

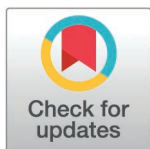
RESEARCH ARTICLE

Therapeutic role of *Cousinia thomsonii* in ameliorating the oxidative stress and inflammation: A comprehensive *in vitro* and *in vivo* investigation

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Abstract

The present study evaluated the antioxidant and anti-inflammatory properties of an edible medicinal herb, *Cousinia thomsonii* (CT), commonly known as Thomson's thistle. Preliminary investigations such as DPPH assay, lipid peroxidation estimation, reducing power assessment, hydroxyl radical scavenging activity, and plasmid DNA damage assessment revealed that ethyl-acetate extract of the plant exhibited significantly higher antioxidant potential compared to the methanolic extract. We further evaluated the antioxidant potential of ethylacetate extract of *Cousinia thomsonii* in model cell systems. Using DCFDA staining, our results showed that ethyl acetate extract of this herb alleviated H₂O₂-induced oxidative stress in HepG2 cells. Further, the extract protected these cells from H₂O₂-induced cell death as demonstrated by PI/DAPI staining-based cell viability assays. These data were further substantiated by the cleavage status of PARP as evaluated by immunoblotting which pointed at the rescue of HepG2 cells from H₂O₂ induced cell death. Inhibition of NO release, corroborated by the downregulation of iNOS, also confirmed the effect of the extract in SNP-induced NO stress. Additionally, co-treatment with different concentrations of the extract led to the upregulation of Nrf2, the master regulator of oxidative stress, while dose-dependently suppressing LPS-induced TNF-α, IL-1β, and IL-6 expression in THP-1 cells. The *in vivo* anti-inflammatory activity of *Cousinia thomsonii* was further assessed through inhibition of paw edema formation and histopathological analysis of paw tissue. Taken together, these findings demonstrate that the ethyl acetate extract of CT exhibit potent antioxidant and anti-inflammatory effects in both *in vitro* and *in vivo* models, supporting its potential therapeutic application.

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Data availability statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

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Introduction

Cousinia represents the largest genus in Asteraceae family with more than 670 species. *Cousinia thomsonii* (CT) is a rare medicinal herb native to the Himalayan region ranging from Afghanistan to Tibet. Traditionally, various species of this genus have been used to treat inflammatory disorders such as arthritis, dermatitis and asthma [1–4,20,21]. Most of these effects have recently been attributed to the phytoactive compounds isolated from these plants like flavonoids, triterpenes, sesquiterpenes, and steroids. Several reports also suggest that extracts of some of the Cousinia species have been used to treat gastrointestinal ulcers and other serious infections. Additionally, compounds isolated from these herbs have demonstrated potent cytotoxic effects in several cancer cell lines with some, such as parthenolide, artemisinin and thapsigargin already progressing to phase I clinical trials. Although *Cousinia thomsonii* has demonstrated potent antioxidant and anti-inflammatory activities due to presence of varied phytochemicals, the underlying molecular mechanisms including Nrf2 signaling, apoptosis, and iNOS regulation have largely remained unexplored. In our earlier research, we reported that CT extracts show potent anti-inflammatory action *in vivo* [5,6]. These anti-inflammatory effects were mostly attributed to some novel compounds such as phytol acetate, 9-octadecenoic acid, n-hexadecanoic acid and 1-dodecanol [5,6]. GC-MS analysis of the CT extracts has also led to the identification of several compounds with potential antioxidant and antiproliferative capacity.

Oxidative stress arising as a result of oxygen-dependent metabolic reactions is defined as an imbalance between pro-oxidant and anti-oxidant systems [7]. This concept implies that under normal conditions cells maintain pro-oxidant/antioxidant systems that continuously generate and neutralize oxidants. When additional oxidative events occur, the pro-oxidant systems outbalance the anti-oxidant, leading to oxidative damage of lipids, proteins, carbohydrates and nucleic acids, ultimately leading to cell death in severe oxidative stress related diseases including chronic inflammation [8].

Chronic inflammation is linked with almost all of the chronic diseases, including cancer, diabetes, cardiovascular diseases and obesity [9–11]. In recent years, researchers focused on inflammation related carcinogenesis and one of the important and well-established links between chronic inflammation and cancer is the colorectal cancer and liver cancer [12].

Aerobic organisms including humans are armoured with intrinsic antioxidant defence systems to protect against oxidant damage. Among natural antioxidants, plant derived phenolic and flavonoids are considered safe and bioactive. Therefore, in recent years considerable attention has been directed towards identification of plants with antioxidant and anti-inflammatory activities that may be used for human consumption to neutralize oxidant related disorders.

The current study aims to bridge these gaps through comprehensive *in vitro* and *in vivo* approaches, including DPPH radical scavenging activity, DNA protection assay, DCFDA and PI/DAPI staining assays, histopathological examination, RT-PCR, and western blotting to assess the anti-inflammatory activity of *Cousinia thomsonii*. This

is the first report that elucidates how *Cousinia thomsonii* extracts ameliorates the oxidative stress through activation of Nrf2 signalling cascade, inhibition of apoptosis and downregulation of iNOS. By employing this multifaceted experimental design, we seek to provide new insights into the mechanisms through which these natural agents may alleviate oxidative stress related disorders including inflammation.

Materials and methods

Collection and identification of plant material

Cousinia thomsonii plants were collected from the upper reaches of Sankoo Kargil province of UT of Ladakh, India. Plant samples were identified and authenticated at the Centre of Plant Taxonomy Centre, University of Kashmir. For future reference, a specimen of *Cousinia thomsonii* was retained in the herbarium of COPT, University of Kashmir with specific voucher number 2620-KASH.

Preparation of extracts

Cousinia thomsonii samples were shade drying at room temperature ($25 \pm 2^\circ\text{C}$) for a period of 15 days. Dried plant sample was powdered and 100 g of the powder material was extracted with 300 ml of solvent using soxhlet extraction at 60°C to 80°C , employing solvents of increasing polarity viz hexane, ethyl acetate, ethanol, methanol and water. Extracts so obtained were concentrated at low pressure in rotary evaporator. All concentrated extracts were kept in refrigerator at 4°C for further investigations. The yield percentage for ethylacetate, ethanolic and aqueous extract was 5%, 3.5% and 3.95% respectively.

DPPH radical scavenging activity

DPPH assay was used to determine antioxidant properties of *Cousinia thomsonii* by using the method of Braca [13]. Different extract concentrations were used (varied from 100–1000 $\mu\text{g/mL}$). As per the standard procedure 1 ml of DPPH solution (dissolved in 0.004% methanol) was added to different concentrations of plant extract. All the samples were kept in dark for about half an hour. The absorbance was measured at 517nm. Catechin (a known free radical scavenger) was taken as Positive control. Calculation for antioxidant activity was done by using formula

$$\% \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

A_0 = absorbance of Catechin (control)

A_1 = absorbance in the presence of *Cousinia thomsonii* extracts

Reducing power

The reducing power assay was performed according to Oyaizu [14]. Varied concentrations of *Cousinia thomsonii* extracts were mixed with 2.5 ml of phosphate buffer (0.2M pH 6.6) and 2.5 ml of 1% potassium hexacyanoferrate ($\text{K}_4(\text{Fe}(\text{CN})_6)$). The mixture was incubated for 25 minutes at 50°C . TCA 2.5 ml (10%) was added to the mixture and centrifuges at 3000 rpm for 10 minutes. The upper layer of the centrifuged solution was taken (2.5 ml) and mixed with 2.5 ml water and ferric chloride (0.5 ml, 0.1%). Absorbance at 700nm were measured. The standard for the assay was catechin. Percentage reduction results of the sample were calculated by using equation

$$\text{Reduction (\%)} = [1 - (1 - A_C / A_S)] \times 100$$

A_C is absorbance of standard at maximum concentration tested and A_S is absorbance of sample.

The reducing power of *Cousinia thomsonii* extract and standard increases with increase in concentration.

Hydrogen peroxide scavenging

Hydroxyl radical generated by Fenton's reaction causes degradation of deoxyribose and produce thiobarbituric acid reactive species (TBARS). Scavenging of hydroxyl radical was evaluated by Halliwell method [15]. Different concentrations of *Cousinia thomsonii* extracts were used, in a reaction mixture (25mM deoxyribose, 10mM ferric chloride, 2.8mM hydrogen peroxide and KH_2PO_4 (10mM pH 7.4). The whole reaction mixture was incubated for about 1 hour at 37°C, then 1 ml TCA (3%), 1 ml (1% TBA) were added and heated for 20 minutes at 100°C. Absorbance was measured for TBARS at 532nm.

Lipid peroxidation

Liver microsomes of rat were used to assay lipid peroxidation. The assay was done in accordance with the method of wright [16]. 1.0 ml of reaction mixture containing phosphate buffer (pH 7.4, 0.1M), 0.58 ml ferric nitrate, 0.2 ml (20mM), ascorbic acid 0.1 ml (100mM) and 0.12 ml hydrogen peroxide (30mM) was incubated in shaking water bath at 37°C for 60 minutes. Lipid peroxide reaction was stopped by adding 1 ml TCA (10% w/v). 1.0 ml TBA (1% w/v) was added to all tubes and placed in water bath for about 20 minutes. The tubes were shifted to Ice bath and centrifuged at around 5000rpm for 20 minutes. The supernatant were collected and optical density were measured at 535nm against reagent blank for the amount of malonaldehyde in each sample.

DNA protection assay

The assay is based on the ability of *Cousinia thomsonii* to protect the plasmid DNA against the hydroxyl radical, which is the most potent radical to induce DNA damage. Hydroxyl radical is generated by Fenton's reaction that leads to DNA strand breaks and forms nicked circular and linear (relaxed) forms. The assay was performed according to Russo [17], in a solution of 20 micro litres, containing super coiled plasmid (0.5 µg), Hydrogen peroxide (1m M), ascorbic acid as positive control (100 µM), Ferric chloride (100µM), Tris-HCl buffer (10mM pH 7.8) and different concentrations of *Cousinia thomsonii* extracts. The whole reaction mixture was incubated at 37°C for an half an hour, 1µl EDTA (0.5M) were added to stop the reaction. The different concentration samples were run on agarose gel electrophoresis (0.7%) followed by ethidium bromide staining. The analysis was done on UV illuminator.

DCFDA staining

2×10^4 cells HepG2 liver cells were plated per well of 96 well plate and allowed to grow overnight. Next day cells were treated with 100 µM H_2O_2 alone or in combination with different concentrations of Ethyl acetate extract for 24 h. Washings with PBS was carried out three times which was followed with incubation of cells with 25 µM DCFDA for 45 min at 37°C in dark. After three washings with PBS, visualization was carried out under EVOS cell imaging system (Life Technologies, USA)

PI/DAPI staining

HEPG2 cells were treated with 100 µM H_2O_2 alone or in combination with different concentrations of Ethyl acetate extract for 24 h. Cells were then washed with PBS three times and then stained with 100 µM DAPI (diluted as 1:1000 in PBS) for 30 minutes. Washing was carried out again three times with PBS followed by counterstaining with 0.5 µg/mL Propidium Iodide. After three washings with PBS, visualization was carried out under EVOS cell imaging system (Life Technologies, USA).

Sodium Nitroprusside Treatment (SNP) and Griess's assay

HEPG2 cells were treated with 400 µM SNP alone or in combination with different concentrations of Ethyl acetate extract for 24 h. Following treatment, culture supernatants were collected. Standards of NaNO_2 were prepared in water to create

a standard curve followed by loading 50 µl of standard and samples to each well of 96 well plate. Then 50 µL of Griess Reagent I was added to each well followed by 50 µL of Griess Reagent II. Absorbance was measured at 540–550 nm.

Immunoblotting

Immunoblotting was carried out in accordance with the procedure as described by Amin [18]. Untreated and treated cells were harvested, lysed and proteins extracted from them which was followed by SDS PAGE and protein transfer using standard methods. Blots were incubated with primary antibody, PARP (46D11), Abcam (9532); iNOS, Cell signalling (2982) and GAPDH, Cell signalling (2118). Subsequently, the blots were washed and incubated with IR-tagged or HRP labeled secondary antibodies, Thermo Scientific Ltd. Imaging was carried out using an infrared imager, Odyssey Licor (Licor Biosciences). Normalization of protein expression was carried out using GAPDH as a loading control and band intensities were quantified by densitometric analysis using ImageJ software.

In vivo anti-inflammatory assays

Experimental animals. Adult male albino Wistar rats (8–10 weeks old) weighing 200–250 g were procured from the Department of Pharmaceutical Science, University of Kashmir, India. A total of 42 male Wistar rats were used for the study. All animal studies were performed according to the Declaration of Helsinki and the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, government of India, established under chapter 4, section 15 (1) of the Prevention of Cruelty to Animals Act 1960. Our study was approved by the Institutional Animal Ethics Committee, Department of Pharmaceutical Sciences, University of Kashmir, Srinagar, India (wide approval F [IAEC approval]KU/2024/10). Experimental animals were acclimatized for 2 weeks before in vivo experimentation. Rats were treated humanely and fed properly with food and water ad libitum. The environment at the institutional animal house was well maintained with a 12-hour light/dark cycle, relative humidity 50% ± 20%, temperature 23°C ± 2°C, and ventilation of 10–15 air changes/hour.

In vivo anti-inflammatory study

Wistar rats were categorized into seven groups of six rats per group: group I normal saline only, group II carrageenan solution only, group III Indomethacin at a dose of 10 mg/kg body weight, group IV carrageenan + 100 mg/kg EA extract, group V carrageenan + 300 mg/kg EA extract, group VI carrageenan + 100 mg/kg ME extract, group VII carrageenan + 300 mg/kg ME extract. Group I was given normal saline for 21 days. Groups IV–VI received plant extracts through oral gavage for 21 days. On the 22nd day, Groups II–VII received a single subplantar injection of 0.1 ml of carrageenan into the left hind paw. Group III was given indomethacin one hour prior to carrageenan injection. Six hours after carrageenan administration, all animals were anesthetized with an intramuscular injection of 80 mg/kg ketamine and 10 mg/kg xylazine to relieve pain. Finally euthanization was carried out through cervical dislocation.

One hour after treatment, paw edema was induced by injecting 0.1 mL of a 1% carrageenan solution into the left hind paw, just beneath the plantar of aponeurosis. Subsequently, the increase in edema in the left paw was measured at hourly intervals for 6 hours after treatment. The percentage inhibition of inflammation was calculated using the following formula:

$$\% \text{ inhibition of inflammation} = (1 - V_r/V_c) \times 10$$

Where V_r represents the mean paw edema in the treated groups, and V_c represents the mean paw edema in the control group.

Histopathological studies. The animals were sacrificed and the paw was extracted and fixed in 10% formalin in normal saline solution. The paw tissue was dehydrated by passing it through a graded series of alcohol and acetone solution, benzene used as clearing agent and tissue samples were then embedded in paraffin blocks melting point

60°C. The sections of 5µm thickness prepared using an automated microtome. The sections were stained in Harris haematoxylin and eosin for examination under microscope (Nikon Phase contrast) in order to reveal the effect of extract and fractions on the aortic tissue.

Statistical analysis

Data was statistically analysed using Graphpad Prism 5. Each experimental data represents the mean ± standard deviation of three biological replicates (n = 3). Tukey's and Bonferroni posttest was performed to calculate significant differences among various groups. Bars represent standard deviation and asterisk significant differences between catechin and various extracts of *Cousinia thomsonii* at *P<0.05; **P<0.01; ***P<0.001

Results

In vitro antioxidant activity

DPPH radical scavenging activity. The capacity of the *Cousinia thomsonii* extracts (ethyl acetate, methanol and aqueous) to scavenge DPPH radical was measured and the results are depicted in (Fig 1A).

Ethyl acetate and methanolic extracts of *Cousinia thomsonii* showed the maximal activity. The scavenging effect of *Cousinia thomsonii* extracts increased significantly in a dose dependent fashion with increasing amounts of extracts. Ethyl acetate extracts at a concentration of 700 µg/mL exhibited ~88% radical scavenging property, a noticeable effect than that of the methanol and aqueous extract which showed only around 45 (P<0.001), and 28% (P<0.001) scavenging effect at the same concentration, compared to catechin. The DPPH scavenging activity of ethyl acetate extract was comparable to that of Catechin as reference free radical scavenger which exhibited around 88.71 percentage inhibition at a concentration of 700 µg/mL. The findings point at the presence of increased antioxidants in the ethyl acetate fraction of *Cousinia thomsonii* compared to other fractions.

FRAP assay. The reducing power of ethyl acetate, methanolic and aqueous extracts of *Cousinia thomsonii* are summarized in (Fig 1B). The data shows that the reducing power of extracts increased in a dose dependent manner. The ability of reducing power ethyl acetate, methanolic and aqueous extracts were almost comparable to the synthetic antioxidant Catechin. At a high concentration of 700 µg/mL, the reducing powers of ethyl acetate, methanolic and aqueous were found to cause 83%, 77% and 50% percentage inhibition when compared to Catechin (P<0.001, compared with catechin) (Fig 1B). The results indicate the presence of the highest reducing capacity in ethyl acetate extract followed by methanolic and aqueous extracts.

Hydroxyl radical scavenging assay (DNA damage). As depicted in (Fig 1C), Fe⁺⁺/ascorbic in the presence of H₂O₂, markedly causes deoxyribose oxidation. The radical scavenging activity of methanolic, aqueous and ethyl acetate extracts of *Cousinia thomsonii* was evaluated and compared with known radical scavenger Catechin. A dose-dependent effect of the known antioxidant and our plant extracts on hydroxyl radical scavenging was observed. At a concentration of 500 µg/mL, we observed 89% (P<0.01), 74.29% and 78.36% hydroxyl radical scavenging activity of ethyl acetate, methanolic and aqueous extracts. Hydroxyl radical activity shown by ethyl acetate extracts was significantly higher than that shown by positive control Catechin (82%). The finding demonstrates that the ethyl acetate extract exhibits the highest activity against hydroxyl radical mediated damage compared to other extracts.

Lipid peroxidation estimation. The exposure of membrane lipids to reactive oxygen species leads to the formation of TBARS, such as malondialdehyde (MDA), through lipid peroxidation (LPO). MDA reacts with thiobarbituric acid (TBA) to produce a pink chromogen that exhibits absorbance at 535 nm. *Cousinia thomsonii* extracts dose dependently inhibited the MDA formation, and thus the lipid peroxidation in liver microsomes. The amount of MDA formed in the presence of known antioxidant and test compounds are shown in (Fig 1D). At a concentration of 100 µg/mL ~ 78, 63 and 44% inhibition in LPO was observed with ethyl acetate, methanolic and aqueous extracts respectively (P<0.001) compared with catechin. In the presence of known antioxidant, Catechin at 100 µg/mL concentration, LPO was inhibited by 85%

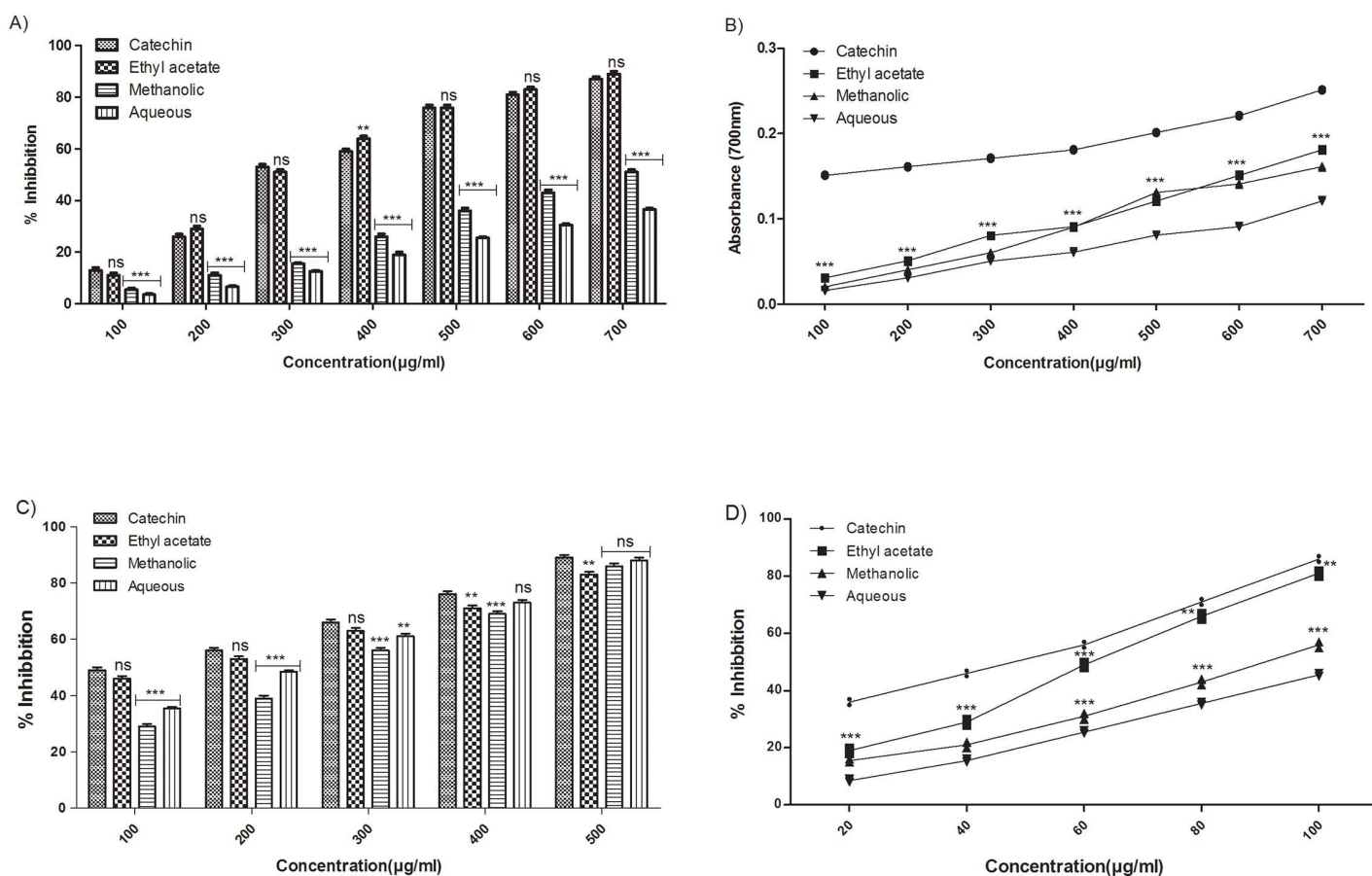


Fig 1. A: Assessment of *in vitro* antioxidant activity of various *Cousinia thomsonii* extracts by DPPH radical scavenging activity. **B:** Ferric iron reducing power to evaluate the reducing power of various extracts, **C:** Hydroxyl radical induced DNA damage to assess the DNA damage protection, **D:** Liver microsomal lipid peroxidation assay to test the lipid damage protection in presence of various extracts. All the assays were carried out with antioxidant Catechin as a positive control. Data is presented as mean $\pm$ standard deviation of three independent replicates ($n=3$). Bonferroni posttest was performed to calculate significant differences among various groups. Bars represent standard deviation and asterisk significant differences between catechin and various extracts of *Cousinia thomsonii* at * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

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respectively. The percentage inhibition of all the antioxidants was observed is in the following descending order at all the concentrations.

Catechin > Ethyl acetate extract > Methanolic extract > Aqueous extract

Overall ethyl acetate extract emerged as the extract with highest antioxidant content.

DNA protective effects of ethyl acetate and methanolic extracts from *Cousinia thomsonii* against H_2O_2 and Fe^{2+} induced DNA Damage

In the DNA protection assay conducted on plasmid pGEX-4T2 and pET-28A, the ethyl acetate and methanolic extracts derived from *Cousinia thomsonii* exhibited significant protective effects against damage induced by known concentrations of hydrogen peroxide (H_2O_2) and ferrous ion (Fe^{2+}). Under normal conditions, in the absence of DNA damaging agents such as H_2O_2 and Fe^{2+} , the plasmid was present in three distinct forms, as depicted in (Fig. 2).

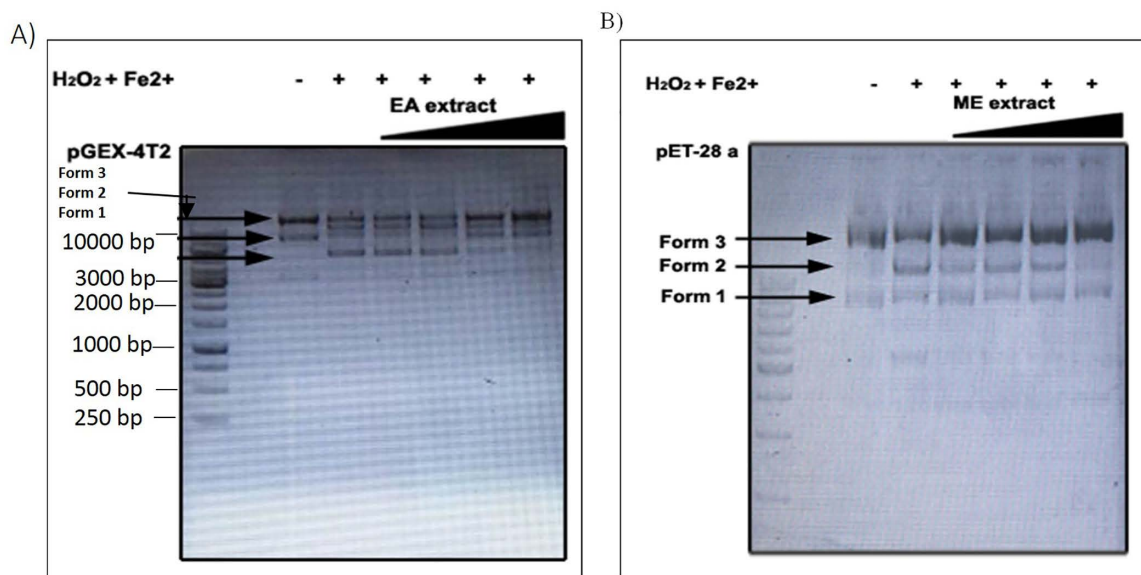


Fig 2. Agarose gel depicting the DNA Protective effects of Ethyl Acetate (A) and methanolic Extracts (B) from *Cousinia thomsonii*. The figure illustrates the DNA protection assay conducted on plasmid pGEX-4T2 and p-ET-28A in the presence of hydrogen peroxide (H₂O₂) and ferrous ions (Fe²⁺).

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However, in the presence of H₂O₂ and Fe²⁺, a substantial damage to the plasmid occurred, which was notably attenuated in the presence of *Cousinia thomsonii* extracts. The results are in agreement with the hydroxyl radical scavenging assay and further underscore the abundance of DNA-protective antioxidants in the *Cousinia thomsonii* extracts. Upon the addition of increased concentrations of ethyl acetate and methanolic extracts, a significant reduction in DNA damage was observed (Fig 2A and 2B).

***Cousinia thomsonii* ablates ROS stress and ROS induced cell death in cellular models**

In order to substantiate our findings in cellular systems, HepG2 cells were exposed to 100 μM of H₂O₂ for 24 h in order to induce oxidative stress. The potency of the extract was evaluated by the co-treatment of HepG2 cells with H₂O₂ and various concentrations of the extract. It was observed that the extract exhibited a dose-dependent effect in overcoming the effect of H₂O₂ as indicated by the staining of cells with DCFDA (Fig 3A). Among the tested concentrations, 250 μg proved to be the best concentration in ablating the oxidative stress induced by H₂O₂ treatment (P<0.001) compared to control. Collectively these results demonstrate the antioxidant efficacy of *Cousinia thomsonii* extracts in mitigation of oxidative stress in cellular models and are in affirmation with cell-free assay based findings.

As higher levels of ROS are known to induce cell death. To further corroborate our results, we carried out PI/DAPI staining of HepG2 cells treated with H₂O₂ or co-treated with H₂O₂ and various concentrations of the extract. The premise of the assay lies in the principle that the DAPI enters all cells while PI only enters necrotic cells, or those undergoing late apoptosis (or secondary necrosis/necrosis) when membrane integrity is lost. The results indicated that the extract exhibited a dose-dependent effect in overcoming the cell death induced by treatment with H₂O₂ (Fig 3C). In coherence with the results obtained in DCFDA assay, 250 μg concentration of the extract proved to be the best performer to ablate the cell death. At molecular level, we assessed the cleavage status of PARP-1, one of several known cellular substrates of caspases. Western blotting of the PARP-1 revealed the cleavage of PARP when cells were treated with H₂O₂, however treatment with various concentrations of extract inhibited this cleavage (Fig 3D). Taken together, lowered number of PI

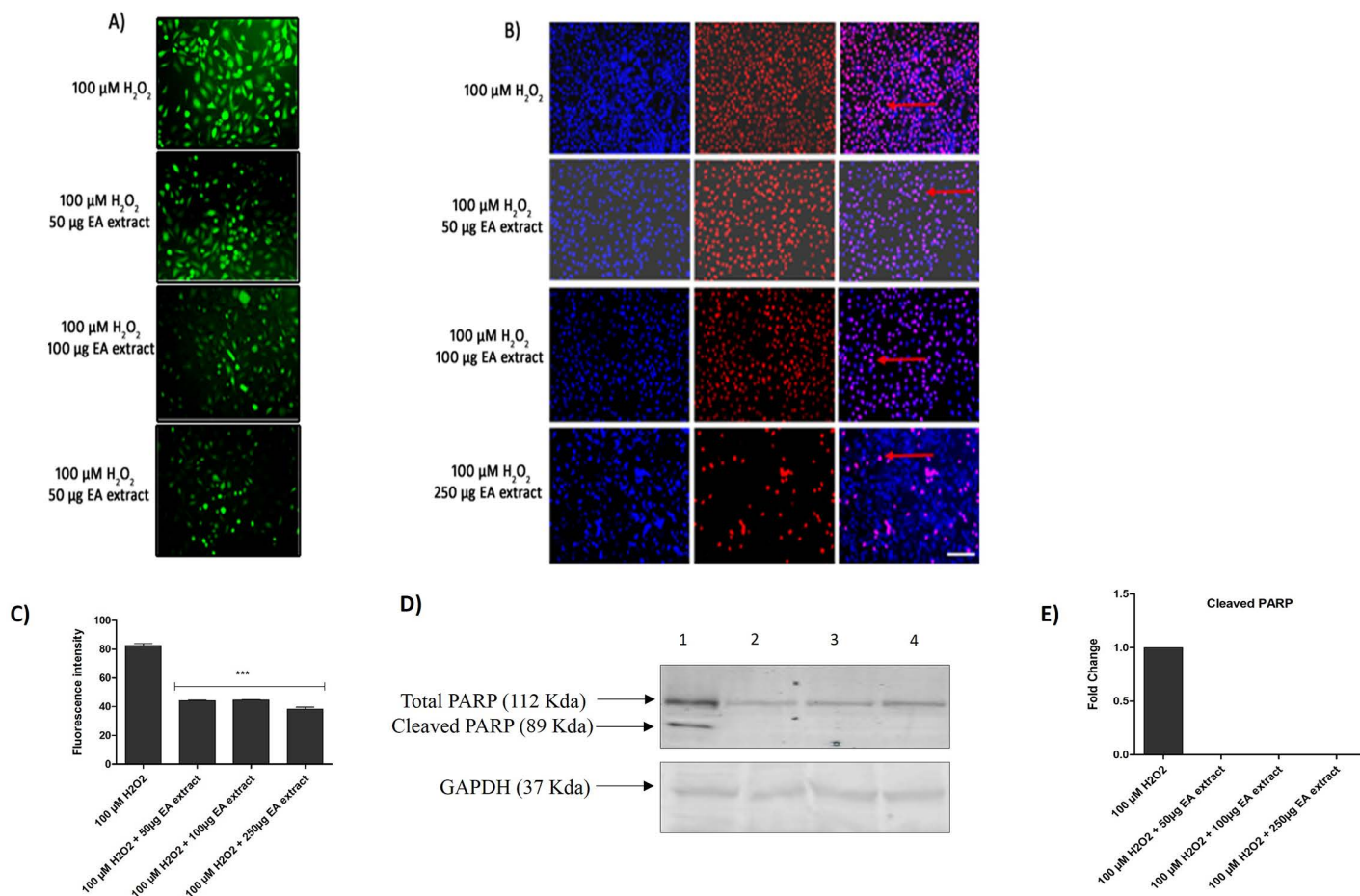


Fig 3. *Cousinia thomsonii* mitigates H_2O_2 -induced oxidative cell death. (A) DCFDA staining indicating the abundance of ROS under indicated conditions (B) Graphical representation of fluorescence intensity observed during DCFDA staining as quantified by ImageJ software. Data is presented as mean $\pm$ standard deviation of three independent replicates (n=3). Tukey's Multiple Comparison Test was performed to calculate significant differences among various groups. Bars represent standard deviation and asterisk significant differences between H_2O_2 treated and H_2O_2 +Ethyl acetate treated groups at *P<0.05; **P<0.01; ***P<0.001 (C) PI/DAPI staining indicating cell death in the extract treated and untreated cells. Arrows indicate cell death, Scale bar, 100 μM . (D) Cleavage status of the PARP under indicated conditions. (E) Expression of PARP in terms of Fold change under indicated conditions.

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positive cells and inhibition of PARP-1 cleavage point at the role of *Cousinia thomsonii* in ablating the oxidative stress triggered apoptosis. In line with the ROS evaluation, 250 μg proved to be the optimal dosage to overcome the oxidative challenge.

Cousinia thomsonii inhibits NO stress under *in vitro* conditions

We also investigated the effect of *Cousinia thomsonii* extracts on scavenging of elevated NO levels during Sodium Nitroprusside-induced toxicity in HepG2 cells. As NO is secreted out by cells, we estimated its level after various treatments in culture supernatants employing standard Griess assay (described in materials and methods). As shown in (Fig 4A), the exposure of HepG2 cells to the 400 μM SNP resulted in the appreciable rise in NO levels after 24 hrs, compared to control untreated cells (control).

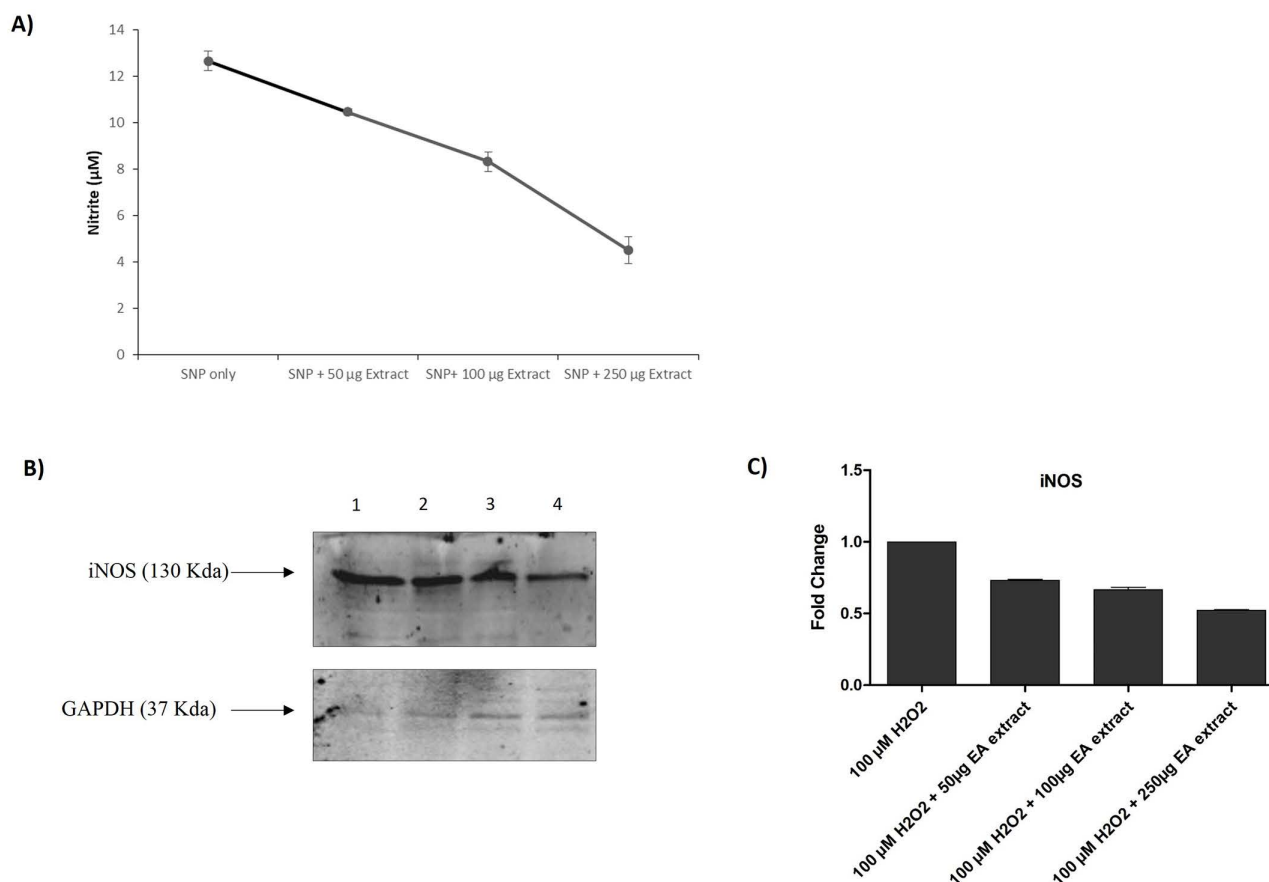


Fig 4. *Cousinia thomsonii* attenuates SNP-induced nitrosative stress in HepG2 cells. (A) Nitrite concentration under indicated conditions as determined by Griess's assay. Data is represented as the mean $\pm$ SEM of three independent experiments ($n = 3$). Tukey's Multiple Comparison Test was performed to calculate significant differences among SNP treated and SNP+Ethyl acetate treated groups at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) Immunoblot indicating the change in iNOS expression upon treatment with (1) SNP (2) SNP + 50 µg extract (3) SNP + 100 µg extract (4) SNP + 250 µg extract. (C) Expression of iNOS in terms of Fold change under indicated conditions.

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However, co-treatment with different concentrations of the extract exhibited a dose-dependent inhibition on elevated NO in HepG2 cells. These results led us to investigate the expression status of iNOS, an enzyme regulating the production of NO. The expression analysis of iNOS was carried out by immunoblotting. As expected, iNOS was significantly up-regulated in HepG2 upon stimulation with SNP (Fig 4B). However, its expression was significantly downregulated when cells were treated with different concentrations of *Cousinia thomsonii* extract.

Cousinia thomsonii modulates the expression of Nrf2, the master regulator of Oxidative stress

Nrf2 (Nuclear factor erythroid 2-related factor 2) is a critical transcription factor that plays a pivotal role in cellular defense against oxidative stress. By activating genes involved in detoxification and antioxidant responses, Nrf2 acts as the body's first line of defence against cellular damage caused by oxidative stress. Nrf2 is known to be regulated at transcriptional and post-transcriptional level. Therefore we set out to investigate the effect of H₂O₂-induced oxidative stress on Nrf2 mRNA and protein expression in HepG2 cells. We observed that the co-treatment with different concentrations of the extract led to increase in the expression of Nrf2 (Fig 5A), in a dose dependent manner, with highest expression being observed at 250 µg concentration ($P < 0.001$). The finding points at the role of *Cousinia thomsonii* extract in mediating

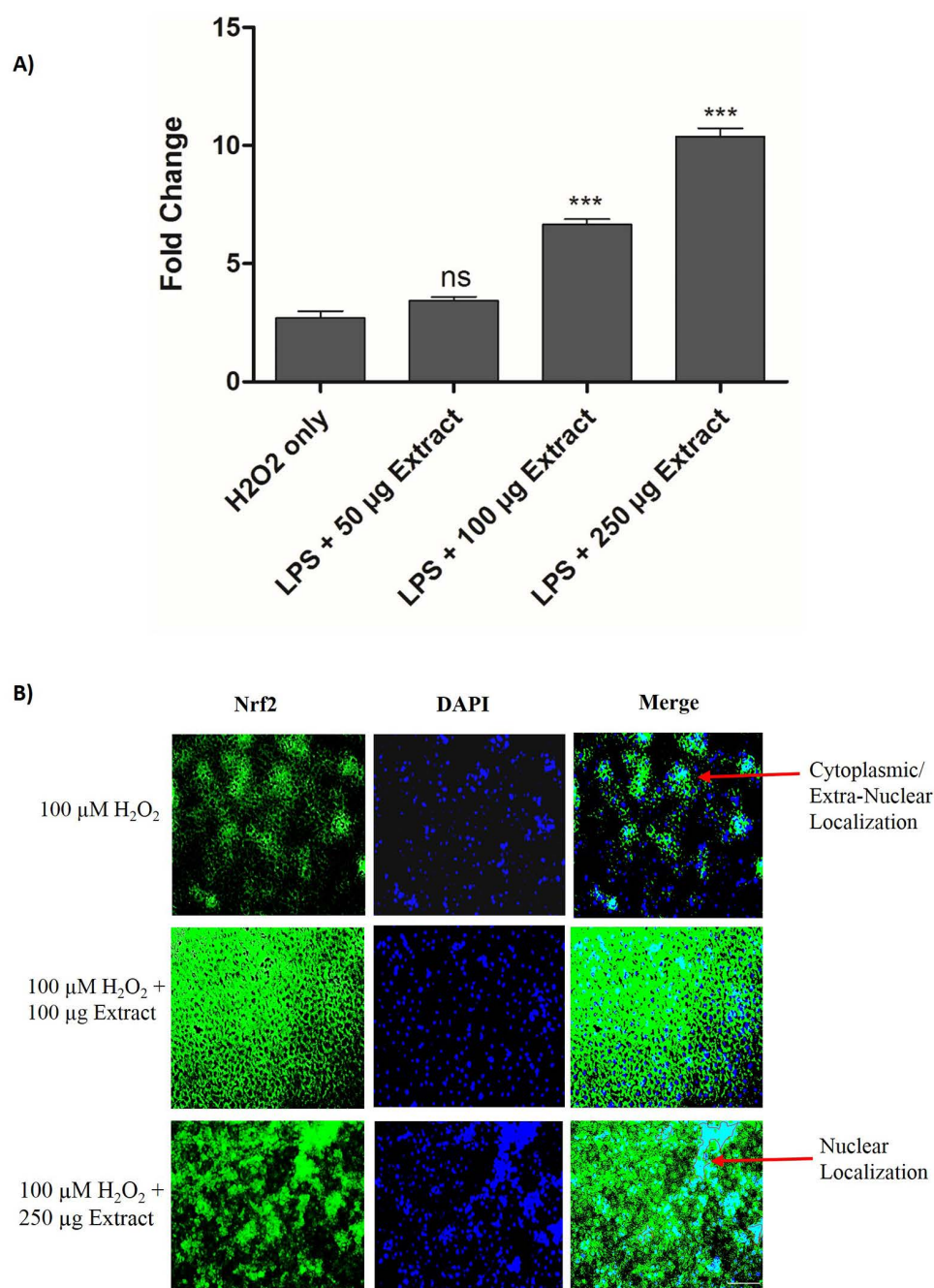


Fig 5. *Cousinia thomsonii* modulates Nrf2 expression and promotes its nuclear localization. (A) Quantitative mRNA expression levels of Nrf2 as carried out Real time PCR. Tukey's Multiple Comparison Test was performed to calculate significant differences among various groups. Data is represented as the mean $\pm$ standard deviation of three independent experiments ($n=3$). Bars represent standard deviation and asterisk significant differences between control and LPS+extract treated groups at *** $P<0.001$. (B) Cytoplasmic and nuclear localization of Nrf2 under indicated conditions.

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the up-regulation of Nrf2 to curb the oxidative stress. Furthermore, treatment with different concentrations of the extract led to the nuclear localization of Nrf2 especially at 250 µg concentration (Fig 5B). Overall the effect was observed to be dose-dependent.

Evaluation of anti-Inflammatory effects of *Cousinia thomsonii* in cellular models

To evaluate the anti-inflammatory effect of *Cousinia thomsonii*, the levels of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 were quantified. For triggering inflammation LPS was used and THP-1 cells were exposed to 1 μ g/mL LPS. We observed that the levels of TNF- α , IL-1 β and IL-6 resulting from the LPS stimulation were inhibited by *Cousinia thomsonii* extract in a dose-dependent manner with highest inhibition being observed at 250 μ g concentration ($P < 0.001$). (Fig 6).

Expression of all the three cytokines showed a similar trend. Collectively, these results suggest that *Cousinia thomsonii* inhibited pro-inflammatory cytokine release, exhibiting an anti-inflammatory effect.

Cousinia thomsonii extracts shows anti-Inflammatory effects on Carrageenan-induced paw edema in rats

The results presented in (Fig 7) demonstrate the anti-inflammatory potential of ethyl acetate extract from *Cousinia thomsonii*, as well as Indomethacin, in a carrageenan-induced inflammation model. Animals treated with 100 and 300 mg/kg b.w. of EA extract and 10 mg/kg b.w. of Indomethacin exhibited a significant dose-dependent inhibition of paw edema over a 6-hour period compared to the untreated control group ($P < 0.001$). Specifically, at the sixth hour, Indomethacin (10 mg/kg b.w.) and EA extract at doses of 100 and 300 mg/kg b.w. demonstrated a remarkable suppression of paw edema by 1.2 ± 0.04 mm (87.14%), 0.98 ± 0.05 mm (81.08%), and 1.16 ± 0.05 mm (88.25%), respectively, when compared to the untreated control group.

Similarly, the results presented in (Fig 8) shows that ME of *Cousinia thomsonii* were also effective in reducing carrageenan induced paw edema but at a lesser efficiency compared to the EA extracts. Indomethacin at 10 mg/kg and ME extract at doses of 100 and 300 mg/kg b.w. suppressed paw edema in rats at the sixth hour by 0.98 ± 0.04 mm (71.13%), 0.58 ± 0.05 mm (63.01%), and 0.63 ± 0.04 mm (68.40%), respectively, when compared to the untreated control group ($P < 0.001$).

Histopathological studies of rat paw

The histopathological changes in the paw of rats were examined to understand the impact of these pro-inflammatory compounds. The normal group (Control) of wistar rats exhibited normal tissue histopathology with no edema or infiltration

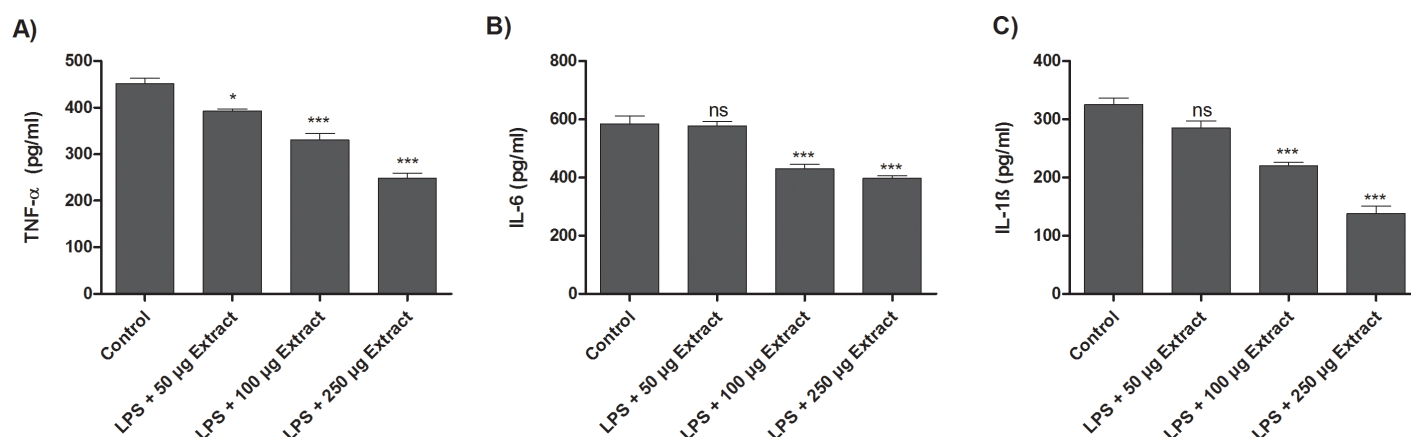


Fig 6. *Cousinia thomsonii* suppresses LPS-induced expression of inflammatory cytokines. (A) TNF- α (B) IL-6 and (C) IL-1 β in THP-1 cells. Tukey's Multiple Comparison Test was performed to calculate significant differences among various groups. Data is represented as the mean $\pm$ standard deviation of three independent experiments ($n = 3$). Bars represent standard deviation and asterisk significant differences between control and LPS+extract treated groups at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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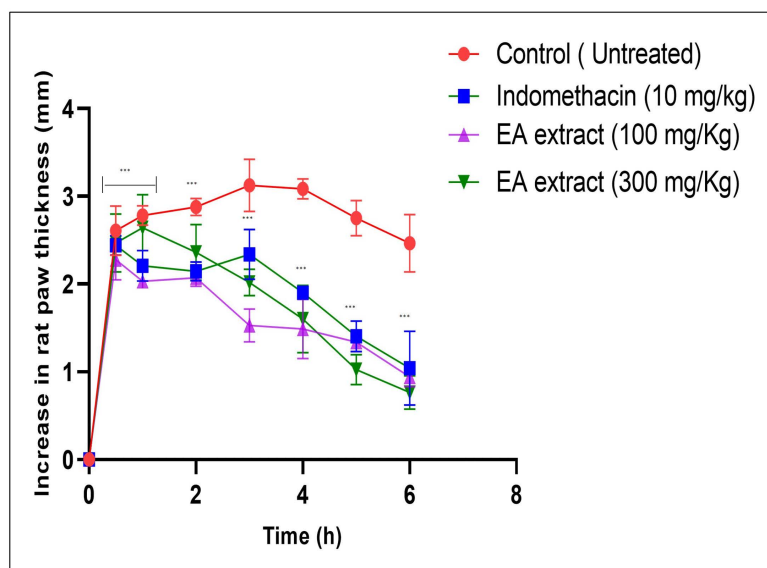


Fig 7. Percentage inhibition of Carrageenan- induced paw edema rats treated with 100 and 300 mg/Kg ethyl acetate (EA) extract of *Cousinia thomsonii* with respect to control. Data are expressed as mean $\pm$ SD. Bonferroni posttest was performed to calculate significant differences among various groups. Data is represented as the mean $\pm$ standard deviation of three independent experiments (n=3). Bars represent standard deviation and asterisk significant differences between control, indomethacin and ethyl acetate extract treated groups at *P<0.05; **P<0.01; ***P<0.001.

<https://doi.org/10.1371/journal.pone.0343450.g007>

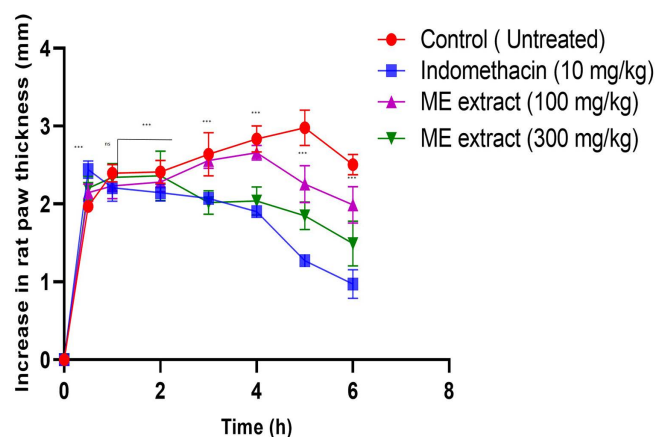


Fig 8. Percentage inhibition of Carrageenan- induced paw edema rats treated with 100 and 300 mg/Kg methanolic (ME) extract of *Cousinia thomsonii* with respect to control. Data are expressed as mean $\pm$ SD. Bonferroni posttest was performed to calculate significant differences among various groups. Data is represented as the mean $\pm$ standard deviation of three independent experiments (n=3). Bars represent standard deviation and asterisk significant differences between control, indomethacin and methanolic extract treated groups at *P<0.05; **P<0.01; ***P<0.001.

<https://doi.org/10.1371/journal.pone.0343450.g008>

of cells (Fig 9A). In contrast, the group treated with Carrageenan displayed evident edema, indicating the induction of inflammatory processes (Fig 9B). Notably, the induction of the inflammatory response was significantly attenuated in samples treated with *Cousinia thomsonii* methanolic and ethyl acetate extracts (at concentrations of 100 mg/kg and 300 mg/kg of body weight) (Fig 9D and 9F). These treated samples exhibited minor cellular infiltration, akin to the group treated with Carrageenan+ Indomethacin (Fig 9C). This underscores the potential anti-inflammatory effects of *Cousinia thomsonii*

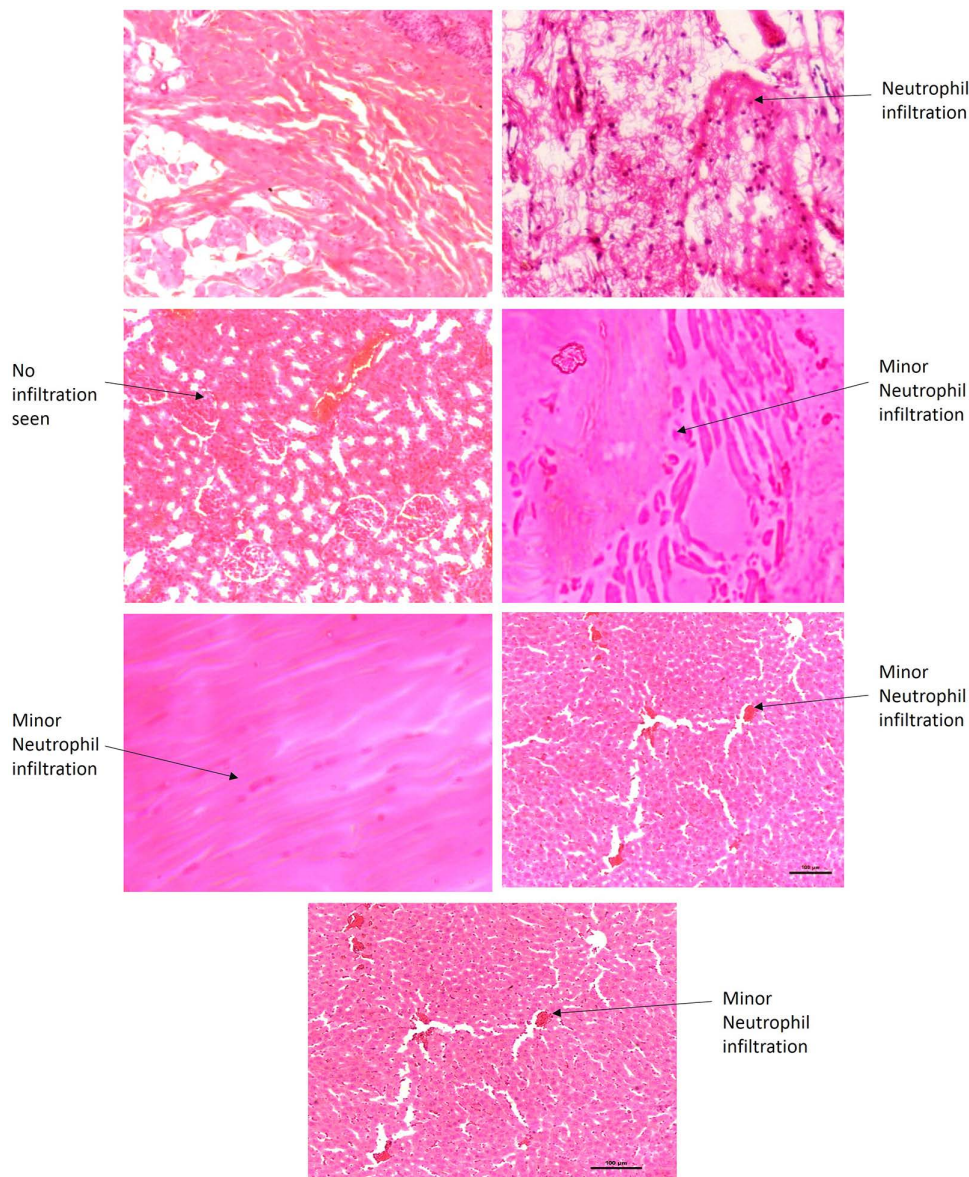


Fig 9. Histopathology of rat paw tissue (H&E stain) in carrageenan paw edema. Value expressed as average of 3 sample. **A)** Control (normal Paw) **B)** Carrageenan induced **C)** Carrageenan+ Indomethacin **D)** *Cousinia thomsonii* methanolic extract 100 mg/kg of body weight **E)** *Cousinia thomsonii* ethyl acetate extract 100 mg/kg of body weight **F)** *Cousinia thomsonii* ethyl extract 300 mg/kg of body weight. **G)** *Cousinia thomsonii* methanolic extract 300 mg/kg of body weight. Magnification level: 10X.

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extracts, suggesting a comparable efficacy to the well-established anti-inflammatory agent, Indomethacin, in mitigating cellular infiltration and highlighting the promise of *Cousinia thomsonii* as a natural anti-inflammatory agent.

Discussion

Multiple complex pathological states such as rheumatoid arthritis, cardiovascular diseases, neurodegeneration and cancer are associated with considerable oxidative stress at both the cellular and organ levels [19]. Mitigating oxidative stress

provides one of the approach to prevent these diseases and associated subsequent mortality. Natural antioxidants have always been preferred as a choice of source due to their ease of use, low toxicity and better therapeutic potential than their man-made counterparts. This study elucidated the mechanistic basis of antioxidant and anti-inflammatory activity of *Cousinia thomsonii* extracts through the modulation of Nrf2 signalling, inhibition of apoptosis and downregulation of iNOS.

In this study, we carried out various *in vitro* assays (DPPH, Fe^{3+} reducing power estimation, LPO and TBARS) of aqueous, ethyl acetate (EA) and methanolic extracts of the whole plant. We observed that ethyl acetate (EA) extract shows maximum antioxidant activity across assays when compared to Catechin, a known antioxidant. This pronounced antioxidant effect seems due to the presence of multiple compounds like α -tocopherol, ascorbic acid derivatives, phytols and other related compounds [6]. Most of the identified compounds have well-documented anti-inflammatory properties and act through diverse mechanisms. For example Phytol has been demonstrated to impede infiltration of neutrophils and total leukocytes, as well as suppress expression of IL-1 β and TNF- α . Likewise β -sitosterol ameliorates inflammation by modulating arachadonic acid pathway and blocks the translocation of NF κ B to nucleus. Based on this study and strong antioxidant activity observed in the present *in vitro* screening, we conducted further assays in cellular model systems of liver disease. The ethyl acetate extract mitigated oxidative stress and subsequent cell death in a concentration dependent manner as evidenced by DCFDA, PI/DAPI staining and PARP cleavage in HepG2 cells. We also assessed the NO scavenging activity of *Cousinia thomsonii* extracts during sodium nitroprusside-induced toxicity in HepG2 cells. The results affirmed the role of *Cousinia thomsonii* in alleviating ROS and NO stress. At molecular level, *Cousinia thomsonii* extract was found to modulate the expression and sub-cellular localization of Nrf2, the master regulator of oxidative stress. The findings indicate that multiple antioxidant compounds in *Cousinia thomsonii* extracts are effectively mitigating peroxide induced oxidative stress in cellular model systems.

Our results are in agreement with previously published data that extracts from *Cousinia thomsonii* have potent antioxidant activity and regulate redox homeostasis as revealed by several *in vitro* and *in vivo* assays [20,21]. The present study unravels the mechanistic details including activation of Nrf-2 pathway by *Cousinia thomsonii* to inhibit oxidative stress in the challenged cells. It is also lays impetus on the role of the herb in overcoming NO stress and inflammatory signalling. The mechanistic profile observed here is consistent with the phytochemicals present in various other plant species including curcumin from *Curcuma longa*, epigallocatechin gallate (EGCG) from *Camellia sinensis*, and withanolides from *Withania somnifera*, all of which have been demonstrated to activate the Nrf2–Keap1 signaling axis. Furthermore, many studies have reported bioactive compounds with antioxidant properties to inhibit inflammatory responses by the activation of Nrf2 signaling cascade [22]. Nrf2 signalling pathway is clinically recognised as an indispensable cytoprotective mechanism that ablates oxidative stress–driven inflammation, a key precursor to various chronic inflammatory disorders and cancers. Several lines of evidence also support the use of plant derived bioactive in modulation of Nrf2 signaling through synergistic effect. These compounds include Naringenin, Curcumin, Quercetin lycopene, Resveratol, lutein, chlorogenic acid, and delphinidin [23,24]. Overall, Our findings demonstrate that the antioxidant and anti-inflammatory effects of *Cousinia thomsonii* extracts are mediated through activation of the Nrf2 pathway, attenuation of apoptosis, and downregulation of iNOS.”.

Conclusion

Cousinia thomsonii extracts exhibit potent antioxidant and anti-inflammatory activity which is evident from its properties to reduce lipid peroxidation, hydroxyl radical and plasmid DNA damage. The herb showed a marked ameliorating effect on H_2O_2 induced oxidative stress and cell death in HepG2 cells. It also exhibited its effects on the expression of Nrf2, the master regulator of oxidative stress as well as the levels of TNF- α , IL-1 β and IL-6 resulting from the LPS stimulation in THP-1 cells. Histopathological analysis further confirmed the protective effects of both the extracts on paw tissue, demonstrating their capacity to restore normal tissue architecture and function. Our study demonstrates for the first time the anti-oxidant potential of *Cousinia thomsonii* through modulation of Nrf2 signalling, inhibition of apoptosis and

downregulation of iNOS. In conclusion, *Cousinia thomsonii* showed a promising approach to manage oxidative stress and inflammation and to enhance treatment outcomes in future. The study further warrants validation of specific bioactive molecules found present in *Cousinia thomsonii* vis-à-vis their mechanistic insights.

Supporting information

S1 Fig. S3D Full length blot indicating the cleavage status of the PARP under indicated conditions. Total and cleaved PARP are indicated by 112 and 89 Kda bands respectively while 37 Kda band represents GAPDH.
(PDF)

S2 Fig. S4B Full length blot indicating the expression of iNOS under indicated conditions. iNOS is represented by 130 Kda band while 37 Kda band indicates GAPDH.
(PDF)

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