ISSN 2304-3415, Russian Open Medical Journal
2025. Volume 14. Issue 4 (December). Article CID e0420
DOI: 10.15275/rusomj.2025.0420

Original article

Efficacy of *Gratiola officinalis* L. (Scrophulariaceae) Extract Against Multidrug-Resistant *Mycobacterium tuberculosis* in Vitro

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Received 23 June 2025, Revised 20 August 2025, Accepted 6 September 2025

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Abstract: This study *aimed* to evaluate the efficacy of a dry extract solution of *Gratiola officinalis L.* against reference and clinical strains of *M. tuberculosis*, including multidrug-resistant (MDR) strains.
Material and Methods — The extract of *Gratiola officinalis* L. was prepared using the authors' original technique. Its effects were assessed *in vitro* against a reference strain of *M. tuberculosis* that was clinically sensitive, a clinical MDR *M. tuberculosis* strain (with nucleotide substitutions in codon 531 of the *rpoB* gene [rifampicin resistance] and codon 315 of the *katG* gene [isoniazid resistance]), and additional sensitive and reference strains.
Results — Minimum inhibitory concentrations (MICs) of the *Gratiola officinalis* L. extract were determined. At 14 mg/mL, the extract exerted a complete bactericidal effect against the *M. tuberculosis* reference strain. At 26.6 mg/mL, growth of the clinically sensitive *M. tuberculosis* strain (sensitive to isoniazid, rifampicin, ethambutol, and streptomycin) was fully suppressed. At 53.1 mg/mL, growth of the MDR *M. tuberculosis* strain (resistant to rifampicin, isoniazid, streptomycin, and ethambutol) was fully suppressed.
Conclusion — The *Gratiola officinalis* L. extract demonstrated high bacteriostatic and bactericidal activity against *Mycobacterium tuberculosis*. This activity was evident against a reference strain (assessed via colony growth dynamics) and, using the serial dilution method, against a sensitive clinical strain and an MDR strain resistant to isoniazid and rifampicin.

Keywords: *Gratiola officinalis* extract, drug resistance, *Mycobacterium tuberculosis.*

Cite as Navolokin NA, Bucharskaya AB, Pankratova LE, Manaenkova EV, Polukonova NN, Maslyakova GN. Efficacy of Gratiola officinalis L. (Scrophulariaceae) Extract against Multidrug-Resistant Mycobacterium tuberculosis in Vitro. *Russian Open Medical Journal* 2025; 14: e0420.

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Introduction

On November 7, 2023, the World Health Organization (WHO) released its annual *Global Tuberculosis Report* for 2023. The report noted that 7.5 million people were diagnosed with tuberculosis in 2022 – the highest number recorded since WHO began global monitoring in 1995. In terms of mortality from a single infectious agent, tuberculosis ranked second in 2022, behind only coronavirus disease 2019 (COVID-19). Tuberculosis mortality was nearly twice that of HIV/AIDS. WHO experts estimate that more than 10 million people develop tuberculosis annually. Multidrug-resistant tuberculosis poses a major threat to public health. Halting the global spread of tuberculosis is a key target in the WHO Sustainable Development Goals through 2030 and the End TB Strategy through 2035. Specifically, tuberculosis mortality should decrease by 90% and incidence by 80% by 2030. Achieving tuberculosis elimination in the next 15 years requires intensified efforts in prevention, detection, and treatment, according to the report [1].

WHO experts further note that the growing global tuberculosis epidemic stems largely from the spread of drug-resistant forms of the disease, particularly multidrug-resistant cases, which are challenging to treat with the limited available anti-tuberculosis drugs.

Treating patients with drug-resistant tuberculosis – especially multidrug resistance to the primary drugs isoniazid and rifampicin – demands more expensive, prolonged regimens involving drugs that cause severe adverse effects and have broad contraindications [2, 3].

Multidrug-resistant (*MDR-TB*) and extensively drug-resistant (*XDR-TB*) *Mycobacterium tuberculosis* remain critical challenges. Standard regimens based on isoniazid and rifampicin achieve only 60% efficacy, underscoring the need for new therapeutic agents [4].

Contemporary research, including the End TB project, shows that combinations of novel drugs can improve survival even in advanced cases [4, 5]. However, global coordination and investment in innovative anti-tuberculosis drugs are essential. This situation highlights the need for new, nontoxic antimicrobial agents with antituberculous activity and minimal mutagenic effects [6]. Medicinal plant extracts represent a promising source for such agents. Among plant-derived compounds, bioflavonoids –

a class of phenolic substances – exhibit notable antimicrobial properties [7].

For this study, we selected leaves and flowers of *Gratiola officinalis* L. as the plant material. Various extraction methods from *Gratiola officinalis* yield biologically active compositions with diverse effects, including laxative, emetic, antispasmodic, diuretic, and digitalis-like cardiac actions [8], as well as anticarcinogenic [9], antioxidant [10], antitumor, and immunomodulatory properties [11-13]. We previously demonstrated the antimicrobial activity of *Gratiola officinalis* extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14]. These findings suggested potential antituberculous activity. To our knowledge, prior literature reports no antibacterial activity of *Gratiola officinalis* L. extracts against *Mycobacterium tuberculosis*.

Objective: To evaluate the efficacy of an aqueous solution of dry *Gratiola officinalis* L. extract against reference *Mycobacterium tuberculosis* strains, including MDR strains with nucleotide substitutions in codon 531 of the *rpoB* gene (rifampicin resistance) and codon 315 of the *katG* gene (isoniazid resistance).

Material and Methods

Characterization of Gratiola officinalis L. Extract

Gratiola officinalis L., a herbaceous plant in the Scrophulariaceae family, is widespread in Eurasia and North America but is highly toxic. The quality of *Gratiola officinalis* L. raw material is regulated by pharmacopoeial article 42-2358-85. We collected flowers and leaves of *Gratiola officinalis* L. in the Saratov region, Russia, for extraction. The extract was produced using our original method [9, 10], which enhances flavonoid yield while removing alkaloid fractions to yield nontoxic products. The chemical composition was analyzed by high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) [10, 11]. The resulting extract contained the following: 4-vinyl-2-methoxyphenol; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; 2,3-dihydrobenzofuran; 3-furancarboxylic acid; 5-hydroxymethyl-2-furaldehyde; ethyl-α-riboside; 4-propylphenol; pyrocatechol; L-rhamnose (a pentose); 6-deoxyhexose L-galactose; benzoylacetic acid ethyl ester; hexadecanoic acid (palmitic acid); homovanillic acid; glucose; 1,4-anhydro-D-mannitol; benzoic acid; and quercetin. In a recent study, the antitumor effects of *Gratiola officinalis* extract on A498 renal carcinoma cells were attributed to flavonoids such as 7-O-glucoside apigenin, 7,3'-di-O-luteolin glycoside, trace eupatilin, and 3-(1-2)-glucoside diosgenin B [11].

Evaluation of Growth Dynamics of the Reference Strain H37Rv Under Gratiola officinalis L. Extract at 14 mg/mL

Observations spanned 28 days. Two *Mycobacterium tuberculosis* (*MBT*) titers (50×10^7 and 500×10^7 microbial bodies per 1 mL) were inoculated onto nutrient medium. Each group had five replicates: group 1 (control, no extract exposure to the reference *MBT* strain); group 2 (exposed to *Gratiola officinalis* L. extract). Growth was scored as follows: 0 – no *MBT* growth; 1+ (1-20 colony-forming units [*CFU*]) – sparse *MBT* growth; 2+ (21-100 *CFU*) – moderate *MBT* growth; 3+ (>100 *CFU*) – abundant *MBT* growth. We modified the standard method by using serial dilutions instead of absolute concentrations to identify extract thresholds beyond which *MBT* growth was undetectable.

Evaluation of Growth Dynamics of MBT Clinical Strains Under Gratiola officinalis L. Extract

Mycobacterium tuberculosis strains were isolated from sputum samples of patients treated at the Tambov Regional Clinical Tuberculosis Dispensary. These strains underwent testing via standard methods for evaluating drug resistance on Löwenstein–Jensen egg-based medium (HiMedia, India), employing absolute concentrations and polymerase chain reaction [15]. The absolute concentration method assessed resistance levels to first-line anti-tuberculosis drugs (rifampicin, isoniazid, ethambutol, and streptomycin). Strains were deemed resistant to the extract if ≥20 *M. tuberculosis* colonies grew and sensitive if ≤20 colonies.

The first *M. tuberculosis* strain was sensitive to these drugs (designated sensitive strain). The second clinical strain was resistant to isoniazid, rifampicin, ethambutol, and streptomycin (designated MDR strain).

PCR Method for Detecting Mutations Conferring Resistance in Mycobacterium tuberculosis

A molecular genetic study evaluated resistance to rifampicin and isoniazid in clinical strains using polymerase chain reaction (PCR) followed by hybridization of *M. tuberculosis* genetic material on TB-BIOCHIP biochips (Biochip-IMB LLC, Russia), per manufacturer recommendations. The method included isolating *M. tuberculosis* DNA from respiratory samples (sputum), conducting two consecutive multiplex PCRs to amplify DNA copies, and hybridizing amplification products from the second-stage PCR on a biochip. Hybridization results were recorded on a portable biochip analyzer (LLC BIOCHIP-IMB, Russia) with dedicated software.

To determine minimum inhibitory and bactericidal concentrations, researchers established four culture lines: the first line was a positive control (a series of two tubes with each strain individually without exposure), and the second line was a series of two tubes without inoculation with strains to exclude accidental growth of MBT (negative control). Serial dilutions of the extract determined the threshold of extract concentrations beyond which MBT growth was not observed. The third and fourth lines were experimental (cultures with two clinical strains with different degrees of drug sensitivity) and contained eight tubes with different dilutions of the extract (from 1.7 to 212.5 mg/mL).

The minimum inhibitory concentration of *Gratiola officinalis* L. extract was determined using the serial dilution method on dense Löwenstein–Jensen starch-free egg medium (HiMedia, India) [15]. An aqueous solution of dry alcohol extract was added to the medium during preparation at 20-25 °C, evenly distributing it throughout. *Mycobacterium tuberculosis* (MBT) was inoculated with a standard titer (cell content corresponding to 1×10^7 microbial bodies/mL). Colony growth of the tuberculosis pathogen was monitored for 28 days.

Statistical Analysis

Statistical calculations were performed using Microsoft Office Excel software. Normality of distribution in the variables was tested using the Shapiro-Wilk test. If the data deviated significantly from a normal distribution and the null hypothesis was rejected, the median, minimum, maximum, 25th, and 75th percentiles were calculated. Researchers used the non-parametric Kruskal-Wallis test for independent samples to determine statistically significant

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differences between the groups. Differences were considered significant when the *p*-value was <0.05.

The authors did not use artificial intelligence or AI-based tools in preparing this article.

Results

Study of the Growth Dynamics of the Reference Strain Mycobacterium tuberculosis H37Rv in the Presence of Gratiola officinalis L. Extract

After processing the results of all replications, there was a complete absence of colony growth of the *M. tuberculosis* reference strain on a medium containing *Gratiola officinalis* L. extract for 28 days at an *M. tuberculosis* titer of 50×10^7 (bactericidal effect). In contrast, colony growth was observed from day 10 in the control groups (*Table 1*).

At a titer of *M. tuberculosis* 500×10^7, single colonies of the *M. tuberculosis* reference strain on a medium with *Gratiola officinalis* L. extract appeared only on the 10th day of observation. In contrast, moderate colony growth was observed in the control from day 7, with abundant colony growth by day 10. Moreover, the absence of colony growth of the *M. tuberculosis* reference strain on a medium with *Gratiola officinalis* L. extract occurred in all repetitions at a titer of *M. tuberculosis* 50×10^7. This absence also occurred in the first two repetitions at a titer of *M. tuberculosis* 500×10^7 from day 1 to 28. Compared with the control, this indicates a pronounced bactericidal effect of the extract. The appearance of weak growth at a titer of *M. tuberculosis* 500×10^7 from day 7 and the preservation of weak growth (less than 20 CFU) until day 28 in the presence of the extract, compared with the control, indicates a bacteriostatic effect of *Gratiola officinalis* L. extract on the reference strain *M. tuberculosis*.

Investigation of Drug Resistance in the Obtained Clinical Strain of M. tuberculosis by PCR, Followed by Hybridization of the Genetic Material

Assessment of drug resistance by this method revealed no mutations leading to resistance to the above-mentioned drugs in the genetic material of the first clinical (sensitive) *M. tuberculosis* strain. In contrast, the corresponding mutations were found in the *M. tuberculosis* strain with multidrug resistance: nucleotide substitutions in codon 531 of the *rpoB* gene (resistance to rifampicin) and in codon 315 of the *katG* gene (resistance to isoniazid), leading to amino acid substitutions (*Table 2*).

Evaluation of M. tuberculosis Sensitivity to the Extract

Per *M. tuberculosis* sensitivity criteria, a sensitive clinical strain (without multidrug resistance) showed a pronounced bacteriostatic effect (2 CFU) at 13.3 mg/mL of *Gratiola officinalis* L. extract and a bactericidal effect at 26.6 mg/mL (*Table 3*).

In the multidrug-resistant *M. tuberculosis* strain, a bacteriostatic effect (18 CFU) was observed at a concentration of 26.6 mg/mL of *Gratiola officinalis* L. extract; complete suppression of culture growth (bactericidal effect) was achieved at 53 mg/mL (*Table 3*).

In summary, *Gratiola officinalis* L. extract at 26.6 mg/mL exerts a bactericidal effect on clinical strains sensitive to anti-tuberculosis drugs and a bacteriostatic effect on those with multidrug resistance. The bactericidal effect on multidrug-resistant strains occurs at 53.1 mg/mL of *Gratiola officinalis* L. extract solution in the medium.

Table 1. Growth Dynamics of the *Mycobacterium tuberculosis* Reference Strain After Exposure to *Gratiola officinalis* L. Extract

Observation Days	*7 Days*		*10 Days*		*17 Days*		*28 Days*	
Titer of Mycobacterium tuberculosis	50×10^7	500×10^7	50×10^7	500×10^7	50×10^7	500×10^7	50×10^7	500×10^7
Control	0 (0-0) [0-0]	0 (0-2) [0-2]	2 (1-2) [1-2]	3 (2-3) [2-3]	3 (2-3) [2-3]	3 (3-3) [3-3]	3 (3-3) [3-3]	3 (3-3) [3-3]
Gratiola officinalis L. extract	0 (0-0) [0-0]	0 (0-0) [0-0]	0 (0-0) [0-0]	0 (0-1) [0-1]	0 (0-0) [0-0]	0 (0-1) [0-1]	0 (0-0) [0-0]	0.5 (0-1) [0-1]
p*	p=1	p=0.13	p=0.005	p=0.007	p=0.005	p=0.005	p=0.005	p=0.007

Data presented as median, quartiles, minimum and maximum – Me (LQ-UQ) [Min-Max]. 0 – no *Mycobacterium tuberculosis* (MBT) growth; 1 – poor MBT growth (1-20 CFU); 2 – moderate MBT growth (21-100 CFU); 3 – abundant MBT growth (>100 CFU). *The significance of differences between groups was determined using the Kruskal-Wallis test for independent samples.

Table 2. Results of PCR Analysis of Clinical Strains of *Mycobacterium tuberculosis*

Tested Mycobacterium tuberculosis Cultures	*Resistance to Rifampicin*			*Resistance to Isoniazid*		
	rpoB Gene Codon	Amino Acid Substitution		Gene	Codon	Amino Acid Substitution
Sensitive Culture	*MBT* DNA was found (fragment IS6110). The *rpoB* gene does not contain mutations responsible for rifampicin resistance. The *katG*, *inhA*, and *ahpC* genes do not contain mutations responsible for isoniazid resistance.					
MBT with MDR	531	Ser→Leu		*katG*	315	Ser→Thr(1)

MBT, *Mycobacterium tuberculosis*; MDR, multidrug-resistant. PCR, polymerase chain reaction.

Table 3. Results of the Analysis of the Anti-Tuberculosis Activity of *Gratiola officinalis* L. Extract on Clinical Strains

Indicators	*CFU**						*Result***					
Extract Concentration in the Medium (mg/mL)	212.5	106.3	53.1	26.6	13.3	6.6	212.5	106.3	53.1	26.6	13.3	6.6
Sensitive strain	-	-	-	-	2	30	S	S	S	S	S	R
Strain with MDR	-	-	-	18	35	80	S	S	S	S/R	R	R

CFU, colony-forming unit; *MBT, Mycobacterium tuberculosis*; MDR, multidrug-resistant. * The CFU number is indicated if it can be calculated; with a large number of CFUs that cannot be calculated, an approximate percentage (%) of the surface of the nutrient medium with *MBT* growth is indicated. ** Result: S, strain is sensitive; R, strain is resistant. In the control (without exposure to the extract), the proportion of the surface of the nutrient medium with *MBT* growth was 95%.

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Discussion

The search for new drugs and optimization of existing regimens remains a priority in combating drug-resistant tuberculosis.

Bedaquiline and delamanid, new-generation drugs recently introduced into clinical practice, demonstrated 85% efficacy in international studies (including patients with HIV and comorbidities) [4]. However, their availability is limited, especially in low-income countries [5]. Known cases of resistance to these drugs also exist. *M. tuberculosis* employs unique survival mechanisms, such as hypermutability in the intracellular environment, which activates resistance genes (e.g., the *mutT* locus) and accelerates dominance of resistant strains through mutations [16]. Persistence in the form of L-forms confers resistance to standard therapy [17,18]. These factors complicate treatment and necessitate development of drugs targeting new sites, such as DNA gyrase inhibitors (fluoroquinolones) or antimutagenic agents [19].

Reserve drugs (kanamycin, cycloserine) offer limited use due to high toxicity. For instance, nephrotoxicity and neuropathy occur in 10-15% of patients, and allergic reactions affect 46.4%, often leading to treatment interruption and further resistance [20]. Several medicinal compositions based on plant extracts exist, including those from rosemary flowers, psyllium leaves, thyme, and nettle, used to treat patients with infiltrative pulmonary tuberculosis [21]. Plant extracts purified through multi-stage methods to remove ballast compounds enhance yield of specific compounds with known effects. We established the efficacy of *Gratiola officinalis* L. extract against *M. tuberculosis* strains, both sensitive and multidrug-resistant. This extract appears to have a distinct antibacterial mechanism, differing from first-line anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, and streptomycin), warranting further study. Our results support investigating the effect of flavonoid-containing *Gratiola officinalis* L. extract on experimental models of tuberculosis. They also suggest exploring its clinical potential in developing new delivery methods for tuberculosis patients. Additional research is needed on the molecular mechanism of *Gratiola officinalis* L. extract action.

Conclusion

The prepared *Gratiola officinalis* L. extract demonstrated pronounced anti-tuberculosis activity *in vitro* against all studied *M. tuberculosis* strains: the reference strain, a clinically sensitive strain, and a multidrug-resistant clinical strain. *In vitro* experiments revealed high antituberculous activity (bacteriostatic and bactericidal) for the *Gratiola officinalis* L. extract. This effect occurred in the reference strain, assessed via colony growth dynamics, and in clinical strains—a sensitive one and a multidrug-resistant one (resistant to isoniazid and rifampicin)—evaluated using the serial dilution method. We determined the minimum inhibitory concentration (MIC) of *Gratiola officinalis* L. extract for these strains. At 14 mg/mL, a complete bactericidal effect occurred against the *M. tuberculosis* reference strain; at 26.6 mg/mL, growth of the clinically sensitive *M. tuberculosis* strain (to isoniazid, rifampicin, ethambutol, and streptomycin) was completely suppressed; and at 53.1 mg/mL, growth of the multidrug-resistant *M. tuberculosis* strain (resistant to rifampicin, isoniazid, streptomycin, and ethambutol) was completely suppressed.

Limitations

This study did not include animal experiments, and the extract's activity against *M. tuberculosis* strains with extensive drug resistance remains unexamined.

Funding

The research was funded by Public Procurement of the Ministry of Health of the Russian Federation No. 1024030100147-3.

Conflict of Interest

None declared.

Ethical Approval

All procedures performed in studies involving human participants complied with the ethical standards of the institutional or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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