Chapter IV

Material & Method

4.1 MATERIALS

4.1.1. Chemicals and Reagents

Precoated silica gel aluminium plate 60 GF254 (20 × 20 cm; E. Merck, Germany), Silica gel 60 (Merck, Germany), Methanol, Dichloromethane, Ethyl acetate, Formic acid, Acetonitrile, Hexane & Formaldehyde (Merck chemicals, India). The HPLC solvents used were of chromatographic grade. All chemicals and reagents used were of analytical grade except ethanol (which was of commercial grade obtained from Excise department, New Delhi, India). FCA (Sigma aldrich), Tripr RNA regent, M-MulV Reverse Transcripts and SYBR Premix Ex Tag (Sigma Aldrich). Cell culture material was obtained from Himedia (Mumbai, India). Fetal bovine serum (FBS) was purchased from biological industries, Israel. MTT reagent was purchased from sigma Aldrich (St. Louis, MO, USA). All water soluble compounds were dissolved in DMEM for in vitro studies and in phosphate buffer saline (PBS) for in vivo studies, whereas insoluble chemicals were dissolved into Dimethyl sulfoxide (DMSO) Sigma (MO, USA); the final concentration of DMSO in each sample was less than 0.1%. All solutions were passed through a 0.22 µM filter (GVMP 01230, Millipore) and stored at 4°C until used. All other chemicals used were of analytical grade.

Cell culture accessories

1. Cell culture flask (25 cm²), Tarsons products Pvt. Ltd., Kolkata, India.
2. Centrifuge tube (15 ml), Tarsons products Pvt. Ltd., Kolkata, India.
3. Medium bottle (250 ml), Schott, Germany.
4. 96 well plate (flat bottom), Nunclon, Nunc, Roskilde, Denmark.
5. 6 well plate (flat bottom), Greiner, Gloucestershire, U.K.
6. Tissue culture dish (35 mm X 10 mm), Costar, Cambridge, MA, USA.
7. Microcentrifuge tube (1.5 ml), Axygen, California, USA.
8. Autoclavable pipet tip (0.1 µl, 200 µl, 1000 µl), Axygen, California, USA.

4.1.2. Drugs

Standard Curcuminoids was provided by Natural remedies, Bangalore, India. Diclofenac sodium was a generous gift from Ranbaxy Labs, India. 6-OHDA was purchased from Sigma-Aldrich Chemicals Co. Pvt. Ltd, India.
4.1.3. Cell lines and cell culture

MCF-7 cells

The human breast adenocarcinoma MCF 7 cells were used for in vitro study. The cells were kindly provided by Dr. Manu Jaggi, Dabur Research Foundation, Ghaziabad (UP), India and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 4.5 g/l glucose, 0.01 mg/ml bovine insulin, 100 U/ml of penicillin, and 100 µg/ml streptomycin in a humidified atmosphere with 5 % CO₂ at 37°C.

4.1.4. ELISA and enzymatic kits

The following kits were used according to the manufactures recommendations.

ELISA kits

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Catalogue No</th>
<th>Kit</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α estimation</td>
<td>KRC3012</td>
<td>TNF-α Rat ELISA Kit</td>
<td>Invitrogen, Whitefield, Bangalore</td>
</tr>
<tr>
<td>IL-1β estimation</td>
<td>KRC0012</td>
<td>IL-1β Rat ELISA Kit</td>
<td>Invitrogen, Whitefield, Bangalore</td>
</tr>
<tr>
<td>IL-6 estimation</td>
<td>KRC0062</td>
<td>IL-6 Rat ELISA Kit</td>
<td>Invitrogen, Whitefield, Bangalore</td>
</tr>
<tr>
<td>GSH estimation</td>
<td>703002</td>
<td>Glutathione (GSH) Assay kit</td>
<td>Cayman Chemical Company, MI, USA</td>
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<tr>
<td>SOD estimation</td>
<td>70600</td>
<td>Superoxide dismutase (SOD) Assay kit</td>
<td>Cayman Chemical Company, MI, USA</td>
</tr>
<tr>
<td>MDA estimation</td>
<td>10009055</td>
<td>TBARS Assay kit</td>
<td>Cayman Chemical Company, MI, USA</td>
</tr>
<tr>
<td>GPx estimation</td>
<td>7032102</td>
<td>Glutathione Peroxidase</td>
<td>Cayman Chemical Company, MI, USA</td>
</tr>
<tr>
<td>GR estimation</td>
<td>703202</td>
<td>Glutathione Reductase</td>
<td>Cayman Chemical Company, MI, USA</td>
</tr>
</tbody>
</table>
4.1.5 Equipment’s:
1. Camera fitted inverted microscope, Nikon Eclipse TS 100 binocular model for image documentation, Nikon, Japan.
2. Carbon dioxide incubator, Shel lab model 3517 laboratory – vertical incubator with digital CO₂ control and water jacketed chamber, Sheldon Manufacturing, Oregon, USA.
3. ELISA microtiter plate reader, Bio- Rad 680 model absorbs over range of 400 nm to 750 nm using high quality interference filters and controlled by Microplate manager Basic software, Bio- Rad laboratories, India.
4. Electronic balance, Axid AGN204PR (pan: 90mm) capable of weighing accurately 0.1 mg to 200 g, Axis, ul. Kartuska, Poland.
5. Spectrophotometer, Beckman DU-640 UV/VIS scanning in the wavelength range 190 nm- 1100nm with single cell holder for 10 mm path length cuvettes controlled by Full Spectrum Quantitation (FSQ) software, USA.
6. Refrigerated centrifuge, Plastocraft, Andheri, Mumbai, India.
7. Membrane filter, Millipore 0.22 µM, GV, Millipore, Bedford, MA 01730, USA.
8. Colorimeter, Yorco, Daryaganj, New Delhi, India.
9. Deepfreezer (-80C), Colton, Delhi, India Caltron, Mayapuri, New Delhi, India.
10. Digital weighing balance – AND- McCandless Drive – Milpitas, USA.
11. Electronic weighing balance, Citizen- Bunker Lake Blvd, USA.
12. pH meter– thermo electronic corporation, India.
13. Autoclave, Colton, Delhi, India.
14. Micropipettes, Eppendorf Research, Germany.
16. Digital plethysmometer, Medicaid systems, India.
17. HPLC, Waters, USA.
18. HPTLC, CAMAG, USA.
19. Stereotaxic apparatus, Inco, Ambala, India.
20. Rota Rod, Inco, Ambala, India.
21. Actophotometer, Inco, Ambala, India.
4.2 METHODS

4.2.1 Isolation, identification & standardization of curcuminoids (CUR, DMC, BDMC)

4.2.1.1 Collection and authentication of crude drug:

Curcuma longa (Zingeberaceae) rhizomes were collected from local market of Delhi, India and were identified at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India.

4.2.1.2 Standardization of crude drug

Proximate analysis

Rhizomes of Curcuma longa was subjected to quantitative physicochemical tests (proximate analysis) for determination of moisture content, total ash value, acid insoluble ash value, alcohol soluble extractive value, water soluble extractive value and foreign matter.

Moisture content (API, 1998)

Moisture content was determined using Infrared moisture balance Model-M-3A Deluxe Voltag-230VAC (Advance Research Instrument Co).

4.2.1.3 Ash values (API, 1998)

I) Total ash value

Principle

The method for determination of total ash is designed to measure the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plant tissue itself, and “non physiological ash”, which is residue of extraneous matter (e.g. sand and soil) adhering to the plant surface. When vegetable drugs are incinerated, they leave an inorganic ash in some plants the total ash is of importance and indicates the extent of care taken in the preparation of the drug. Carbon must be removed at as low a temperature (450 °C) as possible because alkali chlorides, which may be volatile at high temperatures, would otherwise be lost. The total ash usually contains carbonates, phosphates, silicates and silica.
**Procedure**

2g of the ground air-dried sample was weighed into previously ignited, dried and tarred silica crucible. The material was spread evenly as a thin layer. Kept on a gas burner under a low flame and ignited slowly to obtain a carbonized residue. It was then placed in the muffle furnace and the temperature of the muffle was adjusted to 450-500 °C and heated for 3 hours, cooled in a dessicator and weighed. The ash value was calculated and expressed as % Ash.

**II) Acid insoluble ash**

**Principle**

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Total ash treated with dilute hydrochloric acid reacts with minerals to form soluble salts and the residue which consists mainly of silica is the acid insoluble ash.

**Procedure**

To the Silica crucible containing the total ash obtained 25 ml of hydrochloric acid (~70g/l) TS was added, covered with a watch glass and boiled gently for 5 minutes on a hot plate. The watch glass was rinsed with 5 ml of hot water and these washings added to the crucible and filtered. The insoluble matter was collected on an ashless filter paper by filtration. The filter paper was rinsed repeatedly with hot water until the filtrate was neutral /free from acid. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500 °C. The silica crucible was removed from the muffle furnace and allowed to cool in a dessicator for 30 minutes, and then weighed without delay. The content of acid insoluble ash was calculated.

**4.2.1.4. Extractive values** (API, 2004)

**Principle**

The determination of water and alcohol soluble extractive value is used as a means of evaluating the quality and purity of drugs whose constituents cannot be readily estimated by
other means. Extraction of the drug can be done by maceration with cold solvent or by a continuous extraction process as in a Soxhlet extractor.

**Procedure**

**Alcohol soluble & water soluble extractive value**

5 g each of the air dried drug, coarsely powdered, was macerated with 100 ml of 90 % ethanol or water (unless otherwise specified in individual monograph) in a glass stoppered flask for 24 hours, the contents were shaken frequently during the first 6 hours and allowed to stand for 18 hours. There after it was filtered rapidly, taking precautions against loss of ethanol. 25 ml of the filtrate was evaporated to dryness on a water bath in a tarred flat bottomed petri plate, dried at 105 °C for 1 hour in a hot air oven, and weighed. The percentage of ethanol-soluble extractive was calculated with reference to the air-dried drug.

**4.2.1.5 Determination of foreign matter** (API, 1998)

**Principle**

Foreign matter is material consisting of parts of the medicinal plant materials other than those named with the limits specified for the plant material concerned; any organism, part or product of an organism, other than that named in the specification and description of the plant material concerned; mineral admixtures not adhering to the medicinal plant materials, such as soil, stones, sand and dust.

**Procedure**

A sample of plant material was weighed about 5 g. It was spread in a thin layer and the foreign matter was sorted into groups either by visual inspection, or with the help of a suitable sieve. The remainder of the sample was sifted through a No. 250 sieve; dust was regarded as mineral admixture. The sorted foreign matter was weighed and expressed as % foreign matter.

**4.2.1.6. Comparative chemical analysis**

Total alkaloids, starch content and total polyphenols in the three samples were quantitatively analyzed.
Determination of total alkaloidal content (API, 2004)

Principle
Alkaloids are weak bases. Alkaloids in the plant material are extracted based on their solubility. Alkaloidal bases are soluble in non-polar solvents like chloroform, ether and their salts are soluble in water. The total alkaloidal content was determined by the procedure described by Anonyms, BIS standards.

Procedure
20 g of coarse powdered drug was moistened with chloroform and ethanol (30:70), mixed by means of a stirring rod, and allowed to stand for 5 minutes. The mixture was rendered alkaline with 5 % ammonia, and mixed. The stirring rod was rinsed with small portion of the solvent, the drug was allowed to macerate for 6 to 12 hours. The drug was then packed into a thimble, covered with a plug of purified cotton, sufficient quantity of solvent was added, and the drug was extracted until extraction was complete. So obtained extract was filtered. The filtrate was concentrated and again extracted with 2 % sulphuric acid twice. The combined aqueous extract was further extracted with chloroform using a separating funnel. The acid solution was rendered alkaline with 10 % ammonia. The alkaline solution was extracted with chloroform (2-3 times). The completeness of extraction was determined, using Meyer’s reagent. The chloroform layer was evaporated and dried at 105 °C to constant weight. The trace of chloroform solvent was removed by the addition of few ml of neutralised alcohol, followed by evaporation at low temperature.

Estimation of starch (API, 2004)
Starch is the storage form of carbohydrate in plants abundantly in roots, tubers, stems, fruits and cereals. It is determined by hydrolysis into simple sugars using dilute acids and the quantity of simple sugars is measured by a colorimetric method. The starch content was determined by the procedure described by API, 1998.

Extraction procedure
1 g of the powdered tuber roots and rhizomes was homogenized with hot 80 % ethanol to remove sugars. Centrifuged, the residue was washed repeatedly with hot ethanol till the
washings do not give colour with anthrone reagent. To the residue 5 ml of water was added followed by digestion with 6.5 ml of 52% Perchloric acid at 0 °C for 20 min for digestion of starch in the residue. The mixture was centrifuged, the supernatant solution was made up to 100 ml. 0.1 ml of the supernatant was pipetted out and made to 1 ml with water.

**Procedure**

A standard curve was prepared by taking 0.2, 0.4, 0.6, 0.8 and 1.0 ml of 100 µg/ml aqueous glucose solution, made up to 1 ml in each tube with water. 4 ml of anthrone reagent was added to the tube. The contents were heated for 8 min in a boiling water bath and cooled rapidly. To 1 ml of the sugar free extract prepared above, 4 ml anthrone reagent was added and treated similar to the standard solution. A blank, treated similarly was also maintained. The intensity of green colour was measured at 630 nm using UV-Visible spectrometer. The sugar content in each of the extracts was obtained from standard graph as glucose equivalents. The value obtained was multiplied by 0.9 to convert it to starch content.

**Determination of total polyphenol content** (Schanderi, 1970; Sadasiyam 1998)

**Principle**

In the presence of sodium carbonate phenolic compounds react with the Folin-Denis reagent and forms blue color which is measured at 700 nm. The total Polyphenol content was determined by the procedure described by Schanderi et al, 1970.

**Procedure**

0.5 g of the powdered material was weighed and was transferred to round bottom flask. 75 ml of water was added to it. The solution was boiled gently for 30 minutes. The solution was centrifuged at 200 rpm for 20 min and supernatant was collected in 100 ml volumetric flask and volume was made up to 100 ml with distilled water. 1 ml of sample extract was transferred to 100 ml volumetric flask containing 75 ml of distilled water. 5 ml of Folin-Denis and 10 ml of sodium carbonate solution was added, volume was made up to 100 ml with distilled water. The solution was shaken well and after 30 min the absorbance was measured at 700 nm. The blank was prepared without sample. The standard graph was
prepared using 0-100 µg tannic acid. Total Polyphenol content of the sample was calculated as tannic acid equivalents from the standard graph and expressed as %.

4.5. Qualitative chemical tests of extracts (Khandelwal, 2011)
The chloroform and methanolic extracts showing presence of chemical constituents in TLC plates were screened for phytochemical constituents using qualitative chemical tests and results were noted.

(1) Detection of alkaloids
a) Mayer’s test
The extract was treated with a few drops of Mayer’s reagent. Formation of creamy white precipitate indicated the presence of alkaloids.

b) Dragendorff’s test
Extract was treated with a few drops of Dragendorff’s reagent. Formation of red precipitate indicated the presence of alkaloids.

c) Wagner’s test
Extracts were treated with Wagner’s reagent. Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

d) Hager’s test
Extracts were treated with Hager’s reagent. Formation of yellow coloured precipitate indicates the presence of alkaloids.

(2) Detection of carbohydrates
Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch’s test
Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicated the presence of Carbohydrates.

b) Benedict’s test
Filtrates were treated with Benedict’s reagent and heated on water bath. Formation of orange red precipitate indicated the presence of reducing sugars.

c) **Fehling’s test**

Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling’s A & B solutions. Formation of red precipitate indicated the presence of reducing sugars.

(3) **Detection of glycosides**

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

a) **Modified borntrager’s test**

Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicated the presence of anthranol glycosides.

b) **Legal’s test**

Chloroform was added to hydrolyzed extract. The chloroform layer was separated and extracts treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicated the presence of cardiac glycosides.

(4) **Detection of saponins**

a) **Foam test**

Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes it indicates the presence of saponins.

b) **Froth test**

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

(5) **Detection of phytosterols**

**Libermann burchard’s test**

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. sulphuric acid was added carefully along the
sides of the test tube. Formation of brown ring at the junction indicated the presence of phytosterols.

(6) Detection of fixed oils & fats

Stain test
Small quantities of extracts were pressed between folds of filter papers. An oily stain on filter paper indicated the presence of fixed oil.

(7) Detection of flavonoids

Lead acetate test
Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoid.

(8) Detection of proteins

a) Ninhydrin test
To the extract, 0.25 % ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicated the presence of amino acid.

b) Biuret test
The extracts were treated with 1 ml of 10 % sodium hydroxide solution and heated. To this a drop of 0.7 % copper sulphate solution was added. Formation of purplish violet colour indicated the presence of proteins.

(8) Detection of tannins

a) Lead acetate test
Extracts were treated with few drops of lead acetate solution. Formation of white precipitate indicates presence of tannins.

b) Gelatin test
To the extract 1 % gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.
4.2.3. Extraction and Isolation of Individual Curcuminoids

Dried powder of *C. longa* (10 kg) was extracted with 95% ethanol (15 L.) using a soxhlet apparatus for 56 hours. The ethanol extract was dried using a rotary evaporator (Heidolph, Heizbad HB Contr, Germany) and yielded crude ethanol extract. The dried ethanolic extract was further fractionated by silica gel 60 column chromatography (75 x 3 inch) in increasing polarity (Pothitirat & Gritsanapan, 2005). Eluted Curcumin, DMC and BDMC were identified by TLC in the mobile phase chloroform: methanol (48:2); Rf value were noted and compared respectively.

Isolated curcuminoids were standardized and characterized using HPTLC, HPLC, IR, Mass and NMR spectroscopy. These isolated curcuminoids were further used in all experiments.

4.2.2.1. High Performance Thin-Layer Chromatography (HPTLC)

20 μl of each methanolic sample solution were spotted as a band width of 6.0 mm on a pre-activated precoated silica gel aluminium plate 60 GF254 using a Camag Linomat V syringe (application rate, 150 nl/s; space between each band, 16.0 mm; slit dimension, 5.00 mm × 0.45 mm; scanning speed, 20 mm/s) and mobile phase; chloroform: methanol (48:2, v/v). Development was carried out in 10 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) presaturated with the mobile phase. The length of each chromatogram run was 8 cm. Densitometric scanning (Camag TLC Scanner 3) was done at lmax 425 nm, operated by WINCATS software (V 1.2.6, Camag). All spots showed fluorescence under UV light (Paramasivam *et al* 2009).

**Preparation of standard solutions**

Stock solutions of individual curcuminoid standard were separately prepared in methanol at 1.0 mg/ml. One milliliter of the stock solution was transferred to a 10 ml volumetric flask and adjusted to volume with methanol. Calibration curves of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were derived from separately applying five concentrations of each curcuminoid on the TLC plate to obtain final concentration ranges of 100-1,200, 150-800 and 200-1,000 ng/spot, respectively.
**Preparation of sample solutions**

For sample preparation, ten milligrams of each extract was transferred to a 10 ml volumetric flask. The sample was dissolved in methanol and adjusted to a concentration of 1.0 mg/ml. A volume of each sample solution was applied in triplicate on TLC plate and analyzed by the proposed method. Each curcuminoid content was calculated using its calibration curve. The contents of curcumin, demethoxycurcumin and bisdemethoxycurcumin were expressed as gram per 100 grams of the extract. After comparison of various factors involving in the process, i.e. yield of crude extract, extraction time consumed, extraction costs, curcuminoids content, amount of solvent used, the appropriate method will be concluded.

**Preparation of standard and sample solutions:**

Curcuminoids samples (0.1 g each) were transferred into 100 ml volumetric flask and filtered through 0.45 μm, 13 mm HVLP membranes (Millipore Corp., Milford, MA, USA) with volume made upto 100 ml with methanol. From this, 2 ml filtered solution was transferred into 10 ml volumetric flask and diluted up to 10 ml volume with methanol. The final concentration injected was 200 ng/ml14.

**Calculation of % purity:**

The purity of isolated curcuminoids was determined by HPTLC (Kamble et al 2011). The percentage purity (calculated from peak area) was determined using following formula:

\[
\text{% Purity} = \frac{\text{AUCIsolated} \times \text{CStandard} \times 500}{\text{AUCStandard} \times 1000}
\]

Where CStandard: concentration of standard (200 ng/ml), 500: dilution coefficient and 1000: conversion of μg into mg.

**4.2.2.2. High performance liquid chromatography (HPLC)**

The preparation of standard and sample solutions was done as described in HPTLC.
Quantification of curcuminoids was done by Waters HPLC (Milford, Massachusetts, USA), using a photo diode array detector at 425 nm. A spherical ODS Luna 5u C18 (2) 100A column (5 µm, 250 X 4.6 mm, Phenomenex, USA) with an Alltima C18 guard column (Particle size 5 µm, 7.5 mm × 2.1 mm) (Grace, Columbia, MD, USA) and a acetonitrile:1% aqueous formic acid (v/v) mobile phase with a flow rate of 1 ml/min at 35±2°C were used. The percentage purity was calculated using the area under the curve for each curcuminoids (Li et al., 2011).

**Preparation of the standard solution**
Reference standards of curcumin, demethoxycurcumin and bisdemethoxycurcumin were separately weighed (5.00 mg of each) and transferred to a 5 ml volumetric flask. Methanol was added and the mixture was adjusted to a final concentration of 1 mg/ml. From this solution, the concentrations of each curcuminoids (0.24 – 250 µg/ml) were prepared and used for preparation of the calibration curves.

**Preparation of the sample solution**
The extract (10.00 mg) was transferred to a 10 ml volumetric flask. The sample was dissolved in methanol and adjusted to a concentration of 1.0 mg/ml. Aliquot of this solution was diluted with methanol to make the final concentration of 20 µg/ml. The sample volume was 10 µl. Each curcuminoid content was calculated using its calibration curve with regard to the dilution factor. The content of curcumin, demethoxycurcumin and bisdemethoxycurcumin was expressed as gram per 100 grams of extract.

**4.2.2.3. Fourier transform infrared spectroscopy (FT-IR)**
Curcuminoid (0.1 mg each) was mixed with 100 mg KBr and pressed to form a pellet which was analyzed on an IR Spectrophotometer (Perkin- Elmer 180 Spectrometer: Waltham, Massachusetts, USA) (Naama et al 2010)

**4.2.2.4. Nuclear magnetic resonance spectroscopy (NMR)**
Spectra of ¹H and ¹³C were acquired at a central frequency of 300.1318534 MHz and
75.4677490 MHz, respectively, in DMSO, using a Bruker Avance 500 MHz NMR spectrometer equipped with a TCI cryoprobe (Bruker BioSpin Limited, Coventry, UK) (Peret et al 2005)

4.2.2.5. MASS spectroscopy
All MS spectra were acquired in positive ion reflector mode on AB Sciex MALDI-TOF/TOF™ 5800 Mass Spectrometer (California, USA) using Explorer software, version 4.0.0. After drying sample at room temperature, MS data was acquired with a 1 kHz laser. Typically, 2040 shot was accumulated for spectra in MS mode (Park et al 2005).

4.2.3 In vivo studies
4.2.3.1 Anti-arthritic potential of Curcuminoids
4.2.3.1.1 Animals
Male albino wistar rats (200-250 g), procured from the animal house of Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi, were used. Rats were housed in temperature controlled room; 25 ± 2 °C with a 12-hour light/dark cycle and 57 ± 7 % relative humidity under standard hygienic conditions and had free access to fresh tap water & pelleted diet (Amrut rat feed, India). The study was approved by the Institutional Animal Ethics Committee (IAEC/DIPSAR/2008-I/18, 19) of DIPSAR, New Delhi. Animals were divided into seven groups (n=6); Group 1 (Control) animals were administered with saline and served as normal control with no immunization.; Group 2 (AA) animals served as arthritic control; Group 3 (AA + STD) animals were served diclofenac sodium (Standard drug) for 28 days starting from arthritic induction; Group 4,5,6 & 7 (AA + CUR, AA + DMC, AA + BDMC & AA + CM) animals were administered with CUR, DMC, BDMC & Curcuminoids mixture (60 mg/kg/po) starting from day of immunization. The selected dose levels were based on a pilot study (3-100 mg/ kg/per oral) on CUR, DMC and BDMC conducted at the Indian System of Medicine Laboratory, DIPSAR, University of Delhi, Delhi, India. Diclofenac sodium was dissolved in water and Curcuminoids was triturated with CMC (carboxymethyl cellulose), and was administered as a suspension, prepared freshly before administration. Animal body weight changes were observed on weekly basis.
4.2.3.1.2 Induction and evaluation of arthritis
Rat AA was induced as previously described (Butler SH et al., 1992). Briefly, rats were injected intra-articularly with 0.1 ml of FCA into the tibio-tarsal joint of the left hind paw containing heat killed *Mycobacterium tuberculosis* in paraffin oil (10 mg/ml). The paw volume of all the animal groups was measured by digital plethysmometer (Medicaid systems, India) at 0, 7, 14, 21 and 28 days after the FCA administration.

4.2.3.1.3. Biochemical assays
Animals were sacrificed at the end of experiments and blood was collected for plasma/serum separation. Collected samples were subjected to biochemical estimations namely total protein, albumin, globulin, fibrinogen & ceruloplasmin. Hematological parameters like Hb, RBC, WBC, & ESR were determined by usual standardized laboratory method.

4.2.3.1.4. Measurement of related cytokine concentrations in serum
The concentrations of TNF-α, IL-1β, IL-6 and IL-10 in serum were measured by ELISA according to the manufacturer's instructions.

**Tumor necrosis factor alpha (TNF-α) estimation**
The Invitrogen Rat TNF-α kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Rat TNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Rat TNF-α content, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the Rat TNF-α antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Rat TNF-α is added. During the second incubation, this antibody binds to the immobilized Rat TNF-α captured during the first incubation. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Rat TNF-α present in
the original specimen. The stop solution changes the colour from blue to yellow, and the intensity of the colour was measured at 450 nm (BioRad, Hercules, CA).

**Interleukin 1-β (IL-1β)**
Interleukin-1 β is an endogenous pyrogen and it has been shown to induce fever and is thought to contribute to wasting of muscles (Jasin & Dingle, 1981). The IL-1β Kit is a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). A monoclonal antibody specific for IL-1β has been coated onto the wells of the microtiter strips provided. Samples, including standards of known IL-1β concentrations and unknowns are pipetted into these wells. During the first incubation, the IL-1β antigen and a biotinylated monoclonal antibody specific for IL-1β are simultaneously incubated. After washing, the enzyme (streptavidin-peroxydase) is added. After incubation and washing to remove the entire unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of IL-1β present in the samples. Absorbance was measured at 450 nm.

**Interleukin-6 (IL-6) estimation**
The Invitrogen Rat IL-6 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Rat IL-6 has been coated onto the wells of the microtiter strips provided. During the first incubation, standards of known Rat IL-6 content, controls, and unknown samples are pipetted into the wells. After washing, biotinylated secondary antibody is added. After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Rat IL-6 present in the original specimen.

**Interleukin-10 (IL-10) estimation**
The Invitrogen Rat IL-10 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Rat IL-10 has been coated onto the wells
of the microtiter strips provided. During the first incubation, standards of known Rat IL-10 content, controls, and unknown samples are pipetted into the wells. After washing, biotinylated secondary antibody is added. After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Rat IL-10 present in the original specimen.

4.2.3.1.5. Real time RT-PCR assay for cytokines relative mRNA expression in synovium tissue

Tissue RNA isolation

Excised synovium tissue was kept at −80 °C until RNA extraction as per earlier described methods [18]. In short, 500 μl Trizol reagent/100 mg tissue weight was added to the weighed tissue. 250 μl chloroform was added to separate total RNA from DNA and proteins followed by precipitation with isopropanol at -20°C for overnight. The obtained precipitate was rinsed in ethanol and was air-dried before resuspending in 75% diethylpyrocarbonate treated water and quantified (A260 nm). One microgram of total RNA was reverse-transcribed to cDNA using iScriptTM cDNA Synthesis Kit (Bio-RAD Laboratories Inc., CA, USA) in a final volume of 20 μl, according to the manufacturer's instruction

Real-time PCR

Primers express software was employed for designing and synthesizing primers of quantitative PCR. TNF-α sense primer was 5’-CTCAGATCATCTCTCAAAA-3’ and 5’-AGGTACAGCCAATCTGCTAA-3’ for antisense primer with 380-bp product. For IL-1β, Sense primer was 5’TTGTGGCTGTGGAGAAGCTG-3’ and the antisense was 5’-GCC GTC TTT CAT ACA CAG G’ with a bp of 377. For IL-10 sense primer was 5’-ACCTGGTAGAAGTGAT-3’ and antisense primer was 5’-GGAGAGGTACCAAACG-3’, a 119bp. The IL-6 sense primer was with sequence: 5’CGAAAGAGAAGCTCTATCTCCCC-3’ and the antisense sequence was 5’CAAAGGATTCAAAACTGCATAGG-3’, a product of 955 bp. The β-actin sense primer was 5’-AGGGAAATCGTGCATGAC-3’, and antisense
β-actin primer was 5’-AACCGCTCATGGCCGATAGT-3’ a 149bp. Real-time quantitative PCR was performed in the iCycler Thermal Cycler (Bio-RAD Laboratories Inc., Hercules, CA, USA) using SYBR green I detection. The following reaction components were prepared to the indicated end-concentrations: 0.6 μM of each primer, 1X IQ SYBR Green BioRad Supermix (Bio-RAD Laboratories Inc.), 150 ng of cDNA and nuclease free double distilled water were added to a final volume of 25 μl. All samples were performed in duplicate for all genes. The real-time PCR protocol followed according to the manufacturer’s instructions with a heated lid (105ºC), an initial denaturation step at 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. The specificity of the amplified PCR products was verified by analysis of the melting curve. The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed signal threshold (Ct). The Ct value correlates inversely with the amount of target mRNA in the sample. The relative changes in TNFα, IL-10, IL-6, and IL-1β with respect to βactin expressions were examined using the ΔΔCt method ΔCt = Cttarget - Ct βactin. As PCR amplification is an exponential process, a ΔCt difference denotes a shift in regulation by a factor of two (2-ΔΔCt). To validate a real-time PCR, standard curves with r2 >0.95 and slope values between 3.1 and 3.4 were required Real-time efficiencies were acquired by amplification of a standardized dilution series and corresponding slopes and PCR efficiencies were calculated using iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories Inc.). At the end of the PCR reaction, all products reached a plateau. To determine whether other non-expected products were also amplified, the PCR products from each IL-6, IL-10, IL-1β, TNF-α or β-actin after 40 cycles were subjected to a subsequent agarose-4% gel electrophoresis with ethidium bromide to confirm amplification specificity.

**4.2.3.1.6. Radiographic and Histopathology analysis**

Radiographs of Adjuvant injected limb of each rat were taken with x-ray instrument (MBR 1505R, Hitachi, Japan) for evaluating joint spaces, bone erosions and soft tissue swelling. For histopathology, adjuvant injected tibio tarsal joints were excised after sacrificing the animals. Specimens were fixed in 10% formalin before embedding in paraffin and sectioned with microtome at a thickness of 5μm. Sections were stained with hematoxlin and eosin and
were observed under light microscope. The inflamed joints were evaluated for synovial congestion, synovial hyperplasia and inflammatory cell infiltration.

4.2.3.2. Neuroprotective potential of Curcuminoids

4.2.3.2.1 Animals

Male albino Wistar rats (200–250 g), procured from the animal house of Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi, were used. Rats were housed in temperature-controlled room: 25 ± 2°C with a 12-hour light/dark cycle and 57 ± 7% relative humidity understand and hygienic conditions and had free access to fresh tap water and pelleted diet (Amrut rat feed, Pranav Agro Ltd., India). The animals were acclimatized for seven days prior to experimental use. The study was approved by the Institutional Animal Ethics Committee (IAEC/DIPSAR/2008-I/18, 19) of DIPSAR, New Delhi, and experiments were carried out in accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

The animals were randomly divided into 5 groups of 8 animals in each group. Group I served as sham control (S) and received 2 μL of 0.1% ascorbic acid in 0.9% NaCl on 22nd day. Group II animals served as lesion (L) and were treated once with 6-OHDA (10 μg/2 μL; 0.1% ascorbic acid- saline) in the striatum. Group III animals (L+C) received pretreatment with CUR (60 mg/kg body weight per oral suspended in 0.5% CMC) for three weeks followed by single injection of 6-OHDA in the striatum on the 22nd day. Group IV animals (L+DMC) received DMC (60 mg/kg/per oral suspended in 0.5% CMC) for three weeks as a pretreatment followed by 6-OHDA administration as in group III. Group V animals (L+BDMC) received BDMC (60 mg/kg/per oral suspended in 0.5% CMC) as pretreatment for three weeks followed by 6-OHDA administration as in group III. The selected dose levels were based on a pilot study (3-100 mg/ kg/per oral) on CUR, DMC and BDMC conducted at the Indian System of Medicine Laboratory, DIPSAR, University of Delhi, Delhi, India.

4.2.3.2.2. Induction of Brain Lesions by Injection of 6-OHDA

The food was withdrawn 10–12 h before the surgical procedure. The rats were anesthetized
with chloral hydrate 350 mg/kg i.p. After anesthesia, the head of the rat was mounted in a stereotaxic apparatus (Inco, Ambala, India) frame. The skin was cut to expose the skull, and the periosteum (shiny membrane overlying the skull) was removed. The stereotaxic coordinates were measured accurately as anteroposterior 0.5 mm, lateral 2.5 mm, and ventral 5.0 mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. All the animals in the experimental group were lesioned in right unilateral region by injecting 10 μg 6-OHDA/2 μL (in 0.1% in ascorbic acid-saline) into the right striatum through the drilled hole, with the help of a very fine Hamilton syringe (28 Gauge, Hamilton Bonaduz AG, Switzerland). After injection, the syringe was kept in place for an additional 5.0 min before being slowly removed, to prevent oozing out of the drug. The sham group was followed with same surgical procedure, but injection of 6-OHDA was replaced with 2.0 μL of 0.1% ascorbic acid-saline. Post-surgery rats were provided with the necessary postoperative care and were monitored for average weight loss and mortality for 10 days.

Figure 4: Depiction of induction of brain lesions by injection of 6-OHDA
4.2.3.2.3. Behavioral Studies
The behavioral studies were started with standard optimal laboratory conditions after 21 days of surgery and were performed by an individual blind to the experiment.

Apopomorphine-Induced Rotational Behavior Study.
On 22nd day after the lesion, apomorphine was injected subcutaneously at a dose of 0.5 mg/kg (in ascorbic acid-saline) to observe neurodegenerative effect of 6-OHDA and protective effect of curcuminoids on contralateral rotations. The rotational scores were collected over a period of 5-minute interval for three times.

Rota Rod Study
Rota rod motor training was performed at the beginning of the experiment till the learning of each rat. The apparatus (Inco, Ambala, India) consists of a metal rod of 4 cm in diameter, 75 cm in length with 6 equally divided sections, and speed adjusted to 8 rotations/minute. Protective effect of CUR, DMC, and BDMC on motor coordination was studied in rats following a standard procedure (Kelly et al., 1998).

Spontaneous Locomotor Study
The spontaneous locomotor activity of each animal was recorded in square arena of actophotometer (Inco, Ambala, India) individually for ten minutes (Boissier JR & Simon P, 1965). Actophotometer registers of the number of times IR photobeams of light were interfered, as the rat moved inside the cage. Each rat was placed in the center of the metal cage of actophotometer and ambulatory activity was measured. The arena was cleaned with dilute alcohol and dried between trials (Pandey et al., 2010) to avoid any experimental interference.

4.2.3.2.4. Neurochemicals Study
Animals were sacrificed and striatal tissue from lesioned sites was carefully dissected in the light of Rat Brain Atlas (Paxinos G & Watson C, 1981) and homogenized in phosphate buffer (10 mM phosphate buffer, pH 7.0, having 10 μL/mL protease arrests (5 mM
leupeptin, 1.5 mM aprotinin, 2 mM phenyl-ethyl-sulfonylfluoride, 3 mM pepstatin A, 10 mM EDTA, 0.1 mM EGTA, 1 mM benzamidine, and 0.04% butylated hydroxyl toluene) and centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The supernatant was divided into two portions: minor portion was used for estimation of MDA, and the major portion of supernatant was recentrifuged to get pellet fraction and supernatant fraction.

4.2.3.2.5. Enzyme Estimations
Various biochemical enzymes, that is, MDA, GSH, GPx, GR, SOD, and CAT were estimated using spectrophotometer to establish the role of free radicals in the study.

Malondialdehyde (MDA)
Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA. In human platelets, thromboxane synthase also catalyzes the conversion of PGH₂ to thromboxane A₂, 12(S)-HHTrE, and MDA in a ratio of 1:1:1.

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation. Modifications of the TBARS assay by many researchers have been used to evaluate several types of samples including human and animal tissues and fluids, drugs, and foods. Even though there remains a controversy cited in literature regarding the specificity of TBARS toward compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation. If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized, and the test becomes quite specific for lipid peroxidation. Lipids with greater unsaturation will yield higher TBARS values. The assay kit provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in plasma, serum, urine, tissue homogenates, and cell lysates.
Chapter IV  

Material & Method

The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm.

**Glutathione Estimation:**
GSH estimation utilizes enzymatic recycling method, using glutathione reductase, for the quantification of GSH. Sulphydryl group of GSH reacts with 5,5’-dithio-bis-2-(nitro benzoic acid, Ellman’s reagent and produces a yellow colored 5-thio-2-nitrobenzoic acid measured at 405nm with 3.6% intra assay coefficient.

**Glutathione Peroxidase Estimation:**
GPx activity was measured, indirectly by a coupled reaction with GR. Oxidized glutathione (GSSG), produced upon reduction of H2O2 by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. The intra assay coefficient of variation was 5.7%.

**Glutathione Reductase (GR):**
Glutathione Reductase activity was estimated by measuring the rate of NADPH oxidation. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm and was directly proportional to the GR activity in the sample. The intra assay coefficient of variation was 9.3%.

**Superoxide dismutase (SOD) estimation**
The microtiter plate provided in this kit has been pre-coated with an antibody specific to SOD. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for SOD and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3′,5,5′ tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain SOD, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ±2 nm. The concentration of SOD in the samples is then determined.
by comparing the O.D. of the samples to the standard curve.

**Catalase Estimation:**
Catalase estimation utilises the peroxidatic function of catalase for determination of enzyme activity. Method is based on the reaction of enzyme with methanol in the presence of an optimal concentration of H₂O₂ and formaldehyde produced was measured by colorimetry with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color, absorbed at 540 nm. The intra assay coefficient of variation was 9.9%.

### 4.2.3.2.6. Dopamine, DOPAC and HVA Estimation
The supernatants were purified using earlier described method (Haikala H, 1987), and samples were assayed for Dopamine, DOPAC, and HVA using HPLC with electrochemical detection as described earlier (Kilpatrik, 1986) using waters HPLC equipped with 2465 electrochemical detector.

### 4.2.3.2.7. Immunohistochemical Studies and Tyrosine Hydroxylase
5.0 μm thick striatal tissue sections were deparaffinized, rehydrated, and then heated in 10 mM citrate buffer (pH 6) containing triton X-100 (Sigma-Aldrich) 0.1% (v/v). After two washes with PBS, slides were then incubated with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity. After washing with PBS, tissues were incubated with blocking serum at room temperature for 1 h. Subsequently, a primary antibody diluted in blocking serum (TH 1: 200 dilution) (Abcam ab611) was added followed by overnight incubation at 4°C in a humidified chamber. Tissue sections were washed in PBS, and incubated with biotinylated antibody, again washed with PBS and incubated with avidin-biotin complex and horseradish peroxides for 30 min. The tissue sections were counterstained with haematoxylin. TH positive cells were counted and photographed with the help of Motic micro- scope (Motic BA310; Causeway Bay, Hong Kong), and digital camera (Moticam 2500). Area of TH positive cell were measured (Mishra & Palanivelu, 2008) with
the help of Digital Microscopy Software (Motic Images plus 2.0) and expressed in percentage when compare to sham group.

4.2.4. In vitro studies

Preparation of cell culture medium

Basic DMEM

13.5 g of Dulbecco’s Modified Eagle Medium (DMEM) w/L- Glutamine, 4.5 g Glucose per litre and sodium pyruvate was suspended in sterile water with constant, gentle stirring until the powder is completely dissolved. 3.7 g of sodium bicarbonate was added and mixed well. The media was filter-sterilized 0.22 µM sterile filter membrane. The sterility of the medium was confirmed by incubating it for 48 h in the incubator at 37° C with 5 % CO₂. The media was kept at 4° C and the stability of the media prepared is two months.

10 % Supplemented DMEM

This medium was prepared using the following materials and mixed well in a beaker: 90.0 ml of sterile basic DMEM medium, 10.0 ml of fetal bovine serum (FBS), 0.1 µg/ml bovine insulin, 100 U/ml penicillin and 100 µg /ml streptomycin. The media was filter-sterilized 0.22 µM sterile filter membrane and kept at 4° C.

Maintenance of cell culture

Human breast adenocarcinoma MCF-7 cells were used to study the anti-proliferative activity of various drugs. The cells (Passages 16-24) were grown under aseptic conditions using DMEM enriched with 10 % heat inactivated FBS, with 4.5 g/l glucose, 0.01 µg/ml bovine insulin, 100 U/ml of penicillin and 100 µg/ml streptomycin in the tissue culture flask at 37° C in 5.0% CO₂ water jacket incubator. Cells were examined daily using the inverted microscope for signs of bacterial or fungal contamination. The supplemented medium in the flask was replaced with fresh DMEM twice a week or when a colour change was observed. Cultured cells were sub cultivated when the cell concentration exceeds 2 X 10⁶ per ml.
Sub cultivation of cells
The medium in the tissue culture flasks was discharged when the MCFR-7 cells attached themselves onto the surface of the flasks. The flasks were rinsed using 5.0 ml of PBS (pH 7.2). then, 1.0 ml of 0.25 % trypsin-EDTA were added into the tissue culture flasks so that the cells would detach themselves from the surface of the flasks. The cells were incubated at 37°C for 1-2 minutes in 5.0% CO_2 incubator or till they rounded up. Cells were centrifuged at 4000 rpm for five minutes with 1.0 ml of 10.0 % supplemented DMEM medium. Supernatant in the eppendorf tube was discarded and the pellet of MCF 7 cells was resuspended in 2.0 ml 10.0% supplemented DMEM medium. Cells were counted using haemocytometer and the medium was adjusted to give a plating density of 1 X 10^6 cells. the flasks were incubated in 5.0 % CO_2 incubator at 37 °C. the doubling rate for MCF-7 cells is ~29 h.

4.2.4.1. Cell proliferation assay
The effect of treatment with curcuminoids (Curcumin, DMC, BDMC & Curcuminoids Mix) upon proliferation in MCF-7 breast cancer cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenil tetrazolium bromide (MTT) assay (Agrawal et al., 2011c).

Principle
The MTT tetrazolium salt assay determines the cytotoxic effect of a test agent against uninjured cells which is reflected by the reduction of formation of blue formazan from the yellow MTT tetrazolium salt by the succinate dehydrogenase in the mitochondria of the cell (Mosman, 1983). This water insoluble formazan can then be solubilized using an organic solvent such as DMSO. The concentration of the converted dye is directly correlated to the number of metabolically active cells in the culture and this can be measured spectrophotometrically.

Procedure
MCF-7 cells were seeded at a density of 1 X 10^4 cells per well (200 µl) in a 96 well cell culture plate containing 10% supplemented growth medium DMEM and incubated for 24 h. the cells were exposed to increasing concentrations of Cur, DMC BDMC & Cur Mix (20 µl
each) in growth medium for 24, 48 and 72h time intervals. Control wells were incubated with the 0.1 % DMSO (20 µl) and demonstrated no cytotoxicity in cell line. After treatment, MTT reagent (Sigma, USA) was added to a final concentration of 0.5 mg/ml and further incubated for 4 h. After 4h incubation period, the supernatant was discarded and DMSO was added to solubilize the water insoluble formazan. The culture plate was then agitated on the microtiter plate reager (Bio- Rad, USA) and the results were read at 490 nm with a micro plate reader. All test sample treatments were conducted in triplicate and the entire experiment was run in triplicate.

**Calculating the cell death**

The drug induced cytotoxicity was calculated by the following formula. The 50 % inhibitory concentration (IC$_{50}$) of drugs which produces 50% killing was calculated.

\[
\% \text{ Killing} = \frac{\text{Average OD of control well} - \text{Average OD of treatment well}}{\text{Average OD of control well}} \times 100
\]

where, OD = Optical density

**4.2.5. Statistical analysis:**

Results were expressed as mean ± SEM. One way ANOVA followed by Bonferroni’s multiple comparison test was used to analyze the effect of different drugs when compared to the control with the help of graph pad prism 5 software, version 4.01, P<0.05 was considered significant.