Chapter-4
Studies on *Bauhinia variegata* Linn Bark Extract
CHAPTER 4
STUDIES ON BAUHINIA VARIEGATA LINN BARK EXTRACT

4.1 RESEARCH OBJECTIVES

*Bauhinia variegata* (*Leguminosae*) is a medium sized deciduous tree, bark dark brown, nearly smooth. Young shoots brown pubescent. Leaves 10-15 cm long, broader than long, cleft 1/4 to 1/3 of the way down into 2 obtuse lobes, pubescent beneath when young. Flowers large, fragrant, white or purplish, appearing when the tree is leafless [1].

*Bauhinia variegata* Linn is a widely used plant in Ayurvedic medicine for the treatment of several illnesses such as leucoderma, leprosy, menorrhagia, asthma, wounds and ulcers. In various indigenous systems of medicine, bark of the plant is described as astringent to the bowels, tonic to the liver [2, 3]. Despite its extensive use in folk medicine a few phytochemical studies aimed at analyzing the chemical constituents of bark of this plant have been performed until now and there are still no scientific data in the literature which clearly demonstrate the existence of a hepatoprotective and anticataract activity of the bark. Therefore the purpose of the study was:

1. To evaluate potential anticataract activity of the ethanolic extract of *Bauhinia variegata* Linn bark and its fractions.
2. To estimate the hepatoprotective effect of ethanolic extract of *Bauhinia variegata* Linn bark and its fractions.
3. To isolate and identify compounds in the studied extract.

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIAL

Stem bark of *Bauhinia variegata* Linn was collected from G. G. University campus in October 2005 and was authenticated by plant taxonomist Dr. C.
Rajasekharan. A voucher specimen (SLT-Med. Plant. -721) was deposited in the S. L. T. Institute of Pharmaceutical Sciences, Guru Ghasidas University, Bilaspur (Chhattisgarh, India).

**Preliminary Phytochemical analysis of *Bauhinia variegata* L. bark:**

Qualitative phytochemical analysis of *Bauhinia variegata* L. bark powder was tested as follows: **Tannins** (200 mg plant material in 10ml distilled water, filtered). A 2ml filtrate + 2ml FeCl₃, blue-black precipitate indicated the presence of Tannins. **Alkaloids** (200 mg plant material in 10ml methanol, filtered). A 2ml filtrate + 1% HCl + steam, 1ml filtrate + 6 drops of Wagner’s reagent. Brownish-red precipitate indicated the presence of alkaloids. **Saponins** (frothing test: 0.5 ml filtrate + 5 ml distilled water. Frothing persistence meant Saponin present). **Cardiac Glycosides** (Keller-kiliani test: 2 ml filtrate + 1 ml glacial acetic acid+ FeCl₃ + conc. H₂SO₄). Green-blue color indicated the presence of cardiac glycosides. **Steroids** (Liebermann Burchard reaction: (200 mg plant material in 10ml chloroform, filtered). A 2 ml filtrate + 2 ml acetic anhydride + conc. H₂SO₄. Blue-green ring indicated the presence of terpenoids. **Flavonoids** (200 mg plant material in 10 ml ethanol, filtered). A 2 ml filtrate + conc. HCl + magnesium ribbon. Pink-tomato red color indicated the presence of flavonoids. Glycosides were tested by Borntragers test. The preliminary qualitative phytochemical analysis of *Bauhinia variegata* L. bark indicated the presence of tannins, alkaloids, saponins, flavonoids and glycosides. The extractive value of *B. variegata* bark was 12.5%.

**4.2.2 EXTRACTION AND ISOLATION**

The stem bark (2.5kg) was separated from wood, air-dried, powdered and was exhaustively defatted with light petroleum ether (60-80°). The petroleum free mass was then extracted with 70% aqueous ethanol. The ethanol extract was concentrated under reduced pressure in vacuum and dried. The dried extract left a blood red brown substance (275 g). A small part of the crude ethanolic extract (SBE, 15g), obtained from *Bauhinia variegata* bark, was fractioned following the modified Kupchan method [4] as is explained on page 149. Following the pharmacological profile the series of purifications were carried out. The obtained fractions (Chloroform fraction: CSBE and methanol fraction: MSBE) were subjected to biological studies.
and were also subjected to column chromatography over various adsorbents with various solvents in order of their increasing polarity (Scheme 6.1). The crude ethanolic extract (SBE) and fractions (CSBE and MSBE) were tested for anticataract and hepatoprotective activity.

4.3 ANTICATARACT ACTIVITY

India shoulders the largest burden of global blindness. Almost 1.5 million of our 1.4 billion people are visually challenged. The cumulative loss to the country’s GNP is estimated to be about US $ 11.1 billion. The three most common diseases causing visual impairment in our country are cataract, retinal disease and glaucoma. According to a large epidemiological study called the Andhra Pradesh Eye Disease Study (APEDS) conducted by the LVPEI (Hyderabad), cataract accounts for 44% of blindness in the population studied.

Cataract formation cannot be prevented or reversed [5] and can only be cured by surgical replacement of the lens. There have been significant advances in surgical techniques and refinement of intraocular lens implants which have benefited cataract patients [6]. The whole procedure takes only 20 minutes and can be performed under local anaesthetic. More than 1 million cataract operations performed annually in the United States [7] account for over $3 billion in annual expenditure [6]. Thus, although surgical removal of the afflicted lens accompanied by ocular lens implant is highly successful in restoring sight, the procedure is costly, accounting for 12% of the US Medicare budget during the evaluation in 1992 [7]. Estimates indicate that delaying the onset of cataract by ten years could reduce the need for surgery by 45%, thus saving billions of dollars [8]. Therefore, it is desirable to avoid surgery if possible.

In the developing world, where medical services are often unavailable or extremely limited, the situation is even worse. In India alone around 30 million people suffer from cataract [5]. Thus, the expense and unavailability of surgery mean that nonsurgical medical therapy or nutritional treatment to inhibit the formation or slow
the progression of cataracts is an important goal in experimental eye research to benefit patients and reduce the huge economic burden.

Developing anti-cataract agents has been difficult because cataract is not a single disease with a single etiology. There are three major categories of cataract (nuclear, cortical, and posterior sub-capsule), each of which is multifactorial in etiology and highly variable in severity and rate of progression [6]. Further complicating the situation, the factors contributing to age-related cataractogenesis are a combination of pathological and normal aging processes, which have no obvious borders to distinguish them.

Cataracts may be prevented if the mechanisms of formation are known. Based on the available knowledge of the biology of the normal lens and the cataractogenic process, three hypotheses have been proposed for the etiology of cataract and three approaches have accordingly been adopted in the design of anti-cataract agents. The first hypothesis is that chronic oxidative stress is a major factor in the etiology of age-related cataract. Experimental evidence suggests that oxidative stress due to the generation of free radicals plays a role in the pathogenesis of cataracts and that the process can be prevented or ameliorated by antioxidants [9]. The second hypothesis is that “phase separation phenomena” is integral to cataract development [10]. Phase separation results from non-covalent attractive interactions between proteins in concentrated solutions, creating protein-rich and protein-poor regions. In the lens, formation of such domains creates light scattering, leading to cataract. Two putative phase separation inhibitors, pantethine and the radioprotective phosphorothioate WR-77913, were tested in several acute animal models of cataract and displayed the delay of the onset of cataract [6].
The third hypothesis is the "protease hypothesis". Calcium activated neutral enzymes, calpains, can induce proteolysis and truncate crystallins to precipitate and scatter light to form cataract [11]. Therefore, research on calpain inhibitors is another approach to prevent or inhibit cataract formation. In order to study cataracts and possible treatments for cataracts, a number of *in vitro* and *in vivo* animal models have been developed. Studies have established that a lens organ culture model system can be used to screen potential anti-cataract agents. The lens, which is avascular and non-innervated *in vivo*, can be maintained in a fully viable state in organ culture. Opacity can be induced in cultured lenses by various chemical or environmental perturbations, and prevention or inhibition of opacification can be observed after addition of
appropriate agents to counteract the cataractogenic stresses [6]. Sheep or ovine lenses are considered to be more appropriate models of the human lens than the rodent lenses commonly used for lens research.

![Diagrammatic representation of mammalian lens.](image)

**Figure 4.2: Diagrammatic representation of mammalian lens.**

### 4.3.1 ANIMAL MODELS

A role for oxidation as one of the contributing factors to cataract formation has been reported in several animal models including: selenite (an oxidant)-induced rat cataract model [12]; sugar xylose-induced cataract in rhesus monkey lens model [6]; diamide (a thiol-specific oxidant)-induced rat cataract model [13] and hydrogen peroxide-induced rat cataract model [14]. Cataract formation in developing chick embryo by glucocorticoids is widely used nuclear cataract model. When 15 day old chick embryos are administered with 100µg/egg of hydrocortisone acetate an opaque ring develops between the cortical region and the nucleus within 48 hrs [15]. Overall, there is evidence that oxidative damage in the lens is part of cataract formation in models of cataractogenesis.

### 4.3.2 SELECTING AN APPROPRIATE MODEL TO TEST ANTICATARACT ACTIVITY

In order to study cataracts and possible treatments for cataracts, a number of animal models have been developed including guinea pigs and rabbit [16], with rat and mouse being the most common models [17, 18]. However, sheep or goat lenses are more appropriate models of the human lens than the rodent lenses commonly used for lens research. The spherical rat lens with its very high and steep refractive index...
gradient is designed for use at close distances [19]. It cannot accommodate for distance viewing because it is too hard to deform and lacks the necessary accommodative apparatus. By contrast, the biconvex human lens, with its flatter refractive index gradient, is designed to focus further away. The soft tissue is amendable to deformation and this is used during accommodation to change the focal length [19]. The oval ovine lens resembles the human tissue. From the properties of the lens and the anatomy of its suspensory structure, it would be expected that the ovine lens is capable of accommodation. Therefore, the ovine cataract is an alternative model for cataractogenesis. Cataract formation in developing chick embryo by glucocorticoids is widely used nuclear cataract model. Therefore, this is another model for studying cataractogenesis. The decrease of free sulphhydryl (-SH), possibly glutathione, has been suggested to allow the lens protein to polymerize through the formation of disulfide bonds, resulting in loss of transparency [20, 21, 22]. Therefore, determination of glutathione content in lenses at 48 hr after hydrocortisone treatment gives the profile of alteration of sulphhydryl content in lenses which is closely related to that of cataract appearance and recovery from cataract [15].

4.3.3 CHEMICALS AND REAGENTS:
Glutathione, Ascorbic acid, Ellman's reagent and Hydrocortisone succinate were purchased from Hi Media Labs; Mumbai, India. Silymarin was obtained as gift sample from Micro Labs, Hyderabad, India. Other chemicals and reagents (Qualigens, Loba chemicals and CDH Chemicals, Mumbai, India.) used were of analytical grade.

4.3.4 OVINE MODEL FOR CATARACT STUDY:
4.3.4.1 Animals: Whole-eye globes of goat were removed immediately after slaughter were obtained from the local slaughterhouse and was transported in 100ml Tyrode solution to the laboratory. The lenses were dissected from the globes of the animal within an hour of death using a posterior approach. After dissection, the lenses were transferred to the Tyrode medium. The entire lens was submerged with its anterior epithelium upward in the medium.
4.3.4.2 Preparation of doses and administration of test materials: 5, 5'-dithiobis 2-nitrobenzoic acid, DTNB, was prepared as 4mg 10ml of trisodium citrate (Ellman’s reagent).

4.3.4.3 Experimental design: The lens protective effect was determined by culturing freshly excised goat lenses in a Tyrode medium containing H₂O₂ (0.5mM). Ninety lenses were divided into fifteen groups of six lenses each according to different treatment:

1. **Group I** (Positive Control): Six lenses were incubated in a 10ml Tyrode medium only.

2. **Group II** (Negative Control): Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM).

3. **Group III** (Standard): Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.1 mg ascorbic acid.

4. **Group IV**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.1 mg crude ethanolic *B. variegata* stem bark extract (SBE).

5. **Group V**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.2 mg SBE.

6. **Group VI**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.3 mg SBE.

7. **Group VII**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.4 mg SBE.

8. **Group VIII**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.1mg Chloroform fraction of ethanolic *B. variegata* stem bark extract (CSBE).

9. **Group IX**: Six lenses were incubated in a 10ml Tyrode medium containing H₂O₂ (0.5mM) and 0.2 mg CSBE.
10. **Group X:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.3 mg CSBE.

11. **Group XI:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.4 mg CSBE.

12. **Group XII:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.1 mg methanol fraction of ethanolic *B. variegata* stem bark extract (MSBE).

13. **Group XIII:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.2mg MSBE.

14. **Group XIV:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.3mg MSBE.

15. **Group XV:** Six lenses were incubated in a 10ml Tyrode medium containing \( \text{H}_2\text{O}_2 \) (0.5mM) and 0.4mg MSBE.

4.3.4.4 **Parameters measured**

Lenses were evaluated firstly for their ability to transmit light at different time intervals & by measuring the time taken for the lenses to become opaque \( (t_{op}) \) and secondly, for lens glutathione levels [23, 24].

4.3.4.4.1 **Assessment of Opacification**

As discussed above, all the lenses from different groups were photographed and evaluated for opacification by measuring the time taken for the lenses to become opaque \( (t_{op}) \) and lens protective effect by *B. variegata* against peroxide induced damage was calculated by comparing with standard and control group [23, 24].

4.3.4.4.2 **Assessment of Glutathione (GSH)**

This assay was based on the glutathione recycling method of Tietze, 1969 [25] by using 5. 5'-dithiobiis (2-nitrobenzoic acid) (DTNB) and glutathione reductase. DTNB and GSH reacted to generate 2-nitro-5-thiobenzoic acid and GSSG (oxidized glutathione i. e. glutathione disulphide). Since 2-nitro-5-thiobenzoic acid is a yellow
Figure 4.3: Normal lens

Figure 4.4: Opaque lens
Figure 4.5: Opaque lens

Figure 4.6: Opaque lens
colored product, GSH concentration could be determined by measuring absorbance at 412 nm.

Freshly excised goat lenses (six lenses in each group) were incubated in a petridish containing 4 ml of Tyrode solution having 0.5mM H₂O₂ with or without B. variegata extract (SBE, CSBE or MSBE), at 37°C for 3 hrs. The lenses were then homogenized in 1.0 ml of 50μm EDTA. The proteins were precipitated by treating with 1.0 ml of 10% TCA. To 0.5 ml of the supernatant, 4ml of 0.3M Na₂HPO₄ and 0.5 ml of DTNB (Ellman’s reagent) was added; and absorbance was measured at λₘ₃₄ 412 nm.

4.3.5 CHICK EMBRYO MODEL FOR CATARACT STUDY

4.3.5.1 Animals: Fourteen day old fertilized Australorp eggs were purchased from Government hatchery, Koni, Bilaspur (CG) and incubated for 24 hrs in a humidified incubator at 37°C (Jyoti Scientific Industries, Gwalior, M. P., India).

4.3.5.2 Preparation of doses and administration of test materials:
1. 2500μM of Ascorbic acid (Hi Media) was dissolved in 25ml of sterilized water just before use to get 20μM/0.2ml of ascorbic acid.
2. Hydrocortisone succinate was dissolved in sterilized water just before use. All other chemicals were of analytical grade available from commercial sources.

4.3.5.3 Experimental design: Cataracts were induced in chick embryos according to the method of Nishigori et al 1983 [15]. After 24 hrs incubation, at 15 days of age; hydrocortisone (0.25μM 0.2ml/egg) was administered to the chorioallantoic membrane of chick embryos through a small hole in the egg shell on the air sack. Ascorbic acid or B. variegata extract fraction were administered at 3, 10 and 20 hr after hydrocortisone administration at specified dose. The puncture was sealed with a cellophane tape and eggs were incubated for 48 hrs in a humidified incubator at 37°C. After 48 hrs the lenses were isolated for the determination of the extent of opacity and estimation of Glutathione.
Ninety 15 day old Australorp fertilized eggs were divided into fifteen groups of six eggs each according to different treatment:

1. **Group I** (Control): Six fertilized eggs were treated with sterilized distilled water (0.2ml/egg) and incubated for 48 hrs.

2. **Group II** (Negative Control): Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) and incubated for 48 hrs.

3. **Group III** (Standard): Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & ascorbic acid (20μM/0.2ml/egg) and incubated for 48 hrs.

4. **Group IV**: Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & crude ethanolic B. variegata stem bark extract (SBE, 0.1mg/0.2ml/egg) and incubated for 48 hrs.

5. **Group V**: Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & SBE (0.2mg/0.2ml/egg) and incubated for 48 hrs.

6. **Group VI**: Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & SBE (0.3mg/0.2ml/egg) and incubated for 48 hrs.

7. **Group VII**: Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & SBE (0.4mg/0.2ml/egg) and incubated for 48 hrs.

8. **Group VIII**: Six fertilized eggs were treated with hydrocortisone (0.25μM/0.2ml/egg) & Chloroform fraction of ethanolic B. variegata stem bark extract (CSBE, 0.1mg 0.2ml egg) and incubated for 48 hrs.

9. **Group IX**: Six fertilized eggs were treated with hydrocortisone (0.25μM 0.2ml egg) & CSBE (0.2mg 0.2ml egg) and incubated for 48 hrs.

10. **Group X**: Six fertilized eggs were treated with hydrocortisone (0.25μM 0.2ml egg) & CSBE (0.3mg 0.2ml egg) and incubated for 48 hrs.

11. **Group XI**: Six fertilized eggs were treated with hydrocortisone (0.25μM 0.2ml egg) & CSBE (0.4mg 0.2ml egg) and incubated for 48 hrs.
12. **Group XII:** Six fertilized eggs were treated with hydrocortisone (0.25\(\mu\)M/0.2ml/egg) & MSBE (0.1mg/0.2ml/egg) and incubated for 48 hrs.

13. **Group XIII:** Six fertilized eggs were treated with hydrocortisone (0.25\(\mu\)M/0.2ml/egg) & methanol fraction of ethanolic *B. variegata* stem bark extract (MSBE, 0.2mg/0.2ml/egg) and incubated for 48 hrs.

14. **Group XIV:** Six fertilized eggs were treated with hydrocortisone (0.25\(\mu\)M/0.2ml/egg) & MSBH (0.3mg/0.2ml/egg) and incubated for 48 hrs.

15. **Group XV:** Six fertilized eggs were treated with hydrocortisone (0.25\(\mu\)M/0.2ml/egg) & MSBE (0.4mg/0.2ml/egg) and incubated for 48 hrs.

### 4.3.5.4 Parameters measured

After 48 hrs of incubation the lenses were isolated for the determination of the extent of opacity and glutathione level.

#### 4.3.5.4.1 Assessment of Opacification

The extent of opacity was classified as normal (1), mild (2), moderate (3), severe (4) and most severe (5) according to Nishigori et al 1983 [15, 26]. As shown in figure 2.7 the lenses could be visually classified into three typical groups: Stage I (mild) lens with faint opaqueness or insignificant changes in the cortical region. Stage III (moderate) lens with an opaque ring between the cortical region and the nucleus, and Stage V (most severe) lens with an opaque nucleus. In addition to these classes, the lenses with more faint rings than Stage III and the lenses between Stage III and Stage V were called Stage II and Stage IV (severe) respectively. The faint ring between the cortical region and lens appears at 20 hrs and increases with time. After 30 hrs of hydrocortisone treatment about 75\(\%\) of the chick embryos had lenses classified as Stage III. Then the nuclear region begins to become opaque and after 48 hrs of hydrocortisone treatment about 30\(\%\) and 60\(\%\) of the lenses were classified as being at Stage III and Stage V respectively.
4.3.5.4.2 Assessment of Glutathione (GSH)

This assay was based on the glutathione recycling method of Tietze, 1969 [25]. After determination of extent of opacity individual lens was weighed and all the lenses from a specific group were homogenized in 1ml of ice cold 0.05 M citrate buffer (pH 4.0) and deprotenized by adding 1% metaphosphoric acid [26]. Glutathione was determined according to Ellman’s method [27]. Briefly 0.2 ml of supernatant was mixed with 2 ml of 0.3M Na$_2$HPO$_4$ and 0.5 ml of DTNB (Ellman’s reagent). The optical absorption of the resulting yellow colored compound was measured spectrophotometrically at $\lambda_{max}$ 412 nm and GSH content was calculated.
Figure 4.8: NORMAL LENS(C)
(Clear lens)

Figure 4.9: STAGE I
(Lens with faint opaqueness or insignificant changes in the cortical region)
Figure 4.10: STAGE III
(Lens with an opaque ring between the cortical region and the nucleus)

Figure 4.11:
STAGE V
(Lens with an opaque nucleus)
4.3.6 ANTIMICROBIAL ACTIVITY

The unique structure of the human eye as well as exposure of the eye directly to the environment renders it vulnerable to a number of uncommon infectious diseases caused by fungi and parasites. Host defenses directed against these microorganisms, once anatomical barriers are breached, are often insufficient to prevent loss of vision. Therefore, the timely identification and treatment of the involved microorganisms are paramount [28]. Any of the various parts of the eye can be infected. The etiological agents will vary with the type of tissue infected. Following are the most common eye infections [29].

Conjunctivitis: This is one of the most frequently occurring ocular infections caused by viruses. However, bacteria are the causative agents in as many as 80% of the cases. Staphylococcus aureus; Pseudomonas aeruginosa; Chlamydia; Proteus; H. influenzae are the pathogens most frequently causing conjunctivitis.

Blepharitis: Blepharitis is the infection of the eyelid and is one of the common ocular infections. It is usually caused by S. aureus.

Keratitis: This is an infection of the cornea and is usually due to Herpes simplex or Herpes zoster viruses. However bacteria can be the causative agent in immunosuppressed individual or in those who have suffered damage to the eye. in such cases S. aureus; S. pneumoniae; P. aeruginosa are usually involved.

Endophthalmitis: This is a serious infection of the posterior section of the eye that generally results in either partial or complete blindness. Staphylococcus epidermidis; S. aureus; Bacillus cereus are usually involved.

Orbital cellulitis: Orbital cellulitis is a serious infection of the orbital contents that can result in blindness and death. It is uncommon infection. S. aureus; S. pneumoniae; S. pyogen and H. influenzae are indicated.

Dacryocystitis: This is a disease involving one or more of the components of the tear drainage system. Members of the ocular micro biota may infect the system. S. aureus
and *S. pneumoniae* are indicated. The inflammation produced during infection often result in blockage of the nasolacrimal duct.

Therefore, antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* and *Bacillus cereus*; causative agents of majority of infective eye conditions, was determined by estimating the minimum inhibitory concentration (MIC). Antimicrobial activity was determined by making serial dilutions of the crude ethanolic *B. variegata* stem bark extract (SBE). Chloroform fraction of SBE (CSBE) and methanol fraction of SBE (MSBE) in double strength nutrient broth, and inoculating tubes with $1 \times 10^5$ viable cells from a 24 hr broth culture of *S. aureus* (MTCC 29737), *P. aeruginosa* (MTCC 424), *S. epidermidis* (MTCC 6535), *S. pyogenes* (MTCC 442) and *B. cereus* (MTCC 1305). The minimum concentration of the extract inhibiting the growth of microorganisms was taken as minimal inhibitory concentration (MIC).

4.4 HEPATOPROTECTIVE ACTIVITY

The liver is one of the largest and most important organs in the human body. It is located behind the lower right section of the ribs and carries out numerous functions that our body requires to remain healthy. Following are just a few of the liver's many functions:

- Storing important nutrients from the food that we eat.
- Building necessary chemicals that our body needs to stay healthy.
- Breaking down harmful substances, like alcohol and other toxic chemicals.
- Removing waste products from our blood.

Hepatotoxicity implies chemical-driven liver damage. The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may injure the organ. Other chemical agents such as those used in laboratories and industries, natural chemicals (e.g. microcystins) and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins. More than 900 drugs have been implicated
due to unique metabolism and close relationship of liver with the gastrointestinal tract, the liver is susceptible to injury from drugs and other substances. 75% of blood coming to the liver arrives directly from gastrointestinal organs and then spleen via portal veins which bring drugs and xenobiotics in concentrated form. Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria. Its dysfunction releases excessive amount of oxidants which in turn injures hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress [33]. Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver. This promotes further liver damage [34]. Non-parenchymal cells such as Kupffer cells, fat storing stellate cells and leukocytes (i.e. neutrophil and monocyte) also have role in the mechanism.

**Figure 4.12: Human Liver**
Patterns of injury

Chemicals produce a wide variety of clinical and pathological hepatic injury. Biochemical markers (i.e. alanine transferase; ALT, alkaline phosphatase; ALP and bilirubin) are often used to indicate liver damage. Liver injury is defined as rise in either (a) ALT level more than three times of upper limit of normal (ULN), (b) ALP level more than twice ULN, or (c) total bilirubin level more than twice ULN when associated with increased ALT or ALP [35, 36]. Liver damage is further characterized into hepatocellular (predominantly initial alanine transferase elevation) and cholestatic (initial alkaline phosphatase rise) types. However, they are not mutually exclusive and mixed type of injuries are often encountered. Specific histopathological patterns of liver injury from drug induced damage are discussed below.

Zonal Necrosis This is the most common type of drug induced liver cell necrosis where the injury is largely confined to a particular zone of the liver lobule. It may manifest as very high level of ALT and severe disturbance of liver function leading to acute liver failure.
**Hepatitis** In this pattern hepatocellular necrosis is associated with infiltration of inflammatory cells. There can be three types of drug induced hepatitis: (A) viral hepatitis type picture is the commonest, where histological features are similar to acute viral hepatitis. (B) in the focal or non specific hepatitis scattered foci of cell necrosis may accompany lymphocytic infiltrate and (C) chronic hepatitis type is very similar to autoimmune hepatitis clinically, serologically as well as histologically.

**Cholestasis** Liver injury leads to impairment of bile flow and clinical picture is predominated by itching and jaundice. Histology may show inflammation (cholestatic hepatitis) or it can be bland without any parenchymal inflammation. In rare occasions it can produce features similar to primary biliary cirrhosis due to progressive destruction of small bile ducts (Vanishing duct syndrome).

**Steatosis** Hepatotoxicity may manifest as triglyceride accumulation which leads to either small droplet (microvesicular) or large droplet (macrovesicular) fatty liver. There is a separate type of steatosis where phospholipid accumulation leads to a pattern similar to the diseases with inherited phospholipid metabolism defects (e.g. Tay-Sachs disease).

**Granuloma** Drug induced hepatic granulomas are usually associated with granulomas in other tissues and patients typically have features of systemic vasculitis and hypersensitivity.

**Vascular lesions** They result from injury to the vascular endothelium.

**Neoplasm** Neoplasms have been described with prolonged exposure to some medications or toxins. Hepatocellular carcinoma, angiosarcoma and liver adenomas are the ones usually reported.

Liver is the key organ of metabolism and excretion is constantly endowed with the task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Thus, disorders associated with this organ are numerous and varied. While a curative agent has not yet been found in modern medicine, the current usage of corticosteroids and immunosuppressive agents only brought about
symptomatic relief [37]. Furthermore, their usage is associated with risk of relapses and danger of side effects. Herbal medicines have been the oldest forms of healthcare. On the other hand, Ayurveda, an indigenous system of medicine in India, has a long tradition of treating liver disorders with plant drugs [38]. In Ayurveda many indigenous plants have been mentioned and well established as hepatoprotective agents. *Bauhinia variegata* (Leguminosae) bark is astringent to the bowel and tonic to the liver, is traditionally used for liver disorders [39]. Yet there is a paucity of scientific information regarding the activity of *Bauhinia variegata* in liver diseases. This study was undertaken to fill the lacuna in this regard.

### 4.4.1 ANIMAL MODELS

Hepatotoxicity from drugs and chemicals is the commonest form of iatrogenic disease. Some of the inorganic compounds producing hepatotoxicity are arsenic, phosphorus, copper and iron. The organic agents include certain naturally-occurring plant toxins such as pyrrolizidine alkaloids, mycotoxins and bacterial toxins. Following are various models used for screening hepatoprotective drugs.

1. **CCl₄-induced hepatotoxicity**

   Liver injury due to carbon tetra-chloride in rats was first reported in 1936 [40] and has been widely and successfully used by many investigators [41, 42]. Carbon tetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl₃O⁻, a reactive oxidative free radical, which initiates lipid peroxidation [43]. Administration of a single dose of CCl₄ to a rat produces, within 24 hrs, a centrilobular necrosis and fatty changes [40]. The poison reaches its maximum concentration in the liver within 3 hrs of administration. The development of necrosis is associated with leakage of hepatic enzymes into serum.

2. **Galactosamine induced hepatotoxicity**

   D-Galactosamine induced liver damage has been extensively used as an experimental model. Galactosamine produces diffuse type of liver injury simulating viral hepatitis. It presumably disrupts the synthesis of essential uridylate nucleotides resulting in organelle injury and ultimately cell death. Depletion of those nucleotides would impede the normal synthesis of RNA and consequently would produce a
decline in protein synthesis. This mechanism of toxicity brings about an increase in cell membrane permeability leading to enzyme leakage and eventually cell death. The cholestasis caused by galactosamine may be from its damaging effects on bile ducts or ductules or canalicular membrane of hepatocytes. Galactosamine decreases the bile flow and its content i.e. bile salts, cholic acid and deoxycholic acid. Galactosamine reduces the number of viable hepatocytes as well as rate of oxygen consumption [44].

3. **Thioacetamide induced hepatotoxicity**

Thioacetamide interferes with the movement of RNA from the nucleus to cytoplasm which may cause membrane injury. A metabolite of thioacetamide (perhaps s-oxide) is responsible for hepatic injury. Thioacetamide reduce the number of viable hepatocytes as well as rate of oxygen consumption. It also decreases the volume of bile and its content i.e. bile salts, cholic acid and deoxycholic acid [44].

4. **Alcohol induced hepatotoxicity**

Liver is among the organs most susceptible to the toxic effects of ethanol. Alcohol consumption is known to cause fatty infiltration, hepatitis and cirrhosis. Fat infiltration is a reversible phenomenon that occurs when alcohol replaces fatty acids in the mitochondria. Hepatitis and cirrhosis may occur because of enhanced lipid peroxidative reaction during the microsomal metabolism of ethanol. It is generally accepted that alcohol can induce *in vivo* changes in membrane lipid composition and fluidity, which may eventually affect cellular functions. Among the mechanisms responsible for effects of alcohol, an increase in hepatic lipid peroxidation leads to alteration in membrane phospholipid composition. The effects of ethanol have been suggested to be a result of the enhanced generation of oxyfree radicals during its oxidation in liver. The peroxidation of membrane lipids results in loss of membrane structure and integrity. This result in elevated levels of γ-glutamyl transpeptidase, a membrane bound enzyme in serum. Ethanol inhibits glutathione peroxidase, decrease the activity of catalase, superoxide dismutase, along with increase in levels of glutathione in liver. The decrease in activity of antioxidant enzymes superoxide dismutase, glutathione peroxidase are speculated to be due to the damaging effects of free radicals produced following ethanol exposure or alternatively could be due to a
direct effect of acetaldehyde, formed by oxidation of ethanol [45]. Alcohol pre-
treatment stimulates the toxicity of CCl₄ due to increased production of toxic reactive
metabolites of CCl₄, namely trichloro-methyl radical by the microsomal mixed
function oxidative system. This activated radical binds covalently to the
macromolecules and induces peroxidative degradation of membrane lipids of
endoplasmic reticulum rich in polyunsaturated fatty acids. This lipid peroxidative
degradation of biomembranes is the principle cause of hepatotoxicity [46].

5. Paracetamol induced hepatotoxicity

Paracetamol, a widely used analgesic and antipyretic drug, produces acute
liver damage in high doses. Paracetamol administration causes necrosis of the
central lobular hepatocytes characterized by nuclear pyknosis and eosinophilic
cytoplasm followed by large excessive hepatic lesion. The covalent binding of N-
acetyl-P-benzoquinoneimine, an oxidative product of paracetamol to sulphhydryl
groups of protein, result in lipid peroxidative degradation of glutathione level and
thereby, produces cell necrosis in the liver [46, 41].

6. Antitubercular drugs induced hepatotoxicity

Drug induced hepatotoxicity is a potentially serious adverse effect of the
currently used antitubercular therapeutic regimens containing Isoniazid (INH).
Rifampicin and Pyrazinamide. Adverse effects of antitubercular therapy are
sometimes potentiated by multiple drug regimens. Thus, though INH, Rifampicin and
Pyrazinamide each in itself are potentially hepatotoxic, when given in combination,
their toxic effect is enhanced. INH is metabolized to monoacetyl hydrazine, which is
further metabolized to a toxic product by cytochrome P-450 leading to hepatotoxicity.
Patients on concurrent rifampicin therapy have an increased incidence of hepatitis.
This has been postulated due to rifampicin-induced cytochrome P-450 enzyme-
induction, causing an increased production of the toxic metabolites from acetyl
hydrazine (AcHz). Rifampicin also increases the metabolism of INH to isonicotinic
acid and hydrazine, both of which are hepatotoxic. Pharmacokinetic interactions exist
between rifampicin and pyrazinamide in tuberculosis patients, when these drugs are
administered concomitantly. Pyrazinamide decrease the blood level of rifampicin by
decreasing its bioavailability and increasing its clearance. Pyrazinamide, in
combination with INH and rifampicin, appears to be associated with an increased incidence of hepatotoxicity [47].

As liver is a multifunctional organ, a battery of liver function tests are employed to evaluate the effect of drug on liver, which include estimation of Aspartate Transaminase (AST or SGOT), Alanine Transaminase (ALT or SGPT), Alkaline phosphatase, Serum bilirubin, Serum protein, Total proteins, morphological test like Wet weight of liver and histopathology of liver.

4.4.2 SELECTING AN APPROPRIATE MODEL TO TEST HEPATOPROTECTIVE ACTIVITY:

A number of pharmacological and chemical agents act as hepatotoxin and produce variety of liver ailments. Our experiment was designed to use carbon tetrachloride (CCl₄) intoxicated rat liver as model. The procedure, technique and biochemical estimations were carried by using the method of Venukumar and Latha 2002 [48].

4.4.3 CHEMICALS AND REAGENTS

All biochemicals and chemicals used for the experiments were of analytical grade and were purchased from HiMedia & Qualigens Fine Chemicals: Mumbai (India).

4.4.4 CCl₄ INDUCED HEPATOTOXICITY MODEL:

4.4.4.1 Animals: Male Sprague-Dawley rats weighing between 100-120 g were used in present study and were purchased from CCS Haryana Agriculture University, Hisar (Haryana, India). The animals had free access to food and water and were maintained under controlled temperature (27±2°C) and 12 h: 12 h light and dark cycle. Initial body weight of each animal was recorded.

4.4.4.2 Experimental protocol: Institutional Animal Ethics Committee (IAEC) had approved the experimental protocol and the care of animals was taken as per the guidelines of CPCSEA, Department of Animal Welfare, Government of India.

4.4.4.3 Acute toxicity studies: Acute toxicity study was performed as per OECD-423 guidelines. Sprague-Dawley female (nulliparous and non-pregnant) rats weighing
100-120 g between 8 and 12 weeks old were used. The animals had free access to food and water and were maintained under controlled temperature (27±2°C) and 12 h: 12 h light and dark cycle. Initial body weight of each animal was recorded. The test substance was administered in a single dose by an intubation canula. Three animals were used for each step. The test substance was administered orally at a doses ranging from 5- 2000 mg kg body weight. Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, mortality were observed. All the test animals were subjected to gross necropsy. No signs of toxicity were observed. The doses selected were 50, 100 and 200 mg kg [49].

4.4.4.4 Experimental design: Liver damage was induced in rats by administering CCl₄ subcutaneously (SC) in the lower abdomen in a suspension of liquid paraffin (LP) in the ratio 1:2 v/v at the dose of 1 ml CCl₄ /kg body weight of each animal. CCl₄ was administered twice a week, on every first and fourth day of all the 13 weeks.

Ninety six rats were divided into 12 groups of 8 animals each as follows:

1. **Group I** (Control): Animals served as control and received SC administration of LP only at the dose of 3 ml kg body weight, twice a week for duration of 13 weeks (89 days).

2. **Group II** (Negative Control): Animals were treated with SC administration of LP+ CCl₄ twice a week for a total of 13 weeks.

3. **Group III** (Standard): Animals were treated with SC administration of LP- CCl₄ twice a week and silymarin orally at the dose of 25 mg kg body weight daily for a total of 13 weeks.

4. **Group IV**: Animals were treated with SC administration of LP- CCl₄ twice a week and SBE suspension orally at the dose of 50 mg kg body weight daily for a total of 13 weeks.

5. **Group V**: Animals were treated with SC administration of LP- CCl₄ twice a week and SBE suspension orally at the dose of 100 mg kg body weight daily for a total of 13 weeks.
6. **Group VI**: Animals were treated with SC administration of LP+ CCl₄ twice a week and SBE suspension orally at the dose of 200 mg kg body weight daily for a total of 13 weeks.

7. **Group VII**: Animals were treated with SC administration of LP+ CCl₄ twice a week and CSBE suspension orally at the dose of 50 mg kg body weight daily for a total of 13 weeks.

8. **Group VIII**: Animals were treated with SC administration of LP+ CCl₄ twice a week and CSBE suspension orally at the dose of 100 mg kg body weight daily for a total of 13 weeks.

9. **Group IX**: Animals were treated with SC administration of LP+ CCl₄ twice a week and CSBE suspension orally at the dose of 200 mg kg body weight daily for a total of 13 weeks.

10. **Group X**: Animals were treated with SC administration of LP+ CCl₄ twice a week and MSBE suspension orally at the dose of 50 mg kg body weight daily for a total of 13 weeks.

11. **Group XI**: Animals were treated with SC administration of LP+ CCl₄ twice a week and MSBE suspension orally at the dose of 100 mg kg body weight daily for a total of 13 weeks.

12. **Group XII**: Animals were treated with SC administration of LP+ CCl₄ twice a week and MSBE suspension orally at the dose of 200 mg kg body weight daily for a total of 13 weeks.

Replenishing a known quantity of fresh food daily at 8.00 a.m. and thereby measuring the food intake of the previous day carried out measurement of daily food consumption. Body weight of rats was recorded weekly to assess percentage of weight gain of each animal. Animals were kept starved overnight on the 89th day. On the next day, after recording the weight of each animal, pentobarbitone (40 mg kg. i.p) was injected and the sleeping time recorded in all the animals. The same animals were sacrificed by decapitation by making an incision on jugular vein to collect blood. The liver tissue was dissected out, blotted off blood, washed in saline and weighed instantaneously to get the wet weight. This was kept in frozen containers and proceeded for biochemical estimations.
4.4.4.5 Parameters measured

**Biochemical parameters:** The specific and non-specific biochemical parameters which were known to be altered by hepatotoxins were measured as markers for evaluating hepatoprotective activity of *Bauhinia variegata*. Serum was prepared from the collected blood and subjected to biochemical estimations of different parameters like aspartate aminotransferase, AST [50], alanine aminotransferase, ALT [50], alkaline phosphatase, ALP [51], gamma glutamyl transpeptidase, GGT [52], total proteins [53] and total lipids [54]. Liver homogenates were also subjected to various biochemical estimations like total proteins and total lipids.

**Glutathione estimation:** To measure the reduced glutathione (GSH) level, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken. The procedure was followed initially as described by Ellman 1959 [55]. The homogenate was added with equal volume of 20% trichloroacetic acid (TBA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant (200 μl) was then transferred to a new set of test tubes and added 1.8 ml of the Ellman's reagent. Then all the test tubes were made up to the volume of 2ml. After completion of the total reaction, solutions were measured at 412 nm against blank [56].

4.4.5 HISTOPATHOLOGY

A portion of liver tissue in each group was fixed in 10% formalin (formalin diluted to 10% with normal saline) and proceeded for histopathology. Sections were stained with Ehrlich's hematoxylin and eosin.

4.4.6 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was applied for determining the statistical significance of difference in serum marker enzymes, protein and lipid levels (serum and liver) between different groups. Results were considered statistically significant at $P < 0.05$. 

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