

Epigallocatechin Gallate, a Green Tea Polyphenol Inhibits *Mycobacterium smegmatis*: *In silico* and *In vitro* Studies

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Narayanan and Ramesh: Epigallocatechin Gallate Inhibits *M. smegmatis*

Presence of multidrug resistant strains makes treatment of tuberculosis difficult. Conventional frontline antituberculosis therapy has high incidence of treatment failure due to non-adherence and severe side effects, and therefore warrants alternative approaches using safe, natural compounds. (-) Epigallocatechin gallate, a green tea catechin with significant antimicrobial properties, and its derivatives have been studied for their inhibitory effects on *Mycobacterium smegmatis*. Enoyl reductase (Inh A, PDB ID: 2NV6), an important drug target inhibited by the antituberculosis drug, isoniazid, was docked with geometrically optimized conformation of epigallocatechin gallate and two of its derivatives using AutoDock software. Perpropionate and permethyl derivatives of epigallocatechin gallate were synthesized and, along with epigallocatechin gallate, evaluated for their inhibitory and cytotoxic properties against *M. smegmatis* using microplate dilution method and alamarBlue® assay, respectively. Ames test was performed to assess the mutagenic potential of epigallocatechin gallate. It was docked successfully onto the InhA with docking energy of $-9.38 \text{ kcal mol}^{-1}$. Analysis of neighbouring groups revealed 11 amino acid residues common to both isoniazid and epigallocatechin gallate. The minimum inhibitory concentrations for epigallocatechin gallate, perpropionate and permethyl were found to be 128 (58.2% inhibition), 8 (32.9%) and 4 (12.5%) $\mu\text{g/ml}$, respectively. Epigallocatechin gallate showed a cytotoxicity of 18.6% at a concentration of 8 $\mu\text{g/ml}$, while the two derivatives did not show any toxicity, even at 64 $\mu\text{g/ml}$. Ames test revealed the absence of mutagenicity for epigallocatechin gallate, in presence and absence of metabolic activation. Non-mutagenic epigallocatechin gallate inhibits the growth of *M. smegmatis* and warrants further investigation as an adjunct therapy for pathogenic mycobacteria.

Key words: Tuberculosis, *Mycobacterium*, mutagenicity, EGCG, green tea polyphenol, enoyl reductase

Tuberculosis (TB) is one of the most serious diseases and has been the scourge of the mankind since ancient times. As per the WHO estimate of 2015, TB has been responsible for over 1.8 million deaths worldwide annually^[1]. This coupled with the occurrence of multidrug resistant (MDR) strains of the pathogen and co-infection with human immunodeficiency virus (HIV) has rendered TB patients highly susceptible to the disease^[2]. In addition, conventional frontline antituberculosis (antiTB) therapy is known to have high incidence of treatment failure if the prescribed regimen is not followed^[3]. The drugs used to treat TB themselves possess marked side effects, such as blurring or loss of vision and neuritis caused by ethambutol, and abdominal pain, bleeding gums, fever and headache by rifampin^[4,5]. Isoniazid is reported to cause nausea, vomiting, stomach effects and allergic

reactions^[6]. Consequently, there is an urgent need to explore alternative approaches to treatment of TB using compounds derived from natural substances with almost negligible side effects and their possible use in combination therapy.

Mycobacteria are tenacious microorganisms resistant to common disinfectants due to the presence of a lipid coat of mycolic acid. Mycolic acid is synthesized through an elaborate biosynthetic pathway regulated by enzymes of the fatty acid synthase (FAS) complex. One

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of the key enzymes in this pathway is enoyl reductase, also known as InhA. This enzyme has been regarded as an important drug target and is inhibited by the leading anti-TB drug, isoniazid^[7]. This has prompted us to examine inhibition of InhA by Epigallocatechin gallate (EGCG) using *in silico* studies followed by *in vitro* studies.

EGCG is one of the poly phenolic catechins found in green tea. Green tea has been reported to have significant antimicrobial properties^[8]. EGCG has not been evaluated in detail for the inhibition of *Mycobacterium tuberculosis* (MT), although few reports have mentioned some antimycobacterial activity based on enzyme inhibition studies in combination with compounds, such as triclosan^[9]. They have also reported that EGCG, inhibits InhA, the enoyl-ACP reductase of MT and interferes with the binding of NADH to InhA. Kohda *et al.* have reported significant antimicrobial activity of EGCG against *Listeria monocytogenes*, another intracellular bacterium like MT^[10]. Hu *et al.* have observed a potent synergy between EGCG and Ampicillin/Sulbactam in the treatment of Methicillin-resistant *Staphylococcus aureus* infection^[11]. Cho *et al.* have reported that EGCG exhibited synergistic activity with the antibiotic Imipenem in treatment of the pathogenic bacteria, *Klebsiella pneumoniae* implicated in pneumonia infections^[12]. Novy *et al.* have also reported the synergistic and additive effects of EGCG in combination with antibiotics such as oxytetracycline against *S. aureus* strains^[13].

EGCG and three of its derivatives have been synthesized and evaluated for their inhibitory properties against *M. smegmatis* a non-pathogenic bacterium, genetically similar to MT, using the microplate dilution method. AlamarBlue[®] assay, a rapid and highly sensitive method to measure cell proliferation and cytotoxicity in various bacteria, fungi and cell lines has also been used to determine the cytotoxicity of EGCG and its derivatives^[14]. In the long and often tortuous path of drug discovery, a candidate drug needs to be checked for its mutagenic potential before it is taken up for therapeutic evaluation in a clinical setting, since both cancer and mutagenicity are often linked together. EGCG and its derivatives have also been evaluated for mutagenicity using the Ames test (bacterial reverse mutation test)^[15].

MATERIAL AND METHODS

EGCG was purchased from Carbosynth (Berkshire, UK). Three derivatives of EGCG, *viz.* EGCG

perpropionate, permethyl EGCG and pentamethyl EGC were synthesized from EGCG based on the method of Utenova *et al.*^[16]. In the case of pentamethyl EGC, a modified procedure was used, where the permethyl EGCG was hydrolysed with lithium hydroxide (2 equivalent) in a mixture of tetrahydrofuran:methanol:water (2:2:1) at room temperature to yield pentamethyl EGC.

In silico studies:

EGCG structure was drawn using Gauss View package (Gaussian Inc., CT, USA) and subsequently optimized using Gaussian package^[17]. Hartree-Fock theory with “6-31++g (d, p)” as the basis set was used for optimizing the structures. Standard orientation of EGCG after convergence to its global energy minima was visualized using Argus package (Argus Lab, WA USA), and saved in PDB format. Geometrically optimized EGCG was later docked onto the crystal structure of the enoyl reductase receptor protein (PDB ID:2NV6) using the AutoDock ver.4.0 package (The Scripps Research Institute, La Jolla, CA, USA) described elsewhere^[18]. Docking energy was calculated based on the shape and electrostatics using default grid spacing of 0.375 Å. The docked structure was analysed using PyMOL while the neighbouring residues of the docked structure were identified using Swiss PDB Viewer^[19].

Microplate dilution method:

The minimum inhibitory concentration (MIC) of EGCG and its derivatives were determined using the microplate dilution method. Briefly, broth culture of *M. smegmatis* was grown using Muller Hinton (MH) broth. The compounds were serially diluted in the medium (MH broth) and 100 µl of each solution was added into the wells of a 96-well plate. The following concentrations were used-EGCG (1, 2, 4, 8, 16, 32, 64 µg/ml), EGCG perpropionate and permethyl EGCG (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/ml), ciprofloxacin (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/ml), rifampicin (0.29, 0.6, 1.2, 3.1, 6.3, 12.5, 25.0, 50.0, 64 µg/ml), amikacin (0.98, 2, 4, 8, 16, 32, 64, 128 µg/ml). The cells were matched with the McFarland standard 0.5, which represents 10⁸ cells and then were diluted to 10⁵ cells in the medium. Hundred microliters of this cell suspension was added into each well. The plate was incubated at 37° for 48 h and the optical density was measured at 600 nm on a Power Wave X Microplate Reader (BioTek, Winooski, VT, USA). The percent inhibition of cell growth by the given compound was calculated using the Eqn., percent

inhibition=(Av.OD600 (control)-OD600 (test))/
(Av.OD600 (control))×100.

AlamarBlue® assay:

This dye reduction assay was performed in a 96-well plate, using ciprofloxacin and amikacin as positive controls. Briefly, broth culture of *M. smegmatis* was grown using MH broth^[14]. The test compounds and controls were also serially diluted in MH broth. Hundred microliters each of the diluted solutions was added into the wells. The following concentrations were used-EGCG (8, 16, 32, 64 µg/ml), EGCG perpropionate, permethyl EGCG and pentamethyl EGCG (32, 64, 128), ciprofloxacin (0.03, 0.06, 0.12, 0.25, 0.50, 1.0 µg/ml) and amikacin (0.045, 0.09, 0.18, 0.37, 0.75, 1.50 µg/ml). The cells were matched with the McFarland standard 0.5, which represents 10⁸ cells. The cells were then diluted to 10⁵ cells and 100 µl of the diluted cells were added into each well. Ten microliters of alamarBlue dye was then added to the wells. The plate was incubated at 37° for 48 h. The dye reduction was visualized as change of colour from blue (oxidized) to pink (reduced) indicating viability of cells. The fluorescence intensity (FI) was read on a Power Wave X Microplate Reader under excitation at 560 nm and emission at 590 nm. The percent reduction of alamarBlue (= % cell viability) was calculated using the formula, % reduction=(FI590 (test)-FI590 (untreated control))/(FI590 (100% reduced AB)-FI590 (untreated control))×100; percent cytotoxicity=100-percent reduction.

Mutagenicity assay:

Ames assay was performed to assess mutagenicity using the following seven bacterial strains of *Salmonella typhimurium* – TA 98, TA 100, TA 1535, TA1537, TA97a, TA102; *Escherichia coli* – WP2 uvrA (pKM101)^[15]. Seven wells (one each for positive and vehicle control, 5 test compound concentrations) in three replicates were taken for each compound. Maximum concentration taken was up to 1000 µg/plate. Bacteria were exposed to the test item both in the presence and absence of metabolic activation (by the S9 rat liver homogenate). Plating was done in a six well Petri plate. A volume of 0.5 ml top agar containing concentrations of test item/vehicle/positive control (20 µl), bacterial culture suspension (20 µl) and S9 mix/PBS (100 µl) were plated on to 4-6 ml basal agar (Vogel Bonner agar). The plates were incubated from 48 to 72 h and observed for the following parameters: precipitation, bacterial background lawn

and the reverted mutants were counted. The test item EGCG would be considered mutagenic in this assay if, a concentration related increase in revertant numbers of ≥2-fold of the concurrent vehicle control values was observed.

Cytotoxicity and precipitation:

The presence or absence of cytotoxicity was determined as mentioned in OECD Guideline 471, that is, by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures, in presence and absence of metabolic activation. The precipitation was also assessed for each concentration of EGCG as mentioned in the above guidelines.

RESULTS AND DISCUSSION

Energy minimized structure of EGCG after Gaussian optimization represented in fig. 1. Characteristic parameters of the optimized structure are shown in Table 1. Lowest docking energy was -9.38 kcal mol⁻¹ observed in Run 8 of the clustering histogram shown in Table 2. The docked structure of EGCG with 2NV6 is shown in fig. 2. The neighbouring residues for EGCG are depicted in fig. 3a and the 11 amino acid residues marked with an asterisk are common to both EGCG and isoniazid (Zid). These residues are Zid^{300*}, Ile^{122*}, Phe^{97*}, Gly^{96*}, Ile^{95*}, Ala⁹⁴, Gln^{66*}, Val^{65*}, Leu^{63*}, Ile⁴⁷, Leu⁴⁴, Arg⁴³, Asp⁴², Phe^{41*}, Gly⁴⁰, Ile^{16*}, Gly^{14*}.

The neighbouring residues for isoniazid are depicted in fig. 3b and eight residues marked with an asterisk are common to both Zid and EGCG (mol): MOLA^{*},

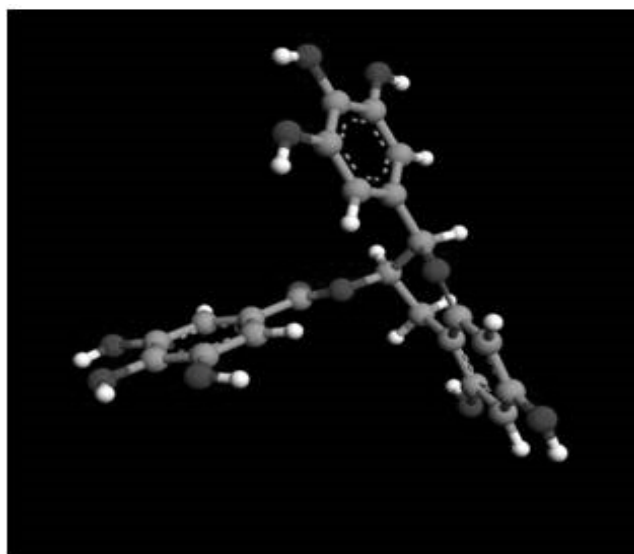


Fig. 1: Geometrically optimized structure of epigallocatechin-3-gallate

TABLE 1: ENERGY MINIMIZATION PARAMETERS OF EGCG

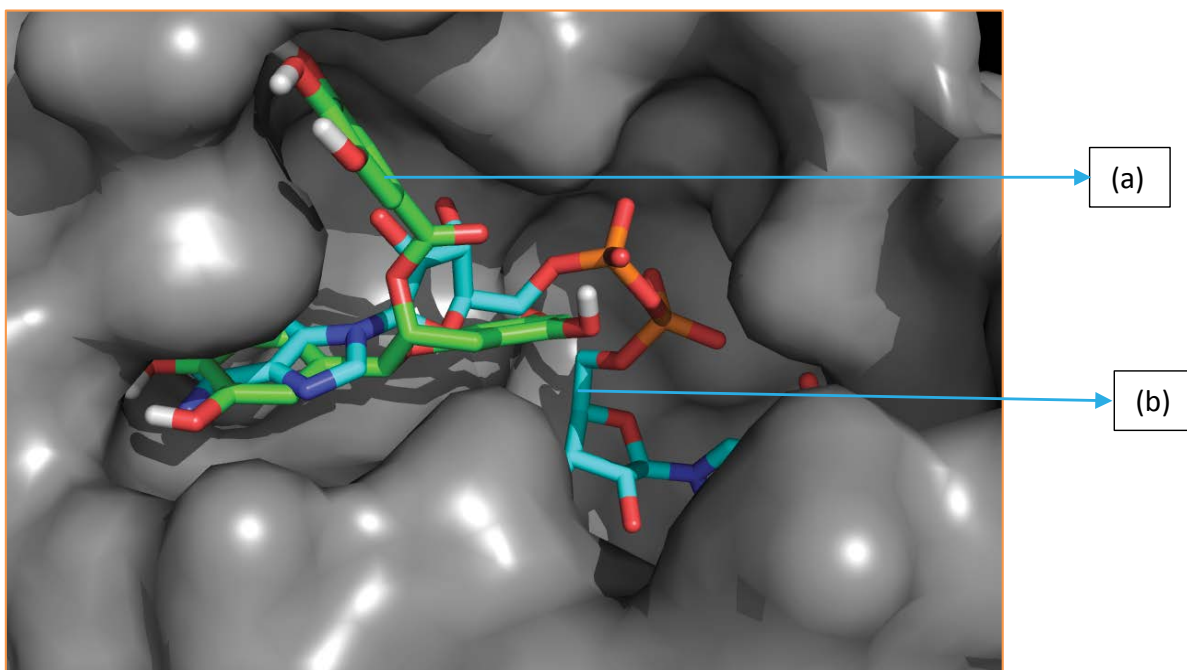
Parameter	Value	Threshold	Convergence
Maximum force	0.000025	0.000450	Yes
RMS force	0.000005	0.000300	Yes
Maximum displacement	0.001478	0.001800	Yes
RMS displacement	0.000334	0.001200	Yes
Predicted change in energy		-1.895627×10 ⁻⁸	
Optimization		Completed	
Stationary point		Found	

The 4 parameters used for the same have achieved convergence as indicated by the YES mentioned against each parameter in addition to calculated value, which is less than threshold value in each case. RMS indicates Root Mean Square (RMS Force/RMS Deviation). Stationary point found indicates that the criteria for mentioned in the optimization calculation have been met successfully

TABLE 2: CLUSTERING HISTOGRAM: EGCG DOCKED WITH 2NV6

Cluster rank	Lowest binding energy	Run	Mean binding energy	Number in clusters
1	-9.38	8	-8.24	7
2	-8.74	11	-8.74	1
3	-8.52	22	-8.52	1
4	-8.47	5	-8.47	1
5	-8.41	7	-8.41	1
6	-8.32	18	-8.21	3
7	-7.65	6	-7.39	2
8	-7.63	1	-6.88	3
9	-7.29	21	-7.29	1
10	-7.27	2	-7.27	1
11	-7.22	23	-7.22	1
12	-6.83	14	-6.83	1
13	-6.61	12	-6.61	1
14	-6.47	17	-6.47	1

Binding energies of EGCG (epigallocatechin3-gallate) docked on to crystal structure of enoyl reductase from MT (PDB ID:2NV6); using AutoDock software, which ranks the docked conformations by energy, lowest (best) to highest. The lowest binding energy of -9.38 kcal.mol⁻¹ was achieved in the 8th docking run and ranked No.1 in the cluster ranks

**Fig. 2: Docking of EGCG with enoyl reductase receptor 2NV6**

Docking of Fluoroamine EGCG onto crystal structure of enoyl reductase from MT (PDB ID:2NV6). EGCG (epigallocatechin-3-gallate), the docked ligand is represented in blue (b) whereas Isoniazid, is the reference molecule within the crystallographic structure of MT enoyl reductase receptor protein (PDB ID: 2NV6) is represented in green (a)

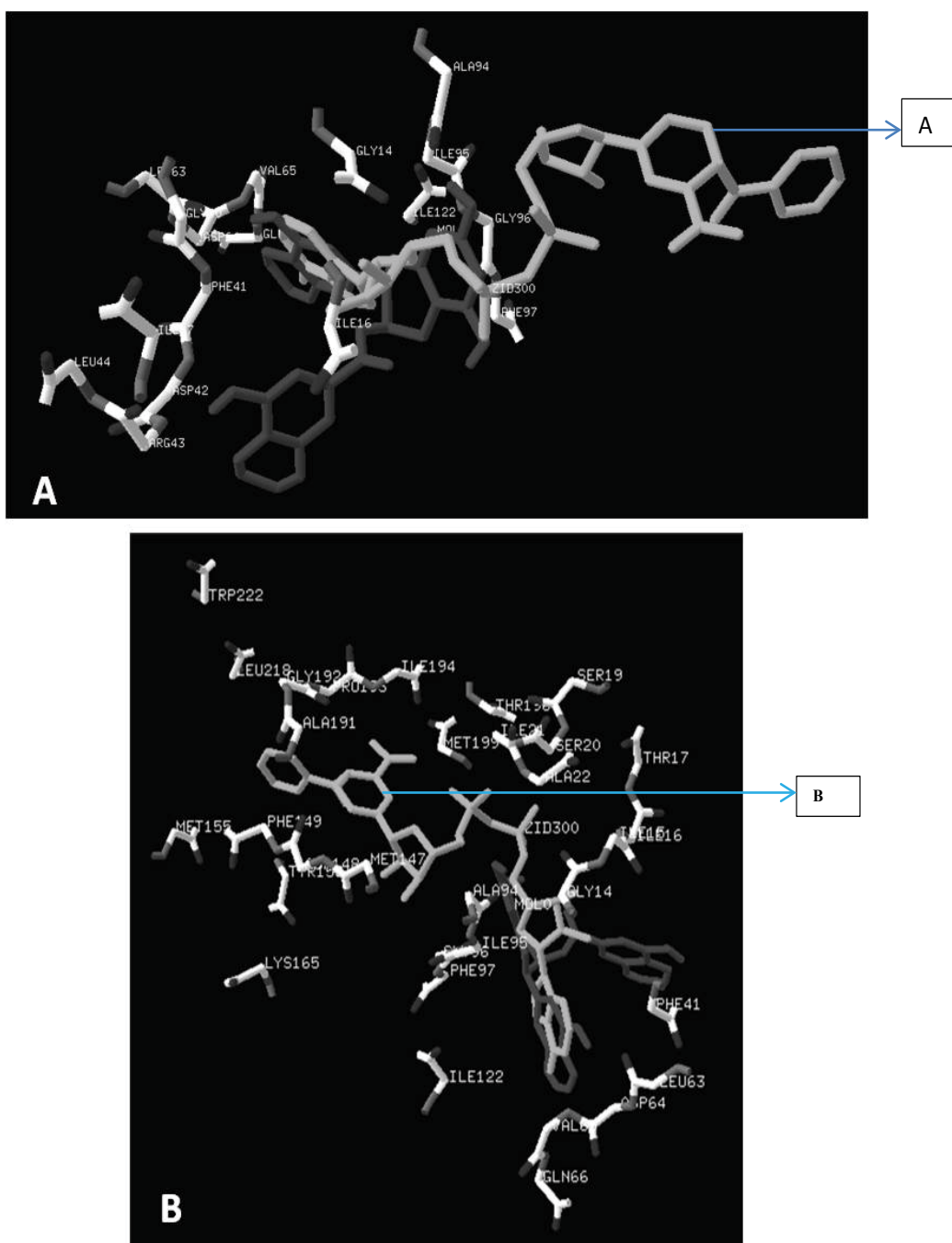


Fig. 3: Neighboring amino acid residues of EGCG and isoniazid

(A) EGCG (epigallocatechin-3-gallate) is the ligand used for docking whereas (B) INH (isoniazid) is the reference molecule within the crystallographic structure of Mtb receptor protein. The amino acid residues observed in the figure are within 5Å of the docked molecules

Trp²²², Leu²¹⁸, Met¹⁹⁹, Thr¹⁹⁶, Ile¹⁹⁴, Pro¹⁹³, Gly¹⁹², Ala¹⁹¹, Lys¹⁶⁵, Tyr¹⁵⁸, Met¹⁵⁵, Phe¹⁴⁹, Asp¹⁴⁸, Met¹⁴⁷, Ile^{122*}, Phe^{97*}, Gly^{96*}, Ile⁹⁵, Ala^{94*}, Gln⁶⁶, Val^{65*}, Asp^{64*}, Leu^{63*}, Phe⁴¹, Ala²², Ile²¹, Ser²⁰, Ser¹⁹, Thr¹⁷, Ile^{16*}, Ile¹⁵, Gly^{14*}. In all, there are 11 neighbouring residues common to both these ligands. There are five hydrogen bonds observed in the docked structure as shown in fig. 4. These are located between Ala94 --- O; Asp64 --- OD1; Leu63 --- O; Asp42 --- OD2; Arg43 --- N.

The extent of inhibition and cytotoxicity of EGCG and its synthesized derivatives, determined by the microplate dilution assay and alamarBlue assay, respectively, are shown in Table 3. Microplate dilution assay results indicate that the antibiotics *viz.* ciprofloxacin, rifampicin and amikacin showed an MIC₅₀ at 0.25, 0.29 and 0.98 µg/ml, respectively, in accordance with the expectation. EGCG showed the MIC at 128 µg/ml (58.2% inhibition), however,

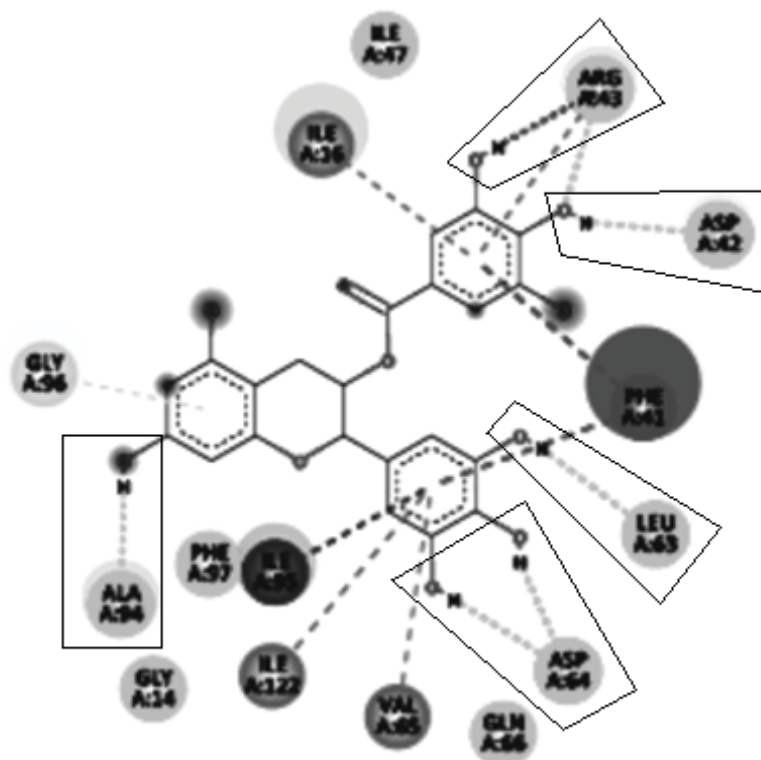


Fig. 4: Hydrogen bonds formed by EGCG with amino acid residues on 2NV6

The numbered amino acid residues of the receptor enoyl reductase protein of MT (PDB ID: 2NV6), which had formed H-bonds with EGCG (epigallocatechin-3-gallate). The same are represented as dotted lines connecting the amino acid residue with the ligand EGCG

TABLE 3: MICROPLATE INHIBITION AND ALAMAR BLUE ASSAYS

Compound	Microplate inhibition assay		AlamarBlue assay	
	Inhibition (%)	Concentration (µg/ml)	Cytotoxicity (%)	Concentration (µg/ml)
EGCG	58.2	128 ^a	82.6	64 ^a
	28.3	64 ^a	85.3	32 ^a
			74.1	16 ^a
EGCG Per propionate	32.9	8 ^a	18.6	8 ^a
	18.8	1 ^a	Not observed	32 to 128
Per methyl EGCG	12.5	4 ^a	Not observed	32 to 128
Penta methyl EGC	ND	ND	Not observed	32 to 128

^aRepresents P<0.05 taken as statistical significance, 95% CI. ND: not done. Statistical tests done-Student T test (unpaired) using Graph pad prism (v. 6). Since no cytotoxicity (AlamarBlue Assay) for EGCG (epigallocatechin-3-gallate) derivatives was observed in the concentrations between 0-32 µg/ml, tests of significance were not conducted for these concentrations. Similarly in the MIC plate assay, tests of significance were performed only for concentrations where MIC was observed

inhibition of bacterial growth could be observed even at 64 µg/ml also (28.3% inhibition). EGCG perpropionate showed a slight inhibition of 18.8% at concentration of 1 µg/ml and 32.9% at 8 µg/ml, and no inhibition thereafter at any other concentration tested. Permethyl EGCG showed slight inhibition of 12.5% at a concentration of 4 µg/ml and no inhibition at other tested concentrations. Pentamethyl EGC did not show any inhibition at the concentrations tested.

In alamarBlue assay, the growth of *M. smegmatis*

was inhibited by ciprofloxacin (72.5%) at 0.031 µg/ml, amikacin (35.8%) at 0.045 µg/ml. In the case of EGCG, the lowest concentration tested (8 µg/ml) exhibited 18.5% inhibition, which increased gradually to 74.1% at 16 µg/ml. At further higher concentrations, viz. 32 and 64 µg/ml, the effect saturated in the range of 82.6-85.3%, as shown in Table 3. EGCG perpropionate, permethyl EGCG, and pentamethyl EGC did not show any cytotoxicity towards *M. smegmatis*.

The viable cell counts were within the required range of

1-2×10⁹ for the cultures. Data from control treatments confirmed that the revertant counts were within the historical as well as in-house spontaneous counts for each of the strains tested. The correctness of strain, functioning of the assay, and the data were accepted as valid since genotypic characterizations had been already verified earlier^[15]. Following treatment with EGCG, no significant increase in revertant colonies (at least 2-fold increase expected) were observed for any of the tested strains under any condition of metabolic activation. The positive controls induced 5 to 25-fold increases in revertant numbers for different strains when compared to the concurrent vehicle controls.

EGCG did not precipitate on the plates at any concentration tested, indicating no cytotoxicity in the preliminary test. Absence of cytotoxicity was determined as mentioned in OECD Guideline 471, that is there was no reduction in the number of revertant colonies, no clearing or diminution of the background lawn, no decrease in degree of survival of treated cultures, in presence and absence of metabolic activation. Taken together, the results of this bacterial reverse mutation assay establish that EGCG does not cause a positive mutagenic response with any of the tester strains, either the presence or absence of metabolic activation, under the conditions of the assay.

Presently, TB continues to be a rampaging global disease with high morbidity and significant mortality, especially in undernourished populations or those already infected with HIV and other concurrent infections, which reduce the immunity of the patient^[1]. Emergence of MDR strains has added to the difficulties of treating this dreaded disease with the existing directly observed treatment short course (DOTS) regimen, which calls for a high degree of compliance^[2]. Further, many of the frontline drugs against TB are known to possess severe side effects. This necessitates a rethink in our approach to the treatment of this insidious disease. EGCG, a polyphenol found in green tea has been reported to have wide antimicrobial properties^[8]. However, this compound has not been evaluated, in detail, for the inhibition of MT, although few reports have mentioned some antimycobacterial activity based on enzyme inhibition studies in combination with compounds, such as triclosan^[9]. It was therefore decided to evaluate EGCG and few derivatives using a combination of approaches comprising *in silico* and *in vitro* studies. The results of *in silico* docking studies indicated good docking energy of the geometrically optimized ligand EGCG with InhA receptor 2NV6, an important link in the FAS enzyme complex responsible

for synthesis of mycolic acid in Mycobacteria. Earlier, Ramesh *et al.* have docked epigallocatechin (EGC) to a homology-modelled domain of ketoacyl-ACP reductase (KR) protein of MT^[20]. In the present study, EGCG, the gallate ester of EGC was able to be successfully docked on to enoyl reductase receptor 2NV6 with good binding energy. Presence of number of common residues between EGCG and isoniazid already present in the receptor further indicates similar binding sites. Presence of five hydrogen bonded residues in the docked structure further substantiates our *in silico* findings. He *et al.* have earlier reported synthetic carboxamides to inhibit the enoyl ACP protein^[21]. However, to the best of our knowledge, there are no reports of inhibition of InhA of Mtb by EGCG alone by docking studies.

The above findings were corroborated by the results of our *in vitro* findings wherein we observed that EGCG was able to inhibit growth of *M. smegmatis*, a *Mycobacterium* genetically similar to MI, both in the microplate dilution inhibition assay as well as the sensitive alamarBlue assay. However, the derivatives permethyl EGCG and EGCG perpropionate showed only slight inhibition of *M. smegmatis* in the microplate dilution assay and no inhibition in the alamarBlue assay. Utenova *et al.*^[16] have earlier tested EGCG and few derivatives, such as permethyl EGCG and EGCG perpropionate for their TB inhibition properties against MT (H37RV) in Bactec 12B medium using the alamarBlue assay^[14]. They have observed that while permethyl EGCG and EGCG perpropionate showed some inhibition, EGCG itself did not show any TB inhibitory activity. On the other hand, present findings regarding the MICs of EGCG (MIC between 8-16 µg/ml) are broadly in agreement with those of Sivakumar *et al.* who have studied the antimycobacterial properties of few medicinal plants, such as *Allium* species by alamarBlue assay^[22]. They have reported the inhibitory properties of these compounds towards *Mycobacteria* at concentrations of 50-100 µg/ml. Ugwu *et al.* have also shown that the inhibitory concentrations of carboxamides against MT is in the range of 25-50 µg/ml^[23]. Cho *et al.* showed that the inhibitory concentrations of EGCG against the antibiotic resistant *Imipenem Klebsiella pneumonia* strains to be in the range of 300-650 µg/ml^[12].

The Ames test is a well-established, robust, *in vitro* mutagenicity assay used by regulatory agencies, such as OECD to screen lead compounds for their mutagenic potential^[24]. EGCG was found to be non-mutagenic by this assay and actively suggests towards being a promising candidate therapeutic molecule for further

investigations. In conclusion, present *in silico* and *in vitro* studies strongly suggests that EGCG is able to inhibit *M. smegmatis*. This compound is also non-mutagenic as observed in the Ames test. Further investigations towards the development of EGCG as an adjunct therapy for TB are recommended based on the present promising findings. Additional studies, such as molecular dynamics simulation of EGCG, 2NV6 and their complex are being performed by the authors.

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Conflict of interests:

None declared.

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