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HISTOPATHOLOGICAL AND POLYPEPTIDE ASSESSMENT DURING DIETHYLNITROSAMINE-INDUCED LIVER DAMAGE AND PROTECTION BY *CAMELLIA SINENSIS* LEAF EXTRACT (CSLE) IN WISTAR RATS

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Abstract: The present study focused on the phytotherapeutic efficacy of *Camellia sinensis* leaf extract (CSLE) against hepatotoxicity induced by diethylnitrosamine in Wistar rats. Rats were divided into 4 groups: control (normal saline, 0.9%/kg bwt), CSLE (20 mg/kg bwt), DEN (diethylnitrosamine, 1%/kg bwt), and D + CSLE (diethylnitrosamine, 1% once, and CSLE, 20 mg/kg bwt daily for 2 weeks). Liver tissue and blood serum were utilized for histological and protein profiling respectively. Administration of DEN significantly reduces rat body weight and alters the liver-to-body weight ratio. It also affects liver function and serum protein content, as assessed by spectrophotometry and gel electrophoresis. Histopathological evaluation marked a detrimental effect by DEN and significant restoration with CSLE as observed by H&E and Masson's trichrome staining. The CSLE restored body and liver weight dynamics and restored the expression of polypeptides in silver-staining liver gel profiles. The findings showed that the CSLE exerts rapid and potent hepatoprotection against DEN-induced liver injury by safeguarding structural integrity and protein expression.

Keywords: Antioxidant, Collagen, Hepatotoxicity, Oxidative stress, Polypeptide profiling.

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INTRODUCTION

Liver injury marks a critical subset of the global index of liver disease. These collectively account for over a million deaths annually. Environmental toxicants and xenobiotics play a crucial role in acute liver diseases, such as acute hepatitis, acute liver failure, liver fibrosis, cirrhosis, and hepatocellular carcinoma, in which drug-induced liver injury (DILI) increasingly accounts for a major proportion of cases (Devarbhavi *et al.*, 2023). Therefore, it is underscoring a dire need of accessible therapeutic interventions. To understand and counter these events experimentally, DEN was chosen as it is a proven standard for inducing liver damage and carcinogenesis (Mukherjee and Ahmad, 2015). DEN is found in several food products, including cheese, processed meats, tobacco smoke, and cosmetics, as well as in environmental effluents, and humans regularly get their exposure (Scanlan, 1983; Rajeshkumar and Kuttan, 2000). It is principally metabolized in the liver by CYP450 enzymes that trigger ROS cascades, such as cell death, disrupt the antioxidant system, cause DNA damage, and promote

progressive liver fibrosis via extracellular matrix deposition, particularly collagen (Ahmad and Ahmad, 2012; George and Chandrakasan, 2000).

To break this progression of damage, studies are shifting towards safe, biocompatible plant-based alternatives. *Camellia sinensis* has received significant attention due to its high polyphenolic content, which exhibits potent antioxidant, anti-inflammatory, anti-fibrotic, and anti-cancerous properties (Xiao *et al.,* 2014; Li *et al.,* 2019). Green tea has been extensively studied for its beneficial properties, which are established and evident by a number of studies, mostly *in vitro*. Additionally, most studies of *Camellia sinensis* focus on long-term metabolic effects in rats; very few have been shown to hold for short-term periods, such as14 days.

Evaluating a treatment requires a perfect, reliable, reproducible and translationally similar animal model for in vivo studies. Indeed, male Wistar rats are the ideal disease model for tracking these rapid hepatoprotective responses at multiple analytical levels. The present study was aimed to evaluate the acute therapeutic efficacy of *C. sinensis* leaf extract (CSLE) in Wistar rats, administered 3 hours after DEN administration. The protective efficacy of CSLE was comprehensively characterized by monitoring body weight throughout the study and liver weight dynamics post-euthanasia. Morphometric and histological alterations, such as architectural variation and collagen extension, were assessed by H & E and MT staining, whereas alterations in total protein were assessed by spectrophotometric and protein profiling with SDS-PAGE using established silver staining protocol.

MATERIALS AND METHODS

Chemicals

Copper sulfate, sodium tartrate, and sodium carbonate; silver nitrate; trichloroacetic acid; formaldehyde; and acetone were purchased from Sigma-Aldrich, whereas hematoxylin, eosin, and aniline blue were purchased from Fisher Scientific. Every additional chemical, reagent, and salt used was of analytical reagent (AR) grade.

Preparation of *Camellia sinensis* leaf extract

Fresh *Camellia sinensis* leaf extract was prepared each time by adding 2 mg/mL *Camellia sinensis* dried leaf powder to warm water for 1-2 minutes, then filtering through Whatman filter paper (#01).

Experimental Animals

Adult male rats (*Rattus norvegicus*), Wistar strain, weighing 205±15 g, were procured from NIB, Noida, New Delhi, and they were kept under the animal house facility of Ajmal Khan Tibbiya College, AMU, Aligarh (India), in controlled conditions (temperature 23±2 ℃, humidity 50-70%, and 12 h for dark/light conditions). They were regularly fed a diet (20 g/rat/day) purchased from Nutri Vet Life Sciences, Pune (Maharashtra), and water *ad libitum*. Experimental procedures were strictly followed in accordance with the Institutional Ethical Committee after due approval (Approval no. 1979/GO/Re/S/ 17/CPCSEA/30) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Treatment Regimen

Post acclimatization of 7 days, all 20 rats were divided into 4 groups with 5 in each, as:

Group-I (Control/C): received 0.9% normal saline orally daily.

Group-II (CSLE): *Camellia sinensis* leaf extract in a dose of 20 mg/kg b. wt., orally daily (Hung *et al.,* 2012).

Group-III (D): diethylnitrosamine at 1% in 0.9% normal saline, per kg b. wt., intraperitoneally once for 14 days (Mukherjee and Ahmad, 2015).

Group IV (D+CSLE): the rats were administered 1% DEN, followed by a 3-hr gap, and treated with CSLE orally as described in group II (Fig. 1).

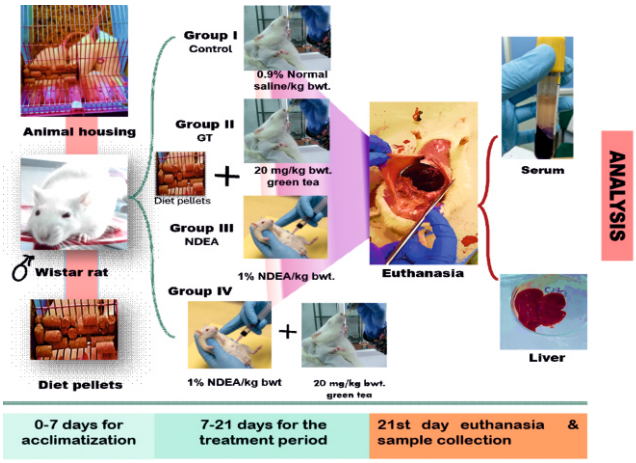

Fig. 1: Treatment regimen.

Collection of blood and liver

On the 15th day of the experiment, animals were euthanized, and blood for serum and the liver were collected following the protocol of Ahmad *et al.* (2014).

Histological assessment

After excision of liver tissue, some of them were fixed in 10% formaldehyde for up to 24 hours, then processed for sectioning. Liver tissue sections (~5μm) were further treated with a gradual series of ethanol and stained with H&E for architectural evaluation and collagen with Masson's trichrome staining following the protocol of Ahmad *et al.* (2009). Photograph were taken from a compound microscope (CX41) assisted with digital camera (Dwinterdigi 1000).

Preparation of tissue homogenate

Part of the liver tissues was homogenized in Tris-HCl buffer (50 mM, pH 7.5) in a volume of 1:3 (w/v) using a mortar and pestle. The homogenized tissues were then centrifuged at 8000 rpm for 10 min. at 4°C. The collected supernatant was then kept at -20°C for further use.

Protein determination with a spectrophotometer

The total protein contents of the samples, serum and liver, were estimated using the Lowry *et al.* (1951) method at 660 nm on a UV-Vis spectrophotometer (Bio-Sync, India).

Polypeptide profiling based on molecular weight in liver homogenates

The tissue homogenate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% vertical slab gel following the protocol of Laemmli's (1970). Equal volume of the sample of proteins was loaded in the wells, and the sample was allowed to slowly enter the resolving gel. Then the gel was allowed to run at 20 mA and 110 V/gel until completion. After completion, gels were stained with the silver staining method of Nesterenko *et al.* (1994), as the protocol is quite sensitive to estimate proteins in nanograms and offers best results in a shortest duration of 30 minutes (Table 1). The molecular weight marker utilized was chicken actomyosin to estimate the molecular weight of unknown polypeptides.

Table 1: Procedure modified to stain denatured polyacrylamide gels, adopted from Nesterenko *et al.* (1994).

Steps	Reagent	time
Fixation	60 ml, 50% Acetone containing 1.5 ml trichloroacetic acid and 25 μl formaldehyde	5 min.
Rinse	Deionized water	3 times for 5 seconds each
wash	Deionized water	5 min.
Rinse	Deionized water	3 times again for 5 sec. each
Pretreatment 1	60 ml Acetone (50%)	5 min.
Pretreatment 2	60 ml of deionized water containing 100 μl sodium thiosulfate pentahydrate	1 min.
Rinse again	Deionized water	3 times again for 5 sec. each
Impregnate	0.8ml silver nitrate and 0.6 ml formaldehyde	8-6 min.
Develop	1.2 g sodium carbonate + 25 μl formaldehyde + 25 μl sodium thiosulphate pentahydrate	10-20 sec
Stop	1% GAA	Leave the gel in GAA

Densitometry and quantitative analysis of PAGE profiles

The quantitative assessment of polypeptide profiles proceeded with densitometry of the gel photographs, which were captured with a visible-range illuminator using a Sony Cybershot digital camera (14.1 megapixels, 4x optical zoom), and analysis was done with GelPro software (Media Cybernetics, USA).

Statistical Analysis

The datasets were analyzed with GraphPad Prism 8.4.3 using one-way ANOVA followed by Tukey's multiple-comparison test across all experimental groups. The results are expressed as mean ± SEM. A significant difference was observed at $p \leq 0.05$ and 0.01.

RESULTS

During this study, the toxicity of DEN and the therapeutic potential of CSLE were evaluated with biochemical, histological, and polypeptide profiling. The study indicated that NDEA damaged the liver, as evidenced by changes in body and liver weight, total protein levels in liver and serum, and expression of polypeptides in liver tissue.

Alteration in body and liver weight

A significant change in body weight and liver weight was observed in DEN-treated rats compared to the control group ($p = 0.001$), whereas in D+CSLE, both liver weight and body weight are significantly improved as compared to DEN ($p = 0.01$). The weight pattern, as noted in CSLE, showed that it supports restoration of animal weight, compared with control rats, which increased significantly from the first day (Fig. 2).

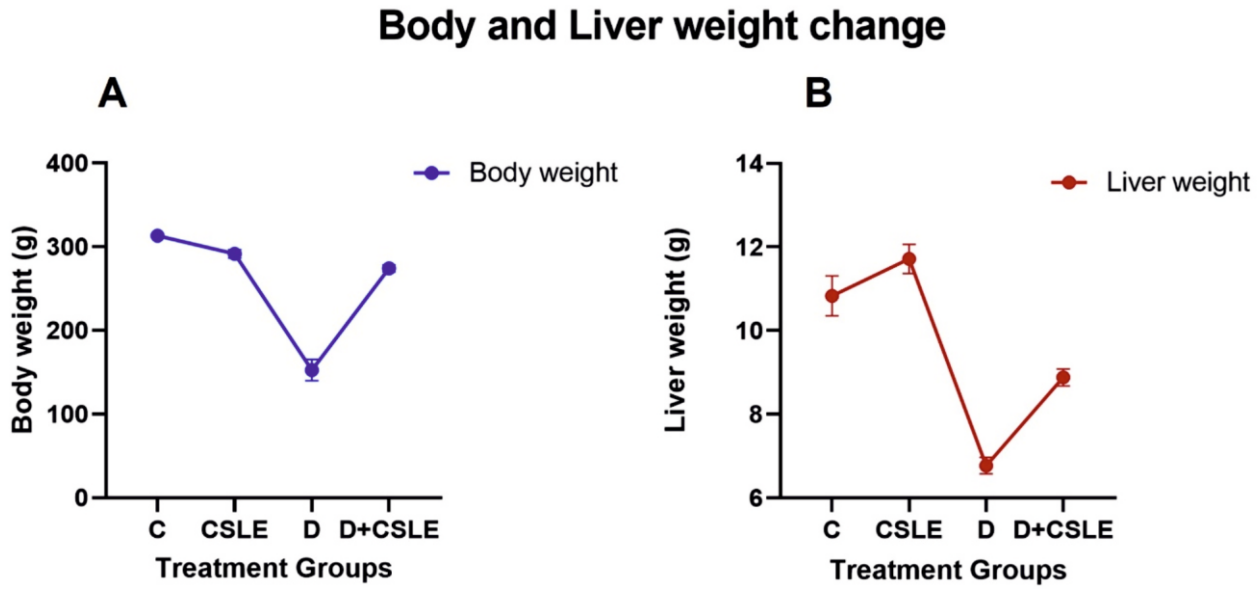

Fig. 2: Alteration in body weight (A) and liver weight (B). The DEN-treated animal shows drastic weight loss, whereas the CSLE supplement restores it. The data are presented as mean ± SEM (n=5).

Histopathological Alteration

The structure of the whole liver of 'C' and 'CSLE' did not show any morphological changes, whereas the DEN-treated livers were stiffened and showed rough surface along with red patches. On the other hand, 'D+CSLE' group showed results recovery in the appearance of color (Fig. 3). Further, the H&E and Masson's trichrome stained section of 'C' and 'CSLE' showed apparently no architectural differences; cells appeared healthy, with no signs of cell degeneration or necrosis (Fig. 4A, B, and Fig. 5A, B). However, in the 'D' group (DEN-treated), prominent damage was observed, including a large number of neutrophil infiltrations, cell death, and collagen accumulation, as indicated by the blue stain in the MT-stained slide (Fig. 4C and Fig. 5C). A significant improvement was observed in the D+CSLE group (Fig. 4D and Fig. 5D).

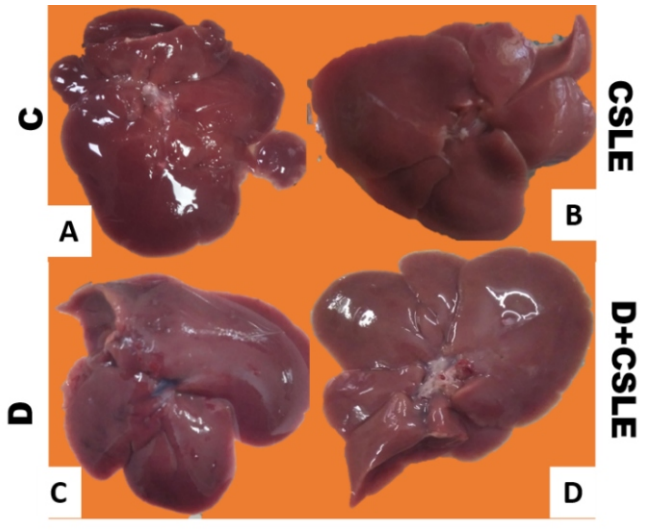

Fig. 3: Photographs showing the dissected liver of healthy and injured animals. (A) C, control; (B) CSLE, (C) D, Diethylnitrosamine; (D) D+CSLE, treated.

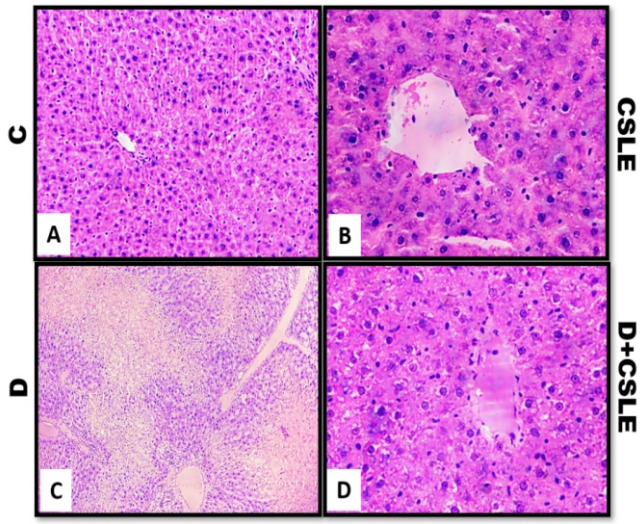

Fig. 4: H&E staining of liver tissue: (A) C, control; (B) CSLE, (C) D, Diethylnitrosamine; (D) D+CSLE, treated. Prominent damage was observed in the 'D' group tissue; however, tissue integrity was maintained in the D+CSLE group.

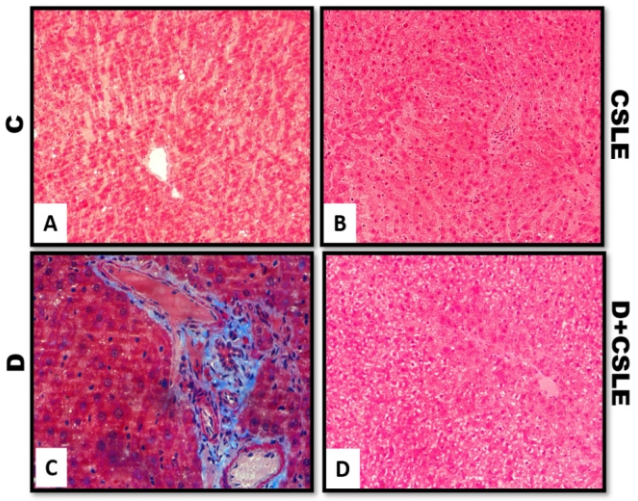

Fig. 5: Masson's Trichrome (MT) staining of liver tissue: (A) C, control; (B) CSLE, (C) D, Diethylnitrosamine; (D) D + CSLE treated. A significant collagen accumulation was displayed in the 'D' group tissue; however, D+CSLE represented normalcy in hepatic architecture.

Assessment of total protein content of serum and liver

Both the liver and serum of the DEN group showed significant declines in total protein content compared to the control ($p \leq 0.01$). On the contrary, rats of group D+CSLE showed a significant recovery as compared to the 'D' group ($p \leq 0.05$) (Fig.6).

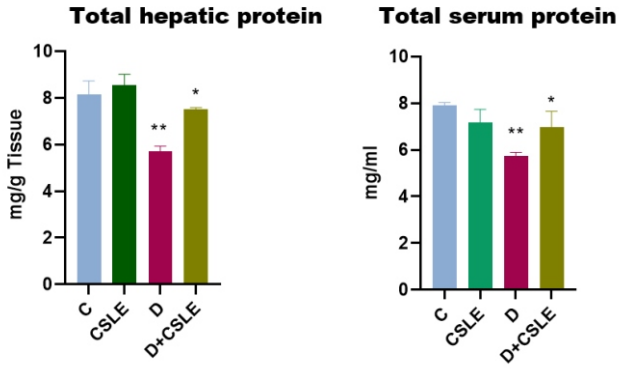

Fig. 6: Bar graph representing total protein evaluation of the liver and serum. Both liver and serum marked a profound decline in protein levels of group 'D' due to the toxicity. Data represented here means ± SEM (n=5), **p ≤ 0.01, and *p ≤ 0.05. Group 'D' was compared with the Control, whereas D+CSLE was compared with group 'D'.

Evaluation of the molecular weight of the protein profile

Polypeptides of molecular weights ~150 kD, ~100 kD, ~42 kD, ~35 kD, ~30 kD, ~18 kD, and ~ 13 kD were ubiquitously present in all the samples of 4 groups, but the band intensity (quantity) differs in each of the samples. However, the sample of the 'D' group (treated with DEN) marked a significant change in the band intensity of the polypeptide of the above-mentioned molecular weight. Further polypeptides of MW ~200 kD, ~25 kD, ~23 kD, and ~16 kD were detected in all groups except the 'D' group samples (Fig. 7).

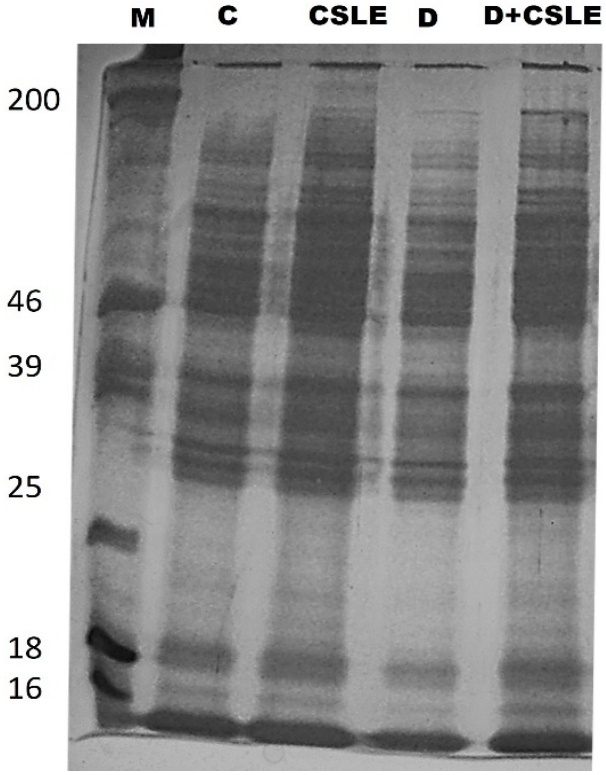

Fig. 7: SDS-PAGE (12%) profile of liver samples of animals belonging to different groups stained with silver. Where M = molecular weight marker, C = control, CSLE = *Camellia sinensis* leaf extract, D = Diethylnitrosamine, D+CSLE = Diethylnitrosamine + *Camellia sinensis* leaf extract. Molecular weights are presented in kD.

DISCUSSION

The results of this study clearly demonstrate that the aqueous extract of *Camellia sinensis* leaves (CSLE) possesses significant hepatoprotective property against diethylnitrosamine-induced liver injury in Wistar rats. The result corroborates previously published findings where DEN has been shown to generate potential toxicity in the liver, causing major damage to the organ morphology and anatomy resulting in liver dysfunction. The observations further document that DEN in the prescribed doses further exacerbates damage to blood cells and plasma proteins, which is in agreement with the documented reports (Mukherjee and Ahmad, 2015; Latief *et al.*, 2016; Hussain *et al.*, 2018).

The findings on total protein content in liver and serum are consistent with DEN-induced suppression

of hepatic protein synthesis, most probably the antioxidant proteins, albumin, and globulin, which constitute an important cellular milieu and may also serve as reliable biochemical marker of liver impairment. Literature shows that DEN causes hepatocellular lysis and damage to the endoplasmic reticulum, disrupting the machinery of protein synthesis and secretion, leading to both intracellular protein depletion and declined hepatic protein secretion into the bloodstream (Ha *et al.,* 2001).

Conversely, the parallel recovery in both liver tissue protein and serum total protein in the 'D+CSLE' group indicates that CSLE not only preserves intracellular protein content but also restores the liver's functional capacity to synthesize and secrete plasma proteins into the systemic circulation. This restoration is a hallmark of effective hepatoprotection, as also demonstrated by other hepatoprotective agents, such as silymarin, resveratrol, β-carotene and phytoextracts, which similarly restore total protein and albumin levels in DEN-intoxicated rats (Ramakrishnan *et al.,* 2006; Mukherjee and Ahmad, 2018).

The morphological and histological data clearly distinguished 'D' group animals from others, showing increased tissue stiffness, along with apparent rough surfaces and hemorrhage. On the contrary, the liver morphology in the other three groups, including the one supplemented with CSLE appeared relatively better without any nodules or hemorrhage (Latief *et al.,* 2016). Treatment of DEN leads to neutrophilic infiltration, hemorrhage, and significant collagen accumulation. These findings are consistent with the metabolic activation of NDEA via action of cytochrome P450 enzymes (specifically CYP2E1), which recruits biomolecules to initiateevent of oxidative stress (Brambilla *et al.,* 1987). The prominent blue-stained collagen near the portal vein is evident in the progression of fibrosis induced by diethylnitrosamine treatment. It has been investigated that the changes in the oxidative environment leads to the activation of hepatic stellate cells and Kupffer cells through the cytokine storm and other important proteins (TNF-α, TGF-β, IL-6, VEGF, and α-SMA) (Friedman, 2008; Ahmad and Ahmad, 2012).

However, a significant recovery in architectural changes was observed by CSLE supplement in animals belonging to 'D+CSLE' group. Anatomical anomalies were not noted in this group, which may be attributed to the antioxidant and anti-inflammatory properties of green tea polyphenols, particularly epigallocatechin-3-gallate (EGCG). It has been well documented that *Camellia sinensis* neutralizes superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide, in order to maintain cellular integrity (Tamura *et al.,* 1997; Badiee *et al.,* 2025).

The present findings of SDS-PAGE reveal critical insights into the patterns of protein expression and modification in polypeptides following DEN treatment and supplement by CSLE. The silver stained SDS-PAGE, as a highly sensitive technique, documented both quantitative and qualitative alterations in the liver protein profile (Nesterenko *et al.,* 1994). The presence of polypeptides of MW ~150 kD, ~100 kD, ~42 kD, ~35 kD, ~30 kD, ~18 kD, and ~13 kD in all four groups (C, CSLE, D, and D+CSLE) represents that these proteins are constitutive in the tissue and appears to be essential for normal hepatic function. On the contrary, the significant change in band intensity evaluated specifically in the 'D' group suggests DEN-induced protein dysregulation or protein degradation (Kurma *et al.,* 2021). The apparent absence of polypeptides with MW ~200 kD, ~25 kD, ~23 kD, and ~16 kD, possibly representing multimeric enzymes (critical for protein synthesis and tissue homeostasis), may have contributed to cellular stress response that exacerbates oxidative damage in the liver of DEN-treated animals (George and Chandrakasan, 2000). However, CSLE supplement significantly restores protein levels suggesting diminished tissue damage, improved liver function, thereby showing anti-proteolytic property of CSLE against DEN-induced liver injury in rats (Safer et *al.,* 2015).

In conclusion, present exploration suggests that CSLE preserves liver architecture against DEN-induced damage by maintaining balanced polypeptide level and reducing oxidative damage, as evident by the present histopathology and biochemical data. Further, CSLE effectively counters the detrimental effects produced by DEN in the liver of Wistar rats within the stipulated time of two weeks, clearly demonstrating hepatoprotective potential of CSLE.

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