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Shikonin-induced secondary metabolite modulation in grape berries (*Vitis vinifera* L. cv. 'Öküzgözü') under salinity stress

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Abstract

Background Salt stress represents a critical challenge in viticulture, significantly impacting grape berry biochemical profiles and potentially threatening crop productivity and quality. In this study, therefore, we systematically investigated the metabolic responses of grape berries (*Vitis vinifera* L. cv. 'Öküzgözü') to salinity stress by applying NaCl at four different concentrations (0, 50, 100, and 150 mM) and shikonin at two levels (0 and 25 µM). Our study analyzed changes in organic acids, phenolic compounds, anthocyanins, and sugar contents, employing a standardized foliar spray technique to explore the individual and interactive effects of salt stress and shikonin on grape berry biochemical composition.

Results Salinity stress induced by NaCl markedly suppressed sugar metabolism in the studied plants, with glucose and fructose contents decreasing by approximately 85% and 82%, respectively, under high salinity conditions (e.g., 150 mM NaCl). This drastic reduction indicates a significant disruption in carbohydrate homeostasis due to ionic and osmotic stress. In contrast, the application of shikonin partially alleviated the deleterious effects of salt stress, particularly by enhancing anthocyanin biosynthesis. Under severe salinity, total anthocyanin accumulation increased by up to 60% with shikonin treatment, suggesting its potential role as a modulator of secondary metabolism and antioxidant defense. Phenolic compound levels exhibited highly variable responses depending on the interaction between NaCl and shikonin, with individual compounds showing changes ranging from a 20% decrease to a 75% increase compared to control conditions. These findings reflect a compound-specific regulation, likely driven by differential activation of phenylpropanoid pathway enzymes under stress and elicitor influence. Furthermore, anthocyanin profiling revealed profound shifts in composition; notably, malvidin-*3-O*-glucoside levels were elevated by more than 200% under combined high-salinity and shikonin treatment, indicating a strong synergistic effect on flavonoid pathway activation.

Conclusions Consequently, the current study provides crucial insights into potential mitigation strategies for salt stress in viticulture, demonstrating that targeted interventions like shikonin treatments can help preserve grape berry metabolic integrity under challenging environmental conditions, potentially offering valuable strategies for sustainable grape production in saline-prone agricultural landscapes.

Keywords Anthocyanin biosynthesis, Salinity stress response, Sugars, Phenolic compounds, *Vitis vinifera* L

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Introduction

Agriculture in the contemporary global landscape faces significant challenges posed by various abiotic stress factors, with salinity stress emerging as a critical environmental constraint that severely impedes plant growth

and development [1]. This ecological challenge predominantly affects agricultural productivity in sensitive ecosystems such as semi-arid and arid regions, inducing substantial crop losses and compromising food security [2, 3]. Salinity stress represents a complex physiological and biochemical phenomenon that fundamentally disrupts plant metabolic processes. By inducing severe ionic toxicity and osmotic stress, salt stress creates a hostile environment within plant tissues, triggering multiple interconnected biochemical perturbations. These disruptions manifest through compromised photosynthetic apparatus, increased oxidative stress, and reactive oxygen species accumulation, ultimately constraining biomass production and overall plant performance [4]. The physiological mechanisms underlying salt stress are multifaceted, involving intricate interactions between ionic imbalances, membrane integrity, and metabolic regulations. Excessive sodium (Na^+) and chloride (Cl^-) ion accumulation in plant tissues induces significant molecular-level disturbances, including membrane structural disruptions, nutrient deficiencies, and metabolic dysfunctions. These alterations directly impact critical physiological processes such as stomatal functioning, photosynthetic capacity, and cellular homeostasis [5, 6].

Reactive oxygen species (ROS) accumulation represents a pivotal mechanism through which salt stress exerts its deleterious effects. The electron transport chain experiences significant leakages, resulting in lipid peroxidation, protein oxidation, and substantial damage to photosynthetic complexes like Photosystem I (PSI) and Photosystem II (PSII). To counteract these oxidative challenges, plants mobilize sophisticated antioxidant enzyme systems designed to regulate and mitigate ROS-induced cellular damage [7, 8]. Grapevine (*Vitis* spp.), an economically significant crop cultivated across diverse climatic conditions, exhibits moderate salt sensitivity. Grapes rank among the world's most economically significant fruit crops, with global production exceeding 77 million tons annually and cultivation spanning approximately 7.4 million hectares across diverse geographical regions [9]. The grapevine industry generates substantial economic value through table grapes, wine production, raisins, and juice, contributing significantly to agricultural economies in both developed and developing nations [10]. This economic importance has intensified research efforts toward understanding and mitigating environmental stresses that threaten sustainable grape production worldwide [11]. Salt stress manifests in grapevines through characteristic physiological and biochemical symptoms, including stomatal closure, chlorophyll degradation, and reduced photosynthetic efficiency [12–14]. Shikonin, a natural naphthoquinone pigment derived from the *Boraginaceae* family, emerges as a promising bioactive compound with multifaceted pharmacological properties. Shikonin exerts its protective effects through multiple molecular mechanisms, including modulation of antioxidant enzyme activities, regulation of ion homeostasis, and activation of stress-responsive transcription factors [15]. Recent studies have demonstrated shikonin's ability to upregulate superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities while simultaneously reducing lipid peroxidation and membrane damage in various plant species under stress conditions [16]. Furthermore, shikonin's naphthoquinone structure facilitates electron transfer processes that can directly neutralize free radicals, potentially providing additional protection against oxidative damage [17, 18]. These molecular actions suggest shikonin could serve as an effective biostimulant for enhancing crop resilience against environmental stresses [19]. Its potential in regulating reactive oxygen species and supporting plant defense mechanisms presents an intriguing avenue for mitigating salt stress-induced physiological perturbations [20–22]. Salt stress profoundly impacts secondary metabolite production in grapevines, particularly anthocyanins and phenolic compounds that serve as crucial quality determinants in grape fruits [23]. Previous research has documented significant alterations in anthocyanin profiles under saline conditions, including reduced accumulation of malvidin−*3-O*-glucoside and peonidin derivatives, which directly affects fruit coloration and quality [24]. Similarly, salt stress disrupts phenolic compound synthesis pathways, resulting in modified flavonoid compositions and compromised antioxidant capacities in grape berries [25]. Additionally, sugar metabolism undergoes substantial reconfiguration under salt stress, with documented reductions in sucrose synthase activity and altered sink-source relationships that ultimately diminish fruit sugar content and quality parameters [26]. These metabolic disruptions collectively contribute to reduced fruit quality and shelf life, underscoring the economic significance of salt stress management in viticulture [9, 26]. Despite extensive research on salt stress mechanisms, significant knowledge gaps persist regarding comprehensive strategies for enhancing plant resilience, particularly in economically important crops like grapevines. Existing literature predominantly focuses on descriptive analyses of stress responses, with limited exploration of targeted interventions using bioactive compounds like shikonin.

This study aims to bridge these critical research gaps by investigating the metabolic regulations and adaptive mechanisms of grape (*Vitis vinifera* L. cv. 'Öküzgözü') under salt stress conditions following shikonin application. By systematically examining changes in antioxidant activities, organic acids, phenolic compounds, and anthocyanin levels, the research seeks to elucidate novel

physiological adaptation pathways. The primary objectives of our study are to: (i) assess shikonin's potential in modulating salt stress responses in 'Öküzgözü' grape cultivar, (ii) characterize some biochemical alterations induced by shikonin under saline conditions, (iii) provide innovative insights into plant adaptation mechanisms that could inform future agricultural biotechnological interventions. By comprehensively exploring these dimensions, the study anticipates generating transformative knowledge that could substantially contribute to developing sustainable agricultural strategies for mitigating salinity-induced crop productivity challenges.

Materials and methods

Experimental site and plant materials

This study was conducted in a producer's vineyard located in the Central Anatolia Region of Turkey (39°27'54.1"N/34°44'33.2"E, elevation 906 m) during the pre-veraison (August 5) and harvest periods (October 17), spanning 74 days in 2022. The plant material consisted of 6-year- old vines (*Vitis vinifera* L. cv 'Öküzgözü') grafted on 1103 Paulsen rootstock (*Vitis berlandieri* × *Vitis rupestris*). The 1103 Paulsen rootstock is characterized by vigorous growth and tolerance to soil salinity up to 0.6 g NaCl. The 'Öküzgözü' grape cultivar features large berries (6 g), elliptical shape, gray-black color, medium skin thickness, and winged-conical clusters weighing 450–550 g. It is suitable for both wine and table grape production (Supp. Figure 1). The vineyard was established with a double-cordon training system, planted at 3 m × 2 m row and inter-row spacing, with a trunk height of 80 cm. During the experimental period, the mean midday temperature was 30.1 °C, the average relative humidity was 48.83%, and the mean wind speed was 1.83 m/s. Climate data were obtained from a meteorological station located 9.2 km from the experimental site. The experimental site's soil characteristics had a sandy-loam texture with slightly alkaline conditions (pH: 8.1) and low organic matter content (1.30%). The electrical conductivity was minimal at 0.01 mmhos/cm, with a relatively low lime content of 1.80%. Nutrient analysis revealed moderate to low mineral composition, with notably high calcium levels (1908 ppm) contrasting with very low concentrations of micronutrients like zinc (0.09 ppm) and copper (0.34 ppm). Nitrogen, phosphorus, and potassium levels were relatively modest, suggesting potential requirements for targeted soil amendments. Throughout the experimental period, standard regional viticultural practices were consistently applied to maintain uniform cultivation conditions.

Salt stress, shikonin applications and experimental design

The experimental design was constructed to address potential methodological critiques by implementing a rigorous 4 × 2 Factorial Randomized Complete Block Design. The experimental site was selected for its homogeneous soil properties, ensuring consistent baseline conditions across treatment plots. The treatments in this study consisted of various combinations of salt stress (NaCl) and shikonin applications. The control group received no salt or shikonin (0 N0S; 0 mM NaCl + 0 µM Shikonin). Mild salt stress was applied without shikonin in the 50 N0S group (50 mM NaCl + 0 µM Shikonin), while moderate and severe salt stresses were applied in the 100 N0S (100 mM NaCl + 0 µM Shikonin) and 150 N0S (150 mM NaCl + 0 µM Shikonin) groups, respectively. Shikonin alone was administered in the absence of salt stress in the 0 N25S group (0 mM NaCl + 25 µM Shikonin). Combined treatments included 50 N25S (50 mM NaCl + 25 µM Shikonin), 100 N25S (100 mM NaCl + 25 µM Shikonin), and 150 N25S (150 mM NaCl + 25 µM Shikonin), representing increasing salt levels supplemented with shikonin. To prepare a 100 mL solution of 25 µM shikonin, 0.72 mg of shikonin was weighed and dissolved in distilled water. This solution was applied at two distinct phenological stages: pre-veraison (EL-34) and post-veraison (EL-36), administering 100 mL per grapevine during each application. Each experimental unit incorporated three biological replicates with three vines per replicate. Uniform irrigation volume (25 mm) was maintained across all treatments to eliminate water-related confounding factors. The experimental timeline, spanning pre- and post-veraison periods (August 5–25), was strategically selected to capture critical physiological responses during grape development. Detailed documentation of experimental protocols, including precise application methods, concentration calculations, and environmental monitoring, was implemented to ensure transparency and reproducibility.

Determination of organic acids in grape berries

Grape berry samples were collected at physiological maturity (approximately 21.0 Brix), with twenty berries selected from the middle sections of three clusters per replicate. The organic acid extraction methodology, adapted from Keskin et al. [27], involved mixing 5 ml of grape juice with 20 ml of 0.009 M NH_2SO_4 solution. The mixture underwent homogenization, continuous agitation for one hour, and centrifugation at 15,000 rpm for 15 minutes. The resulting supernatant was systematically purified through multiple filtration stages: first through filter paper to remove coarse particles, then twice through 0.45 µm membrane filters to eliminate fine impurities. Further refinement was achieved by passing

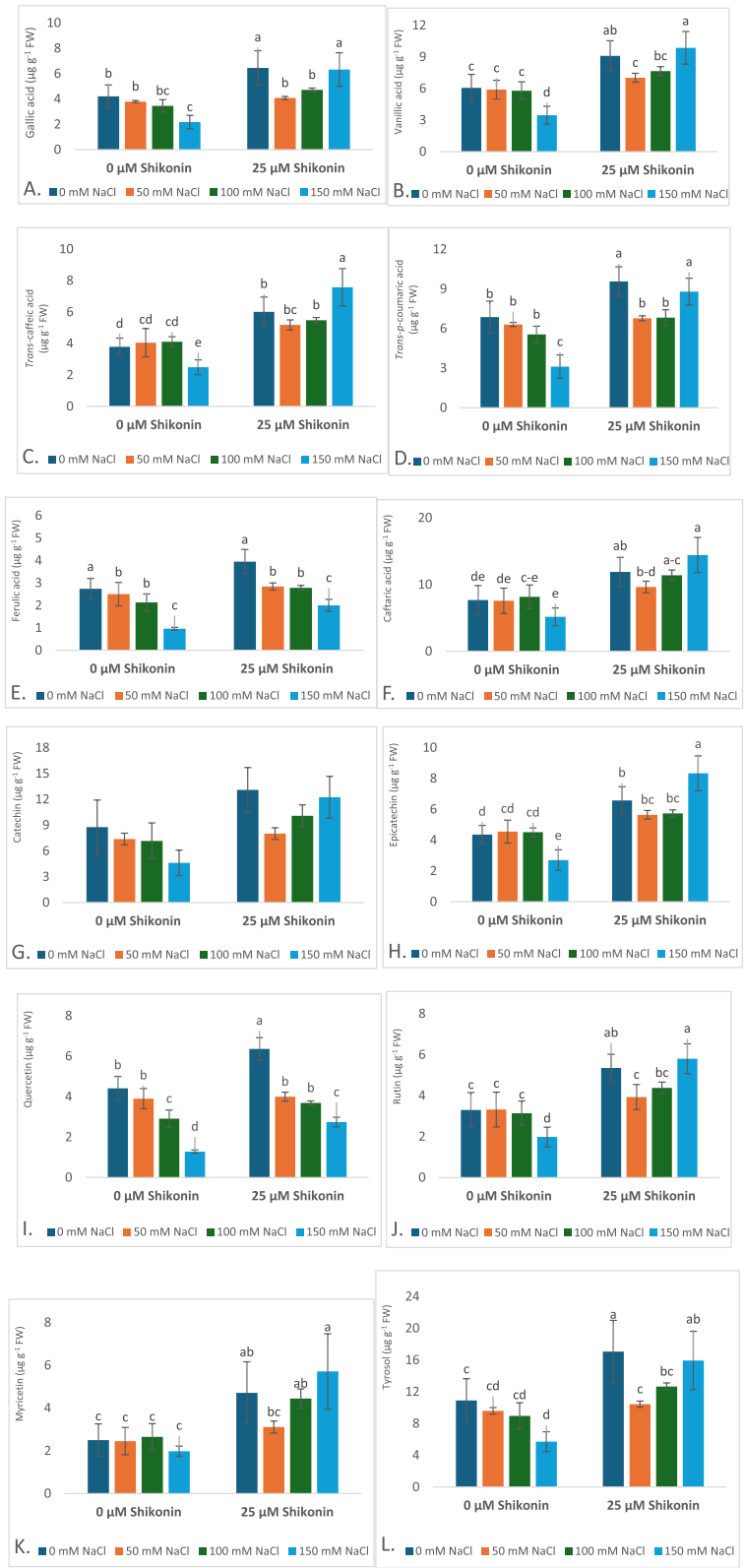

Fig. 1 Effects of different concentrations of shikonin treatments on phenolic compound profile of grapes against salt stress. Different lower case letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$. Data are mean values ± SE

the filtrate through a SEP-PAK C18 cartridge. Organic acid analysis was performed using High-Performance Liquid Chromatography (HPLC, Agilent 1100) with an Aminex column (HPX-87 H, 300 mm × 7.8 mm) for chromatographic separation and quantification. Throughout the analytical process, analytical-grade chemicals were employed, with organic acid standard products sourced from Sigma-Aldrich (St. Louis, USA).

Determination of phenolic compounds in grape berries

The phenolic compounds in 'Öküzgözü' grape cultivar were analyzed using a comprehensive HPLC method adapted from Cosme et al. [28]. The sample preparation involved homogenizing grape berries with pure water, followed by centrifugation at 15,000 rpm for 15 minutes. The resulting supernatant was filtered through 0.45 µm filters and analyzed using HPLC equipped with a diode-array detector and an octadecylsilica column. Compound separation employed a mobile phase consisting of methanol, water, and acetic acid in two different ratios: Component A (10% methanol, 28% water, 2% acetic acid) and Component B (90% methanol, 8% water, 2% acetic acid). Detection was performed at specific wavelengths (254 nm and 280 nm), with 20 µL sample injections at a flow rate of 1 mL per minute. For anthocyanin analysis, the method of Youssef El et al. [29] was utilized, with a mass spectrometer added to the HPLC system to enhance compound identification accuracy.

Determination of anthocyanins in grape berries

Anthocyanin analysis was performed using an advanced HPLC system (Agilent 1100 Series) equipped with a diode array detector and LC/MSD Trap VL ESI-MS/MS system. The analytical method, slightly modified from Kaya et al.'s [30] approach, utilized a Zorbax Eclipse XDB-C18 reversed-phase column maintained at 40 °C. The separation employed a sophisticated mobile phase gradient comprising water, acetonitrile, and formic acid, with varying solvent compositions. The gradient program involved complex phase transitions over 36 minutes, ensuring comprehensive anthocyanin separation. Injection volume was 10 µL, with a flow rate of 0.19 mL/min. Quantification was achieved through external standardization using reference compounds from Sigma–Aldrich Fluka, and Extrasynthese, including rutin, quercetin, Isoquercitin, cyanidin-*3*-*O*-glucoside, malvidin-*3*-*O*-glucoside, Isorhamnetin glucoside, and procyanidin B1. Data processing was conducted using Agilent ChemStation and LC/MS Trap software, enabling precise characterization of anthocyanin profiles in the grape samples.

Determination of sugars in grape berries

Sugar determination followed Liu et al.'s [31] methodology. A 5 g sample was mixed with 40 mL ultrapure water and 25 mL methanol in a 100-mL flask, then volume-adjusted with ultrapure water. After centrifugation at 10,000 rpm for 10 minutes, the filtrates were membrane-filtered (0.45 µm) and collected. The extracts were analyzed using an Agilent 1100 Series HPLC with a refractive index detector, employing a Hypersil GOLD Amino column (5 µm, 250 mm × 4.6 mm). Analysis conditions included a column temperature of 30 °C and a mobile phase of acetonitrile/water (80:20) at a flow rate of 1.3 mL/min, enabling precise sugar quantification.

Statistical analysis

All experiments were designed as a two-factor factorial experimental layout (4 × 2), where the first factor represented NaCl treatments, and the second factor represented shikonin treatments. Each parameter was evaluated using three technical replicates, with three vines per replicate. A Two-Way ANOVA was performed using IBM SPSS software version 22.0 to assess interactions between the two independent variables. Post hoc comparisons were conducted using Duncan's multiple range test at a significance level of $p \leq 0.05$ to determine differences between treatment means. Data were presented as mean values along with their corresponding standard deviations (SD). To visualize the relationships and intensities among factors and examined traits, a hierarchical clustering heatmap was generated [32, 33] using the SRPLOT online platform (https://www.bioinformatics.com.cn/en, accessed on October 11, 2024). Additionally, to determine the directionality of the relationships between factors and the studied traits, Principal Component Analysis (PCA) was performed using GraphPad Prism version 9.3.1 (GraphPad Software, LLC, San Diego, CA, USA). The results were illustrated through a biplot following the methodology outlined by Evgenidis et al. [34].

Results

Phenolic compounds in grape berries

Our analysis revealed significant interaction effects (NaCl × Shikonin) for most phenolic compounds (Fig. 1 and Supplemental Material Table 1). The interaction significantly influenced gallic acid ($p = 0.004$), vanillic acid ($p = 0.002$), trans-caffeic acid ($p = 0.001$), trans-p-coumaric acid ($p = 0.000$), caftaric acid ($p = 0.017$), catechin ($p = 0.049$), epicatechin ($p = 0.000$), quercetin ($p = 0.005$), rutin ($p = 0.004$), and tyrosol ($p = 0.017$). Shikonin application (25 µM) at 0 mM NaCl increased gallic acid concentration by 196% compared to control conditions (from 2.18 to 6.45 µg $g^{-}1$ FW). Under severe salt stress (150

mM NaCl), shikonin application increased vanillic acid levels by 185% (from 3.46 to 9.86 μg g^{-1} FW). Similarly, shikonin treatment enhanced trans-caffeic acid by 204% (from 2.49 to 7.57 μg g^{-1} FW) and trans-p-coumaric acid by 207% (from 3.11 to 9.56 μg g^{-1} FW) compared to their respective controls. Ferulic acid showed a 312% increase with shikonin treatment (from 0.96 to 3.95 μg g^{-1} FW), while caftaric acid levels improved by 179% (from 5.17 to 14.42 μg g^{-1} FW). Flavonoids including catechin and epicatechin increased by 184% and 209% respectively with shikonin application. Quercetin exhibited the most dramatic response to shikonin treatment with a 397% increase (from 1.28 to 6.36 μg g^{-1} FW), while rutin and myricetin increased by 193% and 188%, respectively. Tyrosol concentrations showed a 199% enhancement with shikonin treatment (from 5.71 to 17.05 μg g^{-1} FW) (Fig. 1).

Organic acids in grape berries

The interaction between NaCl and shikonin significantly affected organic acid accumulation in grape berries (Fig. 2 and Supplemental Material Table 2). At 0 mM NaCl, shikonin application (25 μM) increased oxalic acid concentration by 38% (from 10.61 to 14.66 g L^{-1} FW), while under severe salt stress (150 mM NaCl), shikonin improved oxalic acid levels by 37% (from 3.13 to 4.30 g L^{-1} FW). Shikonin treatment enhanced pionic acid content by 33% at 0 mM NaCl and by 119% at 150 mM NaCl compared to their respective controls. For tartaric acid, shikonin application resulted in a remarkable 262% increase under severe salt stress (from 4.41 to 15.95 g L^{-1} FW). Fumaric acid showed a 43% improvement with shikonin at 0 mM NaCl, while the enhancement reached 88% under 150 mM NaCl. Malonic acid levels increased by 55% with shikonin application under control conditions and by 94% under severe salt stress. Lactic acid, the most abundant organic acid measured, increased by 49% with shikonin at 0 mM NaCl (from 145.48 to 216.81 g L^{-1} FW) and by 72% at 150 mM NaCl. Citric acid content improved by 19% and 26% with shikonin application at 0 mM and 150 mM NaCl, respectively. Maleic acid showed an 88% increase with shikonin under control conditions and a 59% improvement under severe salt stress. Maric acid levels were enhanced by 55% at 0 mM NaCl and by 94% at 150 mM NaCl with shikonin treatment. Succinic acid was the only compound that showed better response without shikonin under control conditions, but still showed a 25% improvement with shikonin under severe salt stress (Fig. 2).

Anthocyanins in grape berries

The interaction between NaCl and shikonin significantly ($p \leq 0.001$) affected anthocyanin profiles in grape berries (Fig. 3 and Supplemental Material Table 3). Shikonin application (25 μM) substantially increased all measured anthocyanins: delphinidin-*3-O*-glucoside by 104%, cyanidin-*3-O*-glucoside by 72%, petunidin-*3-O*-glucoside by 71%, peonidin-*3-O*-glucoside by 54%, and malvidin-*3-O*-glucoside by 131%. The interaction effects varied dramatically across different NaCl concentrations. At 50 mM NaCl, shikonin application decreased most anthocyanins, with reductions of 1% in delphinidin-*3-O*-glucoside, 23% in cyanidin-*3-O*-glucoside, 27% in petunidin-*3-O*-glucoside, and approximately 33% in malvidin-*3-O*-glucoside compounds. At 100 mM NaCl, shikonin treatment further reduced anthocyanin content, with delphinidin-*3-O*-glucoside decreasing by 34%, and malvidin-*3-O*-glucoside compounds showing nearly 50% reduction. However, peonidin-*3-O*-glucoside acetyl showed a slight increase of 1.3%. Most notably, under severe salt stress (150 mM NaCl), shikonin application dramatically reversed the negative effects of salinity, increasing delphinidin-*3-O*-glucoside by 264%, petunidin-*3-O*-glucoside by 264%, peonidin-*3-O*-glucoside by 312%, and malvidin-*3-O*-(p-coumaryl)-glucoside by 378%. This demonstrates shikonin's protective role against salt-induced anthocyanin degradation under high salinity conditions (Fig. 3).

Sugars in grape berries

The interaction between NaCl and shikonin significantly affected sugar content in grape berries (Fig. 4 and Supplemental Material Table 4). At 0 mM NaCl, shikonin application (25 μM) increased glucose content by 43% (from 9.42 to 13.47 g L^{-1} FW) and fructose by 41% (from 10.17 to 14.34 g L^{-1} FW). Under increasing NaCl concentrations, the beneficial effect of shikonin diminished, but still provided some protection against salt-induced sugar reduction. Rhamnose content increased by 47% with shikonin application and showed improvements across all NaCl concentrations, with the most pronounced effect at lower salinity levels. Similarly, galactose content increased by 42% with shikonin at 0 mM NaCl (from 2.17 to 3.08 g L^{-1} FW). Xylose levels improved by 47% with shikonin, while arabinose showed a 42% increase (from 2.10 to 2.99 g L^{-1} FW). Under severe salt stress (150 mM NaCl), the protective effect of shikonin was less pronounced for most sugars, but still resulted in modest improvements compared to salt stress alone. Notably, severe salt stress (150 mM NaCl) caused dramatic reductions in all sugar compounds compared to control conditions, with decreases of 84% in sucrose, 88% in glucose, 80% in fructose, 86% in rhamnose, 96% in galactose, 91% in xylose, and 92% in arabinose. Shikonin application partially mitigated these negative effects, especially at moderate salinity levels (Fig. 4).

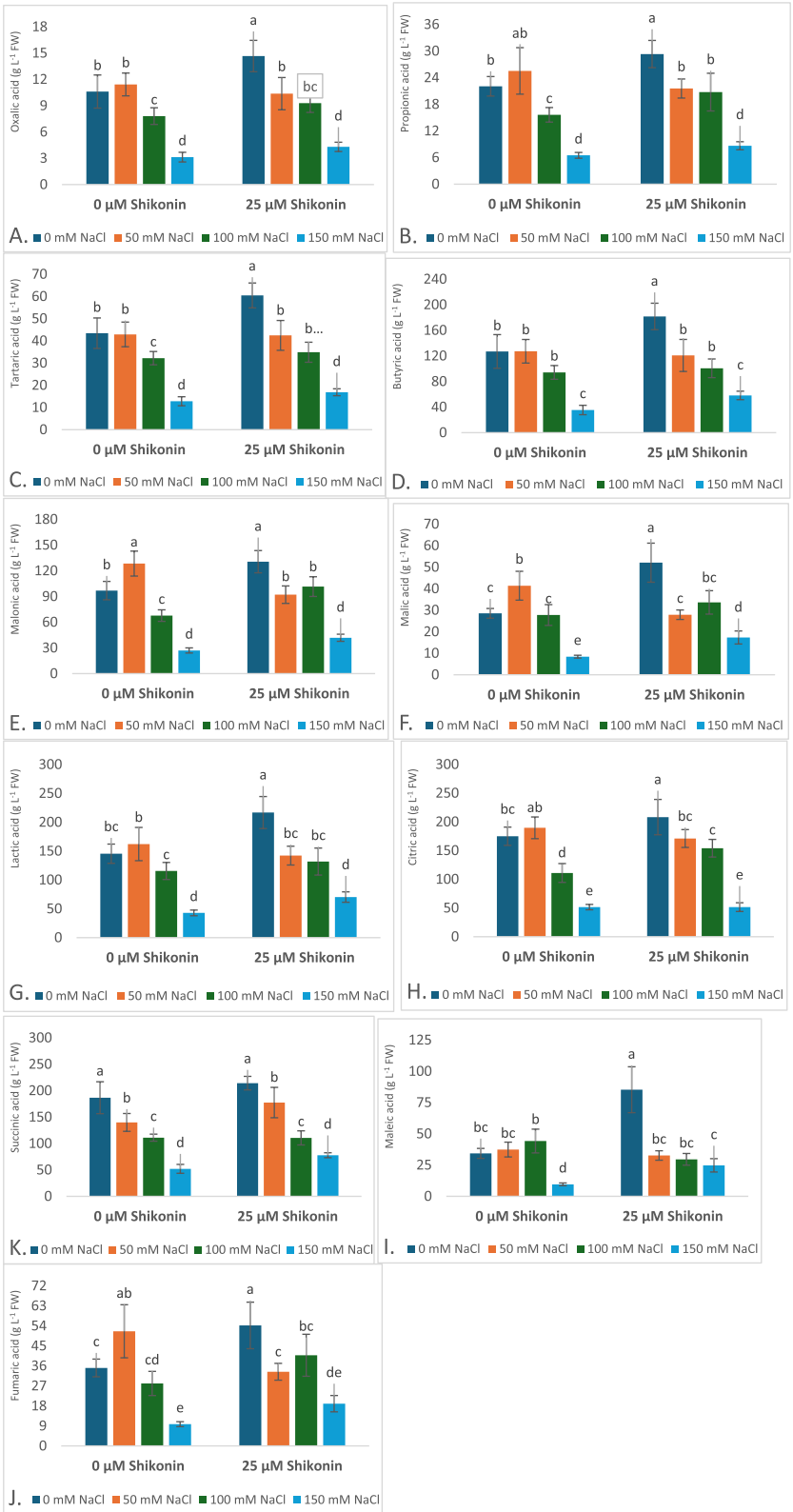

Fig. 2 Effects of different concentrations of shikonin applications on organic acid profile of grapes against salt stress. Different lower case letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$. Data are mean values ± SE

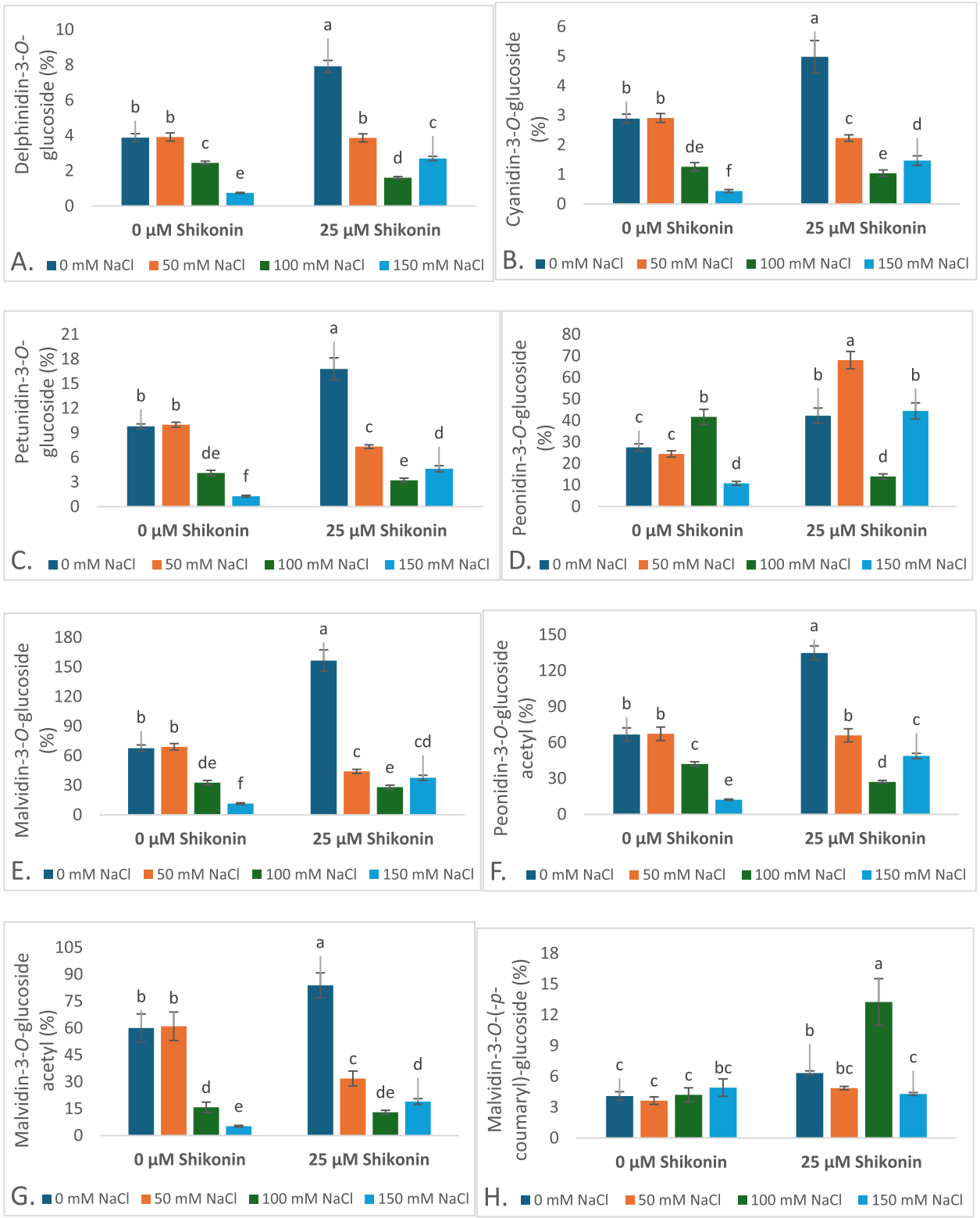

Fig. 3 Effects of different concentrations of shikonin applications on anthocyanin profile of grapes against salt stress. Different lower case letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$. Data are mean values ± SE

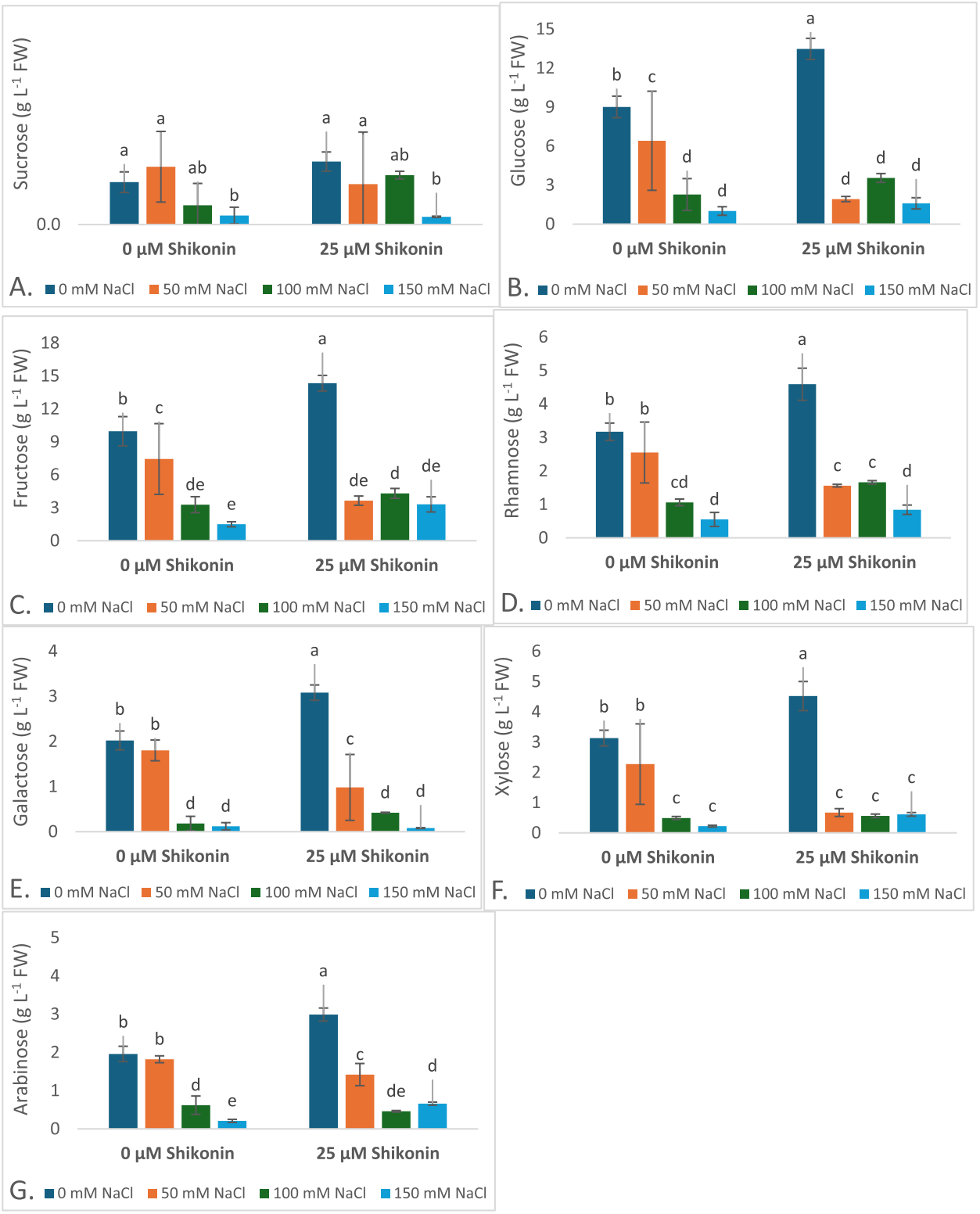

Fig. 4 Effects of different concentrations of shikonin applications on sugar profile of grapes against salt stress. Different lower case letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$. Data are mean values ± SE

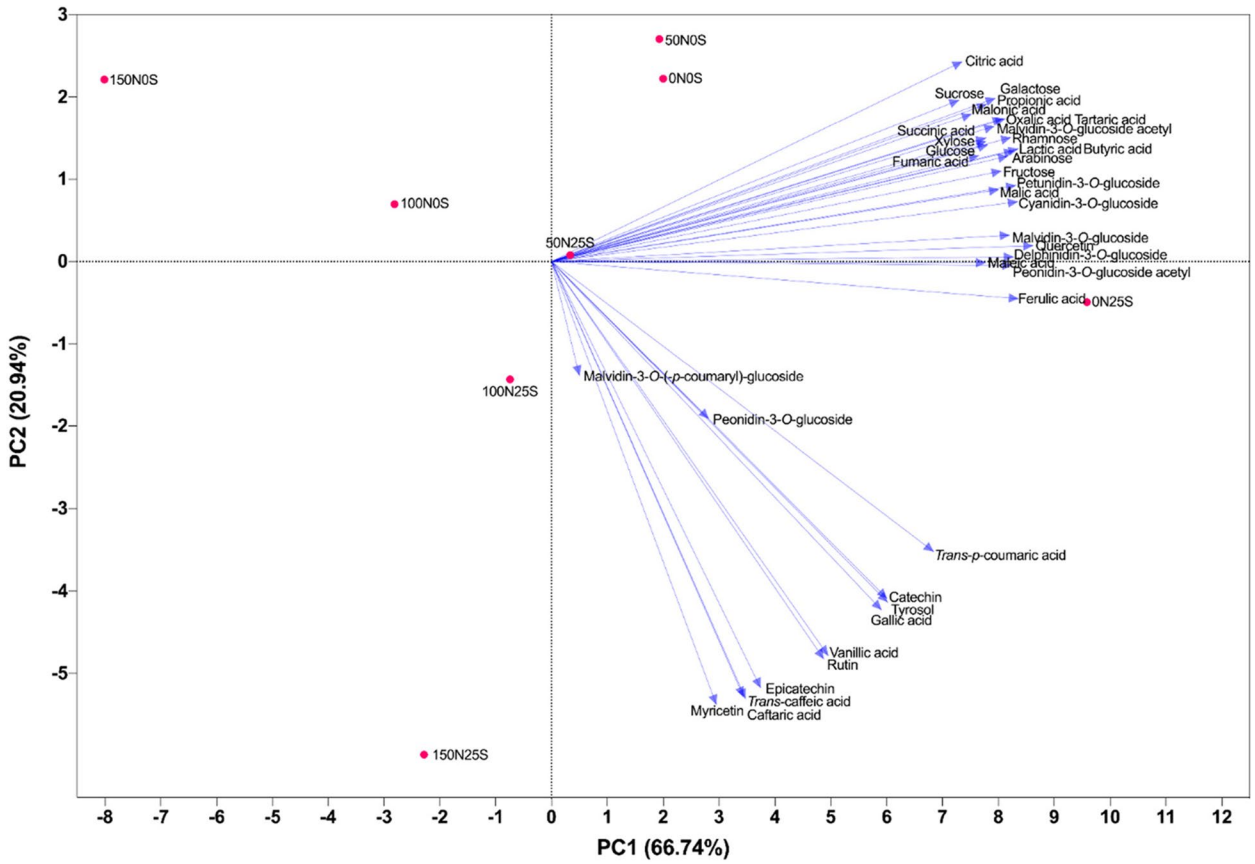

Fig. 5 Principal Component Analysis (PCA) showing the direction of the effects of different concentrations of shikonin treatments on phenolic compounds, organic acids, anthocyanins and sugar profile of grapes against salt stress

The evaluation of PCA and heatmap

Our PCA results indicated that the majority of the data variation was explained by the first two principal components (PC1 and PC2) (Fig. 5). In terms of eigenvalues, PC1 had an eigenvalue of 25.36, while PC2 had an eigenvalue of 7.96. PC1 accounted for 66.74% of the total variance, and PC2 explained 20.94%, with the two components together encompassing 87.68% of the total variance. This demonstrated that most of the data could be summarized by these two components, which play a critical role in understanding the profile of the compounds. The biplot analysis revealed that the position of each compound along the PC1 and PC2 axes reflected the concentrations and relationships of the compounds in the samples. Compounds such as ferulic acid, quercetin, and catechin were concentrated on the positive side of PC1, whereas compounds like malvidin−*3-O*-(-p-coumaryl)-glucoside were located on the negative side of PC1. This indicated that the PC1 axis created a distinction based on the concentrations of these compounds in the samples, with most samples being grouped according to the presence of these compounds. Additionally, a distinct variation was observed along PC2, where compounds such as sucrose and malvidin−*3-O*-glucoside exhibited high positive values, while sugars like glucose and fructose displayed negative values. Differences between samples were also clarified through PCA. The 0 mM NaCl (no salt) + 0 µM Shikonin (no shikonin) (0 N0S) treatment had positive values on both PC1 and PC2, indicating a high concentration of compounds and suggesting that this sample had a rich compound profile, occupying a substantial area on both axes. In contrast, the 150 mM NaCl + 25 µM Shikonin (150 N25S) treatment had highly negative values on PC1 but more positive values on PC2, indicating low concentrations of compounds influenced by PC1 but a more balanced concentration of compounds associated with PC2. Similarly, the 50 N25S treatment showed negative values on both PC1 and PC2, indicating that this sample was differentiated based on varying concentrations of its components. On the other hand, our hierarchical clustering heatmap analysis clearly illustrated the concentrations of compounds in each treatment and their relationships (Fig. 6). Sugars such as glucose and fructose were

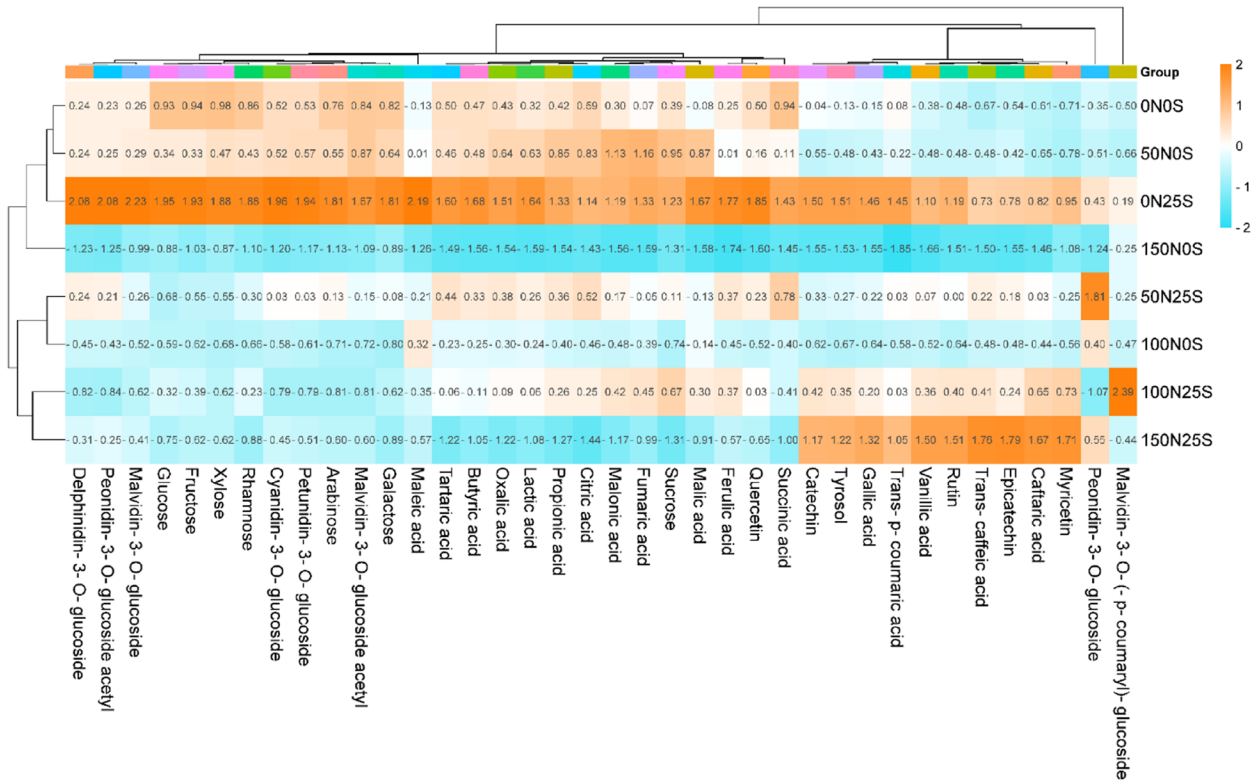

Fig. 6 Hierarchical clustering heat map analysis showing the intensity of effects of different concentrations of shikonin treatments on phenolic compounds, organic acids, anthocyanins and sugar profile of grapes against salt stress. The control group received no salt or shikonin (0 N0S; 0 mM NaCl + 0 µM Shikonin). Mild salt stress was applied without shikonin in the 50 N0S group (50 mM NaCl + 0 µM Shikonin), while moderate and severe salt stresses were applied in the 100 N0S (100 mM NaCl + 0 µM Shikonin) and 150 N0S (150 mM NaCl + 0 µM Shikonin) groups, respectively. Shikonin alone was administered in the absence of salt stress in the 0 N25S group (0 mM NaCl + 25 µM Shikonin). Combined treatments included 50 N25S (50 mM NaCl + 25 µM Shikonin), 100 N25S (100 mM NaCl + 25 µM Shikonin), and 150 N25S (150 mM NaCl + 25 µM Shikonin), representing increasing salt levels supplemented with shikonin

generally observed at lower concentrations, particularly in treatments like 50 mM NaCl + 25 µM Shikonin (50 N25S) and 100 mM NaCl + 0 µM Shikonin (100 N0S), where their levels were notably reduced. In contrast, flavonoid glycosides such as malvidin−*3-O*-glucoside and cyanidin−*3-O*-glucoside were found at higher levels in treatments like 0 mM NaCl (no salt stress) + 25 µM Shikonin (0 N25S), indicating a rich phenolic compound profile in these samples. Organic acids, including tartaric acid, malic acid, and citric acid, were predominantly present at high concentrations across treatments. However, certain organic acids like oxalic acid, succinic acid, and butyric acid were detected at lower levels. The 0 N0S treatment exhibited high concentrations of many compounds and was particularly rich in phenolic compounds, while the 150 N25S treatment showed elevated levels of certain compounds but lower concentrations of others, such as rutin and quercetin, suggesting a more diverse compound profile. Compounds such as gallic acid, vanillic acid, quercetin, and rutin were found at high levels in 0 N0S and 150 N25S treatments, highlighting the phenolic richness of these samples and the co-localization of these compounds. While sugars like glucose, fructose, and sucrose often showed high values alongside organic acids, their distribution varied across treatments. For example, some sugars like xylose were found at very low levels in 150 N25S treatments.

Discussion

Phenolic compounds in grape berries

Our study revealed significant insights into the complex interactions between sodium chloride (NaCl) and shikonin treatments on phenolic compound concentrations in grape berries (Fig. 1 and Supplemental Material Table 1). Berry biochemistry under salt stress and shikonin treatment reveals complex phenolic dynamics intersecting broader viticulture stress response mechanisms. Indeed, Waśkiewicz et al. [35] demonstrated that salt stress significantly alters phenolic profiles in grape berries, consistent with our observation of substantial

variations in compounds like gallic acid and vanillic acid. Similarly, Rienth et al. [36] and Daldoul et al. [13] showed that environmental stressors trigger adaptive biochemical mechanisms, particularly in phenolic biosynthesis pathways, which align closely with our findings of compound-specific responses to NaCl and shikonin treatments. Regarding organic acid metabolism, our results demonstrate that shikonin significantly counteracts the detrimental effects of salt stress through several key mechanisms. The pronounced increase in tartaric acid (262% under severe salt stress) with shikonin application likely results from enhanced activity of L-ascorbic acid catabolism pathway enzymes, particularly L-idonate dehydrogenase, which is typically suppressed under salt stress [37]. Similarly, the observed 72% increase in lactic acid at high NaCl concentrations with shikonin treatment suggests activation of alternative pyruvate metabolism pathways that bypass salt-inhibited TCA cycle enzymes [38]. The protective effect of shikonin on citric acid levels (26% improvement under severe salt stress) can be attributed to its role in stabilizing mitochondrial membrane integrity, as documented by Wang et al. [39], which maintains citrate synthase activity even under ionic stress conditions. The mechanism behind fumaric acid enhancement (88% under 150 mM NaCl) likely involves shikonin-mediated upregulation of succinate dehydrogenase activity, an enzyme particularly vulnerable to salt-induced oxidative damage [40]. Additionally, the significant improvements in oxalic and malonic acid concentrations with shikonin application reflect enhanced regulation of glyoxylate cycle enzymes, which serve as an adaptive mechanism for carbon skeleton preservation under stress conditions [41]. Interestingly, the differential response of succinic acid compared to other TCA cycle intermediates suggests compartment-specific effects of shikonin on mitochondrial metabolism, consistent with findings by Das et al. [42] on subcellular stress response variability. The interaction between salinity and secondary metabolite production represents a critical area of investigation.

Our data shows nuanced concentration changes across multiple phenolic compounds, echoing work by Haider et al. [37], who highlighted the dynamic nature of grape berry biochemical responses under stress conditions. The significant variations observed in *trans-*caffeic acid, *trans-p-*coumaric acid, and quercetin concentrations suggest sophisticated cellular adaptation mechanisms that go beyond simple linear stress responses. Intriguingly, the combinatorial effects of NaCl and shikonin treatments produced highly significant interactions for multiple phenolic compounds, a phenomenon partially explained by Hao et al. [38] in their comprehensive review of plant stress signaling. The observed concentration ranges, such as tyrosol varying from 5.71 to 17.05 μg $g^{-}1$ FW, indicate remarkable biochemical plasticity that could have implications for grape berry quality and stress tolerance. The pronounced effects of shikonin, with nearly all phenolic compounds showing statistically significant changes, represent a novel insight into potential stress mitigation strategies in viticulture. These observations align with research exploring plant stress response mechanisms, particularly the intricate interactions between environmental stimuli and secondary metabolite biosynthesis [37, 38]. On the other hand, our PCA and heat map analyses revealed that tartaric, malic, and citric acids were abundant across treatments, especially in 0 N0S, highlighting their role in flavor and acidity. However, under 150 mM NaCl + 25 μM Shikonin treatment (e.g., 150 N25S) exhibited variations in organic acid profiles (Figs. 5 and 6). Salt stress fundamentally disrupts organic acid metabolism through multiple mechanisms: osmotic effects that reduce water availability for hydrolytic reactions, ionic toxicity that directly inhibits acid-metabolizing enzymes, and energy diversion toward stress response pathways at the expense of normal acid synthesis [43]. Shikonin appears to mitigate these effects by stabilizing enzyme structures against salt-induced conformational changes, enhancing membrane integrity to maintain compartmentalization of acid metabolism, and potentially activating alternative biosynthetic pathways less susceptible to salt inhibition [44]. The observed metabolic resilience in shikonin-treated samples, particularly evident in the maintenance of key flavor-related acids under stress, aligns with Wang et al.'s [39] findings on bioactive compounds capable of reprogramming primary metabolism under adverse conditions.

Organic acids in grape berries

The accumulation of organic acids in grape berries plays a critical role in determining their biochemical properties and resilience to environmental stressors. In this study, we demonstrated that both NaCl and shikonin treatments, along with their interactions, influenced the concentrations of organic acids in grape berries (Fig. 2 and Supplemental Material Table 2). The substantial reductions in phenolic compound levels under high NaCl stress, despite the application of shikonin, underscore the profound impact of salinity on grape metabolism. Our findings align with previous research indicating that salinity stress disrupts phenolic biosynthesis by impairing key metabolic pathways, such as glycolysis and the tricarboxylic acid (TCA) cycle, which provide essential precursors for phenolic synthesis [5, 35]. For instance, tartaric acid, which reached its highest concentration (60.46 ± 5.61 g $L^{-}1$ FW) at 0 mM NaCl with 25 μM shikonin, exhibited a pronounced decline under 150 mM

NaCl. This trend corroborates the observations of Das et al. [42], who reported that salinity stress inhibits organic acid synthesis through a reduction in TCA cycle enzyme activity. Similarly, the sharp decline in fumaric acid levels under high NaCl concentrations is consistent with the findings of Reta et al. [43] and Lu et al. [44], who suggested that osmotic stress significantly limits the flux of the TCA cycle, thereby restricting the synthesis of downstream metabolites. Interestingly, the application of shikonin partially alleviated the negative effects of NaCl stress on specific compounds, such as oxalic acid and lactic acid, supporting the findings of Shi et al. [45], who highlighted shikonin's role in enhancing stress tolerance through modulation of metabolic and antioxidant pathways. However, the relatively modest improvement under severe stress conditions (e.g., succinic acid levels at 150 mM NaCl with 25 µM shikonin) suggests that the protetive efficacy of shikonin is contingent upon both the severity of the stress and the metabolic pathway involved. This observation is in line with Hasanuzzaman et al. [46], who proposed that while secondary metabolites like shikonin bolster antioxidant defenses, they may not fully restore metabolic fluxes under extreme stress conditions. The pronounced reductions in citric acid and malonic acid under high salinity stress further reinforce the notion that salt stress disrupts cellular energy metabolism, leading to resource reallocation away from organic acid biosynthesis [47]. Nevertheless, shikonin was found to enhance citric acid levels under moderate salinity (e.g., 208.28 g L^{-1} FW at 0 mM NaCl with 25 µM shikonin, representing a 11.9 % increase compared to untreated controls), consistent with findings by Almagro et al. [48], who suggested that phenolic modulators can stabilize mitochondrial function, thereby supporting organic acid synthesis under mild to moderate stress conditions. The differential responses of specific organic acids to NaCl and shikonin interactions indicate potential variations in their regulatory pathways. For instance, lactic acid levels were significantly elevated under 0 mM NaCl with shikonin application, suggesting that shikonin may promote the diversion of glycolytic intermediates toward organic acid biosynthesis [49]. However, the dramatic decline in lactic acid concentrations under 150 mM NaCl highlights the limitations of shikonin in counteracting the combined osmotic and ionic stresses associated with severe salinity. The observed variability in phenolic compound profiles across treatments likely reflects genotype-specific differences in stress responses. Grapes are well-known for their diverse phenolic composition, which is influenced by genetic factors, environmental conditions, and agronomic practices [50]. The differential efficacy of shikonin observed in our study showed the importance of optimizing its application based on stress severity and grape genotype to maximize its beneficial effects. Therefore, our findings contributed valuable insights into the complex interplay between salinity stress, phenolic metabolism, and exogenous modulator applications in grape berries. On the other hand, our PCA and heat map analyses revealed that phenolic compounds such as quercetin, rutin, and gallic acid were elevated in salinity-stressed treatments (150 N25S), indicating enhanced phenolic synthesis under salt stress. This agrees with Shiraishi et al. [50] and Almagro et al. [48], who reported increased phenolic content as a defense response to oxidative stress caused by salinity. These compounds are vital for antioxidant activity and contribute to grape and wine quality.

Anthocyanins in grape berries

Our findings revealed that the accumulation of specific anthocyanins in grape berries was significantly ($p \leq 0.001$) influenced by NaCl and shikonin treatments, both individually and in combination, with marked variations in their responses depending on treatment levels (Fig. 3 and Supp. Material Table 3). These results highlighted the intricate interaction between salt stress and the exogenous application of phenolic modulators like shikonin in modulating anthocyanin biosynthesis. Under control conditions (0 mM NaCl and 0 µM shikonin), baseline anthocyanin levels were relatively low, but the application of 25 µM shikonin significantly enhanced the accumulation of key anthocyanins. For example, delphinidin–*3-O*-glucoside increased by 104.4%, cyanidin–*3-O*-glucoside by 72.3%, and malvidin–*3-O*-glucoside by 131.2%. These findings are consistent with previous studies reporting that phenolic inducers like shikonin can stimulate the phenylpropanoid pathway, leading to increased synthesis of anthocyanins and other flavonoids [51]. The dramatic increase in malvidin–*3-O*-glucoside, the most abundant anthocyanin, aligns with observations by Mansour [52], who demonstrated that exogenous phenolic compounds can enhance anthocyanin accumulation by upregulating key genes such as UFGT and DFR in the anthocyanin biosynthetic pathway. Interestingly, under moderate salt stress (50 mM NaCl), shikonin application had mixed effects. While delphinidin–*3-O*-glucoside levels remained relatively stable, other anthocyanins, including cyanidin–*3-O*-glucoside and petunidin–*3-O*-glucoside, showed significant ($p \leq 0.001$) reductions. This decline might be attributed to salt-induced oxidative stress, which disrupts anthocyanin biosynthesis by impairing precursor availability or enzymatic activity [42, 44]. However, peonidin–*3-O*-glucoside acetyl showed only minor reductions under these conditions, suggesting a differential regulatory mechanism for acetylated anthocyanins under stress. Such differential responses among anthocyanins have been reported by de Rosas

et al. [53], who noted that structural modifications like acetylation or coumarylation can influence anthocyanin stability and accumulation under stress conditions. At higher salt concentrations (100 mM NaCl), the inhibitory effects of salinity on anthocyanin synthesis became more pronounced. Significant reductions ($p \leq 0.001$) were observed for delphinidin–*3-O*-glucoside (−34.4%), malvidin–*3-O*-glucoside (−49.1%), and malvidin–*3-O*-glucoside acetyl (−58.6%). These findings are consistent with reports by de Rosas et al. [53], which suggested that high salinity disrupts anthocyanin biosynthesis by limiting carbon flux through the phenylpropanoid pathway and reducing enzymatic activities involved in anthocyanin modifications. However, the slight increase in peonidin–*3-O*-glucoside acetyl under these conditions suggests a possible compensatory mechanism aimed at enhancing anthocyanin stability under stress. Remarkably, under severe salt stress (150 mM NaCl), the application of 25 µM shikonin led to substantial increases in some anthocyanins. For instance, delphinidin–*3-O*-glucoside and petunidin–*3-O*-glucoside increased by over 263%, while peonidin–*3-O*-glucoside showed a remarkable rise of 312.3%. These results suggest that shikonin might activate stress-responsive pathways that enhance anthocyanin biosynthesis even under extreme conditions. Previous studies have proposed that shikonin can modulate antioxidant and hormonal signaling pathways, which may enhance the resilience of biosynthetic processes under stress [54]. The substantial increase in malvidin–*3-O*-(p-coumaryl)-glucoside (378.3%) under severe stress further supports the hypothesis that coumarylation enhances anthocyanin stability, allowing for their accumulation under adverse conditions [44]. On the other hand, our PCA and heat map analyses revealed salinity stress positively influenced anthocyanin levels, with malvidin–*3-O*-glucoside and cyanidin–*3-O*-glucoside more abundant in stressed samples like 150 N25S. This aligns with de Rosas et al. et al. [53], who demonstrated that salt stress enhances anthocyanin biosynthesis by upregulating key enzymes involved in pigment production.

Sugars in grape berries

Based on our results, NaCl and Shikonin treatments significantly influenced the sugar content in grape berries, with distinct effects observed across different sugar types (Fig. 4 and Supplemental Material Table 4). Sucrose content exhibited a notable decline with increasing NaCl concentrations, decreasing by 84.15% from 0.82 g L^{-1} FW at 0 mM NaCl to 0.13 g L^{-1} FW at 150 mM NaCl. This reduction aligns with previous findings suggesting that salinity stress disrupts sucrose metabolism by downregulating biosynthetic enzymes or transporters [55]. Interestingly, shikonin treatments alone did not significantly alter sucrose levels, indicating that sucrose metabolism is primarily governed by salinity stress rather than phenolic compound modulation. Glucose and fructose levels demonstrated similar trends, with the highest concentrations observed under 0 mM NaCl and 25 µM shikonin. Glucose decreased by 88.05% and fructose by 80.28% at 150 mM NaCl compared to 0 mM NaCl. While shikonin enhanced glucose and fructose levels under non-stress conditions, its protective effect was minimal under severe salinity, consistent with studies indicating that phenolic compounds can transiently support metabolic activity but are insufficient to counteract the prolonged effects of high salinity [56, 57]. These results suggest that shikonin may enhance glycolysis and sugar synthesis under mild conditions but is limited by the broader metabolic disruptions induced by osmotic and ionic stress. Rhamnose and galactose contents also declined sharply with increasing NaCl concentrations, showing reductions of 85.80% and 96.08%, respectively, at 150 mM NaCl. Shikonin slightly increased rhamnose levels across all NaCl treatments, particularly under low salinity, possibly through enhanced sugar transporter activity or stabilization of energy metabolism pathways [54]. However, galactose content was more sensitive to NaCl, and shikonin's impact on this sugar was limited, highlighting the differential regulation of individual sugars under stress conditions. Xylose and arabinose followed a similar pattern, with significant reductions under high NaCl conditions. Xylose decreased by 90.71%, and arabinose by 91.53% at 150 mM NaCl, consistent with reports that salinity stress disrupts cell wall biosynthesis and sugar interconversion pathways [53, 55]. While shikonin slightly improved arabinose content under low NaCl conditions, its effect was diminished under severe stress, further suggesting that its protective capacity is constrained by stress severity and the metabolic pathways involved. In general, our findings highlight that the interaction of NaCl and shikonin treatments significantly modulates sugar metabolism in grape berries. Shikonin showed potential in mitigating the adverse effects of mild salinity stress by enhancing sugar accumulation, particularly for glucose, fructose, and rhamnose. However, its efficacy was limited under severe NaCl stress, indicating that the protective effects of phenolic compounds may not fully counterbalance the metabolic disruptions induced by high salinity. On the other hand, our PCA and heat map analyses indicated sugars such as glucose, fructose, and sucrose showed reduced levels in treatments like 50 mM NaCl + 25 µM Shikonin (50 N25S) under salinity stress, indicating an inhibitory effect on sugar metabolism. This is consistent with Griffiths et al. [50], who reported that salinity impacts photosynthesis and

sugar translocation, leading to lower sugar accumulation. As sugars are crucial for sweetness and fermentation, their reduction under salt stress may affect grape quality and wine production potential.

Conclusion

Based on our findings, this study highlighted the metabolic adaptations induced by salt stress in the grape (*Vitis vinifera* L. *cv* Öküzgözü) and demonstrated the regulatory effects of shikonin applications. Salt stress caused significant changes in key metabolites, including phenolic compounds, organic acids, anthocyanins, and sugars. However, shikonin treatments alleviated these effects by strengthening plant defense mechanisms and enhancing metabolic resilience. The combination of 150 mM NaCl and 25 µM shikonin proved to be the most effective treatment, as it significantly increased the levels of phenolic compounds (e.g., ferulic acid, quercetin), organic acids (e.g., tartaric acid, malic acid), and anthocyanins (e.g., malvidin−*3-O*-glucoside). PCA and hierarchical clustering analyses confirmed that shikonin preserved and enhanced metabolic diversity under salt stress conditions, facilitating improved metabolic balance and stress tolerance. Current our results suggested that shikonin acted as a potent biostimulant, alleviating oxidative damage through enhanced phenolic and antioxidant activity while supporting energy metabolism and ion regulation. Findings also provided evidence for shikonin's potential application in sustainable viticulture, offering a novel strategy to manage salinity stress. Further studies should investigate its effects on different grape varieties and agricultural conditions to expand its use in stress management and improve agricultural productivity.

Abbreviations

ROS	Reactive oxygen species
PSI	Photosystem I
PSII	Photosystem II
HPLC	High-performance Liquid Chromatography
LC/MSD	Liquid Chromatography/Mass Spectrometry Detector
ESI-MS	Electrospray Ionisation Mass Spectrometry
PCA	Principal Component Analysis
SD	Standard Deviations
TCA	Tricarboxylic Acid

Supplementary Information

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Supplementary Material 1.

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Plant material and permits

All plant materials used in this study, including 'Öküzgözü' (*Vitis vinifera* L.) grafted onto 1103 Paulsen rootstock, were obtained from a commercial vineyard managed by Adem Yagci in the Central Anatolia Region of Turkey. No wild specimens were used. The study complied with all relevant institutional, national, and international guidelines for cultivated plant research, and no specific collection permits were required.

Author's contributions

A.Y. was involved in the investigation, methodology, conceptualization, draft preparation, software, and formal analysis. The author wrote and reviewed the manuscript and approved the final version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

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Competing interests

The authors declare no competing interests.

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