Discussion
5. DISCUSSION:

Traditions are dynamic entities of unchanging knowledge. Traditional medicine is an evolutionary process as communities and individuals continue to discover new techniques that can transform practise. It has been confirmed by World Health Organization (WHO), those herbal medicines serves the health needs of about 80 percent of the world’s population. Meanwhile, consumers in developing countries are becoming disillusioned with modern health care and seeking alternatives. The recent resurgence of plant remedies results from several factors, such as effectiveness of the plant medicines, the development of science and technology.

Ethnopharmacology and drug discovery using natural products remain important tissue in current target-rich and lead-poor scenario (Patwardhan, et al., 2004). Many modern drugs have their origin in ethnopharmacology. Globally, there is a positive trend in favour of traditional and integrative health sciences in research and practice. There are common approaches to drug discovery including the use of chemical biology, serendipity, chemical synthesis, combinatorial chemistry and genomics. However, the innovative approaches involve ethnopharmacology, reverse pharmacology, holistic systems biology and personalized medicine. There are clear trends to show that the mainstream in pharmaceutical research in moving away from single target approach to combinations and multiple target approaches (Wermuth 2004). The ethnopharmacology knowledge and experimental base allows drug research from ‘clinics to laboratories’ a true reverse pharmacology approach. In this process, ‘safety’ remains the most important starting point and the efficacy becomes a matter of validation. A golden triangle consisting of Traditional Knowledge, Modern Medicine and Modern Science with system orientation will cover to form an innovative discovery engine for newer, safer, affordable and effective therapies (Mashelkar, 2005).

Rapid growth has been seen in the herbal medicine market in recent years, as increasing number of consumers is persuaded by the benefits of plant extracts as an alternative to medicinal products with chemically derived active pharmaceutical ingredients. While the pharmaceutical industry in the developed world will continue to
investigate promising leads from natural products in their effort to produce new entities, the production of new medicines in the developing world may have quite different priorities. In these parts of the world, when a plant is readily available and has the potential to provide inexpensive therapy for the treatment of a disease, then a product may well be developed. Close collaboration is expected between clinical and scientists with common endeavour of production of safe, quality and efficacious medicine.

Today, at the beginning of the 21st century, traditional medicine and ethnopharmacology are attracting more and more attention within the context of healthcare provision and health sector reform. The knowledge gathered by generations was passed on to posterity and this practice is generally termed as traditional medicine or ethnomedicine. According to the WHO, traditional medicine is defined as: health practices, approaches, knowledge and beliefs incorporating plant, animal and / or mineral-based medicines, spiritual therapies to treat, diagnose and prevent illness and maintain well being. Therefore, traditional medicine may be regulated, taught openly and practiced widely and systematically.

Current drug discovery from plants has mainly relied on bioactivity – guided isolation methods, which for example, have led to discoveries of the important anticancer agents, paclitexel from Taxus brevifolia and camptothecin from camptotheca acuminate (Kinghorn, 2005). Other new chemical entities discovered or modified from plants during 2000-2005 are apomorphine, tiotropium, galanthamine, arteether, etc. Therefore, use of natural products has been the single most successful strategy for the discovery of new medicines and leads. Plants represent a vast resource of untapped chemicals. The chemical diversity of plants is far greater than that of microbes. Hence, it is of most utmost importance to involve in herbal drug research. Herbal medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products. With this background, in the present study, Echinops echinatus plant in ethnomedicine was evaluated systematically for its medicinal properties.
5.1 Phytochemical Investigation:

Determinations of preliminary phytochemical analysis are of paramount importance for the purpose of evaluation of crude drugs. Various physicochemical constants (ash values), moisture content of the roots were determined and the values are depicted in table no 2 and also percentage of successive Soxhlet extractives were calculated and results are depicted in table 1. The phytochemical studies, showed the presence of glycosides, terpenoids, steroids, carbohydrate, flavonoids, saponins, tannins and phenolic compounds in ether, chloroform, ethanol extracts of *E. echinatus* Roxb roots except in water extract. Total phenolic content is most important for the purpose of evaluation of crude drugs for its pharmacological activity. Phenol compounds occurring in nature and environment are of interest from many view points (antioxidant, astringency, bitterness, colour, oxidation substrate, protein constituents, anti-tumour etc.). Phenols are responsible for the majority of the oxygen capacity in most plant-derived products (Singleton *et al.*, 1999; Robards *et al.*, 1999). With few exceptions such as carotene, the antioxidants present in foods are phenols. Hence, the quantitative analysis was conducted to determine the concentration of polyphenols in extracts whose presence was qualitatively analysed. This is based on the principles that polyphenols when react with Folin reagent give blue colour chromogen in alkaline media, which can be measured at 750 nm. The concentration of polyphenols in extracts is calculated using standard curve prepared with gallic acid. The total phenolic content of Petroleum ether, chloroform, ethanol and water extracts of *E. echinatus* root were found to be 7.5, 12.5, 20.94 and 3.74 mg gallic acid equivalent per gram of extracts respectively. Ethanol extract has the highest total phenolic content (20.94 % w/w) among all other extracts studied.

The bioactive principle constituents mostly phenols have also been reported in the flower extracts of *E. echinatus* by Chudhruri (1988), they are; as n-hentriacontane, n-hentriacontanol, lupeol, lupeol acetate, β-amyrin, β-amyrin acetate, β-sitosterol, palmitic acid, betulinic acid, betulin, apigenin, luteolin, quercetin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin-7-O-β-D-(4”-p-coumaroyl)-glucoside and echinopsine. Similarly, Shukla (2003) also conducted phytochemical studies and reported the presence
of various classes of bioactive pharmacologically potent compounds viz. alkaloids, terpenoids, flavonoids, lipids, polyacetylenes and steroids in whole plant of *E. echinatus*. Further, Singh *et al.*, (2006) have isolated a new isoflavone glycoside, echinoside, together with 7-hydroxyisoflavone, kaempferol-4'-methylether, kaempferol-7-methylether, myrecetin-3- O -α-l-rhamnoside, kaempferol and kaempferol-3- O -α-l-rhamnoside, from the whole plant of *Echinops echinatus*. The structure of echinoside has been established by chemical and spectral data.

Similar observations have been made by several workers in the related species belonging to the genus Echinops. The phytochemical investigation on the root of *E. grijissi* demonstrated the presence of thiophenes (Lu *et al.*, 1989; Guo *et al.*, 1992; Koike *et al.*, 1999; Lin *et al.*, 1999; Liu *et al.*, 2002) and essential oil (Guo *et al.*, 1994). Bioactive flavonoids, acylflavone-glycoside, echinacin, echinacin and tamarixetin have been isolated from the *E. niveus* by Singh and Pandey (1994). The essential oil, exclusively tricyclic sesquiterpenes, viz. silphiperfol-6-ene, presilphiperfol-7(8)-ene, silphiperfolan-6-ol, were isolated from the roots of *E. giganteus* by Menut *et al.*, (1997). A new neolignan glycoside, 1-(3-methoxy-4-hydroxyphenyl)-3-hydroxy-2-(2-O-beta-D-glucopyranosyl-3-methoxy-5-((1E)-3-hydroxypropenyl)phenyl) propanone has been isolated from the root of *E. grijissii* (Koike *et al.*, 2002). Hymete *et al.*, (2005) studied the hydroalcoholic extracts of the root, flower head, leaf and stem of *E. ellenbeckii* and *E. longisetus* were investigated for their phytochemical constituents and biological activities. The presence of alkaloids, saponins, phytosterols, polyphenols and carotenoids in the different parts of the plants was observed. There are also several reports suggesting the presence of phenols in a variety of plant species belonging to various genera. All these observations clearly substantiate the phenomenon that presence of phenolic compounds certainly attributes to the pharmacological efficacy of the plants. Hence, further studies related to the evaluation of pharmacological properties of this plant have been carried out by employing various parameters as mentioned in the objectives.
5.2 Isolation and characterization of the bioactive constituents:

Based on the primary phytochemical analysis followed by preliminary pharmacological screening of the various crude extracts, the micro analysis or the characterization of the active constituents has been carried out by employing appropriate analytical techniques. In this study the ethanol extract has been subjected for isolation and characterisation as this extract has very prominently exhibited the presence of phenolic compounds and further it has proved itself to be more stronger in exerting the pharmacological abilities compared to other extracts of Pet-ethr, chloroform and water.

A. Characterisation by Column chromatography

Plant constituents which were isolated and may be used as starting material for the synthesis of modified structures or they may serve as model for biologically active compounds in natural products synthesis. Alternatively, they may be used as phytochemicals or therapeutic agents. The isolated phytoconstituents can also serve as marker compounds for the standardization of extracts. The ethanol extract of *E. echinatus* root was selected for isolation of bioactive constituents since this extract exhibited potent both *in vivo* and *vitro* antioxidant, Hepatoprotective, woundhealing anti-inflammatory, antimutagenic and antitumour properties (Figs.4 to 20). The ethanol extract of *E. echinatus* root chromatographed on silica gel column. Elution was carried out with solvents and solvent mixtures of increasing polarity. Fractions were collected in 15 ml portions and monitored by TLC and fractions showing similar spots were combined. In the present study three triterpenoids have been isolated from the ethanol extract of the *E. echinatus* root. The investigations on the IR, $^1$HNMR, $^{13}$CNMR and Mass spectral analysis of the isolated constituents confirmed the identity of the constituents as lupeol, betulin and friedelin.

i. Characterization of Compound 1(PE-1)

The $^1$H NMR spectrum revealed the presence of terminal methyl groups at $\delta$ 0.7589 to $\delta$ 1.0279, a singlet at $\delta$ 4.5654 due to olefinic protons. The $^{13}$C NMR spectrum
of the Code-1 showed 30 signals for 30 carbon atoms. The FAB$^+$ mass spectrum showed the molecular ion peak at m/z 426.3 corresponding to the molecular formula C$_{30}$H$_{50}$O. Therefore, compound 1(PE-1) was characterized as pentecyclic triterperine with an isopropenyl group which is commonly known as lupeol. The IR spectrum of the compound 1 showed absorptions bands at 3332.17 cm$^{-1}$, and 2945.43 cm$^{-1}$, 2872.13 cm$^{-1}$ and 890 cm$^{-1}$ for hydroxyl group and terminal methylene groups respectively. The following investigators have also isolated the same compound from different plants. Kiem et al., (2005) applied the gradient elution technique using Silica gel column for the isolation of lupeol from the methanol extract of the leaves of *Caesalpinia decapetala*. Similar technique has been used by Suksamrarn et al., (2006) to isolate the lupeol from the ethanol extract of *Ziziphus cambodiana* root bark. In another study, the investigators Fotie et al., (2006) adopted a combination of flash Silica gel and Sephadex LH-20 column chromatography technique followed by preparative TLC to isolate the lupeol from the hexane-dichloromethane (7:3) eluted fractions of chloroform extract of the stem bark of *Holarrhena floribunda*.

### ii. Characterization of Compound 2(CE-1)

The IR spectrum of the compound 2 revealed the presence of a C=C stretching at 1644.39 cm$^{-1}$ and the secondary alcoholic group at 1008.81 cm$^{-1}$. It showed a broad absorption band for the OH group at 3467.20 cm$^{-1}$. The $^1$H NMR spectrum of compound 2 showed the signals at $\delta$ 0.752 to 1.618 due to five methyl groups. The signal of hydroxy group was observed as a doublet at $\delta$ 4.679. The $^{13}$C NMR spectrum of Code-2 gave the proof of 30 carbon atoms in the compound. A prominent molecular ion peak at m/z 442.5 (M$^+$) in the MS-MS spectrum corresponds to the molecular weight 442.72 and the assigned formula C$_{30}$H$_{50}$O.

Considering these points from the IR, $^1$H NMR, $^{13}$C NMR and mass spectral data, compound 2 was therefore determined to be the known structure 20(29)-lupene-3,28-diol, more commonly known as Betulin. In other plants also the same bioactive principle have been isolated. Chang et al., 1999 have isolated betulin from *Syzygium formosanum*. Similarly Horiuchi et al., 2007 have reported the isolation of betulin from *Salvia*.
officinalis, by employing TLC technique. Mankini and Krishna (2004) have employed a different method to isolate betulin from the leaves of Diospyros cordifolia. The petroleum ether extract was dissolved in hot benzene and allowed to cool and precipitate and betulin was recovered from ethyl acetate.

iii. Characterization of Code-Compound 3(CE-2)

In IR spectra the KBr band at 1715.76 cm\(^{-1}\) due to C=O stretching; 2925.17 cm\(^{-1}\) due to C-H stretching of CH\(_3\). an absorption at 1389.77 cm\(^{-1}\) due to C-H deformation in gem dimethyl. The \(^1\)H-NMR spectrum of the compound Compound-3 showed the presence 3H at \(\delta\) 0.7228. The absorptions at \(\delta\) 1.0046, 1.178 and 1.2511 indicate the protons of terminal methyl groups. The \(^{13}\)C NMR spectrum showed the presence of 30 carbon atoms in the Code-3. In the mass spectrum the molecular ion peak at 426.4 [M\(^+\)] corresponds to the molecular weight of the compound i.e. 426.72. Based on the above data compound 3(CE-2) was identified as friedelin.

Few reports are also available indicating the presence of friedelin in some plants. Moiteiro et al., (2001) isolated friedelin from the cork extracts of Quercus suber. by employing stepwise gradient elution technique in which the petroleum ether extract was chromatographed on Silica gel column using petroleum ether-dichloromethane mixtures of increasing polarity as elution. Huerta-Reyes et al., (2004) also isolated friedelin from the hexane extract of leaves of Calophyllum brasiliense. In this method friedelin precipitated as crystalline compound directly from the hexane extract during solvent evaporation.

5.3 Pharmacological Investigation:

A. Antioxidant activity:

Reactive oxygen species have been implicated in more than a hundred diseases from malaria to haemorrhagic shock to AIDS (Alho and leinonen, 1999). Oxidative stress causes various forms of tissue damage and inflammation and plays a main role in the development of several degenerative changes in cells and tissue, which lead to several degenerative disorders. Defences from body are not completely efficient to prevent the on
Discussion

going oxidative damage to DNA, lipids and proteins (Matkovics, 2003). Recently, natural products and drugs as antioxidant agents have received much attention. Many plant extracts and plant products have shown antioxidant activities (Couladis et al., 2003; Lee, et al., 2004). In the modern western medicine, the balance between antioxidant and oxidant is believed to be a critical concept for maintaining a healthy biological system (Davies, 2000; Tiwari, 2001). A high intake of plant products is associated with a reduced risk of number of chronic diseases, such as atherosclerosis and cancer (Gossslau and Chen, 2004). These beneficial effects have been partly attributed to the compounds which possess antioxidant activity. Bioactive principles are very interesting as antioxidants because of their natural origin and ability to act as efficient free radical scavengers (Langley-Evans, 2000).

Epigallocatechin-3-o-gallate, lycopene, quercetin, genistein, ellagic acid, ubiquinone and indole-3-carbinol are among the major antioxidants apart from well known antioxidant vitamins, ascorbic acid and α-tocopherol which are used as nutritional supplements for prophylaxis or therapy of various disorders like cancer, diabetes, cardiovascular diseases, autoimmune diseases and neurodegenerative disorders (Venkat Ratnam, et al., 2006). Hence, in the present study all the four extracts of *E. echinatus* root were evaluated for *in vitro* antioxidant activity by employing various protocols.

The assay of scavenging the DPPH radical is a common method to evaluate antioxidant activity. The DPPH test (Wagner, 1996) provides information on the reactivity of compounds with a stable free radical. Because of its odd electron, 2, 2-diphenyl-picryl-hydrazyl radical (DPPH) gives a strong absorption band at 517nm in visible spectroscopy. This DPPH radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and as the indicator for the reaction.

\[
\text{DPPH}^\cdot + \text{AH} \rightarrow \text{DPPH-H + A}^\cdot
\]

As the electron becomes paired off in the presence of a free radical scavenger, the absorption varnishes, thus the consequential decolorization is stoichiometric with respect
to the number of electrons. The scavenging properties of antioxidants are frequently linked with their ability to form stable radicals. DPPH has long been recognized as a convenient reagent to enumerate antioxidants in complex biological systems and has been extensively employed for this purpose (Larrauri et al., 1999; Yen and Wu, 1999).

Data reported in the present study shows the significant antioxidant content of ethanol extract of *E. echinatus* root. Results reveal that the decrease in the concentration of DPPH radical due to scavenging ability of ethanol extract. There is a 50.00% decrease of the DPPH radical at a 36 μg ethanol extract concentration. Other extracts did not exhibit the activity. Due to presence of more phenolic compounds such as terpenoids and flavonoids in ethanol extract compared to other extracts, it exhibited good antioxidant activity. Miguel et al., (2005) reported the DPPH radical scavenging activity of Columbian traditional medicinal plant *Talauma hernandezii*. They studied the activity using the petroleum ether extract of leaves of *T. hernandezii*. The extract showed the inhibition of DPPH radical was about 23.1, 24.3 and 29.5% at the concentrations of 5, 10, 20 μg/ml respectively. They used α-Tocopherol as a reference standard, which exhibited 57.1% of DPPH inhibition at the concentration of 20 μg/ml.

Ramteke et al., (2007) studied the DPPH radical scavenging activity of leaf extracts of *Physalis minima*. They found that 50 and 100 μg/ml of the extracts lowered the radical level to 57% and 80%, respectively. They used Butylated hydroxytoluene (BHT) as a standard reference compound.

Experiments were carried out to investigate the direct superoxide anion scavenging activity of the extracts. A chemical superoxide anion generating system (NADH/PMS/NBT) was chiefly used to determine the scavenging activities of the compounds. In the PMS/NADH -NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Addition of various concentrations of extract, as well as butylated hydroxy toluene (standard) in the above coupling reaction exhibited a decrease in the absorbance. The ethanol extract demonstrated very significant with a maximum
inhibition being 50% at 37 μg concentration in vitro antioxidant activity by suppressing the formation of superoxide anion radical, whereas a moderate superoxide scavenging activity was seen in chloroform extract. Other extracts did not exhibit the activity.

Earlier reports of Coban and Konuklugil, (2005) also suggest similar activity where the superoxide anion scavenging activity of ethanol extract of *Linum arboretum* exhibited 88% inhibition of superoxide at higher concentration of 10 mg/ml. In their study they have taken α-Tocopherol a reference standard, which displayed a significant scavenging effect (85%) at the lower concentration of 0.8 mg/ml. In a related work, Sheetal *et al.*, (2007) reported the free-radical scavenging activity of *Bergia suffruticosa*. They showed that the methanol extract of whole plant possess a significant antioxidant activity, at the concentration of 500 μg exhibiting 71.45 % of superoxide anion inhibition.

Lipid peroxidation is the oxidative deterioration of polyunsaturated fatty acids. The peroxidative process leads to the formation of free radical intermediates. It is may be due to interaction of reactive oxygen spices with polyunsaturated lipids in cell membrane and leads to subsequent changes in the structure, function and permeability of cell membrane, ultimately leading to cellular death. (Callaway *et al.*, 1998).

Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex of compounds. These include reactive carbonyl compounds. The most abundant among them is malondialdehyde (MDA), one of the secondary lipid peroxidation products. MDA is a representative of thiobarbituric acid reacting substances (TBARS). The amount of MDA is a measure of lipid peroxidation which provides an estimation of free radical activity (Bonnefont *et al.*, 1989). This assay is based on the reaction of breakdown products of lipid peroxidation such as MDA (malonodialdehyde) and other aldehydes, which form a pink chromogen with TBA, absorbing at 532 nm (Kosugi *et al.*, 1987). In the present investigation, the all extracts were assessed for the lipid peroxidant activity by TBARS determination method. The percentage of inhibition for ethanol extract with a maximum inhibition being 50% at 38μg concentration.
Sithisarn et al., (2005) has worked on the leaves, fruits, flowers and stem bark extracts from the Siamese neem tree (Azadirachta indica). They assessed the different extracts for antioxidant activity in vitro using the DPPH scavenging assay, total antioxidant activity and inhibition of lipid peroxidation by the thiobarbituric acid reactive substances method. Their results showed that flower ethanolic and leaf aqueous extracts significantly decreased malondialdehyde (MDA) levels (46.0 and 50.6%, respectively) by at 100 µg/ml concentration. Cruz et al., (2007) assessed essential oils of Salvia officinalis for the lipid peroxidant activities by using the TBARS method. Among the isolated essential oils, the 3h-oil showed the best antioxidant activity (79%) at 1000mg/L. They compared the activities with that of standard reference α-Tocopherol.

Antioxidant activity is one of some factors that probably contribute to the mechanisms responsible for the therapeutic efficacy of drugs to reduce chronic diseases. The relative activity of ethanol extract has been calculated under different in vitro model conditions and in all the models of in vitro anti-oxidant assays, the ethanol extract exhibited strong free radical scavenging ability.

Among the all extracts tested for in vitro antioxidant activity using different methods, the ethanol extract of E. echinatus root was found to be more potent. A large number of phenolic compounds are known to possess potent antioxidant activity (Badami, et al., 2003; Lee, et al., 2004). The preliminary tests of the ethanol extract indicated the presence of carbohydrates, terpenoids, steroids, tannins, glycosides and phenolic compounds. The total phenol content of the ethanol extract was found to be higher than all the extracts. Hence, potent in vitro antioxidant activity of the ethanol extract might be due to the presence of higher amounts of these compounds in the extract. Structurally, the characterised compounds viz., lupeol, betulin and friedelin, are possessing lactone rings. It has been presumed that there is a structure – function relationship between the bioactive compounds with their biological properties. The presence of lactone ring in the bioactive compounds has been discussed in the findings of Kamdem et al., (2002) where they suggest that the compounds with lactone ring in their structures are biologically very strong in scavenging the free radicals, they might scavenge free radicals by donating the allylic hydrogen of the lactone ring. Another such
study reports that the lactones involved in anti-aging process via attenuating lipid peroxidation and NO and apoptosis of cerebral cells (Dong et al., 2004).

B. **Hepatoprotective activity:**

In the *in vitro* antioxidant activity studies, the ethanol extract exhibited potent antioxidant activity, so all extracts and isolated bioactive constituents were analyzed for its hepatoprotective and *in vivo* antioxidant studies using CCl₄ intoxicated Wistar rats model and various biochemical parameters in serum was estimated along with histological study of liver.

Liver is one of the vital organs in the body. It is most concerned with the vital functions of the body such as metabolism and detoxification. Moreover, it is one of the most frequently exposed organ and liable for damage in the body and it is indeed fortunate that it has enormous functional routes (Bhat, et. al., 1996). Many hepatotoxic chemicals like paracetamol (Wang, 1983), alcohol (Tripathi, 1991) and CCl₄ (Wagner, 1986) cause damage to hepatocytes. Among these toxins the hepatic damage caused by CCl₄ is more and it simulates the human hepatitis (Recknagel, 1989). Hence CCl₄ was used as a tool to induce toxic hepatitis in the experimental models.

According to Robbins (2008), in toxic condition the formation of free radicals takes place. Free radicals are chemical species that have a single unpaired electron in an outer orbital. Energy created by this unstable configuration is released through reactions with adjacent molecules, such as inorganic or organic chemicals such as proteins, lipids and carbohydrates particularly with key molecules in membranes and nucleic acids. Moreover, free radicals initiate autocatalytic reactions, whereby molecules with which they react are themselves converted into free radicals to propagate the chain of damage.

Free radicals may be initiated within cells in several ways, such as by absorption of radiant energy (UV rays and X-rays) and endogenous oxidative reactions. Enzymatic metabolism of exogenous chemicals/drugs e.g. CCl₃, a product of CCl₄, cause cell injury by (a) lipid peroxidation of membranes usually by oxygen derived free radicals, (b) oxidative modification of proteins and (c) lesions in DNA.
The toxic effect of CCl₄ is due to its conversion by P₄₅₀ to highly reactive toxic free radical CCl₃ that initiates lipid peroxidation.

\[ \text{CCl}_4 + e^- \rightarrow \text{CCl}_3 + \text{Cl}^- \]

The free radicals are produced locally, because autooxidation of polyenoic fatty acids present within membrane phospholipids. There by oxidative decomposition of the lipid is initiated, and organic peroxides are formed after reacting with oxygen (lipid peroxidation). This reaction is autocatalytic in that new radicals are formed from peroxide radicals themselves. Thus, rapid breakdown of structure and function of endoplasmic reticulum is due to decomposition of lipid.

CCl₄ induced liver cell injury is both severe and extremely rapid is onset. Within 30 minutes, there is decline in hepatic protein synthesis, within 2 hours, there is a swelling of smooth endoplasmic reticulum and dissociation of ribosomes from rough endoplasmic reticulum takes place. Lipid export from the hepatocytes is reduced owing to their inability to synthesize apoprotein to complex with triglycerides and thereby facilitate lipoprotein secretion. This leads to the fatty liver due to CCl₄ poisoning. Mitochondrial injury then occurs, and this is followed by progressive swelling of cell due to increased permeability of plasma membrane. Plasma membrane damage is thought to be caused by relatively stable fatty aldehydes, which are produced by lipid peroxidation in smooth endoplasmic reticulum but are able to act at distant sites. This is followed by massive influx of calcium and cell death. Therefore, the examination of preventive action in liver damage caused by CCl₄ may give an indication of the liver protective action of the drug in general. A number of plants have been shown to processes the hepatoprotective activity by improving antioxidant properties. (Torres-Duran, et al., 1999)

The liver contains several types of cells. Hepatocytes are polar epithelial cells and as such have apical and basolateral surfaces. AST (Serum aspartate transaminase) and ALT (Serum alanine transaminase) are aminotransferase group of enzymes, those catalyses the reversal transfer of the amino group from alpha amino acid to an oxo acid.
AST is found in both the cytosol and mitochondria of hepatocytes (Herlong, 1994). High tissue levels are also found in heart, kidney, and skeletal and cardiac muscles. Hepatocellular damage with the subsequent disruption of the plasma membrane allows leakage of intracellular enzymes such as AST and ALT from liver into the blood (Scheig, 1996). Significant raise in serum transaminases concentration is the index of liver damage. ALP is an enzyme present in the cells lining of the biliary ducts of the liver. ALP is also present in bone and placental tissue. Increase in its activity is due to increased synthesis in the presence of increased biliary pressure. (Moss and Butterworth, 1974). In the present investigation it was observed that the levels of the serum marker enzymes was significantly (p<0.001) increased in the animals treated with CCI4. Concomitant administration of the ethanol extract with CCI4 showed significant (p<0.001) reduction in the serum enzyme levels. However, other extracts of *E. echinatus* root failed to protect the liver against the CCI4 damage. Among the isolated constituents of *E. echinatus* root friedelin was highly significant (p<0.001) in reducing the toxic effect of CCI4, by controlling the levels of the serum marker enzymes ALT, AST, ALP compared to other isolated constituents lupeol and butelin. This effect was comparable to that of the standard drug silymarin. However, significant reversal of the enzyme levels was observed in animals treated with the standard drug silymarin. The hepatoprotective effect of the extracts and the constituents were compared with the standard drug silymarin which is a mixture of silibyn related flavonoids extracted from the seeds of *silybum marianum* (Choudhari, 1999). It is a potent hepatoprotective drug commonly prescribed by the physicians to heal jaundice. Reports are also available for the use of silymarin as a reference standard drug (Chun-Chin Lin, et. al., 1995).

Bilirubin is a breakdown product of haemaglobin. The liver is responsible for clearing the blood of bilirubin. Determination of serum bilirubin represents an index for the assessment of hepatic function and any abnormal increase in the bilirubin in the serum indicate hepatobiliary disease and severe disturbance of hepatocellular function. Hence, hyperbilirubinemia is common in hepatitis condition. In the present investigation, it was observed that concomitant treatment of the animals with ethanol crude extract showed significant reduction in the levels of serum bilirubin. In the animals treated with
friedelin there were a significant reduction in the serum level of bilirubin. While the other extracts of *E. echinatus* root and lupeol and betulin isolated constituent extracts had insignificant effect on the serum bilirubin. However, significant reduction in the serum level of bilirubin was observed in animals treated with the standard drug silymarin.

A healthy functioning of the liver is required for the synthesis of the serum proteins except for gamma globulin. The protein synthesis processes in the liver are usually decreased in hepatocellular diseases, but the immunoglobins are increased in viral hepatitis and chronic liver infections (Sherwin and Sobenes, 1996). The oxidative modifications by CCl₄ alter the biological properties of proteins leading to their fragmentation, increased aggregation and enzyme dysfunction (Levine, 2002). A significant decrease in the level of total protein was observed for the CCl₄ treated animals in serum, when compared to normal animals. The significant restoration of the level towards normal by the ethanol extract and friedelin treatment indicates the protection of the vital organs from the damage induced by CCl₄. However, significant restoration in the serum level of protein was observed in animals treated with the standard drug silymarin.

The histopathological studies of the liver tissue also evidenced the hepatic lesions caused by the effect of CCl₄ and the therapeutic efficacy of the extracts and isolated constituents by controlling the toxic effect of CCl₄, which caused peroxidative degradation in the adipose tissue, and as a result, fatty infiltration occurred in the hepatocytes. The increase in the levels of serum bilirubin, transaminases and alkaline phosphatases was the clear indication of cellular leakage and loss of functional integrity of the cell membrane (Saraswath, et. al., 1993). Histological profile of the control animals showed normal hepatocytes. The sections of the animals treated with CCl₄ exhibited severe intense centrilobular necrosis, vacuolization, and macro vesicular fatty change. The sections of liver taken from the animals treated with standard drug silymarin showed the hepatic architecture, which was similar to that observed in the vehicle control. Treatment with the ethanol extracts gave significant protection to the hepatocytes against CCl₄ damage. Among the isolated constituents of friedelin exhibited potent hepatoprotective activity as indicated by the presence of normal hepatic cords, absence of
necrosis and macro vesicular fatty change. While the other extracts of *E. echinatus* root and other isolated constituents were devoid of any hepatoprotective activity.

The hepatic injury caused by CCl₄ is associated with damage to the endoplasmic reticulum and any compound capable of preventing the toxicity of CCl₄ must have some direct or indirect effect on the liver (Maruice *et al*., 1987). Phenolics, flavonoids and tannins are known hepatoprotective and antioxidant agents (Gutteridge, 1994). The investigation of Khalid, *et al.*, (2002) revealed that Rutin, a well-known flavonoid, isolated from *Artemisia scoparia* Thunb. (Asteraceae) has exhibited hepatoprotective activity by inhibiting cytochrome-P₄₅₀ (CYPs) enzymes, which oxidize CCl₄ into the toxic radical. Phytochemical investigation reveals the presence of these compounds in the ethanol extract. The observed hepatoprotective activity of the ethanol extract and its constituent friedelin of *E. echinatus* root are probably due to either the anti-oxidant property or inhibition of the activities of cytochrome-P₄₅₀ (CYPs) enzymes. The following reports also strengthen the present results.

Krishna and Shanthamma (2004) reported the hepatoprotective activity of root extracts of *Boerhaavia erecta*. The 50% ethanol extract at the dose of 100 mg/100g b.w significantly reversed the toxicity induced by the CCl₄. It was evidenced by the decreased level of serum bilirubin, significantly elevated concentration of total proteins in serum and the depletion in the levels of serum markers such as, AST, ALT and ALP. In a similar work by Krishna *et al.*, (2005), the hepatoprotective activity of stem bark of *Diospyros cordifolia* has shown the restoration of levels of serum proteins and the levels of AST, ALT and ALP. Similarly, the results obtained by Vidya *et al.*, (2007) also confirms our results in the extracts of *Clerodendrum serratum* and Ursolic acid (isolated compound), against carbon tetrachloride-induced toxicity in rats.

Meera *et al.*, (2009) reported antioxidant and hepatoprotective activites of *Ocimum basilicum* Linn and *Trigonella foenum-graecum* Linn against H₂O₂ and CCl₄ induced hepatotoxicity in goat liver. Significant hepatoprotective effects were obtained by ethanolic extracts of leaves of these plants against liver damage as evidence by decreased levels of antioxidant enzymes (enzymatic and non enzymatic). The extract also showed
significant anti lipid peroxidation *in vitro*, besides exhibiting significant activity superoxide radical and nitric oxide radical scavenging, indicating their potent antioxidant effects.

C. Wound healing activity:

The wound healing means replacement of destroyed tissue by living tissue. The four basic processes which take place in wound healing are inflammation (early and late), granulation tissue formation and reepithelialization, finally wound contraction. Immediately after disruption of tissue integrity and inflammation starts. Platelets become adherent and with clotting factors form haemostatic plug to stop bleeding from the small vessels. The prostaglandins (PGE₁ and PGE₂) are released in the inflamed area, seem to be the final mediators of acute inflammation, and may play a chemotactic role for white blood cells and fibroblasts. Actively motile white cells migrate into the wound and start engulfing and removing cellular debris and injured tissue fragments. Leukotaxin (a peptide formed in the damaged tissue by the enzymatic destruction of albumin) is thought to be the chemotactic agent, attracting leukocytes into the wounds. Monocytes must be present to create normal fibroblasts production.

Wound contraction begins initially slowly, after 3 or 4 days rapid wound contraction occurs. Collagenation increases rapidly during mid-phase. The myofibroblasts present in the margins of the wounds appear to constitute the machinery for the wound contraction. These are responsible for moving the overlying debris. Epithelialization of the wound mainly occurs by proliferation and migration of the marginal basal cells lying close to the wound margin. The haematoma within the wound is soon replaced by the granulation tissue, which consists of new capillaries and fibroblasts. The newly formed capillary loops leak protein and thus the tissue fluid which is formed is a very suitable medium for fibroblastic growth. Fibroblasts are responsible for the production of the mucopolysaccharide ground substance. The lymphatics develop, new nerve fibers are formed, hyalinization occurs. There is also formation of scar tissue. Collagen turn over increases and remodeling in the scar continues. The gross appearance of remodeling scars suggests that collagen fibers are altered and re woven into different
architectural patterns with time. Initially the strength of the wound is only that of the clot, which cements the cut surfaces together. Later on various changes take place in the wound healing process as mentioned earlier. At the end the tensile strength of the wound corresponds to the increase in the amount of collagen formation.

The study presented a multiscale modelling framework which allows to study the effects of different factors on wound healing, such as contraction of cutaneous wound, its tensile strength, collagen alignment, the hydroxyproline content and the strength of granuloma tissue and scar formation during dermal wound healing. Therefore, in order to examine the above mentioned parameters, the three different models were used in this study to assess the wound healing effect of various extracts and the isolated constituents on various phases of wound healing, which run concurrently, but independent of each other. The standard drug nitrofurazone is used as a standard reference to assess the healing effect of the extracts and the constituents against the controls. Jaswanth, et al., (2001) and Hemalatha, et al., (2001) also used nitrofurazone as the standard drug to evaluate the wound healing effect of *Aegle marmelos* and *Indigofera enneaphylla* Linn.

The results of the present study clearly indicates that ethanol extract and its constituents enhanced healing of all the three types of cutaneous wounds. Application of ointment base prepared from ethanol extract displayed significant wound healing activity. The healing time required for complete epithelialization of the excision wound was found to be much earlier (14.8± 0.48th post wounding day) and it was on par with that of the standard reference drug sulphathiazole. While in placebo treated group animals the duration of epithelialization was delayed by 10 days. In case of ether, chloroform and water extracts treated and the control animals, complete wound contraction occurred on 22nd, 20th, 22nd and 25th day respectively.

In the animals treated with the isolated constituents lupeol and betulin the complete wound contraction observed on 15.33±0.33th day and 17.67±0.33th day respectively. The complete wound contraction was occurred on 21.67±0.49th day in the animals treated with friedelin.
The breaking strength is the strength of a healing wound and is measured experimentally by the amount of force required to disrupt it. In the beginning, a wound will be having little breaking strength because the clot alone will be holding the edges together. Thereafter breaking strength increases rapidly as collagen deposition increases and cross linkages are formed between collagen fibers. By the 8th to 10th day there is sufficient restoration of breaking strength and stitches can be removed. The breaking strength of a wound is a point of practical importance in surgery.

In the incision wound model, the animals treated with ethanol extract of *E. echinatus* root showed significant increase in the tissue breaking strength (619.17±6.64g) on 10th post wounding day. The tissue breaking strength of the animals treated with petroleum ether, chloroform and water extracts of *E. echinatus* root were 503.33±4.59, 544.17±8.00, 505.50±6.64g respectively and comparatively less to ethanol extract. The results of this investigation coincides with the works of Taranahalli, *et al.*, (1996) on *Trigonella foenum gracecum*, Leite, *et al.*, (2002) on *Veronica scorpioides*, and Shirwaikar, *et al.*, (2002) on *Gmelina arborea* Roxb. leaves. In incision wound model the constituent lupeol was found to be more effective in increasing the breaking strength (620.00±4.83g) as similar to that of the standard drug nitrofurazone (634.17±5.83g) and others constituents were comparatively less effective.

The presence of the foreign body in the subcutaneous area initiates the formation of granulation tissue around it. Initially new blood vessels are formed accompanied by lymphatics. These arise from the preserved lymphatics at the margins of the wound. In the initial three days of the injury the intercellular spaces are filled with proteinous fluid. Later the fluid becomes gelatinous and shows increasing quantities of mucopolysaccharides which are either produced locally by fibroblasts or mast cells or come from the blood. The intercellular fibers are laid down in the wound fluid from the 4th day onwards and the concentration of mucopolysaccharides starts declining. At first these are fine thread like, later they coarsen and thicken. In the beginning, a collagen fiber run parallel and in one plane but soon their arrangement is remodeled to suit local mechanical stresses. Finally, it forms a tough membrane of laminated collagen, which is the essential material for healing of the wound. By this time, fibroblasts decrease in
number and appear as shrunken in conspicuous fusiform cells in between rows of collagen fibers. The concentration of mucopolysaccharides becomes normal or even low. The wound has now acquired significant tensile strength.

The newly formed tissue is known as granulation tissue because it has the appearance of pink granules protruding from the floor of the wound. Microscopically these granules show newly formed capillaries, fibroblasts and leukocytes. Because of more vascularization, granulation tissue bleeds easily on the slightest trauma. It lacks nerves and therefore insensitive. It is also resistant to infection because of macrophages present in its interstices. As more and more collagen fibers are laid down, vascularization in the granulation tissue decreases. The conversion of granulation tissue into fibrous scar tissue is known as cicatrisation. The breaking strength of the granulation tissue increases proportionately with the collagen deposition. The hydroxyproline is an amino acid distributed mainly in the collagen tissue. The hydroxyproline content of granulation tissue also increases with the increased collagenation.

The effects of oral administration of suspensions of the root extracts and isolated constituents on the dead space wound models were also assessed by the increase in the weight of granulation tissue, in its breaking strength and hydroxyproline content of the granulation tissue. The increase in breaking strength of the granulation tissue indicates enhanced collagen maturation by increased cross-linking of collagen fibers. While, an increase in granulation tissue weight indicates the presence of higher protein content. Among these treated animals the response was shown to be the best in *E. echinatus* root ethanol extract and lupeol and butelin treated animals. Other extracts and friedelin did not exhibited potent activity compared to ethanol extract and other isolated constituents.

Histopathological study of the granulation tissue provides further evidences on the wound healing efficacy of the extracts and the constituents. The sections of the granulation tissue of the untreated animals showed monocytes and fibroblasts. Incomplete healing was evidenced with lesser epithelialization, fibrosis, and collagen formation. The sections of the granulation tissues of the animals treated with ethanol extract of *E. echinatus* showed complete epithelialization, fibrosis and collagen
formation. Whereas, in the other extracts treated animals the healing activity was comparatively less and it is indicated by the presence of the monocytes and fibroblasts.

Histopathological examination of the sections of the granulation tissues of the animals treated with lupeol and betulin showed lesser monocytes, fibroblasts and increased collagen deposition. This fact suggests its potent wound healing property. Comparatively lesser collagen formation was observed in the animals treated with friedelin.

Several phytoconstituents like triterpenids (Somava, et al., 2003), alkaloids (Marjorie, 1999) and flavonoids (Suchiya et al., 1996) are known to promote wound healing process due to their antioxidant and antimicrobial activities, which seems to be responsible for wound contraction and increased rate of epithelization. In addition, triterpenoids reported to possess an ability to increase the collagen content, which is one of the factors promoting wound healing (Veerapur, et al., 2004). Further more, the wound healing effect can be attributed to free radical scavenging activity of flavonoids and triterpenoids. Both these class of phytoconstituents are known to reduce lipid peroxidation, not only by preventing or slowing the onset of cell necrosis, but also improving vascularity. Lipid peroxidation is an important process in several types of injuries like burns, infected wounds, skin ulcer etc. Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils, which in turn results in increase in the strength of collagen fiber by increasing the circulation, preventing the cell damage and promoting the DNA synthesis (Joshi, et al., 2003). Phytochemical investigation reveals the presence of carbohydrates, terpenoids, steroids, tannins glycosides and phenolic compounds. Hence, the observed wound healing activity of ethanol extract and isolated bioactive constituents lupeol and betulin of E. echinatus root may due to its antioxidant activity and due to presence of such compounds in extract. There are many reports which further strengthens the present observations.

Singh et al., (2005) evaluated the wound healing activity of the leaf extract and deoxyelephantopin isolated from Elephantopus scaber. The ethanol extract and the constituent deoxyelephantopin exhibited significant wound healing activity in all the
three different models of wound. In excision wound model, wound contraction and time taken for the complete epithelialization for extract and constituent were 16.6 days and 14 days respectively. The skin breaking strength in incision model was 380 and 412 g respectively. The dry weight of granulation tissue and its tensile strength was increased considerably after the oral administration of ethanol extract and the constituent deoxyelephantopin. They used Nitrofurazone ointment as standard reference drug.

Similarly Manjunatha et al., (2005) reported the wound healing potency of *Vernonia arborea* using different extracts of leaves assessed for three different wound models. Among them methanolic extract showed excellent wound healing activity which was almost 100% closer of excision wound on 18\textsuperscript{th} post wounding day. This healing activity was on par with that of standard drug Framycetin sulphate. Similar results were obtained in *Catharanthus roseus* by Nayak and Lexley (2006). Using the isolated compound of *Embelia ribes* namely, embelin, Kumara Swamy et al., (2007) reported the wound healing activity where wound closer occurred on 16\textsuperscript{th} day.

D. **Anti-inflammatory activity:**

The results of the present pharmacological investigation revealed that the root of *E. echinatus* possesses a potential anti-inflammatory effect which was evidenced by the significant reduction in paw oedema. Inflammation is a local response of living mammalian tissues to the injury. It is a body defense reaction in order to eliminate or limit the spread of injurious agents. There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Oedema formation, leukocyte infiltration and granuloma formation represent such components of inflammation (Mitchell and Cotran, 2000). Oedema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and/or the mediators that increase blood flow (Ialenti et al., 1995). The enzyme, phospholipase A2, is known to be responsible for the formation of mediators of inflammation such as prostaglandins and leukotrienes which by attracting polymerphonuclear leucocytes to the site of inflammation would lead to tissue damage probably by the release of free radicals. Phospholipase A2 converts phospholipids in the
cell membrane into arachidonic acid, which is highly reactive and is rapidly metabolized by cyclooxygenase (prostaglandin synthesis) to prostaglandins, which are major components that induce pain and inflammation (Higgs et al., 1984; Vane, 1971). It is well known that carrageenan induced paw edema is characterized by biphasic event with involvement of different inflammatory mediators. In the first phase (during the first 2 h after carrageenan injection), chemical mediators such as histamine and serotonin play some role, while in second phase (3–4 h after carrageenan injection). Kinin and prostaglandins are involved (Hernandez et al., 2002). Our results revealed that administrations of ethanol, chloroform extract and isolated lupeol, betulin constituents inhibited the oedema starting from the first hour and during all phases of inflammation, which is probably inhibition of different aspects and chemical mediators of inflammation.

There is increasing evidence that lysosomal enzymes play an important role in the development of acute and chronic inflammation (Anderson et al., 1971; Shen, 1967; Weissmann, 1967; Jannoff and Zweifach, 1964). Most of the anti-inflammatory drugs exert their beneficial effects by inhibiting either release of these enzymes or by stabilizing lysosomal membrane, which is one of the major events responsible for the inflammatory process (Nair et al., 1988). In our investigation, the significant (p<0.01) anti-inflammatory activity of chloroform extract may also be probably due to one of the many ways which hinder or seize the arachidonic acid synthesis, whereas the ethanol extract and lupeol, betulin isolated compounds which are triterpenoids in nature, may act by modulating the enzyme cycloxygenase leading to the inhibition of prostaglandin synthesis. From the above studies it is quite apparent that the chloroform and ethanol extract possesses significant anti-inflammatory activity. The present results also justifies and strengthens the earlier works of Singh et al., (1989), Funda and Bilge (1995) and Tunon et al., (1995) where they have also reported the presence of anti inflammatory activity in *E. echinatus*. However, the in the present investigation the anti-inflammatory parameter was selected and experiments were conducted to further cross verify the earlier reports and confirm the relationship of inflammation with the wound. When a compound is anti-inflammatory in nature, then the possibilities of that compound being more potent in the woundhealing activity will be more. As these parameters are interrelated in
pharmacological properties it is therefore imperative to study the related activities of a
given compound.

E. **Antimutagenic activity:**

Internal and external factors including environmental pollutants induce various
kinds of genetic damages, like mutations, chromosome structural changes, cell death etc.
DNA is quintessential part of the cell required for regulating normal cellular activities
and maintenance of its hereditary properties. The primary target of all the physical,
chemical and biological agents is this DNA, leading to mutations. It is also evident that
mutations lead to several diseases including cancer.

Many number of natural and structurally novel compounds from higher plants and
several compounds in food exhibited antimutagenic activity due to the presence of
phytochemicals like phenolics, quinines, glycosides, terpenoids, alkaloids and several
studies have showed a relationship of these activities with antioxidant properties. Hence,
extensive research is going on to decipher natural antimutagenic agents in both *in vitro*
and *in vivo* systems, though at a much lesser scale on *in vivo* system particularly in mice
(Velanganni *et al.*, 2003). Most of the earlier studies on chromosomal aberrations were
carried out in *Drosophila* and plants. In the present investigation in view of certain
advantages of mouse as an *in vivo* system, experiments were carried out to analyze the
antimutagenic property of *E. echinatus* root.

Mouse as an *in vivo* system has many merits, small size, easy maintenance, well
established karyotype and low frequency spontaneous chromosomal aberrations during
experimental work for maintaining mutagenicity. (Benrischke *et al.*, 1967).

A well known monofunctional alkylating agent, Ethyl methanesulfonate (EMS)
served as a standard mutagen to induce clastogenic effect in the mouse model. Dose of
80mg/kg of b.w of EMS, 200 mg/kg of b.w of root extract and 10mg/kg of b.w of
isolated constituent have been selected.
Studies with EMS revealed that the dose of 80 mg/kg b.w can induce significant chromosomal aberrations when compared to different solvent extracts and isolated constituents.

As described in the results (Table 12: Fig 14) sodium alginate treated animals exhibited lower percentage of chromosomal aberrations indicating that it has no mutagenic effect. But the animals treated with EMS alone and with the combination of sodium alginate showed considerable number of chromosomal aberrations. This result indicates that EMS being a mutagen showed significant mutagenic activity on the test system. The results are in conformity with the earlier reports of Mahmood and Vasudev (1993) who have reported the induction of chromosomal aberrations induced by a dose of 80mg/kg b.w, EMS while studying adaptive response in in vivo mouse.

The frequency of aberrations produced by control 1 and the frequencies observed after the treatment with four different root extracts (Table 13: Fig 14) and isolated constituents (Table 25: Fig 29 B) indicates that there is no significant increase in chromosomal aberrations. From these results it is clear that the different extracts and isolated constituents have no role in inducing mutagenicity and thus, they are non mutagenic. Similarly, the sodium alginate used as a vehicle to carry the plant extract also did not exert any mutagenic effect either alone or in combination with root extract and isolated constituents.

The results obtained from the four different solvent extracts (Table 14: Fig 15) and isolated constituents of E. echinatus root (Table 26: Fig 30) are compared with EMS and EMS+extract, EMS+ isolated constituents treated animals which clearly indicate the non-mutagenic nature of the extracts and the constituents.

Treatment with combination of petroleum ether extract with EMS, chloroform extract + EMS, ethanol extract + EMS and water extract with EMS yields 11.6%, 8.95%, 5.56% and 13.65% chromosomal aberrations respectively, which are low compared to aberrations induced by EMS alone (14.9%) alone suggesting the antimutagenic property of the compounds present in the extracts.
From the above results it is clear that among the four extracts of root employed in the present study ethanol extract and chloroform extract have found to possess strong antimutagenic potential against clastogenic ability of the standard mutagen EMS. Where as the petroleum ether and aqueous extract did not shown significant \( p<0.05 \) reduction in the abbreviation frequency. It is opined from the present results that the chloroform and ethanol extracts of root are capable of imparting more protection against mutagenecity induce by EMS. These results also suggest that the phytochemical constituents presence in ethanol and chloroform extract may possess the antimutagenic properties.

The combination of lupeol+EMS, and betulin+EMS treated animals yielded 4.39\% and 6.58\% of aberrations respectively which were significantly \( p<0.05 \) very lesser than the frequency obtained by EMS alone, showing very strong antimutagenic property of these isolated compounds.

Friedelin with EMS yield 11.35\% of chromosomal aberrations which is low compared to aberrations induced by EMS alone indicating the weak antimutagenic potential compared to other isolate compounds.

Thus, it can be opined from the present results that lupeol and betulin isolated constituents of root are capable of imparting more protection against mutagencity induced by EMS as evidenced by the reduction in the percentage aberrations compared to the friedelin.

The protective effect of chloroform and ethanol extracts, isolated constituents lupeol and butelin may be attributed to their antioxidant properties, trapping of free radicals, formation of complex with mutagens, modulation of mutagen metabolism. Further, it may also be due to the presence of high concentration of polyphenols and trepenoids in the extracts.

There are several reports on the protective effects of polyphenols, theaflavins and flavonoids showing the antimutagenicity potential of plant isolated compounds. Weisburger et al., (1996) reported the effects of specific tea phenols against a number of
genotoxic carcinogens. Anticlastogenic and antinutagenic effects of black tea polyphenols have been studied by Haider et al., (2005).

Natural substances such as flavonoids and tannins and their derivates possess antinutagenic properties (Kada et al., 1985, Edenharder et al., 1993). The hydroxycinnamic acid isolated from plant cell wall which are potent antioxidants are also potent antinutagens. The simplest explanation of their inhibitory effect on the mutagenicity is that they reduce the concentration of mutagenic oxidation products by free radical scavenging activity (Fergusan et al., 2003).

Yen and Chen (1996) studied the relationship between the chemical composition of tea leaves and their antinutagenic activity and found that principle components of tea leaves are catechin which is seen to be responsible for antinutagenic activity. According to Bu-Abbas et al., (1994) high concentration of flavonoids in green tea compared to black tea may mean that these compounds are one of those responsible for antinutagenic and anticarcinogenic properties of tea.

Kun-Young Park (2004) reported that the relationship between the chemical composition of heartwood of Rhus vericiflua and the antinutagenic effect. Pretreatment of the methanolic extract of the heartwood of Rhus verniciflua (Anacardiaceae) to rats prevented the activation of hepatic microsomal cytochrome P_{450} enzymes, inhibition of hepatic glutathione S-transferase by bromobenzene treatment, respectively, and therefore significantly decreased malondialdehyde content in the rat. The Ames test showed that the addition of 1.0 mg/plate of the methanolic extract or the EtOAc fraction of the Rhus verniciflua heartwood extract potentially inhibited the mutagenicity by aflatoxin B_{1}. Column chromatography of the EtOAc fraction yielded four flavonoids, garbanzol, sulfuretin, fisetin, fustin and mollisacasin. When these components were subjected to the Ames test, it was found that sulfuretin might effectively prevent the metabolic activation or scavenge electrophilic intermediates capable of causing mutation. In contrast, fustin showed a dose-independent antinutagenic activity and it has mutagenic/antinutagenic activity. However, a mixture of sulfuretin and fustin (1:1) exhibited dose-dependent antinutagenicity indicating that sulfuretin inhibited the mutagenicity of fustin. These
results suggest that the extract of *Rhus verniciflua* heartwood containing flavonoid complex could also be a potent anticarcinogen.

Flavonoids and polyphenolic compounds ubiquitous in plants and found in significant quantities in vegetables, fruits, seeds, nuts and beverages. Duthie et al., (2000) reported the protective effects of flavonoids, quercetin and myricetin against the hydrogen peroxide induced DNA damage in human lymphocyte.

**F. Antitumour activity:**

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year (Abdullaev et al., 2000) An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or in combination) to block the development of cancer in humans. Plant derived extracts containing antioxidant principles showed cytotoxicity towards tumour cells (Jiau-Jian et al.,1977) and antitumor activity in experimental animals (Ruby et al.,1995). Chemoprevention usually targets various stages in the cancer process (Carry and Engstrom, 1999). A number of endogenous and exogenous cancer risk factors generate oxygen free radicals (OFR) *in vivo* (Nakayama et al., 1985). Therefore, there was much hope that the increase of cancer incidence in an aging population could be reversed by removing avoidable sources of OFR and / or by enhancing the antioxidant defence system. OFR may attach lipids and DNA giving rise to a large number of damaged products (Imaly et al., 1988). Iron is known to be involved in the generation of reactive oxygen species (ROS) and in the formation of highly toxic hydroxyl radicals from other active oxygen species such as hydrogen peroxide (Aruoma et al., 1989). The enhanced generation of ROS *in vivo* could be quite deleterious, since they are involved in mutagenesis, apoptosis, aging, and carcinogenesis (Halliwell et al., 1990). Free radicals also cause DNA strand breaks and chromosome deletions and rearrangements. Further, activated oxygen species most likely play an important role in tumour promotion and progression. In the recent years, convincing evidence has accumulated that OFR are indeed a relevant class of carcinogens (Cerutti, 1994). Cancer development is now commonly recognized as a micro evolutionary process that requires the cumulative action
Discussion

of multiple events (Klein, 1987). These events occur in one cell and include in a simplified three-stage: (Janseen, et al., 1993) the induction of DNA mutation in a somatic cell -initiation (Yu, 1994), the stimulation of tumourigenic expansion of the cell clone-promotion (Davies, 1993) and the malignant conversion of the tumour into cancer-progression. OFR can stimulate cancer development at all the three stages (Salim, 1993).

In the present study to evaluate the antitumour ability of E. echinatus root extracts and its isolated constituents Ehrlich ascitic carcinoma (EAC) mouse model has been employed. Ehrlich tumour is a rapidly growing carcinoma with very aggressive behaviour (Segura, et al., 2000). It is able to grow in almost all strains of mice. The Ehrlich ascitic tumour implantation induces per se a local inflammatory reaction, with increasing vascular permeability, which results in an intense oedema formation, cellular migration, and a progressive ascitic fluid formation (Fecchio et al., 1990). The ascitic fluid is essential for tumour growth, since it constitutes a direct nutritional source for tumour cells.

In the results (Table-17) obtained in the antitumour study the packed cell volume and the number of viable EAC tumour cells in peritoneum were significantly lower in mice treated with ethanol extract and with lupeol and betulin (isolated compounds) when compared to the tumour control group. These results could indicate either a direct cytotoxic effect of ethanol extract and isolated compounds on tumour cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

The reliable criterion for judging the value of any antitumour drug is the prolongation of life span of the animals and the disappearance of leukemic cells from blood (Gupta et al., 2004). The ethanol extract and isolated lupeol , betulin treatment decreased the packed cell volume and viable tumour cell count. It also inhibited the increase in body weight due to tumour burden and increased the average life span of animals when compared to the EAC control (Table 16 : Fig 17). Hence, it may be concluded that, ethanol extract compounds by a direct cytotoxic effect or by decreasing the nutritional fluid volume and arresting the tumour growth, increased the life span of
EAC-bearing mice. The percentage increase in life span by ethanol extract and isolated lupeol, betulin were found to be the highest among the tested extracts and compounds indicating its potent antitumour nature.

A significant decrease in haemoglobin and the number of erythrocytes and a significant increase in total WBC in the tumour-bearing mice are known. Anemia is found frequently in cancer patients (De Vita et al., 1993). Similar results are also observed (Table 18: Fig 19) in the present study in animals of the EAC tumour control group. A significant decrease in the total WBC and neutrophils count is observed by the ethanol extract, isolated lupeol and betulin treatment when compared to the tumour control. A significant increase in the RBC, haemoglobin, and lymphocytes towards the normal values by the ethanol extract, lupeol and betulin treatment is seen (Table 18 and 33: Fig 19 and 32). The reversal of haematological parameters indicates that the ethanol extract, lupeol and betulin may possess protective action on the haemopoietic system.

The implication of free radicals in tumour is well documented (Ravid and Korean, 2003). Lipid peroxide, an autocatalytic free radical chain propagation reaction, is known to be associated with pathological condition of a cell. The presence of tumour is known to affect many functions of the vital organs, especially the liver. This leads to an increase in the level of MDA (malondialdehyde), end product of lipid peroxide, in disease control group (Sinclair et al., 1990). It has been reported that a decrease in super oxide dismutase (SOD) activity in EAC cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver of EAC bearing mice (Sun et al., 1989). Glutathione and catalase were also involved in the free radical scavenging activity. There is a reduction in the levels of the scavengers as a result of tumour growth in disease control animals. Hepatocellular necrosis leads to high levels of AST and ALT, which are released from the liver into the blood. ALP activity, on other hand, is related to the functioning of hepatocytes. Increase in its activity is due to increased synthesis in the presence of increased biliary pressure (Moss and Butterworth, 1974). The inoculation of EAC caused a significant increase in the level of ALT, AST and ALP in serum, when compared to normal animals. Reduction in the level of these towards the respective normal values in serum is an indication of stabilization of plasma membrane as well as repair of hepatic
tissue damaged by tumour inoculation. A significant increase in total bilirubin and protein level was observed for the EAC tumour bearing mice, when compared to normal group. A significant reversal of the above indicators towards the normal values observed after the treatment with ethanol extract, lupeol and betulin indicating the normal functional liver. These findings are significantly comparable to that of the standard drug 5-flurouracil. The free radical hypothesis also supported the fact that the ethanol extract and isolated lupeol, betulin possesses significant antitumour and antioxidant potential against EAC bearing mice. The ether, chloroform, and aqueous extracts and friedelin did not show any significant effect on EAC tumour mice. Similar results were observed in our earlier studies using the same parameters in the CCI4 model. Treatment with ethanol extract, lupeol and betulin brought back the levels of these scavenge.

The liver section of EAC tumour bearing mice showed altered architecture of liver tissue and individual necrosis. The liver section of EAC tumour treated with ethanol extract showed liver tissue with maintained architecture and less necrosis. The treatment with isolated bioactive constituents lupeol and betulin exhibited liver protection, as evidenced by the presence of liver tissue with normal architecture and normal hepatocytes. The liver section of EAC tumour bearing mice treated with 5-fluorouracil also revealed normal architecture with normal chords of hepatocytes. These histopathological results also confirm the antitumour, hepatoprotective and antioxidant nature of ethanol extract and its constituents lupeol and betulin. While as Friedelin another isolated constituent did not show any antitumour effect.

Preliminary phytochemical screening indicated the presence of polyphenolic compounds, triterpenoids, alkaloids and flavonoids in extract. Triterpenoids and flavonoids have been shown to possess antimutagenic and antimalignant effects. Moreover, triterpenoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation (Weber et al., 1996) and angiogenesis (Fotsis et al., 1997). The cytotoxic and antitumor properties of the extract and isolated compounds may be due to the chemical nature of these compounds. The observations made by Misra, et. al., (1989) also support our view, where they have evaluated the anti-tumour activity of betulin, betulinic acid and lupeol. They found that these compounds were active
against the Walker- Carcinoma-256 (intramuscular) tumor system. Betulin inhibited P-388 leukemia growth and was also found to possess highly selective activity against human melanoma in vitro and in vivo.

Comparison between the pharmacological properties of extracts and isolated bioactive constituents of E. echinatus root.

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Pharmacological activities</th>
<th>Different extracts of E. echinatus root.</th>
<th>Bioactive constituents isolated from ethanol extract of E. echinatus root.</th>
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<tr>
<td></td>
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<td>PetEther extract</td>
<td>Chloroform extract</td>
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<tr>
<td>1</td>
<td>In vitro Antioxidant activity</td>
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<td>-ve</td>
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<td>2</td>
<td>In vivo and Hepatoprotective activity</td>
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<td>3</td>
<td>Wound-healing activity</td>
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<td>4</td>
<td>Anti-inflammatory activity</td>
<td>+ve</td>
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<td>5</td>
<td>Anti-mutagenic activity</td>
<td>+ve</td>
<td>++ ve</td>
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<tr>
<td>6</td>
<td>Antitumour activity</td>
<td>+ve</td>
<td>++ ve</td>
</tr>
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</table>

-ve = not potent, +ve = less potent, ++ ve = potent, +++ve = more potent

From above comparison between the extracts and isolated constituents for their pharmacological activities, it is clearly indicated that pharmacological activities exhibited by ethanol extract of E. echinatus root mainly due to the presence of isolated compounds which are found to be triterpenoids in chemical nature. Certainly, all pharmacological actions shown by the ethanol extract and isolated bioactive constituents can be (Table 18: Fig 19) unambiguously attributed to the antioxidant abilities. Several earlier reports also confirm these results. Antioxidants neutralize or trap reactive oxygen species and act as disease preventive agents. Furthermore, human observational studies also provide very strong support, showing, on one hand that oxidant stress increases clinical progression of
disease (Khanzode et al., 2004) and, on the other, a diet rich in antioxidants containing foods reduces the risk of many diseases (Steinmnetz and Potter 1996).

The overall results of this investigation using the crude extracts and isolated compounds from the ethanol extract clearly indicate the presence of pharmacological properties. The plant extract is successful in exhibiting antioxidant activities both in vitro and in vivo which includes hepatoprotective activity. Further, the ethanol extract and its constituents viz., lupeol, betulin and friedelin have also proved to be more strong enough to exhibit wound healing abilities, antimutagenic effects and most importantly anti-tumour effects. Thus, all these observations made provide the supportive scientific evidence to suggest that this plant *Echinops echinatus* Roxb (Fam: compositae) possess all the qualities of a medicinal plant further confirming the ethnomedical claim of the traditional medicine. Hence, it is hoped that, these studies could certainly pave the way for further investigations leading to the designing and authentication of the herbal drugs most suitably required for liver disorders and tumours also.