Thymus vulgaris AS A POTENCIAL SOURCE OF ANTITUBERCULOUS COMPOUNDS

Adelina Jiménez-Arellanes[1], Rosa Martinez[1], Rosa García[1], Rosalba León-Díaz[1], Julieta Luna-Herrera[2], Gloria Molina-Salinas[3], Salvador Said-Fernández[3] .

[1]UIM Farmacología de Productos Naturales, Hospital Pediatría, Centro Médico Nacional Siglo XXI. México, D.F. [2] Depto de Inmunología, ENCB, IPN. [3]CIBIN, Monterrey, N. L; México, D.F.
adelinaj@servidor.unam.mx, rossalba_leon@yahoo.com.mx

Summary

Hexanic and methanolic extracts from aerial parts of *T. vulgaris* inhibited the growth of *M. tuberculosis* H37Rv. Bioassay-guided from the most active extract to led obtained secondary fraction with antimicobacterial activity. From the secondary fractions active compounds were obtained and were identified by spectrometric and spectroscopic data. The mixture of (E and Z)-phytol and ursolic and oleanolic acid results active against *M. tuberculosis* H37Rv (MIC=25 µg/mL) and moderately active against MDR *M. tuberculosis* clinical isolates with MIC values from 6.25 to 12.5 µg/mL. Main compounds from *T. vulgaris*, thymol, carvacrol and mixture of them were inactive against *M. tuberculosis* H37Rv. Both extracts showing LD_{50} >5g/kg and IC_{50} = 646 ppm. Mixture of thymol/carvacrol and ursolic/oleanolic acid exhibited the IC_{50} = 250 and >1000 ppm, respectively.
Key words: *Thymus vulgaris*, antimycobacterial compounds, MDR *M. tuberculosis*

Tuberculosis is the most ancient epidemic disease in the world, and it is mainly an opportunistic disease in HIV/AIDS patients. Approximately, one million patients die each year and it is estimated that for 2020, one billion persons will have acquired the disease, 200 million could develop it, and 70 million will die due to TB, if urgent measures are not taken immediately.[1,2] In 1996, WHO implemented the DOTS strategy to control and eradicate TB. The treatment scheme established through DOTS is the administration of 4 or 5 combined drugs for periods of up to nine months.[3] Actually, TB is caused by multidrugresistant (MDR) strains that do not respond to the actual TB therapy and are hard to eradicate. Some authors describe that approximately 3.2% of the TB cases worldwide are MDR. Second-line drugs are applied for this cases, but they are expensive, cause several and serious secondary effects, and induce resistance easily.[4] On the other hand, for more than three decades, the search for new anti-TB agents was scarcely.[5] Several investigations groups are now focusing in the search of active molecules and/or structural prototypes for the development of new anti-TB agents from natural and synthetic sources, which will shorten the treatment or complement it.[6-8] In Mexico the use of medicinal plants is ancient and very spread and has allow to development of a permanent line of investigation focused in the search of molecules with anti-TB activity.

It has allowed evaluating approximately 50 species, from which seven inhibit the growth of *Mycobacterium tuberculosis* H37Rv.[9,10] and *Thymus vugaris* is one of them. This specie is commonly known as "tomillo" and it is used to treat respiratory and gastrointestinal diseases.[11,12] Previous biological research describe their antispasmodic, antibacterial, antiviral, antifungal, antiinflammatory and antiseptic activity, but the antimycobacterial potential has not been described.[13-16] Many investigations on this species have been limited to the study of the composition and antimicrobial activity of the essential oil. Chemical investigations of the essential oil and low polar extracts have showed the presence of thymol and carvacrol as main components. Many other monoterpenoids and their glycosides, as well as diterpenoids, acetophenone glycosides, biphenyl compounds, and flavonoids are reported in essential oils, chloroformic and methanolic extracts from this species.[15, 17-22]

Material and methods

Plant Material

T. vulgaris was collected in Oaxaca, Mexico, in July, 1999 and was botanically identified and a voucher specimen was deposited at the IMSS Herbarium (code IMSSM No. 2117).

Extraction and Isolation

Hexanic (Hex) and methanolic (MeOH) extracts from dry and powdered aerial parts of *T. vulgaris* were prepared by maceration for 24 hr at room temperature. Both of them were filtered and evaporated under low pressure to dryness at 40 ºC, and later were fractionated by column chromatography on silica gel and eluted with a mixture of organic solvents of increasing polarity. From the Hex extract 20 primary fractions (F1-20) were obtained and 34 from the MeOH extract (F1'-F34'). All fractions were evaluated by microdilution alamar blue assay (MABA). Active fractions (F8 and F9-12 from Hex extract) were further rechromatographed on Si gel to obtain 12 (8a-8l) and 17 secondary fractions (10a-10q), respectively. 8h-8j and 10g-10l showed in tlc a major compounds, were the most active and were analyzed by GC-EM.

Additionally, in primary fraction F12' and F13' (from MeOH extract) a mixture of thymol/carvacrol, β-sitosterol and stigmasterol was detected. From fraction F25' and F26' a mixture of ursolic/oleanolic acid was obtained by acid/basic partition process. All compounds were structurally characterized by spectroscopic and spectrometric data and were compared with those previously described in literature.

Antimycobacterial activity in vitro

The samples were tested for activity against *M. tuberculosis* using MABA, as previously described.[9,23,24] against *M. tuberculosis* H37Rv and 7 MDR *M. tuberculosis* clinical isolates.

Brine Shrimp lethality and acute toxicity

The extracts were evaluated for lethality to brine shrimp larvae, according to previously described protocols.[25] IC_{50} was determined by probit test. The acute toxicity was determined in male Balb/c (±28 gr) mice following the methodology described by Lorke.[26] The study with animals was performed according the guidelines of the local ethics committee for experimentation in animals in Mexico. The samples were solubilized in tween 20:H_2O (3:7) and were administered orally in a volume not higher to 0.3 mL at 1, 1.6, 2.9, and 5 g/K.

Results and Discusion

Hex and MeOH extracts showed MIC=50 µg/mL against *M. tuberculosis* H37Rv. The bioguided assay from Hex extract led to 20 primary fractions (F1-F20) with increasing polarity, only F8 (MIC=50 µg/mL) and F9-12 (CMI=100 µg/mL) showed antimycobacterial activity (Tabla 1). F8 was submitted to rechromatography and secondary fractions (8a-8l) were obtained, in 8h-8j a mixture of thymol/carvacrol was detected with Rt 7.53 min and other minor compounds detected by GC-MS. These were β-sitosterol, α-amyrin, stigmasterol, 9-eicosin, linoleic and hexadecanoic acids with TR 20.13, 19.63, 19.13, 14.48, 12.52 and 11.58 min, respectively. These compounds were identified by comparing spectral data with those reported data in Pub/Nist library.

Table 1. Antimycobacterial activity of Hex and MeOH extracts and primary fraction of *T. vulgaris*.

Fraction	Elusion sistem (%)	MIC* (µg/ml)
Hex. Ext.		50
Met. Ext.		50
F1-F4	Hex (100)	>200
F5-F6	Hex:$CHCl_3$ (90:10)	>200
F7	Hex:$CHCl_3$ (80:20)	>200
F8	Hex:$CHCl_3$ (80:20)	50
F9-F12	Hex:$CHCl_3$ (80:20)	100
F13	Hex:$CHCl_3$ (70:30)	>200
F14-F17	Hex:$CHCl_3$ (50:50)	>200
F18-F20	$CHCl_3$ 100	>200
Rifampin (Control)		0.062

Hex. Ext: hexanic extract; Met Ext.: methanolic extract; ND: No determined

From 10g-10l, thymol was obtained as a major compound with m.p. 47 ºC (described 49 ºC), by crystallization process and was identified by ^{1}H-NMR data. Other compounds detected by GC-MS in this fraction were carvacrol (TR 7.53 min), (E, Z)-phytol (TR 17.07 y 17.32 min), methylic esther of linoleic acid (TR 17.18 min). From F25' and F26' a mixture of ursolic/oleanolic acid was obtained and was chemical identified by ^{1}H-RMN. The antimycobacterial test showed that 8h-8j and 10g-10l inhibited the growth of *M. tuberculosis* H37Rv with MIC values of 50 and 100 µg/mL, respectively.

The rest of fractions were inactive showing MIC >200 µg/mL against *M. tuberculosis* H37Rv. In addition, only fraction 10g-10l showed marginal activity against MDR *M. tuberculosis* clinical isolates (MIC= 100 µg/mL) (Table 2).

Table 2. Effect of secondary fractions against *M. tuberculosis* H37Rv and MDR clinical isolates of *M. tuberculosis*.

Fraction	MIC (µg/mL) *M. tuberculosis*					
	H37Rv	MTY611	MTY652	MTY650	MTY687	MTY616, MTY282, MTY234
8h-8j	50	>100	>100	>100	>100	>100
10g-10l	100	100	100	100	100	>100

Pure compounds were tested and the results showed that (E and Z)-phytol is the most active against *M. tuberculosis* H37Rv (MIC=25 µg/mL) and was very active against MDR *M. tuberculosis* clinical isolates with MIC values from 6.25 to 12.5 µg/mL (Table 3).

Table 3. Antimycobacterial activity of pure compounds.

Strains	MIC* (µg/mL)				
M. tuberculosis	Mixture of thymol/carvacrol	phytol	Oleanolic acid	Ursolic acid	Mixture of OA/UA
H37Rv	>100	25	12.5	12.5	12.5
MTY652	100	6.25	ND	ND	ND
MTY687	100	12.5	50	50	50
MTY650	100	12.5	50	50	50
MTY675	>100	12.5	50	50	50
MTY282	>100	12.5	50	50	50
MTY234	>100	12.5	100	50	50
MTY99	100	12.5	100	50	ND

The antimycobacterial activity of this compound against *M. tuberculosis* H37Rv (MIC= 2 µg/ml) has been previously described and was isolated from *Leucas volkensii*.[6,7] Thymol, carvacrol, and a mixture of them were inactive against *M. tuberculosis* H37Rv (MIC >200 µg/ml), these compounds were abundant in *T. vulgaris* and has been described as antimicrobial, antiseptic, antiviral and antioxidant agents but not antimicobacterial activity. On the other hand, the mixture of ursolic/oleanolic acids showed good antimycobacterial activity against *M. tuberculosis* H37Rv (MIC=12.5 µg/mL) and a marginal effect against MDR *M. tuberculosis* clinical isolates (MIC=50 µg/mL). Mixture of ursolic/oleanolic acids has been isolated in several vegetal include *T. browsonetii* but has not reported in *T. vulgaris.* The antimycobacterial activity was previously described and potency of the effect was MIC=50 µg/ml (64 µM) against *M. tuberculosis* H37Rv by radiorespirometric assay.[6]

Acute toxicity evaluation in mouse showed that the Hex and MeOH extracts were inactive (DL_{50} >5g/kg). Regarding the evaluation of the extracts against *A. salina* larvae, IC_{50} was 646 ppm for the both extracts and the thymol/carvacrol mixture showed IC_{50} =250 ppm. The ursolic/oleanolic acids mixture was not toxic as the maximum concentration tested (IC_{50} >1000 ppm). This is the first reported that described the antimicobacterial and toxicity of *Thymus vulgaris.*

References

1. Ruxin J, Paluzzi JE, Wilson PA, et al. Emerging consensus in HIV/AIDS, malaria, tuberculosis, and access to essential medicines. Lancet. 2005;365:618-620.
2. De-Riemer K, Garcia-Garcia L, Bobadilla-del-Valle ., et al. Does DOTS work in population with drug–resistant tuberculosis? The Lancet. 2005;365:1239-1245.
3. WHO. World Health Organization (2004) Report on the Tuberculosis. Infection and tranmission.World health Organization. Geneva, Switzerland.
4. Zignol M, Hosseini MS, Wright A, et al. Global incidence of multidrug-resistant tuberculosis. J Infect Dis 2006; 194:479-485.
5. Duncan K, Barry CE 3rd. Prospects for new antitubercular drugs. Curr Opin Microbiol 2004;7:460-465.
6. Cantrell CL, Franzblau SG, Fisher NH. Antimycobacterial Plant Terpenoids. Planta Med. 2001; 67:685-695.
7. Copp BR. Antimycobacterial Natural Products. Nat Prod Rep. 2003;20:535-557.
8. Pauli G, Case R, Inui T, et al. New perspectives on natural products in TB drug research. Life Sciences. 2005;78:485-494.
9. Jimenez-Arellanes A, Meckes M, Ramírez R, et al. Activity against multidrug-resistant *Mycobacterium tuberculosis* in Mexican plants used to treat respiratory diseases. Phytother Res. 2003;17:903-908.
10. Jiménez A, Meckes M, Alvarez V, et al. Secondary metabolites from *Chamaedora tepejilote* are active against Mycobacterium tuberculosis. Phytother Res 2005; 19:320-322.
11. Aguilar A, Camacho JR, Chino S. Herbario Medicinal del Instituto Mexicano del Seguro Social. Información Etnobatánica. IMSS. México. 1994. ix-xv, 111.
12. Argueta A, Cano LM, Rodarte ME. Atlas de las plantas de la Medicina Tradicional III. Instituto Nacional Indigenista. Biblioteca de la Medicina Tradicional Mexicana. México; 1994:1352-1353 pp.
13. Hersch-Martinez P, Leaños-Miranda BE, Solórzano-Santos F. Antibacterial effects of commercial essential oils over locally prevalent pathegenic strains in Mexico. Fitoterapia. 2005;76: 453-457.
14. Essawi T, Srour M. Screening of some Palestinian medical plants for antibacterial activity. J Ethnopharmacol. 2000;70:343-349.
15. Bhaskara-Reddy MV, Angers P, Gosselin A. Characterization and use of essential oil from *Thymus vulgaris* against *Botrytis cinerea* and *Rhizopus stolonifer* in strawberry fruits. Phytochemistry.1998; 47:1515-1520.
16. Yamamoto J, Yamada K, Naemura A, et al. Testing various herbs for antithrombotic effect. Nutrition. 2005; 21: 580-587.
17. Kitajima J, Ishikawa T, Urabe A. A new Hydroxijasmone Glucoside and Its Related Compounds from the Leaf of Thyme. Chem Pharm Bull. 2004a:1013-1014.
18. Kitajima J, Ishikawa T, Urabe A, et al. Monotepenoids and their glycosides from the leaf of thyme. Phytochem. 2004b:3279-3287.
19. Duke JA. CRC Handbook of Medicinal Herbs. Ed. CRC Press. 1ra Edición. Florida. 1985: 483 pp.
20. Giordani R, Regli P, Kaloustian J, et al. Portugal H. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. Phytother Res. 2004;18:990-995.
21. Brunentong J. Farmacognosia, Fitoquímica Plantas Medicinales. Ed. Acribia. S.A. Zaragoza, 2ª edición. España. 2001:331, 488, 540 pp.

22. Wang M, Kikazaki H, Lin C, et al. Acetophenone Glycosides from Thyme (*Thymus vulgaris* L.). J Agric Food Chem. 1999; 47: 1911-1914.
23. Molina-Salinas GM, Ramos-Guerra MC, Vargas-Villarreal J, et al. Bactericidal activity of organic extracts from *Flourensia cernua* DC against strains of Mycobacterium tuberculosis. Arch Med Res 2006;37:45-49
24. Collins L, Franzblau S. Microplate Alamar Blue Assay versus BACTEC 460 System for High-Througput Screening of Compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chem. 1997;41:1004-1009.
25. Anderson JE, Goetz CM, McLaughlin JL, et al. A blind comparison of simple bench-top bioassays and human tumor cell cytotoxicities as antitumor pre-screen. Phytochemical analysis. 1991;2:107-111.
26. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol. 1983;54:257-287.