Research Article

Antibacterial Activity of Alkaloids, Phenols Extracts, Estimation of Active Compounds and Profile of Bioactive Compounds by HPLC Method of *Rosmarinus officinalis*

Ekhlass Mashhad Solaq[1]* and Roaa M.H. Shoker[2]

[1]*Department of Biology, College of Science, Waist University, Iraq.*

Abstract | *Rosmarinus officinalis* is being used in folk medicine due to rich of bioactive secondary metabolisms such as alkaloids, phenols, flavonoids, glycoside, tannins, and saponins which have antibacterial activity especially with developing the drug-resistant bacteria. This study investigates antibacterial activity of alkaloid, and phenolic extracts against bacteria of urinary tract infection UTI which obtained from Al-Kut hospital in waist province, and estimation of total active compounds, discover the qualities and quantities of these the constituents by HPLC methods. Alkaloids and phenols extracts were prepared from arial parts of *R. officinalis* which were obtained from local market in waist province and assessed for their antibacterial activity. Total concentration of extracts was analyzed by HPLC method, and estimation of total active compounds in plants. The result showed the highest yield of *R. officinalis* extracts was alkaloids, while phenols extract yields the lowest. Phenols extract was having significant highest antibacterial activity at p≤0.05 against four bacterial strains *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. The results showed *S. aureus* was more sensitive for alkaloid and phenols extracts of *R. officinalis*, while *P. aeruginosa* was more resistance for each extract. Estimation of active compounds in *R. officinalis* involve total phenolic compounds (gallic), total flavonoids compounds (rutin), alkaloid, glycoside, tannins, and saponins. The results of HPLC analysis showed the occurrence of eight phenolic constituent including gallic acid, apigenin, chlorogenic acid, cinnamic acid, ferulic acid, qurcetine, rosemaric acid, and rutin in *R. officinalis*. Nowadays due to occurrence various useful and beneficial bioactive constituents of *R. officinalis*, its requirements to attention on evaluation the effectiveness antimicrobial agents of this plant for developing pharmaceutical and biomedical applications.

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***Correspondence** | Ekhlass Mashhad Solaq, Department of Biology, College of Science, Waist University, Iraq; **Email:** std2023204.eadew@uowasit.edu.iq
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Introduction

Resistance to antibiotics a serious human health issue has emerged in recent years. Antibiotic resistance has grown to be a serious problem for human health in recent years, due to their resistance to several medicines, diverse antimicrobial-resistant bacteria are currently regarded as a serious worldwide

hazard to public health (Spagnolo *et al.*, 2021). Urinary tract infections (UTIs) are a significant public health concern caused by various pathogens, commonly *E. coli*, and classified as either complicated or simple, with the growing emergence of multidrug-resistant strains exacerbating the challenge of treatment. These infections are prevalent, particularly in women, and can cause symptoms like frequent and urgent urination, pain during urination, and general unwellness (Foxman *et al.*, 2025). Today, UTIs pose an increasingly serious problem due to the multidrug resistance exhibited by both Gram-positive and Gram-negative bacteria (Mishra *et al.*, 2015). According to the estimates of the World Health Organization (WHO), more than 80 percent of the world population rely on traditional medicine to meet the primary healthcare needs (Arcopinto *et al.*, 2019). Among the primary interest fields in the search of effective and affordable medicines to address the modern needs is medicinal plants and their bioactive metabolites (Sitesh, 2021). *R. officinalis* was a Lamiaceae plant (Gonzalez-Minero *et al.*, 2020). According to the experimental researches, rosemary has been shown to have a number of positive characteristics, such as antibacterial, antifungal, antidepressant, anti-diabetic, anticancer, antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective (Nazir *et al.*, 2020). *R. officinalis* has a long history of application in traditional medicine in managing various diseases, such as headache, dysmenorrhea, indigestion, rheumatism, muscle and joint pains, fatigue, nervous agitation, and so on (Christopoulou *et al.*, 2021). Abortive, stomachic, carminative, cholagogue, antispasmodic properties of the plants have resulted in using the plants branches in as herbal tea. Experimental studies indicate that the plant has antibacterial, antifungal, antidepressant, anti-diabetic, anticancer, antioxidant, anti-inflammatory, hepato-protective, and neuroprotective properties (Abo-elhamd *et al.*, 2024). Due to its good antibacterial and antioxidant properties, rosemary extracts find application in the food industry as natural preservatives. They offer customers benefits in terms of maintaining the quality of the product, extending shelf life, as well as technological benefits. These extracts are highly active in inhibiting oxidative and microbial rancidity formation in diverse food products, is a clean label substitute to conventional food preservatives like Butylated Hydroxy anisole (BHA) and Butylated Hydroxy touene (BHT), which are under increasing regulatory pressure because of the possible health risks associated (Veenstra and Johnson, 2021). This research intends to assess the anti-bacterial effects of alkaloid and phenolic extracts of *R. officinalis* on bacteria of urinary tract infection UTI, and the number of total active compounds, to identify the phenolic compounds using HPLC techniques.

Materials and Methods

Preparation of plant extracts

Extraction of crude alkaloids

Crude alkaloids were isolated from *Rosmarinus officinalis* following the method described by Harborne (1984). Briefly, 100 g of powdered plant material was homogenized for 5 min with 350 mL of a 4:1 (v/v) ethanol–distilled water mixture using an electric blender. The homogenate was first filtered through muslin cloth and then through Whatman No. 1 filter paper under reduced pressure using a Buchner funnel. The filtrate was concentrated to dryness using a rotary evaporator at 45 °C. The residue was re-dissolved in a minimal volume of distilled water, and the pH was adjusted to 1–2 by the addition of 2% sulfuric acid. The acidic aqueous layer was then subjected to three successive extractions with chloroform in a separating funnel. The lower chloroform layer (containing non-alkaloidal impurities) was discarded, and the aqueous layer was retained.

The pH of the aqueous layer was subsequently raised to 9–10 by dropwise addition of concentrated ammonium hydroxide. Alkaloids were then extracted twice with a chloroform–methanol mixture (3:1, v/v) and once with pure chloroform. The combined organic layers were evaporated to dryness at 40 °C using a rotary evaporator and stored at 4 °C until further use.

Extraction of crude phenolics

Crude phenolic compounds were also extracted according to Harborne (1984). Two hundred grams of powdered plant material was divided into two equal portions. One portion was mixed with 300 mL of 1% HCl, and the other with 300 mL of distilled water. Both mixtures were homogenized for 5 min, then heated in a water bath for 30–40 min. Each of the mixtures was filtered with the help of muslin cloth, and centrifuged at 3000 rpm within 10 minutes after cooling. The supernatants were combined and n-propanol of equal quantity was added with a little bit of sodium chloride to facilitate the phase separation.

A separating funnel was used to extract the lower aqueous layer using ethyl acetate. The ethyl acetate fraction and the rest of the aqueous supernatant were dried at 40 ˚C using rotary evaporation separately. The dried extracts were kept in 4 ˚C until analysis.

Phytochemical quantification

Total phenolic content

The Folin-Ciocalteu colorimetric method was used to determine the total phenolic content. A 100 µL of plant extract, 500 µL of Folin-Ciocalteu reagent (Merck, Germany) and 1.5 mL of 20% sodium carbonate were mixed in a reaction mixture, vortexed and diluted with 10 mL of distilled water. Then, the dark incubation period was 2 h and at the room temperature, absorbance was recorded at 765nm through a UV-Vis spectrophotometer. Gallic acid (Sigma-Aldrich, Germany) was used as a standard to obtain a calibration curve. The results were reported as milligrams of gallic acid equivalents (GAE)/ gram of the dry extract (mg GAE/g DW).

Total flavonoid content

The aluminum chloride colorimetric method was used to measure the total flavonoid content. In short, 4 mL of distilled water was added to 50 µL of extract (diluted to 1 mg/mL using ethanol). Then 0.3 mL of 5% sodium nitrite was added to the mixture, and the mixture was incubated over a period of 5 min. Thereafter, 0.3 mL of 10 percent aluminum chloride were introduced and incubated after 6 minutes. Lastly, 2 mL of 1 M NaOH was placed in it and the volume was brought to 10 mL using double-distilled water. The absorbance was taken after 15 min of reaction at a wavelength of 510 nm. Rutin was used as a standard to form a calibration curve, and the data presented in the form of the number of milligrams of rutin equivalents per gram of dry extract (mg RE/g DW).

Total glycoside content

Maceration of 10 grams of the dried plant powder with 80% methanol at room temperature was performed with solvent replacement after every 48 h until exhaustion. The joint extracts were concentrated at low pressure to get a crude extract. To quantify glycosides, 10% (w/v) solution of the extract was made and 1mL of the solution was mixed with freshly prepared Baljet reagent (0.95mL of 1percent picric acid + 0.05mL of 10percent of NaOH). The incubation period was followed by the addition of distilled water to the 1 h incubation to bring the volume to 20 mL and the absorbance at 495 nm with a Shimadzu UV-1600A spectrophotometer (Kyoto, Japan). Securidaside (12.5-100mg/L) was used to construct a standard curve and the total glycoside content was converted to mg securidaside equivalents per gram of dry extract (mg SE/g DW) by triplicate determination.

Total tannin content

The extract (2gm) was dissolved in water- ethanol (20: 80, v/v) then the solution was heated in a water bath and then the solution was filtered. Initial identification of tannins was done by the addition of ferric chloride that gave a dark green color. To analyse quantitatively, 2 percent solution of sodium chloride and 5 percent solution of gelatin were added to a 1 mM solution of sample. Precipitate thus obtained was centrifuged and the supernatant was subjected to spectrophotometric measurement at 540 nm. The absorbance was compared with a standard curve that is used to determine tannin concentration.

Total alkaloid content

Twenty g of plant material was dried in methanol in a Soxhlet apparatus in 24h. A rotary evaporator was used to wdry the extract under reduced pressure and then filtered. The dried residue was dissolved in acidic aqueous medium (pH ~3), filtered to remove impurities, and then basified to pH 9–10 with ammonium hydroxide. The liberated alkaloids were extracted with chloroform, evaporated, and reconstituted for quantification.

Alkaloid content was determined spectrophotometrically using Bromocresol green reagent in phosphate buffer (pH 4.7). A calibration curve was prepared using atropine as the standard, and results were expressed as milligrams of atropine equivalents per gram of dry extract (mg AE/g DW).

Estimation of total saponins content

Samples that had been air-dried were kept overnight in the lab to allow them to rest, then crushed/pounded to become fine powder using hand grinders prior to determining saponin content by using gravimetric double-extraction methods in which specific amounts (5g) of sample weight were combined with an ethanol mixture of water and heated at various controlled temperatures while repeatedly filtering to clear residue, extracting repeatedly until filtering was clear or defined by prior extraction order and at termination

of identical procedure the partitioning was finished and the values weighed for final percent calculations relating amounts of sample used.

Percentage of saponins= (W2 – W1 / Wt. of sample) X 100

W1= weight of evaporating dish
W2 =weight of evaporating dish + sample

Preparation of different concentrations of plant extracts

Preparation of plant extract concentrations

Alkaloid/phenol extractions formulated dissolving predetermined weights according specified concentrations ranging25–75 mg/ml into ethylene glycol employing equations modeled after Shoker *et al.*(2021)

$$\text{Concentration mg/ml} = \frac{\text{Weight}}{\text{Volume}} \times 1000$$

Analysis of chemical composition of the plants extracts

Compounds were analyzed by injecting 100 µL of each sample into a High-Performance Liquid Chromatography (HPLC) system for identification, following the method described by (Shoker *et al.*,2021).

Analysis of chemical composition and biological activity

Chemical profiling

The chemical constituents of the *Rosmarinus officinalis* extracts were identified using High-Performance Liquid Chromatography (HPLC). Each sample was injected at a volume of 100 µL, following the analytical protocol described by Raheema and Shoker (2020). Chromatographic peaks were compared with reference standards to tentatively identify major phenolic and alkaloidal compounds.

Antibacterial activity assay

The antibacterial activity of the phenolic and alkaloidal extracts was evaluated using the agar well diffusion method. Bacterial suspensions were standardized to a 0.5 McFarland turbidity standard, corresponding to an approximate cell density of 1.5×10^8 CFU/mL (note: the value of 10^{15} CFU/mL cited in the original protocol appears to be a typographical error, as it vastly exceeds typical bacterial concentrations used in antimicrobial assays). No additional dilution of the standardized inoculum was done before the preparation of lawn.

Mueller-Hinton agar plates of aseptic wells (diameter 6-8 mm) were prepared with the aid of a sterile cork borer. The plant extracts were dissolved in ethylene glycol (as the solvent control). Different concentrations of the extracts were loaded in each well. Aerobic incubation of the plates at 37 ˚C was done in 24 hours. After incubation, the diameters of the inhibition zones (including well diameter) were determined in millimeters with the help of the digital caliper. Each of the assays was done in triplicate.

Statistical analysis

Data were analyzed using IBM SPSS Statistics software (version 26). One-way analysis of variance (ANOVA) was employed to assess differences in antibacterial efficacy across extract concentrations and bacterial strains. Post-hoc mean comparisons were considered statistically significant at $p < 0.05$.

Results and Discussion

The present study aerial parts of *R. officinalis* were extracted for detection of the alkaloids and phenols extracts and evaluated the antibacterial activity of these extracts of plant against four clinically relevant bacterial strains, including *S, aureus*, *E, coli*, *K. pneumoniae,* and *P. aeruginosa*, using varying concentrations (25, 50, 75 mg/ml) of each extract**.** The yield of alkaloids and phenolic extracts were represented as percentage (%) of the dry weight. The result showed the yield of alkaloids extract of *R. officinalis* was 8.86 %, while phenol extract yield was 7.96%. The yield of alkaloids and phenolic extracts was shown in Table 1.

Table 1: *Extracts the yield of plant extract of R. officinalis expressed as %*

Plants Name	Extract Types	Yield (%)
R. officinalis	Alkaloids	8.86
	Phenols	7.96

Estimation of active compounds in plants

The phytochemical analysis of the studied plants revealed clear variations in the levels of bioactive compounds estimation of active compounds in *R. officinalis* involve Total Phenolic Compounds TPC (gallic), Total Flavonoid Compounds TFC (rutin), alkaloid, glycoside, tannins, and Saponins in Table 2. The results displayed the higher concentration compound was gallic (125.19 mg/gm), while,

saponins (0.33mg/gm) lower concentration in plant. The rich phenolic content in rosemary aligns with previous studies reporting its strong antioxidant and antimicrobial potential (Nieto *et al*., 2018; Flores-Villa *et al*., 2020). Alamami *et al*. (2023) have shown that rosemary which growing in the east of Libya is rich in phenolic compounds as gallic acid.

Table 2: *Estimation of the active compounds in R. officinalis*

Name	Active compounds					
	Gallic (TPC) (mg/ gm)	Rutin (TFC) (mg/ gm)	Alkaloid %	Glycoside %	Tannins %	Saponins%
R. officinalis	125.19	63.15	2.66	0.63	1.08	0.33

Antibacterial activity

Analysis of antimicrobial susceptibility revealed substantial resistance among the isolated bacterial strains, with resistance patterns differing considerably between Gram-negative and Gram-positive groups which isolated from UTI. The culture yielded *S. aureus* which showed resistance to oxacillin, gentamicin, moxifloxacin, erythromycin, clindamycin, and vancomycin, while remaining sensitive to trimethoprim-sulfamethoxazole and tigecycline. Oxacillin resistance is evidence that the strain is methicillin-resistant *S. aureus* (MRSA), which is generally linked to multidrug resistance. The further resistance to vancomycin is significant, and it can be an indicator of vancomycin-intermediate or vancomycin-resistant *S. aureus* (VISA/VRSA) which is a rare but clinically critical observation. Although this pattern of resistance makes the organism resistant to first-line treatments trimethoprim-sulfamethoxazole and tigecycline, there is a small, though possible, treatment option. The choice of treatment must be taken regarding the place of infection, the severity and patient specific factors. These results align with the findings that were provided by Hussein *et al*. (2023), who also reported the same resistance patterns in clinical isolated S. aureus. *Pseudomonas aeruginosa* (resistant to cefazolin, tigecycline, and gentamicin) and sensitive to piperacillin, cefepime, and imipenem were obtained by the culture. The use of cefazolin and tigecycline is likely to be resisted because these have weak activity against *P. aeruginosa* owing to inherent processes like low outer membrane permeability and efflux pumps. The development of resistance to gentamicin can be explained by such acquired mechanisms as aminoglycoside-modifying enzymes. The resistance of the organism to the use of piperacillin, cefepime, and imipenem means that the use of those antibiotics is still possible to treat severe *P. aeruginosa* infections. The choice of antibiotic must be specific to the location of infection, patient, as well as stewardship. The observed resistance pattern is in line with the results of Al-Saeedi and Raheema, (2019) and, to a greater extent, Raheema *et al*. (2024), who identified the same antimicrobial susceptibility patterns in clinical isolates. Results of antibacterial activity of the *R. officinalis* extracts against selected bacteria species which isolated from the patients suffering from urinary tract infection were assessed depending on the diameters (mm) of inhibition zones according to agar –well diffusion methods. Inhibition zones were diverse depending to bacterial species, extract types, plant aerial part, and their concentrations. Results showed that all extracts from *R. officinalis* displayed significant inhibition at (P≤0.05) against all tested selected bacteria species.

Table 3: *Diameters of inhibition zone caused by diverse concentration of R. officinalis against different species of bacteria*

Plant type	Extract type	Concentration of *R. officinalis*			LSD
		25%	50%	75%	
S. aureus	Alkaloid	C 11.00 ± 1.00^{c}	C 19.67 ± 1.57^{b}	B 25.0 ± 1.00^{a}	2.73
	Phenol	A 25.67 ± 1.15^{b}	A 27.00 ± 1.00^{ab}	A 28.33 ± 1.57^{a}	1.35
E. coli	Alkaloid	CD 8.33 ± 1.15^{c}	D 16.00 ± 1.0^{b}	BC 23.00 ± 1.0^{a}	1.51
	Phenol	AB 22.33 ± 2.08^{a}	B 23.00 ± 1.0^{b}	BC 24.00 ±1.0^{a}	1,91
K. pneumoniae	Alkaloid	D 7.33 ± 0.57^{c}	D 15.00 ± 1.0^{b}	C 22.00 ± 1.00^{a}	2.01
	Phenol	B 20.0 ± 1.00^{a}	BC 20.67 ± 2.08^{b}	BC 22.67 ± 1.15^{a}	1.71
P. aeruginosa	Alkaloid	CD 8.00 ± 1.00^{b}	E 9.00 ± 1.0^{b}	D 11.00 ± 1.00^{a}	1.44
	Phenol	CD 9.33 ± 1.00^{b}	E 11.33± 2.08^{a}	D 13.00 ± 1.00^{a}	1,81
LSD		3.40	3.16	2.71	
P value		0.001**	0.001**	0.001**	

Means in the same row with a different small letter are significantly different at (P≤0.05)
Means in the same column with a different capital letter are significantly different at (P≤0.05)

*†: One way Anova **: significant at P > 0.0*

All inhibition zones mean were increased with

increasing of concentration. Result in Table 3 showed *S. aureus* was more sensitive for alkaloid and phenols extracts of *R. officinalis*, while *P. aeruginosa* was more resistance for each extract, the inhibition zone of phenols extracts against *S. aureus* a was (28.33 ± 1.57mm),(24.00 ± 1.0 mm),(22.67± 1.15 mm), and (13.00± 1.00 mm) while the inhibition zone of alkaloid extract was (25.0 ± 1.00 mm), (23.00 ± 1.0 mm), (22.00 ± 1.00 mm), and (11.00 ± 1.00 mm) at (75 mg/ml) concentration, Phenols were having significant highest antibacterial activity at p≤0.05 against bacteria than alkaloids. Tiwari *et al.* (2011) and Tabit *et al.* (2016) showed that phenolic and alkaloid compounds have significantly antimicrobial activity due to their capability to disrupt cell walls and inhibit enzymatic functions of microbial. Phenolic compounds are active antioxidants compounds created from natural plants due to their metal chelating and free radical scavenging properties (Maduka *et al.*, 2015). Phenolic extract of *Ocimum basilicum,* and *Ocimum sanctum* having high antimicrobial activity against selected Gram-positive\ negative bacteria in all concentrations (Shoker *et al.*, 2021). *R. officinalis* extract showed even greater inhibition of the growth of Gram-positive bacteria including *S. aureus* (Kernou, and Rijo, 2023). Rosmaric acid can effectively disrupt bacterial membranes and suppress quorum sensing in *P. aeruginosa* (Kloy *et al.*, 2020; Bouloumpasi *et al.*, 2024; Jummana *et al.*, 2024). Alkaloids play an essential role in both human medicine and in an organism's natural defense, alkaloids make up approximately 20% of the known secondary metabolites founds in plants (Kaur and Arora, 2025). Alkaloids are particularly well known as anaesthetics, cardioprotective, and anti-inflammatory agents (Kurek, 2019). Results showed phenols, and alkaloids extracts of *Conocarpus lancifolius* have higher antibacterial activity against both gram-positive and gram-negative pathogenic bacteria (Raheema and Shoker, 2020).

Chemical constituents of the plant extracts

Results of HPLC analysis showed the occurrence of eight phenolic constituents in *R. officinalis* (Table 4, Figure 1). All the investigated compounds seemed to have different retention time. The highest concentration of phenols constituent in *R. officinalis* was Gallic acid (145.9 ppm), while Apigenin (70.9 ppm) the lowest concentration. These findings are in agreement with Hcini *et al.* (2021) and Troncoso *et al.* (2005), who reported that in rosemary have phenolic constituents as rosmarinic acid and gallic acid which contain antioxidant and antimicrobial activity. Rosemary which collected from Tunis have phenolic compounds as gallic acid, caffeic acid, ferulic acid, rosmaric acid, coumaric acid, carnosol, carnosic acid, hesperidin, luteolin, apigenin, and genkwanin (Hcini *et al.*, 2013).

Table 4: *Types and concentration of phenols extract in plant.*

Phenolic compounds (ppm)	*R. officinalis*
Gallic acid	145.9
Apigenin	70.9
Chlorogenic acid	90.2
Cinnamic acid	88.7
Ferulic acid	82.6
Qurcetine	74.6
Rosemaric acid	132.7
Rutin	80.7
Sinapic acid	-
Total concentration (µg/ml)	703.3

Conclusions

Highlight to discovery alternative therapeutic approaches which have antimicrobial agent from natural products as rosemary plant especially with rising antimicrobial resistance. Rosemary plant having significantly antibacterial activity due to its rich secondary metabolism products as alkaloid, phenols, glycoside, tannins, saponins rutin, gallic acid, apigenin, chlorogenic acid, cinnamic acid, ferulic acid, qurcetine, rosemaric acid, rutin, and sinapic acid, that have antibacterial and antioxidant activity which may be used in future to enhance the therapeutic potential for healthcare and pharmaceuticals application.

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Novelty Statement

The novelty of this study lies within the discovery of bioactive chemical constituents which have antimicrobial agent from natural products of rosemary *R. officinalis* plant against drug-resistant bacteria. The research introduces new visions into the potential of bioactive chemical constituent from plant natural products which act as natural antimicrobial agents.

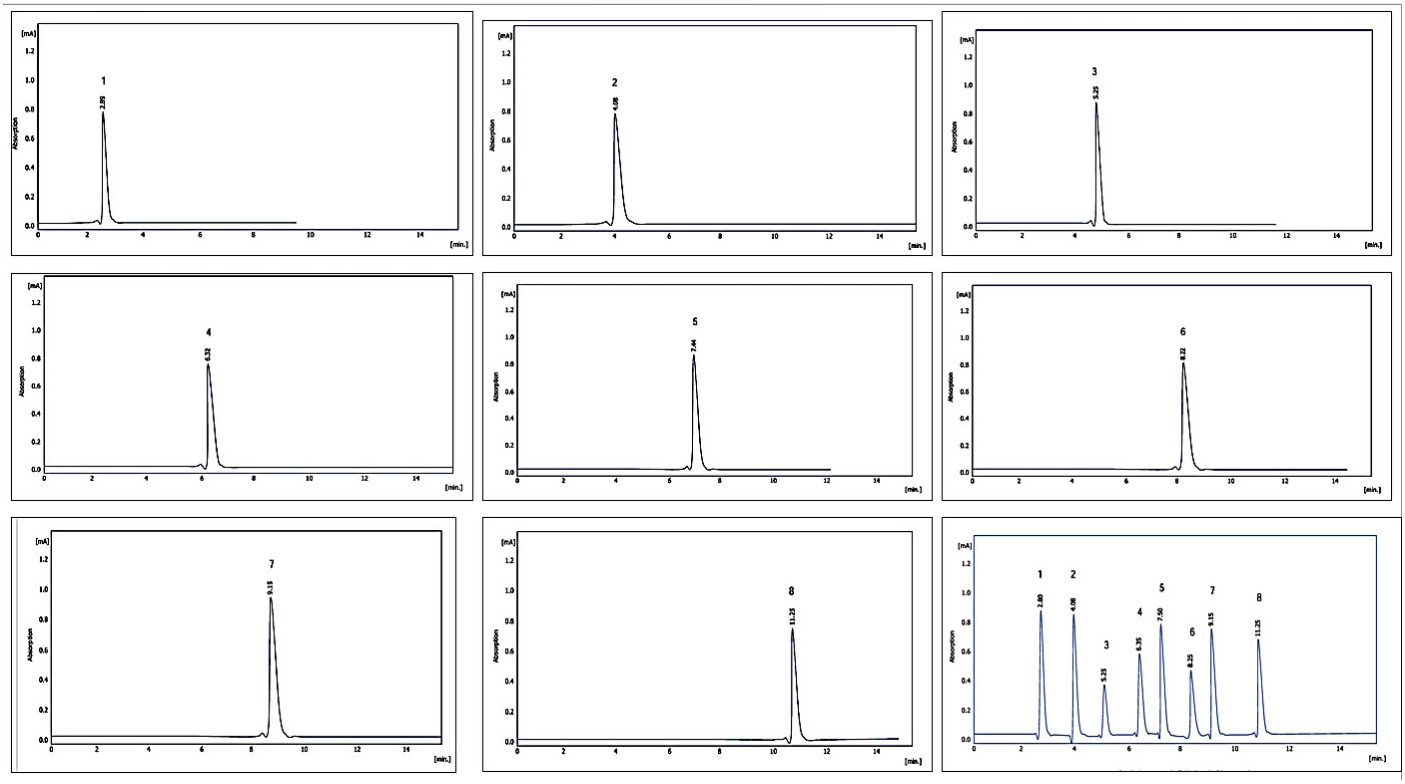

Figure 1: *HPLC profile of R. officinalis phenolic extracts*
Gallic acid (2) Rosemaric acid (3) Quercetin (4) Apigenin (5) Chlorogenic acid (1) (6) Rutin (7) Cinnamic acid (8) Ferulic acid

Author's Contribution

Ekhlass Mashhad Solaq: Article preparation.
Roaa M. H. Shoker: Supervision

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors have avowed conflict of interest.

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