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Assessment of DEN-Induced Albino Rats and Histopathological Changes in Hepatocellular Carcinoma Treated with *Lagerstroemia speciosa* Acetone Leaf Extract

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Abstract

Hepatocellular carcinoma (HCC) is a highly aggressive form of primary liver cancer with increasing global incidence, ranking as the fifth most common malignancy. Diethyl-nitrosamine (DEN), a widespread carcinogen found in various sources including cigarettes, gasoline, cosmetics, and certain foods like milk and meat, is implicated in its development. *Lagerstroemia speciosa*, known as India's pride, is the focal point of this research. The primary objective is to assess the histopathological effects of experimental plant extracts on DEN-induced liver tumours in albino rats. Phytochemical analysis of *Lagerstroemia speciosa* acetone leaf extract (LALE) confirms the presence of alkaloids, flavonoids, saponins, phenols, tannins, proteins and amino acids, reducing sugars, glycosides, phytosterols, and coumarins. The study included different experimental groups: Group I as the control, Group II exposed to DEN, Group III treated with a standard drug (STD) alongside DEN induction, and Groups IV and V treated with LALE at low (250 mg/kg body weight) and high doses (500 mg/kg body weight), respectively, daily for 16 weeks. Results indicated morphological changes and increased liver weight. Histological examination involved biopsy or necropsy tissue collection, fixation, section preparation, staining, and microscopic analysis. Histopathological evaluation of *L. speciosa* leaf extract in Group V, treated with the highest dose, showed minimal response compared to the STD group.

***Key words*:** Hepatocellular carcinoma, Diethyl-nitrosamine (DEN), *Lagerstroemia speciosa*, Biopsy

Hepatocellular carcinoma (HCC) is a highly aggressive primary liver cancer, now ranking as the fifth most common malignancy globally [1]. Diethyl nitrosamine (DEN), a known carcinogen found in various human products such as cigarettes, gasoline, cosmetics, milk, and meat [2-4], is particularly notable for its ability to induce liver tumours, including HCC, in animal research models. DEN is extensively used in laboratory settings to simulate conditions that lead to liver cancer, aiding in the study of disease progression and potential treatments. Due to its hazardous and carcinogenic nature, strict controls govern its use in research.

Natural compounds sourced from medicinal plants, such as terpenoids, phenolic acids, lignans, tannins, flavonoids, quinones, coumarins, and alkaloids, are known for their significant antioxidant properties and other beneficial effects in cancer therapy [5]. These compounds also exhibit anti-inflammatory, antitumor, antimutagenic, and anti-carcinogenic activities [6]. Derived from primary metabolites like steroids, prostaglandins, and sialic acids, these secondary metabolites play crucial roles not only in combating cancer by inhibiting, reversing, or preventing its progression but also in managing diverse conditions such as atherosclerosis, diabetes, obesity, cardiovascular diseases, neurodegenerative disorders, inflammatory conditions, and immune disorders [7-10]. *Lagerstroemia speciosa*, commonly known as queen's flower or crepe myrtle, is native to tropical and subtropical regions of Asia and belongs to the Lythraceae family has been widely studied for its pharmacologically active phytochemicals. These compounds, found in various parts of the plant, exhibit diverse biological properties such as antibacterial, antidiabetic, anti-inflammatory, hepatoprotective, and antioxidant effects. This plant has been studied for its pharmacologically active phytochemicals found in various parts, demonstrating antibacterial, antidiabetic, anti-inflammatory, hepatoprotective, and antioxidant properties [11-12]. This makes *Lagerstroemia speciosa* a valuable resource in exploring new therapeutic approaches, particularly in cancer research and the management of other chronic diseases.

Chemoprotection, which involves using natural foods and phytochemicals externally to enhance the body's natural defences against cancer development, is recognized as effective in mitigating cancer effects [13-15]. Managing hepatocellular carcinoma (HCC) involves complex tasks like accurate tumor classification through histopathological assessment. Early detection of HCC is challenging due to the absence of specific diagnostic markers and the often-asymptomatic nature of the tumor, leading to diagnosis at an advanced stage. Evaluation of both macroscopic (tumor size, nodularity, growth pattern in the liver) and microscopic features (differentiation grade, vascular invasion) is crucial for accurate diagnosis and predicting patient

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outcomes. The study is evaluating the medicinal value of the leaf extract and specifically examining its effects on DEN-induced albino rats, with a focus on histopathological changes related to hepatocellular carcinoma.

MATERIALS AND METHODS

Collection and authentication of experimental plant

The leaves of *Lagerstroemia speciosa* were collected from the PG Girls Hostel at Government Arts College (Autonomous) in Coimbatore District, Tamil Nadu, India. The plant was identified and verified as authentic by the Botanical Survey of India at Tamil Nadu Agricultural University, Coimbatore-03 (Identification No. BSI/ SRC/ 5/ 23/ 2020/ Tech/ 52).

Preparation of plant extracts

The selected plant leaves were meticulously cleaned prior to preparing the extract. They were then placed in a shaded area at room temperature (27±2°C) and allowed to dry completely over a period of approximately two weeks. Once dried, the leaves were ground into a fine powder using a blender. A quantity of 100 grams of the powdered material was immersed in 1000 milliliters of acetone in an airtight, wide-mouthed bottle and left to stand for four days with occasional shaking. Subsequently, the extract was filtered through Whatman No. 1 filter paper and allowed to dry in a plastic tray at room temperature [16].

Phytochemical qualitative analysis

The qualitative phytochemical analysis of the green leaf extracts of *Lagerstroemia speciosa* was performed using acetone, following the methodologies described by Harborne [17] and Trease and Evans [18].

Experimental animals

The study utilized male Wistar rats, aged 2 months and weighing between 100 and 150 grams. These rats were kept at a room temperature of 22°C on a 12-hour light/dark cycle within the animal facility. They were provided with a commercial pellet diet and water freely available throughout the study. All experimental procedures were carried out with approval from the Institutional Animal Ethics Committee (IAEC) of KMCH College of Pharmacy, Coimbatore (Approval No. KMCRET/ReRc/Ph.D./25/2021).

Experimental design

The experimental animals were divided into five groups for the study. Group I served as the control group. Groups II, III, IV, and V were subjected to induction with diethyl nitrosamine (DEN). Two weeks after DEN administration, Phenobarbital (Pb) at a concentration of 0.05% was added to the drinking water, available ad libitum, for up to 16 weeks to promote cancer development. Group III received 5-Fluorouracil (20 mg/kg), administered intraperitoneally twice a week for 28 days, and served as the standard (post-induction) group. Groups IV and V were treated with acetone leaf extracts of *L. speciosa* at low and high doses (250 mg/kg and 500 mg/kg body weight, respectively) daily for 16 weeks.

Assessment of body and liver weight

The rats from all experimental groups were weighed individually to monitor changes in total body weight. Throughout the study, we recorded the mortality rate, growth rate, and dietary intake on a daily basis. Additionally, tumor incidence and changes in liver weight were also monitored.

Histopathology study

Liver tissue samples were excised and rinsed in 0.1 M phosphate-buffered saline (PBS) with a pH of 7.4, followed by fixation in 4% paraformaldehyde (PFA) in PBS. The tissues were then dehydrated in a series of increasing concentrations from isopropanol to xylene. After dehydration, the liver tissues were embedded in melted paraffin wax and sectioned to a thickness of five microns. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Collection of tissue samples

Thin sections, approximately 3 to 5 mm in thickness, were collected from tissues exhibiting noticeable morbid changes, as well as from normal tissue for comparison.

Fixation

The tissue samples are immersed in a fixative for 24 to 48 hours at room temperature. This process hardens the tissues by coagulating cell proteins, prevents autolysis, preserves tissue structure, and reduces shrinkage. The fixative volume used should be ten times greater than the volume of the tissues. A common fixative employed is 10% formalin.

Haematoxylin and eosin staining method

Begin by deparaffinizing the tissue sections using xylene for 5 to 10 minutes, then remove the xylene with absolute alcohol and rinse in tap water. Stain the sections with hematoxylin for 3 to 4 minutes, followed by a tap water wash. Immerse the sections in tap water for 5 to 10 minutes, then wash again with tap water. Counterstain with 0.5% eosin until the sections attain a light pink colour (15 to 30 seconds), then rinse with tap water. Blot the sections and dehydrate them in alcohol. Clear the sections with xylene for 15 to 30 seconds, and mount them using Canada balsam or DPX mounting medium. Ensure the slide remains dry and eliminate any air bubbles.

RESULTS AND DISCUSSION

Result 1: *Qualitative phytochemical analysis*

The qualitative phytochemical analysis revealed the presence of various active compounds in the acetone extract of *Lagerstroemia speciosa* leaves (LALE). These compounds include alkaloids, flavonoids, saponins, phenols, tannins, proteins and amino acids, reducing sugars, glycosides, phytosterols, and coumarins.

Result 2: *Body and liver weight study*

Group II, which underwent induction, showed a significant increase in final body weight compared to the control group. Conversely, Groups III, IV, and V, which received treatments, exhibited a notable decrease in final body weight compared to Group II.

For liver weight changes:

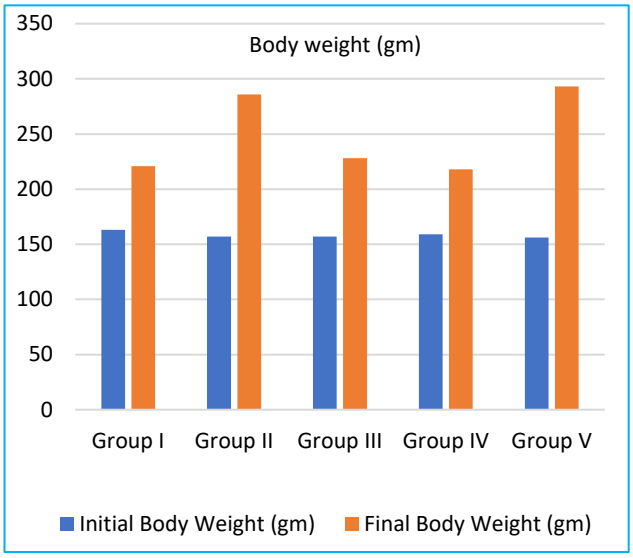
Control group showed a 4.13% increase in liver weight.

Group II (DEN + PB) had a significantly higher liver weight increase of 58.03% compared to the control group.

Group III (DEN + STD) experienced a liver weight increase of 14.85%, also higher than the control group.

Group IV (DEN + LALE low dose, 250 mg/kg) had a 32.30% increase in liver weight compared to the control.

Group V (DEN + LALE high dose, 500 mg/kg) showed a 56.20% increase in liver weight compared to the control.



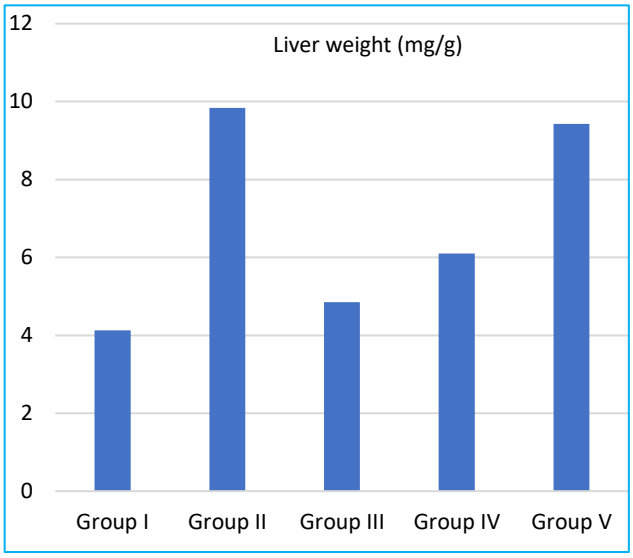


Fig 1 Change in the body weight in control and experimental rats and liver weight

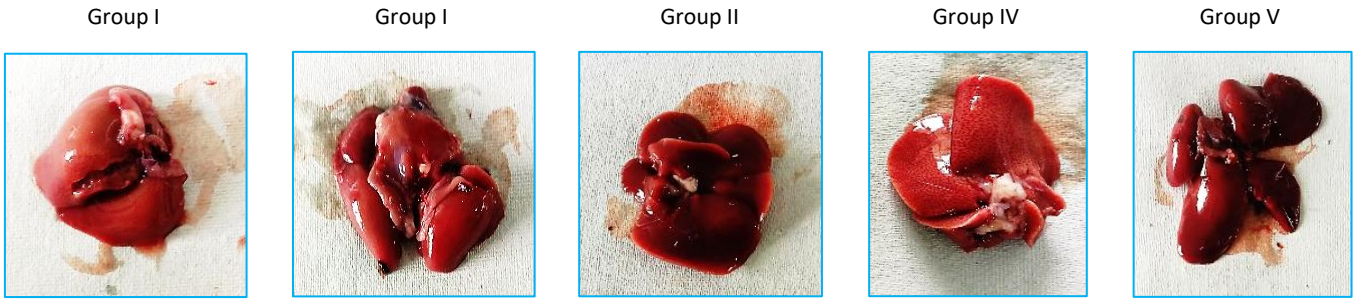


Fig 2 Liver Control Group I, DEN + Pb Group II, DEN + STD Group III, DEN + LALE Low dose (250 mg/ kg) And DEN + LALE High dose (500 mg/kg)

Result 3: *Histopathology study with microscopic appearance*

Control group: Examination of multiple sections from the control group revealed normal liver architecture, with hepatocytes arranged in organized cords and strands. The portal tracts and central vein appeared unremarkable.

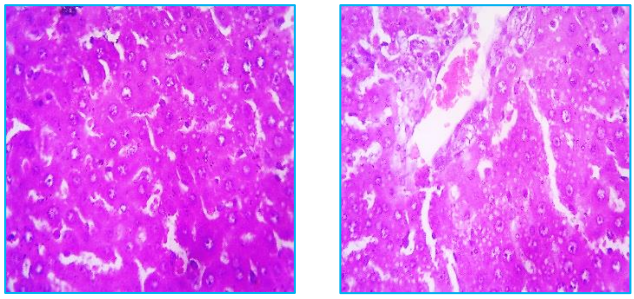
DEN group: Sections from the DEN-only group showed normal liver architecture with hepatocytes in cords and strands. However, there was noticeable lymphocytic infiltration around the portal tracts and sinusoids, and areas of fibrosis around the portal tracts. No zonal necrosis or individual hepatocytic necrosis was observed.

DEN + STD: Sections from Group III (DEN + STD) displayed normal liver architecture with hepatocytes arranged in cords and strands. Inflammatory cells were present around the portal tracts.

DEN + LD: Examination of sections from Group IV (DEN + LD) revealed hepatocytes arranged in cords and strands, with some individual hepatocytes showing ground-glass nuclei. Lymphocytic infiltration was observed around the portal tracts and sinusoids, along with minimal fibrosis around the portal tracts.

DEN + HD: Sections from Group V (DEN + HD) showed normal liver architecture, with hepatocytes in cords and strands. Inflammatory cells were observed around the portal tracts, similar to the DEN + STD group.

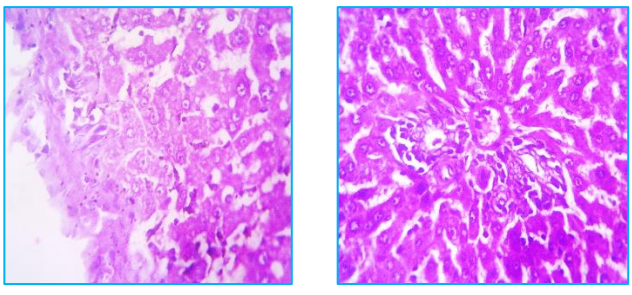
Control group



a) Normal Individual hepatocytes

b) Normal portal tract

DEN group



a) Lymphocytic infiltration and mild

b) Portal tract with inflammation and fibrosis fibrosis

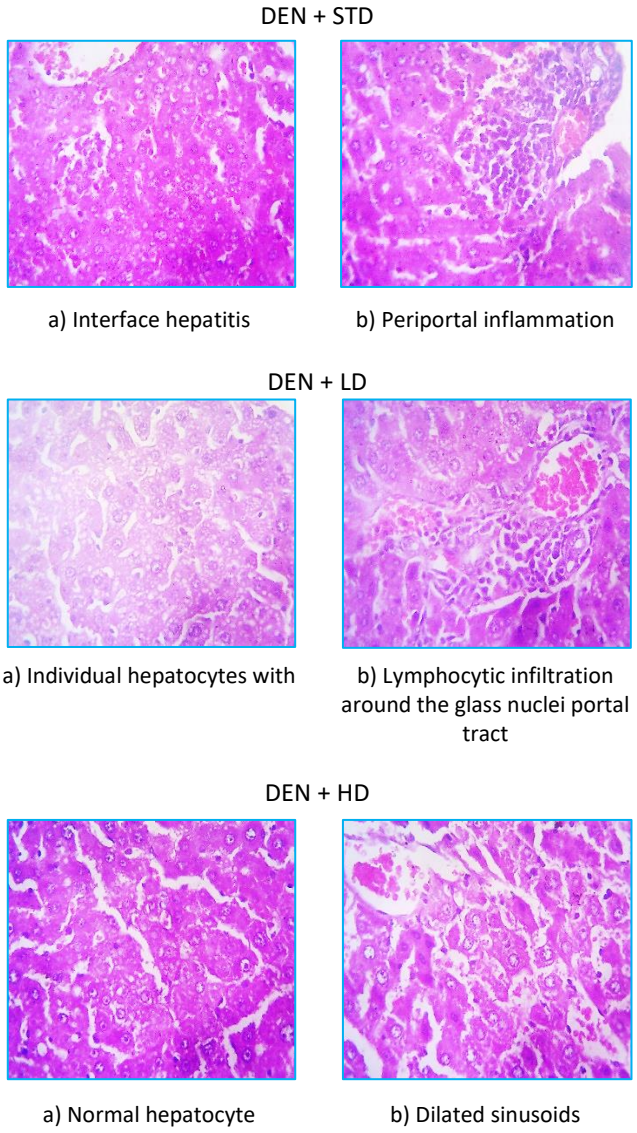


Fig 3 Histoarchitecture of Liver tissues in control and experimental animals stained with H&E (40x)

The qualitative phytochemical analysis of the acetone extract from *Lagerstroemia speciosa* leaves (LALE) revealed the presence of several bioactive compounds, including alkaloids, flavonoids, saponins, phenols, tannins, proteins and amino acids, reducing sugars, glycosides, phytosterols, and coumarins [19]. These findings are significant when considering the potential therapeutic effects of LALE in liver cancer treatment. Alkaloids are a diverse group of naturally occurring compounds known for their pharmacological activities. Flavonoids are a class of polyphenolic compounds with recognized antioxidant, anti-inflammatory, and anti-cancer properties [20]. In liver cancer, oxidative stress and inflammation play significant roles in disease progression.

The flavonoids in LALE could mitigate these processes through their ability to neutralize free radicals and inhibit pro-inflammatory pathways. Furthermore, flavonoids such as quercetin and catechins have shown promising anti-cancer effects, including the suppression of tumour growth and induction of cancer cell apoptosis, making them valuable components in the context of liver cancer. Saponins may help in reducing tumour burden. Phenolic compounds and tannins are well-documented for their antioxidant and anti-inflammatory properties [21]. Phenolic compounds and tannins are well-documented for their antioxidant and anti-inflammatory properties. The differential effects of the treatments on liver weight suggest that the LALE (low and high doses) have a notable impact on liver tumour development or liver damage.

The high dose of LALE (500 mg/kg) provides some degree of protection, as evidenced by a reduced liver weight increase compared to the DEN + PB group, though not completely reverting to control levels.

This indicates that while LALE shows promise as a therapeutic agent, further optimization of the dosage or combination with other treatments may be necessary to achieve more effective outcomes. The histopathological results underscore the effectiveness of high-dose LALE in maintaining normal liver architecture and reducing the extent of pathological changes compared to the DEN group. The preservation of liver architecture in the DEN + HD group suggests that high-dose LALE may offer substantial protective benefits, potentially mitigating some of the adverse effects of DEN-induced liver damage. In contrast, the low dose of LALE, while also beneficial, does not provide as comprehensive a protection, as evidenced by the presence of ground glass nuclei and ongoing inflammation. The standard treatment (STD) showed some efficacy in preserving liver structure but was less effective compared to the high-dose LALE.

CONCLUSION

The diverse array of phytochemicals identified in the acetone extract of *Lagerstroemia speciosa* leaves underscores its potential as a source of bioactive compounds with anti-cancer properties. Overall, these findings suggest that LALE, particularly at a high dose, could be a promising therapeutic agent in liver cancer treatment, offering protection against both inflammation and fibrosis while preserving liver architecture. Further studies are warranted to optimize dosing and explore the underlying mechanisms of LALE's protective effects. This research contributes to the understanding of potential therapeutic strategies for managing liver damage and cancer.

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