RESEARCH ARTICLE

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Antioxidant and Apoptotic Activity of Curcumin Nanoparticles on HEp-2 Cell Line

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Abstract

Introduction: Nanocurcumin is a widely used anti-inflammatory agent due to its antioxidant and anti-inflammatory properties. **Aim of the study:** The current study aimed to examine cytotoxicity, antioxidant and apoptotic Activity of Curcumin nanoparticles on squamous cell carcinoma (HEp-2). **Material and Methods:** The present work conducted to evaluate the cytotoxicity using MTT assay, evaluation of morphological changes post cell treatment with Curcumin nanoparticles, also evaluation of cell apoptosis using Annexin V-FITC and Propidium Iodide (PI) staining. Finally, Genotoxicity was traced via arranging for flow cytometry. **Results:** Data recorded revealed that the inhibitory concentration (IC_{50}) of Curcumin nanoparticles was 40.82 ug/ ml. Also, cytotoxicity was concentration dependent with significant difference ($p<0.05$). Cytotoxicity enhanced apoptosis process accompanied with cell cycle arrest through the induction of G2/M and pre-G1 phases arrest compared with that of control cell. Cytotoxicity accompanied with morphological changes denoting apoptosis and necrosis after treatment with Curcumin nanoparticles. In the meantime, the biochemical marker (ROS) was elevated in a significant way post treatment with Curcumin nanoparticles. **Conclusion:** Nanocurcumin has a potent anticancer effect due to its antioxidant and anticancer activity.

Keywords: Tumeric- Cell line- Anticancer- Antioxidant and Curcumin

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Introduction

Oral squamous cell carcinoma is a destructive disease-causing essential morbidity and mortality in humans. It is the most common subtype of the head and neck squamous cell carcinoma (HNSCC), which is the sixth most common cancer worldwide, with 700,000 cases diagnosed per year. When a patient develops recurrent or metastatic HNSCC that is not suitable to surgical removal or re-irradiation, the goals of care become sedative. Due to the anatomic location and the burden of late toxicities, patients with recurrent, metastatic HNSCC often suffer from symptoms affecting vital human functions, such as talking, eating, and breathing [1]. Despite promising advancements in the usual therapeutic methods currently used for patients with oral cancer, many disadvantages are still to be reported; surgical resection leads to permanent disfigurement, altered sense of self and debilitating physiological consequences, while chemo- and radio-therapies result in significant toxicities, all affecting patient wellbeing and quality of life. Thus, the development of new therapeutic approaches or modifications of current methods is mandatory to improve individual health outcomes and survival [2].

The most common oral problems occurring after radiation and chemotherapy are mucositis, infection, pain, and bleeding. Other possible complications might include dehydration and malnutrition, commonly associated with dysphagia. Radiation therapy to the head and neck may induce xerostomia or cause trismus due to damage the muscles and joints of the jaw and neck. These treatments may also cause a decrease in the blood supply of the bones of the maxilla or mandible. In addition, these treatment methods cause caries, or soft tissue complications, or may cause osteonecrosis [1].

Curcumin is a polyphenol derived from Turmeric; a spice that has long been recognized for its medicinal properties, it has received interest from both the medical and scientific world, as it is the major source of the polyphenol curcumin which is considered a strong anti-inflammatory and antioxidant agent [3]. It helps in the management of oxidative and inflammatory conditions, metabolic syndrome, arthritis, anxiety, and hyperlipidemia. Most of these benefits can be due to its antioxidant and anti-inflammatory effects. Taking curcumin by itself does not lead to the associated health benefits due to its poor bioavailability, which appears to be due to poor absorption, rapid metabolism, and rapid

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elimination. The main reasons for the low bioavailability are supposed to be the poor solubility of curcumin in aqueous media, rapid hydrolysis followed by molecular fragmentation at physiological pH and inactivity of its metabolic products [4, 5]. There are several methods that can increase bioavailability one of them is to change it to nano scale to increase its absorption and dissolution. The slow release of curcumin from nanoparticulate curcumin formulation increased the bioavailability of delivered curcumin unlike native curcumin that made us to prepare curcumin nanoparticles to study their antioxidant and apoptotic effect on cancer cells [4].

Cell lines represent an invaluable resource in modern science including basic and translational cancer research. For example, the Hep-2 cell line that was used in this research due to its availability and reliability in its results.

In this research we studied the antioxidant and apoptotic effect of curcumin nanoparticles against cancer cells in a trial to discover a new conservative anticancer treatment to avoid traditional surgical approaches in the treatment of oropharyngeal cancer can result in morbidity with poor functional and cosmetic outcomes and to overcome the deteriorative side effects of conventional chemotherapy and radiotherapy.

Materials and Methods

Nanocurcumin was purchased from Nanotech Egypt for Photo-Electronics. Size was verified by TEM in central laboratory, south valley university, Qena by the transmitted electron microscope, and Human squamous cell carcinoma cell line (HEp-2) used in the present study was supplied from the international center for training and advanced research (ICTAR- Egypt).

Cell Treatment

Human squamous cell carcinoma cell line (HEp- 2) used in the present study was kindly supplied from the international center for training and advanced Research (ICTAR-Egypt). Cells were supplied in 75 Cm2 surface area tissue culture flasks and grown in Minimum essential medium (Eagle) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 IU/ml penicillin and 100µg/mL streptomycin at 37 º C under a humidified 5% CO2 atmosphere. Cells were treated with different concentrations of Curcumin nanoparticles then the cytotoxicity was observed using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide) assay. IC_{50} value was calculated, and the effective dose was used for further study.

Cytotoxicity Assay (MTT assay)

In the present study, Methyl Thiazole Tetrazolium (MTT) assay as a quantitative colorimetric method to determine cell proliferation. It utilizes yellow tetrazolium salt (3- [4aa, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) which is a water-soluble salt reduced to an insoluble purple formazan complex by cleavage of the tetrazolium ring by lactate dehydrogenase (LDH) within the mitochondria. The cell membrane is impermeable to the formazan product and therefore it accumulates in healthy cells. The resulting intracellular purple formazan crystals can be solubilized and quantified by spectrophotometric means. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, there is reduction in cell viability. For each cell type, there is a linear relationship between cell number and signal produced, thus allowing an accurate quantification of changes in the rate of cell proliferation [6, 7]. The viability of HEp-2 post treatment with Curcumin nanoparticles was determined 24 hours.

The cell viability percentage was calculated using the following formula:

Viability percentage (%) = Mean OD of test dilution × 100/Mean OD of control wells.

For MTT assay, HEp-2 cells were seeded in 96-well culture plates and treated with 2-fold serially diluted 10 mg/ml Curcumin nanoparticles for 24 hours. Dead cells were decanted / washed out using phosphate buffer saline (PBS) MTT as 50µL of 0.5mg/ml stock solution were dispensed to the remaining lived cells. Plate was incubated at 37°C for 4 hours. Thereafter, the staining solution was decanted from the wells, cells were washed using PBS and 50µL of Dimethyl sulfoxide were added to each well to dissolve the purple formazan crystals. The absorbance was conducted at 570nm using the Dynatech MR5000 spectrophotometer. The absorbance values at 570nm were relative to the number of residual viable cells. The IC_{50} value was determined using GraphPad Prism software (v.6, GraphPad Software, La Jolla, CA, USA).

Flow cytometry

Cell cycle analysis was performed by staining the DNA with Propidium Iodide (PI) as described previously with some modifications [8]. The PI fluorescent nucleic acid dye is capable of binding and labeling double-stranded nucleic acids, making possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis and subsequent identification of hypodiploid cells. In brief, cells are seeded in T25 flask at a density of 1×10^6 cells/ml. The IC_{50} concentration of Curcumin nanoparticles were added to each flask incubated for 24 h. Cells were trypsinized, harvested and fixed in 70% ice cold ethanol in blank tubes and stored at +4o C until use. The cells were centrifuged, the cell pellets were resuspended in PI (40 µM mLG1 in PBS) solution containing RNase (100 µM mLG1). The stained cells were analyzed using fluorescence activated cell sorter (FACScan, Becton-Dickinson) with 488 nm argon ion laser using MAC Cell-QuestTM Software. The cell cycle distribution was analyzed using PI signals were collected using the 585/42 band pass filter. The data acquired were analyzed using quest software.

Cell cycle analysis

Annexin V Apoptosis Detection Kit is based on the

observation that soon after initiating apoptosis, cells translocate the membrane Phosphatidyl Serine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. The one-step staining procedure takes only 10 min. Detection can be analyzed by flow cytometry or by fluorescence microscopy. The kit can differentiate between apoptosis and necrosis when performing both Annexin V-FITC and Propidium Iodide (PI) staining.

Microscopic Examination

Detached and adhered cells were collected post treatment using trypsinization as previously described. Pelleted cells were re-suspended in PBS and a part (50µL) was dispended on the clean ethanol washed glass slide, air dried and fixed using methanol as a preparatory step for cytological examination. Fourteen microscopic fields of each group were photomicrographed at power of 1000X (Oil immersion). This was done using a digital video camera (Canon, Japan) which was mounted on a light microscope (Olympus BX60, Japan). Images were then transferred to the computer system for analysis. Fields selection was based on the presence of the highest number of apoptotic cells. The photomicrographs were evaluated for the presence of morphological criteria of apoptosis. The photomicrographed fields were analyzed using image analysis software (Image J, 1.27z, NIH, USA).

Determination of Reactive Oxygen Species

This immunoassay kit allows in vitro quantitative determination of Rat reactive oxygen species, ROS concentrations in serum, tissue homogenates and other biological fluids. The microtiter plate provided in this kit has been pre-coated with an antibody specific to ROS. Standards or samples were added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ROS and Avidin conjugated to Horseradish Peroxidase was added to each microplate well and incubated. Tetramethylbenzidine substrate solution was added to each well. Only those wells contain ROS, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution used as 2% and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of ROS in the samples was determined by comparing the optical density of the samples to the standard curve.

Statistical analysis

All experiments were carried out in three independent tests. Data were expressed as mean standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). Significance difference between treated and untreated cells was determined using one way ANOVA. The results were considered statistically significant at probability ($P<0.05$).

Results

Characterization of nanoparticles

Size was verified by TEM JOEL 2010 in central laboratory unit, South Valley University, Qena, Egypt. The particle size was with an average size of 10 ±5 nm (Figure 1).

Cytotoxicity assay

Regarding the cytotoxic effect of Curcumin nanoparticles on HEp-2 cells, viability was employed using MTT assay. Data recorded revealed that the mean viability percentage of Curcumin nanoparticles treated cells was concentration dependent (Figure 2) and the safe concentration induced 100% cell viability was at ~<20 µM mLG1. And the IC_{50} value determined was 40.82 ug/ml indicating a significant difference ($p<0.05$) (Figure 3).

Flow cytometric analysis

Regarding HEp-2 cells distribution post 24 h treatment

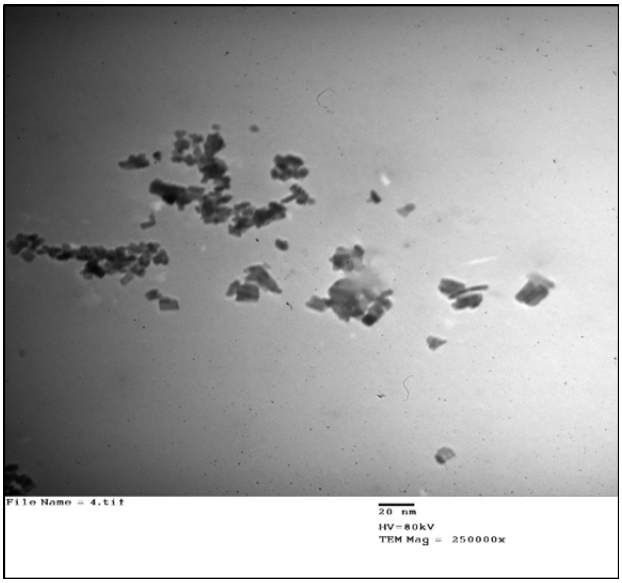

Figure 1. Transmitted Electron Micrograph Showing the Nanocurcumin Particles Character (25000x)

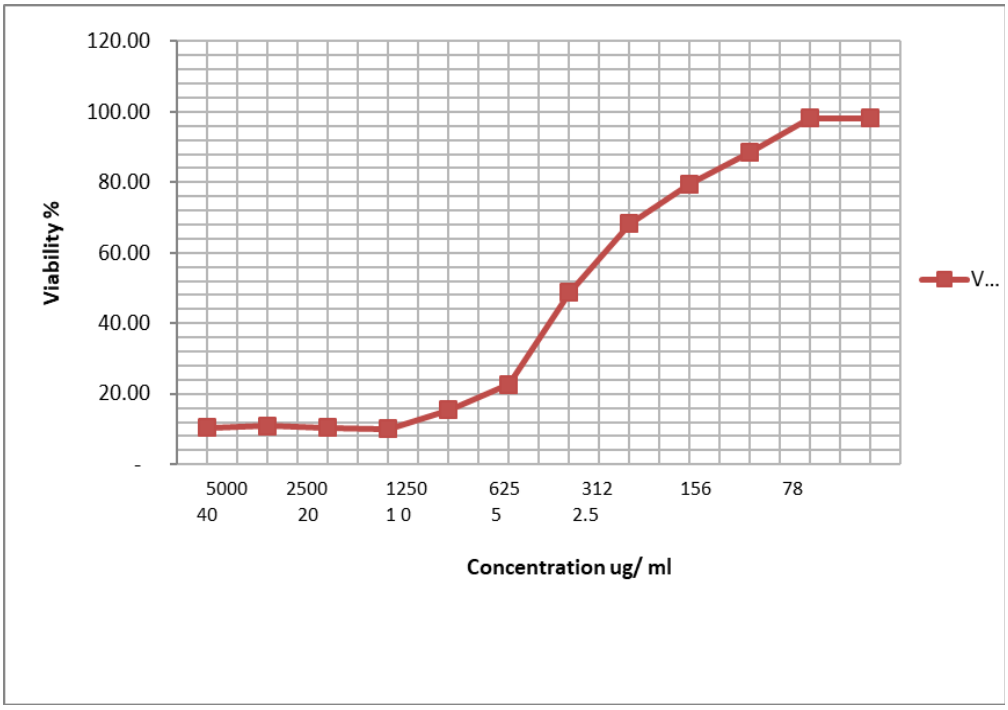

Figure 2. Evaluation of Cell Viability Post Treatment with Curcumin Nanoparticles Using MTT Assay.

with the IC_{50} value of Curcumin nanoparticles, it was noticed that the % of cells arrested at the G0/G1 phase and S-phase were significantly decreased compared with that of nontreated cell control ($p<0.05$). Also, Data recorded revealed that there was a significant elevated arrest during the G2-M phase in case of HEp-2 treatment with Curcumin nanoparticles ($p<0.05$) compared with cell control. Also, there was a significant apoptotic cell in the pre-G1 phase compared to its values in control group (Figure 4-5).

Apoptotic profile

Apoptotic cells were accumulated at G2/M phase which proceed G1 phase, this called pre G1 apoptosis, so the percentage of cells enter G1 phase decreased. Regarding the apoptotic profile of treated cells, there was a significant elevated early and late apoptotic cells, necrotic cells, and total apoptotic cells after cell treatment with Curcumin nanoparticles ($p<0.05$) (Figure 6-7).

Determination of Reactive Oxygen Species

The intracellular ROS level was increased in Curcumin nanoparticles treated cells (740.8 pg/ml) as compared to the untreated cells (197.7 pg/ml) (Figure 8).

Microscopic examination

On cytological features of untreated control HEp-2

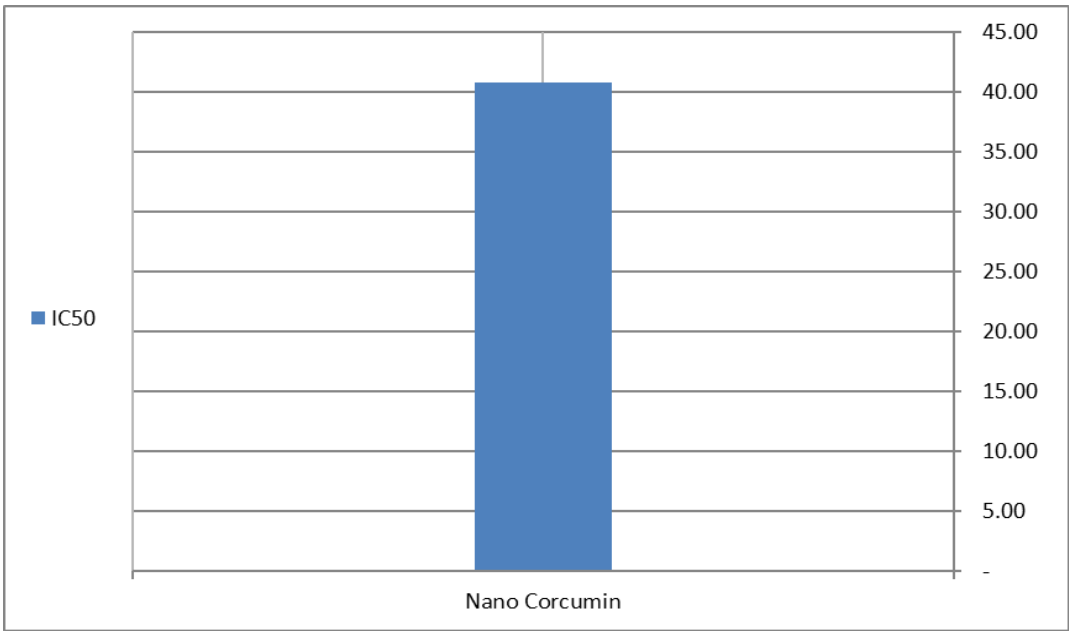

Figure 3. Evaluation of Inhibitory Concentration (IC_{50}) post HEp-2 Cell Treatment with Curcumin Nanoparticles

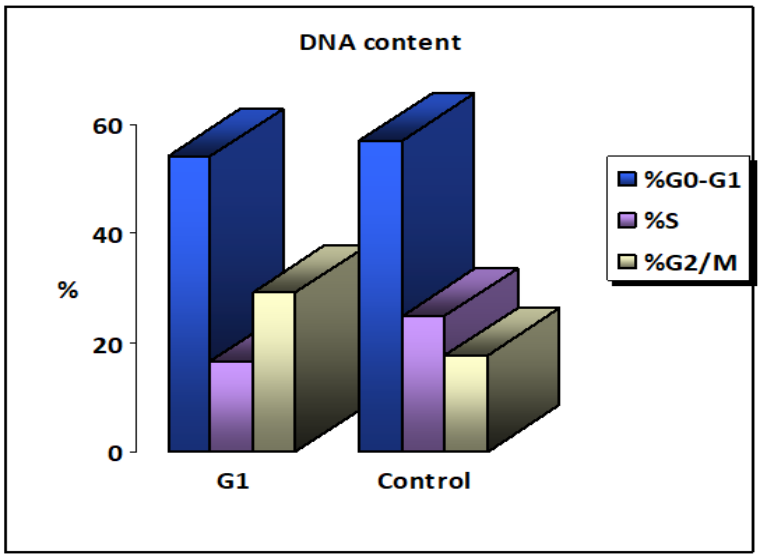

Figure 4. Histograms Demonstrating the Percentage of Cells in Different Phases of Cell Cycle for the Control Versus Curcumin Nanoparticles Treated cells for 24 hr.

cell line after 24 hours, Most of HEp-2 cells showed shows cancer cells with signs of dysplasia such as cellular and nuclear pleomorphism, nuclear hyperchromatism and increased nuclear cytoplasmic ratio and the cellular outline was almost regular without evidence of any folding (Figure 9). After 24 hours post treatment with IC 50 concentration of Curcumin nanoparticles. Some cells of this group show apoptosis, such as cellular and nuclear shrinkage, apoptotic bodies and nuclear fragmentation. Other cells show necrosis in the form of swollen cells with ruptured cell membrane (Figure 10-11).

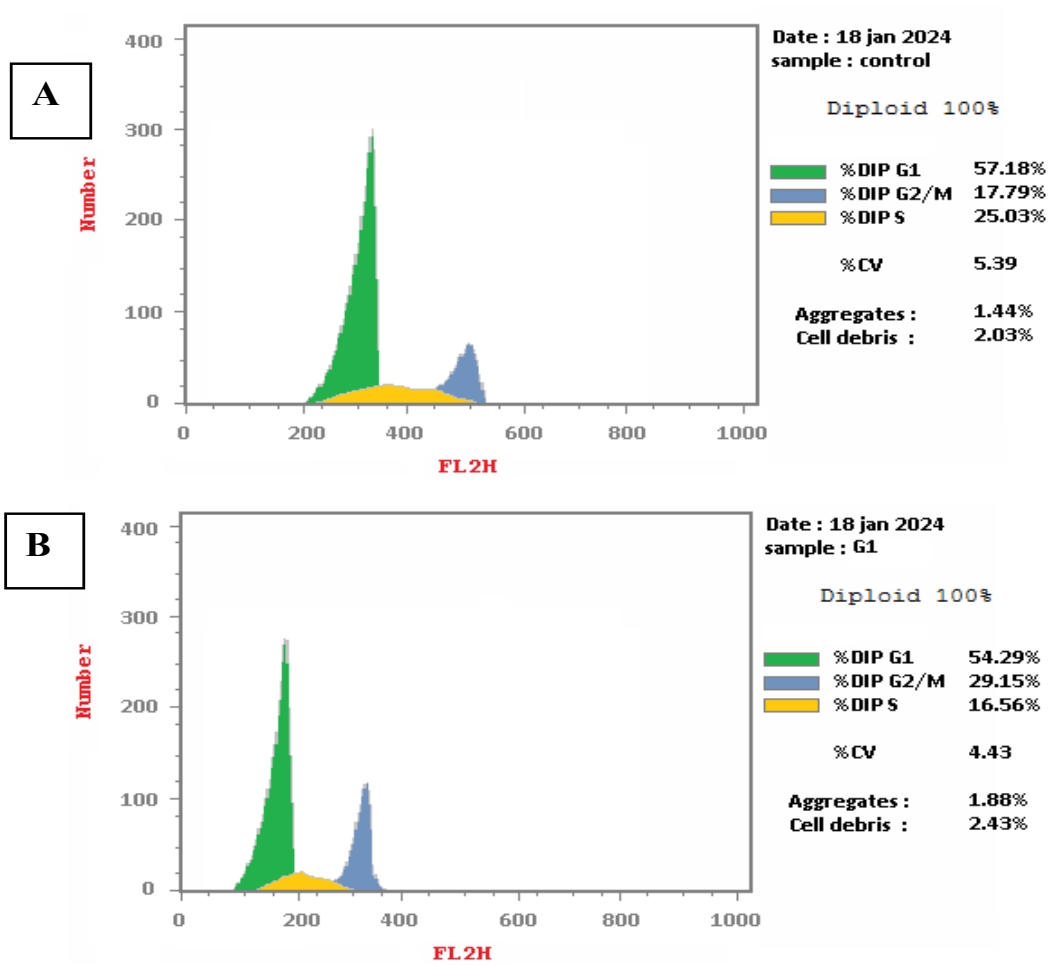

Figure 5. Flow Cytometric Analysis of Cell Cycle with Propidium Iodide Staining of HEp-2 Post Treatment with Curcumin Nanoparticles. A, control cells; B, Curcumin nanoparticles treated cells.

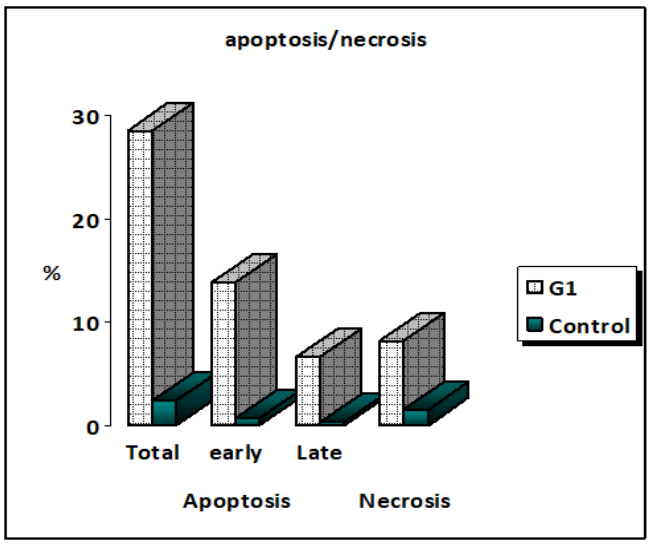

Figure 6. Histogram Showing the Percentage of Apoptosis for Control Cells Versus Curcumin Nanoparticles Treated HEp-2 Cells Using Annexin V- FITC.

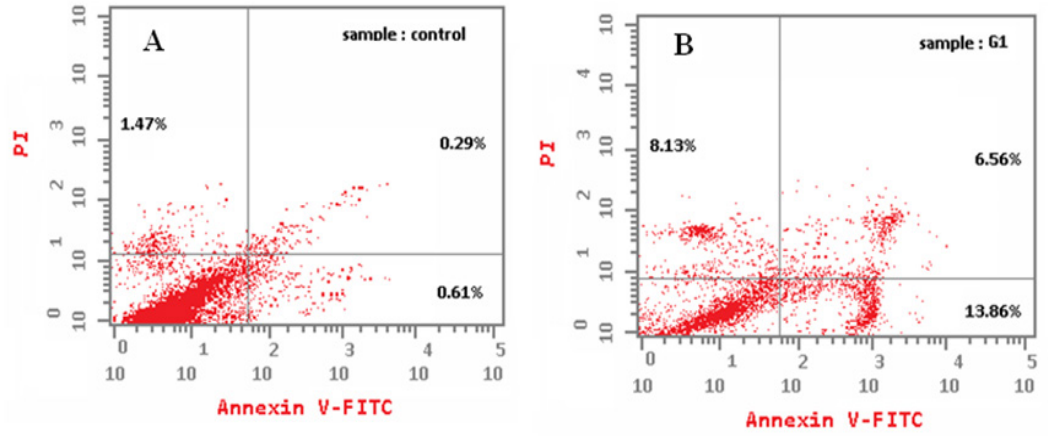

Figure 7. Evaluation of Apoptotic Profile of HEp-2 Post Treatment with Curcumin Nanoparticles Using Flow Cytometry. A, control cells; B, Curcumin nanoparticles treated cells.

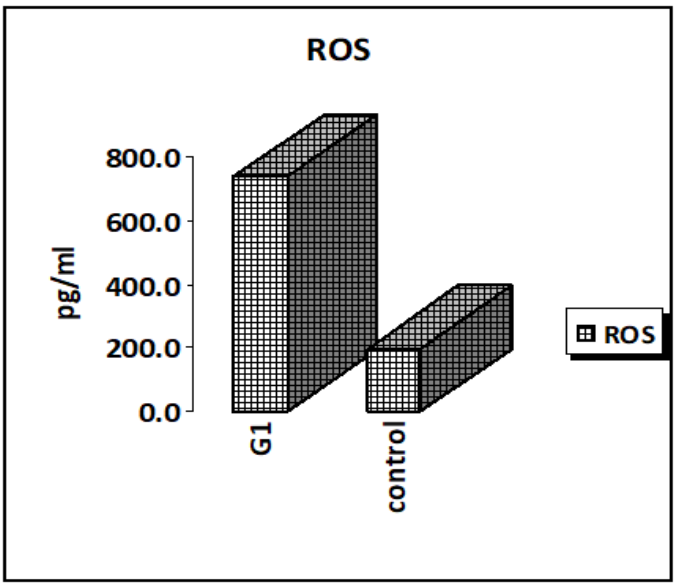

Figure 8. Bar Chart Revealing Generation of ROS Level Post Curcumin Nanoparticles Treatment Measured Spectrofluorimetrically for 24 hr. versus Control Cells

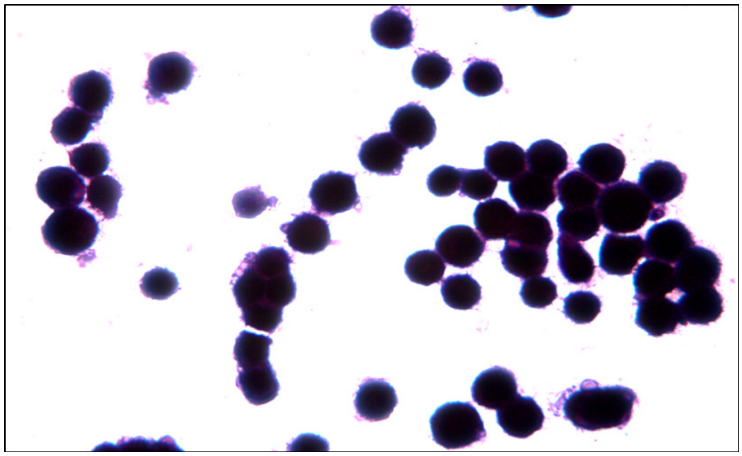

Figure 9. A Photomicrograph Showing Cancer Cells with Hyperchromatic Nuclei, Increased Nuclear and Cytoplasmic Ratio, and Cellular and Nuclear Pleomorphism. (H and E, Original magnification 100X, oil).

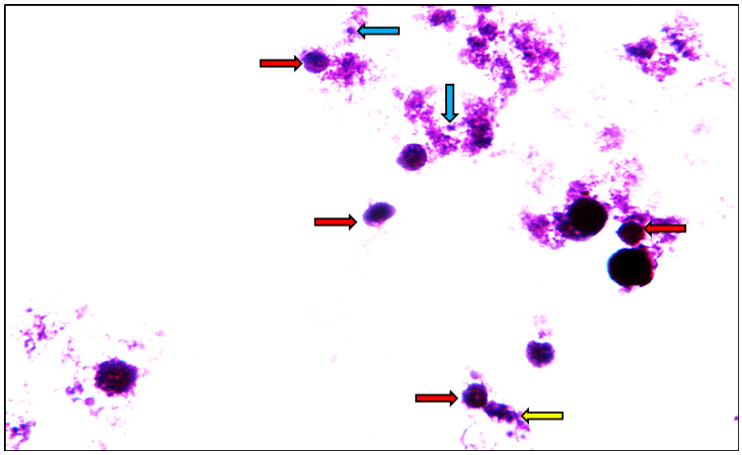

Figure 10. A Photomicrograph Showing Shrunken Apoptotic Cells (Red Arrows), Apoptotic Bodies (Blue Arrows), and Nuclear Fragmentation (Yellow Arrow). (H and E, original magnification 100X, oil).

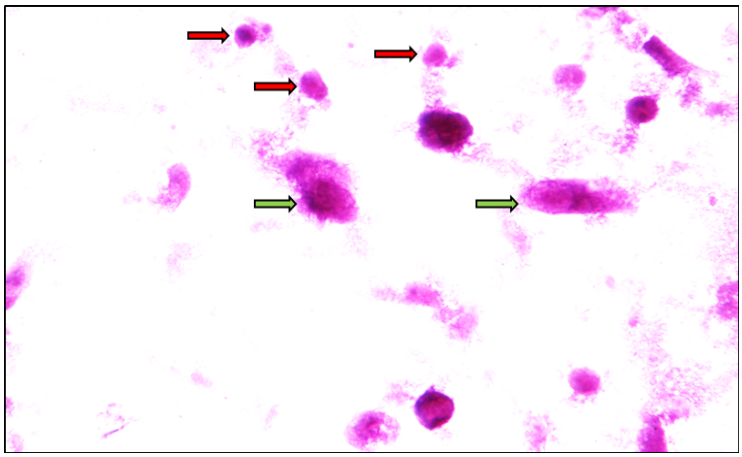

Figure 11. A Photomicrograph Showing Apoptotic Cells (Red Arrows), Swollen Necrotic Cells with Ruptured Cell Membrane (Green Arrows). (H and E, original magnification 100X, oil).

Discussion

Curcumin has been displayed to possess anti-inflammatory, hypo-glycemic, wound-healing, antimicrobial activities, anti-tumoral, and antioxidant agent capable of inducing apoptosis innumerous cellular systems [9]. Moreover, curcumin has strong therapeutic potential and protective effect against a variety type of cancers as well as ability to suppress proliferation, transformation, and metastasis of tumors [10]. It has been reported as one of the most studied chemopreventive agents. Curcumin could not make a clinical impact due to its rapid degradation and poor bioavailability in biological systems [11]. Some research on nanomaterials has shown

that nano-curcumin has better bioavailability, numerous investigations have been conducted to develop more efficient systems for drug delivery. It has been found that the particle size is an important parameter as it can directly affect the physical stability, cellular uptake, and drug release from the nanoparticles [12]. Nanocurcumin demonstrates comparable in vitro therapeutic efficacy to parent's curcumin against a panel of human pancreatic cancer cell lines, as assessed by cell viability [13].

In this study we studied nanocurcumin effect on HEP-2 cell line our results revealed that:

Regarding the cytotoxic effect of Curcumin nanoparticles on HEp-2 cells, viability was employed using MTT assay. Data recorded revealed that the mean viability percentage of Curcumin nanoparticles treated cells was concentration dependent and the safe concentration induced 100% cell viability was at ~<20 µM /mLG1. And the IC_{50} value determined was 40.82 ug/ml indicating a significant difference ($p<0.05$).

These results were found to be in line with a previous study by Afzali et al who found that the CUR-NPs inhibited proliferation and increased apoptosis in spheroid human breast cancer cells [14].

Our results revealed that nano curcumin has a strong cytotoxic effect against HEP-2 cancer cells which was in accordance with previous studies which revealed that the cytotoxic effect of nanocurcumin against normal BHK cells and human Hep-2 cancer cells was investigated and the data obtained using the NRU assay revealed that both the dose and incubation time were critical factors in evaluating the inhibition efficiency of nanocurcumin towards Hep-2 cells.

It was found that the IC_{50} value of nanocurcumin for 48 h was more effective with a higher anti-proliferative effect against Hep-2 cells than the IC_{50} value for 24 h [15].

In addition, using microscopic examination, the nanocurcumin Hep-2 treated were more shrinking, greater decrease of viable cells, more round, and less adherent at a higher concentration for a longer incubation period (48 h) [16]. Which was in line with our microscopic results that revealed that Most of HEp-2 cells showed cancer cells with signs of dysplasia such as cellular and nuclear pleomorphism, nuclear hyperchromatism and increased nuclear cytoplasmic ratio and the cellular outline was almost regular without evidence of any folding. Also, after 24 hours post treatment with IC_{50} concentration of Curcumin nanoparticles. Some cells of this group show apoptosis, such as cellular and nuclear shrinkage, apoptotic bodies and nuclear fragmentation. Other cells show necrosis in the form of swollen cells with ruptured cell membrane.

The percentage of cells arrested at the G0/G1 phase and S-phase significantly decreased compared with that of non-treated cells. Also, it was revealed that there was a significant elevated arrest during the G2-M phase in case of HEp-2 treatment with Curcumin nanoparticles. Also, there was a significant apoptotic cell in the pre-G1 phase.

This was in line with previous results showed that nano-curcumin has a direct anti-proliferative effect on EAC cell lines [17] (Milano, F 2013), as well as previous results showing that free curcumin decreases the proliferation and survival of esophageal cancer cells [18].

Regarding the apoptotic profile of treated cells, there was a significant elevated early and late apoptotic cells, necrotic cells, and total apoptotic cells after cell treatment with Curcumin nanoparticles which was in line with previous studies who revealed remarkable antiproliferative and apoptotic effects of polymeric Nano-micelles of curcumin in colorectal cancer cell lines [19].

The intracellular ROS level was increased in Curcumin nanoparticles treated cells. Which was in line with previous study who concluded that The Cur-coordinated nanoparticles developed in this study improved Cur stability, which could further release Cur in a ROS-dependent manner in cancer cells [20].

Also, our results were in line with a previous study which revealed that the induction of apoptosis in A549 cells by ZnO and Curcumin resulted by AO-EB staining. ZnO and Curcumin activated to promote intracellular ROS overproduction and induced apoptosis [21].

Conclusions

- Curcumin nanoparticles have a strong antioxidant effect
- Curcumin nanoparticles have anticancer effect

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

Ethical Declaration

This study will be carried out after ethical approval of the ethical committee, faculty of medicine, South Valley University.

Availability of data

Data is available upon request.

Conflict of interest

All authors declare that there is no conflict of interest.

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