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Chemical composition, antioxidant, and antibacterial activity of *Ruta graveolens* (Rutaceae)

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Abstract. *Saeed YS, Ali JF, Mohammed MA. 2023. Chemical composition, antioxidant, and antibacterial activity of* Ruta graveolens *(Rutaceae). Biodiversitas 24: 3162-3168.* The present study was carried out to investigate the antioxidant and antibacterial activities of phenolic components isolated from the leaves of *Ruta graveolens* L. The phenolic compounds were extracted in different organic solvents (hexane, ethanol, and ethyl acetate) and analyzed by column chromatography, thin-layer chromatography, and high-performance liquid chromatography. Three discrete fractions were collected and subsequently examined: one from ethyl acetate extraction (I) and two from ethanolic extraction (II and III). No fractions from hexane were obtained. In particular, the ethanolic fractions were analyzed by HPLC analysis which contained the most bioactive compounds. The major phenolic compounds from the ethanol extracts were catechol and coumarin (1.104966 and 16.612227 mg/kg), followed by catechol and hydroquinone (0.144731 and 1.90781 mg/Kg), whereas, resorcinol and coumarin from the ethyl acetate extracts (14.78298 and 21.75253 mg/kg). The ethanol extract had the highest level of antioxidant activity, and other fractions had antioxidant effects at different levels; because the three fractions' phenolic chemicals have varied redox characteristics, their impacts vary in strength. The highest inhibition zones (mm) of *Bacillus cereus* and *Staphylococcus aureus* at 24 µg/mL for fractions II and I were 23 and 22 mm, respectively. At 24 µg/mL, *Escherichia coli* and *Proteus vulgaris* had inhibition zones of 21 and 20 mm, respectively. Fraction I and II did not show Escherichia coli and Proteus vulgaris inhibition zones (mm) at 1.5 µg/mL. Finally, the study's results suggest that using *R. graveolens* ethanol extracts may be a natural source of antioxidants and anti-infectious agents.

Keywords: Antibacterial, antioxidant, HPLC, phenolic compounds, *Ruta graveolens*

INTRODUCTION

Ruta graveolens L. is an herbaceous perennial plant that grows and spreads in the Mediterranean; later spreads to many regions worldwide. It belongs to the Rutaceae family in the order Sapindales, which contains about 160 genera and more than 1,600 species. *Ruta graveolens*, also called "Rue" in English and "*Sodaab*" in Arabic, has been used as a spice and medicine since ancient times (Lingaraju et al. 2016; Elansary et al. 2020; Senica et al. 2020). Rue is a small evergreen shrub or semi-woody. Their length ranges from 0.6 to 0.9 meters, but their width is slightly smaller. The stems become stiffer and more woody near the base while remaining softer near the terminal branches. The leaf venation pinnately ranges from 7.6 to 12.7 cm in length and is shaped like a rectangle or a spoon. They are a little bit meaty. In the middle of summer, small yellow flowers grow in clusters that rise well above the plant's leaves and often cover most of the plant. Each flower measures approximately 1.3 cm across and contains four petals (Reddy and Al-Rajab 2016; Pavić et al. 2019; Jianu et al. 2021; Mokhtar et al. 2022). Various civilizations have historically used Rue in folk medicine despite its bitter taste and strong odor. This plant's fresh and dried leaves can be used for traditional and alternative medicine. Recent pharmaceutical studies have shown that Rue can also be used as an antioxidant, an anti-inflammatory, an anti-diabetic, an antibacterial, an antifungal, an antiandrogenic, and an insecticide (Azalework et al. 2017; Bakour et al. 2021). Rue is an Iraqi medicinal plant with various phenolic compounds responsible for its effectiveness, such as simple coumarins (coumarin, herniarin, umbelliferon, methoxy coumarin, and scopoletin), phenolic acids (vanillic acid, chlorogenic acid, ferulic acid, protocatechuic acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, caffeic acid, and gentisic acid), and flavonoids (rutoside). (Attia et al. 2018; González-Locarno et al. 2020). In addition, *R. graveolens* and its derivatives were used as antimicrobials. Antibiotic resistance is a global public health problem most common in developing countries. Antimicrobial drug resistance in microorganisms, especially bacteria, is primarily caused by clinical and cellular changes. As a result of the preceding, the need has become urgent to find natural sources that act as antibiotics against pathogenic bacteria and are characterized by high efficacy and fewer side effects. (Al-Sokari et al. 2015; Giresha et al. 2015). The phenolic compounds isolated from the leaves of *R. graveolens* are effective antioxidants, which are preferred to chemical antioxidants. Antioxidants are molecules that can protect against the damage caused by free radicals. Antioxidants could work in different ways at different stages (prevention, interception, and repair) by giving hydrogen, reducing singlet oxygen, acting as chelators, and catching free radicals in different ways (Cefali et al. 2019; Bouabida and Dris 2022). These differences in the phytochemical

profile of *R. graveolens* may explain the antimicrobial and antioxidant properties reported in recent pharmacological trials (Shubha and Bhatt 2021). To our knowledge, no investigation of the antimicrobial and antioxidant properties of the phenolic compounds extracted from *R. graveolens* has been previously reported. However, several studies report Rue's in vitro antimicrobial and antioxidant properties as an extract. Therefore, the aim of this study was to identify the phenols that are extracted from *R. graveolens* as having antibacterial and antioxidant properties.

MATERIALS AND METHODS

Materials

Ruta graveolens L. aerial parts (leaves) were collected from northern Iraq in April and June 2019, during the flowering season, when temperatures usually range from 20°C at night to 38°C at day. After the plant's identity was confirmed, it was washed with sterile distilled water to wipe off all the dust on the surface, then dry in dark at room temperature (about 25°C) because phenolics could break down when exposed to light. All solvents and chemicals used in the study from Sigma Aldrich.

Extraction of *R. graveolens* by soxhlet apparatus

The Soxhlet continuous extraction apparatus was used in the extraction process that used hexane, ethyl acetate, and ethanol in a certain order. First, the extraction process was done by adding 100 mL of the used solvent per 10 g of the sample (*R. graveolens* powder). The extraction was done for 48-72 hours. Then RVE (rotary vacuum evaporator) concentrates the extracts and makes the crude extract. Next, keep the crude in the fridge until used and packaged in the dark in sealed glass bottles (Wiktor et al. 2019).

Isolation and fractions of *R. graveolens* extract by column

This separation objects method needs a Stationary Phase (SP), represented by silica gel with mesh (at 60-120), and a mobile phase, represented by the solvent. Each phase has physical characteristics, such as polarity and the ability to hold objects. In the separation column, a glass column of different sizes filled with activated silica gel by heating method and used in the separation process, several solvents with different polarities were used in different quantities to get various parts or fractions (Wiktor et al. 2019).

Thin-Layer Chromatography (TLC)

A previously described approach was used to perform TLC on the crude extract to choose the best solvent system to separate various components contained in the crude extract (Mohammed et al. 2021). First, an aluminum-backed Thin-Layer Chromatography (TLC) equipment was filled with 2 µL of each extract to identify the presence of phytochemicals. The glass TLC plates were 20 cm by 20 cm in size and were pre-coated with silica gel 60 F254. (E. Merck/Millipore, Billerica, MA, USA, 0.2 mm thickness). Next, the TLC plate made a 10:8:2 v/v solution of chloroform, ethyl acetate, and formic acid. The developed plate was then checked for fluorescent compounds with a UV-visible spectrophotometer at 254 nm and 365 nm (Sunny UV.7804C, Tokyo, Japan). Then, vanillin-sulfuric acid was sprayed on the TLC plate and heated to examine the colors of the different compounds. The Rf value was detected and compared to standards for each found spot. Next, each fraction with the same Rf value was concentrated with a rotary evaporator. Therefore, to establish the identity of the phenolic compounds and weigh them after drying, condensed fractions were further tested using HPLC (High-performance Liquid Chromatography) technique.

Analysis of phenolic compounds by HPLC

HPLC technology ensured that the components separated from the plant were phenolic compounds. The analytical HPLC system comprised a detector for SPD-10A UV-VIS, VP pump LC-10AT, auto-injector SIL-10AF, and system controller SCL-10A VP. The Chiralcel® OD-RH analytical column was used (150 mm, roughly 4.6 mm in diameter, and 5 mm particle size; Chiral Technologies Inc. Exton, PA, USA). The mobile phase comprised acetonitrile, water, and phosphoric acid (30:70:0.08, v/v/v), and it flowed at 0.4 mL/min under isocratic conditions at 25°C. Each run took 8 minutes, and cleaning up afterward took 15 minutes. At 288 nm, the compounds that had been separated were picked up by the SPD-10A UV-Vis detector that was built in (Skendi et al.2017). Furthermore, it was possible to identify each compound in fractions by comparing the retention times of relevant standards (Table 1) with those of the peaks in the extract.

Origin and selection of microorganism strains

This study used several very-dangerous microorganisms to test the *R. graveolens* extracts. Four bacteria that are harmful to humans were selected to be tested in a lab setting to study the antimicrobial activities *R. graveolens* extracts. All four microbial strains were taken from our lab's stock culture; Gram-positive strains like *Bacillus cereus* (ATCC 14579) and *Staphylococcus aureus* (ATCC 6538) and Gram-negative strains such as *Escherichia coli* (ATCC 8739) and *Proteus vulgaris* (ATCC 7829). These microbial, the most common foodborne pathogens of concern, were tested in this study. Bead vials were used to keep microorganisms safe while they were frozen (-18°C).

Table 1. Standards of phenolic compounds and their retention time

Standards	Retention time (min)	Conc. (ppm)	Area [1]
Resorcinol	2.686	25	50.9239e2 (0.01)
Coumarin	3.165	25	3179.35669 (0.01)
Catechol	2.715	25	7.75188e4 (0.01)
Hydroquinone	2.511	25	8.76891e3 (0.01)

[1] Area represented as mean (n = 5) with a coefficient of variation in brackets

Preparation of the inoculums

The bacterial strains were grown on Nutrient Agar (NA) at 37°C for 24 hours, which was good for their growth (Mueller-Hinton broth). After being incubated, they were split into new groups before any antimicrobial tests were done. Next, bacteria were mixed with 0.85% NaCl to make inoculums in a sterile saline solution. The suspensions' Optical Density (OD) was changed or kept between 0.4 and 0.6 at 405 nm. This is close to a cell density of 0.5 McFarland and then matched an estimated inoculum of 106 to 108 CFU/mL (Andrews et al. 2009).

Method of disk diffusion on agar

The antibiotic susceptibility test was done with a few changes to the standard disc diffusion method as explained by (Zazharskyi et al. 2020). By using a clean swab, the Mueller-Hinton Agar (MHA) plates were marked with inoculums that had already been made. Then, 5 µL of each of the fractions: the ethyl acetate (F. I) and ethanol (F. II), were put on sterile paper discs (6 mm, Whatman paper N5) in a solvent (10% v/v dimethyl sulfoxide and 1% v/v Tween 80 in deionized water). The antibiotics Amikacin and Gentamycin (5 mg/mL) were used as the positive and negative controls, and the same solvent was used to dilute extract fractions under the same conditions. The plates were left at Room Temperature (RT) and then incubated at 37°C for 24 hours. In the end, the antibacterial activity was graded by measuring the diameter of the nearest inhibition zone in millimeters. In addition, each experiment was repeated three times.

DPPH radical scavenging activity assay

DPPH (2,2-diphenyl-1-picryl hydrazyl) was used as a well-known organic chemical to measure their ability to eliminate free radicals, with a few modifications (Amiri et al. 2020). First, the radical-scavenging activity was measured using the free-radical DPPH assay and the spectrophotometric method. Next, the fractions were prepared in different concentrations of (12.5, 25, 50, 100, and 200) µg/mL and dissolved in 1 mL of ethanol. Then, 20 mg of DPPH dissolved in 100 mL of ethanol. After shaking the mixture, it was left for 30 minutes at room temperature in the dark. A DPPH solution was used as a control, and ascorbic acid was used as a reference standard. Finally, the absorbance level was measured using the UV-Visible spectrophotometer at 517 nm. Therefore, to calculate the antioxidant activity, the following equation was used to determine how much the radical scavenging is:

$$\text{DPPH Inhibition \%} = [(A_o - A_1)/A_o] \times 100$$

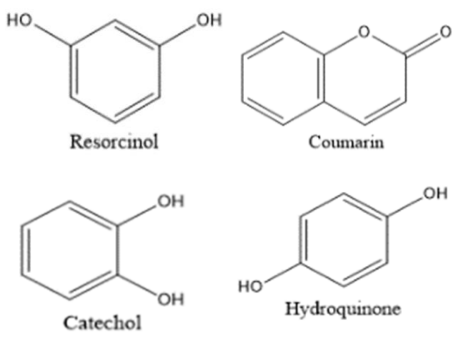
Where:
A_o : Control test absorbance after 30 min
A_1 : Sample extract absorption after 30 min

RESULTS AND DISCUSSION

Phenolic compound estimation

Four phenolic compounds, namely Resorcinol, Coumarin, Catechol, and Hydroquinone, as shown in Scheme 1, were identified using HPLC from different extracts of fresh leaves of *R. graveolens*. The ethyl acetate extract was associated with fraction one (F. I), the ethanol extract was associated with fraction two (F. II), and the last fraction was (F. III), each fraction containing two peaks. Phenolic compounds were determined by their composition and concentration of retention time and peak area, with reference standards data, as shown in Table 2 and Figure 1. Resorcinol and Coumarin were the major phenolic compounds from the ethyl acetate extracts of *R. graveolens,* reaching their concentration amount 14.78298 and 21.75253 mg/kg. At the same time, Catechol and Coumarin were the major phenolic compounds from the ethanol extracts concentrated at 1.104966 and 16.612227 mg/kg, followed by Catechol and Hydroquinone, which reached 0.144731 and 1.90781mg/kg, respectively.

Ouerghemmi et al. (2017) investigated many phenolic compounds in different parts of Rue, such as hydroxycinnamic and hydroxybenzoic acids. Most of the phenols in leaves and flowers are phenolic acids and coumarins. Conversely, flavonoids only represent about 10% of the total phenols (Pavić et al. 2019). Based on previous studies, the Total Phenolic Compounds (TPC) of *R. graveolens* extract are significantly ($P<0.001$) higher in n-butanol extract (2,580.47 mg/gm) than in ethanol (944.63 mg/gm), petroleum ether (720.93 mg/gm), ethyl acetate (717.13 mg/gm), and chloroform extract (18.93 mg/gm). The polarity of the solvent used to extract affects the total phenolic contents. Without a doubt, the total phenolic content is highest in polar solvents and lowest in non-polar solvents, which means that the TPC of Rue is polar and works best with polar solvents. TPC was extracted with methanol at 60°C, showing that all plants have significant phenolic compounds. The TPC of *R. graveolens* leaves and flowers extracted with methanol was 1,328.8 mg /100gm extract. TPC was always much higher in the leaves and stems than in the roots (Al-Ghamdi et al. 2020). The total phenolics in Rue leaves was 13 g/ml of Catechol equivalent. Much attention has been paid to these different groups of compounds as possible natural antioxidants. The Rue's aerial parts extract has a lot of Coumarins, flavonoids, and tannins, which is in line with what we found. On the other hand, it was suggested that flowers and stems of Rue contain a lot of Coumarins; they could be used as a commercial source of natural Coumarins. In addition, Rue leaves can be a natural source of Vanillic acid (Mokhtar et al. 2022).



Scheme 1. Phenolic compounds identified by HPLC

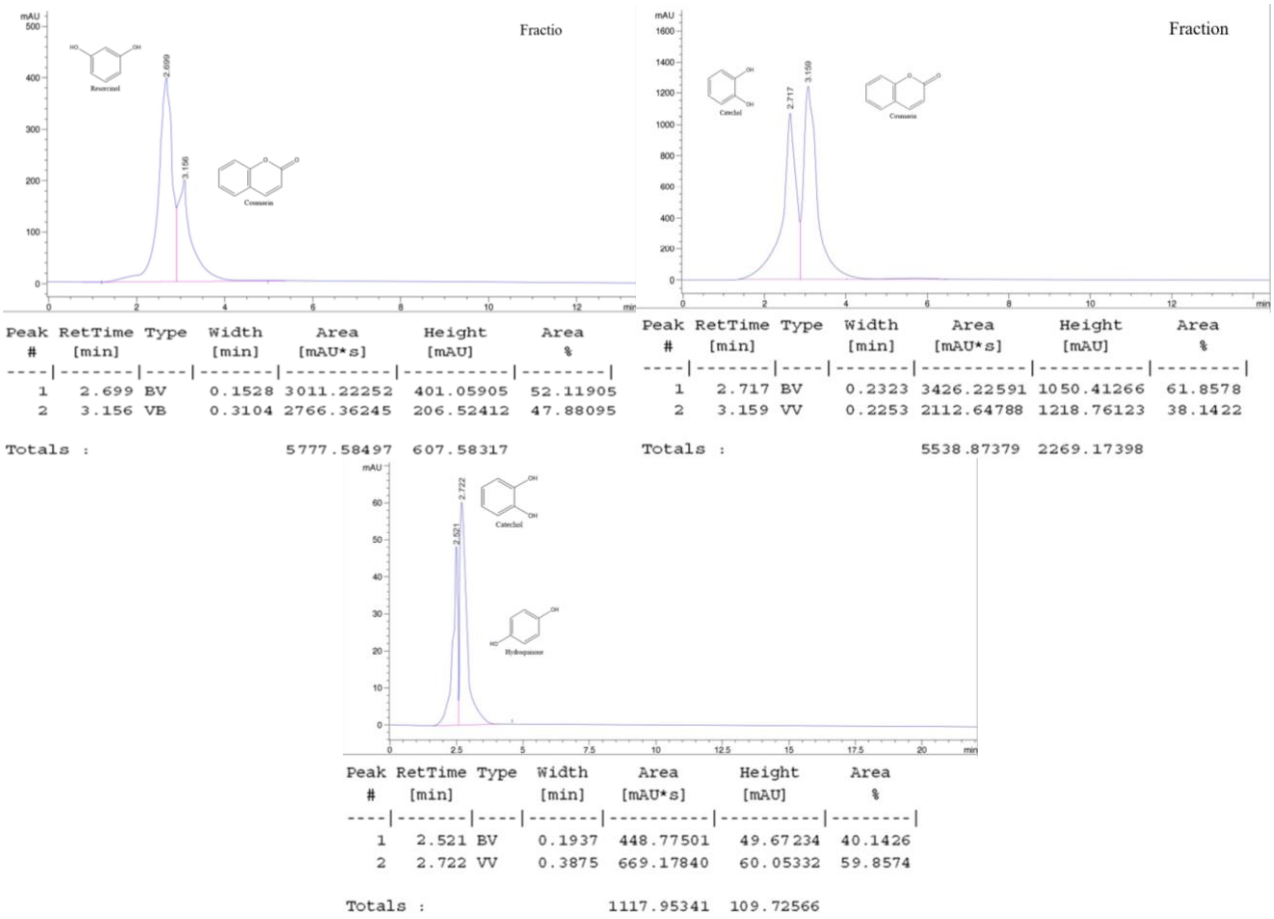


Figure 1. HPLC chromatograms of isolated fractions (I, II and III)

Table 2. Phenolic compounds detected in *R. graveolens* the different fractions by HPLC analysis

Fractions	Number of peaks	Retention time (min)	Concentration (mg/kg)[c]	Identified compounds
I[a]	1	2.699	14.78298	Resorcinol
	2	3.156	21.75253	Coumarin
II[b]	1	2.717	1.104966	Catechol
	2	3.159	16.612227	Coumarin
III[b]	1	2.521	0.144731	Catechol
	2	2.722	1.90781	Hydroquinone

Evaluation of the antibacterial activity

The antibacterial activity of different extracts (fractions) of *R. graveolens* was also tested by the disc diffusion method against four pathogenic bacterial strains. Table 3 shows that fractions were effective against the bacteria used in the study [*Bacillus cereus* and *Staphylococcus aureus* (as gram-positive bacteria), *Escherichia coli*, and *Proteus vulgaris* (as gram-negative bacteria)]. *B. cereus* and *S. aureus* were inhibited at 1.5 µg/mL concentrations for Fractions I, II, and III; *E. coli* and *P. vulgaris* were inhibited at 1.5 µg/mL concentrations for Fraction III. The results showed that Fractions I, II, and III had different effects on the four bacteria that were being studied.

The highest inhibition zone (mm) of each *B. cereus* and *S. aureus* were 23 and 22mm, respectively, at 24 µg/mL for Fractions II and I compared to control that reached 20 and 21 mm, respectively. Likewise, the highest inhibition zones (mm) of each *E. coli* and *P. vulgaris* were 21 and 20mm, respectively, at 24 µg/mL for fractions III and II, compared to the control that reached 20 and 19 mm, respectively. On the other hand, the inhibition zones (mm) of *E. coli* and *P. vulgaris* were not observed at 1.5 g/mL for Fraction I and II. Furthermore, evidence showed that resorcinol, coumarin, catechol, and hydroquinone all play important roles in inhibiting the tested strains in Fractions I, II, and III from growing.

Several studies have been conducted in the past decades focusing on plants as potential natural sources of antimicrobial agents that might be effective against multi-resistant strains without side effects on the environment. The antibacterial activity of the phenolic extract of *R. graveolens* was tested on five pathogenic strains: two Gram-positive strains (*Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC 7644) and three Gram-negative strains (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* ATCC 13311). The disc diffusion and micro dilution methods were used to observe the inhibition zones and the minimum inhibitory concentrations, respectively. *Staphylococcus aureus* was the most sensitive bacteria, with an inhibition zone of 14.37 mm; followed by *Listeria monocytogenes* (11.75 mm) and *Escherichia coli* (10.25 mm). The most resistant strains were *Pseudomonas aeruginosa* and *Salmonella typhimurium*. Gram-negative

bacteria are usually more resistant to antimicrobial agents than Gram-positive bacteria. That is because gram-negative bacteria have a special outer membrane of phospholipids and Lipopolysaccharides (LPS). This structure is important because it protects the bacteria against antimicrobial agents without affecting the material exchange necessary for life (Colucci-D'Amato and Cimaglia 2020). In a different study, the methanolic and ethanolic extracts of wild and cultivated *R. graveolens* were tested against three Gram-positive strains (*B. cereus* ATCC 10876, *L. monocytogenes* ATCC 15313, and *S. aureus* ATCC 6538), three Gram-negative strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Salmonella enteritidis* ATCC 13076). This study revealed that the wild *R. graveolens* collected at the flowering season's start had the most antimicrobial activity (6.25 MIC/25 mg/mL) (Pavlović et al. 2014).

Some of the phenolic compounds found in *R. graveolens*, like rutin, syringic acid, coumarins, and naringenin may be the reason for the antibacterial effect of the extract. Polyphenolic compounds are great antimicrobial substances that come from nature. Even though it is still not exactly understood how polyphenols inhibit the growth of bacteria, there are several possible ways. For example, polyphenols could damage the cell wall and cause ions, ATP, nucleic acids, and amino acids to leak out. It could stop DNA being formed by reducing gyrase activity, changing protein biosynthesis, stopping ATP production, lowering pH levels, and changing how biofilms form (Al-Qurainy et al. 2011).

Estimation of the antioxidant activity by the DPPH method

The present study measured *R. graveolens* fractions using the DPPH free radical method. Therefore, compared to the standard, all of the fractions had antioxidant effects; Resorcinol and Coumarin were the identified phenolic compounds in fraction I, while Catechol and Coumarin were the identified compounds in fraction II, followed by Catechol and Hydroquinone in fraction III. According to the DPPH results, the best effects are 19.74, 37.41, 55.09, 66.35, and 77.67 0 µg/mL with a concentration of (200 0 µg/mL) in fraction III, while the best effect as antioxidants with a concentration (100 0 µg/mL) is 60.87 0 µg/mL in fraction II. In addition, based on previous research, the efficiency of phenolic compounds as anti-radicals and antioxidants depends on many factors. One major factor is the number of hydroxyl groups directly bonded to the aromatic rings. This structure is related to the stability of the hydroxyl radical formed after phenols donate their hydrogen atoms to the radical (Sroka and Cisowski 2003; Szewczyk et al. 2022).

The alcoholic extract of Rue showed the best radical scavenging properties, reaching about 60% anti-radical activity (Molnar et al. 2017). Concerning the antioxidant activity of *R. graveolens*, plant samples extracted with ethanol showed a higher DPPH scavenging activity (% inhibition = 59.3%) than plant samples extracted with hexane (% inhibition = 16.8%) at the same concentration (250 g/mL) (Pavlović et al. 2014). Also, methanolic and ethanolic extracts of *R. graveolens*, which grew in the wild, exhibited significant antioxidant potential (Asgharian et al. 2020; Coimbra et al. 2020). Therefore, *R. graveolens* is a bioactive compound-rich genus. Studies on the phytochemistry of extracts from *Ruta* sp. cultivated in the wild have found the presence of coumarins, phenolic acids, flavonoids, alkaloids, and tannins, among other substances (Gentile et al. 2015; Yu et al. 2021). The antioxidant activity of phenolic compounds is associated with their capability of inactivating reactive radical species. Furthermore, neutralization occurs when the antioxidant transfers an electron and/or hydrogen atom to the radical. The scavenging of free radicals by phenolic compounds can follow four chemical pathways (Sahu and Green 2017). Moreover, as is commonly known, phenolic compounds' antioxidant capability depends on the number of hydroxyl groups in the ring structure and their arrangements. Furthermore, an ortho-position of hydroxyl groups confers high stability to the radical formed after the radical neutralization process. The higher the number of hydroxyl groups, the better their antioxidant properties (Mohammedi et al. 2020). In alcohol extracts (a polar solvent), the reaction between the ten examined phenolic compounds and the radical (the DPPH radical) is faster than in the non-polar solvent (Szewczyk et al. 2022).

Table 3. Antimicrobial activity of fraction I - III

Fractions	Conc. (µg/mL)	Zone of inhibition (mm)			
		B.cereus	*S.aureus*	*E.coli*	*P.vulgaris*
I	1. 5	6	6	0	0
	3	7	9	8	5
	6	10	10	9	8
	12	14	17	11	16
	24	20	22	16	19
II	1. 5	9	8	0	0
	3	14	11	8	5
	6	17	15	8	8
	12	19	19	12	18
	24	23	21	15	20
III	1. 5	7	5	5	6
	3	9	6	8	7
	6	11	8	11	15
	12	18	10	13	18
	24	20	15	21	18
	Gentamycin	20	21	20	19

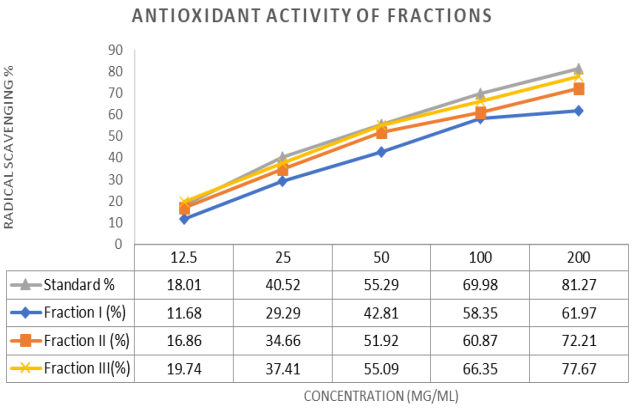


Figure 2. Antioxidant and free radical scavenging activity of isolated fractions

In conclusion, the superiority of the Soxhlet extraction method used in this study was confirmed through the additional extraction optimization, which has higher antioxidant and antibacterial activities. Based on the findings of this investigation, the fractions separated from the ethanolic extract of *R. graveolens* are superior to those separated from the ethyl acetate extract in terms of bioactive compounds, antibacterial capability, and antioxidant activity. Furthermore, four phenolic compounds were positively identified by HPLC analysis compared to the standard. Fraction I had two peaks from the ethyl acetate extract, fraction II from the ethanol extract, and fraction III also showed two peaks. Resorcinol and coumarin were the main phenolic compounds in *R. graveolens* ethyl acetate extracts, reaching 14.78298 and 21.75253 mg/kg, respectively. At the same time, Catechol and Coumarin were the main compounds in the ethanol extracts, 1.104966 and 16.612227 mg/kg, respectively, followed by Catechol and Hydroquinone at 0.144731 and 1.90781 mg/kg, respectively. Therefore, *R. graveolens* leaves could be considered promising as an antibacterial and an antioxidant agents, even though further studies are necessary to propose their role in medicinal and nutritional applications.

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