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. Phytoconstituents were isolated using column chromatography and characterized using 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 total protein, albumin, and 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 in its 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 sotosterol, 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 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 explore something new in the field of herbal drug discovery.

2. MATERIALS AND METHODS

2.1. Preparation of ethanolic extract

The leaves of *Averrhoa bilimbi* were collected from local regions of Mangaluru, Karnataka. The plant of *Averrhoa bilimbi* had been authenticated by botanist Dr.Noeline J. Pinto, Head,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

Standard phytochemical screening test was carried out to detect the presence of secondary metabolites to relate the analgesic stem bark extract of Sapindus trifoliatus linn for the presence or absence of these active constituents [9]

2.2. Fractionation of ethanolic extract

Fractionation was carried out by suspending extract (350 g) in distilled water (1,500 ml) followed by extracting it with pet. ether (60 – 80 °C, 8 X 500 ml). Wash all fractions with the help of distilled water (30 ml), these fractions are then dried using an. sodium sulphate and extract is made solvent free by distillation. Thus, ethanolic extract was fractionated to pet. 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 alumina column (150 g) after evaporation of solvent, preprepared in pet. ether (60–80°C). Pet. ether (60-80°C) was eluted first through the column, pet. ether:$CHCl_3$ (95:5, 90:10, 80:20). TLC was used to monitor elution (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.

Single spot was observed while elution was carried using pet. Ether (60-80°C) and it was named as compound I (50mg). Elutions along with mixture of pet.ether(60-80°C): chloroform gave single spot in TLC and obtained spot was named as compound II (55mg). Remaining elution resulted in only brown resinous masses, it was not processed further.

2.2.2. Acetylation of Compound II

Compound 1 (5 mg) was taken up 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. The solid obtained was crystallized from C_6H_6 as white flakes, m.p. 126-128 ° C.

2.3. Ethyl acetate extract:

The residue (30g) was suspended in a small volume of methanol (5 ml) and was made into slurry with silica gel G. this was then loaded onto a silica gel column (150 g) prepared in ethyl acetate. 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 colored compound. It was recrystallized from methanol obtained as yellow amorphous powder and was designated as compound III. It gave orange color 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.

2.4. Acute toxicity studies: Adult female albino rats were used for acute toxicity studies as per "Up and Down"[10] method and OECD guidelines 425[11] 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 *A.bilimbi*. 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).

2.5. Assessment of hepatoprotective activity: Assessment of hepatoprotective activity[12] 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 -IIIwas considered as standard receiving the drug silymarin at ehe 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 II was induced by paracetamol administered orally at a dose of 2mg/kg on day 5. After the 7th day animals were sacrified under deep anaesthesia. The blood was collected and serum was obtained after centrifugation (2500rpm for 15min) and that serum was used for various biochemical estimation.

2.5.1.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.

2.5.2.Histopathological studies : The rats were sacrificed under deep anaesthesia 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[13].

3.RESULTS

3.1.Analysis of compound I

Physical state: White-cream color crystal, Rf: 0.2 (solvent system;Pet ether: $CHCl_3$; 80:20),Melting point:74-78 ºC, Boiling point:306 ºC

3.1.1.Spectral characteristics of compound I

IR (KBr) :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 ($CDCl_3$)** :δ 0.8532 to δ 0.8967 (m, 3H terminal methyl),δ 1.2095 to δ 1.3180 (m, 38H, 19CH_2),δ 2.1728 (t, 1×CH_2, 2H, CH_2 of C-2);**Mass spectra (GC-Ms**):Molecular formula:$C_{22}H_{44}O_2$,Molecular wt:340,GC-MS (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

3.2. 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

3.2.1.Spectral Characterization of compound II:

IR (KBr cm-1) :3480.1 (O-H),2943.58, 2391.23 (C-H stretching in CH_2-CH_3),1637.6 (C=C stretching),1463.4 (C-H deformation inCH_3) 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, 6xCH_3),δ 1.03 to 1.235 (m, 22H,11xCH_2),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(EI-MS):Molecularformula:$C_{29}H_{50}O$,Molecular wt414,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 H_2O, 6%), 231(10%), 199 (20%),161 (30%), 147 (34%), 133 (24%)105 (50%), 91 (76%), 71 (44%), 57 (100%).

3.3.Analysis of compound III

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

3.3.1.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}HNMR**(DMSO-d6):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 (**DMSO-d6):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%).

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 bilirubinwere 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 *Averrhoa bilimbi* 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.

3.4.1.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).

3.4.1.1.Histopathological slide of liver

Fig-a

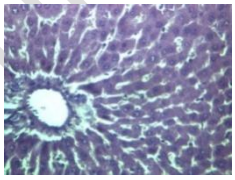


Fig-b

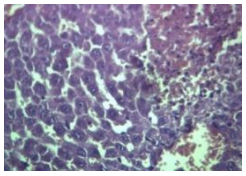


Fig-c

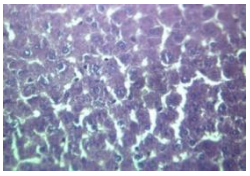


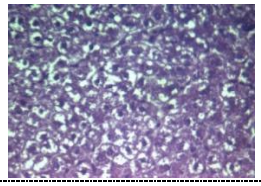
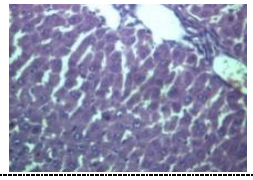
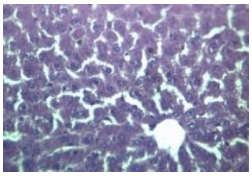
Fig-d	Fig-e	Fig –f
		

Fig 1: Histopathological slide of liver

4.DISCUSSION

4.1.Phytochemical Investigation

The phytochemical investigation of the leaves of *Averrhoa bilimbi* led to the isolation of n-Docosanoic acid , β sitosterol from petroleum ether extract and Apigenin from ethyl acetate extract.

4.1.1.n-Docosanoic acid: m.p. 74-78°C, white-cream colored crystal. 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[14]

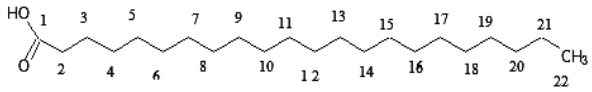


Fig 2: n-docosanoic Acid

4.1.2.ß – Sitosterol: It gave a characteristic color reaction for a sterol. Along with tetranitromethaneyellow color was obtained which confirmed unsaturation. Its acetate matched at 126-128°C . ^{13}CNMR spectramatched 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[15] was further confirmed by IR, ^{1}HNMR, ^{13}CNMR and mass spectral data and co- chromatography with an authentic sample (Sigma chemical company. USA).

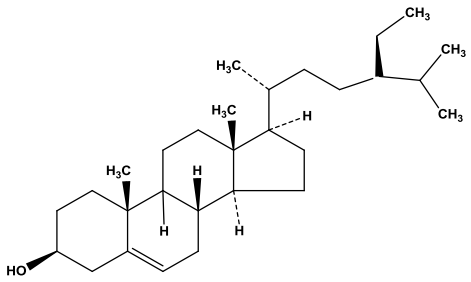


Fig 3: ß – sitosterol

4.1.3.Apigenin: 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[16].

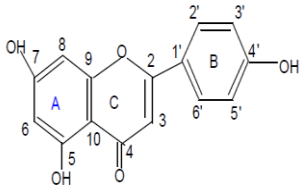


Fig 4: Apigenin

4.2. Hepatoprotectiveactivity

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 *Averrhoa bilimbi* 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 *Averrhoa bilimbi* 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 *Averrhoa bilimbi* which contains flavonoids, triterpenoids, saponins and steroids showed significant hepatoprotective effects [17]

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 *Averrhoa bilimbi* 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.

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