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ORIGINAL ARTICLE

Bioactivity-Guided Isolation, Characterization, and Estimation of Esculetin – A Potential Marker from *Launaea pinnatifida* Cass.

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ABSTRACT

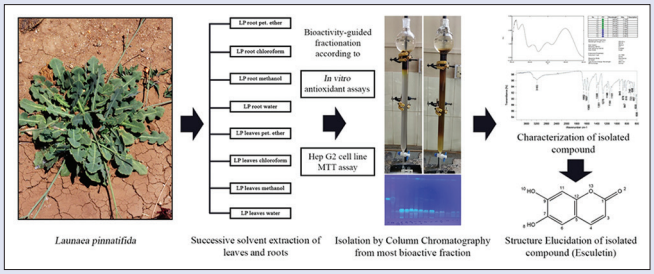
Background: Loss of expertise in Sanskrit language and medicinal plant identification as per ayurvedic texts has resulted in the creation of "Controversial Drugs," i.e., plants with different botanical identities being used by the same name *Launaea pinnatifida* Cass. It is one of the species of one such traditional Indian ayurvedic herb known as "Gojihva." As per the ayurvedic and modern research, Gojihva possesses hepatoprotective, diuretic, galactagogue, and blood-purifying properties. The present study was aimed at bioactivity-guided isolation, characterization, and estimation of a marker compound obtained from *L. pinnatifida*. **Materials and Methods:** Fractions were obtained through the successive solvent extraction technique and subjected to *in vitro* antioxidant and hepatoprotective bioassays which signified that the methanol extract of leaves had significantly better pharmacological activity. Hence, the methanol extract was subjected to further isolation of the active compound. Estimation of the isolated compound was carried out by high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography fingerprinting. **Results:** A marker compound was isolated from chloroform:ethyl acetate (90:10) fraction and elucidated to be esculetin based on its spectral analysis (Fourier transform infrared, liquid chromatography–mass spectroscopy, ^{13}C nuclear magnetic resonance, and ultraviolet). The percentage purity and yield of esculetin in methanol extract of leaf fractions was found to be 99.75% and 0.927%, respectively, by HPLC. **Conclusion:** Esculetin has been reported for the first time in *L. pinnatifida*. Hence, it can be used as the bioactive marker for this plant. This marker can also be useful in the development of hepatoprotective formulations as well as standardization and quality control of *L. pinnatifida* formulations.

Key words: Antioxidant assay, bioactivity-guided isolation, Gojihva, hepatoprotective activity, *Launaea pinnatifida*, spectral technique

SUMMARY

- Methanolic extract of leaves of *Launaea pinnatifida* and esculetin isolated there from possesses marked antioxidant and hepatoprotective activity in a dose-dependent manner. Esculetin may serve as a marker for the standardization of this plant, which can help in differentiating it from other species of the controversial plant Gojihva.

Abbreviations used: NMR: Nuclear magnetic resonance; LC-MS/MS: Liquid chromatography–mass spectrometry; HepG2: Liver hepatocellular carcinoma; DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP assay: Ferric reducing antioxidant power assay; H_2O_2 assay: Hydrogen peroxide assay; TAC assay: Total antioxidant capacity assay; PCM: Paracetamol; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; DMSO: Dimethyl sulfoxide; SSE: Successive solvent extraction; HPLC: High-performance liquid chromatography; HPTLC: High-performance thin-layer chromatography; ^{13}C NMR: Carbon-13 nuclear magnetic resonance; ANOVA: Analysis of variance; TLC: Thin-layer chromatography; UV: Ultraviolet; VLC: Vacuum liquid chromatography; FTIR: Fourier transform infrared; LCMS: Liquid chromatography–mass spectroscopy; NSAIDs: Nonsteroidal anti-inflammatory drugs; IC_{50}: Half-maximal inhibitory concentration; *L. pinnatifida: Launaea pinnatifida;* LPLPE: *Launaea pinnatifida* leaf petroleum ether extract; LPLC: *Launaea pinnatifida* leaf chloroform extract; LPLM: *Launaea pinnatifida* leaf methanol extract; LPLW: *Launaea pinnatifida* leaf water extract; LPRPE: *Launaea pinnatifida* root petroleum ether extract; LPRC: *Launaea pinnatifida* root chloroform extract; LPRM: *Launaea pinnatifida* root methanol extract; LPRW: *Launaea pinnatifida* root water extract; ROS: Reactive oxygen species; GBD: Global burden of disease; WHO: World Health Organization.



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INTRODUCTION

The liver is also known as the fighting organ of the human body, playing a vital role in many physiological processes.[1,2] Many essential body functions were done by the liver, such as storage, secretion, and most vital metabolism.[3] Detoxification of noxious substances is the most fundamental function of the liver.[4] Generation of reactive oxygen species (ROS) leads to the induction of liver diseases such as hepatocellular carcinoma, fever, and hepatitis. Overproduction of ROS plays an essential role in liver damage; many plant antioxidants have been isolated and used as a medicinal agent to prevent liver damage.

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Secondary metabolites such as polyphenols, flavonoids, and coumarins are conscientious for the antioxidant properties of plants, which play a significant role in the neutralization of ROS.[5] Many environmental factors are responsible for the generation of ROS, which leads to cell damage and cell death. Liver cirrhosis is a result of almost all liver diseases identified by necrosis and fibrosis. Currently, alcohol, nonsteroidal anti-inflammatory drugs, and viral hepatitis are the main causative factors that lead to liver damage. According to the information given by the Global Burden of Disease (GBD) project by the WHO,[6] liver disease rates are gradually escalated since the last few years. Recently, almost 50 million peoples, including adults, would be suffering from chronic liver disease.[7] Hence, there is an urgent need to isolate new photochemical and evaluate its potency scientifically. In India, only very few herbal formulations are used to treat liver damage with clinically proven data. Among such herbal formulation, Liv 52 DS tablet from Himalaya Health Care is the more trusted drug in the Indian market for maintaining liver health.[8,9] Clinical trials with human volunteers are not all the time for the herbal formulation to evaluate the efficacy and potency; hence, *in vitro* cell lines are the first choice in such cases. *In vitro* cell line assay is a rapid analytical measure to evaluate the therapeutic potency and efficacy of the herbal drug.

Almost 30% of herbal plants mentioned in the Indian classical system of medicine belong to the class of controversial plants because of its mistaken morphological and botanical identity.[10-13] Thirty-two percent of medicinal plants belong to the controversial drug but are widely used in herbal industries because of the lack of authentification of correct species.[13] Hence, there is a need to isolated phytochemicals from such plants and established the reference standard or phytochemical biomarkers for the standardized plant. *Launaea pinnatifida* (*L. pinnatifida)* also recognized as *Launaea sarmentosa* (Willd.) belongs to family Asteraceae (Compositae).[12,14] It has been traditionally used as a folk herbal remedy for many diseases. As per the ayurvedic literature, *L. pinnatifida* belongs to the class of controversial drug "Gojihva" (shape and texture of the leaves of the plant are similar to cow's tongue).[15] The plant is well known for its medicinal properties but scientifically not much explored. According to the literature survey, six different medicinal plant species are considered as Gojihva, namely *L. pinnatifida*, *Onosma bracteatum, Elephantopus scaber, Anchusa strigosa, Macrotomia benthami,* and *Coccinia glauca*.[15] However, the plant remains largely unexplored. Systematic pharmacological evaluation of the plant by modern methods is not yet done, and the traditional medicinal uses suggest that it may yield important bioactive phytoconstituents; the basic aspect of medicinal plant research is plant authentication as per ayurvedic literature. Our work is mainly concerned with the bioactive-guided isolation, characterization, and estimations of the active compound by *in vitro* assay methods to explore the plant in the direction of phytochemistry and generate authentic data to standardize and distinguish the plant among other species of Gojihva.

MATERIALS AND METHODS

Chemical and reagents

All chemicals and reagents were analytical grades that were purchased from Finar Ltd., Ahmedabad. DPPH and all the high purity standards, namely gallic acid and ascorbic acid, were purchased from Sigma Aldrich. Silica gel G 100–200 mesh was purchased from Finar Ltd., Ahmedabad. Silica gel G 60-120 mesh (for column chromatography) was purchased from Spectrochem Pvt. Ltd., Mumbai. Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline, trypsin, glucose, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid, and hydrogen peroxide were obtained from Hi-Media Laboratories Ltd., Mumbai.

Plant material

The fresh leaves and roots of plant *L. pinnatifida* were collected in May 2017 from Nagoa Beach, Diu (A union territory in West India). The authentification of the plant was done by Dr. Hitesh Solanki, Professor, Department of Botany, School of Science, Gujarat University, Ahmedabad. The geographical coordinate of plant location was 20°42′30.5 ″N, 70°53′35.3 ″E. The voucher specimen (Voucher no. HM1) was deposited at the Herbarium Unit of the Department of Botany, School of Science, Gujarat University, Ahmedabad, Gujarat, India, for future reference. In this study, dried leaves and roots of the plant have been used.

Preparation of extracts and fractions

The powdered shade-dried leaves (300 g) and roots (200 g) were extracted with series of solvents of increasing priority in the Soxhlet apparatus as follows: petroleum ether (60°C–80°C), chloroform, methanol, and water. The material was thoroughly dried each time before extracting it with the next solvent. A total of 8 fractions were prepared in the same manner. The percentage yield of *L. pinnatifida* leaf petroleum ether fraction (LPPE), chloroform fraction (LPLC), methanol fraction (PLLM), and water fraction (LPLW) were found to be 1.706%, 2.121%, 10.128%, and 7.839%, respectively. Furthermore, the percentage yield of *L. pinnatifida* root petroleum ether fraction (LPRPE), chloroform fraction (LPRC), methanol fraction (LPRM), and water fraction (LPRW) was found to be 1.606%, 0.887%, 14.410%, and 12.811%, respectively.

All crude fractions were filtered and dried under reduced pressure at 40°C using a rotary evaporator. Among all fractions, methanol fraction (LPLM) showed more hepatoprotective and antioxidant activity. Therefore, LPLM fraction was subjected to bioactivity-guided fractionation assay for the isolation of more potent compound.[16,17] Further, LPLM (10.128 g) was subjected to vacuum liquid chromatography (VLC1). The bioactive fraction was adsorbed on silica (60–120 mesh size) with a 1:1 ratio. Ethyl acetate and methanol were used with different ratios for isolation of bioactive compound, for example, 100% ethyl acetate (LPLM/F1, 0.6896 g and 4.31%), 50:50 v/v ethyl acetate:methanol (LPLM/F2, 5.2766 g and 32.97%), and 100% methanol (LPLM/F3, 8.9331 g and 55.89%). The volume of each fraction collected was 900 ml. Each fraction was concentrated under reduced pressure at 40°C using a rotary evaporator. The most active fraction of LPLM/F3 was subjected to second VLC (VLC 2). This time only two solvents were used for separation of compounds: 100% ethyl acetate fraction (LPLM/F3/EA) and 100% methanol fraction (LPLM/F3/MEOH). LPLM/F3/EA showed significant bioactivity and subjected to silica gel column chromatography (LPLM/F3/EA 1.7 g with 10 g silica gel 230-400 #), eluting with a gradient mixture of ethyl acetate and chloroform (100/0; 90/10; 80/20; 70/30; 60/40; 50/50; 40/60; 30/70; 20/80; 10/90; 0/100). Glass column length was 18" and diameter of 1". Fraction 46–51 (assigned as F46) shown a single bend on thin-layer chromatography (TLC); hence, all the fractions were pooled together and concentrate on dryness. The TLC was done using chloroform:ethyl acetate:formic acid (7:2.5:0.5) as the mobile phase. Isolated compound esculetin (90 mg) was subjected to the 4–5 solvent system for TLC development to identify the non-polar and polar impurities. The purity of the isolated compound was achieved by a repeated recrystallization technique using methanol. TLC solvent system chloroform:ethyl acetate:formic acid (7:2.5:0.5) indicates the single spot at Rf value 0.30 (under ultraviolet [UV] 366 nm). Hence, a further study was carried out for the elucidation of structure.

Characterization of the isolated compound

The isolated bioactive pure compound was then characterized by the various spectral techniques, namely Fourier transform infrared (FTIR), ^{1}H NMR, liquid chromatography–mass spectroscopy, and UV as well as a comparison of these data with data in the literature.

Ultraviolet-visible analysis

The full scan of 200–800 nm was performed to find out λ_{max} by UV-visible spectrophotometer, Shimadzu 1800, Japan, with serial no. A11455009148.

Liquid chromatography–mass spectroscopy analysis

The isolated compound in LPLM fraction was evaluated using an Agilent UHPLC 1290 instrument coupled to Agilent LC-QToF 6545 mass spectrometer. The mass spectrometric behavior of active fractions was studied for positive ion mode. The sample was prepared in methanol. The following instrument settings were used for analysis: column Agilent C_{18} Poroshell (100 mm × 4.6 mm × 2.7 µm); the column was kept at room temperature; ionizing voltage, 3500 V; nebulizer gas (N2), 35 psig, 11 L/min; fragmentor voltage, 130 V; nozzle voltage, 1000 V; drying gas (N2), 11 L/min; acquisition range, 100–1500 *m/z*; a mixture of ammonium acetate (A) and CAN (B) gradient, 80:10%; and ammonium acetate for 30 min was selected as the mobile phase at a flow rate of 0.4 ml/min.

^{13}C nuclear magnetic resonance analysis

^{13}C nuclear magnetic resonance (NMR) spectra of the isolated compound were measured by Bruker Ultrashield 500 Mz instrument at the National Institute of Pharmaceutical Education and Research (NIPER), Gandhinagar, Gujarat, India. DMSO was used as a solvent and TMS was used as an internal standard. Chemical shift values were recorded δ (ppm) and are depicted in Figure 1.

Fourier transform infrared analysis

FTIR study was performed and FTIR spectra were recorded on Bruker compact FTIR spectrometer with software OPUS version 7.5 at AUM Research Labs., Ahmedabad. Isolated compound C1 were pelleted with potassium bromide (KBr). FTIR spectral data were recorded and are depicted in Figure 1b. Functional group stretching and bending wavelength was matched with literature published data.[18]

Estimation of an isolated compound in *Launaea pinnatifida* leaf methanol extract fraction

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) estimation of the isolated compound was performed using Agilent 1200 infinity instrument (Agilent Technology, USA) and equipped with a DAD detector, an autosampler, a column heater, and Welchrom® C_{18} (4.67 mm ID, 250 mm, 5 µm particle size) column.The mobile phase consisted of two solvents: (A) 0.140 g of anhydrous potassium dihydrogen orthophosphate (KH2PO) dissolved in 900 ml HPLC grade water with addition of 0.5 ml orthophosphoric acid, made up to 1000 ml with water and filtered through a 0.45 µ membrane filter and degassed in a sonicator for 3 min.; and, (B) Acetonitrile (HPLC grade). With a flow rate of 1.5 ml/min, gradient elution was performed using the two solvents at 348 nm detection wavelength. AQ4µ20 µl sample volume was injected into the system by the autosampler. 10.97 mg of the isolated Esculetin was dissolved in 10 ml methanol, whereas 6.26 mg LPLM (considered as sample) was also dissolved in 10 ml methanol. HPLC was then performed for both standard and sample. Analytical HPLC chromatogram of isolated compounds and LPLM are depicted in Figure 2a and b, respectively.

High-performance thin-layer chromatography analysis

HPLTC analysis of LPLM and LPLM/F3 fractions was performed on percolated high-performance thin-layer chromatography (HPTLC) plate silica gel 60 F_{254} (20.0 × 10.0 cm) manufactured by E. MERCK using a Camag Linomat V sample applicator and a Hamilton syringe with 100 µl volume capacity. Total 12 bands were spotted on a silica plate with a 6 mm bandwidth, 15 mm margin at X position, and 8 mm margin from Y position (bottom). A constant application rate of 150 nl/s was used using inert gas. TLC plate was developed in the mobile phase of chloroform:methanol:formic acid (8.5:1:0.5) for separation. Camag twin trough glass chamber (20 × 10 cm) was used for the linear ascending development, which is 30 min presaturated with 10 ml of the mobile phase. After the completion of plate development, the HPTLC plate was dried with a hairdryer at 50°C for 5 min. The slit dimension settings of length 4 mm and width 0.30 mm and a scanning rate of 20 mm/s were employed. Densitometric scanning was performed with reflectance mode at λ_{max} at 348 nm using on Camag TLC scanner 2.01.02 and incorporation with by win CATS software. Deuterium lamp (D2 lamp) was used as a source of radiation. The estimation of the isolated compound was done via height and peak area [Figure 3].

Procedure

The isolated compound served as standard and LPLM fraction as a sample. Sample and standard were applied on the HPTLC plate, and the plate was developed in chloroform:methanol:formic acid (8.5:1:0.5) solvent system. The plate was dried using a hairdryer. The plate was scanned at 348 nm. The R_f value of the selected band was recorded.

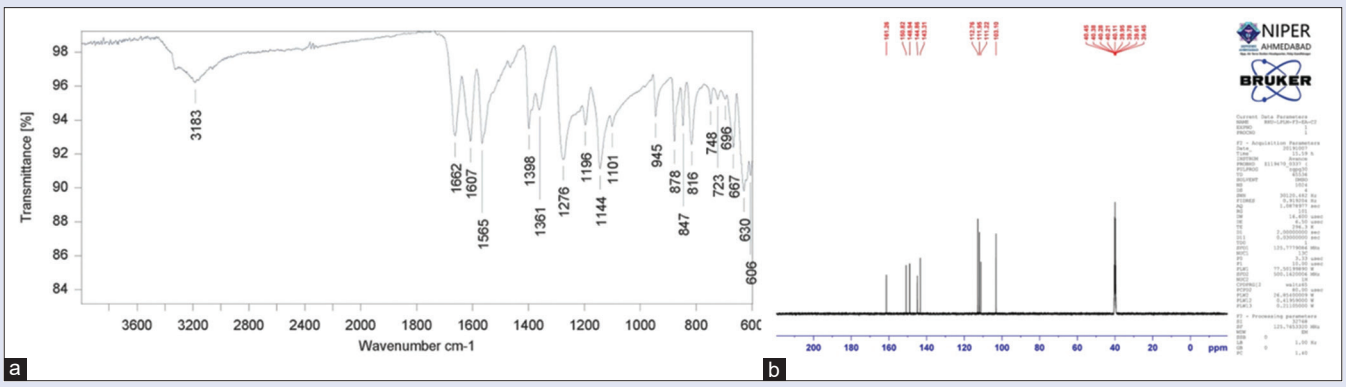

Figure 1: Characterization of an isolated compound by (a) ^{13}C nuclear magnetic resonance spectra of an isolated compound, (b) Fourier transform infrared spectra of an isolated compound

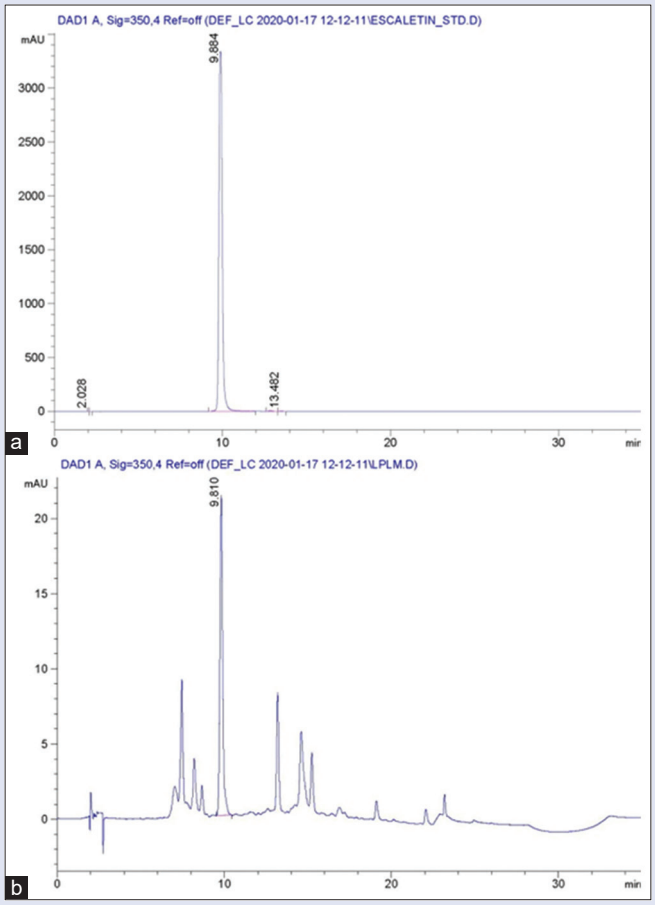

Figure 2: Analytical high-performance liquid chromatography chromatogram of (a) isolated compound from *Launaea pinnatifida* methanol fraction. (b) Crude methanol fraction (LPLM) from *Launaea pinnatifida*

Biological activities

In vitro antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The DPPH-free radical scavenging activity of all fractions obtained from SSE and VLC was carried out as per the methods given by Gyamfi *et al.*[19] Ascorbic acid was the reference standard, and the experiment was performed in triplicate. The IC_{50} value was calculated for each fraction. A decrease in absorbance indicated higher free radical scavenging activity. The following equation calculated the percentage of DPPH scavenging effect:

% inhibition = $[(Abs_{control} - Abs_{sample}]/Abs_{control}) \times 100$.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) model was used as an alternative radical scavenging activity.[20] Ascorbic acid was chosen as a reference standard. Various concentrations of sample and standard were evaluated for % inhibition. Absorbance was measured on an ultraviolet spectrophotometer at 700 nm; results were given in terms of IC_{50}. % inhibition of FRAP by each concentration of fractions was calculated as:

% inhibition = ([Absorbance of control – Absorbance of test]/Absorbance of control) × 100.

Hydrogen peroxide scavenging (H_2O_2) assay

The hydrogen peroxide scavenging (H_2O_2) method was performed to determine the antioxidant activity of all fractions.[5,21] In this method, ascorbic acid was used as a reference standard. Different concentrations of fractions (10, 50, 100, 250, and 500 µg/ml) in distilled water were added to a hydrogen peroxide solution (3 ml, 40 mmol/L). The absorbance value was measured using a UV spectrophotometer at 230 nm. % inhibition of H_2O_2 by each concentration of fractions was calculated as:

% inhibition = $([Abs_{control} - Abs_{sample}]/Abs_{control}) \times 100$.

All tests were performed in a set of triplicates, and results were expressed in terms of IC_{50} value.

Total antioxidant capacity

The total antioxidant capacity assay of all the fractions and extracts of *L. pinnatifida* was carried out by the phosphomolybdenum method employing ascorbic acid as a reference standard.[22] The final absorbance was measured at 695 nm using a UV-visible spectrophotometer against a blank. The IC_{50} value was calculated from the standard calibration curve.

In vitro hepatoprotective cell line assay

HepG2 cells (human hepatocellular carcinoma cell line) were obtained from National Centre for Cell Science NCCS Complex, Savitribai Phule Pune University Campus, Pune, Maharashtra State, India. The cell line was subcultured in minimum essential medium (MEM) and incubated at 37°C in a CO_2 incubator.[5] HepG2 cells were maintained in DMEM supplemented with 100 units/ml penicillin, 10% FBS, and 100 µg/ml streptomycin. Isolated compound and all fractions were subjected to cell line assay. Silymarin (125, 250, 500, and 1000 µg/ml) was used as a positive control, whereas paracetamol (PCM, 25 mM)[23] was used as a negative control. Cytotoxicity was assessed by measuring the percentage cell viability of HepG2 cells in the presence of PCM by MTT reduction assay. Enzyme-linked immune sorbent assay reader measured absorbance at 570 nm, and it is directly proportional to the cell viability of the cell. The percentage of cell viability was calculated using the following formula:

% cell viability= $([Abs_{control}/Abs_{test}] \times 100)$.

Statistical analysis

All the results were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA test followed by Dunnett's multiple comparisons test using GraphPad Prism software with the latest version 8. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Extraction and bioactivity-guided isolation of active compounds

In this study, methanol fraction of *L. pinnatifida* leaves was found to be more active in antioxidants and hepatoprotective *in vitro* assay as compared among all other fractions. Hence, this fraction was selected for further partition. Afterward, VLC1 was carried out and three fractions were obtained. The percentage yield of all the LPLM/F1, LPLM/F2, and LPLM/F3 was found to be 4.31, 32.97, and 55.89, respectively. LPLM/F3 showed more antioxidant and hepatoprotective activity.

Further fractionation was carried out for LMLM/F3 fraction by VLC, which results in two new subfractions (LPLM/F3/EA and LPLM/F1/MEOH). LPLM/F3/EA showed significant hepatoprotective activity as compared to LPLM/F1/MEOH. Further, column chromatography was performed for LPLM/F1/EA fraction, which yielded Esculetin from the fraction F46. Further purification of esculetin was done by repeated recrystallization. In this way, broad bioactivity-guided isolation leads to

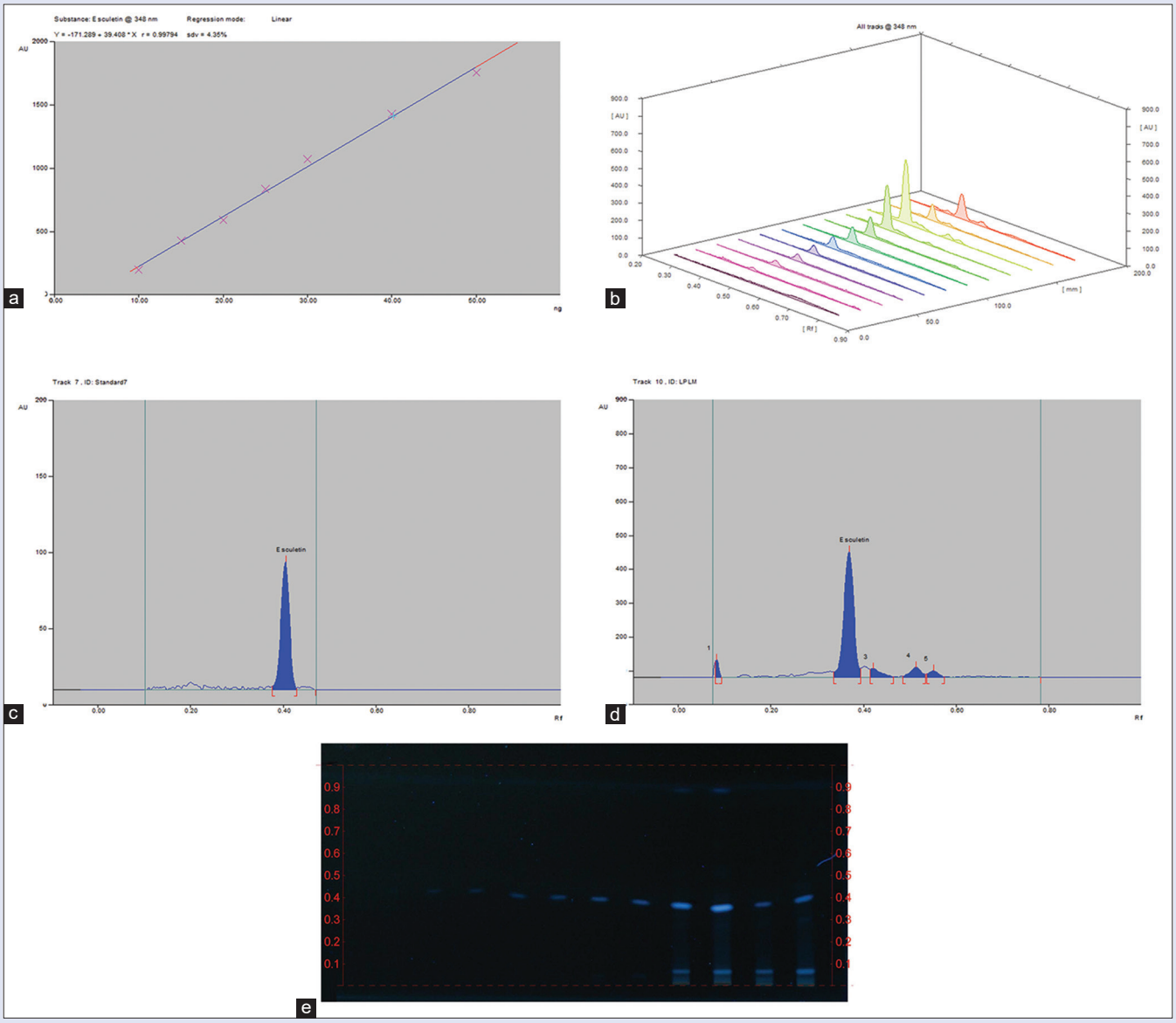

Figure 3: Analytical high-performance thin-layer chromatography chromatogram, (a) Calibration curve as per peak area, (b) 3D chromatogram of various standard concentrations, LPLM and LPLM/F3 fraction, (c) Isolated compound esculetin as standard, (d) LPLM fraction as sample, (e) Thin-layer chromatography plate spotted with standard (1–8 spots), LPLM (9 and 10), and LPLM/F3 (11 and 12)

first-time isolation of florescent coumarin esculetin from *L. pinnatifida* leaves.

Structure elucidation of the isolated compound esculetin

LPLM fraction was fractionated with bioactivity-guided isolation, and one compound was isolated and then elucidated by UV, LC-MS , ^{13}C NMR, and FTIR spectral technique. Esculetin was obtained as off white to pale yellowish color amorphous powder. Melting point decomposes at 272°C–275°C, matched with the Combined Chemical Dictionary database, which was matched with esculetin.[24] UV results confirmed the λ_{max}, 348 nm. The molecular formula, $C_9H_6O_4$, was established based on MS ([M + H] + ion peak at 179.03) and NMR data. Fragmentation pattern indicates the presence of esculetin in LPLM fraction. ^{13}C NMR was carried out in DMSO. Additional structural information was conclusively revealed regarding the pure isolated compounds by the ^{13}C NMR. NMR signals were matched with literature, which showed the

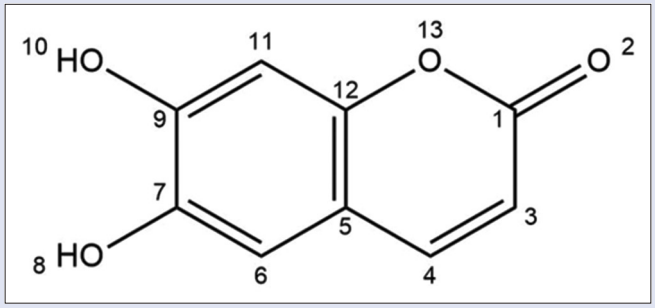

Figure 4: Structure of an isolated compound esculetin

similarity with the 13C NMR spectra of Esculetin. These 13C NMR (125 MHz, DMSO D6) spectra revealed the following δ values which indicated the presence of specific functional groups (mentioned in the brackets): 161.26 (CO), 150.82 (C), 148.94 (C), 144.86 (C), 143.31 (C), 112.76 (CH), 111.95 (CH), 111.95 (CH), 111.22 (CH) and 103.10 (CH). The FTIR spectroscopy is an advanced instrumentation technique for the identification of functional groups in the compound. FTIR (KBr) spectra revealed the peaks at 3183.0239 cm-1, 2416.5986 cm-1, 1564.8022 cm-1, 1464.6614 cm-1 and 1195.9922 cm^{-1}. Analysis and comparison of all the spectral data with already published literature data[25] allowed us to elucidate the structure of esculetin [Figure 4].

Estimation of isolated compound esculetin by high-performance liquid chromatography

The isolated compound was used as a standard for the quantification of esculetin in the LPLM fraction. Samples were analyzed at various concentrations to minimize experimental error and improve the accuracy and precision of the method. All the validation parameters, including the lower limit of quantification and relative standard deviation (RSD), were performed. Standard and LPLM fraction showed a characteristic peak of esculetin at a retention time of 9.8884 and 9.810 min, respectively. *L. pinnatifida* contains the highest amount of esculetin in LPLM fraction, which

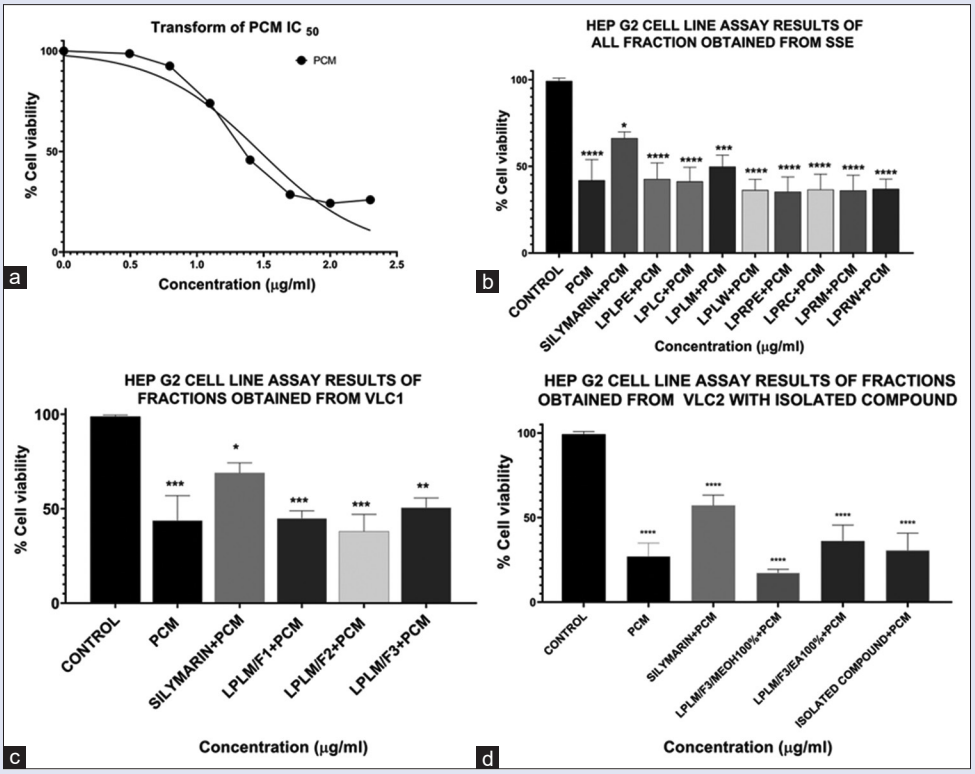

Figure 5: % cell viability of *Launaea pinnatifida* extracts, (a) PCM-treated group with various concentrations, (b) Fractions obtained from SSE, (c) Fractions obtained from VLC1, (d) Fractions obtained from VLC2 and isolated compound

Table 1: Calibration curve data and estimation of esculetin in *Launaea pinnatifida* leaf methanol and *Launaea pinnatifida* leaf methanol/F3 fraction by high-performance thin-layer chromatography analysis

Track	Sample ID	Concentration (µg)	R_f	Peak area	Peak height	Calculated esculetin (µg) as per peak height	Calculated esculetin (µg) as per peak area
1	Esculetin	0.005	-	-	-	-	-
2	Esculetin	0.01	0.43	194.40	12.36	-	-
3	Esculetin	0.015	0.44	424.04	23.79	-	-
4	Esculetin	0.02	0.44	589.35	33.79	-	-
5	Esculetin	0.025	0.42	839.56	46.04	-	-
6	Esculetin	0.03	0.41	1085.20	60.49	-	-
7	Esculetin	0.04	0.41	1454.34	83.56	-	-
8	Esculetin	0.05	0.39	1823.83	105.32	-	-
9	LPLM	Unknown	0.40	4596.31	248.35	-	-
10	LPLM	Unknown	0.40	307.07	17.92	0.1261	0.1232
11	LPLM/F3	Unknown	0.40	1480.10	87.34	0.4204	0.4088
12	LPLM/F3	Unknown	0.40	2561.80	133.30	-	-

LPLM: *Launaea pinnatifida* leaf methanol; LPLM/F3: 100% methanol fraction of LPLM after VLC 1

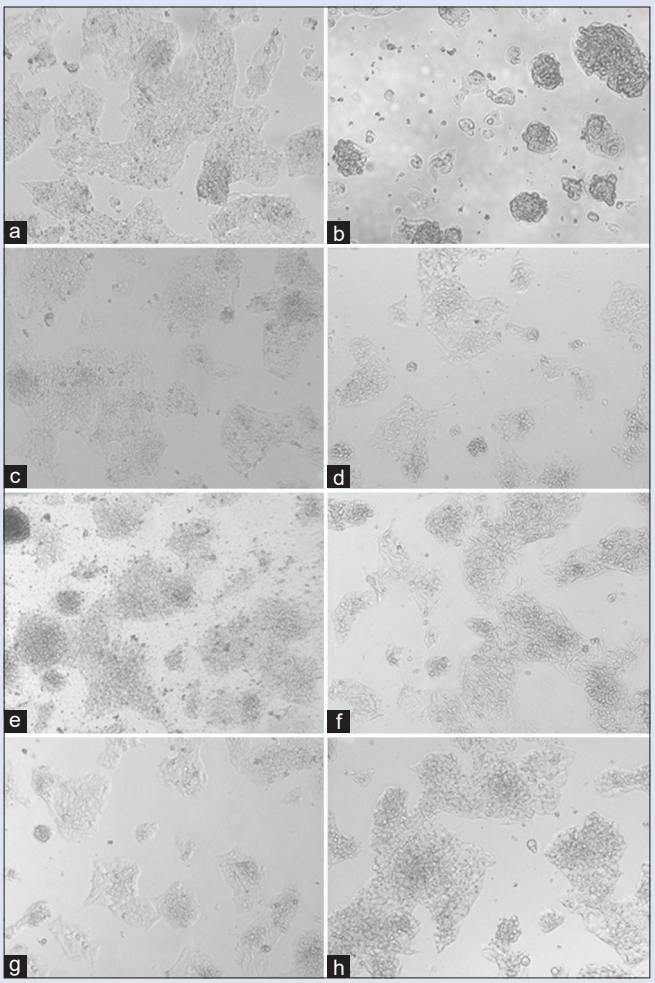

Figure 6: Phase-contrast microscopic analysis: Protective effect of *Launaea pinnatifida* plant extract on paracetamol-induced damage to HepG2 cell line, (a) Control cells, (b) Cell treated with paracetamol (25 mM), (c) Cell treated with silymarin 250 µg/ml in the presence of paracetamol, (d) Cell treated with LPLPE fraction 250 µg/ml in the presence of paracetamol, (e) Cell treated with *Launaea pinnatifida* leaf chloroform extract fraction 250 µg/ml in the presence of paracetamol, (f) Cell treated with LPLM fraction 250 µg/ml in the presence of paracetamol, (g) Cell treated with LPLW fraction 250 µg/ml in the presence of paracetamol, (h) Cell treated with isolated compound (250 µg/ml) in the presence of paracetamol

was calculated as 0.927% concerning 99.75% purity of standard esculetin.

Quantification of an isolated compound in *Launaea pinnatifida* leaf methanol extract and *Launaea pinnatifida* leaf methanol extract/F3 fractions by high-performance liquid chromatography

ICH Q2-R1 guidelines were followed for the quantification of standard marker compounds in plant fractions.[26] Table 1 indicates the calibration curve data (as per peak area) of the isolated compound by HPTLC methods, whereas Figure 3 indicates the chromatograms of standard and fractions. HPTLC fingerprint profile showed that the fluorescent violet color band at R_f 0.40 was in both LPLM and LPLM/F3. The florescent violet band (under 348 nm) at R_f 0.40 was selected as a marker compound and acknowledged as esculetin standard.

The percentage (w/w) amount of isolated compound esculetin was found to be 0.1261% and 0.4204% (as per peak height), respectively, whereas 0.1232% and 0.4088% (as per peak area) in LPLM and LPLM/F3 fractions, respectively.

In vitro antioxidant activity

The antioxidant properties of the *L. pinnatifida* leaf and root fractions were carried out by standard listed methods. In all four antioxidant assays, ascorbic acid showed at least IC_{50} which proves the best antioxidant potential as a standard drug, followed by LPLM/F3 and LPLM fractions.

The DDPH scavenging activity of all the extracts and fractions of *L. pinnatifida* was determined and the IC_{50} values are given in Table 2. Among all the fractions obtained by SSE, LPLM fractions showed significant DPPH scavenging activity with the 17.94 ± 0.37 µg/ml IC_{50} value which is nearest to the IC_{50} value of standard ascorbic acid (16.53 ± 1.28 µg/ml). The LPLM/F3 showed the highest activity (IC_{50} = 21.77 ± 2.47 µg/ml) among all other subfractions obtained through VLC1.

The extract and fractions showed concentration-dependent DPPH discoloration assay with scavenging activity with LPLM and LPLM/F3 showing the least IC_{50} value of 17.94 ± 0.37 µg/ml and 21.77 ± 2.47 µg/ml for LPLM and LPLM/F3, respectively; hence, fraction F3 was considered to be the most potent and shown highest % scavenging of DPPH radicals. FRAP-free radical scavenging increased with increasing concentrations of fractions with LPLM and LPLP/F3 showing the lowest IC_{50} values (26.18 ± 2.67 and 21.92 ± 4.55 µg/ml, respectively). The LPLM extract showed 26.18 ± 2.67 µg/ml, 85.84 ± 9.18 µg/ml, and 14.78 ± 1.84 µg/ml in FRAP, H_2O_2, and TAC assays, respectively. The LPLM/F3 fractions showed IC_{50} 21.77 ± 2.47 µg/ml, 21.92 ± 4.55 µg/ml, 85.29 ± 11.53 µg/ml, and 10.29 ± 3.67 µg/ml in DPPH, FRAP, $H_2O_2$and TAC assays, respectively. The IC_{50} values are recorded in Table 2.

The potency of the extract/fractions was found to be in the following order: LPRC > LPRPE > PLRW > LPLC > LPLPE > LPLM/F1 > LPLW > LPLM/F2 > LPRM in all antioxidant assays. The highest % scavenging activity was observed by standard ascorbic acid (3.442 ± 0.89 µg/ml) followed by LPLM/F3 (10.29 ± 3.67 µg/ml) in TAC assay. The free radical scavenging potential of the entire fraction is directly proportional to the concentration of sample, and a lower IC_{50} value indicates the batter antioxidant potential. Based on *in vitro* assay results, LPLM/F3 fraction was selected and subjected to further isolation of a more potent bioactive compound using column chromatography.

In vitro hepatoprotective activity

In this study, the hepatoprotective effect of bioactive compounds isolated from *L. pinnatifida* on the HepG2 cell line was investigated. The treatment of HepG2 cells with PCM (25 mM) significantly decreases the percentage cell viability to 73.29 ± 4.79, 40.75 ± 0.34, 38.93 ± 0.52, and 14.27 ± 0.4 at concentration of 125, 250, 500, and 1000 µg/ml, respectively, with respect to control cells. Pretreatment with only LPLM with similar concentration significantly protected HepG2 cell death induced by PCM in a dose-dependent manner [Figure 5]. Fraction of LPLM/F3 showed the strongest protective effect of 63.93 ± 0.862 at 1000 µg/ml, whereas the isolated compound showed a significant protective effect of 54.06 ± 1.04 at 1000 µg/ml, as shown in Table 3. Standard hepatoprotective marker drug silymarin showed the highest protective effect of 80.94 ± 3.24 at 1000 µg/ml was further confirmed through the cell morphology by phase-contrast microscopic images [Figure 6]. Exposure of HepG2 cells to PCM significantly ($P < 0.05$) decreased cell viability, whereas exposure of HepG2 cells to an isolated compound in the presence of PCM significantly ($P < 0.05$) increased cell viability concerning

Table 2: *In vitro* antioxidant assay results in terms of IC_{50} (µg/ml) for 1,1-diphenyl-2-picrylhydrazyl, ferric reducing antioxidant power, H_2O_2, and total antioxidant capacity assays

Fractions	*In vitro* antioxidant assays			
	DPPH scavenging assay	FRAP scavenging assay	Hydrogen peroxide scavenging	Total antioxidant capacity
	IC_{50}(µg/ml)			
Standard	16.53±1.28	14.2±1.27	17.19±2.47	3.442±0.89
LPLPE	31.33±2.17	62.2±7.41	630.9±44.31	38.18±3.47
LPLC	30.26±088	89.57±7.55	832.5±61.14	22.08±4.31
LPLM	17.94±0.37	26.18±2.67	85.84±9.18	14.78±1.84
LPLW	28.06±1.28	30.85±3.47	113.7±12.4	21.33±4.22
LPRPE	54.45±6.42	118.9±11.28	618.3±46.7	34.57±5.14
LPRC	37.2±2.67	136.1±14.62	510.6±34.2	36.51±1.47
LPRM	28.25±1.57	28.08±2.47	137.6±14.25	20.94±1.77
LPRW	31.46±2.69	97.87±8.11	302.9±22.45	24.99±3.71
LPLM/F1	37.04±4.62	49.45±4.97	439.3±28.91	15.51±2.83
LPLM/F2	37.71±4.74	29.54±6.14	115.9±17.21	12.67±4.15
LPLM/F3	21.77±2.47	21.92±4.55	85.29±11.53	10.29±3.67

Antioxidant potential of all fractions of *L. pinnatifida* obtained from SSE and VLC1 in terms of IC_{50} with positive control (n=3). LPLPE: *L. pinnatifida* leaf petroleum ether extract; LPLC: *L. pinnatifida* leaf chloroform extract; LPLM: *L. pinnatifida* leaf methanol extract; LPLW: *L. pinnatifida* leaf water extract; LPRPE: *L. pinnatifida* root petroleum ether extract; LPRC: *L. pinnatifida* root chloroform extract; LPRM: *L. pinnatifida* root methanol extract; LPRW: *L. pinnatifida* root water extract; LPLM/F1: 100% ethyl acetate fraction of LPLM after VLC 1; LPLM/F2: 50:50 ethyl acetate and methanol fraction of LPLM after VLC 1; LPLM/F3: 100% methanol fraction of LPLM after VLC 1; IC_{50}: Inhibitory concentration; FRAP: Ferric reducing antioxidant power assay; DPPH: 1,1-diphenyl-2-picrylhydrazyl

Table 3: Percentage cell viability of *L. pinnatifida* fractions by methylthiazolyldiphenyl-tetrazolium bromide assay

Treatment groups	Percentage cell viability (concentration µg/ml)			
	125	250	500	1000
3 (a) Percentage cell viability of *L. pinnatifida* fractions obtained from SSE				
Control	98.97±4.17			
PCM treated	73.29±4.79	40.75±0.34	38.93±0.52	14.27±0.4
Silymarin + PCM treated	59.93±0.342	63.01±0	65.07±2.083	76.71±2.397
LPLPE + PCM treated	15.98±0.523	43.26±0.713	54±0.395	57.19±1.907
LPLC + PCM treated	17.35±0.862	44.18±0.685	48.52±1.886	54.68±1.046
LPLM + PCM treated	36.53±1.384	39.95±1.757	57.76±0.862	64.5±0.395
LPLW + PCM treated	20.78±0.862	32.19±1.235	42.01±0.862	49.89±0.395
LPRPE + PCM treated	10.84±0.713	36.07±1.384	46.92±1.37	47.37±1.203
LPRC + PCM treated	13.81±0.395	31.05±0.862	49.43±0.862	51.94±0.713
LPRM + PCM treated	10.05±0.523	38.36±0.685	48.17±0.523	47.26±1.186
LPRW + PCM treated	22.95±1.235	32.76±1.203	41.67±0.713	50±1.235
3 (b) Percentage cell viability of *L. pinnatifida* fractions obtained from VLC 1				
Control	98.97±4.17			
PCM treated	79±7.13	41.89±1.69	38.93±0.52	14.95±2.52
Silymarin + PCM treated	56.05±1.89	64.5±0.52	73.97±5.18	80.94±3.24
LPLM/F1 + PCM treated	11.07±1.297	36.87±1.101	47.95±0.906	49.77±1.046
LPLM/F2 + PCM treated	12.1±1.757	39.27±1.724	49.09±1.947	51.48±2.064
LPLM/F3 + PCM treated	38.93±0.523	48.29±1.812	50.91±1.046	63.93±0.862
3 (c) Percentage cell viability of *L. pinnatifida* fractions obtained from VLC 2 along with isolated esculetin compound				
Control	98.07±1.21			
PCM treated	47.18±1.52	24.59±1.29	27.69±0.95	8.47±0.41
Silymarin + PCM	42.22±1.13	52.62±1.68	63.71±4.75	69.97±5.27
LPLM/F3/MEOH + PCM	12.12±0.24	16.46±0.24	18.18±0.21	22.45±0.86
LPLM/F3/EA + PCM	16.12±0.21	26.24±0.55	42.77±1.09	59.16±0.63
Isolated compound + PCM	12.6±0.62	14.05±0.21	41.18±0.32	54.06±1.04

3 (a) Fraction obtained from SSE 3 (b) Fraction obtained from VLC1 3 (c) Fraction obtained from VLC2 and isolated compound esculetin by MTT assay. Cytotoxicity effect in terms of IC_{50} of the plant fractions is expressed as mean±SEM (n=3) *L. pinnatifida* obtained from SSE and VLC1 in terms of IC_{50} with positive control (n=3). LPLPE: *L. pinnatifida* leaf petroleum ether extract; LPLC: *L. pinnatifida* leaf chloroform extract; LPLM: *L. pinnatifida* leaf methanol extract; LPLW: *L. pinnatifida* leaf water extract; LPRPE: *L. pinnatifida* root petroleum ether extract; LPRC: *L. pinnatifida* root chloroform extract; LPRM: *L. pinnatifida* root methanol extract; LPRW: *L. pinnatifida* root water extract; LPLM/F1: 100% ethyl acetate fraction of LPLM after VLC 1; LPLM/F2: 50:50 ethyl acetate and methanol fraction of LPLM after VLC 1; LPLM/F3: 100% methanol fraction of LPLM after VLC 1; IC_{50}: Inhibitory concentration; PCM: paracetamol; MTT: Methylthiazolyldiphenyl-tetrazolium bromide

control cells. Hence, it indicated that isolated compounds at a dose of 1000 µg/ml possessed marked hepatoprotective activity. Many studies have suggested the hepatoprotective activity of coumarin compounds because of their strong antioxidant potential. Coumarin has the potency to reduce free radical formations and also leads to scavenging free reactive radicals. Cell damage due to free radicals was effectively

reduced by esculetin by its antioxidant property. Moreover, esculetin remarkably reduced the CCl_4-induced cell damage in rats, probably by free radical scavenging property.

CONCLUSION

This is the first report on the bioactivity-guided isolation of *L. pinnatifida*, which shows that the methanolic extract of leaves and esculetin isolated there from possesses marked antioxidant and hepatoprotective activity in a dose-dependent manner. Esculetin may serve as a phytochemical reference marker for the standardization of the plant. Moreover, because of the limited studies on Gojihva species, these qualitative and quantitative studies combined with bioactivity evaluation will shed new light for advanced studies as well as serve as quality control parameters for this plant. The plant could be an alternative source for the development of a new formulation for free radical-mediated degenerative diseases, mainly regarding the liver. The work also broadens the horizon for further phytochemical and pharmacological research on the plant.

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Conflicts of interest

There are no conflicts of interest.

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