Chapter -2

Materials and Methods

2.1 MATERIALS

2.1.1 Plant material

Fresh leaves and root bark of *Ailanthus excelsa* Roxb. along with flowers of *Butea monosperma* Lam. were collected from local area of Amravati, Maharashtra, India and were identified in the Department of Botany, The M S University of Baroda, Vadodara, Gujarat.

2.1.2 Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′ azo-bis (amidinopropane) dihydrochloride (AAPH), Superoxide dismutase (SOD), bovine serum albumin (BSA), epinephrine bitartarate, isoproterenol, cis-platin, nitroblue tetrazolium (NBT), 2-deoxy ribose, L-ascorbic acid, curcumin, quercetin, rutin, gallic acid, and apigenin were procured from M/s Sigma Chemical Co. (St. Louis, USA). Thiobarbituric acid (TBA), trichloroacetic acid (TCA), tris buffer, 5, 5'-dithiobis (2-nitro benzoic acid) (DTNB), riboflavin and EDTA were obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. While all the reagents and organic solvents used were of analytical grade.

2.1.3 Animals

Balb C females and C57 male mice weighing between 20-25g along with male albino rats of SD strain weighing between 250-280g were used for the study. They were housed under standard environmental condition and fed with commercial diet and water *ad libitum*. All experiments were carried out as per guidelines of Institutional ethics committee, Pharmacy department/ M.S.University, Baroda/404/01/1/CPCSEA and National Center for Cell Sciences (NCCS), Pune

2.2 METHODS

2.2.1 Preparation of powdered material

The selected plant material were collected, cleaned to remove any adhering material and then dried in shade. The dried plant materials were then subjected to size reduction to coarse powder and used for further studies.

2.2.3 Elemental analysis

Elements like manganese, zinc, copper, sodium, potassium, iron, lead and cadmium were analyzed using atomic absorption spectrophotometer. The elements detected were quantified by using standard calibration plot and expressed in PPM.

2.2.4 Extraction of plant material

2.2.4.1 Extraction of Ailanthus leaves

Powdered leaves of Ailanthus were successively extracted in petroleum ether, diethyl ether, chloroform, ethyl acetate, methanol in a soxhlet extractor. The mark was air dried before extracting with next solvent. Finally the mark was extracted with chloroform water by simple decoction.

2.24.2 Extraction of Ailanthus root bark

Powdered root bark was successively extracted in petroleum ether, benzene, chloroform, ethyl acetate and methanol and water by using same methodology as above.

2.2.4.3 Preparation of Alkaloidal fraction of root bark

As most of the extracts prepared from root bark contain alkaloids as common constituent, we prepared here alkaloidal fraction of root bark using reported method. Alkaloidal fraction of root bark was designated as AFRB in this thesis.

2.2.4.4 Extraction of Butea followers

Coarse powder of the shade dried flowers was extracted with methanol in a soxhlet extractor; the extract was concentrated and dried under vacuum. The vacuum dried extract was suspended in water and then extracted with ethyl acetate and n-butanol. The remaining aqueous portion was concentrated and dried. The total methanol extract along with its fractions were taken for further studies.

2.2.5 Qualitative chemical tests

The extracts and fractions obtained above were then subjected to different qualitative chemical tests for presence of various phytoconstituents.

2.2.6 Evaluation of Antioxidant activity

All the extracts and fractions from both Ailanthus and Butea were further screened for their radical scavenging activity using different *in vitro* methods. The selected extracts from these studies found effective were further evaluated for their antioxidant activity using *in vivo* methods.

2.2.6.1 In vitro methods

Radical scavenging activity of various extracts and fractions was determined by measuring their reducing power, percentage inhibition of DPPH radical, superoxide anion radical, nitric oxide radical, hydroxyl radical and erythrocyte hemolysis induced by 2, 2'- azo-bis (amidinopropane) dihydrochloride (AAPH).

Rapid screening for radical scavengers in the extract was also done on TLC using DPPH reagent.

2.2.6.1.1 Determination of reducing power

Reductive ability was measured in terms of Fe^{3+}- Fe^{2+} transformation in the presence of different concentrations of the extracts. Absorbance of the reaction mixture is directly proportional to the reducing power of the test substance. Ascorbic acid was used as a reference standard (Oyaizu M, 1986)

2.2.6.1.2 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Blois with some modifications. In brief, different concentrations of the test samples were incubated with 0.2 m*M* solution of DPPH for 30 min at room temperature and decrease in absorbance of the solution brought about by the sample was measured at 517 nm. Rutin was used as a reference standard (Blois MS, 1958, Navarro M, 1993).

2.2.6.1.3 Superoxide anion radical scavenging activity

Superoxide radical scavenging activity was measured by riboflavin/light/NBT reduction method. The method was based on generation of superoxide radical by autooxidation of riboflavin in presence of light which in turn reduced NBT to a blue colored formazon. The capacity of extracts to inhibit the formazon formation was measured. Ascorbic acid was used as a reference standard (Robak J, 1998; Beauchamp C, 1971).

2.2.6.1.4 Hydroxyl radical scavenging activity

The method described by Halliwell was followed in the studies. It involved *in vitro* generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_2O_2 system by 'Fenton reaction'. Scavenging of hydroxyl radical in presence of different concentrations of test samples was measured at 532 nm. Curcumin was used as a reference standard (Halliwell B, 1987).

2.2.6.1.5 Nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside in aqueous solution under physiological *p*H and the extent of nitric oxide scavenged by the test samples was measured by the Greiss reaction. Curcumin was used as a reference standard (Green L C, 1982).

2.2.6.1.6 Inhibition of Erythrocyte lysis

AAPH, a water-soluble free radical generator was used to simulate the *in vivo* conditions of oxidative stress and to generate peroxyl radicals by thermal decomposition of an azo compound in oxygen. Addition of AAPH to the

suspension of erythrocytes induces the oxidation of membrane lipids and proteins resulting in hemolysis. Inhibition of hemolysis in the presence of test sample was determined by measuring the absorbance of the supernatant fraction of reaction mixture at 540 nm. Ascorbic acid was used as a reference standard (Zhu Q Y,, 2002 and Ko F N, 1997).

In all the above methods, percentage inhibition was calculated using following formula,

% Radical scavenged =

[(Absorbance of control – Absorbance of test) x 100/ Absorbance of control]

Where, control is the sample without test material and test is the sample containing the substance to be tested.

2.2.6.1.7 Rapid screening for antioxidant compounds

To make a semi-quantitative visualization possible, extracts and fractions were applied on a TLC plate (10µg in particular solvent) and developed in suitable solvent system. The plate was then dipped in a 0.2% solution of DPPH in methanol. The appearance of white and yellow colour spots on violet background was the indirect measure of radical scavenging components in the extract (Cuendet M, 1997).

2.2.6.1.8 Determination of total phenolic content

Total soluble phenolics in the extracts were determined according to the method of Shahidi and Wanasundara using Folin-Ciocalteau reagent, percentage of total phenolic content was calculated from calibration curve of gallic acid and was expressed as percentage equivalent to gallic acid (Shahidi F, 1992).

2.2.6.2 In vivo methods

2.2.6.2.1 Acute toxicity study

Toxicity study was carried out on test material as per OECD guidelines in female albino mice. Animals were dosed with single oral dose of 2000mg/kg body weight and observed for mortality. If the animals remained alive additional animals were dosed with the same bolus. In case of mortality fresh mice were administered with lower dose in the range of 1.75, 5.5, 17.5, 55, 175, 550, 2000mg/kg body weight. Histopathological examination of the visceral organs like heart, kidney and liver was done in order to check the toxic effect of test material. Mice were observed for any reactions like tremors,

convulsions, salivation, and diarrhoea. $1/10^{th}$ of the highest tolerable dose was used as a safe dose for further in vivo studies.

The selected extracts and fractions which were found active in the *in vitro* screening were then evaluated for *in vivo* antioxidant activity using isoproterenol-induced myocardial infarction and liver damage in which pathological conditions, associated with the generation of free radicals, were induced.

2.2.6.2.2 Isoproterenol-induced myocardial infarction (MI)

In myocardial infarction there is death of some of the muscle cells of the heart due to lack of supply of oxygen and other nutrients. This lack of supply is due to closure of the coronary artery that supplies blood to that particular part of the heart muscle, this occurs mainly from the process of arteriosclerosis. Atherosclerosis can occur in even minor blockages where there is rupture of the cholesterol plaque. This in turn causes blood clotting within the artery, blocking the flow of blood. The heart muscle, which is injured in this way, can cause irregular rhythms, which can be fatal.

When the injured area heals, it will leave a scar. Other factors responsible for the development of coronary artery disease are smoking, which damages the lining to the arteries, promotes the clotting of blood, lowers the level of "good" cholesterol in the blood and promotes spasm of the vessels, tending to keep it closed. Other factors, which promote coronary artery disease, are high blood pressure, diabetes, male sex, overweight and family history of the problem.

Dietary antioxidants may reduce the risk of ischemic heart disease and the extent of myocardial infarction. The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins contribute to myocardial cell damage, leading to depressed cardiac function and irreversible tissue injury with concomitant depletion of certain key endogenous antioxidant compounds, such as superoxide dismutase (SOD), catalase and reduced glutathione (GSH) (Ferrari, 1991).

Use of plant extracts, food supplement and even drugs which augment major cellular endogenous antioxidants following chronic administration have been identified as a promising therapeutic approach to combat oxidative stress associated with ischemic heart disease (Bhattacharya et al., 1999).

Isoproterenol, a non-selective β-adrenergic agonist, has been reported to cause oxidative stress in the myocardium resulting in infarct like necrosis of the heart muscle (Wexler, 1978). Animals develop infarct-like lesions when injected with isoproterenol (ISO). These lesions are morphologically similar to those of 'coagulative myocytolysis' (COAM) or myofibrillar degeneration, one of the findings described in acute myocardial infarction and sudden death in man. Myocardial necrosis induced by ISO is probably due to a primary act on the sarcolemmal membrane, followed by stimulation of adenylate cyclase, activation of Ca^{2+} and Na^{+} channels, exaggreated Ca^{2+} inflow, excess of excitation-contraction coupling mechanism, energy consumption and cellular death. The close resemblance of human COAM to ISO-induced lesions suggests that similar mechanisms may be involved (Milei, 1978).

During isoproterenol-induced myocardial infarction enhanced free radical formation and lipid peroxide accumulation have been proposed as one of the possible biochemical mechanism for myocardial damage (Sushmakumari, 1989). Myocardial infarction is accompanied by the disintegration of membrane polyunsaturated fatty acids expressed by increase in lipid peroxides and by the impairment of natural scavenging which is characterized by the decrease in the levels of superoxide dismutase, catalase and reduced glutathione (Nirmala, 1996).

Serum levels of creatine kinase, lactate dehydrogenase and transaminases are the diagnostic indicators of myocardial infarction. Isoproterenol treated rats showe extensive necrosis due to lipid peroxidation (Hearse DJ, 1979).

An increase in the activity of these enzymes in serum is due to the leakage of enzymes from the heart as a result of necrosis induced by ISO (Manjula TS, 1992). Increase in serum uric acid could be due to excessive degradation of purine nucleotides and proteolysis (Iriama, 1987).

Antioxidants like carnitine, vitamin E, and curcumin were found to be helpful in ISO induced oxidative stress (Sushmakumari, 1989; Ithayarasi, 1997; Nirmala, 1996).

Study design

Animal were divided into different groups, containing six animals each.

Group-1: Served as a control, received 1% aqueous solution of tween 80 p.o. for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs)

Group-2: Received 1% tween 80 (p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs)

Group-3: Treated with extracts/fractions to be tested alone (100 and 200mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs)

Group 4: Treated with extracts/fractions to be tested (100 and 200mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs).

The body weights of the animals were recorded through out the experimental period. At the termination point of the experiment the rats were sacrificed under light ether anesthesia and blood was collected by cutting the carotid artery and the serum was separated and used for the assay of marker enzymes like lactate dehydrogenase (LDH), creatine phosphokinase isoenzymes (CK-MB), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALKP) and uric acid. Liver and heart were dissected out, washed in ice-cold saline and weighed accurately to calculate the ratio of heart weight/ body weight (HW/BW) for each group. 10% homogenate of the heart was prepared in 0.1M ice cold Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 2500 rpm and the clear supernatant was used for the estimation of total proteins and various antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and lipid peroxides (LPO). Apart from these histopathological studies of liver and heart were also carried out.

2.2.6.2.2.1 Estimation of endogenous antioxidant enzymes and Lipid peroxodes in heart homogenate

a) Determination of Lipid Peroxidation

Tissue homogenate (2.0ml) was mixed with 2ml of freshly prepared 10% w/v trichloroacetic acid and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2.0ml of freshly prepared thiobarbituric acid (0.67%w/v). The resulting solution was heated in

a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank (Slater, 1971)

b) Superoxide Dismutase (SOD)

Tissue homogenate (0.5m) was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform was added. The mixture was mixed well for 5 minutes and centrifuged at 2000 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer (0.05M, pH 10.2) and 0.5ml of 0.49M EDTA solution were added. The reaction was initiated by the addition of 0.4ml of 3mM epinephrine and the change in optical density/minute was measured at 480nm against reagent blank. Take the absorbance for 3mins at 30 seconds interval. SOD activity was expressed as ng/mg protein. Calibration curve was prepared by using 10-125 units of SOD (Misra, 1972).

c) Catalase (CAT)

Diluted homogenate (2ml) was mixed with 1ml of H_2O_2 (30mmol/l) to initiate the reaction. Blank was prepared by mixing 2ml of diluted sample (similar dilution) with 1ml of phosphate buffer (50mmol/l; pH 7.0). The decrease in absorbance was measured at 240nm. Catalase activity was expressed as ng/mg protein (Colowick, 1984).

d) Reduced Glutathione (GSH)

Equal volumes of tissue homogenate and 20% TCA were mixed. The precipitated fraction was centrifuged. To 0.5ml of supernatant add 4ml of DTNB (0.6mM). The colour developed was read at 412nm against reagent blank.

Different concentrations (10-50µg) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as ng/mg protein (Moron, 1979)

e) Estimation of Total Protein

To 0.1ml of supernatant add 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent, allowed to stand for 15 minutes. Then 0.5ml of 1N Folin-Ciocalteau reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640nm against reagent blank containing distilled water instead of sample. Different concentrations (40-200µg) bovine serum albumin were taken and processed as above for standard graph. The values were expressed as mg of protein/g of wet tissue (mg/gm) (Lowry, 1951)

Procedure for preparation of Lowry C reagent given in ANNEXURE-I

2.2.6.2.2.2 Estimation of serum marker enzymes

a) Creatine kinase Isoenzyme (CKMB)

Quantitative estimation of CKMB was done using ENZOPAK-CK-NAC kit [Reckon Diagnostics Pvt. Ltd., Baroda].

In this reaction creatine kinase catalyses the formation of ATP from creatine phosphate and ADP. Glucose is converted to glucose-6-phosphate by hexokinase using ATP as a source for phosphate moiety. Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to 6-phosphogluconate reducing NADP to NADPH. Their action after the lag phase is monitored by the increase in absorbance at 340nm and is directly proportional to the creatine kinase activity (i.e. the formation of NADPH is in equimolar amount as that of formation of creatine.

b) Lactate dehydrogenase (LDH)

Quantitative estimation of lactate dehydrogenase (LDH) was done using ENZOPAK LDH L→P kit [Reckon Diagnostics Pvt. Ltd., Baroda].

LDH catalyses the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD. Units were expressed as U/L.

c) Creatinine

Quantitative estimation of creatinine was done using kit [Span Diagnostics Ltd., Surat, India].

Creatinine in a protein free solution reacts with alkaline picrate and produced a red colored complex, which is measured calorimetrically. Units were expressed as mg/dL.

d) Uric acid

Quantitative estimation of uric acid was done using diagnostic kit [Span Diagnostics Ltd., Surat, India].

Uric acid in alkaline medium reduces phosphotungstic acid to "tungsten blue" a blue colored complex, which is measured calorimetrically. Units were expressed as mg/dL.

e) Alanine aminotransferase (GPT)

Quantitative estimation of serum glutamate pyruvate transaminase (GPT) was done using span diagnostic reagent kit [Span Diagnostics Ltd., Surat, India].

Pyruvate so formed is coupled with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured calorimetrically. Units were expressed as U/L.

f) Aspartate aminotransferase (GOT)

Quantitative estimation of serum glutamate oxaloacetate transaminase (GOT) was done by using span diagnostic reagent kit [Span Diagnostics Ltd., Surat, India]. Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured calorimetrically. Units were expressed as U/L.

g) Alkaline Phosphatase (ALKP)

Quantitative estimation of alkaline phosphatase was done by using span diagnostic reagent kit [Span Diagnostics Ltd., Surat, India].

At pH 10.3 alkaline phosphatase catalyses the hydrolysis of p-nitrophenyl phosphate and the change in absorbance measured at 405 nm is directly proportional to enzyme activity. Units were expressed as IU/L.

h) Total Bilirubin

50µl each of sodium nitrite, sulphanilic acid and serum were mixed with 0.5 ml of caffeine benzoate and 0.5 ml of distilled water in the order mentioned. Blank was prepared similarly by adding saline instead of sodium nitrite. Samples were then immediately incubated in dark for 5 min. and values were directly determined on RA-50 chemical analyzer. Units were expressed as mg/dl.

2.2.6.2.3 Isoproterenol induced hyperlipidemia

Mathew et al. reported an altered lipid metabolism in myocardial necrosis following Isoproterenol administration. High level of circulating cholesterol and its accumulation in heart tissue are well associated with cardiovascular damage. Administration of Isoproterenol mainly raised LDL cholesterol and decreased HDL cholesterol level in serum. High levels of LDL cholesterol have positive relation and high levels of HDL cholesterol have negative relation with myocardial infarction. HDL inhibits the uptake of LDL by arterial wall and also facilitates the transport of cholesterol from peripheral tissue to the liver where it is catabolised and excreted out of the body. LDLc/HDLc ratio considered to be favorable risk factor. Isoproterenol administration resulted in

significant increase in the free fatty acid level. Hypertriglyceridemia seen in isoproterenol treated rats, a condition observed in ischemic heart disease, is due to a decrease in the activity of lipoprotein lipase in the myocardium resulting in decreased uptake of triglycerides from circulation (Mathew S, 1981).

For evaluating isoproterenol induced hyperlipidemia, analysis of serum lipid profile was carried out in isoproterenol induced myocardial infarction. Various factors like, total cholesterol (TC), LDL, HDL, VLDL and triglycerides were analyzed under this study.

a) Cholesterol

Quantitative determination of cholesterol was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Cholesterol esterase hydrolyses cholesterol ester. Free cholesterol is oxidized by the cholesterol oxidase to cholest 4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-amino antipyrine and phenol in the presence of peroxidase to produce pink colored quinoneimine dye. The intensity of colour produced is proportional to the cholesterol concentration. Units were expressed as mg/dL

b) LDL- cholesterol

Quantitative determination of cholesterol was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

The VLDL and LDL fractions of serum are precipitated using precipitating reagent and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase hydrolyses the ester cholesterol. Then cholesterol is oxidised by cholesterol oxidase to cholest 4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-amino antipyrine and phenol in the presence of peroxidase to produce pink coloured quinoneimine dye. The intensity of colour produced is proportional to the concentration of HDL-cholesterol. Units were expressed as mg/dL

c) Triglycerides

Quantitative determination of triglycerides (neutral fat) was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Triglycerides in the sample are hydrolyzed by microbial lipases to glycerol and free fatty acids. Glycerol is phosphorylated by ATP to glycerol-3-phosphate (G-3-P) in a reaction catalysed by glycerol kinase (GK). G-3-P is oxidised to

dihydroxy acetone phosphate (DAP) in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction, hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and 3, 5-dichloro-2-hydroxybenzene sulphonic acid (DHBS) in a reaction catalyzed by peroxidases. The result of this oxidative coupling is a quinoneimine red colored dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides in the sample. Units were expressed as mg/dL.

2.2.6.2.4 Histopathological Studies

After the treatment period, the animals were sacrificed and the organs (heart and liver) were excised, made free of blood and tissue fluids and preserved in 4% v/v buffered normal saline solution. Tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5μ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The hematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus BX40 photomicroscope and photographed.

2.2.6.3 In vitro studies using H9c2 cell lines

The active extracts, fraction and compounds from the above *in vivo* studies were further studied for their protective against H_2O_2 and Xanthine –xanthine (X+XO) generated reactive oxygen species (ROS) in rat myocardial cell lines H9c2.

a) Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO, Invitrogen Corporation, Carlsbad, California. Tetramethylrhodamine methyl ester (TMRM), propidium iodide, were obtained from Molecular Probes (Eugene, OR). Annexin V was purchased from BD Bioscience Pharmingen (San Diego, CA). 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6$), dihydro ethidium (DHE), 3-(4, 5-dimethylthiazol-2-yl)- 2, 5-

diphenyltetrazolium bromide (MTT), 2', 7'– dichlorofluorencein diacetate (DCF-DA), 4',6-diamidino-2-phenylindole (DAPI), penicillin, RNAase A, streptomycin, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest grade available.

b) Cell culture

The H9c2 cell line was originally derived from embryonic rat heart tissue using selective serial passages and was purchased from America Tissue Type Collection (Manassas, VA; catalog # CRL – 1446). Cells were cultured in DMEM medium supplemented with 10% FCS, 100U/ml of penicillin and 100µg/ml of streptomycin in 75cm² tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. Cells were fed every 2–3 days, and sub-cultured once they reached 70 – 80% confluency in order to prevent the loss of differentiation potential.

H_2O_2 and X+XO were dissolved in DMEM without FCS and test materials were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was not more than 0.2% during the experiments.

2.2.6.3.1 Analysis of cell viability

The viability of cells after treatment with test material was assayed by the reduction of 3-(4, 5-dimethylthiazole-2-yl)-2, 5- diphenyl-tetrazoliumbromide (MTT) to formazan as described previously (Plumb, 1989). Briefly, cells were seeded in 96- well microtiter plates (5×10^3 cells per well), and left to adhere to the plates overnight before being exposed to different concentrations of test material. In each experiment, different concentrations of test material (1, 2, 5, 10, 20, 50 and 100µg/ml) were tested in three separate wells and the cytotoxicity curve was constructed from three different experiments. After exposure to test material, 10µl of 5 mM MTT solution was added to each well, and the cells were incubated in the dark at 37°C for an additional 4hrs. Thereafter, the medium was removed, the formazan crystals were dissolved in 100µl of DMSO and the absorbance was measured at 570nm in a microplate reader (Molecular Devices, Spectra MAX 250). The data of the survival curves were expressed as the percentage of untreated controls.

2.2.6.3.2 Effect of test material on reactive oxygen species (ROS)

Antioxidant potential of test material can be studied by analyzing its effect on H_2O_2 and xanthine-xanthine oxidase generated ROS in H9c2 cells after loading cells with ROS specific molecular probes like (DCF-DA, DHE and DHR

123) which emit fluorescence after exciting at particular wavelength. This change in fluorescence intensity can be analyzed with FACs or Confocal microscopy. The detailed methodology is depicted below.

2.2.6.3.2.1 Measurement of intracellular ROS

Generation of ROS was monitored by measurement of hydrogen peroxide (H_2O_2) generated using the fluorescent probe DCF-DA (Kuo, 1998). Briefly, H9c2 cells were harvested from the culture flask by trypsinisation and an aliquot of 2 x 10^5 cells were incubated with test material in 6 well plates for definite time period. Cells were co-incubated with 2,7-Dichlorodihydrofluorescein diacetate (DCF-DA) (5μM) in the absence and also in the presence of H_2O_2 (10μM) separately with different concentrations of test material for 30min at 37°C in the dark. After treatment, cells were immediately washed and resuspended in 1X PBS, filtered through nylon mesh and analyzed by flow cytometry. The fluorescence emitted at 525 nm was measured with a FACS calibur flow cytometer (Beckton Dickinson, San Jose, CA) and analyzed using the CELL Quest ™ software. Ten thousand cells were examined for each sample with a flow rate of 300-600 cells per second. The values are expressed as percentage of fluorescence in control.

The intracellular H_2O_2 was detected by confocal microscopy (Ye, 1999). DCF-DA, dye specific for intracellular H_2O_2, was used for this study. H9c2 cells were plated onto glass cover slips in 6 well plates and exposed to H_2O_2 with and without test material for definite time period. After being stained for 30 min in the dark with DCF-DA, the cells were washed with PBS and fixed with 10% buffered formalin. The glass cover slip was mounted on a glass slide and observed using a Saratro 2000 (Molecular Dynamics, Inc., Sunnyrate, CA) laser scanning confocal microscope (Optiphot-2, Nikon, Inc., Melville, PA).

2.2.6.3.2.2 Measurement of *Cellular Superoxide (O2·)*

The intracellular super oxide anion ($O_2^{\bullet-}$) was detected by confocal microscopy using dihydroethidium (DHE). Dihydroethidium is a $O_2^{\bullet-}$ specific dye. Superoxide radicals were generated in the cells by using xanthine- xanthine oxidase system. Cells were cultured in a 6-well plate at 5x10^5/well. Dihydroethidium was added into the cell culture 15 min before the treatment was completed, and the staining was carried out at 37°C. Then the cells were washed in PBS and harvested for FACS analysis.

In the case of cellular image, the cells (2X10^4/well) were plated onto a glass slip in the 6-well plate. After being stained, the cells were washed in PBS and fixed with 10% buffered formalin. The slip was mounted on a glass slide and observed using a Saratro 2000 (Molecular Dynamics, Inc., Sunnyrale, CA) laser scanning confocal microscope (Optiphot-2, Nikon, Inc., Melville, PA) fitted with an argon-ion laser (Cai J, 1998).

2.2.6.3.2.3 Measurement of endogenous nitric oxide (NO)

Nitric oxide production in the cells was measured by using the NO sensitive dye 4, 5-Diaminofluorescein diacetate (DAF-2DA). DAF-2DA is a cell-permeable molecule and is hydrolyzed to an impermeable non fluorescent active form, DAF-2, in cells by the action of esterases. On reaction with NO, DAF-2 is then converted to a cell-impermeable fluorescent form, DAF-2T, whose presence can be measured as a specific increase in fluorescence signal. Cells were incubated in 6 well plate on cover slips with DAF-2DA for 60 minutes at room temperature and then washed and incubated for another 15 minutes in the experimental buffer. DAF-2T was excited at 480 nm and collected through a 535/40-nm band pass emission filter (Kojima H, 1998; Zhong Zhang, 2004; Roychowdhury, 2002).

2.2.6.3.2.4 Measurement of cellular peroxynitrite

Direct visualization of mitochondrial ROS was made using dihydrorhodamine (DHR123). DHR123 selectively accumulates in the mitochondria, where it is oxidized by mitochondrial ROS to the fluorescent rhodamine derivative. After the treatment period cells were incubated with DHR123 (1mmol/L) for 1 hour before visualization by confocal microscopy. DHR is used as an indicator of peroxynitrite formation. It is oxidized by peroxynitrite to the highly fluorescent product rhodamine in vitro. Neither nitric oxide, superoxide nor hydrogen peroxide alone appears to oxidize DHR. Formation of DHR can be monitored by fluorescence microscopy using excitation and emission wavelength of 500 and 536nm respectively (Crow JP, 1997).

2.2.6.3.2.5 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) with TMRE

The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is a hallmark for apoptosis. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical

gradient (referred to as $\Delta\Psi_m$) across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm (Desagher S, 1999; Narita M, 1998; Basanez G; 1999; Luo X, 1998).

This assay utilizes a derivative of Tetramethyl Rhodamine (TMRE) to visualize mitochondrial membrane potential. The TMRE is a cell permeable cationic dye that has a strong fluorescent signal and exhibits low membrane potential independent binding. In healthy cells TMRE is accumulated by the mitochondria in proportion to the DeltaPsi (membrane potential). In most cell lines, accumulation of TMRE in the mitochondria results in a quenched fluorescence intensity. In apoptotic cells, where the mitochondrial membrane potential is compromised, TMRE is not accumulated in the mitochondria and therefore its fluorescence is not quenched (Ehrenberg B, 1988; Farkas DL, 1990; Russell C, 1999)

In brief, culture cells in 6-well plates ($5x10^5$) cells/ml. After pretreatment with test material induce apoptosis by using 35μM of H_2O_2 in medium. A negative control should also be set up at this time point. After the activation times add 100nm of the 20X TMRE to each well. Incubate the samples for 30 to 60 minutes at the same culture conditions 37°C at 5% CO_2. Transfer cells to a FACS tube and add 500 μL PBS and analyze samples via Flow Cytometer at excitation: 488nm and measure emission on the FL2 Channel.

2.2.6.3.2.6 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) with DiOC6 (3)

Recently, mitochondria have been regarded as the key regulator of cell death [Bernardi P, 1999) Healthy mitochondria maintain a negative membrane potential across the mitochondrial inner membrane, collapse of ΔΨm lead to a reduction of mitochondrial mass which could activate the cell death cascade in turn. 3, 3'- dihexyloxacarbocyanine iodide (DiOC6), a fluorescent dye that incorporate into mitochondria in a ΔΨm dependent manner was used to evaluate the changes of mitochondrial potential during treatment. In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial membrane potential. After treatment of the test material cells cultured on the cover slip (10^6 cells/ml), were incubated at 37°C

for further 15min with DiOC6 (4mol/L). Fluorescence was immediately recorded with Saratro 2000 (Molecular Dynamics, Inc., Sunnyrate, CA) laser scanning confocal microscope (Optiphot-2, Nikon, Inc., Melville, PA). (λex max 484 nm, λem max 501 nm) [Rottenberg H, 1998].

2.2.6.3.2.7 Apoptosis detection by nuclear staining

4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) is known to form fluorescent complexes with natural double-stranded DNA and useful to find the apoptotic nuclei. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining. The cells were seeded on cover slip in 6 well plates and exposed to test material for the definite time. Cells are washed once with PBS, and then incubated in PBS containing 0.1 % Triton X (to induce holes in the cells' membrane and increase permeability) for 10 min on ice. After washing again with PBS cells were incubated in 4% buffered paraformaldehyde solution containing 10μg/ml DAPI. Later on cover slips were fixed on the glass slides and the morphology of the cells' nuclei was observed using a Saratro 2000 (Molecular Dynamics, Inc., Sunnyrate, CA) laser scanning confocal microscope (Optiphot-2, Nikon, Inc., Melville, PA) fitted with an argon-ion laser, at excitation wavelength 350 nm.

2.2.7 Antitumor activity for compound isolated from Ailanthus root bark

2.2.7.1 *In vitro methods for antitumor activity*

Cell lines

The human prostate cancer (PC-3) cells were obtained from the American Type Culture Collection (Manassas, VA). and were cultured in F-12 HAM Nutrient Mixture (Sigma) supplemented with 10% FCS, 100 units/mL penicillin, 100 Ag/mL streptomycin, and 2 mmol/L glutamine in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

2.2.7.1.1 Flow cytometry analysis of DNA content for cell cycle treated with different concentrations of AECHL-1

DNA content and cell cycle distribution are useful to detect variations of growth patterns, to monitor apoptosis, cell growth and defects in cell cycle regulation, oncology research, DNA ploidy determinations, to study tumor behavior and suppressor gene mechanisms. Cell cycle analysis based on measurements of DNA content generates a clear pattern of distribution of G0/G1 phase (one set of paired chromosomes per cell), S phase (DNA

synthesis with variable amount of DNA), and G2/M phase (two sets of paired chromosomes per cell, prior to cell division). DNA content can be measured using fluorescent, DNA-selective stains that exhibit emission signals proportional to DNA mass. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various phases of the cell cycle. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain (propidium iodide).

In brief, about 5×10^5 cells/well in 6-well plates were incubated with AECHL-1 (0, 5, 10, 20, 50 100 µg/ml) for different time periods. The cells were trypsinised and harvested by centrifugation with repeated PBS washing and fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight at -20°C and then after repeated washing with PBS, re-suspended in PBS containing 50µg/ml propidium iodide, 0.1 g/L RNase (Sigma) and Triton X-100. Incubate the cells for 30 min at 37°C in the dark, and then transferred to the tubes, analyzed with flow cytometry (FACS Vantage–BD Sciences, USA) equipped with an argon laser at 488 nm. The data was analyzed using Modfit software for determining the percent population in different phases of cell cycle.

2.2.7.1.2 Annexin V-PI Staining For Apoptosis Detection

Apoptosis is a fundamental mode of cell death which performs a regulatory function during normal development, in tissue homeostasis, and in some disease processes. In normal viable cells phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids in most cell types occurs leading to exposure of PS on the cell surface. Recognition of PS by phagocytes *in vivo* results in the removal of cells programmed to die thus apoptosis is not commonly associated with the local inflammatory response which accompanies necrosis *In vitro* detection of externalized PS can be achieved through interaction with the anticoagulant annexin V. In the presence of calcium, rapid high affinity binding of annexin V to PS occurs. PS translocation to the cell surface precedes nuclear breakdown, DNA fragmentation, and the appearance of most apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis (Fig-2.1).

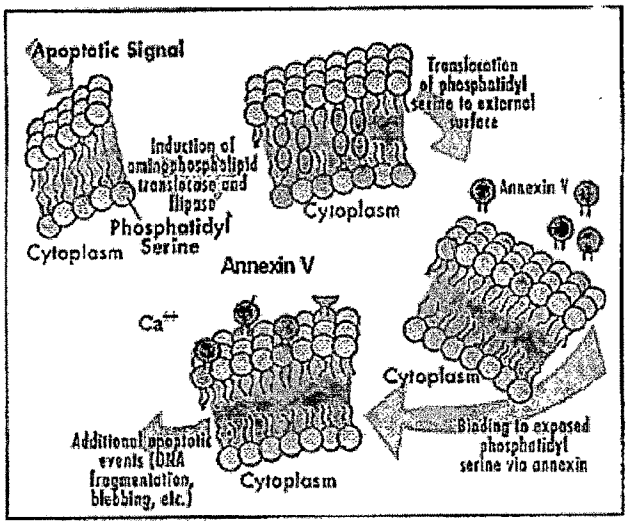

Fig-2.1 Annexin/Phosphatidyl Serine in early stages of Apoptosis

In this assay Annexin V is used for detection of apoptosis by flow cytometry microscopy. Since membrane permeabilization is observed in necrosis, necrotic cells will also bind Annexin V. Propidium iodide is used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. Necrotic cells will bind Annexin V and stain with propidium iodide while propidium iodide will be excluded from viable and early apoptotic cells. In the absence of phagocytosis final stages of apoptosis involve necrotic-like disintegration of the total cell, thus cells in late apoptosis will be labeled with both Annexin V and propidium iodide. (Frey T. 1997)

Procedure for Annexin V binding with adherent Cells

Transfer media from flask of adherent cells to a 15 ml conical tube and place on ice. *(Note: This media will contain cells that have become detached from the flask during the cell death process).* Gently wash cells in flask with 10 ml PBS (pH-7.4). Remove PBS and add 1-2 ml 0.5X trypsin and incubate just until cells appear detached by microscopic evaluation. Release cells from flask with firm tapping. Gently resuspend cells in media from step 1 OR 1X cold binding buffer to approximately 1 x 10^6 cells/ml. Transfer 0.5 ml of cell suspension to a microfuge tube. Add 10µl Media Binding Reagent. Add 1.25µl Annexin V. Incubate for 15 min at room temperature in the dark. Centrifuge at 1000 x g for 5 min at room temperature. Remove media. Gently resuspend cells in 0.5 ml cold 1X Binding Buffer and add 10 µl Propidium Iodide. Place the samples on ice and away from light and analyze by flow cytometry or fluorescence microscopy immediately.

Analysis by Flow Cytometry

A flow cytometer emitting an excitation light at 488 nm from an argon ion laser should be used to quantify the Annexin V and propidium iodide signals. To set up the flow cytometer use apoptosis-induced cells stained with Annexin V only and apoptosis-induced cells labeled with only propidium iodide. The Annexin V signal can be detected by FL1 at 518 nm. Propidium iodide fluoresces at 620 nm and can be detected by FL2. Perform necessary adjustments to minimize overlap between these two measurements. The log of annexin V fluorescence should be displayed on the X axis and the log of propidium iodide fluorescence on the Y axis (Fig-2.2).

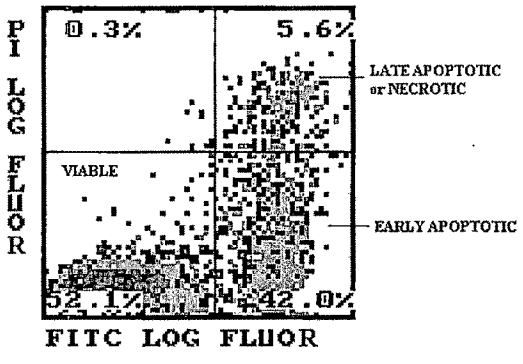

Fig-2.2 Distribution of cells after staining with Annexin V and PI

In the above cytogram viable cells do not bind Annexin V or propidium iodide as reflected in the lower left-hand quadrant of the dot plot. Early apoptotic cells with exposed PS but intact cell membranes bind Annexin V but exclude propidium iodide. Fluorescence from this population is reported in the lower right hand quadrant. Necrotic or apoptotic cells in terminal stages will be both Annexin V and propidium iodide positive and was reported in the upper right-hand quadrant. A small percentage of normal cell death should be expected in routine cultures of untreated cells.

2.2.7.1.3 Tritiated thymidine ([3H]-TdR) incorporation assay

Cell proliferation is the measurement of the number of cells that are dividing in a culture. Cell proliferation was measured by analyzing the DNA synthesis in cell by incorporating the labeled DNA precursors (^{3}H-thymidine) in cells and quantifying with liquid scintillation counter. Amount of the labeled precursor into DNA is directly proportional to the amount of cell division occurring in the culture.

Cell proliferation was measured by tritiated thymidine incorporation assay, based on the incorporation of ^{3}H-thymidine into DNA during cell growth. Cells were grown 96 well plates and treated with serial concentrations of AECHL-1 (5 to 50µg/ml) and incubated for 24, 48 hr, during the last 18 hr before termination of the assay 0.5µCi ^{3}H thymidine was added to wells. Cells in the cultures were trypsinised and harvested using cell harvester (Nunc, Denmark). The radioactivity measured as counts per minute (CPM) was determined using a liquid scintillation counter (Packard, USA). The percent inhibition was calculated by considering incorporation in control (untreated) cells as 100%.

2.2.7.2 In vivo methods for antitumor activity

2.2.7.2.1 Materials

Antibodies

Antibodies like Cyclin D1, CDK4, P53, pP53, β-Actin from mouse and Nf-kB, Bcl2, P21 from rabbit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of B16-F10 cells

Bl6 cells were cultured in and maintained in Dulbecco's minimal essential media (DMEM) containing 10% FBS and penicillin/streptomycin.

Murine Tumor Models

Male C57Bl/6 mice (6–8 weeks of age) were obtained from National Center for Cell Sciences, Pune, India, and kept in standard housing with standard rodent chow and water available *ad libitum* at a 12-hour light/dark cycle. Experiments were performed according to national regulations and were approved by the local animal experiments ethical committee.

Test material

Cis-platin obtained from Sigma chemicals, both AECHL-1 and Cis-platin were dissolved in PBS before administration.

2.2.7.2.2 Method for antitumor activity

Following one week of acclimation, $5x10^{5}$ B16-F10 melanoma cells in 100µl PBS were injected subcutaneously in the flank of C57Bl/6 mice. B16.F10 tumors became palpable around 7 days after tumor cell inoculation. Animals were assessed for the presence of tumors every other day and tumor measurement began 10 days after implantation and continued biweekly until

tumors reached approximately 8–10% of animals' body weights. The length (L) and width (W) of tumors were measured using vernier calipers (Mitutoyo, Kawasaki Kanagawa, Japan) and tumor volume calculated using the formula LxW2/2. After 15 days of inoculation, animals were divided into four groups ie control, AECHL-1 (50µg), AECHL-1 (100µg) and cis-platin (100µg) respectively with (n=6) in each group. The animals were given intratumor injections of AECHL-1 and cis-platin in PBS while control received only PBS for 15 days. Tumor volume was measured at regular interval during the study. At the termination of the experiment animals were sacrificed under etheral anesthesia, tumor was dissected out and small pieces from every tumor were store in liquid nitrogen and used for western analysis (Raymond M, 2005). Tumors were weighed to get the tumor/body weight ratio.

Different organs like kidney, heart, liver, spleen, and pancreas were isolated as a whole and immersed in 4% buffered formalin. Sections were prepared from paraffin-embedded specimens. Samples were stained with haematoxylin and eosin (H & E) stains in order to study the effect of AECHL-1 and cis-platin.

Protocol for western bloat analysis is given in **ANNEXURE-I**

2.2.8 Effect of chloroform extract of Ailanthus root bark (AECHL) on the haemodynamics of rats

Male rats of SD Strain weighing between 250-280 g were administered with a single dose of AECHL 2000mg/kg body weight p.o. Heart and circulatory functions were measured with ultra miniature catheter pressure transducers in spontaneously breathing rat anaesthetized with ketamine 80 mg/kg i.p. After shaving and midline cervical incision, the right carotid artery was exposed. Microcatheter (Millar, Mikro-Tip, Millar Instruments, Houston, TX, USA) was inserted in the right carotid artery. A security suture was then placed around the artery to fix the catheters in place to protect haemostasis. The tip of the Millar catheter was positioned in the left ventricle and cutaneous ECG needles were positioned on the four limbs. Various haemodynamic variables, such as heart rate, ECG, systolic pressure, diastolic pressure, mean pressure, systolic and diastolic duration, left ventricular contractility (dP/dt_{max}) and relaxation (dP/dt_{min}), contractility index, pressure time index were recorded continuously on a PC using the Power Lab

system (Power Lab, AD Instruments). The catheter position for haemodynamic measurement is presented in (Fig-2.3).

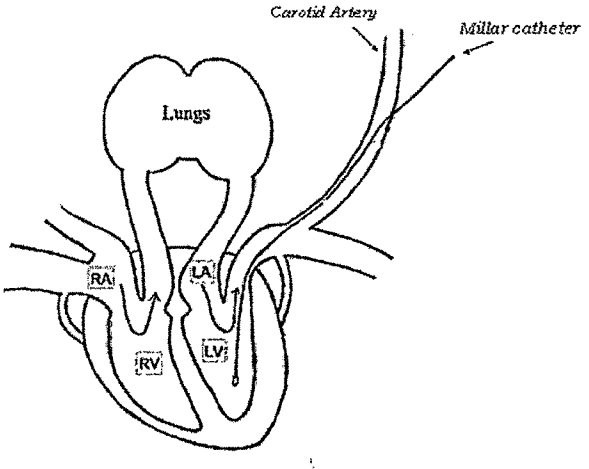

Figure- 2.3 Catheter setting for haemodynamic measurements

The tip of the Millar catheter is placed in the left ventricle (LV)

2.2.8 Effect of compounds isolated from root bark on electrophysiological characteristics of the heart using neonatal rat cardiomyocytes

2.2.8.1 *Isolation of Neonatal Rat Cardiomyocytes*

Wistar rats (1–5-days old) were used for isolation of neonatal cardiomyocytes. Cardiomyocytes adhered to the cultivation plate, and then initiate rhythmical contraction. Moreover, the cardiomyocytes from neonatal rats were more resistant to cellular disintegration than cardiomyocytes obtained from older rats, which are less active and lose contraction their ability quickly. Quality of the cardiomyocyte preparation, as evidenced by an increase of cell debris and a loss of cell viability decreases as the age of the neonatal rat advances. (Harary I, 1963). Newborn rats are sacrificed by decapitation for isolation of cardiomyocytes (Estevez MD, 2000; Post JA, 1988; Lampidis TJ, 1980; Post JA, 1992).

2.2.8.2 Preparation of neonatal rat cardiomyocytes

The heart was removed from 2- to 5-day old rats and minced in a balanced salt solution containing 20 mM HEPES, 120 mM NaCl, 1 mM NaH2PO4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM MgSO4 (pH 7.3–7.4). The minced tissue was washed with balanced salt solution containing trypsin (0.2%) by stirring at 37 °C in a water bath for 20 min. The enzymatic solution with free cells was

then removed and discarded. The pieces of tissue were incubated again with fresh solution containing trypsin (0.2%) for 20 min at 37 °C. The supernatant was collected and centrifuged at 1200 rpm for 10 min. The obtained pellet of cells was resuspended in 2 ml fetal calf serum. The cell suspension was kept at 37 °C in an incubator under an atmosphere of 95% air and 5% CO2. The digestion step was repeated five times.

The cell suspensions from each digestion were combined and centrifuged at 1200 rpm for 10 min. The pellet of cells was resuspended in DMEM supplemented with horse serum (10%), fetal calf serum (5%), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were plated for 1.5–2 h to allow the differential attachment of non-myocardial cells. The non-adhesive cells (cardiomyocytes) were transferred into a centrifugation tube, washed and centrifuged at 1200 rpm for 10 min. After counting the myocyte-enriched suspension was transferred to gelatin coated culture dishes at density of $5x10^4$ cells per cm^2. The viability of cells (85–90%) was determined by exclusion of trypan blue dye. The cells were incubated in 95% air and 5% CO2 at 37 °C. The medium was removed after 72 h and replaced by a medium containing DMEM with penicillin (100 U/ml) and streptomycin (100 mg/ml). The cultured cardiomyocytes were allowed to reach confluence before being used experimentally. The percentage of beating myocardial cells exceeds 85% after 3 d in culture in each experiment.

2.2.8.3 Effect of compounds isolated from Ailanthus root bark on mechanical properties of myocytes measured with IonOptix

Mechanical properties of ventricular myocytes were assessed by using a SoftEdge MyoCam system (IonOptix, Milton, MA). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70, Olympus Optical, Tokyo, Japan). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera, which rapidly scans the image every 8.3 ms. The cells were field stimulated with suprathreshold voltage at a frequency of 0·5 Hz, 8 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE, USA). The myocytes being studied was displayed on a computer monitor using an IonOptix MyoCam camera (IonOptix Corporation, Milton, MA, USA). IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Following

contractile indices like peak shortening (PS) amplitude, maximal velocity of shortening/relengthening ($\pm dL/dt$), time to PS (TPS), and time to 90% relengthening (TR_{90}) were measured (Fig-2.4).

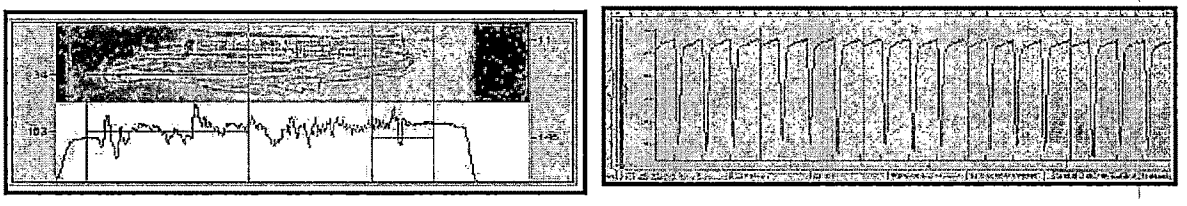

Fig-2.4 SoftEdge™ – Myocyte Cell Length Recording via Digital Imaging (Acquisition of length traces).

2.2.9 Effect of compounds isolated from Ailanthus root bark on cytosolic and nuclear calcium in H9c2 cells (Fluo-3/AM assay)

Fluo-3 fluorescence depends on concentration of free Ca^{2+} (Kao, 1989; Minta, 1989). With the help of calcium-sensitive fluorescent dyes and video microscopic imaging detection of the changes in intracellular calcium in the cytoplasm, in the perinuclear region, and in the nucleus gets possible. However, the presence of calcium in the nucleus is often difficult to ascertain because the fluorescence derived from the perinuclear area interferes with that of the nucleus. We have used confocal microscopy and flow activated cytometry along with calcium-sensitive dyes acetoxymethyl esters of fluo 3 (fluo 3-AM) to analyze the effect of test material on cytosolic and nuclear calcium distribution in H9c2 cells studied at rest and after with calcium stimuli (Burnier M, 1994).

Cells (5×10^5) were incubated in 3ml of medium (without foetal calf serum) in the humidified atmosphere with 5% CO2 at 37°C in the absence or presence of test material. Directly to this medium, 1.8µmol/l fluo 3- AM was added and the mixtures were incubated for additional 30 min at the same condition. Samples were washed twice with PBS; and evaluated by using confocal microscope and flow activated cytometry at an excitation of 488 nm. Emission at the range 493-620 nm was detected. Cells incubated with fluo 3- AM but without test material were used in control experiments. (Orlicky J, 2004)

The experiment was standardized by setting fluorescence with unstained and fluo stained cells. ionomycin and ethylene glycol tetra acetic acid (EGTA) was used as a reference standard.

2.2.10 Statistical Analysis of Data

Results of all the in vivo experiments have been expressed as mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons test and Dunnett multiple comparisons tests.

For *in vitro* experiments values are expressed as mean± SEM.

2.2.11 TLC fingerprint profiles

For HPTLC fingerprint profile, stock solutions of the extracts/fractions were prepared in respective solvents. Different concentrations of samples were applied on pre-coated Silica gel G60 F_{254} TLC plates using CAMAG Linomat V automatic sample spotter and the plates were developed in solvent systems of different polarities to resolve polar and non-polar components of the extracts/fractions. The plates were scanned using TLC Scanner 3 (CAMAG) at 254, 366 and 520nm. The R_f values, spectra, λ_{max} and peak areas of the resolved bands were recorded. Relative percentage of each band was calculated from peak areas (Ravishankar M N, 2002).