Ferulic Acid Inhibits Topoisomerase I of Mycobacterium Tuberculosis

Nihong Lu
The third people's hospital of kunming city
Haiyuan Li
The first Affiliated Hospital of Kunming Medical University
Jingsong Bai
The Third Affiliated Hospital of Kunming city
Yongrui Yang
The Third Affiliated Hospital of Kunming city
Yangbing Ou
The Third Affiliated Hospital of Kunming city
Yangjun Chen
The Third Affiliated Hospital of Kunming city
Yingrong Du (yingrongdu73@hotmail.com)
The Third People Hospital of Kunming City https://orcid.org/0000-0003-4782-929X

Research Article

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Abstract

Introduction

Various drugs are first line anti-TB drugs for more than thirty years. Since ferulic acid have shown promising inhibition growth of M. tuberculosis and M. smegmatis cell.

Objective

In this study, we have explored the inhibitory effect of ferulic acid against Mycobacterium tuberculosis topoisomerase I. We further investigated the mechanism of ferulic acid by various experiments.

Methods

DNA relaxation assays was done for supercoiled pUC18 DNA, oligonucleotide cleavage assay was performed for 5-end-labeled 32-mer harboring the STS (Strong Topoisomerase Site) annealed to a complimentary sequence. Growth inhibition (MIC) values were calculated by performing resazurin reduction microplate assay (REMA).

Results

In this study, we describe the growth inhibition of M. tuberculosis and M. smegmatis through Mycobacterium tuberculosis topoisomerase I (MttopoI) by ferulic acid. We have further investigated the mechanism of ferulic acid by analysing the two step of topoisomerase 1 (topoI) reaction cycle. Ferulic acid was able to stimulate cleavage which further leads to cleavage-relegation equilibrium. Moreover, it was shown that ferulic acid inhibited topoI overexpressing cells at low minimum inhibitory concentration (MIC). Ferulic acid affects the activity of DNA relexation in the mutations in DxDxE motif of metal binding mutants which contributes to the reaction of enzyme with the drug.

Conclusion

In conclusion, our results indicate that ferulic acid leads to combat microbial infection and act as toxins to MstopoI and MttopoI. Moreover, targeting metal coordination to topoisomerases might be a general strategy to develop new lead molecules.

Introduction

Tuberculosis (TB) is among deadly infectious diseases that claims 1.5 million lives per year worldwide [1]. It has been more than sixty years since the discovery of first line anti-TB drugs like pyrazinamide, rifampicin, ethambutol and isoniazid for the treatment of TB [2]. After decades, there are new drugs and regiments that lead to the emergence of delamanid, bedaquiline and clinical candidates like PA-824 and TBA-354 [3]. The current treatments for TB lead to the development of multidrug-resistance tuberclosis (MDR-TB) has further complictaed the treatment [4]. MDR-TB is caused by *Mycobacterium tuberculosis*

strains that is resistant against atleast rifampicin (RIF), isoniazid (INH) and atleast one of three like kanamycin, amikacin and capreomycin worldwide [5]. TB resistance is caused by enabling inadequate selection and growth of drug-resistant strain. Gatifloxacin and moxifloxacin are recently developed drugs against TB that shows the emergence of developing resistance [6]. Many drugs which are in market takes more time to treat TB and they are resistant to the new developing strains. All these problems show that there is an urgent need for the development of molecules that interfere with protein reaction and inhibit its function. In processes like transcription, replication and segregation of chromosome that are involved in DNA transaction, homeostasis of topology is maintained by topoisomerases in cells [7]. On the basis of structure and mechanism topoisimerases has been classified as type I and type I topoisomerases [8]. Type 1 topoisomerases are the enzymes that relax the DNA by generating a single-strand nick in one of the two strands of DNA, relexes and reanneled the strand [9]. Various antitumor drugs were made that specifically inhibits type 1 topoisomerases and delaying relegation DNA step that leads to DNA lesions [10]. Investigations on the single strand breaks through chemical compounds were established to inhibit the fuction of type 1 topoisimerase which leads to the cytotoxicity. While as, double strand break of DNA is generated by type II topoisomerases by the DNA cleavage gate removes the DNA tangles and supercoils [11]. There are various drugs which resealed double strand DNA breaks caused by the inhibition of type II topoisomerase [12]. Type I topoisomerases does not require covalent intermediates like metal divalent for the cleavage of DNA strand but it is required for Type II topoisomerases which form a divalent matel ions [13]. Metal divalent is not used for the cleavage of DNA but its is necessary for the religation of DNA in bacterial topoisomerase I [14]. Therefore, there is an opportunity for selective inhibition of DNA religation by ferulic acid. Previously none of the determined structures have shown DNA and divalent metal ions present in the active site. So there is need to study the role of metal ions in the catalytic site of type I topoisomerase by structural information. Natural products plays a vital role in the drug discovery program [15]. Ferulic acid is an organic compound found abondantly in the plant cell wall which has antioxidant, chemopreventive activity [16]. In this study, we reported that ferulic acid catalyzed DNA relaxation which inhibits MstopoI and MttopoI but not *Escherichia coli.* Ferulic acid inhibits the type 1 topoisomerase and lowers the MIC value. Finally, we demonstrated that ferulic acid disturbs different steps of catalytic reactions and is active against *Mycobacterium* tuberculosis and *Mycobacterium smegmatis* in whole-cell assay.

Materials And Methods

Enzymes and DNA constructs

Purification of *Mycobacterium* tuberculosis topoisomerase I (MttopoI), *MycobacteriumSmegmatis* topoisomerase I (MstopoI) and E. coli topoI (EctopoI) was done as described previously [17]. Ferulic acid was obtained from New England Biolabs. Qiagen miniprep kit was used for purification of plasmid DNA pUC18. Since overexpression of MttopoI and MstopoI genes were excised from their respective constructs, pAVN1 and pPVN123 [18, 19]. These constructs were digested with NdeI and EcoRV and were cloned into the pMIND vector which was linearized with the same restriction enzymes [17]. Further these

constructs were electroporated into *M. smegmatis* mc2 155 or *M. tuberculosis* H37Ra cells, and the positive colonies were selected on kanamycin (25 mg/ml) 7H9 agar plates.

DNA relaxation assays

DNA relaxation assay was carried out for supercoiled pUC18 DNA relaxation as previously described method [20]. Different type of topoisomerases were incubated with 500 ng of DNA for 30 min at 37° C. Ferulic acid at different concentrations and enzyme were pre-incubated for 15 min at 37° C followed by the addition of DNA substrate into the reaction. The reaction were analysed by agarose gel electrophoresis and stained with ethidium bromide which was visualised through UV light.

Oligonucleotide cleavage assay

In this assay 5-end-labeled 32-mer harboring STS (Strong topoisomerase site) annealed to a complimentary sequence. Ferulic acid was pre-incubated with double-stranded substrate in buffer having 30 mM Tris-HCl, 15 mM NaCl, 1.1 mM EDTA and $MgCl_2$, 5 mM for 15 min at 37° C and again incubated the reaction mixtures for 30 min by adding different concentrations of ferulic acid. The reaction were stopped by formamide (45%) and heated for 2 min at 95° C. The samples were resolved into electrophoresis and the images were taken at phosphor imager (model BAS 1800; Fujifilm).

Site directed mutagenesis

D108A and E112A mutants were generated by inverse PCR-based site-directed mutagenesis of Mstopol at DXDXE motif. The amino terminal region were encoded by expression vector pPVN1 which acts as a template. In forward primers, oligonucleotides were designed for respective mutations (5'-CCTCGCCACTGCCGGCGACCGCG-3' for D108; 5'-CACTGACGGTGCGCGCGAGGGTG-3' for E112A) and terminator sequence T7 (GCTAGTTATTGTTCAGCGGTGGG-3') were used as the reverse primer. PVN1 forms of D108 and E112A were subcloned by full length topoisomerase 1 (pPVN123) by NdeI/HindIII restriction site. Restriction digestion and DNA sequencing of plasmid DNA were used for confirmation of mutations.

M.tuberculosis and *M. smegmatis* growth inhibition

M. tuberculosis (H37Ra) or *M. smegmatis* (mc2155) was grown in Middle brook broth supplemented with 0.5% 7H9, 0.06% tween-80 and 0.3% glycerol. Fresh 7H9 broth (10 ml) was used for culture dilutions and aliquoted into100-well growth plate. The serial dilutions of ferulic acid were made and untreated cultures were taken as negative control. The broth were placed into shaker at 200 rpm and the absorbance was taken at 595 nm. The data was analysed by graph pad prism version 5 software. The MIC value of ferulic acid was calculated by resazurin reduction microplate assay (REMA) in which *M. smegmatis* was treated with ferulic acid for 2 days and *M. tuberculosis* for 6 days. *M. tuberculosis* and *M. smegmatis* cultures were stained with resazurine at a concentration of 0.02% for 14 h and 1 h respectively as described earlier [21].

P-gp assay

LS180 cells were seeded into 96 well plates at a density of 40×10^4 for 24 h. After 24 h, cells were treated with ferulic acid at concentration dependent manner (10 µM, 20 µM and 30 µM) for 2 h in presence of rifampicin (10 µM) in hanks media containing rhodamine-123 (10 µM). Cells were washed four times with ice cold PBS and were lysed into 200 µl of lysis buffer containing 0.4 N NaOH and 0.2% Triton X-100. A total of 100 µl of lysate was used for reading fluorescence of Rh123 at 485/529 nm. Statistical analysis values presented here are mean ±SD of three independent experiments. Students T test was used for comparison of three experiments. Treated and untreated samples were compared. P 0.05 was considered to be a statistically significant. Statistical significance were performed by graph pad prism versions 5 software.

Results

Inhibition of DNA relaxation by ferulic acid

The inhibitory potential of ferulic acid against MttopoI was determined by DNA relaxation assay. The activity of the enzyme was inhibited by ferulic acid at a concentration of 1, 2, 3, 4 and 5 µM. Interestingly, ferulic acid at a concentration of 5 µM was able to inhibit completely the activity of MttopoI (Fig. 1). Surprisingly, it was shown that DNA relaxation activity was not inhibited by ferulic acid against EctopoI (Data not shown). Overall, these results indicate that ferulic acid may specifically inhibit the *Mycobacterial* enzyme.

Effect of ferulic acid on Antibacterial activity

To determine the inhibitory potential as well as inhibition specificity by ferulic acid on cell growth. *M. tuberculosis* and *M. Smegmatis* cells were treated with ferulic acid in a concentration dependent manner. The inhibition of *M. Smegmatis* cells were delayed and complete growth inhibition by ferulic acid at a concentration of 500 µM (Fig. 2 A). Resazurin assay was performed to determine the MIC value of ferulic acid for *M. Smegmatis* and *M. tuberculosis* which was 125 µM and 250 µM respectively (Fig. 2 B, C, D).

Ferulic acid triggers the activity of DNA cleavage of topo1

Next, we further investigated the mechanism of ferulic acid by cleavage assay on each step of enzyme in DNA relexation cycle. The Cleavage assays were conceded with different concentrations of ferulic acid, double-stranded 32-mer containing strong topoisomerase site (STS). The cleavage of DNA through topoisomerase I was 2 fold higher in ferulic acid treated samples (Fig. 3 A, B).

Cells overexpressing enzymes affected by ferulic acid

DNA cleavage asaay suggested that ferulic acid may be a cytotoxic agent. In order to find out the cytotoxic potential of ferulic acid, we have overexpressed the topo1 in mycobacterial cells. Interestingly, ferulic acid (125 µM) was able to inhibit the growth of *M. smegmatis* cells with normal level of topoI

(WT). Whileas, the growth inhibition of the topo1 overexpressed cells (OE) was higher at lower concentration of ferulic acid (Fig. 4 A, B). These results suggested that MIC value of ferulic acid was lowed in topo1 overexpressing cells may be because of topoisomerase mediated DNA cleavage.

Effect of ferulic acid on metal binding mutant activity

Type 1A topoisimerases has a metal coordination motif DxDxE and TOPRIM domain in amino acid terminal region [22]. The $Mg2^{+}$ coordination with these motifs is very important for the function of enzyme. The mutations in these acidic residues results in impairment of the catalytic activity and cytotoxicity which leads to the impairment of the enzyme function. The binding of ferulic acid with the topoisomerase may be abrogated in the metal binding site of the enzyme embracing mutations. Ferulic acid at a concentration 1 μM and 5 μM inhibited the activity of mutant D108A as compared with WT enzyme inhibition (Fig. 5). Intrestingly, it was shown that ferulic acid remains unaffected the relaxation activity of E112A (Fig. 5). These results suggested that the interaction of ferulic acids and enzymes proximal to metal coordination motif may be in TOPRIM region.

Ferulic acid enhances efficacy of rifampicin in LS-180 cells

Ferulic acid were tested for P-gp inhibition activity in LS-180 cells overexpressing P-gp by using rhodamine-123 at a concentration dependent manner (10 μM, 20 μM and 30 μM) in the presence and absence of rifampicin. It was observed ferulic acid retains rifampicin in a concentration dependent manner which was indicated by the increasing fluorescence of rhodamine-123 (Fig. 6A). Moreover, ferulic acid was non-toxic at 30 μM in LS180 (Fig. 6B).

Discussion

M. tuberculosis is a pathogen which evades host defence system by controlling its central metabolic pathway [23]. There are various antimicrobial agents which inhibits the metabolic pathay in transcription, translation as well as in cell wall synthesis [24]. However, the extensively drug-resistant TB and multi drug-resistant TB demands new chemotherapeutic agents and new drug targets that will be more effective. Enzyme MttopoI (Rv3646c) is one important target for the inhibition of bacterial growth which can be a potent target [25]. Various research groups are underway to find small molecule inhibitors to topoisomerase1. Here, in this study we discovered ferulic acid as a novel molecule which inhibited MttopoI activity. Ferulic acid was able to increase the lethality as well as DNA cleavage activity in topoI overexpressing cells. Moreover, it was shown that ferulic acid immediately act on enzyme-DNA complex reaction, causes DNA cleavage stimulation which may be clearly visualized in double-stranded DNA. However, ferulic acid does not directly binds with the DNA, but it was able to bind with the DNA enzyme complex system which is preffered target. With this view, we thought that the mode of action of ferulic acid is similar to fluoro quinolones (FQ). Earlier it was shown that FQ inhibits the reaction by binding with the DNA and inhibits DNA gyrase complex but it was shown that FQ inhibits the reaction by directly binding with the DNA gyrase complex [26, 27]. Thus, the effect of ferulic acid was observed in topo1

overexpressed cells which increased accumulation of DNA breaks mediated by topo1 that finally leads to cell death. Observed results showed that there is difference in ferulic acid MIC for *M. tuberculosis* and *M. smegmatis* and this is because the two species have different topoI. Furthermore, some reports showed targeting metal binding motif by small molecules affected the enzyme activity [28, 29]. Ferulic acid inhibited the activity of mutant D108A as compared with wild type enzyme inhibition. Intrestingly, it was shown that ferulic acid remains unaffected the relaxation activity of E112A.

Conclusions

In conclusion, we demonstrated that ferulic acid inhibits *M. tuberculosis* growth by DNA break through topoisimerase 1 in metal binding packet which can be a potent anti- *M. tuberculosis* candidate drug.

Declarations

We declare that the manuscript has not been published previously and is not currently submitted for review to any journal.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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Author contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Nihong Lu, Jingsong Bai and Haiyuan Li: Wrote the paper, performed, participated and designing most of the experiments, Yongrui Yang, Yangbing Ou and Yangjun Chen contribute in various experiments. Yingrong Du designed and conceived the experiments and oversaw all aspects of the study. All the authors have read the manuscript and approved for publication.

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Figures

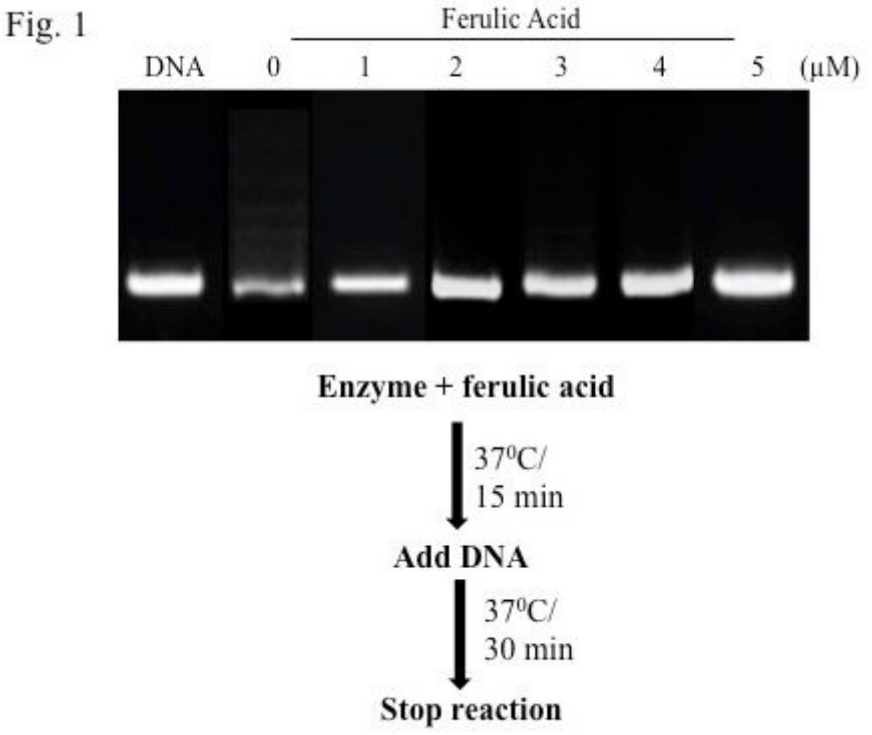
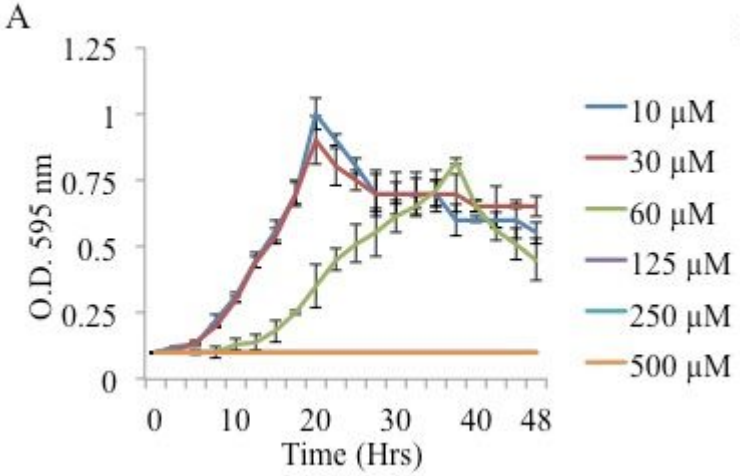


Figure 1

Inhibition of M. tuberculosis topoisomerase I relaxation activity by ferulic acid: Mycobacterium tuberculosis topoisomerase I (1 unit) incubated with ferulic acid at 37º C for 15 min after that pUC18 was added. Again at 37º C for 30 min reaction was incubated and terminated by addition of 0.6 % SDS-agarose dye. Reactions were then analysed by agarose gel electrophoresis. The gels were stained in ethidium bromide. Lane 1, supercoiled pUC18; lane 2, relaxation reaction in absence of the ferulic acid; lanes 3-5, two concentrations (1-5 μM) of ferulic acid.

Fig. 2



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Compound	Mycobacterium *smegmatis* MIC (µM)	Mycobacterium *tuberculosis* MIC (µM)
Ferulic Acid	125	250

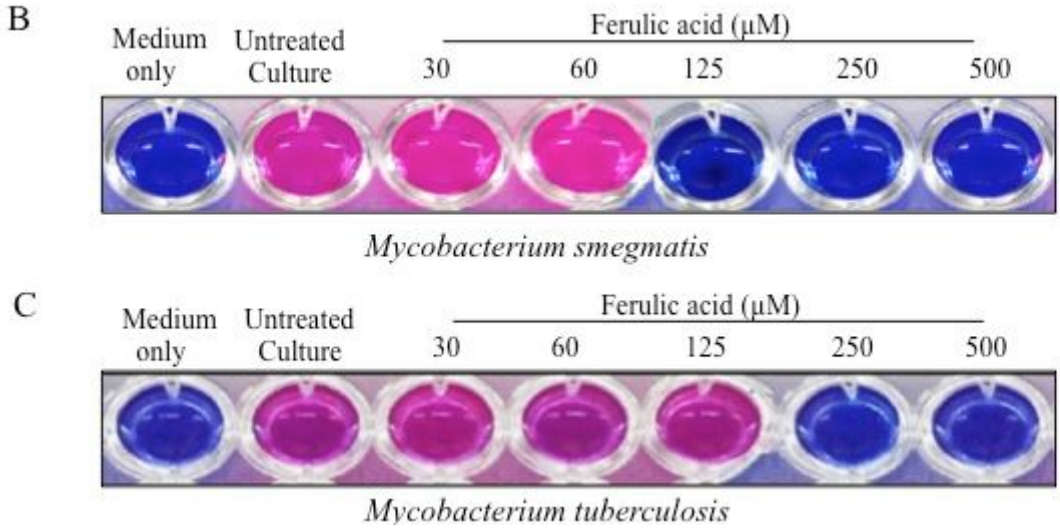

Figure 2

Determination of inhibition of mycobacterial growth by ferulic acid and MIC value: (A) In the presence or absence of ferulic acid M. segmatis cells were grown over a period of 48 h with O.D measured for every 2 h. Untreated cells and sterile media were used as control. (B) M. Smegmatis or (C) M. tuberculosis cells were grown in presence of ferulic acid at a concentration of 1µM and 5 µM followed by the addition of resazurin dye to each well at a concentration of 0.02%. (D) To evaluate the MIC value the plate was incubated at 37º C. The untreated culture and sterile medium were used as control. Error bars indicate the standard deviation obtained in three independent experiments.

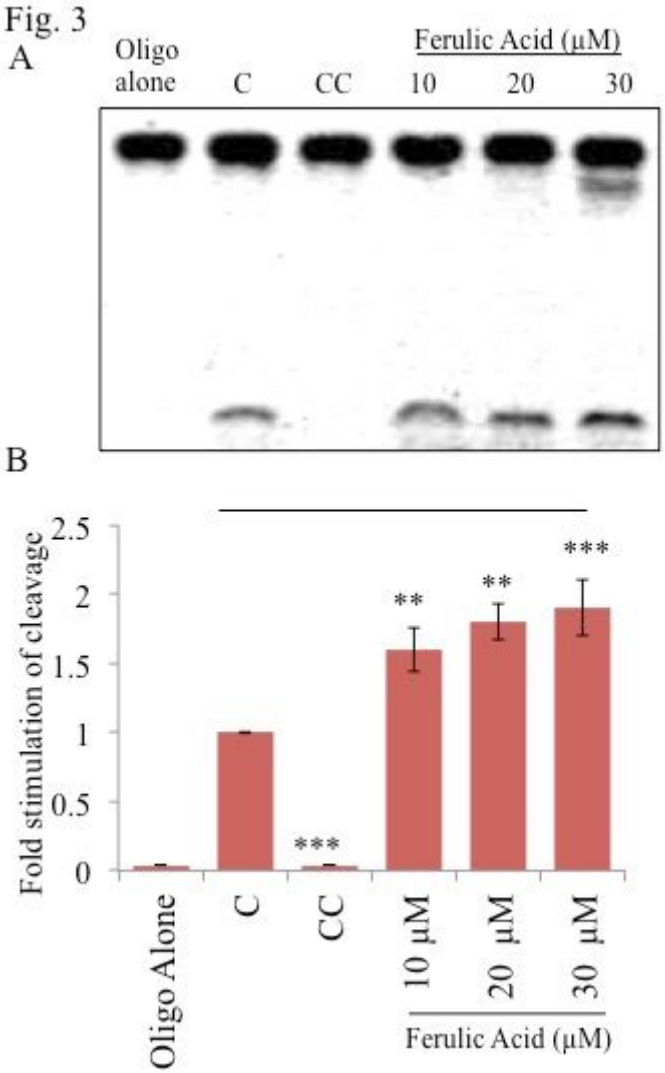

Figure 3

DNA cleavage activity stimulated by ferulic acid: (A) TopoI-mediated cleavage of the DNA stimulated with ferulic by 2-fold. Lane 2 (C) Enzyme cleavage reaction in absence of compound. Lane 3 indicate compound control (CC) for ferulic acid (B) Quantification of cleavage products. Error bars represent the standard deviation obtained in three independent experiments.

Fig. 4

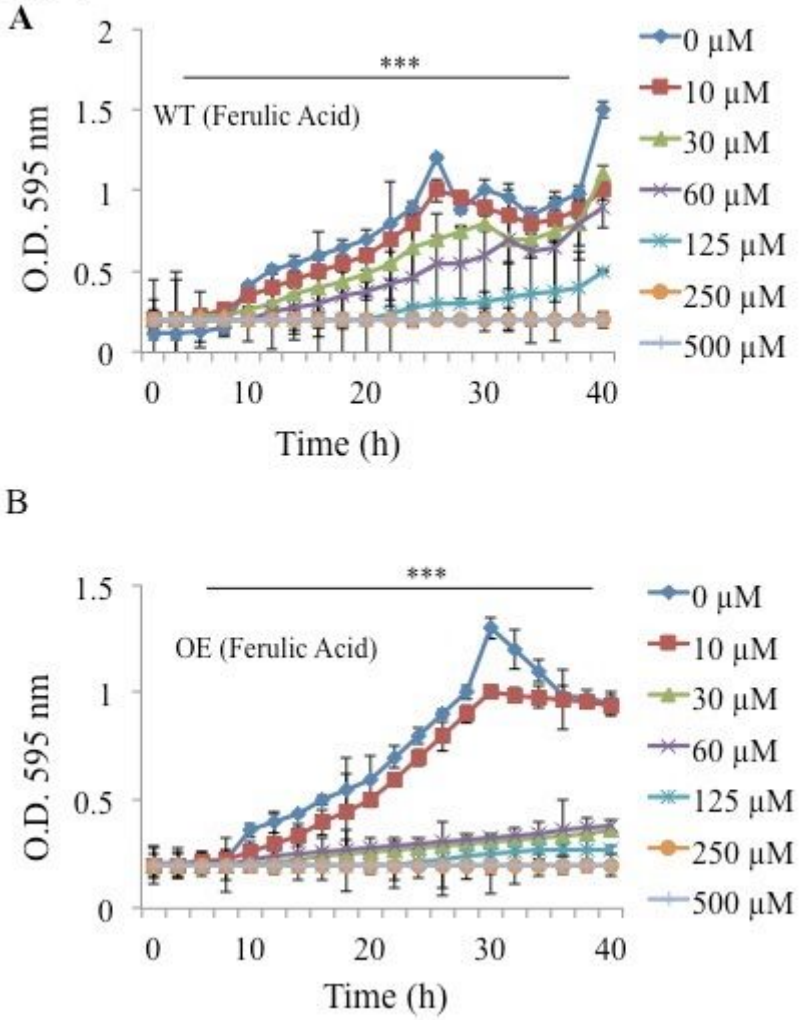

Figure 4

Ferulic acid affect Msm cells overexpressing enzymes: (A, B) In presence or absence of ferulic acid M. Smegmatis cells overexpressing enzymes Mstopol or normal level Mstopol were grown. Growth was monitored for 40 h with O.D being measured every 2 h and untreated cells were used as control.

Fig. 5

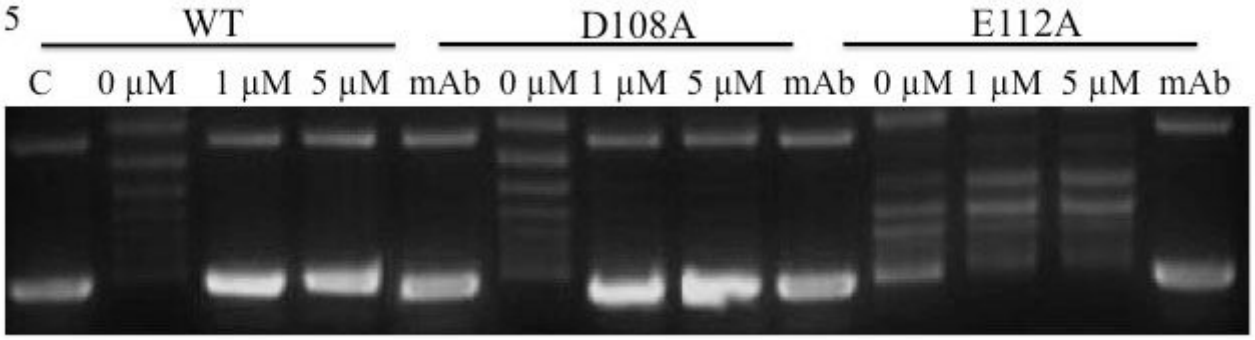

Figure 5

Metal binding mutants of Mstopol are affected differently: Different concentrations of ferulic acid incubated with D108A (lane 5) or E112A at 37º C for 15 min followed by the addition of 500 ng of supercoiled pUC18 and again incubated for 30 min. SDS-agarose dye (0.6%) was used to terminate the reaction. Reactions were then analysed by agarose gel electrophoresis. The gels were stained in ethidium bromide and monoclonal antibody -2F3G4, were used as positive control.

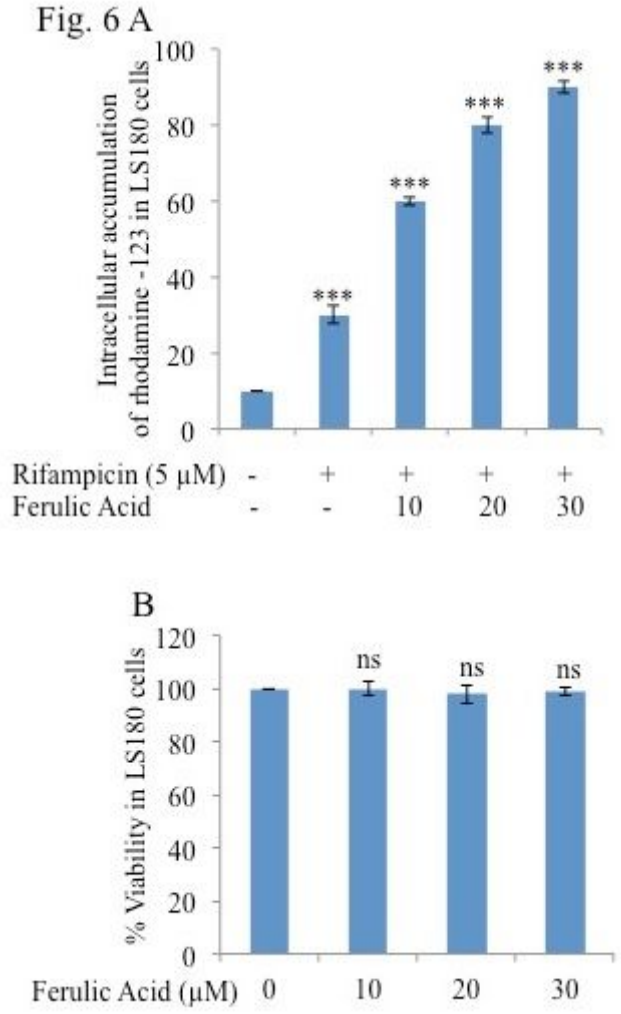

Figure 6

PGP inhibition by ferulic acid in LS180 cells: (A) Ferulic acid at 10 µM, 20 µM and 30 µM treatment of LS180 enhanced the accumulation of rifampicin that is calculated by intracellular accumulation of Rh-123/protein. Statistical comparisons were made between control vs Rifmapicin, ferulic acid (10 µM, 20 µM and 30 µM) by using ANOVA followed by Bonferroni method. The p value <0.05 was considered to be significant. p value *< 0.5, **<0.01,***<0.001. (B) Toxicity of ferulic acid at 10 µM, 20 µM and 30 µM treatment of LS180 cells by MTT assay.