

Original Article



Antibacterial Activities and Chemical Compounds of *Plantago Lanceolata* (Ribwort Plantain) and *Plantago Major* (Broadleaf Plantain) Leaf Extracts

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ABSTRACT

Background: *Plantago lanceolata* L. (ribwort plantain) and *Plantago major* L. (broadleaf plantain) are widely used in ethnobotanical studies and for treating various diseases. This study aims to investigate the antimicrobial activity and chemical compounds of these plants.

Methods: The leaf extracts of *P. lanceolata* and *P. major* were fractioned using different solvents. The phytochemical screening was carried out by the gas chromatography-mass spectrometry (GC-MS) method. The antibacterial activity of extracts was assessed using the disc diffusion method, and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC) were measured by microtiter-broth dilution method.

Results: The dichloromethane leaf extract of *P. lanceolata* and *P. major* showed the highest antibacterial activity against *Salmonella paratyphi* (diameter of the inhibition zone: 18.83 and 20.00 mm, respectively) at 100 mg/mL concentration. The lowest MIC was related to dichloromethane extracts of both plants against *S. paratyphi* (500 µg/mL). The lowest MBC (1000 µg/mL) was related to the dichloromethane extract of *P. major* against *S. paratyphi*. The main compounds of *P. lanceolata* leaf extracts were bis(2-ethylhexyl) phthalate (41.96%), 1-methoxy-3-(2-hydroxyethyl)nonane (32.69%), bicyclo[3.1.1]heptane, 2,6,6-trimethyl-(1.alpha.,2.beta.,5.alpha.)- (10.45%), and cycloheptasiloxane tetradecamethyl- (27.96% and 31.33%). The main compounds of *P. major* leaf extracts were eicosane (23.62%), cyclohexasiloxane dodecamethyl- (18.21%), 1-methyl-3-n-propyl-2-pyrazolin-5-one (18.08%), cycloheptasiloxane tetradecamethyl- (33.85%), and 1,2-benzisothiazole-3-acetic acid, methyl ester (34.26%).

Conclusion: Fractionation of the methanolic leaf extract of *P. lanceolata* and *P. major* can help better isolate active components from these plants. The antibacterial properties of the extracts of two plants may be due to the presence of antibacterial compounds detected in GC-MS.

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Introduction

Plantago, as a genus within the *Plantaginaceae* family, has about 275 species, with a global distribution. The aerial parts of *Plantago* species can be used as herbal medicine for treatment of some diseases related to the skin, respiratory, and digestive systems [1]. The benefits of these aerial parts for treatment of diabetes and cardiovascular diseases have been attributed to the presence of rutin, luteolin 7-o-glucoside, quercetin hexoside, and chlorogenic acid compounds [2]. On the other hand, ruminant microbiome functions are modulated due to their antimicrobial and antioxidant properties [3].

Plantago lanceolata L. and *Plantago major* L. are medicinal plants that are widely used without having significant side effects [4]. The biocompatibility and cytotoxic activity of different extracts of *P. lanceolata* and *P. major* roots and aerial parts have been assessed against human red blood cells and cancer cells using different techniques [5, 6]. The toxicity effects of crude extracts of these plants against *Artemia salina* in mice has also been investigated [4-6]. A study examined the biological activities of the root extracts of these plants by fractionating the crude extracts [7]. These plants contain aucubin and catalpol which are chemotaxonomic markers for examining the quality of extracts [8, 9]. These compounds have also been evaluated in different parts of these plants [10]. Since there is a need to improve the knowledge for the treatment of opportunistic bacterial infections, this in-vitro study aims to investigate the antimicrobial activities of different extracts of *Plantago* species against pathogenic bacteria. To confirm the antibacterial activity of the extracts, phytochemical compounds were also analyzed.

Materials and Methods

Samples

The plants were first obtained from Zanjan, Iran (36°41'15.5"N 48°24'02.2"E). Then, they were authenticated at the Department of Botany, [University of Zanjan](#). All sections of plants were completely washed and separated. The leaves were then cut into small pieces and dried in the shade and at a room temperature for 10 days.

Extract preparation

The leaves of plants (250 g) were extracted by petroleum ether using the reflex method for 16 hours. The

leaves of plants (250 g) were extracted by petroleum ether using the reflex method for 16 hours followed by methanol with the same duration time. The methanol extract was then separated using liquid-liquid extraction with ethyl acetate, n-butanol, and aqueous phases in a separatory funnel [11]. The aqueous extract was filtered using a filter paper to delete the herbal fibers. The extracts were concentrated using a rotary evaporator and then dried at room temperature for 10 days.

Pathogenic bacteria

The culture of three standard pathogenic bacteria, including gram-positive *Bacillus cereus* (ATCC 11778), gram-negative *Salmonella paratyphi* (ATCC 5702), and *Proteus vulgaris* (PTCC 1182) were prepared from the culture collection of Iranian Biological Resource Center. The mentioned bacteria were then cultured in Mueller-Hinton broth (MHB) at 37°C for 18 hours. Subsequently, 0.5 McFarland bacterial suspensions (1.5×10^8 CFU/mL) were prepared.

Antibacterial activity assessment

The disc diffusion method was employed to determine the antibacterial activity of *P. lanceolata* and *P. major* according to the guidelines of the National Committee for Clinical Laboratory Standards [12]. The paper discs (Whatman No. 2) with 6 mm in diameter were then impregnated with 5 μ L of extract dissolved in Dimethyl sulfoxide (DMSO) to obtain the concentration of 100 mg/mL. The sterile blotting paper discs were soaked in the diluted extracts and left to fully dry. The dried discs were then applied for antibacterial tests using the disc diffusion method. The turbidity of inoculums was matched with the 0.5 McFarland turbidity standard. Subsequently, the inoculums were inoculated onto the Mueller-Hinton agar (MHA) plate with a sterile cotton swab to reach uniform microbial growth. Gentamicin (10 μ g/mL) was used as the positive control, while the DMSO-soaked discs were considered as negative controls. The plates underwent 24 hours of incubation at 37°C. The antibacterial properties were then evaluated based on the inhibition zone diameter (mm).

Assessment of minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) of extracts was determined by the broth microdilution method according to the guidelines of Clinical Laboratory Standards Institute guidelines [13, 14]. Growth inhibition assays were performed in the sterile 96-well plates at a final volume of 200 μ L. The cell concentrations were

Table 1. Antimicrobial activities of *P. lanceolata* and *P. major* leaf extracts against pathogenic bacteria

Extracts (100 mg/mL)	Mean±SD					
	Diameter of the Inhibition Zone (mm)					
	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>
	<i>B. cereus</i> (ATCC 11778)	<i>B. cereus</i> (ATCC 11778)	<i>P. vulgaris</i> (PTCC 1182)	<i>P. vulgaris</i> (PTCC 1182)	<i>S. paratyphi</i> (ATCC 5702)	<i>S. paratyphi</i> (ATCC 5702)
Petroleum ether	16.00±1.0 ⁺⁺	-	9.00±0.0	11.50±0.5 ⁺	7.00±0.0	-
Ethyl acetate	12.00±2.0 ⁺	16.75±1.2 ⁺⁺	14.50±1.4 ⁺	17.50±2.5 ⁺⁺	7.33±0.3	-
Dichloromethane	8.75±0.25	-	8.25±0.3	15.00±1.2 ⁺⁺	18.83±0.3 ⁺⁺	20.00±1.5 ⁺⁺⁺
Butanol	9.25±1.2	8.25±0.25	12.25±2.2 ⁺	17.50±1.5 ⁺⁺	7.66±0.6	8.50±1.5
Aqueous	-	-	8.75±1.2	-	-	-
Gentamicin (10 µg/mL)	28.00±2.0 ⁺⁺⁺		26.00±1.3 ⁺⁺⁺		20.00±1.1 ⁺⁺⁺	

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-No inhibition; ⁺Moderate activity (10–15 mm); ⁺⁺Indicates strong activity (15–20 mm); ⁺⁺⁺Very strong activity (>20 mm).

estimated from the optical densities at a 600-nm wavelength. Then, 100 µL of mid-logarithmic-phase bacterial cultures (10⁵ CFU/mL) in MHB were added to 100 µL of serially diluted extracts. The final concentration of extracts in each well was 0.5 to 4 mg/mL. The wells containing MHB with bacterial inoculum only used for the bacterial growth control and those with MHB alone were applied for the control of sterility. All samples were prepared in triplicate. Microplates were incubated at 37°C for 24 hours, and the bacterial cell growth was assessed by measuring the optical density of cultures at a 600-nm wavelength using an ELISA plate reader (Tecan Infinite M200, Austria). The MICs were defined as the lowest concentration that completely inhibits the bacte-

rial growth. To determine the minimum bactericidal concentration (MBC) of the extracts, 100 µL of solution in the clear wells were inoculated on MHA plates and incubated at 37°C for 24 hours.

Phytochemical screening

The findings of gas chromatography-mass spectrometry (GC-MS) were analyzed using a GC-MS device (Agilent technologies 5975c, USA). The leaf extracts of *P. lanceolata* and *P. major* (1 µL) were injected into the GC-MS system equipped with a capillary column (30 m × 250 µm × 0.25 µm). Helium was applied at a flow rate of 1.0 mL/min. The temperature of the injector and the

Table 2. MICs of *P. lanceolata* and *P. major* leaf extracts against pathogenic bacterial strains

Extracts	MIC (µg/mL)					
	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>
	<i>B. cereus</i> (ATCC 11778)	<i>B. cereus</i> (ATCC 11778)	<i>P. vulgaris</i> (PTCC 1182)	<i>P. vulgaris</i> (PTCC 1182)	<i>S. paratyphi</i> (ATCC 5702)	<i>S. paratyphi</i> (ATCC 5702)
Petroleum ether	1000 ^a	3000 ^b	2000 ^b	2000 ^b	3000 ^c	3000 ^c
Ethyl acetate	2000 ^b	2000 ^b	2000 ^b	2000 ^b	2000 ^b	2000 ^b
Dichloromethane	3000 ^c	4000 ^d	2000 ^b	2000 ^b	500 ^a	500 ^a
n-Butanol	3000 ^c	3000 ^c	2000 ^b	2000 ^b	2000 ^b	2000 ^b
Aqueous	4000 ^d	4000 ^d	3000 ^c	4000 ^d	4000 ^d	3000 ^c
Gentamicin	500 ^a		500 ^a		500 ^a	

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Note: Data with the same letters are not statistically significant based on the Duncan test (P<0.05). The results are obtained based on the ratio of bacteria treated with the extract to the growth of untreated bacteria after 24 h.

Table 3. MBCs of *P. lanceolata* and *P. major* leaf extracts against pathogenic bacterial strains

Extracts	MBC (µg/mL)					
	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>	<i>P. lanceolata</i>	<i>P. major</i>
	<i>B. cereus</i> (ATCC 11778)	<i>B. cereus</i> (ATCC 11778)	<i>P. vulgaris</i> (PTCC 1182)	<i>P. vulgaris</i> (PTCC 1182)	<i>S. paratyphi</i> (ATCC 5702)	<i>S. paratyphi</i> (ATCC 5702)
Petroleum ether	2000	R	3000	R	4000	4000
Ethyl acetate	4000	3000	3000	3000	3000	3000
Dichloromethane	R	R	4000	3000	2000	1000
Butanol	R	R	3000	3000	4000	4000
Aqueous	R	R	R	R	R	R

R: MBCs are not determined in concentrations (1-4 mg/mL).

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interface were kept at 350°C. The column temperature was first 50°C for 2 min and then increased to 230°C at a rate of 4°C/min for 2 min. The fragments were detected by comparing mass spectral fragmentation patterns in

MS data, NIST08.L library [11]. To prepare the applying solutions (5 mg/mL), the dried extracts were diluted in methanol (HPLC grade) and then the extracts were filtered by a 0.22-µm sterile filter and kept in a vial at 4°C.

Table 4. Different chemical compounds of *P. lanceolata* leaf extracts based on the GC-MS analysis

Compounds	%				
	Petroleum Ether	Ethyl Acetate	Dichloromethane	n-butanol	Aqueous
Fatty acids	3.6841	-	8.137	-	-
Alkynyl alcohols	1.7136	-	-	-	-
Phytosterols	1.7849	-	-	-	22.1536
Siloxanes	3.3865	28.087	11.7213	53.689	58.627
Esters	15.6941	9.1558	15.786	6.3158	-
Alkanes	5.8507	1.5927	23.7641	-	-
Phthalates	43.7601	-	3.6858	-	-
Terpenoids	0.9937	3.4496	26.5081	-	-
Amines	16.63	-	-	-	-
Ketones	-	-	-	-	7.9534
Organosulfur	1.8828	-	-	-	-
Alkenes	0.2604	19.786	-	-	-
Alcohols	5.6698	32.6911	6.095	20.2967	-
Silanes	-	-	4.3026	-	11.2658
Organo bromines	-	2.2827	-	9.0646	-
Benzoic acid derivatives	-	-	-	10.6336	-
Other	-	2.0348	-	-	-

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Table 5. The compounds obtained by fractionation of *P. lanceolata* leaf extract

Compounds	RT (min)	Petroleum ether (%)	RT (min)	Ethyl acetate (%)	RT (min)	Dichloromethane (%)	RT (min)	n-Butanol (%)	RT (min)	Aqueous (%)
n-Hexadecanoic acid	38.9	2.59	-	-	38.99	8.13	-	-	-	-
9,12-Octadecadienoic acid (Z,Z)-	42.81	0.38	-	-	-	-	-	-	-	-
Cyclohexanol, 1-ethynyl-	36.57	1.71	-	-	-	-	-	-	-	-
Beta.-sitosterol	48.44	1.78	-	-	-	-	-	-	48.18	8.22
Gamma.-sitosterol	-	-	-	-	-	-	-	-	48.54	13.93
Cycloheptasiloxane, tetradecamethyl-	25.72	2.52	26.05	12.91	26.10	8.05	26.13	27.96	26.12	31.33
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	-	-	-	-	47.03	7.73	-	-	-	-
Hexadecanoic acid, methyl ester	37.69	1.27	37.68	4.10	37.69	4.69	37.70	6.31	-	-
Octadecanoic acid, methyl ester	42.36	0.29	-	-	-	-	-	-	-	-
Oleic acid	34.65	0.29	-	-	-	-	-	-	-	-
Eicosane	39.40	2.55	-	-	39.40	8.11	-	-	-	-
Bis(2-ethylhexyl) phthalate	52.46	41.96	-	-	52.25	3.68	-	-	-	-
Phytol	42.07	1.72	-	-	42.06	6.09	-	-	-	-
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	41.78	1.33	41.77	3.42	41.77	5.03	-	-	-	-
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	42.97	0.41	-	-	-	-	-	-	-	-
2-Pentadecanone, 6,10,14-trimethyl-	35.67	0.99	-	-	35.68	3.57	-	-	-	-
Cyclohexasiloxane, dodecamethyl-	19.92	0.86	20.63	12.18	20.83	3.66	20.69	25.72	20.67	27.29
Hexadecane	29.07	0.49	-	-	-	-	-	-	-	-
Octadecane	34.48	1.84	-	-	34.48	2.38	-	-	-	-
Heptadecane	43.91	0.95	-	-	-	-	-	-	-	-
Heneicosane	-	-	-	-	53.98	2.60	-	-	-	-
Docosane	-	-	43.91	1.59	43.91	5.93	-	-	-	-
Tetracosane	-	-	-	-	48.14	4.73	-	-	-	-
Methanamine, N-methoxy-	3.35	0.12	-	-	-	-	-	-	-	-
Cyclohexane, 1-methyl-4-(1-methylethenyl)-, trans-	-	-	-	-	36.57	4.74	-	-	-	-
2-Pentanethiol	3.90	1.15	-	-	-	-	-	-	-	-
9,12-Octadecadienoic acid, methyl ester	-	-	-	-	41.63	1.55	-	-	-	-
2-Butanone, 4-hydroxy-3-methyl-	-	-	-	-	-	-	-	-	3.59	7.95
Benzoic acid, 2,5-bis(trimethylsiloxy)-, trimethylsilyl ester	-	-	-	-	-	-	31.07	10.63	-	-
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	50.19	11.13	-	-	-	-	-	-	-	-

Compounds	RT (min)	Petroleum ether (%)	RT (min)	Ethyl acetate (%)	RT (min)	Dichloromethane (%)	RT (min)	n-Butanol (%)	RT (min)	Aqueous (%)
Methanamine, N-hydroxy-N-methyl-	3.38	16.50	-	-	-	-	-	-	-	-
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	-	-	-	-	35.49	10.45	-	-	-	-
2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	-	-	35.66	4.86	-	-	-	-	-	-
Silane, dimethoxymethyl-	-	-	-	-	3.16	4.30	-	-	-	-
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	38.63	0.96	-	-	-	-	-	-	-	-
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	-	-	39.08	2.98	-	-	-	-	-	-
1,2-Benzenedicarboxylic acid, diisooctyl ester	51.47	0.83	-	-	-	-	-	-	-	-
1-Butanol, 2-methyl-, (+/-)-	-	-	-	-	-	-	3.61	20.29	-	-
9-Octadecyne	-	-	36.56	14.92	-	-	-	-	-	-
5-Ethylcyclopent-1-ene-1-carboxylic acid	-	-	34.16	0.92	-	-	-	-	-	-
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis(trimethyl-	-	-	-	-	-	-	-	-	31.07	11.26
2-Hexadecene, 2,6,10,14-tetramethyl-	-	-	35.34	3.44	-	-	-	-	-	-
10,13-Octadecadienoic acid, methyl ester	41.63	1.66	-	-	-	-	-	-	-	-
1-Methoxy-3-(2-hydroxyethyl)nonane	35.50	3.70	35.50	32.69	-	-	-	-	-	-
1-Undecene, 8-methyl-	32.66	0.26	-	-	-	-	-	-	-	-
11,13-Dimethyl-12-tetradecen-1-ol acetate	-	-	-	-	-	-	35.49	9.06	-	-
6-Methyl-2-(4-methylphenyl)-1H-imidazo[2,1-c][1,4]benzoxazine	-	-	59.39	2.03	-	-	-	-	-	-
i-Propyl tricosanoate	-	-	31.07	1.62	31.07	2.80	-	-	-	-
i-Propyl 9,12,15-octadecatrienoate	-	-	-	-	43.28	1.69	-	-	-	-
1-Octadecanesulphonyl chloride	37.56	0.72	-	-	-	-	-	-	-	-
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	36.11	0.23	-	-	-	-	-	-	-	-
2-Piperidinone, N-[4-bromo-n-butyl]-	-	-	37.55	2.28	-	-	-	-	-	-

RT: Retention time

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Statistical analysis

All graphs were drawn in Excel software 2016. The values were reported as Mean±SD. The mean values were compared using the Duncan test in SPSS software, version 21. P<0.05 was considered as statistically significant.

Results

Antibacterial activity

The leaf extracts of *P. lanceolata* and *P. major* showed varied inhibitory functions against gram-positive and gram-negative bacterial strains (Table 1). The dichloromethane extract of *P. lanceolata* and *P. major* leaves exhibited the highest inhibitory activity against *S. paratyphi* (18.83±0.3 mm and 20.00±1.5 mm). Petroleum

Table 6. Different chemical compounds of *P. major* leaf extracts determined by the GC-MS analysis

Compounds	%				
	Petroleum Ether	Ethyl Acetate	Dichloromethane	n-butanol	Aqueous
Fatty acid	-	2.0677	6.5765	-	-
Ester	20.3732	50.2162	19.7445	-	-
Terpenoid	24.2965	2.5978	7.0805	-	14.5592
Alkane	15.9313	-	25.1938	-	-
Alkene	-	-	4.228	-	-
Alcohol	9.4392	-	3.3156	21.1489	-
Siloxane	23.0752	35.9266	4.4385	65.0266	34.1785
Silane	4.0418	-	10.5071	-	-
Phenol	-	2.5896	-	-	-
Carbazoles	2.8427	-	-	-	-
Organobromine	-	-	0.8303	-	-
other	-	6.6022	18.0852	13.8246	51.2624

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extract of *P. lanceolata* and ethyl acetate and butanol extracts of *P. major* showed very good inhibitory activity against *B. cereus* and *P. vulgaris*, respectively.

The MICs of *P. lanceolata* and *P. major* extracts showed good antibacterial activity against *P. vulgaris* and *S. paratyphi* (Table 2). These MIC values indicated the inhibition of bacterial proliferation and growth after treatment by using different extracts. The lowest MIC was related to the dichloromethane leaf extracts of two plants (500 µg/mL) against *S. paratyphi*. The dichloromethane leaf extracts had strong inhibitory activity against *S. paratyphi* similar to that of gentamicin (Table 2). The petroleum ether extract of *P. lanceolata* leaf showed more effective activity against *B. cereus* with a MIC value of 1000 µg/mL (Table 2). The acceptable antibacterial activity of different extracts on the growth of bacteria after 24 hours indicates their significant antibacterial potential.

Table 3 shows the MBC values. The lowest MBCs (1000 µg/mL) was related to the dichloromethane leaf extract of *P. major* against *S. paratyphi*. On the other hand, *B. cereus* was the most resistant bacteria to dichloromethane, butanol, and aqueous extracts of two plants, because it did not show MBC values at 0.5-4 mg/mL concentrations. The aqueous extract showed the lowest

antibacterial effect, since the MBC values were not determined for extract against tested bacteria.

Phytochemical analysis

The petroleum ether solvent was applied to delete non-polar compounds. In this regard, the crude methanol extract was fractionated to make various extracts. Table 4 shows the main components of *P. lanceolata* leaf extracts. The main chemical compounds of petroleum ether, ethyl acetate, dichloromethane, n-butanol, and aqueous extracts were phthalates (43.76%), alcohols (32.69%), terpenoids (26.50%), Siloxanes (53.68% and 58.62%), respectively. As can be seen, the main component of petroleum ether extract was bis (2-ethylhexyl) phthalate (41.96%), while the main component of ethyl acetate extract was 1-methoxy-3-(2-hydroxyethyl) nonane (32.69%). The major component of dichloromethane, extract was bicyclo [3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)- (10.45%), while for the both of butanol and aqueous extracts, the main component was cycloheptasiloxane tetradecamethyl- (27.96%, and 31.33%, respectively) (Table 5). Cycloheptasiloxane, tetradecamethyl- and cyclohexasiloxane-, dodecamethyl- were common components in all fractions of *P. lanceolata* leaf (Table 5).

Table 7. The compounds obtained from fractionation of *P. major* leaf extract

Compound	RT (min)	Petroleum ether (%)	RT (min)	Ethyl acetate (%)	RT (min)	Dichloromethane (%)	RT (min)	n-Butanol (%)	RT (min)	Aqueous (%)
n-Hexadecanoic acid	-	-	38.88	2.06	39.04	6.57	-	-	-	-
Cyclohexanol, 1-ethynyl-	-	-	-	-	36.55	3.31	-	-	-	-
Cycloheptasiloxane, tetradecamethyl-	26.21	14.29	26.19	12.72	26.22	2.06	26.15	33.85	26.22	9.72
Hexadecanoic acid, methyl ester	37.72	3.87	-	-	37.68	3.36	-	-	-	-
Octadecanoic acid, methyl ester	-	-	42.35	9.65	42.35	1.57	-	-	-	-
9-Octadecenoic acid (Z)-, methyl ester	-	-	41.75	8.03	-	-	-	-	-	-
Eicosane	39.40	23.62	-	-	39.40	10.00	-	-	-	-
Methyl tetradecanoate	-	-	32.54	1.65	-	-	-	-	-	-
Cyclotetracosane	-	-	-	-	48.63	1.82	-	-	-	-
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	35.48	11.30	-	-	35.47	7.08	-	-	-	-
2-Pentadecanone, 6,10,14-trimethyl-	35.68	4.59	-	-	-	-	-	-	-	-
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	41.81	4.07	-	-	-	-	-	-	-	-
Cyclohexasiloxane, dodecamethyl-	20.79	12.64	20.69	18.21	20.83	1.54	20.69	31.17	-	-
Cyclotrisiloxane, hexamethyl-	4.32	13.68	4.15	3.09	-	-	-	-	-	-
Cyclotetrasiloxane, octamethyl-	9.15	10.12	-	-	9.54	0.82	-	-	9.20	24.45
Cyclononasiloxane, octadecamethyl-	-	-	35.30	1.89	-	-	-	-	-	-
Octadecane	34.48	7.34	-	-	-	-	-	-	-	-
Heptadecane	43.90	8.75	-	-	-	-	-	-	-	-
Docosane	-	-	-	-	43.91	7.88	-	-	-	-
Tetracosane	48.13	5.37	-	-	48.13	4.70	-	-	-	-
9-Hexadecenoic acid, methyl ester, (Z)-	-	-	2.85	2.85	-	-	-	-	-	-
3-Acetyl-6-bromocoumarin	-	-	25.41	1.58	-	-	-	-	-	-
Homovanillyl alcohol	-	-	28.56	2.58	-	-	-	-	-	-
7H-Dibenzo[b,g]carbazole, 7-methyl-	9.54	2.84	-	-	-	-	-	-	-	-
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	43.22	16.73	43.16	10.40	43.19	14.81	-	-	-	-
Pentadecanoic acid, 14-methyl-, methyl ester	-	-	37.67	17.60	-	-	-	-	-	-
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	35.48	19.70	35.47	2.59	-	-	-	-	35.48	14.55
5-Octadecene, (E)-	-	-	-	-	44.27	2.79	-	-	-	-
Silane, dimethoxymethyl-	3.21	18.33	-	-	3.16	10.50	-	-	-	-
1,2-Benzisothiazole-3-acetic acid, methyl ester	-	-	-	-	-	-	-	-	5.63	34.26

Compound	RT (min)	Petroleum ether (%)	RT (min)	Ethyl acetate (%)	RT (min)	Dichloromethane (%)	RT (min)	n-Butanol (%)	RT (min)	Aqueous (%)
1-Methyl-3-n-propyl-2-pyrazolin-5-one	-	-	-	-	33.99	18.08	-	-	-	-
1-Butanol, 2-methyl-, (+/-)-	-	-	-	-	-	-	3.57	21.14	-	-
5-Ethylcyclopent-1-ene-1-carboxylic acid	-	-	-	-	34.25	0.78	-	-	-	-
Benzeneacetic acid, .alpha.,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	-	-	31.07	5.01	-	-	31.06	13.82	-	-
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-	31.08	4.04	-	-	-	-	-	-	-	-
7,10,13-Hexadecatrienoic acid, methyl ester	41.80	3.63	-	-	-	-	-	-	-	-
5-Eicosene, (E)-	-	-	-	-	39.67	1.43	-	-	-	-
2,2-Dimethyl-1-diisopropylsilyloxypropane	-	-	-	-	-	-	-	-	3.54	16.99
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	36.56	15.00	-	-	-	-	-	-	-	-
2-Piperidinone, N-[4-bromo-n-butyl]-	-	-	-	-	47.07	0.83	-	-	-	-

RT= Retention time.

PBR

The components of *P. major* leaf extracts are shown in Table 6. The main chemical compounds of petroleum ether, ethyl acetate, dichloromethane, butanol, and aqueous extracts were terpenoid (24.29%), esters (50.21%), alkanes (25.19%), and Siloxanes (65.02%), and Siloxanes (34.17%). Eicosane (23.62%) and Cyclohexasiloxane, dodecamethyl- (18.21%) were the main compounds of petroleum ether and ethyl acetate extracts, respectively. The main components of dichloromethane, n-butanol, and aqueous extracts were 1-methyl-3-n-propyl-2-pyrazolin-5-one (18.08%), cycloheptasiloxane, tetradecamethyl- (33.85%), and 1,2-benzisothiazole-3-acetic acid, methyl ester (34.26%), respectively (Table 7). Cycloheptasiloxane, tetradecamethyl- was present in all fractions of *P. major* leaf. Cyclohexasiloxane, dodecamethyl was also observed in all fractions except for the aqueous extract.

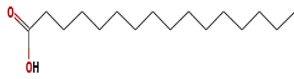
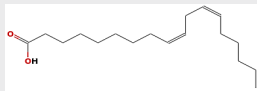

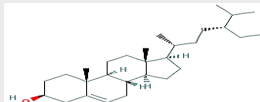
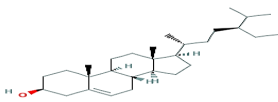
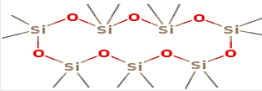
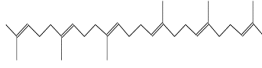

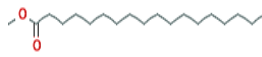


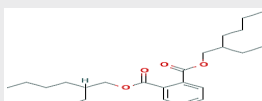
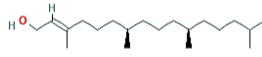

Both *P. lanceolata* and *P. major* extracts contain valuable compounds which exhibited numerous properties, such as anti-microbial, anti-viral, anti-inflammatory, and anti-cancer properties. The biological activities of identified compounds are reported in Table 8.

Discussion

P. lanceolata and *P. major* are two medicinal plants with extensive applications for treatment of various diseases. The antibacterial assessment showed that they could be

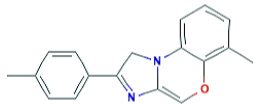
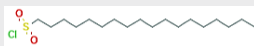
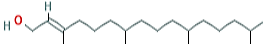
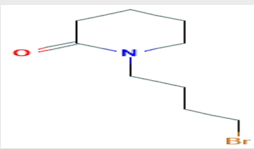
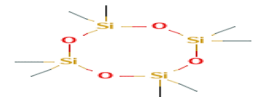
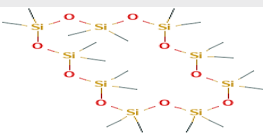
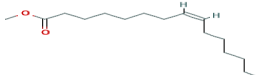



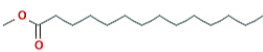
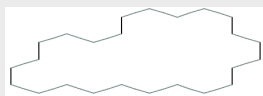


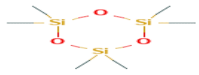
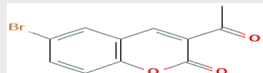
classified as potent inhibitors. Studies have reported varying degrees of antibacterial activity for *P. lanceolata* extract against some human pathogens [15]. However, Karakas et al. reported no significant antibacterial activity for methanolic extract of *P. lanceolata* against gram-positive or gram-negative bacteria. Their aqueous extract offered poor or moderate antimicrobial activity against bacteria such as *P. vulgaris* [16]. The present study also showed the poor antibacterial activity of the aqueous extracts against the bacteria. However, the methanolic extracts of two plants showed good antibacterial activity against some pathogenic bacteria, including *P. vulgaris* [8, 9, 17]. In this regard, *P. major* ethyl acetate extract showed more inhibitory function against gram-positive and gram-negative bacteria compared to the aqueous extract [18]. The results of our study are consistent with the previous studies where ethyl acetate extracts showed a significant inhibitory effect on the bacteria. Abate et al. assessed the antibacterial activity of different extracts such as pure petroleum ether extract of *P. lanceolata* leaf against pathogenic bacteria [19]. In our study, the petroleum ether extract of *P. lanceolata* showed a suitable inhibitory effect against *B. cereus*. The difference in the antibacterial activities of extracts for different species of the same genus can be attributed to differences in the growth and harvesting locations, loss of compounds during extraction and extraction methods, and thus the presence of various secondary metabolites in them [20]. MICs of extracts are different in each study based on the density of bacterial suspension (CFU/mL) and tested concentrations.

Table 8. Compounds in different extracts of *P. lanceolata* and *P. major* detected by the GC-MS analysis

Library/ID	Chemical Formula	Activity	Structure
n-hexadecanoic acid or palmitic acid	$C_{16}H_{32}O_2$	Antioxidant, hypocholesterolemic, nematocide, pesticide, antiandrogenic, hemolytic inhibitor, antifungal, flavour, 5-alpha reductase inhibitor, antimicrobial, antimalarial; antifibrinolytic, antiallopecic	
9,12-octadecadienoic acid (Z,Z)- or linolenic acid	$C_{18}H_{32}O_2$	Antiinflammatory, antibacterial, antiarthritic, hepatoprotective, antihistaminic, anticoronary, hypocholesterolemic, cancer preventive, insectifuge, antiarthritic, antiandrogenic, nematocide, antieczemic, antiacne, 5-alpha reductase inhibitor	
Cyclohexanol, 1-ethynyl-	$C_8H_{12}O$	No activity	
Beta-sitosterol	$C_{29}H_{50}O$	Antidiabetic, hypolipidemic, anticancer, antiarthritic, hepatoprotective role, antimicrobial, antioxidant, antiinflammatory, antiasthma, diuretic	
Gamma-sitosterol	C29H50O	Antioxidant, antibacterial, prophylactic	
Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	Antiperspirants, deodorants, antibacterial, antifungal, antimicrobial, antiseptic, hair conditioning agent, skin-conditioning agent-emollient, solvent	
2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- or squalene	$C_{30}H_{50}$	Antioxidant, chemopreventive, antitumor, hypocholesterolemic, antibacterial, cancer preventive, immunostimulant, chemo preventive, lipoxygenase-inhibitor pesticide	
Hexadecanoic acid, methyl ester or palmitic acid, methyl ester	$C_{17}H_{34}O_2$	Antibacterial, antifungal, antiinflammatory, hypocholesterolemic, cancer preventive, antieczemic, hepatoprotective, nematocide, insectifuge, antihistaminic, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, antioxidant, pesticide, hemolytic	
Octadecanoic acid, methyl ester, or Stearic acid methyl ester	$C_{19}H_{38}O_2$	Antiinflammatory, anticancer, antibacterial, antifungal, antimicrobial, emulsifier, perfumery industry	
Oleic Acid	$C_{18}H_{34}O_2$	Antifungal, solution phase synthesis of nanoparticles, hypocholesterolemic, anticancer	
Eicosane	$C_{20}H_{42}$	Antitumour, antifungal, antibacterial, used in petrochemical industry	
Bis(2-ethylhexyl) phthalate or Di-(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	Antibacterial, antifungal, cytotoxicity (LC ₅₀) against brine shrimp nauplii (<i>A. salina</i>)	
Phytol	$C_{20}H_{40}O$	Anticancer, antioxidant, antimicrobial, antiinflammatory, diuretic	
9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- or Linolenic acid methyl ester	$C_{19}H_{32}O_2$	Antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematocide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary	

Library/ID	Chemical Formula	Activity	Structure
9,12,15-octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	Preventive against cardiovascular diseases	
2-pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	Allelopathic, antibacterial	
Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	Antifungal, emollient, in personal care products, lubricant and de-foaming agent	
Hexadecane	$C_{16}H_{34}$	Antibacterial, antifungal, antioxidant	
Octadecane	$C_{18}H_{38}$	Anticancer, antioxidant, antimicrobial, antifungal	
Heptadecane	$C_{17}H_{36}$	Antioxidant	
Heneicosane	$C_{21}H_{44}$	Antimicrobial	
Docosane	$C_{22}H_{46}$	Antibacterial	
Tetracosane	$C_{24}H_{50}$	Cytotoxicity against cancerous cells, antidiarrheal, antibacterial, cardiotoxic, laxative, anthelmintic and removes fatigue, antiinflammatory, used in peptic ulcer treatment, anticorrosive, antioxidant, antitrichomonas	
Methanamine, N-methoxy-	C_2H_7NO	Antifungal, antibacterial	
Cyclohexane, 1-methyl-4-(1-methylethenyl)-, trans- or p-Menth-8-ene, trans-	$C_{10}H_{18}$	No activity	
2-Pentanethiol	$C_5H_{12}S$	substantial 2-pentanethiol identified for the strong antioxidant capacity displayed	
9,12-octadecadienoic acid, methyl ester or Linoleic acid methyl ester	$C_{19}H_{34}O_2$	Antioxidant, antimicrobial	
2-butanone, 4-hydroxy-3-methyl-	$C_5H_{10}O_2$	Useful for the treatment of pain, cold menthol receptor antagonist, antiviral activity	
Benzoic acid, 2,5-bis(trimethylsiloxy)-, trimethylsilyl ester or gentisic acid (tms)	$C_{16}H_{30}O_4Si_3$	It is used to treat skin irritant, cosmetic, antifungal activity of the essential oil	
1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	Antimicrobial, cytotoxicity, antioxidant, antiinflammatory, antiviral	
Methanamine, N-hydroxy-N-methyl-	C_2H_7NO	No activity	
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)- or Pinane, cis	$C_{10}H_{18}$	Antimicrobial	

Library/ID	Chemical Formula	Activity	Structure
2-hexadecene, 3,7,11,15-tetra-methyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}$	Antimicrobial, anti-inflammatory, analgesic	
Silane, dimethoxymethyl-	$C_3H_9O_2Si$	No activity	
1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester or Phthalic acid, butyl isobutyl ester	$C_{16}H_{22}O_4$	Antimicrobial, antifouling, antiviral, plasticizer compound	
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	$C_{14}H_{42}O_6Si_7$	Identified in highly medicinal plants	
1,2-benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	Antimicrobial, antioxidant	
1-butanol, 2-methyl-, (+/-)-	$C_5H_{12}O$	Biocontrol activity, total inhibition of the phytopathogens	
9-octadecyne	$C_{18}H_{34}$	Antioxidant, antimicrobial	
5-ethylcyclopent-1-ene-1-carboxylic acid	$C_7H_{10}O_2$	No activity reported	
Benzeneacetic acid, .alpha.,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	$C_{20}H_{40}O_5Si_4$	No activity reported	
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)] bis[trimethyl-	$C_{20}H_{42}O_4Si_4$	No activity reported	
2-hexadecene, 2,6,10,14-tetra-methyl-		No activity reported	
10,13-octadecadienoic acid, methyl ester or Linoleic acid esters	$C_{19}H_{34}O_2$	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematocide, insectifuge, antieczemic, anticancer, antiarthritic, antihistaminic, anticorony	
1-methoxy-3-(2-hydroxyethyl) nonane	$C_{12}H_{26}O_2$	No activity reported	
1-undecene, 8-methyl-	$C_{12}H_{24}$	No activity reported	
N-(trifluoroacetyl)-N,O,O',O''-tetrakis(trimethylsilyl)norepinephrine	$C_{22}H_{42}F_3NO_4Si_4$	No activity reported	
11,13-dimethyl-12-tetradecen-1-ol acetate	$C_{18}H_{34}O_2$	No activity reported	

Library/ID	Chemical Formula	Activity	Structure
6-methyl-2-(4-methylphenyl)-1H-imidazo[2,1-c][1,4]benzoxazine	$C_{18}H_{16}N_2O$	No activity reported	
i-Propyl tricosanoate		No activity reported	Not found
i-propyl 9,12,15-octadecatrienoate		No activity reported	Not found
1-octadecanesulphonyl chloride	$C_{18}H_{37}ClO_2S$	Not activity reported (2020)	
3,7,11,15-tetramethyl-2-hexadecen-1-ol or Phytol	$C_{20}H_{40}O$	Antimicrobial, Anti-inflammatory	
2-piperidinone, N-[4-bromo-n-butyl]-	$C_9H_{16}BrNO$	No activity reported	
Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	Antibacterial, antiseptic, hair conditioning agent, skin conditioning agent-emollient	
Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	Antioxidant, antifungal	
9-hexadecenoic acid, methyl ester, (Z)-	$C_{16}H_{30}O_2$	Antioxidant	
Pentadecanoic acid, 14-methyl, methyl ester	$C_{17}H_{34}O_2$	No activity reported	
7,10,13-hexadecatrienoic acid, methyl ester	$C_{17}H_{28}O_2$	No activity reported	
2,2-dimethyl-1-diisopropylsilyloxypropane	$C_{11}H_{25}OSi$	No activity reported	
Methyl tetradecanoate	$C_{15}H_{30}O_2$	Nematicidal	
Cyclotetracosane	$C_{24}H_{48}$	No activity reported	
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	$C_{10}H_{18}$	No activity reported	
9,12,15-octadecatrien-1-ol, (Z,Z,Z)-	$C_{18}H_{32}O$	No activity reported	
Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	Antioxidant, antimicrobial, antibacterial	
3-acetyl-6-bromocoumarin	$C_{11}H_7BrO_3$	No activity reported	

Library/ID	Chemical Formula	Activity	Structure
Homovanillyl alcohol	$C_9H_{12}O_3$	No activity reported	
7H-dibenzo[b,g]carbazole, 7-methyl-	$C_{21}H_{15}N$	No activity reported	
5-octadecene, (E)-	$C_{18}H_{36}$	Stronger sexual characters	
1,2-benzisothiazole-3-acetic acid, methyl ester	$C_{10}H_9NO_2S$	No activity reported	
1-methyl-3-n-propyl-2-pyrazolin-5-one	$C_7H_{12}N_2O$	No activity reported	
5-eicosene, (E)-	$C_{20}H_{40}$	Antibacterial, antifungal, antitumor, antidiabetic	

PBR

A study reported that n-hexane leaf extract of Iraqi *P. lanceolata* contains a high amount of hydrocarbons, fatty acids, steroids, terpenoids, and other compounds [21]. The various components of *P. major* leaf extracts found in the study by Jamileh et al. included 13.22% phytol, 10.48% benzofuranone, 10.26% pentyndiol and 10.18% benzene propanoic acid in petroleum ether extract; 30.70% glycerin, 21.81% benzene, and 16.22% dibutyl phthalate in ethyl acetate extract; 24.62% phthalic acid, 16.83% benzene propanoic acid, and 10.20% phenol in butanol extract; and 27.47% phenol, 14.53% diathiapentene, 14.13% naphthalene, and 12.02% glycerine in aqueous extract [11]. In the current study, 1.27% and 6.09% phytol were found in petroleum ether and dichloromethane extracts of *P. lanceolata* leaf, respectively. All these extracts had different chemical compositions due to the different polarities of the extraction solvents. *P. lanceolata* leaf essential oil is mainly composed of fatty acids (28%-52%), especially myristic acid, palmitic acid, linoleic acid, and linolenic acid [22]. In the present study, fatty acids (2%-39%) and esters (2%-74%) were found in all extracts, except for the aqueous leaf extracts. Fatty acids have been recognized as antimicrobial agents. Fatty acids like n-hexadecanoic acid, 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-; and 9,12-octadecadienoic acid (Z,Z)- were present in the most of extracts. These components have reported antimicrobial, and anti-inflammatory activities [23, 24]. The

analysis of leaf extracts of *Plantago* species showed that they contain a considerable amount of alkanes such as docosane, eicosane, heneicosane, heptadecane, octadecane, and tetracosane whose antibacterial properties have been reported in previous studies [25-28]. Siloxanes that are present in the leaf extracts of plants are also known as antimicrobial phytochemicals. Hexadecane, which is present in the petroleum extract of *P. lanceolata*, has been reported to be effective against *Pseudomonas aeruginosa* [23].

Rahamouz-Haghighi et al. reported various components for the root extracts of *P. lanceolata*. For its ethyl acetate extract, the main component was 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (60.93%). For the dichloromethane extract, the main component was 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (60.64%); and for the butanol extract, the main component was 1-butanol, 2-methyl-, (+/-)- (17.85%) [7]. Volatiles produced by *Saccharomyces cerevisiae*, Fialho et al. reported that the mycelium growth of the phytopathogen was inhibited almost 100% by the compound 2-methyl-1-butanol [29]. In the present study, 2-methyl-1-butanol was identified in the butanol extracts of two *Plantago* species that can be considered the main compound responsible for the antimicrobial activity. We could not determine whether di-(2-ethylhexyl) phthalate is synthesized by *P. lanceolata*, is absorbed from the at-

mosphere, or is absorbed by the roots; however, it seems that the compound, regardless of its origin, is probably present in the *P. lanceolata* leaf [30]. This compound has been reported in *Euphorbia cyparissias*, *Euphorbia seguieriana* [31], *Aloe vera* [32], *Alchornea cordifolia* [33], and *Calotropis gigantea* flowers [30]. According to the studies, this compound is not a contaminant, which was further confirmed by the GC-MS analysis. *C. gigantea* flowers can not be seen in plastic bags; therefore, they can be discounted as a source of di-(2-ethylhexyl) phthalate. Indeed, it may have taxonomic significance [30].

The antibacterial properties of *P. lanceolata* and *P. major* leaf extracts may be attributed to the presence of antibacterial compounds found in the GC/MS analysis, including palmitic acid [34], linolenic acid [24], gamma.-sitosterol [35], squalene [36], cycloheptasiloxane, tetradecamethyl- [37], hexadecanoic acid, methyl ester [38], phytol [39], 9,12-octadecadienoic acid, methyl ester [40], 1,2-benzenedicarboxylic acid, diisooctyl ester [41], 9-octadecyne [42], stearic acid [43], 9-octadecenoic acid (Z)-, methyl ester [44], cyclotetrasiloxane, octamethyl- [45], 2-methoxy-4-vinylphenol [24], pentadecanoic acid, ethyl ester [46], trans-vaccenic acid [40], Z-10-octadecen-1-ol acetate [47], 9,17-octadecadienal, (Z)- [48], phthalic acid, butyl undecyl ester [49], cyclotrisiloxane, hexamethyl- [50], octadecanoic acid [51], and other compounds.

Conclusions

Fractionation of the methanolic leaf extract of *P. lanceolata* and *P. major* can help better isolate active components from these plants, so that the antibacterial activity of the extracts can be carefully evaluated. We found numerous compounds in two *Plantago* species with antibacterial properties.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, project administration, investigation, formal analysis and writing the original draft: Samaneh Rahamouz-Haghighi; Funding acquisition and supervision: Khadijeh Bagheri and Ali Sharafi.

Conflict of interest

The authors declared no conflicts of interest.

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