Article

J. Mex. Chem. Soc. **2014**, *58(4)*, 431-434

ISSN 1870-249X

Volatile Constituents Identified in Hexane Extract of *Citrus sinensis* Peel and Anti-Mycobacterial Tuberculosis Activity of Some of its Constituents

Patricia C. Esquivel-Ferriño,[1] Aldo F. Clemente-Soto,[1] Mayela Y. Ramírez-Cabriales,[1] Elvira Garza-González,[2] Laura Álvarez,[3] and María del Rayo Camacho-Corona[1]*

[1] Universidad Autónoma de Nuevo León, Facultad de Ciencias Químicas. Av. Universidad s/n, Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, C.P. 66451. México. maria.camachocn@uanl.edu.mx; Tel: +52-81-83294000 (ext 3463)

[2] Universidad Autónoma de Nuevo León, Servicio de Gastroenterología y Departamento de Patología Clínica, Hospital Universitario, Dr. José Eleuterio. Madero y Aguirre Pequeño, Mitras Centro, Monterrey, Nuevo León, C.P. 64460. México.

[3] Universidad Autónoma del Estado de Morelos, Centro de Investigaciones Químicas, Avenida Universidad 1001, Chamilpa 62209, Cuernavaca, Morelos, México.

Received April 14th, 2014; Accepted June 23rd, 2014

Abstract. Tuberculosis (TB) is a great cause of morbidity and mortality around the world. The increasing incidence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* and co-infection TB-AIDS led to the need to develop new TB drugs, and plants could be a source of them. The hexane extract of *Citrus sinensis* with anti-TB activity was analyzed by GC-MS. This analysis showed the presence of 40 volatile components, including monoterpenes (81.74%), sesquiterpenes (1.32%), fatty acids (1.15%), and some other oxygenated non-aromatic compounds (15.73%). Some identified compounds were tested against *M. tuberculosis* H37Rv, and one clinical isolated MDR *M. tuberculosis* strain. Results showed that palmitic acid, decanal, caryophyllene oxide, and *cis*-limonene oxide contributed to the anti-TB activity of hexane extract of *C. sinensis* peel.
Keywords: *Citrus sinensis*, GC-MS, *Mycobacterium tuberculosis,* anti-TB compounds.

Resumen. La tuberculosis (TB) es causa de gran mortalidad y morbilidad en todo el mundo. El incremento en la incidencia de *Mycobacterium tuberculosis* multifármaco resistente (MFR) y la co-infección TB-SIDA, lleva a la necesidad de desarrollar nuevos fármacos para el tratamiento de la TB, y las plantas pueden ser una fuente de éstos. El extracto hexánico de *Citrus sinensis* con actividad anti-TB fue analizado por GC-EM. Este análisis mostro la presencia de 40 compuestos volátiles, los cuales incluyen monoterpenos (81.74%), sesquiterpenos (1.32%), ácidos grasos (1.15%), y algunos compuestos oxigenados no aromáticos (15.73%). Algunos de los compuestos identificados fueron ensayados en contra de *M. tuberculosis* H37Rv, y un aislado clínico de *M. tuberculosis* MFR. Los resultados mostraron que el ácido palmítico, el decanal, el óxido de cariofileno, y el óxido de *cis*-limoneno contribuyen con la actividad anti-TB del extracto hexánico obtenido de la cáscara de *C. sinensis*.
Palabras clave: *Citrus sinensis*, CG-EM, *Mycobacterium tuberculosis,* compuestos anti-TB.

Introduction

Globally, tuberculosis (TB) remains a major public health problem. The World Health Organization has estimated that more than one-third of the world's population is infected with *M. tuberculosis* and 1.7 million of these infections result in death each year. Factors that contribute to this health problem include the concurrent HIV epidemic, and the increasing incidence of infections with multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains [1]. To counteract the global problems of TB, there is an urgent need to identify new drugs to combat it. Therefore, ongoing research is focused on the search for new anti-tuberculosis drugs and natural products are an important source of them [2].

Citrus sinensis (Rutaceae) peel has yielded polymethoxyflavones, hydroxylated polymethoxyflavones and hydroxylated polymethoxychalcones, which inhibited the development of arthritis [3]. Furthermore, polymethoxyflavones and hydroxylated polymethoxyflavones have displayed antioxidant [4] and anticancer activities [5]. Oligosaccharides derived from orange peel have shown prebiotic properties [6].

Essential oil extracted from orange peel has shown antibacterial [7] and insecticidal activities [8], as well as decreased oxidative injury in acute otitis media rats [9]. Ethanol extract of *C. sinensis* peel showed hipoglucemic effects in mice [10] as well as larvicidal, pupicidal, and insecticidal activities [11]. Hexane extract of *C. sinensis* peel showed activity against chloroquine (CQ)-sensitive (3D7) strain of *Plasmodium falciparum* [12]. Our research group reported that hexane extract of *C. sinensis* peels exhibited activity against one sensitive (MIC 200 μg/mL) and two monoresistant strains (MIC 25-50 μg/ml) of *M. tuberculosis* H37Rv [13]. Therefore, the aim of this study was to identify some of the active compounds present in hexane extract.

Results and discussion

GC-MS analysis of the hexane extract of *C. sinensis* fruit peel led to the identification of 40 volatile components, including monoterpenes (81.74%), sesquiterpenes (1.32%), fatty acids (1.15%), and some other oxygenated non-aromatic compounds (15.73%) (Table 1). The main components were identified as d-limonene (24.39%), 1-methyl-4-(methylethenyl)-1,2-cyclohexanediol (9.80%), 2-methyl-5-(1-mehylethenyl)-2-cyclohexen-1-one (8.71%), *trans*-L-carveol (7.10%), dodecanal (4.61%),

Table 1. Volatile chemical constituents from the hexane extract of *C. sinensis* fruit peel.

Peak	Compound	RT[a]	%[b]	RI[c]
1	3-Carene[a]	5.46	0.063	846
2	3-Methyl-2-cyclopenten-1-one[d]	6.22	0.29	964
3	β-Myrcene[a]	6.86	0.16	990
4	Octanal[d]	7.19	2.18	1003
5	d-Limonene[a]	7.98	24.39	1029
6	*cis*-β-Terpineol[a]	9.13	0.27	1068
7	1-Octanol[d]	9.21	1.42	1070
8	Trans-Linalool oxide[a]	9.29	1.68	1073
9	*cis*-Linalool oxide[a]	9.78	1.40	1089
10	2-Hydroxy-3-methyl-2-cyclopenten-1-one[d]	10.01	0.73	1097
11	Linalool[a]	10.14	2.79	1101
12	Nonanal[d]	10.26	0.48	1105
13	Trans-p-mentha-2,8-dienol[a]	10.82	1.19	1122
14	1-Vinyl-1-cyclohexenol[d]	10.97	1.25	1126
15	*cis*-Limonene oxide[a]	11.23	0.99	1134
16	*cis*-p-menth-2,8-dienol[a]	11.29	1.39	1136
17	Trans-limonene oxide[a]	11.38	2.42	1139
18	2-Methylene-5-(1-methylethenyl)-cyclohexanol[a]	13.03	0.99	1189
19	α-Terpineol[a]	13.15	1.35	1192
20	*trans*-p-mentha-2,8-dienol[a]	13.30	1.31	1197
21	Decanal[d]	13.59	1.09	1206
22	*trans*-L-Carveol[a]	14.10	7.10	1221
23	1,3,3-Trimethyl-2-oxabicyclo(2.2.2)octan-6-ol[d]	14.22	0.44	1225
24	*cis*-L-Carveol[a]	14.48	2.26	1233
25	2-Methyl-5-(1-mehylethenyl)-2-cyclohexen-1-one[a]	14.91	8.71	1246
26	3-Methyl-6-(1-methylethenyl)-(S)-2-cyclohexen-1-one[a]	15.82	1.33	1273
27	4-(1-methylethenyl)-1-cyclohexene-1-carboxaldehyde[a]	15.89	0.99	1276
28	*cis*-carvone oxide[a]	16.00	0.77	1280
29	α-Limonene diepoxide[a]	16.60	2.77	1297
30	4-(1-methylethenyl)-1-cyclohexene-1-methanol[a]	16.72	1.37	1301
31	2-Ethenyl-1,3,3-trimethyl-ciclohexene[a]	17.01	0.99	1310
32	1-Methyl-4-(methylethenyl)-1,2-cyclohexanediol[a]	18.11	9.80	1344
33	5-Isopropenyl-2-methyl-7-oxabicyclo(4.1.0)-heptan-2-ol[a]	18.71	1.11	1363
34	(2E)-Undecenol[d]	18.83	3.05	1367
35	Dodecanal[d]	20.16	4.61	1408
36	*cis*-p-menth-2,8-dienol[a]	21.35	4.15	1447
37	Caryophyllene oxide[b]	25.50	0.60	1586
38	Nootkatone[b]	31.58	0.72	1809
39	Palmitic acid[c]	35.45	1.15	1964
40	Isopropyl palmitate[d]	36.94	0.19	2046
a: Monoterpenes		81.74		
b: Sesquiterpenes		1.32		
c: Fatty acids		1.15		
d: Other oxygenated compounds		15.73		
TOTAL		99.94		

[a]RT Retention time (min); [b]% Relative abundances from the peak area integration; [c]RI Retention index calculated for each compound.

and *cis*-*p*-menth-2,8-dienol (4.15%). It is important to mention that this is the first report of the chemical composition of hexane extract of *C. sinensis* peel.

Previous GC-MS chemical studies of essential oils obtained from fruit peel of *C. sinensis* from Brazil led to the identification of 28 components. Limonene was the main constituent. Other minor components were α-pinene (1.5%), myrcene (5.9%) and linalool (2.4%) [14]. Chemical composition of essential oil from fruit peel of *C. sinensis* from Greece was determined by GC-MS analysis, yielding in high proportion limonene (94%), and in lower quantities α-pinene (0.7%), sabinene (2%), β-pinene (1.3%), myrcene (1.5%), and linalool (0.6%) [15]. GC-MS analysis of *C. sinensis* essential oil from fruit peel originated from China led to the identification of 32 volatile compounds, of which limonene was the main component (90.85%); some minor components were α-thujene (0.04%), α-pinene (0.76%), sabinene (0.49%), β-myrcene (1.88%), octanal (0.34%), δ-3-carene (0.14%), *cis*-β-ocimene (0.26%), γ-terpinene (1.21%), terpinolene (0.08%), linalool (0.92%), nonanal (0.08%), *trans*-limonene oxide (0.01), decanal (0.21%), geranial (0.11%) and nootkatone (0.01%) [16]. The above reports showed that limonene is the most common and abundant component of the essential oil of *C. sinensis* peel. In addition, we observed that some minor constituents are also similar in the essential oils obtained from orange peel sourced from several regions in the world, for example α-thujene, α-pinene, sabinene, β-myrcene and linalool. In accordance with previous reports we found that chemical composition of hexane extract of *C. sinensis* peel, studied in this research, was similar to the essential oils obtained from orange peel fruit from different regions of the world. In contrast, we found the presence of fatty acids in the hexane extract of *C. sinensis* peel.

Furthermore, some of the constituents identified in the hexane extract of *C. sinensis* peel were evaluated against *M. tuberculosis* H37Rv and one clinical multidrug resistant *M. tuberculosis* strain. Results in Table 2 showed that all tested compounds exhibited less activity than the positive control ethambutol. The best activity was shown by palmitic acid with MIC values of 100 and 50 μg/mL against the sensitive and multidrug resistant strains of *M. tuberculosis*, respectively. Seidel and Taylor [17] reported that saturated fatty acids including palmitic acid, with the exception of C12:0, were devoid of activity against fast-growing mycobacteria (*M. aurum*, *M. smegmatis*, *M. fortuitum*, etc). However, we found that palmitic acid (C16:0) was active against slow-growing strains (*M. tuberculosis* H37Rv and multidrug resistant clinical isolated *M. tuberculosis*), which is in agreement with that reported for Sandoval-Montemayor *et al*., [18]. Kondo and Kanai [19] suggested that mycobactericidal activity of long-chain fatty acids is due to their detergent-like action on the cytoplasmic membrane. Kondo and Kanai [20] found that carboxylic group in fatty acids is necessary for antimycobacterial activity due that fatty acids methyl ester were devoid of activity.

Table 2. MIC values (μg/mL) of some constituents of *C. sinensis* against *M. tuberculosis*.

Compound	H37Rv[a]	M26[b]
d-Limonene	>200	NT
cis-Limonene oxide	>200	50
Decanal	200	25
Caryophyllene oxide	200	100
Palmitic acid	100	50
Ethambutol	3.125	1.25

[a]*M. tuberculosis* H37Rv strain is sensitive to isoniazid, rifampicin, streptomycin and ethambutol;
[b]*M. tuberculosis* M26 clinical strain is resistant to isoniazid and rifampicin. NT: no tested

Decanal showed good (MIC 25 μg/mL) and weak (MIC 200 μg/mL) activities towards the multidrug resistant and sensitive strains of *M. tuberculosis,* respectively. This is the first report of its anti-TB activity. Studies have shown that aldehydes are intrinsically very reactive compounds and readily react with biologically important nucleophilic groups in the membrane, because the carbonyl group is subjected to a variety of addition and condensation reactions, thereby creating disorder in the bilayer of the membrane [21]. Other authors have suggested that the electronegativity on the aldehyde oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane. Thus aldehydes could enter the molecular structure of the membrane with the polar aldehyde group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase, thus affecting lipid bilayer permeability of bacteria [22].

Caryophyllene oxide had weak activity (MIC 100-200 μg/mL) against the sensitive and multidrug resistant strains of *M. tuberculosis*. This study is the first report of its anti-TB activity. It has been reported that sesquiterpene caryophyllene oxide exhibited antimalarial activity against *P. falciparum* [23], and it was found as a constituent of the essential oil of the leaves of *Protium confusum.*

Limonene, the most abundant constituent (24.39%) in the hexane extract of *C. sinensis,* was inactive at concentrations tested. However, its analogue *cis*-limonene oxide displayed moderate (MIC 50 μg/mL) and null activities (MIC >200 μg/mL) against multidrug resistant and sensitive strains of *M. tuberculosis*, respectively. It is important to mention that this is the first report of its anti-TB activity. The activity of this monoterpene is probably due to the presence of the epoxide group.

Previous results on the chemical composition of hexane extract of *C. aurantifolia* peel showed that coumarins, fatty acids and terpenes are responsible of anti-TB activity of this extract [18]. These results are in agreement with our results because we found activity in fatty acids and terpenes, however, we did not find coumarins in hexane extract of *C. sinensis* peel.

From the above we concluded that palmitic acid, decanal, caryophyllene oxide, and *cis*-limonene oxide contained in hexane extract of *C. sinensis* peel contributed to the anti-TB activity in the above extract.

Experimental

Chemicals. Standard solution of 20-*n*-alkanes, *d*-limonene, *cis*-limonene oxide, decanal, caryophyllene oxide, palmitic acid, and ethambutol were obtained from Sigma Co. (St. Louis, MO, USA).

Plant material. *C. sinensis* was collected from Montemorelos, Nuevo León, Mexico in 2010. Voucher (024770) specimen was deposited at the herbarium of Faculty of Biology, Universidad Autónoma de Nuevo León.

Extraction. *C. sinensis* fruit peels (1071 g) were cutted with scissors. Vegetal material was then macerated two times with 8 L of hexane for 24 h at room temperature. Extract was concentrated using a vacuum evaporator. Dried extract (3.54 g; 0.33%) was collected and stored in a refrigerator at 4 °C for further use.

***GC-MS analysis*.** Hexane extract was analyzed on a HP Agilent Technologies 6890 gas chromatograph equipped with a mass spectrometer (MSD 5973) using a dimethylpolysiloxane HP-1 fused silica capillary column (25m x 0.2 mm i.d.; film thickness, 0.3 μm). The carrier gas was helium with a flow rate of 1 mL/min, the injector and detector temperatures were 250°C; injection in split mode (5:1). The oven temperature was held at 60 ºC for 2 min, and then programmed from 60 to 200 ºC at 4°C/min. The mass spectra were recorded on a selective quadrupolar type Hewlett-Packard model 5973; ionization was obtained by electronic impact under a potential of 70 eV. A standard solution of 20-alkanes was used to obtain the index retention time. Identification of components was based on GC retention times and retention index, computer matching with NIST 1.7 library, and comparison of the fragmentation patterns with those reported in the literature.

Mycobacterium cultures. *M. tuberculosis* H37Rv (ATCC 27294; sensitive to streptomycin, isoniazid, rifampicin, ethambutol and pyrizanamide) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Multidrug-resistant clinical strain of *M. tuberculosis* (M-26) was obtained from Hospital Universitario, Dr. José Eleuterio González, Universidad Autónoma de Nuevo León. Both strains were cultured a 37 ºC in Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% OADC (oleic acid albumin dextrose catalase, Difco) until logarithmic phase growth was achieved in order to be used for biological assay.

***Anti-tuberculosis activity*.** Anti-tuberculosis activity was determined as previously described in literature [12]. Assays were run twice on different days. Ethambutol was used as positive control. An institutional ethical committee approved this work.

Acknowledgements

This project was supported financially by Consejo Nacional de Ciencia y Tecnología (CONACYT, grant number CB-106107). We thank to M. Medina from CIQ-UAEM for technical assistance in GC-MS analysis.

Conflict of interest

The authors have declared that there is no conflict of interest.

References

1. Word Health Organization. Global Tuberculosis Report 2013.
2. Camacho-Corona, M.R.; Favela-Hernández, J.M.J.; González-Santiago, O.; Garza-González, E.; Molina-Salinas, G.M.; Said-Fernández, S.; Delgado, G.; Luna-Herrera, J. *J. Mex. Chem. Soc*. **2009**, *53*, 71-75.
3. Li, S.; Lo, C.Y.; Ho, C.T. *J. Agric. Food. Chem*. **2006**, *54*, 4176-4185.
4. Chen, Z.T.; Chu, H.L.; Chyau, C.C.; Chu, C.C.; Duh, P.D. *Food Chem.* **2012**, *135*, 2119-2127.
5. Al-Ashaal, H.A.; El-Sheltawy, S.T. *Pharm. Biol*. **2011**, *49*, 276-282.
6. Manderson, K.; Pinart, M.; Tuohy, K.M.; Grace, W.E.; Hotchkiss, A.T.; Widmer, W.; Yadhav, M.P.; Gibson, G.R.; Rastall, R.A. *Appl. Environ. Microbiol*. **2005**, *71*, 8383-8389.
7. Pittman, C.I.; Pendleton, S.; Bisha, B.; O'Bryan, C.A.; Belk, K.E.; Goodridge, L.; Crandall, P.G.; Ricke, S.C. *J. Food. Sci.* **2011**, *76*, M433-438.
8. Ribeiro, N.C.; da Camara, C.A.; Born, F. de S.; de Siqueira, H.A. *Nat. Prod. Commun.* **2010**, *5*, 1819-1822.
9. Lv, Y.X.; Zhao, S.P.; Zhang, J.Y.; Zhang, H.; Xie, Z.H.; Cai, G.M.; Jiang, W.H. *Int. J. Biol. Macromol.* **2012**, *50*, 1144-11450.
10. Parmar, H.S.; Kar, A. *Biofactors* **2007**, *31*, 17-24.
11. Murugan, K.; Mahesh, K.P.; Kovendan, K.; Amerasan, D.; Subrmaniam, J.; Hwang, J.S. *Parasitol. Res.* **2012**, *11*, 1757-1769.
12. Bagavan, A.; Rahuman, A.A.; Kamaraj, C.; Kaushik, N.K.; Mohanakrishnan, D.; Sahal, D. *Parasitol. Res.* **2011**, *8*, 1099-10109.
13. Camacho-Corona, M.R.; Ramírez-Cabrera, M.A.; González-Santiago, O.; Garza-González, E.; Palacios, I.; Luna-Herrera, J. *Phytother. Res.* **2008**, *22*, 82-85.
14. Araújo, C.P. Jr,; da Camara, C.A.; Neves, I.A.; Ribeiro, N.; Gomes, de Morales, M.M.; Botelho, P. de S. *Nat. Prod. Commun.* **2010**, *5*, 471-476.
15. Michaelakis, A.; Papachristos, A.; Kimbaris, G.; Koliopoulos, A.; Giatropolus, A.; Polissiou, M.G. *Parasitol. Res.* **2009**, *105*, 769-773.
16. Qiao, Y.; Xie, B.J.; Zhang, Y.; Zhang, Y.; Fan, G.; Yao, X.L.; Pan, S.Y. *Molecules* **2008**, *13*, 1333-1344.
17. Seidel, V.; Taylor, P. *Int. J. Antimicrob. Agents* **2004**, *23*, 613-619.
18. Sandoval-Montemayor, N.E.; García, A.; Elizondo-Treviño, E.; Garza-González, E.: Alvarez, L.; Camacho-Corona, M.R. *Molecules* **2012**, *17*, 11173-11184.
19. Kondo, E.; Kanai, K. *Jpn. J. Med. Sci. Biol.* **1976**, *29*, 25-37.
20. Kondo, E.; Kanai, K. *Jpn. J. Med. Sci. Biol.* **1977**, *30*, 171-178.
21. Bisignano, G.; Lagana, M.; Trombetta, D.; Arena, S.; Nostro, A.; Uccella, N.; Mazzanti, G.; Saija, A. *FEMS Microbiol. Lett.* **2001**, *198*, 9-13.
22. Kubo, I.; Fujita, K.; Nihei, K.; Kubo, A. *J. Appl. Microbiol.* **2004**, *96*, 693-699.
23. Kanokmedhakul, S.; Kanokmedhakul, K.; Lekphrom, R. *J. Nat. Prod.* **2007**, *70*, 1536-1538.