Chapter-2

LITERATURE SURVEY

Chapter – 2

Chapter – 2. Literature Survey

2.1 Anticancer

Controlled proliferation of living cells is the secret of growth and development in living organisms, when once body loses control over the cell growth becomes a major problem for mankind and termed as *cancer*. It is considered as second major cause of death after myocardial infarction as millions of people die every year despite tremendous amount of efforts and economy is placed for the development of possible cure to it.[121]

Breast cancer and liver cancer are the second and fifth leading cause of death from cancer, in men and women in the United States. In Britain as many as 20% of cases of cancerwere breast cancer, as well as causing the most deathsin women who falls in the age group of 35-55 years. The hospital data indicate that breast cancer ranked first among othercancers in women [121] and Hepatic cancer is the fifth most life threatening type of cancer with the incidence and mortality rates of 554,344 and 536,904 cases globally.[121] In India breast cancer is the second most common type of cancer. A survey carried by ICMR (Indian council of medical research) for a period of 23 years has revealed that the incidences of breast cancers have doubled in last few years. As much as 100000 new cases of breast cancer are being detected every year.[122] Cancer is a condition in which cells have lost normal growth control mechanisms, which results in the unusual, fast and control less growth of cells which is a neoplastic disease derived from the parenchyma or connective tissues.

4.2 million new cancer cases which accounts for about 39% of new cases worldwide, were diagnosed among 3.2 billion persons about 48% of the world's population, living in the developing countries in Asia.[123] 22.9% cases in India, 21.97%cases in USA, 26.0% cases in China, 26.08% cases in European union, 54.41% cases in African region, 25.6% cases in American region were identified as cancer cases. In the year 2008; the incidence of cancer in USA was 182000 and in India was 115000. Cancer cases in India seems to be less in comparison to USA but it is equivalent to 2/3rd of cancer burden of USA.[124] The number of deaths because of cancer in India may climb up to 106124 by 2015 123634 by 2020. The cancer incidence is highest in Christian and parsi community where as lowest in hindu and muslim community.[125]

Various kinds of anticancer treatments are available including surgery, chemotheraphy, radiation theraphy but none is free of unwanted effects like bonemarrow depression, secondary infections, nausea, vomiting, internal bleeding etc. Hence, it has become a need to explore other forms of medicine to derive active ingredients which shall treat cancer with least or no side effects.[126]

2.1.1 In Vitro cell growth assay (Cell development and Viability: Micro culture assay)

2.1.1.1 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Alternatively three assays for cellular growth, development and viability are likely utilized in the new primary screen were extensively employed. Two are metabolic assays; in both the cellular cutback

(reduction) of a colourless tetrazolium salt (MTT) yielded a chromified foramen in relation to viable cell number. The intensity of colour could be measured suitably in a computerized colorimeter. The improvement of the XTT tetrazolium assay was encouraged by the objective to simplify further the MTT procedure by eliminating the solubilization step; the reduction of MTT yielded an unsolvable foramen, which had to be dissolved in dimethylsulfoxide prior to computerise colorimetric estimation. In difference, the MTT reagent is enzymatically degraded by viable cells directly to a water-soluble foramen, allowing the urgent reading of optical density in the culture wells with no further processing. Even though easy and handy, the MTT method shall give comparatively elevated surroundings readings little "signal-to-noise" fraction ratio. MTT as well shared the quality of an unstable (i.e., time critical) end point, down grading the possible exercise of either of the tetrazolium assays in a high-flux anticancer screen employing in a big section of cell lines.[16]

2.1.1.2 SRB (Sulphorhodamine B assays)

The sulforhodamine B (SRB) assay was proposed by Skehan et.al to assess drug-developed cellular toxicity and cell division for momentus drug-screening applications. Its theory is based on the capability of the protein pigment sulforhodamine B to unite electro statically and pH dependent on protein vital amino acid residues of trichloroacetic acid-fixed cells. In gentle acidic environment it binds to and under gentle basic environment it can be detached from the proteins of cells and solubilized for estimation. Results of the SRB assay were linear with cell

figure and cellular protein considered at cellular densities ranging from 1 to 200% of union. Its accuracy is similar with that of quite a few fluorescence assays and higher to that of Lowry or Bradford. The signal-to-noise ratio is approving and the resolution is 1000-2000 cells/well. It is carried out similarly as compared to other cellular toxic assays such as MTT or clonogenic assay. The SRB assay develops a computed colorimetric end point and is non corruptible and forever stable. These matter-of-fact advances make the SRB assay a suitable and responsive assay to measure drug-induced cellular toxicity even at noteworthy application. The observation is made at 488 nm.[17]

2.1.2 Cell lines available:

MCF (Human breast cancer cell line), HepG2 (human liver cancer cell line), T47D (breast cancer cell line), P815 (Murine mastocytoma cell line), Vero (Kidney carcinoma cell line of monkey), BSR (Kidney carcinoma cell line of hamster), ICO 1 (Sheep heart carcinoma), PDA (Pancreatic ductus adenocarcinome), EAT (Ehrlich ascites tumor cell line), 143B (osteosarcoma cell line), 184A1 (Breast carcinoma cell line), 1A2 (Lymphoma cell line), 2.040pRSV-T (Cornea sarcoma cell lines), 20H11 (Spleen carcinoma cell line), 293/CHE-FC (Kidney carcinoma cell line), A549 (lung cancer cell line), HCT15 (Colon cancer cell line), BT549 (Breast cancer cell line) and PC3 (Prostate cancer cell line).

2.2 Antiosteoporosis

2.2.1 Models available for screening of Antiosteoporotic activity

2.2.1.1 Inflammation induced osteoporosis

The inflammation mediated osteoporosis model utilizes the subcutaneous injection of non-specific irritants such as talc or cotton wool, typically on the back of rat at sites distant from the skeleton to stimulate an acute phase response. Granulomatous reactions are noted at the injection sites along with the accumulation of chronic inflammatory cells. In the skeleton, at sites of distant from the inflammatory lesions, loss of trabecular bone volume and significant decrease in osteoblast numbers are observed. Decrease in osteoblast numbers and bone formation is the major features of this model. In growing rats, decrease in osteoprogenitor numbers and bone elongation are observed. Elevated tumor necrosis factor-α (TNF-α) level have been shown to play a cause role in the bone loss and the effects can be neutralised with anti-TNF-α antibodies. TNF-α is known to stimulate NO production. Increased osteoblast apoposis associated with the production of high levels of NO synthesized by the inducible nitric oxide synthase pathway contributes to the pathogenesis.[18]

2.2.1.2 Glucocorticoid induced Bone Resorption

It is caused by administering glucocorticoid medicines such as Prednisone, Prednisolone, Dexamethasone and Cortisone. These medicines have both direct and indirect effects on bone tissue that lead to bone loss. These drugs have a negative effect on bone cells, resulting in reduced rate of forming new bone. They interfere with the body`s

handling of calcium and effect levels of se hormones which can lead to increased bone loss.[19]

2.2.1.3 Induction of bone loss by Ovariectomisation (OVX)

Ovariectomisation is the surgical removal of both the ovaries. There is no single animal model that identically represents the stages of osteoporosis in humans as a whole currently. But there are some that provide a relatively close comparison. Both small and large animals are used depending on the aspects of the osteoporotic condition being studied. Such animals include rats, rabbits, and sheep.

Of these animal models, the ovariectomised (OVX) rat model remains the most popular choice as it has been validated to represent the most important clinical features of estrogen deficiency induced (or postmenopausal) bone loss in the adult human,[20] particularly during the early stages of osteoporosis.[21] They include increased rate of bone turnover with resorption exceeding formation, an initial rapid phase of bone loss followed by a much slower phase, greater loss of cancellous bone than cortical bone; reduced intestinal calcium absorption, some protection against bone loss by obesity and similar skeletal response to therapy with estrogen, tamoxifen, bisphosphonates, parathyroid hormone, calcitonin and exercise.[22]

It also offers certain advantages compared the other animal models like rabbits and sheep. These include the ability to use peripheral micro-computed tomography to perform *in vivo* analyses, cost-effectiveness, and ease of handling and housing. Because of these considerations, the OVX rat model is widely used for the studies relating to the prevention and

treatment of osteoporosis in general as well as studies relating to the healing of osteoporotic fractures.

2.2.1.4 Heparin administration induced osteoporosis

Heparin also known as unfractionated heparin, a highly sulfated glycosaminoglycan, is widely used as an injectable anticoagulant. Long-term treatment with heparin causes osteoporosis through an as not well defined mechanism. A work published in 1996 revealed that heparin treatment produced a dose-dependent decrease in serum alkaline phosphatase and a transient increase in urinary PYD and thus decreases the rate of bone formation and increases the rate of bone resorption.[23] The exact pathomechanism is unclear, but there is a hypothesis which states that the transcription axis of Cbfa-1 and osteocalcin is crucial in maintaining an equilibrium of bone formation and resorption in vivo. At high doses, dalteparin caused a significant inhibition of both osteocalcin and Cbfa-1 expression in vitro.[24]

2.2.1.5 Rosiglitazone induced osteoporosis

Osteoblasts and bone marrow fat cells are derived from a familiar mesenchymal tissue. Increased growth of adipocytes may perhaps take place at the cost of osteoblasts, leading to bone loss. Beginning of the proadipogenic transcript factor peroxisome proliferator-stimulated receptor isoform gama 2 with rosiglitazone deactivated osteoblast differentiation. It shows bone loss related with an increase in bone marrow adipocytes, a reduction in the fraction of osteoblasts to osteoclasts, a decrease in bone configuration pace and a decrease in wall thickness. It decreases the expression of the key osteoblastogenic

transcription factors Runx2 and Osterix in cultures of marrow-derived mesenchymal progenitors. These effects are associated with diversion of bipotential progenitors from the osteoblast to the adipocyte lineage and suppression of the differentiation of monopotential osteoblast progenitors. Hence, rosiglitazone attenuates osteoblast differentiation and thereby reduces bone formation rate *in vivo*, leading to bone loss.[25]

2.3 Urolithiasis

2.3.1 Invitro Models for Anti-Urolithiasis screening:

2.3.1.1 Nucleation assay

The method used was similar to that described by Hennequin *et al.* with some minor modifications.[26] Solutions of calcium chloride and sodium oxalate were prepared at the final concentrations of 3 and 0.5 mmol/l, respectively, in a buffer containing Tris 0.05 mol/l and NaCl 0.15 mol/l at p^H 6.5. 33 µl of calcium chloride solution was mixed with 3.3 ml of extract at different concentrations. Crystallisation was started by adding 33 ml of sodium oxalate solution. The final solution was magnetically stirred at 800 rpm using a Polytetrafluoroethylene (PTFE)-coated stirring bar. The temperature was maintained at 37 ^{o} C. The absorbance of the solution was monitored at 620 nm after every 1 min. The percentage inhibition produced by the herb extract was calculated as Inhibition percentage = $[1 - (Tsi / Tsc)]X\ 100$, where *Tsc* was the turbidity slope of the control and *Tsi* the turbidity slope in the presence of the inhibitor.

2.3.1.2 Growth assay

Inhibitory activity against CaOx crystal growth was measured using the seeded, solution-depletion assay described previously by Nakagawa and colleagues Nakagawa Y, *et al.*[27] Briefly, an aqueous solution of 10 mM Tris-HCl containing 90 mM NaCI was adjusted to p^H 7.2 with 4 N HCl. Stone slurry (1.5 mg/ml) was prepared in 50 mM sodium acetate buffer (p^H 5.7). COM crystal seed was added to a solution containing 1 mM $CaCl_2$ and 1 mM sodium oxalate ($Na_2C_2O_4$). The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed would lead to deposition of CaOx on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometry at wavelength 214 nm. When aqueous extract is added to this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30-second incubation with or without test sample. The relative inhibitory activity was calculated as follows: % relative inhibitory activity = $[(C - S)/C]$X 100, where C is the rate of reduction of free oxalate without any test sample and S is the rate of reduction of free oxalate with a test sample.

2.3.1.3 Cell culture

Normal rat epithelial derived renal tubular epithelial (NRK 52E) cells were maintained as monolayer in DMEM with 2.0 mM l-glutamine adjusted to contain 3.7 G/l sodium bicarbonate and 4.5 G/l glucose. Medium was supplemented with 1% penicillin (100 units/ml),

streptomycin (10,000 µg/ml) and 10% fetal bovine serum. Cells were cultured in 25 cm ^{2} tissue-culture treated flasks at 37 ^{o} C and 5% CO_2 in humidified chambers.[28]

2.3.1.4 Oxalate-induced cell injury

Normal rodent (rat) epithelial derived renal tubular epithelial NRK 52E cells be preserved in DMEM bearing 1mM sodium oxalate solution in the company of different concentrations of the aqueous extract of the test sample (100, 250 and 500 µg/l) for 72 hours.[29,30] Cell damage shall be assessed by means of measuring the cell viability with trypan blue and spotting the leakage of lactate dehydrogenase (LDH) within the test medium.

2.3.1.5 Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase leak assay was performed by the means of Wagner *et al* method.[31] Briefly, 6.6 mM NADH and 30 mM sodium pyruvate were prepared in Tris (0.2 M, p^H 7.3). The reaction was initiated with the addition of 50 µl of the test sample and the disappearance of NADH was monitored at 340 nm for 5 min at an interval of 1 min. The percentage of LDH release was calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication.

2.3.1.6 Kinetic study

The effect of the test material on kinetics of calcium oxalate (CaOx) crystallization was determined by the time course measurement of turbidity changes due to the crystal nucleation and aggregation after

mixing metastable solutions of Calcium (Ca^{++}) and Oxalate (Ox). Stock solutions of $CaCl_2$ (8.5 mM) and $Na_2C_2O_4$ (1.5 mM), containing 200 mM NaCl and 10 mM sodium acetate were adjusted to p^H 5.7.[32] An aggregometer (Chrono-Log Corporation, USA) devised for platelet aggregation studies based on the measurement of optical density at 620 nm was used to investigate the event of CaOx crystallization.[33] The $CaCl_2$ solution (0.5 ml) was stirred constantly both in the absence and presence of different concentrations of the test material or reference drug: potassium citrate at 37°C. After obtaining a stable base line, crystallization was induced by the addition of $Na_2C_2O_4$ solution (0.5 ml) to obtain the final concentration of Ca^{++} as 4.25 and Ox as 0.75 mM. The time course measurement of turbidity was simultaneously started on a chart, moving at the speed of 30 mm/h, and continued for 15 minutes with constant stirring of the solutions. All experiments were run in triplicate. The slopes of nucleation (SN) and aggregation phases (SA) were calculated using linear regression analysis. Using the slopes, the percentage inhibition was calculated as [(1-Sm/Sc) x 100], where Sm is slope in the presence of modifier and Sc is slope of the control experiment.

2.3.1.7 Incubation study

To determine the effect of incubation on CaOx crystal formation, stock solutions of $CaCl_2$ and $Na_2C_2O_4$ similar to those in the kinetic study were used. $CaCl_2$ solutions, containing different concentrations of the test material or potassium citrate, were aliquoted (0.5 ml) to the flat-

7 - bottomed tubes in a 24 well plate. To each of these tubes $Na_2C_2O_4$ solution (0.5 ml) was added.[34] Each concentration of the test material was prepared in triplicate. The plates were then incubated in a shaking water bath at 90 oscillations/ min at a temperature of 37°C for 45 min. Abundance and the morphology of the crystals in each tubes were then observed under inverted microscope.

2.3.2 *In vivo* methods for antiurolithic screening

2.3.2.1 Ethylene glycol model

Ethylene glycol is used to induce stones. The major metabolite of Ethylene glycol is oxalate. This oxalate combines with the calcium and form insoluble complex. Normally 0.75% ethylene glycol (through drinking water) is used for 28 days to induce stones.[35]

2.3.2.2 Ethylene glycol ammonium chloride model

Ethylene glycol is used for 21 days to induce the stones. In the first 5 days of the treatment ammonium chloride is administered parallel with ethylene glycol which acidifies urine thereby enhancing the stone formation

2.3.2.3 Sodium oxalate model

Calcium oxalate nephrolithiasis in rats was induced by intraperitoneal injection of sodium oxalate (7 mg/100 g) daily for 7 days.[36] Sodium oxalate combines with the calcium and form insoluble complex.

2.3.2.4 Glyoxalate induced urolithiasis model

Glyoxalate is injected100mg/kg intra abdominally for 7 days, it forms stones by forming intermediate metabolite oxalic acid which inturn forms calcium oxalate crystals.[37]

2.3.2.5 Ammonium oxalate feed method

Calcium oxalate urolithiasis was induced by administration of gentamycin and calculi producing diet (5%ammonium oxalate) in standard rat pellet feed.

2.4 Antihyperglycemic

2.4.1 Animal models in experimental diabetes mellitus[38]

For the screening of hypoglycemic agents, diabetes can be produced in animals like mouse, rat, guinea pig, cat, dog, hamster, rhesus monkey etc.

Animal models are classified as follows

- Insulin Dependent Diabetes Mellitus (IIDDM) resembling animal models.
- Non-Insulin Dependent Diabetes Mellitus (NIDDM) resembling animal models.
- IDDM resembling animal models.

  IDDM is due to the destruction of β-cells of islets of Langerhans. This can be brought about by viral infection, injection of diabetogens or by introduction of transgenes. Streptozotocin and alloxan were found to

be more selective in β-cells destruction than other diabetogenic agents.

2.4.1.1 Alloxan- induced model

Alloxan is derived by oxidation of uric acid and chemically it is 2, 4, 5, 6 - tetra-oxo-hexa hydropyrimidine. It has the property of inducing diabetes mellitus in experimental animals. In non-fasted animals, the blood glucose level after alloxan injection fluctuates in a triphasic pattern. Following its injection there is a rise in the blood sugar which is followed by a reduction below normal and which in turn is again followed by hyperglycemia. The initial rise in blood sugar is probably due to a direct action of the drug on the liver. The hypoglycemia which follows the initial rise is not due to the liberation of insulin as formerly believed, but is probably an extra-pancreatic effect due to the inability of the liver to produce glucose. The drug causes destruction of the β-cells of the islets of Langerhans and thus gives permanent diabetes which is responsible for the final observed rise in blood sugar. The drug is toxic and affects other tissues than the liver and pancreas.

2.4.1.2 Streptozotocin induced diabetes model

Streptozotocin is specifically cytotoxic to β-cells of the pancreas. For inducing diabetes in rats, 60 mg/kg of streptozotocin is injected intravenously. As with alloxan, three phases of blood glucose changes are observed. Initially, blood glucose is increased, reaching values of 150-200 mg % after 3 hours, 6-8 hours after streptozotocin, the serum insulin valves are increased up to 4 times, resulting in a hypoglycemic phase

which is followed by persistent hyperglycemia. Severity and onset of diabetic symptoms depend on the dose of streptozotocin. After the dose of 60 mg/kg iv, symptoms occur after 24-48 hours with hyperglycemia upto 800 mg%, glucosuria and ketonemia. Histologically, the β-cells are degranulated or even necrotic. A steady state is reached after 10-14 days,' which provides to use the animals for pharmacological tests.

2.4.1.3 NIDDM resembling animal models

NIDDM animal models can be prepared by injecting streptozotocin intravenously at a dose of 100 mg/kg to neonatal wistar rats on the day of birth, through sapheneous vein which is accessible by transcutaneous puncture.

Another model of NIDDM is by injecting streptozotocin (90 mg/kg,IP) to two days old Sprague Dawley rats resulting in transient hyperglycemia followed by recovery Post prandial hyperglycemia, as well as oral glucose intolerance, in the diabetic range is noticeable at 4-6 weeks of age.

NIDDM along with hypertension can be developed by injecting streptozotocin neonates of the spontaneous hypertensive rats. The different diabetogenic agents used and the other animal models used in experimental diabetes mellitus are mentioned in table-3 and table-4 respectively.

Table No. 2.1: List of diabetogenic agents

Sl.No	Compound	Dose	Animal
1	Dehydrogluco ascorbic acid	3.5 to 3.9 gm/kg	Rat
2	Dehydro ascorbic acid	650 mg/kg three days	Rat
3	Dehydroisoascorbic acid	1.5 mg/kg	Rat
4	Ethyl Alloxan	50-130 mg/kg	Rat
5	Methyl Alloxan	53 mg/kg	Rat
6	Oxine and dithizone	50 mg/kg	Rabbit
7	Potassium xanthate	200 to 350 mg/kg	Rabbit
8	Sodiumdiethyl-dithiocarbonate	0.5 – 1 g/kg	Rabbit
9	Uric acid	1 mg/kg	Rabbit

Table No. 2.2: Animal models in experimental diabetes mellitus

1	Surgical diabetes • Total excision of the pancreas • Partial excision of the pancreas
2	Chemically induced diabetes : Produced by chemical agents such as • Alloxan • Streptozotocin • Cyproheptadine • Uric acid • Pentamidine • Oxidative free radicals • Lithium • Cyclosporine A
3	Endocrine diabetes • Hypophysical diabetes • Metahypophysical diabetes • Adrenocorticoid diabetes
4	Starvation diabetes
5	Physiological diabetes • Growth hormone induced diabetes • Steroid induced diabetes • Genetically induced diabetes • Immunogenic diabetes
6	Virus induced diabetes

2.5 Antihyperlipidaemic

2.5.1 Triton WR-1335(a non-ionic detergent, iso octyl polyoxy ethylene phenol, formaldehyde polymer) induced hyperlipidaemia

Triton WR-1335 is a surfactant which is used to reduce surface tension in various processes. If this molecule is administered in to either animal or human it alters the lipid metabolism and results in the accumulation of lipids in the body. This condition is termed as state of hyperlipidaemia.[39, 40]

2.5.2 Propylthiouracil induced hyperlipidaemia

Propylthiouracil is a drug used in the treatment of hypothyroidism. Thyroid gland secretes T3 (triiodothyroxine) and T4 (tetraiodothyronin) which are calorigenic/energy producing hormones in nature. When the secretion of T3 and T4 is decreased the energy maintenance of the system disturbs with decreased breakdown of energy liberating molecules and results in their storage, which in turn increases in lipid profile of the subject termed as hyperlipidaemia.[41]

2.5.3 High fat diet induced hyperlipidaemia

Fats and lipids are the richest source of energy for the human body. These molecules are metabolised regularly to satisfy the energy needs of the human system. If not metabolised these molecules undergo transformation into other energy releasing molecules namely sugars through a process known as gluconeogenesis. If the molecules are present in excess in the human body the remaining part of the used molecules starts getting stored in the body and develops a condition associated with increased levels of lipids known as hyperlipidaemia.[41]

2.6 Antiparkinsons

2.6.1 Chemical models available for screening neuroprotective agents/antiparkinsonism agents

2.6.1.1 MPTP induced Parkinsonism

The unintentional self-administration of 1-mehty1-4-pheny1-1,2,3,6-tetrahydropyridine (MPTP) by addicts of heroin in the 1880s resulted in an acute form of parkinson's disease (PD). The medical and biochemical character were impossible to differentiate from that of idiopathic PD.[42] MPTP cohort (idiopathic disease developed characters of patients were compared against the MPTP induced PD patient's charecters) established an outstanding answer to levodopa and dopamine agonist management however resulted in motor nervous problems within a small phase of period.[42]

2.6.1.2 Hydroxy dopamine Induced Parkinsonism

6-Hydroxydopamine (6-OHDA or 2,4,5-trihydroxyphenylethylamine) is a precise catecholamine deriving excitatory neurotoxin characteristically resembling dopamine and noradrenaline equally.

2.6.1.3 Reserpine antagonism

The first ever animal model resembling human characteristics for PD was established by Carlsson in the 1950s by means of rabbits treated by reserpine. Reserpine is a catecholamine concentration decreasing agent which hinders the vesicular storage of monoamines.[43]

2.6.1.4 Haloperidol induced tardive dyskinesia

Haloperidol (HAL) a widely used neuroleptic for the treatment of psychosis is limited by its tendency to produce a range of exrapyramidal movement disorders like tardive dyskinesia (TD), akathisia, dystonia and parkinsonism.[44]

2.6.1.5 Rotenone induced Parkinson's disease

Rotenone (an inhibitor of mitochondria complex I) is pesticide which causes PD by precisely producing neurochemical imbalance, physiological changes and creating pathological damage to dopamine secreating neurons in the brain.

2.6.1.6 Paraquat model

Paraquat (N,N-di4-4-4′-bypiridium) is a herbiside. Structurally similar to MPP^+. It has the ability to induce lewy bodies like structures in dopamine neurons of SNPC.

2.6.1.7 Methamphetamine induced Parkinson's disease

Amphetamine and its other synthetic products (including methamphetamine) show the way to continuing reduction of both dopamine and serotonin in the brain (neurons) when feed to nonhuman like rats and primates.[45]

2.6.1.8 Other models

- ✓ Alpha methyl para tyrosine induced parkinsonism (AMPT)
- ✓ Genetic models of parkinson's disease
- ✓ Spontaneous rodent models for parkinson's disease Transgenic mouse models

2.7 Invitro antioxidant

2.7.1 Models for evaluating Invitro antioxidant activity

2.7.1.1 Preparation of sample solution required for the tests

4.1ml of 2.5% linolenic acid in absolute alcohol is added with 4mg of plant extract in 4ml of absolute alcohol. To this mixture 8ml of 0.05M phosphate buffer of P^H 7 was added and the final volume of the preparation was adjusted to 20 ml bythe addition of 3.9ml of water. The prepared sample solution was kept in a vail with screw cap and was placed in an oven at 40°C for half an hour in dark.[46,47,48]

2.7.1.2 FTC Method:

0.1ml of 30% ammonium thiocyanate and another solution 9.7ml of 75% ethanol along with 0.1ml of 0.02M ferric chloride in 3.5%Hcl was added to 0.1ml of above solution and absorbance was measured at 500nm at regular intervals of 24 hrs untill standard absorbance reaches maximum. [46,47]

2.7.1.3 TBA method:

TBA method test was performed on the final day of FTC method. 1ml of same above sample solution was added with 2ml of 20% trichloro acetic acid and 2ml of 0.67% Thio barbituric acid. Then the mixture was placed in boiling water bath for 10 mins then removed and cooled to room temperature. After cooling, the solution was subjected for centrifugation at 3000 rpm for 20 mins and absorbance was measured at 552 nm. [46,47,48]

2.7.1.4 DPPH method:

50µl of standard test extract was added with 1ml of 0.1mM DPPH in methanol and 450µl of 50mM Of Tris Hcl buffer of pH 7.4. Then the mixture was incubated at room temperature for 30 mins and absorbance was measured at 517 nm.

While performing all the tests ascorbic acid (Vit – C) and tocoferol (Vit – E) was taken as standard and was termed as positive control where as water and alcohol was taken as negative control. [46,47,48] In the below given formula absorbance of either water or alcohol should be taken as absorbance of control.

% Inhibition can be calculated by applying the following formula.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test Sample}}{\text{Absorbance of Control}} \times 100$$

2.7.1.5 Hydroxyl radical scavenging activity assay

The scavenging action for hydroxyl ions shall be measured by the means of Fenton reaction. The reaction mixture is blended with 60 µl of 1.0mM FeCl3 along with 90 µl of 1mM 1,10- phenanthroline along with 2.4 ml of 0.2 M phosphate buffer (pH 7.8) for the maintenance of proper P^H and also 150 µl of 0.17 M H_2O_2 finally 1.5 ml of test material and standard at different concentrations. Adding H_2O_2 immediately starts the reaction. Past constant standing at controlled temperature for 5 min the absorbance of the combination at 560nm shall be considered with a spectrophotometer.[49]

2.7.1.6 Assay for Nitric Oxide Scavenging Activity

The scheme of reactions was followed for the research, sodium nitroprusside (10 mM) in buffer of phosphate in saline shall be mixed with dissimilar concentrations of the test extract mixed in ethanol and incubated at constant room temperature for a period of 2½ hours. The similar reaction with no test sample but equivalent amount of ethyl alcohol serves as control. After standing undisturbed for a period, 0.5 ml of Griess reagent must be added. Absorbance of the coloured compound shall be measured at 546 nm. Ascorbic acid should be used as helpful control. The method is based on the belief that, Sodium nitro prusside solution impulsively generates nitric oxides, which instantaniously reacts with oxygen to fabricate nitrite ions which can be sketched using Greiss reagent. Neutralisers of nitric oxides race with oxygen leading to decrease assembly of nitrite ions.[50]

2.7.1.7 Assay for Superoxide Radical Scavenging Activity

The test for superoxide ion neutralising action was performed as per standard procedure. The reion mixture bearing 50 milli Molar buffer of phosphate (pH 7.6), 20 µgram riboflavin, 12 milli Molar EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all mixed in chronological succession. The reaction shall start by creating sub reactions in the reaction mixture bearing different amounts of the test substance for 1.5 minutes and then measuring the absorbance at 590nm. Ascorbic acid shall be taken as the comparative standard.[51]

2.7.1.8 Total antioxidant activity (Phosphomolybdic acid method)

The antioxidant/free radical scavenging activity of the test sample shall be determined by the conversion of Mo (VI) to Mo (V) to form phosphomolybdenum compound. An aliquot of 0.4 ml of test solution shall be pooled in a vial with 4 ml of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate (Na_2 $(PO_4)_2$) and 4 mM ammonium molybdate). The vial should be replaced with cap and incubated in a simulated water bath/jacket at 950 ˚C for $1^1/_2$ hours. Following the samples had chilled to control room temperature; the absorbance of the combination mixture shall be calibrated at 695 nm against a blank. The antioxidant/free radical scavenging activity shall be expressed relative to that of ascorbic acid.[52] (standard)

2.7.2 Quantitative antioxidant activity:

TLC plate with precoated silica gel was taken and the spots of different solvent extracts were placed on the plate 1 cm from bottom of the plate. Methanol (95): Chloroform (5) was selected as mobile phase to develop a chromatogram of different solvent extracts. After 30 mins of development of chromatogram whole plate was sprayed with 0.15% w/v DPPH solution using automiser. Any colour differentiation (yellowish colour on the pinkish background on TLC plate) shows the presence of antioxidants.[53]

2.8 Pharmacovigilance[54]

2.8.1 Different models for carrying out a clinical study

The traditional systems of medicine have been in use for the treatment of much suffering human ailments for ages. Though it is a

common belief that the traditional systems of medicine are devoid of untoward reactions or adverse drug reactions (ADR) many attempts have been made to evaluate the different herbs used in these medicines systems for their ADR potency. When an general population was counselled for the knowledge, attitude and perception of people towards ADR produced by natural products most of them were found to be denying. Laxation or diarrhoeal like symptoms when tamarind is consumed in excess, gastritis when capscicum is consumed excess can be considered as possible ADR, which are neglected in day to day life. Hence, an attempt has been made to evaluate the ADR of seeds of *Nigella Sativa* through clinical application.

The study models are,

2.8.1.1 Randomised or Non randomised

In this type of study the study participants are selected basing up on specific reasons for the purpose of study. To evaluate the ADR of antihypertensives, anti arrhythmics, anti angina agents subjects suffering from cardio vascular disorders are selected. In the case of sedative and hypnotics, antiparkinson's, anti alzheimer's subjects suffering from either psychiatric or psychological disorders are selected. On the other hand, in the non randomised study the participants for ADR study were selected basing on no particular diseased state.

In randomised control trials the selection of the study population will be as similar to the reference population as possible based on suitable inclusion and exclusion criteria.

Protocol with details of the study should be prepared and presented for ethical considerations to which the researchers must stick till the completion of the study. Studies shall be terminated in the mid of the study of the unpredicted adverse events occurs which are not at all anticipated before the study.

After explaining the intent of the study the inform consent must be taken from either the participant or the guardian in case of minors before the beginning of the study mandatory.

Randomisation of the study population must be done properly as it is a statistical study which bears high weight age for all the criterias except the intervention. This will nullify various interfering factors in the sudy as possible.

Ascertaing the outcome for simple variables shall be easy in the study.

2.8.1.2 Prospective or Retrospective

The prospective study proceeds from the point of beginning to forward with time, the retrospective study includes the proceedings from beginning of study to backward in time.

2.8.1.3 Observational or Interventional

Observational study limits the research co coordinators other than physician from intervening the study in regard with study subjects. Whereas, interventional study gives liberty to the research coordinators to intervene in the study for the better outcome of the result. For example: A qualified pharmacist, very well pays the role of a research coordinator by the side of a physician to overturn the work burden from

the shoulders of physician through providing patient counselling (bedside counselling, discharge counselling), helping attaining drug adherence through bringing the awareness among diseased subjects by preparing and distributing pre evaluated patient information leaflet (PIL) etc.

2.8.1.4 Single blind or Double blind

In single blind study the drug to be administered is known to the administrator where as in the double blind study the secrecy of drug is neither revealed to the physician nor to the receiver (Patient/subject).

2.8.2 Specific Models for Clinical Study are

2.8.2.1 Descriptive study

2.8.2.1a Cross sectional studies

Cross sectional studies measures the prevalence of diseases and hence called as prevalence studies also. In this study, the measurements of exposure and effect are made at the same time. In this study we are able to get information on the relationship between a disease and other variables of interest as they exist at one point of time. In this a set of individuals are chosen who may be a representative sample of a general population or of people in a particular community or a sample of members of some special sub groups like school children, the arm forces, workers in a particular industry. Data from cross sectional studies are useful in assessing the healthcare needs of population.

2.8.2.1b Longitudinal studies

When a descriptive study is conducted over a long period of time, it can be called as longitudinal study. These studies are usually done on samples drawn from the population and the observations are made at

periodic intervals. In this study the same individuals are examined upon repeated occasions and the changes within the group recorded in terms of elapsed time between observations. This study is extremely useful for identifying the risk factors associated with the disease, for calculating the incidence rate of the disease.

2.8.2.2 Analytical studies

2.8.2.2a Case control studies

The approach of beginning with the disease and searching for the causes in the past is referred to as case control study. Here one starts with a group of individuals who suffered from a particular episode of illhealth. enquiries are taken up to find out what caused the episode. This has to be done in conjunction with the study of another group of individuals who have not fallen prey to the condition called 'controls'. This is a retrospective study which is primarily used to assess risk and to study the causes of diseases in general.

2.8.2.2b Cohort study

The approach of beginning with the exposure and searching for effects in a prospective manner in time is referred to as cohort study. These studies constitute on important form of epidemiologic investigation undertaken to test the hypothesis regarding the causation of disease. This study is an observational epidemiological study which attempts to study the relationship between a purported cause (exposure) and the subsequent risk of developing disease. The distinguishing features of cohort studies are the group or the groups of persons to be studies are defined in terms of characteristics manifest prior to the appearance of

the disease under investigation. The study groups so defined are observed over a period of time to determine the frequency of disease among them. Cohort studies are also called by various names like incidence studies, prospective studies, follow up studies, longitudinal studies and panel studies.

2.8.2.3 Field trials

2.8.2.3a Preventive trials

When one has to derive disease free status in a health population using preventive techniques like vaccination, one has to resort to large scale field trials. Here in again individuals belonging to such population are selected the outcome of interest however will be the proportion of disease which was prevented are death averted.

2.8.2.3b Risk factor trials

Here instead of a vaccine or a drug, specific risk factors are averted in groups of population and the reduction in disease incidence observed For example: dietary patterns and caries.

2.8.2.3c Community trials

Instead of few people whole community is taken as study group.

2.9 Plant profile of *Nigella sativa:*

Figure 2.1: Plant of *Nigella sativa*

Figure2.2: Seeds of *Nigella sativa*

2.9.1 Etymology

The scientific name was *Latin* originated *Nigella* (Black)

2.9.2 Biological source:

It consists of seeds of *Nigella Sativa.*

2.9.3 Habitat and ecology:

It is native of south East Asia and Mediterranean

2.9.4 Classification:

Kingdom	:- Plantae (Plants)
Sub-kingdom	:- Tracheobionta (Vascular plants)
Super division	: - Spermatophyta (Seed plants)
Phylum	: - Magnoliophyta (Flowering plants)
Class	: - Magnoliopsida (Dicotyledons)
Sub-class	:- Magnolidae
Order	: - Ranunculales
Family	: - Ranunculaceae (Buttercup family)
Genus	: - Nigella
Species	: - N.Sativa

2.9.5 Common Names

English :- Black caraway, Roman coriander, fennel flower

Hindi : - Kalonji

Telugu : - Nallajelakara

Kannada : - Krishna jeerige

Tamil : - Reske

Malayalam : - Karim jeerakam

2.9.6 Description

It is an annual herb which bears flowers in the flowering season, native of south west Asia. It grows to a height of 20-30 cms with linear and divided leaves. The flowers are delicate with pale yellow petals (5-10). Seven unified follicular fruit is the characteristic feature of this plant with numerous seeds which are used as spice in different parts of the world. The seeds taste bitter still it is used as condiment in food preparations to alter the taste desiringly.[87]

2.9.7 Chemical constituents

thymoquinone, γ-terpinene, thymol, β-caryophyllene, thymohydroquinone, ρ-cymene, Lauric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid, Linoleic acid, Linolenic acid, Eicosadienoic acid, n-Nonane, 3-Methyl nonane, 1,3,5-Trimethyl benzene, n-Decane, 1-Methyl-3-propyl benzene, 1-Ethyl-2,3-dimethyl benzene, n-Tetradecane, n-Hexadecane, Nonterpenoid hydrocarbons, α-Thujene, α-Pinene, Sabinene, Pinene, Myrcene, α-Phellandrene, p-Cymene, Limonene, γ-Terpinene, Monoterpenoid hydrocarbons, Fenchone, Dihydrocarvone, Carvone, Monoterpenoid ketones, Terpinen-4-ol, p-Cymene-8-ol,

Carvacrol, Monoterpenoid alcohols, α-Longipinene, Longifolene, Sesquiterpenoid hydrocarbons, Estragole, Anisaldehyde, trans-Anethole, Myristicin, Dill apiole, Apiole. These compounds were identified in the oil of seeds of Nigella sativa.[87]

2.9.8 Uses

The seed and seed oil of *Nigella Sativa* has medicinal value. The massage of seed oil is useful in arthritis. In skin diseases, the local application of seed oil is beneficial as it is antiseptic and anti-inflammatory in nature. The seed oil with salt is an effective gargle in dental infections. The paste of seeds is applied in cases of backache, arthritis, paralysis, oedema of the lungs and liver.

Internally, the seed powder is used in various diseases. It is commonly used as a household remedy. In hiccups, seed powder is given along with the ghee and honey. The seed powder with sugar and ghee augments the appetite. Abdominal pain is relieved when it is given along with jaggery.

The seeds are edible and are used in Indian cuisine. If carefully cultured, it can be used as iron, sodium and minerals food supplement.[87]

2.10 Earlier work done on *Nigella sativa:*

1) The work entitled "The active fractions from *Nigella Sativa* and its activity against T47D cell line" by Heny Ekowati. *et.al.* published in Indo J Chem 2011 vol II Issue 3 states that the cytotoxicity of chloroform extract's third fraction has a better activity at IC 50 of 68.56 µg/ml against T47D (breast cancer) cell line using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.[55]
2) The manuscript entitled "Anti tumor property of black seed (*Nigella Sativa)* extract" carried out by L.Ait.Mbarek *et.al.* published in Brazilian journal of medical and biological research 2007, Vol 40, states that the essential oil at a concentration of 0.6% v/v and ethyl acetate extract at a IC 50 concentration of 0.75% v/v had better cytotoxicity activity against cancer cell lines namely P815 (Murine mastocytoma cell line), Vero (Kidney carcinoma cell line of monkey), BSR (Kidney carcinoma cell line of hamster), ICO 1 (sheep heart carcinoma cell line)against adriamycin as standard.[56]
3) Navdeep chehl *et.al.* published a study titled "anti-inflammatory effect of the *Nigella Sativa* seed extracts, thymoquinone in pancreatic cancer cells". In HPB 2009, Vol II states that the thymoquinone (25-75µM) fraction of oil of seeds of *Nigella Sativa* suppresses inflammation in pancreas which is considered to be the primary triggering factor for development of pancreatic ductus adenocarcinoma as it has successfully decreased inflammation in hepatic – pancreatic ductus adenocarcinoma in comparison with

trichostatin a hepatic – pancreatic ductus adenocarcinoma inhibitor. Thymoquinone has successfully decreased TNFα, IL II, Cox 2 with in 24 hours of administration.[57]

4) Ahmed O Kaseb *et.al.* states in their manuscript titled "Androgen receptor and E2F-1 targeted thymoquinone therapy for hormone – refractory prostrate cancer" published in cancer res 2007, Vol 16, that E2F-1 an androgen receptor is the major culprit in the relapse of prostrate cancer in males. Thymoquinone of *Nigella Sativa* down regulated the Androgen receptor and E2F-1 and constricted the continuation of cell cycle G1-S Phase by inhibiting DNA replication in mice. It also induced apoptosis in cancerous cells of mice.[58]
5) Sameera R Samarakoon *et.al.* reported in their manuscript "a Comparison of the cytotoxic potential of standardized aqueous and ethanolic extract of a polyherbal mixture comprises of *Nigella Sativa* seeds, *Hemidesmus Indicus* (roots), *Smilax Glabra* (rhizome) in the year 2010, Vol II, Issue 6, states that the decoction of above titled work was made and evaluated against HepG2 cell lines by MTT and SRB (sulphorodamine) assay. The decoction yielded dose dependant cytotoxicity against HepG2 cell line. Aqueous extract showed better activity than alcoholic extract.[59]
6) Protection against diethylnitrosoamine induced hepatocarcinogenesis by an indigenous medicine comprises of *Nigella Sativa, Hemidesmus Indicus, Smilax Glabra*: a preliminary study is an is an invivo antineoplastic study by Samantha and Iddamaldehiya *et.al.* to demonstrate the effect of decoction of

entitled plants can inhibit carcinogenesis in liver of male wister rats even in presence of toxicant was published in journal o carcinogenesis 2003, Vol II.[60]

7) A long term investigation of anti hepato carcinogenesis potential of decoction of Nigella *Sativa, Hemidesmus Indicus, Smilax Glabra* was proved by Samantha and Iddamaldehiya *et.al* in male wister rats. The study period comprises of 9 months in which only one rat of 12 developed liver carcinoma and by the end of 16 months all rats developed overt tumor and histopathological changes leading to cancer development on daily administration of toxicant and test decoction.[61]
8) Daoud Musa *et.al.* published heir work "antitumor activity of a ethanol extract of *Nigella Sativa* seeds" in Biologia 2004, Vol 56, Issue 6, means that the effect of ethanolic extract of *Nigella Sativa* seeds on Ehrlich ascites tumor (EAT) cells in vivo in mice showed the increase in lifespan and antioxidant concentration. The cells were observed to be intact with cell membrane.[62]
9) The work entitled "Emerging clinical and therapeutic application of *Nigella Sativa* in gastroenterology" by shalinder kapoor in the world journal of gastroenterology 2007,Vol 15, issue 17 explains the benefits of *Nigella Sativa* in the treatment of colon cancer, diabetes and liver diseases.[63]
10) Hala gali muhtasib *et al.* explained the components and medical benefits of *Nigella Sativa* in their manuscript "the medicinal potential of black seed *(Nigella Sativa*) and its components" stating

that the seeds are extensively used in the eastern countries because of their good pharmacological values, the aqueous extract processes good antioxidant activity as well.[64]

11) The article entitled "Effect of *Nigella Sativa* oil on various clinical and biochemical parameters of insulin resistant syndrome" by Ahmed Najmi *et.al.* published in the Int.J.Diab.Dev Ctries 2008, 28, states that the treatment of patient with metabolic syndrome clinically showed significant improvement in comparison with cholesterol, LDL-C, and fasting blood glucose levels. It can be taken as add on theraphy in patients of Insulin resistant syndrome.

12) Afaf Jamal Ali *et.al.* explained in their manuscript entitled "*Nigella sativa* oil has significantly repaired ability of damaged pancreatic tissue occurs in induced type I Diabetes Mellitus" published in Global journal of pharmacology 2013, Vol 7, Issue 1, that the degeneration beta cells of langerhans in pancreas of streptozotocin induced diabetes rats appears to be regenerating almost equivallant to that of normal group which proves that the oil of *Nigella Sativa* is found to be active against streptozotocin induced Diabetes mellitus in rats.[65]

13) Evaluation of antioxidant effect of *Nigella Sativa* extract on the ultra structure of neural tube defects in diabetes rat offsprings by Panahi M and submitted in the Jundishapur Journal of National Phamaceutical Products 2011, Vol VI, Issue 1, explains the effect of *Nigella Sativa* extract on oxidative stress induced gestationsl

diabetes in rats. Which can protects the neuronal tube defects in new born pubs of rats. *Nigella Sativa* has shown protective effect on embryo and decreased foetal loss.[66]

14) The effect of *Nigella Sativa* seed on biochemical parameters in diabetic rats was explained by M Kaleem *et al.* in their work titled "Biochemical effects of *Nigella Sativa* seeds in diabetic rats". Alcoholic extract when orally administered for 30 days to a diabetic rat the lipid profile, glucose levels were found decreased tranciently.[67]
15) In an attempt by Mohtashami *et.al.* on the effect of *Nigella Sativa* clinically on healthy volunteers resulted in fall in fasting blood glucose without gastric, renal or hepatic side effects. Clinically, the dose of black seed oil used was 2.5m/day.[68]
16) A work published by A Najmi *et.al.* in the international journal of diabetes and metabolism 2008, Vol XVI, explains the effect of *Nigella Sativa* oil on metabolic syndrome with the difference among groups in case of cholesterol, LDL-C, fasting blood glucose was clearly seen in different groups.[69]
17) Aftab ahmed *et.al.* explained that the seeds of *Nigella Sativa* has wide applications as antihypertensives, liver tonic, diuretics, digestives, antidiarrhoeal, analgesic, antibacterial etc. The whole credit of good activity of *Nigella Sativa* is attributed to the presence of essential oils in their manuscript entitled "A review on therapeutic potential of *Nigella Sativa: A miracle herb".*[70]

18) A work of Ahmed Gorbani *et.al.* published in Pakistan journal of biology 2013, explains the effect of polyherbal formulation bearing *Nigella Sativa* along with 12 other herbal extracts showed that the differences in the groups in water intake, urine formation, triglycerides, total cholesterol which was found to be lower in polyherbal treated rats than non treated rats groups.[71]

19) A Randomised, placebo controlled clinical trial was performed by Ali mohammed S using seeds of *Nigella Sativa* and reported that the work was concentrated to estimate cholesterolemia which is major risk factor in atherosclerosis/arteriosclerosis. A significant reduction in the levels of total cholesterol, LDL and triglyserides without altering fasting blood glucose and HDL levels.[72]

20) M H Al Nazwi and S M El Bhar in their research work on hypolipidaemic and hypocholesterolemic effect evaluation of black cumin seeds and turmeric. The work explains results of combo theraphy on 24 rats. There was increase in globulin levels in the blood with decreased in LDL levels. Remaining all other parameters including biochemical remained the samein comparision with normal which can be well explained as unchanged. It also resulted in unchanged renal or hepatic functions.[73]

21) A meta analysis was done by shuchi jain on the topic of mortality because of dyslipidaemia includes the explanation of all randomized control trials, this was done by collecting all the matter required for the study. The study revealed the association between

coronary artery disease, cardiovascular disease and mortality in 19 trial study proved to be significant.[74]

22) Kuldeep S Sandhu in his research on "evaluation of antiparkinson's activity of seeds of *Nigella Sativa"* has presented that the ethanolic extract of *Nigella Sativa* has shown increase in glutathione, total protein concentration and with decreased catalepsy. It is justified that the antiparkinson's activity of *Nigella Sativa* in drug induced disease condition shall be explained.[75]

23) Beheshteh Babazadeh published their research work regarding usefulness of seeds of *Nigella Sativa* in preventing in preventing serum low glucose level induced DNA damage in cells. In this state terminal DNA tail is seen where as, the DNA damage in controlled cells to DNA is lessbecause of Ischaemia. It explains the protective effect of seeds of *Nigella Sativa.*[76]

24) The effect of hydroalcoholic extract of *Nigella Sativa* and thymoquinone on depression induced by lipopolysaccharides was studied by Mahmoud Hosseini and stated that *Nigella Sativa* inhibits depression like behavior because of lipopolysaccharides in wister rats. The conclusion was made basing on the results obtained during forced swim test and open field test.[77]

25) Dr Galib *et.al.* carriedout an experimental explanation of drug discovery of inflammation. The article explains the importance of drugs present in Ayurveda. It explains the need of hour is to carryout research in alternate system of medicine to come out with better agents with therapeutic importance.[78]

26) Rupali A Patel *et.al.* studies "the reversal of reserpine induced orofacial dyskinesia and catalepsy by *N.Jatamansi* in animals". This was predicted to be associated with free radical induced neurodegenaration. Treatment with *N.jatamansi* clearly decreased the dyskinesia and catalepsy as well as increased the antioxidant concentration in the body of animals, which was considered as protective behavior of the test herb.[79]

27) The in vitro antioxidant activity of a herb "*Chrozophora plicata"* was performed by K Sunil kumar *et.al.* for the evaluation of reducing power, superoxide anion scavenging activity, nitric oxide scavenging potency and hydroxyl radical neutralizing strength. The herb was effective in eradicating free radicles to equivalent effect with standard, only in case of nitric oxide it was one forth the potency of standard.[80]

28) *Gallus Gallus Domesticus* was in vivo evaluated for its antioxidant potency in cold stressed animals. Superoxide dismutase, glutathione peroxide, glutathione reductase in the serum was significantly high in animals even after the stress induction when compared to untreated group.[81]

29) A H M Zulfikar *et.al.* performed an Invitro antioxidant activity concomitant with hypoglycemic activity of "*Ficus racemosa"*. The DPPH free radicle scavenging activity of ethanolic extract of plant was at twice the dose of the standard. The study concluded that the plant processes anti oxidant activity.[82]

30) The Invitro antioxidant activity of different parts of a plant was done by Zaveri maitreya *et.al.* gave a detailed procedure to carryout in vitro said study. It explains anti lipid peroxidation assay (TBA), in the study was successfully concluded and defines its antioxidant potency.[83]

31) Urmila M Aswar *et.al.* in their study related to antiosteoporotic activity in ovariectomized rats explains the effect and procedure of ovariectomization and also the study goes on explaining the benefits of phyto estrogens which gets separated in ethanolic fraction of the plant extract over the prevention of osteoporosis in wister rats. The study resulted in increased levels of serum Estradiol concentration of diseased group.[84]

32) A study of evaluation of osteoporosis, which was induced by ovariectomization was done in turkey by using calcitonin to evaluate bone quality and bone collagen structure in rats. The study explains the procedure of Ovariectomisation, treatment schedule and benefits of clacitonin during osteoporosis.[85]

33) Ethylene glycol is the primary choice of toxicant in the induction of urolithiasis in an experimental study. The same was done by Marzieh Ahmedi *et.al.* in studing curative and preventive effect of their test drug. The drug successfully stands to the expectations of researchers.[86]