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ORIGINAL ARTICLE

Antityrosinase activity and LC-MS/MS analysis of optimized ultrasound-assisted condition extracts and fractions from strawberry tree (*Arbutus unedo* L.)

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Abstract

Investigation of utilization possibilities of natural sources has been an important area for research. Tyrosinase inhibitory activity plays a key role in food and medicine industry. Strawberry tree (*Arbutus unedo*), a widely distributed plant among Mediterranean countries, possess fruits and leaves with rich bioactive phytochemicals, especially polyphenolic compounds. In this study, we aimed to investigate the antityrosinase activity of the fruit and leaf extracts of the plant, and to determine the phenolic compounds that contribute to the antityrosinase activity. In this regard, we evaluated the effect of solvent composition on the extraction of phenolic compounds from *A. unedo* and on its antityrosinase activity using a simplex centroid design approach, and used chromatographic and LC-MS/MS techniques. The leaf extracts prepared using EtOH:water (50:50) provided higher TPC (456.39 mg GAE/g extract) and acetone:EtOH:water (33:33:33) provided higher TFC (56.15 mg QE/g extract) values than of fruit extracts. LC-MS/MS analysis revealed 23 phenolic/flavonoid compounds in leaf extracts (L1-8), and major metabolites were detected as quercitrin, quinic acid, catechin, tannic acid, isoquercitrin, gallic acid, and ellagic acid. Among the leaf extracts, L3 (aceton:water, 50:50) exhibited 72.01% tyrosinase inhibition at 500 μg/mL. After fractionation studies guided by antityrosinase activity, its subfraction L3-Fr2 exhibited 40.06% inhibition at 50 μg/mL concentration (IC_{50}: 146 ± 7.75 μg/mL), and catechin (113.19 mg/g), tannic acid (53.14 mg/g), ellagic acid (22.14 mg/g), gallic acid (10.27 mg/g), and epicatechin gallate (8.65 mg/g) were determined as major metabolites. Its subfraction L3-Fr2-sub7 exhibited better antityrosinase activity (IC_{50}: 206.23 ± 9.87 μg/mL), and quantitative analysis results revealed the presence of tannic acid (127.40 mg/g), gallic acid (13.96 mg/g), ellagic acid (7.66 mg/g), quercetin-3-*O*-glucuronide (5.06 mg/g), and quinic acid (3.2 mg/g) as major metabolites, and correlation analysis showed that ellagic acid and quinic acid were positively correlated with antityrosinase activity.

Keywords: Antityrosinase, *Arbutus unedo*, Chemical composition, LC-MS/MS, Simplex centroid design

1. Introduction

Arbutus unedo L. (Ericaceae) is an evergreen small tree, up to 4 m. tall, and distributed around the Mediterranean basin, mostly Western, partially Southern and Eastern Europe, and North-West Africa [1]. *A. unedo* is known as strawberry tree, for its red, spherical fruits with conical papillae, and it has drooping panicles with glabrous, greenish-white, campanulate flowers, eliptic/oblanceolate leaves with prominently serrate margins, and dark brown bark that is falling in small pieces [2]. The flowering period is usually between September and December, and the fruits ripen in a year, so the inflorescences can be seen along with mature fruits during autumn and early winter. *A. unedo* is a source of wild edible fruits, and besides its use as a food, the plant is of increasing importance in breeding

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programs, beekeeping facilities, horticultural and ornamental purposes. Cultivation studies to obtain high quality fruits, and selection studies based on some pomological and chemical characteristics have also been carried out from natural populations [3].

Ethnobotanical studies have revelaed that different parts of the plant have been used for various medicinal purposes among people. As a food, the ripe fruits are eaten fresh, or used to prepare jam, marmalades, and pastry, or to flavor alcoholic beverages [4]. In traditional medicine, raw fruits or decoctions prepared with fruits have been used to relieve the symptoms of gastrointestinal disorders, and urinary tract ailments, such as stomachache, diarrhea, kidney stones and as diuretics [5]. Decoction, or infusion prepared from the leaves, or the roots of the plant have been used internally for hypertension, urinary tract disorders, diarrhea, and hemorrhoids [6].

Bioactivity studies have shown that the plant exhibits different bioactivities, which support the ethnobotanical data. The fruits were reported to have antioxidant [7], antimicrobial [8], and cytotoxic [9] activities. The leaves have antispasmodic [10], antihypertensive [11], and antiurolytic [12] activities as well. *In vivo* and *in vitro* studies revealed that the roots and/or root bark of the plant have antihypertensive [13] and antidiabetic [14] activities.

Phytochemical investigations were mostly performed on fruits of the plant, and generally revealed the presence of phenolic compounds, such as flavonol glycosides (e.g. quercetin-3-rutinoside), anthocyanins (e.g. cyanidin-3-galactoside), flavan-3-ols and proanthocyanidins (e.g. catechin, epicatechin, gallocatechin and their oligomers), ellagic acid derivatives (e.g. ellagic acid glucoside), and phenolic acids (e.g. benzoic acid, gallic acid, and cinnamic acid derivatives) [15,16]. *A. unedo* leaves are also rich in phenolic compounds, such as flavonoids (e.g. quercitrin, hyperoside) and polyphenolic compounds (e.g. arbutin, ethylgallate, catechin, gallocatechin, galloylarbutin). Additionally, triterpene derivatives such as betulinic acid and lupeol have also been reported previously [17−19].

Tyrosinase plays an important role in melanin biosynthesis by transformation of L-tyrosine to L-DOPA (Levodopa, or L-3,4-dihydroxyphenylalanine), and L-DOPA to dopaquinone by oxidation. Dopaquinone is the precursor of melanin pigment, and the melanin production normally gives color to skin, and hair, but in some cases, defects in melanin synthesis causes dermatological disorders, like overproduction or accumulation of melanin results in hyperpigmentation. The tyrosinase enzyme is an important target to decrease the melanocyte function, and to overcome pigmentation problems, which also have a cosmetic importance [20,21]. Melanin pigment is also found in the brain, as neuromelanin, in the substantia nigra, and the tyrosinase is related to neuromelanin formation. High levels of neuromelanin have been associated with dopamine neurotoxicity, and the loss of dopaminergic neurons is related to the neurodegenerative disorders, and the Parkinson's disease [22].

Therefore, research in the area of cosmetics and medicine has focused on various compounds that inhibit tyrosinase activity, and the medicinal plants attract attention as a natural source of effective compounds [23]. Most of the tyrosinase inhibitors have phenolic structures, and the structure−activity relationship was explained by the connection of hydroxyl groups to the two copper ions at the active site of the enzyme [24].

Different extraction techniques have been used for the extraction of secondary metabolites from plants, and statistical design models were benefited for the interpretation of synergistic capability of solvent composition, which was mainly attributed to solvating power and polarity. Simplex centroid design (SCD) is one of the statistical models and has been used to optimize the extraction efficiency, solvent systems to be used, chemical content, and bioactivity exhibited [25].

Based on the rich phytochemical content and the phenolic compounds of the *A. unedo*, we aimed to investigate the antityrosinase activity of the fruit and leaf extracts of the plant, and to determine the compounds that contribute to the activity. In this regard, we evaluated antityrosinase activity, total phenolic, and total flavonoid content of different extracts that were prepared using different solvent systems according to the simplex centroid design, and performed various chromatographic techniques and a detailed LC-MS/MS analysis to detect the phenolic compounds that contribute to the activity.

2. Materials and methods

2.1. Chemicals

Gallic acid, quercetin, Folin-Ciocalteu reagent, levodopa, kojic acid and tyrosinase were purchased from Sigma. Trifluoroacetic acid, potassium phosphate dibasic, ethanol, sodium carbonate, aluminum chloride, potassium acetate, sodium acetate, potassium chloride were purchased from Merck (Germany). Extraction and fractionation studies were performed with analytical grade solvents, and LC-MS/MS analysis was performed with HPLC grade solvents (Merck). Column chromatography was carried out on silica gel 60 (40−63 mm-

Merck), and Lichroprep RP-C18 (25–40 mm, Merck).

2.2. Plant material and extract preparation

A. unedo fruits and leaves were collected from Karaburun-İzmir in November 2018. Plant was authenticated by Professor Dr. Sura Baykan Ozturk, and a voucher specimen was deposited in the IZEF Herbarium, Ege University, Faculty of Pharmacy (IZEF 6705). The leaf samples were transferred without absorbing moisture, and fruit samples were transferred in a cool box. The fruits were lyophilized, and the leaves were dried at room temperature on racks with air flow.

Ultrasound-assisted extraction was performed with a fixed-frequency (35 kHz) ultrasonic bath (Sonorex Super RK514BH, Bandelin Electronic GmbH & Co., Berlin, Germany). Plant materials were extracted using mixtures of varying amounts of acetone, ethanol, and water, according to a simplex centroid design (SCD). Solvent ratios used are given in Table 1. Ten grammes of air-dried leaves, or lyophilized fruits were grounded and extracted three times with 250 mL of solvent mixture using an ultrasonic bath for 1 h at room temperature. Extracts were filtered and separately concentrated with an evaporator to dryness at 40 °C, and kept at −20 °C for further experiments.

2.3. Phytochemical analysis

2.3.1. Determination of total phenolic content

To determine the total phenolic content (TPC) of the extracts, a modified 96-well procedure was developed based on Folin-Ciocalteu method described by Singleton et al. [26,27]. Briefly, 100 μL distilled water, 20 μL extract and 10 μL Folin-Ciocalteu reagent (×10 diluted with distilled water) were mixed in the well. The mixture was kept in dark for 4 min. Then, 100 μL saturated sodium carbonate solution was added to the mixture, and kept in dark for 2 h at room temperature. Absorbance of the solution was determined against the blank (distilled water) with a microplate reader at 760 nm (BMG Labtech Multimode Reader, Germany). A seven-point calibration curve in the range of 10–250 μg/mL was produced using gallic acid as the reference standard. All analysis were done in triplicate and total phenol contents were expressed as mg gallic acid equivalent (GAE)/g dry extract.

2.3.2. Determination of total flavonoid content

For the determination of the total flavonoid content (TFC) a modified 96-well procedure was developed based on aluminum chloride method, which was previously described by Ercisli and Orhan [27,28]. Briefly, 25 μL extract was mixed with 200 μL EtOH:-water (7,5:13), 10 μL aluminum chloride (10%), 10 μL potassium acetate (1 M) in the well and the mixture

Table 1. Solvent ratios according to SCD, extraction yields, total phenol, and flavonoid contents, and tyrosinase enzyme inhibition ratios of fruit (F) and leaf (L) extracts.

SCD extracts	Acetone %	EtOH %	Water %	Yield % dry material	TPC[a] ± SD	TFC[b] ± SD	TAC[c] ± SD	Tyrosinase Inhibition ± SD %
F1	–	–	100	58.3	18.28 ± 0.27	0.16 ± 0.02	<0.01	20.34 ± 0.37[d]
F2	33	33	33	58.9	50.72 ± 5.63	0.61 ± 0.01	0.02 ± 0.008	46.72 ± 2.32[d]
F3	50	–	50	61.3	52.64 ± 2.45	0.39 ± 0.07	<0.01	53.47 ± 3.72[d]
F4	–	50	50	58.4	36.87 ± 3.11	0.68 ± 0.01	<0.01	38.84 ± 0.77[d]
F5	100	–	-	14.9	21.44 ± 0.28	0.47 ± 0.02	0.53 ± 0.021	21.57 ± 0.53[d]
F6	100	–	-	14.7	21.84 ± 1.73	1.22 ± 0.21	0.50 ± 0.020	32.42 ± 0.60[d]
F7	–	100	-	43.4	28.01 ± 1.57	1.42 ± 0.19	0.09 ± 0.010	43.79 ± 1.61[d]
F8	50	50	-	43.6	37.41 ± 2.44	1.59 ± 0.18	0.03 ± 0.003	62.78 ± 1.57[d]
L1	–	–	100	39.2	416.11 ± 12.51	35.16 ± 1.80		67.88 ± 1.35[e]
L2	33	33	33	40.5	373.75 ± 8.27	56.15 ± 3.10		32.81 ± 1.94[e]
L3	50	–	50	43.7	389.72 ± 21.15	40.77 ± 1.41		72.01 ± 6.36[e]
L4	–	50	50	42.2	456.39 ± 13.11	39.14 ± 0.78		79.06 ± 3.34[e]
L5	100	–	-	12.6	331.74 ± 7.82	29.73 ± 1.86		46.67 ± 3.02[e]
L6	100	–	-	12.9	304.31 ± 14.15	39.04 ± 3.54		29.00 ± 2.55[e]
L7	–	100	-	19.0	297.01 ± 39.87	29.36 ± 2.80		61.15 ± 8.12[e]
L8	50	50	-	22.8	368.19 ± 37.01	35.91 ± 4.18		44.44 ± 0.13[e]

-: Not tested.
[a] TPC: Total phenolic content (mg GAE/g dry extract); GAE: Gallic acid equivalent.
[b] TFC: Total flavonoid content (mg QE/g dry extract); QE: Quercetin equivalent.
[c] TAC: Total anthocyanin content (mg Cya3Glu E/g extract); Cya3Glu E: Cyanidin-3-glucoside equivalent.
[d] At 1250 μg/mL concentration.
[e] At 500 μg/mL concentration.

was kept in darkness (40 min). Absorbance was determined against the blank (distilled water) at 415 nm. A seven-point calibration curve in the range of 10–250 μg/mL was produced using quercetin as the reference standard. Triplicate analysis were carried out and results were expressed as mg quercetin equivalent (QE)/g dry extract.

2.3.3. Determination of total anthocyanin content

To determine the total anthocyanin content (TAC), a modified 96-well procedure was developed based on pH change method [29]. Briefly, 40 μL of extract sample was mixed with 160 μL pH 1.0 (0.025 M potassium chloride) buffer. In a separate well, another 40 μL of same extract sample was mixed with 160 μL pH 4.5 (0.4 M sodium acetate) buffer. The mixtures were kept in the dark at room temperature for 20 min. Absorbance values were determined against the blank (distilled water) at 520 nm and 700 nm. For the determination of total anthocyanin content absorbance value was calculated according to Eq. (1), and total anthocyanin content was expressed as cyanidin-3-glucoside equivalent by using the formula (Eq. 2).

$$\text{Abs} = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (1)$$

$$\text{TAC} = \frac{Abs \times DF \times MW \times 1000}{e \times l} \quad (2)$$

where Abs is absorbance value calculated from previous formula, DF is dilution factor, MW is molecular weight of cyanidin-3-glucoside (449 g/mol), 1000 is the factor to convert g into mg, e is molar extinction coefficient of cyanidin-3-glucoside (26.900 L/mol. cm) and l is the calculated pathlength of the studied volume in well (calculated via Thermo Scientific Application Note). Triplicate analysis were carried out and results were expressed as mg cyanidin-3-glucoside equivalent (Cya3Glu E)/g dry extract.

2.3.4. Activity guided fractionation studies based on tyrosinase inhibitory activity

Approximately 1.8 g of extract to be fractioned was dissolved in 2 mL of water and chromatographed over RP-C18 column (20 g) using H_2O:EtOH (100:0, 75:25, 50:50, 25:75, 0:100; each 100 mL) and 5 fractions were collected for each extract. Most active fraction was chromatographed over silica gel column using $CHCl_3$:MeOH:H_2O systems (90:10:1, 85:15:0.5, 80:20:2, 75:25:2.5, 70:30:3, 61:32:7, and 0:100:0, each 200 mL) and 7 subfractions were collected. Fractions were monitored by thin layer chromatography (TLC), using UV light and vanillin-sulphuric acid reagent.

2.3.5. Quantitative analysis using LC-MS/MS system

LC-MS/MS analysis of the all leaf extracts, and subfractions of the most active samples were performed by comparison of 53 natural phenolic compounds, according to the previously described and validated method by Yilmaz [30]. A Shimadzu-Nexera model ultrahigh performance liquid chromatograph (UHPLC) coupled with a tandem mass spectrometer (Shimadzu LCMS-8040) equipped with an electrospray ionization (ESI) source (Shimadzu Europa GmbH, Germany), was used to accomplish quantiative evaluation of the samples. LC-ESI-MS/MS data were processed by LabSolutions software (Shimadzu), and the MRM (multiple reaction monitoring) mode was used for the quantification of the phytochemicals. The analysis results were presented as mg analyte/g dry extract (or fraction). Mass spectrometer, chromatography conditions, and analytical method validation parameters that belong to the LC-MS/MS method are presented in the supplementary file (S1.materials and methods, Fig. S1, and Table S5).

2.4. In vitro tyrosinase inhibitory activity

Tyrosinase inhibitory activity assay was adopted from a previously described method, and optimized for 96-well microplate [27,31]. Samples were dissolved in DMSO:water (2:3) and 25 μL of sample solution was mixed with 150 μL L-DOPA (2 mM, dissolved in 50 mM phosphate buffer, pH 6.8), and kept in dark for 5 min at 25 °C. 25 μL tyrosinase enzyme (50 Unit/mL in phosphate buffer) was added to start the reaction. During 10 points (5 min), dopachrome formation was monitored at 475 nm with a microplate reader (BMG Labtech Multimode Reader), and kinetic readings with 30 s intervals were taken to follow the linear change of the absorbance. Kojic acid was used as the positive control. Kojic acid is an inhibitor of mushroom tyrosinase, and shows inhibitory effect on monophenolase and diphenolase activity of the enzyme, and is used as a positive control for comparing the inhibitory strength of different samples [23]. Activity results for tyrosinase inhibition was calculated by the formula (Eq. 3) where ΔA_{blank} is the linear absorbance change with DMSO:water (2:3) and $\Delta A_{extract}$ is the linear absorbance change with the extract at 475 nm during the kinetic readings.

$$\text{Tyrosinase inhibition (\%)} = (\Delta A_{blank} - \Delta A_{extract}) / \Delta A_{blank} \times 100 \quad (3)$$

2.5. Statistical design and analysis

Extraction optimization was studied through the investigation of effect of solvent composition in the mixture via SCD [25,32]. Equal or limit percentages of included solvents in design were examined to find out the optimum ratios aiming to maximize the selected responses. Acetone, ethanol, and water were the selected solvents for the extraction mixture due to differing solvating power and polarity while preserving miscibility. Regarding composition and activity of the extracts; total phenol, total flavonoid, total anthocyanin contents and tyrosinase inhibitory activity were evaluated (Design-Expert version 7.0.0 Software). The Pearson correlation test was used to examine the relationship between phytoconstituents in tested subfractions and antityrosinase activity [33,34]. Obtained data were analyzed using GraphPad Prism version 5.03 program (GraphPad Software). The data were given with the standard deviation of the mean. Analysis results of tested samples were calculated from triplicate analysis.

3. Results and discussion

3.1. Extraction efficiency

In this study, acetone, ethanol, and water mixtures were used to evaluate the composition and antityrosinase activity of *A. unedo* fruit and leaf extracts.

Extraction yields of fruit and leaf extracts are presented in Table 1. Fruit extracts yielded between 14.7 and 61.3%, and the leaf extracts yielded 12.6–43.7% of the dried materials. Among the fruit extracts, higher amounts were obtained with F3 (61.3%), followed by F2 (58.9%), and F4 (58.4%). Similar results were obtained for the leaf extracts (L3, 43.7%; L4, 42.2%; and L2, 40.5%). On the other hand, using only acetone for the extraction also resulted in the decrease of extraction yields. Fruit extracts F5, and F6; and leaf extracts L5, and L6 provided yields of 14.9, 14.7, 12.6, and 12.9% respectively. In a previous study extraction with EtOH (100%) afforded 45% (w/w) extract from dried fruits [35], and for the leaf samples, extraction process using polar solvents provided higher extract yields, compared to apolar solvents [water (32.14%), MeOH (22.85%), EtOH (15.03%), and diethylether (2.86%)] [36]. As a result, using or adding a more polar solvent would be suggested to obtain higher amounts of extracts from the leaves and fruits of *A. unedo*.

3.2. Phytochemical analysis

3.2.1. Optimization of extracts for the total phenol, total flavonoid, and total anthocyanin contents

In the optimization study, correlation coefficients (R^2) indicated the correlation between the experimental work and the fitted models for all responses where R^2 values were found as 0.9995 for TPC (model *p*-value: 0.0151), 0.8408 for TFC (model *p*-value: 0.0101), 0.9695 for TAC (model *p*-value: 0.0216), and 0.9977 for tyrosinase inhibitory activity (model *p*-value: 0.0339) for fruit extracts. For the leaf extracts, R^2 values were 0.9953 for TPC (model *p*-value: 0.0483), 0.8411 for TFC (model *p*-value: 0.0448), and 0.9955 for tyrosinase inhibitory activity (model *p*-value: 0.0478). For all responses as composition and activity results, significant *p*-values indicated the model fitness in both fruit and leaf extracts.

Total phenol, flavonoid, and anthocyanin contents of the fruit extracts, and the leaf extracts are presented in Table 1. Effect of solvent mixture components on TPC, TFC, and TAC values of *A. unedo* fruit, and leaf extracts are depicted with 3D plots (Fig. 1). Most of the previous research were focused on fruits of *A. unedo* [7,37,38]. Among the fruit samples, extracts prepared using acetone:EtOH mixtures provided higher TPC values, as determined in F3 (Acetone:water, 50:50) and F2 (Acetone:EtOH:water, 33:33:33) (TPC: 52.64, and 50.72 mg GAE/g extract, respectively). Apart from these compositions, none of the other mixtures or a single solvent in maximum amount could provide such result for TPC. In terms of TFC, fruit extracts F8 (acetone:EtOH 50:50) and F7 (EtOH 100%) were quantified as 1.59 mg QE/g extract and 1.42 mg QE/g extract, respectively, despite providing lower TPC. According to the results, presence of ethanol in solvent mixture while keeping the polarity as moderate may be concluded as mandatory for the extraction of flavonoid compounds. As singularly acetone could derive 1.22 mg QE/g extract TFC, but presence of 50–100% ethanol managed to get the highest flavonoid amounts. Compared to the others, anthocyanin compounds were highest obtained with F5, and F6 as 0.53, and 0.50 mg cya-3-gluE/g extract where both systems were the same as acetone 100%, although they provided lower extract yields (14.9 and 14.7%) and TPC (21.44 and 21.84 mg GAE/g). In a previous study, TPC of fruit extracts obtained by different techniques were reported as 25.72 mg GAE/g for supercritical carbon dioxide extract, 24.89 mg GAE/g for conventional water extract, and 15.12 mg GAE/g for EtOH extract [37]. In another study TPC and TFC of methanol extract

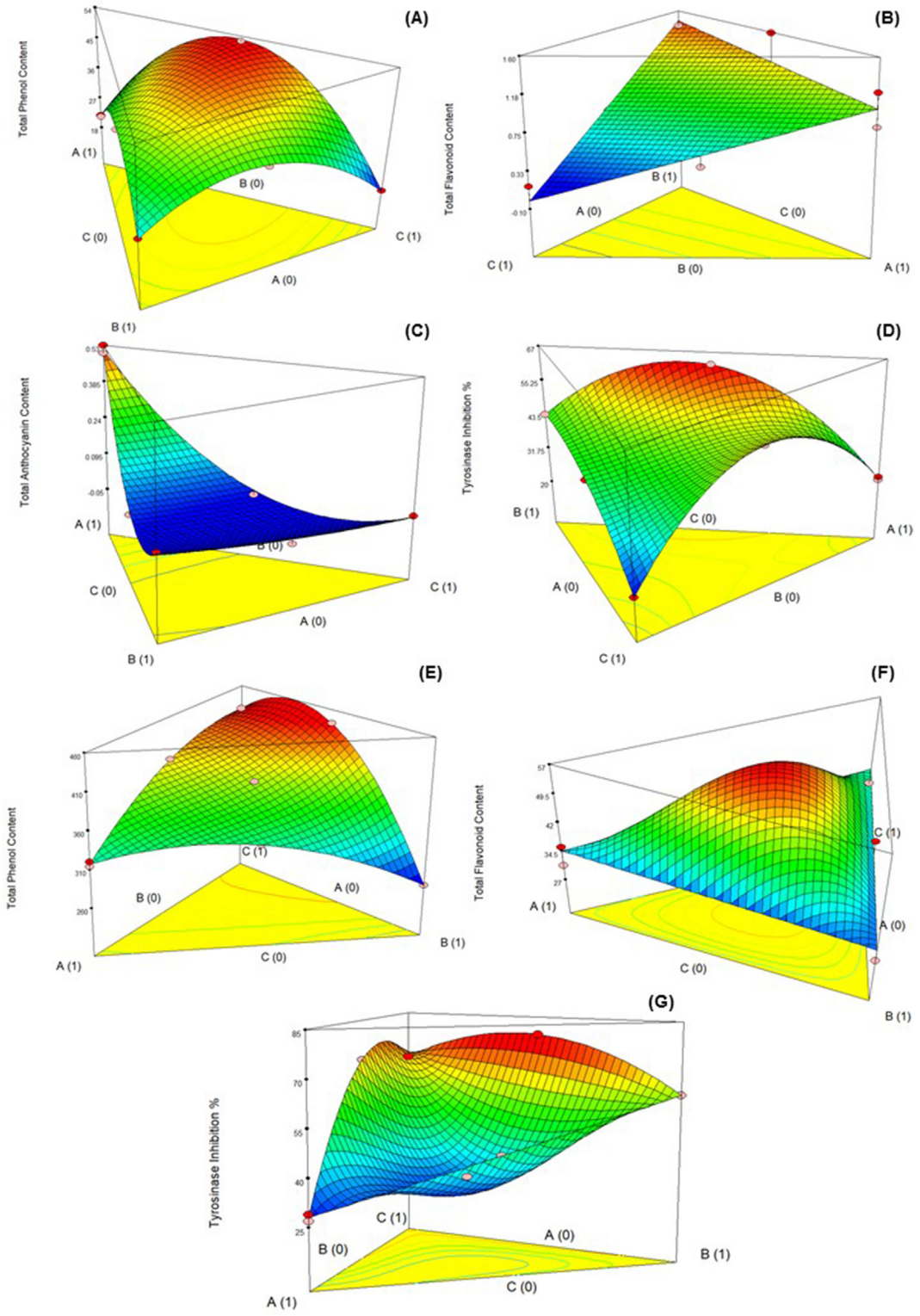

Fig. 1. 3D plots showing effect of solvent composition on total phenol (A), flavonoid (B), anthocyanin (C) contents and antityrosinase activity (D) of A. unedo fruit extracts, and 3D plots showing effect of solvent composition on total phenol (E), flavonoid (F) contents and antityrosinase activity (G) of A. unedo leaf extracts. (In the 3D plots, X1 = A: Acetone, X2 = B: Ethanol, and X3 = C: Water).

from the fruits were reported to be 126.83 mg GAE/g and 34.99 mg catechin equivalent (CE)/g, respectively [38]. While not including the effect of solvent pH, extraction of highest anthocyanin amounts by acetone within the extraction design conformed with literature data [39].

According to the TPC and TFC values, the extracts obtained from leaves provided higher

phytochemical contents than of fruit extracts. This can be related to the relatively lower concentration of bioactive compounds due to the high sugar content of fruits [9,38]. The highest TPC value was determined in L4 (EtOH:water, 50:50) as 456.39 mg GAE/g extract, which was higher than the previously reported data where the TPC values of the water, MeOH, EtOH, and diethyl ether extracts form the leaves were determined as 172.21, 149.28, 192.66, and 14.93 mg GAE/g extract, respectively [36]. Increasing water and ethanol content higher than 50% and addition of acetone into the solvent mixture negatively affected the extraction of phenolic content. In terms of TFC, L2 (Acetone:EtOH:water, 33:33:33) was quantified as 56.15 mg QE/g extract. Equivalent presence of acetone, ethanol and water in solvent mixture provided the most effective extraction of flavonoid content. Changing the solvent composition in favor of any of the solvents resulted with the decline of flavonoid amounts. Our results suggest that, instead of fruits of *A. unedo*, the leaf extracts prepared by using the acetone:water and EtOH:water mixtures, and the adjustment of extraction solvent composition could be useful to obtain extracts that provide higher TPC and TFC values.

3.3. Activity guided fractionation studies based on antityrosinase activity

Tyrosinase enzyme inhibition ratios of the fruit extracts, and the leaf extracts are presented in Tables 1, and 3D plots showing effect of solvent mixture components on tyrosinase inhibitory activity of *A. unedo* fruit, and leaf extracts are presented in Fig. 1D and G. Tables of analysis of variance (ANOVA) with Mixture Special Cubic Model for tyrosinase inhibitory activity of the fruit and leaf extracts are presented in supplementary file (Tables S1 and S3). Monitoring the linear absorbance change with kinetic readings of 30 s intervals enabled the observation of reaction without the effect of absorption due to the sample's content. The linear absorbance change (ΔA) between the initial and final point of the kinetic readings were taken into account for the calculation of tyrosinase

Table 2. Tyrosinase enzyme inhibitory activity of fractions of F3, F8, L3, and L4; and IC_{50} values of F3-Fr2, F8-Fr2, L3-Fr2, and L4-Fr2.

Sample (OA[a])	Tyrosinase inhibition ± SD[b]%	SE[c]	RSD%[d]
F3-Fr1 (1662.3 mg)	9.89 ± 0.47[e]	0.33	4.73
F3-Fr2 (300.3 mg)	23.50 ± 2.11[e]	1.22	8.98
F3-Fr3 (11.7 mg)	8.58 ± 1.00[e]	0.71	11.71
F3-Fr4 (5 mg)	1.64 ± 0.25[e]	0.15	15.37
F3-Fr5 (20 mg)	11.25 ± 0.99[e]	0.70	8.77
F8-Fr1 (1559.8 mg)	9.11 ± 0.99[e]	0.57	10.84
F8-Fr2 (126.1 mg)	17.70 ± 2.13[e]	1.23	12.05
F8-Fr3 (10.3 mg)	1.67 ± 1.11[e]	0.64	66.74
F8-Fr4 (6.8 mg)	ND[e]	–	–
F8-Fr5 (54.1 mg)	ND[e]	–	–
L3-Fr1 (744.4 mg)	24.70 ± 1.43[f]	0.82	5.78
L3-Fr2 (631.5 mg)	40.06 ± 1.02[f]	0.59	2.55
L3-Fr3 (132.6 mg)	19.00 ± 2.08[f]	1.20	10.92
L3-Fr4 (21.9 mg)	20.41 ± 1.07[f]	0.62	5.23
L3-Fr5 (27.2 mg)	17.78 ± 0.62[f]	0.36	3.48
L4-Fr1 (688.5 mg)	21.74 ± 1.37[f]	0.79	6.31
L4-Fr2 (619.6 mg)	36.30 ± 1.70[f]	0.98	4.69
L4-Fr3 (98.3 mg)	14.78 ± 1.89[f]	1.33	12.76
L4-Fr4 (17.7 mg)	17.63 ± 0.97[f]	0.56	5.51
L4-Fr5 (23.5 mg)	12.72 ± 1.83[f]	1.06	14.41
	IC_{50} ± SD μg/mL (R^2)		
F3-Fr2	816 ± 47.13 (0.9574)	33.38	5.78
F8-Fr2	1076 ± 109.13 (0.9854)	77.25	10.15
L3-Fr2	146 ± 7.75 (0.9560)	4.50	5.29
L4-Fr2	168 ± 13.13 (0.9721)	7.63	7.81
Kojic acid	4 ± 0.25 (0.9983)	0.13	4.96

[a] OA: Obtained amount after fractionation.
[b] SD: Standard deviation.
[c] SE: Standard error.
[d] RSD: Relative standard deviation.
[e] At 250 μg/mL concentration.
[f] At 50 μg/mL concentration; ND: Not determined; R^2: Coefficient of determination.

inhibitory activity. Therefore, the effect of the initial absorbance differences due to varying phenolic contents were prevented to have an effect on the activity results. Among the tested samples, fruit extracts F8, and F3 provided 62.78 and 53.47% inhibition ratios at 1250 μg/mL concentration, respectively, but other fruit extracts could not provide inhibition ratio greater than 50% at the tested concentration. On the other hand, leaf extracts L1, L3, L4, and L7 exhibited 67.88, 72.01, 79.06, and 61.15% inhibition ratios at 500 μg/mL concentration, respectively. Suggested solutions by the optimization model for tyrosinase inhibitory activity of the fruit and leaf extracts are presented in supplementary file (Tables S2 and S4). Majority of the model solutions as 45:55, 43:57 or 48:52 acetone:ethanol mixtures indicated the tyrosinase inhibitory activity by the fruit extracts with a maximum inhibition of 63.67%, which was also confirmed by the experimental results with 50:50 acetone:ethanol mixture as 62.78%. Similarly, for the leaf extracts, suggested model solutions with the highest activity included 45:55 (79.25% inhibiton), 47:53 (79.19% inhibition) or 44:56 (79.18% inhibition) ethanol:water mixtures, which was confirmed by the experimental results of the 50:50 ethanol:water extract that the tyrosinase enzyme was inhibited by 79.06%. As the activity results were within the 5% RSD range of the experimental results, additional studies with the specific suggested points were not performed.

TPC and TFC values could be concluded as the prominent factors affecting the tyrosinase inhibitory activity potential of *A. unedo* fruit and leaf extracts, but when compared to L3 and L4 samples, L2 provided 32.81% inhibition, despite having the highest TPC value (56.15 mg QE/g extract). According to the results obtained, it can be speculated that the anti-tyrosinase activity may not be directly related to the phenolic or flavonoid contents. Based on the anti-tyrosinase activity, the fruit extracts F3, and F8, and the leaf extracts L3, and L4 were selected for fractionation studies to identify the bioactive moiety for the inhibitory activity.

After fractionation of F3 (2.04 g), F8 (1.89 g), L3 (1.84 g), and L4 (1.72 g) extracts using RP-C18 column, each fraction was tested against the tyrosinase enzyme (Table 2). Among the tested samples, fractions of fruit extracts exhibited lower activity (inhibition values were between 1.64 and 23.5%, at 250 μg/mL concentration). Elution with water (100%) through RP-C18 column also revealed the high sugar content of the fruit extracts, as the yield percentages of F3-Fr1 and F8-Fr1 were approximately 80% of the samples [9]. F3-Fr2, and F8-Fr2 were relatively active fractions, and the IC_{50} values were calculated as 816 ± 47.13, and 1076 ± 109.13 μg/mL, respectively.

Fractions of leaf extracts exhibited promising activities (12.72–40.06% inhibition at 50 μg/mL concentration), and L3-Fr2 was selected for further analysis (IC_{50}: 146 ± 7.75 μg/mL). L3-Fr2 (514 mg) was chromatographed over silica gel column, and among the collected 7 subfractions, L3-Fr2-sub7 exhibited 32.35 ± 0.78% inhibitory activity at 62.5 μg/mL, and IC_{50} value was determined as 206.23 ± 9.87 μg/mL (Table 3).

3.4. LC-MS/MS analysis of leaf extracts (L1-L8), and subfractions of L3, and L3-Fr2

According to the TPC and TFC values, the leaf extracts provided higher phytochemical contents, and exhibited better activity than of fruit extracts. Phytochemical studies have mostly been carried out on the fruits, but studies on the leaves of the plant are limited. *A. unedo* leaves were reported to contain

Table 3. Tyrosinase enzyme inhibition ratios at 62.5 μg/mL concentration, and IC_{50} values of subfractions of L3-Fr2.

Samples (OA[a])	Inhibition ± SD[b] %	SE[c]	RSD%[d]	IC_{50} ± SD[b] μg/mL (R^2)
L3-Fr2-sub1 (17 mg)	ND	–	–	–
L3-Fr2-sub2 (26 mg)	ND	–	–	–
L3-Fr2-sub3 (53 mg)	10.76 ± 0.73	0.42	6.81	NA
L3-Fr2-sub4 (37 mg)	15.79 ± 1.37	0.79	8.71	NA
L3-Fr2-sub5 (14 mg)	16.35 ± 1.43	0.83	8.78	NA
L3-Fr2-sub6 (27 mg)	18.38 ± 1.46	0.84	7.94	457.73 ± 27.37 (0.9854)
L3-Fr2-sub7 (236 mg)	32.35 ± 0.78	0.45	2.41	206.23 ± 9.87 (0.9603)
Kojic acid				4 ± 0.25

[a] OA: Obtained amount after fractionation.
[b] SD: Standard deviation.
[c] SE: Standard error.
[d] RSD: Relative standard deviation, R^2: Coefficient of determination, ND: Not determined, NA: Not active (IC_{50} value was over 500 μg/mL).

various secondary metabolites, including mainly phenolic compounds, such as flavonoids, tannins, and polyphenolic compounds, including arbutin, ethyl gallate, galloyl arbutin, gallocatechin, and their derivatives [17–19]. Studies have shown that hydroquinone derivatives and arbutin, quercitrin, isoquercitrin, hyperoside and chlorogenic acid were present in the leaves in varying amounts throughout the year [18]. In another study terpenoid and steroid compounds were reported from the leaves of the plant, such as 7β-hydroxystigmast-4-en-3-one, α-amyrin acetate, betulin, betulinic acid, 6β-hydroxystigmast-4-en-3-one, lupeol, platanic acid, pomolic acid 3-acetate, and β-sitosterol [17]. The bioactivity of the plant has often been attributed to polyphenolic compounds. Antioxidant [7] and cytotoxic [9] activities of the fruit extracts were previously associated with phenolic components such as gallic acid, flavan-3-ol, and anthocyanin derivatives that were present in the extracts. Similarly, Pavlovic et al. [10] reported that *in vitro* antioxidant activity of *A. unedo* leaves was related to high levels of phenols, tannins, arbutin and flavonoids that were found in the extracts. In another study, vasorelaxant activity of *Arbutus* leaf extract (and its subfractions) was assigned to polyphenolic components such as oligomeric condensed tannins and catechin gallate [11]. Therefore, we performed a detailed LC-MS/MS analysis on all leaf extracts (L1-8), and subfractions of the most active ones (L3-Fr1-5, and L3-Fr2-sub1-7) to identify the phenolic compounds that contribute to antityrosinase activity. The components according to the quantitative analysis result are presented in Tables 4–6.

LC-MS/MS analysis of leaf extracts (L1-8) revealed 23 phenolic/flavonoid compounds in varying amounts, and quercitrin, quinic acid, catechin, tannic acid, isoquercitrin, gallic acid, and ellagic acid were determined as major metabolites (Table 4). In addition, total amounts of the phenolic and flavonoid compounds were in good agreement with the TPC and TFC values of the extracts. It can be suggested that the equivalent presence of acetone, ethanol and water in solvent mixture is an effective way of providing higher amounts of quercitrin (162.87 mg/g extract), catechin (73.49 mg/g extract), tannic acid

Table 4. Quantitative analysis results for leaf extracts (L1-8).

Analytes[a]	L1	L2	L3	L4	L5	L6	L7	L8
Quercitrin	**119.56 ± 3.20**	**162.87 ± 4.36**	**141.34 ± 3.79**	**134.31 ± 3.60**	**113.66 ± 3.05**	**96.65 ± 2.59**	**95.90 ± 2.57**	**115.98 ± 3.11**
Quinic acid	**107.81 ± 4.01**	**47.42 ± 1.76**	**92.90 ± 3.46**	**91.48 ± 3.40**	**26.09 ± 0.97**	**22.54 ± 0.84**	**55.07 ± 2.05**	**37.85 ± 1.41**
Catechin	**62.38 ± 1.38**	**73.49 ± 1.62**	**64.70 ± 1.43**	**57.07 ± 1.26**	**73.81 ± 1.63**	**62.88 ± 1.39**	**53.61 ± 1.18**	**62.20 ± 1.37**
Tannic acid	**25.79 ± 0.49**	**50.45 ± 0.96**	**23.75 ± 0.45**	**24.65 ± 0.47**	**34.35 ± 0.65**	**29.91 ± 0.57**	**26.98 ± 0.51**	**42.70 ± 0.81**
Isoquercitrin	**14.93 ± 0.33**	**22.32 ± 0.49**	**19.11 ± 0.42**	**17.73 ± 0.39**	**21.27 ± 0.47**	**17.56 ± 0.39**	**15.10 ± 0.33**	**16.66 ± 0.37**
Gallic acid	**14.95 ± 0.17**	**9.98 ± 0.11**	**9.58 ± 0.11**	**13.30 ± 0.15**	**6.35 ± 0.07**	**5.44 ± 0.06**	**7.35 ± 0.08**	**7.65 ± 0.09**
Ellagic acid	**5.16 ± 0.19**	**6.39 ± 0.23**	**8.95 ± 0.33**	**7.47 ± 0.27**	**5.18 ± 0.19**	**4.74 ± 0.173**	**5.00 ± 0.18**	**5.25 ± 0.19**
Epicatechin gallate	**2.77 ± 0.063**	**5.63 ± 0.13**	**4.50 ± 0.103**	**3.92 ± 0.090**	**5.31 ± 0.12**	**4.27 ± 0.098**	**3.18 ± 0.073**	**3.92 ± 0.090**
Miquelianin	2.62 ± 0.058	1.80 ± 0.039	2.31 ± 0.051	2.32 ± 0.051	0.80 ± 0.018	0.70 ± 0.015	1.09 ± 0.024	1.09 ± 0.024
Astragalin	1.72 ± 0.020	2.72 ± 0.031	2.23 ± 0.025	2.06 ± 0.023	2.56 ± 0.029	2.17 ± 0.025	1.69 ± 0.019	1.96 ± 0.022
Rutin	2.32 ± 0.057	2.67 ± 0.066	2.23 ± 0.055	2.10 ± 0.052	1.19 ± 0.029	0.93 ± 0.023	1.09 ± 0.027	1.15 ± 0.028
Nicotiflorin	1.91 ± 0.021	2.23 ± 0.024	1.81 ± 0.020	1.67 ± 0.018	0.99 ± 0.011	0.81 ± 0.009	0.91 ± 0.010	0.98 ± 0.011
Hesperidin	0.99 ± 0.033	1.26 ± 0.042	1.11 ± 0.037	1.02 ± 0.034	0.66 ± 0.022	0.58 ± 0.019	0.61 ± 0.020	0.73 ± 0.024
Epigallo catechin gallate	0.37 ± 0.005	0.68 ± 0.010	0.56 ± 0.008	0.49 ± 0.007	0.60 ± 0.009	0.54 ± 0.008	0.46 ± 0.007	0.51 ± 0.007
Quercetin	0.41 ± 0.007	0.60 ± 0.011	0.48 ± 0.008	0.50 ± 0.009	0.38 ± 0.007	0.29 ± 0.005	0.31 ± 0.005	0.40 ± 0.007
Protocatechuic acid	0.40 ± 0.014	0.41 ± 0.014	0.28 ± 0.010	0.30 ± 0.010	0.24 ± 0.008	0.19 ± 0.007	0.34 ± 0.012	0.28 ± 0.009
Gentisic acid	0.05 ± 0.0008	0.13 ± 0.002	0.12 ± 0.002	0.09 ± 0.0015	0.06 ± 0.0010	0.05 ± 0.0008	0.07 ± 0.0012	0.08 ± 0.0013
Kaempferol	0.04 ± 0.0008	0.06 ± 0.0013	0.06 ± 0.0013	0.06 ± 0.0013	0.06 ± 0.0013	0.04 ± 0.0008	0.05 ± 0.0011	0.05 ± 0.0011
Piceid	0.04 ± 0.0008	0.04 ± 0.0008	0.02 ± 0.0004	0.03 ± 0.0006	0.04 ± 0.0008	0.03 ± 0.0006	0.03 ± 0.0006	0.03 ± 0.0006
Protocatechuic aldehyde	0.02 ± 0.0008	0.03 ± 0.0012	0.02 ± 0.0008	0.03 ± 0.0012	0.04 ± 0.0016	0.04 ± 0.0016	0.04 ± 0.0016	0.04 ± 0.0016
Cosmosiin	0.01 ± 0.0001	0.02 ± 0.0002	0.02 ± 0.0002	0.01 ± 0.0001	0.03 ± 0.0002	0.02 ± 0.0002	0.01 ± 0.0001	0.02 ± 0.0002
Cynaroside	N.D.	0.01 ± 0.0004	0.01 ± 0.0004	N.D.	0.01 ± 0.0004	0.01 ± 0.0004	0.01 ± 0.0004	N.D.
Naringenin	0.01 ± 0.0004	0.005	0.003	0.005	0.004	N.D.	0.003	0.004
Total mg/g dry extract	**364.26**	**391.186**	**376.068**	**360.593**	**293.673**	**250.369**	**268.906**	**299.509**

N.D.: Not detected.

[a] The analytes are sorted according to the major metabolites of L3, Quantitative analysis results are presented as mg analyte/g dry extract.

Table 5. Quantitative analysis results for subfractions of L3.

Analytes[a]	L3-Fr1	L3-Fr2	L3-Fr3	L3-Fr4	L3-Fr5
Catechin	**0.40 ± 0.009**	**113.19 ± 2.50**	1.34 ± 0.030	0.43 ± 0.010	N.D.
Tannic acid	**6.20 ± 0.12**	**53.14 ± 1.01**	8.19 ± 0.16	**1.17 ± 0.022**	**0.10 ± 0.002**
Ellagic acid	**1.36 ± 0.050**	**22.14 ± 0.81**	6.69 ± 0.24	0.21 ± 0.008	N.D.
Isoquercitrin	N.D.	**20.40 ± 0.45**	**124.18 ± 2.73**	**1.60 ± 0.035**	0.09 ± 0.0020
Gallic acid	**11.31 ± 0.13**	**10.27 ± 0.12**	2.17 ± 0.024	**5.28 ± 0.06**	**0.20 ± 0.002**
Epicatechin gallate	0.03 ± 0.0007	**8.65 ± 0.20**	0.13 ± 0.003	0.10 ± 0.002	N.D.
Miquelianin	N.D.	5.60 ± 0.12	0.03 ± 0.0007	0.01 ± 0.0002	N.D.
Rutin	N.D.	3.36 ± 0.083	**9.07 ± 0.224**	0.07 ± 0.0017	N.D.
Quercitrin	0.14 ± 0.004	2.78 ± 0.075	**750.56 ± 20.12**	**17.31 ± 0.46**	**1.35 ± 0.036**
Hesperidin	N.D.	2.06 ± 0.069	5.90 ± 0.20	0.05 ± 0.0016	N.D.
Quinic acid	**166.69 ± 6.20**	2.02 ± 0.075	0.28 ± 0.010	0.64 ± 0.024	**0.43 ± 0.016**
Epigallocatechin gallate	N.D.	1.15 ± 0.017	N.D.	0.04 ± 0.0006	N.D.
Protocatechuic acid	0.07 ± 0.0025	0.64 ± 0.022	0.06 ± 0.0021	**0.69 ± 0.024**	**0.12 ± 0.004**
Gentisic acid	N.D.	0.17 ± 0.003	N.D.	N.D.	N.D.
Piceid	N.D.	0.08 ± 0.0016	N.D.	N.D.	N.D.
Protocatechuic aldehyde	0.01 ± 0.0004	0.06 ± 0.0024	0.02 ± 0.0008	0.10 ± 0.004	0.01 ± 0.0004
Acacetin	N.D.	0.03 ± 0.0011	0.002	0.48 ± 0.017	0.01 ± 0.0004
Nicotiflorin	N.D.	0.02 ± 0.0002	**20.55 ± 0.22**	0.13 ± 0.001	N.D.
Cynaroside	N.D.	0.02 ± 0.0007	0.03 ± 0.0011	N.D.	N.D.
Astragalin	N.D.	0.01 ± 0.0001	**27.28 ± 0.31**	0.35 ± 0.004	0.03 ± 0.0003
Salicylic acid	N.D.	0.01 ± 0.0002	N.D.	0.01 ± 0.0002	N.D.
Apigenin	N.D.	0.008	0.004	0.11 ± 0.002	0.003
Chrysin	N.D.	0.005	N.D.	0.11 ± 0.004	0.003
Naringenin	N.D.	0.005	0.05 ± 0.0020	0.04 ± 0.0016	N.D.
Quercetin	N.D.	N.D.	5.08 ± 0.09	0.42 ± 0.007	N.D.
Kaempferol	N.D.	N.D.	0.64 ± 0.014	0.10 ± 0.002	N.D.
Luteolin	N.D.	N.D.	0.005	0.03 ± 0.0009	N.D.
Cosmosiin	N.D.	N.D.	0.21 ± 0.0017	0.005	N.D.

N.D.: Not detected.

[a] The analytes are sorted according to the major metabolites of L3-Fr2. Quantitative analysis results are presented as mg analyte/g dry fraction.

Table 6. Quantitative analysis results for subfractions of L3-Fr2.

Analytes[a]	Arb L3-Fr2-sub1	Arb L3-Fr2-sub2	Arb L3-Fr2-sub3	Arb L3-Fr2-sub4	Arb L3-Fr2-sub5	Arb L3-Fr2-sub6	Arb L3-Fr2-sub7
Tannic acid	**384.10 ± 7.30**	**159.87 ± 3.04**	**100.69 ± 1.91**	**55.20 ± 1.05**	**53.07 ± 1.01**	**150.34 ± 2.86**	**127.40 ± 2.42**
Gallic acid	0.11 ± 0.001	0.06 ± 0.0007	1.02 ± 0.011	**40.31 ± 0.45**	**36.80 ± 0.41**	**15.47 ± 0.17**	**13.96 ± 0.16**
Ellagic acid	**1.01 ± 0.037**	N.D.	N.D.	0.34 ± 0.012	N.D.	N.D.	**7.66 ± 0.28**
Miquelianin	N.D.	N.D.	N.D.	N.D.	N.D.	**23.91 ± 0.53**	**5.06 ± 0.11**
Quinic acid	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**3.20 ± 0.119**
Catechin	**0.76 ± 0.017**	**144.71 ± 3.20**	**17.50 ± 0.39**	2.84 ± 0.062	2.35 ± 0.052	4.28 ± 0.095	1.87 ± 0.041
Epicatechin gallate	N.D.	N.D.	**46.72 ± 1.07**	**21.53 ± 0.49**	4.12 ± 0.094	2.26 ± 0.052	0.61 ± 0.014
Isoquercitrin	N.D.	1.38 ± 0.030	**93.76 ± 2.06**	**17.63 ± 0.39**	**5.71 ± 0.13**	**4.42 ± 0.097**	0.53 ± 0.012
Rutin	N.D.	N.D.	N.D.	**11.57 ± 0.29**	**31.06 ± 0.77**	**6.66 ± 0.16**	0.22 ± 0.005
Epigallocatechin gallate	N.D.	N.D.	N.D.	1.08 ± 0.016	3.43 ± 0.050	2.13 ± 0.031	0.13 ± 0.002
Hesperidin	N.D.	N.D.	N.D.	6.59 ± 0.22	**17.08 ± 0.57**	3.56 ± 0.119	0.12 ± 0.004
Protocatechuic acid	0.10 ± 0.004	**4.69 ± 0.164**	1.88 ± 0.066	0.20 ± 0.007	0.15 ± 0.005	0.19 ± 0.007	0.10 ± 0.004
Quercitrin	0.04 ± 0.0011	**5.19 ± 0.14**	**12.32 ± 0.33**	1.40 ± 0.038	0.61 ± 0.016	0.38 ± 0.010	0.10 ± 0.0027
Protocatechuic aldehyde	**1.57 ± 0.062**	0.14 ± 0.006	0.04 ± 0.0016	0.04 ± 0.0016	0.04 ± 0.0016	0.05 ± 0.0020	0.03 ± 0.0012
Cosmosiin	0.03 ± 0.0002	0.01 ± 0.0001	N.D.	N.D.	N.D.	N.D.	0.02 ± 0.0002
Apigenin	0.17 ± 0.003	N.D.	N.D.	N.D.	N.D.	N.D.	0.002
Gentisic acid	N.D.	N.D.	N.D.	3.10 ± 0.051	N.D.	0.10 ± 0.002	N.D.
Quercetin	0.27 ± 0.005	N.D.	N.D.	N.D.	0.07 ± 0.0012	0.09 ± 0.0016	N.D.
Astragalin	0.21 ± 0.002	0.07 ± 0.0008	0.02 ± 0.0002	0.03 ± 0.0003	N.D.	0.03 ± 0.0003	N.D.
Salicylic acid	0.06 ± 0.0009	0.02 ± 0.0003	0.04 ± 0.0006	0.12 ± 0.002	N.D.	N.D.	N.D.

(*continued on next page*)

Table 6. (continued)

Analytes[a]	Arb L3-Fr2-sub1	Arb L3-Fr2-sub2	Arb L3-Fr2-sub3	Arb L3-Fr2-sub4	Arb L3-Fr2-sub5	Arb L3-Fr2-sub6	Arb L3-Fr2-sub7
Nicotiflorin	N.D.	N.D.	0.11 ± 0.001	0.05 ± 0.0005	N.D.	N.D.	N.D.
Cynaroside	N.D.	N.D.	0.14 ± 0.005	0.02 ± 0.0007	N.D.	N.D.	N.D.
Piceid	0.10 ± 0.002	0.50 ± 0.010	0.22 ± 0.004	N.D.	N.D.	N.D.	N.D.
Epicatechin	N.D.	**15.53 ± 0.34**	N.D.	N.D.	N.D.	N.D.	N.D.
Caffeic acid	N.D.	0.10 ± 0.002	N.D.	N.D.	N.D.	N.D.	N.D.
p-Coumaric acid	**0.62 ± 0.012**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acacetin	0.53 ± 0.019	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Vanillin	0.46 ± 0.006	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Syringic aldehyde	0.18 ± 0.004	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Chrysin	0.11 ± 0.004	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Kaempferol	0.07 ± 0.0015	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Naringenin	0.06 ± 0.0024	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Luteolin	0.04 ± 0.0013	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.: Not detected.

[a] The analytes are sorted according to the major metabolites of L3-Fr2-sub7. Quantitative analysis results are presented as mg analyte/g dry fraction.

(50.45 mg/g extract), and isoquercitrin (22.32 mg/g extract). Among the tested samples, L3 provided the highest extract yield (43.7%), and the second highest total amount of phenolic/flavonoid compounds determined (376.07 mg analyte/g extract). Tyrosinase is a copper-containing, mixed function oxidase enzyme that can catalyze both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones, and the quinone product is a reactive precursor for the synthesis of melanin pigment. Most of the tyrosinase inhibitors have phenolic structures, and the structure–activity relationship was explained by the connection of hydroxyl groups to the two copper ions at the active site of the enzyme [40]. The major metabolites were in similar amounts among the total extracts (L1-8). The bioactivity results and components of the sub-fractions were compared to establish a relationship between antityrosinase activity and phenolic contents.

After a simple fractionation, L3-Fr1 provided a quinic acid rich fraction (166.69 mg/g fraction); L3-Fr2 yielded a catechin rich fraction (113.19 mg/g fraction); L3-Fr3 yielded a flavonoid rich fraction with higher amounts of quercitrin (750.56 mg/g extract) and isoquercitrin (124.18 mg/g extract). L3-Fr4 and L3-Fr5 yielded lower amounts of phenolic compounds (29.45, and 2.35 mg analyte/g fraction, respectively) (Table 5). In terms of antityrosinase activity, L3-Fr2 exhibited better activity (40.06% inhibition at 50 μg/mL concentration, IC_{50}: 146 ± 7.75 μg/mL). LC-MS/MS analysis of L3-Fr2 revealed the presence of 245.79 mg/g phenolic/flavonoid content, where quercitrin and quinic acid were eluted, and catechin (113.19 mg/g), tannic acid (53.14 mg/g), ellagic acid (22.14 mg/g), gallic acid (10.27 mg/g), and epicatechin gallate (8.65 mg/g) were concentrated.

Among seven subfractions of L3-Fr2, the relatively highest amounts of the compounds were as follows; tannic acid (384.10 mg/g) in L3-Fr2-sub1; catechin (144.71 mg/g) in L3-Fr2-sub2; isoquercitrin and quercitrin (93.76, 12.32 mg/g respectively) in L3-Fr2-sub3; gallic acid (40.31 mg/g) in L3-Fr2-sub4; rutin (31.06 mg/g), and hesperidin (17.08 mg/g) in L3-Fr2-sub5; miquelianin (syn. quercetin-3-*O*-glucuronide) (23.91 mg/g) in L3-Fr2-sub6; and ellagic acid (7.66 mg/g), and quinic acid (3.20 mg/g) in L3-Fr2-sub7 (Table 6). Among the tested samples L3-Fr2-sub7 yielded higher amount (236 mg from 631.5 mg fraction), and exhibited better antityrosinase activity (32.35% inhibition at 62.5 μg/mL, IC_{50}: 206.23 ± 9.87 μg/mL).

The major metabolites, tannic acid and gallic acid, were previously reported to have antityrosinase activity, and IC_{50} values were determined as 22 μM and 4.5 mM, respectively. Tannic acid exhibited much more potent tyrosinase inhibitory activity with the characteristics of a competitive inhibitor, but was not indicated to be a direct inactivator of the enzyme [41]. In another study, the antityrosinase mechanism of gallic acid was explained by the conversion of L-DOPA to dopaquinone by enzymatic oxidation, and the oxidation of gallic acid by dopaquinone to the *o*-quinone structure before L-DOPA, and the reduction of dopaquinone back to L-DOPA by a redox cycle similar to ascorbic acid [42]. Tannic acid rich fraction (L3-Fr2-sub1), and tannic acid + catechin rich fraction (L3-Fr2-sub2) did not exhibit antityrosinase activity at 62.5 μg/mL concentration. Presence of isoquercitrin, epicatechin

gallate, gallic acid, and rutin provided a minor effect on the activity at the tested concentration. L3-Fr2-sub6 exhibited 18.38% inhibition, in which gallic acid, miquelianin, and rutin were major metabolites (15.47, 23.91, and 6.66 mg/g, respectively). When compared to others L3-Fr2-sub7 provided 32.35% inhibition, and was found to contain relatively higher amounts of ellagic acid and quinic acid. The Pearson correlation test was performed to evaluate the relationship between phytoconstituents and the antityrosinase activity of the subfractions (L3-Fr2-sub1-7) [33,34]. Correlation heat map is presented in supplementary file (Fig. S2), and the analysis suggested that quinic acid (R:0.7), ellagic acid (R:0.7), gallic acid (R:0.5), and miquelianin (R:0.4) were positively correlated with antityrosinase activity.

Ellagic acid is a heterotetracyclic polyphenol derivative presents in foods and vegetables, and a previous study showed that, a pomegranate extract rich in ellagic acid (90.16%) exhibited antityrosinase activity with an IC_{50} value of 182.2 μg/mL concentration, and orally administered extract inhibited UV-irradiated pigmentation on brownish guinea pig skin [43]. The mechanism of action was explained as, the ellagic acid acted as an alternative substrate to L-tyrosine and L-DOPA in the melanogenesis pathway, and also showed antioxidant activity by reacting with the *o*-quinones and semiquinones that were formed in the pathway [44]. Flavonoids are known for their antioxidant activities, and the antityrosinase activity of flavonoids were similar to the ellagic acid, and the flavonoid core with a free hydroxyl at C-7 was reported to be a key component for interactions with the active site of the tyrosinase enzyme, and quercetin-3-*O*-glucuronide was reported to have inhibitory activity on tyrosinase (IC_{50}: 220.10 ± 1.14 μM) [45].

Considering all the results and the relevant data, *A. unedo* fruit and leaf extracts exhibit antityrosinase activity, and the polyphenolic compounds contribute to the activity. During the experiments, antityrosinase activity was determined to decrease with fractionation, as the IC_{50} value of the subfraction (L3-Fr2-sub7, 206.23 ± 9.87 μg/mL) was lower than the main fraction (L3-Fr2, 146 ± 7.75 μg/mL) obtained from the leaf extracts. Therefore, instead of a single compound, or a crude extract, a combination of bioactive compounds concentrated by chromatographic techniques from optimized extract mixtures would be suggested.

4. Conclusion

A. unedo fruits and leaves are rich in bioactive phytochemicals, and especially polyphenolic compounds, which make it an important plant for food industry to develop functional foods, and dietary supplements. In addition, bioactive compounds that have antityrosinase activity promotes its use as a food additive to prevent the browning of fruit and vegetables, and also in medicinal, and cosmetics industry to develop nutraceuticals and pharmaceuticals related to neurodegenerative disorders, and skin whitening cosmeceutical preparations against melanogenesis.

The bioactivity of the plant has often been related to polyphenolic compounds, but these relations have mostly been correlated with total phenolic and flavonoid contents, and further analysis reports are limited. Herein, we report a systematic study evaluating the solvent composition that effects chemical composition and enzyme inhibitory activity of *A. unedo* fruit and leaf extracts. A detailed LC-MS/MS analysis were also performed in an activity-guided approach to identify bioactive content of the leaf extracts. Quantitative analysis results showed that *A. unedo* leaf extracts could be regarded as a source for quinic acid, catechin, quercitrin, tannic acid, and isoquercitrin, which were the major metabolites. These results would support further studies on the chemical composition and biological activity of *A unedo*.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix

Supporting Information

Table S1. Table of anaysis of variance (ANOVA) with Mixture Special Cubic Model for tyrosinase inhibitory activity of the fruit extracts

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probability (p) > F
Model	1528.38	6	254.73	509.46	0.0339
Linear Mixture	368.36	2	184.18	368.36	0.0368
AB (acetone-EtOH)	560.05	1	560.05	1120.09	0.0190
AC (acetone-water)	643.68	1	643.68	1287.36	0.0177
BC (EtOH-water)	32.67	1	32.67	65.33	0.0784
ABC (acetone-EtOH-water)	86.25	1	86.25	172.51	0.0484
Pure error	0.50	1	0.50		
Cor total	1528.88	7			
R^2: 0.9997					
R^2 adj.: 0.9977					

The Model *F*-value of 509.46 implied the model was significant for the response as tyrosinase inhibitory activity for the optimization of extraction of fruits. There was only a 3.39% chance that a "Model *F*-Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicated model terms were significant where linear mixture components, AB, AC and ABC were significant model terms.

Table S2. Suggested Solutions by the Optimization model for tyrosinase inhibitory activity of the fruit extracts.

Solutions	Acetone	Ethanol	Water	Tyrosinase Inhibition%
1	0.376	0.624	0.000	63.46
2	**0.451**	**0.549**	**0.000**	**63.59**
3	0.396	0.604	0.000	63.62
4	**0.434**	**0.566**	**0.000**	**63.67**
5	**0.483**	**0.517**	**0.000**	**63.27**
6	**0.419**	**0.581**	**0.000**	**63.69**
7	0.352	0.648	0.000	63.15
8	**0.500**	**0.500**	**0.000**	**63.00**
9	0.527	0.000	0.473	53.08

Suggested solutions by the optimization model were confirmed with the experimental results, where 50:50 acetone:ethanol extract were determined with the highest tyrosinase inhibitory activity of 62.78%.

Table S3. Table of anaysis of variance (ANOVA) with Mixture Special Cubic Model for tyrosinase inhibitory activity of the leaf extracts

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probability (p) > F
Model	3075.50	6	512.58	256.29	0.0478
Linear Mixture	2039.62	2	1019.81	509.91	0.0313
AB (acetone-EtOH)	0.18	1	0.18	0.091	0.8136
AC (acetone-water)	401.64	1	401.64	200.82	0.0448
BC (EtOH-water)	130.67	1	130.67	65.33	0.0784
ABC (acetone-EtOH-water)	803.23	1	803.23	401.62	0.0317
Pure error	2.00	1	2.00		
Cor total	3077.50	7			
R^2: 0.9994					
R^2 adj.: 0.9955					

The Model *F*-value of 256.29 implied the model was significant. There was only a 4.78% chance that a "Model *F*-Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicated model terms were significant where linear mixture components, AC and ABC were significant model terms.

In the equation presented below A represents acetone, B represents ethanol concentration and C represents water (Table S2);

Tyrosinase Inhibition% = (26.50 × A) + (44.00 × B) + (20.00 × C) + (111.00 × A × B) + (119.00 × A × C) + (28.00 × B × C) − (319.50 × A × B × C).

In the equation presented below A represents acetone, B represents ethanol concentration and C represents water (Table S4);

Tyrosinase Inhibition% = (28.00 × A) + (61.00 × B) + (69.00 × C) − (2.00 × A × B) + (94.00 × A × C) + (56.00 × B × C) − (975.00 × A × B × C).

Table S4. Suggested Solutions by the Optimization model for tyrosinase inhibitory activity of the leaf extracts.

Solutions	Acetone	Ethanol	Water	Tyrosinase Inhibition%
1	**0.000**	**0.488**	**0.512**	**79.09**
2	0.000	0.384	0.616	79.18
3	0.000	0.362	0.638	79.04
4	**0.000**	**0.454**	**0.546**	**79.25**
5	**0.000**	**0.470**	**0.530**	**79.19**
6	**0.000**	**0.435**	**0.565**	**79.18**
7	0.000	0.405	0.595	79.25
8	0.282	0.000	0.718	76.47
9	0.000	1.000	0.000	61.00

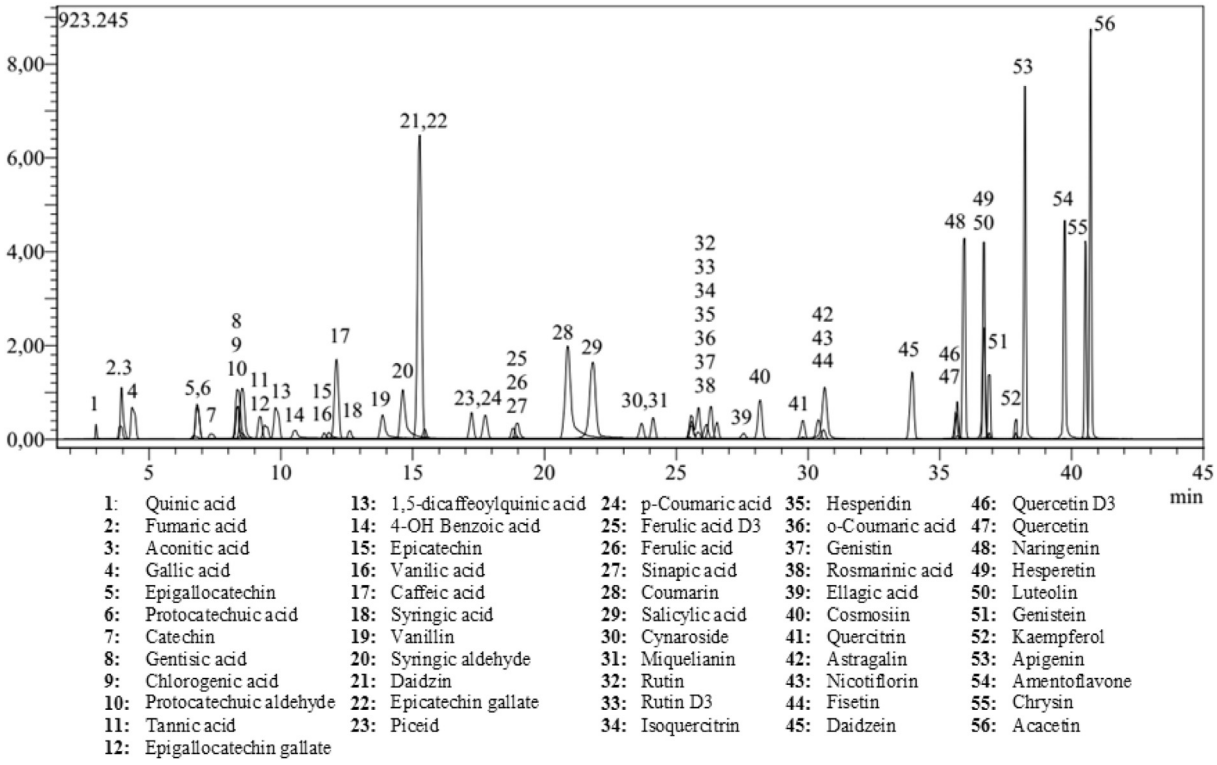
Suggested solutions by the optimization model were confirmed with the experimental results, where 50:50 ethanol:water extract were determined with the highest tyrosinase inhibitory activity of 79.06%.

S1. Material and methods

S1.1. Quantitative analysis using LC-MS/MS system

Mass spectrometer and chromatograph conditions. A Shimadzu-Nexera model ultrahigh performance liquid chromatograph (UHPLC) coupled with a tandem mass spectrometer was used to accomplish quantiative evaluation of 53 phytochemicals. The reversed-phase UHPLC was equipped with an autosampler (SIL-30AC model), a column oven (CTO-10ASvp model), binary pumps (LC-30AD model), and a degasser (DGU- 20A3R model). The chromatographic conditions were optimized in order to achive optimum separation for 53 phytochemicals and overcome the suppression effects. The chromatographic separation was performed on a reversed phase Agilent Poroshell 120 EC-C18 model (150 mm × 2.1 mm, 2.7 μm) analytical column. The column temperature was set to 40 °C. The elution gradient was composed of eluent A (water + 5 mM ammonium formate + 0.1% formic acid) and eluent B (methanol + 5 mM ammonium formate + 0.1% formic acid). The following gradient elution profile was used: 20–100% B (0–25 min), 100% B (25–35 min), 20% B (35–45 min). Furthermore, the solvent flow rate and injection volume were settled as 0.5 mL/min and 5 μL, respectively. Total ion chromatogram (TIC) of standard phenolic compounds is presented in Fig. S1.

The mass spectrometric detection was carried out using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI-MS/MS data were acquired and processed by LabSolutions software (Shimadzu). The MRM (multiple reaction monitoring) mode was used for the quantification of the phytochemicals. The MRM metot was optimized to selectively detect and quantify phytochemical compounds based on the screening of specified precursor phytochemical-to-fragment ion transitions. The collision energies (CE) were optimized in order to generate optimal phytochemical fragmentation and maximal transmission of the desired product ions (Table S5). The MS operating conditions were applied as: drying gas (N2) flow, 15 L/min; nebulizing gas (N2) flow, 3 L/min; DL temperature, 250 °C; heat block temperature, 400 °C, and interface temperature, 350 °C.



1:	Quinic acid	**13:**	1,5-dicaffeoylquinic acid	**24:**	p-Coumaric acid	**35:**	Hesperidin	**46:**	Quercetin D3
2:	Fumaric acid	**14:**	4-OH Benzoic acid	**25:**	Ferulic acid D3	**36:**	o-Coumaric acid	**47:**	Quercetin
3:	Aconitic acid	**15:**	Epicatechin	**26:**	Ferulic acid	**37:**	Genistin	**48:**	Naringenin
4:	Gallic acid	**16:**	Vanilic acid	**27:**	Sinapic acid	**38:**	Rosmarinic acid	**49:**	Hesperetin
5:	Epigallocatechin	**17:**	Caffeic acid	**28:**	Coumarin	**39:**	Ellagic acid	**50:**	Luteolin
6:	Protocatechuic acid	**18:**	Syringic acid	**29:**	Salicylic acid	**40:**	Cosmosiin	**51:**	Genistein
7:	Catechin	**19:**	Vanillin	**30:**	Cynaroside	**41:**	Quercitrin	**52:**	Kaempferol
8:	Gentisic acid	**20:**	Syringic aldehyde	**31:**	Miquelianin	**42:**	Astragalin	**53:**	Apigenin
9:	Chlorogenic acid	**21:**	Daidzin	**32:**	Rutin	**43:**	Nicotiflorin	**54:**	Amentoflavone
10:	Protocatechuic aldehyde	**22:**	Epicatechin gallate	**33:**	Rutin D3	**44:**	Fisetin	**55:**	Chrysin
11:	Tannic acid	**23:**	Piceid	**34:**	Isoquercitrin	**45:**	Daidzein	**56:**	Acacetin
12:	Epigallocatechin gallate								

Fig. S1. Total ion chromatogram of standard phenolic compounds.

Table S5. Analytical method validation parameters that belong to the LC-MS/MS method

No	Analytes	RT[a]	M.I. (*m/z*)[b]	F.I. (*m/z*)[c]	Ion. mode	Equation	r^{2d}	*RSD%*[e]		Linearity Range (mg/L)	*LOD/LOQ* (μg/L)[f]	Recovery (%)		*U*[g]	Gr. No[i]
								Interday	Intraday			Interday	Intraday		
1	Quinic acid	3.0	190.8	93.0	Neg	$y = -0.0129989 + 2.97989\times$	0.996	0.69	0.51	0.1–5	25.7/33.3	1.0011	1.0083	0.0372	1
2	Fumaric aid	3.9	115.2	40.9	Neg	$y = -0.0817862 + 1.03467\times$	0.995	1.05	1.02	1–50	135.7/167.9	0.9963	1.0016	0.0091	1
3	Aconitic acid	4.0	172.8	129.0	Neg	$y = -0.7014530 + 32.9994\times$	0.971	2.07	0.93	0.1–5	16.4/31.4	0.9968	1.0068	0.0247	1
4	Gallic acid	4.4	168.8	79.0	Neg	$y = 0.0547697 + 20.8152\times$	0.999	1.60	0.81	0.1–5	13.2/17.0	1.0010	0.9947	0.0112	1
5	Epigallocatechin	6.7	304.8	219.0	Neg	$y = -0.00494986 + 0.0483704\times$	0.998	1.22	0.73	1–50	237.5/265.9	0.9969	1.0040	0.0184	3
6	Protocatechuic acid	6.8	152.8	108.0	Neg	$y = 0.211373 + 12.8622\times$	0.957	1.43	0.76	0.1–5	21.9/38.6	0.9972	1.0055	0.0350	1
7	Catechin	7.4	288.8	203.1	Neg	$y = -0.00370053 + 0.431369\times$	0.999	2.14	1.08	0.2–10	55.0/78.0	1.0024	1.0045	0.0221	3
8	Gentisic acid	8.3	152.8	109.0	Neg	$y = -0.0238983 + 12.1494\times$	0.997	1.81	1.22	0.1–5	18.5/28.2	0.9963	1.0077	0.0167	1
9	Chlorogenic acid	8.4	353.0	85.0	Neg	$y = 0.289983 + 36.3926\times$	0.995	2.15	1.52	0.1–5	13.1/17.6	1.0000	1.0023	0.0213	1
10	Protocatechuic aldehyde	8.5	137.2	92.0	Neg	$y = 0.257085 + 25.4657\times$	0.996	2.08	0.57	0.1–5	15.4/22.2	1.0002	0.9988	0.0396	1
11	Tannic acid	9.2	182.8	78.0	Neg	$y = 0.0126307 + 26.9263\times$	0.999	2.40	1.16	0.05–2.5	15.3/22.7	0.9970	0.9950	0.0190	1
12	Epigallocatechin gallate	9.4	457.0	305.1	Neg	$y = -0.0380744 + 1.61233\times$	0.999	1.30	0.63	0.2–10	61.0/86.0	0.9981	1.0079	0.0147	3
13	1,5-dicaffeoylquinic acid	9.8	515.0	191.0	Neg	$y = -0.0164044 + 16.6535\times$	0.999	2.42	1.48	0.1–5	5.8/9.4	0.9983	0.9997	0.0306	1
14	4-OH Benzoic acid	10.5	137.2	65.0	Neg	$y = -0.0240747 + 5.06492\times$	0.999	1.24	0.97	0.2–10	68.4/88.1	1.0032	1.0068	0.0237	1
15	Epicatechin	11.6	289.0	203.0	Neg	$y = -0.0172078 + 0.0833424\times$	0.996	1.47	0.62	1–50	139.6/161.6	1.0013	1.0012	0.0221	3
16	Vanilic acid	11.8	166.8	108.0	Neg	$y = -0.0480183 + 0.779564\times$	0.999	1.92	0.76	1–50	141.9/164.9	1.0022	0.9998	0.0145	1
17	Caffeic acid	12.1	179.0	134.0	Neg	$y = 0.120319 + 95.4610\times$	0.999	1.11	1.25	0.05–2.5	7.7/9.5	1.0015	1.0042	0.0152	1
18	Syringic acid	12.6	196.8	166.9	Neg	$y = -0.0458599 + 0.663948\times$	0.998	1.18	1.09	1–50	82.3/104.5	1.0006	1.0072	0.0129	1
19	Vanillin	13.9	153.1	125.0	Poz	$y = 0.00185898 + 20.7382\times$	0.996	1.10	0.85	0.1–5	24.5/30.4	1.0009	0.9967	0.0122	1
20	Syringic aldehyde	14.6	181.0	151.1	Neg	$y = -0.0128684 + 7.90153\times$	0.999	2.51	0.77	0.4–20	19.7/28.0	1.0001	0.9964	0.0215	1
21	Daidzin	15.2	417.1	199.0	Poz	$y = 9.45747 + 152.338\times$	0.996	2.25	1.32	0.05–2.5	7.0/9.5	0.9955	1.0017	0.0202	2
22	Epicatechin gallate	15.5	441.0	289.0	Neg	$y = -0.0142216 + 1.06768\times$	0.997	1.63	1.28	0.1–5	19.5/28.5	0.9984	0.9946	0.0229	3
23	Piceid	17.2	391.0	135/106.9	Poz	$y = 0.00772525 + 25.4181\times$	0.999	1.94	1.16	0.05–2.5	13.8/17.8	1.0042	0.9979	0.0199	1
24	*p*-Coumaric acid	17.8	163.0	93.0	Neg	$y = 0.0249034 + 18.5180\times$	0.999	1.92	1.43	0.1–5	25.9/34.9	1.0049	1.0001	0.0194	1
25	Ferulic acid-D3-IS[h]	18.8	196.2	152.1	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0170	1
26	Ferulic acid	18.8	192.8	149.0	Neg	$y = -0.0735254 + 1.34476\times$	0.999	1.44	0.53	1–50	11.8/15.6	0.9951	0.9976	0.0181	1
27	Sinapic acid	18.9	222.8	193.0	Neg	$y = -0.0929932 + 0.836324\times$	0.999	1.45	0.52	0.2–10	65.2/82.3	1.0031	1.0037	0.0317	1
28	Coumarin	20.9	146.9	103.1	Poz	$y = 0.0633397 + 136.508\times$	0.999	2.11	1.54	0.05–2.5	214.2/247.3	0.9950	0.9958	0.0383	1
29	Salicylic acid	21.8	137.2	65.0	Neg	$y = 0.239287 + 153.659\times$	0.999	1.48	1.18	0.05–2.5	6.0/8.3	0.9950	0.9998	0.0158	1
30	Cynaroside	23.7	447.0	284.0	Neg	$y = 0.280246 + 6.13360\times$	0.997	1.56	1.12	0.05–2.5	12.1/16.0	1.0072	1.0002	0.0366	2
31	Miquelianin	24.1	477.0	150.9	Neg	$y = -0.00991585 + 5.50334\times$	0.999	1.31	0.95	0.1–5	10.6/14.7	0.9934	0.9965	0.0220	2
32	Rutin-D3-IS[h]	25.5	612.2	304.1	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	2
33	Rutin	25.6	608.9	301.0	Neg	$y = -0.0771907 + 2.89868\times$	0.999	1.38	1.09	0.1–5	15.7/22.7	0.9977	1.0033	0.0247	2
34	Isoquercitrin	25.6	463.0	271.0	Neg	$y = -0.111120 + 4.10546\times$	0.998	2.13	0.78	0.1–5	8.7/13.5	1.0057	0.9963	0.0220	2
35	Hesperidin	25.8	611.2	449.0	Poz	$y = 0.139055 + 13.2785\times$	0.999	1.84	1.35	0.1–5	19.0/26.0	0.9967	1.0043	0.0335	2
36	*o*-Coumaric acid	26.1	162.8	93.0	Neg	$y = 0.00837193 + 11.2147\times$	0.999	2.11	1.46	0.1–5	31.8/40.4	1.0044	0.9986	0.0147	1
37	Genistin	26.3	431.0	239.0	Neg	$y = 1.65808 + 7.57459\times$	0.991	2.01	1.28	0.1–5	14.9/21.7	1.0062	1.0047	0.0083	2
38	Rosmarinic acid	26.6	359.0	197.0	Neg	$y = -0.0117238 + 8.04377\times$	0.999	1.24	0.86	0.1–5	16.2/21.2	1.0056	1.0002	0.0130	1
39	Ellagic acid	27.6	301.0	284.0	Neg	$y = 0.00877034 + 0.663741\times$	0.999	1.57	1.23	0.4–20	56.9/71.0	1.0005	1.0048	0.0364	1

(*continued on next page*)

Table S5. (continued)

No	Analytes	RT[a]	M.I. (*m/z*)[b]	F.I. (*m/z*)[c]	Ion. mode	Equation	r^{2}[d]	*RSD%*[e]		Linearity Range (mg/L)	*LOD/LOQ* (μg/L)[f]	Recovery (%)		*U*[g]	Gr. No[i]
								Interday	Intraday			Interday	Intraday		
40	Cosmosiin	28.2	431.0	269.0	Neg	$y = -0.708662 + 8.62498\times$	0.998	1.65	1.30	0.1–5	6.3/9.2	0.9940	0.9973	0.0083	2
41	Quercitrin	29.8	447.0	301.0	Neg	$y = -0.00153274 + 3.20368\times$	0.999	2.24	1.16	0.1–5	4.8/6.4	0.9960	0.9978	0.0268	2
42	Astragalin	30.4	447.0	255.0	Neg	$y = 0.00825333 + 3.51189\times$	0.999	2.08	1.72	0.1–5	6.6/8.2	0.9968	0.9957	0.0114	2
43	Nicotiflorin	30.6	592.9	255.0/284.0	Neg	$y = 0.00499333 + 2.62351\times$	0.999	1.48	1.23	0.05–2.5	11.9/16.7	0.9954	1.0044	0.0108	2
44	Fisetin	30.6	285.0	163.0	Neg	$y = 0.0365705 + 8.09472\times$	0.999	1.75	1.19	0.1–5	10.1/12.7	0.9980	1.0042	0.0231	3
45	Daidzein	34.0	253.0	223.0	Neg	$y = -0.0329252 + 6.23004\times$	0.999	2.18	1.73	0.1–5	9.8/11.6	0.9926	0.9963	0.0370	3
46	Quercetin-D3-IS[h]	35.6	304.0	275.9	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	3
47	Quercetin	35.7	301.0	272.9	Neg	$y = +0.00597342 + 3.39417\times$	0.999	1.89	1.38	0.1–5	15.5/19.0	0.9967	0.9971	0.0175	3
48	Naringenin	35.9	270.9	119.0	Neg	$y = -0.00393403 + 14.6424\times$	0.999	2.34	1.69	0.1–5	2.6/3.9	1.0062	1.0020	0.0392	3
49	Hesperetin	36.7	301.0	136.0/286.0	Neg	$y = +0.0442350 + 6.07160\times$	0.999	2.47	2.13	0.1–5	7.1/9.1	0.9998	0.9963	0.0321	3
50	Luteolin	36.7	284.8	151.0/175.0	Neg	$y = -0.0541723 + 30.7422\times$	0.999	1.67	1.28	0.05–2.5	2.6/4.1	0.9952	1.0029	0.0313	3
51	Genistein	36.9	269.0	135.0	Neg	$y = -0.00507501 + 12.1933\times$	0.999	1.48	1.19	0.05–2.5	3.7/5.3	1.0069	1.0012	0.0337	3
52	Kaempferol	37.9	285.0	239.0	Neg	$y = -0.00459557 + 3.13754\times$	0.999	1.49	1.26	0.05–2.5	10.2/15.4	0.9992	0.9990	0.0212	3
53	Apigenin	38.2	268.8	151.0/149.0	Neg	$y = 0.119018 + 34.8730\times$	0.998	1.17	0.96	0.05–2.5	1.3/2.0	0.9985	1.0003	0.0178	3
54	Amentoflavone	39.7	537.0	417.0	Neg	$y = 0.727280 + 33.3658\times$	0.992	1.35	1.12	0.05–2.5	2.8/5.1	0.9991	1.0044	0.0340	3
55	Chrysin	40.5	252.8	145.0/119.0	Neg	$y = -0.0777300 + 18.8873\times$	0.999	1.46	1.21	0.05–2.5	1.5/2.8	0.9922	1.0050	0.0323	3
56	Acacetin	40.7	283.0	239.0	Neg	$y = -0.559818 + 163.062\times$	0.997	1.67	1.28	0.02–1	1.5/2.5	0.9949	1.0011	0.0363	3

[a]R.T.: Retention time, [b]MI (*m/z):* Molecular ions of the standard analytes (*m*/*z* ratio), [c]FI (*m/z):* Fragment ions [d]r^2: Coefficient of determination, [e]*RSD*: Relative standard deviation, [f]*LOD*/*LOQ* (μg/L): Limit of detection/quantification, [g]*U* (%): percent relative uncertainty at 95% confidence level ($k = 2$), [h]IS: Internal standard, [i]Gr. No: Represents grouping of internal standards, these numbers indicate which IS stands for which phenolic compound.

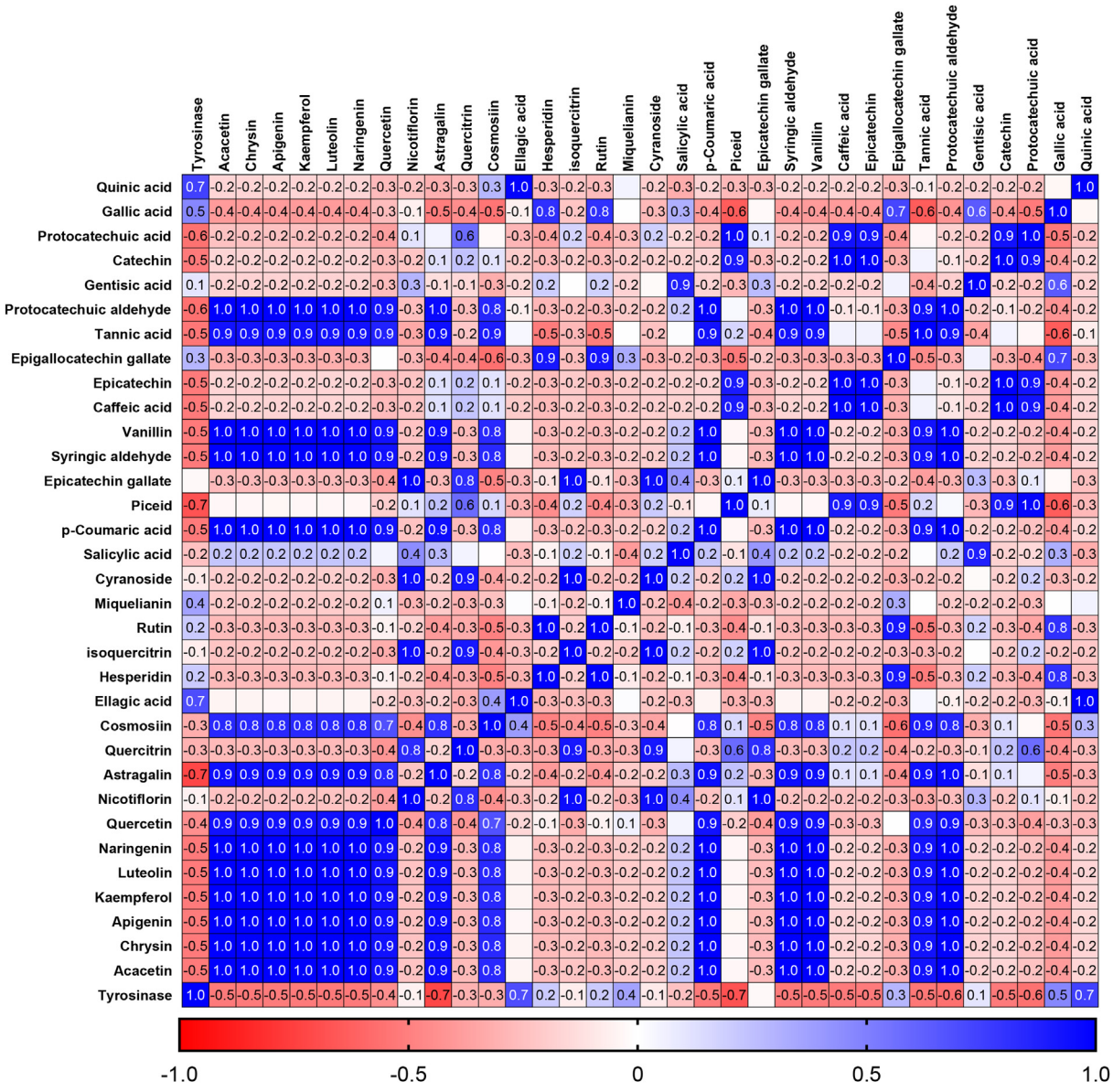


Fig. S2. Correlation heat map between chemical components and biological activity of the tested subfractions of L3-Fr2 (sub1 to sub7) ($p < 0.05$).

References

[1] Torres JA, Valle F, Pinto C, Garcia-Fuentes A, Salazar C, Cano E. *Arbutus unedo* L. communities in southern Iberian Peninsula mountains. Plant Ecol 2002;160:207–23.

[2] Stevens PF. *Arbutus* L. In: Davis PH, editor. Flora of Turkey and east aegean islands, vol. 6. England: Edinburgh University Press; 1978. p. 99–100.

[3] Celikel G, Demirsoy L, Demirsoy H. The strawberry tree (*Arbutus unedo* L.) selection in Turkey. Sci Hortic 2008;118: 115–9.

[4] Tardio J, Pardo-De-Santayana M, Morales R. Ethnobotanical review of wild edible plants in Spain. Bot J Linn Soc 2006;152: 27–71.

[5] El-Hilaly J, Hmammouchi M, Lyoussi B. Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province (Northern Morocco). J Ethnopharmacol 2003;86:149–58.

[6] Ziyyat A, Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M, Benjelloun W. Phytotherapy of hypertension and diabetes in oriental Morocco. J Ethnopharmacol 1997;58:45–54.

[7] Ruiz-Rodriguez BM, Sanchez-Moreno C, De Ancos B, Sanchez-Mata MD, Fernandez-Ruiz V, Camara M, et al. Wild *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits are underutilized sources of valuable bioactive compounds with antioxidant capacity. Fruits 2014;69:435–48.

[8] Malheiro R, Sa O, Pereira E, Aguiar C, Baptista P, Pereira JA. *Arbutus unedo* L. leaves as source of phytochemicals with bioactive properties. Ind Crop Prod 2012;37:473–8.

[9] Guimaraes R, Barros L, Calhelha RC, Carvalho AM, Queiroz MJ, Ferreira IC. Bioactivity of different enriched phenolic extracts of wild fruits from Northeastern Portugal: a comparative study. Plant Foods Hum Nutr (Dordr) 2014;69: 37–42.

[10] Pavlovic DR, Brankovic S, Kovacevic N, Kitic D, Veljkovic S. Comparative study of spasmolytic properties, antioxidant

activity and phenolic content of *Arbutus unedo* from Montenegro and Greece. Phytother Res 2011;25:749−54.
[11] Legssyer A, Ziyyat A, Mekhfi H, Bnouham M, Herrenknecht C, Roumy V, et al. Tannins and catechin gallate mediate the vasorelaxant effect of *Arbutus unedo* on the rat isolated aorta. Phytother Res 2004;18:889−94.
[12] Kachkoul R, Housseini TS, Mohim M, El Habbani R, Miyah Y, Lahrichi A. Chemical compounds as well as antioxidant and litholytic activities of *Arbutus unedo* L. leaves against calcium oxalate stones. J Integr Med 2019;17:430−7.
[13] Afkir S, Nguelefack TB, Aziz M, Zoheir J, Cuisinaud G, Bnouham M, et al. *Arbutus unedo* prevents cardiovascular and morphological alterations in L-NAME-induced hypertensive rats - Part 1: cardiovascular and renal hemodynamic effects of *Arbutus unedo* in L-NAME-induced hypertensive rats. J Ethnopharmacol 2008;116:288−95.
[14] Mrabti HN, Faouzi ME, Mayuk FM, Makrane H, Limas-Nzouzi N, Dibong SD, et al. *Arbutus unedo* L., (Ericaceae) inhibits intestinal glucose absorption and improves glucose tolerance in rodents. J Ethnopharmacol 2019;235:385−91.
[15] Ben Salem I, Ouesleti S, Mabrouk Y, Landolsi A, Saidi M, Boulilla A. Exploring the nutraceutical potential and biological activities of *Arbutus unedo* L. (Ericaceae) fruits. Ind Crop Prod 2018;122:726−31.
[16] Pallauf K, Rivas-Gonzalo JC, del Castillo MD, Cano MP, de Pascual-Teresa S. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. J Food Compos Anal 2008;21:273−81.
[17] Carcache-Blanco EJ, Cuendet M, Park EJ, Su BN, Rivero-Cruz JF, Farnsworth NR, et al. Potential cancer chemopreventive agents from *Arbutus unedo*. Nat Prod Res 2006;20:327−34.
[18] Males Z, Saric D, Bojic M. Quantitative determination of flavonoids and chlorogenic acid in the leaves of *Arbutus unedo* L. using thin layer chromatography. J Anal Methods Chem 2013:385473.
[19] Fiorentino A, Castaldi S, D'Abrosca B, Natale A, Carfora A, Messere A, et al. Polyphenols from the hydroalcoholic extract of *Arbutus unedo* living in a monospecific Mediterranean woodland. Biochem Systemat Ecol 2007;35:809−11.
[20] Inoue Y, Hasegawa S, Yamada T, Date Y, Mizutani H, Nakata S, et al. Analysis of the effects of hydroquinone and arbutin on the differentiation of melanocytes. Biol Pharm Bull 2013;36:1722−30.
[21] Tada M, Kohno M, Niwano Y. Alleviation effect of arbutin on oxidative stress generated through tyrosinase reaction with L-tyrosine and L-DOPA. BMC Biochem 2014;15.
[22] Hori I, Nihei K, Kubo I. Structural criteria for depigmenting mechanism of arbutin. Phytother Res 2004;18:475−9.
[23] Chang TS. An updated review of tyrosinase inhibitors. Int J Mol Sci 2009;10:2440−75.
[24] Zolghadri S, Bahrami A, Hassan Khan MT, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, et al. A comprehensive review on tyrosinase inhibitors. J Enzym Inhib Med Chem 2019;34:279−309.
[25] Munhoz VM, Longhini R, Souza JRP, Zequi JAC, Mello EVSL, Lopes GC, et al. Extraction of flavonoids from *Tagetes patula*: process optimization and screening for biological activity. Rev Bras Farmacogn 2014;24:576−83.
[26] Singleton VL. Citation classic - colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Cc/Agr Biol Environ 1985:18.
[27] Koyu H, Kazan A, Demir S, Haznedaroglu MZ, Yesil-Celiktas O. Optimization of microwave assisted extraction of *Morus nigra* L. fruits maximizing tyrosinase inhibitory activity with isolation of bioactive constituents. Food Chem 2018;248:183−91.
[28] Ercisli S, Orhan E. Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits. Food Chem 2007;103:1380−4.
[29] Lee J, Durst RW, Wrolstad RE. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J AOAC Int 2005;88:1269−78.
[30] Yilmaz MA. Simultaneous quantitative screening of 53 phytochemicals in 33 species of medicinal and aromatic plants: a detailed, robust and comprehensive LC-MS/MS method validation. Ind Crop Prod 2020:149.
[31] Chang TS, Ding HY, Tai SSK, Wu CY. Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with *Aspergillus oryzae* BCRC 32288. Food Chem 2007;105:1430−8.
[32] Scheffe H. The simplex-centroid design for experiments with mixtures. J R Stat Soc Ser B Methodol 1963;25:235−63.
[33] Demiroz Akbulut T, Aydin Kose F, Demirci B, Baykan S. Chemical profile and cytotoxicity evaluation of aerial parts of *Marrubium vulgare* L. from different locations in Turkey. Chem Biodivers 2023;20:e202201188.
[34] Uba AI, Zengin G, Montesano D, Cakilcioglu U, Selvi S, Ulusan MD, et al. Antioxidant and enzyme inhibitory properties, and HPLC−MS/MS profiles of different extracts of *Arabis carduchorum* Boiss.: an endemic plant to Turkey. Appl Sci 2022;12:6561.
[35] Oliveira I, Baptista P, Malheiro R, Casal S, Bento A, Pereira JA. Influence of strawberry tree (*Arbutus unedo* L.) fruit ripening stage on chemical composition and antioxidant activity. Food Res Int 2011;44:1401−7.
[36] Oliveira I, Coelho V, Baltasar R, Pereira JA, Baptista P. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. Food Chem Toxicol 2009;47:1507−11.
[37] Akay S, Alpak I, Yesil-Celiktas O. Effects of process parameters on supercritical CO_2 extraction of total phenols from strawberry (*Arbutus unedo* L.) fruits: an optimization study. J Separ Sci 2011;34:1925−31.
[38] Barros L, Carvalho AM, Morais JS, Ferreira ICFR. Strawberry-tree, blackthorn and rose fruits: detailed characterisation in nutrients and phytochemicals with antioxidant properties. Food Chem 2010;120:247−54.
[39] Garcia-Viguera C, Zafrilla P, Tomas-Barberan FA. The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. Phytochem Anal 1998;9:274−7.
[40] Liu J, Chen Y, Zhang X, Zheng J, Hu W, Teng B. Hop tannins as multifunctional tyrosinase inhibitor: structure characterization, inhibition activity, and mechanism. Antioxidants 2022;11:772.
[41] Kubo I, Kinst-Hori I, Nihei K, Soria F, Takasaki M, Calderon JS, et al. Tyrosinase inhibitors from galls of *Rhus javanica* leaves and their effects on insects. Zeitschrift fur Naturforschung C, J Biosci 2003;58:719−25.
[42] Kubo I, Chen Q, Nihei K. Molecular design of antibrowning agents: antioxidative tyrosinase inhibitors. Food Chem 2003;81:241−7.
[43] Yoshimura M, Watanabe Y, Kasai K, Yamakoshi J, Koga T. Inhibitory effect of an ellagic acid-rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation. Biosci Biotechnol Biochem 2005;69:2368−73.
[44] Ortiz-Ruiz CV, Berna J, Tudela J, Varon R, Garcia-Canovas F. Action of ellagic acid on the melanin biosynthesis pathway. J Dermatol Sci 2016;82:115−22.
[45] Jakimiuk K, Sari S, Milewski R, Supuran CT, Sohretoglu D, Tomczyk M. Flavonoids as tyrosinase inhibitors in *in silico* and *in vitro* models: basic framework of SAR using a statistical modelling approach. J Enzym Inhib Med Chem 2022;37:421−30.