Research Article

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The exogenous application of naringenin and rosmarinic acid modulates functional traits in *Lepidium sativum*

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Abstract

BACKGROUND: Phenolic modulators have attracted attention for their potential in shaping functional traits in plants. This work investigated the impact of naringenin (Nar) and rosmarinic acid (RA) on the functional properties of *Lepidium sativum* leaves and roots.

Results: Untargeted metabolomics identified a diverse phenolic profile, including flavonoids, phenolic acids, low molecular weight phenolics, lignans, and stilbenes. Cluster, analysis of variance multiblock orthogonal partial least squares (AMOPLS), and orthogonal projection to latent structures discriminant analysis (OPLS-DA) multivariate analyses confirmed tissue-specific modulation of bioactive compounds. The tissue was the hierarchically most influential factor, explaining 27% of observed variability, while the treatment and their interaction were statistically insignificant. Thereafter, various *in vitro* assays were employed to assess antioxidant capacity, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), and ferric ion reducing antioxidant power (FRAP), metal chelating ability, and phosphomolybdenum (PMD) assays. Extracts were also tested for inhibitory effects on cholinesterase, amylase, glucosidase, and tyrosinase enzymes. RA application positively impacted antioxidant and enzyme inhibitory activities, holding valuable implications in shaping the health-promoting properties of *L. sativum*.

Conclusion: The untargeted metabolomics analysis showed a significant tissue-dependent modulation of bioactive compounds, determining no synergistic effect between applying phenolic compounds in combination. Specifically, the sole application of RA increased anthocyanins and hydroxyphenyl propanoic acid content on leaves, which was strictly related to enhancing the biological activities.

Supporting information may be found in the online version of this article.

Keywords: phenolic profiling; elicitors; chemometrics; functional foods; nutraceutical; bioactive constituents

INTRODUCTION

Lepidium sativum L., a fast-growing, edible annual herbaceous plant of the *Lepidium* genus and Brassicaceae family, is known as a garden cress in several world regions. *Lepidium sativum* has long been used traditionally to treat several illnesses, including asthma, uterine tumors, ulcers, hemorrhoids, cough, sores, dermatomycosis, dysmenorrhea, sciatica, and nasal polyps. Additionally, *L. sativum* leaves have diuretic and mild stimulant properties, and they are also used therapeutically for liver issues, edema, and other inflammatory diseases.[1] According to recent reports, *L. sativum* aqueous extract possesses several pharmacological properties, including antioxidant, anticancer, antibacterial, bronchodilator, allopathic, hypotensive, and hypoglycemic properties.

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Lepidium sativum has a phytochemical profile that includes flavonoids, phenols, cardiotonic glycosides, alkaloids, and coumarins, among others. In addition to these characteristics, roots, leaves, and seeds are used as vegetables in the kitchen.[2]

Important plants like *L. sativum* require eco-friendly agricultural methods to meet future food demand in the face of today's environmental concerns, and sustainable solutions must also be used. This brings up plant phenolic chemicals, which may promote plant development, thus increasing crop productivity, and protecting plants from environmental challenges.[3] Phenolics are aromatic chemicals frequently found in plants and are produced through the phenylpropanoid pathway as secondary metabolites. Recent research has demonstrated the role of phenolic compounds in promoting quick seed germination, balanced plant growth, rapid expansion in plant biomass, and more effective plant metabolism.[4] Additionally, they are naturally occurring bioactive chemicals that have key functions in various metabolic activities, including plant growth, cell differentiation, photosynthetic productivity, saccharization, and the modulation of defense responses.[5] Besides, these compounds serve as natural defense mechanisms against pathogens, acting as phytoalexins to inhibit the growth of harmful microorganisms, and contribute to allelopathy by enabling plants to release chemicals that suppress neighboring plant growth. The phenolic compounds elicit the production and activation of bioactive compounds through intricate molecular mechanisms via regulating gene expression and influencing the synthesis of various secondary metabolites. They also modulate enzyme activity, either activating enzymes involved in bioactive compound synthesis or inhibiting those responsible for their degradation. Acting as signaling molecules, phenolics stimulate cascades of biochemical reactions that lead to the production of bioactive molecules as part of plant defense responses.[6-8] Rosmarinic acid (RA) is a phenolic compound frequently found in many plant species, from hornworts to eudicotyledonous plants, having several uses in the pharmaceutical, food, and cosmetics industry.[9] According to the findings reported by Marchev *et al*.,[10] RA, a phenolic ester of caffeic acid, has several health-promoting properties and has been identified as a useful antioxidant having the ability to bind to biomacromolecules, chelate pro-oxidant ions, capture free radicals, and inhibit lipid peroxidation.[11] Additionally, it has been claimed that RA has no genotoxic effects and suppresses primary DNA damage.[12] Naringenin (4′,5,7-trihydroxyflavanone) (Nar) is another phenolic with antioxidant, free radical scavenger, anti-inflammatory, immune system modulation properties, widely distributed in citrus fruits.[13] It has recently been demonstrated that Nar contributes to enhanced plant growth, increased osmotic regulation, and increased vegetative development, biochemical accumulation, and protein bioavailability.[14]

Phenolic-derived compounds may have various synergistic or antagonistic effects on plants when combined,[15-17] but these effects are not currently well known. Therefore, the aim of this study is to investigate the potential synergistic, neutral, and antagonistic effects of exogenous RA and Nar application on the metabolic processes of *L. sativum*, aiming to enhance our understanding of how these compounds influence the overall bioactive compounds in plants. To this end, an untargeted metabolomics analysis was performed. This approach allows researchers to analyze the entire metabolite profile of plant tissues, providing a holistic view of the plant's metabolic response to exogenous treatments and then revealing which bioactive compounds are induced or altered.

MATERIALS AND METHODS

Plant material and experimental design

In the sterilization process of garden cress seeds (*L. sativum* L.), 5% sodium hypochlorite was used for 10 min, and the seeds were germinated. Next, 7 day-old *Lepidium* plants were grown in 1/4 Hoagland solution including 1 mmol/L KNO_3, 1 mol/L $CaNO_3$, 0.4 mol/L $MgSO_4$, 0.2 mol/L KH_2PO_4, 0.03 mol/L H_3BO_3, 0.002 mol/L $CuSO_4$, 0.004 mol/L $ZnSO_4$, 0.005 mol/L $MnCl_2$, 0.001 mol/L $(NH_4)Mo_7O_{24}$ and 0.2 mol/L Fe-EDTA under controlled conditions for 14 days. The solution was refreshed every 5 days. The RA (100 µmol/L) and Nar (100 µmol/L) were applied individually and in combination by adding to the growth medium. These concentrations were determined based on the reports observed by the authors Khaleghnezhad *et al*.[18] and Bido *et al*.[19]

Untargeted metabolomics analysis of bioactive compounds

Plant samples were prepared for untargeted metabolomics analysis by homogenizer-assisted solvent extraction. For this purpose, 1 g of dried samples was combined with 10 mL of a mixture consisting of 80% methanol (MeOH), 19.9% water (H_2O), and 0.1% formic acid (HCOOH) (purity ≥ 95%, Sigma-Aldrich, St Louis, MO, USA). The resulting extracts were then homogenized for 10 min and were centrifuged at 8000 × *g* for 15 min at a temperature of 4 °C. Subsequently, the supernatants (liquid above the sediment) were filtered using a 0.22-µm syringe filter and transferred into high-performance liquid chromatography (HPLC) vials. The analysis was carried out utilizing an ultra-high-pressure liquid chromatography quadrupole-time-of-flight mass spectrometry instrument (UHPLC-QTOF-MS) from Agilent Technologies, located in Santa Clara, CA, USA. The separation of compounds was accomplished using an Agilent InfinityLab Poroshell 120 pentafluorophenyl (PFP) column (2.1 mm × 100 mm, 1.9 µm) as the stationary phase and a combination of water and acetonitrile as the mobile phase. The parameters for mass spectrometry were optimized in previous studies by Zhang *et al*.[20]

For the analysis of bioactive compounds, the acquired data underwent a filtering process using Agilent Profinder B.10.0 software. Only compounds potentially identified in at least 75% of the replications under any given condition were retained. Phenolic compounds were identified using the 'find by-formula' algorithm, which compared the data against the Phenol-Explorer database (http://phenol-explorer.eu/)[21] and employed a confidence level of 2 for annotation.[22] The semi-quantitative analysis of various bioactive compound classes was performed by utilizing standard curves of cyanidin, catechin, luteolin, quercetin, gluconapin, ferulic acid, sesamin, resveratrol, and tyrosol (obtained from Merck KGaA, Darmstadt, Germany). The results were expressed as milligrams of phenolic equivalent per gram of dry extract. The detailed descriptions are provided as Supporting Information Methods S1.

Profile of bioactive compounds

Folin–Ciocalteu and aluminum chloride ($AlCl_3$) assays were utilized to determine the total phenolic and flavonoid contents.[23] Results were expressed as gallic acid equivalent (mg GAE/g extract) and rutin equivalent (mg RE/g extract) for respective assays.

Determination of antioxidant and enzyme inhibitory effects

The antioxidant and enzyme inhibitory activity of the tested extracts was determined according to the previously described Zhang *et al*.[20] 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), and

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ferric ion reducing antioxidant power (FRAP) were expressed as milligrams Trolox equivalent (TE) per gram of extract. The metal chelating ability (MCA) was reported as milligrams EDTA equivalent (EDTAE) per gram of extract, whereas the total antioxidant activity [phosphomolybdenum (PMD) assay] was expressed as mmol TE per gram of extract. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities were given as milligrams galanthamine equivalent (GALAE) per gram of extract; tyrosinase inhibitory activity was expressed as milligram kojic acid equivalent (KAE) per gram of extract; amylase and glucosidase inhibitory activities were presented as mmol acarbose equivalent (ACAE) per gram of extract.

Statistical analysis

The spectra obtained from UHPLC-QTOF-MS analysis were aligned and normalized using Mass Profiler Professional B.15.1 software from Agilent Technologies. For chemometric interpretation, unsupervised hierarchical cluster analysis (HCA) was then performed using the same software as described in Zhang *et al*.[24] The analysis of variance (ANOVA) multiblock orthogonal partial least squares (AMOPLS) was performed following the methodology outlined in a prior study,[25] following unit variance scaling, within the R studio environment. To validate the AMOPLS model and evaluate the statistical significance of the effects, a set of 10^4 random permutations was executed. Given that multiple components can be linked to a particular effect, the variable importance in the projection (VIP) metric is employed to assess the overall contribution of a specific variable to each effect. AMOPLS efficiently decomposes data variation by considering experimental design structure and multivariate characteristics. This enables more straightforward interpretation of metabolic variations resulting from multiple influences. AMOPLS provides a convenient and automated approach to evaluate experimental factors' main effects and potential interactions. Interpretation of effects is done through scores and loadings plots. Additionally, AMOPLS provides information on the proportion of total variation associated with each effect.[25] The supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) and validation models were conducted using SIMCA 16 software (Umetrics, Malmo, Sweden), as previously outlined by Zhang *et al*.[24] The OPLS-DA models for prediction were combined with VIP analysis to identify the metabolite markers involved in distinguishing between treatments for each plant organ. Pearson's correlation analysis was carried out using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) to examine the correlation between different phenolic classes and biological activities.

In the antioxidant and enzyme inhibitory assays, the values were expressed as mean ± standard deviation (SD) of three parallel experiments. One-way ANOVA with Tukey's *post hoc* was performed to determine between tested extracts in terms of antioxidant and enzyme inhibitory abilities. The statistical analysis was performed using XlStat 16.0 software.

RESULTS AND DISCUSSION

Total bioactive compounds and the untargeted phenolic profiling of leaf and root tissues treated with RA and Nar

Phenolic modulators have attracted interest in developing functional plant materials for agricultural applications.[26] These compounds have significant biological properties, including antioxidants and antimicrobial agents, and therefore could be considered versatile biological agents to improve human health.[26] In addition, these compounds are involved in the defense system of plants against biotic and abiotic stress.[27] With this in mind, we first tested the effect of Nar and RA applications (individually and combined) on the total content of phenols and flavonoids in *L. sativum* (cress) leaves and roots. The results are shown in Table 1. In the leaf groups, the total content of phenols was not affected by the application of both individual and combined applications of Nar and RA. However, the applications positively affected the roots, and the combination of Nar and RAs provided the highest value (32.37 mg GAE/g). Furthermore, we found no statistical differences between combined and individual RA applications (31.39 mg GAE/g) in root treatments ($P > 0.05$). Similarly, the total flavonoid content (TFC) was increased by applying the tested phenolic modulators, and these values ranged from 1.41 mg RE/g (in the control) to 3.70 mg RE/g (in the combined treatment). Similar to the total phenolic content (TPC), the TFC of the individual RA application was very close to that of the combined group ($P > 0.05$). In addition, the TFC of the leaves was reduced in all applications, with the highest total flavonoid found in the control group (4.87 mg RE/g). As a comparison between Nar and RA groups for both parts, RA was more effective than Nar. Consistent with our findings, several researchers have reported that the application of RA has been found to effectively modulate responses to biotic and abiotic stress.[5,28,29] The results show that the combination of Nar and RA treatment had lower total phenols and flavonoids levels than the individual treatments in the leaf groups. The finding could be explained by the antagonistic effect of these compounds.

An untargeted metabolomics UHPLC-QTOF-MS method was used to assess the impact of exogenous Nar, RA, and their combination (Nar + RA) on the phenolic profile of root and leaf tissues in *L. sativum*. The deconvolution of raw data using a specific bioactive compounds database resulted in annotating 524 compounds (Supporting Information, Table S1). Subsequently, an HCA that relied on fold-change values normalized by the median was applied to explore similarities and dissimilarities in the metabolic profiles. Supporting Information, Fig. S1 shows that the most differentiating factor was the type of plant tissues, as indicated by two main clusters (one for roots and the other for leaves) observed, indicating a distinct tissue type-dependent response. Moreover, there were distinct similarities among treatments depending on tissue types. In root tissues, there was a higher degree of similarity among the treatments, as they have been clustered within a single cluster. However, in leaf tissues, the Nar + RA treatment has been grouped into a single cluster compared to the other two treatments and the control group, confirming the effectiveness of a combination of Nar and RA on the phenolic profile of leaves.

Considering the bioactive compound classes, two main categories of phenolic compounds (including a total of 272 flavonoids, 104 phenolic acids, 58 lignans, 20 stilbenes, and 84 compounds associated with other polyphenols), and glucosinolates (40 compounds) were putatively identified, indicating the prevalence of flavonoids. In agreement, it has been reported that flavonoids are major constituents of the *L. sativum*.[30,31] In detail, anthocyanins were mostly represented by pelargonidin, cyanidin, delphinidin, malvidin, and petunidin derivatives. Among others, flavanones exhibited higher heterogeneity, represented by sakuranetin, eriodictyol, Nar, naringin, eriocitrin, and so forth (Table S1). The derivatives of apigenin and luteolin constituted most of the compounds related to flavones. In the same class, flavonols were mainly represented by kaempferol, patuletin, quercetin, isorhamnetin, and myricetin. Other frequent classes were phenolic

Table 1. The impact of rosmarinic acid (RA), naringenin (Nar) and their synergistic combination on the concentration of total phenolic content (TPC), total flavonoid content (TFC), as well as antioxidant properties (DPPH, ABTS, CUPRAC, FRAP, PMD, and MCA) in the leaves and roots of *Lepidium sativum*

Treatment – parts	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	PMD (mmol TE/g)	MCA (mg EDTAE/g)
Control – leaves	27.67 ± 1.66^{a}	4.87 ± 0.04^{a}	3.39 ± 0.31^{b}	26.70 ± 0.51^{a}	51.53 ± 0.94^{b}	26.68 ± 0.37^{b}	1.44 ± 0.09^{a}	34.31 ± 0.36^{a}
Nar – leaves	26.73 ± 0.41^{a}	2.07 ± 0.04^{d}	4.01 ± 0.92^{b}	27.06 ± 1.11^{a}	56.82 ± 0.57^{a}	28.93 ± 0.31^{a}	1.40 ± 0.06^{a}	31.46 ± 0.25^{b}
RA – leaves	27.28 ± 0.25^{a}	3.21 ± 0.17^{b}	5.83 ± 0.96^{a}	28.03 ± 1.26^{a}	48.55 ± 1.26^{c}	24.54 ± 0.59^{c}	1.42 ± 0.08^{a}	32.07 ± 0.27^{b}
Nar + RA – leaves	26.28 ± 0.38^{a}	2.39 ± 0.04^{c}	3.73 ± 0.23^{b}	23.11 ± 1.00^{b}	51.26 ± 0.99^{c}	25.57 ± 0.26^{c}	1.32 ± 0.04^{a}	31.79 ± 0.33^{b}
Control – roots	21.25 ± 1.03^{c}	1.41 ± 0.13^{c}	11.02 ± 0.33^{d}	31.54 ± 0.83^{d}	44.95 ± 1.59^{d}	28.41 ± 0.63^{c}	1.61 ± 0.13^{b}	31.81 ± 0.22^{c}
Nar – roots	28.11 ± 0.22^{b}	2.44 ± 0.01^{b}	18.04 ± 0.31^{c}	44.03 ± 1.01^{c}	62.33 ± 2.48^{c}	35.72 ± 0.80^{b}	1.79 ± 0.15^{b}	35.92 ± 0.57^{b}
RA – roots	31.39 ± 0.47^{a}	3.39 ± 0.30^{a}	23.85 ± 0.32^{a}	48.53 ± 0.63^{b}	86.97 ± 1.95^{a}	40.45 ± 1.21^{a}	1.84 ± 0.07^{ab}	36.59 ± 0.04^{ab}
Nar + RA – roots	32.37 ± 0.35^{a}	3.70 ± 0.34^{a}	20.44 ± 0.40^{b}	51.27 ± 0.74^{a}	70.94 ± 2.91^{b}	41.57 ± 0.37^{a}	2.10 ± 0.01^{a}	36.80 ± 0.10^{a}

Note: Values are reported as mean ± standard deviation of three parallel measurements. Different supercript lowercase letters indicate significant differences in the tested group for each part ($P < 0.05$).
Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; CUPRAC, cupric ion reducing antioxidant capacity; FRAP, ferric ion reducing antioxidant power; PMD, phosphomolybdenum; MCA, metal chelating ability; GAE, gallic acid equivalent; RE, rutin equivalent; TE, Trolox equivalent; EDTAE, EDTA equivalent.

acids (mainly represented by hydroxybenzoic acids such as gallic acid and ellagic acid), and hydroxycinnamic acids like cinnamic, caffeic, and sinapic acid. Other polyphenols were also followed by various compounds such as alkylphenols, hydroxycoumarins, and hydroxyphenylpropenes. Consistent with our study, prior research has documented the presence of a diverse array of bioactive compounds in *L. sativum*. These compounds encompass phenolic acids such as gallic acid, coumaric acid, caffeic acid, caffeoylquinic acid, flavonoids (quercetin and kaemferol), as well as isoflavonoids like daidzin, genistein, and daidzein.[32,33] Additionally, several kaempferol-derivatives have been also identified.[34]

Following that, the levels of phenolic compounds in different parts of the *L. sativum* treated with RA, Nar, and their combination were assessed using a semi-quantitative approach. The findings for various components such as anthocyanins, isoflavonoids, flavanols, flavonols, glucosinolate lignans, tyrosols, hydroxyphenylpropanoic acids, and stilbenes are provided in Fig. 1(A)–(I). While the main focus of cultivating *L. sativum* is its seeds, other components possess significant economic value due to their content of various bioactive compounds.[35] In our trial, leaf tissues were found to be the richest tissues in terms of all compound classes except for tyrosols. In leaf tissues, anthocyanins and hydroxyphenylpropanoic acids were the only significantly regulated classes, while anthocyanins, isoflavonoids, glucosinolates, lignans, and tyrosols were significantly regulated in the roots. The root's direct exposure to the treatments may have resulted in a more pronounced variation in phenolic profile. Accordingly, all treatments increased the anthocyanin content in leaves compared to the control group (up to +13%). In agreement, the positive correlation of anthocyanins and RAs has been previously reported,[36] owning to share a common early biosynthetic pathway started from phenylalanine. Regarding hydroxyphenylpropanoic acids, Nar application resulted in a higher amount (+16%) of this class of phenolic compounds compared to the control, as well as RA and their combination. Notably, in root tissues, all treatments led to a reduction in the levels of anthocyanins (up to −27% in Nar + RA), isoflavonoids (−53% in RA), lignans (−53% in RA), and tyrosols (−43% in Nar + RA) compared to the control group. This can be explained by the hypothesis that these compounds (Nar and RA) might have induced enzymatic or chemical reactions that led to the breakdown or conversion of phenolic compounds into other forms. Moreover, the exposure to RA and Nar + RA treatments caused a significant increase (up to +19%) in glucosinolates. The hyperaccumulation of glucosinolates has been reported in *L. sativum* sprouts grown under elevated carbon dioxide (CO_2).[37] The authors explained that this increase could be due to increased amino acid production and their hydrolysis by myrosinase. It is worth mentioning that upon considering Fig. 1, it can be inferred that there are no significant differences between the effects of treatments on the majority of phenolic compound classes. These findings suggest that Nar, RA, and their combination as elicitors can potentially increase specific phenolic compound classes. However, they may also lead to a decrease in other phenolic compound classes, particularly in the root.

To enhance our understanding of metabolic changes influenced by multiple factors (i.e., matrix and treatments), we conducted AMOPLS analysis, which aided in the interpretation of these variations. The AMOPLS score plot is reported in Fig. S2. The results from the AMOPLS model indicated that the matrix factor significantly influenced the overall observed variability (Table S2). The matrix (i.e., tissue type) accounted for 27% of the total variability, while the treatment with Nar and RA elucidated 15%. The primary influence of the matrix was associated with variations in metabolite levels between different tissue types, averaging across all three treatments (Nar, RA, and their combination). Since treatments are not considered in this situation, the observed patterns are thus associated with the specific tissue type. On average, although non-significant across both tissues, the treatment's main effect was related to metabolic changes resulting from exposure to Nar, RA, and Nar + RA. The interaction between matrix and treatment, related to potentially different metabolic changes due to different treatment exposure depending on the tissue type, accounted for 15% of the observed variations. Regardless, the statistical insignificance of this interaction implies minimal discernible patterns of specific alterations associated with the different treatments, depending on the tissue type in the system. The remaining 43% of the total observed variability was represented by the residuals, indicating the existence of additional sources of variation within the dataset.

To interpret the model, the distribution of observations (groupings) and the contributions of variables to the specific

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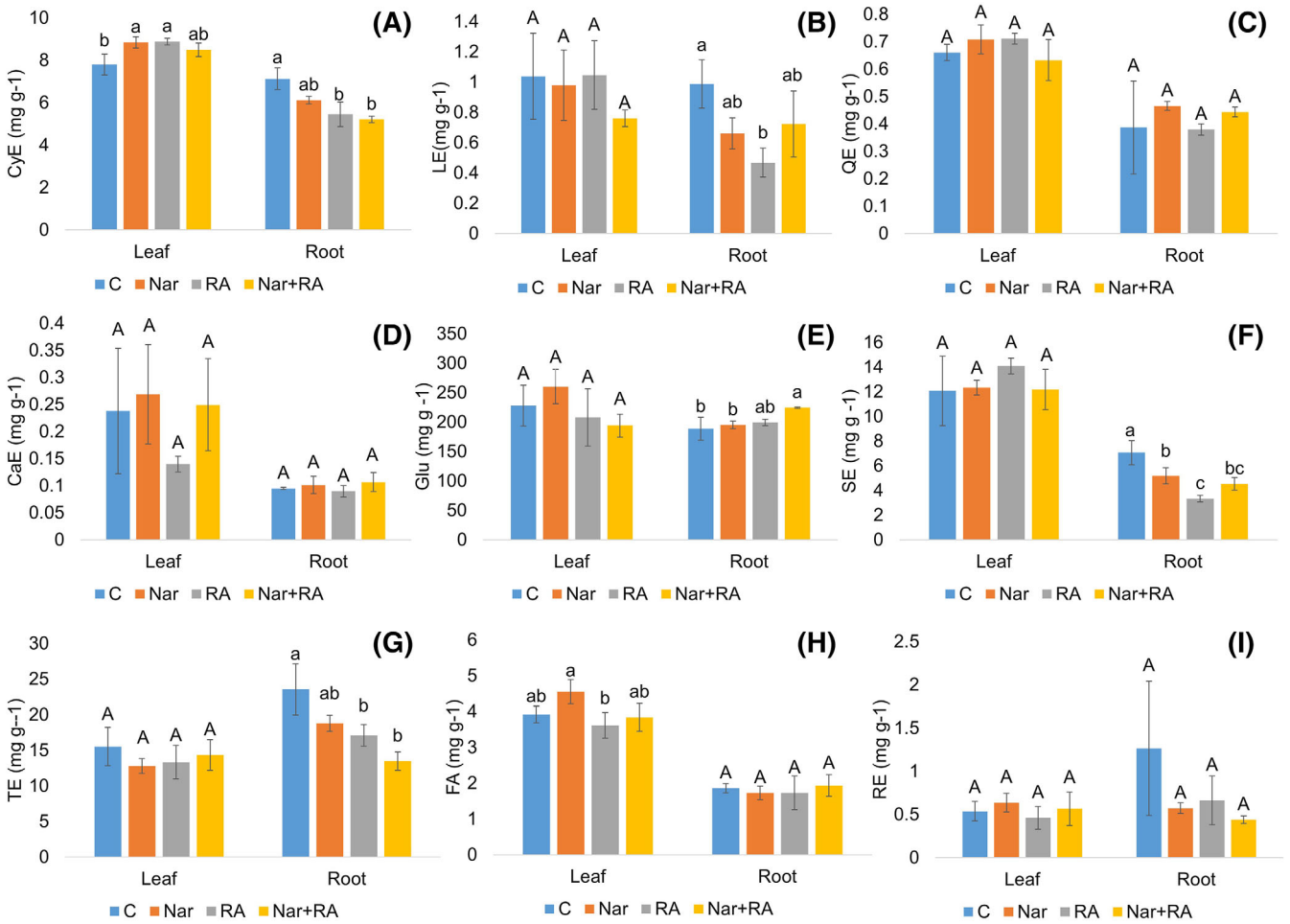

Figure 1. Semi-quantitative analysis of various classes of phenolic compounds in leaf and root tissues of *Lepidium sativum* upon treatment with Nar, RA, and Nar + RA combination, C (control). The quantified classes were: (A) anthocyanins expressed as mg cyanidin equivalent (CyE)/g; (B) isoflavonoids expressed as mg luteolin equivalent (LE)/g; (C) flavonols expressed as mg quercetin equivalent (QE)/g; (D) flavanols expressed as mg catechin equivalent (CaE)/g; (E) glucosinolate expressed as mg gluconapin equivalent (Glu)/g; (F) lignans expressed as mg sesamin equivalent (SE)/g; (G) tyrosols expressed as mg tyrosols equivalent (TE)/g; (H) phenolic acids expressed as mg ferulic acid equivalent (FA)/g; (I) stilbenes expressed as mg resveratrol equivalent (RE)/g. The data represents the average of three independent replicates. Lowercase letters indicate statistical differences between different treatments, while uppercase letters signify no statistical differences among the treatments. The data were drawn from the analysis of variance (ANOVA) and Duncan's *post hoc* test, with a significance level of $P < 0.05$.

predictive components linked to each main effect were examined (Fig. S2(A)–(C)). Consistent with the contribution shown in Table S2, a distinct grouping of observations based on the matrix was observed on the initial predictive component (tp1) of the AMOPLS model. Leaf tissue exhibited a positive score on tp1, while root tissues were distinguished by negative score values (as shown in Fig. S2(A)). These separations exhibited a certain level of coherence with the findings obtained from the quantification analysis (Fig. 1), confirming the influence of the matrix. The tp2 *versus* tp4 score plot (Fig. S2(B)) also displayed a clear separation of the four groups of samples: control, Nar, RA, and Nar + RA, highlighted by the block contributions.

Subsequently, to offer a supplementary analysis specific to each variable, the VIP^2 was calculated using the AMOPLS decomposition (Fig. 2). The calculated VIP^2 for the predictive components of the model enabled the evaluation of the combined contribution of each metabolite based on its explained variance.[38] The complete list of metabolites, along with their VIP^2 scores, is presented in Table S3. Figure 2 displays the VIP^2 values for 50 metabolites, illustrating their overall significance and specific contributions within the global model concerning the matrix effect. Larger VIP^2 values show which effect(s) are the most important for each annotated compound.[39] The calculated VIP^2 for the matrix effect summarizes the significance of each metabolite in the predictive components tp1 and tp5. Upon further examination of the VIP^2 values, it becomes apparent that metabolites associated with flavonoids could hold significant importance. 24-Methylcholesterol ferulate, kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside, quercetin 3-*O*-glucosyl-rhamnosyl-galactoside, petunidin 3,5-*O*-diglucoside, and so forth, were among the top-ranked metabolites (Table S3, Fig. 2). However, the treatments also impacted other compounds primarily linked to lignans, including 1-acetoxypinoresinol, 7-oxomatairesinol, and sesamolinol (Fig. 2). Previous research has indicated that lignan production can be stimulated by exposure to certain exogenous materials, such as chitosan and putrescine.[40,41]

After that, a supervised OPLS-DA approach was utilized to identify discriminating compounds for each tissue in response to Nar, RA, and Nar + RA treatments, using the respective predictive models (Fig. 3(A),(B)). Both models showed high quality regarding

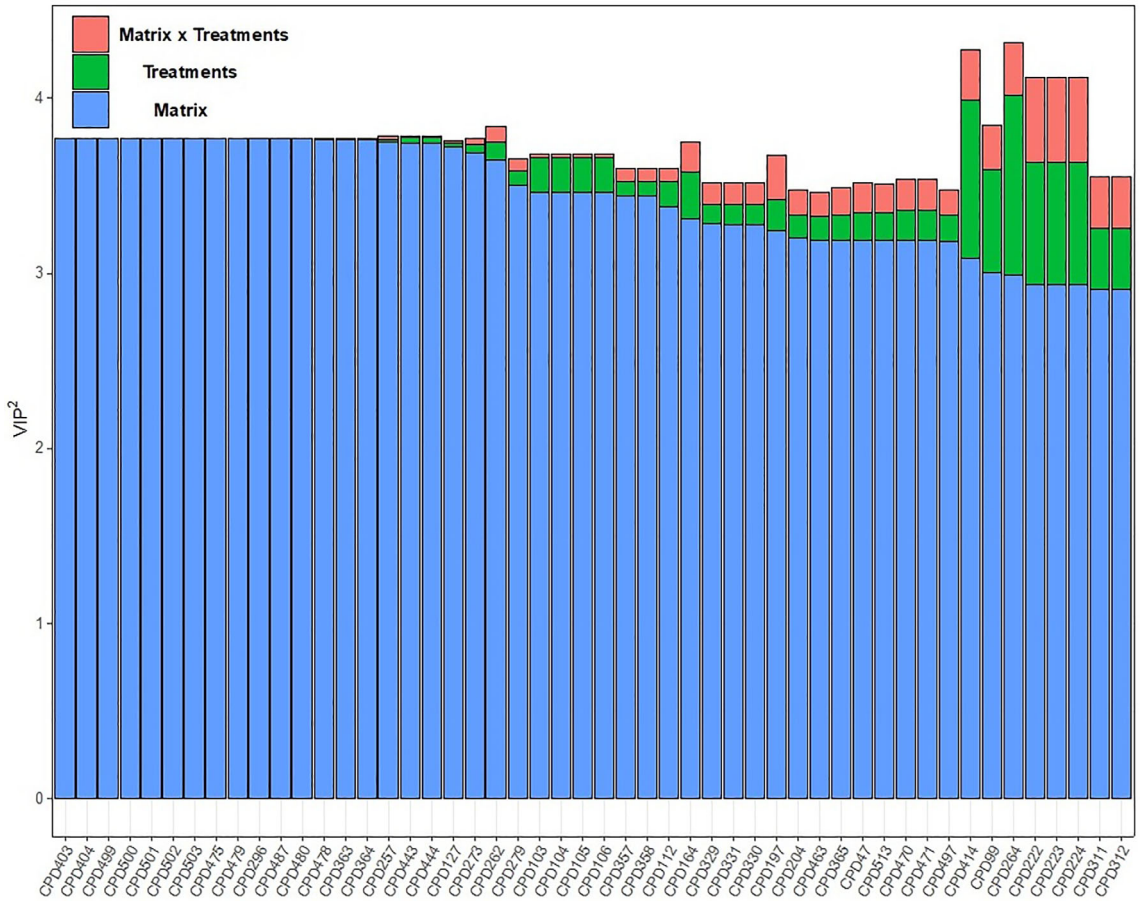

Figure 2. Total and effect-specific VIP^2 values for the 50 compounds show the most significant contribution to the global model through the matrix effect. The total VIP^2 values serve as valuable tools for prioritizing compounds and identifying the metabolites that have a significant influence on the model. By utilizing the effect-related VIP^2 values, it becomes feasible to dissect the total contributions to the model based on various experimental factors. This enables the evaluation of the significance of each metabolite in distinct effects (here, matrix and treatments) allowing for a comprehensive assessment of their individual importance. The comprehensive list of 50 discriminant compounds can be found in Supporting Information, Table S2.

goodness-of-fit (R2Y) and goodness-of-prediction (Q2Y) parameters and good performance after the permutation test (Fig. S3). In root tissues, the OPLS-DA exhibited a clear separation of the control group from those treated by Nar and RA according to the first latent vector, then RA treatment according to the second vector. On the contrary, this separation was not as distinct in leaves compared to roots. However, it can be observed that the two treatments of RA and RA + Nar are noticeably separated from each other (Fig. 3(A),(B)). Afterward, the variables that played a significant role in the aforementioned discrimination were selected, specifically VIP markers (Tables S4 and S5). The most discriminant compounds reported in root tissues were represented by anthocyanins (16 compounds), phenolic acids (15 compounds), and other polyphenols (12 compounds), respectively (Fig. 3(C)). In detail, the compounds having the highest VIP score were chrysoeriol 7-*O*-glucoside, isorhamnetin 3-*O*-rutinoside (as flavonols), 4-benzoyloxybutylglucosinolate (a glucosinolate), and the anthocyanins petunidin 3-*O*-galactoside, cyanidin 3-*O*-glucoside. However, in leaves, phenolic acids (20 compounds), and other polyphenols (11 compounds) were the most abundant classes among others (Fig. 3(D)). As episesamin, sesamin (as lignans), isorhamnetin 7-*O*-rhamnoside (as flavonols), *p*-coumaroyl glycolic acid (as phenolic acids), esculin (other polyphenols), and so forth, were among the most discriminant compounds represented. A diverse spectrum of compounds is anticipated, as previous studies have documented the abundance of phenols, flavonoids, glucosinolates, and glycosides in various parts of *L. sativum*.[30,42,43] It has been reported that bioactive compounds can be modulated in response to external stimuli. For instance, Ullah *et al.*[44] reported that exposure to white light resulted in an increased production of various metabolites in *L. sativum*, including chlorogenic acid, quercetin, kaempferol, ferulic acid, sinapic acid, protocatechuic acid, vanillic acid, and caffeic acid. In agreement, our findings support the notion that the composition of *L. sativum* tissues can be influenced by Nar and RA treatments, leading to distinct profiles of discriminant compounds.

Antioxidant effects

Antioxidants have a positive effect on human health and play a crucial role in the defense system of plants against stressful conditions. Recently, there has been increasing interest in improving plants' antioxidant properties through using phenolic modulators to develop functional foods.[26] In the current study, we investigated the effect of Nar and RA on the antioxidant properties of *L. sativum*. The results are summarized in Table 1. ABTS and DPPH were selected radical quenching assays and all root treatments were higher than leaves in both assays. In leaves treatments, the individual applications of RA (DPPH: 5.83 mg TE/g; ABTS:

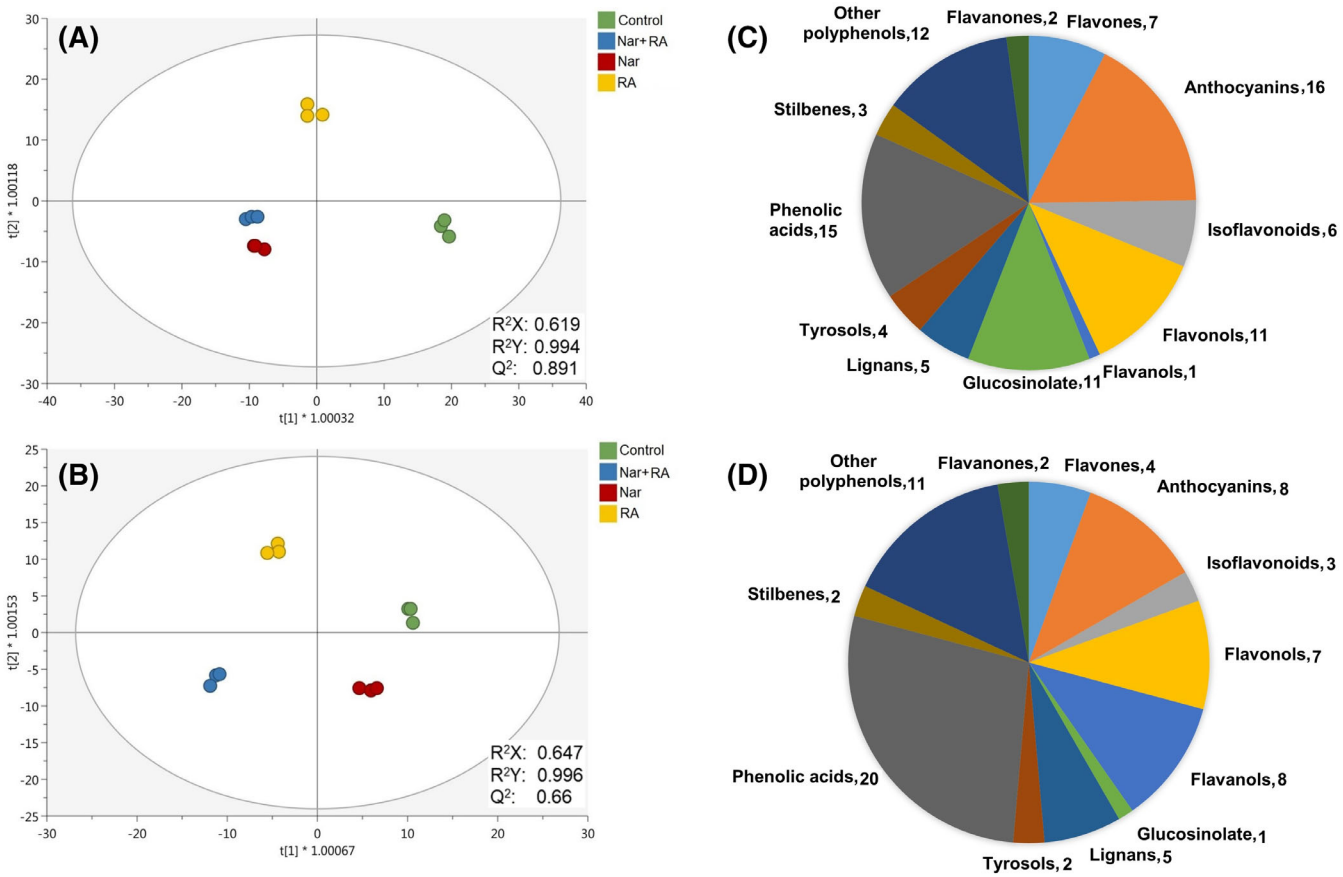

Figure 3. Score plot of orthogonal projection to latent structures discriminant analysis (OPLS-DA) supervised modeling performed on untargeted phenolic profiles of *Lepidium sativum* roots (A) and leaves (B). The pie charts depict different classes of variable importance in the projection (VIP) compounds chosen from the OPLS-DA model: (C) root tissue, and (D) leaf tissue.

28.03 mg TE/g) and Nar (DPPH: 4.01 mg TE/g; ABTS: 27.06 mg TE/g) slightly increased radical quenching ability, but this was not statistically different compared to the control (DPPH: 3.39 mg TE/g; ABTS: 26.70 mg TE/g). However, the combination of these modulators reduced the ABTS scavenging ability. In contrast to the leaf treatments, all of the applications on roots showed a positive effect on the ability to scavenge free radicals compared to the control. Overall, the application of RA was found to be more effective than Nar in both leaf and root treatments. These favorable effects can be attributed to increased total phenolic and flavonoid content from RA applications. Studies have shown that RA can up-regulate the production of secondary metabolic pathways, such as phenylalanine ammonia lyase (PAL) activity in plants.[45] Ullah *et al.*[44] investigated the effect of melatonin on UV-C radiation-induced stress in *L. sativum*, and melatonin treatments showed greater ABTS and DPPH inhibitory effects compared to the control group. The treatments had stronger ABTS and DPPH properties compared to the control. Following radical scavenging assays, reducing power assays are the most used, as they reflect the electron-donating ability of antioxidant compounds. For this purpose, CUPRAC and FRAP assays were performed. As seen in Table 1, in both parts, the effects of the tested modulators on reducing power were similar to the radical scavenging results. All treatments for root samples improved reducing abilities compared to the control. The highest CUPRAC value in the root samples was found in the individual application of RA with the value of 86.97 mg TE/g, followed by combined (70.94 mg TE/g), Nar (62.33 mg TE/g) and control (44.95 mg TE/g) groups. Among leaf groups, the best ability for both reducing power assays was recorded in the individual application of Nar (CUPRAC: 56.52 mg TE/g; FRAP: 28.93 mg TE/g). However, in the combination applications for leaves (CUPRAC: 51.26 mg TE/g; FRAP: 25.57 mg TE/g), the abilities were reduced compared to the control group (CUPRAC: 51.53 mg TE/g; FRAP: 26.68 mg TE/g). Several applications to improve the reducing ability of some cress varieties and their seeds or leaves have been described in the literature. Ullah *et al.*[44] found that the application of melatonin positively impacted the ferric-reducing abilities of cress cultures. Similarly, in another study, the FRAP abilities of three cress cultivars increased under high CO_2 levels compared to atmospheric CO_2 levels.[37] The PMD (phosphomolybdenum) assay is another reducing power assay as it involves the transformation of molybdenum(VI) to molybdenum(V) by antioxidants. As shown in Table 1, the results of the PMD assays were similar to those of the FRAP and CUPRAC assays. For the leaf treatments, the results of the PMD were adversely affected by single and combined applications, but no statistical differences were observed. All applications improved the reducing power of the PMD in the root samples compared to the control. The chelation of transition metals is an important antioxidant to inhibit hydroxyl radicals' production in the Fenton reactions.[46] In leaf treatments, the obtained metal chelating ability can be ranked as control > RA > Nar + RA > Nar. However, we observed no statistically significant differences in the modulator groups. The best metal chelating ability was

recorded in the combined group with 36.80 mg EDTAE/g, followed by RA (36.59 mg EDTAE/g), Nar (35.92 mg EDTAE/g) and the control (31.81 mg EDTAE/g). Although several studies have reported phosphomolybdenum (PMB) and metal chelating abilities of *L. sativum*,[47-50] there is no data on the effects of modulators on these abilities. After combining and analyzing all the results, it became clear that the modulators significantly improved the antioxidant ability of the root samples. In addition, the combined group showed a higher activity level than the individual groups in the root treatments. Therefore, our results can be a new starting point for developing agricultural products that promote health and wellbeing.

Enzyme inhibition effects

The effects of *L. sativum*'s bioactive compounds on the enzyme-inhibitory properties were accessed. Cholinesterase (against Alzheimer's disease), tyrosinase (against hyperpigmentation), amylase, and glucosidase (against diabetes mellitus) were chosen as targets. The results are shown in Table 2. For AChE inhibition, the applications of both parts did not have a positive impact on the inhibition process. Although individual Nar and combined applications in root samples showed a slightly increased AChE inhibition ability, these did not differ from the control group. Interestingly, RA had a negative impact on AChE inhibitory activity in both parts compared to the control groups. However, for both parts, the individual RA application greatly affected BChE inhibition. In particular, the application of individual RA resulted in an almost 2.5-fold increase in BChE inhibition compared to the control. In addition, the combined application increased BChE inhibition for both parts compared to controls. The synergetic effects of these modulators can explain the results. As shown in Table 2, the combined applications of both parts resulted in a significant increase ($P < 0.05$) in tyrosinase inhibitory abilities (leaves: 57.72 mg KAE/g; roots: 67.65 mg KAE/g) when compared to the controls (leaves: 56.45 mg KAE/g; roots: 56.07 mg KAE/g). For both parts, the application of individual RA reduced the observed tyrosinase-inhibitory effects of the controls. Similar to BChE and tyrosinase inhibition, applying Nar and RA (individual or combined) to both parts enhanced the α-amylase inhibition. The amylase inhibition in the root control was 0.60 mmol ACAE/g, while the value increased to 0.82 mmol ACAE/g through the individual application of RA. Interestingly, we found different results of α-glucosidase inhibition. All treatments of leaves exhibited glucosidase inhibition, but only the combined group for roots was active on glucosidase. Individual Nar application improved the glucosidase inhibitory potential in leaves, compared to the control. In a previous study by Ullah *et al.*,[44] the application of melatonin enhanced the anti-diabetic properties of *L. sativum* under UV-C radiation stress. The authors also suggested that the enhancement could be explained by the increase of phenolic in the samples. In this study, we found that amylase inhibition was closely related to the TPC of the tested groups. Although the effect of some modulators on the enzyme inhibitory effects of some plants has been reported, to our knowledge, no data are available for *L. sativum*. Thus, the results presented here could serve as a crucial basis for developing health-promoting pharmaceuticals and nutraceuticals.

Correlation of bioactive compounds, antioxidant capacity, and enzyme inhibition

Accordingly, Pearson's correlation analysis of all annotated bioactive compounds, along with assays for antioxidant and enzyme inhibitory capacity, revealed a distinct correlation pattern between the root and leaf tissues (Fig. 4, Tables S6 and S7). This finding aligns with the outcomes from HCA and OPLS-DA, substantiating the divergent reactions exhibited by root and leaf tissues towards the applied treatments. In the root, a strong negative correlation was observed between lignans and nearly all parameters associated with antioxidant and enzyme inhibitory capacity (reaching a maximum correlation coefficient of $r = -0.976$; $P < 0.01$ with BChE), except for AChE, isoflavonoids, tyrosols, anthocyanins, and stilbenes. The negative correlation between lignans and most parameters related to antioxidant and enzyme inhibitory capacity in the root can be attributed to their potential role as reactive oxygen species (ROS) scavengers or antioxidant modulators. Lignans may exert antioxidant effects by neutralizing ROS and protecting cellular components from oxidative damage.[51] Similarly, the same pattern was also observed for tyrosols, anthocyanins, and stilbenes (Fig. 4). On the contrary, the most positive correlations were observed between ABTS, TPC, FRAP, TFC, amylase, DPPH, CUPRAC, and chelating (up to $r = 0.989$; $P < 0.01$). Among the bioactive compounds identified through metabolomics annotation, glucosinolates exhibited the highest positive correlation with antioxidant and inhibitory enzymes (up to $r = 0.902$ with FRAP).

Table 2. The measurements of the total enzyme inhibitory properties of the rosmarinic acid (RA), naringenin (Nar) and their combination (Nar + RA) in both leaves and roots of the *Lepidium sativum* plant

Treatment – parts	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
Control – leaves	1.96 ± 0.07^{a}	0.90 ± 0.04^{d}	56.45 ± 0.42^{ab}	0.66 ± 0.01^{b}	0.15 ± 0.03^{ab}
Nar – leaves	1.86 ± 0.13^{ab}	2.48 ± 1.11^{b}	57.35 ± 0.85^{a}	0.74 ± 0.02^{a}	0.21 ± 0.05^{a}
RA – leaves	1.72 ± 0.09^{b}	2.51 ± 0.83^{a}	54.38 ± 1.26^{b}	0.70 ± 0.01^{ab}	0.11 ± 0.04^{b}
Nar + RA – leaves	1.96 ± 0.03^{a}	1.76 ± 0.76^{c}	57.72 ± 0.85^{a}	0.74 ± 0.02^{a}	0.17 ± 0.01^{ab}
Control – roots	1.92 ± 0.06^{a}	3.56 ± 0.41^{b}	56.07 ± 0.89^{b}	0.60 ± 0.01^{c}	—
Nar – roots	2.19 ± 0.09^{a}	3.39 ± 0.51^{b}	49.00 ± 0.88^{d}	0.71 ± 0.01^{b}	—
RA – roots	0.49 ± 0.02^{b}	5.33 ± 0.03^{a}	51.36 ± 0.24^{c}	0.82 ± 0.02^{a}	—
Nar + RA – roots	2.06 ± 0.24^{a}	5.24 ± 0.02^{a}	67.65 ± 0.77^{a}	0.79 ± 0.02^{a}	0.85 ± 0.05

Note: Values are reported as mean ± standard deviation of three parallel measurements. Different superscripts indicate significant differences in the tested group for each part ($P < 0.05$).
Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GALAE, galanthamine equivalent; KAE, kojic acid equivalent; ACAE, acarbose equivalent.

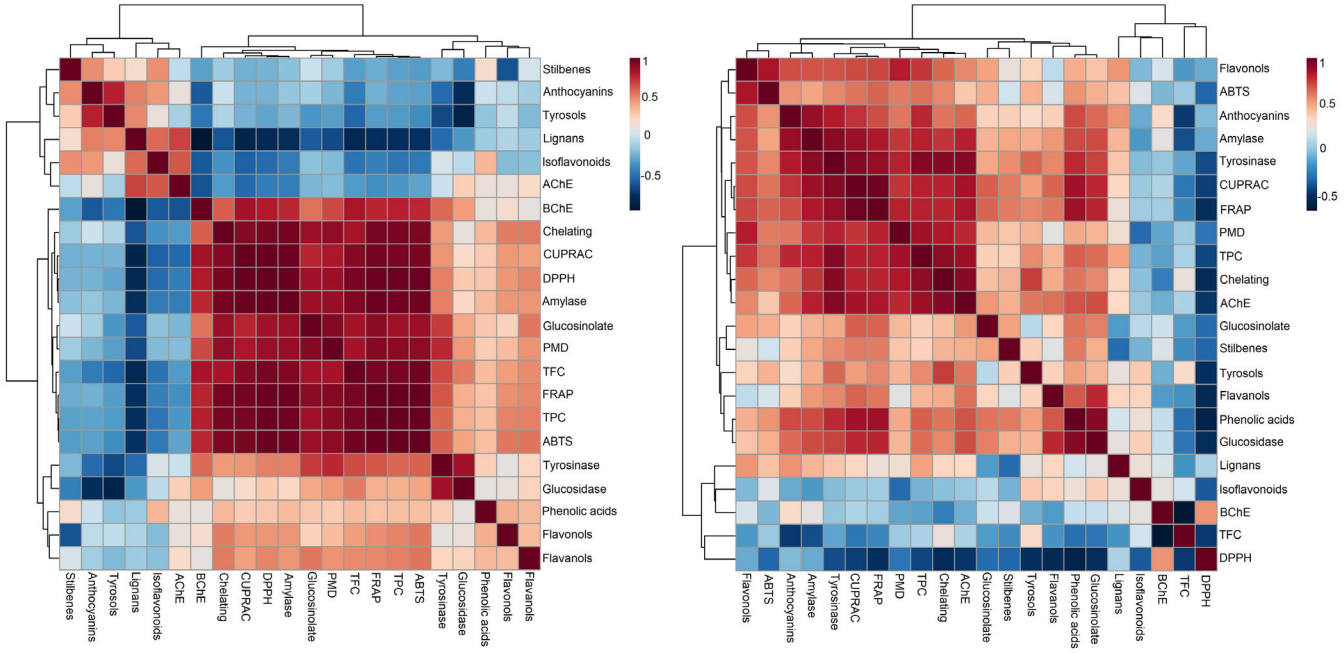

Figure 4. The correlation heatmap of root (left) and leaf (right) tissues were generated by analyzing the prominent phenolic classes identified, along with the antioxidant capacity and enzyme inhibitory capacity. Abbreviations: DPPH: 1,1-diphenyl-2-picrylhydrazyl; TFC, total flavonoid content; BChE, butyryl-cholinesterase; AChE, acetylcholinesterase; TPC, total phenolic content; PMD, phosphomolybdenum; FRAP, ferric ion reducing antioxidant power; CUPRAC, cupric ion reducing antioxidant capacity; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid.

Considering the leaf extracts, the most notable negative correlation was observed between TFC and BChE, displaying correlation coefficients of $r = -0.674$ ($P < 0.01$). Conversely, tyrosinase, amylase, CUPRAC, chelating, TPC, FRAP, and AChE exhibited positive correlations among themselves, with the highest correlation coefficient observed between tyrosinase and CUPRAC, reaching $r = 0.930$ ($P < 0.01$). Furthermore, anthocyanins were found to be positively correlated with amylase. The positive and linear correlation of phenolic compounds as redox properties responsible for antioxidant activity has been documented in the literature.[52-55] Overall, these findings highlight the complex and diverse interactions between bioactive compounds and their impact on antioxidant and enzyme-inhibitory activities in different tissues.

CONCLUSION

In summary, this study investigated the impact of Nar and RA on the metabolomic profile and biological activities of *Lepidium sativum* leaves and roots. The untargeted metabolomics analysis showed a significant tissue-dependent modulation of bioactive compounds confirmed by HCA, AMOPLS, OPLS-DA, and VIP markers. Indeed, the matrix (tissue type) is the prevalent factor, contributing 27% of the observed variability. In leaf tissues, only anthocyanins and hydroxyphenylpropanoic acids showed significant regulation, whereas in the roots, significant regulation was observed for anthocyanins, isoflavonoids, glucosinolates, lignans, and tyrosols. In detail, compared to the control group, all treatments increased the anthocyanin content in leaves. Conversely, in root tissues, all treatments led to decreased levels of anthocyanins, isoflavonoids, lignans, and tyrosols. The results demonstrate that the individual application of RA significantly enhanced the biological activities of the leaves. Furthermore, when RA was applied in combination, the root samples exhibited notable biological activities. These findings could provide valuable information for cultivating *L. sativum* with enhanced functional properties, making it suitable for agro-food applications.

AUTHOR CONTRIBUTIONS

Hajar Salehi: investigation, methodology, formal analysis, writing – original draft, writing – review and editing. Leilei Zhang: conceptualization, methodology, formal analysis, writing – review and editing. Fatma Nur Alp-Turgut: methodology, writing – review and editing. Busra Arikan: methodology, writing – review and editing. Fevzi Elbasan: methodology, writing – review and editing. Ceyda Ozfidan-Konakci: methodology, writing – review and editing. Melike Balcı: methodology, writing – review and editing. Gökhan Zengin: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, funding acquisition, resources, supervision. Evren Yildiztugay: methodology, writing – review and editing. Luigi Lucini: writing – review and editing, funding acquisition, resources, supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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