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Evaluation of the active constituents, Antioxidant, and Antimicrobial Activities of Iraqi Euonymus japonicus leaves using Ethyl Acetate Extract

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Abstract

Background: *Euonymus japonicus* is one species of celastraceous family used as decorative plant and in traditional Chinese medicine. The lack of information about the main active constituents and the possible biological activities of Iraqi *Euonymus japonicus* leaves isconsidered a motivation to start this *in vitro* study

Aim of the study: to identify the phytochemical components and to evaluate antioxidant and antimicrobial activities

Material and Methods: The chemical composition of Iraqi *Euonymus japonicus* leaves was identified and analyzed using the Reversed-Phase High-Performance Liquid Chromatography approach and the antioxidant properties were measured by free radical - scavenging assay DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate). Furthermore, the antibacterial properties were evaluated via theagar well diffusion method against two pathogenic bacteria *Staphylococcus- aureus, E. coli,* and *Candida albicans*.

Results: The results showed that the main active constituents of *Euonymus japonicus* leaves in ethyl acetate fraction were Naringenin, vitexin, Kaempferol, Apigenin, and quercetin respectively.in addition , antioxidant activity ethyl-acetate fraction had the greatest antioxidant activity with the IC50 value of 54.89 µg /mL while the highest antimicrobial efficacy of the ethyl acetate extract (**3.125, 6.25, 12.5, 25, 50, and 100 µg/ml**) was demonstrated by the inhibitory zones (12-19 mm for Staphylococcus- aureus,16-20 mm for *E. coli*,16-21 mm for candida albicans) compared with positive control Augmentin (19 mm) and ketoconazole (16mm) respectively

Conclusion: The first identification of antimicrobial and antioxidant activity of *E. japonic* in vitro, show this plant has thehighest activity compared to standard, this activity isrelated to the polyphenolic compound.

Keywords: Antibacterial, antioxidant and ethyl acetate fraction, *Euonymus japonicus*, polyphenolic compounds.

Introduction:

Many novel compounds with definite uses have been discovered as a consequence of study into natural source elements with biomedical applications Because it has the largest plant diversity in the environment, Euonymus stands out as a signifi cant source of many natural elements, and the majori ty of species have yet to be identified in terms of thei r medicinal capabilities(1). Natural polyphenol chemicals have potential activity in the treatment of different illnesses, including cardiovascular issues, antioxidant, anticancer, anti-inflammatory, and antibacterial capabilities, and pharmaceutical and food industries (2). Additive antioxidants to food to prevent food degradation. Other compounds bind to free radicals, minimizing or inhibiting their adverse effects on the human body(3).

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The degradation of fats and oils caused by lipid oxidation leads to differences in appearance, tas te, and nutritional qualities, whereas oxidative stress is linked to the development of a wide range of diseases(4). Alternative antibiotics, such as plant-based drugs, are being researched as potential substitutes for standard antibiotics. As a result, extensive study has been performed to evaluate the antibacterial impact of polyphenolics, which have been proven to inhibit the growth of a range of hazardous pathogens(5). One of Euonymus cultivated Iraqi species is *Euonymus japonicus* that had been used as a decorative garden plant and as traditional Chinese medicine, *Euonymus japonicus* was used to cure several diseases including liver disorders. The bark has diuretic, tonic, and antirheumatic properties, and the cooking leaves are used to improve difficult deliveries. To the finest of our knowledge, just a few research studies have been done on *Euonymus japonicus* which sparked the interest in conducting scientific research on the polyphenolic composition, andantioxidant and antibacterial activity of Iraqi *Euonymus japonicus* ethyl acetate leaves extract.

Materials and Methods:

Chemicals and Apparatus: All of the compounds and solvents used were of analytical quality and obtained from Scharlab S.L., Spain, except acetonitrile and methanol, that HPLC quality and obtained from (Sigma-Aldrich), Germany. Naringenin, Vitexin, Apigenin, quercetin, and Kaempferol standards were given by Chengdu Biopurify Phytochemicals/China. In this study, High-Performance Liquid Chromatography [HPLC] [Knauer Germany 10AV-LC], a rotary evaporator (BCHI Rotavapor R-205, Swiss), and a Sonicator (Baranson Sonifier, USA) were used. Plant sample Collection and Identification: *Euonymus japonicus L*. plant material consists of fresh leaves cultivated in Iraq and manually harvested between October and November 2021, from Zayona plant nurseries in Baghdad, Iraq. Taxonomist is Dr. Israa Abdulrazaq, this plant was found and authenticated in the biology department of the College of Science/University of Baghdad. Before extraction, the plants were dried in the shade at room temperature and pulverized using an electrical mill, and quantified. Extraction of *Euonymus japonicas* fifty grams of the powdered plant material were extracted via maceration in hexane for seven days with frequent shaking, at room temperature, the extract was filtrated, this procedure was repeated three times for da defatting purposes, then the plant material was left to dry and suspected to extraction by ahot extraction method that was done by using Soxhlet with 85% methanol as a solvent.The extract of methanol was filtrated, and the solvent was dried at a low pressure utilizing a rotary vacuum evaporator at a temperature no higher than 40 oC, yielding a dark green residue known as acrude extract. Ethyl acetate was used to partition the crude extract (3x200 ml), dried over anhydrous sodium sulfate, filtrated, vacuum-dried, weighed, and submitted to qualitative and quantitative analyses, as well as biological evaluation(6) Qualitative and quantitative estimation of polyphenolic compounds by RP-HPLC: An accurate, simple, and rapid RP-HPLC method may reveal numerous the composition of a plant extracts. It is also used for qualitative and quantitative extraction content evaluation such as through calibration (linear least square regression equation) of studied component for validated reference standards under the same chromatographic circumstances through ofh us frapprochement of retention time and UV spectrum shape. In this work, the ethyl acetate fraction of Euonymus japonicus leaf portions was determined using this approach. Preparations of standard and samples for analysis: Five polyphenol and dried leaves fractions of ethyl acetate reference standards for RP-HPLC analysis were prepared by being dissolved in methanol and sonicated at 60% obligation cycles for 30 min. at 25°C, followed by 15 minutes centrifugation at 3000 rpm. The resultant solution from the sample was dried, and the remainders were individually suspended in 1 ml of HPLC grade methanol, crushed with a homogenizer, and passed through a 0.45 mm membrane filter before being stored at 4°C for future analysis. For analysis, 20L of each sample and standard were put into the HPLC apparatus(7). RP-HPLC conditions for qualitative and quantitative analysis: Using a Knauer Germany 10AV-HPLC system, the ethyl acetate fraction of Euonymus japonicus leaves and four concentrations of each reference standard were analyzed. The results were carried out at 280 nm using an RP- C18-ODS column (250 mm x 4.6 m particle size), which was kept at room temperature. Solvent A (1% acetic acid in HPLC grade water) and solvent B comprised the mobile phase (acetonitrile). The descent elution mode was used with a flow rate of 1ml/min. B:60%,40-50min A:10% B:90%,50-55min A:90% B:10%,60% B:10%,60% B:10%,60% B:10%,60% B: The volume of the implanted standard and sample was 50 µL (8). Evaluation of Antioxidant Activity. The antioxidant capability of the extracts was evaluated via the (DPPH)free radical scavenging assay (8,9). The decolorization of the DPPH methanol solution revealed the plant extractives' capacity to donate hydrogen atoms. When antioxidants are present, the mauve color of [2, 2-diphenyl-1-picrylhydrazyl (DPPH)] in methanol is solutionmodified t iso a creamy color. which involved dissolving 10mg of DPPH in a methanol-DMSO combination (9/1 v/v) and adding 0.5 ml of ethyl acetate sample examined at different concentrations (200mg, 100mg, 50mg, 25mg, 12.5mg) to 3.5 ml of DPPH solution. The sample is incubated in the dark for one hour at 37°C. The absorption was measured at 517nm. Ascorbic acid was employed as a reference standard(10). Calculation of IC50: The IC50 of each analytical sample was calculated as follows: At each of the six sites, inhibition ratios (y) were plotted versus sample concentrations (x), and the associated regression line (y = ax + b) was generated (figure 1). The regression line did not have to pass through the basis. Because the inhibition curve was not precisely straight but somewhat curved, the IC50 value may alternatively be calculated using interpolation by joining the two points around the 50% inhibition point with a straight line(1) .

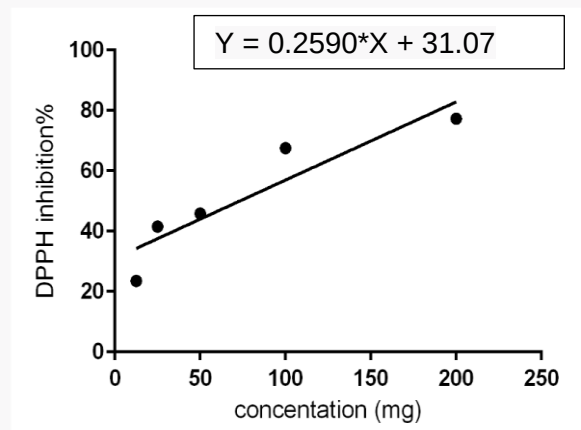

Figure (1): **Scavenger activity of Euonymus japonicus ethyl acetate extract**

Evaluation of Antimicrobial Activity:
Antimicrobial test: Two groups of bacteria (one gram-positive: S. aureus, one ggram-negative E. coli, and one fungus: C. albicans) were obtained from the Alrazi Research Center's/ Department of Biology, Microbiology Laboratories.
Preparation of sample solution: To assess bactericidal activity, 0.1 g of ethyl acetate fraction was dissolved in 5 mL of DMSO separately. The resultant concentration was then diluted to provide six concentrations (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, and 3.125mg/ml). The antimicrobial activity of an ethyl-acetate fraction of *Euonymus japonicus*. Leaves against infection-causing fungal and bacterial strains were investigated. All bacterial strains were cultivated on Mueller Hinton agar (MHA) for 24 hours at 37°C. The extracts' antibacterial activity was then tested using the Agar well diffusion technique (12). For the production of Petri plates, sterile MHA was utilized as a medium. The test strains were swabbed over the top of the hardened medium and allowed to dry at room temperature. For ten minutes before forming 5 wells (6 mm in diameter) on the surface of the solidified petri plate using a sterilized cork borer. The inoculation plates were incubated at 37°C for 24 hours after 12–15 minutes of diffusion time at room temperature. Finally, of the incubation period, the antibacterial Antimicrobial activity was identified by measuring the zone of inhibition in millimeter (mm) units of length. Plant fractions were investigated for antibacterial Antimicrobial efficacy against three microorganisms: *S. aureus*, *E. coli*, and *Candida albicans*. The negative control was DMSO solvent, while the positive control was ketoconazole,e, amoxicillin, and clavulanic acid. The inhibitory zones' sizes were measured to the nearest millimeter (mm) (12-(14))

Results:
Quantity and Percentage yield of fractions:
Based on defatting, extraction, and fractionation dry weight by different solvents, the extracts of *Euonymus japonicus* leaves gave varying percentages. The amounts and percent yields of extracts in n-hexane, methanol, and ethyl acetate were determined and presented in Table 1.

Table (1): The quantities and percentage yield of the extracts with n- ,hexane, methano,l, and ethyl acetate:

Fraction of plant extract	Quantity	Percentage yield
n-hexane	5gm	0.05%
Methanol	18gm	0.18%
Ethyl acetate	1gm	0.01%

3.2 RP-HPLC analysis: A qualitative characterization of flavonoids was performed on RP-HPLC fingerprinting method based on the distribution and relative amount of five bioactive flavonoids was established for the quality evaluation of Iraqi *Euonymus japonicus*, Separation of the crude flavonoid extract was achieved on a column filled with C_{18} material with high carbon content, as identity in Figure (2).

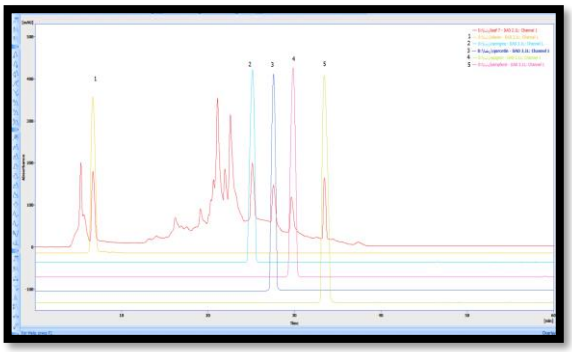

Figure (2): HPLC chromatogram of *Euonymus japonicus* leaves (ethyl- acetate fraction).
An excellent matching fitness was adopted between the UV spectra of the ethyl acetate and five standards of phytochemicals; which are Naringenin, Vitexin, Kaempferol, Apigenin, and quercetin as shown in Figure (3) respectively revealing a clue to the existence of these phytochemicals in *Euonymus japonicus* Iraqi plant(15).

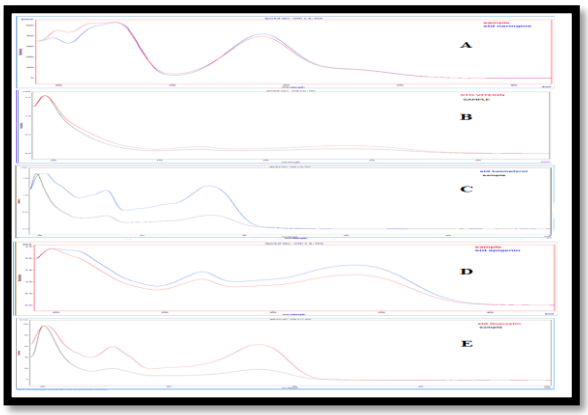

Figure (3): UV spectrum matching fitness of ethyl acetate fraction with A: Naringenin, B: Vitexin, C: Kaempferol, D: Apigenin, and E: quercetin standards.
It is worth treferringto thechromatogram of ethyl acetate fraction which has many significant compounds that didn't fit the standards used reflecting a ford to more extensive studies with additional standards. Because the area under the peak in the HPLC technique is proportional to the concentration of certain compounds, calibration curves for five matched standards were utilized for quantitative assay, and ethyl-acetate fraction peak areas of noticed compounds were utilized to calculate the concentration of each one in the fraction using the first line equation Y = aX + b, slop = Y (area under the curve)/X (concentration mg/mL) (16). The

quantitative concentration of the five polyphenolic components in the ethyl-acetate fraction indicated that Naringenin had the greatest concentration, while quercetin had the lowest as in tTable2

Table (2): The concentrations of the detected polyphenolic compounds from Iraqi *Euonymus japonicus* leaves ethyl acetate fraction:

Experimental Sample	Peak Area	Concentration in ethyl-acetate fraction µg /mg	Concentration in Plant µg /g
Vitexin	225.275	0.297333053	2.824664
Quercetin	53.537	0.056177663	0.5336878
Naringenin	164.092	0.328453811	3.1203112
Apigenin	270.157	0.231369444	2.19800972
Kaempferol	142.11	0.280214779	2.6620404

Analysis of DPPH Scavenging Activity: Natural product researchers see the DPPH scavenging test as the most valuable approach for determining the antioxidant activity of plant extracts. DPPH (2,2-dipheny-l-picrylhydrazyl) is a dark violet/ purple stable free radical that reacts with phenolic compounds to form yellow to olorless 2,2-dipheny-l-picrylhydrazine (DPPH-H) (17) . The DPPH quenching intensity is proportional to the phenolic content. Figure 3 depicts the DPPH scavenging ability of *Euonymus japonicus* leaves (ethyl acetate fraction). By increasing the extract concentration, all of the tested leaf sample extracts showed a progressive fading of the purple-colored radical DPPH into yellow-colored DPPH-H(18) . Among plant elements, phenolic and flavonoids are regarded as powerful free radical scavenging chemicals, and hence may be used as a measure of free radical scavenging capability. The antioxidant activity of *Euonymus japonicus* ethyl-acetate fraction was assessed by its capacity to scavenge DPPH free radicals in comparison to vitamin C. The DPPH radical scavenging effects of both plant fractions and the standard were represented as half maximum inhibitory concentration (IC50) values, and the findings are shown in Table 3. A lower IC50 value indicates more DPPH radical scavenging activity. According to the data, the ethyl acetate fraction had strong DPPH activity with an IC50 value of 54.89 g mL^{-1}, whereas the IC50 of vitamin C as a control was 26.35 g mL^{-1}

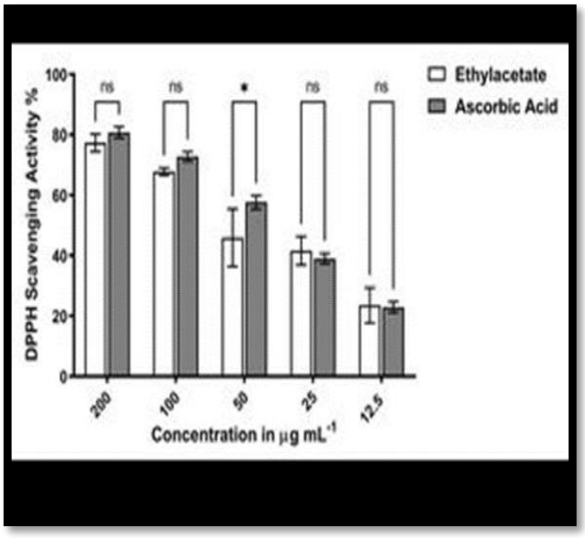

Figure (4): Barograph of %DPPH inhibition of ethyl-acetate fractions of leaves of *Euonymus japonicus* L. and Ascorbic Acid

Table 3: Percentages and IC50 values for free radical scavenging activity of ethyl acetate fraction and ascorbic acid standard.

Concentration(µg /ml)	Ethyl acetate fraction(mean ± standard deviation)	Ascorbic acid standard(mean ± standard deviation)
12.5	23.50±5.824	22.90±1.836
25	41.59±4.690	39.00±1.732
50	45.87±9.562	57.60±2.207
100	67.75±1.270	72.73±1.617
200	77.31±2.912	80.73±1.872
IC50 value	54.89	26.35

Antibacterial and antifungal activity of Euonymus japonicas:

In recent years the inappropriate and overuse of antibiotics has increase resistance to currently accessible antibiotics. due to this development, a critically important task to investigate a new and better antimicrobial drug that are more efficient and less harmful. plant rich in active constituent that require to be isolated and scrscreenedr their therapeutic activity (19). The existence of inhibitory zones was utilized to evaluate the antibacterial activity of Euonymus japonicus leaves (ethyl acetate fraction) against the microorganisms employed in the study (20). As shown in figure 4 and table 4, ethyl acetate fraction at 25 **µ**g/ml exhibited a higher inhibitory effect against Staphylococcus aureus with respective inhibition zones of 19 mm, displaying a similar potent inhibitory effect of antibacterial activity as compared to the positive control antibiotic (Augmentin®) with an inhibition zone of 19 mm. Furthermore, the antibacterial examination revealed that the ethyl acetate fraction at 100 **µ**g/ml has a possible antibacterial impact against gram-negative

bacteria. This impact demonstrated a powerful inhibitory effect of antibacterial activity against Escherichia coli with corresponding inhibition zones of 20 mm as compared to a positive control antibiotic (Augmentin®) with an inhibition zone of 19 mm. In this study, *Euonymus japonicus* leaves (ethyl acetate fraction) presented higher antibacterial activity versus gram-negative bacteria. In this study, *Euonymus japonicus* leaves (ethyl acetate fraction) had more antibacterial action against gram-negative bacteria than gram-positive bacteria. For antifungal activity, the ethyl acetate fraction exhibited strong effect against *Candida albicans* pathogens with inhibition zone diameter 21mm of at 25 µg /ml as compared to theositive control antibiotic (Ketoconazole) aandinhibitiontion zone of16mm. these findings might be attributed to the major components that weret discovered for the first time in this study and involved Naringenin, Vitexin, Kaempferol, Apigenin and quercetin in Iraqi *Euonymus japonicus* leaves ethyl acetate fraction.

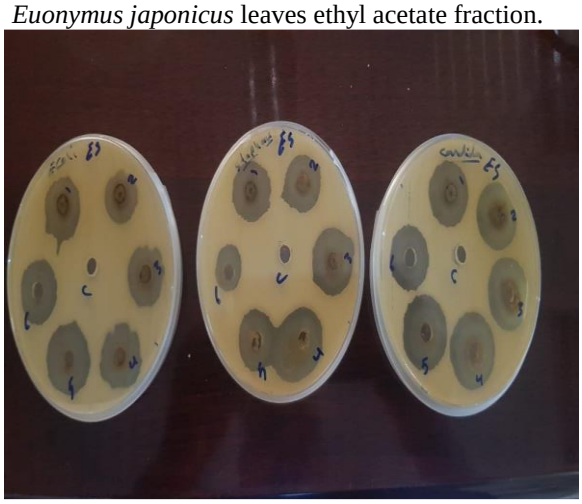

Figure (5): Antibacterial activity of ethyl acetate

Table (4): The antibacterial activity tration of ethyl acetateconcentrations

The concentration			
Concentration of extract µg/ml	S*taphyloc occus aureus*	*Escherichia coli*	*Candida albicans*
100	18	20	20
50	17	18	20
25	19	16	21
12.5	18	17	18
6.25	15	18	18
3.125	12	16	16
DMSO (Negative control)			NA
Augmentin ® (positive control)			19

means mean not ***activmeansoncentration the of positive control 10µg/µ >***

Discussion:

The evaluation of the medicinal value of a plant; considered as biosynthetboratory stock; depends on the biologically active constituents and the amounts of these constituents. To check the presence or absence of these bioactive constituents, basic operation steps include pre-washing, drying of plant materials, grinding, and extraction the desired chemical components from the plant materials for further separation and identification by multiple techniques were performed(21). HPLC is a dominant higher performance diverse analytical applications technique for its versatility, reproducibility, higher resolution, and sensitivity precision. Five polyphenolics; Naringenin, Apigenin, Vitexin, quercetin, and Kaempferol had been found in good amounts in Iraqi *Euonymus japonicus*. Apigenin and Vitexin are widely recognized for their anti-oxidant, anti-inflammatory, cytotoxic, antinociceptive, and neuro-protective properties (22), while quercetin has a main role in activities like cytotoxic, antioxidant, anti-inflammatory, anti-aging and neuro-protective (23) and Naringenin acts as anticancer (24). All of the above activities suggest that *Euonymus japonicus* has a potential future in pharmaceutical research since it includes a variety of secondary metabolites, particularly polyphenolic components, which were discovered for the first time in this study. Two evaluation biological studies had been done, the first Iraqi study and perhaps worldwide about the antioxidant and antimicrobial activities of Iraqi *Euonymus japonicus* leaves. *Euonymus japonicus*, a traditional Chinese medicinal plant known to cure several diseases, however its pharmacological and chemical bases of action are not well understood. For antioxidant activity, the results of the current study are concordant with a previous study which evaluated the antioxidant activity of aqueous and methanol extracts from *Euonymus japonicus* aerial parts(2). For the first time, this study deals with the evaluation of antimicrobial effect of *Euonymus japonicus* leaves with regard to the (Augmentin®) and Ketoconazole standards group, the leaves extract (ethyl acetate fraction) had more antibacterial action against gram-negative bacteria than gram-positive bacteria at concentration 100 µg/ml. For antifungal activity, the ethyl acetate fraction exhibited strong effect against Candida albicans pathogens at concentration 25 µg /ml as compared to positive control (Ketoconazole). These findings might be attributed to the major components that discovered for the first time in this study and involved Naringenin, Vitexin, Kaempferol, Apigenin and quercetin in Iraqi *Euonymus japonicus* leaves ethyl acetate fraction.

Conclusions:

To the best of our knowledge, this is the first study on the detection of five flavonoids from *Euonymus japonicus* leaves by RP-HPLC with quantity estimation and evaluation of antimicrobial and antioxidant activities. The findings demonstrate that main active constituents of *Euonymus japonicus*

leaves in ethyl acetate fraction were Naringenin, vitexin, Kaempferol, Apigenin and quercetin respectively; in addition to, the high antimicrobial and antioxidant potential of this plant for *in vitro* manner, which is attributed mainly to the occurrence of these flavonoids detected via agar well diffusion method and free radical - scavenging assay DPPH respectively.

Authors' declaration:

Conflicts of Interest: None

We hereby confirm that all the Figures and Tables in the manuscript are mine/ ours. Besides, the Figures and images, which are not mine /ours, have been given permission for re-publication attached with the manuscript.-

Ethical Clearance: The project was approved by the local ethical committee in College of pharmacy, University of Baghdad, according to the code number (5.10.2022).

Author's contributions:

Rasha abdulrida : MSC

Thukaa Z. Abdul-Jalil : Supervisor

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تقييم المكونات النشطة و مضادات الاكسدة ومضاد الميكروبات لاوراق نبات الشمشار العراقي باستخدام مستخلص خلات الاثيل

د. رشا عبد الرضا / كلية الصيدلة / جامعة بغداد
د. تقى عبد الجليل / كلية الصيدلة / جامعة بغداد

الخلاصة:
خلفية البحث: ق واحد من نوع من عائلة السلاسية المستخدمة كنبات الزخرفية والطب الصيني التقليدي. يعتبر نقص المعلومات حول المكونات النشطة الرئيسية والأنشطة البيولوجية المحتملة للشمشار العراقي دافعا لبدء هذه الدراسة في المختبر
الأهداف: تحديد المكون الكيميائي النباتي وتقييم النشاط المضاد للبكتيريا المضاد للأكسدة
طرق العمل: تم تحديد التركيب الكيميائي وتحليله باستخدام نهج الكروماتوغرافيا السائلة المعكوسة - المرحلة عالية الأداء وتم قياس الخصائص المضادة للأكسدة بواسطة مقايسة الجذور الحرة - الكسح (DPPH). علاوة على ذلك ، تم تقييم الخصائص المضادة للبكتيريا عن طريق طريقة نشر الأجار بشكل جيد ضد اثنين من البكتيريا المسببة للأمراض والمبيضات البيضاء
النتائج: أظهرت النتائج أن المكونات النشطة الرئيسية لأوراق الشمشار في جزء خلات الإيثيل كانت Naringenin و vitexin و Kaempferol و Apigenin و quercetin على التوالي. كان للنشاط المضاد للأكسدة لجزء من خلات الإيثيل أكبر نشاط مضاد للأكسدة مع قيمة IC50 54.89 ميكروغرام / مل في حين أن أعلى فعالية مضادة للميكروبات لمستخلص خلات الإيثيل (3.125 و 6.25 و 12.5 و 25 و 50 و 100 ميكروغرام / مل) من خلال المناطق المثبطة (12-19 مم للمكورات العنقودية الذهبية ، 16-20 مم للإشريكية القولونية ، 16-21 مم للالمبيضات البيضاء) مقارنة بالتحكم الإيجابي (19 Augmentin مم) والكيتوكونازول (16 مم) على التوالي
الاستنتاجات: في هذه الدراسة ، استخدام تقنية RP-HPLC التي وفرت الأشعة فوق البنفسجية مرفق ومجموعة من معايير البوليفينول الناتجة ؛ لأول مرة ، في تحديد وقياس خمسة مكونات من البوليفينول التي تنتمي إلى فئة الفلافونويد في الأوراق العراقية جزء خلات الإيثيل ، ، حيث كان لدى نارينجينين أعلى تركيز يليه فيتكسين وكيمبفيرول وأبيغينين بينما كان كيرسيتين أقل تركيز. بالإضافة إلى الإمكانات العالية المضادة للأكسدة والمضادة للبكتيريا في المختبر للنبات والتي قد تعزى بشكل رئيسي إلى حدوث مركبات البوليفينول المكتشفة عن طريق فحص الكسح الجذري (DPPH) وطريقة نشر آجار جيدا على التوالي.
الكلمات المفتاحية: الشمشار ، مركبات البوليفينول ، المضادة للبكتيريا ، مضادات الأكسدة وجزء خلات الإيثيلز