JOTCS A
Journal of the Turkish Chemical Society
Section: A
Chemistry

TURKISH CHEMICAL SOCIETY

Determination of Antioxidant Capacities of Some Dietary Supplements by Spectrophotometric and Chromatographic Methods

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Abstract: The main objective of this study is to propose standard analytical methods for the determination and comparison of plant-specific antioxidant components found in some herbal products (sold as dietary supplements). Numerous studies indicate that nutritional supplements can offer medical benefits due to their content of hydrophilic and lipophilic molecules as well as natural extracts or synthetic compounds with antioxidant properties. While these products are marketed as antioxidant boosters, there is a limited amount of data available on the antioxidant activity and bioactive compound content of commercially available formulations. Therefore, in our study, tablet and capsule forms of ground grape seed, rosemary, bitter melon, and ginkgo biloba plants containing polyphenol-type compounds known for their antioxidant properties were investigated. The total polyphenol contents (TPC) of these products were determined by the Folin-Ciocalteu method, and total antioxidant capacities (TACs) were determined by CUPRAC and ABTS methods. The HPLC system was used to detect and quantify the components responsible for antioxidant capacity, and the most appropriate chromatographic analysis methods were suggested for each sample. Total antioxidant capacity values as trolox (TR) equivalent (mmol TR/g) determined by CUPRAC and ABTS methods are 0.90 ± 0.07 and 0.72 ± 0.17 for grape seed, 0.79 ± 0.05 and 0.41 ± 0.09 for rosemary, 0.08 ± 0.006 and 0.11 ± 0.05 for bitter melon, 0.12 ± 0.01 and 0.14 ± 0.02 for ginkgo biloba, respectively. These findings were correlated with HPLC data, and components contributing to the antioxidant capacity were identified.

Keywords: Dietary supplements, grape seed, rosemary, bitter melon, ginkgo biloba.

Submitted: January 3, 2025. **Accepted:** April 15, 2025.

Cite this: Demir A, Sözgen Başkan K, Demirci Çekiç S. Determination of Antioxidant Capacities of Some Dietary Supplements by Spectrophotometric and Chromatographic Methods. JOTCSA. 2025;12(2): 129-40.

DOI: https://doi.org/10.18596/jotcsa.1506003

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1. INTRODUCTION

Food and good nutrition are the basis of human existence and healthy life (1,2). To live a quality life, the food we eat must have high nutritional quality. Recently, the consumption of commercially prepared and processed foods has become widespread because they are practical. These foods are exposed to physical and chemical transformations as a result of some processes (for example, refined sugar, bran-free flour, skimmed milk, margarine, etc.). These operations mean that their important properties are lost. As a result, wrong eating habits develop in society, and accordingly, health problems caused by malnutrition appear. Because the basic substances the body needs (vitamins, proteins, carbohydrates, minerals, oils, etc.) can only be met by a sufficient and balanced diet (3,4). The opposite situation causes the immune system to weaken gradually. Numerous epidemiological and clinical studies link the consumption of antioxidant-rich fruits, vegetables, plant-based drinks, and whole grains with lower incidence and mortality rates of chronic diseases such as diabetes, atherosclerosis, rheumatoid arthritis, neurodegenerative and coronary diseases, and cancer (5-12). In addition to fruits and vegetables, herbs of no specific nutritional value can also constitute an important source of antioxidants (13-15). The term herb includes not only herbaceous plants but also the leaves, bark, roots, seeds, fruits, and flowers of shrubs and trees. There are also products called nutraceuticals, which are often mixed with dietary supplements. The term "nutraceutical" is derived from the words "nutrition" and "drug" and is used for nutritional products that are also used as medicine. Nutraceuticals include probiotic and prebiotic food substances and foods for special medical purposes, either individually or in combination. In contrast, dietary supplements include minerals, vitamins, protein supplements, functional foods, and herbal products, either individually or in combination. It is stated in the

literature that nutraceuticals and nutritional supplements are collectively referred to as dietary supplements (16,17).

Due to the reasons we explained above and the environmental conditions, the decrease in the quality of most of the foods consumed has caused consumers to turn to products sold under the name of dietary supplements to maintain a healthy life. Dietary supplements are used worldwide and represent a broad category of ingestible products that are distinguishable from conventional foods and drugs (4). These are not medical medicines; they are used extensively to support a healthy life and to compensate for some nutritional elements not taken enough by diet. The US Food and Drug Administration (FDA) states that dietary supplements are beneficial for a healthy diet. It has been reported that until relatively recently, limited scientific research has been done on dietary supplements, and there is not much information on this subject. The same authors also pointed out that the prevalence of supplement use has increased significantly in the last 20 years (18). Dietary supplements are prepared from different parts of a plant, have different compositions, and have different concentrations of bioactive compounds; hence, it is to be expected that they will vary widely in their antioxidant powers. There is no information about antioxidant activities on the label of herbal supplement products sold on the market. In addition, there are very few studies in the world literature to determine the antioxidant activities of these products (19-22). Since humans use these products to support natural antioxidant intake, their activities should be known and standardized. Therefore, manufacturers should use standard analytical methods to standardize their products and determine effective doses and their antioxidant capacity so that they can market their products with confidence.

The dietary supplements we examined in our study were grape seed extract, rosemary, bitter melon, and ginkgo biloba, sold in capsule or tablet form. The grape seed extract is known as a powerful antioxidant that protects the body from premature aging and disease (23). Scientific studies have shown that the rosemary plant has antioxidant, antibacterial, antiviral, and immune system-enhancing effects due to its compounds (24). It is known that some of the bioactive substances found in bitter melon have hypoglycemic, antiulcer, antioxidant, antibiotic, antidiabetic, anticarcinogenic, and antimutagenic activities that are proven by clinical studies (25). The use of ginkgo biloba products has become widespread all over the world due to the effects of their components on the brain vessels in situations such as age-related memory impairment and dementia (26). Commercial herbal products are in tablet or capsule form but are often not standardized, and quality can vary from manufacturer to manufacturer and batch to batch (27,28). Assessments of the safety, quality, and efficacy of nutrients and other bioactive compounds are needed to provide the scientific information that regulators need (28,29). Indeed, data on the antioxidant activity of products currently on the market are scarce. These are called antioxidant boosters while recommending, but their labels lack information on effective antioxidant capacity values.

The main purpose of this study is to propose standard analytical methods to determine the originality and antioxidant capacities of selected dietary supplements, which are widely used for various reasons and are closely related to human health. Another purpose is to develop and apply chromatographic methods that will provide data that can be used to determine the bioactive components they contain and, thus, the effective dose to be used according to the needs of the person. Based on chromatographic data, plant-specific antioxidant components can be identified in plant products, and these can be compared with original plant samples.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Instrumentation

Trolox (TR), quercetin (QUE), rosmarinic acid (RA), flavone, apigenin (APG), luteolin (LUT), kaempferol (KAM), rutin (RUT), caffeic acid (CA), carnosol (CAR), carnosic acid (CRA), catechin (CAT) hydrate, epicatechin (ECAT), gallic acid (GA), chlorogenic acid (CLA), copper(II) sulfate pentahydrate, Folin–Ciocalteu (FC) reagent, potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$), neocuproin (2,9-dimethyl-1,10-phenanthroline) (Nc), sodium hydroxide (NaOH), sodium chloride (NaCl), hydrochloric acid (HCl) (37%) were supplied from Sigma-Aldrich (Steinheim, Germany); procyanidin B2, resveratrol (RES), cyanidin (CYD) chloride, ABTS {2,2′-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid)} from Fluka Chemie AG (Buchs, Switzerland); copper(II) chloride dihydrate ($CuCl_2.2H_2O$), ortho-phosphoric acid (*o*-H_3PO_4), formic acid (HCOOH) from Merck KGaA (Darmstadt, Germany), ammonium acetate (NH_4Ac), sodium carbonate (Na_2CO_3), potassium persulfate ($K_2S_2O_8$), methanol (MeOH) (HPLC grade), ethanol (EtOH) (96%) from Honeywell Riedel-de Haën GmbH (Seelze, Germany). All chemicals used were of analytical reagent grade.

The instruments and equipment used were as follows: Radwag WAS 220/X (Bracka Poland) analytical balance for weighing chemicals and real samples**,** Bandelin Sonorex model ultrasonic bath for preparation of solutions and extracts (Bandelin electronic GmbH & Co. KG, Berlin, Germany), IKA HB4 Basic brand water bath (IKA-Werke Gmbh & Co. KG, Staufen, Germany), Elektro-Mag vortex (İstanbul, Turkey), Millipore brand bidistilled water device (EMD Millipore Corp., Burlington, MA, USA), Varian Cary 100 UV-visible spectrophotometer for absorbance measurements (Varian, Inc., Palo Alto, CA, USA), PerkinElmer Series 200 UV-Vis. HPLC System (detector, pump, vacuum degasser) (Shelton, USA) for chromatographic analysis.

2.2. Preparation of Solutions

Trolox, luteolin, kaempferol, rutin, caffeic acid, quercetin, rosmarinic acid, carnosic acid, carnosol, flavone, gallic acid, procyanidin B2, and chlorogenic acid stock solutions were prepared in 80% (v/v)

MeOH; apigenin in 0.2 M ethanolic sodium hydroxide. Catechin, epicatechin, resveratrol, and cyanidin chloride stock solutions were prepared in 80% (v/v) MeOH containing 2% (v/v) HCl. All antioxidant solutions were stored at −20 °C.

CUPRAC (Cupric Reducing Antioxidant Capacity) assay reagents; 1.0×10^{-2}M $CuCl_2$ solution was prepared by dissolving $CuCl_2{\cdot}2H_2O$ in water; 1.0 M ammonium acetate (NH_4Ac) buffer (pH 7.0) was prepared from NH_4Ac in water and 7. 5×10^{-3}M neocuproine (Nc) solution was prepared daily by dissolving Nc in 96% ethanol (EtOH).

ABTS (2,2′-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid))/TEAC assay reagents; ABTS radical cation ($ABTS^{+\bullet}$) chromogenic reagent (7.0 mM) was prepared by dissolving this compound in water and adding $K_2S_2O_8$ to this solution such that the final persulfate concentration in the mixture is 2.45 mM. The resulting solution was left to mature at room temperature in the dark for 12-16 h and then used for ABTS/TEAC assays. The reagent solution was diluted with EtOH at a volume ratio of 1:10 before use.

Folin-Ciocalteu assay reagents; Lowry A solution: 2% (w/v) Na_2CO_3 was prepared in 0.1 M NaOH solution, Lowry B solution: 0.5 M $CuSO_4$ was prepared in 1% (w/v) $KNaC_4H_4O_6$ solution, Lowry C: 1 mL of Lowry B solution was added to 50 mL of Lowry A solution.

2.3. Preparation of Dietary Supplement Products for Analysis

2.3.1. Samples and extraction procedures

Grape seed extract, rosemary, and ginkgo biloba samples, which are sold in capsule or tablet form, and bitter melon samples in paste form (dried and in capsules) were obtained from companies selling such products in İstanbul (Türkiye) (Fig. 1).

Figure 1: Studied samples and their contents.

Aqueous solutions of methanol at 80, 70, and 50% (v/v) concentrations were used as the possible solvent for the extraction of powdered rosemary and ginkgo biloba samples in capsule form. The paste content of the bitter melon capsule was cut into pieces with a plastic knife and then extracted with the same solvents at 65 °C. For grape seed extract, the same solvents were used to contain 1% (v/v) HCl. 0.5 or 1.0-gram amount of the powder samples were extracted in stoppered flasks placed in an ultrasonic bath first with 10 mL solvent for 30 min, then with added 10 mL solvent for 30 min, and finally with 5 more mL solvent for 30 min, in conclusion overall extraction taking 90 min. The extracts were first filtered through a filter paper, then through a GF/PET (glass fiber/polyethylene terephthalate) 1.0/0.45 μm microfilter, and analyzed.

2.3.2. Hydrolysis of extract

The hydrolysis of all glycosides to aglycones provides convenience for the quantitative determination of flavonoids in samples. For this reason, grape seed extract containing 80% (v/v) MeOH and 1% (v/v) HCl was adjusted to 50% (v/v) MeOH and 1.2 M HCl in the final volume and then heated under reflux at 80 ºC for 4 h for hydrolysis (30). Extracts from other samples prepared with 80% (v/v) MeOH were diluted to 50% (v/v) MeOH. Hydrochloric acid was added to reach a final concentration of 1.2 M HCl, and the hydrolysis process was carried out as described above. At the end of the process, the resulting hydrolysates were filtered through a 1.0/0.45 μm GF/PET microfilter. The volumes of the filtered solutions were then adjusted with 50% (v/v) MeOH.

2.4. Spectrophotometric Total Antioxidant Capacity Assays

2.4.1. CUPRAC assay

The CUPRAC method, as described by Apak et al. (31) was applied as follows: A mixture comprised of 1 mL of 1.0×10^{-2} M $CuCl_2$ solution, 1 mL of 1 M NH_4Ac buffer at pH 7.0, and 1 mL of 7.5×10^{-3} M Nc solution was prepared, x mL sample solution and (1−x) mL distilled water were added, and well mixed (total volume: 4.0 mL). This final mixture in a stoppered test tube was stood at room temperature for 30 min. At the end of this period, the absorbance at 450 nm was measured against a reagent blank. This method was applied to the extracts and hydrolysate of the studied samples. The pH of the hydrolysate solution was first brought to pH 6.0 with the addition of NaOH solution, and then analysis was performed.

The total antioxidant capacity (TAC) values of the samples analyzed using this method were calculated as mmol TR/g of dry matter.

2.4.2. ABTS/TEAC assay

The blue-green solution prepared as described above was diluted 1:10 (v/v) with EtOH. The reference solution was prepared with diluted 1 mL of $ABTS^{\bullet+}$ solution and 4 mL of EtOH (total volume: 5.0 mL). The sample solutions were prepared as follows: x mL of extract, (4-x) mL of 80% (v/v) MeOH, and 1 mL of $ABTS^{\bullet+}$ solution. The absorbances of all solutions were recorded at 734 nm against EtOH at the end of the 6th min. (32) The absorbance of the reference diminished in the presence of antioxidants, the absorbance decrease (ΔA) being proportional to antioxidant concentration.

The total antioxidant capacity (TAC) values of the samples analyzed using this method were calculated as mmol TR/g of dry matter.

2.5. Determination of Total Phenolic Content by Folin-Ciocalteu Assay

According to the Folin-Ciocalteu (FC) method measuring total phenolic content (TPC), x mL of extract, (2-x) mL of distilled water, and 2.5 mL of Lowry C solution (the preparation is explained above) were added to a test tube. After 10 min, 0.25 mL FC reagent (diluted with water at a 1:3 (v/v) ratio) was added (total volume: 4.75 mL). The tubes were kept at room temperature for 30 min, and absorbance was measured at 750 nm against a reagent blank (33).

The TPC values of the samples analyzed using this method were calculated as mmol GA/g of dry matter.

2.6. Chromatographic Analyses

Different gradient elution programs were modified for chromatographic analysis of the polyphenolic compounds found in the studied samples. The elution programs were formed using the ACE 5 C18 (25 cm × 4.6 mm, 5 µm particle size) HPLC column (ACE Ltd, Aberdeen, Scotland) and one of the suitable binary mobile phase systems (0.2% (v/v) formic acid and MeOH or 0.2% (v/v) *o*-H_3PO_4 and MeOH).

The gradient elution program (I), which is modified for the analysis of grape seed capsule components and consisted of 0.2% formic acid (A) and MeOH (B) binary solvent system was applied as follows: initially and for 5 min 93% A, 10 min from 93% to 90% A (curve 6), 5 min from 90% to 87% A (curve 6), 5 min from 87% to 82% A (curve 6), 15 min from 82% to 79% A (curve 10), 10 min from 79% to 76% A (curve 10), 6 min from 76% to 73% A (curve 10), 12 min from 73% to 0% A (curve 10). Curve numbers in parentheses are the slope (change rate of solvent) codes of the Empower Software (Waters Corporation) program. The flow rate was 1 mL/min; analytical detection wavelengths were selected as 280 and 520 nm.

The gradient elution program (II), which is modified for the analysis of rosemary capsule components and consisted of 0.2% formic acid (A) and MeOH (B) binary solvent system was applied as follows: initially 80% A, 3 min from 80% to 65% A (curve 6), 10 min from 65% to 50% A (curve 6), 15 min from 50% to 40% A (curve 6), 10 min from 40% to 20% A (curve 6), 15 min from 20% to 0% A (curve 10). The flow rate was 1 mL/min, and the analytical detection wavelength was selected as 280 nm.

The gradient elution program (III), which is modified for the analysis of bitter melon capsule components and consisted of 0.2% *o*-H_3PO_4 (A) and MeOH (B) binary solvent system was applied as follows: initially and for 3 min 100% A, 17 min from 100% to 70% A (curve 6), 10 min from 70% to 45% A (curve 6), 10 min from 45% to 0% A (curve 6). The flow rate was 0.7 mL/min, and the analytical detection wavelength was selected as 320 nm.

The gradient elution program (IV), which is modified for the analysis of ginkgo biloba capsule components and consisted of 0.2% *o*-H_3PO_4 (A) and MeOH (B) binary solvent system was applied as follows: initially and for 1 min 20% A, 3 min from 20% to 35% A (curve 6), 5 min from 35% to 45% A (curve 6), 12 min from 45% to 50% A (curve 6), 3 min from 50% to 55% A (curve 6), 2 min from 55% to 60% A (curve 6), 2 min from 60% to 80% A (curve 6), 13 min from 80% to 100% A (curve 6). The flow rate was 1.0 mL/min; analytical detection wavelengths were selected as 280 and 320 nm.

In the HPLC analysis of studied sample extracts, retention times were compared with those of standards. Analysis results were evaluated according to the calibration graphs, which were drawn between peak areas and concentrations of standard compounds.

2.7. Combined HPLC-CUPRAC and HPLC-ABTS Methods

The contribution of the sample components determined by chromatographic analysis to the measured spectrophotometric total antioxidant capacity was calculated using the equation (1) (34-36). In this equation, the component concentrations were multiplied by the TEAC (Trolox equivalent antioxidant capacity) coefficients determined by the spectrophotometric methods, and by summing these values, the theoretical TACs of the samples were calculated. As a result, HPLC–CUPRAC refers to the capacity calculated by multiplying the concentrations determined in HPLC by the TEAC coefficients of the CUPRAC method.

$$Theoretical\ TAC = \sum_{i=1}^{n} C_i\,(TEAC)_i \qquad \text{(Eq. 1)}$$

C_i: concentration of ith component determined by HPLC; *(TEAC)i*: TEAC coefficient of ith component calculated by the selected TAC measurement method (i.e., CUPRAC and ABTS).

2.8. Statistical Analysis

Spectrophotometric assays were applied in three repetitions for each sample and standard. Descriptive statistical analyses were performed using Excel

software (Microsoft Office 2016) to calculate the mean and the standard error of the mean.

3. RESULTS AND DISCUSSION

3.1. Sample Preparation and Selection of Suitable Solvent for Extraction

The spectra of the extracts, which were prepared as specified in section 2.3.1 and diluted at appropriate rates, were taken in the range of 200-600 nm (the spectra were not given), and the solvent ratios that provided the highest extraction efficiency were determined. The most suitable solvents were determined according to spectra: 80% MeOH containing 1% (v/v) HCl for grape seeds sample and 80% (v/v) MeOH for rosemary, bitter melon, and ginkgo biloba samples.

3.2. The Results of Spectrophotometric Methods

Since the results obtained by applying the spectrophotometric methods (CUPRAC, ABTS, and Folin-Ciocalteu) to the samples will be given as TR and GA equivalents, firstly, calibration graphs were created with the related standard compounds. Thus, molar absorption coefficients were calculated for each compound (Table 1).

Table 1: Molar absorption coefficients of reference standard compounds used in spectrophotometric methods.

Spectrophotometric method	Reference standard compound	Molar absorption coefficient (mol/L.cm)
CUPRAC	TR	1.67×10^4
ABTS/TEAC	TR	2.60×10^4
Folin-Ciocalteu	GA	6.10×10^3

According to the literature data, the compounds with antioxidant properties expected to be found in the highest amount in grape seeds are CAT, ECAT, GA, flavone, procyanidin B2, RES, and CYD (37). The main antioxidant compounds that are likely to be found in our other studied samples are; CA, RA, CAR, and CRA in rosemary (38); GA, CAT, CA, and CLA in bitter melon (39,40); RUT, LUT, KAM, APG, and QUE in ginkgo biloba (41,42). Using the standards of these substances, calibration graphs (drawn as absorbance vs molar concentration) were created with spectrophotometric total antioxidant capacity measurement methods (CUPRAC and ABTS), and the molar absorption coefficients of each compound were calculated. As a result, the molar absorption coefficients of tested compounds were divided into the molar absorption coefficient of standard reference TR, and the TEAC coefficient of each compound was calculated (Table 2). The TEAC coefficient expresses the mM concentration of the TR solution, which is equivalent to the activity of a 1 mM solution of the antioxidant compound whose reducing power is to be measured (43).

Table 2: TEAC coefficients of antioxidants tested with CUPRAC and ABTS methods.

Antioxidant	$TEAC_{CUPRAC}$	$TEAC_{ABTS}$
CAT	3.13	3.14
ECAT	2.77	2.65
GA	2.97	3.84
Flavone	0.34	0.05
Procyanidin B2	7.72	5.45
RES	1.30	3.63
CYD	1.04	1.18
CA	3.00	1.39
RA	5.40	5.65
CAR	1.47	2.31
CRA	2.16	1.09
QUE	4.49	4.23
RUT	2.99	3.15
CLA	3.05	1.35
APG	0.25	0.65
LUT	2.83	1.58
KAM	2.00	1.12

3.3. Total Phenolic Contents and Total Antioxidant Capacities of Samples

The total antioxidant capacities of the sample extracts and their hydrolysates were determined spectrophotometrically using the CUPRAC and ABTS/TEAC methods. The total phenolic content of the same samples was determined using the Folin-Ciocalteu method. All results are listed in Table 3.

Although there are not many studies in the literature measuring the antioxidant activities of plant-based dietary supplements, when examining a few studies for this purpose, it has been found that DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Capacity), ORAC (Oxygen Radical Absorbance Capacity) and HORAC (Hydroxyl Radical Antioxidant Capacity) methods are used (19-21,40,44). Among these methods, ORAC and HORAC have a hydrogen atom transfer (HAT) mechanism, while FRAP and DPPH have a single electron transfer (SET) mechanism. The CUPRAC and ABTS methods used in our study are based on the SET mechanism. The main advantage of the CUPRAC method over other similar assays is that the reagent is more stable and easier to prepare than other chromogenic reagents (e.g., ABTS, DPPH). The TAC values of the antioxidants determined with CUPRAC are perfectly additive, i.e., the TAC of a mixture is equal to the sum of the TAC values of its components. The CUPRAC reagent is selective because it has a lower redox potential; accordingly, non-antioxidant reducing compounds, such as simple sugars and citric acid, are not oxidized with the CUPRAC reagent. The standard potential of the Cu(II, I)-Nc redox couple is about 0.6 V and thus close to that of $ABTS^{\bullet+}$/ABTS, i.e., 0.68 V (43). For this reason, ABTS was preferred as a comparison method. As can be seen in Table 3, the TAC values determined with both methods were close to each other. It can be seen that the highest TAC value among the analyzed products belongs to the acidic and non-acidic grape seed extracts. Özcan et al. (44) reported that the content of phenolic compounds in the skin and pulp of certain grape varieties grown in Turkey was lower than in the seeds. In this study, 15 mL of a methanol-water-formic acid mixture (5:4.85:1.5, v/v) was added to approximately 2 g of a grape seed sample. The mixture was sonicated for 2 minutes. The sample was then centrifuged at 4500 rpm for 15 minutes. After centrifugation, 10 mL of n-hexane was added to the separated supernatant and mixed with a vortex mixer. The extract was then concentrated using a rotary evaporator at 50 °C. The resulting residue was dissolved in methanol, and analytical methods were applied. The TACs of the samples were evaluated using the DPPH assay, the TPCs were determined using the Folin-Ciocalteu assay, and the chromatographic analyses were performed using an HPLC system with a PDA detector. The determined TAC and TPC values of the grape seeds were between 86.688-90.974% and 421.563-490.625 mg GAE/100 g, respectively. The phenolic compounds of the grape seeds were determined to be significantly higher compared to the other parts of the grapes. The major phenolic compounds quantified were 1,2-dihydroxybenzene, rutin, apigenin-7-glucoside, caffeic acid, (+)-catechin, gallic acid, quercetin, and resveratrol. The authors compared their results with those of similar studies and attributed the observed differences in the results to diversity, cultural factors, or analytical conditions. In a study by Krasteva et al. (45), the TPC, the compositions, and the antioxidant and antibacterial activities of four grape seed extracts (Cabernet Sauvignon, Marselan, Pinot Noir, and Tamyanka) were investigated. The antioxidant capacity of these extracts was analyzed using DPPH and ABTS assays. An HPLC system with a PDA detector was used for the chromatographic analysis of the extracts. The total phenol content was determined using the Folin-Ciocalteu assay. In this study, the samples were prepared as follows: 5 g of grape seed powder was mixed with 25 mL of 70% aqueous ethanol using a magnetic stirrer at a constant speed of 500 rpm at room temperature and pressure for 3 hours. This procedure was repeated twice, and the collected supernatants were centrifuged and concentrated to 1 mL in an evaporator. The total phenolic content of the extracts was determined in the range of 79.06–111.22 mg GAE/g DW. The total antioxidant capacity values of the same samples were determined in the range of 245.60 ± 3.23 - 597.23 ± 4.12 (μM TE/g DW) using the DPPH assay and in the range of 1907.24 ± 9.56 - 2273.92 ± 12.32 (μM TE/g DW) using the ABTS method. The differences between the results of the two assays were attributed to the different mechanisms used in the determination of antioxidant capacity. The components identified in the HPLC analysis of the same samples were gallic acid, gallic acid glucoside, (+)-catechin and (-)-epicatechin, procyanidins B1, B2, and B3, and procyanidin C1. The results of these two studies, which include analyses of grape seeds, are in close agreement with our findings on the phenolic compounds identified. The TEAC coefficient values of these components in Table 2 explain the high antioxidant capacities of the grape seed extracts. In addition, our results show that the grape seed extracts had the highest total phenolic component contents (Table 3). On the other hand, the values obtained with the ABTS method are relatively low compared to the results obtained with the CUPRAC method. This finding can be explained by the different responses of the components to these methods. Indeed, this is confirmed by the TEAC coefficients obtained (Table 2). The order of the other commercial products we examined according to their antioxidant capacity values from highest to lowest is rosemary > ginkgo biloba > bitter melon. This ranking also applies to the total phenolic content. The FC method is used to determine the total phenolic content of a sample, but it is not specific to phenolic compounds or antioxidants in general. This method suffers from several interfering substances (sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, Fe(II), etc.) (46). For this reason, the values obtained with this method are generally higher than the TAC values.

The antioxidant activity of rosemary is mainly attributed to its phenolic compounds, particularly phenolic diterpenes such as carnosol, carnosic acid, rosmanol, epirosmanol, and isorosmanol. Additionally, rosmarinic acid, a caffeic acid ester, is also recognized as an important component of

rosemary due to its superior antioxidant properties (24,38). Olah et al. (47) conducted a study on the polyphenol content and antioxidant capacity of three different rosemary extracts prepared from both fresh and dried plants. The phenolic compounds in these samples were identified using thin-layer chromatography (TLC) and an HPLC system with a diode array detector (DAD). The polyphenols were determined using sodium molybdate reagent at 505 nm, and the total antioxidant activity and capacity values were evaluated by DPPH, ABTS, FRAP, and CUPRAC assays. The analyzed extracts were prepared by cold extraction with 100 mL 70% EtOH (1:5 - dry plant: solvent) from dry and fresh plants. The TLC and HPLC analyses revealed that rosmarinic acid was the component with the highest concentration. The study showed that the total polyphenol content and rosmarinic acid content in the alcoholic extract of fresh plants were two to three times higher than in extracts from dried plants. From these results, the authors concluded that the drying process can lead to changes or degradation of the polyphenolic compounds. In this study, the high polyphenol content determined for the hydroalcoholic extract (0.601 mg/mL rosmarinic acid) was confirmed by the highest values determined for antioxidant activity. Namely, the IC_{50} values of the DPPH and ABTS methods were determined as 39.1 µL and 7.7 µL, respectively, while the values determined as TE µM/100 mL for the FRAP and CUPRAC methods were 698 and 1947, respectively.

The highest antioxidant capacity of rosemary capsules was also measured in our study using the CUPRAC method.

The main phenolic components found in bitter melon extracts are gallic acid, epicatechin, chlorogenic acid, catechin, and gentisic acid (40,48,49). Anilakumar et al. (50) reported that the products of bitter melon fruit and seed extracts in capsule or tablet form are widely used in many countries. Still, the Food and Drug Administration has not evaluated these products, and they need further validation. Various products made from bitter melon (in the form of powder, paste, capsules, or tablets) are sold on the market and are widely used in our country. In our previously published study, the total phenolic content and antioxidant capacity of some commercial bitter melon products (powder, packaged powder, capsule, paste in olive oil) and of unripe and ripe fruits were determined spectrophotometrically (Folin-Ciocalteu, CUPRAC, and ABTS) and chromatographically (51). In this study, the bitter melon samples were extracted with 80% MeOH. In addition, most research studies on bitter melon have found EtOH, MeOH, or water to be suitable extraction agents (52-54). In this study, the ranking of TAC (total antioxidant capacity) of the samples analyzed by CUPRAC and ABTS method was determined as follows: capsule (CUPRAC value, 140.8; ABTS/HRP value, 143.6 µmol TRE/g) > packaged powder (129.6; 126.1) > powder (52.3; 64.3) > unripe fruit (42.5; 36.3) > paste in olive oil (17.6; 14.4) > ripe fruit (8.7; 7.0). On the other hand, the order of phenolic content of the same samples was determined as follows: unripe fruit (193.2 µmol GAE (gallic acid equivalent)/g) > capsule (162.0) > packaged powder (160.6) > powder (83.6) > paste in olive oil (38.3) > ripe fruit (14.6) (51). The TAC and TPC values of the bitter melon extract examined in the present study (see Table 3) are also within the range of the values mentioned above.

Table 3: The total antioxidant capacity (TAC) and total phenolic content (TPC) values of the examined samples were determined by spectrophotometric methods.

Sample	CUPRAC (mmol TR/g)[a]	ABTS/TEAC (mmol TR/g)[a]	TPC (mmol GA/g)[a]
Grape seed extract	0.910±0.070	0.720±0.170	1.710±0.470
Grape seed extract hydrolysate	0.420±0.030	0.300±0.030	0.740±0.150
Acidic grape seed extract	1.240±0.050	0.710±0.210	2.040±0.430
Acidic grape seed extract hydrolysate	0.570±0.030	0.330±0.020	0.760±0.220
Rosemary extract	0.790±0.050	0.410±0.090	0.550±0.130
Rosemary extract hydrolysate	0.180±0.030	0.120±0.070	0.160±0.040
Bitter melon extract	0.080±0.006	0.110±0.050	0.240±0.030
Bitter melon extract hydrolysate	0.070±0.010	0.070±0.010	0.150±0.040
Ginkgo biloba extract	0.120±0.008	0.140±0.020	0.400±0.160
Ginkgo biloba extract hydrolysate	0.050±0.002	0.070±0.006	0.160±0.040

The results are presented as mean (n=3) ± standard deviation.

The results of the CUPRAC and ABTS assays results showed that the antioxidant capacity values of the Ginkgo biloba extract were close to each other. In a study, the total phenolic content and antioxidant capacity (FRAP method) of Ginkgo biloba L. leaves and various commercial Ginkgo teas were determined and compared. For this purpose, different water extracts (infusions and decoctions) were prepared by varying the steeping, boiling, and infusion times, while an aqueous ethanolic extract (water/ethanol 80/20, v/v) was also prepared. It was found that the total phenolic content and FRAP values of the collected ginkgo leaves were similar to those of commercial ginkgo teas. Contrary to the preparation methods recommended by the manufacturers, decoction was found to be more effective than infusion in extracting the antioxidant compounds. It was also reported that aqueous ethanolic extracts had significantly higher total phenolic content and antioxidant capacity than water extracts. The phenolic concentrations of the water extracts ranged from 21.11 to 34.22 mg/g GAE for leaf samples and from 15.84 to 27.31 mg/g GAE for commercial teas. The phenolic content of the

aqueous ethanol extracts for the collected ginkgo leaf samples was 75.74 mg/g GAE, while these values for the commercial ginkgo teas ranged from 85.51 to 98.28 mg/g GAE. It was found that the antioxidant capacity of the aqueous ethanol extracts of the ginkgo samples was about three times higher than that of the water extracts. FRAP values of the water extracts ranged from 16.68 to 31.91 mmol/g AAE for the collected leaf sample and from 14.10 to 31.34 mmol/g AAE for the commercial teas. The antioxidant capacity of the aqueous ethanolic extracts was 69.12 mmol/g AAE for the collected leaf sample, and its values ranged from 62.76 to 69.99 mmol/g AAE for commercial tea samples (55). Aybastier conducted a study on Ginkgo biloba samples available for sale in Türkiye, examining three different forms: medicine, food supplement, and leaf (56). The study involved the ultrasonic extraction of all three sample types in both acidic and non-acidic environments, and the antioxidant properties of the extracts were evaluated. The antioxidant capacities were measured using the ABTS method, while the total phenolic content was assessed using the Folin-Ciocalteu method. Additionally, HPLC was used to identify the antioxidant compounds present in the samples. In this study, the drug, Ginkgo biloba leaf, and food supplement products were each extracted using a 60% methanol solution. For the acidic extraction, a 60% methanol solution containing 2 M HCl was utilized. The extraction process was conducted in an ultrasonic bath at 50°C for 100 minutes. It has been stated that the extraction was also carried out in an acidic environment to facilitate the hydrolysis of antioxidant compounds in the glycoside structure and convert them into aglycones. It is known that a compound with a glycoside structure and its aglycone, which is formed through acidic hydrolysis, displays different antioxidant properties (57). The results indicated that the drug form exhibited superior antioxidant properties compared to both the food supplement and leaf forms. According to the Folin-Ciocalteu method, the total phenolic content of the drug sample was measured at 68.16 ± 1.83 mg GAE/g for the non-acidic extract and 80.27 ± 6.55 mg GAE/g for the acidic extract. Additionally, the antioxidant capacity value for the same sample, determined using the ABTS method, was found to be 59.75 ± 3.57 mg TE/g for the non-acidic extract and 56.48 ± 8.66 mg TE/g for the acidic extract. As a result of HPLC analyses, rutin (quercetin-3-rutinoside) was determined as the main phenolic substance in all extracts, while the others were protocatechuic acid, p-hydroxybenzoic acid, kaempferol-3-glucoside, kaempferol, and quercetin. The differences in antioxidant content among the Ginkgo biloba products studied can be attributed to several factors. First, the plants were sourced from different locations, which may have influenced their composition. Second, the methods used to prepare these products varied. Namely, the leaves were collected from the tree and dried; the dietary supplement was created by encapsulating the processed product; and the drug formulation underwent a standardized preparation method after verifying its composition. As a result, it was concluded that the most reliable form of Ginkgo biloba is the tablet sold as a pharmaceutical drug.

3.4. Chromatographic Analysis

In the chromatographic analysis of the studied samples, the phenolic compositions were determined by comparing the retention times of the standards and/or by adding standard solutions to the diluted sample extracts in an appropriate ratio.

Compounds detected in the methanol extract of the grape seed sample at 280 nm include epicatechin, resveratrol, and flavone, while the hydrolysate contains catechin, procyanidin B2, and resveratrol. In the acidic methanol extract and hydrolysate of the same sample, catechin, gallic acid, and resveratrol were detected at 280 nm, along with cyanidin chloride at 520 nm. The extract prepared from the rosemary capsule contained caffeic acid, rosmarinic acid, carnosol, and carnosic acid, as determined by HPLC at 280 nm. The phenolic compounds identified in the other studied samples are as follows: in the bitter melon extract (at 320 nm), chlorogenic acid, rutin, and quercetin; in the ginkgo biloba extract (at 340 nm), rutin, luteolin, kaempferol, apigenin, and quercetin.

Calibration graphs were created using the standards of the relevant phenolic substances, and the findings are reported in Table 4. In the calibration equations provided in Table 4, y refers to the peak area, c to the concentration, and r to the correlation coefficient. The individual antioxidant concentrations of the studied samples were determined using calibration curves in HPLC. The recovery percentage values of the HPLC methods were assessed by adding standard antioxidant compounds to the sample extracts and hydrolysates. These values ranged from 94.6% to 99.0% in grape seed extracts and hydrolysates, 97.0% to 99.7% in rosemary extract, 95.2% to 98.9% in bitter melon extract, and 92.0% to 99.8% in ginkgo biloba extracts.

3.5. Theoretical TAC Values Determined by HPLC-CUPRAC and HPLC-ABTS Methods

Concentration calculations for each component were conducted through chromatographic analysis, applying the calibration equations found in Table 4. The theoretical total antioxidant capacity (TAC) values for each sample were calculated using equation (1) from section 2.7. By leveraging the additivity property of TAC in complex samples, we determined the theoretical TAC by multiplying the concentration of each identified antioxidant by its corresponding Trolox Equivalent Antioxidant Capacity (TEAC) coefficient value, then summing all obtained values. The ratio of the theoretically calculated TAC values to those measured via spectrophotometric methods allowed us to assess the contribution of each component identified through chromatographic analysis to the overall antioxidant capacity. These contributions were expressed as percentages in parentheses in Table 5 and were found to range from 33% to 57%. These results suggest that when the analytical standards for antioxidant compounds in similar plant-based products are available, it is feasible to accurately determine the contribution of each compound to the total antioxidant capacity through chromatographic analysis.

Table 4: Calibration equations, correlation coefficients, and linear ranges of some phenolic compounds determined from HPLC chromatograms.

Phenolic compound	Retention time (min)	Detection wavelength (nm)	Calibration equation	r	Linearity range (mol/L)
GA[a]	6.0	280	$y = 6.8\times10^{9}\ c - 3.0\times10^{4}$	0.9684	1.0×10^{-5}–2.0×10^{-4}
CAT[a]	27.5	280	$y = 4.0\times10^{8}\ c - 2.7\times10^{3}$	0.9945	1.0×10^{-5}–2.0×10^{-4}
ECAT[a]	39.1	280	$y = 9.0\times10^{7}\ c - 5.6\times10^{2}$	0.9987	1.0×10^{-5}–2.0×10^{-4}
RES[a]	60.8	280	$y = 8.2\times10^{9}\ c - 5.0\times10^{3}$	0.9765	1.0×10^{-5}–2.0×10^{-4}
CYD[a]	61.5	520	$y = 8.0\times10^{9}\ c - 4.0\times10^{4}$	0.9413	1.6×10^{-5}–1.6×10^{-4}
Flavone[a]	64.1	280	$y = 7.4\times10^{9}\ c - 1.6\times10^{4}$	0.9984	1.6×10^{-5}–1.6×10^{-4}
Procyanidin B2[a]	32.0	280	$y = 5.0\times10^{8}\ c - 4.0\times10^{2}$	0.9898	1.6×10^{-5}–1.6×10^{-4}
CA[b]	9.0	280	$y = 9.0\times10^{8}\ c - 5.1\times10^{4}$	0.9728	1.0×10^{-5}–2.0×10^{-4}
RA[b]	14.2	280	$y = 9.2\times10^{8}\ c - 4.0\times10^{3}$	0.9992	1.0×10^{-5}–2.0×10^{-4}
CAR[b]	39.8	280	$y = 3.2\times10^{8}\ c - 2.7\times10^{3}$	0.9928	1.0×10^{-5}–2.0×10^{-4}
CRA[b]	46.3	280	$y = 1.4\times10^{8}\ c - 2.2\times10^{3}$	0.9824	1.0×10^{-5}–2.0×10^{-4}
CLA[c]	25.9	320	$y = 6.0\times10^{9}\ c - 3.9\times10^{4}$	0.9986	1.0×10^{-5}–2.0×10^{-4}
RUT[c]	36.8	320	$y = 7.0\times10^{9}\ c - 3.2\times10^{4}$	0.9958	1.0×10^{-5}–2.0×10^{-4}
QUE[c]	44.7	320	$y = 5.2\times10^{9}\ c - 1.1\times10^{4}$	0.999	1.0×10^{-5}–2.0×10^{-4}
LUT[d]	20.8	340	$y = 6.0\times10^{9}\ c - 1.3\times10^{4}$	0.9844	1.0×10^{-5}–2.0×10^{-4}
KAM[d]	23.8	340	$y = 7.2\times10^{8}\ c - 2.1\times10^{4}$	0.9830	1.0×10^{-5}–2.0×10^{-4}
APG[d]	25.5	340	$y = 6.4\times10^{9}\ c - 2.1\times10^{4}$	0.9749	1.0×10^{-5}–2.0×10^{-4}

a, b, c, and d indicate that the chromatograms of these compounds were taken by gradient elution programs called I, II, III, and IV, respectively.

Table 5: Theoretical total antioxidant capacity values (mmol TR/g) were determined by HPLC-CUPRAC and HPLC-ABTS methods.

Sample	HPLC-CUPRAC (mmol TR/g)	HPLC-ABTS (mmol TR/g)
Grape seed extract	0.42 (46%)	0.37 (51%)
Grape seed extract hydrolysate	0.18 (43%)	0.11 (37%)
Acidic grape seed extract	0.71 (57%)	0.34 (48%)
Acidic grape seed extract hydrolysate	0.32 (56%)	0.16 (48%)
Rosemary extract	0.31 (39%)	0.19 (46%)
Rosemary extract hydrolysate	0.04 (22%)	0.05 (42%)
Bitter melon extract	0.03 (38%)	0.04 (36%)
Bitter melon extract hydrolysate	0.03 (43%)	0.04 (57%)
Ginkgo biloba extract	0.04 (33%)	0.05 (36%)
Ginkgo biloba extract hydrolysate	0.02(40%)	0.03 (43%)

4. CONCLUSION

In our study, we examined dietary supplements that include grape seed extract, rosemary, bitter melon, and ginkgo biloba, all sold in capsule or tablet form. For the first time, we employed the CUPRAC and HPLC-CUPRAC methods to assess the antioxidant capacity of these products, comparing our findings with those obtained from the ABTS/TEAC method. The analytical techniques used to evaluate the specificity and antioxidant capacity of the selected dietary supplements can be recommended as standard methods.

To determine the effective dose based on human requirements, we can measure the types and amounts of bioactive substances using the methods developed or modified for the chromatographic analysis of the samples in our study. Furthermore, the chromatographic data obtained will allow us to identify plant-specific antioxidant compounds in raw plants and ascertain whether there is imitation or adulteration in products falsely claimed to contain these plants.

5. CONFLICT OF INTEREST

The authors declare there is no potential conflict of interest concerning the research, authorship, and/or publication of this article.

6. ACKNOWLEDGMENTS

This study was funded by the Scientific Research Projects Coordination Unit of Istanbul University-Cerrahpaşa. Project number: 18516.

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