THE CHEMICAL COSTITUENTS OF EGYPTIAN CITRUS SPECIES. AN EXAMINATION OF THE VOLATILE OIL AND LIGHT PETROLUM FRACTION OF ROUGH LEMON (CITRUS JAMBAHIRI LUSH.) LEAF

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Abstract

A steam distilled oil obtained from rough lemon leaf (*Citrus jambhiri* Lush.) grown in Egypt was analyzed by gas liquid chromatography (GLC) and gas liquid chromatography –mass spectrometry (GLC- MS). A total of 139 chemical constituents were detected in the leaf oil. (*E*)-Caryophyllene was the major constituent in the oil with a peak area (%) of 14.47 followed by linalool (12.20 %), nerol (7.41%), limonene (7.06 %), γ-elemene (5.11%), (*Z*)-α-bisabolene (4.14%), δ-elemene (4.12%) and citronellal (3.37 %). Research was carried out to determine the aroma profile of Egyptian *C. jambhiri* leaf oil constituents as a contribution in the species taxonomy. In addition, the present study achieved a success of the isolation and characterization of four polymethoxyflavones (nobiletin, 5-*O*-demethylnobiletin, tangeretin, 5-hydroxy-3,6,7,8,3´,4´ hexamethoxyflavone), four sterols (a mixture of common sterols: stigmasterol and *β*-sitosterol, *β*-sitosterol-3-*O*-*β* - (6'-*O*-acetyl)-D - glucopyranoside, *β*-sitosterol-3-*O*-*β*-D-glucopyranoside), palmitic acid and long chain alcohol (1-octacosanol) from the light petroleum fraction obtained from the ethanol extract of the dried leaves. The chemical structure of the isolated compounds was established using MS (EI and FAB) and NMR (APT, H-COSY, HSQC and HMBC). The cytotoxic activity of 5-*O*-demethylnobiletin and 5-hydroxy-3,6,7,8,3´,4´-hexamethoxyflavone (5-HHxMF) was evaluated against human lung cancer (A549), human breast canc-er (MCF7) and human prostate cancer (PC3) cell lines. Both compounds showed significant growth inhibitory effects on the tested cell lines. 5-HHxMF exhibited more pronounced activity with IC_{50} 40.4, 10.72 and 13.90 µM, respectively.

Keywords: Citrus jambhiri, leaf essential oil, sterols, polymethoxyflavones, cytotoxicity

Introduction

Genus *Citrus* (Rutaceae) is the most important in term of their nutritional value and essential oils production. Essential oils from these plants are widely used in flavours, fragrances, cosmetics, soaps, detergents and owing to their typical lemon-like aroma. Also, *Citrus* oils have been known to possess impressive antibacterial, antifungal, insecticidal and insect repellent activities for a long time (Bakkali et al., 2008; Adorjan and Buchbauer, 2010). However, the biological and pharmacological significance of these essential oils has been rapidly expanded in the last decades as anti-inflammatory, anticancer, allelopathic, antioxidant and other useful biological activities. Applications in industrial products particularly food packaging have now been demonstrated (González-Molina et al., 2010; Crowell, 1999). A large part of these oils come from the leaves, and branches of *Citrus* tree which manicured annually after fruit harvesting and represent a recycling waste. Flavonoids are rich in Citrus species and have important effects on cancer chemoprevention and chemotherapy through many mechanism of actions including carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, antioxidation, and reversal of multidrug resistance or a combination of these mechanisms (Kandaswami et al., 2005; Sergeev et al., 2007; Kawaii et al., 1999; Chahar et al., 2011, Meiyanto et al., 2012).

The chemical composition of leaf oils from *C. jambhiri* has been studied previously (Scora et al., 1969; Lund et al., 1981; Agarwal et al., 1989; Nemec and Lund 1990; Lota et al., 2002 and Kasali and Olaniyan 2009). The major constituents of Floridian oils were limonene (33.7%) and other components included sabinene (7.8%), γ-terpinen (7.4%), β-ocimene (7.3%) and linalool (5.3%), cironellal (7.3%), linalool (7.2%) and (*E*)-β-ocimene (5%) (Lund et al., 1981). In another study, a total of 48 compounds were chemically identified (Nemec and Lund, 1990). The leaf oil of a variety from Nigeria consisted mainly of limonene (58.1%), geranial (11.7%) and neral (9.8%) together with other noticeable constituents viz. (*E*)-β-ocimene (2.3%), geranial (2.0%) and sabinene (1.8%) (Kasali and Olaniyan, 2009). Lota et al., (2002) reported on the composition of an oil of France origin while, Agrawal et al., (1989) studied the effect of seasonal variation on the composition of *C. jambhiri* leaf oil produced in India. The chemical composition of oils from 110 *Citrus* species, cultivars, hybrids and varieties of Chinese origin, among them the rough lemon were studied (Huang et al., 2000) and a comprehensive report about the volatile constituents of some

Citrus species was published (Lawrence, 2002). It is obvious that the leaf oil of this plant shows extremely variable composition by different localities.

In the framework of our chemical and biological investigations of the volatile components of *Citrus* species cultivated in Egypt (Hamdan et al., 2010, 2013a, b, c), the current work represents the study of the volatile oil and lipophilic fraction of the leaf of *C. jambhiri* grown in Egypt. A survey of literature reveals that the leaf of Egyptian variety has not been the subject of previous study.

Thus, the aim of this study is to explore and analyse the composition of the essential oil of the leaf and isolation of the secondary metabolites from the light petroleum fraction from of the Egyptian *C. jambhiri*. In addition, the cytotoxic activity of the isolated polymexoxyflavones, 5-*O*-demethylnobiletin and 5-hydroxy-3,6,7,8,3´,4´-hexamethoxyflavone (5-HHxMF) was evaluated against human lung cancer (A549), human breast cancer (MCF7) and human prostate cancer (PC3) cell lines.

Materials and methods

Plant material

The leaves of *C. jambhiri* Lush (Rutaceae) were collected from the Research Station of the Faculty of Agriculture, Banha University, Egypt in January 2011. The plant was kindly verified by Dr. B. Holyel, Professor of Pomolgy, Banha University. A voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University.

Essential oil preparation

The fresh leaves (100g) of *C. jambhiri* Lush. were subjected to hydrodistillation using Clevenger-type apparatus for six hours producing fragrant yellowish oil with 0.2%, yield which dried over anhydrous sodium sulphate and kept in brown vial in the refrigerator at 4ºC until further analyses.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The volatile oil constituents were analyzed by high-resolution capillary GLC and GLC-MS. Samples of the oil (2 µl each was dissolved in 1ml n-hexane) were injected (1µl volume) into a gas chromatograph (TRACE GC ULTRA, Thermo Scientific, Milan, Italy) under the following conditions: column, RTX-5MS® fused silica capillary equivalent to DB-5 (30 m × 0.32mm ID, film thickness 0.25 µm); carrier gas He (2 ml/min); detector FID, temperature 300°C, injection temperature 250°C; oven temperature program: initial temperature 45°C, 2 min isothermal, 300°C, 4°C/1 min, then 20 min isothermal; split ratio, 1: 15. Kovat's retention indices (RI) were

calculated with respect to a set of co-injected standard hydrocarbons (C10–C28). The identified components quantified using the GLC "Peak Simple" software. GLC-MS data were recorded on a Clarus 600 gas chromatograph (Connecticut, USA) equipped with an identical column used for separation and quantification. The capillary column was directly coupled to a quadrupole mass spectrometer Clarus 600T. Ionization energy was 70 eV. Split ratio was 1: 30; other conditions were identical to those mentioned for GLC.

Identification of volatile components

Compounds were identified by comparing their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), and the literature (Adams, 2007; Hamdan et al., 2010; Hamdan et al., 2013). Where possible, retention times and mass spectra were also compared with those of authentic pure samples. Most of non-identified components are present as traces with relative abundances of less than 0.01%. The identified constituents are grouped and listed in Table **1**.

Extraction with light petrolum

The dried leaves powder (700g) of *C. jambhiri* Lush. was exhaustively extracted with 90% aqueous ethanol (5×3 L). The total alcoholic extract was filtered, concentrated under reduced pressure to yield 101g of viscous dark green residue. The residue was suspended in methanol–water (1:9 v/v) and partitioned aganist light petroleum (b.p. 60–80 ^{o}C), dichloromethane and ethyl acetate successively to yield 14, 6.5 and 5g of final residue, respectively.

Isolation of compounds

Dry mixed initial zone of light petroleum fraction (10g) was chromatographed on a Silica gel column (150 x 2.5cm, 300g). The column packed in light petroleum and the polarity was gradually increased using dichloromethane followed by methanol. Fractions of 250 ml each were collected, concentrated under reduced pressure and monitored by TLC using pre-coated Silica gel GF_{254} (Merck). Mixture of dichloromethane-acetone (9: 1 or 8.5: 1.5 v/v) or dichloromethane-methanol (9.6:0.4 or 9:1 v/v) were used for development. Spots were visualized by spraying with 10% aqueous H_2SO_4 followed by heating at 105˚C for 5 minutes. Further column chromatography, crystallization and/or preparative TLC revealed the isolation of nine compounds (**1-9**).

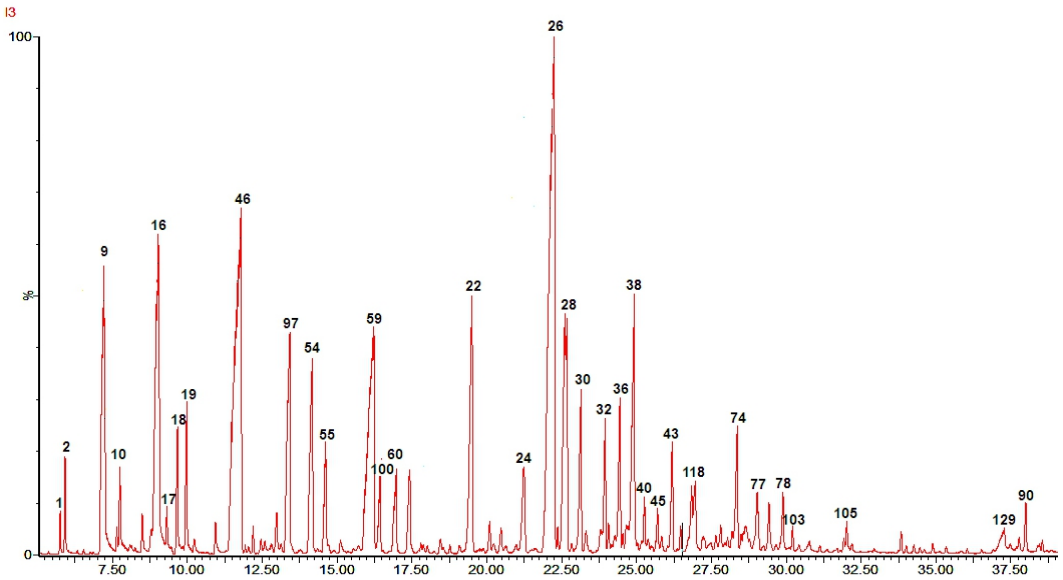

Figure 1: Total ion current (TIC) chromatogram for *C. jambhiri* leaf oil. The identification of the numbered peaks is presented in Table **1**.

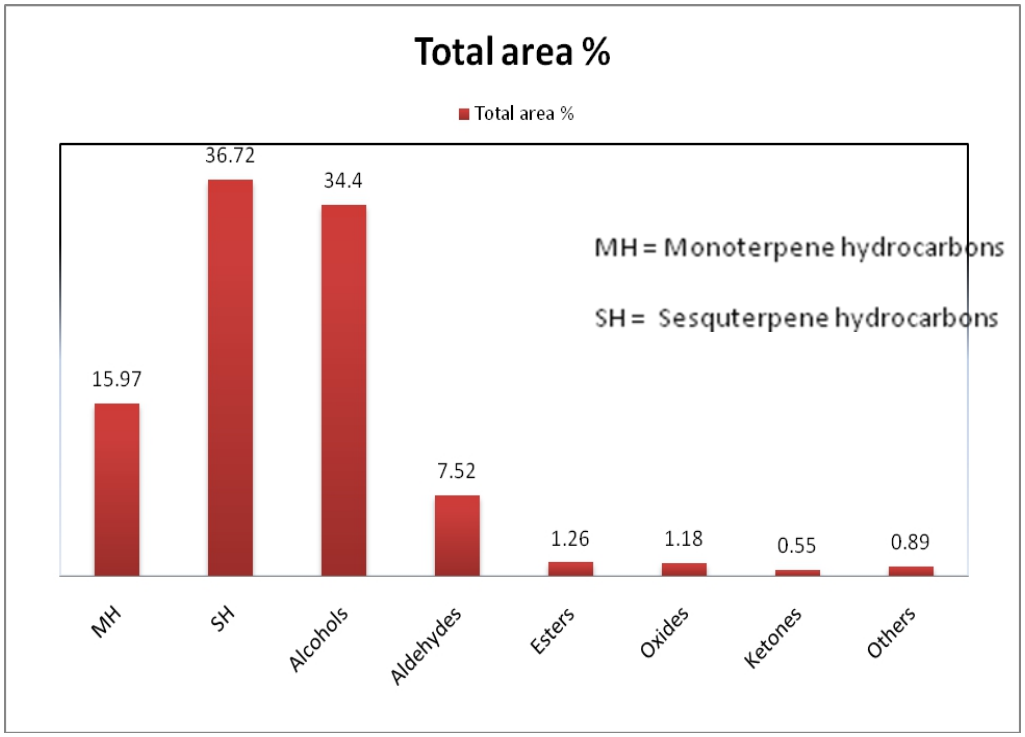

Figure 2: Relative volatile oil composition *C. jambhiri* leaf oil.

Table 1: Chemical composition of the essential oil of *C. jambahiri* Lush. leaf.

No	Components	RI	RRI*	Area %
Hydrocarbons				
Monoterpene hydrocarbons				
1	Tricyclene*	926	926	Tr.
2	*α*-Thujene	930	930	0.01
3	Cumene*	931	930	Tr.
4	Allyl isovalerate*	938	938	Tr.
5	*α*-Pinene	939	939	0.03
6	*α*-Fechene*	953	952	0.01
7	Sabinene	973	975	0.02
8	*β*-Pinene	979	979	0.15
9	Myrcene	990	990	4.13
10	6-methyl-5-Hepten-2-one	991	991	0.01
11	*δ*-2-Carene*	1002	1002	0.11
12	*α*-Phellandrene	1003	1002	0.10
13	*iso*-Sylvestrene*	1007	1008	0.03
14	*α*-Terpinene	1015	1017	0.33
15	*ρ*-Cymene	1024	1024	Tr.
16	Limonene	1029	1029	7.06
17	(*Z*)-*β*-Ocimene	1037	1037	0.32
18	(*E*)-*β*-Ocimene	1049	1050	1.17
19	*γ*-Terpinene	1058	1059	1.24
20	Terpinolene	1087	1088	0.32
21	*allo*-Ocimene	1131	1132	0.10
	Total			15.97
Sesquterpene hydrocarbons				
22	*δ*-Elemene*	1338	1338	4.12
23	*β*-Panasinsene*	1383	1382	0.12
24	*β*-Elemene*	1391	1390	1.22
25	(*Z*)-Caryophyllene*	1407	1408	0.08
26	(*E*)-Caryophyllene	1419	1419	14.47
27	*β*-Copaene*	1431	1432	0.12
28	*γ*-Elemene*	1436	1436	5.11
29	6,9-Guaiadiene*	1444	1444	0.06
30	*α*-Humulene	1454	1454	1.17
31	Sesquisabinene*	1460	1459	0.27
32	*γ*-Muurolene*	1478	1479	0.31
33	*γ*-Himachalene*	1482	1482	1.47
34	Germacrene D	1485	1485	0.01
35	*δ*-Selinene*	1492	1492	0.12
36	*β*-Alaskene*	1497	1498	1.62
37	*α*-Muurolene*	1501	1500	0.08
38	(*Z*)-*α*-Bisabolene*	1507	1507	4.14
39	(*Z*)-*γ*-Bisabolene*	1515	1515	0.12
40	*δ*-Cadinene*	1523	1523	0.46
41	(*E*)-*γ*-Bisabolene*	1530	1529	0.13
42	*γ*-Cuprenene*	1532	1533	0.06

43	Germacrene B*	1560	1561	1.17
44	Abietadiene*	2088	2087	0.02
	Total			36.72
Alcohols				
45	*cis*-Sabinene hydrate*	1070	1070	0.14
46	Linalool	1096	1096	12.20
47	*endo*-Fenchol*	1117	1116	0.05
48	*exo*-Fenchol*	1121	1121	0.19
49	1-Terpineol*	1133	1133	0.08
50	*iso*-3-Thujanol*	1139	1138	0.09
51	*cis*-*β*-Terpineol*	1144	1144	0.42
52	*trans*-Verbenol*	1145	1144	0.08
53	*trans*-*β*-Terpineol*	1163	1163	0.05
54	Terpinen-4-ol	1178	1177	3.00
55	*α*-Terpineol	1188	1188	1.36
56	*cis*-Piperitol*	1197	1196	0.04
57	*trans*-Carveo*l	1215	1216	0.07
58	*cis*-Carveol*	1228	1229	0.09
59	Nerol	1229	1229	7.41
60	Geraniol	1250	1252	0.02
61	*E*-Anethole*	1284	1284	0.16
62	*ρ*-Cymen-7-ol*	1290	1290	0.05
63	Carvacrol*	1301	1299	0.21
64	*ρ*-Vinyl-guaiacol*	1309	1309	0.08
65	*cis*-Sesquisabinene hydrate*	1544	1544	0.37
66	Elemol*	1549	1549	0.12
67	*E*-Nerolidol	1563	1563	0.22
68	Spathulenol*	1578	1578	0.91
69	Virdiflorol*	1591	1592	0.23
70	Rosifoliol*	1599	1600	0.11
71	*epi*-Cedrol*	1618	1619	0.20
72	1,10-di-*epi*-Cubenol*	1620	1619	0.12
73	10-*epi*-*γ*-Eudesmol*	1624	1623	0.16
74	Muurola-4,10(14)-dien-1-*β*-ol*	1631	1631	1.50
75	Caryophylla-4(12),8(13)-dien-5*β*-ol*	1639	1640	0.11
76	Hinesol*	1645	1644	0.60
77	*α*-Cadinol*	1655	1654	0.87
78	*epi*-*β*-Bisabolol*	1671	1671	0.57
79	Khusinol*	1680	1680	0.06
80	*epi*-*α*-Bisabolol*	1684	1684	0.60
81	Nootkatol*	1715	1715	0.19
82	(*2E,6E*)-Farnesol*	1743	1743	0.04
83	(*Z*)-Lanceol*	1761	1761	0.12
84	14-Hydroxy-*α*-muurolene*	1780	1780	0.03
85	*α*-Chenopodiol*	1855	1856	0.08
86	Phytol*	1944	1943	0.05
87	(*E,Z*)-Geranyl linalool	1986	1987	0.05
88	(*6Z,10Z*)-Pseudo phytol*	1988	1988	Tr.

89	(*Z*,*E*)-Geranyl linalool*	1998	1998	0.15
90	(*6E*,*10Z*)-Pseudo phytol*	2018	2018	0.46
91	(*E*,*E*)-Geranyl linalool*	2027	2027	0.12
92	(*6Z*,*10E*)-Pseudo phytol*	2032	2031	0.10
93	(*Z*)-Falcarinol*	2037	2036	0.04
94	Abienol*	2148	2149	0.08
95	Incensole*	2159	2159	0.34
96	3-*α*-Hydroxy-manool*	2299	2297	0.01
	Total			34.40
Aldehydes				
97	Citronellal	1153	1153	3.37
98	*E*-Isocitral*	1180	1180	0.03
99	*n*-Decanal	1201	1201	0.17
100	Neral	1239	1238	0.84
101	Geranial	1266	1267	2.42
102	Dimethoxy-*E*-citral*	1341	1341	0.05
103	*β*-Sinensal*	1700	1699	0.20
104	*α*-Sinensal*	1755	1756	0.05
105	*β*-Bisabolenal*	1769	1769	0.39
Total				7.52
Esters				
106	*cis*-Chrysanthenyl acetate*	1265	1265	Tr.
107	(*2E*)-Nonenol acetate*	1307	1307	0.07
108	*neo*-Verbanol acetate*	1321	1321	0.08
109	*α*-Terpinyl acetate*	1350	1349	0.07
110	Citronellyl acetate*	1352	1352	0.48
111	Neryl acetate	1361	1361	0.30
112	Methyl hexadecanoate*	1921	1921	0.07
113	Methyl linoleate*	2086	2085	0.06
114	Octadecanol acetate*	2208	2209	0.03
115	(*E*)-Phytol acetate*	2218	2218	0.04
116	Mehyl sandaracopimarate*	2261	2256	0.06
Total				1.26
Oxides				
117	*cis*-Rose oxide*	1108	1108	0.04
118	Caryophyllene oxide*	1582	1583	1.03
119	Humulene epoxide II*	1608	1608	0.11
Total				1.18
Ketones				
120	*γ*-Valerolactone*	948	948	Tr.
121	(*Z*)-*β*-Damascenone*	1364	1364	0.10
122	*β*-Atlantone*	1668	1669	0.06
123	(*E*)-Apritone*	1708	1708	0.08
124	(*E*)-*α*-Atlantone*	1778	1778	0.01
125	(*Z*,*Z*)-Farnesyl acetone*	1861	1861	0.08
126	dehydro Juvibione*	2084	2085	0.05

127	Grandiflorene*	2175	2175	0.14
128	α-Santonine*	2203	2203	0.03
Total				0.55
Others				
129	Hexadecanoic acid*	1960	1960	0.59
130	7-Isoprenyl oxycoumarin*	2114	2116	0.14
131	1-Docosene*	2189	2189	0.05
132	*n*-Tricosane*	2300	2300	0.01
133	*n*-Tetracosane*	2399	2400	Tr.
134	*n*-Pentacosane*	2501	2500	0.02
135	Hinokienone*	2559	2557	0.02
136	Hexacosane*	2601	2600	0.01
137	Heptacosane*	2702	2700	0.02
138	Octacosane*	2800	2800	0.03
139	Nonacosane*	2900	2900	Tr.
Total				0.89
Total area (%) of identified components				97.66

* = newly identified components in leaf oil of *C. jambhiri*
RRI*= Reported RI (Adams 2007).
Tr. = trace

Spectral analysis

Electron impact mass spectra (EIMS) were recorded on a MAT 8200 instrument with electron energy 70eV. 3-Nitrobenzyl alcohol was used as a matrix in FAB-mass. NMR spectra (^{1}H and ^{13}C) were recorded on a Mercury 300 and VARIAN 500 at 300 and 500 MHz for ^{1}H measurements and 75 and 125 MHz for ^{13}C measurements, respectively. $CDCl_3$ and DMSO-d_6 were used as solvents. Chemical shifts are given in ppm with TMS as internal standard. APT, 2DNMR, H-H COSY and HMPQC experiments were applied to gain reliable assignments. Experimental data were processed using MestRe-C software.

Compound 1: was obtained as a white amorphous powder (50mg, dichloromethane/ methanol); EI/MS (rel. int.): *m/z* 410 $[M]^+$ (2), 392 (31), 364 (16), 336 (5), 321 (3), 307 (3), 392 (5), 279 (4), 251 (4), 237 (5), 209 (7), 181 (9), 153 (16), 125 (35), 111 (57), 97 (90), 83 (80), 69 (67), and 57 (100). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.64 (2H, t, *J*= 6.6, H-1), 1.56 (2H, m, H-2), 1.25 (48H, br. s, H-3:H-26), 1.32 (2H,m, H-27), 0.88 (3H, t, *J*= 6.0, H-28). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 63.3 (CH_2, C-1), 33.1 (CH_2, C-2), 32.2 (CH_2, C-26), 29.9-29.8 (CH_2, C-4: C-25), 25.9 (CH_2, C-3), 22.9 (CH_2, C-27), 14.3 (CH_3, C-28). From the spectral data and physical properties, compound **1** was identified as long chain alcohol with molecular formula $C_{28}H_{58}O$ (1-octacosanol) (Firdous et al., 2014; Masoodi et al., 2010).

Compound 2: was obtained as a white crystals (60mg, dichloromethane/ methanol); EI/MS (rel. int.): *m/z* 414 $[M1]^+$ (100), 412 $[M2]^+$ (22), 396 $[M1\text{-}H_2O]$ (26), 394 $[M1\text{-}H_2O]$ (1), 381 (13), 379 (2), 255

(17), 213 (12). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 3.53 (1H, tt, *J* = 4.5, 11.1 Hz, H-3), 5.37 (1H, br. s., H-6), 0.69 (3H, s, H-18), 0.99 (3H, s, H-19), 0.91 (3H, d, *J* = 6.57 Hz, H-21), 5.15 (1H, dd, *J* = 8.61, 15.18 Hz, H-22), 5.02 (1H, dd, *J* = 8.69, 15.17 Hz, H-23), 0.79 (3H, d, *J* = 6.8 Hz, H-26), 0.83 (3H, d, *J* = 7.4 Hz, H-27), 0.84 (3H, t, *J* = 7.6 Hz, H-29); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm): 37.2 (C-1), 31.66 (C-2), 71.79 (C-3), 42.29 (C-4), 140.74 (C-5), 121.69 (C-6), 31.89 (C-7), 31.89 (C-8), 50.15 (C-9), 36.49 (C-10), 21.10 (C-11), 39.77 (C-12), 42.31 (C-13), 56.76 (C-14), 24.29 (C-15), 28.23 (C-16), 56.05 (C-17), 11.85 (C-18), 19.39 (C-19), 36.13 (C-20), 18.77 (C-21), 33.94, 138.21 (C-22), 26.08, 129.31 (C-23), 45.83, 51.22 (C-24), 29.15, 31.66 (C-25), 19.02 (C-26), 19.80 (C-27), 23.06, 24.32 (C-28), 11.97, 12.22 (C-29). Compound **2** was identified as a mixture of *β*-sitosterol (**2′**) and stigmasterol (**2″**)from these spectral data and physical properties (Goad and Akihisa, 1997) which may have maximum portion of *β*-sitosterol. The only difference between the two compounds is the presence of C22=C23 double bond in stigmasterol and C22-C23 single bond in β-sitosterol.

Compound 3: was obtained as a white needle-shaped crystals (55mg, dichloromethane/ methanol); HR-ESI/MS [M^{+}+1] at *m/z* 257, EI/MS (Rel. Int.): *m/z* 256 [M] $^{+}$, 213 (20), 199 (5), 185 (12), 169 (15), 157 (11), 143 (10), 129 (34), 115 (12), 97 (24), 73 (77), 55 (72), 43 (100) and 29 (30). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.23 (OH), 2.35 (2H, m, H-2), 1.62 (2H, m, H-3), 1.31 (2H, m, H-4), 1.25 (16H, m, H-5: 12), 1.28 (2H, m, H-13), 1.29 (2H, m, H-14), 1.33 (2H, m, H-15), 0.88 (3H, t, *J*=6.9, H-16); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 179.69 (C-1, COOH), 34.2 (C-2), 32.2 (C-14), 29.28: 29.92 (C-4: C-13) 24.91 (C-3), 22.92 (C-15), 14.34 (C-16). Compound **3** was identified as palmitic acid from co-TLC with authentic material and spectral data (Hamdan et al., 2011).

Compound 4: was obtained as a yellow amorphous powder (10mg, dichloromethane/ methanol); FAB-MS [M^{+}+1] at *m/z* 373, EI/MS (rel. int.): *m/z* 372 [M] $^{+}$, 357 (M-15) (100), 314 (329-15) (10), 197 (8) and 83 (5). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.88 (1H, d, *J*=8.9 Hz, H-2`), 7.01 (1H, d, *J*= 8.9 Hz, H-3′), 7.01 (1H, d, *J*=8.9 Hz, H-5′), 7.88 (1H, d, *J*= 8.9 Hz, H-6′), 6.6 (1H, s, H-3), 4.09, 4.01, 3.96×2, 3.88 (each 3H, s, 5 OMe at C-5, -6, -7, -8, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 177.6 (s, C-4), 162.5 (s, C-2), 161.4 (s, C-4′), 151.6 (s, C-7), 148.6 (s, C-8), 147.9 (s, C-9), 144.3 (s, C-5), 138.3 (s, C-6), 127.9 (d, C-2′, C-6′), 124.0 (s, C-1′), 115.1 (s, C-10), 114.7 (d, C-3', C-5′), 106.9 (d, C-3), 62.5, 62.2, 62.0, 61.9, 55.7 (5 q, OMe at C-5, -8, -6, -7, -4′). Compound **4** was identified as 5,6,7,8,4′-pentamethoxyflavone (tangeretin) from these spectral data and physical properties (Uckoo et al., 2011).

Compound 5: was obtained as a yellowish white needle-shaped crystals (15mg, chloroform/ acetone); FAB-MS [M^{+}+1] at *m/z* 403, EI/MS

(rel. int.): *m/z* 402 [M] $^{+}$, 387 (100), 372 (4), 357 (10), 314 (1), 197 (8) 165 (8), 162 (3), 147 (8) and 83 (0.3). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.4 (1H, d, *J*=2.1 Hz, 2′), 6.97 (1H, d, *J*=8.5 Hz, 5′) 7.55 (1H, dd, J=2.1, 8.5 Hz, 6′) 6.59 (1H, s, H-3), 4.08, 4.06, 3.98, 3.93, 3.92×2 (each 3H, s, 6 OMe at C-5, -6, -7, -8, -3′, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 177.3 (s, C-4), 160.9 (s, C-2), 151.8 (s, C-4′), 151.3 (s, C-7), 149.1 (s, C-3′), 148.3 (s, C-8), 147.6 (s, C-9), 143.9 (s, C-5), 137.9 (s, C-6), 123.9 (s, C-1′), 119.5 (d, C-6′), 114.7 (s, C-10), 111.1 (d, C-5′), 108.4 (d, C-2′), 106.8 (d, C-3), 62.2, 61.9, 61.8, 61.6, 55.9, 55.8 (6 q, OMe at C-5, -8, -6, -7, -3′, -4′). Compound **5** was identified as 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin) from these spectral data and physical properties (Uckoo et al., 2011).

Compound 6: was obtained as a yellow granules (7mg, dichloromethane/ methanol); FAB-MS [M^{+}+1] at *m/z* 389, EI/MS (rel. int.): *m/z* 388 [M] $^{+}$, 373 (100), 343 (5), 327 (6), 211 (14), 183 (15) and 163 (7). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.39 (1H, d, *J*= 2.2 Hz, H-2′), 6.9 (1H, d, *J*= 8.7 Hz, H-5′), 7.56 (1H, dd, *J*= 2.2, 8.7 Hz, H-6′), 6.6 (1H, s, H-3), 12.5 (OH, s, H-5), 4.1, 3.95, 3.94, 3.90, 3.85 (5 s, OMe at C-6, -7, -8, -3′, -4′). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 182.9 (s, C-4), 163.9 (s, C-2), 152.9 (s, C-7), 152.4 (s, C-4′), 149.5 (s, C-3′), 149.3 (s, C-5), 145.8 (s, C-9), 136.5 (s, C-6), 132.9 (s, C-8), 123.7 (s, C-1′), 120.1 (d, C-6′), 111.7 (d, C-2′), 108.8 (d, C-5′), 106.9 (s, C-10), 103.9 (d, C-3), 62.0, 61.7, 61.1, 56.1, 55.9 (5 q, OMe at C-7, -8, -6, -3′, -4′). Compound **6** was identified as 5-*O*-demethylnobiletin from these spectral data and physical properties (Li et al., 2006).

Compound 7: Dark yellow needle-shaped crystals which crystallized ($CHCl_3$/ MeOH); FAB-MS [M+1]$^{+}$ at m/z 419, EI/MS (rel. int.): m/z 418 [M] $^{+}$, 403 (100), 373 (6), 359 (1), 209 (5) and 165 (3). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.7 (1H, d, *J* = 2.2 Hz, H-2´), 7.0 (1H, d, *J* = 8.7 Hz, H-5´) 7.9 (1H, dd, *J* = 2.2, 8.7 Hz, H-6´), 12.4 (OH, s, H-5), 4.1, 3.92, 3.91, 3.94, 3.90, 3.80 (6 s, OMe at C-3,-6,-7,-8,-3´,-4´); ^{13}C NMR (75MHz, $CDCl_3$): δ (ppm): 179.2 (s, C-4), 155.8 (s, C-2), 152.9 (s, C-7), 151.5 (s, C-3´), 149.1 (s, C-4´), 148.7 (s, C-5), 144.8 (s, C-9), 138.7 (s, C-3), 136.1 (s, C-6), 132.7 (s, C-8), 122.9 (s, C-1´), 122.3 (d, C-6´), 110.9 (d, C-5´), 110.9 (d, C-2´), 107.4 (s, C-10), 62.0, 61.7, 61.1, 60.1, 55.9 and 55.8 (6 q, OMe at C-3, -7, 8, 6, 4´, 3´). Compound **7** was identified as 5-hydroxy- 3, 6, 7, 8, 3´, 4´-hexamethoxyflavone from these spectral data and physical properties (Li *et al*., 2006).

Compound 8: was obtained as a yellowish sticky residue (10mg, dichloromethane/ methanol); EI/MS (Rel. Int.): *m/z* 414, M^{+}- acetyl sugar moiety (3), 396 (100), 381 (8), 303 (3), 275 (8), 255 (13), 213 (9), 175 (9), 164 (2), 135 (11), 133 (10), 93 (10), 85 (14), 81 (20) and 43 (32). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.32 (1H, m, C- 3), 5.29 (1H, m, C- 6), 0.61

(3H, s, CH_3 - 18), 0.93 (3H, s, CH_3 - 19), 0.86 (3H, d, J = 6.5 Hz, CH_3 - 21), 0.79 (3H, d, J = 6.4 Hz, CH_3 - 26), 0.80 (3H, d, J = 6.5 Hz, CH_3 - 27), 2.7 (2H, q, J = 7.1 Hz, C-28), 0.75 (3H, t, J = 7.1 Hz, CH_3- 29), 1.19 (29 H, m, CH and -CH_2), 3.25-3.52 (4H, m, sugar protons), 4.35 (1H, d, J = 7.5, H-1´), 4.25, 4.31 (2H, m, H-6´), 0.25 (3H, s, Ac, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 36.8 (C-1), 29.8 (C-2), 79.9 (C-3), 40.0 (C-4), 140.2 (C-5), 122.1 (C-6), 32.0 (C-7), 31.9 (C-8), 50.1 (C-9), 37.4 (C-10), 21.3 (C-11), 39.5 (C-12), 42.3 (C-13), 56.9 (C-14), 26.2 (C-15), 25.9 (C-16), 56.1 (C-17), 12.0 (C-18), 20.0 (C-19), 36.1 (C-20), 19.9 (C-21), 34.1 (C-22), 30.1 (C-23), 46.1 (C-24), 28.3 (C-25), 18.9 (C-26), 19.0 (C-27), 27.5 (C-28), 12.1 (C-29), 101.8 (C-1´), 73.9 (C-2´), 74.2 (C-3´), 70.2 (C-4´), 76.2 (C-5´), 63.8 (C-6´), 174.2 (Ac, C=O), 14.1 (Ac, Me).

Compound 9: was obtained as a white amorphous powder (50mg, dichloromethane/ methanol); EI/MS (Rel. Int.): *m/z* 414 [M^+ - sugar moiety] (4), 396 (91), 381 (12), 329 (2), 303 (3), 273 (3), 255 (22), 213 (14), 175 (11), 159 (23), 145 (41), 107 (29), 81 (45) and 43 (100). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm): 3.5 (1H, tt, J = 4.5, 11 Hz, C- 3), 5.3 (1H, m, C- 6), 0.6 (3H, s, CH_3 - 18), 0.9 (3H, s, CH_3 - 19), 0.9 (3H, d, J = 6.5 Hz, CH_3 - 21), 0.8 (3H, d, J = 6.6 Hz, CH_3 - 26), 0.8 (3H, d, J = 7.3 Hz, CH_3 - 27), 0.84 (3H, t, J = 7.5 Hz, CH_3- 29), 1-2.5 (29H, m, -CH_2- and CH of the steroid nucleus and side chain); 3-3.4 (4H, m, sugar protons, 2´, 3´, 4´ , 5´), 4.9 (1H, d, J = 7.5, H-1'), 3.51, 3.7 (2H, m, H-6´), 3.6 (OH, s, hydrogen bonding). ^{13}C NMR (125 MHz, DMSO-d_6): δ (ppm): 37.5 (C-1), 29.8 (C-2), 77.7 (C-3), 40.6 (C-4), 141.1 (C-5), 122.0 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.9 (C-10), 21.5 (C-11), 38.6 (C-12), 42.2 (C-13), 56.4 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 12.0 (C-18), 19.6 (C-19), 36.1 (C-20), 18.9 (C-21), 33.9 (C-22), 25.9 (C-23), 45.9 (C-24), 29.2 (C-25), 19.3 (C-26), 20.0 (C-27), 23.3 (C-28), 12.2 (C-29), 101.5 (C-1´), 74.0 (C-2´), 77.8 (C-3´), 71.1 (C-4´), 77.7 (C-5´), 61.9 (C-6´). Comparing the MS, ^{1}H- NMR and ^{13}C- NMR data of this compound with the reported ones (Rai et al., 2006; Khatun et al., 2012), it was concluded that compound **9** was *β*-Sitosterol-3-*O*-*β* -D-glucopyranoside.

Cell lines and normal culture conditions

Human lung carcinoma (A549), human breast cancr (MCF7), and human prostste cancer (PC3) cell lines were used in this study. The cell lines were obtained from the Egyptian holding company for biological products and vaccines (VACSERA; Giza, Egypt). Cells were cultured in either RPMI 1640 medium, or DMEM procured from Sigma (St. Louis, MO, USA) and supplemented with 10% fetal bovine serum from Gibco® (Invitrogen; Karlsruhe, Germany) , 100 U penicillin G/mL and 100 U streptomycin/mL. The cells were grown under humidified conditions in an incubator at 37°C

with 5% CO_2 and were maintained as "monolayer culture" by serial subculturing. All tissue culture plates and other plastic-ware were from Nunc (USA). Solvents used were of analytical grade unless mentioned and were purchased from Merck (Darmstadt, Germany), J.T. Backer® (Deventer, Netherlands) and Theo Seulberger® (Karlsruhe, Germany).

Cytotoxicity assay

Relative toxicity was determined by the sulforhodamine (SRB) assay as described by Skehan et al. (1990). Breifely, the growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at 1000-2000 cells/well in supplemented medium. After 24 h, cells were incubated for 72 h with various concentrations (0.1, 1, 10, 100, 100 µM) of the tested compounds (5-*O*-demethylnobiletin and 5-hydroxy-3,6,7,8,3´,4´-hexamethoxyflavone) in DMSO. Following 72 h treatment, the cells will be fixed with 10% trichloroacetic acid for 1 h at 4 ºC. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris-HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). All experiments were performed in triplicate .The IC_{50} values were calculated according to the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models (Graph Pad, Prism Version 5). Doxorubicin was used as a reference stanrdard.

Chemicals

All solvents for extraction and separation were of analytical grade. Chemicals were purchased from AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerland) and Sigma Aldrich GmbH (Sternheim, Germany).

Statistical analysis

Data are presented as mean ± SEM of at least three replicates for each sample (or standard). Statistical analysis was performed by ANOVA followed by Tukey post hoc test using a computer-based curve fitting program (prism 5, Graphpad,CA, USA). P value <0.05 was considered statistically significant.

Results and discussion

Phytochemical results

Essential oil obtained from the leaf of *C. jambhiri* Lush. was subjected to detailed GLC/MS and GLC/FID analysis. The chemical composition of leaf oil is listed in Table **1**. One hundred fifty five peaks were

detected in this oil of which 139 were identified and quantified representing 97.66% of the total oil (Figure **1**). The most abundant volatile classes were sesquiterpene hydrocarbons (36.72%), alcohols (34.40%), monoterpene hydrocarbons (15.97%) and aldehydes (7.52%) (Figure **2**). Interestingly, (*E*)-caryophyllene was the major constituent (14.47%) in the oil followed by linalool (12.20 %), nerol (7.71%), limonene (7.06 %), γ-elemene (5.11%), (*Z*)-α-bisabolene (4.14%), δ-elemene (4.12%) and citronellal (3.37 %). In addition, eleven esters (1.26%), three oxides (1.18%), nine ketones (0.55%) and ten constituents belonging to long chain hydrocarbons, coumarin derivatives and fatty acid (0.89 %) were also identified. The obtained results showed considerable differences in the oil composition of the studied Egyptian species in comparison with other localities. A number of compounds not previously reported in *C. jambhiri* leaf were identified (Table **1**). The data could be useful in taxonomic studies and identification of new aroma in the oil. Obviously, the oil components are greatly influenced by genetic, environmental and geographical conditions (Figueiredo et al., 2008).

Column chromatography of the light petroleum extract fraction of the leaves afforded four polymethoxyflavones include: tangeretin **(4),** nobiletin **(5),** 5-*O*-demethylnobiletin (**6),** 5-hydroxy-3,6,7,8,3´,4´-hexamethoxyflavone (5-HHxMF) (**7**). Unlike other flavonoids, the absence of hydroxyls and sugar moieties in polymethoxyflavones make them less polar. 1-Octacosanol **(1),** a mixture of *β*-sitosterol (**2′**) and stigmasterol **(2′′),** palmitic acid **(3),** and *β*-sitosterol-3-*O*-*β*-D-glucopyranoside (**9**) were also isolated (Figure **3**)**.** Their structures were determined by means of spectroscopic methods, including 1D and 2D NMR experiments and MS analysis together with comparison to published values in the literatures.

Compound **8** shows a great similarity in ^{1}H- and ^{13}CNMR data to that of **9** with extra methyl and carbonyl groups at δ 14.1 and 174.2, respectively in ^{13}C and . Acylation at 6′ position of glucose with acetyl moiety is evident by the presence of the downfield shift with δ 63.8 compared with **9** (δ 61.9). The same situation in ^{1}H-NMR compound **8** shows a δ 3.51, 3.7 compared with **9** (δ 4.25 and 4.31). Comparing the spectral data of compound **8** with that of **9**, published data (Potocka and Zimowski 2008; Khatun et al., 2012), and from the biogenetic consideration, compound **8** was identified as *β*-sitosterol-3-*O*-*β*-(6'-*O*-acetyl)-D-glucopyranoside**.**

To our knowledge, all isolated and identified compounds in the current study are reported for the first time in *C. jambahiri* leaf. This is first time to report *β*-sitosterol-3-*O*-*β*-(6'-*O*-acetyl)-D-glucopyranoside (**8**) in genus Citrus**.** However, tangeretin, nobiletin, palmitic acid and sitosterol mixture (*β*-sitosterol and stigmasterol) were previously reported in the peel of the same species (Hamdan, *et al.,* 2011, Chaliha, *et al*. 1965, Tatum and Berry, 1972).

In vitro cytotxic activity

Hydroxylated polmethoxyflavones (PMFs) aer a natural class mainly found in Citrus species and exert a broad biological activity including antiproliferative and proapoptotic effects in cancer cells (Kawaii et al., 1999; Nishino et al., 2004; Ju-Ichi, 2005; Sergeev et al., 2007; Walle et al. 2007; Sekiguchi et al., 2008; Meiyanto et al., 2012). To date, a relatively large number of studies have investigated the antiproliferative effect of nobiletin and tangeretin on different types of cancer cells (Kawaii et al., 1999; Rooprai et al., 2001, Yáñez et al., 2004; Tang et al., 2007; Morley et al. 2007, Luo et al. 2008; 2009; Kim et al., 2010; Chiang et al., 2012; Uesato et al. 2014; Chen et al., 2007, 2014; Baek et al., 2012; Surichan et al., 2012; Tang et al., 2011; Ma et al., 2014). However, few studies have focused on the biologically active substances of Citrus 5-*O*-demethylnobiletin and 5HHxMF. 5-HHxMF involved in the induction of death receptor-associated apoptosis in human gastric AGS carcinoma cells (Kim et al., 2012). It induces apoptosis through reactive oxygen species production, growth arrest and DNA damage-inducible gene 153 expression, and caspase activation in human leukemia cells (Pan et al., 2007). Its activity was more potent in growth inhibition of human lung cancer H1299, H441, and H460 cells than its permethoxylated counterpart nobiletin (Xiao et al., 2009) and potently inhibited the growth of multiple human colon cancer cells (Quin et al., 2010, 2011). 5-*O*-Demethylnobiletin inhibits delayed-type hypersensitivity reactions, human lymphocyte proliferation and cytokine production (Bas et al., 2007). The present study was the first to evluate the therapeutic potential of of 5-*O*-demethylnobiletin and 5HHxMF from *C. jambhiri* leaf aganist A549, MCF7 and PC3 human tumor cell lines **(Figure 4).** Both compounds showed significant growth inhibitory effects on the tested cell lines in a dose dependent manner compared with standard anticancer doxorubicin with IC_{50} 0.411, 1.172 and 0.925 µM, respectively. 5-*O*-Demethylnobiletin exerts IC_{50} with 77.20, 73.63 and 58.06 µM, respectively. 5-HHxMF was found to remarkably inhibition effect on A549, MCF7 and PC3 tumor cells with an IC_{50} of 40.4, 10.72 and 13.90 µM, respectively.

Conclusion

The leaf of C. Jambahiri cultivated in Egypt was investigated for its volatile oil and light petrolum farction content. The oil was analysied by GLC and GLC-MS and 139 compounds were identified. (*E*)-Caryophyllene was the major component in the oil. Column chromatography of the petrolum ether soluble fraction resulted in the isolation of 1-octacosanol**,** a mixture of *β*-sitosterol and stigmasterol**,** palmitic acid**,** and *β*-sitosterol-3-*O*-*β*-D-glucopyranoside, *β*-sitosterol-3-*O*-*β*-(6'-*O*-acetyl)-D-glucopyranoside and four polymethoxyflavones (nobiletin, 5-*O*-demethylnobiletin, tangeretin

and 5-hydroxy-3,6,7,8,3´,4´ hexamethoxyflavone). 5-*O*-demethylnobiletin and 5-hydroxy-3,6,7,8,3´,4´ hexamethoxyflavone treated with A549, MCF7 and PC3 human tumor cell lines and exhibited significant cytotoxic activity.

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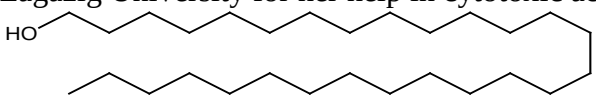
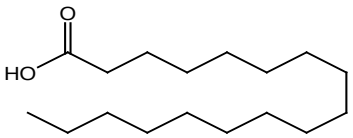
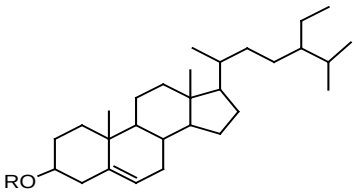


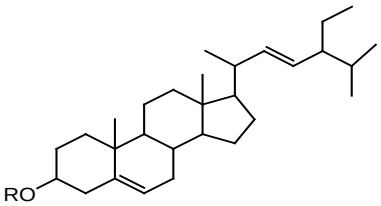
(**1**)



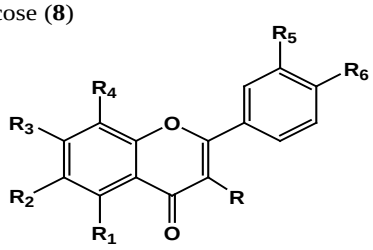
(**3**)



R = H (**2'**)
R = Glucose (**9**)
R = 6'-O-acetylglucose (**8**)



R = H (**2''**)



R	R_1	R_2	R_3	R_4	R_5	R_6	
H	OMe	OMe	OMe	OMe	H	OMe	(**4**)
H	OMe	OMe	OMe	OMe	OMe	OMe	(**5**)
H	OH	OMe	OMe	OMe	OMe	OMe	(**6**)
OMe	OH	OMe	OMe	OMe	OMe	OMe	(**7**)

Figure 3: Chemical structures of active compounds purified from a light petroleum fraction of C. jambahiri leaf.

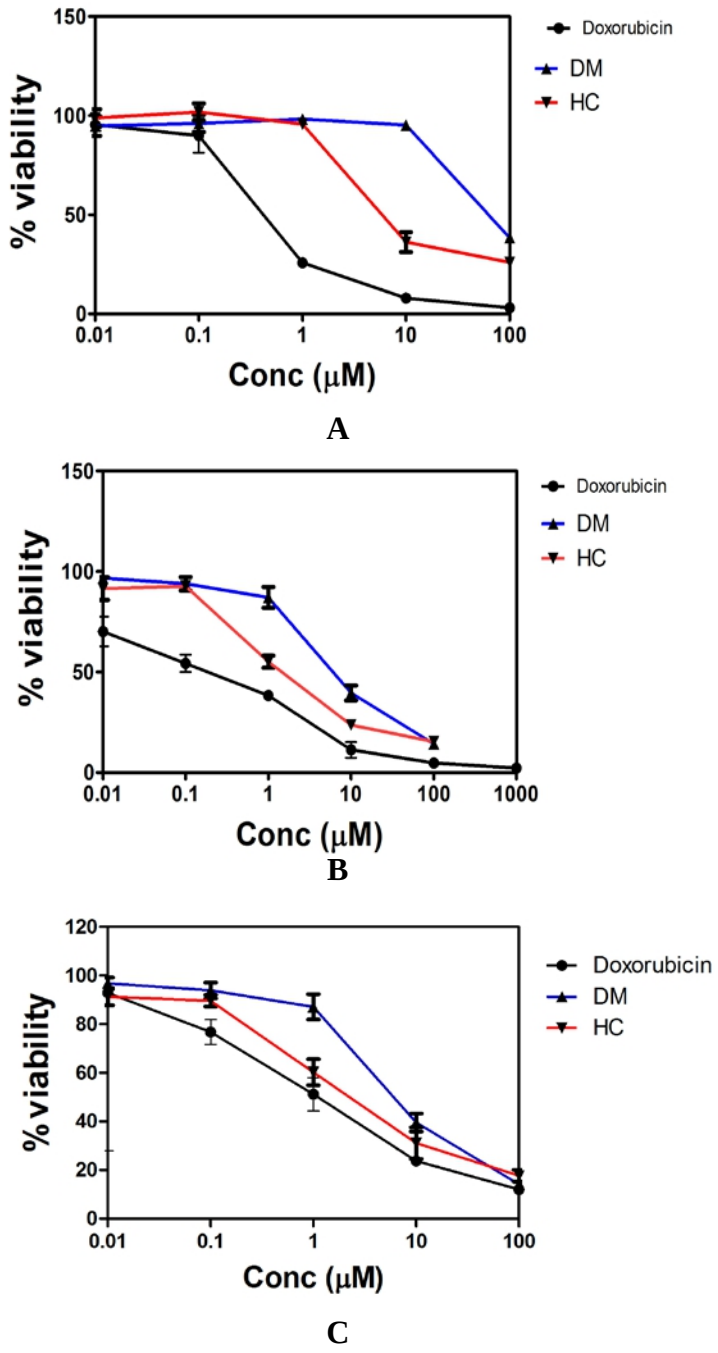


Figure 4: Effect of 5-*O*-demethylnobiletin (DM) and 5-hydroxy-3,6,7,8,3´,4´-hexamethoxyflavone (HC) on the viability of human cell lines: (A) A549, (B) MCF7 and (C) PC3. Doxorubicin was used as standard compounds. The results represent the mean ±SD of 3 independent determinations.

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