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COMPARATIVE STUDY ON THE EFFECT OF NANO-CURCUMIN COMPLEXES AS ANTITUMOR IN MICE

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ABSTRACT

This study was undertaken to compare the antitumor activity of copper oxide nanoparticles (CuO-NPs), hydrazine sulphate-copper nanoparticle (HS-Cu-NPs), nano-curcumin (Nano-Cur), curcumin capped copper nanoparticles (Cur-Cu-NPs) and native curcumin (Native-Cur) with ascorbate *in vivo* and *in vitro* using breast carcinoma cell line (MCF-7). **Methods:** CuO-NPs and HS-Cu-NPs were injected intratumorally at dose 10 mg/Kg b.wt., Cur-Cu-NPs was injected intratumorally at dose 50 mg/Kg b.wt., Nano-Cur and Native-Cur were injected intratumorally (G6, G7) or administered orally (G8, G9) at dose 50 mg/Kg b.wt. *In vivo*, Female mice were divided into 9 groups: **G1:** Healthy control. **G2:** tumor bearing mice (TBM). **G3:** TBM+CuO-NPs + ascorbate. **G4:** TBM+ HS-Cu-NPs + ascorbate. **G5:** TBM+Cur-Cu-NPs + ascorbate. **G6, G8:** TBM+Nano-Cur + ascorbate **G7, G9:** TBM+Native-Cur +ascorbate. *In vitro*, Cell lines were treated with the different nano complexes for 48h. **Results:** *In vivo*, Different nano-complexes significantly reduced tumor markers and inflammation immunologic markers and significantly elevated the caspase-3 activity and P53 gene expression**.** These results were confirmed by histopathological examination and immunohistochemistry. *In vitro*, all nano-complexes showed a clear cytotoxic effect on (MCF-7). **In conclusion:** All nano-complexes administration significantly inhibited tumor growth and reduced tumor markers but Cur-Cu nano-complex was the most effective treatment.

KEYWORDS: Curcumin, Nanocurcumin, Curcumin capped copper nanoparticles, EAC, tumor bearing mice.

INTRODUCTION

Cancer is the second most common cause of disease-related death in the United States.[1] Recent advances in nanoparticle-based cancer drug delivery present a promising strategy to achieve high therapeutic efficiency of anticancer agents by providing protection during circulation and enhancing their bioavailability.[2] Nanoparticles (NPs) are typically defined as particles with diameter from 1 to 100 nm and have been exploited for both diagnostic and therapeutic purposes. Therefore, NPs must be sufficiently small to perfuse out of the blood stream, penetrate the vessels, and reach the tumor site.[3]

Turmeric (*Curcuma longa*) belongs to the family *Zingiberaceae.* The most important part of the turmeric tuber is a group of bioflavonoids, i.e. curcumins. Curcumin (Cur) has significant anti-inflammatory, antioxidant, chemoprotective, anticancer, and gastroprotective properties.[4] Reducing the particle size of Cur in the nanometer range not only improves its aqueous phase solubility and cellular uptake but also enhances its activity as anticancer agent.[5]

Cur forms strong complexes with most of the known metal ions. It has been observed that complexation with Cur reduces the toxicity of metals and some Cur complexes with metals like Cu^{2+}, act as new metal-based antioxidants.[6] Previous studies have shown that the different metal oxide nanoparticles induce cytotoxicity in cancer cells, but not in normal cells.[7] Recent study demonstrated that copper oxide nanoparticles (CuO-NPs) selectively induce apoptosis of tumor cells *in vitro.*[8]

Hydrazine sulphate (HS) is a chemical compound that has been studied as a treatment for cancer and certain side effects caused by cancer. HS may block the tumor from taking glucose that tumor cells need to grow.[9]

Vitamin C (Vit C) is a naturally occurring potent antioxidant and cofactor for many enzymes. Vit C has been demonstrated to induce apoptosis in cancer cells by creating oxidative stress via upregulation of reactive oxygen species (ROS) release.[10] High dosage of vit C as an anti-cancer therapy has shown to lessen chemo-therapy side effects.[11]

MATERIALS AND METHODS

1. Materials

1.1. Curcumin: Curcumin was purchased from HiMedia Laboratories, Mumbai, India.

1.2. Tumor cell line: MCF-7 (breast carcinoma cell line) was obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection. The murine Ehrlich ascites carcinoma (EAC) cells were provided by the oncology unit, from the National cancer institute (NCI), Cairo University, Egypt.

1.3. Animals: One hundred and eighty healthy adult female Swiss albino mice weighing between 20-25g were supplied from oncology unit – (NCI), Cairo University, Egypt. Mice were maintained on standard commercial pellets diet [12] and tap water *ad libitum*, and kept individually in stainless cages in constant conditions.

1.4. Chemicals: Copper oxide nanoparticles, Hydrazine sulphate, Vitamin C, Poly vinyl alcohol and Sodium Borohydride were purchased from (Sigma, USA). All other chemicals were purchased from El- Gomhouria Company, Cairo, Egypt.

2. Methods

2.1. Synthesis of nanocomplexes: The present study includes 4 nanocomplex (NC) compounds:

2.1.1. Copper oxide Nanocomplex (CuO-NCs): Copper oxide Nanocomplex was synthesized using copper oxide nanoparticles (CuO-NPs) with ascorbate as described by ***Kimoto et al.***[13]

2.1.2. Hydrazine Sulphate-Copper Nanocomplex (HS-Cu-NCs): The (HS-Cu-NPs) with ascorbate was prepared according to the method described by ***Morsy et al.***[14]

2.1.3. Curcumin capped copper Nanocomplex (Cur-Cu-NCs): The synthesis of (Cur-Cu-NPs) with ascorbate was carried out according to the method described by ***Kamble et al.***[15]

2.1.4. Curcumin Nanocomplex (Cur-NCs): the highly basic nanocurcumin salt with ascorbate was prepared according to the method described by ***Hassan et al.***[16]

2.2.Nanoparticles characterization: Transmission electron microscopy (TEM): Observations in the Nano scale to the corresponding nanoparticles for determination of the size and shape were shown from TEM as described by ***Basniwal et al.***[17]

2.3. *In vitro* study: (Cytotoxicity assay): Evaluation of the cytotoxicity of different nano-complexes against breast carcinoma cell line (MCF-7) was carried out by Sulphorhodamine-B assay of cytotoxic activity as described by ***Muthuraman et al.***[18]

2.4. *In vivo* study

2.4.1. Assessment of LD50 for different nanocomplexes: LD50 was examined for all different nanocomplexes using healthy adult female Swiss albino mice according to the method described by ***Narang and Desavi.***[19]

2.4.2. Ehrlich Ascites Carcinoma (EAC) cells preparation: EAC cells were maintained *in vivo* according to the method recommended by the Egyptian National Cancer Institute, Cairo University. Cells were harvested and the desired concentration of tumor cells was obtained by dilution with saline (0.9% NaCl).

2.5. Experimental design: Throughout this study, a total number of 180 adult female Swiss albino mice were subjected to experimentation. The animals were divided into 9 groups (20 mice /group) as follows:

Group I: Healthy Control group (HC): healthy mice were fed on standard pellet diet and water *ad libitum* until the end of the experiment.

Group II: Tumor bearing mice group (TBM): mice in this group were inoculated with a single intramuscular (IM) injection of 2.5 $x10^6$ EAC/ml in the right thigh to form a solid tumor as described by ***Abd ElDayem et al.***[20]

Group III: Copper oxide nanocomplexes group (CuO-NCs): mice in this group were inoculated with a single intramuscular injection of 2.5 $x10^6$ EAC/ml in the right thigh, then treated with CuO-NCs intratumorally (10 mg/Kg b.wt.) suspensed in 0.3 ml distilled water three times per week for two weeks.

Group IV: Hydrazine sulphate-copper nanocomplexes group (HS-Cu-NCs): mice in this group were inoculated with EAC as mentioned before, then treated with (HS-Cu-NCs)

intratumorally (10 mg/Kg b.wt.) suspensed in 0.3 ml distilled water solution three times per week for two weeks.

Group V: Curcumin Capped Copper nanocomplexes group (Cur-Cu-NCs): mice in this group were inoculated with EAC as mentioned before, then treated with Cur-Cu-NCs intratumorally (50 mg/Kg b.wt.) dissolved in 0.3ml distilled water solution three times per week for two weeks.

Group VI: Curcumin nanocomplexes group (Cur-NCs): mice in this group were inoculated with EAC as mentioned before, then treated with Cur-NCs intratumorally (50 mg/Kg b.wt.) suspensed in 0.3ml distilled water solution three times per week for two weeks.

Group VII: Native Curcumin complexes group (Native-Cur-Cs): mice in this group were inoculated with EAC as mentioned before, then treated with Native-Cur-Cs intratumorally (50 mg/Kg b.wt.) suspensed in 0.3ml distilled water three times per week for two weeks.

Group VIII: Oral curcumin nanocomplexes group (O-Cur-NCs): mice in this group were inoculated with EAC as mentioned before, then treated orally with Cur-NCs (50 mg/Kg b.wt.) suspensed in 0.3ml distilled water by gavage tube three times per week until the end of the experiment.

Group IX: Oral native Curcumin complexes group (O-native-Cur-Cs): mice in this group were inoculated with EAC as mentioned before, then treated orally with (50 mg/Kg b.wt.) of native curcumin complexes suspensed in 0.3ml distilled water by gavage tube three times per week until the end of the experiment.

2.6. Blood sample collection: At the end of experimental period, 10 mice from each group were scarified after 12 hours fasting with water *ad libitum*. Blood was allowed to stand for the separation of serum. Serum was kept in plastic vials at -20°C until used for biochemical analyses.

2.7. Tissues sampling: Liver and tumor were separated and cleaned, rinsed and washed by saline solution. Part of the liver and tumor were stored frozen at -20°C until used for tissue biochemical analyses. Another portion of tumor tissues were stored frozen at -80°C for gene expression analysis.

2.8. Tumor assessment: The effect of different NCs on tumor growth inhibition and host's survival time was examined by studying the following parameters:

2.8.1. Assessment of tumor weight: After solid tumors were separated and cleaned, tumor weight (g) was recorded immediately using sensitive digital scale.

2.8.2. Assessment of tumor volume: After dissection, the size of solid tumor was measured using Vernier caliper to measure the two axes; the tumor volume was calculated using the following formula as described by ***Jensen et al***.[21]

$$\text{Tumor volume } (mm^3) = 0.52 \text{ X } (\text{length x width}^2)$$

Where length is the greatest longitudinal diameter and width is the greatest transverse diameter.

2.8.3. Measurement of tumor growth inhibition

Tumor growth inhibition ratio (T/G %) was recorded as described by ***Abd El Dayemet al.***[20] using the following formula:

T/G (%) = (Mean tumor weight of TBM group – Mean tumor weight of treated group/ Mean tumor weight of TBM group) X 100

2.8.4. Measurement of life span

Ten mice of each group were kept alive to measure the mean survival time (MST). Mice were monitored by daily recording the mortality. The percentage of increased life span (%IL) was calculated using the following equations as described by ***Ayyad et al.***[22]

Mean survival time (MST) = [(day of first death + day of last death)/2]

ILS (%) = [(MST of treated group/MST of EAC group) -1] X 100

2. 9. Biochemical measurements

2.9.1. Assessment of tumor markers

2.9.1.1. Assessment of serum CA15-3 (carbohydrate Antigen 15-3)

CA15-3 was determined quantitatively in serum samples using sandwich enzyme-linked immune-sorbent assay according to the method of ***Luftner et al.***[23] by CA15-3 ELISA kit.

2.9.1.2. Assessment of serum Alkaline Phosphatase (Alp) activitiy

Serum ALP activity was measured using the method described by ***Zawta et al.***[24]

2.9.2. Assessment of cell apoptosis

a. ***Apoptotic assay In vitro***

Apoptosis Detection with Hoschet 33342 staining assay: Apoptosis in the breast cancer cell lines (MCF-7) was screened through fluorescence microscopy visualization. Hoschet 33342 is a fluorochrome for nuclear staining which permits uniqueness between viable, apoptotic and necrotic cells. In this work, the breast cancer cell lines (MCF-7) were subjected to Hoschet staining after treatment with different nanocomplexes, Images of the cells were taken by a UV-fluorescence microscope within 30 min to study the apoptosis-induction potential according to the method described by ***Lovine et al.***[25]

b. ***Apoptotic assay In vivo***

1- Assessment of Caspase-3 activity in tumor tissues

The activity of caspase-3 was determined quantitatively in tumor tissues using Caspase-3 ELISA kit according to the method described by ***Kaushal et al***.[26]

2- P53 gene expression analysis RNA extraction

Total cellular RNA was extracted from frozen tissue samples of solid Ehrlich tumor. First strand cDNA was generated from 1µ of total RNA according to the method described by ***Bassiony et al.***[27]

Quantitative real time (qRT-PCR): Synthesized cDNA was quantified using SYBR green-based real-time PCR according to the method described by ***Bassionyet al.***[27]

3- P53 Immunohistochemical analysis

Immunohistochemical examination of p53 was performed using Streptavidin-Biotin method by Histostain-plus kit (Zymed, USA). For counterstaining, sections were stained with hematoxylin, then dehydrated and mounted. Sections were examined using light microscope (Olympus, CX41, Japan) to evaluate p53 immunostaining. Positive nuclei for p53 accumulation were stained brown.[28]

2.9.3. Assessment of inflammation/immunologic markers

2.9.3.1. Assessment of serum interleukin-6 (IL-6)

The determination of serum IL-6 was performed following the quantitative immunoassay techniques according to the method described by ***Gaines Das and Poole.***[29]

2.9.3.2. Assessment of C-reactive protein (CRP)

CRP in serum was measured by Enzyme-Linked Immunosorbent Assay (ELIA) according to the method described by ***Ridker et al.***[30]

2.9.4. Assessment of oxidative stress markers

Oxidative stress markers measured in liver tissues included: (GSH) concentration,[31] (CAT) enzyme activity,[32] and (MDA) level.[33]

2.10. Statistical analysis

Data were analyzed using the Statistical Package for Social Science (SPSS) program, version 17.0. The data were expressed as mean ± standard deviation (S.D) of the mean. Statistical differences between groups were performed using one way analysis of variance (ANOVA). The mean difference was significant at $P < 0.05$ level according to ***Levesque.***[34]

RESULTS

1. Nanoparticles characterization

High Resolution Transmission Electron Microscopy (HR-TEM): The morphology and diameter of Nanoparticles were analyzed using HR-TEM. TEM average diameter was calculated from measuring over 100 particles in random field of TEM view. TEM image revealed that average particle size of CuO-NPs, HS-Cu-NPs, Cur-Cu-NPs and the formed Nano-cur sodium salt were approximately around 39.46 - 62.47 nm, 16.81 - 23.77 nm, 67.55 -79.07 nm and 0.6 - 5.26 nm, respectively (fig.1).

2. *In vitro* study

Cytotoxic assay: Cell lines were treated with the different concentrations (25, 50, 100, 200 ug/ml) of different nano-complexes for 48h.The different nano complexes showed a clear cytotoxic effect on breast carcinoma cell line (MCF-7) and a clear concentration-response relationship. As shown in (fig.2), the calculated IC50 indicated that CuO-NCs and HS-Cu-NCs were more cytotoxic to MCF-7, then Cur-NCs followed by native-Cur-Cs. The least effect exerted by Cur-Cu-NCs.

3. *In vivo* study

3.1. Effect of different nano-complexes on tumor assessment: The incidence of solid tumor in G2, G3, G4, G5, G6, G7, G8 and G9 was 100%. The tumors were very prominent

and fast growing in G2, While G3, G4, G5, G6, G7, G8 and G9 showed a markedly smaller and slower tumor growth compared to G2 (Fig.3).

Remarkably, as shown in table (1), the significant reduction in tumor weight were -78.7% for Cur-Cu-NCs treatment followed by -69.1% for Cur-NCs treatment then G3 and G4 (chemical treatments) recorded -64.2% and -61.8% respectively and finally G8 recorded -60% when compared with G2. Whereas, groups administrated native-Cur-Cs either injection or oral showed less impact on tumor weight reduction reached -58.2% and -43.6% respectively as compared with G2 ($P<0.05$).

Moreover, significant and progressive tumor suppression in G3, G4, G5, G6, G7, G8 and G9 was also recorded with a percentage of significant reduction in tumor volume, -98.6%, -98.6%, -99.2%, -98.9%, -98.5%, -98.5% and -97.5% respectively, when compared with G2 *($P<0.05$)* (table.1).

Similarly, results illustrated in table (2), show the inhibition of tumor growth (T/G %) in different nano-complexes treatments in G3, G4, G5, G6, G7, G8 and G9. It was 54.8%, 51.4%, 70.37%, 62.9%, 51.9%, 37.04% and 33.3% when compared to G2 in the same order. The previous results indicated that there was a significant increase in mean survival time (MST) and increased life span (%ILS) in all TBM treated groups especially in G5. Cur-Cu NCs treatment showed the highest prolongation of MST; (46 days) after that G6, G7 caused a remarkable prolongation of MST (44 days) and (41days) respectively, then G8, G9 recorded (37 days) and (36 days) respectively and finally G3 and G4 (chemical treatments) caused a slight prolongation of MST; (32 days) and (34 days) respectively as compared with G2 (27 days), $P<0.05$.

3.2. Effect of different nano-complexes on serum tumor marker (CA 15-3) level and (ALP) activity

Table (3) shows the Serum (CA 15-3) levels in different nano-complexes groups. Cur-Cu-NCs treatment in G5 was the most effective treatment that caused a significant reduction in serum CA 15-3 level by -87.1% as compared to G2. whereas, There was a significant decrease in serum (CA 15-3) levels in G3 and G4 by -79.3% and -78.8% respectively while Cur-NCs and native-Cur-Cs treatments caused a significant decrease in serum (CA 15-3) levels in G6, G7, G8 and G9 by -79.6%, -41.7%, -63.7% and -23.1% respectively as compared to G2 *($P<0.05$)*.

As indicated in table (3), regarding to ALP activity, ALP activity was significantly and noticeably decrease in G5 (Cur-Cu- NCs) and G6 (Cur-NCs) to the control level by -61.2% and -60.3% when compared to G2 *(P<0.05)*. Chemical treatments in G3 and G4 caused a significant decrease in ALP activity by -45.9% and -42.6% respectively while Cur-NCs and native-Cur-Cs treatments in G7, G8 and G9 caused a significant decrease by -37.2%, -47.5% and -30.6% respectively as compared to G2 (*P<0.05*).

3.3. Effect of different nano-complexes on cell apoptosis

a. *In vitro* study

As shown in figure (4), the alterations in nuclear morphology in response to different nano-complexes treatments were assessed by nuclear counterstain (Hoschet 33342). Different nano-complexes significantly reduced the number of colonies of breast carcinoma cell line (MCF-7). Cur-Cu-NCs was the most effective cytotoxic treatment to MCF-7 as there were less colonies and more nuclear condensation as compared to the control. Noteworthy, CuO-NCs and HS-Cu-NCs treatments were more toxic to (MCF-7), then Cur-NCs and native-Cur-Cs compared to the control.

b. *In vivo* study

1- Effect of different nano-complexes on Caspase-3 activity (Casp-3) in tumor tissues

Table (4) shows the results of caspase-3 activity in tumor tissues. There was a highly significant increase of 481.4%, 431.4%, 550%, 262.9%, 190%, 171.4% and 111.4% of caspase activity in tumor tissues of treated groups in G3, G4, G5, G6, G7, G8 and G9 respectively compared to G2 *(P<0.05)*. Noticeably, Cur-Cu-NCs treatment was highly apoptotic that caused a significant increase in caspase-3 activity in G5 compared to untreated TBM group.

2-Effect of different nano-complexes on P53 gene expression in tumor tissues

Changes of the gene expression by different nano-complexes were shown in table (4). Expression of P53 was examined by real time PCR. Results showed that the expression levels of P53 were down regulated in TBM (G2) as compared to the control muscle (G1). Furthermore, the levels of P53 was significantly increased more than the control level in TBM treated with Cur-Cu-NCs in G5 and Cur-NCs in G6 by 2686.7% and 2240.7% respectively as compared to G2 *(P<0.05)*. In addition, increase in expression of P53 was noticed in G3, G4, G7, G8 and G9 by 273.3%, 286.7%, 526.7%, 553.3% and 146.7%

respectively as compared to G2, however, this increase still significantly less than the control level.

3- P53 Immunohistochemistry of Ehrlich solid tumor

Results of P53 gene expression were significantly confirmed by immunohistochemical analysis where TBM received Cur-Cu-NCs and Cur-NCs highly expressed P53 (score 3, +++) in G5 and G6, respectively (Fig. 5) compared to moderate expression (score 2, ++) of P53 in TBM received CuO-NCs, HS-Cur-NCs, native-Cur-Cs and Cur-NCs in G3, G4, G7 and G8 respectively. Weak positive expression of P53 (score 1, +) was observed in G9 treated with oral Native-Cur-Cs as compared to negative staining of P53 in untreated TBM.

3.4. The effect of different nano-complexes on serum inflammation/immunologic markers C-reactive protein (CRP) and interleukin-6 (IL-6) in experimental groups

Regarding to the inflammation immunologic markers, there was a significant increase in CRP and IL-6 levels in untreated TBM. In contrast, Mice in G5 treated with Cur-Cu-NCs exhibited a significant decrease in CRP and IL-6 levels by -82.8% and -39.5%, respectively, while CuO- NCs and HS-Cur-NCs treatments caused a significant decrease in CRP levels by -72.2% and -73.1% respectively and a marked significant decrease in IL-6 levels by -30.4% and -31.02% respectively as compared to untreated group. A statistically significant reduction in CRP levels by -69.2%, -43.6%, -59.03% and -26.4% in G6, G7, G8 and G9 as compared to G2. Also there was a significant reduction in IL-6 levels by -31.7%, -22.5%, -26.7% and -10.6% in G6, G7, G8 and G9 as compared to G2 (table. 5).

3.5. The effect of different nano-complexes on oxidative stress markers in liver tissues

Table (6) illustrates oxidative stress markers measured in liver tissue homogenates. GSH content was considerably decreased in G2 by 5.4% relative to G1. Remarkably, CuO-NCs, HS-Cu-NCs and Cur-Cu-NCs treatments caused a significant decrease in GSH content in G3, G4 and G5 by -41.1%, -31.8% and -23.3%, respectively compared to G2. Cur-NCs and native-Cur-Cs treatments caused a substantial elevation of GSH content in groups 6, 7, 8 and 9 by 37.3%, 33.6%, 29.9% and 28.2%, respectively as compared to G2, *($P<0.05$)*.

Regarding to CAT enzyme activity, it was found to be significantly lowered in G3, G4 and G5 by- 34.9%, -29.7% and -24.1% correspondingly, when compared with G2. In G6, G7, G8 and G9, CAT activity in liver tissue homogenates was expressively increased by 52.9%, 41.9%, 32.9% and 43.3% respectively, as compared to G2 *($P<0.05$)*(table.6).

As exhibited in table 6, a significant increase in MDA levels in G3, G4 and G5 by 226.6%, 188.2% and 170.4%, respectively which associated with a significant decrease in GSH content and catalase activity. Predictably, Hepatic MDA levels in Cur-NCs and native-Cur-Cs treatments were significantly lowered by -17.2%, -11.2%, -11.2% and -13.6% for G6, G7, G8 and G9, respectively as compared to G2 *(P<0.05)*.

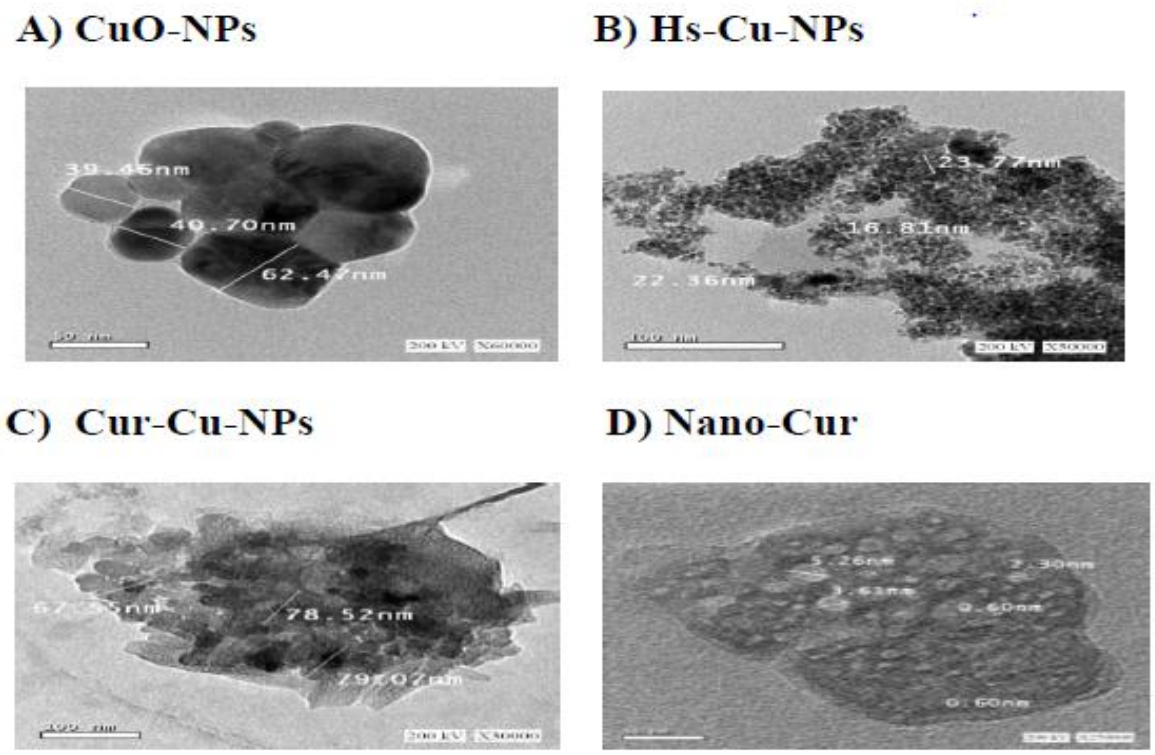

Figure 1: HR-TEM image of the prepared NPs shows that: A) CuO-NPs with average size 39.46 - 62.47 nm. **B)** HS-Cu-NPs with average size 16.81 - 23.77nm. **C)** Cur-Cu-NPs with average size 67.55 - 79.07nm. **D)** Nano-Cur particles with average size 2.30 - 5.26 nm.

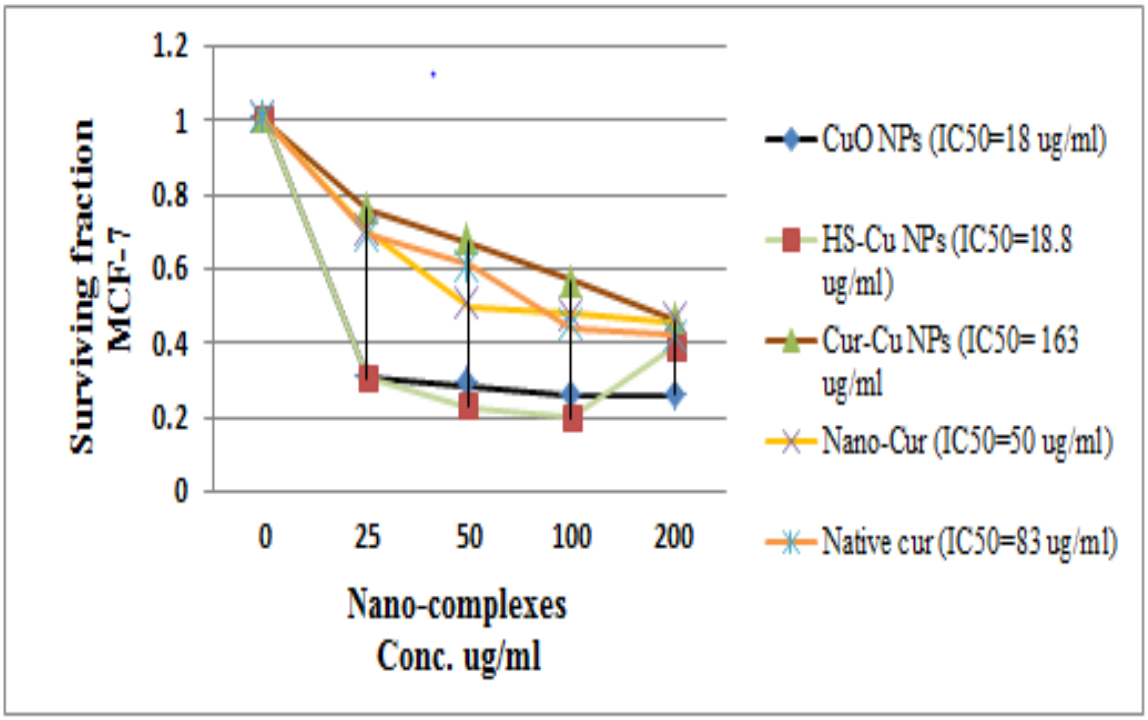

Figure 2: Cytotoxic assay indicates the effect of different nano-complexes on surviving fraction of MCH-7 cell line after 48 h at different concentrations.

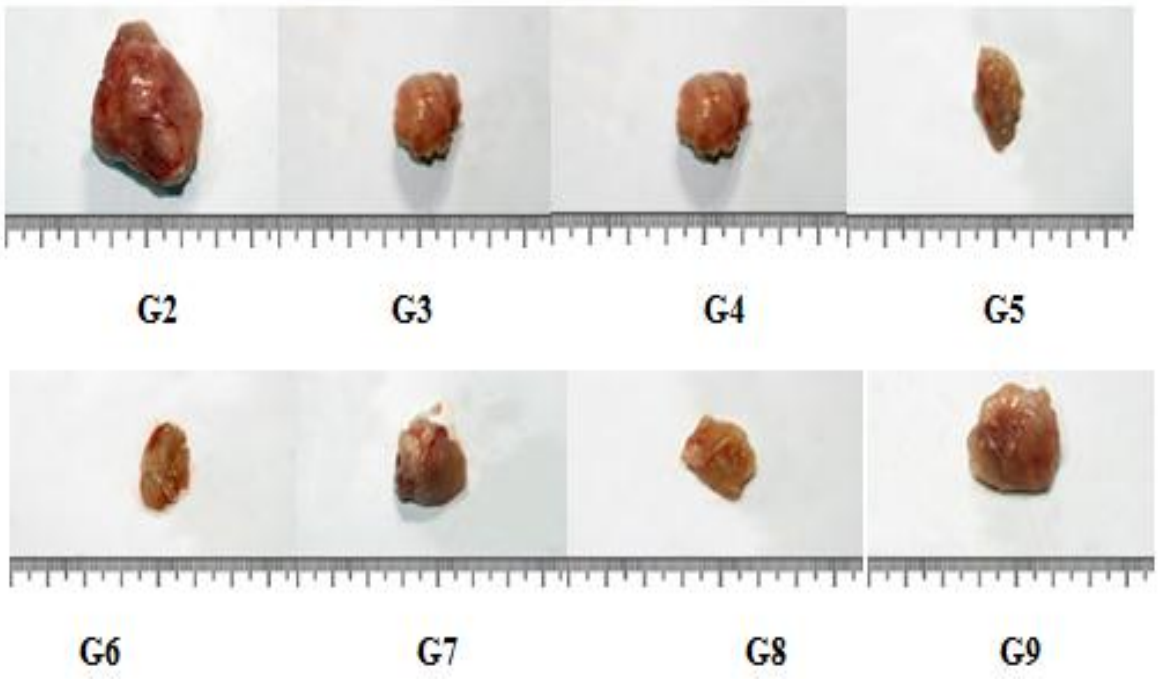

Figure 3: Representative images illustrating the effect of different nano-complexes treatments on solid tumor volume in different experimental groups (G2: TB, G3: CuO-NCs, G4: HS-Cu-NCs, G5: Cur-Cu-NCs, G6: Cur-NCs, G7: Native-Cur-Cs, G8: O-Cur-NCs, G9: Native-O-Cur-Cs).

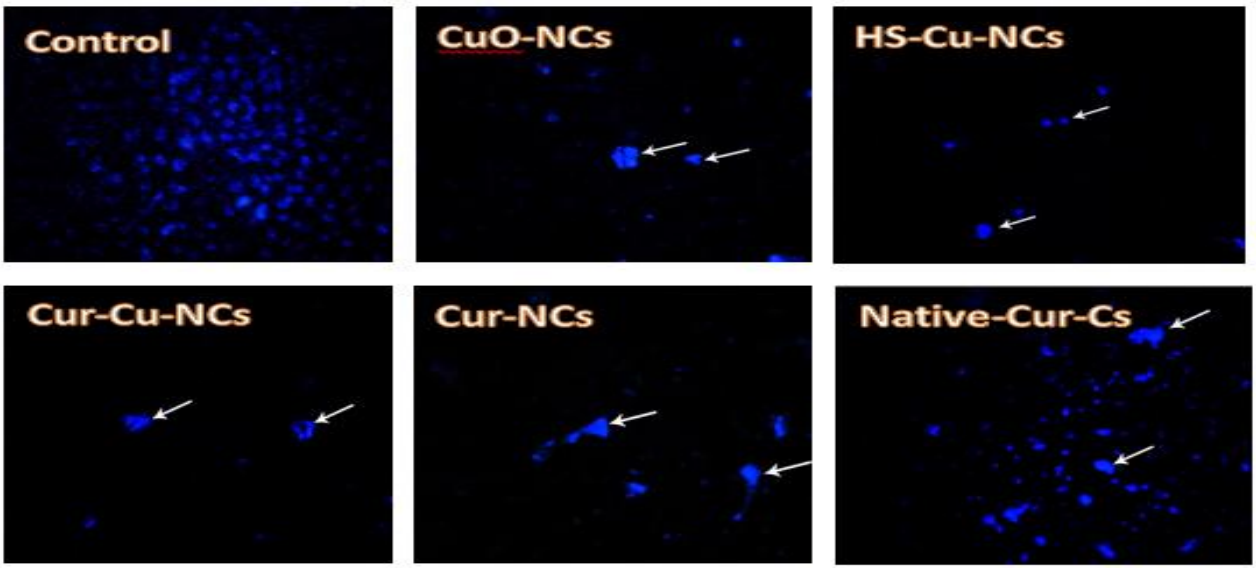

Figure 4: Effect of different nano-complexes on cell nuclei. Cells were stained with Hoechst 33342 to image the nuclei. Condensed nuclei of (MCF-7) cell line indicate apoptotic cells.

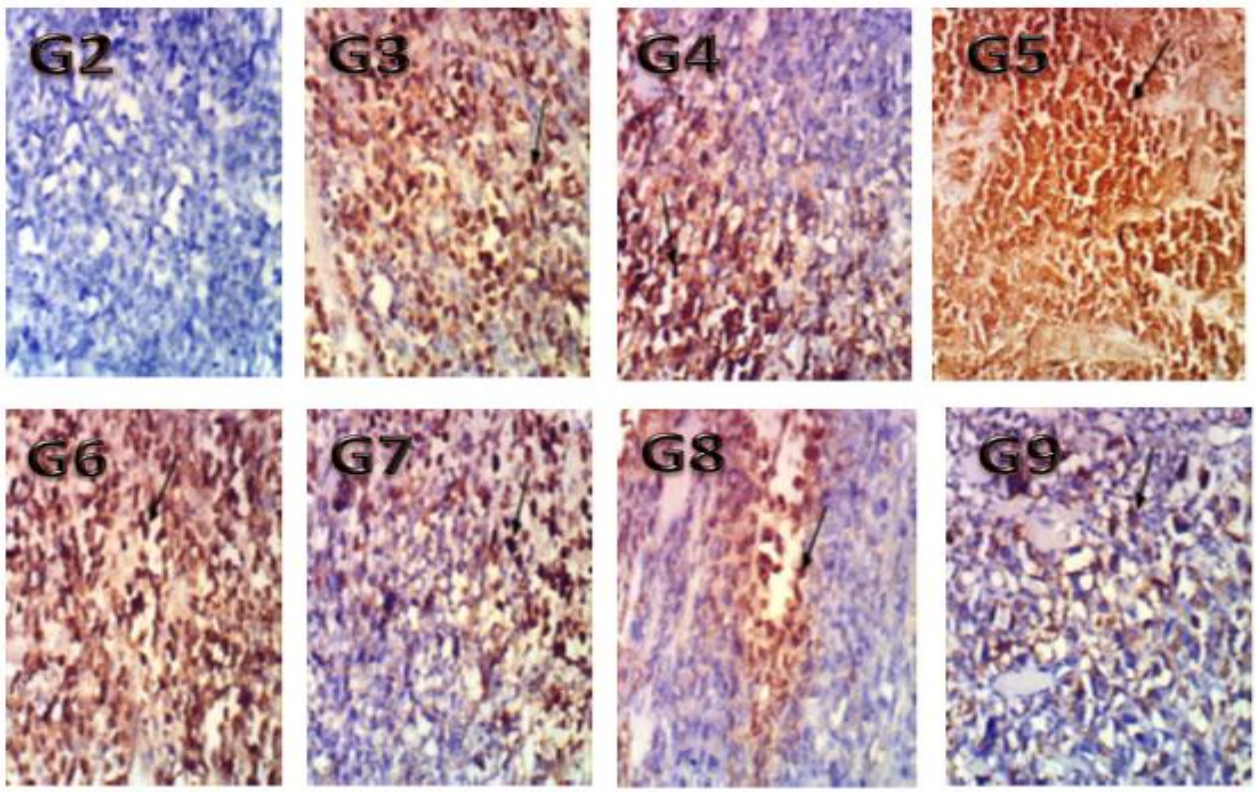

Figure 5: Photomicrographs represent immunohistochemistry staining of p53 expression of Ehrlich solid tumor from mice. G2: Shows negative immunohistochemical reaction (no expression of P53) (score 0) in TBM. **G3, G4, G7, G8:** shows moderate positive expression of P53 (score 2, ++) in TBM treated IT with CuO-NCs, HS-Cu-NCs, Native cur-Cs and orally with O-Cur-NCs respectively. **G5, G6:** shows over positive expression of P53

(score 3, +++) in TBM treated (IT) with Cur-Cu-NCs and Cur-NCs. **G9** shows weak positive expression of P53 (score 1, +) in TBM treated with Native O-Cur-Cs. Magnification is (X400).

Table 1: Effect of different nanocomplexes on tumor weight (g) and tumor volume (mm^3) in experimental groups.

Groups	Parameters	
	Tumor weight (g)	Tumor volume (mm^3)
G2: Tumor bearing (TB) group	3.85 ± 0.35^{a}	4785.00 ± 539.86^{a}
G3: Copper oxide nano-complexes (CuO-NCs) group	1.38 ± 0.29^{b}	66.77 ± 36.17^{b}
G4: Hydrazine sulphate copper nano-complexes (HS-Cu-NCs) group	1.47 ± 0.26^{bc}	66.98 ± 35.02^{b}
G5: Curcumin Capped Copper nano-complexes (Cur-Cu-NCs) group	0.82 ± 0.30^{d}	38.38 ± 12.32^{b}
G6: Curcumin nano-complexes (Cur-NCs) group	1.19 ± 0.32^{b}	54.96 ± 45.83^{b}
G7: Native curcumin complexes (Native-Cur-Cs) group	1.61 ± 0.23^{bc}	73.79 ± 45.29^{b}
G8: Oral curcumin nano-complexes (O-Cur-NCs) group	1.54 ± 0.19^{bc}	70.10 ± 42.37^{b}
G9: native Oral curcumin complexes (Native-O-Cur-Cs) group	2.17 ± 0.30^{c}	117.42 ± 83.64^{b}

• Values are mean ±SD.

•There is no significant difference between means having the same letter in the same column ($p \leq 0.05$).

Table 2: Effect of different nanocomplexes on life span and tumor growth inhibition in tumor bearing mice groups.

PTI (days)	Mortality							
	TB	CuO-NCs	HS-Cu-NCs	Cur-Cu-NCs	Cur-NCs	Cur-Cs	O - Cur-NCs	O-Native-Cur-Cs
15	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
21	1/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
24	1/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10
26	2/10	2/10	1/10	0/10	0/10	0/10	0/10	1/10
27	4/10	2/10	2/10	0/10	0/10	0/10	1/10	1/10
31	4/10	2/10	3/10	0/10	0/10	0/10	1/10	2/10
35	7/10	3/10	3/10	0/10	0/10	2/10	2/10	2/10
39	10/10	4/10	3/10	1/10	2/10	3/10	3/10	3/10
40		7/10	4/10	1/10	2/10	4/10	3/10	5/10
43		10/10	10/10	2/10	3/10	6/10	6/10	7/10
45				3/10	5/10	7/10	9/10	10/10

46				**6/10**	**8/10**	**10/10**	**10/10**	
48				**8/10**	**10/10**			
52				**10/10**				
MTW	**5.90**	**2.67**	**2.87**	**1.21**	**1.83**	**2.44**	**2.28**	**3.20**
MST	**27**	**32**	**34**	**46**	**44**	**41**	**37**	**36**
ILS%		**18.52**	**25.93**	**70.37**	**62.96**	**51.85**	**37.04**	**33.33**
T/G%		**54.75**	**51.36**	**79.49**	**68.98**	**58.64**	**61.36**	**45.76**

PTI: Post Tumor Inoculation MTW: Mean Tumor Weight

MST: Mean Survival Time ILS%: Increase in life span%

T/G%: Tumor growth inhibition

Table 3: The effect of different nano-complexes on serum tumor markers (CA 15-3) and Alkaline phosphatase (ALP) activity in different experimental groups.

Groups	**Parameter**	
	CA 15-3	**ALP**
	ng/ml	**U/L**
G1: Control normal (CN) group	0.05 ± 0.05^{a}	46.70 ± 2.22^{a}
G2: Tumor bearing (TB) group	3.72 ± 0.11^{b}	116.40 ± 9.53^{b}
G3: Copper oxide nano-complexes (CuO-NCs) group	0.77 ± 0.09^{c}	62.90 ± 5.63^{c}
G4: Hydrazine sulphate copper nano-complexes (HS-Cu-NCs) group	0.79 ± 0.07^{cd}	66.85 ± 5.64^{c}
G5: Curcumin Capped Copper nano-complexes (Cur-Cu-NCs) group	0.48 ± 0.03^{e}	45.20 ± 7.50^{a}
G6: Curcumin nano-complexes (Cur-NCs) group	0.76 ± 0.08^{cd}	46.25 ± 7.01^{a}
G7: Native curcumin complexes (Native-Cur-Cs) group	2.17 ± 0.09^{f}	73.10 ± 1.72^{d}
G8: Oral curcumin nano-complexes (O-Cur-NCs) group	1.35 ± 0.07^{g}	61.10 ± 5.09^{c}
G9: native Oral curcumin complexes (Native-O-Cur-Cs) group	2.86 ± 0.05^{h}	80.80 ± 9.27^{e}

• Values are mean ±SD.

•There is no significant difference between means having the same letter in the same column ($p \leq 0.05$).

Table 4: Effect of different nanocomplexes on P53 gene expression in experimental groups.

Groups	**Parameter**	
	P53 gene expression	**Caspase-3 ng/100 mg**
G1: Control normal (CN) group	1.00 ± 0.00^{a}	---------
G2: Tumor bearing (TB) group	0.15 ± 0.15^{a}	0.70 ± 0.01^{a}
G3: Copper oxide nano-complexes (CuO-NCs) group	0.56 ± 0.18^{a}	4.07 ± 0.10^{b}
G4: Hydrazine sulphate copper nano-complexes (HS-Cu-NCs) group	0.58 ± 0.34^{a}	$3.72 \pm .09^{c}$
G5: Curcumin Capped Copper nano-complexes (Cur-Cu-NCs) group	4.18 ± 1.21^{b}	4.55 ± 0.05^{d}

G6: Curcumin nano-complexes (Cur-NCs) group	3.51 ± 1.99[b]	2.54 ± 0.06[e]
G7: Native curcumin complexes (Native-Cur-Cs) group	0.94 ± 0.40[a]	2.03 ± 0.06[f]
G8: Oral curcumin nano-complexes (O-Cur-NCs) group	0.98 ± 0.01[a]	1.90 ± 0.01[g]
G9: native Oral curcumin complexes (Native-O-Cur-Cs) group	0.37 ± 0.25[a]	1.48 ± 0.02[h]

• Values are mean ±SD.

•There is no significant difference between means having the same letter in the same column ($p \leq 0.05$).

Table 5: The effect of different nano-complexes on serum inflammation/immunologic markers C-reactive protein (CRP) and interleukin-6 (IL-6) in experimental groups.

Groups	Parameters	
	CRP	IL-6
	ng/ml	Pg/ml
G1: Control normal (CN) group	0.11 ± 0.01[a]	115.10 ± 9.09a
G2: Tumor bearing (TB) group	2.27 ± 0.13[b]	254.07 ± 11.60b
G3: Copper oxide nano-complexes (CuO-NCs) group	0.63 ± 0.08[c]	176.82 ± 5.30c
G4: Hydrazine sulphate copper nano-complexes (HS-Cu-NCs) group	0.61 ± 0.05[c]	175.25 ± 1.30cd
G5: Curcumin Capped Copper nano-complexes (Cur-Cu-NCs) group	0.39 ± 0.01[d]	153.81 ± 6.60e
G6: Curcumin nano-complexes (Cur-NCs) group	0 .70 ± 0.04[e]	173.51 ± 4.38cd
G7: Native curcumin complexes (Native Cur-Cs) group	1.28 ± 0.05[f]	196.95 ± 2.92[f]
G8: Oral curcumin nano-complexes (O-Cur-NCs) group	0.93 ± 0.07[g]	186.30 ± 5.68[g]
G9: native Oral curcumin complexes (Native O-Cur-Cs) group	1.67 ± 0.10[h]	227.08 ± 2.62[h]

• Values are mean ±SD.

•There is no significant difference between means having the same letter in the same column ($p \leq 0.05$).

Table 6: The effect of different nano-complexes on oxidative stress markers in liver tissues of experimental groups.

Groups	Parameters		
	GSH	CAT	MDA
	µM/mg	mU/mg	nmol/mg
G1: Control normal (CN) group	12.89 ± 0.11[a]	9.97 ± 0.48[a]	1.01 ± 0.03[a]
G2: Tumor bearing (TB) group	12.20 ± 0.13[b]	9.63 ± 0.36[b]	1.69 ± 0.07[b]
G3: Copper oxide nano-complexes (CuO-NCs) group	7.18 ± 0.10[c]	6.26 ± 0.07[c]	5.52 ± .06[c]
G4: Hydrazine sulphate copper nano-complexes (HS-Cu-NCs) group	8.32 ± 0.20[d]	6.77 ± 0.06[d]	4.87 ± 0.11[d]
G5: Curcumin Capped Copper nano-complexes (Cur-Cu-NCs) group	9.36 ± 0.15[e]	7.31 ± 0.05[e]	4.57 ± 0.06[e]
G6: Curcumin nano-complexes (Cur-NCs) group	16.75 ± 0.04[f]	14.73 ± 0.06[f]	1.40 ± 0.02[f]

G7: Native curcumin complexes (Native-Cur-Cs) group	16.30 ± 0.01^{g}	13.67 ± 0.14^{g}	1.49 ± 0.02^{g}
G8: Oral curcumin nano-complexes (O-Cur-NCs) group	15.85 ± 0.15^{h}	12.80 ± 0.57^{h}	1.50 ± 0.03^{gh}
G9: native Oral curcumin complexes (Native-O-Cur-Cs) group	15.64 ± 0.22^{i}	13.80 ± 0.31^{g}	1.46 ± 0.03^{gh}

• Values are mean ±SD.

•There is no significant difference between means having the same letter in the same column ($p \leq 0.05$).

DISCUSSION

1. Nanoparticles Characterization

Bioavailability of a drug to the cells, whether *in vitro* or *in vivo*, is critical for its optimal efficacy. To enhance the solubility of drugs in aqueous solvents, increase their bioavailability, enhance serum half-life, for tumor cell targeting and bioimaging, nanotechnology has recently emerged as a new technology of choice.[35]

Characterization of different NCs was done using HR-TEM technique in order to provide clear insight into morphology and particle size. The present study revealed that average particle size of CuO-NPs were approximately around 39.46-62.47 nm and spherical in shape with a smooth surface. This is very similar to those described in the previous studies.[36] Whereas, TEM image showed that average particle size of HS-Cu-NPs were 16.81-23.77 nm and decahedran in shape with irregular surface. It was reported that Cu activates hydrazines to free radical species.[37]

Our study revealed that TEM image for Cur-Cu-NPs and the formed Nano-cur sodium salt were 67.55-79.07 nm and 0.6-5.26 nm respectively. The formed nanocurcumin sodium salts were nanorods with smooth surface. Basicity showed high pH = 9.5 due to the formed Nano-Cur sodium salt and excess of sodium bicarbonate. This high basicity has been characterized by quick solubility in water and its high penetration through cell wall. Confirming this result, ***Basniwal et al. (2011)*** found that Nano-Cur prepared by wet-milling technique, in size range of 2–40 nm was shown to express stronger antimicrobial potential and anti-cancer activity as well.[38]

2. Cytotoxic effect of different nanocomplexes

Data presented in the current study demonstrated that different nano complexes showed a clear cytotoxic effect on breast carcinoma cell line (MCF-7) and a clear concentration-response relationship.

In a similar study, ***Khosropanah et al. (2016)*** formulated Cur nanoparticles to increase its bioavailability and to study the effect on breast cancer cells. More than 50% of the tumor cells died within 48 hours after the administration of Cur. The dosage of Nano-Cur in this study was effective in half the dosage of the regular preparation of native-Cur.[39]

In a previous study, Breast cancer cells treated with different concentrations of native-Cur resulted in the inhibition of cell proliferation in a dose- and time-dependent manner. The literature reveled that Cur is a potent anticancer agent because of its ability to obstruct various biochemical pathways which are associated with the proliferation of cancer cells by binding with the various targets.[40]

It is evident from the current study that chemical treatment by CuO-NPs generated cytotoxicity. CuO-NPs Oxidative stress has been suggested to play an important role in the toxicity mechanisms of nanoparticles. This has been attributed due to their small size and large surface area which is generally thought to generate ROS. ROS such as superoxide anion (O_2^-), hydroxyl radical ($HO^\bullet$) and hydrogen peroxide (H_2O_2) elicit a variety of physiological and cellular events including inflammation, DNA damage and apoptosis.[7] CuO-NPs were found to induce cytotoxicity in a human liver carcinoma cell line (HepG2) in a dose-dependent manner, which was probably mediated through ROS generation and oxidative stress.[41]

Regarding to HS-Cu-NCs treatment, an important distinguishing feature of cancer cells is their propensity to obtain energy through the anaerobic metabolism of glucose. The enzyme phosphoenol pyruvate carboxykinase played very important role in gluconeogenesis and proposed that inhibition of this enzyme would impede gluconeogenesis and reduce the severity of cachexia. Hydrazine sulfate is thought to interfere with gluconeogenesis. Hydrazine, a metabolite of HS, has been reported to have cytotoxic effects on hepatocyte cell cultures.[42]

Our result demonstrates the efficacy of Cur with promising antiangiogenic and antiproliferative potential as compared to Cur-Cu-NPs. While describing the possible mechanism, it has been illustrated that Cur analogues are found to be less active than Cur in suppressing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation and tetrahydro-curcumin was found to be less active than Cur in preventing chemical-induced skin tumor promotion in mice due to the reduction of the carbonyl group.[43]

3. Antitumor effect of different nanocomplexes

Our data also showed that there was significant reduction in tumor weight and volume in all treated TBM groups. Moreover, the previous results indicated that there was a significant increase in mean survival time (MST) and increased life span (%ILS) in all TBM treated groups especially in G5. Cur-Cu-NCs were the most effective treatment followed by Cur-NCs then CuO-NCs then HS-Cu-NCs and finally Native-Cur-Cs. These results summarized that intratumoral (IT) treatment with Cur-Cu-NCs and Cur-NCs have a stronger antitumor effect than chemical IT treatments (CuO-NCs and HS-Cu-NCs) and native curcumin treatment either IT or oral.

The transcription factor (NF-κB) has the main role in the creation of tumors and inflammation, and the goal of most pharmaceutical and entomological preparations is to reduce its hyper productivity. Cur is a natural product which reacts with a large variety of compounds in the downstream of the NF-κB pathway. Cur blocks IKB kinase (IKK) activation, phosphorylation, and the degradation of nuclear factor kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IκBα). A large number of Cur analogues were investigated in order to improve its efficiency in blocking NF-κB.[44]

The current study goes with the study of ***Shahani et al***. who found that A number of previous studies have shown that Cur exhibit poor bioavailability in the human body when administered orally. It is due to their rapid degradation and poor absorption in the gastrointestinal tract, which results in low plasma concentrations and a very low distribution in tissues. In addition to oral intake, injection entry was investigated and has proven effective in keeping curcumin in tissues for a longer period. Intravenous intake nanoparticles has been proven to be effective for the treatment of tumors in animals.[45]

In a similar study, ***Wanninger et al.*** also reported that Cur and the curcuminoids should be ideally suited to act as chelating ligands toward a variety of metals and to form stable

complexes. Cu (II) curcumin complexes turned out to exhibit the highest selective cytotoxicity *in vitro*.[46] The Cu (II) complexes have increased solubility and crystallinity due to blocking of the phenolic –OH groups through alkylation. These complexes showed significantly enhanced antitumor activity against human cancer cell lines in comparison with the free ligands.[47] These findings indicate why Cur-Cu NCs was the effective treatment as antitumor in our study.

Cu is an essential trace element that is widely distributed throughout the body and forms the essential redox–active reaction center in a variety of metalloenzymes. Cu concentration is obviously altered in tumors, and that serum concentrations are correlated with tumor incidence, progression and recurrence in a large number of human tumors.[49] Previous study reported that CuO-NPs can induce cancer cells apoptosis through a mitochondrion-mediated apoptosis pathway, which raises the possibility that CuO-NPs could be used to cure melanoma and other cancers.[48]

Gratefully, tumor hypoxia can be exploited to develop prodrugs that become activated in the reducing environment of cancer cells. In this concern, Cu is very appealing because it can exist under two different oxidation states in cells. The anoxic character of cancer cells promotes the reduction of Cu (II) to Cu (I), which is not possible in normal healthy cells and thus provides a therapeutic opportunity to target tumors.[49] Cu (I) can catalyze the formation of reactive oxygen and nitrogen species (ROS and RNS), to induce a pro-apoptotic oxidative stress.[50]

HS is active metabolite and that it may normalize the carbohydrate metabolism of cancer patients with cachexia. Our results are consistent with previous result suggesting that HS administered to rats with transplanted tumors inhibited tumor growth and increased survival.[51]

Regarding to tumor marker, in the current study, Cur-Cu-NCs treatment in G5 was the most effective treatment that caused a significant reduction in serum CA 15-3 level. Chemical treatmens (G3 and G4) recorded a significant decrease in serum (CA 15-3) levels less than Cur-Cu-NCs treatment in G5. Also, Cur NCs treatment in G6 caused a significant decrease in serum (CA 15-3) levels very close to chemical treatments levels compared to TBM group. Predictably, Native-Cur treatment either IT or oral caused a less decrease in CA15-3 level

compared to Cur NCs and other NCs treatments. These results were supported by our results.[52]

CA15-3 (also known as mucin 1) is overexpressed in human breast cancers and in their subsequent metastases.[53] CA15-3 promotes tumor invasion and metastasis through activation of the mitogen-activated protein kinase signaling pathway[53] and down regulation of E-cadherin.[54] Previous studies suggested that carbohydrate antigen 15-3 (CA15-3) is predictive marker of radiological response in metastatic breast cancer.[55]

Regarding to ALP activity, ALP activity was significantly and noticeably decreased due to Cur-Cu-NCs and Cur-NCs treatments to the control level. Chemical treatments (CuO-NCs and HS-Cu NCs) caused a significant decrease in ALP activity but not to the control level while Cur NCs (oral) and native Cur Cs (IT or oral) treatments caused less decrease in ALP activity as compared to TBM.

Our results are in agreement with an *in vivo* study reported that Nano-Cur supplementation prevented the increase in such hepatic enzymes, especially in groups received Nano-Cur after tumor induction, suggesting that Nano-Cur may have a potential protective effect against liver damage.[56]

It is clear from the current study that Cur-Cu-NCs was the most effective treatment and decreased ALP activity to the control level, that may be due to the protective and antioxidant effect of nanocurcumin which abolished almost the harmful effects of CuO-NPs treatments. Our results are in agreement with previous results investigating oxidative stress role of N-acetyl-cystein (NAC) in the cytotoxicity of CuO NPs, results showed that NAC abolished almost fully the harmful effect of CuO NPs at all concentrations studied when HepG2 cells were exposed to CuO NPs in the presence of the NAC.[41]

Previous studies demonstrated that vit C induce apoptosis in cancer cells by creating oxidative stress via upregulation of reactive oxygen species (ROS) release.[57] Furthermore, using high dosage of vit C as an anti-cancer therapy has shown to reduce cancer cell growth and lessen chemo-therapy side effects such as nausea, fatigue, pain and depression.[11]

4. Apoptotic effect of different nanocomplexes

The p53 gene acts as a guardian of the genome and is one of the major factors controlling cell proliferation, growth suppression and transformation. Inactivation of the p53 tumor

suppressor gene is a frequent event in tumorigenesis. Interestingly, mutations in the p53 gene were shown to occur at different phases of malignant transformation, thus contributing differentially to tumor initiation, promotion, aggressiveness, and metastasis.[58]

The role of Cur in triggering apoptosis has been investigated in numerous studies, and there is a range of evidence demonstrating its potential to activate different pathways related to apoptosis. Interestingly, it has been revealed that Cur-mediated apoptosis induction in cancer cells occurs in a p53-dependent mode.[59]

Moreover, ***Balasubramanyam et al.*** previously demonstrated that Cur could inhibit p300-specific acetylation of p53, which may be helpful in the acetylation-dependent regulation of p53 function; this causes Cur, which targets p300 to serve as a lead compound in cancer suppression [60]. Therefore, it is concluded that one of the Cur pathways which play a role in cancer suppression is modulations of the transcriptional co-activating proteins mediating the p53 gene level, which promotes invasion, metastasis and a metabolic shift to an anaerobic process known as the 'Warburg effect'. Gratefully, tumor hypoxia can be exploited to develop pro-drugs that become activated in the reducing environment of cancer cells.[61]

Current study illustrated that the expression levels of P53 were down regulated in TBM (G2) as compared to the control muscle (G1). Furthermore, the levels of P53 were significantly increased more than the control level in TBM treated intratumorally with Cur-Cu NCs and Cur NCs in G5 and G6, respectively as compared to G2. Moreover, increase in P53 expression was noticed in TBM treated with chemicals (G3, G4), TBM treated with Cur NCs orally and TBM treated with Cur Cs (IT or oral) as compared to G2, but, the increase in P53 expression was less than the control level.

Results of P53 gene expression were significantly confirmed by immunohistochemical analysis where TBM received Cur-Cu NCs and Cur NCs highly expressed P53 in G5 and G6 respectively compared to moderate expression of P53 in TBM received chemical treatments (CuO-NCs and HS-Cur-NCs), native Cur-Cs (IT) and Cur-NCs (oral) in G3, G4, G7 and G8 respectively. Whereas, G9 treated with oral native Cur-Cs observed minimized alteration in expression of p53 as compred to negative staining of P53 in untreated TBM.

Moreover, our results are consistent with the previous results suggesting 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 downregulated in HepG2 cells treated with CuO-NPs. It was suggested that bax is up-regulated by p53. Since an increase in bax expression was noticed, the role of p53 in the upregulation of bax upon CuO-NPs exposure can be postulated. The insertion of bax into the mitochondrial membrane possibly leads to p53-mediated apoptosis.[62] Caspases are activated during apoptosis in many cells and are known to play a vital role in both initiation and execution of apoptosis.[41]

Furthermore, other authors observed that CuO-NPs targeted the mitochondria of HeLa cells *in vitro*, which resulted in the release of cytochrome C from the mitochondria and the activation of caspase-3 and caspase-9 after the CuO-NPs entered the cells.

Our result revealed that there was a highly significant increase of caspase-3 activity in tumor tissues of treated groups in G3, G4, G5, G6, G7, G8 and G9 respectively compared to G2. Noticeably, Cur-Cu-NCs treatment was highly apoptotic that caused a significant increase in caspase-3 activity in G5 more than other treatments as compared to untreated TBM group. Increase in caspase-3 activity in tumor tissues of TBM treated with chemicals was significantly more than its activity in TBM treated with Cur-NCs (IT or oral) and native-Cur-Cs (IT or oral). Furthermore, Cur-NCs (IT) treatment caused a significant increase in caspase-3 activity very close to its activity exerted by chemical treatments.

In the present study, it was observed that the changes in nucleus following NCs treatment on MCF-7 cell line. Hoschet 33342 is cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. For this reason, this dye was used to distinguish condensed pycnotic nuclei in apoptotic cells. It is clear that Cur-Cu-NCs was the most effective cytotoxic treatment to MCF-7 as there were less colonies and more nuclear condensation as compared to the control. Noteworthy, CuO-NCs and HS-Cu-NCs treatments were more toxic to (MCF-7), then Nano-Cur-NCs and Native-Cur-Cs compared to the control. This observation is consistent with the data obtained by ***Wang et al.***[48]

5. Anti-inflammatory activity of different nanocomplexes

Regarding to the inflammation immunologic markers, there was a significant increase in CRP and IL-6 levels in untreated TBM. In contrast, Mice in G5 treated with Cur-Cu-NCs exhibited a significant decrease in CRP and IL-6 levels. Then, CuO-NCs and HS-Cur-NCs treatments caused a significant decrease in CRP levels and IL-6 levels as compared to untreated group

but the reduction was less than Cur-Cu-NCs treated group. A statistically significant reduction in CRP and IL-6 levels was observed in all groups treated with Cur-NCs or native Cur-Cs as compared to G2.

Cur is described as a potent inhibitor of angiogenesis. It is reported that Cur can down-regulate all positive regulators of angiogenesis including cytokines, such as IL-6 can act either as stimulators or inhibitors, depending on their amounts, the tumor site, and the tumor microenvironment. These cytokines play a pivotal role in promotion of tumor growth, particularly by recruiting massive vasculature.[63]

Moreover, our results are consistent with previous results suggesting that Nano-Cur, polymeric nanoparticle encapsulated Cur, readily dispersed in aqueous media and with confirmed anti-cancer potentials in preclinical *in vivo* models. Nano-Cur retained the mechanistic specificity of free Cur, inhibiting the activation of the seminal transcription factor NF-κB and reducing steady state levels of pro-inflammatory cytokines like ILs and TNF-α.[64]

Previous study demonstrated that one of the main metabolic pathways for hydrazine derivatives leads to formation of various free radical species *in vitro* and *in vivo*. Cu, is known to activate hydrazines to free radical species and have been shown to induce DNA damage. Thus, the oxidative metabolism of hydrazines by copper and consequent formation of reactive species may contribute significantly to the pathophysiology of hydrazines in humans.[37]

6. Antioxidant effect of different nanocomplexes

Regarding to GSH content and CAT enzymes activity, it was found to be significantly lowered in chemically treated groups (G3, G4) and G5 which is treated with Cur-Cu NPs. when compared with G2. Also, there was a significant increase in MDA levels in G3, G4 and G5 which associated with a significant decrease in GSH content and catalase activity. Predictably, Hepatic MDA levels in Cur-NCs and Native Cur-Cs treated groups were significantly lowered.

Cur has been found to be an excellent scavenger of most ROS.[65] The reaction of peroxyl radicals with Cur produces Cur phenoxyl radicals, which are less reactive than the peroxyl radicals and thereby cause protection from ROS-induced oxidative stress. The regeneration

reaction of phenoxyl radicals back to Cur by water soluble antioxidants like ascorbic acid, impart the molecule with a chain breaking antioxidant ability.[66] It was also documented that Cur has a property of donating electrons in order to neutralize free radicals by creating stable products, and thus breaking a chain reaction of creating free radicals in a living organism. Cur's ability of capturing hydrogen peroxide is higher than that of the commercial antioxidants at the same concentration.[67]

Recently, ***Assadian et al***., also reported that *in vitro* cytotoxicity of CuO-NPs was associated with significant increase at intracellular ROS level with effective induction of oxidative stress.[68] The capability of NP to produce free radicals is one of the primary mechanisms of NPs toxicity. It may result in oxidative stress, inflammation, and consequent damage to proteins, membranes, and DNA.[69]

In a recent study, ***Thit et al.*** aimed to determine the role of ROS release and establish the sequence of events during CuO-NP toxicity. Results showed that CuO-NPs were more toxic than Cu^{2+}, due to the increase in the generation of ROS, DNA damage and decrease levels of GSH compared to control.[70]

It is well documented that free radical species are very reactive and bind irreversibly to cellular macromolecules, causing inhibition of cellular functions and inducing profound cellular damage. Primary free radicals, e.g., alkyl radicals, react with molecular O_2, leading to the formation of reactive oxygen-derived species, superoxide anion radical ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2) and, eventually, to the highly reactive hydroxyl radical ($^{\cdot}OH$). Alternatively, primary radicals can eliminate hydrogen atoms from membrane lipids, inducing peroxidation and decomposition of lipid membranes and compromising cellular functions. Reactive oxygen species formation has been shown to induce "oxidative stress" where the production of oxidant overwhelms antioxidant defense mechanisms. Oxidative stress is known to exhaust reduced glutathione in cells, compromising cellular integrity.[71]

Hydrazine derivatives have been shown to deplete glutathione and cause oxidative stress. Therefore, formation of free radical species during the biotransformation of hydrazines may be very important in the toxicity and pathophysiology of hydrazines. The formation of oxygen radicals and metal/peroxo species from hydralazine has been involved in DNA strand scission, and it has been suggested that these reactive species may also form oxidation products of guanosine bases in DNA.[72]

CONCLUSION

Drug conveyance frameworks utilizing nanoparticles are appreciated as a promising methodology for enhancing the safety and bioavailability of curcumin. The basic aim of the present study was to compare the efficacy of different nanocomplexes as a potential anticancer and antiangiogenic agent in concert with native curcumin. Cur-Cu-NCs treatment was the most effective one as antitumor. Our results confirmed that Cu (II) curcumin complexes turned out to exhibit the highest selective cytotoxicity and also showed significant reduction in solid tumor volume in ascites tumor-bearing mice without harmful effects on liver and kidney function. Also, Nano-Cur appeared to be an effective free radical quencher with antioxidant activities, and capable of inhibiting oxidative stress, as it could protect mice liver from chemical treatments induced altered hepatic functioning. Our study provided valuable insights into the possible mechanism of CuO-NCs and HS-Cu-NCs cytotoxicity in tumor cells. Furthermore, the antitumor effect of chemical treatments was very close to Nano-Cur effect, but chemical treatment adversely affected liver and kidney function. We suggest that Cur-Cu-NCs, which are a potentially safe and inexpensive for clinical use, may be considered as an effective chemopreventive agent against tumors with more protective rather than therapeutic action.

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