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Anti-oxidant and hepatoprotective effects of *Lactuca serriola* and its phytochemical screening by HPLC and FTIR analysis

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Abstract:*Lactucaserriola*(*L. serriola*), commonly known asprickly lettuce, is an annual and biennial herb. The plant is traditionally used as sedative, hypnotic, cough suppressant, expectorant, purgative, diuretic, vasorelaxant, demulcent, antiseptic and antispasmodic. Aim of present study was to evaluate protective effect of *L. serriola* in paracetamol-induced hepatotoxicity. Silymarin was used as a standard drug. Hepatoprotective effect of extract was determined by liver biochemical markers, anti-oxidant enzymes and blood lipid profile. Methanolic extract was found to be the most potent in *in-vitro* antioxidant assays and used for further studies. Treatment with paracetamol increased the level of hepatic biomarkers, blood lipid profile and decreased anti-oxidant enzyme levels. Pre-treatment with *Lactuca serriola* caused restoration of hepatic biomarkers, blood lipid profile and antioxidant enzymes. FTIR represented that *L. serriola* contains hydroxyl (-OH), carboxylic acid (C=O) and alkene (C=C) groups. HPLC analysis showed presence of polyphenolic compounds in the methanolic extract of *L. serriola*. It is concluded that the methanolic extract of *Lactuca serriola* possesses hepatoprotective potential that might be linked to the presence of polyphenolic compounds and their anti-oxidant activities.

Keywords: Antioxidant, hepatoprotective, *Lactuca serriola*, paracetamol.

INTRODUCTION

Metabolism of lipids, proteins and carbohydrates occurs in liver that is continuously exposed to xenobiotic and environmental pollutants. When the protective defence mechanisms of liver fail to cope with these chemicals, it will cause hepatic injury. A number of drugs are available to treat liver problems, however, these often fail to treat and have serious side effects(Robinson and Zhang, 2011; Mushtaq *et al*., 2016). Now a days, herbal drugs are being preferred for protection and treatment of liver disorders (Wangkheirakpam, 2018). It is need of time that the traditional and herbal approach must be established on the modern scientific perspectives to further search for new drugs (Gilani, 2005; Mushtaq and Akhtar, 2015).

*Lactuca serriola*L. (*L. serriola*) belongs to Compositae family, is known for its medicinal significance(Dogan *et al*., 2004).*L. serriola* is commonly known prickly lettuce, jagged lettuce, kahu and khas. *L. Serriola*is locally found in temperate zone of Europe and Asia. The plant is an annual and biennial herb with the light green, glabrous, round, erect and prickly stems. Its leaves are alternate and pinnately lobed with few dentate margins. Major compounds isolated from *L. Serriola* are lupeol, lupeol acetate, germinol, alpha amyrin, beta amyrin and other oleanans(Mohammad, 2013,Elsharkawy and Alshathly, 2013; El-Esawi *et al*., 2017).The phytochemical screening showed the presence of lactucone, lactucin,lactucic acids, vitamins, beta-carotene, iron, alkaloids, oxalic acid, lactucopicrin, sesquiterpene esters, and phenolic contents. The plant is traditionally used as sedative, hypnotic, cough suppressant, expectorant, diuretic, vaso-relaxant, antispasmodic, antiseptic, demulcent and purgative (El-Esawi *et al*., 2017). The present study aimed to investigate hepato-protective activity of *L. serriola* using paracetamol-induced hepatotoxic rat model. FTIR and HPLC analysis has also been carried out for phytochemical screening of the plant.

MATERIALS AND METHODS

Collection of plant

The plant was collected from district Kasur of Punjab, Pakistan in March, 2017. Specimen was confirmed by Prof. Dr. Zaheer-ud-Din, Department of Botany, Govt. College University,Lahore (voucher number; Bot. Herb # 2969).

Preparation of extract

The shoots and leaves of the plant were washed and shade dried for 45 days. *L. serriola* dried parts were ground into the coarse powder and kept in a well closed glass bottle. First of all, the dried coarse powder of the plant was extracted by maceration for three days and solvents used were in order of increasing polarity (petroleum ether,

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chloroform and methanol). The same process was repeated at least three times. After filtration filtrate was dried, using rotary evaporator followed by placement in the incubator and stored in well closed cellophane bags.

Antioxidant activity

Anti-oxidant tests such as DPPH scavenging and hydrogen peroxide assays for each extract were performed to estimate the antioxidant activity and the most active extract was selected for *in vivo* studies.

DPPH scavenging assay

DPPH (0.5mM) was added in test tubes containing 1 ml of three extracts and standard separately. After that, 2 ml of the methanol was addedin each test tube. By adding 1 mL of DPPH and 3 ml of methanol control sample was prepared. All test tube samples were incubated at room temperature for 30 min and absorbance was taken at 515 nm. L-ascorbic acid and quercetin were used as the standard antioxidant. The control was prepared by adding 50 μL deionized water to 950μL 100μM DPPH reagent and the analysis was followed as described above. Antioxidant activities(AA) of the three extracts and reference control were measured by using following formula(Kamali *et al*., 2016):

$$\% AA = \frac{\text{Absorption (Control)} - \text{Absorption (sample)}}{\text{Absorption (Control)}} \times 100$$

Hydrogen peroxide scavenging assay

Sample solution (5 ml) and the reference control (ascorbic acid and quercetin) were mixed with 0.6 ml of the hydrogen peroxide solution and absorbance was calculated at 230 nm against a solution of blank. Activity percentage was calculated by using the following formula:

$$\% \text{Activity} = \frac{\text{Absorption (Control)} - \text{Absorption (sample)}}{\text{Absorption (Control)}} \times 100$$

Animals used

Male Wistar albino rats having weight 150-200 g were used. All rats were maintained at a temperature of 22 ± 2°C with a 12 hours light/dark cycle and were given pellet diet with water ad libitum. In the new environment of the laboratory, the animals were acclimatized for at least 6 to 7 days before the start of experiment. The experiments were started after the approval of experimental protocols by the institutional animal ethical committee, University of Sargodha (UOS/ORIC/1441).

Study design

This experiment was performed according to the previously described method with slight modifications (Mahmood *et al*., 2014). Rats randomly divided into six groups and each group containing of six animals, were kept in separate plastic cages. Drugs/extracts were administered orally. Group 1 (Control) received single dose of distilled water for nine days. Group II (Diseased control); received distilled water in single dose for nine days and also one dose of paracetamol on 8th day (2.5 g/Kg). Group III (Standard drug treated group); received silymarin (100mg/Kg) for all nine days and on day 8 paracetamol (2.5 g/Kg) two hours after silymarin was administered. Group IV, V and VI (Extract treated-LS-100, LS-250, LS-500); received methanolic extract of *L. serriola* (100, 250 and 500mg/kg respectively) for nine days plus single dose of paracetamol (2.5g/Kg) on day eight, 2 hours after administration of *L. serriola* extract (Asif *et al*., 2020).

Blood sample collection and analysis

The animals were anesthetized by chloroform and with the help of needle of disposable syringe, cardiac puncture was made to withdraw the blood (5ml) on 10thday of the experiment. At 4ºC blood was allowed to clot for12 hours, then it was centrifuged at 3000 rpm for 15 min. Collected serum was used for estimation of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total proteins, albumin, bilirubin, triglycerides (TG), cholesterol, low-density lipoproteins (LDL), high density lipoproteins (HDL), glutathione reductase (GR), superoxide dismutase (SOD), malondialdehyde (MDA) and catalase. SOD, GR, MDA and catalase (CAT) were determined by enzyme linked immunosorbent assay (ELISA) using automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA).

Histopathological study

Livers from animals were preserved in 10% formalin. Slides were made by cutting tissues in sections of 4-5µm thickness and were stained with dye hematoxylin and eosin. These slides were examined under light microscope.

Fourier transform infra-red (FTIR) analysis

FTIR spectrometer of Nicolet iS5, iD3 ATR (Thermo Scientific, USA) was used during spectra acquisition equipped with a detector and beam splitter (DTGS KBr). Software of the OMNIC (version 8.2 Thermo Nicolet iS5) operating system was used for spectrum. Sample was placed on attenuated total reflectance (ATR) crystal and spectrum was obtained at 4,000-600 cm-1 frequency having 32 scans and at 4 cm-1 resolutions. Spectra were displayed as absorbance value at least in three measurements.

HPLC analysis

Chemicals and Reagents

All chemicals and solvents used were of analytical grade such as methanol and acetonitrile (RCI Labscan Ltd., Thailand), tetrahydrofuran and acetic acid (E-Merk, Germany), sodium acetate trihydrate (BDH Laboratory, England), chlorogenic acid (China), caffeic acid and

ferulic acid (Sigma Aldrich). Sterlitech nylon syringe filters (0.45µm) were used to filter the samples.

Instrument and Chromatographic conditions

HPLC system of Agilent Technologies, 1200 series (Waldronn, Germany) equipped with iso-pump (G1310 A), auto-sampler (G1329 A), column oven (G1316 A), diode array detector (G1315B) was used for phenolic compounds determination. A reversed phase HPLC method was used for the simultaneous determination of three phenolic compounds (chlorogenic acid, caffeic acid and ferulic acid). 20µl of each samples and mix standard solutions were eluted at 0.8ml flow rate through a column (Agilent 5 TC-C18 250x4.6 mm), maintained at 30˚C with mobile phase comprising acetate buffer, methanol, acetonitrile, tetrahydrofuran (65: 20: 10: 5, v/v/v/v). The detection wavelength was 300 nm (reference beam 360nm). Data acquisition was performed by LC/LC-MS software for Windows, attached with HP laser jet P2015 printer.

Preparation of acetate buffer (pH 3.6)

Solution A: Preparation of 0.2M Acetic acid

11.55 ml of glacial acetic acid was added to a volumetric flask (1 L) and made up volume with HPLC grade water.

Solution B: Preparation of 0.2M sodium acetate

27.2 g of sodium acetate was dissolved in 1000 mL of distilled water.The acetate buffer was then prepared by mixing 46.3 ml of solution A and 3.7 ml solution B and made the volume 100 ml. The pH was adjusted to 3.6 by adding 1 M solution of HCl drop wise.

Preparation of mobile phase

Mobile phase was prepared by mixing acetate buffer: methanol: Acetonitrile and tetrahydrofuran. (65: 20: 10: 5, V/V/V/V). The mobile phase was filtered by filtration assembly and degassed by sonication for 20 min.

Preparation of standard solutions

Standard stock solutions

Stock solutions of chlorogenic, caffeic and ferulic acids having concentration 1mg/ml were prepared in mobile phase separately.

Preparation of sample solution

75 g powder of plant was extracted with 3 litres of methanol. 4 mg of this dried extract was mixed with 1 ml of mobile phase to prepare the sample solution and filtered through 0.45µm syringe filters.

STATISTICAL ANALYSIS

The data were analyzed by one-way ANOVA followed by Bonferroni test whereas $P<0.05$ was considered as statistically significant. The GraphPad Prism (version 8.0.2) software has been used for data analysis.

RESULTS

Free radical scavenging ability of L. serriola

Free radical scavenging ability of different extracts of *L. Serriola* was determined by DPPH and hydrogen peroxide assay. The methanolic extract was found to be more potent as shown in table 1.

Effect of L. serriola on liver profile

Treatment with paracetamol increased ALT, AST and ALP levels as compared to control group. However, treatment with the methanolic extract of *L. Serriola* significantly decreased paracetamol-induced rise of serum enzymes at dose of 100mg/kg but at higher dose the result was unexpectedly different and serum enzyme level was higher as compared to 100mg/kg. Paracetamol-induced hepatotoxicity model showed reduction in plasma proteins levels while the methanolic extract of different doses of *L. serriola* increased plasma proteins levels (table 2).

Effect of L. serriola on blood lipid profile in paracetamol induced toxicity

Treatment with paracetamol increased cholesterol, TG and LDL levels whereas decreased the HDL level as compared to control group. However, treatment with the methanolic extract of *L. serriola* (LS-100, LS-250 and LS-500mg/Kg) significantly decreased paracetamol-induced rise in TC, TG and LDL and increased HDL levels as shown in table 3.

Effect of L. Serriola on oxidative stress

The methanolic extract of *L. serriola* showed increase in SOD, GR and catalase levels but reduced MDA level as compared with diseased control group. For GR and catalase, results of all the plant groups were more significant than the standard drug (table 4).

Histopathological study

Histopathological studies have been performed for Control, Diseased control and *L. serriola* treated group of rats. The liver of rats from control group revealed that hepatocytes, portal triads and vasculature appeared normal. The liver from diseased control group showed massive necrosis, haemorrhage and inflammation characterized by lymphocytes infiltration as shown in fig. 1. However, *L. serriola* treated (100, 250 and 500 mg/Kg) group of rats showed reversal of paracetamol intoxications

FTIR

The spectrum of *L. serriola* powder showed a characteristic peak at 3333 cm-1 indicating the presence of hydroxyl (-OH) group which depicts the presence of phenols, alcohols in form of carboxylic acids in the plant. The band from 2400-3400 cm-1 is attributed to the hydroxyl bond stretching vibration (Eibagi *et al*., 2020). Similarly, peak 2360 cm-1 range is near to 2400 so might be OH group. The peak 1726 cm-1, 1615, 1397 are

Table 1: Free radical scavenging ability of *Lactuca serriola*

Treatments	DPPH assay (% inhibition)	Hydrogen peroxide scavenging assay (% inhibition)
Petroleum ether extract	93.45±0.56	66.51±1.02
Chloroform extract	84.33±0.42	68.30±0.95
Methanol extract	97.15±0.87	69.86±0.74
Ascorbic acid	98.07± 1.03	88.60± 0.89
Quercetin	96.26± 0.55	84.93± 0.87

Values are expressed as Mean ± SEM of three replicate determinations (n = 3)

Table 2: Effect of *L. serriola* on liver biomarkers and plasma proteins

Parameters	Control	Diseased control	Standard drug treated group	Extract treated (LS-100)	Extract treated (LS-250)	Extract treated (LS-500)
ALT	40.67±4.39	195.67 ±33.0	90.67 ±18.40**	86.00 ±8.41**	107.67±16.54*	104.00 ±15.98*
AST	39.17 ±5.93	210.83 ±27.8	96.83±19.58***	82.50 ±8.35***	86.83±9.67***	117.00 ±16.24**
ALP	213.33±12.60	297.17 ±18.2	200.00 ±6.34**	190.83 ±28.30**	200.33 ±26.91**	194.33 ±2.04**
Albumin	3.93±0.10	3.17 ±0.05	3.77 ±0.08###	3.82 ±0.12###	3.85 ±0.08###	3.82 ±0.05###
Bilirubin	0.50 ±0.04	0.73 ±0.04	0.45 ±0.04**	0.55 ±0.04*	0.48 ±0.04*	0.57 ±0.07*
Total Protein	6.37±0.15	5.28 ±0.11	6.02 ±0.08###	5.95 ±0.09##	6.055 ±0.08###	5.98 ±0.05###

Values are mean ± SEM (n = 6). Where * = P<0.05, ** = P<0.01, *** = P<0.001 significant decrease; # = P>0.05, ## = P<0.01 and ## = P< 0.01 significant increase compared to disease control (LS; *Lactuca serriola*; ALT: amino transferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase)

Table 3: Effect of *L. serriola* on blood lipid profile

Parameters	Control	Diseased control	Standard drug treated group	Extract treated (LS-100)	Extract treated (LS-250)	Extract treated (LS-500)
TC	71.00±4.58	105.50 ±5.31	74.83 ±7.93**	72.50 ±3.97**	69.50 ±3.91**	67.83 ±5.22***
TG	62.83±7.44	91.00 ±4.24	59.50 ±2.95**	59.00 ±6.02**	55.33 ±3.84**	64.67 ±6.92*
HDL	16.67±0.84	12.17 ±0.79	16.83 ±0.65##	17.50 ±0.99##	19.83 ±1.17###	18.67 ±0.71###
LDL	52.33±6.85	72.17 ±3.22	59.17 ±0.95*	54.67 ±4.39*	52.17 ±3.00*	51.33 ±3.34*

Values are mean ± SEM (n = 6). Where *= P<0.05, ** = P<0.01, *** = P<0.001 significant decrease; ## = P<0.01 and ## = P<0.01 significant increase as compared to disease control (LS: *Lactuca serriola;* TC: total cholesterol; TG: triglycerides; HDL; high density lipoproteins; LDL: low density lipoproteins)

Table 4: Effect of *L. serriola* on oxidative stress

Parameters	Normal control	Disease control	Standard drug treated group	Extract treated (LS-100)	Extract treated (LS-250)	Extract treated (LS-500)
SOD	106.37±6.44	70.94 ±2.99	109.71±7.78#	122.63 ±7.26##	142.96±13.36###	96.57±5.33#
GR	201.70±7.45	151.56 ±3.45	194.59±2.96#	195.99 ±5.58#	221.52±11.93###	225.76±18.02###
MDA	1.38±0.06	2.54 ±0.19	1.42±0.03***	1.89 ±0.03**	1.50±0.07***	1.57±0.11***
CATALASE	4.99 ±0.17	3.72 ±0.06	4.19±0.03#	5.13 ±0.07###	4.68±0.09###	4.51±0.11###

Values are mean ± SEM (n = 6). Where * = P< 0.05, ** = P< 0.01, *** = P< 0.001 significant decrease; ## = P< 0.01 and ## = P< 0.01 significant increase compared to Diseased control (LS: *Lactuca serriola;* SOD: superoxide dismutase; GR: glutathione reductase; MDA: malondialdehyde)

indicating C=O Carboxylic acid, and C=C alkene respectively as peaks range is (1725-1700) for carboxylic acid, 1680-1600 (C=C) and 1400-1000 (C-X). The peak at 1055.00 represent C-O group because of the range of this group (1300-1000).

HPLC analysis

HPLC analysis for phenolic compounds present in the methanolic extract of *L. serriola* has been performed. HPLC analysis showed the presence of polyphenolic compounds such as chlorogenic acid and caffeic acid but ferulic acid was not detected (figs. 3 and 4).

DISCUSSION

One of the important causes of liver dysfunction is oxidative stress that is pathological basis of hepatic cell injury (Cichoż-Lach and Michalak, 2014). It has been reported previously that oxidative stress leads to lipid peroxidation, inflammation, abnormal function of organelles and DNA damage in cells (Mello *et al*., 2016).

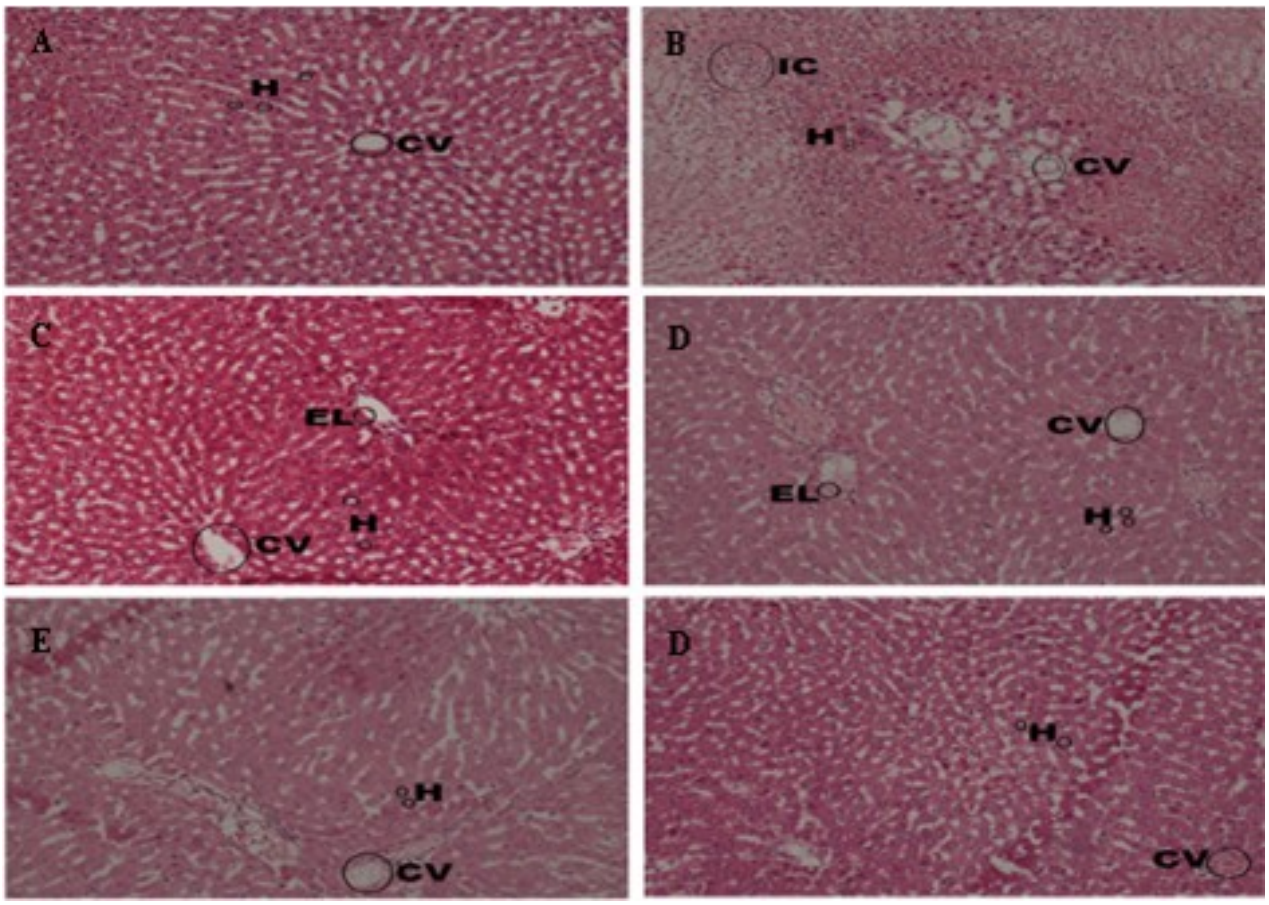

Fig. 1: Histopathological study of livers from control (A), Diseased control (B), standard drug treated group (silymarin) (C) and *L. serriola* extract (100, 250 and 500mg/Kg) treated group of rats (D, E and F respectively) (n; 6) (H; hepatocytes, CV = Central Vein, EL; epithelial lining cell)

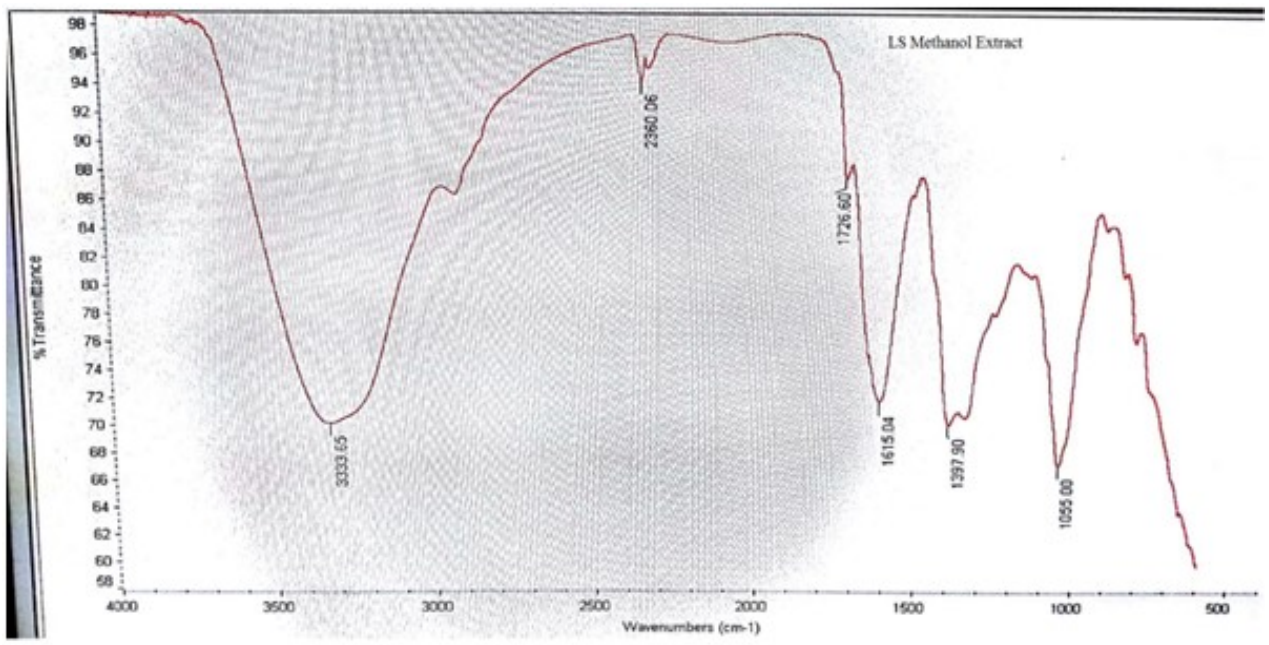

Fig. 2: FTIR spectra of the methanolic extract of *Lactuca serriola*.

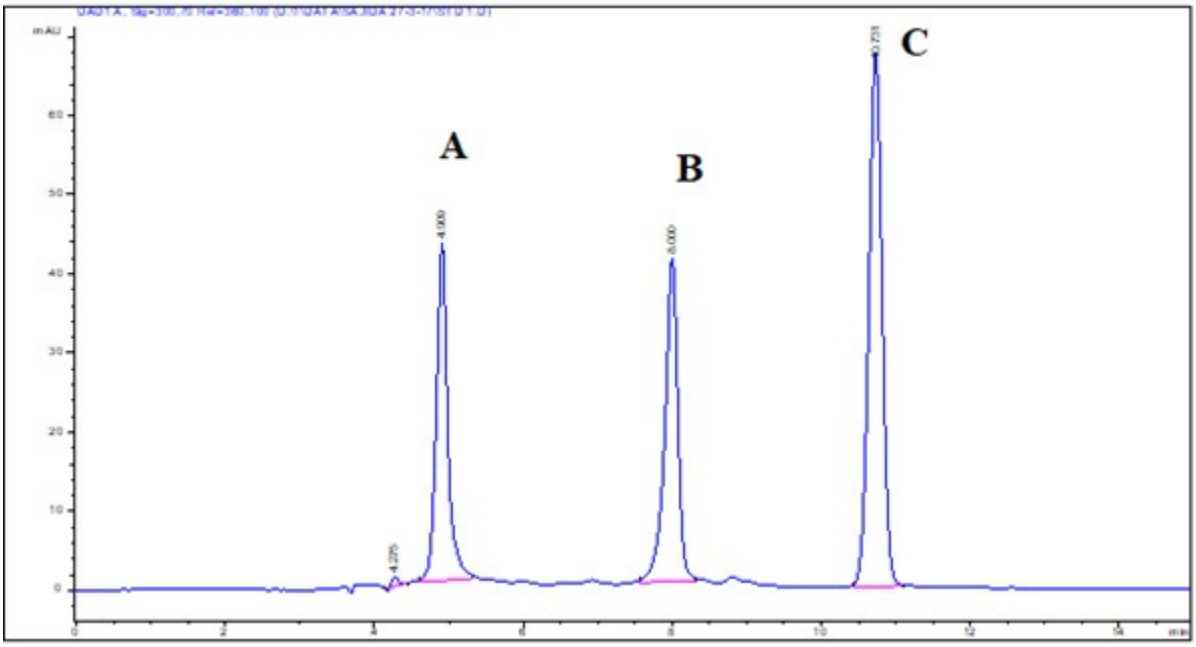

Fig. 3:Standard chromatogram for Chlorogenic acid (A), Caffeic acid (B) and Ferulic acid (C)

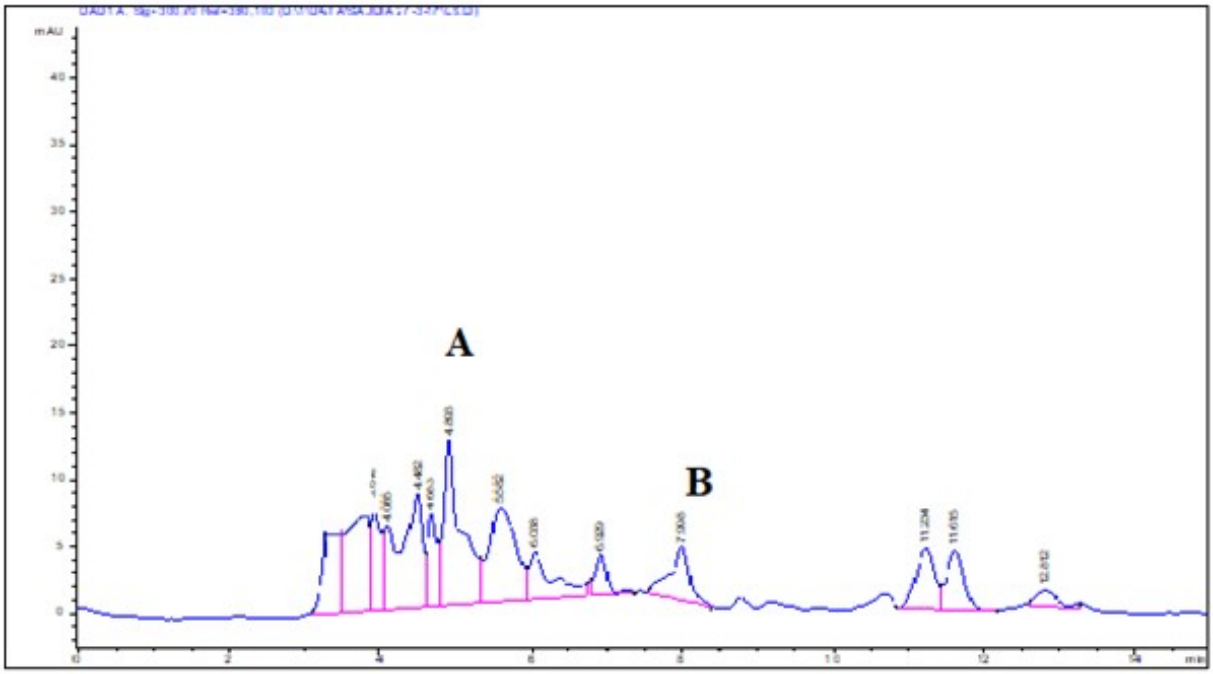

Fig. 4: HPLC chromatogram of methanolic extract of *L. serriola*, chlorogenic acid (A) and caffeic acid (B).

The effectiveness of hepato-protective substance depends on its ability to reduce harmful effects of toxins or maintaining normal liver physiology. The plants are used for multiple purposes in traditional medicines (Wangkheirakpam, 2018). *L. Serriola* has not been yet investigated for its hepatoprotective activity. In the present study, hepatoprotective activity of the plant has been evaluated in paracetamol-induced hepatotoxicity animal models. Free radical scavenging ability of different extracts of the aerial parts of *L. serriola* such as petroleum ether, chloroform and methanol extracts were investigated first. The methanolic extract was found to be more effective as shown in table 1 hence further studies were carried out by using methanolic extract of *L. serriola.*

The use of paracetamol in high doses leads to liver damage. Metabolism of paracetamol results in the

production of secondary metabolites including N-acetyl p-benzoquinoneimine (NAPQI) that binds covalently to tissue macromolecules. The binding of NAPQI with tissues results in oxidation of lipids and sulfhydryl groups and modifies homeostasis of calcium ions. The resulting high number of reactive species causes consumption of protective moieties such as glutathione and α-tocopherol, which may lead to cell membrane damages and hence liver injury(Jaeschke and Ramachandran, 2018). Cell membrane damage leads to leakage of cytoplasmic enzymes such as ALT, AST, ALP and bilirubin, total cholesterol and decreased synthesis of plasma proteins(Mushtaq, 2017). A rise in serum levels of ALT, AST, ALP, bilirubin, and cholesterol as well as decrease in total protein and albumin levels have been found in diseased control group of rats presenting the damage to liver cells by paracetamol as shown in table 1. Pre-treatment with *L. Serriola*r estored the serum levels of enzymes in paracetamol-induced hepatotoxic rat model. This hepatoprotective effect of *L. Serriola* might be due to its membrane stabilizing effect that prevented the leakage of enzymes from cytoplasm to serum. Our findings agree with the previous study(Vijayakumar *et al*., 2020).

Treatment with paracetamol raised the level of cholesterol, TG and LDL while decreased HDL level as compared to control group. However, treatment with *the* methanolic extract of *L. serriola* (LS-100, LS-250 and LS-500mg/Kg) significantly decreased paracetamol-induced rise in TC, TG and LDL and increased HDL levels. It has been reported that paracetamol causes deterioration of hepatic cells, which leads to slow metabolism of lipids(Saied and Hamza, 2014). Reduction in triglycerides level by the methanolic extract of *L. Serriola* might be due to its protective ability against paracetamol-induced deleterious effects.

The methanolic extract of *L. serriola* showed increase in SOD, GR and catalase levels but reduced MDA level as compared with control group. For GR and catalase, results of all the extract-treated groups were more significant than the standard drug as shown in table 3. This effect might be due to the ability of *L. Serriola* to reduce oxidative damage. Histopathological studies confirmed the biochemical findings of the present study.

FTIR of the methanolic extract of *L. serriola* represented hydroxyl (-OH), C=O carboxylic acid, and C=C alkene groups while HPLC analysis showed chlorogenic and caffeic acid (polyphenols) might be responsible for anti-oxidant and free radical scavenging ability of *L. serriola*.

CONCLUSION

In conclusion, phytochemical constituents such as polyphenols present in *Lactuca serriola* might be responsible for hepatoprotective effect, accompanied by anti-oxidant mechanisms. Isolation, identification of respective phytochemicals and determination of their pharmacological parameters become necessary in future.

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