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独山瓜馥木提取物促休眠型结核杆菌复苏的活性研究

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摘 要:结核杆菌具有休眠的特性,休眠型结核杆菌对现有抗痨药物均不敏感,因此研发抗休眠型结核杆菌药物对根治结核病具有重要意义。本试验采用 Wayne 模型,发现独山瓜馥木根的乙醇提取物能复苏休眠型结核杆菌,独山瓜馥木的促复苏作用可能与刺激休眠型结核杆菌利用 C_{10} 化合物作为能量来源有关。在体外试验时,独山瓜馥木根的乙醇提取物(2 mg/mL)与异烟肼(8 μg/mL)联合用药,可杀死休眠型结核杆菌。结果提示,传统抗痨药物与促进休眠型结核杆菌复苏的药物联合用药可能是根治结核病的一种新策略。

关键词:独山瓜馥木;复苏;休眠型结核杆菌

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Resuscitating Activity of *Fissistigma cavaleriei* Extract on Dormant Mycobacteria

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Abstract: *Mycobacterium tuberculosis* could go into nonreplicative dormancy. This stage of mycobacteria posed a significant problem for effective therapy as these persistent bacilli were resistant to most of the currently available drugs for the treatment of tuberculosis. The development of drugs that specifically target dormant bacilli could have a profound impact on tuberculosis therapy. In the present study, the extract of *Fissistigma cavaleriei* root showed a potential activity to resuscitate dormant tubercle bacillus into active bacilli based on Wayne model. It seemed that *F. cavaleriei* extract might stimulate dormant *M. tuberculosis* to use compounds of C_{10} as energy sources for resuscitation. The combination of *F. cavaleriei* extract (2 mg/mL) and isoniazid (8 μg/mL) showed powerful killing effect on dormant *M. tuberculosis*. The findings of this study suggested that conventional anti-mycobacterials in combination with 'dormancy-regrowth' drugs might be considered as a novel strategy to eradicate latent *M. tuberculosis*.

Key words: *Fissistigma cavaleriei*; resuscitation; dormant *Mycobacterium tuberculosis*

Introduction

Tuberculosis continues to be a major cause of morbidity and mortality throughout the world. In 2010, there were 8.6 million people fell ill with tuberculosis bacilli (TB) and 1.3 million died from TB[1]. Current TB therapy, also known as DOTS (directly observed treatment, short-course) consists of an initial phase of treatment with 4 drugs, isonizid, rifampicin, pyrazinamide and ethambutol, for 2 months daily, followed by treatment with isonizid and rifampicin for another 4 months, three times a week[2]. In particular, the long chemotherapy regimen can be attributed to patients' noncompliance and poor case management in resource-limited countries with a high burden of the disease. An incomplete course of treatment is considered to be one of the primary contributors to the emergence of multidrug-resistant TB and extremely drug-resistant TB [3,4].

Mycobacteria has the unique property of becoming persistent or dormant for very long periods. This stage of mycobacteria poses a significant problem for effective therapy as these persistent bacilli are resistant to most of the currently available drugs for the treatment of tuberculosis. A key challenge in TB control therefore is to

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clear the community of dormant TB infection. The second major challenge is the lack of drugs effective against latent TB infection.

To achieve global control of this epidemic, there is an urgent need for new strategies to tackle the latent TB infection. Recently Seidi and Jahanban-Esfahlan proposed a novel cost-effective approach, resuscitation-promoting factor (Rpf) + antibiotics, which could effectively eradicate both active and latent TB in a short period of time without having any risk of reactivation[5]. *Mycobacterium tuberculosis* contains five resuscitation-promoting factor (Rpf)-like proteins, RpfA-E, that are implicated in resuscitation of this organism from dormancy via a mechanism involving hydrolysis of the peptidoglycan by Rpfs and partnering proteins. The bulk of the evidence suggests that Rpf (resuscitation-promoting factors) proteins are not required for general viability. However, under certain conditions, Rpf does appear to be vital for growth. Strains of *M. tuberculosis* lacking combinations of three rpf genes were defective for growth *in vivo* and in an *in vitro* resuscitation assay[15]. Inspired by above idea, we hypothesized that there might be natural products which can reactivate the dormant *M. tuberculosis* into active type. More than seventy crude extracts of Chinese herbal medicines, which collected from Dushan, Guanling, Liping and Leishan counties of Guizhou province, were investigated to resuscitate dormant tubercle bacillus. These traditional Chinese medicines mainly belonged to the types of heat-clearing, blood-moving, wind-damp-dispelling, stopping coughing and blood-nourishing. Among them, the extract derived from roots of *Fissistigma cavaleriei* (Levl) Rehd (Annonaceae) showed resuscitating activity in Wayne model. It is interesting that the extract combined with isoniazid can eradicate the dormant tubercle bacillus in *in vitro* assay. *F. cavaleriei* is a perennial shrub, which is distributed mainly in Guizhou province of China. This plant has been used as a folklore medicine for anti-inflammatory, anti-arthritic effects, and also used as antitubercular agent by Miao people [6]. Previous studies on this plant yielded a beta-lactamase inhibitor named salsalate and a fractionation with antiangiogenic activity [7,8]. To the best of our knowledge, this is the first report on the anti-dormant mycobacterial activities of *F. cavaleriei*.

Materials and Methods

Plant material and extraction

The dried roots of *F. cavaleriei* were collected on April 19, 2008 from the Dushan County, Guizhou province, China, and identified by authors. A voucher specimen (accession number GZ49) has been deposited at the Pharmacy Laboratory, Guizhou University, China. Air-dried, powdered root of *F. cavaleriei* was extracted with 80% MeOH/H_2O three times in the reflux extraction unit at 70 ℃ to yield a dark-brown residue. The stock solutions of the extract and isoniazid were dissolved in DMSO and kept in refrigerator.

M. tuberculosis in vitro oxygen depletion assay

The experimental procedure used for the *M. tuberculosis in vitro* oxygen depletion assay was a slight modification of the method described firstly by Wayne and Hayes [9,10]. Briefly, mid-log-phase aerobic *M. tuberculosis* H37Rv cultures were diluted 100-fold in Dubos medium and transferred to tubes (150 by 20 mm) in 21 mL volumes. The tubes were closed with sterile 8.0 mm silicone rubber septa (Aldrich, Milwaukee, WI). The cultures were grown at 37 ℃ with slow stirring for 24 days. Control tubes contained methylene blue dye (1.5 μg/mL) as an indicator of oxygen depletion. The blue dye fades and finally disappears under anaerobic conditions, as described by Wayne and Hayes [9]. The extracts of *F. cavaleriei* were injected at final concentrations ranging from 0.5 to 4 mg/mL through the septa of 24-day-old cultures. The reference drug, isoniazid alone, was injected at final concentrations ranging from 4 to 32 μg/mL. The extract (2 mg/mL) + isoniazid (8 μg/mL) were injected through the septa of 24-day-old cultures. Culture tubes were prepared in duplicate for each concentration of drug. Control tubes received DMSO without drugs, with a final concentration of DMSO in the cultures of 1%. After drug treatment, the cultures were incubated at 37 ℃ with slow stirring for 4 days. After 96 h of drug exposure, the septa were removed and 1 mL samples were drawn from the middle of the culture tubes. The cultures were serially diluted

in saline and plated onto 7H11/OADC agar plates (Difco). The plates were incubated at 37 ℃ under normal atmospheric conditions for 21 days, at which time the colonies were counted. Also the viability of the cultures was monitored by FDA-EB staining. The FDA-EB staining was performed as described previously[11,12]. Briefly, mycobacterial cultures (100 μL) were stained for about 20 min with 50 μL of FDA-EB working solution containing FDA and EB. A wet mount was prepared and sealed to prevent evaporation and observed under fluorescent microscope at a magnification of 1000 × (Olympus BX 40, Japan) with blue filter. A total of 200 cells were counted in replicate and differentiated on the basis of their color. Green cells were considered to be live; whereas red-stained cells were considered to be dead. Percent viability was calculated by dividing the number of green cells by the total number of cells observed and multiplying by 100.

GC-MS analysis of the cell wall extract of *M. tuberculosis*

Over 60% of the mycobacterial cell wall is lipid. In order to investigate whether the extract of *F. cavaleriei* may intervene the synthesis of cell wall, GC-MS analyses was used to identify the differences of lipids between the *M. tuberculosis* treated with *F. cavaleriei* extract and untreated one. A spadeful of bacteria (10 mg [wet weight]) was collected from the above described culture that treated with *F. cavaleriei* extract (2 mg/mL) or treated with only DMSO. The mycobacterial lipids were extracted and derivatized to methyl esters by a modification of the method of Luquin, *et al*.[13]. The cells were mixed with 1 mL of a reagent composed of 30 mL of methanol, 15 mL of toluene, and 1 mL of concentrated H_2SO_4 in a screw-cap test tube (14 by 120 mm) fitted with a Teflon-lined cap. The mixture was heated in a covered bath at 80 ℃ for 16 h (overnight). After being cooled at room temperature, the samples were extracted twice with 2 mL of n-hexane. The hexane extracts were combined, transferred to another test tube, and mixed with an equal volume of 0.3 M phosphate buffer (42.57 g of Na_2HPO_4 and 12.0 g of NaOH per liter of distilled water [pH 11 to 12]). The hexane upper layer was then removed, placed in a clean tube, and evaporated to dryness in a water bath at 40 ℃ under a stream of nitrogen. The residue was dissolved in n-hexane. GC-MS analyses were performed using HP 5890 GC and HPMSD 5973 GC-MS instruments with an HP 5 column (30 m × 0.25 mm × 0.25 μm) and using helium as a carrier gas at a linear flow rate of 1 mL/min. The injector temperature was set to 280 ℃, and the interface was at 280 ℃. The oven temperature was programmed from 60-280 ℃ at 5 ℃ per min. The electronic impact energy was set at 70 eV, and mass spectra were collected in the range of 10-550 atomic mass units.

Results and Discussion

Resuscitating effect of *F. cavaleriei* extract on dormant *M. tuberculosis*

It was observed (Table 1) that *F. cavaleriei* extract can increase the CFU of *M. tuberculosis* and isoniazid obviously decreased the CFU of *M. tuberculosis*, both in a dose-dependent manner based on Wayne model at same time. Fluorescein diacetate-ethidium bromide (FDA-EB) staining can rapidly assess the viability status of *M. tuberculosis* cultures[11]. The results showed that a high degree of correlation existed between the two methods. It is very interesting that almost all the tubercle bacillus were killed by the combination of extract of *F. cavaleriei* (2 mg/mL) and isoniazid (8 μg/mL).

Table 1 Comparison of mycobacterial cell viability as determined by plating and staining with FDA-EB

Group	Concentration	Viability determined by Plating (CFU/mL)	FDA/EB (%)
F. cavaleriei extract	0.5 mg/mL	4.4×10^6	96
	1 mg/mL	5.8×10^6	94
	2 mg/mL	6.2×10^6	96
	4 mg/mL	6.4×10^6	96

Isoniazid	4 μg/mL	3.8×10^6	64
	8 μg/mL	3.4×10^6	52
	16 μg/mL	2.2×10^6	46
	32 μg/mL	1.8×10^6	34
Extract + isoniazid	2 mg/mL + 8 μg/mL	0.4×10^2	2
Control (DMSO)	–	4.2×10^6	94

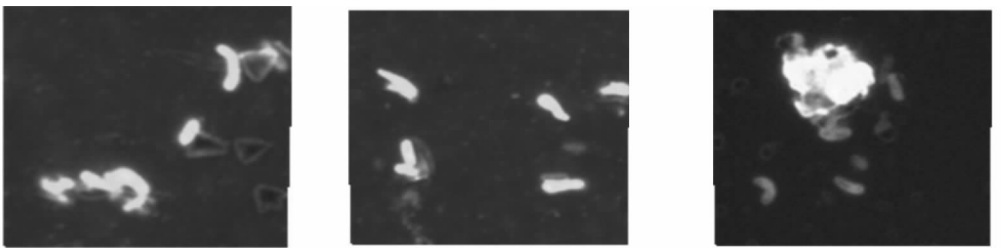

Fig. 1 Images of *M. tuberculosis* cultures treated with extract of *F. cavaleriei* (2 mg/mL) (A), DMSO (1%) (B), and mixtures of *F. cavaleriei* extract (2 mg/mL) + isoniazid (8 μg/mL) (C)

The pictures showed that the shapes of *M. tuberculosis* cultures treated with extract of F. cavaleriei (Fig. 1A) were same as that of control (Fig. 1B). It suggested that the extract of *F. cavaleriei* is not toxic for *M. tuberculosis*. The red-orange fluorescence (Fig. 1C) showed that the *F. cavaleriei* extract + isoniazid can disrupt the membrane integrity of *M. tuberculosis*.

GC-MS analysis of the cell wall extract of *M. tuberculosis* cultures

Twenty-one components were identified in the extract of *M. tuberculosis* cultures of control, representing 82.57% of the total compounds (Table 2). The most abundant compounds were linoleic acid (38.4%, C_{18}), palmitic acid (11.537 %, C_{16}), (E,Z)-2,4-Decadienal (11.267 %, C_{10}), and (E,E)-2,4-Decadienal (3.714% , C_{10}).

Only nine components were identified in the extract of *M. tuberculosis* cultures treated with *F. cavaleriei* extract, representing 68.06% of the total compounds (Table 3). The most abundant compounds were linoleic acid (21.407%, C_{18}), methyl palmitate (18.076% , it is actually the palmitate that be methylated in sample preparation, the number of carbon should be sixteen, C_{16}), methyl 6-Octadecenoate (9.158% , actually the number of carbon should be sixteen, C_{18}), methyl stearate (8.187% , actually the number of carbon should be sixteen, C_{18}), and palmitic acid (4.489 % , C_{16}).

From the data (Table 4) we can deduced that C_{16} and C_{18} were used to synthesis of mycolic acids that are the main ingredients of mycobaterial cell wall. *F. cavaleriei* extract may stimulate dormant *M. tuberculosis* to use compounds of C_{10} as energy sources for resuscitation.

Table 2 GC-MS analysis of the cell wall extract of *M. tuberculosis* cultures of control

RT	Name	MF	MW	Total (%)
9.86	(E)-2-Heptenal	$C_7H_{12}O$	112	0.643
11.09	Dihydromyrcene	$C_{10}H_{18}$	138	0.076
11.63	(E)-2-Octenal	$C_8H_{14}O$	126	0.156
12.98	(E,E)-2,4-Nonadienal	$C_9H_{14}O$	138	0.214
13.92	Decanal	$C_{10}H_{20}O$	156	0.275
14.28	(E,Z)-2,4-Nonadienal	$C_9H_{14}O$	138	0.307
16.98	(E)-2-Decanal	$C_{10}H_{18}O$	154	2.356
18.41	(E,E)-2,4-Decadienal	$C_{10}H_{16}O$	152	3.714

19.39	(E,Z)-2,4-Decadienal	$C_{10}H_{16}O$	152	11.267
20.97	(Z)-2-Decanal	$C_{10}H_{18}O$	154	2.285
24.89	Pentadecane	$C_{15}H_{32}$	212	0.239
26.94	Lauric acid	$C_{12}H_{24}O_2$	200	3.27
27.44	Diethy phthalate	$C_{12}H_{14}O_4$	222	0.625
29.10	6,9-Heptadecadiene	$C_{17}H_{32}$	236	1.625
29.25	8-Heptadecene	$C_{17}H_{34}$	238	1.326
29.36	(Z,Z)-10,12-Hexadecadienal	$C_{16}H_{28}O$	236	0.303
31.22	Myristic acid	$C_{14}H_{28}O_2$	228	0.57
35.53	Palmitic acid	$C_{16}H_{32}O_2$	256	11.537
39.02	Linoleic acid	$C_{18}H_{32}O_2$	280	38.4
45.39	Octoil	$C_{24}H_{38}O_4$	390	2.407
51.30	Methy linoleate	$C_{19}H_{34}O_2$	294	0.976

Table 3 GC-MS analysis of the cell wall extract of *M. tuberculosis* cultures treated with *F. cavaleriei* extract (2 mg/mL)

RT	Name	MF	MW	Total (%)
16.31	endo-Borneol	$C_{10}H_{18}O$	154	0.959
34.49	Methyl palmitate	$C_{17}H_{34}O_2$	270	18.076
35.15	Palmitic acid	$C_{16}H_{32}O_2$	256	4.489
35.33	Methyl glycol phthalate	$C_{14}H_{18}O_6$	282	2.574
36.42	Methyl margarate	$C_{18}H_{36}O_2$	284	0.992
37.73	Methyl 9,15-Octadecadienoate	$C_{19}H_{34}O_2$	294	2.22
37.83	Methyl 6-Octadecenoate	$C_{19}H_{36}O_2$	296	9.158
38.28	Methyl stearate	$C_{19}H_{38}O_2$	298	8.187
38.51	Linoleic acid	$C_{18}H_{32}O_2$	280	21.407

Table 4 Relative percentage of compounds based on carbon number

	Carbon Number (%)										
	C_7	C_8	C_9	C_{10}	C_{12}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{24}
Control	0.64	0.15	0.52	19.97	3.89	0.57	0.24	11.84	2.95	39.37	2.41
Treatment	0	0	0	0.96	0	2.57	0	22.56	0.99	31.82	0

Discussion

In the present study, it was found that exogenously added *F. cavaleriei* extract induce the resuscitation of dormant *M. Tuberculosis* obtained in stationary phase after cultivation under hypoxia conditions. Shleeva have previously reported the reactivation of dormant mycobacteria in the presence of free fatty acids[14]. They have shown that an optimum FA concentration is needed to produce the observed resuscitation effect, higher concentrations being ineffective or even inhibitory. However, our experimental results (Table 1) showed that the *F. Cavaleriei* extract at higher concentration (4 mg/mL) or lower concentration (0.5 mg/mL) had no significant difference in the effect on the resuscitation of dormant *M. Tuberculosis*. Therefore, we hypothesized that the active components of the *F. Cavaleriei* extract may not be fatty acids.

In particular, exogenous small molecules, which may induce the resuscitation of dormant mycobacteria, have not yet been described. The evidence from this study suggested that *F. cavaleriei* extract could resuscitate dormant *M. tuberculosis* into active type. So it might be the first report that plant extract containing small mole-

cules showed resuscitating effect on dormant *M. tuberculosis in vitro*.

Previous studies have reported that dormant bacteria might alter cell wall structure and require unique metabolic pathways for regrowth to occur[15]. In order to investigate the mechanism of *F. Cavaleriei* extract recovery of dormant *M. tuberculosis*, GC-MS analyses was used to identify the differences of lipids between the *M. tuberculosis* treated with *F. cavaleriei* extract and untreated one. In this study, we found obvious evidence for a difference in chemical compositions of dormant *M. tuberculosis* cultures treated with *F. cavaleriei* extract in comparison with the control group. In general, therefore, it seemed that the compounds of C_{10} contained in *M. tuberculosis* may be used as a critical energy sources to resume cell division. We hypothesized that *F. cavaleriei* extract could activate a new metabolic pathway for resuscitating dormant *Mycobacterium tuberculosis*.

Any given drug regimen capable to achieve the culture-negative state of the host does not guarantee eradication of infection and reactivation prevention. Human beings may need to change their strategies to fight against the dormant *M. tuberculosis*. This study confirms that the combination of *F. cavaleriei* extract and isoniazid showed powerful killing effect on dormant *M. tuberculosis*. It is possible to hypothesis that *F. cavaleriei* extract resuscitate dormant *M. tuberculosis* into active type which more susceptible to isoniazid treatment. Because it is difficult to know the precise conditions encountered by pathogens in the host, several *in vivo* experiments should be employed for studying this combining strategy.

In conclusion, we showed that *F. cavaleriei* extract could resuscitate dormant *M. tuberculosis* into active type, and the combination of *F. cavaleriei* extract and isoniazid exhibited powerful killing effect on dormant *M. tuberculosis*. The finding could provide new ideas for the treatment of latent tuberculosis.

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