Antibacterial activity of olive extracts (*Olea europaea*) against multidrug-resistant Pseudomonas aeruginosaI isolated from patient wounds and burns.

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Abstract

Background : Multidrug-resistant Pseudomonas aeruginosa (MDR-PA) with multiple and intricate mechanisms of resistance is considered a significant clinical problem for burn and surgical units. It is one of the most difficult bacteria to eliminate since it has developed an extensive resistance against antibiotics that doctors frequently use and also, recently identified ones. **Methods**: A total of 130 swabs were collected from burn and surgical wards in various clinical areas. Besides multiplex PCRs detection for resistance genes (oprD, mexB and algD), confirmatory bacterial identification was done by the VITEK 2 system . Antibacterial activity was evaluated by the Kirby–Bauer disk diffusion method and MIC and MBC were determined. Quantitative assessment of biofilm inhibition was performed using the ubiquitous microtiter plate assay. **Results**: Out of 130 samples, 57 MDR-P. The prevalence of confirmed *P. aeruginosa* isolates was found to be 43.8%. In the plant extracts tested, the ethanolic extract demonstrated Maximum inhibitory acivity at experimental concentration of 200 mg/ml (23.1 ± 1.4 mm inhibition zone vs reference antibiotics). MIC and MBC values were between 6.25 to 12.5 mg/ml Maximum biofilm inhibition was 76.2% at varying concentrations as well. **Conclusions**: The HPLC-MS analysis revealed oleuropein , hydroxytyrosol and apigenin as dominant bioactive compounds. These results indicate that olive extracts are a potential source of anti-MDR P. aeruginosa therapeutic agents from nature.

Keywords: *Olea europaea*, MDR-*Pseudomonas aeruginosaI*, oleuropein, MIC, burn wounds .

Introduction

Pseudomonas aeruginosaI bacteria are among the top ten challenging pathogenic microorganisms in hospital settings, according to the World Health Organization Critical Priority list of pathogens that urgently require the discovery of new medicines [1]. This bacterium's exceptional danger stems from a combination of three characteristics: its opportunistic nature, which selectively targets immunocompromised patients, multidrug resistance against almost all antibiotic classes, and an unprecedented ability to form biofilm on both living and non-living surfaces. [2]. Burn wounds represent the most susceptible environment for P. aeruginosa infection; international statistics reveal that this bacterium is responsible for 17-30% of infections in specialized burn units and 60-70% of deaths associated with surgical infections in burned patients [3]. In the Iraqi context, a study by [4] indicated that *P. aeruginosa* is the primary cause of infections in burn units, accounting for 38.4%, with carbapenem resistance rates exceeding 65%. P. aeruginosa possesses a wide arsenal of resistance mechanisms: loss of the OprD porin channel responsible for carbapenem resistance, efflux of antibiotics via pumps such as MexAB-

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OprM, MexCD-OprJ, and MexXY-OprM, production of extended-spectrum beta-lactamases (ESBL) and metallo-beta-lactamases (MBL), as well as target site modification and formation of a highly dense biofilm that increases MIC values by 10-1000 times [5] [6] .

Given this alarming situation, there is a growing need to investigate natural sources with antimicrobial activity. olive plant (*Olea europaea* L.) stands out From the Oleaceae family, it is considered one of the richest available plant sources of active phenolic compounds, particularly oleuropein, hydroxytyrosol, and apigenin, which have proven broad antimicrobial efficacy in multiple studies [7]. Oleuropein is a secoiridoid glycoside that represents the main phenolic compound in olive leaves, with a concentration reaching up to 140 mg/g in dry matter. Molecular studies reveal that oleuropein acts thru multiple-target mechanisms: it inhibits the quorum sensing system responsible for the production of virulence factors and biofilm formation in P. aeruginosa, disrupts bacterial cell membrane integrity, inhibits vital enzymes such as DNA gyrase and topoisomerase IV, and obstructs the MexAB-OprM efflux pumps [8] [9]. Promising activity of olive extracts against P. aeruginosa was reported in recent scientific literature; [8] that showed 78% inhibition of carbapenem-resistant P. aeruginosa isolates with MIC between3.9-15.6 mg/ml'. At a concentration below the minimum inhibitory concentration (MIC), hydroxytyrosol inhibits biofilm formation with 68.4% reductions in density [9] On the other hand, local studies related to olive extracts associated with clinical P. aeruginosa isolates originated from environment of Kirkuk are limited which lead us to conduct this study.

Herein this study aims to (1) isolate and identify MDR-PA strains from patients' wounds and burns at Kirkuk Teaching Hospital with molecular characterization for resistance genes; (2) prepare three different extracts of olive leaves chemically analyzed by HPLC-MS;(3)evaluate their antibacterial efficiency using inhibition zones, MICs, MBCs;(4)evaluating the capacity effective of extracts on biofilm formation and finally (5)detection effect of all those extracts in gene expression toward quorum sensing genes.

Material and Methods

Source of Samples and Ethical Approval

A total of 130 wound and burn swabs were collected from patients residing in the burn unit and surgical ward at Kirkuk Teaching Hospital during the period from January to July 2025. The samples included: burn swabs (38%), surgical wounds (32%), chronic wounds (20%), and traumatic wounds (10%).

Bacterial Isolation and Identification

The samples were cultured on selective Cetrimide Agar , Mueller-Hinton Agar, and King B Agar. The distinctive green colonies were identified by: Gram staining, metabolic characteristics (glucose oxidation not fermentation), oxidase test, and production of fluorescent pigments (pyoverdine, pyocyanin). The final identification was confirmed using the VITEK 2 Compact system (bioMérieux, France).

Molecular detection of resistance genes by multiplex PCR

DNA was extracted using the boiling method, and multiplex PCR was performed to detect three resistance genes: oprD (carbapenem resistance, amplification product of 393 base pairs), mexB (MexAB-OprM efflux pump, 394 base pairs), and algD (biofilm/alginates

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formation, 571 base pairs). It was classified as MDR according to the criteria of [10] with resistance to three or more classes of antibiotics.

Preparation of plant extracts

Fresh olive leaves were collected from certified orchards in the Dubz district/Kirkuk, dried in the shade at 35°C for 96 hours, and then ground. Each extract was prepared by dissolving 100 grams in 1000 ml of solvent (distilled water, 70% ethanol, 80% acetone) with continuous stirring for 72 hours at 25°C. Filtered thru Whatman No. 1 paper and then evaporated using a rotary evaporator at 40°C. The dry extract was dissolved in 1% DMSO at concentrations of (25, 50, 100, 200 mg/ml).

Chemical analysis and efficacy evaluation

The chemical analysis was conducted using the HPLC-MS/MS technique (Agilent 1260 + AB SCIEX QTRAP 5500). The efficacy was evaluated using the Kirby-Bauer method and the determination of MIC and MBC thru serial dilution. Biofilm inhibition was evaluated using 96-well microtiter plates and crystal violet staining. Imipenem (10 micrograms) was used as a positive control. The statistical analysis was conducted using SPSS v.26 and GraphPad Prism 9 (One-Way ANOVA + Tukey, $P < 0.05$).

Results and Discussion

Prevalence and Clinical Distribution

Figure (1) and Table (1) show that *Pseudomonas aeruginosaI* bacteria were isolated from 79 out of 130 clinical samples, accounting for 60.8%. Among these isolates, 57 were classified as multidrug-resistant (MDR), representing 43.8% of the total samples and 72.2% of the *Pseudomonas aeruginosaI* isolates. Molecular analysis using multiplex PCR showed that 91.2% of the isolates carried the mexB gene, while the oprD gene associated with carbapenem resistance was recorded at 78.9%. Meanwhile, the prevalence of the algD gene associated with biofilm formation reached 84.2% of the total multidrug-resistant isolates.

Chemical analysis using HPLC-MS technique

The results shown in figure (5) and table (5) of the ethanolic extract analysis using the HPLC-MS technique revealed the presence of seven main active compounds, which were: oleuropein at a concentration of 28.4 mg/g, hydroxytyrosol at a concentration of 18.2 mg/g, apigenin at a concentration of 11.6 mg/g, gallic acid at a concentration of 9.3 mg/g, luteolin at a concentration of 7.4 mg/g, quercetin at a concentration of 5.8 mg/g, in addition to ellagic acid at a concentration of 4.2 mg/g.These results indicate the richness of the ethanolic extract in phenolic and flavonoid compounds with high biological activity, which may contribute to antibacterial efficacy and biofilm inhibition against multidrug-resistant *Pseudomonas aeruginosaI* isolates

Results of inhibition zones (Kirby–Bauer method)

Figure (2) and Table (2) show that the ethanolic extract exhibited higher antibacterial activity than the other extracts at all tested concentrations ($P < 0.001$), with an average inhibition zone diameter of 23.1 ± 1.4 mm at a concentration of 200 mg/ml, which is close to the inhibitory effect of the antibiotic imipenem, recorded at 26.8 ± 0.8 mm. The acetone extract also showed moderate efficacy, while the aqueous extract recorded the lowest inhibition values. The statistical analysis showed that the increase in the extract concentration correlated significantly with an expansion of inhibition halos ($r = 0.98$,

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P<0.001), which indicates a clear dependence between antibacterial activity and concentration level within this study range.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The ethanolic extract have the least values of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration were 6.25 mg/ml,12.5mg /ml in this study as shown in Table (3), The MBC/MIC ratio was 2 suggesting bactericidal effect. On the other hand, the least antimicrobial activity was demonstrated by aqueous extract with MIC values from 50–100 mg/ml which showed that this extract is less efficient than ethanolic and acetonic extracts in inhibiting, growing and killing multidrug resistant *Pseudomonas aeruginosaI* isolates..

Biofilm Inhibition

The results in figure (4) and table (4) of the microtiter test showed dose-dependent inhibition for all extracts. The inhibition rate of the ethanolic extract was (76.2 ± 3.1%) at 200 mg/ml, which is considered to be of high clinical significance. Statistical analysis confirmed a strong positive correlation between concentration and inhibition ($r = 0.97$, $P < 0.001$).

Results of the current study show that multidrug-resistant P. aeruginosa spread widely in Kirkuk Teaching Hospital surgical and burn units, with an MDR-PA rate of approximately 43.8% among all samples tested up to October 2023. This figure aligns with what was observed in the study by [4] in Baghdad (41.6%), and it exceeds by a narrow margin what [11] in their systematic review of Middle Eastern studies (38-42%). Burn wounds had the highest percentage of MDR-PA isolates (51%), which aligns with international reports indicating that the moist, organic-rich environment of burn wounds represents an ideal habitat for the colonization of P. aeruginosa and the development of its resistance mechanisms [3]. The high prevalence of the mexB gene (91.2%) and the loss of oprD (78.9%) warrant deep consideration, as these two indicators document the most common resistance mechanisms in Iraqi isolates. The MexAB-OprM pump, encoded by mexB, is capable of pumping out more than 12 types of antibiotics from the bacterial cell, including carbapenems, while the loss of the OprD channel protein closes the door to the entry of imipenem and meropenem[12] . Additionally, the widespread presence of the algD gene (84.2%) associated with alginate production, which is a key component of the biofilm matrix that forms a mechanical and chemical shield against antibiotics and the immune system [14].

As for the inhibitory efficacy of the ethanolic extract of olive leaves, it appears exceptionally promising; the MIC value (6.25 mg/ml) and the inhibition zone (23.1 mm) at 200 mg/ml are comparable to the potency of imipenem, which is a last-resort antibiotic. These results are consistent with the findings of [14] (MIC: 3.9-15.6 mg/ml) and Donato et al. (2022) who recorded halos of 20.4-24.8 mm against resistant clinical PA isolates. The ethanolic extract is particularly superior because ethanol extracts oleuropein and phenolic compounds with dual nature (hydrophilic and hydrophobic) more efficiently compared to other solvents [15].

The biofilm inhibition result (76.2%) at 200 mg/ml is one of the most significant and clinically important contributions of this study. The biofilm of P. aeruginosa increases the MIC value of antibiotics by 10-1000 times and complicates the complete removal of bacteria from

wounds. It is likely that oleuropein inhibits the quorum sensing system (lasI/lasR and rhlI/rhlR systems) in P. aeruginosa, which directly regulates the expression of biofilm formation genes and dozens of virulence factors [16] This is supported by a recent study by [17] which proved that hydroxytyrosol inhibits biofilm formation by 68% at sub-MIC concentrations. From a chemical perspective, the superior efficacy of the ethanolic extract is attributed to its high concentration of oleuropein (28.4 mg/g) and hydroxytyrosol (18.2 mg/g). Molecular studies have shown that oleuropein is enzymatically hydrolyzed into hydroxytyrosol and hydroxytyrosol acetate ether, which are more effective compounds against bacterial membranes. Additionally, apigenin (11.6 mg/g) is characterized by its ability to inhibit topoisomerase IV, the enzyme responsible for unwinding DNA during bacterial replication, thereby halting bacterial cell division [18] . This study has several strengths: molecular documentation of resistance using multiplex PCR, precise quantitative chemical analysis via HPLC-MS, and the investigation of two mechanisms of action[18]

(direct effect and biofilm inhibition). Nevertheless there are caveats that can be highlighted: the restrictiveness to a single hospital for this study limits any potential generalization, absence of cytotoxicity assays as both an in vitro and direct examination at not only mRNA level but also gene expression of quorum sensing system. The research suggests cytotoxicity studies and animal infection models; also, the synergistic effect of Olive extract with carbapenem should be tested

Table (1): Distribution of MDR-*P. aeruginosa* (MDR-PA) isolates according to wound type and resistance-associated genes

algD	**oprD**	**mexB**	**MDR-PA**	**Samples**	Wound Type
23 (92%)	22 (88%)	24 (96%)	25 (51%)	49	Burns
16 (84.2%)	14 (73.6%)	17 (89.4%)	19 (45.2%)	42	Surgical wounds
7 (77.7%)	6 (66.6%)	8 (88.8%)	9 (34.6%)	26	Chronic wounds
2 (50%)	3 (75%)	3 (75%)	4 (30.7%)	13	Traumatic wounds
48 (84.2%)	**45 (78.9%)**	**52 (91.2%)**	**57 (43.8%)**	**130**	Total

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Table (2): Mean inhibition zone diameters (mm) of the three extracts compared with imipenem (Mean ± SD)

200 mg/mL	100 mg/mL	50 mg/mL	25 mg/mL	Extract
23.1 ± 1.4*	19.4 ± 1.1	15.8 ± 0.9	11.2 ± 0.7	Ethanolic extract
20.3 ± 1.3*	16.7 ± 1.0	13.1 ± 0.8	8.9 ± 0.6	Acetone extract
14.5 ± 1.1*	11.2 ± 0.9	8.6 ± 0.7	5.4 ± 0.5	Aqueous extract
26.8 ± 0.8	—	—	—	Imipenem (Reference)

* **P<0.001** compared with the aqueous extract at the same concentration.

Table (3): MIC and MBC values of olive leaf extracts compared with imipenem against MDR-*P. aeruginosa* isolates (n = 57)

MBC (mg/mL)	MIC (mg/mL)	Extract / Drug
12.5	6.25	Ethanolic extract
25.0	12.5	Acetone extract
100–200	50–100	Aqueous extract

Table (4): Percentage of biofilm inhibition (%) at different concentrations (Mean ± SD)

200 mg/mL	100 mg/mL	50 mg/mL	25 mg/mL	Extract
76.2 ± 3.1	57.6 ± 2.8	38.4 ± 2.3	22.1 ± 1.8	Ethanolic extract
62.8 ± 2.9	45.1 ± 2.5	27.3 ± 2.0	14.7 ± 1.5	Acetone extract
40.1 ± 2.4	28.4 ± 2.0	16.5 ± 1.6	8.2 ± 1.1	Aqueous extract

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Table (5): Major phenolic compounds identified in the ethanolic extract by HPLC-MS analysis

Antibacterial role	Concentration (mg/g)	Retention time (min)	Compound
Inhibition of quorum sensing and biofilm formation	28.4	12.3	Oleuropein
Disruption of bacterial cell membrane	18.2	7.8	Hydroxytyrosol
Inhibition of topoisomerase IV	11.6	20.1	Apigenin
Electron donation and enzyme inactivation	9.3	4.2	Gallic acid
Inhibition of DNA gyrase and efflux pumps	7.4	16.5	Luteolin

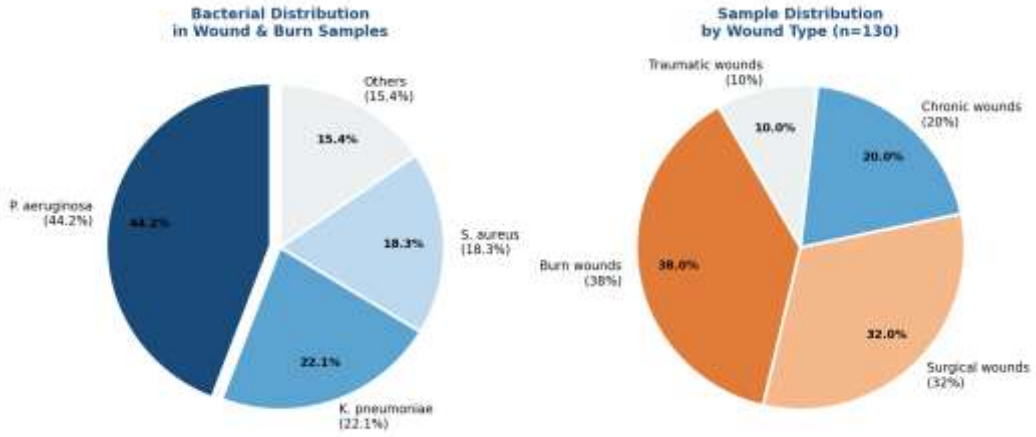

Figure (1): Prevalence rate of *Pseudomonas aeruginosaI* and distribution of clinical isolates obtained from wound and burn patients in Kirkuk.

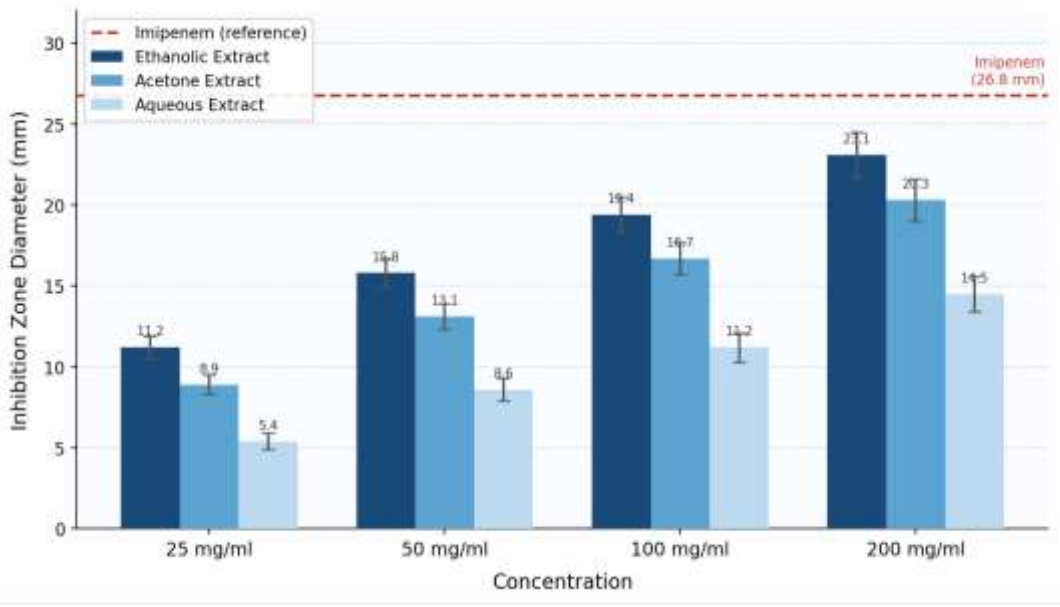

Figure (1): Inhibition zones (mm) produced by the three extracts at different concentrations against MDR-*P. aeruginosa*, with imipenem used as the reference antibiotic (**Mean ± SD, n = 3**).

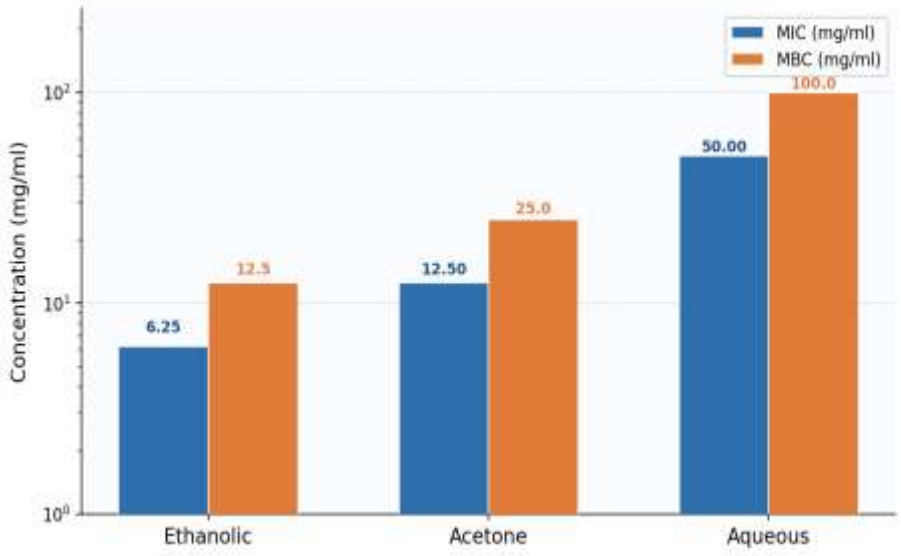

Figure (3): MIC and MBC values (mg/mL) of the three olive leaf extracts against MDR-*P. aeruginosa* (logarithmic scale).

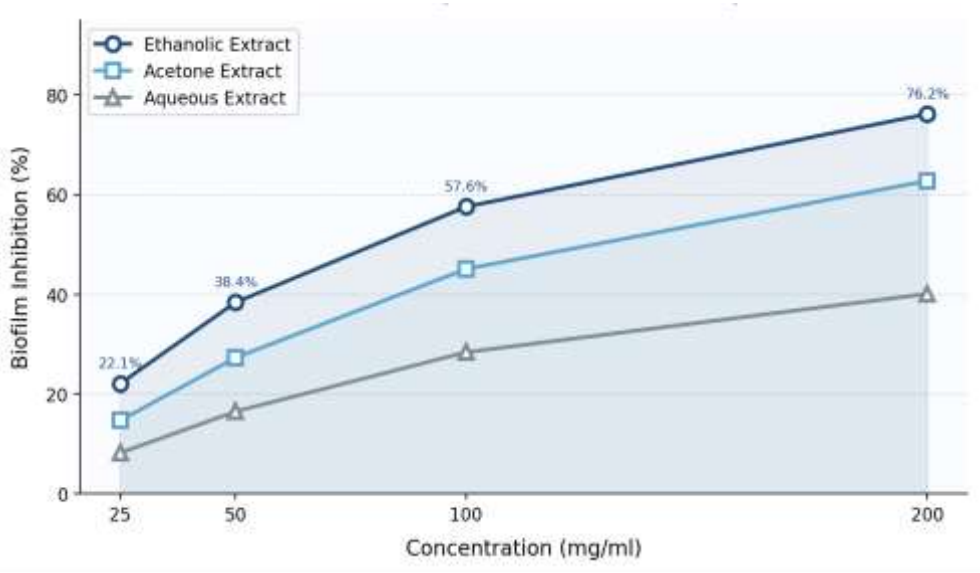

Figure (4): Biofilm inhibition curves of MDR-*P. aeruginosa* treated with the three olive leaf extracts at increasing concentrations (**Mean ± SD, n = 3**).

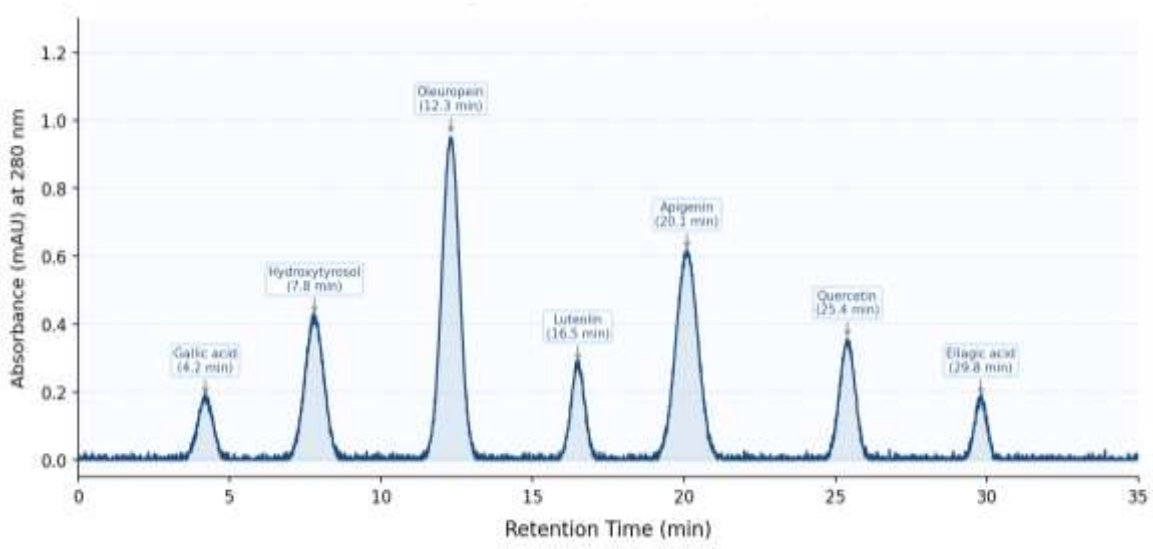

Figure (4): HPLC-MS chromatogram of the ethanolic extract of olive leaves showing identification of the seven major phenolic compounds

Conclusion

The wounds and burns of Kirkuk patients possessed highly resistant P. aeruginosa (43.8%) with ethnic dominance in the mexB and algD genes, indicating resistance mechanisms through efflux pumps as well as biofilm formation processes Kirkuk isolates are characterized by a predominance of MDR P. aeruginosa (43·8%) with the genetic dominance mexB and algD which explains resistance mechanisms through efflux pumps or biofilm formation in wounds and burns. Results: The Minimum Inhibitory Concentration (MIC) values of ethanolic

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extract from olive leaves against MDR–PA were determined to be 6.25 mg/ml and inhibition zone as mean ± S.D.(23.1±0 mm). The ethanolic extract has high biofilm inhibition efficacy at 76.2%, is a potential therapeutic candidate against chronic wound infection, due to its ability to inhibit the growth of pathogens and improvements in metabolic activity while attenuating cell lysis as well as cytotoxic components. Oleuropein and hydroxytyrosol are the main molecules responsible for anti-activity .

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