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Cerium Oxide Nanoparticles Ceo_2np and Retinoic Acid Trigger Cytotoxicity and Apoptosis Pathway in Human Breast Cell Lines

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Abstract

Tumors are the most types of diseases that still kill human life, many treatments have been used, including chemotherapy and others, but the side effects were exponentially severe. The research has tended towards the use of nanoparticles and plant products. The nanoparticles used in the current study are CeO2 with a size of ≤5 nm in addition to the use of Retinoic acid (RA) as a synthetic natural chemical. The role of these materials in breast cancer has been investigated by using cancerous and natural cell lines by assay MTT and via qPCR technique. Five concentrations were used for each of the CeO2NP (0, 52, 70,100, 150, 200 µg / ml) and for RA (0,150,180,200,250,300µg / ml). To discuss the relationship between the joint actions of the two articles, they were merged after taking three concentrations for each substance and the CompuSyn software was adopted to determine that. The Results revealed that there is cytotoxicity of the two substances CeO2NP and RA on the cell lines (MCF-7, CaL51, & HBL-100) and that the levels of vitality decrease with increasing concentration, except for the normal cell line HBL-100, that their vitality increases with the increase in the concentration of the substance. The combined treatment of both substances did not show synergistic activity on the three cell lines. After 48 hours, CeO2NP and RA were caused different cytopathic changes in cell lines, those changes are outcome of biochemical and molecular events happening in apoptotic cells. In addition, cell death was seen after staining with the AO-EB dye after being treated for 48 hours. The current study examined the cellular pathways that depend on activating the gene expression of the caspase genes (Caspase 8& 9) in two cell lines (Cal51, HBL-100) showed that the CeO2NP have apoptosis-promoting effects by activation of the Caspase8 in both the normal HBL-100 and carcinogenic lines Cal51, while the retinoic acid, its action was similar to CeO2NP by activating Caspase8 in the normal cell line HBL-100, except that it stimulated the intrinsic pathway of apoptosis in cell line Cal51 through activation of the Caspase9. Our finding, that RA have a similar cytotoxicity on cell lines, while CeO2NP was more effective on MCF-7 and CaL51 lines, despite its obvious toxicity on normal cell line HBL-100. The substances did not show any synergy with each other when they were combined and treated with cells. It was also found that each substance has a different cell death pathway.

Keyword: CeO_2NPs, retinoic acid, breast cell lines, cytotoxicity, apoptosis

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Introduction

Chemotherapy is one of the methods used in treating cancerous tumours, but over recent years, it has become clear that this therapy causes extremely high side effects (Aslam *et al*., 2014), therefore there is great interest to develop t chemotherapy as alternative treatment that prevent the growth of tumour; such as using a natural product or combination of these products to treat cancerous tumours with this in mind the mitigating side effect levels (Mann, 2002). Among the natural materials that have been praised by studies and research as potential therapeutic materials, either individually or in combination, are nanoparticles due to their exceptional properties that they have such as their small size and shape (Baalousha *et al*., 2014). Nanotherapy is one of the modern methods used to limit the spread many diseases, especially cancer, therefore the researchers sought to conduct more studies on different types of nanoparticles. Many nanoparticles, such as silver, gold and cerium nanoparticles have been used as anti-cancer agents for the qualities of these nanoparticles (Abdel-Fattah and Ali, 2018). There was little local and international studies about CeO2NP, and the toxicity of CeO2NP remains controversial as conflicting results have been reported in the literatures.

Cerium oxide nanoparticles (CeO2NP) are promising therapy for some types of cancer diseases .Tiny volume of CeO2NP has more effectiveness in treatment than large. Its surface energetic more redox +3 and +4 processes. Several studies have stated the effect of the cerium oxide nanomaterial on cell lines: Nasiri *et al*. (2017) showed that using CeO2NP in several concentrations decreased cell line HT29 vitality, Kim *et al*.(2010*)* explained the toxicity of CeO2NP on the A549 carcinoma cell line causing damage in cell membrane of the streak cells,Ahamed *et al*.(2019*)* found occurrence of phenotypic changes in the A5A9 cell line by using different concentration of CeO2NP.

Several previous studies have confirmed that CeO_2NP has toxic effects on different human and animal normal cell lines (Benameur *et al*., 2015; Forest *et al*., 2017; Al-Ali *et al*., 2021). Conversely, Abbas *et al*.(2015) and pesic,(2015) have revealed that CeO_2NP targets cancer cells without normal cells. Some studies have showed that the affectivity of CeO2NP could be increased by used in combination with other products such as; anti-cancer drugs Oxalipatir, 5-flubrouracil, and Adriamyein León-Silva *et al*.(2018), these drugs have a side effects, so studies have directed to use of substances that a high efficacy with little or no side effects including metabolites such as the Retinoic acid (RA). (Pettinelli *et al*.,2018).

Retinoic acid (RA) is one of the retinoids, which is derivative of A vitamin.It is characterized by its ability to suppress tumor chemoprevention (Peehl and Feldman, 2003). Ramos *et al*. (2016) study demonstrated the effect of RA on neuroblastoma cells (SH-sy5y), where the rate of tumor cell proliferation decreased in concentration of 40µM. Lee *et al*. (2000) showed that the treatment of five cell lines for human colon cancer (DLD-1, HT-29, HCT-15,

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Colo-201, WiDE) with two concentrations of RA 1and 10 µM can prevent the growth of these lines and induce cell death in them. RA also could prevent the growth in cell cultures of the leukemia cell line in addition to inhibiting their migration *(*Idres *et al*.,2001. Six breast cancer cell lines (MCF-7 and ZR, 75.1, MDA, MB, 231) treated successfully by RA with different concentration (Toma *et al*. 1997), the study showed the ability of RA to inhibit the growth of tumor cells. The present study aimed to find the concentration is required to inhibit, in vitro, some biological processes by 50%. (IC50) of CeO2NP and RA, and investigate the role of CeO2NP whether having selectivity to cancer cell lines or not. On the other hand, our study focused on investigating the synergism or antagonism relationship between CeO2NP and RA in cancer and normal cell lines using the CompuSyn software, and determination possibility of the potency these natural materials inducing cellular death pathways in normal and cancerous cell lines.

Materials and methods

Cell maintaining

The cell lines in the current study were obtained from Cell Bank in the Tissue Culture Laboratory at Department of Biology College of Education for applied science Basrah University. They represent the human breast epithelial tissue MCF7 carcinoma cell line and the Cal51 carcinoma cell line derived from the human breast duct tissue and the normal human breast cell line HBL-100 derived from the breast normal epithelial tissue), and its cultivation media (25ml tissue culture flasks contain RPMI-1640 culture medium containing 10% of bovine serum supplemented with antibiotics of 2.50 ml of gentamicin and 3 ml of penicillin. Cell lines incubated at 37°C and 5% CO2 humidity,after that when the cell line reached to 70-80 % confluent the subculture was conducted (Freshny, 2015).

Cytotoxicity assay

After a trypsinization process , cells are suspended by the RPMI1640 10% Fetal Bovine Serum medium, the suspended cells were cultured at 1 * 10^4 cells / well using special culture plates of 96 well, the plate then transfered into the incubator at 37°C and 5% CO2 humidity. The plates were incubated until the monolayer was formed. After 24 hours of incubation (a monolayer was formed), cell lines were treated with six concentrations of CeO2NP (0,52,70,100,125,200 µg / ml), and six concentrations of RA (0,150,180,200,250,300 µg / ml). 100µl of each concentration put in each well of the culture plate with four replications for each concentration, then it was return to the incubator at of temperature 37°C and 5% CO2 humidity and left for 72 hours (Dutta *et al*.,2009). The experiment was replicated three times to ensure accurate results, this is as recommended (AL-Shammery *et al*.,2019).

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The MTT (Methyl Thiazolyl Tetrazolium) assay used to study the efficacy of materials on cell lines. After discard the old medium, 10µl (50 mg / ml PBS) of MTT stain from sigma company and 90µl of Media Free Serum were added to each well. The plates were incubated for 2.5 hours (Darroudi *et al* .,2013). The dye was removed and replaced with 100 µl of DMSO (Dimethyl sulphoxide) per well. And left at room temperature for 20 minutes in the dark, after which the absorbance was read by the ELIZA device at a wavelength of 490 nm (Olia *et al*.,2019). The efficacy of the materials was calculated according to the equation below:

$$PA = \frac{B}{A*100}$$

Where PA represents the percentage of cell viability, B represents the absorbance of material-treated wells and A well absorbency for a non-treated control group. The inhibition concentration that kills 50% of cells were calculated according to GraphPad prism (version 8.0).

Exposure cell line to combination of retinoic acid and CeO_2 nanoparticles

Three cell lines were exposed to CeO2NP and RA together with three concentrations of each one (52, 100 & 200 µg / mL) and (150, 200& 300 µg / ml) respectively, 50 µl of CeO2NP and 50 µl of RA) are placed together in one well of the culture plate, three repeatedreplications of treatments, in addition to the control group (untreated cells) , After completing the distribution of the required concentrations in wells of the plate using micropipette, then was covered with lid and tightened with a tape of parafilm, then incubated at 37°C with 5% humidity of CO2 and left for 72 hours, the experiment was repeated four times (AL-Shammery *et al*.,2019). Eventually, the MTT assay used to calculate the percentage of cell viability, then the data were analyzed according to isobologram technique using CompuSyn software (Version 2011) (Gao *et al*., 2003).

Morphological analysis

After a trypsinization and cells suspension with RPMI1640 10% FPS medium, the cells were cultured $1 * 10^5$ on the slide cover using a 5 ml / well Petri dish. Then, the Petri dish was entered into the incubator at 37°C and humidity of 5% CO2 for a period of 24 hours, and the Petri dish was left in the incubator until the monolayer layer was formed. Then the cell lines (MCF7, Cal51& HBL-100) were exposed to the IC50 of CeO2NP (136, 85.9& 106.7 µg / ml), respectively, and the IC50 of RA (212, 188& 197 µg / ml) respectively. Then, the Petri dishes were incubated for 48 hrs, so that the cover slide was extracted. Some of it fixed with formalin 4% for 5 minutes, after which they have washed with PBS solution for 2 minutes and treated with ethyl alcohol and xylene, stained with hematoxylin and eosin , then cells were examined under an light microscope and photographed with a CANON digital camera under different powers magnification (Khoshdeli *et al*.,2017).

Some cells were stained that grown on the cover slide utilizing acridine orange- ethidium

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bromide (AO-EB) dye, these cells were treated with the IC50 of CeO2NP & RA for 24. The (AO-EB) dye prepared according to liqu *et al*.(2015) , then examined directly under a fluorescent microscope.

Study of apoptosis pathway

Quantitative PCR (qPCR) was conducted for cell lines Cal51 and HBL-100 cells that cultured at 100 x 105 cells / well in 12-well plates for 24 hours, after that exposed to the half-lethal concentrations IC50 for CeO2NP (µg / ml 86, 136) and µ g / ml 197, 212) from RA, after 48 hours of incubation of temperature at 37 Celsius and 5% CO2 humidity, RNA was extracted from cell lines according to the GENEzolTM TriRNA Pure Kit, the RNA was transformed into cDNA using Accupwr @ Rocketscripttm using the reverse transcriptase enzyme. Go Taq qPCR Master Mix was used in qPCR reaction , then adjusted as shown in (Table.1)

Table 1 Quantitative PCR (qPCR)

NO.	Steps	Temperature	Time	No. of Cycles
I	Pre-Denaturation	95 ^{o}C	2 min	1
II	Denaturation	95 ^{o}C	30 sec	45
III	Annealing	60 ^{o}C	30 sec	
IV	Extension 1	72 ^{o}C	30 sec	
V	Final Extension	72 ^{o}C	10 min	1

Table 2 Sequences of caspase8, Caspase 9 and P53 used in real time

Gene			bp
Caspase8	F	5- CATCCAGTCACTTTGCCAGA-	128bp
	R	5- GCATCTGTTTCCCCATGTTT-3	
Caspase9	F	5-GTTTGAGGACCTTCGACCAGCT-3	129bp
	R	5-CAACGTACCAGGAGCCACTCT-3	
P53	F	5-CCTCAGCATCTTATCCGAGTGG-3	128bp

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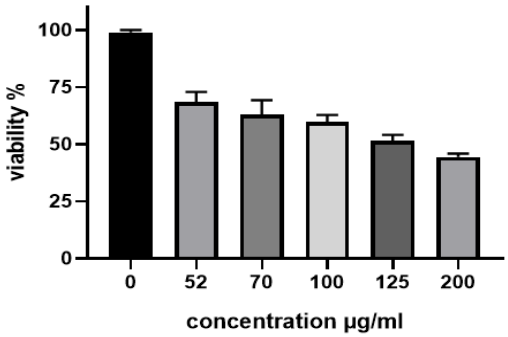
	R	5-TGGATGGTGGTACAGTCAGAGC-3	
Hmn rRNA18s	F	5-GGA GTA TGG TTG CAA AGG TGA-3	128bp
	R	5-ATC TGT CAA TCC TGT CCG TGT-3	

Results

After 72 hours of reading the absorbance by the ELIZA method, the results of the MTT assay showed that the vitality of cancer cell lines is affected by the CeO2NP. The used concentration of this nanomaterial causes growth inhibition of normal and cancerous cells. The growth inhibition of cell lines was increased concentration-dependent manner, except for the HBL-100 cell line. (Figure.1) shows the vitality of the cell line Mcf-7 when exposed to the CeO2NP in the concentrations 0,52, 70, 100, 125and 200 µg/ml. The highest vital percentage was 68% at the low concentration (52 µg/ml), while the lowest rate of vital percentage was 44% at 200 µg/ml. The IC50 of CeO2NP in the MCF-7 breast cancer cell line reached up to 106.7µg/ml as shown in (Table.3).

Table 1. Half- lethal concentration(IC50) for CeO2NP and RA in three types of cell lines.

Cell line	IC50 µg\ml		
	HBL-100	MCF-7	CAL51
RA	211.5	188	197
CeO2NP	136	107	86



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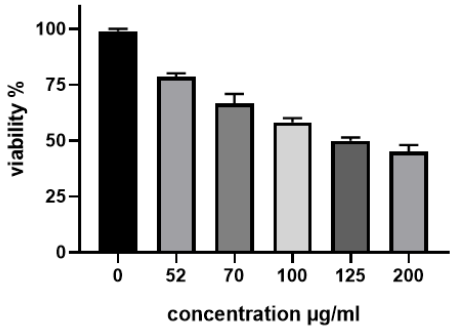

Figure 2 Vital ratio in the cell line normal cell line type Cal51 when exposed to CeO2NP after 72 hours, (n = 4)

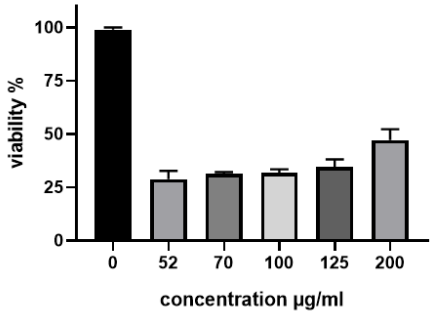

Figure 1 Vital ratio in the cell line normal cell line type HBL-100 when exposed to CeO2NP after 72 hours, (n = 4)

When the cancer cell line Cal51 was exposed to the CeO_2NP for 72 hours, it was observed that the highest rate of vitality was 78% at concentration of 52 µg/ml, While the lowest vitality percentage was 45% at concentration of 200 µg/ml as shown in (Figure.2). Where the IC50 of these cells was 86µg / ml as shown in (Table.3).

The normal cell line, HBL-100, which exposed to a series of concentrations of CeO2NP (0,52,70,100,125,200 µg/ml) for 72 hours, the results showed that IC50 also affected the vitality normal lines cells. The cells also followed an opposite behavior to increase the concentration in its growth. It was found that as the concentration decreased, the inhibition of cell growth increased, so the rate of cell viability was approximately 29% at the low concentration52 µg/ml. The rate of cell viability gradually increased than that at higher concentrations up to approximately 47% at concentration of 200µ g / ml (Figure.3), and it reached 136 µg/ml for the IC50, as shown in (Table.3).

Cytotoxicity of Retinoic acid on cell lines

Figure.4 showed the rate of vitality ratios of cancer cell line Mcf-7 when exposed to a series of concentrations of RA (0,150,180,200,250,300 µg/ml) for 72 hours. It was found that the highest rate of vital percentage reached 68% in the low concentration (150 µg/ml), while the rate of vitality was the lowest about 39% at 300 µg/ml. The IC50 of cell line Cal51 reached 197 µg/ml as shown in Table.3. When exposing the cancer cell line Cal51 to a series of concentrations of RA (0,150,180,200,250,300 µg / ml) for 72 hours, the results showed that the cell viability rate was 78% in low concentration (150 g/ml) compared to 47% in high concentration (300 µg/ml), as shown in (Figure.5). The IC50 of this substance was 188 µg/ml of Mcf-7, as shown in Table.3. While when exposing the normal cell line HBL-100 to a series of concentrations of RA (0,150,180,200,250,300 µg / ml) for 72 hours, the rate of the vitality was 46% at the low concentration 150 µg / ml, compared to the highest rate 39% at concentration of 300 µg / ml, as

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shown in Figure.6. The IC50 was 212 µg / ml, (Table.3).

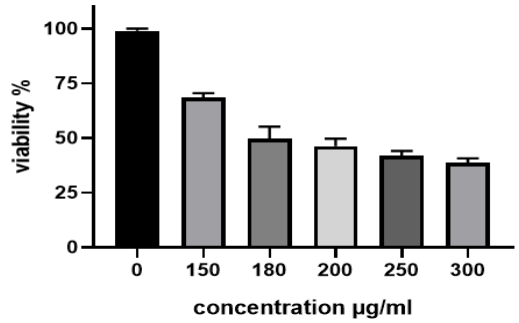

Figure 4 Average vital ratios in the cell line of breast cancer type Mcf-7 when exposed to RA after 72 hours, (n = 4)

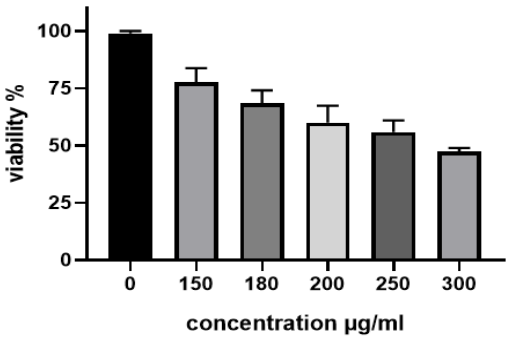

Figure 5 Average vital ratios in the cell line of breast cancer type Cal51 when exposed to RA after 72 hours, (n = 4)

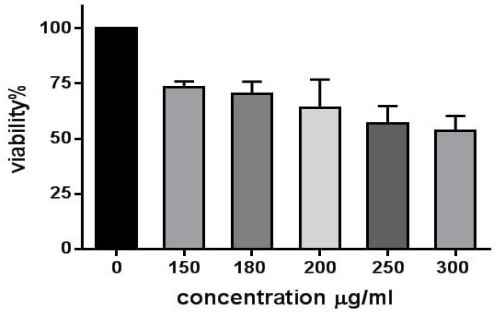

Figure 6 Average vital ratios in the cell line of breast cancer type HBL-100 when exposed to RA after 72 hours, (n = 4)

On the other hand, the statistical analysis found a significant difference between CeO2NP and RA in the first cell line in each lines (CaL51, MCF-7) at a significant level of $P < 0.05$, as can be seen in the Figs.7A, B,while there wasn't any significant difference between the substances for the normal cell line HBL-100. This indicates that both materials had a clear convergent effect at the level on the cell line.

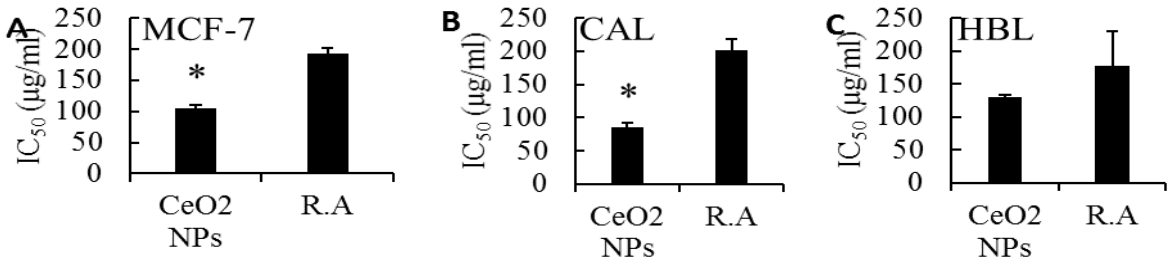

Figure 7 Significant difference between the effect of substances on a single cell line

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Synergistic or antagonistic effect of CeO2NP and RA on cell line

The cytotoxicity data extracted from MTT assay for cell lines were entered for three concentrations of RA and CeO2NP besides three concentrations of mixture of the two substances together, this to determine the synergistic or antagonism action of the two substances on the different cell lines. The results obtained from the CompuSyn program showed that the two substances did not have a synergistic action. Together in the three concentrations and on different lines, if the values of the combination index (CI) are greater than 1, the action of the two substances together is antagonism in the concentrations used on the three cell lines, (Tables 4,5,6 and *Figure* 8,9, 10).

Table 4 (CI) combination index values for three concentrations of a mixture of CeO2NP and RA obtained from the CompuSyn software program in Mcf-7 cancer cell line (n = 3).

CI	effect	con. RA	con. CeO2NP	POINT
2.71539	**0.275**	**150**	**52**	**1**
1.03537	**0.62**	**200**	**100**	**2**
1.39712	**0.672**	**300**	**200**	**3**

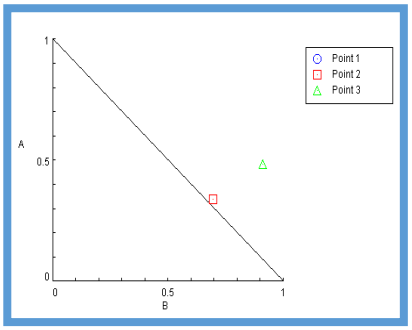

Figure (8) Shows the antagonism action for a mixture of CeO2NP and RA on the Mcf-7 cancer cell line and for three concentrations (CeO2NP 52µg / ml + RA 150 µg \ ml), (CeO2NP 100µg \ ml + RA 200 µg / ml) and (CeO2NP 200µg / ml + RA 300 µg / ml) for 72 h exposure, n = 3.

Table 5 (CI) combination index values for three concentrations of a mixture of CeO2 and RA obtained from the CompuSyn software program (n = 3).

CI	effect	con. RA	CeO2NP con.	POINT
1.92433	**0.34**	**150**	**52**	**1**
1.12759	**0.6**	**200**	**100**	**2**
1.41247	**0.675**	**300**	**200**	**3**

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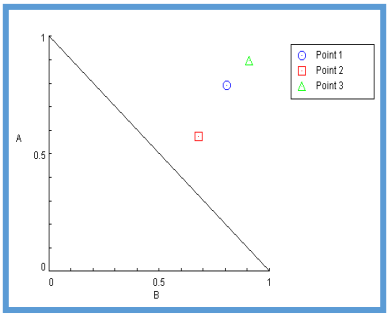

Figure (9) Shows the antagonism action for a mixture of CeO2NP and RA on the Cal51 cancer cell line and for three concentrations (CeO2NP 52µg / ml +(RA 150 µg \ ml), (CeO2NP 100µg \ ml + RA 200 µg / ml) and (CeO2NP 200µg / ml + RA 300 µg / ml) for 72 h exposure, n = 3.

Table 6 (CI) Combination index values for three concentrations of a mixture of CeO2NP and RA obtained from the CompuSyn software program (n = 3).

CI	effect	con. RA	CeO2NP con.	point
2.13462	**0.4**	**150**	**52**	**1**
3.82638	**0.319**	**200**	**100**	**2**
6.75070	**0.28**	**300**	**200**	**3**

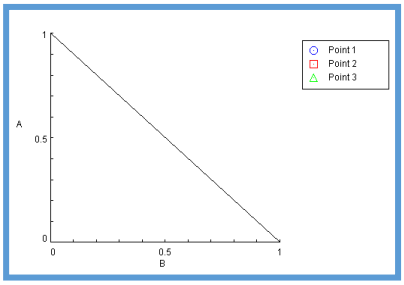

Figure (10) Shows the antagonism action for a mixture of CeO2NP and RA on the HBL-100 cell line and for three concentrations (CeO2NP 52µg / ml + (RA 150 µg \ ml, CeO2NP 100µg \ ml + (RA 200 µg / ml) and) CeO2NP 200µg / ml + (RA 300 µg / ml for 72 h exposure, n = 3.

Morphological analysis

The cell cultures of the three cell lines were dyed on the slide cover after treatment with the IC50 of two substances, CeO2NP and RA for 48 hr. They stained with the H&E dye and examined under a microscope. Pathological changes were diagnosed in the cell cultures. RA and CeO2NP leads to clear cellular pathological changes, including the distribution and shape of cells in the cell culture, in addition to changes in cell composition (nucleus and cytoplasm). The pathological changes are found in normal, and cancerous cell lines are very similar.

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Untreated and cultured Mcf-7 cells line appear on the slide cover, as a monolayer after 48 h of culture in medium free serum MFS (Figure 11 A, B), the cells appear in elongated forms, they had - oval nucleus. The cell cytoplasm was pink and its nucleus was light blue (Figure 11 A, B), the microscopic examination of the Mcf-7 cells treated with CeO2NP and RA and cultured onto slide cover disintegrates the cell culture cells. The emergence of large spaces devoid the cells. A change in the apparent shape of the cells and their atrophy was observed. The karyopyknosis was appear in some treated cells. It was clear that the most cells in the cell culture suffering from degeneration and necrosis at different stages, also the decomposition of other cells have been observed as rounded cells (Figure 11 D, C).

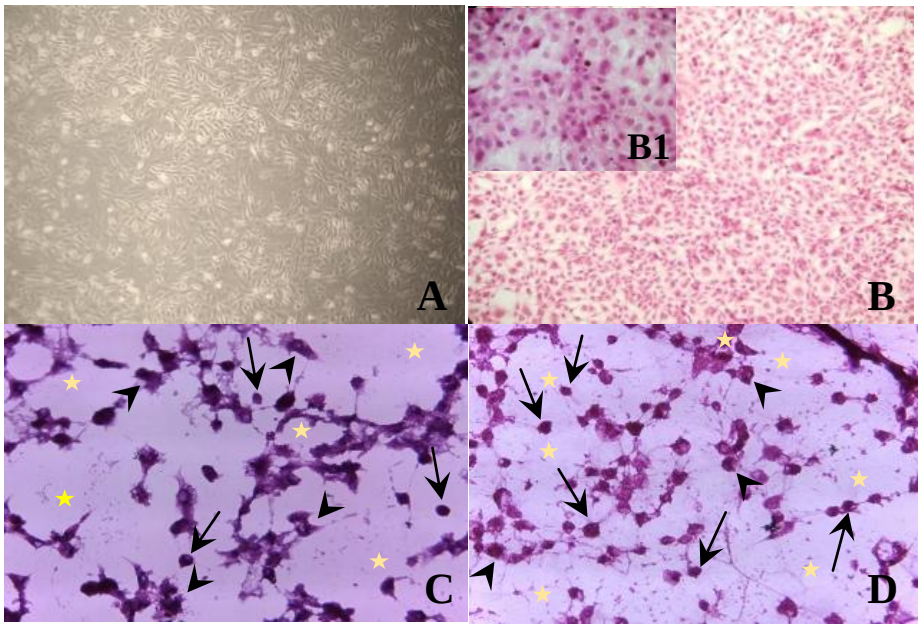

Figure (11) MCF-7 breast cancer cell line treated with CeO2NP and RA and implanted on the slide cover after 72 hours, (A and B) untreated cells appear as a single cell layer monolayer, at magnification 10x, A non-pigmented cells, B1 stained cells, B1 non-treated Mcf-7 cells, H&E, at magnification 40X. (C) Mcf-7 cells treated with CeO2, H&E, at magnification 40X, stain showing apoptosis (arrow), arrowheads indicating degradation of the cytoplasm of cells. (D) Mcf-7 cells treated with RA staining material, showing cellular pathological changes, arrows indicate necrotic cells, arrowheads indicating cytoplasmic degradation, H&E, at magnification 40X.

The Cal51 cell line implanted on the slide cover slide, untreated appears in the form of a monolayer, as the cells appear as a compact, enlarged, so they appear to be polygonal. The single cells appear to be somewhat elongated, the hematoxylin & eosin dye stained their components,

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so light pink cytoplasm and light blue nucleus were appeared (Figure A, B)

The examination of treated Cal51 cell line with CeO2NP and RA have revealed that this cell line suffered obviously cytopathic changes, represented by the rounded of cells, areas devoid of cells appeared as a result of the loss of their ability to adhere and communicate to each other. Furthermore, The microscopic examination showed the presence of cells suffering from vacuole degeneration. While necrotic cells appeared that containing a karyopyknotic nucleus that was difficult to distinguish their details and surrounded by lysis cytoplasm. Some parts of the cell culture contained remnants of dead and lysis cells (Figure. 12 D, C)

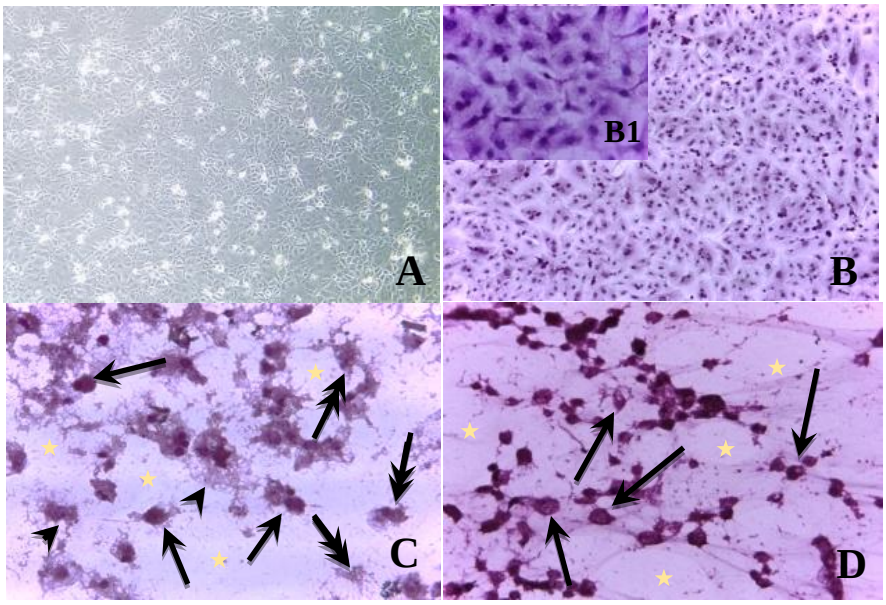

Figure (12) Cal51 cells treated and untreated with CeO2NP and RA grown on the cover slide after 72 hours. (A, B) Cal51 cells are untreated, A - stained cells, Note the compactness of cells and their shape, at magnification 10X. B- Cells stained, note the shape of cells and the staining of their compartment, H&E, at magnification 20X, B1- Magnified image for non-treatment cell culture, H&E, at magnification 40X. (C) Staining cells showing cellular changes such as vacuole degeneration (arrowheads), cell necrosis (arrows), and the emergence of cell-free spaces (asterisk). Note the presence of lysed cells (double arrow), H&E, at magnification 40X. (D) cells treated with RA. Note the morphological changes in cells treated with RA. The cells suffer from necrosis (arrows), vacuole degeneration (double arrows), in addition to the presence of empty spaces between cells (asterisk)

The untreated normal cell line HBL-100, after 72 hours of culture on the cover slide, appears as monolayer, the cells appeared as an elongated with an oval nucleus. The cell was stained with H&E, the cytoplasm was stained pink, and the nucleus was light blue (Figur*e*. 13A, B). Upon examining the cell culture cells treated with CeO2NP and RA. It was found that they suffer from cytopathic changes. Some treated cells with CeO2NP appeared as a cluster cells, formed a cell mass that has lost their normal and distinctive shape. The other cells suffered from necrosis and

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appeared as dark balls, with small size (as a atrophied cells), and it seems that the cells go through a series of continuous processes that ultimately lead to the degradation of the cell and its disappearance. These processes begin with the lysis of parts of the cytoplasm, accompanied by the thickening of the nucleus and its atrophy In some cells in cultured cells, we found its suffered from gradual lysis, its appeared as a necrotic cells. the event of necrosis processes ended with completely cell lysis leaving in place decomposed residues in large areas of the culture devoid of cells (Figure C). The cells treated with RA undergo almost a similar change, the shape of the culture cells changes. Therefore, It appears as a rounded cells with a dark nucleus, these cells are represented necrotic cells, while other cells aggregated in the form of a cell mass made up of spherical cells with little or degeneration cytoplasm, other cells appeared with long, very thin cytoplasmic protrusions, which connected them to nearby cells, appeared large areas without cells were observed in the cell culture (Figure D).

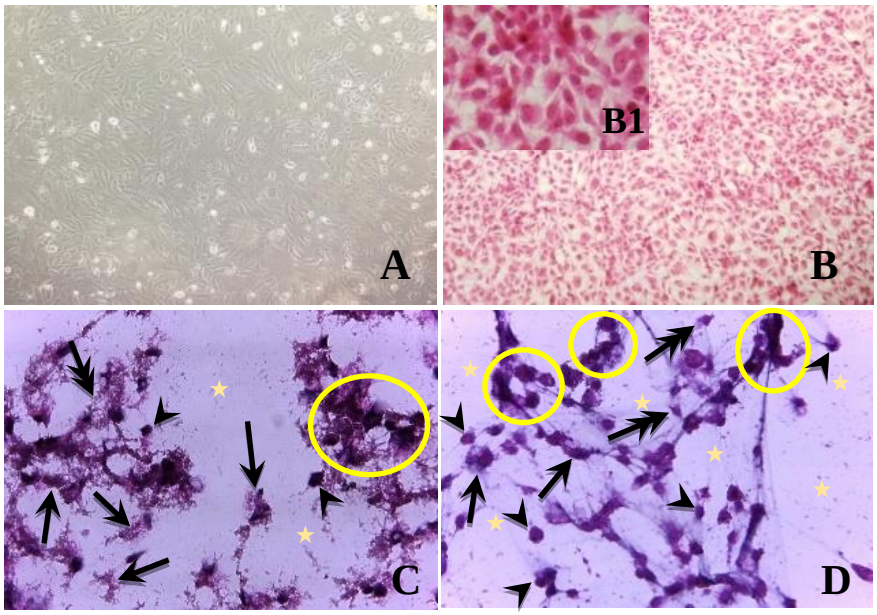

Figure (13) HBL-100 cells grown on the treated and untreated cover slide after 72 hours. (A, B) Untreated cells appear as a monolayer, A- unstained cells 10X, B- stained cells showing cell stains and details with H&E stain, at magnification 20X power. B1- H&E, at magnification 40X. (C) Cells treated with CeO2 showing the cytopathic on the cell line, the stages of cell necrosis (arrowheads), double arrows indicate cytoplasmic degeneration as a stage of necrosis, the arrow indicates residual decomposed of cells in the middle of spaces free of cells (asterisk), the circle refers to the grouping of cells as a mass, H&E, at magnification 40X. (D) cells treated with RA, stained cells showing necrosis (arrowheads) and cells with degraded cytoplasm (arrows), (double arrows) refer to dissolved cell remnants. Note the cell clustering in the form of a mass (circle),

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Study of programmed cell death by using an acridine tincture

Acridine Orange-Ethidium Bromide (AO-EB) dye was used to staining cells were cultivated on coverslide after treated with CeO2NP and RA. AO-EB assay utilized to diagnose stages of programmed cell death. the treated coverslide shown the differential absorption of fluorescent DNA-binding stain AO-EB, the cells that have undergone apoptosis are non-viable cells colored with red while the viable cells colored with green. (Figure. A14, A15, A16). Different stages of the cell death process were diagnosed, as the nuclei of some cells appeared. A lumpy chromatin formed in yellow, which represents one of the early stages of nucleus fragmentation and the appearance of cell death bodies. While those blocks were formed in red in the advanced stages of cell death. The results showed that some dead cells coloured all their components (the nucleus and cytoplasm) in red, and in the cell cultures of the three treated lines the emergence of large spaces devoid of cells as a result of cell death and separation from the cultivated cells (Figure 14-16).

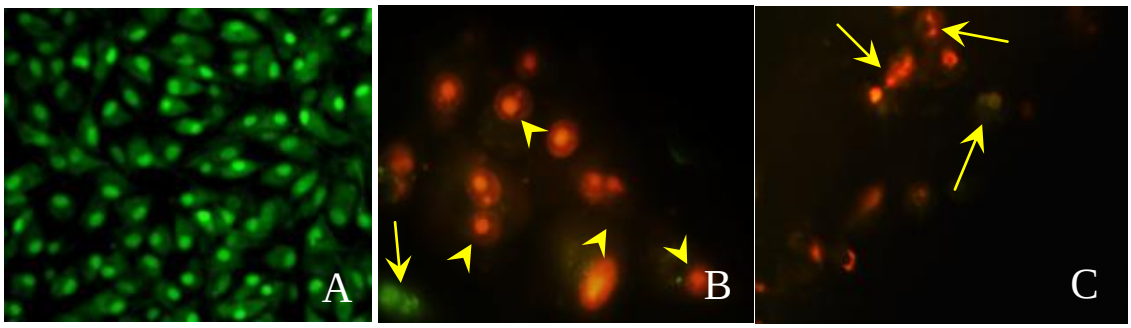

Figure (14) Mcf-7 cell line stained and untreated AO-EB stain cells culture on the slide cover after 48 hours. (A) Untreated cells appear unaffected by green colour, AO-EB at magnification 20X. (B) Cells treated with CeO2 appear to be affected. Note that the chromatin material mass into the nucleus and colours it in red (arrowheads). The arrow indicates the fragmentation of the nucleus and its appearance as green clumps representing an early stage of programmed cell death, AO-EB at magnification 20X. (C) Cells treated with RA show that most of the transplanted cells are affected, and large areas devoid of cells appear. Note the advanced stages of programmed cell death by the appearance of the chromatin material for the nucleus and its red colour (arrow). Arrowheads indicate the condensation of the chromatin material into dead cells and its red colour, AO-EB at magnification 20X.

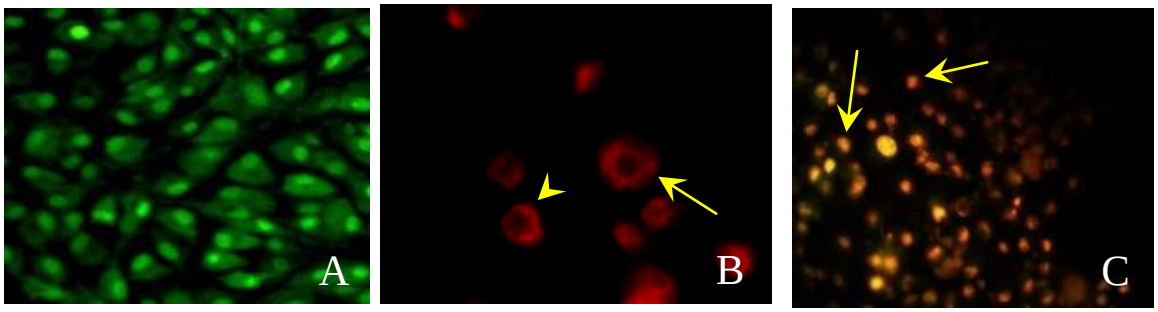

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Figure (15) Cell-line Cal51 Dye AO-EB Double stained with treated and untreated AO-EB stain on the slide cap after 48 h. (A) Untreated cells are considered healthy, and their contents (nucleus and cytoplasm) are coloured green and cells form integral monolayer, AO-EB at magnification 20X. (B) Cells treated with CeO2 appear to be affected. Note the dead cell staining red (arrow) and the arrowhead indicates a cell in repeated stages of cell death AO-EB at magnification 20X. (C) Cells treated with RA showing red coloured dead cells (arrows) , AO-EB at magnification 20X.

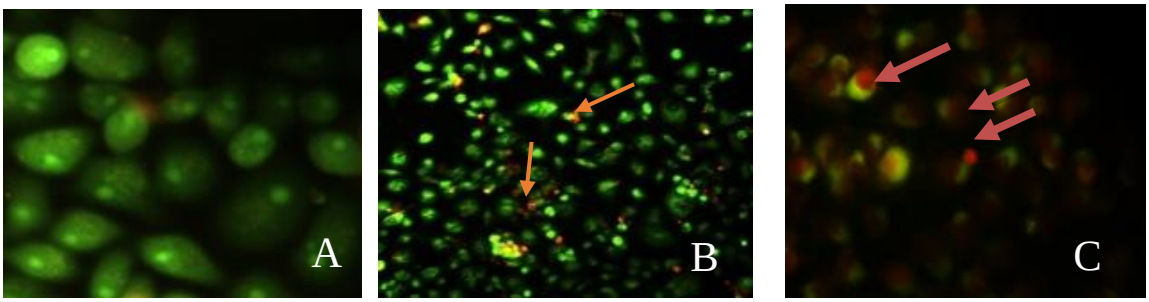

Figure (16) HBL-100 cells grille with acridine orange dye - ethidium bromide treated, untreated and duplex, on slide cover, after 48 hours (A)Untreated cells appear unaffected and form a single layer and stain green with acridine stain AO-EB at magnification 40X. (B) Cells treated with CeO2 appear affected by the treatment, and their cells are coloured red (arrows) AO-EB at magnification 20X. (C) The effect of cells treated with RA evident if the cells lose their normal characteristics and become discoloured to coloured red (arrows) AO-EB at magnification, 40X.

Gene expression of apoptosis in Cal51& HBL-100

1- Gene expression of programmed cell death genes in the HBL-100 cell line

Gene expression of programmed cell death genes in HBL-100 cell line was studied, after treatment for 24 hours with IC50 of CeO2NP and RA in addition to a mixture of the two substances together. The results showed that the two substances could stimulate the gene expression of programmed cell death genes in the normal cell line HBL-100. The results showed a similarity in the pathway taken by the two substances, each of them alone, in the cell line HBL-100 to stimulate the genes of programmed cell death, as significant stimulation of Caspase 8 gene expression was observed when cells were treated with the two substances, as shown in (Figure 17). It is evident in the figure that the two substances stimulated apoptosis in the caspase 8-dependent external pathway and did not show any stimulation of caspase 9 and P53 gene. The co-treatment also did not show any expression of the Caspase,9 and P53 genes (Figure 17)

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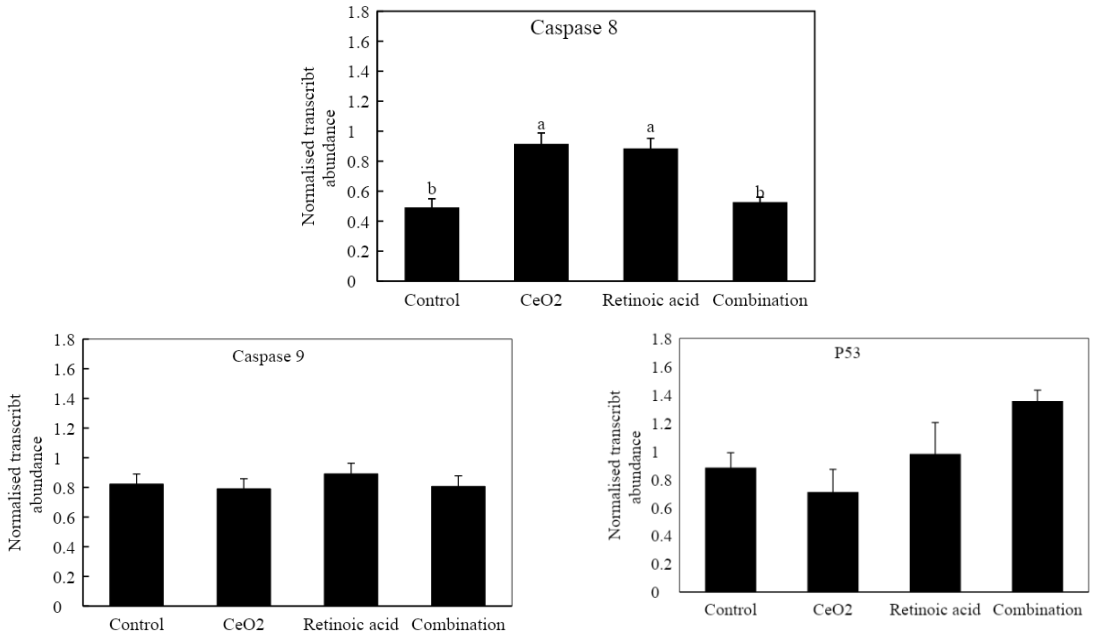

Figure (17) Gene expression of caspase 8, caspase 9 and the P53 genes for both ceo2NPand RA and the two compounds together in addition to the control group in the cell line HBL100, P≤ 0.05, n = 3.

2- Gene expression of apoptotic genes in the cal51 cell line

Gene expression of apoptotic genes was followed up at cell line Cal51, as in the cell line HBL-100, where the cells were treated for 24 hours with the half-lethal concentration of both CeO2NP and RA, in addition to the combination of the two substances together, and the results showed that the two substances were stimulated expression of apoptosis genes in cell line Cal51. The apoptotic pathway that induces by the CeO2NP was different from that one of the pathways which induce by RA . The CeO2NP was triggered by the extrinsic apoptosis pathway, so a significant increase in the stimulation of gene expression for the caspase 8 gene was observed when cells were treated with CeO2NP in cell line Cal51(Figure18), the gene expression of Caspase 9 and P53 genes did not show stimulation when treated with CeO2NP (Figure18). Whereas, treatment with RA resulted in significant stimulation of gene expression for Caspase 9 gene (Figure 18) No stimulation of the Caspase 8 and P53 genes was shown after 24 hours of treatment with RA, meaning that this substance stimulated the endogenous Caspase 9 dependent pathway (Figure 18).

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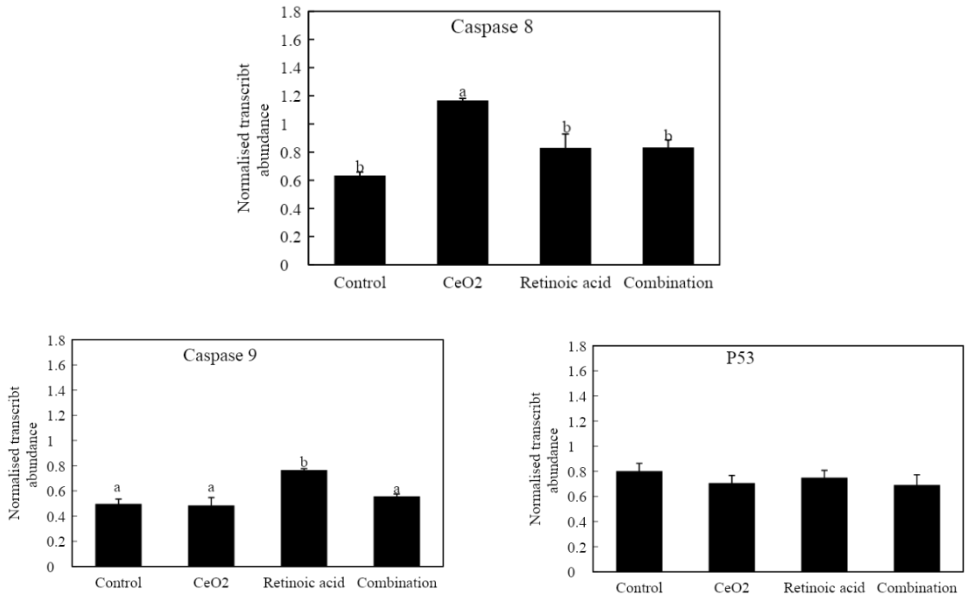

Figure (18) Gene expression of caspase 8, caspase 9 and the P53 genes for both ceo2NP and RA and the two compounds together in addition to the control group in the cell line Cal51, P≤ 0.05, n = 3.

Discussion

Understanding the nature of activity of different substances inside and outside the body is one of the important aspects that have been addressed in many studies. This is in order to provide accurate information about the areas in which these substances may be included. That is what the present study is designed to analyze and understand the effectiveness of nanoparticles of cerium oxide and retinoic acid individually or together in both normal and cancer cell lines.

The results of MTT assay showed that CeO_2NP has toxicity effects on various normal and cancer cell lines, which indicates that CeO_2NP has the ability to enter both normal and cancerous cells alike. As it is known that the nanoparticles enter the cells through their membranes via pinocytosis process (Behzadi *et al*.,2017). This entry into the cells seems to cause major changes in the structure as well as the function of these cells, and leading to cell death eventfully. The toxicity of CeO_2NP depends on particle characteristics of these substances, where is the surface valence ratio Ce3/Ce4 the most important one, which determines the action nature of these particles during their entrance into the cells (Hochella *et al*.,2008).

The physical and chemical properties of CeO_2NP enable it to interfere with the cellular pathways at the molecular level. It is possible that the properties of CeO_2NP stimulate cellular signals inside cells to inhibit a specific biological pathway (Anderson *et al*.,1994 and Flohé *et al*.,1997). This could interpret the cytotoxic effects caused by nanoparticles (CeO_2NP) in different cell lines. It was found that stopping the change of the valence ratio ($Ce3^+/Ce4^+$) may

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lead to the accumulation of some forms of ROS, this cause a harmful oxidative stress in the cells, which threatens the balance in them, then make them weakens more and eventfully causes a cellular death (Pizzino *et al*.,2017 ; Fiaschi and Chiarugi,2012). These ROS may act as early cellular signals that stimulate the pathways of cell death (Vassie *et al*.,2018). Some studies on SCL-1, CRL 1541, MCF-7 such as: Alili *et al*.,2011; Babu *et al*.,2010 ; Colon *et al*.,2010 ; Tarnuzzer *et al*.,2005 have been indicate the ability of CeO_2NP to impair the viability of human cancer cells, as a result of the damage caused by CeO_2NP in the plasma membrane to human bronchial carcinoma cells (Lin *et al*.,2006), which also confirmed by this study when staining the nuclei of different cells with the ethidium tincture, where usually, the normal membranes are impermeable to this tincture (Hayashi *et al*.,1990).

The size of the nanoparticles has a role in their bioactivity and often the sizes smaller than 10nm have higher toxicity than the larger ones (Schubert *et al*.,2006 ; Kumar *et al*.,2014). This may also explain the cytotoxicity behavior of CeO_2NP in this study, which used volume less than 5nm, where the small volumes are characterized by an increase in the surface size ratio Ce+3\Ce+4 Yokel *et al*.(2014), and also an increase in cellular absorption (Sajid *et al*.,2015). In contrast to the results of this study and previous studies, Schubert *et al*.,(2006) and Kumar *et al*.(2014) showed that the large volumes are more toxic than small volumes because of their accumulation in the biological system.

The CeO_2NP toxicity varied according to the different cancer and normal cell lines. The present study proved that CeO_2NP toxicity is not selective against cancerous or normal cells, where the half-lethal concentration (IC_{50}) has been reached in all lines and with the same concentrations for all lines. The variety in the toxicity of CeO_2NP on normal and cancerous lines or its targeting cancerous rather than natural lines is due to the different nature of the cells themselves, and then the different mechanism followed by CeO_2NP within the vital system of cells to induce the toxic effects (Kim *et al*.,2010). This was evidence in pathways of apoptosis during study the gene expression of apoptotic genes.

Based on some references, CeO_2NP toxicity may differ according to different cell lines, which in turn differs depending on the mechanism in which CeO_2NP affects cell lines and that are associated with cell membrane damage (Kim *et al*.,2010). Moreover, CeO_2NP has no toxicity on normal cells (Alili *et al*.,2011; Babu *et al*.,2010 ; Colon *et al*.,2010 ; Tarnuzzer *et al*.,2005). while other studies have proved its toxicity to normal cells such as pneumocyte of rat Cho *et al*.(2010), human cell line (THp-1) Jiang *et al*.(2018), and rat liver cells (Nalabotu *et al*.,2011). Studies differ on the effects of CeO_2NP on normal cells.

In this study, several concentration of CeO_2NP and MTT assay have been used to get the half lethal concentrations (IC_{50}) of CeO_2NP in normal and cancerous cell lines, and IC_{50} values varied between cell lines. The results of the statistical analysis showed that normal cells (HBL-100), cancerous cells (Mcf-7) have a high IC_{50} rate compared to the rest lines, which is indicate

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that these lines are less sensitive to CeO_2NP than Cal51. This is due to the significant difference between Cal51 cell and both of HBL-100 and Mcf-7 cells . In three experiments, the higher value of half lethal concentration was for HBL-100 cells, followed by the lethal concentration rate of IC_{50} for the cell line Mcf-7.

The results revealed that the toxicity of CeO_2NP depends on the used concentration, where the toxicity increased on cell lines with increasing of the concentration, which is also confirmed by several studies (e.g. Nasiri *et al*.,2017 ; Mittal and Pandey,2014 ; Milani *et al*.,2017). Only the HBL-100 was an exception, where its toxicity increased at lower concentrations. The reason of that may be due to the nature of the HBL-100 and the activity of CeO_2NP and its properties in the normal cell line (Cho *et al*.,2010).

Plant compounds possess a set of properties that enable them to carry out vital activities within the cells. Among these compounds are the terpenoids to which the RA belongs (Álvarez *et al*.,2008). The later plays several functional roles in various stages of cell differentiation, this in addition to its role in differentiated cells (Lee *et al*.,2010). Consequently, it inhibits the cell growth in the tumour masses (Al-Sheddi *et al*., 2015). So, the results proved that RA leaded to decrease the viability of cell lines in the used concentrations. Reducing the viability of cancer and natural cell lines indicates that RA has cellular toxicity on those lines. RA and its derivatives have often been used in inhibiting various cancerous tumours such as; lung cancer (Magalhães *et al*,2020), breast cancer (Al-Sheddi *et al*,2015), cervix cancer (Chinapayan and Prabhakaran,2019) and gastrointestinal cancer (Rafa *et al*., 2017).

Despite the regulatory role of RA for many genes in different cells and tissues and during various embryogenesis processes as indicated by several previous studies, however, the present study showed that it has cellular toxicity on the normal lines also. The reason for this may be due to the rate of used concentrations as reported by Ramos *et al*.(2015), where the high concentrations of RA led to malformations and toxic effects during embryogenesis and in cell cultures of human stem cells, which are normal cells (Chen and Khillan,2010 and Zhang *et al*.,2011) .

The toxic effects of retinoic acid may be due to its role in cells as a generator of oxidative stress (De Oliveira and Moreira, 2008). It is observed that it has the ability to cause a disturbance in the balance of oxidation and reduction status through increasing fat oxidation and increasing of oxidative proteins, which are related to the generation of ROS and oxidation of the Thiol group in proteins (De Oliveira *et al*., 2007). Also could be from the ability of retinoic acid to make changes in the activity of the antioxidant enzymes. It is found that it increases the activity of the SOD enzyme and reduces the level of the activity of the enzyme Catalase, therefore, increasing the level of hydrogen peroxide ($H_2O_{2)}$, which leads to increase the hydroxyl radical (HO) in the tissues (De Oliveira and Moreira, 2008). Moreover, at higher doses, RA consumes higher amounts of antioxidant proteins such as GSH by increasing the activity of (GSH) transferase

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enzyme (De Oliveira *et al*., 2012). Also, it has role causing mitochondrial dysfunction and electron leakage, and cause increasing of the O_2^- root and reducing ATP energy. Eventually, it decrease cell vitality and stimulate cell death pathways (Halliwell, 2006).

Also, several concentrations of RA and MTT assay have been used in this study to get the half-lethal concentration in cancer and normal cell lines, while IC_{50} values varied between cell lines. The results of the statistical analysis showed that the IC_{50} rate for all normal and cancer cell lines is almost the same, and the normal cells (HBL-100) are the more sensitive among the other cells.

The efficacy of RA depends on its Retinoic A Receptor (RAR) and Retinoic X Receptor (RXR) (Masi *et al*., 2015), these receptors making RA able to inhibit tumours (Zhou *et al*.,2016). The results of the present study displayed the ability of RA to induce apoptosis in cell lines, but the pathway stimulated by RA to activate apoptosis in the normal cell line HBL-100 differs from the one in the cancer cell line Cal51. The ability of RA to stimulate the external pathway of apoptotic cell death is attributable to the binding of RA receptors in the classic site (RARE) of the gene (Connolly *et al*., 2013), which is the primary promoter of the apoptotic Caspase Cascade,consequently cleavage and inhibition of procaspase-8 happens that ends to apoptosis (Jiang *et al*., 2008). In another unusual pathway, RA receptors inhibit the Wnt/b-catenin pathway, and then induce apoptosis or these receptors may bind to the CREB region on the gene to stimulate gene expression of the Caspase8 gene (Jiang *et al*., 2008).

The combined effect of CeO_2NP and RA on cell lines for 72 hours was investigated. The obtained results by using COMPUSIA program showed that there is no synergy between the two substances, despite the synergy between CeO_2NP or RA with other substances in different cell lines. Previouse studies have demonstrated the synergy of CeO_2NP nanoparticles with DOX and on different lines 4T1, SKOV3, A2780, MDA-MB-468LN (Cohen *et al*., 2011; Sack *et al*.,2014 ; Das *et al*.,2017). Furthermore, synergism of RA has been confirmed with DHA on cell line Mcf-7 Abdolahi *et al*.(2016), DOX on CSCs Sun *et al*.(a 2015), and paclitaxel on Mcf-7 cells (pratt *et al*.,2006).

By study the shape of the cells which are treating with CeO_2NP and RA for 48 hours. The results showed changes in the cell shape and the cell culture habits of these lines. These changes related to toxicity of both substances on the cell lines. Empty spaces related to separated cells after their death. Cells separation may occur as a result to cells manufacture of extracellular materials, especially extracellular matrix proteins, and partially due to defect in the secretion of non-collagenous substances that work to bind cells with their ground substance, such as multi adhesive glycoproteins, of which Fibronectin is the most important one (Hayrapetyan and Sarvazyan,2020).

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Among these changes is the change in cell shapes that may negatively affect the cell functional performance, where the shape is consistent with the functional performance (Fletcher and mullins, 2010). The shape changes to spherical might be due to changes in the connections of the cytoskeleton components, which give the specific shape to the cells. This occurs as a result of the stress that the cells are subjected to, as a form of adaptation to that stress such as pulling out protrusions or atrophies (Kroemer *et al*., 2005). Also, the plasma membrane contributes to a disturbance in the ionic balance of the cells, consequently this leads to their balled up and shrinkage (Kroemer *et al*., 2005).

The pathological changes observed on the treated cell of the culture cells, such as thickening of the chromatin material and the degradation of the cytoplasm, indicate irreversible phenotypic changes that end to cell death. The thickening and condensation of chromatin material are one of the early signs of apoptosis (Lonard and O'Malley, 2005). The CeO_2NP enters into the cells through normal pinocytosis of cells and accumulates in the cytoplasm near the nucleus. It may behave like other nanomaterials in terms of their effect on the nucleus causing cell death (Asati *et al*.,2010). Also, the reason might be related to connect the receptors in the cell after their simulation by the RA, which in turn stimulate histone unwrapping l (Lonard and O'Malley, 2005).

The results of stained cells with acridine orange ethidium bromide confirmed that both CeO_2NP and RA lead to changes in the permeability of the plasma membrane, where the healthy membrane is usually impermeable to the ethidium bromide tincture (Hayashi *et al*, 1990), and the staining of the treated cells with the red colour indicates that membrane is not intact. Consequently, it became permeable to that tincture.

Cells treated with various substances enter the pathway of cell death and are usually of the type of programmed cell death or necrosis (Fink and Cookson, 2005). Programmed cell death occurs in three different pathways. Firstly, the casepase-dependent pathway, which has two pattrens: the external path, and the internal path. Secondly, the caspase-independent pathway, when stimulating a number of proteins such as AIF. Third, apoptosis occurs when the endoplasmic reticulum is exposed to stress, and a number of proteins are stimulated, such as UPA (Hongmei,2012).

The gene expression was performed on the two cell lines (Cal51 and HBL-100). The results showed that both CeO_2NP and RA have the ability to induce apoptosis in normal and cancer cells alike, but with different pathways. CeO_2NP induces apoptosis by stimulating the caspase8 dependent external pathway. A significant increase in the gene expression level of the caspase8 gene was observed. Simultaneously there was no difference in the gene expression of both caspase9 and P53 genes. Whereas, previous studies have revealed that CeO_2NP stimulates the Caspase9 dependent internal pathway in different cell lines A5A9, HT29, and HCT116 (Mittal *et al*., 2014 ; Nasiri *et al*.,2017 ;Datta *et al*.,2020). This may lead to stimulation of gene expression level in the Caspase-independent pathway through an increase in gene expression of the P53

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gene (Mittal *et al*. 2014 as a result of DNA damage Datta *et al*.(2020), which was not observed in the this study. This could be due to the difference in the cellular pathway that CeO_2NP adopt to cause apoptosis, the difference of cell lines or because of the physical properties of CeO_2NP such as size and surface equivalence (Yokel *et al*., 2014).

The pathway stimulated by RA has differed in cell line Cal51 to induce apoptosis, as an increase in the gene expression of the caspase9 gene is observed, and this may be due to the difference between the nature of cancer and normal cells. Also, the molecules associated with RA works on the release of cytochrome C, and then activates the gene expression of caspase9, gene as happened in jurkat cells (Ortiz *et al*,2001), NB4 line (Zhao *et al*,2013), NPC line (Lee *et al*,2012), MCF-7 line (Donato and Noy, 2005), and HEPG2 line (Heo *et al*.,2015). RA can stimulate the internal and external pathways in a single cell line as it happened in the Mcf-7 cell line (Kim *et al*., 2009).

The results of the present study conflict with what Heo *et al*.(2015*)* reported about the ability of RA to stimulate gene expression of the P53 gene in the HePG2 cell line, where the gene expression of the P53 gene has not been stimulated by exposing the cells to both CeO_2NP and RA. According to Heo *et al*. (2015*)*, the P53 gene is stimulated when all the internal and external apoptotic pathways are turned on together, while the present study proved that each substance (CeO_2NP and RA) leads to stimulate only one pathway, the reason for stimulating P53 may be that the internal and external pathways of apoptosis are not stimulated at the same time.

Conclusion

According to the toxicity shown by cerium oxide nanoparticles to both normal and cancerous lines, it has no selective or directed action to cancerous cell. RA shows clear toxicity on normal and carcinogenic line at high concentrations. Though, CeO2NP is more toxic than RA in carcinogenic lines (MCF-7, Cal51).The absence of antagonism between RA and CeO2NP in all cancer and normal lines. It was concluded from the gene expression results that the action of the two substances alone is more active than their combined action on the cell line HBL-100, while the combined action simulates one of the substances in the cell lines carcinogenic. RA induces programmed cell death in cancer cell lines with a pathway that differs from inducing programmed cell death in normal lines ,while the programmed cell death induced by CeO2NP in both normal and cancer cell lines was the same.

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