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ORIGINAL PAPER

Integrative analysis of *Cichorium intybus* L. Extracts: from NADES-Based extraction and UHPLC-Orbitrap®-HRMS to bioinformatics and machine learning

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Abstract

This study aimed to comprehensively evaluate the phytochemical composition, antioxidant, and antimicrobial activities plans aerial parts of *Cichorium intybus* L. extracts obtained using conventional solvents (dichloromethane, n-hexane, methanol, ethanol) and a natural deep eutectic solvent (NADES). Antioxidant capacity was assessed through DPPH, ABTS radical scavenging, and Fe^{3+}/Cu^{2+}reducing assays, while antimicrobial potential was evaluated using the microdilution method against *Staphylococcus aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae.* Chemical profiling of methanol and NADES extracts was performed via UHPLC-Orbitrap®-HRMS/MS, revealing that NADES extraction enriched glucuronidated flavonoids and hydroxycinnamic acids, including luteolin-7-O-glucuronide, quinic acid, and chlorogenic acid. Chemometric approaches (principal component analysis and heatmap clustering) confirmed distinct metabolite enrichment patterns between extracts. To further elucidate biological relevance, bioinformatic analyses (SwissTargetPrediction, Kyoto Encyclopedia of Genes and Genomes (KEGG), DisGeNET, and GO enrichment) suggested potential molecular targets and pathways linked to antioxidant and antimicrobial activity. Moreover, a machine learning model (Random Forest with leave-one-out cross-validation) combined with Shapley additive explanations (SHAP) identified quinic acid and flavonoid glucuronides as the most predictive determinants of antioxidant capacity, demonstrating > 92% variance explanation with minimal error ($R^2 = 0.9225$, RMSE = 0.0219). Collectively, these integrative findings highlight NADES as a sustainable extraction medium and underscore the utility of combining chemometrics, bioinformatics, and machine learning for a systems-level understanding of plant-derived bioactive compounds.

Keywords Cichorium intybus L. · NADES · Antioxidant activity · Bioinformatics · Machine learning

The NADES method is thought to better extract components with antimicrobial properties from plants. Studies support this.

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Introduction

The chicory (*Cichorium intybus*) in the genus *Cichorium* of the family *Asteraceae* is economically important an dicot herbaceous plant [1]. The *Cichorium* genus is distributed across various regions, including Türkiye, South America, India, the USA, and European countries [2].

Generally, chicory is quite well-known as a coffee alternative [2]. The leaves or roots of *C. intybus* are roasted and used in coffee or as a coffee additive. The fresh roots are also used in milk soups. The young leaves of *C. intybus* are mainly used in salads [3]. It is also an important ingredient in tea mixtures [4]. On the other hand, it is used in animal feed, as a source of inulin, and for ethanol production by direct fermentation [5].

All plant parts are highly appreciated due to the accumulation of numerous biologically active substances such as unsaturated sterols, sesquiterpene lactones, vitamins, alkaloids, inulin, coumarins, chlorophyll pigments, among which flavonoids, saponins and tannins can be found [6–9]. The plant *C. intybus* L. also contains significant amounts of polyphenols (i.e., flavonoids, stilbenes, phenolic acids, lignans) that have the potential to prevent cardiovascular disease, cancer, and inflammation [10]. In chicory, cycoric acid, the main phenolic acid, is a phenolic derivative with significant antioxidant properties. Its high antioxidant activity stems from the presence of a catechol ring in the structure of this bioactive compound. It has also been reported that complexes of cicoric acid with Cu(II), Ni(II), Co(II), and Zn(II) exhibit higher antimicrobial activity as a result of increased absorption [11]. The aqueous extract of chicory seeds has been shown to exhibit antibacterial activity against *Staphylococcus aureus* but is ineffective against *Escherichia coli*[12]. The antimicrobial activity of aqueous extract of *C. intybus* against *S. aureus*, ethyl acetate and ethanol extracts against *S. aureus*, *Pseudomonas aeruginosa* and red chicory leaves against *Saccharomyces* yeasts was also observed [3].

C. intybus extracts have been reported to have cytotoxic effects on breast cancer (MCF-7), amelanotic melanoma (C32), prostate cancer (LNCaP), renal adenocarcinoma (ACHN), leukemia cells, Ehrlich ascites carcinoma, prostate cancer (PC-3), breast cells (T47D and SKBR3), and cervical cells (HeLa). It has also been reported that sesquiterpenes obtained from *C. intybus*exhibit cytotoxic effects on ovarian cancer cells, mouse lymphoma, and leukemia cells [13].

Although *C. intybus* has a long history of human use, the possibility of high levels of secondary metabolites causing potential toxicological effects has been investigated [14]. An oral toxicity study in rats indicated no death or toxicological signs related to the extract [12, 14]. The study by Habibollahi et al., [15], stated that the average concentrations of each toxic heavy metal detected in 15 plant species, including *C. intybus*, were significantly below the legal limit published by the World Health Organization. Conversely, it has been suggested that aqueous extract of *C. intybus* seeds may have antioxidant effects due to its phenolic compounds and flavonoids (such as kaempferol, quercetin, and cycoric acid), which reduce oxidative load and increase levels of good cholesterol in the blood of diabetic rats [12]. The major constituents of chicory, chlorogenic and caffeic acids, are known to stimulate glucose uptake in muscle cells and are suspected antidiabetic agents. These agents induce insulin release in insulin-secreting cell lines and Langerhans islets [14, 16].

The efficiency of the extraction of natural deep eutectic solvents (NADES) for polyphenolic bioactive compounds like phenolics, flavonoids, and anthocyanins have been widely tested, showing better results in comparison to organic solvents, traditional methanol, and ethanol [17]. NADES and Deep eutectic solvents (DESs) have been employed more and more in the chemical industry and biotechnology in recent years. NADES is a eutectic mixture of two or more solid or liquid compounds and has a melting point that is lower than any of the components [18]. It is prepared from natural ingredients and approved by US Food and Drug Administration (FDA) [19].

In addition, NADES application on the study of the *C. intybus* has not been done yet, and no previous study has tried to combine advanced analytical and computational methods of developing a systematic association between extraction chemistry, phytochemical composition, and bioactivity.

To fill this gap, the current study pursues a multi-layered analytical approach, which is integrated. The UHPLC-ESI-Orbitrap®-HRMS/MS is used to obtain precise and thorough profiling of phenols, which provides the chemical basis of the analysis. Solvents driven compositional patterns and functional antioxidant responses are visualised using chemometric methods. Bioinformatic studies extend these results by placing prevalent phytochemicals in an environmental context and in the predicted molecular targets and biological pathways. Lastly, the relationship between phenolic composition and antioxidant activity is quantitatively modelled using machine learning, which allows identifying those compounds that have the most significant functional importance. This is a crucial combined approach to go beyond solvent-level novelty, and a mechanistic, systems-level appreciation of the bioactive potential of extracts of *C. intybus*.

Materials and methods

Chemicals

All solvents and chemicals were purchased from Sigma-Aldrich (Germany). All chemicals used were of analytical grade.

Plant material

The plant material utilized in this study comprised specimens of *C. intybus* collected from the green spaces within the Bingöl University campus on July 18, 2023. *C. intybus* is a widely distributed cosmopolitan species, commonly found throughout the geographical boundaries of the Bingöl region [20, 21].

The plant specimens employed in this study were sourced from the Central Laboratory of Bingöl University and have been deposited in the Bingöl University Herbarium (BIN) for preservation. The samples were collected on July 18, 2023, from an area near the Central Application and Research Center of Bingöl University, situated in a humid microclimate at an altitude of 1,158 m. These specimens were cataloged under the laboratory code "Dursun 08." Herbarium records and visual documentation of the collected material are provided in Fig. 1.

Plant specimens were identified at the Bingöl University Herbarium, taking into account the flora of Turkey and the Aegean Islands, after which the plants were harvested and cleaned of weeds. *C. intybus* L. plans aerial parts were sliced into 1–2 cm segments with pruning shears. *C. intybus* L. plans aerial parts was then transferred to 250 mL glass flasks and frozen. The samples were then lyophilized using a Telstar LyoQuest apparatus. The temperature was set at −80 °C and the pressure at 0.01 mbar. The process lasted for 48 h, during which time the moisture was completely removed. The dried samples were ground into a fine powder using a mill and kept in amber glass bottles under a nitrogen atmosphere at + 4 °C until the extraction process began.

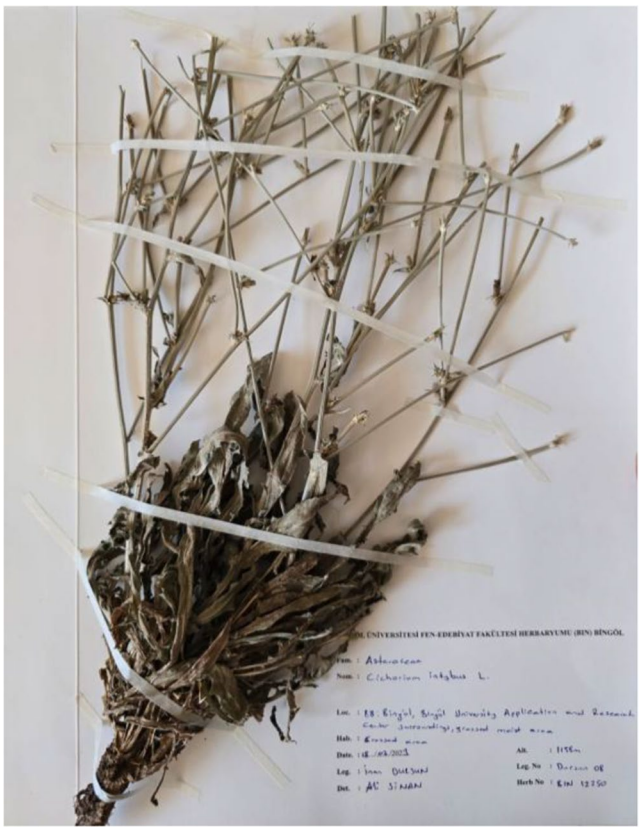


Fig. 1 Herbarium documentation of *C. intybus* L. Locality (B8): Vicinity of Bingöl University Application and Research Center, grassy area; collected on 18 July 2023; coordinates: 38°54'05.36" N, 40°29'19.21" E; altitude: 1,158 m; collector: Dursun 08; life form: hemicryptophyte; herbarium voucher: BIN 12,250

Preparation of NADES

Choline chloride (ChCl, Sigma-Aldrich, USA) was used as a hydrogen bond donor, and glycerol was used as a hydrogen bond acceptor. ChCl was kept at 40 °C until its moisture was removed before use. Then, glycerol and ChCl were mixed in a specific ratio (1:2 M), and this mixture was kept at 90 rpm in a shaking water bath at 80 °C for approximately 12 h until a homogeneous colorless liquid was formed. The dilution process was performed by adding 20% distilled water to this homogeneous colorless liquid. Dilution was performed to increase fluidity. Thus, a non crystallizing, homogeneous, transparent, colorless NADES solution was obtained [22].

Preparation of extracts

The extracts were prepared by weighing 1 g of the dry and ground plant material in Falcon tubes and adding 20 mL of solvent [NADES (N), n-hexane (H) (Sigma-Aldrich, USA), dichloromethane (DCM) (Sigma-Aldrich, USA), methanol (M) (Sigma-Aldrich, USA), and ethanol (E) (Sigma-Aldrich, USA). The solution was sonicated at 55 ± 2 °C in an ultrasonic water bath for 30 min. After centrifugation for 10 min at 6000 rpm, the supernatants were collected. Sequential extraction was carried out thrice, and the extracts were pooled.

Antimicrobial activity

C. intybus plant extract was prepared with different solvents (specified in 2.4 section), using the microdilution method to measure the minimum inhibitory concentration (MIC) value [23, 24]. Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Klebsiella pneumoniae*) bacteria were used as test bacteria.

In the application, 20 µL of 10^6log test bacteria were added to the wells of a 96-well sterile microplate containing 150 µL of double scraped double medium and 150 µL of plant extracts. The microplates were incubated at 37 °C. Following 24 h of incubation, the presence of live bacteria was assessed using TTC (2,3,5-triphenyl tetrazolium chloride, Sigma, TTC 20 mg/mL in water). The minimum

concentration at which no color change occurred was noted as the MIC value. The precision of the MIC value was verified by inoculating the samples into nutrient broth medium [22, 25]. Gentamicin sulfate (Sigma-Aldrich, CAS number:1405-41-0) was used as a positive control for all test bacteria at a 20 µg/mL concentration. % 10 dimethyl sulfoxide was used as a negative control.

Antioxidant activity analysis

Fe^{3+} reduction capacity

For the purpose of this analysis, 1 mL of the sample solutions was partitioned and diluted to concentrations ranging from 10 to 30 µg/mL. Subsequently, a phosphate buffer solution (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%) were introduced. The reaction mixture was then incubated at 50 °C for 20 min. Following incubation, the reaction was halted by the addition of trichloroacetic acid (2.5 mL, 10%). Ferric chloride (0.1%, 0.25 mL) was subsequently added to this solution. The samples and standards were analyzed at a wavelength of 700 nm using a spectrophotometer [26].

Cu^{2+} reduction capacity (CUPRAC)

The reducing potential of cupric ions (Cu^{2+}) in plant extracts and NADES was evaluated according to a modified version of the protocols delineated by Apak et al. [27] and Gülçin et al. [28]. Subsequently, the absorbance of the NADES and extract samples, assessed at concentrations ranging from 10 to 30 µg/mL, was measured at a wavelength of 450 nm. All measurements were conducted in triplicate to ensure statistical reliability and experimental reproducibility.

DPPH radical scavenging activity

The DPPH (1,1-Diphenyl-2-Picryl-Hydrazyl) radical scavenging activity of the plant extracts was quantified by the methodology established by Blois [29], which is a widely recognized procedure for evaluating the radical scavenging potential of plant-derived substances. The absorbance of each sample was determined spectrophotometrically at a wavelength of 517 nm. The percentage inhibition of DPPH radicals was calculated, and the IC_{50}(Inhibitory Concentration 50%) values were derived from the corresponding inhibition percentages, as outlined by Zor et al. [30].

$ABTS^{+}$ scavenging activity

The $ABTS^{\bullet+}$ (2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activities of the plant extracts were evaluated according to the method described by Gülçin et al. [28]. A spectrophotometer detected the absorbance of the samples at 734 nm. The percentage inhibition of $ABTS^{\bullet+}$ radical was determined, and IC_{50}values were calculated from the percentage inhibition curves according to the method of Zor et al. [30].

Phenolic profile analysis

Working conditions of liquid chromatography

Methanol and NADES extracts obtained from the *C. intybus* plant were injected at a concentration of 1000 ppm, diluted in MeOH/H_2O (50:50). The phenolic composition was determined using a ultra high performance liquid chromatograph (UHPLC) system (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA). A 20 µL volume of methanol and NADES extracts was injected and separated on a Purospher® STAR RP-18 endcapped Hibar®HR 3 µm, 2.1 × 100 mm column (Merck, KGaA, Darmstadt, Germany). The mobile phase A consisted of 0.5% acetic acid in water, and mobile phase B consisted of LC-MS grade methanol. Gradients were applied according to the studies of Dursun [22, 31].

The operating conditions of the high-resolution mass spectrometer (HRMS)

The analysis detections were performed using a high-resolution mass spectrometer (HRMS) equipped with electrospray ionization (ESI) (Thermo Fisher Scientific Exactive Plus Orbitrap®mass spectrometer (MS), Waltham, MA, USA). The HRMS operating conditions were based on the parameters used in the studies by Dursun [22, 31]. In all measurements, the mass accuracy remained below 5 ppm [22, 31].

Phytochemical constituents present in the methanol and NADES extracts of *C. intybus* were identified and characterized using UHPLC-ESI-Orbitrap®-HRMS/MS. To ensure reliable compound identification, three distinct selection criteria were employed. The first was a mass accuracy threshold of ± 5 ppm relative to the theoretical m/z of the molecular ion widely accepted standard for signal identification in high-resolution mass spectrometry (HRMS), particularly in targeted analyses, as described by Moschet et al. [32]. This mass tolerance is considered critical for confirming the presence of an analyte based on the concordance between measured and calculated mass values [33]. The second criterion involved visual evaluation of the retention times and isotopic patterns of the target analytes in comparison with those of authentic standards, using extracted ion chromatograms (EICs) within a ± 5

ppm mass window and full-scan MS data. At this step, elution times of detected compounds were compared to those of corresponding reference standards. Lastly, the third criterion focused on comparing tandem mass spectrometry (MS/MS) fragmentation spectra of detected analytes with those of known reference compounds, using diagnostic product ions. The integration of these three stringent evaluation parameters significantly improved the confidence in compound identification [22, 31].

Statistical analysis

The results of Fe^{3+} reduction capacity, Cu^{2+} reduction capacity (CUPRAC), and phenolic profile analyses were evaluated using statistical analysis. All statistical evaluations were conducted using SPSS software (version 25.0; SPSS Inc., Chicago, IL, USA). Group differences were assessed through one-way analysis of variance (ANOVA), and Duncan's multiple range test was applied for post hoc comparisons to determine statistically significant differences among groups. Data are expressed as mean values ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant (*), while values below 0.01 were regarded as highly significant (**).

Principal component analysis (PCA) and heatmap visualization

PCA was performed to evaluate the distribution and variability of the top phenolic compounds between extraction systems [34]. Quantitative data of the ten most abundant phenolic constituents identified in both the M and NADES extracts were used as input variables. The data matrix was standardized (mean-centered and scaled to unit variance) prior to analysis. PCA was carried out using the scikit-learn package in R (version 4.5.1, R Core Team, Vienna, Austria) The first two principal components (PC1 and PC2) were retained for graphical interpretation based on the proportion of explained variance. A two-dimensional PCA score plot was generated to visualize group clustering and compound-level variation. Compounds were color-coded based on their relative abundance in each extraction type: red for NADES-enriched and blue for M-enriched components. All visualizations were created using matplotlib and seaborn libraries. Heatmap-based hierarchical clustering was carried out to visualize the collective phenomenon of antioxidant activities of *C. intybus* extracts and standard antioxidants. The antioxidant data were composed by ferric (Fe^{3+}), and cupric (Cu^{2+}) reducing powers of all of the extracts and for butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), Trolox, and α-tocopherol standards. Before heatmap construction, all data were log-transformed using the log1p(x) function to normalize the distribution and reduce skewness. The heatmap was generated in R software using the pheatmap package, with Euclidean distance as the clustering metric and the complete linkage method applied. Color scaling was based on a gradient from navy (low activity) to red (high activity), providing a chemometric visualization of antioxidant efficiency among samples.

Bioinformatic target and pathway interpretation

The dominant phenolic compound identified in the methanol and NADES extracts of *C. intybus* L. by LC-MS/MS analysis (Luteolin-7-O-glucuronide) were subjected to in silico target prediction using the SwissTargetPrediction platform (http://www.swisstargetprediction.ch/) with *Homo sapiens* as the selected species. The predicted protein targets were compiled, and duplicates were removed prior to enrichment analysis. Disease association mapping was performed using the DisGeNET database to identify pathological conditions linked to the predicted targets. Functional enrichment of Gene Ontology (GO) biological processes, and KEGG pathways was conducted in R (4.5.1, R Core Team, Vienna, Austria) using the clusterProfiler, and org.Hs.eg.db packages. Bubble plots illustrating enrichment results were generated using the ggplot2 package, with bubble size representing the number of genes mapped to each term and color scale reflecting the adjusted p-value.

Machine learning–based modeling of antioxidant activity

In order to explore the relative role of phytochemical contents in antioxidant activity of extracts of *C. intybus* extracts, a supervised machine-learning strategy was used. The half-maximal inhibitory concentration (IC_{50}) of DPPH and ABTS radical scavenging experiment was used to measure the antioxidant activity. As the lower values of IC_{50} denote greater antioxidant effects, the logarithmic values of (IC_{50}_DPPH_$\log_{10}$ and IC_{50}_ABTS_$\log_{10}$) were established as dependent variables. The major phenolic and flavonoid concentrations were added as the explanatory variables in the model, which included luteolin-7-O-glucuronide, chlorogenic acid, quinic acid, luteoloside, eriodictyol, isoquercitrin, esculin hydrate, apigenin-7-O-glucuronide, nicotiflorin, astragalin, narcissin and rutin hydrate. All the predictor variables were first normalised before being modelled and missing values were checked and imputed accordingly.

The implementation of the algorithm of a Random Forest was performed in the ranger package of R, and the hyperparameter tuning was conducted in accordance with a leave-one-out cross-validation (LOOCV) scheme because of the limited size of the sample. LOOCV guarantees that every

sample is used as a test sample in turn and the other observations are taken as the training set hence maximising the use of data and reducing the chances of over-fitting. The performance of the models was estimated based on root mean squared error (RMSE), mean absolute error (MAE), and the coefficient of determination (R^2). Observed vs. Predicted plots were also used to visualise the predictive performance to allow graphical analysis of model fit. To understand model predictions and determine the compounds of the strongest impact on antioxidant capacity, permutation-based variable importance scores as well as Shapley additive explanations (SHAP) values were computed with the help of the fastshap package. The importance of permutation gave a global score of variables in terms of their relative ability to reduce the error of prediction, SHAP analysis allowed a finer insight into the direction and strength of the effect of each compound on the IC_{50} values. Negative SHAP values were viewed as the compounds that reduce the IC_{50} (i.e. antioxidant potential) and the positive SHAP values denote the rise in IC_{50} (i.e. reduced activity). The R (version 4.5.1) was used in all the analyses with the assistance of the libraries, which are, caret, ranger, fastshap, and ggplot2.

Results and discussion

Antimicrobial activity results

Table 1 summarizes the antimicrobial effects of *C. intybus* extracts obtained through different solvent extractions, including Ethanol (E), Methanol (M), Hexane (H), Dichloromethane (DCM), and NADES, against a selection of pathogenic bacterial strains: *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Bacillus subtilis*. According to the analysis results, *C. intybus* plant ethanol extract showed antimicrobial activity against *B. subtilis* and *S. aureus* bacteria (MIC values 9.25–17.50 mg/mL, respectively). Sales et al. [35] determined the MIC value of ethanol extract of the same plant as 6.50 mg/mL against*S. aureus* bacteria. E and M extracts of leaf and root powder of *C. intybus* were gamma irradiated at different doses (0, 4, 8, and 12 kGy), and their antioxidant and antimicrobial activities were evaluated. It was reported that the extracts showed significant inhibitory activity against potent bacteria such as *B. cereus*, *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *K. Pneumoniae*[36].

Table 1 MIC values of different extracts of C. intybus (mg/mL)

Solvents	K. pneumoniae	B. subtilis	S. aureus
Ethanol (E)	-	9.25	17.5
Methanol (M)	-	-	-
Hexane (H)	-	13.5	10.25
Dichloromethane (DCM)	-	-	-
NADES	0.71	1.43	2.85
Positive Control (Gentamicin, 20 µg/mL)	No growth occurred	No growth occurred	No growth occurred

The hexane extract of *C. intybus* also showed antimicrobial activity against the test bacteria (*B. subtilis* and *S. aureus*) (MIC values 13.5-10.25.5.25 mg/mL, respectively). Hexane extract of the roots of *C. intybus* L. plant was used against some pathogenic bacteria (*B. subtilis*, *S. aureus*, *Micrococcus luteus*, *E. coli*, and *Salmonella typhi*) by Nandagopal and Kumari [37]. As a result of the study, hexane extract showed antimicrobial activity against*B. subtilis* and *Staphylococcus aureus* (Inhibition zone diameters, 12.30–9.80 mm, respectively).

According to the analysis results (Table 1), methanol and dichloromethane extracts of the plant did not provide any inhibition against the test bacteria. However, NADES extract of *C. intybus* plant showed extremely low MIC values (0.71–2.85 mg/mL) against the test bacteria (*K. pneumoniae*, *B. subtilis*, and *S. aureus*).

In the antimicrobial activity study of NADES extracts of *Tragopogon dubius* and *Tragopogon porrifolium* L. subsp. *longirostris*, MIC values (0.18–0.71 mg/mL) were determined against test bacteria (*K. pneumoniae*, *B. subtilis*, and *S. aureus*) [22]. In a study on the antimicrobial effects of NADES extracts of black currant (*Ribes nigrum* L.) leaves, the extracts showed significant activity against Gram-positive bacteria and *Candida albicans*. In a recent study investigating the antimicrobial properties of NADES-based extracts obtained from the leaves of black currant (*Ribes nigrum* L.), the extracts were reported to exhibit notable inhibitory effects against Gram-positive bacteria (6.75–23.68 mm inhibition zone), Gram-negative bacteria (9.26–19.03 mm inhibition zone), and *C. albicans* (6.69–10.07 mm inhibition zone). The study further emphasized that NADES represents a more sustainable and efficient alternative to conventional organic solvents in the extraction of bioactive compounds from plant materials [38].

In parallel with previous studies, NADES extract from *C. intybus* showed significantly better antimicrobial activity against test bacteria (*K. pneumoniae*, *B. subtilis*, and *S. aureus*) than the extracts prepared with solvents such as ethanol, methanol, dichloromethane, and hexane. The NADES method is thought to better extract components with antimicrobial properties from plants.

Reducing capacity and radical scavenging ability results

The antioxidant activity of *C. intybus* extracts and NADES: DPPH and ABTS radical scavenging, Fe^{3+} and Cu^{2+} reducing power, and CUPRAC were studied. Synthetic extracts extracts obtained using conventional solvents, that is, BHA,

BHT, and the natural analogue α-tocopherol and its analogue Trolox, were also included in the study. An antioxidant activity assay determined the IC_{50}, and values were calculated. The results are summarized in Tables 2 and 3.

Sesquiterpene found in the leaves and the roots of *C. intybus*, known as chicory, contains various natural bioactive metabolites associated with health benefits, such as fatty acids, polyphenols, and lactones [39]. In addition,*C. intybus* is rich in terms of phenolic compounds (coumarins such as esculetin, 7-dihydroxycoumarin, scopoletin, esculin, chicoriin A-B-C, and 6-hydroxycinnamic acids) with antioxidant properties, phenolic acids, vitamins, flavonoids, and sesquiterpene lactones [40]. Due to this rich composition, chicory has been reported to have hepatoprotective, antibacterial, and anti-inflammatory activities [2].

IC_{50} values for DPPH scavenging activity of *C. intybus* extracts and standards were determined in the following order: Trolox (9.67 µg/mL)>BHA (10.59 µg/mL)>BHT (18.73 µg/mL) > α-Tocopherol (21.00 µg/mL)>*C. intybus* -NADES (35.54 µg/mL)>*C. intybus* -M (51.14 µg/mL)>*C. intybus* -E (63.00 µg/mL)>*C. intybus* -DCM (192.50 µg/mL)>*C. intybus* -H (216.13 µg/mL) (Table 2). A lower IC_{50} value indicates greater antioxidant power. As shown in Table 2, the *C. intybus* extracts exhibited lower radical scavenging activity than the standard antioxidants. At this point, a comparison of the IC_{50} values for all extracts in the DPPH assay revealed that the NADES, ethanol, and methanol extracts demonstrated the strongest radical scavenging capacities. It was determined that hexane and DCM extracts had the lowest radical scavenging effect (Table 2).

All plant extracts' ABTS radical scavenging test IC_{50} values were calculated with standard antioxidants. It was found that NADES, methanol, and ethanol extracts were much more effective than the other extracts (Table 2). IC_{50} values for ABTS scavenging activity of *C. intybus* extracts and standards were determined in the following order: Trolox (7.00 µg/mL) > BHA (8.93 µg/mL) > α-tocopherol (10.42 µg/mL) > BHT (16.94 µg/mL) > *C. intybus*-NADES (18.33 µg/mL) > *C. intybus*-M (21.69 µg/mL) > *C. intybus*-E (25.96 µg/mL) > *C. intybus* -DCM (169.02 µg/mL) > *C. intybus*-H (198.00 µg/mL) (Table 2). *C. intybus*-NADES extracts showed strong ABTS and DPPH radical scavenging activities compared to other extracts. In addition, both methods determined DCM and hexane extracts to have the lowest radical scavenging effect. When looking at the literature, limited studies were found on different parts of this plant with different solvents. Additionally, no study has been found in which NADES was used as a solvent for this plant. Matvieieva et al. [2] reported that ethanol extract showed a higher level of antioxidant activity in their study for quantifying and identifying bioactive compounds in aqueous and ethanol extracts of*C. intybus* "hairy" roots. In the study in which the antioxidant activity of ethanol and aqueous extracts of chicory "hairy" roots was determined by DPPH analysis, it was respectively reported that the IC_{50}values were 0.174 and 0.346 mg, and according to the results obtained, chicory "hairy" roots had high antioxidant activities [2]. Abbas et al. [41] reported a relatively low EC_{50} value (67.20 ± 2.6 µg/mL) for the DPPH radical scavenging activity of the hydroalcoholic extract of*C. intybus*leaves. Shad et al. [42] reported in their study in which they determined the DPPH radical scavenging capacities of different parts (stem, root and leaves) of *C. intybus* that leaves showed higher DPPH radical inhibition; however, the DPPH radical inhibition percentage of all parts was lower compared to trolox and ascorbic acid, which are the standard antioxidants.

The Fe^{3+} reduction ability of *C. intybus* at a concentration of 30 µg/mL showed absorbance in the range of 0.171 ± 0.010 to 1.442 ± 0.017 at 700 nm. In addition, the order of absorbance values of Fe^{3+} reduction capacity of the extracts is as follows: *C. intybus* -NADES (λ_{700}: 1.442 ± 0.017) > *C. intybus*-M (λ_{700}: 0.973 ± 0.043) > *C. intybus*-E (λ_{700}: 0.528 ±

Table 2 IC_{50} (µg/mL) values for DPPH and ABTS radical scavenging activities of *C. intybus* extracts and standard antioxidants

Antioxidants	DPPH		ABTS	
	IC_{50}(µg/mL)	r^2	IC_{50} (µg/mL)	r^2
BHA	**10.59**	**0.998**	**8.93**	**0.929**
BHT	**18.73**	**0.996**	**16.94**	**0.973**
Trolox	**9.67**	**0.921**	**7.00**	**0.940**
α-tocopherol	21.00	0.923	10.42	0.974
Extracts				
C. intybus L. DCM	192.50	0.913	169.02	0.967
C. intybus E	63.00	0.952	25.96	0.998
C. intybus H	216.13	0.923	198.00	0.929
C. intybus M	**51.14**	**0.999**	**21.69**	**0.994**
C. intybus NADES	**35.54**	**0.988**	**18.33**	**0.995**

Table 3 Fe^{3+} and Cu^{2+} reducing activities of *C. intybus* extracts and standards at 30 g/mL

Antioxidants	Fe^{3+} Reducing		Cu^{2+} Reducing	
	λ_{700}	r^2	λ_{450}	r^2
BHA	2.39 ± 0.02^{a}	0.959	2.03 ± 0.02^{a}	0.996
BHT	1.49 ± 0.00^{b}	0.956	1.64 ± 0.03^{c}	0.965
Trolox	1.24 ± 0.02^{c}	0.995	0.53 ± 0.03^{i}	0.951
α-Tocopherol	1.04 ± 0.05^{d}	0.987	1.20 ± 0.02^{e}	0.998
Extracts				
C. intybus DCM	0.19 ± 0.00^{g}	0.923	0.65 ± 0.01^{g}	0.938
C. intybus E	0.53 ± 0.01^{f}	0.999	1.03 ± 0.02^{f}	0.942
C. intybus H	0.17 ± 0.01^{h}	0.934	0.58 ± 0.02^{h}	0.906
C. intybus M	$\mathbf{0.97 \pm 0.04^{e}}$	**0.999**	$\mathbf{1.48 \pm 0.05^{d}}$	**0.957**
C. intybus NADES	$\mathbf{1.44 \pm 0.02^{b}}$	**0.976**	$\mathbf{1.74 \pm 0.065^{b}}$	**0.935**
Significance	**		**	

a-ı: means with different letters in the same line are significantly different ($p<0.05$); **; $p<0.01$

0.006) > *C. intybus* -DCM (λ_{700}: 0.193 ± 0.004) > *C. intybus* -H (λ_{700}: 0.171 ± 0.010). The Fe^{3+} reducing capacity of the standards at the same concentration (30 µg/mL) was found as BHA (λ_{700}: 2.385 ± 0.016) > BHT (λ_{700}: 1.494 ± 0.003)> Trolox (λ_{700}: 1.240 ± 0.022)> α-Tocopherol (λ_{700}: 1.044 ± 0.047) (Table 3). The results obtained from this test showed that *C. intybus*-NADES extracts had better Fe^{3+}reducing properties compared to Trolox and α-tocopherol standard antioxidants. Dalar & Konczak [43] established the FRAP values of the leaf and flower extracts of *C. intybus* from Eastern Anatolia as 251.60 and 240.80 µmol Fe^{+2}/g dry weight, respectively, and these values were determined to be higher than those of the root and stem.

CUPRAC antioxidant method was determined by measuring absorbance at 450 nm at a concentration of 30 µg/mL (Table 3). In our research, the reducing powers of *C. intybus* and standard antioxidants were compared with each other and the following results were obtained: BHA (λ_{450}: 2.027±0.019)>*C. intybus*-NADES (λ_{450}: 1.737±0.065)>BHT (λ_{450}: 1.638±0.030)>*C. intybus*-M (λ_{450}: 1.475±0.048) > α-Tocopherol (λ_{450}: 1.200±0.020)>*C. intybus*-E (λ_{450}: 1.030±0.018)>*C. intybus*-DCM (λ_{450}: 0.645±0.011)>*C. intybus*-H (λ_{450}: 0.583±0.017) > Trolox (λ_{450}: 0.527±0.032). When NADES extracts were compared with the standards in this method, it was found that they showed lower reducing properties compared to the BHA standard, but higher reducing properties compared to the other standards.

UHPLC-ESI-Orbitrap®-HRMS/MS-Based method validation for comprehensive phenolic characterization

In literature, the UHPLC-ESI-Orbitrap®-HRMS/MS method is frequently employed in quantification of the phenolic compounds [22, 31]. In this regard, an accurate quantitative method was established in a mass spectrometer with an Orbitrap®-HRMS/MS analyzer to measure 71 phenolic compounds (Table 4). UHPLC-ESI-Orbitrap®-HRMS/MS strategy to estimate 71 the concentration of these compounds in the extracts of *C. intybus* L., methanol and NADES. A total of 71 phytochemical profiles were visually explored in the samples analyzed using UHPLC-ESI-Orbitrap®HRMS/MS. The standards solution for the phenolic compounds in this analysis were prepared at concentrations ranging 10–100 ng/mL. These different levels of standards were analyzed in triplicate to make an external calibration curve.

Figure 2 shows the parent ions (peak) for quantification, the fragment ions (m/z) for qualitation 1, chromatograms and calibration curves of the main peak of substance luteolin 7-O-glucuronide with the concentration of solutions of 100 µg/mL. Additionally, the UHPLC-ESI-Orbitrap®-HRMS/MS total ion chromatogram (TIC) is provided for the 71 phenolic compound standards at a concentration of 100 ng/mL and the *C. intybus* methanol extract. *C. intybus* NADES extract, is provided in Fig. 3.

The primary components identified in the extract of *C. intybus* M were ascertained as such: luteoloside 666.13±28.63 µg/g, quinic acid 542.98±32.73 µg/g, eriodictyol 437.20±19.14 µg/g, luteolin-7-o-glucuronide 415.11±21.47, isoquercitrin 308.25±14.82 µg/g, esculin hydrate 291.04±22.93 µg/g, chlorogenic acid 271.64±21.23 µg/g, astragalin 141.78±4.56 µg/g, nicotiflorin 95.32±5.43 µg/g, apigenin 7-glucuronide 94.20±8.58 µg/g, µg/g, rutin hydrate 82.91±23.96 µg/g and narcissin 75.24±5.00 µg/g.

The main compounds found in the NADES extract of C. intybus were identified as: luteolin-7-o-glucuronide 806,70±26,06 µg/g, luteoloside 449.59±9.19 µg/g, eriodictyol 402.38±13.24 µg/g, chlorogenic acid 310.28±14.06 µg/g, esculin hydrate 243.06±12.35 µg/g, isoquercitrin 182.75±5.49 µg/g, apigenin 7-glucuronide 162.63±10.26 µg/g, quinic acid 119.65±4.00 µg/g, nicotiflorin 79.94±4.11 µg/g, astragalin 76.08±4.69 µg/g, narcissin 69.90±2.70 µg/g and rutin hydrate 65.07±3.72 µg/g.

In the analysis of phenolic compounds obtained from M and NADES extracts, it was observed that these compounds were commonly detected in both extracts. However, some phenolic compounds were higher in M extract, while others were higher in NADES extract. Upon examining this, it was determined that phenolic compounds such as 4-hydroxybenzoic acid, protocatechuic acid, coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, apigenin 7-glucuronide, schaftoside, luteolin-7-o-glucuronide, Orientin, and fisetin hydrate were quantitatively higher in the NADES extract compared to the M extract. In the extract obtained with M, quinic acid, apigenin 7-glucoside, rutin hydrate, luteolin, luteoloside, luteolin 7-rutinoside, isoquercitrin, narcissin, nicotiflorin, astragalin, eriodictyol, and esculin hydrate were found to be in higher quantities compared to the NADES extract. Additionally, phenolic compounds were detected in the UHPLC-ESI-Orbitrap®-HRMS/MS analysis of the *C. intybus* NADES extract. Among the screened phenolic compounds, the highest detected compound was luteolin-7-O-glucuronide at 806.70 ± 26.06 µg/g. Flavonoids detected in *C. intybus*contain flavanol, flavonol, flavone, and anthocyanin groups. The compounds that have been identified are quercetin, luteolin, kaempferol, myricetin, apigenin, chrysoteriol, jaquelin, and isorhamnetin glycosides [14].

The leaves of *C. intybus* are known to contain large concentrations of phenolic compounds including hydroxycinnamic acid derivatives such as chlorogenic acid and chicoric acid [44, 45]along with caffeic acid, quercetin glucuronide, and gallic acid [46]. Phenolic acids and flavonoids, such as camptoric acid, chlorogenic acid, chicoric acid, isoquercitrin, rutin, quercitrin, luteolin, and apigenin, are found in

Table 4 Analytical parameters of the UHPLC-ESI-Orbitrap®-HRMS/MS method and phenolic compounds identified and quantified in M and NADES extracts of *C. intybus* L. (mg analyte/g dry extract)

No	Phenolic Compounds	RT	Quan Peak (Parent Ion) (m/z)	Confirming Ions (Fragment Ions) (m/z)	Ion Mode	Adduct Ions	R^2	Liner Range (ng/mL)	LOD (ng/mL) (n=10)	LOQ (ng/mL) (n=10)	% Recovery (10 ng/mL) (n=10)	% Recovery RSD	C. intybus L M (μg/g dry extract) (n=3)	C. intybus L NADES (μg/g dry extract) (n=3)
1	Benzoic acid	10.86	121.02950	121.02950	-	M-H	0.9913	10–100	0.83	2.75	103.24	2.67	ND	ND
2	4-Hydroxybenzoic acid	7.82	137.02442	93.03471	-	M-H	0.9921	10–100	1.10	3.66	116.16	3.15	1.04±0.04[a]	1.34±0.06[b]
3	Salicylic acid	10.93	137.02442	93.03468	-	M-H	0.9946	10–100	1.22	4.08	115.33	3.54	ND	ND
4	3-hydroxybenzoic acid	8.66	137.02442	93.03471	-	M-H	0.9926	10–100	1.81	6.03	117.14	5.14	ND	ND
5	3-hydroxyphenylacetic acid	8.67	151.04007	107.05045	-	M-H	0.9923	10–100	2.65	8.84	103.65	8.52	ND	ND
6	Syringic acid	8.90	197.04555	197.04555	-	M-H	0.9949	10–100	0.99	3.29	111.85	2.94	ND	ND
7	Gallic acid	3.70	169.01425	125.02461	-	M-H	0.9903	10–100	3.03	10.09	108.42	9.30	ND	ND
8	Protocatechuic acid	6.24	153.01933	109.02949	-	M-H	0.9908	10–100	0.78	2.59	80.98	3.20	0.78±0.17[a]	0.99±0.08[a]
9	Protocatechuic acid ethyl ester	7.29	137.02442	136.01671	-	M-H	0.9909	10–100	1.43	4.76	65.77	7.24	ND	ND
10	Protocatechuic aldehyde	8.42	153.01933	67.01888	-	M-H	0.9935	10–100	0.48	1.60	71.64	2.23	ND	ND
11	Vanillic acid	8.57	167.03498	108.02173	-	M-H	0.9938	10–100	1.97	6.58	126.60	5.20	ND	ND
12	Homovanillic acid	8.67	181.05063	122.03749	-	M-H	0.9950	10–100	2.10	7.02	91.30	7.68	ND	ND
13	Vanillin	9.23	151.04007	108.02178	-	M-H	0.9913	10–100	1.66	5.54	110.58	5.01	ND	ND
14	Gentisic acid	7.40	153.01933	153.01933	-	M-H	0.9913	10–100	0.55	1.82	104.56	1.74	ND	ND
15	Trans-Cinnamic acid	12.31	147.04515	147.04515	-	M-H	0.9952	10–100	2.32	7.72	105.72	7.30	ND	ND
16	Coumaric acid	9.80	163.04007	119.05027	-	M-H	0.9928	10–100	1.07	3.56	98.67	3.61	0.85±0.12[a]	3.49±0.21[b]
17	Caffeic acid	8.62	179.03498	135.04509	-	M-H	0.9917	10–100	0.67	2.22	108.32	2.05	3.27±0.47$_a$	4.45±0.27[b]
18	Caffeic acid phenhyl ester	14.28	283.09758	135.04526	-	M-H	0.9980	10–100	1.98	6.61	100.95	6.55	ND	ND
19	Ferulic acid	10.05	193.05063	134.03751	-	M-H	0.9933	10–100	1.46	4.88	103.10	4.73	11.96±2.11[a]	25.99±1.08[b]
20	Sinapic acid	10.02	223.06120	193.01436	-	M-H	0.9934	10–100	0.89	2.95	105.15	2.81	ND	ND
21	Chlorogenic acid	8.07	353.08781	191.05624	-	M-H	0.9976	10–100	0.75	2.50	105.49	2.37	**271.64±21.23[a]**	**310.28±14.06[a]**
22	Quinic acid	0.93	191.05611	85.02962	-	M-H	0.9939	10–100	0.77	2.58	105.39	2.45	**542.98±32.73[a]**	**119.65±4.00[b]**
23	3-(4-Hydroxyphenyl) propionic acid	9.26	165.05572	108.02195	-	M-H	0.9927	10–100	2.46	8.20	94.69	8.66	ND	ND
24	α-Cyano-4-hydroxycinnamic acid	10.09	188.03532	93.03470	-	M-H	0.9925	10–100	0.47	1.57	102.33	1.54	ND	ND
25	Catechin	7.62	289.07176	109.02975	-	M-H	0.9943	10–100	0.58	1.92	109.74	1.75	ND	ND
26	Chrysin	14.62	253.05063	253.05063	-	M-H	0.9907	10–100	0.64	2.12	105.54	2.01	ND	ND
27	Apigenin	13.24	269.04555	117.03464	-	M-H	0.9932	10–100	1.07	3.57	104.44	3.42	ND	ND
28	Acacetin	14.93	283.06120	268.03717	-	M-H	0.9911	10–100	1.99	6.63	111.14	5.97	ND	ND
29	Vicenin 2	9.05	593.15119	473.10941	-	M-H	0.9945	10–100	1.11	3.68	113.88	3.24	ND	ND
30	Apigenin 7-glucuronide	11.21	445.07763	269.04535	-	M-H	0.9923	10–100	1.32	4.39	103.56	4.24	**94.20±8.58[a]**	**162.63±10.26[b]**
31	Apigenin 7-glucoside	11.14	431.09837	269.04544	-	M-H	0.9947	10–100	0.82	2.74	109.48	2.50	25.91±1.78[a]	15.71±1.05[b]
32	Genkwanin	14.93	283.06120	268.03745	-	M-H	0.9911	10–100	1.29	4.31	105.69	4.08	ND	ND
33	Apiin	10.97	563.14063	269.04498	-	M-H	0.9923	10–100	0.85	2.84	113.72	2.50	ND	ND
34	Vitexin	10.09	431.09837	311.05603	-	M-H	0.9939	10–100	0.96	3.19	108.34	2.94	ND	ND
35	Schaftoside	9.66	563.14063	443.09949	-	M-H	0.9908	10–100	1.80	5.98	102.72	5.83	15.68±0.60[a]	19.16±0.51[b]
36	Rutin hydrate	10.67	609.14611	300.02777	-	M-H	0.9912	10–100	1.31	4.36	100.01	4.36	**82.91±23.96[a]**	**65.07±3.72[a]**
37	Luteolin	12.53	285.04046	175.04039	-	M-H	0.9932	10–100	1.51	5.05	98.65	5.12	8.16±2.37[a]	1.93±0.79[b]
38	Luteolin-7-O-glucuronide	10.52	461.07255	285.04056	-	M-H	0.9938	10–100	0.86	2.85	97.37	2.93	**415.11±21.47[a]**	**806.70±26.06[b]**
39	Orientin	9.76	447.09328	327.05148	-	M-H	0.9955	10–100	0.88	2.95	104.32	2.83	0.45±0.11[a]	1.52±0.06[b]
40	Luteoloside	10.50	447.09328	285.04059	-	M-H	0.9946	10–100	0.75	2.50	101.24	2.47	**666.13±28.63[a]**	**449.59±9.19[b]**
41	Luteolin 7-rutinoside	10.40	593.15119	285.04065	-	M-H	0.9923	10–100	0.72	2.42	104.98	2.30	39.34±2.35[a]	35.67±1.12[a]
42	Galangin	14.68	269.04555	269.04555	-	M-H	0.9936	10–100	1.43	4.77	96.72	4.93	ND	ND
43	İsoquercitrin	10.74	463.08820	300.02768	-	M-H	0.9939	10–100	0.74	2.46	104.52	2.36	**308.25±14.82[a]**	**182.75±5.49[b]**
44	Narcissin	11.41	623.16176	315.05139	-	M-H	0.9918	10–100	1.35	4.50	98.70	4.55	**75.24±5.00[a]**	**69.90±2.70[a]**

Table 4 (continued)

No	Phenolic Compounds	RT	Quan Peak (Parent Ion) (m/z)	Confirming Ions (Fragment Ions) (m/z)	Ion Mode	Adduct Ions	R^2	Liner Range (ng/mL)	LOD (ng/mL) ($n=10$)	LOQ (ng/mL) ($n=10$)	% Recovery (10 ng/mL) ($n=10$)	% Recovery RSD	C. intybus L M (µg/g dry extract) ($n=3$)	C. intybus L NADES (µg/g dry extract) ($n=3$)
45	Quercetin 3-rutinoside 7-glucoside	11.53	287.05536	135.04512	-	M-H	0.9934	10–100	0.80	2.67	97.62	2.74	ND	ND
46	Isorhamnetin	13.21	315.05103	300.02780	-	M-H	0.9958	10–100	0.62	2.06	96.17	2.14	ND	ND
47	Kaempferol	13.08	285.04046	136.01686	-	M-H	0.9924	10–100	0.59	1.98	96.15	2.06	ND	ND
48	Afzelin	11.98	431.09837	285.04028	-	M-H	0.9951	10–100	1.95	6.49	102.18	6.35	ND	ND
49	Kaempferide	14.80	299.05611	284.03265	-	M-H	0.9973	10–100	0.42	1.41	95.28	1.48	ND	ND
50	Nicotiflorin	11.31	593.15119	285.04041	-	M-H	0.9915	10–100	0.79	2.63	96.23	2.74	**95.32±5.43[a]**	**79.94±4.11[b]**
51	Astragalin	11.34	447.09328	284.03281	-	M-H	0.9936	10–100	1.61	5.37	96.85	5.54	**141.78±4.56[a]**	**76.08±4.69[b]**
52	Leucoside	11.53	287.05536	151.00372	-	M-H	0.9934	10–100	0.52	1.73	101.51	1.71	ND	ND
53	Fisetin hydrate	11.48	285.04046	135.00900	-	M-H	0.9913	10–80	1.27	4.22	104.62	4.03	15.01±0.82[a]	16.89±0.94[a]
54	Naringenin	12.34	271.06120	119.05035	-	M-H	0.9932	10–100	1.43	4.77	102.76	4.64	ND	ND
55	Sakuranetin	14.01	285.07685	119.05032	-	M-H	0.9921	10–100	1.03	3.44	105.44	3.26	ND	ND
56	Narirutin	10.40	579.17193	271.06107	-	M-H	0.9926	10–100	1.42	4.72	102.69	4.60	ND	ND
57	Eriodictyol	10.68	287.05501	287.05501	+	M+H	0.9907	10–100	2.40	7.99	123.45	6.47	**437.20±19.14[a]**	**402.38±13.24[a]**
58	Liquiritigenin	11.59	255.06628	255.06592	-	M-H	0.9943	10–100	0.57	1.89	96.19	1.97	ND	ND
59	Daidzin	9.35	415.10346	253.05191	-	M-H	0.9947	10–100	1.10	3.68	106.04	3.47	ND	ND
60	Formononetin	13.60	267.06628	252.04263	-	M-H	0.9917	10–100	1.94	6.46	97.74	6.61	ND	ND
61	Kuromanine	8.19	447.09328	285.03970	-	M-H	0.9993	10–100	0.88	2.95	114.15	2.58	ND	ND
62	Ellagic acid	10.93	300.99899	300.99872	-	M-H	0.9943	10–100	0.71	2.37	106.20	2.24	ND	ND
63	Esculin hydrate	7.43	339.07216	177.01930	-	M-H	0.9960	10–100	0.90	2.99	105.55	2.83	**291.04±22.93[a]**	**243.06±12.35[b]**
64	Phloridzin	10.98	435.12967	273.07648	-	M-H	0.9924	10–100	1.04	3.46	112.06	3.09	ND	ND
65	Rosmarinic acid	10.83	359.07724	161.02451	-	M-H	0.9935	10–100	0.68	2.26	106.07	2.13	ND	ND
66	Glabridin	15.16	323.12888	135.04533	-	M-H	0.9967	10–100	1.10	3.67	103.47	3.55	ND	ND
67	Arbutin	2.99	271.08233	108.02180	-	M-H	0.9904	10–100	1.43	4.77	98.30	4.85	ND	ND
68	Emodin	16.35	269.04555	225.05573	-	M-H	0.9909	10–100	1.40	4.66	97.80	4.76	ND	ND
69	Etoposide	11.65	611.17351	405.09308	-	M-H	0.9906	10–100	1.96	6.54	101.52	6.44	ND	ND
70	Doxorubicin Hydrchloride	11.78	542.16678	395.07828	-	M-H	0.9929	10–100	2.01	6.69	93.55	7.15	ND	ND
71	Ethylgallate	9.40	197.04555	124.01682	-	M-H	0.9920	10–100	0.49	1.64	96.40	1.70	ND	ND

RT Retention time, *RSD* Relative standard deviation, *LOD* Limit of detection, *LOQ* Limit of quantification, R^2 Correlation coefficien. *Quan Peak* Parent Ion (m/z) of phytochemical compound standards, *Confirming Ions* fragment ions (m/z) of phenolic compound standards, *ND* Not detected

The means of *C. intybus* L. M and *C. intybus* L. NADES and the differences between groups were evaluated using one-way analysis of variance (ANOVA), and Duncan's multiple comparison test was applied for post-hoc comparisons to determine statistically significant differences between groups. Data are expressed as mean±standard deviation (SD). Differences between groups with different letters (a–b) were considered statistically significant at $p<0.05$ (*)

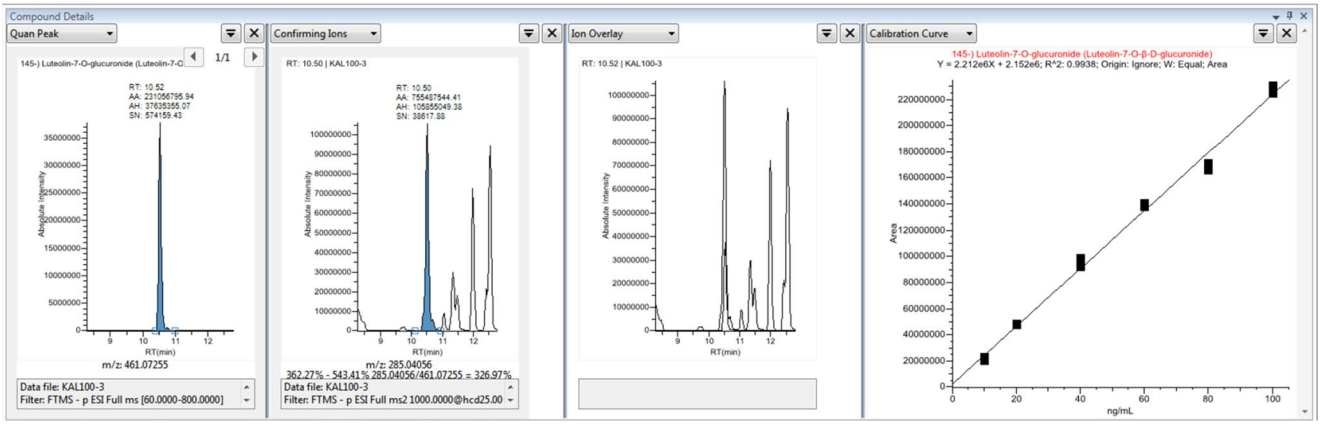


Fig. 2 UHPLC-ESI-Orbitrap®-HRMS/MS Quan Peak (Parent Ion), Confirming Ion (m/z) (Fragment Ions) chromatograms of 100 ng/mL major compound Luteolin-7-O-glucuronide and calibration curve

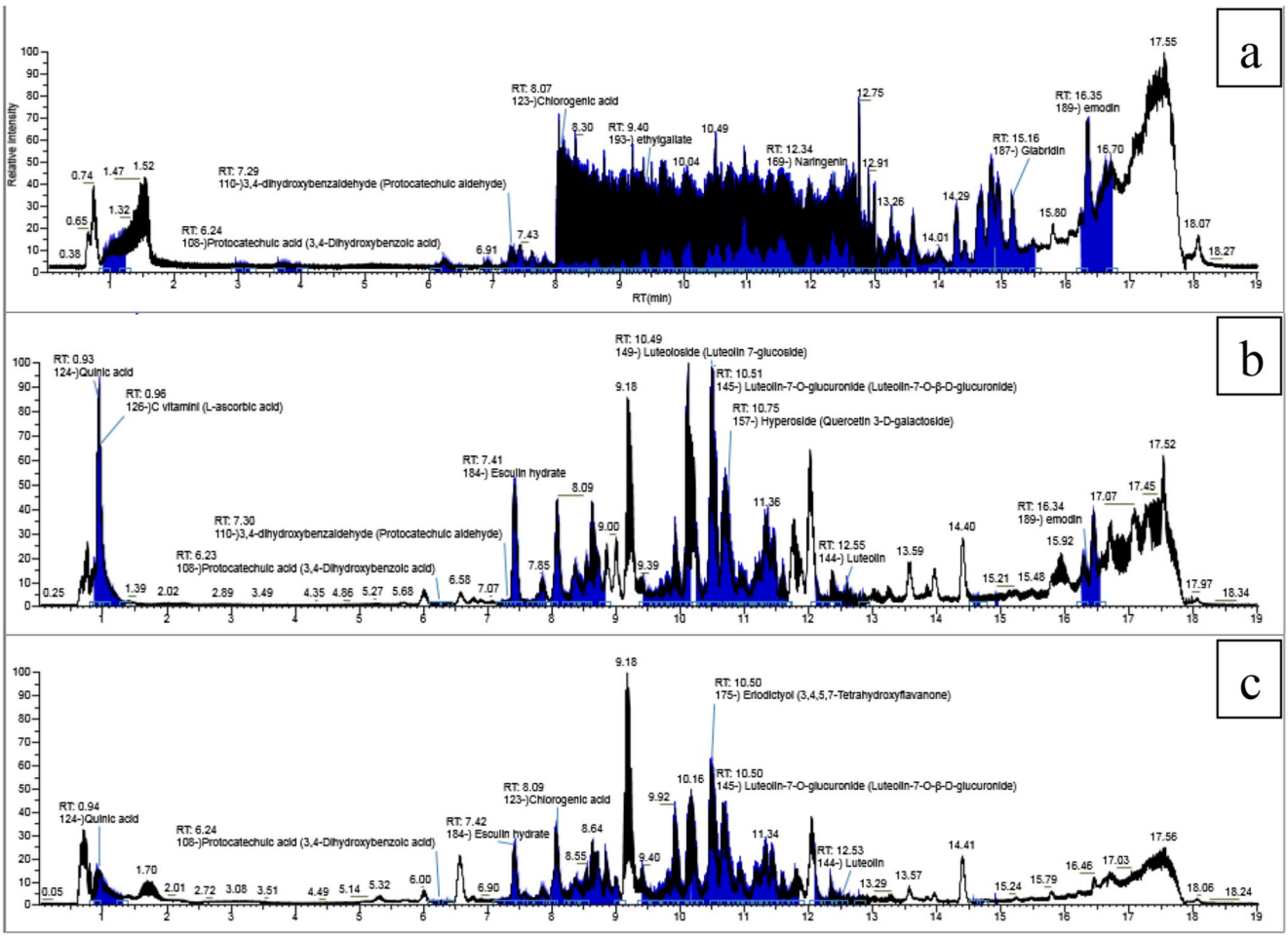


Fig. 3 UHPLC-ESI-Orbitrap®-HRMS/MS TIC of **a**: 100 ng/mL 71 phenolic compounds; **b**: *C. intybus* methanol extract; **c**: *C. intybus* NADES extract

the aerial parts of the plant [46]. Jancic et al. [47] obtained fresh material (leaves) of *C. intybus* genotypes from different geographic area in Montenegro. A total of 9 samples of *C. intybus* were analyzed: 7 wild plant samples (collected from Zoganje, Risan, Podgor, Tivat, Pricelje, Plavnica and Pljevlja) and 2 plants cultivated in greenhouses (from Komani and Susanj). In wild samples the highest content of CGA (total isomers) was determined in the Zoganje sample (1034 µg/100 g), while the average value was 568 ± 4 µg/100 g, and the lowest were recorded in the ones from cultivated

material in the areas of Komani and Susanj (104 µg/100 g). The caffeic acid content generally ranged between 1.4 and 7.0 µg/100 g, with an average value of 3.2 µg/100 g.

Epure et al. [46] investigated the methanol extract and tincture obtained from the spontaneously growing *C. intybus* species in Romania, evaluating their polyphenol content. Using HPLC-UV-MS analysis, the following compounds were identified in the methanol extract: chlorogenic acid 910.69 ± 36.15 µg/g d.w., cichoric acid 18450.00 ± 732 µg/g d.w., isoquercitrin 427.30 ± 16.43 µg/g d.w., rutin 14.51 ± 0.66 µg/g d.w., quercitrin 104.61 ± 4.01 µg/g d.w., and luteolin 3.98 ± 0.12 µg/g d.w. In the tincture, the contents were as follows: cichoric acid 6122.58 ± 232.10 µg/g d.w., isoquercitrin 296.31 ± 11.85 µg/g d.w., rutin 15.99 ± 0.56 µg/g d.w., quercitrin 54.13 ± 1.95 µg/g d.w., luteolin 21.96 ± 0.82 µg/g d.w., and apigenin 6.77 ± 0.2 µg/g d.w.

PCA analysis of phenolic compounds and heatmap-based comparison of antioxidant profiles

PCA was explicitly performed using only the ten most abundant phenolic compounds quantified in both methanol and NADES extracts, thereby focusing the multivariate interpretation on the dominant metabolites most likely to drive the observed biological activities. This targeted approach ensures that the PCA reflects biologically relevant variation rather than noise arising from low-abundance constituents.

As shown in Fig. 4, the first two principal components together explain nearly all of the variance in the reduced dataset, indicating that the compositional differences between extraction systems are largely governed by this limited set of high-impact phenolics. The PCA score plot reveals a clear separation between methanol and NADES extracts along PC1, demonstrating that solvent polarity and hydrogen-bonding capacity selectively influence the extraction of specific dominant compounds.

The NADES extract is primarily associated with higher relative contributions from luteolin-7-O-glucuronide, chlorogenic acid, and apigenin-7-glucuronide, which load positively on PC1. These compounds are well-recognized for their strong redox activity, attributable to their conjugated aromatic structures and multiple hydroxyl groups, which facilitate electron donation and radical stabilization. Their dominance within the top-10 profile of the NADES extract provides a direct chemical basis for the superior antioxidant performance observed in radical scavenging and reducing power assays.

Conversely, the methanol extract clusters on the opposite side of PC1 and is more strongly associated with quinic acid, luteoloside, isoquercitrin, nicotiflorin, astragalin, and eriodictyol, which constitute the majority of its top-10 phenolic composition. Although these compounds also possess antioxidant potential, several are glycosylated flavonoids or cyclitol derivatives whose antioxidant efficacy may be less pronounced on a molar basis or more dependent on synergistic interactions. This compositional distinction explains why methanolic extracts, while active, consistently exhibit weaker antioxidant responses compared to NADES extracts across multiple assays.

The heat map analysis is an effective but concise visualisation of the antioxidant performance of the extracts and the reference antioxidants in terms of Fe^{3+} and Cu^{2+} reducing

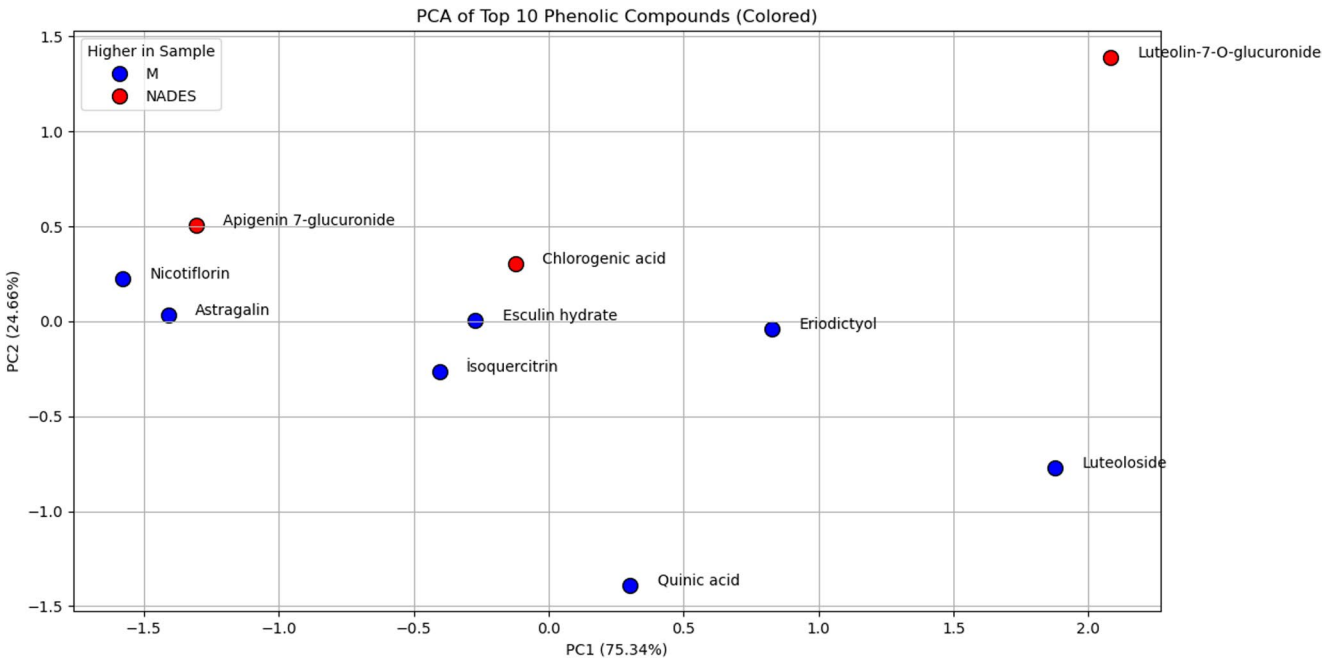


Fig. 4 PCA of the top 10 phenolic compounds detected in the M and NADES extracts

capacities of the antioxidant performance of the extracts and the reference antioxidants. The heatmap allows a comparative assessment of the overall redox behaviour instead of the assay specific results because both assays are run at the same time.

As shown in the pattern of clustering, extracts made with non-polar solvents (hexane and dichloromethane) constitute a well-separated low-activity group, which is typified by systematically weak reducing power of Fe^{3+} and Cu^{2+}. This grouping indicates their low ability to remove polar phenolic compounds that had strong electronic donating power and thus affirms the fact that the polarity of solvents is a decisive factor in determining antioxidant efficiency.

Conversely, the NADES and methanol extracts of *C. intybus* are more similar to those of synthetic antioxidants, especially BHT, meaning the reducing ability is much greater. It is noteworthy that NADES extract has a higher color than the methanol extract in both reducing assays indicating that the redox profile is stronger and more balanced (Fig. 5). This observation agrees with the enhancement of glucuronidated flavonoid and hydroxycinnamic acids in the NADES extract, which has been previously reported to have high reducing potential because of its conjugated structures and multiple hydroxyl groups.

Regarding the standards, high-activity cluster is expected to consist of BHA and BHT, and this is the case, with Trolox and α-tocopherol taking intermediate positions. Notably, the closeness of the NADES extract to the BHT in the hierarchical clustering highlights the fact that its antioxidant activity is close to that of commonly used synthetic antioxidants even though it is obtained using a green extraction methodology.

The methanolic extract has a moderate reducing capacity and clusters between NADES and α-tocopherol, indicating that despite the good ability of methanol to extract antioxidant compounds, its extract composition is not as optimised towards high-impact reducing agents as NADES (Fig. 5). The difference is consistent with compositional results that methanol is more selective in favor of some flavonol glycosides and quinic acid and NADES is more particularly enriches compounds with stronger intrinsic redox active properties.

Bioinformatic target and pathway interpretation

DisGeNET disease association

DisGeNET enrichment analysis of the key compound luteolin-7-O-glucuronide, which has been identified in *C. intybus* L. in NADES extract, provides strong evidence of its hypothetical disease-relatedness. The findings suggest that the given flavonoid derivative is not restricted to antioxidant and anti-inflammatory functions but might have extensive biological functions in neurological, metabolic, and oncological pathways. Among others, it is of particular significance that the neurological and pain-related disorders, including hyperalgesia, amnesia, headache, and airway disease, are enriched. The high gene ratios, and the significantly high adjusted p-values indicate that luteolin-7-O-glucuronide may bind to molecular targets of nociception and neuroprotection (Fig. 6A). This observation is supported by the earlier research that has established that luteolin derivatives have a neuroprotective effect, neurotransmitter regulation, and synaptic plasticity which is mediated by the regulation of neuroinflammation. Another interesting relationship appears with the caffeine-related disorders and drug use that involves interactions with adenosine receptor mechanisms and other neuromodulatory mechanisms. These results support the previous report that flavonoids have an effect on purinergic signalling, which could be behind their established action on cognition, fatigue, and stimulant-like behaviours. Similarly, the acute lung injury and bone marrow disease enrichment shows

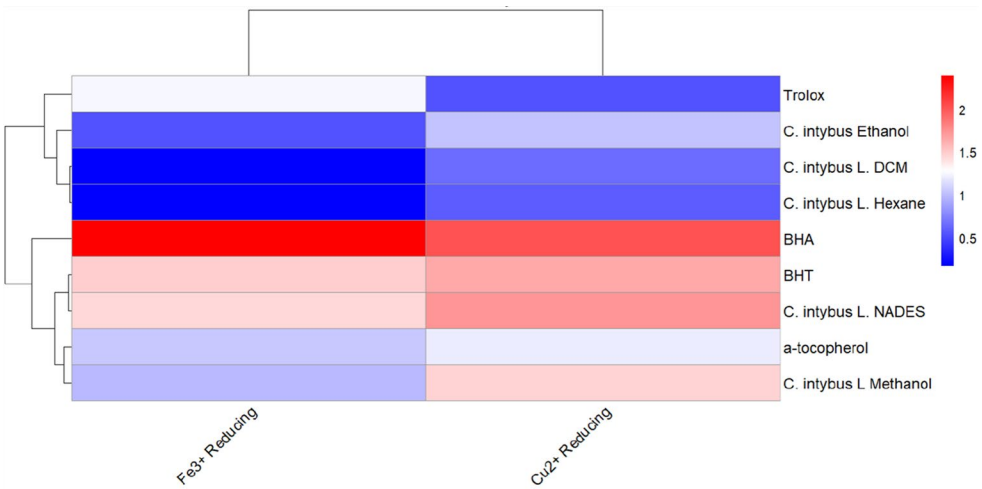


Fig. 5 Heatmap visualization of antioxidant activities (Fe^{3+} and Cu^{2+} reducing power) of *C. intybus* extracts and standard antioxidants. The color gradient indicates the activity intensity, with red representing higher and blue representing lower antioxidant values

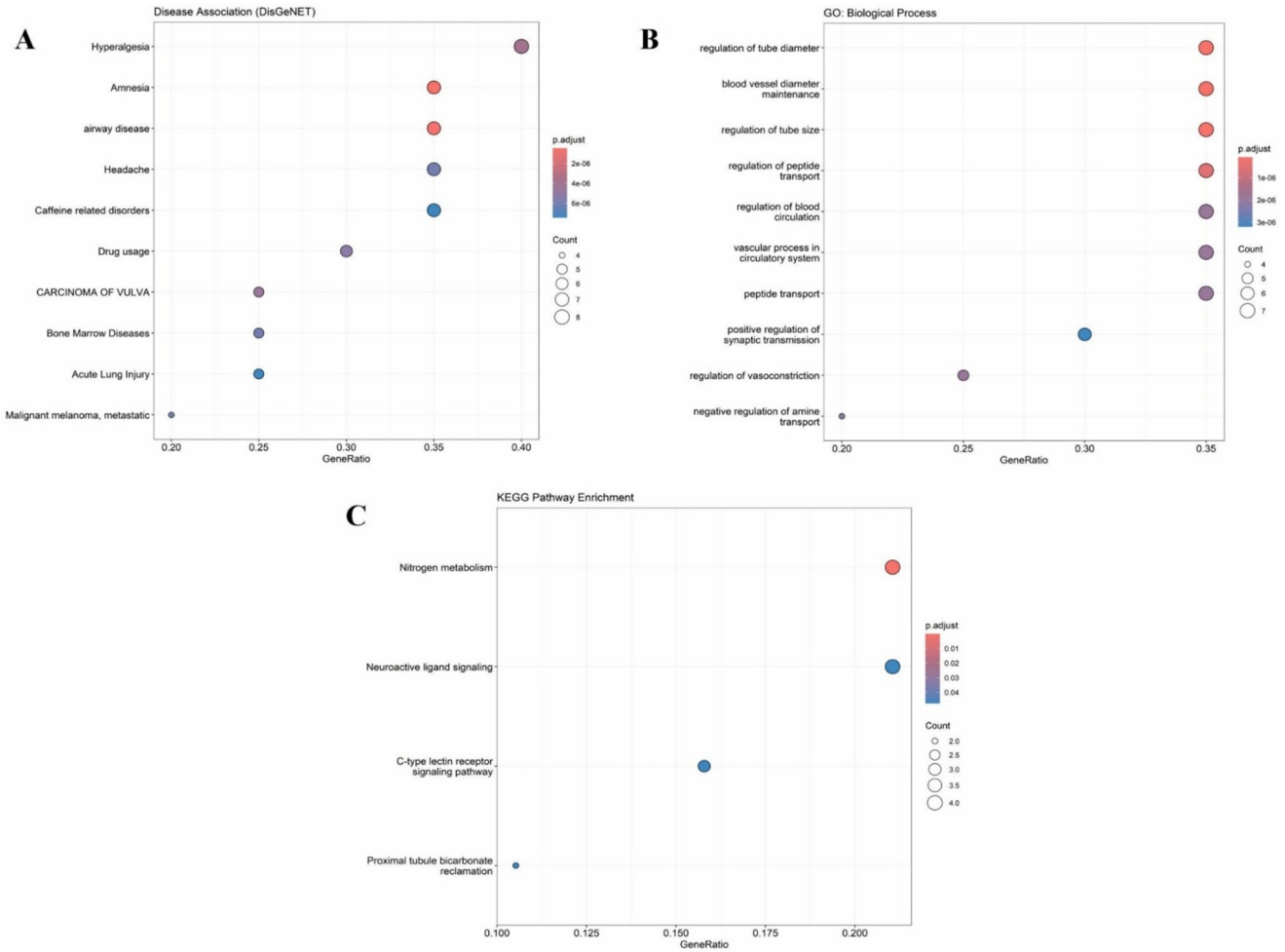


Fig. 6 Functional enrichment studies on the predicted targets of luteolin-7 -O-glucuronide of *C. intybus* L. after NADES extraction. (**A**) DisGeNET disease association analysis. (**B**) Gene Ontology (GO) Biological Process analysis. (**C**) KEGG pathway enrichment. The x-axis in all panels is the gene ratio, the dot size is the number of related genes and the colour gradient is the adjusted p -value

the possible immunomodulatory and tissue-protective advantages of the investigated phytochemical, which supports the anti-inflammatory and cytoprotective potential of this phytochemical.

In addition to neurological and inflammatory diseases, the analysis also revealed the relation with malignant melanoma (metastatic) and vulvar carcinoma. Such findings are of interest especially considering the growing evidence that luteolin analogues regulate tumour micro-environment, apoptosis and proliferative signalling. Therefore, luteolin-7-O -glucuronide can be suggested as a promising candidate for further exploration in the context of cancer chemoprevention or as the additional therapeutic agent.

GO biological process enrichment

The GO Biological Process enrichment analysis of the most abundant metabolite found by NADES extraction in *C. intybus* L. luteolin-7-O-glucuronide indicates a number of biologically significant pathways. The most involved terms, as shown on the dot plot, include response to drugs, regulation of apoptotic signalling, oxidative stress response, and cellular response to chemical stress. The relatively large gene ratios and highly significant adjusted p-values suggest that luteolin-7-O-glucuronide can be at the centre of controlling the cellular defensive, and homeostasis in stress conditions (Fig. 6B).

Interestingly, the mechanisms associated with the inflammatory response and regulation of the immune-system are also represented significantly, which supports the anti-inflammatory and immunomodulatory effects of luteolin derivatives, which are well-documented. The cell-cycle regulation, DNA-damage response and apoptotic pathways enrichment can be considered an extra mechanistic connection with anticancer activity, consistent with previous results that flavonoids can suppress tumour growth with pro-apoptotic and anti-proliferative effects. In addition, the

enriched allotropy of the synaptic signalling and neurotransmitter regulation implies possible neuroprotective properties, thus, indicating the suitability of the compound in the health of the nervous system.

KEGG pathway analysis

We interpret the KEGG pathway enrichment built from the high-scoring SwissTargetPrediction genes of luteolin-7-O-glucuronide (the most abundant metabolite from *C. intybus* L. after NADES extraction) (Fig. 6C). The dot plot shows four significant pathways. The top signals by gene ratio (~0.21) are Nitrogen metabolism and Neuroactive ligand signaling; both also display low adjusted p-values, indicating robust enrichment. The nitrogen-metabolism signal, together with the enrichment of Proximal tubule bicarbonate reclamation (gene ratio≈0.10), points to targets involved in acid–base handling and renal physiology most plausibly carbonic anhydrase isoforms and bicarbonate transporters suggesting a mechanistic basis for renal and systemic pH modulation by this flavonoid. Neuroactive ligand signaling implicates GPCR-mediated neurotransmission (e.g., aminergic and purinergic receptors), compatible with reported neuromodulatory actions of luteolin scaffolds and consistent with potential analgesic/neuroprotective effects. The enrichment of the C-type lectin receptor signaling pathway (gene ratio≈0.16) indicates interaction with innate-immune sensing hubs that converge on NF-κB/MAPK modules, providing a rationale for the compound's anti-inflammatory/immunomodulatory profile. Overall, the KEGG pattern supports pleiotropic pharmacology spanning renal acid–base regulation, innate-immune signaling, and neurotransmission. These results prioritize experimental validation along three axes: (i) enzymology/docking against carbonic anhydrase and bicarbonate transport machinery, (ii) receptor-level assays within neuroactive-ligand families, and (iii) cytokine/NF-κB readouts downstream of C-type lectin receptors.

Machine learning

Interpretation of random forest variable importance (DPPH Assay)

The Random Forest model, as calibrated on a LOOCV scheme, showed that different phytochemical constituents made different contributions to the prediction of DPPH radical scavenging activity. The most significant predictor of antioxidant potential was quinic acid, with a permutation importance score of the highest magnitude of all the predictors. This observation is an indication that the change in the levels of quinic acid has a significant effect on the IC_{50} values of the DPPH assay thereby indicating its central role in quenching free radicals. Luteolin-7-O-glucuronide and astragalin were the second and third most pertinent compounds, respectively, and both compounds were flavonoid subclass, which is famous due to antioxidant properties (Fig. 7A). Other contributors were apigenin-7-glucuronide, luteoloside, isoquercitrin, eriodictyol and rutin hydrate which proved to be moderately important which supports the hypothesis that several flavonoid glycosides might work together to regulate antioxidant activities. Conversely, chlorogenic acid and narcissin showed insignificant significance in the model, which suggests that the concentrations did not explain the DPPH scavenging capacity of the extracts that were studied. Taken together, these results highlight the fact that not every phenolic component has a similar impact on antioxidant activity, and that quinic acid and certain flavonoid glucuronides are the most important factors in predicting DPPH-derived IC_{50} values in *C. intybus*.

Interpretation of SHAP analysis for DPPH assay

It should be mentioned that feature importance attributable to SHAP is the absolute intensity of the contribution of a variable to the model predictions as opposed to the direction of its effect. In line with this, though quinic acid has positive SHAP values, that is, it leads to an increase in IC_{50} and to the decrease in antioxidant activity, their high ranking implies a strong predictive effect, but not a positive biological effect. The SHAP summary plot gives additional understanding of the directional contribution of individual phytochemicals to predicting the IC_{50} values in DPPH assay. In this model, luteolin-7-O-glucuronide always had negative values of SHAP at higher concentrations, which means that higher concentrations of this compound are closely related to lower values of IC_{50} and, hence, greater antioxidant properties. On the same note, luteoloside, as well as apigenin 7-glucuronide also revealed a negative effect on IC_{50}, albeit with medium effects. On the other hand, quinic acid, even though it is also of high significance in the variable importance analysis, exhibited positive SHAP values at elevated concentrations, implying that the higher the level of quinic acid, the more the increase in IC_{50} value, and hence, the reduced radical-scavenging activity (Fig. 7B). It is important to note that SHAP-based feature importance shows the absolute strength of a variable in making predictions of the model and not the direction of the effect of the variable. Therefore, despite mostly positive values of SHAP, 0.560 indicating an increase in the IC_{50} and, consequently, a decrease in antioxidant activity, quinic acid is the most important because it has a significant and consistent effect on the variability of the prediction.

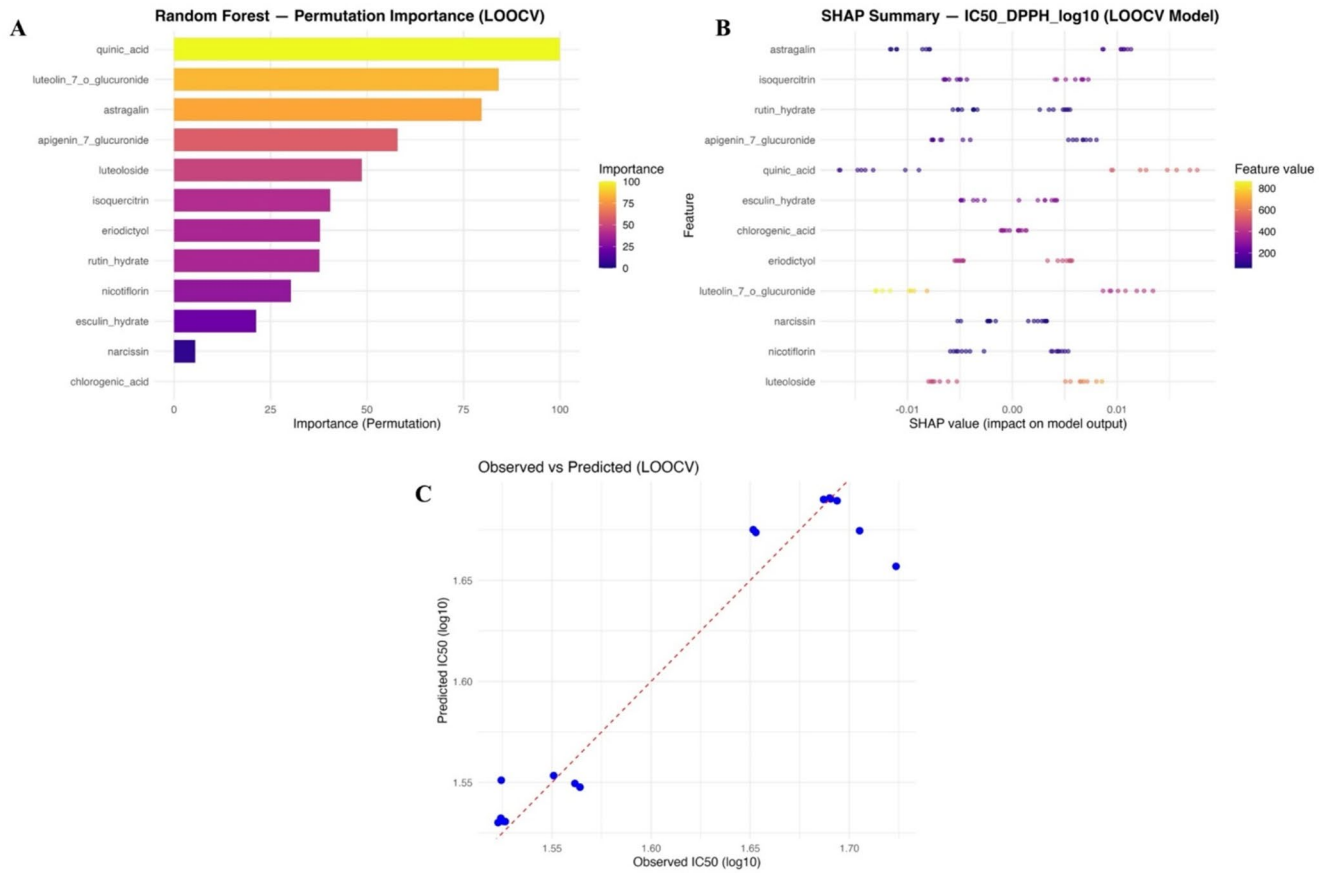


Fig. 7 Machine learning–based modeling of antioxidant activity using the DPPH assay. (**A**) Permutation importance ranking derived from the Random Forest model under a LOOCV scheme (**B**) SHAP summary plot illustrating the direction and magnitude of each variable's contribution to IC_{50} predictions. (**C**) Observed versus predicted IC_{50}values (log_{10}) from the LOOCV model, showing a strong alignment ($R^2 = 0.9225$, RMSE = 0.0219, MAE = 0.0146)

Model accuracy assessment (DPPH Assay, LOOCV)

The random Forest model predictive accuracy was assessed through the use of the leave-one- out cross-validation (LOOCV). A scatter plot of observed and predicted values of IC_{50} (Fig. 7C) showed a high linear relationship, meaning that the model can well describe the difference between the samples in the DPPH radical-scavenging activity. This conclusion was also supported by performance measures, which gave a RMSE of 0.0219, a MAE of 0.0146, and a R^2 of 0.9225. These values reveal that the amount of variance in experimental IC_{50} values that is explained by the model is more than 92%, with little error in prediction. All these findings confirm the idea that the Random Forest model offers a strong and solid predicting system based on analyzing phytochemical profiles to predict antioxidant activity (Fig. 6C). Further, the high level of agreement between the experimental and the predicted results supports the possible advantages of machine-learning-based interventions to support the analysis of small yet information-rich biochemical information and, thus, supplement classical statistical methods.

Conclusion

Summarizing, the results of this paper show that NADES-based extraction is one of the green and efficient methods to enrich bioactive metabolites of *C. intybus* L. especially, glucuronidated flavonoids and hydroxycinnamic acids, which had effective antioxidant and antimicrobial activity. UHPLC-ESI-Orbitrap®-HRMS/MS profiling with chemometric analyses established that NADES selectively enhanced the diversity and abundance of the phenolic compounds as compared to the traditional solvents. In addition to the phytochemical characterisation, bioinformatic studies were conducted on relevant biological pathways and molecular targets, positing a mechanistic relationship between a particular phenolic and its associated bioactivities. Moreover, predictive accuracy ($R^2 = 0.9225$) was obtained with the help of the integration of a machine-learning framework (Random forest with SHAP interpretation), and quinic acid and flavonoid glucuronides were revealed as the most influential determinants of antioxidant potential. Together, these multidisciplinary results indicate the synergistic

application of the green extraction technologies, high-tech metabolomics, chemometrics, bioinformatics, and artificial intelligence in studying natural products. This approach of integrative type does not only legitimize the pharmacological utility of the species of *C. intybus* but provides a model that can be generalised, in future research to assess medicinal plants systematically. This study is limited in several ways, although the analytical framework used is a complete one. The in vitro antioxidant and antimicrobial assays were used to evaluate the biological activity, and biological analysis of the results would be reinforced by in vivo or cell assays. Moreover, the machine-learning model itself was trained using a rather small sample, which could limit generalisability even with the help of cross-validation. Future researches can build upon this framework by including more data sets, more bioactivity outcomes, and experimental validation of the predicted molecular targets to reveal more roles of common phytochemicals in their mechanistic functions.

Author contributions Rüya Sağlamtaş, İnan Dursun, Melek Zor, Abdullah Demirci, Kübra Fettahoğlu and Ali Sinan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. Yeliz Demir: Formal analysis, Investigation, Writing – original draft, Writing – review & editing İhami Gülçin: Investigation, Methodology, Writing – review & editing.

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

Declarations

Consent for publication Not applicable.

Consent to participate Not applicable.

Generative AI statement Our research team utilized AI-assisted tools solely for language enhancement and readability improvement during the manuscript preparation process.

Competing interest 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 paper.

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