

Combined effect of anti-mycobacterial plant extracts and their inhibitory effect on RNA polymerase and DNA gyrase gene of *Mycobacterium tuberculosis*

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Abstract

Background

Tuberculosis continues to be a serious health problem, and drug-resistant tuberculosis is an increasing threat that directly impacts the tuberculosis control program. These can be prevented or controlled by combining several drugs with antituberculous activity. In the present study, an attempt is made to understand the combinatorial effect of two medicinal plant extracts i.e. *Piper longum* and *Cressa cretica* against *Mycobacterium tuberculosis*.

Methodology

2D checkerboard assay was used to study the combinatorial effect of the crude extracts. The $2^{-\Delta\Delta Ct}$ method was used to determine the changes in gene expression of selected genes *rpoB*, *gyrA* and *gyrB* involved in transcription and DNA replication and intrinsic resistance of *Mycobacterium tuberculosis* to front-line drugs.

Results

Determination of the effect on gene expression at the subminimum inhibitory concentration of the crude extracts. The required MIC concentration, 125 µg/ml, was inversely reduced up to 7 µg/ml for *P. longum*. Similarly, the required MIC concentration of 250µg/mL of *C. cretica* is reduced to 10µg/mL.

Conclusions

The relative change in gene expression for *rpoB* and *gyrA* was 2 and 4-fold, respectively, and 1-fold for *gyrB*. Further characterization of the downstream target genes and in vivo studies may provide the mode of action of these two anti-mycobacterial agents.

Background

Tuberculosis (TB) was declared a global emergency in 1993 by the World Health Organisation[1]. TB is considered a severe public health problem and is a leading cause of death globally. An estimated 10.0 million people were infected and 1.3 million TB deaths occurred in 2019.[2] In the last decade, the global incidence rate of tuberculosis has grown at approximately 0.4%/year.[3]. In such a scenario, multidrug-resistant tuberculosis (MDR-TB) offers a big challenge to TB control programs. Globally approximately 3.8% of all new tuberculosis cases are MDR-TB. Among these MDR-TB cases, 20% of patients have been previously treated for tuberculosis or have a TB history. MDR-TB is at critical levels in Eastern Europe and Central Asia.[4] Although in recent years, several new TB cases have shown a decline, not at a pace to attain the end TB strategy milestone [2]. The anti-TB drug therapy uses streptomycin, isoniazid, rifampicin and ethambutol which constitute a potent drug combination for infections against susceptible strains of *M. tuberculosis*. However, there is growing resistance to all first-line and second-line drugs.[5] Drug resistance mechanisms involved in resistance to all five first-line anti-mycobacterial drugs-

isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB), and streptomycin (STM) have been elucidated. This resistance is due to a series of chromosomal mutations.[6] Despite identifying resistance causes, intrinsic/natural drug resistance mechanisms include cell barrier permeability, antibiotic-inactivating enzymes, and non-specific efflux pump.[7]. Furthermore, treatment expense, patient compliance, and the necessity to eliminate persistent infection underscore the need to develop new anti-mycobacterial agents to combat MDR-TB. Moreover, the essential anti TB drugs such as RIF, INH and PZA are known to cause liver damage besides neuropathy, hypersensitivity, nephrotoxicity, nausea, vomiting, gastritis and hepatotoxicity[8]. Many herbal extracts have been known to have hepatoprotective characteristics[9]. Being a natural product, it is believed that these may possess a lesser probability of developing resistance and hepatotoxicity, thus playing a crucial role in effective treatment[8]. Many factors drive the requirement of multiple drug combinations in treating *M. tuberculosis* but are not restricted to recalcitrance. *M. tuberculosis* has a unique cellular structure and metabolic functions that enable the emergence of resistance post-exposure to drugs and/or attack by the host immune system.[9]. To counter this, it is necessitate to develop combinatorial therapies, preferably using natural products.

Piper Longum (family – Piperaceae), locally called Peepali, is a medicinal plant that is effective in treating respiratory infections and is traditionally used by Ayurveda practitioners as a remedy for TB-like symptoms. The extract of *P. longum* has been shown to increase circulating antibody titers and antibody-forming cells, indicating its stimulatory effect on the humoral immune system.[10] The immunomodulatory activity of *P. longum* and piperine could combine the results of humoral and cell-mediated immune responses.[11]

Similarly, *Cressa cretica* (family – Convolvulaceae), locally called Rudravanti, another medicinal plant implicated in having anti-tubercular and anti-bilious properties. According to Ayurvedists, *C. cretica* is widely accepted as an anti-tubercular herb used to treat respiratory infections due to its anti-bronchodilatory, antitussive, antibacterial, antipyretic and analgesic properties. *C. cretica* inhibits the growth of *M. tuberculosis* through both bacteriostatic and bactericidal activity.[12]

RNA polymerase beta subunit (*rpoB*) and DNA gyrase subunits; *gyrA* and *gyrB*, are the essential genes that can significantly upregulate several important metabolic functions in *M. tuberculosis*. [13, 14] RNA polymerase catalyzes transcription. The *rpoB* gene alters the structure and integrity of the cell wall by modifying the *M. tuberculosis* response during transcription.[15] DNA gyrase catalyzes the unwinding of DNA strands essential for efficient DNA replication, transcription, and recombination, while topoisomerase IV has a specialized role in chromosome segregation. Subunit A carries the active site for re-joining, while subunit B promotes ATP hydrolysis, which is required for energy transduction. *gyrA* and *gyrB* encode subunits A and B, respectively. DNA gyrase is thought to be the only topoisomerase target for quinolones in *M. tuberculosis*. [14] This study describes the combinatorial effect of plant extract against *M. tuberculosis* growth and inhibition of the *rpoB*, *gyrA* and *gyrB* genes.

Materials and methods

Plant Collection and Extraction

Taxonomically verified plants were collected from the herbal garden of Jawaharlal Nehru Agricultural University, Jabalpur district of Madhya Pradesh, India. The methanolic extract was prepared using soxhlet apparatus.[16]

Qualitative phytochemical screening

Preliminary phytochemical screening for sterols, alkaloids, phenols, tannins, glycosides, protein, carbohydrate, flavonoids, steroids, anthraquinones and terpenoids was carried out using standard protocol followed by [17] to identify the phyto-constituents present in *P. longum* and *C. cretica*.

Bacterial strain and growth conditions

This study was conducted at tuberculosis containment laboratory (BSL-III facility) in the division of communicable diseases of ICMR-National Institute of Research in Tribal Health, Jabalpur, India using standard strain of *M. tuberculosis*-H37Rv (ATCC 25618). *M. tuberculosis* was cultured in Middlebrook 7H9 broth supplemented with oleic acid, albumin dextrose catalase (OADC supplement) (Himedia, India) and 1 percent glycerol at 37°C as inoculum as reported earlier[18].

Determination of minimum inhibitory concentrations (MICs)

Colorimetric Redox Indicator Assay (Resazurin dye assay) was performed [19] to determine the MICs of *P. longum* and *C. cretica* against *M. tuberculosis*. Laboratory strain H37Rv was used as a positive control and inoculum was prepared using 1:20 dilution of 1 MacFarland H37Rv. Two-fold serial dilutions ranging from 1mg/mL to 0.0625 mg/mL of *C. cretica* and *P. longum* were used directly in the wells. All tests were conducted twice with three replicates of each.

2D Checkerboard assays

Standard “two-dimensional” (2D) drug-drug interactions were followed in a checkerboard titration in a 96-well plate (Fig. 1A). The 2D microdilution was carried out as described by[20]. Briefly, the first serially diluted extract (100 µL) was distributed along the x-axis (columns 2–9) at an initial concentration 100 times the final well concentration and the second serially diluted 100 µL extract solution was distributed along the y-axis (rows B–G) with an initial concentration 100-fold higher than the final concentration in a 96-well microtiter plate. 100µL of 1:20 dilution of 1 MacFarland H37Rv was added as inoculum. The last two columns (10 and 11) contained drug-free positive and negative controls, respectively. The first row B2–B9 and column B2–G2 contained the extracts separately, giving the MIC value of each extract separately in each assay. The plates were sealed with parafilm and incubated at 37°C for seven days. After the incubation, 30µL of 0.02% resazurin dye was added to each well and the plate sealed again and further incubated for 24 hours for color development. A change in color from blue to pink indicates a growth of *M. tuberculosis* at that extract concentration and blue represents the absence of growth. The color of the test was compared to the color intensity level with controls for growth estimation.

Fractional inhibitory concentration index (FICI)

The combinatorial effect of *P. longum* and *C. Cretica* was calculated as below [21]

$$FICI = \frac{\text{MIC of P. longum in combination}}{\text{MIC of P. longum alone}} + \frac{\text{MIC of C. Cretica in combination}}{\text{MIC of C. Cretica alone}}$$

For FICI of value_0.5, the effect of the combination was considered to be synergistic, whereas for values in the range of 0.5–4, the effect was supposed to be additive and for values > 4, the result was considered to be antagonistic[22]

Expression studies

RNA extraction and reverse transcription

H37Rv cultured in Middlebrook 7H9 broth with OADC (Oleic Albumin Dextrose Catalase) supplement, and for stress, *P. longum* and *C. cretica* were added to these cultures individually at sub-inhibitory concentrations (half of the MIC), incubated at 37°C upto 30 days, and fractions of samples for RNA extraction were collected at defined time intervals (i.e., 0, 6, 12, 24, 72 hours, 7, 15 and 30 days). Total bacterial RNA was isolated using the Trizol extraction method (Invitrogen) according to Simões AE, et al [23]. The RNA samples were DNase treated and cleaned up on RNeasy Mini-Columns (Qiagen, USA). The quality and integrity of the total RNA were ascertained spectrophotometrically. RNA from treated and untreated samples (1.5µg) was reverse transcribed by using sensiscript reverse transcriptase (Qiagen USA) according to the manufacturer’s recommendations and the thermal cycling conditions were as follows: 70°C for 5 min, 37°C for 90 min. Two cDNA were prepared for each strain and cDNA was stored at -20°C.

Quantification of gene expression using real-time quantitative PCR (qPCR)

Specific primers of targeted genes and housekeeping genes of *M. tuberculosis* were designed and synthesized by Eurofins Genomics, were used (Table.1). The assay was performed using a Luna Universal qPCR Master Mix (New England Bio Labs Company) in QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). Briefly, 0.2-mL reaction mixture tube contained 10µL 2× qPCR mix, 1µL each primer, 1µL cDNA, and RNase-free water to a final volume of 20µL. Thermal cycling conditions were as follows: 95°C for 1 minute and 95°C for 15 seconds, then 45 denaturation cycles at 95°C for 15 seconds, extension and plate reading at 60°C for the 30s and the last step consisted of a melting curve analysis (65–95°C).

Table.1: Primers used to amplify and sequence rpoB, gyrB, gyrA, 16sRNA and ITS

<i>Gene</i>	<i>Primer</i>	<i>Sequence (5'→3')</i>	<i>Amplicon size (bp)</i>
<i>rpoB</i>	<i>F</i>	ACACCGCAGACGTTGATCA	369
	<i>R</i>	TCGTCCGGCGGTCAGGTAC	
<i>gyrB</i>	<i>F</i>	GGCGCCGAAGTCGGCAAGT	157
	<i>R</i>	CCGGAAAAGTCCACGATGCTGCTA	
<i>gyrA</i>	<i>F</i>	CGTCGTTGTCACTATCACCG	207
	<i>R</i>	GATCAAATCGTGGGTGGAGC	
<i>16sRNA</i>	<i>F</i>	CCTTCGGGTTGTAAACCTCTTTC	132
	<i>R</i>	GGACAACGCTCGCACC	
<i>ITS</i>	<i>F</i>	TGCGGCTGGATCACCTCCTT	101
	<i>R</i>	CCGGCTCTCGCCCACTACAG	

F, forward primer; R, reverse primer

Gene Expression in *M. tuberculosis*

Real-time PCR was performed using SYBR green chemistry in triplicate for each gene and a mean expression level of each gene was obtained. 16 sRNA and internal transcribed spacer (ITS) sequences were used as housekeeping gene /endogenous control and data were normalized with the same. Relative mRNA expression and fold-change were calculated using the $2^{-\Delta\Delta C_t}$ method. The changes in expression levels of the genes with and without extract treatment were compared.[24]

Statistical analysis

All experiments were performed in triplicates. For Statistical analysis significance effect determination two-way ANOVA, two treatment groups were compared with the one control group. Further, significance between the control and treated group was quantified using student's-test with equal variance, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, ns- not significant.

Results

In the present study, an attempt was made to determine the effect of combinations of *P. longum* and *C. cretica* on the growth of *M. tuberculosis*. In addition, an attempt was made to determine their effect on the beta subunit of RNA polymerase and DNA gyrase gene activity.

Phytochemical Analysis

Qualitative phytochemical screening of *P. longum* and *C. cretica* revealed the presence of various bioactive compounds, i.e. sterols, alkaloids, phenols, tannins, glycosides, proteins, carbohydrates,

flavonoids, anthraquinones, and terpenoids, in *P. longum* and *C. cretica*. (Table- 2)

Table 2
Results of the phytochemical tests done of the three selected medicinal plant extracts

Sr.	Name of metabolites	<i>Piper longum</i>	<i>Cressa cretica</i>
1	Phenols	-	+
2	Terpenoids	+	-
3	Sterols	+	+
4	Tannins	-	+
5	Flavonoids	-	+
6	Alkaloids	+	+
8	Carbohydrates	+	+
9	Glycosides	-	+
10	Protein	+	+
11	Anthraquinone	-	-
12	Phytosteroids	-	-
The symbol “+” means “present” while the symbol “-” means “absent”			

Quantitative Determination of the MIC of *P. longum* and *C. cretica*

The MIC was determined as the lowest drug concentration that prevents color change of the dye. Isolates with MICs of 0.125 mg/mL (125 µg/mL) and 0.25 mg/mL (250 µg/mL) were defined as active concentrations for *P. longum* and *C. cretica*, respectively [18]

The combinatory effect of *P. longum* and *C. cretica* against *M. tuberculosis*.

To further quantitate the interaction between *P. longum* and *C. cretica*, an adaptation of an isobolograph [25] was plotted (Fig. 1B). The line of connecting 125 µg/mL *P. longum* and 250µg/mL *C. cretica* denotes the line of additivity. The interactional treatment resulting in growth inhibition was obtained under the connecting line of additivity with these concentrations of *P. longum* and *C. cretica*. The interconnection between the two is considered as a synergistic effect. 18 fold reduction in the MIC of *P. longum* was observed in combination with a sub-inhibitory concentration of 250µg/mL of *C. cretica* and 25 fold reduction was observed in MIC of *C. cretica* in combination with a sub-inhibitory concentration of 125 µg/mL of *P. longum*.

A FICI value of < 0.5 indicates a combinatorial effect between *P. longum* and *C. cretica* to be synergistic. [22] In the presence of a sub-inhibitory concentration of 125µg/mL of *C. cretica*, the required

concentration of *P. longum* was reduced to 0.007mg/mL (7µg/mL). Similarly required concentration of *C. cretica* was decreased to 0.01 mg/mL (10µg/mL) in the presence of a sub-inhibitory concentration of 0.06mg/mL (60µg/mL) of *P. longum*. FICI calculation value of 0.096 and isobolographic analysis confirmed the interaction between the two to be synergistic.

Inhibitory effect of *P. longum* and *C. cretica* in the Gene Expression level on RNA polymerase beta subunit and DNA gyrase gene of *Mycobacterium tuberculosis*

Expression of *rpoB* and gyrase genes was performed in two experimental groups with and without plant extracts. In the treated group, inhibition of *rpoB*, *gyrA*, and *gyrB* genes was observed, as shown in Fig. 2 (A), (B), and (C). These plant extracts showed a decreased expression that began in the early exponential phase and persisted until the late stationary phase. The observed effect on gene expression is shown in Fig. 2. In *P. longum*, the inhibition of *rpoB* starts after 48 hours of treatment up to 15 days, and the maximum effect was observed after 72 hours, whereas *C. cretica* inhibited *rpoB* after 72 hours of treatment up to 15 days. In *P. longum*, maximal inhibition of DNA gyrase expression was detected after 72 hours for *gyrA* and after 48 hours for the *gyrB* gene. In *C. cretica*, effective inhibition was detected after 48 hours for both *gyrA* and *gyrB*. According to the results, the relative change in gene expression was 2- and 4-fold for *rpoB* and *gyrA*, respectively, and 1-fold for *gyrB*.

Results show *P. longum* and *C. cretica* extract have the ability to inhibit the expression of the DNA-dependent RNA polymerase beta subunit (*rpoB*) and DNA gyrase subunits (*gyrA* and *gyrB*) gene of *M. tuberculosis*. While the growth of *M. tuberculosis* was affected, the extracts have the ability to inhibit the growth of organisms at the concentrations tested.

Discussion

Currently, tuberculosis treatment is faced with ever-increasing drug resistance in strains of *M. tuberculosis*. [4] Naturally derived anti-mycobacterial compounds have been identified to play an important role and are effective against MDR and XDR tuberculosis. However, their bioactive compounds have not been characterized for their effect on MDR-TB. [26] Alkaloids and phenolic compounds have numerous identified therapeutic uses [27]. The current efforts are focused on scanning for novel and effective compounds against tuberculosis. Phenols are structurally and chemically diverse compounds, showing new targets for inhibiting *M. tuberculosis*. [28] Another bioactive compound, piperine, a rich and natural source from *piper longum*, inhibits the efflux pumps. However, the mechanisms of action of piperine are yet to be identified, particularly against tuberculosis [29]. Phytochemical results from this study found various bioactive compounds (i.e., phenols, terpenoids, sterols, tannins, flavonoids, glycosides and alkaloids) in *P. longum* and *C. cretica*. Among these compounds, alkaloids, terpenes, resin, monoterpenoids, sesquiterpenoids and phenols have been shown to possess anti-mycobacterial activity. Both *C. cretica* and *P. longum* have anti-mycobacterial potential. Several tribal populations [10, 12] use these plants as anti-tubercular agents. Evidence to their inhibition needs to be improved in the case of *M. tuberculosis*. In the present studies, MIC was determined as the lowest drug concentration that prevented

a color change in the resazurin dye. Isolates with MICs of 125µg/mL and 250µg/mL were defined as active concentrations to *P. longum* and *C. cretica*, respectively.[18] A typical growth curve of *M. tuberculosis*-H37Rv shows three growth phases; mid-log (14-day culture), early-stationary (28-day culture) and late-stationary (50-day culture)[30]. Reduction on the expression of *rpoB*, *gyrA* and *gyrB* gene was observed in *M. tuberculosis* when cultured with plant extracts in each growth phase. Most antibiotics work on bacteria in its potential log phase[31] and it was observed that the inhibitory effect of *P. longum* and *C. cretica* extract also occurred in the log phase (7–15 days post-treatment). Several medicinal plants have been shown to exhibit anti-mycobacterial properties; *Hibiscus sabdariffa*, *Kaempferia galanga* and *Piper crocatum*, plant extracts have good synergistic effects with rifampicin to combat MDR strains.[32] Here, we have demonstrated the inhibitory potential of *P. longum* and *C. cretica* against *M. tuberculosis* and their effect on reduction of DNA gyrase activities. This study revealed that *C. cretica* lowered the MIC of *P. longum* for inhibition of growth and vice versa. With checkerboard assay, an 18-fold reduction in the MIC of *P. longum* was observed in combination with a sub-inhibitory concentration of 250µg/mL *C. cretica*. The reverse showed about a 25-fold decrease in the MIC of *C. cretica* in combination with a sub-inhibitory concentration of 125µg/mL *P. longum*. Moreover, the FICI calculations and isobolographic analysis confirmed the association between the two compounds to have a combinatorial effect. Overexpression of genes that encode proteins that regulate the efflux pumps is the primary mechanism by which bacteria develop resistance against antibacterial drugs. Inhibition of efflux pumps reduced the emergence of MDR phenotype in *M. tuberculosis*[20], [33]. Figure 2 shows the relative expression of each gene at the defined time intervals. These genes are responsible for the high tolerance of *M. tuberculosis* to available anti-mycobacterial drugs [34]. These plant extracts inhibited the expression of the *rpoB*, *gyrA* and *gyrB* genes of *M. tuberculosis* in different stages of growth. Its short use of *P. longum* and *C. cretica* looks promising for developing plant-based anti-tubercular molecules to combat tuberculosis, particularly MDR-TB.

Conclusions

In the present situation of increasing DRTB cases, there is an urgent need to review the treatment strategy and prognosis of tuberculosis. *P. longum* and *C. cretica* are promising and can be designed as anti-mycobacterial agents that are selectively toxic to *M. tuberculosis* and not to the host cell. Further study is needed to identify the specific active phytochemicals of these extracts and evaluate their potential against *M. tuberculosis*. In vivo studies are also required for further understanding of the effect of plant extract against *M. tuberculosis* in the preclinical setup and their mechanism of action.

Declarations

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Research Fellowship. The manuscript has been approved by the Publication Screening Committee of ICMR-NIRTH, Jabalpur, and assigned the number ICMR-NIRTH/PSC/04/2023.

Authors' contributions

VT, JB and SR conceived the design of the study. VT and NP, carried out the experiments. VT, JB and RS were involved in drafting and revision of the manuscript. All the authors have read and agreed to the manuscript.

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Availability of data and materials

All generated and analysed data during the current study are available within the article.

Ethics approval and consent to participate

The Institutional Ethical Committee (IEC) of ICMR–NIRTH, Jabalpur approved the study with a reference number NIRTH/IEC/539/2018 dated 5/06/2018 and consent from participate is not required because there is no direct patient samples were used. Taxonomically verified plants were collected from Jawaharlal Nehru Agricultural University, Jabalpur, Madhya Pradesh, India, in July-August 2019, with approval from the Faculty of Botanical Garden, Department of Plant Physiology, Jawaharlal Nehru Agricultural University, Jabalpur, following institutional guidelines and regulations. The nomenclature of the selected plants was compared with the TROPICOS database.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

References

1. Najima, H., *The World Health Organisation report*. 1996, World Health Organisation: Geneva, Switzerland. <https://apps.who.int/iris/handle/10665/178481>
2. Global tuberculosis report 2020. Geneva: World Health Organization; 2020 <https://apps.who.int/iris/bitstream/handle/10665/336069/9789240013131-eng.pdf>,
3. Espinal, M.A., *The global situation of MDR-TB*. Tuberculosis (Edinb), 2003. **83**(1-3): p. 44-51. [https://doi.org/10.1016/s1472-9792\(02\)00058-6](https://doi.org/10.1016/s1472-9792(02)00058-6)

4. Seung, K.J., S. Keshavjee, and M.L. Rich, *Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis*. Cold Spring Harb Perspect Med, 2015. **5**(9): p. a017863.
<https://doi.org/10.1101/cshperspect.a017863>
5. Umar, F., et al., *The effect of anti-tuberculosis drugs therapy on mRNA efflux pump gene expression of*. New Microbes New Infect, 2019. **32**: p. 100609. <https://doi.org/10.1016/j.nmni.2019.100609>
6. Leonard, B., et al., *Inter- and intra-assay reproducibility of microplate Alamar blue assay results for isoniazid, rifampicin, ethambutol, streptomycin, ciprofloxacin, and capreomycin drug susceptibility testing of Mycobacterium tuberculosis*. J Clin Microbiol, 2008. **46**(10): p. 3526-9.
<https://doi.org/10.1128/JCM.02083-07>
7. Silva, P.E., et al., *Characterization of P55, a multidrug efflux pump in Mycobacterium bovis and Mycobacterium tuberculosis*. Antimicrob Agents Chemother, 2001. **45**(3): p. 800-4.
<https://doi.org/10.1128/AAC.45.3.800-804.2001>
8. Jiménez-Arellanes, M.A., et al., *Medical plant extracts and natural compounds with a hepatoprotective effect against damage caused by antitubercular drugs: A review*. Asian Pac J Trop Med, 2016. **9**(12): p. 1141-1149. <https://doi.org/10.1016/j.apjtm.2016.10.010>
9. Kerantzas, C.A. and W.R. Jacobs, *Origins of Combination Therapy for Tuberculosis: Lessons for Future Antimicrobial Development and Application*. mBio, 2017. **8**(2).
<https://doi.org/10.1128/mBio.01586-16>
10. Sunila, E.S. and G. Kuttan, *Immunomodulatory and antitumor activity of Piper longum Linn. and piperine*. J Ethnopharmacol, 2004. **90**(2-3): p. 339-46. <https://doi.org/10.1016/j.jep.2003.10.016>
11. Barua, C., et al., *In vitro Antioxidant and Antimycobacterial Activity of Seeds of Piper longum Linn: A comparative Study*. Scholarena Journal of Pharmacy and Pharmacology, 2014. **1**.
<https://doi.org/10.18875/2375-2262.1.101>
12. Chaudhary Sudhir, K.R.L., Priyank, Rani Sangeeta, *A report on Pharmacognostical and quality control parameters of stem and root of Cressa cretica Linn, Convolvulaceae*. 2012, Journal of Pharmacy Research.
13. Miotto, P., et al., *Role of Disputed Mutations in the*. J Clin Microbiol, 2018. **56**(5).
<https://doi.org/10.1128/JCM.01599-17>
14. Aubry, A., et al., *Mycobacterium tuberculosis DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity*. Antimicrob Agents Chemother, 2004. **48**(4): p. 1281-8.
<https://doi.org/10.1128/AAC.48.4.1281-1288.2004>
15. Hu, Y., et al., *Mycobacterium tuberculosis RbpA protein is a new type of transcriptional activator that stabilizes the σ A-containing RNA polymerase holoenzyme*. Nucleic Acids Res, 2012. **40**(14): p. 6547-57. <https://doi.org/10.1093/nar/gks346>
16. Redfern, J., et al., *Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties*. J Microbiol Biol Educ, 2014. **15**(1): p. 45-6.
<https://doi.org/10.1128/jmbe.v15i1.656>

17. Ali, S., et al., *Phytochemical investigation and antimicrobial appraisal of Parrotiopsis jacquemontiana (Decne) Rehder*. BMC Complement Altern Med, 2018. **18**(1): p. 43.
<https://doi.org/10.1186/s12906-018-2114-z>
18. Tamrakar, V., et al., *In vitro evaluation of anti-mycobacterial active plant extracts against Mycobacterium tuberculosis using mycobacterium growth indicator tubes (MGIT-960) BACTEC system*. 2022, Research Square. <https://doi.org/10.21203/rs.3.rs-1609878/v1>
19. Singh, S., et al., *Rapid Identification and Drug Susceptibility Testing of Mycobacterium tuberculosis: Standard Operating Procedure for Non-Commercial Assays: Part 3: Colorimetric Redox Indicator Assay v1.3.12*. J Lab Physicians, 2012. **4**(2): p. 120-6. <https://doi.org/10.4103/0974-2727.105594>
20. Chen, P., et al., *Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro*. J Antimicrob Chemother, 2006. **58**(2): p. 332-7.
<https://doi.org/10.1093/jac/dkl227>
21. Botelho, M.G., *Fractional inhibitory concentration index of combinations of antibacterial agents against cariogenic organisms*. J Dent, 2000. **28**(8): p. 565-70. [https://doi.org/10.1016/s0300-5712\(00\)00039-7](https://doi.org/10.1016/s0300-5712(00)00039-7)
22. Odds, F.C., *Synergy, antagonism, and what the chequerboard puts between them*. J Antimicrob Chemother, 2003. **52**(1): p. 1. <https://doi.org/10.1093/jac/dkg301>
23. Simões, A.E., et al., *Efficient recovery of proteins from multiple source samples after TRIzol(®) or TRIzol(®)LS RNA extraction and long-term storage*. BMC Genomics, 2013. **14**: p. 181.
<https://doi.org/10.1186/1471-2164-14-181>
24. Li, G., et al., *Efflux pump gene expression in multidrug-resistant Mycobacterium tuberculosis clinical isolates*. PLoS One, 2015. **10**(2): p. e0119013. <https://doi.org/10.1371/journal.pone.0119013>
25. Gessner, P.K., *Isobolographic analysis of interactions: an update on applications and utility*. Toxicology, 1995. **105**(2-3): p. 161-79. [https://doi.org/10.1016/0300-483x\(95\)03210-7](https://doi.org/10.1016/0300-483x(95)03210-7)
26. Gupta, R., et al., *Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant Mycobacterium tuberculosis isolates*. Indian J Med Res, 2010. **131**: p. 809-13.
27. Mishra, S.K., et al., *Drug development against tuberculosis: Impact of alkaloids*. Eur J Med Chem, 2017. **137**: p. 504-544. <https://doi.org/10.1016/j.ejmech.2017.06.005>
28. Mazlun, M.H., et al., *Phenolic Compounds as Promising Drug Candidates in Tuberculosis Therapy*. Molecules, 2019. **24**(13). <https://doi.org/10.3390/molecules24132449>
29. Hegeto, L.A., et al., *Promising Antituberculosis Activity of Piperine Combined with Antimicrobials: A Systematic Review*. Microb Drug Resist, 2019. **25**(1): p. 120-126.
<https://doi.org/10.1089/mdr.2018.0107>
30. Ang, K.C., P. Ibrahim, and L.H. Gam, *Analysis of differentially expressed proteins in late-stationary growth phase of Mycobacterium tuberculosis H37Rv*. Biotechnol Appl Biochem, 2014. **61**(2): p. 153-64. <https://doi.org/10.1002/bab.1137>
31. Preiss, L., et al., *Alkaliphilic Bacteria with Impact on Industrial Applications, Concepts of Early Life Forms, and Bioenergetics of ATP Synthesis*. Front Bioeng Biotechnol, 2015. **3**: p. 75.

32. Fauziyah, P.N., E.Y. Sukandar, and D.K. Ayuningtyas, *Combination Effect of Antituberculosis Drugs and Ethanolic Extract of Selected Medicinal Plants against Multi-Drug Resistant Mycobacterium tuberculosis Isolates*. Sci Pharm, 2017. **85**(1). <https://doi.org/10.3390/scipharm85010014>
33. Kardan-Yamchi, J., et al., *Expression analysis of 10 efflux pump genes in multidrug-resistant and extensively drug-resistant Mycobacterium tuberculosis clinical isolates*. J Glob Antimicrob Resist, 2019. **17**: p. 201-208. <https://doi.org/10.1016/j.jgar.2019.01.003>
34. Zhu, J.H., et al., *Rifampicin can induce antibiotic tolerance in mycobacteria via paradoxical changes in rpoB transcription*. Nat Commun, 2018. **9**(1): p. 4218. <https://doi.org/10.1038/s41467-018-06667-3>

Figures

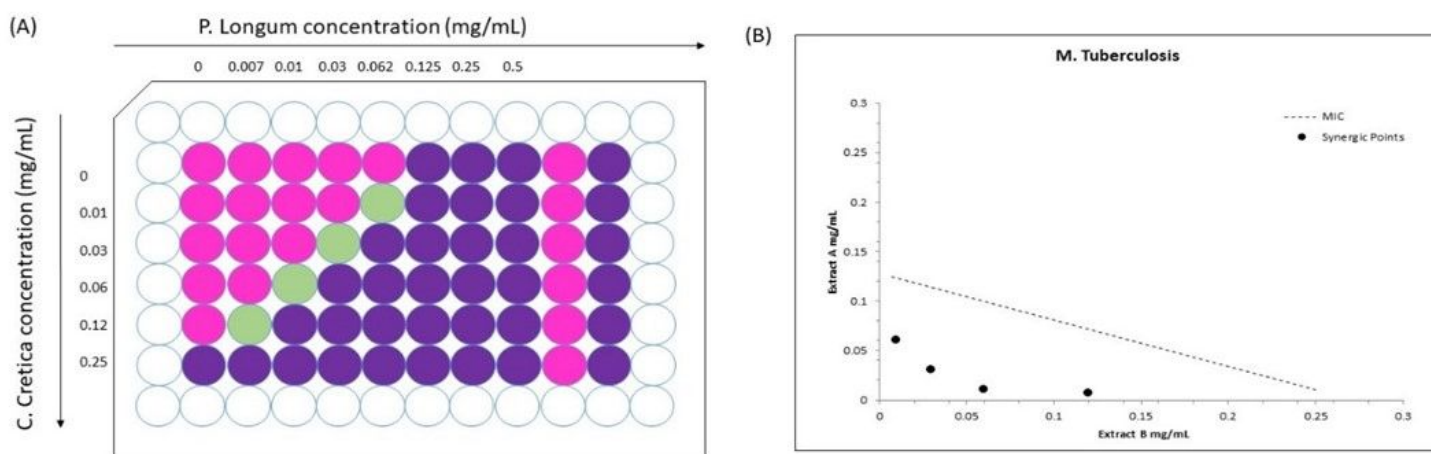


Figure 1

Combinatorial effect of *P. longum* and *C. cretica* on minimum inhibitory concentration (MIC) of *Mycobacterium tuberculosis*

(A) Representation of checkerboard assay where columns indicate *P. longum* concentration and rows indicate *C. cretica* concentration.

(B) Adaptation of Isobolographic analysis where the concentrations plotted here show growth inhibition.

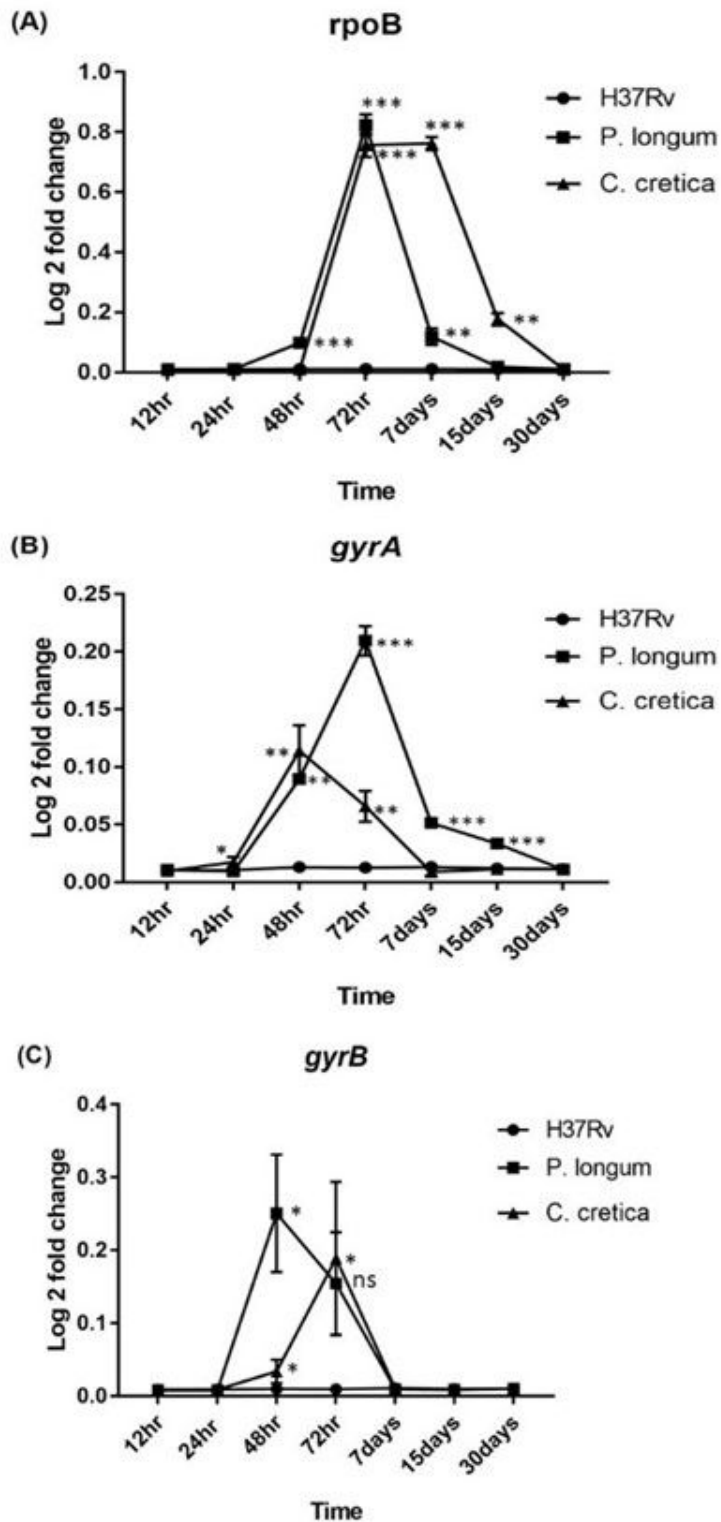


Figure 2

The relative expression level of *rpoB* (A), *gyrA* (B) and *gyrB* (C) after the treatment with stress conditions. The significance between the expression ratios from 12hr to 30 days has been plotted here. The expression of *rpoB*, *gyrA* and *gyrB* in the presence of *P. longum* and *C. cretica* were inhibited. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$