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Effect of *Lactuca sativa* on Oxidative Stress, Proinflammatory Cytokines in Carrageenan Induced Inflammation in Rats

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ABSTRACT

Objective: To evaluate the effect of aqueous leaf extract of *Lactuca sativa* (ALLS) on oxidative stress and inflammatory cytokines induced with carrageenan in Wistar albino rats. **Materials and Methods:** Carrageenan-induced paw edema was used to induce the inflammation. The paw thickness was measured at different time intervals. At the end of 5 hrs all the treatment group animals were sacrificed. The paw levels of SOD, CAT, GSH and MDA were estimated as a marker of oxidative stress. The pro-inflammatory markers such as TNF α, IL 6 and CRP levels were estimated in paw homogenates. Finally, histopathology of paw was conducted in all the treatment groups. The functional group analysis of *Lactuca sativa* was conducted by fourier transform infrared spectrophotometer (FTIR) and thermostability was observed by using Differential Scanning Calorimetry (DSC).**Results:** The ALLS showed significant dose dependent manner reduction of paw thickness in carrageenan-induced edema during 1,2,3,4 and 5 hrs. The ALLS showed significant ($p<0.05$) elevation of reduced oxidative stress markers (SOD, CAT, GSH) and reduction of MDA levels in paw homogenates in carrageenan-induced edema in rats. The ALLS showed subsequent reduction of TNF α, IL 6 and CRP in a dose dependent manner. The histopathological studies indicating ameliorated structure of paw tissue to normal in carrageenan-induced inflammation. **Conclusions:** The present study indicating that the anti-inflammatory activity of *Lactuca sativa* due to the effect on antioxidant enzymes and pro-inflammatory cytokines.

INTRODUCTION:

The inflammation is characterized by release of reactive oxygen species (ROS) from activated neutrophils and macrophages[1]. ROS interact with tissue proteins, carbohydrates, lipid and nucleic acids due to highly reactive in nature (unpaired electron). These ROS trigger the production of pro-inflammatory cytokinin (TNF α and IL 6) and chemokinin mediators (CRP). [2]Thus free radicals are important mediators stimulate inflammatory responses and these ROS are neutralized by antioxidants and radical scavengers like superoxide dismutase, catalase and reduced glutathione to the control inflammation.[3] Chronic inflammation is a pathological condition characterized by continued active inflammation response and tissue destruction.[4]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the first line drugs among all anti-inflammatory agents. Now a day, the usage of NSAID is common to treat mild and moderate types of pain. Because of NSAIDs significantly reduced the risk of gastrointestinal ulceration, however, increased rates of myocardial infarction, heart failure, hypertension and acute renal insufficiency remained.[5] Aside of the pharmacological response extensive use of anti-inflammatory agents leads to toxicity and untowards effects on liver, kidney and GIT upon long term usage. [6]To overcome the above problem researchers looking on plant based medicine with potent anti-inflammatory activity with lesser side effects.

The *Lactuca sativa* belongs to the family Asteraceae. The leaves of L. sativa reported having antioxidant activity[7], antimicrobial activity [8], anticancer activity [9], anticonvulsant activity[10], analgesic activity[11], antiviral [12]and cardioprotective acivity[13] (Nicolle C., 2004). All the activities might be due to the presence of carotene, Vitamin C and Vitamin E [14], triterpenoids, saponins and simple phenols [11], sesquiterpene lactones, lactucin, deoxylactucin and lactucopicrin [15]. Based on the literature, the study was designed to evaluate the effect of aqueous leaf extract of *Lactuca sativa* (ALLS) for antioxidant activity and anti-inflammatory activity in carrageenan induced inflammation in Wistar albino rats.

MATERIALS AND METHODS:

Plant Material

The lettuce (*Lactuca sativa*) were obtained from local market. The leaves were manually separated and shade dried. The leaves were powdered in a grinder to get 40-mesh size

powder. The moisture content of the dried powder was found to be 13.5%. The extract was prepared with methanol using Soxhlet apparatus. The extract was suspended in 2% gum acacia and used in the experimental studies.

Animals

Animals were obtained from the Tina laboratories, Hyderabad. Albino Wistar rats (180-200 g) of male were used in the present study. The animals were housed under standard environmental conditions (23±1ºC) with relative humidity of 50±10% and maintain 12:12 dark and light cycle, maintained with free access to water and *ad libitum* standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids (Hindustan liver Bangalore). After randomization before the experiment, the rats were acclimatized for a period of two weeks. The animal housing and handling were in accordance with CPSCEA guidelines.

Carrageenan-Induced Paw Edema

The carrageenan-induced rat paw edema was performed according to the previously described technique [16]. Briefly, carrageenan (1.5% w/v, 0.1 ml/paw) was injected into right hind paw at the plantar side. Rats were observed for abnormal behavior and physical condition after carrageenan injection. The ALLS (150 and 300mg/kg bd.wt) were administered orally 60 min prior to carrageenan administration. The paw edema was measured (cm) before and then at 1, 2, 3, 4, 5 h, after carrageenan injection. Indomethacin (10 mg/kg) was used as the reference drug. The weight of the left (non-carrageenan injected) and right (carrageenan injected) paw was measured at every time interval after carrageenan injection [17]. Difference between the left and the right paw weight was obtained for assessment of edema at all the time points. The percentage increase in paw weight was calculated by dividing edema, weight after 1st, 2nd, 3rd, 4th and 5th hours by weight of left paw multiplied by 100. Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Animals were sacrificed treatment period. Blood was collected, allowed to clot and serum was separated at 3500rpm for 15 min and used for assessment of different enzyme activities.

Biochemical Analysis of Anti-inflammatory Activity

The rats were anesthetized; the paws were dissected out and rinsed with ice cold saline. Each tissue was homogenized in a glass homogenizer with Teflon pestle in 2ml of ice cold

phosphate buffer (20mM, pH 7.4), centrifuged and the supernatant was used for the analysis. The amount of total protein and soluble protein present in the lens homogenate was estimated by the method of Lowry [18].

Estimation of Antioxidant Enzymes:

Superoxide dismutase (SOD) activity was determined by the inhibition of auto catalyzed adrenochrome formation in the presence of the homogenate at 480 nm [19].Catalase (CAT) activity was estimated by the catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 and recorded at 240 nm [20]. Reduced glutathione (GSH) activity was measured[21].

Estimation of Inflammation Markers:

The levels of TNF-α, IL-6and C-reactive Protein (CRP) in paw tissue homogenates were determined using commercially available ELISA kits in accordance with the manufacturer's instructions (eBioscience Inc., San Diego, CA, USA).

Histopathology:

Organ such as paw was dissected out from the rats and fixed in 10% neutral buffered formalin for 48 hours. The organs were then washed in running tap water. The organs were then trimmed and were processed in Yorco automatic tissue processor. The processed tissue sections were then embedded in paraffin wax using Leica embedding station. Three micron thick sections were prepared using Leica microtome. The sections were stained using routine haematoxylin and eosin technique. The stained sections were observed for any changes under light microscope (Max Erb light microscope).

Differential Scanning Calorimetry (DSC):

A Perkin Elmer DSC-6 calorimeter (Perkin Elmer Corp., Norwalk, CT, USA) was employed to study the oxidation stability of the samples according to the method described by Gortzi et al., 2008[22]. Samples (4 mg) were placed in DSC aluminum crucibles closed with lids perforated by a hole [internal diameter: 1 mm] in the center in order to allow the sample to be in contact with the oxygen stream. The purge gas foaming the reaction atmosphere was oxygen. An empty crucible, hermetically sealed, was used as reference. The starting temperature of oxidation was determined as the onset temperature of the oxidation peak. The

temperature program was: heat from 30 °C to 180 °C (100 °C/min), hold for 1 min at 180 °C and finally heat from 180 °C to 370 °C (10 °C/min).

Fourier Transform Infrared Spectrophotometer (FTIR):

Dried powder of APPG was used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FTIR spectroscopy (Shimadzu, IR Affinity 1, Japan), with a Scan range from 400 to 4000 cm 1 with a resolution of 4 cm.

Statistical Analysis:

All the data were expressed as mean±SEM. Statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison tests in comparison with disease control group.

RESULTS AND DISCUSSION:

Inflammation is the reaction of body to local injury and infection. Inflammation is a characterized by releases of various inflammatory mediators such as prostaglandins, leukotrienes and kinins of platelet activating factor, etc. Inflammation is a characteristic feature in many degenerative diseases such as rheumatoid arthritis, heart disease, asthma, cancer, and inflammatory bowel disease[23]. The treatment for inflammatory disorders become important nowadays, the drug of choice is nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. These drugs exert their anti-inflammatory activities through inhibition of the biosynthesis of prostaglandins and some pro-inflammatory cytokines [24].

`Acute inflammation is a process that involved the generation of excessive free radical production, activation enzymes involved in inflammation and release of inflammatory and pro-inflammatory cytokines. The standard and widely used acute model of inflammation to screening novel anti-inflammatory compounds is carrageenan-induced paw edema. It induced inflammation by two phases. During this early phase is characterized by release of histamine, serotonin, bradykinin, and prostaglandins around at 1 hour and the delayed phase (after 1 h) is characterized as release of neutrophil derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), and interleukin-1 β (IL-1 β) [25]. Carrageenan injection also provokes the release of some important pro-inflammatory

cytokines during early and delayed phases of inflammation[26].Hence, the present study was carried in carrageenan-induced paw edema to evaluate the anti-inflammatory activity. The ALLS and Indomethacin showed significant reduction in thickness of edema during both first phase and second phase of inflammation (fig 1).

Oxidants or free radicals are playing a significant role in the pathogenesis of a number of chronic disorders such as inflammation. Oxidative stress is defined as an imbalance between cellular production of ROS and antioxidant defense mechanisms. ROS (e.g., superoxide radical, peroxynitric, hydroxyl radical, and hydrogen peroxide) are key signaling molecules in the progression of inflammatory disorders. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and nonenzymatic antioxidants as reduced glutathione (GSH) showed protective mechanism over oxidative stress induced inflammation. Among all the GSH is plays a major role in coordinating the body's antioxidant defense process [27]. Inflammation leads to the reduced levels of GSH and promoted lipid peroxidation (MDA) in paw tissues. Excessive peroxidation causes increased GSH consumption. Recently, several studies showed that lack of antioxidant systems can cause many inflammatory diseases [28]. Carrageenan induced rats showed reduction of paw levels of SOD, CAT and GSH and elevation of MDA. But the treatment with ALLS and Indomethacin showed significant ($p<0.05$) elevation of SOD, CAT and GSH and significant ($p<0.05$) reduction in MDA levels in carrageenan-induced edema in paw homogenates (table 1). However, it showed that various roles of enzymatic and nonenzymatic antioxidants help to protect organisms from excessive generation of ROS in the inflammatory states.

Increased pain sensitivity is a common feature of the inflammatory response and occurs after tissue injury. The peripheral sensitization is triggered by NF-κB-related pro-inflammatory mediators, including the cytokines TNF-α and IL-6[29], several pathways involved in inflammation of paw might be due to stimulation m of proinflammatory chemokines which are responsible for the infiltration and activation of various leukocyte population in joint tissue. The ALLS showed marked reduction in cytokine levels in paw homogenates in carrageenan-induced rats compared with edema group (figure 2 and 3).And the proinflammatory cytokines TNF-alpha and IL-6 are the central mediators for the regulation of several biomarkers such as C reactive protein (CRP) during acute phase response in carrageenan-induced edema[30]. The ALLS treatment showed reduction in CRP levels in Carrageenan induced edema (figure 4). The results obtained from the present study indicate

the ALLS showed potent antioxidant and anti-inflammatory agent by acting on several mechanisms. Normal rats show the normal appearance of epidermis and dermis without any lesion. Carrageenan induced rat showed heavy Polymorphonuclear infiltration (PMN) and a spongy-like appearance and bulla in the epidermis. Treatment with Indomethacin showed significant reduction in the migration of PMN and oedematasis in dermis without any spongy-like feature and bulla. The ALLS showed moderate reduction in PMN and near to normal appearance of dermis (figure 7). The ALLS showed marker regeneration of Polymorphonuclear infiltration induced with carrageenan.

All the activities might be due to the presence of active phytoconstituents of ALLS. Further, it can be supported by the FTIR analysis of ALLS for the presence of functional groups present in it. The FTIR analysis reveals that ALLS contains alkanes, alkenes and amines as shown in figure 5. In general thermal behavior of the extracts was studied using DSC. Thermogram of ALLS exhibited endothermic peak starts around at 126.0^{o}C (Figure 6).

Table 1: Effect of ALLS on tissue parameters in carrageenan-induced paw edema in rats.

	Protein	**SOD**	**CAT**	**GSH**	**MDA**
Control	44.59±0.99	3.11±0.10	1.34±0.03	18.85±0.96	1.39±0.16
Carrageenan	32.40±0.68	1.02±0.05	0.21±0.01	11.48±0.32	13.59±0.22
Indomethacin	45.24±0.93	2.97±0.05	1.17±0.02	17.31±0.46	3.24±0.15
ALLS (150mg/kg)	38.32±0.80	2.28±0.05	0.67±0.03	14.58±0.22	5.18±0.04
ALLS (300mg/kg)	39.28±0.47	2.30±0.05	0.83±0.16	15.47±0.25	4.38±0.06

Protein was expressed as mg, SOD, CAT were expressed as IU/min/mg of protein, GSH expressed as ηmol/mg of protein, MDA was expressed as n moles of MDA/mg of protein $P<0.05^{*}$ significance followed by one way ANOVA followed by DUNNETT's multiple comparison tests when compared with toxicant group.

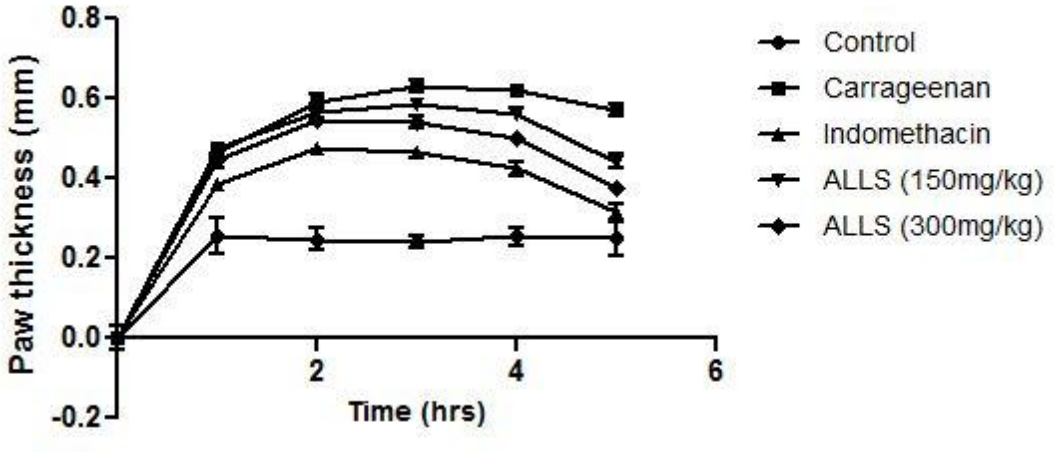

Figure 1: Effect of ALLS on carrageenan-induced paw edema in Wistar albino rats.

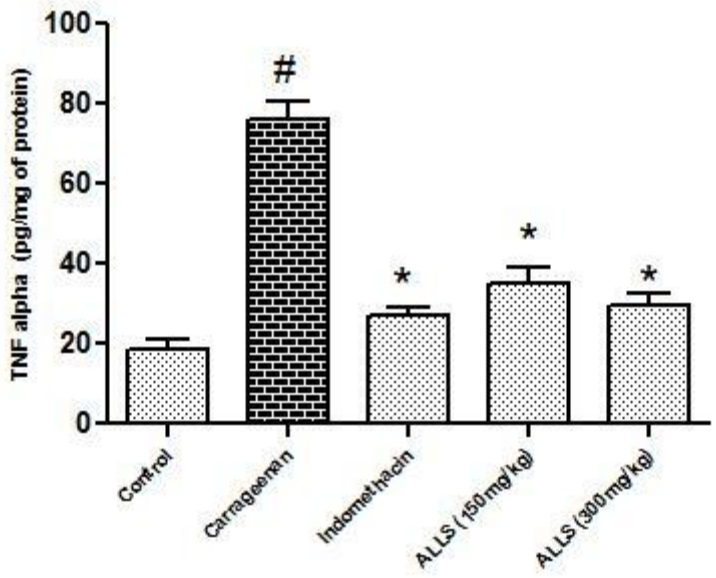

Figure 2: Effect of ALLS on TNF alpha in carrageenan-induced paw edema, Data were expressed as mean±SEM. one way ANOVA followed by Dunnet's multiple comparison tests *p<0.001 in comparison with disease control group, #p<0.01 compared with control group.

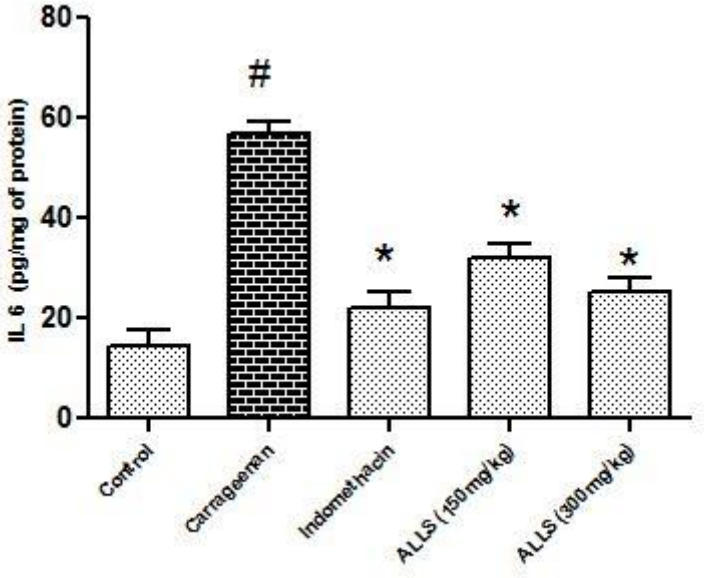

Figure 3: Effect of ALLS on IL 6 in carrageenan-induced paw edema, Data were expressed as mean±SEM. one way ANOVA followed by Dunnet's multiple comparison tests *p<0.001 in comparison with disease control group, #p<0.01 comparison with control group.

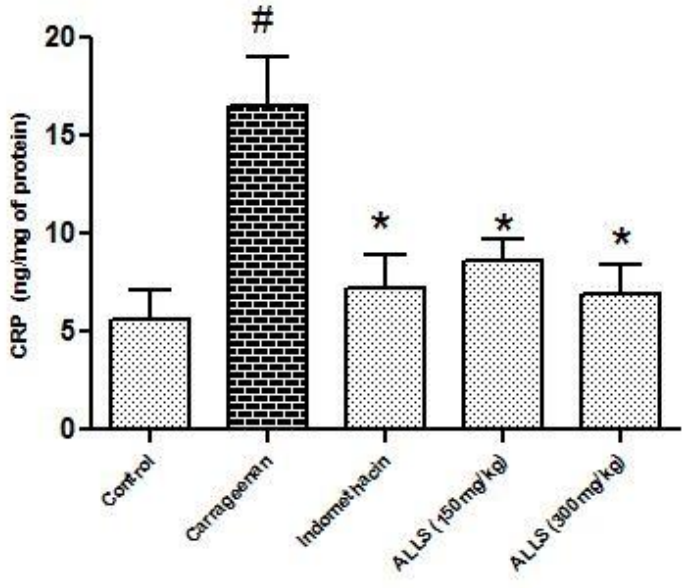

Figure 4: Effect of ALLS on CRP in carrageenan-induced paw edema, Data were expressed as mean±SEM. one way ANOVA followed by Dunnet's multiple comparison tests *p<0.001 in comparison with disease control group, #p<0.01 compared with control group.

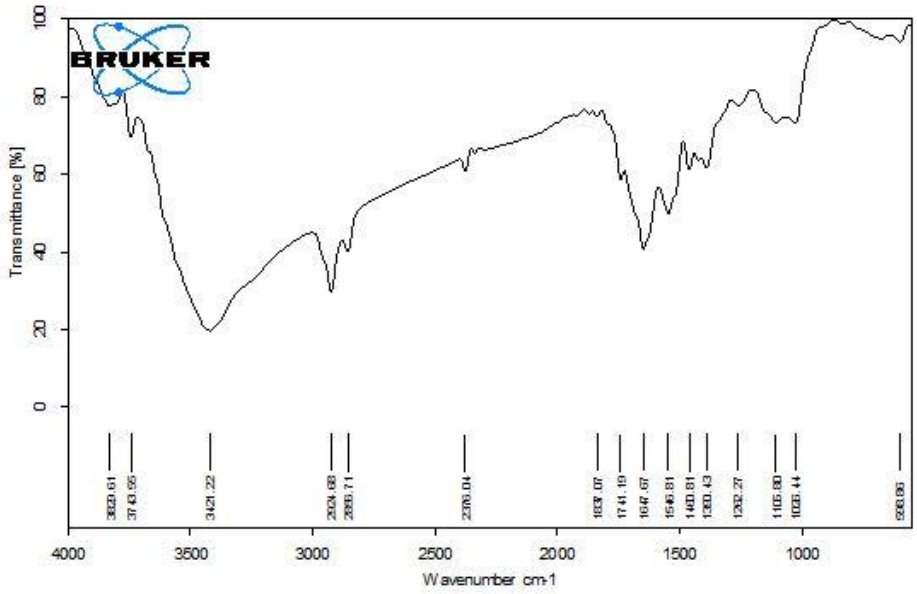

Figure 5: FTIR of *Lactuca sativa*

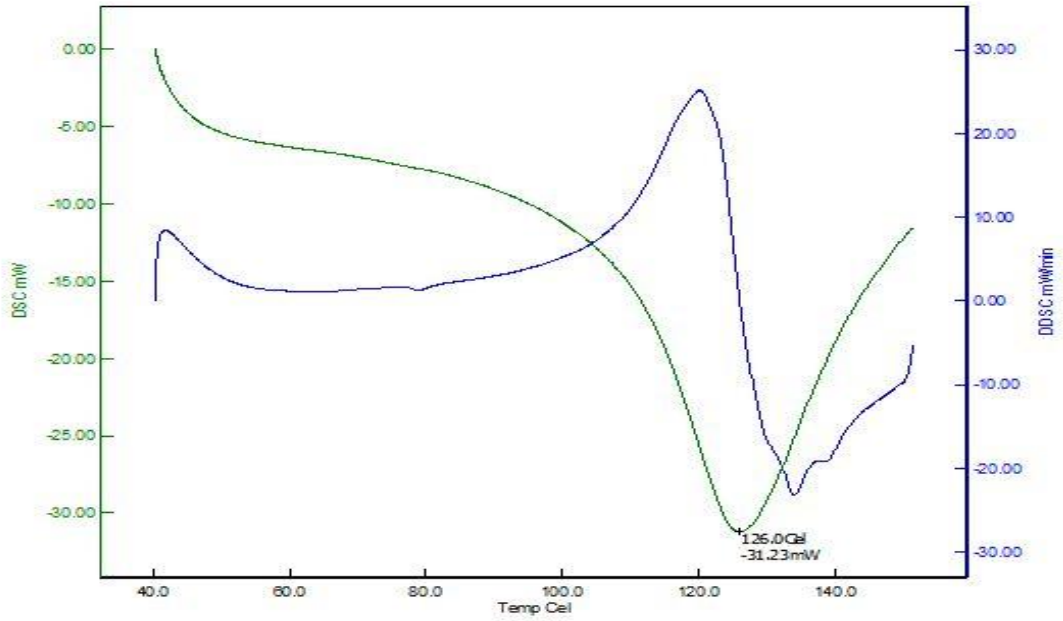

Figure 6: DSC of *Lactuca sativa*

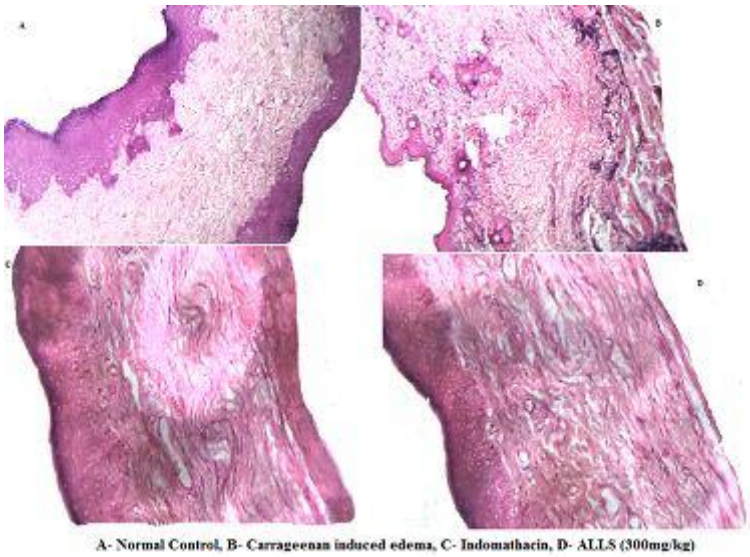

Figure 7: Effect of ALLS on histopathology of paw

CONCLUSION:

The present study indicates that the aqueous leaf extract of *Lactuca sativa* showed to have effect on *In vivo* antioxidant enzymes, proinflammatory cytokines involved in both acute and delayed phases of inflammation and amelioration of damaged structure of carrageenan-induced paw edema. The activities of *Lactuca sativa* might be due to the presence of phenols and terpenoids in leaves.

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