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Phytochemical Constituents and Antibacterial Activity of the Medicinal Herb *Deverra tortuosa* (Desf.) DC.

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ABSTRACT

Deverra tortuosa (Desf.) DC. is an aromatic medicinal plant belonging to the family Apiaceae. The plant was collected from two different topographic regions (Wadi Hagul (WH) and West Alexandria (Alex), Egypt at 29° 59' 906'' north to 32° 5' 707'' east and 30° 56' 358'' north to 29° 35' 373'' east respectively). A comparative study of the composition of essential oils and volatile constituents of petroleum ether and methylene chloride fractions from two samples of the two localities were done using GC/MS analysis. The phytochemical investigation of the species collected from Wadi Hagul was carried out. Bioassay-guided separation has revealed the identification of two phytosterols from pet. ether fraction and three flavonoid glycosides from butanol fraction, which were identified using NMR analyses as luteolin 7-O-α-Lrhamnopyranoside **(1)**, isorhamentin-3-O- rutinoside **(2)** and luteolin 7-O-rutinoside **(3)**. Additionally, the antibacterial activity of the two essential oils along with four fractions, pet. ether, methylene chloride, ethyl acetate and butanol fractions were investigated. The essential oils, E.Os, of the two ecological niches (WH and Alex) as well as butanol fraction gave promising activity towards *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Klebsiella pneumoniae.*

Keywords: *Deverra tortuosa*, Apiaceae, Essential oils, Flavonoids, Antibacterial activity.

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INTRODUCTION

Phytotherapeutic activities derived from aromatic and medicinal plant extracts showed a considerable interest as sources of agents to fight microbial diseases [1].The antimicrobial activities of aromatic plant extracts have been shown to depend not only on the plant species but also on the method used to extract bioactive compounds and the method employed for measuring antimicrobial capacity. In addition, the chemical composition of the extract can vary according to geo-climatic location, growing conditions (soil type, amount of water, season) and the plant's genetics [2, 3]. *Deverra tortuosa* (Desf). DC (syn. *Pituranthos tortuosus* (Desf)), known in Arabic as "Guezzah" " Shabat El-gabal", strongly aromatic glabrous shrub belonging to family Apiaceae; it is densely branched of bushy appearance with numerous blue-green slender tortuose branched umbel-rays few or numerous, always thin, flowers hardy opening. This plant is predominant in sandy and stony places [4]. *D. tortuosa* is used traditionally as analgesic, carminative, diuretic, against intestinal parasites [5], constipation, bites and for the treatment of hypertension [6,7], it is also used to relieve headache and fever. High palatability for grazing animals and used as seasoning [4]. Flavonoids, phenylpropanoids, terpenoids, unsaturated sterols and coumarins are characteristic chemical constituents which have characterized previously from *D. tortuosa* [8,9]. The objective of the current study is extraction, isolation, purification and structural Characterization of the bioactive constituents from *D. tortuosa*, in addition to, *in vitro* antibacterial assessment for its all fractions and isolated compounds.

MATERIALS AND METHODS

General experimental procedures:

NMR spectra were recorded on 500 MHz (JEOL). Chemical shifts are given in δ (ppm) relative to TMS as internal slandered material at NMR Unit of Faculty of Science, Mansoura University, Mansoura, Egypt. GC/MS analyses were performed on a Varian GC interfaced to Finnegan SSQ 7000 mass selective Detector (SMD) with ICIS V2.0 data system for MS identification of the GC components. The column used was DB-5 (J&W Scientific, Folosm, CA) cross-linked fused silica capillary column (30 m long, 0.25 mm internal diameter) coated with ploy dimethyl siloxane (0.5µmfilm thickness). The oven temperature was programmed from 50^oC for 3 min. isothermally, then heating by 7^oC/ min. to 250^oC and isothermally for 10 min., at 250^oC. Injector temperature was 200oC and the volume injected was 0.5 µl. Transitionline and ion source temperature were 250^oC and 150^oC, respectively. The mass spectrometer had a delay of 3 min. to avoid the solvent peak and then scanned from *m/z* 50 to *m/z* 300. Ionization energy was set at 70 eV. (Agriculture Research Center, National Research Center (NRC), Dokki, Cairo, Egypt). Thin layer chromatography and preparative (TLC) were performed on silica gel (Kieselgel 60, GF 254) of 0.25 thickness. Petroleum ether (60-80^oC), methylene chloride, ethyl acetate, butanol and methanol were obtained from Adwic Company.

Plant materials

The dried aerial parts of *D. tortuosa* was collected from two regions Wadi Hagul and West Alexandria, Egypt at 29^o 59' 906'' north to 32^o 5' 707'' east and 30^o 56' 358'' north to 29^o 35' 373'' east respectively in August 2017 and identified by the 3rd and the 4th authors .Voucher specimens (DT-1 and DT-2) were deposited at the Herbarium of the Botany and Microbiology Department, Faculty of Science, Damietta University, Egypt.

Extraction and isolation

Two samples of freshly aerial parts of *D. tortuosa* from Wadi Hagul (400 g) and Alexandria (300 g) were subjected to hydro-distillation for 8 h using a Clevenger-type apparatus giving their essential oils, which yielded after drying over anhydrous sodium sulphate 0.04 wt/wt (E.O W.H) and 0.03 wt/wt (E.O Alex). The essential oils were stored in dark glass tubes under refrigeration (4°C) until use.The air-dried and powdered aerial parts of the sample collected from Wadi Hagul plant (1 kg) was soaked in MeOH (3x4 L), then filtrated and evaporated to its 1/3 volume. Exhaustive solvent extraction gave pet. ether fraction (21.95 g), CH_2Cl_2 fraction (12.13 g), EtOAc fraction (5 g) and n-BuOH fraction (7 g). The pet. ether fraction (21.95 g) was defatted with cold methanol to yield defatted pet. ether fraction (7.41 g) which was chromatographed over silica gel CC using mixtures of increasing polarities from pet. ether/ ethyl acetate. Five sub-fractions were obtained after comparing the TLC patterns. Sub-fraction I afforded a mixture of two compounds by using PTLC (silica gel, pet. ether/ ethyl acetate, 9:1, R*f* 0.41). Butanol fraction has been applied on PTLC (silica gel, EtOAc/MeOH/H_2O, 45:3:2) to afford compound **(1)** (*Rf* 0.66), compound **(2)** (*Rf* 0.5) and compound **(3)** (*Rf* 0.58).

Luteolin 7-O-α-L-rhamnopyranoside (1) Yellow powder; ^{1}H NMR (CD_3OD, 500 MHz): δ 7.55 (1H, d, *J* =1.8 Hz, H-2'), 7.56 (1H, dd, *J*= 1.8, 8.5 Hz, H-6'), 6.87 (1H, d, *J*= 8.5 Hz, H-5'), 6.70 (1H, s, H-3), 6.86 (1H, d, *J*= 1.8 Hz, H-8), 6.51 (1H, d, *J*= 1.8 Hz, H-6), 4.96 (1H, brs, H-1''), 1.14 (1H, d, *J*= 6.0 Hz, H-6'').

Isorhamentin 3-O-rutinoside (2) Yellow powder; ^{1}H NMR (CD_3OD, 500 MHz): δ 7.95 (1H, d, J 1.6 Hz, H-2'), 7.62 (1H, dd, *J*= 1.6, 8.5 Hz, H-6'), 6.90 (1H, d, *J*= 8.5 Hz, H-5'), 6.14 (1H, d, *J*= 1.8 Hz, H-6), 6.32 (1H, d, *J*= 1.8 Hz, H-8), 3.94 (3H, s, OCH_3), 5.15 (1H, d, *J*= 7.5 Hz, H-1''), 4.52 (1H, brs, H- 1'''), 1.11 (1H, d, *J*= 6.2 Hz, H-6'''); ^{13}C NMR (CD_3OD, 125 MHz): δ 158.6 (C-2), 135.4 (C-3), 179.2 (C-4), 163.0 (C-5), 100.3 (C-6), 167.2 (C-7), 95.1 (C-8), 158.8 (C-9), 105.4 (C-10), 123.0 (C-1'), 114.5 (C-2'), 148.3 (C-3'), 150.9 (C-4'), 116.1 (C-5'), 124.0 (C-6'), 104.5 (C-1''), 75.9 (C-2''), 77.4 (C-3''), 71.6 (C-4''), 78.2 (C-5''), 68.5 (C-6''), 102.5 (C- 1'''), 72.1 (C-2'''), 72.2 (C-3'''), 73.8 (C-4'''), 69.8 (C-5'''), 17.9 (C-6'''), 56.7 (OCH_3).

Luteolin 7-O-rutinoside (3) Yellow powder; ^{1}H NMR (CD_3OD, 500 MHz): δ 7.42 (1H, d, *J* = 2.2 Hz, H-2'), 7.50 (1H, dd, *J* = 2.2, 8.0 Hz, H-6'), 6.85 (1H, d, *J* = 8.0 Hz, H-5'), 6.66 (1H, s, H-3), 6.53 (1H, d, *J* = 2.2 Hz, H-6), 6.78 (1H, d, *J* = 2.2 Hz, H-8), 5.04 (1H, d, *J* = 7.1 Hz, H-1''), 4.52 (1H, d, *J* = 1.4 Hz, H-1'''), 1.18 (1H, d, *J* = 6.4 Hz, H-6'').

Antibacterial activity assay

The pathogenic microbial Gram-positive strains [*Streptococcus pyogenes* (ATCC19615), *Staphylococcus aureus* (ATCC6538)] and Gram-negative strains [*Salmonella typhimurium* (ATCC25566), *Escherichia coli* (ATCC10536), *Klebsiella pneumonia* (ATCC10031)] were obtained from American Type Culture Collection (ATCC). The assays were performed at Genetic Engineering and Biotechnology Unit of Faculty of Science, Mansoura University, Mansoura, Egypt. Antibacterial effectiveness was assessed using filter paper disc method [10, 11].

RESULTS AND DISCUSSION

Previously phytochemical screening of Deverra tortuosa focused on the essential oil composition and its biological activities, herein the essential oils of *D. tortuosa* belonging to two different geographical sources (Wadi Hagul and Alexandria) were extracted using hydro-distillation Clevenger-type apparatus and phytochemically characterized using GC/MS technique. In addition, the aerial parts of this plant were extracted and afforded four different fractions which were investigated for their biological activity as antibacterial and the most promising bioactive fraction were also phytochemically investigated as a trial to reach to the active ingredients.

GC/MS allowed the identification of forty compounds in both *D. tortuosa* essential oils (E.O WH and E.O Alex.) (Table.1). The identified compounds were grouped into monoterpenes and sesquiterpenes through retention time (R.T) and comparing the obtained EI-MS spectra with a series of previously reported EI-MS spectra deposited in NIST library. *D. tortuosa* E.O WH locality contains higher terpene content (84.23 %) than E.O Alex (71.37 %) with interestingly noticeable increase in the sesquiterpenes content, E.O WH predominant compounds were terpinen-4-ol (16.74 %), cis-verbenol (5.11 %), 1.6-[1-(hydroxymethyl)vinyl]- 4,8adimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenol (5.19 %) and Perhydrofarnesyl acetone (4.63%). Other major compounds in the E.O Alex locality were γ-terpinene(12.27 %), terpinen-4-ol (17.14 %), cis-p-menth-2-en-1- ol (5.15 %) and p-mentha-1,4-dien-7-ol (7.07 %). The results showed different chemo-types of both *D. tortuosa* essential oil localities. These variations in chemical composition are probably due to the differences in the plant ecotype, geographic origins, genotype and climatic (temperature, altitude, rainfall, solar radiation, etc.) which may lead to the predominance of a particular biosynthetic pathway [12]. Pet. ether and CH_2Cl_2fractions were also analyzed by GC/MS technique and various volatile constituents were characterized. The obtained results (Table. 2) represented the chemical constituents of pet. ether fraction which revealed the presence of nineteen components belonging to three main classes; terpenes (7.75%), shikimates (20.01%) and acetogenines (3.72%), while CH_2Cl_2 fraction revealed the presence of twenty one components belonging to terpenes(3.25%), shikimates (34.57%) and acetogenines (4.07%), myristicin was the dominant component (32.86%) in CH_2Cl_2 fraction. Phytochemical investigations of *Deverra tortuosa* through application of various chromatographic methods (CC and PTLC) and spectral measurements as GC/MS, ^{1}H and ^{13}C NMR spectra led to separation and structure elucidation of five main constituents from the most active antibacterial fractions pet. ether and n-BuOH fractions. Pet. ether fraction afforded two steroid compounds in a mixture, which were identified by ^{1}H NMR as *β*-sitosterol and stigmasterol [13]. Three flavonoid glycosides were isolated from butanol fraction using preparative TLC and identified using

NMR spectra. Two flavone glycoside skeletons were identified as luteolin **7-O-α-L-rhamnopyranoside (1)** and **luteolin 7- O-rutinoside (3)** as well as one flavonol glycoside namely **isorhamentin-3-O-rutinoside (2)** and confirmed by comparison with the previously reported spectra. [14-17] It is worthy to mention that all three identified flavonoid glycosides reported from this plant species for the first time (Fig.1).

All the obtained essential oils (E.O WH and E.O Alex), fractions and three isolated flavonoids were assessed for their antibacterial activity against *Streptococcus pyogenes, Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae*. Of all the tested materials (Fig. 2) essential oils were the most powerful broad antibacterial agents against all tested pathogenic microbial strains. E.O WH and E.O Alex. for *S. pyogenes*, showed the highest antibacterial activity (18 and 19 mm) while for *S. aureus* (17 and 19 mm), *K. pneumoniae* (16 and 16 mm), *S. typhimurium* (15.3 and 12 mm) and finally *E. coli* (12.6 and 13.3 mm), respectively. Among the rest tested agents pet. ether, butanol, **luteolin 7-*O*-α-L-rhamnopyranoside (1)** and **luteolin 7- O-rutinoside (3)** gave a moderate antibacterial activity against *S. pyogenes* with inhibition zone (8, 12, 11 and 9 mm), respectively and finally pet. Ether fraction showed antimicrobial activity of (9 mm) toward *S. aureus*.

The obtained antimicrobial activity results of the essential oils which revealed a significant activity against Gram -ve, Gram +ve bacteria pathogens were in agreement with those reported by [5].

Table 1: GC/MS analysis: Chemical constituents of *Deverra tortuosa* essential oils.

SN	Component name	R.T	%		M.F	M.W
			E.O W.H	E.O Alex		
Monoterpene hydrocarbons						
1	γ-terpinene	5.65	1.45	12.27	$C_{10}H_{16}$	136
Oxygenated monoterpenes						
2	3,5-heptadienal, 2-ethylidene-6-methyl	6.35	2.79	-	$C_{10}H_{14}O$	150
3	cis-p-menth-2-en-1-ol	6.74	-	5.15	$C_{10}H_{18}O$	154
4	bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)-	6.77	1.97	-	$C_{10}H_{18}O_2$	170
5	6-camphenol	6.87	1.96	-	$C_{10}H_{16}O$	152
6	cis-verbenol	7.27	5.11	1.12	$C_{10}H_{16}O$	152
7	terpinen-4-ol	7.77	16.74	17.14	$C_{10}H_{18}O$	154
8	cis-sabinene hydrate	8.01		4.52	$C_{10}H_{18}O$	154
9	exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	8.03	1.26	-	$C_{10}H_{18}O$	154
10	(-)-myrtenol	8.12	3.20		$C_{10}H_{16}O$	152
11	cis-p-menth-1-en-3-ol	8.26		1.19	$C_{10}H_{18}O$	154
12	2-pinen-4-one	8.41	2.97	-	$C_{10}H_{14}O$	150
13	cis-carveol	8.52	2.57	-	$C_{10}H_{16}O$	152
14	(E)-p-menth-2,8-dien-1-ol	8.76	1.49	2.74	$C_{10}H_{16}O$	152
15	(Z)-p-mentha-1(7),8-dien-2-ol	9.15	1.62	-	$C_{10}H_{16}O$	152
16	citral	9.34	-	2.45	$C_{10}H_{16}O$	152
17	1.(4-Isopropyl-1,3-cyclohexadien-1-yl)methanol	9.62	-	1.89	$C_{10}H_{16}O$	152
18	p-cymen-7-ol	9.80	1.14	2.91	$C_{10}H_{14}O$	150
19	p-mentha-1,4-dien-7-ol	10.49	-	7.07	$C_{10}H_{16}O$	152
Total monoterpenes			**44.27**	**58.45**		
Sesquiterpenes hydrocarbons						
20	α-ylangene	10.82	1.21	-	$C_{15}H_{24}$	204
21	α-copaene	11.36	2.22	-	$C_{15}H_{24}$	204
22	β-copaene	11.54	0.62	-	$C_{15}H_{24}$	204
23	α-selinene	13.37	1.08	1.10	$C_{15}H_{24}$	204

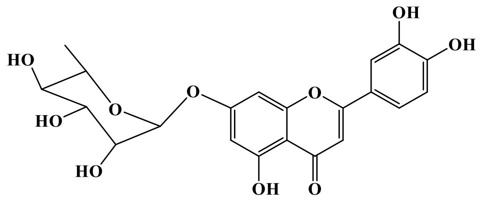
24	α-calacorene	14.29	1.49	-	$C_{15}H_{20}$	200
25	7-isopropyl-1,4-dimethyl-Azulene	16.49	4.63	-	$C_{15}H_{18}$	198
Oxygenated sesquiterpenes						
26	6-epi-shyobunol	12.52	2.42	-	$C_{15}H_{26}O$	222
27	4-epi-cubedol	13.47	2.12		$C_{15}H_{26}O$	222
28	Ledene oxide-(II)	14.94	2.17	3.57	$C_{15}H_{24}O$	220
29	caryophyllene oxide	15.04	2.97	-	$C_{15}H_{24}O$	220
30	isoaromadendrene epoxide	15.31	2.95	-	$C_{15}H_{24}O$	220
31	1.6-[1-(Hydroxymethyl)vinyl]-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenol	15.57	5.19	-	$C_{15}H_{24}O_2$	236
32	aromadendrene oxide-(2)	15.88	1.22	-	$C_{15}H_{24}O$	220
33	.β-eudesmol	16.17	-	4.56	$C_{15}H_{26}O$	222
34	cis-Z-α-bisabolene epoxide	16.28	2.35	1.39	$C_{15}H_{24}O$	220
35	8-isopropenyl-1,3,3,7-tetramethyl-bicyclo[5.1.0]oct-5-en-2-one	16.63	2.43	-	$C_{15}H_{22}O$	218
36	perhydrofarnesyl acetone	19.06	4.89	2.30	$C_{18}H_{36}O$	268
Total sesquiterpenes			**39.96**	**12.92**		
Miscellaneous						
37	4-methoxy-6-(2-propenyl)-1,3-benzodioxole	13.86	-	0.65	$C_{11}H_{12}O_3$	192
38	butylidenephthalide	16.59	-	3.13	$C_{12}H_{12}O_2$	188
39	trans-ligustilide	17.67	-	4.85	$C_{12}H_{14}O_2$	190
40	2,5-octadecadiynoic acid, methyl ester	16.89	2.17	-	$C_{19}H_{30}O_2$	290
Total essential oils constituents			**86.4**	**80.0**		

Table 2: Volatile constituents of pet. ether and methylene chloride fractions of *Deverra tortuosa*

S.N	Compound name	R.T	%		M.F	M.W
			Pet. ether Fr.	CH_2Cl_2 Fr.		
Terpenoids						
Monoterpenes hydrocarbon						
1	*p*-cymene	15.09	0.71	-	$C_{10}H_{14}$	134
2	γ-terpinene	17.65	0.67	-	$C_{10}H_{16}$	136
Total			**1.38**			
Oxygenated Monoterpenes						
3	terpinen-4-ol	20.35	-	0.26	$C_{10}H_{18}O$	154
4	thymol	20.43	-	0.14	$C_{10}H_{14}O$	150
5	cymen-8-ol	20.43	1.73	-	$C_{10}H_{14}O$	150
6	(-)-myrtenol	20.58	0.99	0.1	$C_{10}H_{16}O$	152
7	1,4-dihydroxy-*p*-menth-2-ene	23.27	1.30	-	$C_{10}H_{18}O_2$	170
8	*p*-cymen-7-ol	23.73	-	0.22	$C_{10}H_{14}O$	150
9	2-hydroxy-*p*-cymene	23.81	-	0.15	$C_{10}H_{14}O$	150
10	2,3-epoxygeraniol	29.18	0.29	-	$C_{10}H_{18}O_2$	170
11	dihydroactindiolide	30.89	0.65	-	$C_{11}H_{16}O_2$	180
12	citronellyl Propionate	36.73	-	0.89	$C_{13}H_{24}O_2$	212
13	dehydrovomifoliol	36.92	1.20	-	$C_{13}H_{18}O_3$	222
14	dihydroionone	37.95	0.21	-	$C_{13}H_{22}O$	194
Total			**6.37**	**1.76**		

Sesquiterpenes hydrocarbon						
15	β-seliene	29.83	-	0.48	$C_{15}H_{24}$	204
Oxygenated Sesquiterpenes						
16	β-eudesmol	34.20	-	1.69	$C_{15}H_{26}O$	222
17	phytone	37.98	-	0.90	$C_{18}H_{36}O$	236
Total				**2.59**		
Oxygenated diterpenes						
18	phytol	43.64	-	0.40	$C_{20}H_{40}O$	296
Total terpenes			**7.75**	**5.23**		
Shikimates derivatives						
19	2-methoxy-4-vinylphenol	24.30	3.80	-	$C_9H_{10}O_2$	150
20	methyl Eugenol	26.76	-	1.29	$C_{11}H_{14}O_2$	178
21	vanillin	26.86	3.17		$C_8H_8O_3$	152
22	myristicin	30.30	4.49	32.86	$C_{11}H_{12}O_3$	192
23	elemicin	30.76		0.42	$C_{12}H_{16}O_3$	208
24	4-hydroxy-3,5-dimethoxybenzaldehyde	33.70	1.77	-	$C_9H_{10}O_4$	182
25	coniferyl alcohol	35.73	5.49	-	$C_{11}H_{16}O_2$	180
26	3-methylcatechol, diacetate	36.98	1.55	-	$C_{11}H_{12}O_4$	208
27	4,7-dimethoxy-2H-1-benzopyran-2-one	39.43	1.74	-	$C_{11}H_{10}O_4$	206
Total shkimates			**20.01**	**34.57**		
Acetogenines (Fat and fatty acid derivatives)						
28	2-butoxyethanol	10.97	1.11		$C_6H_{14}O_2$	118
29	2-octanone	21.38		0.10	$C_8H_{16}O$	129
30	hexadecanoic acid, methyl ester	39.75		0.87	$C_{17}H_{34}O_2$	270
31	n-hexadecanoic acid	40.71		3.61	$C_{16}H_{31}O_2$	256
32	(Z,Z)-9,12-octadecadienoic acid methyl ester	43.30		0.47	$C_{19}H_{34}O_2$	294
Total			**1.11**	**5.05**		
Phthalides						
33	3-butylidene-3H-isobenzofuran-1-one	34.40	1.07	1.14	$C_{12}H_{12}O_2$	188
34	(E)-ligustilide	35.86	2.65	-	$C_{12}H_{14}O_2$	190
35	3-n-butylphthalide	35.95	-	2.93	$C_{12}H_{14}O_2$	190
Total			**3.72**	**4.07**		
Miscellaneous						
36	androst-5, 16-diene-3-β-ol	46.83	-	1.44	$C_{19}H_{28}O$	272
37	5-methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(3H)-furanone	48.40	-	0.50	$C_{21}H_{40}O_2$	324
Total volatile constituents			**40.34**	**55.2**		

Figure 1: Chemical constituents of the isolated natural products from *Deverra tortuosa*



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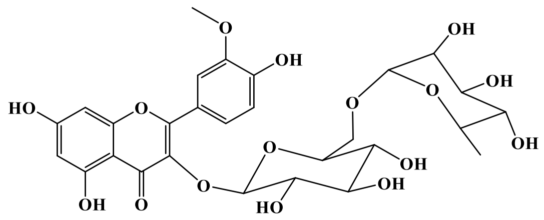

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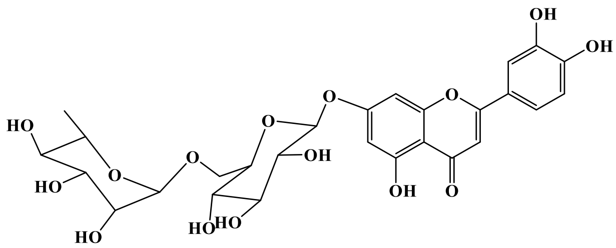
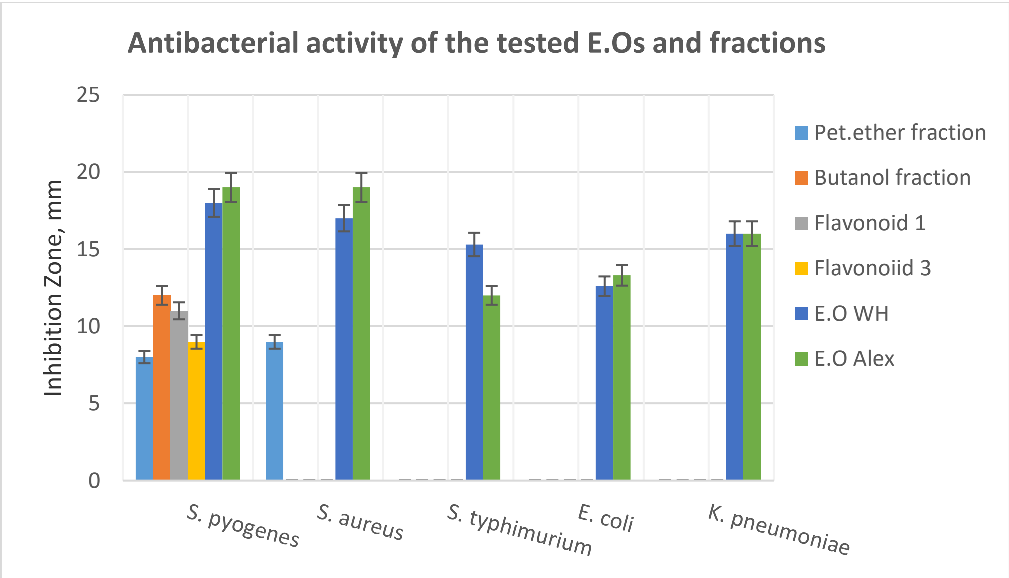


(3)

Figure 2: Antimicrobial activity of *Deverra tortuosa* essential oils, extracted fractions and isolated compounds.



CONCLUSION

A comparative study of the composition of essential oils of *Deverra tortuosa* collected from two different topographic regions (Wadi Hagul (WH) and West Alexandria (Alex) using GC/MS analysis was conducted. In addition to, phytochemical investigation of the species collected from Wadi Hagul based on bioassy-guided fractionation was accomplished to deduce the active fractions towards five pathogenic bacterial strains. Isolation and structural elucidation of three known flavonoid glycosides from butanol fraction were reported from the plant under investigation for the first time. The extracted essential oils as well as the obtained fractions and isolated compounds were evaluated for their antibacterial activity and the obtained essential oils (E.O WH and E.O Alex) were the most effective agents.

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