Chapter -4

Summary

SUMMARY

Antioxidants are the compounds of exogenous or endogenous origin which, either prevents the generation free radicals or intercept any that are generated and inactivate them, thereby blocking the chain propagation reaction produced by these oxidants

Free radicals are chemical species associated with an odd or unpaired electron which reacts with membrane lipids, nucleic acids proteins, and enzymes, resulting in cellular damage. These are the major contributor to aging; cancer, atherosclerosis, parkinson's disease, hepatic damage, cardiovascular disease, cataracts, immune system decline, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress.

Free radicals are generated from various endogenous and exogenous sources which include tobacco, smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. Natural antioxidants like catalase, superoxide dismutase, glutathione peroxidase, serve to protect the body from generated free radicals.

The moieties having antioxidant potential are supplemented in the case of lack of endogenous antioxidants.

Synthetic antioxidants, such as butylated hydroxyl toluene and butylated hydroxyl anisole, are to be restricted use in foods as these are suspected to be carcinogenic in nature.

Therefore, the present studies were conceived to explore antioxidant potential of two medicinal plants *Ailanthus excelsa* Roxb (Simaroubaceae) and *Butea monosperma* Lam (Leguminosae), based on their traditional claims. Leaves and root bark from *Ailanthus*, while flowers of Butea were selected based on the literature and use in the ayurvedic formulations.

A. excelsa Roxb (*Tree of Heaven*) is a tree, indigenous to central and southern India, belongs to family Simaroubaceae is widely used in Ayurveda and evidence based phytotherapy. The roots of this plant also serve as one of the constituents of an ayurvedic formulation *Dasmularista*. The root bark is used to cure epilepsy and heart troubles.

The leaves are used by the tribal women in debility after child birth. Different parts of the tree were worked out for their antifertility, antifungal, anticancer, antihypertensive and antipyretic activity. In our previous study we found *A. excelsa* leaves as a good hepatoprotective agent. Traditionally the plant is used to cure cardiac disorders. Based on these traditional claims regarding its role in cardiac disorder, isoproterenol induced myocardial infarction was used as an ideal model for evaluating its cardioprotective and antioxidant property.

B. monosperma Lam (Flame of the forest), is an erect tree inhabiting mountainous regions of India, Burma and Ceylon, traditionally used in leprosy, gout, skin disease and biliousness. Flowers contain butein, butin, butrin, Isobutrin, coreopsin, isocoreopsin (butin 7-glucoside), sulphurein, monospermoside (Butein-3-β-D-glucoside) and isomonospermoside. Different parts of the tree are worked out for their antifertility, antifungal, anthelmintic, hepatoprotective, antinflammatory, antidibetic, antidiarrhoel, antipyretic, antimicrobial, antiulcer, and anticonvulsant activity. Literature revealed the usage of these flowers in liver disorders.

Therefore the present investigation were undertaken to explore the antioxidant potential of these plants to verify their traditional claims.

The plant material was collected from the local area of Amravati, Maharashtra and Baroda, Gujarat and authenticated in the Botany department, of the M.S. University of Baroda.

Successive extracts of *Ailanthus* leaves and root bark in (petroleum ether, diethyl ether, chloroform, ethyl acetate, methanol, water) were prepared. Flowers of Butea were extracted with methanol and fractionated in ethyl acetate, butanol and water, while successive extracts of stem bark in (petroleum ether, benzene, chloroform, ethyl acetate and methanol) were prepared.

All the above extracts were subjected to preliminary phytochemical analysis which revealed the presence of flavonoids, steroids, triterpenoids, sugars, amino acids in various extracts. Percentage phenolic content was determined by using Folin-Ciocalteau reagent, where ethyl acetate and diethyl ether extract were found to be rich in phenolic content.

All the above extracts were subjected to rapid screening for the presence of free radical scavengers by developing on TLC plates and dipping in DPPH solution, which showed that diethyl ether (AEDT), chloroform (AECL), ethyl acetate (AEEA) and successive methanol (AEME) extract were rich in radical scavenging compounds.

All the successive extracts along with total methanol and aqueous extracts were further screened for in-vitro antioxidant activity, by using reducing power assay and DPPH, superoxide, nitric oxide anion, hydroxyl radical scavenging assay, which reveals AEDT, AEEA, AECL, and AEME more active compared to others. AEDT, AECL, AEEA and AEME showed significant reduction in erythrocytes lysis mediated by 2, 2'-azo-bis (2-amidinopropane) dihydropropane. All the above results were compared with reference standards like, ascorbic acid, rutin, quercetin, curcumin respectively.

In order to carryout biological studies toxicity study was carried out on these selected extracts, as per OECD guideline in female Balb/C mice, where all the extracts were found to be non toxic at a dose of 2000mg/kg b.w. (p.o) which was supported by histopathological study. Safe dose 200mg/kg body weight was taken for further studies.

The selected extracts were further screened for *in vivo* antioxidant activity using Isoproterenol (ISO) induced myocardial infarction (MI) and liver damage in male albino rats of SD strain. Endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and lipid peroxides (LPO) were analyzed in heart homogenate along with serum marker enzymes like glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALKP), creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), and uric acid.

Subcutaneous injection of ISO (25 mg/kg b.w./ml saline) to rats, at 24hrs interval for two consecutive days caused myocardial damage in rat, which was determined by the increased levels of endogenous antioxidant enzymes along with serum marker enzymes.

The study was further supported by the histopathological examination of the heart and liver and comparing with that of control and ISO treated groups.

The treatment with AEEA and AEDT was found to increase the ISO affected levels of endogenous antioxidant enzymes. AEEA showed pronounced effect by significantly decreasing the ISO elevated levels of cardiac and liver marker enzymes along with uric acid, while AEDT significantly increases the CKMB, LDH level with non significant change in liver marker enzymes and just manage to reduce the serum uric acid level, compared with ISO group. Even AEDT alone pretreated group showed significant increase in CKMB, LDH level. Thus AEDT failed to protect the ISO induced cardiac and liver damage. When compared with ISO group, AECL treated group was found to reverse the ISO affected levels of endogenous antioxidant enzymes except in SOD as the change was not significant but it showed significant increase in LPO level, while AEME pretreated group did not showed any significant change in ISO affected antioxidant enzymes except non significant reduction in SOD and increase in LPO level when compared with ISO group. Both AECL and AEME alone pretreated group showed non significant reduction in SOD and CAT level with significant increase in GSH and LPO level when compared with control group. In case of AECL there was significant increase in LPO level compared to control. AECL alone showed non significant reduction in CKMB and significant reduction in LDH, with significant increase in the levels of SGPT and non significant increase SGOT, ALKP, and uric acid, while AEME showed non significant increase in the level of CKMB, LDH, SGOT, and ALKP with significant increase in SGPT, and uric acid when compared with control group. Thus only AEEA pretreated group showed significant protection against ISO induced myocardial infarction, with increase in endogenous antioxidant enzyme levels.

AEEA was further subjected to study its effect on myocardial cell lines H9c2. Percentage cell viability was checked after incubation of H9c2 cells with different concentration of AEEA (2-100µg/ml) at different time interval.
AEEA showed concentration and time dependent effect on cell viability. As both the concentration and time increased cell viability was found to be decreased. These results obtained for AEEA provides a lead for dose fixation for further studies carried.

Antioxidant activity of AEEA was studied on H_2O_2 induced stress in H9c2 cell lines, where H_2O_2 treatment reduces the cell viability. AEEA reverse the H_2O_2 effects in concentration and time dependent manner. As concentration and incubation period increased cell viability was found to be decreased. Decreased viability with increasing concentration might be due to prooxidant effect of drug.

AEEA was also studied for its effect on reactive oxygen species (ROS) generated by using H_2O_2 and xanthine-xanthine oxidase system in H9c2. AEEA pretreatment provides significant protection against H_2O_2 and Xanthine-xanthine oxidase induced stress in H9c2 cells, when analyzed by flow activated cytometry and confocal microscopy after loading the cells with radical specific molecular probes like DC-FDA, DHE, DHR-123.

AEEA also protects the H_2O_2 induced loss of membrane potential in H9c2 cells analyzed after loading cells with membrane potential specific probes like TMRE and DiOC6.

Above study revealed the antioxidant potential of ethyl acetate extract of *A. excelsa* leaves (AEEA), where it showed significant radical scavenging effect by various in vitro and ex-vivo methods. AEEA showed high concentration of phenolic content. It also provides significant protection against ISO induced MI without showing any toxic effect. AEEA also showed concentration dependent effect on H9c2 cells where at higher concentration it showed prooxidant effect like most of the well known antioxidants. AEEA prevents the generation of H_2O_2 and xanthine–xanthine oxidase induced ROS in H9c2 cells and thus showed protective effect.

Chromatographic studies showed the presence of phenolic and specifically flavonoids in AEEA, which are previously known to possess antioxidant activity.

Phytochemical and analytical data showed the presence of various flavonoids like apigenin, kaempferol, luteoline and for the first time we found the presence of quercetin in the leaves. These all flavonoids are present in glucoside form and are previously reported to be responsible for the antioxidant effect. Quantitative analysis by HPTLC and spectrofluorimetry showed high concentration of quercetin and apigenin in the ethyl acetate fraction. Flavonoids in leaves are

responsible for the antioxidant effect of *A. excelsa*, by increasing the endogenous antioxidant levels these compounds protects liver and cardiovascular system thus fulfilling the claims made in traditional literature.

Successive petroleum ether, benzene, chloroform, ethyl acetate, methanol, and aqueous extracts of root bark of *A. excelsa* were prepared and subjected to preliminary phytochemical analysis, which reveals the presence of alkaloids, steroids, triterpenoids and tannins. Percentage phenolic content was determined by using Folin-Ciocalteau reagent, where chloroform fraction showed high percentage of phenolics. Rapid screening for radical scavengers (TLC/DPPH) revealed the presence of free radical scavengers in chloroform, ethyl acetate and benzene extracts.

All the extracts along with total methanol and aqueous, were further screened for in-vitro and ex vivo antioxidant activity using (AAPH/ RBC) system, as mentioned earlier, which revealed chloroform, ethyl acetate, and benzene extract more active.

Literature revealed conflicting reports on the safety of root bark and their use in the ayurvedic formulations. All the extracts from root bark were subjected for toxicological evaluation as mentioned earlier. Chloroform extract was found to be non lethal at the dose of 55mg/kg body weight orally. While successive aqueous extract was found to be non toxic at a dose of 2000mg/kg b.w. which was supported with histopathological examination of different organs.

TLC study of chloroform, ethyl acetate, benzene and methanol extract showed alkaloids and quassinoids as common constituents present in chloroform, ethyl acetate, benzene and methanol extract which were absent in aqueous extract. The chromatographic profile of all the three extracts was nearly similar.

The TLC plate developed above was dipped in DPPH solution, where chloroform, ethyl acetate and benzene extracts were found to contain the antioxidant constituents. The antioxidant present in all the three extracts had same Rf and λ max. As these extracts were also found to contain alkaloids in common, a total alkaloidal extract of root bark was prepared separately and screened for in-vitro antioxidant activity. Although the extract was found to have good antioxidant activity also inhibit the AAPH induced erythrocytes lysis as

happened to be cytotoxic in nature so not take for further studies. The *in vitro* antioxidant activity of the ethyl acetate, chloroform, benzene extract and alkaloidal fraction may be attributed to the phenolic nature of the alkaloids and quassinoids.

Chloroform extract (AECHL) based on its radical scavenging potency was further evaluated for chronic toxicity study. The long term treatments of chloroform extract of root bark AECHL (5.5mg/kg) in rats showed increase in WBC and platelet counts with decreased RBC and hemoglobin content.

AECHL also increase the serum cortisol, CKMB and brain dopamine level. An increased level of serum cortisol indicates that administration of AECHL may affect the normal carbohydrate metabolism and increased stress in rats, in response to this serum cortisol level get increased or may also be due to increased secretion of adrenocorticotropic hormone through stimulation of pituitary gland which may leads to increased cortisol level with excessive stimulation of adrenal glands. This increased cortisol may be the reason for intestinal ulcer, muscle damage and initial increased blood pressure in rats occurred during study. Excess of creatinine in serum after AECHL administration may be due to muscular break down, which leads to loss of high amount of energy stored in the form of ATP which is required for the normal muscle contraction.

Dopamine is an intermediate in the synthesis of norepinephrine and occurs in caudate nucleus. Increased dopamine level might be due to the stimulation of sympathetic stimulation caused by AECHL.

Uric acid is an end product of nucleic acid metabolism, increased serum levels of uric acid with AECHL indicates increased nucleic acid metabolism.

Increased bilirubin with AECHL may be due to break down of blood pigment hemoglobin, which in tern showed increase levels of serum bilirubin and decrease hemoglobin content in AECHL treated rats.

Increased level of CKMB with AECHL treatment might be due to loss of normal myocardial architecture.

Long term treatment with AECHL showed damaged liver with lots of necrotic areas. Sevier myocardial damage was observed with loss of muscle integrity and

displacement of nuclei. Development of lesions from the inner side of intestine occurred which grows slowly to form tumor like mass, this lesions formation might be due to increased serum cortisol level. Normal anatomy of stomach showed secretary epithelial sheath containing secretory cells on the inner wall of the stomach, along with surface mucosal cells, AECHL treatment showed loss of this secretory epithelial sheath of stomach.

A gradual increase in systolic, diastolic and mean blood pressure, with decreased systolic, diastolic and cycle duration was observed with single dose administration of AECHL (2000mg/kg b.w. p.o). AECHL also increased the heart rate with reduction in the contractility and pressure time index. An increase in Max dP/dt and Min dP/dt was observed. These data supports that the AECHL shows sympathomimetic effect on cardiovascular system. However this effect was abolished with time as shown by reduced systolic, diastolic and mean blood pressure increased systolic, diastolic and cycle duration time. The heart rate was decreased with a reduction in contractility index and increase in pressure time index. Thus AECHL showed reversal of its sympathomimetic effect with time.

The initial sympathomimetic effect of AECHL may be due to the stimulation of sympathetic nervous system, releasing the sympathetic neurotransmitter responsible for increasing the cardiac functions. The effect may also be due to increase in intracellular calcium level which further leads to release of calcium from the storage, sarcoplasmic reticulum, leading to increased brushing action of myocardial contractile proteins actin and myosin resulting in increased contraction.

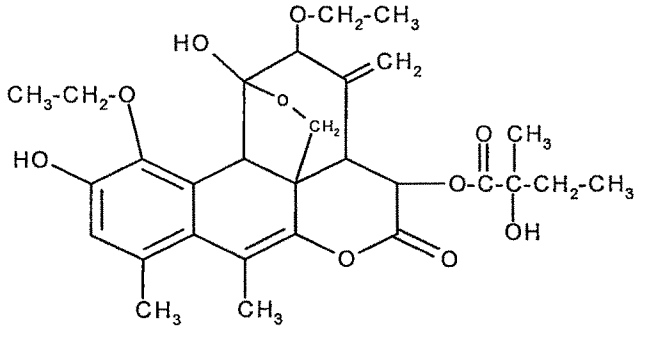
The reversal of sympathomimetic action with time may be due to aggravation of intracellular calcium level causing fatigueness of myocardium, leading to heart failure. This effect of AECHL was clearly indicated in the ECG recording, where it showed elevation of QRS complex wave with a decrease in time taken for wave formation as correlated with decrease in systolic, diastolic duration, contractility and pressure time index.

A gradual dip in the QRS complex peak height accompanied by increase in the refractory period, followed by a sharp decline in the QRS wave, dips down

drastically with an occurrence of Bigemini which is a sign of ectopic focus or ventricular arrhythmia. These changes can be correlated with increased systolic, diastolic and cycle duration along with increased contractility and pressure index. All these changes lead to drastic fall in blood pressure and heart rate causing death of animals.

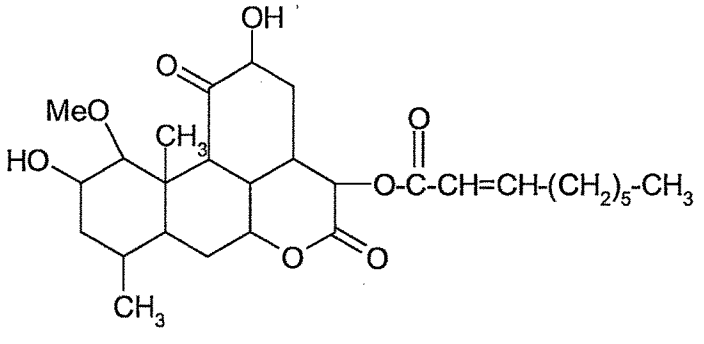
Compounds AECHL-1 and AECHL-2 were isolated from the chloroform extract of Ailanthus root bark in order to explore the possible chemical moiety responsible for the cardiovascular effects.

Compound AECHL-1 is a solid, mp. 248-250°C possessed a molecular formula of C_{29} $H_{36}O_{10}$ as indicated by EI and ES mass spectra. The IR spectrum showed the presence of hydroxyl (s) (3425nm, 3419nm), δ lactone (1733nm), and aromatic moiety (1600nm). The UV spectrum gave a characteristic absorption maximum at 235nm, indicating the presence of auxochromic groups like hydroxyl and ketone. The ^{1}H- NMR spectrum of AECHL-1 revealed the presence of an aromatic proton δ 6.89 and a singlet at δ 5.30 which is characteristic of the ester function at C-15. H-22 appeared as an AB system as a singlet at δ 4.05 and doublet at δ 3.65 and H-12 appeared as a triplet at δ 3.95. The methyl group H-19 on the aromatic ring appeared as singlet at δ 2.3. A doublet at δ 1.235 for six protons is assigned at H-5'. H- 4' appeared as a triplet at δ 0.95. The methyl group, H-18 appeared as a singlet at δ 2.16. Possible structure of AECHL-1 is shown below.



AECHL [m/z : 543 C $_{29}H_{36}O_{10}$]

Compound AECHL-2 is an orange color solid gives very bright green fluorescence at 366nm, mp. 180-184°C, has a molecular formula of C_{28} $H_{40}O_8$ as indicated by EI and ES mass spectra. The IR spectrum showed the presence of hydroxyl (s) (3683nm, 3072nm), unsaturated ester (1728 nm) and methoxy group (1110 nm). The UV spectrum gave characteristic absorption maxima at 243 nm, indicating the presence of auxochromic groups like hydroxyl and ketone. The ^{1}H- NMR spectrum of AECHL-2 revealed the presence of terminal methyl groups as doublets at δ-0.85 and δ-0.89. A doublet at δ 1.28 for six protons was assigned to H-5' to H-8' and H-4' appeared as a singlet at δ 1.33. Possible structure of AECHL-2 is shown below.



m/z : 504 [$C_{28}H_{40}O_8$]

AECHL-1was studied for its effect on % viability of H9c2 cells using MTT assay where it showed decreased cell viability in concentration and time dependent manner, showing it to be cytotoxic.

AECHL-1 generates reactive oxygen species in H9c2 cell lines when analyzed by confocal microscopy and flow activated cytometry after loading with fluorescent molecular probes.

Annexin-V and PI staining of the H9c2 cells treated with AECHL-1 for 48hrs was done and analyzed by FACs at the excitation wavelength 488nm. Treatment with AECHL-1 showed increased both apoptotic and necrotic/late apoptotic cell death necrosis was found to be more prominent compared to apoptotic cells.

To check the effect of AECHL-1 on cardiovascular system, rat neonatal ventricular myocytes were isolated and the mechanical properties of myocytes

were assessed using an IonOptix MyoCam system. Cell mechanics were assessed using the peak shortening (PS), time-to-90% PS (TPS), time-to-90% relengthening (TR_{90}) along with maximal velocities of shortening (+dL/dt) and relengthening (-dL/dt).
AECHL-1 showed initial stimulation of neonatal cardiomyocytes which gets abolished with increasing concentration.

Flow activated cytometric and confocal analysis of H9c2 cells after treatment with AECHL-1 and loading with the cells with calcium specific molecular probe fluo-3 AM showed increased intracellular calcium level in perinuclear area. The results were compared with the standards like Ionomycin, and with calcium cheater EGTA.

AECHL-1 showed the cell cycle arrest in H9c2 cells when stained with propidium iodide (PI). 24 hrs treatment of AECHL-1 showed cell growth arreste in G0/G1 phase confirmed by increased cell count in this phase and decreased cell count in S, G2/M phase. At higher concentration AECHL-1 showed necrotic cell death.
After 48 hrs of treatment cell cycle gets arrested in G0/G1 phase at the low concentration, but as the concentration increased necrosis occurs. After 72 hrs of treatment again the cell growth was found to be arrested due to the necrosis. No signs of apoptosis were observed. Thus AECHL-1 induced G2/M arrest in a concentration- and time-dependent manner.
During study on H9c2 cells AECHL-1 arrests the growth of cells during treatment, which was concentration dependent. Hence we carried out the antitumor activity of AECHL-1 using different cancer cell lines like, PC3- prostate cancer, MDA-MB- breast cancer and B16- melanoma cells.

AECHL-1 showed concentration and time dependent effect on the viability of PC3, B16 and MDA-MB cells. As comcentration and incubation time increased viability of these cells decreased.

Cell proliferation is the measurement of the number of cells that are dividing in a culture. It is 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. AECHL-1 showed concentration and time dependent effect on the growth of these cancer cell lines. The effect was very much significant in PC3 and B16 cells, compared to MBA-MB cells. AECHL-1 showed significant inhibition in cell proliferation.

Effect of AECHL-1 on cancer cells was carried with PI staining and analyzing the cells FACs. Treatment of PC3 (prostate cancer) cell lines with higher concentration of AECHL-1 showed apoptotic cell death with decreased cell count in G2/M phase thus inhibiting the cell cycle proliferation. While at lower concentration it showed apoptotic cell death and the cell growth was found to be arrested in G0/G1 phase, confirmed from increased cell count in G0/G1 and decreased cell count in S-G2/M phase. Thus AECHL-1 showed inhibition in PC3 cell proliferation by arresting cell cycle in G0/G1 with significant apoptotic cell death.

Treatment of MDA-MB (breast cancer) cells with different concentration of AECHL-1, showed cell cycle arrest in S-G2/M phase, confirmed by accumulation of cells in these phases and decreased cell count in G0/G1 phase. Most of the cells were found to be arrested in G2/M phase of the cell cycle. The effect was found to be concentration dependent. Also AECHL-1 did not show any signs of necrosis in MDA-MB cells.

In B16 cells AECHL-1 arrest the cell cycle in G0/G1 phase, marked with increased cell count in this phase and decrease cell counts in S- and G2/M-phase. At higher concentration marked signs of apoptosis occur. Thus AECHL inhibits cell proliferation in concentration dependent manner, showing G0/G1 phase arrest at lower concentration while Go/G1 phase arrest with marked apoptosis at higher concentration.

In vivo antitumor activity of AECHL-1 was studied in C57 mice at dose level of 50 and 100µg. Tumor volume was measured with caliper through out the experiment. Intra tumor injection of AECHL-1 for 15 days reduced the tumor volume significantly when compared with that of control. AECHL-1 also showed significant reduction in tumor volume and tumor/body mass index an important factor in tumor treatment. Cis-platin was used as a reference standard (100µg).

Histopathological examination of the tumor showed increased neovascularization with increased cell density with presence of hemorrhagic areas which showed probable signs of angiogenesis with increased threat of metastasis. The blood vessels were well developed.

AAECHL-1 treated group showed decreased cell density and neovasulization and increased necrotic cells.

AECHL-1 (50µg) did not affect the normal architect of the liver while (100µg) hypertrophic cell nucleus with normal liver architect. Hepatocytes necrosis was seen here in a case of cis-platin. AECHL-1 at 50µg showed necrosis of myocardial fiber with lymphocytic infiltration, while at 100µg extensive myocardial fiber necrosis with contraction bands and loss of nuclei. The fragmentation and smudging of the muscle fibers occur which is characteristic of coagulative necrosis. Same condition occurs with cis-platin treated animals. AECHL-2 at 50 and 100µg showed tubular vacuolization and tubular dilation with hemorrhagic condition in kidney which are scattered chronic inflammatory cell infiltrates while cis-platin showed proliferative glomerulo nephritis. Microscopic examination of spleen showed increased numbers of granulocytes in the marginal zones.

The absolute number of blood vessels determines the number of tumor cells that can be supported. Acquisition of blood vessels is a critical step in the survival and progression of many types of cancer. Through production of pro-angiogenic factors and down-regulation of angiostatic factors, tumors recruit new blood vessels from surrounding host tissue through both angiogenesis and vasculogenesis. These blood vessels enable tumor growth, survival, and a point of entry into circulation for metastatic dissemination. AECHL-1 showed

significant reduction in neovasulization thus minimizing the risk of angiogenesis and metastasis.

Western blot analysis of tumor cells isolated from animals was carried out using SDS–polyacrylamide-gel electrophoresis gel. The proteins from the gel were transferred to a nitrocellulose membrane, blocked and probed with the appropriate primary antibodies and secondary horseradish peroxidase-labeled antibodies, detected by chemiluminescence and quantified with a Kodak 2000 gel-imaging system. Treatment of solid tumors with AECHL-1 reduced cyclin D1 protein levels, as detected by a pan D-cyclin antibody, increased p53 expression levels and decreased Bcl-2 levels, which suggests that inhibition of cyclin D1 expression and expression of p53 contributes to the growth inhibition induced by AECHL in B16F10 tumors through a cyclin D1/Cdk4/pRB signaling pathway.

AECHL-2 isolated from chloroform extract of Ailanthus root bark was subjected for its effect on H9c2 cells.
AECHL-2 showed concentration dependent repose on the viability of H9c2. It did not showed any significant effect on cell viability at lower concentration, even after treatment up to 48 hrs, but at higher concentration cell viability was found to be decreased with respect to increased concentration and time. Thus AECHL-2 was found to be cytotoxic at higher concentration.

Treatment with AECHL-2 showed increased both apoptotic and necrotic/late apoptotic cell death analyzed with Annexin-V and PI staining of the H9c2 cells.

AECHL-2 generates reactive oxygen species in H9c2 cell lines when analyzed by confocal microscopy and flow activated cytometry after loading with ROS specific fluorescent molecular probes.

AECHL-2 increases the intracellular calcium concentration in H9c2 and also showed significant changes in mechanical properties of primary culture of

neonatal myocytes measured by using IonOptix. The effect of AECHL-2 was similar to that of AECHL-1 on neonatal myocytes.

In the present study *B. monosperma* flowers were extracted with methanol. The vacuum dried methanol extract was then fractionated in ethyl acetate, butanol and water respectively. The total methanolic extract, along with its ethyl acetate, butanol and aqueous fractions were then used for the further study. Preliminary phytochemical investigation of ethyl acetate and butanol fractions showed the presence of phenolics including flavonoids as a major class of components along with steroids and sugars as other components.

The above extracts were subjected to screen for *in vitro* antioxidant activity as mentioned earlier.

From the results it was observed that the methanol extract of the *B. monosperma* and its various fractions were found to act as radical scavengers against different free radicals under the conditions of oxidative stress. Most nonenzymatic antioxidative activity like scavenging of free radicals, inhibition of lipid peroxidation, etc. is mediated by redox reactions. The reducing power determined in the present study depends on the redox potentials of the compounds present in different fractions. The highest amount of reducing power was observed in the ethyl acetate and butanol fractions followed by total methanol extract. Thus, it can be expected that the fractions may have scavenging activity against other oxidizing agents. Ethyl acetate and butanol fractions along with total methanolic extract showed a concentration dependent antiradical activity by reducing DPPH radical. Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in initiating oxidation reactions associated with aging. It plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA. Ethyl acetate and butanol fractions were found to be good scavenger of superoxide radicals. Hydroxyl radicals are reported to cause oxidative damage to various biomolecules like DNA, lipids and proteins. Ethyl acetate and butanol fractions were capable of reducing the damage to deoxyribose more effectively

than the total methanol extract. Sodium nitroprusside serves as a main source of nitric oxide radicals. Butanol and ethyl acetate fractions scavenge the NO formed from the sodium nitroprusside by inhibiting the chromophore formation.

The present study was further supported by the finding that the ethyl acetate and butanol fractions strongly inhibited erythrocyte hemolysis induced by AAPH, where lysis occurs mainly by two events, lipid peroxidation and redistribution of oxidized band 3 proteins within the cell membrane. It is considered that AAPH attack the membrane to induce the chain oxidation of lipids and proteins leading to the damage of spectrin, a membrane protein, by oxidative cross linking which may ultimately result in hemolysis. Band 3 in erythrocyte membranes plays an important role in a rapid exchange of $HCO3^-$ and Cl^- across the membrane. Butea fractions may block the hemolysis by inhibiting the formation of hemolytic holes in the erythrocytes cell membrane by blocking oxidation and redistribution of band 3 proteins.

Rapid screening for antioxidants by TLC revealed the presence of many constituents with radical scavenging properties in varying proportions in all the fractions other than aqueous. These constituents can be isolated and can be used for future structural elucidation.

HPTLC fingerprinting profile of ethyl acetate and butanol fractions was generated in solvent systems of different polarities in order to ascertain the total number of chemical moieties which will also help in designing the method of isolation and characterization of the bioactive components.

These studies suggest that specific constituents in the ethyl acetate and butanol fractions contribute to the radical scavenging activity of the flowers. Phenolic compounds are reported to be potent free radical scavengers and thus, the results are further supported by the varying amounts of total phenolic content in different fractions of flowers.

In the present study, ethyl acetate and butanol fractions were found to have potent free radical scavenging activity and can also act by inhibiting the AAPH mediated erythrocyte hemolysis. The observed activity may be mainly due to their total phenol content. However, in case of erythrocyte hemolysis, butanol

fraction with lower phenolic content was found to be more effective than ethyl acetate fraction with higher phenolic content, which may be due to the presence of two antihepatotoxic principles *viz.*, butrin and isobutrin.

Toxicity study was carried out for ethyl acetate and butanol fraction of Butea flowers, as per OECD guideline in female albino mice. Both the fractions were found to be non toxic at a dose of 2000mg/kg body weight supported with histopathological study. The safe dose of 100 and 200mg/kg b.w. was taken for further studies.

The ethyl acetate and butanol fractions were subjected to screen for *in vivo* antioxidant activity using Isoproterenol (ISO) induced myocardial infarction in rats.

Butanol fraction of the flowers showed marked increase in antioxidant enzyme levels in the heart homogenate with significant decrease in serum cardiac marker enzymes when compared with ISO group. Butanol fraction alone pretreated rats showed a non-significant change when compared to control rats. No variation in body weight and heart to body weight ratio was observed in butanol fraction treated group. It also reverses the ISO induced histopathological changes in cardiac muscles.

The ethyl acetate fraction (200mg/kg b.w.) showed decline in antioxidant enzyme levels in heart homogenate when compared to both control and ISO control group. The fraction also failed to decrease the ISO induced myocardial damage, marked with increase serum marker enzymes like LDH and uric acid, but only manage to reduce the serum CK-MB level as compared to ISO control. There was a severe change in body weight and heart to body weight ratio in ethyl acetate treated group. Even ethyl acetate fraction alone pretreated group showed significant weight loss.

At lower dose of 100mg/kg b.w. ethyl acetate fraction was found to reverse the ISO induced damaging effect marked with increase in endogenous antioxidant enzymes with decreased serum cardiac marker enzymes. The study was further confirmed from the histopathological observations.

Ethyl acetate fraction of flowers showed significant antioxidant activity against H_2O_2 and xanthine-xanthine oxidase induced stress in H9c2 cells after loading with ROS specific molecular probes and analyzing with confocal microscopy and flow activated cytometry.

As the weight loss occurs in ethyl acetate fraction treated group, we studied here the effect of *B. monosperma* flowers in ISO induced hyperlipidemia. ISO treated group (25mg/kg.b.w) showed significant increase in serum total cholesterol, LDL, VLDL and triglycerides with marked decrease in HDL cholesterol level. It also showed the increase in TC/HDL and LDL/HDL value which are considered to be the risk factor for the heart.

Butanol extract of flower showed significant decrease in serum TC, LDL, TG, and VLDL with marked increase in HDL level. It also reduced the risk factor associated with the TC/HDL and LDL/HDL value, compared to ISO control group. Butea fraction alone pretreated group did not show any change in serum lipid profile of the rat when compared to control group.

Ethyl acetate extract (200mg/kg) of Butea flower showed significant increase in serum TC and LDL level. The fraction also increased the risk factor associated with TC/HDL and LDL/HDL value. But the extract reduced the serum TG and VLDL level significantly with marked increase in serum HDL level when compared to ISO control group.

At (100mg/kg b.w.) ethyl acetate extract of flowers showed significant protection against ISO induced hyperlipidemia.

Compound BFEA-1 was isolated by preparative thin layer chromatography (PTLC) of ethyl acetate extract of flowers. BFEA-1was characterized by UV, ^{1}H-NMR, ^{13}C NMR, and IR.

Compound BEFA, is a colourless solid, mp. 278-281°C was identified as 3, 4, 5-trihydroxy benzoic acid, i.e., *Gallic acid.* The UV spectrum gave the characteristic absorption maximum of gallic acid at 215nm and 272nm, further the R_f value of the compound was matched with gallic acid. On TLC dark black bands of BEFA-1 and pure gallic acid were observed with methanolic $FeCl_3$, which further confirmed BEFA-1 to be gallic acid. The IR spectrum

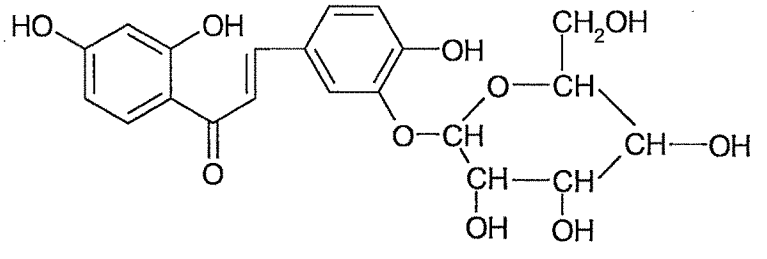
fingerprinting matched exactly with that of pure gallic acid. BFEA-1 showed the presence of hydroxyl(s) (3365nm, 3287nm), aromatic moiety (1619nm), and carboxylic acid (1703nm). The ^{1}H NMR spectrum of BEFA showed presence of aromatic protons at δ 6.99. The ^{13}C NMR showed peak at δ 109.98 for C-2 and C-6 and at δ 170.14 for carboxylic acid.

While extraction of *B. monosperma* flowers, with methanol, we found the yellow colored residual mass, which was separated and washed several times with methanol and designated as BM1.
Phytochemical investigation of BM1 revealed the presence of flavonoids and sugars with 16.7% of phenolic content.
Rapid screening showed the presence of radical scavengers in BM1 and showed significant antioxidant activity when screened by using various *in vitro* and *ex vivo* methods.
Toxicity study was carried out for BM1 fraction of Butea flowers, as per OECD guidelines. The fraction was found to be non toxic at a dose of 2000mg/kg body weight. The safe dose of 200mg/kg body weight was used for biological studies.

The BM1 fraction was subjected to screen for *in vivo* antioxidant activity using isoproterenol (ISO) induced myocardial infarction in rats. Pretreatment with BM1 was found to reverse the ISO induced changes in antioxidant enzymes along with cardiac and liver marker enzymes. The study was further supported by the histopathological examination of the heart and liver.
TLC fingerprint profile of the BM1 fractions was established in solvent system of different polarities and scanned.

Compound BM was isolated from the fraction BM1 as a granular white solid, m.p.176-178°C, possessed a molecular formula of $C_{21}H_{22}O_{10}$ as indicated by EI and ES mass spectra. The colour reaction (deep orange red fluorescence in UV light when exposed to ammonia) and U.V. spectra indicated it to be chalcone glycoside. Polyhydroxychalcones absorb strongly in the 300-400 nm region (Band I) and in 220-270 nm region (Band II). Band I consists of an intense peak (Band Ia) and a minor inflection (Band Ib). The UV spectra of BM showed

maxima at 270 nm, 312 nm, and 372 nm. Addition of shift reagents showed free hydroxyl groups at 2', 4', 4 positions of the chalcone ring. The UV maxima shifted with $AlCl_3$ to 422 nm indicating that 2'position is free. Bathochromic shift with increased intensity (Band Ia shifted to 431 nm) on addition of sodium ethoxide showed the presence of free hydroxyl group at 4 position. Lack of change in the UV spectrum with sodium acetate and boric acid indicated that the glucose unit may be in the B- ring, linked to 3– hydroxyl group.
From the above data BM was identified as β-D- monospermoside.



Butein-3-beta-D-glucoside (Monospermoside)

In cell viability, 3 and 6hrs of treatment, BM did not show any significant change in viability of H9c2 cell at 2-100μg/ml concentration, but after 12 hrs of incubation cell viability was found to be reduced.

BM showed concentration and time dependent protection against H_2O_2 induced stress in H9c2 cells. As the concentration of BM increased cell viability was found to be increased, but above 20μg/ml, BM showed reduction in cell viability. This effect might be due to its prooxidant activity on H9c2 cells.
Compound BM showed significant antioxidant activity against H_2O_2 and Xanthine-xanthine oxidase induced stress in H9c2 cells after loading with ROS specific molecular probes and analyzing with confocal microscopy and flow activated cytometry.

The present study showed the antioxidant potential of *A. excelsa* and *B. monosperma* in a more concentric form of AEEA, BFEA and BM when studied

by using various in vitro, ex vivo, and in vivo methods along with their effect on neutralizing ROS in H9c2 cell lines.

BFEA from Butea flowers proved its importance in the treatment of hyperlipidemia, thus minimizing the risk of cardiac failure in today's fast moving life style.

Compound AECHL-1 from Ailanthus root bark comes out with its valuable role in the treatment of tumor.

Compound AECHL-2 can provide an excellent tool for research due to its tremendous radical generating property and can again fetch an attention of research community towards valuable natural wealth.

Although there are number of queries remain unanswered like appropriate time of administration, choice of therapy, delivery at specific sight and specificity of targeting free radicals, antioxidants play a very important role in combating pathophysiological state due to generation of free radicals/ROS, an attempt was made to evaluate two important plants designated as medicaments for number of disorders caused due to generation of free radicals/ROS. These include the leaves and root bark of *A. excelsa* and flowers of *B. monosperma.*

The studies supported utility of *A. excelsa* and its isolated compounds as antioxidant as well as potential antitumor agent, when tested against cancer cell lines.

The flowers of *B. monosperma* showed potential antioxidant effect along with significant antihyperlipidemic activity, justifying their traditional usage.