Chapter – 7

DISCUSSION

In modern medicine nearly 25% of them are based on plants or plant derived drugs. [1] The world trade of herbal medicine is now estimated at US$ 70 billion with an annual growth rate of 7%. [2] India is one of the mega biodiversity centers of the world and the north eastern region of India belongs to the biodiversity hotspot. WHO is promoting traditional, complementary and alternative medicines in health policies because of its positive features, which include diversity and flexibility, accessibility and affordability in many parts of the world; broad acceptance among many populations in developing countries, comparatively low cost and growing economic importance. A good number of known and unknown medicinal plants are found in the forests of Darjeeling Hill district of West Bengal and the state of Sikkim. [3-4] The state contains over 4000 species of flowering plants, which includes over 600 species of orchids and more than 100 species of medicinal plants. [5]

Cancer is the second common cause of death in the developed countries next to cardiovascular diseases. According to WHO, out of an estimated total of 50 million death occurs annually in the world, more than 5 million are attributed to cancer and the number of death from cancer throughout the world is increasing. [6] Leukemia is a heterogeneous hematological malignancies characterized by unregulated proliferation of the blood forming cells of the bone marrow. Acute Lymphocytic Leukemia (ALL) and Chronic Myeloid Leukemia (CML) are the most common types of Leukemia. ALL is predominantly a disease of childhood with 75% of all cases occurring in patients younger than 15 years of age. [7] Medicinal plants and their phytoconstituents have always been a better choice for leukemia. The experimental studies on nutraceuticals have proved many of them as antileukemic agents. [8]

Bischofia javanica Blume (Euphorbiaceae), known as 'kainjal' in Nepali and used by the tribes of Sikkim for various ailments like cancer, tuberculosis, stomach ache, and ulcer. [9] It is an evergreen tree widely distributed over the Southeast Asia, Japan and Australia. In India it is distributed over the Sub-Himalayan region and South West coast from Konkan to Nilgiris. [10] This plant has been utilized significantly for various other ailments like topical treatment of sores and boils. [11] It also possesses antimicrobial,

anthelmintic and antidysenteric activities. [12] *B.javanica* has been reported to contain the phytoconstituents like triterpenoids, glycosides, flavonoids and tannins. [13]

Fraxinus floribunda Wallich (Oleaceae) is a tree, occurring in eastern Himalayas and khasi hills. Leaves are pinnate, leaflets 7-9, opposite, stalled, ovate-oblong. Manna is obtained by incision from the stem of the tree and it is used as laxative. [14] The barks and leaves of the plant have been traditionally used for the treatment of fracture and dislocation. [15] The leaves are employed as diuretic and for the treatment of gout [16]. Some coumarins have been isolated from the leaves in early 1980. [17]

7.1 Phytochemical Studies:

In this study the plants *Bischofia javanica* and *Fraxinus floribunda* have been chosen on basis of their traditional utility. The plants were authenticated at Botanical survey of India (BSI) Gangtok. The dried, powdered leaves were extracted with methanol by continuous hot extraction procedure. [18] The extracts were subjected to preliminary qualitative analysis for determination of different secondary metabolites. The methanol extract was fractionated with different solvents like petroleum ether, chloroform and water. The chloroform fraction was found to have better anti-inflammatory and anticancer activity. Moreover it gave the positive test for triterpenoids, therefore, chloroform fraction was further used for the isolation of pure phytoconstituents .The standard protocol was used for the isolation of triterpenoids from the Chloroform fraction of *B.javanica* and *F.floribunda.* [19] The structure of the isolated compounds were elucidated by their IR, ^{1}H NMR, ^{13}C NMR, DEPT-90, DEPT-135 and Mass spectra [20-22] **(Chapter 3)**.

The IR spectrum of the compound LS-1 revealed the presence of carbonyl group (1738 cm^{-1}), C-H stretching and bending were confirmed by absorption at 2870 and 1461 cm^{-1}. The ^{1}H NMR data revealed the eight singlet methyl protons in the compound showed the signals at 1.16, 1.18, 1.13, 1.04, 0.99, 0.82, 0.76 and 0.98 ppm. The signal at 4.36 ppm revealed the acetyl substitution at C-3 and the singlet at 2.03ppm confirmed the acetyl group.^{13}C NMR data revealed the 32 carbons in the skeleton with eight methyl groups.

The acetyl group at C-3 showed the chemical shift at 75.17 and the C=O group has provided the δ_C at 171. Further DEPT-90 and DEPT-135 studies confirmed singlet, doublet, triplet and quadralet carbons of ^{13}C NMR studies and inferred the pentacyclic triterpenoid skeleton for the test compound LS-1. [23-25] The Mass spectra (ES-MS) confirmed the molecular weight of the compound as 470.77. In conclusion the compound LS-1 structure was found to be Friedelin-3α-acetate with molecular formula $C_{32}H_{54}O_2$.

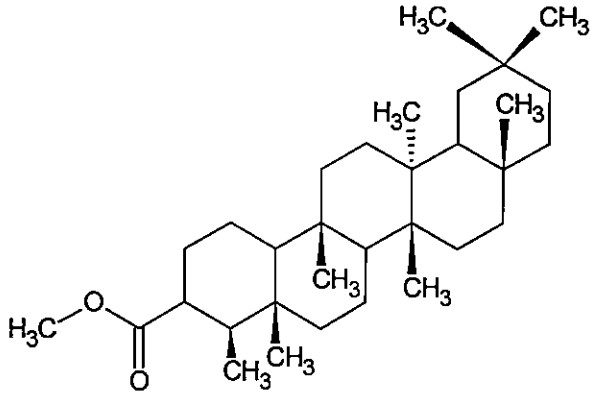

Figure 3.5 Compound LS-1 (Friedelin-3α-acetate)

For the compound LS-2 the IR spectrum revealed the presence of hydroxyl group (1465 cm^{-1}),-CH=CH- (Alkene) (1054cm^{-1}) and C-H stretching (3424 cm^{-1}). The ^1H NMR spectrum revealed the presence of eight CH_3 proton and these signals are at 0.80, 0.82, 0.91, 1.18, 1.01, 0.97, 0.85 and 0.87ppm. H-3 gave signal at 5.36 ppm, it inferred the OH group attached steric hinderance at C-3 carbon. The proton in alkene bond at H-12 provided the chemical shift at 2.27ppm. The ^{13}C NMR spectrum of the compound showed signals at 72.2 ppm for C-3 in which the OH group is attached. The alkene bridge between C-12 and C-13 showed the signals at 122 and141ppm. ^{13}C NMR spectrum confirmed the hydroxyl group and alkene bridge of the compound LS-2. The CH_3 group of the compound provided the chemical shift between 19-42 ppm. The signals for C-30 carbons confirmed the total molecular structure belongs to the pentacyclic triterpenoids. [26-27] DEPT-90 and DEPT-135 studies have provided the supportive evidence for the Singlet and doublet in the carbon skeleton of the compound LS-2. ES

Mass spectrum revealed the molecular weight of the compound as 427.23. Finally it was concluded that the structure of the compound LS-2 is β-amyrin have the molecular formula $C_{30}H_{51}O$.

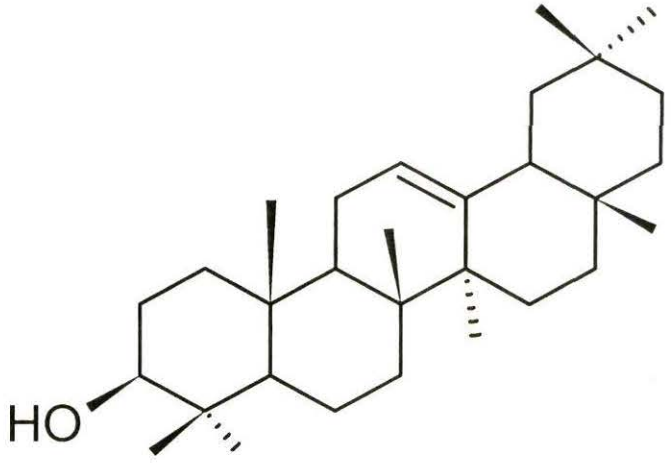

Figure 3.9 Compound LS-2 (β-amyrin)

7.2 Toxicity and Anti-inflammatory Studies:

Acute toxicity studies revealed that the LD_{50} value of methanol extract of leaves of *Bichofia javanica* (BJ) is about 3,200mg/kg b.w, p.o and for the leaves of *Fraxinus floribunda* (FF) it is about 2,400mg/kg b.w, p.o. This report provided the information on dose in toxic range for both the extracts of the plants (0.5-5g/kg b.w, p.o). [28]

Carrageenan-induced paw edema studies involves three distinct phases of mediator release. The early phase is attributed to the release of histamine and serotonin, an intermediate phase is mediated by kinin like substances and the last phase is due to the release of prostaglandin like substances. [29] The results of the carrageenan induced paw edema studies evident that the test drugs inhibited the paw edema in a dose and time dependent manner (**Chapter 4**). The isolated compounds (Pentacyclic triterpenoids) of *B.javanica* and *F.floribunda* i.e., Friedelin-3α-acetate and β-amyrin were shown to have significant anti-inflammatory, anti-nociceptive and anti-pyretic activities. BJ at the dose

of 600mg/kg b.w inhibited the paw edema by 41.83% after 4 hr of drug administration. Friedelin-3α-acetate, the isolated compound of *B.javanica* LS-1, at the dose of 20mg/kg b.w inhibited the paw edema by 53.08% in comparison to the standard drug aspirin (45.83%). FF at the dose of 200 and 400mg/kg b.w inhibited the paw edema by 51.40% and 55.14% after 4 hr of drug administration. β-amyrin the isolated compound of *F.floribunda* LS-2, at the dose of 20mg/kg b.w inhibited the paw edema by 69.02% which was found to be more potential than the standard drug aspirin (64.48%) at 100mg/kg b.w p.o. The carrageenan induced inflammation in rat was inhibited by FF and its isolated constituent, β-amyrin in better way compared to BJ and its isolated compound. The results of this study was similar to the anti-inflammatory activity of ethanol extract of the aerial parts of *Pothomorphe umbellate* in carrageenan induced paw edema by Fabio F, *et al*, the ED_{50} (oral) for the inhibition of carrageenan-induced rat paw edema for *Pothomorphe umbellate* was determined to be 550 mg/kg, while the LD_{50} was higher than 2.0 g/kg. At a dose of 550 mg/kg, inhibited the inflammation by 48.7% on the third hour of the assay (edema peak) when compared to the untreated control. [30]

The percentage inhibition of cotton pellet induced granuloma in rats was determined and from the study it was evident that the isolated compound of BJ, Friedelin-3α-acetate at 20mg/kgb.w shown to have 55% inhibition and the isolated compound of FF, β-amyrin at 20mg/kgb.w shown to have 49.33% inhibition (**Chapter 4**). The anti-inflammatory activity of an alcohol extract of *Achyranthes aspera* in this model studied by Vetrichelvan *et al* revealed that the extract exhibited a 40.03% and 45.32% reduction in granuloma weight [31] at the dose of 375 and 500mg/kg respectively. BJ and isolated compound of *B.javanica,* LS-1, have shown significant anti-inflammatory effect in Freund's adjuvant induced arthritis. BJ at the dose of 600mg/kg b.w inhibited arthritis by 50.24% and the isolated compound LS-1, at the dose of 20mg/kg b.w inhibited by higher rate of 65.21%, which was higher than that of FF and its isolated compound β-amyrin (13.91% and 51.23%). Triterpenoids isolated from the plant *Trichodesma amplexicaule* Roth at the dose of 5, 10,15mg/kg p.o significantly reduced the carrageenan and Freund's adjuvant induced inflammation in rats similar to *Bischofia javanica* and *Fraxinus floribunda.* [32]

Arachidonic acid-induced paw edema in rats is an *in vivo* model to establish the dual inhibitors COX and LOX. [33] BJ inhibited the arachidonic acid induced paw edema in rats significant manner (75.27%) at the dose of 600mg/kg b.w compared to the isolated compound Friedelin-3α-acetate 20mg/kg b.w (59.27%). FF at the dose of 400mg/kg b.w and the isolated compound LS-2, at the dose of 20mg/kg b.w inhibited the arachidonic acid induced paw edema in rats by 30.86 and 34.97% respectively. The standard dual-inhibitor drug Phenidone has the maximum inhibition of arachidonic acid induced paw edema (77.81%) in this study. This study has established the probable mode of action of BJ and FF for their anti-inflammatory activities. BJ and its isolated compound LS-1 are dual inhibitors whereas FF and its isolated compound LS-2 are not dual inhibitors in their anti-inflammatory pathways.

Pain is one of the cardinal signs of inflammation. Hence, it is necessary to evaluate whether a new drug modify the inflammatory pain which appears to be the most relevant test because, this type of pain is prevalent in most of the conditions for which anti-inflammatory drugs are prescribed. Anti-nociceptive activity by tail immersion method is adopted for opioid analgesics, it is known that centrally acting analgesic drugs elevate the pain threshold of animals towards heat and pressure. Moreover the acetic acid induced writhing response inhibited by drugs which act peripherally by inhibiting the release of mediators at the local site. [34] The results of anti-nociceptive studies revealed that the test drugs have significant anti-nociceptive activities in both Tail-immersion test and Writhing test. In Tail-immersion test, BJ at the higher dose of 600mg/kg b.w and FF at the dose of 400mg/kg b.w showed tail-flick after 12 and 13 seconds at 60 and 120 minutes which was higher than that of the isolated compounds. Inhibition of writhing by the test drug BJ and FF was 50.26% and 48.27% at the dose of 600 and 400 mg/kg b.w p.o. respectively and the isolated compounds also have significant inhibition towards writhing induced by acetic acid on mice (45.25% by LS-1and 58.35% by LS-2). The test drugs BJ, FF and their isolated compounds shown to have anti-nociceptive activities by both peripheral and central mechanisms. Similar type of study was performed by Niele MG *et al*, here anti-nociceptive activity of two Amazonian Copaiba oils *Copaifera*

multijuga Hayne and *Copaifera reticulata* Ducke, were studied for peripheral (acetic acid-induced abdominal writhing), spinal (tail flick) and supra-spinal (hot plate) models of nociception. Results demonstrated that the Copaiba oils ranging from 30 to 150 mg/kg were enough to significantly develop peripheral antinociceptive effect. All Copaiba oils demonstrated central activity but with less effect on supra-spinal regions of the brain. Administration of the opioid receptor antagonist, naloxone completely inhibited the antinociceptive effect induced by both Copaiba oils. The results indicated that Copaiba oils demonstrate both peripheral and central antinociceptive effect. [35]

Drugs having anti-inflammatory activity generally possess anti-pyretic activity e.g. non-steroidal anti-inflammatory drugs (NSAIDs).The mode of action of NSAIDs is not clear. It has been suggested that prostaglandins (PGE) mediates pyrogen fever; the ability of NSAIDs to inhibit prostaglandin synthesis could help to explain their antpyretic activity. [36] The isolated compound of *B.javanica* LS-1, at the dose of 20mg/kgb.w and the standard drug paracetamol at the dose of 100mg/kg b.w decreased the elevated rectal temperature of rats after Brewer's yeast administration by 1.1°C after 1 hr of (19hr after yeast administration) of drug administration. The isolated compound of *F.Floribunda* LS-2, at the dose of 20mg/kgb.w decreased the elevated rectal temperature of rats after Brewer's yeast administration by 1.2 and 1.3°C after 1 hr of (19hr after yeast administration) of drug administration. In the similar way different extracts of *Aegle marmelos* at the dose of 50mg/kg i.p, *Emblica officinalis* at the dose of 500mg/kg i.p, *Venonia cinerea* leaf at the doses of 100,200 and 400mg/kg i.p shown to have significant anti-pyretic activity on brewer's yeast induced pyrexia in rats. [37-39] **(Chapter 4).**

7.3 Anticancer Studies:

Cancer chemotherapy may be indicated a primary, palliative, adjuvant or neo adjuvant modality. Treatment with cytotoxic drugs is the primary curative modality for a few diseases including leukemia, lymphomas, choriocarcinomas, and testicular cancer. Programmed cell death (PCD) is an important mechanism in both development and homeostasis in adult tissues. [40] One form of PCD is apoptosis, which is characterized by maintenance of intact cell membranes during the suicide process so as to allow adjacent

cells to engulf the dying cell so that it does not release its content and trigger a local inflammatory reaction. Experimental studies revealed the apoptotic pathway of cell death is one of the most common mechanism of action of anticancer agents. [41-44] Cells undergoing apoptosis usually exhibit fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of the DNA into small oligonucleosomal fragments. [40] In this study we have studied the anticancer activity of methanol extract of leaves of *B. javanica* (BJ), *F.floribunda*(FF) and their isolated compounds, Fredelin-3α-acetate and β-amyrin on Human Leukemia cell lines U-937, K-562 and HL-60. Cell viability studies of BJ and its isolated compound, Friedelin-3α-acetate shown to have time and dose dependent inhibition on cell growth **(Chapter 5)**. In U-937, K-562 and HL-60 cell lines BJ at the dose of 5, 10 and 15μg/ml and LS-1at the dose of 5 and 10μg/ml inhibited the cell growth in 24, 48 and 72 hrs in significant manner. Whereas FF and its isolated compound β-amyrin does not shown to have significant effect on cell viability of U-937, K-562 and HL-60 cell lines.

Cytotoxicity study (MTT assay) of BJ at the dose of 10, 15μg/ml and its isolated compound, Friedelin -3α-acetate at the dose 5 and 10μg/ml of shown to have significant cytotoxicity at 24 ,48 72 hr of incubation, the percentage of cytotoxicity was found to be 52.65,65.27 and 69.33 for BJ at 15 μg/ml in 24, 48 and 72 hrs ($P<0.001$) and for Friedelin -3α-acetate at the dose of 10 μg/ml it was19.40, 33.33, 44.00 % ($P<0.05$) in U-937 cell line. In K-562 cell line in 72 hrs of incubation BJ at the dose of 15 μg/ml and Friedelin-3α-acetate at the dose of 10μg/ml provided the cytotoxicity by 59.01 and 29.56%. In HL-60 the percentage of cytotoxicity for BJ at the dose of 15 μg/ml and Friedelin -3α-acetate at the dose of 10μg/ml were 77.41 and 69.35% ($P<0.001$) respectively. Whereas FF and the isolated compound, β-amyrin does not shown to have significant cytotoxicity on U-937 and K-562 cell lines. Cytotoxicity of FF at the dose of 15 μg/ml and β-amyrin at the dose of 10μg/ml in HL-60 in 72 hrs of incubation shown to have 26.15 and 40.00% respectively. The IC_{50} value of BJ and isolated compound, Friedelin-3α-acetate was found to be less compared to FF and its isolated compound, β-amyrin. BJ provided the dose dependent inhibition in all the cell lines studied. At 72 hrs the IC_{50} value of BJ in U-937, K-562 and HL-60 were found to be 4.1, 12.9 and 3.5 μg/ml respectively, the

isolated compound LS-1 yielded 12.2, 20.0 and 3.7 μg/ml of IC_{50} value for the cell lines U-937, K-562 and HL-60. In *F.floribunda* the IC_{50} value of FF and β-amyrin are higher than BJ and Friedelin -3α-acetate i.e. at 72 hrs in K-562 cell line the isolated compound, β-amyrin has shown the IC_{50} value 63.0 μg/ml, whereas Friedelin -3α-acetate has shown the value as 20 μg/ml. In statistical analysis FF did not show a significant cytotoxicity in any of the cell line. Therefore, the drug FF and its isolated compound have not been considered for further anti cancer studies.

In the search of cytotoxicity studies on leukemia cell lines by natural products, Methylene chloride fraction of *Scutellariae barbatae* was studied for apoptosis related experiments on human U-937 leukemia cells by 2,3-bis[2-4-nitro-5-sulphophenyl]2*H*-tetrazolium-5-carboxanilide (XTT) assay for cytotoxicity. The extract inhibited the proliferation of human U-937 leukemia cells in a dose-dependent manner (IC_{50}=10 μg/ml). [45] L-Ascorbic acid (LAA) was investigated clinically for the treatment of patients with acute myeloid leukemia (AML) based on the observed effects of LAA on AML progenitor cells *in vitro*. However, the mechanism for LAA-induced cytoreduction remains to be elucidated. LAA at concentrations of 0.25–1.0mM induced a dose and time-dependent inhibition on proliferation in three AML cell lines and also in leukemic cells from peripheral blood specimens obtained from three patients with AML.[46] Different terpenoids have cytotoxicity effects on cancer cell lines for anti cancer activity e.g. a formylated triterpene named cladocalol has been isolated from the leaves of *Eucalyptus cladocalyx* together with ursulolactone acetate, ursolicacid, 3β-acetate-12,20(29)-lupadien-28-oic acid, β-sitosterol. In this study it is reported that Cladocalol has potent cytotoxic effect in myeloid leukemia cell line HL-60 by MTT assay.[47] Sclareol is a labdane-type diterpene that has demonstrated a significant cytotoxic activity against human leukemia cell lines.[48]

During apoptotic cleavage of nuclear DNA at internucleosomal sites yielding the DNA fragments in multiples of 180bp, which upon electrophoresis yields a ladder pattern. This is a biochemical hallmark of apoptosis. [49] DNA fragmentation assay of BJ have shown ladder formation in concentration dependent manner in the leukemia cell lines, however,

the isolated compound Friedelin-3α-acetate at the dose of 10μg/ml did not show the effect in predictable way. The reason may be that the cytotoxicity of BJ is through the mode of action of apoptosis and the isolated compound, Friedelin-3α-acetate presumably causing cytotoxicity by other mechanisms. Morphological characterization of treated cells by fluorescence microscopy and confocal microscopy studies confirmed the test drug, BJ having anticancer activity mediated through induction of apoptosis. Typical features of apoptosis like chromatin condensation and nuclear fragmentation of treated cells were clearly evident from the confocal microscopic studies **(Chapter 5)**.

The effect of trichostatin-A (TSA), a histone deacetylase (HDAC) inhibitor, on the cell growth and apoptosis and its effect on the telomerase activity in human leukemic cell line U-937 was carried out by Hyunjoo *et al.* Exposure of U-937 cells to TSA resulted in growth inhibition and induction of apoptosis in a dose-dependent manner as measured by hemocytometer counts, fluorescence microscopy, agarose gel electrophoresis and flow cytometry analysis. The increase in apoptosis was associated with the up-regulation in proapoptotic Bax expression and down-regulation of anti apoptotic Bcl-2 and Bcl-XL. It is therefore concluded that TSA demonstrated antiproliferative and apoptosis-inducing effects on U-937 cells *in vitro*, and that changes in Bcl-2 family protein levels as well as telomerase activity may play an important role in its mechanism of action. [50] Realgar (Arsenic-containing mineral drug in Chinese medicine) was shown to have a therapeutic effect against acute promyelocytic leukemia (APL) by inducing apoptosis as studied by Han-Qing Y. *et al.* However, there is little data about the effects of it on plasma membrane. In a study, the cytotoxicity of realgar to HL-60 cells including its inhibiting cell growth, inducing apoptosis and bringing about membrane toxicity was investigated. It was suggested that realgar could significantly suppress the proliferation of HL-60 cells in a dose-dependent manner in 3-(4,5-dimethylthiazol-2-diphenyl-tetrazolium bromide (MTT) assay and the IC_{50} value of realgar was 5.67M. [51] The effects of 1,8-cineole in two human leukemia cell lines, Molt 4B,HL-60 and stomach cancer KATO III cells was studied by Hiroyuki M. and his coworkers. Specific induction of apoptosis by 1, 8–Cineole was observed in human leukemia Molt 4B and HL-60 cells but not in human stomach cancer KATO III cells. Morphological changes showing apoptotic bodies were

observed in the human leukemia HL-60 cells treated with 1, 8 cineole. The fragmentation of DNA by cineole to oligonucleosomal sized fragments i.e. a characteristic of apoptosis were concentration and time-dependent in Molt 4B and HL-60 cells, but not in KATO III cells. [52] **(Chapter 5).**

7.4 Antioxidant Studies:

Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and ageing.[53] Antioxidants are the drugs offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and other mechanisms and thus prevent disease.[54] DPPH radical scavenging assay, lipid peroxidation assay and hydroxyl radical scavenging assay are the three *in vitro* methods of antioxidant activities performed for the methanol extract of *B.javanica, F.floribunda* and for their isolated constituents, Friedelin-3α-acetate and β-amyrin respectively. The doses of the test drugs were chosen between 20-320μg/ml. DPPH is a relatively stable radical, it is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance. Radical scavenging activity increased with increasing percentage of the free radical inhibition. [55] The test drugs induced dose dependent inhibition of DPPH radical formation. The percentage of DPPH radical scavenging of BJ at 320μg/ml, Friedelin-3α-acetate at 320μg/ml, FF at 320μg/ml, β-amyrin at 320μg/ml and α-tocopherol at 320μg/ml were 84.26, 68.21, 61.42, 72.14 and 78.67 respectively. Moreover the study revealed that the test drug BJ is more effective (IC_{50}-118.29μg/ml) to scavenge the DPPH radical than the other test compounds **(Chapter 6)**. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver. Initiation of lipid peroxidation by ferrous sulphate takes place through hydroxyl radical by Fenton's reaction. [56] The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxyl radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. In this study, *in vitro* lipid peroxidation was induced in rats brain by ferrous sulphate and ascorbic acid. The results of Lipid peroxidation assay revealed that the test drugs induced dose-dependent prevention

towards generation of lipid peroxides. BJ is more effective in the control of Lipid peroxidation than the isolated compound, Friedelin-3α-acetate. However in *F.floribunda* the isolated constituent, β-amyrin was shown to have more activity (IC_{50}-137.92μg/ml) than the methanol extract of FF (IC_{50}-172.54μg/ml). The hydroxy radical scavenging activity is measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture, by studying the competition between deoxy ribose and the extract for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxy ribose which eventually results in TBARS formation. In this assay the leaves of *F.floribunda* shown to have more efficacy than the *B.javanica*. The IC_{50} value of the isolated constituent of *F.floribunda i.e.,* β-amyrin is 129.85μg/ml whereas for the isolated constituent of *B.javanica i.e.,* Friedelin-3α-acetae is 163.37μg/ml.

The antioxidant properties and total phenolic contents of two varieties of cowpea (*Vigna unguiculata*) were examined by Perumal S. *et al.* The extracts were screened for their potential antioxidant activities using tests such as di-phenyl-picryl hydrazyl (DPPH), ABTS, FRAP, linoleic acid emulsion and beta-carotene–linoleic acid *in vitro* model systems. At 800 μg of extract in the reaction mixture, the superoxide anion radical scavenging activity was found to be significantly higher in the raw and dry heated seed extracts than the hydro thermally processed seed samples of the respective varieties. [57] The antioxidant activity of methanol extracts of five plants from the genus Phyllanthus was evaluated by various antioxidant assays by Kumaran A. *et al* [58], including total antioxidant, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide scavenging, reducing power and metal ion chelating activities. Among the five plants, *Phyllanthus debilis* has been found to possess the highest activity in all tested models. In addition to the antioxidant activity of these plants, the total phenolic compounds, flavonoids and flavonols were measured in the extracts. A correlation between the antioxidant activity and total phenolic content was observed. Similar type of *in vitro* antioxidant activity was performed by Baskar R. *et al*, on the leaves of Annona species. The ethanol extract of *A.muricata* at 500μg/ml showed maximum scavenging activity (90.05%) of ABTS radical cation followed by the

scavenging of hydroxyl radical (85.88%) and nitric oxide (72.60%) at the same concentration. However the extract showed only moderate lipid peroxidation inhibition activity. In contrast, the extract of *A.reticulata* showed better activity in quenching DPPH (89.37) and superoxide radical (80.88%) respectively. [59] **(Chapter 6).**

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