells. The result showed an increase in the level of gene expression of bax in HePG2 cells with increasing the sample concentration as shown in Table 4. HePG2 shows an increase in the level of gene expression of Caspase- 9 level after its treatment with different concentrations of the sample. Fold change increase as compared to control sample. We normalized the results according to house keeping gene as GAPHD gene that expressed by all cells.

Table 4: the level of genes used in RT-PCR for (CuO-C+B) sample.

	control	2.5	5	10	15	25
	group I	group II	group III	group IV	groupV	group VI
bax	1	0.438303	0.650671	1.132884	2.361985	4.594793
bcl-2	1	0.260616	0.171943	0.14161	0.140632	0.092142
caspase 3	1	0.554785	0.574349	1.765406	2.928171	3.09513
caspase 9	1	1.125058	1.231144	1.670176	2.084932	2.143547
p53	1	0.486327	0.707107	0.82932	0.852635	0.907519

The examination of the level gene expression of anti-apoptotic bcl-2 was done by using a real time PCR in existence of different concentrations of nano-copper oxide (sample prepared by using NaOH and aloe vera extract (CuO-C+B)) in HePG2 cells. The results of HePG2 cell line by using real time PCR indicate a noticeable decrease in the level of gene expression of bcl-2 in HePG2 cells by increasing the concentration of nano-copper oxide. Also the examination of the level gene expression of caspase 3 is by using a real time PCR in the presence of different concentrations of nano-copper oxide in HePG2.

The HePG2 showed increase in the level of gene expression of Caspase- 9 level after treatment with different concentrations of nano-copper oxide (CuO-C+B) .The toxicity of CuO NPs has been assumed as a serious limitation for their applications and prior mechanistic toxicological characterization of this material is needed. This study clarify the cytotoxicity, and apoptosis response of CuO NPs on human hepatocellular carcinoma HepG2 cells. In this study, it has observed that the expressions

of both mRNA and protein levels of tumor suppressor gene p53 and apoptotic genes (bax and cleaved caspase-3) were up-regulated while the expression of anti-apoptotic gene bcl-2 was down-regulated in HepG2 cells treated with CuO NPs. It has been supposed that bax is up-regulated by p53 [27]. When an increase in bax expression was recognized, the role of p53 in the up-regulation of bax upon CuO NPs exposure can be postulated.

The insertion of bax into the mitochondrial membrane possibly leads to p53-mediated apoptosis [27].On the other hand Caspases are activated during apoptosis in many cells and are known to play a vital role in both initiation and execution of apoptosis. It was reported that activated caspase-3 (cleaved caspase-3) is essential for cellular DNA damage and apoptosis [28]. The obtained findings suggest that CuO NPs has anticancer role against HePG2 cell line through its effect on cell viability of the cells and also through up regulation of p53, bax, Caspase-9 /caspase3 and down regulation of bcl-2 gene expression.

The HePG2 cell lines showed decrease in the level of expression of bcl-2 by increasing copper oxide NPs concentration.The cytotoxicity in HepG2 cells in dose-dependent manner of CuO NPs was found to be encouraging. Tumor suppressor gene p53 and apoptotic gene caspase-3 were up-regulated due to the exposure CuO NPs.

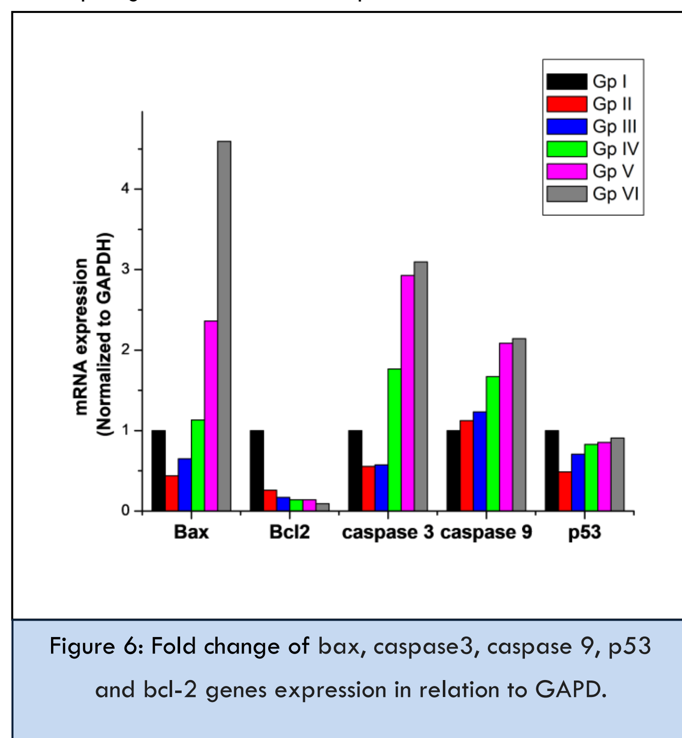

Figure 6: Fold change of bax, caspase3, caspase 9, p53 and bcl-2 genes expression in relation to GAPD.

CONCLUSION

An eco-friendly, low-cost biosynthetic method for the preparation of CuO NPs using *aloe vera* is presented in this work CuO NPs exhibit very low cytotoxicity towards normal cells ,while stimulate apoptosis in hepatocellular carcinoma.

The cytotoxicity in HepG2 cells in dose-dependent manner of CuO NPs was found to be encouraging. Tumor suppressor gene p53 and apoptotic gene caspase-3 were up-regulated due to the exposure CuO NPs. This study provided valuable insights into the possible mechanism of liver toxicity caused by the prepared material at *in vitro* level. The short-term exposure study of high level induction of apoptotic response of CuO NPs will need to be further investigated to determine whether long-term exposure consequences may exist for CuO NPs application.

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