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Inorganic complex intermediate Co_3O_4 nanostructures using green ligation from natural waste resources

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MATERIALS AND METHODS

Co_3O_4 nanostructures using rambutan extract

Rambutan fruit exported from Thailand was collected from Daejeon supermarket, South Korea. Cobalt nitrate [$Co(NO_3)_2.6H_2O$] and ethanol were purchased from Merck chemicals. Double distilled water was used throughout the experiment. Manually separated rambutan peels were washed with running water and subsequently incised into small pieces and placed in circulating oven at 50°C until complete dryness. Finely dried rambutan peels (3g) were boiled with ethanol and double distilled water mixture (1:2 ratio) for 10 min. The extract was filtered through Whatman No. 1 filter paper. 0.1 M $Co(NO_3)_2.6H_2O$ was prepared in 50 mL and 10 mL rambutan extract was slowly added drop wise into the solution under magnetic stirring at 80°C for 2 h and then incubated at room temperature for 1, 4 and 7 days to form cobalt-ellagate complex formation. Complex formed after adequate time of incubation was collected and Co_3O_4 nanostructures were obtained due to direct decomposition of cobalt-ellagate complexes in muffle furnace at 450°C in static air atmosphere.

MTT assay for cell proliferation

Dulbecco's Modified Eagles Medium (DMEM) (pH 7.4)

DMEM medium was added to 900 ml of sterile double distilled water. To this solution, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate and 10 ml of antibiotic-antimycotic (50 U/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphoterecin B) solution were added; the pH was adjusted to 7.4 using 1 N NaOH and the final volume was made up to one liter with distilled water. The medium was then filtered through 0.22 μm filter with the membrane dispensed into sterile container and stored at $4^{o}C$.

Growth medium (10% FBS)

100 ml of growth medium was prepared by adding 10 ml FBS in 90 ml RPMI-1640 and was stored in a sterile container.

Phosphate buffered saline (PBS) (pH 7.4)

0.63 g of sodium phosphate monobasic (NaH_2PO_4), 0.17 g of sodium phosphate dibasic (Na_2HPO_4) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of sterile double distilled water. The pH was adjusted to 7.4 with 0.1 N NaOH and stored in a refrigerator.

Culture medium

Human Breast cancer cell line (3T3) were cultured in 75-cm^2 flask containing Dulbecco's modified Eagle's medium (DMEM; Sigma). The mediums were supplemented with 10% Fetal Bovine Serum (FBS; invitrogen), 1.5 g/L sodium bicarbonate (Gibco), 10,000 U/ml penicillin (Gibco), 10 mg/ml streptomycin (Gibco) and 25μg/ml Ampotericin B (Gibco). Cells were cultured as monolayers in culture flasks at $37^{o}C$ under a humidified atmosphere of 5% CO_2 in air. All experiments were performed using cells from passage 20 or less. During the experiment time, the serum containing medium was replaced by serum free medium containing 20–2.5μg/ml of

the crude, which were dissolved in DMSO and the stock maintained in -20°C. The final working concentration of DMSO was less than 1.0 %.

Passaging the cells

The cells reaching 80-90% of confluence, the cells were trypsinized and used for subculture. The medium from the culture flask was aspirated; cells were rinsed with 2 ml of PBS and aspirated quickly and 0.5 ml of trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt) solution was added and incubated at room temperature (in the laminar hood) for 30 - 60 sec. Then the trypsin-EDTA solution was aspirated quickly and the flask was incubated in CO_2 incubator for 2 min and tapped gently at the bottom for complete detachment of cells from the surface of the flask. The cells were then gently re-suspended in fresh growth medium and transferred to sterile 75 cm^2 flasks and the volume of medium was made up to 20 ml with growth medium.

Drug preparation

The extract was dissolved in Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 5% and did not affect cell survival.

Cell viability test

The viability of cells was assessed by MTT assay (Mosmann, 1983) using Human Breast cancer cell line (3T3). Reagents used are MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5 mg MTT/ml of serum-free DMEM medium, solubilizing solution: dimethyl sulfoxide and phosphate buffered saline (PBS) (pH 7.4). The cells were plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of test compound (10-500 µg/ml) for 24 h. At the end

the treatment period, the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37ºC in a CO_2 incubator. The 50% inhibitory concentration value (IC_{50}) of the crude extracts was identified in different cancer cell lines and a normal cell line. The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipettting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software.

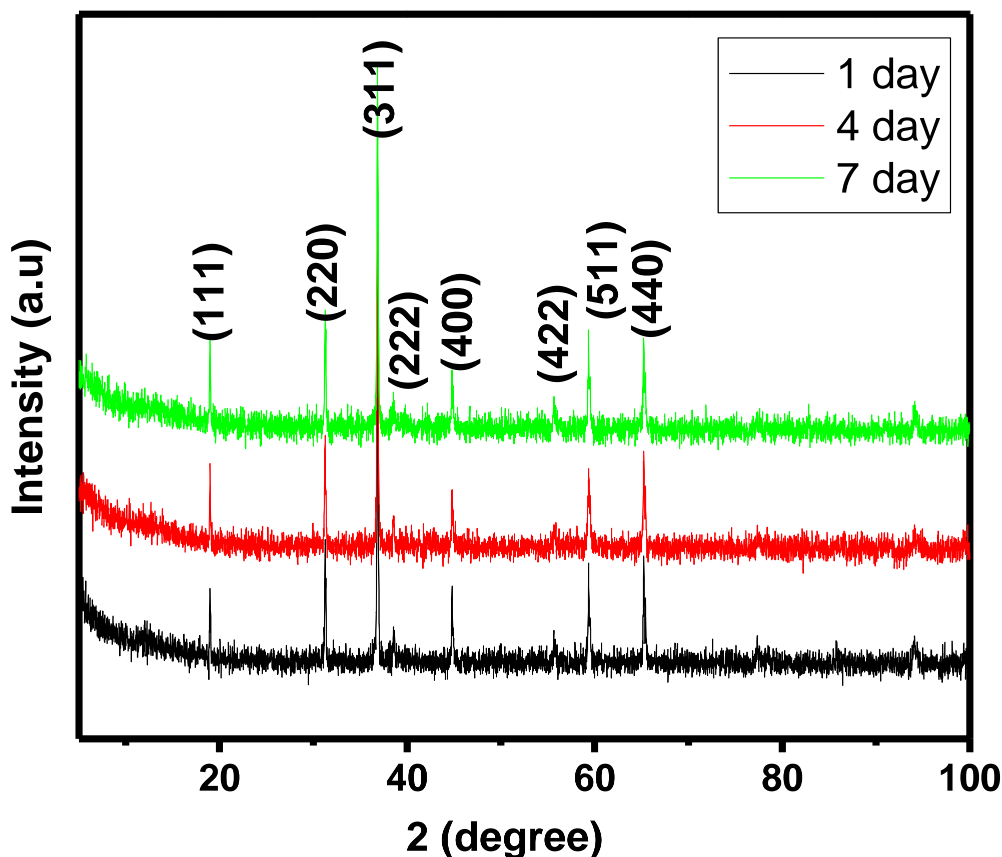

Figure S1. XRD pattern: (a) 1, (b) 4, (c) 7 days of reaction product from the mixture of 0.1M $Co(NO_3)_2.6H_2O$ and 10 ml extract

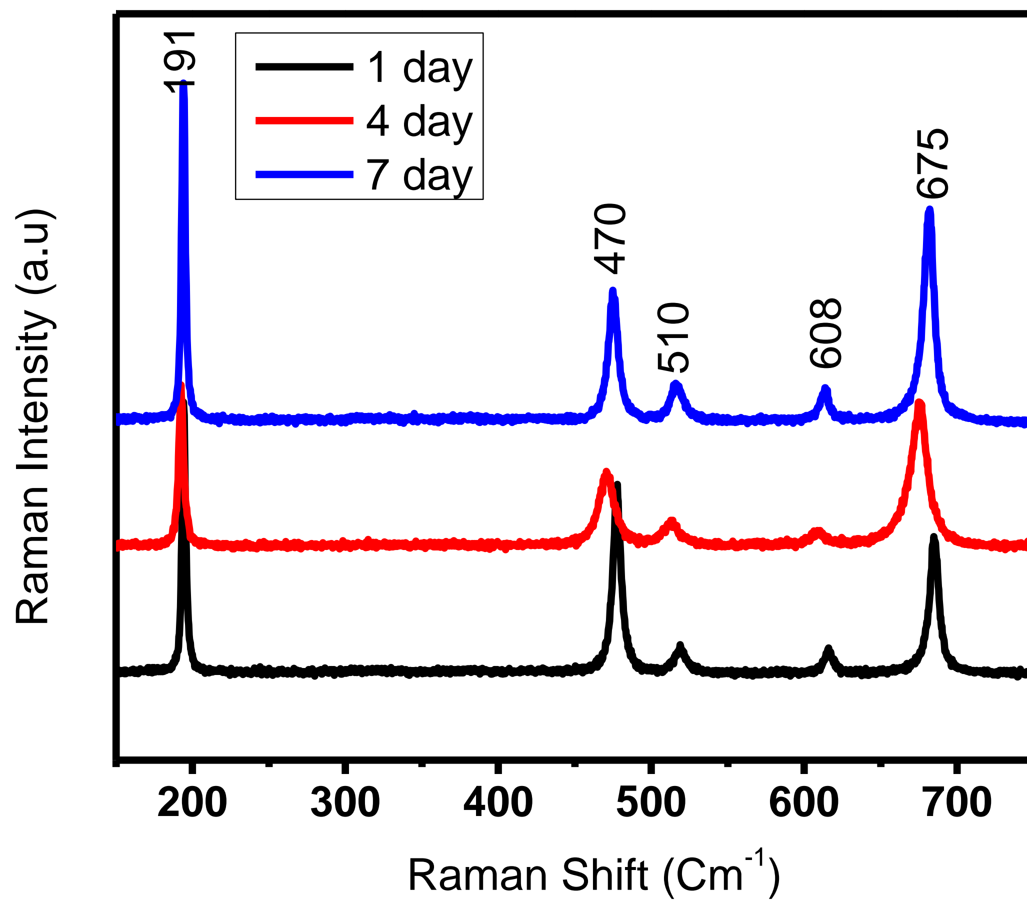

Figure S2. Raman pattern: Reaction product from the mixture of 0.1M $Co(NO_3)_2.6H_2O$ and 10 ml extract (a) 1, (b) 4, (c) 7 days

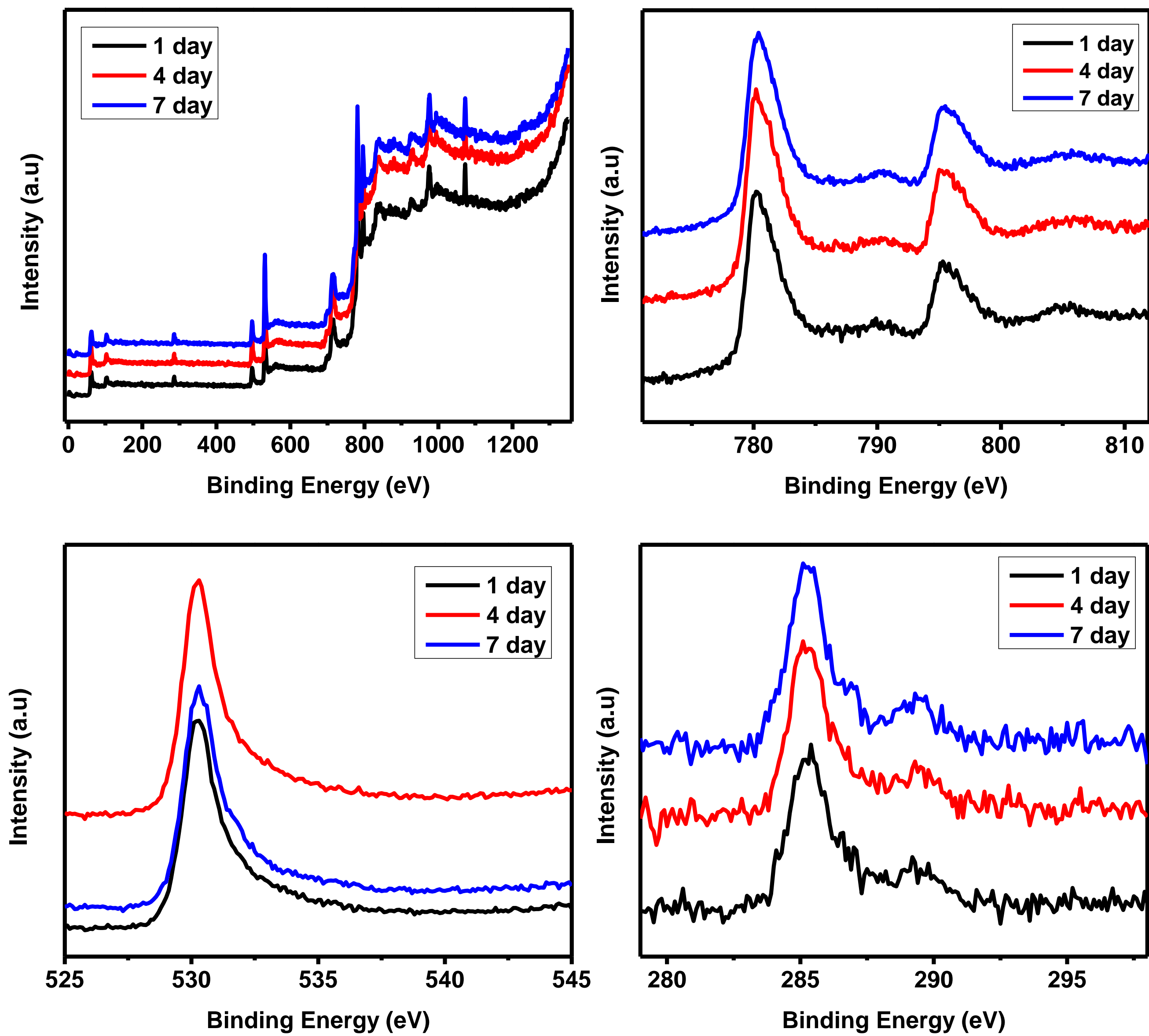

Figure S3. XPS pattern (Survey spectrum, Co $2p_{3/2}$ and $_{1/2}$, O_{1s} and C_{1s} scan): (a) 1, (b) 4, (c) 7 days of reaction product from the mixture of 0.1M $Co(NO_3)_2.6H_2O$ and 10 ml extract