

Evaluation of the effect of *Pulicaria gnaphalodes* and *Perovskia abrotanoides* essential oil extracts against *Mycobacterium tuberculosis* strains

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Abstract

Background: *Mycobacterium tuberculosis* (MTB) is the causative agent of tuberculosis (TB), which remains one of the major public health problems in the world. The increasing incidence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) worldwide highlights the urgent need to search for alternative antimycobacterial agents. More and more people in developing countries utilize traditional medicine for their major primary health care needs. It has been determined that the medicinal plants *Pulicaria gnaphalodes* and *Perovskia abrotanoides* possess strong antibacterial effect.

Materials and Methods: In this study, the antimycobacterial effects of *P. gnaphalodes* and *P. abrotanoides* essential oil on MTB were examined. Essential oil was prepared from *P. gnaphalodes* aerial parts and *P. abrotanoides* flower. The effects of six different concentrations (20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml, and 640 µg/ml) were examined against sensitive isolates of MTB and MTB H37Rv (ATCC 27294).

Results: The results showed that *P. gnaphalodes* and *P. abrotanoides* essential oil extracts have strong inhibitory effects on MTB. This activity for *P. gnaphalodes* was observed from very low (4%) to good (70.9%) effect; meanwhile, this activity for *P. abrotanoides* was observed from very low (4%) to strong (86%) effect.

Conclusion: The mean of inhibition percentage for *P. gnaphalodes* and *P. abrotanoides* in 640 µg/ml was 58.1% and 76.2%, respectively. So, *P. abrotanoides* plant is more effective against MTB than *P. gnaphalodes*. Identification of the effective fraction against MTB is a further step to be studied.

Key Words: Antimycobacterial effect, essential oil, *Perovskia abrotanoides*, *Pulicaria gnaphalodes*

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INTRODUCTION

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is one of the top killer diseases in the world. In 2011, 8.7 million people were overtaken with TB, including 1.1 million cases among people with HIV. It was estimated that 1.4 million people died from TB in this year.^[1] Today, the emergence of resistance to antimycobacterial

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agents has become an important public health issue in many developing countries. The increasing incidence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant TB (XDR-TB) worldwide highlights the urgent need to search for newer antituberculosis compounds.^[2] More and more people in developing countries utilize traditional medicine for their major primary health care needs.^[3] Medicinal plants offer a great hope to fulfill these needs and have been used for curing diseases for many centuries.^[4] The scientific experiments that have been carried out on antimicrobial properties of plant components were first documented in the late nineteenth century.^[5] Plants have been a source of effective chemotherapeutic agents for various infectious diseases, and there is a growing interest in the development of drugs of plant origin. A number of plants have shown significant *in vitro* antimycobacterial activities. These findings have therefore stimulated further research toward the isolation of new antimycobacterial agents from natural products.^[6]

The *Pulicaria* genus belongs to the family *Compositae* (*Asteraceae*), tribe *Inuleae*, which contains more than 77 species that are widely distributed across Asia, Europe, and Africa. *P. gnaphalodes* is effective on *B. anthracis*, *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *Sh. sonnei*, *S. paratyphi A*, and *V. cholerae*.^[7] This species completely inhibited lettuce seed germination, thus it has allelopathic potential.^[8] *Perovskia* is a genus that belongs to the *Lamiaceae* family, which is allied to *Salvia*, *Nepeta*, and *Rosmarinus*. *Perovskia* is represented in the Flora Iranica by only three species. *Perovskia abrotanoides* is an aromatic erect herb used in Iranian folk medicine as an analgesic for rheumatic pains. Previous investigations of this plant resulted in the isolation of bioactive tanshinones with leishmanicidal, antiplasmodial, and cytotoxic activities.^[9] There are also other reports of the isolation and identification of two triterpenes with a novel carbon skeleton from *P. abrotanoides*.^[10] Essential oil from the flowers of *P. abrotanoides* showed *in vitro* antibacterial activity on *B. cereus*, *S. aureus*, *K. pneumoniae*, and *E. coli*.^[11] This plant is often used as a fortifier, as an antiseptic, and for rheumatic pains, and is known to be antiinflammatory, leishmanicidal, and anthelmintic.^[9] However, what has not yet been reported is the antimycobacterial effect of *P. gnaphalodes* and *P. abrotanoides* plants essential oils toward *MTB* that has been recognized as the major organism involved in deaths worldwide. In this study, the effect of the essential oils of these plants on *MTB* isolates and *MTB* H37Rv (ATCC 27294) were evaluated.

MATERIALS AND METHODS

Plant material

P. gnaphalodes and *P. abrotanoides* were collected from Khorasan province on May 2012 and identified by botanist Dr. Rahiminejad, and the voucher specimen has been deposited in the herbarium of the School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.

Preparation of essential oils

The plant's aerial part including leaves, stem, and flower were air-dried for 3 days in the shade at temperatures 23°C-27°C, then powdered mechanically using a blender; 550 gm of plant aerial part powder was mixed with water; 1:10 plant material/water volume ratio was maintained, and the mixture was distilled for hours. Essential oils were obtained from the plant samples using a Clevenger-type apparatus where the plant powder is subjected to hydro-distillation. The oils were collected in a screwed tube glass and stored in at 4°C. The essential oils of these plants were prepared for polar and semipolar compound extraction.

MTB strains

Fifty isolates of *MTB* species were collected from tuberculosis centers of Isfahan. Isolates subcultured on Löwenstein-Jensen (LJ) medium and incubated for 21 days at 37°C, and characterized by conventional methods including staining, colony characteristics, pigmentation, growth temperature, and time of growth.^[12]

Susceptibility testing of MTB

The susceptibility of isolates to isonicotinyldiazine (INH) (0.2 µg/ml), rifampin (RIF) (40 µg/ml), streptomycin (STR) (4 µg/ml), and ethambutol (EMB) (2 µg/ml) was tested using the proportion method.^[13,14] Briefly, the isolates were subcultured on LJ medium and incubated for 21 days. A standard suspension of 10⁷ colony-forming units (CFU)/ml (equivalent to a 1 McFarland standard) of *MTB* isolates was prepared by dissolving of 3-5 colonies of *MTB* in distilled water.^[15] Subsequently, LJ media without and with subjected antibiotics were prepared and inoculated with 0.2 ml of 10⁻⁴ dilutions of *MTB* strains. The inoculated plates were then incubated for 42 days at 37°C (1st reading after 28 days, 2nd after 42 days), and the percentage of inhibition was determined.^[16] Resistance was defined as growth on drug-containing tubes greater than 1% of the growth of drug free control medium for INH, RIF, EMB, and 10% for STR.^[17,18] *MTB* H37Rv (ATCC 27294) was set as the control in all culturing and sensitivity testing processes.

Determination of antimycobacterial activity

The antimycobacterial effects of six concentrations (20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml, and 640 µg/ml) of *P. gnaphalodes* and *P. abrotanoides* essential oil on *MTB* isolates and *MTB* H37Rv (ATCC 27294) were determined according to the standard procedures.^[14] LJ medium containing and free of subjected plants were prepared and inoculated with 0.2 ml of 10⁻⁴ dilutions of a 1 McFarland standard of each isolate. RIF and INH were used as the positive control and water as the negative control. The percentage of inhibition was determined after 3-6 weeks of inoculation by the following formula:^[19,20]

$$\frac{\text{Number of colony on control medium} - \text{Number of colony on medium with extract}}{\text{Number of colony on control medium}} \times 100 = \text{percentage of inhibition}$$

The *P* value was used to quantify the idea of statistical significance of evidence.

RESULTS AND DISCUSSION

All isolates of were confirmed as *MTB* species by conventional biochemical and phenotypic methods (staining, colonial morphology, pigmentation, biochemical profiles, and growth rate). Drug susceptibility testing was done using the proportion method with INH (0.2 µg/ml), RIF (40 µg/ml), STR (4 µg/ml), and EMB (2 µg/ml), and two sensitive isolates that were susceptible to all drugs and *MTB* H37Rv (ATCC 27294) were selected for the study. The antimycobacterial effect of *P. gnaphalodes* and *P. abrotanoides* against sensitive isolates of *MTB* were determined using the proportion method. The results showed that the essential oils of the plants had a strong inhibitory effect against *MTB* strains [Tables 1 and 2, Figure 1]. The maximum

percentage inhibition for these plants (*P. gnaphalodes* and *P. abrotanoides*) was related to the highest concentration. So the essential oils of these plants were more effective in the 640 µg/ml concentration.

The prevalence of MDR strains of *M. tuberculosis* is an important reason for the resurgence of TB as a major disease in many parts of the world. There is, therefore, an urgent need for new, inexpensive TB drugs that are effective and have fewer side effects.^[21] In many countries, medicinal plants are used by traditional medical practitioners to combat TB. Medicinal plants are an important natural source of novel leads in the field of antimycobacterial therapeutics.^[22,23] Various biological activities have been reported for some species of *Pulicaria*, such as antibacterial, antifungal, and insecticidal properties.^[24] Khani *et al.* showed that the essential oil of this plant has good insecticidal activity against *Callosobruchus maculatus*.^[25] Mahboubi reported that *P. abrotanoides* flower oil shows antifungal activity

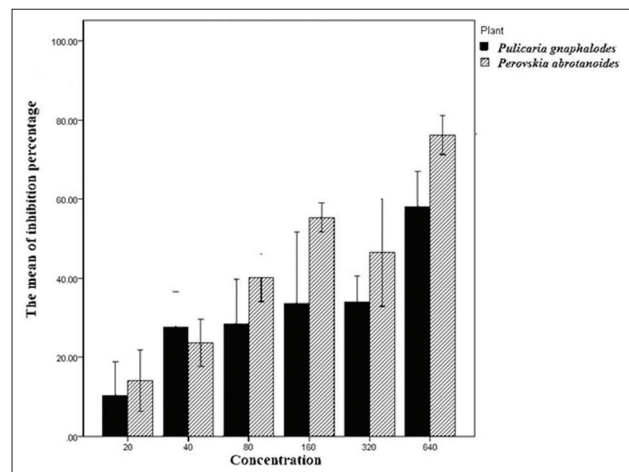


Figure 1: The mean of *M. tuberculosis* inhibition percentage according to the kind of plant and concentration

Table 1: Efficiency of essential oil extracted from *P. gnaphalodes* against *M. tuberculosis* strains

Strain of <i>MTB</i>	Percentage of inhibition in different concentration (%)					
	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	320 µg/ml	640 µg/ml
Sensitive isolates (No. 1)	0	17.6	9.4	5.8	23.5	41.1
Sensitive isolates (No. 2)	4	20	28	28	32	62.4
<i>M. tuberculosis</i> H37Rv (ATCC 27294)	27.2	45.4	48.1	67.2	46.3	70.9
Mean of inhibition	10.4±8.4	27.6±8.8	28.5±11.1	33.6±17.9	33.9±6.6	58.1±8.8

Table 2: Efficiency of essential oil extracted from *P. abrotanoides* against *M. tuberculosis* strains

Strain of <i>MTB</i>	Percentage of inhibition in different concentration (%)					
	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	320 µg/ml	640 µg/ml
Sensitive isolates (No. 1)	29.4	31.7	47	51.7	54.1	70
Sensitive isolates (No. 2)	4	12	28	ND	20	86
<i>M. tuberculosis</i> H37Rv (ATCC 27294)	9	27.2	45.4	59	65.4	72.7
Mean of inhibition	14.1±7.7	23.6±5.9	40.1±6	55.3±3.6	46.5±13.6	76.2±4.9

against *Aspergillus flavus*, *Candida albicans*, and *Trichophyton mentagrophytes*.^[26]

In this study, the antimycobacterial activity of the *P. gnaphalodes* and *P. abrotanoides* essential oils in concentrations of 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml, and 640 µg/ml on sensitive isolates of *MTB* was determined by the proportion method according to standard procedures.^[13,27] These plants showed a noticeable inhibitory activity against *MTB* strains [Tables 1 and 2]. This activity for *P. gnaphalodes* was observed from very low (4%) to good (70.9%) effect. Meanwhile, this activity for *P. abrotanoides* was observed from very low (4%) to strong (86%) effect. These plants showed similar activity on *MTB* H37Rv (ATCC 27294) strain of *MTB* in 640 µg/ml (70.9% for *P. gnaphalodes* plant and 72.7% for *P. abrotanoides* plant). Maximum percentage inhibition for these plants was related to 640 µg/ml concentration, and for *P. gnaphalodes* and *P. abrotanoides* was 70.9% and 86%, respectively. So, the essential oils of these plants were more effective in the 640 µg/ml concentration. The mean of inhibition percentage for *P. gnaphalodes* and *P. abrotanoides* in 640 µg/ml was observed to be 58.1% and 76.2%, respectively. So the *P. abrotanoides* plant is more effective on *MTB* than *P. gnaphalodes*. Renu Gupta et al. evaluated the antituberculosis activity of *Allium sativum* and *Adhatoda vasica* by the proportion method. *Allium sativum* and *Adhatoda vasica* showed 63% and 70% inhibition on the *MTB* H37Rv (ATCC 27294) strain, respectively. They showed that *P. gnaphalodes* and *P. abrotanoides* in comparison with *Adhatoda vasica* have similar activity on *MTB*; meanwhile they showed stronger antituberculosis activity than *Allium sativum*.^[16] The identification of the potent constituents of these essential oils against *MTB* is a problem for further study.

CONCLUSION

The two-way analysis of variance (ANOVA) showed that *P. gnaphalodes* and *P. abrotanoides* ($P < 0.04$) in different concentrations ($P < 0.001$) are effective against *M. tuberculosis*. The mean of inhibition percentage of the *P. gnaphalodes* plant is more than that of the *P. abrotanoides* plant.

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