SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2014; 31; 336-342

In vitro and in vivo study of anti-tuberculosis effect of extracts isolated from *Ranunculi Ternati Radix*

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Abstract. *Aim:* This study was designed to investigate the anti-tuberculosis activities of *Ranunculi Ternati Radix* extracts to demonstrate the effect of active part of *Ranunculi Ternati Radix*, which could be enriched through macroporous resin, on mycobacterium tuberculosis infections. *Materials and methods:* In vitro, the anti-tuberculosis activity of its water extract (WE), 70% ethanol extract (EE), water eluted part of EE from D101 macroporous resin (WEPMR), 70% ethanol eluted part of EE from D101 macroporous resin (EEPMR) was conducted using H37Rv. Then EEPMR of better anti-tuberculosis activity was chosen to carry out anti-tuberculosis activity test against MDR2314-2 and XDR1220. In vivo, the anti-tuberculosis activities of EEPMR, Ranunculi Ternati Capsules and Isoniazid alone or in combination with different doses were evaluated on mouse model infected H37Rv. *Results:* In vitro, EEPMR had inhibitory effect on H37Rv, MDR2314-2 and XDR1220. In vivo study, both medium and high dose of EEPMR alone had therapeutic effect on chronic tuberculosis in mouse. No acute toxicity was identified of EEPMR at a dose of 12.0 g·kg-1. *Conclusions:* EEPMR possessed better anti-tuberculosis effects than other extracts and Radix Ranunculi Ternati Capsules. This supported the use of macroporous resin to enrich the active part of *Ranunculi Ternati Radix* to cure mycobacterium tuberculosis infections. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 336-340)*

Key words: *Ranunculi Ternati Radix*, anti-tuberculosis activity, Fractionation methods, mice

Abbreviations

WE: Water extract
EE: 70% Ethanol extract
WEPMR: Water eluted part of EE from D101 macroporous resin
EEPMR: 70% ethanol eluted part of EE from D101 macroporous resin
MIC: Minimum inhibitory concentration
NT: Model group
A: Radix Ranunculi Ternati Capsules
I: Isoniazid
B1: high dose of EEPMR
B2: moderate dose of EEPMR
B3: small dose of EEPMR
I+B1: Combined therapy of Isoniazid and high dose of EEPMR
I+B2: Combined therapy of Isoniazid and moderate dose of EEPMR
I+B3: Combined therapy of Isoniazid and small dose of EEPMR
I+A: Combined therapy of Isoniazid and Radix Ranunculi Ternati Capsules.

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Introduction

Ranunculus ternatus is a species of genus Ranunculus, which is distributed in Henan, Zhejiang, Hubei, Guangxi and Sichuan provinces in China, and also spreads out in Tanwai and Japan .The earliest written document about Ranunculus ternatus was in Handbook of Chinese Herbal Medicines . As an approved traditional Chinese herbal medicine, Ranunculus ternatus was listed in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 1977) (1). The root of Ranunculus ternatus has been used alone or combined with other herbal medicines in the treatment of tuberculosis, neck scrofula, M. scrofulaceum, lymphadenitis, faucitis, tumor etc (2, 3).

The traditional use of the plant as anti-tuberculosis agents has been supported by several studies (4, 5), which indicated that the extracts or fractions of the plant possessed anti-tuberculosis activity. Radix Ranunculi Ternati Capsules, based on Ranunculus ternatus, is now the only commercially available clinical medicine for the treatment of tuberculosis in China. Current studies showed the combination of Radix Ranunculi Ternati Capsules and first-line tuberculosis drugs can shorten the treatment and strengthen the efficacy within no side effect (6-9).

Despite chemical investigations showing the presence of sugar, fatty acid esters, alkaloids, flavonoids, Glycosides etc in this plant (10-14), no correlations between the effective parts and active ingredients of treatment of tuberculosis have been reported before.

In this study, our objective was to investigate the anti-tuberculosis activity of the different extracts and fractions of *Ranunculi Ternati Radix* by H37Rv, MDR2314-2 and XDR1220 in vitro and in mouse models infected H37Rv in vivo. Preliminary study was taken to determine the phytochemical compositions of the four preparative extracts and Radix Ranunculi Ternati Capsules.

Materials and methods

Plant material

Ranunculi Ternati Radix was obtained from Huadong Medicine Co., Ltd in Zhejiang Province, China and was authenticated by vice Professor Lin Zhang of Zhejiang University. A voucher specimen (No. 20090805) is kept at College of Biomedical Engineering & Instrument Science, Zhejiang University.

Preparation of plant extracts and fractions

For WE, 1kg dried herb samples was extracted exhaustively in 10L water for 3 times (for 1.5h, 1h and 1h respectively) by refluxing extraction method. The obtained extracts were filtered and evaporated under reduced pressure on a rotary evaporator (temperature at 55-60). Then the solvent-reduced extract was cooled down to 25 and diluted into needed concentration in volumetric flask. For EE, a similar extraction procedure to WE was used except that the solvent was 70% ethanol instead of water. Then another 70% ethanol extract of 10kg dried herb was obtained using the same procedure above. This extract was evaporated under reduced pressure until ethanol was exhausted to get an aqueous extract. The aqueous part was absorbed on D101

macroporous resin for 1h, and then eluted with water to get the water eluted part, and then eluted with 70% ethanol to get the 70% ethanol eluted part. The water eluted part and 70% ethanol eluted

part were both reduced on a rotary evaporator and diluted into needed concentrations in volumetric flasks which were WEPMR and EEPMR respectively. The yields of the two extracts were 20.7% and 17% while the two fractions were 14.6% and 1.2% (Supplementary Fig.S1) Radix Ranunculi Ternati Capsules was bought from Kaikai Yuansheng Medicine CO., LYD. All the samples were stored at 4 before use (15).

Chromatographic analysis of WE, EE, WEPMR, EEPMR and Radix Ranunculi Ternati Capsules

Waters 2695 Alliance HPLC system (USA)-diode array detection (DAD) equipped with an online degasser and auto-sampler was used for solvent delivery. The measurements were carried out on an RP-18 column (250mm×4.6mm Agilent USA) with column temperature of 40 and flow rate of 1ml·min^{-1} (15). The solvents used for separation was water containing 0.4% acetic acid as solvent B and methanol as solvent C. The gradient of HPLC analysis for the six preparative extracts is as follows: 0.00 min, 95.0% B

(5.0% C); 35.00 min, 81% B (19% C); 45.00 min, 81% B (19%C); 50.00 min, 5% B (95% C); 55.00 min, 5% B (95% C); 55.01 min, 95.0% B (5.0% C); 60.00 min, 95.0% B (5.0% C). And all the HPLC chromatograms were extracted under the wavelength of 292 nm.

Experiment strains

H37Rv, standard strains of mycobacterium tuberculosis, was obtained from National Culture Collection (ATCC 95054). MDR-TB (2314-2), multidrug resistant strains and XDR-TB (1220), extensively drug-resistant strains, were obtained from Shanghai Pulmonary Hospital. Strains were cultured at 37 for two weeks in liquid medium made from Middlebrook 7H9 dehydrated medium(BD, USA) and nutritional additive(OADC, BD, USA). Then some of the strains were added to a 4 ml liquid medium. After that, ten sterile glass beads of 2 or 3mm diameter were added, oscillated for 20 ~ 30 seconds, and placed for l0 ~ 20min. The supernatant of bacterial suspension were drawn and strains of H37Rv were diluted to 10^5CFU/ml with the liquid medium, while stains of MDR-TB (2314-2) and XDR-TB (1220) were diluted to 10^7CFU/ml with the liquid medium.

Experiment animals

Vivo experiments were performed on C57BL/6 mice (SPF, 6~8weeks old), which were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and the number of Animal Production License is SCXK (JING) 2006-0009. The animals were housed five per cage under a 12h light/dark cycle in a room with controlled temperature (20.0-23.6), fed with normal mice chow and water ad libitum. All animals were allowed to acclimate for 7 days prior to the first treatment. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of the Zhejiang University (SYXK (ZHE) 2007-0098).

Assessment of anti-tuberculosis activity in vitro

The preparative extracts and fractions as well as the reference drugs were diluted to different concentrations under sterile conditions. Then 100μl of above drug solution were added to 96-well microplates following the addition of 100μl bacterial liquid and cultured at 37 . Three groups of parallels were set at the same drug concentration. The control groups were established with bacteria amount of 1%, 10% and 100% respectively without any drug solution and blank groups were set without any drug solution or bacterial liquid. MIC (Minimum inhibitory concentration) was used to evaluate the anti-tuberculosis activities.

Assessment of anti-tuberculosis ability in vivo

C57BL/6 mouse models infected with H37Rv were used to evaluate the effectiveness of the tuberculosis treatment of the preparative extracts (EEPMR). Two hundred and fifteen C57BL/6 mice were divided into eleven groups randomly with animal number of 10,25,20,20,20,20,20,20,20,20 and 20 which were orally treated with water (control and model=NT), Radix Ranunculi Ternati Capsules (A=positive control with 1240 mg/kg/d), Isoniazid (I=positive control with 25 mg/kg/d), Radix Ranunculi Ternati Capsules+Isoniazid (A+I=positive control with 1240+25 mg/kg/d), three doses (B1=400,B2=200,B3=200mg/kg/d) of EEPMR and three doses (B1+I=400+25, B2+I=200+25, B3+I= 100+25 mg/kg/d) of combination therapy (EEPMR+Isoniazid) of respectively (supplemental data, Table S1). For control and model groups, each mouse was injected with 200μl sterile water, and the same volume of drug was injected (oral administration) to each mouse for drug administration group. 5 mice of the model group were put to death before treatment. After 4 weeks and 8 weeks of treatment, 10 mice in each group were put to death. Pathological changes of the vital organs, organ index (lung and spleen, =weight of the organ/ weight of the mice ×100) and bacterial counts in mice were observed. Histological examination was also taken in lung. Both isoniazid and commercially available drug Radix Ranunculi Ternati Capsules were used as reference drugs.

Acute toxicity

Two groups of BALB/c each weighing 17-19g were used (n=20 for each group). The preparative

($12.0 g \cdot kg^{-1}$) was orally administered and the general behavior and the toxic symptoms were observed 30min,1h,1 14d after drug treatment. All mice were further maintained with free access to food and water to see if there is any sign of toxicity or mortality for a time span of two weeks after treatment.

Statistical analysis

Results were reported as Mean±S.D and Mean $\log_{10}$CFU±SEM. Data were analyzed statistically using t-test with probability values of $p \leq 0.05$ which were considered to be statistically significant.

Results and discussion

In vitro study, the anti-tuberculosis activities of the four preparative extracts with different concentrations were investigated by the strain of H37Rv with Isoniazid, Rifampicin and Radix Ranunculi Ternati Capsules as reference drugs. Our results (Table 1) showed that Isoniazid and Rifampicin can significantly inhibit H37Rv with the MIC of 0.5 1.0 μg/mL and <0.0625μg/mL. For WE, the MIC was a little lower than that of EE but closed to EE, which meant that the active components of WE and EE were similar. While WEPMR showed little activity according to MIC, which indicated that most undesirable constituents might be concentrated in this part, and the enhanced anti-tuberculosis activity of EEPMR which was better than Radix Ranunculi Ternati Capsules sold in the market in China indicated that after elimination of unwanted ingredients, most active compounds maybe concentrated in the 70% ethanol eluted part.

According to the above results, the anti-tuberculosis activity of EEPMR was then carried out by MDR-TB (2314-2) and XDR-TB (1220). Our results (Table 2) showed that Isoniazid and Rifampicin can significantly inhibit MDR-TB (2314-2) and XDR-TB (1220). EEPMR had stronger anti-tuberculosis effects than Radix Ranunculi Ternati Capsules

Toxicity study was carried out for EEPMR to confirm its safety. Results showed that no acute toxicity (16) was observed with oral administration of the extract at a dose of 40.0mg/kg body wt.

Finally, we started to determine anti-tuberculosis activity of EEPMR in mouse model infected with H37Rv. Radix Ranunculi Ternati Capsules and Isoniazid were used as two reference drugs. In order to make sure that the mouse models were successfully established, five of the mouse models were put to death before treatment. The model mice showed vascular congestion, severe inflammatory cell infiltration around "http://dict.bioon.com/detail.asp?id=901d483156" venule and in alveolar septa, infiltration cells conclude monocytes and lymphocytes, alveolar septa increased in width, alveolar number reduced and lost their normal structure. (Fig.1)

The trends of weight gain or lose of each treatment group and model control group was approximately consistent. Food in each group showed no significant difference at each time point, as well as water intake. Drug group and control group also showed no significant difference. After 4 weeks of treatment, compared to the model group, the number of lung bacteria in mice of each treatment group decreased to a certain extent except B1, B3, I+A

Table 1. Inspection of the inhibition of the standard strains

No.	Tested drugs	Tested strain	MIC
1	WE	H37Rv	60 mg/mL
2	EE	H37Rv	40 mg/mL
3	WEPMR	H37Rv	160 mg/mL
4	EEPMR	H37Rv	6.25 mg/mL
5	Radix Ranunculi Ternati Capsules	H37Rv	12.5 mg/mL
6	Isoniazid	H37Rv	0.5-1.0 μg/mL
7	Rifampicin	H37Rv	<0.0625 μg/mL

Abbreviations: WE, water extract; EE, 70% ethanol extract; WEPMR, water eluted part of EE from D101 macroporous resin; EEPMR, 70% ethanol eluted part of EE from D101 macroporous resin; MIC, minimum inhibitory concentration. Ranunculi Ternati Capsules, Isoniazid and Rifampicin were used as reference drugs.

Table 2. Inspection of the inhibition of the resistant strains

No.	Tested drugs	Tested strain	MIC
1	EEPMR	MDR	1.0 mg/mL
2	Radix Ranunculi Ternati Capsules	MDR	25 mg/mL
3	Isoniazid	MDR	4 μg/mL
4	Rifampicin	MDR	>32 μg/mL

Abbreviations: EEPMR, 70% ethanol eluted part of EE from D101 macroporous resin; MIC, minimum inhibitory concentration. Ranunculi Ternati Capsules, Isoniazid and Rifampicin were used as reference drugs.

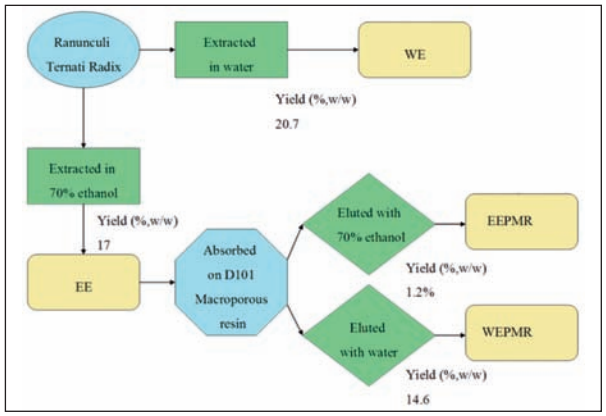

Fig. 1. 2 weeks after infected with H37Rv, mouse models were successfully established. (A) control group; (B) model group

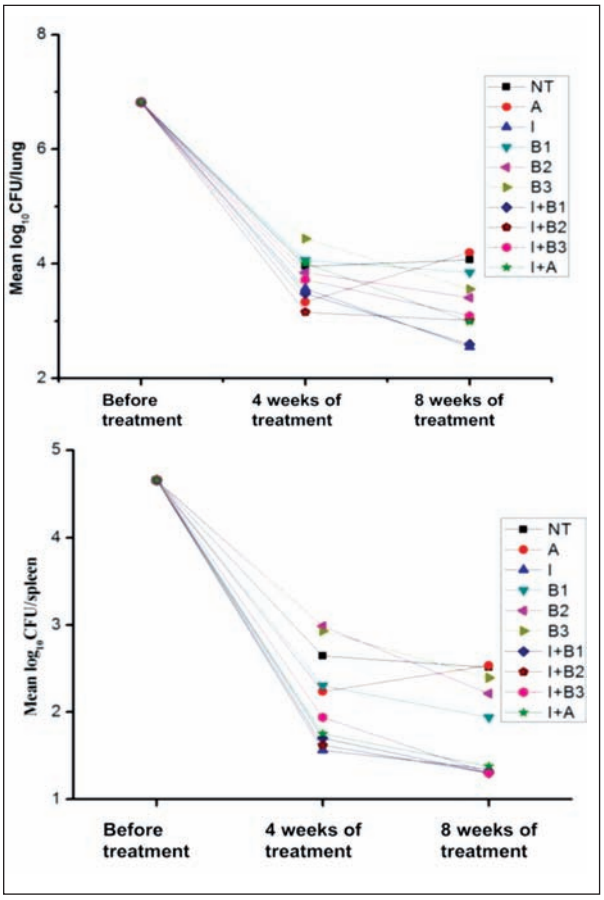

Fig. 2. C57BL/6 mouse models infected with H37Rv were used to evaluate the effects of the preparative extracts(EEPMR) using bacterial counts and organ index (lung and spleen). (A)Lung bacterial counts after 8 weeks of treatment. (B)Spleen bacterial counts after 4 weeks of treatment. (C) Spleen bacterial counts after 8 weeks of treatment. (D) lung index after 8 weeks of treatment(Mean±SD).
Abbreviations: NT, model group; A, Radix Ranunculi Ternati Capsules; I, Isoniazid; B1, high (400mg/kg/d) dose of EEPMR; B2, moderate (200mg/kg/d) dose of EEPMR; B3, low (100mg/kg/d)dose of EEPMR.
I+B1, I+B2, and I+B3, Combined therapy of EEPMR and Isoniazid of different doses; I+A, Combined therapy of Radix Ranunculi Ternati Capsules and Isoniazid.
Data are expressed reported as Mean log10CFU±SEM (A,B,C)and Mean±S.D.(D). *Significantly different from model group with the value of $p \leq 0.05$.

group, but not significantly. Fig. 2A shows the number of lung bacterial after 8 weeks of treatment. The results showed that the number of lung bacterial declined to a great extent in each treatment group except Radix Ranunculi Ternati Capsules group when compared to model group. B and C in Fig.2 are spleen bacterial counts after 4 weeks and 8 weeks of treatment. The results showed that EEPMR had a good effect on chronic tuberculosis treatment and appeared dose-dependent as a single drug. The efficacy of a combination with isoniazid showed no significant difference when compared with the use of isoniazid alone. After 4 weeks of treatment, compared with model group, lung index in treatment group all declined except group B1 and B3, but not significantly. After 8 weeks of treatment, lung index in each treatment group decreased compared with the model group, but significant difference showed only between the combined therapy treatment group and model group. (Fig.2D)

Also, Fourth weekend and eighth weekend after administration, no significant pathological changes were visible to naked eyes in important organs except lung with gross anatomical observation of visible pathological changes. Pathological changes in treatment groups of high EEPMR doses were better than that of the medium and low doses. The lung pathology of the high dose EEPMR treatment groups relatively reduced when compared to the model group and A group, similar to the INH group. (Fig.3 and Fig.4)

Besides, after 4 weeks of treatment, both the number of lung bacterial and spleen bacterial decreased in treatment groups while rebound rise of bacterial counts appeared in minority groups after 8 weeks of treatment. The antibacterial speed was faster and more stable in both EEPMR and the combined therapy (Supplementary Fig.S2.).

Based on the above results, a preliminary study was carried out to get some information of the phy-

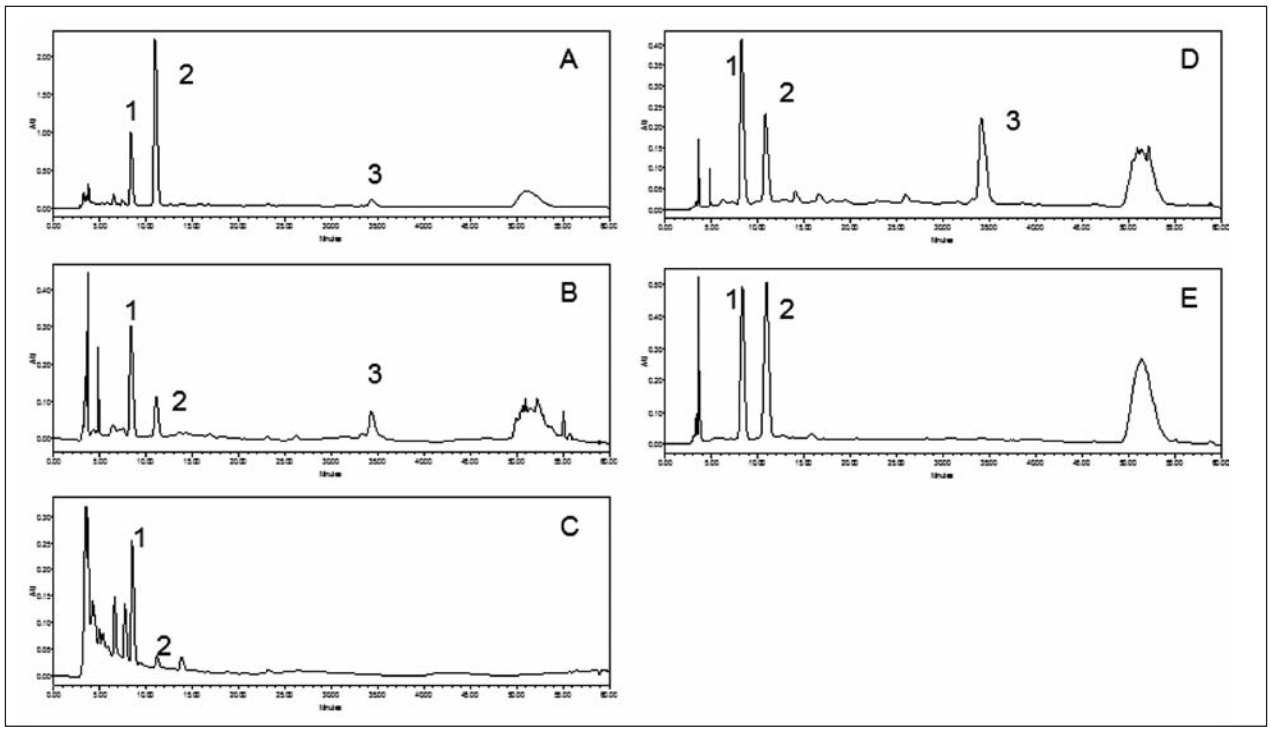

Fig. 3. The lung pathology of NT(A),A(B),I(C),B1(D),B2(E),B3(F),I+B1(G),I+B2(H),I+B3(I),I+A(J) after 4 weeks treatment.
Abbreviations: NT,model group; A, Radix Ranunculi Ternati Capsules; I, Isoniazid; B1, high (400mg/kg/d) doses of EEPMR; B2, moderate (200mg/kg/d) doses of EEPMR; B3, small (100mg/kg/d)doses of EEPMR
I+B1,I+B2, and I+B3, combined therapy of different doses of EEPMR and Isoniazid; I+A, combined therapy of Radix Ranunculi Ternati Capsules and Isoniazid

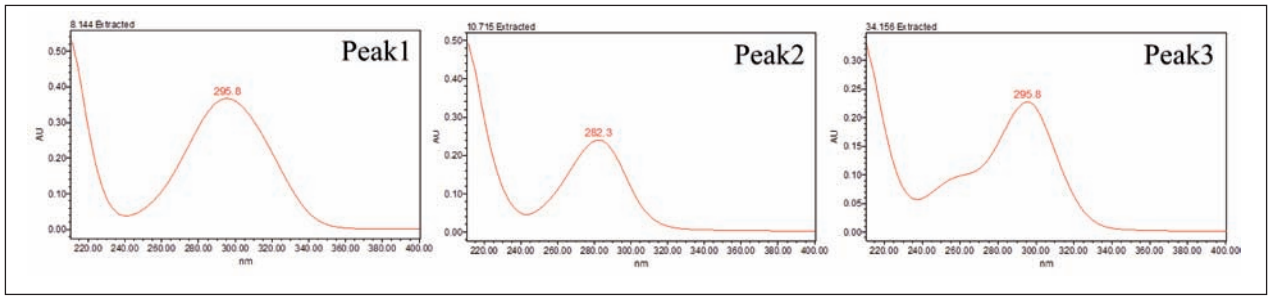

Fig. 4. The lung pathology of NT(A),A(B),I(C),B1(D),B2(E),B3(F),I+B1(G),I+B2(H),I+B3(I),I+A(J) after 8 weeks treatment.
Abbreviations: NT, model group; A, Radix Ranunculi Ternati Capsules; I, Isoniazid; B1, high (400mg/kg/d) doses of EEPMR; B2, moderate (200mg/kg/d) doses of EEPMR; B3, small (100mg/kg/d)doses of EEPMR
I+B1, I+B2, and I+B3, combined therapy of different doses of EEPMR and Isoniazid; I+A, combined therapy of Radix Ranunculi Ternati Capsules and Isoniazid

tochemical compositions (Fig.S3, Fig.S4) of the extracts and Radix Ranunculi Ternati Capsules by HPLC-MS. Compound 2 and 3 were identified as furfural and 4-[formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoic acid respectively. Further study need to carry out to get a full understanding of the compounds in these extracts and fractions.

Conclusions

In vitro, our results show that crude extracts and WEPMR have low antimycobacterial activities against H37Rv while EEPMR demonstrated relatively high activity, which indicated that EEPMR was active part of *Ranunculi Ternati Radix* and the anti-tuberculosis effect was better than Radix Ranunculi Ternati Capsules. The result of antibacterial experiment against MDR-TB (2314-2) and XDR-TB (1220) showed that EEPMR had stronger inhibitory effect on MDR2314-2 and XDR1220 and the MIC of MDR-TB (2314-2) and XDR-TB (1220) were smaller than that of H37Rv. Besides, its antimycobacterial activity was better than that of the Radix Ranunculi Ternati Capsules. Thus, D101 macroporous resin is a desirable material to accumulate active compounds in *Ranunculi Ternati Radix*.

Moreover, in vivo experiment, both medium and high dose of EEPMR alone had therapeutic effect on chronic tuberculosis in mice and the effect was better than Radix Ranunculi Ternati Capsules. Combined therapy of medium or high dose of EEPMR and Isoniazid could sterilize mycobacterium tuberculosis in mice lung and spleen, which had anti-inflammatory (lung index decreased) effects. No significant side effects were found in vital organs of tuberculosis mouse when using EEPMR alone or the combined therapy. The effect dose to treat tuberculosis mouse was from B2 to B1 group, which was 200mg/kg/d to 400mg/kg/d.

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