Extraction and Purification of Resveratrol from Grape Waste

Esraa Shanan Jabbar Al-Jubouri[1], Wedad Fadhil Abas[1], Azhar Jawad Al- Mousawi[2]

[1]Scholar Researcher, Department of Home Economics, College of Education for Women, University of Baghdad, Iraq, [2]Scholar Researcher, Department of Food Sciences, College of Agricultural Engineering, University of Baghdad, Iraq

Abstract

The current study aimed to extract and purify the phenolic compound Resveratrol from dried black grape residues, Vitis vinifera, which were purchased from the local market in Iraq. Resveratrol was extracted from sun-dried grape residues with 2.5 liters of 80% ethanol. Then the compound resveratrol was concentrated using a rotary evaporator and dried in very high freezing conditions after being filtered with animal charcoal. A partially purified material was obtained after a column chromatography process, where 9 grams were obtained for every 600 grams of grape residues as a result of these steps. Chemical tests were conducted to detect resveratrol, and they included: general tests for polyphenols, detection of unsaturated cyclic compounds, high-efficiency liquid chromatography (HPLC) method. The results showed the appearance of two peaks at a wavelength of 280 nm for the compound resveratrol extracted from grape residues, where the time of its appearance was at 4.267 /min compared with the standard compound resveratrol which was at 4.356 /min. The concentration of the resveratrol extract was 107.6 ppm. The total antioxidant activity of resveratrol extracted from grape residues and the standard compound resveratrol was estimated. The total antioxidant activity of resveratrol extracted from grape residues was 71.14%, while it was 62.21% for the standard compound resveratrol. The results showed that the resveratrol compound extracted from grape residues was purified in the dark to prevent the conversion of resveratrol from trans to cis. The study also shed light on the quantities of resveratrol contained in grape residues, and this depends on the extraction steps as well as the purpose of its applications.

Keywords: *Resveratrol, grape waste, Antioxidants, Polyphenols, HPLC.*

Introduction

Resveratrol (3,5,4-trans-trihydroxyestilbine) is a polyphenolic phytoalexin belonging to the stilbene family. It is a natural dietary plant compound that occurs main-ly in grape skin and seeds but is also found in wines and various other types of plant foods, especially pea-nuts, berries, and tea [(1)]. Resveratrol is synthesized by more than 70 species of plants in response to infection, stress, injury, bacteria or fungal infections, and UV-irradiation [(2)]. Synthesis of this molecule in plants is catalysed by resveratrol synthase in the phenylpropanoid pathway in a process similar to that of flavonoids [(3)]. Resveratrol possesses two phenol rings (monophenol and diphenol) bonded together by a double styrene bond and it exists in both cis and trans isomeric forms, Trans-resveratrol appears to be the more abundant and stable natural form [(4)]. This molecule has three hydroxyl groups which are involved in free radical scavenging and metal chelation [(5)]. The presence of hydroxyl groups also facilitates interaction with macromolecules.

Esraa Shanan Jabbar Al-Jubouri
Email: israa.shanan1210a@coeduw.uobaghdad.edu.iq

Resveratrol reveals a wide range of biological properties including anti-glycation, antioxidant, anti-inflam- mation, neuroprotective, anti-cancer, and anti-aging ac- tivity in various in vitro and in vivo experimental models [6]. For industrial purposes, resveratrol is generally obtained by chemical or biotechnological synthesis from yeasts [7]. Fresh grape skin contains (50-100) µg/g of trans- resveratrol [8].

[9]studied the effect of resveratrol extracted from black grapes and its derivatives on some physiological and histological characteristics of experimentally infected female rabbits with type 2 diabetes. This study was also used to evaluate resveratrol and its derivatives in female rabbits at a concentration of 1 mg / kg of body weight given orally for 42 days after induction of type 2 diabetes. Histological sections of the liver and pancreas were studied, and the statistical results showed a significant decrease in glucose, Urea, creatinine, cholesterol, triglycerides, asparagine transporter enzyme (AST). Resveratrol and its derivatives when used in the treatment of rabbits showed a statistically significant increase in high-density lipoproteins (HDL) and insulin in serum levels. [9] also studied the effect of the compound Resveratrol extracted from the peels of grapes Vitis vinifa on some cell lines in the laboratory in order to extract and purify the compound Resveratrol from the peels of the black grape plant. Where a partially purified substance was obtained after performing a column chromatography process and examining the purity of the resveratrol compound by high-efficiency liquid chromatography. The study included an ex vivo evaluation of the cytotoxic activity of the purified and partially purified substance and their effect on some normal and cancerous lines. The study showed a comparison of the cytotoxic effect of both partially purified and fully purified extracts in the three cell lines, and it appeared that different concentrations gave a difference in effect, and that the pure substance had a more inhibitory effect than the partially purified. The cytogenetic study of the completely purified extract on the division of normal human blood lymphocytes showed that Resveratrol inhibited the action of the cleaved substance (PHIA) and showed significant differences within the level ($P < 0.01$) for all concentrations as an anti-cell fission substance and anti-oblastogenesis in a significant manner. It is proportional to the concentration increase used and the exposure time.

Materials and Methods

Resveratrol extraction and purification

Resveratrol was extracted and purified according to the method approved by [5].

Preparation of grape residue extract

Iraqi black grapes were collected from the local market and classified as belonging to the variety Vitis vinifera by Herbarium, Department of Life Sciences, College of Science, University of Baghdad.

Preparation of grape residues

After the grapes were obtained, they were washed well, then they were mixed with an electric mixer for a period of (5-7 minutes). The mixture was filtered using a sieve lined with a fine cloth, discard the juice produced from it, while the remaining residues were taken in the strainer and dried solar, then put in bags of polyethylene. And the samples were preserved by freezing (at -20 ° C) until extraction steps were carried out for it.

Extract preparation

Grape residue extract was prepared with a weight of 500 g of sun-dried grape residue and mixed with 2.5 liters of ethanol at a concentration of 80%, the mixture was left for 72 hours in a cool and dark place, then the extract was filtered using a vacuum filtration unit and then concentrated using a rotary evaporator at of 30-40 ° C. The concentrated filtrate was stored at -20 ° C for subsequent steps, as all the above steps were carried out away from direct light.

Identification of Polyphenols

Phenolic group (C6H5-OH) in phenolic compounds, which are colourless but attain colour due to oxidation, are soluble in 5% NaOH solution and insoluble in 5% sodium carbonate solution, and the phenolic groups in the molecule can be determined by the following general tests:

Ferric Chloride Test

The classic procedure for detecting phenolic compound is by means of the intense green, purple, blue or black colours, many of them give in solution when 1% aqueous or alcoholic ferric chloride is added [9].

Liebermann Reaction

Only those phenols which posses a free para position respond to this test. The test includes the additional 1 ml of conc. H_2SO_4 to the phenolic compound in a dry test tube and addition of a few crystal of $NaNO_2$, a blue green or blue- violet colour is immediately formed which changed to red on dilution [5].

Detection of the Aromatic Ring

The aromatic ring was detected in the extract of grape tailings by conducting the aluminum chloride test mentioned by [7,8] as follows: 0.1 gm of the extract of prepared grape residues was taken and 1 ml of chloroform was added to it, then aluminum chloride crystals were added to it. Anhydrous (ALCl3) and using a spoon added along the sides of the test tube, formed yellow colored aluminum chloride crystals which turned dark orange within a few minutes, with the appearance of a colorless chloroform layer detachment.

Specific Test for double Bond:

In order to find out unsaturated compound, the following test is applied [6].

Bromine Decolourisation Test:

0.1 g of unknown resveratrol in 2 ml carbon tetrachloride is added with shaking, then a drop wise of 5% solution of bromine was added. The discharge of reddish brown colour of bromine without evolution of hydrogen bromide represents a positive test for unsaturated compound [9].

Isolation and Purification of Resveratrol:

The following steps were followed for the isolation and purification of resveratrol: Acid hydrolysis, Liquid portion, Column chromatography (Partial purification), HPLC.

Acid Hydrolysis

Acid hydrolysis was done using 10% V/V conc. HCl for (10-30) min on a water bath. This step led to the hydrolysis of the glycosidic linkage and got the aglycone moiety, cool and filter.

Liquid – Liquid Partion

The filtrate was transferred to seperatory funnel. An organic solvent like chloroform was added in a quantity equal to the aqueous phase, with gently shaking and reapting the process three times. The chloroform layers were collected together and washed from the access acid with distilled water. The collected chloroform layers were evaporated to dryness under vacuum with a rotary evaporator at 30°C. The residue was green viscous alquest stored in dark umber vessels at –20°C untill use [9].

Column Chromatography (partial purification) of resveratrol:

a method was adopted [5]. To obtain the pure resveratrol compound, the partial purification of the concentrated grape tailings extract was carried out using using open glass column (2.5 x 30) cm filled with silca gel G60 special for column chromatography. The residue was dissolved in 1-2 ml methanol and the mobile phase is benzene: methanol: acetic acid, 20:4:1. The elutions were collected in 40 separated tube each

filled with 3ml eluent. All fractions were tested for $FeCl_3$ 1% solution as a colourimeteric method for polyphenols identification [6]. then the ethyl acetate solvent was used at a ratio of 1:1 to obtain partially purified resveratrol, then the purified sample was treated with charcoal to get rid of the color, then the extract was concentrated in a rotary evaporator and dried in an electric convection oven.

Diagnosis of Resveratrol using High-performance Liquid Chromatography(HPLC):

Resveratrol was diagnosed according to [9]. Resveratrol was detected in the sample obtained using a high-sufficiency liquid chromatography device of SYKAMN HPLC under the following conditions:

Column type	C18-OSD a Zorbax Eclipse
Column length	cm x 4.6 mm 0.25
Flow Rate	ml/min 0.7
Wave length	nm 280
Mobile phase	Mobile phase Methanol (A) and formic acid 1% (B) diluted with water v /v as follows: 0-13 minutes 40% of formic acid 1% and 14-20 minutes, 50% of formic acid solution
time Rotation	It is set according to the result obtained
Injected sample size	µl 100

Estimation of total antioxidant activity

The total antioxidant activity of natural and synthetic resveratrol was estimated according to the method used by [2].

Results and Discussion

Extraction and Partial Purification of Resveratrol:

Since grape skins are one of the sources of resveratrol [5], mentioned that each gram of fresh grape skin contains 100-50 micrograms of pure resveratrol. Therefore, the process of extraction and partial purification of the compound resveratrol was carried out using 600 gram of dried black grape residues with 2.5 liters of 80% ethanol. Then the extract was filtered and concentrated in a rotary evaporator, then the acid hydrolysis process was carried out using 10% hydrochloric acid in a water bath for (10-30) minutes and extracted with the organic solvent chloroform, Then the resveratrol was separated using column chromatography by an open column filled with silica gel G60. The remaining filtrate was dissolved in 1-2 ml of methanol, the extract was added to the column and washed by the mobile phase (benzene, methanol, acetic acid, 1:4:20), After that, ethyl acetate solvent was used in a ratio of 1:1, then the purified sample was treated with charcoal, then the extract was concentrated using a rotary evaporator and dried in the oven, In this step, partially purified resveratrol was obtained, Then the sample was kept in opaque glass containers in order to preserve the resveratrol from oxidation , since the naturally occurring trans-resveratrol easily oxidized and converted to the cis – configuration by day or UV light and with the presence of heat, heavy metals and atmospheric oxygen [4]. The results of separation and purification showed the following:

Resveratrol Extract

Resveratrol was extracted from grape residues according to (6) using ethanol at a concentration of 80%, As this alcohol is a good solvent for primary extraction purposes, all extraction steps were carried out in conditions far from light. 9 g was obtained from every 600 g of grape residue used, this method is consistent with the method used by [5] when using 80% ethanol alcohol to extract resveratrol for 30 minutes at a temperature of 60 °C to prevent the enzymatic oxidation of the compound. [3] also indicated that resveratrol is hydrolyzed using hydrochloric acid at a concentration of 10% after being placed in a water bath for 10-30 minutes, and it dissolves easily in organic solvents such as chloroform.

Purification of Resveratrol

Since the grape skin contains numerous amounts of chemical compounds, therefore, the extract cleaning up is necessary by liquid-liquid partion technique for separation compounds according to the different distribution coefficients and the solvent affinity to solutes [6]. Ethylacetate is a good solvent for resveratrol taking up as viewed in many studies [7].

Chemical Identification of Resveratrol:

Table (1) shows the most important general phenolic tests for resveratrol, it was clear that the resveratrol compound was soluble in 5% sodium hydroxide solution, while it was insoluble in 5% sodium carbonate solutionWhen tested with 1% ferric chloride solution, it gave a positive result, which resulted in a green color. It also gave a positive result with Liebermann test, which resulted in a blue-green color. It also gave a positive result with the aluminum chloride test, which depends on the presence of the aromatic ring. It also gave a positive result with the bromine decolorization test, which indicates the presence of the double bond. These results are consistent with the findings of [8].

Table (1): Tests for the general phenolic compound

Test	Result
%5 NaOH solution %5	Soluble
5% Sodium carbonate solution	Insoluble
1% Ferric chloride solution	Green colour
Libermann reaction	+ ve
Aluminum chloride test for the aromatic ring	+ ve (yellow to orange colour)
Bromine decolourisation test for the double bond	+ ve (Discharge of reddish – brown colour

High Performance Liquid Chromatography (HPLC) for Resveratrol

The HPLC method was used to analyze the resveratrol compound extracted from grape residues and compare it with the standard resveratrol compound according to what was mentioned in [9]. And through the scheme obtained for the compound resveratrol in Figure (1,2) and the results obtained in Table (2), Two peaks at 280 nm wavelength were observed for the compound resveratrol extracted from grape residues, and when compared with the standard resveratrol

compound, the presence of resveratrol was confirmed in the extract at a concentration of 107.6 ppm. It was also noted that the time of appearance of the extracted resveratrol compound was 4.356 minutes compared to the standard compound where the time of appearance was 4.267 minutes.

Table (2): HPLC results for resveratrol extracted from grape residues and standard resveratrol at 280 nm wavelength.

Resveratrol	Retention time (min)	Peak area
Standard	4.267	603.61
Extracted	4.356	1087.948

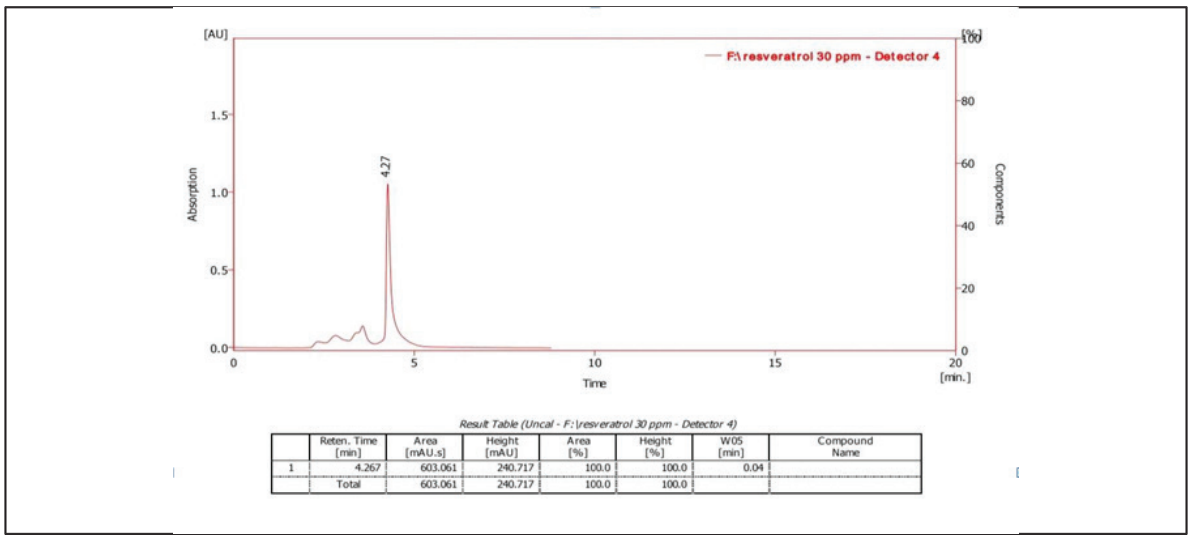

Figure (1) Chromatographic analysis of resveratrol standard by HPLC technique.

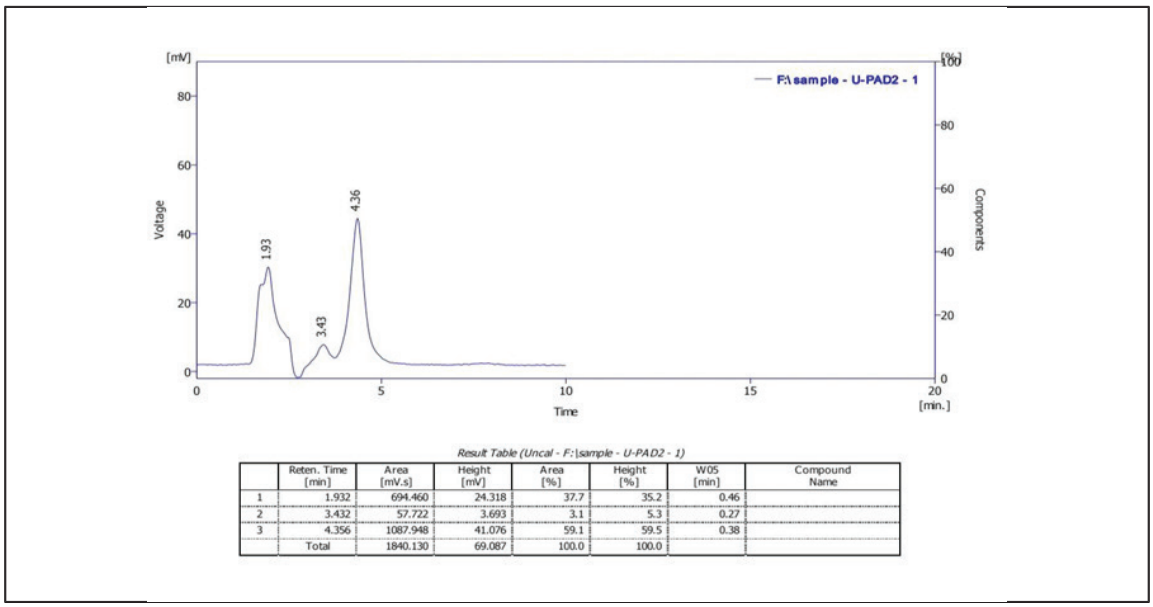

Figure (2) Chromatographic analysis of standard resveratrol by HPLC technique

Determination of the total antioxidant activity of Resveratrol

In order to detect the ability of resveratrol to show the antioxidant activity, the antioxidant activity of both resveratrol extracted from grape residues and synthetic resveratrol was estimated. The results showed that the resveratrol extracted from grape residues gave a higher antioxidant power compared to the synthetic resveratrol, as the percentage of the total oxidative activity of the extracted resveratrol was 71.14%, while the percentage was 62.21% in the synthetic resveratrol compound. When comparing the antioxidant power of resveratrol extracted from grape residues, which was mentioned above, we find that it was good when compared with the antioxidant power of ascorbic acid as one of the powerful natural antioxidants. [9] reported that the antioxidant power of this acid was 87.33%.

Conclusion

The current study concluded the possibility to extract and purify the phenolic compound Resveratrol from dried black grape residues, and its quantities depends on the extraction steps as well as the purpose of its applications.

Conflict of Interest: None

Funding: self

Ethical Clearance: Not required

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