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Research Article

BERGAPTEN FROM *LEUCAS URTICIFOLIA* MITIGATES ISOPROTERENOL-INDUCED CARDIOTOXICITY THROUGH NRF2/HO-1 SIGNALLING PATHWAY

NEMALAPALLI YAMINI[1], JUTURU MASTANAIAH*[2]

[1]Department of Pharmacology, Jawaharlal Nehru Technological University-Oil Technological and Pharmaceutical Research Institute, Anantapur, Andhra Pradesh, India. [2]Department of Pharmacology, Balaji College of Pharmacy, Anantapur, Andhra Pradesh, India.
*Corresponding author: Juturu Mastanaiah; Email: masthpharma@gmail.com

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ABSTRACT

Objectives: This study evaluated whether an ethanolic extract of *Leucas urticifolia* (ELU) attenuates isoproterenol (ISO)-induced cardiac damage in Wistar rats.

Methods: The cardioprotective potential of ELU was assessed using *in vitro* and *in vivo* experiments, and its bioactive component bergapten was identified using gas chromatography-mass spectrometry and quantified by reversed-phase high-performance liquid chromatography. The safety profile and effects of ELU (50 and 100 mg/kg) on cardiac biomarkers, lipid profiles, oxidative stress markers, inflammatory cytokines, Nrf2/Keap1/HO-1 pathway, and cardiac histopathology were evaluated in experimental rats.

Results: ELU showed significant tissue antioxidant activity, no substantial cytotoxicity in H9C2 cells ($IC_{50:}$ 82.04 μg/mL), and was safe in acute oral toxicity studies up to 2000 mg/kg in rats. ELU significantly reduced the levels of cardiac biomarkers, including creatine kinase-MB, lactate dehydrogenase, and cardiac troponin T. ELU also enhanced myocardial antioxidant enzyme activity and decreased malondialdehyde levels. ELU downregulated the inflammatory mediators tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, nuclear factor kappa B, and C-reactive protein, while upregulating anti-inflammatory IL-10. Gene expression and western blot analysis showed that ELU restored redox homeostasis by activating the Nrf2/HO-1 antioxidant axis and suppressing Keap1 expression in a dose-dependent manner. Histopathological analysis confirmed cardiac architecture preservation in the ELU-treated groups.

Conclusion: The present study demonstrates that ELU exerts cardioprotective effects by attenuating oxidative stress, suppressing inflammation, reactivating Nrf2 signaling, and minimizing myocardial injury in ISO-induced myocardial infarction in Wistar rats, with bergapten identified as a likely contributing constituent supporting its development as a natural cardioprotective agent and providing a rationale for further investigations.

Keywords: Isoproterenol, *Leucas urticifolia*, Gas chromatography-mass spectrometry, Cardiac markers.

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INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide. Myocardial infarction (MI) is the most common manifestation of CVD. CVD remains a substantial health burden worldwide, with an estimated 50 million individuals suffering from various cardiac conditions annually. CVDs contribute to 17.9 million deaths annually worldwide [1]. Among CVDs, MI is a life-threatening condition because of its late diagnosis and acute or sudden onset of symptoms [2]. MI occurs due to an acute reduction in coronary blood flow, leading to ischemic damage to cardiac tissue. The occurrence of necrosis in the myocardium is related to the formation of free radicals that trigger the release of cytokines. The cytokines that affect myocardium cell injury are tumor necrosis factor alpha, interleukin (IL)-6, IL-1, and IL-8 [3]. This acute event can have long-lasting consequences on cardiac function and overall health, often leading to complications such as heart failure, arrhythmias, and reduced quality of life [4].

The use of experimental models, particularly the isoproterenol (ISO)-induced MI model, has helped us understand the pathophysiology of MI and create new therapeutic options for MI. When administered at high doses, ISO, a synthetic catecholamine, induces myocardial damage that closely mimics the effects of naturally occurring MI in humans [5]. This model replicates the key features of MI, including oxidative stress, which results from an imbalance between free radical production and inflammatory responses characterized by immune cell infiltration and the release of proinflammatory mediators, as well as changes in lipid profiles that exacerbate cardiovascular risk and myocardial necrosis [6,7]. By studying these processes in controlled experimental settings, researchers can evaluate potential cardioprotective agents and develop novel therapeutic approaches to prevent, mitigate, or reverse the damaging effects of MI, ultimately improving patient outcomes in clinical settings.

Natural products have long been used as cornerstones in the search for new therapeutic agents because of their diverse bioactive constituents and favorable safety profiles. Among these, the medicinal plant *Leucas urticifolia* is of ethnopharmacological relevance as a promising candidate for developing cardioprotective interventions. *L. urticifolia*, a member of the Lamiaceae family, is extensively used in the Indian medicinal system to cure various maladies, such as cough, cold, diarrhea, and inflammatory skin conditions [8,9]. Recent studies have led to the isolation and identification of novel compounds from *L. urticifolia*, highlighting their potential as sources of bioactive compounds. Two novel flavonoid glucosides, leufolins A and B, were extracted from the ethyl acetate fraction of the entire plant, and both demonstrated considerable inhibition of butyrylcholinesterase [10]. In addition, a new steroid, leucisterol, and a new peroxy acid, urticic acid, along with other known compounds, were isolated from the chloroform-soluble fraction, along with other known compounds [11]. The phytochemical profile of *L. urticifolia* is consistent with the general characteristics of the genus *Leucas*, which includes a variety of chemicals, including lignans, flavonoids, coumarins, steroids, terpenes, fatty acids, and aliphatic long-chain compounds [8,9]. These phytoconstituents contribute to the diverse pharmacological effects of *Leucas* species, including anti-inflammatory, analgesic, antidiarrheal, antibacterial, antioxidant, and insecticidal activities. Despite its historical therapeutic use and intriguing phytochemical profile, the cardioprotective potential of *L. urticifolia* remains unclear.

Although direct cardiovascular outcome data for *Leucas* species are limited, several *Leucas* spp. (e.g., *Leucas aspera*, *Leucas cephalotes*, *Leucas zeylanica*) exhibit robust antioxidant, anti-inflammatory, and metabolic (antidiabetic/antihyperlipidemic) activities *in vitro* and *in vivo*, and contain flavonoids, phenolics, and related phytochemicals known to modulate vascular oxidative stress and endothelial function. These observations provide a mechanistic rationale to investigate cardioprotective effects of *L. urticifolia* in the present study.

This study explored the cardioprotective potential of ethanolic extract *L. urticifolia* (ELU) in a rat model of ISO-induced MI. Given that ISO administration generates free radicals, leading to oxidative stress and direct myocardial injury, we hypothesized that ELU attenuates this damage and restores cardiac function. To validate this hypothesis, we evaluated the cardioprotective efficacy of ELU by analyzing changes in cardiac biomarkers, lipid profiles, oxidative stress markers, and histopathological features. Bergapten, a major furocoumarin in *L. urticifolia*, has antioxidant and anti-inflammatory activities (and promotes mitophagy), and while related psoralens have been reported to activate Nrf2, direct evidence for bergapten → Nrf2/HO-1 activation is lacking; thus, Nrf2/HO-1 involvement here is plausible but not proven. We attempted to elucidate the mechanism of action by modulating the Keap1/Nrf2/HO-1 pathway. The biological effects of ELU were further examined through cytotoxicity and antioxidant assays, including the MTT assay on H9C2 cells to assess cytotoxicity and the 2, 2-dipheny l-1-picrylhydrazyl (DPPH) assay to evaluate its free radical scavenging capacity. Antioxidants are chemicals that have the power to either entirely reverse the oxidation process or to slow its progression toward completion [12]. Gas chromatography-mass spectrometry (GC-MS) analysis was conducted to characterize the bioactive constituents of ELU and provide insights into its potential therapeutic mechanisms.

METHODS

Plant collection and extraction

Whole *L. urticifolia* plants were collected in January 2024 from a local area and authenticated by a botanist with voucher number 0889. The herbarium was deposited at the Department of Botany, Sri Venkateswara University, Tirupati. The dried plant material was ground and extracted by maceration with 90% alcohol at a 10:4 ratio. The solvent was removed using a rotary evaporator at decreased pressure and 60°C, yielding a dark green ethanolic extract. The extract was stored at 4°C until needed. ISO (CAS number 5984-95-2) was purchased from Sigma-Aldrich. All chemicals used in this study were of analytical grade and purchased locally.

Total polyphenols and total flavonoids content estimation

The total polyphenol content of the ELU was evaluated using the Folin-Ciocalteu technique, and the results were reported as milligrams of gallic acid equivalent per gram of extract. The extract stock solution (1 mg/mL) was combined with Folin-Folin-Ciocalteu's reagent, and 20% w/v sodium carbonate solution was added. The absorbance was measured at 765 nm after 30 min of incubation. Similarly, the total flavonoid content was determined using the aluminum chloride colorimetric method, which involved adding 5% sodium nitrite and 0.3 mL of 10% aluminum chloride to the ELU (1 mg/mL), incubating for 10 min, and then adding 1 M sodium hydroxide. The absorbance of the pink chromogen was measured at 510 nm, and the values were expressed as milligrams of rutin equivalent per gram of extract [13].

GC-MS analysis

GC-MS analysis was conducted using an Agilent 7890B gas chromatograph (Agilent Technologies, USA) paired with a mass-selective detector (MSD). The separation process was performed using an HP-5 capillary column (30 m × 0.32 mm, thickness 0.25 μ). The oven temperature started at 50°C, where it was held for 1 min, then increased at a rate of 15°C/min until reaching 280°C, where it was maintained for 10 min. The injector was set to 230°C, and helium served as the carrier gas at a steady flow rate of 1.2 mL/min. A 1 μL sample was injected in split mode with a split ratio of 50:1, and the purge flow was maintained at 2.0 mL/min. The detector was operated at 250°C, and the auxiliary gases were provided at flow rates of 40 mL/min for hydrogen and 400 mL/min for air.

Quantification of bergaptene by reversed-phase high-performance liquid chromatography (RP-HPLC) method

The confirmation and identification of bergaptene in the ELU was performed using a GC-MS 5977A MSD (Agilent Technologies). Bergapten quantification was conducted using RP-HPLC on an Agilent system equipped with an HPH C-18 column (4.6×150 mm, 2.7 μm) at a temperature of 40°C. The mobile phase consisted of 100% methanol with 0.072% phosphate buffer, and the flow rate was set at 1 mL/min. A 10 μL sample was injected via an automatic injector, and the compounds were analyzed using a ultraviolet (UV) detector. An analytical method was devised to establish a chemical profile and quantitatively evaluate bergapten in *L. urticifolia* using a combination of solvent systems. The effectiveness of the solvent system was assessed based on the run time, cost, and method sensitivity.

Preparation of standard and sample solutions

A stock solution of bergapten at a concentration of 14.8 mg/L was prepared using high-performance liquid chromatography (HPLC)-grade methanol in a 10 mL volumetric flask. Bergapten quantification was performed using an external standard, as described previously [14,15]. Dilutions ranging from 0.5 to 10 μg/mL were prepared from stock solutions. A calibration curve was established by plotting the peak areas against the injected concentrations of the analytes. The stability of the standard in solution during analysis was assessed by repeatedly analyzing the sample on the same day and after 48 h of storage under laboratory conditions and in a refrigerator.

In vitro studies

DPPH scavenging assay

ELU's radical ion-scavenging potential of ELU was determined using the DPPH assay. 50 μL of 0.1 mM DPPH in methanol was incubated for 30 min in the dark with ELU at various doses, in triplicate. Ascorbic acid was used as the reference molecule, and the absorbance was measured at 517 nm against a blank (methanol) using a UV-Vis spectrophotometer (Shimadzu UV-2401PC). A 5 mL sample of methanolic DPPH solution without an antioxidant was used as the control. The half-maximal inhibitory concentration (IC_{50}) was calculated.

Cell culture

H9C2 cells were cultured in RPMI medium supplemented with 10% inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified environment with 5% CO_2 until they reached confluency. To remove the cells, 0.05% trypsin was used, followed by centrifugation at 1000 rpm for 5 min. The supernatant was removed, and the cell pellet was resuspended in 2 mL of freshly prepared RPMI medium. After assessing cell viability, a single-cell suspension containing 5.0×105 cells/mL was prepared.

MTT assay

H9C2 cells were cultured in 96-well plates at a density of 1×104 cells/well for 24 h. Cell viability was evaluated after exposure to different ELU concentrations (0, 5, 10, 20, 40, and 100 μg/mL). After adding 20 μL of MTT to each well, the cells were incubated for 4 h at 37°C. After removing the medium, 150 μL DMSO was added to each well to dissolve the formazan crystals [16]. Optical density was measured at 570 nm using a SpectraMax i3X microplate reader (Molecular Devices, USA).

In vivo experimentation

Animals

Wistar albino rats of both sexes were housed in polycarbonate cages with autoclaved corncobs as bedding materials under standard conditions (25±2°C, 60–70% relative humidity). They were kept on a 12 h light and dark cycle with free access to food and water *ad libitum*. Animals were acclimatized to laboratory conditions for 7 days before the start of each experiment. This study followed the Animals Research Reporting *in vivo* Experiments Criteria. The experimental design and implementation strictly followed the ethical criteria established by the Committee for Control and Supervision of Experiments on Animals and the Institutional Animal Ethical Committee (1423/PO/Re/S/11/CPCSEA) of P. Rami Reddy Memorial College of Pharmacy, Kadapa, Andhra Pradesh, India.

Acute toxicity study of ELU

An acute oral toxicity study was conducted using 12 female Wistar albino rats in accordance with OECD 423 guidelines, with slight changes. The control group was administered 0.5% carboxymethyl cellulose (CMC) (10 mL/kg), whereas the test group received ELU (2000 mg/kg in 0.5% CMC). The test was carried out in two stages; with clinical observations taken at various time intervals of up to 24 h. Mortality was analyzed for prospective dose modification. After 14 days, all surviving animals were euthanized, and a gross necropsy was performed.

ISO induced MI in experimental rats

To assess cardioprotective activity, 24 Wistar albino rats of either sex (6–8 weeks, 150–200 g) were randomly allocated into four groups of six animals each. The experimental animals in Group I received vehicle (0.5% CMC) and served as the normal control, whereas those in Group II were administered ISO 85 mg/kg s.c. to induce myocardial toxicity for the last 2 days of the study and served as the disease controls. Groups III and IV were administered ELU at low (50 mg/kg, p.o.) and high (100 mg/kg, p.o.) dosages, respectively, for 28 days, with ISO (85 mg/kg, s. c.) on the last 2 days of the study.

Biochemical analysis

At the end of the experiment, blood was collected from the retro-orbital plexus under isoflurane anesthesia and centrifuged at 3,000 rpm for 10 min to obtain serum, which was stored at −4°C until analysis. Cardiac troponin was measured using an Enzyme-linked immunosorbent assay (ELISA) kit (RK04950; Abclonal, USA). Creatine kinase-MB (CK-MB) (Cat. no. 120619), lactate dehydrogenase (LDH) (Cat. no. 121020), alanine aminotransferase (Cat. no. 120616), aspartate aminotransferase (Cat. no. 120617), C-reactive protein (CRP) (Cat. no. 121505), and lipid profile parameters were analyzed using ERBA diagnostic kits on an automated biochemistry analyzer (ERBA EM 360, TransAsia, India).

Proinflammatory markers

Proinflammatory markers, including IL-1β (Cat. no. RK00098), IL-6 (Cat. no. RK00020), IL-10 (Cat. no. RK00050), tumor necrosis factor-alpha (TNF-α) (Cat. no. RK00029), and nuclear factor kappa B (NF-Kb) (Cat. no. RK08775), were analyzed using ELISA kits (Abclonal, USA) according to the manufacturer's instructions.

Estimation of oxidative stress markers

At the end of the experiment, the rats were euthanized, and their hearts were removed, rinsed with ice-cold phosphate-buffered saline (PBS) to eliminate any remaining blood, and promptly homogenized with 0.1 M Tris buffer (pH 7.4). The homogenate was centrifuged at 4500 rpm, and the supernatant was used to assess the amount of tissue antioxidants, such as superoxide dismutase (SOD) [17], glutathione (GSH), catalase [18], and malondialdehyde (MDA) [19].

Gene expression analysis of Nrf2, Keap1, and HO-1 in cardiac tissue

Total RNA was isolated from cardiac tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA purity and concentration were assessed using an H1 synergy multimode microplate reader (Agilent, USA), and RNA integrity was confirmed through agarose gel electrophoresis. Subsequently, 1 μg of RNA was reverse-transcribed into cDNA using a high-capacity cDNA reverse-transcription kit (Bio-Rad, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green Master Mix (Bio-Rad, USA) on a Bio-Rad Opus 96 real-time PCR system (Bio-Rad). Gene-specific primers were designed and validated for Nrf2, Keap1, and HO-1. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control to normalize the expression data for each gene. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold-change compared to the normal control group. The primer sequences are presented in Table 1.

Western blot analysis of Nrf2/HO-1 expression in cardiac tissue

The levels of Nrf2/HO-1 expression in the heart tissue of rats treated with ELU were measured using a Bio-Rad Dc Protein Assay kit. Protein extraction: Isolated rat myocardial tissues were cleaned with ice-cold PBS and homogenized using radioimmunoprecipitation buffer containing 50 mM tris-HCL pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitor. Centrifugation was performed after incubation for 20 min, and the supernatant was collected. Electrophoresis: The supernatant was heated for 5 min at 70°C for denaturation and then loaded (approximately 20 μg of protein) onto a 4% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel along with a pre-stained protein ladder and run at 100–120 V through electrophoresis. Membrane transfer: The protein was transferred onto a polyvinylidene fluoride membrane using a wet transfer system, and the quality of the transfer was verified using Ponceau staining. Blocking was conducted in 3% skim milk in 20 mM Tris buffer saline, and 0.1% Tween 20 in 1X PBS for 1 h at room temperature to reduce non-specific binding. The cells were washed thrice with phosphate-buffer saline Tween 20 solution for 5 min at a constant temperature. The cells were then incubated with primary antibodies (1:1000 of Nrf2 and HO-1) in 1X PBS overnight at 4°C with continuous shaking. Membranes were further incubated with horseradish peroxidase-conjugated secondary antibody for about 2 h and washed with 1X PBST 3 times for 5 min. Detection: The membrane was visualized using the enhanced chemiluminescent substrate 3,3'-diaminobenzidine, and the visible bands were photographed using a ChemiDoc MP system (BioRad). Densitometry: Quantitative densitometry analysis of band intensities was performed using ImageJ software. Each target protein band was normalized to the corresponding β-actin- band from the same lane, provided that β-actin- expression was under similar experimental conditions [20].

Histopathology

Histopathological investigation was performed to assess the structural and morphological alterations in the cardiac tissue. Heart tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. The sections were examined using a light microscope to detect structural alterations in cardiac tissue.

Table 1: RT-PCR primer sequences used in the present study

Gene	Primer sequence (5'→3')
Nrf2	F: TGATTTAAGCAGCATACAGCAG R: GTATTAAGACACTGTAACTCGGG
Keap1	F: TAGATGGCCACATCTACGC R: TCTCGATCTGGCTCATATCTC
HO-1	F: TGCTGACCCATGACACCAAG R: GGGCAGAATCTTGCACTTTGTT
GAPDH	F: AGGTCGGTGTGAACGGATTTG R: GGGGTCGTTGATGGCAACA

RT-PCR: Real-time polymerase chain reaction

Statistical analysis

Data are expressed as mean±standard error of the mean. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. All datasets met the assumptions for parametric analysis. Accordingly, comparisons among groups were performed using one-way analysis of variance, followed by Tukey's *post hoc* test. Statistical analyses were conducted using GraphPad Prism software (version 5.0), and statistical significance was set at $p<0.05$.

RESULTS

Total phenolic and flavonoid content and *in vitro* antioxidant activity of ELU

The ELU had a total phenolic content of 7.47±2.82 mg gallic acid equivalents per gram dry weight. The estimated flavonoid concentration was 5.36±1.46 mg rutin equivalents per gram of dry extract. The results of the DPPH radical scavenging assay of ELU demonstrated significant radical ion scavenging activity at all concentrations compared to that of the control sample.

Cytotoxicity assessment of ELU on H9C2 cells

The effect of ELU on H9C2 cell viability was validated using the MTT assay at doses ranging from 0.78125 to 200 μg/mL. Cell viability decreased as ELU concentration increased (Fig. 1), with an IC_{50} value of 82.04 μg/mL and no substantial cytotoxicity.

Acute oral toxicity assessment of ELU

Throughout the trial, no atypical clinical signs or symptoms of toxicity, death, or moribundity were detected in the animals administered 2000 mg/kg ELU. Animals treated with ELU showed no statistically significant changes in absolute body weight compared to the control group. The external surfaces of the ELU-treated animals, orifices (anal, urethral, vaginal, and nasal), and cavities (thoracic and abdominal) were examined and confirmed to be normal. Gross pathological examination revealed no anomalies; therefore, histopathology was not performed (for details, see the Supplementary Material). The globally harmonized method classifies ELU as Category 5 or unclassified because the LD_{50} is >2000 mg/kg of rat body weight. Hence, to evaluate the cardioprotective potential of ELU, doses of 50 and 100 mg/kg (i.e., 1/40th and 1/20th of the LD_{50}, respectively) were selected.

Metabolite profiling of ELU by GC-MS analysis

GC-MS analysis revealed the presence of 20 secondary metabolites in the ELU, including bergapten (Fig. 2 and Table 2). A major peak found at retention time (RT) 13.475 corresponds to a mixture of coumarin derivatives, such as bergapten and methoxsalen. The correlation of the obtained data with the NIST library confirmed the presence of bergapten (7H-Furo[3,2-g][1] benzopyran-7-one 4-methoxy; $C_{12}H_8O_4$match factor 945) and methoxalen, (9-methoxy-7H-furo[2-g] benzopyran-7-one; $C_{12}H_8O_4$ with match factor 885). There is no specific threshold confirmation for the impurities listed in Table 2, other than bergapten and methoxalen, where the match factor is <800. Hence, we did not confirm the identity of these impurities using a match factor.

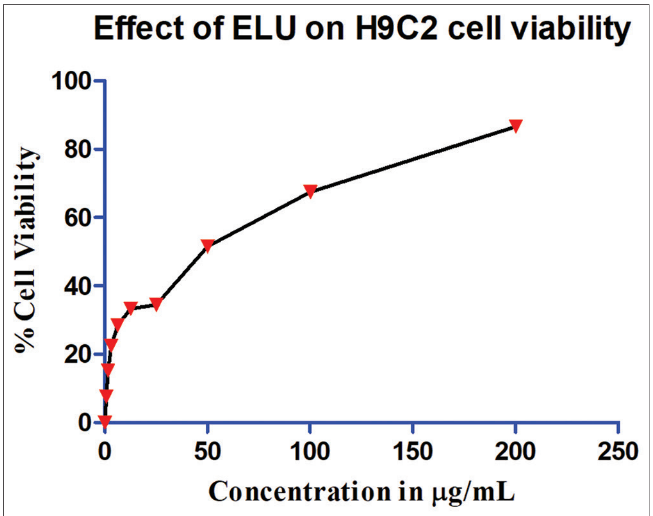

Fig. 1: Effect of ethanolic extract of *Leucas urticifolia* on H9C2 cell viability (MTT assay). Data are presented as mean±standard error of the mean (n=3). Data were analysed using one-way analysis of variance followed by Tukey's *post hoc* test

Quantification of bergapten from ELU

The quantitative assessment of bergapten in *L. urticifolia* extract produced consistent and precise results, confirming the reliability of the detection and measurement. Calibration involved five different concentrations of bergapten standards, ranging from 14.8 to 740 mg/L, and the results showed excellent linearity (supplementary material), with a correlation coefficient (R2) of 0.9997. The RT for bergapten was 12.652±0.004 min, indicating high reproducibility. The quantified bergapten value was 1.898% by HPLC using a reference standard. Furthermore, the RT in the ELU precisely matched that of the bergapten standard, confirming the presence of bergapten in the *L. urticifolia* extract (Fig. 3).

Cardioprotective effect of ELU on serum biomarkers

The levels of the cardiovascular biomarkers CK-MB, LDH, and cardiac troponin T (CTnT) were significantly ($p<0.05$) higher in the disease control group than in the normal group. ELU treatment at 50 and 100 mg/kg significantly reduced these biomarkers ($p<0.05$) compared to the disease control group, indicating a dose-dependent cardioprotective effect in experimental rats (Fig. 4).

Table 2: Tentative identification of phytochemical compounds from ELU through GCMS analysis

RT	Compound Name	Formula
10.202	Rheadan-8-ol, 2,3,10,11-tetramethoxy-16-methyl-, (6α,8α)-	$C2_2H_{27}NO_6$
	Thiourea, N-pentyl-N'-phenyl	$C_{12}H_{18}N_2S$
12.705	Rheadan-8-ol, 2,3,10,11-tetramethoxy-16-methyl-, (6α,8α)	$C_{22}H_{27}NO_6$
	Thiourea, N-pentyl-N'-phenyl	$C_{12}H_{18}N_2S$
12.812	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$
	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$
13.475	7H-Furo[3,2-g][1] benzopyran-7-one, 4-methoxy (Bergapten)	$C_{12}H_8O_4$
	Methoxsalen	$C_{12}H_8O_4$
13.862	9,12-Octadecadienoyl chloride, (Z, Z)	$C_{18}H_{31}C_lO$
	9,12-Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$
15.4	4-(3,3-Dimethyl-but-1-ynyl)-4-hydroxy-2, 6,6-trimethylcyclohex-2-enone	$C_{15}H_{22}O_2$
	3-Oxo-10 (14)-epoxyguai-11 (13)-en-6,12-olide	$C_{15}H_{18}O_4$
15.76	1-Heptatriacotanol	$C_{37}H_{76}O$
	Cedran-diol, (8S,14)	$C_{15}H_{26}O_2$
15.930	1b, 4a-Epoxy-2H-cyclopenta[3,4] cyclopropa[8,9]cycloundec[1,2-b] oxiren-5 (6H)-one, 7-(acetyloxy) decahydro-2,9,1	$C_{22}H_{32}O_8$
	4-(1-Hydroperoxy-2,2-dimethyl-6-methylene-cyclohexyl)-pent-3-en-2-one	$C_{14}H_{22}O_3$
16.238	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl) oxy]-1-[[(trimethylsilyl) oxy] methyl] ethyl ester, (Z, Z, Z)	$C_{27}H_{52}O_4Si_2$
	Cedran-diol, (8S,14)	$C_{15}H_{26}O_2$
17.922	Squalene	$C_{30}H_{50}$
	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (Z, E)	$C_{15}H_{26}O$

ELU: Ethanolic extract of *Leucas urticifolia*, GC-MS: Gas chromatography-mass spectrometry. *The dual listings for retention times represents co-eluting compounds not isomers

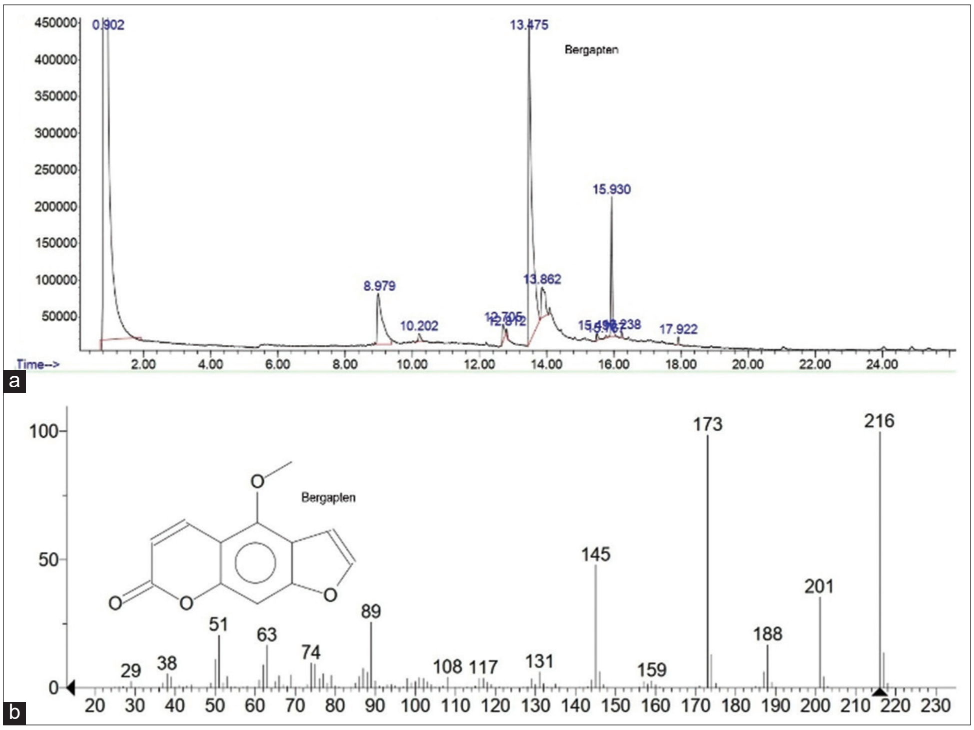

Fig. 2: Tentative identification of bergapten from ethanolic extract of *Leucas urticifolia* using gas chromatography–mass spectrometry. (a) Total chromatogram (b) Mass spectrum of bergapten

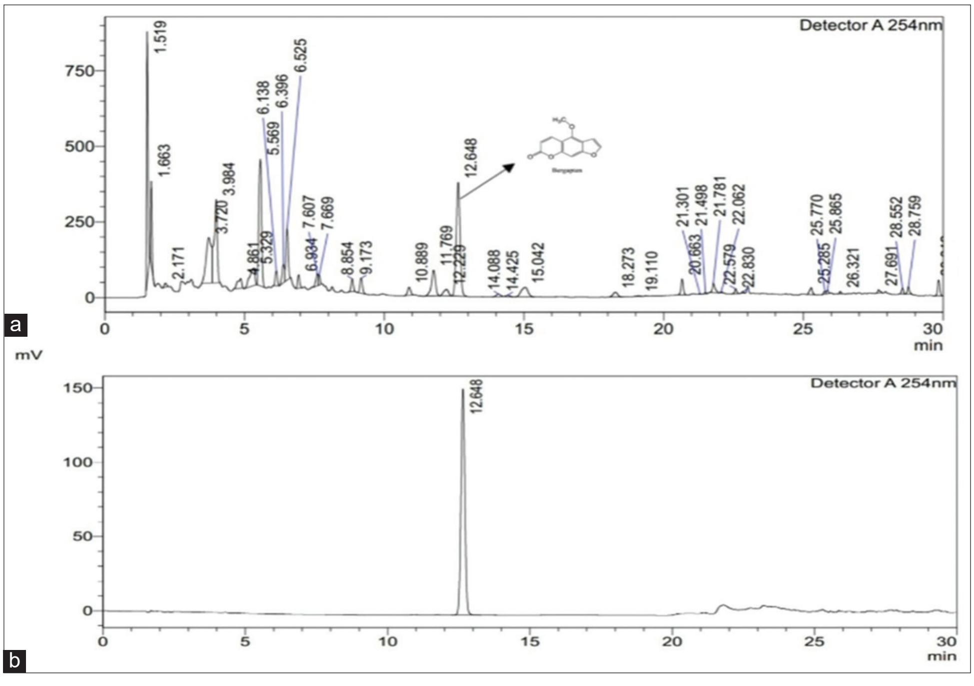

Fig. 3: (a) Total reversed-phase high-performance liquid chromatography (RP-HPLC) chromatogram of ethanolic extract of *Leucas urticifolia* (ELU) (b) RP-HPLC chromatogram of standard bergapten. The ELU was injected at a concentration of 30 mg/mL, while the reference standard bergapten was used at 20 mg/mL. The peak with a retention time of 12.648 in the ethanolic extract of *Leucas urticifolia* chromatogram (Panel a) was matched and quantified against a similar retention time of the standard bergapten (Panel b)

Effect of ELU on cardiac antioxidant enzymes and lipid peroxidation

To evaluate ELU's cardioprotective benefits of ELU, the antioxidant enzyme activity of cardiac tissue was evaluated. In the disease control group, the tissue antioxidant enzyme levels of SOD, GSH, and catalase were considerably lower ($p<0.05$), whereas the MDA levels were higher than those in the normal control group ($p<0.05$) (Fig. 4). Pre-treatment with ELU at 50 and 100 mg/kg significantly ($p<0.05$) restored antioxidant levels to near normal and enhanced MDA levels compared to the disease control group (Fig. 5).

ELU attenuated the inflammatory cytokines against ISO induce cardiotoxicity

In the disease control group, the levels of TNF-α, IL-1β, IL-6, NF-κB, and CRP were significantly higher ($p<0.05$), whereas those of IL-10 were

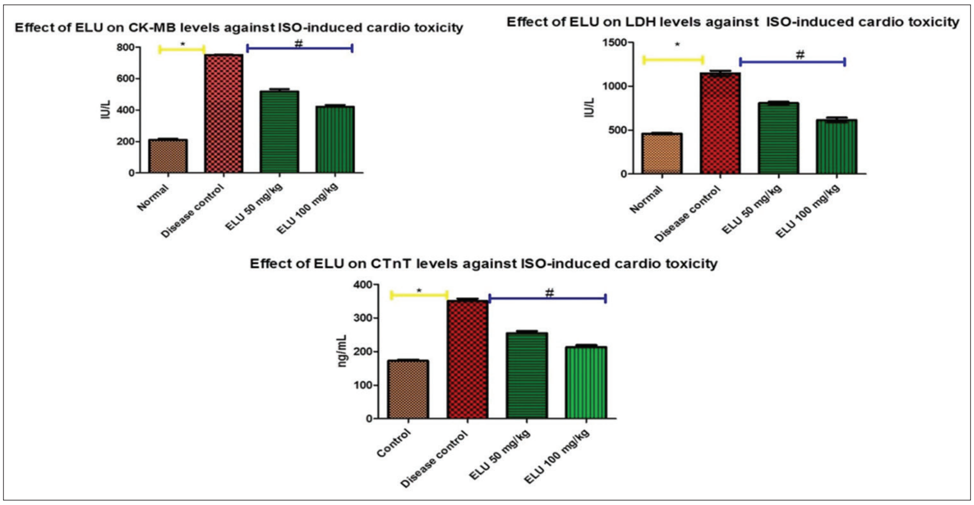

Fig. 4: Effect of ethanolic extract of *Leucas urticifolia* on cardiac biomarkers (creatine kinase-MB, lactate dehydrogenase, and cardiac troponin T) in isoproterenol (ISO)-induced cardiotoxicity in Wistar albino rats. Values are expressed as mean±standard error of the mean (n=6). Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. p<0.05 versus normal control; #p<0.05 versus disease (ISO) control

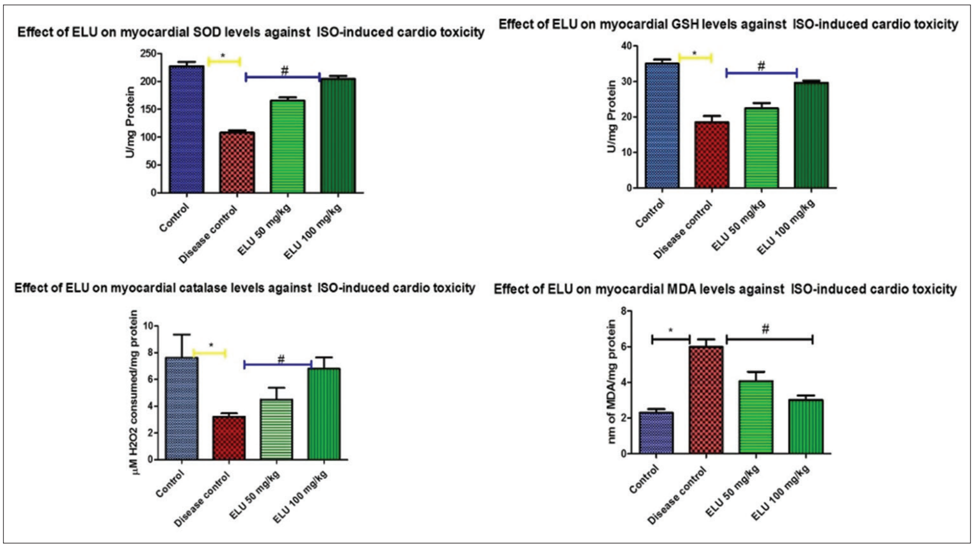

Fig. 5: Effect of ethanolic extract of *Leucas urticifolia* on tissue antioxidant enzymes in isoproterenol (ISO)-induced cardiotoxicity in Wistar albino rats. Values are expressed as mean±standard error of the mean (n=6). Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. p<0.05 versus normal control; #p<0.05 versus disease (ISO) control

significantly lower (p<0.05) than those in the normal group. Treatment with ELU at 50 and 100 mg/kg significantly (p<0.05) restored the elevated levels of inflammatory markers and increased IL-10 levels compared to the disease control (Fig. 6).

Dose-dependent modulation of the Nrf2/Keap1/HO-1 antioxidant pathway by ELU

Quantitative RT-PCR analysis revealed significant dysregulation of the Nrf2/Keap1/HO-1 antioxidant pathway in the ISO control group. Compared to the normal control, ISO administration markedly downregulated Nrf2 (p<0.05) and HO-1 (p<0.05) expression levels, while significantly upregulating Keap1 (p<0.05), indicating impaired antioxidant defense and elevated oxidative stress in the heart. Co-treatment with ELU 50 mg/kg significantly reversed these changes, as evidenced by the upregulation of Nrf2 (p<0.05 vs. ISO control) and HO-1 (p<0.05 vs. ISO control), along with a reduction in Keap1 expression (p<0.05 vs. ISO control), suggesting partial restoration of redox balance. Notably, ELU 100 mg/kg co-treatment elicited a more pronounced effect, with Nrf2 expression elevated and HO-1 levels (p<0.05 vs. ISO control), while Keap1 levels were normalized (p<0.05 vs. ISO control), indicating robust activation of the Nrf2/HO-1 antioxidant axis (Fig. 7). These findings indicate that ELU exerts dose-dependent protective effects by reactivating the Nrf2 signaling pathway and mitigating oxidative stress induced by ISO.

Western bolt analysis Nrf2/HO-1

Nrf2/HO-1 proteins were quantified from myocardial tissues experimental animals. Rats that received only ISO treatment showed a significant (p<0.05) reduction in Nrf2/HO-1 levels compared to normal rats. In contrast, pretreatment with ELU significantly (p<0.05) increased the expression and levels of these proteins compared to the disease control group (Fig. 8).

Effect of ELU on myocardial histopathological change in ISO induced cardio toxicity

Histopathological analysis of normal rat hearts revealed a normal texture with no inflamed cells, whereas the disease control group exhibited severe damage, including tissue breakdown, swelling, and

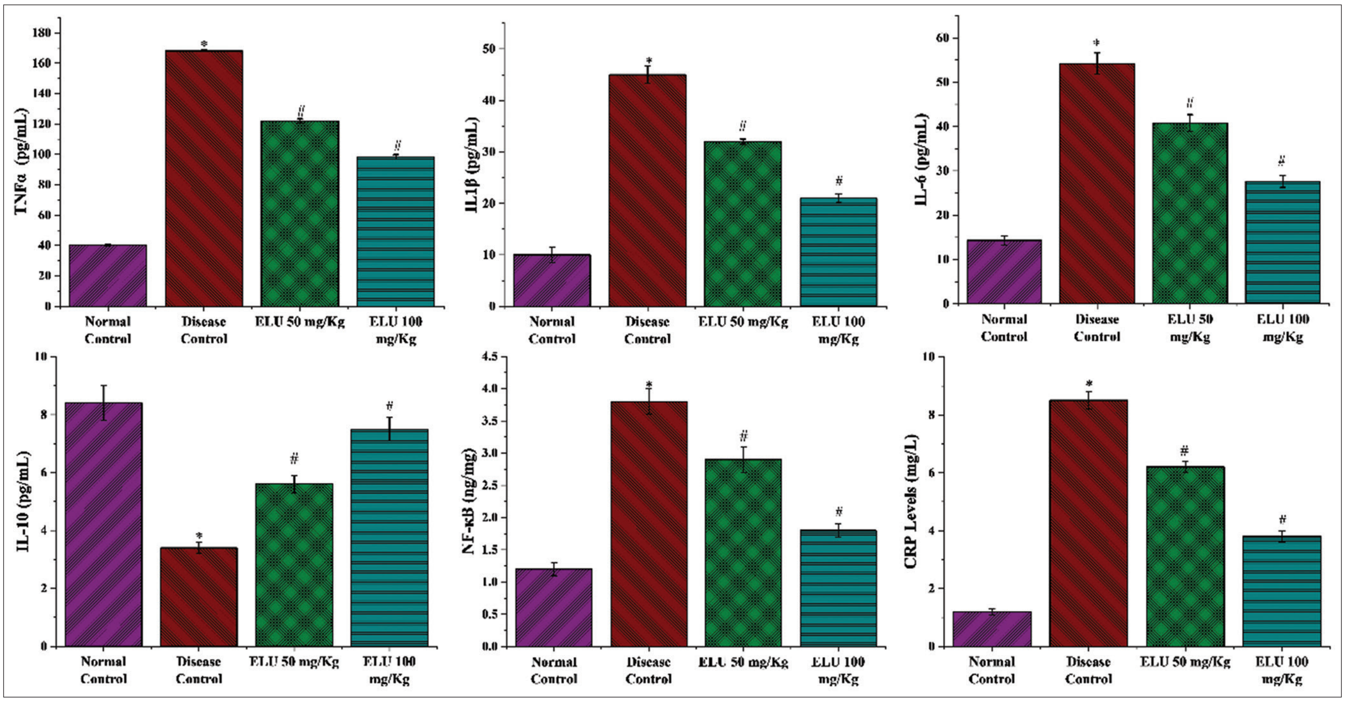

Fig. 6: Effect of ethanolic extract of *Leucas urticifolia* on inflammatory cytokines in isoproterenol (ISO)-induced cardiotoxicity in Wistar albino rats. Values are expressed as mean±standard error of the mean (n=6). Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. p<0.05 versus normal control; #p<0.05 versus disease (ISO) control

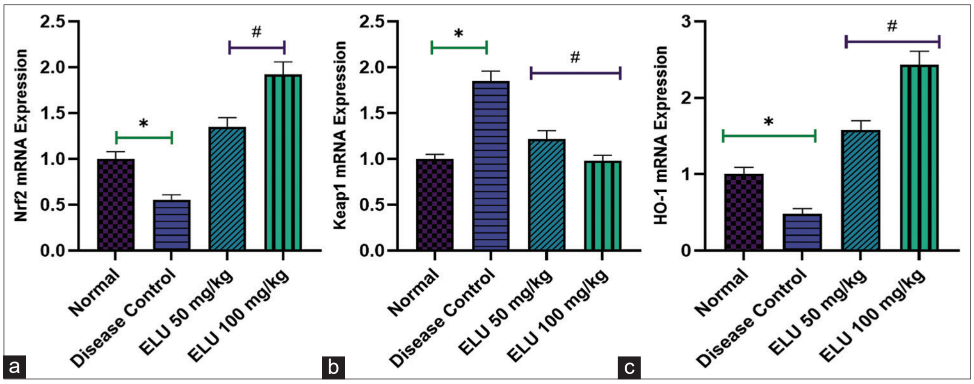

Fig. 7: Effect of ethanolic extract of *Leucas urticifolia* on (a) Nrf2, (b) Keap1, and (c) HO-1 mRNA expression in cardiac tissue. Values are expressed as mean±standard error of the mean (n=6). Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. p<0.05 versus normal control; #p<0.05 versus disease (isoproterenol) control

recruitment of inflammatory cells. In contrast, the hearts of the ELU-treated groups (50 and 100 mg/kg) showed low-grade cellular damage, fluid accumulation, and fewer inflammatory cells than those of the disease control group (Fig. 9).

DISCUSSION

CVD is currently the main trigger of morbidity worldwide [21]. It is caused by a cascade of interdependent pathological events characterized by ischemia-induced myocardial necrosis, which disrupts the integrity of cardiac cell membranes, leading to the leakage of intracellular enzymes and proteins into the bloodstream [22]. Various therapeutic approaches, including the use of medicinal plants with cardioprotective properties, have been explored to address this condition. In this study, we assessed the cardioprotective effects of ELU against ISO-induced MI in rats, which is a well-established experimental model closely resembling the pathophysiology of human MI.

L. urticifolia, a renowned medicinal plant in traditional Indian medicine, has been used to treat various health conditions due to its rich phytochemical composition. This species contains a diverse range of secondary metabolites, including phenolics, steroids, terpenoids, and coumarins, which exhibit therapeutic properties such as anti-inflammatory, antioxidant, and anticancer effects [9]. To further understand the bioactive compounds responsible for these effects, GC-MS analysis of the ELU was performed, revealing the presence of a diverse range of phytochemicals, including coumarins, such as bergapten (as the maximum peak) and methoxsalen. Bergapten is a biologically active compound known for its potential nephroprotective, anti-inflammatory and antioxidant activity [23].

Based on this phytochemical profile, the biological effects of *L. urticifolia* were explored using cytotoxicity and antioxidant activity assays. The MTT assay on H9C2 cells revealed a high IC_{50} value (82.04 μg/mL), indicating that the extract exhibited low cytotoxicity and was safe even at higher concentrations. These findings align with those of previous studies on related species, in which the hexane extract of *L. aspera* showed no adverse effects on MDA-MB-231 cell viability [24]. The *in vitro* antioxidant potential of ELU was further confirmed using the DPPH assay, which demonstrated its radical ion quenching ability.

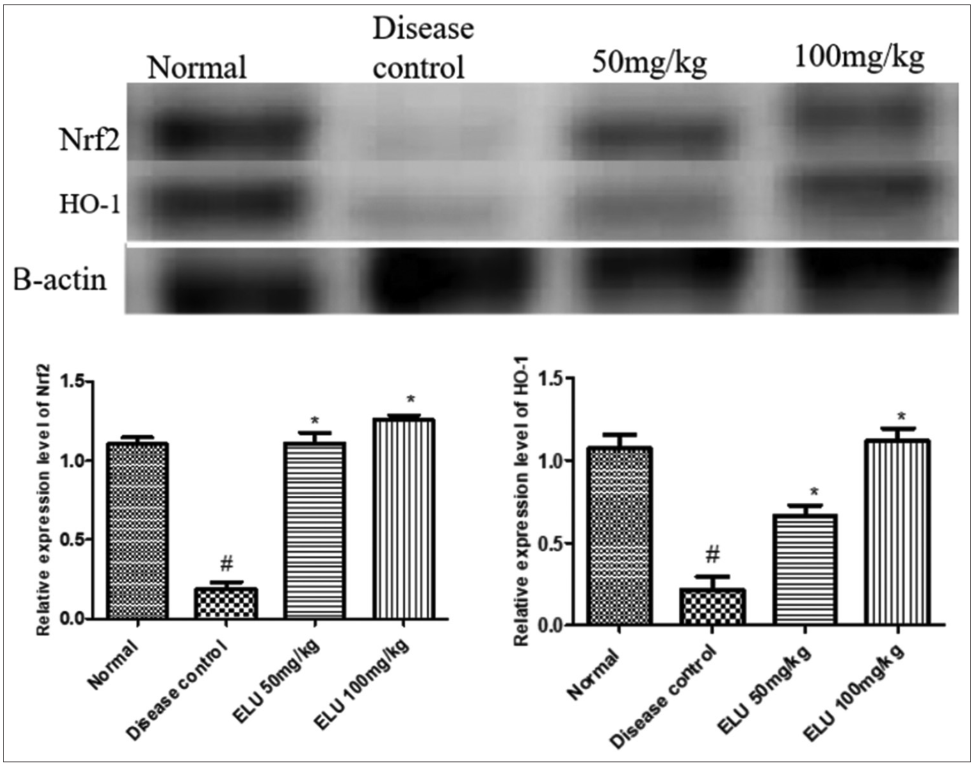

Fig. 8: Effect of ethanolic extract of *Leucas urticifolia* on Nrf2 and HO-1 levels. All data are presented as the mean±standard error of the mean and were subjected to one-way analysis of variance with Tukey's *post hoc* test. #p<0.05, when normal compared with disease control; *p<0.05, when test groups compared with disease control

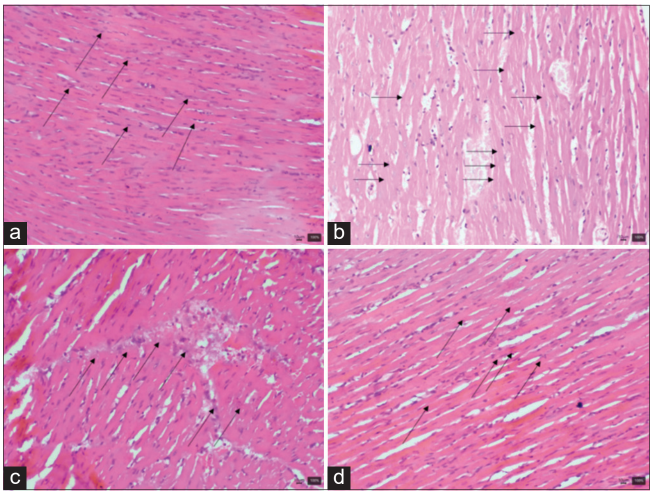

Fig. 9: Histopathology of heart stained with hematoxylin and eosin observed at ×100. (a) normal control (b) disease control (c) ethanolic extract of *Leucas urticifolia* (ELU) 50 mg/kg (d) ELU 100 mg/kg. The black arrow indicates the infarction region with enucleation, fibrosis, and fluid in panel B, and normal architecture or low-level damage in the heart muscle in panels a, c, and d

Previous studies have identified 11 biologically active polyphenols in the alcoholic extract of *L. urticifolia*, which contribute to its antioxidant properties [25]. In addition, acute toxicity testing in rats indicated no mortality or symptoms of toxicity at a single dose of 2000 mg/kg, verifying the safety profile of the extract.

The cardiotoxicity model employed in this study was based on ISO, a synthetic catecholamine that stimulates β-adrenergic receptors, leading to increased myocardial oxygen consumption, excessive reactive oxygen species (ROS) production, and intracellular calcium overload [26]. These effects collectively induce oxidative stress, leading to myocardial cell damage, which is reflected by elevated levels of key cardiac biomarkers, such as CK-MB, LDH, and CTnT, which are hallmark indicators of myocardial injury [27]. The present study confirmed a significant increase in these biomarkers in the ISO-treated group, indicating extensive myocardial damage. Furthermore, oxidative stress-induced damage amplifies the pro-inflammatory response by recruiting immune cells, triggering cytokine release, and exacerbating tissue injury [28]. ISO-treated rats had higher levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, NF-κB, and CRP) and lower levels of the anti-inflammatory cytokine IL-10. This proinflammatory milieu contributes to myocardial fibrosis and adverse tissue remodeling, thereby aggravating cardiac dysfunction [29]. Pre-treatment with ELU significantly mitigated the adverse effects of ISO. A dose-dependent reduction in CK-MB, LDH, and CTnT levels was observed, with the most pronounced effect observed at 100 mg/kg. Furthermore, ELU treatment effectively suppressed pro-inflammatory cytokines while restoring IL-10 levels, thereby disrupting the vicious cycle of inflammation and oxidative stress. The suppression of NF-κB signaling by ELU highlights its molecular mechanism in modulating inflammation, as NF-κB activation drives the expression of multiple pro-inflammatory cytokines [30].

Mohsin *et al*. observed a significant anti-inflammatory effect of bergapten by restoring proinflammatory markers such as IL-1β, IL-6, and TNF-α against cyclophosphamide-induced nephrotoxicity in rats [31]. Similar results were observed in the present study on pretreatment with the ELU against ISO-induced cardiotoxicity in rats. In addition, the present study identified and quantified bergapten in L. *urticifolia*, confirming its use in alternative medicinal systems as a potential cardioprotective agent. Furthermore, Kripa *et al*. validated the anti-inflammatory properties of *Leucas* species by observing a significant reduction in TNF-α, IL-2, and CRP levels in an adjuvant-induced arthritis model, thereby reinforcing the anti-inflammatory or immunomodulatory potential of ELU [32].

At the cellular level, the body's defense against oxidative stress and inflammation is mediated by antioxidant enzymes such as SOD, GSH, and catalase. ISO-induced auto-oxidation generates highly toxic free radicals, including quinones and ROS, which disrupt the balance of antioxidant enzyme activity, resulting in extensive myocardial damage [33]. Although previous studies have primarily focused on the *in vitro* antioxidant potential of *L. urticifolia* [34], this study is the first to report its *in vivo* antioxidant efficacy in rats. Pre-treatment with ELU significantly reduced MDA levels and restored tissue antioxidant enzyme (SOD, GSH, and catalase) activities in a dose-dependent manner, thereby neutralizing ROS and enhancing the antioxidant defense. GC-MS analysis identified the presence of bergapten, which has been shown to have a potential antioxidant effect [35] and may play a key role in attenuating oxidative stress and restoring tissue antioxidant enzyme levels in ELU pretreated rats.

The antioxidant potential of ELU was further investigated by modulating the Nrf2/Keap1/HO-1 antioxidant pathway, which is closely linked to its rich phytochemical composition, as identified by GC-MS analysis. ELU contains a diverse array of bioactive compounds, including coumarins and other unidentified secondary metabolites that potentially contribute to the upregulation of the Nrf2/HO-1 axis in ISO-induced myocardial infarcted rats [36]. Similarly, in another study, bergaptene successfully inhibited LPS-induced inflammation in RAW cells by activating the Janus kinase/signal transducer and activator of transcription signaling pathway, along with exhibiting potential antioxidant activity by reducing intracellular ROS levels [37]. Supporting this mechanistic basis, gene expression analysis in the present study revealed that ELU co-treatment significantly restored Nrf2 and HO-1 mRNA levels while downregulating Keap1 in ISO-induced myocardial injury in rats. The dose-dependent upregulation of Nrf2 and HO-1 suggests that ELU enhances the transcription of cytoprotective genes involved in maintaining redox balance. Notably, the higher dose of ELU (100 mg/kg) elicited a more pronounced effect, indicating its greater efficacy in reestablishing oxidative homeostasis.

Furthermore, ELU-pretreated rats exhibited increased SOD and GSH levels in the heart tissues, which are part of the downstream pathways of Nrf2/HO-1 [15]. Thus, quantifying these proteins helps to understand the precise mechanism by which ELU protects against ISO-induced cardiotoxicity. ELU treatment may induce Nrf2 activation, allowing the activation of antioxidant-responsive elements and enhancing tissue antioxidant enzyme [38]. One of the important phytochemicals quantified from ELU is bergapten, which has been proven to have antioxidant activity against scopolamine-induced memory impairment in rats [39] and may also be involved in the activation of the Nrf2/HO-1signaling pathway. However, a limitation of this study was that we did not isolate and elucidate the role of bergapten in the activation of the Nrf2/HO-1 signaling pathway. A recent study found that supplementation with bergapten, a biologically active furocoumarin, in ISO-induced MI rats effectively restored the tissue antioxidant and cardiac biomarkers, along with the attenuation of ISO-induced myocardial apoptosis through the activation of the AMPK/eNOS/AKT signaling pathway [40]. The present study corroborates that bergapten from ELU may contribute potential for the cardioprotective activity of *L. urticifolia* by activating the Nrf2/HO-1 signaling pathway against ISO-induced MI.

Collectively, these findings imply that the cardioprotective effects of ELU are at least partly mediated through phytochemical-induced activation of the Nrf2/Keap1/HO-1 axis, thereby contributing to the attenuation of oxidative stress and myocardial injury. Finally, histopathological analysis provided direct evidence of ELU's cardioprotective effects of ELU. Severe necrosis, inflammation, and myocardial disarray were observed in the ISO-treated group, whereas ELU pretreatment preserved the myocardial architecture, reduced necrotic damage, and decreased inflammation. These findings further reinforce the therapeutic potential of ELU in mitigating myocardial injury and preventing cardiac deterioration.

CONCLUSION

The present study demonstrated the cardioprotective efficacy of ELU in mitigating ISO-induced MI in rats through marked reduction of elevated cardiac biomarkers, suppression of pro-inflammatory cytokines, and improvement of lipid profiles, while simultaneously enhancing antioxidant defenses. GC-MS analysis revealed the presence of biologically active furocoumarins, such as bergaptene, which were quantified using RP-HPLC. These compounds may have contributed to the modulation of the Keap1/Nrf2/HO-1 signaling axis, exerting cardioprotective effects of ELU. In addition, histopathological analysis confirmed substantial restoration of myocardial architecture in the ELU-treated groups, underscoring ELU's multifaceted therapeutic potential. This study found that *L. urticifolia* extract enhances antioxidant defense mechanisms, regulates inflammatory cytokines, and preserves cardiac tissue architecture, thereby underscoring its therapeutic potential. Nevertheless, further research is required to isolate, purify, and characterize the individual bioactive constituents, elucidate the specific molecular mechanisms of ELU, and evaluate its efficacy in other cardiovascular and inflammatory models before clinical investigation.

ETHICS APPROVAL

The experimental design and implementation strictly followed the ethical criteria established by the Committee for Control and Supervision of Experiments on Animals and the Institutional Animal Ethics Committee.

DATA AVAILABILITY

The data generated during the study are provided upon request.

AUTHOR'S CONTRIBUTION

Nemalapalli Yamini: Methodology, data analysis, and drafting original manuscript. Juturu Mastanaiah: Conceptualization, Supervision, Writing - review and editing.

COMPETING INTEREST

The authors declare no conflicts of interest.

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