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Quantitative Determination and Cytotoxic Effect of Oleanolic Acid from *Olea europaea* Leaves Extract Cultivated in Iraq

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Abstract

Since its first description as a cytotoxic agent, *Olea europaea* leaves extract gained significant popularity against human breast cancer, ethyl acetate extract of *Olea europaea* leaves obtained by acid hydrolysis method was evaluated *in vitro* as cytotoxic agent against new human breast cancer (AMJ13) cell line, using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. One of the main pentacyclic triterpenoid; oleanolic acid, which was extracted from leaves of *Olea europaea* by well-known two different methods, , the acidic hydrolysis method and basic acidic method, the presence of oleanolic acid was proved in both methods with qualitative and quantitative determination using high performance liquid chromatography (HPLC), the former extract was active against breast cancer AMJ13 cell line with IC50 value of 0.8936 µg/ml.

Key words: Oleanolic acid, Acidic hydrolysis method, Basic acidic method, HPLC, AMJ13 cell line.

التحديد الكمي والتأثير السمي للخلايا لحمض الأولينوليك من أوراق الزيتون المزروعة في العراق

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الخلاصة

منذ وصفه الاولي كعامل سمي للخلايا، اكتسب مستخلص أوراق الزيتون (Olea europaea) شعبية كبيرة ضد سرطان الثدي البشري، وتم تقييم مستخلص أسيتات الإيثيل من أوراق الزيتون التي تم الحصول عليها بطريقة التحلل المائي الحمضي في المختبر كعامل سام للخلايا ضد خط خلايا سرطان الثدي البشري الجديد (AMJ13) باستخدام فحص ٣- [٤،٥-ثنائي ميثيل ثيازول-٢-يل] -٢،٥-ثنائي فينيل تيترازوليوم بروميد (MTT). واحد من اهم التراينيربينويد خماسية الحلقه (oleanolic acid)، الذي تم استخلاصه من أوراق الزيتون بطريقتين مختلفتين معروفتين وهما طريقة التحلل المائي الحمضي والطريقة الحمضية القاعدية، وقد تم إثبات وجود حمض الأولينوليك في كلتا الطريقتين مع التحديد النوعي والكمي باستخدام الفصل اللوني السائلة عالية الأداء(HPLC)، كان المستخلص بالطريقة الاولى نشطًا ضد خط خلايا AMJ13 لسرطان الثدي بقيمة IC50 تبلغ ٠,٨٩٣٦ ميكروغرام / مل.

الكلمات المفتاحية: حمض الأولينوليك، طريقة التحلل المائي الحمضي ، الطريقة الحمضية الأساسية ، الفصل اللوني السائلة عالية الأداء ، خط الخلايا AMJ13

Introduction

Olea europaea (olive), family Oleaceae, is an evergreen small tree[(1)]; native to the Mediterranean region, regarded as a significant crop owing to the remarkable nutritional and therapeutic oil effects[(2)]. Steroids, terpenoids, reducing sugar flavonoids and tannins were detected in olive tree leaves[(3)]. Oleuropein(phenolic compound) and oleanolic acid(triterpene) are the most abundant key components phytochemicals in olive leaves[(4,5)]. Leaves and fruits content of oleanolic acid differ among varieties, olive fruit maturation leads to acid content drastically decreases by 70-80%[(6)].

Oleanolic acid is a secondary metabolite that belongs to an Oleanane type pentacyclic triterpenic acid[(7)], widely distributed in the plant kingdom in greater than 1620 plant species; particular in Oleaceae family, as do in spices, edible crops and medicinal plants; clove, apple, olive, and lanata shrub are included[(8-10)].

Chemically oleanolic acid is 3β-hydroxyolean-12-en-28-oic acid[(3)], naturally, oleanolic acid present in free form (aglycone of many saponins) and as glycoside derived from the mevalonic acid pathway[(9,11,12)]. Oleanolic acid along with its natural and synthetic derivatives attracts much attention because of its diverse pharmacological effects as hepatoprotective, anti-osteoporosis, anti-inflammatory, neuroprotective, hypoglycemic, antitumor and other effects[(13)]. Oleanolic acid demonstrated proved anticancer effect versus lung, gallbladder, pancreatic cancers, leukemia, and osteosarcoma[(14)].

An exhaustive literature survey revealed that quantitative estimation of oleanolic acid from this plant has been recruited by different methods recently,[(15)] however, this study articulates the frame to view the first report with regard to extract, isolate, identify oleanolic acid using TLC and HPLC, to estimate its quantity using HPLC, and evaluate its cytotoxic effect on breast cancer (AMJ13) cell line.

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Materials and method

Plant materials and Extraction of oleanolic acid

Olea europaea leaves were collected in December 2020 from Baghdad, the identification was done by the college of pharmacy/ University of Baghdad while authentication by prof. Dr Sukaena Abbas\ Department of Biology\ College of the Science \University of Baghdad. The leaves were washed with water, and then shade dried at room temperature for three weeks. 40 gm of milled leaves were placed in thimble and extracted with 400 ml methanol for 3 hours using soxhelt apparatus. The solvent was evaporated in a rotary evaporator till dryness to give crude methanolic extract 8.22 gm[16, 17].

Extraction of oleanolic acid

Two methods were used for extraction

Method 1: Acidic hydrolysis of crude methanolic extract

Four grams of methanolic extract was reflexed using 100 ml of 5% HCl for 2 hours. Crude hydrolysis products were extracted with 100ml ethyl acetate two times. Ethyl acetate extract was concentrated in rotary evaporator to give 1.543 gm and the percentage yield was 3.86%. Ethyl acetate extract was stored for further examination[18, 19].

Method 2: Basic –acidic extraction method

Four grams of methanolic extract was reconstituted with an appropriate amount of water and dissolve in the water bath. To about 30 ml of aqueous extract, add10% KOH dropwise till basification (pH about 9). Centrifuge to remove precipitate, take supernatant, and add 20% HCl drop by drop to get an acidic medium (pH about 4) and the precipitate is formed. Centrifuge to get precipitate which contains oleanolic acid and related compounds[20].

Identification of oleanolic acid by chromatographic techniques

Two simple and accurate chromatographic techniques were developed for the determination of oleanolic acid in Iraqi Olea europaea leaves:

Qualitative identification by thin layer chromatography

An analytical TLC was performed for oleanolic acid identification in which Silica gel TLC (GF 254) plate was used as a stationary phase, Toluene: methanol (18:2) was the solvent system, standard solution of oleanolic acid (1 mg/ml), solution of ethyl acetate fraction and extracted oleanolic acid were applied on TLC plate. After development, detection the chromatogram was done by spraying 5% ethanolic H2SO4 and heated in the oven. Oleanolic acid was observed as a purple spot and Rf value was calculated[21].

Qualitative and quantitative identification by RP-HPLC

The oleanolic acid isolated from Olea europaea leaves was analyzed using SYKMN germane HPLC system. The detection was performed at 215 nm, RP- C18-ODS column (250cm x 4.6mm,) was used and the column was maintained at room temperature, the mobile phase was composed of acetonitrile: water (85:15 v/v), the flow rate was 1ml/min and the isocratic mode of elution was used. The volume of the injected standard and sample was 0.1 ml[22, 23].

Evaluation of bioactivity of the ethyl acetate fraction using MTT cytotoxicity assay

To determine the cytotoxic activity of ethyl acetate fraction; human breast cancer cell (AMJ13) was obtained from the Iraq biotech Cell Bank Unit and preserved in RPMI-1640 (Capricorn, Germany), supplemented with 10% fetal bovine (Capricorn, Germany), 100 unit/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA (Capricorn, Germany), reseeded at 50% confluence twice per week, and incubated at 37 °C. The MTT cell viability assay was performed on 96-well plates, Cell lines were seeded at 1 × 104cells/well, 24 hrs later or when the confluent monolayer was done, cells were treated with the tested compound (serial concentrations of ethyl acetate fraction). After 72 hrs of treatment, the viability of the cell was measured by removing the medium or supernatant, MTT solution was added to each well (and incubating the cells for 1.5 hrs. at 37 °C; The MTT test is a sensitive colorimetric and quantitative test, reflects cell viability, proliferation, and toxicity and is based on the ability of the mitochondrial NADPH-dependent oxidoreductase enzymes to reduce the soluble yellow MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) to insoluble purple formazan crystals, the darker the solution the greater number of viable cells[24]. After eliminating the MTT solution, the remaining crystals in the wells were dissolved by adding 130 µL of DMSO (Dimethyl Sulphoxide) followed by incubation at 37 °C for 15 min with shaking[25]. The absorbance was measured on a microplate reader at 492 nm (test wavelength); the assay was carried on in triplicate. The inhibitory rate of cell growth (the percentage of cytotoxicity) was calculated according to the following equation[26]:

% Cytotoxicity = 100 – [(A 492 in extract treated cell / A 492 in extract untreated cell) (100)]

Statistical analysis

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism [27]. The values were presented as the mean ± SD of triplicate measurements [28].

Results and Discussion

Extraction is the crucial primary step in the analysis of medicinal plants to get the desired chemical components for further separation and identification. Olea europaea leaves are regarded to be economical and appreciable source of oleanolic acid; therefore, Different extraction methods

(conventional and non-conventional) were utilized for extraction and separation of oleanolic acid from Olea europaea leaves but for the first time the acidic hydrolysis method and basic acidic method were applied for assessing oleanolic acid presence. Methanol was used as extracting solvent because oleanolic acid is freely soluble in alcoholic solvents with optimum solubility in methanol[17, 29-31].

TLC method development

Analytical TLC (Figure 1) revealed the presence of oleanolic acid in ethyl acetate fraction (method 1) and an oleanolic acid fraction (method 2) since one of the separated spots in these fractions has similar color and Rf value to that of oleanolic acid standard (Rf for oleanolic acid in toluene: methanol (18:2) is 0.43.

HPLC identification and quantification of oleanolic acid

HPLC was chosen as a qualitative and quantitative method for oleanolic acid determination as it is a simple, rapid, efficient, and comprehensive method for triterpenic acids separation in plant extracts(31) . The retention time for the oleanolic acid standard was 9.69 min (Figure 2, A). The chromatogram for ethyl acetate and extracted oleanolic acid fractions (Figure 2, B and 2, C) demonstrated the presence of distinct peaks at 9.70 and 9. 79 min which reflects the presence of oleanolic acid respectively in both examined fractions

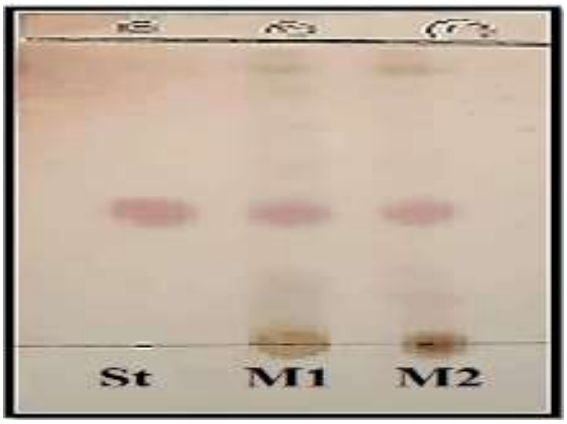

Figure 1. TLC chromatogram of (St: oleanolic acid standard, M1: ethyl acetate fraction by method 1, M2: extracted oleanolic acid by method 2) after derivatization with 5 % H2SO4 reagent and observation at daylight.

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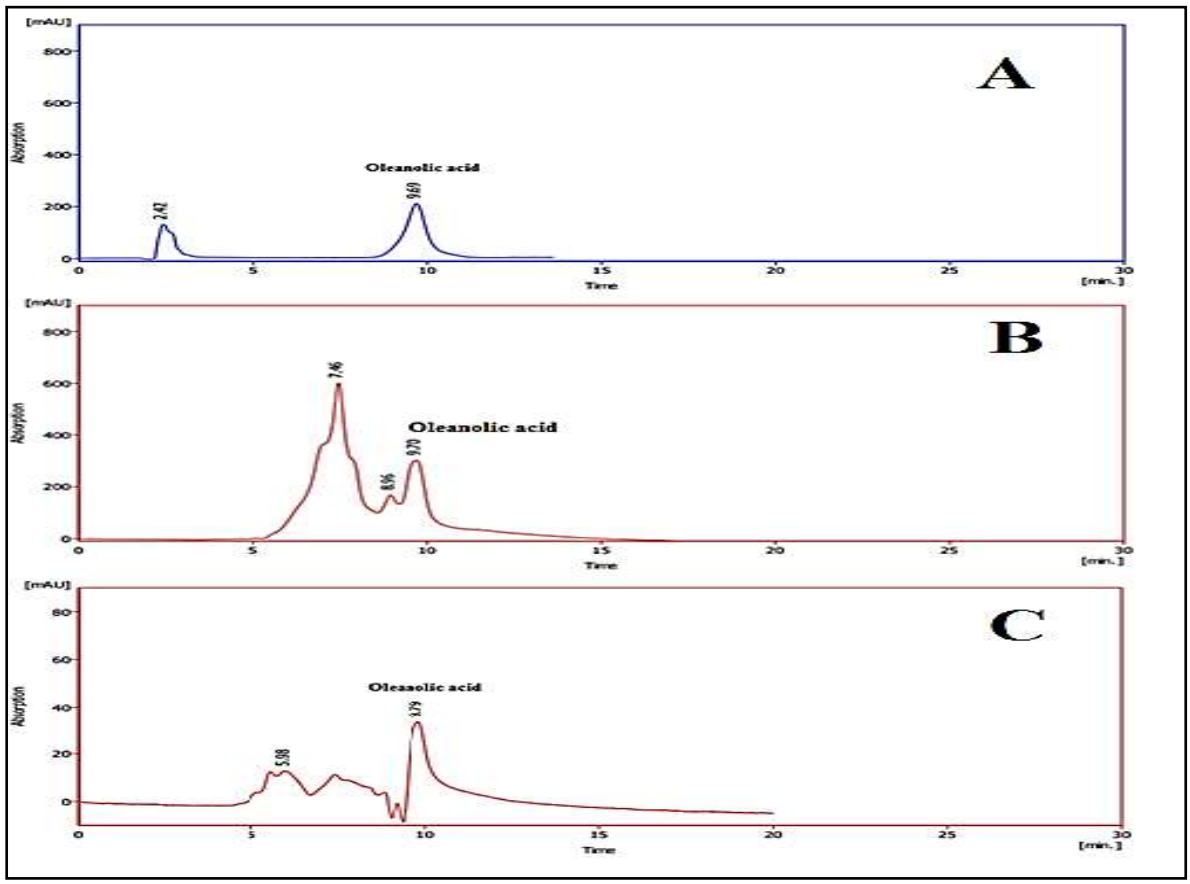

Figure 2. HPLC chromatogram of A: Standard oleanolic acid, B: Ethyl acetate fraction (method 1), C: extracted oleanolic acid fraction (method 2)

Concerning quantitative determination of oleanolic acid in the desired fractions serial dilutions of standard oleanolic acid in methanol were prepared (10, 20,30, and 40 ppm). A plot of area vs concentration of oleanolic acid standard shows a linear fit (Figure 3). This calibration chart was subsequently used for the quantification of oleanolic acid in the analyzed fractions.

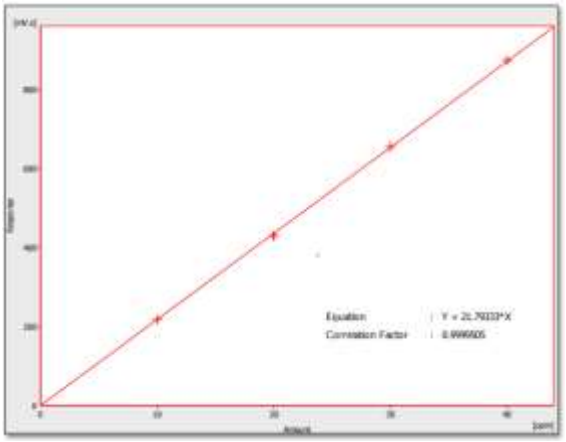

Figure 3. HPLC calibration of oleanolic acid standard

Oleanolic acid amount in the method 2 fraction is lower than its amount in the ethyl acetate fraction; illustrated in Table 1, as the area under the curve and concentration of both fractions prove. This may be attributed to the fact that the oleanolic acid in Olea europeae leaves may be present in bound forms (monodesmosidic and didesmosidic type of glycoside) and acidic hydrolysis of glycosides release aglycone from glycone(s)[19, 32, 33]. In Olea ferruginea leaves oleanolic acid glycosides had been isolated[34].

Table 1. AUC and concentration of ethyl acetate and extracted oleanolic acid fractions

Fraction	AUC (mAU.s)	Concentration (ppm)
Ethyl acetate (method 1)	6111.985	70.414
Extracted oleanolic acid (method 2)	1866.144	21.4

Cytotoxic test

The cytotoxic test was performed to evaluate the toxicity of ethyl acetate fraction of Olea europaea leaves on human tumor cell line; breast ductal carcinoma AMJ13 cells by the MTT test.

As shown in Figure 4, ethyl acetate fraction revealed a cytotoxic activity against the AMJ13 cell line with a minimum cytotoxic effect at 6.25 µg/mL and a maximum cytotoxic effect at 200 µg/mL (Table 2). This effect is concentration dependent.

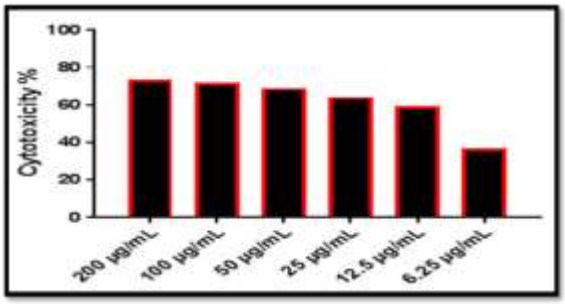

Figure 4. Effect of ethyl acetate fraction over AMJ13 cell growth. The results are expressed as % of cytotoxicity.

Table 2. % Cytotoxicity of ethyl acetate fraction at various concentration

Concentration	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL
% Cytotoxicity	73.40	71.70	69.00	64.30	59.20	36.60

The (IC50) concentrations inducing 50% cell growth inhibition for examined fraction was 0.8936 µg/ml (Figure 5). Morphology of AMJ13 cell before treatment, and after treatment with ethyl acetate fraction is seen in Figure 6.

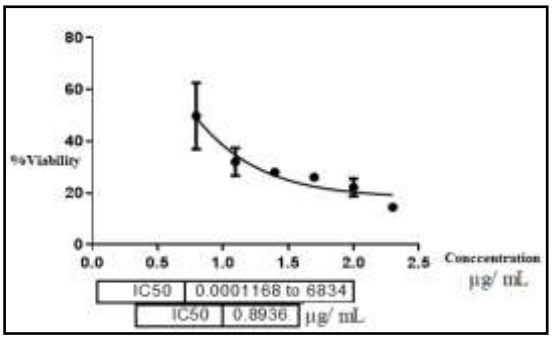

Figure 5. IC_{50} of ethyl acetate fraction on AMJ13 cell line

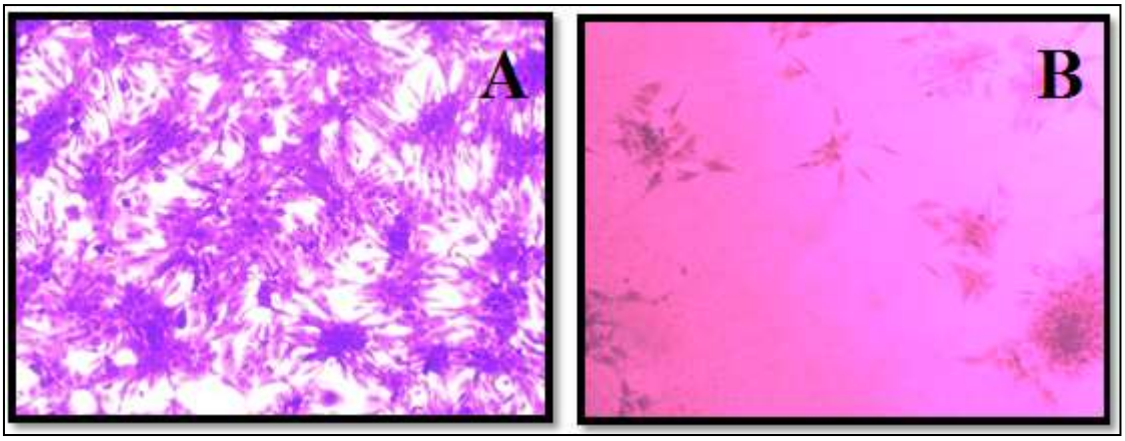

Figure 6. Morphology of AMJ13 cell A: before treatment, B: After treatment

Previous studies demonstrated the anti-proliferative and pro-apoptotic effect of oleanolic acid against breast cancer MCF-7, MDA-MB-231 cells line [35,36]. Oleanolic acid inhibits the cell cycle at various phases and promote apoptosis in the cancer cells via alteration in the expression of the tumor cell cycle regulatory proteins differently [37].

Conclusion

To the best of our knowledge, this is the first study on the extracted oleanolic acid from Olea europaea leaves by acidic hydrolysis and basic acidic methods with HPLC quantification and evaluation of cytotoxic activity against new human breast cancer (AMJ13) cell line. The findings demonstrate that acidic hydrolysis method was better than basic acidic method for oleanolic acid extraction; in addition to, the high cytotoxic potential of this plant for in vitro manner, which is attributed mainly to the occurrence of oleanolic acid and other related compounds detected via MTT cytotoxicity assay.

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Conflict of interest

The authors declare no conflict of interest

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