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Unveiling the dual antioxidant power of *Androsace sempervivoides* L.: in vitro, in vivo and histopathological insights in LPS-induced inflammation in albino Wistar rat models

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Abstract

Background Inflammation and oxidative stress are pivotal in the initiation and progression of several chronic diseases. Plant-derived bioactive compounds rich in phytochemicals are increasingly being explored as safer alternatives to synthetic drugs. *Androsace sempervivoides*, a native medicinal plant of the Ladakh region, possesses promising bioactive potential and has been extensively employed in traditional medicinal practices; however, its therapeutic activities remain largely unexplored. In vitro antioxidant assays, including DPPH, reducing power, hydroxyl radical scavenging, metal chelation (ferrozine), levels of total phenolics (TPC) and total flavonoids (TFC), and DNA damage protection, were carried out using standard procedures. Forty two (42) adult male albino Wistar rats (170–200 g, 6–8 weeks old) were randomly allocated to seven groups. Six rats per group were used to determine antioxidant enzyme levels and to perform histopathological studies. Western blotting and qRT-PCR analyses were conducted to evaluate the protein and gene expression of various inflammatory markers.

Results Among all the extracts evaluated, the ethanol extract (ASE) and ethyl acetate extract (ASEA) displayed the strongest antioxidant potential. The DPPH radical scavenging activity was $86.11 \pm 2.21\%$ and $78.46 \pm 2.56\%$, reducing power was 4.630 ± 0.46 and 4.626 ± 0.34 , hydroxyl radical scavenging activity was $85.89 \pm 1.88\%$, and $73.07 \pm 2.05\%$, and metal chelation activity was $95 \pm 2.48\%$ and $88 \pm 4.31\%$, for the ASE and ASEA, respectively ($P < 0.0001$, $n = 3$). The TPC of the same extracts was 784.68 ± 9.39 and 713.7 ± 8.14 mg GAE g⁻¹ extract, while TFC was 537.59 ± 6.38 and 489.08 ± 5.02 mg QUE g⁻¹ extract, respectively ($P < 0.001$, $n = 3$). Both extracts also effectively protected DNA from Fenton reaction-induced DNA damage, confirming their strong antioxidant and protective potential. In vivo, these extracts significantly restored elevated superoxide dismutase (SOD) and catalase (CAT) levels, with the maximum effect observed with ethanol, followed by ethyl acetate extract. Western blot and qRT-PCR analyses showed marked suppression ($P < 0.05$) of the inflammatory markers TNF α and IL1 β at both the protein and mRNA

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levels. Histopathological evaluation showed that ethanol (100 mg/kg bw), followed by ethyl acetate (100 mg/kg bw), protected against tissue damage and blood vessel rupture, comparable to that of standard dexamethasone.

Conclusion Overall, *Androsace sempervivoides*, particularly its ethanol and ethyl acetate extracts, revealed remarkable antioxidant and anti-inflammatory capacities, underscoring its potential as an emerging natural source for formulating therapeutic agents against oxidative stress and inflammation-related disorders.

Keywords *Androsace sempervivoides*, Antioxidant activity, In vitro assays, Inflammation, Oxidative stress, Phytochemical screening

Background

Oxidative stress (OS) describes a biological state in which the formation of reactive oxygen species (ROS) outstrips endogenous antioxidant defence mechanism. Under normal physiological conditions, ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide are produced in cells as metabolic by-products; however, they are crucial mediators of signal transduction and homeostasis (Zhang et al., 2022). The excessive build-up of ROS triggers oxidative stress, leading to damage of key biomolecules including proteins, nucleic acids, and lipids, thereby compromising cell function and viability (Juan et al., 2021). Mounting evidence indicates that oxidative stress has a pivotal implication in the development and progression of chronic and degenerative disease conditions including, diabetes mellitus, heart disease, stroke, cancer, rheumatoid arthritis, and neurodegenerative disorders (Leyane et al., 2022). Antioxidant enzymes such as SOD, CAT, and GPx, together with non-enzymatic compounds like vitamin E, vitamin C and glutathione, constitute the body's defence against oxidative stress (Irato & Santovito, 2021). These systems neutralize ROS and balance redox potential. However, prolonged or intense oxidative stress neutralizes these protective systems and causes the scale to tip towards cellular damage and inflammation (Chatterjee, 2016). This crosstalk between ROS and inflammation advocates a vicious cycle: the expression of pro-inflammatory mediators is activated by redox-sensitive transcription factors such as NF- κ B, which stimulate themselves by their active complex formation, along with amplification of tissue injury and disease progression (Kostenko et al., 2023).

To cope with oxidative stress, several synthetic antioxidants, particularly BHA and BHT, are utilized in food preservation and therapeutic applications (Stoia & Oancea, 2022). Nonetheless, emerging concerns about their toxicity, putative carcinogenicity, and narrow safety margins preclude their long-term use (Awulachew, 2024). On the other hand, bioactive compounds, especially those of plant origin, have received greater attention as functional foods because they are safe, abundant, and possess multifunctional health-promoting properties (Mondal et al., 2021). Phytochemicals such as polyphenols, flavonoids, tannins, and terpenoids possess potent free radical

scavenging activities, regulate endogenous antioxidant enzymes, and suppress inflammatory signaling pathways, making them promising substitutes for synthetic agents (Chaudhary et al., 2023).

Androsace sempervivoides (family *Primulaceae*), locally called evergreen rock jasmine, is a perennial plant of the Alpine belt that grows in the alpine areas of Tibet, Afghanistan, Pakistan, and Ladakh at an altitude of 3000–4000 m (Zeng et al., 2010). In Tibetan medicine, it is extensively acknowledged due to its potent anti-tumor, inflammation-modulating properties and in treating diseases associated with serous fluid accumulation (Li et al., 2023). Ethnopharmacological reports list this as a resolutive, with a bitter taste and cooling properties. Although the plant has been used for medicinal purposes since ancient times, it has not yet been scientifically scrutinized, and its pharmacological features, particularly its antioxidant and anti-inflammatory effects, are not extensively documented.

The present investigation assessed the anti-inflammatory and antioxidant potential of *Androsace sempervivoides* extracts. In vitro (DPPH, radical scavenging, reducing power, hydroxyl radical scavenging, ferrozine metal chelation, and DNA protection) and in vivo studies on lipopolysaccharide (LPS)-induced inflammatory Wistar rat models were performed. Western blotting and qRT-PCR were used to determine the regulation of pro-inflammatory cytokines at the molecular level. Overall, the current study presents the first scientific evidence validating the traditional use of *Androsace sempervivoides*, highlighting its potential as natural leads for the development of therapeutic agents combating oxidative stress and inflammation-associated pathologies.

Materials and methods

Chemicals

Ascorbic acid, DPPH and gallic acid were bought from Sigma-Aldrich. Himedia provided all additional analytical-grade chemicals.

Plant material

The *Androsace sempervivoides* were collected in July and August 2021 from the higher altitudes of the Kargil District, Ladakh (Union Territory), India. Its identification

was done at Department of Botany (Centre for Biodiversity and Taxonomy), University of Kashmir, Srinagar, India. A reference specimen, designated as specimen No. 4185-KASH bot was placed in the herbarium affiliated with the Department of Botany.

Extract preparation

Whole plant samples were shade-dried at room temperature until a constant weight was achieved. A mortar and pestle were used to initially crush the material, which was then dried and sieved over a 0.3 mm mesh screen. Soxhlet extraction of the powder was carried out with multiple solvents, namely hexane, ethyl acetate, ethanol, methanol, and distilled water for 48 h within a temperature of 60 to 80 °C. Each extract underwent concentration under reduced pressure employing a rotary evaporator. The solidified extracts were then refrigerated at -20 °C.

Phytochemical screening

Following Soxhlet extraction, the extracts were subjected to phytochemical screening. Phytochemicals, including flavonoids, phenols, tannins, terpenoids, anthracenoides, saponins, sterols, and steroids, were identified using established methods, as described by Anees et al. (2018).

In vitro anti-oxidant analysis

DPPH free radical-scavenging assay

The DPPH free radical-quenching activity of plant extracts was executed as per the method outlined by Anees et al. (2018), with slight modifications. Plant extracts at different concentrations (100–600 µg/mL) were blended with one mL of 0.02% DPPH solution and vortexed. After 30 min incubation under dark conditions at room temperature, the absorbance was taken at 517 nm. Reaction combinations with lower absorbance are more efficient in scavenging free radicals. Butylated hydroxytoluene functioned as the reference control. The inhibition percentage was computed using the equation presented below:

$$\text{Percentage inhibition} = \{(1 - (A_S/A_C)) \times 100\}.$$

A_C denotes control sample's absorbance and A_S reflects absorbance measured with the standard or *Androsace sempervivoides* extract.

Reducing power assay

The reducing capacity of the plant extracts was evaluated using a slightly modified procedure described by Anees et al. (2018). A range of concentrations of the plant extracts were combined with equivalent volume (2.5 mL each) of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferrocyanide $K_4[Fe(CN)_6]$, allowed to incubate for 20 min at a temperature of 50 °C. After incubation, 2.5 mL of TCA solution (10%) was incorporated, and

the resulting mixture was then centrifuged for 10 min at 3000 rpm. An aliquot of the supernatant (2.5 mL) was subsequently combined 0.5 mL of 0.1% $FeCl_3$ solution and 2.5 mL distilled water. The absorbance readings were recorded at 700 nm with a spectrophotometer (Thermo Scientific, USA). BHT was used as the standard. The development of Perl's Prussian blue complex, denoting Fe^{2+} generation, corresponded to higher absorbance values.

Hydroxyl radical scavenging assay

The efficacy of the plant extracts to counteract hydroxyl radicals was determined using the method adopted from Anees et al. (2018), with minor refinements. The Fenton reaction system (Fe^{3+} -ascorbate- H_2O_2) generates hydroxyl radicals that degrade deoxyribose, forming thiobarbituric acid reactive substances (TBARS). The sample mixture comprised 25 mM deoxyribose, 20 mM ferric nitrate, 100 mM ascorbic acid, and 30 mM H_2O_2 in the presence of 10 mM potassium phosphate buffer (pH 7.4) with different concentrations of plant extracts (100–600 µg/mL). After incubation at 37 °C for one hour, equal volumes (1 mL each) of 10% trichloroacetic acid and 10% thiobarbituric acid were added and the reaction mixture was heated for 20 min at 100 °C. The mixture was cooled under running water, and the absorbance measurement was performed at 532 nm using a spectrophotometer (Thermo Scientific, USA). The extent of hydroxyl radical suppression was quantified using the equation provided below:

$$\text{Percentage inhibition} = \{(1 - (A_S/A_C)) \times 100\}$$

A_C denotes control sample's absorbance and A_S reflects absorbance measured with the standard or *Androsace sempervivoides* extract.

Metal chelating activity using ferrozine

Ferrous ion sequestration by the extracts and the standard was analysed following the procedure reported by Dinis et al. (1994). A range of different extract concentrations (100 to 600 µg/mL) were combined with 0.05 mL of 2 mM $FeCl_2$. To initiate the reaction, 0.2 mL (5 mM) of ferrozine was added. After thorough mixing the solution was incubated at 25 °C for ten minutes and the volume was made up to 1 mL using methanol. The absorbance measurement was taken at 562 nm with a spectrophotometer (Thermo Scientific, USA). The percentage chelating impact of the active extracts and the standard was determined employing the equation presented below:

$$\text{Percentage chelating effect} = \{(1 - (A_S/A_C)) \times 100\}$$

A_C denotes control sample's absorbance and A_S reflects absorbance measured with the standard or *Androsace sempervivoides* extract.

Total phenolic content determination

The Folin-Ciocalteu assay was employed to quantify the phenolic content, using gallic acid as the standard. Different extract concentrations (100–600 µg/mL), were carefully added to 2.5 mL of Folin-Ciocalteu reagent (10%), with the subsequent addition of 2 mL of sodium carbonate solution (2.5%). Following thorough shaking, the mixture was maintained at 45 °C for 45 min. Absorbance was recorded at 700 nm, and the total phenolic content was evaluated by comparing sample absorbance to a Gallic acid (GAE) standard calibration curve.

Total flavonoid content determination

The aluminium chloride based colorimetric method was employed to estimate total flavonoid content. An aliquot of 0.3 mL of each plant extract at concentrations ranging from 100 to 600 µg/mL was combined with 3.4 mL of 30% methanol, 0.15 mL of 0.3 M aluminium chloride and 0.15 mL of sodium nitrite (NaNO_2 , 0.5 M). After incubation for 5 min at room temperature, one mL of 1 M sodium hydroxide was introduced into the reaction mixture and the final volume was brought to 10 mL using distilled water. Upon homogenization, the solution's absorbance was measured at 505 nm using a spectrophotometer (Thermo Scientific, USA) against a reagent blank. Flavonoid concentration was measured based on a Quercetin standard calibration curve (0–100 mg/L) and expressed as milligrams of quercetin equivalents (mg QUE/g) of dried extract.

Antioxidant assay evaluating protection against oxidative DNA damage

The ability of the plant extracts to protect against DNA damage was evaluated using the procedure outlined by Anees et al. (2018), with small adjustments. The assay mixture containing 5 µL plant extract at different concentrations (30, 50, and 80 mg/mL) along with 15 µL of calf thymus DNA was incubated with 5 µL of Fenton reagent formulation (30 mM hydrogen peroxide, 100 mM ascorbic acid and 20 mM ferric nitrate solution). The final reaction volume of 25 µL was incubated at 37 °C for 30 min. Afterward, the samples were loaded onto 0.7% ethidium bromide stained agarose gel and visualized with a Gel Doc™ XR+ Imaging System (Bio-Rad, USA).

In vivo antioxidant analysis

Experimental animals

Adult male Wistar albino rats (170–200 g, 6–8 weeks old) were obtained from the Indian Institute of Integrative Medicine (IIIM), Jammu, India. Forty-two rats were

employed for this study and randomly assigned to seven groups, each consisting of six rats. Ethical clearance for the experimental protocol was granted under the authorization of the IAEC, University of Kashmir (approval no. F [IAEC approval] KU/2023/09, dated January 24, 2023). Prior to the commencement of the study, all animals were acclimatized for two weeks under standard laboratory conditions. Rats were maintained under controlled environmental conditions kept at 23 ± 2 °C with $50 \pm 20\%$ relative humidity, and a 12-hour light/dark photoperiod. The animals were treated humanely throughout the study and were provided ad libitum access to a standard pellet diet and water.

Grouping

The study comprised seven groups, containing six Wistar rats per group, as illustrated in (Fig. 1).

Treatment

The control group received normal saline throughout the experiment. On day 15, the negative control group was administered a subplantar injection of 100 µL lipopolysaccharide (LPS) at a concentration of 0.5 mg/kg into the right hind paw producing localized inflammation/edema and oxidative stress. Experimental groups 4–7 were treated with plant extracts (ethanol and ethyl acetate extract dissolved in normal saline) administered through oral gavage at dosage levels of 50 and 100 mg/kg body weight for 14 consecutive days. The doses of 50 and 100 mg/kg bw were selected according to acute oral toxicity testing conducted in our laboratory, where no adverse effects or mortality were observed up to 2500 mg/kg, confirming the safety of the extracts in accordance with OECD-423 guidelines (data not shown). On day 15, these groups also received a subplantar injection of 100 µL of LPS. The positive control group underwent dexamethasone treatment and was similarly challenged with LPS on day 15. Three hours after LPS administration, all animals were humanely euthanized for collection of blood and paw tissue samples. Paw tissue was collected for collected for histopathological and molecular analyses, serving as a validated model to investigate the in vivo modulation of inflammatory and oxidative responses by *Androsace sempervivoides*.

Histopathological studies

Excised paw tissues were fixed in neutral-buffered formalin (10%) for preservation. Following fixation, the tissues were dehydrated using a graded series of ethanol and acetone solutions and cleared using benzene as a clarifying agent. The processed tissue specimens were then mounted in paraffin wax with a melting point of 60 °C to form tissue blocks. Sections of 5 µm thickness were obtained using an automated rotary microtome (Leica

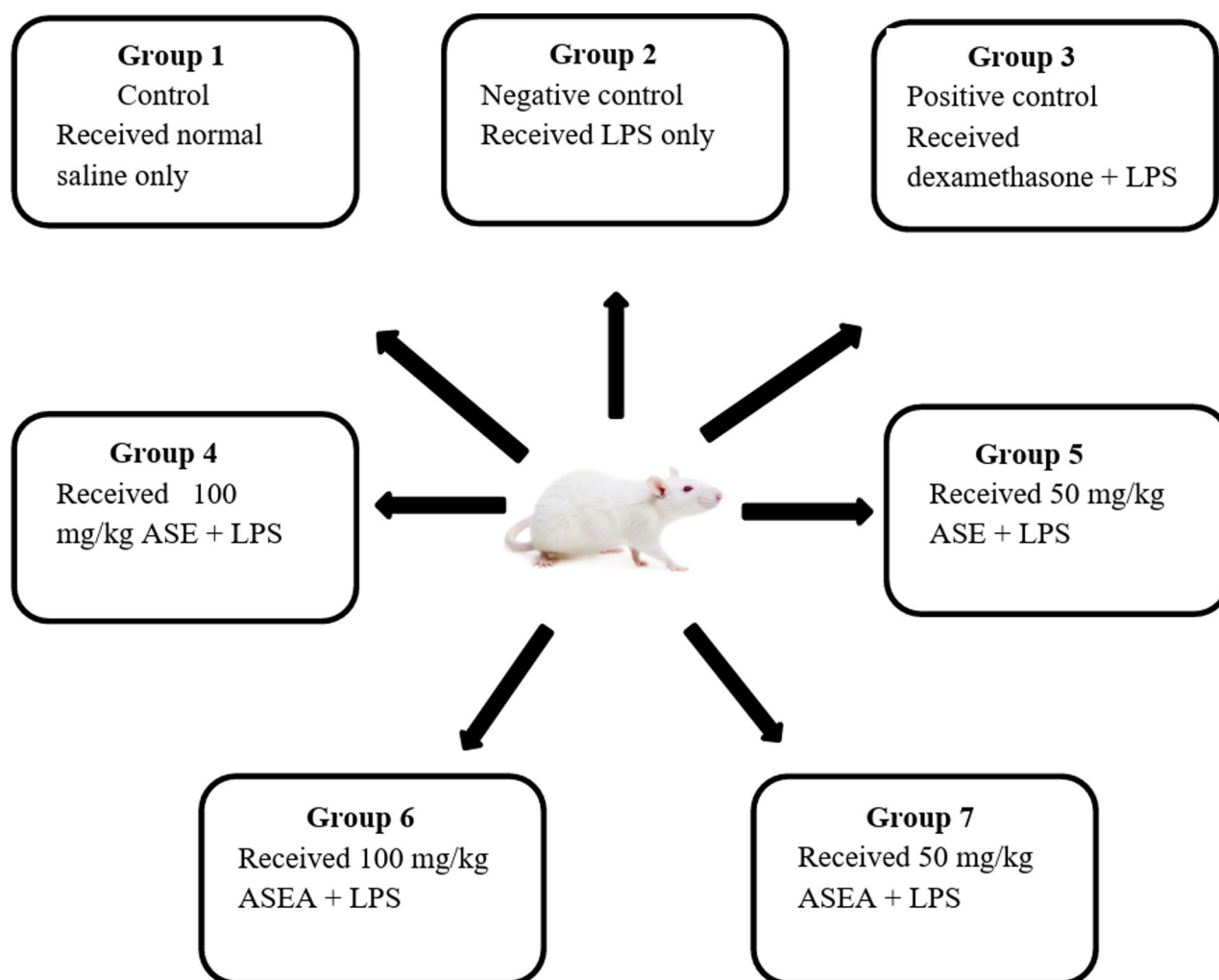


Fig. 1 Grouping of experimental animals- A total of seven groups, each consisting of six Wistar rats

Biosystems, Germany). These sections were subsequently stained using Harris's H&E stain and examined under an inverted light microscope (Carl Zeiss, Germany) to assess the histological alterations and the impact of the active plant extracts on tissue morphology.

Estimation of oxidative stress markers

To evaluate the contribution of oxidative stress in the pathophysiology of inflammation and to analyze the ameliorative effects of the active plant extracts, the enzymatic activities of key oxidative stress biomarkers, CAT and SOD, were measured in paw tissue samples. Excised paw tissues were first rinsed with normal saline followed by ice-cold 1.15% KCl solution to remove blood and debris. The tissues were then homogenized in a homogenizing buffer using a mortar and pestle, followed by Centrifugation at 9,000 rpm for 20 min at 4 °C to separate cell debris. The resulting supernatant underwent a second centrifugation step at 15,000 rpm for a duration of 20 min at 4 °C to collect the post mitochondrial supernatant (PMS), for

enzymatic assays. Protein levels in PMS were analysed with a NanoDrop spectrophotometer (Thermo Scientific, USA). CAT and SOD activities were determined using standard biochemical assay protocols.

Estimation of catalase (CAT)

Catalase activity was assessed based on its capacity to degrade hydrogen peroxide (H_2O_2), as described by Trawczyńska (2020). The assay mixture contained 100 μ L of the tissue sample (post-mitochondrial supernatant) and 100 μ L of H_2O_2 substrate. After an incubation period of 60 s, the reaction was terminated by adding 200 μ L of ammonium molybdate (32.4 mM) solution. The change in absorbance was monitored at 405 nm with a spectrophotometer (Thermo Scientific, USA). CAT activity was expressed as the enzyme quantity that decomposes 1 μ mol of H_2O_2 per minute per milligram of protein.

Estimation of super oxide dismutase (SOD) activity

Superoxide dismutase activity was analysed based on the procedure outlined by Weydert and Cullen (2010). The formulation of the reaction mixture included 20 μ L of 0.1 mM EDTA, 100 μ L of sodium carbonate buffer (50 mM, pH 10.2), 40 μ L of 25 μ M nitro blue tetrazolium (NBT) and 50 μ L of PMS derived from the paw tissue homogenate. The reaction commenced upon the addition of 40 μ L of hydroxylamine hydrochloride (1 mM). The measurement of absorbance was performed at 560 nm through a Thermo Scientific spectrophotometer (USA), and a control reaction was simultaneously performed under identical conditions without the tissue homogenate. One unit of SOD activity reflects the enzyme concentration that suppresses NBT reduction by half.

Protein extraction and Western blotting

The expression levels of the inflammatory markers (TNF- α and IL-1 β), were evaluated in vivo using western blotting. Proteins were extracted from paw tissue samples using a lysis buffer composed of Tris-HCl (50 mM, pH 8.0), NaF (10 mM), NaCl (150 mM), phenylmethylsulfonyl fluoride (1 mM), EDTA (2 mM, pH 8.0), 0.25% sodium deoxycholate, 0.1% SDS, 1% NP-40, 10% glycerol, and 10 μ L/mL protease inhibitor cocktail. Tissue samples were placed on ice in lysis buffer for 40 min and subsequently centrifuged at 10,000 rpm at 4 °C for 10 min. Total protein was carefully collected in fresh Eppendorf tubes, by collecting supernatant and the pellet was discarded. Protein concentrations were determined with a NanoDrop spectrophotometer (Thermo Scientific, USA). Equivalent protein amounts were loaded onto the SDS-PAGE gel and electrophoretically separated. Following transfer onto nitrocellulose membranes, the blots were incubated in blocking solution for one hour at room temperature prepared using 5% non-fat dry milk formulated in Tris-buffered saline (TBS). After blocking step, overnight incubation at 4 °C was performed with primary antibodies specific to TNF- α and IL-1 β (Abcam) at a dilution of 1:1000 in blocking buffer. After incubation, the membranes were subjected to three washes with TBS containing 0.1% Tween-20 (TBS-T) and subsequently

incubated for three hours with the appropriate secondary antibody (Cell Signalling Technology, CST), diluted to a 1:1000 ratio in TBS. After another series of three washes with TBST, the protein bands were visualized using an LI-COR imaging system (Odyssey CLx).

Total RNA extraction

Expression analysis of TNF- α and IL-1 β was performed following total RNA extraction from tissue samples using the conventional TRIzol method (Rio et al., 2010). The tissue was finely minced and homogenized in 1 mL of TRIzol reagent and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was carefully decanted into a new Eppendorf tube, discarding the pellet, and 200 μ L of ice-cold chloroform was added. After a 15-second vortex, the mixture underwent centrifugation at 12,500 rpm for duration of 15 min at 4 °C, resulting in the formation of three distinct layers. The upper aqueous phase was carefully collected and placed into a fresh Eppendorf tube, after which 500 μ L of ice-cold isopropanol was added. After brief vortexing, the mixture was centrifuged for 15 min at 4 °C to precipitate the RNA. The RNA pellet was subsequently washed twice with ice-cold 70% ethanol, followed by centrifugation at 10,000 rpm for 10 min. Upon completion of the final wash, the pellet was allowed to air-dry and then resuspended in nuclease-free water. RNA purity and concentration were quantified with a NanoDrop spectrophotometer (Thermo Scientific, USA). For downstream gene expression analysis, 2 μ g of total RNA was converted into complementary DNA (cDNA) using a cDNA synthesis kit (Thermo Fisher Scientific, USA) in accordance with the manufacturer's guidelines.

Quantitative real-time polymerase chain reaction

Proinflammatory gene expression was measured using qRT-PCR. As directed by the manufacturer, 10 μ L of KAPA SYBR® FAST SYBR green, 0.3 μ L of forward and 0.3 μ L of reverse primers, and cDNA containing fewer than 100ng (1 μ L) in a 20 μ L reaction were utilized. The reaction procedure comprised of 40 amplification cycles involving 95 °C for 20s, 62 °C for 15s, and 72 °C for 15s, followed by a pre-incubation step of 5 min at 95 °C. The melting curve was analyzed after amplification at 95 °C for 5 s, 60 °C for 1 min, and 95 °C continuously. To confirm the size of the amplified product, it was tested on a 2% agarose gel. A relative quantification approach ($\Delta\Delta C_t$) was used to analyze the results. The mRNA expression of the target mRNAs of TNF α and IL1 β was assessed using GAPDH, which serves as a constitutively expressed reference gene. The primer nucleotide sequences employed in the present study are provided in Table 1. Each qRT-PCR experiment was carried out in triplicate.

Table 1 Phytochemical screening of various extracts of *Androsace sempervivoides*

S.No.	Gene	Primers	Product size (bp)
1.	TNF- α	Forward: GAGCACGGAAAGCATGATCC Reverse: TAGACAGAAGAGCGTGCTGG	148
2.	IL1- β	Forward: GGGATGATGACGACCTGCTA Reverse: TGTCGTTGCTTGTCTCTCCT	192
3.	GAPDH	Forward: CAAGTTCAACGGCACAGTCA Reverse: CCCCATTTGAGTTTAGCGGG	102

Table 2 Phytochemical screening of various extracts of *Androsace sempervivoides*

S.No.	Phytochemicals	Hexane extract	Ethyl acetate extract	Ethanol extract	Methanol extract	Aqueous extract
1.	Flavonoids	+	++	+++	++	++
2.	Phenols	–	++	+++	++	+
3.	Tannins	–	+	+	+	+
4.	Terpenoids	–	+++	+++	++	+
5.	Anthracenosids	–	+	++	+	–
6.	Sterols and steroids	–	+	++	–	–
7.	Saponins	–	–	+	–	–

The table summarizes the presence (+) or absence (–) of major phytochemical constituents across different solvent extracts

(–) absent; (+) weakly present; (++) present; (+++) strongly present

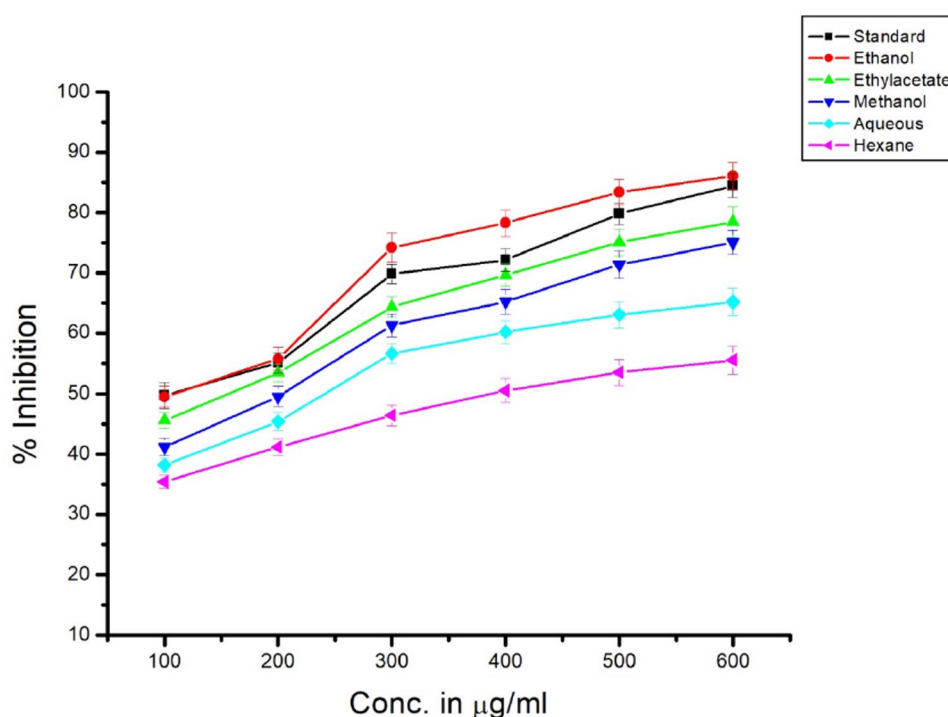


Fig. 2 DPPH radical scavenging activity of *Androsace sempervivoides* ethanol, ethyl acetate, methanol, aqueous, and hexane extracts. Data are presented as mean $\pm$ standard deviation ($n=3$)

Statistical analysis

Data are summarized as the mean $\pm$ standard deviation (SD). One-way ANOVA was employed for data analysis using SPSS. The antioxidant properties displayed by the active extracts of *Androsace sempervivoides* was assessed using in vitro and in vivo approaches, each carried out in triplicate.

Results

Phytochemical screening

The results of the preliminary phytochemical constituents identified in *Androsace sempervivoides* extracts are summarized in Table 2.

DPPH radical scavenging activity

The DPPH radical-quenching potential of the ethanolic extract of *Androsace sempervivoides* demonstrated a clear

dose dependent response, with increasing concentrations exhibiting enhanced free radical scavenging potential. At the uppermost tested concentration of 600 $\mu\text{g/mL}$, the ethanolic extract demonstrated peak DPPH inhibition of $86.11 \pm 2.21\%$, which was marginally greater than that of the standard antioxidant BHT, showing inhibition of $84.48 \pm 1.97\%$, followed by ethyl acetate extract showing $78.46 \pm 2.56\%$ at the same concentration. The present findings demonstrate the strong antioxidant efficacy of the *Androsace sempervivoides* extracts. The DPPH radical quenching activity is demonstrated in Fig. 2.

The IC_{50} values highlighted that the ethanol extract evidenced the greatest antioxidant potential, with the lowest IC_{50} value ($53.32 \pm 3.05 \mu\text{g/mL}$), followed by the standard antioxidant BHT ($68.86 \pm 1.10 \mu\text{g/mL}$) and the ethyl acetate extract ($126.55 \pm 6.42 \mu\text{g/mL}$). The lower IC_{50} value of the ethanol extract reflects its superior

free radical scavenging efficiency compared to the other tested extracts.

Reducing power assay

Among the extracts evaluated, the ethanolic extract exhibited the highest reducing power in a dose-dependent manner. At 600 $\mu\text{g/mL}$, the ethanol extract showed a significantly higher reducing power (4.630 ± 0.46) compared to the ethyl acetate (4.626 ± 0.34) and methanol extracts (4.591 ± 0.39). These results indicate the strong electron-donating ability of the ethanol extract, suggesting its potential as an effective antioxidant. The dose-dependent reducing power curves for all extracts are shown in Fig. 3.

Hydroxyl radical scavenging assay

Within the concentration range of 100–600 $\mu\text{g/mL}$, the hydroxyl radical scavenging ability of the three *Androsace sempervivoides* extracts followed the order: ethanol > ethyl acetate > methanol (Fig. 4). At the highest tested concentration (600 $\mu\text{g/mL}$), the ethanolic extract exhibited the strongest scavenging activity ($85.89 \pm 1.88\%$), followed by the ethyl acetate ($73.07 \pm 2.05\%$) and methanolic ($65.27 \pm 2.78\%$) extracts. These findings suggest that the ethanol extract possesses superior efficacy in neutralizing hydroxyl radicals, which is critical for preventing the propagation of

lipid peroxidation and mitigating oxidative stress. The observed scavenging activities reflect the potential of the extracts to counteract reactive oxygen species (ROS) and contribute to their antioxidant properties.

The IC_{50} values from the hydroxyl radical scavenging assay revealed a clear sequence of activity, where the standard antioxidant BHT exhibited the highest scavenging potential with the lowest IC_{50} value ($32.55 \pm 2.03 \mu\text{g/mL}$), followed by the ethanol ($49.49 \pm 3.40 \mu\text{g/mL}$), ethyl acetate ($73.87 \pm 2.32 \mu\text{g/mL}$), and the methanol extract ($196.82 \pm 4.36 \mu\text{g/mL}$). This order indicates that BHT was the most potent hydroxyl radical scavenger, while among all evaluated plant extracts, ASE showed the strongest activity followed by ASEA and methanol extract.

Metal chelating activity using ferrozine

The standard antioxidant BHT, ethanol and the ethyl acetate extract effectively disrupted the formation of the ferrous-ferrozine complex, indicating their ability to chelate Fe^{2+} ions by competing with ferrozine for binding sites resulting in a significant reduction in complex formation, confirming their chelating activity. The ethanol extract demonstrated a dose-dependent reduction in the absorbance of the Fe^{2+} -ferrozine complex at 400, 500, and 600 $\mu\text{g/mL}$, suggesting an efficient metal-binding capacity. At 600 $\mu\text{g/mL}$, the metal-chelating activity reached $95 \pm 2.48\%$ for the ethanol extract, $88 \pm 4.31\%$ for

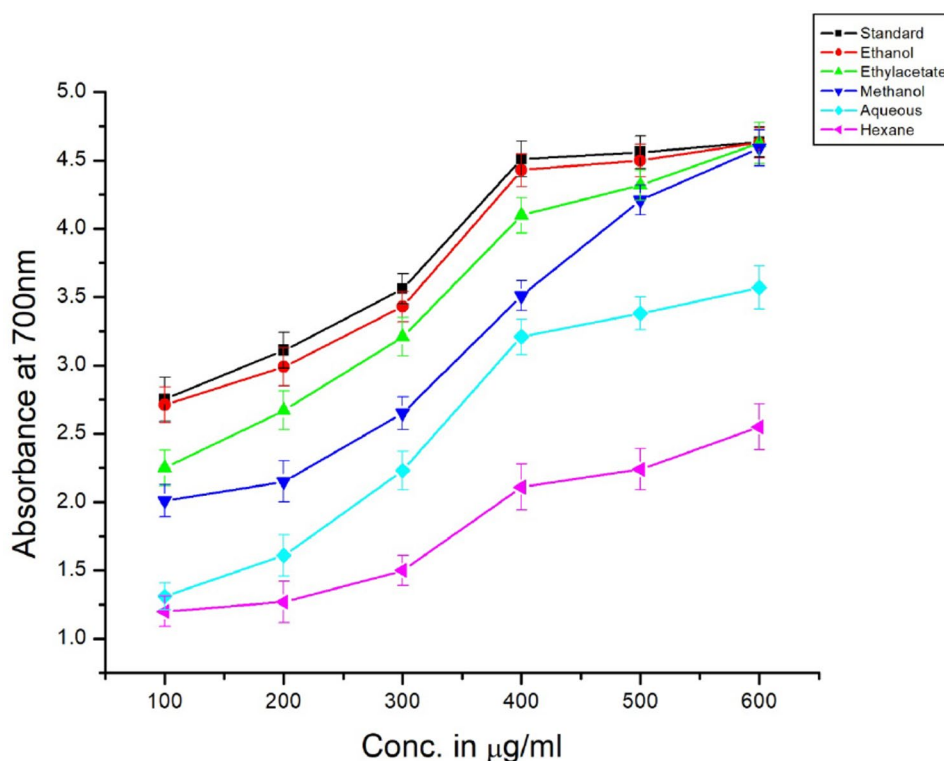


Fig. 3 Reducing power of *Androsace sempervivoides* extracts (ethanol, ethyl acetate, methanol, aqueous, and hexane). Data are presented as mean $\pm$ standard deviation ($n=3$)

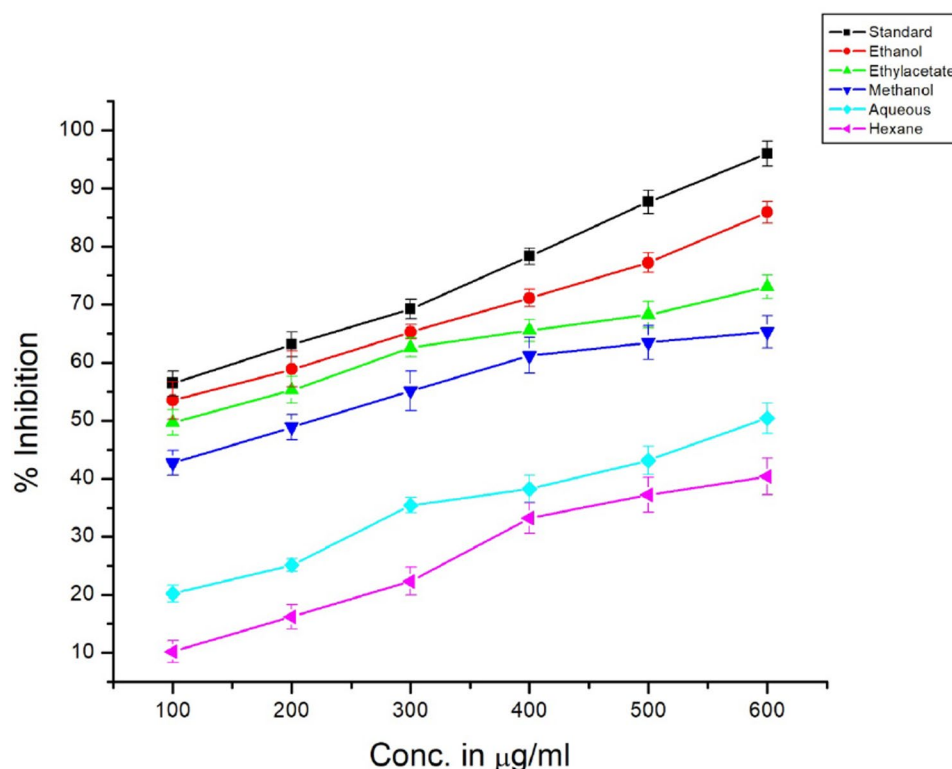


Fig. 4 Hydroxyl radical scavenging activity of *Androsace sempervivoides* extracts (ethanol, ethyl acetate, methanol, aqueous, and hexane). Data are presented as mean $\pm$ standard deviation ($n=3$)

the ethyl acetate extract and $93 \pm 3.41\%$ for BHT. Based on the observed chelating efficiencies, the extracts were ranked as follows: ethanol > BHT > ethyl acetate. The results, illustrated in Fig. 5, highlight the potential of *Androsace sempervivoides* extracts, particularly the ethanol extract as effective metal ion chelators. Chelating agents reduce the catalytic activity of transition metals in oxidative reactions by stabilizing them in their oxidized form through sigma bond formation, thereby functioning as potent auxiliary antioxidants.

The IC_{50} values derived from the metal chelation assay revealed that the ethanol extract exhibited the strongest ferrous ion chelating ability, with an IC_{50} value of $(34.59 \pm 2.01 \mu\text{g/mL})$, followed by the BHT ($79.33 \pm 1.92 \mu\text{g/mL}$) and the ethyl acetate extract ($123.55 \pm 4.21 \mu\text{g/mL}$). The relatively lower IC_{50} value of the ethanol extract indicates its higher metal ion chelating potential compared to the other extracts.

Total phenolic content

The phenolic content of ethanol, ethyl acetate, methanol, and aqueous extracts were determined to be $(784.68 \pm 9.39, 713.7 \pm 8.14, 563.48 \pm 7.09, \text{ and } 408.31 \pm 5.11)$ mg gallic acid equivalents/g of extract, respectively. These results indicate that all extracts contain substantial amounts of phenolic compounds, with

ASE exhibiting the highest phenolic content relative to all other tested extracts.

Total flavonoid content

The flavonoid content of ethanol, ethyl acetate, methanol, and aqueous extracts was found to be $(537.59 \pm 6.38, 489.08 \pm 5.02, 426.38 \pm 8.65, \text{ and } 304.21 \pm 7.62)$ mg quercetin equivalents/g of extract. Among these, the ethanol extract demonstrated the greatest flavonoid content, followed by ethyl acetate, methanol, and aqueous extracts, indicating a substantial presence of flavonoid compounds in *Androsace sempervivoides* extracts.

Antioxidant assay evaluating protection against oxidative DNA damage

The ethanol and ethyl acetate extracts of *Androsace sempervivoides* demonstrated noteworthy mitigation of hydroxyl radical-mediated damage to calf thymus DNA. As shown in Fig. 6, lane 1 represents calf thymus DNA in its native, undamaged form whereas lane 2 represents the DNA damage control, treated with 20 mM ferric nitrate, 100 mM ascorbic acid and 30 mM H_2O_2 . Lanes 3–5 included the same pro-oxidant mixture along with increasing concentrations of *Androsace sempervivoides* extracts (30 mg, 50 mg, and 80 mg, respectively). At 30, 50, and 80 mg, the extracts provided near-complete protection, as evidenced by the preservation of DNA

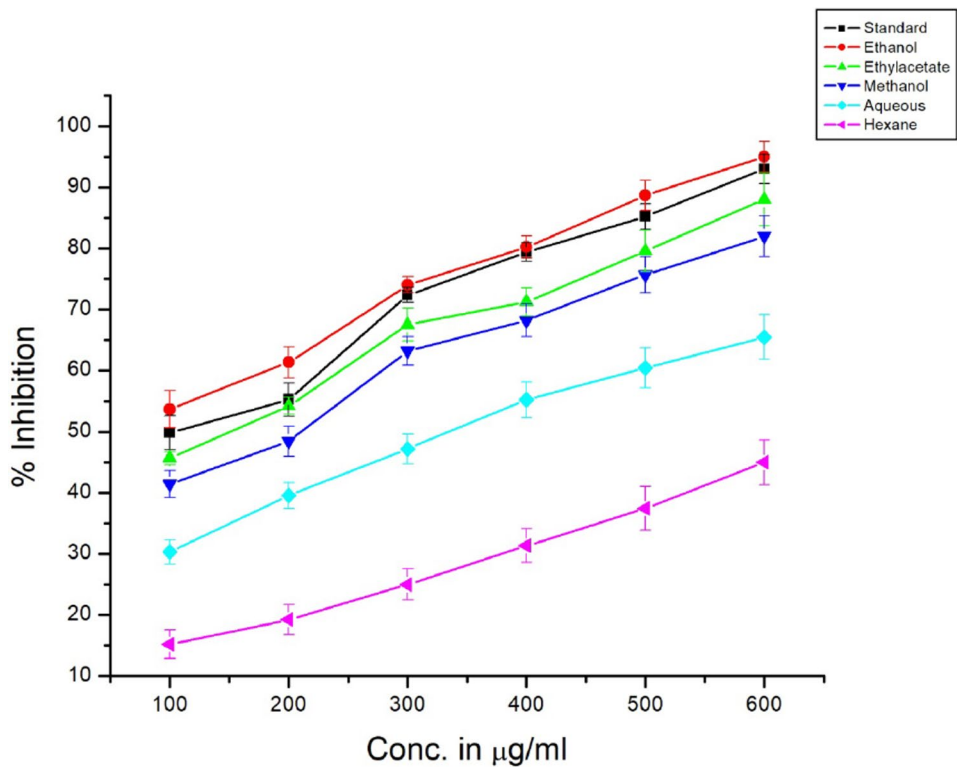


Fig. 5 Metal chelating activity on ferrous ions at varying concentrations (100–600 µg/mL) of BHT (standard) and *Androsace sempervivoides* extracts (ethanol, ethyl acetate, methanol, aqueous, and hexane). Data are presented as mean ± standard deviation (n = 3)

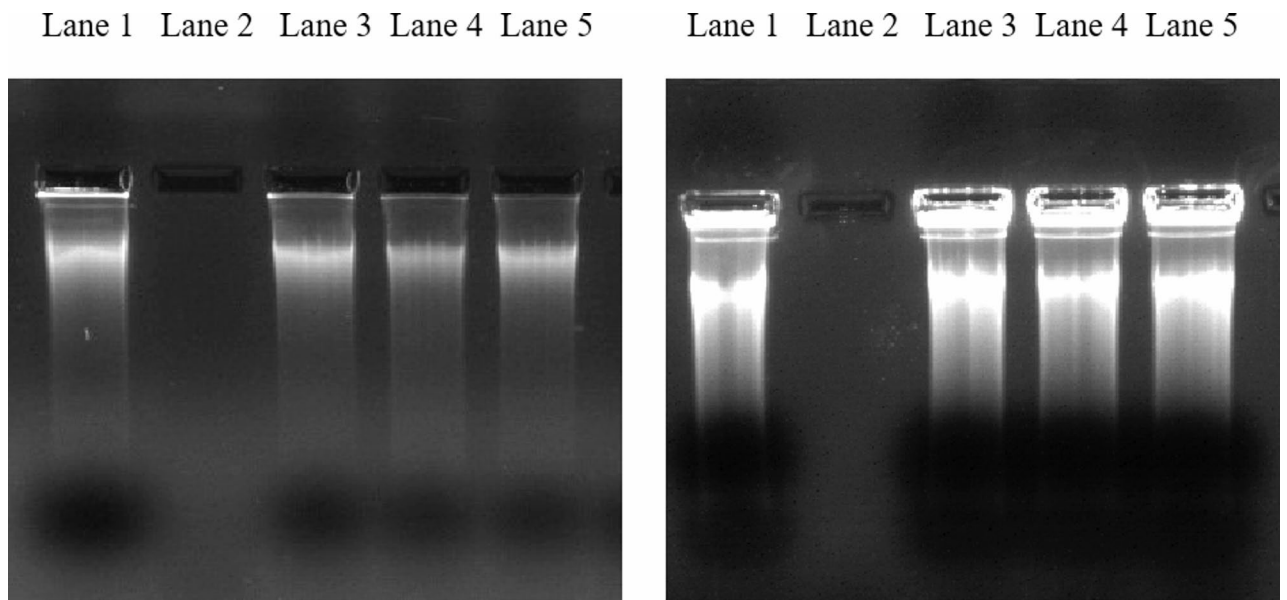


Fig. 6 Protective effects of ethanol and ethyl acetate extracts of *Androsace sempervivoides* against oxidative damage to calf thymus DNA

integrity in lanes 3–5. Among the tested extracts, the ethanolic extract demonstrated superior protective activity, followed by the ethyl acetate extract. These results indicate that the DNA-protective effects may be attributed to the polyphenolic constituents of the plant, which can effectively scavenge hydroxyl radicals and mitigate oxidative stress. This finding supports the potential therapeutic application of *Androsace sempervivoides* in oxidative stress-related disorders.

Histopathology of paw tissue

To evaluate the therapeutic potential of the active extracts, histopathological examination of the paw tissues was performed. LPS stimulation initiates a localized immune response characterized by macrophage infiltration and subsequent tissue inflammation, leading to visible swelling and redness. In the LPS-only group (Group 2), pronounced histopathological alterations were observed, including blood vessel rupture, extensive edema, and tissue necrosis, in stark contrast to the normal histoarchitecture observed in the control group (Group 1). Group 3, which was treated with a standard anti-inflammatory drug (dexamethasone), exhibited marked attenuation of these inflammatory features. Notably, Groups 4–7, which received different *Androsace sempervivoides* extracts, demonstrated varying degrees of histological improvement. Among these, the group treated with the ethanol extract at 100 mg/kg (ASE 100) showed the greatest protective effect, with minimal necrosis and vascular damage, closely resembling the standard treatment group. As shown in Fig. 7, the ASE extract significantly preserved paw tissue integrity compared to the LPS-only group. Overall, the histopathological findings support the inflammation-reducing efficacy of *Androsace sempervivoides*, particularly the ethanol extract, in mitigating LPS-induced tissue injury (Fig. 7).

Oxidative stress markers

As opposed to the control group, the LPS-treated group showed a marked decline in key antioxidant enzymes indices, CAT and SOD, indicating elevated oxidative stress. Dexamethasone administration effectively restored the activity of these enzymes. Similarly, treatment with ASE at 100 and 50 mg/kg, as well as ASEA at 100 mg/kg pronouncedly reduced elevated CAT and SOD activities when contrasted to the LPS-only group ($P < 0.05$). These findings are illustrated in Fig. 8, highlighting the antioxidant potential of the extracts in ameliorating LPS-induced oxidative damage.

Protein and gene expression analysis

Western blot analysis demonstrated that treatment with both ethanol and ethyl acetate extracts of *Androsace sempervivoides* resulted in a concentration-dependent

downregulation of TNF- α (55 kDa) and IL-1 β (31 kDa) protein expression, relative to LPS-only treated group. GAPDH (36 kDa) served as the internal loading control. Notably, the groups administered 100 mg/kg body weight of ethanol and ethyl acetate extracts showed expression levels comparable to the positive control (dexamethasone-treated) group, as illustrated in Fig. 9. Complementary real-time PCR analysis (Fig. 10) confirmed a significant reduction in the mRNA expression of both genes in the extract-treated groups. LPS administration markedly increased the transcript levels of TNF- α and IL-1 β (mRNA) by 24.13-fold and 22.12-fold, respectively, compared with the control. Treatment with ASE (100 mg/kg bw) reduced expression of TNF- α and IL-1 β to 4.95 and 6.07 fold, respectively. Similarly, ASEA at the same dose reduced expression levels by 12.55-fold and 13.72-fold, respectively. These findings indicate that *Androsace sempervivoides* extracts, particularly the ethanol extract, exert a robust anti-inflammatory property, likely by attenuating the expression of key pro-inflammatory cytokines. The ethanol extract consistently outperformed the ethyl acetate extract in mitigating LPS-induced inflammatory responses at both the transcriptional and translational levels.

Discussion

This study offers the first comprehensive assessment of the antioxidant and anti-inflammatory activities of *Androsace sempervivoides*, a Tibetan ethnomedicinal plant. Both in vitro assays and an LPS-induced inflammatory rat model demonstrated significant bioactivity, with the ethanol extract showing the highest and most consistent potency. Phytochemical analysis demonstrated the occurrence of flavonoids, phenolics, tannins, terpenoids, and saponins, all of which are well documented for their antioxidant and inflammation-modulating effects (Muhamad et al., 2022). Phenolics and flavonoids likely contribute to free radical scavenging and DNA protection, whereas tannins and terpenoids may be involved in metal chelation and cytokine suppression. Variations in extract activity emphasize the role of solvent polarity, with ethanol extracting a broader range of bioactive phytochemicals than ethyl acetate or methanol.

In vitro antioxidant assays revealed that the ethanol extract exhibited notable DPPH radical scavenging activity at 600 $\mu\text{g/mL}$, surpassing that of the standard antioxidant BHT. This finding aligns with studies linking high phenolic and flavonoid content in the ethanol and ethyl acetate extracts to enhanced radical-scavenging potential (Jafri et al., 2023). Reducing power assays confirmed the robust electron donation capacity of the ethanol extract, indicating its ability to terminate radical chain reactions. Metal chelation assays revealed that ASE and ASEA efficiently sequestered Fe^{2+} ions, inhibiting the Fenton

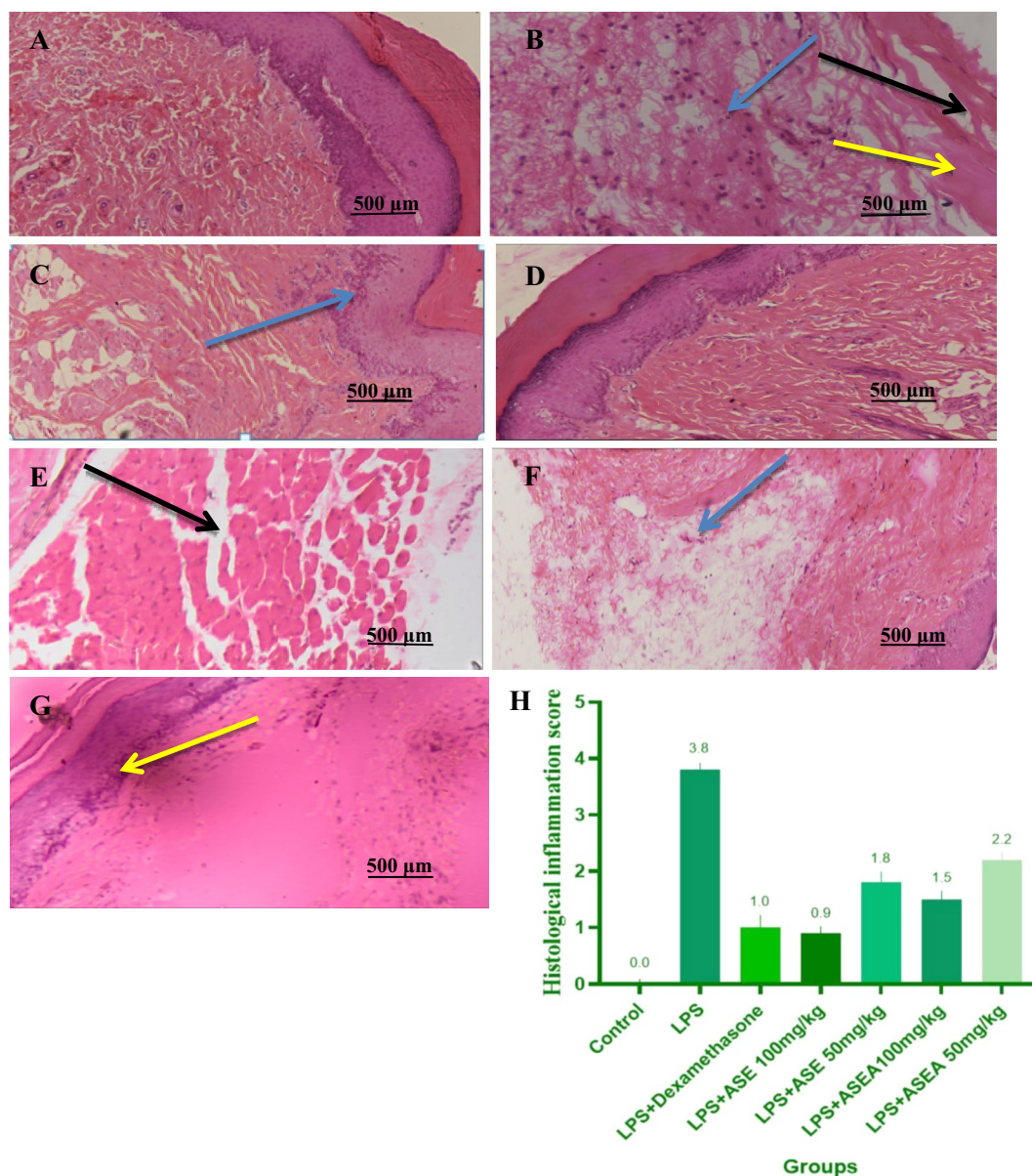


Fig. 7 Histological analyses of rat paw tissue sections from different treatment groups. **A** Normal control, **B** LPS-treated group, **C** LPS+Dexamethasone (10 mg/kg), **D** LPS+Ethanol extract 100 mg/kg, **E** LPS+Ethanol extract 50 mg/kg, **F** LPS+Ethyl acetate extract 100 mg/kg, **G** LPS+Ethyl acetate extract 50 mg/kg, **H** Bar graph presenting histological inflammation score (0–4 scale), expressed as mean $\pm$ SD ($n=3$), with significance set at $p < 0.05$ relative to the LPS treated group. Images were captured at 4 $\times$ magnification, scale bar = 500 μ m. Blue arrows indicate neutrophil infiltration (inflammation), black arrows indicate blood vessel rupture, and yellow arrows indicate tissue necrosis

reaction and subsequent ROS generation, which is critical given the role of transition metal-catalyzed radicals in lipid peroxidation and degenerative diseases (Valgimigli, 2023). Hydroxyl radical scavenging and DNA protection assays further validated the capacity of the extracts to prevent oxidative DNA strand breaks, possibly through polyphenolic stabilization of hydroxyl radicals. The strong correlations among the antioxidant assays reinforce the reliability of these findings.

Histopathological analysis of paw tissues *in vivo* supported the antioxidant and anti-inflammatory potential

of *Androsace sempervivoides* extract. LPS-induced inflammation caused tissue necrosis, vascular rupture, and edema, which were significantly alleviated by the 50 and 100 mg/kg extract treatments. The 100 mg/kg ethanol extract group exhibited the most pronounced protection, preserving the cellular architecture and reducing necrosis. These outcomes align with reports of ethanol extracts from other medicinal plants, such as *Prasiola japonica* and *Paridis rhizoma*, which attenuate LPS- or carrageenan-induced inflammation by preserving tissue

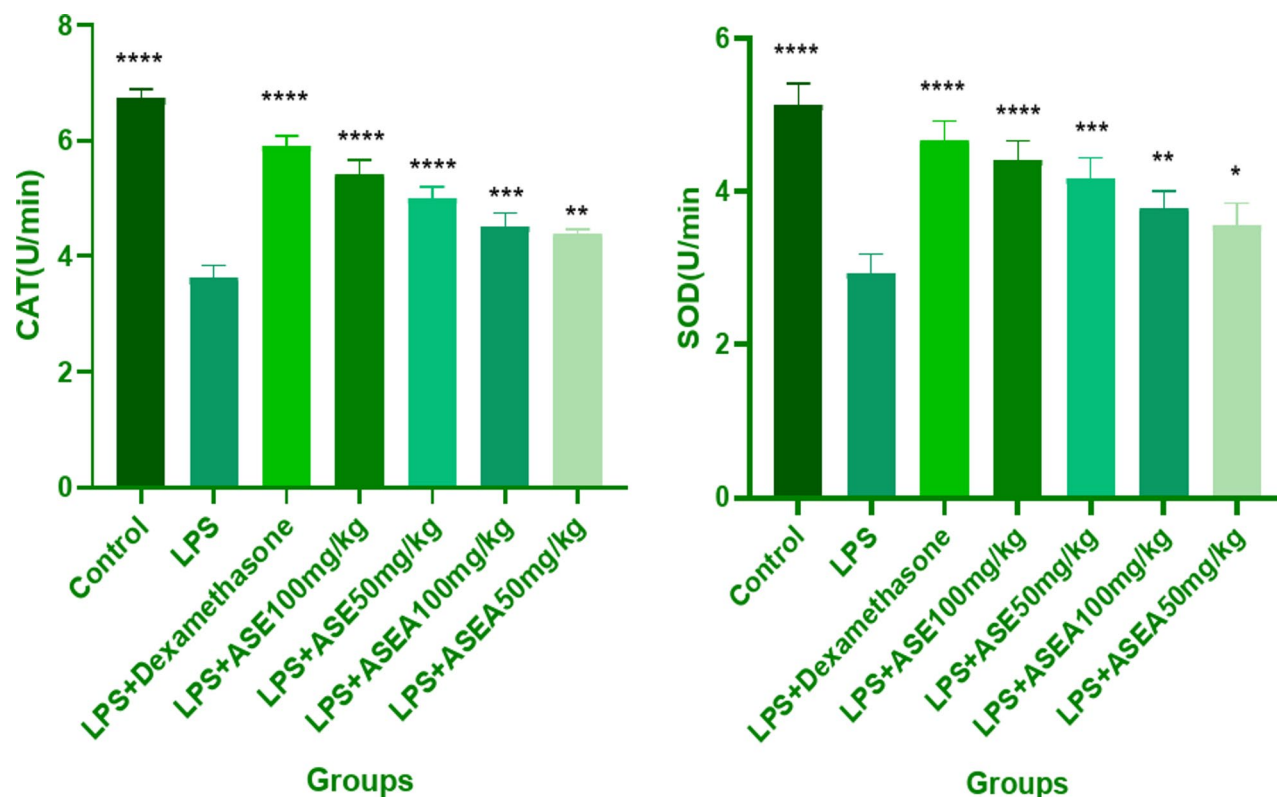


Fig. 8 Effect of *Androsace sempervivoides* extracts on CAT and SOD levels across different groups. Group 2 (LPS treated only) was compared to Group 1 (control), and treatment groups (Dexamethasone, ASE 100 and 50 mg/kg, ASEA 100 and 50 mg/kg; Groups 3–7) were compared to Group 2. Data are presented as mean $\pm$ standard deviation ($n=3$). ns : $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

integrity and modulating proinflammatory pathways (Rahmawati et al., 2021; Xiang et al., 2023).

The LPS-induced paw edema model adopted in this study is a widely recognised *in vivo* approach for evaluating the antioxidant and anti-inflammatory efficacy of plant extracts (Patil et al., 2019). In this model, subplantar injection of LPS triggers localized inflammation, accompanied by oxidative stress, edema, and immune cell infiltration. The significant modulation of inflammatory markers and oxidative responses observed in paw tissue following treatment with *Androsace sempervivoides* extracts indicates its capacity to attenuate local inflammatory damage. Although this model primarily reflects a localized response, the molecular mechanisms involved, such as cytokine signalling and ROS generation, are also central to systemic inflammation, suggesting that the extracts may exert broader protective effects against oxidative stress-mediated disorders (Dar et al., 2020). The potent *in vivo* antioxidant effects likely stem from the high level of phenolic and flavonoid content of the ethanol and ethyl acetate extracts, which are known to scavenge free radicals and bolster endogenous antioxidant defences (Tumilaar et al., 2024; Zhang & Tsao, 2016).

LPS-induced oxidative stress significantly reduced catalase (CAT) and superoxide dismutase (SOD) activities, reflecting elevated ROS production and disrupted redox homeostasis in the cells. Treatment with *Androsace sempervivoides* extracts restored these enzyme activities, demonstrating their ROS-neutralizing capacity and mitigation of oxidative cellular damage. This restoration reduces ROS-mediated activation of redox-sensitive transcription factors NF- κ B and AP-1, as evidenced by decreased nuclear translocation of the NF- κ B p65 subunit and reduced phosphorylation of c-Jun, thereby inhibiting their transcriptional activation of pro-inflammatory cytokines IL-6, IFN- γ , TNF- α , and IL-1 β . Consequently, the extracts suppress downstream inflammatory responses and cellular apoptosis associated with chronic inflammation (Ye et al., 2016; Ramesh et al., 2013; Zielinski et al., 2019; Al-Manthari, 2022). The phenolic and flavonoid compounds in the extracts likely mediate these effects by modulating the signalling pathways involved in oxidative stress and inflammation.

Consistent with this mechanism, western blotting and qRT-PCR analyses revealed significant suppression in the expression of TNF- α and IL-1 β , by the active extracts. The superior efficacy of the ethanol extract may be ascribed to its higher flavonoid and tannin content,

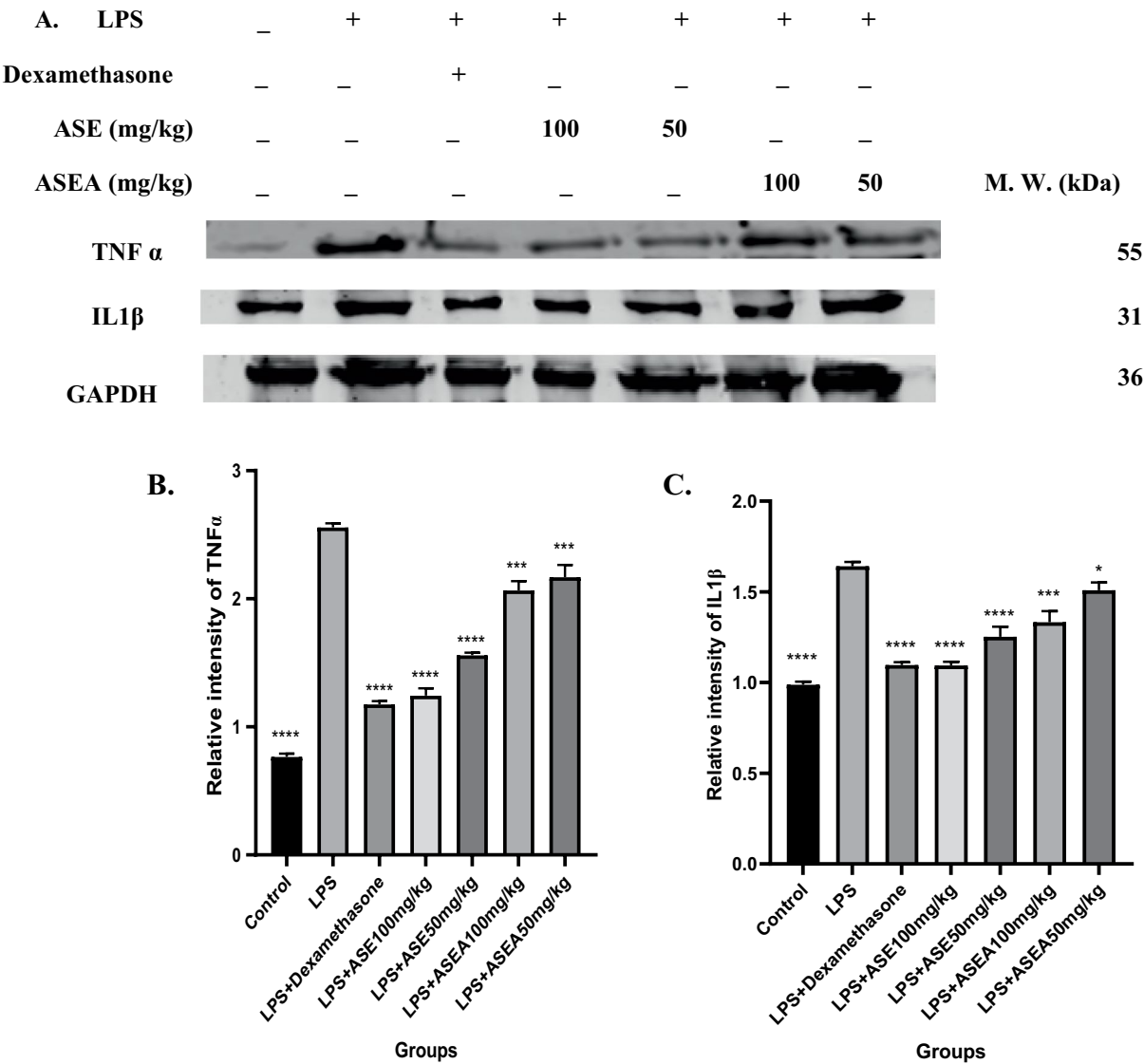


Fig. 9 Western blot and densitometric evaluation of TNF- α and IL-1 β . **A** Representative blots showing TNF- α and IL-1 β levels across different treatment groups: Lane 1: Normal control; Lane 2: LPS only; Lane 3: LPS + Dexamethasone; Lane 4: LPS + ASE 100 mg/kg; Lane 5: LPS + ASE 50 mg/kg; Lane 6: LPS + ASEA 100 mg/kg; Lane 7: LPS + ASEA 50 mg/kg. **B** Densitometric analysis of TNF- α , **C** Densitometric analysis of IL-1 β . Data are presented as mean $\pm$ SD ($n=3$). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

which are documented to exhibit both antioxidant and anti-inflammatory properties (Singh et al., 2023). These findings correspond with prior studies demonstrating that ethanol extracts of *Illicium henryi* and *Parkia speciosa* reduce LPS-induced proinflammatory cytokine expression (Islam et al., 2019; Samrit et al., 2024).

Study limitations and future perspectives

Despite these encouraging results, this study has several limitations. First, the absence of isolation and characterization of individual bioactive compounds limits a detailed understanding of the distinct constituents attributing to the observed effects and their precise mechanistic pathways at the molecular level. Second, the exclusive

use of an acute LPS-induced inflammation model may not entirely reflect the complexity of chronic oxidative stress and inflammatory conditions in humans. Third, the pharmacokinetic properties and long-term efficacy of the extracts remain unexplored, which are important for potential therapeutic applications. Future research should focus on isolating active compounds, elucidating detailed molecular pathways, evaluating their effects in chronic disease models, and conducting comprehensive pharmacokinetic and efficacy studies.

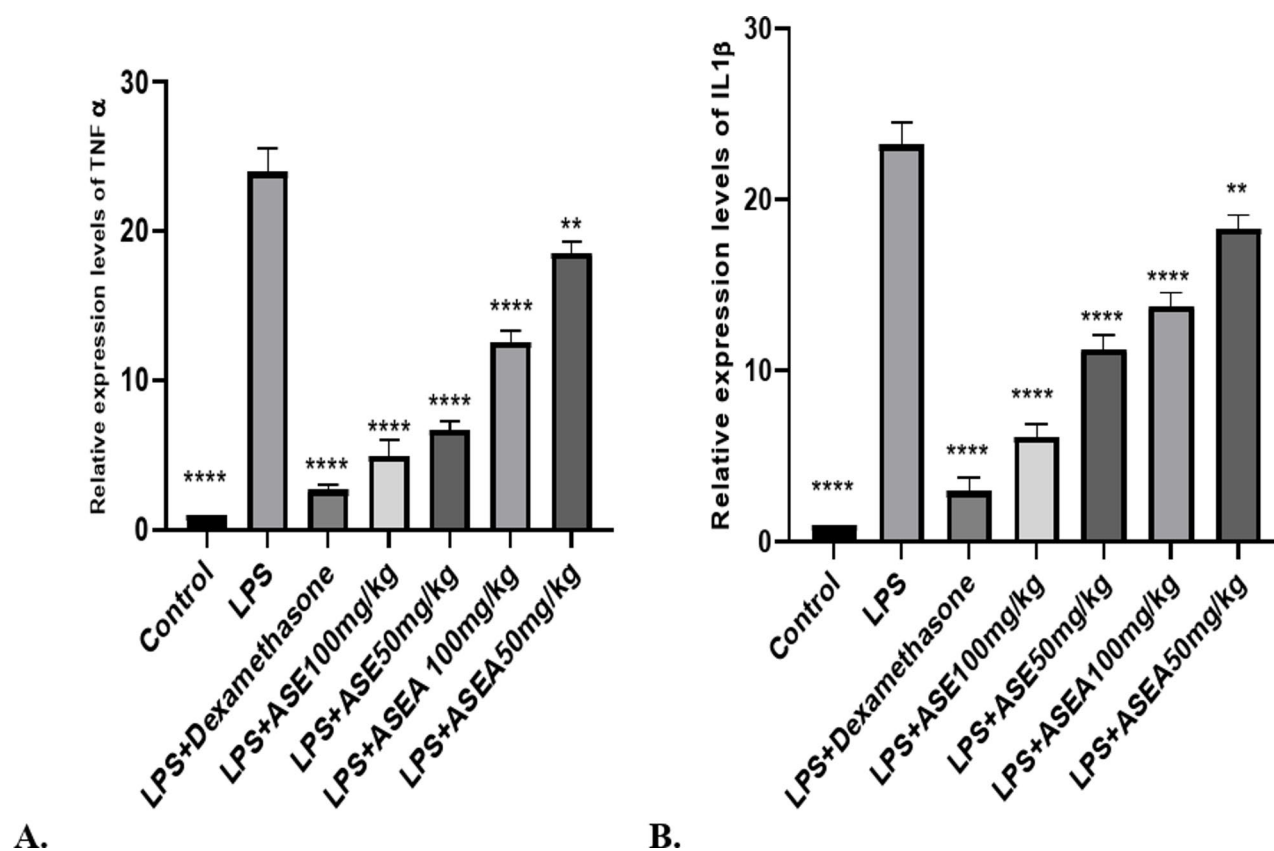


Fig. 10 Relative mRNA expression levels of inflammatory markers in different experimental groups. **A** TNF- α **B** IL-1 β . Data are presented as mean $\pm$ standard deviation ($n=3$). ns: $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Conclusion

The present study provides the first experimental evidence supporting the traditional medicinal use of *Androsace sempervivoides*. Both ethanol and ethyl acetate extracts, particularly ASE, demonstrated significant antioxidant potential through free radical scavenging, DNA protection and metal chelation. In vivo, these extracts enhanced antioxidant defences and down regulated TNF- α and IL-1 β , thereby mitigating LPS-induced oxidative stress and inflammation while preserving tissue architecture. Overall, the study supports the therapeutic promise of *Androsace sempervivoides* as a natural reservoir of bioactive agents for mitigating oxidative stress and inflammatory disorders. Expanded research efforts are warranted to isolate the compound, understand its molecular mechanism, and ensure its overall safety for therapeutic use.

Abbreviations

LPS	Lipopolysaccharide
TBA	Thiobarbituric acid
BHT	Butylated hydroxytoluene
FeCl ₃	Ferric chloride
H ₂ O ₂	Hydrogen peroxide
BHA	Butylated hydroxyanisole
NBT	Nitro blue tetrazolium
DPPH	1,1-diphenyl-2-picrylhydrazyl

H&E	Hematoxylin and eosin
SOD	Super oxide dismutase
CAT	Catalase
GPX	Glutathione peroxidase
PBS	Phosphate buffer saline
OECD	Organization of Economic Co-operation and Development
bw	Body weight
TNF- α	Tumour necrosis factor alpha
IL-1 β	Interleukin 1-beta
CST	Cell Signalling Technology
qRT-PCR	Quantitative real-time polymerase chain reaction
PMS	Post mitochondrial supernatant

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Author contributions

WYK : Writing- original draft, Software, Experimental work, Methodology, Formal analysis, Data curation. SR: Validation, Formal analysis. FJ, SA, IAD, and NK: Formal analysis. SA : Reviewing, supervision, and formal analysis. SAG : Validation, Supervision, Project administration, Investigation, and Conceptualization.

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Data availability

The data supporting the findings of this study will be provided by the authors upon request. The authors report no conflicts of interest.

Declarations

Ethical approval and consent to participate

All experimental procedures involving animals adhered to the regulations of the Institutional Animal Ethics Committee (IAEC), University of Kashmir, with Approval No: (IAEC approval) KU/2023/09.

Competing interests

The authors declare no competing interests.

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