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Seasonal thyroid hormone alterations in Kermani sheep: An adaptive thermoregulatory mechanism for heat stress survival

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ABSTRACT

Elevated ambient temperatures adversely affect livestock production. Among domestic animals, sheep in southern Iran experience heat stress for seven months annually based on the Temperature-Humidity Index (THI), sheep in this region experience heat stress conditions for seven months of the year. Understanding the endocrine mechanisms that regulate metabolic homeostasis may provide insights into how sheep cope with such stress. Thyroid hormones—namely triiodothyronine (T3) and thyroxine (T4)—play fundamental roles in metabolic processes and thermoregulation. In this study, plasma levels of T3 and T4 were measured in Kermani sheep during winter and summer. Both hormones were significantly lower in summer than in winter ($P < 0.007$). However, the calculated T3/T4 ratio was significantly higher in summer ($P < 0.001$), indicating a 15-fold increase in the conversion of T4 to T3 compared to winter. Although overall thyroid hormone production declined during summer, although overall thyroid hormone concentrations declined during summer, the increased T3/T4 ratio suggests possible alterations in peripheral thyroid hormone metabolism and conversion, which may contribute to the adaptive response to high ambient temperatures. Further studies measuring rT3 levels and deiodinase activity are required to confirm the underlying mechanisms. In conclusion, seasonal modulation of thyroid hormone dynamics represents a key thermoregulatory strategy in Kermani sheep.

Keywords: Kermani sheep, thyroid hormones, heat stress, adaptation, thermoregulation

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Introduction

Livestock productivity is adversely affected by extreme climatic conditions. High environmental temperatures combined with forage scarcity significantly impair the well-being and performance of ewes during summer months. In sheep, rectal temperature rises when ambient temperature exceeds 32 °C, and hyperthermia develops when heat production surpasses heat loss. The efficacy of thermoregulatory mechanisms in sheep is largely

dependent on breed and individual genetic makeup (Sevi and Caroprese, 2012). Under high-temperature exposure, sheep activate a series of physiological compensatory mechanisms that facilitate acclimatization to extreme environments and help maintain vital functions through substantial changes in biological functioning. Extensive rearing systems remain the most common method of sheep production in developing countries. In such systems, sheep often graze over long distances under intense solar radiation, with restricted grazing time during the hottest months. Under the influence of multiple concurrent stressors, grazing small ruminants have evolved a suite of adaptive mechanisms that promote survival under harsh environmental conditions. Sheep tolerate these stressors primarily by modifying their thermoregulatory pathways. Thyroid hormones are critical regulators of metabolic adjustments, thermogenesis, growth, organ function, wool follicle maturation, and cartilage-to-bone differentiation. Their dynamics are influenced by sex, breed, age, physiological state, dietary iodine supplementation (Nudda et al., 2013), feed composition (Katole et al., 2011), and treatment with propylthiouracil (PTU). For instance, pregnant ewes treated with PTU for 35 days exhibited significantly lower T3 and T4 concentrations compared to controls (Gifford et al., 2007). Similarly, ewes treated with PTU for 66 days postpartum had reduced thyroid hormone levels (Wells et al., 2003).

A phytoestrogen-rich diet also lowered blood T4 levels relative to control sheep (69.7 vs. 57.2 ng/mL) (Skipor et al., 2012). Interestingly, thyroid hormone levels in twin-pregnant ewes decreased more rapidly than in single-pregnant ewes during gestation (Abdollahi et al., 2013). In pregnant Merino ewes, blood T4 (62.9–64.4 ng/mL) and T3 (1.41–1.88 ng/mL) have been reported (McDowall et al., 2011). Nellore sheep showed no significant seasonal differences in T4 (48.39–53.42 ng/dL) and T3 (2.07–2.25 ng/dL) between summer and winter (Karthik et al., 2021). From early to late gestation, T3 decreased from 1.49 to 1.27 ng/mL and T4 from 38.74 to 37.92 ng/mL in pregnant ewes, whereas non-pregnant ewes had significantly higher T3 (1.81 ng/mL) and T4 (48.5 ng/mL) concentrations (Colodel et al., 2010). Reduced thyroid hormone levels, particularly T4, during hot summer months have been reported in male lambs, with cooling systems restoring blood concentrations (Koluman and Daskiran, 2011). In shaded versus unshaded sheep, blood thyroid hormone levels were higher in the unshaded group (Liu et al., 2012). The T3/T4 ratio is considered an indirect indicator of alterations in thyroid hormone metabolism because it reflects the relative balance between circulating T4 availability and its peripheral conversion to T3. This conversion is regulated by deiodinase enzymes, which play a central role in determining thyroid hormone bioavailability and tissue-specific responses.

Therefore, seasonal changes in the T3/T4 ratio may provide insights into adaptive modifications of thyroid hormone metabolism under environmental stress, although direct evaluation of deiodinase activity is required to confirm these mechanisms. To date, seasonal patterns of serum thyroid hormone concentrations in the Kermani sheep breed have not been characterized. Establishing the seasonal profile of thyroid hormones in this locally adapted breed may provide a better understanding of endocrine mechanisms associated with heat tolerance and adaptation. Such information could be valuable for developing appropriate management strategies and improving production efficiency of sheep raised in hot and semi-arid environments. Therefore, this study aimed to investigate thyroid hormone levels in Kermani sheep during summer and winter in the southern part of Kerman province (Jiroft).

Materials and Methods

Study Site and Animals

The study was conducted during summer and winter at the research station of the University of Jiroft, Iran (28°4′ N latitude, 57°4′ E longitude, 712 m altitude). Based on meteorological data, the average monthly Temperature-Humidity Index (THI) values for April, May, June, July, August, September, October, November, December, February, and March were 23.4, 26.4, 29.4, 31.6, 29.6, 28.0, 26.4, 19.7, 18.0, 14.0, and 13.8,

respectively. According to Marai et al. (2007), a THI below 22.2 indicates no heat stress. These data indicate that sheep in this region experience heat stress from April through October (seven months), with no heat stress from November to March. Twenty-nine mature male lambs aged 11–13 months, weighing 29–35 kg, were selected for the study The same 29 animals were followed throughout the study and blood samples were collected from the same individuals during both summer and winter seasons. All animals received routine vaccinations (enterotoxemia, foot-and-mouth disease) and anthelmintic treatment (via drench) one month prior to the experiment. During the study period, sheep grazed on 270 hectares of land belonging to the university research station. They were housed in well-ventilated sheds with asbestos roofing (2.4 m height) and open sides, under proper hygienic conditions. Animals had ad libitum access to high-quality drinking water and salt blocks. All experimental procedures were conducted in accordance with the guidelines of the Iranian Council of Animal Care and were approved by the Animal Ethics Committee of the University of Jiroft.

Blood Sampling and Hormone Assays

Blood sampling was conducted from mid-June to mid-September (summer) and from December to March (winter). Sheep were held in a corral in the morning before grazing. Blood samples were collected in four sessions per month via jugular venipuncture using 7 mL evacuated blood collection tubes (Medical Polymer Co., Ltd., China). Samples for serum analysis were placed in an ice bath and immediately transported to the laboratory. Serum was separated by centrifugation at 3,000 × g for 15 minutes at 4°C and stored at −20°C until analysis. Thyroid hormone concentrations (total T3 and total T4) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Monokit ELISA kits, Monobind Inc., USA) according to the manufacturer's instructions. The analytical sensitivity of the T3 assay was 0.1 ng/mL, with intra-assay and inter-assay coefficients of variation (CV) of 5.2% and 8.1%, respectively. For the T4 assay, the sensitivity was 0.2 µg/dL, with intra-assay and inter-assay CVs of 4.8% and 7.6%, respectively. All samples were analyzed in duplicate, and the mean values were used for statistical analysis.

Statistical Analysis

Data were analyzed using SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA). The normality of data distribution was assessed using the Univariate procedure. As the data were normally distributed, parametric tests (T test procedure) were employed. Differences in serum thyroid hormone concentrations (T3 and T4) and T3/T4 ratios between summer and winter seasons were compared using paired-sample t-tests, as the same animals were sampled in both seasons. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

Results

Meteorological data and THI values are presented in Figure 1. According to THI thresholds, sheep were under heat stress for seven months of the year and free from heat stress for five months. Serum concentrations of both thyroid hormones were significantly lower in summer than in winter ($P < 0.001$ for T3; $P < 0.007$ for T4). Mean T3 levels were 0.62 ± 0.03 ng/mL in summer versus 2.23 ± 0.12 ng/mL in winter, representing a 3.6-fold increase in winter. Mean T4 levels were 1.5 ± 0.21 µg/dL in summer versus 9.37 ± 0.41 µg/dL in winter, a 6.2-fold increase in winter. In contrast, the calculated T3/T4 ratio was significantly higher in summer (0.3 ± 0.029) than in winter (0.02 ± 0.001, $P < 0.001$). These results are illustrated in Figure 2 and Figure 3.

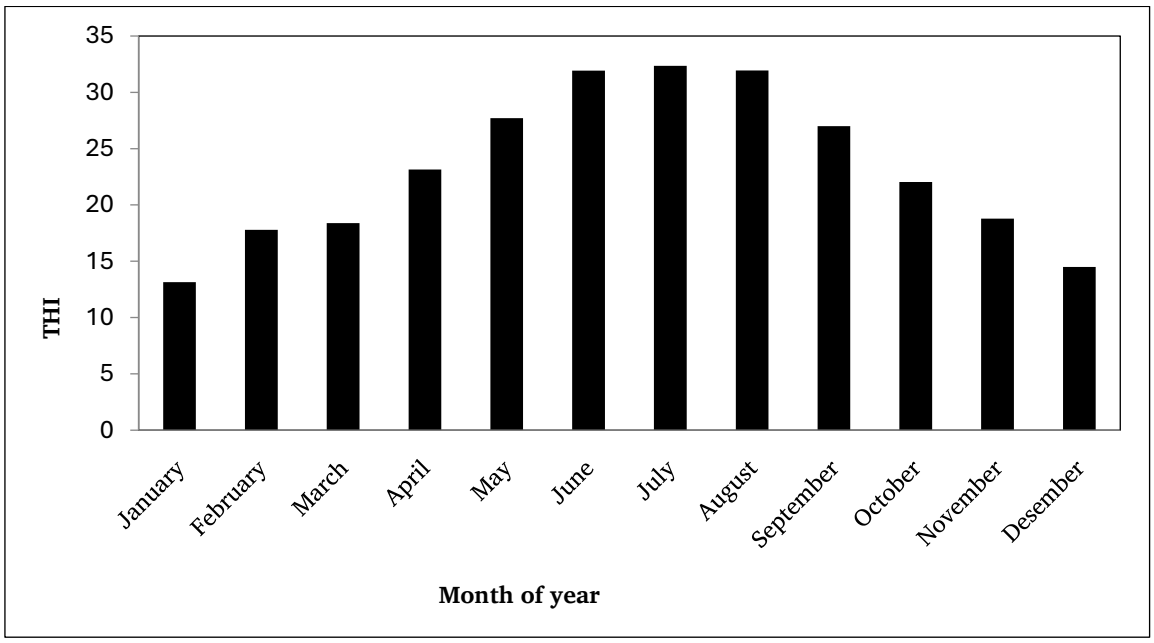

Figure 1. Monthly Temperature-Humidity Index (THI) values at the study location (Jiroft, Iran) throughout the year. The horizontal dashed line at THI = 22.2 indicates the heat stress threshold according to Marai et al. (2007), with values above this line representing heat stress conditions. Data indicate that sheep experienced heat stress from April through October (seven months) and were free from heat stress from November through March (five months). THI values were calculated using monthly temperature and relative humidity data obtained from the Jiroft Meteorological Station.

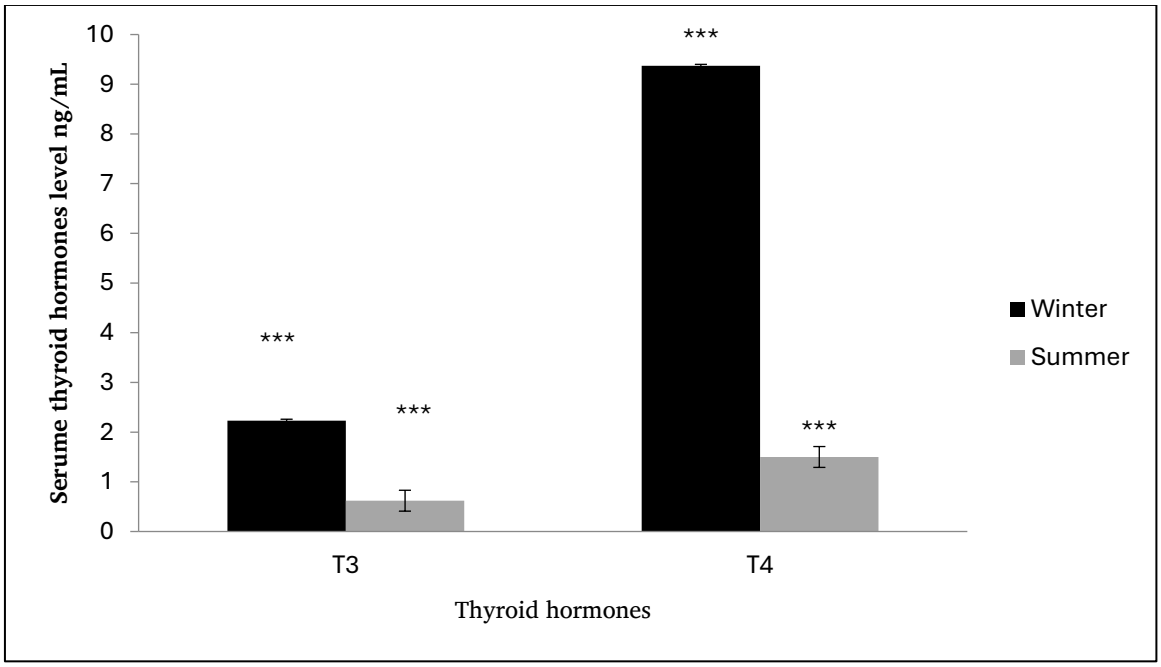

Figure 2. Serum thyroid hormone concentrations in Kermani sheep during summer and winter seasons. (A) Triiodothyronine (T3) concentration; (B) Thyroxine (T4) concentration. Data are presented as mean ± SEM (n = 29). ***P < 0.001 for T3; **P < 0.01 for T4,

comparing summer vs. winter using paired t-tests. Both T3 and T4 concentrations were significantly lower during summer compared to winter, indicating seasonal modulation of thyroid hormone levels in response to heat stress.

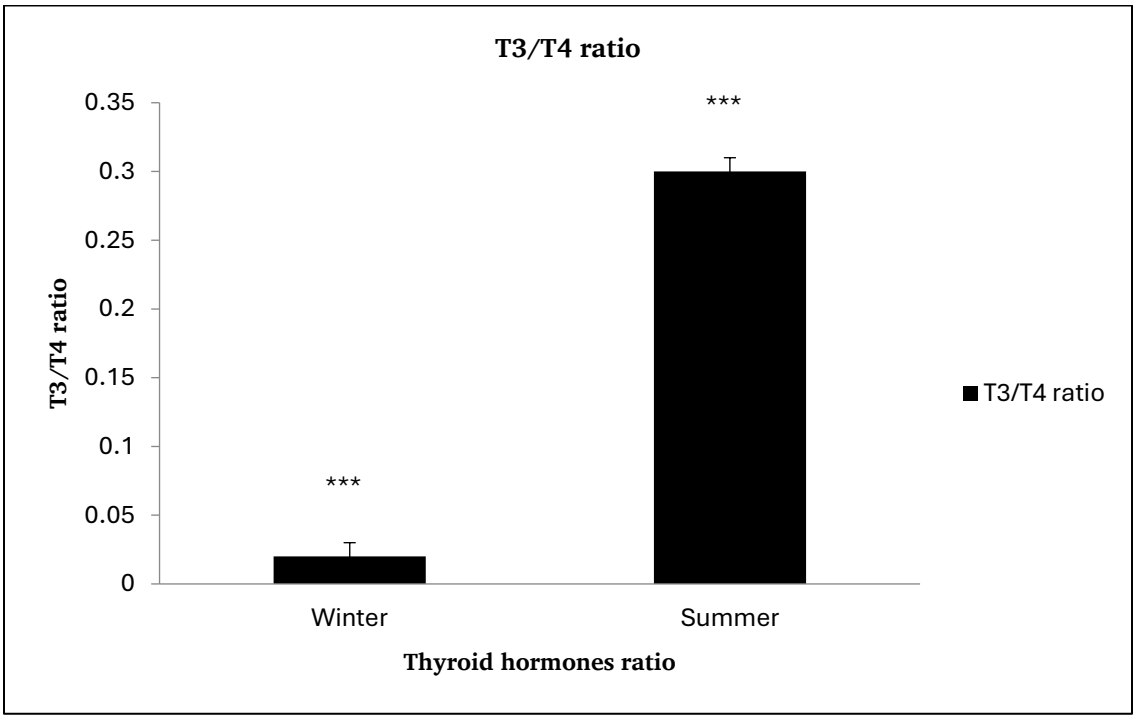

Figure 3. T3/T4 ratio in Kermani sheep during summer and winter seasons. Data are presented as mean ± SEM (n = 29). ***P < 0.001, comparing summer vs. winter using paired t-tests. The T3/T4 ratio was significantly higher during summer compared to winter, suggesting possible alterations in peripheral thyroid hormone metabolism and enhanced conversion of T4 to T3 under heat stress conditions.

Discussion

The present study demonstrated significant seasonal variations in circulating thyroid hormone concentrations in Kermani sheep, with markedly lower T3 and T4 levels during summer compared to winter. These findings are consistent with previous reports in various sheep breeds and other livestock species exposed to heat stress conditions (Koluman and Daskiran, 2011; Liu et al., 2012; Marai et al., 1995). The observed reduction in thyroid hormone levels during summer likely represents an adaptive thermoregulatory response aimed at decreasing basal metabolic rate and endogenous heat production, thereby facilitating heat dissipation and maintaining thermal homeostasis (Sevi and Caroprese, 2012).

Changes in thyroid hormone levels under thermal stress may involve alterations in deiodinase activity and other regulatory mechanisms. Deiodinases regulate thyroid hormone signaling by controlling the conversion of T4 to active T3 and the inactivation pathways involving T2 and reverse T3 (rT3). Although changes in these pathways may contribute to seasonal adaptation, direct measurements of rT3 concentration and deiodinase activity are required to confirm their involvement in Kermani sheep under heat stress conditions. Hypothyroidism can result from nutritional, environmental, or psychological changes. Reduced thyroid hormone levels in sheep have been reported following rapeseed consumption (Mandiki et al., 1999), attributed to glucosinolates and their derivatives, particularly goitrin, which inhibit thyroid hormone biosynthesis. Given the crucial role of thyroid hormones in thermoregulation and metabolic adjustments, even short-term temperature fluctuations can impact these endocrine pathways and their responsiveness to acute stressors.

Hot summer conditions exert a rapid and profound effect on thyroid hormone concentrations. Our findings are consistent with previous reports of declining T4 levels in male lambs during hot summers, with cooling systems reversing this decline (Koluman and Daskiran, 2011). Similarly, unshaded sheep had higher thyroid hormone levels than shaded ones (Liu et al., 2012). Changes in thyroid hormone levels under thermal and psychological stress are primarily mediated by alterations in deiodinase activity. Deiodinases modify the biological activity of thyroid hormones by converting T4 to active T3 or inactivating T3 to T2 and T4 to reverse T3 (rT3). These enzymes not only regulate circulating T3 levels but also provide dynamic control of thyroid hormone signaling. Among the three deiodinase types, type 3 deiodinase (DIO3) is responsible for converting T4 to rT3, which reduces T3 signaling and metabolic rate (Russo et al., 2021). A short period of severe hyperthermia has been shown to produce a fivefold rise in rT3 and a halving of T3 levels (Konits et al., 1984). The most notable finding of this study was the significantly higher T3/T4 ratio during summer despite lower absolute T3 and T4 concentrations. This observation suggests potential alterations in peripheral thyroid hormone metabolism, possibly involving enhanced conversion of T4 to the more biologically active T3 or increased production of reverse T3 (rT3).

However, it is important to note that the T3/T4 ratio is influenced by multiple factors, including hormone synthesis, secretion, peripheral conversion, and clearance rates. Therefore, the observed increase in this ratio should be interpreted as an indirect indication of altered thyroid hormone dynamics rather than definitive evidence of enhanced T4-to-T3 conversion. Interestingly, reverse T3 has been reported to exert protective effects against cerebral ischemia injury in both in vitro and in vivo models (Rastogi et al., 2018). It likely exerts negative feedback on the hypothalamic-pituitary-thyroid axis without mediating other T3 functions, thereby reducing circulating T4 levels. The brain expresses relatively high levels of DIO3, which enhances local T4 inactivation via rT3 production (Bianco et al., 2019).

Previous studies have reported that short-term heat stress in steers significantly decreased T4 and T3 levels while increasing the T3/T4 ratio, with rT3 levels remaining unchanged (Kahl et al., 2015). Similarly, in cattle adapted to long-term heat conditions, plasma T3 reduction was associated with decreased thyroid activity and downregulation of DIO1 and DIO2, accompanied by upregulation of DIO3 (Pereira et al., 2008). In agreement with these findings, our results showed a significantly higher T3/T4 ratio during summer despite lower absolute T3 and T4 levels, suggesting enhanced rT3 synthesis. Similar patterns have been observed in heat-stressed female goats, where T4 decreased but T3 remained unchanged (Sharma et al., 2013), and in Friesian calves, where summer thyroid hormone levels were significantly lower than in winter (Marai et al., 1995). In agreement with previous studies, our results showed a significantly higher T3/T4 ratio during summer despite lower absolute T3 and T4 concentrations. This finding may indicate alterations in peripheral thyroid hormone metabolism; however, the T3/T4 ratio is influenced by multiple factors, including hormone synthesis, conversion, and clearance. Therefore, the observed increase in this ratio should be interpreted as a possible indication of altered thyroid hormone dynamics rather than direct evidence of enhanced T4-to-T3 conversion or increased rT3 production.

Several limitations of this study should be acknowledged. First, we did not measure rT3 concentrations or deiodinase enzyme activities, which would have provided direct evidence for the proposed mechanisms. Second, we did not assess other physiological parameters such as rectal temperature, respiratory rate, or heart rate, which could have provided additional context for interpreting the endocrine responses. Third, the study was conducted on male lambs only; sex and age-related differences in thyroid hormone responses to heat stress warrant further investigation. Fourth, while we observed significant seasonal differences, the relatively small sample size ($n = 29$) may limit the generalizability of our findings. Despite these limitations, our findings contribute to the understanding

of endocrine adaptations in sheep breeds indigenous to hot and semi-arid environments. The observed seasonal modulation of thyroid hormone dynamics in Kermani sheep likely represents an adaptive thermoregulatory strategy that facilitates survival under prolonged heat stress conditions. These results may have practical implications for developing management strategies to improve productivity and welfare of sheep in similar climatic regions.

Conclusion

In summary, these results demonstrate a significant effect of hot summer conditions on circulating thyroid hormone levels in Kermani sheep. The observed changes may be associated with alterations in thyroid hormone regulation and metabolism under seasonal thermal stress. Seasonal modulation of thyroid hormone profiles may contribute to the thermoregulatory adaptation of this breed; however, further studies are required to clarify the underlying mechanisms

Conflict of Interest

The authors declare that they have no conflicts of interest regarding the publication of this manuscript. No financial or personal relationships with other people or organizations have inappropriately influenced this work.

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Screening of Safflower Genotypes for Enhanced Flavonoid and Anthocyanin Contents under Water-Limited Conditions

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ABSTRACT

Drought is the main abiotic stress limiting safflower production in semi-arid regions, with flavonoid and anthocyanin pathways playing a key role in antioxidant defense. A two-year field experiment in Arak, Iran, evaluated 38 safflower genotypes under full irrigation and terminal drought stress, measuring total flavonoid and anthocyanin contents. Drought increased average flavonoids by 6.3% (from 0.1395 to 0.1483 mg QE g^{-1} DW) and anthocyanins by 8.1% (from 0.00382 to 0.00413 mg g^{-1} DW). A three-way ANOVA showed significant effects of irrigation and year on both traits, with year accounting for 62.8% of anthocyanin variance, while genotype effects were not significant ($p = 0.953$ and $p = 0.496$), indicating high residual variability (75.8% and 23.2%). Using eight drought-tolerance indices, Variety 31 ranked first for flavonoid stability (STI = 1.311), and Variety 14 ranked first for anthocyanin stability (STI = 1.952). Variety 14 was the only genotype among the top five for both traits. The correlation between flavonoid and anthocyanin contents was weak and non-significant ($r = -0.06$, $p = 0.187$), suggesting independent regulation of these two phenylpropanoid branches. These results introduce Variety 14 as a promising multi-trait parent for drought-resilience breeding and highlight that interannual climatic variation, rather than genotype, is the primary factor influencing anthocyanin accumulation in safflower petals.

Keywords: Anthocyanin; Drought stress; Environment interaction; Flavonoid; Genotype

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Introduction

Water scarcity is the foremost constraint on global crop productivity, and its severity is increasing under

climate change: rain-fed systems that sustain roughly 60% of global food production are projected to face intensifying green-water deficits, with food production for hundreds of millions of additional people at risk under 1.5 ˚C and 3 ˚C warming scenarios (He and Rosa, 2023). Arid and semi-arid regions of the Middle East, including the Iranian plateau, are disproportionately exposed to this trend, and oilseed systems in these regions increasingly depend on genotypes that combine agronomic productivity with physiological drought resilience.

Safflower (Carthamus tinctorius L., Asteraceae) is a deep-rooted, thermophilic oilseed crop that is intrinsically well suited to drought-prone cultivation because of its extensive taproot system and capacity for osmotic adjustment (Golkar et al., 2021). Its seed oil is valued for a high linoleic and oleic acid content and is used in edible-oil, pharmaceutical, and cosmetic applications, while its flowers are a traditional source of natural dyes and bioactive flavonoids with documented antioxidant, hepatoprotective, and cardiovascular pharmacological activity (Cheng et al., 2024). Because of this dual industrial and pharmaceutical value, there is growing interest in identifying safflower genotypes that maintain high phytochemical quality under water-limited conditions, particularly in major production regions such as Iran, where safflower is cultivated as a rotational crop in rain-fed and supplementary-irrigation systems (Rahimi, 2021).

At the biochemical level, drought stress triggers the accumulation of reactive oxygen species (ROS), and plants counteract this oxidative burden through both enzymatic antioxidant systems and non-enzymatic secondary metabolites (Bao et al., 2024; Fathi et al., 2025). Flavonoids and anthocyanins, downstream products of the phenylpropanoid pathway, are among the most effective non-enzymatic ROS scavengers: they quench singlet oxygen and hydroxyl radicals directly, function as protective compounds that limit photo-oxidative damage, and their biosynthesis is transcriptionally up-regulated under water deficit through the coordinated induction of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and downstream branch-point enzymes such as dihydroflavonol-4-reductase and anthocyanidin synthase (Li and Ahammed, 2023; Zhou et al., 2023). In Arabidopsis, drought induces a rapid and reversible remodelling of the flavonol and anthocyanin pool that is tightly coupled to the plant's water-loss trajectory (Fidel et al., 2022), and in purple-leaf oilseed rape genotypes, constitutively higher anthocyanin accumulation is directly associated with lower ROS burden, better membrane integrity, and superior physiological performance under drought relative to green-leaf counterparts (Chen et al., 2022). A comparable protective role for anthocyanins has been reported across a wide range of unrelated taxa subjected to water deficit, reinforcing the generality of this defence mechanism (Yan et al., 2022).

Safflower itself possesses a well-characterised flavonoid biosynthetic capacity, and recent functional work has shown that a safflower flavonol synthase gene, CtFLS1, is sufficient to enhance drought tolerance in a heterologous system by driving flavonol and anthocyanin accumulation (Ma et al., 2024), providing direct mechanistic support for flavonoid- and anthocyanin-based drought defence in this species. Parallel evidence from the aromatic crop anise (Pimpinella anisum L.), evaluated under water-deficit stress in Kerman province using drought-tolerant and drought-sensitive genotypes, showed that up-regulation of the same core phenylpropanoid genes (PAL, 4CL, CHI, F3H) accompanied large increases in total flavonoid and anthocyanin content specifically in the tolerant genotype (Mehravi et al., 2025), underscoring that genotypic variation in phenylpropanoid pathway activation, rather than drought exposure alone, determines the magnitude of the phytochemical stress response.

Screening large genotype panels for drought tolerance is most informative when trait values under stressed (Ys) and non-stressed (Yp) conditions are combined into complementary selection indices rather than interpreted independently. The stress susceptibility index (SSI; Fischer and Maurer, 1978) and stress tolerance index (STI; Fernandez, 1992) remain the most widely used metrics,

and are typically interpreted alongside mean productivity (MP), geometric mean productivity (GMP), harmonic mean, tolerance (TOL), and the yield stability index (YSI) to distinguish genotypes that are merely stress-avoidant from those that combine high productivity with genuine stress tolerance. This multi-index framework has been validated across a wide range of crops under recent field conditions, including maize (Shojaei et al., 2022), dry bean (Mutari et al., 2023), and Bambara groundnut (Odesola et al., 2023), and has been applied specifically to safflower yield screening in worldwide germplasm collections (Golkar et al., 2021) and in Iranian marginal-area trials (Pasban Eslam et al., 2024), as well as to genome-wide association mapping of drought-tolerance loci in safflower (Hassani et al., 2024). However, most safflower drought-tolerance index studies to date have focused on seed yield and morphological traits rather than on the biochemical, antioxidant-related traits that determine both stress physiology and downstream product quality.

Furthermore, drought responses in phenylpropanoid-derived metabolites are frequently confounded by seasonal climatic variation, since temperature and radiation load during flowering can independently drive large shifts in anthocyanin accumulation irrespective of the imposed irrigation treatment, as demonstrated in a two-year rain-fed trial of high-oleic safflower genotypes in Sicily in which year, rather than genotype, was the dominant source of variation in several productive traits (Licata et al., 2023). Disentangling the relative contributions of irrigation regime, genotype, and year is therefore essential for correctly attributing phytochemical variation to drought stress rather than to background seasonal effects, and requires multi-year trials with adequate genotypic replication.

Building on this body of evidence, the present study evaluated 38 safflower genotypes under full irrigation and terminal drought stress across two growing seasons at Arak, Iran, with the following objectives: (i) to quantify the effect of drought stress, year, and genotype on total flavonoid and anthocyanin content in safflower flag leaves using a three-way factorial ANOVA; (ii) to identify genotypes combining high productivity and stability under both irrigation regimes using eight complementary drought-tolerance indices; (iii) to test whether flavonoid and anthocyanin accumulation are correlated, as an indicator of shared versus independent regulation of the two phenylpropanoid branches; and (iv) to identify candidate parental genotypes for breeding programmes targeting drought-resilient phytochemical quality in safflower.

Materials and Methods

Site description

The study was conducted over two growing seasons (2015 and 2016) at the Research Farm of the Faculty of Agriculture, Islamic Azad University, Arak Branch (Khomein Road), Iran (36°15′ N, 59°23′ E; 1708 m a.s.l.). The soil was a loam with pH ≈ 7.7 and electrical conductivity ≈ 1.3 dS m^{-1}. Mean annual precipitation at the site was approximately 350 mm and mean annual temperature 12.5 °C, characteristic of the semi-arid climate typical of central Iranian safflower production areas.

Experimental design and treatments

A split-plot design within a randomized complete block framework, with three replications, was used following standard agronomic field-experiment protocols (Gomez and Gomez, 1984). The main-plot factor comprised two irrigation regimes: (i) full irrigation (control), in which irrigation was scheduled at 20% allowable soil moisture depletion throughout the growing season; and (ii) drought stress, in which full irrigation was maintained until stem elongation and then withheld until harvest (approximately 70% allowable depletion).

The sub-plot factor comprised 38 safflower genotypes (coded G1–G38), obtained from the Dryland Agricultural Research Center, Kermanshah, Iran. Each sub-plot measured 3 × 1.8 m (5.4 m^2) and contained six rows spaced 30 cm apart. A drip (tape) irrigation system with 5-cm emitter spacing at 1.5 atm pressure was used to apply irrigation treatments precisely. Sowing was

performed on 22 February in both years. Standard agronomic practices, including manual weeding and fertilization (70 kg N ha^{-1} as sulfur-coated urea, applied in two splits), followed local recommendations for the region.

Traits measured

Leaf samples were collected from the flag leaf at full flowering (50% flowering) in both seasons. Two flavonoid-pathway biochemical traits were quantified:

Total flavonoid content (mg QE g^{-1} DW) was determined by the aluminium chloride colorimetric method using quercetin as the calibration standard (Zhishen et al., 1999).

Anthocyanin content (mg g^{-1} DW) was measured by the pH-differential method at pH 1.0 and 4.5 (Giusti and Wrolstad, 2001).

Statistical analysis

All statistical analyses were performed in Python 3.12 using the pandas, NumPy, SciPy, statsmodels, and openpyxl libraries, with visualisations generated in matplotlib. Descriptive statistics included sample size (N), mean, standard deviation (SD), standard error (SE), coefficient of variation (CV%), minimum, first quartile, median, third quartile, maximum, skewness, and kurtosis, computed overall and stratified by irrigation regime, year, and genotype.

Inferential analysis employed a three-way fixed-effects factorial analysis of variance (ANOVA) using Type II sums of squares, fitted as Trait ~ Year × Irrigation × Variety. This model simultaneously estimates the main effects of each factor and all two-way and three-way interactions; the percentage contribution of each term to total variance was computed as Source SS / Total SS × 100.

Drought tolerance was quantified for each genotype using eight complementary indices calculated from genotype means under normal (Yp) and drought (Ys) irrigation, averaged across years and replications. The stress susceptibility index (SSI; Fischer and Maurer, 1978) expresses the proportional reduction in trait value under stress relative to the mean population reduction, with lower SSI indicating greater tolerance. The stress tolerance index (STI; Fernandez, 1992), mean productivity (MP), geometric mean productivity (GMP), and harmonic mean (HARM) reward genotypes that maintain high trait values under both normal and stress conditions. Tolerance (TOL = Yp − Ys) measures the absolute reduction, while the yield stability index (YSI = Ys / Yp) and drought tolerance index (DTI) provide dimensionless rankings of relative stability under stress. This multi-index approach follows established practice for drought-tolerance screening in field crops (Shojaei et al., 2022; Mutari et al., 2023; Odesola et al., 2023) and has previously been applied to safflower yield evaluation (Golkar et al., 2021; Pasban Eslam et al., 2024).

The relationship between total flavonoid and anthocyanin content was assessed using both Pearson (linear) and Spearman (rank-based) correlation coefficients, computed overall and within each combination of irrigation regime and year. Pearson correlations assume linearity and bivariate normality, whereas Spearman correlations are robust to non-linear monotonic relationships and outliers, providing complementary information.

Assumption testing

Two key assumptions of parametric ANOVA were formally tested. Normality of residuals from the full ANOVA model was assessed using the Shapiro–Wilk test. Homogeneity of variances across the four year-by-irrigation groups was evaluated with Levene's test using the median-centred estimator, which is robust to non-normal distributions. Both tests returned significant results ($p < 0.001$ for both traits in the Shapiro–Wilk test; $p < 0.001$ for flavonoid and $p < 0.001$ for anthocyanin in Levene's test), indicating that the strict distributional assumptions of parametric ANOVA were violated. Nevertheless, the F-test is known to be robust to moderate departures from normality and variance homogeneity, particularly in balanced designs with large sample sizes ($n = 456$ in the present study). Interpretive conclusions are therefore reported with appropriate caution, and

effect sizes (percentage contribution to total variance) are emphasized alongside p-values throughout the Results.

Results

Effect of irrigation regime

Drought stress increased mean total flavonoid content from 0.1395 ± 0.0359 mg QE g^{-1} DW under normal irrigation to 0.1483 ± 0.0318 mg QE g^{-1} DW under drought, an absolute increase of 0.0088 (6.3% relative increase). The coefficient of variation was 25.8% under normal irrigation and 21.4% under drought. Anthocyanin content increased from 0.00382 ± 0.00278 mg g^{-1} DW under normal irrigation to 0.00413 ± 0.00259 mg g^{-1} DW under drought, an 8.1% relative increase (Figure 1).

Inter-annual variation

Mean flavonoid content was 0.1397 mg QE g^{-1} DW in Year 1 and 0.1481 mg QE g^{-1} DW in Year 2, a 6.0% increase (Figure 2); the coefficient of variation was 31.0% in Year 1 and 14.0% in Year 2. Mean anthocyanin content was 0.00611 mg g^{-1} DW in Year 1 and 0.00185 mg g^{-1} DW in Year 2, a 69.7% reduction. The Year main effect accounted for 62.8% of total variance in the ANOVA for anthocyanin content (Section 3.4, Table 2).

Varietal variation

For total flavonoid content under normal irrigation, Variety 31 had the highest mean (0.1553 mg QE g^{-1} DW) and Variety 24 the lowest (0.1006 mg QE g^{-1} DW), a 54.4% range. Under drought, Variety 32 had the highest mean (0.1706 mg QE g^{-1} DW) and Variety 20 the lowest (0.1178 mg QE g^{-1} DW) (Figure 4). For anthocyanin content under normal irrigation, Variety 14 had the highest mean (0.0055 mg g^{-1} DW) and Variety 13 the lowest (0.0030 mg g^{-1} DW); under drought, Variety 5 had the highest mean (0.0065 mg g^{-1} DW), while several varieties had means as low as 0.0031 mg g^{-1} DW (Figure 3).

Factorial ANOVA

For total flavonoid content, the three-way factorial ANOVA (Table 1) showed significant main effects of Year ($F = 6.003$, $p = 0.0148$) and Irrigation ($F = 6.558$, $p = 0.0109$), each accounting for approximately 1.5–1.6% of total variance. The Year × Irrigation interaction was not significant at $\alpha = 0.05$ ($F = 3.047$, $p = 0.0819$). The Variety main effect was not significant ($p = 0.953$), nor were any interactions involving Variety significant. Residual variance accounted for 75.8% of the total sum of squares.

For flavonoid content, mean values by year showed a larger drought-associated increase relative to normal irrigation in Year 1 than in Year 2 (Figure 6; Year × Irrigation interaction, $p = 0.0819$). For anthocyanin content, the corresponding Year × Irrigation interaction was $p = 0.087$, with higher normal-irrigation anthocyanin levels in Year 1 than in Year 2 (Figure 6).

Correlation between flavonoid and anthocyanin content

Pearson and Spearman correlation coefficients between total flavonoid and anthocyanin content were computed overall and within each combination of irrigation regime and year (Figure 7, 8). All coefficients were weak ($|r| < 0.13$) and not statistically significant at $\alpha = 0.05$, with one exception approaching significance (drought stress overall: $r = -0.114$, $p = 0.084$). The overall correlation across all conditions was $r = -0.062$ ($p = 0.187$) (Figure 5).

Trait variability and stability

Total flavonoid content had an overall coefficient of variation (CV) of 23.7%; CV was 25.8% under normal irrigation and 21.4% under drought, and 31.0% in Year 1 and 14.0% in Year 2. Anthocyanin content had higher CV values across all groups, with a maximum of 73.4% in Year 2 (Figure 9).

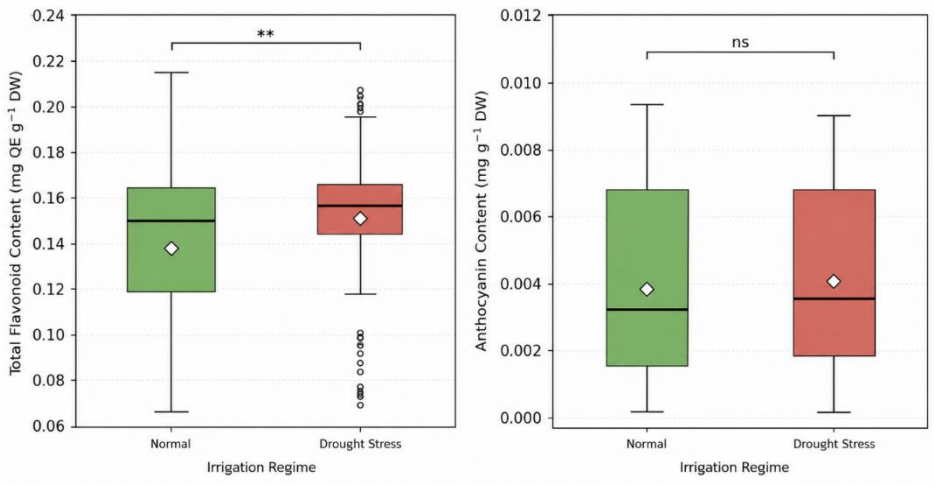

Figure 1. Boxplot distributions of total flavonoid and anthocyanin content under normal versus drought irrigation. White diamonds mark group means; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $p < 0.1$, ns = not significant (independent samples t-test).

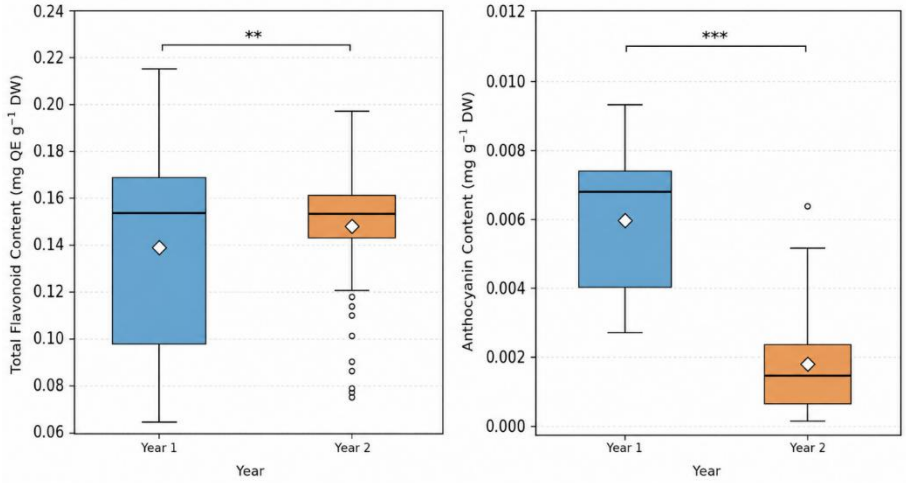

Figure 2. Boxplot distributions of phytochemical traits between the two experimental years. The Year 1 versus Year 2 contrast is especially pronounced for anthocyanin content (***).

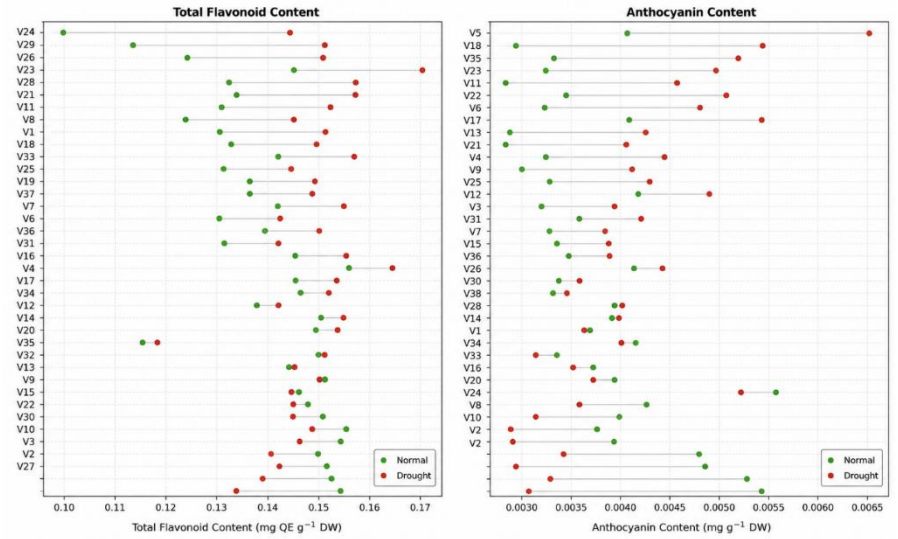

Figure 3. Lollipop chart comparing each variety's mean value under normal (green) versus drought (red) irrigation for both traits. Varieties are sorted by the drought − normal difference.

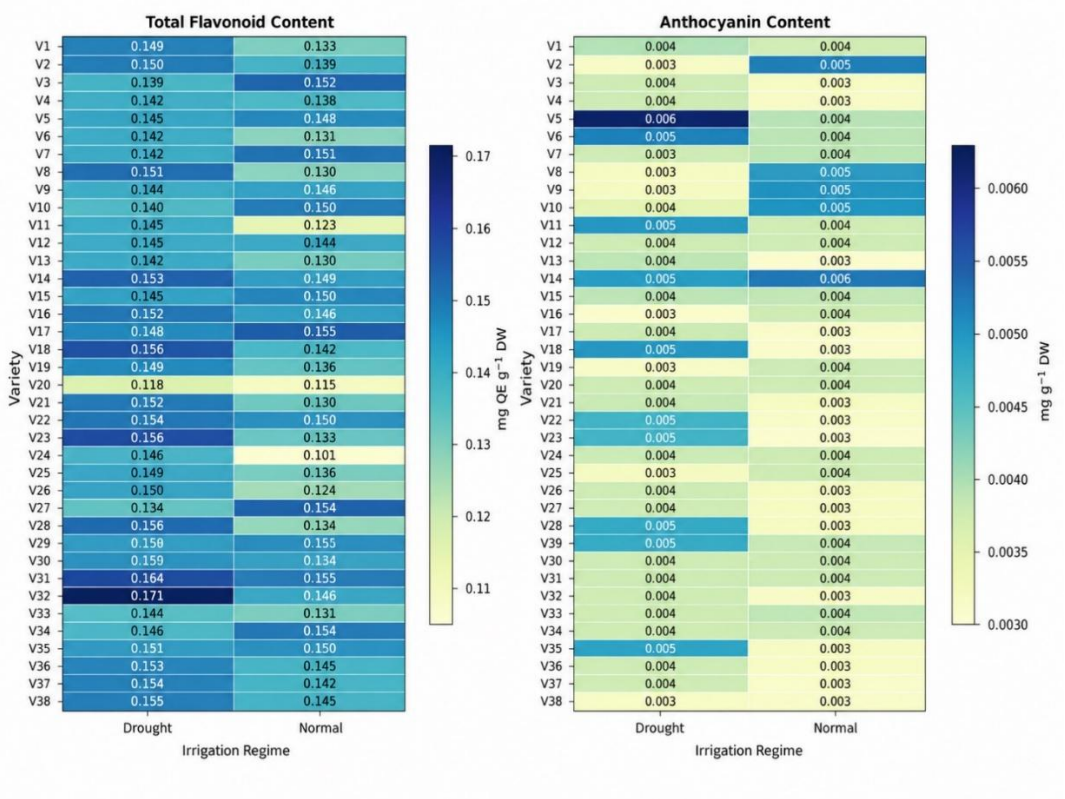

Figure 4. Heatmap of variety means across irrigation regimes. Cell values are mean trait values across years and replications; colour intensity scales linearly with magnitude.

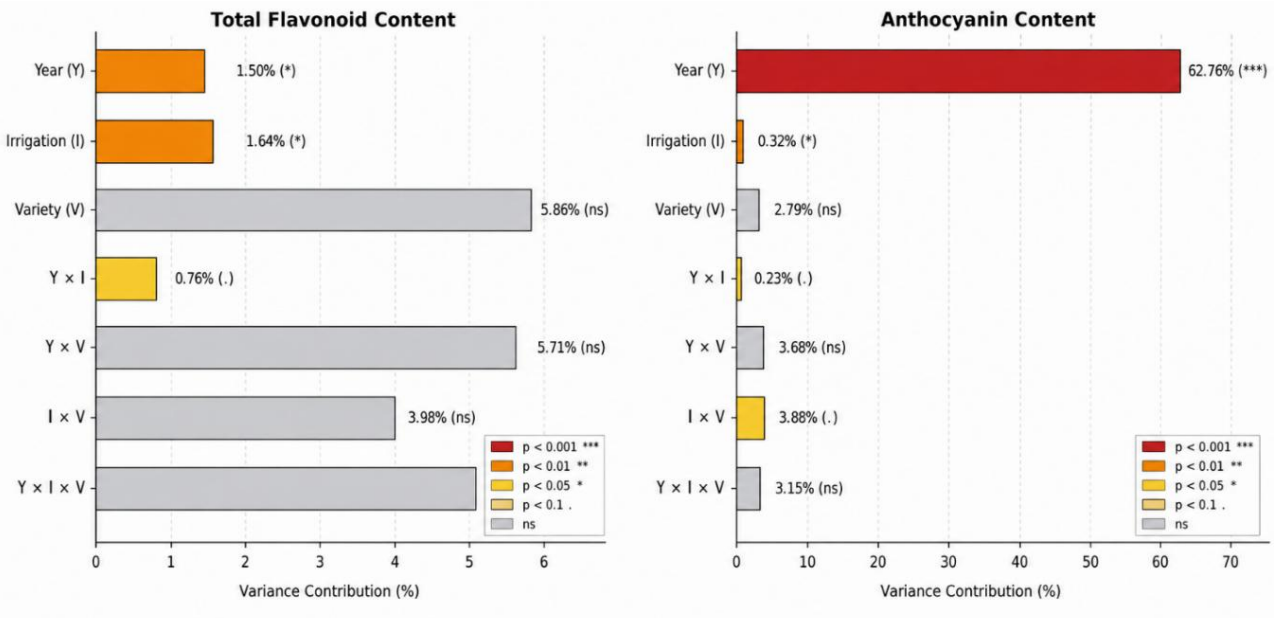

Figure 5. Percentage variance contribution of each experimental factor and their interactions, with significance codes from the three-way factorial ANOVA.

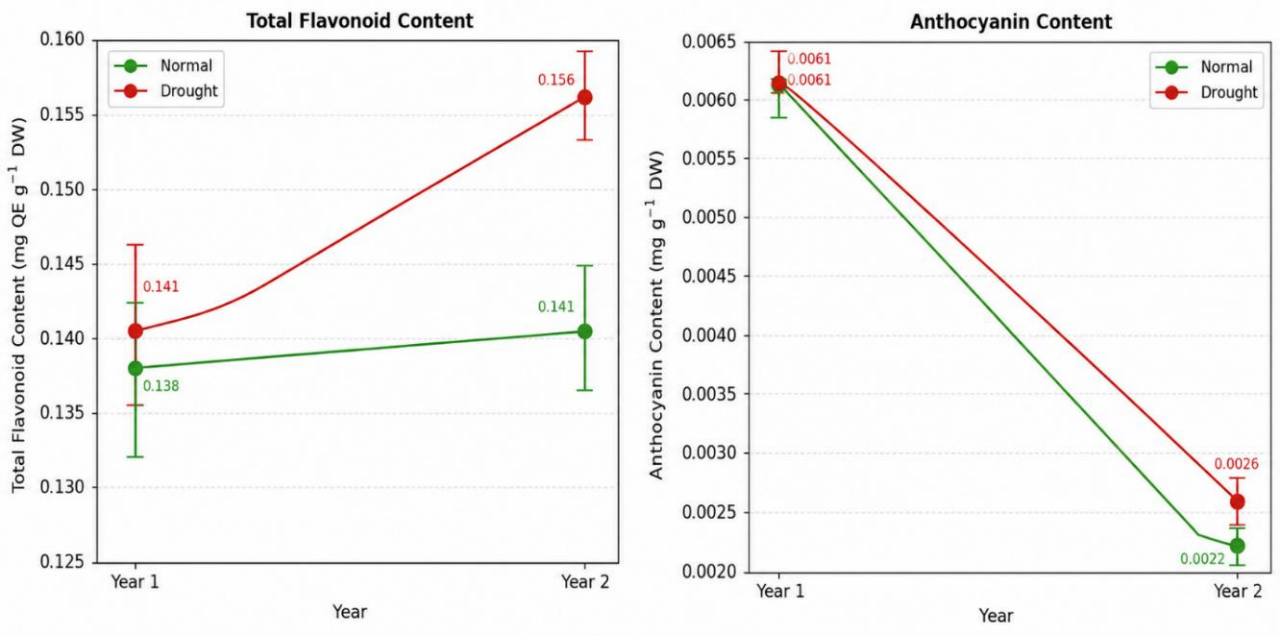

Figure 6. Year × Irrigation interaction plots for both phytochemical traits. Error bars denote ± 1 standard error of the mean.

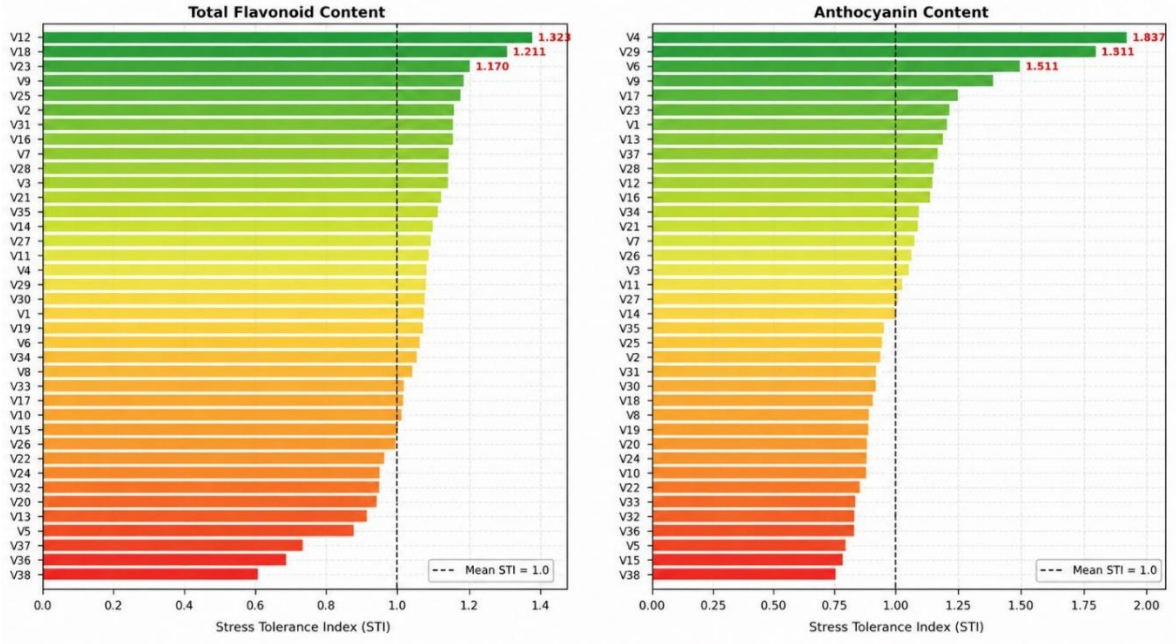

Figure 7. Full STI ranking of all 38 varieties for both traits. The vertical dashed line marks STI = 1.0 (population-mean tolerance); bars to the right indicate above-average tolerance.

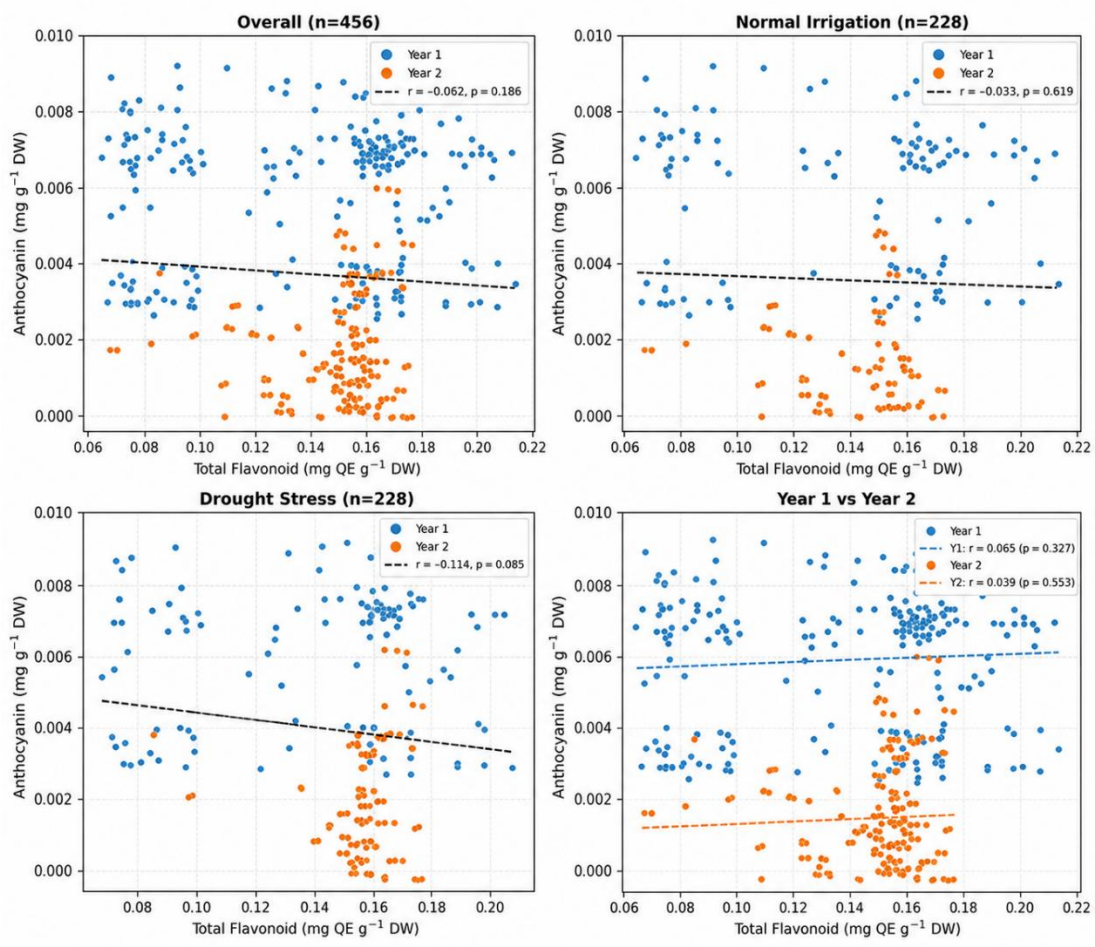

Figure 8. Scatter plots of total flavonoid versus anthocyanin content overall, by irrigation regime, and by year. Black dashed lines show linear fits; Pearson correlation statistics are reported in each panel

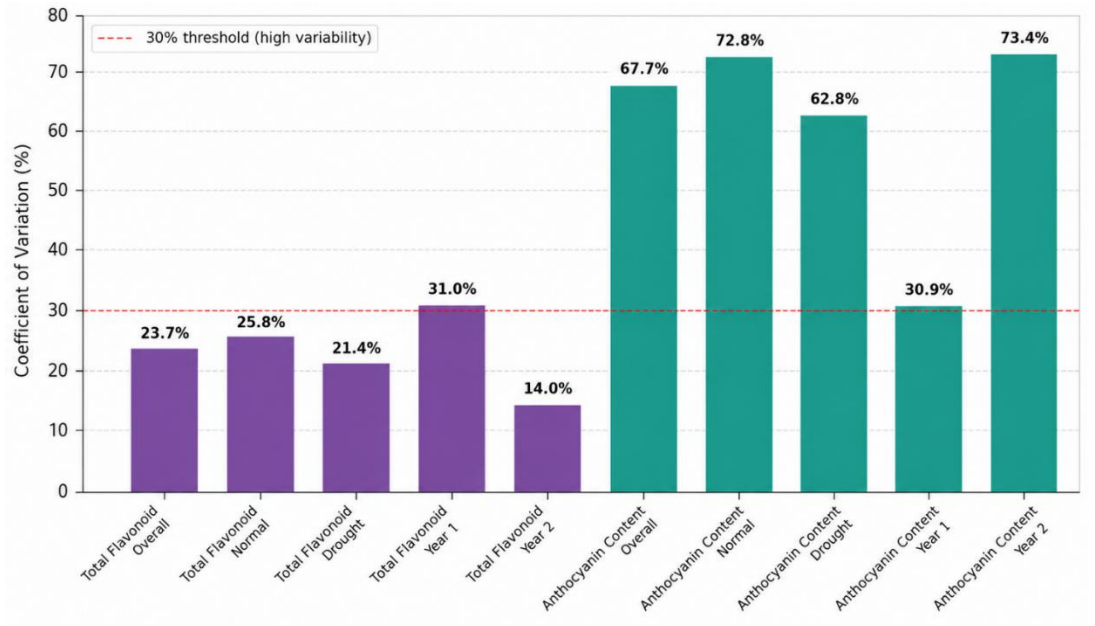

Figure 9. Coefficient of variation (CV%) for both traits across experimental groups. The red dashed line at 30% marks a common threshold above which trait variability is considered high.

Table 1. Three-way factorial ANOVA for total flavonoid content (Type II SS).

Source of Variation	df	sum of squares	F-value	p-value	% Contribution	Sig.
Year (Y)	1	0.008687	6.003	0.0148	1.50%	*
Irrigation (I)	1	0.031118	6.558	0.0109	1.64%	*
Variety (V)	37	0.004036	0.635	0.9526	5.86%	ns
Y × I	1	0.030310	3.047	0.0819	0.76%	.
Y × V	37	0.021136	0.618	0.9612	5.71%	ns
I × V	37	0.025226	0.431	0.9986	3.98%	ns
Y × I × V	37	0.402678	0.515	0.9919	4.75%	ns
Residual	304	0.008687	—	—	75.81%	—
ns: not significant, *: significant at $p < 0.05$, significant at $p < 0.1$						

For anthocyanin content, the ANOVA (Table 2) showed a significant Year main effect ($F = 822.277$, $p < 0.001$), accounting for 62.8% of total variance, and a significant Irrigation main effect ($F = 4.145$, $p = 0.0426$), accounting for 0.32% of variance. The Irrigation × Variety interaction was not significant at $\alpha = 0.05$ ($F = 1.374$, $p = 0.0797$). The Variety main effect ($p = 0.496$) and remaining interactions were not significant. Residual variance accounted for 23.2% of the total sum of squares.

Table 2. Three-way factorial ANOVA for anthocyanin content (Type II SS).

Source of Variation	df	sum of squares	F-value	p-value	% Contribution	Sig.
Year (Y)	1	0.002068	822.277	<0.001	62.76%	***
Irrigation (I)	1	1.04e-5	4.145	0.0426	0.32%	*
Variety (V)	37	9.18e-5	0.986	0.4964	2.79%	ns
Y × I	1	7.41e-6	2.948	0.0870	0.23%	.
Y × V	37	1.21e-4	1.301	0.1212	3.68%	ns
I × V	37	1.28e-4	1.374	0.0797	3.88%	.
Y × I × V	37	1.04e-4	1.115	0.3040	3.15%	ns
Residual	304	7.64e-4	—	—	23.20%	—
ns: not significant, significant at $p < 0.1$, *: significant at $p < 0.05$, ***: significant at $p < 0.001$						

For flavonoid content, mean values by year showed a larger drought-associated increase relative to normal irrigation in Year 1 than in Year 2 (Figure 6; Year × Irrigation interaction, $p = 0.0819$). For anthocyanin content, the corresponding Year × Irrigation interaction was $p = 0.087$, with higher normal-irrigation anthocyanin levels in Year 1 than in Year 2 (Figure 6).

Discussion

Drought-induced activation of secondary metabolism

A central finding of this study is the modest but consistent increase in both flavonoid and anthocyanin content under drought stress. This pattern aligns with the well-established role of flavonoids and anthocyanins as ROS scavengers and photoprotectants that accumulate in plant tissues in response to abiotic stress (Bao et al., 2024; Zhou et al., 2023). The 6.3% increase in flavonoid content and 8.1% increase in anthocyanin content under drought, while modest in absolute magnitude, are biologically meaningful because they represent population-level

responses averaged across 38 genotypes and two years, and are consistent with drought-induced up-regulation of the phenylpropanoid pathway reported for other species (Fidel et al., 2022; Mehravi et al., 2025), in which flavonoids and anthocyanins function as ROS scavengers and photoprotectants. Comparable stress-induced increases in total flavonoid and anthocyanin content have been reported under water-deficit conditions in other economically important species, including anise (Mehravi et al., 2025) and purple-leaf oilseed rape (Chen et al., 2022), in which the constitutively higher-anthocyanin genotype also displayed lower ROS accumulation and better membrane integrity under drought, supporting a direct protective function for these pigments rather than a purely correlative association with stress exposure.

The lower coefficient of variation for flavonoid content under drought relative to normal irrigation (21.4% vs. 25.8%) suggests a degree of convergence in genotypic response toward a common stress-induced phenotype. Similarly, the marked narrowing of flavonoid CV between years (31.0% in Year 1 to 14.0% in Year 2) suggests that Year 2 environmental conditions were more favourable or more uniform, constraining the expression of genotypic variation in that season.

The substantial inter-annual variation observed here, particularly the 69.7% reduction in anthocyanin content between Year 1 and Year 2, and the dominant contribution of Year to total ANOVA variance for anthocyanin (62.8%), indicate that anthocyanin accumulation in safflower petals is governed primarily by seasonal climatic drivers — most plausibly temperature, radiation, and precipitation timing — rather than by irrigation regime or genotype. This is an exceptionally large effect size for a single factor in a multi-factor field experiment, and it echoes conclusions drawn from other multi-year phenylpropanoid-pathway studies under field conditions (Mehravi et al., 2025) as well as from a two-year rain-fed trial of high-oleic safflower genotypes in Sicily, in which year, rather than genotype, was the dominant source of variation in several productive traits (Licata et al., 2023).

The marginal Year × Irrigation interaction observed for both traits ($p = 0.0819$ for flavonoid; $p = 0.087$ for anthocyanin) suggests that the magnitude of the drought response differed between years — more pronounced in Year 1 for both traits — consistent with reports that seasonal climate can modulate the magnitude of stress-induced phenylpropanoid responses independently of the imposed water treatment (Licata et al., 2023). Together, these findings indicate that the biochemical impact of drought on flavonoid and anthocyanin accumulation cannot be fully interpreted without reference to the prevailing environmental conditions of each growing season.

Genotypic differentiation versus residual variance

The non-significant Variety main effect in the ANOVA ($p = 0.953$ for flavonoid, $p = 0.496$ for anthocyanin) appears to contradict the substantial variation observed among variety means (Section 3.3), and indicates that the 38 genotypes responded similarly to irrigation treatment at the population level. This apparent paradox is explained by the high residual variance (75.8% for flavonoid, 23.2% for anthocyanin), which reflects substantial within-treatment variation rather than an absence of underlying biological variation among genotypes, and is likely driven by plot-level micro-environmental heterogeneity, biological replication noise, and possibly temporal variation in sampling within each plot, including factors such as floret position or diurnal sampling effects that were not explicitly modelled.

Independence of the flavonoid and anthocyanin branches

The weak and non-significant correlations between flavonoid and anthocyanin content across all subgroups (overall $r = -0.062$, $p = 0.187$) indicate that these two classes of phenolic compounds respond largely independently to environmental and genetic factors in safflower petals. While both compound classes share the phenylpropanoid precursor pathway, their downstream branches are regulated by distinct enzymatic cascades and, in several species, by partially non-overlapping

transcription-factor networks (Li and Ahammed, 2023). Functional evidence from a safflower flavonol synthase gene, CtFLS1, which drives flavonol and anthocyanin accumulation when expressed heterologously (Ma et al., 2024), demonstrates that individual branch-point genes can be manipulated to enhance one pathway output without necessarily co-regulating the other, reinforcing the biological plausibility of the observed independence. This finding implies that selection for one trait will not indirectly improve the other, and that multi-trait selection indices or marker-assisted approaches targeting both branches simultaneously are required for their combined improvement in breeding programmes.

Implications for breeding programme design

The drought-tolerance indices converge on a small number of elite candidates. Variety 31 is the most drought-tolerant genotype for flavonoid content, having actually increased flavonoid content under drought (a 5.8% gain), supporting the interpretation that flavonoid biosynthesis in this and other top-ranked genotypes (Varieties 32, 22, and 14, which also showed drought-induced gains) is up-regulated rather than suppressed by water deficit. Variety 14 is the most tolerant genotype for anthocyanin content, maintaining high anthocyanin content under both irrigation regimes (only a 6.2% reduction under drought) and indicating broad stress stability, and is the only genotype ranking in the top five for both traits. Variety 5 displayed the largest drought-induced gain in the panel for anthocyanin content (55.9%). The high STI values (> 1.2) of the top-five varieties for each trait indicate strong candidacy for breeding programmes targeting flavonoid and anthocyanin stability under water scarcity. These varieties are recommended as priority parents in crosses aimed at improving the drought resilience of safflower phytochemical profiles, in line with the broader recommendation that STI-based multi-index screening is the most effective way to identify genotypes combining productivity and tolerance under both irrigation regimes (Golkar et al., 2021; Shojaei et al., 2022; Mutari et al., 2023).

The marginally non-significant Irrigation × Variety interaction for anthocyanin ($p = 0.0797$) provides weak evidence for genotypic differences in drought responsiveness for this trait, and suggests that such differences could be exploited in targeted selection once sample sizes are increased. The dominant Year effect for anthocyanin (62.8% of variance) further implies that multi-year evaluation, rather than single-season screening, is essential to obtain robust estimates of genotypic value for this environmentally sensitive trait, a caution that applies broadly to phenylpropanoid-derived quality traits in field-grown oilseed and aromatic crops (Licata et al., 2023; Mehravi et al., 2025).

More broadly, these results support the growing case for incorporating antioxidant phytochemical traits, and not only yield, into safflower drought-tolerance screening pipelines. Given the pharmaceutical and nutraceutical value attached to safflower flavonoid content (Cheng et al., 2024), genotypes that couple agronomic drought tolerance with stable or enhanced flavonoid and anthocyanin accumulation, such as Variety 14 in the present panel, offer a route to breeding cultivars with dual value under the water-limited conditions that are expected to intensify across semi-arid production regions in the coming decades (He and Rosa, 2023).

Trait variability and environmental sensitivity

The coefficient of variation (CV) provides a dimensionless measure of relative dispersion and is widely used in plant breeding to assess trait stability. The narrowing of flavonoid CV under drought (21.4% vs. 25.8% under normal irrigation) and in Year 2 (14.0% vs. 31.0% in Year 1) suggests that both environmental stress and favourable growing seasons constrain genotypic expression of this trait, albeit through different mechanisms. Anthocyanin content displayed substantially higher CV values across all groups, peaking at 73.4% in Year 2 — a counterintuitive finding given that Year 2 also had the lower mean anthocyanin content — indicating that the very low anthocyanin values observed in Year 2 were accompanied by disproportionately high relative dispersion. These patterns reinforce the conclusion that

anthocyanin is a less stable and more environmentally sensitive trait than total flavonoid content in safflower, consistent with the dominant Year effect identified in the ANOVA (Section 3.4).

Conclusion

This study quantified the effects of irrigation regime, genotype, and year on two pharmaceutically important phytochemical traits in a 38-genotype safflower panel evaluated over two field seasons. The principal conclusions are: (i) drought stress significantly up-regulated both total flavonoid and anthocyanin content, supporting the role of these compounds in stress defence; (ii) the year effect was dominant for anthocyanin, contributing nearly 63% of total variance, while irrigation and genotype played secondary roles; (iii) varietal differences were not statistically significant at the population level owing to high residual variance, but a subset of varieties — notably Variety 31 for flavonoid and Variety 14 for anthocyanin — consistently exhibited superior drought tolerance across multiple indices, with Variety 14 the only genotype ranking in the top five for both traits; (iv) flavonoid and anthocyanin contents were uncorrelated, indicating independent genetic and biochemical regulation of the two phenylpropanoid branches; and (v) the assumptions of normality and variance homogeneity required for parametric ANOVA were violated, warranting cautious interpretation and suggesting that non-parametric or robust alternatives (e.g., aligned-rank-transform ANOVA, permutation tests) should be considered in confirmatory analyses.

For breeding programme deployment, we recommend: (a) prioritising Variety 14 as a multi-trait, drought-tolerant parent given its top-five ranking for both flavonoid and anthocyanin content; (b) incorporating Variety 31 into crosses targeting flavonoid enhancement under drought; (c) conducting multi-year, multi-environment trials with increased replication and spatially adjusted designs to improve statistical power for detecting genotypic differences; (d) employing multi-trait selection indices that explicitly account for the independence of the flavonoid and anthocyanin pathways; and (e) extending the analytical framework to include meteorological covariates (cumulative growing-degree days, total evapotranspiration) that may explain the dominant year effect on anthocyanin accumulation. Together, these findings provide a phytochemically informed complement to conventional yield-based drought-tolerance screening in safflower and identify concrete genotypic targets for breeding programmes operating in water-limited production environments.

Conflict of Interest

All authors declared that they have no conflict of interest

Acknowledgments

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Short-term effect of zinc oxide nanoparticles (ZnO-NPs) on *Carum copticum* growth and physiological effects under hydroponic condition

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ABSTRACT

This study investigated the growth and physiological response in medicinal plant *Carum copticum* exposed to zinc oxide nanoparticles (ZnO-NPs). Treatments of 0.1, 0.2, 1, 5 and 25µM of ZnO-NPs compared with 0.2 µM $ZnSO_4$ (control; Zn form in Johnson solution) were applied in culture media under hydroponic conditions. The plants shoot/root length, and water content, H_2O_2, anthocyanin and activity POD enzyme were measured. The root length was more sensitive to ZnO-NPs than shoot length. The 0.2 and 1 µM NPs increased plant fresh weight by 96.8 and 115.2%, respectively. The shoot and root water content increased at 0.1-25 µM NPs treatments by 40-51% and 26-34% compared to the control group, respectively. The increase in POD enzyme was accompanied by increased length and fresh weight and anthocyanin contents under ZnO-NPs. The 25 µM ZnO NPs (high level) resulted in 36% shoot length, 44% root length, 38% plant fresh weight and 27% anthocyanin decline, while increased 40% shoot water content, 26% root water content, 17% H_2O_2 and 69% POD activity compared with control. Overall, 25 µM ZnO-NPs treatment induced higher damages to the growth parameters due to H_2O_2 increase (oxidative stress) and the failure of the enzymatic and non-enzymatic mechanisms (anthocyanin and POD) to cope with the H_2O_2 enhancement induced by ZnO-NPs.

Keywords: Ajwain, nano metal oxide, nano toxicity

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Introduction

Nanoparticles (NPs; at least one dimension < 100 nm) are widely have been widely used in various industries due to their enhanced and multidimensional effects. It is predicted that, the amount of nanotechnology production will reach more than $1 trillion within the next 10 to 15 years. The rapid expansion of nanotechnology in various industries leads to their intentional or unintentional release into soil, air, and ultimately plants, animals and humans. The effects of NPs are being widely studied, with both positive and negative impacts on living organisms

(Ochoa-Chaparro et al., 2026; Islam et al., 2025). Some general mechanisms of interaction of NPs with plant have been identified over the past decade. Some general mechanisms of NPs' interaction with plants have been elucidated over the last decade (Islam et al., 2025; Zeng et al., 2025). Oxide metal NPs are among the most often used forms of NPs in various section of industries. Metallic nanoparticles might release lower concentrations of soluble metal ions in the environment, but they could be more efficiently taken up by plants. Metal oxide NPs have attracted attention due to their potential positive and negative ecological and biological impacts on living organism and ecosystem (Ochoa-Chaparro et al., 2026; Zeng et al., 2025). Zinc element is an important micronutrient that plays important roles in many integrated metabolic processes (Ochoa-Chaparro et al., 2026; Mousavi et al. 2024). Zinc is essential for optimal plant growth regulation; zinc toxicity leads to oxidative stress and disruption of physiology and molecular processes in cells (Ochoa-Chaparro et al., 2026; Mousavi et al., 2024; Singh et al. 2018). The remarkable properties of zinc oxide nanoparticle (ZnO-NPs) may contribute to increase agricultural yield. On the other hand, ZnO-NPs are widely used in many commercial applications and may leach into the surrounding environment (Amin and Erfan, 2025; Lebaka et al., 2025). The ZnO-NPs had cost-effective and less toxic NP types that reportedly benefit from "quantum size effects" as a result of their special physical properties. Plants of response to ZnO-NPs had reported to be dose-dependent, and in various species, it showed positive impacts on seed germination, growth, development and flowering under various concentrations (Lebaka et al., 2020; Mazaheri-Tirani and Dayani, 2020). It has been reported that the life-long effect of ZnO-NPs five months after seed germination at concentrations of 1 and 5 µM on the growth in *Carum copticum* under hydroponic condition (perlite-base) significantly improved growth and flowered earlier than the control, while concentrations of 25 and 125 µM resulted in toxic responses (Mazaheri-Tirani et al., 2024). ZnO toxicity produces significant amounts of H_2O_2, as a strong oxidizing agent and consequently leads to hydroxyl radicals' production. Antioxidant systems scavenged ROS (Such as H_2O_2) and can be classified into main classes including non-enzymatic (carotenoids, flavonoids, ascorbic acid and anthocyanin) and enzymatic (superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), and peroxidase (POD)) (Mustafa et al., 2024; Mazaheri-Tirani et al., 2024).

Soil supports plant life by providing a physical environment for roots and a source of macro- and micro-nutrients needed for growth and development. However, the nutrient availability it provides varies across soils and depends on conditions at the level of roots and plant tissues. However, soil cultivation environments may have many limitations. The soil limitations such as reduced gas exchange, pesticide residue, high salt concentrations and excessive nitrates, and can impair plant growth and development. Hydroponic conditions can be a suitable alternative approach (Xing et al., 2025; Pandao et al., 2024; Thapa et al., 2024).

Carum copticum L. (known as Ajwain) is an important medicinal plant, native to India and Iran. *C. copticum* is widely cultured because of its beneficial biochemical composition that results in its high antioxidant (Monfared et al. 2020; Firoozi et al. 2019; Nagalakshmi et al. 2000). The production of agro-products under controlled environments, such as hydroponics systems can contribute to food safety, and increase the efficiency of land and fresh water resources (Barbosa et al. 2015). Therefore, this study investigated the impact of ZnO nanoparticles in hydroponics conditions (water base) and reported its influence on plant species growth and physiological traits of ajwain medicinal plant.

Materials and Methods

Plant material and growth conditions

The seeds of *Carum copticum* L. were collected from Pakan-bazr Company, Isfahan, Iran. The seeds were planted in plastic pot containing sterilized perlite. The two-leaf stage of seedlings were transferred to soilless culture with three plants as one replication in 1 L containers. Ten days later, ZnO NPs treatments were applied at 0.1, 0.2, 1, 5, and 25 µM levels (after

optimization) in the growth media. The ZnO-NPs [average particle size: 25 nm, specific surface area: 4.8-6.8 m^2 g^{-1}, purity: ≥99.9 wt%, volume density: 0.3 g cm^{-3}, spherical shaped and without covering, Nanotec, Itay] were provided by Nanotec, Italy. The solutions of ZnO-NPs were prepared in deionized water and were sonicated for 20 min, two times at 40°C, using a sonicator instrument (Ultrasonic cleaner 2200 MH, Soltec, Italy). The base nutrient solution (without $ZnSO_4$) was prepared, then different concentrations of ZnO-NPs were added (solutions pH: 5.7 ± 0.05). The control group was 0.2 µM $ZnSO_4$ (amount in Johnson nutrient solution). The solutions were continuously aerated at a rate of about 0.5 L min^{-1} with the light intensity of ~100 µM m^{-2} s^{-1}. The solutions were replaced once a week. The analyses were conducted under controlled environment at 25-27°C for 16 h light and 18-21°C for 8 h darkness. Three weeks later, the seedlings were harvested and dried in an oven for dry weight. Fresh samples were collected and frozen in liquid nitrogen and stored at -80ºC for later analyze.

Growth parameters

The plants root and shoot were cut and separated. The root/shoot (length) and fresh/dry weight (FW/DW) were calculated. Then, the water content of root (WCR) and shoot (WCS) was calculated as fallows (Shah et al. 2002):

$$WC(\%) = \frac{(FW - DW)}{FW} \times 100$$

Measurement of Hydrogen peroxide

The H_2O_2 content was measured according to Tirani and Haghjou (2019). The 50 mg leaves tissues were homogenized in 1 ml of 0.1% trichloroacetic acid (TCA), and were centrifuged at 5000 × g for 15 min. Then, 750 µl supernatant was added to 750 µl of 1 M KI. The absorbance's each sample were read at 390 nm using UV/VIS spectrophotometer (UV-1601, Rayleigh, China). H_2O_2 (30%) was used as the standard.

Measurement of anthocyanin contents

The amount of anthocyanins were evaluated using acidified methanol according to the method reported by Wagner (1979). The 20 mg leaves were homogenized in 5 mL of acidified-methanol [pure methanol: HCl 37% = 99:1 (v/v)]. The sample kept for two days at room temperature in full dark conditions. Then, they were centrifuged at 5000 × g for 15 min at 4 ºC temperature. The absorptions of the supernatants were read by a UV-VIS spectrophotometer (Rayleigh, China) at 550 nm. The anthocyanin contents were measured using an extinction coefficient of 33000 M–1 cm–1.

Determination of POD enzymatic activity

The activity of peroxidases (POD) on plant leaf tissues were determined according to Mazaheri-Tirani et al. (2019). The 100 mg leaves were homogenized in 4 ml solution containing phosphate buffer (50 mM, pH = 7.8). The homogenate was centrifuged at 10,000 rpm for 15 min at 4ºC. The reaction mixture contained 20 mM guaiacol and 40 mM H_2O_2 were measured. The increase in the absorbance at 470 nm were measured for 60 s. Peroxidase (POD) activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Statistical analysis

The experiment was planned as completely randomized design (CRD) with three replicates (3 pots). One pot with three individual plants was assigned for each replicate. The analysis of variance (one-way ANOVA) using the Duncan test was applied on mean separations by SPSS software package 22 ($P \leq 0.05$). Error bars are the standard deviation (StD).

Results and discussion

Plant biomass production and water content

The impacts of different levels of ZnO-NPs on shoot/root length (SL/Rl), plant fresh weight (PFW), shoot/root fresh weight (SFW/RFW) ratio, shoot/root water content (SWC/RWC), anthocyanin, H_2O_2 and peroxidase (POD) of *Carum copticum* seedlings compared to the control (0.2 µM $ZnSO_4$) under hydroponic conditions are reported (Table 1). The results showed that the impact of different levels of zinc oxide nanoparticles on the measured parameter was significant

Table 1- The ANOVA results of the effects of different levels of zinc oxide nanoparticles on shoot length (SL), root length (RL), plant fresh weight (PFW), shoot/root fresh weight (SFW/RFW) ratio, shoot water content (SWC), root water content (RWC), anthocyanin (Antho), H2O2 and polyphenol oxidase (POD) of Carum copticum seedlings compared with the control (0.2 µM ZnSO4) grown under hydroponic condition.

		Means square								
Source of variation	**df**	**LS**	**LR**	**PFW**	**FWS/FWR**	**SWC**	**RWC**	**H_2O_2**	**Antho**	**POD**
Treatment	5	37.32**	43.90**	0.129**	5.037**	0.027**	0.019**	0.021**	4599.0**	0.048**
Error	12	4.18	2.33	0.002	0.213	0.001	0.001	0.001	53.1	0.009

** respectively significant at 1 percent probability

Table 2. The effect of ZnO nanoparticles (NPs) treatments on shoot length (SL), root length (RL), plant fresh weight (PFW), shoot/root fresh weight (SFW/RFW) ratio, shoot water content (SWC) and root water content (RWC) of *Carum copticum* seedlings compared with the control (0.2 µM $ZnSO_4$) grown under hydroponic condition.

Treatments (µM)	**SL (cm)**	**RL (cm)**	**PFW (g)**	**SFW / RFW**	**SWC (%)**	**RWC (%)**
Control	16.09 ± 1.57^{a}	20.01 ± 2.03^{ab}	0.336 ± 0.058^{c}	5.22 ± 0.79^{ab}	47.5 ± 2.6^{b}	66.8 ± 3.2^{b}
0.1	19.66 ± 1.81^{a}	18.04 ± 0.56^{b}	0.662 ± 0.034^{ab}	4.70 ± 0.42^{bc}	73.2 ± 2.8^{a}	83.8 ± 3.4^{a}
0.2	17.09 ± 2.16^{a}	21.52 ± 1.71^{a}	0.724 ± 0.069^{a}	5.71 ± 0.29^{a}	70.1 ± 0.8^{a}	83.3 ± 2.0^{a}
1	19.95 ± 1.50^{a}	21.53 ± 1.97^{a}	0.635 ± 0.051^{b}	4.03 ± 0.36^{c}	76.4 ± 1.1^{a}	87.3 ± 1.7^{a}
5	17.83 ± 3.41^{a}	19.08 ± 1.13^{ab}	0.616 ± 0.018^{b}	4.91 ± 0.45^{ab}	74.4 ± 1.5^{a}	83.7 ± 1.8^{a}
25	10.28 ± 0.91^{b}	11.28 ± 1.22^{c}	0.208 ± 0.023^{d}	2.05 ± 0.23^{d}	70.7 ± 1.9^{a}	81.8 ± 3.8^{a}

The values presented are the mean of three independent experiments and different letters indicate significant differences among treatments in each parameter, at $p < 0.05$ according to the Duncan's test.

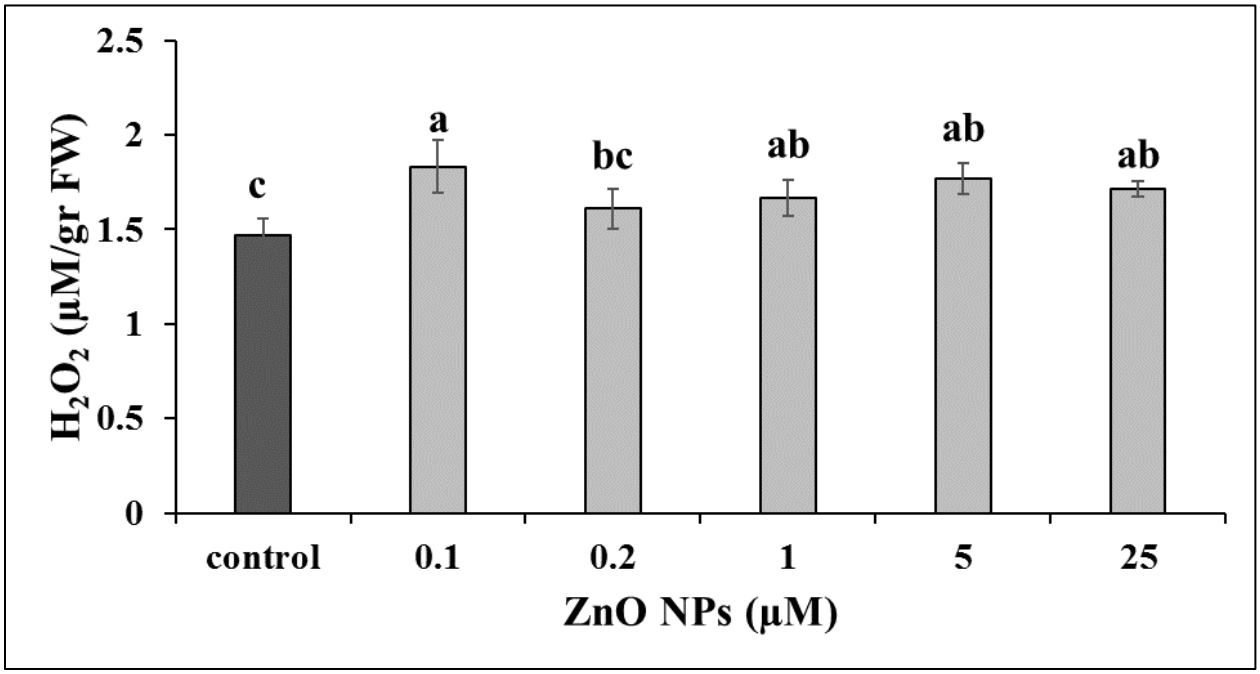

Figure 1. Mean comparison of the effects of different levels of zinc oxide nanoparticles on H_2O_2 content of Carum copticum seedlings compared with the control (0.2 µM ZnSO4) grown under hydroponic condition. The values presented are the average of three

at the 1% level. In this study, the ZnO-NPs treatments at 25 µM negatively affected almost all the growth parameters studied compared to the control. Thus, this level of ZnO-NPs could indicate a toxicity threshold for *Carum copticum* seedlings (Table 2). Exceptionally, the 25µM ZnO-NPs decreased shoot and root length by 36 and 44% compared with the control, respectively. The plant FW increased 97, 115, 89 and 83% in 0.1, 0.2, 1 and 5 µM, respectively but decreased 38% in 25 µM of ZnO-NPs treatments (Table 2). Shoot FW to root FW ratio decreased in 5 and 25 µM of NPs (Table 2). All levels of ZnO-NPs treatments increased the shoot and root water content compared to the control. These parameters were increased by 39-51% in shoot and 26-34% in root compared with control. The lowest and highest water contents reported at control and 1 µM ZnO-NPs groups, respectively (Table 2). There were conflicting results about the effects of NPs on plant growth among various studies. The shoots of ryegrass grown in hydroponic conditions were reduced when treated with 1000 mg/L (about 12.3 mM) of ZnO-NPs. While this concentration caused spot damage in the roots (Lin and Xing 2008). On the other hand, the 50 mg L^{-1} ZnO-NPs improved plant biomass in *Triticum aestivum*. The ZnO-NPs at 20 and 50 mg L^{-1} significantly increased lateral roots by 19 and 32%, respectively (Awasthi et al. 2017). It has been reported that ZnO-NPs lead to higher bioavailability of Zn^{2+} in tobacco callus cultures and show lower metal toxicity compared to non-nano ZnO (ZnO bulk) (Mazaheri-Tirani and Dayani 2020). Zinc regulated auxin bio-synthesis by tryptophan synthetase, and improved in plant cells expansion and growth and development in plant (Begum et al. 2016; Castillo-González et al. 2018). Changes in growth indices may likely due to alteration in the plant growth regulations.

Oxidant and antioxidant system parameters

The oxidative stress under high zinc concertation is due to imbalanced generation and removal of hydrogen peroxide (H_2O_2) in the intracellular cell (Huang et al. 2023; Sachdev et al. 2021; Nadarajah 2020). All levels of ZnO-NPs (except 0.2 µM) significantly increase H_2O_2 content (Figure 1). The H_2O_2 contents were recorded at 0.1, 1, 5 and 25 µM ZnO-NPs by 24, 13, 20 and 17.0% increase compared to the control, respectively. H_2O_2 content showed positive correlation with SWC (r = 0.680) and RWC (r = 0.672) treatments (Table 3). Any stimulant that increased ROS and upset the redox balance; resulted in increased oxidative stress. The oxidative explosion due to increased H_2O_2 has led to cell death (Hasanuzzaman et al. 2020; Nadarajah 2020). Plants enzyme activity (e.g. POX, POD and SOD) non-enzymatic (e.g. phenolic compound and anthocyanin) mechanisms to dampen the NPs toxic impacts by oxidative stress via ROS-scavenging (Ghosh et al. 2016; Hasanuzzaman et al. 2020; Mazaheri-Tirani et al. 2019). The 25 µM ZnO-NPs declined the oxidative stress (H_2O_2) in *Cicer arietinum* L. plant under chromium toxicity (Singh et al. 2024). Under ZnO-NPs treatment, only 25 µM treatment decreased anthocyanin content by 27.4%, but the 0.1, 1 and 5 µM ZnO-NPs treatments were increased by 130.6 58.1 and 56.9% compared to control, respectively (Figure 2). The anthocyanin content showed positive correlations with shoot length (r = 0.762) and plant FW (r = 0.681) (Table 3). The POD enzyme activity showed an increase at 5 and 25 µM nanoparticle treatments by 15.4 and 69.3%, while 1 µM ZnO-NPs decreased it about 30.9% compared with the control, respectively. The others NPs levels didn't meaningfully change the POD enzyme activity compared to the control (Figure 3). The POD enzyme activity showed negative correlations with shoot length (r = -0.790), root length (r = -0.829) and plant FW (r = -0.682) treatments (Table 3). Figure 4 shows the summary of the effects of different levels of zinc oxide nanoparticles on *C. copticum* seedlings under hydroponic condition. Various abiotic and biotic stresses increased H_2O_2 significantly. It was reported that the antioxidant system plays important role in supporting plants against negative impacts induced by ZnO toxicity (Mazaheri-Tirani et al. 2019). Singh et al. (2024) and Pullagurala et al. (2018) reported improved antioxidant defense system

Table 3- Correlation matrices showing relationships between measured parameters of *C. copticum* seedlings grown in hydroponic under ZnO nanoparticles (NPs) treatment. SL: shoot length; RL: root length; PFW: plant fresh weight; SWC: shoot water content; RWC: root water content; Antho: Anthocyanin; H_2O_2: Hydrogen peroxide; POD: Polyphenol oxidase.

	LS	LR	Plant FW	WC shoot	WC root	H_2O_2	Antho
LR	0.794**						
Plant FW	0.769**	0.699**					
WC shoot	0.242	-0.013	0.498*				
WC root	0.216	0.006	0.508*	0.952**			
H_2O_2	0.052	-0.316	0.262	0.680**	0.672**		
Antho	0.762**	0.329	0.681**	0.402	0.353	0.451	
POD	-0.790**	-0.829**	-0.682**	-0.038	-0.08	0.145	-0.564*

*and** respectively significant at 1 and 5 percent probability.

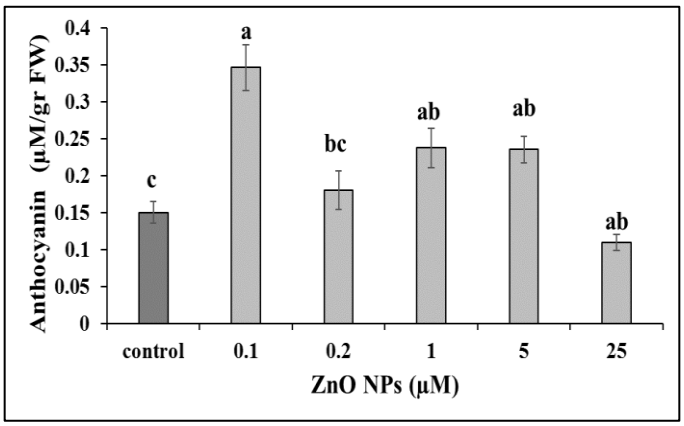

Figure 2. Mean comparison of the effects of different levels of zinc oxide nanoparticles on anthocyanin content of *C. copticum* seedlings compared with the control (0.2 µM $ZnSO_4$) grown under hydroponic condition. The values presented are the average of three independent experiments and different letters indicate significant differences between treatments in each parameter, at $p < 0.05$ according to Duncan's range test.

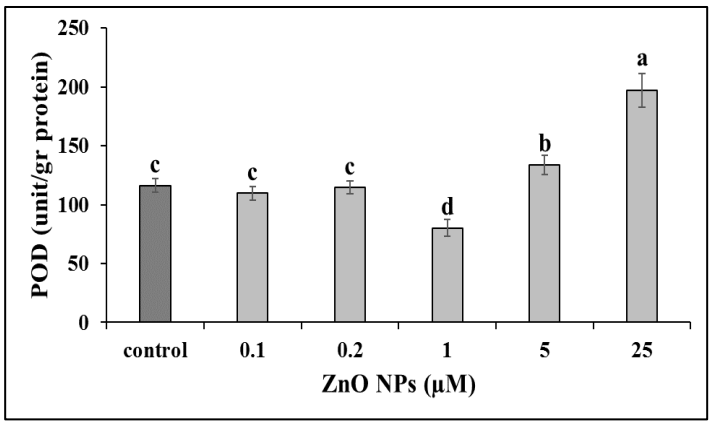

Figure 3. Mean comparison of the effects of different levels of zinc *to* oxide nanoparticles on POD enzyme activity of *C. copticum* seedlings compared with the control (0.2 µM $ZnSO_4$) grown under hydroponic condition. The values presented are the average of three independent experiments and different letters indicate significant differences between treatments in each parameter, at $p < 0.05$ according to Duncan's range test.

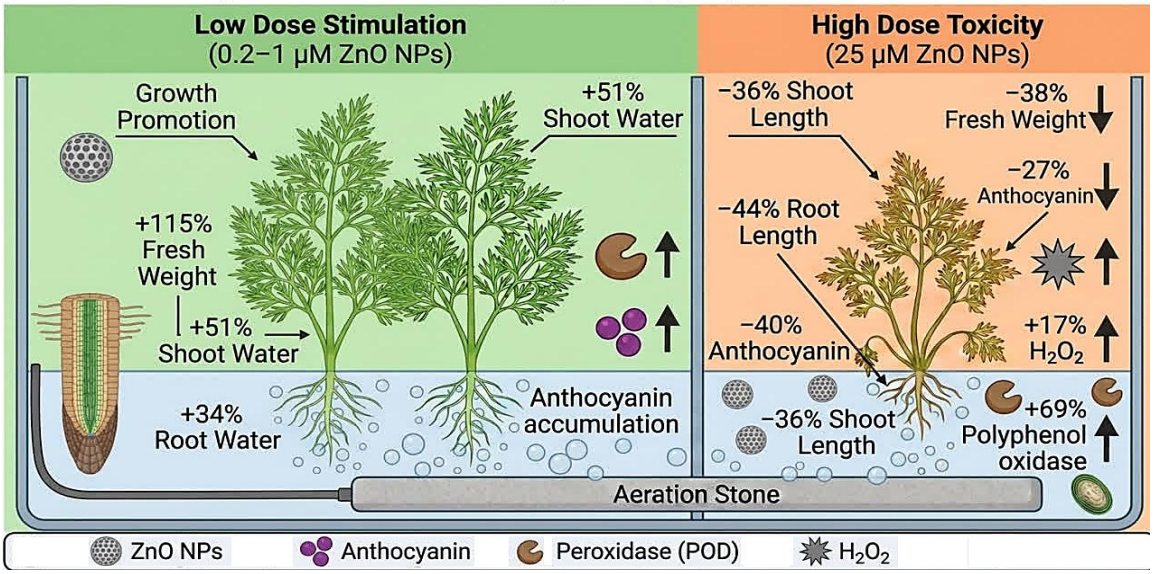

Figure 4. The summary of the effects of different levels of zinc oxide nanoparticles on C. copticum seedlings under hydroponic condition.

with ZnO-NPs treatment on C*icer arietinum* and *Coriandrum sativum*, respectively. It could be the result of the genes expression (Nair and Chung 2017) or the activity of different oxidative processes (Mazaheri-Tirani et al. 2019; Li et al. 2016). The alteration in anthocyanin (major phenolic class) content was compensated with modification in phenolic compounds at ZnO-NPs treatment. Therefore, ZnO-NPs might affect the phenolic compound's biosynthesis pathways. Plants' response to stresses might vary depending on the plant species, forms and intensity of stress, and other environmental parameters (e.g. temperature, media pH and culture substrate).

Conclusion

This study demonstrated that ZnO-NPs increased POD activity but H_2O_2 content in *Carum copticum* seedling under hydroponic (water-based; without solid culture medium) conditions, thus leading to increased H_2O_2 and induction of oxidative stress. Plants of response to ZnO-NPs had reported to be dose-dependent. Important oxidative stress biomarkers, such as H_2O_2 generation, were different under ZnO-NPs concentrations in these plants. It should be noted that due to its thin stem and fragile root systems, *Carum copticum* plant is not suitable for cultivation in a hydroponic (water-base) system.

Conflict of Interest

The authors have not declared any conflict of interests.

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Abscisic acid attenuates pentylenetetrazole-induced seizures and oxidative stress in adult male rats: evidence for GABAergic involvement

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ABSTRACT

Epilepsy is a common neurological disorder, and many patients remain resistant to available antiepileptic drugs. This study investigated the effects of intracerebroventricular (ICV) administration of abscisic acid (ABA), a plant-derived compound with antioxidant and anti-inflammatory properties, in a pentylenetetrazole (PTZ)-induced seizure model. Fifty-six adult male rats were divided into eight groups: Control, Sham + PTZ, PTZ, diazepam + PTZ, three ABA-treated groups (2.5, 5, and 10 µg/rat for five days), and a bicuculline + ABA + PTZ group to assess GABAergic involvement. Seizure severity was assessed using Racine's scale, anxiety-like behavior by the elevated plus maze (EPM) and open field tests (OFT), and hippocampal oxidative stress markers, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), using ELISA. PTZ induced severe seizures, increased anxiety-like behaviors, elevated MDA levels, and reduced SOD activity (all $p < 0.001$). ABA, particularly at 10 µg/rat, attenuated seizure severity, prolonged seizure latency, improved anxiety-like behaviors, decreased MDA levels, and restored SOD and CAT activities, with the greatest effects observed at 10 µg/rat. Bicuculline partially attenuated the anticonvulsant and antioxidant effects of ABA, indicating the involvement of GABAergic signaling. ABA exerts anticonvulsant, anxiolytic, and antioxidant effects in PTZ-induced seizure, at least partly through modulation of the GABAergic system, suggesting its potential as a therapeutic candidate for epilepsy.

Keywords: Abscisic Acid, Seizure, Pentylenetetrazole, Oxidative Stress, GABA

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Introduction

Epilepsy is a chronic neurological condition marked by recurrent, unprovoked seizures that arise from abnormal, excessive, and synchronized electrical discharges within the brain. Affecting nearly 50 million individuals across the globe, it represents one of the most common neurological disorders and continues to impose a substantial burden on public health systems worldwide (Kosenkov et al., 2025; Ji et al., 2025a).

In addition to recurrent seizures, individuals with epilepsy frequently experience psychiatric comorbidities, particularly anxiety and depression, which significantly affecting their quality of life (Lima et al., 2021, Mula et al., 2021, Kwong et al., 2016, Piazzini et al., 2008). Anxiety is one of the most common psychiatric comorbidities in patients with epilepsy, with a prevalence up to three times higher than that in the general population (Gavrilovic et al., 2024). The bidirectional relationship between epilepsy and anxiety is complex and is thought to involve several overlapping neurobiological mechanisms, including GABAergic dysfunction, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, chronic neuroinflammation, and oxidative stress (Basu et al., 2021, Suleymanova, 2021, Zhu et al., 2018, Peek et al., 2024).

Pentylenetetrazole (PTZ) is a widely used chemoconvulsant agent that reliably induces seizures by acting as a noncompetitive antagonist of GABA_A receptors, thereby disrupting inhibitory neurotransmission and increasing neuronal excitability (Monteiro et al., 2024). Accordingly, the PTZ model has become a well-established experimental platform for investigating seizure mechanisms and evaluating potential anticonvulsant therapies, as well as seizure-associated behavioral abnormalities.

Seizure activity induces mitochondrial dysfunction and excessive production of reactive oxygen species (ROS), leading to disruption of cellular homeostasis and neuronal damage (Pearson-Smith and Patel, 2017). Elevated MDA levels, together with the decreased activities of SOD, glutathione peroxidase (GPx), and reduced glutathione (GSH), have been consistently reported in both experimental models and patients with epilepsy. Furthermore, seizures stimulate the release of pro-inflammatory cytokines, creating a vicious cycle of oxidative stress and inflammation that further exacerbates seizure severity (Aguiar et al., 2012, Puttachary et al., 2015). Therefore, targeting oxidative stress and neuroinflammation represents a promising therapeutic strategy for epilepsy.

Given the central role of oxidative stress and inflammation in epileptogenesis, antioxidant and anti-inflammatory approaches have attracted increasing attention as potential adjunctive or alternative therapies. However, currently available antiepileptic drugs primarily modulate ion channels or synaptic transmission and often fail to prevent epileptogenesis or alleviate the cognitive and emotional comorbidities associated with epilepsy. Moreover, approximately one-third of patients remain resistant to pharmacological treatment (Löscher et al., 2020). These limitations highlight the need for novel therapeutic agents with multimodal mechanisms of action that target both seizure activity and its associated neuropsychiatric complications.

ABA is a plant-derived phytohormone traditionally recognized for its role in regulating plant stress responses. In both plant and

mammalian tissues, its biological effects are mediated, at least in part, through activation of Lanthionine synthetase C-like protein 2 (LANCL2) and peroxisome proliferator-activated receptors (PPARs) (Guri et al., 2010, Sturla et al., 2009). Recently, ABA has attracted considerable attention as a potential therapeutic agent for several diseases in mammalian systems (Gharib et al., 2024). ABA is also endogenously present in mammals and has demonstrated beneficial effects in experimental models of neuropsychiatric and neurodegenerative disorders. Central administration of ABA has been shown to alleviate anxiety- and depression-like behaviors in rodents exposed to stress (Shabani et al., 2022). In addition, ABA improves spatial learning, memory, and anxiety-like behaviors (Naderi et al., 2017). Several studies have reported that ABA enhances antioxidant defense by increasing the activities of antioxidant enzymes, including SOD and GSH, while reducing ROS production and lipid peroxidation in rats (Soti et al., 2019, Jiang and Zhang, 2001, Bellaire et al., 2000, Celik et al., 2007). Furthermore, accumulating evidence indicates that ABA exerts potent anti-inflammatory and analgesic effects (Ahmadi et al., 2025, Bassaganya-Riera et al., 2010).

Despite significant advances in understanding the pathophysiology of epilepsy and the development of numerous antiepileptic drugs, there remains an urgent need for novel therapeutic strategies with greater efficacy and fewer adverse effects. We hypothesized that central administration of ABA would protect against PTZ-induced seizures by reducing oxidative stress and improving anxiety-like behaviors. Therefore, this study evaluated the anticonvulsant effects of ABA by investigating: its efficacy against PTZ-induced seizures, effects on seizure-associated behavioral alterations, its antioxidant capacity in the hippocampus, and finally the involvement of GABAergic neurotransmission using pharmacological antagonism. Although the antioxidant and anti-inflammatory properties of ABA have been demonstrated in several neurological disorders, its interaction with GABAergic signaling during PTZ-induced seizures remains largely unexplored.

Materials and Methods

Animals and Housing

Fifty-six adult male Wistar rats (weighing 250–270 g) were obtained from the Animal House of Kerman University of Medical Sciences (KUMS), Iran. Animals were housed in groups of four per cage under controlled environmental conditions (22 ± 2 ˚C, 50–60% humidity, 12 h light/dark cycle), with free access to standard laboratory chow and tap water. All experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Committee of Shahid Bahonar University of Kerman (IR.UK.VETMED.REC.1403.012).

Experimental Design and Grouping

Animals were randomly divided into eight experimental groups (n = 7/group). The sample size of seven animals per group was justified based on previous published studies and pilot experiments conducted in our laboratory evaluating anticonvulsant effects in acute PTZ-induced seizure models (Zamyad et al., 2019). The groups were designated as follows:

- Control group: Intact animals with no surgical intervention and no drug administration
- PTZ group: Animals receiving PTZ (50 mg/kg, i.p.) without any surgical intervention
- Sham + PTZ group: Animals undergoing complete stereotaxic surgery and ICV injection of vehicle (DMSO + saline, in a ratio of 2:1) followed by PTZ administration (50 mg/kg, i.p.)
- Diazepam + PTZ group: Animals receiving diazepam (2 mg/rat, i.p.) administered 20 minutes before PTZ
- ABA (2.5 µg/rat) + PTZ group: Animals receiving ICV ABA at 2.5 µg/rat once daily for 5 days, followed by PTZ.
- ABA (5 µg/rat) + PTZ group: Animals receiving ICV ABA at 5 µg/rat once daily for 5 days, followed by PTZ.
- ABA (10 µg/rat) + PTZ group: Animals receiving ICV ABA at 10 µg/rat once daily for 5 days, followed by PTZ.
- Bicuculline + ABA (10 µg/rat) + PTZ group: Animals receiving ICV bicuculline (6 µg/rat) 30 min prior to ABA (10 µg/rat, ICV), followed by PTZ

In addition, treatment administration was performed by a separate investigator not involved in outcome assessment. All behavioral assessments (seizure scoring, EPM, OFT) were performed by investigators blinded to treatment allocation. Biochemical sample processing and analysis were conducted by laboratory personnel unaware of group assignments. Unblinding occurred only after completion of all statistical analyses. A schematic representation of the experimental design is provided in Figure 1.

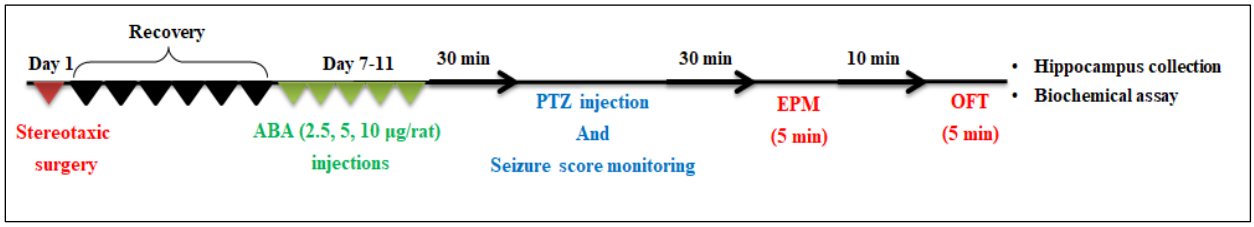

Figure 1. Schematic representation of experimental groups and treatment timeline.

Stereotaxic Surgery and Drug Administration

Rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) combined with xylazine (3 mg/kg) and mounted in a stereotaxic frame (Stoelting Co., USA). Bilateral 22-gauge guide cannulae were stereotaxically implanted into the lateral ventricles according to the Paxinos and Watson rat brain atlas, using the following coordinates relative to bregma: AP, −1.6 mm; ML, ±0.8 mm; and DV, −3.4 mm. The cannulae were fixed in place with stainless-steel screws and dental acrylic cement. Following surgery, the animals were allowed to recover for seven days before the experiments.

For intracerebroventricular administration, ABA was dissolved in DMSO and delivered in a total volume of 3 µL through a Hamilton microsyringe once daily for five consecutive days. Thirty minutes after the final administration of ABA or its vehicle (DMSO + saline, ratio 2:1), seizures were induced by intraperitoneal injection of pentylenetetrazol (PTZ; 50 mg/kg). Bicuculline was dissolved in normal saline and the final injection volume was considered 1 µL per side per rat. The injection volume for PTZ and diazepam were 1 mL/kg.

Seizure Induction and Behavioral Assessment

Seizures were induced with a single i.p. injection of PTZ (50 mg/kg), administered 30 minutes after the final treatment. Each rat was placed in an individual transparent Plexiglas chamber (30 × 30 × 30 cm), and behavior was video-recorded for 40 minutes. Seizure severity was scored using the Racine scale as follows: Stage 0: No response, Stage 1: Facial automatisms, Stage 2: Myoclonic jerks, Stage 3: Forelimb clonus, Stage 4: Rearing and Stage 5: Rearing and falling (generalized tonic–clonic seizures). In this study, latency to seizure onset and the duration of myoclonic, tonic, and tonic-clonic seizures were recorded for each animal.

Anxiety-Like Behavioral Tests

Elevated Plus Maze (EPM)

Anxiety-like behavior was assessed using the Elevated Plus Maze (EPM) test, conducted 30 minutes after PTZ administration. The EPM apparatus comprised two opposing open arms (50 × 10 cm) and two opposing closed arms (50 × 10 × 40 cm), positioned 54 cm above the floor. At the start of each trial, each rat was placed in the central platform facing one of the open arms and allowed to explore the maze freely for 5 minutes. Behavioral activity was recorded using an overhead video camera, and the time spent in the open arms and the number of open-arm entries were quantified during subsequent video analysis. To eliminate odor cues, the maze was thoroughly cleaned with 70% ethanol between successive trials (Shahsavari et al., 2020).

Open Field Test (OFT)

The OFT was conducted in a square arena (60 × 60 × 50 cm) with the floor divided into 16 equal squares, including a central zone. Each rat was placed in one corner and allowed to explore for 5 minutes. Time spent in the central area and the number of entries was measured using a video tracking system. Testing occurred under standardized light and noise conditions (Shahsavari et al., 2020).

Biochemical Analysis of Oxidative Stress Markers

At the end of behavioral testing, animals were euthanized using CO_2 inhalation. Brains were rapidly removed, and the hippocampi were dissected on ice and stored at −80 °C until analysis. Approximately 25 mg of tissue was homogenized in cold PBS and centrifuged at 1,200 ×g for 15 minutes at 4 °C. The supernatants were stored at −80 °C.

Oxidative stress parameters were evaluated using commercially available colorimetric assay kits from Carmania Pars Gene Company (Kerman, Iran). Hippocampal MDA, CAT, and SOD levels were measured according to the manufacturer's instructions using an ELISA reader. MDA levels were determined using the Lipid Peroxidation Assay Kit (Catalog No. KPG-Nob) based on the thiobarbituric acid reactive substances (TBARS) method. Serum samples (100 µL) were incubated with TBA reagent (200 µL) at 95°C for 60 minutes, centrifuged at 3000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 532 nm. MDA concentration was calculated from a standard curve and expressed as µmol/L. CAT activity was measured using the Catalase Activity Assay Kit (Catalog No. KPG-CAT) based on colorimetric detection of residual H_2O_2 at 570 nm.

Absorbance was measured and catalase activity calculated using the formula. SOD activity was determined using the Superoxide Dismutase Assay Kit (Catalog No. KPG-SOD) based on the inhibition of pyrogallol auto-oxidation method at 420 nm. The inhibition percentage was calculated as: Inhibition (%) = [1 – (A2sample – A1sample) ÷ (A2standard – A1standard)] × 100, and SOD activity was expressed as: SOD activity (U/mL) = Inhibition (%) × 0.42.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison post hoc test. All analyses were performed with GraphPad Prism software (version 10). A p-value < 0.05 was considered statistically significant.

Results

Effects of ABA on PTZ-Induced Seizure Parameters

Seizure Severity

Administration of PTZ induced a characteristic progression of seizure behaviors in adult male rats, encompassing myoclonic jerks through generalized tonic–clonic convulsions. One-way ANOVA demonstrated significant treatment effects on the duration of myoclonic ($F(2.299, 13.80) = 27.63$, $P<0.0001$), tonic ($F(2.669, 16.02) = 19.40$, $P<0.0001$), and tonic–clonic ($F(2.620, 15.72) = 24.91$, $P<0.0001$) seizure episodes. Post hoc analysis using Tukey's test revealed that pretreatment with ABA at the highest dose (10 µg/rat) attenuated significant anticonvulsant protection, as evidenced by substantial reductions in the duration of myoclonic ($P=0.001$), tonic ($P=0.01$), and tonic–clonic ($P=0.002$) episodes relative to the PTZ control group. Furthermore, the anticonvulsant efficacy of ABA at 10 µg/rat was dose-dependent, demonstrating significantly greater protective effects compared to lower doses of ABA (2.5 and 5 µg/rat) ($P<0.05$, $P<0.01$, and $P<0.001$). Notably, co-administration of bicuculline, a competitive GABA_A receptor antagonist, significantly attenuated the anticonvulsant effects of ABA (10 µg/rat), resulting in increased seizure behaviors compared to both ABA (10 µg/rat) and diazepam treatment groups ($P<0.05$, $P<0.01$) (Figure 2).

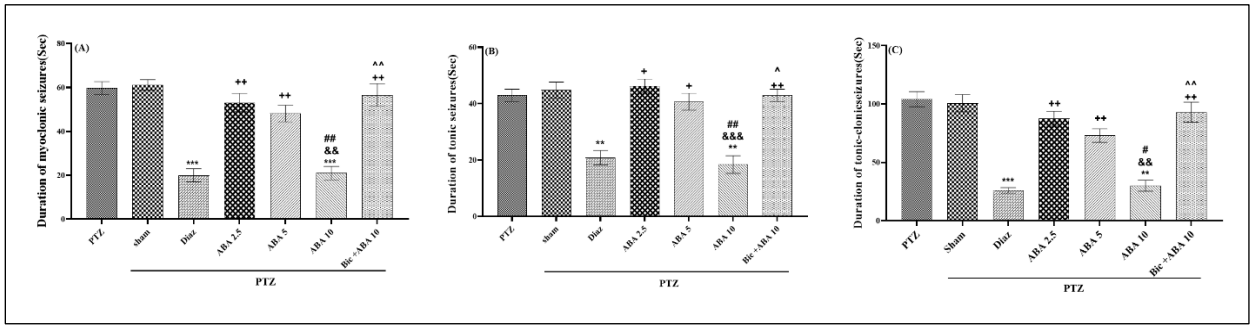

Figure 2. Duration of myoclonic (A), tonic (B), and tonic–clonic (C) seizures across treatment groups. Data are presented as mean ± SEM (n = 7). PTZ: pentylenetetrazole, Diaz: Diazepam, ABA: Abscisic acid, and Bic: Bicuculline. $^{**}p<0.01$ and $^{***}p<0.001$ *vs. Sham and PTZ groups,* $^{+}p<0.05$ and $^{++}p<0.01$ *vs. Diaz group,* $^{\hat{}}p<0.05$ and $^{\hat{}\hat{}}p<0.01$ *vs. ABA 10 group,* $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs. ABA 5 group,* $^{\&\&}p<0.01$ and $^{\&\&\&}p<0.001$ *vs. ABA 2.5 group.*

Latency to Seizure Onset and Total Seizure Duration

One-way ANOVA indicated significant between-group differences in latency to first seizure ($F(2.312, 13.87) = 25.30$, $p < 0.001$) and cumulative seizure duration ($F(3.229, 19.38) = 19.36$, $p < 0.001$). Post hoc comparisons revealed that animals receiving PTZ alone exhibited rapid seizure onset with a mean latency of less than 200 seconds. In contrast, pretreatment with either diazepam or ABA at 10 µg/rat significantly prolonged seizure latency compared to the PTZ group ($p = 0.007$ and $p = 0.008$, respectively). This protective effect was reduced by bicuculline pretreatment, which significantly shortened seizure latency relative to both diazepam ($p = 0.004$) and ABA 10 µg/rat ($p = 0.005$) groups (Figure 3A).

Consistent with these findings, total seizure duration was significantly attenuated in animals treated with ABA 10 µg/rat or diazepam compared to PTZ controls ($p = 0.021$). Conversely, bicuculline pretreatment significantly prolonged total seizure duration relative to both diazepam ($p = 0.003$) and ABA 10 µg/rat ($p = 0.004$) treatments (Figure 3B).

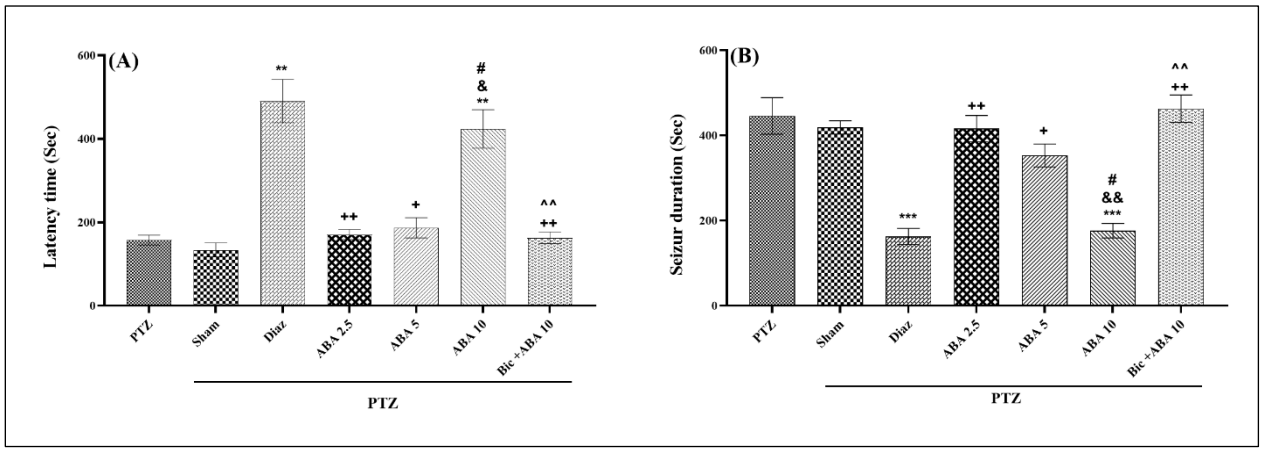

Figure 3. Seizure onset latency (A) and total seizure duration (B) across treatment groups. Data are mean ± SEM (n = 7). PTZ: pentylenetetrazole, Diaz: Diazepam, ABA: Abscisic acid and Bic: Bicuculline. $^{**}p < 0.01$ *and* $^{***}p < 0.001$ *vs. Sham and PTZ groups,* $^{+}p < 0.05$ *and* $^{++}p < 0.01$ *vs. Diaz group,* $^{\wedge\wedge}p < 0.01$ *vs. ABA 10 group,* $^{\#}p < 0.05$ *vs. ABA 5 group,* $^{\&}p < 0.05$ *and* $^{\&\&}p < 0.01$ *vs. ABA 2.5 group.*

Effects of ABA on Anxiety-Like Behavior in the Elevated Plus Maze

One-way ANOVA revealed significant treatment effects on time spent in open arms ($F(2.673, 16.04) = 14.42$, $p < 0.001$) and the frequency of open-arm entries ($F(3.376, 20.25) = 14.92$, $p < 0.001$). Post hoc analysis demonstrated that PTZ administration induced a pronounced anxiogenic response, characterized by a significant reduction in time spent exploring the open arms compared to control animals ($p = 0.004$). Although ABA at 10 µg/rat produced a notable increase in open-arm time, demonstrating an anxiolytic profile comparable to diazepam, this effect did not reach statistical significance when compared to either PTZ or sham-operated groups ($p = 0.561$). Bicuculline pretreatment reduced the anxiolytic tendency of ABA, significantly reducing open-arm time compared to both ABA 10 µg/rat and diazepam groups ($p < 0.05$) (Figure 4A).

A parallel pattern emerged in the analysis of open-arm entries. Post hoc testing indicated that PTZ significantly reduced the number of entries relative to controls ($p = 0.025$), whereas ABA at 10 µg/rat significantly increased entries compared to PTZ ($p = 0.0003$), ABA 2.5 µg/rat ($p = 0.0025$), and sham groups ($p = 0.017$). However, bicuculline administration significantly diminished open-arm entries relative to both control ($p = 0.011$) and ABA 10 µg/rat ($p = 0.0151$) groups (Figure 4B).

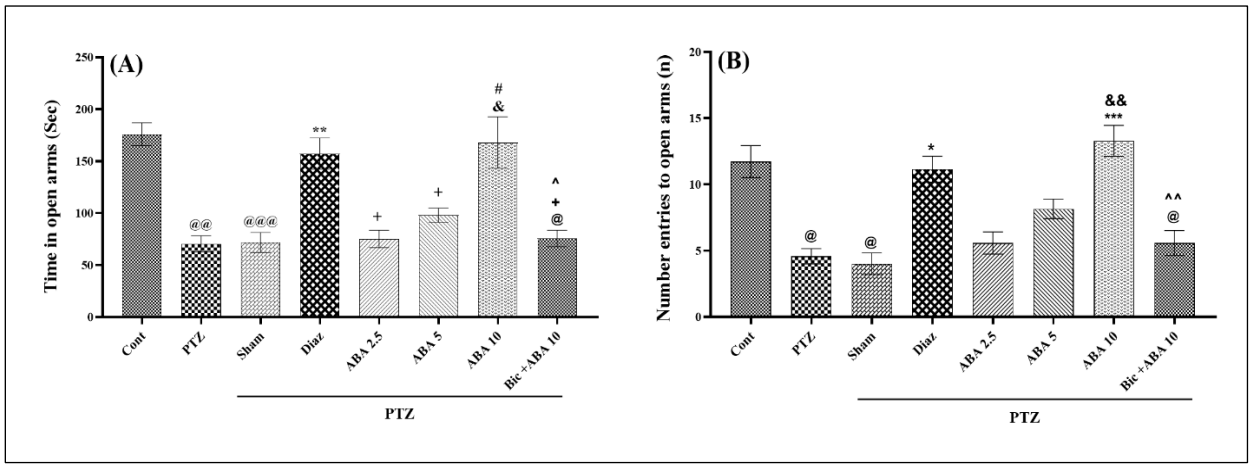

Figure 4. Time spent (A) and number of entries (B) into open arms in the EPM. Data are mean ± SEM (n = 7). Cont: Control, PTZ: pentylenetetrazole, Diaz: Diazepam, ABA: Abscisic acid and Bic: Bicuculline. *$^{@}p < 0.05$, $^{@@@}p < 0.001$ vs. Cont group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Sham and PTZ groups, $^{+}p < 0.05$ vs. Diaz group, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ vs. ABA 10 group, $^{\#}p < 0.05$ vs. ABA 5 group, $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. ABA 2.5 group.*

Effects of ABA on Exploratory Behavior in the Open Field Test

In the OFT paradigm, one-way ANOVA demonstrated significant between-group differences in time spent in the central zone ($F(2.299, 13.80) = 27.63$, $p < 0.0001$) and the number of center entries ($F(2.978, 17.87) = 16.43$, $p < 0.0001$). Post hoc comparisons revealed that PTZ-treated animals exhibited significantly reduced central zone exploration time compared to controls ($p = 0.021$), indicative of heightened anxiety-like behavior. Conversely, ABA at 10 µg/rat significantly increased center zone duration relative to PTZ ($p = 0.001$), sham ($p = 0.003$), and ABA 5 µg/rat ($p = 0.027$) groups. This anxiolytic-like effect was reduced by bicuculline, which significantly decreased central zone time compared to both ABA 10 µg/rat ($p = 0.021$) and diazepam ($p = 0.010$) treatments (Figure 5A).

Similarly, analysis of center zone entries revealed that PTZ administration significantly reduced the frequency of entries compared to controls ($p = 0.021$). Both diazepam ($p = 0.006$) and ABA 10 µg/rat ($p = 0.001$) significantly increased center entries relative to the PTZ group. Bicuculline decreased this effect, significantly reducing center entries compared to control ($p = 0.046$), diazepam ($p = 0.01$), and ABA 10 µg/rat ($p = 0.021$) groups (Figure 5B).

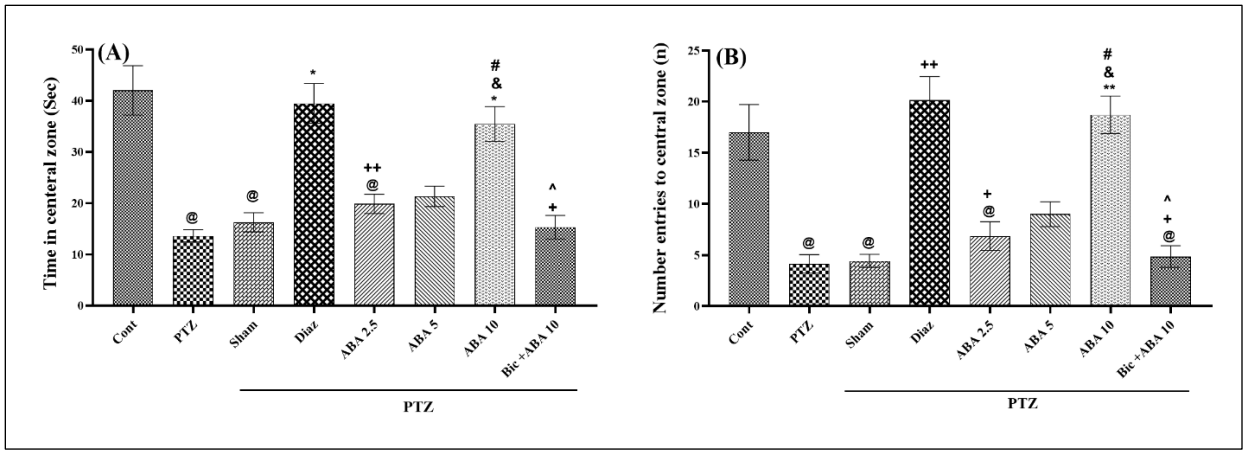

Figure 5. (A) Time spent and (B) entries into the central zone of the OFT. Data are mean ± SEM (n = 7). Cont: Control, PTZ: pentylenetetrazole, Diaz: Diazepam, ABA: Abscisic acid and Bic: Bicuculline. $^{@}p<0.05$ vs. Cont group, $^{*}p<0.05$ and $^{**}p<0.01$ vs. Sham and PTZ groups, $^{+}p<0.05$ and $^{++}p<0.01$ vs. Diaz group, $^{\wedge}p<0.05$ vs. ABA 10 group, $^{\#}p<0.05$ vs. ABA 5 group, $^{\&}p<0.05$ vs. ABA 2.5 group.

Effects of ABA on Hippocampal Oxidative Stress Markers

One-way ANOVA revealed significant treatment effects on hippocampal oxidative stress biomarkers, including CAT ($F(7, 32) = 9.623$, $P<0.0001$), SOD ($F(3.133, 12.53) = 29.73$, $P<0.0001$), and MDA ($F(2.618, 10.47) = 20.50$, $P<0.0001$). Post hoc analysis demonstrated that ABA treatment significantly modulated the hippocampal antioxidant defense system. Specifically, CAT activity was significantly elevated in animals receiving ABA at 10 µg/rat compared to both PTZ ($P=0.005$) and sham-operated ($P=0.030$) groups. This enhancement was decreased by bicuculline, which significantly reduced CAT activity relative to both ABA 10 µg/rat ($P=0.003$) and diazepam ($P=0.015$) groups (Figure 6A).

Analysis of SOD activity revealed significant reductions in PTZ ($P=0.011$) and ABA 2.5 µg/rat ($P=0.013$) groups compared to controls, potentially reflecting seizure-induced oxidative burden. ABA at 10 µg/rat significantly enhanced SOD activity compared to PTZ ($P=0.007$), sham ($P<0.0001$), and ABA 2.5 µg/rat ($P=0.014$) groups. However, bicuculline pretreatment significantly attenuated SOD activity relative to both diazepam ($p=0.013$) and ABA 10 µg/rat ($p=0.014$) (Figure 6B).

Evaluation of MDA levels, a well-established marker of lipid peroxidation and oxidative damage, demonstrated significant elevations in both PTZ ($P=0.010$) and sham ($P=0.007$) groups compared to untreated controls. ABA at 10 µg/rat significantly attenuated hippocampal MDA accumulation relative to both PTZ ($P=0.042$) and sham ($P=0.014$) groups. This antioxidant effect was reduced by bicuculline, which markedly increased MDA levels compared to ABA 10 µg/rat ($P=0.013$) and diazepam ($P=0.010$) treatments (Figure 6C).

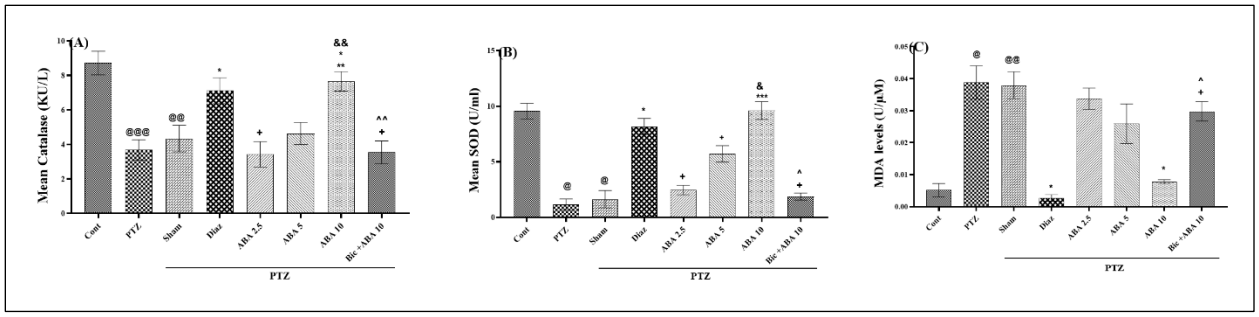

Figure 6. Hippocampal oxidative stress biomarkers: Catalase (CAT) (A), Superoxide dismutase (SOD) (B), and Malondialdehyde (MDA) (C). Data are expressed as mean ± SEM (n = 7). Cont: Control, PTZ: pentylenetetrazole, Diaz: Diazepam, ABA: Abscisic acid and Bic: Bicuculline. $^{@}p<0.05$, $^{@@}p<0.01$ and $^{@@@}p<0.001$ vs. Cont group, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Sham and PTZ groups, $^{+}p<0.05$ vs. Diaz group, $^{\hat{}}p<0.05$ and $^{\hat{}\hat{}}p<0.01$ vs. ABA 10 group, $^{\&}p<0.05$ and $^{\&\&}p<0.01$ vs. ABA 2.5 group.

Discussion

The current study demonstrates that central administration of ABA significantly attenuated PTZ-induced seizure activity while simultaneously improving anxiety-like behavior and restoring hippocampal antioxidant status. Specifically, ABA at 10 µg/rat prolonged seizure latency, reduced seizure duration, improved behavioral performance in the EPM test, decreased MDA levels, and increased the activities of the endogenous antioxidant enzymes SOD and CAT. Furthermore, the partial attenuation of these protective effects by bicuculline suggests that activation of GABAergic signaling contributes, at least in part, to the potential neuroprotective actions of ABA. Collectively, these findings indicate that ABA at 10 µg/rat produced the most pronounced effects. In addition, ABA may exert anticonvulsant effects through multiple interacting mechanisms rather than through modulation of a single molecular pathway (Monteiro et al., 2024, Gharib et al., 2024).

Epileptic seizures are characterized by excessive and synchronous neuronal firing resulting from an imbalance between excitatory and inhibitory neurotransmission. In the PTZ model, seizure generation primarily occurs through antagonism of GABA_A receptors, leading to reduced inhibitory tone and increased neuronal excitability (Psarropoulou et al., 1994). Therefore, the significant prolongation of seizure latency together with the reduction in seizure duration observed in the present study suggests that ABA at 10 µg/rat effectively attenuated PTZ-induced neuronal hyperexcitability. Because bicuculline markedly attenuated the protective effects of ABA, our findings further support the hypothesis that enhancement of GABAergic neurotransmission is involved in the anticonvulsant action of ABA. Although the present study was not designed to determine whether ABA directly interacts with GABA_A receptors or indirectly regulates GABAergic signaling, previous investigations have demonstrated that modulation of inhibitory neurotransmission represents one of the principal mechanisms underlying seizure suppression (Tipton and Russek, 2022). The present findings therefore provide evidence suggesting that GABAergic signaling may contribute to the anticonvulsant effects of ABA.

Interestingly, bicuculline did not completely abolish the anticonvulsant effects of ABA. This

observation indicates that additional signaling pathways are likely involved. Increasing evidence suggests that epilepsy is a multifactorial process involving oxidative stress, neuroinflammation, mitochondrial dysfunction, altered calcium homeostasis, excitotoxic glutamate release, and apoptotic signaling (Sumadewi et al., 2023, Xu et al., 2025, Sun et al., 2025, Ji et al., 2025). Consequently, compounds capable of simultaneously targeting several pathological mechanisms may provide greater therapeutic efficacy than agents acting exclusively on neuronal excitability. The present findings support this concept and suggest that ABA may act as a potential neuroprotective agent rather than as a conventional anticonvulsant drug.

Oxidative stress is increasingly recognized as one of the major contributors to both seizure initiation and seizure-induced neuronal injury. During epileptic seizures, excessive neuronal activity dramatically increases cerebral metabolic demand, resulting in mitochondrial dysfunction and overproduction of reactive oxygen species (ROS). These reactive molecules subsequently induce lipid peroxidation, protein oxidation, DNA damage, and impairment of endogenous antioxidant defense systems, thereby establishing a vicious cycle that further lowers seizure threshold and exacerbates neuronal injury (Pearson-Smith and Patel, 2017, Ji et al., 2025, Borowicz-Reutt and Czuczwar, 2020). Consistent with this mechanism, the present study demonstrated a marked elevation of MDA together with significant reductions in CAT and SOD activities following PTZ administration. ABA effectively reversed these biochemical alterations, indicating restoration of redox homeostasis within the hippocampus.

The antioxidant effects observed in the present study are in agreement with previous investigations demonstrating that ABA activates endogenous antioxidant pathways through LANCL2-mediated signaling and subsequent activation of peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent transcriptional mechanisms (Parajuli et al., 2024, Spinelli et al., 2021). However, while our study demonstrates significant antioxidant effects of ABA through direct measurement of oxidative stress markers (MDA, SOD, and CAT) and provides evidence for GABAergic pathway involvement via bicuculline antagonism experiments, we acknowledge that the underlying molecular mechanisms require cautious interpretation. Previous studies in other experimental models have suggested that ABA may exert its protective effects through activation of the LANCL2 receptor, PPARγ, and Nrf2 signaling pathways (Parajuli et al., 2024, Spinelli et al., 2021); however, these molecular targets were not directly measured in the present investigation. While existing literature suggests ABA may activate endogenous antioxidant pathways through these mechanisms, our findings remain hypothetical within our experimental model. Therefore, while the existing literature provides a theoretical framework suggesting that ABA may engage these pathways, the involvement of LANCL2, PPARγ, and Nrf2 in the anticonvulsant and antioxidant effects observed in the present study remains speculative and requires direct experimental validation in future studies. The GABAergic modulation demonstrated in our study through bicuculline antagonism represents the primary mechanistic insight directly

supported by our data, whereas the involvement of LANCL2, PPARγ, and Nrf2 pathways constitutes a plausible hypothesis derived from previous research that warrants future investigation. Further studies employing molecular techniques such as Western blotting, quantitative PCR, immunohistochemistry, or receptor binding assays are needed to directly investigate whether these proposed signaling cascades contribute to the neuroprotective and antioxidant effects of ABA observed in our PTZ-induced seizure model. We recognize this distinction between demonstrated findings and literature-based hypotheses as an important limitation that should guide the interpretation of our results and inform future mechanistic research.

Previous investigation also demonstrates activation of Nrf2 promotes transcription of numerous antioxidant enzymes, including SOD, CAT, glutathione peroxidase, and heme oxygenase-1, thereby enhancing cellular resistance against oxidative injury (Ngo and Duennwald, 2022, Sahu and Jain, 2025, Costa et al., 2026). Although these molecular pathways were not directly examined in the present study, the significant restoration of antioxidant enzyme activities strongly supports the hypothesis that activation of endogenous antioxidant defense mechanisms contributes substantially to the anticonvulsant properties of ABA.

Another important finding of the present study was the improvement in anxiety-like behavior following ABA administration. Moreover, significant effects were primarily observed with the 10 μg/rat dose. Anxiety is one of the most common neuropsychiatric comorbidities in patients with epilepsy and substantially reduces quality of life, even in individuals with adequate seizure control (Gavrilovic et al., 2024). The coexistence of epilepsy and anxiety is thought to reflect shared pathological mechanisms, including hippocampal dysfunction, oxidative stress, neuroinflammation, and impaired GABAergic neurotransmission (Espinosa-Garcia et al., 2021, Gulyaeva, 2021). Consequently, the behavioral improvement observed after ABA treatment is unlikely to be independent of its anticonvulsant activity. Instead, restoration of hippocampal redox balance together with normalization of inhibitory neurotransmission may contribute simultaneously to seizure suppression and anxiety reduction.

This study has several important limitations that should be considered. First, the acute PTZ-induced seizure model does not fully replicate the pathological complexity of chronic epilepsy; therefore, extrapolation of our findings to chronic epileptogenesis should be made cautiously. Future studies utilizing chronic seizure models or repeated PTZ administrations are necessary to assess whether ABA's antioxidant effects extend to sustained neuroinflammatory responses. Second, only male animals were included; sex-related differences in ABA's potential effects warrant investigation. Third, the absence of a bicuculline + PTZ control group limits our ability to distinguish between ABA's GABAergic antagonism and bicuculline's intrinsic proconvulsant properties. Finally, this study lacked direct assessment of neuroprotection through histological examination, apoptosis markers, or neuronal survival assays. Future studies incorporating morphological, electrophysiological, immunohistochemical, direct GABA quantification, inflammatory marker

measurements and biochemical measures of neuronal viability are necessary to substantiate the potential neuroprotective properties of ABA.

In conclusion, the present findings demonstrate that central administration of ABA significantly attenuates PTZ-induced seizures, improves anxiety-like behavior, and restores hippocampal antioxidant capacity. The partial attenuation of ABA's effects by bicuculline suggests potential GABAergic involvement in its neuroprotective actions, while restoration of oxidative balance may provide an additional mechanism underlying its anticonvulsant efficacy. Collectively, these findings support ABA as a multifunctional antioxidant agent that warrants further investigation in chronic seizure models.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

H.Akbari: Data curation and Formal analysis; F.Shahsavari: Writing–original draft, M. Abbasnejad: Conceptualization and Supervision, S. Esmaeili Mahani: Editing–original draft.

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The biological functions of copper in ruminant animals with emphasis on its influence on the rumen microbiota: A narrative review

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ABSTRACT

Trace elements play a significant role in the health, metabolism, and productivity of ruminants. Copper (Cu) is an essential mineral required for various biological functions in ruminant animals. The dietary requirements and the maximum tolerable concentration for Cu in ruminants are subject to variation based on multiple factors such as animal breed, production type, Cu source, and the concurrent presence of antagonistic minerals. The role of Cu within the rumen microbial ecosystem is of considerable importance in ruminant species. The source and concentration of Cu can modulate the population dynamics and metabolic activities of rumen microorganisms, particularly bacteria and protozoa. Additionally, the resulting alterations in microbial metabolite production and feed digestibility can ultimately influence ruminant production. Organic and inorganic sources of Cu have distinct effects on rumen microorganisms. An optimal Cu amount is advantageous for rumen fermentation. Conversely, elevated Cu levels, particularly those derived from inorganic sources such as oxides and sulfates, can adversely affect feed digestibility, while hydroxychloride formulations and organic amino acid chelates have lower negative impacts on this parameter at high concentrations. Primary effects of Cu on rumen microbiota include alterations in bacterial populations, particularly an increase in fiber-degrading bacteria, a decrease in protozoan communities at elevated levels of Cu, changes in the production and composition of volatile fatty acids, and the potential reduction of methane emissions. This review discusses the biological effects of Cu in ruminants, encompassing Cu requirements, deficiency manifestations, and toxicity concerns, while emphasizing the interactions between Cu and rumen microbial populations and their metabolism.

Keywords: Metabolism, Methane emission, Microorganisms, Minerals, Rumen fermentation

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Introduction

The rapid increase in the global population has resulted in a corresponding rise in the demand for food, particularly in relation to animal proteins. This population growth represents the principal driver of domestic animal population expansion, imposing immense pressure on the livestock sector. Consequently, the efficiency of animal production plays a vital role in fulfilling global food requirements (Valizadeh, 2014; Mousaie et al., 2025; Jimenez-Islas et al., 2026). To sustain the health and productive performance of animals at an optimal level, micronutrients specifically minerals, hold significant importance in ruminants. Minerals possessing structural, physiological, catalytic, and regulatory functions within biological systems, and as metabolic modifiers, they influence ruminants' metabolism, growth and production (NRC, 2005; Suttle, 2021, Mousaie et al., 2026). The Cu element is a trace mineral that is widely distributed among animal, plant, and microbial cells. In adult humans, it is present in quantities of approximately 100 milligrams, with Cu-binding proteins making up about 0.3% of bacterial proteomes (Li et al., 2019). This mineral serves a critical function in ruminant animals' physiology, as it exerts significant impacts on health status and productive performance. Copper is essential for numerous biological functions, including antioxidant system activity, wool and hair pigmentation, cellular respiration, immune system function, bone development, erythrocyte formation, and connective tissue development in ruminants (NRC, 2007; NASEM, 2021; Suttle, 2021). Both Cu deficiency and excess can result in growth retardation, lowered productive performance as well as morbidity and mortality in ruminant animals (NRC, 2005; Wang et al., 2025). Ruminants exhibit varying Cu requirements based on their productive status, breed, age, environmental conditions and nutritional plans. Furthermore, the bioavailability of Cu differs between organic and inorganic sources (Ward et al., 1996; Wang et al., 2012). However, the utilization of various Cu sources with differing concentrations, coupled with inconsistent and sometimes contradictory results reported in the scientific literature concerning the effect of this mineral on rumen microbial populations, metabolic byproducts, dietary digestibility, and ultimately livestock production performance, creates considerable uncertainty within both the farming and research communities. Therefore, this situation requires comprehensive evaluation and critical discussion of existing research evidence. The primary objectives of this review were to clarify the diverse biological functions of Cu and to assess the existing knowledge regarding Cu requirements, supplementation strategies, and their respective influences on productive performance of ruminants and rumen microbial communities. Additionally, this review aimed to address inconsistencies or conflicting results in the literature and to provide evidence-based and practical recommendations for researchers, veterinary practitioners, and livestock producers.

Biological importance of Cu in ruminant animals

Copper is essential for various biological functions in ruminants. As demonstrated in Table 1 (Aupperle et al., 2001; McDonald et al., 2010; NRC, 2005; Engle, 2011; Suttle, 2021; Postma et al., 2021; NASEM, 2021; Ranaweera et al., 2023), Cu is a component of certain enzymes and proteins such as cytochrome C oxidase (CCO), superoxide dismutase (SOD), ceruloplasmin, tyrosinase, lysyl oxidase and dopamine-β-hydroxylase that facilitate physiological and metabolic processes in these animals. This mineral is present in the environment and circulates from soil to the cells of animal bodies as illustrated in Figure 1. Most of the soluble Cu in surface soils is organically complexed, causing the total Cu in soil solution to be large, and in most cases assuming about 90% with the available Cu occupying only 10%. This element is also known to play important roles in the mineral nutrition, biochemistry, and physiology of the plant. An important function of Cu in plant metabolism is concerned with chlorophyll formation and as a component of a number of different plant enzymes

(Haque et al., 1993). The Cu content of pastures and forages is influenced by factors such as plant species, maturity, specific soil conditions and the type of fertilizers applied. In general, temperate grasses have been observed to contain lower levels of Cu compared to legumes when grown under identical conditions, with reported values of 4.7 mg/kg dry matter for grasses and 7.8 mg/kg for legumes, respectively (Suttle, 2010). Seeds and seed by-products are usually rich in Cu, whereas straws generally contain little amounts of this element (McDonald et al., 2010). Increased quantities of Cu have been documented as varying according to plant variety, maturity, soil conditions, fertilizer application, and environmental factors (Suttle, 2021). The consumption of feed containing Cu by ruminants leads to significant interactions with rumen microorganisms, which in turn influences its absorption from the digestive system (Zhang et al., 2007; McDonald et al., 2010; Shete et al., 2023), and has implications for the health and productivity of these animals. Copper metabolism functions through regulated pathways of Cu trafficking rather than relying on pools of Cu lability. Within the cell, Cu is transported to facilitate enzyme production, retained in metallothionein, or excreted via the Golgi apparatus into the bloodstream. In hepatocytes, the synthesis of Cu-containing caeruloplasmin allows for the systemic supply of Cu, while excess Cu is excreted through bile (Clarkson et al., 2020).

Table 1. Copper-containing proteins, as well as the biological functions of Cu and the consequences of its deficiency in ruminant animals.

Cuproenzymes and Cu-binding proteins	Biological function	Hypocuprosis/Cu-deficiency consequences	References
Cytochrome C oxidase	Cellular respiration	Anorexia Encephalomyopathy Swayback disease Reduced cellular immunity Impaired energy metabolism Reduced animal's production	Aupperle et al., 2001; McDonald et al., 2010; NASEM, 2021; Suttle, 2021
Ceruloplasmin	Cu and Fe transport, ROS scavengering	Anemia	NRC, 2005; McDonald et al., 2010; Suttle, 2021
Superoxide dismutase	Antioxidant, ROS scavenger	Increased susceptibility to oxidants Elevated risk of infection	NRC, 2005; Suttle, 2021
Tyrosinase	Tyrosin to Melanin conversion	Wool and hair depigmentation	Suttle, 2021; Ranaweera et al., 2023
Lysyl oxidase	Cross linkages in connective tissues	Bone and Joint abnormalities Reduced wool quality	Postma et al., 2021; Suttle, 2021
Dopamine-β-hydroxylase	conversion of dopamine into norepinephrine	Nervous system disorders	NRC, 2005; Engle, 2011; Suttle, 2021

The Cu deficiency has been extensively documented as an etiology of enzootic ataxia, anemia, and reduced growth performance in ruminant animals particularly in ovine species. The associated clinical manifestations are characteristically attributed to neurological degeneration, skeletal malformations, and compromised immune function, with disease severity demonstrating considerable variation across species and age groups (NRC, 2005). Cattle can experience Cu deficiency under unsuitable nutritional circumstances. A study conducted by Dargatz et al. (1999) examined 2,007 beef cows and heifers from 256 herds across 18 states, revealing that 38.9% of the subjects were identified as marginally

deficient in Cu, with serum Cu concentrations equal to or less than 0.65 mg/L (Spears et al., 2022). Research has indicated that dietary Cu deficiency in cattle compromises the capacity of neutrophils to eliminate Candida albicans thereby indicating its significance in immune system function and disease prevention (Li et al., 2019). In addition, secondary Cu deficiency is primarily precipitated by antagonistic interactions with molybdenum (Mo), sulfur (S), and iron (Fe), which substantially diminish Cu bioavailability and metabolic utilization (Suttle, 2021). The manifestation of Cu deficiency observed in the small ruminants was characterized by variable severity levels of lethargy, swayback, ataxia, progressive weight reduction, emaciation, loss of hair and wool pigmentation, alopecia, marked anemia, diarrhea, reproductive anestrus, embryonic resorption, placental retention during the postpartum period, as well as extended recumbency (Almeida et al., 2022; Byrne and Murphy, 2022). The effects of Cu on livestock performance exhibit variability across studies. One investigation (Solaiman et al., 2007) indicated that increased Cu concentrations in caprine subjects improved growth performance, while another study (Zhang et al., 2008) reported that optimal growth performance and feed efficiency were achieved in goats fed diets containing 10 mg of Cu from copper sulfate (CuSO4) supplementation per kg of feed.

Conversely, goats receiving diets with higher Cu concentrations of 30 mg/kg displayed the lowest weight gain, indicating the variability in outcomes based on the quantity and source of Cu supplementation. Copper, particularly from organic sources at concentrations of 10 and 20 mg/kg of feed, has been shown to reduce carcass adiposity in sheep by promoting lipolysis and inhibiting lipogenesis (Cheng et al., 2010). A study showed that elevated dietary Cu concentrations exceeding recommended amounts may enhance the digestion of low-quality forage (Lopez-Guisa et al., 1992). The evidence implies that dietary Cu has an additive effect on rumen nutrient digestion and microbial proliferation. In contrast, some findings confirmed that free Cu ions derived from rumen-soluble CuSO4 may react with S, Mo, and Fe to form insoluble complexes in the rumen, which could reduce intestinal Cu absorption (Gould and Kendall, 2011; Wilk et al., 2022). Coated CuSO4 has been reported to be beneficial in dairy cows by improving milk performance and feed digestion (Wang et al., 2021). However, research on dairy cows has indicated that the addition of dietary Cu did not influence feed intake or milk yield but altered the composition of milk fatty acids (Engle and Spears, 2000; Morales et al., 2000). This suggests potential variations in the responses to Cu supplementation based on different productive conditions among animals and various form and quantity of administered Cu supplements.

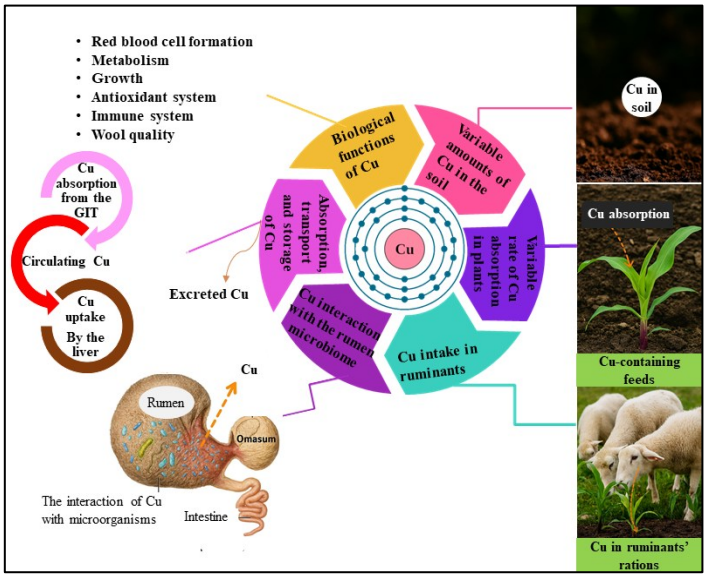

Figure 1. The circulation of Cu from the soil into plants, followed by its transfer through the digestive system of ruminants, its absorption, and subsequent entry into ruminant cells, along with its associated roles in their metabolic processes and production (Haque et al., 1993; NRC, 2005; Zhang et al., 2007; McDonald et al., 2010; Clarkson et al., 2020; Suttle, 2021; Shete et al., 2023).

Although Cu is an essential trace element for livestock, excessive concentrations in the diet can lead to toxicity (Suttle, 2021). Consequently, various organisms, depending on their species and biological conditions, have evolved specialized homeostatic mechanisms to effectively absorb, transport, and excrete Cu, while also mitigating its toxic effects (Lopez-Alonso and Miranda, 2020; Byrne and Murphy, 2022). Copper's high reactivity permits it to readily engage in redox reactions,

influencing cellular processes, and this characteristic contributes to its toxicological impacts on both animal and prokaryotic cells (Solioz, 2018). Ruminants exhibit a heightened vulnerability to Cu toxicity compared to non-ruminant species, likely due to their grazing habits on pastures with low Cu content, which may also be rich in Cu antagonist minerals such as S, Mo, and Fe. Given the limited regulatory control ruminants possess over Cu absorption, they have adapted mechanisms for accumulating excess Cu in the liver by minimizing its biliary excretion (Lopez-Alonso and Miranda, 2020). The liver serves as the primary storage organ for Cu allowing for the mobilization of Cu into the bloodstream to fulfill various biochemical functions (Grace et al., 2012). When animals encounter Cu concentrations that surpass their physiological requirements, this can lead to an excessive storage of Cu in liver tissue; symptoms of Cu toxicity include diarrhea, abdominal pain, liver dysfunction, anemia, and hemolysis, with manifestations occurring either acutely or chronically based on the exposure level (NRC, 2005; Suttle, 2021; Wang et al., 2025). Instances of acute Cu toxicity are primarily linked to unintentional ingestion of Cu-containing substances, such as CuSO4 or copper chloride (CuCl2); Chronic Cu toxicity is mainly attributed to excessive dietary Cu intake and genetic predispositions linked to specific mutations (Wang et al., 2025). Research indicated that optimal Cu levels can positively affect the population of rumen microorganisms and their metabolic activities; however, the detrimental effects of Cu toxicity on rumen cellulose-degrading bacteria have been reported (Hubbert et al., 1958). Additionally, elevated levels of inorganic Cu in animals' nutrition can have negative implications for human health through increased excretion and its subsequent entry into soil and plant systems (Zhen et al., 2022).

Copper requirements of ruminants

As demonstrated in Figure 2, the Cu requirements of some industrial ruminant species such as cattle, sheep, and goats are not constant and vary according to breed, age, sex, stage of production, amount and type of production, environmental conditions, and other dietary components, particularly Cu antagonist elements such as Fe, Mo, S, and to a lesser extent Zn. Notably, liver Cu concentration serves as a superior indicator of body Cu status compared to blood Cu levels (NRC, 2005, NASEM, 2021; Suttle, 2021). However, there is limited information regarding Cu status of soils, Cu content in plants, and blood Cu concentration of ruminants in different regions of Iran. In one study, the optimal plasma Cu level was reported to be 63-127 µg/Dl (Ganjkhanloo et al., 2025). In an experiment conducted on male Kermani lambs, the blood Cu level of the control group was reported to be 58 µg/dL (Seifalinasab et al., 2022), which indicates the necessity to increase dietary Cu, particularly from organic sources, in wool-producing sheep. Additionally, the Cu concentration in the diet of fattening lambs completely mixed with alfalfa and a concentrate consisting of barley grain, soybean meal, and bran was approximately 5.8 mg/kg of dry matter, which increased to 8.2 mg with CuSO4 supplementation, thereby partially meeting livestock requirements (Mousaie, 2021). However, it seems that the Cu requirement in wool-producing sheep is considerably higher (Suttle, 2021).

While maximum tolerable levels (MTL) of Cu have been established at 30, 20, and 35 mg/kg feed for cattle, sheep, and goats, respectively (Figure 3), the actual MTL for ruminants remains uncertain, and higher Cu concentrations have been documented for these animals in various productive and nutritional conditions (NRC, 2005; Solaiman et al., 2007; Marsh et al., 2026). This uncertainty arises from factors such as animal sex and species, the quantity of other dietary elements, particularly Mo, S, and Fe along with the Cu source regarding its solubility and absorption, which all influence the amount of Cu entering circulation and body stores (Suttle, 2021; Zhou and Shen, 2025). For instance, researchers have reported that Angus cattle, in the absence of Cu supplementation, demonstrate higher plasma Cu quantities and ceruloplasmin activity than Charolais and Simmental cattle, and that Angus bulls exhibit superior performance in Cu absorption and retention (Wang et al., 2025).

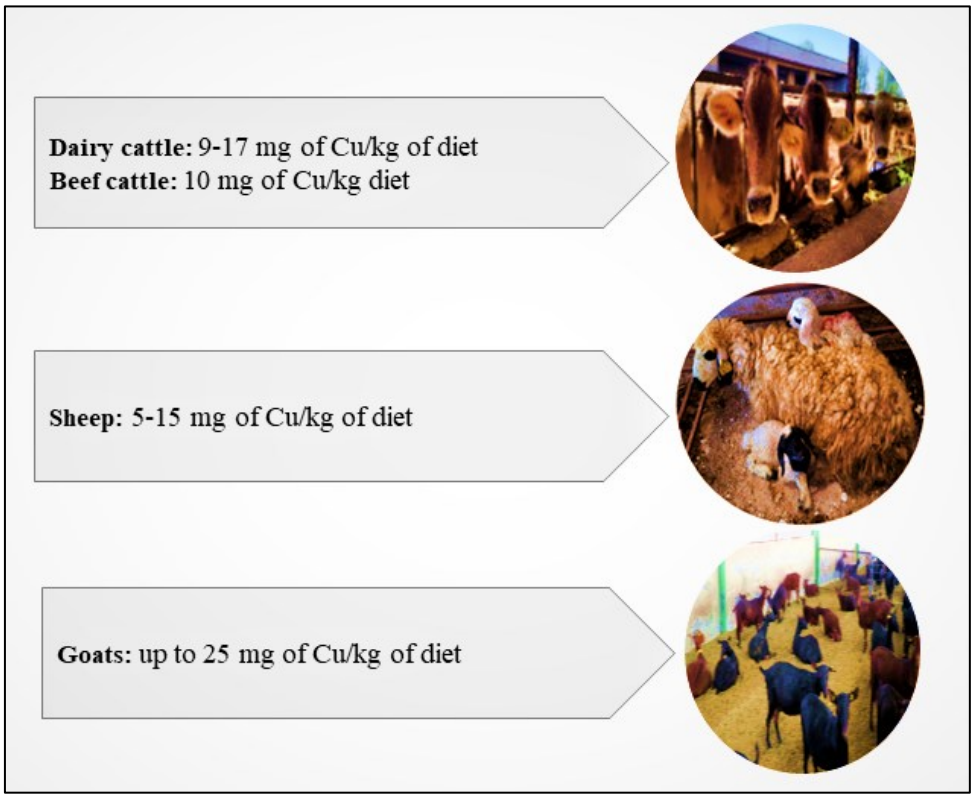

Figure 2. The Cu requirements of cows, sheep and goats (NRC, 2005, NRC, 2007; NASEM, 2021; Suttle, 2021).

Empirical evidence demonstrated that Cu tolerance varies significantly among different sheep breeds (Lopez-Alonso and Miranda, 2020). Merino sheep breed exhibits heightened resistance to elevated and toxic Cu concentrations, whereas breeds including Suffolk, Leicester, Charolais, and Texel demonstrate greater susceptibility to Cu toxicity when compared to the Cambridge breed, which shows relative resistance (Saeed et al., 2025). Nonetheless, a notable knowledge gap exists regarding the tolerable Cu threshold in indigenous Iranian sheep breeds. In comparison to bovine and caprine species, sheep display increased sensitivity to Cu toxicity. The higher sensitivity of sheep to Cu toxicity is attributed to their comparatively diminished capacity to increase bile secretion, thereby reducing Cu excretion under conditions of elevated dietary Cu intake. Owing to impaired Cu excretion through biliary pathways, sheep possess limited capacity to tolerate dietary Cu excess, resulting in hepatic Cu accumulation and subsequent hepatotoxicity (Wang et al., 2025).

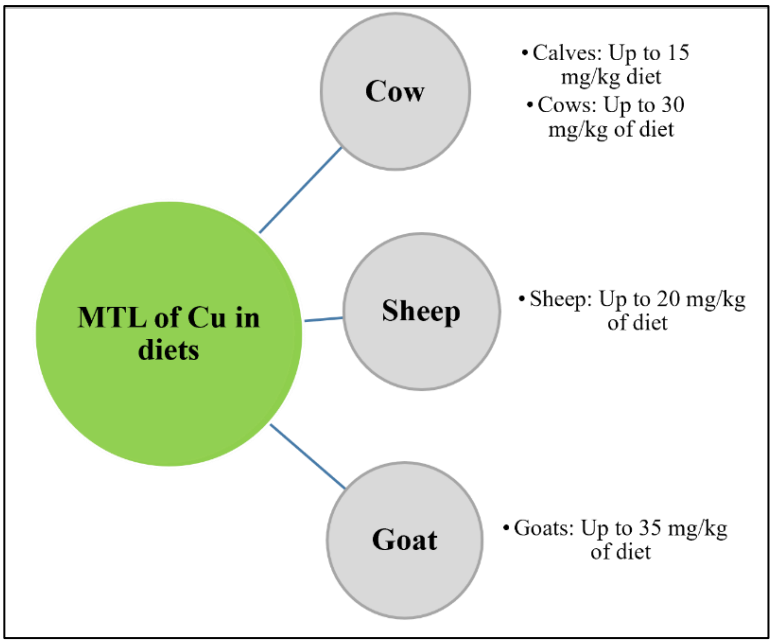

Figure 3. The maximum tolerable level (MTL) of Cu in the diet of ruminants (NRC, 2005; Solaiman et al., 2007; Lopez-Alonso and Miranda, 2020; Suttle, 2021; Marsh et al., 2026).

Copper absorption and bioavailability of different Cu sources in the digestive tract

Ruminants possess a distinctive digestive system that differentiates them from other mammalian species which consequently influences Cu metabolism. In these organisms, Cu metabolism occures in the rumen, and the tripartite interaction among Cu, Mo, and S represents one of the most significant physiological distinctions between ruminants and monogastric animals, thereby affecting animal health and productivity. This noncompetitive interaction mechanisms result in diminished intestinal absorption of this mineral, ranging from less than 1 to 10%, when compared with nonruminant and suckling calf species, which demonstrate absorption rates reaching up to 70%; however, the degree of absorption varies substantially based on the relative concentration of Cu antagonistic compounds. Although there is some Cu absorption within the rumen environment, intestinal absorption demonstrates greater bioavailability efficiency (Lopez-Alonso and Miranda, 2020; Byrne and Murphy, 2022). Inorganic and organic S compounds undergo microbial metabolism in the rumen, resulting in sulfide production, as illustrated in Figure 4. Concurrently, S and Mo undergo a chemical reaction to form thiomolybdates, which exist in mono-, di-, tri-, and tetrathiomolybdate forms.

These compounds exhibit strong binding affinity to Cu, with tri- and tetrathiomolybdates demonstrating irreversible binding capacity, thereby forming Cu-thiomolybdate complexes. Copper bound to thiomolybdates exhibits low solubility and remains unabsorbed in the intestinal tract. In the absence of Cu in the rumen environment, thiomolybdates are absorbed through the rumen epithelium and, at a reduced rate, through the small intestinal mucosa. Following absorption into the circulatory system, these compounds may bind to Cu present in biological compounds (Lopez-Alonso and Miranda, 2020; Zhou and Shen, 2025). Ceruloplasmin, a glycoprotein, functions as the primary Cu carrier in blood plasma, transporting approximately 90% of circulating Cu, and serves an essential role in facilitating Fe utilization for hemoglobin synthesis (NRC, 2005; Suttle, 2021). The liver is the primary storage site for Cu, and this mineral can be readily mobilized from hepatic tissue into the bloodstream to meet the biochemical requirements of the animal. The liver Cu quantity is greater in newborn ruminants than adults with developed rumen. Depending on hepatic Cu concentrations and the level of dietary Cu antagonists, liver Cu reserves can sustain normal plasma Cu levels for extended periods, even in ruminants consuming Cu-deficient diets (Spears et al. 2022).

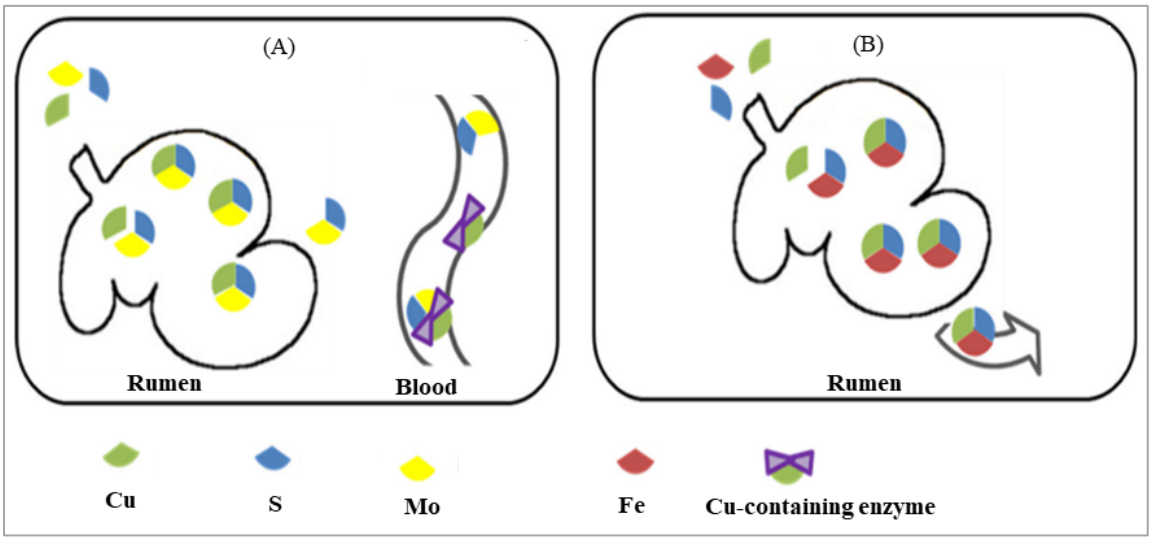

Figure 4. The interaction of Cu with S and Mo in the rumen and blood (a), and with Fe and S in the rumen (b) of ruminants (Lopez-Alonso and Miranda, 2020).

Dietary Cu absorption in adult dairy cattle ranges from 1 to 5 percent (Wang et al., 2021). Copper sulfate represents the predominant Cu supplement employed within the livestock industry. Although Cu absorption primarily occurs in the small intestine, CuSO4 demonstrates solubility in the rumen environment and may establish insoluble complexes with S, Mo, and Fe, thereby hindering Cu absorption (Wang et al., 2021). Although elevated concentrations of elemental Zn may potentially diminish Cu absorption, a study in lambs indicated that utilizing three Zn sources—nano-zinc oxide (25 mg of Zn/ kg of diet), zinc-methionine (50 mg of Zn/ kg of diet), and zinc oxide (100 mg of Zn/ kg of diet)—produced no adverse effects on Cu absorption or blood Cu concentrations (Hashemzaei et al., 2025). Among inorganic Cu formulations, sulfate and chloride forms exhibit greater bioavailability than carbonate and oxide forms. Organic Cu supplements, including amino acid chelates and Cu-proteinate, demonstrate enhanced intestinal bioavailability compared to inorganic Cu supplements due to absorption via specific transporters and hydrochlorides, attributed to diminished rumen interactions, particularly with Mo and S (Byrne and Murphy, 2022). Additionally, using organic Cu supplement (Cu-methionine) in sheep revealed superior Cu absorption and reduced gastrointestinal excretion compared to CuSO4 (Pal et al., 2010). Conversely, a study reported equivalent bioavailability between Cu-proteinate and CuSO4 at low Mo concentrations (Ward et al., 1996). However, sulfate supplements, encompassing CuSO4, have been documented to exert negative effects on feed cell wall digestibility within the ruminant animals' gastrointestinal tract (Arce-Cordero et al., 2020). Therefore, the source and concentration of Cu as well as the presence of other antagonistic minerals, significantly influence its absorption.

The effect of Cu on microbial fermentation in the rumen

The impact of Cu on microbial community in the rumen of cattle has produced inconsistent findings. Some studies have reported negative effects of dietary Cu supplementation at levels ranging from 5 to 25 mg/kg of diet on the feed digestibility (Genther and Hansen, 2015; Faulkner et al., 2017; Caldera et al., 2019). In a study, decreased neutral detergent fiber (NDF) and crude protein digestibility was observed throughout the entire gastrointestinal tract of cross-bred bulls administered an intraruminal tablet containing 12.5 g of Cu-oxide (Arthington, 2005). This particular study documented mean liver Cu concentrations exceeding 600 mg/kg in bulls receiving these tablets, substantially higher than concentrations observed in cattle receiving CuSO4 through dietary supplementation, indicating that the Cu tablet formulation produces elevated rumen Cu levels with greater effects on nutrient digestibility. These findings suggest that adverse effects manifest at markedly high Cu concentrations, particularly when derived from inorganic sources such as oxide and sulfate, whereas hydroxychloride formulations and organic chelate sources do not adversely affect digestibility. Conversely, it has been reported either an absence of Cu effects on fermentation or improvements in fermentation parameters when Cu-supplemented diets were provided. Research indicated that molar ratios of volatile fatty acids (VFA) in the rumen were not affected by Cu supplementation, and supplemental Cu at 10 or 20 mg of Cu/kg of high-concentrate diet containing 4.9 mg of Cu/kg of dry matter, produced no effect on rumen fermentation (Engle and Spears, 2000). However, in vitro investigations using culture media containing rumen microorganisms demonstrated that different Cu sources at varying concentrations affected microbial fermentation, with in vitro dry matter degradability and microbial amylase activity being elevated with Cu-methionine compared to CuCl2 and tribasic CuCl2 (Zhao et al., 2022). In addition, ammonia nitrogen concentration, microbial protein, and propionate were higher in the Cu-methionine treatment relative to the tribasic CuCl2 treatment. In a study, the Cu supplementation at 10 mg/kg of feed from CuSO4, with a basal diet containing approximately 7.5 mg of Cu/kg, reduced rumen pH in goats while enhancing microbial fermentation, resulting in increased total VFA concentration, acetate ratio, and NDF digestibility

throughout the gastrointestinal tract (Zhang et al., 2007). Similarly, lambs receiving 10 or 20 mg of Cu from an organic source, specifically Cu-proteinate, demonstrated improved NDF and acid detergent insoluble fiber (ADF) digestibility in the gastrointestinal tract relative to those receiving an inorganic source (Dezfoulian et al., 2012). In another research, cattle receiving 7.5 mg of Cu/kg of feed exhibited higher apparent digestibility of dry matter, organic matter, crude protein, NDF, and ADF compared with control animals (Wang et al., 2021). Similarely, bulls provided with a diet containing 7.68 mg of supplemental Cu from CuSO4, showed increased total rumen VFA, particularly acetate, with concurrent improvements in dry matter, organic matter, protein, and NDF digestibility (Shang et al., 2020), while application of 15 mg of Cu as coated CuSO4 within a continuous culture system showed that microencapsulation of Cu reduced the molar ratio of acetate and increased the butyrate ratio, though feed nutrient digestibility remained unaffected (Arce-Cordero et al., 2020). These researchers proposed that CuSO4 may modify the metabolism and production of VFA and their molar ratios in the rumen without altering nutrient digestion, thereby increasing the butyrate molar ratio relative to acetate.

Copper and the activity of rumen bacteria

The mechanism by which Cu exerts its effects on rumen bacteria remains incompletely understood. Approximately 18 to 27% of the supplemented Cu was analyzed in a bacteria-enriched fraction, indicating that Cu might be assimilated by rumen bacteria (Vigh et al., 2023). This mineral influences the activity of numerous rumen bacteria through its participation as a cofactor for essential enzymes, including CCO, NADH dehydrogenase, and SOD (Kenney and Rosenzweig, 2012). In one study (Shang et al., 2020), male cattle receiving diets supplemented with 7.68 mg of Cu from CuSO4 demonstrated increased activity of microbial enzymes, namely carboxymethyl cellulase, cellobiase, xylanase, and pectinase, as well as elevated total bacterial populations, particularly Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Prevotella ruminicola, and Ruminobacter amylophilus. The enhancement of bacterial populations, especially cellulose-degrading bacteria, resulted in increased feed digestibility and daily weight gain in studied animals. In another study (Wu et al., 2021), the administration of 10 mg of Cu/kg of feed from a protected CuSO4 source in Holstein bulls increased cellulase and protease activities while decreasing alpha-amylase activity in rumen microorganisms. Additionally, coated CuSO4 increased populations of Butyrivibrio fibrisolvens and Ruminococcus flavefaciens while decreasing populations of Prevotella ruminicola and Ruminobacter amylophilus. The researchers reported that these alterations in microbial populations enhanced feed digestibility and VFA production, particularly the molar ratio of acetate. In this regard, it has been documented that among rumen bacteria, Butyrivibrio has greater sensitivity to CuSO4 compared to other rumen microorganisms and can make changes in VFAs molar ratios without exerting substantial effects on substrate fermentation (Arce-Cordero et al., 2020). Copper has the ability to diminish methanogenic microorganisms by adversely impacting rumen protozoa (Shete et al., 2023). In addition, elevated Cu concentrations, particularly from inorganic sources, may produce negative effects on certain rumen bacterial species. In one investigation (Hernandez-Sanchez et al., 2019), high Cu levels (80 and 100 µg of Cu/g dry matter) reduced bacterial populations. The researchers attributed this effect to intracellular mineral accumulation. Fluctuations in cytoplasmic Cu between oxidized ($Cu2+$) and reduced ($Cu1+$) states can convert it into a toxic substance. Gram-negative bacteria exhibit heightened sensitivity to Cu due to membrane permeability characteristics; consequently, Cu accumulation in the periplasm results in bacterial lysis and cellular death. Bacterial capacity to tolerate high quantities of Cu correlates with bacterial homeostatic systems, and specific genes within bacterial cells mediate Cu resistance; furthermore, proteins associated with Cu-efflux pumps function within bacterial cells. Moreover, Cu resistance has been documented through plasmid DNA

transfer among rumen bacteria (Hernandez-Sanchez et al., 2019).

The interaction of Cu with rumen protozoa and fungi

Rumen protozoa constitute approximately 50% of the microbial biomass. Nonetheless, their functional role within this ecosystem remains poorly understood. Initial studies indicated a potential involvement of protozoa in carbohydrate and protein metabolism, however, due to their close association with bacteria, has made it difficult to conclusively assign these functions to protozoa (Williams et al., 2020). The existing literature on the effects of Cu on rumen protozoa reveals contradictory results. In one investigation, supplementing the diet of bulls with CuSO4 increased the total rumen protozoa population (Shang et al., 2020), whereas in another study, elevated Cu levels (100 and 200 mg of Cu per day) reduced rumen protozoa populations in goats, with researchers attributing improved growth performance to this reduction (Solaiman et al., 2007). The antiprotozoal effects of Cu have been demonstrated to a certain extent. Nanocopper administration has been shown to eliminate rumen ciliated protozoa, consequently reducing methanogen populations and methane production (Shete et al., 2023). Consistent with these findings, another experiment indicated that the application of 10 and 20 mg of nanocopper/kg of feed reduced rumen protozoa in cattle (Palangi et al., 2024). Notably, a foundational study conducted approximately 40 years ago, addressed the protective role of protozoa in mitigating Cu toxicity in livestock (Ivan et al., 1986), a topic that has received limited attention in subsequent research.

These researchers reported that introducing rumen ciliated protozoa into defaunated sheep reduced Cu absorption and hepatic accumulation. Consequently, the prevalence of chronic Cu toxicity is elevated in protozoa-depleted ruminants. Limited information exists regarding potential effects of Cu on rumen fungi. Although fungi represent a modest proportion of total rumen microbiota, accounting for approximately 8% of rumen biomass, they play a significant role in feed degradation; the composition of ruminant diets, particularly elevated fiber content, may promote rumen fungal population development (Krol et al., 2023). A study reported that adding CuSO4 to the diet increases anaerobic rumen fungi populations in cattle (Shang et al., 2020). Similarly, it was found that using organic Cu sources (up to 15 mg of Cu/kg of diet) positively influenced rumen fungal populations and fiber digestion (Adedeji et al., 2024). In contrast, no significant effect of Cu on rumen fungal populations of cattle was reported (Hanauer, 2017), suggesting that the source and concentration of dietary Cu may influence fungal response. Nevertheless, additional research is necessary to elucidate the relationship between Cu intake and rumen fungi. In general, the potential effects of Cu on the population and activity of rumen microorganisms can be summarized as presented in Figure 5.

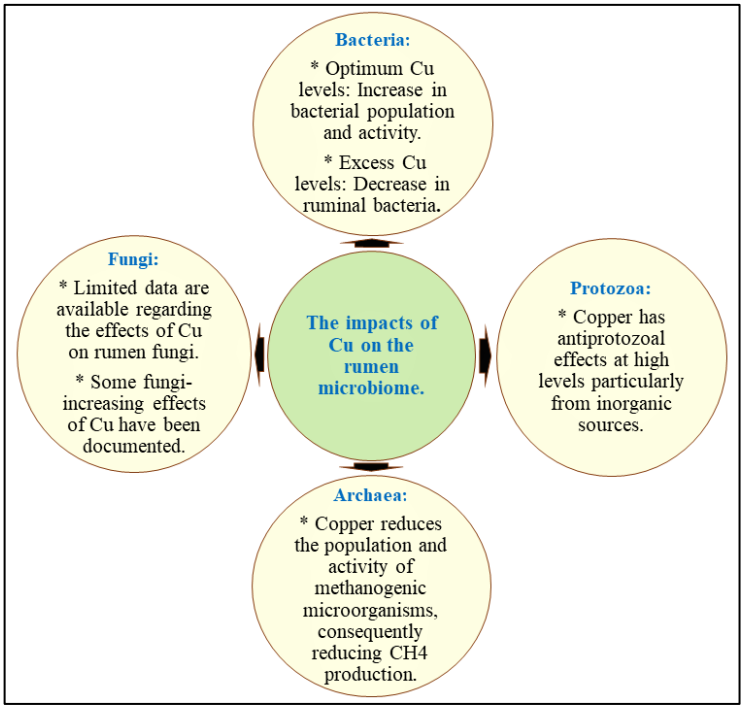

Figure 5. A summary of the effects of Cu on rumen microbiota, including bacteria, protozoa, fungi, and archaea (Hernandez-Sanchez et al., 2019; Shang et al., 2020; Wu et al., 2021; Shete et al., 2023; Palangi et al., 2024; Adedeji et al., 2024).

Conclusion

The findings of this review demonstrated that the administration of Cu to ruminants at appropriate levels is positively associated with their health and productive performance. This mineral is crucial for the optimal functioning of antioxidant and immune systems, nutrient metabolism, nervous system activity, as well as skin and wool health. A deficiency of Cu can lead to disease occurrence and diminished productive performance in ruminants. Conversely, excessive Cu intake, especially in sheep, can result in severe toxicity. The absorption rate of organic Cu supplements is higher than that of inorganic forms. The use of organic Cu supplements, particularly amino acid chelates at recommended concentrations, may yield beneficial effects on feed digestibility and reduce methane production while enhancing energy efficiency in ruminants by decreasing protozoa and certain methanogenic species. However, elevated levels of Cu, particularly from inorganic sources, may adversely affect specific microbial species and decrease their populations; hence, the incorporation of CuSO4 supplement in ruminants' diets should be minimized. It is therefore recommended that during drought conditions and thermal stress, when animals may not receive adequate nutrition, or in circumstances where dietary Cu levels are insufficient or S and Mo concentrations are high, Cu supplements should be added to diets, particularly in the form of organic compounds such as Cu-methionine and Cu-proteinate, or mineral forms such as Cu-hydroxychloride. In addition, the MTL for ruminant breeds should be taken into account to avoid Cu toxicity. Nevertheless, a more comprehensive understanding of Cu's effects on rumen microbiota necessitates further research, particularly utilizing genomics-based and metabolomics technologies to elucidate the potential roles of Cu in influencing the population and activity of rumen microorganisms. Moreover, further studies may be required to revise the optimal dietary Cu concentrations for ruminants when employing Cu sources with greater bioavailability than inorganic alternatives, such as amino acid-chelated and nanoparticle formulations of Cu.

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