



SELECTED PURIFIED PLANT COMPOUNDS AS POSSIBLE INHIBITORS OF RV1819C A DRUG EFFLUX PUMP (ABC PROTEIN) FROM MYCOBACTERIUM TUBERCULOSIS

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Abstract: Multidrug-resistant tuberculosis (MDR-TB) is among the most worrisome aspects of the pandemic of antibiotic resistance because TB patients that fail treatment have a high risk of death. The active multidrug efflux pump (EP) has been described as one of the mechanisms involved in the natural drug resistance of bacteria, such as mycobacteria. *Rv1819c* a putative efflux pump ATP binding cassette (ABC) protein gene from *Mycobacterium tuberculosis*, was cloned and transformed into *Corynebacterium glutamicum*. Susceptibility to standard anti-TB drugs and purified plant compounds, in the presence or absence of standard efflux pump inhibitors (EPIs), (carbonyl cyanide m-chlorophenylhydrazone (CCCP), reserpine and verapamil) was determined. A fluorometric method was used to assess the ability of the purified plant compounds to inhibit efflux pumps in comparison with three standard EPIs: reserpine, verapamil, and CCCP. Three of the plant compounds coded Ma8, IXLE1B and IXLE2FA were found to have potent antibacterial activity with the extract from *Mammea africana* (Ma8) being the most potent with an MIC of 4 mg/L. The three purified plant extracts were also shown to reduce the efflux of ciprofloxacin from the mycobacteria cells. The plant extracts have the potential to augment conventional drugs in the treatment of drug resistant *M. tuberculosis* upon further studies.

Keywords: *Mycobacterium tuberculosis*, ATP mediated drug extrusion, drug resistance, *Corynebacterium glutamicum*, *Rv1819c*, ciprofloxacin

INTRODUCTION

Tuberculosis (TB) remains a serious public health threat around the world, and according to the World Health Organization^[1], nearly two billion people are infected with *Mycobacterium tuberculosis*, with about 8.8 million being new TB cases and 1.1 million deaths among HIV-negative cases of TB and an additional 0.35 million deaths among people who were HIV-positive^[2]. Moreover, the emergence of multidrug resistant tuberculosis (MDR-TB), caused by *M. tuberculosis* simultaneously resistant to isoniazid and rifampicin, the two most effective anti-bacillary drugs used in TB therapy, represents a challenge to the control of the disease since 650,000 of the TB cases in 2010 are estimated to be MDR-TB cases^[1]. Most of the estimated number of cases in 2010 occurred in Asia (59%) and Africa (26%). Zimbabwe is amongst the top 20 most TB burdened nations in the world. MDR-TB when it becomes resistant to any of the fluoroquinolones and any of the second line injectable drugs is termed extensively drug resistant TB (XDR-TB)^[2]. In India, a hospital recently reported tuberculosis caused by strains of *Mycobacterium tuberculosis* that were classified as “totally drug resistant” (TDR-TB)^[3].

Chromosomal gene mutation has been considered the single cause for antibiotic resistance in *M. tuberculosis*, with multidrug resistance arising as a

consequence of sequential accumulation of spontaneous mutations in genes encoding drug targets^[2, 4]. The analysis of genome sequences has shown that mycobacteria have multiple putative efflux pumps^[5] and to date, several pumps have been identified in various species of mycobacteria in association with low level resistance to various compounds, including isoniazid and rifampicin^[6,7,8]. In general, increased activity of efflux systems is responsible for conferring low-level resistance to antibiotics, contrasting with the high-level resistance caused by mutations in genes encoding for the primary targets of these antibiotics^[8]. Increased activity of efflux systems results in the reduction of intracellular levels of the antibiotic, which may enable the survival of a bacterial subpopulation under constant stress promoted by a sub-lethal level of antibiotic. During this period, mutants with alterations in the genes that favor resistance can be selected, therefore insuring the establishment of an antibiotic resistant population that is clinically significant^[2]. It is this sub-population of bacteria that may then accumulate mutations with prolonged exposure to a constant concentration of antibiotic^[9]. There is strong evidence that when challenged with antibiotics, *M. tuberculosis* reacts by a prompt efflux mediated response^[3]. Efflux pumps are attractive antimycobacterial targets and inactivation or silencing of the efflux pumps could be a possible mechanism for controlling drug resistance^[8]. Co-

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administration of an efflux pump inhibitor (EPI) with an antibiotic has progressed to human trials^[10]. Due to the complexity of the pathology of *M. tuberculosis* it is unlikely that a single new drug will be enough^[11].

Many drugs today are derived from plant natural products or depend upon a plant natural product for their development^[12,13]. The discoveries of the antimalarials and the anticancer agent taxol indicate the continuing importance of plant species in drug discovery. Anti-tuberculosis compounds from natural sources have an enormous potential for the development of new drugs, which have shown not only antimicrobial activity per se but also inhibition of the mechanism of resistance such as efflux pumps or modulation of the immune response (e.g. macrophage stimulation). The isoflavone biochanin A exhibited efflux pump inhibiting activity comparable to that of verapamil in *M. smegmatis* mc²¹⁵⁵^[14]. To our knowledge, few EPIs for mycobacteria have been identified so far^[15]. Even though several experimental compounds, such as reserpine, chlorpromazine, verapamil and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), have been shown to have EPI effects against mycobacteria both in vitro and ex vivo^[16], these compounds have not yet fulfilled certain requirements of clinical relevancy such as serum concentration, toxicity, immunosuppression, and stability and solubility concerns in medicine^[17]. Therefore, it is important to explore new EPIs for mycobacteria. Using *Corynebacterium glutamicum* as a model for expressing *Rv1819c*, a putative membrane protein from *M. tuberculosis*, we investigated the interaction of purified natural plant compounds with the efflux pump.

MATERIALS AND METHODS

Materials

Ciprofloxacin was obtained from Fluka BioChemica (Steinheim, Germany). Efflux pump inhibitors, reserpine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), verapamil and all other antibiotics were obtained from Sigma Aldrich (Steinheim, Germany). All the chemicals used were of the highest grade. Plant compounds coded Ma8, IXLE1B and IXLE2FA were extracted from *Mammea africana*, *Chrysophyllum albidum* and *Ixora coccinea* respectively [Figure 1]. These compounds were isolated by flash chromatography, followed by sephadex LH-20 column chromatography and finally by preparative thin layer chromatography. The purity of each was established by thin layer chromatography and then by NMR. Characterization of the structures was achieved using full spectroscopic techniques, NMR (¹H, ¹³C, COSY, HMBC and HMQC) as well as HRMS. The compounds were selected after bio guided

fractionation of the crude extracts revealed strong antimycobacterial activity.

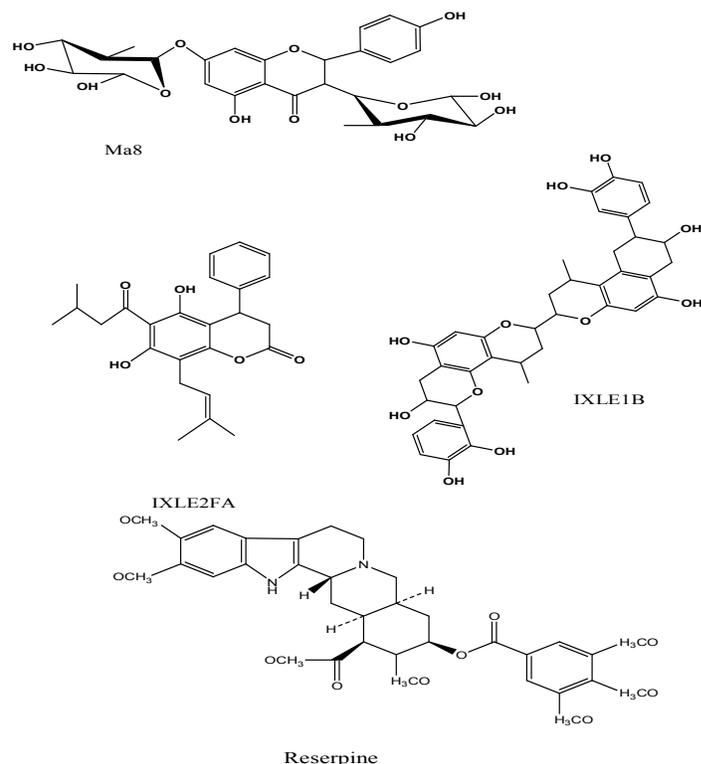


Figure.1: Structures and code names of the most active plant compounds. Ma8 was extracted from *Mammea africana*; IXLE1B and IXLE2FA were extracted from *Chrysophyllum albidum* and *Ixora coccinea* respectively. These compounds were isolated by flash chromatography, followed by sephadex LH-20 column chromatography and finally by preparative thin layer chromatography. The purity of each was established by thin layer chromatography and then by NMR. Characterization of the structures was achieved using full spectroscopic techniques, NMR (¹H, ¹³C, COSY, HMBC and HMQC) as well as HRMS.

Bacterial strains, culture media and growth conditions

Luria-Bertani (LB) broth supplemented with 0.05% glucose was used to culture the *C. glutamicum* strains. Kanamycin A (50µg/ml) was also added since the *C. glutamicum* strains used in this experiment carried a gene for kanamycin resistance as a selection marker. All the cultures were incubated in a shaking incubator (Labcom Labdesign Engineers, Germany) at 30°C rotating at 200 revolutions per minute (rpm).

Cloning of *Rv1819c* from *Mycobacterium tuberculosis* in *C. glutamicum*

Cloning and transformation: *E. coli* (J109, Promega; Top 10 Invitrogen or BL21 (DE3) Novagen) was transformed by conventional heat shock

transformation and plated on LB agar (Biolab, Merck, Gauteng, South Africa) containing appropriate antibiotics (hygromycin B, Roche, Mannheim, Germany) at 150 µg/ml, kanamycin (Roche, Mannheim, Germany) at 50 µg/ml, Ampicillin (Sigma-Aldrich,) at 100 µg/ml. For blue-white selection, 0.5 mM IPTG (Sigma-Aldrich,) and 40 µg/ml X-Gal (Promega, Madison, USA) were added. All sub-cloning and cloning were carried out in the three strains of *E. coli* unless otherwise stated. *E. coli* cultures were grown in LB medium (Merck) containing appropriate antibiotics. *M. tuberculosis* ABC Rv1819c was amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template using Expand High Fidelity DNA polymerase™ (Roche) [Figure 2]. The Primer Designer software version 3.0 was used to design primers and the primer sets for Rv1819 were as follows; the forward primer was 5'-GGATCC-TTGGGCCCGAAATTGTTAAG-3' and reverse primer was 5'-GGATCCATACACTTCGGCGGGCCG-3'. For insertion into expression plasmids, the *M. tuberculosis* ORFs was cut out from the cloning vectors using *Bam*HI and inserted into *Bam*HI-digested and dephosphorylated expression plasmids. Once the correct orientation of the insert had been determined by restriction analysis, *C. glutamicum* was transformed. *C. glutamicum* ATCC13032 was electroplated with the recombinant plasmid as described below and plated on LB agar (Difco, Becton Dickinson, Sparks, USA) containing kanamycin (25 µg/ml)^[18]. *C. glutamicum* cultures were grown in LB medium supplemented with 0.5% glucose. Sequence identity and determination of lack of mutations introduced by PCR was verified by sequencing the entire insert. Plasmid pECXK99E is a *C. glutamicum* / *E. coli* shuttle expression vector. Plasmid pECXK99E (his+) was derived from pECXK99E and was a generous gift from Prof. D. Steenkamp (Chemical Pathology, University of Cape Town, South Africa). Plasmids were isolated from *E. coli* Top 10 cells (Invitrogen) using the Plasmid Midi Kit according to the manufacturer (QIAGEN, Germany). Small-scale plasmid isolations were performed according to the protocol described by Schürch^[19]. The DNA concentration was determined by measuring absorbance at 260 and 280 nm using an Agilent 8453 diode array UV-spectrophotometer (Agilent Technologies Deutschland, GmbH, Waldbronn, Germany).

Electroporation: The electroporation medium (Epo) was made as described by van der Rest et al.,^[18]. A microgram (1µg) DNA was added to the mixture on ice and transferred to an electroporation cuvette (Biorad Laboratories, CA. USA) interelectrode distance of 0.2 cm. Electroporation was performed with parameters set at 25 µM, 600 Ω and 2.5 kV, yielding

pulse duration of 11.6-14.6 ms a gene pulser II (Biorad Laboratories, CA. USA). Immediately after electroporation, LB glucose (LB containing 2 % glucose) at room temperature was added and the suspension transferred to a 1.5ml Eppendorf tube. The tube was incubated for 6 minutes at 46°C in a water bath to guarantee optimal heat transfer. After the heat shock, cells were then incubated for 1 hour at 30°C to allow for recovery and expression of the kanamycin resistance marker. The cells were plated on LB medium containing 25 µg kanamycin/ml. The recombinant strain of *C. glutamicum* now harboured the plasmid pECXK99E (His+) that possessed an insert that coded for the Rv1819 ATP Binding Cassette (ABC) protein pump from *M. tuberculosis*. A schematic diagram of the cloning and transformation procedure is shown in Figure 2.

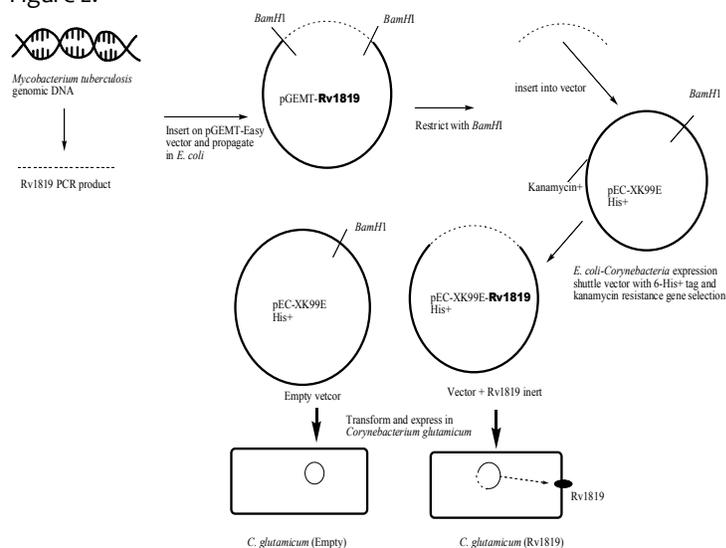


Fig.2: Schematic representation of the cloning of Rv1819c gene from *Mycobacterium tuberculosis* into *Corynebacterium glutamicum*. The *M. tuberculosis* ORFs was cut out from the cloning vectors using *Bam*HI and inserted into *Bam*HI-digested and dephosphorylated expression plasmids. Plasmid pEC-XK99E His+ with the Rv1819c gene was then electroplated into *C. glutamicum* ATCC13032 as described in the text.

Antibiotic susceptibility testing

The susceptibility of *C. glutamicum* derivatives containing the plasmid pEC - XK99E to ciprofloxacin, isoniazid, ethambutol, streptomycin, erythromycin and the purified natural plant compounds was tested. The MIC was determined in *C. glutamicum* strains containing the cloning vector (pEC-XK99E) and the recombinant plasmid pEC-XK99E/Rv1819c after induction using 1mM IPTG. Both the microdilution method and the agar disc diffusion methods were used in the determination of minimum inhibitory concentration determination (MICs). The disc diffusion method was used to screen out plant compounds that

had activity against *C. glutamicum*. MICs were determined at least three times for each compound.

The microdilution method was modified by performing the dilutions in micro-titre plates instead of test tubes. The antibiotic concentration ranges studied were 128mg/L to 0.0625mg/L for standard antimycobacterial drugs. The MIC of plant compounds, Ma8, IXLE1B and IXLE2FA were also determined. The plates were incubated at 30°C for 24 hours at 200 rpm after an initial reading of the optical density at 600 nm was done on a Biotek EL-340 (Pegasus Scientific Inc. Columbia Pike, Burtonsville) microplate reader. The MIC was determined as the value of the concentration of the compound that gave the same reading as the negative growth control after 24 hours. The MICs were also determined in the presence of standard EPIS; reserpine, CCCP and verapamil. A fixed concentration of EPIS (100 µM) was used with a varying concentration of standard anti-Tuberculosis drugs. To determine the activity of the purified plant compounds as efflux pump inhibitors, MICs of standard TB drugs were performed in the presence of half of the MIC values of the plant compounds.

In the disc diffusion method paper discs impregnated with the test compounds were placed on agar plates which were inoculated with the *C. glutamicum* strains^[20]. The agar plates were put at 4°C for one hour to allow the test compounds to diffuse onto the agar. After one hour the plates were placed in an incubator set to 30°C overnight^[21]. The effect of the standard efflux pump inhibitors (EPI) and the purified plant products on growth inhibition were also determined. A fixed concentration of EPIS (100 µM) was used with a varying concentration of standard anti-tuberculosis drugs. Minimum inhibitory concentration (MIC) in this study was taken as the concentrations where no zone of inhibition was observed^[21].

Accumulation of ciprofloxacin in *C. glutamicum*

Accumulation of ciprofloxacin was measured by the method of Xia et al.,^[22] with a few modifications. Bacteria were grown in LB broth with 1mM IPTG to induce the expression of the Rv1819c gene, at 30°C to an A₆₆₀ of 0.6 - 0.8 and harvested by centrifugation at 8 000 rpm for 5 minutes. Bacteria were then washed once with 50 mmol/L sodium phosphate buffer (pH 7.0) at 4°C and resuspended in that same buffer at about 40 mg (dry weight) cells/ml. The cells were placed in a water bath set at 30°C for 15 minutes. Samples were split into sample A and B and maintained at the same temperature. Sample B was the baseline control where EPIS were not added. Ciprofloxacin was added to both samples to a final concentration of 20mg/L. Samples of 750 µL were

removed at 10, 20, 40 seconds, 1, 2, 3, 4, 5, 15, 30, 45 and 60 minutes after the addition of the fluoroquinolone to the bacteria from both A and B. The active efflux of fluoroquinolone was then examined in the presence of the purified plant compounds and standard efflux pump inhibitors reserpine, verapamil and CCCP from stock solutions of 100µM in DMSO. The inhibitors were added to sample A only, to a final concentration of 100µmol/L^[21]. Aliquots of 750µL were removed at 5, 15 and 30 minutes after the addition of the efflux pump inhibitors.

Samples were diluted immediately with 750µL chilled sodium phosphate buffer (pH 7.0) on ice, and then centrifuged in a Hermle Z233 MK-2 microcentrifuge (MIDSCI, St. Louis, USA) at 8000 r/min, 4°C, for 5 minutes. The bacteria were washed again in the chilled buffer and re-centrifuged for 5 minutes. The cell pellet was then suspended in 3.0ml glycine hydrochloride (0.1 mol/L, pH 3.0) for 2 hours at 30°C, and centrifuged at 8000 rpm for 10 minutes. The supernatant was centrifuged for another 5 minutes. Fluorescence of the ciprofloxacin in the final supernatant was determined at the excitation and emission wavelengths of 270 nm and 452 nm respectively spectra using a fluorescence spectrophotometer RF-1501 Shimadzu spectrofluorometer (Shimadzu cooperation, Kyoto, Japan). A standard curve of ciprofloxacin in glycine hydrochloride (0.1 mol/L, pH 3.0) was prepared in order to quantify the accumulation of ciprofloxacin in the bacteria.

Statistical Analysis

All values are expressed as mean ± standard deviation and the comparison of ciprofloxacin concentration for the standard EPI's was evaluated by applying t-test. P ≤ 0.05 values were considered to indicate statistically significant difference.

RESULTS AND DISCUSSION

Cloning of Rv1819c in *Corynebacterium glutamicum*

Using the *Corynebacterium glutamicum*-*Escherichia coli* shuttle plasmid pECXK99E (His+), the gene encoding drug transporters; Rv1819c, from *M. tuberculosis* was successfully cloned into *C. glutamicum*. The presence of Rv1819c gene in *C. glutamicum* was verified by sequencing, colony PCR and restriction digestion of the recombinant plasmid (data not shown). The expression of the Rv1819c gene in *C. glutamicum* was verified by SDS PAGE [Figure 3].

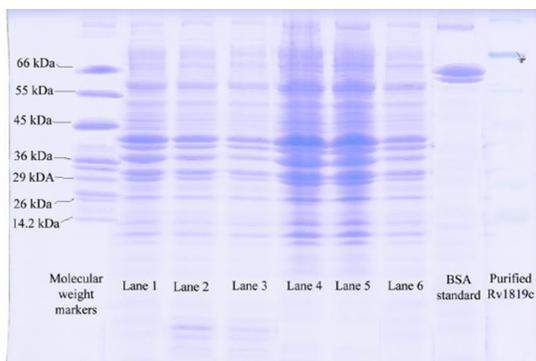


Figure.3: SDS-PAGE analysis of aliquots from different stages of purification of *C. glutamicum* membrane proteins showing the evidence of expression of the Rv1819c protein. 15 % SDS/polyacrylamide gel was stained with Coomassie Blue. Approximately 1 µg of protein was loaded in each lane. Lanes 1, 2, 3, 4 and 5 represent the non-bound fraction, wash fraction, affinity pool, culture lysate, crude membrane fraction and the enriched crude membrane fractions respectively. Standard molecular weight markers, Bovine serum albumin (BSA) and the purified membrane fraction are as labelled in the diagram. The MW of the purified Rv1819c membrane protein was approximately 70 kDa.

Antibiotic susceptibility testing

Minimum inhibitory concentration is defined as the lowest concentration of the drug causing complete inhibition of bacterial growth. The disc diffusion method was used to screen out purified plant compounds that had activity against *C. glutamicum*. *M. tuberculosis* and *C. glutamicum* are Gram-positive bacteria which share characteristic cell walls common to all other corynebacteriaceae and have a DNA of high G+C content [12]. Drug permeability of the *C. glutamicum* cell wall is comparable to that of *M. tuberculosis* making it a good model for studying drug susceptibility of *M. tuberculosis*. *C. glutamicum* is easier to manipulate genetically, grows faster and does not require cumbersome labs for its containment compared to *M. tuberculosis* [23]. The MICs of the solvents used were also determined (results not shown). During MIC determination, solvent

concentration did not exceed 1% of the final volume. It was, therefore, concluded that the solvents had no significant effect on the growth of *C. glutamicum*.

The MIC of standard TB drugs and the purified plant compounds was determined for the strain transformed with the Rv1819c gene and another strain with an empty plasmid; both strains were induced with IPTG to increase expression of the insert. *C. glutamicum* strain carrying an empty plasmid was more susceptible to test compounds than the strain that was actively expressing the Rv1819c (Table 1) showing that the inserted efflux pump was active. The susceptibility of the *C. glutamicum* strains were also carried out in the presence of sub inhibitory concentrations (experimentally determined to be 100µM) of the standard efflux pump inhibitors verapamil, CCCP and reserpine (Table 1). Generally, susceptibility to test compounds in the presence of efflux pump inhibitors increased. The EPIs generally decreased the MIC of test compounds by a factor of 2 for the *C. glutamicum* strain carrying the Rv1819c gene. The observation that known inhibitors of ABC transporters are able to inhibit efflux activity thereby increasing susceptibility to drugs indicates the presence of an active efflux system [24]. Compounds affecting efflux pumps would be expected to react synergistically or modulate the activity of antibiotics with targets located within the bacterial cell (i.e. ciprofloxacin), the internal concentrations of which depend on the efficacy of efflux pumps [25]. Cumulative research efforts into the study of antibiotic resistance mechanisms have implicated efflux pumps as a major underlying factor in intrinsic resistance [7]. ATP-dependent drug transporters have been characterized in other bacteria, which mediate the resistance to structurally different compounds [26]. All together, these data suggest that the Rv1819c pump may contribute to the intrinsic resistance of *C. glutamicum* to drugs by an efflux mechanism and that the efflux can be reversed by the use EPIs.

Table.1: Minimum inhibitory concentrations (MICs) of *C. glutamicum* strains against standard TB drugs and the most effective plant compounds in the presence and absence of standard EPIs.

Antibiotic	MICs (mg/L)				
	<i>C. glutamicum</i> with a blank plasmid	<i>C. glutamicum</i> with induced Rv1819c gene	In the presence of 100 µM CCCP	In the presence of 100 µM verapamil	In the presence of 100 µM CCCP
Isoniazid	>0.0625	>0.0625	>0.0625	>0.0625	>0.0625
Ethambutol	10 ± 2	12 ± 3	14 ± 2	14 ± 4	15 ± 3
Ciprofloxacin	4	8 ± 1	2	4	4
Erythromycin	8 ± 1	20 ± 3	8 ± 1	4 ± 1	8 ± 2
Streptomycin	18 ± 2	64 ± 4	14 ± 3	17 ± 2	17 ± 2
Ma8	6 ± 1	16 ± 2	8	4	4
IXLE1B	20 ± 2	64 ± 3	52 ± 4	32	64
IXLE2FA	32	64	32	32	32

C. glutamicum was incubated with test compounds for 24 hours in the presence and absence of efflux pump inhibitors (EPIs), reserpine, CCCP and verapamil. *C. glutamicum* with induced Rv1819c gene was tested in the presence and absence of efflux pump inhibitors (EPIs). Values are mean \pm SD for n = 6.

In order to further the search for novel TB therapeutics as well as elucidate the role of an efflux pump from *M. tuberculosis* in drug resistance, we looked at the effects of purified plant compounds on *C. glutamicum* expressing Rv1819c, an ABC efflux pump from *M. tuberculosis*. We determined the MICs of the plant compounds on *C. glutamicum*, a suitable model for *M. tuberculosis*. MICs are used by diagnostic laboratories, mainly to confirm resistance, but most often as a research tool to determine the in-vitro activity of new antimicrobials^[27]. Of all the plant compounds tested, three were found to have potency comparable to drugs currently being used for the treatment of TB. Ma8 extracted from *Mammea africana* proved to be the most potent of all the four compounds with an MIC of 16 ± 2 mg/l against the *C. glutamicum* that was actively expressing an efflux pump (Table 1). Ma8 is a phenolic compound which is a flavanone derivative [Figure 1]. The other two compounds had significant activity in comparison with

the standard TB drugs used in the study. All compounds under study were extracted from plants which have antibacterial properties; IXLE1B and IXLE2FA were extracted from *Chrysophyllum albidum* and *Ixora occinea* respectively [Figure 1]^[28, 29, 30, 31]. The MICs of all the compounds compared well with the MICs of other natural plant compounds studied against *M. tuberculosis* like those of diospyrin extracted from *Euclea natalensis* (MIC of 50mg/L)^[31]. A series of flavonoids, chalcones and chalcone-like compounds were evaluated for inhibitory activity against *M. tuberculosis* H37Rv and flavanones were amongst the most active compounds^[32]. To elucidate the probable mechanism of action of the three plant compounds we incubated sub inhibitory concentrations of the compounds together with test compounds on the *C. glutamicum* that actively expressing an efflux pump. All three compounds showed the ability to reverse the increase caused by the expression of the efflux pump on the *C. glutamicum* strains (Table 2); hence they can be classified under inhibitors of ABC efflux pumps as their activities also compared with standard efflux pump inhibitors (Table 1). This study shows new potential efflux pump inhibitors that could augment TB chemotherapy albeit after more rigorous studies to ascertain efficacy on actual *M. tuberculosis*.

Table.2: Effect of plant compounds as efflux pump inhibitors on the MICs of *C. glutamicum* strains when tested in synergy with standard anti-tuberculosis drugs.

Antibiotic	MICs (mg/L)				
	<i>C. glutamicum</i> with a blank plasmid	<i>C. glutamicum</i> with induced Rv1819c gene	In the presence of 8 mg of Ma8	In the presence of 10 mg of IXLE1B	In the presence of 32 mg of IXLE2FA
Isoniazid	>0.0625	>0.0625	0.063	>0.0625	>0.0625
Ethambutol	10 ± 2	12 ± 3	7 ± 2	10 ± 4	12 ± 3
ciprofloxacin	4	8 ± 1	4	4	4
erythromycin	8 ± 1	20 ± 3	11 ± 1	15 ± 1	14 ± 2
streptomycin	18 ± 2	64 ± 4	10 ± 3	17 ± 2	17 ± 2
Ma8	6 ± 1	16 ± 2	*nd	4	4
IXLE1B	20 ± 2	64 ± 3	24 ± 4	*nd	32
IXLE2FA	32	64	20 ± 2	32	*nd

Cells with *nd represent cases where the test compound would also be the EPI under test. *C. glutamicum* with induced Rv1819c gene was incubated with test compounds for 24 hours in the presence and absence of purified plant compounds, Ma8, IXLE1B and IXLE2FA at half their MICs. Values are mean \pm SD for n = 6.

Ciprofloxacin accumulation

To conclusively ascertain the mode of action of the plant compounds as efflux pump inhibitors, the accumulation of ciprofloxacin in *C. glutamicum* cells expressing the efflux pump in the presence and absence of the plant compounds was monitored by a fluorometric method, as previously described above. Ciprofloxacin is a very hydrophobic quinolone, with a partition coefficient in n-octanol-phosphate buffer (pH 6.8) of 0.062^[33]. The uptake of quinolones by gram-negative bacteria is proposed to occur by passive diffusion through porin channels or the lipid bilayer, depending on the hydrophobicity of the quinolone^[34], and the uptake of quinolones by gram-positive

bacteria is thought to involve simple diffusion across the cytoplasmic membrane^[35].

C. glutamicum cells accumulated ciprofloxacin rapidly, reaching steady-state levels within 5 minutes of incubation [Figure 4 and 5]. As antibiotic efflux pump systems are energy-dependent, bacterial cells accumulate larger amounts of antibiotic in the presence of uncouplers. Ciprofloxacin uptake experiments were carried out in the presence of CCCP, verapamil and reserpine. Upon CCCP addition, ciprofloxacin accumulation increased (Figure 4) compared to the control where the inhibitor was not

added. Ciprofloxacin accumulation also increased in the presence of reserpine and verapamil. The result shows that there were increases in the intracellular accumulation of ciprofloxacin when inhibitors of efflux pumps were added, as shown in Figure 4. The most widely used efflux inhibitors are CCCP, a PMF uncoupler, ortho-vanadate and reserpine are two inhibitors of ATP-dependent efflux pumps, and verapamil is a well-known inhibitor of human P-glycoprotein and bacterial efflux pumps^[36]. These compounds generally abolish the increase in resistance produced by efflux pumps, as proven for the Rv2686c–2687c–2688c efflux pump^[24]. We expected the plant compounds to also cause an increase in the intracellular accumulation of ciprofloxacin via the same mechanism as standard EPIs. The three plant compounds also caused an increase in the intracellular accumulation of ciprofloxacin [Figure 5]. The observation confirmed that the plant compounds achieve their antibiotic activity by inhibiting efflux mechanisms.

The results obtained in this study confirmed that the Rv1819c efflux pump of *M. tuberculosis* contributes to intrinsic ciprofloxacin resistance and we showed that the effect can be blocked by three purified novel plant compounds. The use of bacterial resistance modifiers such as EPIs could facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use such as ciprofloxacin and might even suppress the emergence of MDR strains^[37]. One general strategy to reduce the health threat of resistant bacteria is to block a major bacterial resistance mechanism such as efflux while at the same time interfering with another bacterial pathway or target site. New developments of this approach in the context of dual-action prodrugs and dual-action (or hybrid) drugs in which one action is targeted at blocking the efflux pump and the second action at an alternative bacterial target site (or sites) for the antibacterial action are already showing promise^[38].

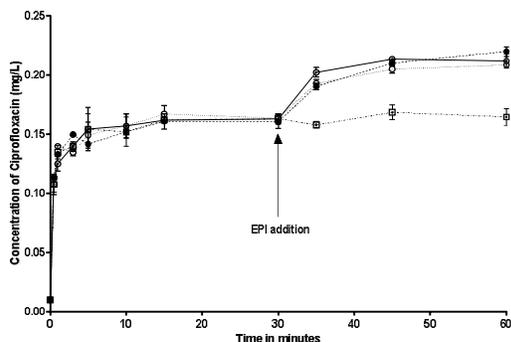


Figure.4: Plot of the intracellular accumulation of ciprofloxacin with time after adding ciprofloxacin to *C. glutamicum* expressing Rv1819c to a final concentration of 20 mg/L and incubating in lysis buffer,

glycine-HCl, for 2 hours. After 30 minutes standard efflux pump inhibitors CCCP (solid line and dotted circles ○), verapamil (dashed and solid circles ●) and reserpine (faint dashed line with an unfilled circles ○) were added to a final concentration of 100 μM. No inhibitor was added to the control (dotted line and dotted squares □). The intracellular concentration of ciprofloxacin was measured by fluoremetry and the results are the average of three experiments, and error bars indicate standard deviations n = 3.

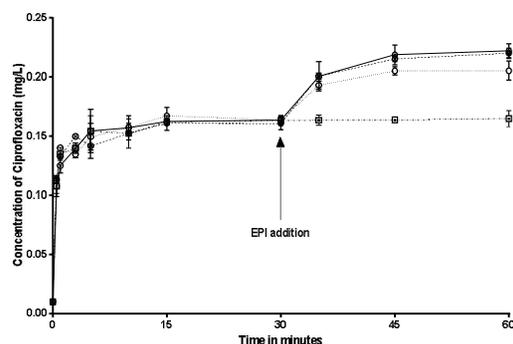


Figure.5: Plot of the intracellular accumulation of ciprofloxacin with time after adding ciprofloxacin to *C. glutamicum* expressing Rv1819c to a final concentration of 20 mg/L and incubating in lysis buffer, glycine-HCl, for 2 hours. After 30 minutes plant compounds Ma8 from *Mammea africana* (solid line and dotted circles ○), IXLE1B (dashed and solid circles ●) and IXLE2FA (faint dashed line with an unfilled circles ○) extracted from *Chrysophyllum albidum* and *Ixora coccinea* respectively were added to a final concentration at half their MIC values. No inhibitor was added to the control (dotted line and dotted squares □). The intracellular concentration of ciprofloxacin was measured by fluoremetry and the results are the average of three experiments, and error bars indicate standard deviations n = 3.

CONCLUSION

This study confirms the role of Rv1819c protein in effluxing ciprofloxacin when expressed in *C. glutamicum* and the ability of the purified plant compounds Ma8, IXLE1B and IXLE2FA to act as efflux pump inhibitors. Our study has revealed new efflux pump inhibitors and antimycobacterial compounds that have potential application against antimycobacterial drug resistance through drug efflux.

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