Chapter-2
Material and methods
Chapter 2

Materials and methods

Materials

(all-E)-Lycopene (>90%), propidium iodide, toluene, potassium permanganate, poly-D-lysine, ethidium bromide, acridine orange, dichloro-dihydro-fluorescein diacetate (DCHF-DA) dye, 4,6-diamidino-2-phenylindole dilactate (DAPI) and butylated hydroxytoluene (BHT), tetrahydrofuran (stabilized with BHT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Glutathione, glutathione reductase, 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), NADPH, EDTA, silica G (particle size 70-230 mesh) were purchased from Sisco Research Laboratories (Mumbai, India). Acetone, hexane, methanol, acetonitrile and tetrahydrofuran of HPLC-grade solvents, PVDF syringe filters (0.45 µm) were procured from Merck (Mumbai, India). Dulbecco's minimal essential medium (DMEM), Minimum essential medium (MEM), Ham's F-12 media, fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), antibiotic antimycotic solution, calcium magnesium free phosphate buffer saline (PBS), all other cell culture consumables were purchased from Hi-Media Chemical Laboratories (Mumbai, India). FITC Annexin-V apoptosis detection kit was purchased from BD pharменging (BD Bioscience, San Diego, CA), JC-1 dye (5,59,6,69 tetrachloro 1,193,39 tetracythylbenzimidazol-carbocyanine iodide; Calbiochem, USA). All other chemicals and solvents of analytical grades were purchased from Sisco Research Laboratories (Mumbai, India).

Extraction of Lycopene

Ripened stage tomatoes (Indian hybrid) were chosen randomly from a local supermarket, a known quantity of fruits was taken and washed with deionised water and only epicarp portions were taken for the preparation of tomato puree using a grinding mixer. Extraction of lycopene (LYC) was carried out by using following solvents system: Solvent 1, methanol: acetone: hexane in the ratio of 25:25:50, v/v/v was used as per the existing procedures (Lin et al. 2003). Further, solvent 1 was modified into solvent 2, 25:50:25 and solvent 3, 50:25:25 v/v/v ratios, respectively and compared the yield and purity of LYC. Based on the analysis of LYC content in these extracts, solvent 2 was considered as a better solvent system for isolation. In
brief, a portion of the tomato puree (n=5) (2.5 g) was mixed with solvent 2 (25 mL) in the ratio of 1:10 with 0.1% BHT (w/v) in ethanol was added to minimize isomerization/oxidation. The sample extract was vortexed vigorously and kept in the dark at 4 °C for 20 minutes. Then top hexane layer was separated by adding equal volume of deionized water in a separating funnel. These procedures were repeated for three times, otherwise until the hexane layer becomes colourless. The pooled hexane layers were collected and evaporated to dryness under N₂ gas and residue was redissolved in a known volume of THF (3 mL) for further aliquots. The extracted LYC was quantified and its % purity was checked by using UPLC with PDA detector. The peak identity, absorption maxima (λmax) and characteristics UV-Visible spectra of LYC isomers were confirmed by UPLC-PDA detector. Extraction and preparation of samples and standards were carried out under dim yellow light to prevent isomerization and degradation.

**UPLC analysis of Lycopene**

Chromatographic separation of LYC was performed by using an ACQUITY UPLC® system (Waters Corp., Milford, MA) consisting of binary solvent manager and sample manager, coupled with PDA detector. LYC and its isomers were separated by using ACQUITY UPLC® CSH Phenyl-hexyl column (100 X 2.1 mm; 1.7 μm) and compared with ACQUITY UPLC® BEH C18 (100 X 2.1 mm; 1.7 μm) and ACQUITY UPLC® BEH shield RP18 columns (100 X 2.1 mm; 1.7 μm) (Waters Inc., US). Mobile phase contained 10 mM ammonium acetate in Milli-Q water (A) and 5% of THF in acetonitrile (B) with flow rate of 0.4 mL/min and was monitored at 471 nm by using PDA detector. The gradient condition was maintained as follows: 25% of A and 75% of B solvents was fixed initially, then increased solvent B to 85% in 3 minutes, 90% in 8 minutes and returned to 75% in 8.2 minutes. Needle was washed with weak and strong solvents to avoid the cross contamination and analytical error during analysis. The injection volume was 2 μL and column temperature was set at 45 °C in column heater and sample manager was maintained at 4 °C. LYC was quantified from its peak area by plotting a calibration curve with analytical reference standard. The peak identity of the components was further confirmed by its characteristic UV-Visible spectra recorded with PDA detector. Data acquisition and processing were carried out by using Empower software (Waters, USA)
Method validation

A stock solution of standard LYC and β-carotene (1 mg/mL) was prepared in THF separately. Working standard solutions were aliquoted by diluting the stock solution with 50% THF and 50% acetonitrile to attain the concentrations ranging from 0.125 to 64 μg/mL. These stock and working solutions were analysed immediately or stored at -80 °C. The UPLC method was validated by using various parameters such as LOD (signal-to-noise ratio 3:1), LOQ (signal-to-noise ratio 10:1), reproducibility was checked with six replicates where the percent relative standard deviation is less than 5% and the linear dynamic range of LYC is greater than 0.998.

UPLC-MS conditions

Qualitative analysis of carotenoids was done by using Waters Xevo TQD mass spectrometer interfaced with the ACQUITY UPLC® system via an APCI source operated in positive ion mode. An aliquot (2 μL) of LYC sample containing ~40 μg/mL was injected onto the column. The gradient conditions used are the same as mentioned in the section UPLC analysis. Instrument was conditioned by following parameters: corona voltage 0.9 kV, cone 35 V, RF 2.50 V, extractor 3.00 V, source temperature 150 °C, probe temperature 450 °C, cone gas flow 50 L/hr, desolvation gas flow 900 L/hr. Mass spectra of cis/trans LYC and β-carotene were acquired with an m/z 50-1000 scan range. The MS identity of standard LYC and β-carotene was compared with the extracted sample. Data were processed with Mass Lynx 4.1 software (Waters, USA).

Tandem mass spectrometry (MS/MS) analysis

Argon was used as collision gas and collision-induced dissociation energies were acquired from 10-40 eV. The instrumental operating parameters for the Tandem Quadrupole were the same as mentioned in the MS conditions. Positive ion collision induced dissociation of ions were recorded and compared for the molecular ions of cis/trans LYC and β-carotene. Different collision-induced dissociation energies were used 10, 15, 18, 20, 30 & 40 eV to check the fragmentation pattern. MS/MS analysis was repeated three times to check the reproducibility.

Lycopene oxidation by KMnO₄

LYC was subjected to oxidation by using KMnO₄ as per the procedure of Caris-Veyrat et al. (2003) with slight modification. In brief, LYC (30 mg) and cetyltrimethylammonium bromide (6 mg) were dissolved in 30 mL DCM/toluene (1:1
ratio) and made 6 aliquots (5 mg/5mL) separately for degradation study. The oxidation of LYC was initiated by adding 1.5 mL of aqueous solution of KMnO₄ stock (135 mg in 9 mL water) to the LYC sample. The reaction mixture was stirred on a magnetic stirrer and incubated for different time points (5, 10, 15, 20, 30 & 40 minutes) at room temperature (26 ± 2 °C). Then, separation of the organic phase was done by repeated washing with double distilled water (10 mL). The organic solvent phase of LYC oxidized sample was filtered through a PVDF filter (0.45 μm) and dried on sodium sulphate under nitrogen environment. The residue of LYC oxidised mixture was re-dissolved in petroleum ether and filtered again with PVDF filter to remove the KMnO₄ remnant. Oxidative products obtained were analysed by MS - APCI +ve mode. The degradation time point ODT₁₀₀ and degradation half ODT₅₀ of LYC incubated with KMnO₄ was calculated, and the optimal ODT₅₀ was preferred for further cell culture treatments. The aliquots of LYC and its oxidative products were sealed under nitrogen environment and used for cell culture treatments or stored in - 80 °C in amber vials.

Optimization of TLC for separation of lycopene oxidation products (KMnO₄ induced)

TLC Silica gel 60 F254 aluminium sheet 20 X 20 cm was used and the mobile phase consists of petroleum ether: acetone (7:3 ratio v/v) for the separation of LYC oxidative products. The separated bands on TLC were scraped separately and used for further characterization and evaluation of bioactivity.

Column chromatography

LYC oxidized products were separated by open column chromatography (OCC, 20 cm X 1.5 cm) on silica G (particle size 70 230 mesh) by use of specific gradient solvent systems. An aliquot of the LYC oxidized products was evaporated to dryness under nitrogen, re-dissolved in a known volume of Hexane/Acetone (50/50 v/v), and applied onto the silica G. The oxidized products was eluted using hexane/acetone in the following ratios (90:10, 80:20, 70:30 and 60:40 v/v) each fractions were eluted and collected separately. The purity of an individual elute was analyzed by HPLC. The peak identity, their respective spectra, and absorption maxima (λ_max) of each fractions were confirmed by HPLC.
MS conditions and analysis of lycopene oxidation products

MS analysis of LYC and chemical oxidised lycopene (COL) products was performed on the Thermo Scientific, LC-MS system coupled to an Ion Trap mass spectrometer (LCQ Deca XP Max). The capillary and vaporizer temperatures were set at 298 °C and 300 °C. The corona discharge current was (5 μA), Entrance lens (34.6 V) and nitrogen was used as a sheath and drying gas at 18.85 and 58.89 L/min and ion gauge was 0.76 X 10^-5 Torr. The spectrometer was calibrated in the positive ion mode. Mass spectra of LYC and its oxidised products were acquired with an m/z 0-700 scan range. Due to unavailability of reference standards for LYC oxidative products and constraint in the sample size, the possible structure of COL products were predicted and characterized using Chemsketch 8.0 software (ACD Labs, USA).

HPLC analysis of lycopene oxidation products

Chromatographic separation of LYC cleavage products was performed by using an AB SCIEX API 2000 system (Applied Biosystems, USA). LYC cleavage products was separated by using C30 column (5 μM; 250 X 4.6 mm; Princeton, Cranbury, USA). Mobile phase consisted of acetonitrile: methanol: isopropyl alcohol (40:40:20, v/v/v), under the isocratic condition with flow rate of 0.8 mL/min and was monitored at 420 and 450 nm by using UV detector. The injection volume was 20 μL and column temperature was set at 40 °C in column heater. Data acquisition and processing were carried out by using analyst 1.5 version software (Applied Biosystems, USA)

LC-MS conditions for analysis of lycopene oxidation products

Analysis of LYC and oxidation products were done by using AB SCIEX API 2000 mass spectrometer system (Applied Biosystems, USA) via an ESI source operated in positive ion mode. An aliquot (20 μL) of LYC and their oxidation products sample containing ~20 μg/mL was injected onto the column. The conditions used are the same as mentioned in the section HPLC analysis. Instrument was conditioned by following parameters: ion source voltage (3,500 V), declustering potential (20 V), focusing potential (400 V), entrance potential (10 V), source temperature (420 °C), GS-I (50 psi), GS-II (60 psi), curtain gas (nitrogen) (30 psi). Mass spectra of lycopene and their oxidation products were calibrated in the positive ion mode (M + H) 

signals were recorded. Mass spectra of LYC and its oxidized
products were with an m/z 50-1000 scan range were acquired at 450 nm. Data were processed with by using Analyst 1.5 version software (Applied Biosystems, USA). Further, collision-induced dissociation of ions were recorded and compared for the molecular ions of LYC and oxidation products. Collision-induced dissociation energies with 6 V were used to check the fragmentation pattern.

**Cell lines and culture conditions**

Cancer cell lines (PC-3, MCF-7, A431, HepG2, HeLa and A549) were purchased from National Centre for Cell Science, Pune, India. Cells were grown in DMEM, MEM or Ham's F-12 media, containing 10% FBS (GIBCO BRL, USA), 4 mM L-glutamine, and antibiotics (40 μg/mL penicillin and 40 U/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO₂ in the air.

**Cell viability assay**

Exponentially growing cells (70-80%) were seeded (5 X 10³ cells/well) in the 96-well plate containing 200 μL of culture medium. After 24 h of incubation, media was removed and replenished with 200 μL of the media containing purified LYC (1-50 μM) dissolved in THF (0.5%) and incubated for 48 h (Kotake-Nara et al. 2001). Based on the effect of LYC on cell viability, three cell lines (PC-3, MCF-7 and HeLa) were chosen for further experimentation. Cells treated separately either with LYC (50 μM), or its equivalent relative concentration of AOL or COL or without carotenoid (control) were incubated for 24 h. The reason for selection of higher concentration of LYC for cell treatment was, LYC may lead to generation of cleavage products at a lower concentration with lesser stability. These compounds are reported to decompose quickly than the major fragments (Lakshminarayna et al. 2013). The final concentration of THF in the culture medium was 0.5%, and control culture received a same concentration of THF alone. Then, The different fractions (1-4) of lycopene oxidized products obtained by OCC purification were dissolved in cell culture grade tetrahydrofuran (THF) and added to the cells in the medium at different concentrations. Experiments were repeated three times independently.

**Trypan blue exclusion assay**

Trypan blue assay was carried out to assess the effect of LYC and COL on the viability of MCF-7 cells. Briefly, the cells were seeded (0.75 X 10⁵/mL) in six-well plates and different concentrations of LYC and COL (25, 50 or 100 μM) was added to
the cells. After 24 h, cells were mixed with trypan blue (Sigma), counted under a microscope and plotted.

**LDH leakage assay**

Lactate dehydrogenase (LDH) activity in the extracellular medium (an indicator of membrane leakage) was measured by ELISA plate reader using a LDH assay kit (Biovision Research Products, USA) following manufacture’s specification.

**Crystal violet/Giemsa staining**

Cells were seeded in a density at a 5 X 10⁴ cells/well in a six well plate, and incubated for 24 h to grow in a confluent monolayer. The cells were treated with LYC and COL and incubated for 24 h. After 24 h of incubation, cells were fixed with 4% paraformaldehyde and stained with crystal violet and giemsa (0.1%). The morphological changes in the treated cells were compared with control and photographed using a bright field microscope (CKX 41, Olympus Inverted Trinocular Microscope, Japan).

**In vitro wound healing assay**

The wound healing assay was performed as described previously by Liang et al. 2007. In brief, cells were seeded in a six well plate at a density 5 X 10⁴ cells/well and incubated at 37 °C in a humidified 5% CO2 incubator for 24 h to grow the cells in a confluent monolayer. To this wounds were induced or scratched through the cell monolayer using a sterile 200 μL pipette tip. The cells were washed gently with PBS to remove the detached cells, the fresh media with the LYC and COL (Fraction 3) was added. After incubation for 24 h, cells were fixed with 4% paraformaldehyde and stained with crystal violet (0.1%). Each well was photographed using a bright field microscope (CKX 41, Olympus Inverted Trinocular Microscope, Japan).

**Activity of catalase and superoxide dismutase**

Enzyme activities was determined in the supernatant from cells harvested after incubation with carotenoids. Catalase activity was determined by the decomposition of H₂O₂ can be followed directly by the decrease in absorbance at 240 nm (Aebi, 1984). Briefly, to the 0.1 M PBS (pH, 7.0) sample (50-200 μg protein) and 50-100 μL of H₂O₂ (8.8 mM) was preferable for enzyme assay. Superoxide dismutase activity was measured by the inhibition of cytochrome C reduction mediated via superoxide dismutase.
anions generated by xanthine-xanthine oxidase and monitored at 550 nm (Flohe et al. 1984). One unit of superoxide dismutase will be defined, as the amount required inhibiting the reduction of cytochrome C by 50%. Briefly, 20 to 50 μL of 1:10 diluted supernatant samples was added to the solution containing 50 μM xanthine and 20 μM cytochrome C in 50 mM phosphate buffer (0.1 mM EDTA), reaction will initiated by adding 20 μL of xanthine oxidase (0.2 U/mL in phosphate buffer, pH 7.8), measured at 550 nm for minutes by using spectrophotometer (Shimadzu-Japan, 1601).

**Measurement of glutathione and malondialdehyde (MDA) levels in cells**

A standard solution of glutathione, as well as cell lysates from control and cells treated with LYC, AOL or COL were incubated with the solution containing NADPH (12 mM), 0.1 mM DTNB and 50 U/L glutathione reductase (GR). The working solution was prepared in 100 mM sodium-phosphate buffer with 5 mM sodium EDTA (pH 7.4). Oxidized glutathione is reduced by NADPH in the presence of GR and sequentially oxidized by DTNB. The rate of 5-thio-2-nitrobenzoic acid formation was measured at 412 nm and glutathione levels (nmoles/mg protein) present in the sample were recorded by using a standard curve (Tietze, 1969). For lipid peroxides estimation (nmoles MDA/mg protein), cells were trypsinized and the pellets were lysed using tris-buffer (50 mM), EDTA (15 mM) with the protease inhibitor. The cell lysate was used to measure MDA (Ohkawa et al.1979). Protein was estimated according to Lowry’s method (Lowry et al. 1951).

**Cell cycle analysis**

Cell cycle analysis was done by flow cytometry as per Sowmya et al. (2015). In brief, control and treated cells were rinsed with PBS and detached with trypsin-EDTA at room temperature and centrifuged at 2500 rpm for 5 minutes. The cells were washed twice with PBS and resuspended in 1 mL ice-cold hypotonic solution, containing 0.1% triton X-100, 0.1% citrate buffer and 0.1 mg/mL RNase and 50 μg/mL propidium iodide and incubated for 15 minutes at 37 °C in the dark. After incubation, the cell suspension was analysed for DNA content (less than 2N). The cell cycle distribution were measured with FACS verse flow cytometer (BD BioSciences, San Jose, CA, USA) and analysed by FACS Diva analysis software.

**Apoptosis detection**

Apoptosis detection was made with an FITC Annexin-V apoptosis detection kit according to the manufacturer’s instructions. Briefly, control and treated cells after
24 h of incubation were collected, washed with ice-cold PBS and centrifuged at 2500 rpm for 5 minutes. The cell pellet was resuspended in the ice-cold 1X binding buffer and incubated with FITC-conjugated annexin V and propidium iodide (PI) for 15 minutes at room temperature in the dark. The samples were immediately analysed on FACS verse flow cytometer using the Diva analysis software.

Confocal microscopy

Cells untreated and treated with LYC oxidation products (50 and 100 μM) were harvested after 24 h incubation and used for annexin V-FITC and PI staining. The cells were then observed under inverted confocal laser scanning microscope (Ziess LSM 510 MK4, Germany) and images were captured. DAPI was used as a nuclear marker.

Fluorescence morphological examination by AO & EB and DAPI

The cellular morphological changes were studied by using fluorescence microscope (CKX 41, Olympus Inverted Trinocular Fluorescence Microscope, Japan). Cells were seeded (50,000 cells/100 μl) onto a poly-D lysine coated cover slips and cultured to about 75% confluency. Cells with and without COL was incubated for 24 h at 37 °C and 5% CO₂, dual stained with 1:1 ratio of acridine orange (100 μg/mL) and ethidium bromide (100 μg/mL) for 5 min. For further confirmation, nuclei staining were done with 1 μg/mL DAPI for 3 min in the dark. The cells were then washed with PBS three times. The morphology of cell nuclei was observed and images were documented using Q-Imaging MP3.3 cooled colour camera with Q-Capture Pro7 imaging software (Canada).

ROS detection by flow cytometry

Control and treated cells (2 X 10⁶) were harvested after 24 h of incubation, washed with PBS, and suspended in 1 mL PBS. Followed by DCFH-DA (10 μM) was added and incubated for 15 minutes in 5% CO₂ at 37 °C. After incubation, cells were washed and resuspended in PBS and were analysed within 1 h on FACS Verse Flow Cytometer. The results were expressed as fluorescence intensity of dichlorofluorescein compared with control, treatment and cells with H₂O₂ (positive control).
Detection of mitochondrial transmembrane potential (MTP, Dym)

The changes in the mitochondrial potential were detected by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide/chloride (JC-1), a cationic dye that exhibits potential dependent accumulation in mitochondria, indicated by fluorescence emission shift from red (~590 nm) to green (~525 nm). In brief, MCF-7 cells (1 X 10^5 cells/mL) were treated with LYC and different concentrations of COL for 24 h. Cells treated with 4 mM of 2,4-Dinitrophenyl hydrazine (Sigma-Aldrich, USA) for 24 h were used as a positive control. After treatment, cells were stained with JC-1 and analyzed by a fluorescence-activated cell sorter (FAC Verse, Becton Dickinson, USA). The ratio of mean fluorescence intensity (MFI) of red to green fluorescence was calculated for each treatment and plotted.

Gap junctional intercellular communication (GJIC) assay

A scrape loading-dye-transfer method was performed using Yeh et al. (2003) method. In brief, control and treated cells washed 3 times in PBS, and scraped with a sharp blade in the presence of Lucifer yellow dye (0.5 mg/mL of PBS), followed by incubation in the dark for 5 min at room temperature. The lucifer yellow medium was then discarded and cells were washed three times with PBS. The amount of lucifer yellow dye transferred from the scraped edge to the neighboring cells was examined using a fluorescent microscope (Olympus BX40). To quantify GJIC, we calculated the coupling index, which was the ratio of the number of lucifer yellow-labeled cells to the number of cells on scraped edge; the latter was determined by the corresponding phase-contrast image.

Caspase-3, -8, and -9 assay

Cells were cultured in 25 cm² flasks with 5 X 10^6 cells/5 mL media. Cells were treated with LYC and its oxidised products. After harvesting, cells were washed twice with cold PBS and resuspended with cell lysis buffer following the manufacturer’s protocol. Protein concentrations in all cell lysates were determined using Bio-Rad protein assay (Bio-Rad Laboratories), and 200 μg of total protein were used for each examined sample. Samples were added to the reaction buffer with the appropriate caspase fluorogenic substrate. Reactions were incubated for 2 h at 37 °C, and fluorescence was determined using the Tecan pro 200 fluorescence microplate.
reader that allowed for light excitation at 400 nm wavelength and collected emitted light at a wavelength of 505 nm.

**Semi-quantitative and quantitative RT-PCR**

Cancer cells treated with COL (fraction-3), and cells without treatment was considered as control. Cells were plated at a density of $2 \times 10^6$ cells/well in a 6 well plate. At stage of 80% confluency, cells were washed twice with PBS. Total RNA was isolated by using TRI reagent protocol and dissolved in diethyl-pyrocarbonate-treated water. Then the RNA concentrations was determined by A260 measurements using spectrophotometer (Chalabi et al. 2004). RNA was reverse transcribed to cDNA in 20 µL final volume containing 1 µg of extracted RNA and mixed with 200 ng of random hexamer and 0.5 mM dNTP. The mixture was heated to 65 °C for 5 min, and then add 4 U RNase inhibitor, RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2), 10 mM DTT and 200 unit M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany). This mixture was incubated for 10 minutes at 25 °C followed by 50 min incubation at 37 °C. The prepared cDNA was heated at 75 °C for 15 min to denature the MMLV-RT and then stored at -20 °C for PCR. PCR was performed by GAPDH (internal control), Cyclin D, E, A, D, p21, p27, Bax, Bcl-2, p53, ku 70 and connexin 43 primers. After preparation of the first-strand cDNA, the reaction solution was mixed with PCR reagents to make a 20 µL reaction solution containing 1 units Taq DNA polymerase, 5 pM of each primer, 2.5 mM of each dNTP, 10 mM Tris - HCl, 50 mM KCl, and 1.5 mM MgCl2 or SYBR green master mix (semi quantitative and qualitative PCR). PCR was performed by using thermal cycler or real time by incubating at 95 °C for 2 min (initial melt), followed by 30 cycles as follows: 95 °C for 1 min (denature), 55 °C for 1 min (anneal) 72 °C for 2 min (extend). PCR will be completed with a final extension of 72 °C for 7 min. The reaction product was kept at -20 °C for further analysis. For semi quantitative PCR, the PCR products was analyzed on a 1% agarose gel in tris-acetate/EDTA buffer and molecular weight was confirmed with the 100-bp DNA ladder. Gels will be stained with ethidium bromide and photographed (Guo et al. 1998) and for real time PCR the change in Ct values was calculated to understand the fold changes between control and treatments.
### Table 2.1. Primers for semi-quantitative and quantitative polymerase chain reaction

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### Western blotting analysis of β-tubulin and connexin 43 expression from whole cell lysates

The cells were seeded at a density of 2 X 10^5 cells/mL/well in a 6 well plate and treated with COL (fraction-3). The control and treated cells were harvested using trypsin-EDTA, washed twice with PBS and was lysed in a lysis buffer containing 500 mM Tris-HCl, pH 6.8, 10% SDS, and 10% glycerol. The trypsinized-cells was suspended in 1 mL of isotonic buffer containing 10 mM HEPES-KOH (pH 7.4) 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF on ice for 30 min. The cells was homogenized on ice using a Dounce homogenizer with a tight pestle, and subjected to centrifugation at 3,500 g for 5 min to obtain post-nuclear supernatant. About 50 μg for whole cell proteins was separated on 7.5% or 12% for SDS-polyacrylamide minigels. Gels were further electroblotted onto nitrocellulose membrane (Bio-rad). The nitrocellulose membrane was incubated in milk block buffer (TBS-T) containing 20 mM Tris-HCl (pH 7.6) 270 mM NaCl, 0.1% of Tween-20 involving 5% non-fat milk at RT for 1 h, and probed with each of the primary antibodies in milk block buffer (TBS-T and 5% nonfat milk) overnight at 4 °C. The antibodies used is as follows: β-tubulin and connexin 43. The membranes was rinsed and washed twice for 10 min in TBS-T and incubated with alkaline phosphatase-conjugated anti-mouse (sigma) or anti-rabbit (Santa Cruz Biotechnology) secondary antibodies in TBS-T. The membranes was washed twice in TBS-T for 10 min and subsequently in alkaline phosphatase buffer containing 100 mM Tris-HCl (pH 9.5)
100 mM NaCl, and 5 mM MgCl$_2$. Protein was visualized by incubating with nitro blue tetrazolium and bromochloroindolyl phosphate (Nahum et al. 2001, Watts et al. 1995; Terasaki et al. 2007)

**Statistical analysis**

Values are means ± SD of three/five samples. Data were tested for homogeneity of variances by the Bartlett test. When homogenous variances were confirmed, the data were tested by ANOVA (Assistat software, v.7.7 and Graphpad Prism 5) and significant differences between the groups were evaluated by Tukey’s test. The difference in mean was considered significant at $p<0.05$. 
Figure 2.1. Overall experimental plan.