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3. MATERIALS AND METHODS

3.1. In Vitro

3.1.1. Source of chemicals

Umbelliferone (UMB), N-Nitrosodiethylamine (DEN), Vitamin C, 3-(4, 5-
dimethylthiazol- 2-yi) -2, 5-diphenyl-2H-tetrazolium bromide (MTT), DEPC were
purchased from Sigma chemical Co., (St. Louis, MO, USA). Dulbecco’s Modified
Eagle Medium (DMEM), fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO),
aacidine orange (AO), ethidium bromide (EB), phosphate buffer solution (PBS) and
trypsin were purchased from Himedia laboratories (Mumbai, India). All the other
chemicals and solvents used in this study were of analytical grade.

3.1.2. Culture and maintenance of HepG2 cells

Human HCC cells (HepG2 cells) were obtained from National Centre for
Cell Science (NCCS), Pune, India and cell culture reagents were purchased from
Himedia laboratories (Mumbai, India). The cells were grown in T-25 culture flasks
maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with
10% fetal bovine serum (FBS), 10mg/mL of streptomycin and 10000U/mL of
penicillin, and grown in 5% CO₂ atmosphere at 37°C.

3.1.3. MTT Assay

MTT assay was performed by the method of Mosmann, (1983).

Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-
dimethylthiazol-2 yl) -2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial
succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria
where it is reduced to an insoluble, coloured (dark purple) formazan product. The
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cells are then solubilised with an organic solvent (e.g. Isopropanol, DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Reagents

1. MTT [3, (4,5-dimethylthiazol-2-yl) -2,5,-diphenyltetrazolium bromide]: 5mg in 1X PBS.
2. DMSO

Procedure

HepG2 cells were seeded in a 96 well plate, at a cell density of 1X 10^6 cells/well and incubated overnight at 37°C and 5% CO₂. Complete medium was then replaced with 100μL DMEM medium containing different concentrations of the drug dissolved in 0.1% DMSO and incubated for 24 and 48 hours respectively. After the incubation period, medium with drugs was replaced with 100μL DMEM medium containing 10μL of MTT and incubated in the dark for four hours at 37°C. Medium with MTT was discarded and 100μL DMSO was added to solubilize the dark blue formazan crystals. The plate was read on an ELISA plate reader at 570nm and percentage viability was calculated as given below:

\[
% \text{ Viability} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of control}} \times 100
\]

3.1.4. Acridine orange/ Ethidium bromide dual staining

Acridine orange/ethidium bromide dual staining was performed by the method of Kasibhatla, et al., (2006).
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Reagents

1. 100X staining solution: 50mg of acridine orange and 15mg of ethidium bromide were dissolved in 1.0mL of 95% ethanol and made up to 50mL with distilled water, mixed well and stored as 1.0mL aliquots in freezer. To prepare 1X staining solution, 1mL of aliquot was thawed and diluted with 99mL of 1X PBS.

Procedure

After incubation time, the control and UMB, Vitamin C and UMB+Vitamin C treated HepG2 cells were fixed in ice cold methanol and washed in 1X PBS. Then, 10μL of the (1X) staining solution was added and allowed to stand for 3-5 minutes at room temperature. Again the cells were washed with 1X PBS loaded on a microscopic slide carefully without forming any air bubbles. The slide was then observed under microscope using long band pass filter combination (Acridine orange filter). Viable cells were seen as green, dead cells as red and compromised, but viable cells as yellow.

3.1.5. Morphological analysis after Propidium iodide stain


Principle

Propidium iodide is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population and as a counter stain in multicolor fluorescent techniques. Propidium iodide is suitable for fluorescence microscopy, confocal, flow cytometry and fluorometry.
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Reagents

1. Propidium iodide: 1.0mg/mL in PBS

Procedure

For the identification of the apoptotic cells, cell culture and drug treatment were done as described. The cells were washed with PBS and stained with a hypotonic PI solution containing 50µg/mL PI in 0.1% sodium citrate plus 0.1% triton X-100. Morphological analysis of apoptosis was performed after staining with PI under fluorescence microscopy (Zeiss, Axiovert 200, Germany).

3.1.6. DNA fragmentation assay

DNA fragmentation analysis was performed by the method of McGee, et al., (2002).

Reagents

1. Lysis buffer: (pH- 8.3)
   a. 200mM NaCl
   b. 10mM Tris
   c. 20mM EDTA
   d. 0.2% Triton-X-100

2. Proteinase K

3. Tris saturated phenol

4. Chloroform: Isoamyl alcohol (24:1 v/v)

5. Ice cold isopropanol
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6. 3M Sodium acetate

7. 70% Ethanol

8. 10X TE buffer: (pH- 8)
   a. 100 mM Tris base
   b. 10 mM EDTA

9. 1X PBS- pH- 7.4

Procedure

Control, UMB, Vitamin C and UMB+Vitamin C treated HepG2 cells were trypsinized and washed with 1X PBS. 200µL of cell lysis buffer and 20µL proteinase-K were added to the cell pellet and mixed well by vortexing. It was then incubated at 65°C for 1 hour. DNA was then extracted with 400µL of phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v/v) and then centrifuged at 12,000 rpm for 15 minutes. Upper aqueous layer containing DNA was transferred into another microfuge tube containing 500µL of ice-cold isopropanol and 50µL of sodium acetate. DNA was pelleted down by centrifugation at 12,000 rpm for 15 minutes. DNA pellet was then washed with 70% ethanol at 13,000 rpm for 5 minutes. The air-dried pellet was then dissolved in 20µL of 1X TE buffer. DNA fragmentation was visualized by agarose (1.5%) gel electrophoresis.

3.1.7. Agarose gel electrophoresis

Principle

Agarose gel electrophoresis is used for the separation of DNA fragments ranging from 50 base pairs to several mega bases (millions of bases). The distance
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between DNA bands of a given length is determined by the percent agarose in the gel.

Reagents

1. 2% Agarose

   2g agarose in 100mL of TBE buffer

2. TBE Buffer (1X) Tris -

   0.8g Borate -

   5.5g EDTA -

   372mg

3. Ethidium bromide

4. Loading dye buffer

   4g of sucrose, 2.5mg of bromophenol blue was completely dissolved

   in 10mL

   TBE buffer

Procedure

2.0g of agarose was dissolved into 100mL of TBE buffer, mixed well and the contents were boiled. The clear agarose solution was allowed to cool for 5 minutes at room temperature and 1.0μL of ethidium bromide was added and mixed well. The solution was poured into gel tray and allowed to set for 30 minutes. 10μL of DNA sample was transferred into microfuge tube and 2μL of loading dye was added. The gel was run at 50V and once the loading dye crossed to reach the bottom of the gel the power was turned off and the gel was carefully removed. The gel was placed on the UV transilluminator and photographed.
3.1.8. Flow cytometric analysis

Principle

Cell cycle analysis using propidium iodide staining by flowcytometry is used as a diagnostic tool to measure DNA ploidy as well as to measure alterations in cell cycle profiles characteristic of DNA fragmentation (necrosis) compared to patterned DNA cleavage (apoptosis). The information obtained includes the visualization of cell subpopulations with differing DNA contents. For each nucleus subpopulation identified, the parameters of population size, fractions of nuclei in each phase of the cell cycle and computation of DNA ratios can be discerned. DNA hypoploid changes are characteristic of apoptosis.

Reagents

1. PBS (pH 7.4)
2. Propidium iodide (0.1 mg/mL; 50 μL)
3. Triton X-100
4. Sodium citrate,

Procedure

Briefly, 2.5 × 10^5 cells were incubated in 24 - well plates with and without UMB as well as with UMB and Vitamin C in 0.5 mL of medium at 37°C for 24 hours. Cells were collected by centrifugation at 600 g for 10 minutes at 4°C, washed twice with PBS, and then resuspended in 100 μL of lysis solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/mL propidium iodide. Apoptosis was quantitatively determined by flow cytometry after incubation at 4°C in the dark for at least 24 hours as cells containing nuclei with subdiploid
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DNA content. The number of cells at each stage of the cell cycle was estimated by
fluorescence-associated cell sorting (FACS) and monitored by flow cytometry. The
data were analyzed to determine the percentage of cells at each phase of the cell
cycle (G₀/G₁, S and G₂/M) or in aneuploid peak.

3.1.9. Western blot analysis

Western blot analysis was done as previously described with minor
modifications to detect the expressions of caspases-3, Bcl-2 and Bax proteins (Chen,
et al., 2008). After treatment for 24 hours, the cells were washed thrice with ice-cold
PBS. The total proteins were solubilized and extracted with 100µl lysis buffer
(20mmol/L Tris (pH 7.5), 150mmol/L NaCl, 1% (v/v) Triton X-100, 1mmol/L
sodium orthovanadate, 100μmol/L β-glycerophosphate, 5mmol/L EDTA,
0.2mmol/L sodium orthovanadate, 10μg/ml leupeptin, and 2mmol/L
phenylmethanesulfonyl fluoride (PMSF)). The lysates were used to estimate their
protein content with bicinchoninic acid (BCA) protein assay. Equal amounts of
protein (50µg) from each sample were subjected to electrophoresis on a sodium
dodecylsulfate (SDS) polyacrylamide gel, using 10%, 12%, or 15% (v/v) acrylamide
gels. After electrophoresis, proteins were electroblotted to a Hybond-C Extra
nitrocellulose membrane (Amersham, USA). The membrane was blocked at room
temperature with 5% (w/v) nonfat dry milk in Tris buffered saline (TBS) containing
0.3% (v/v) Tween (TBS-T). Then the membrane was washed thrice with TBS-T and
incubated overnight at 4°C with the primary antibody, rabbit monoclonal anti-
caspases-3 and 9, (1:5000, v/v), anti-Bax (1:1000, v/v), anti-Bcl-2 (1:1000, v/v),
followed by 1-hour incubation with a 1:5000 (v/v) dilution of the appropriate
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horseradish peroxidase-conjugated secondary antibody. After incubation, the membrane was washed with TBS-T for three times, and the antigen-antibody complexes were visualized by enhanced chemiluminescence and exposure to X-ray film (Kodak, Eastman Kodak, USA) for 0.5 to 30 minutes.

3.2. Experiment I (In vivo studies): Dose dependent study

3.2.1. Chemicals

Umbelliferone (UMB), N-Nitrosodiethylamine (DEN), Vitamin C, nitrobluetetrazolium (NBT), riboflavin, Schiff reagent, AgNOR staining fluid, were purchased from Sigma-aldrich Chemical Company, MO, USA. The antibodies for NF-κBp65, p53, Bax, Bcl2, Caspase-9, Caspase-3 and β-actin were purchased from Santa Cruz Biotechnology (USA) and cell signaling technology (USA). The PCR primers for TNF-alpha, and COX-2 were purchased from Integrated DNA Technologies (Coralville, IA). TRI reagent, random hexamer primers, deoxy nucleotide triphosphate (dNTP), reverse transcriptase (RT) enzyme and PCR ready master mix were purchased from GeNeiTM (India). All other chemicals and solvents were of analytical grade.

3.2.2. Tumour induction

N-Nitrosodiethylamine (DEN) at a concentration of 0.01% was treated in drinking water for 16 weeks for the entire study (Ha, et al., 2001).

3.2.3. Preparation of UMB

Umbelliferone (UMB) was suspended in 10% of dimethylsulphoxide and each rat received at the daily of dose of 10, 20 and 30mg/kg (body weight) via
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intra gastric intubation. (Ramesh and Pugalendi, 2006a and Vasconcelos, et al., 2009).

3.2.4 Preparation of Vitamin C

Vitamin C was given by intragastric intubation, at a daily dose of 200mg/kg body weight for 16 weeks (Almino Cardoso Ramos, et al., 2009).

3.2.5. Animals and Diet

Male Wistar rats, weighing about 100-120 g were obtained from Vinayaka Mission College of Pharmacy, Salem. The animals were cared for in compliance with the principles and guidelines of the Indian Animal Ethical Committee of Periyar University in accordance with the Indian National Law on Animal Care and Use (Reg.No: PU-IAEC/JULY2011/04). The animals were housed in polypropylene cages, 6 rats per cages, under controlled conditions of 12 hours dark/light cycle, 50 ± 10% relative humidity and 24 2°C temperature. The rats were held in quarantine for 1 week and had access to tap water ad libitum. A commercial pellet diet containing 56.17% of nitrogen free extract (carbohydrates), 22.02% of crude protein, 7.5% of ash, 4.25% of crude oil, 3.02% of crude fibre, 2.46% of glucose, 1.8% of Vitamins, 1.38% of sand silica, 0.8% of calcium and 0.6% of phosphorus. This diet was fed to rats throughout the experimental period of 17 weeks (Including 1 weeks of acclimatization).
3.2.6. Experimental design

Rats were randomly distributed into 8 groups of 6 rats each as follows.

Group I - Rats were received normal control rats received standard pellet diet and pure drinking water for 16 weeks.

Group II - Rats were induced with hepatocellular carcinoma by providing 0.01% DEN through drinking water for 16 weeks.

Group III - Rats were received UMB via intra gastric intubation at a daily dose of 10mg/kg (body weight).

Group IV - Rats were received UMB via intra gastric intubation at a daily dose of 20mg/kg (body weight).

Group V - Rats were received UMB via intra gastric intubation at a daily dose of 30mg/kg (body weight).
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Group VI - Rats were received UMB via intra gastric intubation at a daily
dose of 10mg/kg (body weight) and 0.01% DEN through
drinking water for 15 weeks.

Group VII - Rats were received UMB via intra gastric intubation at a daily
dose of 20mg/kg (body weight) 0.01% DEN through drinking water for 15 weeks.

Group VIII - Rats were received UMB via intra gastric intubation at a daily
dose of 30mg/kg (body weight) 0.01% DEN through drinking water for 16 weeks.

At the end of the experimental period of 16 weeks, the rats were anesthetized
by ketamine hydrochloride (30mg/kg body weight, i.m) and all the animals were
sacrificed by cervical dislocation after an overnight fast. The dose of UMB was
fixed on the basis the results of histopathology studies and the changes in the levels
of liver marker enzymes.

3.3. Experiment II (In vivo studies)

3.3.1. Experimental design

Rats were randomly distributed into 6 groups of 6 rats each as follows.

Group I - Rats were received normal control rats fed with standard diet and
pure drinking water for 16 weeks.

Group II - Rats were induced with hepatocellular carcinoma by providing 0.01%
DEN through drinking water for 15 weeks.

Group III - Rats were received UMB by intragastric intubation at daily dose of
30mg/kg (body weight) and Vitamin C was at a daily dose of 200mg/kg
(body weight) through intra gastric intubation for 16 weeks.

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Group IV - Rats were pretreated with UMB by intragastric intubation at a daily
dose of 30mg/kg (body weight) and Vitamin C 200mg/kg (body
weight) 1 week prior the administration of 0.01% DEN and continued
till the end of the experiment (i.e., 16 weeks).

Group V - Rats were post-treated with UMB by intragastric intubation at a daily
dose of 30mg/kg (body weight) and Vitamin C 200mg/kg (body
weight) for 5 weeks after the administration of DEN for 10 weeks (i.e.,
after administering DEN alone for 10 weeks the rats will be treated with
UMB and Vitamin C 200mg/kg (body weight) along with DEN for
another 5 weeks) and continued till the end of experiment.

Group VI - Entire period rats received UMB by intragastric intubation at a daily
dose of 30mg/kg (body weight) and Vitamin C 200mg/kg (body
weight) along with DEN for 16 weeks of experimental period.

Fig.11.Schematic representation of experimental design-II
3.3.2. Preparation of hemolysate

Blood was collected in heparinized tubes and plasma was separated by centrifugation at 2000 ×g for 10 minutes. After the separation of plasma, the buffy coat was removed and packed cells (RBCs) were washed thrice with cold physiological saline. To determine the activity of RBC antioxidant enzymes, RBC lysate was prepared by lysing a known volume of RBCs with hypotonic phosphate buffer, pH 7.4. Centrifuging at 3000×g for 10 minutes at 2°C separated the hemolysate.

3.3.3. Preparation of tissue homogenate

Tissue samples were immediately transferred to ice-cold containers weighed and homogenized using the appropriate buffer in a tissue homogeniser.

3.3.4. Estimation of haemoglobin

Haemoglobin was determined by the method of Drabkin and Austin, (1932).

Principle

The basis of the method was dilution of blood in an alkaline solution containing potassium cyanide and potassium ferricyanide. Haemoglobin was oxidised to methaemoglobin that combines with cyanide to form cyanmethaemoglobin that was measured at 540nm.

Reagents

1. Drabkin’s reagent

2. Cyanmethaemoglobin standard solution (16g/dL): This was obtained commercially
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Procedure

0.02mL blood was mixed well with 5.0mL of drabkin’s reagent and allowed to stand for 10 minutes. The colour developed was read colorimetrically at 540nm together with the standard solution of cyanmethemoglobin, against a reagent blank. The amount of haemoglobin was expressed as mg/dl blood.

3.3.5. Estimation of protein

Protein content was determined by the method of Lowry, et al., (1951).

Principle

Proteins react with folin-ciocalteau reagent to give a coloured complex. The colour so formed was due to the reaction of alkaline copper with protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present.

Reagents

1. Alkaline copper reagent
   
   i. Solution A: 2% sodium carbonate in 0.1N NaOH
   
   ii. Solution B: 0.5% copper sulphate
   
   iii. Solution C: 1% sodium potassium tartarate 50mL solution A was mixed with 0.5mL solution B and 1.0mL solution C just before use.

2. Folin's phenol reagent: This commercial Folin's phenol reagent was diluted 1:2 with double distilled water just before use.

3. Standard bovine serum albumin (BSA): A stock solution was prepared by dissolving 100mg of BSA in 100mL water in a standard flask. 10mL of the stock was diluted to 100mL, to obtain a working standard concentration 100µg/mL.
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Procedure

0.1mL of tissue homogenate was made up to 1.0mL with saline, then 1.0mL 10% TCA was added. The mixture was centrifuged, the supernatant discarded and the precipitate was dissolved in 1.0mL of 0.1 N NaOH. From this aliquots were taken for the estimation. 4.5mL of alkaline copper reagent was added to all the tubes and the contents were allowed to stand at 37°C for 10 minutes. Then 0.5mL diluted folin's phenol reagent was added and mixed. The colour intensity was measured in a spectrophotometer at 660nm. A series of standards with a concentration range of 20-100µg and a blank were processed in a similar manner. The total protein content was expressed as mg/dl in serum and mg/g tissue in the liver.

3.3.6. Estimation of plasma thiobarbituric acid reactive substances (TBARS)

Plasma TBARS was determined by the method of Yagi, (1978).

Principle

The colour formation with thiobarbituric acid was used as the index. Plasma was deproteinised with phosphotungstic acid and the precipitate was treated with TBA at 90°C for one hour. The pink colour formed gives a measure of the TBARS in spectronic 20 colorimeter at 535nm.

Reagents

1. 0.83N H₂SO₄
2. 10% phosphotungstic acid
3. TBA reagent-670mg in 100mL water. To this 100mL acetic acid was added.
4. Standard malondialdehyde: A stock solution (184µg/mL) of 1, 1', 3, 3'-tetraethoxy propane was prepared.

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Procedure

To 0.5mL of plasma, 4.0mL of 0.83N H₂SO₄ and 0.5mL of 10% phosphotungstic acid were added and mixed. After standing for 5 minutes the mixture was centrifuged at 3000xg for 10 minutes. The supernatant was discarded and the sediment was mixed with 2.0mL of H₂SO₄ and 0.3mL of 10% phosphotungstic acid. The mixture was shaken well and centrifuged at 3000xg for 10 minutes. The sediment was suspended in 4.0mL of distilled water and 1.0mL of TBA reagent was added. The reaction mixture was heated at 95°C for hours. After cooling, 5.0mL of butanol was added and the mixture was shaken vigorously and centrifuged at 3000xg for 15 minutes. The colour extracted in the butanol layer was read at 535nm. Standard solution in the concentration range of 1-5 nmoles and blank containing distilled water were processed along with test samples. The values are expressed as nmoles/mL.

3.3.7. Estimation of tissue thiobarbituric acid reactive substances (TBARS)

The extent of lipid peroxidation in tissues was determined by the method of Ohkawa, et al., (1979).

Principle

Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) in acidic conditions to generate a pink coloured chromophore, the absorbance of which was read in a spectronic 20 colorimeter at 535nm.
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Reagents

1. 5% TCA
2. 0.25N HCl
3. TBA: 0.375% in hot distilled water
4. TCA-TBA-HCl reagent: Solutions 1, 2 and 3 were mixed freshly in the ratio of 1: 1:1.
5. Stock standard: 0.16mL of 3.0 M solution of 1, 1', 3, 3' tetraethoxy propane was made up to 100mL with double distilled water.
6. Working standard: 50 nmoL/m in distilled water

Procedure

The tissue homogenate was prepared in Tris-HCl buffer (0.025 M, pH 7.8). 1.0mL of the tissue homogenate was mixed with 2.0mL of TCA-TBA-HCl reagent. The mixture was kept in a boiling water bath for 15 minutes, cooled and the tubes were centrifuged at 1000xg for 10 minutes and the colour developed in the supernatant was measured in a spectronic 20 colorimeter at 535nm against a reagent blank. A series of standard solutions in the concentration range of 2.5-10 nmoles were treated in a similar manner. The values are expressed as nmoles/mg protein.

3.3.8. Estimation of conjugated dienes

Conjugated dienes were assayed by the method of Rao and Recknagel, (1968).

Principle

Lipid peroxidation is associated with rearrangement of the double bonds in the poly unsaturated fatty acids leading to the formation of conjugated dienes, which
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absorb at 233nm. The measurement of the formation of conjugated dienes reflects the extent of lipid peroxidation taking place.

Reagents

1. Chloroform
2. Methanol
3. Cyclohexane

Procedure

To 1.0mL of plasma or tissue homogenate, 5.0mL chloroform-methanol reagent (2:1v/v) was added, mixed thoroughly and centrifuged for 5 minutes. To this, 1.5mL of cyclohexane was added and the absorbance was read at 233nm against a cyclohexane blank. The amount of conjugated dienes formed was calculated using a molar extinction coefficient of 2.52 x 10^4cm^{-1}. The concentration of conjugated dienes was expressed as μmoles/mL plasma or mmoles/mg protein.

3.3.4. Estimation of enzymatic and non enzymatic antioxidants

3.3.4.1. Estimation of superoxide dismutase (SOD, EC 1.15. 1. 1)

Superoxide dismutase was assayed by the method of Kakkar, et al., (1984).

Principle

The assay was based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan. The reaction was initiated by the addition of NADH and incubated for 90 seconds. Glacial acetic acid was added to arrest the reaction. The colour developed at the end of the reaction was extracted into the n-butanol and the absorbance was measured in a colorimeter at 520nm.
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Reagents

1. Sodium pyrophosphate buffer: 0.052M, pH 8.3.
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS): 186µM
6. Nitroblue tetrazolium (NBT): 300µM
7. Reduced nicotinamide adenine dinucleotide (NADH): 780µM
8. Glacial acetic acid

Procedure

Plasma/Tissue was homogenised by using sodium pyrophosphate buffer (0.025 M, pH). 0.5mL of the erythrocytes or tissue homogenate was diluted to 10mL with water, followed by the addition of 2.5mL of ethanol and 1.5mL chloroform (chilled reagents were added). This mixture was shaken for 90 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2mL of sodium pyrophosphate buffer, 0.1mL of PMS and 0.3mL of NBT and appropriately diluted enzyme preparation in a total volume of 3.0mL. The reaction was started by the addition of 0.2mL NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0mL n-butanol. The contents were left aside for 10 minutes, centrifuged and the n-butanol layer was separated. The colour intensity of the chromogen in n-butanol layer was

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measured in a spectronic 20 colorimeter at 560nm. A system devoid of enzyme
served as control. The enzyme concentration required to inhibit the chromogen
produced by 50% in one minute under standard conditions was taken as one unit.
The specific activity is expressed as units/min/mg Hb or protein.

3.3.4.2. Estimation of catalase (CAT, EC 1. 11. 1. 6)

The activity of catalase was determined by the method of Sinha, (1972).

Principle

Dichromate in acetic acid when heated in the presence of H₂O₂ was
converted to perchromic acid and then to chromic acetate. The chromic acetate
formed was measured at 620 nm. The enzyme catalase was allowed to split H₂O₂ for
various periods of time. The reaction was stopped at different time intervals by the
addition of dichromate-acetic acid mixture and the remaining H₂O₂ as chromic
acetate was measured using a spectrophotometer at 620nm.

Reagents

1. Phosphate buffer: 0.01M, pH 7.0

2. Hydrogen peroxide: 0.2M

3. Potassium dichromate: 5%

4. Dichromate-acetic acid reagent: 1:3 ratio of potassium dichromate and
   glacial acetic acid.

5. Standard hydrogen peroxide: 0.1mL/100mL in distilled water.
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Procedure

Tissue homogenate was prepared by using phosphate buffer (0.01 M, pH 7.0). 0.9mL phosphate buffer was mixed with 0.1mL erythrocytes or tissue homogenate and 0.4 mL H$_2$O$_2$. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0mL of dichromate-acetic acid reagent. The tubes were kept in a boiling water bath for 10 minutes cooled and the absorbance of the colour developed was read in a spectronic colorimeter at 620nm. Standards in the concentration range of 20-100 µmoles were processed as for the test. The specific activity of catalase is expressed as µmoles of H$_2$O$_2$ utilized/min/mg Hb or protein.

3.3.4.3. Estimation of glutathione peroxidase (GPx, EC. 1.11.1.9)

Glutathione peroxidase was assayed by the method of Rotruck, et al., (1973).

Principle

A known amount of enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period. The amount of GSH was utilized measured colorimetrically.

\[
2 \text{ GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2 \text{H}_2\text{O}
\]

Reagents

1. Tris-HCl buffer: 0.4 M, pH 7.0
2. Sodium azide solution: 10mM
3. TCA: 10%
4. EDTA: 0.4mM
5. 0.4mM H$_2$O$_2$ solution
6. Glutathione (GSH): 2.0mM
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Procedure

0.2mL Tris buffer (0.4M pH 7), 0.2mL EDTA, 0.1mL sodium azide and 0.5mL haemolysate or tissue homogenate were mixed together. To this mixture, 0.2mL of GSH followed by 0.1mL H₂O₂ was added. The contents were mixed well and incubated at 37°C for 10 minutes along with a control containing all reagents except the homogenate or erythrocyte. After 10 minutes the reaction was arrested by the addition of 0.5mL of 10% TCA. The contents were centrifuged and the supernatant was assayed for GSH by the method of Ellman. The activities are expressed as μmoles of GSH utilized/min/mg Hb or protein.

3.3.4.4. Estimation of glutathione reductase (GR, EC. 1. 6. 4. 2)

Glutathione reductase activity was assayed by the method of Carlberg and Mannervik, (1985).

Principle

The enzyme activity was assayed by measuring the GSH formed when the oxidised glutathione (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Reagents

1. Phosphate buffer: 0.1M pH 7.4
2. Sodium bicarbonate solution: 0.1M
3. GSSG: 250μM
4. FAD: 250 mM
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5. NADPH: 4mM

6. EDTA: 80mM

Procedure

2.0mL phosphate buffer, 0.1mL enzyme sample (plasma or tissue homogenate), 0.1mL FAD and 0.5mL EDTA solution were taken in a test tube. A blank was set up using all the reagents except FAD$. The tubes were incubated at 37°C for 15 minutes, followed by the addition of 0.1mL of NADPH solution to each tube. The reaction rate was then continuously monitored at 340 nm for 5 minutes and the linear absorbance change was measured. The values are expressed in $\mu$moles of NADPH oxidised/min/mg Hb or protein.

3.3.4.5. Estimation of glutathione-S-transferase (GST, EC. 2. 5. 1. 18)

The activity of glutathione-S-transferase (GST) was assayed by the method of Habig, et al., (1974).

Principle

GST activity was measured by following the increase in absorbance at 340nm using 1 - chloro, 2, 4 dinitrobenzene (CDNB) as the substrate.

$$2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Non-Se-GPx}} \text{GSSG} + 2\text{H}_2\text{O}$$

Reagents

1. Phosphate buffer: 0.3M, pH 6.5

2. GSH: 30mM

3. 30mM CDNB in 95% ethanol
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Procedure

1.0mL phosphate buffer, 0.1mL CDNB and 0.1mL of tissue homogenate or erythrocytes were taken in a test tube. The volumes were adjusted to 2.9mL with water. The reaction mixture was preincubated at 37°C for 5 minutes and the reaction started by the addition of 0.1mL of 30mM glutathione. The absorbance was followed for 5 minutes at 540 nm. A system devoid of enzyme served as the blank. The specific activity of GST is expressed as μmoles of CDNB-GSH conjugate formed/min/mg Hb or protein that was calculated using the formula

\[
\text{Activity} = \frac{\text{O.D} \times 3 \times 1000}{9.6 \times 5 \times \text{proteinin mg}}
\]

9.6 is the difference in the molar extinction co-efficient between CDNB-GSH conjugate and CDNB.

3.3.5. Estimation of non enzymatic antioxidants

3.3.5.1. Estimation of reduced glutathione

Reduced glutathione (GSH) was determined by the method of Ellman, (1959).

Principle

This method was based on the development of yellow colour when dithio-nitrobenzoic acid (DTNB) is added to compounds containing sulphhydryl groups.

Reagents

1. Phosphate buffer: 0.1M, pH 8.0

2. TCA: 5%
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3. Ellman’s reagent: 34mg of DTNB was dissolved in 10mL of 0.1% sodium citrate.

4. 0.3M Disodium hydrogen phosphate

5. Stock standard glutathione: 1000µg/mL in distilled water

6. Working standard: 100µg/mL in distilled water.

Procedure

0.5 mL of tissue homogenized in phosphate buffer (0.1M, pH 7.0), 2.0mL of 5 % TCA was added to precipitate the proteins. After centrifugation, to 1.0mL of supernatant, 3.0mL of 0.2 M phosphate buffer and 0.5mL of Ellman’s reagent were added. The yellow colour developed was read in a colorimeter at 412nm. A series of standards (20-100µg) were treated in a similar manner along with a blank containing 1.0mL buffer. The amount of glutathione is expressed as mg/dL or mg/g tissue.

3.3.5.2. Estimation of Ascorbic acid [Vitamin C]

Vitamin C was measured according to the method of Roe and Kuether, (1943).

Principle

Ascorbic acid is converted to dehydroascorbic acid in the presence of thiourea, a mild reducing agent and then coupled with 2, 4 dinitrophenyl hydrazine (DNPH). The coupled dinitrophenyl hydrazine is converted into a red coloured compound when treated with sulphuric acid, which is read at 520nm.
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Reagents

1. Trichloroacetic acid (TCA)-10%
2. Sulphuric acid-65%
3. DNPH, thiourea, copper sulphate reagent (DTC): This reagent was prepared by dissolving 0.4g of thiourea, 0.05g of copper sulphate and 3.0g of 2, 4 dinitrophenyl hydrazine in 100mL of 9N H₂SO₄.
4. Stock ascorbic acid: 100mg L-ascorbic acid was dissolved in 100mL of 5% TCA.
5. Working ascorbic acid: 1 in 10 dilutions with 5% TCA to obtain a concentration of 0.1mg/mL.

Procedure

To 0.5mL of tissue homogenate 1.5mL of ice-cold 10% TCA was added, mixed and centrifuged for 10 minutes at 1800xg. To 0.5mL of the supernatant, 0.1mL of DTC reagent was added and mixed well. The tubes were incubated at 37°C for exactly 3 hours. 0.75mL of ice-cold 65% sulphuric acid was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standards containing 10-50μg of ascorbic acid were taken and made upto 0.5 mL and processed in a similar manner along with a blank containing 0.5mL of 10% TCA. The colour developed was read at 520nm. The amount of Vitamin C was expressed as μmoles/mg protein.

3.3.5.3. Estimation of α-tocopherol (Vitamin E)

Plasma/Tissue Vitamin E was determined by the method of Baker, et al., (1980).
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Principle

The method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red colored complex with 2, 2’-dipyridyl. Absorbance of the chromophore was measured at 520nm.

Reagents

1. Petroleum ether, 60-80°C
2. Double distilled ethanol
3. 0.2% dipyridyl in ethanol (w/v)
4. 0.5% ferric chloride in ethanol (w/v)
5. Stock Standard: 10mg of α-tocopherol in 100mL of distilled ethanol.
6. Working Standard: Stock solution was diluted to a concentration of 10µg/mL in distilled ethanol.

Procedure

To 0.5mL of plasma or tissue homogenate, 1.5mL ethanol and 2.0mL petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C. To this, 0.2mL of 2, 2’-dipyridyl solution and 0.3mL ferric chloride solution were added mixed well and kept in dark for 5 minutes and 4.0mL of butanol added. The intense red colour developed was read at 520nm. Standard α-tocopherol in the concentration range of 10-100µg and treated in a similar manner along with a blank containing only the reagent. The amount of Vitamin E was expressed as µmoles/mg protein.
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3.3.6. Estimation of liver marker enzymes

3.3.6.1. Assay of aspartate aminotransferase (AST, EC. 2. 6. 1. 2)

Aspartate aminotransferase (AST) was assayed by the method of King, et al., (1965).

Principle

Serum aspartate aminotransferase catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate.

AST catalyses the following reaction:

$$\text{L-aspartate + } \alpha\text{-oxoglutarate } \rightarrow \text{oxaloacetate + L-glutamate}$$

Reagents

1. 0.1M Phosphate buffer, pH 7.4
2. 1.0mM DNPH in 2N HCl
3. 0.4N NaOH
4. Substrate solution 1.33g of aspartic acid and 15mg of α-oxoglutarate were dissolved in 100mL of phosphate buffer containing 0.5mM NaOH
5. Standard Pyruvate

Procedure

1.0mL of buffered substrate was taken and incubated at 37°C for 10 minutes. The 0.1mL of serum was added and the mixture was incubated at 37°C for one hour. The reaction was stopped by the addition of 1.0mL of DNPH reagent and incubated for 20 minutes. In the case of the blank tubes, 0.1mL of enzyme was added after the addition of DNPH reagent. The tubes were kept at room temperature
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for 20 minutes. Then 10mL of sodium hydroxide was added. The standard pyruvates in the tubes were also treated in a similar manner. The color developed was read at 540nm using a spectrophotometer. The enzyme activity was expressed as IU/ L in serum.

3.3.6.2. Assay of alanine aminotransferase (ALT, EC. 2. 6. 1. 1)

Alanine aminotransferase (ALT) was assayed by the method of King, et al., (1965).

Principle

The oxaloacetate is measured by the reaction with 2,4 dinitrophenylhydrazine giving a brown colored hydrazone after the addition of sodium hydroxide. The color developed is read at 520nm. The enzyme catalyses the following reaction:

\[
\text{L-alanine} + \alpha\text{-oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate}
\]

Reagents

1. 0.1M Phosphate buffer, pH 7.4
2. 1mM DNPH in 2N HCl
3. 0.4N NaOH
4. Substrate solution 1.78g of D-L-Alanine and 38mg of 2-ketoglutaric acid were dissolved in 0.5mL of 0.4N NaOH and made upto 100mL with phosphate buffer.
5. Standard Pyruvate
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Procedure

1.0mL of buffered substrate was taken and incubated at 37°C for 10 minutes. Then 0.1 mL of serum was added and the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1.0mL of DNPH reagent and allowed to stand for 20 minutes. In the case of the blank tubes, the 0.1mL of enzyme was added after the addition of DNPH reagent. The tubes were kept at room temperature for 20 minutes. Then 10mL of sodium hydroxide was added. The pyruvate standards in the tubes were also treated in a similar manner. The color developed was read at 540nm using a spectrophotometer. The enzyme activity was expressed as IU/ L in serum.

3.3.6.3. Activity of alkaline phosphatase

Alkaline phosphatase was assayed by the method of King, et al., (1965) using disodium phenyl phosphate as the substrate.

Principle

Disodium phenyl phosphate is hydrolysed by ALP with the liberation of phenol, which reacts under alkaline condition with Folin-phenol reagent to form blue colored complex, which was estimated colorimetrically at 680nm.

Reagents

1.  Bicarbonate buffer - 0.1M, (pH 10)
2.  Substrate - 0.01M phenylphosphate disodium salt solution
3.  Folin - phenol reagent
4.  Sodium carbonate - 10%
5.  Standard phenol solution - 5µg/mL
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Procedure

An incubation mixture containing 150μmoles of bicarbonate buffer and 10μmoles of substrate in 2.9mL distilled water was preincubated at 37°C for 10 minutes. 0.2mL of serum was added to this and incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0mL of Folin-phenol reagent. The suspension was centrifuged and 2.0mL of 10% sodium carbonate was added to the supernatant. The solution was incubated at 37°C for 10 minutes. Standard phenol solutions (2.5μg - 10.0μg) were also treated with Folin-phenol reagent and sodium carbonate. The blue colour developed was read at 680nm. The enzyme activity was expressed as IU/L.

3.3.6.4. Assay of lactate dehydrogenase

The activity of lactate dehydrogenase (LDH) was measured by the method of King, et al., (1965).

Principle

The lactate is acted upon by lactate dehydrogenase to form pyruvate in the presence of nicotinamide adenine dinucleotide (NAD\(^+\)). The pyruvate forms pyruvate phenyl hydrazone with 2, 4-dinitrophenyl hydrazine. The colour developed is read in a spectrophotometer at 440nm.

Reagents

1. Glycine buffer :100mM
2. Buffered substrate
3. NAD\(^+\) : 20mM
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4. DNPH: 0.2%
5. Sodium hydroxide: 0.4N
6. Standard pyruvate solution

Procedure

To a set of tubes, 1.0mL of the buffered substrate and 0.1mL of serum was added and the tubes were incubated at 37°C for 15 minutes. After adding 0.2mL of NAD solution, the incubation was continued for another 15 minutes. The reaction was then arrested by adding 1.0mL of DNPH reagent and the tubes were incubated for a further period of 15 minutes at 37°C. 0.1mL of serum was added to blank tubes after arresting the reaction with DNPH. 7mL of sodium hydroxide solution was added and the colour developed was measured at 420nm spectrometer. Suitable aliquots of the standards were also analysed by the same procedure. The enzyme activity is expressed as IU/L.

3.3.6.5. Estimation of total bilirubin

Bilirubin was estimated by the method of Melloy and Evelyn, (1937).

Principle

Bilirubin reacts with the diazo reagent to form azobilirubin. The resulting red-purple colour is measured spectrometrically at 540nm.

Reagents

1. Diazo reagent:

Solution A: Dissolved 0.1gm of sulphanilic acid in 100mL of 1.5% (v/v) hydrochloric acid and made upto 1.0 litre with distilled water.
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Solution B: Dissolved 0.5gm of sodium nitrite in 100mL of distilled water.

Diazoo reagent, prepared by adding 0.3mL of solution B and 10mL of solution A

2. Absolute methanol

3. Standard bilirubin: A stock solution was prepared by dissolving 10mg of bilirubin in 100mL of chloroform in a standard flask. 10mL of the stock was diluted to 100mL, to obtain a working standard concentration 100µg/mL.

Procedure

0.2mL of serum was taken in two test tubes and was made upto 2.0mL with distilled water. To the test added 0.5mL diazo reagent and to the blank added 0.5mL of hydrochloric acid. Finally to each added 2.5mL methanol. Into a series of tubes standard bilirubin solution in the concentration range of 10-50µg was taken and a blank were processed in a similar manner. All the tubes were incubated for 30 minutes and color developed was read at 540nm. The amount of bilirubin in the serum was expressed as mg/dL.

3.3.6.6. Estimation of total albumin

Principle

Albumin binds quantitatively with bromocresol green (BCG) at pH 4.15 resulting in the formation of a green colour which shows maximum absorbance at 630nm.

Reagents

1. Bromocresol green (BCG) reagent
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Procedure:

Pipette out 0.2mL of serum into a test tube. Add 1.8mL distilled water to it to achieve a dilution of 1.0 in 10. Then 0.1mL of diluted serum added to test tube and add 3.0mL BCG reagent. Mix all tubes by tapping them, one by one, against the palm and stand for 10 minutes at room temperature. After completion of incubation period, the absorbance was measured at 600 nm. The activity was calculated by using the formula:

\[
\text{Total serum albumin in g/dL} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 4.5.
\]

3.3.7. Extraction of lipids

Lipids were extracted from liver tissues by the method of Folch, et al., (1957) using chloroform: methanol mixture (2:1 v/v).

The tissues were rinsed in ice-cold physiological saline thoroughly and dried by pressing between the folds of filter paper. The samples were weighed and homogenized in ice cold chloroform-methanol (2:1 v/v) and the contents were extracted after 24 hours. The extraction was repeated for four times. The combined filtrate was washed with 0.7% potassium chloride and the aqueous layer was discarded. The organic layer was made up to a known volume with chloroform and used for various biochemical estimations.

3.3.7.1. Estimation of total cholesterol

Total cholesterol was determined by the method of Zlatkis, et al., (1953).
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Principle

Lipid extract was treated with ferric-chloride acetic acid reagent to precipitate the proteins. The protein free supernatent was treated with concentrated sulphuric acid. A reddish purple colour formed was read at 560nm.

Reagents

1. Ferric chloride-acetic acid reagent: 0.05% ferric chloride in acetic acid.
2. Concentrated sulphuric acid.
3. Cholesterol stock standard: 1mg/mL in acetic acid.

Procedure

0.1mL of extract was evaporated to dryness and 5.0mL ferric chloride acetic acid reagent was added, mixed and centrifuged. To the supernatent 3.0mL of concentrated sulphuric acid was added and the absorbance was read after 20 minutes at 560nm against a reagent blank. Standards in the range of 40-200µg were treated similarly. The values are expressed as mg/100g of tissue.

3.3.7.2. Estimation of phospholipids

Tissue phospholipids were determined by the method of Zilversmit and Davis, (1950).

Principle

Phospholipids were digested with concentrated sulphuric acid to liberate the lipid bound phosphorous inorganic phosphorous and then made to react with ammonium molybdate to form phosphomolybdic acid. This on treatment with
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1-amino 2-naphthol 4-sulfonic acid (ANSA) forms a stable blue colour, which was read in a colorimeter at 680nm.

Reagents

1. 5N H₂SO₄
2. Ammonium molybdate
3. 1-Amino-2-naphthol-4-sulphonic acid (ANSA): 0.2g of ANSA was mixed with 1.2g of sodium sulphate and 1.2g sodium sulphite. 0.25g was taken from this mixture and dissolved in 10mL of double distilled water.
4. 4. Standard phosphorus solution: 35.1g of potassium dihydrogen phosphate was dissolved in water. To this 1.0mL of 10N H₂SO₄ was added and made up to 100mL with distilled water. 10mL of this solution was diluted to 100mL, to prepare a working standard containing 8µg phosphorus/mL.

Procedure

An aliquot of the lipid extract was pipetted into a kjeldhal flask and evaporated to dryness. 1.0mL of 5N H₂SO₄ was added and digested in a digestion rack till light brown. Then 2 to 3 drops of concentrated nitric acid was added and the digestion continued till it became colorless. The kjeldhal flask was cooled, 1.0mL water was added and heated in a boiling water bath for about 5 minutes. Then 1.0mL of 25% ammonium molybdate and 0.1mL of ANSA were added. The volume was then made upto 10mL with distilled water and the absorbance was measured at 660nm within 10 minutes. Standards in the concentration range of 2-8µg were treated in the similar manner. The values obtained were multiplied with a factor 25
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to convert inorganic phosphorus to its phospholipids equivalents. The values are
expressed as mg/dl of Hb or tissue.

3.3.7.3. Estimation of triglycerides

Triglycerides were determined by the method of Foster and Dunn, (1973).

Principle

The triglycerides were extracted by isopropanol, which upon saponification
with potassium hydroxide yielded glycerol and soap. The glycerol liberated was
treated with metaperiodate, which released formaldehyde, which inturn was made to
react with acetyl acetone and ammonia to form yellow coloured compound, the
absorbance of which was read colorimetrically at 405nm.

Reagents

1. Isopropanol

2. Activated aluminium oxide (neutral)

3. Saponifying agent: 50g of potassium hydroxide was dissolved in 600mL
   water and 400mL isopropanol.

4. Acetyl acetone reagent: 0.75mL of acetyl acetone was dissolved in 60mL of
distilled water and 40mL of isopropanol was added.

5. Sodium metaperiodate reagent: 77g anhydrous ammonium acetate was
dissolved in 700mL water, 60mL glacial acetic acid and 650mg sodium
metaperiodate were added and made upto a liter with distilled water.

6. Standard triolein solution: 1g of triolein was dissolved in 100mL
   isopropanol. 1.0mL of stock standard was diluted to 100mL to prepare a
   working standard of concentration 100μg of triolein/mL.
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Procedure

An aliquot (0.5mL) of lipid extract was evaporated to dryness. Added 0.1mL of methanol followed by 4.0mL of isopropanol. About 0.4g of alumina was added to all the tubes and shaken well for 15 minutes. Centrifuged and then 2.0mL of the supernatant was transferred to appropriately labelled tubes. The tubes were placed in a water bath at 65°C for 15 minutes for saponification after adding 0.6mL of the saponification reagent followed by 0.5mL of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for an hour. A series of standards of concentration 8-40μg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405nm. The amount of triglycerides was expressed as mg/100g of tissue.

3.3.7.4. Estimation of free fatty acids

Free fatty acids were determined by the method of Falholt, et al., (1973).

Principle

Free fatty acids were extracted with chloroform-heptane-methanol mixture to eliminate interference from phospholipids and the extract was shaken with a high density copper reagent at pH 8.1. The copper soap remained in the upper organic layer from which an aliquot was removed and the copper content determined colorimetrically by treating with diphenyl carbazide.

Reagents

1. Extraction solvent: Chloroform-heptane-methanol solvent (5:5:1)
2. Stock copper solution: 50mM
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3. Triethanolamine (TEA) solution: 1.0M
4. Sodium hydroxide solution: 1.0M
5. Copper reagent (Cu-TEA solution): 10mL of copper solution was mixed with
   10mL triethanolamine, 6 mL sodium hydroxide and made up to 100mL, and
   the pH adjusted to 8.1 by adding 33g of sodium chloride.
6. Diphenyl carbazide solution; 1.5M in ethanol
7. Standard palmitic acid; A solution containing 2.0mg/mL of palmitic acid in
   chloroform was used as stock. Working standard containing 200μg/mL was
   prepared by diluting 1.0mL of stock to 10mL with chloroform.

Procedure

0.5mL of lipid extract was evaporated to dryness and dissolved in 6mL
extraction solvent and 2.5mL of copper reagent was added. All the tubes were
shaken vigorously for 90 seconds and were kept aside for 15 minutes. The tubes
were centrifuged and 3.0mL of the copper layer was transferred to another tube
containing 0.5mL of diphenyl carbazide and mixed carefully. The colour developed
was read at 540nm against a reagent blank containing 3mL solvent and 0.5mL
diphenyl carbazide. The free fatty acid content was expressed as mg/100g of tissue

3.3.8. Enzyme linked immunosorbent assay of AFP and CEA

Quantitative estimation of tumor markers viz., alpha-feto protein (AFP) and
carcinoembryonic antigen (CEA) was carried out by solid phase enzyme linked
immunosorbent assay (ELISA) using the UBI MAGIWELL (USA) enzyme
immunoassay kit (Sell and Becker, 1978; Macnab, et al., 1978). The desired
numbers of coated wells were secured in the holder. 10μL of standards, controls or

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serum samples were then dispensed into appropriate wells. One well was saved for
the blank, to which standards or enzyme conjugate should not be added. 50μL of
antibody solution was dispensed into each well, except the blank well. 50μL of
enzyme conjugate (conjugated to horseradish peroxidase) was dispensed in to each
well except the blank well. The wells were then incubated for one hour at room
temperature. After incubation mixture the wells were rinsed with working washing
buffer (50mL, 20X) five times. Then, 100μL of Solution A (phosphate buffer
solution containing hydrogen peroxide) and 100μL solution B
(Tetramethylbenzidine solution) was added into each well including the blank well.
This was then incubated for 30 minutes at room temperature, after which, the
enzyme reaction was stopped by the addition of 50μL of stop reagent and the
intensity of the color measured with microreader at 450nm.

3.3.9. Estimation of MMP-2/9 by ELISA

Protein concentrations of MMP-2/9 in homogenized liver tissue were
determined using commercial ELISA kits (R&D Systems, Minneapolis, MN). All
samples were analyzed in triplicate. Standard curves were generated according to the
manufacturer’s instructions. MMP-2 levels could be measured in the range 1.5 to 24
ng/mL; the sensitivity of the assay was 0.37ng/mL. MMP-9 levels could be
measured in the range 0.156 to 10ng/mL; the sensitivity of the assay was
0.028ng/mL.

3.3.10. Preparation of slides for mast cell staining

Mast cell staining was performed on a section of liver tissue of both
tumorous and non-tumorous portion (i.e., the number of sections prepared for groups
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2, 4, 5, 6 and 7 were 12 each, whereas for groups 1 and 3 it was six each) of the liver
tissue would be fixed in 10% formalin and embedded in paraffin wax and the
sections were cut at 5µm in thickness and then stained.

3.3.10.1. Mast cell staining

Histochemical analysis of mast cells was carried out by the method of Ranieri, et al., (2002).

Reagents

1. Toluidine blue: 1g in 70mL of absolute alcohol added to 0.5mL of HCl
   and made up to 100mL.

Procedure

Briefly, the 5µm thickness sections were dewaxed in xylene and rehydrated
through decreasing concentrations of ethanol to distilled water. Stained with
toluidine blue for 2 minutes and washed with distilled water, dehydrated in
increasing concentrations through alcohol series, xylene and mounted using DPX.
High power objective field (40×) was chosen for counting total number of mast cells
in ten different fields/slide.

3.3.11. AgNOR staining

AgNOR staining was performed according to the method of Ploton, et al.,
(1986).

Reagents

1. Solution A: 1% formic acid and 2% gelatin

2. Solution B: 50% (w/v) silver nitrate
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3. Solution A and Solution B were mixed in the ratio 1:2 (v/v) to form working solution.

**Procedure**

Liver sections of 5μm, obtained from each paraffin block were stained by the one step silver colloid method. Briefly, slides were kept in oven for 30 minutes at 56°C, dewaxed in xylene and rehydrated through decreasing concentration of ethanol to distilled deionised water. The AgNOR solution was freshly prepared by dissolving gelatin at a concentration of 2g/dL in 1 g/dL aqueous formic acid. This solution was added to 50g/dL aqueous silver nitrate solution (1:2, v/v).

This final solution was then immediately poured on to the slides, which were left in the dark at room temperature for 45 minutes. The silver colloid was washed from the section with deionised water and the sections were dehydrated through a graded series of ethanol to xylene. For quantification, a mean of 10 different areas of sections were chosen to determine the homogenous AgNOR quantification throughout all groups and at least 500 cells were counted, the quantification was always performed in well preserved cells, the AgNOR dots were easily identified as black points within the nuclei.

3.3.11. Isolation of RNA for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The phenol - guanidinium thiocyanate based trizol (TRI) Reagent was used for the isolation of total RNA. 100 mg of liver tissue was taken and homogenized using 1mL of TRI solution and left undisturbed for 5 minutes. To this, 200μl of chloroform was added for phase separation and vigorously vortexed for 15 to 30
Individual and synergetic effects of Umbelliferone with Vitamin C ................................................................. an in vivo and in vitro study seconds and allowed to stand for 15 minutes, followed by centrifugation at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to another tube, to this 250μL of isopropanol (ice cold) was added to precipitate RNA. Then the tube was incubated for 10 minutes and centrifuged at 14,000×g for 10 minutes at 4°C. The RNA pellet was washed in 75% ethanol twice, and dissolved in 25μL of DEPC water. The total RNA was stored at -20°C until use.

3.3.11.1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A reverse transcription reaction solution containing, 1.0μg total RNA in RNase/DNase-free water and 1.5μL of random hexamer primer, were incubated for 10 minutes at 72°C and chilled immediately to synthesize cDNA. To this, 5.0μL of premixed 10mmol/L deoxy nucleotide triphosphate (dNTP) solution, 3.0μL, 10× M-MLV RT buffer, and 1.0μL (200 units/μL) M-MLV RT were added and made up to 50μL using sterile RNase/DNase free water. The PCR ready mix was used according to the manufacturer’s instruction for amplifying the cDNA. All PCR samples were denatured at 94°C for 5 minutes before cycling and were extended for 10 minutes at 72°C after cycling. The PCR assay using primers was performed for 39 cycles at 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds. Primers for, NF-κB p65, COX-2, TNF-α were shown below (Table). Primers were designed using primer 3 software available free at http://fokker.wi.mit.edu/primer3/input.htm, and nucleic acid sequence was accessed from http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi.
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Table-1: List of primer sequences, product size and annealing temperature
used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence Product</th>
<th>Size (bp)</th>
<th>Annealing temperature(°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κBp65</td>
<td>Forward 5’-acgatctgttctccctatcct-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-tgtttcttccccaggaata-3’</td>
<td>150</td>
<td>57.0</td>
<td>40</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Forward 5’-aaagctcgtcagatgtct-3’</td>
<td>249</td>
<td>61.0</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-atgctggttgtggagggag-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward 5’-agatgtgggagctggcagggagg-3’</td>
<td>178</td>
<td>58.0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-cccgattgggagttctctt-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-1: List of primer sequences, product size and annealing temperature used for
NF-κBp65, Cox-2 and TNF-α

3.3.12. Immunoblot analysis

Immunoblotting was performed by the method of Towbin, et al., (1979) to
detect the expression pattern of a specific protein.

Principle

Immunoblotting is a rapid and sensitive assay for the detection and
characterization of proteins. Immunoblotting allows one to identify particular
proteins by utilizing the specific inherent in antigen-antibody recognition. This
procedure involves the solubilization and electrophoretic separation of
macromolecules by SDS-PAGE followed by quantitative transfer and irreversible
binding to nitrocellulose paper. This technique has proven to be useful in
identifying, specific antigens recognized by polyclonal or monoclonal antibodies
and it is highly sensitive, i.e. ng of antigen can also be detected.
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Reagents

1. Transfer buffer: pH 8.3
   a. Tris-Hcl: 25mM
   b. Glycine: 192mM
   c. Methanol: 20%

2. 10 X Tris buffered saline (pH 7.6)
   a. Tris base: 24.2g
   b. Nacl: 80g

   Dissolved in 1 liter of distilled water

   10X TBS: 5mL
   Distilled water: 45mL
   b. BSA: 1.5g

4. Ponceau’s solution
   a. Ponceau’s: 0.5g
   b. Glacial acetic acid: 1mL

   Dissolved in 100mL of distilled water

5. Wash buffer TBS/T
   a. 1X TBS, 0.1% Tween 20

6. Developing solution

7. Fixative solution

8. Distilled water
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Procedure

Samples were separated on different percentage of separating gel in SDS-PAGE gel for the detection of proteins with 5% stacking gel. After SDS-PAGE, the gel was equilibrated in blotting buffer for 20 minutes at room temperature. While the gel was equilibrating, a piece of PVDF membrane was cut to the same dimension as the gel and it was wet slowly by sliding it at 45° angle into the methanol for 2 minutes followed by transfer buffer was soaked for 20 minutes.

The pieces of What Mann No.1 filter paper, four pads were also soaked in transfer buffer for 20 minutes. One sheet of then, the pads, filter paper, PVDF membrane and gel were assembled in the semi-dry blot apparatus (HoeferSemiphor, semi-dry transfer unit) in the following order. One sheet of mylar mask was spread for electric conductivity followed by the one presoaked pad was placed at the bottom and a glass pipette was rolled over the surface of the pad to remove air bubbles. Then, buffer soaked What Mann No. 1 filter paper was placed followed by the PVDF membrane. Carefully, the equilibrated gel was placed on top of the PVDF membrane. The second Whatman No. 1 filter paper and following it, the second set of pad where placed on top of the gel. (After each step care was taken to remove the bubbles). The transfer cell and electrophoretic power supply unit was assembled and the gel was transferred for 1 hour and 30 minutes at 130 milli amperes and 25V. After transfer, the unit was disconnected, the blotting pads and paper removed, and the PVDF membrane was used for further processing. The transferred proteins were visualized by staining in Ponceau’s solution for 1 minute, and destained with 1X transfer TBS. Membrane blocking and Antibody incubations
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- Incubate membrane in blocking buffer for 1 hour at room temperature.
- Wash 3 times for 5 minutes each with 15mL of TBS/T.
- Incubate membrane with primary for overnight at 4°C.
- Wash 3 times for 5 minutes each with 15mL of TBS/T.
- Incubate membrane with HRP-conjugated secondary antibody for 3 hours at
  room temperature.
- Wash 3 times for 5 minutes each with 15mL of TBS/T.

3.3.13. Native-Polyacrylamide gel electrophoresis (Native-PAGE)

Reagents

1. 30% Acrylamide-Bisacrylamide solution
2. Resolving gel buffer (1.5M Tris-HCl, pH 8.8)
3. Stacking gel buffer (1.0M Tris-HCl, pH 6.8)
4. N,N,N'-Tetramethylethylenediamine (TEMED)
5. 10% Ammonium per sulphate-100mg of APS in 1.0mL of distilled water.
6. Sample Buffer
7. Pre-run buffer (187.5 mM Tris, 1mM EDTA)
8. Running buffer (50mM Tris, 300mM Glycine, 1.8mM EDTA)

Procedure

A constant current was applied to gel prior to the application of the sample in
the presence of Tris (187.5mM) and EDTA (1mM) to remove the oxidants which
might alter the enzyme activity. The proteins were then separated by electrophoresis
in the presence of Tris (50mM), Glycine (300mM), and EDTA (1.8mM) at constant
current 50mA for 3-5 hours. Gel lanes were loaded with equal concentration of
3.3.14. Immunofluorescence staining of liver tissue

Principle

Immunofluorescence is a technique for localization and visualizing an antigen in a section by using an antibody specific for the target proteins. The Immunofluorescence procedure consists of tissue preparation antibody incubation and series of detection reactions. The section is then dewaxed, treated with a target retrieval solution, blocked with a protein based blocking solution and then incubated with a primary and corresponding secondary antibody FITC/TRITC conjugates.

Reagents

1. Absolute ethanol

2. Phosphate buffered saline (1X PBS) (100mL, pH 7.4):
   i. NaCl - 0.8g
   ii. KCl - 0.02g
   iii. Na$_2$HPO$_4$ - 0.115g
   iv. KH$_2$PO$_4$ - 0.02g

3. They were dissolved in a low volume of double distilled water, adjusted the pH to 7.4 and made up to 100mL with double distilled water.

4. Xylene (clearing agent)

5. 30% Hydrogen peroxide (H$_2$O$_2$)

6. 3% BSA

7. Primary antibodies and corresponding secondary antibodies FITC/TRITC conjugates were diluted in 1X PBS containing 1% BSA.
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Procedure

4μm paraffin embedded liver sections were deparaffinized in two changes of xylene for 10-15 minutes. The slides were then rehydrated in a series of graded ethanol solution (100%, 90%, 70%, 50% and 30%) for 10 minutes and then brought to distilled water and then to PBS for 10 minutes. The tissue sections were incubated in blocking buffer (3% BSA solution in PBS) and were kept inside the moisture chamber for one hour to block non-specific endogenous peroxidase activity. The slides were treated with primary antibodies at a range of 1:200 dilutions for 16-18 hours at 4°C. After overnight incubation of primary antibodies, the slides were washed with PBS twice for 5 minutes and then incubated with their corresponding secondary antibodies (FITC/TRITC conjugated) (1:400 dilution) at room temperature followed by washing twice with PBS for 5 minutes. Finally the slides were viewed and photographed in an Axioskope 2d, Carl Zeiss Microscope (Germany).

3.3.15. Assessment of caspase-3 and caspase-9 by ELISA

Protein concentrations of caspase-3 and caspase-9 in homogenized liver tissue were determined using commercial rat caspase-3 ELISA kit (Uscn Life Science E90626Ra, China) and rat caspase-9 ELISA kit (Uscn Life Science SEA627Ra, China) were applied utilizing 96-well plates precoated with monoclonal antibody specific to caspase-3 and caspase-9 for the evaluation of the levels of caspase-3 and caspase-9 respectively, in the serum aliquots collected from all rats. All samples were analyzed in triplicate. Standard curves were generated according to the manufacturer’s instructions. The caspase-3 could be measured in the range of 1.563 to 100 ng/mL; the sensitivity of the assay was 0.55ng/mL. The caspase-9
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could be measured in the range of 1.563 to 100ng/mL; the sensitivity of the assay
was 0.69pg/mL.

3.3.16. Isolation of DNA

DNA was isolated according to the method of Higuchi and Linn, (1995).

Reagents

1. TE buffer (pH 7.4)
2. 10% SDS
3. Proteinase K
4. Saturated Phenol
5. Phenol/chloroform (1:1)
6. 3 M sodium acetate
7. 99.5% ethanol

Procedure

The liver homogenate was suspended in 3mL of TE buffer. 330µL of 10%
SDS and 400µL of Proteinase K (10mg/mL) were added to the above and incubated
at 50°C for 3 hours. After incubation, the digest was extracted with 3.0mL of phenol
saturated with TE buffer by shaking gently for 30 minutes. The extraction was
repeated twice and once with 3.0mL of phenol/chloroform (1:1). The DNA in the
aqueous phase was precipitated by the addition of 370µL of 3M sodium acetate and
8.0mL of 99.5% ethanol followed by incubation for 30 minutes at -80°C. The
precipitate was collected by centrifugation, washed with 3mL of 70% ethanol, dried
under a stream of nitrogen and resuspended in TE buffer.
3.3.16.1. Agarose gel electrophoresis (Analysis of DNA fragmentation)

Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation according the method of Herzig, et al., 2002).

Reagents

1. TE buffer, pH 8.0
2. 10X TBE buffer, pH 8.3
3. Ethidium bromide (10mg/mL)
4. 6X Gel loading buffer (0.25% bromophenol blue, 40% sucrose solution in Water)

Procedure

1% agarose gel was prepared in 0.5X TBE buffers (pH 8). DNA samples were loaded into the wells. The gel was run in TBE buffer and was stained with the solution containing 0.5μg/mL ethidium bromide. The gel was then visualized under UV-Transilluminator and documented.

3.3.16.2. In situ assays for apoptosis

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay kit was used to detect nicked or fragmented DNA in cryostat sections of the liver. TUNEL assays were performed using [FITC] dUTP and terminal deoxynucleotidyl transferase. To detect nuclear conformation sections were further stained with propidium iodide (0.5μg/mL) for 2 minutes at room temperature. The slides were rinsed thoroughly in PBS, cover-slipped and examined by fluorescence microscopy [Roy, et al., 2010]

3.3.17. Transmission electron microscope (TEM)

The changes in the ultra structure of the liver tissues were studied by TEM according to the method of Mosses and Claycomb, (1982).
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Reagents

1. Osmium tetroxide: 1% osmium tetroxide in 0.1M sodium phosphate buffer, pH -
    7.4.
2. Acetone
3. Uranyl acetate: 1%
4. Lead citrate: 3%

Procedure

The liver samples were fixed in Karnovsky’s fixative immediately after
euthanization of rats for 6-8 hours at 4°C. These were post-fixed in 1% osmium
tetroxide in 0.1M phosphate buffer for 2 hours at 4°C, dehydrated in ascending
grades of acetone, infiltrated and embedded in araldite CY212 and polymerized at
60°C for 72 hours. Thin (60-70nm) sections were cut with an ultra-microtome. The
sections were mounted on copper grids and stained with uranyl acetate and lead
citrate and observed under a transmission electron microscope.

3.3.18. Histopathological staining studies (HE staining)

Procedure

A portion of the liver tissue after dissection was fixed in 10% buffered
formalin solution for histological studies. After fixation, 4μm of these tissues were
cut and stained with Hematoxylin and Eosin stains.

1. The slides were dewaxed using xylene and then treated with absolute 90%
   and 70% grades of alcohol and then dipped in water.
2. Stained with Mayer’s haematoxylin for 30 minutes.
3. Stained slides are washed well with water until the sections colour
   changed to blue.
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4. Differentiated in 0.1% HCl for 50 seconds.

5. Again washed well in tap water until the sections colour changed to blue.

6. Then sections were stained with 1% eosin for 1 minute.

7. Washed well in running water for 4 -5 minutes.

8. Dehydrated in ascending grades of alcohol, cleared in xylene and sections
   were mounted using dibutylphthalate polystyrene xylene (DPX).

9. The sections were then viewed under a light microscope for
   histopathological changes in the liver.

3.3.19. Statistical analysis

Statistical analysis was performed by one-way analysis of variance
(ANOVA) followed by Duncan's Multiple Range Test (DMRT) using Statistical
Package for the Social Sciences (SPSS) software package version 16.0. Results were
expressed as mean ± S.D for six rats in each group. p values < 0.05 were considered
as significant (Duncan, 1957).