Analysis and Evaluation of Hydroxytyrosol in Olive Leaf Extract

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ABSTRACT

There is a group of phenolic compounds and their glycosidic forms responsible of antioxidant effect in the olive leaf extract. Hydroxytyrosol is one of these compounds considered as building unit for the other phenolic molecules in the extract. A normal phase chromatographic methodwas established for analysis of hydroxytyrosol. It generates clean chromatograms suitable for analytical and preparative purposes and better resolved than those obtained from reversed phase systems. Mobile phase of (1:1) acetonitrile and 1% acetic acid aqueous solution was used. Flow rate was kept constant at 0.5 mL/min. Injection volume was 20 µL and UV detector was set atʎ=280 nm. The developed HPLC method was found linear within the range of 0.82-4.12 mg%, precise with RSD less than 2% and accurate with a range of 97.6-101.2 %. LOQ and LOD of the method were calculated to be 8 and 0.8 µg/ml respectively.Furthermore, antioxidant degree of hydroxytyrosol and its derivatives in the extract was evaluated by Follin-ciocalteu'sreagent and found equivalent to 40 mg gallic acid.

Keywords: Hydroxytyrosol, Olive leaf and Antioxidant.

INTRODUCTION

Olive leaf has a reported use in preserving food and also it has strong potential to be used in pharmaceutical preparations [1-3]. Leaf extract is used in treatmentof diabetes and cardiovascular diseases [4-7]. The activity of extracts could be attributed to phenolic constituents which possess antimicrobial and antioxidant effects[8,9]. According to the literature, olive leaf extract contains group of antioxidant compounds[10-12] . Hydroxytyrosol and Oleuropein are the major ones **Figure.1**[13-15]. Hydroxytyrosol is the primary structure of antioxidantsin olive leaf which might be further modified and glycosylated to oleuropein and other glycosides [16,17].

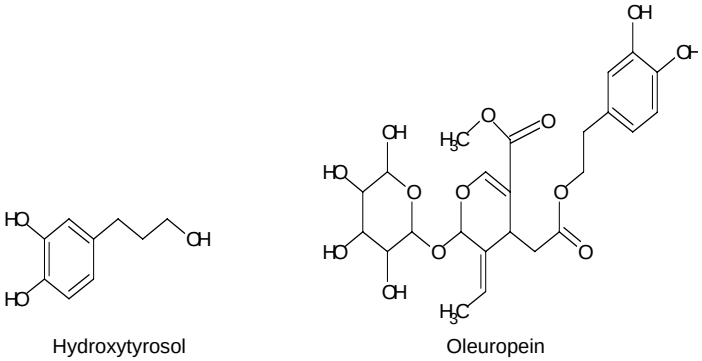

Figure.1Structure of major metabolites in olive leaves; Hydroxytyrosol and oleuropein.

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Antioxidants are quite susceptible to decomposition due to heat and light [18-20]. Thus, the total content of phenolic compounds in the extract areanalysedby non-destructive test such as Folin-Ciocalteu phenol test [21-23]. However, stability-indicating method is also essential to periodically evaluate the stability of products containing the plant extract [24]. Since hydroxytyrosol is one of major compounds in the extract and it is responsible of part of antioxidant activity, it is used for standardizing the extract relying on an HPLC-dependent method [24,25]. Hydroxytyrosol analysis was found challenging because of its amphiphilic nature[26,27].

However, several chromatographic separations of hydroxytyrosol from different reversed phase systems were reported[28-30]. Separation of hydroxytyrosol from these systems is often required gradient mobile phase.The latter often crumbles the stability of chromatographic systems and the repeatability of results[28,29]. Additionally, the published work on simple isocratic systems with reversed phase column generates weakly resolved peaks for hydroxytyrosol[30]. Even if resolution is enough for analytical purposes, it might not be satisfactory for preparative application by which hydroxytyrosol can be isolated.

This work introduced anew normal phase chromatographic method obtained the advantage of amphiphiliccharacter of hydroxytyrosol in chromatographic separation. Clean chromatograms obtained from the method and the system stability due to isocratic flow enableaccurate standardization of hydroxytyrosol. Also clean and well-resolved chromatograms facilitate the application of the method in preparative analysis to produce hydroxytyrosol in pure form and with cheap cost. This is expected to generate positive impact on countries relying on olive tree as one of their economic resources including pharmaceutical and food industries.

Material and methods

Reagents and chemicals

All chemicals were purchased from Sigma- Aldrich (St. Louis, MO) unless otherwise indicated. Also all the organic solvents were obtained from Fisher Scientifics, Pittsburgh, PA, USA

HPLC standardization of Olive extract according to the level of hydroxytyrosol.

Preparation of working standard

0.5Kg of fresh leaves were collected from the campus of The University of Jordan and taxonomically identified. Then they were cut and soaked in 500ml methanol to enhance the extractability of hydroxytyrosol. The mixture left for 24 hours with frequent stirring. The extract was completely evaporated and the residue was reconstituted with 200ml ether. Subsequently, the solution was filtered to remove ether-insoluble compounds and evaporated. 100ml of water was added to reconstitute the hydroxytyrosol residue which possessing amphiphilic solubility character. The identity of hydroxytyrosol was confirmed by several systems of HPLC-UV against a relatively-expensive standard material of hydroxytyrosol (phytoplan GmbH, Heidelberg). The standardized working standard solution was entirely dried by freeze drying technique (lyophilization) and stored in refrigerator until used.

Preparation of samples

Methanolic extract of olive leaves were filtered to remove the generated insoluble particles. The filtrate was dried completely andthe residue was then reconstituted with 20ml ether. After that the solution was filtered to remove ether-insoluble compounds and then evaporated. 1ml of water was added to reconstitute the residue. The resulted solution was used to quantify the amount of hydroxytyrosol in extract samples against the working standard.

Chromatographic conditions

HPLC Instrument (Shimadzu, Japan) equipped with a LC-20AT Pump, and SPD-20A UV detector. The chromatographic experiments were performed on a normal phase column (Hypersil silica column 25X4.6cm, 5µm). Mobile phases were applied with different ratios of acetonitrile and 1% acetic acid aqueous solution namely, 20, 50, 80 % acetonitrile for standardizing and confirming the identity of hydroxytyrosol working

standard whereas 50% acetonitrile was selected to monitor the sample stability against the working standard. Flow rate was kept constant at 0.5 mL/min. Injection volume was 20 µL and UV detector was set atλ=280 nm. Additionally, outcomes of this normal phase system were compared with reversed phase system applied under the same conditions by using of C18 column (Ultrasphere, 25X4.6cm, 5µm)**.**

Evaluation of total phenolic content in the olive extract by using Follin-ciocalteu's reagent.

Preparation of the sodium carbonate reagent

200 g of anhydrous sodium carbonate (Laboratory Rasayan, India) was dissolved in 800ml water and heated to boil then left for 24 hours at room temperature after that the solution was filtered and the volume completed to one liter.

Test procedure

14g of fresh olive leaves were cut and soaked in 100ml methanol for 24 hours with frequent shaking. 1ml of it was further diluted to 100ml with methanol. 0.5ml of the final solution was added to 8ml of distilled water and 0.5ml of Follin-ciocalteu's phenol reagent, 2N. The mixture was shaked promptly (<8min). 1.5 ml of sodium carbonate reagent was added and the samples were incubated for 2 hours. Blank preparation was carried out with the same procedure with using methanol instead of extract. Additionally, group of gallic acid solutions treated with same manner were used to establish calibration curve suitable for calculatinggallic acid equivalent of the total phenols in the extract. Final concentrations of gallic acid solutions were 0.06425, 0.1285, 0.257, 0.514 and 1.285mg%. Absorbances of solutions were measured by UV-spectrophotometer.The background absorbance (extract without treatment) has no light absorption within the region of 500-800 nm.

UV-spectrophotometer

All spectroscopic measurements were undertaken using a Spectro UV-VIS Double Beam PC Scanning Spectrophotometer (Model UVD-2950, Labomed , Inc., USA). The analysis was carried out at λ=760 nm.

Results and discussion

There is a group of phenolic compounds and their glycosidic forms responsible of antimicrobial effect in the olive leaf extract. Thus it has been decided to evaluate the content of the total phenols by colorimetric assay method. Meanwhile, it is necessary to also establish a chromatographic method to standardize olive leaf extract. Because hydroxytyrosol is one of the major compounds in olive leaf extract and also it is considered as building unit for the other phenolic molecules in olive leaves, it was decided to use it in standardization of the extract.

HPLC standardization of the extract according to the level of hydroxytyrosol.

The reference standard of hydroxytyrosol was tested depending on reversed phase chromatography with several ratios of water and acetonitrile as mobile phase. It was found that the retention and elution of hydroxytyrosol is not satisfactory enough. Fortunately, hydroxytyrosol is amphiphilic compound so it might be better retained in a normal phase column. Thus a normal phase chromatography using silica column was established for hydroxytyrosol, the results show a well retained peak for hydroxytyrosol promising to pass the validation requirements as shown in **Figure 2**, **A**.

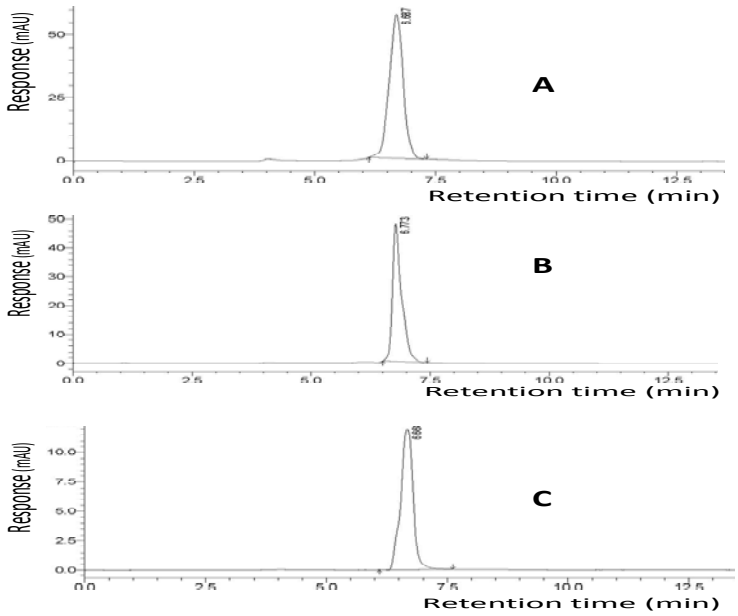

Figure. 2Chromatogramsfrom a normal phase HPLC system forhydroxytyrosol reference standard (A), hydroxytyrosol working standard extracted from olive leaves (B) and hydroxytyrosol in a sample of olive leaf extract (C).

However, the reference standard is quite expensive and sometimes unavailable for routine analysis use. Thus it has been decided to extract hydroxytyrosol from the olive leaves as working standard after standardizing it with the reference standard material. The amphiphilic character of hydroxytyrosol, which gives it a good solubility in polar and non-polar solvents, enables its purification from the other compounds in the extract. **Figure 2, B** depicts a chromatogram of the working standard prepared from the olive leaf extract**.**

Working standard identity was confirmed by retention time similarity with the reference standard material under several compositions of the mobile phase as described in materials and methods topic. Although the working standard is not entirely pure, its content of hydroxytyrosol was determined quantitatively against a pure reference standard material and kept dried until used. HPLC analysis of extract samples depicts pure sharp peak for hydroxytyrosol as shown in **Figure 2, C**. Samples preparation helps in removing water soluble antioxidants such as oleuropein which has strong potential to overwhelm the obtained chromatogram.Additionally, chromatographic separation by normal phase system resolves hydroxytyrosol peak from other interfering compounds along relatively long time period (*ca* 15 min). Interfering compounds were washed from the system between run times of samples. The obtained baseline resolution of hydroxytyrosol peak in a clean chromatogram enables transferring this method from analytical type to preparative one. Additionally, the applied isocratic system is able to keep better stability and precision than any gradient system. Simplicity and stability of the method as well as clean chromatogram facilitate the application of the method in preparative mode to produce hydroxytyrosol in pure form and with cheap cost. This should have a positive impact on countries relying on olive tree as one of their economic resources includingpharmaceutical and food industries.

However, the vast majority of published work about chromatographic separation of hydroxytyrosol was implemented by reversed phase system[28-30]. It was found that baseline resolution of hydroxytyrosol under simple isocratic was rather difficult. The best obtained separation for hydroxytyrosol from reversed phase system is shown at **Figure 3**. This separation will not be satisfactory unless gradient systems are used. However, applying gradient systems make upgrading the analytical method to preparative analysis noisy and troubleshooting.

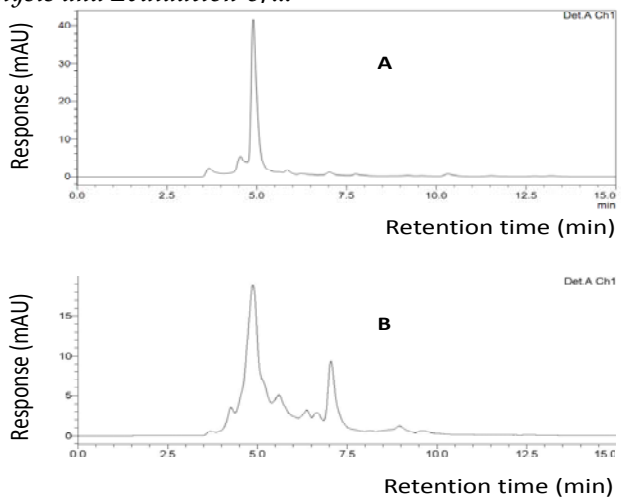

Figure.3 Chromatograms from a reversed phase HPLC system for hydroxytyrosol reference standard (A) and hydroxytyrosol in a sample of olive leaf extract (B). Baseline resolution of hydroxytyrosol was not achieved by applying a reversed phase system.

Validation of HPLC method

Linearity

Stock solution of working material equivalent to 10.3mg% of hydroxytyrosol was prepared and further diluted to six solutions with the following concentrations: 4.12, 3.29, 2.47, 2.06, 1.24 and 0.82 mg%. Each solution was injected triplicate and the average responses were plotted against concentrations as shown in **Figure 4**.

The regression coefficient shows that the method is linear over the range (0.82-4.12) mg%.

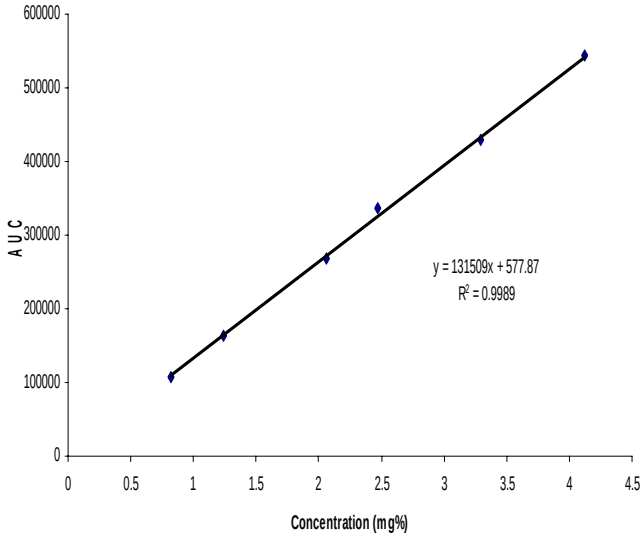

Figure. 4 linearity study of HPLC method of analysis. Response versus different concentrations of hydroxytyrosol prepared from working standard material.

Precision and Accuracy

Six solutions of working standard material with 2 mg/ml equivalent concentration of hydroxytyrosol were prepared to evaluate method precision. Moreover, other solutions of the working standard incorporated in pretested olive leaf extract were tested with the same manner to evaluate the recovery of hydroxytyrosol as shown in **Table 1**. Triplicate injections were carried out for each solution. It was found that relative standard deviations (RSD) of inter-day and intra-day were less than 2.0%(**Table 1**). And also percentages of recovery for all prepared samples are within the range of 97.6-101.2 % (**Table 1**).

Table 1.The results of hydroxytyrosol analytical validation including accuracy and precision.

Trial#	Recovery of added 50%	Recovery of added 100%	Recovery of added 200%	Inter-day Precision (RSD)	Intra-day precision(RSD)
1	49.0±0.5	100.2±0.8	199.2±0.9	1.9%	0.7%
2	48.8±0.6	98.6±1.1	202.4±1.3	1.6%	1.5%
3	51.2±0.2	99.2±1.7	201.9±1.5	1.1%	0.6%

Limits of quantification (LOQ) and detection (LOD)

The limit of quantification (LOQ) was determined according to the least concentration below which linearity of calibration curve deviate from the norm. It was found that LOQ = 8 µg/ml. The limit of detection(LOD) was determined according to three times signal-to- noise ratio (3S/N) procedure. It was found to be 0.8 µg/ml.

Selectivity and Specificity

Selectivity of the method was assessed by comparing chromatograms of blank solvent with sample chromatograms. Specificity of the developed method was assessed by performing forced degradation study. Three solutions were prepared and heated at 80°C for 15 minutes. One of the solutions contained few drops of concentrated HCl solution. A second solution contained few drops of H_2O_2 (30% w/v) and the third one containedNaOH (40% w/v). The purity of the hydroxytyrosol peak was tested by purity test of photodiode array detector. The purity indices of the results were above the purity threshold limit (>0.996). This confirms the purity of chromatographic peak from its degradation products.Consequently, the purity of peak from the interference of degradation products confirms the suitability of it as a stability indicating method of analysis.

Evaluation of total phenols by Follin-ciocalteu's phenol reagent

A group of Gallic acid solutions was prepared according to the procedure mentioned in experimental section. The samples show linear curve suitable to interpolate the gallic acid equivalent value of the olive leaf extract as shown in **Figure 5**.

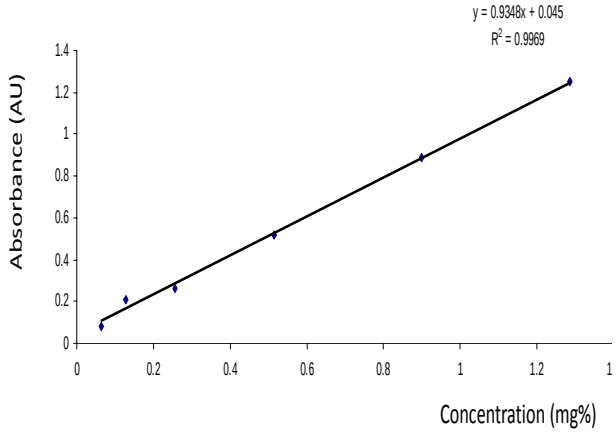

Figure 5 Linear curve of gallic acid tested according to Follin-Ciocalteu's method

On the other hand, the olive leaf extract prepared according to the method mentioned in the experimental part gave rise an absorbance value equal to 0.32. Interpolation of this value in the gallic acid curve shown at **Figure 5** resulted in gallic acid equivalence of 0.3mg%. Depending on this result, phenol content in each 1g olive leaf is equivalent to 40mg of gallic acid.

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تحليل وتقييم لمادة الهيدروكسي تيروزول في مستخلص اوراق الزيتون

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ملخـص

هنالك مجموعه من المركبات الفينوليه ونظائرها الجلوكوسيدية مسؤوله عن الفعل المضاد للاكسدة؛ حيث إن الفعل المضاد للاكسدة من قبل الفينولات يتحلل عادة باستخدام محلول فولين سيكالتو. ولانه من الضروري انشاء طريقة تحليل داله على الثباتكي، ويتم التحقق من الثباتية لمستخلص اوراق الزيتون في المستحضرات الدوائية والغذائية. ولأن مادة الهيدروكسي تيروزول هي من المواد الاكثر توافرا داخل مستخلص اوراق الزيتون والبنية الاساسية للفينولات الاخرى في المستخلص؛ فقد تقرر استخدامه كمؤشر تحليلي للمستخلص.تم استحداث طرقة تفريق لوني ذات طور عادي باستخدام عمود فصل سيليكا هايبرسيل لمادة الهيدروكسي تيروزول. أما الطور المتحرك فيتكون من 1% حمض اسيتيك و اسيتونايتريل بنسبة 1:1. أما سرعة الجريان للطور المتحرك؛ فهي 0,5 مل\دقيقة. وأما حجم العينة المحقنه داخل الجهاز؛ فهي 20 ميكروليتر وطول موجة مقداره 280 نانوميتر.ولقد تبين أن الطريقة خطية ضمن المدى من 0,82 ولغاية 4,12 ملغرام\100 مل. وكذلك فان الطريقة دقيقة، حيث ان الانحراف المعياري اقل من 2% والطريقة صحيحة بنتائج من 97,5 وحتى 101,2 % . كما ان اقل تركيز كمي هو 8 ميكروغرام\ مل و اقل تركيز تحسسي هو 0,8 ميكروغرام\ مل. ولقد تم تطبيق طريقة فولين سيكالتو باستخدام جهاز الاشعة فوق البنفسجية، وتبين ان المحتوى الفينولي في غرام واحد لاوراق الزيتون يعادل قدره 40 ملغراما حمض غاليك في منع الاكسدة.

الكلمـات الدالـة: الهيدروكسي تيروزول، اوراق الزيتون، مضادات الاكسدة.

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