EFFECT OF AQUEOUS EXTRACT OF *Lawsonia inermis* LEAVES ON THE LIVER FOLLOWING ACUTE ETHANOL-INDUCED HEPATIC DAMAGE IN WISTAR RATS

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DEPARTMENT OF HUMAN ANATOMY,
FACULTY OF MEDICINE,
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MAY, 2016

DECLARATION

I declare that the research work reported in this dissertation entitled '**Effect of aqueous extract of *Lawsonia inermis* leaves on the liver following acute ethanol-induced hepatic damage in Wistar rats**' has been carried out by me in the Department of Human Anatomy, Faculty of Medicine, A.B.U, Zaria, under the supervision of Dr. B. Danborno and Dr W. O. Hamman. The information derived from the literatures has been duly acknowledged in the text and a list of references provided. No part of this work was previously presented for another degree or diploma at this or any other institution.

Fareha MUZAFFAR	Signature	Date

CERTIFICATION

This dissertation titled EFFECT OF AQUEOUS EXTRACT OF *Lawsonia inermis* LEAVES ON THE LIVER FOLLOWING ACUTE ETHANOL-INDUCED HEPATIC DAMAGE IN WISTAR RATS by Fareha MUZAFFAR meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my father Engr. Muzaffar Ahmad, and to those afflicted with liver ailments.

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LIST OF ABBREVIATIONS

°C	centigrade
µg	microgram
µl	microlitre
A	absorbance
AELI	aqeous extract of *Lawsonia inermis* leaves
AO-EB	acridine orange – ethidium bromide
Bp	base pairs
BSA	Bovine serum albumin
Cat	Catalase
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffer saline
DPX	Distyrene Plasticizer Xylene
g	gram
GSH	Reduced glutathione
H&E	Haematoxylin and eosin
H_2O_2	hydrogen peroxide
L. inermis	*Lawsonia inermis*
M	Molar
MDA	Malondialdehyde
mg	milligram
Mins	minutes
Ml	millilitre
mM	millimolar
nm	nanometer

NSDUH	National survey on drug use and health
PBS	phosphate buffer saline
rpm	rotations per minute
s	seconds
SOD	Superoxide dismutase
TAE	triacetate acid
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TES	Tris buffer saline
U	international unit
V	volts
WHO	World health organisation

ABSTRACT

Lawsonia inermis commonly known as henna has been used traditionally, especially in ayurvedic medicine, for various conditions including liver ailments, and reported to have hepatoprotective properties. This study aims to study the effect of aqueous extract of *L. inermis* leaves in acute ethanol induced hepatic damage in adult Wistar rats. Thirty (30) female rats were equally divided into five (5) groups (I-V). Group I which served as negative control received distilled water (2 ml) for 8 days. Group II which served as positive control received 40 % ethanol (20 ml/kg) on the 8^{th} day after seven days of distilled water. Group III which served as prophylactic group received 400 mg/kg of aqueous extract of *Lawsonia inermis* for seven days, and 40 % ethanol (20 ml/kg) on the 8^{th}. Group IV served as the therapeutic group, and received 400 mg/kg of the extract for seven days after receiving 20 ml/kg of 40 % ethanol on the first. Group V received silymarin (70 mg/kg) for seven days before 20 ml/kg of 40 % ethanol on the 8^{th} day, to serve as the reference drug. All animals were sacrificed on the ninth (9) day. Body weight changes and liver body weight index were determined. Liver tissues were collected for assessment of oxidative stress markers (superoxide dismutase, catalase, reduced glutathione and malondialdehyde), protein concentration, DNA fragmentation, and also for haematoxylin and eosin staining, and acridine orange-ethidium bromide staining. Body weight increased in all groups from initial mean weight of 121.5 g, though significantly ($p<0.05$) only in Group I and Group IV. Liver body weight index was highest in Group II, and was significantly ($p<0.05$) different from Group I and Group IV. Ethanol administration reduced levels of Superoxide dismutase, catalase, reduced glutathione and caused an increase in lipid peroxidation (Malondialdehyde), though insignificantly. Increase in levels of Superoxide dismutase, catalase, reduced glutathione and decrease in lipid peroxidation (Malondialdehyde) was observed in the groups

receiving extract, more so prophylactically, though statistically insignificant. Silymarin significantly increased the level of Superoxide dismutase in comparison to Group II, levels of catalase, reduced glutathione, and Malondialdehyde were insignificantly less than Groups III and IV. Protein concentration was significantly higher in Group II, Group III and Group V, with Group II having the highest. Group III and Group V also differed significantly ($p<0.05$) from Group II. DNA fragmentation was observed to be most in Group II, while Group III and Group V had Fragmentation pattern comparable to that of Group I. H&E staining revealed attenuation of the effects of ethanol administration by the extract and silymarin, though the extract proved more effective prophylactically at 400 mg/kg dose and duration of seven days. Acridine orange-ethidium bromide (AO-EB) staining revealed reduced necrotic and apoptotic cells in Groups III, IV and V. The extract of *Lawsonia inermis* at 400 mg/kg for seven days proved to have more effective hepatoprotection prophylactically than therapeutically, and provided comparable hepatoprotection with that of silymarin.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Alcohol consumption is customary in most cultures and alcohol abuse is common worldwide. For example, more than 50 % of Americans consume alcohol and 23 % of Americans participated in heavy and/or binge drinking at least once in a month (Substance Abuse and Mental Health Services Administration, 2011). The most commonly recognized symptoms of alcohol consumption are associated with chronic alcoholism, and it is a casual/risk factor in about 60 major types of diseases (Massey and Arteel, 2012). These, and other effects of alcohol consumption, has made alcohol the third leading risk factor globally for disease and disability (WHO, 2011).

Acute Alcohol exposure or binge drinking is defined in National Survey on Drug Use and Health (NSDUH) 2010 as five or more drinks on a single occasion within a time period of three hours (Massey and Arteel, 2012). However, it can also include a period of heavy drinking that may span several days or periods of intermittent, repeated episodes of heavy drinking (Massey and Arteel, 2012). Reports by NSDUH show that more than 58 million Americans participated in binge drinking on at least one occasion within thirty days in 2010 (Substance Abuse and Mental Health Services Administration, 2011) and acute alcohol consumption is a major underlying cause of morbidity and mortality during hospital admittance (Jones *et al.,* 1991). Due to this, and increasing prevalence of binge drinking in people aged 18-25 (Massey and Arteel, 2012), greater interest has turned towards the effect of acute alcohol exposure.

The liver is an organ of immense complexity that has fascinated mankind since antiquity. It is essential for survival as no other organ can compensate for its multiplicity of functions (Sirica *et al*., 2008). Ethanol is toxic, and the body begins to dispose of it immediately upon its consumption (Shakashiri, 2009). The liver, and to a lesser extent, the gastrointestinal tract are the main sites of alcohol metabolism (Lieber, 1970), with 90 % of it occurring in the liver (Shakhashiri, 2009). The susceptibility of the liver to alcohol-induced toxicity is due to both the high concentration of alcohol found in the portal blood (versus systemic), as well as the metabolic consequence of ethanol metabolism (Massey and Arteel, 2012). Potential mechanisms by which acute alcohol consumption causes damage include steatosis, dysregulated immunity and inflammation, and altered gut permeability (Massey and Arteel, 2012).

Medicinal plants are a part of human society to combat diseases, from the dawn of civilization (Bandyopadhyay *et al*., 2002). Among the estimated 2,500,000 higher plant species on earth, only 35,000 to 70,000 species (less than 1%) have been used for medicinal purpose (Ponnu *et al*., 2003). Medicinal plants have contributed immensely to health care in Nigeria (Adebolu and Oladimeji, 2005). This is due in part to the recognition of the value of traditional medical systems, and the identification of medicinal plants from indigenous pharmacopoeias, which have significant healing power (Adebolu and Oladimeji, 2005). Plants provide an alternative strategy in the search for new drugs. There is a rich abundance of plants reputed in traditional medicine to possess protective and therapeutic properties. It is likely that plants will continue to be a valuable source of new molecules which may, after possible chemical manipulation, provide new and improved drugs (Shah *et al*., 2006). There are plenty of chances to find out a new compound of plant origin (Farnsworth, 1988), including those used as ornamentals, amongst which is *Lawsonia inermis* (Latha *et al*., 2004).

L. inermis commonly called henna is used all over the world, and is abundantly available in tropical and subtropical areas (Simon *et al*., 1984; Rao *et al*., 2005). Henna is a flowering plant, two to six meters in height. It's the sole specie in the genus *Lawsonia* in the family *Lythraceae* (Endrini *et al.,* 2012). Some common names of *L. inermis* in different languages are: Henna (English), lalle (Hausa), lali (Yoruba), mehndi/heena (Urdu), mehndi (Hindi). Ancient history of India describes diverse uses and appreciable role of henna in Ayurvedic or natural health medicines (Lavhate *et al*., 2007). It has been used both cosmetically and medicinally for over 9,000 years. Traditionally in India, it is applied to hands and feet as a symbol of fertility (Chopra *et al*., 1956; Chetty, 2008).

1.2 Statement of Problem

Alcohol consumption is on the rise and health care is becoming less affordable, especially to majority of people in the third world countries. Thus a cheap, readily available way for management of liver damage caused by alcohol consumption is needed.

1.3 Justification

Worldwide, about 16 % of drinkers engage in heavy episodic drinking, often referred to as binge-drinking, which is the most harmful to health (WHO, 2014). Alcohol consumption increases the risk of developing more than 200 diseases (WHO, 2011). Lower-income groups are most affected by social and health consequences of alcohol, they often lack quality health care and are less protected by functional family or community networks (WHO, 2014). An alternative way of management of liver damage that is cheap and affordable, as well as easily accessible can be developed especially for the places where poverty is prevalent and tertiary health care is less affordable, using a plant that is cheap and found almost all over the world.

1.4 Significance of Study

Medicinal plants may serve as vital sources of potentially useful new compounds for the development of effective therapies to combat a variety of health problems (Adebolu and Oladimeji, 2005). Different parts of *L. inermis* have been used traditionally in ayurvedic medicine in management and treatment of ailments including liver ailments (Lavhate *et al.,* 2007). This study will help to determine if *L. inermis* can be used to provide a cheap affordable and easily available way of management of liver damage caused by alcohol consumption.

1.5 Aim and Objectives of the Study

1.5.1 Aim of the study

This study aims to investigate the effects of *Lawsonia inermis* leaves in acute Ethanol-induced liver injury in rats.

1.5.2 Objectives of the study

The study will investigate the following:

i. changes in histology of the liver, in Wistar rats administered aqeous extract of *Lawsonia inermis* (AELI) leaves and rats not treated with AELI, in acute ethanol-induced liver injury, using haemotoxylin and eosin stains.
ii. the presence of apoptotic, necrotic and normal hepatocytes histologically, in Wistar rats administered AELI and rats not treated with AELI, in acute ethanol-induced liver injury, using acridine orange and ethidium bromide stains.
iii. changes in superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), and reduced glutathione (GSH) in the liver lysate of Wistar rats

administered AELI and rats not treated with AELI, in acute ethanol-induced liver injury, as markers of oxidative stress using antioxidant assays.

iv. the level of DNA damage in the hepatocytes, in Wistar rats administered AELI and rats not treated with AELI in acute ethanol-induced liver injury, using gel electrophoresis.

v. the changes in protein concentration of the hepatocytes in Wistar rats administered AELI and rats not treated with AELI in acute ethanol-induced liver injury, using Bradford reagent.

1.6 Hypothesis

Lawsonia inermis provides hepatoprotection in acute ethanol-induced liver injury.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Lawsonia inermis*

2.1.1 Taxonomy of *Lawsonia inermis*

Kingdom: Plantae-Plants

Division: Magnoliophyta

Class: Magnoliospida

Order: Myrtales

Family: Lythraceae

Genus: Lawsonia

Species: inermis (Jiny *et al.,* 2010)

2.1.2 Origin and distribution of *Lawsonia inermis*

Lawsonia is a monotypic genus, represented by *Lawsonia inermis*, native of North Africa and south-west Asia, widely cultivated as an ornamental and dye plant (Grieve, 2005; Ashnagar and Shiri, 2011). *L. inermis* (Lythraceae) is commonly called as Henna and the synonym is *L. alba* Linn. It is a glabrous, much branched shrub or small tree and grows wild. Henna is cultivated in many tropical and warm temperate regions as a hedge plant (Larson, 2005; Ashnagar and Shiri, 2011).

2.1.3 Local names of *Lawsonia inermis*

The different local names of *L. inermis* as listed in the Agroforestry database by Orwa *et al.,* 2009 are:

Amharic- *hina*

Arabic- *yoranna, hinná, hena, henna*

Bengali - *mendi, mehedi*

Burmese – *dan*

Creole - *flè jalouzi, ene*

English - *mignonette tree, henna tree, camphire*

Egyptian - *privet, Zanzibar bark*

Filipino – *cinamomo*

French - *jalousie, fleurs, henné, réséda de France*

Hausa - *Lalle*

Hindi – *mehndi*

Indonesian - *inai, pakar kuku*

Javanese - *pacar kuku*

Khmer – *krâpéén*

Lao (Sino-Tibetan) - *kaaw*

Malay -*inai, pacar kuku, hinna*

Sanskrit - *mendika, ragangi, raktgarbha*

Somali - *erip*

Spanish - *resedá, henna*

Swahili - *mheni, mhina, mkokoa, mkokoa, muhina, muina*

Tamil - *maruthani, marithondi*

Thai - *thian daeng, thian khaao, thian king*

Tigrigna - *hina, elam*

Vietnamese - *nhuôm móng tay, lâ mòn*

2.1.4 Morphology of *Lawsonia inermis*

L. inermis is a glabrous branched shrub or small tree (2 to 6 m in height) (Habbal *et al.,* 2005; Ashnagar and Shiri, 2011). Leaves are small, opposite, entire margin elliptical to broadly lanceolate, sub-sessile, about 1.5 to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, petiole short and glabrous acute or obtuse apex with tapering base (Ashnagar and Shiri, 2011). New branches are green in colour and quadrangular, turn red with age (Muhammed and Muhammad, 2005). Young barks are greyish brown (Rahmoun *et al.,* 2010), older plants have spine-tipped branchlets. Inflorescence has large pyramid shaped cyme. Flowers are small, numerous, aromatic, white or red coloured with four crumbled petals. Calyx has 0.2 cm tube and 0.3 cm spread lobes. The fruits are small, brown globose capsule, opening irregularly and split into four sections with a permanent style (Borade *et al.,* 2011; Deborah *et al.,* 2011). Seeds have typical, pyramidal, hard and thick seed coat with brownish coloration (Sastri, 1962; Vasudevan and Laddha, 2003; Habbal *et al.,* 2005; Chauhan and Pillai, 2007; Rahmoun *et al.,* 2010).

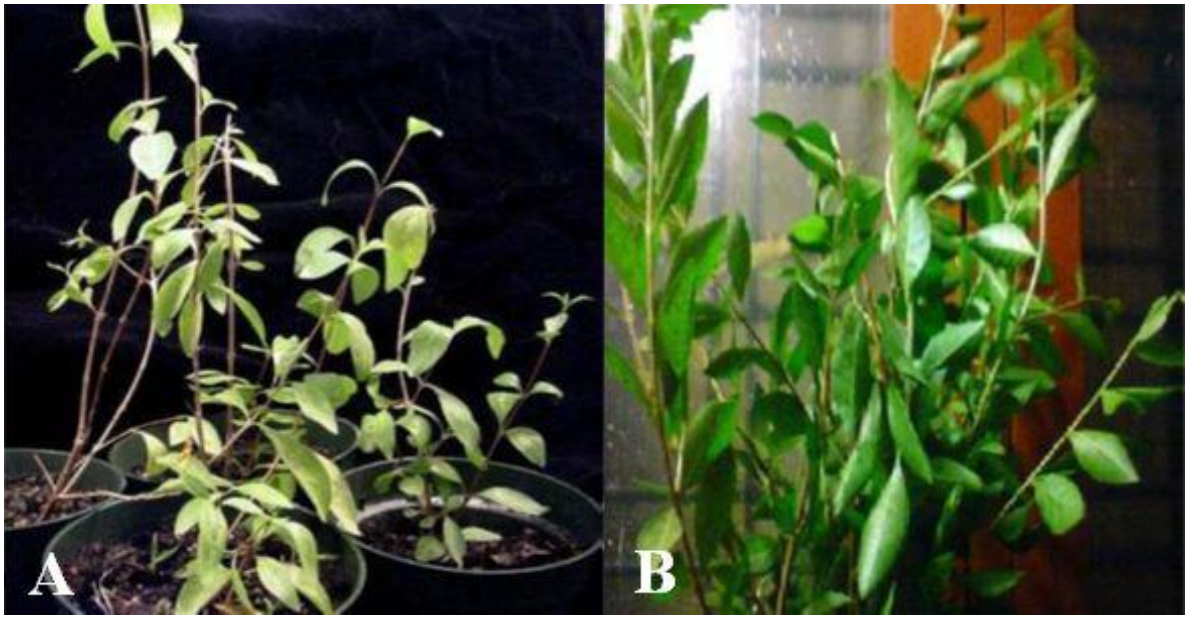


Figure 2.1: (A) *Lawsonia inermis* plant and (B) Aerial parts of *Lawsonia inermis* (Varghese *et al.*, 2010).

2.1.5 Phytochemistry of *Lawsonia inermis*

Much work has been done in the field of phytochemical investigation of the plant. The chemical constituents isolated from henna according to Varghese *et al.* (2010), are divided into the following categories:

i. Naphthoquinone derivatives
ii. Aliphatic components, triterpenes, and sterols
iii. Phenolic derivatives, coumarins, xanthones and flavonoids
iv. Other chemical constituents

2.1.5.1 Naphthoquinone derivatives

The colouring matter in the leaves of Henna is lawsone and identified as 2-hydroxy-1, 4-naphthoquinone ($C_{10}H_6O_3$, m.p.1900 decomp.) which is present in dried leaves in a concentration of 1.0-1.4 %. The compound was extracted from the leaves and preparation of various derivatives of lawsone was reported (Ashnagar and Shiri, 2011).

Spectrophotometric method has been developed for determination of lawsone in Henna. Colorimetric methods have also been described for the estimation of lawsone in leaves (Hanna *et al.*, 1998). A TLC system for identification of the natural red dye has been evaluated (Kharbude and Agarwal, 1985).

Resolution of chlorophyll, lawsone, 1, 3- dihydroxy naphthalene, 1,4- naphthoquinone and 1,2-dihydroxy-4-glucosyloxynaphthalene has been isolated from water insoluble extract of Henna leaves (Hanna *et al.*, 1998). A naphthoquinone derivative isoplumpagin has been isolated from the stem bark of Henna (Gupta *et al.*, 1993; Al-Rubiay *et al.*, 2008).

2.1.5.2 Aliphatic components, triterpenes, and sterols

The structure of new phenolic glycosides, lawsoniaside and lalioside isolated from the leaves has been established as 1,2,4-trihydroxynaphthalene 1,4- di- -D-glucopyranoside and 2,3,4,6-tetrahydroxyacetoxy-2--D-glucopyranoside (Takeda, 1988). An aliphatic ester n-tricontyl n-tridecanoate, -sitosterol, lupeol, 30-norlupan-3-ol-20-one, betulin and betulinic acid have been isolated from the bark of *L.inermis* (Chakraborthy *et al.,* 1977; Badoni *et al.*, 2014).

A glucoside of -sitosterol has been reported from the leaves (Al-Rubiay *et al.,* 2008). Methanol extract of leaves of henna also yielded -sitosterol and stigmasterol (Afzal *et al.,* 1984). Two pentacyclic triterpenes isolated from the bark of Henna were identified as 3--30-dihydroxylup-20 (29)-ene-one (hennadiol) and (20 S) - 3-, 30-dihydroxylupane (Chakraborthy *et al.*, 1982; Gull *et al.,* 2013).

The seeds of the plant yielded 7.5 % viscous oil containing behenic, arachidic, stearic, palmitic, oleic and linolenic acids. Among the fatty acids the linolenic occurred in the predominant amount (Badoni *et al.,* 2014). From the stem bark, an aliphatic constituent 3-methyl-nonacosan-1-ol (Gupta *et al.,* 1992) and the sterols viz. lawsaritol (Gupta *et al.,* 1992) and lawsaritol A (Hanna *et al.,* 1998) have been reported. Bioassay guided fractionation of methanolic extract of the defatted seed led to isolation of two new triterpenoids, lawnermis acid and its methyl ester (Handa *et al.,* 1997; Gull *et al.,* 2013).

2.1.5.3 Phenolic derivatives, coumarins, xanthones and flavonoids

The ethanolic extract of the whole of the aerial parts of Henna was found to be devoid of tannins (Gull *et al.,* 2013). Galic acid has been reported in the leaves (Al-Rubiay *et al.,*

2008). The structure of coumarins, lacoumarin isolated from the whole plant, was established as 5-allyoxy-7-hydroxycoumarin (Bharadwaj *et al.,* 1976).

Three laxanthones, viz laxanthones I, II, and III which were characterized as 1, 3-dihydroxy-6, 7-dimethoxy xanthone, 1-hydroxy-3, 6-diacetoxy-7-methoxyxanthone and 1-hydroxy-6- acetoxy xanthone, respectively have been reported (Bharadwaj *et al.,* 1977; Bharadwaj *et al.,* 1978). Apigenin-7-glucoside, apigenin-4-glycoside, luteolin-7-glucoside and luteolin-3-glucoside were the flavonoids isolated from Henna (Afzal *et al.,* 1973). Luteolin, luteolin-7-O-glucoside and acacetin-7-O-glucoside have been isolated from the leaves (Varghese *et al.,* 2010).

2.1.5.4 Other chemical constituents

The leaves contain a brown substance of a resinoid fraction having the chemical properties, which characterize the tannins (Al-Rubiay *et al.,* 2008), and therefore named hennotannic acid. Tannin and chlorogenic acid are absent. The leaves also contain glucose but starch and pyrogallol are absent (Varghese *et al.,* 2010). Gallic acid (Al-Rubiay *et al.,* 2008) is present to the extent of 5-6 %. Amino acid composition of Henna has been reported (Khan *et al.,* 1991).

The presence of mannitol in Henna stem, flowers and roots has been investigated. An albumin inhabitable lectin was obtained from Henna (Al-Rubiay *et al.,* 2008). Trace elements in henna powder were Copper, Nitrogen, Molybdenum, Vanadium, Manganese, Serenium, Barium, Iron and Aluminium including mineral substances like Sodium Oxide, Calcium Oxide and Potassium oxide (Muhammad and Muhammad, 2005).

The essential oil of flowers of Henna contains -ionone and -ionone as the chief constituents. The immunomodulatory bioassay-guided fractionation of the methanolic

extract of henna leaves resulted in the isolation of seven compounds; three have been isolated for the first time from the genus, namely *p*-coumaric acid , 2-methoxy-3-methyl-1,4-naphthoquinone and apiin, along with the previously isolated compounds: lawsone, apigenin, luteolin, and cosmosin (Muhammad and Muhammad, 2005; Al-Rubiay *et al.*, 2008; Ashnagar and Shiri *et al.*, 2011), chemical structures of which can be seen in figure 2.2 and figure 2.3.

The compounds found in different parts of *Lawsonia inermis* are summarized in Table 2.1.

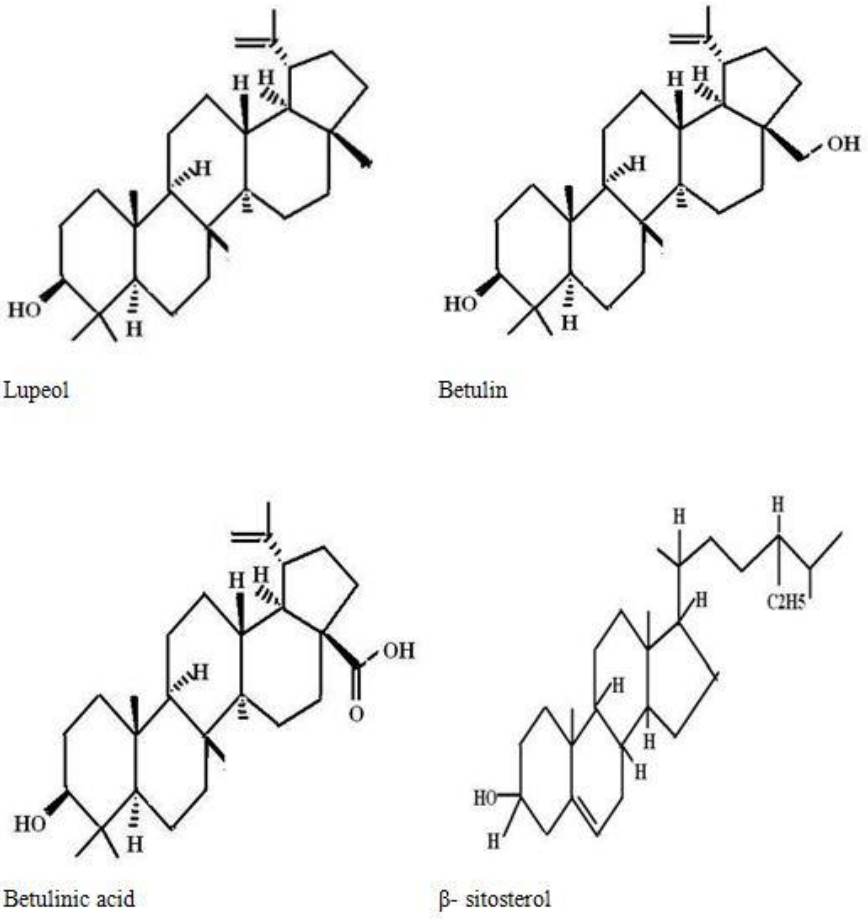


Figure 2.2: Structures of phytochemicals of *L. Inermis* (Lupeol, Betulin, Betulinic acid and β-sitosterol) (Varghese *et al.*, 2010)

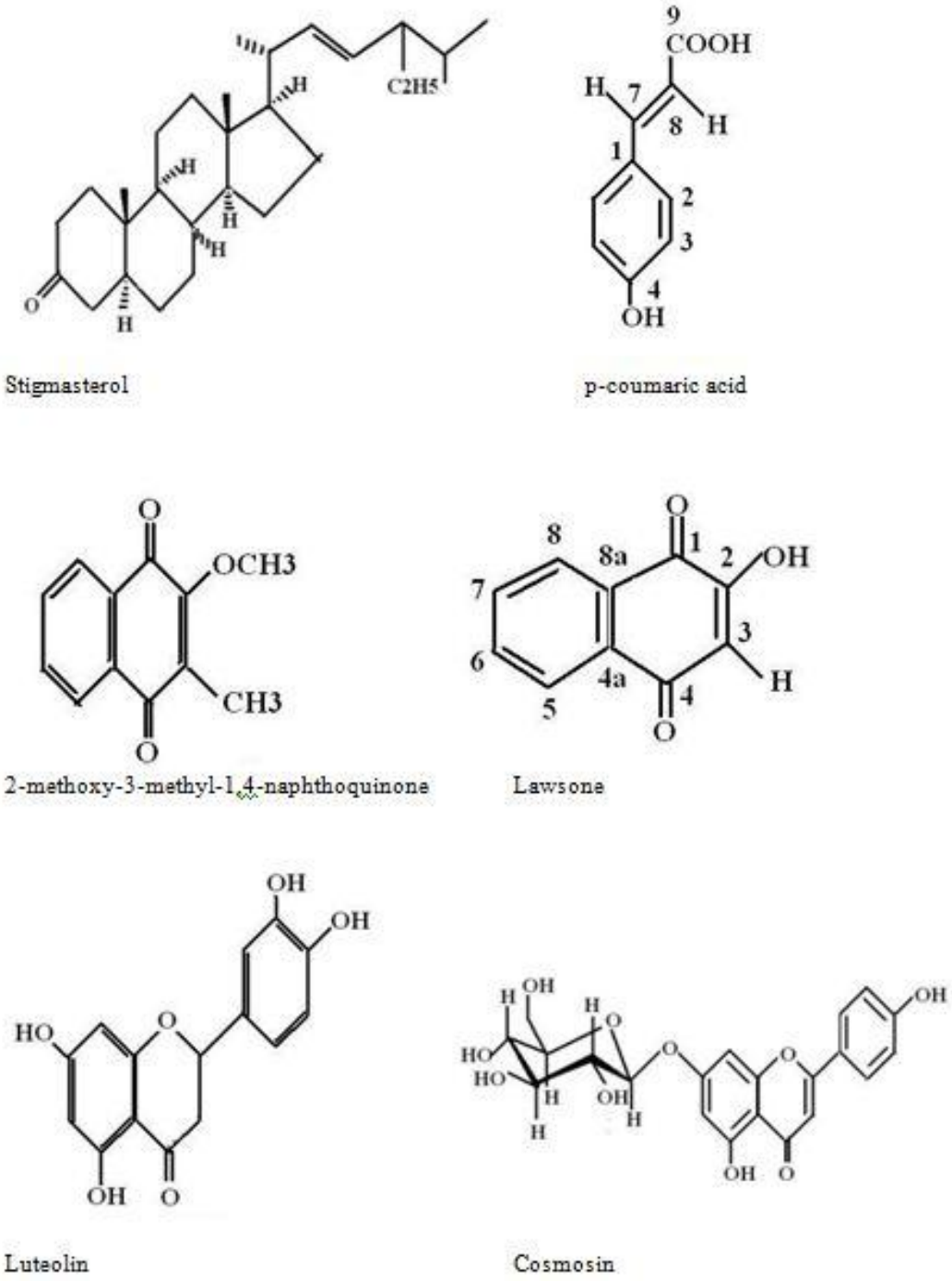


Figure 2.3: Structures of phytochemicals of *L. Inermis* (2-methoxy-3-methyl-1,4-naphthoquinone, Lawsone, Luteolin and Cosmosin) (Varghese *et al.*, 2010).

Table 2.1: Phytochemicals found in various parts of *L. inermis*

Compounds	**Plant Parts**	**References**
Napthoquinone derivatives Lawsone (2-hydroxy 1,4-naphthoquinone)	Leaves	Dixit *et al.*, 1980; Ashnagar and Shiri *et al.*, 2011; Hikmat *et al.*, 2011
1,3-dihydroxy naphthalene, 1,4-napthaquinone, 1,2-dihydroxy-4-glucosylnaphthalene	Leaves	Afzal *et al.*, 1984; Hanna *et al.*, 1998
Isoplumbagin	Stem bark	Gupta *et al.*, 1993
Phenolic conpounds Lawsoniaside (1,3,4-trihydroxynaphthalene 1,4-di-β-D-gluco-pyronoside), Lalioside (2,3,4,6-tetrahydroxyacetoxy-2-β-D-glucopyranoside) Lawsoniaside B (3-(4-O-a-D-glucopyranosyl-3,5-dimethoxy) phenyl-2E-propenol), syringinoside, daphneside, daphnorin, agrimonolide 6-O-β-D-glucopyranoside, (+)-syringaresinol O-β-D-glucopyranoside, (+)-pinoresinol di-O-β-D-glucopyranoside, syringaresinol di-O-β-D-glucopyranoside, isoscutellarin	Bark, Leaves	Takeda and Fatope, 1988; Cuong *et al.*, 2010.
Terpenoids 3β, 30-dihydroxylup-20(29)-ene (hennadiol), (20S)-3β, 30-dihydroxylupane, Lupeol, 30-*nor*-lupan-3β-ol-20-one, betulin, betulinic acid, lawnermis acid (3β-28β-hydroxy-urs-12,20-diene-28-oic acid) and its methyl ester	Bark, Seeds	Chakraborthy *et al.*, 1982; Handa *et al.*, 1997.
Sterols Lawsaritol (24β-ethycholest-4-en-3β-ol) Stigmasterol and β-sitosterol	Roots, Leaves	Gupta *et al.*, 1992.
Aliphatic constituents 3-methyl-nonacosan-1-ol, n-tricontyl n-tridecanoate	Stem bark	Gupta *et al.*, 1992; Chakraborthy *et al.*, 1977

Source: (Kumar, 2011)

Table 2.1: Phytochemicals found in various parts of *L. inermis cont'd*

Compounds	**Plant Parts**	**References**
Xanthones Laxanthone I (1,3 dihydroxy-6,7 dimethoxy xanthone), Laxanthone II (1-hydroxy-3,6 diacetoxy-7-methoxyxanthone), Laxanthone III (1-hydroxy-6-acetoxy xanthone)	Whole plant	Bhardwaj *et al.*, 1977; Bhardwaj *et al.*, 1978.
Coumarins Lacoumarin (5-allyoxy-7-hydroxycoumarin)	Whole plant	Bhardwaj *et al.*, 1976
Flavonoids Apigenin-7-glucoside, apigenin-4-glycoside, luteolin-7-glucoside, luteolin-3-glucoside	Leaves	Afzal *et al.*, 1980
Essential oil -(Z)-2-hexenol, linalool, α ionone, β ionone, α-terpineol, terpinolene, δ-3-carene and γ-terpineol	Leaves	Wong and Teng, 1995; Reichling *et al.*, 1999.
Other chemical constituents Hennotannic acid, glucose, gallic acid, amino acid Trace metal – Cu, Ni, Mo, V, Mn, Sr, Ba, Fe and Al Minerals – Na_2O, CaO and K_2O	Whole plant	Muhammad and Muhammad, 2005; Ashnagar and Shiri *et al.*, 2011.

Source: (Kumar, 2011)

2.1.6 Ethnomedicinal uses of *Lawsonia inermis*

L. inermis is a well known ethnomedicinal plant used cosmetically and medicinally for over 9,000 years. Its use in the Indian traditional folk medicine is well documented (Kumar, 2011). Table 2.2 indicates the use of different parts of *L. inermis* in traditional system of medicines.

Table 2.2: Ethnomedicinal uses of different parts of *Lawsonia inermis*

Plant Parts	Traditional Uses (as/in)
Root	Bitter, depurative, diuretic, emmenagogue, abortifacient, burning sensation, leprosy, skin diseases, amenorrhoea, dysmenorrhoea and premature greying of hair (Singh and Singh, 2001; Warrier, 2004)
Leaves	Bitter, astringent, acrid, diuretic, emetic, edema, expectorant, anodyne, anti-inflammatory, constipating, depurative, liver tonic, haematinic, styptic, febrifuge, trichogenous, wound, ulcers, strangury, cough, bronchitis, burning sensation, cephalalgia, hemicranias, lumbago, rheumatalgia, inflammations, diarrhoea, dysentery, leprosy, leucoderma, scabies, boils, hepatopathy, splenopathy, anemia, hemorrhages, hemoptysis, fever, ophthalmia, amenorrhoea, falling of hair, greyness of hair, jaundice (Reddy, 1988; Ahmed *et al.,* 2000; Bich *et al.,* 2004; Warrier, 2004)
Flowers	Cardiotonic, refrigerant, soporific, febrifuge, tonic, cephalalgia, burning sensation, cardiopathy, amentia, insomnia, fever(Warrier, 2004).
Seeds	Antipyretic, intellect promoting, constipating, intermittent fevers, insanity, amentia, diarrhoea, dysentery and gastropathy (Singh and Singh, 2001; Warrier, 2004).

Source: (Kumar, 2011)

2.1.7 Pharmacology of *Lawsonia inermis*

In the indigenous system of medicine, Henna leaves are used as a prophylactic against skin diseases. It has been used for the treatment of boils, burns, bruises and skin inflammations (Varghese *et al.,* 2010). Although a lot of pharmacological investigations have been carried out based on the ingredients present but a lot more can still be explored, exploited and utilized.

2.1.7.1 Antiviral activity

The ethanol soluble fraction of the fruits displayed highly potent activity against Semliki Forest Virus (SFV) in Swiss mice and chick embryo models exhibiting 65-100 % activity after 10-25 days of virus challenge (Khan *et al.,* 1991).

2.1.7.2 Antibacterial activity

50 % ethanolic extract of the whole aerial part did not show antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Agrobacterium tumerfaciens* (Varghese *et al.,* 2010). In another investigation, lawsone showed some inhibition against Proteus species and *S. aureus* (Kelmanson *et al.,* 2002). In another study, the leaf extract was active against a wide range of microbes such as Staphylococcus, Streptococcus, Brucella and Salmonella. The aqueous extract of the leaves of Henna was shown to possess reproducible activity *in vitro* against a wide variety of microorganisms (Habbal *et al.,* 2005; Al-Rubiay *et al.,* 2008). The effects of water and chloroform extracts of the leaves of *L. inermis* (henna plant) against the primary invaders of burnt wounds was investigated. Clinical isolates of *Staphylococcus aureus, Streptococcus* sp, *Pseudemonas aeruginosa, Candida albicans, Fusarium oxysporum*, and *Aspergillus niger* were treated with extracts of the leaves of *L. inermis* (Gull *et al.,* 2013)

for antimicrobial activity. The henna leaves extracts were able to inhibit the growth pattern of *A. niger* and *F. oxysporum*. *Streptococcus* sp. and *S. aureus* were also inhibited by the extracts. Inhibition of the microorganisms' growth suggests that henna may be valuable in the management of burnt wound infections (Muhammad and Muhammad, 2005). *L. inermis* displayed noteworthy antimicrobial activity against both gram positive and gram negative bacterial strains (Bhuvaneshwari *et al.,* 2002; Habbal *et al.,* 2005; Hussain *et al.,* 2011; Gull *et al.,* 2013).

2.1.7.3 Antifungal activity

The ethanolic extract of the whole plant also showed Antifungal activity against *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes*, *Microsporum canis* and *Aspergillus niger* (Muhammad and Muhammad, 2005). Alcoholic and aqueous extracts of henna leaves and lawsone were tested separately *in vitro*. No Antifungal activity was determined for the henna extract *in vitro*. On the other hand, the lawsone aqueous solution showed a fungistatic activity. Bark decoction of henna inhibited the activity of protopectinase and polygalactouronase (Bhakuni *et al.,* 1971)

2.1.7.4 Nootropic activity

The acetone fraction of the petroleum ether extract of Henna inhibited prominent nootropic activity, potentiating clonidine induced hypodermia and decreased lithium induced head twitch. The fraction modified 5-HT and NA mediated behaviour. However haloperidol induced catalepsy was not modified (Prasad and Gupta, 1967).

2.1.7.5 Immunomodulatory activity

Methanolic extract of Henna leaves showed significant immunostimulant activity in mice (Varghese *et al.,* 2010). Lawsone at a dose of 100 g/ml had suppressive effect on the

induction of antibodies in lymphocytes *in vitro*. The naphthoquinone fraction at the dose of 5 mg/kg was shown to increase phagocytosis of carbon particle (Iyer *et al.,* 1998).

2.1.7.6 Anti-inflammatory activity

Isoplumbagin and lawsaritol, isolated from stem bark and root of *L. inermis* screened for anti-inflammatory activity against carrageenan-induced paw edema in rats. The compounds phenylbutazone, isoplumbagin and lawsaritol at the oral dose of 100 mg/kg exhibited 61, 60 and 40 % inhibition respectively. The results showed that isoplumbagin exhibited significant activity, comparable to that of phenylbutazone (Singh *et al.,* 1982; Gupta *et al.,* 1993).

2.1.7.7 Antimitotic activity

In studies on the antimitotic actions of methanolic extracts of Henna and standard lawsone in onion root tips, Henna and standard lawsone inhibited mitosis in a concentration- dependent manner. The methanolic extract of Henna showed concentration-dependent inhibition of mitosis, which was less than that produced by the standard lawsone at corresponding concentrations. The methanolic extract of Henna showed about 50 % mitotic inhibition, whereas standard lawsone showed about 60 % mitotic inhibition at 200 g/ml (Varghese *et al.,* 2010).

2.1.7.8 Tuberculostatic activity

The tuberculostatic activity of the herb henna (Lawsonia inermis Linn.) was tested *in vitro* and *in vivo* (Sharma, 1990). On Lowenstein Jensen medium, the growth of tubercle bacilli from sputum and of Mycobacterium tuberculosis H37Rv was inhibited by 6 micrograms/ml of the herb. In vivo studies on guinea pigs and mice showed that the herb

at a dose of 5 mg/kg body weight led to significant resolution of experimental tuberculosis following infection with M. tuberculosis H37Rv (Mikhaeil *et al.*, 2004).

2.1.7.9 Activity of L. inermis on drug metabolism

The effect of 200 and 400 mg/kg body weight of 80% ethanolic extract of the fresh leaves of *L. inermis* were examined on drug metabolizing phase-I and phase-II enzymes, antioxidant enzymes, glutathione content, lactate dehydrogenase and lipid peroxidation in the liver of 7 weeks old Swiss albino mice. Also anticarcinogenic potential of Henna leaf extract was studied adopting the protocol of benzo(a)pyrene induced forestomach and 7,12dimethylbenz(a)anthracene (DMBA)-initiated and croton oil-promoted skin papillomagenesis. Primary findings reveal the 'duel-acting' nature of henna leaf as deduced from its potential to induce only the phase-II enzyme activity, associated mainly with carcinogen detoxification in liver of mice and inhibit the phase I enzyme activities. The hepatic glutathione S-transferase and DT-diaphorase specific activities were elevated above basal level by *Lawsonia inermis* extract treatment. With reference to antioxidant enzymes the investigated doses were effective in increasing the hepatic glutathione reductase (GR), superoxide dismutase (SOD) and catalase activities significantly at both the dose levels. Reduced glutathione (GSH) measured as non-protein sulphydryl was found to be significantly elevated in liver and in all the extrahepatic organs studied Among the extrahepatic organs examined (forestomach, kidney and lung) glutathione S-transferase and DT-diaphorase level were increased in a dose independent manner. Chemopreventive response was measured by the average number of papillomas per mouse (tumor burden) as well as percentage of tumor bearing animals and tumor multiplicity. There was a significant inhibition of tumor burden in both the tumor model

systems studied. Tumor incidence was also reduced by both the doses used in our experiment in both the model systems (Sharma, 1990).

2.1.7.10 Anticarcinogenic properties and antioxidant activity

The investigation was conducted to examine the anticarcinogenic properties of henna (*L. inermis*) extract (using chloroform as a solvent) by the microculture tetrazolium salt (MTT) assay on the human breast (dependent- hormone, MCF-7; non-dependent hormone, MDA-MB-231), colon (Caco-2), liver (HepG2) carcinoma cell lines and normal human liver cell lines (Chang Liver). The preliminary results showed that the henna extract displayed the cytotoxic effects against HepG2 and MCF-7 with IC -value of 0.3 and 24.85 µg mlG, respectively. However, no IC values were obtained from Caco-2 and MDA-MB-231 cell lines at the concentration studied. This extract also did not show the IC value against normal human liver cell lines, Chang 50 Liver, indicated the selectivity of its cytotoxic properties. The antioxidative activities of this extract which could contribute to its cytotoxic properties were also studied. Antioxidant activity in henna was found to be the highest as compared to vitamin E or " -tocopherol. The strong cytotoxic properties of this extract could be due to its high antioxidant activities (Dasgupta *et al.,* 2003; Kamal and Jawaid, 2010). Pink *et al.,* (2000) also declared that several research reports confirmed cancer treatment activity of *Lawsonia inermis.*

2.1.7.11 Molluscicidal activity

Leaf, bark and seed of henna against *Lymnaea acuminata* and *Indoplanorbis exustus* were studied. Seed powder was more toxic than leaf and bark against *I. exustus.* Binary combinations of henna seed with *Cedrus deodara Roxh* and *Azadirachta indica* A. Juss oil, or powdered *Allium sativum*, or *Zingiber officinale* rhizome oleoresin was more toxic to snails *L. acuminata* and *I. exustus* than their single treatment. The highest increase in

the toxicity was observed when henna seeds powder and *C.deodara* oil (1:1) were tested against both the snails. The combination with neem oil was also more toxic than their individual components and other combinations (Susi *et al.,* 2002).

2.1.7.12 Wound healing activity

The ethanol extract of *L. inermis* (200 mg/kg/day) was used to evaluate wound healing activity on rats using excision, incision and dead space wound models. The following differences were noted in experimental animals which were treated with an extract of *L. inermis* when compared with the control and reference standard animals: The extract-treated animals showed 71 % reduction in the wound area when compared with controls which was 58 %. Enhanced wound contraction, increased skin breaking strength, hydroxyproline and histological findings; increased well organized bands of collagen, more fibroblasts and few inflammatory cells, suggest the use of *L. inermis* in the management of wound healing (Amrita and Singh, 2001; Muhammad and Muhammad, 2005).

2.1.8 Safety evaluation of *Lawsonia inermis*

Most of the toxicological studies report that toxic effects due to the use of herbal medicine are associated with hepatotoxicity. Other toxic effects on the kidney, nervous system, blood and cardiovascular system, as well as mutagenecity and carcinogenicity have also been published (Nadjib *et al.,* 2013). Methanolic extract of *L. inermis* roots induced abortion in mice, rats and guinea pigs, though the effect was dosage dependent (Aguwa, 2008). Significant toxicity for male Wistar rats administered orally with *L. inermis* seeds was observed with dose dependent damage of vital organs exemplified by necrosis, fatty changes, hemorrhage and congestion (Abdelgadir *et al.,* 2010). Intraperitoneal administration of high dose of aqeous root extract of *L. inermis* actively induced spontaneous abortion, and toxic effects such as partial paralysis, temporary amnesia, loss of appetite and dizziness (Mudi *et al.,* 2011).

2.1.9 Ethnobotanical uses of *Lawsonia inermis*

Henna has been used cosmetically and medicinally for over 9,000 years. Traditionally in India, henna is applied to hands and feet (Hanna *et al.,* 1998). Henna symbolizes fertility. Its use became popular in India because of its cooling effect in the hot Indian summers. Henna leaves, flowers, seeds, stem bark and roots are used as/in colouring agent. Henna leaf has an orange-red dye and leaf paste or powder is widely used for decorating hands, nails and feet with patterns. It is also used as a hair dye. (Chopra *et al.*, 1956; Reddy, 1988; Kelmanson, 2002; Chetty, 2008; Chaudary *et al.,* 2010; Cuong *et al.,* 2010; Jiny *et al.,* 2010).

Flowers are very fragrant and used to extract a perfume, which is used as base for local scents. An infusion of the flowers is a valuable application to bruises (Warrier *et al.,* 2004; Rahmoun *et al.*, 2010).

Seeds are deodorant and used for extraction of fragrance. (Chopra *et al.*, 1956; Reddy, 1988; Chetty, 2008).

2.2 The Liver

2.2.1 Embryology of the liver

The liver develops from an endodermal bud that arises from the ventral aspect of the gut, at the point of junction between foregut and midgut,in the early 4th week of intrauterine life (Saddler, 1998). This bud grows into the ventral mesogastrium and passes through it into the septum transversum. It enlarges, and soon shows a division into a larger cranial part called the pars hepatica and a smaller caudal portion called the pars cystic. The pars hepatica divides into right and left parts each of which forms one lobe of the liver (Singh, 2003).

As the right and left divisions of the pars hepatica enlarge and extend into the septum transversum, the cells arising from them are broken up into interlacing columns called hepatic trabeculae. In this process, the umbilical and vitelline veins which lie in the septum transversum are broken up to form the sinusoids of the liver. Sinusoids are also formed from the mesenchyme of the septum transversum. The endodermal cells of the hepatic bud give rise to parenchyma of the liver and to bile capillaries. The mesoderm of the septum transversum forms the capsule and fibrous tissue basis of the liver.

The fetal liver is an important centre of blood formation (heamopoiesis). Large aggregations of blood forming cells are present between hepatic cells and blood vessels. Bile formation begins when the fetus is about 3 months old (Singh, 2003).

2.2.2 Gross anatomy of the liver

The liver is a reddish brown organ with four lobes of unequal size and shape. A human liver normally weighs 1.44–1.66 kg (Costran *et al.*, 2005), and is a soft, triangular organ. It is both the largest internal organ and the largest gland in the human body. The liver possesses four surfaces which are superior, inferior, anterior, and posterior. The liver has four lobes right, left, quadrate and caudate. These lobes are separated by the ligaments of the liver. It is connected to the diaphragm and abdominal walls by five ligaments viz the membranous falciform ligaments, coronary ligament, right and left triangular ligament, and the fibrous round ligament. The large right lobe and smaller left lobe are visible anteriorly. They are separated from each other by the falciform ligament, a sheet of mesentery that suspends the liver from the diaphragm and anterior abdominal Wall. The round ligament (ligamentum teres), also visible anteriorly, is a fibrous remnant of the umbilical cord to the liver of the foetus. In the interior aspect, we see a squarish quadrate lobe next to the gallbladder and a tail-like caudate lobe posterior to that. The gallbladder

adheres to the depression on the interior surface of the liver between the right and quadrate lobe. The posterior aspect of the liver has a deep groove that accommodates the inferior vena cava. Most of the liver is covered by a serosa but this is lacking from the bare area of its superior surface, where the liver is attached to the diaphragm (Gray, 2004).

2.2.2.1 Blood supply

The liver receives blood from two sources i.e. the portal vein (70%) and the hepatic artery (30%) (Dalley and Moore, 2006). The portal vein is a short wide vein formed by the superoior mesenteric and splenic veins posterior to the neck of the pancreas and ascends superior to the inferior vena cava, and divides at the right end of the porta hepatis into right and left branches that ramify within the liver (Dalley and Moore, 2006). The hepatic artery is a branch of the celiac trunk and may be divided into the common hepatic and the hepatic artery proper (Dalley and Moore, 2006).

The hepatic artery carries well oxygenated blood from the aorta and the portal vein carries poorly oxygenated but nutrient filled blood from the gastrointestinal tract to the sinusoids of the liver (Dalley and Moore, 2006). The hepatic artery and the portal vein terminate at the porta hepatis by divivding into right and left branches which supply the right and left parts of the liver respectively (Dalley and Moore, 2006).

2.2.2.2 Nerve supply

The nerves of the liver are derived from the hepatic nerve plexus which is the largest derivative of the celiac plexus. The hepatic plexus accompanies the branches of the hepatic artery and portal vein to the liver (Dalley and Moore, 2006). It consists of fibres

from the anterior and posterior vagal trunks. Nerve fibres accompany the vessels and bile duct of the portal triad within the liver (Dalley and Moore, 2006).

2.2.2.3 Lymphatic drainage

The liver is a major lymph-producing organ. It produces between one-quarter and one-half of the lymph received by the thoracic duct. The lymphatic vessels of the liver occur as superficial lymphatics in the subperitonaeal fibrous capsule of the liver and as deep lymphatics in the connective tissue and accompany the ramification of the portal triad and hepatic veins (Dalley and Moore, 2006). The lymphatics on the anterior aspect of the diaphragm and viscera surface of the liver and those accompanying the portal triad drain into the hepatic lymph nodes. Efferent nodes drain into celiac lymph nodes and the chyle cistern (Dalley and Moore, 2006). The lymphatics on the posterior aspect of the diaphragmatic and viscera surfaces drain into the phrenic lymph nodes. Efferents go to the thoracic duct while a few drain into the left gastric, parasternal and lymphatics of the anterior abdominal wall (Dalley and Moore, 2006).

2.2.3 Histology of the liver

The liver exhibits repeating hexagonal units called liver lobules. In the middle of each lobule is a central vein, from which radiates plates of liver cells, called hepatocytes, sinusoids towards the periphery. Here, the connective tissue forms the portal triads, or portal areas where branches of hepatic artery, hepatic portal vein, and bile duct can be seen. Venous and arterial blood first mix in the liver sinusoids as it flows towards the central vein. From here, the blood enters the general circulation through the hepatic veins (Eroschenko, 2000).

The hepatic sinusoids are tortuous, dilated blood channels lined by a discontinuous layer of fenestrated endothelial cells, which are separated from the underlying liver cells, the hepatocytes, by a perisinusoidal space (of Disse). As a result, ingested materials carried in the sinusoids percolates through the discontinuous endothelial wall and comes in direct contact with the hepatocytes. This allows for a more efficient exchange of materials in the blood with those in the hepatocytes and vice versa (Eroschenko, 2000.) Hepatocytes secrete bile into tiny channels called bile canaliculi that are located between the hepatocytes. These canaliculi converge at the periphery of each lobule in the portal areas as bile ducts. The bile ducts then drain into larger hepatic ducts that carry bile out of the liver (Eroschenko, 2000.)

2.2.4 Functions of the liver

According to Singh (2002), the functions of the liver include:

i. it acts as an exocrine gland for the secretion of bile.
ii. it plays a prominent role in the metabolism of carbohydrate, proteins and fats.
iii. it acts as a store for various substances including glycogen, lipids, vitamins and irons.
iv. it plays a protective role by detoxifying substances including drugs and alcohol.
v. during fetal life, the liver serves as a centre for haematopoiesis.

2.2.5 Liver diseases

Liver disease is a term used for a collection of conditions, diseases and infections that affect the cells, tissues, structures or functions of the liver. It is also called hepatic disease (Shah, 2010). Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders (Kumar *et al.,* 2010). Most of the

hepatotoxic chemicals damage the liver mainly by inducing lipid peroxidation and other oxidative damages (Chattopadhay, 2003; Valarmathi *et al.,* 2010).

Drug-induced liver injury is a major health problem that challenges not omly health care professionals but also the pharmaceutical industry and drug related agencies (Mohammed *et al.,* 2007). More than 900 drugs have been implicated in causing liver injury (Friedman *et al*., 2003) and it is the most common reason for a drug to be withdrawn from the market. Drug induced liver injury is responsible for 5 % of all hospital admissions and 50 % of all acute liver failures (Ostapowicz *et al*., 2002).

2.2.5.1 Mechanism of liver damage

Due to its unique metabolism and close relationship 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 near-undiluted form. Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria, an intracellular organelle that produce energy. Its dysfunction releases excessive amount of oxidants which, in turn, injure hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also lead to oxidative stress (Jaeschke *et al*., 2002). Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside the liver. This promotes further liver damage (Patel *et al*., 1998). Non-parenchymal cells such as Kupffer cells, fat storing stellate cells, and leukocytes (i.e. neutrophil and monocyte) also have a role in the mechanism. However, the liver has a great capacity to regenerate and has a large reserve capacity. In most cases, the liver only produces symptoms after extensive damage.

2.2.5.2 Causes of liver damage

The most common causes of chronic liver failure (where the liver fails over months to years) include: Hepatitis B, Hepatitis C, Long term alcohol consumption, Cirrhosis, Hemochromatosis (an inherited disorder that causes the body to absorb and store too much iron) and Malnutrition.

The causes of acute liver failure, when the liver fails rapidly, however, are often different. These include: Acetaminophen (Tylenol) overdose, Viruses including hepatitis A, B, and C (especially in children), reactions to certain prescription and herbal medications, ingestion of poisonous wild mushrooms. Adverse drug reactions are classified as type A (intrinsic or pharmacological) or type B (idiosyncratic) (Rawlins, 1991). Type A drug reaction accounts for 80 % of all toxicities (Pirmohammed *et al*., 1998).

Drugs or toxins that have a pharmacological (type A) hepatotoxicity are those that have predictable dose-response curves (higher concentrations cause more liver damage) and well characterized mechanisms of toxicity, such as directly damaging liver tissue or blocking a metabolic process. As in the case of acetaminophen overdose, this type of injury occurs shortly after some threshold for toxicity is reached.

Idiosyncratic (type B) injury occurs without warning, when agents cause non-predictable hepatotoxicity in susceptible individuals which is not related to dose and has a variable latency period (Zimmerman, 1978). This type of injury does not have a clear dose-response or temporal relationship, and most often does not have predictive models. Idiosyncratic hepatotoxicity has led to the withdrawal of several drugs from market even after rigorous clinical testing as part of the FDA approval process.

2.3 Ethanol

Ethanol, also known as ethyl alcohol, is the main component of all alcoholic drinks (Shakashiri, 2009). It is the single most widely used pharmacological agent in the world (Guenthner, 2012).

2.3.1 Uses of ethanol

Ethanol is widely used in many different types of products such as alcoholic beverages, solvents, perfumes, toiletries, disinfectants, preservatives, polishes, as a fuel additive and in the manufacturing of plastics, rubber and drugs. It is also increasingly being used as a biofuel. Alcohol-based hand gels in the form of liquids, foams and gels can contain up to 95% ethanol (Bull, 2010).

2.3.2 Pharmacology of ethanol

Alcohol is metabolized by the enzyme alcohol dehydrogenase to acetaldehyde, a sympathomimetic toxin, which in turn is rapidly metabolized by another enzyme, aldehyde dehydrogenase (Comporti *et al.,* 2010), to acetic acid, and eventually to carbon dioxide and water (Pohorecky and Brick, 1990).

Ethanol, like all drugs undergoes four scientific or pharmacokinetic processes in the body:

i. absorption
ii. distribution
iii. metabolism
iv. excretion

These four processes occur contemporaneously until;

a. all the ethanol is absorbed from the GI tract, and there is no more absorption phase
b. all the ethanol has been metabolized, and there is no more metabolism of Ethanol and it is no longer detectable in the blood.

2.3.2.1 Absorption of ethanol

Absorption is the process by which ethanol is made available to the fluids of distribution of the body (e.g., blood, plasma, serum, aqueous humor, lymph, etc.). Approximately 80% of orally ingested ethanol is absorbed from the small intestines, and the remainder is absorbed from the stomach (Comporti *et al.*, 2010).

In the fasting state, the majority of ethanol (>50 %) will be absorbed within 15 minutes and a "peak" or maximum blood level will occur in approximately 20 minutes, with 80-90 % complete absorption achieved within 30-60 minutes. The rate of absorption of ethanol and subsequent appearance of ethanol in the blood is dependent on the following factors:

i. the rate of consumption (chugging vs. sipping),
ii. the volume consumed (1 shot, ~1.25 oz. vs. a 12 oz. beer),
iii. the concentration or proof of ethanol in the drink(beer = ~3.5 %, wine = ~12 %, whiskey = ~43 % or 86 proof),
iv. the presence or absence of carbonation (e.g., champagne vs. wine, or scotch and soda vs. scotch and water, carbonation increases the rate of absorption, but absorption of alcohol from beer is delayed).
v. the presence or absence of food in the stomach (food delays absorption).
vi. is the person taking any medication(s) that can interfere with gastrointestinal (gi) motility (e.g., reglan slows, aluminum antacids slow, drugs like atropine or scopolamine used for ulcers or "queasy stomachs" slow the motility keeping the alcohol in the stomach slowing absorption, while drugs like tagamet, zantac and

pepcid-ac decrease gastric acid production increasing the rate of gastric emptying and increasing the rate of ethanol absorption (DiPadova *et al.,* 1992).

2.3.2.2 Distribution of ethanol

Once a drug has been absorbed from the stomach and/or intestines (GI Tract) into the blood, it is circulated to some degree to all areas of the body to which there is blood flow. This is the process of distribution (David, 2015).

2.3.2.3 Metabolism of ethanol

Alcohol in the blood and tissues must be inactivated and excreted from the body. This process is initiated by altering the chemical structure of the alcohol in such a way as to promote its excretion. The transformation of the alcohol molecule into a chemically related substance that is more easily excreted from the body is called metabolism or detoxification (David, 2015).

In the case of Ethanol, it is metabolized in the liver by the enzyme alcohol dehydrogenase, to acetaldehyde which causes dilatation of the blood vessels and, after accumulation, is responsible for the subsequent hangover which ensues. The acetaldehyde is subsequently metabolized by the enzyme aldehyde dehydrogenase to acetate, a substance very similar to acetic acid or vinegar (David, 2015).

2.3.2.2 Excretion of ethanol

The kidney and lungs excrete only 5-10 % of an absorbed dose of ethanol unchanged (unmetabolized). The rest must be metabolized prior to excretion. In most individuals, the rate of excretion ranges from 0.01 % (10 mg/100 ml) to 0.025 % (25 mg/100 ml) per hour, with a mean of 0.0175 (frequently rounded off to 0.017 or 0.018) (David, 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection and identification of plant

The leaves of *L. inermis* were obtained from Kundu village, Giwa, Kaduna State, Nigeria. The plant was identified and authenticated at the Department of Biological Sciences, Ahmadu Bello University, Zaria and a voucher number 900270 was given.

3.1.2 Experimental animals

Thirty female Wistar rats of weight between 110-130 g were purchased from the Department of Human Anatomy, Ahmadu Bello University Zaria. The animals were kept in the animal holding of the Department of Human Anatomy for two weeks to acclimatize. They were housed in well ventilated aluminum cages at room temperature in hygienic conditions under natural light (13hours) and dark (11 hours) schedule and were fed on standard laboratory diet. Food and water were given *ad libitum*.

3.1.3 Equipment/instruments

Laboratory mortar and pestle, separating funnel, filter paper, water bath, evaporating dish, spatula, weighing scale, plastic tubes, syringes, canula, timer, chloroform chamber, scalpels, scissors, forceps, blades, icebox, ice, centrifuge (HERMLE MR-2 BY Labnet), Pasteur pipettes, Apetman Micropipettes, test tubes, curvettes, spectrophotometer, beakers, measuring cylinders, eppendorf microcentrifuge tubes, plastic homogenizer, incubator (Thermolyne Type 17600 Dri-bath), microwave oven (Binatone MWO-2519EG), gel electrophoresis machine, gel documenting machine, couplin jars, funnel,

rotary microtome (RM2125RTS, Leica Biosystems, Germany), glass slides, cover slips, drying racks, light microscope (Olympus BX43), Fluorescence microscope (Olympus BX51) , Armscope MD900 and Scoptek 2.0 M digital camera.

3.1.4 Reagents

Distilled water, Absolute Ethanol (Analytical grade by SIGMA-ALDRICH), Silymarin, chloroform, 10% formalin, 4% paraformaldehyde, Phosphate buffer saline (PBS), carbonate buffer, trichloroacetic acid (TCA), thiobarbituric acid (TBA), Ellman's reagent, potassium phosphate, hydrogen peroxide, Bradfords reagent, Bovine Serum Albumin (BSA), Tris buffer saline (TES) lysis buffer, Proteinase K (amnesco, Biotechnology Trade Lot no. 1483C496), DNA loading dye, agarose gel powder (Alkali scientific inc), Triacetate (TAE) buffer, Dulbecco's phosphate buffer (Cambrex Bio Science Walkersville inc, Walkersville, USA), xylene (Analytical grade by LOBAL chemie), paraffin wax, DPX, Haematoxylin, Eosin, Acridine orange (SIGMA-ALDRICH), Ethidium Bromide (SIGMA-ALDRICH).

3.2 Methods

3.2.1 Preparation of plant extract

Aqueous extraction of the plant was done by cold water maceration. *L. inermis* leaves were washed with tap water, shade dried and then pounded. The powder obtained was soaked in distilled water for 24 hours in a separating funnel with occasional shaking. The solution was then filtered. The filtrate was allowed to settle and then decanted. The decant was placed in an evaporating dish and then put in a water bath at 60 $^{\circ}$C for excess water to evaporate gently after which the dried residue was scraped out and stored in a refridgerator.

3.2.2 Determination of dose

The oral LD_{50} of aqueous extract of *L. inermis* leaves is >3,000 mg/kg (Reddy *et al.,* 2011). 10-15 % of LD_{50} was used to determine the dose.

3.2.3 Experimental design

The thirty female (30) rats were assigned into five groups with six (6) animals each, using the block method, with an average weight of 121.5 g across the groups.

Group I: negative control group; was given distilled water (2ml) for 8 days

Group II: positive control group; was given distilled water for 7 days, and 20 ml/kg of 40 % ethanol on the 8^{th} day.

Group III: Prophylactic group; was given aqueous extract of *L. inermis* (AELI) (400 mg/kg) for 7 days then 20 ml/kg of 40 % ethanol on the 8^{th} day.

Group IV: Therapeutic group; was given 20 ml/kg of 40 % ethanol on day 1, then AELI treatment (400 mg/kg) for 7 days

Group V: was given Silymarin (70 mg/kg) for 7 days, then 20 ml/kg of 40 % ethanol treatment on day 8

3.2.4 Hepatotoxicity induction

Hepatotoxicity was induced by following a modification of binge-drinking model designed by Carson and Pruett (Carson *et al.,* 1996). After six (6) hours of fasting, animals were administered 40 % ethanol at a total accumulative dosage of 20 ml/kg body weight (Garba *et al.,* 2006) by four equally divided gavages in 20-minute intervals orogastrically.

3.2.5 Sacrifice of animals

The animals were humanely sacrificed on the ninth (9) day, eighteen (18) hours after hepatotoxicity was induced, using chloroform anaesthesia. The liver was harvested, through a longitudinal incision made in the abdominal cavity, and processed respectively for the analysis.

3.2.6 Morphological studies

The rats were weighed in grams using a weighing scale, before the start of the experiment and before their sacrifice. After sacrifice, the liver weight was also taken in grams. The final and initial bodyweights were compared to get the weight gain. The liver weight and body weight was used to determine the liver-body weight ratio.

3.2.7 Biochemical studies

Biochemical studies were done at Department of Biochemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

3.2.7.1 Estimation of oxidative parameters

Preparation of homogenate: A 10 % fresh tissue homogenate was prepared by mincing the liver tissues in cold phosphate buffer saline, pH 7.4 using mortar and pestle. At centrifugation at 10,000 rpm for 10 mins, the resulting supernatant was pipetted for the different oxidative stress parameters.

Determination of superoxide dismutase activity: Superoxide dismutase (SOD) activity was determined by the method described by Fridovich (1989). 0.1 ml of supernatant was diluted in 0.9 ml of distilled water to make 1:10 dilution of serum. An aliquot mixture of 0.20 ml of the diluted serum was added to 2.5 ml of 0.05 M carbonate buffer. The

reaction was started with the addition of 0.3 ml of 0.3 mM adrenaline. The reference mixture contains 2.5 ml of 0.05 M carbonate buffer. 0.3 m1 of 0.3 mM adrenaline and 0.20 ml of distilled water. Absorbance was measured over 30 up to 150 s at 480 nm.

Calculations

Increase in absorbance per minute = (A5 - A1)/2.5

A1 is absorbance at 30s and A5 is absorbance at 150s

$$\% \text{ inhibition} = 100 - \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50 % inhibition of the oxidation of adrenaline to adrenochrome in one minute.

Assay of reduced glutathione concentration: Reduced glutathione (GSH) concentration measurements was done according to ElIman (1959) as described by Rajagopalan *et al.*, (2004).

To 150 µl of supernatant (in phosphate saline pH 7.4), 1.5 m1 of 10 % TCA was added and centrifuged at 1500 rpm for 5 min. 1 ml of supernatant was treated with 0.5 ml of ElIman's reagent and 3 ml of phosphate buffer (0.2 M, pH 8). The absorbance was read at 412 nm.

Table 3.1: Reduced glutathione standard curve.

Absorbance (412nm)	GSH Conc. (µg/ml)
0.040	8
0.101	20
0.194	40
0.380	80
0.572	120
0.749	160

Lipid Peroxidation assessment: Lipid peroxidation was determined according to Okhawa *et al.,* (1979) with slight modification by Atawodi *et al.,* (2011) using trichloroacetic acid (TCA) and thiobarbituric acid (TBA).

Exactly 2 ml of 15 % trichloroacetic acid was measured into a test tube, 2 ml thiobarbituric acid was added and 100 µl of the tissue homogenate was added. The mixture was incubated at 80 $^{\circ}$C for 30 minutes in a water bath and allowed to cool for some time followed by centrifugation at 3000 rpm for 10 mins. A clear supernatant was collected and the absorbance of it was determined at 535 nm on a spectrophotometer.

Molar extinction coefficient is $1.56 \times 10^5\ cm^{-1}M^{-1}$

$$\text{MDA Concentration} = \frac{\text{absorbance of sample}}{1.56 \times 10^5\ cm^{-1}M^{-1}}$$

Catalase (CAT) activity: Catalase activity was measured using Abei's method (1974). Exactly 10 µl of tissue homogenate was added to test tubes containing 2.8 ml of 50 mM potassium phosphate (buffer pH 7.0). The reaction was initiated by adding 0.1 ml of freshly prepared 30 mM Hydrogen peroxide and the decomposition rate of H_2O_2 was measured at 240nm for 5 minutes using a spectrophotometer. A molar extinction coefficient (E) of 0.041 $mM^{-1}cm^{-1}$ was used to calculate the catalase activity.

Catalase concentration = absorbance of sample/molar extinction coefficient (E)

3.2.7.2 Determination of protein concentration

Protein concentration of the lysate of the liver tissue was determined using Bradfords reagent.

Preparation of Tissue Lysate: Approximately 300 mg of liver tissue was homogenized with 3 ml of cold lysis buffer, in a mortar and pestle. This was centrifuged, and the resultant supernatant used for the analysis.

Preparation of bovine serum albumin standard (BSA) curve: A standard curve was prepared by making a series of dilutions using distilled water from a stock solution of 10 mg/ml. 900 microlitre (µl) of Bradford reagent was added to 100 µl of each concentration, and was left to incubate for 5 mins. Absorbance was taken at 595 nm.

Table 3.2: Bovine serum albumin standard curve

concentration(mg/ml)	Absorbance
2	0.04
4	0.08
6	0.121
8	0.144
10	0.177

Determination of protein concentration in samples: Bradford reagent (900 µl) was added to 100 µl of the tissue homogenate. This was allowed to incubate at room temperature for 5 mins and the absorbance was taken at 595 nm using a spectrophotometer, against a blank (Bradford reagent without any protein added). The absorbance readings were then used to calculate the concentration from the standard curve.

Dilution factor of sample is 10, thus

Protein concentration = concentration from standard curve x 10

3.2.8 Molecular study

Analysis of DNA fragmentation was carried out at DnaLabs, Kaduna, Kaduna State, Nigeria.

3.2.8.1 Determination of DNA fragmentation using gel electrophoresis

DNA Extraction: liver tissue (30-60 mg) was cut using scissors and forceps, and homogenized with 100 µl of Tris buffer saline (TES) lysis buffer using a plastic homogenizer. The homogenate was left to incubate at room temperature for 30 minutes, and then 20 µl of Proteinase K was added. This was then incubated at 50 $^{\circ}$C for two (2) hours.

Agarose gel electrophoresis: 1.5 g of agarose gel powder was dissolved in 100 ml of 1X TAE (triacetate) buffer to prepare 1.5 % agarose gel. It was then heated in a microwave oven for 2-3 minutes, till fully dissolved and the liquid turned clear. It was allowed to cool for a bit, before 5 µL of 10 mg/ml ethidium bromide was added and mixed. This was then gently poured into the gel cast with the combs in place and allowed to solidify.

DNA (15 µl) was mixed with 3 µl of 5 X loading dye, loaded into wells, and the electrophoresis was set to run for 4 hours at 40 V. The gel was viewed under UV and snapped.

3.2.9 Histological studies

Excised Liver section was rinsed in cold phosphate buffer saline (PBS, pH $^{7.4}$), and then processed through routine histological procedure for tissue preparation.

3.2.9.1 Tissue preparation

Tissues were histologically prepared in the histology laboratory, Department of Human Anatomy, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The tissue sections were fixed in 10 % formalin for Haematoxylin and eosin staining for 48 hours, and in 4 % paraformaldehyde for acridine orange-ethidium bromide staining for 18-20 hours. The sections were then passed through grades of alcohol, 30 %, 50 %, 70 % and two changes of 95 % alcohol, for four hours each to dehydrate. Next the sections were cleared in two changes of xylene for two hours each and impregnated with two changes of molten paraffin wax for two hours. The tissue sections were then embedded in paraffin wax, before being mounted on a wooden block and trimmed. Sections were made using the rotary microtome. The sizes of tissue sections were between 3-5 microns. The cut sections were floated on hot water bath and were picked on clean albuminized glass slides, and left to dry for 3 hours before staining.

3.2.9.2 Staining of tissues sections

Haemotoxylin and Eosin (H&E) staining: Tissues sections were stained at histology laboratory, Department of Human Anatomy, ABU Zaria. The sections were dewaxed in two changes of xylene for three minutes each. Then the sections were rehydrated by placing in absolute alcohol, 95 %, 70 %, and 50 % alcohol for three minutes each. Next, the sections were placed in Haemaotoxylin for ten minutes, rinsed with distilled water, placed in acid alcohol for 30 seconds, rinsed with distilled water for a minute, and placed in alkaline alcohol for 30 seconds. The sections were then counterstained with Eosin for three minutes, and then rinsed in distilled water. The coverslips were mounted with D.P.X. The sections were viewed under light microscope at a magnification of ×250. Photomicrographs were snapped with digital Armscope MD900.

Acridine orange-ethidium bromide staining: Tissue sections were stained at DnaLabs Kaduna, Kaduna State, Nigeria. The slides were prepared for staining following the protocol for the preparation and fluorescent IHC staining of Paraffin-embedded tissue sections by R&D systems. The slides were cleared in two changes of xylene for 10 minutes each. Then passed through two changes of absolute alcohol for ten minutes each, then 95 %, 70 % and 50 % alcohol for five minutes each. Distilled water was used to rinse the slides, and then immersed in buffer saline (DPBS) for 10 minutes.

A dye mixture (50 µg/ml) of Acridine orange and ethidium bromide was made by dissolving the dyes in DPBS. The dye mixture was dropped onto the section using a Pasteur pipette, and a coverslip was placed, and viewed at a magnification of ×400 under blue light immediately. The fluorescence observed was captured using Scoptek 2.0M camera within 10 minutes.

3.2.10 Statistical analysis

Results obtained were expressed as Mean ± SEM (standard error of mean). Differences among the means were determined by one-way analysis of variance (ANOVA). Values were considered statistically significant if P value is less than 0.05 ($p < 0.05$). Tukey's Post Hoc test was used to determine where the significance lay. Statistical package SPSS version 20 (IBM Corporation, NY) for Windows was used to analyze the data obtained in the study.

CHAPTER FOUR

4.0 RESULTS

4.1 General Observation

No mortality was observed throughout the experiment. The animals across the groups showed similar behavior, except when 40 % ethanol was administered. Group II (positive control group) and Group IV (therapeutic group) exhibited sleepiness, drowsiness, less mobility for up to 18 hours after administration of ethanol. Group III (Prophylactic group), receiving the aqueous extract before ethanol was administered, showed similar signs, and returned to normal behaviour faster than Group II. Group V (Silymarin) took longer than any group to display similar signs, and recovered fastest.

4.2 Morphological Studies

4.2.1 Body weight change

There was increase in body weight observed in all groups, though significant weight gain ($p<0.05$) was observed in only Group I (Negative control), and Group IV (Treatment group), as shown in Figure 4.1.

4.2.2 Liver-body weight ratio

There was an increase in the liver-body weight ratio across the groups. The highest increase was in Group II (Positive control), while Group I (Negative control) and Group IV (Therapeutic group) were significant ($p<0.05$) when compared to Group II, as shown in Figure 4.2.

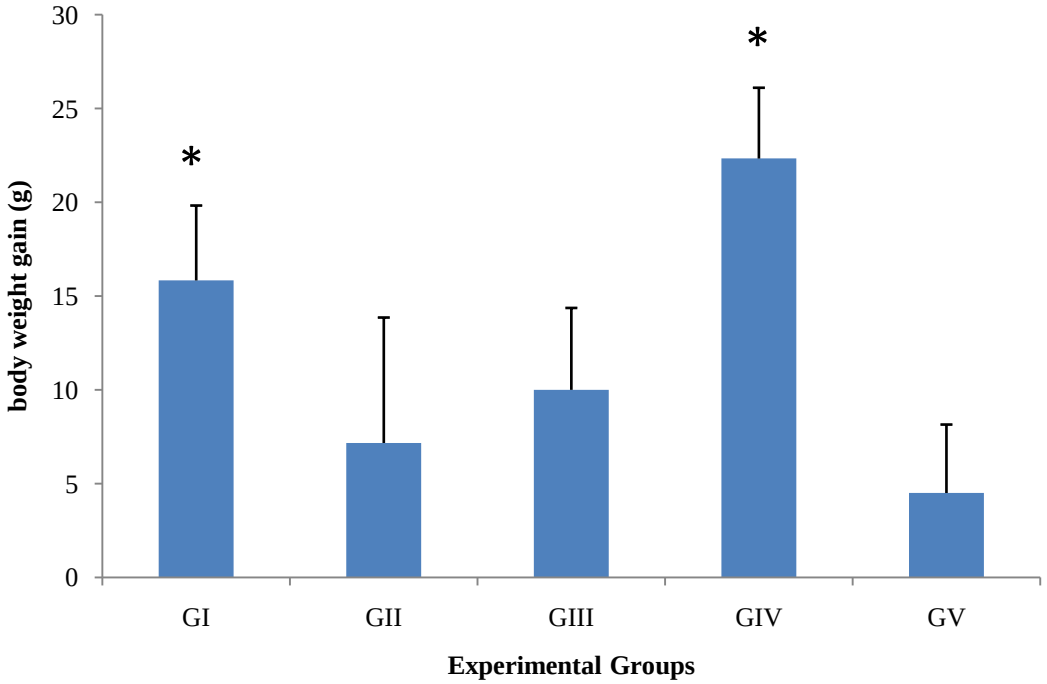


Figure 4.1: Mean body weight gain (g) of Wistar rats in the control and experimental groups.

*- significant increase in weight ($p < 0.05$)
GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

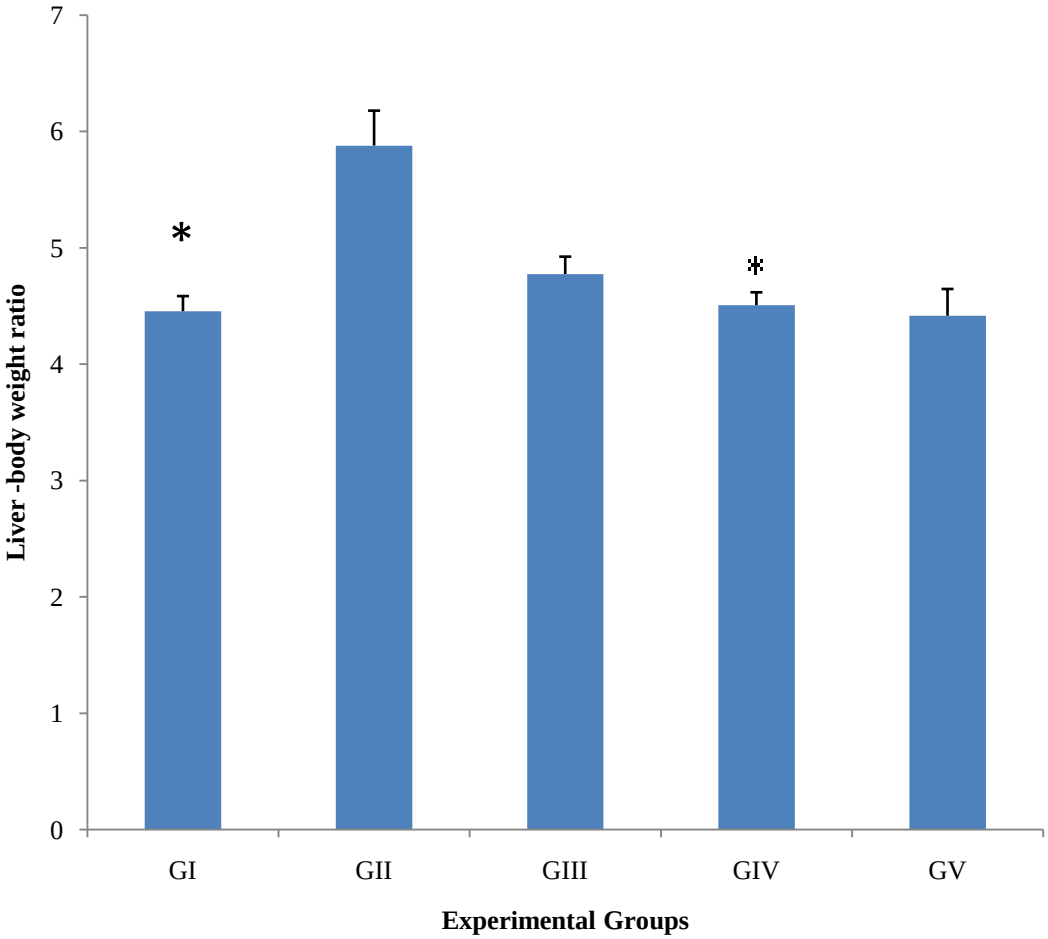


Figure 4.2: Liver- body weight index of Wistar rats in control and experimental groups.

*- statistically significant difference compared to GII (ethanol group) ($p<0.05$)
GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

4.3 Biochemical Studies

4.3.1 Oxidative stress markers

4.3.1.1 Superoxide dismutase

Insignificant reduction of the level of superoxide dismutase in Group II (positive control) was observed, while insignificant increases in the levels were observed in groups receiving Aqueous extract of *Lawsonia inermis* (AELI), that is Group II (Prophylactic group) and Group IV (Therapeutic group). Group V (Silymarin) showed a significant increase in the level of SOD when compared to Group II, as shown in Figure 4.3.

4.3.1.2 Reduced glutathione

Insignificant increases in the level of reduced glutathione was observed in the groups receiving AELI that is Group III (prophylactic group) and Group IV (therapeutic group), while an insignificant decrease was seen in Group II (Positive control), as shown in Figure 4.4.

4.3.1.3 Malondialdehyde

There was no significant difference observed in the levels of Malondialdehyde across the groups, though an increase was seen in Group II (positive control) as shown in Figure 4.5.

4.3.1.4 Catalase

Insignificant reduction in level of catalase was observed in Group II (positive control), while insignificant increase was seen in groups that received Aqueous extract of *L. inermis* i.e. Group III and Group IV, as shown in Figure 4.6.

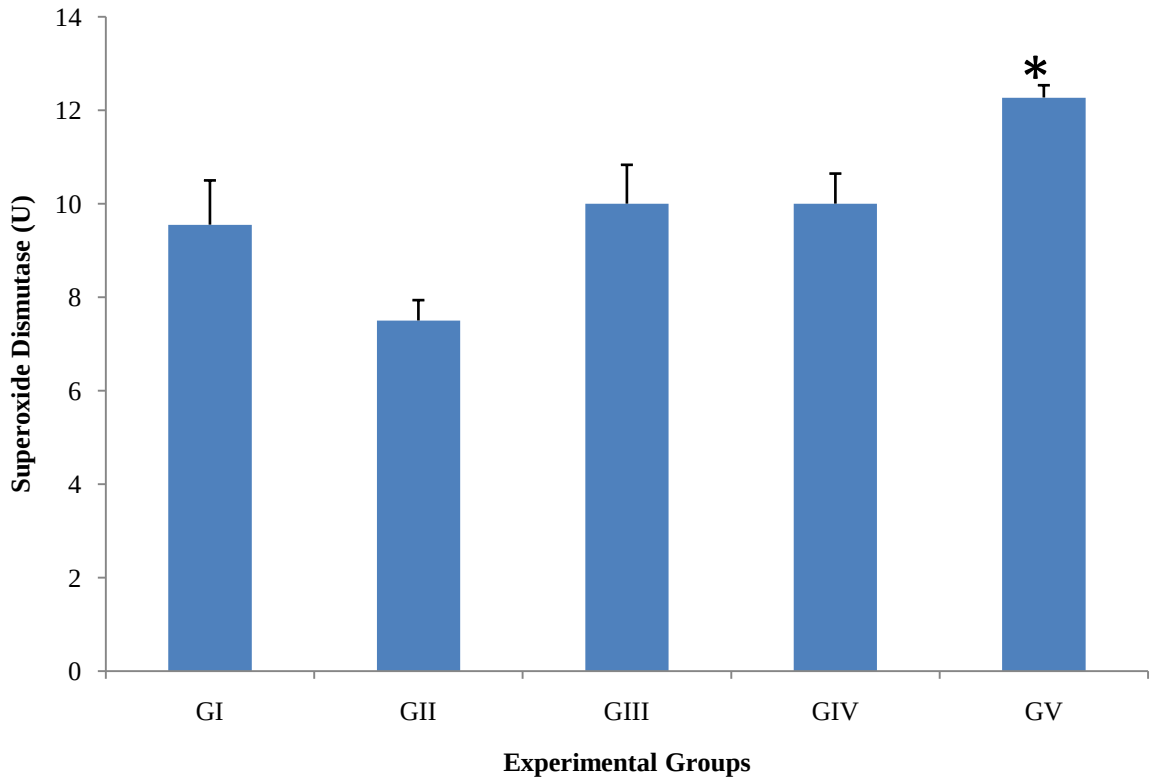


Figure 4.3: Mean level of superoxide dismutase (U) in the liver of Wistar rats in control and experimental groups.

* - $p<0.05$ when compared to GII
GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

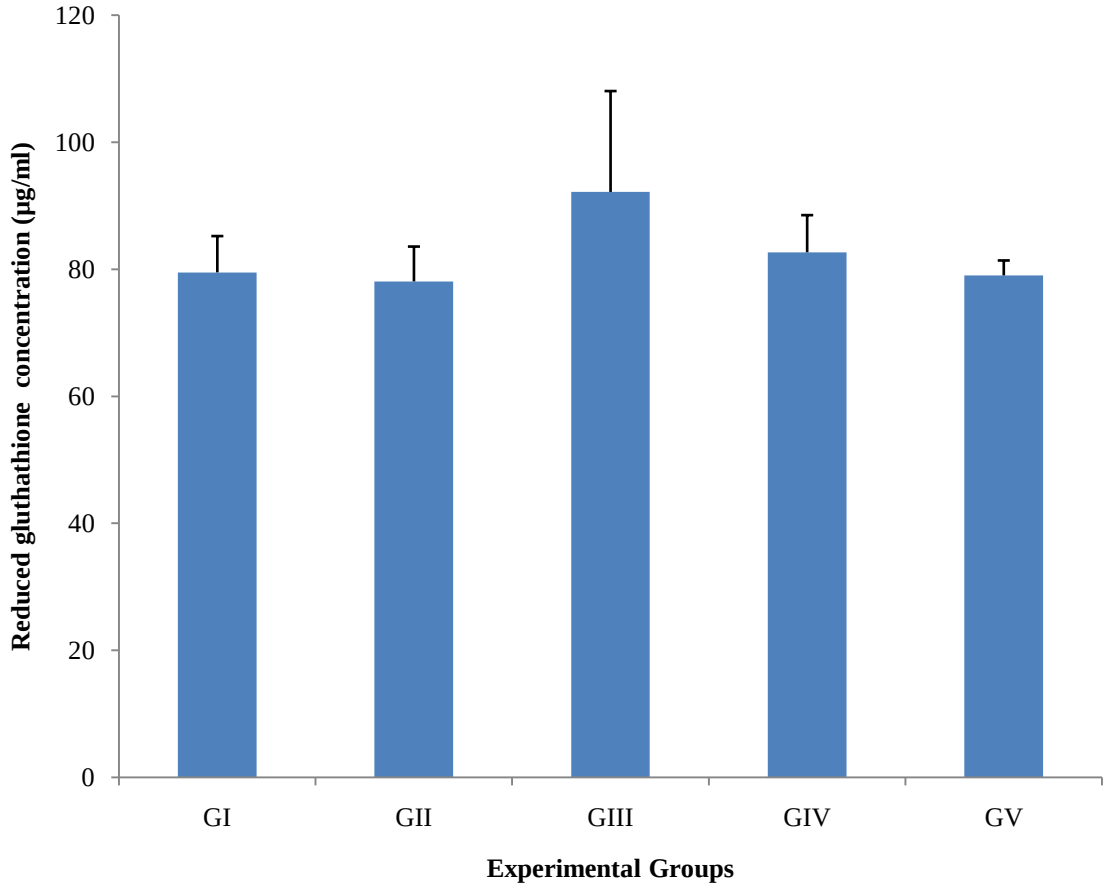


Figure 4.4: Mean concentration of reduced glutathione (µg/ml) in the liver of Wistar rats in control and experimental groups

GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

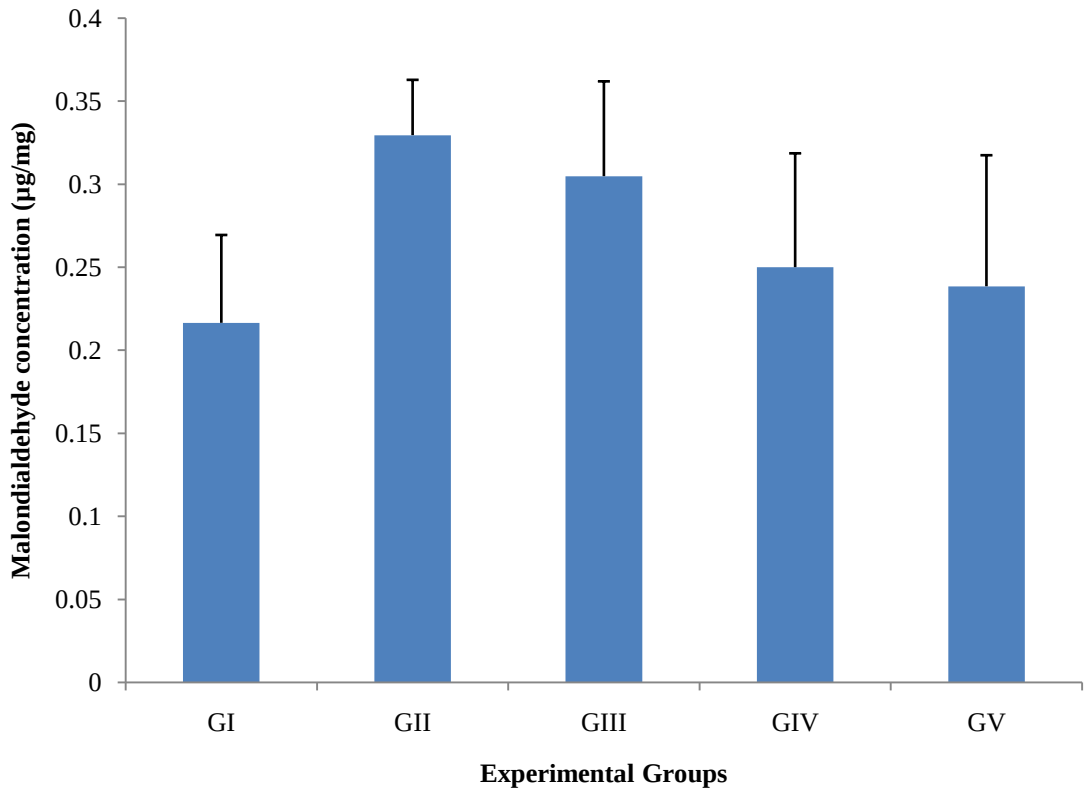


Figure 4.5: Mean concentration of Malondialdehyde (µg/mg) as indicator of lipid peroxidation in liver of Wistar rats in control and experimental groups

GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

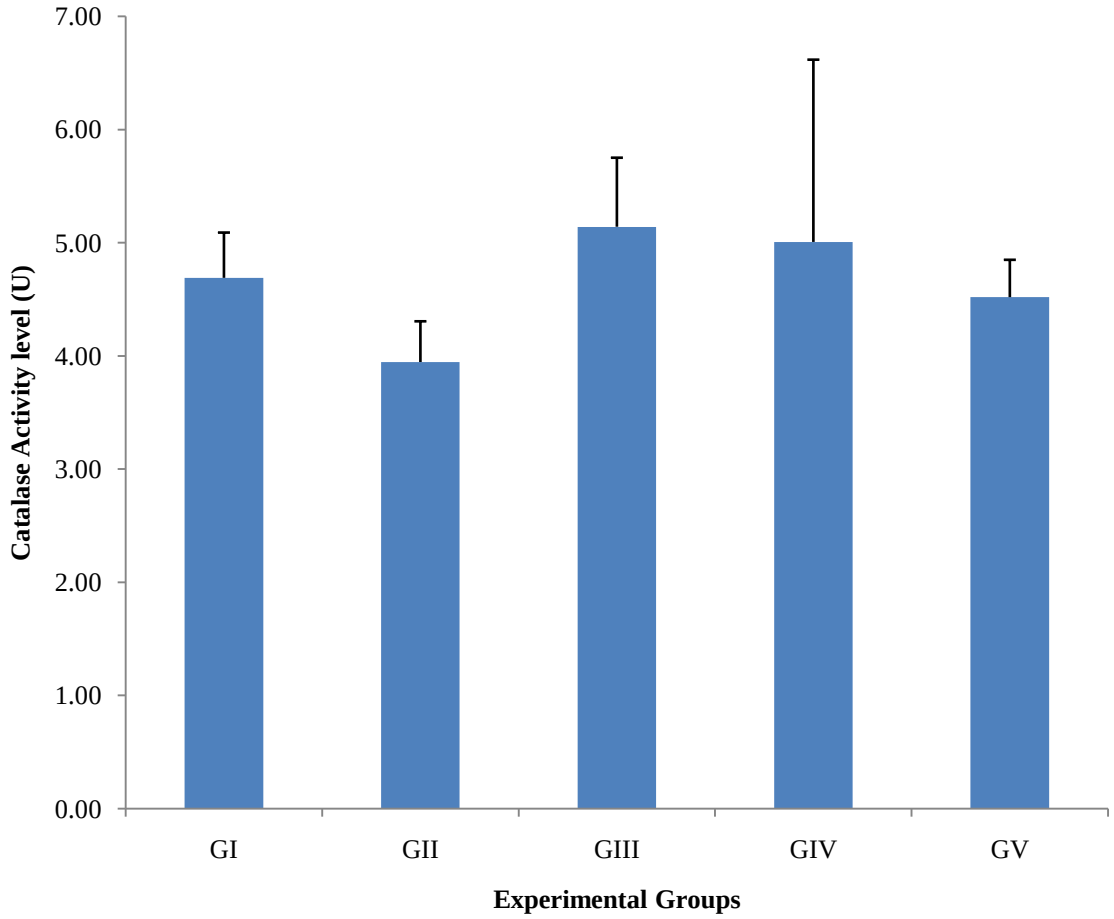


Figure 4.6: Mean level of catalase activity (U) in liver of Wistar rats in control and experimental groups

GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

4.3.2 Protein concentration

There was significant increase in protein concentration of Group II (Positive control), Group III (Prophylactic group), and Group V (Silymarin) compared to Group I (Negative control). A significant difference was observed within the groups, as shown in Figure 4.7

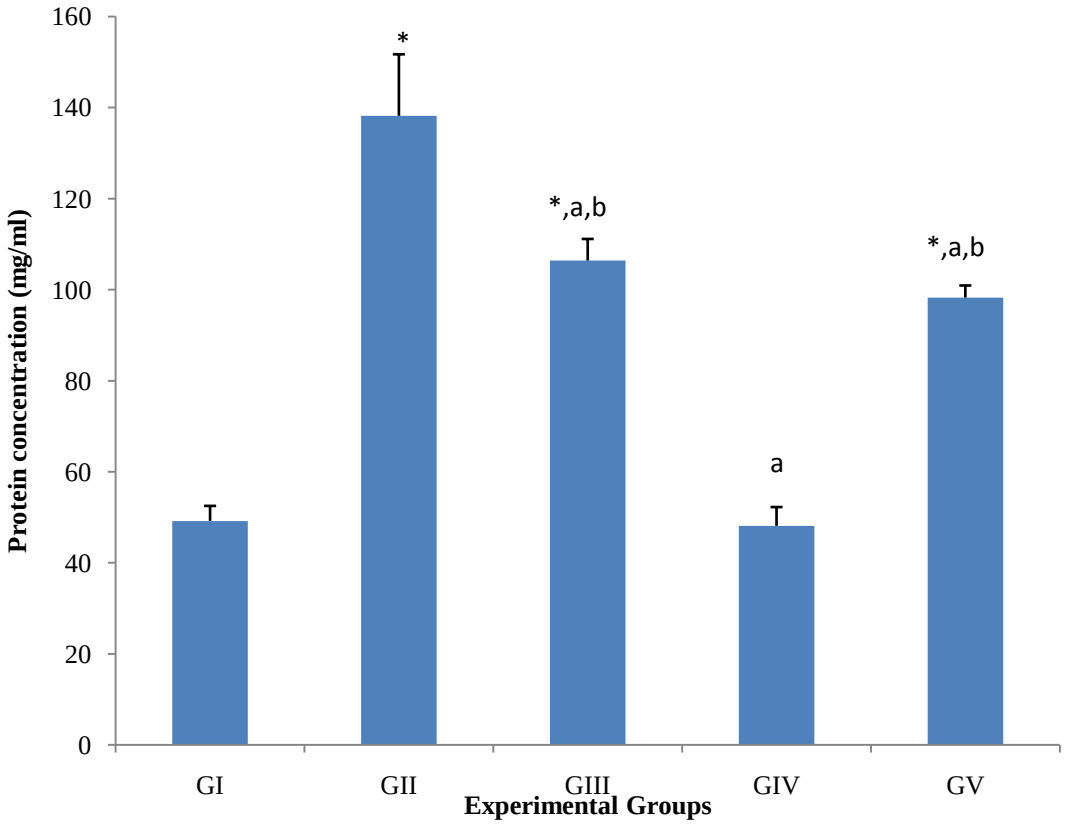


Figure 4.7: Mean serum protein concentration (mg/ml) in hepatocytes of Wistar rats in control and experimental groups.

*: statistically significant compared to Group I ($p<0.05$)
a: significant compared to Group II ($p<0.05$)
b: significant compared to Group IV ($p<0.05$).
GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

4.4 Molecular Study

4.4.1 DNA fragmentation

The agarose gel electrophoresis showed DNA fragmentation occurring in all the groups. Group I (Negative control) showed fragments of 700 to 500 base pairs; with the highest molecular weight DNA. The fragmentation seen in Group II (positive control) had most fragments about 800 bp and less, and no high molecular weight DNA. High molecular weight DNA and fragments of 500 bp and less was observed in Group III (Prophylactic group), IV (therapeutic group) and V (Silyamrin). Band was observed at 500 bps in all groups. In addition the agarose gel electrophoresis revealed sharper smears in Group III, and Group V, indicating high DNA content, compared to the dull smear of Group II indicating low DNA content, as shown in Plate I.

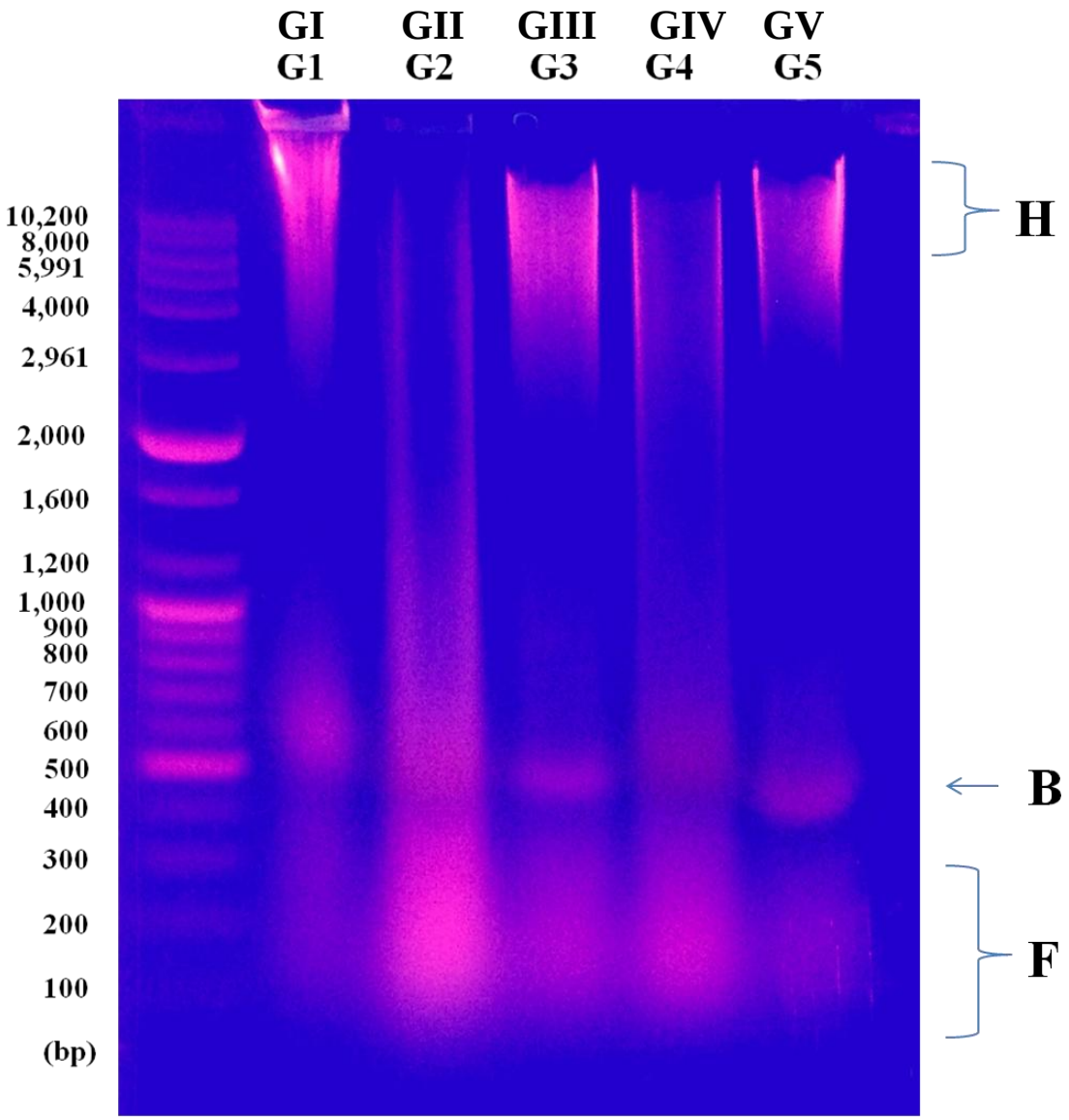


Plate I: Agarose gel electrophoresis showing DNA fragmentation, high molecular weight fragments (H), band of 500 bp (B), and small fragments of 100-400 bp (F) in liver of Wistar rats in control and experimental groups.

GI: Negative control (distilled water for 8 days)
GII: Positive control (7 days distilled water + 20 ml/kg of 40 % ethanol on day 8)
GIII: Prophylactic group (7 days 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8)
GIV: Therapeutic group (20 ml/kg of 40 % ethanol on day 1 + 7 days 400 mg/kg of AELI)
GV: Silymarin (7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40% ethanol on day 8)

4.5 Histological Studies

4.5.1 Haematoxylin and eosin (H&E) staining

Liver sections from Group I (negative control) (Plate II) showed normal hepatic structure of hepatocytes having normal nuclei, and well defined sinusoids and central vein, though mild vacuolation was also observed. Liver sections from Group II (positive control) (Plate III) showed areas of necrosis and intense vacuolation, and hepatocytes with pyknotic nuclei. Liver sections of Group III (prophylactic group) (Plate IV) showed protection of hepatic structure except for areas of vacuolation suggestive of fatty accumulation, and mild necrosis. Liver sections from Group IV (therapeutic group) (Plate V) receiving the extract showed histology comparable to Group II, though the hepatocytes had normal nuclei rather than the pyknotic nuclei, and there was more prominent necrosis in Group II. Group V (Silymarin) (Plate VI) showed liver sections with histology comparable to that of Group III.

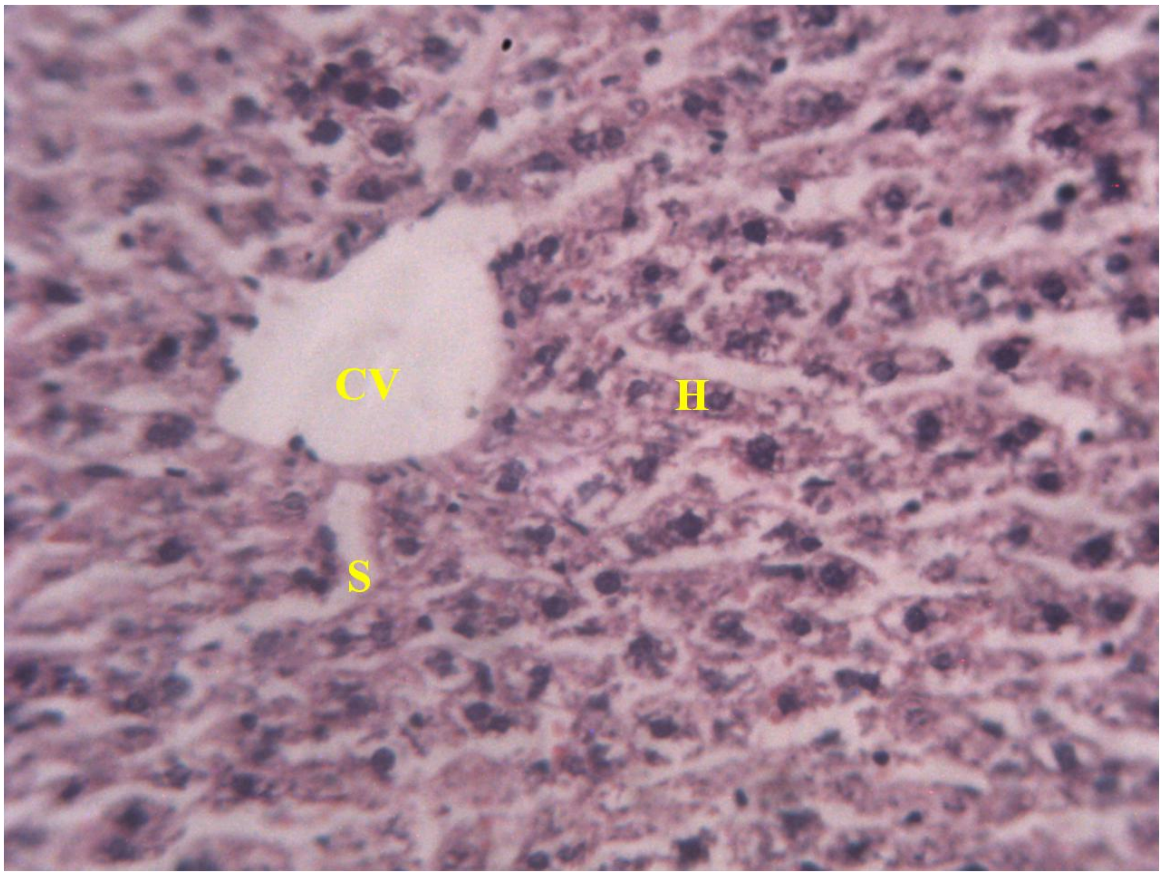


Plate II: Photomicrograph of longitudinal section of liver of Wistar rat in Group I (Negative control: distilled water for 8 days) showing normal hepatic architecture; central vein (CV), sinusoids (S), normal hepatocytes (H). (H & E stain ×250).

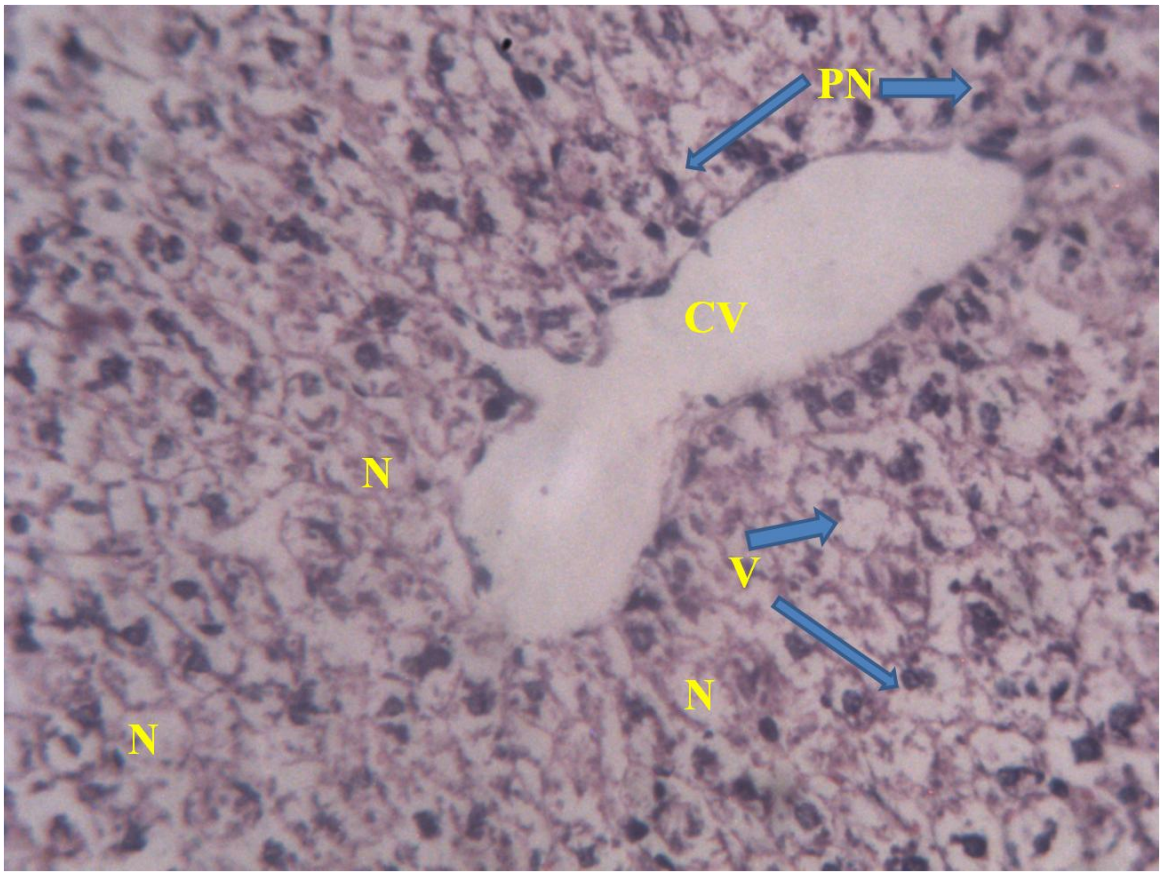


Plate III: Photomicrograph of longitudinal section of liver of Wistar rat in Group II (positive control: 7 days distilled water + 20 ml/kg of 40 % ethanol on day 8) that shows central vein (CV), vacuolation (V), pyknotic nucleus (PN) and necrosis (N). (H & E stain × 250)

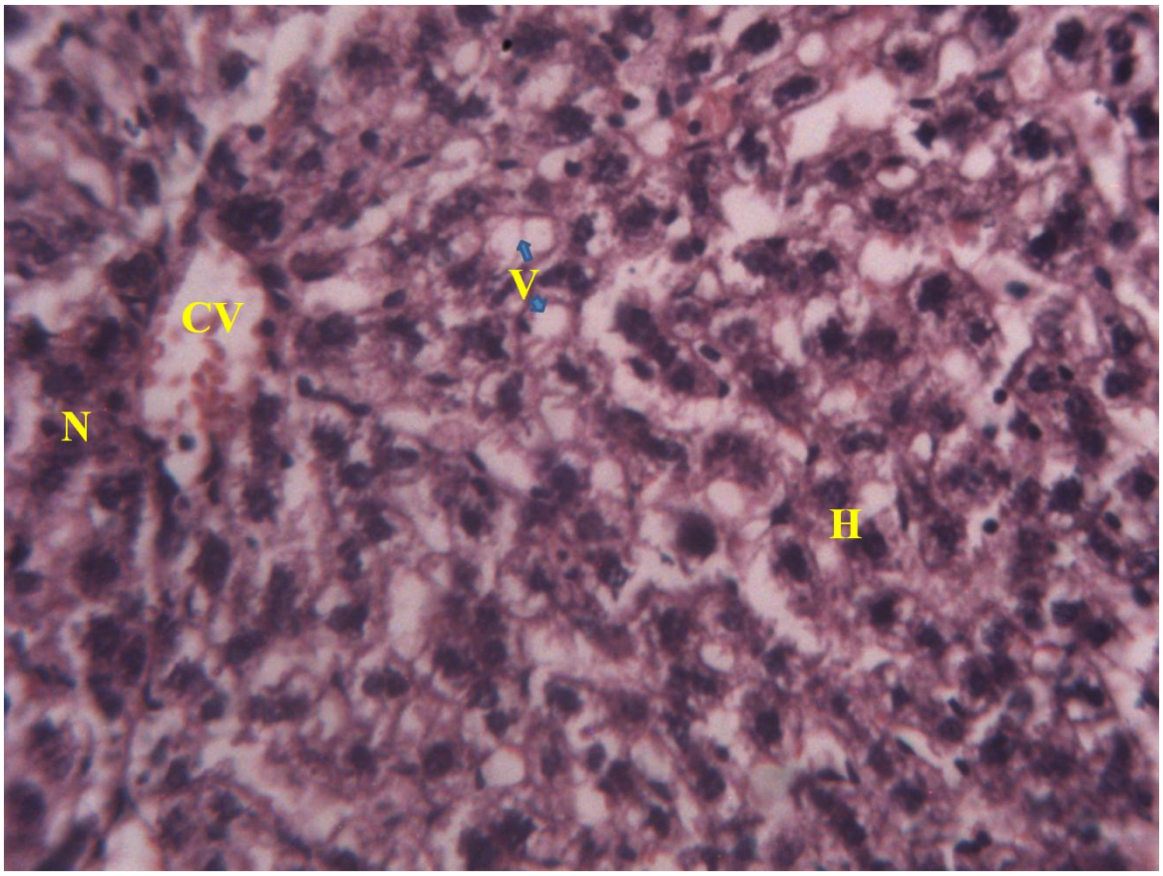


Plate IV: Photomicrograph of longitudinal section of liver of Wistar rat in Group III (Prophylactic group: 7 days of 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8), showing central vein (CV), areas of vacuolation (V), necrosis (N), and normal hepatocytes (H). (H & E stain × 250).

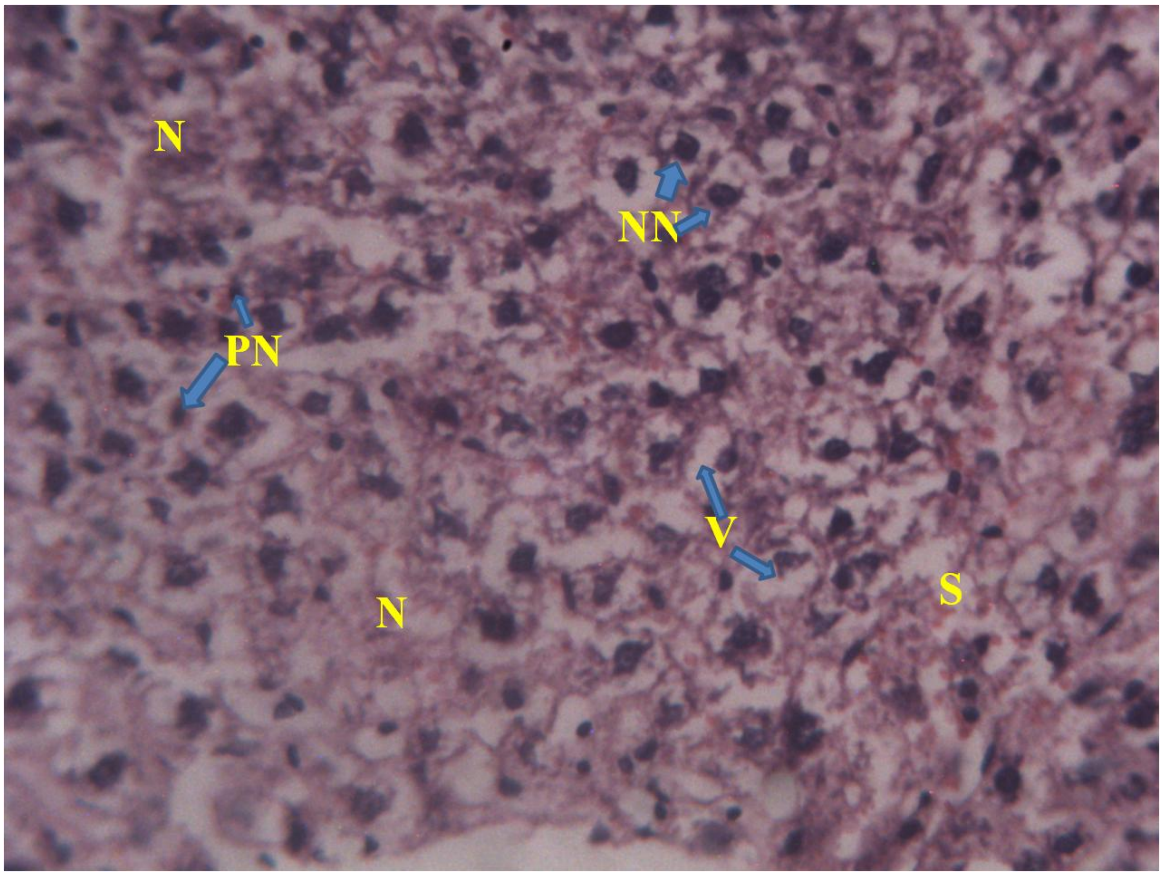


Plate V: Photomicrograph of longitudinal section of liver of Wistar rat in Group IV (Therapeutic group: 20 ml/kg of 40 % ethanol for 1 day + 7 days of 400 mg/kg of AELI), showing vacuolation (V), sinusoid (S), pyknotic nucleus (PN), necrosis (N) and normal nuclei. (H & E stain × 250).

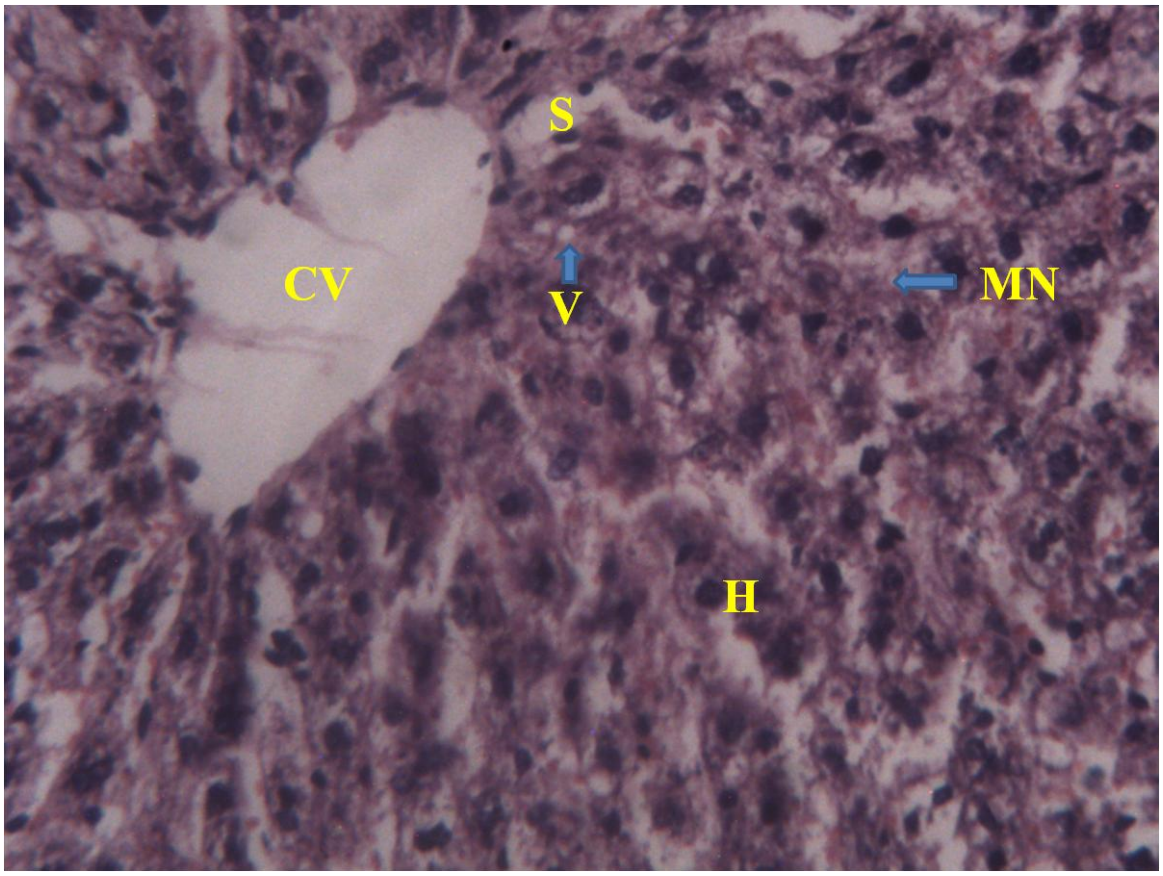


Plate VI: Photomicrograph of longitudinal section of liver of Wistar rat in Group V (Silymarin: 7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40 % ethanol on day 8) showing central vein (CV), sinusoid (S), normal hepatocytes (H), vacuolations (V), and mild necrosis (MN). (H & E stain × 250).

4.5.2 Acridine orange and ethidium bromide (AO-EB) staining

Liver sections from Group I (Plate VII) showed normal cells giving green fluorescence and normal morphology of the nucleus. Cells of Group II (Plate VIII) showed necrotic cells exhibiting a uniform yellow fluorescence, some apoptotic cells at different stages exhibiting condensation and fragmentation of the nucleus and giving yellowish green fluorescence. Cells from Group III (Plate IX) showed normal cells giving green fluorescence, with some apoptotic cells. Cells from Group IV (Plate X) showed more necrotic cells than Group III, but less than Group II. Group V (Plate XI) showed necrotic and apoptotic cells comparable to Group IV.

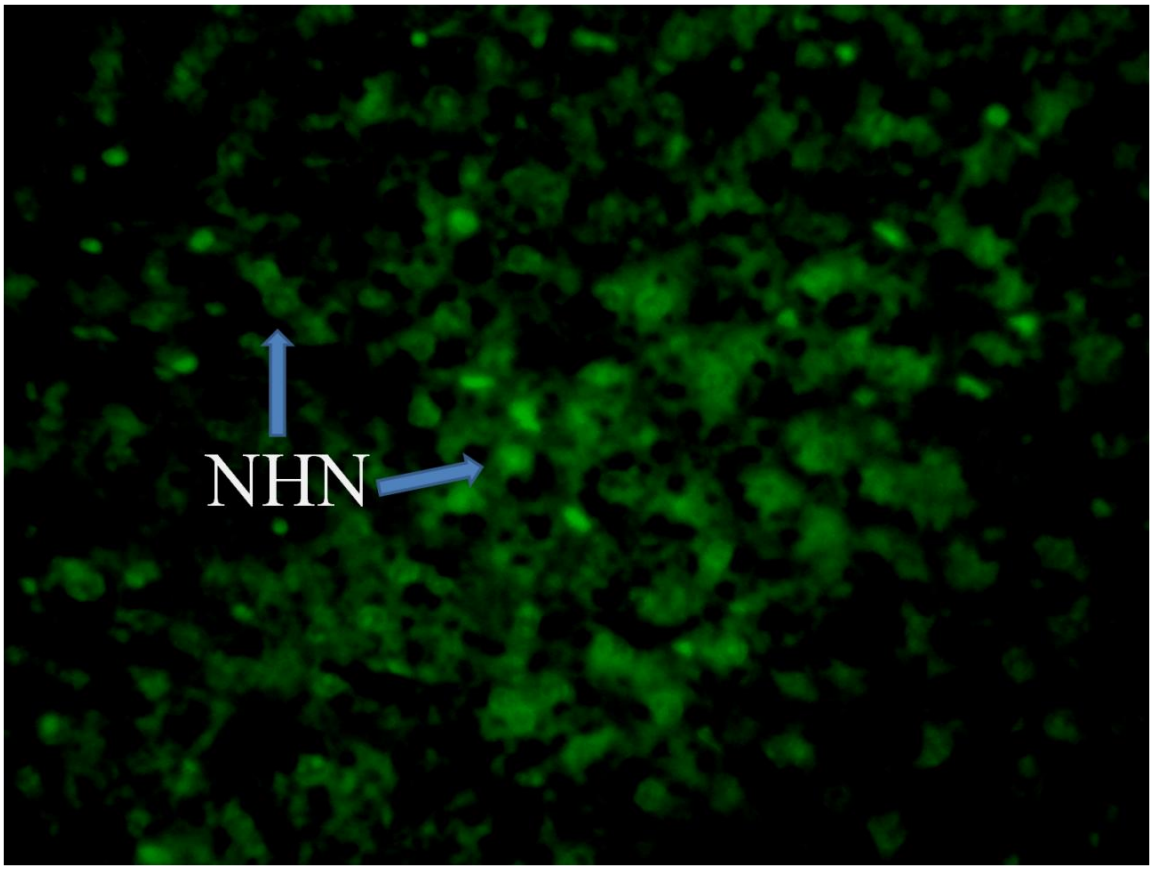


Plate VII: Photomicrograph of longitudinal section of liver of Wistar rat in Group I (negative control: distilled water for 8 days) showing normal hepatocyte nucleus (NHN) exhibiting green fluorescence. (AO-EB Staining × 400).

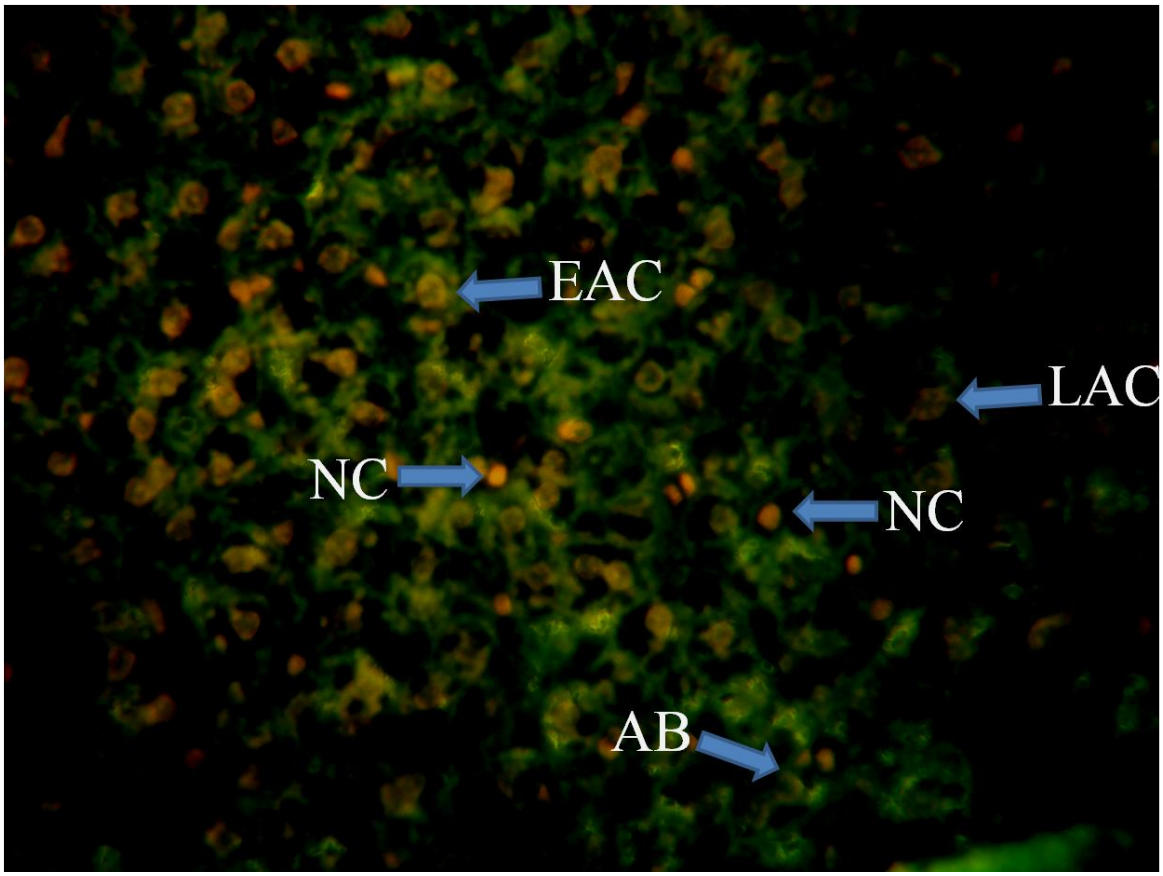


Plate VIII: Photomicrograph of longitudinal section of liver of Wistar rat in Group II (Positive control: 7 days distilled water + 20 ml/kg of 40 % ethanol on day 8) showing necrotic cells (NC) exhibiting uniform yellow fluorescence, early apoptotic cells (EAC) exhibiting yellow greenish fluorescence and Late apoptotic cells (LAC) exhibiting fragmented yellow fluorescence, with cluster of apoptotic bodies (AB). (AO-EB Staining × 400).

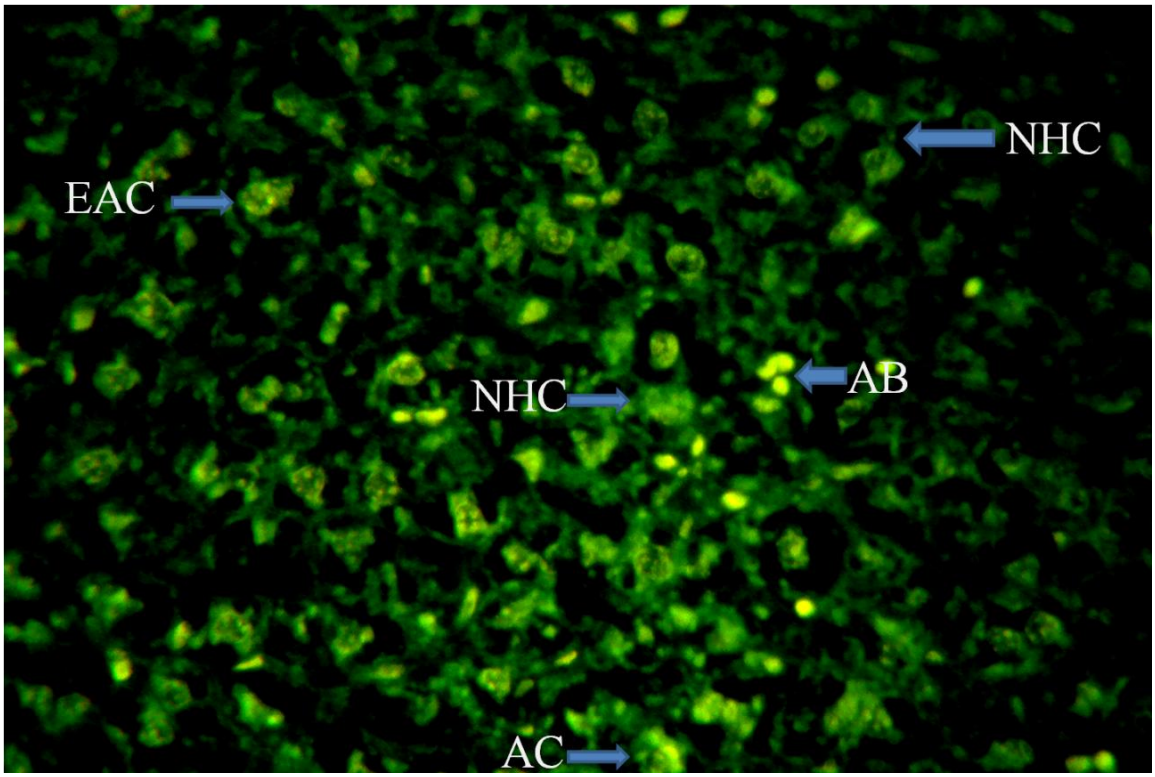


Plate IX: Photomicrograph of longitudinal section of liver of Wistar rat in Group III (Prophylactic group: 7 days of 400 mg/kg of AELI + 20 ml/kg of 40 % ethanol on day 8) showing normal hepatocyte cell nucleus (NHC) exhibiting green fluorescence, early apoptotic cell (EAC) exhibiting fragmentation in green fluorescence, apoptotic cell (AC) exhibiting yellowish-green fluorescence, a cluster of apoptotic bodies (AB). (AO-EB Staining × 400).

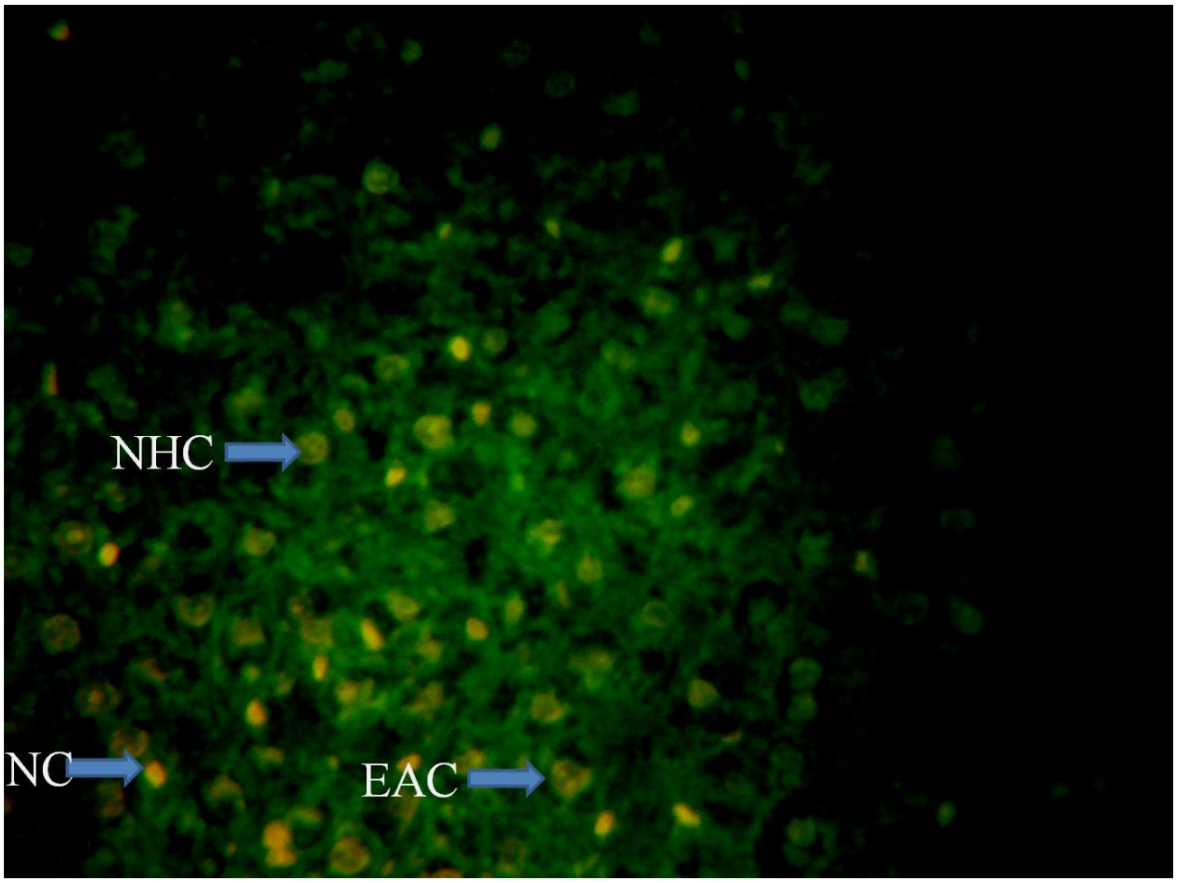


Plate X: Photomicrograph of longitudinal section of liver of Wistar rats in Group IV (Therapeutic group: 1 day of 20 ml/kg of 40 % ethanol + 7 days of 400 mg/kg of AELI) showing normal hepatocyte nucleus (NHC), necrotic cell (NC) exhibiting uniform yellow fluorescence, and early apoptotic cell (EAC) (AO-EB Staining × 400).

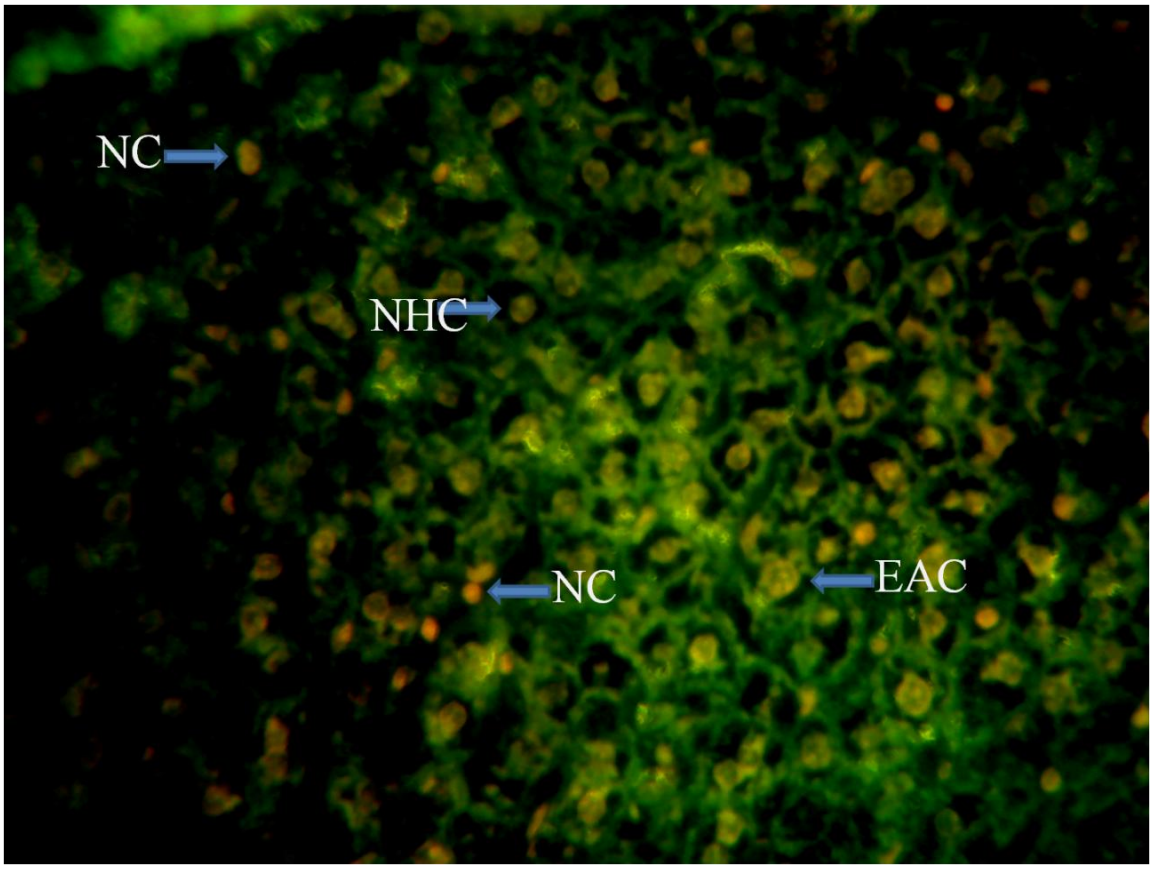


Plate XI: Photomicrograph of longitudinal section of liver of Wistar rat in from Group V (Silymarin: 7 days of 70 mg/kg of Silymarin + 20 ml/kg of 40 % ethanol on day 8) showing normal hepatocyte (NHC), necrotic cell (NC) exhibiting uniform yellow fluorescence, and early apoptotic cell (EAC) exhibiting fragmentation and yellowish green fluorescence. (AO-EB Staining × 400).

CHAPTER FIVE

5.0 DISCUSSION

5.1 Morphological Studies

A higher increase in body weight was observed in all the groups compared to the positive (ethanol) groups, though a significant increase was only in the negative (water) control and the therapeutic (AELI) group. This suggests that AELI like Silymarin helped protect against ethanol toxicity, and does not affect food intake.

It has been reported that increase or decrease in either absolute or relative weight of an organ after administering a chemical or drug is an indication of the toxic effect of that chemical (Orisakwe *et al.,* 2003). The hepatic index of the positive (ethanol) group was the highest, and can be explained as such: metabolism of ethanol leads to production of hydrogen ions that replace fatty acids as fuels leading to an increase in quantity of fatty acids and triglycerides in liver thereby leading to fatty liver (Sherlock and Dooley, 2004). In addition, as the body processes ethanol, activity in the liver increases thereby causing an increase in liver mass (Sherlock and Dooley, 2004; Infante, 2008). The increase in Liver-Body weight Index without significant Body weight gain could have been inflammation rather than growth (Hart and Ihemelandu, 2015). The liver-body weight ratio of the positive group being significantly higher than the negative control, and the therapeutic group shows that AELI was effective in restoring the normal index. Prophylactically, AELI though insignificant also showed hepatic index closer to normal than the positive group. So it can be said that the extract protected from effects of ethanol metabolism that might result in inflammation.

5.2 Biochemical Studies

5.2.1 Oxidative stress markers

Alcohol administration causes accumulation of reactive oxygen species (ROS), including superoxide, hydroxyl radical, and hydrogen peroxide (Nordmann *et al.*, 1992). Reactive oxygen species, in turn, cause lipid peroxidation of cellular membranes and protein and DNA oxidation, which result in hepatocyte injury (Kurose *et al.*, 1997; Rouach *et al.*, 1997; Navasumrit *et al.,* 2000). Antioxidants are agents that scavenge free radicals, and prevent their formation or repair the damage they cause (Halliwell, 1991). Superoxide dismutase is considered as the first line of defense against deleterious effects of oxygen radicals in the cells (Johnson *et al.,* 2005; Okado-Matsumoto and Fridovich, 2011), and it scavenges ROS by catalizing the dismutation of superoxide to hydrogen peroxide (Okado-Matsumoto and Fridovich, 2011). Hydrogen peroxide is catalized by catalase to water and oxygen (Baudhuin, 1969). Reduced GSH is the main antioxidant found in cells that plays a critical protective role in the metabolism of a large number of toxic agents, including Ethanol. Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation (Laura *et al.*, 2003). Results obtained from the present study confirmed previous observations that acute ethanol administration decreases the levels of Superoxide dismutase (SOD), catalase, the hepatic reduced GSH level (Song *et al.*, 2003; Zhou *et al.,* 2003), along with enhanced lipid peroxidation (Zhenyuan *et al.*, 2006). The study also showed increase in levels of SOD, catalase, reduced glutathione, and decreased lipid peroxidation in prophylactic and therapeutic group, suggesting antioxidant activity of AELI, which is consistent with previous studies that showed ethanolic extract of *L. inermis* increasing SOD and catalase

activities, increased reduced glutathione, and decreased lipid peroxidation (Dasgupta *et al.,* 2003).

5.2.2 Protein concentration

Nearly all proteins are synthesized in the liver, and measurement of total serum protein concentration is used to assess the synthesis and maintenance of proteins in circulation (Roger, 2014). Any change in the concentration of serum protein indicate a change in normal liver functions (Ahmed *et al.,* 2000; Imafidon and Okunrobo, 2012). Elevation of serum protein concentration has two principal causes; dehydration, in which there is less water in the body and the blood volume decreases, thereby concentrating the proteins, and overproduction of specific proteins which is more common (Marshall, 2012; Roger, 2014). Most commonly overproduced proteins are immunoglobulins (Marshall, 2012), which elevate in infections and in hematological neoplasms (Roger, 2014). A variety of proteins are classified as acute-phase reactants because their concentrations increase rapidly in response to inflammation (Roger, 2014).

Increased urination that usually occurs after ethanol consumption could have led to dehydration, and also increased production of enzymes and reactive substance as a result of ethanol metabolism and inflammation, may be the reason for the increased serum protein concentration seen in the positive control group. Prophylactically AELI and Silymarin decreased the serum protein concentration while therapeutically AELI showed no significant difference from the negative control. This suggests that the extract and the silymarin protected against toxic effects of ethanol metabolism that may have been the reason for the inflammation. This might be due to increased antioxidants levels as indicated by oxidative stress markers, which incase of Silymarin is confirmed in acute ethanol toxicity (Zhenyuan *et al.,* 2006), and/or reduction in the production of

acetaldehyde. Acetaldehyde generated by the ethanol metabolism is a highly reactive compound that can react with amino group of proteins resulting in acetaldehyde-protein adducts that may play a role in pathogenesis of alcohol toxicity (Brooks, 1997).

5.3 Molecular Study (DNA Fragmentation)

DNA damage in alcohol toxicity can be produced either by reactive oxygen species (ROS), acetaldehyde and products of lipid peroxidation, which are all generated by alcohol metabolism (Brooks, 1997). Brooks, 1997 also reported that DNA is highly susceptible to attack from hydroxyl radicals, which can produce strand breaks and a variety of different types of oxidative base damage with both mutagenic and cytotoxic potential. Lipid peroxidation products, resulting from attack of ROS on lipids, also react with DNA, forming ethenobases and other adducts with known carcinogenicity and miscoding potential. Acetaldehyde also reacts with DNA to produce N2-Et-dG, which is yet to be studied with respect to miscoding potential. Thus, during alcohol abuse, where levels of ROS and lipid peroxidation products are elevated, and antioxidant levels are reduced there is the potential for significant damage to DNA (Brooks, 1997) and acute exposure to ethanol causes cellular fragmentation (Ishii *et al.,* 1996) which is observed in Group II (Positive control). AELI prevented the extensive fragmentation of DNA both prophylactically and therapeutically, comparable with that of Silymarin. Its seen that AELI produced higher values of anti-oxidants than ethanol though insignificantly in this study, it suggests AELI may have also protect the DNA from products of lipid peroxidation, and acetaldehyde. Thus AELI has DNA protecting property, which is consistent with the previous study in which aqeous and methanolic extracts of *L. inermis* showed properties of DNA-protection chromium (VI)-induced DNA toxicity (Guha *et al.,* 2009), and in a study that compared potential of 56 different extracts for prevention of

DNA damage due to oxidative stress, aqeous extract of *Lawsonia inermis* proved to be most effective (Guha *et al.*, 2009).

5.4 Histological Study

Acute ethanol administration causes prominent microvesicular steatosis, and mild necrosis (Zhenyuan *et al.*, 2006). Findings in the histological studies showed that AELI when administered prophylactically attenuated the effects of acute ethanol toxicity to certain extent by decreasing the extent of steatosis, severity of necrosis, and vacuolation in hepatocytes. AELI prophylactically also showed lesser number of apoptotic cells. Therapeutically AELI wasn't as effective. Silymarin also protected against the effects of ethanol hepatotoxicity, by reducing the severity of necrosis, and steatosis which is consistent with study that show that Silymarin attenuates the adverse effects of ethanol administration showing less extensive steatosis and necrosis (Zhenyuan *et al.*, 2006).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From the present study, it is seen that Aqueous extract of *Lawsonia inermis* had the following effects in acute ethanol-induced liver injury:

i. reduced vacuolation, necrosis and presence of pyknotic nuclei in the liver as shown by Haematoxylin and Eosin staining
ii. Reduced number of apoptotic and necrotic cells, as shown by Acridine Orange – Ethidium Bromide staining.
iii. Insignificant decrease in oxidative stress, as shown by markers of oxidative stress; Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and reduced glutathione (GSH).
iv. Prevention of DNA damage as indicated by less fragmentation and retaining of high molecular weight DNA, as shown with gel electrophoresis
v. Reduced protein concentration of hepatocytes, indicating less damage as shown by assessment with Bradford reagent.

It can be concluded that the Aqueous extract of *L. inermis* leaves at the dose of 400 mg/kg for seven days attenuated the effects of acute ethanol induced hepatotoxicity more effectively prophylactically than therapeutically. Also, hepatoprotection by Aqueous extract of *L. inermis* leaves at the dose of 400 mg/kg for seven days, is comparable to that by the reference drug Silymarin.

The possible mechanism through which AELI provides hepatoprotection in acute ethanol induced hepatotoxicity is prevention of nucleic damage, with reduced apoptotic and necrotic cell death. This may have some contribution from anti-oxidant effect.

6.2 Recommendations

From the present study, the following recommendations are made;

i. different concentrations of AELI should be used to test the effectiveness of hepatoprotection, and determine the most effective dose.
ii. Different extract procedures should be carried out to assess the most effective extraction process.
iii. Different extracts of *Lawsonia inermis* should be tested
iv. different durations of treatment should also be tested.
v. the effects in chronic ethanol toxicity should be studied.
vi. further detailed studies should be carried out to confirm if AELI has an anti-apoptotic effect, and its specific mechanism.
vii. further studies should be done to investigate the main active principle responsible for hepatoprotection by AELI.

CONTRIBUTIONS TO KNOWLEDGE

1. This study showed that the Aqueous extract of *Lawsonia inermis* leaves (AELI), when administered in acute ethanol-induced liver damage, provided DNA protection and was more effective as a prophylactic than a treatment.
2. This study also showed that the degenerative effects of acute ethanol induced toxicity on the liver histology, were ameliorated by the administration of aqueous extract of *Lawsonia inermis* leaves. This was better seen when AELI was used a prophylactic than as treatment.

REFERENCES

Abdelgadir, E. H., Ahmed, R. H., adam, S. I. Y. and Hussein, A. M. (2010), "evaluation of toxicological activity (acute and sub-chronic toxicities) of the aquoes extract of *Lawsonia inermis* seeds on wistar rats". *Journal of Pharmacology and Toxicology,* 5:324-333.

Abei, H. (1974), "Catalase", Method of enzymatic analysis, Academic Press, New York, USA, pp 673-684.

Adebolu, T. T. and Oladimeji, S. A. (2005), "Antimicrobial activity of leaf exracts of *Ocimum gratissimum* on selected diarrhea causing bacteria in south western Nigeria", *African Journal of Biotechnology,* 4(7):682

Afzal, M., Al-Oriquat, G., Al-Hassan, J. M. and Muhammad, N (1980), "Flavone glycosides from *Lawsonia inermis*". *Heterocycles,* 14:1973-1976

Afzal, M., Al-Oriquat, G., Al-Hassan, J. M. and Muhammad, N (1984), "Isolation of 1,2-Dihydroxy-4-gucosyloxynaphthalene from *L. inermis*". *Heterocycles,* 22(4): 813

Aguwa, C. N. (2008), "Toxic effects of the methanolic extract of *Lawsonia inermis* roots". *International Journal of Crude Drug Research,* doi:10.3109/13880208709055201.

Ahmed, S., Rahman, A., Alam, A., Saleem, M., Athar, M. and Sultana, S. (2000), "Evaluation of the efficacy of *Lawsonia alba* in the alleviation of carbon tetrachloride induced oxidative stress". *Journal of Ethnopharmacology,* 69:157-164.

Al-Rubiay, K. K., Jaber, N. N., Al-Mhaawe, B. H. and Alrubaiy, L. K. (2008), "Antimicrobial efficacy of Henna extracts". *Oman Medical Journal*, 23(4):1-4.

Amrita, S. and Singh, D. K. (2001), "Molluscicidal activity of *Lawsonia inermis* and its binary and tertiary combinations with other plant derived molluscicides". *Indian Journal of Experimental Biology,* 39:263-268.

Ashnagar, A. and Shiri, A. (2011), " Isolation and characterization of 2-hydroxy 1-1,4 – napthoquinone (lawsone) from the powdered leaves of henna plant marketed in; Ahwaz city of Iran"; *International Journal of chemtech research* 3: 1941-1944.

Atawodi, S. E., Adekunle, O. O. and Bala, I. (2011), "Antioxidant, organ protective and ameliorative properties of methnol extract of *Anogeissus leiocarpus* stem bark against carbon tetrachloride-induced liver injury". *International Journaal of Pharmaceutical Sciences Research,* 2:748-753.

Badoni, S.R., Semwal, D.K., Combrink, S., Cartwright-Jones, C. and Viljoen, A. (2014), "*Lawsonia inermis* L. (henna): ethnobotanical, phytochemical and pharmacological aspects". *Journal of Ethnopharmacology,* 155 (1): 80-103, doi: 10.1016/J.Jeo 2014.05.042. Epub.

Bandyopadhyay, U., Biswas, K., Chattopadhyay, I., and Banerjee, R.K. (2002). "Biological Activities and medicinal properties of neem (*Azadirachta indica*)". *Current Science,* 82(11):1336-1345.

Baudhuin, P. (1969), "Liver peroxisomes, cytology and function". *Annals of New York Academy of Sciences,* 168:214–228

Bhakuni, D.S, Dhar, M.I, Dhar, M.M., Dhawan, B.N., Gupta, B. and Srimal, R.C. (1971), "Screening of Indian plants for biological activity, part III". *Indian Journal of Experimental Biology*, p91.

Bharadwaj, D. K., Jain, R. K., Jain, B. C. and Mehta, C. K. (1978), "1-hydroxy-3,7-dimethoxy-6-acetoxyxanthone; a new xanthone from *Lawsonia inermis*". *Phytochemistry,* 17:1440-1441

Bharadwaj, D. K., Murari, R., Seshadri, T. R. and Singh, R. (1976), "Lacoumarin from *Lawsonia inermis*". *Phytochemistry,* 15:1789

Bharadwaj, D. K., Seshadri, T. R. and Singh, R. (1977), "Xanthones from *Lawsonia inermis*". *Phytochemistry,* 16:1616-1617

Bhuvaneshwani, K., Poongothai, S.G., and Kunivilla, A. (2002)," Appalaraju, B: Inhibitory concentrations of *L.inermis* dry powder for urinary pathogens." *Indian Journal of pharmacology,* 34:260-263.

Bich, D. H., Chung, D. Q., Chuong, B. X., Dong, N. T., Dam, D. T., Hien, P. V., Lo, V. N., Mai, P. D., Man, P. K., Nhu, D. T., Tap, N. and Toari, T. (2004), The medicinal plants and animals in Vietnam, Science and Technology publishing house, Hanoi Vietnam, p215.

Borade, A. S., Babasaheb, N. K. and Rajkumar, V.S. (2011), "A phytopharmacological review on *Lawsonia inermis* (Linn)". *International Journal of Pharmacy and Life Sciences,* 2(1):536-539.

Brooks, P. J. (1997), "DNA damage, DNA repair and Alcohol-toxicity- A Review". *Alcoholism: Clinical and Experimental Research,* 21:6

Bull, S. (2010), "Ethanol- general information", Health Protection Agency, 1:2-4.

Carson, E. J., Prutt, S. B. (1996). "Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol", *Alcoholism Clinical and Experimental Research,* 20:132–138

Chakraborthy, D., Poddar, G. and Deshmukh, S. K. (1977), "Triterpenoids and other constituents of *Lawsonia inermis*". *Indian Journal of Chemistry,* 15:96-97

Chakraborthy, T. Poddar, G. and St-Pyrek, J. (1982), "Isolation of dihydroxylupene and dihydroxylupane from the bark of *Lawsonia inermis*". *Phytochemistry,* 21(7):1814-1816

Chattopadhay, R.R. (2003). "Possible mechanism of the hepatoprotective activity of *Azadirachta indica* leaf extract". *Journal of Ethnopharmacology*, 104:124-128.

Chaudary, G., Goyal, S. and Poonia, P. (2010), "*Lawsonia inermis* Linnaeus: A phytopharmacological review". *International Journal of Pharmaceutical Science and Drug Research*, 2(2):91-98.

Chauhan, M. G. and Pillai, A. P. G. (2007), "Henna – *Lawsonia inermis*", Microscopic profile of powdered drugs used in Indian system of medicine, Jamnagar Gujrat, pp84-85

Chetty, K. M. (2008). "Flowering plants of Chittoor". *Andhra Pradesh,* 1:132.

Chopra, R. N., Nayer, S. L., and Chopra, I. C. (1956). *Glossary of India Medicinal Plants*, CSIR Publications, New Delhi, pp 151.

Comporti, M., Signorini, C., Leoncini, S., Gardi, C., Ciccoli, L., Giardini, A., Vecchio, D. and Arezzini, B. (2010), "Ethanol-induced oxidative stress: basic knowledge". *Genes and Nutrition*, 5:101-109, doi:10.1007/s12263-009-0159-9

Costran, R. S., Kumar, V., Fausto, N. F., Robbins, S. L. and Abul K. (2005). Robbins and Cotran pathologic basis of disease, St Louis, Elsevier Saunders, 7th Ed, p878

Cuong, N. X., Nguyen, N. X., Thao, N. P., Nam, N. H., Dat, N. T., Anh, H. L. T., Huong, L. M., Kiem, P. V., Minh, C. V., Won, J. H., Chung, W. Y. and Kim, Y. H. (2010), "Inhibitors of osteoclastogenesis from *Lawsonia inermis* leaves". *Bioorganic and Medicinal Chemistry Letters*, 20(6):4782-4784.

Dalley, A. F. and Moore, K. L. (2006), "Liver". Clinically oriented anatomy, Lippincott Williams and Wilkins, 5th edition, pp 293-298

Dasgupta, T., Rao, A. R. and Yadava, P.K. (2003), "Modulatory effect of henna leaf (*Lawsonia inermis*) on drug metabolising phase I and phase II enzymes, antioxidant enzymes, lipid peroxidation and chemically induced skin and *forestomach papilloma* genesis in mice". *Molecular and Cellular Biochemistry*. 245:11–22.

David (2015), Absorption of ethanol, retrieved from www.doctorbenjamin.com/areas-of-focus/blood-breath-alcohol-levels/ on 06/08/2015

Deborah, D.E, bhavani, R.S., Kumar, R R-Y and Bharath, K A (2011)," Formulation and Evaluation of *Lawsonia inermis* paste". *International Journal of Research in Pharmaceutical and Biomedical sciences*, 2 (2): 687-690.

DiPadova, C., Roine, R., Frezza, M., Gentry, R. T., Baraona, E. and Lieber, S. C. (1992), "Effects of ranitidine on blood alcohol levels after ethanol ingestion, comparison with other H-2 receptors antagonists". *Journal of the American Medical Association*, 267:83-86

Dixit, S. N., Srivastava, H. S., Tripathi, R. D. (1980), "Lawsone, the antifungal antibiotic from the leaves of Lawsonia inermisand some aspects of its mode of action". *Indian Phytopathology,* 31:131-133

Ellman, G. L. (1959), "Tissue sulfhydryl groups". *Archives of Biochemistry and Biophysics*, 82:70-74.

Endrini, S., Rahmat, A., Ismail, P., Taufiq-Yap, Y. H. and Othamn, F. (2012), "Effects of Henna (*Lawsonia inermis*) on the Apoptotic pathway of human liver carcinoma cell lines". *Journal of Biological Chemistry*, 7(3):321

Eroschenko, V.P., (2000). "Digestive system: Liver, gallbladder, and pancreas". *Atlas of histology with functional correlations.* Lippincot Williams and Wilkins. (9): 219

Farnsworth, N.R. (1988), "Screening plants for new medicine". *National Academy Press,* pp 83

Fridovich, I. (1989), "Superoxide dismutases – An adaptation to a paramagnetic gas", *Journal of Biological Chemistry,* 264:7761-7764

Friedman, Scott, E., Grendell, James, H., McQuaid, and Kenneth, R. (2003). *Current Diagnosis Treatment in Gastroenterology*. New York: Lang Medical Books/McGraw-Hill. pp. 664–679.

Garba, S. H., Ahmadu, S. and John, I. A. (2006), "The effect of aqeous stem bark extract of *Sclerocarya birrea* (Hochst) on alcohol-carbon tetrachloride induced liver damage in rats". *Pakistan Journal of Biological Sciences,* 9(12): 2283-2287.

Gray (2004). The anatomical basis for clinical practice. Elsevier Churchill Livingstone. 39th ed, pp 970-976, 1218-1222.

Grieve, M. (2005), "Henna", www.botanical.com/botanical/mgmh/h/henna-24.html retrieved on 26/01/2014

Guenthner, T. M. (2012). "Pharmacology and toxicology of ethyl alcohol". Epidemiology, retrieved from www.uic.edu/classes/epidemiology/DENTALETOH on 22/01/2014

Guha, G., Rajkumar, V., Kumar, R. A. and Mathew, L. (2009), "Antioxidant activity of *Lawsonia inermis* extracts inhibits chromium(VI)-induced cellular and DNA toxicity". *Evidence-based Complementary and Alternative Medicine,* 7:4.

Guha, G., Rajkumar, V., Mathew, L. and Kumar, R. A. (2009), "The antioxidant and DNA protection potential of Indian tribal medicinal plants". *Turkish Journal of Biology,* 35:233-242, doi:10.3906/biy-0906-64

Gull, I., Sohail, M., Shahbaz, M.A. and Muhammed, A.A. (2013), " Phytochemical, toxicological and antimicrobial evaluation of *Lawsonia inermis* extracts against clinical isolates of pathogenic bacteria". *Annals of clinical microbiology and Antimicrobials,* 12:36. http:www.ann.clinmicrob.com/content/12/1/36.

Gupta, S. S. (1994), "Prospects and perspectives of natural plants products in medicine". *Indian Journal of Pharmacology*, 26:1-12.

Gupta, S., Ali, M. and Alam, M. S. (1993),"A naphthaquinone from *Lawsonia inermis* stem bark". *Phytochemistry,* 33(3):723-724

Gupta, S., Ali, M. M., Alam, M. S., Niwa, M. and Sikai, T. (1992), "24β-ethylcholest-4-en-3-β-ol from the roots of *Lawsonia inermis*". *Phytochemistry,* 31(7):2558-2560

Gupta, S., Ali, M., Alam, M. S., Sakai, T. and Niwa, M. (1992), "A new aliphatic hydrocarbon from *Lawsonia inermis* bark". *Indian Journal of Chemistry,* 31:705-707

Gupta, S., Ali, M., Pillai, K. K. and Alam, M. S. (1993), " Evaluation of anti-inflammatory activity of some constituents of *Lawsonia inermis*". *Fitoterapia*, 64(4):365-366.

Habbal, O. A., Al-Jabri, A. A., El-Hag, A. H., Al-Mahrooqi, Z.H. and Al-Hashmi, N. A. (2005). "In vitro antimicrobial activity of *Lawsonia inermis* Linn (Henna); A pilot study on the Omani Henna". *Saudia Medical Journal*, 26:69-72.

Halliwell, B. (1991), "Reactive oxygen species in living systems; source, biochemistry and role in human disease". *American Journal of Medicine*, 91:14-22.

Halliwell, B. (1994), "Free radicals, anti-oxidants and human diseases: Curiosity, cause or consequence". *Lancet,* 344:721-724

Halliwell, B. and Gutteridge, J. (1989). Free radicals in biology and medicine. Oxford, Clarendon, 2nd edition.

Handa, G., kapil, A., Sharma, S. and Jagdev, S. (1997), "Lawnermis acid; a new anticomplementary triterpenoids from *Lawsonia inermis* seeds". *Indian Journal of Chemistry,* 36(3):252-256

Hanna, R., Maciej, J.N., Lapinsky, l and Adamowicz, L. (1998), "Molecular structure and infra-red spectre of 2-hydroxy-1-4-naphthquinone; Experimental matrix isolation and theoretical Hatree-Fock and post Hatree-Fock study". *Spectrochimica Acta,* 54: 1091: 1103.

Hart, J. S. and Ihemelandu, F. C. (2015), "The effect of Alpha-Tocophenol on Paraquat-Induced Hepatotoxicity of Male Wistar rats". *The Journal of Anatomical Sciences*, 6(1):24.

Hikmat, U.J, Khan, Z.S. and Ashfaq, A.K.C (2011), " staining effect of dye extracted from dry leaves of *Lawsonia inermis* Linn (Henna) on Angiospermic stem tissue". *Pakistani Journal of Botany;* 43 (1): 383-389.

Hussain, T., Arshad, M., Khan, S., Saltar, H. and Qureshi, M.S. (2011)," In vitro screening of methanol plant extracts for their bacterial activity" *Pakistani Journal of Botany*, 43:531-538.

Infante, S. (2008), “Symptoms of alcohol-induced liver and heart diseases in rats that regularly drink alcohol”. *Ethnicity and disease*, vol 18

Ishii, H., Thurman, R., Ingelman-Sundberg, M., Cederbaum, A**.,** Fernandez-Checa, J., Kato, S**,** Yokoyama, H. and Tsukamoto, H. (1996), “Oxidative stress in alcoholic liver injury”. *Alcoholism: Clinical and experimental Research*, 20:162A-l67A

Iyer, M. R., Pal, S. C., Kasture, V. S. and Kasture, S. B. (1998), “Effectof Lawsonia inermis on memory and behavior mediated via monoamine neurotransmitters”. *Indian journal of Pharmacology*, 30:181-185.

Jaeschke, H., Gores, G .J., Cederbaum, A. I., Hinson, J. A., Pessayre, D. and Lemasters, J. J. (2002). "Mechanisms of hepatotoxicity". *Toxicological Sciences.* 65 (2): 166–76.

Jiny, V.K., Silvipinya, K.S, Resmi, S and Jolly, C.I. (2010),” *Lawsonia inermis* (henna): a natural dye of various therapeuticuses a review”. *Inventi Impact: Cosmeceuticals.* Article ID- inventi: CC/3/10,2010.

Johnson, W. T., Johnson, L. A. K., Lukaski, H. C. (2005), “Superoxide dismutase 3 (extracellular superoxide dismutase) activity is a sensitive indicator of Cu status in rats”. *Journal of Nutritional Biochemistry,* 16:682-692

Jones, J. D., Barber, B., Engra, V. L., and Heimback, D. (1991), “Alcohol use and burn injury”. *Journal of Burn Care and Rehabilitation,* 12:148-152

Kamal, M. and jawaid, T (2010),” pharmacological activities of *Lawsonia inermis* Linn: a review.” *International Journal of Biomedical Research,* 2:62-68.

Kelmanson, J. F., Jager, A. K. and Staden, J. V. (2002), “Zulu Medicinal plants with antibacterial activity”. *Journal of Ethnopharmacology,* 69:241-246.

Khan, M. M., Ali, A., Jain, D.C., Bhakuni, R. S., Zaim, M., Thakur, R. S. (1991), “Occurrence of some antiviral sterols in *Artemisia annua*”. *Plant Science,* 75(2):161-165.

Kharbude, B. V. and Agarwal, O. P. (1985), “TLC system for identification of natural dye”. *Journal of Chromatography,* 347:447

Kumar, I.M. (2011), “*Lawsonia Inermis*- From traditional use to scientific assessment”. *PharmaTutor- pharmacy Infopedia,* retrieved on August 15th, 2014.

Kumar, K. V. A., Satish, R., Rama T., Kumar, A., Babul, D., and Samhitha, J. (2010). “Hepatoprotective effect of *Flemingia strobilifera* R.Br. on paracetamol induced hepatotoxicity in rats”. *International Journal of PharmTech Research,* 2(3): 1924-1931.

Kurose, I., Higuchi, H., Kato, S., Miura, S., Watanabe, N., Kamegaya, Y., Tomita, K., Takashi, M., Horie, Y., Fukuda, M., Mizukami, K. and Ishii, H. (1997),

"Oxidative stress on mitochondria and cell membrane of cultured rat hepatocytes and perfused liver exposed to ethanol". *Gastroenterology,* 112:1331–1343.

Larson, G. E. (2005), "Vascular plants", www.npwre.usgs.gov/resource/vascplnt/familythr.south retrieved 23/03/2015

Latha, P. G., Suja, S. R., Shyamal, S. and Rajaseldharan, S. (2004). "Some hepatoprotective Garden plants". *Green page:Article, Tropical Botanic Garden and Research Institute,* Palode, Trivandum– 695 562, p 1.

Laura, J., Niedernhofer, J., Scott, D., Carol, A. R., Rachel E. G.and Lawrence J. M. (2003), "Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells". *The American Society for Biochemistry and Molecular Biology, Inc,* retrieved from http://www.jbc.org/content/early/2003/05/29/jbc.M212549200.full.pdf on august 4th, 2015

Lavhate, M. S. and Mishra, S.H. (2007). "A review: nutritional and therapeutic potential of *Ailanthus excels*", *Pharmacognosy Review,* 1(1):105-113.

Lieber, C. S. (1970), "New pathway of ethanol metabolism in the liver". *Gastroenterology,* 59:930-937

Marshall, W. (2012), "Total protein". *Association for clinical Biochemistry, p* 1-3.

Massey, V. L. and Arteel, G. E. (2012), "Acute alcohol-induced liver injury – Review article". *Frontiers in Physiology,* doi: 10.3389/fphys.2012.00193

Mikhaeil, B. R., Badria, F. A., Maatooq, G. T. and Mohammed, M. A. (2004), "Antioxidant and immunomodulatory constituents of Henna leaves". *Zeilschift fur Naturforsch,* 59C:468-476.

Mohammed, A., Tanko, Y., Okasha, M. A., Magaji, R. A. and Yaro, A. H. (2007). "Effects of aqueous leaves extract of *Ocimum gratissimum* on blood glucose levels of streptozotocin induced diabetic wistar rats". *African Journal of Biotechnology* 6: 2087-2090.

Mudi, S. Y., Ibrahim, H. and Bala, M. S. (2011), "Acute toxicity studies of the aqueous root extract of *Lawsonia inermis* Linn. in rats". *Journal of Medicinal Plants Research,* 35: 5123-5126

Muhammad, H. S. and Muhammad, S. (2005). "The use of *Lawsonia inermis* Linn (Henna) in the management of burn wound infections". *African Journal of Biotechnology,* 4:934-937

Nadjib, M. R., Zahia, B. A., Mohammed, B., Kebir, B. and Noureddine, C. B. (2013), "Antimicrobial activities of Henna extract and some synthetic naphthoquinones derivatives". *American Journal of Medical and Biological Research,* 1(1): 16-22

Navasumrit, P., Ward, T. H., Dodd, N. J. and O'Connor, P.J. (2000). "Ethanol-induced free radicals and hepatic DNA strand breaks are prevented in vivo by antioxidants: effects of acute and chronic ethanol exposure". *Carcinogenesis*, 21:93–99.

Nordmann, R., Ribiere, C. and Rouach, H. (1992). "Implication of free radical mechanisms in ethanol-induced cellular injury". *Free Radical Biology and Medicine,* 12:219–240.

Nuha, A. M. E., Shaddad, S. A. I., and Mudathir, A. K. (2014), "Safety of *Lawsonia inermis* ethanolic seeds extract". *Journal of Pharmaceutical and Biomedical Sciences*, 4(4):303-309.

Okado-Matsumoto, A. and Fridovich, I. (2011), "Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu^{-}, Zn-SOD in mitochondria". *Journal of Biology and Chemistry,* 276:38388-38393

Okhawa, H., Qohishi, N. and Yagi, K. (1979), "Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction". *Analytical Biochemistry,* 95:351-358.

Orisakwe, O. E., Hussaini, D. C. and Afonne, O. J. (2003), "Testicular effects of subchronic administration of *Hibiscus sabdariffa* calyx aqeous extract in rats". *Reproductive Toxicology*, 18:295-298.

Orwa, C., Mutua, A., Kinat, R., Jamnadass, R., Anthony, S. (2009), "Lawsonia inermis", Agroforestree Database: a tree reference and selection guide 4.0, www.worldagroforestry.org/sites/treedatabase.asp retrieved 24/05/2015

Ostapowicz, G., Fontana, R.J., Schiødt, F.V. (2002). "Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States". *Annals of International Medicine,* 137 (12): 947–54.

Patel, T., Roberts, L. R., Jones, B. A. and Gores, G. J. (1998). "Dysregulation of apoptosis as a mechanism of liver disease: an overview". *Seminar. Liver Disease,* 18 (2): 105–114.

Pink, J., Planchon, J.S.M., Tagliarino, C., Varnes, M.E, Siegel, D. and Boothman, D.A. (2000), "NAD(P)H, Quinone oxidoreduotae activity is the principal determinant if beat-lapachone cytotoxicity". *Journal of Biological chemistry*, 275:5410-5424.

Pirmohammed, M., Breckenridge, A. M., Kitteringham, N. R. and Park, B. K. (1998). "Adverse drug reactions". *British Medical Journal,* 316 (7140): 1295–1298.

Pohorecky, L. and Brick, J. (1990), "Pharmacology of Ethanol", *International Enclycopedia of Pharmacological Therapeuties: Psychotic drugs of Abuse,* Pergamon Press, 189-254

Ponnu, S., Santhi, D. K., Jacob, N. and Suresh, B. (2003). "Safety measures with herbs". *Indian Pharmacist*, 2, pp:9-12.

Prasad, V. and Gupta, S.C. (1967), "Inhibitory effect of bark and leaf decoctions on the activity of pectic enzymes of Alteriaria tenuis". *Indian Journal of Experimental Biology,* 192.

Rahmoun, M. N., Benabdallah, M., Villemin, D., Boucherit, K., Mostefa-Kara, B., Ziani-Cherif, C. and Choukchou-Braham, N. (2010), "Antimicrobial screening of *Lawsonia inermis* (henna)". *Der Pharma Chemica,* 2:320-326

Rajagopalan, R., Kode, A., Penumatha, S. V., Kallikat, N. R. and Venugopal, P. M. (2004), "Comparative effects of curcumin and an analog of curcumin on alcohol and PUFA induced oxidative stress". *Journal of Pharmacy and Pharmaceutical Sciences,* 83:2741-2752.

Rao, S.S., Regar, P. L. and Singh, Y. V. (2005). "Agrotechniques for henna (Lawsonia inermis L.) cultivation, improvement and trade". *Central Arid Zone Research Institute,* Pali-Marwar, pp: 25-27.

Rawlins, M. D. and Thompson, J. W. (1991),"Mechanisms of adverse drug reaction". Textbook of adverse drug reactions, Oxford University Press, Oxford,pp 18-45.

Reddy, K. R. (1988), "A survey of Plant crude drugs of Anantapur District, India". *International Journal of Crude Drug Research*, 26:137-140.

Riechling, U.J., Harkenthal, M., Brandt, H. and Bayeri, C. (1999), " Temporary Henna Tattoos". *Deutsche Apotheker Zei tung*, 33(139): 35-41.

Roger, L. B. (2014), "Clinical significance of protein and albumin measurements". *Laboratory Medicine,* 45(1):e25-e41.

Rouach, H., Fataccioli, V., Gentil, M., French, S. W., Mirimoto, M. and Nordmann, R. (1997), "Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology". *Hepatology*, 25:351–355.

Sastri, B. N. (1962), "the wealth of India: Raw materials", Council of scientific and industrial research, New Delhi, pp47-50

Shah, J. S., Shah, M. B., Goswami, S. S., and Santani, D. D. (2006), "Mechanism of action of antiulcer activity of bark extracts of *Manikara hexandra* against experimentally induced gastric ulcer in rats". *Pharmacognosy Magazine,* 2:40-45

Shah, M. (2010). "Hepatoprotective drugs and its biological screening", retrieved from http://www.doyouknow.in/articles/pharmaceutical on 28th January 2015.

Shakhashiri, B. (2009), "Chemical of the week – Ethanol". General Chemistry, retrieved on 23rd september, 2014 from www.scifun.org

Sharma, V.K. (1990), " Tuberculostatic activity of henna *Lawsonia inermis* Linn.) " *Tubercle,* 71: 293-295.

Sherlock, S. and Dooley, J. (2004), Diseases of the liver and biliary system. Malden USA Blackwell Publishing, 11th edition

Sies, H. (1991), "Oxidative stress: oxidants and antioxidants". Academic press, London, San Diego. p 10-13.

Simon, J. E., Chadwick, A. F. and Craker, L. E. (1984). *Herbs an indexed bibliography, The scientific literature on selected herbs aromatic and medicinal plants of the temperate zone*. Archon Books, Hamden, pp: 1971-1980.

Singh, I. (2002). Textbook of human histology with colour atlas. JAYPEE publishers. 4th Edition.

Singh, I. (2003). An introduction to Human Embryology for Medical Students. Jaypee Brothers, New Delhi, India. (7):197-198.

Singh, S., Shrivastava, N.M, Modi, N.T, and Saifi, A.Q. (1982)," Anti-inflammatory activity of *Lawsonia inermis." Current Science 51: 470-471.*

Sirica, A. E., Nathanson, M. H., Gores, G. J., Larusso, N. F. (2008), "Pathobiology of biliary epithelia and cholangiocarcinoma: proceedingsof the Henry M and Lilian Stratton Basic Research Single-topic conference". *Hepatology,* 48:2040-2046

Song, Z., Zhou, Z., Chen, T., Hill, D., Kang, J., Barve, S. and McClain, C. (2003). "S-adenosylmethionine (SAMe) protects against acute alcohol induced hepatotoxicity in mice". *Journal of Nutritional Biochemistry*, 14:591–597

Substance Abuse and Mental Health Services Administration (2011), "Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings". *Rockville, MD: Substance Abuse and Mental Health Services Administration.*

Susi, E., Asmah, S. R., Fatimah, I. and Taufiq-Yap, Y. H. (2002), "Anticarcinogenic properties and antioxidant activity of Henna (*Lawsonia inermis*)". *Journal of Medical Sciences*, 2:194-197.

Takeda, Y. and Fatope, M. O. (1988), "New phenolic glucosides from *Lawsonia inermis*". *Journal of Natural Products*, 51(4):725-729

Trevisan, M., Browne, R., Ram, M., Muti, P., Freudenheim, J., Carosella, A. N. and Armstrong, D. (2001), "correlates of markers of oxidative stress in general population". *American Journal of Epiddemiology*, 154:384-356

Valarmathi, R., Rajendra, A., Gopal, V., Senthamarai, R., Akilandeswari, S. and Srileka, B. (2010). "Protective effect of the whole plant of *Mollugo pentaphylla* Linn. against carbon tetrachloride induced hepatotoxicity in rats". *International journal of PharmTech Research* 2(3): 1658-1661.

Varghese, J. K., Silvipriya, K. S., Resmi, S., Jolly, C. I. (2010), "*Lawsonia inermis* (Henna): A natural dye of various therapeutic uses – A Review", *Inventi Rapid: Cosmoceuticals,* 1(1): 1-5.

Vasudevan, T. N. and Laddha, K. S. (2003), "*Lawsonia inermis*", Herbal Drug microscopy, Yucca Publication Dombivli, 68-69

Warrier, P. K. (2004), "*Lawsonia inermis*". Indian Medicinal Plants – a compendium of 500 species, Orient Longman private limited, Chennai, pp 303-304.

Wong, K. C. and Teng, Y. E. (1995), "Volatile components of *Lawsonia inermis* Linn. flowers", *Journal of Essential Oil Research,* 7(4):425-428.

World Health Organisation (2011), "Global status report on alcohol and health", Geneva: WHO press.

World Health Organization (2014), "Global status report on alcohol and health", Geneva: WHO Press.

Zhenyuan, S., Ion, D., Ming, S., David, Y.W. L, Yanze, L., Xiaosheng, J. and Craig, M. (2006), "Silymarin Protects Against Acute Ethanol-Induced Hepatotoxicity in Mice". *Alcoholism: Clinical and Experimental Research*, 30(3): 407–413.

Zhou, Z., Wang, L., Song, Z., Lambert, J. C., McClain, C. J. and Kang, Y. J. (2003). "A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production". *American Journal of Pathology,* 163:1137–1146.

Zimmerman, H. J. (1978). "Drug-induced liver disease". *Drugs,* 16 (1): 25-45.