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Protective potential of *Carduus marianus* extract against p-dimethylaminoazobenzene (pDAB) induced hepatocarcinogenesis in mice through apoptosis induction and antioxidant pathway

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ABSTRACT

Objective: In homeopathy, ethanolic extract of *Carduus marianus* (EECM), is used against various liver disorders including cancer. This investigation aims at evaluating hepatoprotective potential of EECM, if any, against p-dimethylaminoazobenzene (pDAB)-induced hepatocarcinogenesis in mouse models *in vivo* and elucidating its possible underlying mechanism(s).

Methods: Randomized sets of inbred mice were chronically fed with different food regimens for varying periods of time and divided accordingly, 6 mice in each group, into control (Normal I and Alcohol II) and treated groups (III-V); group I: fed Normal diet, group II: Normal diet + Alcohol, group III: pDAB + Phenobarbital (PB), group IV: pDAB + PB + Alcohol, group V: pDAB + PB + EECM. They were sacrificed at day 30, 60, 90 and 120. All routine protocols were deployed for cytogenetical, enzymatic, and histopathological studies. Expressions of B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xl), Bcl-2 associated X protein (Bax), Cysteine aspartic acid protease-3 (Caspase-3), and Matrix metalloproteinase 9 (MMP-9) were evaluated at day 90 and 120 only. The DPPH free-radical scavenging activity of EECM was estimated to determine the antioxidant properties.

Results: No mice of groups I and II developed tumors in liver at any fixation intervals while all mice of groups (III-IV) developed liver tumors at three fixation intervals. But in group V mice, 4 each of 6 mice at 90 and 120 days, did not show tumor nodules in their livers, signifying that feeding of EECM could combat carcinogenesis. EECM reduced genotoxic effects and favorably modulated expression of Caspase 3 and MMP-9 as compared to control.

Conclusion: The treatment of EECM clearly demonstrated protective action against pDAB induced hepatocarcinogenesis in mice for delaying tumor progression, decreasing total tumor load and genotoxic effects, and also evidenced by favourable modulations of the apoptotic signal proteins like Bcl2, Bcl-xl. Bax, Caspase 3 and other marker enzymes AST (Aspartate amino transferase), ALT (Alanine amino transferase) etc. However, the molecular mechanism of this protective action still needs to be further elucidated.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent (5th in serial order) cancer types that leads to a high mortality rate (about 1/3rd of affected persons) worldwide.[1] The treatment for hepatic cancer is regarded as a challenging task. Some drugs have adverse side-effects or sometimes cancer cells acquire resistance to them after repeated use. Further, because of high toxicity, some chemotherapeutic drugs are not recommended for advanced stages of HCC treatment. Therefore, searching for effective new medicines, particularly in the plant kingdom, to combat this dreadful disease has been on for several decades and is still needed. The medicinal plant, *Carduus marianus*, which has been chosen for the current experimental study, has a geographical distribution from Europe to Central Asia and India. *Carduus marianus* is also known as milk thistle. Numerous substances, including silybin, silibinin A and B, silicristin, silidianin, apigenin, dehydrosilybin, deoxysilyncristin, and deoxysilyndianin have been detected in the seeds of this plant. Silymarin has also been reported in the crude extract; it has antioxidant properties, scavenging free radicals and mitigating oxidative stress in liver cells. Silymarin also has been reported to have anti-inflammatory effects by

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modulating cytokine levels and inhibiting inflammatory pathways, contributing to its hepatoprotective potential.[2–4] It has been used for reducing hypercholesterolemia and hypertriglyceridemia as well.[5]

To our knowledge, *Carduus marianus* had not been scientifically tested *in vivo* so far for its possible anti-carcinogenic effect in any mammalian model. It has earlier been reported that chronic feeding of both p-dimethylaminoazobenzene (pDAB) and Phenobarbital (PB) usually generate cytogenetical damage manifested as chromosomal aberrations, various nuclear anomalies including induction of micronuclei, and sperm head anomaly (SHA),[6,7] which can be used as valid parameters of degree of genotoxicity. Hence, the present study was undertaken on hepatocellular carcinoma mice (*Mus musculus)* model induced by pDAB and PB, with the aim to ascertain if ethanolic extract of *Carduus marianus* (EECM) has any potential anti-cancerous activity against pDAB and PB induced hepatocellular carcinoma and to understand some key mechanisms underlying their effects.

2. Materials and methods

2.1. Animals

The procedure and methods of how an inbred strain of Swiss albino mice (*Mus musculus*), that served as materials for the present study was reared and maintained with standard food and water in the animal house of the Department of Zoology, Kalyani University, have been described in detail elsewhere.[6] Ethical clearance was duly procured from the Institutional Ethical Committee, University of Kalyani (Approved Proposal No. KU/IAEC/Z-11/07 dt. May 18, 2007), and the animal experiments were conducted under supervision of the Animal Welfare Committee, Department of Zoology. For induction of hepatic liver nodules and subsequent hepatocarcinogenesis, the chronic dietary feeding method routinely used by earlier workers was adopted.[7] The azo dye, 0.06% pDAB was chronically fed followed by chronic feeding of 0.05% phenobarbital (PB).

2.2. Experimental protocol

A total of 120 Swiss albino mice were used in this study. Twenty-four healthy mice weighing between 25 and 30 g were randomized for each group corresponding to the four long term fixation intervals 30, 60, 90 and 120 days. Six mice of each group were sub-divided into the following sets:

I. The first set that served as a negative control was fed with a normal diet; six of them were sacrificed at each of four fixation intervals.
II. The second set of mice was provided with a low protein diet mixed with 0.06 mL alcohol (Alc).
III. The third set of mice were allowed to have a normal low protein diet mixed with 0.06% pDAB (Sigma, D-6760) at a daily dose of 165 mg/kg body weight ("initiator" of hepatocarcinoma), and with 0.05% aqueous solution of PB ("promoter"), till they were sacrificed at different fixation intervals.
IV. The fourth set of mice were provided with a low protein diet with 0.06% pDAB and 0.05% aqueous solution of PB plus 0.06 mL alcohol till they were sacrificed.
V. The fifth set of mice were given pDAB and PB in the same way as that of the fourth group but were additionally fed 0.06 mL of EECM thrice a day (7 a.m., 1 p.m., 7 p.m.) from first day onward of pDAB feeding till they were sacrificed. This non-toxic and efficacious dose was arbitrarily selected on the basis of standard recommended clinical dose administered to human subjects varying from 0.9 to 1.5 mL in a fairly dose equivalent manner in mice weighing about 30 g each.

2.3. Preparation of extract

Carduus marianus (used as a homeopathic mother tincture) was procured from "HAPCO", 165, Bipin Behari Ganguli Street, Kolkata. A government registered homeopathic drug manufacturer who produces high quality drugs under stringent quality control. The mother tincture is best for human use within 2 years from manufacturing date. During the preparation, dried plant seeds were macerated, mixed with 90% alcohol and filtered. The filtrate was then evaporated to thick residue at 50 °C. The yield of the extract was about 60%. The extract was re-suspended in 90% alcohol and used for *in vivo* experiments.

2.4. 2,2-Diphenyl-1-picrlhydrazyl (DPPH) free-radical scavenging assay

The DPPH free-radical scavenging activity of EECM was estimated as per procedure described earlier.[8] The test extracts were prepared in different concentrations and added to reaction mixture containing 0.1 mM DPPH in ethanol, 0.95 mL of 0.05 mol/L Tris-HCl buffer pH 7.4. The mixture was shaken vigorously and allowed to stand at ambient temp for 30 s. The absorbance was measured at 517 nm by using spectrophotometer (UV–VIS Shimadzu). Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was estimated by following the equation: DPPH scavenging effect (%) or percent inhibition = (A0 - A1/A0) × 100, where A0 was the absorbance of control reaction and A1 was the absorbance in presence of test sample. For *in vitro* studies here, three doses, 50, 100 and 200 μg/mL were selected from a preliminary range-finding trial using varying number of doses (50, 75, 100, 150 and 200 μg/mL) (Supplementary Fig. S1) on a single parameter where these three doses showed low, middle and high efficacy, respectively.

2.5. Cytogenetic assay

The standard cytogenetic protocols like endpoint analysis of chromosome aberrations, micronuclei, mitotic index, and sperm head anomaly have been deployed in the present study.[6] Mice were intra-peritoneally injected with 0.03% colchicine @ 1 mL/(100 g) body weight 1 h and 30 min prior to sacrifice. Marrow of the femur was flushed out in 1% sodium citrate solution at 37 °C and fixed in freshly prepared acetic acid/ethanol (1:3). Slides were prepared by the conventional flame drying method followed by Giemsa staining for carefully checking all forms of bone marrow chromosome aberrations. A total of 500 bone marrow cells were observed, more or less equally from each of 6 mice of a set.

For micronucleus preparation, a part of the suspension of bone marrow cells in 1% sodium citrate was smeared on clean grease free slides, briefly fixed in methanol and subsequently stained with May-Grunwald followed by Giemsa. Approximately 5000 bone marrow cells, comprising both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scanned and the ratios between PCE and NCE were ascertained.

The mitotic index (MI) was determined from the same slide which was scanned for micronucleus. Both non-dividing and dividing cells were observed and their ratios were ascertained.

For SHA, the epididymis from each side of testis of mouse of both (control and treated) sets was dissected out and its inner content was macerated and flushed out into 10 mL of 0.87% normal saline separately. The content was made free of fats, vas deferens and other adjoining tissue. The content was then thoroughly shaken, filtered through a silken cloth and dropped on grease free clean slides. The slides were allowed to air dry. Then air dried slides were flooded with diluted Giemsa stain (1 mL Giemsa in 10 mL distilled water) for an hour, washed in tap water, and again air dried before microscopical observation.

2.6. Estimation of biochemical parameters

Mice were sacrificed and their livers were quickly taken out. The tissues were homogenized with cold 0.87% normal saline, followed by centrifugation at 3000 g for 20 min in a cooling centrifuge (C24, Remi Instruments). Before carrying out the enzymatic estimations the quantitative estimation of total protein was made by the method of Lowry et al.[9] Estimation of hepatic parameters such as aspartate amino transferase (AST),[10] alanine aminotransferase (ALT),[10] lactate dehydrogenase (LDH)[11] and gama glutamyl transferase (GGT)[12] was done by following routine protocols.

2.7. Estimation of lipid peroxidase (LPO)

Lipid peroxidation was analyzed from the supernatant by the method of Buege and Aust.[13] 1 mL of sample (homogenate containing 0.1–0.2 mg of protein) was mixed thoroughly with 2 mL of TCA-TBA-HCl (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl). The absorbance of the sample was determined at 535 nm in a double beam spectrophotometer against a suitable blank. The malonaldehyde concentration of the sample was calculated by using an extinction coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$.

2.8. Determination of enzymatic and non-enzymatic antioxidant parameters

Liver homogenates were utilized to determine the various enzymatic antioxidant parameters such as catalase (CAT),[14] superoxide dismutase (SOD),[15] Glutathione peroxidase (GPx),[16] Glutathione reductase (GR)[17] and non-enzymatic antioxidant parameters such as glutathione (GSH).[18]

2.9. Histological study of liver tissue

The liver tissue was preserved in 10% formalin solution before use. For the histology study, areas of 5 μm were embedded in paraffin, deparaffinized, subjected to rehydration and stained with hematoxylin and eosin. The slides were observed under a light microscope.

2.10. Western blot after treatment with crude extract

Protein was isolated for the analysis by western blot. For protein isolation, livers were collected from all groups at 90 and120 days fixation intervals, homogenized in lysis buffer.[19] Expression of Bcl-xl, Bcl2, Bax, cleaved caspase-3, and β-actin in all groups after 90 and 120 days of EECM treatment was monitored. Western blot analysis was performed as described previously.[20] For quantitative analysis of each band, density was determined using Gel Doc system (UltraLum Inc, Claremont, CA, USA).

2.11. Level of MMP-9 expression

MMP activity was determined by Gelatin Zymography as described.[21] For the study of MMP expression, liver tissue was collected from the sacrificed mice in an ice bucket and homogenized with phosphate buffer saline, followed by centrifugation in a cooling centrifuge (REMI, C24, India). The supernatant was collected and subjected to gelatin zymography. Metalloproteinase activity was analyzed by Image analyzer (Total LAB-2.01) from Ultra Lum, 1D Image.

2.12. Statistical analysis and scoring of data

The significance test between different series of data was conducted by student's *t*-test. During observation of all protocols, the "observer" was kept "blinded" in order to remove any "bias" in observation of data of both treated and control sets of mice.

3. Result

3.1. DPPH free-radical scavenging assay

The free radical scavenging activity of EECM was tested at different concentrations (50, 100 and 200 μg/mL) on the basis of preliminary results (Supplementary data). The EECM showed a concentration-dependent rise in the scavenging of DPPH free radicals and the highest scavenging activity was observed at the 200 μg/mL concentration (Fig. 1).

3.2. Impact of EECM on liver morphology

Out of the total number of 24 mice fed with pDAB plus PB and sacrificed at four fixation intervals, livers in 10 mice showed distinct signs of tumor formation in the form of pale reddish multiple nodules (Fig. 2), while the remaining ones did not develop such nodules. All mice fed pDAB plus PB but no drug developed tumorous nodules in liver and also had appreciably enlarged spleens. The total number of mice and incidences of liver nodules have been represented in Table 1 pDAB + PB + Alc mice showed the presence of hepatic nodules, while there were no noticeable nodules observed in the Normal + Alc group mice. pDAB + PB mice when treated with EECM declined the hepatic nodules when contrasted with pDAB + PB + Alc groups. However, 4 out of 6 mice that received EECM alongside pDAB plus PB did not show tumorous nodules in 90 and 120 days, respectively.

3.3. Cytogenetical studies

3.3.1. Chromosomal studies

Various types of chromosome aberrations of both major and minor nature were encountered in certain metaphase plates of mice that received pDAB and PB treatments as compared to normal metaphase plates which did not show any aberrations (Table 2A). The total percentage of aberrations was found to be maximum in the pDAB and PB fed mice and the aberrations were considerably reduced in EECM fed series (Table 2A). EECM appeared to protect the bone marrow cells at a higher scale at 90 days ($P < 0.001$; Table 2A).

3.3.2. Micronucleated erythrocytes

Data on occurrence of micronuclei in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) have been provided in Table 2B. The percentages of micronuclei were highest in the pDAB and PB fed mice. pDAB + PB + EECM feeding reduced the occurrence of micronuclei. EECM showed more pronounced action ($P < 0.01$, $P < 0.001$) at 90 and 120 days (Table 2B).

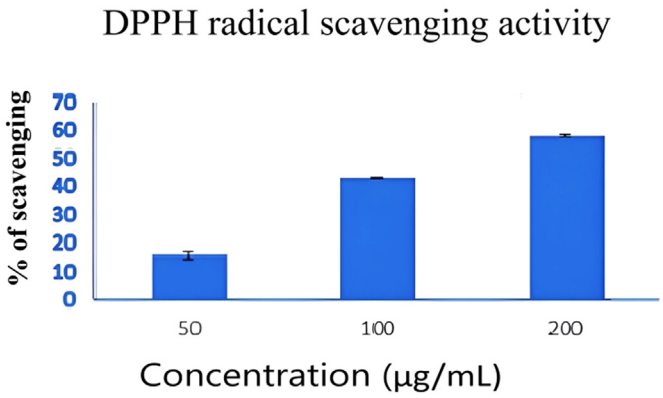

Fig. 1. DPPH radical scavenging activity of EECM. The values are expressed as mean ± Standard error (SE) (n = 3). DPPH: 2,2-Diphenyl-1-picrlhydrazyl; EECM: ethanolic extract of *Carduus marianus*.

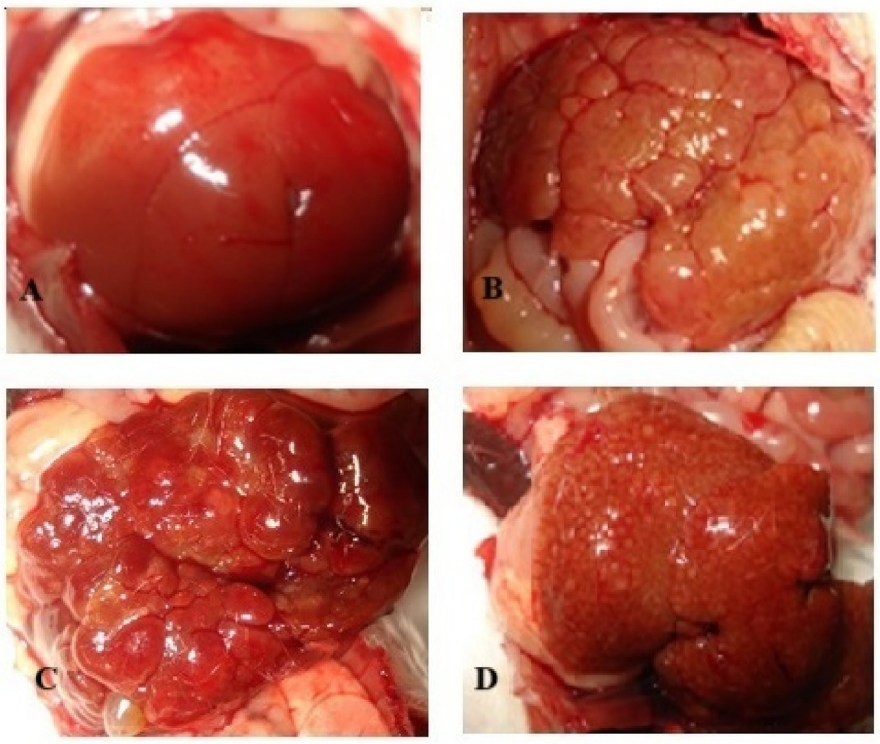

Fig. 2. Gross morphology of liver of different groups. A. Normal; B. pDAB + PB; C. pDAB + PB + Alc; D. pDAB + PB + EECM.
pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

Table 1
Number of mice with tumors at different fixation intervals after chronic feeding of pDAB plus PB for 30, 60, 90 and 120 days ($n = 6$).

Groups	No. of mice	30 days	60 days	90 days	120 days
Normal	24	0/6	0/6	0/6	0/6
Normal + Alc	24	0/6	0/6	0/6	0/6
pDAB + PB	24	0/6	2/6	3/6	5/6
pDAB + PB + Alc	24	0/6	4/6	5/6	6/6
pDAB + PB + EECM	24	0/6	0/6	2/6	2/6

pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

3.3.3. Mitotic index (MI)

In pDAB + PB + EECM fed mice, the MI was much less than in the pDAB plus PB fed mice, and the protection was statistically significant ($P < 0.05$ through $P < 0.001$) (Table 2C).

3.3.4. Sperm head anomaly (SHA)

Mice on the pDAB + PB diet showed significantly elevated SHA levels compared to normal untreated controls. Incorporating EECM into the diet (pDAB + PB + EECM) resulted in a significant decrease in SHA levels ($P < 0.05$ through $P < 0.001$) (Table 2D).

3.4. Histology of hepatic tissue

Normal structure with well-defined architecture contains small uniform nuclei scattered in cytoplasm and uniform polyhedral-shaped hepatocytes were observed in liver tissue of normal control mice. pDAB + PB + Alc fed mice showed cell necrosis, cytoplasm formed an irregular shape and confirmed the presence of focal proliferation. pDAB + PB + EECM groups showed improvement in histological features of hepatic tissue with improving cytoplasm architecture, less necrotic cells, altered hepatocytes, and hepatocellular architecture (Fig. 3).

3.5. Effects of EECM on hepatic toxicity markers

The activity of various enzyme markers in different groups of mice is shown in Fig. 4A–D. In mice treated with pDAB + PB + Alc, a significant increase in the activities of toxicity enzyme markers such as AST, ALT, GGT, and LDH was noted when compared to normal controls. However, treatment of animals with EECM (Groups V) resulted in a significant reduction in AST, ALT, GGT, and LDH levels ($P < 0.001$) at 90 and 120 days, with respect to the positive control group pDAB + PB + Alc (Fig. 4A–D).

Table 2A
Percentage (%) of chromosomal aberrations in different groups at different fixation intervals.

Groups	30 days	60 days	90 days	120 days
Normal	3.00 ± 0.120	3.66 ± 1.200	4.33 ± 0.667	4.32 ± 1.330
Normal + Alc	3.99 ± 0.577	3.67 ± 0.310	5.33 ± 0.177	5.66 ± 0.190
pDAB + PB	15.32 ± 1.764	17.33 ± 2.404	20.00 ± 2.646	25.66 ± 2.906
pDAB + PB + Alc	17.66 ± 1.453	19.33 ± 1.453	22.33 ± 1.453	27.67 ± 1.856
pDAB + PB + EECM	10.33 ± 0.882**	13.33 ± 0.882**	11.67 ± 0.780***	16.99 ± 1.528**

Outcomes were depicted as mean ± SE of triplicate measurements. $n = 6$, statistically significant: $^{**}P < 0.01$, $^{***}P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

Table 2B
Percentage (%) of micronucleated erythrocytes in different groups at different fixation intervals.

Groups	30 days	60 days	90 days	120 days
Normal	0.17 ± 0.033	0.17 ± 0.033	0.20 ± 0.058	0.20 ± 0.020
Normal + Alc	0.19 ± 0.010	0.19 ± 0.010	0.22 ± 0.030	0.20 ± 0.058
pDAB + PB	0.87 ± 0.067	0.90 ± 0.100	1.00 ± 0.058	1.30 ± 0.115
pDAB + PB + Alc	0.93 ± 0.088	0.97 ± 0.067	1.03 ± 0.088	1.36 ± 0.088
pDAB + PB + EECM	0.60 ± 0.058*	0.70 ± 0.058*	0.57 ± 0.033**	0.77 ± 0.033***

Outcomes were depicted as mean ± SE of triplicate measurements. $n = 6$, statistically significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

Table 2C
Percentage (%) of mitotic indices (MI) in different groups at different fixation intervals.

Groups	30 days	60 days	90 days	120 days
Normal	3.36 ± 0.178	3.66 ± 0.140	3.39 ± 0.154	3.56 ± 0.227
Normal + Alc	3.40 ± 0.050	3.73 ± 0.201	3.56 ± 0.080	3.83 ± 0.320
pDAB + PB	5.93 ± 0.202	7.83 ± 0.117	6.49 ± 0.217	10.63 ± 0.486
pDAB + PB + Alc	6.99 ± 0.217	8.79 ± 0.520	7.36 ± 0.343	11.06 ± 0.643
pDAB + PB + EECM	6.23 ± 0.154*	6.39 ± 0.135**	5.59 ± 0.171**	7.59 ± 0.146***

Outcomes were depicted as mean ± SE of triplicate measurements. $n = 6$, statistically significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

Table 2D
Percentage (%) of abnormal sperm head shapes (SHA) in different groups at different fixation intervals.

Groups	30 days	60 days	90 days	120 days
Normal	0.33 ± 0.075	0.36 ± 0.062	0.43 ± 0.085	0.42 ± 0.040
Normal + Alc	0.38 ± 0.020	0.38 ± 0.020	0.48 ± 0.040	0.50 ± 0.118
pDAB + PB	1.13 ± 0.110	1.39 ± 0.085	1.36 ± 0.098	1.66 ± 0.118
pDAB + PB + Alc	1.26 ± 0.154	1.59 ± 0.113	1.49 ± 0.074	1.86 ± 0.194
pDAB + PB + EECM	0.76 ± 0.112*	1.03 ± 0.178*	0.96 ± 0.133**	1.29 ± 0.064***

Outcomes were depicted as mean ± SE of triplicate measurements. $n = 6$, statistically significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

3.6. Impact of lipid peroxidation and antioxidant parameters

Mice treated with pDAB showed increased lipid peroxidation (LPO), as evidenced by higher levels of malondialdehyde (MDA), a byproduct of lipid peroxidation. Mice that received the EECM demonstrated a reduction in lipid peroxidation compared to those treated with pDAB + PB + Alc. This suggests that EECM has antioxidant properties and can effectively reduce oxidative damage induced by pDAB and PB + Alc (Fig. 5A). The results shown in Fig. 5B–F confirmed that the pDAB-fed group of animals had significantly reduced levels of antioxidant parameters, including Catalase, SOD, GPx, GR, and GSH. However, these levels were significantly ($P < 0.001$) restored by EECM treatment at both 90 and 120 days. These findings suggest that EECM effectively regulates pDAB induced hepatocarcinogenesis by modulating antioxidant parameters.

3.7. Changes in expression level of pro and anti-apoptotic proteins after treatment

To investigate the regulation of apoptosis-related proteins by EECM, we analyzed the expression levels of Bcl-2, Bcl-xl, Bax, and Cleaved Caspase-3 using Western blotting after 90 and 120 days of treatment (Fig. 6A and B). β-actin was used as a house keeping protein to normalize the data. The ratio of Bcl-2 to Bax is a critical determinant of whether cells undergo apoptosis or survival. In our study, EECM treatment resulted in a notable decrease in Bcl-2 expression and an increase in Bax expression in pDAB + PB + Alc group mice. The alteration in the Bcl-2/Bax ratio and the activation of cleaved caspase-3 clearly indicate a positive shift towards the apoptotic pathway in response to EECM treatment.

3.8. Metalloproteinase activity

At 90 and 120 days, normal mice exhibited a single band in the expression analysis, which is suspected to correspond to MMP-9. In contrast, mice fed a diet containing pDAB + PB + Alc showed two distinct bands, whereas drug-fed mice only displayed a single band. This suggests that the mice fed with the pDAB + PB + Alc diet had differential gene expression compared to the drug fed group at the same time points (90 and 120 days), as illustrated in Fig. 7A and B.

4. Discussion

It was convincingly demonstrated that feeding of carcinogenic amino-azo dyes chronically induced liver damage and nodules, and some of these nodules at later stages can turn into malignant tumors.[22] It was also reported earlier that pDAB can produce reactive electrophiles[23] and free radicals which subsequently may trigger formation of reactive oxygen species (ROS), which in turn can generate hepatotoxicity.[24] Further, dietary PB was also reported to have a positive carcinogenic effect only when fed with the azo dye 2-methyldiaminoazobenzene, but neither of these two when fed alone showed positive hepatocarcinogenesis in both mice and rat.[25] Ohnishi et al., 2001[23] also demonstrated that when these two chemicals were chronically fed to mice or rats, liver tumors usually develop after 60 days, some of which may subsequently transform into neoplastic growth. pDAB and its metabolites have been reported to cause oxidative DNA damage manifested in the form of the various types of chromosome aberrations encountered in the present investigation. The formation of adducts, DNA-copper-hydroperoxo complexes, as suggested

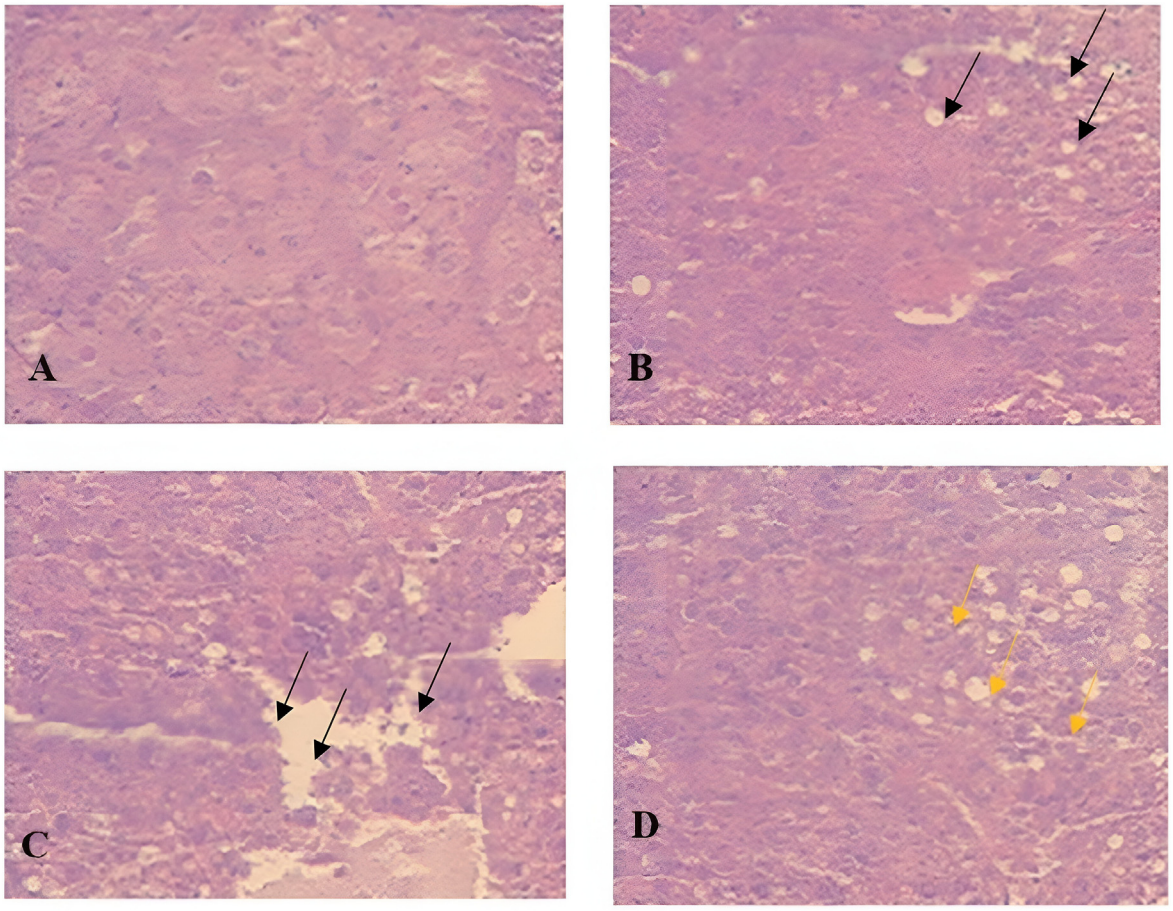

Fig. 3. Histological section of liver in different groups. A. Normal; B. pDAB + PB; C. pDAB + PB + Alc; D. pDAB + PB + EECM. The pathological changes were observed using a light microscope (Olympus) at a magnification of 100×. Normal cell arrangements were noted in the control (Group I), pDAB + PB and pDAB + PB + Alc treated groups (III and IV) demonstrating hepatic injury (marked with black arrows). The treatment with EECM (Group V) displayed the reduced hepatocellular degeneration (yellow arrows). pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

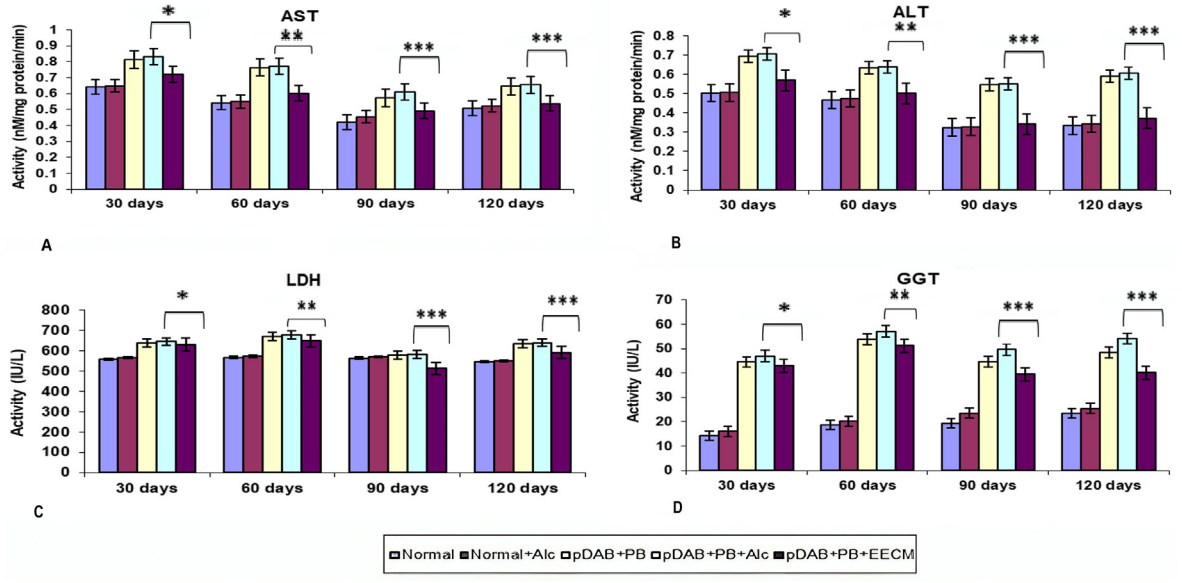

Fig. 4. Activities of different toxicity biomarker enzymes in liver tissues in different groups of mice at 30, 60, 90 and 120 days. (A) Aspartate amino transferase (AST), (B) Alanine aminotransferase (ALT), (C) Lactate dehydrogenase (LDH), (D) Gamma glutamyl-transferases (GGT). $n = 6$, statistically significant: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

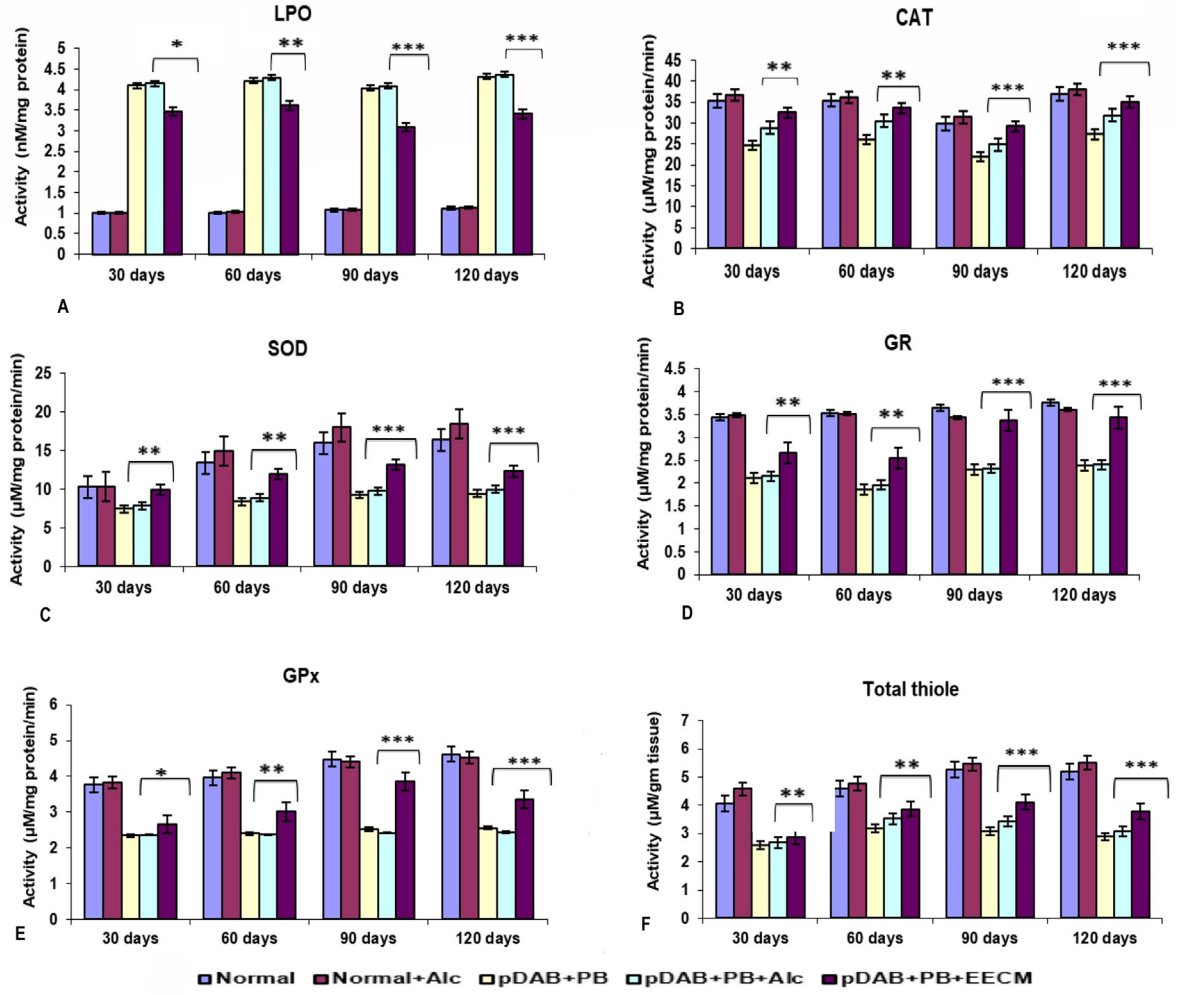

Fig. 5. Effect of EECM on lipid peroxidation (A), and the activities of antioxidant enzymes CAT (catalase, B), SOD (superoxide dismutase, C), GR (Glutathione reductase, D), GPx (Glutathione peroxidase, E), total thiole (F) in livers of control and experimental mice. The data are presented as Mean ± SE of five independent biological replicates. $n = 6$, statistically significant: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus pDAB + PB + Alc. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

by Ohnishi et al., 2001[23] could also play an important role in the carcinogenic processes of pDAB. In the present study, we also did not encounter any tumor nodules in mice fed with only PB, while those fed with pDAB plus PB developed tumors. However, interestingly enough, every 4 out of 6 mice, that received both pDAB and PB along with EECM did not develop tumors in liver while all mice of other groups fed pDAB plus PB showed tumors at 120 days. This is a significant finding that would indicate protective potential of EECM in reducing/delaying tumor growth in mice. In this context, it may be mentioned that antioxidants may protect membranes from ROS toxicity by combating ROS attack, through scavenging the reactive metabolites and converting them to less reactive molecules.[26] Natural antioxidants are also capable of inhibiting the ROS production and thereby reducing the associated intracellular oxidative stress.[27] On the basis of these facts, we performed DPPH assay which is regarded as one of the most widely employed methods for screening antioxidant activities of plant extracts.[28] In this study, we observed that the purple color of DPPH solution was reduced to a yellow colored product, diphenylpicryl hydrazine on the addition of EECM in a concentration-dependent manner. Thus, our study suggested that the free radical scavenging capacity of EECM could contribute either moderately or strongly to their anticancer activity, which is supported by the fact that antioxidants are known as "free radical scavengers" by preventing and repairing damage caused by ROS and thus can lower the risk of cancer.

Extensive toxicological investigations have now established that an increase in lipid peroxidation, alkaline and acid phosphatase activities along with a decreased level of glutathione actually signifies cytotoxicity and hepatocellular dysfunction.[29] Lipid peroxidation also plays a significant role in the process of carcinogenesis and can lead to the production of several toxic products, like malondialdehyde (MDA) and 4-hydroxynonenal which can attack DNA, thus inducing mutagenicity and carcinogenicity.[30] The results of the present study revealed that EECM inhibits lipid peroxidation. The inhibition of LPO may be due to the antioxidant effects of flavonoids, tannins, and phenolic compounds present in EECM.

Any structural damage in the liver is reflected by an enhancement in the level of serum transaminases, because these are cytoplasmic in location and after cellular damage they are released into circulation.[31] *Carduus marianus* extract used in the present study has protective ability and can maintain the structural integrity of hepatic cells. This was evident from the significant reduction in serum levels of AST, ALT, GGT, and LDH.

Decreases in the serum levels of SOD, CAT, GP, GR, and GSH were recorded in pDAB plus PB treated group. These would also bear testimony that EECM can scavenge excessive free radicals and may also contribute to hindering the process of hepatocarcinogenesis. Such studies support our findings that activities of the enzymatic antioxidants are reverted to near normal in EECM fed mice and hence prevent the initiation of hepatocarcinogenesis by pDAB.

Induction of apoptosis in cancer cells is considered as one of the key

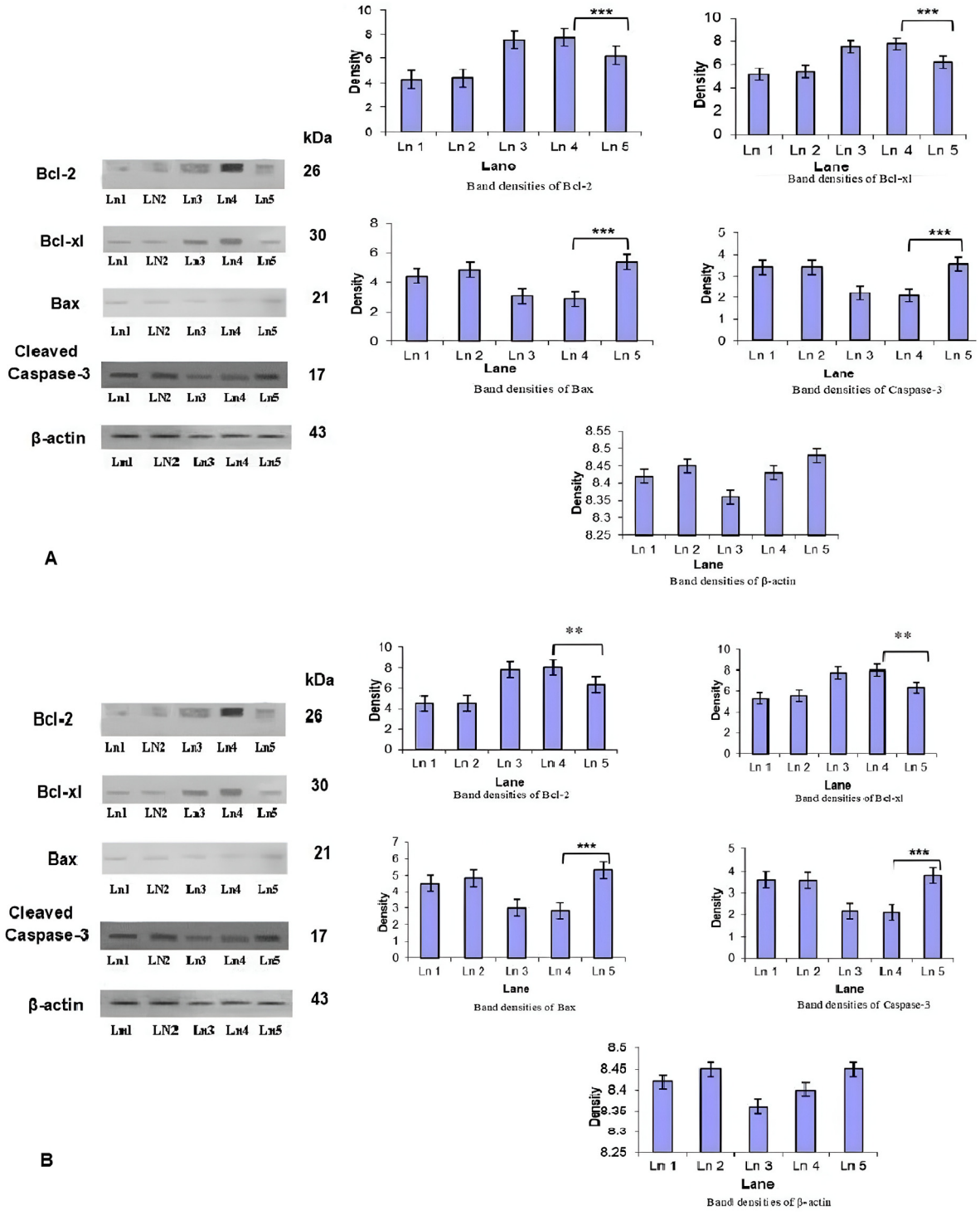

Fig. 6. The effect of EECM on the expression of β-actin, Bcl-2, Bcl-xl, Bax, Cleaved Caspase-3 during liver carcinogenesis induced by pDAB + PB in mice at day 90 (A) and at day120 (B). The quantification of expression of these genes (*Bcl-2*, *Bcl-xl*, *Bax*, *Cleaved Caspase 3*) with respect to internal control (β-actin) is represented in this figure also. Each experiment is representative of three different sets of experiments. Lane 1: Normal; Lane 2: Normal + Alc; Lane 3: pDAB + PB; Lane 4: pDAB + PB + Alc; Lane 5: pDAB + PB + EECM. $n = 6$, statistically significant: $^{**}P < 0.01$, $^{***}P < 0.001$, versus pDAB + PB + Alc. Ln: Lane. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

strategies to kill cancer cells.[32] Bcl-2 protein is known to have anti-apoptotic qualities. The antiapoptotic activity of Bcl-2 correlates with its intracellular ratio to Bax. Results of the current study imply that EECM interfered with the process of programmed cell death presumably through modulation of cleaved caspase-3 expression. Notably, EECM upregulated the expression of the pro-apoptotic gene Bax and down regulated the expression of anti-apoptotic gene Bcl-2. Therefore, the EECM treatment increased Bax/Bcl2 ratio. An increase in Bax/Bcl2 ratio generally makes the mitochondrial membrane porous to release the cytochrome-c and triggers a series of reaction cascades, for example, the formation of apoptosome followed by activation of caspase-9, caspase-3, and nuclear fragmentation, leading to apoptotic cell death.[33] This also

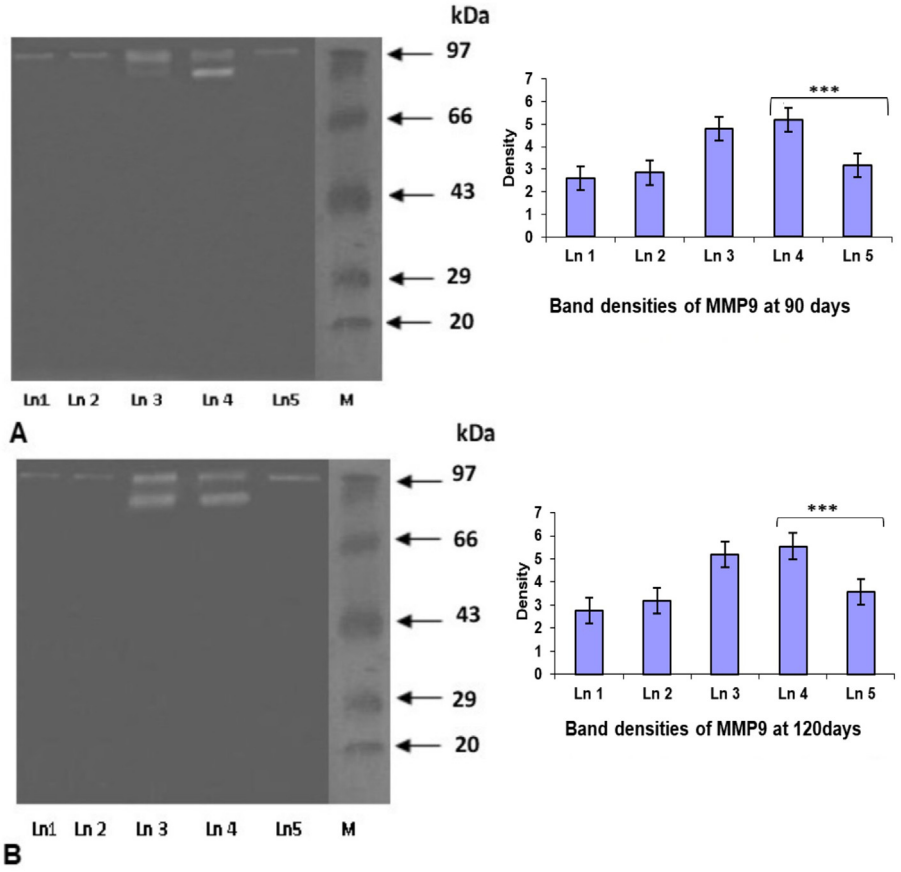

Fig. 7. Gelatin zymographic profile of liver tissue of carcinogen intoxicated and EECM fed mice (A) at 90 days and (B) 120 days fixation interval. Lane 1: Normal; Lane 2: Normal + Alc; Lane 3: pDAB + PB; Lane 4: pDAB + PB + Alc; Lane 5: pDAB + PB + EECM. $n = 6$, statistically significant: ***$P < 0.001$, versus pDAB + PB + Alc. Ln: Lane, M = Molecular weight marker. pDAB: p-dimethylaminoazobenzene; PB: Phenobarbital; Alc: alcohol; EECM: ethanolic extract of *Carduus marianus*.

might have happened in our study. The results of this study suggested the crude extract EECM showed a very good anti-cancer effect through apoptosis induction in pDAB plus PB fed mice, and had no appreciable toxic effect when fed to normal mice. Several previous studies[34–36] confirmed the presence of phytochemicals like silybin, silibinin A and B, silicristin, silidianin, apigenin, etc. in EECM. Incidentally, all these compounds when treated individually also have manifested varying degree of apoptosis-inducing properties as per earlier reports.[37,38] But future studies are warranted to determine the precise role played by each of these ingredients. However, in this study, we could clearly demonstrate the overall anti-cancer effects of crude extract of *Carduus marianus* with a focus on the key underlying mechanistic principle involving apoptotic and anti-oxidant pathways.

5. Conclusion

From the present investigation, it reveals that EECM can demonstrate a hepato-protective effect against pDAB and PB induced hepatocarcinogenesis. It has potential to be considered as a possible health supplement to prevent progression of suspected liver cancer or combating hepatoxicity generated by the carcinogenic agent(s) in future. However, further studies are warranted to see if EECM could also show similar protective effects in some other higher mammals and also on *in vitro* cancer cells for recommending its possible clinical use against cancer.

CRediT authorship contribution statement

Saili Paul: Software, Investigation, Formal analysis, Data curation. **Anisur Rahman Khuda-Bukhsh:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of interest statement

No conflict of interest to declare by both authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2025.02.005.

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