

Anti-icteric effect mediated by hepatoprotective activity of *Amaranthus spinosus* L. evaluated through phytochemicals analysis, liver biochemical parameters, antioxidant assays and PKC pathway modulation

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Protein kinase C (PKC) and its active forms, phospho-PKC (pPKC), are key mediators of Hepatic jaundice. Despite modern drugs, plant-based therapies are preferred for safer and effective alternatives. Although *Amaranthus spinosus* L. roots are traditionally used for jaundice management in Assam, the scientific validation of hepatic anti-icteric activity by PKC pathway mechanism remains limited. This study systematically evaluated the hepatic anti-icteric activity of *A. spinosus* roots through phytochemical screening, liver biochemical analysis, antioxidant activity and PKC pathway modulation. *A. spinosus* roots fractionation was performed by Soxhlation with Chloroform, Ethyl acetate, Methanol and Hydro-alcohol (1:9). The methanol extract of *Amaranthus spinosus* roots (MEAS) showed greater bioactive compounds mainly alkaloids and phenolics and significantly higher antioxidant potential. MEAS contained 14 compounds identified by GC-MS, in which “(22E)-Ergosta-5,22-dien-3-ol” served as a key bioactive compound with strong binding affinity (-6.20 kcal/mol) against PKC. MEAS showed Significant ($p < 0.01$) reductions in ROS and PKC/pPKC activity in HepG2 cells. MEAS at 400 mg/kg markedly ($p < 0.001$) restored serum biochemical markers and improved hepatic histoarchitecture. These findings validate the traditional use of *A. spinosus* roots and highlight its potential as a therapeutic agent for hepatic anti-icteric potential via PKC pathway mechanism.

Keywords: *Amaranthus spinosus* L., HepG2 cell, Protein kinase C, Hepatic anti-icteric effect, Molecular docking, Antioxidant effect.

Introduction

Jaundice is characterized by yellowish pigmentation of the skin, mucous membrane, and sclera that causes changes in skin colour, liver inflammation, abdominal pain, fever, chills, etc¹. Hepatic jaundice is the most common form other types in adults². This occurs by the severe hepatic injury through mechanisms involving oxidative stress, inflammation, insulin resistance, apoptosis, and fibrosis³. It can be caused by oxidative stress, nuclear factor kappa B (NF- κ B) activation, hepatic stellate cell activation, activation of Transforming growth factor- β (TGF- β) signaling rising extracellular matrix deposition, hepatocellular apoptosis due to mitochondrial dysfunction, etc⁴. The protein kinase C (PKC), a key protein that is distributed in multiple cell types and subcellular locations of the liver, plays an important role in hepatic function and disease

progression⁵. The localization and function of PKC depend on its isoform, which determines its specific involvement in liver physiology and pathology. When PKC phosphorylated to its active form phospho-protein kinase C (pPKC), plays direct role in the hepatic jaundice causing liver injury⁶. The pPKC activates NF- κ B, causes the release of a number of inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). These further cause inflammation of hepatocytes and ultimately lead to liver injury⁷. PKC (a family of serine or threonine kinases) and its active form play crucial roles in hepatic damage through their involvement in signaling pathways related to inflammation, oxidative stress, and fibrosis^{6,8}.

In the liver, activation of PKC is implicated in several pathological processes such as- Oxidative Stress: enhances reactive oxygen species (ROS) production, contributing to lipid peroxidation and hepatocyte injury⁹; Inflammation: the isoforms (such as PKC α , PKC β , and PKC δ) activate NF- κ B and other

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transcription factors, promoting release of pro-inflammatory cytokine, e.g., IL-6, TNF- α ¹⁰; Insulin Resistance: PKC θ and PKC ϵ contribute to insulin resistance in hepatocytes, one of the main characteristics of nonalcoholic fatty liver disease (NAFLD)¹¹; Fibrosis: stimulates activation of hepatic stellate cell, increasing extracellular matrix (ECM) deposition and leading to fibrosis¹².

While, pPKC plays more direct role in liver injury such as- Activation of Cell Death Pathways: pPKC δ and pPKC ζ contribute to hepatocyte apoptosis by activating caspases¹³; Mitochondrial Dysfunction: alters mitochondrial membrane permeability, leading to cytochrome c release and hepatocyte apoptosis¹⁴; Endoplasmic Reticulum (ER) Stress: exacerbates ER stress, which is linked to liver diseases like alcoholic liver disease (ALD) and NAFLD¹⁵; Hepatic Fibrogenesis: enhances TGF- β signaling, a key driver of liver fibrosis. Likewise, PKC and pPKC are central key molecules to hepatic injury. Thus, targeting these pathways could be a potential strategy for treatment of hepatic jaundice.

Despite modern medical interventions, the traditional therapies that involve the use of medicinal plants are playing an important role in their ability to restore health and well-being¹⁶. *Amaranthus spinosus* is one of the common plants used traditionally to treat kidney stone, jaundice in various regions of Assam¹⁷. Thus, the study aimed to scientifically analyze the traditional claim by evaluating the beneficial effect of *A. spinosus* root against hepatic jaundice. This research uniquely investigated by integration of experimental and computational approaches to evaluate the potential of *A. spinosus* root extract in treating hepatic jaundice via Protein Kinase C (PKC) signaling pathway.

Materials and Methods

Preparation of extracts

Roots from mature plant *A. spinosus* L. were collected by hand-picking method from the Fulgasa, a village of the district Dhubri, Assam, India in the month of September 2020. The roots were washed thoroughly using purified water, sun dried to remove external water content. Further, air dried at room temperature and pulverized to fine powder. This powdered material was then packed in thimble, defatted with petroleum ether (40°C – 60°C) and extracted by soxhlet apparatus using solvents such as Chloroform, Ethyl acetate, Methanol and Hydro-

alcohol (1:9) in their increased order of polarity^{18,19}. Each extraction cycle was continued for 6-8 hours, or until the solvent in the siphon arm became colorless. Each extract obtained was carefully collected and subsequently evaporated under reduced pressure until complete dryness. All solvent extracts were further analyzed by using different chemical test for the occurrence of phytoconstituents present in the extracts^{18,19}.

Quantitative analysis of *A. spinosus* root extracts

Determination of total alkaloid content (TAC)

A solution of 1 mg/ml of each solvent extracts were prepared. In the other hand, 50, 100, 150, 200 and 250 μ g/ml solutions of atropine sulphate was prepared. 1 ml of each solution was transferred to a 25 ml volumetric flask, in which 5 ml phosphate buffer of pH 4.7 solution followed by 5 ml of bromo cresol green solutions were added. To this, 4 ml chloroform was added and shaken well. The volume was made up to the mark with chloroform. The absorbance of the resulting solution was measured by UV-visible spectrophotometer (SHIMADZU CORP., 80866) at a wavelength of 470 nm^{20,21}. Finally, total alkaloid content of each extracts was calculated from the standard curve.

Determination of total flavonoid content (TFC)

A solution of 1 mg/ml of each solvent extracts were prepared. In the other hand, 10, 20, 30, 40, 50 μ g/ml, 60, 70, 80, 90 and 100 μ g/ml solutions of rutin was prepared. For 6 minutes, a mixture of 1 ml of all solution, distilled water (4 ml), and 5% sodium nitrite solution (0.3 ml) was left in a dark area. The reaction mixtures were incubated in a dark area for 5 minutes after adding 0.3 ml of 10% aluminum chloride. Next, 0.5 ml of distilled water and 1ml of sodium hydroxide (1M) were added. The solution's absorbance was measured at 510 nm using a UV-visible spectrophotometer (SHIMADZU CORP., 80866) after vigorously shaking the reaction mixture^{20,21}. The standard curve was used to determine the total flavonoid concentration of each extract.

Determination of total phenolic content (TPC)

A solution of 1 mg/ml of each solvent extracts were prepared. In the other hand, 10, 20, 30, 40, 50 μ g/ml, 60, 70, 80, 90 and 100 μ g/ml solutions of gallic acid was prepared. 0.5 ml solution of extracts and each solution of gallic acid were mixed 2 ml of Folin-Ciocalteu reagent and 4 ml of 7.5% sodium

carbonate solution. The reaction mixture was shaken well and incubated at room temperature for 30 minutes. Absorbance of the resulting solutions was measured by UV-visible spectrophotometer (SHIMADZU CORP., 80866) at the wavelength of 765 nm^{20,21}. Finally, total phenolic content of each extract was calculated from the standard curve.

Antioxidant activity of *A. spinosus* root extracts

DPPH radical scavenging assay

0.1mM DPPH (39.4mg of DPPH in 1000ml) solution was prepared in methanol. Estimation was done on 1:1 ratio as 2.5 ml of methanol and 2.5ml DPPH solution as the control. 2.5 ml methanol used as the blank while, 2.5 ml ascorbic acid along with the with 2.5 ml DPPH solution as the standard at different concentrations like 1, 2, 4, 8, 16 and 32µg/ml. Similarly, 2.5 ml solution of plant extracts were mixed with 2.5ml DPPH as sample at different concentrations as standard. There was an instant effort to keep all of these solutions out of the light. After 30 minutes, the absorbance was measured at 517 nm using a UV-Visible spectrophotometer (SHIMADZU CORP., 80866)²². The IC₅₀ was then determined using the calibration curve.

Hydrogen peroxide (H₂O₂) radical scavenging assay

1 mg/ml stock solutions of all solvent extracts and standard (Ascorbic acid) were prepared using distilled water. Then 0.4M solution of hydrogen peroxide and 0.5M phosphate buffer of pH 7.4 were prepared. Then each solution of extracts and ascorbic acid were further diluted to prepare 10, 20, 40, 80, 160, 320, 640 and 1280 µg/ml solutions. To 100 µl of each solution, 600 µl of hydrogen peroxide solution and 4 ml of phosphate buffer was added and incubated for 10 minutes. The control was prepared by addition of 600 µl of hydrogen peroxide solution in 4 ml of phosphate buffer solution. UV-Visible spectrophotometer (SHIMADZU CORP., 80866) absorbance were recorded at 230 nm utilizing phosphate buffer as the blank, and the calibration curve yielded IC₅₀ values²³.

Superoxide (O₂⁻) radical scavenging assay

1 mg/ml stock solutions of all solvent extracts and standard (Ascorbic acid) were prepared using distilled water. Then each solution of extracts and ascorbic acid were further diluted to prepare 10, 20, 40, 80, 160, 320, 640 and 1280 µg/ml solutions. To 0.1 ml of each solutions, 0.1 ml of 6 µM ethylene diamine tetra

acetic acid containing sodium cyanide, 0.1 ml of 50 µM nitro blue tetrazolium and 0.05 ml of 2 µM riboflavin were added, and ultimate volume was prepared up to 3 mL using phosphate buffer of pH 7.8. The reaction mixture was then incubated at 25°C for 5 minutes. The control was prepared by addition of phosphate buffer solution without drug or extract solution. The absorbance was measured at 560 nm in UV-Visible spectrophotometer (SHIMADZU CORP., 80866) against phosphate buffer as the blank and IC₅₀ values were calculated from the calibration curve²⁴.

Characterization of phytoconstituents of MEAS by GC-MS

1 gram of methanol extract of roots of *Amaranthus spinosus* L. (MEAS) was solubilized in 10 ml of solvent methanol, it was then filtered and dilute and qualitatively identified by GC-MS analysis fitted with TG-5MS capillary column (30m × 0.25mm × 0.25µm), Thermo Scientific. The optimization parameters for the study of roots extract by the column alignment were as such: carrier gas hydrogen was utilized at 1 mL/min flow rate, the injection temperature was set 200°C, injection capacity was fixed as 1 µl and operation mode was the splitless (1:100). Programmer temperature was set 50°C (detained for two minute) then raised at 5°C/min that reached up to 200°C (detained for 5 minutes) that further increased to 320°C at 150C/min, which detained for 5 minutes. Mass spectrometry detection was done by electron ionization technique in which the temperatures were set as 200°C and 250°C for ion source detector and interface correspondingly. The sort of scanning of mass to charge ratio (m/z) of ion was set 40-700 with scanning intermission of 0.5 second at a scanning rate of 1.5 scans/sec²⁵. Mass spectra obtained from the methanol extract of the plant were then matched with Mass Spectral Library (ISQ-7000) of University of Science and Technology, Meghalaya. Thus, compounds that are present in the MEAS were identified.

In-silico molecular docking study of phytoconstituents of MEAS

Preparation of protein

3D structures of the Protein Kinase C (PKC) (PDB ID: 1KPA), was downloaded from RCBS protein data bank (PDB) in PDB format. The proteins were then optimized by removing heteroatoms using Notepad and energy was minimized using Swiss PDB viewer.

Then the protein was prepared for docking using Discovery Studio 2020 Client. All the water molecules, the drugs bound to protein and all the chains other chain-A were deleted and finally saved in PDB file.

Preparation of ligands

The chemical structure of phytoconstituents of MEAS were drawn using ChemSketch (ACD labs) and saved as MDL mol files which were further converted to pdb files utilizing the software Open Babel GUI.

Docking and visualization of compounds

The compound was docked against PKC using Autodock Tools 1.5.6. Ligands and the protein was made visible in the software and water molecules deleted, further added Kolmann charges and assigned the atom to AD4 type and then docked against the desired protein PKC. The docking of the compounds were analyzed and visualized utilizing Discovery Studio 2020 Client Visualizer²⁶.

Effect of MEAS in HepG2 cells

Safety study of MEAS on HepG2 cell line by MTT assay

HepG2 Cells from National Centre for Cell Science (NCCS), Pune were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS) and 1% penicillin and Streptomycin (100 µg/ml) in 5% carbon dioxide (CO₂) in controlled conditions. HepG2 cells were seeded at a density of 4×10⁵ cells in 96 well plates and treated with 10-200 µg/ml of MEAS or only with 0.1% dimethyl sulfoxide (DMSO) and incubated for 24 hours. Then 20 µl solution of 2.5 mg/ml concentration of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each of the well plates and incubated again for 4 hours. After dissolving MTT formazan crystals in 500 µl DMSO, the optical density was measured at 570 nm using an enzyme-linked immuno sorbent assay (ELISA) plate reader²⁷.

Effect of MEAS on intracellular reactive oxygen species (ROS) by LPS-induced ROS- DCFDA dye fluorescent analysis

HepG2 Cells (NCCS Pune) were grown in DMEM medium supplemented with 10 % FBS in 5% CO₂ in controlled conditions. More than 95% of viable cells were treated with or without lipopolysaccharide (LPS) (25 µg/ml) and different concentrations of MEAS

(0-100 µg/ml) in the same controlled condition in 96 healthy plates. The level of intracellular reactive oxygen species (ROS) was estimated using 2',7'-dichlorofluorescein diacetate (DCFDA) fluorescence analysis²⁷.

LPS-induced PKC and phospho-PKC activity of MEAS in HepG2 cells

HepG2 Cell (NCCS Pune) were grown in DMEM medium supplemented with 10 % FBS in 5% CO₂ and controlled conditions. More than 95% of viable cells were treated with or without LPS (25µg/ml) and different concentrations of MEAS (0-100 µg/ml) in the same controlled condition in 96 healthy plates. The Protein kinase C (PKC) and Phospho-PKC levels were estimated using a commercially available Sandwich ELISA kit (KBH0816 and KBH15203)²⁸. The unknown concentration of PKC and pPKC in the treated and untreated cells were interpolated using a standard curve using linear regression analysis in Graph Pad prism, version 5, considering X=0, Y=0.

Animals

We picked healthy adult Wistar albino rats of both sexes that weighed between 140g and 160g. Animals were kept in clean, sterile polypropylene cages at room temperature (25 ± 2°C) with a 12-hour light and dark cycle. They had unfettered access to commercial pellet food and water. The rats were randomly put into different groups and kept apart for a week to get used to their new habitat and to handle them before the experiments started²¹.

Acute toxicity study

Acute toxicity study was executed in accordance to OECD Guideline number 423, adopted in 2001 in female rats of 8-12 weeks. Female rats were retained in chamber temperature maintaining 12 hours of bright and 12 hours of black for 5 days prior to dosing. Animals were provided normal food and drinking water. Three female rats were fasted overnight with food while drinking water was withheld for 3-4 hours then administered the initial dose of MEAS as 2000 mg/kg of body weight in the volume 0.1 ml/10 grams of bodyweight by oral route with oral feeding cannula. All 3 female rats were observed independently for the initial 30 minutes after MEAS administration, then for 4 hours at 1 hour interval and periodically for 24 hours and then for 14 days regularly²⁹.

Evaluation of hepatic anti-icteric activity of MEAS in experimental rats

Rats were grouped into five groups consisting six rats in each group both male and female in the same ratio as follows;

Group-1: 0.5% Carboxy methyl cellulose (CMC), per oral (p.o.)

Group-2: Paracetamol (PCM) (2000 mg/kg) + 0.5% CMC, per oral (p.o.)

Group-3: Paracetamol (PCM) (2000 mg/kg) + Silymarin (100 mg/kg), per oral (p.o.)

Group-4: Paracetamol (PCM) (2000 mg/kg) + MEAS (200 mg/kg), per oral (p.o.)

Group-5: Paracetamol (PCM) (2000 mg/kg) + MEAS (400 mg/kg), per oral (p.o.)

The rats were administered with paracetamol (2000 mg/kg, per oral) for first five days to grow jaundice in accordance of standard protocol²¹. The rats were casually scattered into five groups (n = 6). The treatment for hepatic jaundice with Silymarin and MEAS was for 5 days and was begun from 6th day up to 10th day. Plasma samples were taken through tail veins of all groups of rats on 1st day, 5th day (6 hours of paracetamol post-intake), and on the 10th day and plasma concentration of total bilirubin (TB), serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) were determined (using kits from Span Diagnostic Ltd.) following standard procedure²¹.

At the end of the therapy term, histopathological analyses were performed²¹. Carbon dioxide (CO₂) asphyxiation was used to euthanize both control and experimental animals. Livers from all groups were isolated, fixed in saline containing 10% formalin, dehydrated in ethanol, cleaned with xylol, and mounted in molten paraplast at 58°C to 62°C. Five-micron specimens were produced, dyed with haematoxylin and eosin, and analyzed with a bright-field microscope at 100X magnification for structural alterations.

Evaluation of *in-vivo* antioxidant potential of MEAS in rat liver tissue

According to the standard protocol³⁰, 1 gram of liver tissue isolated from the rats was homogenized in 9 ml of 20 mM Tris-hydrochloric acid using a homogenizer. The homogenized liver tissue was then centrifuged for 30 minutes at 15000 rpm at 4°C. The supernatant liquid was then shifted to eppendorf tubes and kept at (-80)°C until the biochemical investigation was executed. lipid peroxidation (LPO) activity was

measured in which absorbance values were checked at the wavelength of 412 nm. Similarly, glutathione (GSH) activity was measured and absorbance recorded at time intervals of 30 seconds at the wavelength of 340 nm for 3 minutes. Catalase (CAT) activity was checked at the wavelength of 405 nm, and superoxide dismutase (SOD) activity at the wavelength of 560 nm using the colorimetric method based on the degree of blockage of nitroblue tetrazolium.

Statistical analysis

Data were statistically studied for determination of mean value and their standard deviation. In addition, the data were processed for one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc for conclusion of significance level.

Result

Pharmacognostic and physicochemical studies of plant materials

Physicochemical evaluation plays a crucial role in confirming the identity, purity, and quality of crude plant materials. In the present investigation, the results were found optimum and within permissible range. The moisture content (8.03 ± 0.68 %), Water soluble extractive value (1.29 ± 0.10 % w/w), Alcohol soluble extractive value (2.31 ± 0.24 % w/w), Total ash (8.63 ± 0.32 % w/w), Acid insoluble ash (1.89 ± 0.12 % w/w) and Water soluble ash (2.72 ± 0.05 % w/w). The observed findings support the purity of the sample and its suitability for further pharmacological investigations.

Further, successive extraction of roots of the plant with different solvents using soxhlet apparatus, the methanol extract of roots of *Amaranthus spinosus* L. (MEAS) showed the highest yield value (3.80 % w/w) than other solvent extracts. The yield obtained for other solvent extracts are chloroform extract (0.45 % w/w), Ethyl acetate extract (2.22 % w/w) and hydro-alcoholic extract (0.97 % w/w). All the solvent extracts were analyzed by phytochemical screening and showed numerous important phytoconstituents such as alkaloids, glycosides, terpenoids, phenolic compounds, etc (Table 1). The MEAS contained most of them.

Quantitative analysis of *A. spinosus* root extracts

The abundance of phenolics and flavonoids suggests potential antioxidant and other phytoconstituents may

Table 1 — Phytochemical analysis of several solvent extracts of roots of *Amaranthus spinosus* L.

Phytoconstituents	Chloroform extract	Ethyl acetate extract	Methanol extract	Hydro-alcoholic extract
Terpenoids	-	-	-	-
Glycosides	-	-	+	+
Carbohydrates	-	-	+	-
Proteins	-	-	-	-
Alkaloids	-	-	+	-
Fixed oils and Fats	+	+	+	-
Flavonoids	-	+	-	-
Tannins & phenolic compounds	+	+	+	+

Note: “+” represents Present, “-” represents Absent.

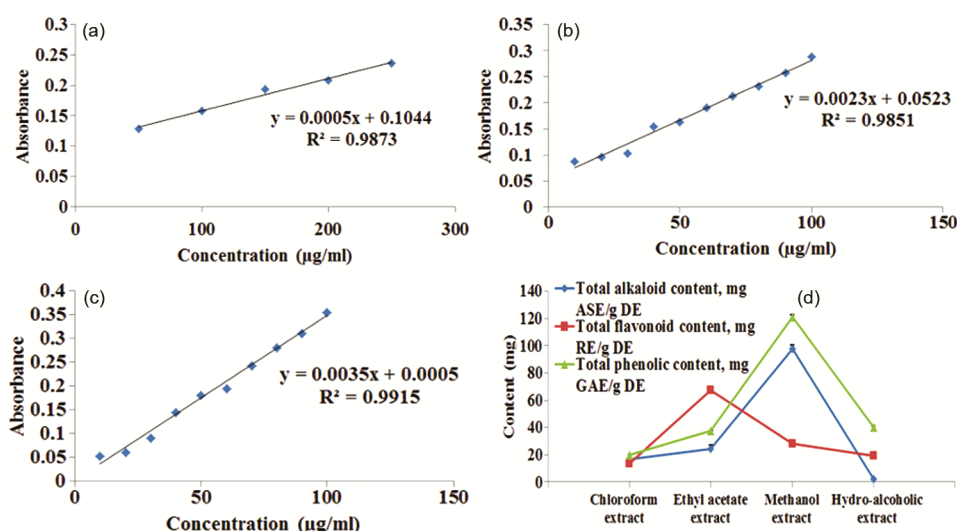


Fig. 1 — Quantitative analysis of extracts of roots of *Amaranthus spinosus* L. a) Standard calibration curve of Atropine sulphate for estimation of total alkaloid content. b) Standard calibration curve of Rutin for estimation of total flavonoid content. c) Standard calibration curve of Gallic acid for estimation of total phenolic content. d) Total alkaloid, flavonoid and phenolic contents of extracts of roots of *Amaranthus spinosus* L. Data presented as mean $\pm$ standard deviation (SD). “ASE”- Atropine sulphate equivalent; “RE”- Rutin equivalent; “GAE”- Gallic acid equivalent; “DE” Dry extract.

further contribute to diverse biological activity. On analysis, *A. spinosus* showed the presence of significant amount of alkaloids, flavonoids and phenolic contents equivalent to atropine sulphate, rutin and gallic acid respectively when calculated from the standard calibration curves as shown in Fig. (1). Significant findings of MEAS and presence of other bioactive compounds could be responsible for the therapeutic potential.

Antioxidant activity of *A. spinosus* root extracts

All solvent extracts of roots of *A. spinosus* were analyzed for their *in-vitro* antioxidant efficacy by DPPH free radical scavenging assay, hydrogen peroxide scavenging assay and superoxide radical scavenging assay as in the Fig. (2). MEAS showed promising antioxidant property when compared to the standard drug Ascorbic acid. These findings reflected

that MEAS is the potent extract of all other solvent extracts. Hence, MEAS could be responsible for promising activity against hepatic aundice.

Phytoconstituents of MEAS identified by GC-MS

GC-MS chromatogram obtained showed a number of peaks for the presence of chemical components as shown in the Fig. (3)., which were identified by the mass spectral library. The GC-MS spectrum identified 14 compounds in MEAS (Table 2). These compounds further subjected to docking with specific protein (PKC) responsible for hepatic jaundice to confirm the possible mechanism of action.

In-silico molecular docking of phytoconstituents of MEAS

On docking of all compounds of MEAS enlisted in Table 4 against PKC. The only compound “(22E)-

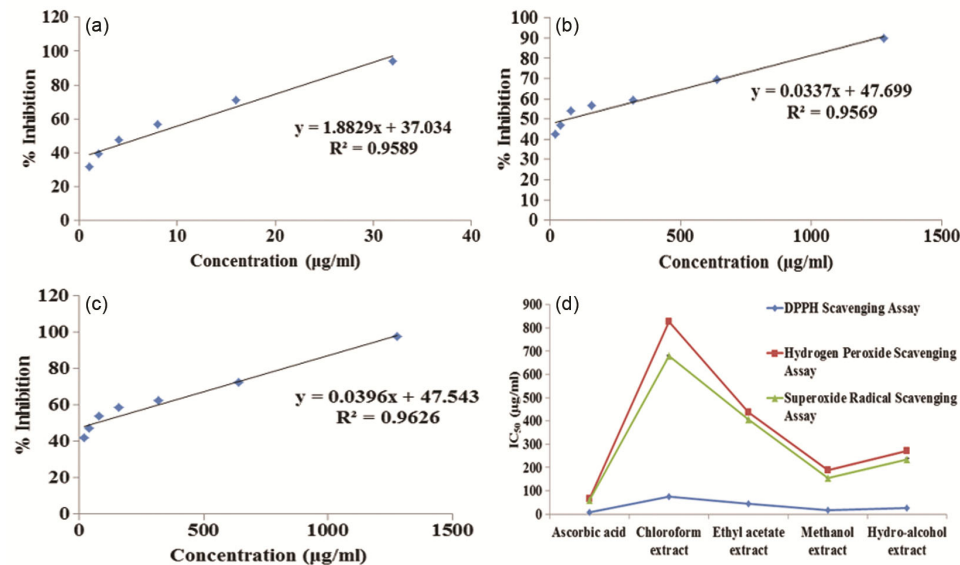


Fig. 2 — Antioxidant study of extracts of *Amaranthus spinosus* L. in-vitro. a), b) and c) Standard inhibitory calibration curve by DPPH, Hydrogen peroxide and superoxide radical scavenging assay. d) Antioxidant property of extracts of *Amaranthus spinosus* L. Data are shown as mean $\pm$ standard deviation (SD) of three variables.

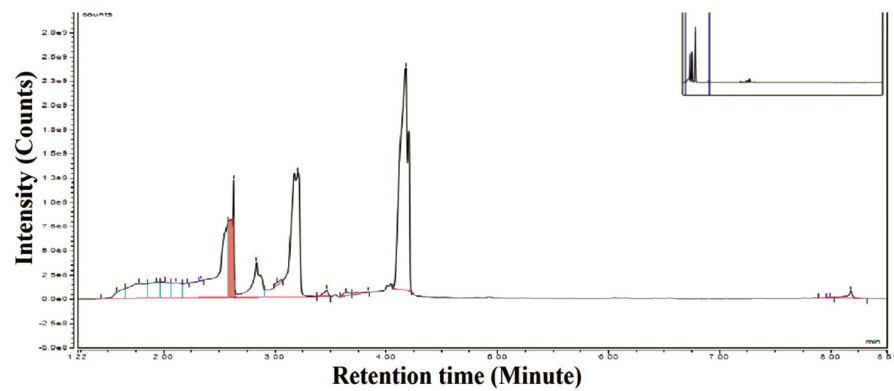


Fig. 3 — GC-MS chromatogram of MEAS.

Table 2 — Phytoconstituents of MEAS identified by GC-MS analysis.					
Sl. No.	Retention time (Min)	Name of compound	Similarity index (SI)	Reverse similarity index (RSI)	Molecular formula
1	2.920	Methyltartronic acid	792	802	C ₄ H ₆ O ₅
2	3.548	2-Methyl-1-propanol	921	930	C ₄ H ₁₀ O
3	4.184	1,1-Diethoxy- ethane	843	857	C ₆ H ₁₄ O ₂
4	5.228	3,3-Dimethyl-1,2-epoxybutane	817	848	C ₆ H ₁₂ O
5	6.201	Ethyl butanoate	884	890	C ₆ H ₁₂ O ₂
6	7.316	1-Butanol-3-methyl acetate	857	862	C ₇ H ₁₄ O
7	8.776	Ethyl hexanoate	905	905	C ₈ H ₁₆ O ₂
8	9.626	Ethyl heptanoate	842	850	C ₉ H ₁₈ O ₂
9	10.354	Ethyl octanoate	853	861	C ₁₀ H ₂₀ O ₂
10	11.187	Tetradecamethyl-cycloheptasiloxane	831	851	C ₁₄ H ₄₂ O ₇ Si ₇
11	12.319	2,4-Di-tert-butylphenol	883	907	C ₁₄ H ₂₂ O ₂
12	12.789	Phthalic acid, di-(1-hexen-5-yl) ester	678	715	C ₂₀ H ₂₆ O ₄
13	14.588	Ethyl hexadecanoate	877	884	C ₁₈ H ₃₆ O ₂
14	15.326	(22E)-Ergosta-5,22-dien-3-ol	609	640	C ₂₈ H ₄₆ O

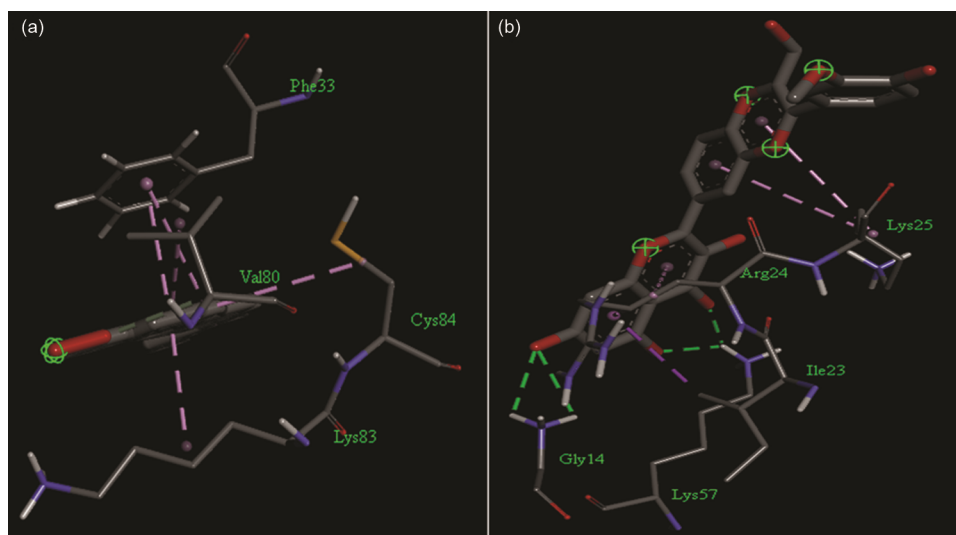


Fig. 4 — *In-silico* molecular docking analysis. a) Interaction of MEAS compound “(22E)-Ergosta-5,22-dien-3-ol” against PKC. b) Interaction of Silymarin against PKC. Green dashed lines represent Hydrogen bond; Purple dashed lines represent π - π and π -cation interactions.

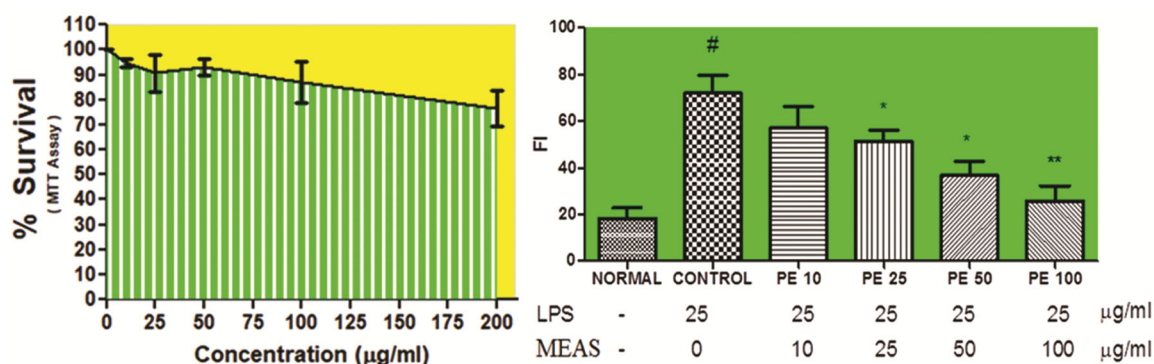


Fig. 5 — Effect of MEAS on HepG2 cell line. a) Safety study of MEAS by MTT assay. b) Effect of MEAS on ROS production. Data are shown as mean $\pm$ standard deviation of four variables. “LPS”- Lipopolysaccharide; “MEAS”- Methanol extract of *Amaranthus spinosus* L.; “FI”- Fluorescence intensity. # represents $p < 0.05$ when compared with normal; * represents $p < 0.05$, ** represents $p < 0.01$, when compared with control.

Ergosta-5,22-dien-3-ol” showed binding affinity (-6.20 kcal/mol) towards the PKC interacting with 4 amino acids such as Val80, Lys83, Cys84 and Phe33 with carbon-hydrogen bond, alkyl and pi-alkyl bonds as in Fig. (4). While the standard drug Silymarin interacted with -5.65 kcal/mol with 5 amino acids such as Lys57, Ile23, Gly14, Arg24 and Lys25 with conventional hydrogen bond, and pi-bonds.

Effect of MEAS in HepG2 cell

The MEAS showed decreasing HepG2 cells viability with increasing concentration from 10 μg/ml to 200 μg/ml as represented in the Fig. (5). More than 70% cell survival was observed at the 200 μg/ml concentration. This represented the extract is safe for the study. Further, reactive oxygen species (ROS)

were estimated in the HepG2 cell line by the LPS-induced ROS- DCFDA dye fluorescent analysis in which the MEAS showed decreased formation of ROS in the higher concentrations as in the Fig. (5).

The Protein kinase C (PKC) and Phospho-PKC (pPKC) levels were also estimated from the standard curve using linear regression analysis by utilizing commercially available Sandwich ELISA kit. The MEAS showed a significant and promising effect inhibiting the phosphorylation of PKC as shown in the Fig. (6).

Acute toxicity study

After the observation for 14 days rats were free from any toxic signs and symptoms and no mortality was seen. Thus, the evaluations of *in-vivo*

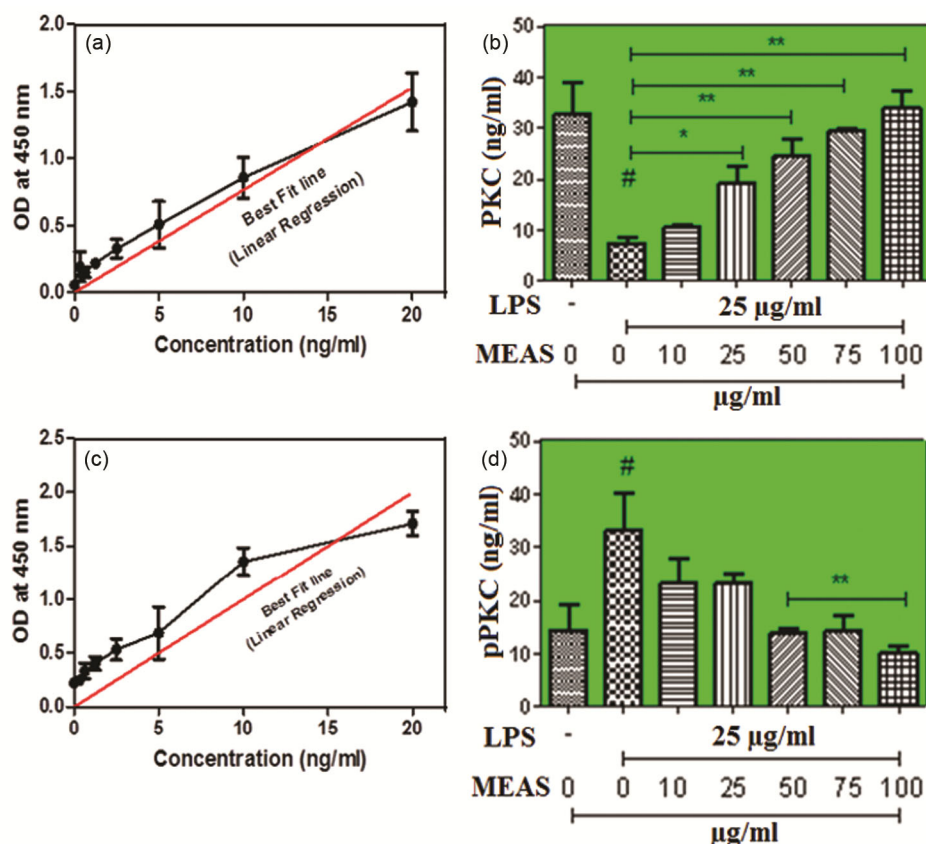


Fig. 6 — Mechanistic approach of MEAS on HepG2 cell line by Sandwich ELISA. a) Standard curve for Protein Kinase C. b) Effect of MEAS on Protein Kinase C (PKC) c) Phospho-Protein Kinase C. d) Effect of MEAS on phospho-Protein Kinase C (pPKC). Data are shown as mean $\pm$ standard deviation of four variables. “LPS”- Lipopolysaccharide; “MEAS”- Methanol extract of *Amaranthus spinosus* L. # represents $p < 0.05$ when compared with normal (without LPS); * represents $p < 0.05$, ** represents $p < 0.01$, when compared with control (with LPS).

pharmacological activities were carried out using 200 mg/kg bodyweight as low dose and 400 mg/kg bodyweight as the high dose.

Hepatic anti-icteric effect of MEAS

MEAS was administered orally to the jaundiced rats and the potency was compared using Silymarin as the standard drug determining biochemical parameters such as total bilirubin, SGOT, SGPT and ALP. The total bilirubin of jaundiced rats was significantly normalized by MEAS ($p < 0.001$). Similarly, SGOT, SGPT and ALP levels of jaundiced rats was significantly normalized by MEAS ($p < 0.001$) as shown in Fig. (7).

Effect of MEAS on liver architectures

In the histopathological study of the isolated livers of the experimental rats under digital microscope at 100X magnification, MEAS showed significance recovery of the hepatic cells and other histological characteristics as shown in Fig. (8).

Antioxidant property of MEAS on isolated liver tissue

The isolated livers of the experimental rats were analyzed for antioxidant property by determining LPO, GSH, SOD and CAT. The jaundiced animals treated with the MEAS and showed the significant recovery of the level of LPO, GSH, SOD and CAT ($p < 0.001$) as shown in Fig. (9).

Discussion

The current work offers extensive data on the efficacy against hepatic jaundice of the methanol extract from the roots of *Amaranthus spinosus* L. (MEAS) in the context of paracetamol-induced hepatic jaundice, including *in-vitro*, *in-vivo*, and *in-silico* investigations. Our results corroborate the ethnomedicinal use of *A. spinosus* roots in the treatment of jaundice and elucidate the mechanisms behind its protective benefits by mitigating oxidative stress and modulating PKC signaling pathways.

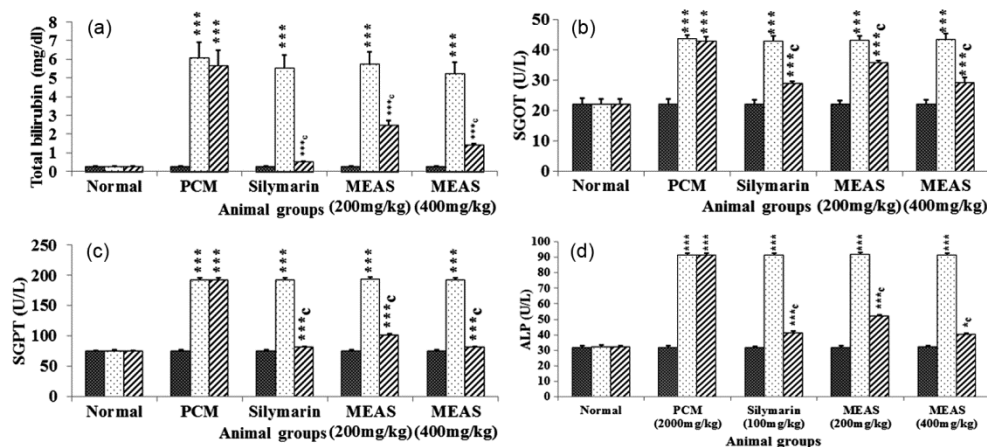


Fig. 7 — Effect of MEAS against hepatic jaundice in experimental rat model. a) Effect of MEAS on total bilirubin level. b) Effect of MEAS on SGOT level. c) Effect of MEAS on SGPT level. d) Effect of MEAS on ALP level. Data are shown as mean $\pm$ standard deviation of six variables. PCM: Paracetamol; MEAS: Methanol extract of *Amaranthus spinosus* L.; SGOT: Serum Glutamic Oxaloacetic Transaminase; SGPT: Serum Glutamic Pyruvic Transaminase; ALP: Alkaline Phosphatase. '#' represents $p < 0.05$. '##' represents $p < 0.01$, '###' represents $p < 0.001$, when compared with normal control; '*' represents $p < 0.05$, '**' represents $p < 0.01$, '***' represents $p < 0.001$, when compared with positive control (PCM).

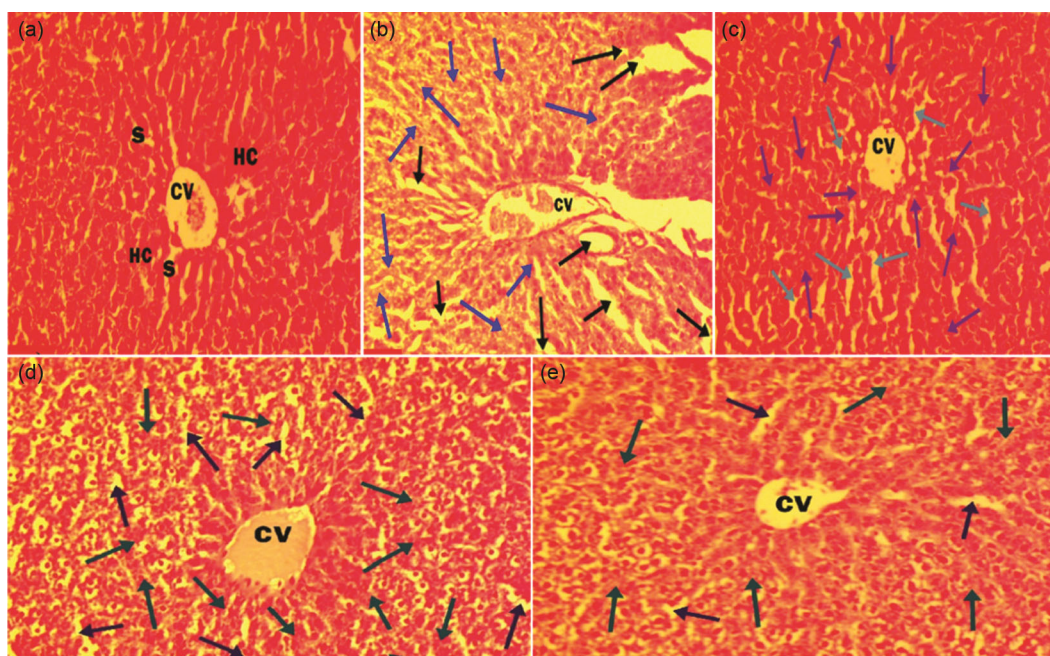


Fig. 8 — Photomicrograph of liver tissues of experimental rats. a) The untreated rats exhibited with the central vein (CV) surrounded by normal hepatic cells (HC) and sinusoids (S) among the HC. b) Jaundiced induced rats exhibited with high damage of HC characterized by the blue arrows, breakdown of the CV followed by infiltration as well as oversize of the S (black arrows). c) Treatment with the standard drug rats showed speedy recovery of the liver and exhibited with the normal HC, S and the CV. d) Treatment with the MEAS at low dose HC regeneration was observed (Dark Green arrow), recovery of S (Grey arrow) and appearance of the CV. e) Whereas at high dose normal like CV, HC (Dark Green arrow) and S (Dark violet arrow) was observed.

Phytochemical analysis revealed that MEAS is abundant in alkaloids, flavonoids, and phenolic compounds, which are well-documented for their hepatoprotective and antioxidant effects^{20,21}. The GC-MS study also found bioactive compounds, such as

(22E)-ergosta-5,22-dien-3-ol, which demonstrated a robust binding affinity with PKC in docking tests, indicating a direct involvement in the modulation of PKC-mediated inflammatory and apoptotic pathways. This is important because dysregulation of PKC and

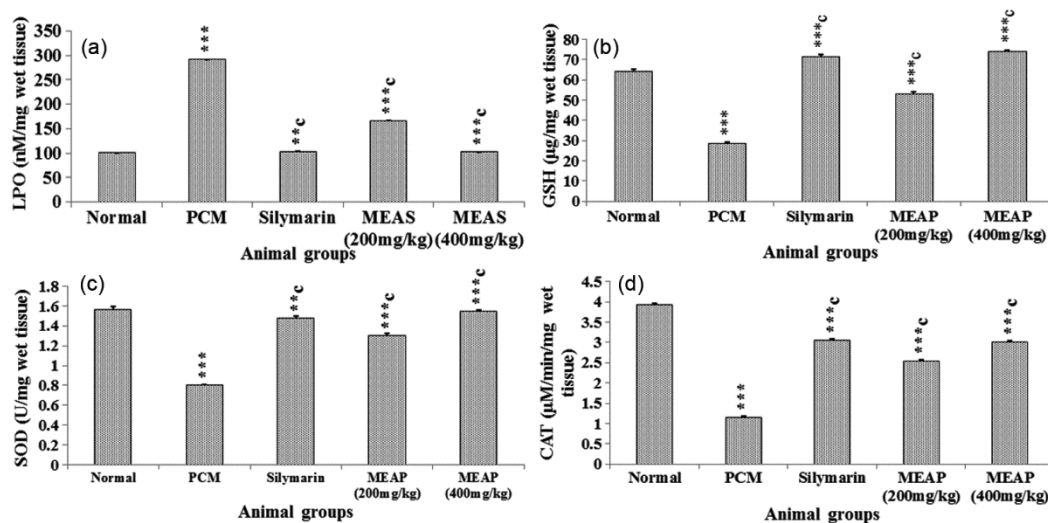


Fig. 9 — Antioxidant properties of MEAS in wet liver tissues of experimental rats. a) Effect of MEAS on LPO level. b) Effect of MEAS on GSH level. c) Effect of MEAS on SOD level. d) Effect of MEAS on CAT level. Data are shown as mean $\pm$ standard deviation of six variables. LPO: Lipid Peroxidation; GSH: Glutathione; SOD: Superoxide Dismutase; CAT: Catalase; PCM: Paracetamol; MEAS: Methanol extract of *Amaranthus spinosus* L. ‘#’ represents $p < 0.05$. ‘###’ represents $p < 0.01$, ‘####’ represents $p < 0.0001$, when compared with normal control; ‘*’ represents $p < 0.05$, ‘**’ represents $p < 0.01$, ‘***’ represents $p < 0.001$, when compared with positive control (PCM).

its phosphorylated version (pPKC) is a major cause of damage to liver cells via the production of ROS, mitochondrial dysfunction, and the formation of fibrous tissue^{6,14}.

The antioxidant tests showed that MEAS was as good as ascorbic acid in getting rid of free radicals such DPPH, hydrogen peroxide, and superoxide. These findings aligned with the reported restoration of endogenous antioxidant defenses (SOD, CAT, GSH) and the decrease of lipid peroxidation in rat liver tissues, suggesting that MEAS provides hepatoprotection predominantly via reducing oxidative stress.

Cell line tests in HepG2 cells confirmed the antioxidant results, showing that MEAS dramatically reduced LPS-induced ROS production and blocked PKC phosphorylation. These findings corroborate the concept that MEAS disrupts ROS–PKC cross talk, hence inhibiting the amplification of inflammatory cascades that culminate in hepatocellular damage. This combined action of removing ROS and blocking PKC could be a good way to treat hepatic jaundice.

The safety profile of MEAS was determined using acute toxicity testing, which showed no deaths or other toxic effects up to 2000 mg/kg. This confirmed that it has a broad therapeutic range. *In-vivo* research further substantiated the effectiveness of MEAS in hepatic jaundice. Oral treatment of MEAS (200 and 400 mg/kg bodyweight) significantly normalized Total bilirubin, SGOT, SGPT, and ALP levels in

paracetamol-induced jaundiced rats, equivalent to the standard medication silymarin. Histopathological analysis corroborated the biochemical results, revealing that MEAS-treated groups exhibited substantial restoration of normal hepatic architecture, characterized by hepatocyte regeneration and the maintenance of central veins and sinusoids. The high dose group (400 mg/kg bodyweight) exhibited recovery patterns similar to silymarin, indicating a dose-dependent benefit in the treatment of hepatic jaundice.

Nonetheless, this research has certain limitations. The specific contributions of particular phytoconstituents were not specified. Additional bioactivity-guided fractionation, separation of active components, and validation in chronic liver damage models are necessary. Moreover, molecular investigations focusing on downstream signaling pathways (e.g., NF- κ B, TGF- β , mitochondrial apoptosis) may provide more comprehensive mechanistic insights.

Conclusion

The current study shows that the methanol extract of *Amaranthus spinosus* L. roots (MEAS) has significant properties against paracetamol-induced hepatic jaundice. MEAS markedly normalized biochemical indicators, reinstated antioxidant enzyme levels, decreased oxidative stress, and maintained

liver histoarchitecture in rats. In HepG2 cells, MEAS efficiently decreased ROS production and lowered PKC phosphorylation. Molecular docking revealed ergosta-5,22-dien-3-ol as a possible PKC inhibitor. These results corroborate the ethnomedicinal usage of *A. spinosus* for hepatic jaundice and show its therapeutic potential as a safe and natural drug. Additional research concentrating on the separation of bioactive compounds, chronic liver disease models, and the clarification of molecular pathways is essential to facilitate its progression into a phytopharmaceutical for liver illnesses.

List of abbreviations

ALD- Alcoholic liver disease
 ALP- Alkaline phosphatase
 ANOVA- One-way analysis of variance
 CAT- Catalase
 DCFDA- 2',7'-Dichlorofluorescein diacetate
 DMEM- Dulbecco's modified eagle medium
 DMSO- Dimethyl sulfoxide
 DPPH- 2,2-Diphenyl-1-picrylhydrazyl
 ECM- Extracellular matrix
 ELISA- Enzyme-linked immuno sorbent assay
 ER- Endoplasmic reticulum
 GC-MS- Gas chromatography-mass spectrometry
 GSH- Glutathione
 HepG2- Hepatocellular Carcinoma
 IL-Interleukin
 LPO- Lipid peroxidation
 LPS- Lipopolysaccharide
 MEAS- Methanol extract of roots of *Amaranthus spinosus* L.
 MTT- 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
 NAFLD- Nonalcoholic fatty liver disease
 NCCS- National Centre for Cell Science
 NF- κ B- Nuclear factor kappa B
 PDB- Protein data bank
 PKC- Protein kinase C
 pPKC- Phospho Protein kinase C
 ROS- Reactive oxygen species
 SGOT- Serum glutamic oxaloacetic transaminase
 SGPT- Serum glutamate pyruvate transaminase
 SOD- Superoxide dismutase
 TAC- Total alkaloid content
 TB- Total bilirubin
 TFC- Total flavonoid content
 TGF- β - Transforming growth factor- β

TNF- α - Tumor necrosis factor- α

TPC- Total phenolic content

Ethics approval

In this study, the plant specimen was authenticated from Department of Botany, Gauhati University and identified as *Amaranthus spinosus* L. with the Accession no. 19794.

The experimental procedures were permitted by Institutional Animal Ethics Committee (IAEC) of Assam down town University, Guwahati, Assam, India with IAEC approval no. AdtU/IAEC/2022/10 on 24/09/2022.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Availability of data and materials

The original data analyzed in this study are available from the corresponding author upon reasonable request for further inquiries.

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