# PHYTOCONSTITUENTS ISOLATION AND HEPATOPROTECTIVE ACTIVITY POTENTIAL OF *AVERRHOA BILIMBI* LEAF EXTRACT


## ABSTRACT

Objective: To isolate and evaluate the hepatoprotective activity of the crude ethanolic leaf extract of *Averrhoa bilimbi*

Methods: The leaves of *Averrhoa bilimbi* were extracted by cold maceration using ethanol as a solvent, and the solvent fractions were obtained with petroleum ether and ethyl acetate. Preliminary phytochemical tests were performed for the presence or absence of secondary metabolites. Plant chemical constituents were isolated using column chromatography and characterized by IR,$^{1}$HNMR,$^{13}$CNMR and mass spectroscopic values. Albino rats were treated with the vehicles (distilled water or 2% Tween 80), three different doses (100, 200 and 400 mg/kg) of the crude ethanol extract and the standard drug (silymarin 100 mg/kg), and the hepatotoxicant paracetamol. Then, the levels of biomarkers of liver injury – such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) – and liver function such as bilirubin were measured along with histopathological examination.

Results: Preliminary phytochemical studies shown the presence of n-docosanoic acid and beta sitosterol from petroleum extract and from ethyl acetate a flavonoid apigenin.The ethanol extract suppressed the plasma levels of AST, ALT and ALP (P=0.05) in the aforementioned doses. Maximum hepatoprotective activity was observed at the dose of 400 mg/kg body weight.

Conclusion: *Averrhoa bilimbi* is endowed with hepatoprotective activity, probably with the presence its chemical constituents like sterols,flavonoids and terpenoids



*Keywords:Averrhoa bilimbi, Oxalidaceae, hepatoprotective, paracetamol, apigenin, beta sitosterol, n- docosanoic acid*


## 1. INTRODUCTION

Herbal medicine is the oldest form of health care known to humans. In healthcare herbs are used by all cultures. Later, in modern civilization this proved to be a vital part of development. Most of the drugs we usually use are of plant origin. In United States more than 25% of the total prescribed drugs dispensed contain at least one active ingredient from plant material. Some are prepared directly from plants; others are synthesized to mimic action of a plant compound. The trend has been changed from synthetic to natural medicine in the developing world citing the safety principles. Trees and plants act as source for various drugs. The use of plant materials as drugs were compiled in Ayurveda which listed more than 8000 herbal remedies. Some compounds like starch, cane sugar, camphor and benzoic acid had long been known, as their preparation was simple. Complex mixtures such as fats, volatile oils, fixed oil, resins and tars had been prepared and used even though virtually nothing was known regarding their composition. India with over 45,000 different plant species is one of world's twelve biodiversity centers. Among these 45,000 plants only about 15,000-20,000 plants were found to possess good drug like properties out of which only 7,000-7,500 plants are being used by traditional practitioners. As a result of growing

interest in the plants as a probable source of newer drugs, approaches for the fractionation of plant extracts on biological activity instead of concentrating on a specific class of compound have been developed. After isolation, chemical examination will be performed for the active fraction. The phytochemical study of a plant may thus include the following: extraction of the plant material; separation and isolation phytoconstituents; investigation of the biosynthetic pathways to particular compounds; and quantitative tests [1].

Plants are found to be good source of phenolic antioxidants. Phenolic antioxidants are produced by the stimulation of protective secondary metabolite pathways, due to the plants' adaptation to biotic and abiotic stress. Many plants affording hepatoprotection do so by inducing endogenous antioxidant enzymes.

*Averrhoa bilimbi* (AB) is among oldest and holy trees of India, belongs to the family *Oxalidaceae*[2]. Fruits and leaves of this plant are usually utilized for therapeutic purposes [3]. *Averrhoa bilimbi* has been extensively reported as anti-inflammatory, antioxidant [4], anti-scorbutic, astringent, antidiabeic[5] anti-bacterial[6], antihypertensive[7], antimicrobial[8], anti-atherogenic, antihyperlipidemic and postpartum protective properties India has diverse plant wealth and these plants are extensively employed in ayurvedic science for their drug like nature. Present study aims to isolate the active constituents and screen the invivo hepatoprotective activity of ethanolic leaf extract of AB.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of ethanolic extract

The leaves of AB were collected from local regions of Mangaluru, Karnataka. The plant of AB had been authenticated by the Department of Botany,St. Agnes College, Mangaluru. The leaves were washed, cleaned, dried and powdered into a coarse powder by using mechanical grinder and obtained coarse powder was extracted using ethanol by maceration. By the aid of flash evaporator under reduced pressure extract was concentrated to dryness.

#### 2.1.1. Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out to detect the presence of secondary metabolites to co-relate the hepatoprotective activity of stem bark extract of AB. *[9]*

### 2.2. Fractionation of ethanolic extract [10]

Fractionation was carried out by suspending the ethanolic extract (350 g) in distilled water (1,500 ml) followed by extracting it with petroleum ether (60 – 80 °C, 8 X 500 ml),diethyl ether and followed by ethyl actate. Washed all fractions with the help of distilled water (30 ml), these fractions are then dried using anhydrous sodium sulphate and extract is made solvent free by distillation. Thus, ethanolic extract was fractionated to petroleum ether soluble fraction (60 g) diethyl ether soluble fraction (40 g) and ethyl acetate soluble fraction (35 g).

#### 2.2.1. Petroleum ether extract

$CHCl_3$ (20 ml) was used to dissolve pet.ether extract (20 g) and alumina was used as adsorbent. It was loaded onto column packed with alumina (150 g).First it was eluted with Petroleum ether (60-80°C) alone and followed by mixture of petroleum ether:$CHCl_3$ (95:5, 90:10, 80:20). Eluates were monitored using TLC (Silica gel G; visualization was done by using vanillin-sulphuric acid reagent heated at 110°C). Each time 10 ml was collected in a test tube,equivalent volume of eluates (TLC monitored) were combined and was concentrated to 15 ml and placed in a dessicator.Concentrated eluates given a single spot in the TLC and it was designated as compound I (50mg). Elutions along with mixture of petroleum ether (60-80°C): chloroform (95:5, 90:10, 80:20) gave another single spot in TLC and obtained spot was named as compound II (55mg). Remaining elutions resulted in only brown resinous masses, it was not processed further.

Acetylation of Compound II

Compound 1 (5 mg) was taken in dry pyridine (0.2 ml) and freshly distilled $Ac_2O$ (1 ml) was added to it. The mixture was kept overnight under room temperature, then added to crushed ice, stirred, after 2 h resultant mixture was filtered and dried.

**2.2.2.. Ethyl acetate extract**:
The residue (30g) was suspended in a small volume of methanol (5 ml) and was made into a slurry. This was then loaded onto a silica gel column (150 g) prepared in ethyl acetate. First 100 % ethyl acetate was eluted through the column followed by graded mixture of 1 %, 5 % and 10 % methanol in ethyl acetate. TLC was used in monitoring elutes of different fraction [silica gel g; ethyl acetate: methanol and visualized by UV/$NH_3$].The 100% ethyl acetate and 99:1 (ethyl acetate: methanol) elutes showed similar spot. On concentration this was deposited as a yellow coloured compound. It was recrystallized from methanol obtained as yellow amorphous powder and was designated as compound III. It gave orange colour with Shinoda's test for flavonoids and a yellowish green color with $NH_3$ and melting point was found 348 $^0$C. Remaining fractions resulted in resinous masses which were not processed further.

All the melting points were recorded in a Toshniwal melting point apparatus and were uncorrected. IR spectra of the compounds were recorded using the KBr pellet method on a Perkin-Elmer model 700 IR spectrophotometer. $^1$HNMR spectra of the compounds were taken on varian EM-360 (270 MHZ) NMR spectrometer using $CDCl_3$ as a solvent. Chemical shifts are expressed in ppm values using TMS as an internal standard. Mass spectra were recorded on EI-MS and TLC was carried out using Silica-gel G (Merck 220- 240 mesh). All chemicals used were of analytical grade and distilled prior to use

**2.3. Acute toxicity studies:** Adult female albino rats were used for acute toxicity studies as per "Up and Down"[11] method and OECD guidelines 425[12] were followed. The animals tested with oral dose starting from 100mg/kg body weight upto 2000mg/kg body weight of ethanolic extract of leaves of AB The animals were continuously observed for 2-3 h for general behavioural, neurological and autonomic profile and death for a period of 24 h and for 14 d, after administration of the leaf extract. Upto 2000mg/kg of body weight there was no mortality or signs of toxicity and found to be safe. All the experiments were carried out within the guidelines of the IAEC of KSHEMA, Deralakatte, Mangalore (KSHEMA/AEC/27/2010).

**2.4. Assessment of hepatoprotective activity:** Assessment of hepatoprotective activity [13, 14] was carried out on 6 albino rats. The rats of either sex were used and the animlas were segregated into six groups each of six rats and maintained on normal pellet and water ad libitum. Group- I was considered as vehicle control. Group- II were given paracetamol. Group –III was considered as standard receiving the drug silymarin at the dose of 25 mg/kg b.w p.o., for 7 days. Group –IV, V, VI: received the different doses of Ethanolic extract of ethanolic extract of A. bilimbi 100, 200 and 400mg/kg b.wt, p.o.,respectively for a week.

Hepatotoxicity in all groups (except group I) was induced by paracetamol administered orally at a dose of 2mg/kg. After the 7$^{th}$ day animals were sacrified under anaesthetic ether. The blood was collected and serum was obtained after centrifugation (2500rpm for 15min) and that serum was used for various biochemical estimation.

**2.4.1.Biochemical estimation:** Serum was separated from the blood and subjected to various biochemical parameters like aspartate aminotranaminase (AST), alkaline phosphatase (ALP) ,alanine transaminase (ALT), and total bilirubin.

**2.4.2. Histopathological studies:** The rats were sacrificed using anaesthetic ether and livers were excised quickly, washed with normal saline and preserved in 10% buffered neutral formalin solution for histopathological studies. Conventional methods were used in embedding liver pieces in paraffin, they were cut into thick sections of 5µm, haeomatoxyline-eosin dye was used for staining and finally mounted in diphenyl xylene. Histopathological changes in liver structure were observed using microscope [15].

### 2.5.Statistical Analysis

The results of biochemical estimation were expressed as mean ± SEM. The total variation present in the data was analyzed by one way analysis of variance (ANOVA) followed by Post hoc Dunnett's test. 'P' value less than 0.05 was considered as statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1.Preliminary Phytochemical Investigation

The preliminary phytochemical investigation of the leaves of AB shown the presence of sterols,flavonoids,faaty acids,carbohydrates and isolation followed by charecterization led to the presence of n-Docosanoic acid , β sitosterol from petroleum ether extract and Apigenin from ethyl acetate extract.

### 3.2. Analysis of compound I

**Physical state: Cream color crystals, Rf : 0.2 (solvent system;Petroleum ether: $CHCl_3$; 80:20),Melting point:74-78 ºC, Boiling point:306 ºC**

| **Spectral Characterization of compound II** | |
|---|---|
| IR (KBr)$cm^{-1}$ | 1711.3 $cm^{-1}$ (C=O str.),1462.2 $cm^{-1}$ (C-H deformation in $CH_3$),1017.8 $cm^{-1}$ (C-H deformation in $CH_2$) |
| $^1$H NMR (CDCl3) | δ 0.8532 to δ 0.8967 (m, 3H terminal methyl),δ 1.2095 to δ 1.3180 (m, 38H, 19$CH_2$),δ 2.1728 (t, 1×$CH_2$, 2H, $CH_2$ of C-2) |
| **Mass** spectra | Molecular formula:$C_{22}H_{44}O_2$, |
| | Molecular weight:340, |
| | EIMS (m/z):340 ($M^+$, $C_{22}H_{44}O_2$), the other peaks appeared at 256,227 ($M^+$ - 29), 213 ($M^+$ - $CH_2$), 199 ($M^+$ - $CH_2$), 43 ($C_3H_7^+$), 40 |

The IR spectrum showed characteristic peak at 1711.3 $cm^{-1}$showed the existence of C=O. IR peak at 1462.2 $cm^{-1}$ and 1017.8 $cm^{-1}$showed the existence of C-H deformation in $CH_3$ and $CH_2$ respectively. The $^1$HNMR signal at δ0.85 showed terminal methyl protons δ1.2-1.3 showed $CH_2$ protons, and δ2.17 showed the existence of $CH_2$ protons adjacent to carboxylic group. According to GC-MS spectral data molecular ion peak was seen at m/z 340 [$M^+$] corresponding to the mol. formula $C_{22}H_{44}O_2$ comparision of spectra data with the known fatty acid supported it's characterization as n-Docosanoic acid[16] (Fig 1)

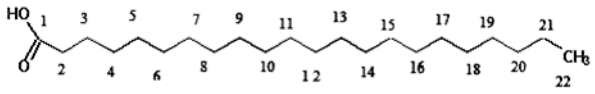


**Fig 1**

## Analysis of compound II

**Physical state: Pearl white crystals, Rf: 0.7 (solvent system; Pet ether:$CHCl_3$,80:20), Melting Point : 138-140°C**

**Spectral Characterization of compound II:**

| | |
|---|---|
| IR (KBr)$cm^{-1}$ | 3480.1(O-H), 2943.58, 2391.23 (C-H stretching in CH2-CH3),1637.6 (C=C stretching),1463.4 (C-H deformation inCH3) 1381.56 (C-H deformation in gem dimethyl)(C-O stretching) ,1061.5 (C-O str of secondary alcohol) |
| $^1$H NMR ($CDCl_3$) | δ 0.64 to 1.007, (18H, 6xCH3),δ 1.03 to 1.235 (m, 22H,11xCH2),δ 1.44 to 2.26 (m, 8H, methane protons),δ 3.55 (br, 1H, OH),δ 5.35 (m, 1H,Vinylic proton at C-12) |
| **$^{13}$C** NMR ($CDCl_3$) | 140.7(C-5), 121.67 (C-6), 56.02 (C-17), 45.79 (C-13) 36.09 (C-22) 33.9 (C-1),35.46 (C-8), 33.9 (C-23),36.0(C-10), 32.36 (C-16), 31.81(C-7), 29.11(C-25), 30.23 (C-24), 40.14(C-29.11(C-25), 30.23 (C-24), 40.14(C-31.5(C-26),77.7(C-28) |
| Mass spectra | Molecularformula:$C_{29}H_{50}O$, Molecular wt414, |
| | Mass spectra EIMS (m/z):414 (M+, $C_{29}H_{50}O$, 54%), 397 (18%) 329 (12%), 303 (10%), 288 (4%),273 (10%), 255(M+- side chain H2O, 6%), 231(10%), 199 (20%),161 (30%), 147 (34%), 133 (24%)105 (50%), 91 (76%), 71 (44%), 57 (100%). |

Compound II showed characteristic color reaction for a sterol. Along with tetranitromethane yellow color was obtained which confirmed unsaturation. Its acetate matched at 126-128°C .$^{13}$CNMR spectra matched with that of ß- sitosterol. The most downfield signals at δ 140 was accommodated for sp2 (olefinic) carbon at C-5and the next downfield signal at δ 121 to C-6 carbon the oxygenated carbon at C-3 gave a downfield signal at δ 77 ppm. The next downfield at δ 56 was accommodated for C-17 other carbon atoms of the steroidal skeleton except that in the side chain appeared in the range δ 45-δ 30 ppm. The angular methyl groups and the side chain methyl carbon gave signal in the region δ 19.8-δ 8.4 ppm. Its identity as ß – sitosterol [17](Fig 2) was further confirmed by IR, $^1$HNMR, $^{13}$CNMR and mass spectral data and co- chromatography with an authentic sample (Sigma chemical company. USA).

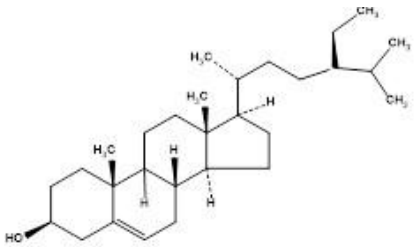

**Fig 2**

## Analysis of compound III

Physical state: Yellow Crystals, Rf Value: 0.52 (Solvent system: EtOAc: MeOH (99:1)), Melting Point: 345 °C

Spectral Characterization of compound III:

| | |
|---|---|
| UV λmax | MeOH: nm 266, 296 sh, 335; +NaOMe: 276, 324, 392; +$AlCl_3$: 276, 302, 349, 384; + $AlCl_3$ + HCl: 274, 300, 340, 381, +NaOAc: 275, 301sh, 339, +NaOAc + $H_3BO_3$ : 258, 303sh, 338 |
| IR (KBr) $cm^{-1}$ | 3312.36 (br. O-H str), 3093.43 (Ar. C-H str) 1603.0 (C= Cstr), 1667.12 (C=Ostr) |
| $^1$H NMR ($CDCl_3$) | δ 10.80 (s, 1H,3- OH),δ 10.33 (s, 1H.4'-OH),δ 10.33 (s, 1H.4'-OH),δ 12.94 (s. 1H.7-OH),δ 7.92 (d.2H.H-2', H-6'),δ 6.92 (d,2H, H-3',H-5'),δ 6.77 (d,1H,H-8),δ 6.47 (d,1H,H-6) ,δ 6.18 (s,1H,H-3) |
| $^{13}$**C** NMR ($CDCl_3$) | δ 164.0 (C-2), 103.6 (C-3), 181.6 (C- 4), 161.34 (C-5) 98.71 (C-6), 163.64 (C-7), 93.83 (C-8), 157.18 (C-9),102.75 (C-10), 121.08 (C-1'),115.84 (C-3',5'), 128.32 (C-6'-2'), 161.03 (C-4') |
| Mass spectra | Molecular formula:$C_{15}H_{10}O_5$ ,Molecular weight:270; |
| | EIMS (m/z):270 (M+, $C_{15}H_{10}O_5$, 100%), 242 (18%), 152 (22%), 121(14%), 96 (8%) 69(9%). |

The flavonoid m.p.345 °C and reflected positive responses to Shinoda test and a yellowish green color on treatment of $NH_3$.The UV spectral data showed the characteristic pattern of flavone with 5, 7, 4' trihydroxy flavone. A large bathochromic shift of 44nm in band of the $AlCl_3$ spectral, relative to the MeOH spectrum indicated the presence of 5 hydroxyl group. NaOAc induced shift of 8 nm in ring A indicated presence of 7-OH group. $^{13}$CNMR spectra matched with that of apigenin. Its identity as apigenin was further supported by the mass spectral data and confirmed by co-chromatography with an authentic sample of apigenin[18].

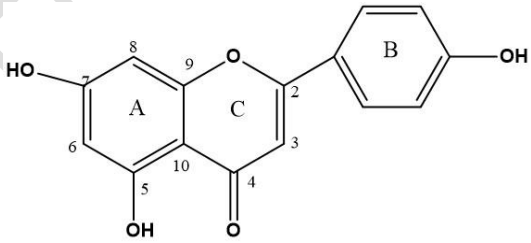


**Fig 3**

### 3.3. Acute Toxicity Studies

No death was observed even at the maximum administered dose 2000 mg/kg body weight. However there was a slight increase in the magnitude of certain autonomic responses as the dose increased. Irritability and decreased pain response were observed.
The result of acute toxicity study revealed that the ethanolic extract leaves of the plant AB is safe with maximum dose of 2000 mg/kg body weight in rats.

### 3.4. Hepatoprotective Activity

Serum levels of alanine aminotransaminase, aspartate amino trasaminase, alkanine phosphatase, total bilirubinwere elevated significantly in paracetamol intoxicated normal rats.Significant reduction in alanine aminotrans-aminase, aspartate aminotrasaminase alkaline phosphatase, total bilirubin were observed in all three groups of rats that were treated with ethanolic extract of *Averrhoa bilimbi* as compared to the Paracetamol treated group.

**Table 1.Results showing effect of ethanolic extract of AB on paracetamol induced liver damage**

| Group No: | Treatment Groups (n=6) | ALT (IU/L) | AST (IU/L) | ALP (IU/L) | Total Bilirubin (mg/dL) |
|---|---|---|---|---|---|
| I | Control | 86.50± 1.66 | 109.1± 3.08 | 140.6± 2.23 | 0.82± 0.02 |
| II | Pcml | 285.4± 1.58* | 380.2± 1.89* | 387.6± 1.15* | 2.16± 0.13* |
| III | Pcml +Silymarin (25mg/kgb wt.) | 94.18± 1.68** | 120.4± 3.23** | 149.5± 1.35** | 0.85± 0.05** |
| IV | Pcml+AB (100mg/ kgbwt) | 161.3± 2.03** | 185.4± 1.63** | 198.6± 1.21** | 1.29± 0.14** |
| V | Pcml + AB(200mg/ kg bwt.) | 132.8± 2.17** | 167.0± 1.22** | 177.2± 1.71** | 0.92± 0.05** |
| VI | Pcml + AB(400mg/ kgbwt.) | 108.9± 1.89** | 159.1± 1.61** | 168.6± 1.35** | 0.85± 0.05** |

Values are expressed as mean±SEM, significant,(P=0.05) compared to control,
**significant (P=0.05) compared to toxic control.

**Histological profile:** On microscopic investigation of group I liver displayed a normal portal triad, sinusoids, and cord arrangement of hepatocytes (Fig a)**.**Microscopic investigation of group II displayed extensive necrosis of liver along with inflammation (Fig b). Microscopic investigation of group VI displayed almost normal hepatocytes (Fig f). Thus the finding of this group are comparable with the finding of silymarin treated group, suggesting the hepatoprotection at this dose. Mild fatty changes were observed on microscopical investigation of group V liver (Fig e). On Microscopic investigation it was discovered that test drug used in 100mg/kg body weight was unable to provide required protection from fatty change in liver as the sections of liver at this dose showed various hydropic degeneration( Fig d). Almost normal appearing hepatocytes were observed in microscopic investigation of group III liver (Fig c).

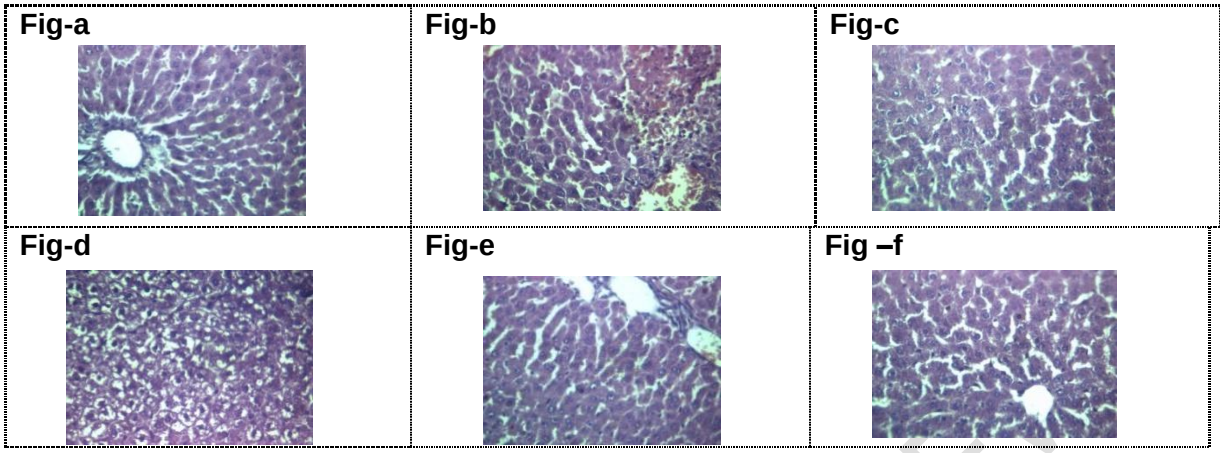


Fig 4: Histopathological slide of liver

Paracetamol is normally eliminated mainly as sulfate and glucoronide. Only 5% of the paracetamol is converted into N-acetyl-p-benzoquineimine. Paracetamol in larger doses produces liver necrosis after undergoing bio-activation to a toxic electrophile, N-acetyl-p-benzoquinone-imine (NAPQI) by cytochrome P450 monooxygenase. NAPQI binds to macromolecules and cellular proteins, and also oxidizes lipids and alters homeostasis of calcium after depletion of glutathione. Pretreatment with ethanol extract of leaves of AB brought down the elevated levels of ALT, AST, ALP and Total Bilirubin. These biochemical restorations may be due to the inhibitory effects on cytochrome P450 or/and promotion of its glucuronidation. Administration of Ethanolic extract of leaves of AB at all the dose levels viz., 100, 200, and 400 mg/kg/d, showed significant hepatoprotective activity but the results obtained from the dose level of 400 mg/kg/d statistically comparable with the results obtained from the standard drug silymarin in support histopathological reports also revealed that there is a marked hepatoprotection in group III, IV, V and VI. Though the ethanolic extract of leaves of AB which contains flavonoids, triterpenoids, saponins and steroids showed significant hepatoprotective effects [19]

## 4. CONCLUSION

The results of serum biochemical markers and histopathological studies in the crude ethanol extract treated group support the hepatoprotective effect and provide evidence for the traditional use of AB for treatment of liver disorders. The larger dose of ethanol leaf extract produced a remarkable hepatoprotective activity, which was comparable to silymarin. The presence of natural phytoconstituents like n-docosanoic acid, beta-sitosterol and apigenin.

## ETHICAL APPROVAL

“All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee” (KSHEMA/AEC/27/2010).

The study highlights the efficacy of " Ayurveda " which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## REFERENCES


1. Trease, Evans. Text book of pharmacognosy. 1989; 14:119.
1. Khory RN, Katrak NN. Materia medica of India & Their therapeutics. NewDelhi: Komal Prakasham; 1999.p.151-152
2. Faridah Abas, Nordin H, Lajis, DA Israf, Khozireh S, Kolsam YU. Antioxidant and nitric oxide inhibition activities of selected malay traditional vegetables. *Food chemistry* 2006;95(4):566-573. https://doi.org/10.1016/j.foodchem.2005.01.034
3. Pushparaj P, Tan CH. Tan BKH. Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic rats. *Journal of Ethnopharmacology* 2000;72(1,2):69-76. PMID: 10967456. DOI: 10.1016/s0378-8741(00)00200-2
4. Zakaria ZA, Zaiton H, Henie EFP, Jais AMM, Engku ENH. In vitro Anti bacterial activity of *Averrhoa bilimbi* L. leaves and fruits Extracts. *International Journal of Tropical Medicine* 2007;2(3):96-100. https://medwelljournals.com/abstract/?doi+ijtmed.2007.96.100
5. Bipat R., Toelsie JR., Joemmanbaks, RF,Jill MG,Julio k, etal.Effects of plants popularly used against hypertention on norepinephrine-stimulated guinea pig atria.*Pharmacognosy Magazine.* 2008;4(13):12-19
6. Nurul HB, Abdul W, Effendy MB,Wahid A. Phytochemical screening and antimicrobial efficacy of extracts from *Averrhoa bilimbi (*Oxalidaceae) fruits against human pathogenic bacteria.*Pharmacognosy Journal.* 2009; 1(1):62-64.
7. Ambili S, Subramoniam A, Nagarajan NS. Studies on the antihyperlipidemic properties of A*verrhoa bilimbi* fruit in rats. Plant Medica 2009; 75(1):55-58. Doi:10.1055/s-0028-1088361
8. Anonymous, useful Indian Medicinal Plant. PID (CSIR), New Delhi; 1994; 53
9. Rajisha K,Fernanandes J.Pharmacognostic and Preliminary Phytochemical Investigation of *Exacum bicolor Roxb.Research J.Pharm.and Tech.2020*;13(4):1752-56 doi:10.5958/0974-360X.2020.00316.9
10. Ahmad F,Ali M,Alam P. New phytoconstituents from the stem bark of Tinospora cordifolia Miers.*Natural Product Research*.2010;24(10):926-34.DOI10.1080/14786410802435679
11. Ghosh MN. Fundamentals of Experimental Pharmacology. 2nd ed. Calcutta: scientific Book Agency; 1984; p.153-157.
12. OECD/OCED 425 OECD guidelines for testing of chemicals acute oral toxicity up and down procedure. 2001; 26: 1-26.
13. Ramachandra Setty, Absar Ahmed Quereshi, A.H.M. Viswanath Swamy Tushar Patil , T. Prakash , K. Prabhu , A. Veeran Gouda.Hepatoprotective activity of Calotropis procera flowers against paracetamol-induced hepatic injury in rats. *Fitoterapia*.2007; 78:451–54. PMID: 17600635,DOI: 10.1016/j.fitote.2006.11.022

14. Harish R,Shivanandappa T.Antioxidant activity and hepatoprotective potential of Phyllanthus niruri. *Food Chemistry* 2006;95(2):180-85.https://doi.org/10.1016/j.foodchem.2004.11.049
15. Chaudhari NB, Chittam KP, Patil VR**.** Hepatoprotective Activity of *Cassia fistula* Seeds against Paracetamol-Induced Hepatic Injury in rats. *Arch Pharm Sci & Res*.2009;1(2)218 – 221
16. Hansen RP,Shorland FB ,June Coke N. Isolation and identification of the high molecular weight saturated fatty acids of butterfat. *Journal of Dairy Research*.1959;26(2):190-195.DOi:https://doi.org/10.1017/S0022029900009882.
17. Aliba MO, Ndukwe IG, Ibrahim H. Isolation and Characterization of B-Sitosterol from Methanol Extracts of the Stem Bark of Large- Leaved Rock Fig (Ficus Abutilifolia Miq). J. *Appl. Sci. Environ. Manage.* 2018; 22 (10): 1639–1642. DOI: https://dx.doi.org/10.4314/jasem.v22i10.19.
18. Huma AB, Rubina N. Isolation of Apigenin by solute-solvent extraction from Symphotrichum novea anglea*. Integrative Food, Nutrition and Metabolism*.2019;6:1-3.
19. Chattopadhyay RR, Possible mechanism of hepatoprotective activity of Azardica indica leaf extract. *Journal of Ethnopharmacology* 2003; 89(2-3):217-9 PMID: 14611885,DOI: 10.1016/j.jep.2003.08.006