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In Vitro Molecular Evaluation of *Capparis spinosa* Leaf Extract Against *Leishmania tropica*

Evaluasi Molekuler secara In Vitro terhadap Ekstrak Daun Capparis spinosa dalam Melawan Leishmania tropica

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ABSTRAK

Latar Belakang: Leishmaniasis adalah penyakit tropis terabaikan yang ada di seluruh dunia. Resistensi parasit terhadap kemoterapi konvensional menyulitkan pengontrolannya. Oleh karena itu, ada kebutuhan mendesak untuk menemukan agen terapeutik alternatif yang berasal dari tanaman. **Tujuan:** Penelitian ini dilakukan untuk mengevaluasi efek *in vitro* dari ekstrak etanol daun *Capparis spinosa* terhadap ekspresi dua gen pertahanan antioksidan utama pada promastigot *Leishmania tropica*, yaitu *iron-containing superoxide dismutase* (Fe-SOD) dan *trypanothione reductase* (TryR). **Metode:** Promastigot *L. tropica* dikultur dalam media RPMI-1640 pada kondisi laboratorium yang optimal. Selanjutnya, mereka diobati dengan berbagai konsentrasi ekstrak etanol daun *C. spinosa*, sementara promastigot yang tidak diobati dianggap sebagai kontrol. Ekspresi relatif dari Fe-SOD dan TryR ditentukan menggunakan *real-time reverse transcription polymerase chain reaction* (RT-qPCR). **Hasil:** Ekstrak *C. spinosa* memberikan perubahan yang signifikan pada ekspresi Fe-SOD dan TryR dibandingkan dengan kelompok kontrol. Hal ini menunjukkan bahwa ekstrak tersebut mengganggu homeostasis redoks parasit dan mempromosikan stres oksidatif. **Kesimpulan:** Ekstrak etanol daun *C. spinosa* memodulasi sistem pertahanan antioksidan pada promastigot *L. tropica*. Efek ini mungkin dimediasi oleh fitokimia bioaktif yang menginduksi stres oksidatif dan mengkelat zat besi, sebuah kofaktor esensial dari Fe-SOD. Inang mamalia tidak memiliki salah satu dari enzim tersebut, menjadikan keduanya sebagai target yang menarik untuk obat selektif. Hasil ini menunjukkan bahwa *C. spinosa* adalah sumber alami potensial untuk agen antileishmanial. Penelitian lebih lanjut diperlukan untuk mengisolasi prinsip aktifnya dan memvalidasi khasiatnya secara *in vivo*.

ABSTRACT

Background: Leishmaniasis is a neglected tropical disease, present worldwide. The resistance of the parasite to conventional chemotherapy makes its control difficult. Therefore, there is an critical need to find alternative therapeutic agents of plant origin. **Objective:** This study was carried out to evaluate the in vitro effect of Capparis spinosa ethanolic leaf extract on the expression of two major antioxidant defense genes in *Leishmania tropica* promastigotes, iron-containing superoxide dismutase (Fe-SOD) and trypanothione reductase (TryR). **Methods:** The *L. tropica* promastigotes were cultured in RPMI-1640 media in optimum laboratory conditions. Later, they were treated with different concentrations of the ethanolic extract of C. spinosa leaves and untreated promastigotes were considered as control. The relative expression of Fe-SOD and TryR was determined by real time reverse transcription polymerase chain reaction (RT-qPCR). **Results:** *C. spinosa* extract had significant change in expression of both Fe-SOD and TryR compared with control group. This demonstrates that the extract disturbs the redox homeostasis of the parasite and promotes oxidative stress. **Conclusion:** Ethanolic extract of leaves of *C. spinosa* modulates the antioxidant defense system of *L. tropica* promastigotes. This effect may be mediated by bioactive phytochemicals that induce oxidative stress and chelate iron, an essential cofactor of Fe-SOD. Mammalian hosts do not have either enzyme, making them both attractive targets for selective drugs. These results suggest C. spinosa is a potential natural source of antileishmanial agents. More work is needed to isolate its active principles and validate their efficacy *in vivo*.

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INTRODUCTION

Leishmaniasis is a complex of neglected tropical diseases caused by obligate intracellular protozoan parasites of the genus Leishmania, with more than 20 species infective to humans and transmitted by the bite of infected female phlebotomine sand flies (WHO, 2023). The disease presents in three main clinical forms: visceral, mucocutaneous and cutaneous leishmaniasis (CL), the latter being the most common form and responsible for skin ulcers in exposed areas of the body that can result in permanent scars, disability and social stigma (WHO, 2023) . It is estimated that 600,000 to 1 million new cases of CL occur globally each year, of which only approximately 200,000 cases are reported to the World Health Organization (WHO). Approximately 95% of cases occur in the Americas, the Mediterranean basin, the Middle East, and Central Asia (WHO, 2023). In 2022, leishmaniasis was endemic in 99 countries and lands (WHO., 2026). The Eastern Mediterranean Region has the greatest burden, accounting for 81% of the global CL cases, with Iraq being one of the main contributing countries (WHO, 2025).

Cutaneous leishmaniasis is caused by *Leishmania tropica* and *L. major* and is an endemic national health problem in Iraq (Flaih et al., 2024; Bayram et al., 2021). During a six-year research in Al-Muthanna Governorate, *L. tropica* was detected in 61.9% of the CL cases confirmed by genetic methods (Flaih et al., 2024; Bayram et al., 2021). *L. tropica* causes anthroponotic CL, produces chronic and recurrent (lupoid) lesions, and is less responsive to treatment than other dermotropic species, making new treatments for it essential.

Pentavalent antimonials, such as sodium stibogluconate and meglumine antimoniate, have been the first-line treatment for all forms of leishmaniasis for more than seven decades (Chakravarty, J., & Sundar, 2010). However, their clinical application is increasingly limited due to major side effects as cardiotoxicity, pancreatitis, hepatotoxicity, and nephrotoxicity, in addition to extended parenteral administration and high cost (Zhang et al., 2025; Bhusal et al., 2025).The second-line medicines, including amphotericin B, miltefosine, paromomycin and pentamidine are likewise limited by toxicity, expense, limited availability and a rising number of cases of treatment failure (Zhang et al., 2025; Bhusal et al., 2025). More crucially, parasite resistance to antimonials has arisen in many endemic countries (Bhusal et al., 2025). This is mostly due to poor dose and incomplete treatment routine. These limitations underline the pressing need for novel, safe, inexpensive and selective antileishmanial medicines and medicinal plants are a viable source of such agents because of their structural variety and long history of ethnomedicinal usage (Sakyi et al., 2021; Hassan et al., 2022).

Leishmania lacks glutathione reductase and catalase, and depends on trypanothione reductase (TryR) to keep trypanothione reduced under oxidative stress from its own metabolism and from the macrophage respiratory burst (Roy et al., 2025; Onwah et al., 2026). TryR is required for virulence, and its substrate specificity differs from that of host glutathione reductase, which allows selective inhibition (Onwah et al., 2026). Moreover, Leishmania depends on iron-containing superoxide dismutases (Fe-SODs) as a primary defense mechanism against superoxide radicals, and the mitochondrial isoform Fe-SODA has been demonstrated to be necessary for parasite virulence and resistance to oxidative stress (Mittra et al., 2017). Mammals do not have Fe-SOD but have copper/zinc and manganese SODs and so Fe-SOD is viewed as an interesting parasite-specific chemotherapeutic target (Santi et al., 2022; Martín-Montes et al., 2021). Interestingly, the overexpression of Fe-SOD and TryR has been related with antimony resistance in Leishmania, including clinical isolates of *L. tropica* (Nery et al., 2024). The dual inhibition of Fe-SOD and TryR has been recently proposed as a viable multitarget method against Leishmania (Bora et al., 2023). Therefore, monitoring of transcriptional expression of these two genes is a sensitive molecular marker of the disturbance of parasite redox homeostasis by potential chemicals.

Capparis spinosa L. (caper; family Capparaceae) is a perennial shrub widely found in the Mediterranean basin and the Middle East including Iraq, with a long history of usage in traditional medicine for the treatment of numerous diseases (Annaz et al., 2022; Zhang, H., & Ma, 2018). Phytochemical studies have revealed that *C. spinosa* is rich in flavonoids, especially rutin, quercetin and kaempferol glycosides, phenolic acids, alkaloids, glucosinolates and terpenoids, with the largest level of rutin in the leaves among the plant organs (Zhang, H., & Ma, 2018). The herb has antioxidant, antibacterial, anti-inflammatory, antidiabetic, hepatoprotective and anticancer effects. It also has shown antileishmanial potential as aqueous and methanolic extracts of *C. spinosa* reduced the growth of *L. tropica* promastigotes with IC50 values of 28.5 and 44.6 µg/mL, respectively without significant cytotoxicity toward macrophages (Nazer et al., 2021). Also, methanolic and aqueous extracts of the plant inhibited *L. major* promastigotes and intramacrophage amastigotes. This was linked to increased nitric

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oxide generation and inhibition of arginase (Darif et al., 2023). In addition, flavonoids such as quercetin and rutin are strong iron chelators (Sen et al., 2008). Quercetin has been found to interfere with iron metabolism in Leishmania and to block iron-dependent parasite enzymes (Wang et al., 2021). Iron chelation by phytochemicals of *C. spinosa* may potentially impair the antioxidant defense of the parasite since iron is the key catalytic cofactor of Fe-SOD.

However, the molecular mechanisms of the antileishmanial activity of *C. spinosa* are still largely unknown. To our knowledge, the effect of *C. spinosa* leaf extract on the expression of the key redox-regulating genes in *L. tropica* has not been studied before. Therefore, the current study was conducted to assess the in vitro effect of the ethanolic leaf extract of *C. spinosa* on the transcriptional expression of Fe-SOD and TryR genes in *L. tropica* promastigotes using real-time reverse transcription quantitative PCR (RT-qPCR) to provide molecular insight into its mechanism of action and potential as a natural source of antileishmanial agents.

RESEARCH METHOD

Collection and preparation of plant extract

The leaves of *Capparis spinosa* L. were collected from different locations in the city of Tikrit, Salah al-Din Governorate, Iraq and transferred to the laboratory of the College of Science, University of Tikrit and the botanical identity was confirmed. Leaves were dried in air at room temperature and ground to a fine powder. The powder (100 g) was placed in a cellulose thimble and extracted with 250 mL of 96% ethanol using a Soxhlet apparatus (Germany). Finally, the solvent was evaporated in a rotary evaporator under reduced pressure at 45 °C. The concentrated extract was freeze-dried in a lyophilizer (Alpha 1-2 LD plus, Martin Christ, Germany; supplied by KARL KOLB) and the dried residue was stored at 4 °C until further use (Darif et al., 2023; Almulaiky, 2025). The phytochemical constituents of the ethanolic leaf extract were identified by gas chromatography–mass spectrometry (GC-MS) at the Central Laboratory, College of Applied Sciences, University of Samarra, Iraq.

Parasite isolation ,Identification and Culture

Leishmania tropica was obtained from a clinical isolate from a patient with cutaneous leishmaniasis attending (Dermatology Outpatient Clinic, Tikrit Teaching Hospital, Tikrit, Salah Al-Din, Iraq),. Lesion aspirates were cultured initially on Novy-MacNeal-Nicolle (NNN) biphasic medium at 25°C. The promastigotes were then cultured in bulk in RPMI-1640 supplemented with 10-15% heat inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (pH 7.2) at 25 ± 1 °C with sub-culture after every 3-4 days (Bahrami et al., 2022). The identity of the species was confirmed by ITS1-PCR and restriction fragment length polymorphism (RFLP) using HaeIII (Schönian et al., 2003). Viability and gene-expression assays were performed on promastigotes in logarithmic growth phase (day 3–4).

Morphological study

Smears of promastigotes, treated and untreated, were made on clean glass slides, fixed in absolute methanol, stained with 10% Giemsa and observed under light microscope at ×1000 magnification. Cell rounding, flagellar shortening or loss, cytoplasmic vacuolation and membrane blebbing and cell shrinkage were recorded. At least 300 cells/slide were scored.

Molecular evaluation of gene expression by quantitative real-time PCR

Experimental groups and treatment for RNA extraction

Eight experimental groups were prepared in triplication as shown in Table 1. Promastigotes at 1 × 10^7 cells/mL were treated as indicated for 24 h and 48 h, collected by centrifugation at 3000 × g for 10 min at 4 °C, washed twice with ice-cold RNase free PBS and either processed immediately or stored in RNA stabilization solution at −80 °C.

Table 1. Experimental groups used in the gene-expression arm of the study.

Group	Treatment	Concentration	Purpose / expected role
G1	Vehicle control (DMSO)	0.5% (v/v)	Excludes any solvent-attributable effect on viability and transcription
G2	*C. spinosa* ethanolic leaf extract	50ug /ml	Sub-lethal dose; detects early transcriptional responses
G4	*C. spinosa* ethanolic leaf extract	100ug/ml	Principal test dose for the gene-expression arm

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Total RNA extraction and quality assessment

Total RNA was extracted using TRIzol reagent (or equivalent silica column total RNA purification kit) according to manufacturer's protocol and using RNase free materials at all steps. Residual genomic DNA was eliminated by DNase I digestion either in column or in solution (37 °C for 30 min) and subsequent inactivation of the enzyme with EDTA (65 °C for 10 min). RNA concentration and purity were determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A260/A280 ratios between 1.8 and 2.1 and A260/A230 ratio above 2.0 were considered as acceptable. RNA integrity was assessed on a 1.5% denaturing agarose gel and visualized for intact ribosomal RNA bands and where applicable by Bioanalyzer RNA integrity number of 7 or higher. The RNA was purified, stored at −80 °C, and not subjected to more than one freeze–thaw cycle.

Synthesis of complementary DNA cDNA

RNA samples were reverse transcribed in a 20 µL reaction mix containing random hexamers, oligo(dT) primers, dNTPs, RNase inhibitor and M-MuLV reverse transcriptase. The reaction was incubated at 25 °C for 10 min, 42-50 °C for 60 min and 85 °C for 5 min to inactivate the To ensure the absence of genomic DNA contamination, a no-reverse-transcriptase (-RT) reaction was performed for each sample. The cDNA was diluted 1:5 in nuclease free water and stored at -20 °C until amplification.

Quantitative Real-Time PCR (RT-qPCR)

We measured the relative transcript levels of two genes encoding components of the parasite antioxidant system, iron superoxide dismutase (Fe-SOD) and trypanothione reductase (Try R). Fe-SOD catalyzes the conversion of superoxide radicals to H 2 O 2 . TryR maintains trypanothione in the reduced form required for Leishmania to detoxify host-derived reactive oxygen species. There is no TryR equivalent in mammalian hosts. Both enzymes are well-known drug targets and markers of antimony resistance in *L. tropica* (Mohebali et al., 2019; Ahmadpour et al., 2025 ; Bustin et al., 2025). The endogenous reference gene was 18sRNA was designed.

Table 2. Oligonucleotide primers used for RT-qPCR.

Gene	Description	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size (bp)	Ref.
Fe-SOD A	Mitochondrial iron superoxide dismutase A	TGTGCTACCATACCCTTCCTC	CCGAGCCTGTTCAACTTGT	146	
Fe-SOD B (optional)	Glycosomal iron superoxide dismutase B	ATGTCGAAGGAGCAGGTCAC	CAGACTTGATGATGTCCACCA G	127	(Bahrami et al 2022)
TryR	Trypanothione reductase (ortholog of *L. major* LmjF.05.0350)	TTGAGTTTGCCGGCATCTTCA ACG	CTTCGTCAGGCTCTTGCGCAC CTC	121	(Fozongari et al 2020)
18s	Reference gene	GCGAAACGCCAAGCTAATAC	CCAGTGAAGGCATTGGTTTT	116	desig ned

Each qPCR reaction (20 µL) contained 10 µL of 2× SYBR Green master mix, 0.4 µL of each primer (10 µM; final 200 nM), 2 µL of diluted cDNA and 7.2 µL of nuclease-free water. Reactions were conducted on a [real-time PCR instrument, e.g., Bio-Rad CFX96 / Applied Biosystems StepOnePlus] in technical triplicate. The cycling settings were 95 °C for 3 min (initial denaturation) and 40 cycles of 95 °C for 10 s and 60 °C for 30 s (annealing and extension with fluorescence collection) adopted from Bahrami *et al*. [2022]. After amplification, a melt curve (65–95 °C, 0.5 °C increments) was constructed to demonstrate a single unique product and absence of primer dimers. Each run contained a no-template control (NTC) and NRT controls. The amplification efficiency (E) for each primer pair was measured by E = 10^(−1/slope) − 1 using a five-point tenfold serial dilution standard curve of pooled cDNA. Primer pairs were allowed only if efficiency was 90-110% and R2 was > 0.98. The experimental design, validation and reporting was carried out according to the MIQE 2.0 criteria (Bustin et al., 2025).

Relative Quantification of Gene Expression

Relative transcript abundance was calculated by the comparative threshold-cycle (2^−ΔΔCt) method [31]:

ΔCt = Ct (target gene) − Ct (geometric mean of the reference genes)

ΔΔCt = ΔCt (treated group) − ΔCt (untreated control)

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Relative expression (fold change) = 2^−ΔΔCt

The untreated promastigote group (G1) served as the calibrator, to which an expression value of 1.0 was assigned. A fold change greater than 2.0 was interpreted as significant up-regulation and a value below 0.5 as significant down-regulation, provided that the corresponding statistical test reached significance.

Statistical analysis

All experiments were performed in eight biological replicates (n=8). Each biological replicate was done in triplicates (n=3). Technical replicates were averaged and the biological replicate was the unit of analysis. Data are expressed as mean ± standard deviation (SD). Normality was tested by Shapiro-Wilk test. Differences between concentrations were analyzed by one-way analysis of variance (ANOVA) and effects of concentration and time by two-way ANOVA . Post hoc tests were performed using Dunnett's test for comparisons with untreated control, and Tukey's test for all pairwise comparisons. Statistical analysis of gene expression was performed using ΔCt values and the results were then converted to fold change using the 2^−ΔΔCt method. Statistical analysis was performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA) and SPSS version 26 (IBM Corp., Armonk, NY, USA). Two-tailed p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The ethanolic leaf extract of *C. spinosa* up-regulates *Fe-SOD* transcription in *L. tropica* promastigotes

The relative transcript abundance of the iron-dependent superoxide dismutase gene (Fe-SOD) was significantly increased in promastigotes treated with the ethanolic leaf extract of *C. spinosa* (Fig. 1; Table 3). The mean relative expression in untreated parasites was 1.937 ± 0.406 (mean ± SD; n = 8; median = 1.879; range 1.350–2.528). Exposure to 50 µg/mL increased the mean to 3.273 ± 0.765 (median 3.097; range 2.450–4.475) and exposure to 100 µg/mL increased it to 3.596 ± 0.714 (median 3.650; range 2.670–4.780). These values correspond to 1.69- and 1.86-fold increase compared to untreated control, respectively.

One-way analysis of variance indicated treatment explained a large and highly significant proportion of the total variance in Fe-SOD expression ($F(2, 21) = 14.75$, $P < 0.001$; $\eta^2 = 0.584$; $\omega^2 = 0.534$), suggesting that around 58% of the observed variation could be explained by extract exposure rather than inter-replicate noise. Tukey's honestly significant difference test localized this effect to the two extract-treated groups. The increase over the control was significant at 50 µg/mL (mean difference = +1.336; 95% CI 0.520 to 2.152; $P = 0.001$; Cohen's $d = 2.18$) and at 100 µg/mL (mean difference = +1.659; 95% CI 0.843 to 2.475; $P < 0.001$; $d = 2.86$). Conversely, the extra gain achieved by increasing the concentration from 50 to 100 µg/mL was minor and not statistically different from zero (mean difference = +0.323; 95% CI −0.493 to 1.139; $P = 0.587$; $d = 0.44$). Dispersion was similar in the three groups (coefficient of variation 19.8-23.4%). The mean and the median were very close together in all groups, indicating the distributions were approximately symmetrical and supporting the use of parametric analysis for this data set.

The extract simultaneously upregulates the transcription of TryR

The trypanothione reductase gene (TryR), exhibited a similar qualitative trend but with lower amplitude (Fig. 2; Table 3). The mean relative expression was 1.855 ± 0.321 for untreated promastigotes (median 1.907; range 1.370–2.300), 2.694 ± 0.565 for 50 µg/mL (median 2.550; range 1.870–3.475), and 3.035 ± 0.493 for 100 µg/mL (median 3.040; range 2.423–3.670), corresponding to a 1.45- and 1.64-fold increase compared to the control, respectively.

The total treatment effect was again profoundly significant ($F(2, 21) = 13.31$, $P < 0.001$; $\eta^2 = 0.559$; $\omega^2 = 0.506$). Post hoc analyzes showed significant up-regulation compared to untreated parasites at 50 µg/mL (mean difference = +0.839; 95% CI 0.246 to 1.432; $P = 0.005$; $d = 1.83$) and at 100 µg/mL (mean difference = +1.180; 95% CI 0.587 to 1.773; $P < 0.001$; $d = 2.84$), while the two extract concentrations did not show significant difference between them (mean difference = +0.341; 95% CI −0.252 to 0.934; $P = 0.335$; $d = 0.64$). In-group variability was moderate and constant (coefficient of variation 16.2-21.0%).

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Comparison of the two transcription response

The two genes together presented a coherent biphasic pattern (Table 4). In both transcripts, the change from untreated control to 50 µg/mL induced a significant and pronounced increase whereas the change from 50 to 100 µg/mL only caused a small, non-significant increase. The second dosage increment accounted for only 19.5% of the increase in Fe-SOD expression and 28.9% of the increase in TryR expression when expressed as a proportion of the total induction seen at the peak concentration; the remaining 80.5% and 71.1% respectively had already been attained at 50 µg/mL. Essentially, the increase in the extract concentration resulted in only an additional 10–13% increase in the transcript levels at the reduced concentration.

In all instances, the response to Fe-SOD was greater than that to TryR, whether stated as a fold change (1.69 and 1.86 vs. 1.45 and 1.64 for TryR), an absolute mean difference (+1.336 and +1.659 vs. +0.839 and +1.180) or the proportion of variance accounted for (η2 = 0.584 vs. 0.559) at both concentrations. All effect sizes relative to the untreated control were large according to conventional standards (d = 1.83–2.86) and the 95% confidence intervals of the four corresponding effect sizes significantly excluded zero, indicating that activation of both genes was not only statistically significant, but also of a magnitude unlikely to be biologically trivial.

Table **3**. Descriptive statistics for the relative expression of the *Fe-SOD* and *TryR* genes in *L. tropica* promastigotes exposed to the ethanolic leaf extract of *C. spinosa* (n = 8 per group).

Gene	Group	Min.	Q1	Median	Q3	Max.	Mean ± SD	SEM	CV (%)	Fold vs. control
Fe-SOD	Control	1.350	1.650	1.879	2.364	2.528	1.937 ± 0.406	0.143	20.9	1.00
	50 µg/mL	2.450	2.558	3.097	4.051	4.475	3.273 ± 0.765	0.270	23.4	1.69
	100 µg/mL	2.670	2.838	3.650	4.013	4.780	3.596 ± 0.714	0.252	19.8	1.86
TryR	Control	1.370	1.554	1.907	2.100	2.300	1.855 ± 0.321	0.114	17.3	1.00
	50 µg/mL	1.870	2.286	2.550	3.285	3.475	2.694 ± 0.565	0.200	21.0	1.45
	100 µg/mL	2.423	2.528	3.040	3.505	3.670	3.035 ± 0.493	0.174	16.2	1.64

SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation (SD/mean × 100). Fold change is the ratio of the group mean to the mean of the untreated control.

Table 4**.** One-way analysis of variance and Tukey's post hoc pairwise comparisons of relative *Fe-SOD* and *TryR* expression.

Gene	Comparison	Mean difference	95% CI	P value	Cohen's d	Outcome
Fe-SOD	ANOVA: F(2, 21) = 14.75	—	—	< 0.001	$\eta^2 = 0.584$	Large overall effect
	Control vs. 50 µg/mL	+1.336	0.520 to 2.152	0.001	2.18	Significant
	Control vs. 100 µg/mL	+1.659	0.843 to 2.475	< 0.001	2.86	Significant
	50 vs. 100 µg/mL	+0.323	−0.493 to 1.139	0.587	0.44	Not significant
TryR	ANOVA: F(2, 21) = 13.31	—	—	< 0.001	$\eta^2 = 0.559$	Large overall effect
	Control vs. 50 µg/mL	+0.839	0.246 to 1.432	0.005	1.83	Significant
	Control vs. 100 µg/mL	+1.180	0.587 to 1.773	< 0.001	2.84	Significant
	50 vs. 100 µg/mL	+0.341	−0.252 to 0.934	0.335	0.64	Not significant

CI, confidence interval. Mean differences are expressed in units of relative expression; confidence intervals are Tukey-adjusted. Cohen's d values of 0.2, 0.5 and 0.8 denote small, medium and large effects, respectively.

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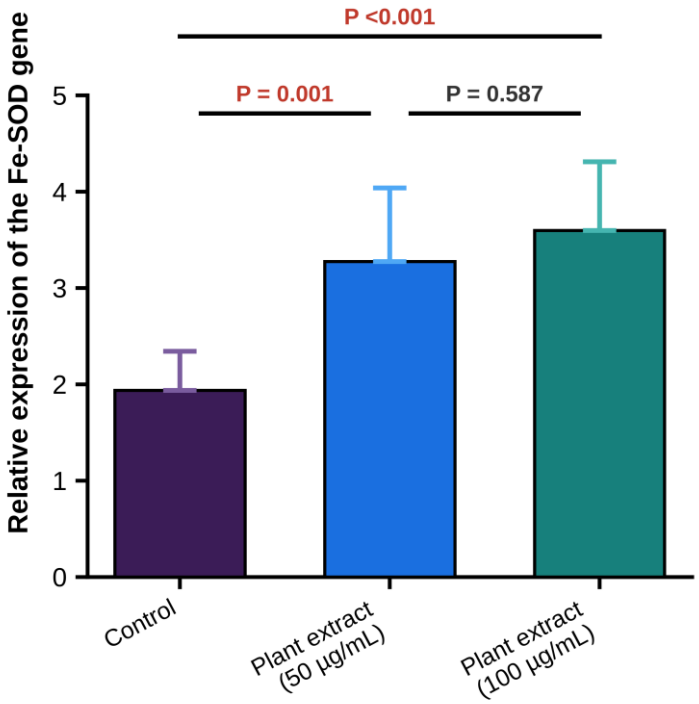


Figure 1. Relative expression of the *Fe-SOD* gene in *L. tropica* promastigotes after exposure to the ethanolic leaf extract of *C. spinosa* at 50 and 100 µg/mL compared with untreated controls. Bars represent the mean of eight independent determinations and error bars represent one standard deviation. P values are derived from one-way analysis of variance followed by Tukey's multiple comparisons test.

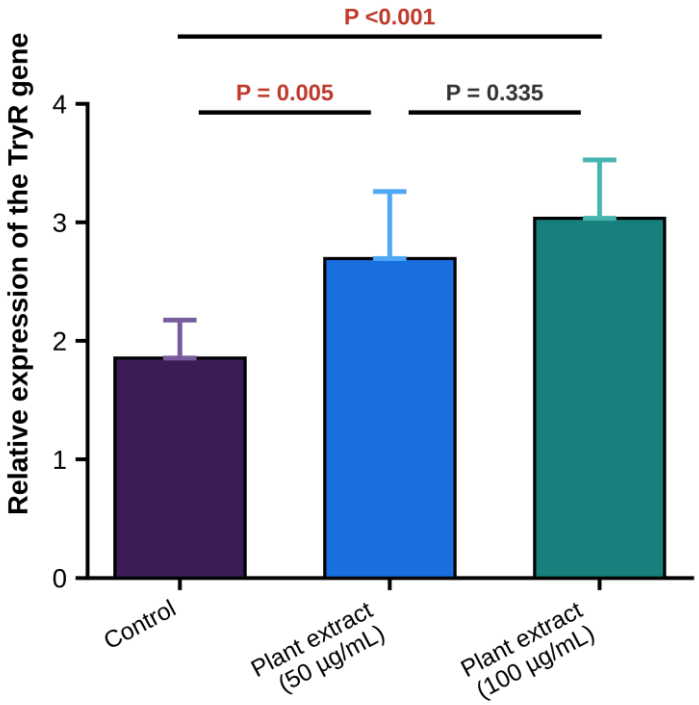


Figure 2. Relative expression of the *TryR* gene in *L. tropica* promastigotes after exposure to the ethanolic leaf extract of *C. spinosa* at 50 and 100 µg/mL compared with untreated controls. Bars represent the mean of eight independent determinations and error bars represent one standard deviation. P values are derived from one-way analysis of variance followed by Tukey's multiple comparisons test.

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Cutaneous leishmaniasis due to *L. tropica* remains a serious public health problem in the endemic band of the Old World. In 2024, the World Health Organization received reports of 211 466 new cases of cutaneous leishmaniasis from 99 endemic countries and territories, with Iraq being one of the 12 countries to report more than 2000 cases (WHO, 2025).

Molecular surveys performed in several governorates of Iraq have repeatedly identified *L. tropica* as a major or co-major agent of the anthroponotic form of the disease (Flaih et al., 2024). In this epidemiological setting the therapeutic armamentarium is still limited and old: pentavalent antimonials, amphotericin B, miltefosine, paromomycin and pentamidine are all limited by parenteral administration, cumulative toxicity, high cost, variable regional efficacy and the constant degradation of susceptibility, whereas the clinical development pipeline has been essentially empty for decades (Zhang et al., 2025). This makes the search for natural products with low toxicity and chemical complexity, which also target parasite-specific biochemistry, not just an academic exercise but a practical necessity. In the present study, this need was addressed by going beyond the viability end-points that dominate the ethnopharmacological literature and by asking at the transcriptional level how *L. tropica* promastigotes responded to the ethanolic leaf extract of *C. spinosa*.

The two genes selected for this purpose were not randomly chosen. Mammalian hosts possess an antioxidant architecture that trypanosomatids lack. They have no catalase or selenium-dependent glutathione peroxidases and have substituted the universal glutathione/glutathione reductase pair with the dithiol trypanothione and its corresponding flavoenzyme, trypanothione reductase (Roy et al., 2025).

In this simplified system, superoxide anions generated by aerobic metabolism or host-derived oxidative attack are dismuted by iron-dependent superoxide dismutases, and the resulting hydrogen peroxide is reduced by the tryparedoxin/tryparedoxin peroxidase relay, which takes its reducing equivalents from the trypanothione pool that is continuously regenerated by TryR at the expense of NADPH. Thus, Fe-SOD and TryR occupy sequential and non-redundant positions within a single detoxification cascade, the former at its entry point and the latter at its reducing terminus. Both enzymes are considered as selectively druggable targets, since there is no structural or functional counterpart in human cells, which rely on Cu/Zn- and Mn-superoxide dismutases and on glutathione reductase (Santi et al., 2021). Genetic data supports this view: deletion of FeSOD-A in L. infantum failed, indicating its essentiality, and clones with decreased FeSOD-A expression showed impaired tolerance to oxidative stress and reduced intracellular multiplication in macrophages whereas overexpression of TryR in *L. tarentolae* conferred resistance to hydrogen peroxide and a striking improvement in infectivity (Fonseca et al., 2026).

The simultaneous overexpression of these two transcripts reported here is probably a defensive response to stress rather than an indication of the inactivity of the extract or, bizarrely, its fortifying effect on the parasite. The canonical transcriptional response of a trypanosomatid to redox stress is upregulation of antioxidant defenses (Roy et al., 2025) and the fact that two functionally distinct steps in the same cascade are induced in parallel with comparable effect sizes and near-identical dose profiles suggests that this is a true perturbation of redox homeostasis as opposed to a gene-specific artifact. The parasite seems to have been forced to spend in its own defenses, and the size of that investment provides an indirect but useful estimate of the oxidative challenge of the extract.

The phytochemistry of *C. spinosa* provides a possible reason for such a problem. Caper leaves are extremely rich in flavonols, mainly rutin and quercetin, as well as chlorogenic acid, vanillin, and a variety of other phenolic acids, alkaloids, and glucosinolates (Santi et al., 2021). These elements are classically known as antioxidants based on cell-free radical scavenging studies, yet in a parasite they often act in the opposite way. Quercetin causes concentration-dependent generation of reactive oxygen species and collapse of the mitochondrial membrane potential in *L. amazonensis* promastigotes and intracellular amastigotes, which is abolished by glutathione and N-acetylcysteine (Fonseca et al., 2026); quercetin, rutin and gallic acid induce apoptosis-like death and DNA damage in *L. donovani* while inhibiting trypanothione reductase and trypanothione synthetase (El Finou et al., 2024); and a significant body of work now identifies flavonoid-driven pro-oxidant activity, mitochondrial dysfunction and iron deprivation as the main antiprotozoal mechanisms of this chemical class (Fonseca-Silva et al., 2024). Of particular interest to the present findings is the ability of quercetin to chelate iron and to deprive iron-dependent enzymes of their cofactor, a trait used to block ribonucleotide reductase in L. donovani (Torkian et al., 2024) and often suggested to be involved in flavonoid leishmanicidal activity (Memariani et al., 2024). As Fe-SOD is itself an iron metalloenzyme, partial sequestration of the intracellular labile iron pool should impair holoenzyme assembly, and may induce a compensatory increase in transcript abundance even in the absence of a commensurate gain in functional enzyme. The observed disproportionately large induction of Fe-SOD relative to TryR is at least compatible with such multiple

pressures, with the gene encoding the iron-requiring enzyme susceptible to both generalized oxidative signaling and a particular cofactor limitation.

This amount of induction is similar to drug-unresponsive field isolates in which the same two redox arms are chronically elevated. Antimony-resistant *L. tropica* isolates from anthroponotic cutaneous leishmaniasis patients overexpress 3.8 and 4.81 fold SODA and SODB transcripts (Memariani et al., 2024), trypanothione biosynthesis pathway genes 1.56 to 2.33 fold and intracellular thiol content 2.17 fold [37].Transcripts for cytosolic tryparedoxin, tryparedoxin peroxidase and ascorbate peroxidase are up 1.69 to 2.51 fold (Memariani et al., 2024). The extract-induced 1.45- to 1.86-fold induction is thus at the lower end of the spectrum of known resistance phenotypes, supporting two complementary readings. First and most quickly the extract produces a true oxidative insult like antimonial pressure. The second caveat is that any treatment that generates the defensive response underlying antimony unresponsiveness could select cross-tolerant parasites if used frequently at sub-inhibitory levels. Any claim of translation should be preceded by comprehensive characterization of the extract for dose–response behavior and exposure regimens designed to rapidly kill the parasite rather than impose prolonged sub-lethal stress.

These results are in line with an increasing body of evidence indicating natural products converge on this redox axis. Curcumin photodynamic therapy alters FeSODA expression in L. major promastigotes without affecting several unrelated transcripts, confirming the specificity of this axis as a readout of oxidative injury (Marcolino et al., 2024); epigallocatechin-3-gallate competes with trypanothione reductase in *L. infantum* and kills the parasite via hydrogen peroxide accumulation (Inacio et al., 2021); and virtual screening campaigns have begun to identify FeSOD-binding lig In C. spinosa, methanolic and aqueous extracts inhibit L. major promastigotes and intramacrophagic amastigotes while stimulating nitric oxide production and inhibiting arginase in silico ,copper nanoparticles green-synthesised with caper extract amplify nitric oxide output and parasite clearance (Albalawi et al., 2021; Mohebali et al., 2019).The present results show that the leaf extract hits the parasite at numerous points, such as direct oxidative damage with stress-protein and antioxidant responses, which are the hallmarks of the damage.

These limitations should be considered when interpreting these results and also indicate directions for further research. First, the steady-state abundance of trypanosomatid transcripts is not a good measure of enzyme activity. These organisms transcribe their genomes in a polycistronic fashion and regulate gene expression nearly solely post-transcriptionally through untranslated region-mediated modulation of mRNA stability and translational efficiency (Requena, 2011; Schönian et al., 2003). Thus, a 1.86-fold increase in Fe-SOD mRNA does not always imply an increase in functional superoxide dismutase. Assays of enzyme activity, immunoblotting and detection of non-protein thiols are needed to bridge this gap. Second, the study focused on the promastigote stage, while the clinically relevant amastigote resides in the phagolysosome and encounters a host-derived oxidative burst. The transcriptional set point of the redox system may be altered. Third, the test sample was a crude extract thus bioassay guided fractionation and LC-MS/MS profiling need to be carried out to identify the chemicals responsible and to rule out additive or antagonistic interactions. The fourth is that the design only sampled one time point and two concentrations, leaving the induction kinetics and the system behavior below 50 µg/mL uncertain. Fifth, reactive oxygen species, mitochondrial membrane potential and intracellular thiol content were not explicitly measured, although the convergent literature strongly supports the oxidative process hypothesized here. Selectivity against mammalian cells was not determined in our research, but past results suggest that *C. spinosa* extracts at leishmanicidal dosages are poorly cytotoxic for J774-A1 macrophages (Darif et al., 2023; Almulaiky, 2025; Bahrami et al., 2022).

CONCLUSION

To summarize, the ethanolic leaf extract of *C. spinosa* up-regulated two non-redundant components of the parasite-specific antioxidant defense system of L. tropica with substantial effect sizes and obvious saturation at concentrations over the inhibitory threshold. These data are the first molecular evidence that caper leaf extract perturbs the Fe-SOD/trypanothione axis in this species, which makes it a rational target for combination approaches. The questions now are if the transcriptional response is accompanied by an increase in enzyme activity, whether it rescues the parasite and whether it can be pharmacologically subverted.

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ETHICS AND DECLARATIONS

Ethical Approval and Consent

This in vitro study evaluated the effect of an ethanolic leaf extract of Capparis spinosa on gene expression in cultured Leishmania tropica promastigotes.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare no competing interests in this work or in its publication financial or non-finansial

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Author Contributions

Ali Mohammed Abe conceptualised and designed the study, coordinated the research, and drafted the manuscript. Hajir Zaidan Khala conducted the in vitro experiments and documented the gene expression measurements. Noor Tala Ali analysed the data, interpreted changes in Fe-SOD and TryR expression, and contributed to writing the Results and Discussion sections. All authors reviewed and revised the manuscript and approved the final version for publication.

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