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Estimation of some total secondary metabolites and profile of polyphenols of grape seeds by HPLC techniques

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ABSTRACT

Chemical analysis of Vitis vinifera grape seed phytochemicals was carried out in this study. Seeds were acquired from six different cultivars obtained from the local market of Najaf AL Ashraf, Iraq, shade-dried, ground, and extracted with 99.8% methanol by shaking maceration for 24 h during the harvest season in 2023. HPLC analyses of methanolic extracts indicated the presence of several phenolic and flavonoid compounds with different retention times and concentrations. These compounds were catechin, epicatechin, gallic acid, quercetin, and rutin. Calibration was external for each standard ($R^2 > 0.999$) validated through precision intra-day and inter-day recovery studies and limits of detection (LOD) and quantification (LOQ) determined by signal-to-noise ratios. In terms of concentration levels, all detected compounds were highest in V. vinifera cv. Black magic with catechin having the highest amount. The total phenolic content was determined by the Folin-Ciocalteu method. Total flavonoid content was determined by the aluminum chloride colorimetric assay. Total anthocyanin content was determined by the pH differential method. total alkaloid content by BCG, tannins by precipitation with gelatin, saponins by gravimetric method, terpenoids by colorimetric method using linalool standard, and steroids by Zak's method. The results indicated that there was a highly significant difference in all chemical contents for the six cultivars studied ($p< 0.05$, ANOVA).

1-Introduction

Grapes (Vitis vinifera L.) are among the most widely consumed fruits worldwide,appreciated not only for its taste but also for its health benefits [1]. It comprises a vast array of bioactive compounds such as proanthocyanidins, anthocyanins, flavanols, phenolic acids, and stilbenes. The levels of these compounds vary greatly among the different grape tissues, that is, skin, pulp, and seeds [1]. Grape seeds, in particular, are rich in flavonoids, including monomers, dimers, trimers, oligomers, and polymers [2]. Most studies have reported that grape seed extracts main bioactive constituents are gallic acid, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, dimeric and trimeric procyanidins, and trans-resveratrol. The composition profile of these compounds is yet to be known specifically that gives the GSEs their antioxidant and antibacterial activities [3-5]. For instance, the essential oil of Cinnamomum zeylanicum bark has very good antioxidant and antimicrobial activities related to its phenolic content, which further demonstrates the general bioactivity of phenolic compounds in plants. Similarly, the extracts of Echinops setifer have been found to possess significant in vitro antioxidant, antimicrobial, and cytotoxic activities based on their phytochemical profiles This study presented the use of pulegone essential oil as an edible coating on basil seed mucilage to improve the quality and extend the shelf life of refrigerated veal, in actualizing the application of such bioactive compounds in food preservation.These phenolic compounds play pivotal roles in enhancing the functional and nutritional qualities of human diets. Also, both the edible parts of the fruit and the remaining by-products have significant amounts of phenolic compounds and other bioactive phytochemicals [6,7].

Apart from phenolic compounds, constituents of nutritional value in grape extracts include ash (2.47%), lipids (1.75%), protein (21%), carbohydrates (60.72%), and fiber (2.20%) [8]. They also contain vitamins (B2, C, K, E) and essential minerals, such as calcium, copper, iron, magnesium, manganese, phosphorus, potassium, silicon, and sulfur [9,10].

Owing to this rich biochemical profile, the present study was designed to: (1) characterize the chemical composition of Vitis vinifera L. grape seeds through methanolic extraction, (2) carry out a qualitative and quantitative analysis of phenolic and flavonoid compounds by high-performance liquid chromatography (HPLC), and (3) determine the total content of various active secondary metabolites, including total phenolics, flavonoids, anthocyanins, alkaloids, tannins, saponins, terpenoids, and steroids in six different cultivars. It was hypothesized that HPLC profiling would show catechin to be the most abundant polyphenol in grape seeds, and that total phenolic content and individual polyphenol concentrations would vary significantly among cultivars.

2- Material and Methods

2.1. Plant Material and Sample Preparation

The seeds of six Vitis vinifera cultivars (Family: Vitaceae) were purchased from the local market of Najaf AL Ashraf, Iraq, during the 2023 harvest season. The supplier identified the specific cultivars as V. vinifera cv. Black rose, V. vinifera cv. Black magic, V. vinifera cv. Red roomy, V. vinifera cv. Kamali, V. vinifera cv. French, and V. vinifera cv. Dis-al-Anz. The geographic origin of all grapes was confirmed as commercial vineyards in the Najaf province, Iraq. On receipt, the seeds were separated from the grapes manually, washed with plenty of fresh distilled water to remove residues of pulp, and shade-dried in a dark well-ventilated room at ambient temperature of about 25-30°C for nine The number of days required to attain constant weight was noted. The dried seeds were powdered using an electric laboratory mill. The powder obtained was sieved through a 40-mesh to ensure that the particle size was uniform. The ground seed was stored in airtight containers that were also protected from light at a temperature of 4°C until one week of extraction.

2.2. Preparation of Grape Seed Extract

One gram of dried sieved seed powder was weighed and mixed with 10 mL of 99.8% methanol (solid-liquid ratio 1:10, w/v) in a 25 mL amber beaker. A beaker containing the sample was then placed in a shaking incubator at 120 rpm and 25°C for 24 h. The extracts were filtered through Whatman No. 1 filter paper and a 0.45 µm nylon syringe filter. Two more

extractions were performed on the same marc with fresh solvent (2 × 10 mL, 24 h each) and the combined filtrates were evaporated to dryness under reduced pressure at 40°C with a rotary evaporator. The extract was then dried in vacuo at 40°C using a rotary evaporator. The dried extract was reconstituted in 10 mL of methanol to obtain a final concentration of 100 mg/mL (based on the initial weight of the seed) and then stored at -20 degrees Celsius until the time of analysis. The extraction yield was calculated gravimetrically as a percentage. The general approach was adapted from Dowek [11], with the modifications stated above.

2.3. Estimation of Total Secondary Metabolite Compounds

2.3.1. Assessment of Total Phenolic Content

The total phenolic content was determined using the Folin-Ciocalteu reagent method as described by Laouini and Ouahrani. One hundred microliters of the reconstituted extract were mixed with 500 µL of Folin-Ciocalteu reagent and 1.5 mL of a 20% sodium carbonate solution. The contents were mixed and then made up to 10 mL with distilled water. The absorption was read after incubating for 2 hours at ambient temperature in the dark; a blank was included in the measurement. All readings were taken in triplicate. The concentration was obtained from a standard curve of gallic acid (10-500 mg/L) expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight (DW).

2.3.2. Total Alkaloid Content

The total alkaloid content was estimated by the method reported by Ajanal et al. [13] using bromocresol green (BCG) with atropine as the standard. 1 mL of the extract is dissolved in NH_4Cl solution, then filtered, and its pH brought to neutrality with 0.1 N NaOH; chloroform is used for the extraction of alkaloids. Measure the absorbance at 470 nm. Read the colored complex resultant of BCG with phosphate buffer at a wavelength of 470 nm. Express the findings as milligrams of atropine equivalents per gram of dry weight.

2.3.3. Total Flavonoid Content

The TFC was assayed using the colorimetric method of aluminum chloride as outlined by Habibatni et al. [14]. An aliquot of 50 µL of the extract was first diluted with methanol then added 5% $NaNO_2$, 10% $AlCl_3$, and 1 mol/L NaOH in sequence. The absorbance was read at 510 nm after incubation. The TFC was expressed in milligrams of rutin equivalents per 100 g of dry weight; the calculation was based on a standard curve of rutin with a linearity range from 10 to 200 mg/L.

2.3.4. Total Saponin Content

Total saponin content was determined gravimetrically according to the method of Ezeabara et al. [15]. Five grams of the seed powder (pre-extraction) was extracted with 20% aqueous ethanol at 55°C; the saponins were then evaporated and weighed after being partitioned into n-butanol. The percentage of crude saponins was calculated based on the initial sample weight (%).

2.3.5. Determination of Total Tannins Content

The total content of tannins was determined according to the method of Abdelkader et al. [16] which makes use of precipitation with a 1% gelatin solution. The absorbance of the supernatant obtained was read at 540 nm. Tannin content was expressed as milligrams of tannic acid equivalents per 100 grams of dry weight, based on a standard curve.

2.3.6. Determination of Total Anthocyanin Content

The content of anthocyanin was measured as described by Sutharut and Sudarat [17] by the method of pH differential. Readings of absorbance were recorded at 510 nm and 700 nm with the use of buffer potassium chloride at pH 1.0 and buffer sodium acetate at pH 4.5. The results were expressed in mg/kg of dry weight as cyanidin-3-glucoside equivalents, calculated assuming molar absorptivity of 26,900 L/mol•cm and molecular weight of 449.2 g/mol.

2.3.7. Determination of Total Steroids

The total steroid content was determined by the method of Zak (18) by measuring the pink-

colored complex formed at 540 nm with ferric chloride and concentrated sulfuric acid. The results were expressed as milligrams of cholesterol equivalents per gram of dry weight. Cholesterol was used as a reference standard.

2.3.8. Determination of Total Terpenoids

Total terpenoid content was determined by the method of Ghorai et al. [19] using linalool as a standard. The extract was prepared in methanol and acetonitrile (1:1), then it was reacted with chloroform and concentrated sulfuric acid. Absorbance was measured at 538 nm. Results were expressed as mg linalool equivalents per gram dry weight.

2.4. HPLC-DAD Analysis of Individual Polyphenols

The HPLC system for individual phenolic compound quantitative analysis was an Agilent 1200 HPLC system. It consisted of a quaternary pump, autosampler, column oven, and diode array detector (DAD). A reversed-phase chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus-C18 column (2.1×50 mm, 1.8 μm particle size) maintained at 30°C. The mobile phase was HPLC-grade methanol (A) and ultrapure water with 1% (v/v) formic acid (B). Elution was carried out at a constant flow rate of 0.8 mL/min with a gradient program as follows: 0-2 min, 100% B; 2-4 min, 100%-98% B; 4-6 min, 98%-95% B; 6-7 min, 95%-73% B; 7-10 min, 75%-48% B; 10-12 min, 12 minutes at 48% B; 12-20 min, 12 minutes from 48%-40% B; and finally, 20-22 min, 40% B followed by 5 minutes post-run equilibration at initial conditions. The injection volume was 5 μL, and the total run time was 22 minutes.

Flavanols (catechin, epicatechin) and gallic acid were detected at 280 nm, Phenolic acids at 320 nm, and flavonols, quercetin, and rutin at 360 nm. The retention times and UV-Vis absorption spectra of standards solutions prepared under the same conditions were used to identify the compounds. Calibration curves obtained from standard solutions of appropriate dilutions of the respective compounds were used for quantification.

2.5. Analytical Standards, Calibration, and Method Validation

Reference standards of high purity (≥95%) were purchased as authentic materials: gallic acid, (+)-catechin, (−)-epicatechin, quercetin, rutin, p-coumaric acid, caffeic acid, kaempferol, and trans-resveratrol (Sigma-Aldrich, USA). Prepare individual stock solutions (1 mg/mL) in HPLC-grade methanol and store them at -20°C. Make serial dilutions to prepare working mixed standard solutions for building five-point calibration curves. The concentration ranges were linear for all analytes with correlation coefficients of (R^2) greater than 0.999.

The signal-to-noise (S/N) ratios used to set the limits of detection (LOD) and quantitation (LOQ) were 3:1 and 10:1, respectively. Intra-day and inter-day analyses were applied to check method precision; they used six replicate injections on the first day and one triplicate injection on each of three consecutive days, respectively. All results were reported as % RSD. Recovery was determined from three levels of standards spiked into a known sample matrix; an average recovery percentage was then calculated.

System suitability was assured by the injection of a mixed standard solution before each batch to confirm the stability of retention times and column efficiency.

A blank (methanol) injection was performed after every 10 sample injections to monitor carryover.

2.6. Experimental Design and Statistical Analysis

Three independent biological replicates (different seed extractions from the same pooled batch per cultivar) were carried out. The analysis of each extract was done in duplicate (technical replicates). Results were expressed as the mean ± standard deviation (SD). All statistical analyses were carried out Utilizing SPSS software (version 26.0), a one-way analysis of variance (ANOVA) accompanied by Tukey's HSD post hoc test was employed to ascertain significant differences among the cultivars for each phytochemical parameter. A *p*-value of less than 0.05 was deemed statistically significant.

3- Findings and Analysis

3.1. Parameters for Extraction Yield and Validation.

The extraction yield was between 12.5% and 15.8% (w/w) for all six cultivars, and there was no statistically significant difference. Linearity was excellent for all five standards over the ranges tested with R^2 values between 0.9991 and 0.9998. The LODs were between 0.08 and 0.25 mg/L, and the LOQs were between 0.25 and 0.80 mg/L (Table S1). Intra-day and inter-day precision (% RSD) for all analytes were <3.5% and <5.2%, respectively. Recovery mean percentages at three spiked levels ranged between 94.6% and 103.8%, thus accuracy was confirmed to be acceptable.

3.2. Quantitative Assessment of Total Secondary Metabolite Compounds

The amounts of secondary metabolites in the six cultivars under study showed significant differences ($p < 0.05$) in the contents of total phenolics, flavonoids, anthocyanins, alkaloids, tannins, saponins, terpenoids, and steroids as shown in Table 1.

Table 1. Quantitative assessment of secondary metabolite compounds in six *V. vinifera* cultivars

Compound Name	*V. vinifera* c v. Black rose	*V. vinifera* c v. Black magic	*V. vinifera* c v. Red roomy	*V. vinifera* c v. Kamali	*V. vinifera* c v. French	*V. vinifera* c v. Dis al-Ans
Total phenolic content (mg GAE/100 g)	445.9	469.8	410.6	420.6	414.5	436.9
Total flavonoid content (mg Rutin/100 g)	98.8	108.9	78.6	83.6	95.6	90.5
Total anthocyanin content (mg/kg)	62.6	74.9	45.6	50.4	60.2	54.5
Total alkaloid content (%)	0.80	0.98	0.58	0.63	0.88	0.74
Total tannin content (%)	1.19	1.30	0.87	0.98	1.56	1.10
Total saponins	0.42	0.65	UDL	0.15	0.43	0.32

Compound Name	*V. vinifera* c v. Black rose	*V. vinifera* c v. Black magic	*V. vinifera* c v. Red roomy	*V. vinifera* c v. Kamali	*V. vinifera* c v. French	*V. vinifera* c v. Dis al-Ans
content (%)						
Total terpenoid content (%)	0.33	0.55	0.08	0.13	0.30	0.20
Total steroid content (%)	0.28	0.39	UDL	0.11	0.20	0.18

UDL: Under Detection Limit

The total phenolic content ranged from 410.6 to 469.8 mg GAE/100 g. The highest phenolic content was found in V. vinifera cv. Black magic (410.6 mg GAE/100 g), whereas the lowest was found in V. vinifera cv. Red roomy (469.8 mg GAE/100 g). The highest total flavonoid content was recorded in Black magic (108.9 mg Rutin/100 g) and the lowest in Red roomy (78.6 mg Rutin/100 g). Anthocyanin content ranged from 45.6 mg/kg (Red roomy) to 74.9 mg/kg (Black magic). Alkaloid concentrations varied between 0.58% and 0.98%, with the lowest in Red roomy and the highest in Black magic. Tannin content was highest in Black magic (1.30%) and lowest in Red roomy (0.87%). Black magic also showed the highest saponin content (0.65%). Saponins and steroids were undetectable in Red roomy. The terpenoid content ranged from 0.08% to 0.55%.

The total phenolic content values in our study (410.6–469.8 mg GAE/100 g DW) were higher than those of sun-dried seeds (170 mg/100 g) as reported by Ghosh and Kalpana [21], which is probably because our methanol maceration with shaking was more exhaustive and the choice of cultivar and shade-drying better preserved the thermolabile phenolics that degrade under direct sunlight. Soxhlet extraction gave higher tannin values (105.37 mg/g) in the study of Buvaneswari et al. [22], while our maceration method gave lower but similar tannin percentages. This difference can be explained by the higher efficiency of heat-assisted Soxhlet extraction for tannins although our gentle method minimizes degradation of monomeric flavanols like catechin. Jyothi et al. [23] The above tests also established the existence of alkaloids, flavonoids, anthocyanins, saponins, terpenes, and phenols in methanolic extracts of black grape seeds. This piece of information concurs with our qualitative findings. It further proves that there is a wide range of secondary metabolites throughout V. vinifera cultivars.

3.3. Identification and Quantification of Individual Polyphenols by HPLC

The HPLC-DAD analysis of grape seed extracts separated and identified five major phenolic compounds; their retention times and UV spectra matched those of authentic standards (Figures 1-6). The compounds were summarized in Table 2, which included their names and the corresponding concentrations.

Table 2. Concentrations (mg/L) of phenolic compounds across six cultivars of V. vinifera. by HPLC

Compound	Black rose	Black magic	Red roomy	Kamali	French	Dis al-Ans
Catechin (mg/L)	174.6	186.9	141.5	152.4	174.6	166.9
Epicatechin (mg/L)	163.5	179.8	125.6	133.6	159.3	148.7
Gallic acid (mg/L)	115.4	124.7	88.9	95.7	112.6	99.5
Quercetin (mg/L)	105.6	111.2	74.9	80.5	101.6	92.6
Rutin (mg/L)	100.2	109.8	80.8	86.9	97.5	90.6

Black magic consistently showed the highest levels of all the five compounds with gallic acid at 124.7 mg/L and quercetin at 111.2 mg/L, while Red roomy showed the lowest. Retention times were highly reproducible (RSD < 0.1%), thus confirming that the method used was appropriate. Tarek et al. [24] found gallic acid, catechin, and rutin to be the major compounds with gallic acid as the most abundant. This variance may be attributed to differences in extraction solvents or HPLC detection wavelengths; our method is more sensitive to flavanols, using a wavelength of 280 nm. Wongnarat and Srihanam [25] found catechin and epicatechin to be the major compounds, which is in agreement with our study. Catechin was found to be the most abundant flavonoid in all the cultivars. Epicatechin closely followed it in all the cultivars, which further confirms the assumption of monomeric flavanols in the polyphenolic profiles of grape seeds. Quercetin was exclusively found in white grapes in their results, while in our study, we detected quercetin in all six cultivars. This observation agrees with the results of Diab et al. and validates the use of aqueous methanol for extracting flavonol glycosides. These differences may be due to factors such as cultivar, geographic origin, harvest maturity, extraction methods and chromatographic conditions, as well as the use of different techniques for assessing quercetin and kaempferol levels. The observed differences could be ascribed to such factors as cultivar, geographic origin, and harvest maturity; extraction methods and chromatographic conditions used; and the different techniques applied in determining the levels of quercetin and kaempferol. Further studies should widen the polyphenol profile to include procyanidin dimers (B1 and B2), epicatechin gallate, and resveratrol, thus requiring the setting of more standards and a longer gradient elution.

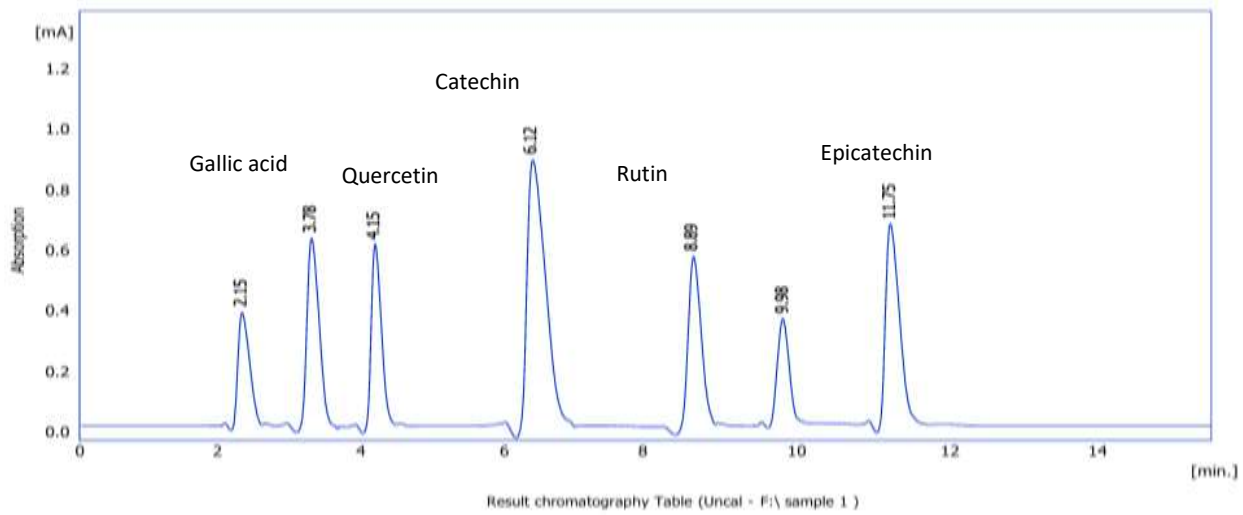

Figure1. Chemical composition profile of methanolic seed extract from the Vitis vinifera cultivar cv. Black rose

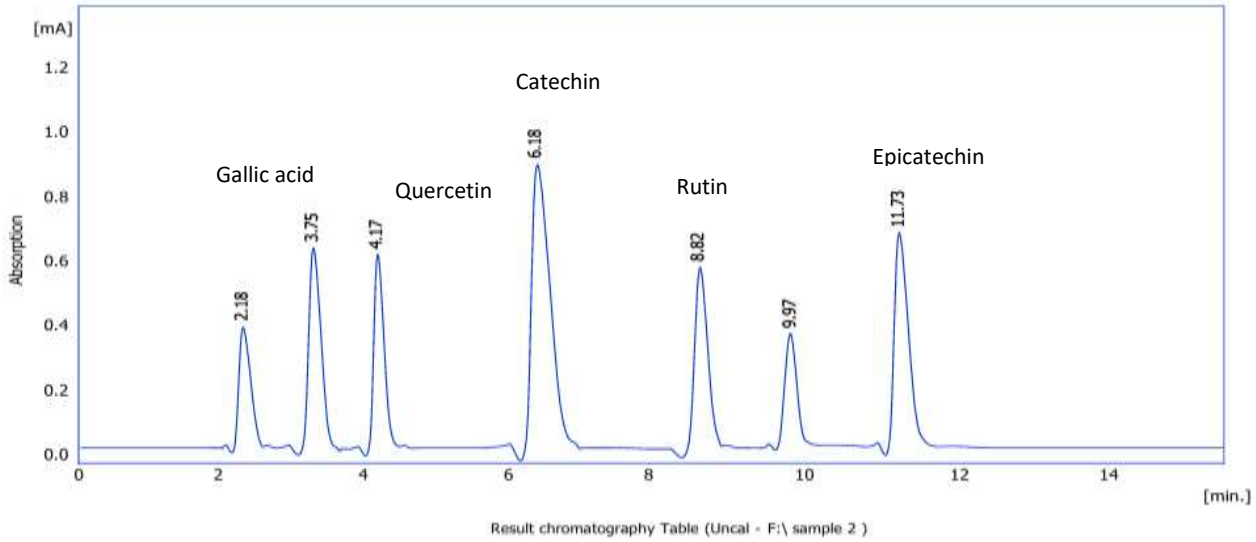

Figure 2. Chemical composition profile of the methanolic seed extract from the Vitis vinifera cultivar cv. Black magic

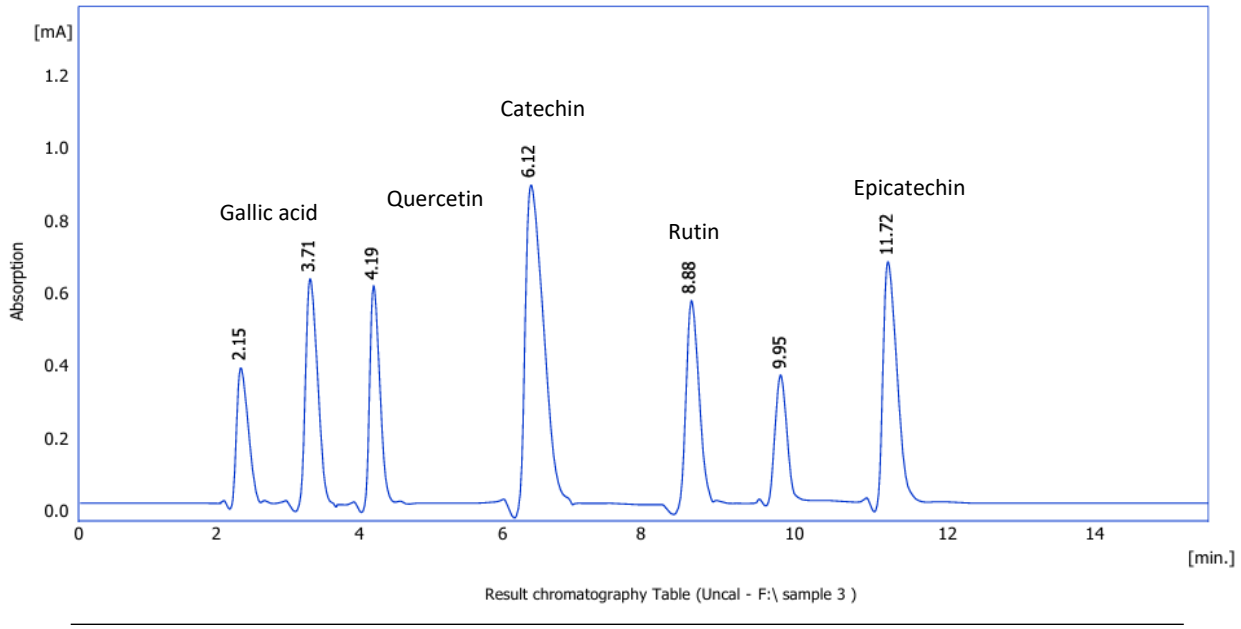

Figure3. Chemical composition profile of the methanolic seed extract from the Vitis vinifera cultivar. Red roomy

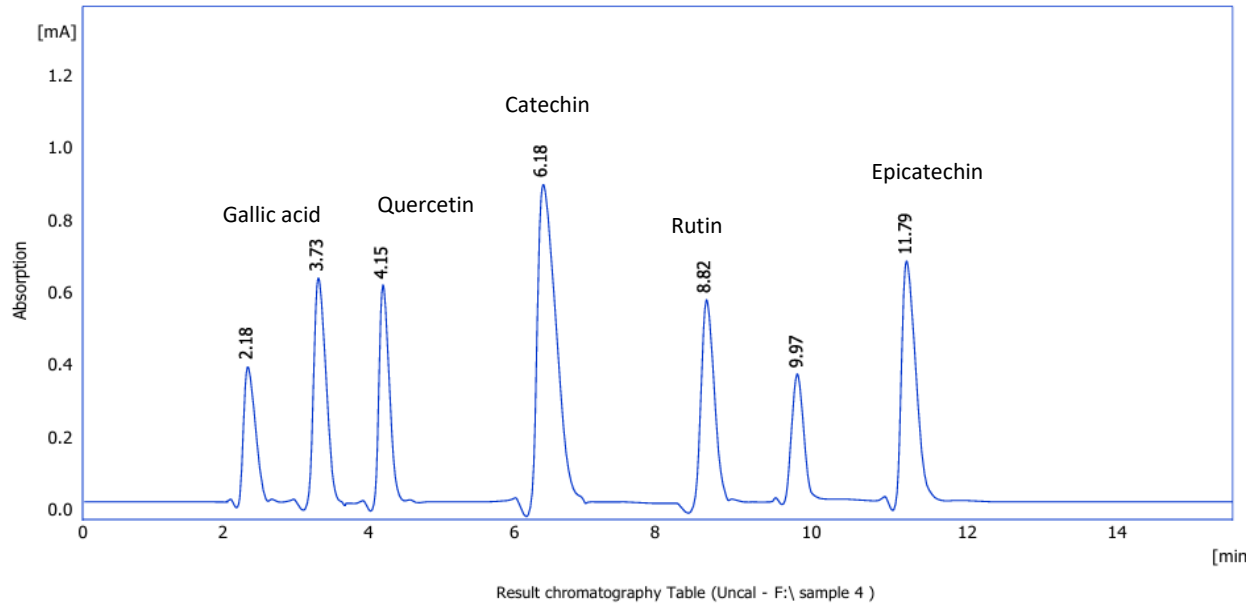

Figure4. Chemical composition profile of the methanolic seed extract from Vitis vinifera cultivar Kamali intact.

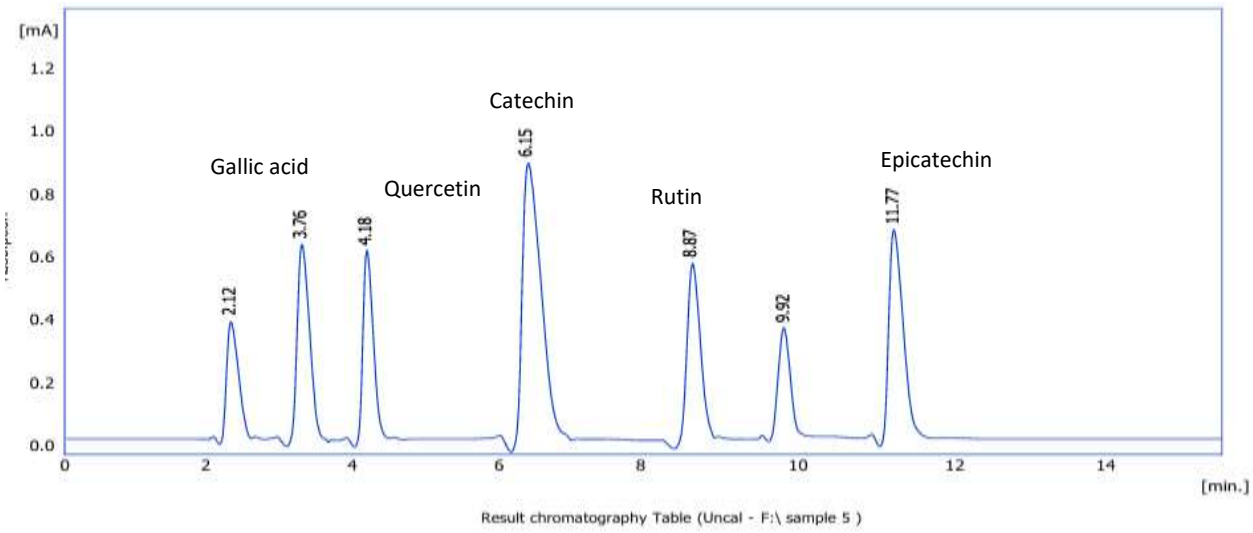

Figure5. Chemical composition profile of the methanolic seed extract from the Vitis vinifera cultivar.French

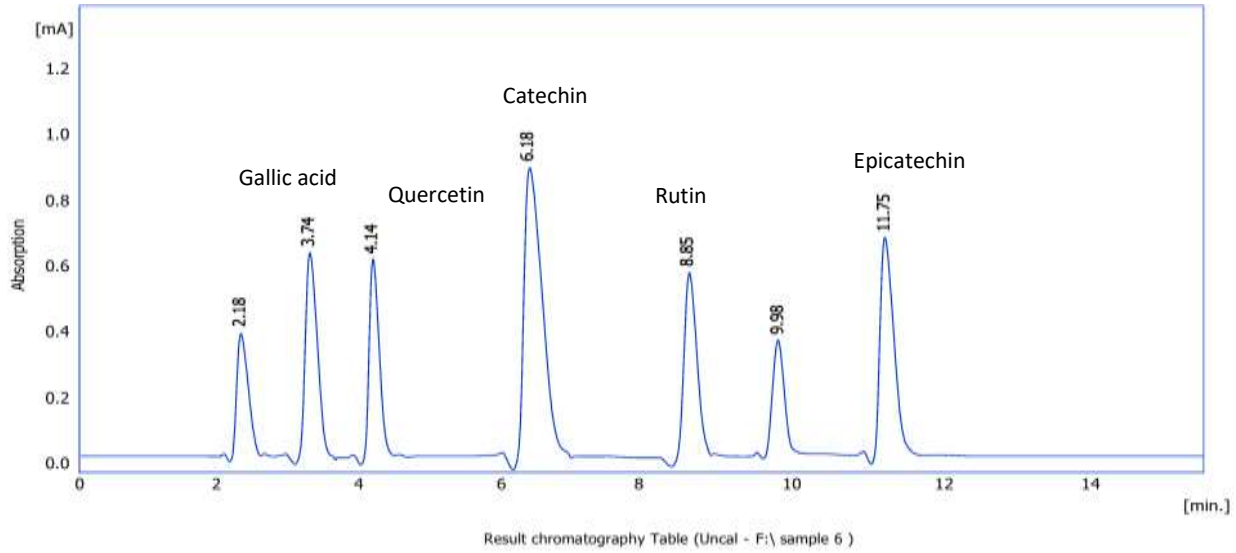

Figure6 Chemical composition curve of methanolic extract of seeds of the cultivar V. vinifera cv. Dis al-Anz.

(Note: The original figures (media/image1.png to media/image6.png) are placed in the same positions in the file of the manuscript.)

4-Conclusion

The six cultivars of Vitis vinifera from Najaf, Iraq, were found to have high amounts of bioactive secondary metabolites. Validation of the HPLC-DAD method was highly successful for the identification and quantification of five major polyphenols; catechin was determined to be the most abundant compound in all samples. The total content assays revealed a broad diversity of compounds belonging to phenolics, flavonoids, tannins, and alkaloids. Black Magic consistently presented the highest total and individual phenolic compound concentrations among the cultivars, showing great promise for use as a nutraceutical and functional food. Such an approach would facilitate the extraction and analytical validation steps that are necessary to further identify and establish specific polyphenol profiles having in vitro antioxidant activities. (e.g., DPPH, FRAP) and on examining the antimicrobial and food preservation potential of these extracts in line

with applications cited for Cinnamomum and Mentha species.

Ethical Approval

This study did not involve human participants or animals. All experimental procedures were conducted in accordance with institutional and laboratory biosafety guidelines.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

All data supporting the findings of this study are available within the manuscript.

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