Electronic Supplementary Material (ESI) for RSC Advances.

Fluorescent delivery vehicle containing cobalt oxide-umbelliferone nanoconjugate: DNA/protein interactions studies and anticancer activity on MF7 cancer cell line

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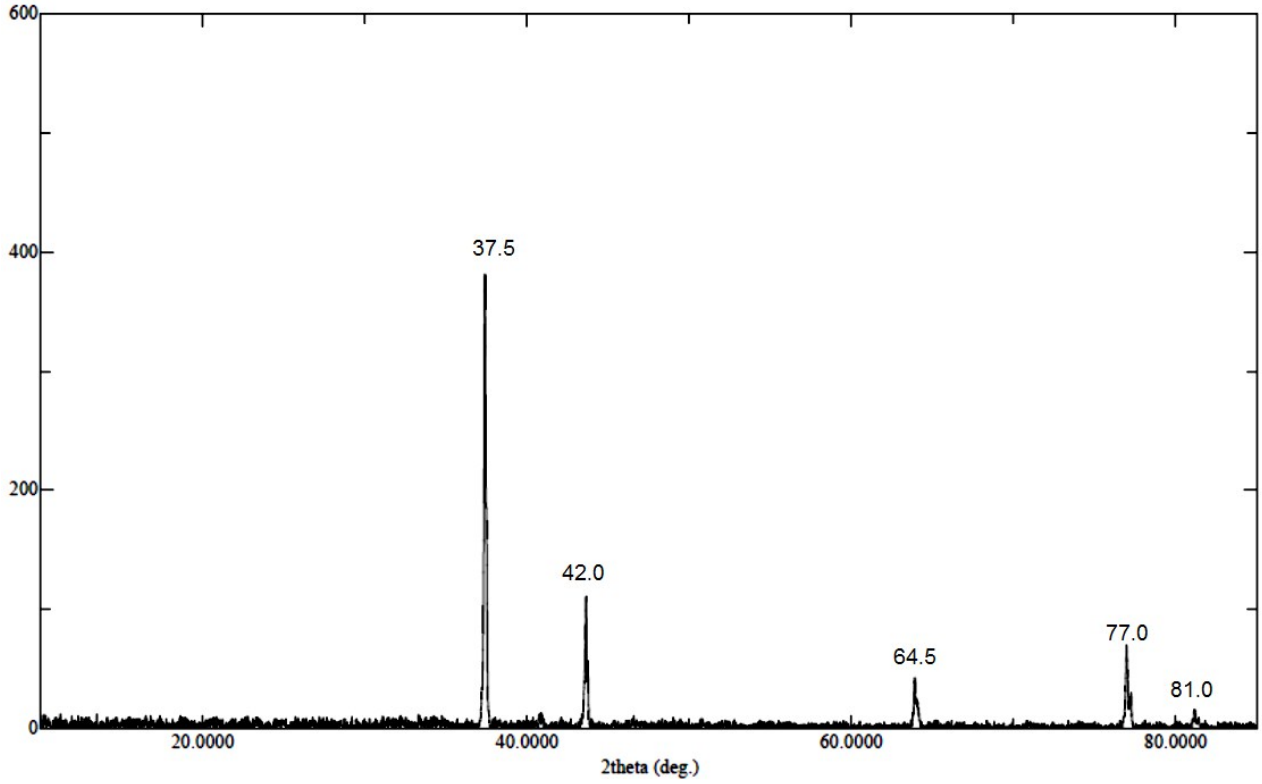

Fig. S1. XRD pattern of nanoconjugate.

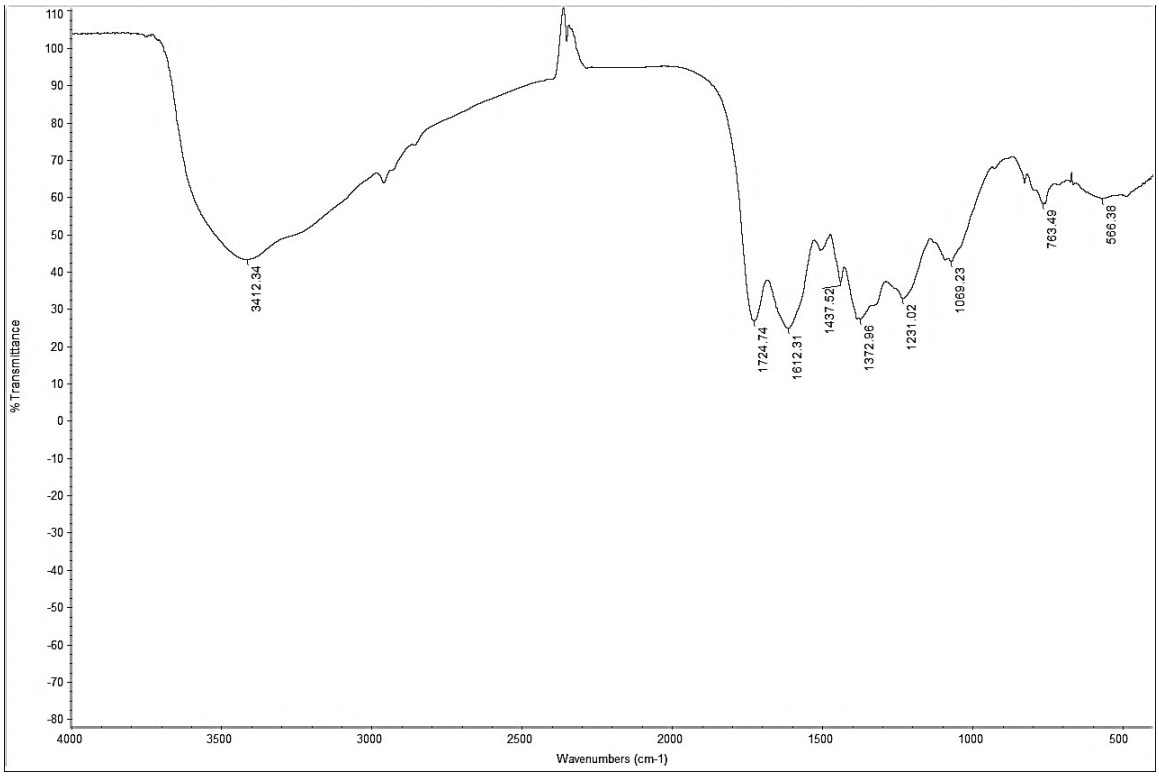

Fig. S2. FT IR of nano conjugate.

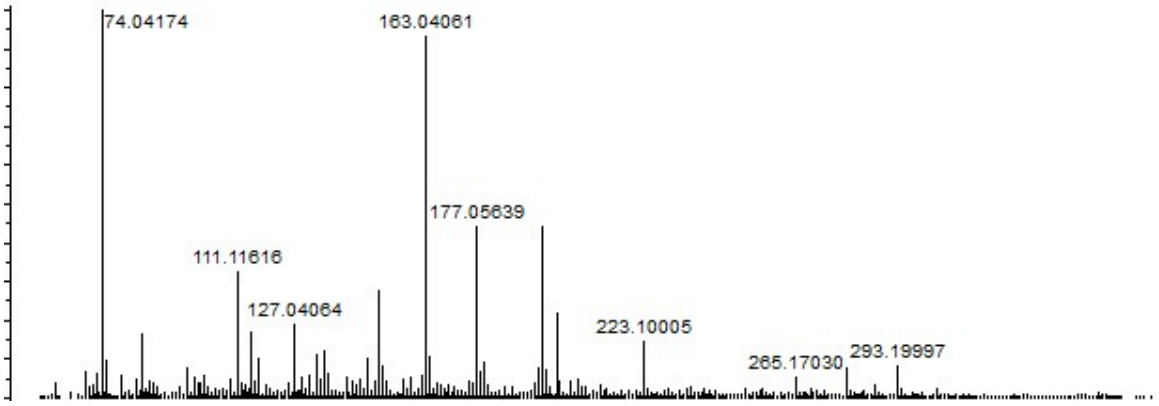

Fig. S3. Mass spectrogram**.**

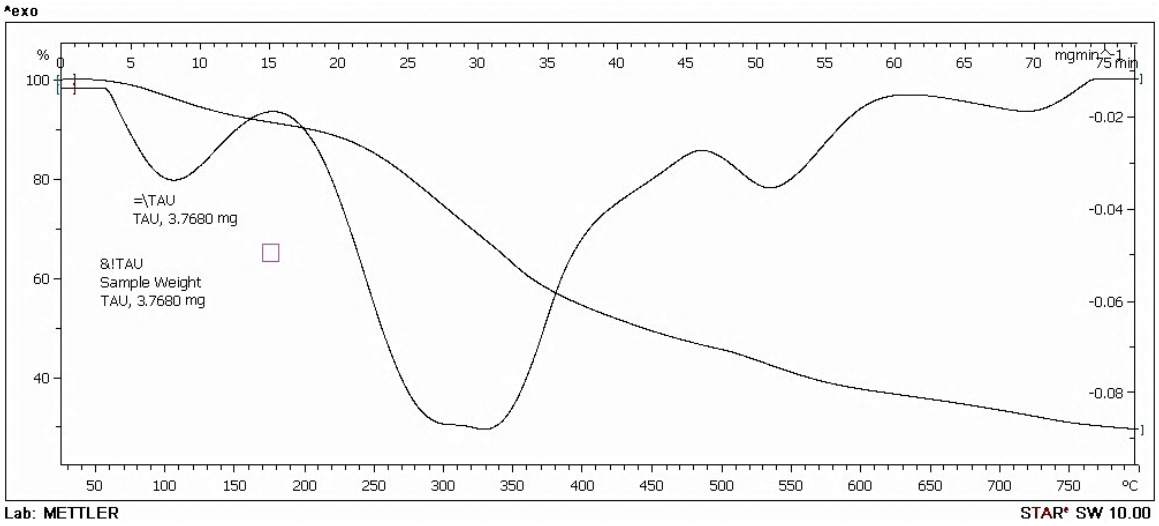

Fig S4. TGA curve showing dehydration (100 to 120 °C), conjugate degradation (150 °C) and complete decomposition of umbelliferone (500 °C).

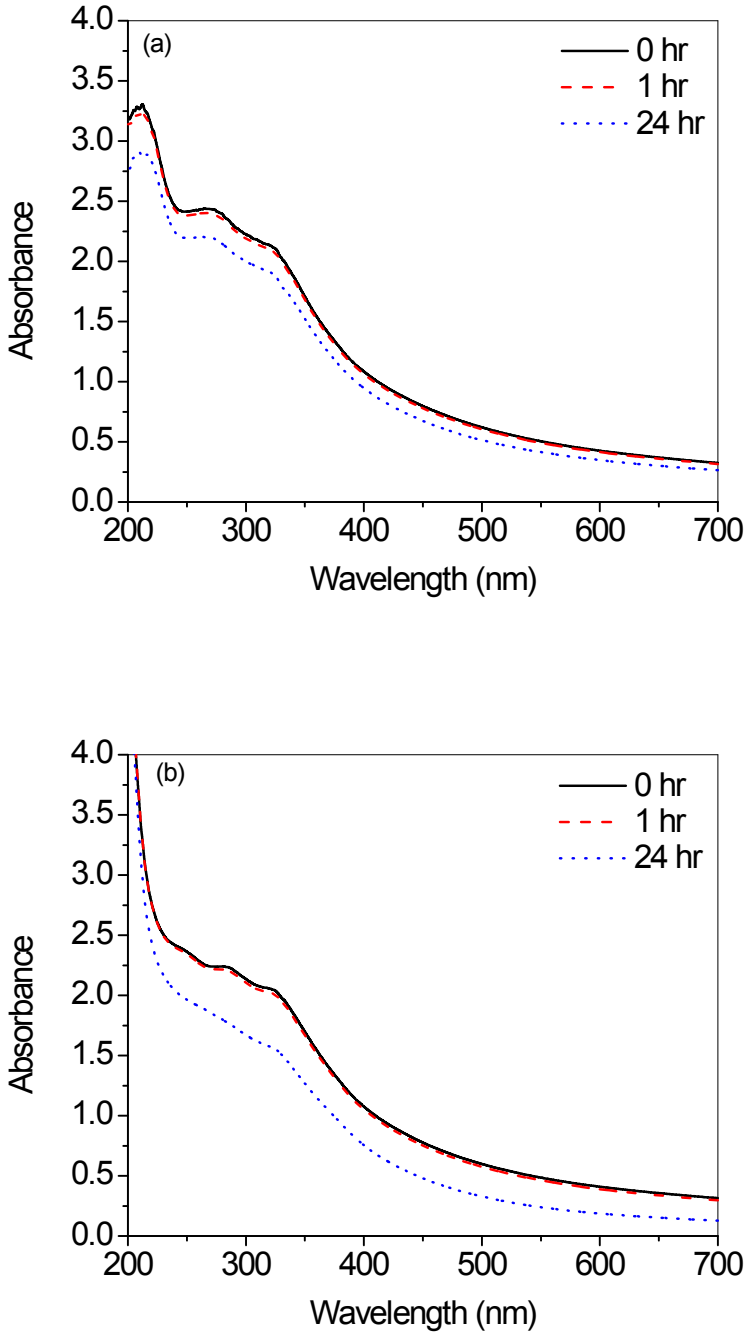

Fig. S5 . UV-visible spectra of nano-conjugate in water (a) and in medical grade saline (b) at physicological pH.

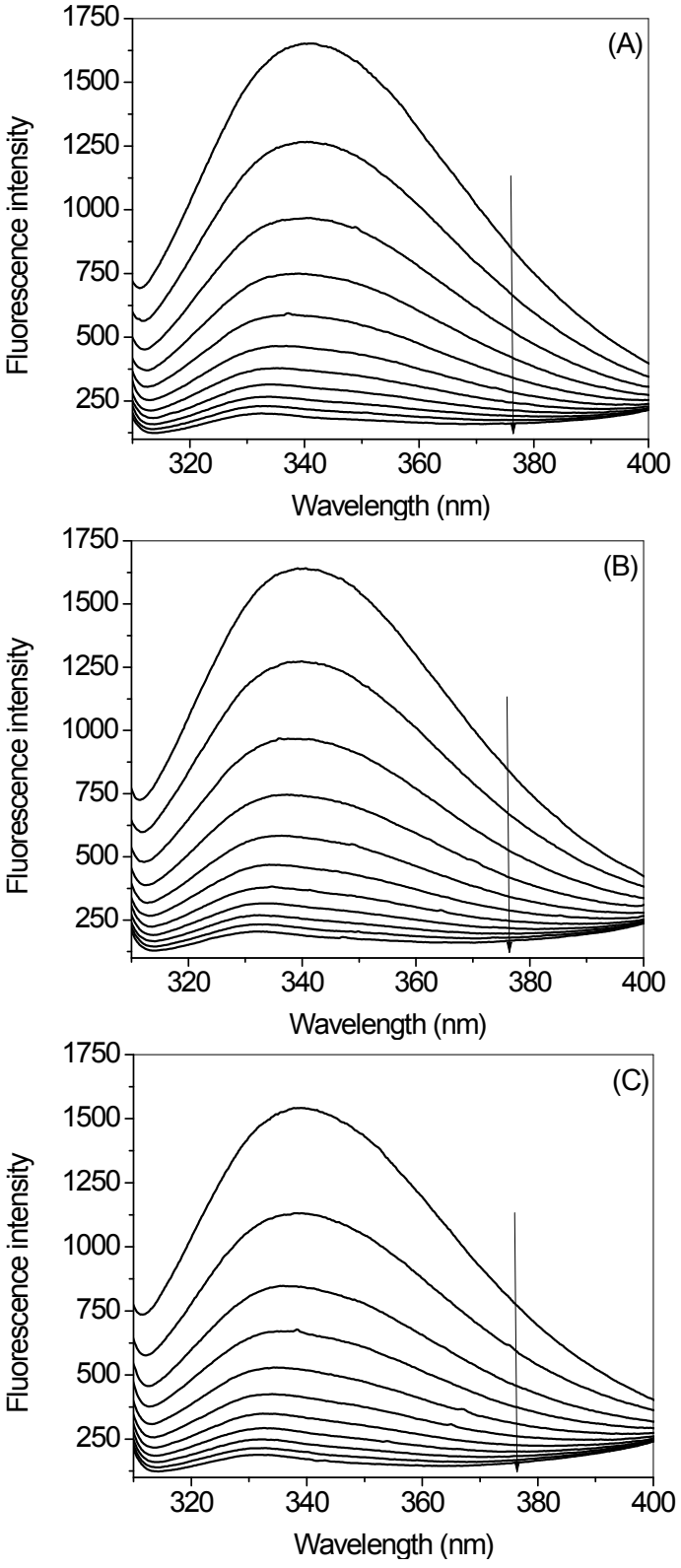

Fig. S6 . Fluorescence emission spectra of HSA in presence of complex **(**(0-50 μM) at 25 (A), 35 (B) and 45 °C (C).

Table S1 Non-covalent interactions of drug nano conjugate with the DNA.

Name	Distance (Å)	Category	Type
B:DA18:H7 - :UNK1:O	2.54759	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - A:DG2:OP2	3.26434	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - B:DA18:O5'	3.09537	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - B:DT20:O4	2.68346	Hydrogen Bond	Conventional Hydrogen Bond
B:DA18:C8 - :UNK1:O1	3.44795	Hydrogen Bond	Carbon Hydrogen Bond
B:DT19:C6 - :UNK1:O	3.78895	Hydrogen Bond	Carbon Hydrogen Bond
:UNK1:CO1 - A:DC3:OP2	3.2737	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:OP2	2.26097	Other	Metal-Acceptor
:UNK1:CO1 - A:DG4:OP2	3.21028	Other	Metal-Acceptor
:UNK1:CO1 - A:DC3:OP1	3.37747	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:OP2	2.97496	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:O5'	2.90564	Other	Metal-Acceptor
:UNK1:CO1 - B:DT20:O4	2.89256	Other	Metal-Acceptor
:UNK1:CO1 - A:DG4:O6	2.94738	Other	Metal-Acceptor
:UNK1:CO1 - B:DT20:O4	2.97297	Other	Metal-Acceptor
:UNK1:CO1 - B:DT19:O4	2.96698	Other	Metal-Acceptor
B:DA17:OP2 - :UNK1	2.93723	Electrostatic	Pi-Anion
B:DA17:OP2 - :UNK1	2.74523	Electrostatic	Pi-Anion
B:DT20:OP2 - :UNK1	4.78854	Electrostatic	Pi-Anion
B:DA17:O5' - :UNK1	2.85204	Other	Pi-Lone Pair
B:DA18:H7 - :UNK1:O	2.54759	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - A:DG2:OP2	3.26434	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - B:DA18:O5'	3.09537	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - A:DC3:OP2	2.03019	Hydrogen Bond	Conventional Hydrogen Bond
:UNK1:O1 - B:DT20:O4	2.68346	Hydrogen Bond	Conventional Hydrogen Bond
B:DA18:C8 - :UNK1:O1	3.44795	Hydrogen Bond	Carbon Hydrogen Bond
B:DT19:C6 - :UNK1:O	3.78895	Hydrogen Bond	Carbon Hydrogen Bond
:UNK1:CO1 - A:DC3:OP2	3.2737	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:OP2	2.26097	Other	Metal-Acceptor
:UNK1:CO1 - A:DG4:OP2	3.21028	Other	Metal-Acceptor
:UNK1:CO1 - A:DC3:OP1	3.37747	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:OP2	2.97496	Other	Metal-Acceptor
:UNK1:CO1 - A:DG2:O5'	2.90564	Other	Metal-Acceptor
:UNK1:CO1 - B:DT20:O4	2.89256	Other	Metal-Acceptor
:UNK1:CO1 - A:DG4:O6	2.94738	Other	Metal-Acceptor
:UNK1:CO1 - B:DT20:O4	2.97297	Other	Metal-Acceptor
:UNK1:CO1 - B:DT19:O4	2.96698	Other	Metal-Acceptor
B:DA17:OP2 - :UNK1	2.93723	Electrostatic	Pi-Anion
B:DA17:OP2 - :UNK1	2.74523	Electrostatic	Pi-Anion
B:DT20:OP2 - :UNK1	4.78854	Electrostatic	Pi-Anion
B:DA17:O5' - :UNK1	2.85204	Other	Pi-Lone Pair

Note: UNK1 = NANO-DRUG-CONJUGATE

Cell culture and treatments

The MCF-7 human breast adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM with 10% FBS and 1% penicillin/ streptomycin in a completely humidified atmosphere with 95% air and 5% CO_2 at 37 ^oC. The exponentially growing cells were sub-cultured into 6-well or 96-well plates or Nunc Lab-Tek II chamber slides according to the experimental requirements. The viability of the cells was determined by trypan blue test. The cells were counted using a cell counter (Bio Rad TC20 automated cell counter) and diluted in medium at a density of 1×10^5 cells/ mL to be used throughout the experiments. A stock solution of Test compounds TiO_2, Complex and mix (TTU) was prepared in Millipore water (w/v) and was then diluted in cell culture medium to obtain the desired concentrations for cell treatment.

Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay with modification was used to analyze the cytotoxic activity of test compounds. A CellTitre 96® non-radioactive cell proliferations assay kit (Promega, Madison, WI, USA) was used following the manufacturer's instructions. Briefly, the MCF-7 cells (1×10^4 cells/ well) were grown overnight in 96-well flat bottom cell culture plates, and were then exposed to six different concentrations of TiO_2, Complex and mix (TTU) (250 µg/ mL, 200 µg/ mL, 150 µg/ mL, 100 µg/ mL, 50 µg/ mL and 25 µg/ mL) for 24 hrs. A negative control (untreated) was also maintained for comparison. After the completion of the desired treatment, 15 µl of MTT reagent, provided in the kit, was added to each well and further incubated for 3 hrs at 37 °C. Finally, the medium with MTT solution was removed, and 200 µl of solubilization solution was added to each well and further incubated for 30 min by occasional vortexing. The optical density (OD) of each well was measured at 550 nm by using a Synergy microplate reader (BioTek, Winooski, VA, USA). Results were generated from three independent experiments and each experiment was performed in triplicate. The percentage of cytotoxicity compared to the untreated

cells was estimated in order to determine the IC_{50} value (the concentration at which 50% cell proliferation is inhibited).

Morphological changes analysis

MCF-7 cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and allowed to grow overnight. Morphological changes were observed to determine the alterations induced by half of IC_{50} concentrations of TiO_2, nano conjugate and mix TiO_2 respectively. After the end of the incubation period (24 h), cells were washed with PBS (pH- 7.4) and observed under a phase contrast inverted microscope equipped with a CCD camera (Olympus IX51, Tokyo, Japan) at 100× magnification.

Intracellular reactive oxygen species (ROS) measurement

The generation of intracellular ROS was monitored using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2 DCFDA). For the quantitative estimation of intracellular ROS by spectrofluorometry, 1×10^4 cells per well were seeded in 96-well culture plates (black-bottomed) and allowed to adhere for overnight in a CO_2 incubator at 37°C. Then, cells were treated with half of IC_{50} concentrations of TiO_2 (75µg/mL), complex (65µg/mL) and Mix (52µg/mL) followed by incubation for 24 hours at 37 °C. At the end of the respective treatment period, cells were washed twice with PBS and then incubated with 25 µM working solution of carboxy-H_2 DCFDA in a serum free medium at 37 °C for 30 min. The reaction mixture was discarded and replaced by 100 µL of PBS in each well. The green fluorescence intensity was detected using a Synergy microplate reader (BioTek, Winooski, VA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The values were averaged from multiple wells and expressed as a percentage of fluorescence intensity relative to the control wells. Similarly, the qualitative analysis of intracellular ROS by fluorescence microscopic imaging was achieved by seeding the cells on Nunc Lab Tek II chamber slides at 1×10^5 cells/ chamber. Then the cells were treated and processed as indicated above. Finally, after washing with PBS the stained cells in chambers were covered with cover slips and images were collected using appropriate filter settings in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and a CCD camera.

Detection of autophagy by acridine orange staining

As a marker of autophagy induction, acidic vesicular organelles (AVOs), which consist predominantly of autophagosomes and autolysosomes, were observed by fluorescence microscopy after staining of cells with acridine orange (AO). AO is a fluorescent weak base that accumulates in acidic vesicular spaces and fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of AVO formation. MCF-7 cells were grown in Nunc Lab Tek II chamber slides and treated with half of IC_{50} concentrations of TiO_2 (75µg/mL), complex (65µg/mL) and Mix (52µg/mL) and incubated for 24 hours at 37 ^oC. Then, the cells were washed twice with PBS and then incubated with 5 µg/ mL working solution of AO in a serum free medium at room temperature for 15 min in the dark. The reaction mixture was discarded and replaced by 100 µL of PBS in each chamber. Finally, the AO stained cells were observed in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and a CCD camera.

DNA Fragmentation assay:

Apoptotic DNA fragmentation was analyzed by extracting genomic DNA from MCF-7 cells by using Apoptosis DNA ladder kit (Roche Diagnostics, Mannheim, Germany). Briefly, $1x10^5$ cells/ mL were grown for overnight and exposed to half of IC_{50} concentrations of TiO_2 (75µg/mL), complex (65µg/mL) and Mix (52µg/mL) in 6-well plates for 24 h. The concentration and purity of the DNA thus extracted was determined by reading the absorbance at 260 nm, and protein contamination estimates were based on the ratio of absorbance at 260/280 nm. For the analysis of DNA laddering, 1.5% agarose gel was prepared, and the DNA was separated by electrophoresis. An equal amount of DNA from the treated and control group was mixed with 1X tracking dye (bromophenol blue) and loaded into wells of agarose gel. A standard 100 base pair DNA marker was also loaded in order to compare the DNA laddering. After the electrophoresis, the DNA was stained with 20 µg/ mL of ethidium bromide and the gel was visualized under UV light and photographed using a Gel Doc XR System (Bio-RAD Lab. Milan, Italy).