

Hepatoprotective and Antioxidative Activity of *Avicennia marina* Forsk

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Abstract

Avicennia marina Forssk. is a common mangrove tree found around estuarine coasts of India. The literature survey on this plant so far has reported the presence of several novel compounds with prospective medicinal value. *Avicennia marina* has been reported to possess flavones and flavonoids along with triterpenoids like betulin, lupeol etc. and has been reported for antiviral activity *in vitro* on hepatitis B virus. But scientific data on its hepatoprotective potential is unavailable.

Thus the present study was aimed to evaluate hepatoprotective activity of standardized ethyl acetate extract of the leaves of *Avicennia marina*. The extract was standardized using validated HPTLC method for betulinic acid, betulin, lupeol, ursolic acid and β -sitosterol content. The antioxidant activity of *Avicennia marina* leaves was assessed by DPPH and Galvinoxyl free radical scavenging activity. Hepatoprotective activity was assessed against CCl₄ induced liver intoxication. CCl₄ induced albino Wistar rats were treated with three different doses of the standardized extract for three days. The results were compared with the established positive control, Silymarin.

The extract provided concentration dependent percent protection which was evident by the changes in the levels of biochemical parameters (SGOT, SGPT, ALP, TPN, TG, CHO, HDL, LDL, direct and total bilirubin and liver glycogen content) in albino Wistar rats. The observations of biochemical parameters, antioxidant assays and histopathology, endorse an overall promising effect against liver disorders.

Keywords: *Avicennia marina*, Hepatoprotection, Carbon tetrachloride, Antioxidant

Introduction

Liver damage is a common complication. Liver has to detoxify many toxic substances. Most of the hepatotoxic chemicals damage liver cells by producing reactive species which form covalent bond with the lipids of the tissue. Due to excessive exposure to hazardous chemicals like CCl₄, alcohol, etc., the number of free radicals

generated is so high that they overpower the natural defense system of the body causing hepatic damage and lead to jaundice, cirrhosis and fatty liver etc. [1-3].

Till date, the synthetic hepatoprotective agents used in clinical practices are therapeutically non-promising and may they lead to hepatotoxicity. Herbal medicines and their bioactives are considered to be relatively safe and

have been used in the treatment of liver diseases for a long time [4]. Modern medicines have little to offer for alleviation of hepatic disorders and diseases, and only limited number of drugs like Ribavirin, alpha interferon; Zeffix, etc are available for the treatment of the same [5].

The studies based on the knowledge of minority ethnic groups and literature search revealed that the mangroves have been neglected for their scientific investigation [6]. Mangrove species have been reported to be efficacious in the treatment of toothache, sore throat, constipation, fungal infections, fever, kidney stone, rheumatism, dysentery and malaria [7]. Traditionally, many of the folk remedies of plant origin have long been used for treatment of liver diseases [8,9].

Mangroves and related species have also been reported to have hepatoprotective activity [10]. Hepatoprotective activity of *Rhizophora mucronata* [5], *Lumnitzera racemosa* [11], *Acanthus ilicifolius* [12] etc has been analyzed on CCl₄ intoxicated rats. Hepatoprotective activity of *Suaeda maritima* has been reported against conalavalin-A induced hepatotoxicity in albino Wistar rats [13]. Methanolic extract of *Avicennia marina* has also been reported to possess antiviral properties against hepatitis B virus [14].

The current research work focuses on, *Avicennia marina* Forssk., a common mangrove which has not been thoroughly and scientifically evaluated so far. It has been reported to possess phytoconstituents like betulinic acid, lupeol etc [15]. The study aims to evaluate the efficacy of the ethyl acetate extract of the leaves of *Avicennia marina* against CCl₄ induced hepatic damage in albino Wistar rats.

Materials and methods

Plant material

The leaves of *Avicennia marina* Forssk. Were collected from Airoli, Mumbai and authenticated at Agharkar Research Institute, Pune, (Voucher no. Auth. 13-017). The leaves were shade dried for a week and further oven dried at 37°C ± 2°C to constant weight, powdered in a mixer grinder, sieved (85 mm mesh – BSS Sieve) and stored in airtight plastic containers.

Apparatus and chemicals

CCl₄ (GR grade, Batch no.: IG8G580178, Merck Specialties Pvt. Ltd) and Silybon tablets (Silymarin as silybin 70 mg, Batch no. SIAD0025, Micro Labs Limited) were procured from market. DPPH, and Galvinoxyl

radicals were procured from Sigma-Aldrich Chemical Company, (Steinheim, Germany). All other chemicals used were of analytical grade.

Extract preparation

Ethyl acetate extract of the leaves of *Avicennia marina* was prepared by adding 1000 mL ethyl acetate to 10 g of plant powder using soxhlet extraction method (yeild = 3.04%). The extract was standardized using a validated HPTLC method and content of reported hepatoprotective triterpenoid markers like; Ursolic acid [16], β-sitosterol [17], Lupeol [18] Betulin [19], betulinic acid [20], was estimated for the standardization of the extract. The content was found to be 17.73 %, 5.33 %, 13.59 %, 3.28 % and 4.97 % respectively. The extract was further evaporated to dryness in a water-bath preset at 65°C to remove all traces of ethyl acetate from the extract prior to animal studies. Animals were dosed by weighing this dried extract accurately as per the weight of the animal and suspending it in distilled water (total volume > 1 mL/ 100 g).

Animals

Adult albino Wistar rats (females, 180-220 g) and Swiss Albino mice (females, 18-22 g) were procured from Haffkines Biopharmaceuticals Pvt. Ltd., Mumbai. The animals were maintained under standard laboratory conditions at an ambient temperature of 25 ± 2° C, relative humidity of 50 – 55 % and with 12-h light and dark cycle in an animal house with standard facilities as per CPCSEA guidelines (CPCSEA/315). They were fed with commercially available standard pellet diet (AMRUT feed) and filtered drinking water was provided *ad libitum*. After one week of acclimatization, the animals were used for experimentation. The approval from institutional animal ethics committee of Ramnarain Ruia College, Matunga, for the usage of animals in the experiment was obtained as per Indian CPCSEA guidelines (Approval number: MJ – 130624 – 02).

Safety evaluation

Safety study of standardized extract of *Avicennia marina* was conducted on albino Swiss mice as per OECD guidelines (No. 420, fixed dose procedure). The mice were fasted overnight for 10-14 hours and administered with standardized extract of the leaves of *Avicennia marina* (2.0 g/kg) orally. The animals were observed individually during the first 30 min for all reflexes, periodically during the first 48 hours with special attention given during the first 4 hours (short-term toxicity) and daily thereafter for a total of 14 days (long-term toxicity) for alteration from

general behavior and clinical symptoms like alteration of skin and fur texture, ptosis, diarrhea etc. Daily body weight, food and water intake record was also maintained. The results were compared with control group (orally administered with D/W).

Assessment of hepatoprotective activity

For the study, albino Wistar rats were randomly divided into seven groups with six animals in each. The group details are as follows:

Group I: Normal control (NC)

Group II: CCl₄ control (0.7 ml/kg body weight) (IC)

Group III: CCl₄ control as natural recovery group (NR)

Group IV: CCl₄ + Silymarin (70 mg /kg body weight) (POS)

Group V: CCl₄ + Extract of *Avicennia marina* leaves (50 mg/kg body weight) (AM 1)

Group VI: CCl₄ + Extract of *Avicennia marina* leaves (150 mg/kg body weight) (AM 2)

Group VII: CCl₄ + Extract of *Avicennia marina* leaves (300 mg/kg body weight) (AM 3)

The animals were fasted overnight before the initiation of the study. Animals from Group I received an intraperitoneal injection of 0.5 ml liquid paraffin/animal on the zeroth day of the study and were treated as normal control. Animals from groups II, III, IV, V, VI and VII received an intraperitoneal injection of 0.7 ml/kg body weight of CCl₄ [21] in 0.5 ml liquid paraffin/animal on the zeroth day of the study. The animals from Groups I, II and III received an oral dose of 2 ml of distilled water (D/W) once daily. A dose of 70 mg / kg Silymarin [22] (in the form of Silybon tablets) suspended in 2 ml of D/W was administered orally to each animal of Group IV once daily, starting 24 h post induction on the first day. The animals from Group V, VI and VII received an oral dose of extract of leaves of *Avicennia marina* suspended in distilled water (50 mg/kg, 150 mg/Kg and 300 mg/kg body weight respectively) / animal, daily and 24 h post induction on the first day of the study. The animals from Groups I, II, IV, V, VI and VII were sacrificed on the fourth day (72 hr after first dosing) and those from Group III were sacrificed on seventh day of the study for comparative evaluation of the natural recovery in the study. Daily record of body weight and food and water intake was maintained.

Prior to the sacrifice, blood was collected from retro orbital plexus in heparinized vials and plasma was separated. Biochemical parameters like Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alkaline

phosphatase (ALP), direct (D. Bil) and total Bilirubin (T. Bil), Cholesterol (CHO) and Triglycerides (TG), Total Proteins (TPN), Low-density lipoprotein (LDL) High-density lipoprotein (HDL) content, and Liver glycogen content was analyzed from the plasma samples obtained. During autopsy, liver was excised, rinsed in saline, blotted and weighed. A small piece of liver tissue from the largest lobe was cut and fixed into bouin's fixative. Fixed tissues were processed for routine haematoxylin and eosin staining and evaluated. The extent of liver recovery was compared with positive control.

Percent protection in individual biochemical parameters was calculated as $100 \times (\text{values of CCl}_4 \text{ Control} - \text{values of sample}) / (\text{values of CCl}_4 \text{ control} - \text{values of vehicle})$ [23].

Assessment of antioxidant activity

The antioxidant activity of the extracts, based on the scavenging activity of the stable 1,1 - diphenyl - 2 - picryl hydrazyl (DPPH) free radical, was determined [24] Also, a similar method with a different stable radical (Galvinoxyl) was carried out [25]. Percent inhibition (I) was calculated by the following equation:

$$I = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100 \%$$

Where A_{control} is the absorbance of the ethanol containing control, and A_{sample} is the absorbance of the reaction mixture with the tested sample. EC₅₀ values were determined to be the concentration at which DPPH radical and Galvinoxyl radical are scavenging by 50 %. Ascorbic acid was used as reference standard.

Statistical analysis

The results were expressed as mean \pm SE. The statistical analysis of the values was carried out by using Microsoft Excel and GraphPad Prism 5.0 software for one-way analysis of Variance (ANOVA) followed by Dunnett's *t*-test. P value of < 0.001 was considered to be significant.

Results

The standardization of the ethyl acetate extract *Avicennia marina* using HPTLC showed that the extract is rich in markers like ursolic acid, lupeol, β - sitosterol, betulinic acid and betulin, all of which have been reported to possess hepatoprotective activity. Before the animal experimentation, the extract was evaporated to dryness to remove all the traces of ethyl acetate. This dried extract was administered to the animals during the experimentation using distilled water as the vehicle. The extract was first screened for its safety profile in Albino

Wistar rats following OECD Guidelines No. 420 and was found to be safe up to the maximum dose of 2.0 g/ Kg body weight. No mortality was observed during the study [26].

The antioxidant activity of the extract was assessed in DPPH and Galvinoxyl radical scavenging models. In order to quantify the antioxidant activity, the IC_{50} value, i.e., the concentration of sample required to decrease the absorbance of specific free radical at specific λ_{max} by 50%, was calculated [27]. The extract showed concentration dependant antiradical activity by inhibiting DPPH and Galvinoxyl radicals with an IC_{50} value of 519.24 μ g/mL and 540.09 μ g/mL for both the radicals respectively (Table 1).

Parameters	A. marina IC_{50} (μ g/mL)	Ascorbic acid IC_{50} (μ g/mL)
DPPH radical scavenging activity	519.24 \pm 1.17	2.28 \pm 0.98
Galvinoxyl radical Scavenging activity	540.09 \pm 1.41	1.54 \pm 0.77

Table 1: Antioxidant activity of the standardized extract expressed in IC_{50} (μ g/mL) value.

Hepatic toxicity caused by the induction of CCl_4 (0.7 mL/ Kg body weight) caused acute liver injury which was indicated by the sudden increase in the concentration of biomarkers like SGOT, SGPT, direct and total bilirubin, ALP, CHO, TG, LDL and liver glycogen along with decrease in the concentration of HDL and TPN in the induction control (IC) group of animals when compared with the animals of normal control (NC). The fluxed levels of the biomarkers were significantly brought back to normal by the treatment of standardized extract of the leaves of *Avicennia marina* (AM 1) (AM 2) (AM 3) and in the animals treated as positive control (POS). The results are tabulated in table 2 and 3. The percent protection offered by the extract shows a dose dependant increase with the highest dose at 300 mg/ Kg body weight showing significant recovery ($P < 0.001$ using Dunnett's test). Thus indicating the ability of the extract to reverse the damage caused by CCl_4 intoxication and significantly enhance the recovery process as indicated by the comparison of the treated group of animals (AM 1) (AM 2) (AM 3) (POS) with the animals of the natural recovery group (NR).

	SGOT (UI/ L)	SGPT (UI/ L)	ALP (UI/ L)	T. Bil (mg/ dl)	D. Bil (mg/ dl)
NC	41.38 \pm 0.289	33.42 \pm 0.183	25.17 \pm 0.165	0.56 \pm 0.070	0.08 \pm 0.100
IC	117.58 \pm 0.451@	132.00 \pm 0.126@	89.15 \pm 0.269@	4.36 \pm 0.254@	1.68 \pm 0.960@
NR	105.05 \pm 0.472	112.74 \pm 0.204	70.58 \pm 0.248	3.26 \pm 1.332	1.42 \pm 0.700
% protection	18.92	20.18	29.32	28.86	15.96
POS	40.53 \pm 0.249*	31.93 \pm 0.190*	26.73 \pm 0.125*	0.87 \pm 0.078*	0.19 \pm 0.110*
% protection	101.54	103.1	97.62	91.87	93.39
AM 1	79.55 \pm 0.104	63.29 \pm 0.445	55.16 \pm 0.171	1.95 \pm 1.059	0.84 \pm 0.480
% protection	50.59	70.93	53.33	63.47	52.13
AM 2	64.87 \pm 0.326	60.11 \pm 0.240	50.11 \pm 0.301	1.56 \pm 0.770	0.44 \pm 0.377*
% protection	69.31	74.19	61.21	73.63	77.71
AM 3	51.81 \pm 0.402*	34.86 \pm 0.222*	41.29 \pm 0.207*	1.46 \pm 0.102*	0.40 \pm 0.232*
% protection	86.75	100.11	74.95	76.32	79.91

Table 2: Effect of standardized extract on biochemical parameters. (Values are expressed in mean \pm S.E, n=6 rats in each group)

Values are statistically analyzed for significance using dunnetts test

*= $p < 0.001$ when compared with values of CCl_4 control and @ = $p < 0.001$ against NC.

	HDL (mg/ dl)	LDL (mg/ dl)	CHO (mg/ dl)	TG (mg/ dl)	TPN (mg/ dl)	L.Gly (mg/ dl)
NC	10.11 ± 0.078	30.29 ± 0.278	52.27 ± 0.264	56.22 ± 0.443	6.48 ± 0.077	8.41 ± 0.079
IC	1.92 ± 0.164@	79.20 ± 0.473@	117.00 ± 0.526@	177.33 ± 0.300@	2.48 ± 0.254@	4.33 ± 0.342@
NR	3.65 ± 0.411	73.55 ± 0.252	104.52 ± 0.490	130.29 ± 0.304	3.01 ± 0.133	4.87 ± 0.074
% protection	16.95	12.34	19.64	38.84	13.83	13.24
POS	11.52 ± 0.724*	26.88 ± 0.237*	52.03 ± 0.168*	63.13 ± 0.322*	6.98 ± 0.780*	7.55 ± 0.412*
% protection	112.1	106.46	99.71	94.29	110.98	78.92
AM 1	8.00 ± 1.263	54.00 ± 0.336	81.61 ± 0.220	100.54 ± 0.300	3.82 ± 0.105	5.23 ± 0.363
% protection	69.51	51.77	54.59	63.4	33.67	22.06
AM 2	8.30 ± 1.899	50.11 ± 0.404	71.71 ± 0.303	54.95 ± 0.288*	4.31 ± 0.077	5.66 ± 0.304
% protection	73.18	59.61	69.7	101.05	45.54	32.6
AM 3	8.79 ± 0.456*	38.06 ± 0.354*	58.00 ± 0.294*	53.32 ± 0.373*	4.82 ± 0.102	5.87 ± 0.386
% protection	79.06	83.91	90.62	102.4	58.13	37.75

Table 3: Effect of standardized extract on biochemical parameters.

(Values are expressed in mean ± S.E, n = 6 rats in each group)

Values are statistically analyzed for significance using dunnetts test

*= p<0.001 when compared with values of CCl₄ control and @ = p<0.001 against NC

The hepatoprotective effect of the standardized extracts of the leaves of *Avicennia marina* was further confirmed by histopathological observations of the excised liver tissue. The histoarchitecture of extract treated and POS group animals showed significant recovery to normalcy in comparison to IC group (Figure: 1-7). The liver tissue of the animals of NR group did not show significant improvement when compared to IC. The current study thus demonstrates the hepatoprotective potential of standardized extract of the leaves of *Avicennia marina*.

Discussion

Carbon tetrachloride is one of the most commonly used hepatotoxins. CCl₄ is biotransformed under the action of cytochrome P₄₅₀ enzyme in the microsomal compartment of liver to trichloro methyl radical (CCl₃) which readily reacts with molecular oxygen to form trichloromethylperoxy radical [28]. Both the radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides [29]. This lipid peroxidation leads to a cascade of reactions leading to liver necrosis [30]. This liver damage can be detected by measuring the content of liver function marker enzymes which are released into blood from damaged cells and liver tissues for example, transamines, which are cytoplasmic enzymes in nature and are released into circulation after cellular damage [31]. Thus,

these markers also form the basic indicators of liver damage.

The hepatoprotective activity of doses of *Avicennia marina* was monitored by estimating the transamines, ALP, Bilirubin, etc biomarkers, which gave a good idea about the functional state of the liver [32]. The increase in the levels of both direct and total bilirubin reflected the depth of jaundice and increase of transamines and alkaline phosphatase was a clear indication of cellular leakage and loss of functional integrity of cell membrane [33]. The normalization of these marker enzymes (Table 2 and 3) after the administration of standardized ethyl acetate extract of the leaves of *Avicennia marina* establishes its hepatoprotective potential which may also be due to the ability of the extracts to accelerate regeneration of liver cells, thus reducing the leakage of marker enzymes into the rat blood. The investigation also statistically reveals that the protection offered by the extracts is comparable to the positive control used i.e silymarin. The recovery towards normalcy of enzyme markers content and that of tissue histoarchitecture caused by the extracts was found to be similar to that caused by silymarin (POS) in the present study.

Among the two transmarine, 300 mg dose of the extract showed better result in the SGPT levels with a recovery of 100.11%. GPT is a better index of liver injury as liver GPT represents 90% of the total enzyme in the body and it catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner [34].

The reduction in total protein content (TPN) post induction can be attributed to the initial damage produced and localized in the endoplasmic reticulum which leads to functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver [35]. The plant treatment groups show increased content of total protein post treatment indicating enhanced synthesis of proteins which corresponds to acceleration in the regeneration process. This is also supported by the significant reduction in the

triglyceride content in serum. High dose of the extract has shown better protection in case of TG (102.4 %) when compared with the positive control (94.29 %). Inhibition of the bile acids synthesis from cholesterol leads to the increase in cholesterol levels, which is also a result of CCl₄ intoxication [36]. Significant suppression of cholesterol levels (90.62 %) by the test groups suggests that the bile acids synthesis inhibition was also successfully reversed by the standardized extracts of *Avicennia marina*.

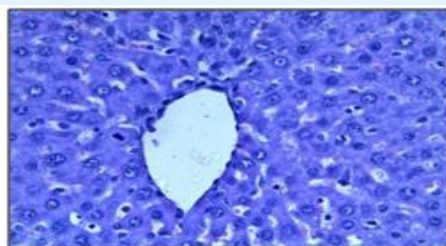


Figure 1: Normal control showing normal cellular histoarchitecture and prominent hepatocytes

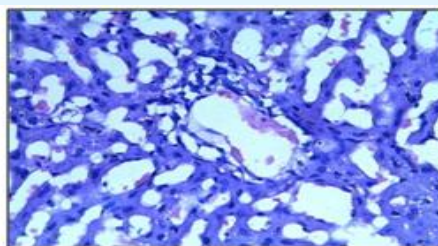


Figure 2: Induction control (IC) showing cellular necrosis, distortion of central vein and vacuolation of hepatocytes

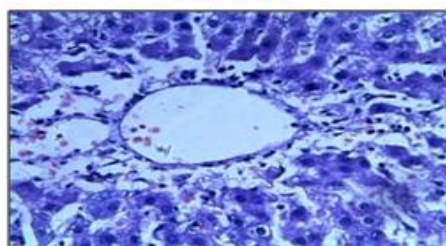


Figure 3: Natural Recovery (NR) showing extensive necrosis around central vein leading to other necrotic areas

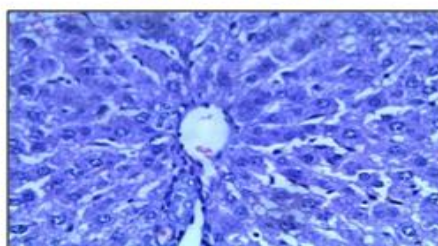


Figure 4: Positive control (POS) showing histoarchitecture returning to normalcy as seen in normal control

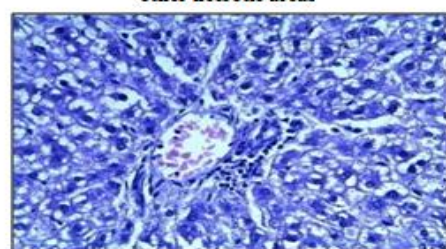


Figure 5: Treated group (AM1) at 50 mg/kg BW showing marginal recovery of histoarchitecture

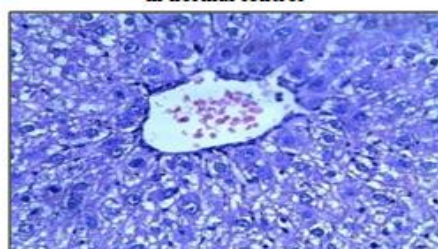


Figure 6: Treated group (AM2) at 150 mg/kg BW showing recovery of hepatocytes and reduction in area under necrosis

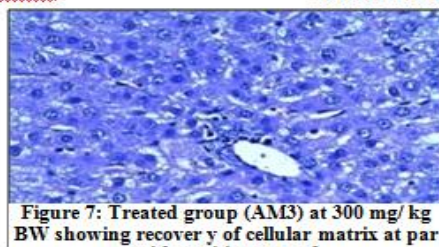


Figure 7: Treated group (AM3) at 300 mg/kg BW showing recovery of cellular matrix at par with positive control

Figure 1-7: Effect of standardized extract on CCl₄ induced rat liver (H & E, X400).

The histopathological profiles of CCl₄ induced liver (Figure 1-7) revealed extensive necrosis near the central veins leading to other necrotic areas. The sections also show distorted hepatocytes along with vacuolated sinusoids in the liver tissue. The liver tissues of the animals of POS group (Figure 4) show significant recovery of all the above observations. Albino Wistar rats treated with plant extract (Figure: 5, 6, 7) also exhibit significant improvement of hepatocellular architecture over IC group (Figure 2) as evident from considerable reduction observed in the areas under necrosis and distortion caused by CCl₄. The liver tissue of the animals of NR group (Figure 3) did not show significant improvement when compared to IC, which also seconds the hypothesis that the treatment groups and silymarin control showed enhanced recovery from the hepatic damage in comparison to the natural rate of liver recovery.

The extract of leaves of *Avicennia marina* also showed potential antioxidant property as observed from DPPH and Galvinoxyl radical scavenging activity assay (Table 1). Hence, this antioxidant property of the extract in turn might have prevented formation of trichloromethyl peroxy radical thereby reducing tissue damage. Therefore, the hepatoprotective activity shown by the extract can also be attributed to its antioxidant potential. Furthermore, the presence of phytoconstituents such as betulinic acid, betulin, ursolic acid, β -sitosterol and lupeol, which have been reported to possess hepatoprotective potential can also partially bear claim of the triterpenoid rich standardized extract's hepatoprotective activity.

Conclusion

From the above results it can be stated that the standardized ethyl acetate extract of the leaves of *Avicennia marina* shows dose dependant hepatoprotective activity which at the dose of 300 mg/ kg body weight showed results at par with established positive control, silymarin. The extract lowered the levels of biochemical markers and showed significant recovery in histopathological analysis. Though the exact mode of action is not yet clear, the presence of favorable phytochemical markers and antioxidant potential of the extract justify the hepatoprotective activity. Further studies can be undertaken to understand the specific pharmacokinetic profile of these markers and to understand in detail the mechanism of action of the standardized extract.

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