"Isolation and characterization of bioactives from roots of *Hemidesmus indicus* with special reference to its antioxidant and hepatoprotective activity"

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UNDER THE GUIDANCE OF

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CERTIFICATE

This is to certify that **Ms. Preeti Giri** has prepared her thesis entitled "**Isolation and characterization of bioactives from roots of hemidesmus indicus with special reference to its antioxidant and hepatoprotective activity**", in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under our guidance. She has carried out the work at the Department of Phytopharmaceuticals and Natural products, Institute of Pharmacy, Nirma University.

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DECLARATION

I declare that the thesis "***Isolation and characterization of bioactives from roots of Hemidesmus indicus with special reference to its antioxidant and hepatoprotective activity***" has been prepared by me under the guidance of Dr. Sanjeev Acharya, Associate Professor and Mrs. Nagja V Tripathi, Assistant Proffesor, Department of Phytopharmaceuticals & Natural Products, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The liver is a vital organ of paramount importance involved in the maintenance of metabolic functions and detoxification from the exogenous and endogenous challenges like xenobiotics, drugs, viral infections and chronic alcoholism (Dienstag JL *et al).* It is the key organ regulating homeostasis in the body and involved in almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction (Piper DW, *et al).* Diverse homeostatic mechanisms are affected if liver function is impaired, with potentially serious consequences for the individual concerned.

Drug induced liver injury is an unresolved problem and often limits drug therapy in clinical practice. Liver injury may follow the inhalation, ingestion or parenteral administration of a number of chemical and pharmacological agents (Dienstag JL *et al).* The nature and extent of liver damage varies depending on the type and stage of the disease. Not all liver diseases produce the same patterns of change, at least while they are evolving, although many forms of chronic liver disease will ultimately lead to the typical clinical and histological picture of cirrhosis. The effects of liver disease on hepatic metabolism of drugs are complex and difficult to predict, particularly when multiple drugs are administered simultaneously (Piper DW, *et al).*

It is well known that drugs are structurally altered in the liver to form biologically inactive/active or toxic metabolites. Indiscriminate uses of certain category of drugs such as analgesics (Bhanwra S et al, 2000) antimalarials (Dass EE et al, 2000), anti-tubercular (Saraswathy SD, *et al*,1998), antidepressants or immunosuppresants (Josephine A et al, 2008) etc. are potential threats to the integrity of liver. Quite often certain drugs even in therapeutic dose may cause hepatic damage in susceptible individual. The spectrum of drug induced liver injury ranges from asymptomatic increase in enzyme (markers of hepatic damage) levels to fulminant hepatic failure. It can occur in a number of different forms including acute drug-induced hepatitis, steatohepatitis, cholestasis, chronic hepatitis and may lead to liver failure. Many drugs may cause more than one type of hepatic injury patterns (Johnson-Fannin AM, 1996) .

It appears that, the nature and extent of drug-induced hepatic damage is dependant on dose and duration of exposure to the causative agent. Accordingly, single overdose of paracetamol (PCM) and alcohol certainly produce hepatotoxicity. Such heaptotoxicity

though clinically important can be preventable, while hepatic damage due to isoniazid (INH) an effective anti-tubercular drug which is administered chronically, often poses problem in the clinical practice. Measures to prevent and use of effective agents to treat the drug induced hepatotoxicity is essential to ensure safety and efficacy of certain drugs like INH. Tuberculosis (TB) is one of the most common infectious disease, in India pulmonary tuberculosis is one of the major cause for adult deaths (Garner P, et al, 2004). Over one-third of the world's population is estimated to be infected with Mycobacterium tuberculosis and over 2 million peoples a year will die of the disease (Shishoo CJ, et al, 2006). The INH and rifampicin (RMP) the first line drugs used for tuberculosis chemotherapy are associated with hepatotoxicity (Tasduq SA, et al, 2005). The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8%-30%) compared to that in advanced countries (2%-3%) with a similar dose schedule. After introduction of RMP, several reports suggested that hepatitis was more frequent and severe in patients receiving both INH and RMP than in those receiving INH alone (Steele MA, et al, 1991).

Paracetamol is a common antipyretic agent being OTC product. The large number of population misuses the drug and increases the risk of hepatotoxicity accordingly. It is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses. (Mitchell JR, et al).

Cyclosporine-A (Cs-A) is a cyclic undecapeptide of fungal origin, which has been used successfully in organ transplantation and in the treatment of autoimmune disorders (Borel JF, *et al,* 1996). It has been reported that impaired hepatic function occurs in 20 to 50 % of Cs-A treated patients (Deters M, et al, 1997) which may limits the therapy in some individuals.

Liver disease is a worldwide problem. Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects (Mohamed BA, et al, 2001). It is therefore, necessary to search for alternative drugs for the treatment of liver disease to replace currently used drugs of doubtful efficacy and safety. In the absence of a reliable liver protective drug in modern medicine there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders (Chatterjee TK, 2000).

Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs (Chattopadhyay RR, et al, 2007). Medicinal plants play a key role in the human health care. About 80% of the world populations rely on the use of traditional medicine, which is predominantly based on plant materials. The traditional medicine refers to a broad range of ancient, natural health care practices including folk/tribal practices as well as Ayurveda, Siddha and Unani. These medical practices originated from immemorial time and developed gradually, to a large extent, based upon practical experiences without significant references to modern scientific principles. These practices incorporated ancient beliefs and were passed on from one generation to another by oral tradition and/or guarded literature. Although herbal medicines are effective in the treatment of various ailments very often these drugs are unscientifically exploited and/or improperly used. Liver diseases are among the important diseases affecting mankind, in allopathic medicine still no effective hepatoprotective medicine is available. In addition, usage of many folklore remedies, mainly plant products, is also quite common throughout India (Anonymous, 1993).

Inspite of tremendous strides in drug discovery and modern medical practice, there are hardly any drugs that stimulate liver functions or offer protection to the liver against injurious substances or help in regeneration of hepatic cells. The presently used agents like folic acid, multivitamins and few polyherbal preparations provide only a supportive therapy and do not play an effective role in providing hepatic protection (Gulati RK, et al, 1995).

Hemidesmus indicus R. Br (Asclepiadaceae), commonly known as 'Anantamul' or 'Indian Sarasparilla', is one of such drug in Ayurvedic system of medicine. It has been used as a antidiabetic, tonic, alternative, demulcent, diaphoretic, and diuretic, in the treatment of syphilis, chronic rheumatism and urinary disorders and liver toxicity, anti-microbial potent anti-inflammatory activity. *(Satyavati, et al., 1987, Dutta, et al., 1982; Alam, et al., 1998, Prabhakan and Gomes, 2000)*

Some important chemical constituents of the root include hemidesmin I, hemidesmin II, α-amyrin, β- amyrin, lupeol, 2-hydroxy-4methoxy-benzoic acid and some triterpenes.*(Satyavati et al., 1987; Das et al., 1992; Alam et al., 1994; Roy et al., 2001)*

2. AIM AND OBJECTIVE

The root of ***Hemidesmus indicus,*** is well established for antioxidant and hepatoprotective activity. It is also proved that plant contains several phenolic and coumarino-lignan compounds that are generally responsible for such activity.

In addition, in the reviews reported for hepatoprotective activity of roots of *H. indicus*, hydro-alcoholic/alcoholic extract was shown as an effective fraction (Nadana Saravanan, et al, 2007, Rao G.M, et al, 2005, Baheti J.R., 2005). Further, most of the reports for characterization of phytoconstitutents showed the use non polar solvents ie. Petroleum ether, Benzene and Hexane fractions (Das *et al.* 1992, Gupta, et al, 1981, Darekar RS, et al, 2009). Thus we have noticed this ambiguity the between extract used for the pharmacological activity and extracts used for phytochemical work were different.

This has turned as a rational to initiate isolation of bioactives which are responsible for the hepatoprotective activity of roots of *Hemidesmus indicus* by bioassay guided fractionation.

With the study of phytochemical and the pharmacological activity done for *H. indicus* we have decided to isolate and subsequently on that basis to characterize the responsible phytoconsitutents or group of phytoconsitutents from the plant. So as to develop a relationship between the constituents of the plants and pharmacological activity reported.

REVIEW OF LITERATURE

3.1 Hemidesmus indicus

3.1.1 Introduction to plant

Hemidesmus indicus (L.) R. Br. (Asclepediceae) commonly known as Indian Sarsaparilla, is a diffusely twining undershrub having numerous slender wiry laticiferous branches with purplish brown bark. This plant is found throughout India growing under mesophytic to semi dry conditions in the plains and up to an altitude of 600 m. It is quite common in open scrub forests, hedges, uncultivated soil etc (Sasidharan, 2004; Siddique et al, 2004; Nayar et al., 2006).

Taxonomical Classification

Kingdom	Plantae
Division	Angiosperms
Subdivision	Eudicots
Class	Asterids
Order	Gentianales
Family	Apocynaceae
Subfamily	Asclepediaceae
Genus	*Hemidesmus*
Species	*H. indicus*

Vernacular Names

Beng.: Anantmool; **Eng.:** Indian Sarsaparilla**; Guj.**: Sariva; **Hindi:** Magrabu, Salsa, Kapooree, Anantamool; **Kan.:** Sogadeberu, Namadaberu; **Konkani**: Dudvali; **Mar**.: Anantmool, Upalsari, Dudhasali; **Ori.** : Onontomulo; **Persian**: Ushbanindi, Yasmine barri, Aushbahe nindi; Punj.: Anantmool; **Sans.**: Anantamula, sariva, naga jihva, gopakanya; **Tam**.: Nannari and **Tel.:** Gadisugandhi, Sugandhipala

Ayurvedic Properties

Rasa-tikta, Madhura; Snigdha; Veerya-sheeta; Vipaka-madhura.

Doshaglmata: Tridoshashamaka; Rogaghnata: Daha, Shotha, Netrabhisyanda, Aruchi, Agnimandya, Atisara, Pravahika, Vatarakta, Phiranga, Upadansha, Amvata, Gandmala, Pradara, Garbhasrava, Stanyavikara, Shukradaurbalya, Mootrakrichchhra, Paittika prameha, Kushtha, Visarpa, Visphota, Jwara, Daurbalya, Pandu, Visha, Kasa, Shwasa; Karma: Rochana, Deepana, Pachana, Anulomana, Raktashodhaka, Shothahara, Kaphaglma, Vrishya, Stanyashodhana, Garbhasthapana, Mootrajanand, Mootravirajaniuhya, Kushthagna, Jwaraghna, Dahaprashamana, Rasayana and Vishaglma (*Sharma et al, 2000).*

Marketed preparation

H. indicus forms an ingredient of about 46 Ayurvedic preparations either alone or in combination with other drugs *(Iyer et.al, 1983)*. The lists of important Ayurvedic preparations are given below:

Dasamoolarishta, Dhanwamthararishta, Balamritham, Saribadyasavam, Anuthaila, Amrithadi enna, Aswagandhadi yamaka, Gandha taila, Chandanadi taila, Triphaladi taila, Dhanwamthara taila, Neeleedaladi taila, Pinda taila, Balaswagandhadi taila, Manjishtadi taila, Madhuyashtyadi taila, Mahabala taila, Lakshadi taila, Sanni enna, Sidharthadi taila, Agragrahyadi kashaya, Jeevanthyadi kashaya, Triphalamarichadi mahakashaya, Dasamoolabaladi maha kashaya, Drakshadi kashaya, Dhanwamthara kashaya, Mahathiktha kashaya, Mridweekadi kashaya, Vidaryadi kashaya, Satavaryadi kashaya, Saribadi kashaya, Marmagudika, Manasamithra vataka, Kalyanaka ghritha, Jathyadi ghritha, Dadhika ghritha,

Naladadi ghritha, Panchagavya ghritha, Pippalyadi ghritha, Brihachagaladi ghritha, Mahakalyanaka ghritha, Mahakooshmandaka ghritha, Mahathiktha ghritha, Vasthyamayanthaka ghritha, Varahyadi ghritha, Madhusnuhi rasayana.

3.1.2. **Introduction to family** (Aslcepediaceae)

The plants under family Asclepiadaceae are mostly herbs and shrubs with white sap comprising about 250 genera and 2,000 species, many of which are lianous and some of which are cactuslike succulents with reduced leaves. The leaves are simple and nearly always opposite or whorled; minute stipules are present. The flowers are bisexual, nearly always actinomorphic, and usually include an elaborate crown or corona of nectariferous appendages between the corolla and sexual parts. The calyx consists of 5 distinct or basally connate sepals. The inner perianth is a 5-lobed sympetalous corolla. The androecium and gynoecium are nearly always adnate into a gynostegium with five highly modified stamens and a massive, 5-lobed stigma. The anthers usually produce paired sacs of pollen called pollinia that are transferred as a unit during pollination. The gynoecium consists of a single compound pistil of two nearly distinct carpels that are separate at the level of the ovaries and styles and are united only by a single massive stigma. The ovaries are distinct, nearly always superior, and each has a single locule with numerous marginal ovules. The fruit is a follicle. Seeds usually have a tuft of hairs at one end.

3.1.3 Morphology (*Aiyer, 1951; Prasad and Wahi, 1965; Warrier et al, 2000*)

Roots: are 30 cm. or more long , 3 to 6 mm. thick, rigid, tortuous, cylindrical, and little branched, consisting of a ligneous center, and a brownish, corky bark, marked with longitudinal furrows and transverse fissures.

Stems and branches: twine anticlockwise, profusely laticiferous, elongate, narrow, terete and wiry of a deep purple or purplish brown colour with the surface slightly ridged at the nodes.

Leaves: simple, petioled, exstipulate, opposite, entire, apiculate acute or obtuse, dark green above but paler and sometimes pubescent below. Leaves of the basal parts of the shoots are linear to lanceolate

Flowers: Greenish yellow to greenish purple outside, dull yellow to light purplish inside, calyx deeply five lobed, corolla gamopetalous, about twice the calyx, Stamens five, inserted near base of corolla with a thick coronal scale, with distinct filaments and small connate oblong anthers ending in indexed appendages. Pistil is bicarpellary, ovaries are free and many ovuled with distinct styles.

Fruits: two straight slender narrowly cylindrical widely divergent follicles.

Seeds: many in number, fiat, oblong, with a long tuft of white silky hairs

3.1.4 Microscopy *(Aiyer, 1951; Prasad and Wahi, 1965; Warrier et al, 2000; ayurvedic pharmacopeia of India)*

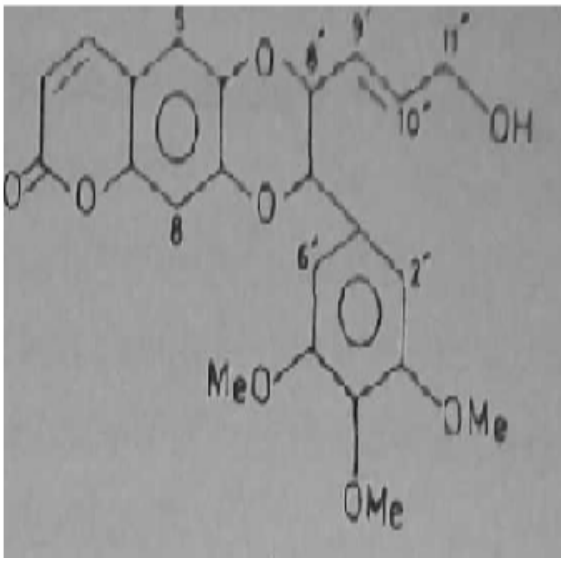
Periderm: consists of three layers of tissues, cork, cork cambium and secondary cortex.
Cork: radially flattened and rectangular in appearance filled with dark brown contents giving reactions of tannins.
Cork cambium: consist of 2 or 3 layer of cells, compressed, and filled with deep brown contents that's gives positive test for tannin.
Secondary cortex: consist of 3-4 layers of cells, similar to cork cells, with very little or no dark brown contents
Secondary phloem: consist of 3-4 layers of cells, similar to cork cells, with very little or no dark brown contents or tannin
Parenchymatous cells: filled with starch grains, diameter 7-10 μ and occasional prismatic crystals of calcium oxalate, laticiferous ducts scattered in parenchymatous tissue
Cambium: very narrow
Vascular bundles: xylem traversed by narrow medullary rays, vessels and tracheids characterised by the presence of pitted markings
Pith: Absent and central region occupied by woody tissues

3.1.5 Phytochemistry

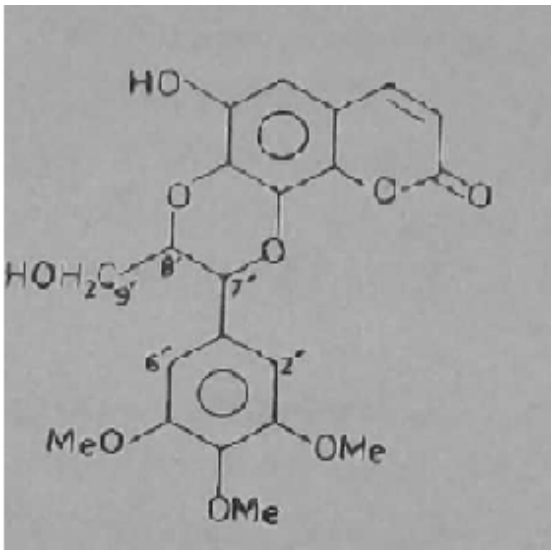
Roots: Many phytochemical studies have been carried out on *H. indicus*. From the roots of *H. indicus,* hemidesmol, resin and glucoside, tannin and resin (Murthi and Seshadri, 1941), lupeol, a and (3-amyrins, [3-sitosterol (Chatterjee and Bhattacharya, 1955), lupeol, a-amy r in, lupeol acetate, [3-amyrm acetate, hexa triconate acid and lupeol octacosonate (Padhy *et al,* 1973), a coumarino lignoid like hesmidesmine (Mandal *etal.,* 1991), hemidesmin-1 and hemidesmin-2 (Das *et al.,* 1992) were isolated (Fig 2.11.A). The constituents of oil obtained from the roots of *H. indicus* contains 80% crystalline matter, glucose, hemidesmol, hemidesterol, 2-hydroxy-4-methoxy benzaldehyde, resin acid, glucoside, sterol and tannins (Anonymous, 1997). Mukherjee and Ray (1980) reported that roots of *H. indicus* contain

steroid, terpenoid, flavonoid and saponin, but alkaloid is absent. Oberai *et al.* (1985) noted that dried twigs of *H. indicus* yield a pregnane ester diglycoside desinine.

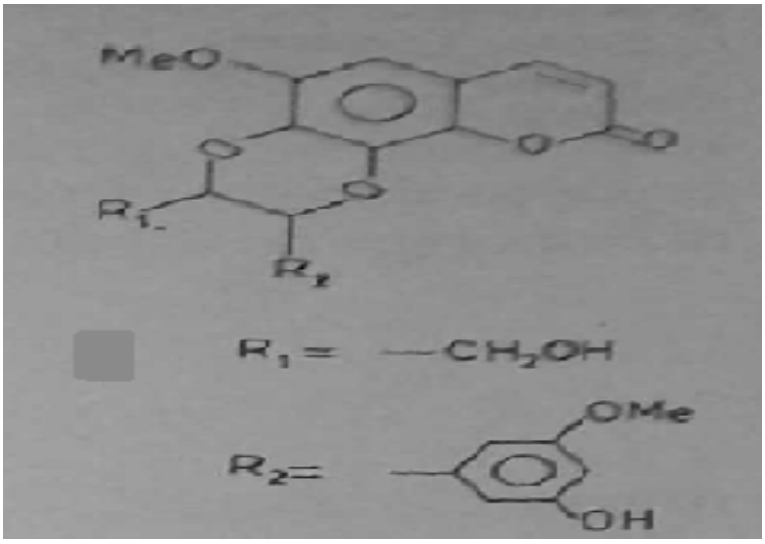
The presence of a-amyrin triterpene, [3-amyrin triterpene and benzaldehyde, 2-hydroxy-4-methoxy benzenoid in the root of Indian *H. indicus* were reported by Gupta (1981). Alam *et al.* (1994) reported the presence of benzoic acid, 2-hydroxy-4-methoxy benzenoid in the roots. Coumarin derivatives namely hemidesmin-1-coumarin and hemidesmin-2- coumarin in the roots were reported by Das *et al.* (1992). Nagarajan and Rao (2003) isolated 2-hydroxy-4- methoxybenzaldehyde from the roots of *D. hamiltonii* and *H. indicus* which is responsible for its aromatic nature, was found to be >90% in their volatile oil, which was isolated from both, fresh and dried roots of different origin. Among the methods adopted, steam hydrodistillation was suitable for extraction of the volatile oils and the quantity varied from 0.03 to 0.54%..



Hemidesminine



Hemidesmin 1



Hemidesmin 2

Fig 2.A: Coumarino-lignans isolated from benzene extract of roots of *H.indicus*

Saravanan N et al, reported 2-hydroxy 4-methoxy benzoic acid, isolated from aqueous alcoholic extract, as active principal against ethanol induced hepatotoxicity.

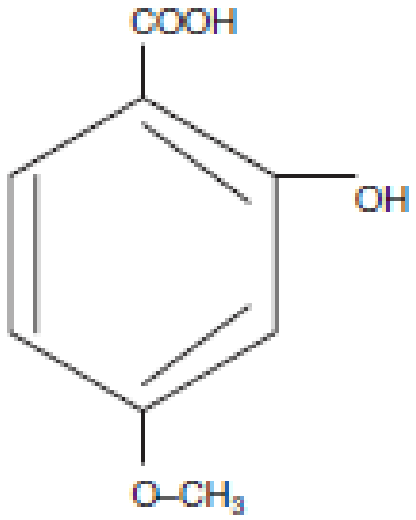


Fig. 2.B. Structure of 2-hydroxy 4- methoxy benzoic acid

Stem: Glycosides like indicine andhemidine were isolated from stem (Prakash *et al*., 1991). Gupta *et al.* (1992) found that hexane soluble portion of ethanol extract of stem yielded lactone, lupanone, Δ^{12}-dehydrolupanyl-3-[3- acetate, Δ^{12}-dehydro lupeol acetate, 4-hydroxy-3-methoxy benzaldehyde. Chloroform and alcohol extracts of stem yielded two novel pregnane glycosides, hemidescine and emidine (Chandra *et al.*, 1994). Oligoglycosides, indicusin and medidesmine, hemisine and desmine were isolated from the plant (Deepak *et al.*, 1995, 1997). Deepak *et al.* (1997) reported that the plants belonging to the family Asclepiadaceae are rich in pregnane and cardiac glycosides. The benzenoid derivatives in the stem were reported by Gupta *et al.* (1992). It contains benzaldehyde, 2-hydroxy-4-methoxy benzenoid, benzaldehyde, 3-hydroxy-4-methoxy benzenoid and benzaldehyde, 4-hydroxy-3-methoxy benzenoid.

Various steroidal compounds are also found in *H. indiucs*. 0.0004% of ca logenin-3 - O - [3 -D - digitoxopyrano steroid (Prakash *et al*., 1991), side-desinine steroid (Oberai *et al.*, 1985), 0.0009% of desmimne steroid (Deepak *et al.*, 1997), Emidine and hemidescine steroids (Chandra *et al.*, 1994), 0.0004% of hemidine steroid (Prakash *etal*., 1991) and hemisine steroid (Deepak *et al.,* 1997) are reported from the stem.

The percentage of triterpene in the stem from India was reported by Gupta *et al.* (1992). It also contains 0.0007% of lup-12-en-21 -28-olide, 3-keto-triterpene, 0.01 % of lup-12-en-3-(3-ol acetate triterpene, 0.00666% of lupanone triterpene, *0.03333%* of lupeol acetate triterpene and 0.03333% of (3-sitosterol. Deepak *et al.* (1997) reported the presence of 0.0008%) of medidesmine steroid in the stem from India. The presence of *0.2666%* of palmitic acid, a lipid in the stem from India was reported by Gupta *et al.* (1992). The presence of alkaloids in the stem and root were reported by Arseculeratne *et al.* (1985). He also reported the absence of pyrrolizidine alkaloids in the stem and roots. Das *et al.* (2003) revealed the presence of tannins, steroids, triterpenoids and carbohydrates. Sigler *et al.* (2000) isolated two novel pregnane glycosides, denicunine and heminine.

Leaves: *2.50%* of tannins were present in the leaves (Daniel *et al.,* 1978). Coumarinolignoids like hemidesminine (Mandal *et al*., 1991), hemidesmin, hemidesmin 1 and hemidesmin 2 were isolated by Mandal *et al.* (1995) and they reported that

coumarinolignoids are new and rare group of naturally occurring compound with cytotoxic and antihepatotoxic properties. Subramanian andNair (1968) further, reported the presence of flavonoids viz., hyperoside and rutin.

Flowers: The flavanoid glycosides identified in the flowers of *H. indicus* were hyperoside, isoquercitin and rutin *(Subramanian and Nair, 1968*). He also reported the presence of iso-quercitrin flavonoid and rutin flavonoid in the flowers from India.

3.1.6 Pharmacology of *Hemidesmus indicus*

Nadana et al, 2007 suggested that ethanolic root extract of *Hemidesmus indicus* (*H. indicus*) and its active principle 2-hydroxy 4-methoxy benzoic acid (HMBA) on liver fibrotic markers and characteristics such as collagen content, matrix metalloproteinases (MMPs) 2 and 9 in ethanol-fed rats. On treatment with *H. indicus* and HMBA the ethanol-fed rats showed significantly reduced levels of liver collagen and hydroxyproline content, cross-linked fluorescence, shrinkage temperature and lipid peroxidation and enhanced solubility of liver collagen and ascorbic acid levels when compared with untreated ethanol-fed rats. MMPs were extracted from the liver of control, *H. indicus*-treated, HMBA-treated, ethanol-administered, ethanol with *H. indicus* coadministered and ethanol with HMBA-coadministered rats. The inhibition was analyzed by gelatin zymography and the percentage of expression was determined by a gel documentation system. The activities of MMPs 2 and 9 were significantly increased in ethanol-supplemented rats. Cotreatment of *H. indicus*/HMBA with ethanol showed significantly decreased activities of these enzymes when compared with those of the untreated rats. *H. indicus*/HMBA alone treatment showed no such significant alterations. *H. indicus* and HMBA improve the quantitative and qualitative properties of hepatic collagen and also MMPs involved in the extracellular matrix degradation during ethanol intoxication. *Hemidesmus indicus* has been shown to possess significant activity against immunotoxicity and other pharmacological and physiological disorders. Application of ethanolic extract of *H. indicus* at a dose level of 1.5 and 3.0 mg/kg body weight in acetone prior to that of cumene hydroperoxide treatment resulted in significant inhibition of cumene hydroperoxide-induced cutaneous oxidative stress, epidermal ornithine decarboxylase activity and enhanced DNA synthesis in a dose-dependent manner. Enhanced susceptibility of cutaneous microsomal membrane for lipid

peroxidation and xanthine oxidase activity were significantly reduced (P<0.01). In addition the depleted level of glutathione, inhibited activities of antioxidants and phase II metabolizing enzymes were recovered to significant level (P<0.05). *Nadana et al, 2007* investigated that 2-Hydroxy-4-methoxy benzoic acid (HMBA), the active principle of *Hemidesmus indicus*, is significantly inhibit the development of liver injury in ethanol administration. It is reduce the severity of liver damage in terms of body weight, hepatic marker enzymes, oxidative stress, antioxidant status and histological changes in ethanol-induced hepatotoxic rats.

HMBA was co-administered at a dose of 200 μg kg^{-1} daily for the last 30 days of the experiment to rats with alcohol-induced liver injury, which significantly increased body weight, significantly decreased the liver-body weight ratio, transaminases, alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase, significantly decreased the levels of lipid peroxidative markers, significantly elevated the activity of enzymic and non-enzymic antioxidants in plasma, erythrocytes and liver and also increased levels of plasma and liver vitamin C and α-tocopherol at the end of the experimental period as compared with untreated ethanol.

Khanna et al, 2007 found the larvicidal effect of aqueous extracts of *Hemidesmus indicus* roots*, Gymnema sylvestre* and *Eclipta prostrata* leaves were tested against *Culex quinquefasciatus*larvae at the concentrations of 1, 2, 3, 4 and 5% up to three days. All extracts showed larval mortality. Larval mortality was 100% with the use of 5% concentration of root extract of *H. indicus*, leaves extracts of *G. sylvestre* and *E. prostrata* after 2 days. Qualitative analysis of the phytochemicals of aqueous extracts revealed the presence of carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins in all the plants. Quantitative analysis showed that the crude saponin was the major phytochemical constituent present in highest percentage followed by crude tannin in all three plants. It is suggested that all the three plants possess larvicidal properties that could be developed and used as natural insecticides for mosquito control. *Shetty et al, 2005* investigated radioprotective effect of *H. indicus* root aqueous extract on lipid peroxidation in rat liver microsomes and plasmid DNA was examined. *Hemidesmus indicus* (HI) root extract was found to protect microsomal membranes as evident from reduction in lipid peroxidation values. The extract could also protect DNA from radiation induced strand breaks.

Das S et al, 2003 Methanolic extract of H . indicus root (MHI) was screened for its antimicrobial activity against S. typhimurium, E . coli and S. flexneri, in vitro and in experimentally induced diarrhoea in albino rats, in vivo . MHI had an anti enterobacteriae effect as evident from agar well diffusion method and decrease in CFU/ml in MHI treated LB broth culture. MHI inhibited the castor oil induced diarrhoea in rats as judged by a decrease in the amount of wet faeces in MHI-pretreated rats at a dose of 500-1500 mg/kg . The results indicated that MHI was more active than standard antidiarrhoeal drug, lomotil . Phytochemical tests revealed the main constituents as tannins, steroids, triterpenoids and carbohydrates . Present findings suggested that MHI might elicit an antidiarrhoeal effect by inhibition of intestinal motility and by its bacteriocidal activity. *Verma et al, 2005* The alcoholic extract of roots of *Hemidesmus indicus* R.Br. (family: Asclepiadaceae) was investigated for possible antinociceptive effect in mice. Three models were used to study the effects of extracts on nociception, which was induced, by acetic acid (Writhing test), formalin (Paw licking test) and hot plate test in mice. *Hemidesmus indicus* R.Br. extract was administered in the dose range of 25, 50 and 100 mg/kg orally 1 h prior to pain induction. Oral administration of *Hemidesmus indicus* extract revealed dose-dependent antinociceptive effect in all the models for antinociception and it blocked both the neurogenic and inflammatory pain and the nociceptive activity was comparable with the reference drug.

3.1.7 Ethnopharmacological studies

Ethnobotanical studies on *H. indicus* revealed its benefits towards various ailments, like scorpion sting, snake bite, fever (Sharma *et al., 1979)* and as a blood purifier (Malhotra and Murthy, 1973; Sharma *et al*, 1979; Pullaiah and Kumar, 1996). It has cooling effect and used in venereal diseases including gonorrhoea (Singh and Maheswari, 1983), stomach ulcer (Jain and Singh, 1994; Jain, 1996), diabetes and fever (Khan *et al,* 1983) increases lactation in mothers (Shukla and Verma, 1996), spermatorrhoea (Singh and Prakash, 1996), biliousness (Balasubramanian and Prasad, 1996) and headache (Khanna *et al*, 1996). Root decoction is useful for curing high fever and skin diseases (Sudhakar and Rao, 1985; Vyas, 1993). The rind of the root is chewed for sore mouth (Prasad *et al.,* 1964, 1996). Fresh root paste with neem oil applied on scalp of children for development of skull bones to enable carrying head-loads in adult age (Banerjee and Pal, 1996). The root is used to make sweet smelling

drink, which is used in the place of coffee and tea (Prasad *et al.*, 1996). The dried entire plant is used for skin diseases (Shah and Gopal, 1985; Pushpangadan and Atal, 1984). Along *with Piper longum* and *P. nigrum*, it is used for postpartum recovery and also in diarrhoea and to improve appetite (Girach *et al.,* 1994; Reddy *et al.,* 1988). This is also used for impotency (Paul, 1979) and to reduce body heat, as a stimulant and as food (Ramachandran and Nair, 1981). This is said to increase blood circulation and acts as a cure for diarrhoea (Jain and Singh, 1994). The roots are peeled and eaten raw as a blood purifier and as cooling beverage (John, 1984) and for treating skin diseases (Arseculeratne *etal,* 1985).

Along with *Mimosa pudica,* it is taken orally, during the menstrual period to treat leucorrhoea and also as a body tonic (Bhandary *et al,* 1995). With black pepper, it is used for fevers of long duration and with milk is taken for anemia (Singh and Prakash, 1996). This is used as an anti-venom (Selvanayahgam *et al.,* 1994). Decoction taken thrice daily checks menorrhagia (Nagaraju and Rao, 1990). It is also used as anti-rheumatic, diuretic, antiinflammatory and to treat snake bite (Alam *et al.,* 1994). Externally it is applied to provide relief from scorpion stings (Singh and Ali, 1992). This is also used for venereal diseases (Shah and Gopal, 1985) and as blood purifier (Reddy *et al.,* 1988), to cure stomach pain and diarrhoea (Sabnis and Bedi, 1983). A decoction with *Elephantopus scaber, H. indicus* and *P. nigrum* are used for gonorrhoea (Sahu, 1984). This is also used for skin affections, syphilis and as a tonic (Atal *et al.,* 1986). With honey, the fresh roots are used for health and vitality. Along with the woods of *Acacia sundra* and *Cinnamomum zeylanicum,* it is used to make a soft, nourishing beverage to promote youthfulness, health and vitality (Pushpangadan and Atal, 1984; Gupta *etal,* 1992).

3.2.Liver

The liver is the heaviest gland of the body, weighing about 1.4 kg in the average adult and after skin is the second largest organ of the body. It is located under the diaphragm and occupies most of the right hypochondrium and part of the epigastrium of the abdomen (Tortora GJ, et al, 1991).

Anatomy

The liver is almost completely covered by peritoneum and by a dense irregular connective tissue layer that lies beneath the peritoneum. It is divided into two principal lobes- a larger right lobe and a smaller left lobe- separated by the falciform ligament. The right lobe is considered by many anatomists to include an inferior quadrate lobe and a posterior caudate lobe. The falciform ligament is reflection of the parietal peritoneum. It extends from the undersurface of the diaphragm to the superior surface of the liver, between the two principal lobes of the liver. In the free border of the falciform ligament is the ligamentum teres (round ligament).

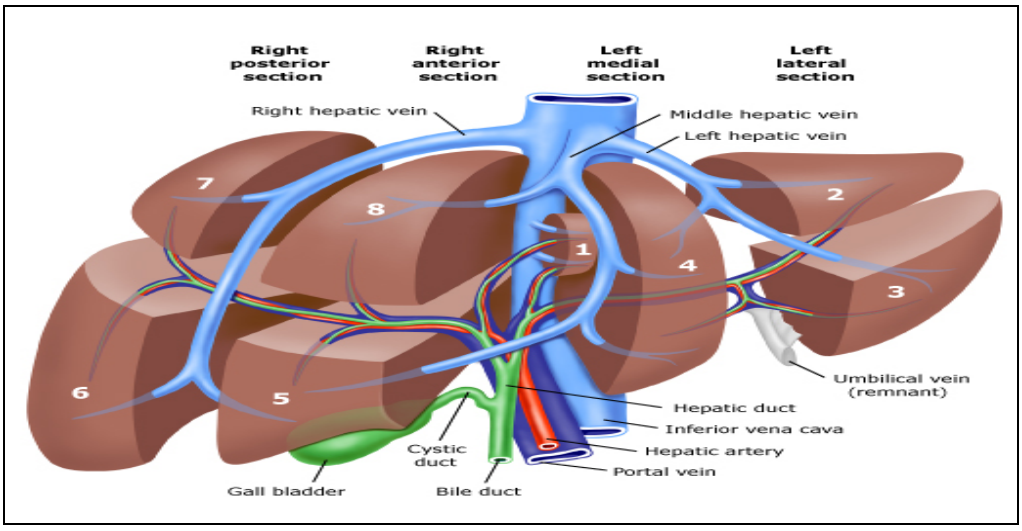


Figure3: Liver segments - physiology of liver

Histology

The hepatic parenchyma is composed of numerous hexagonal or pyramidal classical lobules; each with a diameter of 0.5 to 2 mm. Each classical lobule has a central tributary from the hepatic vein and at the periphery are 4 to 5 portal tracts or triads containing branches of bile ducts, portal vein and hepatic artery. Cords of hepatocytes and blood containing sinusoids radiate from the central vein to the peripheral portal triads. The functioning lobule or liver acinus as described by Rappaport has a portal triad in the centre and is surrounded at the periphery by portions of several classical lobules.

The blood supply to the liver parenchyma flows from the portal triads to the central veins. Accordingly, the hepatic parenchyma of liver lobule is divided into 3 zones.

Zone 1 or the periportal (peripheral) area is closest to the arterial and portal blood supply and hence bears the brunt of all forms of toxic injury.

Zone 3 or the centrilobular area surrounds the central vein and is most remote from the blood supply and thus suffers from the effects of hypoxic injury.

Zone 2 is the intermediate midzonal area.

The blood containing sinusoids between cords of hepatocytes are lined by discontinuous endothelial cells and scattered flat Kupffer cells belonging to the reticuloendothelial system. The space of Disse is the space between hepatocytes and sinusoidal lining endothelial cells. A few scattered fat storing cells lie within the space of Disse. The portal triad or tract besides containing portal vein radical, the hepatic arteriole and bile duct, has a few mononuclear cells and a little connective tissue considered to be extension of Glisson's capsule. The portal triads are surrounded by a limiting plate of hepatocytes. The hepatocytes are polygonal cells with a round single nucleus and a prominent nucleolus. A hepatocyte has 3 surfaces one facing the sinusoids and the space of Disse, the second facing the canaliculus, and the third facing neighbouring hepatocytes.

Bile secreted by hepatic cells, enters the bile capillaries or canaliculi that empty into small bile ducts. These small ducts eventually merge to form the larger right and left hepatic ducts, which unite and exit the liver as the common hepatic duct (Harsh Mohan, et al, 2005).

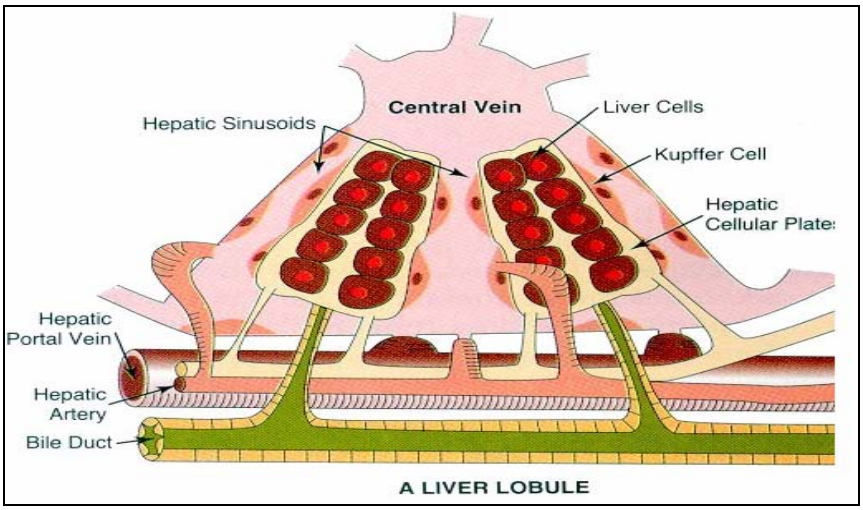


Figure 4: Hepatocyte

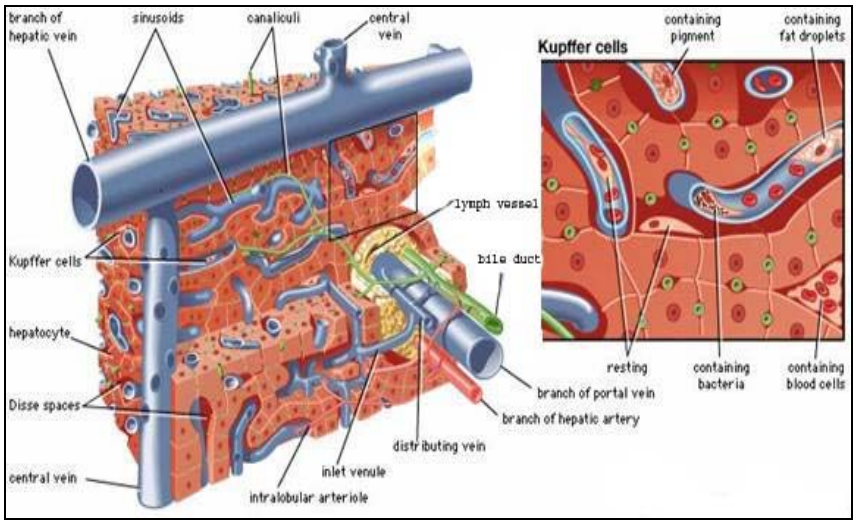


Figure 5: Histology of liver

Blood supply

The liver receives blood from two sources. From the hepatic artery it obtains oxygenated blood, and from the hepatic portal vein it receives deoxygenated blood containing newly absorbed nutrients. Branches of both the hepatic artery and the hepatic portal vein carry blood into liver sinusoids, where oxygen, most of the nutrients and certain poisons are extracted by the hepatic cells. The reticuloendothelial (kupffer's) cells lining the sinusoids phagocytize microbes and bits of foreign matter from the blood (Tortora GJ, et al, 1991).

Functions

The liver performs number of vital and complex functions like, (Tortora GJ, et al, 1991)

1. Carbohydrate metabolism
2. Lipid metabolism
3. Protein metabolism
4. Removal of drugs and hormones
5. Excretion of bile
6. Secretion of bile salts
7. Storage of vitamin A, D, E, K, iron, copper, vitamin B_{12}, folic acid etc.
8. Phagocytosis
9. Activation of vitamin-D

2.3. Hepatic diseases

Various types of hepatic diseases are as follows,

a) Hepatitis

- Acute hepatitis
- Chronic hepatitis
- Viral hepatitis
- Neonatal hepatitis
- Alcoholic hepatitis
- Drug induced hepatitis

b) Hepatic encephalopathy

c) Portal hypertension

d) Alcoholic liver diseases

- Fatty liver (Alcoholic steatosis)
- Alcoholic hepatitis
- Alcoholic cirrhosis

e) Jaundice

f) Haemochromatosis

h) Wilson's disease

i) Non-alcoholic fatty liver disease (NAFLD)

Hepatitis

It is an inflammation of liver. Hepatotrophic viral inflection, excessive use of certain hepatotoxic drugs, prolongs consumption of alcohol, etc are some of the causes of hepatitis.

- Acute hepatitis

The most common consequence of all hepatotropic viruses is acute inflammatory involvement of the entire liver. In general, type A, B, C, D and E run similar clinical course and show identical pathologic findings. It is categorised into 4 phases.

1) Incubation period
2) Pre-icteric phase
3) Icteric phase
4) Post-icteric phase

- Chronic hepatitis

  Chronic hepatitis is defined as continuing or relapsing hepatic disease for more than 6 months with symptoms along with biochemical, serologic and histopatholgic evidence of inflammation and necrosis. Majority of cases of chronic hepatitis are the result of infection with Hepatotrophic viruses- hepatitis B, hepatitis C and combined hepatitis B and hepatitis D infection. It is divided into 2 types, (Harsh Mohan, et al, 2005)

  1) Chronic persistent hepatitis
  2) Chronic active hepatitis

- Viral hepatitis

It is the most common type of hepatitis caused by one of six viruses. The duration of hepatitis can further be classified as acute viral hepatitis (less than 6 months) and chronic viral hepatitis (more than 6 months).

It includes various types of hepatitis (Bodhankar SL, et al, 2006).

- Hepatitis A
- Hepatitis B

- Hepatitis C
- Hepatitis D
- Hepatitis E
- Hepatitis G

Causes

Most cases of acute hepatitis are due to viral infections: Hepatitis A , Hepatitis B , Hepatitis C , Hepatitis B with D , Hepatitis E , Hepatitis G viruses. Other viruses can also cause hepatitis including Yellow fever, Cytomegalovirus, Epstein-Barr virus, Mumps virus, Rubella virus etc (Bodhankar SL, et al, 2006).

- Neonatal hepatitis

It is also termed as giant cell hepatitis or neonatal hepatocellular cholestasis. It is a general term used for the constant morphologic changes seen in conjugated hyperbilirubinaemia as a result of known infectious and metabolic causes or may have idiopathic etiology. The idiopathic neonatal hepatitis is more common and accounts for 75% of cases. The condition usually present in the first week of birth with jaundice, bilirubinuria, pale stool and serum alkaline phosphatase (Harsh Mohan, et al, 2005).

Causes

A. Unconjugated hyperbilirubinaemia
- Physiologic and prematurity jaundice
- Haemolytic diseases of newborn and kernicterus
- Congenital Haemolytic disorders
- Perinatal complications (e.g. hemorrhage, sepsis)
- Gilberts syndrome

B. Conjugated hyperbilirubinaemia
- Hereditary (Rotors syndrome)
- Infections (Hepatitis B, C)
- Metabolic (Galactosemia)
- Idiopathic (Neonatal hepatitis)
- Reye's syndrome

- Alcoholic hepatitis

It is an inflammation of liver induced by alcohol. It develops acutely following a bout of heavy drinking. It is more common in spree drinkers than regular drinkers, because in latter case, alcohol metabolizing capacity is increased which ultimately leads to rapid detoxification. Alcoholic hepatitis has three different histological features that are necrosis of hepatocytes, formation of Mallory bodies (sharp and hyalined material in the cytoplasm of hepatocyte) and neutrophilic infiltration (Bodhankar SL, et al, 2006).

- Drug induced hepatitis

Various drugs can produced hepatitis which may be indistinguishable from viral hepatitis. The diagnosis is usually made in the absence of known exposure to viral hepatitis and with known exposure to hepatotoxic drug. The different mechanisms of hepatic damage are,

- Drugs act as a vehicle for transmission of viral hepatitis
- Drugs act as a direct hepatotoxins
- Drugs may induce hypersensitivity reaction which may further results in hepatic damage (Bodhankar SL, et al, 2006).

*Table 1: Examples of drugs causing hepatotoxicity (*Ingawale D, et al, 2009)

Category	**Examples of drugs**
Antidepressant	Amitriptyline, Nortriptilne, etc.
Antiarrhythmic	Amiodarone
Non steroidal anti-inflammatory drugs (NSAIDs)	Paracetamol, indomethacin, Aspirin, etc.
Anti-tuberculosis	INH, RMP, PZA, etc.
Antifungal	Ketoconazole, Fluconazole, etc.
Antihistamine	Loratadine, Fexofenadine, etc.
Antihypertensive	Methyldopa, Nifedipine
Immune suppressant	Cyclosporine, Methotrexate
Tetracycline antibiotic	Minocycline

Antiepileptics	Phenytoin, Mephytoin, etc.
Antiretroviral	Zidovudine
Anticancer	Azathioprine, 6-Thioguanine, etc.

Hepatic encephalopathy

It is a neuropsychiatric syndrome caused by liver disease. It is often secondary to cirrhosis of liver but also seen in other types of hepatitis. The brain function deterioration takes place due to accumulation of toxic substances, which would be normally removed by the liver. The pathogenesis of hepatic encephalopathy is not clearly understood but attributed to central nervous system derangement due to direct entry and accumulation of toxic substances like ammonia, free fatty acids, false neurotransmitter (otapamine), etc. Increased ammonia level in blood and central nervous system is considered to be prime cause (Bodhankar SL, et al, 2006).

Precipitating factors of hepatic encephalopathy

- High protein intake
- GI hemorrhage
- Infections
- Constipation
- Excessive diuretic therapy
- Sedatives and hypnotics
- Hypokalaemia
- Trauma or surgery

Portal hypertension

Portal hypertension is abnormally high blood pressure in the portal vein (over 10 mm of Hg). Portal venous pressure above 12 mm of Hg usually develops clinical features and complications. The increase in portal blood pressure is either due to increase in the volume of blood flowing through the vessels or increased resistance to blood flow through liver (Bodhankar SL, et al, 2006). Portal hypertension can be classified according to the main site of obstruction to the blood flow in the portal venous system.

1) Pre-hepatic (5-20%) - It is due to blockage of portal vein before veins. e.g. Portal vein thrombosis, trauma and tumours pressing on portal veins.

2) Hepatic (80%) - It is due to distortion of liver architecture and further classified as

- Sinusoidal
- Pre-sinusoidal
- Post-sinusoidal

3) Post-hepatic (< 10%) - It is due to venous blockage outside the liver. e. g. Right sided heart failure, pericardiasis.

Alcoholic liver diseases

- Fatty liver

It is result of accumulation of fat mainly due to disproportion between fat deposition and removal from liver. The accumulation large quantities of triglycerides result in distension of hepatocytes to several times like fat cysts. The fatty liver is a result of changed metabolic activity of hepatocyte and diversion of hepatic enzymes to metabolized alcohol from fat and carbohydrate metabolism (Bodhankar SL, et al, 2006).

- Alcoholic cirrhosis

It is the destruction of normal hepatocyte that leaves non-functioning scar tissues, resulting from excessive alcohol consumption. The quantity and quality of alcohol is most important. The common causes are exposure to hepatotoxic drugs or chemicals, bile duct obstruction, α_1-antitrypsin deficiency, glycogen storage disease and certain autoimmune diseases.

Important features of alcoholic cirrhosis are (Bodhankar SL, et al, 2006),

- Involvement of entire liver
- Disorganization of lobular structure of liver
- Formation of nodules
- Alternate areas of necrosis and regenerative nodules.

Jaundice

This is the yellow pigmentation of the skin, mucous membrane and deeper tissues due to increased bilirubin level in blood. The normal serum bilirubin level is 0.5 to 1.5 mg %. When this exceeds 2 mg %, jaundice occurs (Bodhankar SL, et al, 2006).

Jaundice may result from one or more of the following mechanism.

- Increased bilirubin production
- Decreased hepatic intake of bilirubin
- Decreased hepatic conjugation of bilirubin
- Decreased excretion of bilirubin into bile.

Jaundice mainly classified into 3 types;

- Pre-hepatic (Hemolytic)

During this, the excretory function of liver is normal. But, there is excessive destruction of red blood cells and thus the bilirubin level in blood is increased.

- Hepatic

The jaundice due to the damage of liver cells is called hepatocellular or hepatic jaundice.

- Obstructive (Cholestatic)

It is due to the obstruction of bile flow at any level of biliary system. The bile can not be poured into small intestine and bile salts and bile pigments enter the circulation.

Haemochromatosis

Haemochromatosis is a hereditary disease, characterized by excessive absorption of dietary iron resulting in a pathological increase in total body iron stores. Excess iron accumulates in tissues and organs disrupting their normal function. The most susceptible organs include the liver, adrenal glands, the heart and the pancreas; patients can present with cirrhosis, adrenal insufficiency, heart failure or diabetes (Jones H, et al 1983*)*.

Wilson's disease

Wilson's disease or hepatolenticular degeneration is an autosomal recessive genetic disorder in which copper accumulates in tissues; this manifests as neurological or psychiatric symptoms and liver disease. It is treated with medication that reduces copper absorption or

removes the excess copper from the body, but occasionally a liver transplant is required. The main sites of copper accumulation are the liver and the brain and consequently liver disease and neuropsychiatric symptoms are the main features that lead to diagnosis (Ala A, et al, 2007).

Non-alcoholic fatty liver disease (NAFLD)

The NAFLD is the occurrence of fatty liver in people who have no history of alcohol use. It is most commonly associated with obesity. About 80% of all obese people have fatty liver. It is more common in women. Severe NAFLD leads to inflammation, a state referred to as non-alcoholic steatohepatitis (NASH), which on biopsy of the liver resembles alcoholic hepatitis (with fat droplets and inflammatory cells, but usually no Mallory bodies).

3.4. Mechanism of hepatotoxicity

Certain drugs will produce predictable liver damage in the majority of cases, such as after overdoses. In some cases the mechanism may involve the parent compound; in others a metabolite may be responsible. Direct cytotoxicity is known to be the underlying cause of liver damage in certain cases whereas in others immunological mechanisms or even a mixture of both cytotoxicity and immunogenicity may be involved.

The various mechanisms will now be discussed using specific examples, according to the type of injury.

- **Interference with bilirubin transport and conjugation**

A number of drugs interfere with bilirubin transport and lead to elevated plasma bilirubin or hyperbilirubinaemia. Novobiocin inhibits Uridyl diphosphate (UDP) glucuronosyl transferase and may lead to elevated plasma levels of unconjugated bilirubin especially in neonates (Kenright S, et al 1974, Cox RP et al 1959)

.

Rifampicin: Rifampicin, the antibiotic used in the treatment of tuberculosis inhibits both uptake and excretion of bilirubin in a dose related manner, giving rise to elevated plasma levels of both conjugated and unconjugated bilirubin. This is due to blockade of uptake at the plasma membrane of the hepatocyte. As well as causing hyperbilirubinaemia, rifampicin

may also cause overt hepatic damage. The lesion is characterized as hepatocellular with centrilobular necrosis which may be accompanied by cholestasis. Effects of rifampicin include interference with DNA synthesis and the ability to induce hepatic microsomal enzymes and cause proliferation of the smooth endoplasmic reticulum (Capelle P, et al , 1972; Zimmerman HJ et al 1978)

- **Cytotoxic injury**

Direct, overt damage to hepatic parenchyma may be caused by a number of drugs. It may have a variety of underlying mechanisms.

Paracetamol: This minor analgesic causes predictable centrilobular hepatic necrosis in both experimental animals and man after overdoses. Doses of 10 g may lead to liver damage, and doses greater than 15 g may be sufficient for fatal hepatic damage. Liver damage may be detected as raised serum transaminases, Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) which may reach levels of 5000 IU/L (Boyd EM, et al, 1966; James O, et al 1975; Prescott LF 1971). However, bilirubin levels may be only moderately elevated. The reactive metabolite N-acetyl-p-benzoquinone imine then reacts covalently with cellular macromolecules as revealed by measurement of covalent binding of radiolabelled paracetamol to hepatic protein and autoradiographic studies which indicate binding primarily in necrotic areas. Nucleophiles and increasing glutathione levels protects against the hepatic injury (Mitchell JR, *et al, 1975)*.

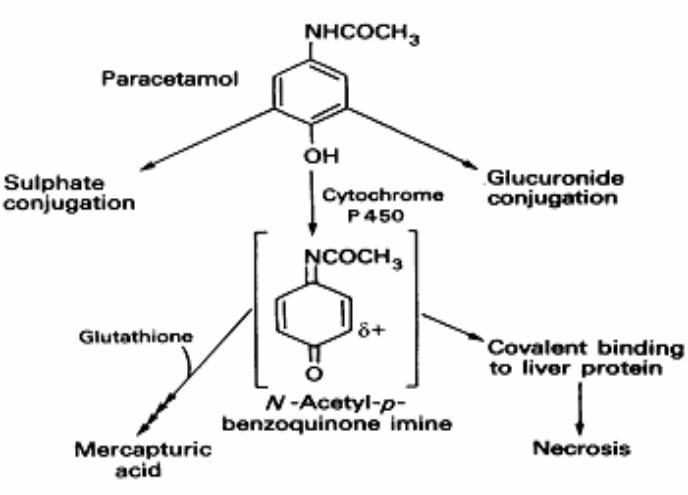


Figure 6: Metabolism of paracetamol showing proposed metabolic activation and its involvement in the toxicity

- **Cholestatic injury**

This type of hepatic toxicity may be seen with a number of drugs, and may be either the mild, canalicular form or the more severe hepatocanalicular variety.

Chlorpromazine: This major tranquilizer is an important cause of drug-induced jaundice. The incidence is estimated at between 0.5 and 1% of recipients of therapeutic doses although up to 50 % of patients receiving large doses may have minor abnormalities of liver function (Zimmerman H, et al, 1974; Ishak KG, et al 1972). Jaundice commonly develops after 3 weeks of therapy, with often the development of fever, itching, abdominal pain, nausea and anorexia. This is often similar to extrahepatic obstructive jaundice, with elevated serum cholesterol and alkaline phosphatase levels more than 4 times normal. SGOT and SGPT levels may also be moderately raised. Chlorpromazine is an amphipathic, cationic tertiary amine detergent. It is highly concentrated in bile during biliary excretion and the concentration in bile may exceed that intrinsically toxic in vitro to cell membranes (Boyer JL, et al, 1981; Ros E, et al, 1979)

.

Anabolic steroids: Steroids with an alkyl group at C_{17} (Figure 7) such as methyl testosterone may give rise to mild hepatotoxicity. This is normally a cholestatic injury generally without parenchymal damage (canalicular type). Thus alkaline phosphatase is only slightly raised (2 times normal) as are transaminases SGOT but jaundice may be marked, with elevated bilirubin levels.

There are precise structural requirements for the steroids which produce this hepatic damage. Thus a C_{17}-methyl substituent is more active than an ethyl or vinyl group (Delorimier AA, et al, 1965; Lennon DH, et al, 1966). The keto group at C_3 confers greater activity than a hydroxyl group and saturation of the A ring of the steroid reduces its ability to cause hepatic dysfunction. Although the exact mechanism of hepatotoxicity is obscure, anabolic steroids clearly cause a dose related blockage of bile secretion

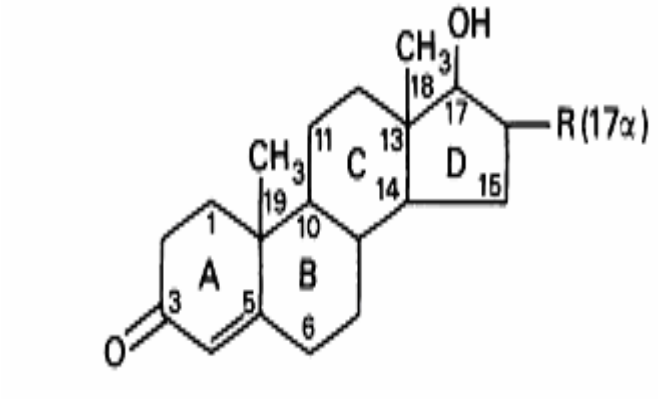


Figure 7: Basic structure of steroids related to testosterone.
For testosterone R = H

- **Mixed cytotoxic/cholestatic injury**

This type of liver injury covers damage with varying proportions of cytotoxic and cholestatic involvement. For example chlorpromazine may cause mixed hepatocanalicular jaundice with parenchymal injury as well as cholestasis. Conversely, p-aminosalicylic acid may cause mixed hepatocellular liver injury.

- **Fatty liver (Steatosis)**

Tetracycline: This antibiotic may cause fatty liver after large (1.5 g/day) intravenous doses (Davis JS,et al, 1968) . Histologically hepatocytes are packed with small fat droplets. This microvesicular steatosis occurs initially in the centrilobular area. There is little inflammation, necrosis or cholestasis. The major effect seems to be inhibition of transport of lipid out of the hepatocyte. This effect may be due to the inhibition of protein synthesis caused by tetracycline which will inhibit the production of the apolipoprotein complex involved in transport of the very low density lipoprotein (VLDL) out of the hepatocyte (Breen L, et al, 1972). Alternative or additional mechanisms may involve decreased fatty acid oxidation, increased triglyceride uptake or increased fatty acid uptake. (Figure 8)

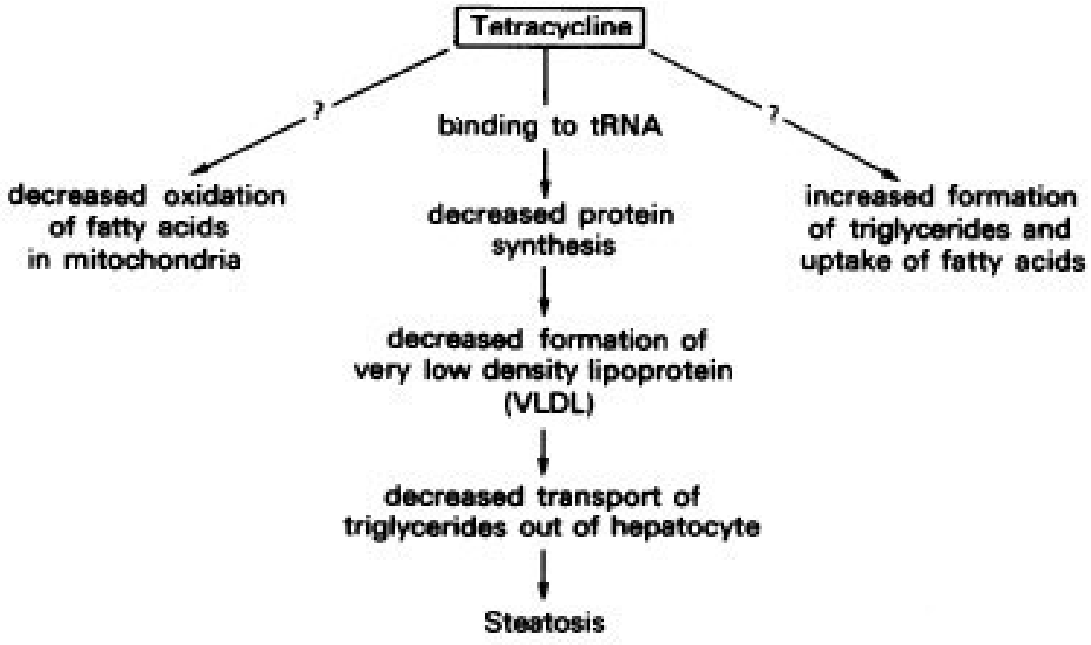


Figure 8: Postulated mechanism for tetracycline induced fatty liver

- **Chronic active hepatitis, cirrhosis and sub-acute necrosis**

Chronic active hepatitis, sometimes associated with cirrhosis is associated with the use of a number of drugs such as oxyphenisatin, α-methyldopa, nitrofurantoin and isoniazid. The INH has been chosen as the example because the mechanism is at least partially understood.

Isoniazid: Long term administration of the antitubercular drug isoniazid leads to hepatic dysfunction in a significant proportion of recipients (10-20%). However, some 0.1-1% of patients develops severe hepatic injury. Although this is generally acute hepatocellular damage, in about 10 % of cases chronic active hepatitis may develop with or without cirrhosis. SGOT and SGPT values are elevated, (up to 10-20 times normal), but alkaline phosphatase (ALP) levels are only moderately raised, except in about 10% of patients when mixed hepatocellular jaundice occurs, giving 4 times normal values for ALP. The serum bilirubin may therefore also be raised. Preexisting liver dysfunction, such as alcoholic cirrhosis, also increases susceptibility (Black M, et al, 1975; Mitchell JR, et al, 1976).

The mechanism of INH induced hepatic damage involves production of a toxic metabolite. The reports of some finding suggested that rapid acetylation might be a predisposing factor as it was reasoned that this would produce more of the metabolite acetylisoniazid and hence more acetylhydrazine. Acetyl isoniazid and especially acetylhydrazine are extremely hepatotoxic causing centrilobular hepatic necrosis, in experimental animals in which the microsomal enzymes are induced by phenobarbitone. The hepatotoxicity of acetyl isoniazid depends on its metabolism to acetylhydrazine. This metabolite in turn is metabolically activated by the microsomal enzymes to a reactive acylating species which reacts covalently with liver protein. The role of the acetylator phenotype in this is complex, as studies in human volunteers indicated that although rapid acetylators produced more potentially toxic acetylhydrazine, this was then further acetylated to non-toxic diacetylhydrazine (Timbrell JA, et al 1977, Bahri AK, et al, 1981, Mitchell JR, et al, 1975)

- **Phospholipidosis**

This syndrome may be caused by a number of different drugs, and various organs may be affected. Hepatic phospholipidosis has been caused by the drug Coralgil, a coronary dilator, in Japan. The features of this form of hepatic damage are an accumulation of phospholipids in hepatocytes, bile duct proliferation and inflammation in the portal area. SGOT and SGPT values may be elevated. The lesion may progress to liver cirrhosis and have a fatal outcome. The mechanism is thought to involve the formation of complexes between lipid micelles or liposomes, and the drug (Lullmann-Rauch R, et al ,1975, Lullman H, et al, 1977)

- . **Liver tumours**

Anabolic steroids have been implicated as responsible for primary hepatocellular carcinomas and adenomas. Similarly use of contraceptive steroids has been connected with liver tumours, particularly the estrogenic components . The mechanism(s) is unknown although interference with the metabolism of foreign compounds or bile salt derivatives so as to increase their tumour genicity has been suggested (Neuberger JM, et al, 1981, Goldfarb S, et al 1976).

3.5. Role of free radicals in hepatotoxicity

Free radicals generated in the body have ability to attack healthy cell and are capable of producing several diseases like liver disorder, diabetes, cancer etc. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases (Anbazhakan S, et al, 20008, Gyamfi MA, et al, 1999).

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cell. This radical has the capacity to join nucleotide in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, this species is considered to be one of the quick initiator of lipid peroxidation process, abstracting hydrogen atom form the unsaturated fatty acids (Singh RP, et al, 2004, Lee J, et al, 2004, Gow-Chin Y, et al, 2002).

The nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals (Lata H, et al, 2003, Ialenti S, et al, 1993, Ross r, et al, 1993).

The hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore, biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate (Miller MJ, et al, 1993).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Diplock AT, et al, 1997, Meir S, et al, 1995).

Ascorbic acid activates the functions of all the cells. It is a powerful antioxidant. It favours the absorption of iron in the intestine, protects against infections, neutralizes blood toxins and intervenes in the healing of wounds. The vitamin-C acts as an antioxidant in biological systems and scavenge the free radicals thereby increase the antioxidant defense in the body. The effect of vitamin-C and other putative antioxidants on biomarkers of oxidation have been studied in many pathological states that are thought to result from or result in oxidative stress (Riemersma RA, et al, 2000).

Total polyphenolic assay by using Folin-Ciocalteu reagent is employed routinely in studying phenolic antioxidants. The total polyphenols play a vital role in anti-oxidization as well as in the biological functions of the plant. Other studies have also indicated that the anti-oxidative properties of polyphenols in edible plants and plant products may help prevent diseases (Forney CF, *et al, 1999,* Burns J, *et al, 2000).*

The flavonoids exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral, anticancer activities. They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants. The presence of high phenolic and flavonoid content in the extract has contributed directly to the antioxidant activity by neutralising the free radicals (Cao G, et al, 1997).

3.6. Clinical Management

In view of multiplicity and complexity of the liver functions, it is obvious that no single test can establish the disturbances in liver function. Thus batteries of liver function tests are employed for accurate diagnosis, to assess the severity of damage, to judge prognosis and to evaluate therapy. These tests are classified below according to their functions.

The presently used agents like folic acid, multivitamins and few polyherbal preparations provide only a supportive therapy and do not play an effective role in providing hepatic protection (Gulati RK, 1995). Hence, the search is towards finding an effective herbal hepatoprotective drug.

Evaluation of hepatotoxicity

Liver function tests

The liver function tests are described below according to the major liver functions (Harsh Mohan, et al, 2005).

- **Tests for manufacture and excretion of bile**

Bilirubin

a) Serum bilirubin estimation

b) Urobilinigen

c) Bromsulphalein excretion

d) Bile acids (Bile salts)

- **Serum enzyme assays**

Alkaline Phosphatase (ALP)

Gamma- glutamyl transpeptidase (γ-GT)

Transaminases (Aminotransferases)

a) Serum Glutamate oxaloacetate transaminase (SGOT)

b) Serum Glutamate Pyruvate transaminase (SGPT)

Other serum enzymes

a) 5'-Nucleotidase

b) Lactic dehydrogenase (LDH)

c) Choline esterase

- **Tests for metabolic functions**

Amino acid and plasma protein metabolism

a) Serum proteins

b) Immunoglobulin

c) Clotting factors

d) Serum ammonia

Lipid and lipoprotein metabolism

Carbohydrate metabolism

- **Immunologic tests**

Nonspecific immunologic reactions

a) Smooth muscle antibody

b) Mitochondrial antibody

c) Antinuclear antibody

Antibodies to specific etiologic agents

a) Hepatitis B surface antigens (HBsAg)

b) Hepatitis B core antibody (HBc)

c) Hepatitis B e antigens (HBeAg)

d) Amoeba antibodies to Entamoeba histolytica

- **Ancillary Diagnostic tests**

Ultrasonography

Precutaneous liver biopsy

In drug discovery and modern medical practice, there are few drugs that stimulate liver functions or offer protection to the liver against injurious substances or help in regeneration of hepatic cells.

3.7. Significance of Parameters

In keeping with the multiplicity of the liver function, a variety of tests are available to access them. The choice of the test is influenced by its simplicity, reliability, and sensitivity as well as particular function one is interested in accessing (Sarada S, et al, 1990).

1. Test of bilirubin metabolism

a) Estimation of serum bilirubin

Bilirubin levels are elevated in all types of Jaundice. The ratio of the conjugated to unconjugated bilirubin giving an idea of the type of Jaundice.

b) Urinary bilirubin

Elevation of water soluble conjugated bilirubin glucoronides in urine occur in obstructive jaundice.

c) Urine urobilinogen

Abnormally low level or absence of urobilinogen in urine in diagnostic of biliary obstruction, while it may be increased in other types of jaundice.

2. Test of protein synthesis and metabolism

a) Estimation of plasma protein

Electrophoresis is very useful in detection of impaired liver function or hepatic failure.

b) Albumin Globulin ratio (A/G ratio)

It is normally 1.2 to 1.4 and may be reversed in liver disorders.

c) Flocculation tests

The most commonly used are the Thymol turbidity and Zinc sulphate turbidity tests.

d) Plasma prothrombin and prothrombin time

In severe liver dysfunction, prothrombin synthesis impaired due to poor absorption of vitamin K leading to low plasma prothrombin levels and a prolonged prothrombin time. Coagulation time also prolonged.

3. Test based on excretory function

a) Serum alkaline phosphatase (ALP)

It is an enzyme excreted normally by liver. The ALP levels in serum are abnormally high in biliary obstruction.

b) Bromsulphthalein (BSP) Excretion

The BSP is a dye which is rapidly excreted by the liver. It is chiefly used to assess the liver cell dysfunction in the absence of jaundice.

4. Test to assess hepato-cellular damage

- **Serum enzyme estimation**

Intracellular enzyme such as SGOT and SGPT are released in hepato cellular damage, thus elevating the serum level of this enzyme. Isocitrate dehydrogenase and certain isozymes of lactic dehydrogenase are also elevated in liver cell damage. Serum transaminase levels (SGOT & SGPT) are markedly elevated in active hepatitis. As a general rule liver function test are employed either to assist in the differential diagnosis of jaundice or to detect and assess the extent of hepatocellular damage.

Serum glutamate oxaloacetate transaminase (SGOT)

Principle

SGOT catalyzes the transfer of the amino group from L-aspartate to α-ketoglutarate to yield oxaloacetate and L- glutamate. Malate dehydrogenase (MDH), then converts oxaloacetate and NADH to malate and NAD. The conversion of NADH to NAD decreases the absorbance at 340 nm, the rate of which proportional to the SGOT activity (Sood R, 1994).

SGOT

L-aspartate + α-ketoglutarate ---------- Oxaloacetate + L-glutamate

MDH

Oxaloacetate + NADH +H^+----------L- malate +NAD^+

Clinical significance

Organ rich in SGOT are heart, liver and skeletal muscle. When any of these organs are damaged, the SGOT level rises in proportion to the severity of damage. In myocardial infraction SGOT starts increasing by 3-9 hours, peaks on second day return to normal on 4^{th}-6^{th} day. In hepatitis, SGOT peaks usually between 7-12 days and any increase upto 100 times. Increased levels SGOT are also found in mononucleosis, pancreatitis, trauma of skeletal muscle, renal necrosis and cerebral necrosis.

Serum glutamate pyruvate transaminase (SGPT)

Principle

SGPT catalyzes the transfer of amino group from L- alanine to α- ketoglutarate to yield pyruvate and L-glutamate. Lactate dehydrogenase (LDH) then converts pyruvate and NADH into lactate and NAD. The conversion on NADH to NAD decreases the absorption at 340 nm. The rate of decrease in absorbance is measured and is proportional to the SGPT activity (Sood R, 1994).

SGPT

L-alanine + α- ketoglutarate ---------- L-Glutamate + pyruvate

LDH

Pyruvate +NADH +H^+ ---------- Lactate + NAD^+

Clinical significance

Elevation of SGPT activity is found in liver and kidney diseases such as infectious or toxic hepatitis, infectious mononucleosis and cirrhosis. Moderate increase is also found in obstructive jaundice, metastasis carcinoma, hepatic congestion and myocardial infraction. The SGPT levels may be decrease in patients undergoing long term hemodialysis without supplemental vitamin therapy.

Alkaline Phosphatase (ALP)

Principle

The substrate, p-nitrophenyl phosphate (PNPP) is hydrolysed by ALP to p- nitrophenol and phosphoric acid. Some divalent ions like Mg^{++} are added to the system which acts activators. PNPP is colourless in acid or alkaline medium while PNP is yellow in colour in the alkaline medium and colourless in the acid medium (Sood R, 1994).

ALP

P-Nitrophenyl phosphate +H_2O ---------- p- nitrophenol + H_3PO_4

Colourless Yellow

Clinical significance

Increased ALP activity may be related to hepatobiliary bone disease. Very high ALP activity in serum is seen in patient with bone cancer and marked increased also occur in obstructive jaundice and biliary cirrhosis.

Gamma-Glutamyl Transpeptidase (GGTP)

Principle

The GGTP catalyses transfer of gamma- glutamyl group from the substrate gamma-glutamyl para–nitroanilide to glycylglycine releasing free P- nitroaniline which absorbs light at 405 nm. Enzyme activity is proportional to the increase absorbance at this wave length (Sood R, 1994).

GGTP

G-glutamyl nitroanilide + glycylglycine ---------- P-nitroaniline +

Glutamyl glucylglycine

Clinical significance

Elevated serum GGTP levels appear to be indicative of disease of liver, biliary tract and pancrea. Serum GGTP activity is usually elevated in the cases of cholesystitis, cholangitis, cholelithiasis, chronic hepatitis, viral hepatitis and metastatic carcinoma.

GGTP is particularly helpful in clinical assessment of alcoholic cirrhosis. Since serum GGTP is not elevated in any form of bone disorder, it assay has been valuable in differentiating bone and liver disease in conjunction with alkaline phosphatase determination.

Total Protein (TP)

A healthy functioning of the liver is required for the synthesis of the serum proteins, except for the gamma globulins. The proteins synthesized in the liver are usually decreased in hepatocellular disease, but the immunoglobulins are increased in viral hepatitis and chronic liver infections (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Albumin

Albumin is decreased in chronic liver diseases and is generally accompanied by an increase in the beta and gamma globulins as a result of production of immunoglobulin-G (I_gG) and immunoglobulin-M (I_gM) in chronic active hepatitis and of I_gM and immunoglobulin-A (I_gA) in biliary or alcoholic cirrhosis, respectively (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Total Bilirubin (TB)

Bilirubin has been used to evaluate chemically induced hepatic injury. It is the principle pigment in the bile and is derived from the breakdown of heamoglobin when senescent red blood cells (RBCs) are phagocytozed. As most of the liver diseases are accompanied by jaundice, the differential diagnosis of jaundice plays an important role in elucidating hepatic dysfunction. An elevated level of serum bilirubin may be produced. It shows severe parenchymal injury (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Direct Bilirubin (DB)

Unconjugated bilirubin is not water-soluble. It is transported in the blood stream bound to albumin. It accounts for 30-50% of bilirubin rise in hepatocellular disease or cholestasis. Unconjugated hyperbilirubenemia is most often due to either haemolysis or Gilbert's

syndrome, an inherited abnormality of bilirubin metabolism (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Lactate Dehydrogenase (LDH)

The LDH is localized in the cytoplasm of the cells and this is extruded into the serum when the cells are damaged or necrotic. When only a specific organ, such as liver is known to be involved, the measurement of total LDH is useful (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Total Cholesterol (TC)

Serum cholesterol comprises two forms, free cholesterol and esterified cholesterol. In jaundice and paranchymatous liver disease, serum cholesterol level will fall. Drug administration will rectify the defective mechanism associated with carbon tetrachloride administration (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

Triglycerides (TG)

Immediately after carbon tetrachloride administration, the TG level in the liver is elevated. The defect in the transport of TGs into the plasma is the cause for accumulation of lipids in the liver during carbon tetrachloride intoxication. Within 3-5 hours after administration of carbon tetrachloride, decrease in serum TG level occurs in rats. Carbon tetrachloride intoxication evokes a defect in the secretory mechanism of TGs in the liver, resulting in accumulation of lipid in liver. A reduction in the synthesis of lipoproteins will result in the lower transport of TGs, which is associated with lipoprotein (Dwivedi, S, 2008, Dr. Nanjaian Mahadevan, 2007).

3.8. Preclinical screening models of hepatotoxicity

- ***In-Vivo* evaluation of hepatic injury**

Various drugs and chemicals such as PCM, CCl_4, INH + RMP, Cs-A, Alcohol, Galactosamine and Thioacetamide produce hepatotoxicity. The various models of hepatotoxicity are as follows,

Acute Model

- PCM induced hepatotoxicity in rat (Jaswanth M, et al , 2008)
- PCM induced hepatotoxicity in mice (Nahid T, et al, 2005)
- CCl_4 induced hepatotoxicity in rat (Padhy BM, et al, 2007)
- Alcohol induced hepatotoxicity in rat (Lu ZM, et al, 2007)
- Alcohol induced hepatotoxicity in mice **(**Fleurentin J, et al, 1986)
- Isoniazid induced hepatotoxicity in rabbit (Sarich TC, et al, 1995)
- Rifampicin induced hepatotoxicity in mice (Upadhyay G, et al, 2007)
- Aflatoxin B1-induced liver damage in rat (Preetha SP, et al, 2006)
- Concavalin-A induced hepatotoxicity in mice (Gantner F, et al, 2005)

Sub-acute Model

- Thioacetamide induced hepatotoxicity in rat (Mitra SK,et al,1998)
- INH + RMP induced hepatotoxicity in rat (Prabakan M, et al, 2000)

Chronic Model

- INH + RMP + PZA induced hepatotoxicity in rat (Tandon VR, et al, 2008)
- INH + RMP + PZA induced hepatotoxicity in guinea pigs (Adhvaryu MR, et al, 2007)
- Hepatitis in Long Evans Cinnamon rats (Hawkins RL, et al, 1995)
- Alcohol induced hepatotoxicity in rat (Pramyothin P, et al, 2007)
- Bile duct ligation induced liver fibrosis in rats (Cameron GR, et al, 1932)
- Galactosamine induced liver necrosis (Keppler D, 1968)
- Aflatoxin B1-induced liver damage in mice (Naaz F, et al, 2007)

- **Paracetamol induced hepatotoxicity**

Paracetamol is a widely used analgesic-antipyretic drug produces acute hepatic damage on accidental over dosage. It is established that, a fraction of PCM is converted via the cytochrome P450 (CYP450) pathway to a highly toxic metabolite; N–acetyl–p–benzoquinamine (NAPQI) which is normally conjugated with glutathione and excreted in urine. Overdose of acetaminophen depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction and the development of acute hepatic necrosis. Several cytochrome P450 enzymes are known to play an important role in PCM bioactivation to NAPQI. The CYP450 2E1 have been suggested to be primary enzyme for PCM bioactivation in liver microsomes . Studies demonstrated that PCM induced hepatotoxicity can be modulated by substances that influence P450 activity. In the assessment of liver damage by PCM the determination of enzyme levels such as SGOT, SGPT is largely used. The necrosis or membrane damage releases the enzyme into circulation and hence, it can be measured in the serum (Dahlin D, et al, 1984, Parmar D, et al, 1995).

Dose of PCM: 3 g/kg p.o.

- **CCl_4 induced hepatotoxicity**

The CCl_4 induced hepatotoxicity is common model for study of experimental liver diseases. In CCl_4 induced toxic hepatitis, toxicity begins with the changes in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures. The toxic metabolite trichloromethyl radical (CCl_3) produced by microsomal oxidase system, binds covalently to the macromolecule and causes peroxidative degradation of lipid membranes of the adipose tissues. The extent of hepatic damage is assessed by the elevated levels of serum markers which are attributed to the generation of trichloromethyl free radicals which in turn causes lipid peroxidation.

$$CCl_4 \longrightarrow CCl_3O^- + O^-$$

Administration of a single dose of CCl_4 to a rat produces within 24 hrs a centrilobular necrosis and fatty changes. The poison reaches its maximum concentration in the liver within 3 hrs of administration. Thereafter, the level falls and by 24 hrs there is no CCl_4 left

in the liver. The development of necrosis is associated with leakage of hepatic enzymes into serum (Recknagel RO, et al, 1983, Datta S, et al, 2001).

Dose of CCl_4: 0.1 ml/100 g, I.P.

3.9. Phenolics as antioxidant:

Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, with more than 8000 phenolic structures currently known. Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group (Bravo. L, et al, 1998). Phenolics are widely distributed in the plant kingdom and are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits and beverages (Bohuran T, et al, 2003; A. Luximon-Ramma; 2005). Polyphenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (E. Middleton Jr, 2000) many of these biological functions have been attributed to their free radical scavenging and antioxidant activity.

Molecular studies have revealed that phenolics can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These include activation of mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serine/threonine protein kinase Akt/PKB, phase II antioxidant detoxifying enzymes, downregulation of pro-inflammatory enzymes (COX- 2 and iNOS) through the activation of peroxisome proliferator-activated receptor gamma (PPAR_), regulation of calcium homeostasis, inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, NF-_B, c-JUN, as well as modulation of several cell survival/cell-cycle genes (R.J. Williams,2004). The redox status of the cell has profound effect on the cell signaling pathways, in particular the MAP kinase cascade (Fig.9)

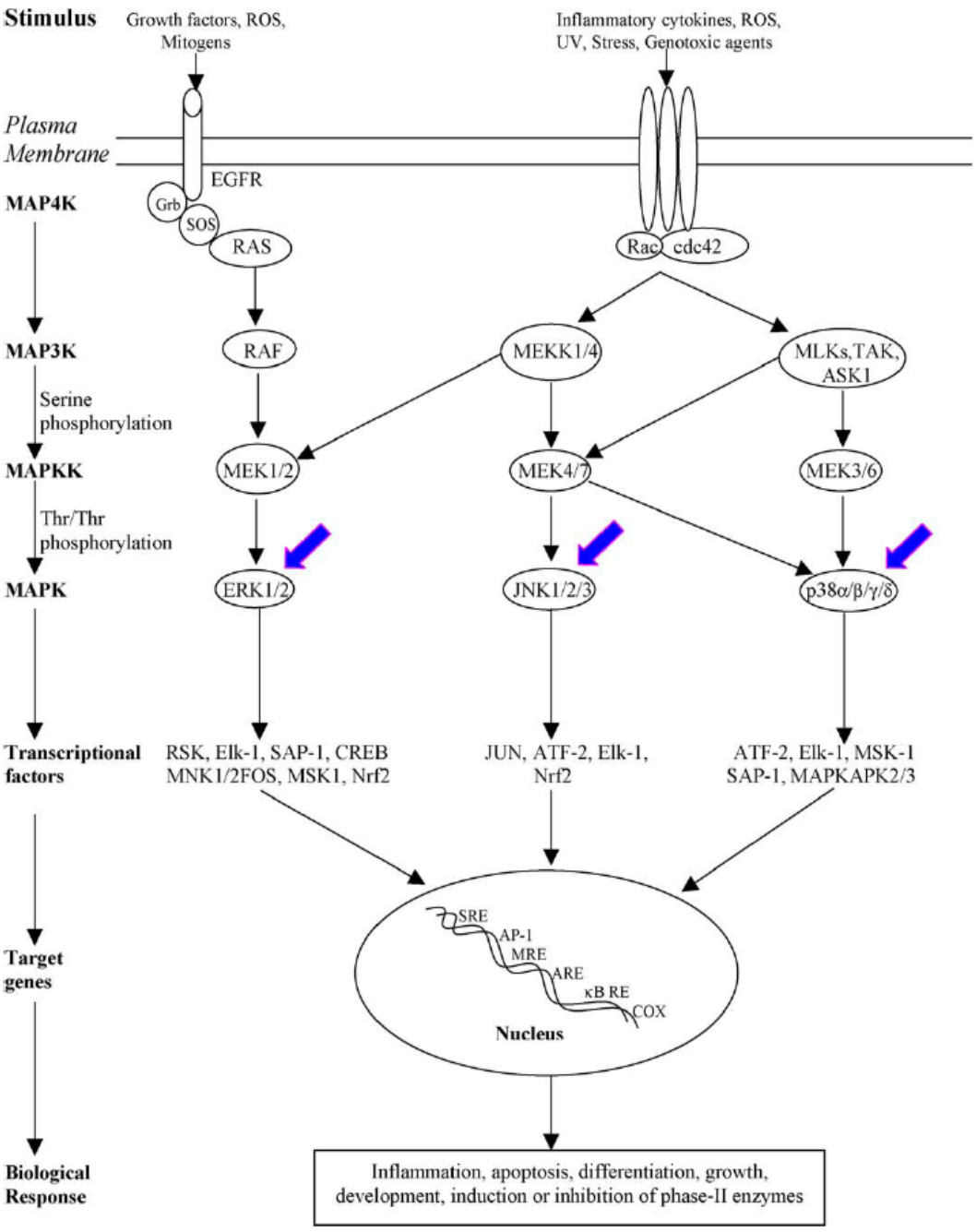


Fig.9. Molecular mechanism of antioxidants

3.10. Silymarin as a Standard hepatoprotecive drug

Silymarin is a flavonolignan which is extracted from the seeds and fruit of *Syllabus marianum* (Compositae) and is a mixture of three structural components: silibinin, silydianine and silychristine (figure 10). Of the three isomers that constitute silymarin, silibinin is the most active. From a medical point of view, silymarin and silibinin have been found to provide cytoprotection and above all, hepatoprotection. Silymarin is used for the treatment of numerous liver disorders characterised by degenerative necrosis and functional impairment. Furthermore, it is able to antagonise the toxin of *Amanita phalloides* and provides hepatoprotection against poisoning by phalloidin galactosamine thioacetamide halothane and CCl_4. The compound also protects hepatocytes from injury caused by ischaemia, radiation, iron overload and viral hepatitis. Silymarin is used as supportive therapy in food poisoning due to fungi and in chronic liver disorders, such as steatosis and alcohol-related liver disease (Valenzuela A, et al, 1994, Morazzoni P et al 1995, Wagner H, et al, 1974).

Chemistry of silymarin

Silymarin is extracted from the dried seeds of milk thistle plant, where it is present in higher concentrations than in other parts of the plant. Later the biochemical effects of silymarin on RNA, protein and DNA synthesis was reported by Sonnenbichler and Zetl. Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin (Figure10).

The structural similarity of silymarin to steroid hormones is believed to be responsible for its protein synthesis facilitatory actions. Among the isomers silybin is the major and most active component and represents about 60-70 %, followed by silychristin (20%), silydianin (10%), and isosilybin (5%). Silipide is the silybin-phosphatidylcholine complex which ensures a large increase in the bioavailability of silybin (Skottova N, et al, 1998, Ferenci P, et al, 1989, Vailati A, et al, 1993).

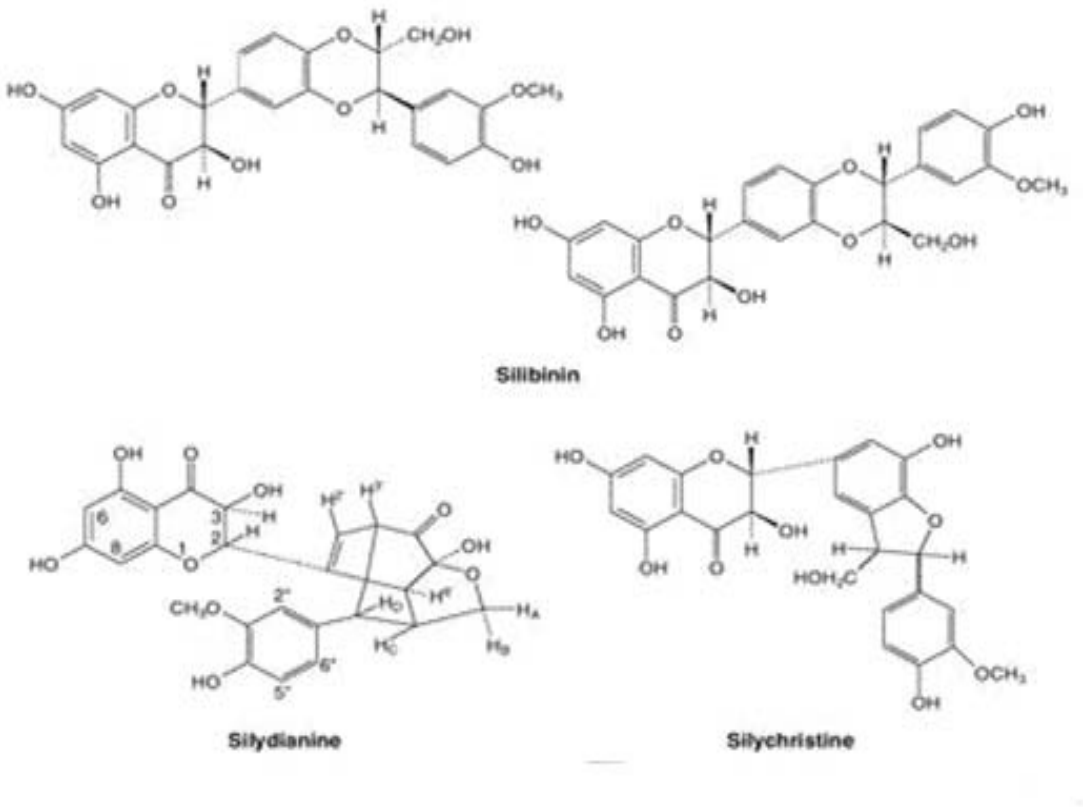


Figure 10: Components of silymarin: silibinin, silydianine and silychristine

Mechanism of action

The preclinical studies using different hepatotoxic substances showed that silymarin has multiple actions as a hepatoprotective agent. The antioxidant property and cell-regenerating functions as a result of increased protein synthesis are considered as most important.

(i) Antioxidant properties: Free radicals, including the superoxide radical, hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and lipid peroxide radicals have been implicated in liver diseases. These reactive oxygen species (ROS) are produced as a normal consequence of biochemical processes in the body and as a result of increased exposure to xenobiotics.The mechanism of free radical damage include ROS- induced peroxidation of polyunsaturated fatty acid in the cell membrane bilayer, which causes a chain reaction of lipid peroxidation, thus damaging the cellular membrane and causing further oxidation of membrane lipids and proteins. Subsequently cell contents including DNA, RNA, and other cellular components are damaged. The cytoprotective effects of silymarin are mainly attributable to its

antioxidant and free radical scavenging properties. Silymarin can also interact directly with cell membrane components to prevent any abnormalities in the content of lipid fraction responsible for maintaining normal fluidity (Miguez MP, et al, 1994, Miller AL, et al, 1996, Muriel P, et al, 1990)

(ii) Stimulation of protein synthesis: Silymarin can enter inside the nucleus and act on RNA polymerase enzymes resulting in increased ribosomal formation. This in turn hastens protein and DNA synthesis. This action has important therapeutic implications in the repair of damaged hepatocytes and restoration of normal functions of liver (Sonnenbichler J, et al, 1986).

(iii) Anti-inflammatory actions: The inhibitory effect on 5-lipoxygenase pathway resulting in inhibition of leukotriene synthesis is a pivotal pharmacological property of silymarin. Leukotriene (B_4) synthesis was reduced while prostaglandin (E_2) synthesis was not affected at higher concentrations of use of silibinin. A study which evaluated the action of silibinin in isolated Kuppfer cells indicated a strong inhibitory effect on leukotriene B4 (LTB_4) formation with the IC_{50} value of 15 μmoles/l. But no effect was observed on tumour necrosis factor-alpha (TNF-α) formation. The NF-kB is a key regulator of inflammatory and immune reactions. Silymarin is found to suppress both NF-kB DNA binding activity and its dependent gene expression induced by okadaic acid in the hepatoma cell line HEP G_2. But, the NF-kB activation induced by TNF-α was not affected by silymarin, demonstrating a pathway dependent inhibition by silymarin (Saller R, et al, 2001, Dehmlow C, et al, 1996, Saliou C, et al, 1998).

(iv) *Antifibrotic action*: Liver fibrosis can result in remodeling of liver architecture leading to hepatic insufficiency, portal hypertension and hepatic encephalopathy. These processes involve complex interplay of cells and mediators. In the initial phase there will be proliferation of hepatic parenchymal cells. The conversion of hepatic stellate cells (HSC) into myofibroblast is considered as the central event in fibrogenesis. Silymarin inhibits NF-kB and also retards HSC activation. It also inhibits protein kinases and other kinases

involved in signal transduction and may interact with intracellular signaling pathways (Gebhardt R, et al, 2002).

Pharmacodynamics

1) Antioxidant properties:

The flavonoids usually possess good antioxidant activity. The water-soluble dehydrosuccinate sodium salt of silibinin is a powerful inhibitor of the oxidation of linoleic acid-water emulsion catalysed by Fe^{2+} salts. It also inhibits in a concentration-dependent way the microsomal peroxidation produced by NADPH - Fe^{2+} -ADP, a well known experimental system for the formation of hydroxyl radicals. In studies performed in rat hepatic microsomes, it has been demonstrated that lipid peroxidation produced by Fe (III)/ascorbate is inhibited by silibinin dihemisuccinate; the inhibition is concentration-dependent. It has been shown that silymarin is as active as quercetin and dihydroquercetin and more active than quercitrin in terms of antiperoxidant activity, independent of the experimental model used to produce peroxidation. It has recently been reported that in rat hepatocytes treated with *tert*-butyl hydroperoxide (TBH), silymarin reduces the loss of lactate dehydrogenase (LDH), increases oxygen consumption, reduces the formation of lipid peroxides and increases the synthesis of urea in the perfusion medium. Furthermore, silymarin is able to antagonise the increase in Ca^{2+} produced by TBH, reducing ion levels down to below 300 nmol/L. The protective effect of silymarin is mediated by the inhibition of lipid peroxidation and the modulation of hepatocyte Ca^{2+} content seems to play a crucial role (Valenzuela A, et al, 1986, Mira L, et al, 1984, Bosisio E, et al, 1992).

2) Protective Effects in Models of Oxidative Stress:

Oxidative stress is defined as structural and/or functional injury produced in tissues by the uncontrolled formation of pro-oxidant free radicals. Oxidative stress usually develops when the pro-oxidant action of an inducer exceeds the anti-oxidant capacity of the cell defense system, altering its homeostatic capacity. Numerous substances induce oxidative stress, including carbon tetrachloride, TBH, ethanol, paracetamol and phenyl hydrazine. It has been shown in rats that silibinin protects neonatal hepatocytes from cell damage produced by erythromycin, amitriptyline, nortriptyline and TBH. Erythrocytes obtained from rats treated

with silymarin exhibited high resistance against the haemolysis produced by phenyl hydrazine and the lysis induced by osmotic shock. This suggests that silymarin may act by increasing the stability of the erythrocyte membrane. The cytoprotective activity of silymarin has also been shown in hepatocytes of rats subjected to osmotic stress produced by hypotonic saccharose solutions. The perfused liver experimental model, it has been shown that phenyl hydrazine produces an increase in oxygen consumption in rat liver *in vitro* and in the release of thiobarbituric acid reactive substances (TBARS) in the perfusate. This stress is associated with a reduction in the amount of reduced glutathione (GSH) in the liver; GSH exerts important protective activity against chemically induced oxidative stress. Using liver from rats pre-treated *in vivo* with silibinin 50 mg/kg intravenously, a significant reduction in the oxygen consumption stimulated by phenyl hydrazine and in the release of TBARS was observed, without any changes in GSH levels. The antioxidant effect of silibinin was observed in rats with acute intoxication caused by ethanol or paracetamol, which are peroxidation inducers that produce marked GSH depletion in the liver. Treatment with silymarin or silibinin was able to protect animals from oxidative stress produced in the liver by ethanol or paracetamol. Furthermore, it has been reported that treatment with silibinin attenuates the increase in plasma levels of AST, ALT and γ-glutamyl transpeptidase (GGT) observed after intoxication by paracetamol. The hepatoprotective activity of silibinin has also been studied in rats with liver cirrhosis induced by the long-term administration of carbon tetrachloride. Muriel & Mourelle have shown that silibinin preserves the functional and structural integrity of hepatocyte membranes by preventing alterations of their phospholipid structure produced by carbon tetrachloride and by restoring alkaline phosphatase and GGT activities. Another interesting property of silibinin and silymarin is their role as regulators of the content of GSH in various organs. In rats treated with silibinin intravenously or silymarin intraperitoneally, a significant increase in the amount of the GSH contained in the liver, intestine and stomach was found, whereas there were no changes in the lungs, spleen and kidneys (Valenzuela A, et al, 1985, Videla LA, et al, 1982, Campos R, et al, 1989).

3) Activity against Lipid Peroxidation:

Lipid peroxidation is the result of an interaction between free radicals of diverse origin and unsaturated fatty acids in lipids. Lipid peroxidation involves a broad spectrum of alterations and the consequent degeneration of cell membranes may contribute towards the development of other disorders of lipoprotein metabolism, both in the liver and in peripheral tissues. The silymarin appears to act as an antioxidant not only because it acts as a scavenger of the free radicals that induce lipid peroxidation, but also because it influences enzyme systems associated with glutathione and superoxide dismutase. It has been shown that all the components of silymarin inhibit linoleic acid peroxidation catalysed by lipoxygenase and that silymarin protects rat liver mitochondria and microsomes *in vitro* against the formation of lipid peroxides induced by various agents (Fiebrich F, et al, 1979).

4) Effects on Liver Lipids:

The influence of silymarin on cellular permeability is closely associated with qualitative and quantitative alterations of membrane lipids (both cholesterol and phospholipids. This suggests that silymarin may also act on other lipid compartments in the liver; this may influence lipoprotein secretion and uptake. It has been shown that silymarin and silibinin reduce the synthesis and turnover of phospholipids in the liver of rats. Furthermore, silibinin is able to neutralize two effects of ethanol in rats: the inhibition of phospholipid synthesis and the reduction in labeled glycerol incorporation into lipids of isolated hepatocytes. In addition, silibinin stimulates phosphatidylcholine synthesis and increases the activity of cholinephosphate cytidyltransferase in rat liver both in normal conditions and after intoxication by galactosamine. Data on the influence of silymarin on triglyceride metabolism in the liver are scanty. It is known that in rats silibinin is able to partly antagonise the increase in total lipids and triglycerides produced in the liver by carbon tetrachloride and, probably, to activate fatty acid ß-oxidation. It has also been suggested that silymarin may diminish triglyceride synthesis in the liver. Letteron *et al*. studied the mechanisms of action of silymarin that provide protection against lipid peroxidation and the hepatotoxicity of carbon tetrachloride in mice, and came to the conclusion that silymarin works by reducing metabolic activation by carbon tetrachloride and by acting as an

antioxidant that prevents chain rupture authors have shown that silymarin affords hepatoprotection against specific injury induced by microcystin (a hepatotoxin), paracetamol, halothane and alloxan in several experimental models (Bindoli A, et al, 1977, Muriel P. et al, 1991, Soto CP, et, al, 1998, Mereish KA, et al, 1991).

5) Effects on Plasma Lipids and Lipoproteins:

The administration of silymarin reduces plasma levels of cholesterol and low-density lipoprotein (LDL) cholesterol in hyperlipidaemic rats, whereas silibinin does not reduce plasma levels of cholesterol in normal rats; however, it does reduce phospholipid levels, especially those transported in LDL Data obtained in experimental models of hepatic injury have shown that silymarin is able to normalize the increase in plasma lipids observed after administration of carbon tetrachloride and to antagonise the reduction in serum free fatty acids induced by thioacetamide. In the experimental model of hepatic injury produced by thioacetamide, silymarin did not appear to be able to normalize the reduction in triglycerides in serum. In the experimental model of hepatic injury produced by paracetamol in rats, it was evident that silymarin improves LDL binding to hepatocytes, an important factor for the reduction of LDL in plasma.

6) Stimulation of Liver Regeneration*:*

One of the mechanisms that can explain the capacity of silymarin to stimulate liver tissue regeneration is the increase in protein synthesis in the injured liver. In *in vivo* and *in vitro* experiments performed in the liver of rats from which part of the organ had been removed, silibinin produced a significant increase in the formation of ribosomes and in DNA synthesis, as well as an increase in protein synthesis. Interestingly, the increase in protein synthesis was induced by silibinin only in injured livers, not in healthy controls. The mechanism whereby silibinin stimulates protein synthesis in the liver has not been defined; it may be the physiological regulation of RNA polymerase I at specific binding sites, which thus stimulates the formation of ribosomes. In rats with experimental hepatitis caused by galactosamine, treatment with intraperitoneal silymarin 140 mg/kg for 4 days completely abolished the inhibitory effect of galactosamine on the biosynthesis of liver proteins and glycoproteins. These data support the results of previous experiments in a similar model of

acute hepatitis in the rat, in which silymarin protected hepatic structures, liver glucose stores and enzyme activity *in vivo* from injury produced by galactosamine. The capacity of silymarin to stimulate protein synthesis has also been studied in neoplastic cell lines, in which no increase in protein synthesis, ribosome formation or DNA synthesis has been found after treatment with silymarin (Magliulo E, et al, 1973, Tyutyulkova N, et al, 1983, Barbarino F, et al, 1981).

3.11. Hepatoprotective Herbs

Table 3: Herbal Drugs Used As Hepatoprotective

Botanical name	***Part used***	***Chemical constituents***
Calotropis gigantea	Leaves	Flavonoids, cardiac glycosides.
Leucophyllum Frutescens (Balderas-Renteria I, *et al*, 2007)	Aerial part	Diayangambin, Epiyangambin, Diasesartemin
Foeniculum vulgare (Ozbek H, et al, 2006)	seeds	Trans-anethole, Fenchone, β-pinene , Limonene, α-pinene, Camphene, β -myrcene, α –phellandrene.
Physalis peruviana (Arun M, et al, 2007)	Whole plant	28-hydroxywithanolide, Phygrine, Withanolides, Kaempferol, Quercetin
Lawsonia alba (Ahmed S, et al, 2000)	Bark	β-sitosterol, Flavanoids, Quinoids, Luteolin, Betulin, Lupeol, Garlic acid, Coumarins, Xanthones
Ficus carica (Mohan K, et al, 2007)	Leaves	Psoralen, Bergapten, β-sitosterol, Campesterol, Stigmasterol, Fucosterol, Calotropenyl.
Picrorrhiza kurroa (Anandan R, et al, 1999)	Rhizome and roots	Kutkin, kurrin, kutkiol, kutkisterol and 2 alkaloids.

Schouwia thebica (Amani, et al, 2006)	Aerial part	Chrysoeriol , Quercetin , Quercetin-7-O-rhamnoside.
Woodfordia fruticosa (Chandan BK, et al, 2007)	Flowers	Woodfordin A, B, C, E, F, G, H and I, Myricetin-3-*O*-arbinopyranoside.
Cassia occidentalis (Jafri MA, et al , 1999)	Leaves	Anthraquinones, Flavonoids, Phytosterol
Phyllanthus emblica (Pramyothin P, et al, 2006)	Fruit	Tannin and Flavonoids
Operculina turpethum (Suresh Kumar SV, et al, 2006)	Roots	Glycosidic resin, Coumarins, Beta-sitosterol.
Calotropis procera(Setty SR, et al, 2007)	Flowers	Flavonoids, Alkaloids, Cardiac glycosides, Sterols, Triterpene.
Solanum fastigiatum (Sabir SM, et al, 2008)	whole plant	Flavonoids and Glycoside
Cistus laurifolius (Upeli EK, et al, 2006)	Leaves	Quercetin, Kaempferol, Apigenin, Luteolin, Diterpenoids.
Solanum nigrum (Hsieh CC, et al, 2008)	Whole plant	Total alkaloid, Steroid, Steroidal saponins.
Aloe barbadensis (Chandan BK, et al, 2007)	Leave	Barbaloin, chrysophanol, Glycoside aloe-emodin,
Cuscuta chinensis (Yen FL, et al, 2007)	Seeds	Flavonoids, Lignans, Quinic acid.
Andrographis paniculata (Singha PK, et al, 2007)	Whole plant	Andrographolide

Glycyrrhiza glabra (Lee CH, et al, 2007)	Roots	Glycyrrhizin
Sargassum wightii	Marine brown algae	Sulphated polysaccharides
Bacopa monnieri (Ghosh T, et al, 2007)	Aerial part	Saponins

3.12. Polyherbal preparations

Table 4: Polyherbal Preparations Used As Hepatoprotective

Polyherbal Preparations	***Composition***
Tefroli (Nair SP,et al, 2006)	*Phyllanthus niruri, Ocimum sanctum Tephrosia purpurea, Eclipta alba Andrographis paniculata, Terminalia chebula*
Liv.52 (Vijaya P, et al, 2009)	*Capparis spinosa,Cichorium intybus Solanum nigrum, Cassia occidentalis Terminalia arjuna,Achillea millefolium Tamarix gallica.*
Hep-I (Savadi RV, et al, 2009)	*Lawsonia alba, Eclipta alba Berberis aristata, Aloe vera, Tephrosia purpuria ,Andrographis paniculata.*

Hep-II (Savadi RV, et al, 2009)	*Boerhavia diffusa, Melia azadirachta, Phyllanthus niruri, Croton oblongifolius, Picrorrhiza kurroa, Plumbago zeylanica.*
Enliv Â® **(**Bhar M, et al, 2005)	*Aphanamixis polystachia, Eclipta alba, Phyllanthus niruri, Picrorhiza kurroa, Tinospora cordifolia, Naregamia alata Emblica officinalis,Andrographis paniculata.*

4. MATERIAL AND METHODS

4.1. Plant materials and standard drug

The drug was procured from DMAPR, Boriavi, Anand (Gujarat). The drug was authenticated with morphological and microscopical characters mentioned in literature review. The drug was powdered and stored in airtight plastic container at room temperature until needed.

4.2. Chemicals

All the solvents were procured from CDH chemicals and enzymatic kits were acquried from Labcare diagnostic Pvt. Ltd.

4.3. Preliminary evaluation

4.3. A **Physical Profile (Ayurvedic Pharmacopoeia of India)**

4.3. A.1. **Determination of Foreign Matter**

100–500 g of the drug sample to be examined was weighed and spread on a thin layer.
The foreign matters have been examined by the use of a lens.

4.3. A.2. **Determination of Total Ash**

About 2 to 3 g accurately weighed drug was incinerated, in a tared platinum or silica dish at a temperature not exceeding 450º until free from carbon, cool and weigh. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 450º.The percentage of ash with reference to the air-dried drug was calculated.

4.3. A.3. **Determination of Acid Insoluble Ash**

The ash obtained in 3.3.A.2 was boild for 5 minutes with 25 ml of dilute hydrochloric acid; collect the insoluble matter in a Gooch crucible or on an ashless filter paper, wash with hot

water and ignite to constant weight. The percentage of acid-insoluble ash with reference to the air dried drug was calculated.

4.3. A.4. **Determination of Alcohol Soluble Extractive value**

5 g of the air dried drug, coarsely powdered, was macerated with 100 ml of Alcohol of the specified strength in a closed flask for twenty-four hours, shaking frequently during six hours and allow to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105º, to constant weight and weigh. The percentage of alcohol-soluble extractive with reference to the air-dried drug was calculated.

4.3.A.5. **Determination of Water Soluble Extractive value**

5 g of the air dried drug, coarsely powdered, was macerated with 100 ml of water in a closed flask for twenty-four hours, shaking frequently during six hours and allow to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105º, to constant weight and weigh. The percentage of water-soluble extractive with reference to the air-dried drug was calculated

4.3 B **Phytochemical screening**

4.3. B.1. **Tests for Alkaloids**

About 500 mg of each of the dried extract was stirred with about 5 ml of dilute hydrochloric acid and filtered. The filtrate was tested with the following reagents:

(i) Mayer's Reagent: Few drops of Mayer's reagent (Potassium mercuric iodide solution) were added separately to each filtrate and observed for the formation of white or cream colored precipitates.

(ii) Dragendroff's Reagent: Few drops of Dragendroff's reagent (solution of potassium bismuth iodide) were added separately to each filtrate and observed for the formation of orange yellow precipitates.

(iii) Hager's Reagent: Few drops of Hager's reagent (saturated aqueous solution of picric acid) were added separately to each filtrate and observed for the formation of yellow precipitates.

(iv) Wagner's Reagent: Few drops of Wagner's reagent (solution of iodine in potassium iodide) were added separately to each filtrate and observed for the formation of reddish brown precipitates.

4.3. B.2. **Tests for Carbohydrates**

(i) Molisch's Test: A small amount of each extract was dissolved in ethanol and two drops of a 20% w/v solution of α-napthol in ethanol were added to it. Now, about 1 ml of concentrated H_2SO_4 was slowly added along the sides of the test tube. Appearance of red-violet ring at the junction of the two layers indicated the presence of carbohydrates.

(ii) Fehling's Test: A small amount of the each extract was dissolved in about 2 ml of distilled water and filtered. An equal amount of Fehling's solution was added to the filtrate and the contents were boiled. Appearance of brick red precipitates confirmed the presence of reducing sugars.

(iii) Benedict Test: A small amount of the each extract was dissolved in about 2 ml of distilled water and filtered. About 1 ml of Benedict solution was added to the filtrate. The contents were boiled and observed for the appearance of brick-red precipitates which confirmed the presence of reducing sugars.

4.3. B.3. **Tests for Glycosides**

(i) Borntrager's Test: A small amount of each extract was hydrolysed with dilute HCl for a few min on water bath. To the hydrolysate, about 1.0 ml of benzene and 0.5 ml of dilute ammonia solution was added. Appearance of reddish-brown color at the junction of the two layers confirmed the presence of glycosides.

4.3. B.4. **Tests for Sterols**

(i) Liebermann-Burchard's Test: A small amount of each extract was dissolved separately in chloroform and few drops of acetic anhydride were added. Now, concentrated sulphuric acid was added drop-wisely along the sides of the test tube and observed for the appearance of blue to blood red color as the indication of sterols.

(ii) **Salkowski Test:** A small amount of each extract was dissolved in chloroform. Concentrated Sulphuric acid was added dropwise along the sides of test tube and observed for presence of red or yellow colour at lower layer.

4.3. B.5. **Tests for Saponins**

(i) Foam Test: About 1 ml of each extract (in the respective solvents) was separately diluted to 20 ml with distilled water and further shaken in a graduated cylinder for 15 minutes. Formation of about 1 cm thick layer of foams confirmed the presence of saponins.

4.3. B.6. **Tests for Phenolic Compounds and Tannins**

(i) Ferric Chloride Test: Small amount of each extracts were separately shaken with water and warmed. Now about 2 ml of 5% ferric chloride solution was added and observed for the formation of green or blue color.

(ii) Lead acetate Test: A few milligrams of each extract were separately stirred with about 2 ml distilled water and filtered. To the filtrate, few drops of 10% w/v lead acetate solution was added and observed for the formation of white precipitates.

4.3. B.7. **Tests for Amino Acids and Proteins**

(i) Millon's Test: A small amount of each extract was separately dissolved in about 5 ml of distilled water and filtered. To 2 ml of the filtrate, 5-6 drops of Million's reagent (solution of mercury nitrate and nitrous acid) were added and observed for formation of red precipitates as an indication of the presence of proteins.

4.3. B.8. **Tests for Flavonoids**

(i) Shinoda Test: A few milligrams of each extract were separately shaken with ethanol in different test tubes. Now, small pieces of metallic magnesium or zinc were added followed by addition of 2 drops of concentrated HCl and observed for the formation of pink color.

(ii) Aqueous NaOH test: To test solution add 10 % NaOH, yellow color is obtained.

(iii) Mineral acid test: To test solution add conc. H_2SO_4, yellow-orange color is obtained.

(iv) Lead acetate solution test: To the test solution add 10 % of lead acetate solution, yellowish precipitates are obtained.

3.4. Preparation of Extract

The powdered drug was sieved through 40 # mesh sieve. The measured amount of the powder was then subjected to continuous and sequential; hot solvent extraction using petroleum ether, ethyl acetate, methanol and water, in a soxhlet apparatus. All the filterates were concentrated and subjected to furthur studies and % yield were 0.4 % w/w, 1.2 % w/w, 2.4 % w/w and 0.56 % w/w for pet.ether, ethyl acetate, methanol and water respectively.

4.5. In-vitro Antioxidant activity

All extracts were subjected to various antioxidant activities such as,

4.5.1. DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by spectrophotometric method (*Qureshi NN, et al, 2009*). 2 ml of methanolic solution of the extracts of various concentrations (0-500 µg/ml) were mixed with 1 ml of ethanolic solution of DPPH (1.5 mg/10ml). A mixture of 1 ml of ethanol and 1 ml of ethanolic solution of DPPH served as control. After mixing, all the solutions were incubated in dark for 20 min and then absorbance was measured at 517 nm. The experiments were performed in triplicate and EC_{50} was measured.

4.5.2. Nitric oxide radical scavenging activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions which were measured by Griess Illosvoy reaction. The 3-ml reaction mixture contained 10-mM SNP, phosphate buffered saline (pH 7.4) and various doses (0-500 µg/ml) of test solution. After incubation for 150 min at 25°C, 1-ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore formed during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against blank sample. All tests were performed thrice and EC_{50} was measured.(*sourav mandal et al 1999*, *A. Kumaran 2006)*

4.5.3. Lipid peroxidation inhibition

The effect of extracts on $FeCl_2$-ascorbic acid induced lipid peroxidation in rat liver homogenate was determined by the method of *Wong et al. (1987).* A mixture containing 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid and 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of various concentrations of extracts, were

incubated for 1 h at 37 °C. After incubation, 0.9 mL of distilled water and 2 mL of 0.6% thiobarbituric acid and 15% TCA mixture were added and then shaken vigorously. The mixture was heated for 30 min in a boiling water bath (100 °C). After cooling, supernatent was collected by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm against a blank, which contained all the reagents except the liver homogenate.

4.5.4. Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of *Oyaizu*. Different concentrations (0-500µg/ml) of extract (0.5 ml) were mixed with 0.5-ml 0.2 M phosphate buffer (pH 6.6) and 0.5-ml 0.1% potassium hexacyanoferrate, followed by incubation at 50°C in water bath for 20 min. After incubation, 0.5-ml 10% TCA was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water and 0.1-ml 0.01% $FeCl_3$ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against appropriate blank solution. All tests were performed thrice. A higher absorbance of the reaction mixture indicated greater reducing power.

4.5.5 Total flavonoids determination

Total flavonoid content was measured by method given by Jia J et al (1999) using cathecin as a standard. The plant extract of 0.1 ml was added to 0.3-ml distilled water followed by 0.03-ml 5% $NaNO_2$. After 5 min at 25°C, 0.03-ml 10% $AlCl_3$ was added. After another 5 min, the reaction mixture was treated with 0.2-ml 1-mM NaOH. Finally the reaction mixture was diluted to volume (1 ml) with water. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercitin standard curve.

4.5.6. Total phenolic compounds determination

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of *Singleton and Rossi.* Briefly, 0.1 ml of extract was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C;

then 0.06% Na_2CO_3 solution was added to the mixture. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. The phenolic content was evaluated from ellagic acid standard curve.

4.5.7. *Fe^{2+} Chelation*

The chelating activity of the extracts for ferrous ion was evaluated by a standard method given by *Ros G et al 2006*. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations of plant extracts (0–300 μg/ml) were added to 12.5 μM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 μM). The mixture was shaken vigorously and incubated for 20 min at room temperature, and the absorbance was measured at 562 nm. All tests were performed for six times. EDTA was used as a positive control.

4.6. *In vitro* evaluation of hepatoprotective effect of drug

Fresh isolated hepatocytes were used to study direct anti-hepatotoxic activity of drugs. Hepatocytes were treated with hepatotoxin (0.25 w/v % CCl_4) and the effect of the plant drug on the same was evaluated. Following steps were involved:

A). Isolation of rat hepatocytes (Marı´a del Carmen Garcı´ade Leo´n, et al, 2005)

B). Effect of test drug on CCl_4 -induced cytotoxicity.

4.6. A. **Isolation of hepatocytes**

The abdomen of the rat was opened under ether anesthesia and 0.2 ml of 0.2 % w/v heparin in 0.9 % w/v NaCl was injected into the tail vein to prevent blood from clotting. A midline incision was made and the portal vein was cannulated and perfused using Ca^{+2} free HBSS (pH 7.4). When liver was completely bleached and freed of blood it was perfused with 100ml HBBS containing 4500 IU Hylauronidase. After 10 –15 min perfusion, the liver was transferred to a beaker containing Ca^{+2}-free Hank's buffer (50 ml) and gently dispersed. The preparation was centrifuged at 50 g for 1 min. The supernatant was removed and the loosely packed pellets of cells were gently resuspended in Ca^{+2}- free Hank's buffer. The washing procedure was repeated 3-5 times.

4.6.B. **Effect of test drug on CCl_4 -induced cytotoxicity**.

Various concentration of extracts were prepared and incubated with aliquot amount of cell suspension for 30 min at 37^0C. Then preincubated with drug, cell suspension were treated with toxicant (0.25% w/v CCl_4) and incubated for 30 min at 37^0C and number of viable and dead cells were scored using Trypan blue exclusion assay.

Group 1 received only vehicle and group 2 received only toxin without drug.

Table.4.6.1. Treatment pattern

Sr. No	Groups	Drug concentration (µg/ml)	Toxicant CCl_4
1.	Control	-	Vehicle
2.	CCl_4	-	0.25 %
3.	EtAc01	10 50 100 250	0.25 %
4.	MeOH01	10 50 100 250	0.25 %
5.	Aq01	10 50 100 250	0.25 %

EtAC01- Crude ethyl acetate extract, MeOH01- Crude methanolic extract, Aq01- Crude aqueous extract.

Trypan Blue Exclusion Assay

Principle:

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not.

Procedure:

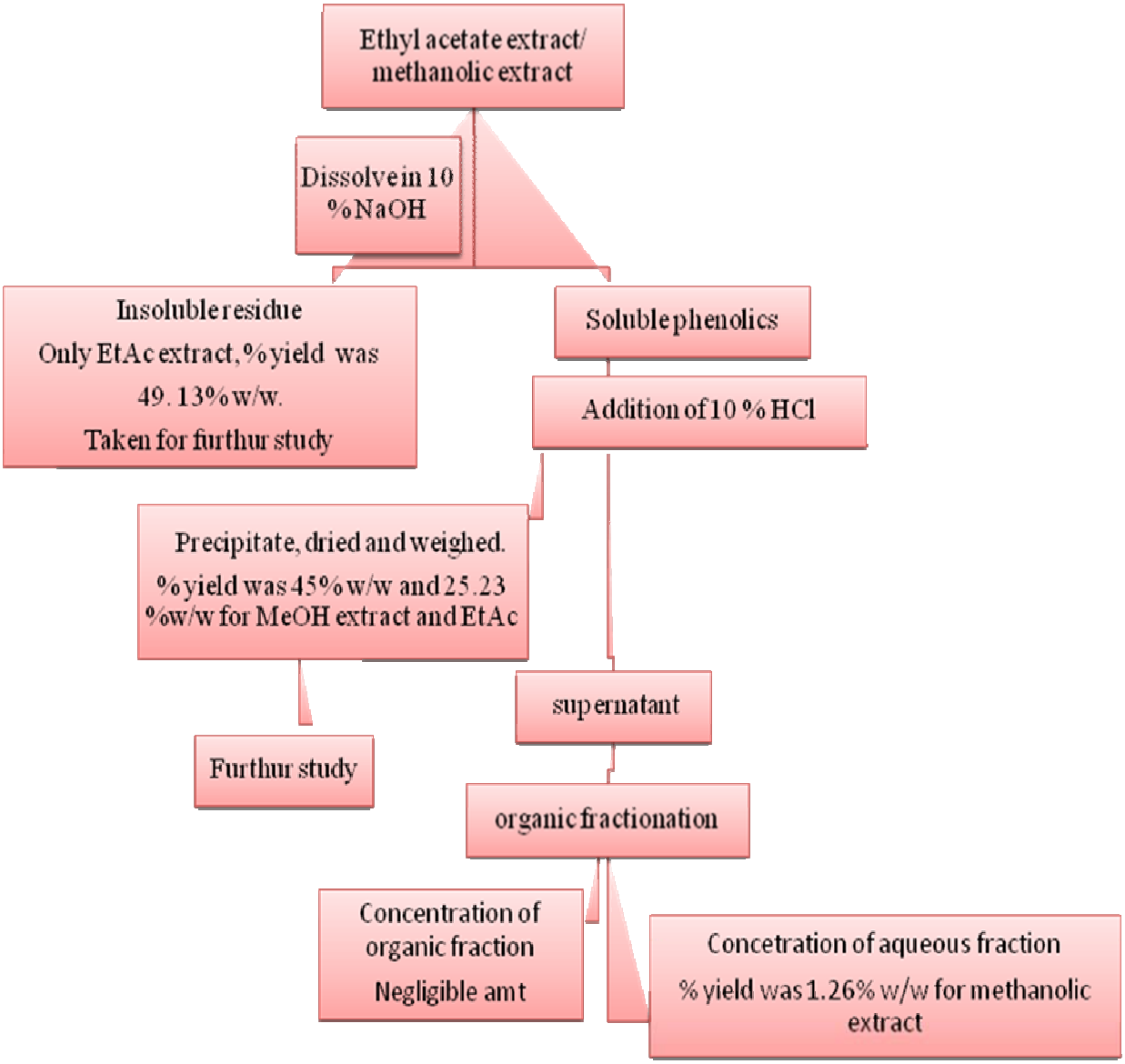
To 1 ml of above suspended cells, 10 µl of trypan blue dye (0.4 % w/v) was added and allowed to stand for 2-3 min.

Cells were then loaded onto a heamocytometer or on a glass slide, covered with cover slip and number of viable and dead cells were counted.

A minimum of 100 cells were counted in three separate fields. Percentage viability was calculated as follows:

$$\text{\% of viable cells} = \frac{\text{Number of cells excluding dye}}{\text{Total no of cells counted}} \times 100$$

4.7. Isolation of phenolics



4.8. IN VIVO STUDY

Paracetamol induced hepatotoxicty

4.8.1. Animals

Healthy adult female albino rats of Sprague Dawley strain weighing between 200-250 gm were selected for the study. Animals were maintained at 25 ± 2^{o}C and kept in well ventilated animal house under natural photoperiodic condition in polypropylene cages with free access to food and water *ad libitum*. During the period of experiment the animals were fed with the standard rat diet. Animals were acclimatized for 10 days before starting the study.

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute Of Pharmacy, Nirma University, Ahmedabad and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol number is **IPS/PCOG/MPH10/003.**

4.8.2. Chemicals

All the enzymatic kits were acquried from (Lab care Diagnostics (India) Pvt.Ltd). Standard drug Silymarin was procured from Ranbaxy.

4.8.3. Experiment

Proedure

PCM induced hepatotoxicity model was employed to evaluate the hepatoprotective activity of various extracts and isolated phenolics. A total of 54 animals were equally divided into 9 groups having 6 animals in each group. Group 1 was assigned as normal control, received .5 % CMC (1ml/kg) for 7 days. Group 2 served as diseased control, received PCM (3g/kg) on 5th day of the study. Group 3 served as reference control received Silymarin (50mg/kg) for 7 days. Group 4 -9 received various extracts and isolated phenolics for 7 days as shown below in treatment protocol. All the animals except Group1, had received PCM on 5th day to induce toxicity. On 7th day blood was collected from retro-orbital plexus under light aneasthesia, which was then subjected to estimation of serum aenzymatic levels. After collection of blood, animals were sacrificed, livers excised and seperated. A fraction of all

the livers were preserved for histopthalogical studies and remaining was stored for estmation of levels of antioxidants and prooxidants.

Treatment protocol

Rats (n=54) were randomized into following groups:-

1) **Normal Control (NC)**:- They were administered with vehicle (0.5 % CMC) for 7 days. They were fed with standard laboratory diet and water *ad libitum.*

2) **Disease control group**:- They were administered with vehicle (0.5 % CMC) for 7 days and administered with 3g/kg dose of paracetamol on 5th day.

3) **Silymarin treated group**: They were administered with silymarin 50mg/kg for 7 days along with paracetamol administered (3g/kg) on 5th day.

4) **10PRGSRA*hi*extMeOH01treated group:** administered with crude alcoholic extract 250 mg/kg along with paractamol 3g/kg on 5th day.

5) **10PRGSRA*hi*isoMeOH02 treated group:** They were administered with phenolics obtained from alcoholic extract 150mg/kg for 7 days along with paracetamol administered (3g/kg) on 5th day.

6) **10PRGSRA*hi*isoMeOH03 treated group:** administered with water soluble phenolics obtained from ethanolic extract 150mg/kg for 7 days along with paractamol 3g/kg on 5th day.

7) 10PRGSRA*hi*extEtAc01 treated group: admininstered with crude ethyl acetate extract 250mg/kg along for 7 days along for 7 days with paractamol 3g/kg on 5th day.

8) 10PRGSRA*hi*isoEtAc02 treated group:- They were administered with phenolics obtained from ethyl acetate fraction 150mg/kg for 7 days along with paracetamol administered(3g/kg) on 5th day.

9) 10PRGSRA*hi*extEtAc03 treated group: administered with alkaline insoluble fraction of ethyl acetate 150mg/kg for 7 days along with paractamol 3g/kg on 5th day.

Note:

10PRGSRA*hi*extEtAc01- crude ethyl acetate extact, 10PRGSRA*hi*isoEtAc02- precipitated phenolics from EtAc crude extract, 10PRGSRA*hi*isoEtAc03- alkaline insoluble fraction of EtAc crude extract, 10PRGSRA*hi*extMeOH01- crude methanolic extract, 10PRGSRA*hi*isoMeOH02- precipitated phenolics from methanolic crude extract, 10PRGSRA*hi*isoMeOH03- water soluble phenolics from crude methanolic extract.

Parameters assesed

Serum: serum gulamate pyruvate transaminase levels (SGPT), serum glutamte oxaloacetate tranaminase SGOT), serum alkaline phosphatase (ALP)

Liver: malondialdehyde, superoxide dismutase, catalase, reduced glutathione and total protien

Collection of serum:

The blood samples were withdrawn from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed to clot for 10 min at room temperature. It was centrifuged at 2500 rpm for 20 min. The serum was kept at 4^0C untill used.

4.9. BIOCHEMICAL ESTIMATIONS

4.9.1 Estimation of Serum Glutamate Pyruvate Transaminase Levels (SGPT)

In vitro quantitative determination of activity of SGPT in serum was done using enzymatic kit (Lab care Diagnostics (India) Pvt.Ltd)

Principle

Glutamate-pyruvic Transaminase (GPT_ALP) catalyses the reaction between alpha ketoglutaric acid and alanine giving L-glutamic acid and pyruvic acid. Pyruvic acid in presene of lactate dehydrogense (LDH) reacts with NADPH giving lactate acid and NAD. The rate of NADPH consuption is determined photometrically and is directly proportional to the GPT activity in the sample.

Procedure: Pipette into test tube

Sample	***20µl***
Reagent	**1000µl**

Mixed well and allowed to stand for 1min at 37^0C. Measure absorbance decrease per min during 3 min (pA/min)

Calculation:

pA/min. x 1746 = U/l ALT

4.9.2. Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT)

In vitro quantitative determination of activity of SGOT in serum was done using enzymatic kit (Lab care Diagnostics (India) Pvt.Ltd)

Principle:

Aspartate transaminase (SOT_AST) catalyses the reaction between alpha ketoglutaric acid and L-aspatrate giving glutamate and oxaloacetate. Oxaloacetate, in the presence of malatedehydrogenase (MDH) reacts with NADPH giving malate and NAD. The rate of NADPH decrease is determined photometrically and is directly proportional to the GOT activity in the sample.

Procedure: Pipette into test tube

Sample	**100µl**
Reagent	**1000µl**

Mix well and let stand for 1min at 37^0C. Measure absorbance decrease per min during 3 min (pA/min)

Calculation:

pA/min. x 1746 = U/l AST

4.9.3 Estimation of serum Alkaline Phosphatase level (ALP)

In vitro quantitative determination of activity of ALP in serum was done using enzymatic kit(Lab care Diagnoostics (India) Pvt.Ltd)

Principle:

p-Nitrophenyl Phosphateis converted to p-nitrophenol and phosphate by alkaline phosphatase. The increase of absorption at 405 nm is proportional to the alkaline Phospahtase concentration in the sample.

Procedure: Pipette into test tube

Sample	**20µl**
Reagent	**1000µl**

Mix well and let stand for 1min at 37^0C. Measure absorbance increase every 30 sec for 2 min determine (pA/min)

Calculation:

pA/min. x 2720 = U/l ALP

4.9.4 Preparation of the tissue for enzyme assay

Liver, kept in cold conditions (precooled in inverted petridish on ice) was removed. It was cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for other enzymes assays.

4.9. E.1. **Superoxide dismutase (SOD)**

SOD was estimated by the method of Mishra and Fridovich, 1972.

Principle:

Rate of auto oxidation of epinephrine & the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8 – 10.2, O_2, generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced. The auto oxidation of epinephrine proceeds by least two distinct pathways only one of which is free radical chain reaction involving O_2 and hence inhabitable by SOD.

Reagent:

1. Carbonate buffer (0.05 M pH 10.2):
   16.8 gm of $NaCO_3$ was dissolved in 500 ml of distill water & the final volume was made up to 1000 ml with distill water.
2. EDTA 0.49 M:
   1.82 gm of EDTA was dissolved in 1000 ml of distill water.
3. Epinephrine (3 mM):
   0.99 gm of epinephrine bitartarate was dissolved in 1000 ml of distill water.
4. SOD standard:
   Dissolve 1 mg (1000 units /mg) of SOD from bovine liver in 100 ml of carbonate buffer.

Procedure:

All the reagents required were kept in cold condition to maitain the reaction condition.

Blank	Test
0.1 ml DW	0.1 ml sample
0.1 ml EDTA	0.1 ml EDTA
0.5 ml carbonate buffer	0.5 ml carbonate buffer
1 ml of epinephrine	1 ml of epinephrine
Absorbance at 480 nm for 3 minat 30 sec interval	

The reaction was initiated by the addition of epinephrine and the change in optical density / min. was measured at 480 nm, readings were taken for 3 min with 30 second interval.

Calculation:

$$SOD = (0.025 - \mathbf{Y}) \div (Y \times 50) \times 100$$

$\mathbf{Y}$ = Final reading – Initial reading

Units = Units/ mg of protein.

4.9. E.2. **Catalase:**

Catalase was estimated by the method of Aebi et al, 1987.

Principle:

In the ultra-violet range H_2O_2 shows a continuous increase in absorption with decreasing wavelength. The décor position of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in the absorbance per unit time is a measure of the catalase activity.

Reagent:

1. Phosphate buffer (50 m mol/L pH 7)

   a) 6.81 gm of KH_2PO_4 dissolved in distill water and make up volume to1000 ml with distill water.

   b) 8.9 gm of Na_2HPO_4 dissolved in distill water and make up volume to 1000 ml with distill water.

2. Hydrogen peroxide (30 n mol/L)

Procedure:

Blank	**Test**
2910 µL of phosphate buffer pH 7	2910 µL of phosphate buffer pH 7
50 µL of Distilled water	5µ uL of Homogenate.
40 µL of hydrogen peroxide solution	40 µL of hydrogen peroxide solution

Add H_2O_2 just before taking OD at 240 nm; readings were taken for 3 min. with 15 second interval.

Calculation:

Log $A_1/A_2 \times 229.7$ (factor) A_1= initial Absorbance, A_2= final Absorbance.

Unit = units / mg of protein.

4.9. E.3. **Reduced glutathione:**

Reduced of glutathione (GSH) was estimated by the method of Moran et. al., 1979.

Principle:

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimerically at 412 nm.

Reagents:

1. Trichloroacetic acid (10%):
10 gm of TCA was dissolved in 100 ml of distill water.

2. Dithiobis nitro benzoic acid (DTNB) :
40 gm of DTNB was dissolved in1% Sodium citrate solution.

3. Phosphate buffer (0.2 M, pH 8.0) :
1.36 gm of KH_2PO_4 was dissolved in 100 ml of distill water and dissolve in 0.8 gm NaOH in 100 ml distill water.

4. Reduced of glutathione standard :
10 gm of GSH standard was dissolved in 100 ml of distill water (100µg/ml).

Procedure:

Blank	**Test**
1 ml of D.W.	1 ml of Homogenate
1 ml of TCA (10%)	1 ml of TCA (10%)
Cool for 10 min and centrifuged at 2000 rpm take 0.5 ml of supernatant	
0.5 ml of above supernatent	0.5 ml of above supernatent
2 ml sodium hydrogen phosphate	2 ml sodium hydrogen phosphate
0.25 ml DTNB	0.25 ml DTNB

Mixed well and allowed to stand for at room temprature, readings were taken against blank at 412 nm using spectrophotometer.

Calculation:

$Y = 0.0002X + 0.0049$

X = Conc. of reduced of glutathione

Y = Abs of test sample.

Units: µg of GSH / mg of protein.

4.9. E.4. **Lipid peroxidation:**

Malondialdehyde formation (MDA) was estimated by the method of Ohkawa et al., 1979.

Principle:

The method estimates malondialdehyde (MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Reagents:

1. Thiobarbituric acid (1% in Tris hydrochloride, pH 7):
   1 gm of thiobarbituric acid was dissolved in 100 ml of Tris hydrochloride buffer.
2. Trichloroacetic acid (10%):
   10 gm of trichloroacetic acid was dissolved in distilled water.
3. SLS (8%)
   8 gm of SLS in 100 ml of water.

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
0.2 ml of SDS	0.2 ml of SDS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^0 C and cool	
2ml mixture + 2 ml TCA	2ml mixture + 2 ml TCA
Centrifuge on 1000 rpm for 5 min	
Pink color measure at 532 nm	

A = a * b * c

A = abs.

a = mol. Extinction coefficient ($1.56 * 10^5$ cm^{-1})

b = Path length (1 cm^2)

c = conc. of sample

Units: nm of MDA / gm of tissue.

4.10. Statistical significance

All the values are expressed as mean ± S.E.M. statistics was applied using SPSS software version 16.0. Statistical significance between normal control and induced control group was tested using student's t- test. Differences were considered to be statitically significant at $P < 0.05$

4.11. High Performance Thin Layer Chromatography (HPTLC):

System:

- Camag Linomat 5
- Semiautomatic application, band application by spray on technique (2 - 500µl)
- Camag twin trough glass chamber (10 x 10 and 20 x 10)
- Camag TLC scanner 3
- Scanning speed upto 100mm/s, Spectral range 190 – 800nm
- Camag Reprostar 3 with digital camera
- Camag UV cabinet with dual wavelength UV lamp
- Dual wavelength 254 / 366nm
- Stationary Phase: Silica gel G60 F_{254} coated on aluminum sheet.
- Hamilton 100µl HPTLC syringe.

The analysis was performed with HPTLC (Camag, Switzerland). The sample extracts were applied with the linomat III applicator on the HPTLC silica gel 60 F_{254} plates (E. Merck, Germany). The plates were developed with a twin-trough developing chamber. After development, the plates were scanned with a Camag TLC scanner 3, and the data were processed with WINCATS software.

Test Sample: ethanolic solution of sample 1, 2, 3, 4, 5; hexane solution of sample 6 and petroleum ether solution of sample 7 were prepared.

Solvent system1: toluene: ethyl acetate

1 : 1

Solvent system1: chloroform: acetone: acetic acid

3 : 1 : 0.2

Sample preparation: all dried extracts and phenolics were dissolved in methanol

Densitometer Scan: 254 nm, 366nm and 500nm

Note:

Sample1- 10PRGSRAhiMeOH01- crude methanolic extract

Sample2- 10PRGSRAhiMeOH02- precipitated phenolics from methanolic crude extract,

Sample3- 10PRGSRAhiMeOH03- water soluble phenolics from crude methanolic extract

Sample4- 10PRGSRAhiEtAc01- crude ethyl acetate extact,

Sample5- 10PRGSRAhiEtAc02- precipitated phenolics from EtAc crude extract,

Sample6- 10PRGSRAhiEtAc03- alkaline insoluble fraction of EtAc crude extract,

Sample7- Petroleum ether extract.

6. DISCUSSION

The liver is largest organ in the body is being evolved to maintain the body's internal milieu and also protect itself from the challenges it faces during its functioning. The human beings are exposed to these compounds via environmental exposure, contaminated food or during exposure to chemical in the occupational environment (AbdulRahman *et al)*. Liver diseases such as cirrhosis, fatty liver and chronic hepatitis are important world health issues (Balderas-Renteria I, *et al)*. Drug induced hepatotoxicity is one of major concern which limits the therapy and use of drugs. About 2% of all causes of jaundice in hospitalized patients are drug induced and a quarter of cases of fulminant hepatic failure are thought to be drug related. (Ingawale D *et al*).

Plants have been the basis of many traditional medicines throughout the world for thousands of years and continue to provide new remedies to mankind. Numerous medicinal plants and their formulations are used for liver disorders in ethno medical practices as well as in traditional systems of medicine in India.

Hemidesmus indicus *R. Br* (Asclepiadaceae), commonly known as 'Anantamul' or 'Indian Sarasparilla', is one of such drug in Ayurvedic system of medicine. Recently various studies have reported to validate the claim of drug *H. indicus* as a potent hepatoprotective agent. (Nadana Saravanan, et al, 2007, Rao G.M, et al, 2005, Baheti J.R., 2005). Further, most of the reports for characterization of phytoconstitutents showed the use non polar solvents ie. Petroleum ether, Benzene and Hexane fractions (Das *et al.* 1992, Gupta, et al, 1981, Darekar RS, et al, 2009). Thus we have noticed this ambiguity the between extract used for the pharmacological activity and extracts used for phytochemical work were different.

Pharmacognostical evaluation

Roots occur in pieces, about 3-5 cm long and 5-10 mm in diameter, cylindrical, thick, hard, somewhat tortuous, sparcely branched, provided with few thick rootlets and secondary roots, externally dark brown, sometimes with centre yellow, woody, surrounded by a mealy white cortical layer. Bark was brownish, corky, marked with transverse cracks and longitudinal fissures and easily detachable from the hard central core. Roots posseses characteristic odour, sweetish, slightly acrid and aromatic taste.

Transverse section of root showed periderm consisting of three layers of tissues viz. cork, cork cambium and secondary cortex. Cork cells were radially flattened and rectangular in appearance, filled with dark brown matter. Cork cambium was indentified as 2- 3 layered, compressed in shapr and filled with deep brown contents followed by secondary cortex, 3-4 layers of cells, similar to cork cells, with very little or no dark brown contents. Secondary phloem found to be consists of sieve elements, parenchyma, phloem ray cells along with several laticiferous ducts. Parenchyma cells were filled with starch grains, occasional prismatic crystals of calcium oxalate, and scaterred laticiferous ducts. Lignified vessels and tracheids were characterised by the presence of pitted markings. Pith was absent and central region was occupied by woody tissues. All these characters were found similar to that of metioned in the literature. In addition to the literature reports, the roots also showed presence rhomboidal calcium oxalate cryatals.

Physical evaluation

In our study powdered was subjected for physicochemical evaluation such as ash values, extractive values and LOD. Results of our study were found to be comparable to that of reported value.

Phytochemical evaluation

Roots of *H.indicus* has been found to be rich in flavanoid, lignans, essential oil, tannins saponins and sterols (Satyavati et al., 1987; Das et al., 1992; Alam et al., 1994; Roy et al., 2001). In preliminary phytochemical screening all the crude extract (pet. Ether, ethyl acetate,methanolic and aqueous extract) and isolated phenolics showed presence of sterols, flavanoids, sugar, tannins and phenolics. Petroleum ether extract showed presence essential oils. All the extracts and isolated phenolics showed absence of alkaloids, reducing sugar and saponins.

Antioxidant activity of different extracts of roots of *H. indicus*

In healthy organisms, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is approximately balanced by antioxidant defence systems. However, an organism can be suffering from so-called ‘oxidative stress’ while it is experiencing disturbance in the prooxidant–antioxidant balance in favour of the former, leading to potential damage (Halliwell & Gutteridge,1999).

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical because of its unpaired electron delocalization over the whole molecule. The delocalization causes a deep violet color with substrate at 517 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable nonradical form of DPPH is obtained with simultaneous change of the violet color to pale yellow (M. R. Szabo *et al* 2007). The various extract and isolated phenolics i.e EtAc 01, MeOH01 and Aq01 showed a concentration-dependent antiradical activity by inhibiting DPPH radical with an IC_{50} value of 174.29µg/ml, 27.40µg/ml and 191.90 µg/ml respectively. They were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (M. R. Szabo *et al* 2007). It appears that they possesses hydrogen donating capabilities and acts as an antioxidant. However MeOH01 was found to be more active than other extracts.

The extracts also showed a moderate nitric oxide-scavenging activity in a dose dependent manner with an IC_{50} 385.65 µg/ml and 378.02 µg/ml for EtAc 01, MeOH01 respectively while for Aq01 it was insignificant. The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (A. Kumaran *et al*, 2007). The EtAc01 and MeOH01 may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation. Further, the scavenging activity of above extracts may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

Free radicals and oxidants can trigger lipid peroxidation in the liver tissue treated with $FeCl_2$-ascorbic acid, as well as oxidize proteins and DNA, causing extensive damage to body cells. Products of lipoperoxidation such as malondialdehyde have been reported to react with nucleic acids (Brooks & Klamerth, 1968) and to be mutagenic. There was significant decline in lipid peroxide content in the liver tissue treated with different extracts of roots of *H. indicus*.Various concentration of extracts had shown dose dependant inhibhition of lipid peoxidation with an IC_{50} value of 478.89 µg/ml, 80.30 µg/ml and 476.75 µg/ml respectively for EtAc 01, MeOH01 and Aq01. However MeOH01 (crude MeOH extract) was found to be the most active extract.

Fig. 2.4. and table 2.4. showed the reductive capabilities of various extracts of *H. indicus* ascorbic acid as srandard. For the measurements of the reductive ability, the Fe^{3+} - Fe^{2+} transformation in the presence of various extracts was investigated, using the method of Oyaizu (1986). Earlier authors (Pin-Der-Duh, 1998; Pin-Der-Duh, Pin-Chan-Du, & Gow-Chin Yen, 1999; Tanaka, Kuie, Nagashima, & Taguchi, 1988) have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and also by reacting with certain precursors of peroxides (Gordon, 1990). Our data on the reducing power of EtAc 01, MeOH01, and Aq01 suggested that it is likely to contribute significantly towards the observed antioxidant effect. Like the antioxidant activity, the reducing power of extracts of *H. indicus* increased increasing the dose. However, the reducing power of ascorbic acid was relatively more pronounced than that of Aq01 while EtAc 01, MeOH01 were comparable to ascorbic acid.

The two oxidation states of iron, Fe^{2+} and Fe^{3+} donate or accept electrons through redox reactions that are significant for biological reactions, but they also may be harmful to cells (Yamaji Y *et al*, 2004). In excess, iron helps superoxide anion ($O^{\bullet 2-}$) and hydrogen peroxide to convert into the extremely reactive hydroxyl radical ($OH^{\bullet}$) (Haber–Weiss reaction) that cause severe injury to membranes, proteins and DNA. It decomposes lipid hydro-peroxides into peroxyl and alkoxyl radicals responsible for the chain reaction of lipid peroxidation (Chang LW *et al*, 2002). The

results from Fig.2.5 and Table 2.5 suggested that the decrease in the colour formation with ferrozine in presence of extract, in dose dependant manner, indicated its iron chelating property. However, compared to standard EDTA, they showed less pronounced activity.

Phenolics and flavanoids may be directly related to antioxidant potential and hence to hepatoprotective activity (E. Middleton Jr, 2000). Flavonoids show their antioxidative action through scavenging or chelating process. Phenolic content is also very important plant constituent because of their scavenging ability due to their hydroxyl groups. Both of these compounds have good antioxidant potential and their effects on human nutrition and health are significant. Total phenolics was determined by F-C reagent by method mentioned by Singleton and Rossii and flavanoid content was measured by method given by Jia J et al. It was found that roots of *H. indicus* contains a significant amount of phenolics and flavanoid. Total phenolics content was found to be maximum in MeOH01 while maximum amount of flavanoid was found to be in EtAC01 suggesting the wide of range of phenolics other than flavanoids were present in MeOH01. The colour of MeOH01 suggests the presence of phlobatannins.

Evaluation of *In Vitro* Hepatoprotective Activity

Carbon tetrachloride, a well-documented liver toxic chemical, is converted to trichloro free radical and trichloro peroxide by the cytochrome P450 system in liver cells. While the trichloro peroxides further involve lipid peroxidation, the trichloro radicals will bind covalently to protein and lipids. Thus, the overall toxic effects of carbon tetrachloride on liver cells include cell membrane malfunctions, mitochondrial swelling and cell death. In preliminary studies, the protective effect of extracts on hepatocytes treated with CCl_4 was analysed.. It was found that cellular damage (assessed by trypan blue exclusion assay) was decreased with increased concentration of extracts. From the data obtained we can conclude that all the extracts have hepatoprotective action against CCl_4 induced toxicity. However MeOH01 (crude MeOH extract) was most active followed by EtAc 01, however Aq01 was found least active.

HPTLC analysis of different extracts and isolated phenolics of *H. indicus*

HTLC fingerprinting for various extract and isolated phenolics was developed. As shown in Fig. 3.4 (solvent system1), track 4 (EtAc01) showed good seperation with 14 spots resolved at different Rf values although furthur amelioration is required for more accuracy. On observing it was found that all tracks except track 3 (MeOH03), showed four spots resolved at almost similar Rf (around .86, .74, .65, .62) indicating possible similarity in their nature. Spot with Rf value around 0.95 was very prominent in all crude extract (track 1, 4, 7), indicates presence of its in higher amount and its solubility in wide range of solvents. Now if we compare track 1, 2 and 3 ((crude MeOH extract and isolated phenolics), there were six common spots in trak 1(MeOH01) and 2 (MeOH02) while only one spot was common in track 3 (MeOH03), suggesting most of compound were of higher molecular weight and came in MeOH02 from parent extract (MeOH01). Similarly on comparision of track 4, 5 and 6 it can be concluded that higher no of compounds seperated in precipitated phenolics (EtAc02) from crude ethyl acetate extract (EtAc01).

As shown if Fig.3.5 (solvent system 2), on derivatization with methanolic $FeCl_3$ very few spots were seen. There was no significant inference drawn from that, so require further developmet of an apt solvent system for resolution of tannins.

PARACETAMOL INDUCED HEPATOTOXICITY

In the present study PCM (Acetaminophen), which is widely used drug and has hepatotoxic potential, was employed as hepatotoxicant to evaluate hepatoprotective effect of roots of *Hemidesmus indicus*. Administration of single dose of acetaminophen (3g/kg) exhibited a significant elevation of serum enzyme levels like SGOT, SGPT and ALP as compared to normal control. The paracetamol (Acetaminophen) a widely used antipyretic-analgesic drug which produces acute hepatic damage on overdosage. A fraction of acetaminophen is converted via CYP450 pathway to a highly toxic metabolite; N–acetyl–p– benzoquinamine (NAPQI) (Dahlin D *et al*) which is normally conjugated with glutathione and excreted in urine. Overdose of acetaminophen depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction (Parmar D *et al*) and the development of acute hepatic necrosis. The elevated serum enzyme levels are indicative of cellular leakage and loss of functional integrity of liver cell membrane (Drotman R *et al*).

There was a significant reduction of these raised enzyme levels on pre and post-treatment with extracts and isolated phenolics of *H. indicus* and silymarin, suggesting the protection of structural integrity of cell membrane or regeneration of damaged hepatic cells. Table 5.1 showed that MeOH01, MeOH02 are highly active followed by EtAc02, EtAc01, EtAc03 and MeOH03. This reveals that higher content of phlobatannins present in MeOH may be responsible for its higher activity. The reversal of increased serum enzyme levels by extracts and isolated phenolics in acetaminophen induced liver damage may be due to the prevention of the intracellular enzymes leakage by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases such as SGOT and ALP return nearer to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew M *et al)*. The CYP450 2E1 have been suggested to be primary enzymes for acetaminophen bioactivation in liver microsomes (Raucy JL *et al*). Studies demonstrated that acetaminophen induced hepatotoxicity can be modulated by substances that influence CYP450 activity (Mitchell J R *et al*).

Our study also reveal that paracetamol significantly decreases the antioxidant levels and increases pro-oxidant levels in diseased rats compared to control rats. Table 5.2 shows that MeOH01, MeOH02 are highly active followed by EtAc02, EtAc01 however EtAc03 and MeOH03 were not significantly active. The possible mechanism behind this is the involvement of reactive oxygen species. The reactive oxygen species (ROS) such as superoxide anion radical (O_2•-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) have been implicated in the pathophysiology of various clinical disorders, including hepatotoxicity (Hemnani, and Parihar, 1998). They play an important role in the inflammation process after intoxication carbon tetrachloride or carrageenan and paracetamol (Yoshikawa, *et al*., 1983; Halliwell, and Gutteridge, 1984; Yuda, *et al*., 1991). It is well reported that these free radicals and the reactive species derived from them cause damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury (Brattin, et al., 1985). The various in vitro studies showed that certain flavanoids and other phenolics are the potent inhibitors of the oxidative stress. Phytochemical profile of individual extacts and isolated phenolics suggests that the presence of phenolic may account for the above mentioned activities.

Histopathology also supported our present findings, that MeOH01, MeOH02 were most beneficial in paracetamol induced cell necrosis and cell death followed by EtAc01 and EtAc02. However EtAc03 and MeOH03 were not found to be signicantly effective.

7. Summary and Conclusion

In present investigation we found that among various extracts and isolated phenolics examined for hepatoprotective potential, MeOH01, MeOH02 were most active fraction followed by EtAC01 and EtAc02 as they had significantly decreased serum enzymes such as SGOT, SGPT and ALP levels. They also showed significantly increase in various antioxidants levels (Glutathione and Catalase) and decrease in pro-oxidant levels (MDA). However effects produced MeOH03 and EtAc03 were not significant. The improvement in enzyme levels, antioxidant and pro-oxidant levels indicates its beneficial effect on paracetamol induced hepatotoxicity.

The phenolics precipitated (MeOH02) from MeOH01 were preliminary identified as tannins, while precipitated phenolics (EtAc02) from EtAc01 were preliminary identified as lower phenolics like flavolignans, flavanoids etc.

Further in our study we received maximum activity from MeOH02 as it was given 150mg/kg body weight compared to crude methaolic extract (MeOH01) 250 mg/kg body weight. This revealed clearly that tannins of *H. indicus* have potent hepatoprotective activity comapared to all other groups of constituent.

Furthur a strong cornerstone is provided to the hepatoprotective effect of extract by *in-vitro* antioxidant and *in- vitro* hepatoprotective activity. Results of *in-vitro* antioxidant activity showed the free radical scavanging, lipid peroxide inhibition and nitric oxide scavanging activity of various extract. The % viability of isolated hepatocytes was also increased by administration of extracts of roots of *H. indicus.*

Preliminary phytochemical revealed the presence of flavanoids, tannins, carbohydrate, coumaro-lignans and sterols. Alkaloids were found to be absent. Furthur literature has already documented the antioxidant and hepatoprotective value of phenolics. Thus the hepatoprotective value of roots of *H. indicus* may be related to its phenolic contents.

In, conclusion our study apparently validate the folk medicinal use of *H.indicus* against hepatotoxicity. Furthur studies in progress for isolation and charaterization of phytoconstituents may lead to development of lead nucleus for hepatic dysfunction.

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