

**Evaluation of the spasmolytic activity and safety of  
*Thymus serrulatus* in laboratory animals**

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## LIST OF ABBREVIATIONS

AIDS	Acquired immuno-deficiency syndrome
ALP	Alkaline phosphatase
ALT	Alanine amino transferase
AST	Aspartate amino transferase
CAM	Complementary and alternative medicine
CD	Cluster of differentiation (T-lymphocytes)
Co-A	Co-enzyme-A
CYP-450	Cytochrome P-450
Cyt-c	Cytochrome c
FDA	Food and drug administration.
GSH	Glutathione
Hsp	Heat shock protein
IMM	Inner mitochondrial membrane
iNOS	Inducible nitric oxide synthase
LD <sub>50</sub>	Lethal dose in 50 % of animals
LKM	Liver Kidney Microsomes
mtDNA	Mitochondrial DNA
mtGSH	Mitochondrial GSH
MPT	Mitochondrial permeability transition
NAPQI	N-acetyl-p-benzoquinone imine
NAT	N-acetyl transferase
NO	Nitric oxide
NO <sub>2</sub>	Nitrite
OMM	Outer mitochondrial membrane
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TGF- $\beta$ 1,	Transforming growth factor-beta 1
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TPN	Total parenteral nutrition
UDPGA	Uridine diphosphate glucuronic acid
UDPGT	Uridine diphosphate glucuronyl transferase

VOD	Veno-occlusive disease
XIH	Xenobiotic-induced hepatotoxicity

## ABSTRACT

Thymus (Labiatae) is one of the herbs used in the traditional medicine practice in Ethiopia. The claimed actions of thymus are carminative, anti-microbial, anti-spasmodic, relaxing expectorant, astringent, anthelmintic and anti-oxidative. But some people claim and have concern, that thymus causes problems to liver in herbivorous animals that graze on it and human beings who ingest it for different purposes.

The aim of the present study was to evaluate the claimed spasmolytic effect and safety of *Thymus serrulatus* on liver of rats.

Experimental study was done in laboratory animals. Charcoal meal test was used to evaluate its spasmolytic effect on mice. Acute toxicity was evaluated by determining LD<sub>50</sub> of the plant extracts on mice. Subchronic toxicity was done on rats. Rats were divided into eight groups of five animals each. Four groups were female and the other four were male. Three different doses, 0.2 gm/kg, 1 gm/kg and 2 gm/kg of the aqueous extract were given to the six treatment groups via oral gavage for 90 days. During the study period the animals were observed for any sign of liver toxicity and their body weight was measured weekly. At the end of the study period clinical chemistry parameters, gross morphology and histopathology of the liver were evaluated.

The results showed the presence of spasmolytic effect, which is statistically significant difference ( $P < 0.05$ ) for both plant extracts when compared to the negative controls. The 200 mg/kg and 400 mg/kg doses of the methanolic extract have shown more effect than the positive control. No statistically significant difference was observed between the different doses of the aqueous extract and 100 mg/kg dose of the methanolic extract when compared with atropine 10 mg/kg, p.o., which is used as positive control ( $P > 0.05$ ). The oral LD<sub>50</sub> for the extract was seen to be higher than 5 gm/kg for both aqueous and methanolic extracts, which is indicative of the plants safety in case of acute high dose exposure. Cage side observation in subchronic toxicity study revealed no sign of liver toxicity. No statistically significant difference ( $P > 0.05$ ) was seen in the clinical chemistry results between the different groups. However, the histologic evaluation revealed cholestatic change of the liver in most of the animals in the treatment group. This may be explained by the presence of phytosterol compounds, in the extract given, which are known to cause cholestasis. Cholestasis was not accompanied by necrosis/degeneration, which could be due to the anti-oxidant and hence protective effect of the active ingredient thymol. In conclusion *T. serrulatus* has spasmolytic activity and is safe for oral use.

Key words: *T. serrulatus*, toxicity, laboratory animals, extracts.

# **1. Introduction**

## **1.1. Herbal medicine**

### **1.1.1. Historical overview of herbal medicine use**

Early humans used to depend on nature to get relieved from their ailments. Led by instinct, taste and experience, primitive people used plants, animal parts and minerals to treat illnesses. Physical evidence of use of herbal remedies goes back some 60,000 years to a burial site of a Neanderthal man uncovered in 1960 (Solecki, 1975). In a cave in northern Iraq, scientists found what appeared to be ordinary human bones. An analysis of the soil around the bones revealed extraordinary quantities of plant pollen that could not have been introduced accidentally at burial site (Bensky and Gamble, 1993).

All cultures have long folk medicine histories that include the use of plants. Even in ancient cultures, people methodically and scientifically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20<sup>th</sup> century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs including strychnine, aspirin, vincristine, taxol, curare and ergot are of herbal origin (Farnsworth, 1976).

In the Middle East the history of traditional medicine extends to 2000 B.C. when there was compilation of the first known ancient pharmacopoeia containing 250 herbal drugs in Sumeria. The Ebers Papyrus, the most important of the preserved Egyptian manuscripts, was written around 1500 B.C. and includes much earlier information (Ackerknecht, 1973).

In Greece and Rome there were many compilations of *materia medica* by many writers, but none was as important or influential as that written by Dioscorides in the 1st century A.D. This text contains 950 curative substances, of which 600 are plant products and the rest are of animal or mineral origin. China is known for its rich history of traditional medicine use. The earliest written evidence of the medicinal use of herbs in China consists of a corpus of 11 medical works recovered from a burial site in Hunan province. The burial itself is dated 168 B.C., and the texts (written on silk) appear to have been composed before the end of the 3rd century B.C. Some of the texts discuss exercise, diet, and channel therapy. The largest, clearest, and most important of these manuscripts, called by its discoverers 'Prescriptions for Fifty-Two Ailments', is predominantly a pharmacological work. More than 250 medicinal substances are named. Most are substances derived from herbs (Ackerknecht, 1973).

The traditional healing knowledge is usually passed on verbally from one generation to another; however, traditional medicine has been practiced in Africa for centuries, as in the other parts of the world. Plants have been used for medicinal purpose for years. Even at the present time herbal medicines are the staple of medical treatment in many

developing countries including Africa. They are used for virtually all-minor ailments. Visits to Western-trained doctors or prescription pharmacists are reserved for life threatening or hard-to-treat disorders (Adewunmi and Ojewole, 2004).

In Ethiopia, the antiquity of traditional medicine could not be established with certainty due to lack of adequate historical sources. However, it is mentioned that traditional medicine has long been practiced in Ethiopia (Pankhurst, 1965). The majority of the population relies on traditional medicine for various problems associated with health and most of the traditional medicines constitute preparations from herbs. It is not unusual for Ethiopians to treat common ailments using plants available around them (Mirutse, 2001). Fresh and dried leaves, flowers, roots, bark, seeds, etc of medicinal plants are displayed in most markets in Ethiopia along with spices such as pepper, cardamom, ginger, etc.

### **1.1.2. Prevalence of herbal medicine use.**

The world health organization estimates that four billion or 80% of the world's population presently use herbal medicine for some aspects of primary health care (Farnsworth, *et al.*, 1985).

The trend towards the use of traditional or complementary medicine is getting attention these days and the use even in the Western countries has increased. In the United States consumers spend over \$34 billion per year on Complementary and alternative medicines (CAM) therapies, dollars spent outside the conventional health care financing system. Such evidence on out-of-pocket expenditures is a testament to the widely held belief that CAM therapies have benefits that outweigh their costs (Patricia, *et al.*, 2005).

The introduction of chemistry, the development of numerous synthetic drugs, and the possibilities opened up by experimental pharmacology has caused herbal knowledge to be neglected. A new climate was created by methods evolved on the basis of modern physiology, concerned predominantly with effects that were measurable under experimental conditions. In this framework it was difficult to know what to do with medicinal plants. Chemical compounds could always be exactly analyzed giving results in parts by weight of so and so many milligrams, etc (Weiss, 1991).

Despite FDA's skepticism about herbal remedies, a growing number of Americans are again getting interested in herbal preparations. The number of CAM users rose from 34% to 42% from 1990 to 1997 (Boon *et al.*, 2004). This surge in interest is fueled by factors like:

- Availability of traditional herbal medicines, from different societies and cultures, in most U.S. health food stores.

- An increasingly over prescription, cost of medicines, and even dangerousness of the side effects associated with modern drugs.
- Exposure to exotic foreign foods prepared with non-European culinary herbs has led many Euroethnic Americans to examine and often consider using medicinal herbs that were brought to the United States along with ethnic culinary herbs.
- People increasingly are willing to "self-doctor" their medical needs by investigating and using herbs and herbal preparations. Many Americans, especially those with chronic illnesses such as arthritis, diabetes, cancer, and AIDS--are turning to herbs as adjuncts to other treatments (Cuellar, *et al.*, 2003; Kuo. *et al.*, 2004).

Of all the organs in human body the liver is the most commonly affected organ when a chemical or drug is introduced into the body. This is because of its unique function in processing the chemicals from the blood stream by changing them into products that can be readily removed through the bile or urine (Quinn and Johnston, 1997).

## **1.2. Thymus (Labiatae)**

Thymus, commonly known as garden thyme, is one of the traditional herbal medicines that are commonly used in Ethiopia and all over the world (Junichi *et al.*, 2004). The genus Thymus (Labiatae) includes about 350 species worldwide and is widely distributed in temperate zones. Two species, *T. schimperi* and *T. serrulatus*, are indigenous to Ethiopia while *T. vulgaris* has been recently introduced. *T. vulgaris* is widely cultivated as a potherb in many tropical and sub-tropical parts of the world (Demissew, 1993).

Though the proportion varies between seasons, the main components of the essential oils in *T. vulgaris* are the phenol thymol (30-70%) and carvacrol (3-15%). The other components are thymol methyl ether, cineol, cymen, alpha-pinen, borneol and esters of the latter two (Junichi *et al.*, 2004; Hudaib *et al.*, 2002). And the claimed actions of thymus are carminative, anti-microbial, anti-spasmodic, relaxing expectorant, astringent, anthelmintic and anti-oxidative (Junichi *et al.*, 2004).

Different studies regarding the claimed pharmacological effects have been done. A study done to see antibacterial effect of *T. vulgaris* on 39 species of hemolytic *E. choli* isolates using disk diffusion method showed remarkable effect (Jugl-Chizzola *et al.*, 2005). A study on the anti-spasmodic action of *T. vulgaris* to spasms induced by different spasmogens on isolated trachea has shown the antagonizing action of this herb in a concentration dependent manner (Meister *et al.*, 1999). A study done on rats to show causation of *T. vulgaris* leaves on enterohepatonephropathy over six weeks period showed that 2% or 10 % *T. vulgaris* leaves are not toxic to rats (Haroun, *et al.*, 2002). One study done to see the effect of *T. vulgaris* leaves and its phenolic compounds, thymol

and carvacrol, on the activities of xenobiotic metabolizing enzymes, i.e., phase I enzymes such as 7-ethoxycoumarin O-deethylase and phase II enzymes, such as glutathione S-transferase and quinone reductase, on mice showed that it is an inducer for the stated enzymes (Sasaki *et al.*, 2005). The effect of constituents of *T. vulgaris*, thymol, carvacrol and gamma-terpinene, on genotoxicity was studied on human lymphocytes by evaluating the DNA damage it causes. The result showed that at higher concentrations all the three constituents cause significant damage (Aydin *et al.*, 2005). On the other hand one study demonstrated the hepatoprotective effect of thymol to carbon tetrachloride induced hepatotoxicity in mice, which was seen *in vitro* test to be due to inhibition of lipid peroxidation (Alam *et al.*, 1999).

Of the endemic thymus Spp. in Ethiopia, *T. schimperi* grows on a hillside in brown soil. The plant from Dinshu (Bale province) was found to contain thymol (50%), gamma-terpine, carvacrol and p-cymene as the major constituents, where as carvacrol (66.2%) and gamma-terpine (13.2%) are dominant in the same species obtained from Addis Ababa (Dagne *et al.*, 1998).

The other endemic species is *T. serrulatus*, which grows in mountainous area, with an altitude of 2800-3600 m, on rocky outcrops on slopes near roadside. It is a branched prostrate perennial herb, woody at the base, 15-30 cm long (Demissew, 1993). The oil from *T. serrulatus* (collected from Tigray region) was found to contain p-cymene (13%), gamma-terpinene (13%) and thymol (49%) as major components (Asfaw *et al.*, 2000). It has been shown to have taenicidal action being second among 33 taenicidal herbs that are compared in one study. The LD<sub>50</sub> determined for the aqueous and hydroalcoholic extracts after intraperitoneal administration showed 4682 +/- 406 mg/kg and 4876 +/- 308 mg/kg, respectively (Belachew, 1995).

People in Ethiopia use thymus (known as 'Tosign' in Amharic) for different health related problems, such as hypertension, abdominal cramp, protection of animals against parasitic infections; as additives in foods, and as a spice in flavoring tea. But some people claim and have concern, that thymus causes problems to liver in herbivorous animals that graze on it and human beings who ingest it for different purposes. This idea comes from observation that most slaughtered animals in the Debre Brahan locality, where the plant grows widely, showed damaged liver. On the contrary the veterinarian in the locality attributes the liver damage to the higher prevalence of liver fluke infection among the animals that reside in the locality. On the basis of this claim, the present study is initiated to assess the toxicological and the spasmolytic effects of *T. serrulatus*.

### **1.3. Xenobiotic-induced hepatotoxicity**

Xenobiotic-induced hepatotoxicity (XIH) is an injury to the different liver cells (mainly hepatocytes) by a drug/chemical, which is introduced to the body. It is an important adverse effect and a relatively common reason for termination of the development of a new drug (Olson, *et al.*, 2000). In the last few years, the US Food and Drug Administration (FDA) has withdrawn 2 drugs (bromfenac and troglitazone) from the

market for causing severe liver injury. It denied approval of ximelagatran, a promising oral anticoagulant medicine in September 2004 due to several cases of hepatotoxicity, including three liver injury-associated deaths. Recently, kava kava, an herb used for anxiety, was reported to be hepatotoxic and withdrawn from the German market (Mehta and Ozick, 2005; Kim, 2005).

More than 1,000 drugs have been implicated in drug-induced liver injuries, rendering the treatment of drug-induced hepatotoxicity an important but challenging task for health care professionals. At present, drug induced liver injury accounts for more than 50% of cases of acute liver failure in the United States. Recent data have shown that liver disease represents a larger percentage of adverse drug reactions than previously reported and that the incidence and severity of drug-induced liver injury is underestimated among the general population (Lee, 2003; Kim, 2005). This is due, in part, to the idiosyncratic nature of toxicity in humans and inherent physiological differences between humans and preclinical species leading to limited correct prediction of adverse responses in humans (Dambacha and Andrews, 2005).

Signs and symptoms in XIH depend on the degree and period of exposure, type of the offending drug/chemical and the time of presentation. It includes non-specific symptoms like, anorexia, weakness, fever, rash, pruritis, arthralgia, weight loss or gain, jaundice, right upper quadrant pain and edema. Signs include icterus, tender hepatomegally, and ascites with peripheral edema, petechia and ecchymosis.

### **1.3.1 Herbal medicine-induced Hepatotoxicity**

Herbal remedies, like conventional medications, carry a risk of adverse reactions. There are many factors contributing to the potential toxicity of herbs. These include misidentification of the plant, variability in the time and place of collecting the plant, use of the wrong part of the plant, incorrect storage, and contamination during preparation, and inconsistency in nomenclature and labeling of the final product. Adulterants such as corticosteroids have been added to some preparations. The remedies may have multiple ingredients, creating difficulty in determining the causative agent and possible mechanism of injury. The identification of an herbal remedy as being responsible for hepatotoxicity often depends on demonstrating a temporal relationship between

consumption of the product and development of the illness and improvement after discontinuation, after excluding other causes of liver disease (Peter, *et al.*, 2002).

In general, a key point to keep in mind is that any herb containing pyrrolizidine alkaloids is potentially hepatotoxic. Pyrrolizidine alkaloids have been found in approximately 350 different plant species and some of them are related to induction of hepatotoxicity (Melissa, 2005; Swain, 2000). Other herbs that contain tannin compounds are also known to cause hepatotoxicity. It causes centilobular necrosis in humans. Pathological findings in experimental animals show evidence of gastritis, liver damage (the usual cause of death) and kidney damage (Evans, 1996).

Some examples of plants those cause hepatotoxicities are: *Mentha pulegium* (i.e., pennyroyal oil, "squaw" mint) is a plant from Labiatae family. Teas of this herb have been mistaken for other mint teas and have been used intentionally as abortifacients. These teas contain the hepatotoxin, pulegone, which causes hepatocellular necrosis. Pulegone toxicity can result in multisystem organ failure (Hirshon, 2004).

Kava kava: extracts of kava kava, *Piper methysticum*, were considered to be very safe alternatives to anxiolytic drugs and to possibly exert a wide range of other benefits. But, by January 2003 kava extracts had been banned in the entire European Union and Canada, and were subject to cautions and advisories by the US FDA as a result of 11 cases of hepatic failure leading to liver transplants, including four deaths (Clouatre, 2004). Other common herbs that are suspected to induce hepatotoxicity are shown in table 1.1(Peter, *et al.*, 2002).

Table 1.1.common herbal remedies suspected of causing hepatotoxicity in human

	Botanical name	Potential toxic constituents	Indications	Hepatic disease
Chaparral	<i>Larrea tridentata</i>	Nordihydroguaiaretic acid	Free radical scavenger Delays aging	Hepatitis
Comfrey	<i>Symphytum officinale</i>	Pyrrrolizidine alkaloids	Herbal tea Poultice	Veno-occlusive disease Hepatic adenomas
Germander	<i>Teucrium chamaedrys</i>	Furano neoclerodane Flavonoids	Antipyretic Weight control	Hepatitis
Greater celandine	<i>Chelidonium majus</i>	Unknown	Gallstones Dyspepsia	Hepatitis
Jin Bu Huan	<i>Lycopodium serratum</i>	Unknown	Sedative Analgesic	Hepatitis
Kombucha tea	Kombucha "mushroom"	Unknown	Arthritis Cancer cure	Hepatitis
Mistletoe	<i>Viscum album</i>	Unknown	Antihypertensive Sedative	Hepatitis
Mixtures of valerian and skullcap	<i>Valeriana officinalis</i> <i>Scutellaria lateriflora</i>	Alkylating agents Crystalline glycoside and a volatile oil	Sedative Sedative	Hepatitis Hepatitis
Sassafras	<i>Sassafras albidum</i>	Safrole	Arthritis	Hepatitis Hepatocarcinogen
Senna	<i>Cassia angustifolia</i>	Sennosides	Laxative	Hepatitis
White chameleon	<i>Atractylis gummifera</i>	Potassium atractylate Gummiferin	Antipyretic Purgative	Hepatitis

### **1.3.2. Types of Xenobiotic-induced hepatotoxicity**

According to a classification scheme formulated by the U.S. Public Health Service in 1979 for drug- and chemical- induced liver injury; hepatic lesions are divided into two categories

*Type I lesions (Intrinsic)* are those that are predictable, dose- and time-dependent, occurring in most, if not all, subjects exposed to appropriate doses of the causative substance; the lesions are usually readily reproducible in animals. These injuries, generally, are the result of reactive metabolites of drugs.

*Type II lesions (Idiosyncratic)* are those that are unpredictable, dose-and time-independent, occurring sporadically and often becoming apparent only after monitoring a large number of exposed individuals; the lesions usually are not reproducible in animals. It is usually associated with classical signs of hypersensitivity, including fever or rash, and liver biopsy specimens reveal evidence of monocytic or eosinophilic infiltrates. These reactions tend to occur only after repeated exposure, suggesting the need for initial sensitization, and drug rechallenge generally elicits prompt appearance of symptoms (Sturgill and Lambert, 1997).

### **1.3.3. Factors affecting xenobiotic-induced hepatotoxicity.**

The liver plays a critical role in promoting excretion of lipophilic compounds by transforming them into metabolites of greater water solubility. In so doing these compounds undergo different phases of enzymatic reactions. Variability in the nature of these metabolizing enzymes in different individuals leads to variability in susceptibility to XIH (Weinshilboum, 2003).

#### *1.3.3.1. Variability in phase I enzymatic activity*

Three CYP gene families designated *CYP1*, *CYP2* and *CYP3*; encode the cytochrome P450 enzymes that play major role in human xenobiotic metabolism. Genetic, physiologic, pathophysiologic and xenobiotic-induced factors that affect cytochrome P450 enzyme activity may help account for the increased susceptibility of certain individuals to XIH. As a rule, women are at increased risk of drug-induced liver injuries, particularly chronic ones. Oral contraceptives are known inducers of cytochrome P450

enzyme activity, whereas pregnancy has been shown to induce certain isoenzymes, such as P450III A4, and inhibit others. Cytochrome P450IA2 activity is gender related (with males consistently exhibiting higher enzyme activity). However, parity may be an important determinant of P450IA2 activity. Parous females who lactated appear to exhibit activity similar to that of males. Genetic polymorphism, characterized by poor and extensive metabolizer phenotypes, have been identified in the P450IIC18, P450IID6, P450IIE1 and possibly the P450III A4 isoforms and can alter susceptibility to xenobiotic-induced liver injury (Sturgill and Lambert, 1997).

Many drug-induced liver injuries are clearly age-related. The activity of some cytochrome P450 isoenzymes (such as P450IA2 and P450IID6) is reduced by 70% in neonates, followed by a rapid increase in activity during the first few weeks to months after birth to an amount two- to threefold more (for P450IA2) than that of adults. The activity of other P450 isoforms, e.g., P450III A enzymes, can be higher in newborn infants than in adults, and certain P450III A isoforms are primarily expressed only in the developing fetus (Sturgill and Lambert, 1997).

The classic example in which altered activity of cytochrome P450 isoenzymes can increase the risk of liver injury is acetaminophen toxicity. Ordinarily, greater than 90% of an acetaminophen dose undergoes phase II glucuronidation and sulphation, yielding inactive conjugates that are excreted in urine and bile. About 5% of the dose is oxidized by cytochrome P450IIE1 isoenzymes and to a lesser degree by other P450 isoenzymes, to the hepatotoxic intermediate N-acetyl-p-benzoquinone imine (NAPQI). Hepatocellular damage is ordinarily prevented by phase II conjugation, which converts NAPQI to the inactive metabolite mercapturic acid. Acute ingestion of greater than 10 gm of acetaminophen saturates the normal glucuronidation and sulphation pathways, leading to increased production of NAPQI, which rapidly depletes available glutathion stores. The risk of damage increased, and the threshold dose lowered, with concomitant use of compounds such as alcohol or Phenobarbital that are capable of inducing P450IIE1 activity (Peter *et al.*, 2000).

#### *1.3.3.2 Variability in phase II enzyme activity*

The functional capacity of phase II detoxification pathways is equally important to host susceptibility. The most common type of phase II reaction is glucuronidation, where glucuronic acid is transferred from uridine diphosphate glucuronic acid (UDPGA) to a drug or phase I metabolite by the enzyme uridine diphosphate glucuronyl transferase (UDPGT). The capacity of glucuronidation process can be inhibited by the temporary depletion of the available UDPGA stores by drugs such as acetaminophen and chloramphenicol. Other drugs, including naproxen, ethinyl estradiol and certain benzodiazepins have been shown to directly inhibit UDPGT enzyme activity. Age can also alter UDPGT activity, which is low at birth but increases steadily to nearly adult values by age 1-3 months. Nutritional deficiency is another potential relevant cause of deficient UDPGA stores (Sturgill and Lambert, 1997).

Sulfation reactions are catalyzed by three families of cytosolic sulfotransferase enzymes that represent important detoxification pathways for alcohols and phase I intermediates containing phenol groups. The efficiency of sulfation reaction can be compromised by temporary depletion of inorganic sulfate pools by ingestion of drugs such as salicylamide (Sturgill and Lambert, 1997).

Glutathion conjugation is critical in preventing liver injury from several agents, including acetaminophen and bromobenzene epoxides, by acting as a free radical scavenger. Acetaminophen overdose causes liver injury secondary to the temporary depletion of glutathion stores in the liver. Administration of N-acetylcysteine prevents further injury by stimulating glutathion synthesis, thereby replenishing liver stores. Glutathion stores are also sensitive to fasting and alcohol ingestion and, as in most phase II pathways except sulfation, glutathion conjugating activity is depressed in neonates, even though glutathion transferase enzyme activities are apparently within the normal reference interval (Sturgill and Lambert, 1997).

Amine or hydrazine-containing drugs or phase I metabolites are detoxified primarily by phase II acetylation reactions, catalyzed by cytosolic N-acetyl transferase (NAT)

enzymes. NAT1 and NAT2 represent the two gene families currently known to exist in the human liver. Polymorphism in NAT-2 results in the rapid or slow acetylator phenotype, which has been implicated in host susceptibility to liver damage by drugs such as isoniazid. Isoniazid undergoes extensive NAT-2-catalyzed acetylation to acetylisoniazide, which is then hydroxylated by cytochrome P450 enzymes to the hepatotoxic intermediate acetylhydrazine, a metabolite capable of forming covalent cellular adducts. The risk of liver toxicity is higher in slow acetylators, in the elderly, and is associated with concomitant use of cytochrome P450 inducers such as alcohol or rifampin (Ohno *et al.*, 2000).

#### *1.3.3.3. Co-morbid states*

Certain co-morbid states have also shown to increase the risk of hepatotoxicity from certain drugs. A recent study showed that HIV and HCV infections were shown to increase the risk of anti-tubercular drugs induced hepatotoxicity by four and five fold, respectively, and when there is co-infection the risk increased to fourteen times (Jaime *et al.*, 1998). Diabetes has been shown to potentiate hepatotoxicity of certain compounds, such as thioacetamide, chloroform, trichloroethane, carbon tetrachloride and benzene. A study done on streptozocin induced-diabetic rats showed potentiation of thioacetamide induced hepatotoxicity with increase in the activity of CypIIE1 enzyme when compared to the negative controls (Wang *et al.*, 2000).

#### **1.3.4. Diagnosis of xenobiotic-induced hepatotoxicity**

Diagnosis is made after taking history, doing physical examination, checking for biochemical markers of liver function and most of all ultrasound-guided biopsy for histologic examination. The temporal relation between exposure to the incriminated drug/chemical and the appearance of sign and symptoms of hepatitis is important to the diagnosis. This may not be difficult when a person is taking conventional medicines some of which are already described to cause hepatitis. The problem arises when herbal medicines are the cause. People tend not to disclose the use of herbs when they appear to their physicians making the diagnosis difficult. One should also rule out the presence of other causes of hepatitis, such as infections, autoimmune and metabolic disorders.

Biochemical evaluations using serum levels of enzymes that are indicators of hepatocellular damage gives a clue as to functional integrity of hepatocytes. The definitive diagnosis, however, relies on histologic findings after ultrasound guided percutaneous biopsy. The histological study also allows characterizing the causative process and the severity of the disease (Beaugrand, 1997).

### **1.3.5 Biochemical evaluation of xenobiotic-induced hepatotoxicity**

Serum concentrations of different enzymes and other biochemical markers show injury to the liver. Most enzymes are non-specific except alanine aminotransferase (ALT), which is specifically present in hepatocytes. Recent increase in serum ALT is an indicator of damage to the hepatocytes. These markers give an idea as to the type of disturbance and the possible site of lesion (Table 1.2.). Other biomarkers include aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and 5' -nucleotidase. The serum concentrations of bilirubin, bile acids and albumin also give information about the biliary secretion and status of the liver's functional mass (Giannini *et al.*, 2005).

Studies to identify non-invasive ways of diagnosing hepatotoxicity have come up with different serum biomarkers associated with certain lesions. Different biomarkers were seen to indicate injury to the sinusoidal cells. This includes an increase in plasma Creatinine kinase level of the brain type (BB) isoenzyme (Vaubourdolle *et al.*, 2001; Georg *et al.*, 2005). Increased plasma levels of Immunoreactive Endothelin-1 were also seen to be associated with significant sinusoidal constriction in experimental animals. This constriction led to significant decrease in perfusion rate, local tissue oxygen tension and erythrocyte flux (Uhlmann *et al.*, 2001). Serum hyaluronic acid concentration elevates during sinusoidal cell injury and it is a useful adjunctive test in the assessment of certain fibrotic liver diseases including alcohol induced ones (Phillips *et al.*, 2003; Takafumi *et al.*, 2002).

Table 1.2. Classification of liver changes by Functional effect. Changes in functional effect are a result of the inciting process (FDA working group, 2000).

Classification	Parameters used to detect	
	Clinical pathology*	Morphologic pathology
Hepatocellular injury	Yes (ALT, AST)	Yes
Cholestasis	Yes (bilirubin, ALP, GGT)	Yes
Altered Kupffer Cell activity	No	Suggestive
Decreased functional hepatic mass	Yes (albumin, urea nitrogen, Coagulation factors, bile acids)	Suggestive
Acute hepatic failure	Yes (acid: base, electrolyte, ALT, AST)	Yes
Altered hepatic blood flow	Yes (albumin, urea nitrogen, bile acids)	Suggestive

\* Parameters listed are representative, and not all-inclusive.

### 1.3.6. Histologic patterns of xenobiotic-induced hepatotoxicity

It depends on type of chemical/drug, intensity of the insult, the population of cells affected and type of the exposure i.e. acute or chronic (Treinen-Moslen, 2001). The following are histologic types of injuries that are observed during hepatotoxicity.

1. *Fatty liver (Steatosis)*: it is an appreciable increase in the hepatic lipid content; which is less than 5% by weight in normal human liver (Treinen-Moslen, 2001). It is a common histological finding in human liver biopsies. The lipid content of hepatocytes is regulated by the integrated activities of cellular enzymes that catalyze lipid uptake, synthesis, oxidation, and export. When "input" of fats into these systems (either because of increased fatty acid delivery, hepatic fatty acid uptake, or fatty acid synthesis) exceeds the capacity for fatty acid oxidation or export (i.e., "output"), then hepatic steatosis occurs (Koteish and Diehl, 2001). The distribution of lipid can be macrovesicular, with hepatocytes being distended by a single vacuole displacing the nucleus or it can be microvesicular with numerous droplets surrounding centrally placed nucleus (Plate I b). Widespread microvesicular steatosis is characteristically of an acute condition in which impairment of fatty acid  $\beta$ -oxidation reflects a more general perturbation of mitochondrial

and ribosomal function both within and outside the liver. Regardless of the etiology, microvesicular steatosis is widely acknowledged as having a poor prognosis with death caused by both liver failure and extra-hepatic causes. Macrovesicular steatosis, by contrast, is typically associated with a more long-standing disturbance of hepatic lipid metabolism and has, until recently, been considered a benign condition (Day and James, 1998).

In some cases steatosis can be associated with hepatocyte inflammation and necrosis, and it is known as steatohepatitis. Steatohepatitis may progress to liver fibrosis and cirrhosis and may result in liver-related morbidity and mortality (Sears and Patel, 2005).

The most common association with fatty liver disease is metabolic syndrome. This includes carrying the diagnosis of type II diabetes, obesity, and/or hypertriglyceridemia. Other factors, such as drugs, metabolic abnormalities (eg, galactosemia, glycogen storage diseases, homocystinuria, tyrosemia), nutritional status (eg, overnutrition or severe malnutrition, total parenteral nutrition [TPN], starvation diet), or other health problems (eg, celiac sprue, chronic hepatitis C infection), may contribute to fatty liver disease (Sears and Patel, 2005).

2. *Cell death*: different types of cell death were defined in purely morphological terms, with limited reference to the underlying biochemical changes. Mammalian cells can die from 'apoptosis' (showing nuclear condensation and fragmentation, DNA fragmentation and cytoplasmic condensation), 'necrosis' (generalized swelling of cells and organelles with limited or no chromatin condensation and increased porosity of the membranes), and 'autophagic cell death' (death with accumulation of autophagosomes and subsequently vacuoles). Several intermediate or mixed cell death types have been reported, such as 'apoptosis-like' cell death with a less pronounced, subapoptotic chromatin condensation, paraptosis, oncosis, necrapoptosis with cytoplasmic vacuolization and pronounced mitochondrial swelling not seen in apoptosis, and apoptosis with autophagic vacuolization. Importantly, different types of cell death can be induced in a context-dependent fashion by the very same initiating stimulus, for instance

by the binding of specific ligands to so-called death receptors anchored in the plasma membrane (Golstein and Kroemer, 2005).

As stated previously, cell death by necrosis is followed by leakage of cytosolic enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), to the systemic circulation. Hence biochemical assays done for hepatocyte specific enzymes can give information to the necrotic processes in the liver (Dufour *et al.*, 2000).

Hepatocyte death can occur in a focal, zonal, or panacinar (pan lobular) pattern. Focal cell death is characterized by the randomly distributed death of single hepatocytes or small cluster of hepatocytes. Zonal necrosis is death to hepatocytes predominantly in zone 2 (periportal) / zone 3 (Centrolobular) (Treinen-Moslen, 2001). Information about zonal location of injury by a given chemical helps identify a sensitive, non-invasive index of functional change (for example serum level of bile salts are more likely to be elevated after damage to zone 1 than zone 3 due to bile salts gradient (Jaeschke *et al*, 2002; Treinen-Moslen, 2001).

3. *Canalicular cholestasis*: it is defined as a decrease in the volume of bile formed or an impaired secretion of specific solutes into bile. It is characterized biochemically by elevated serum levels of compounds normally concentrated in bile particularly bile salts and bilirubine. This in turn colors the different tissues and fluids of the body as yellow (Lee, 2003; Treinen-Moslen, 2001). The histologic features of cholestasis can be very subtle and difficult to detect without ultrastructural studies. Structural changes include dilation of the bile canaliculus and the presence of bile plugs in bile ducts and canaliculi (Plate II c), (Macarri *et al*, 1995). Toxicant-induced cholestasis can be transient or chronic: when substantial it is associated with necrosis/cell death (Treinen-Moslen, 2001, Sturgill and Lambert, 1997).

Some of the drugs that are incriminated in causing cholestasis are amiodarone and ethynylestradiol. Accidental ingestion in humans and administration in experimental animals of phalloidin, one of the toxic peptides of the mushroom *Amanita phalloides*, leads to rapid and sustained cholestasis (Hamada *et al*, 1995).

4. *Bile duct damage*: various drugs have been implicated in the development of a particular form of liver damage, predominantly involving the bile ducts. Bile duct damage can be mild, but also result in progressive ductopenia. Clinically it may be acute or prolonged (Gianfranco *et al*, 2003). Initial lesions following a single dose of cholangiodestructive agents include swollen biliary epithelium, debris of damaged cells within ductal lumens and inflammatory cell infiltration of portal tracts. Chronic administration of drugs/chemicals that cause bile duct destruction can lead to biliary proliferation and fibrosis resembling biliary cirrhosis (Lee, 2003; Treinen-Moslen, 2001). Approximately 1% of patients who develop drug associated cholestatic hepatitis develop progressive destruction of cholangiocytes, with the result so-called Vanishing Bile Duct Syndrome. In this syndrome the lesion of the biliary epithelium involves the loss of interlobular ducts located between the cholangioles and second- or third-generation septal bile ducts, causing ductopenia; this is defined as severe destruction of at least half of the interlobular ducts (Capra *et al.*, 2005).

5. *Sinusoidal damage*: the functional integrity of the sinusoids can be compromised by dilation or blocked of its lumen or by progressive destruction of its endothelial cell wall. Dilation of sinusoid occurs whenever efflux of hepatic blood is impaired. Blockade will occur when the fenestrae enlarges to such an extent that red blood cells become caught in them or pass through with entrapment in the interstitial space of Disse.

Progressive destruction of endothelial cells leads to the loss of sinusoidal fenestrations and the appearance of gaps in the lining, which is followed by extravasation of red cells into the space of Disse. When sinusoidal endothelial cells are injured extensively, it will result in widespread denudation of the sinusoidal lining. These disruptions of the sinusoids are considered the early structural features of the vascular disorder known as Venous-occlusive disease (VOD), (Shaji *et al*, 2003; Bras and Brandt, 1987).

A consequence of extensive sinusoidal blockade is that the liver becomes engorged with Red Blood Cells while the rest of the body is in shock. Main Causes of sinusoidal damage are chemotherapeutic agents such as actinomycin D, mithramycin, dacarbazine, cytosine arabinoside, and 6-thioguanine used at conventional doses and with long-term

use of the immunosuppressive agent, azathioprine. More recently, VOD has been seen after therapy for acute myelogenous leukemia with the monoclonal anti-CD33 antibody gemtuzumab ozogamicin (Mylotarg). Other causes include alkaloid-containing herbs like *Senecio* and *Crotalaria* (Shaji *et al*, 2003).

6. *Cirrhosis*: - represents the final common histologic pathway for a wide variety of chronic liver diseases. It is the end, often fatal, stage of chronic progressive liver injury and is histologically characterized by the accumulation of extensive amounts of fibrous tissue, specifically, collagen fibers, and nodular regeneration (Treinen-Moslen, 2001). The development of hepatic fibrosis reflects an alteration in the normally balanced processes of extracellular matrix production and degradation. Extracellular matrix, the normal scaffolding for hepatocytes, is composed of collagens (especially types I, III, and V), glycoproteins, and proteoglycans. Stellate cells, located in the perisinusoidal space, are essential for the production of extracellular matrix. Stellate cells become activated into collagen-forming cells by a variety of paracrine factors, such as transforming growth factor beta 1 (TGF- $\beta$ 1). Hepatocytes, Kupffer cells, and sinusoidal endothelium following liver injury release such factors. TGF-  $\beta$ 1, in turn, stimulates activated stellate cells to produce type I collagen. Increased collagen deposition in the space of Disse and the diminution of the size of endothelial fenestrae lead to the capillarization of sinusoids. Activated stellate cells also have contractile properties. Both capillarization and constriction of sinusoids by stellate cells contribute to the development of portal hypertension (Wolf, 2005). Drugs that cause cirrhosis when used on chronic basis include ethanol, methotrexate and nitrofurantoin.

7. *Tumors*: drug-induced liver tumors rarely occur. It could arise from hepatocytes, bile duct cells or sinusoidal lining cells. For example hepatocellular cancer has been linked to hormonal contraceptive use, abuse of androgen and a high prevalence of aflatoxin-contaminated diets (Kim, 2005). Angiosarcomas have been tightly associated with occupational exposure to vinyl chloride and arsenic. Exposure to Thoratrast, used in the past as a contrast for radiographic procedures, has been linked to tumors derived from the different cells of the liver (Treinen-Moslen, 2001).

### **1.3.7. Mechanisms of xenobiotic-induced hepatotoxicity**

Progress in hepatology has continued steadily in recent years. Routine use of molecular biology techniques has helped explain the mechanisms of liver diseases with genetic and viral origins and provided insight into susceptibility to other common liver disorders (McCarthy and Wilkinson, 1999).

Cell death is the crucial event leading to the clinical manifestations of drug-induced hepatotoxicity. It should be emphasized that the pathogenesis of clinical drug hepatitis reflects either an immune-mediated attack on the liver or a biochemical effect of toxic metabolites leading to a loss of cell viability. Immune attack involves the participation of death receptors such as Fas or the porin-mediated introduction of granzyme to activate the death cascade distal to the death receptor. The outcome is apoptosis of hepatocytes (or perhaps in some circumstances bile duct or endothelial cells) (Neil, 2002).

The following are the mechanisms by which xenobiotics induce hepatotoxicity.

*1.3.7.1. Cytoskeleton disruption:* cytoskeleton controls the spatial organization of a cell. It is made up of three major types of fibers: microtubules those originate from the centrosome and terminate in the cytoplasm and are used as “railways” for motor proteins to position organelles properly and transport some proteins to the nucleus, by way of phosphorylation and dephosphorylation. Actin filaments control the shape and movement of the cell through directional polymerization and depolymerization; and intermediate filaments give a cell its tensile strength and are components of the cell junctions that interface with neighboring cells and with basal laminae (Fenton and Longo, 1998).

The integrity of hepatocytes cytoskeleton can be disrupted by affecting proteins that are vital to its dynamic nature. Tight binding of Phalloidin, a toxin, to actin filaments prevents the disassembly phase of the normally dynamic rearrangement of the actin filament. Its uptake in hepatocytes leads to striking alteration in the actin-rich web of cytoskeleton adjacent to the canalicular membrane; the actin web becomes accentuated and the canalicular lumen dilates leading to a decrease in bile secretion (Elias *et al*, 1980). But recent studies demonstrate that the effect of Phalloidin on the ATP-binding

cassette proteins (ABC-proteins) that are located on the canalicular membrane of hepatocytes to be the reason for the cholestasis caused by this toxin (Loranger *et al*, 1996; Rost *et al*, 1999). The recent identification and characterization of several genes that are mutated in inherited forms of cholestatic liver disease have provided new insight into the normal physiology of bile secretion, the pathophysiology of intrahepatic cholestasis, and an unexpected major role for a novel group of P-type ATPases in human biology and disease (Arrese *et al*, 1998).

It was also demonstrated that Phalloidin uncouples secretion of cholesterol and phospholipids, which causes a redistribution of fatty acyl chain species among canalicular membrane phospholipids that alters membrane fluidity (Hyogo *et al*, 2000). The commonest causes for cholestatic changes are ethynyl estradiol and the naturally occurring hydrophobic bile acid lithocholic acid and its conjugate tauroolithocholate. Recently, Milkiewicz., *et al*. (2002) using hepatocyte couplets, demonstrated both to cause a decrease in the fluidity of the canalicular membrane and accumulation of cholesterol within the membrane, thus decreasing the phospholipids/cholesterol ratio, inhibiting  $\text{Na}^+/\text{K}^+$ -ATPase, a key driving force of the bile-salt-independent bile flow, and increasing the leakiness of the paracellular barrier.

*1.3.7.2. Bile acid-induced cytotoxicity:* retention of bile constituents within the hepatocyte during cholestasis is associated with hepatocytes injury. One primary factor implicated in cholestasis is the accumulation of hydrophobic bile acids within the liver, which have been demonstrated to be toxic to freshly isolated and cultured hepatocytes, liver mitochondria, and whole animals. Hepatocytes that are exposed to lower concentrations of hydrophobic bile acids (100  $\mu\text{M}$ ) typically undergo apoptotic cell death, whereas at higher concentrations, these compounds promote cell necrosis through oxidative stress, ATP depletion, hepatocyte swelling, and disruption of the plasma membrane. Both modes of bile acid-induced cell death are believed to be important in the pathogenesis of cholestatic liver disease through generation of oxidative stress.

Mechanistic studies of bile acid-induced apoptosis and/or necrosis have also focused attention on induction of the mitochondrial permeability transition (MPT) as a critical event preceding cell death. The MPT is characterized by large-amplitude swelling and loss of the electrochemical potential across the inner mitochondrial membrane caused by opening of a megachannel (MPT pore) spanning both the inner and the outer mitochondrial membranes. Upon induction of the MPT, several intermembranous space proteins are released into cytosol, including the pro-apoptotic signal cytochrome *c* and several inhibitors of apoptosis. Of particular interest are findings correlating increased generation of reactive oxygen species (ROS) with stimulation of the MPT by bile acids, most likely *via* the oxidative modification of MPT pore proteins. Commensurate with their ability to inhibit the MPT and cytochrome *c* release from mitochondria, antioxidants such as tocopherol, ebselen, idebenone and  $\beta$ -carotene also reduce bile acid-induced cellular necrosis and apoptosis in rat hepatocytes (Eric *et al*, 2004).

*1.3.7.3. Mitochondrial toxicity:* mitochondria are the main source of energy for the cell and its dysfunction obviously leads to a decrease in the ATP for the cell. It is also an important mechanism of xenobiotic-induced liver injury. Different molecules including reactive oxygen species (ROS), reactive nitrogen species (RNS) and different reactive drug metabolites are known to cause mitochondria dysfunction. The mitochondrial respiratory chain on the inner mitochondrial membrane (IMM) is a major intracellular source of reactive oxygen species (ROS). ROS cause nonspecific damage to lipids, proteins, and DNA, leading to alteration or loss of cellular function. Mitochondria are continuously exposed to ROS and accumulate oxidative damage more rapidly than the rest of the cell, especially because ROS are highly reactive and short-lived (Kesheng *et al*, 2004).

ROS play a key role in promoting cytochrome *c* release from the mitochondria, which is normally bound to the IMM by an association with cardiolipin. The release of cytochrome *c* proceeds by a two-step process: dissociation of cytochrome *c* from cardiolipin in the IMM, followed by release of cytochrome *c* through the outer mitochondrial membrane (OMM). ROS cause the peroxidation of cardiolipin, which is

associated with the release of cytochrome c to the cytosol. One study done in animals has shown association of ROS with a decrease in mitochondrial GSH (mGSH). A drop in mGSH leads to rapid protein damage, cascading to ATP fall, elevation of matrix  $\text{Ca}^{2+}$  concentration, inversion of the inter-membrane potential and subsequent release of the pro-apoptotic factors, cyt-c, Hsp10, and Hsp60 (Stefan *et al*, 2001).

RNS also play role in mitochondrial dysfunction caused by xenobiotics. The most commonly studied toxicity is that of acetaminophen. In this toxicity serum concentration of nitrate and nitrite were seen to increase in serum dramatically. This in turn leads to production of peroxynitrite which is formed by the rapid reaction of NO and superoxide. Peroxynitrite is detoxified by GSH under normal condition. But the depletion of GSH leads to nitration of Tyrosine residues. Studies using inducible nitric oxide synthase (iNOS) knock out mice showed no increase in NO in the serum and increased lipid peroxidation due to superoxide than that of the wild types. But the histologic result showed toxicity in both groups. Thus acetaminophen toxicity may be mediated by lipid peroxidation in iNOS mice and by nitration in wild mice. Depending on the availability of GSH NO may act as a cause of toxicity or detoxify the superoxide preventing lipid peroxidation (Jaeschke *et al*, 2002). Other studies also showed that nitrite ( $\text{NO}_2$ ) radical, produced by oxidation of  $\text{NO}_2$  ion by myeloperoxidase and free metals, as a nitrating species (James *et al*, 2003).

Reactive metabolites of xenobiotics form covalent binding to cellular macromolecules. A number of protein adducts have been isolated and identified in hepatotoxicity caused by acetaminophen. But events that produce hepatocellular death following the formation of acetaminophen protein adducts are poorly understood. One possible mechanism of cell death is that covalent binding to critical cellular proteins results in subsequent loss of activity or function and eventual cell death and lysis. Primary cellular targets have been postulated to be mitochondrial proteins and mitochondrial DNA (mtDNA), with resulting loss of energy production, as well as proteins involved in cellular ion control. Alterations of plasma membrane ATPase activity following toxic doses of acetaminophen was also seen in some studies (James *et al*, 2003).

Direct damage to mtDNA by reactive metabolites and ROS cause mtDNA deletions in humans. Oxidative stress after ethanol causes damage to mitochondrial proteins, lipids, and DNA. Interferon-alpha and nucleoside analogues impair mtDNA transcription and replication, respectively. DNA polymerase-g incorporates nucleoside reverse transcriptase inhibitors into mtDNA, an event that blocks mtDNA replication, eventually causing mtDNA depletion (Jaeschke *et al*, 2001). Sequestration of Co-A, which is needed to form thio esters with fatty acids, may also lead to inactivation of B-oxidation. Drugs such as salicylic acid and valproic acid are incriminated to sequester Co-A (Jaeschke *et al*, 2001).

#### 1.3.7.4. Immune mechanisms in hepatotoxicity.

A) Innate immune system: the role of cytokines, chemokines and the different cells of the innate immune system were exemplified in different clinical and experimental settings. It is apparent from intensive investigation that Kupffer cells are stimulated to produce a number of inflammatory mediators including TNF- $\alpha$ , a proinflammatory cytokine known to cause hepatocellular inflammation and cell death, various interleukins (IL-1, IL-6) and reactive metabolites of oxygen, superoxide specifically. TNF- $\alpha$  was seen to increase in early phases of hepatotoxicity from alcohol or acetaminophen. A study done to relate this increase of TNF- $\alpha$  with ethanol-induced liver injury using mice deficient in TNF- $\alpha$  receptor 1 exhibited essentially no pathological changes due to ethanol and no increase in serum transaminases when compared with wild-type control mice (Hines and Wheeler, 2004; James *et al*, 2003).

Kupffer cells are also capable of producing large amounts of potentially damaging free radicals including superoxide. One potential source of superoxide within macrophages including Kupffer cells is the phagocytic NADPH oxidase system. To study the role of NADPH oxidase in alcohol-induced liver injury mice deficient in p47<sup>phox</sup> (a regulatory subunit of the oxidase) fed with ethanol for 4 weeks presented with significantly reduced liver injury as assessed by serum transaminase levels and histopathological assessment of tissue damage (Hines and Wheeler, 2004).

## B) Adaptive immune system

Both humoral and cellular mechanisms are implicated in causing hepatotoxicity after exposure to xenobiotics. Hypothesis in explaining idiosyncratic hepatotoxicity rely on the adaptive immune reactions. There are two hypotheses that try to explain the mechanisms of involvement of immunity in XIH. One is the "hapten" hypothesis and the other is the "danger" hypothesis. "Hapten" hypothesis considers the formation of neoantigen because of reaction between a reactive metabolite and a hepatocyte macromolecule as an initial step towards immune sensitization against those macromolecules. "Danger" hypothesis rather claims the 'danger signals' such as cell damage or infection to be the cause for the activation of the immune system and not the foreignness (Sanderson *et al*, 2005).

Many metabolites are so reactive that they covalently bind almost exclusively with enzymes, usually cytochrome P450. The cells of innate immune system will consider this covalently bound cytochrome P450 protein as foreign. This deformed protein will be processed and presented to the B and T lymphocytes by antigen presenting cells. Activation of B-lymphocytes results in production of antibodies against isoenzymes responsible for the metabolism of the offending drug compound. Examples of anti-microsomal antibodies include anti-liver/kidney microsomal (LKM) antibodies directed against cytochrome P450IID6 isoenzymes (anti-LKM1 antibodies), and anti-LKM2 antibodies, which recognize P450IIC9. Anti-LKM3 antibodies are also identified against uridine diphosphate glucuronyl transferase enzymes (Sturgill and Lambert, 1997).

At the same time antigen presenting cells activate T-lymphocytes resulting in drug antigen-specific T-cells that are isolated, cloned and characterized from hypersensitive patients in terms of their cellular phenotype and functionality. Isolated T-cells can express either CD4 or CD8 co-receptor or both. Activated T-cells against sulfamethoxazole, lidocaine, lamotrigine and carbamazepine have been isolated (Sanderson *et al*, 2005).

### **1.3.8. Non-clinical assessment of hepatotoxic nature of Xenobiotics**

A primary purpose of nonclinical studies is to discover target organ toxicity and from this information stop the development of the compound or to utilize this information for monitoring possible toxicities in human studies. The liver is a major target organ of early

screening efforts in the pharmaceutical industry and a major target organ in the repeated dose nonclinical safety studies used to support clinical trials. If a compound is hepatotoxic in animals, it is only after the toxicity is assessed and an adequate safety margin is estimated that such a compound is administered to humans (FDA working group, 2000).

There are *in vitro* and *in vivo* studies for nonclinical assessment of xenobiotics for hepatotoxicity and each is done in a tier approach. The process in tier approach starts by prioritizing those classes of chemicals that have no information regarding their toxicity and may be with higher production volume or exposure to humans. This information could be gathered from literature and by determination of its structural-activity relationship. Chemicals with sufficient information to determine hazard or risk will not be tested. Those with no or insufficient information will be subjected to testing.

Tier I studies are standard screening studies designed to detect and initially characterize a hepatic change, while tier II studies are specialized and used to initially characterize a change or address a specific nonclinical or clinical safety. Tier III is also developed to further characterize the mode of action and biological activity of individual chemicals positive at tier II (Green and Goldberg, 2004).

#### **1.3.8.1. *In vitro* studies**

*In vitro* preclinical study is needed in two conditions: when one anticipates reactive metabolites to be the cause of the hepatotoxicity and / or when one wants to understand the mechanism of hepatotoxicity observed during *in vivo* study. The current methods for *in vitro* studies of hepatotoxicity include tissue slices and isolated hepatocytes, either in suspension or in primary culture. Isolated organ model is difficult to handle and cannot be used for human studies. They reflect the *in vivo* situation, at least for a few hours, and can be obtained from various species including humans and fish (Spielmann *et al*, 1998).

##### **1.3.8.1.1 Tier I *in vitro* studies**

The purpose of this tier is to screen in a qualitative manner for presences of potential reactive metabolites of a compound. Tier I tests for identifying the presence of a reactive metabolite include, screening for glutathione conjugates, determination of covalent binding and mechanism-based inhibition of cytochrome P450 enzymes (Green and Goldberg, 2004).

##### **1.3.8.1.2 Tier II *in vitro* mechanistic studies**

If evidence of reactive metabolites is found during *in vitro* or in subsequent *in vivo* testing, it would be necessary to perform follow up investigations to better assess the hepatotoxic potential of the reactive metabolites. The studies should include determination of the reactive metabolites, the amount of the reactive metabolites formed, the enzymes responsible for formation of the reactive metabolites and the chemical

reactivity of these metabolites. In addition, further *in vivo* studies would be required (Green and Goldberg, 2004).

### **1.3.8.2 *In vivo* animal studies**

It is done by administering the drug / chemical in relevant models covering the range of treatment for proposed clinical trial. Identification of hepatotoxicity is done primarily by clinical and morphological pathology assessment measured at multiple time intervals. If hepatotoxicity occurs, the assessment must identify the changes and their magnitude, provide a *NOEL* (no observed effect level), *NOAEL* (no observed adverse effect level), and determine a mechanism/pathogenesis for the lesion such that one can formulate a development risk and monitoring recommendation for future clinical use (FDA working group, 2000).

#### **1.3.8.2.1 Tier I *in vivo* animal studies**

It typically includes administration of drug to two laboratory animal species (1 rodent and 1 nonrodent – dog/monkey) for multiple durations and doses exceeding those used in clinical trials. Some drugs necessitate additional nonclinical testing in special animal models when these animal models more appropriately mimic the sensitivity of man to certain classes of hepatotoxins, e.g., woodchuck for nucleoside analogs. The duration of nonclinical studies is dependent on multiple factors, including: 1) duration and dosing regimen of the clinical trial; 2) the drug's chemical structure and its relation to known toxic entities or liabilities; and, 3) regulatory guidance (FDA working group, 2000).

The aim of this study is to get as detailed and complete information about drug/chemical disposition (including absorption, distribution, metabolism and excretion) as possible and to see the toxicity of the drug/chemical in different organ systems of the test animals.

Tier I parameters used for detection of hepatic changes are clinical pathology, morphologic pathology, enzymes induction, and *in-life* findings. These parameters are determined at phases of the study that allow identification of acute, chronic, persistent, transient and/or reversible hepatic change. Clinical and morphologic pathology evaluations are the 'gold standard' for identification of hepatic toxicity in animals, each complimentary to the other and each with its advantages. Microscopic evaluation of liver sections by light microscopy is required. The pattern of cellular damage, the presence of cellular infiltrates and the presence of necrotic and/or apoptotic cells should all be studied. It is also important to rule out other causes of liver damage or disease (FDA working group, 2000).

#### **1.3.8.2.2 Tier II *in vivo* animal studies**

Tier II studies and tests may vary considerably depending on the issue to be addressed. The goal of such studies is to determine as well possible mechanism. It is in this area that most of the efforts for the identification of immune mediated responses have been

directed. These studies are done when the previous *in vitro* or *in vivo* studies suggest further studies. Tier II assessments are made with ultrastructural pathology, morphometrics, histologic special stains, or methods for antibody detection (FDA working group, 2000).

### 1.3.8.3 Tier III studies

The purpose of this tier is to further characterize the mode of action and biological activity of individual chemicals positive at tier II. The criterion for tests at this tier is that they have an explicit relevance to humans in terms of the pathway of toxicity. In all likelihood, tests at this level will use *in vitro* systems, as well as animals. As such use of non-invasive approaches in animals should be sought (Green and Goldberg, 2004).

Table 1.3 Findings in selected primary hepatotoxicity indicator parameters that are

Histologic pathology**	Clinical pathology*
Hepatocellular degeneration	ALT: >3-5X increase
Hepatocellular necrosis (including apoptosis)	AST: >3-5X increase
Cholestasis	GGT: >2X increase
Inflammation	ALP: >3-5X increase
Fibrosis/cirrhosis	Bilirubin: absolute value > 1 mg/dl
Hepatocyte or Biliary proliferation	

generally considered adverse (FDA working group, 2000).

\* Increases in clinical pathology parameters, even in the absence of histologic changes, are considered adverse, unless the pathogenesis indicates to the contrary

\*\* The occurrence of any histologic liver pathology change at or above the 'minimal to slight' level is considered adverse, unless the pathogenesis indicates to the contrary

For many reasons predictivity of the nonclinical toxicity tests to humans is not perfect.

The data on Predictivity of animal studies for human toxicities have been limited and a complete analysis has not been done. However, experiences within pharmaceutical industries indicate that the appropriate identification of hepatotoxicity is high. Most compounds found not to be hepatotoxic in animals are not hepatotoxic in humans (FDA working group, 2000). However, most that are severe in animals never proceed to clinical trials as it is judged that there is unacceptable risk for hepatotoxicity in man. The lack of information on the human correlate for these compounds prevents the predictivity of the

animal model to be calculated. Although the animal models are thought to be highly effective in keeping most hepatotoxic compounds from being tested in humans, failures do occur where hepatotoxicities are observed in clinic despite thorough testing by these models. The underlying reasons for the misidentification of an adverse effect to be of a low risk to humans need investigation. A limited number of surveys studying these failures have been done. In one survey where liver toxicity was the cause of project termination for seven compounds after testing in humans, hepatotoxicity was demonstrated in four of these compounds. For the remaining three compounds, liver toxicity was not observed in animal studies (FDA working group, 2000).

As this survey demonstrates there are compounds that show no significant evidence of liver toxicity in animals but cause liver injury in humans. These false negative results in animal studies may be related to insufficient systemic exposure to the drug either because the doses were too low or because intestinal absorption was poor or the metabolism of the compound or the route of elimination differed in the animal species tested compared to humans. The occurrence of other toxicities or the extensions of the pharmacological effect may have prevented testing the compounds at a hepatotoxic dose in the animals. The small number of animals used in toxicity studies may make it difficult to detect hepatotoxicity occurring at a low incidence. The normal animals in nonclinical studies do not mimic the patient population or the potential drug: drug interactions, which may occur in humans, which further complicates interpretation of non-clinical results. Finally, immunoallergic or autoimmune type liver toxicity can only rarely be accurately evaluated in non-clinical studies (FDA working group, 2000).

## **2. Objectives**

### ***2.1 General***

To evaluate the claimed spasmolytic effect and safety of *Thymus serrulatus* to the liver in mice and rats, respectively.

### ***2.2 Specific***

1. To evaluate the spasmolytic effect of *Thymus serrulatus* in mice
2. To evaluate the acute toxicity and determine LD<sub>50</sub> of *Thymus serrulatus* in mice.
3. To evaluate the safety of *Thymus serrulatus* after subchronic oral administration by using clinical, clinical chemistry and histopathological evaluations in rats.
4. To conduct phytochemical screening of the aqueous and methanolic extracts of the plant.

### **3. Materials and Methods**

#### **3.1 Materials**

##### **3.1.1 Collection of plant material**

The leaves of the dried plant material used for this study were collected around 'Debresina', 190 Km North of Addis in November 2004 during the non-flowering season. Voucher specimen for identification purpose was collected from the natural habitat ('Debresina'), authenticated by a Taxonomist and deposited at the EHNRI herbarium (Ethiopia) under the deposit number TS-2103.

##### **3.1.2. Animals.**

Healthy adult male and female albino mice and Wister rats bred in the animal house of Faculty of Medicine, Addis Ababa University, were used for the experiment. The animals were kept under room temperature with light and dark alternating 12 hourly. They were given normal rodent chow and their bedding was wood scrape. The mice were used for spasmolytic and acute toxicity studies while the rats were used for the subchronic study.

##### **3.1.3. Chemicals and drugs.**

Atropine sulfate from Sigma, Absolute alcohol from JOSEPH MILLS (England), 37% Formaldehyde solution from EL NASIR (Egypt), Xylene from CARLOERBA (Italy), Sodium phosphate, monobasic and dibasic from Riedle-DE HAENAG SEELZE-HANOVER (Germany) were used in the present study.

#### **3.2 Methods**

##### **3.2.1 Extraction of the plant**

**Aqueous extract:** the dried leaves were garbled, powdered and soaked in distilled water while shaking using electric shaker for one hour. The suspension was filtered through gauze and the aqueous extract freeze-dried or lyophilized to give an amorphous powder that was collected in a vial and kept in a desiccator until used for the experiment.

**Methanol extract:** the powdered plant material was percolated in 80 % methanol for three days. This was then filtered through Watmann filter paper. The filtrate was then concentrated using Rota vapor to give gummy residue. The gummy crude extract was then weighed and kept at four degree centigrade until used for the experiment.

### **3.2.2 Phytochemical screening study**

Phytochemical screening was done on the extracts, powdered and dried leaves of the plant. It was first screened for presence of different compounds such as Alkaloids, Polyphenols, Tannins, Saponnins, Phytosterols and withanoids by color method using testing reagents indicative for a given class of compounds. Specific tests were done to check for the presence of pyrrolizidine alkaloids and condensed tannins that are reported to cause hepatotoxicity.

Thin layer chromatography was performed following color test to resolve the different components of the detected classes of compounds (Debella, 2002). This was done using different mixture of solvents as mobile phase. The spots on the TLC plate were visualized by spraying the plate with appropriate reagents for the specific chemical compound. The  $R_f$  for each spot was then measured to know the different constituents of the extract.

### **3.2.3 In vivo spasmolytic test.**

Adult male and female albino mice weighing 30 – 40gm were grouped into nine; 6 animals per group. Six groups of animals were used for testing three different doses of the two extracts - aqueous and methanolic extract; one group was used as a positive control (atropine) and two groups as negative controls (distilled water and 3% Tween 80 solution).

The animals were made to fast for 18 hours before the test, but had access to water. On the day of experiment 100 mg/kg, 200 mg/kg and 400 mg/kg doses of the aqueous and

methanolic extracts were reconstituted in fresh distilled water or 3% Tween 80, respectively, before administering to the animals. Each dose was given in 1cc of the prepared solutions using oral gavage. After five minutes 1cc of 5% charcoal solution in 5% Gum acacia was administered. Thirty minutes later the animals were sacrificed by cervical dislocation, laparatomized and the small intestine from the duodenum to the caecum was taken out and stretched on a table. The length from duodenum to the illeocaecal junction was measured. The distance the charcoal plug traveled along the small intestine was measured and expressed as a percent to the total small intestinal length (Ghosh, 1984).

### **3.2.4 Acute toxicity test.**

Adult male and female albino mice weighing 25 – 35 gm were used for this study. Before the day of the experiment the animals were deprived of food for 18 hours. Water was given *ad libitum*. On the day of the experiment the animals were weighed and different doses of the extract were freshly prepared right before administration to the animals.

The animals were observed for any sign of toxicity for a period of two hours continuously and death at the end of 24 hours was registered. After 2 hours the animals were given food. The route of administration was intraperitoneal. The doses given intraperitoneally were 0.5 gm/kg, 1 gm/kg, 1.5 gm/kg, 2 gm/kg and 3 gm/kg of both extracts. Each dose was given to a group of ten animals and each group constituted five female and five male mice. After administration of each dose the animals were observed for any change in behavior for the first 2 hours continuously and observed signs were recorded. Death within 24 hours of administration was also recorded.

The percent of death in each dose was calculated and changed to PROBIT by using PROBIT scale. The doses also were changed to log dose and PROBIT curve constructed for both aqueous and methanol extracts. The LD<sub>50</sub> were determined from the curves.

### **3.2.5 Subchronic toxicity test**

#### **3.2.5.1 Dosing of animals**

Male and female Wistar rats of 4 – 6 weeks age weighing 90 – 160 gm were used for this experiment. The animals were grouped into four. Each group contained five male and five female rats. One group was given distilled water while the other three groups were given 200 mg/kg, 1000 mg/kg and 2000 mg/kg doses of the aqueous extract of *T.serrulatus* dissolved in 2 ml of distilled water, daily, for a total of 90 days by oral gavage. All the animals were fed with pellet and water *ad libitum*.

### **3.2.5.2 Clinical observation and body weight measurements**

The animals were observed prior to, during and 30 minute to one hour after daily dosing. Cage side observation was done throughout the treatment period except where they were found dead. All observations were systematically recorded, with individual records being maintained for each animal. Observation included evaluation of the skin, mucosal surfaces and all the oss (nasal and oral) for pallor, jaundice and any bleeding; change in bowel habit and behavior. Each animal was weighed weekly and the dose was calculated and adjusted accordingly. Weight changes were calculated and recorded.

### **3.2.5.3 Clinical chemistry parameters determination**

At the end of the treatment period the animals were made to fast overnight. In the morning enough amount of blood sample was collected by cardiac puncture after the animals were anaesthetized with Diethyl ether. The blood was centrifuged at 3000 rpm (revolution per minute) for 10 minutes; serum was collected for analysis of clinical chemistry. The collected serum was evaluated for the concentrations of the following; ALT, ALP (BM-AUTOLAB-PM 400, AMS, Italy), total protein, albumin, creatinine (Autohumalyzer, 900S Plus, Germany). Determinations for serum concentrations of Glucose, cholesterol (DR LANGE – LP300, Germany), calcium, sodium and potassium (Electrolyte analyzer 9180, Roche, Germany).

### **3.2.5.4 Gross Morphologic evaluation of the liver**

The anaesthetized animals were sacrificed by strangulation and laparatomized. The liver was then taken out and seen for any gross abnormality, weighed and fixed in 10% neutral buffered formalin solution (see annex- 4) immediately and kept for further work.

**A. Gross pathologic evaluation:** - includes evaluation for any observable gross pathologic conditions of the liver; the color, architecture, texture or tumors. The nature of the observed gross abnormalities was elaborated in terms of site, size and consistency and recorded. The weight of the liver was also measured after stripping off of the extra-tissues and blotting using tissue paper. The relative liver weight was also calculated and compared among the groups.

**B. Histopathologic evaluation:** - small part of the liver tissue was taken from the same site of the median (cystic) lobe of the liver of individual animals of each group and processed manually and embedded using paraffin wax. The embedded tissue was sectioned into 5 - 7 mm size by microtome (Leica RM 2125). The section was put in a warm water bath before mounted into glass slide. Baker's egg albumen adhesive was used to adhere the tissue to the slide (see annex- 3). The slides were put into dry oven overnight. The section was then stained by hematoxyline-eosine (see annex- 2); DPX added and covered using cover slip. It was finally viewed under light microscope with different magnifications for any abnormality. Any observed abnormalities were characterized, elaborated and recorded. Also the differences among the groups were evaluated.

In addition, part of the liver which were seen to have gross pathological abnormalities were also taken out and processed manually, stained and viewed under different magnification of light microscope by a pathologist.

### **3.3 Statistical analysis**

Data were expressed as mean +/- standard error of the mean (SEM). The distribution of data, in each study, was assessed using the Kolmogorov Smirnov test (when  $n \geq 5$ ) and Levene test (when  $n < 5$ ) for homogeneity of variance. Subsequent statistical evaluations were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Chi-square test was used to see the relation between the histopathologic findings. The level of statistical significance was set at  $P < 0.05$ . Statistical analysis was done by using Prism 4.0, graph pad instat<sup>®</sup> and SPSS 11.0.

## 4. Results

### 4.1. Extract's yield

The yield of aqueous extract was 23 % and that of the methanolic extract was 18 %.

### 4.2. Phytochemical screening

#### 4.2.1 Chemical method

Screening the plant extracts for the presence of secondary metabolites using chemical method of detection revealed the presence of polyphenols, phytosterols and withanoids as shown in the table 4.1. However, tests done for tannins and pyrrolizidine alkaloid were negative.

Table 4.1 Secondary metabolites of the extracts of *T.serrulatus* detected by the chemical method.

Secondary metabolites and Detection Reagents / Techniques		Plant materials used for the test		
Compound class	Reagent / Technique	Lyophilized Extract	Crude powder Extract	Unprocessed plant material
Alkaloid	Dragendorff's Mayers	Negative	Negative	Negative
Tannins	1% $K_3Fe(CN)_6$	Negative	Negative	Negative
Polyphenols	Mixture of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$	Positive	Positive	Positive
Saponins	Honey comb like froth formation	Negative	Negative	Negative
Cardiac glycosides	Kedde reagent	Negative	Negative	Negative
Phytosterols and Withanoids	Liebermann and Bruchard's reagent	Positive	Positive	Positive

Condensed Tannins	Test for Tannins after decomposing with mixture of HCl and Formaldehyde	Negative	Negative	Negative
Pyrrolizidine alkaloids	5 % sodium nitroprusside followed by modified Ehrlich reagent	Negative	Negative	Negative

N.B. Positive – presence, Negative - absence

#### 4.2.2 Thin layer chromatography (TLC) method

Identification of the number of components and their relative position ( $R_f$  value) on TLC plate for phytosterols showed three different components.

TLC done for identification of number of components and their relative position ( $R_f$  value) for polyphenols.

#### 4.3 *In vivo* spasmolytic test

The result for the *in vivo* spasmolytic tests done at different doses for both aqueous and methanolic extracts were statistically significantly different ( $P < 0.05$ ) from that of the negative controls, but no statistical difference was observed between the different doses of the extracts and the positive control ( $P > 0.05$ ) (Tables 4.2 and 4.3). The inhibitory effect is not dose dependent.

Table 4.2. Inhibitory activity of the aqueous extract of *T.serrulatus* (100 – 400 mg/kg, p.o.) on intestinal motility in mice (charcoal meal test).

Group	Dose (mg/kg)	% Maximum distance traveled by charcoal plug
Negative control	Distilled water	81.96 +/- 2.51
Positive control (Atropine)	10	65.04 +/- 2.08
<i>T.serrulatus</i>	100	59.99 +/- 3.73***
<i>T.serrulatus</i>	200	63.06 +/- 2.83***
<i>T.serrulatus</i>	400	64.81 +/- 2.86**

Data expressed as mean +/- SEM, \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

Table 4.3. Inhibitory activity of the methanolic extract of *T.serrulatus* (100-400 mg/kg, p.o.) on intestinal motility in mice (charcoal meal test).

Group	Dose (mg/kg)	% Maximum distance traveled by charcoal plug
Negative control	0.3% Tween 80	85.01 +/- 2.69
Positive control (Atropine)	10	65.04 +/- 2.08
<i>T.serrulatus</i>	100	63.65 +/- 2.65*
<i>T.serrulatus</i>	200	50.71 +/- 7.76***
<i>T.serrulatus</i>	400	59.59 +/- 2.27**

Data expressed as mean+/- SEM, \*P<0.05, \*\* P< 0.01, \*\*\*P<0.001

#### 4.4 Acute toxicity test

**4.4.1 Aqueous extract**, the early signs observed after administering a dose of this extract intraperitoneally were decreased movement, writhing reflex, staggered walking, piloerection, decreased and labored respiration. The signs observed late were decreased appetite and dried fecal matter attached to the anal oss.

The LD<sub>50</sub> was determined to be 1322 +/- 1.5 mg (Figure 4.1).

**4.4.2 Methanolic extract**: the early signs observed after injecting the extract intraperitoneally were writhing reflex, decreased movement and staggering. The signs observed late were loss of rightening reflex, piloerection, decreased and labored respiration (gaspings like), which was followed by death of the animal. The LD<sub>50</sub> was determined to be 1791 +/- 2.9 mg (Figure 4.2).

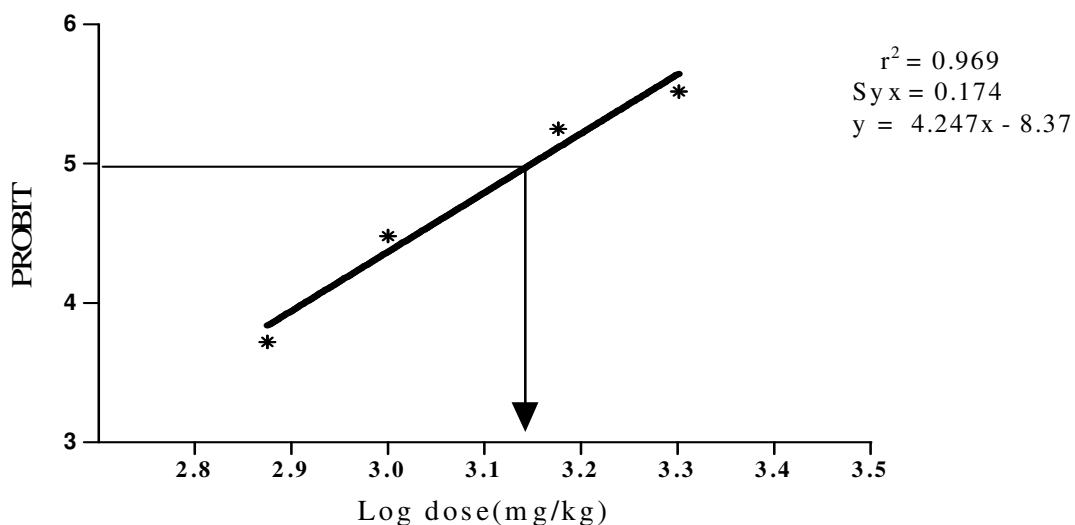


Figure 4.1, LD<sub>50</sub> for i.p. aqueous extract of *T.serrulatus* on mice.

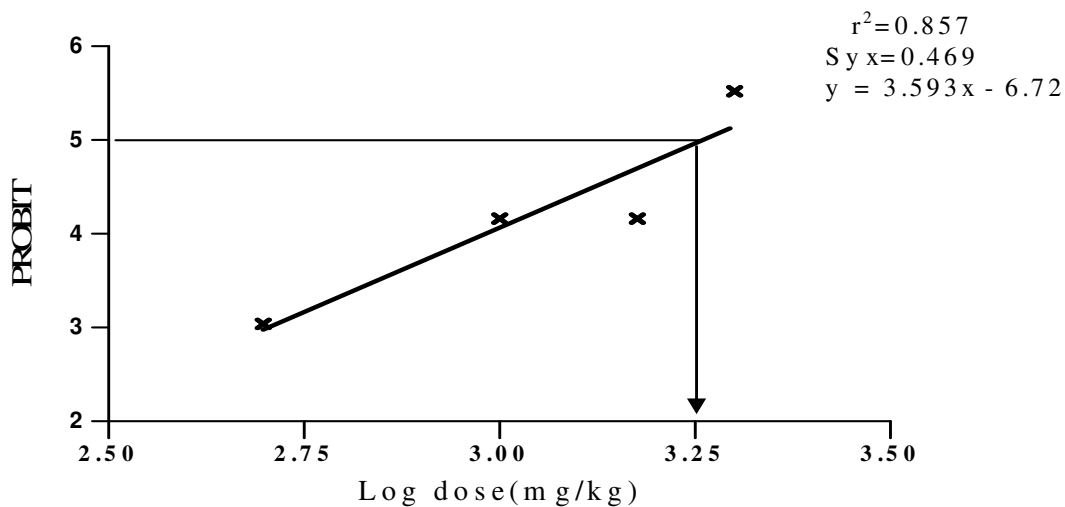


Figure 4.2, LD<sub>50</sub> for i.p. methanolic extract of *T.serrulatus* on mice

## 4.5. Subchronic toxicity test

**4.5.1 Clinical observation and body weight measurement:** - clinically the animals in both the control and treatment groups were observed to have occasional diarrhea and

were seen to pass whitish, 2-4 cms long, flat, segmented worm-like structure (proglottid of cestode). Two animals were found dead during the follow up period. One was from the female control group, which died at the last week of the study. Part of the body of this animal was lost making it difficult for gross examination. The other was from the treatment group, which were getting 2000mg/kg. The animal died at the fourth week of the study period and the body was lost.

Generally there was body weight gain in most of the groups at the end of the study period as compared to the pretreatment weight of the animal (Figure 4.3). The weight gain was marked in the male groups; in the order of 2000 mg/kg > 1000 mg/kg > 200 mg/kg > 0 mg/kg. The weight gain was statistically significant (P<0.05).

The same order of weight gain was observed in the female groups except in the control group where there is a loss of weight (Figure 4.4). This change in weight was statistically significant (P<0.05).

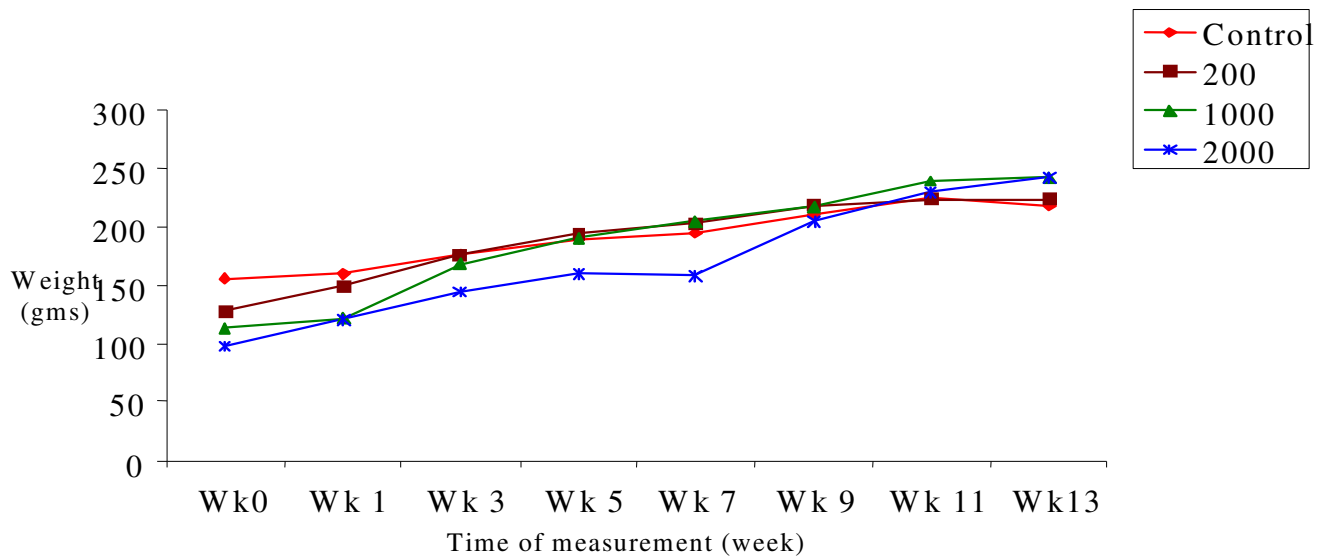


Figure 4.3, Growth curve for male rats.

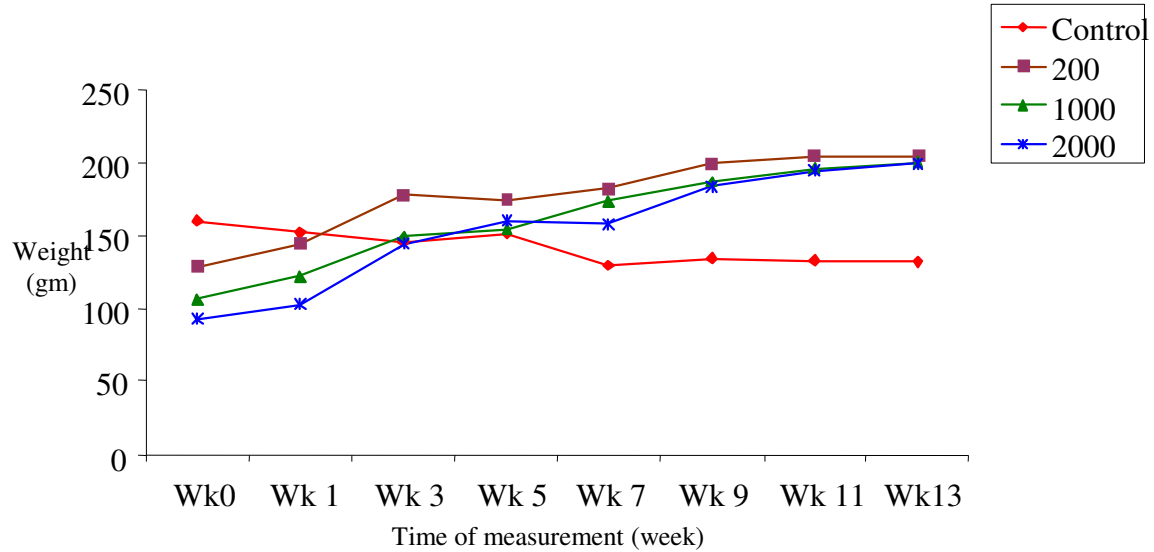


Figure 4.4, Growth curve for female rats.

#### 4.5.2 Gross morphologic evaluation of the liver.

Gross liver morphologic examination at necropsy showed different sized whitish, firm to hard and cystic mass over the surface of the different lobes of the liver. This was observed in eleven animals. This mass was solitary in most of the cases but two were observed in one of the livers of the animals. The mass was observed in 2 out of 9 animals of the control group; in 6 out of 10 animals that were given 200 mg/kg; in 2 out of 10 animals that were given 1000 mg/kg; in 1 out of 9 animals that were given 2000 mg/kg and two masses were observed in the same group over two different lobes.

The masses found in the control group were relatively bigger in size; 0.5 – 0.6 cm, colorless to whitish in color and extending the whole layer of the lobe (plate I a). One was found on the median (cystic) lobe of the liver while the other was found on the right lobe of liver. On dissecting the mass, worm like structure within a colorless fluid was observed.

The other masses were smaller ranging between 0.1 – 0.3 cm, whitish in color and on different lobes of the liver (Plate I b). There was colorless fluid on dissection of the mass but no worm like structure was found unlike those observed in the bigger masses.



Plate I a, relatively bigger liver mass found in the control groups when viewed posteriorly (arrow).

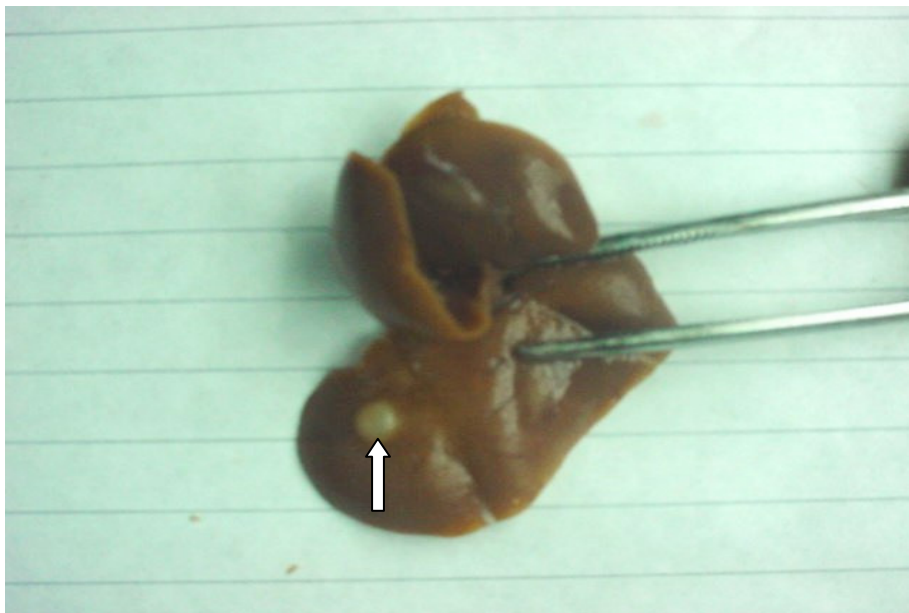


Plate I b, the type of liver mass found in the treatment group when viewed anteriorly (arrow).

The ratio of the liver weight to the gross body weight at the end of the study showed no pattern with the dose in both male and female animals as shown in table 4.4 and 4.5. A higher ratio was seen in the female control group (table 4.5) but statistical comparison between the ratios showed no difference between the groups ( $P > 0.05$ ).

Table 4.4, absolute and relative organ weight for male rats after subchronic administration of the aqueous extract of *T. serrulatus*.

Weight	Dose of the extract (mg/kg)			
	Control (n = 5 )	200 (n = 5)	1000 (n = 5)	2000 (n = 4)
Absolute weight (gm)	5.51 +/- 0.42	6.39 +/- 0.35	7.63 +/- 0.47	7.25 +/- 0.54
Per body weight (%)	2.52	2.84	3.15	2.95

Data expressed as mean +/- SD.

Table 4.5, absolute and relative organ weight for female rats after subchronic administration of the aqueous extract of *T. serrulatus*.

Weight	Dose of the extract (mg/kg)			
	Control (n = 4 )	200 (n = 5)	1000 (n = 5)	2000 (n = 5)
Absolute weight (gm)	6.49 +/- 0.68	5.67 +/- 0.28	5.55 +/- 0.26	5.70 +/- 0.38
Per body weight (%)	4.97	2.77	2.77	2.86

Data expressed as mean +/- SD

### 4.5.3 Clinical chemistry

As shown in table 4.6 in male rats the value of alanine amino transferase (ALT) decreased in a dose dependent manner, but no statistically significant difference was observed ( $P>0.05$ ). There was an increase in serum albumin (ALB) and fasting blood sugar level in a dose dependent manner. But no statistically significant difference was seen for ALB ( $P> 0.05$ ) while statistically significant difference was seen for FBS between the control and medium and highest doses ( $P< 0.01$  and  $0.001$ , respectively). In the rest of the clinical chemistry results neither a pattern among the specific tests nor statistically significant difference among the different doses was observed ( $P> 0.05$ ).

Table 4.6, clinical chemistry data for male rats

Biochemical parameters	Dose (mg/kg)			
	Control (n = 5)	200 (n = 5)	1000 (n = 5)	2000 (n = 4)
ALT (IU/L)	116.8 +/- 72.7	129.2 +/- 99.3	92.2 +/- 9.1	82.5 +/- 30.0
ALP (IU/L)	123.2 +/- 98.6	88.8 +/- 54.6	184.8 +/- 52.1	117.3 +/- 40.3
Total bilir. (mg/dl)	0.58 +/- 0.13	0.56 +/- 0.05	0.54 +/- 0.13	0.70 +/- 0.14
Direct bilir (mg/dl)	0.14 +/- 0.05	0.14 +/- 0.05	0.12 +/- 0.04	0.15 +/- 0.06
FBS (gm/dl)	67.4 +/- 27.9	101.8 +/- 25.4	119.4 +/- 16.7**	139.5 +/- 8.6***
TP (gm/dl)	6.08 +/- 0.96	6.02 +/- 0.61	6.00 +/- 0.12	6.25 +/- 0.33
ALB (gm/dl)	4.80 +/- 0.22	4.82 +/- 0.52	4.92 +/- 0.44	5.05 +/- 0.37
CHO (gm/dl)	23.0 +/- 2.92	23.8 +/- 7.22	26.4 +/- 12.03	27.0 +/- 8.68
Ca <sup>2+</sup> (mEq/L)	9.27 +/- 1.09	9.85 +/- 0.83	9.40 +/- 0.81	9.69 +/- 0.55
Na <sup>+</sup> (mEq/L)	140.2 +/- 9.3	142.8 +/- 3.0	140.4 +/- 0.9	140.8 +/- 2.6
K <sup>+</sup> (mEq/L)	5.25 +/- 1.28	5.96 +/- 1.49	6.08 +/- 0.73	5.60 +/- 1.27

Data were expressed as mean +/- SD, \*\* $P<0.01$ , \*\*\* $P<0.001$

(ALT= Alanine amino transferase, ALP= alkaline phosphatase, bilir. = Bilirubin, FBS= Fasting blood sugar, TP= Total protein, ALB= Albumin, CHO= Cholesterol)

In female rats the value of ALT, direct and total bilirubin appears to decrease in a dose dependent manner among the treatment group, but no statistically significant difference

among the different doses was seen ( $P > 0.05$ ) as shown in table 4.7. There was an increase in the values of alkaline phosphatase (ALP) and total protein among treatment groups in a dose dependent manner, but no statistically significant difference observed among the different doses ( $P > 0.05$ ). The result of FBS showed no pattern to the dose given to the animal, but at medium dose it is statistically significantly lower than both the lower and higher dose ( $P < 0.05$ ). The other results were seen to have neither dose dependent pattern nor statistically significant difference among the different doses ( $P > 0.05$ ).

Table 4.7, clinical chemistry data for female rats.

Biochemical parameters	Dose (mg/kg)			
	Control (n = 4)	200 (n = 5)	1000 (n = 5)	2000 (n = 5)
ALT (IU/L)	146.5 +/- 81.4	150.6 +/- 90.3	87.8 +/- 42.8	85.4 +/- 38.9
ALP (IU/L)	141.3 +/- 64.9	51.6 +/- 28.8	88.4 +/- 62.9	94.4 +/- 21.7
Total bilir (mg/dl)	0.60 +/- 0.08	0.66 +/- 0.21	0.62 +/- 0.08	0.54 +/- 0.11
Direct bilir (mg/dl)	0.15 +/- 0.06	0.16 +/- 0.05	0.14 +/- 0.05	0.12 +/- 0.04
FBS (gm/dl)	109.8 +/- 17.0	129.0 +/- 25.0	93.8 +/- 8.11*	130.4 +/- 17.7
TP (gm/dl)	6.18 +/- 2.25	5.58 +/- 0.51	5.88 +/- 0.18	5.92 +/- 0.29
ALB (gm/dl)	5.35 +/- 2.10	4.60 +/- 0.16	4.60 +/- 0.14	4.70 +/- 0.25
CHO (gm/dl)	32.0 +/- 13.98	27.0 +/- 4.36	24.8 +/- 5.26	25.0 +/- 4.58
Ca <sup>2+</sup> (mEq/L)	10.26 +/- 1.19	9.32 +/- 0.83	9.26 +/- 0.69	9.99 +/- 0.61
Na <sup>+</sup> (mEq/L)	140.8 +/- 20.43	135.4 +/- 10.14	138.8 +/- 1.30	139.6 +/- 1.14
K <sup>+</sup> (mEq/L)	5.25 +/- 0.70	5.78 +/- 1.45	5.72 +/- 1.36	5.34 +/- 0.99

Data were expressed as mean +/- SD, \* $P < 0.05$

(ALT= Alanine amino transferase, ALP= alkaline phosphatase, bilir. = Bilirubin, FBS= Fasting blood sugar, TP= Total protein, ALB= Albumin, CHO= Cholesterol)

#### 4.5.4 Histopathologic findings

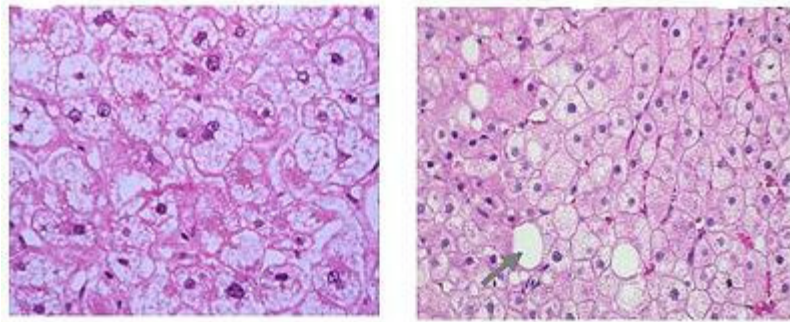
Different histopathologic findings with different degrees of presentation (focal/uniform) were observed. The finding includes dilated and congested sinusoids, yellowish-green pigmentation; at the perivenular and periportal areas, inside the macrophage and

hepatocytes (Plate III a, b). No sample with hepatocyte necrosis, fatty liver infiltration or proliferative conditions of the liver was found.

The finding from and around the area of the mass observed at gross morphologic evaluation showed well delineated (capsulated) circular structure with empty central space and thin membrane like lining. This capsular structure is formed by proliferating fibroblasts. Infiltration of inflammatory cells such as lymphocytes (marked), neutrophils, eosinophils and plasma cells was noticed in the area near to and inside this structure. Loss of hepatocytes in the surrounding was also noted (Plate IV). Histiocytes were seen attached to the inner lining wall. Table 4.8 shows the number of animals with the particular finding in the respective group.

Table 4.8, Histopathologic features seen at the end of the study period.

Histopathologic finding	Dose of the extract (mg/kg)							
	Control		200		1000		2000	
	Male (n=5)	Female (n=4)	Male (n=5)	Female (n=5)	Male (n=5)	Female (n=5)	Male (n=4)	Female (n=5)
Dilated and congested sinusoids	0	2	1	0	1	0	0	0
Yellowish-green pigmentation	2	1	2	5	1	5	0	5
Infiltration with inflammatory cells	0	2	3	3	0	0	1	0



A

B

Plate II a, types of steatosis; a) microvesicular b) macrovesicular (taken from Medscape).

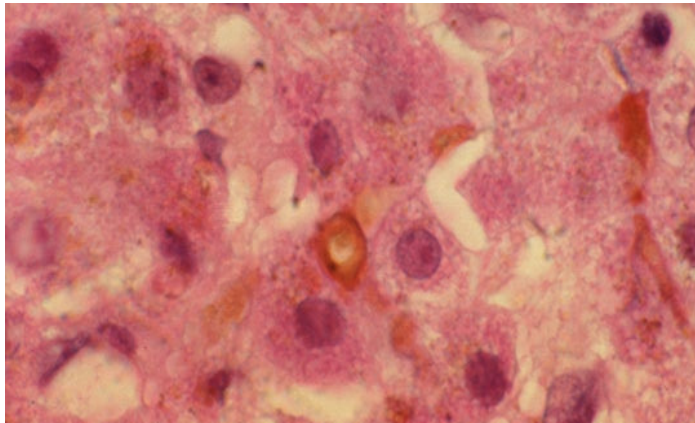


Plate II b, Bile spillage into the surrounding tissue seen in cholestatic change of the liver (taken from Medscape).

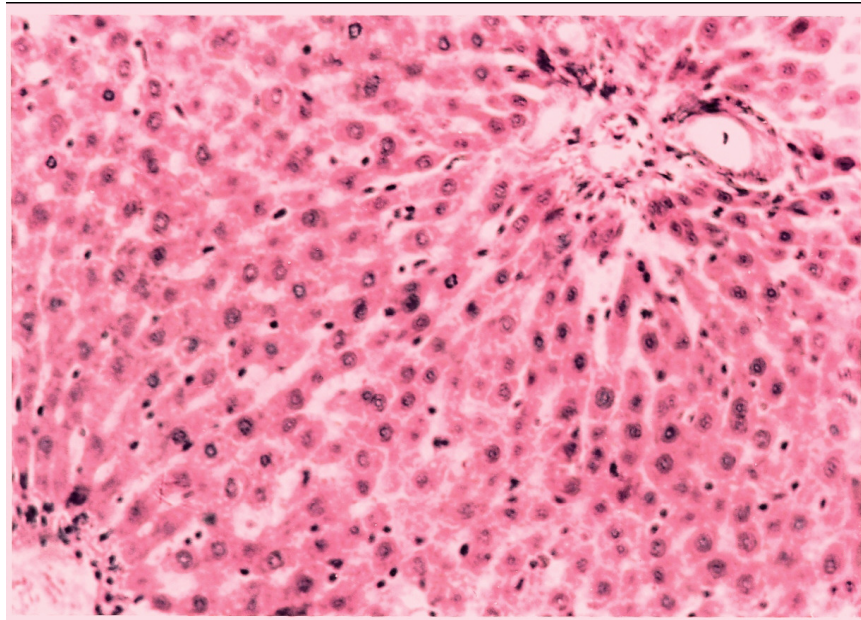


Plate III a, normal hepatic histology finding (central vein and portal triad) seen in rats administered with *T. serrulatus* for a period of 90 days, hematoxyline-eosine stain, (250 X).

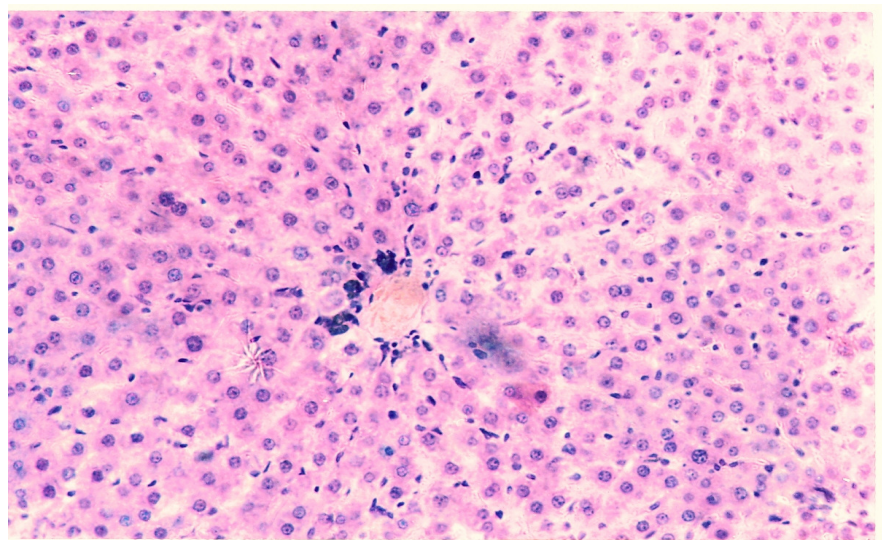


Plate III b, centrilobular, cholestatic change of liver seen in rat fed with *T.serrulatus* for 90 days, hematoxyline-eosine stain, (250 X).

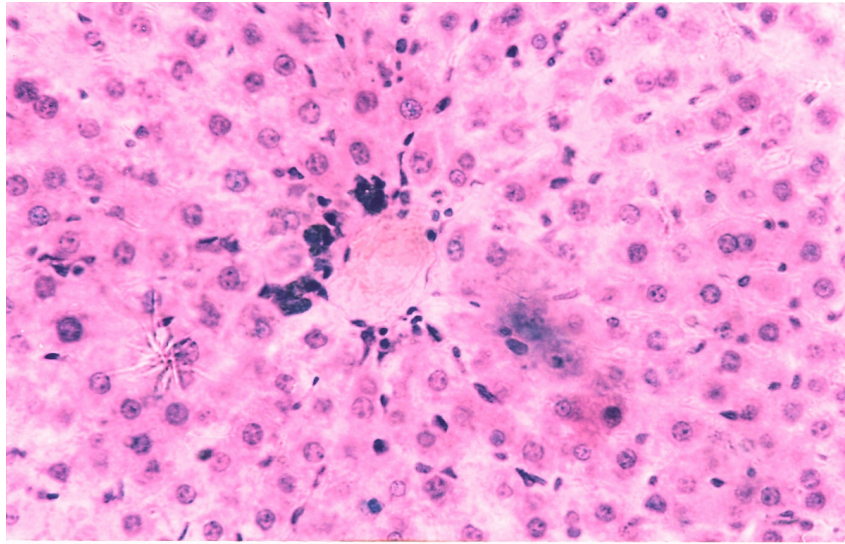


Plate IV a, centrilobular cholestatic change of liver seen in rat fed with *T.serrulatus* for 90 days, hematoxyline-eosine stain, (400 X).

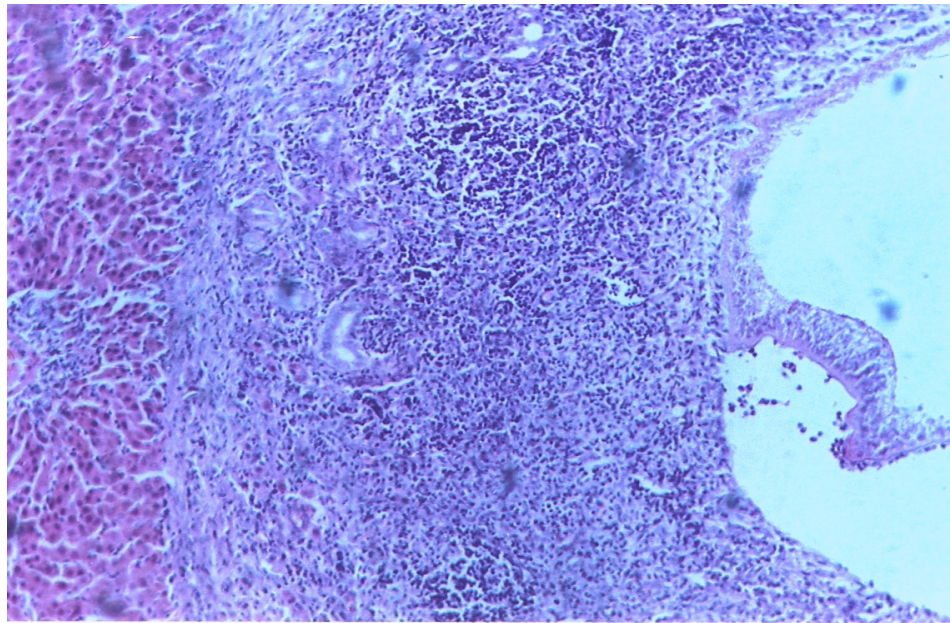


Plate IV b, parasitic wall like structure seen in the liver of rats infected with? Cestode.  
Hematoxyline-eosine stain (100 X).

## **5. Discussion.**

### **5.1 Phytochemical screening**

Phytochemical screening result showed presence of polyphenols and phytosterols. The fact that the preservation of the scent of the plant and the identification of different compounds of polyphenols in the aqueous extract could tell the possible presence of monoterpenes, thymol being the major component of *T.serrulatus* (Dagne *et al.*, 1998).

Different phytosterol compounds are the other classes of compounds found in the extracts of this plant. These are common findings in many plants. They have steroidal skeleton like cholesterol, ethynyl estradiol and many natural hormones. The similarity in structure between phytosterols and cholesterol made phytosterols act as competitive inhibitor of cholesterol at intestinal absorption site. In so doing, it decreases the serum cholesterol level in humans and the cardiac complications associated with it (Law, 2000).

### **5.2 *in vivo* spasmolytic test**

In this study both the aqueous and organic extracts showed spasmolytic activity, which is comparable to the known spasmolytic agent, atropine when given at a dose of 10 mg/kg, orally? This might justify its use as spasmolytic agent in folk medicine.

Thymol, which is lipid-soluble compound, is the active constituent of thymus and it is incriminated for the spasmolytic activity noticed by this plant. In this study no statistical difference was noted in the effect among the respective doses of the aqueous and methanolic extracts of *T.serrulatus*. This comparable effect in spasmolysis suggest the presence of water soluble compounds that are as effective as the active component thymol or the presence of thymol as described earlier.

The mechanism for muscle relaxant effect of thymol was studied on different muscle types. Closure of the calcium ion ( $\text{Ca}^{2+}$ ) channel (L-type) on the myocytes surface thereby decreasing the entrance of  $\text{Ca}^{2+}$  ions into the cell was supposed to be one of the possible mechanisms. Accordingly, Fusi, *et al* (2001) showed the inhibitory effect of some sterically hindered phenol compounds on calcium current, which is qualitatively similar to that of calcium channel blockers using guinea-pig gastric fundus. This

inhibition was seen to be partially reversible. Szentandrassy, *et al* (2003) also studied the mechanism by which thymol causes relaxation on isolated rat skeletal muscle and found marked suppression of calcium and potassium currents at different concentrations. The closure of potassium channels was observed at a lower concentration than that of calcium channels.

Reeve *et al* (1995) showed the opposing effect of oxidant and antioxidants on the potassium channel of rat vascular smooth muscle. Hence part of the effect of thymol on smooth muscle relaxation may also be explained by its antioxidant effect.

The inhibitory effect seen in the present study was not dose dependent. From the mechanism for spasmolysis stated earlier, as the dose (concentration) increases the effect also should increase. But it is difficult to explain this phenomenon.

Interestingly, the taenicidal effect of this plant was demonstrated by Belachew (1995) on human beings. In this regard the spasmolytic effect noticed in this research combined with its taenicidal effect could make this herb the choice in treating taeniasis as some other conventional taenicidal medicines frequently cause abdominal cramp as side effect.

### **5.3 Acute toxicity**

Study of acute toxicity is done to see the effect of acute exposure of an individual to high doses of chemical/drug. This acute exposure could happen accidentally or intentionally.

In general, acute oral exposure of a drug/chemical exceeding 5 gm/kg is associated without development of toxicity signs, including death, is considered safe (Ghosh, 1984).

In this study oral exposure of the test animals greater than 5 gm/kg was associated with neither development of any sign of toxicity nor death. This suggests that the plant is safe for oral use in terms of its acute toxicity effect when taken at large dose.

However, the LD<sub>50</sub> determined in this study for both aqueous and methanolic extracts were far less (less than fifty percent) than those previously reported by Belachew (1995). This could be due to the different seasons and areas of collection of the plant material, though not reported on the previous research. The other reason could be the change in the

concentration of constituent of the plant through time. The difference in the procedures employed could as well contribute to the discrepancy in the LD<sub>50</sub>.

In addition the behavior of the animals used in this study could be different from those used in the previous study or could change through time. The accidental parasitic infection noticed in this study could also be the reason for this discrepancy in LD<sub>50</sub>.

#### **5.4. Subchronic toxicity**

##### **5.4.1. Dose selection**

The dose for subchronic administration is based on the effective dose of the chemical/drug in fifty percent of the population (ED<sub>50</sub>) for a particular effect that chemical/drug shows. The doses for subchronic study are then calculated as twice, five times and ten times that of the ED<sub>50</sub>. However, no data regarding ED<sub>50</sub> of *T.serrulatus*, for any of its claimed activities, were available. Hence, the ED<sub>50</sub> of *T.serrulatus* in this study was considered to be the spasmolytic study dose. This dose was found to be 100 mg/kg. As a result 200 mg/kg, 1000 mg/kg and 2000 mg/kg were chosen as the three doses to be administered for the subchronic study.

##### **5.4.2 Clinical observation and body weight measurement**

Clinical observation of the animals in subchronic study is to see if there are signs and symptoms which could tell about the health condition of the animals possibly brought about by the administered substance. These observations and other results seen at the end of the study period will later be used to explain the effect observed.

The prominent observed signs and symptoms in the subchronic study were occasional diarrhea and passage of proglottid, irrespective of the group. The commonest parasitic infection in wild but not laboratory rats are cestodes. Three Hymenolopis species are identified; *H.nana*, *diminuta* and *microstoma*. Infection is by oro-fecal transmission. This infection is usually asymptomatic but when there is heavy load of infection loss of appetite, diarrhea and weight loss may be seen. Death of the animal is also expected. The laboratory animals could acquire this infection before separation from the older rats that already have the infection, from the infected pellets and water they were fed or from the wild animals they could come in contact with (Jan McArthur, 1999). Diagnosis is made

by microscopic examination for the ova or identification of the adult worm in the intestine. Hence infection with one of the cestodes is the most likely reason for the diarrhea and passage of proglottid with the fecal matter.

The biological rationale behind analyzing both organ weight and organ weight to the body weight ratio (relative organ weight) is that the weight of organs of the animal, except for brain, will change in proportion to the whole body weight (Hothorn and Hajian, 1999). Hence a change in this parameter could tell about the effect or the adverse effect a chemical/drug could bring on that particular organ.

In this study most of the groups have gained weight and the change in weight (weight gain) was statistically significant ( $P < 0.001$ ). On the other hand the female control group lost weight and this loss of weight was statistically significant ( $P < 0.001$ ). The loss of weight could be attributed to the possible cestode infection as described earlier. But this loss in weight was not seen in the male control groups. At the same time no liver mass was noticed in this group on gross morphologic evaluation at necropsy. Hence it is possible to say that the animals in this group were having mild infection or they are not at all infected with this parasite. In this regard microscopic examination of the fecal matter of the animals for the presence of the ova and its load of infection, if at all present, would have cleared this issue.

During the study period two animals were found dead. Part of the bodies of both animals, including the liver, was lost. But no signs of illness, except stated earlier, were noticed on the day prior to their death. Though difficult to conclude, the cause of death of the animal from the female control groups could be related to the same reason for the gross body weight loss, as stated earlier. But it is difficult to comment on the cause of death of the other animal.

#### **5.4.3 Gross morphologic evaluation of the liver**

Gross morphologic evaluation parameters consist of the color, the architecture, the consistency, and the absolute and relative weight of the liver. It gives clue to the possible

pathological processes and etiologies to cause a specific liver disease. For example, yellowish big liver could be due to steatosis where as shrunken and irregular liver could mean cirrhosis.

In this study, gross liver morphologic evaluation revealed presence of white, different sized, cystic, firm to hard masses on the different lobes of the liver. The big masses were noticed in the female control groups and had worm like structure inside the mass.

Etiologies for mass on the liver in humans could range from infections by different organisms, such as bacteria (liver abscess) and parasites (Amoeba and Ecchinococcus), to tumors arising from the liver, such as cysts (benign) and hepatoma (malignant). The occurrence of these masses is associated with clinical signs and symptoms. Diagnosis is by combining the associated signs and symptoms with the description of the mass, the epidemiology of the etiologic agents and laboratory investigations including histologic examinations.

Rats act as an intermediate host for the cat tapeworm, *T.teaniaeformis*. Infection of rats with this parasite upon ingestion of the ova excreted with the faeces of infected cat leads to formation cysticercus on the liver, which is a pea sized cystic liver mass. As an intermediate host the adult form of this cestode will not be formed in rats. But passage of proglottid noticed in the animals studied may suggest co-infection. From what was seen in the clinical observation, the description of the mass at gross morphologic evaluation and histopathologic finding, which will be discussed later, the cause for the liver masses found could be the parasitic infection with cestodes.

Considering the greater size in liver mass and the weight loss in the female control groups, and demonstration of the taenicidal effect of this plant by Belachew (1995) one may suggest the partial protective effect of the plant extract on the possible cestode infection. Further experimental study on the antiparasitic activity, especially on cestodes, of this plant would clear this doubt.

Higher relative liver weight was observed in the female control group, but this was not statistically significant from the rest of the groups ( $P < 0.05$ ). Considering the loss of gross

body weight and the possible increase in liver weight of the two animals in the group due to bigger masses noticed at necropsy, this increase in relative liver weight is expected.

#### **5.4.4 Clinical chemistry**

Clinical chemistry tests are done to evaluate effect of a chemical/drug on the functional status of the different organs and systems of the body. In subchronic studies the clinical chemistry evaluation is done not only at the end but also at different times during the study period. This will help pick those abnormalities that could normalize without discontinuing the offending chemical/drug. The results for all the liver function test done in this study, including alanine amino transferase, alkaline phosphatase, direct and indirect bilirubin, and albumin, for treatment groups was not stastically different from those of the controls. This could suggest the safety of the plant extract to the liver after exposure for long period, even at higher doses. Renal function test was also done to see the effect of the extract on the renal system but no statistically significant difference was seen between the controls and the treatment groups. The only result with statistically significant difference between those taking medium dose (in both male and female) and the rest of the group was fasting blood sugar (FBS). It is difficult to explain this phenomenon. Hence further experimental test on diabetic-induced animals is needed to clarify it.

Free phytosterols are known to decrease the serum and liver levels of cholesterol mainly by competing for absorption at the intestinal surface. Hayes, *et al* (2002) demonstrated cholesterol lowering effect of phytosterols by using Gerbils as animal model. Rath and Walkey, (1987) showed infection of mice with one of the cestodes of rat, *H.microstoma*, to be associated with a decrease in the serum cholesterol level. Owing to this the serum cholesterol level is expected to decrease, may be in all groups. And the result in our study showed no statistically significant difference between any of the groups.

No statistically significant difference was seen between the potassium, calcium and sodium ion concentrations among the different groups suggesting the absence of abnormal effects of the plant extract on serum electrolytes.

In general the absence of abnormal clinical chemistry results between the control groups and the treatment groups may suggest the possible safety of the plant extract in the different systems of the body.

#### **5.4.5 Histopathologic findings**

Long-term total parenteral nutrition (TPN) treatment in children with short bowel syndrome (SBS) has been associated with development of cholestasis of the liver. Moss and Amii, (1999) suggested two hypotheses to explain the occurrence of this phenomenon. The first hypothesis suggests a combination of altered gut hormone production and endotoxins produced by bacterial translocation due to enteral fast during this treatment as a possible cause for cholestasis while the second implicates the direct toxicity of TPN solution, such as intralipid amino acids and phytosterols. More and more studies suggest the presence of phytosterols as a cause for the development of cholestasis. Clayton, *et al* (1998) observed correlation between the increased serum level of phytosterols and development of cholestasis in children with TPN for SBS. Lead by this Iyer, *et al* (1998) demonstrated the presence of the same relationship in animal model using pig neonates and concluded that contaminants of commercial lipid emulsions (phytosterols) as a possible cause of cholestasis. This idea was further supported by Colomb, *et al* (2000), in a retrospective study, which showed the temporal relationship between changes in the lipid delivery and the development of this complication in more than half of the children's record in that study.

In the present study the striking histologic finding was the accumulation of contents of bile in the perivenular, periportal, intrahepatic and intramacrophage. Most of these histologic results were seen in the treatment group. The presence of three different phytosterol compounds in this extract could be the reason for the cholestatic change of the liver in the treatment group. The presence of this feature in the normal female controls could be explained by the obstruction to the bile transport pathways resulting from the mass effect leading to accumulation of bile. However, the presence in the control male groups could not be explained.

Despite the presence of cholestasis, neither associated cell death nor abnormalities in the liver enzymes were noticed in this study. The reactive compounds found in the bile contribute for the hepatocyte cell death. Antioxidants have shown protection against hepatocyte cell death associated with cholestasis. Hence the antioxidant effect of the thymol present in the extract might be responsible for the protection against cell death in the present study.

The other histologic finding was similar to that of chronic infectious processes caused by parasitic infections of the liver. Passage of proglottid noticed during the study period by the animals seems to be related with this histologic finding. One of the commonest parasitic infections in rodents is hymenolopiasis. It is caused by three species. One of the species, *H.microstoma*, resides in the liver, bile duct and pancreas of rodents. But its association with development of cystic liver mass is not described. The co-infection of the rats with the cat tapeworm, as described earlier, could be the reason for these findings.

## 6. Conclusions

- *T. serrulatus* has remarkable spasmolytic activity on gastrointestinal smooth muscle.
- Both aqueous and methanolic extracts of *T. serrulatus* appear to be safe upon acute exposure of higher doses orally.
- The ip LD<sub>50</sub> value found in this study is much lower than the one done previously for the same plant extracts.
- *T. serrulatus* may cause cholestatic change of the liver upon subchronic oral administration of the aqueous extract.

## 7. Recommendations

- Studies on the taenicidal effect of this plant in laboratory animals should be carried out.
- Investigation on the specific compound responsible for the cholestasis should be performed.
- Further *in vitro* studies on ascertaining cholestasis and the mechanism by which it occurs after administration of this plant subchronically should be pursued.

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## ANNEX

### Annexes-I

Preparation of 10 % buffered neutral formalin solution.

1. Formaldehyde, 37% - 40%.....100.00 ml
2. Distilled water.....900.00 ml
3. Sodium phosphate, monobasic.....4.0 gm
4. Sodium phosphate, dibasic (anhydrous).....6.5 gm

The PH at the end of preparation ranges between 6.7 and 7.0

### Annex- II

Tissue processing (Total processing time is 3 – 4 hours)

1. Rinse briefly in running water.
2. Hold in 80% alcohol.
3. 95% alcohol, 3 changes, 15 - 20 minutes each.
4. Absolute alcohol, 3 changes, 15 minutes each.
5. Equal parts of absolute alcohol and xylene, 15 minutes.
6. Xylene, 2 changes, 15 minutes each.
7. Paraffin, 3 changes, 15 minutes each.
8. Paraffin embedding.

## Annex- III

### Preparation of Baker's adhesive albumen

1. Egg white	50 cc
2. Distilled water	50 cc
3. NaCl	0.5 gm
4. Thymol	0.1 gm

The egg white is added after the other ingredients were well mixed. This is further centrifuged using 3000 rpm. The supernatant is used as adhesive. It is stored in refrigerator.

## Annex- IV

### Hematoxylen-eosin staining

1. Deparaffinize, the sectioned tissue by passing it through xylene, 2 changes, 2 minutes each.
2. Rehydrate the tissue, 2 changes of absolute alcohol, 95% alcohol, 80% alcohol, 50% alcohol and distilled water; 2 minutes each.
3. Ehrlich's Hematoxylin, 15 minutes.
4. Wash in running water, 2 – 5 minutes.
5. Differentiate it by dipping in 1% acid alcohol, 1 –2 dips.
6. Briefly wash it in running water.
7. Put it in a bluing solution, weak (0.1 %)  $\text{NaHCO}_3$ , till the tissue gets bright blue.
8. Wash thoroughly in running water, 10 minutes.
9. 80 % alcohol, 1 – 2 minutes.
10. Counter stain in Eosin, 2 – 5 minutes.
11. Wash briefly in running water.
12. Rehydrate, 50% alcohol, 80% alcohol, 95% alcohol, absolute alcohol; each for 2 minutes.
13. Xylene, 2 changes.

