Original Article

Volatile Constituents and Toxicity of Essential Oils Extracted From Aerial Parts of *Plantago Lanceolata* and *Plantago Major* Growing in Iran

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ABSTRACT

Background: *Plantago lanceolata* L. *(P. lanceolate)* and *Plantago major* L. *(P. major)* belong to the Plantaginaceae family and are widely used in traditional medicine.

Objectives: This study aims to qualitatively identify the crucial compounds and evaluate the toxicity effects of essential oils of two Plantago species.

Methods: The plantains were collected from Zanjan Province, Iran. The essential oils were extracted by hydrodistillation and then analyzed using gas chromatography coupled with mass spectrometry (GC/MS). The toxicity effects of the essential oils were evaluated on HCT-116 and HEK-293 cell lines (*in vitro* MTT assay) and *Artemia salina (A.salina)* (*in vivo* assay). The constituents of the essential oils were identified by calculating their retention indices under temperature-programmed conditions for n-alkanes (C_8-C_{20}) in the Agilent 19091S-433 column.

Results: The main identified constituents were metaraminol (14.04%), bifemelane (8.73%), metossamina (8.16%), and pterin-6-carboxylic acid (5.11%) in *P. lanceolata* and 2-dodecen-1-yl (-) succinic anhydride (15.29%), benzenemethanol, α-(1-aminoethyl)-2,5-dimethoxy-(11.83%), dl-phenylephrine (7.51%), and nortriptyline (5.15%) in *P. major*. The essential oils of *P. major* exhibited more antiproliferative properties on HCT-116 at 72 h compared to *P. lanceolata* (IC_{50}: 102.66 μg/mL). At 400 μg/mL of *P. lanceolata* and *P. major*, the percentage of the lethality of nauplii was 8% and 12%, respectively (LC50:2242.57 μg/mL and 1783.7 μg/mL). The present study showed that the most of constituents of oils were alcohols and amines.

Conclusion: Some of the compounds identified in the Plantago species essential oils have important pharmaceutical properties. This study reported the cytotoxicity of essential oils on the colon cancer cell line. However, the essential oils were not toxic against *A.salina* at the examined concentrations.

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Introduction

Essential oils are used as additives in many types of foods and beverages and various food supplements [1]. The Plantago genus of the Plantaginaceae family includes approximately 300 annual and perennial species, growing worldwide, and specially cultivated in the subtropical regions [2]. According to Iran's traditional medicine, Plantago species have many medical applications without serious side effects; however, some of the medicinal effects of *Plantago lanceolata* L. (*P. lanceolata*) and *Plantago major* L. (*P.major*) in Iran's traditional medicine have not been discovered in modern medicine [3].

P. lanceolata and *P. major* are used to treat wounds, infectious diseases, digestive and respiratory problems, fever, pain, dermatitis, and tumors [4, 5]. Furthermore, Plantago species were used to cure burns, ulcers, and eye diseases, as anti-inflammatory, antipyretic agents, antitussive, and purgative for snakebites [6]. Researchers have also reported that P.major mucilage can optimize the drug release in propranolol buccoadhesive tablets [7]. Additionally, they can be used in cosmetics to produce face masks, creams, or lotions for acne-prone and oily skins because of their astringent, anti-septic, and anti-bacterial properties [6].

GC/MS is one of the most important instruments used to analyze a sample with volatile constituents as it combines both the chromatographic technique for the efficient separation of sample constituents and mass spectroscopy that identifies the compounds according to their mass-to-charge ratio (m/z) [8]. The above-mentioned properties of these plants provide us with significant reasons to analyze their volatile composition. To date, only a few Plantago species have been investigated for their chemical constituents and biological activities of extracts. Previous studies on the chemical investigation of Plantago L. leaves and seeds extracts demonstrated the presence of polysaccharides, phenolic acids, flavonoids, iridoid glycosides, and vitamins [2].

There are few valid studies on the essential oil compositions of *P. lanceolata* and *P. major*, considering that these plants contain very small amounts of essential oil. Therefore, in the current study, following our previous studies on these plants, their essential oil compositions were examined. In addition, we evaluated the toxicity effects of the essential oils on colon cancer cells and *Artemia salina (A.salina)*. To the best of our knowledge, there are no reports on the cytotoxicity assay of *P. lanceolata* and *P. major* essential oils on colon cancer cell lines.

Materials and Methods

Herbal material

The aerial parts (leaf and stem) of *P. lanceolata* and *P. major* were collected from Zanjan Province, Iran (the geographical coordinates of the collection sites are as follows: 36°41'15.5"N 48°24'02.2"E). The taxonomic identity of species was authenticated at the Department of Botany, University of Zanjan, Iran. All sections were cut into small pieces and were dried in shade and at room temperature separately for one week.

Isolation of essential oils

The aerial parts of *P. lanceolata* and *P. major* (100 g) were ground to a coarse powder and extracted with 1500 mL of distilled water for hydrodistillation in a Clevenger-type apparatus for 5 to 6 h to arise the volatile composition in the form of essential oils. The essential oils were collected into 1 mL of n-pentane and then poured into a glass and stored at 4°C until further analysis [1].

Gas chromatography-mass spectrometry analysis

The essential oils of the aerial parts of *P. lanceolata* and *P. major* were used for GC/MS analysis. GC/MS analysis was performed using the Agilent technologies 5975c. GC/MS analysis was carried out by 1 µL of the materials subjected to analysis. The GC/MS system has been equipped with a capillary column (30 m×250 µm×0.25 µm, Agilent). Helium as the carrier gas was used at the flow rate of (1 mL/min). The injector and the interface temperature were maintained at 250°C. The column temperature was programmed as follows: the initial temperature was 40°C (1 min) and then it increased at a rate of 2°C/min up to 200°C (10 min). The identification of the constituents of *P. lanceolata* and *P. major* was performed by comparison with MS literature data (NIST08.L) and retention index (RI) [1]. The mixtures of n-alkanes (C_8-C_{20}) were injected using the above temperature program to calculate the RI for each peak. The RI of the compounds was calculated using the following equation:

$$1.\ Ix = 100n + 100\frac{[\log(tx) - \log(tn)]}{[\log(tn+1) - \log(tn)]}$$

Where: (Ix) is the Kovats retention index; (n) is the number of carbon atoms in the alkane; (tn) and (tn+1) are the retention times of the reference n-alkane hydrocarbons with n and n + 1 carbon atoms; and (tx) is the retention time of the peak of the unknown compound.

Several peaks did not have RIs for the calculated mixtures of n-alkanes (C_8-C_{20}). Thus, compounds with a formula structure less than C_8 and more than C_{20} could not be calculated (these compounds were considered unknown).

Cell line culture

Human embryonic kidney cell (HEK-293) as a normal cell line and colorectal cancer cell line (HCT-116) provided by the Pasteur Institute of Iran, Tehran were cultured in the Dulbecco's Modified Eagle Medium with supplementation of penicillin-streptomycin (1%) along with 10% fetal bovine serum incubated in 5% CO_2 incubator at 37°C.

Cytotoxicity assay

The MTT assay was performed to evaluate the cytotoxicity of *P. lanceolata* and *P. major* essential oils on the cell lines [9]. A 96-well plate with a density of 7 × 103 cells/well were used for cell seeding. The cells were allowed to attach and grow for 24 h. The cells underwent treatment with 25-400 µg/mL concentrations. The HCT-116 were treated with 5-fluorouracil (5-FU) (Austria, Ebewe Pharma) in different doses (2.5-10 µg/mL) for 72 h. The 5-FU and untreated cells were utilized as the positive and negative control, respectively. The addition and incubation of 20 µL of MTT (5 mg/mL) for 4 h took place after 24 to 72 h, followed by removing the medium and adding 200 µL of dimethyl sulfoxide to dissolve the obtained formazan. An ELISA plate reader (Tecan Infinite M200, Austria) at 570 and 690 nm read the absorbance. The cell growth inhibition rates were examined by the following formula:

$$2.\ Viability(\%) = \frac{A\ sample}{A\ \ negetive\ control} \times 100$$

Where: (A) indicates the absorbance.

Toxicity assay on *artemia salina*

The larvae of *brine shrimp* (*A.salina* Leach) were employed to examine the *P. lanceolata* and *P. major* essential oils' overall toxicity [10]. A. salina eggs were provided by Urmia University, the West Azerbaijan Province, Iran. A flask with 35 g of NaCl dissolved in 1 L of distilled water was used for cyst culture, followed by 48 h incubation at 28°C and the larvae hatching after 48 h. Every well in the 96-well microtiter plates having the Roswell Park Memorial Institute (RPMI-1640) received the essential oils (25-400 µg/mL). The next step included the addition of 10 nauplii per well to the 96-well plates and incubation at a temperature of 25°C for 24 h. A binocular microscope was employed to calculate the number of live nauplii in every well after 24 h. All experiments were repeated 3 times. Additionally, the negative control contained only 10 nauplii and artificial seawater. Potassium dichromate ($K_2Cr_2O_7$) was used as a positive control at the same concentrations as the essential oils. The number of survived samples in the experimental and control wells was used to calculate the percentages of the nauplii morality. The Abbott formula determined the lethality:

Statistical analysis

The data were analyzed using the SPSS software, version 21. The significant differences between means were calculated. Values were expressed as the mean of the 3 replications ± Standard Deviation (SD). The Duncan test at P value<0.05 was used to determine significant differences among treatments. IC_{50} and LC_{50} values were analyzed with the *ED50* plus v1.0 Software.

Results

Many peaks were detected in the chromatogram of the essential oils extracted from *P. lanceolata* and *P. major* aerial parts by GC/MS and their compositions were identified according to the NIST08.L library. Figure 1 shows the main chromatograms of the essential oils of *P. lanceolata* and *P. major*. The essential oils were rich in amine derivations, alcohols, alkenes, and fatty acids. The essential oils also showed the presence of acids, alkaloids, amino acids, carboxylic acid derivatives, esters, ketones, monoterpenoids, nitriles, oximes, phenols, phenethylamine derivatives, and others (Table 1).

Volatile constituents of *P. lanceolata* essential oil

Most component of *P. lanceolata* essential oil is generated by metaraminol (14.04%), bifemelane (8.73%), metossamina (8.16%), and pterin-6-carboxylic acid (5.11%).

In the present study, 106 components belonging to main chemical groups were identified in *P. lanceolata* essential oil: alcohols (17.56%) with benzyl alcohol; .α.-(1-aminoethyl)-m-hydroxy-, (-)-(14.04) as the main component; amines (14.70%) with phenylephrine (3.71%); alkenes and alkenes (12.28%) with bifemelane (8.73%); ketones (8.70%) with bicyclo [2.2.1] heptan-2-one, 4,7,7-trimethyl-, semicarbazone (2.97%); acids

(8.05%) with pterin-6-carboxylic acid (5.11%); alkaloids (5.76%) with 2H-1,2,3-triazole-4-carboxylic acid; 2-(2-fluorophenyl)- (2.12%); esters (4.02) with 2-thiopheneacetic acid; 3,5-difluorophenyl ester (1.53%); amides (3.55%) with propanamide (0.58%); amino acids (2.71%) with histidine; 1, N-dimethyl-4-nitro- (1.76%); monoterpenoids (2.45%) with Linalool (0.97%); phenol (Benzeneethanamine, 2-fluoro-.beta.,5-dihydroxy-N-methyl-) (0.45%); nitriles (0.21%) with propanenitrile, 3-(methylamino)- (0.17%); oximes with ethanone, 1-(4-pyridinyl)-, oxime (0.13%) as the main components and others (21.03%) (Table 2 and 3). The biological activities of the volatile constituents of *P. lanceolata* oil are reported in Table 4.

Volatile constituents of the essential oils of *p. major*

The present study showed that 2-dodecen-1-yl (-) succinic anhydride (15.29%), benzenemethanol,. α.-(1-aminoethyl)-2,5-dimethoxy- (11.83%), dl-phenylephrine (7.51%), nortriptyline (5.15%) were the major constituents (Tables 2 and 3).

In the present study, 79 components belonging to main chemical groups were identified in *P. major* essential oil: amines (35.74%) with phenylephrine (11.66%) as the main component; alkenes and alkanes (24.88%) with 2-dodecen-1-yl(-)succinic anhydride (15.29%); phenols (10.49%) with dl-phenylephrine (7.51%); esters (6.96%) with sarcosine, N-valeryl-, butyl ester (2.02%); alcohols (5.14%) with cyclobutanol, 2-ethyl- (1.72%); alkaloids (3.97%) with ethylamine, 2-(adamantan-1-yl)-1-methyl- (0.28%); ketones (3.61%) with 3-(E)-hexen-2-one, (5S)-5-[(t-butoxycarbonyl-(R)-alanyl)amino]- (2.65%); amides (2.2%) with [(2,5-dimethoxyphenyl)sulfonyl] ethylamine (0.69%); monoterpenes with isoborneol (1.17%); amino acids (glycine, N-(N-L-alanylglycyl)-) (0.35%) and acid (0.16%) with imidazole-5-carboxylic acid, 2-amino- as the main component. P.major essential oil has many properties and applications that are provided in Table 4.

The essential oils of *P. lanceolata* and *P. major* species showed that the predominant compounds were present in both species; however, the amounts of these compounds (%) were different. For example, (-)-Benzyl alcohol, .α.-(1-aminoethyl)-m-hydroxy (14.04% and 1.37%), metossamina (8.16% and 0.17%), benzenemethanol, .α.- (1-aminoethyl) -2,5-dimethoxy- (3.71% and 11.66%), dl-phenylephrine (0.15% and 7.51%), nortriptyline (0.95% and 5.15%) were present in *P. lanceolata* and *P. major*, respectively (Figure 2). Bifemelane (% 8.73), pterin-6-carboxylic acid (5.11%) existed only in *P. lanceolata* while 2-dodecen-1-yl (-) succinic anhydride (15.29%) were only found in *P. major*.

Cytotoxic activities

Table 1. Major compound groups obtained from extracted essential oil of *plantago lanceolata* and *plantago major* aerial parts

Classification of Compositions	*Plantago Lanceolata* (%)	*Plantago Major* (%)
Alcohols	17.5694	5.14
Alkaloids	5.7652	3.97
Alkanes and alkenes	12.2893	24.88
Amides	3.5522	2.2
Amines	14.7012	35.74
Amino acids	2.711	0.35
Esters	4.0211	6.96
Ketones	8.7041	3.61
Phenols	0.4593	10.49
Terpenes	2.4556	1.17
Others	29.4376	7.09

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Table 2. Identified compositions in *plantago lanceolata* essential oil by hydrodistillation

RI	Area Pct (%)	Library/ID – (*Plantago Lanceolata*)	Formula	Molecular Weight	RI	Area Pct (%)	Library/ID – (*Plantago Major*)	Formula	Molecular Weight
1230.29	0.043	Uramil-N,N-diacetic acid	$C_8H_9N_3O_7$	259.17	1352.44	1.37	Benzyl alcohol, .alpha.-(1-aminoethyl)-m-hydroxy-, (-)-	$C_9H_{13}NO_2$	167.20
1234.07	0.2343	Phosphonic acid, (1-aminoethyl)-, bis(trimethylsilyl) ester	$C_8H_{24}NO_3PSi_2$	269.43	1354.88	0.85	Benzeneethanamine, 4-chloro-.alpha.-methyl-	$C_9H_{12}ClN$	169.65
1251.93	0.126	Adrenalone	$C_9H_{11}NO_3$	181.19	1358.38	0.13	1,2-Benzenediol, 4-[2-(methylamino)ethyl]-	$C_9H_{13}NO_2$	167.2
1262.13	0.0875	1-Methyl-2-phenoxyethylamine	$C_9H_{13}NO$	151.21	1365.88	1.8	Benzeneethanamine, 2-fluoro-.beta.,5-dihydroxy-N-methyl-	$C_9H_{12}FNO_2$	185
1273.40	0.9972	Quinoline, 4-methyl-, 1-oxide	$C_{10}H_9NO$	159.18	1368.89	0.43	Phenethylamine, p,.alpha.-dimethyl-	$C_{10}H_{15}N$	149.23
1290.69	0.1024	2-Amino-1-(o-methoxyphenyl)propane	$C_{10}H_{15}NO$	165.2322	1379.10	0.79	Epinephrine	$C_9H_{13}NO_3$	183.2
1292.05	1.1679	[2,7]Naphthyridine-1,3,6,8-tetraol	$C_8H_6N_2O_4$	194.14	1380.34	0.28	Benzeneethanamine, N-methyl-	$C_9H_{13}N$	135.2
1296.54	0.0628	1,2-Benzenediol, 4-(2-amino-1-hydroxy-propyl)-	$C_9H_{13}NO_3$	183.2	1384.20	0.15	2-(5-Methylaminopentyl)-5-methylthio-1,3,4-thiadiazole	$C_9H_{17}N_3S_2$	231.4
1307.43	1.761	Histidine, 1,N-dimethyl-4-nitro-	$C_8H_{12}N_4O_4$	228.21	1384.98	0.19	Phenol, 4-(2-aminopropyl)-	$C_9H_{13}NO$	151.21
1319.98	2.1206	2H-1,2,3-Triazole-4-carboxylic acid, 2-(2-fluorophenyl)-	$C_9H_6FN_3O_2$	207.16	1397.16	0.93	2-Amino-1-(o-methoxyphenyl)propane	$C_{10}H_{15}NO$	165.23
1330.54	0.6905	Phenylpropanolamine	$C_9H_{13}NO$	151.21	1424.89	0.39	3,4-Methylenedioxy-amphetamine	$C_{10}H_{13}NO_2$	179.22
1345.22	0.2952	l-Alanine, N-(1-oxopentyl)-, methyl ester	$C_9H_{17}NO_3$	187.24	1457.00	0.19	Propanamide, N-(1-cyclohexylethyl)-	$C_{11}H_{21}NO$	183.29
1346.19	0.1483	dl-Phenylephrine	$C_9H_{13}NO_2$	167.2	1466.05	1.26	3-Methoxyamphetamine	$C_{10}H_{15}NO$	165.23
1353.01	0.9611	Racepinephrine	$C_9H_{13}NO_3$	183.2	1468.92	2.68	Phenethylamine, p-methoxy-.alpha.-methyl-, (.+/-.)-	$C_{10}H_{15}NO$	165.23
1356.10	0.0546	2-(5-Aminohexyl)furan	$C_{10}H_{17}NO$	167.25	1479.59	0.17	Benzeneethanamine, 3,4-dimethoxy-N-methyl-	$C_{11}H_{17}NO_2$	195.62
1362.62	0.4638	Epinephrine	$C_9H_{13}NO_3$	183.2	1481.73	0.32	Metanephrine	$C_{10}H_{15}NO_3$	197.23
1366.25	0.4593	Benzeneethanamine, 2-fluoro-.beta.,5-dihydroxy-N-methyl-	$C_9H_{12}FNO_2$	185.2	1514.77	0.54	3-Buten-2-one, 4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)-	$C_{14}H_{22}O$	206.32
1368.84	0.0522	Metanephrine	$C_{10}H_{15}NO_3$	197.23	1522.72	0.3	Mexiletine	$C_{11}H_{17}NO$	179.25
1375.33	3.7122	Benzenemethanol, 3-hydroxy-.alpha.-[(methylamino)methyl]-, (R)-	$C_9H_{13}NO_2$	167.205	1548.55	0.72	3-Ethoxyamphetamine	$C_{11}H_{17}NO$	179.26
1382.87	0.301	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	$C_{13}H_{18}O$	190.2814	1551.58	0.06	2-Ethoxyamphetamine	$C_{11}H_{17}NO$	179.26

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RI	Area Pct (%)	Library/ID – (*Plantago Lanceolata*)	Formula	Molecular Weight	RI	Area Pct (%)	Library/ID – (*Plantago Major*)	Formula	Molecular Weight
1390.68	2.1026	m-Menth-1(7)-ene, (R)-(-)-	$C_{10}H_{18}$	138.25	1576.31	0.12	Benzeneethanamine, 3,4-dimethoxy-.alpha.-methyl-	$C_{11}H_{17}NO_2$	195.26
1408.33	0.2024	Sarcosine, N-valeryl-, ethyl ester	$C_{10}H_{19}NO_3$	201.26	1587.37	0.55	2-Hexanamine, 5-methyl-	$C_7H_{17}N$	115.22
1431.35	0.2885	8-Azabicyclo[4.3.1]decan-10-one, 8-methyl-	$C_{10}H_{17}NO$	167.25	1612.19	1.06	1-(2-Cyano-2-ethyl-butyryl)-3-isopropyl-urea	$C_{11}H_{19}N_3O_2$	225.29
1439.41	0.0497	N-2,4-Dnp-L-arginine	$C_{12}H_{16}N_6O_6$	340.29	1616.34	0.17	Benzenemethanol, .alpha.-(1-aminoethyl)-2,5-dimethoxy-	$C_{11}H_{17}NO_3$	211.26
1453.15	0.0837	N-Isopropyl-3-phenylpropanamide	$C_{12}H_{17}NO$	191.27	1707.88	0.44	2,5-Dimethoxy-4-(methylthionyl)amphetamine	$C_{12}H_{19}NO_3S$	257.35
1456.58	1.7548	8-Amino-6-methoxyquinoline	$C_{10}H_{10}N_2O$	174.2	1749.06	2.02	Sarcosine, N-valeryl-, butyl ester	$C_{12}H_{23}NO_3$	229.31
1457.67	0.1953	Tocainide	$C_{11}H_{16}N_2O$	192.26	1766.30	0.13	Actinobolin	$C_{13}H_{20}N_2O_6$	300.31
1465.31	0.9648	L-Aspartic acid, N-(2,4-dinitrophenyl)-	$C_{10}H_9N_3O_8$	299.19	1786.65	0.11	2-(2-N-Methylaminoethyl)-4-hydroxy-5-methoxyphenylaceticacid, methyl ester	$C_{13}H_{19}NO_4$	253.29
1494.26	0.8048	3,5-Dimethylamphetamine	$C_{11}H_{17}N$	163.26	1787.68	3.52	2-(3-Phenyl-piperidin-1-yl)-ethylamine	$C_{13}H_20N_2$	204.31
1494.91	0.2261	Tricyclo[4.3.1.1(3,8)]undecane-1-carboxylic acid	$C_{12}H_{18}O_2$	194.27	1813.29	1.24	Sarcosine, N-valeryl-, pentyl ester	$C_{13}H_{25}NO_3$	243.34
1526.32	0.4584	Benzenepropanoic acid, .alpha.-(1-aminoethyl)-, [R-(R*,R*)]-	$C_{11}H_{15}NO_2$	193.24	1826.57	0.93	5-Isoxazolepropanamine, N-methyl-3-(4-nitrophenyl)-	$C_{13}H_{15}N_3O_3$	
1532.76	0.1697	Benzeneethanamine, 2-fluoro-.beta.-hydroxy-4,5-methoxy-.alpha.-methyl-	$C_{11}H_{16}FNO_3$	229.25	1900.78	1.74	Sarcosine, N-valeryl-, isohexyl ester	$C_{14}H_{27}NO_3$	257.37
1542.60	2.9722	Bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl-, semicarbazone	$C_{11}H_{19}N_3O$	209.29	1915.13	5.15	Nortriptyline	$C_{19}H_{21}N$	263.38
1573.85	0.3682	(2-Indol-1-yl-ethyl)-methyl-amine	$C_{11}H_{14}N_2$	174.24	1916.40	0.73	1-[.alpha.-(1-Adamantyl)benzylidene]thiosemicarbazide	$C_{18}H_{23}N_3S$	313.5
1592.46	8.1614	Benzenemethanol, .alpha.-(1-aminoethyl)-2,5-dimethoxy-	$C_{11}H_{17}NO_3$	211.26	2032.74	0.8	Benzeneethanamine, .alpha.-methyl-3-[4-methylphenyloxy]-	$C_{16}H_{19}NO$	241.32
1598.97	0.5233	Propanamide, 3-(3,4-dimethylphenylsulfonyl)-	$C_{11}H_{15}NO_3S$	241.31	2053.33	0.06	Ethanamine, N-methyl-2-[(2-methylphenyl)phenylmethoxy]-	$C_{17}H_{21}NO$	255.35
1633.16	0.1986	Acetamide, 2-(adamantan-1-yl)-N-(1-adamantan-1-ylethyl)-	$C_{14}H_{22}ClNO$	355.6	2150.11	0.21	l-Alanine, N-octanoyl-, pentyl ester	$C_{16}H_{31}NO_3$	285.42
1633.68	0.3392	Folic Acid	$C_{19}H_{19}N_7O_6$	441.4	2154.26	0.47	Desmethyldoxepin	$C_{18}H_{19}NO$	265.3
1639.54	0.3155	3-Propoxyamphetamine	$C_{12}H_{19}NO$	193.28	2194.96	1.56	1-Octadecanamine, N-methyl-	$C_{19}H_{41}N$	283.53
1699.53	1.3231	3-Methyl-3,5--(cyanoethyl)tetrahydro-4-thiopyranone	$C_{12}H_{16}N_2OS$		2198.92	0.26	1-Methyl-4-[nitromethyl]-4-piperidinol	$C7H_{14}N_2O_3$	174.2

RI	Area Pct (%)	Library/ID – (*Plantago Lanceolata*)	Formula	Molecular Weight	RI	Area Pct (%)	Library/ID – (*Plantago Major*)	Formula	Molecular Weight
1732.19	0.2144	l-Alanine, N-capryloyl-, methyl ester	$C_{12}H_{23}NO_3$	229.3159	2411.60	0.42	3,3-Dimethyl-4-methylamino-butan-2-one	$C_7H_{15}NO$	129.2
1831.57	0.4466	2-(2-N-Methylaminoethyl)-4-hydroxy-5-methoxyphenylaceticacid, methyl ester	$C_{13}H_{19}NO_4$	253.29	2482.03	0.35	Glycine, N-(N-L-alanylglycyl)-	$C_7H_{13}N_3O_4$	203.19
1833.98	1.0636	5-Isoxazolepropanamine, N-methyl-3-(4-nitrophenyl)-	$C_{13}H_{15}N_3O_3$	261.28					
1915.13	0.9451	Nortriptyline	$C_{19}H_{21}N$	263.4					
1943.73	0.6324	Benzeneethanamine, .alpha.-methyl-3-[4-methylphenyloxy]-	$C_{16}H_{19}NO$	241.33					
1963.23	0.2011	2,5-Dimethoxy-4-propylamphetamine	$C_{14}H_{23}NO_2$	237.34					
1993.21	0.4336	Benzofuran-5-ol, 3-(2-furanoyl)-4-dimethyl-aminomethyl-	$C_{16}H_{15}NO_4$	285.29					
2051.87	0.2945	3,3-Dimethyl-4-methylamino-butan-2-one	$C_7H_{15}NO$	129.2					
2075.78	2.9079	Atomoxetine	$C_{17}H_{21}NO$	255.35					
2092.67	0.5213	8-Methyl-2,3,3a,4,5,6-hexahydro-1H-pyrazino[3,2,1-jk]carbazole-3-carboxamide	$C_{16}H_{19}N_3O$	269.34					
2120.85	1.0019	Desmethyldoxepin	$C_{18}H_{19}NO$	265.3					
2137.14	0.2377	Pentanamide, N-decyl-N-methyl-	$C_{16}H_{33}NO$	255.44					
2163.98	1.2397	2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoic acid	$C_{17}H_{13}N_5O_5$	367.3					
2176.19	8.7366	Bifemelane	$C_{18}H_{23}NO$	269.4					
2226.56	0.4172	Northiaden	$C_{18}H_{19}NS$	281.4					
2236.63	0.053	1-Methyl-4-[nitromethyl]-4-piperidinol	$C_7H_{14}N_2O_3$	174.2					
2420.85	0.7689	Ethyl isopropyl dimethylphosphoramidate	$C_7H_{18}NO_3P$	195.2					
2452.66	5.1189	Pterin-6-carboxylic acid	$C_7H_5N_5O_3$	207.15					

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Notes: Non-isothermal Kovats retention indices (from temperature-programming, using definition of Van den Dool and Kratz); RI, retention index on Agilent 19091S-433. Ix=100n+100[log(tx)-log(tn)]/[log(tn+1)-log(tn)]; (n), the number of carbon atoms in the alkane; (tn) and (tn+1), the retention times of the reference n-alkane hydrocarbons with n and n + 1 carbon atoms; tx, retention time of peak of unknown compound.

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Table 3. Unidentified compositions in *plantago lanceolata* essential oils by hydrodistillation

RT	Area Pct	Library/ID – (*Plantago Lanceolata*)	Formula	Molecular Weight	RT	Area Pct	Library/ID – (*Plantago Major*)	Formula	Molecular Weight
3.1401	0.0989	Sarcosine, n-hexanoyl-, pentadecyl ester	$C_{24}H_{47}NO_3$	397.6	3.15	15.29	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$	266.38
3.5759	0.1056	Cyclopropanecarboxamide	C_4H_7NO	85.1	13.21	1.72	Cyclobutanol, 2-ethyl-	$C_6H_{12}O$	100.16
3.9266	0.0734	1-[alpha-(1-Adamantyl)benzylidene]thiosemicarbazide	nd	nd	20.74	1.56	Thiophene-3-ol, tetrahydro-, 1,1-dioxide	$C_4H_8O_3S$	136.17
4.2667	0.1183	Benzenemethanol, alpha-(1-aminoethyl)-, (R*,R*)-	nd	nd	21.03	0.47	Cyclopropyl carbinol	C_4H_8O	72.1
4.3199	0.1706	Propanenitrile, 3-(methylamino)-	$C_4H_8N_2$	84.12	26.67	0.33	Acetamide, 2-chloro-	C_2H_4ClNO	93.512
6.977	0.0332	L-Alanine, methyl ester	$C_4H_9NO_2$	103.12	50.75	0.43	2-Amino-1-(o-hydroxyphenyl)propane	-	-
7.7211	0.0328	2-Isopropoxyethylamine	$C_5H_{13}NO$	103.16	60.24	0.28	Ethylamine, 2-(adamantan-1-yl)-1-methyl-	-	-
20.8793	0.0445	Propanenitrile, 3-amino-2,3-di(hydroxymino)-	$C_3H_4N_4O_2$	128.09	60.74	0.83	4-Fluorohistamine	$C_5H_8FN_3$	129.13
40.2128	0.2184	2,4-Dimethylamphetamine	nd	nd	64.27	0.75	Cycloserine	$C_3H_6N_2O_2$	102.09
43.4439	0.0565	Adipamide	$C_6H_{12}N_2O_2$	144.17	64.97	0.13	3-Hydroxy-N-methylphenethylamine	-	-
45.0488	0.423	4-Fluorohistamine	$C_5H_8FN_3$	129	72.23	0.29	Acetamide, 2,2-dichloro-	$C_2H_3CL_2NO$	127.95
47.7166	0.2813	Acetamide, 2-cyano-	$C_3H_4N_2O$	84.08	74.16	0.26	Propanamide	C_3H_7NO	
52.7971	0.081	2-Bromoacetamide	C_2H_4BrNO	137.96	75.99	0.23	dl-3-Aminoisobutyric acid, N-methyl-, methyl ester	$C_6H_{13}NO_2$	131.17
55.5605	0.0734	Carbamic acid, N-[(N-cyanomethylpropanamide)-2-yl]-, 1-methyl-1-(3,5-dimethoxyphenyl)ethyl ester	nd	nd	76.18	0.34	2,2-Dichlorocyclopropanecarboxamide	$C_4H_5CL_2NO$	153.99
56.4108	0.3166	Cyanoacetylurea	$C_4H_5N_3O_2$	127.1	77.09	1.87	Methylpent-4-enylamine	$C_6H_{13}N$	99.17
56.8572	0.3047	Propan-1-one, 2-amino-1-piperidin-1-yl-	nd	nd	77.84	0.24	1,4-Benzenedicarboxamide, N,N'-bis(2-hydroxy-1-methyl-2-phenylethyl)-	$C_{26}H_{28}N_2O$	432.5
57.4205	0.2208	Acetamide, 2,2,2-trichloro-	$C_2H_2C_{13}NO$	162.4	77.96	0.16	4H-1,3-Dioxino[5,4-c]pyridine, hexahydro-6-methyl-8a-phenyl-		
57.8138	0.0713	2,4-Bis(hydroxylamino)-5-nitropyrimidine	$C_4H_5N_5O_4$	187.11	79.54	0.19	2,4-Bis(hydroxylamino)-5-nitropyrimidine	$C_4H_5N_5O_4$	187.11
62.3629	0.9949	Propylamine, 3-(furan-2-yl)-1-methyl-	nd	nd	80.13	0.16	Imidazole-5-carboxylic acid, 2-amino-		
62.6817	1.9931	3-Chloro-N-methylpropylamine	$C_4H_{10}ClN$	107.58	83.05	2.65	3-(E)-Hexen-2-one, (5S)-5-[(t-butoxycarbonyl-(R)-alanyl) amino]-	$C_{14}H_{24}N_2O_4$	284.35

RT	Area Pct	Library/ID – (*Plantago Lanceolata*)	Formula	Molecular Weight	RT	Area Pct	Library/ID – (*Plantago Major*)	Formula	Molecular Weight
63.6064	0.7875	3,6-Methano-8H-1,5,7-trioxacyclopenta[ij]cycloprop[a]azulene-4,8(3H)-dione, hexahydro-9-hydroxy-8b-methyl-9-(1-methylethyl)-, [1aR-(1a.alpha.,2a.beta.,3.beta.,6.beta.,6a.beta.,8aS*,8b.beta.,9R*)]-	nd	nd	84.87	0.82	l-Alanine, N-octanoyl-, decyl ester	$C_{21}H_{41}NO_3$	355.6
63.7127	0.562	N-(3-Methylaminopropyl)-N-methylformamide	$C_6H_{14}N_{20}$	130.19	86.2	0.2	Acetamide, 2,2,2-trichloro-	$C_2H_2Cl_3NO$	162.4
64.5949	0.5809	Propanamide	C_3H_7NO	73.09	87.53	0.69	N-Ethyl-2,5-dimethoxy-benzenesulfonamide	-	-
65.1157	0.2673	Benzenemethanol, .alpha.-(1-aminoethyl)-, (R*,R*)-(.+/-.)-	nd	nd	88.87	0.59	l-Alanine, N-valeryl-, tridecyl ester	$C_{21}H_{41}NO_3$	355.6
67.5922	3.9122	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	$C_6H_7N_3O_2$	153.14					
70.7489	0.294	2-Propen-1-amine, 2-bromo-N-methyl-	C_4H_8BrN	150.02					
72.4282	0.505	2-Methylaminomethyl-1,3-dioxolane	$C_5H_{11}NO_2$	117.15					
72.5026	0.536	Methanesulfonamide, N,N-dimethyl-	$C_3H_9NO_2S$	123.174					
74.5645	0.1525	Sarcosine, N-valeryl-, butyl ester	nd	nd					
75.5636	0.2446	Pyridine-3-carboxamide, 1,2-dihydro-4,6-dimethyl-2-thioxo-	nd	nd					
76.4777	0.7274	Benzyl alcohol, p-hydroxy-.alpha.-[(methylamino)methyl]-	nd	nd					
78.7841	0.2749	8-[N-Aziridylethylamino]-2,6-dimethyloctene-2	$C_{13}H_{20}O$	192.3					
80.7079	1.6954	Phenol, 4-(2-aminopropyl)-, (.+/-.)-	$C_9H_{13}NO$	151.21					
81.4307	2.4441	3-(E)-Hexen-2-one, (5S)-5-[(t-butoxycarbonyl-(S)-alanyl)amino]-	$C_{14}H_{24}N_2O_4$	284.35					
83.5032	14.0414	Benzyl alcohol, alpha.-(1-aminoethyl)-m-hydroxy-, (-)-	nd	nd					
88.4243	1.5345	2-Thiopheneacetic acid, 3,5-difluorophenyl ester	nd	nd					

Notes: These compounds were obtained from the NIST08.L library and identified by CAS numbers.

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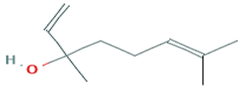
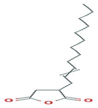
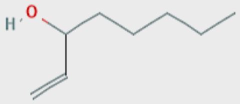
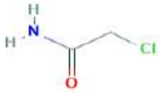
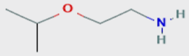
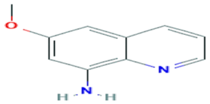
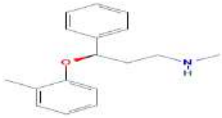
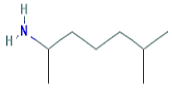
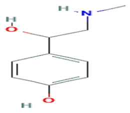
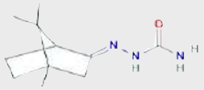
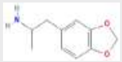
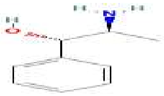
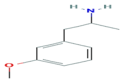
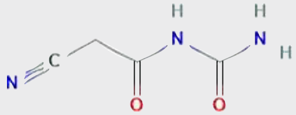
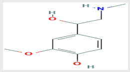
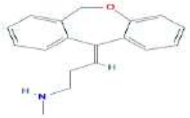
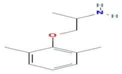
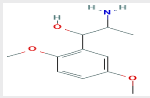
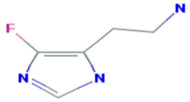
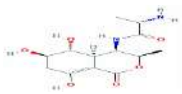
Table 4. Biological Activities of Volatile Compositions of *Plantago Lanceolata* and *Plantago Major*

Library/ID – (*Plantago Lanceolata*)	Biological Activity	Structure	Library/ID – (*Plantago Major*)	Biological Activity	Structure
1,6-Octadien-3-ol, 3,7-dimethyl- or Linalool	Anti-inflammatory, anti-cancer activities [11]		2-Dodecen-1-yl(-) succinic anhydride	Anti-convulsant, anti-neoplastic agents, anti-oxidants, anti-microbial activities [12]	
1-Octen-3-ol	A strong anti-bacterial, inhibition of the growth of insects [13], a profound influence on protein expression patterns, blocking isotropic growth, mild physiological effects on germinating conidia in solution [14]		Phenylephrine	Alpha-adrenergic agonist, decongestant, anti-bacterial activity [15]	
2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis	Anti-viral, anti-oxidative activities [16]		2-Chloroacetamide	Anti-microbial agent, [17] herbicides [18]	
2-Isopropoxyethyl-amine	Anti-microbial activity [19]		2,5-Norbornadiene	To block the ethylene receptor of plant tissues [20]	
8-Amino-6-methoxy-quinoline	Anti-malaria activity [21]		Isoborneol	Anti-viral, [22] antibacterial effects, [23] anti-bacterial activities [24]	
Arginine	Anti-microbial activity [25]		1-Methyldecylamine	Insecticidal activity [26]	
Atomoxetine (brand name Strattera)	A non-stimulant drug in the treatment of attention-deficit hyperactivity disorder and a selective noradrenaline reuptake inhibitor [27]		Octodrine	To treat Bronchitis, Laryngitis, [28] anti-fungal,[29] anti-microbial, [30] anti-tumor activities [28]	

Library/ID – (*Plantago Lanceolata*)	Biological Activity	Structure	Library/ID – (*Plantago Major*)	Biological Activity	Structure
Benzyl alcohol, p-hydroxy-.alpha.-[(methylamino) methyl]- / Synephrine	Synephrine is a primary synthesis drug developed as a sympathomimetic agent with pharmacological activities, such as vasoconstriction, blood pressure elevation, and bronchial muscle relaxation [31].		Epinephrine	To treat bronchiolitis, [32] and anaphylaxis [33]	
Bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl-, semicarbazone	Anti-candida, anti-inflammatory activities [34]		3,4-Methylenedioxyamphetamine	An empathogen-entactogen, psychostimulant, and psychedelic drug of the amphetamine family, as a recreational drug [35]	
(+)-Norpseudoephedrine / Cathine	Cathine and norephedrine, phenylpropanolamines structurally related to amphetamine [36]		3-Methoxyamphetamine	A designer drug alternative to MDMA [37]	
Cyanoacetylurea	As a starting material for the synthesis of a variety of heterocycles1 is easily prepared from low-cost materials [38], a key intermediate in the synthesis of 6-aminouracils, which possess several biological activities such as anti-cancer [39], anti-viral [40], anti-hypertensive [41], insecticidal, herbicidal, acaricidal activities [42]		Metanephrine	Inactive metabolite of epinephrine [43]	
Desmethyldoxepin	Anti-depressant properties [44]		Mexiletine	Anti-arrhythmic activity [45]	
endo-Borneol	Anti-bacterial, anti-fungal activities [46]		Benzenemethanol, alpha.-(1-aminoethyl)-2,5-dimethoxy- / Methoxamine	A blood-pressure increasing drug commonly used for maintaining intraoperative hemodynamics [47]	
4-Fluorohistamine	Substrate for several enzymes and inhibitor for histidine ammonia lyase [48]		Actinobolin	Antibiotic, antitumor, antibacterial [49]	

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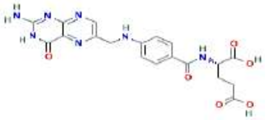
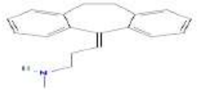
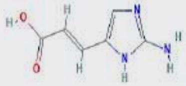
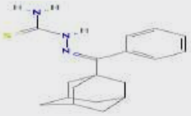
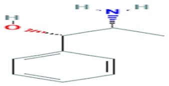
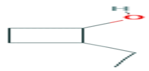
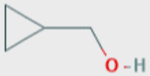
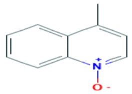
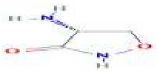
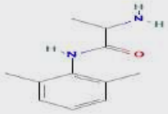
Library/ID – (*Plantago Lanceolata*)	Biological Activity	Structure	Library/ID – (*Plantago Major*)	Biological Activity	Structure
Folic Acid	Free radical scavenging, and anti-oxidant activities [50]		Nortriptyline	Antidepressant, as an analgesic in chronic back pain [51]	
Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	Anti-microbial, anti-inflammatory,[52] anti-cancer activities [53]		1-[alpha-(1-Adamantyl)benzylidene] thiosemicarbazide	Thiosemicarbazone derivatives present a great variety of biological activities, such as anti-viral, anti-cancer, anti-tumor, anti-inflammatory, anti-amoebic, and anti-microbial activities [54].	
N-2,4-Dnp-L-arginine	An activating effect on hepatocellular carcinoma receptor B4 [55]		Desmethyldoxepin	Desmethyldoxepin is the major active metabolite of doxepin (doxepin showed anti-oxidant activities), [56] an anti-depressant, and a drug metabolite [57].	
Northiaden	A major active metabolite of the tricyclic anti-depressant (TCA) dosulepin [58]		4-Fluorohistamine	Substrate for several enzymes and inhibitor for hisitidine ammonia lyase [48]	
Phenylpropanol-amine	A decongestant, appetite suppressant, [59-61] cough, cold preparations [62-63]		Cyclobutanol, 2-ethyl-	Cyclobutanol as an anti-microbial activity [64]	
Pterin-6-carboxylic acid	Anti-cancer, anti-viral [65] anti-psychotic, Moodstabilizer, anti-parasite,[66] anti-oxidant, anti-inflammatory activities [67]		Cyclopropyl carbinol	Biomedicine, flavor, skin care and cosmetic, skin-care and cosmetic, and bioenergy fungicides and insecticides, [68] an intermediate used in chemical laboratory research and development of organic compounds and pharmaceuticals [69]	
Quinoline, 4-methyl-, 1-oxide	Anti-cancer activity [70]		Cycloserine	An antibiotic used to treat tuberculosis [71]	
Tocainide	Anti-arrhythmic, local anesthetics,[72] anti-arrhythmic agent [73]		Methylpent-4-enyl-amine	Flavor indicating volatiles characterized by ripening [74]	

PBR

Table 5. IC$_{50}$ values of colorectal cancer cells and embryonic kidney normal cells and LC50 values of *artemia salina* by *plantago lanceolata* and *plantago major* essential oils

Essential Oils /Cell	HCT-116 (µg/mL)			HEK-293 (µg/mL)			*Artemia Salina* (µg/mL)
	Mean±SD						
	24 h	48 h	72 h	24 h	48 h	72 h	24 h
Plantago lanceolata	622.54^{d}±13.0	322.5^{b}±17.5	158.33^{ab}±12.9	508.65^{b}±1.3	280.5^{ab}±2.2	152.45^{ab}±1.5	2242.57^{b}±8.7
Plantago major	458.62^{a}±8.5	262.45^{a}±10.1	102.66^{a}±9.3	566.82^{c}±2.5	245.32^{a}±7.0	224.45^{b}±13.7	1783.7^{a}±15.3

PBR

Notes: The analysis was performed separately every time. IC$_{50}$ and LC$_{50}$ values are the mean of the 3 replications±standard deviation at 24, 48, and 72 h. The Duncan test was used for mean comparison (P<0.05). Charts with the same letters are not statistically significant. Values were calculated for 5-fluorouracil (IC$_{50}$:4.136 µg/mL) and Potassium dichromate (LC50:58.22 µg/mL) as positive controls.

Colorectal cancer cells were incubated after treatment with essential oils to study the cytotoxic activities of *P. lanceolata* and *P. major*. The essential oils of *P. major* exhibited more antiproliferative properties on HCT-116 at 72 h compared to *P. lanceolata* (IC$_{50}$: 102.66 µg/mL). IC$_{50}$ values showed that *P. major* essential oil had a greater cytotoxic effect on HCT-116 than HEK-293; however, *P. lanceolata* showed almost the same effect on cancer and normal cells (Table 5). The results indicated that a very low IC$_{50}$ of 5-FU (4.136 µg/mL) was required to inhibit HCT-116 cell viability compared to the essential oil of *P. lanceolata* and *P. major*.

Toxicity assay on *artemia salina*

The general toxicity of the essential oils was assessed against A. salina. At 25-100 µg/mL of the essential oils, all of the nauplii were alive, indicating no toxicity *(LC50*:2242.57 µg/mL and 1783.7 µg/mL) (Table 5). At 400 µg/mL of *P. lanceolata* and *P. major*, the percentage of lethality was 8% and 12%, respectively. Although, the $K_2Cr_2O_7$ has shown to have a toxic effect (*LC50* of 58.22 µg/mL).

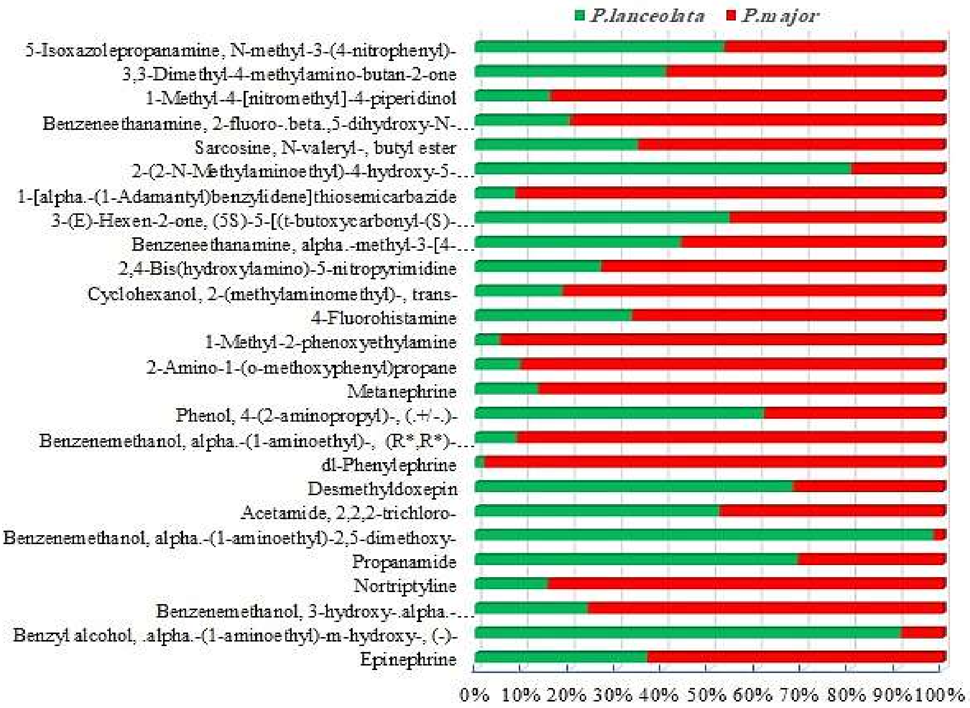


Figure 1. Chromatogram of essential oils of the aerial part of plantago species
(A) *Plantago Lanceolata* and (B) *Plantago Major*

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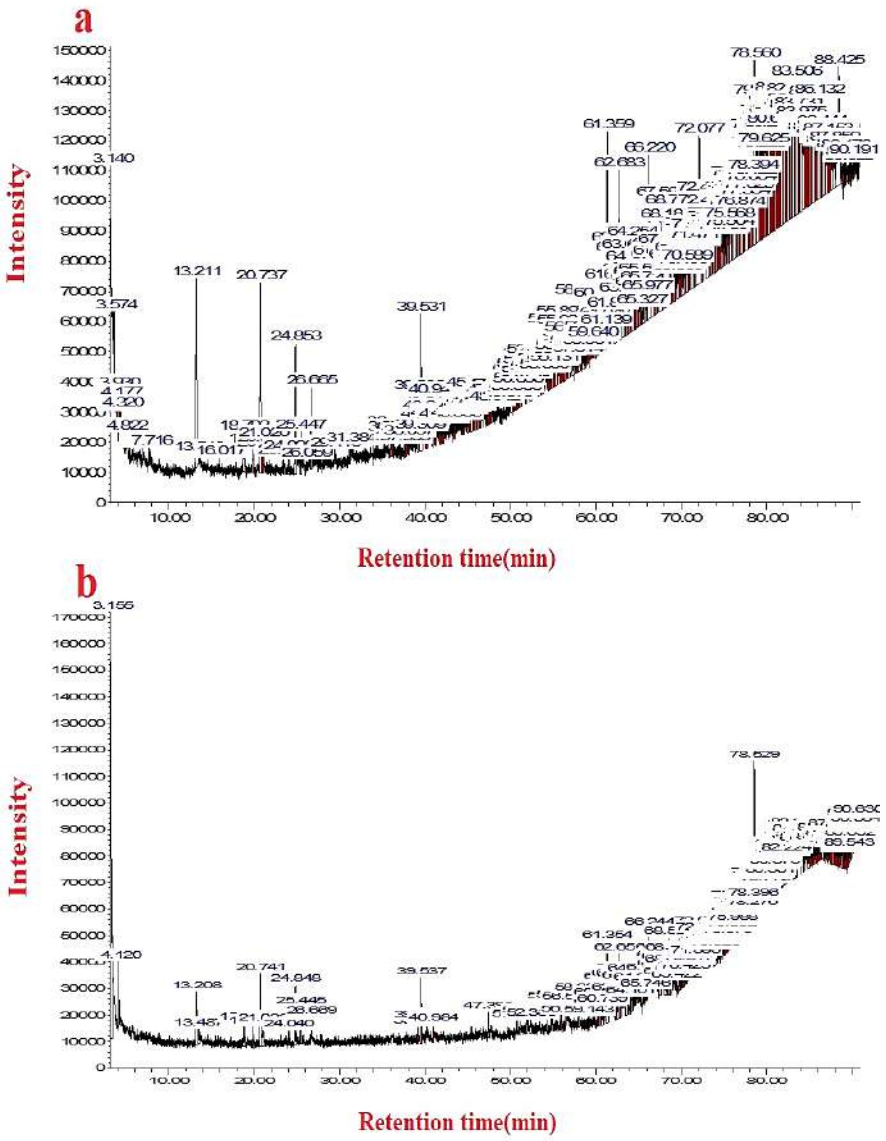

Figure 2. Common volatile composition of *plantago lanceolata* and *plantago major*

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Discussion

The presence of valuable compounds in *P. lanceolata* can be a putative candidate for its application in modern medicine, as it has been used in traditional medicine for many years. The following compounds were present in this species: the anti-cancer compounds reported in Table 4, such as linalool [11]; cyanoacetylurea [39]; imidazole, 2-amino-5-[(2-carboxy)vinyl]- [53]; pterin-6-carboxylic acid [65]; quinoline, 4-methyl-, 1-oxide [70]; anti-microbial compounds, including 1-octen-3-ol [13]; 2-isopropoxyethylamine [19]; arginine [25]; endo-borneol [46]; and imidazole, 2-amino-5-[(2-carboxy) vinyl]- [52]. The anti-viral compounds, including 2-furanmethanol, 5-ethenyltetrahydro-.α., .α.,5-trimethyl-, cis [16]; cyanoacetylurea [40]; pterin-6-carboxylic acid [65]; anti-oxidant compounds, such as 2-furanmethanol, 5-ethenyltetrahydro-.α., .α.,5-trimethyl-, cis [16]; folic Acid [50]; pterin-6-carboxylic acid [67]; anti-inflammatory, such as linalool [11], imidazole, 2-amino-5-[(2-carboxy)vinyl]- [52]; bicyclo[2.2.1] heptan-2-one, 4,7,7-trimethyl-, semicarbazone [34]; pterin-6-carboxylic acid [67]. Meanwhile, the anti-malaria compound 8-amino-6-methoxyquinoline [21] was found in the analysis of *P. lanceolata* essential oil. It was revealed that the common components of essential oil are fatty acids [75]. For instance, Fons reported palmitic acid in the essential oil of *P. lanceolata* leaves [76]. Bajer et al. used GC/MS and GC/FID techniques to study the qualitative and semi-quantitative content of volatile constituents in the essential oil, respectively. In their study, the main aroma constituents of *P. lanceolata* leaves were groups of fatty acids 28.0% – 52.1% (the most abundant palmitic acid 15.3% –32.0%), oxidated monoterpenes 4.3% – 13.2% with linalool 2.7% – 3.5%, ketones and aldehydes 6.9%–10.0% with pentyl vinyl ketone 2.0% –3.4%, and alcohols 3.8%–9.2% with 1-octen-3-ol 2.4%–8.2%. They pointed out that apocarotenoids (1.5%–2.3%) are the important constituents because of their intense fragrance and they were identified in a relatively high amount. The importance is in its potential manufacture control of raw material to supply food supplements [1]. The high content of 1-octen-3-ol (up to 8.2%) has been observed in the Bajer et al., 2016 study [1] in accordance with Fons [76]. This compound in the present study was about 1.27%.

Other studies showed that *P. major* essential oil has anti-tumor and anti-cancer activities because octodrine [28] and 1-[α-(1-adamantyl) benzylidene] thiosemicarbazide [54] were present in *P. major* essential oil. The anti-microbial components, i.e., 2-dodecen-1-yl(-) succinic anhydride [12]; 2-chloroacetamide [17]; isoborneol [23]; octodrine [30]; actinobolin [49]; 1-[α-(1-adamantyl) benzylidene] thiosemicarbazide [54]; cyclobutanol, 2-ethyl- [64]; antiviral compounds, including isoborneol [22]; 1-[α-(1-adamantyl) benzylidene] thiosemicarbazide [54]; antioxidant and anti-inflammatory compounds, such as 2-dodecen-1-yl(-)succinic anhydride [12]; desmethyldoxepin [56] and 1-[α-(1-adamantyl) benzylidene] thiosemicarbazide [54] were observed in the analysis of *P. major* essential oil. Some of the compounds identified in the analysis of the *P. major* essential oil showed important characteristics, such as cycloserine [71] and actinobolin [49] which are antibiotic drugs (0.75% and 0.13%) and isoborneol is anti-infective (1.17%) [22] (Table 4). The percentage and differences in the amount of these compounds depend on many factors, such as climatic conditions, type of region, plant growth conditions, and harvesting methods.

The present study indicated that a very low IC_{50} value of 5-FU was required to inhibit HCT-116 cell viability compared to the essential oil of *P. lanceolata* and *P. major*. However, the IC_{50} obtained for the essential oil of P.lanceolata and P.major were valuable and has increasingly important medical applications. Our previous studies reported the cytotoxic effects of alcoholic and acetonic extracts of P.major leaf and root on HCT-116 and HEK-293. The *P. major* root extract was more effective than the aerial parts, and IC_{50} values for ethanolic, methanolic, and acetonic root extracts were 405.59, 470.16, and 82.26 µg/mL, respectively on HCT-116 at 72 h [77].

In a study by Velasco-Lezama (2006), the cytotoxic activity of *P. major* methanolic extract has been reported on HCT-15 [78].

For the lethality of nauplii, if *LC50*, detected for each sample, is more than 1000 µg/mL, it will be non-toxic [79]. At 400 µg/mL of *P. lanceolata* and *P. major*, the percentage of the lethality of nauplii was 8% and 12%, respectively. Thus, the essential oils were not toxic.

Other researchers have also evaluated the toxicity effect of *P. major* methanolic extract on A. salina and A. uramiana with *LC50* of 303.7 µg/mL [80]. The *LC50* values of Plantago squarrosa Murray extracts were more than 1000 µg/mL; therefore, the extracts were non-toxic in the Artemia franciscana bioassay [81]. Our previous study showed that at all concentrations of ethanolic extracts of P.major aerial parts and roots, no toxicity was observed [77].

Conclusions

Given the non-aromatic nature of *P. lanceolata* and *P. major* and the very small amount of essential oil in these plants, most phytochemical studies are usually performed on their extracts. Therefore, in the present study, the essential oils analysis of two well-known species of Plantago was conducted to discover the valuable compositions. The hydrodistillation method enabled us to gain a great number of volatile constituents, which is evident from the number of peaks that occurred in chromatograms. The most abundant family of compounds was amines. There were also identified acids, alcohols, alkaloids, alkanes, alkenes, amides, amino acids, esters, ketones, phenols, and terpenes that most of the terpenes were oxidated as monoterpenes. On the other hand, nitriles, oximes, and organic compounds were found in a relatively small amount.

Regarding the chemical compounds identified in the *P. lanceolata* and *P. major* essential oils, these components could be employed as an important economical source in the pharmaceutical and chemical industries. We intend to study their biological activities in the future.

Ethical Considerations

Compliance with ethical guidelines

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

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Authors' contributions

Project administration, investigation, formal analysis, and writing-original draft: Samaneh Rahamouz-Haghighi; Formal analysis, methodology, and validation: Alireza Yazdinezhad; Funding and supervision: Khadijeh Bagheri; Funding, supervision, conceptualization, and editing of the English version of the manuscript: Ali Sharafi.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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