

Synthetic quercetin inhibits mycobacterial growth possibly by interacting with DNA gyrase

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Abstract

*Gyrases are DNA topology modifying enzymes, present only in prokaryotes which make them an attractive target for various antimicrobials. Quercetin, a major flavonoid, is known to inhibit supercoiling activity of DNA gyrase. Docking studies were performed to check the effect of quercetin against DNA gyrase of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. The results show that, quercetin effectively binds with the subunit B of DNA gyrase through interaction with residues that are in the Toprim domain of the protein. This domain is found to be very much essential for the cleavage and reunion of DNA. Using resazurin microtitre plate assay, the Minimum Inhibitory Concentration (MIC) of quercetin against both *M. smegmatis* and *M. tuberculosis* was found to be 100 µg/ml. The effect of quercetin against cytoplasmic membrane integrity of bacilli was assayed by potassium ion loss both in *M. smegmatis* and *M. tuberculosis*. The results imply that there is no cytoplasmic membrane damage caused by quercetin. Therefore, we report that DNA gyrase might be a probable target for the quercetin in mycobacterium.*

Keywords: - *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, DNA gyrase, Quercetin.

Introduction

Tuberculosis (TB) has become a major health problem. According to the global tuberculosis report in 2012 of WHO, nearly 8.7 million people throughout the world are affected. The control over the disease has become extremely difficult due to the association of *Mycobacterium tuberculosis* with that of HIV [1]. In addition, the length and complexity of current TB regimens, poor patient compliance and the emergence of drug resistant forms has worsened the conditions. In this scenario, there is a great need in discovery of novel drugs and of newer targets to have an effective control over the bacilli. Several antimycobacterial compounds are available from marine microbes and plants [2, 3]. Rational development of antimicrobials mainly relies on development of new compounds against specific targets. Gyrase is DNA topology modifying enzymes that are widely present in prokaryotes [4]. These are bacterial type II topoisomerases, which are involved in catalysing reactions such as DNA supercoiling /relaxation, catenation / decatenation and knotting/un knotting [5]. The core protein is of two subunits namely A and B. The subunit A consists of two domains N-terminal breakage and reunion domain and carboxy terminal domain (CTD). The subunit B has ATP hydrolysis domain followed by the Toprim domain [6]. Each domain is found to have a unique role in maintenance of DNA topology. Hence each of the subunit and their respective domain remain an attractive target in development of various antimicrobials. Many of the

present day antimicrobials such as novobiocin are found to target DNA gyrase subunit B. Toprim domain of this subunit also play an important role in breakage and reunion of DNA strands [7]. There are naturally occurring and synthetic flavonoid compounds with immense biological properties which have activity against various groups of bacteria and viruses by different mechanisms; especially found effective against different species of mycobacterium [8]. Currently, research is being focussed by various groups in elucidating the mechanism of action of these compounds in mycobacterium [2, 3]. The compounds are effectively inhibiting the targets which are found essential for the growth and virulence. Brown et al, in 2007 reported the ability of flavonoids which inhibit Rv0636, an enzyme present in the fatty acyl synthase complex II [9]. Myricetin and quercetin-3-*O*- β -D-glucoside extracted from *Pelargonium reniforme* were found to increase the intracellular uptake of mycobacterium by macrophages for removal of bacilli [10]. Quercetin is an important compound among the flavonoid group. The activity of quercetin against various bacteria and viruses are reported [11]. Interaction of quercetin with DNA gyrase has been previously reported in *Escherichia coli* [12] but not in mycobacterium. In this study, we report that quercetin effectively dock with subunit B of DNA gyrase in *M. tuberculosis* and *M. smegmatis* *in silico* and the compound effectively inhibits the bacilli *in vitro*.

Materials and Methods

Quercetin (Catalogue No. Q4951) and resazurin were obtained from Sigma Aldrich & Co (USA). Bovine Serum Albumin, Luria Bertani Broth, Middlebrook 7H9 broth, Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from HiMedia & Co (India). DMSO from Rankem (India). The *M. smegmatis* mc² 155 and *M. tuberculosis* (H37Ra) were purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. The macrophage J774A.1 cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune India.

Molecular Docking

Protein preparation

The protein preparation wizard in Maestro software, Schrödinger was used [13]. For *M. tuberculosis*, the protein structure was retrieved from protein data bank (PDB) (PDB ID: - 3M4I). Since, the DNA gyrase of *M. smegmatis* do not had a crystal structure in PDB, the structure was modelled using Prime module (Schrödinger). The modelling was done using template 2ZJT [14]. The protein was treated to add missing hydrogen atoms, missing amino acid residues and removed the ligand present. Finally the protein structure was minimised to default Root Mean Square Deviation (RMSD) value of 0.30.

Ligand preparation

The ligand quercetin was prepared by Ligprep module to clean the structure and generate tautomers as described [15].

Active site prediction and grid generation

The receptors in the protein were predicted with the help of Sitemap. Then the active site was defined as an enclosing box with the centre of predicted receptors. The prepared ligand was allowed to dock with the predicted active site.

Docking

The docking was performed using Schrödinger Glide (XP) [16]. The receptor grid defined was selected for the docking of quercetin prepared by Ligprep. Flexible docking was performed with extra precision (XP) feature in the Glide module. Finally, the images of the docked structures were rendered through UCSF Chimera [17].

Resazurin Microtitre Plate Assay

The Minimum Inhibitory Concentration (MIC) of quercetin against *M. tuberculosis* and *M. smegmatis* were determined by resazurin microtitre plate assay (REMA) [18]. The assay was performed in 96 well microtitre plates (Nest Biotech, China) with each well containing different dilutions of quercetin (3.125, 6.25, 12.5, 25, 50, 100, 200 and 300 µg/ml). *M. smegmatis* and *M. tuberculosis* cells containing 5×10^4 CFU/ml was used. The cells were incubated at 37°C for 2 days in case of *M. smegmatis* and 7 days for *M. tuberculosis* respectively. To each well 30 µl of 0.02% resazurin and 12.5 µl of 20% Tween80 were added and plates were kept for further incubation for 24 hrs. Then the fluorescence count was measured by spectrofluorimeter (TECAN, M200) with excitation at 530 nm and emission at 590 nm.

Cytotoxicity assay

The cytoprotective effect of quercetin against macrophage was assayed [19]. The macrophage cell lines J774A.1 were maintained in complete DMEM medium supplemented with 10% heat inactivated foetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified CO₂ incubator (NuAire, USA). Then 200 µl of complete medium containing 10^5 cells were seeded in 96 well tissue culture plates (Nest Biotech, China) and was incubated at 37°C under 5% CO₂ in incubator for 24 hours for complete adherence. From stock solution (10mg/ml) of quercetin, subsequent dilutions of 25, 50, 100, 150 and 200 µg/ml were prepared and added to cells. The cells were incubated along with quercetin for a period of 24 hours. Resazurin (0.02%, 10 µl) were added to cell and incubated for 6 hours. The cell viability was noted by measuring the absorbance at 570 nm.

Potassium ion loss assay

The effect of quercetin on the cytoplasmic membrane integrity of *M. smegmatis* and *M. tuberculosis* were determined by potassium ion loss assay [20]. To the sterile polymethyl pentene conical flask (Tarson, India) containing 0.625% BSA and 1% (v/v) DMSO solution, *M. tuberculosis* and *M. smegmatis* culture (1×10^9 cells/ml) were added and kept at 37°C shaker for incubation at 200rpm. Cells were collected from the time of inoculation (0 hr) for 10 hours at regular intervals of 1 hour. The samples were centrifuged and 0.8 ml of supernatant were collected and stored at -20 °C. Then the potassium ion concentration in the sample was measured (Ion sensitive electrodes, Seimen).

Results and Discussion**Molecular Docking**

The interaction between ligand quercetin and DNA gyrase subunit B of *M. tuberculosis* and *M. smegmatis* was studied by molecular docking analysis. The glide score obtained for *M. tuberculosis* was -7.9 which shows higher affinity between the ligand and protein. The interaction between quercetin and DNA gyrase subunit B of *M. tuberculosis* is shown in figure no.1A. The ligand was found to preferentially interact with the residues Ala (531), Thr (542) and Ala (564) of the protein. A similar result was obtained in case of *M. smegmatis* (figure no.1B). Since the experimental crystal structure of DNA gyrase subunit B was not available in PDB database, the protein was modelled (figure.no.1C) with the help of PRIME (Schrödinger). The protein (PDB ID 2ZJT) with maximum identity was chosen as structural template. This modelled protein was then used for further docking analysis. The glide score obtained here was -4.9, which suggests higher binding affinity of quercetin with that of protein. The ligand was found to interact with the residues Glu (490), Asp (551) and Glu (646) through hydrogen bond formation. Gyrase is a type II DNA topoisomerase that introduces negative supercoil into the DNA at the expense of a ATP molecule. The protein

has subunits A and B, each with distinct functions. In this study we preferentially tried to target subunit B because of its importance in maintaining the super helical density of DNA. This protein has 639 amino acids and molecular weight of 70.3 kDa in *M. smegmatis* and of 675 amino acids and molecular weight of 74.05 kDa in *M. tuberculosis*. The subunit has ATPase domain, which catalyses the hydrolysis of ATP, followed by Toprim domain. The Toprim domain of subunit B with that of breakage reunion domain subunit A forms the catalytic core which are responsible for the cleavage and reunion of DNA double helix [21]. The Toprim domain in *M. tuberculosis* and *M. smegmatis* encompasses amino acid residues 448-564 and 453-564 respectively. Our results show that quercetin binds to amino acid residues which are in the Toprim domain of the protein both in *M. tuberculosis* and *M. smegmatis*. Therefore, we hypothesize DNA gyrase as a probable target for quercetin in mycobacterial growth inhibition.

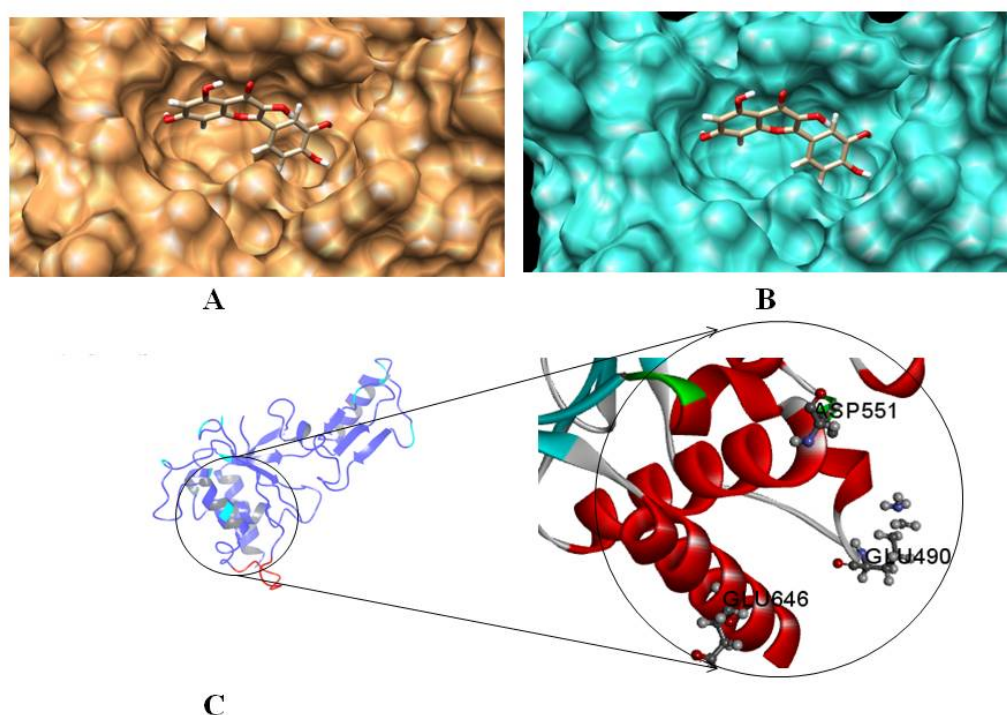


Figure 1. Figure showing interaction of quercetin with DNA gyrase subunit B (A) *M. tuberculosis*; (B) *M. smegmatis* and (C) Modelled structure of DNA gyrase subunit B of *M. smegmatis* containing Toprim domain. The modelled domain depicted where few breaks in loops are due to dissimilar sequences used for modelling from query and template sequences. This region doesn't span the drug binding cavity. Our results show that quercetin dock with the DNA gyrase Toprim domain of *Mycobacterium smegmatis* by interacting with the residues Glu (490), Asp (551) and Glu (646). (zoomed portion in figure 1C)

Resazurin Microtitre Plate Assay

The MIC of quercetin against both *M. smegmatis* and *M. tuberculosis* were done by the experiment resazurin microtitre plate assay. The experiment is based on the principle that the live cells reduce the non fluorescent resazurin dye into resorufin (fluorescent dye). Therefore the fluorescent counts obtained were directly proportional to the number of viable cells. Figure no.2 shows that there is a continuous decrease in fluorescence counts with increase in concentration of quercetin. This indicates that there is a decrease in cell viability of both *M.*

smegmatis and *M. tuberculosis* in response to higher concentrations of quercetin. A continuous decrease in cell viability can be seen from 3.125 to 50 µg/ml with maximum decrease at 100 µg/ml from where no further decrease in cell viability was observed. Therefore concentration of 100 µg/ml was taken as the MIC value of quercetin against *M. smegmatis* and *M. tuberculosis*.

Cytotoxicity assay

The cytoprotective effect of quercetin against macrophage was done using macrophage cell lines (J774A.1). The result shows increased absorbance at 570 nm with increase in concentration of quercetin (figure no. 2C). Therefore the cell viability is increased in higher concentration of quercetin. This can also be related to the antioxidant nature of quercetin. Reddy et al, in 2012 reported the increase of cytoprotective enzymes such as superoxide dismutase, catalase and glutathione S-transferase in response to quercetin [22]. This might be a reason for the increased cell viability at higher concentration of quercetin when compared to the control. The overall result shows cytoprotective nature of quercetin against macrophages cells.

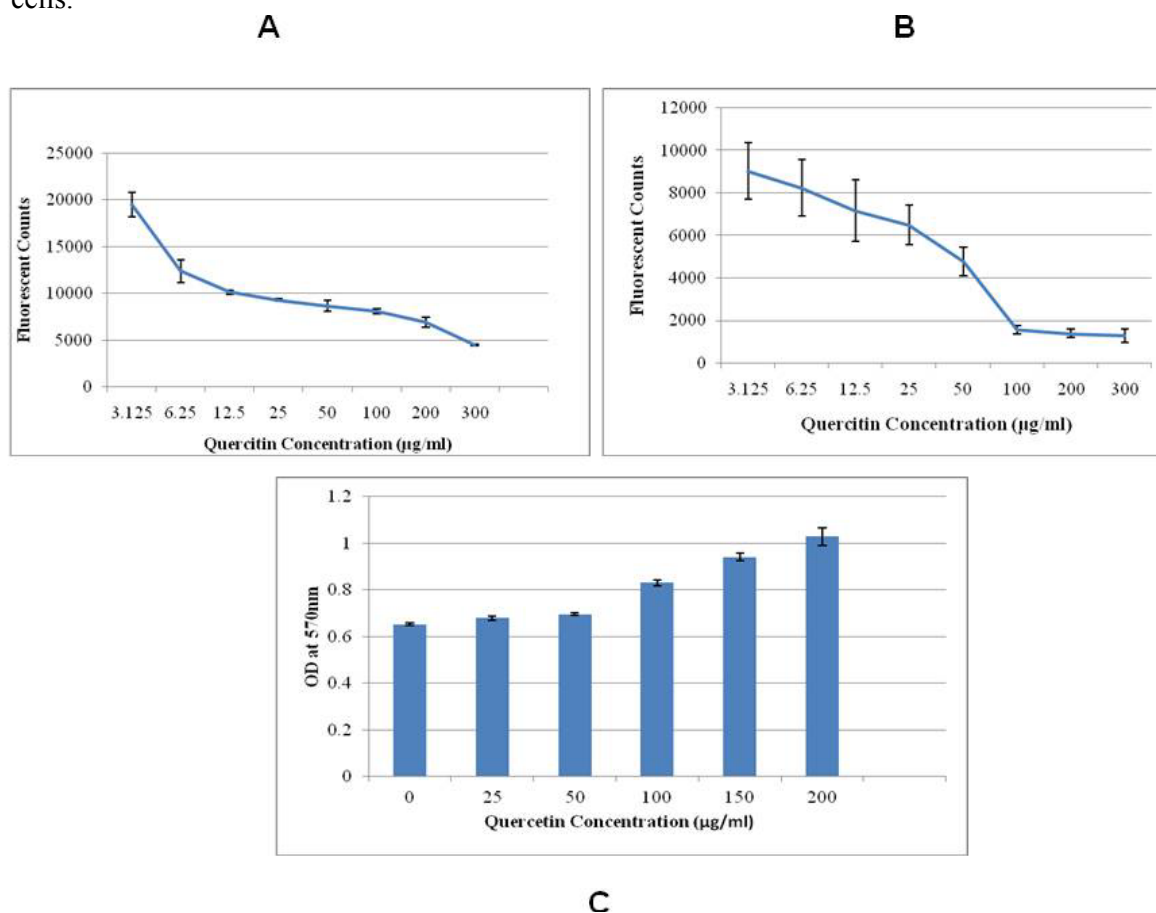


Figure 2. Effect of different concentration of quercetin against (A) *M. tuberculosis*; (B) *M. smegmatis* shows that minimum inhibition concentration is 100 µg/ml and (C) Cytoprotective effect of different concentrations quercetin against macrophages, using macrophage cell lines J774.1.

Potassium ion loss assay

Quercetin effectively inhibits mycobacterial cell growth at concentration of 100 µg/ml (figure. no.2). The effect of quercetin against cell membrane integrity of the bacilli was assayed using loss of potassium ion. Potassium ions are intracellular, present in higher concentration inside

the cells. Therefore the loss of potassium ion is an indication for cell membrane damage. In our experiment, the effect of quercetin on cell membrane integrity of both *M. smegmatis* and *M. tuberculosis* (H37Ra) was checked through potassium ion leakage. Quercetin concentration of 100µg/ml (MIC) was used for experiment in parallel with untreated cells as control. Figure no.3 shows that there is generalised increase in potassium ion concentration observed both with control and test. But there was no significant difference observed in quercetin treated cells and in control of *M. smegmatis* and *M. tuberculosis* (H37Ra). The generalised increase in potassium observed might be due to the autolysis of the cells caused by peptidoglycan hydrolases digestion. This occurs during stressful physiological conditions such as in presence of antibiotics [23]. The prolonged incubation of cells in potassium free media should have resulted in considerable autolysis resulting in generalised increase of potassium ions in both control and quercetin treated cells.

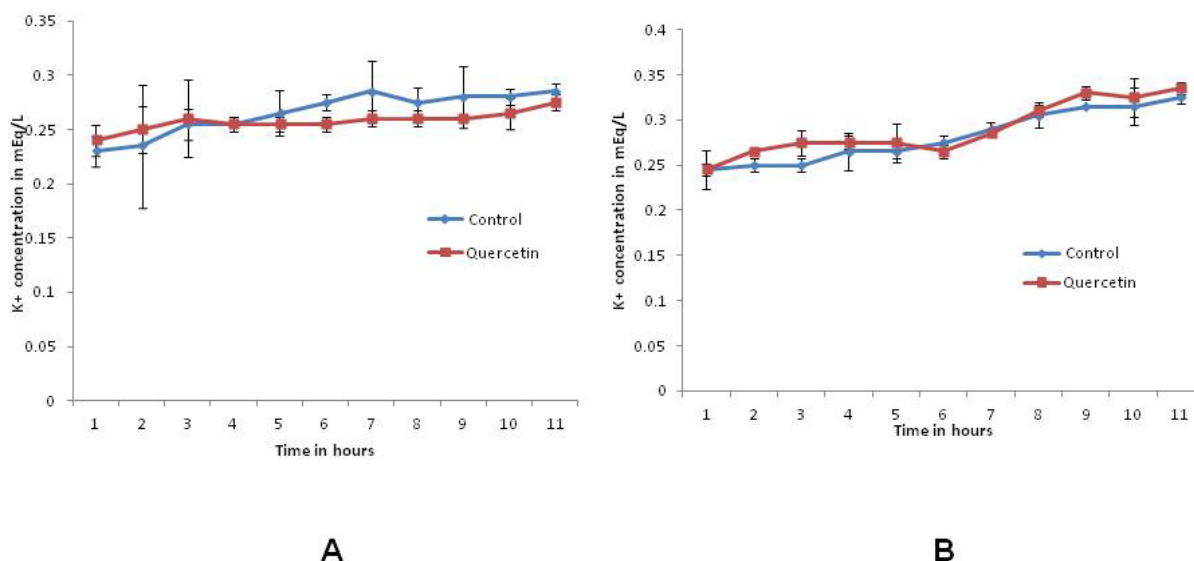


Figure 3. Potassium ion leakage at various time intervals in (A) *M. tuberculosis* and (B) *M. smegmatis* in response to quercetin at concentration of 100µg/ml.

Conclusion

1. Among many targets, Toprim domain of subunit B of DNA gyrase might be a probable target for the action of quercetin in both *M. smegmatis* and *M. tuberculosis* as evidenced by *in silico* analysis.
2. The MIC of quercetin against both *M. smegmatis* and *M. tuberculosis* was found to be 100µg/ml.
3. Quercetin is found to have cytoprotective effect against macrophages.
4. Quercetin does not affect cytoplasmic membrane integrity in both *M. smegmatis* and *M. tuberculosis*.

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