ANTI-TUBERCULAR ACTIVITY OF EDTA AND HOUSEHOLD CHEMICALS AGAINST *MYCOBACTERIUM SMEGMATIS,* A SURROGATE FOR MULTI-DRUG RESISTANT TUBERCULOSIS

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Abstract
Drug discovery efforts are increasingly being directed at re-purposing old drugs for use in treatment of multidrug resistant tuberculosis (MDRTB) which continues to pose serious health challenges worldwide. Recent studies suggest that *Mycobacterium smegmatis*, with innate resistance to isoniazid, could be used as a surrogate for MDRTB screening in laboratories without adequate containment facilities. The current study utilized resazurin microtiter assay plate (REMA) to screen common household chemicals and non-steroidal anti-inflammatory drugs (NSAIDs) for anti-tubercular activity against *Mycobacterium smegmatis,* as a non-pathogenic surrogate of MDRTB. A ligand-based approach was adopted in selecting household chemicals; using molecular docking tools to probe for binding affinity to Enoyl-[acyl-carrier-protein] reductase (InhA), the main target of isoniazid. Among household chemicals and analgesics studied, EDTA and lauric acid showed the highest activity with minimum inhibitory concentration (MIC) of 31 µg/mL and 7.8 µg/mL respectively, while Ibuprofen and aspirin exhibited activity with MIC of 125 µg/mL. Taken together, this study demonstrates the potential use of EDTA, lauric acid or NSAIDs in treatment of drug-resistant tuberculosis, a major contribution of the current study

Keywords: Multi-drug resistance, repurposing old drugs for TB, resazurin assay, mycobacterium tuberculosis, mycobacterium smegmatis

Introduction

Tuberculosis (TB) is a curable disease that continues to pose major health challenges worldwide, mainly due to increasing incidence of drug resistance and co-infection with HIV/AIDS. Each year, TB is responsible for the death of nearly 1.4 million people across the globe (The Union, 2012). An estimated one-third of the world population is believed to be infected with *Mycobacterium tuberculosis (MTB)*, the causative agent of TB, mostly in the latent infestation form (WHO, 2013; O'Garra *et al*., 2013). As for people living with HIV/AIDS, TB remains the main cause of death yearly. According to the World Health Organization (WHO, 2013), about 13% (1.1 million) of the 8.6 million people who developed TB in 2012 were HIV positive. Of the 1.3 million deaths attributable to TB in 2012, for example, 320 000 deaths (about 20%) were from people living with HIV/AIDS, evidence that this co-infestation with HIV/AIDS has further complicated the management of the TB (WHO, 2013).

Drug resistance arises mostly due to inappropriate use of antibiotics by patients undergoing treatment for drug-susceptible active TB, though a person may also be directly infected by someone with multi-drug resistant TB (MDRTB). The combined effect of potentially severe side effects and required prolonged use make patient compliance to treatment regimen very difficult (Umesiri *et al*., 2010). As a result, a lot of resources – human, financial, and laboratory – are dedicated to adherence counseling and infectious control. Further complicating the control and management of MDRTB is the high cost associated with second-line drugs used for treatment of MDRTB. These factors have led to increasing incidence of drug-resistant strains of MTB. For instance, in 2012, 450,000 people developed MDRTB globally, and 170,000 deaths were reported from MDRTB. Although more than half of these cases were from Brazil, Russia, India and China (WHO, 2013; O'Garra *et al*., 2013); the global picture is still rather alarming. The challenges posed by severe side effects, huge cost and required prolonged use of current MDRTB drugs present a unique opportunity to start exploring new approaches to treating MDRTB.
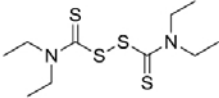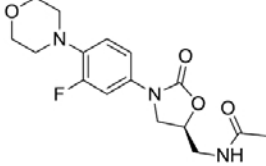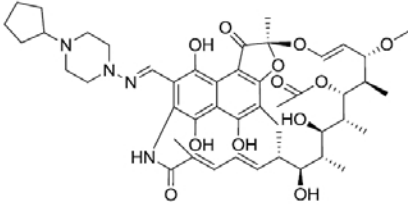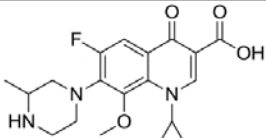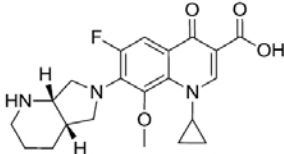
Repurposing Old Drugs for TB

An increasingly popular approach to discovering new TB drugs, especially new drugs against MDRTB, is to re-position and repurpose old drugs that are already approved for other indications. For example, linezolid is currently in advanced clinical trial as a treatment for MDRTB (Nzila *et al*., 2011), even though it has long been used for the treatment of infections resistant to vancomycin, penicillin and methicillin strains. Other drugs currently repurposed for TB treatment (Table 1) include rifapentine (approved for treatment of latent TB in combination with isoniazid for 3

months), disulfiram, the fluroquinolones (Gatifloxacin and Moxifloxacin) seeking regulatory formal registration for use in TB treatment, including MDRTB (Nzila *et al.*, 2011; Stop TB Alliance, 2014). Repurposing old drugs for treatment of TB has significant advantages, namely, existing knowledge of their safety, toxicity, pharmacological profiles lead to huge savings in time, effort and expenses (Guzman *et al.*, 2013).

Repurposing old drugs for new TB treatments take either one of two paths: testing known antibacterial agents against other infections (spectrum expansion), or testing agents with no prior antibacterial profile against mycobacterium species (Nzila *et al.*, 2011).

Table 2: Drugs being repurposed for TB or MDRTB

Drug name	Structure	Original Purpose	TB Indication
Disulfiram		Used in treatment of chronic alcoholism	Yes; against MDRTB and XDRTB
Linezolid		Gram-positive bacterial infections resistant to many other antibiotics	Yes; against MDRTB and XDRTB
Rifapentine		A derivative of rifampicin, general anti-mycobacterial agents	Yes, approved for latent TB infection
Gatifloxacin		4th generation fluoroquinolone antibiotic	Yes, a second-line TB drug
Moxifloxacin		4th generation fluoroquinolone antibiotic	Yes; for use against drug-active TB

Either approach requires whole-cell phenotypic screening. Not surprisingly, whole-cell screening is getting renewed attention in the drug discovery process, compared to the more popular target-based method (Swinney and Anthony, 2011; Schulz *et al.*, 2012; Visnyei *et al.*, 2011).

135

Whole-cell phenotypic screening approach does not require clear understanding of underlying MMOA to be successful, and therefore has the unique advantage that it is able to interrogate all biochemical targets simultaneously in a specific physiological environment (Swinney and Anthony, 2011; Schulz *et al*., 2012; Visnyei *et al*., 2011). This is even more important when dealing with a disease like TB that may involve different biological targets, and therefore presents a more efficient method to screen for actual biological activity against MDRTB as available data seem to suggest. For example, the first FDA approved antitubercular drug in over 40 years, bedaquiline (Mahajan, 2013), and others in advanced clinical development (delamanid, sutezolid and SQ109) were discovered through phenotypic screening (Horita *et al*., 2012; Sotgiu *et al*., 2013). Thus, no truly new anti-TB lead seem to have successful emerged from target-based discovery approach.

Resazurin Microtiter Assay: Simple, Inexpensive Phenotypic Screening Method

Consequently, phenotypic cell viability screens have become the primary high-throughput method for identification of potentially new TB leads. The utility of Alamar Blue and other cell viability assays for screening of clinical isolates of MTB have been demonstrated: BACTEC TB-460 system (Collins and Franzblau, 1997), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (Abate *et al*.,1998; Franzblau *et al*., 1998, Mycobacterium Growth Indicator Tube (Walters and Hanna, 1996), uracil uptake (Chung *et al*., 1995), E-test (Wagner and Mills, 1996), and microplate Alamar blue assay Martin *et al*., 2003; Taneja and Tyagi, 2007), among others. In addition, several studies have clearly shown the reliability of Alamar blue cell viability assay for detection of drug resistant MTB (Palomino *et al*., 2002; Bwanga *et al*., 2009). The resazurin-based assay is a simple, inexpensive method with very high diagnostic accuracy, and lends itself to easy use in countries or even laboratories with limited resources (Chauca *et al*., 2007).

1.3 Mycobacterium smegmatis as potential surrogate for MDRTB

Expectedly, the highly infectious nature of MDRTB is a limiting factor in its use in high throughput screening of probable new TB agents. Fortunately, the utility of *M. smegmatis* in screening for compounds active against MDRTB was recently demonstrated by Chaturvedi and coworkers (Chaturvedi *et al*., 2007). *M. smegmatis* does not require specialized containment facilities, grows relatively fast, and is a good predictor of activity against MDRTB (Chung *et al*., 1995; Chaturvedi *et al*., 2007; Li *et al*., 2004). In addition, *M. smegmatis* is known to exhibit natural resistance

to isoniazid (INH) and rifampicin (Chung *et al*., 1995; Chaturvedi *et al*., 2007; Quan *et al*., 1997). Therefore, in screening selected household chemicals for activity, we have chosen *M. smegmatis* based on previous studies establishing the reliability of *M. smegmatis* as a selective, non-pathogenic, fast growing surrogate for clinical isolates of MDRTB.

In the current study, we investigate the anti-tubercular activity of common household chemicals (boric acid, disodium ethylene diamine tetracetic acid - EDTA, benzoyl peroxide, lauric acid), paracetamol and nonsteroidal anti-inflammatory analgesic (aspirin, ibuprofen, Aleve) against *M. smegmatis*, as a non-pathogenic, fast growing surrogate for multidrug resistant TB. These are safe, cheap, readily available chemicals with known pharmacological profiles, which can be easily and safely deployed as anti-tubercular agents. The objective then is to investigate their potential application for treatment of MDRTB.

Selection of household chemicals was partially based on a ligand-based approach. Since multidrug resistant strains of *MTB* are resistant to INH and RMP, our running hypothesis is that drugs that effectively inhibit the Enoyl-[acyl-carrier-protein] reductase (InhA), with a different mechanism of action from that of INH's role on the same enzyme, may circumvent this resistance. Indeed, several studies seem to bear out this rationale (Vilchèze *et al*., 2011; Sullivan *et al*., 2006)). So, an initial virtual screening was done by docking selected ligands into the active site of InhA receptor (PDB id= 2nsd; resolution of 1.900), to predict drugs likely to bind enzyme target very well. Also, as part of our continued search for potent inhibitors of antigen 85 complex, an acyltransferase enzyme implicated in the synthesis of cord factor and final assembly of mycobacterial cell wall, household chemicals with functional groups known to either inhibit serine or related proteases were chosen (Umesiri *et al*., 2010). Therefore, selected ligands were also docked into antigen 85C receptor (PDB id= 1va5; resolution of 2.020). This approach of combining initial ligand-based rationality with phenotypic whole-cell screening may help to provide preliminary insight into the possible molecular mechanisms of action of select molecules.

Materials and Methods
Materials

M. smegmatis (ATCC 607*)* was procured from American Type Culture Collection Company (ATCC). INH and other drugs and chemicals were sourced directly from manufacturers through VWR International, and used without further purification. Fresh stock of drugs and chemicals were prepared and used fresh for each experiment. For disodium ETDA, lauric acid, boric acid, aspirin, ibuprofen, naproxen (Aleve), and benzoyl peroxide, original stock of drug was prepared in dimethyl sulfoxide

(DMSO) or water at 10 mg/mL. For INH, original stock was prepared in DMSO at 1 mg/mL.

Molecular docking

Molecular docking was carried out with 1-Click Docking tool (Mcule, 2014), a web-based docking application based on Autodock Vina configuration (Trott and Olson, 2010). Virtual screening was done by docking selected ligands differently into the active site of InhA receptor (PDB id= 2nsd; resolution of 1.900) and antigen 85C receptor (PDB id= 1va5; resolution of 2.020). Since our internal docking experiment shows that docking results obtained with either AutoDockVina directly or the 1-Click Docking application were almost identical, we have chosen to use the 1-Click Docking application due to the fact that it is easier, more user-friendly, and meets our current needs. Its limitation is that it may not be adequate for customized advanced docking studies.

Preparation of 7H10 Agar

Preparation of 7H10 Middlebrook Agar medium for *M. smegmatis* growth followed protocols provided by manufacturer, Difco[TM] Middlebrook. To make a half liter batch, 9.5g 7H10 agar was dissolved in 450 mL ultra-purified water in a 1 liter bottle, and 2.5 mL glycerol was added to mixture pipetted using a P-1000 micropippette. The whole mixture was dissolved in microwave for 2 minutes, autoclaved at 121°C for 20 min, and cooled in a 50°C water bath. Afterwards, 50 mL of Middlebrook OADC (oleic acid dextrose catalase) enrichment was poured into the medium aseptically. The enrichment was gently swirled into the agar solution to prevent bubble formation. Sterile plates were set out and agar was poured halfway up the plate, enough to keep the bacteria growing longer. If bubbles appeared, they were flamed gently using a Bunsen burner. It is important to keep the top surface of the agar flat. After all plates were filled, they were stacked upright and left out in room temperature for one day to solidify, and then stored at 4°C.

Preparation of 7H9 broth

Using aseptic methods, and following manufacturer's instructions, Middlebrook 7H9 Broth was prepared. To make a 500 mL batch, 0.94g 7H9 broth was dissolved in 200 mL ultra-purified water in a 1 liter bottle. Using a P-1000 micropipette with a cut tip, 0.4 mL glycerol was added to the mixture, heated in the microwave for 2 minutes to ensure complete dissolution. The broth was autoclaved at 121°C, and was cooled in a 45°C water bath. Next, 40 mL of Middlebrook ADC Enrichment was poured into

the medium and stirred. 5mL 7H9 broth was quickly added to sterile tubes and then capped, and stored at 4°Cbefore using in bacterial cultures.

Bacterial Culture and CFU Counting

Fresh isolates of *Mycobacterium smegmattis* (ATCC 14468) were cultured in Middlebrook 7H11 agar for every experiment. Capped tubes containing pre-made sterile 7H9 broth were warmed to room temperature before inoculating. Meanwhile, an agar plate containing colonies of *M. smegmatis* was already prepared. A colony of the bacteria was transferred aseptically to 7H9 broth, and incubated at 37°C for 3 days.

CFU counting

Bacterial cultures were diluted to an optical density of $OD_{595} = 0.01$ (=4.7 x 10^5 Colony forming units (CFU) per mL). 100 µL of different dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were spread (in triplicate) on Petri dishes containing 25 mL of OADC supplemented Middlebrook 7H10. The cultures were incubated at 37°C for 3 days and the number of colonies was counted.

Resazurin microtiter assay (REMA)

The resazurin microtiter assay (REMA) plate method was performed in 7H9 medium containing Middlebrook broth and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase (Difco^{TM}). Clear- 96-well plates were inoculated with 100 µL of drug diluted with 90 µl of 7H9 medium and 10 µL DMSO (giving essentially 50 µL of drug). Serial twofold dilutions of each drug in 100 µL of 7H9 medium were prepared at concentrations of 0.5 to 0.0039 mg/mL for all drugs, and 0.05 to 0.00039 mg/mL for INH. Growth controls containing no antibiotic and sterility controls without inoculation were also included (Figure 2). To the column containing the control with no bacteria, 10 µL DMSO and 190 µL 7H9 medium was added. To the column containing the control with no drug, 10 µL DMSO, 90 µL 7H9 medium, and 100 µL of bacteria culture were added. Finally, 200 µL of sterile deionized water was added to the outer perimeter wells to prevent evaporation during incubation of the plate. The lid was placed back on the plates and partially sealed with parafilm to allow for air circulation. The plates were incubated plate at 37°C for 3-4 days. After incubation, the plates were removed and 10 µL of resazurin was added to each well that contained bacteria. More water was added to the outer perimeter wells when necessary. The plates were reincubated at 37°C for 24-48 hours and visually assessed for color development. A change from blue to pink indicates reduction of resazurin to resorufin, and therefore an indication

of bacterial growth. The MIC was defined as the lowest drug concentration that prevented this color change, by direct visual examination.
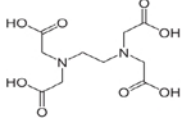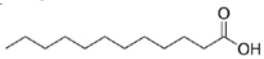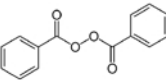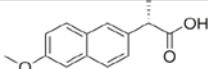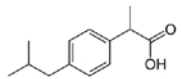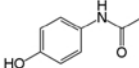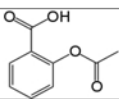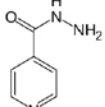
Results
Molecular docking

The best fit pose with the lowest binding energy from initial molecular docking is chosen, and the binding affinity for each ligand with antigen 85C and InhA is reported in kcal/mol (Table 2). Interestingly, the binding affinity of each ligand is very similar for both InhA and antigen 85C.

Minimum inhibitory concentration (MIC)

MIC results from the resazurin-based screening are tabulated in Table 3; showing that lauric acid and EDTA had the lowest MIC values, at 7.8 µg/mL, and 3.1 µg/mL respectively. The MIC for INH, the positive control is at 6.2 µg/mL. As for acetaminophen and the three over-the-counter NSAIAs, aspirin and ibuprofen showed the highest activity against *M. smegmatis*, with MIC of 125 µg/mL (Table 3, and Figure 1).

Table 3: Binding affinity of ligands with Antigen 85C and InhA

Drug:	Chemical Structure:	Ag 85C: Binding Affinity (kcal/mol)	InhA: Binding Affinity (kcal/mol)
EDTA		-5.3	- 6.0
Lauric acid		-5.9	- 5.4
Benzoyl Peroxide		-8.3	-8.3
Naproxen (Aleve)		-7.5	-8.2
Ibuprofen		-7.6	-7.7
Acetaminophen		-6.0	-5.8
Aspirin		-6.5	-6.7
Isoniazid (INH)		-5.2	- 5.3

Discussion

Adopting a ligand-based approach, four over-the-counter analgesics (aspirin, Aleve, acetaminophen, ibuprofen) and four household chemicals (benzoyl peroxide, lauric acid, boric acid, and disodium salt of Ethylenediaminetetraacetic acid, EDTA) were chosen for antibacterial activity against MDRTB using *M. smegmatis* as a non-pathogenic, fast growing surrogate. Our approach involved selection of common household chemicals for anti-tubercular screening based on their ability to successfully bind and potentially inhibit InhA (Table 2).

Benzoyl peroxide (BP) is a common over-the-counter medication for acne vulgaris with broad antibacterial activity. BP has certain characteristics that make it potentially an attractive agent to investigate for anti-MDRTB activity: the ability to penetrate high-lipid environments typical of the mycobacterial cell wall (Decker *et al.* 989), successful use of 5% concentration in treating drug-resistant strains of *Propionibacterium acnes* (Dutil, 2010), and its ability to prevent the selection of erythromycin-resistant *Staphylococcus epidermidis* (Eady *et al.*, 1996). Initial molecular docking carried out with Mcule's 1-Click Docking tool showed that benzoyl peroxide binds well to the active site of InhA, a target of isoniazid; with a binding affinity of -8.3 kcal/mol (Table 2).

Table 4: MIC values for household chemicals screened

Drug:	Current Use, and delivery route	MIC Value (µg/mL)
EDTA	lead poisoning; Intravenously	31.3
Boric acid	Antiseptic; Topically	500
Lauric acid	Food, Viral Infections; Orally.	7.8
Benzoyl Peroxide	Treats acne; Topically	250
Naproxen (Aleve)	Analgesic; Orally.	250
Ibuprofen	Analgesic; Orally.	125
Acetaminophen	Analgesic; Orally.	250
Aspirin	Analgesic; Orally.	125
Isoniazid (INH)	First-line TB drug; Orally.	6.2

As it turns out, the MIC for benzoyl peroxide using REMA was rather high at 250 µg/mL (Table 3 and Figure 1); although its zone of inhibition (17 mm) on Kirby-Bauer disk diffusion assay was decent compared to INH (29 mm), Table 4 and Figure 2. As a radical initiator, its potential application as a fumigation agent against drug-susceptible and drug-resistant MTB in hospitals and related facilities may be more important. 5% vaporized hydrogen peroxide (50,000 µg/mL) has been shown to be effective as fumigation agent against clinical isolates of MTB (Kahnert *et al.*, 2005; Grare *et al.*, 2008). With MIC of 250 µg/mL, benzoyl peroxide appears to be a more potent anti-TB fumigation agent than hydrogen peroxide.

The antimicrobial activity of lauric acid and other medium-chain fatty acid have been reported (Kabara *et al.*, 1972; Nakatsuji *et al.*, 2009; Kanetsuna, 1985). For example, lauric acid was shown to inhibit *Propionibacterium acnes* at a MIC of 3.9 µg/mL. But here, we effectively demonstrate the potential of lauric acid to inhibit drug-resistant TB using *M. smegmatis* as a surrogate, based on resazurin-based microtiter assay plate. At a MIC of 7.8 µg/mL against *M. smegmatis,* lauric acid appears potent enough to be considered a cheap and practical treatment option for MDRTB, especially in combination with other TB drugs. As a naturally occurring chemical which is present in coconut oil, breast milk, among others, lauric acid is nontoxic, safe, inexpensive, and has a long shelf-life.

Even though EDTA has been known to exhibit general antibacterial activity, especially against metalloenzymes and proteases at 32 µg/ml (Upadhye *et al.*, 2009; Aoki *et al.*, 2010), this study demonstrates, for the first time as far as we know, its potential utility in treating MDRTB by utilizing the innate resistance *M. smegmatis* against INH. EDTA showed significant antitubercular activity against *M. smegmatis* with MIC of 31 µg/mL.
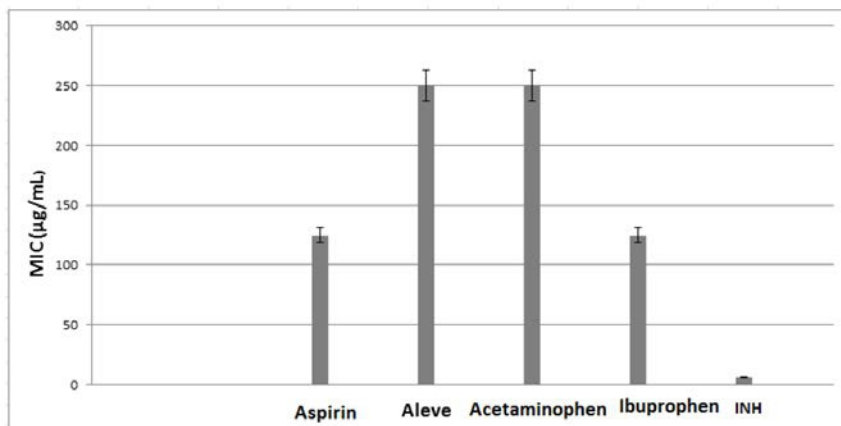
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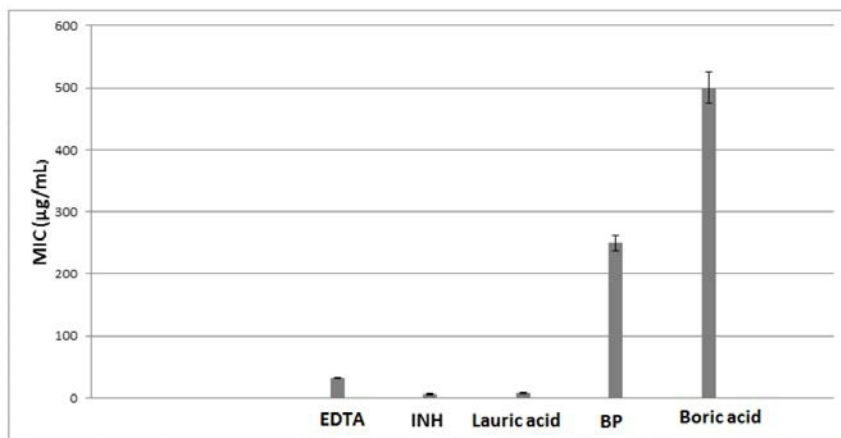
b)

Figure 7: Minimum inhibitory concentration values of a) common household chemicals (EDTA, lauric acid, benzoyl peroxide, boric acid), and b) acetaminophen and NSAIDs; with INH as positive control.

The structural resemblance of EDTA to disulfiram, an alcoholism drug which has shown potent anti-tubercular activity against drug-resistant TB at MIC of 1.56 µg/mL is not lost on us (Horita *et al*., 2012). In that study, disulfiram exhibited activity against *M. smegmatis* at MIC of 25 µg/mL, which is very comparable to the results we obtained with EDTA. Further *in vitro* and *in vivo* studies involving EDTA against clinical isolates of MDRTB is therefore warranted.

Table 5: Zone of inhibition from Kirby-Bauer disk assay

Compound	Zone of Inhibition (mm)
Isoniazid (INH)	29 mm
Lauric Acid	9 mm
Benzoyl Peroxide	17 mm
Aleve (naproxen)	10 mm
Ibuprofen	13 mm
EDTA	0 mm
Boric Acid	0 mm
Acetaminophen (Paracetamol)	0 mm
Aspirin (Acetyl salicylic acid)	0 mm

Boric acid has long been used for medical applications due to its antiseptic, phagocyticidal, antibacterial properties, especially as a topical application for burns or acne, typically at MIC of 2% (Novak & Taylor, 1951). Boric acid showed some activity against *M. smegmatis* at MIC of 500 µg/mL which is more potent than a 2% concentration (20,000 µg/mL) of boric acid used for other indications. Still, 500 µg/mL MIC appears to be too large compared to NIH. Therefore, boronic acid derivatives of larger molecules might be better alternatives than boric acid itself, as shown by our current synthetic efforts with boronic-aurone derivatives (unpublished data).

Figure 8: Kirby-Bauer disk showing antimycobacterial activity of benzoyl peroxide (4) compared to INH (5).

Some of the chemicals tested (EDTA, boric acid, aspirin and acetaminophen) showed little or no inhibition using Kirby-Bauer disk assay (Table 4) but exhibited some mycobacterial activity using resazurin microtiter assay. This may be due to the fact that precipitation was observed for each of those four chemicals on the disk assay, which may have affected rate of diffusion and therefore inhibition. A similar effect was also observed by Juárez-Hernándeza *et al.* (2012) when screening synthetics using both Kirby-Bauer diffusion and MIC methods. The result also suggests that

REMA may be a more sensitive susceptibility test, especially with regard to *M. smegmatis* (Jorgensen & Ferraro, 1998).

Conclusion

Using *M. smegmatis* as a potential surrogate for MDRTB, we have screened a range of household chemicals, including EDTA, lauric acid, benzoic acid, and common over-the-counter analgesics for anti-tuberculosis activity through resazurin-based microtiter assay. Of the household chemicals examined, EDTA and lauric acid showed the highest activity with MIC of 31 µg/mL and 7.8 µg/mL respectively. Of the four analgesics tested, ibuprofen and aspirin showed the highest activity against *M. smegmatis,* with MIC of 125 µg/mL. Naproxen, and acetaminophen showed the same MIC at 250 µg/mL. Results obtained suggest that the use of these chemicals in combinatorial therapy against drug-susceptible and MDRTB needs to be further investigated. In fact, a recent study showed that the use of 20 mg/mL of ibuprofen (oral dose of 80 mg/kg) in adjuvant tuberculosis treatment in murine model of active TB was effective (Vilaplana *et al.*, 2013). Taken together, the current study is important because it demonstrates, for the first time, the potential of EDTA, lauric acid and common analgesics in the treatment of drug-resistant tuberculosis, using *M. smegmatis* as surrogate for MDRTB. Finally, there is a need to carry out further testing using clinical isolates of MDRTB; which constitutes future direction of the above study.

Acknowledgement

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