

EXTRACTION AND PURIFICATION OF RESVERATROL FROM GRAPE SKIN FRUIT *VITIS VINIFERA*

Zainab Y. Mohammed¹ Essam F. Al-Jumaily² Ali O. A. Al-Shammary²

¹Ibn Sina Research Center, Ministry of Industrial and Minerals.

²Genetic Engineering and Biotechnology Institute for Postgraduate Studies,
Baghdad University

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ABSTRACT

This study was conducted with the aim to extract and purify a polyphenolic compound "Resveratrol" from the skin of black grapes *Vitis vinifera* cultivated in Iraq. The partial purified resveratrol is obtained after chromatography on silica gel G60 column. The preparative thin layer chromatography elution yields pure crystals identified as resveratrol (mixture of the two isomers cis and trans) in relation to resveratrol standard trans- resveratrol (35 mg resveratrol crystals/0.5kg fresh grape skin obtained as a result of these processes). Chemical investigations and tests for identification and qualification of the extracted partial and purified crystals involved: general tests for polyphenoles, aromatic unsaturated compounds, spectrophotometric scanning for λ_{\max} screening, High Performance Liquid Chromatography (HPLC) analysis, Fourier Transform Infra-Red (FTIR) assay and melting point estimation in relation to resveratrol standard for the all applied tests.

Key words : Resveratrol , Black Grape skin, Polyphenoles

استخلاص وتنقية مادة Resveratrol من قشور ثمرة العنب *Vitis vinifera*

زينب ياسين محمد¹ عصام فاضل الجميلي² علي عبيس عبد الشمري²

¹ مركز ابحاث ابن سينا، وزارة الصناعة والمعادن

² معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا، جامعة بغداد.

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الخلاصة

هدفت هذه الدراسة استخلاص وتنقية المركب الفينولي Resveratrol من قشور نبات العنب الأسود *Vitis vinifera* المزروع في العراق. تم الحصول على مادة منقاة جزئياً بعد إجراء عملية الفصل الكروماتوغرافي على عمود من سليكا جيل 60 (Column Chromatography) وباستعمال كروماتوغرافي الطبقة الرقيقة التحضيرية PTLC تم الحصول على بلورات المادة النقية والتي تم التعرف على كونها مزيج من النظيرين *trans resveratrol* و *cis resveratrol* مقارنة مع المادة القياسية *Trans-Resveratrol standard* (تم الحصول على 35 ملغم لكل نصف كيلوغرام قشور العنب الطرية نتيجة لهذه الخطوات). أجريت كشوفات كيميائية واختبارات للتعرف على نوعية البلورات النقية وقد شملت: اختبارات عامة لمركبات الفينولات المتعددة وكشف المركبات الحلقية غير المشبعة والكشف بالمطياف الضوئي لمعرفة منحنى أقصى امتصاص وطريقة تحليل كروماتوغرافيا السائل عالي الكفاءة و تحليل FTIR ودرجة انصهار المادة مع المقارنة بالمادة القياسية لجميع الاختبارات.

INTRODUCTION

Many botanicals have been used for the treatment of various human disease throughout history and play a role in disease prevention(1). Epidemiologic studies have suggested that a reduced risk of cancer is associated with high consumption of vegetables and fruits(2). The search for novel and effective cancer chemopreventive agent has led to the identification of various naturally occurring phytochemicals, one of which is resveratrol (trans- 3,4', 5-trihydroxy stilbene) a phytoalexin derived from grape skin and other fruits(3). Black grape cultivated in Iraq is rich with resveratrol(4).

Resveratrol is shown to have a potent anti-inflammatory(5), antioxidant(6), anti platelet aggregation and cardiovascular protection effects(7). Its potential chemopreventive and chemotherapeutic activities have been demonstrated in all three stages of carcinogenesis in numerous *in vitro* and *in vivo* studies(8). It has the ability to modulate various targets and signaling pathways (9). Resveratrol: belongs to a class of polyphenolic compound called stilbene. It is a non flavonoid and non steroidal phytochemical estrogenic agent(10). Some types of plants produce resveratrol and other stilbenes in response to stress, injury, fungal infection and ultraviolet (UV) radiation(11). Resveratrol is a fat-soluble compound that occurs in a trans and a cis configuration shown in Fig.(1). Both cis- and trans-resveratrol also occur as glucosides (bound to a glucose molecule). Resveratrol-3-O-beta-glucoside is also called piceid .Fresh grape skin contains 50-100µg trans-resveratrol/g (12). Trans-resveratrol is found in the skin of young unripe red grapes and it is also found in peanuts, eucalyptus , spruce lily , mulberries and ground net (13).

The aim for this study to extract and purify resveratrol from red grape skin.

MATERIALS AND METHODS

Collection of samples

Native black grapes cultivated in Iraq were collected from the local market and classified as *Vitis vinifera* family (*vitaceae*) by the herbarium of the Biology Department, College of Science, Baghdad University. The skin was separated from the fruit to be then kept in a dark cool place , till the following steps.

Preparation of grape skin extract

Preparation of grape skin extract was carried out according to Harborne(14). The grape skin extract was prepared; all steps were done away from direct light and extensive stress that led to oxidation of the plant extract. About 500 grams of fresh skin grapes was shaken with 2.5 liters (80%) ethanol in cool dark place for 72 hrs. The extract was filtered and the filtrate was dried at 30-40°C by a rotary evaporator to get 1/10 (one tenth) its original volume. The crud extract was stored at -20°C till the followings steps.

Identification of Polyphenols

General tests for phenolic group (C_6H_5-OH) in phenolic compounds, which are colourless but attain colour due to oxidation, are soluble in 5% NaOH solution, insoluble in 5% sodium carbonate solution, and the phenolic group in the molecule was determined by the following tests(15):

- (a) 1% solution of ferric chloride
- (b) Libermann reaction
- (c) Phthalein test

Isolation and purification of resveratrol

Acid hydrolysis was done using 10% V/V conc. HCl for 10-30min at 60°C. This step led to the hydrolysis of the glycosidic linkage and production of the aglycone moiety. The mixture was cooled then filtered(14).

The filtrate was transferred to a separating funnel. An organic solvent like chloroform was added in a quantity equal to the aqueous phase, with gentle shaking. This step was repeated three times. The chloroform layers were collected together and washed from the excess acid with distilled water. The collected chloroform layers were evaporated to dryness under vacuum with a rotary evaporator at 30°C. The residue, which was green viscous alquest; was stored in dark amber vessels at – 20°C until use (14).

Partial purification by using adsorption column chromatography

A partial purification of the residue was performed using open glass column (2.5 x21cm) filled with silica gel G60 special for column chromatography. The residue was dissolved in 1-2ml methanol. The mobile phase was a mixture of benzene: methanol:acetic acid in a ratio of 20:4:1 (14).

The elutions were collected in 100 fractions of 3ml each. All fractions were subjected to polyphenol assay by FeCl₃ 1% solution as a colorimetric method (14,15). Fraction which gave positive results were collected and dried under vacuum by a rotary evaporator. The resveratrol spots were detected on a TLC aluminum sheet silica gel 60F₂₅₄ in comparison with the standard spot using the same mobile phase that had been used in the column chromatography. Both the standard and the corresponding extract spots showed a light violet fluorescence when exposed to U.V. 254 nm lamb (14).

Preparative Thin Layer Chromatography (P.T.L.C.)

The dried collected fractions of the partial purified resveratrol were redissolved in 1ml methanol then applied by a micropipette on silica gel G60 plate as continuous straight line with a width range 2-4mm wide. Plates were inserted into a saturated T.L.C chamber containing benzene:methanol: acetic acid, 20:4:1 as a mobile phase. The chamber was kept in cold dark place.

When the solvent system reached the upper edge, the plate was removed then left to dry for few minutes. The pure resveratrol sample was appeared as dark straight line. The purified resveratrol was scratched and eluted with methanol (10ml three times) then it was stored at –20 for two days. Amorphous white pure crystals were formed, which collected rapidly in a cool, dark place and kept in amber container at –20°C. These crystals were referred as " pure resveratrol ". The crystals were identified using the following tests:

- A) U.V. absorption: Stilbenes show intense purple fluorescence in U.V. light that was changed to blue with NH₃. Trans resveratrol has λ_{max} at 305 (or 307) with an inflection at 320nm (14).
- B) Thin layer chromatography: using TLC plate of silica gel G60 with fluorescence. The mobile phase was benzene:methanol:acetic acid, in the ratio 20:4:1 (14).
- C) HPLC method using the following system (16):
Column: C18–reverse phase.
Mobile phase:acetonitrile:water, 60:40
Flow rate: 0.6ml/min

Standard concentration: 0.6mg/ml (exposed to sun light)

Sample concentration: 0.6mg/ml

Wave length: 307nm for trans and 280nm for cis isomer

- D) FTIR assay: to detect the functional groups in resveratrol structure and to be compared with the standard chart. Resveratrol was reported to contain many functional groups: aromatic benzene ring, aromatic hydroxyl groups and the C=C double bond (16).
- E) Specific reaction of benzene (aromatic ring)
Aluminum chloride $AlCl_3$ test "Friedle graft" (15).
- F) Specific test for double bond: In order to find out unsaturated compound the following two tests are applied (15):
(I) Bromine decolourisation test.
(II) The Baeyer test.
- G) Melting point: melting point apparatus (Glascoo,U.K.specially for this process).The melting point of the extracted compound was measured.

RESULTS AND DISCUSSION

Fresh black grape skin 500g was hydrolysed with acid and extracted with an organic solvent " Chloroform", then resveratrol was separated with liquid – solid adsorption chromatographic technique using silica gel column to yield " partially purified resveratrol", then to isolate pure resveratrol crystals by preparative thin layer chromatography (P.T.L.C) method.

The yield of the pure crystals is about "35mg" for the 500g grape skin used; there may be some loss during the processing of extraction, 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 (10,17).

The fresh skin of black grapes(*Vitis vinifera*) is rich with a non- flavonoid polyphenol, resveratrol(4) and each gram of fresh grape skin contains 50-100 microgram of pure resveratrol (18).

The extraction procedure

The procedure for extraction and purification was concluded from different studies, all the processes have been carried out in the dark. According to Harboren (14), general process for plant extraction, the grape skin is extracted with 80% ethanol to extract the trans and cis resveratrol O-D-glycoside (piceid) which are water soluble. Alcohol, in any case, is a good all-purpose solvent for preliminary extraction. The acid hydrolyses with 10% conc. HCl at 60°C for 10-30min. was done to breakdown the glycosidic linkage(14).

The free aglycon moiety is water insoluble and can be easily taken up with organic solvent such as chloroform (10).

Partial Purification

It was done using silica gel G60 column chromatography technique with a mixture of benzene:methanol:acetic acid in the ratio of (20:4:1) as a mobile phase to elute fractions according to their affinity to the mobile phase. The resultant fractions that gave positive ferric chloride test 1% solution, were detected by T.L.C silica gel 60 F_{254} plate with the same mobile phase indicated in Fig.(1). In chromatogram, the fractions of the positive result and the standard gave fluorescence spots with R_F

value = 0.43. While the negative fractions did not. The dried collected fractions was designated as “ Partial purified resveratrol”.

Purification of resveratrol

Resveratrol purification in this study was done using benzene:methanol:acetic acid, 20:4:1 as a mobile phase on the silica gel G60 glass plate of 0.75mm thickness. After the application of the extract and running the procedure on; a pure resveratrol line appeared as a dark straight line (the silica gel G60 used is without fluorescence). The pure resveratrol crystals and the partial purified resveratrol were subjected to general and specific tests for the identification and analysis of the compound.

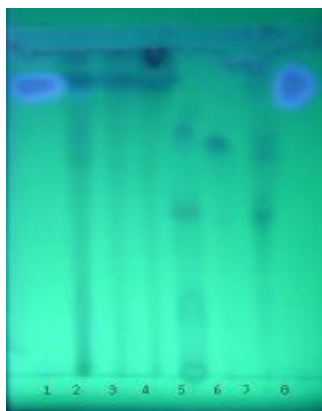


Fig. (1): T.L.C chromatogram for detection partial purified resveratrol under UV lamp (254nm) the positive and negative results of 1% FeCl₃ test.

Spot (1) and (8): 1µl of 0.1% solution trans- resveratrol standard.

Spot (2), (3) and (4): 1µl of partially purified resveratrol that gave positive results with 1% FeCl₃ solution after column chromatography.

Spot (5), (6) and (7): 1µl of extract after column chromatography that gave negative results with 1% FeCl₃ solution.

Chemical Identification of Resveratrol

Resveratrol (partial and pure crystals) was tested for general phenolic tests (Table 1).

Table (1): General phenolic compound tests

Test	Result
5% NaOH solution	Soluble
5% Sodium carbonate solution	Insoluble
1% Ferric chloride solution	Green colour
Liebermann reaction	+ ve
Phthalein reaction	+ ve (red colour)
Aluminum chloride test (Friedle graft) “for the aromatic ring”	+ve (yellow to orange colour)
Bromine decolourisation test “ for the double bond”	+ ve Discharging of redish – brown colour
Baeyer test “ for the double bond”	+ve Disappearance of the purple colour

Spectrophotometric identification of resveratrol

The purified resveratrol was identified by spectrophotometric scanning to investigate the maximum absorption wave length " λ_{\max} ". Fig.(2) demonstrates λ_{\max} of trans-resveratrol standard curve at 304nm. When the substance were exposed to UV or day light for several hours, about 50% of trans isomer was converted to cis configuration (18,19). Fig.(3) shows the two isomer configurations λ_{\max} after sun light exposure. Fig.(4) declares the extracted resveratrol crystals consisting of the two isomer cis and trans- resveratrol. The λ_{\max} for trans- resveratrol 304nm and λ_{\max} for the cis isomer is about 239 for the extracted resveratrol and 237nm for the standard.

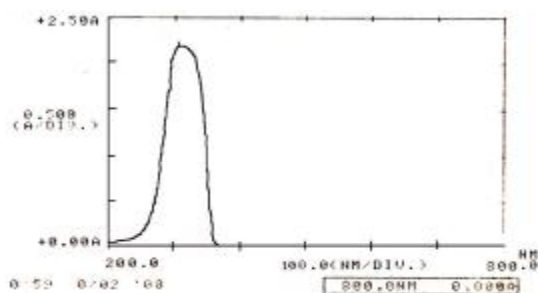


Fig.(2): Demonstrates λ_{\max} of trans-resveratrol standard curve at 304nm

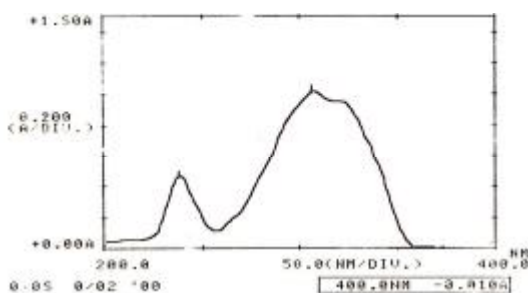


Fig.(3): Demonstrates λ_{\max} of trans and cis resveratrol standard curve exposed to sun light

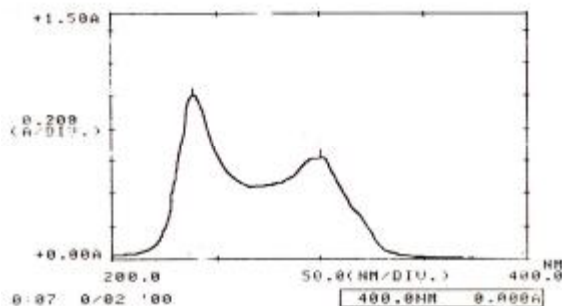


Fig.(4): Demonstrates λ_{\max} of extracted resveratrol crystals

Fourier Transform Infra-Red (FTIR) for resveratrol

Trans-resveratrol has many functional groups. The typical frequencies regions for the functional groups are compared for both the standard and the extracted resveratrol (Table 2).

Table (2): The IR frequencies region for the functional groups of the standard resveratrol and the extracted pure resveratrol

The Functional Group	I.R Frequencies of Resveratrol Standard	I.R. Frequencies of Extracted Resveratrol
Phenolic- OH group stretching	3294.19	3386.67
Aromatic C-H group stretching	3016.46	2970.67
Aromatic C=C and of Alkene C=C stretching	1589.23	1643.24
OH group bending	1149.50	1141.78

High Performance Liquid Chromatography (HPLC) for Resveratrol

In HPLC method which applied for resveratrol detection two wave lengths: 307 nm for trans- resveratrol and 280nm for cis-resveratrol were used.

The resultant chromatogram for trans and cis standard is shown in Fig.(5) and Fig.(6) and for the extracted purified resveratrol is shown in Fig.(7) and Fig.(8) respectively.

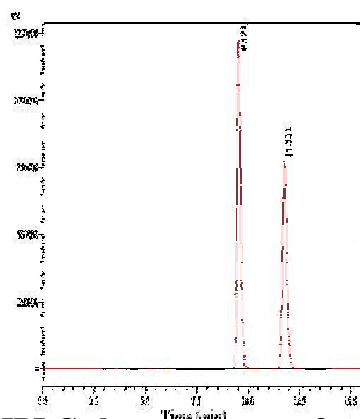


Fig.(5): HPLC chromatogram of trans- resveratrol standard at 307nm after sunlight exposure.

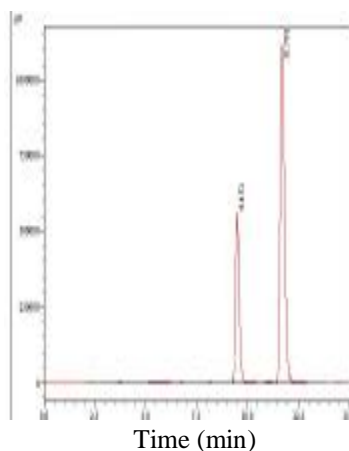


Fig.(6): HPLC chromatogram of resveratrol standard at 280nm after sunlight exposure.

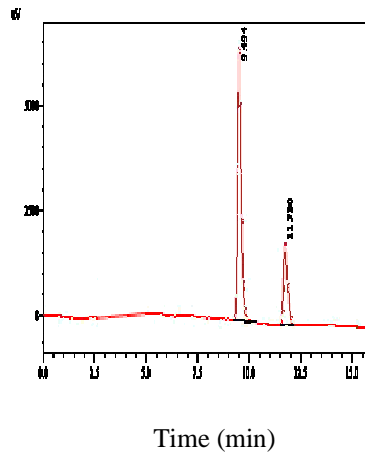


Fig.(7): HPLC chromatogram of the extracted resveratrol at 307nm.

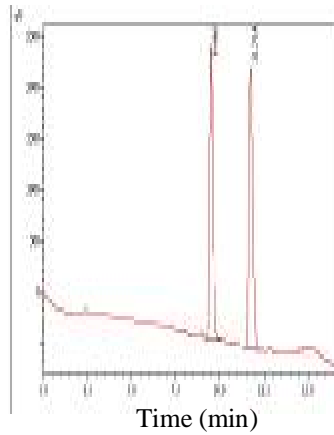


Fig.(8): HPLC chromatogram of the extracted resveratrol at 280nm.

The area under the peak and peak height with corresponding retention time are shown in Table (3):

Table (3): HPLC results for pure extracted and standard resveratrol after sun light exposure for the standard.

	Wave length (nm)	Type isomer	Retention time (min)	Peak area
Resveratrol standard	307	Trans	9.491	15037833
	280	Cis	11.70	16778865
Resveratrol extracted	307	Trans	9.494	81791
	280	Cis	11.714	41368

These results emphasize that even almost all the conditions for extraction and purification occur in dark as much as possible, the substance may be converted from trans configuration to the cis isomer.

Although preparative thin layer chromatography. method for purification, is proved to be a high efficiency method and the isolated substance can be determined by the specific λ_{\max} and HPLC technique.

The identity of standard and extracted resveratrol chromatogram for both cis and trans isomers in the maximum wave length absorption and the retention time explain the selectivity and accuracy of the applied method.

Melting point of resveratrol

Pure trans- resveratrol melting point is between the range of 253–255°C (10). Since the extracted purified resveratrol appears to be a mixture of the two isomers (cis and trans) according to U.V. scan and HPLC chromatogram, and the melting point using Glascoo Apparatus (U.K.) is about 250°C with some decomposition.

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