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THE EFFECT OF DIFFERENT CARBOHYDRATES AND AMINO ACIDS ON THE ACTIVITY OF SELECTED ANTIMICROBIAL COMPOUNDS AGAINST MYCOBACTERIUM SMEGMATIS

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DECLARATION

I, Michael Acheampong Debrah (Department of Biochemistry, Cell, and Molecular Biology, University of Ghana, Legon-Accra), hereby declare and certify that this thesis was done by me under the supervision of Dr. Patrick Kobina Arthur and Dr. Vincent Amarh. All references have been duly cited.

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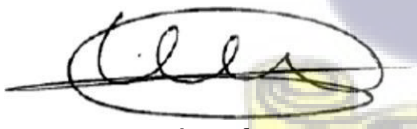

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ABSTRACT

Antibiotics play a significant role in the medical treatment of bacterial diseases. Bacteria respond to antibiotic stress by several means including the initiation of stress responses that promote recruitment of resistance determinants or physiological changes that compromise antimicrobial activity. The overdependence and misuse of these drugs have led to the emergence of resistance in microorganisms which complicates patient management and as such, different strategies are being sought to alleviate this threat that such resistant pathogens pose. One such strategy is chemo-sensitization where compounds are used to potentiate the effect of antibiotics. This study investigates the effect of selected carbohydrates and amino acids on the efficacy of antimicrobial across different classes, against *Mycobacterium smegmatis* strains to provide insight into key cellular processes associated with the functioning of antibiotics, and whose modulation would be key in sustaining antibiotic action and preventing resistance development. Seventeen (17) compounds across fifty-three (53) triple-combinations were screened in an experimental set-up from which 29 combinations were identified that robustly break resistance and 24 that robustly induce resistance. The top two (2) conditions that break resistance (SOR (3X) and MAL (3X)) led to the activation of fifteen (15) antibiotics and the top two (2) that induce resistance (LAC (3X) and SIA+ADO (1.5X)) led to the loss of efficacy of twelve (12) antibiotics. The top two (2) compounds in each category (resistance breaking & resistance inducing) were then prioritized for gene expression analysis looking at a landscape of stress response regulons in both *Mycobacterium smegmatis* (wt) and multidrug-resistant strains. The resistant breaking compounds induced genes such as *vapC* that's responsible for toxicity in the organism, which correlates with its resistant breaking property but also the expression of virulence genes, that is *lsr* is in contrast.

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List of Abbreviations

ADO	Adonitol
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
CELL	Cellobiose
CV	Crystal Violet
DAL	Dulcitol
eMsA	erythromycin-resistant Mycobacterium smegmatis A
eMsB	erythromycin-resistant Mycobacterium smegmatis B
ESKAPE	E- Enterococcus faecium, S- Staphylococcus aureus, K- Klebsiella pneumonia, A: Acinetobacter baumannii, P: Pseudomonas aeruginosa, and E: Enterobacteriaceae
EtBr	Ethidium Bromide
FTC	Functional Triple Combination
GAL	Galactose
GLU	Gluconic Acid
HIV	Human Immunodeficiency Syndrome
INU	Inulin
LAC	Lactose
MAL	Malonic Acid
MALT	Maltose

MDR	Multidrug resistant
MRSA	methicillin-resistant Staphylococcus aureus
Ms wt	M. smegmatis wild type
Mtb	Mycobacterium tuberculosis
MYO	Myo-inositol
NaP	Sodium Pyruvate
NPT	non- phosphotransferase
OD	Optical Density
PBS	Phosphate Buffered Saline
PC	Phenotypic compounds
RAF	Raffinose
RB	Resistance Breaking
RHA(M)	Rhamnose
RI	Resistance Inducing
RNA	Ribonuclease activity
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SIA	Sialicin
SOR(B)	Sorbose
STC	Structural Triple Combination

TB	Tuberculosis
TRY	Tryptophan
XYL	Xylose
ZoI	Zone of Inhibition

DEDICATION

I dedicate this work to all members of Chemical Systems Biology Lab.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Microorganisms are microscopic-sized organisms that include bacteria, archaea, fungi, and protists. These organisms can either be pathogenic or non-pathogenic. In the 20th Century, during the pre-antibiotic era, microbial infections accounted for a significant percentage of morbidities and mortalities (Dougherty & Pucci, 2014). The discovery of penicillin by Alexander Fleming began an antibiotic era, a period of redemption for human health. This addressed challenges with sepsis in advanced procedures like invasive surgery and chemotherapy subsequently increasing life expectancy (Silver, 2011; Tacconelli et al., 2018). During the years 1950 through 1970 described as the "Golden age", many new antibiotic classes having different cellular targets were discovered. Humans seemed to be winning the evolutionary race against microbial infection till resistance to these antibiotics among microbes emerged (Hutchings et al., 2019).

Antimicrobial resistance (AMR) is the evolution of microbes to nullify the effect of antimicrobials. Microbes employ different mechanisms to survive the killing or growth inhibitory effects of antimicrobials. The resistance gained by microbes may be acquired; that is gene transfer among organisms through vectors such as transposons, plasmids, bacteriophages, and integrons, for which plasmids are a major vector for the transfer of resistance genes among bacteria and associated with most resistant phenotypes (C Reygaert, 2018). The resistance may also be inherent through genetic (chromosomal) mutations resulting in cross-resistance (Li & Webster, 2018). Several factors can give rise to antimicrobial-resistant phenotypes such as the extent of expression of resistant genes by microorganism and their ability to endure antimicrobial pressure, just to state a few (Li & Webster, 2018). Microbes also make use of other biochemical types of resistance mechanisms to

elude the effect of antimicrobials such as altering antibiotic targets, biofilm formation, enzymatic degradation, efflux pump overexpression, and so on (Džidić et al., 2008).

AMR, now considered a global health concern, continues to increase primarily because of the non-compliance of health professionals and individuals in adhering to antimicrobial drug prescriptions (Ayukekbong et al., 2017). The problem is exacerbated by the widespread exploitation of antibiotics in aquaculture and farming practices (Manyi-Loh et al., 2018) and a drastic decrease in the discovery and development of new antimicrobial agents against an increasing rate in microbial evolution (Ragheb et al., 2019). The incidence and mortality rate as a result of AMR keeps increasing globally, approximately 700,000 individuals die annually from AMR infections; a number which has been extrapolated to increase to 10 million by 2050 exceeding cancer, which is the leading cause of global mortality (Neill, 2014).

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb) is a global health threat and was the leading worldwide cause of mortality by an infectious disease prior to the COVID-19 pandemic and also the leading cause of death among HIV/AIDS individuals. Over 10 million TB cases and 1.5 million deaths were estimated in 2018(MacNeil et al., 2020) (. The prevalence of TB decreased when anti-mycobacterial drugs such as isoniazid (INH), rifampicin (RIF), pyrazinamide (PZD), ethambutol (EMB) were produced fifty years ago (Chakraborty & Rhee, 2015). Resistance to these anti-tuberculosis drugs occurred soon after their introduction, greatly increasing the difficulty of tuberculosis chemotherapy(Ahmad Khan et al., 2017). This has necessitated the understanding of the molecular mechanism underlying the resistance evolved by Mtb.

Studies have implicated metabolism undertones in antibiotic resistance microbes, especially the ones involved in biofilm formation and latency. According to this view, microbe sensitivity to antibiotics (phenotypic resistance) can be modulated by alterations in microbe's metabolism, signifying an association between microbial metabolism and antibiotic resistance.

Antimicrobial drugs that have lost their efficacy because of AMR, can be revived through the utilization of adjuvants (metabolically relevant non-antibiotic compounds that improve the activity of antibiotics by breaking resistance or by enhancing bacterial susceptibility). Some examples include β-lactamase inhibitor compounds used in medical practices. The synchronal use of antimicrobials and adjuvants aside from enhancing efficacy, also provides a lower toxicity level since less amount of the antibiotic and adjuvant are used (Gill et al., 2015; Sanhueza et al., 2017).

A lot of studies conducted recently have proven the increase in efficacy of antimicrobial agents when combined with certain adjuvants such as amino acids and carbohydrates (Bernal et al., 2013). This current study seeks to examine the effect of selected carbohydrates and amino acids on the bioactivity of different antimicrobial agents and how they modulate *Mycobacterium smegmatis*, *C. albicans,* and *E. coli* phenotypically and genetically.

1.2 Aim

To investigate the effect of certain carbohydrates and amino acids on the antibiotic profile of *Mycobacterium smegmatis* (WT) and two multi-drug resistant strains, Erythromycin-resistant *Mycobacterium smegmatis* A & B.

1.3 Specific Objectives

1. Determine the effect of different carbohydrates and amino acids on the antimicrobial activity of antifungal and antibacterial compounds using phenotypic drug screening assay on *Mycobacterium smegmatis and the two MDR strains, C. albicans,* and *E. coli*.
2. Determine the efficacy of selected compounds against the efflux and biofilm activity of, and *Mycobacterium smegmatis* (WT) and two multi-drug resistant strains, Erythromycin-resistant *Mycobacterium smegmatis* A & B.

3. Analyze the expression patterns of stress response genes in cells treated with growth conditions that increase and decrease drug resistance in *Mycobacterium smegmatis and the two MDR strains*.

CHAPTER TWO

LITERATURE REVIEW

2.1 DISCOVERY AND CHALLENGES REGARDING ANTIMICROBIALS

Alexander Fleming's serendipitous discovery of penicillin some decades ago transformed the course of medicine. The advent of antibiotics and other antimicrobial drugs has served for years as the only effective therapeutic option for the elimination of microbial infection (Dougherty & Pucci, 2014; Silver, 2011). These infections had previously translated to high morbidity and mortality before the discovery of antibiotics. However, the continuous use and misuse of these agents have resulted in the emergence of microbes that are no longer sensitive to these antibiotics (Jackson et al., 2018). The inefficacy of most antimicrobial drugs is a result of over-dependence and inappropriate use of these drugs (Mobarki et al., 2019). AMR can generally be categorized into three main types: acquired (inheritable), inherent (intrinsic), and adaptive (Alekshun & Levy, 2007).

Acquired AMR develops from bacteria's ability to obtain and express additional genetic material excluding genes already present within their primary genome. Mobile genetic elements which cary these new genes include plasmids, transposons, or some other yet-to-be-identified carrier in the immediate environment of the bacteria or other bacteria. This method of transfer and incorporation of new genetic elements is described as horizontal gene transfer (HGT) and resulting resistance may or may not be passed on to offspring.

Intrinsic resistance on the other hand, describes an evolutionary change caused by the interactions of the microbe with its environment. The environment serves as a habitat for natural antimicrobials which interact with bacteria. Bacteria tend to harbour inherent genetic elements of resistance as a result of these natural interactions (Smith, 2017). These resistance patterns in the bacteria evolve

over many years within the bacteria's primary genome and can be directly transferred to new offspring during reproduction (Dcosta et al., 2011).

Finally, adaptive AMR describes more tolerance-focused mechanisms bacteria have taken to withstand the effect of antibiotics. It is characterized by a quick development of resistance and fast reversibility to the non-resistant phenotype when the antibiotic is removed from the medium. Adaptive AMR is highly driven by bacterial exposure to subinhibitory concentrations of antibiotics or gradually increasing antimicrobial concentrations. (Sandoval-Motta & Aldana, 2016).

2.2 ANTIMICROBIAL ADJUVANTS & MECHANISM OF ACTION

In the event that a compound is able to create an environment or trigger signaling pathways that convince these transiently resistant cells exhibiting adaptive AMR properties of the absence of a still-present antibiotic, this leads to a rapid transformation of bacterial cells back to sensitive phenotypes and consequently exposes them to antibiotic concentrations that would otherwise have been non lethal to the resistant phenotype. The compound is described to have potentiated the action of the antibiotic. Such compounds are described as adjuvants, molecules which on their own have none or marginal antibiotic effects, but synergistically potentiate the efficacy of an antibiotic. (Ejim et al., 2011; Sanhueza et al., 2017; Wright, 2016; Yang et al., 2017). The concept of one drug-one target in infectious disease has waned over the years because of the evolution of resistance (Worthington & Melander, 2012). Combination therapies involving adjuvants are now increasingly employed in clinical settings to deal with microbial infections that tend to be resistant to available antibiotics. This concept has long been applied for the treatment of some diseases such as cancer and HIV-AIDS (Bock & Lengauer, 2012). There are generally two forms of these combinatory therapies currently; combinations of two or more antibiotics (Drug-Drug combinations) or a drug and an adjuvant.

The effects of combinations present three outcomes; Additivity (wherein there is no overall change as a result of the combination), antagonism (wherein the action of one agent acts against the effects of the other producing an overall negative result), and synergism (the action of one agent potentiates the activity of the other agent, increasing its overall efficacy). Most combination therapies are therefore geared towards synergistic outcomes (Wright, 2016). Antibiotics in separate classes are usually combined to tackle single drug-resistant strains to reduce the chances of antibiotic resistance to the combination. Common drug combinations are between aminoglycosides or quinolone drugs and beta-lactams (Tamma et al., 2012). However, drug-drug combinations are usually not preferred because it promotes the emergence of multi-drug resistance (MDR). However, adjuvant-drug (AD) combinations are preferred because it either breaks resistance or compliments antibiotic effect thereby reducing the development of resistance against such combinations (Gill et al., 2015; Wright, 2016). A combination of amoxicillin which is a Beta-lactam antibiotic and clavulanic acid, an inhibitor to beta-lactamase serving as the adjuvant is one common example of an AD combination (Worthington & Melander, 2012).

The mechanisms by which antibiotic adjuvants potentiate the activity of antibiotics against resistant microbes have not been widely studied. Some of the currently known mechanisms include enhancing the intake of antibiotic agents through the bacteria plasma membrane, altering bacterial physiological/metabolic state, and efflux pump inhibition (Wright, 2016). Unlike antibiotics, adjuvants are not necessarily required to target specific components of the bacteria but rather potentiate the activity of the antibiotic against its target (Gill et al., 2015).

2.2.1 REGULATION OF MICROBIAL CELLULAR METABOLISM

The metabolic state of bacteria determines their sensitivity to antibiotic therapy and certain metabolic patterns match with AMR. Molecules that can change the metabolic state of resistant bacteria to that of a sensitive one could reverse the antibiotic susceptibility profile (Maugeri et al.,

2019). In a study conducted by Claudi and colleagues in 2014, antibiotic therapy against fast-dividing *Salmonella* spp cells was more effective as compared to non-dividing or slow-growing cells and hence the latter cells were able to tolerate the antibiotic stress to form persisters. They concluded that the growth pattern of a microbe in infection can influence the efficacy of the antibiotic used. Therefore, the reversibility of the growth pattern can influence the activity of antimicrobial drugs (Claudi et al., 2014). Molecules that can increase the metabolic or growth rate of microbes can serve as antibiotic adjuvants.

Studies in this area have identified some carbohydrate moieties to influence metabolic rate systems found within microbes and as such can be employed as antimicrobial adjuvants. Myoinositol, a carboxylic sugar was found to enhance the proliferation of *Corynebacterium glutamicum* when provided exogenously by serving as the source of energy and carbon to the *Corynebacterium* spp (Krings et al., 2006). In contrast to this observation, galactose intake and breakdown in *Saccharomyces cerevisiae* is lower as compared to fructose and glucose and as such decreases yeast proliferation (De Jongh et al., 2008).

The bacterial metabolic milieu and antibiotic sensitivity pattern of *Escherichia coli* can be modified by the addition of exogenous small molecules such as nitric oxide, indole, prebiotics, vitamin-like nutrients, and so on (Allison et al., 2011). Allison and colleagues concluded in their research that the chemotherapeutic potential of aminoglycosides is enhanced against *E. coli* when exogenic metabolites especially of carbon sources are added. Moreover, the bactericidal activity of tobramycin against *Pseudomonas aeruginosa* is potentiated significantly in the presence of mannitol (Allison et al., 2011). Myoinositol, a vitamin-like nutrient has been reported to ameliorate the host's defense system to eradicate resistant *E. coli* when used either alone or as an adjuvant with florfenicol (Chen et al., 2015).

Microbial intracellular enzymes require optimum conditions within their immediate environment for proper physiological functioning (Robinson, 2015). Malonic acid, an organic acid, inhibits endodontic pathogens by decreasing intracellular pH. Undissociated molecules found within the cytosol of the pathogen-free up hydrogen ions (protons) in the presence of organic acids such as malonic acid (Ballal et al., 2011).

Certain amino acids, such as tryptophan, proline, and arginine have been reported to be effective in the elimination of bacterial pathogens (Mishra et al., 2018; Zhu et al., 2014). The immune system secretes certain peptides with antimicrobial activity to eliminate pathogenic bacteria. These antimicrobial peptides (AMPs) consisting preponderantly of proline or tryptophan can eradicate pathogenic microbes by targeting metabolic pathways (Zhu et al., 2014) and hence are an auspicious source of potentiators of antibiotics. The robustness of these peptides is because of the aromaticity of tryptophan sidechains which is capable of quickly forming hydrogen bonds with the plasma membrane of these microbes (Bacalum et al., 2019). Aside from the hydrogen bonding, some literature has also reported that the bulky nature of tryptophan's indole-sidechains can insert deeply into the lipid bilayer's hydrocarbon core and interfere with the lipid acyl group interactions (Mishra et al., 2018).

2.2.2 INFLUENCING CELL MEMBRANE PERMEABILITY

The impermeable membrane barrier of bacteria especially gram-negative bacteria serves as a major hurdle in the field of drug discovery (Peng et al., 2015). In a previous study, Peng and colleagues used exogenic glucose and alanine to revive the activity of kanamycin against resistant *Edwardsiella tarda*. A mixture of glucose and alanine as adjuvants to kanamycin altered the permeability of the plasma membrane increasing drug intake and eliminating resistant strains (Peng et al., 2015).

Cellobiose lipids obtained from the yeast *Cryptococcus* spp displayed antifungal activity against *Candida tropicalis* by damaging their cell membrane. The fatty acid composition of the cellobiose residue is acetylated and contains hydroxyl groups that confer its membrane damaging activity (Kulakovskaya et al., 2014).

2.2.3 INHIBITION OF EFFLUX PUMPS

Efflux pumps are membrane-bound proteins present in the cell membrane and originally evolved for use in expelling out cellular wastes as well as other toxic compounds and can generally be classified into five groups based on their composition, source of energy and substrate, several transmembrane regions: the Major Facilitator superfamily (MFS), small multidrug resistance family (SMR), which is a member of the much bigger drug/metabolite transporter superfamily (DMT), resistance-nodulation-cell division (RND) family, multidrug and toxic compound extrusion family (MATE) and the Adenosine Triphosphate (ATP)- Binding cassette families.

Resistant microbes have adapted the capability of quickly expelling drugs or chemicals Using these efflux pumps which have a poly-substrate specificity (Sun et al., 2014). This decreases the intracellular concentrations of these antibiotics, thereby enhancing the probability of resistance mutations at sub-therapeutic doses of the antibiotic (Sun et al., 2014).

Studies have proven that hindering the action of these pumps will lead to an increase in drug uptake to break antimicrobial resistance. Villagra and colleagues determined the effect of non-phosphotransferase (non-PTS) carbohydrate (xylose) on the antimicrobial resistance pattern of *Salmonella Typhimurium*. They concluded there is the possibility of an increase in the production of carbohydrate permeases which could modify the bacteria's inner-membrane protein constituent. Their data also showed a resistance-breaking effect as a result of an alteration of the efflux pumps mediating the resistance by the carbohydrate permeases (Villagra et al., 2012).

Ying Gong and colleagues conducted a study to determine the synergistic effect of a non-antifungal drug (NBP) and fluconazole (FLC) against fluconazole-resistant *Candida albicans.* Their data demonstrated an increase in fluconazole uptake and a decrease in the efflux activity by the fungi, leading to increased sensitivity of the fungal cells to FLC. Reverse Transcriptase-PCR analyses suggested a down-regulation of the expression of *C. albicans* drug resistance genes CDR1 and CDR2 transporter genes by the synergistic effect of the NBP and FLC (Ying Gong et al., 2019).

2.2.4 SELECTIVE PRESSURE FOR NATURAL GUT FAUNA

Prebiotics are food ingredients that have beneficial functions in the human gut system; they are non-digestible and affect the activity of specific gut bacteria (Doran & Verran, 2007; Mandal et al., 2016). Inulin is an example of a prebiotic that ensures the survival of lactobacilli cells and induces their antimicrobial activity (Balthazar et al., 2018). Inulin can be found in a lot of foods like bananas, garlic, onions, and so on. It is classified under fructans, which are non-digestible carbohydrates (James, 2014). Aside from inulin being utilized mostly by gut *lactobacilli* spp, there have been some reports of its usage by some strains of *Streptococci* spp found in the oral cavity (Doran & Verran, 2007).

A recent study conducted by Önal Darilmaz and colleagues against *Escherichia coli* implicated inulin as a key player in the antibacterial activity of a combination of inulin (prebiotic) and *Lactobacillus fermentum* (probiotic). The synergy between inulin and *L. fermentum* led to an increase in oxalate-mediated degradation of the *E. coli* cells (Önal Darilmaz et al., 2019). This data was consistent with a previous study conducted by Kareem and colleagues where they found a synergistic effect of inulin and *Lactobacillus plantarum* with inhibitory activity against various microbial pathogens (Kareem et al., 2014).

From these, it is apparent that adjuvants exist as a wide variety, from simple sugars, amino acids, and fatty acids to other organic or inorganic molecules, and when used in combination with antibiotics can revert resistance to built-in naturally sensitive microbes or sensitize microbes that are intrinsically resistant to antimicrobial drugs (Bernal et al., 2013). Such combination therapies represent an untapped potential to repurpose obsolete antibiotics with limited or diminished antibiotic activity (Taylor et al., 2012) and increase our repertoire of antimicrobial drugs (Sanhueza et al., 2017).

2.3 ADAPTIVE AMR AND STRESS RESPONSE

There is a general melding between adaptive AMR and stress response in bacteria because the general trigger for adaptive resistance, in this case the antibiotic, is also considered a stressor. Stress response describes a number of complex and dynamic process that involves the production of protective molecules (e.g. antibiotic degrading enzymes), changes in metabolism and behavior, induce the expression of multidrug efflux pumps or modify the permeability of the bacterial cell envelope, which can decrease the intracellular concentration of antibiotics and confer temporary resistance. Other stress response mechanisms, such as the induction of SOS response or stringent response, can cause temporary or reversible changes in bacterial DNA replication, transcription, and translation, leading to the transient inhibition of cell growth and metabolism. Taken together, these act toward enhancing bacterial 'fitness' against adverse conditions, but also impose a 'fitness cost' on growth and reproduction under non-stressful conditions (Johnsen et al., 2009).

For instance, in Pseudomonas aeruginosa, mutations in stress response genes can mitigate the fitness cost of fluoroquinolone resistance by improving bacterial growth and survival under oxidative stress. Similarly, in Salmonella enterica, mutations in stress response genes can increase the fitness of multidrug-resistant strains under low-nutrient conditions. These findings suggest that stress response can play a key role in modulating the trade-offs between resistance and fitness cost,

and may contribute to the spread and persistence of resistant bacteria in different environments (Agnello et al., 2016; Fàbrega & Vila, 2013).

It is important to note however, that not all adaptive AMR mechanisms are related to stress response. One such example of temporary resistance observed in bacteria that is independent of stress response is the phenomenon of "persistence" or "tolerance" to antibiotics. Persister cells are a subpopulation of bacteria that exhibit a transient and non-inheritable resistance to antibiotics, even in the absence of any obvious stressor or environmental cue. Persister cells are thought to arise through stochastic processes of genetic or phenotypic variation, and can be characterized by a slowed or arrested metabolism, reduced growth rate, and altered expression of genes involved in stress response, metabolism, and virulence. Persister cells have been identified in various bacterial species and clinical isolates, and are believed to play a significant role in the persistence and recurrence of chronic infections, as well as in the emergence and spread of antibiotic resistance. The exact mechanisms underlying persister formation and persistence are not yet fully understood, but are believed to involve complex interactions between genetic, biochemical, and physiological factors (Lewis, 2010).

Overall, the relationship between adaptive AMR mechanisms, fitness cost and stress response is a complex and dynamic process that depends on multiple factors, including the type and severity of stress, the genetic and environmental context, and the availability of resources. By understanding the mechanisms and trade-offs involved in this interplay, we can develop new strategies for controlling AMR, such as targeting stress response pathways to enhance the fitness cost of resistance, or manipulating environmental conditions to reduce the selective pressure for resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS & CULTURE CONDITIONS

The model organisms used for this experiment were *Mycobacterium smegmatis* mc^2155 (Ms wt acquired from ETH-Zurich, Switzerland) and two other multi-drug resistant (MDR) strains, erythromycin-resistant *Mycobacterium smegmatis* A and B (eMsA and eMsB). These multi-drug *Mycobacteria spp* were obtained from the Laboratory for Chemical Systems Biology of Infectious Disease and kept on a 7H10 slant at 4°C. Before use, each organism was sub-cultured from the slants onto agar plates and incubated at 37°C for 48 h. The cells were acid-fast stained to serve as a quality control before seeding them into 7H9 broth to make starter cultures. These were incubated with shaking at 180 rpm. Seed cultures were made from the starter cultures at optical density (OD) of 0.1 and incubated for another 48 h for growth until the mid-exponential growth phase was obtained before every bioassay. The OD reading taken at an absorbance of 600nm was adjusted to 0.7 as the working OD. Some limited drug screening assays were done using the *C. albicans*, and *E. coli*. The 7H9 media constituted monopotassium phosphate (1 g/L), magnesium sulphate (0.05 g/L), ammonium sulphate (0.5 g/L), calcium chloride (0.0005 g/L), disodium phosphate (2.5 g/L), copper sulphate (0.001 g/L), biotin (0.0005 g/L), L-glutamic acid (.5 g/L), ferric ammonium citrate (0.04 g/L), and pyridoxine (0.001 g/L). The broth was prepared by weighing 0.1305 g of 7H9 broth base into a clean and sterile conical flask containing 25 ml of distilled water. This solution was complimented with 0.02125 NaCl, 62.5 uL (0.25%) of 20% Tween 80, and 110 uL (0.44%) glycerol and autoclaved at 121°C for 15 min.

The components of 7H10 media used include; L-glutamic acid (0.5 g/L), ammonium sulphate (0.5 g/L), sodium citrate (0.4 g/L), magnesium sulphate (0.025 g/L), zinc sulphate (0.001 g/L), ferric ammonium citrate (0.04 g/L), monopotassium phosphate (1.5 g/L), disodium phosphate (1.5 g/L),

malachite green (0.00025 g/L), sodium citrate (0.4 g/L), calcium chloride (0.0005 g/L), copper sulphate (0.001 g/L) and agar (15 g/L).

Per manufacturer's instructions, 1.9g of Middlebrook 7H10 agar base was weighed into a sterile conical flask containing 100 ml of distilled water to make a 1.9% solution. This solution was augmented with 0.085% NaCl and 0.5% dextrose.

3.2 PREPARATION OF ANTIBIOTICS AND PHENOTYPIC COMPOUNDS

The selected standard antimicrobial drugs used were, Ampicillin, Amoxicillin, Vancomycin, Isoniazid, Ethambutol, Ethionamide, Pyrazinamide, Methicillin, Rifampicin, Linezolid, Tetracycline, Chloramphenicol, Erythromycin, Streptomycin, Cycloserine, Gentamycin, Paromomycin**,** 5- Fluorouracil, Clindamycin, and Moxifloxacin. Stock and working concentrations of each compound were prepared and kept in the freezer (0°C) for storage. These Antimicrobial drugs were pipetted based on the working concentrations as shown on the table.:

Table 3.0: List of antibiotics and their working amounts used in this work

Antibiotic	Abbreviation	Working amounts (ug)
Rifampicin	Rif	10
Tetracycline	Tet	20
Streptomycin	Strep	30
Isoniazid	INH	20
Erythromycin	Ery	40
Ampicillin	Amp	40
Moxifloxacillin	Moxi	1
Linezolid	Lin	40
Vancomycin	Van	5
Ethambutol	Emb	10
Pyrazinamide	PZD	40
Amoxacillin	Amx	40
Chloramphenicol	Chlo	40
Cycloserine	Cyser	20
Gentamycin	Gen	10
Metronidazole	Met	30
Paromomycin	Para	20
5-Fluorouracil	5-FU	1
Clindamycin	Clind	40
Ethionamide	Eth	40

The list of the phenotypic compounds is in the Table below.

Table 3.1: Groupings of the seventeen (17) biomolecules

1. DI- AND TRI-SACCARIDES
❖ LACTOSE (4)
❖ MALTOSE
❖ RAFFINOSE
❖ CELLOBIOSE
❖ INULIN
2. FIVE (5) AND SIX (6)-CARBON ALCOHOLS
❖ DULCITOL
❖ ADONITOL
3. FIVE (5) AND SIX (6)-CARBON MONOSACCHARIDES
❖ SORBOSE
❖ GALACTOSE

❖ RHAMNOSE
❖ **XYLOSE**
4. ACIDIC GLYCOSIDES
❖ GLUCONIC ACID
5. AMINO ACID
❖ TRYPTOPHAN
6. SHORT CHAIN FATTY ACID
❖ MALONIC ACID
❖ PYRUVATE
7. SIX (6)-CARBON COMPOUNDS AND ALCHOLIC GLYCOSIDES
❖ MYOINOSITOL
❖ SALICIN

A mass of 10 mg of each compound was weighed into a 15 ml falcon tube containing 10 ml of 7H9 broth for *Mycobacterium* culturing. The mixture was filter sterilized with a Whatman filter and a syringe. The compounds were stored in the refrigerator.

3.3 DETERMINATION OF THE EFFECTS OF PHENOTYPIC COMPOUNDS ON ANTIBIOTIC ACTIVITY & MORPHOLOGY

As previously described, seed and starter cultures were set up from slants and adjusted to their working OD. The 17 single compounds were tested in the first set of assays performed. After this, 10 structural triple combinations were generated based on the structure and diversity of the phenotypic compounds. From the results obtained, another set of 34 functional triple combinations (FTC) was generated based on the antibiotics the phenotypic compounds affected by breaking or inducing resistance. A volume of 100 ul of each phenotypic compound (1 ug/ul) was spread on agar plates and allowed to dry. The cells at the working OD were spread on the modified agar plates and known amounts of antibiotics pipetted onto sterile filter discs and allowed to dry were placed on the inoculated plates. The zones of inhibition created by the antibiotics were measured after incubating the assay plates at 37°C for 48 hr. A control was set up where no phenotypic compound was spread on the plates and all experiments were set up in duplicates.

A triple combination system of the phenotypic compounds (PC) was established depending on their structure and effect on antibiotic activity. The structural triple combination (STC) had ten groups while the functional triple combination (FTC) had 34 groups, each group containing a combination of 3 PCs. The activity of the compounds was grouped into resistance breaking (compounds that increased activity of the antibiotic) and resistance inducing (compounds that reduced the activity of the antibiotic compound compared to the control).

For the phenotypic assays, the 17 single compounds and the top 5 resistant breaking of the 35 FTC, as well as the top 5 resistant inducing combinations of the FTC, were used. The effect of the FTC was ranked by considering the number of different antibiotics they affected.

The organism was sub-cultured from the slants onto agar plates and incubated at 37°C for 48 h. The cells were acid-fast stained to serve as a quality control before seeding them into 7H9 broth to make starter cultures. These were incubated with shaking at 180 rpm. Seed cultures were made from the starter cultures at optical density (OD) of 0.1 and incubated for another 48 h until the mid-exponential growth phase was obtained before every bioassay. The OD reading taken at an absorbance of 600nm was adjusted to 0.7 as the working OD. A volume of 100 ul of each phenotypic compound (1 ug/ul), both single PCs and triple PC combinations, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10^{-4} dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h and images of the plates were captured. After, a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments.

3.4 DETERMINATION OF THE EFFECTS OF PHENOTYPIC COMPOUNDS ON EFFLUX AND BIOFILM ACTIVITY

To assess accumulation, the three strains of *M. smegmatis* were cultured in 7H9 broth for 48 hr at 37°C in a shaking incubator at a speed of 180 rpm. The OD was adjusted to 0.8 and the cultures were centrifuged at 13, 000 rpm for 3 min and the cell pellet was washed twice with 0.8% Phosphate Buffered Saline (PBS). The pellets were resuspended in 0.8% PBS and the OD adjusted to 0.4. A volume of 3 ul ethidium bromide (EtBr) with a concentration of 3 ug/ul was added to all the cell suspensions. An amount of 990 ul of cells containing EtBr at an OD of 0.4 was pipetted into 2 ml Eppendorf tubes and 10 ul of each PC at a concentration of 20 mg/ul were added to each reaction tube to give a total amount of 0.1 mg. Verapamil at the same amount was added to one of the tubes to serve as the positive control. an amount of 10 ul of 0.8% PBS was added to one reaction set up to serve as a negative control and all the reactions for the PC plus the controls were set up in triplicates. The reaction tubes were incubated for one hour in a shaking incubator after which 100 ul of the content of each reaction tube was pipetted into a 96-well plate. Fluorescence of EtBr at excitation and emission wavelengths at 530 at 585 (nm) respectively were read within time intervals of 30, 60, and 120 min using a Varioskan microplate reader.

For the induction of efflux pump activity, 0.4% glucose was added to each reaction tube containing EtBr cells. This was done by preparing a 0.8% PBS solution containing the 0.4% glucose solution. The protocol as described in the accumulation assay was followed. That is, the reaction setup was incubated for an hour and 100 ul of each tube were aliquoted into a 96 well plate and fluorescence read at 0, 15, 30, 60, and 120 min.

Finally, adhesion and biofilm assays were done as described by Sandberg *et al* (2008) with slight modification. *M. smegmatis* mc^2155, eMsA, and eMsB cultures were incubated for 48 hr and their OD_{600} readjusted to 0.5.

To determine whether the PC inhibits adhesion and biofilm formation, two different assays were set up for each aspect. A volume of 990 ul of the overnight cultures (OD = 0.5) was aliquoted into a 2 ul Eppendorf tube in both setups. To each reaction tube, 10 ul of PC (10 ug/ ml) was added. For the PC combinations, the volume of cells used was adjusted to 970 ul to make up for the additional volume of PCs used. A volume of 200 ul from each reaction tube was aliquoted into 96 well plates and incubated at 60 rpm for 24 h for adhesion and 72 h for biofilm. After the stated period of incubation for the respective assay, the cultures in the 96 well plate were washed off with 0.8% PBS and allowed to air dry. A volume of 20 ul of 1% crystal violet (CV) was added to each well and allowed to stand for 15 min. The plates were washed off with distilled water to remove any unbound CV to cells. A volume of 20 ul of 95% ethanol was added to each well to solubilize the CV. The absorbance was read at 595 nm with the Varioskan plate reader. The inhibitory effect of the PC against cell adhesion and biofilm formation was determined.

The inhibition assays are then followed by disruption assays in which a volume of 200 ul of Mycobacterial culture of OD = 0.5 was aliquoted into 96 well microtiter plates. The plates were incubated with shaking at 60 rpm for 24 h and 72hr to study adhesion and biofilm respectively. After the incubation period, the plates were washed twice with distilled water and replaced with 200 ul of 7H9 containing PC at an amount of 0.1 mg. The two setups were incubated for 2 h with shaking at 60 rpm. The microtiter plates were washed twice with distilled water and 20 ul of 95% ethanol was added. The absorbance was read at 595 nm. Signified compounds that disrupted adhered cells and biofilm formation.

3.5 ANALYSIS OF STRESS RESPONSE EXPRESSION PATTERNS WITH ADJUVANTS

The two top hit treatment conditions that were resistant breaking and resistance inducing were used in the setup for the RNA extraction. Thus, there was a control (no PC added), two resistance-inducing combinations (LAC (3X) and SIA+ADO (1.5X)), and two resistance breaking

combinations (SOR (3X) and MAL (3X)). The top two resistant breaking and resistant inducing combinations were chosen based on the ranking order where these compounds had the highest effect on the number of antibiotics they affected.

The three *M. Smegmatis* strains were cultured as described in section 3.1, a seed plate was made from stored culture slants by streaking and incubating overnight. The cells were acid-fast stained before subculturing into 50 ml 7H9 broth and incubated in a shaking incubator for 72 h. This first culture (day 1 culture) was not treated. Fresh 50ml 7H9 broths containing 0.1 mg of the various PCs were prepared and seeded using the first culture to obtain a day 2 culture. The starting OD of the day 2 culture was set at 0.05 and incubated for another 72 h. For the extraction, 50 ml falcon tubes were pre-weighed, and the treated cell cultures were transferred into them. The tubes were centrifuged at 13,000 rpm for 5 minutes at 4°C. The pellet obtained was left to dry in the hood and the tubes measured. The difference in the weights measured is the weight of the cells pelleted. A kit was used for the extraction, the ZR Fungal/Bacterial MiniPrep kit from Zymo Research. The weight of the cells ranged from 400 – 600 mg. To each falcon tube, 2 ml of RNA lysis buffer was added to resuspend the cells into solution. Of this, 1 ml was pipetted into the tubes containing the bashing bead and vortexed for 10 minutes. The tubes were then centrifuged at 15,000 rpm for 1 minute. A volume of 400ul of the lysate (supernatant) was carefully transferred into the Zymo-Spin™ IIICG column placed in collection tubes and centrifuged at 14,000 rpm for 30 secs. This step was repeated with the same column to obtain a total volume of 800 ul of flow through. To this collection, an equal amount of 96% ethanol was added and mixed thoroughly and 600 ul pipetted into the Zymo-Spin™ IICR in a collection tube. This was centrifuged at the previous speed and for the same period. The flow-through was discarded. To the column, 400ul of the RNA Prep Buffer was added and the flow-through discarded. The column was washed twice by adding 700 ul and 400 ul of RNA Wash Buffer and centrifuged at each point for 30 secs and 2 minutes

respectively. The columns were transferred into sterile 1.5 ml Eppendorf tubes and 10 ul of warm Dnase/Rnase-Free Water was added and eluted time for the same column to give a final volume of 30 ul of extracted RNA. The RNA extracted was then quantified and its purity checked using a nanodrop. Extracted RNA samples were stored at -24 ^{0}C before running the RT-qPCR the following day. Stored RNA samples were diluted to 25 ng/ul using Dnase/Rnase-Free Water. The Luna® Universal One-Step RT-qPCR kit was the kit used for the RT-qPCR experiments. For a 1X reaction of 20 ul RT-qPCR reaction mix, 10ul of the Luna Universal One-Step Reaction Mix (2X), 1ul of the Luna WarmStart® RT Enzyme Mix (20X), 0.8ul each of the 10 uM forward and reverse primers and nuclease-free water added to top up to the required volume. For each primer pair, 20ul of each triplicate per treatment was prepared for 10 selected stress response genes. The assay was performed on ice. The plates were sealed carefully using a transparent plate sealer and centrifuged in a microcentrifuge for 1 min.

The Applied Biosystems real-time machine was programmed using the thermocycling protocol as stated in the Luna® Universal One-Step RT-qPCR Kit.

Table 1.4: PCR Thermocycler conditions

Cycle Step	Temperature	Time	Cycles
Reverse Transcription	55 ^{0}C	10 minutes	1
Initial Denaturation	95 ^{0}C	1 minute	1
Denaturation	95 ^{0}C	10 seconds	40
Extension	60 ^{0}C	1 minute	
Melt Curve	60 – 95 ^{0}C	various	1

The generated data were then analyzed.

CHAPTER FOUR

RESULTS

4.1 RESULTS OF THE EFFECT OF DIFFERENT CARBOHYDRATES AND AMINO ACIDS ON THE ANTIMICROBIAL ACTIVITY OF ANTIFUNGAL AND ANTIBACTERIAL COMPOUNDS

4.1.1 EFFECT OF SINGLE PHENOTYPIC COMPOUNDS ON ANTIBIOTIC ACTIVITY

The ability of modifier compounds to augment or suppress the antibiotic activity of standard in-use antibiotics in three different strains of mycobacteria was set up for 17 different compounds.

The phenotypic interaction assays using single compounds against Ms wt showed most of the compounds used had an accumulated activity lower than the control. All the compounds except cellobiose decreased the antibiotic activity either by decreasing the activity of the individual antibiotics or reducing the number of antibiotics that had activity. Most of the compounds could be said to be resistance-inducing activity. Contrary to all the phenotypic modifying compounds used, cellobiose had the highest accumulated antibiotic impact relative to the control. In the presence of cellobiose, it was observed that there was an increase in the antibiotic activity of vancomycin, linezolid, and gentamicin. It is also noteworthy to point out that cycloserine which is inactive in the control got revived in the presence of cellobiose. Unlike the other antibiotics, Moxifloxacin had a reduction in activity when cellobiose was added. Since most of the antibiotic's activity increased (13 out of 20 antibiotics) after cellobiose modification, it can be said to be resistant-breaking. Maltose had the least accumulated activity on the antibiotics used, having changed 8 out of 20 antibiotics. In the presence of maltose, amoxicillin, vancomycin, ethambutol, and chloramphenicol all had a reduction in their antibiotic activity. 5-fluorouracil being the only exception, had a slight increase in activity. Maltose is largely expressed as resistant-inducing activity (Fig 4.0A).

Results from testing the individual phenotypic compounds against eMsA showed that all the compounds used had an accumulated activity higher or equal to the control. Most of the compounds aside from increasing the activity of individual antibiotics revived the activity of the other antibiotics that were not active in the control. Most of the compounds can be classified as resistant-breaking. Cellobiose, just like in Ms wt had the highest accumulated bioactivity. In the presence of this compound, not only did it increase 5-fluorouracil slightly but revived the activities of ampicillin, amoxicillin, erythromycin, gentamicin, and ethionamide. Contrary to this, it reduced slightly the activity of paromomycin and streptomycin. Cellobiose can be classified as a resistant breaking agent. Adonitol having the least accumulated impact did not have much effect on the antibiotics, with ethionamide being the only antibiotic to be revived while chloramphenicol, streptomycin, and 5-fluorouracil were slightly reduced (Fig 4.0B).

Against eMsB, most of the phenotypic compounds had accumulated activities higher than that of the control (10 out of 17 compounds). Hence most of the compounds can be said to be resistant-breaking compounds. Inulin modified media had the highest effect on antibiotics used against eMsB. Amoxicillin, ampicillin, vancomycin, and chloramphenicol all had their activities revived in the presence of inulin. Streptomycin, gentamicin, and 5-fluorouracil had a decrease in activity. Sorbose had the least accumulated activity among the compounds and fell below the control line. This compound can be said to be a resistant inducing agent which caused Tetracycline to lose its activity while streptomycin, gentamicin, and 5-fluorouracil had a decline in their bioactivity. Cellobiose, which has consistently been the highest in both wt and eMsA, was not the most potent here with eMsB but was among the resistant-breaking compounds (Fig 4.0C).

This interaction study, though designed to address the antimicrobial resistance phenomenon, turns out to demonstrate the significantly different cellular metabolism in the two MDR strains. Also provides a path forward for a more detailed study of the basis of the high level of resistance

acquired by these two strains. The same analysis also applied to *E. coli* and *Candida albicans* and surprisingly, *E. coli* did not respond at all while *C. albicans* responded to an extent similar to that of the *Mycobacteria* cells and provide background data for the future.

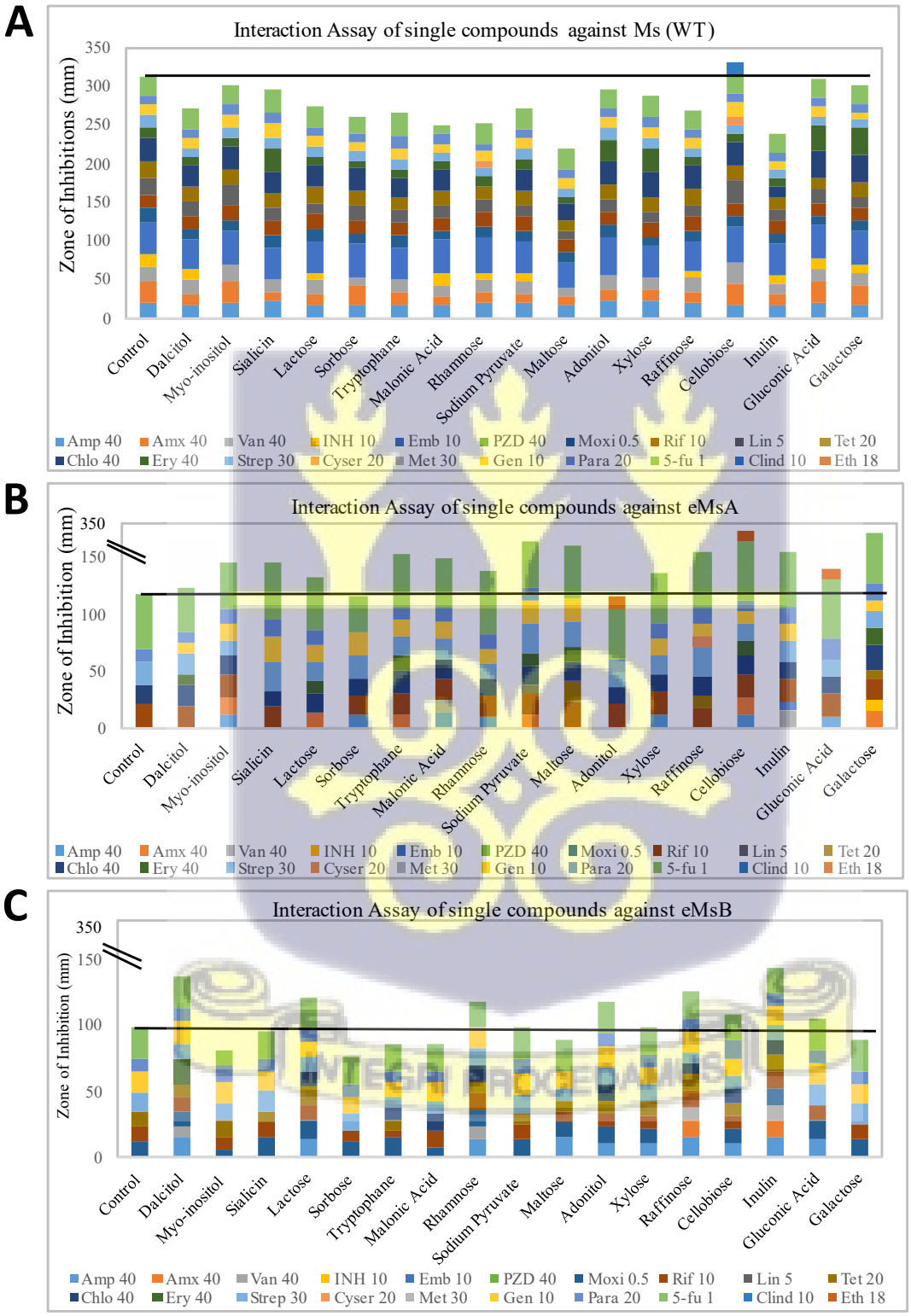


Figure 4.0: Antibiotic activity profile for M. smegmatis treated with modifier compounds*. 7H10 agar plates were treated by spreading 100ul of 1mg/ml of the various PCs. A working OD of 0.7 of cells was prepared and spread on the modified plates. Discs impregnated with the working amounts of antibiotics were placed on the inoculated plate and incubated for 48 h. The zones of inhibition of the active antibiotics were measured and a plot of measured zones of inhibition under controlled conditions and in the presence of each modifier compound for (A) Ms wt (B) eMsA and (C) eMsB*

4.1.2 EFFECT OF STRUCTURAL TRIPLE PHENOTYPIC COMPOUND COMBINATIONS ON ANTIBIOTIC ACTIVITY

To increase the activities observed with the individual compounds, a triple combination of the compounds based on structural similarities was generated. Following from the primary effects of the modifier compounds on the antibiotic activity profiles across the organisms, compounds were grouped based into different structural classes; Di and tri-saccharides (lactose-4, maltose, raffinose, cellobiose, inulin), 5&6 carbon alcohols (dulcitol, adonitol), 5&6 carbon sugars (sorbose, galactose, rhamnose, xylose), Acid alcohol (gluconic acid), Amino acid (tryptophan), Fatty acid (malonic acid and pyruvate) and lastly 6 carbon compounds (myo-inositol and sialicin). The triple 26eaction26onns used were representatives of each class and across groups. Ten structural triple combinations were created to test against the wt and two resistant mutant strains (Table 3.3).

From the assay where the STC compounds were used to test Ms wt, all the ten triple combinations had cumulative activity lower than the control. These combinations can collectively be more resistant inducing, even though some had resistant breaking activities with individual antibiotics (Fig 4.1A).

Against eMsA, five out of the structural triple combination compounds had activities slightly higher than the control, while the other half were slightly below the control. Also, the triple

combination Raf+Cel+Inu had the highest effect, affecting 9 out of 20 antibiotics as compared to the control (Fig 4.1B). The interaction assay of STC compounds with antibiotics against eMsB showed similar observations as seen in eMsA where all the compounds had effects equal to or slightly lower than the control (Fig 4.1C). The overall effects of this STC work were not as interesting as that of the single compounds. For this reason, the combinations were not pursued further in this study.

Table 2.0: List of Structural Triple Combination and their groups

1st criterium; three (3) compounds in each group		
1. Raffinose	Cellobiose	Inulin
2. Dulcitol	Adonitol	D-gluconic acid
3. L-(-)-sorbose	L-rhamnose	Xylose
2nd criterium; one (1) compound in each group		
4. Raffinose	Adonitol	L-rhamnose
5. Cellobiose	D-gluconic acid	Tryptophan
6. D-gluconic acid	Malonic acid	Myoinositol
7. Tryptophan	D-gluconic acid	Galactose
3rd criterium; two (2) compounds from one group		
8. Malonic acid	Sodium-pyruvate	D-gluconic acid
9. Myo-inositol	Sialicin	Tryptophan
10. Dulcitol	Adonitol	Tryptophan

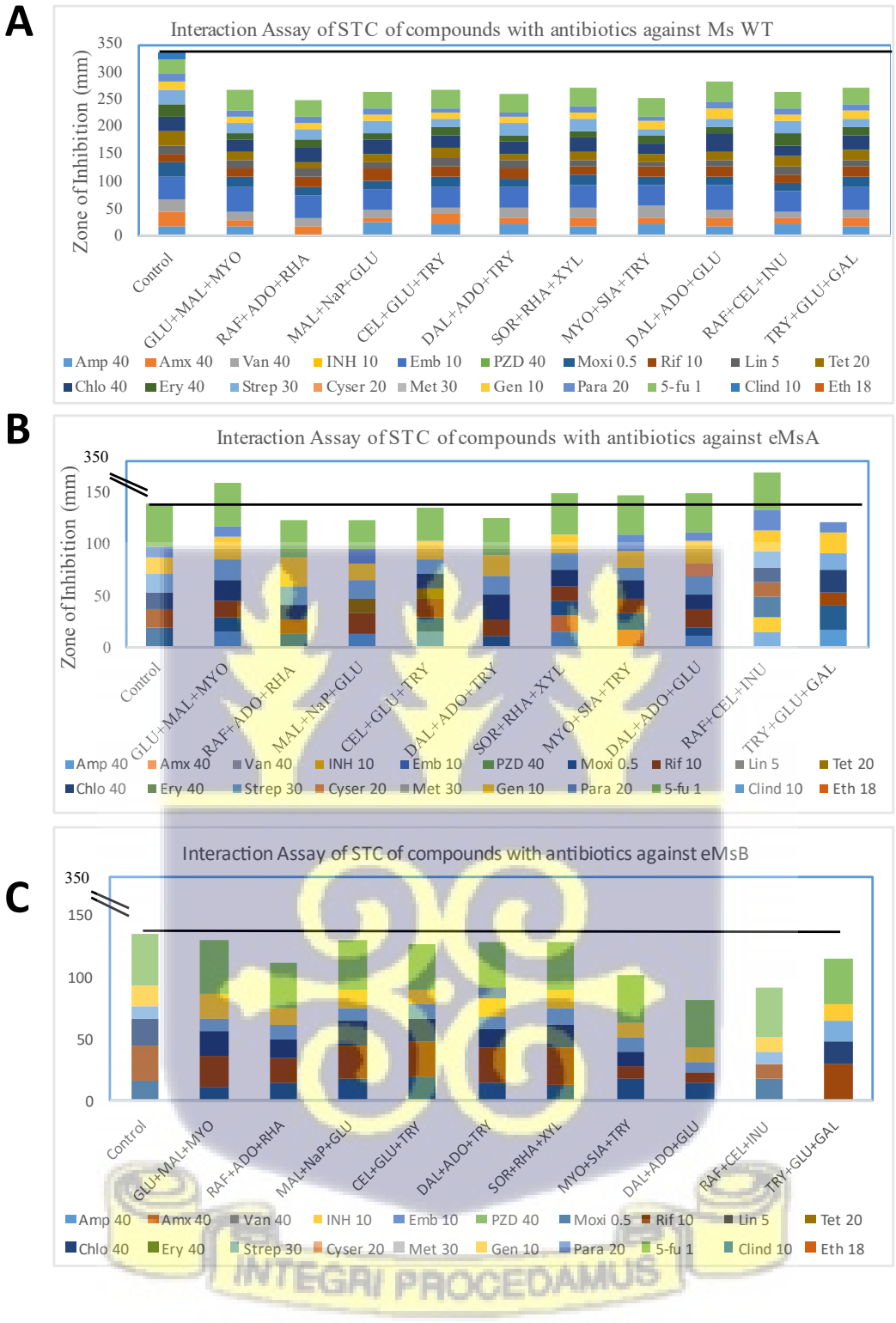


Figure 4.1: Antibiotic activity profile for M. smegmatis WT with structurally grouped modifier compounds*. The disc diffusion method as described above was used to obtain the plot of measured zones of inhibition under controlled conditions and in the presence of structurally grouped modifier compounds for (A) Ms wt, (B) eMsA, and (C) eMsB. On an agar plate 100ul of each PC in the STC was spread on it before inoculation.*

4.1.3 EFFECT OF FUNCTIONAL TRIPLE PHENOTYPC COMPOUND COMBINATIONS ON ANTIBIOTIC ACTIVITY

Concurrently, following the investigation of the role of structural similarities and presence of specific functional groups in potentiating the activity of the antibiotics, the modifier compounds are then organized based on the antibiotic(s) which they affect and grouped in triples toward a combined synergistic effect resulting in 34 different functional triple combinations. The compounds were grouped into triple sets based on the individual activities of the single compounds on the antibiotics.

In the assays where the functional triple combination compounds were used against the wt strain, most of the compounds' cumulative activities were higher than the control. This observation demonstrates a higher resistant-breaking effect when the compounds were combined based on their functional similarities. For example, Myo+Rham+Raf is the triple combination with the highest effect compared to the control. Even though the number of antibiotics impacted by this combination and control are the same, the combination increased the activity of the antibiotics (Fig 4.2A and B). The eMsA bioassay showed that some of the compounds (13/34) had cumulative activities higher than the control while most had cumulative activities lower than the control (18/34). In the presence of Rham (3X), 10/20 antibiotics got their activity changed compared to the control. Most of them had an increase in activity suggesting Rham(3X) is resistant breaking (Fig 4.3A and B). Against eMsB, most of the compounds (23/34) had cumulative activities higher than that of the control as compared to 6/24 compounds having activities lower than the control and Myo+Sia+Lac had the highest accumulative antibiotic impact, changing 8 out of 20 antibiotics. Even though Tryp+Malt+Raf also changed the activity of 8 out of 20, the effect on the individual antibiotics was higher in the former than the latter (Fig 4.4A and B).

Variations in antibiotic activity were more pronounced in both resistant strains in terms of both gain and loss of activity under different treatment combinations. Fourteen of thirty-seven different triple combinations used showed an overall increase in antibiotic activity as plotted of a cumulative stacking of zones of inhibition measured from active antibiotics out of a total of 20 antibiotics used.

The list for the FTC generated is as shown below:

Table 4.1: List of functional Triple Combination and the antibiotics they affect

Functional Triple Combination (FTC)	**Dose**	**The amount for each (mg)**	**Effect**
Mal+Rha+NaP	1X	0.1	(+) Increases activity of Ampicillin, Amoxicillin, and Vancomycin of eMsA and B
Cel+Inu+Glu	1X	0.1	(+) Increases activity of Ampicillin, Amoxicillin, and Vancomycin of eMsA and B
Myo+Sia+Lac	1X	0.1	(-) Decreases activity of Isoniazid against WT
Sor+Try+Mal	1X	0.1	(-) Decreases activity of Isoniazid against WT
Ado+Xyl+Raf	1X	0.1	(-) Decreases activity of Isoniazid against WT
Dal+Myo+Raf	1X	0.1	(-) Decreases activity of Moxifloxacin against WT and eMsB
Myo+Rha+Raf	1X	0.1	(-) Decreases activity of Moxifloxacin against WT and eMsB
3Lac	3X	0.3	(-) Decreases activity of Rifampicin
Malt+Raf+Gal	1X	0.1	(+) Increases activity of Tetracycline against eMsA
Sor+Mal+Glu	1X	0.1	(-) Decreases activity of Tetracycline eMsB

Lac+Try+Mal	1X	0.1	(+) Increases activity of Chloramphenicol against eMsB
Ado+Raf+Cel	1X	0.1	(+) Increases activity of Chloramphenicol against WT
1.5Rha+1.5Inu	1.5X	0.15	(+) Increases activity of Chloramphenicol against eMsB
			(-) Decreases activity of Chloramphenicol against MS
3Myo	3X	0.3	(+) Increases activity of Erythromycin against eMsA and eMsB
Lac+Try+NaP	1.5X	0.15	(+) Increases activity of Erythromycin against eMsA
1.5Xyl+1.5Glu	1.5X	0.15	(+) Increases activity of Erythromycin against WT
1.5Sia+1.5Ado	1.5X	0.15	(+) Increases activity of Erythromycin against WT
1.5Malt+1.5Cel	1.5X	0.15	(+) Increases activity of Erythromycin against eMsA
3Glu	3X	0.3	(+) Increases activity of Erythromycin against WT and eMsA
Sia+NaP+Raf	1X	0.1	(+) Increases activity of Streptomycin against eMsA
Try+Malt+Raf	1X	0.1	(+) Decreases activity of Streptomycin against eMsA
Sor+Rha+Malt	1X	0.1	(-) Decreases activity of Paromomycin of eMsA and B
1.5Myo+1.5Mal	1.5X	0.15	(-) Decreases activity of 5-Fluorouracil of WT and eMsB
3Myo	3X	0.3	(-) Decreases activity of 5-Fluorouracil of eMsB
3Mal	3X	0.3	(-) Decreases activity of 5-Fluorouracil of WT
3Inu	3X	0.3	(+) Increases activity of Clindamycin of WT
Ado+Cel+Glu	1X	0.1	(+) Increases activity of Ethionamide of eMsA

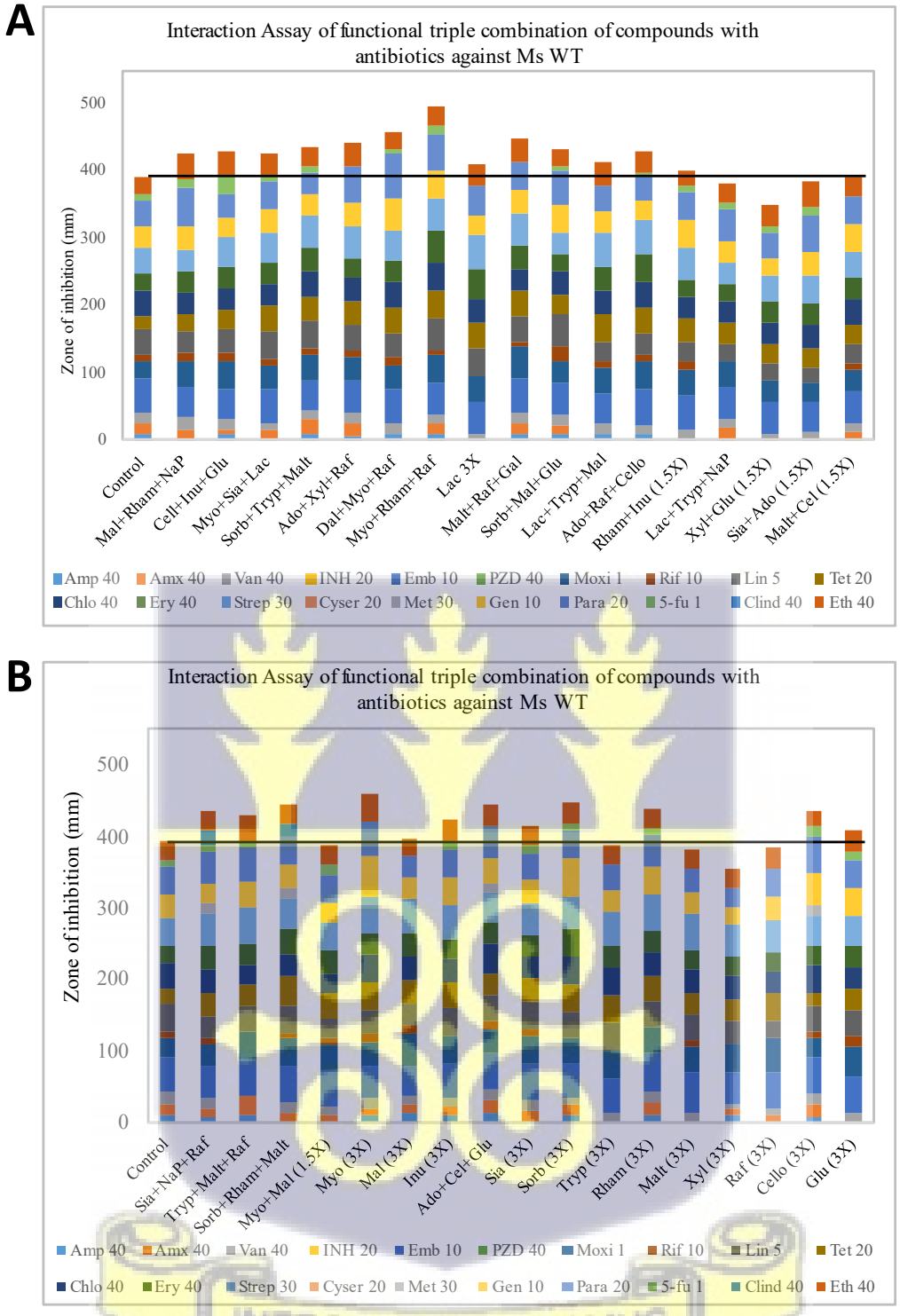


Figure 4.2: Antibiotic activity profile for M. smegmatis WT with functionally grouped modifier compounds. *The plot of measured zones of inhibition under controlled conditions and in the presence of functionally grouped modifier compounds for Mycobacterium smegmatis wild-type. Modified agar plates were prepared by spreading the FTC on the plate and allowed to dry. Cells of working OD 0.7 were used for inoculation and antibiotic-impregnated discs were placed. The plates were incubated for 48h and the ZOI was measured.*

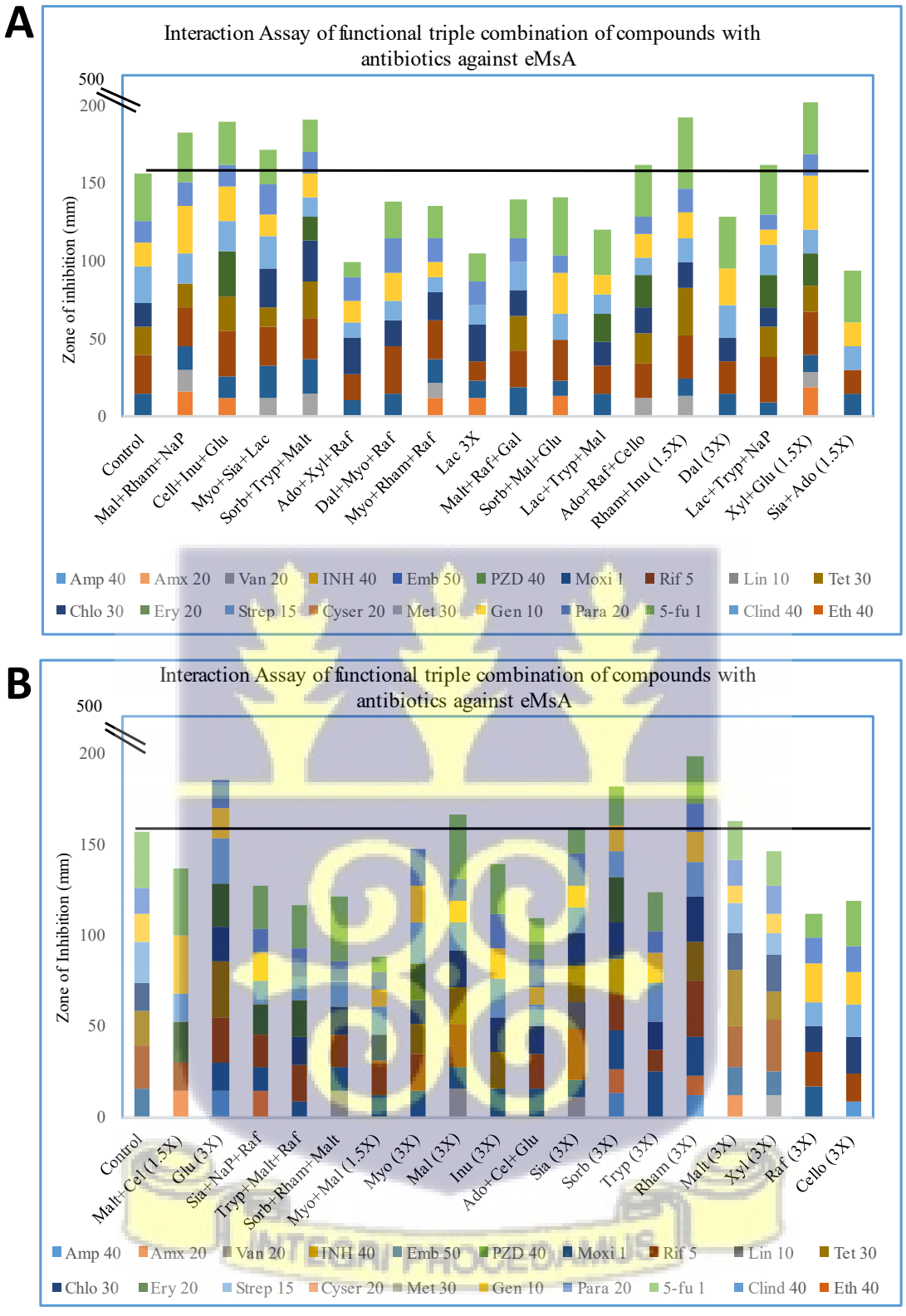


Figure 1.3: Antibiotic activity profile for eMsA with functionally grouped modifier compounds. *The plot of measured zones of inhibition under controlled conditions and in the presence of functionally grouped modifier compounds for erythromycin-resistant Mycobacterium smegmatis A.*

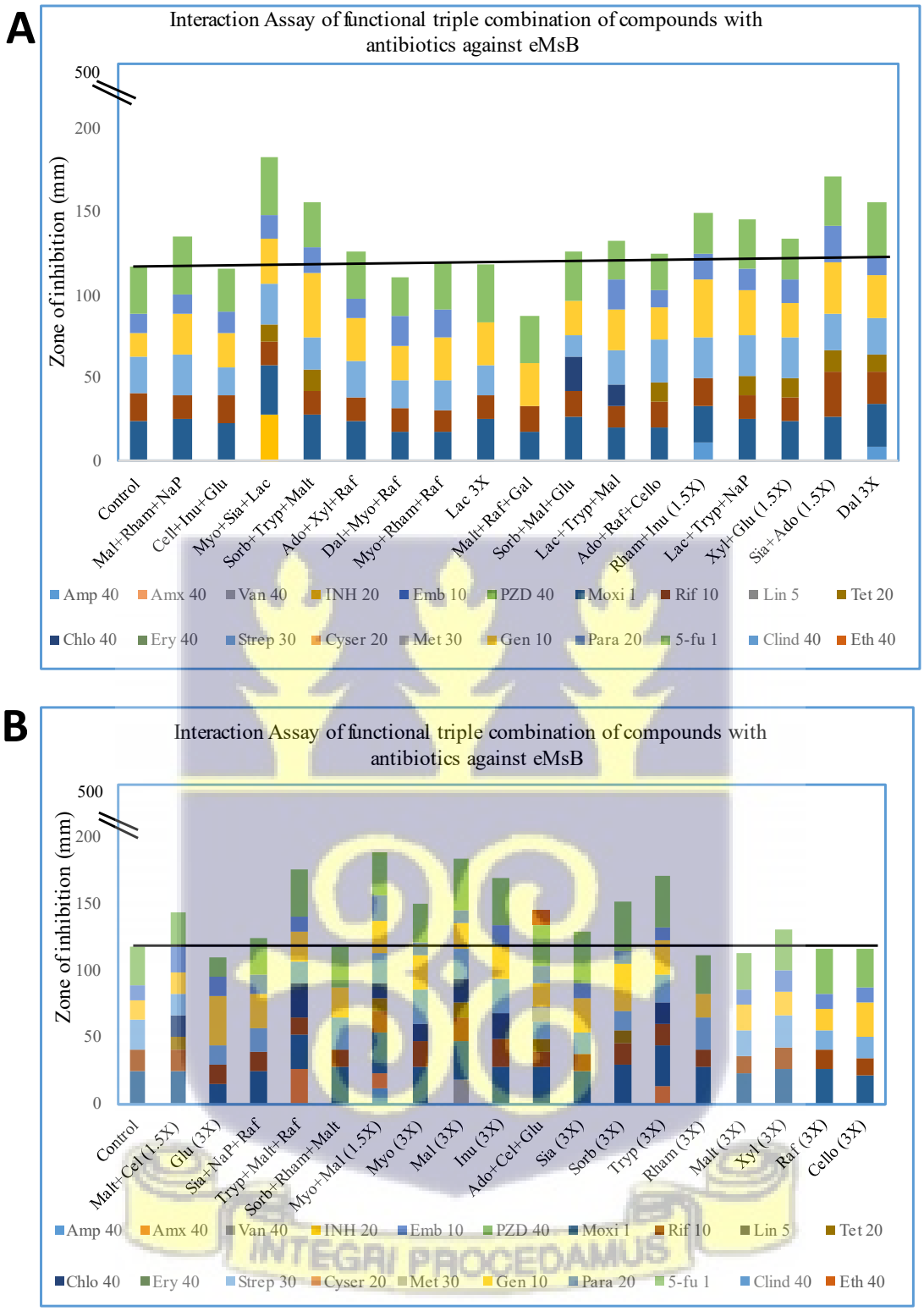


Figure 4.4: Antibiotic activity profile for eMsB with functionally grouped modifier compounds. *The plot of measured zones of inhibition under controlled conditions and in the presence of functionally grouped modifier compounds for erythromycin-resistant Mycobacterium smegmatis B.*

4.1.4 RANKING SYSTEMS FOR THE ANTIBIOTIC RESISTANCE BREAKING AND INDUCING EFFECT

The overall efficacy of the resistant-breaking and resistant-inducing phenotypic compounds against the three strains of *M. smegmatis* (wt, eMsA, and eMsB) were ranked based on certain derived parameters. The parameters used were; 1. The number of antibiotic classes affected (# classes) 2. The number of antibiotics that gained activity, from zero to a number (# 0-n), the number of antibiotics that lost activity, from a number to zero (# n-0) 3. The quantum of change of the zone of inhibition (ZoI) 4. The sum of these parameters to give an overall value for ranking.

The number of antibiotic classes (Table 4.2) affected by each resistant-breaking or resistant-inducing compound was recoded. Different classes of antibiotics were used for this study to understand the activity of the compounds. This parameter shows whether the compounds have specific or broad-spectrum efficacy on the antibiotics. Most of the compounds had an effect on multiple antibiotics, which shows their broad effect while a few had a specific effect on particular antibiotics.

In this study, most of the antibiotics had an effect against the Ms wt just a few of the antibiotics had no activity (cycloserine, pyrazinamide, and ethionamide). Contrary to this most of the compounds were inactive against the mutant strains (eMsA and eMsB) and just a few had activity (rifampicin, moxifloxacin, gentamicin, paromomycin, and 5-fluorouracil). One noteworthy feature was the ability of the compounds to be able to either revive these compounds or make them lose their activity. The number of antibiotics whose ZoI moved from a particular number to zero (n-0) in the presence of resistant-breaking compounds was recorded. Similarly, the number of antibiotics whose activity got revived, that is moved from zero to a number (0-n) in the presence of resistant-inducing compounds were recorded.

The magnitude of the change of the zone of inhibition was recorded by summing the difference obtained, increase for resistant breaking, and decrease in resistant inducing treatment. Some of the changes were increased or decreased and others were either from zero to a number or from a number to zero. The quantum of change here measures the extent of change of antibiotic activity in the presence of the phenotypic compounds.

These three parameters (# of classes, # 0-n/n-0, and quantum of change) were summed up to get the overall value. This total value obtained was used to rank the resistant breaking and resistant inducing compounds in descending order, from the highest to the lowest. An initial ranking was done using the number of antibiotics affected by each phenotypic compound. These numbers were placed in a bracket of each phenotypic compound and were used for the initial ranking.

Table 4.2: The various classes of antibiotics used in the experiment

	Antibiotic Class	Example of Antibiotics
1	Aminoglycoside	Gentamycin, Streptomycin, Paramomycin
2	Fluoroquinolones	Moxifloxacin
3	Glycopeptide	Vancomycin
4	Macrolide	Erythromycin
5	Penicillin	Amoxicillin, Ampicillin
6	Antimycobacterial	Rifampicin, Isoniazid, Pyrazinamide, Cycloserine, Ethambutol
7	Tetracycline	Tetracyline
8	Amphenicol	Chloramphenicol
9	Nitroimidazole	Metronidazole
10	Lincosamide	Clindamycin
11	Thioamine	Ethionamide
12	Antimetabolite	5-Fluorouracil
13	Oxazolidinone	Linezolid

4.1.5 SINGLE PENOTYPIC COMPOUND RANKING BY RESISTANCE-MODIFYING EFFECT AGAINST MYCOBACTERIA

Each modifier compound was assessed based on its resistance-breaking and resistance-inducing effect. The resistance breaking effect either is a return of antibiotic activity or an increase in the extent of antibiotic activity as measured by zones of inhibition in millimeters and vice versa for the resistance-inducing effect. Two ranking systems were applied to each of the 17 compounds as presented in tables (Tables 4.3-4.6); A general rank based on the previously defined resistance-breaking or resistance inducing potential of the compound, and an overall rank representing a total of the number of different classes of antibiotics affected, the total loss or gain of activity to and from a reference point 0 and the quantum change in the antibiotic zone of inhibition (ZoI). The top 5 resistance-breaking compounds by general rank were inulin, cellobiose, raffinose, rhamnose, and lactose respectively and by overall rank; inulin, cellobiose, raffinose, lactose, and sodium pyruvate whilst the top 5 resistance inducing compounds by general rank were maltose, sorbose, rhamnose, sialicin, lactose and by overall rank rhamnose, maltose, sorbose, lactose, and sialicin.

Table 4.3: Resistant-breaking treatment of single compounds against M. smegmatis (Rank 1-6)

RESISTANT-BREAKING TREATMENT OF SINGLE COMPOUNDS								
Overall ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	Quantum change of ZoI	Overall
1	1	Inulin(8)	Ms	Clind 0-18	6	8	107.5	121.5
			eMsA	Van 0-15				
				Emb 0-8				
				Gen 0-15.5				
			eMsB	Amp 0-16				
				Amx 0-13				
				Van 0-11				
				Chlo 0-11				
2	2	Cellobiose(7)	Ms	Cyser 0-9	5	7	82.5	94.5
			eMsA	Amp 0-13				
				Amx 0-14				
				Ery 0-14				
				Eth 0-11				
			eMsB	Amp 0-10.5				
				Chlo 0-11				
3	3	Raffinose(7)	eMsA	Tet 0-11	6	7	80	93
				Cyser 0-10				
				Gen 0-13				
			eMsB	Amp 0-15				
				Amx 0-14				
				Van 0-9.5				
				Chlo 0-7.5				
6	4	Rhamnose(5)	eMsA	Amp 0-10	4	5	58.5	67.5
				Gen 0-12				
			eMsB	Amp 0-14.5				
				Van 0-9.5				
				Chlo 0-12.5				
4	5	Lactose(5)	eMsA	Amx 0-14.5	4	5	65	74
				Ery 0-11				
				Gen 0-14.5				
			eMsB	Amp 0-14				
				Chlo 0-11				
5	6	Sodium Pyruvate(5)	eMsA	Amx 0-12	5	5	61	71
				Emb 0-10				
				Lin 0-8				
				Ery 0-11				
				Gen 0-20				

Table 4.4: Resistant-breaking treatment of single compounds against M. smegmatis (Rank 7-17)

RESISTANT-BREAKING TREATMENT OF SINGLE COMPOUNDS								
Overall ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	Quantum change of ZoI	Overall
6	7	Galactose(5)	eMsA	Amx 0-15	5	5	57.5	67.5
				INH 0-11				
				Tet 0-7				
				Ery 0-14.5				
				Gen 0-10				
9	8	Dalcitol (4)	eMsA	Ery 0-10	3	4	53	60
			eMsB	Amp 0-15				
				Van 0-9				
				Ery 0-19				
8	9	Maltose(4)	Ms	Van 10.5-21	4	3	60	67
				Lin 11.5-23				
			eMsA	Gen 0-22				
			eMsB	Amp 0-16				
9	10	Malonic Acid(4)	eMsA	Amp 0-13	4	4	52	60
				Van 0-12				
				Gen 0-15				
			eMsB	Chlo 0-12				
11	11	Myo-inositol(3)	eMsA	Amp 0-12	2	3	41	46
				Amx 0-15				
				Gen 0-14				
14	12	Adonitol(3)	eMsA	Eth 0-11	3	3	35	41
			eMsB	Amp 0-11				
				Chlo 0-13				
13	13	Xylose(3)	eMsA	Amp 0-13	2	3	37	42
				Gen 0-13.5				
			eMsB	Amp 0-10.5				
12	14	Tryptophane (3)	eMsA	Amx 0-12	3	3	38.5	44.5
				Ery 0-12				
				Gen 0-14.5				
15	15	Gluconic Acid(3)	eMsA	Amp 0-10	2	3	34	39
				Eth 0-10				
			eMsB	Amp 0-14				
16	16	Sorbose(2)	eMsA	Amp 0-12	2	2	32	36
				Gen 0-20				
17	17	Sialicin(1)	eMsA	Gen 0-22.5	1	1	22.5	24.5

Table 4.5: Resistant-inducing treatment of single compounds against M. smegmatis (Rank 1-5)

RESISTANT-INDUCING TREATMENT OF SINGLE COMPOUNDS								
Overall ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# n-0	Quantum change of ZoI	Overall
2	1	Maltose(4)	Ms	INH 14-0	3	3	49.5	55.5
				Amx 27-13				
			eMsA	Para 12-0				
			eMsB	Para 9.5-0				
3	2	Sorbose(3)	Ms	INH 14-0	3	3	36	42
			eMsA	Para 12-0				
			eMsB	Tet 10-0				
1	3	Rhamnose(3)	Ms	Chlo 32-0	3	3	55.5	61.5
				Amx 27-13				
			eMsB	Para 9.5-0				
5	4	Sialicin(2)	Ms	INH 14-0	2	1	29	32
				Amx 27-12				
4	5	Lactose(2)	Ms	Amx 27-13	2	1	35	38
				Rif 21-0				

Table 4.6: Resistant-inducing treatment of single compounds against M. smegmatis (Rank 6-15)

RESISTANT-INDUCING TREATMENT OF SINGLE COMPOUNDS								
Overall ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# n-0	Quantum change of ZoI	Overall
6	6	Myo-inositol(2)	Ms	INH 14-0	2	1	27.5	30.5
			eMsB	5fu 24-10.5				
7	7	Tryptophane(2)	Ms	INH 14-0	2	1	21.5	24.5
			eMsB	Strep 14.5-7				
9	8	Cellobiose(1)	Ms	INH 14-0	1	1	14	16
14	9	Malonic Acid(1)	eMsB	Tet 10-0	1	1	10	12
8	10	Sodium Pyruvate(1)	Ms	Amx 27-11	1	0	16	17
11	11	Dalcitol (1)	Ms	Amx 27-13.5	1	0	13.5	14.5
9	12	Adonitol(1)	Ms	INH 14-0	1	1	14	16
12	13	Raffinose(1)	eMsB	Moxi 12-0	1	1	12	14
13	14	Gluconic Acid(1)	eMsB	Tet 10-0	1	1	10	12
14	15	Galactose(1)	eMsB	Tet 10-0	1	1	10	12

4.1.6 STRUCTURAL AND FUNCTIONAL TRIPLE PHENOTYPIC COMPOUND COMBINATION RANKING BY RESISTANCE-MODIFYING EFFECT AGAINST MYCOBACTERIA

Triple compound combination effects were assessed based on their resistance-breaking and resistance-inducing effect. Again, the resistance breaking effect either being a return of antibiotic activity or an increase in the extent of antibiotic activity as measured by zones of inhibition in millimeters and vice versa for the resistance-inducing effect. Two ranking systems were applied to generate a different number of combinations based on their resistance-breaking and resistance-induction respectively as presented in tables (Tables 4.7-4.8); A general rank based on the previously defined resistance-breaking or resistance inducing potential of the compound, and an overall rank representing a total of the number of different classes of antibiotics affected, the total loss or gain of activity to and from a reference point 0 and the quantum change in the antibiotic zone of inhibition (ZoI). The top 5 resistance-breaking compounds by general rank were Raf+Cel+Inu, Mal+NaP+Glu, Cel+Glu+Try, Sor+Rha+Xyl, and Dal+Ado+Glu respectively and by overall rank; Raf+Cel+Inu, Sor+Rha+Xyl, Mal+NaP+Glu, Cel+Glu+Try, and Dal+Ado+Glu whilst the top 5 resistance inducing compounds by general rank were Raf+Cel+Inu, Sor+Rha+Xyl, Dal+Ado+Try, Myo+Sia+Try, Mal+NaP+Glu and by overall rank Raf+Cel+Inu, Myo+Sia+Try, Dal+Ado+Try, Sor+Rha+Xyl, and Mal+NaP+Glu.

Table 4.7: Resistant-breaking treatment of compounds (STC) against M. smegmatis (Rank 1-9)

RESISTANT BREAKING TREATMENT OF STRUCTURAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	quantum change of ZoI	Overall
1	1	Raf+Cel+Inu (3)	eMsA	Amp 0-15 Inh 0-14 Para 9-20	3	2	40	45
3	2	Mal+NaP+Glu (2)	eMsA	Amp 0-14 Tet 0-15	2	2	29	33
4	3	Cel+Glu+Try (2)	eMsA	Amp 0-14.5 Tet 0-10	2	2	24.5	28.5
2	4	Sor+Rha+Xyl (2)	eMsA	Amp 0-15 Amx 0-15	2	1	30	33
5	5	Dal+Ado+Glu (2)	eMsA	Amp 0-11 Cys 0-12	2	2	23	27
9	6	Dal+Ado+Try (1)	eMsB	Para 0-9.5	1	1	9.5	11.5
8	7	Glu+Mal+Myo (1)	eMsA	Amp 0-15	1	1	15	17
6	8	Myo+Sia+Try (1)	eMsA	Amx 0-17	1	1	17	19
6	9	Try+Glu+Gal (1)	eMsA	Amp 0-17	1	1	17	19

Table 4.8: Resistant-inducing treatment of compounds (STC) against M. smegmatis (Rank 1-10)

RESISTANT INDUCING TREATMENT STRUCTURAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Inducing	# classes	# n-0	quantum change of ZoI	Overall
1	1	Raf+Cel+Inu (5)	Ms	Clind 11-0	5	2	74	81
				Van 22-11				
				Amx 26-12				
			eMsB	Rif 29-11				
				Chlo 20-0				
4	2	Sor+Rha+Xyl (4)	Ms	Clind 11-0	4	2	45	51
				Ery 22-11				
				Amx 26-12				
			eMsA	Para 9-0				
3	3	Dal+Ado+Try (4)	Ms	Clind 11-0	4	2	45.5	51.5
				Amx 26-12				
				Ery 22-10.5				
			eMsA	Para 9-0				
2	4	Myo+Sia+Try (4)	Ms	Clind 11-0	4	1	56	61
				Strep 27-13				
				Amx 26-13				
			eMsB	Rif 29-11				
5	5	Mal+NaP+Glu (3)	Ms	Clind 11-0	3	2	43	48
				Amx 26-11				
			eMsA	Chlo 17-0				
6	6	Dal+Ado+Glu (3)	Ms	Amx 26-13	3	1	34	38
				Clind 11-0				
			eMsA	Moxi 19-9				
8	7	Glu+Mal+Myo (2)	Ms	Clind 11-0	2	1	26	29
				Amx 26-11				
9	8	Cel+Glu+Try (2)	Ms	Clind 11-0	2	2	20	24
			eMsA	Para 9-0				
9	9	Raf+Ado+Rha (2)	Ms	Clind 11-0	2	2	20	24
			eMsA	Para 9-0				
7	10	Try+Glu+Gal (2)	eMsA	5Fu 13-0	2	2	30	34
				Moxi17-0				

Table 4.9: Resistant-breaking treatment of compounds (FTC) against M. smegmatis (Rank 1-10)

RESISTANT BREAKING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	quantum change of ZoI	Overall
1	1	Sorb (3X) (5)	eMsA	Amp 0-13.5	4	4	81	89
				Amx 0-13				
				Ery 0-24.5				
			eMsB	Tet 0-9				
				Gen 15-36				
5	2	Mal (3X) (4)	eMsA	Van 0-16	3	3	63	69
			eMsB	Van 0-18				
				Tet 0-12				
				Chlo 0-17				
3	3	Sorb+Try+Malt (4)	eMsA	Van 0-14.5	4	3	66.5	73.5
				Ery 0-15				
			eMsB	Tet 0-13				
				Gen 15-39				
16	4	Myo+Mal (1.5X) (4)	eMsB	Amp 0-11	3	4	43.5	50.5
				Amx 0-11.5				
				Tet 0-10				
				Chlo 0-11				
17	5	Ado+Cel+Glu (4)	Ms	Met 0-11	4	4	42	50
			eMsB	Tet 0-9				
				Met 0-11				
				Eth 0-11				
2	6	Xyl+Glu (1.5X) (4)	eMsA	Amx 0-19	4	3	68.5	75.5
				Van 0-9.5				
				Ery 0-21				
				Gen 15.5-34.5				
14	7	Myo+Rha+Raf (3)	Ms	Ery 24-48	3	2	46.5	51.5
			eMsA	Amx 0-12				
				Van 0-10.5				
7	8	Mal+Rha+NaP (3)	eMsA	Amx 0-17	3	2	60	65
				Van 0-13				
				Gen 15-30				
4	9	Cel+Inu+Glu (3)	Ms	5-Fu 10.5-28.5	3	2	65	70
			eMsA	Amx 0-17				
				Ery 0-30				
11	10	Myo+Sia+Lac (3)	eMsA	Van 0-12.5	3	3	50.5	56.5
			eMsB	INH 0-28				
				Tet 0-10				

Table 4.10: Resistant-breaking treatment of compounds (FTC) against M. smegmatis (Rank 11-20)

RESISTANT BREAKING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	quantum change of ZoI	Overall
9	11	Sorb+Mal+Glu (3)	Ms	Rif 8-24	3	2	58	63
			eMsA	Amx 0-14				
			eMsB	Chlo 0-20				
15	12	Ado+Raf+Cel (3)	eMsA	Van 0-12.5	3	3	45	51
				Ery 0-21				
			eMsB	Tet 0-11.5				
7	13	Rham+Inu (1.5X) (3)	eMsA	Van 0-14	3	2	60	65
			eMsB	Amp 0-12				
				Gen 15-34				
6	14	Glu (3X) (3)	eMsA	Amp 0-15	3	2	61.5	66.5
				Ery 0-24				
			eMsB	Gen 15-37.5				
10	15	Sia+NaP+Raf (3)	Ms	Clind 0-20	3	3	51.5	57.5
			eMsA	Amx 0-14				
				Ery 0-17.5				
12	16	Sorb+Rha+Malt (3)	Ms	Met 0-15	3	3	48	54
				Clind0-18				
			eMsA	Van 0-15				
27	17	Dal (3X) (2)	eMsB	Amp 0-8.5	2	2	20	24
				Tet 0-11.5				
22	18	Malt+Cel (1.5X) (2)	eMsB	Tet 0-11.5	2	2	28	32
				Chlo 0-16.5				
18	19	Sia+Ado+ (1.5X) (2)	eMsB	Tet 0-11.5	2	1	42	45
				Gen 15-30.5				
19	20	Lac+Try+NaP (2)	eMsA	Ery 0-21.5	2	2	33.5	37.5
			eMsB	Tet 0-12				

Table 4.11: Resistant-breaking treatment of compounds (FTC) against M. smegmatis (Rank 21-30)

RESISTANT BREAKING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Breaking	# classes	# 0-n	quantum change of ZoI	Overall
21	21	Lac+Try+Mal (2)	eMsA	Ery 0-18	2	2	30	34
			eMsB	Chlo 0-12				
13	22	Try+Malt+Raf (2)	eMsB	Amx 0-22.5	2	2	49	53
				Chlo 0-26.5				
25	23	Cel (3X) (2)	Ms	Met 0-15	2	2	23.5	27.5
			eMsA	Amp 0-8.5				
20	24	Myo (3X) (2)	eMsA	Ery 0-20	2	2	33	37
			eMsB	Chlo 0-13				
24	25	Sia (3X) (2)	eMsA	Van 0-11	2	2	26	30
				Lin 0-15				
22	26	Try (3X) (2)	eMsB	Amx 0-13	2	2	28	32
				Chlo 0-15				
26	27	Rham (3X) (2)	eMsA	Amp 0-12	1	2	23	26
				Amx 0-11				
28	28	Inu (3X) (1)	eMsB	Chlo 0-19	1	1	19	21
29	29	Xyl (3X) (1)	eMsA	Van 0-12	1	1	12	14
29	30	Lac (3X) (1)	eMsA	Amx 0-12	1	1	12	14

Table 4.12: Resistant-inducing treatment of compounds (FTC) against M. smegmatis (Rank 1-7)

RESISTANT INDUCING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Rank	Treatment	Organism	Resistant Inducing	# classes	# n-0	quantum change of ZoI	Overall
1	1	Lac (3X) (9)	Ms	Amp 9-0	6	6	111.5	123.5
				Amx 15-0				
				Van 18.5-8				
				Rif 8-0				
				5-fu 10.5-0				
			eMsA	Tet 19-0				
				Gen 15.5-0				
				Rif 24-12				
			eMsB	Para 12-0				
2	2	Sia+Ado+ (1.5X) (6)	Ms	Amp 9-0	5	6	80.5	91.5
				Amx 15-0				
				Rif 8-0				
			eMsA	Tet 19-0				
				Chlo 15.5-0				
				Para 14-0				
3	3	Try (3X) (6)	Ms	Amp 9-0	4	5	73.5	82.5
				Amx 15-0				
				Rif 8-0				
				5-fu 10.5-0				
			eMsA	Tet 19-0				
				Rif 24-12				
4	4	Raf (3X) (5)	Ms	Amp 9-0	4	4	65.5	73.5
				Rif 8-0				
				5-fu 10.5-0				
			eMsA	Tet 19-0				
				5-fu 31.5-12.5				
5	5	Ado+Xyl+Raf (4)	Ms	5-fu 10.5-0	3	2	64.5	69.5
			eMsA	Tet 19-0				
				Strep 22.5-9.5				
				5-fu 31.5-9.5				
6	6	Malt+Raf+Gal (4)	Ms	5-fu 10.5-0	2	4	59.5	65.5
			eMsA	Gen 15.5-0				
			eMsB	Strep 21.5-0				
				Para 12-0				
7	7	Try+Malt+Raf (4)	Ms	Van 18.5-0	4	4	61	69
				Rif 8-0				
			eMsA	Tet 19-0				
				Gen 15.5-0				

Table 4.13: Resistant-inducing treatment of compounds (FTC) against M. smegmatis (Rank 8-20)

RESISTANT INDUCING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Ranking	Treatment	Organism	Resistant Inducing	# classes	# n-0	quantum change of ZoI	Overall
10	8	Xyl+Glu (1.5X) (4)	Ms	Amp 9-0	3	4	47.5	54.5
				Amx 15-0				
				Rif 8-0				
			eMsA	Chlo 15.5-0				
11	9	Sorb+Mal+Glu (3)	eMsA	Tet 19-0	3	3	46.5	52.5
				Chlo 15.5-0				
			eMsB	Para 12-0				
13	10	Lac+Try+Mal (3)	Ms	5-fu 10.5-0	3	3	43.5	49.5
			eMsA	Tet 19-0				
				Para 14-0				
8	11	Glu (3X) (3)	Ms	Amp 9-0	2	3	55.5	60.5
				Amx 15-0				
			eMsA	5-fu 31.5-0				
11	12	Sorb+Rha+Malt (3)	eMsA	Tet 19-0	3	3	46.5	52.5
				Gen 15.5-0				
			eMsB	Para 12-0				
9	13	Myo+Mal (1.5X) (3)	Ms	Amp 9-0	3	3	50.5	56.5
			eMsA	Tet 19-0				
				5-fu 31.5-9				
16	14	Malt (3X) (3)	Ms	Amp 9-0	2	3	34.5	39.5
				Amx 15-0				
				5-fu 10.5-0				
15	15	Xyl (3X) (3)	Ms	Van 18.5-0	3	3	37	43
				Rif 8-0				
				5-fu 10.5-0				
28	16	Malt+Cel (1.5X) (2)	Ms	Amp 9-0	2	2	19.5	23.5
				5-fu 10.5-0				
30	17	Lac+Try+NaP (2)	Ms	Amp 9-0	2	2	17	21
				Rif 8-0				
26	18	Rham+Inu (1.5X) (2)	Ms	Amp 9-0	1	2	24	27
				Amx 15-0				
17	19	Sia+NaP+Raf (2)	eMsA	Tet 19-0	2	2	34.5	38.5
				Chlo 15.5-0				
21	20	Ado+Raf+Cel (2)	Ms	Amx 15-0	2	2	30.5	34.5
			eMsA	Moxi 15.5-0				

Table 4.13: Resistant-inducing treatment of compounds (FTC) against M. smegmatis (Rank 21-34)

RESISTANT INDUCING TREATMENT FUNCTIONAL TRIPLE COMBINATION COMPOUNDS								
Overall Ranking	Ranking	Treatment	Organism	Resistant Inducing	# classes	# n-0	quantum change of ZoI	Overall
21	21	Myo+Rha+Raf (2)	eMsA	Tet 19-0	2	1	31.5	34.5
				Strep 22.5-10.5				
14	22	Myo (3X) (2)	Ms	5-fu 10.5-0	1	2	42	45
			eMsA	5-fu 31.5-0				
20	23	Inu (3X) (2)	Ms	Rif 8-0	1	2	32	35
			eMsA	Rif 24-0				
23	24	Ado+Cel+Glu (2)	Ms	5-fu 10.5-0	2	2	29.5	33.5
			eMsA	Tet 19-0				
29	25	Myo+Sia+Lac (2)	Ms	Amp 9-0	2	1	19.5	22.5
				Van 18.5-8				
24	26	Sia (3X) (2)	Ms	Amp 9-0	2	1	26.5	29.5
			eMsA	5-fu 31.5-14				
25	27	Sorb (3X) (2)	Ms	Rif 8-0	2	2	22	26
			eMsA	Para 14-0				
27	28	Rham (3X) (2)	Ms	Rif 8-0	2	2	20	24
			eMsB	Para 12-0				
17	29	Cel (3X) (2)	eMsA	Moxi 15.5-0	2	2	34.5	38.5
				Tet 19-0				
19	30	Dal (3X) (2)	eMsA	Tet 19-0	2	2	33	37
				Para 14-0				
33	31	Mal (3X) (1)	Ms	5-fu 10.5-0	1	1	10.5	12.5
30	32	Dal+Myo+Raf (1)	eMsA	Tet 19-0	1	1	19	21
34	33	Mal+Rha+NaP (1)	Ms	Amp 9-0	1	1	9	11
32	34	Cel+Inu+Glu (1)	eMsA	Chlo 15-0	1	1	15	17

4.1.7 DIRECT COMPARISON AND EVALUATION OF SINGLE PHENOTYPIC COMPOUNDS TESTED AT 1X AND 3X DOSES TO GUIDE THE SELECTION OF TOP FOR FURTHER STUDIES

The triple combination compounds based on the individual activities of the single compounds were generated to obtain a more robust interaction. To validate this, the functional triple combination graph was plotted side by side with the single combination (Fig 4.5).

Most of the single compounds had a higher resistant breaking activity compared to the triple dose. The 1X single dose treatment by inulin, cellobiose, raffinose had significantly high values compared to the 3X counterparts. Some compounds like malonic acid, gluconic acid, and sorbose had their 3X effect having a higher resistant-breaking effect. In general, the single compound treatment had an overall greater resistant-breaking effect compared to the 3X.

For the resistant-inducing effects, almost all the 3X functional triple combinations had higher effects compared to the single compounds except rhamnose. 3X of lactose, tryptophane, and raffinose are among the compounds with the highest resistant inducing effects. Since the 3X treatments had the highest effect on the resistant inducing compounds and some of the resistant breaking treatments, the 3X treatments can be said to have been more robust than the single treatments.

A scoring system was obtained for both the single and triple combinations based on the magnitude of change, the number of classes of antibiotics affected and the number of antibiotics revived or lost. This ranking system from the tables and the side-by-side comparison of 1X and 3X was used to generate top 5 resistant breaking and inducing compounds to proceed with for the phenotypic assays. They were also used to obtain the top 2 resistant breaking and inducing compound treatments to proceed with for the gene expression analysis.

Sor (3X) and Mal(3X) were among the few functional triple combinations to have a higher effect over the single compounds for the resistant breaking effect. Mal (3X) was also higher than 1X counterpart for resistant inducing effect. Aside from the 3X of sorbose and malonic acid dominating their 1X counterpart in the resistant-modulating effect, they also had the highest resistant breaking effect number of effects on different antibiotics for functional triple combination. These treatments were therefore selected as the top two resistant breaking treatments.

The 3X treatments of lactose, tryptophane, and raffinose were selected based on their significant resistant-inducing effect (Fig 4.6). Even though these treatments had lower resistant breaking effects compared to their single counterparts. Aside from their dominant effect in the resistant inducing activity compared to their single compound treatment, these compounds had the highest resistant inducing effect based on the number of antibiotics affected (Table 4.11).

These served as the scoring and selection system for the resistant breaking and inducing compounds for the further phenotypic and gene expression assay.

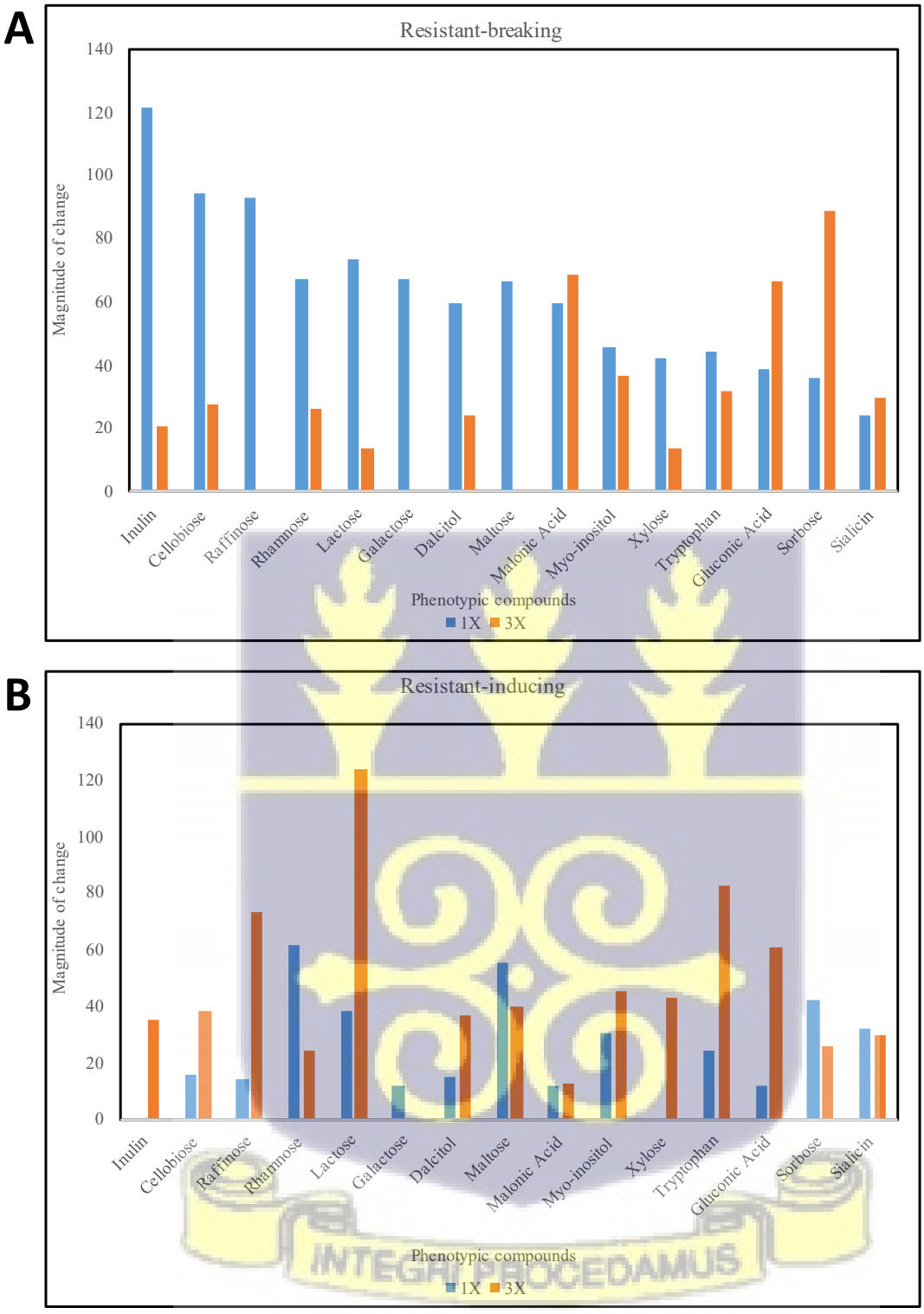


Figure 4.5: Antibiotic activity profile comparing 1X and 3X of the phenotypic compounds among the three strains. *The plot of measured zones of inhibition under controlled conditions and in the presence of single and functionally grouped modifier compounds for the three strains.*

4.1.8 EFFECTS OF SINGLE PHENOTYPIC COMPOUND ON CELL AND COLONY MORPHOLOGY

The effect on the phenotype, colony formation, and cellular morphology of *M. smegmatis* was assessed in the presence of the compounds. Since the compounds are carbohydrate and amino acid, we hypothesized that the growth and metabolic pattern of the organism might be affected which might affect the colony formation and cellular morphology. The single compounds were tested against wt, and ten (10) compounds were selected based on their robust function. These ten compounds were grouped into resistant-breaking (RB) and resistant-inducing (RI).

The morphology of cells in the control were classical *M. Smegmatis* cells, long, slender, consistent big widths, and pink rods. Most of the cells maintained the pink coloration in the presence of the compounds but compounds like inulin, gluconic acid, rhamnose, and malonic acid made some of the cells shorter. Sodium pyruvate made almost all the cells uniformly shorter compared to the control. Dalcitol treated cells were much tinnier and shorter than the control and had pale pink staining. Myoinositol treated cells like dalcitol, had shorter and pale pink coloration of the cells. Salicin similarly had cells with shorter lengths and width but distinctively had a higher staining intensity. Lactose-treated cells had shorter lengths but maintained the pink coloration of the cells. In the presence of tryptophan, the cells had shorter lengths and normal pin coloration compared to the control. Sorbose treated cells maintained pink coloration but had variability of cell length and some of the cells formed groups. Malonic-acid treated cells had shorter cells with normal pink coloration. Sodium pyruvate cells showed significantly smaller cells with a normal pink coloration the cells. Raffinose treated cells had shorter cells with normal staining compared to the wt. Cells found in inulin treatment were short, pale stained, and sparsely arranged. Both gluconic acid and galactose slightly reduced the length of the rods but maintained the pink coloration. Cellobiose, malonic acid, and adonitol had normal cell morphology- with classical rod shapes and normal pink

staining like the wt. Slight differences were observed with the colonies formed in the presence of the compounds. But one noteworthy feature was the halo space that was formed at the center. All

the compounds increased this space compared to the control, which we believe could be linked to how the cells compact themselves (Fig 4.6-4.8).

Colony And Cell Morphology of *M. Smegmatis* In The Presence of Single Compounds

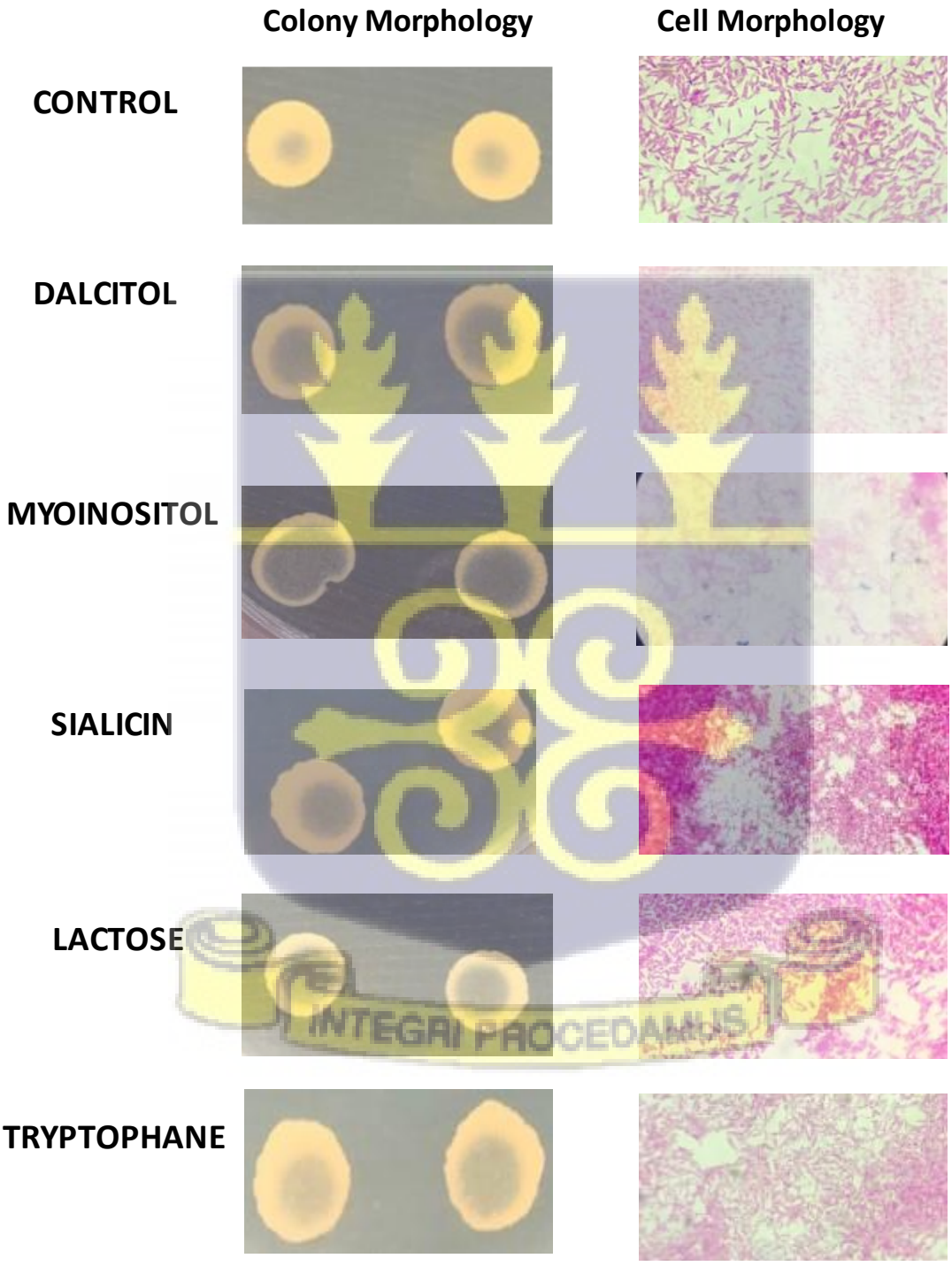


Figure 4.6: Effect of single PCs (dalcitol, myo-inositol, sialicin, lactose, and tryptophane) on the colony and cell morphology of Ms wt. *A volume of 100 ul of each phenotypic compound (1 ug/ul), both single PCs and triple PC combinations, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology.*

Colony And Cell Morphology of *M. Smegmatis* In The Presence of Single Compounds

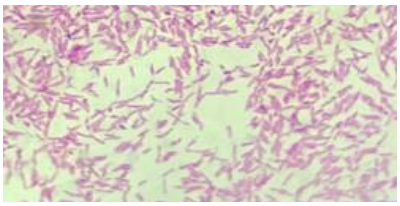
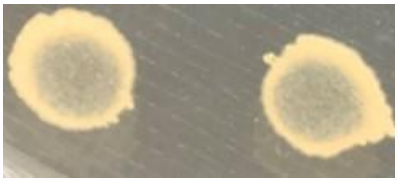
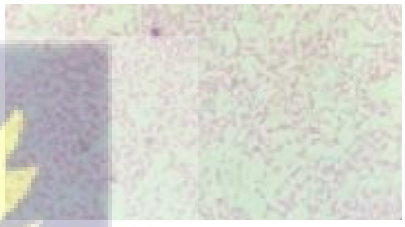
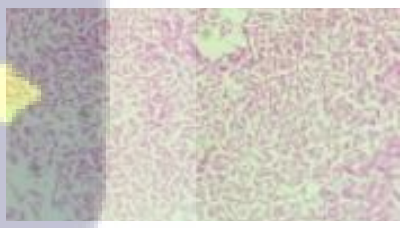
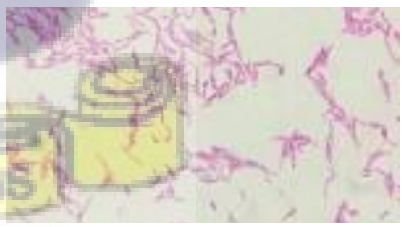
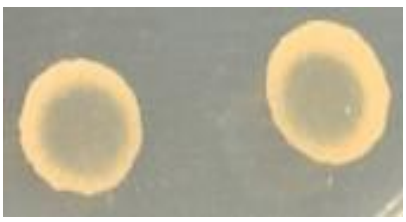
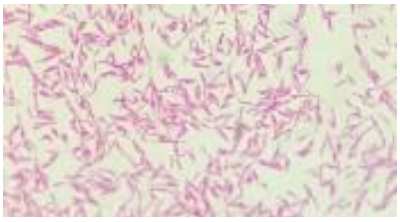
	Colony Morphology	Cell Morphology
CONTROL		
SORBOSE		
MALONIC ACID		
RHAMNOSE		
SODIUM PYRUVATE		
MALTOSE		
ADONITOL		

Figure 4.7: Effect of single PCs (sorbose, malonic acid, rhamnose, sodium pyruvate, maltose, and tryptophane) on the colony and cell morphology of Ms wt. *A volume of 100 ul of each phenotypic compound (1 ug/ul), both single PCs and triple PC combinations, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology.*

Colony And Cell Morphology of *M. Smegmatis* In The Presence of Single Compounds

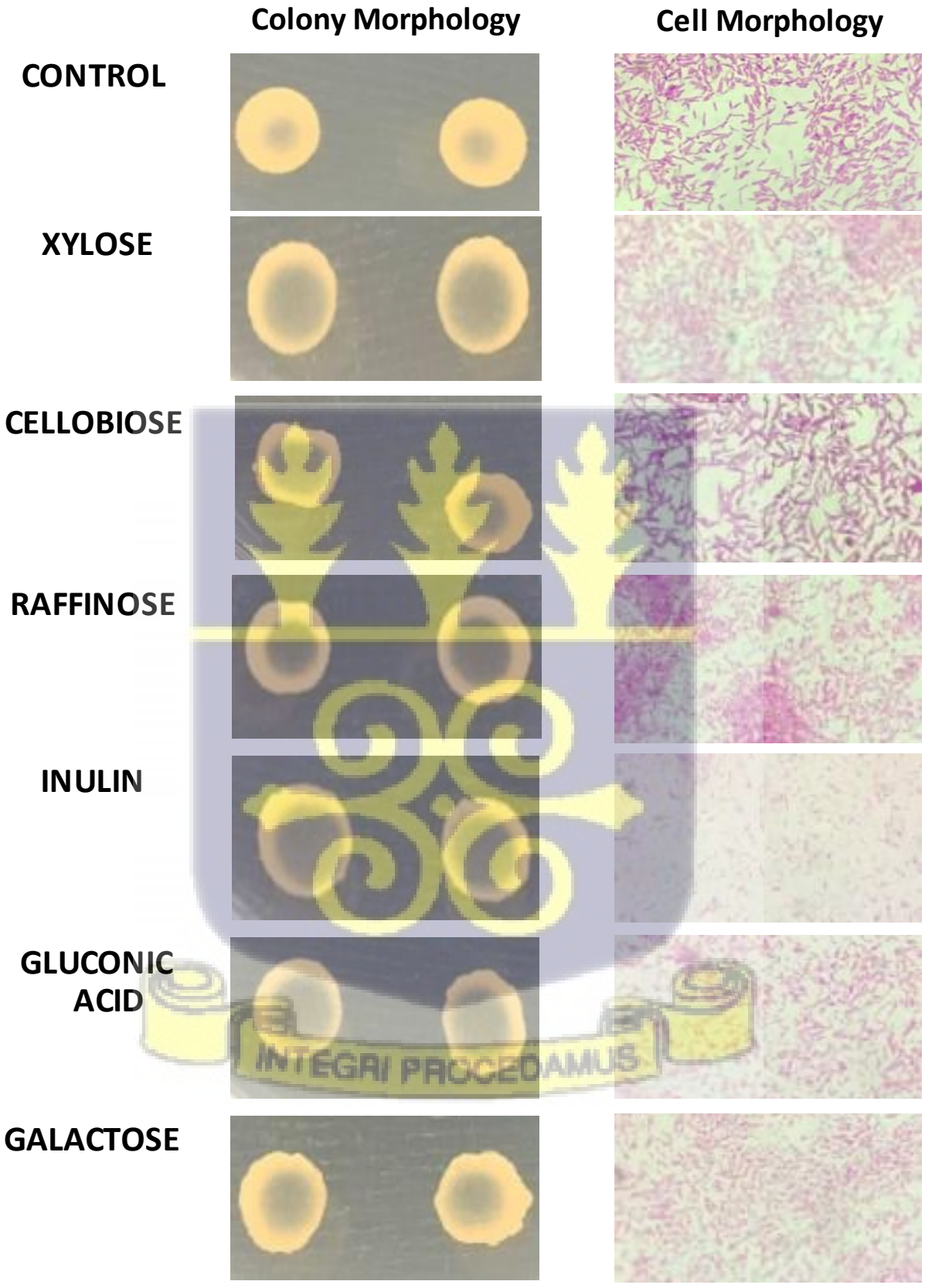


Figure 4.8: Effect of single PCs (xylose, cellobiose, raffinose, inulin, gluconic acid, and galactose) on the colony and cell morphology of Ms wt. *A volume of 100 ul of each phenotypic compound (1 ug/ul), was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

4.1.9 EFFECTS OF TRIPLE COMPOUND COMBINATIONS ON CELL AND COLONY MORPHOLOGY

Most of the cells maintained their typical rod shape with pink coloration. Cells treated with SOR+TRY+MALT, in the resistant-breaking group, not only had shorter cells but also had pale pink coloration of cells. All the resistant-breaking combinations, except SOR+TRY+MALT, had similar cell morphology compared to the control, which is rod-shaped and pink staining. ADO+XYL+RAF and RAF (3X) in the resistant-inducing group also had short rods and pale pink coloration. Aside from these two treatments, all the other combinations (Lac (3X), Sia+Ado (1.5X), and Try (3X)) had normal rod shape and staining intensity. The colonies did not look different in the presence of the different compounds just like was observed in the single treatment. Like the initial observation, larger halo spaces were found within the center. One other feature that stands out here is the wrangling edges of the colonial phenotype compared to the control especially in the resistant inducing groups but also in some of the resistant breakings. Another distinctive feature worth noting is the scattering effect of Sor (3X) on the colony formation in wt (Fig 4.9 and 4.10).

The control eMsA cells had short and pink rods with a bit of purple coloration of the background. Most of the cells in the resistant-breaking treatment had pink and short rods like the control but SOR+TRY+MALT had a pale pink coloration of the cells similar to the wt. Most of the cells in the resistant-inducing treatment had short and pink coloration of cells but ADO+XYL+RAF had a purple coloration in the background. The colony morphology phenotype was a bit hard to see because of the green coloration of the plate caused by pH affecting malachite green in the media. Notwithstanding that, the colony morphology in the treatments had changes in terms of morphology compared to the control. Another noteworthy feature is the formation of smaller colonies within the bigger cell colony in Sia+Ado (1.5X). Even though we are still uncertain of the cause, we implicate cell motility to be the cause of this (Fig 4.11 and 4.12)

The control eMsB cells had short and pink rods which were classical of erythromycin-resistant M. smegmatis B cells. All the cells in both resistant breaking and inducing in the treatment had short rod cells like the control with pink coloration except SOR (3X) which was pale pink. Sor (3X) treated cells showed aggregation of cells in groups. The colony morphology phenotype was difficult to see just like in eMsA, but just slight differences were observed in the colony morphology across the treatments. Similar to eMsA, Try (3X) and Lac (3X) in the resistant inducing group showed signs of cell motility as smaller colonies were seen within the bigger colonies. (Fig 4.13 and 4.14).

Colony and Cell Morphology of Ms WT In The Presence of selected Functional Triple Combination Compounds based on Resistant-breaking activity

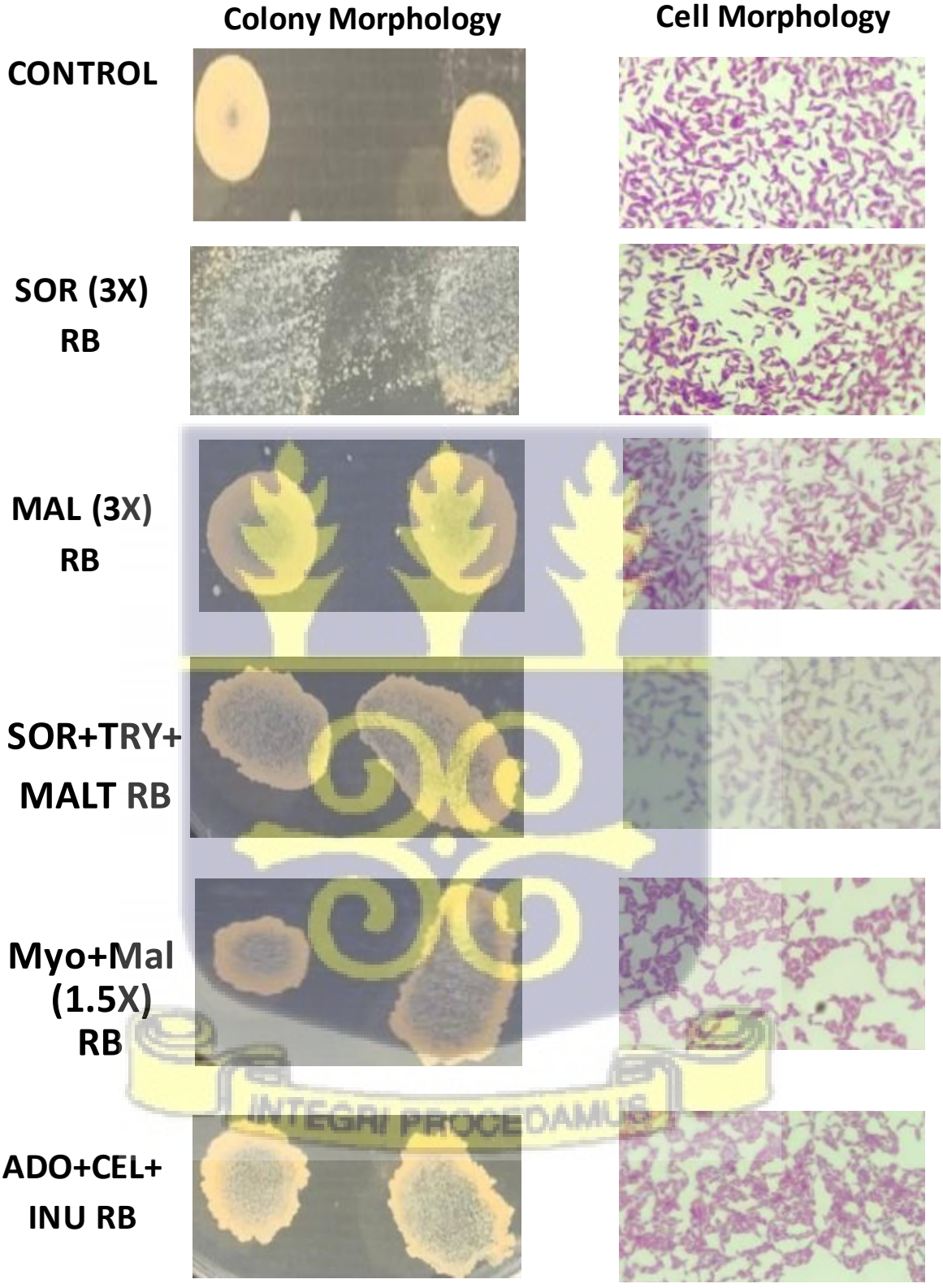


Figure 4.9: Effect of selected resistance-breaking FTC compounds (SOR (3X), MAL (3X), SOR+TRY+MALT, MYO+MAL (1.5X) and ADO+CEL+INU) on the colony and cell morphology of Ms wt. *A volume of 300 ul FTC (3X), 150ul of FTC (1.5X), and 100ul of each compound in the other FTC, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

Colony and Cell Morphology of Ms WT In The Presence of selected Functional Triple Combination Compounds based on Resistant -inducing activity

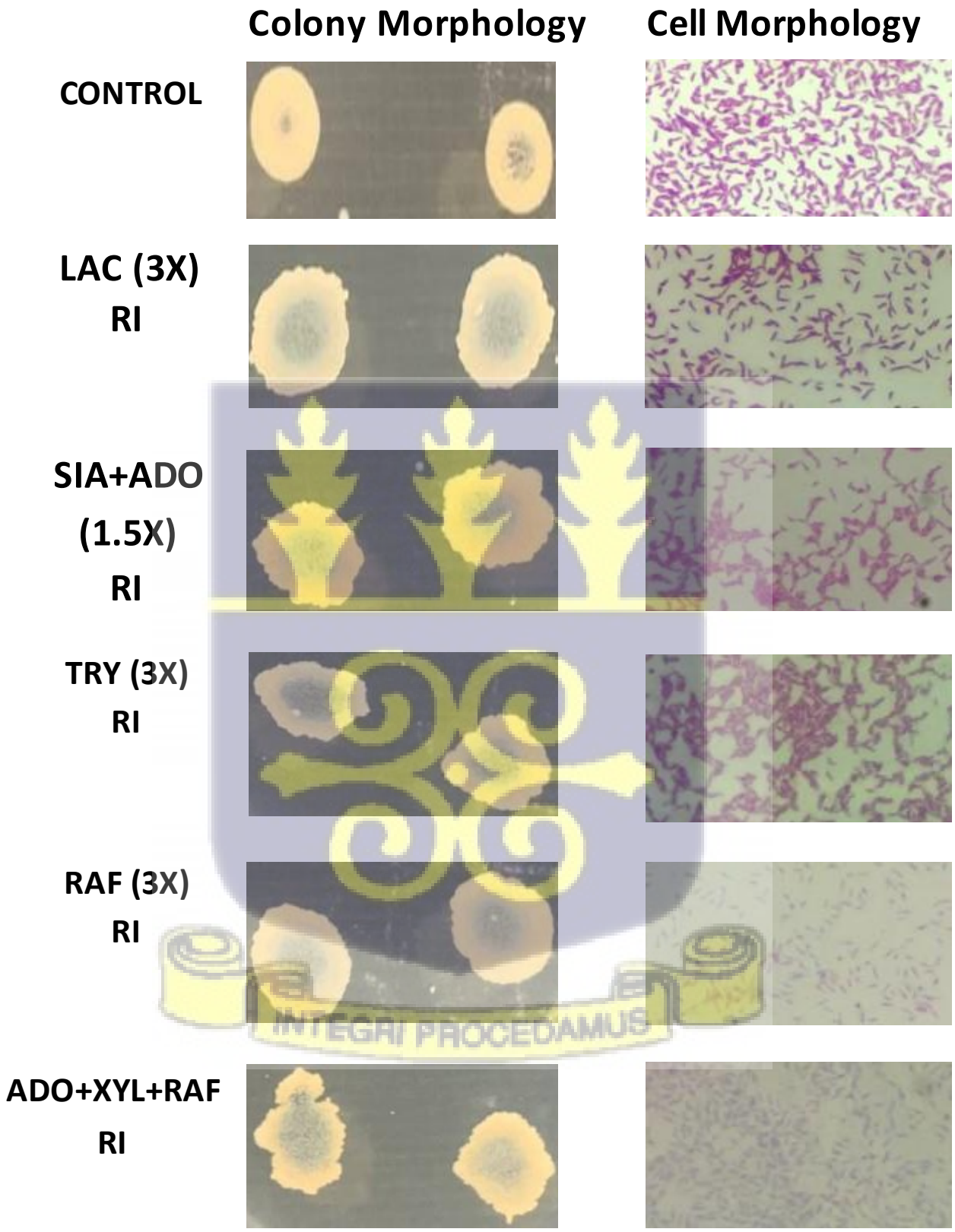


Figure 4.10: Effect of selected resistance-inducing FTC compounds (LAC (3X), SIA+ADO (1.5X), TRY (3X), RAF (3X), and ADO+XYL+RAF) on the colony and cell morphology of Ms wt. *A volume of 300 ul FTC (3X), 150ul of FTC (1.5X), and 100ul of each compound in the other FTC, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

Colony and Cell Morphology of eMsA In The Presence of selected Functional Triple Combination Compounds based on Resistant-breaking activity

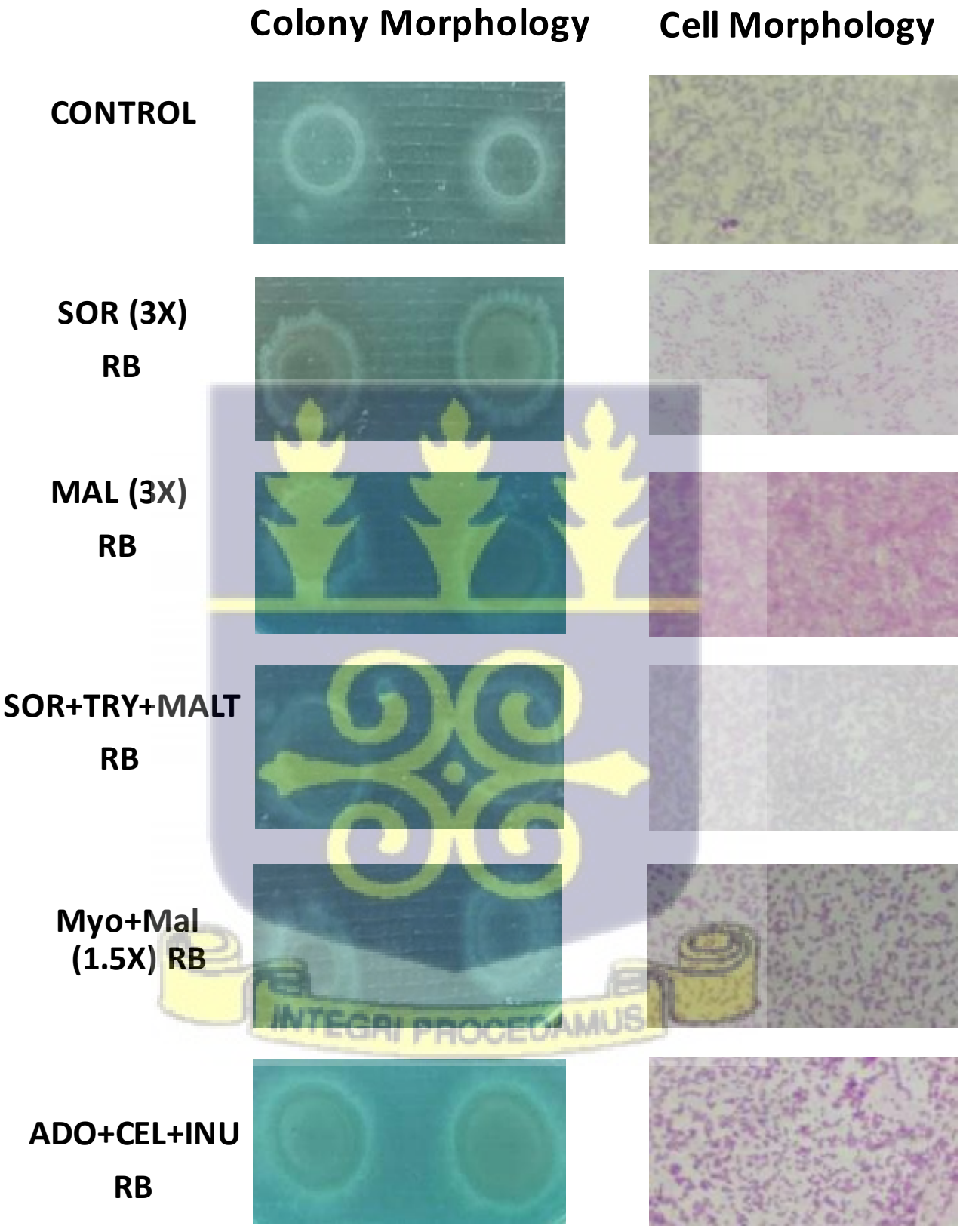


Figure 2: Effect of selected resistance-breaking FTC compounds (SOR (3X), MAL (3X), SOR+TRY+MALT, MYO+MAL (1.5X) and ADO+CEL+INU) on colony and cell morphology of eMsA*. A volume of 300 ul FTC (3X), 150ul of FTC (1.5X) and 100ul of each compound in the other FTC , was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

Colony and Cell Morphology of eMsA In The Presence of selected Functional Triple Combination Compounds based on Resistant-inducing activity

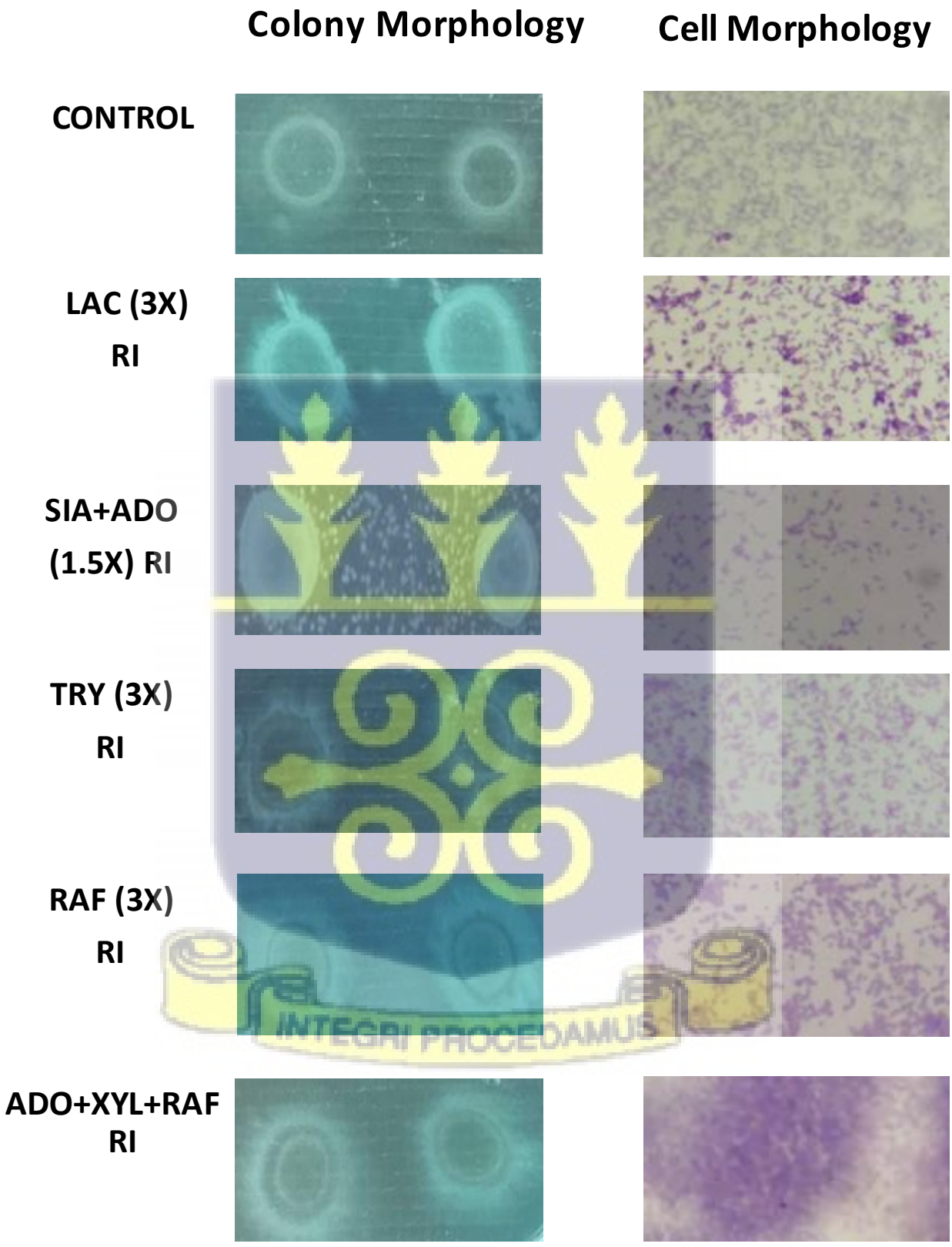


Figure 4.12: Effect of selected resistance-inducing FTC compounds (LAC (3X), SIA+ADO (1.5X), TRY (3X), RAF (3X), and ADO+XYL+RAF) on the colony and cell morphology of eMsA. *A volume of 300 ul FTC (3X), 150ul of FTC (1.5X), and 100ul of each compound in the other FTC, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

Colony and Cell Morphology of eMsB In The Presence of selected Triple Combination Compounds based on Antibiotic Modulating activity

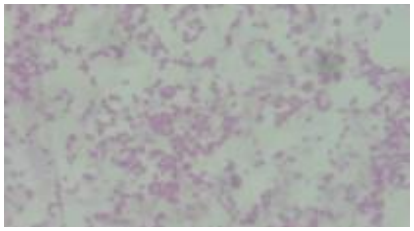
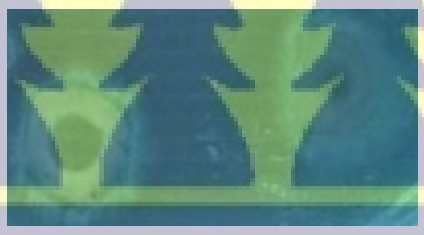
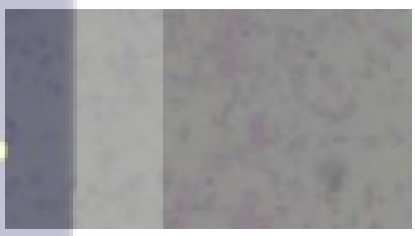
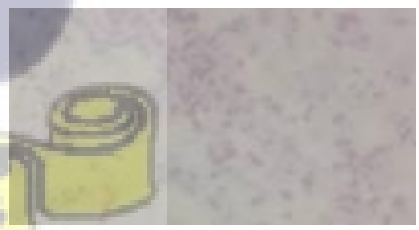
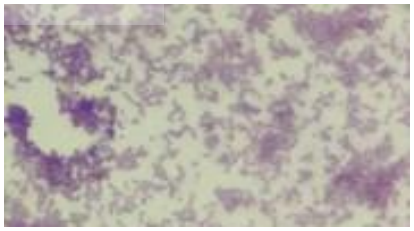
	Colony Morphology	Cell Morphology
CONTROL		
SOR (3X) RB		
MAL (3X) RB		
SOR+TRY+MALT RB		
Myo+Mal (1.5X) RB		
ADO+CEL+INU RB		

Figure 3.13: Effect of selected resistance-breaking FTC compounds (SOR (3X), MAL (3X), SOR+TRY+MALT, MYO+MAL (1.5X) and ADO+CEL+INU) on the colony and cell morphology of eMsB. *A volume of 300 ul FTC (3X), 150ul of FTC (1.5X), and 100ul of each compound in the other FTC, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10-4 dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

Colony and Cell Morphology of eMsB in The Presence of selected Functional Triple Combination Compounds based on Resistant inducing activity

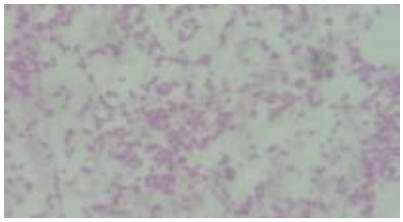
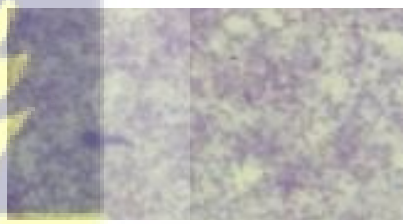
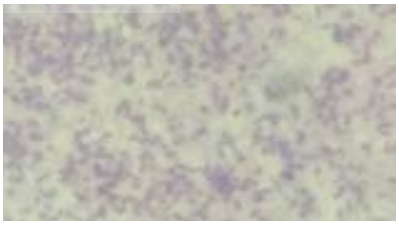
	Colony Morphology	Cell Morphology
CONTROL		
LAC (3X) RI		
SIA+ADO (1.5X) RI		
TRY (3X) RI		
RAF (3X) RI		
ADO+XYL+RAF RI		

Figure 4.14: Effect of selected resistance-inducing FTC compounds (LAC (3X), SIA+ADO (1.5X), TRY (3X), RAF (3X), and ADO+XYL+RAF) on the colony and cell morphology of eMsB. *A volume of 300 ul FTC (3X), 150ul of FTC (1.5X), and 100ul of each compound in the other FTC, was spread on agar plates and allowed to dry. For colony morphology, 10 ul of the 0.7 OD of cells was spotted twice on one half the plate. A 10^{-4} dilution was prepared from the stock cells (OD of 0.7) and 100 ul was spread on the other half of the plate. The plates were incubated for 48 h. The spots (colonies) were observed, and a single colony was picked from the spread and acid-fast stained to determine the effect of the various treatments on the cell morphology*

4.2 RESULTS ON THE EFFICACY OF SELECTED COMPOUNDS AGAINST THE EFFLUX AND BIOFILM ACTIVITY

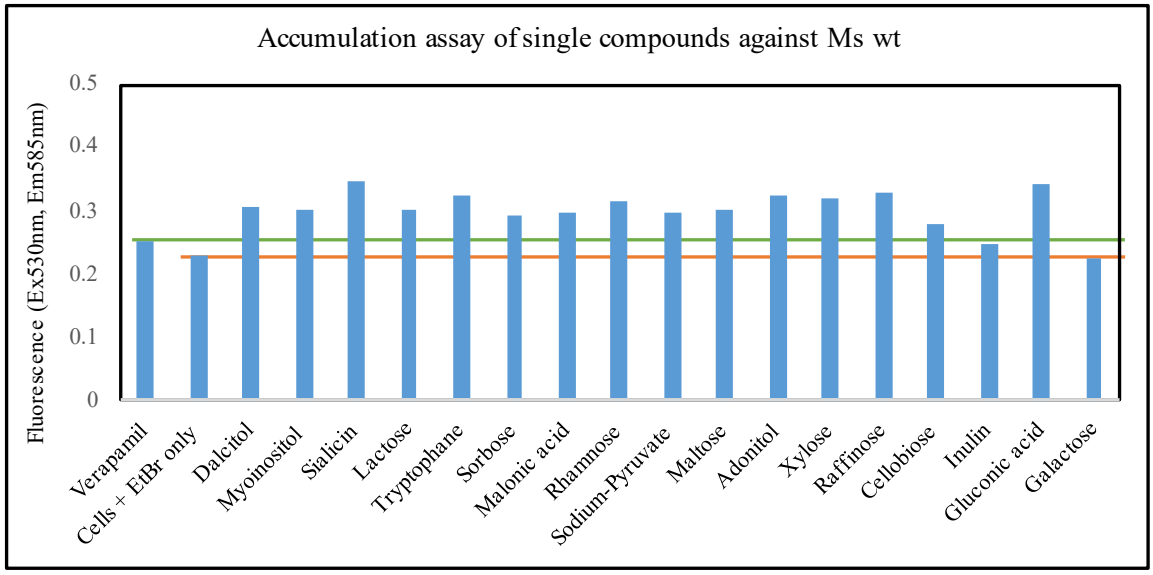
4.2.1 EFFECTS OF SINGLE COMPOUND ON CELLULAR TRANSPORT; ACCUMULATION IN MYCOBACTERIA

One major hallmark of antimicrobial resistance is the ability of microbes to upregulate the expression of efflux pumps to extrude antibiotics. This is done to prevent the accumulation of antibiotics within the bacteria, leading to the buildup of resistance (Tran et al., 2020). The compounds' efficacy to inhibit efflux pump-action and accumulation was investigated using ethidium bromide as the accumulating material and verapamil, a known efflux inhibitor as positive control and cells without any treatment as the negative control. Both the single and selected triple combination compounds were tested against *M. smegmatis* and the two mutant strains.

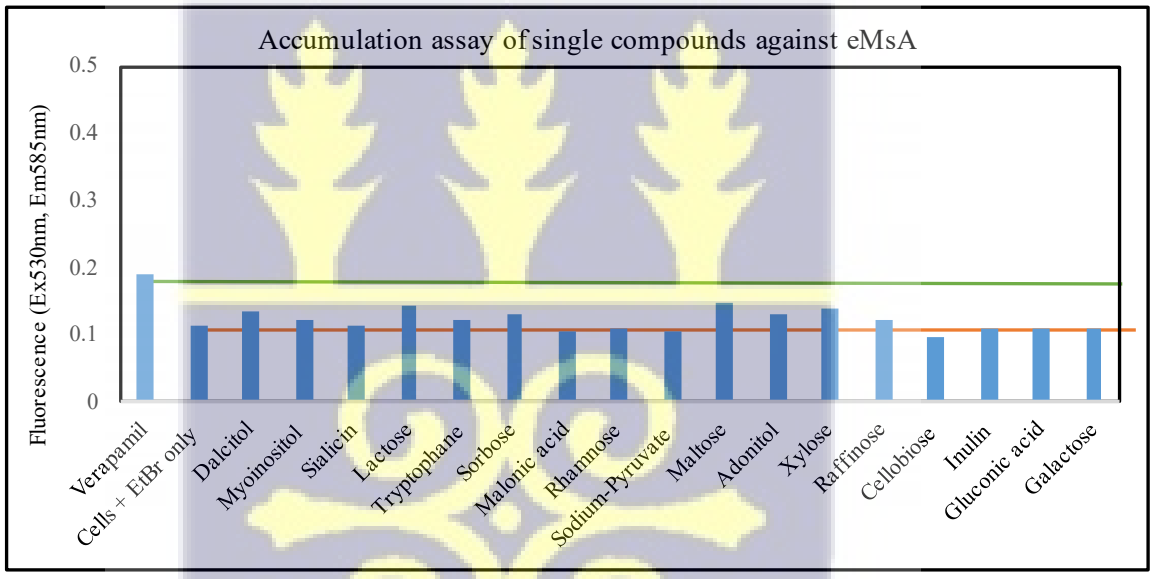
To assess the ability of each compound to potentiate antibiotics by allowing their accumulation within the target cell. The accumulation of ethidium bromide (EtBr); a DNA intercalating dye fluorescing between the wavelengths 530 (Excitation wavelength) and 585 (Emission wavelength) in the presence of each compound, a known efflux pump inhibitor (verapamil) serving as a positive control and a set up containing cells and the dye only as a negative control was used for this assessment. The extent of ethidium bromide accumulation was measured employing fluorescence spectroscopy. The Ms wt cells treated with the single compounds plus ethidium bromide only,

negative control served as the cut-off point to know how the compounds affect the accumulation and were compared to verapamil, the positive control. All the compounds except galactose increased the accumulation of ethidium bromide compared to the negative control. Also, all the compounds except galactose and inulin increased the accumulation of ethidium bromide compared to the positive control in the wt. Most of the compounds are effective in drug/ethidium bromide accumulation, hence can be described as resistant-breaking compounds. Salicin and gluconic acid showed the highest impact on ethidium bromide accumulation. Galactose and inulin even though were not higher, had almost the same level of impact compared to the negative and positive control respectively. For eMsA, most of the cells had activity either slightly higher or equal to the negative control but none of the compounds had activity higher than the positive control. Lactose and maltose had the highest activity above the negative control but were not close to the positive control. For eMsB, all the compounds except malonic acid had higher ethidium bromide accumulation activity compared to the negative control. Out of the seventeen (17) compounds, four (4) salicin, sorbose, xylose, and gluconic acid had higher activity than the positive control. Gluconic acid increased the accumulation activity by 1.5-fold compared to the control (Fig 4.15).

A



B



C

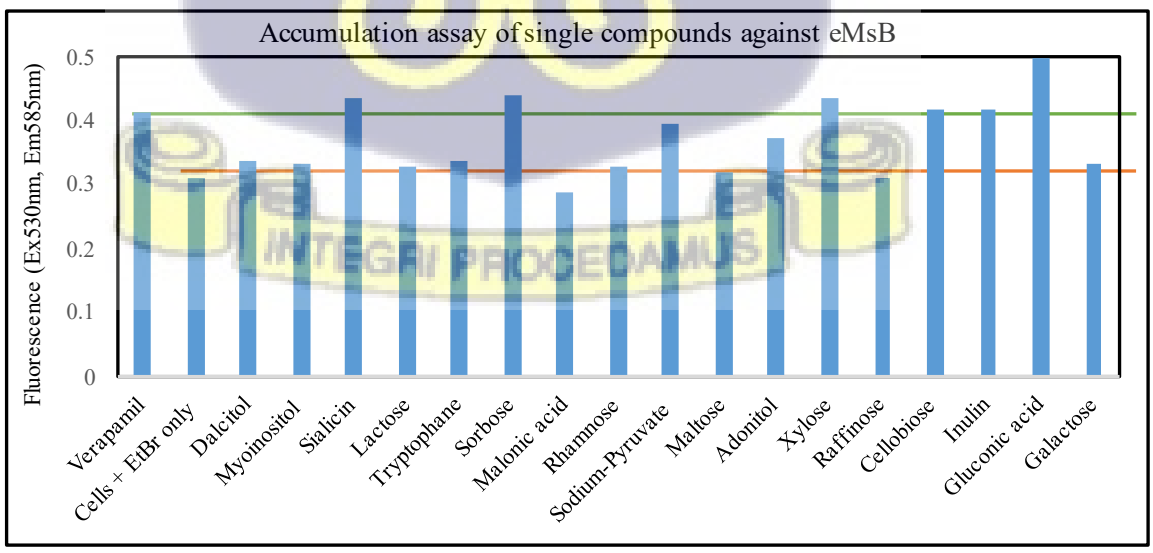


Figure 4.15: Fluorescence values for ethidium bromide accumulation in Ms wt with single compounds. *The plot of fluorescence values under controlled conditions and in the presence of individual modifier compounds for (A) Ms wt (B) eMsA and (C) eMsB. The cell pellets were resuspended in 0.8% PBS and the OD adjusted to 0.4. Then 3 ul of 3 ug/ul EtBr was added to all the cell suspensions. Into 2 ml Eppendorf tubes, 990 ul of cells suspension and 10 ul of 20 mg/ul PC were added. Verapamil served as the positive control and the PBS as the negative control. All the reactions for the PC plus the controls were set up in triplicates. The reaction was incubated for one hour in a shaking incubator, 100 ul of the content of each reaction tube was pipetted into a 96-well plate. Fluorescence of EtBr at excitation and emission wavelengths at 530 at 585 (nm) respectively were read within time intervals of 30, 60, and 120 min using a Varioskan microplate reader.*

4.2.2 EFFECTS OF TRIPLE COMPOUND COMBINATION ON CELLULAR TRANSPORT; ACCUMULATION IN MYCOBACTERIA

In the accumulation assay of selected triple combination compounds against Ms wt, most of the selected triple combination compounds grouped into resistant-breaking and resistant-inducing had activities below the negative control. Contrary to our expectations, the triple combination compounds were not robust compared to the effect of the single compounds. Sor (3X) was the only resistant-breaking compound that had activity against the wt compared to the control. Mal (3X) had a drastic reduction of activity. Most of the compounds in the resistant breaking group had lower activity, contrary to our previous results. This observation could suggest that drug accumulation may not be the mode of action of these compounds even though they are resistant to breaking. All the resistant-inducing compounds except lac (3X) had activities below the negative control. Most of the compound's activity being low as anticipated could signify lower drug accumulation could be one way these compounds induce resistance. This result was consistent with our previous phenotypic interaction assay. Against eMsA, four (4) of the five (5) resistant-breaking compounds had higher ethidium bromide accumulation compared to the control. Sor+Try+Malt and Myo+Mal (1.5X) had activities higher than verapamil, the positive control. This could suggest drug accumulation be a mode by which these compounds break resistance in the bacteria. Four (4) of the five (5) resistant-inducing compounds had accumulation higher than

the negative control. Ado+Xyl+Raf had higher fluorescence greater than the positive inhibitor. Even though these compounds are resistant-inducing groups, as observed in the result, they induce a higher compound accumulation. When eMsB was treated with the selected triple combination compounds for the accumulation assay, most of the compounds in the resistant-breaking group had79eactiities higher than the negative control with Mal (3X) having the highest reading. This result is consistent with the group of being resistant breaking. Most of the compounds in the resistant-inducing had lower or the same accumulation readings compared to the cell-only control. Ado+Xyl+Raf, just like in eMsA had higher readings but not as high as verapamil (Fig 4.16).

RESISTANT -BREAKING RESISTANT -INDUCING

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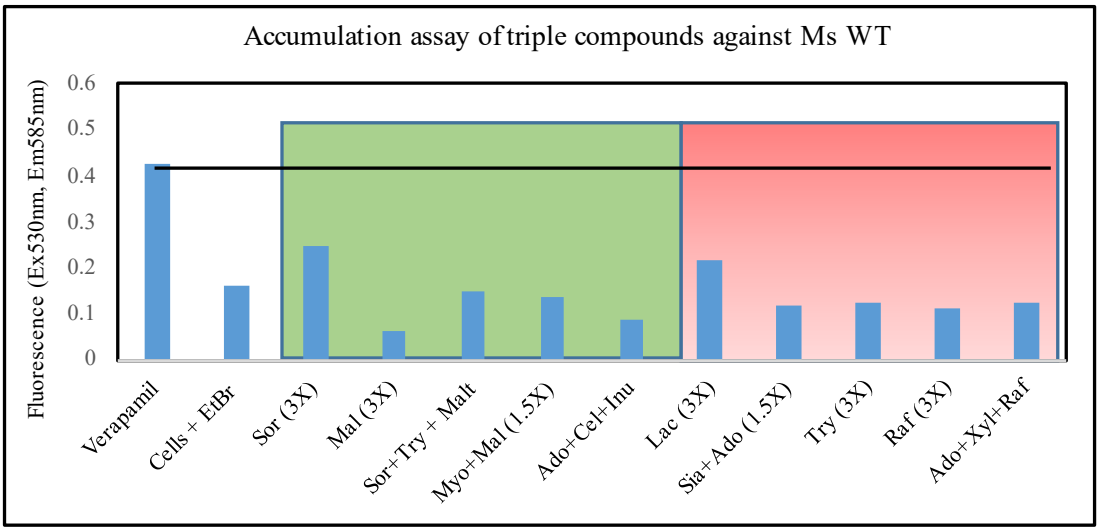

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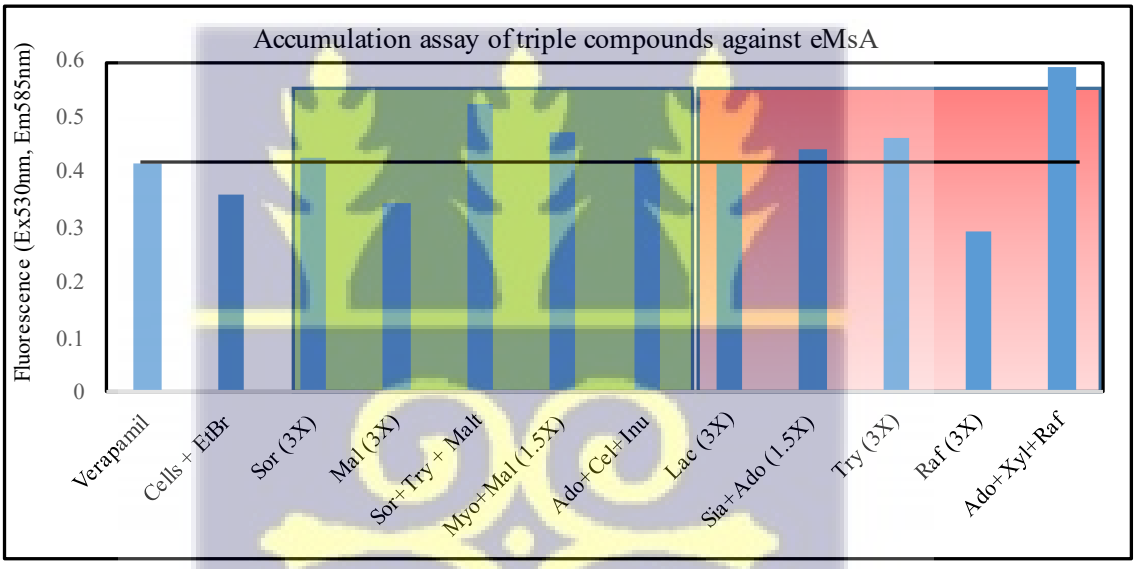

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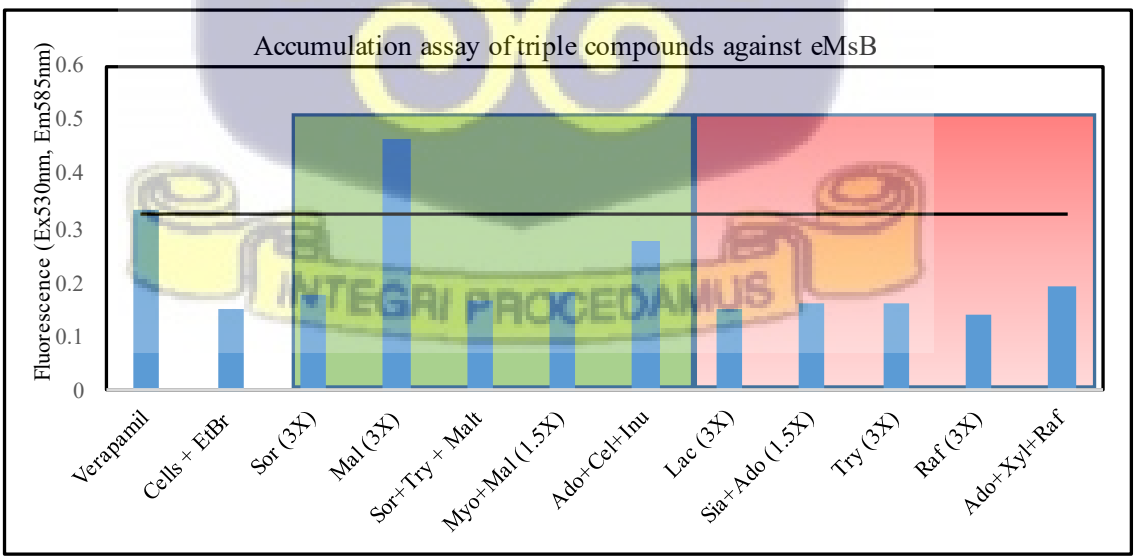

Figure 4.16: Fluorescence values for ethidium bromide accumulation in Ms wt with triple compound combinations. *Plot of fluorescence values under controlled conditions and in the presence of triple modifier compound combinations for (A)Ms wt. (B) eMsA and (C) eMsB. The cell pellets were resuspended in 0.8% PBS and the OD adjusted to 0.4. Then 3 ul of 3 ug/ul EtBr was added to all the cell suspensions. Into 2 ml Eppendorf tubes, 970 ul of cells suspension and 10 ul of 20 mg/ul of each PC in the combination were added. For a 1.5x dose, 15 ul was picked whereas a 3x was 30ul. Verapamil served as the positive control and the PBS as the negative control. All the reactions for the PC plus the controls were set up in triplicates. The reaction was incubated for one hour in a shaking incubator, 100 ul of the content of each reaction tube was pipetted into a 96-well plate. Fluorescence of EtBr at excitation and emission wavelengths at 530 at 585 (nm) respectively were read within time intervals of 30, 60, and 120 min using a Varioskan microplate reader.*

4.2.3 EFFECTS OF SINGLE COMPOUND ON CELLULAR TRANSPORT; EFFLUX IN MYCOBACTERIA

All the single compounds inhibited efflux action in Ms wt better than the cell-only control (negative), with dalcitol, malonic acid, rhamnose, and gluconic acid coming on top of the group. No compound had activity greater than verapamil. In the efflux assay of single compounds against eMsA, all the compounds had activities higher than the negative control but gluconic acid and dalcitol had higher readings than the positive control. For eMsB, most of the compounds had activities higher than the cell-only control, with tryptophane and maltose being slightly higher than verapamil. Gluconic acid had the same reading as verapamil, with just rhamnose and adonitol being the only compounds to have activities lower than the cell-only control. This result is consistent with what was obtained for eMsA (Fig 4.17).

This result suggests that the single compounds have a higher ability to inhibit efflux action in the resistant mutants than in the wt.

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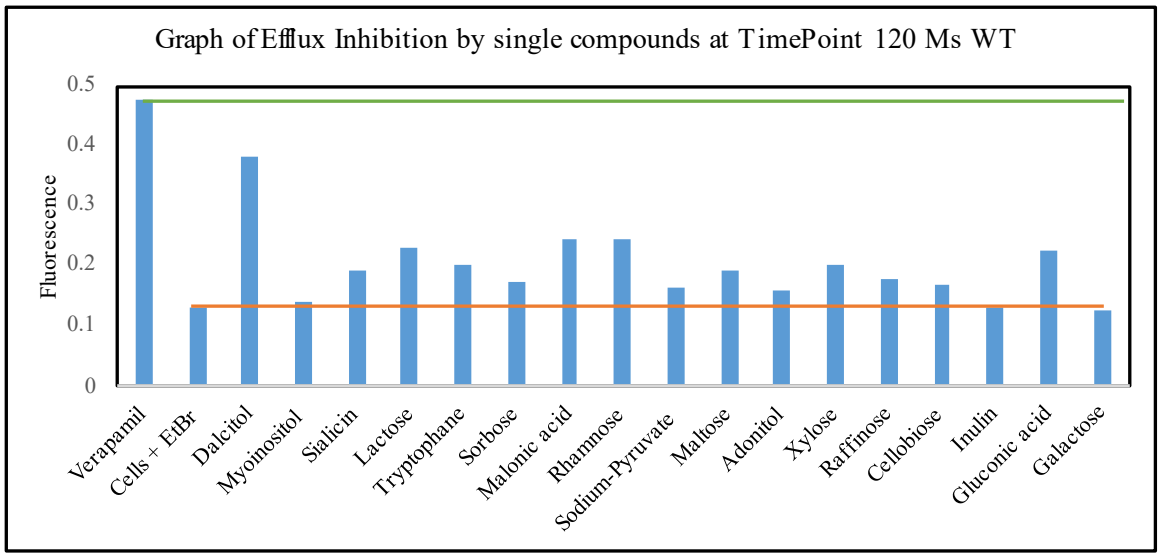

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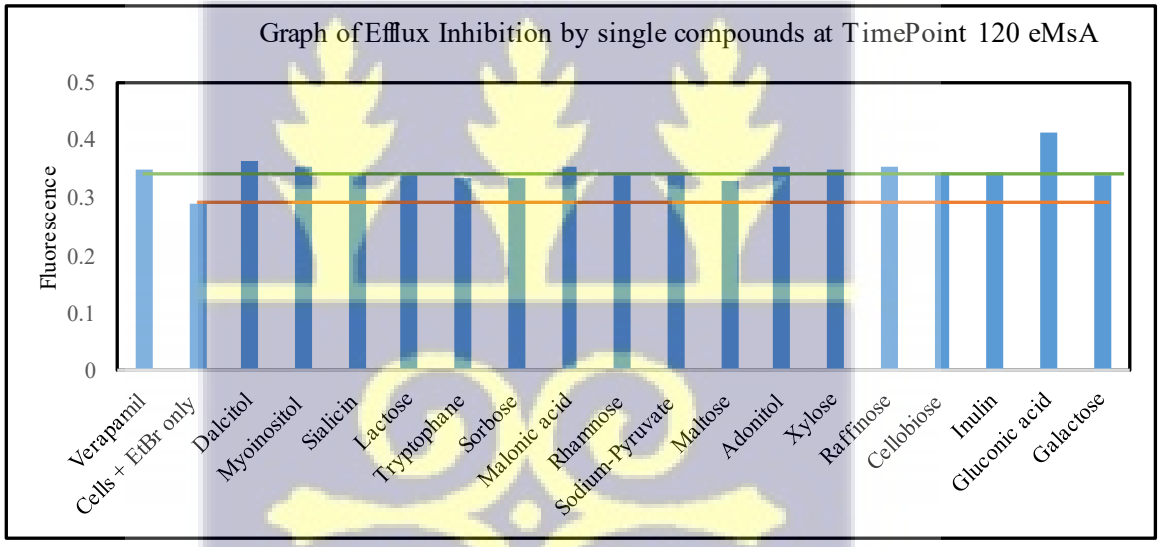

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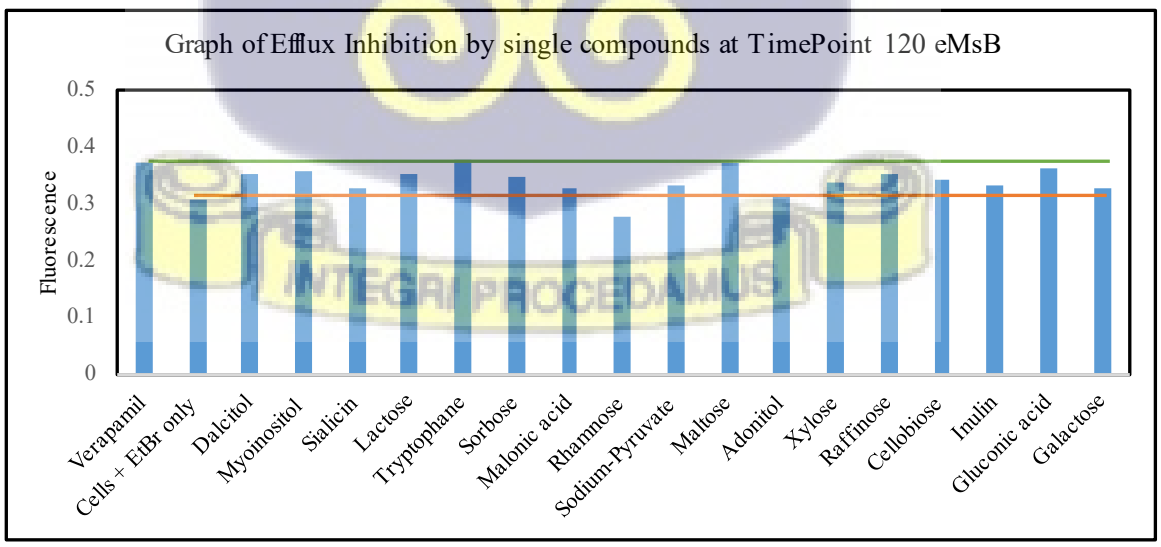

Figure 4.17: Fluorescence values for ethidium bromide efflux in (A)Ms wt. (B) eMsA and (C) eMsB. With single compounds. *The cell pellets were resuspended in 0.8% PBS containing 0.4% glucose and the OD adjusted to 0.4. Then 3 ul of 3 ug/ul EtBr was added to all the cell suspensions. Into 2 ml Eppendorf tubes, 990 ul of cells suspension and 10 ul of 20 mg/ul PC were added. Verapamil served as the positive control and the PBS as the negative control. All the reactions for the PC plus the controls were set up in triplicates. The reaction was incubated for one hour in a shaking incubator, 100 ul of the content of each reaction tube was pipetted into a 96-well plate. Fluorescence of EtBr at excitation and emission wavelengths at 530 at 585 (nm) respectively were read within time intervals of 30, 60, and 120 min using a Varioskan microplate reader. A plot of fluorescence values under controlled conditions and in the presence of individual modifier compounds for Mycobacterium smegmatis wild-type (Ms wt).*

4.2.4 EFFECTS OF TRIPLE COMPOUND COMBINATION ON CELLULAR TRANSPORT; EFFLUX IN MYCOBACTERIA

In the presence of the triple combination compounds against Ms wt, most of the activities of the compounds were similar to that cell-only control. Not much difference was observed. On the other hand, in the efflux assay using the triple combination compounds against eMsA, almost all the compounds in the resistant-breaking group had activities higher than the cell-only control with Mal(3X) being the only compound to be higher than verapamil. Some of the resistant-inducing compounds had activities higher than cell-only control. Ado+Xyl+Raf had the highest activity in the group even though it was not higher or equal to verapamil. The resistant inducing activity was much lower compared to the resistant-breaking. For eMsB, not much difference was observed in the result after the compound treatment. Most of the resistant breaking and inducing compounds had lower activities except Sor(3X) (Fig 4.18).

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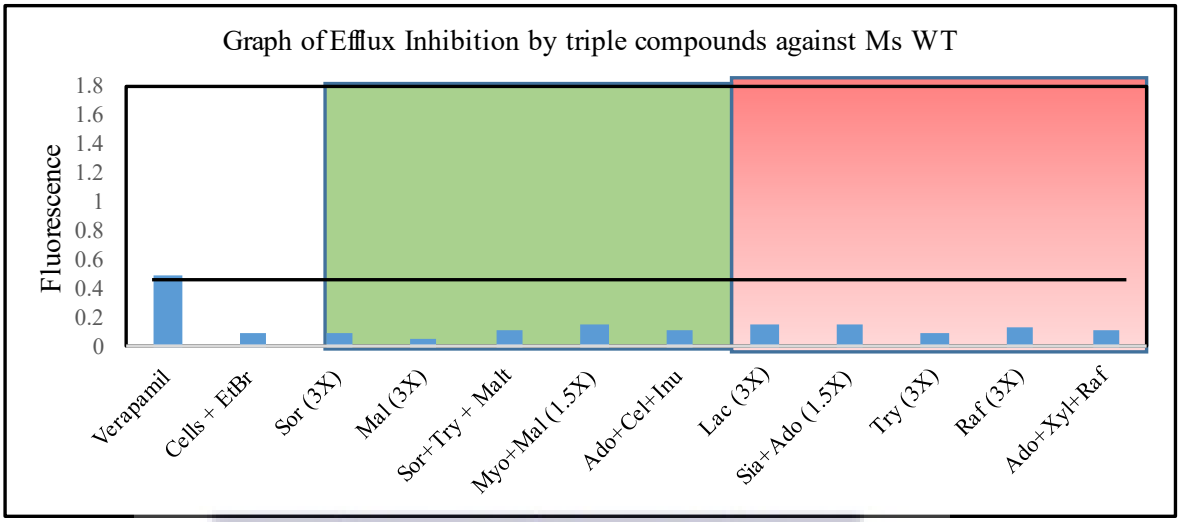

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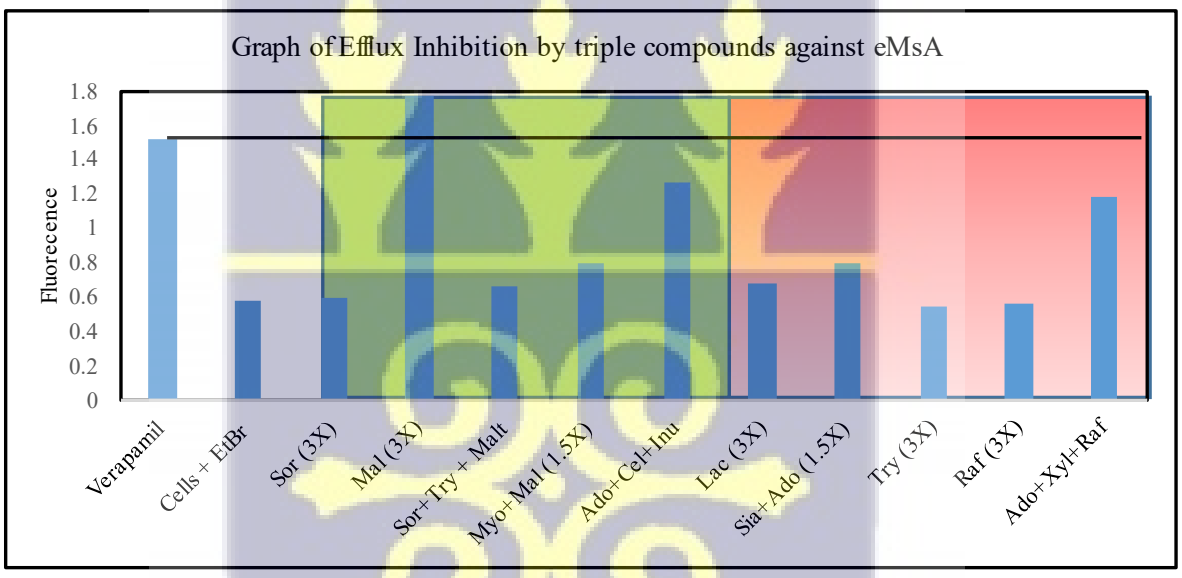

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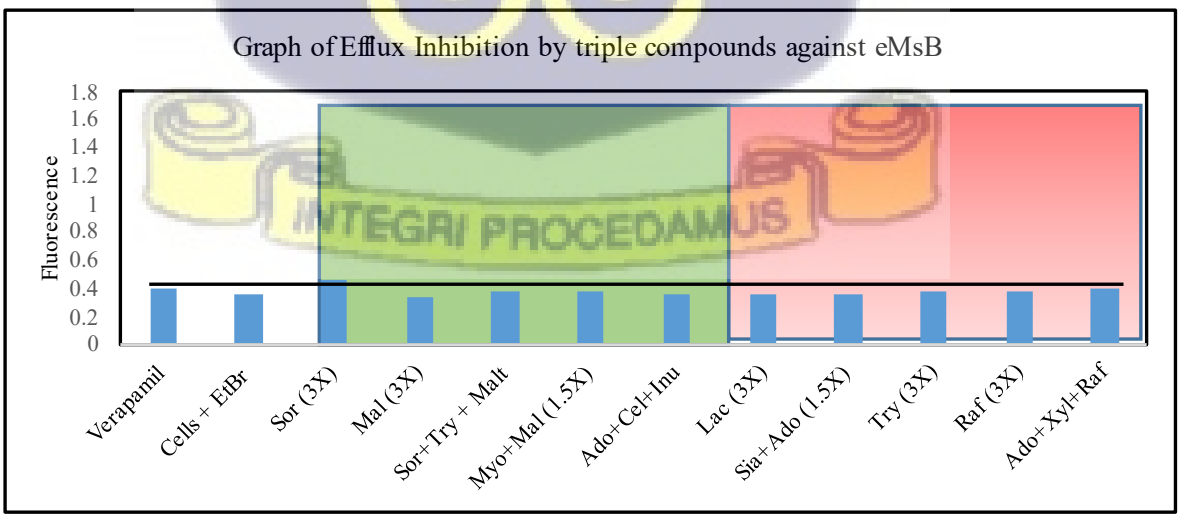

Figure 4.18: Fluorescence values for ethidium bromide Efflux in (A)Ms wt. (B) eMsA and (C) eMsB. With triple compound combinations. *The plot of fluorescence values under controlled conditions and in the presence of triple modifier compound combinations for Mycobacterium smegmatis wild-type (Ms wt). The cells were pelleted and resuspended in 0.8% PBS. The OD was adjusted to 0.4 and 3 ul of 3 ug/ul EtBr was added to all the cell suspensions. Into 2 ml Eppendorf tubes, 970 ul of cells suspension and 10 ul of 10 mg/ul of each PC in the combination were added. For a 1.5x dose, 15 ul was picked whereas a 3x was 30ul. Verapamil served as the positive control and the PBS as the negative control. All the reactions for the PC plus the controls were set up in triplicates. The85eactionn was incubated for one hour in a shaking incubator, 100 ul of the content of each reaction tube was pipetted into a 96-well plate. Fluorescence of EtBr at excitation and emission wavelengths at 530 at 585 (nm) respectively were read within time intervals of 30, 60, and 120 min using a Varioskan microplate reader.*

4.2.5 EFFECTS OF SINGLE COMPOUND ON CELLULAR TRANSPORT; ADHESION INHIBITION IN MYCOBACTERIA

Another distinctive feature of antimicrobial resistance is the adherence of bacteria to each other to form biofilm. Biofilm formation by Mycobacterium has been reported in much literature and this feature has contributed to the resistance acquired by this bacterium to antibiotics (Bhunu et al., 2017). This biofilm layer forms a coat that is impermeable to antibiotics (Ojha & Hatfull, 2007). We sought to investigate the effect of these compounds and their combinations on inhibition and disruption of adhesion and biofilm formation.

Most of the compounds had activities against eMsA higher than the control. Rhamnose, cellobiose, galactose, gluconic acid were the compounds to have the highest effect. Dalcitol is the only compound to be lower than the control (Fig 4.19B). Most of the compounds had slightly lower activity when eMsA cells were treated compared to the control. Rhamnose and gluconic acid were among the compounds with the highest activity consistent with their activity against eMsA (Fig 4.19C). The single compounds against Ms wt for the adhesion inhibition assays, not much activity was observed in the wt for both the cell-only control. This result is consistent with that of adhesion disruption where not much difference was observed for the single compounds compared to the control (Fig 4.19A).

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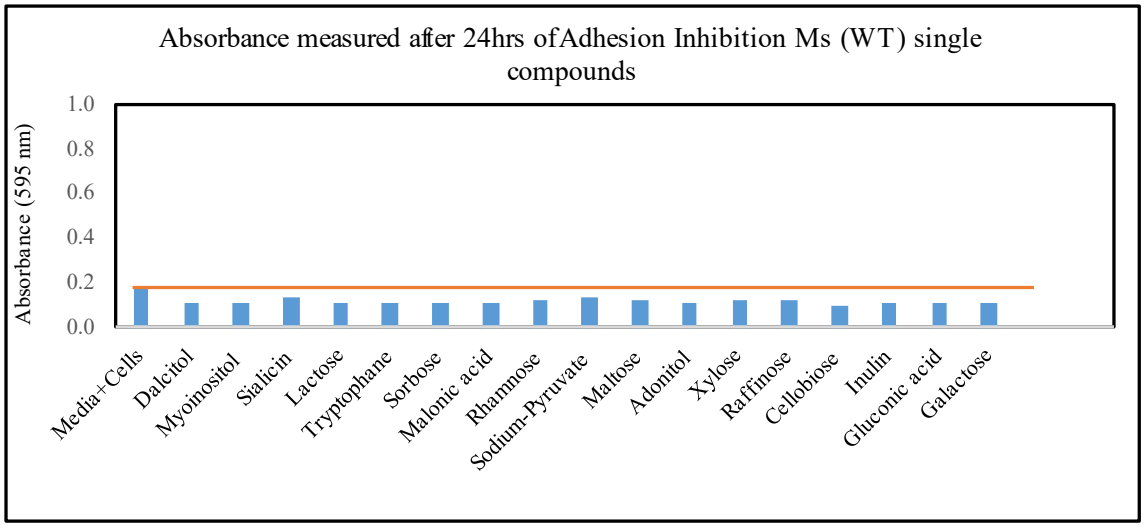

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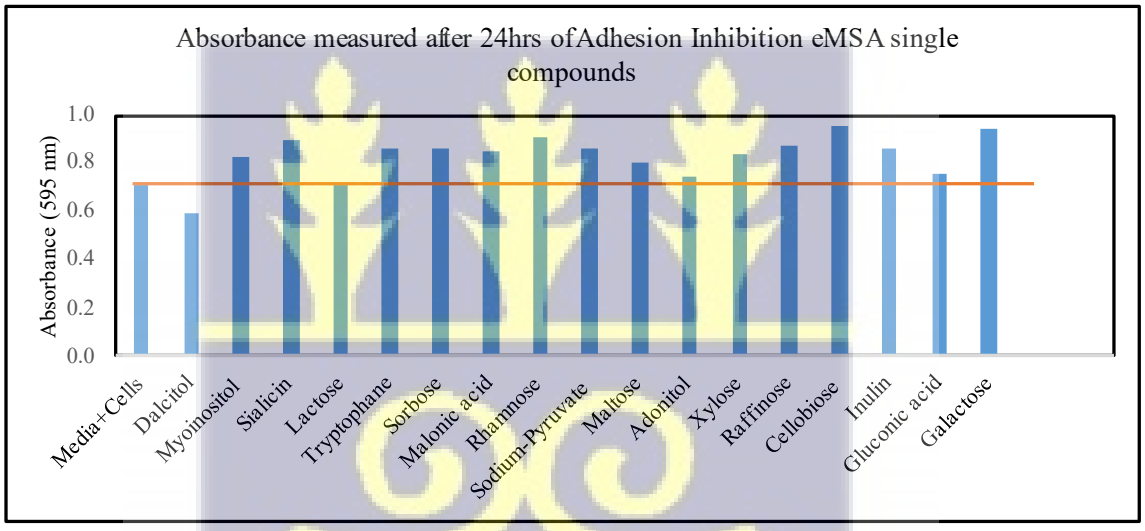

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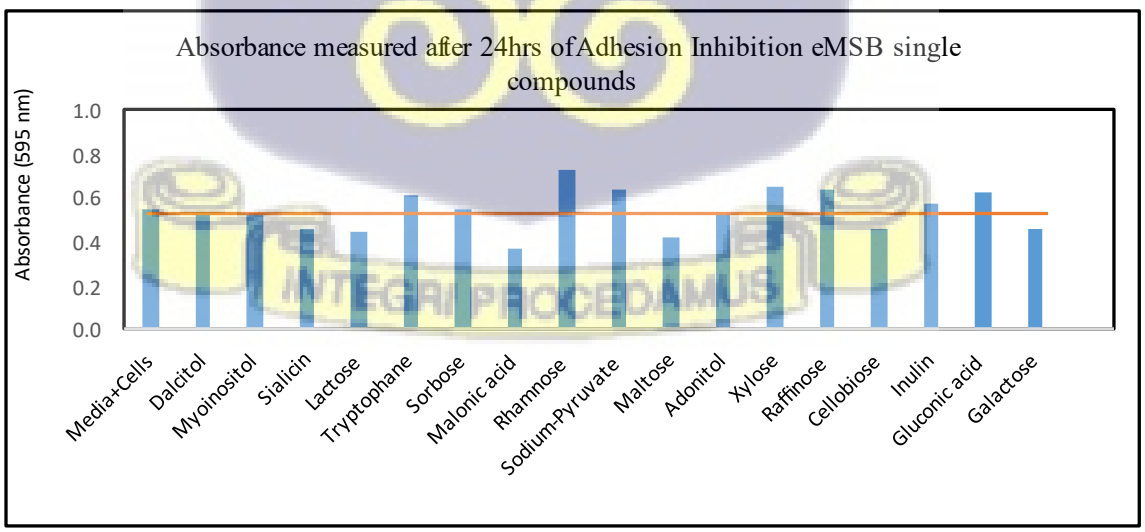

Figure 4.19: Absorbance values for adhesion inhibition in (A)Ms wt. (B) eMsA and (C) eMsB with single compounds. *A volume of 990 ul of the overnight cultures (OD = 0.5) was aliquoted into a 2 ul Eppendorf tube in both setups. To each reaction tube, 10 ul of PC (10 ug/ ml) was added. A volume of 200 ul from each reaction tube was aliquoted into 96 well plates and incubated at 60 rpm for 24 hr. After the stated period of incubation for the respective assay, the cultures in the 96 well plate were washed off with 0.8% PBS and allowed to air dry. A volume of 20 ul of 1% crystal violet (CV) was added to each well and allowed to stand for 15 min. The plates were washed off with distilled water to remove any unbound CV to cells. A volume of 20 ul of 95% ethanol was added to each well to solubilize the CV. The absorbance was read at 595 nm with the Varioskan plate reader.*

4.2.6 EFFECTS OF TRIPLE COMPOUND COMBINATION ON CELLULAR TRANSPORT; ADHESION INHIBITION IN MYCOBACTERIA

In the adhesion inhibition assays using triple combination compounds against Ms wt, all the resistant breaking compounds except Myo+Mal (1.5X) had lower activity compared to the control. Also, all the resistant inducing compounds had lower activity compared to the control (Fig 4.20A).

Against eMsA, all the resistant breaking compounds had higher activities compared to the control which is contrary to their resistant breaking property observed. Sor(3X) had significantly high activity. Lac(3X) and Sia+Ado(1.5X) had higher activity compared to the control strengthening their resistant inducing property. Aside from these two treatments, the other RI compounds had lower readings signifying adhesion inhibition property (Fig 4.20B).

For the eMsB cells treated with the triple combinations, both resistant breaking and resistant inducing compounds had lower absorbance readings compared to the control (Fig 4.20C). This observation implies both groups of compounds reduce the ability of the cells to adhere to each other.

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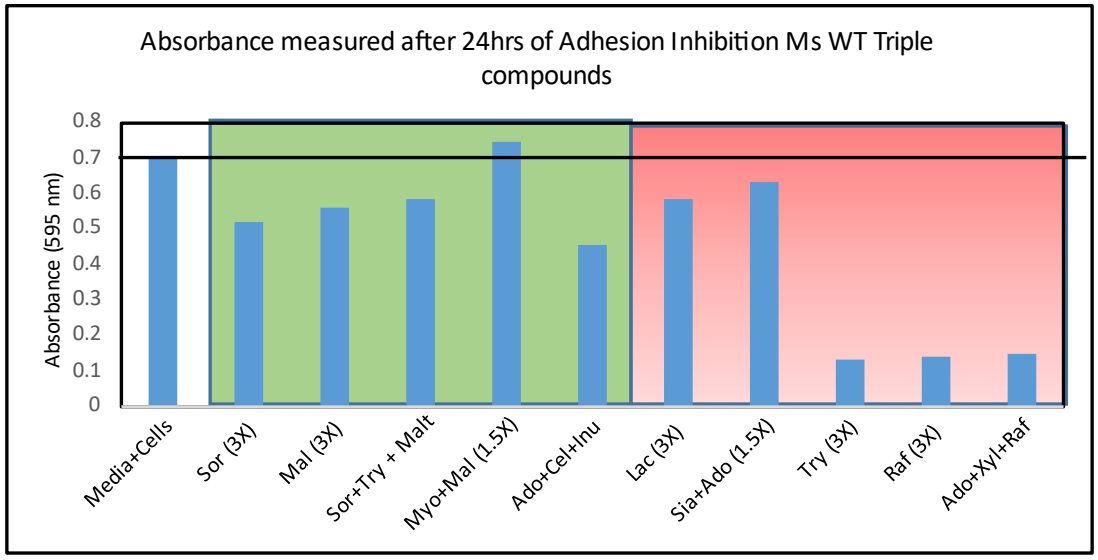

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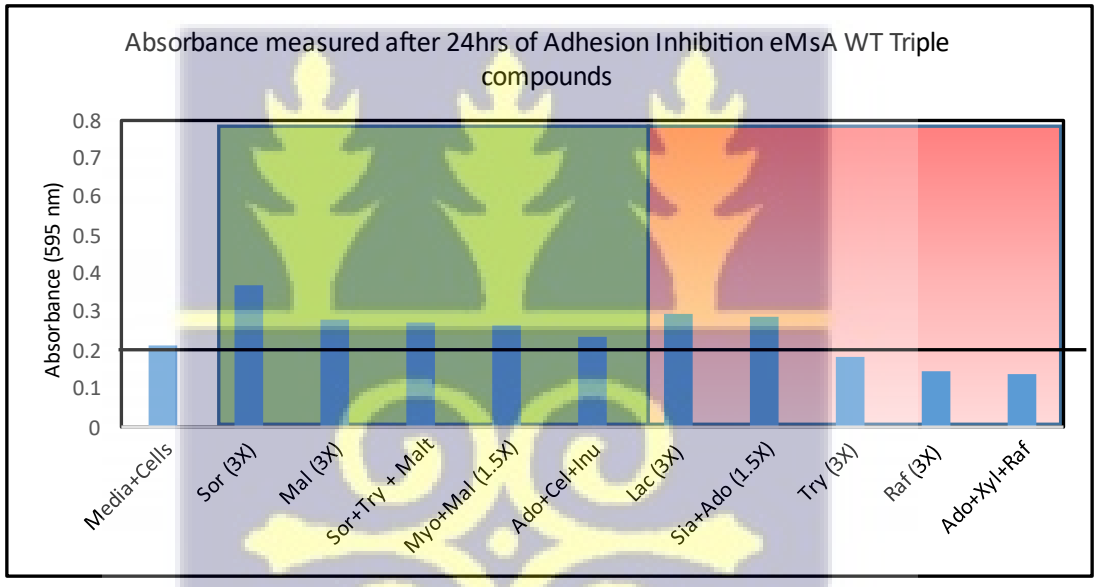

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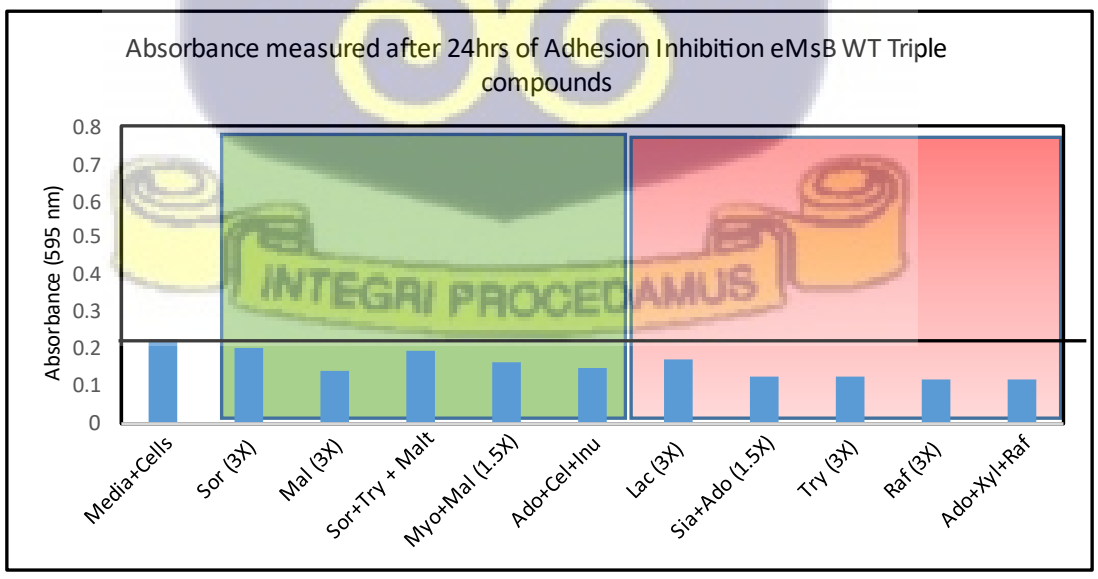

Figure 4.20: Absorbance values for adhesion inhibition in (A)Ms wt. (B) eMsA and (C) eMsB. With triple compound combinations. *10 ul of 10 mg/ul of each PC in the combination were added. A volume of 990 ul of the overnight cultures (OD = 0.5) was aliquoted into a 2 ul Eppendorf tube in both setups. To each reaction tube, 10 ul of each PC in a 3X FTC, for a 1.5x dose, 15 ul was picked whereas a 3x was 30ul. Was added. For the PC combinations, the volume of cells used was adjusted to 970 ul to make up for the additional volume of PCs used. A volume of 200 ul from each reaction tube was aliquoted into 96 well plates and incubated at 60 rpm for 24 h for adhesion. After the stated period of incubation for the respective assay, the cultures in the 96 well plate were washed off with 0.8% PBS and allowed to air dry. A volume of 20 ul of 1% crystal violet (CV) was added to each well and allowed to stand for 15 min. The plates were washed off with distilled water to remove any unbound CV to cells. A volume of 20 ul of 95% ethanol was added to each well to solubilize the CV. The absorbance was read at 595 nm with the Varioskan plate reader.*

4.2.7 EFFECTS OF SINGLE COMPOUND ON CELLULAR SURVIVAL; ADHESION DISRUPTION IN MYCOBACTERIA

In the adhesion disruption by single compounds against Ms wt, most of the compounds had activities lower than the cell-only control, suggesting these compounds are disrupting cell adhesion. Gluconic acid surprisingly was higher than the cell-only control signifying it was inducing resistance in the Ms wt cells. Lower activity was observed across the control and treatments compared to the mutant strains (Fig 4.21A). Using single compounds against eMsA, most of the compounds had activities slightly higher than the cell-only control, suggesting the compounds were inducing resistance. This is not surprising since these strains are highly resistant. Malonic acid had the highest activity compared to the control. Gluconic acid also had activity higher than the control like its activity in the wt (Fig 4.21B). Against eMsB, most of the compounds had slightly lower activity compared to the control. A couple of the compounds still had activities higher than the cell-only control (6/17). Rhamnose had the highest activity among the compounds (Fig 4.21C).

It can be observed that most of the compounds did not play a significant role in disrupting the adhesion of the cells since most of the activities seen are slightly different from the control.

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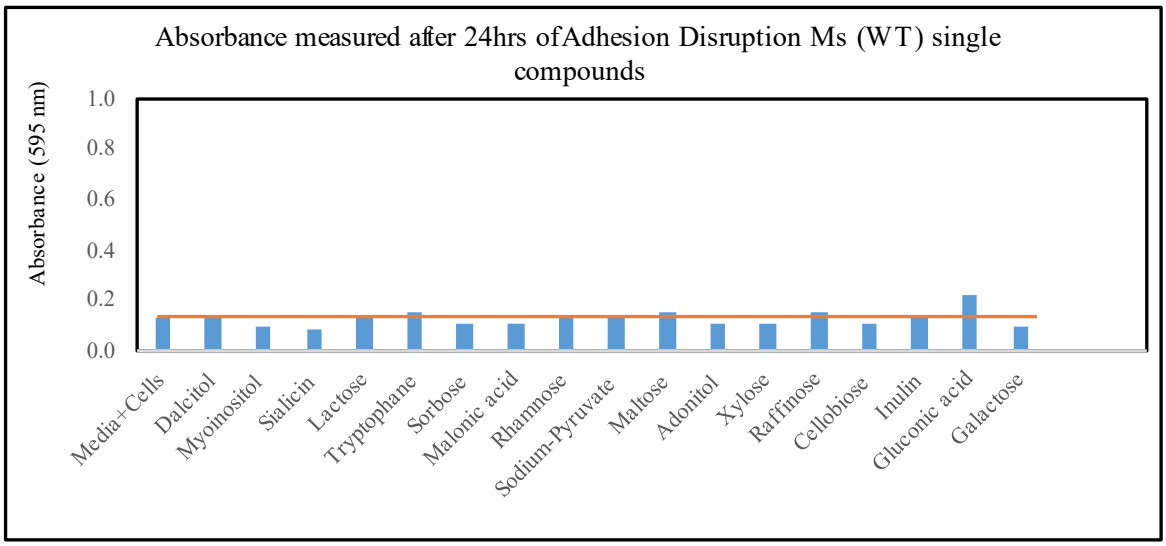

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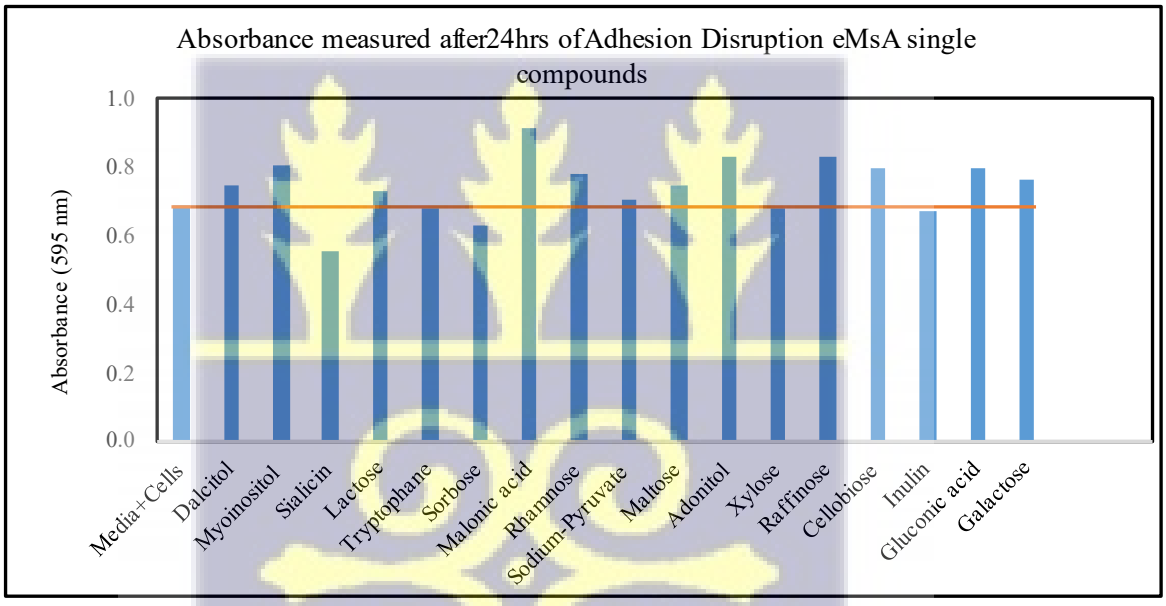

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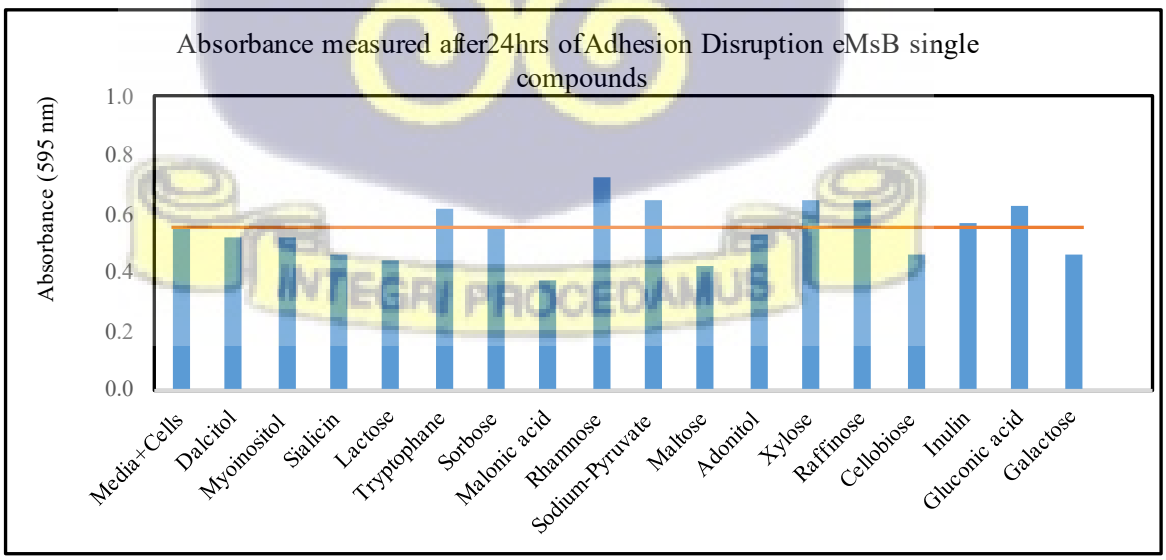

Figure 4.21: Absorbance values for adhesion disruption in (A)Ms wt. (B) eMsA and (C) eMsB. With single compounds*. The plot of absorbance values under controlled conditions and in the presence of individual modifier compounds for Mycobacterium smegmatis. A volume of Mycobacterial culture of OD = 0.5 was aliquoted into 96 well microtiter plates. The plates were incubated with shaking at 60 rpm for 24 hr. After the incubation period, the plates were washed twice with distilled water and replaced with 200 ul of 7H9 containing PC at an amount of 0.1 mg. The two setups were incubated for 2 h with shaking at 60 rpm. The microtiter plates were washed twice with distilled water and 20 ul of 95% ethanol was added. The absorbance was read at 595 nm*

4.2.8 EFFECTS OF TRIPLE COMPOUND COMBINATIONS ON CELLULAR SURVIVAL; ADHESION DISRUPTION IN MYCOBACTERIA

For the adhesion disruption using triple combination compounds against Ms wt, most of the resistant breaking compounds had activities equal to the cell-only control except Mal(3X) which was extremely lower. Some resistant-inducing had higher activity than the control, that is Lac(3X) and Sia+Ado (1.5X) but the rest all had lower activities (Fig 4.22A). Against eMsA, most of the resistant breaking compounds were lower than the cell-only control except Myo+Mal (1.5X), which was higher. This is consistent with the resistant breaking effect observed in this group of compounds. All the resistant inducing groups of compounds had lower activity than the control except Sia+Ado (1.5X) even though they were inferred to induce resistance are resistant inducing compounds from our previous phenotypic interaction assay (Fig 4.22B). For eMsB treated cells, all the resistant breaking compounds had lower activity except Sor (3X) which was higher and all the resistant inducing compounds had lower activities than the control except Raf (3X), which was slightly higher (Fig 4.22C).

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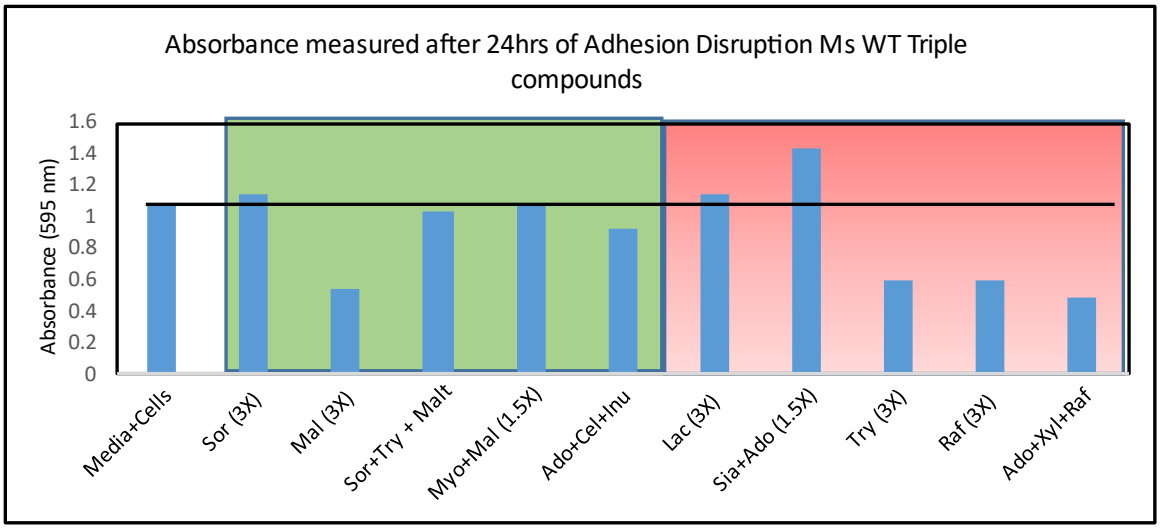

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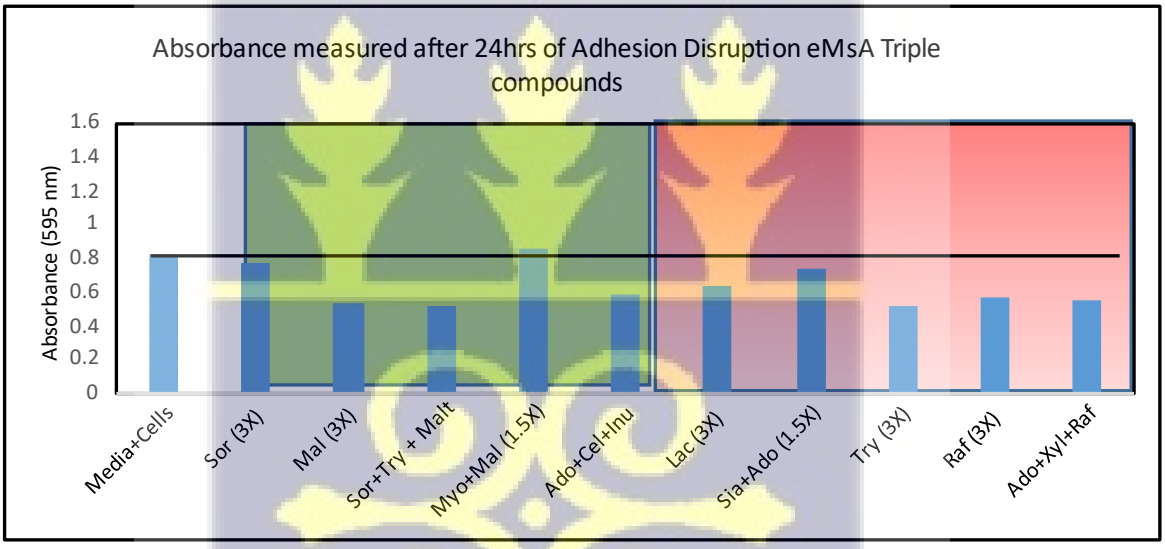

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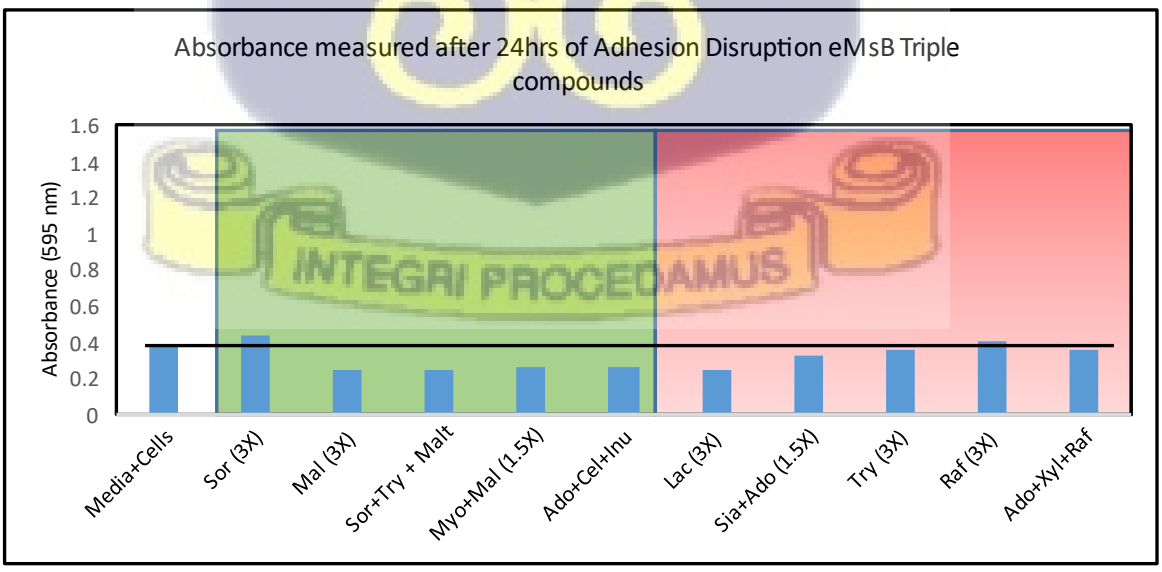

Figure 4.22: Absorbance values for adhesion disruption in (A)Ms wt. (B) eMsA and (C) eMsB. With triple compound combinations. *The plot of absorbance values under controlled conditions and in the presence of triple compound for Mycobacterium smegmatis. A volume of 200 ul of Mycobacterial culture of OD = 0.5 was aliquoted into 96 well microtiter plates. The plates were incubated with shaking at 60 rpm for 24 hr. After the incubation period, the plates were washed twice with distilled water and replaced with 200 ul of 7H9 containing PC at an amount of 0.3 mg. The two setups were incubated for 2 h with shaking at 60 rpm. The microtiter plates were washed twice with distilled water and 20 ul of 95% ethanol was added. The absorbance was read at 595 nm*

4.2.9 EFFECTS OF SINGLE COMPOUND ON CELLULAR SURVIVAL; BIOFILM INHIBITION IN MYCOBACTERIA

When the single compounds were tested to inhibit biofilm formation in Ms wt, the results showed that most of the compounds had higher activity compared to the control. Sodium pyruvate and adonitol had the highest absorbance readings (Fig 4.23A). The eMsA treatments, showed most of the compounds did not show much difference when compared to the control. Malonic acid, raffinose, and gluconic acid were slightly higher than the control (Fig 4.23B). Against eMsB, apart from tryptophan, most of the compounds had lower activity compared to the control. Even though they were lower, most of them were closer to the control (Fig 4.23C).

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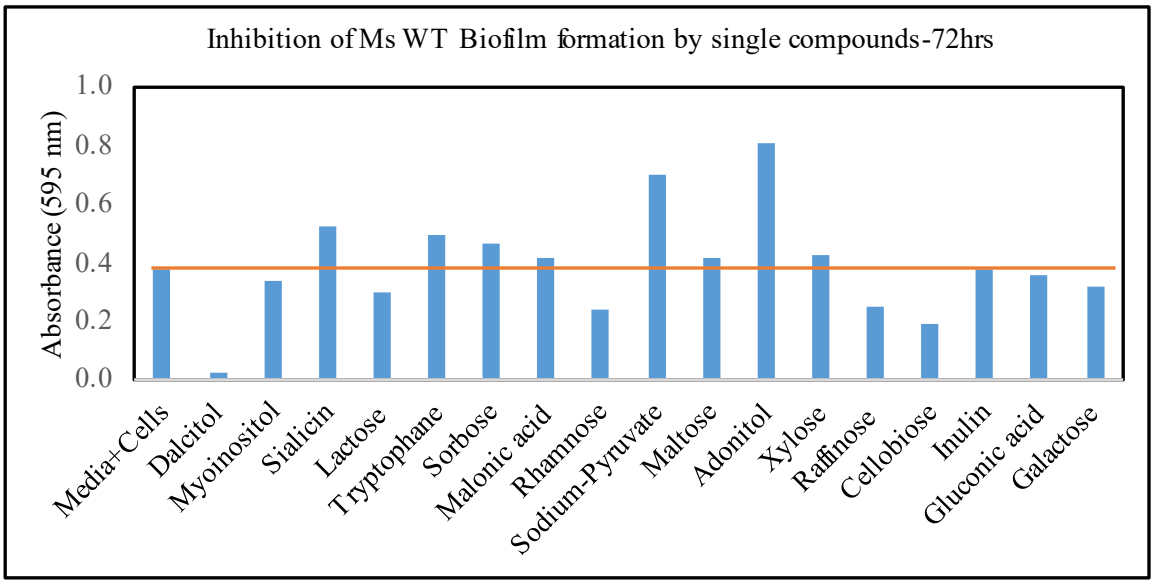

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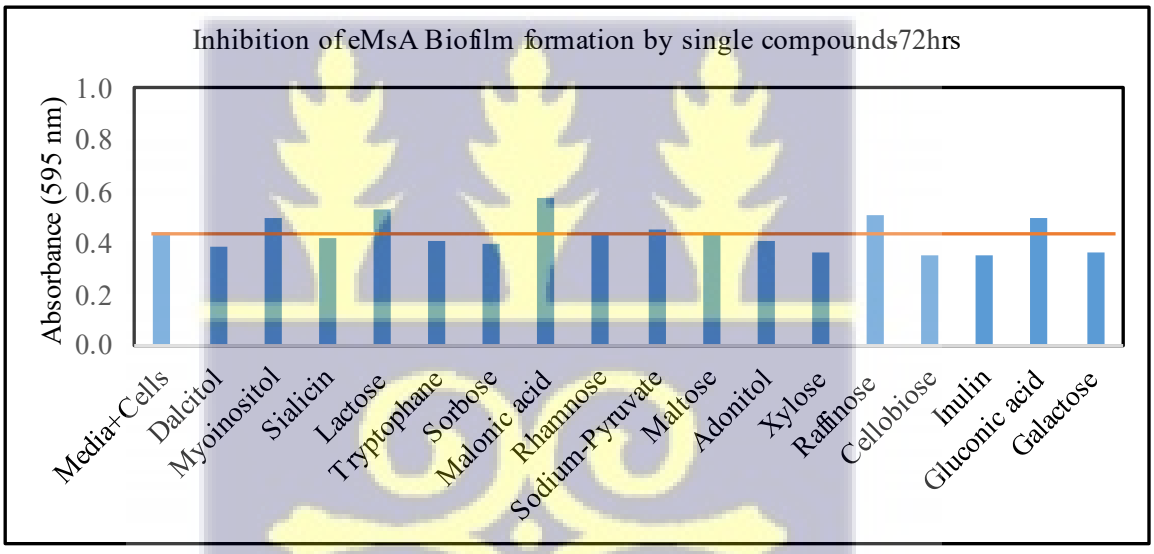

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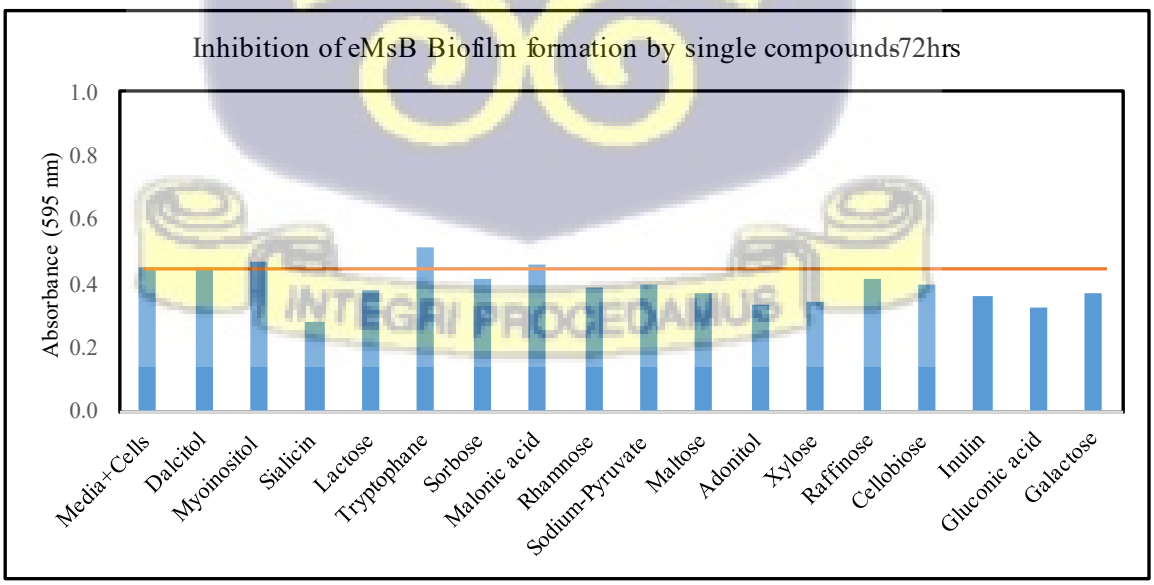

Figure 4.23: Absorbance values for biofilm inhibition in (A)Ms wt. (B) eMsA and (C) eMsB with single compounds*. A volume of 990 ul of the overnight cultures (OD = 0.5) was aliquoted into a 2 ul Eppendorf tube in both setups. To each reaction tube, 10 ul of PC (10 ug/ ml) was added. A volume of 200 ul from each reaction tube was aliquoted into 96 well plates and incubated at 60 rpm for 72 hr. After the stated period of incubation for the respective assay, the cultures in the 96 well plate were washed off with 0.8% PBS and allowed to air dry. A volume of 20 ul of 1% crystal violet (CV) was added to each well and allowed to stand for 15 min. The plates were washed off with distilled water to remove any unbound CV to cells. A volume of 20 ul of 95% ethanol was added to each well to solubilize the CV. The absorbance was read at 595 nm with the Varioskan plate reader.*

4.2.10 EFFECTS OF TRIPLE COMPOUND COMBINATION ON CELLULAR SURVIVAL; BIOFILM INHIBITION IN MYCOBACTERIA

The results from the biofilm inhibition by triple combination compounds against Ms wt, all the compounds, both resistant breaking, and resistant breaking had lower activity compared to the control, except Myo+Mal & Sia+Ado (1.5X) were higher than the control unlike the biofilm disruption by the single compounds (Fig 4.24A). Against eMsA, all the RB and RI compounds had lower activities compared to the control. RB group had significantly lower activities compared to the RI combination compounds (Fig 4.24B). For the eMsB treatments, both RB and RI compounds had lower absorbance readings compared to the control (Fig 4.24C). The results of the resistant-breaking compounds correlated with that of the interaction assay.

Most of the compounds both single and functional triple combinations reduced the formation of adhesion and biofilm and also disrupted the already formed biofilm. Even though the resistant-inducing compounds did not correlate with the interaction assay, these compounds may use other mechanisms in inducing resistance.

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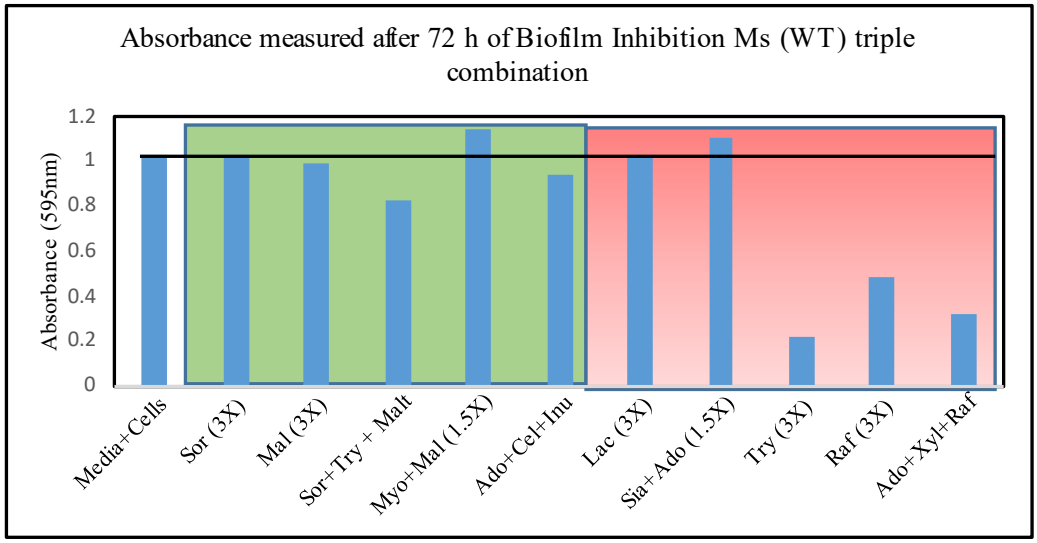

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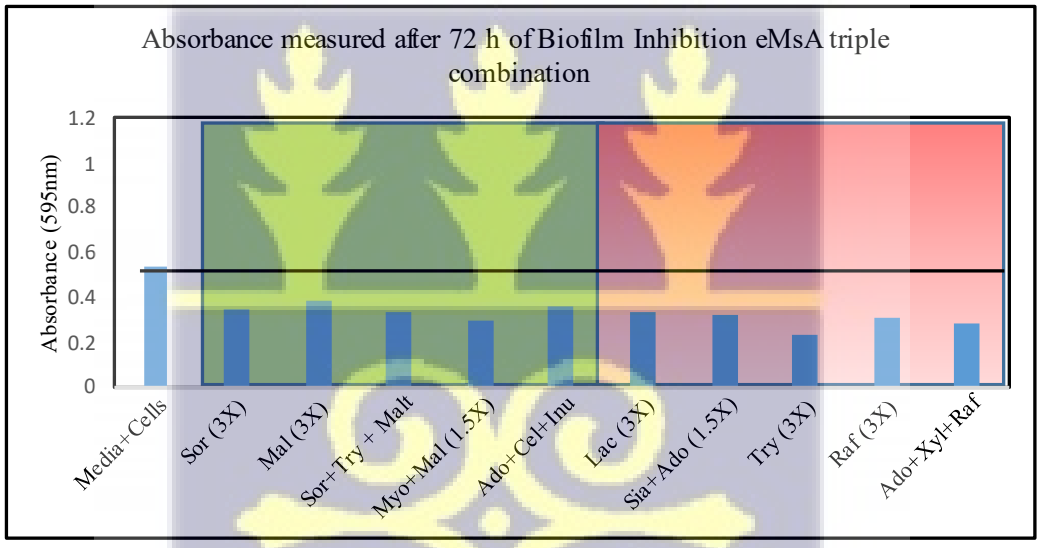

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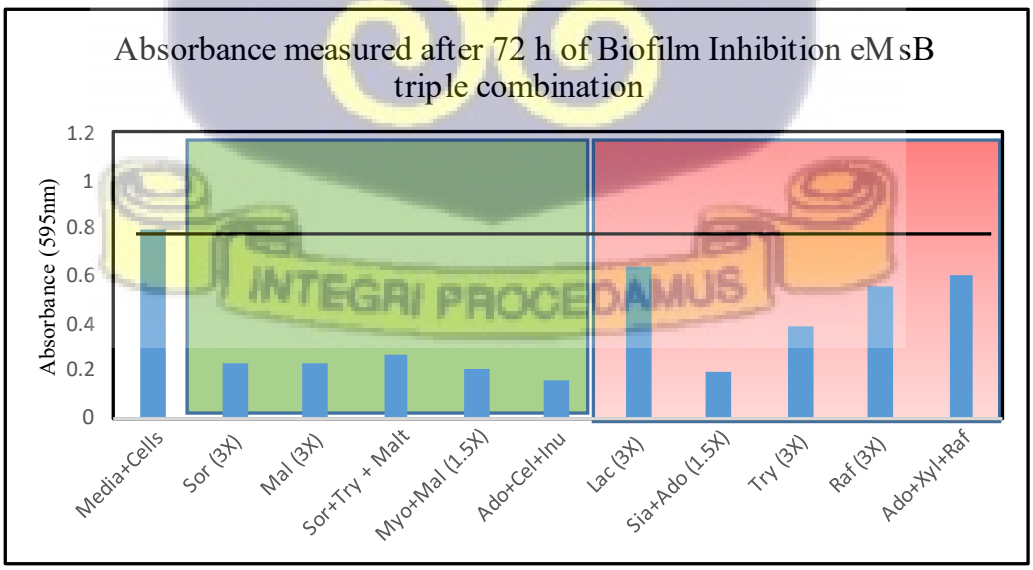
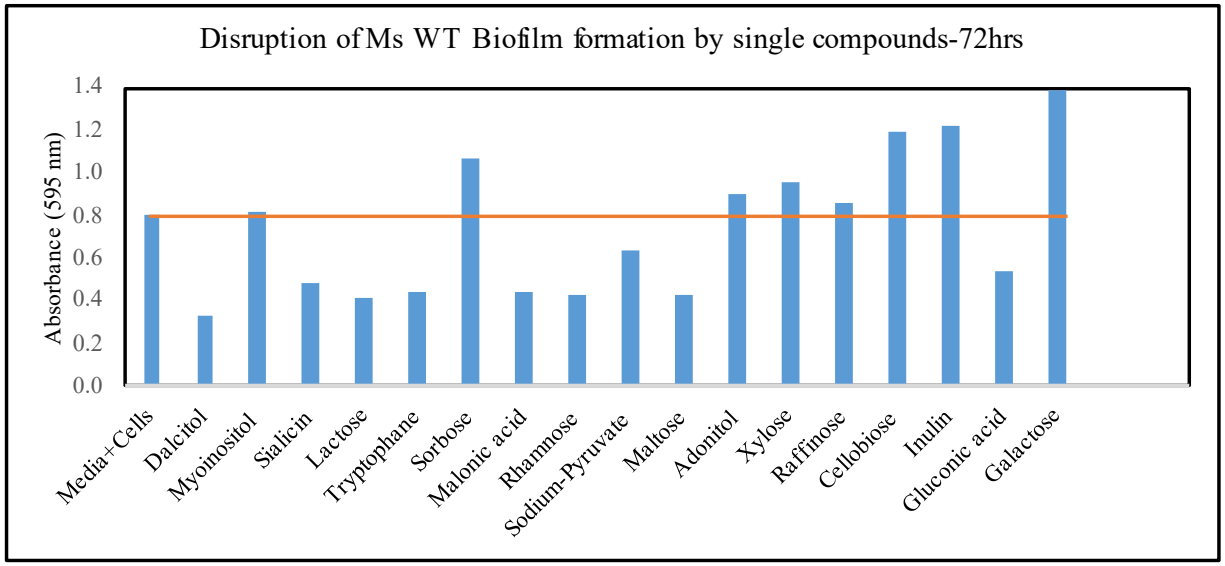


Figure 4.24: Absorbance values for biofilm inhibition in (A)Ms wt. (B) eMsA and (C) eMsB. With triple compound combinations. *10 ul of 10 mg/ul of each PC in the combination were added. A volume of 990 ul of the overnight cultures (OD = 0.5) was aliquoted into a 2 ul Eppendorf tube in both setups. To each reaction tube, 10 ul of each PC in a 3X FTC, for a 1.5x dose, 15 ul was picked whereas a 3x was 30ul. Was added. For the PC combinations, the volume of cells used was adjusted to 970 ul to make up for the additional volume of PCs used. A volume of 200 ul from each reaction tube was aliquoted into 96 well plates and incubated at 60 rpm for 72 h for adhesion. After the stated period of incubation for the respective assay, the cultures in the 96 well plate were washed off with 0.8% PBS and allowed to air dry. A volume of 20 ul of 1% crystal violet (CV) was added to each well and allowed to stand for 15 min. The plates were washed off with distilled water to remove any unbound CV to cells. A volume of 20 ul of 95% ethanol was added to each well to solubilize the CV. The absorbance was read at 595 nm with the Varioskan plate reader*

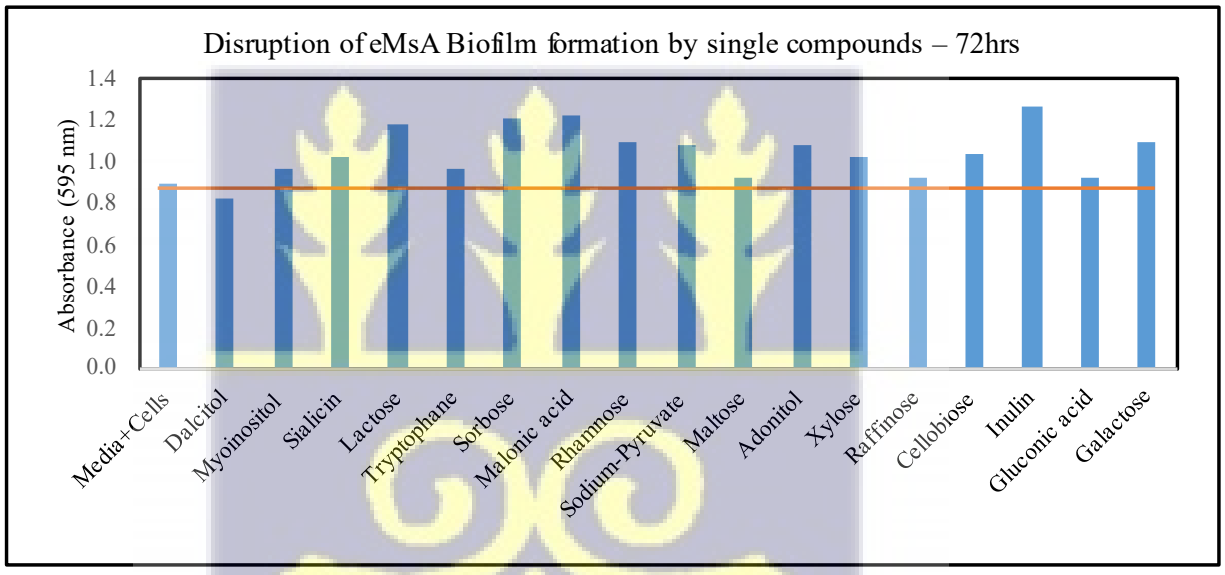
4.2.11 EFFECTS OF SINGLE COMPOUND ON CELLULAR SURVIVAL; BIOFILM DISRUPTION IN MYCOBACTERIA

Investigating biofilm disruption by single compounds against Ms wt, it was observed that most of the compounds had activities lower than the cell-only control, suggesting these compounds are disrupting biofilm. Gluconic acid was lower than the cell-only control signifying it was disrupting cell adhesion contrary to its effect in cell adhesion (Fig 4.25A). In the eMsA treatment assays, all the compounds except dalcitol had activities higher than the cell-only control, suggesting the compounds could be inducing biofilm formation. This is not surprising since these strains are highly resistant. This observation is consistent with what was obtained in the adhesion assay (Fig 4.25B). Most of the compounds had slightly lower activity against eMsB compared to the control. Tryptophane, sodium pyruvate, and xylose were slightly above the cell-only control (Fig 4.25C).

A



B



C

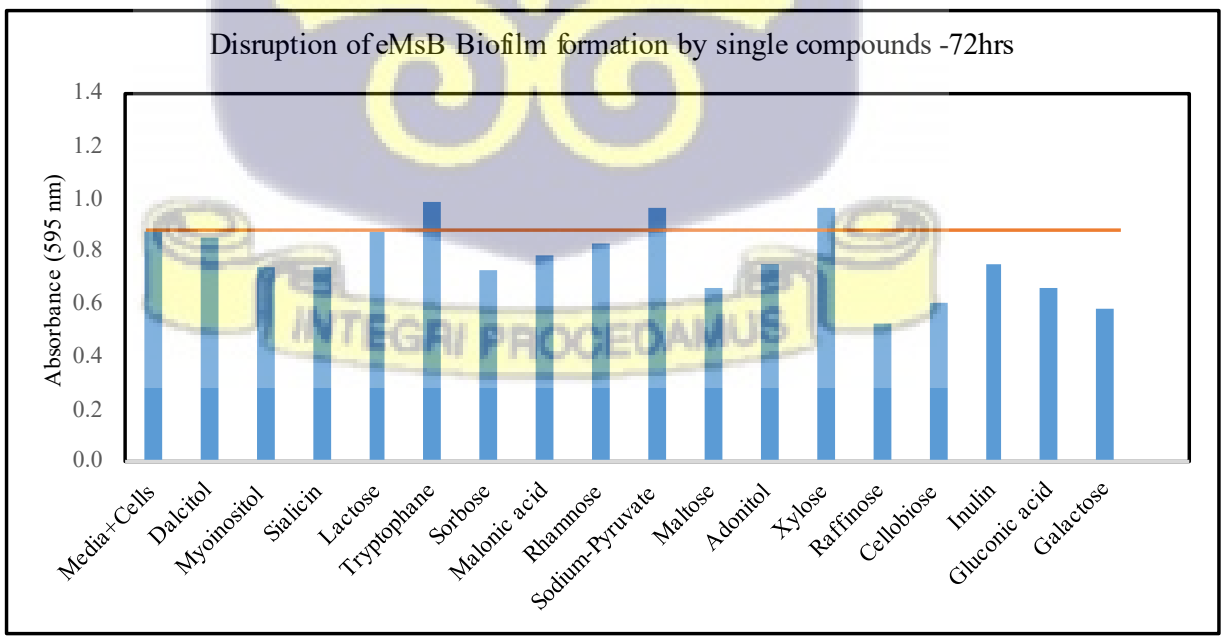


Figure 4.25:Absorbance values for biofilm disruption in (A)Ms wt. (B) eMsA and (C) eMsB. With single compounds. *The plot of absorbance values under controlled conditions and in the presence of individual modifier compounds for Mycobacterium smegmatis. A volume of Mycobacterial culture of OD = 0.5 was aliquoted into 96 well microtiter plates. The plates were incubated with shaking at 60 rpm for 72 hr. After the incubation period, the plates were washed twice with distilled water and replaced with 200 ul of 7H9 containing PC at an amount of 0.1 mg. The two setups were incubated for 2 h with shaking at 60 rpm. The microtiter plates were washed twice with distilled water and 20 ul of 95% ethanol was added. The absorbance was read at 595 nm*

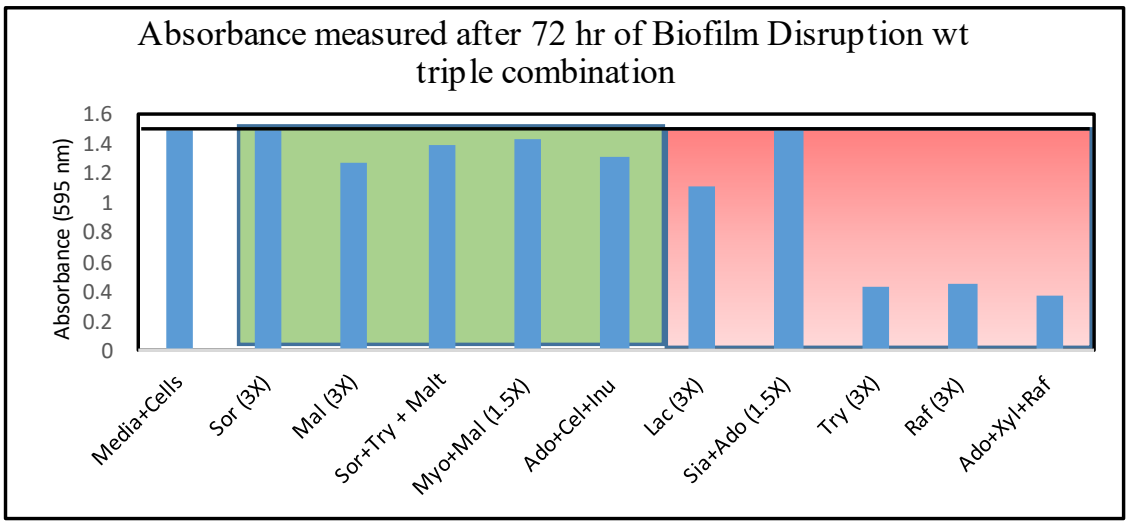
4.2.12 EFFECTS OF TRIPLE COMPOUND COMBINATION ON CELLULAR SURVIVAL; BIOFILM DISRUPTION IN MYCOBACTERIA

In treating the cells with the triple combination compounds against Ms wt, most of the compounds had activities lower than the cell-only control except Try(3X), Raf(3X), and Ado+Xyl+Raf which was extremely lower similar to the adhesion assay (Fig 4.26A). Against the eMsA, all the compounds had lower activity than the control except Sor(3X) and Sia+Ado (1.5X) which were slightly higher. Try(3X), Raf(3X), and Ado+Xyl+Raf, consistent with the wt data, had the lowest absorbances (Fig 4.26B). For eMsB, most of the compounds had a lower or equal activity compared to the control. Consistent with wt and eMsA, Try(3X), Raf(3X), and Ado+Xyl+Raf had lower activities (Fig 4.26C).

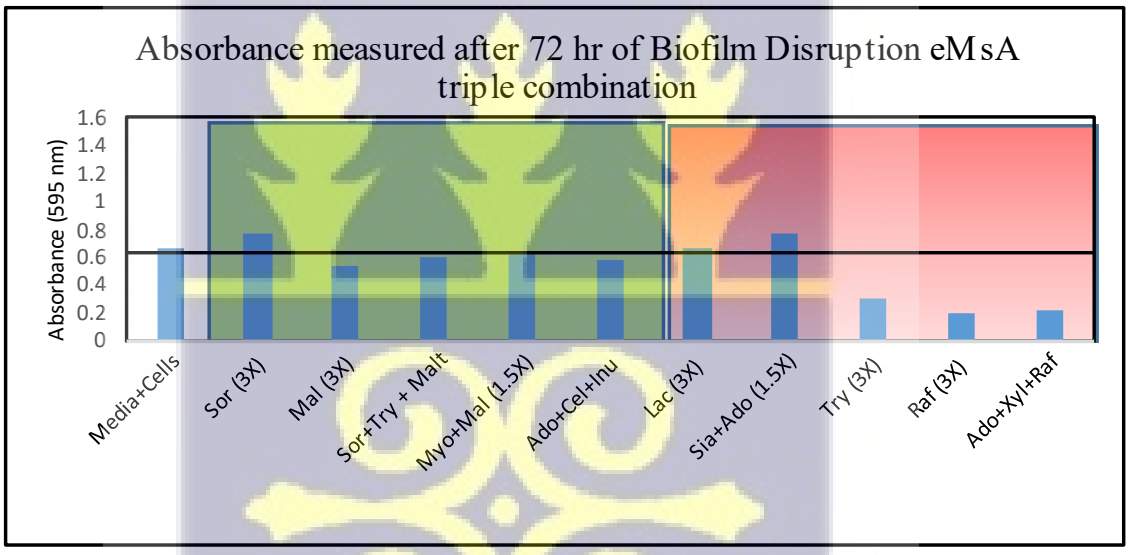
This suggests that these three combination compounds, Try(3X), Raf(3X), and Ado+Xyl+Raf disrupt biofilm formation in all three strains.

RESISTANT -BREAKING | RESISTANT -INDUCING

A



B



C

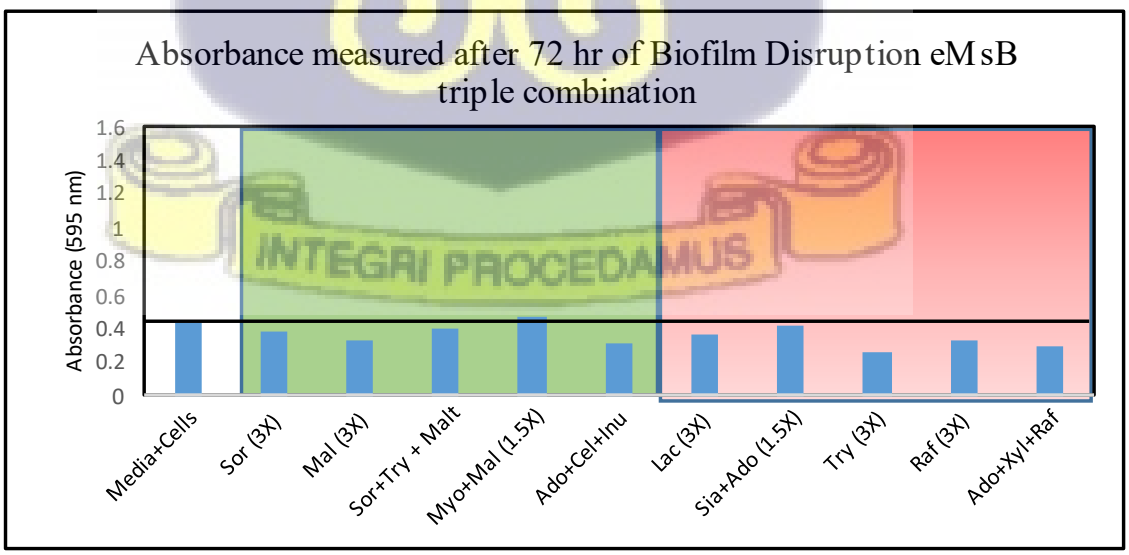


Figure 4.26: Absorbance values for biofilm disruption in (A)Ms wt. (B) eMsA and (C) eMsB. With triple compound combinations. *The plot of absorbance values under controlled conditions and in the presence of triple compound for Mycobacterium smegmatis. A volume of 200 ul of Mycobacterial culture of OD = 0.5 was aliquoted into 96 well microtiter plates. The plates were incubated with shaking at 60 rpm for 72 hr. After the incubation period, the plates were washed twice with distilled water and replaced with 200 ul of 7H9 containing PC at an amount of 0.3 mg. The two setups were incubated for 2 h with shaking at 60 rpm. The microtiter plates were washed twice with distilled water and 20 ul of 95% ethanol was added. The absorbance was read at 595 nm*

4.3 CORRELATION TABLES OF INTERACTION ASSAY WITH PHENOTYPIC ASSAYS

The results of the phenotypic assays were summarized in a table. This is to correlate their interaction assay result with the phenotypic, cell, and colony morphology assay results. This was done for the seventeen (17) individual compounds and the ten (10) selected functional triple combination. For the accumulation assay, a higher fluorescence (+) implies a higher accumulation property of phenotypic compounds. Likewise, a higher fluorescence (+) in efflux implies a higher ability of compounds to inhibit efflux activity. Contrary to this, a lower absorbance (-) in adhesion and biofilm implies compounds can inhibit cells to adhere and form biofilm.

The values in the brackets represent their interaction assay result (either resistant-breaking or resistant inducing) results. For the single compound treatments, Inulin (RB-121.5) and Cellobiose (RB-94.5) were the highest resistant breaking compounds and Sorbose (RI-32) and Sorbose (RI-40) were the highest resistant inducing single compounds.

Table 3: Correlation table of single compounds against Ms wt

A

Ms WT	Control	Dalcitol (RB-60)	Myoinositol (RB-46)	Sialicin (RI-32)	Lactose (RB-74)	Tryptophane (RB-44.5)
Cell and colony morphology	long rods, pink	long rods, pink	short rods, pink	short rods, pink	long rods, pink	long rods, pink
Accumulation assay	0.23	++++	++++	+++++	++++	+++++
Efflux assay	0.13	+++++	+	+++	+++++	+++++
Adhesion	0.17	---	---	--	---	---
Adhesion Disruption	0.14	-	----	-----	0	+
Biofilm	0.37	-----	--	+++++	----	'+++++
Biofilm Disruption	0.8	-----	+	-----	-----	-----

B

Ms WT	Sorbose (RI-40)	Malonic acid (RB-60)	Rhamnose (RB-67.5)	Sodium-Pyruvate (RB-71)	Maltose (RB-67)	Adonitol (RB-41)
Cell and colony morphology	long rods, pink	short rods, pink	short rods, pink	short rods, pink	long rods, pink	long rods, pink
Accumulation assay	++++	++++	+++++	++++	++++	+++++
Efflux assay	++	+++++	+++++	++	+++	+++
Adhesion	---	---	---	--	--	---
Adhesion Disruption	----	---	-	-	+	---
Biofilm	+++	++	-----	+++++	++	+++++
Biofilm Disruption	+++++	-----	-----	-----	-----	++++

C

Ms WT	Xylose (RB-42)	Raffinose (RB-93)	Cellobiose (RB-94.5)	Inulin (RB-121.5)	Gluconic acid (RB-39)	Galactose (RB-67.5)
Cell and colony morphology	long rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	+++++	+++++	+++	+	+++++	0
Efflux assay	+++++	+++	++	0	+++++	0
Adhesion	---	---	---	---	---	---
Adhesion Disruption	--	+	---	-	+++++	----
Biofilm	++	-----	+++++	+	-	---
Biofilm Disruption	+++++	++	+++++	+++++	-----	+++++

Assay Key of the single treatments against Ms (WT)

Key Accumulation
Control (0.23)
+ (0.24-0.25) - (0.22-0.21)
++ (0.26-0.27) -- (0.20-0.19)
+++ (0.28-0.29) --- (0.18-0.17)
++++ (0.30-0.31) ---- (0.16-0.15)
+++++ (>0.31) ----- (<0.15)

Key Adhesion Disruption
Control (0.14)
+ (0.15-0.16) - (0.13)
++ (0.17-0.18) -- (0.12)
+++ (0.19-0.20) --- (0.11)
++++ (0.21-0.22) ---- (0.10)
+++++ (>0.23) ----- (<0.09)

Key Efflux
Control (0.13)
+ (0.14-0.15) - (0.12)
++ (0.16-0.17) -- (0.11)
+++ (0.18-0.19) --- (0.10)
++++ (0.20-0.21) ---- (0.09)
+++++ (>0.22) ----- (<0.08)

Key Biofilm Disruption
Control (0.80)
+ (0.81-0.83) - (0.79-0.77)
++ (0.84-0.86) -- (0.76-0.74)
+++ (0.87-0.89) --- (0.73-0.71)
++++ (0.90-92) ---- (0.70-0.68)
+++++ (>0.93) ----- (<0.67)

Key Adhesion
Control (0.17)
+ (0.18) - (0.16-0.15)
++ (0.19) -- (0.14-0.13)
+++ (0.20) --- (0.12-0.11)
++++ (0.21) ---- (0.10-0.09)
+++++ (>0.21) ----- (<0.09)

Key Biofilm
Control (0.37)
+ (0.38-0.40) - (0.36-0.35)
++ (0.41-43) -- (0.34-0.33)
+++ (0.44-0.46) --- (0.32-0.31)
++++ (0.47-0.49) ---- (0.30-0.29)
+++++ (>0.50) ----- (<0.28-0.27)

Table 4:Correlation table of single compounds against eMsA

A

eMsA	Control	Dalcitol (RB-60)	Myoinositol (RB-46)	Sialicin (RI-32)	Lactose (RB-74)	Tryptophane (RB-44.5)
Accumulation assay	0.114	++	+	0.00	+++	+
Efflux assay	0.29	+++++	+++++	+++++	+++++	++++
Adhesion	0.71	-----	++++	+++++	+	+++++
Adhesion Disruption	0.69	++	++++	-----	++	-
Biofilm	0.44	---	+++	-	++++	--
Biofilm Disruption	0.89	-----	+++	++++	+++++	+++

B

eMsA	Sorbose (RI-40)	Malonic acid (RB-60)	Rhamnose (RB-67.5)	Sodium-Pyruvate (RB-71)	Maltose (RB-67)	Adonitol (RB-41)
Accumulation assay	++	-	0.00	-	+++	++
Efflux assay	++++	+++++	+++++	+++++	++++	+++++
Adhesion	+++++	+++++	+++++	+++++	++++	++
Adhesion Disruption	--	+++++	+++	+	++	+++++
Biofilm	--	+++++	-	+	0.00	--
Biofilm Disruption	+++++	+++++	+++++	+++++	++	+++++

C

eMsA	Xylose (RB-42)	Raffinose (RB-93)	Cellobiose (RB-94.5)	Inulin (RB-121.5)	Gluconic acid (RB-39)	Galactose (RB-67.5)
Accumulation assay	+++	+	-	0.00	0.00	0.00
Efflux assay	+++++	+++++	+++++	+++++	+++++	+++++
Adhesion	+++++	+++++	+++++	+++++	++	+++++
Adhesion Disruption	-	+++++	++++	-	++++	+++
Biofilm	----	+++	-----	-----	+++	----
Biofilm Disruption	+++++	+	+++++	+++++	++	++++

Assay Key tables of the single treatments against eMsA

Key Accumulation

Control (0.11)

+ (0.12)	- (0.10)
++ (0.13)	-- (0.09)
+++ (0.14)	--- (0.08)
++++ (0.15)	---- (0.07)
+++++ (>0.16)	----- (<0.06)

Key Adhesion Disruption

Control (0.69)

+ (0.70 -0.72)	- (0.68-0.66)
++ (0.73-0.75)	-- (0.65-0.63)
+++ (0.76-0.78)	--- (0.62-0.60)
++++ (0.79-0.81)	---- (0.59-0.57)
+++++ (>0.82)	----- (<0.56)

Key Efflux

Control (0.29)

+ (0.30)	- (0.28)
++ (0.31)	-- (0.27)
+++ (0.32)	--- (0.26)
++++ (0.33)	---- (0.25)
+++++ (>0.34)	----- (<0.24)

Key Biofilm Disruption

Control (0.89)

+ (0.90 -0.92)	- (0.88)
++ (0.93-0.95)	-- (0.87)
+++ (0.96-0.98)	--- (0.86)
++++ (0.99-0.10)	---- (0.85)
+++++ (>0.11)	----- (<0.84)

Key Adhesion

Control (0.71)

+ (0.72 -0.73)	- (0.69-0.67)
++ (0.74-0.76)	-- (0.66-0.64)
+++ (0.77-0.79)	--- (0.63-0.61)
++++ (0.80-0.82)	---- (0.60-0.58)
+++++ (>0.83)	----- (<0.58)

Key Biofilm

Control (0.44)

+ (0.45 -0.46)	- (0.43-0.42)
++ (0.47-0.48)	-- (0.41-0.40)
+++ (0.49-0.50)	--- (0.39-0.38)
++++ (0.51-0.52)	---- (0.37-0.36)
+++++ (>0.53)	----- (<0.35)

Table 4.11: Correlation table of single compounds against eMsB

A

eMsB	Control	Dalcitol (RB-60)	Myoinositol (RB-46)	Sialicin (RI-32)	Lactose (RB-74)	Tryptophane (RB-44.5)
Accumulation assay	0.31	++	+	+++++	+	++
Efflux assay	0.31	++++	+++++	+	++++	+++++
Adhesion	0.4	-----	++	+	--	---
Adhesion Disruption	0.55	-	-	---	----	+++
Biofilm	0.45	0	++	-----	-----	+++++
Biofilm Disruption	0.87	-	-----	-----	0	+++++

B

eMsB	Sorbose (RI-40)	Malonic acid (RB-60)	Rhamnose (RB-67.5)	Sodium-Pyruvate (RB-71)	Maltose (RB-67)	Adonitol (RB-41)
Accumulation assay	+++++	-	+	++++	+	+++
Efflux assay	++++	++	----	++	+++++	+
Adhesion	--	+++++	+	---	--	+++++
Adhesion Disruption	-	-----	+++++	+++++	-----	-
Biofilm	'--	+	--	--	---	----
Biofilm Disruption	-----	---	--	+++++	-----	----

C

eMsB	Xylose (RB-42)	Raffinose (RB-93)	Cellobiose (RB-94.5)	Inulin (RB-121.5)	Gluconic acid (RB-39)	Galactose (RB-67.5)
Accumulation assay	+++++	0	+++++	+++++	+++++	+
Efflux assay	+++	++++	+++	++	+++++	+
Adhesion	-----	---	+	----	-----	-----
Adhesion Disruption	+++++	+++++	---	+	++++	---
Biofilm	----	-	--	---	---	---
Biofilm Disruption	+++++	-----	-----	----	-----	-----

Assay Key tables of the single treatments against eMsB

Key Accumulation

Control (0.31)	
+ (0.32 -0.33)	- (0.30-0.29)
++ (0.34-0.35)	-- (0.28 -0.27)
+++ (0.36-0.37)	--- (0.26-0.25)
++++ (0.38-0.39)	---- (0.24-0.23)
+++++ (>0.40)	-----(>0.22)

Key Adhesion Disruption

Control (0.55)	
+ (0.56 -0.57)	- (0.54-0.52)
++ (0.58-0.59)	-- (0.51-0.49)
+++ (0.60-0.61)	--- (0.48-0.46)
++++ (0.62-0.63)	---- (0.45-0.43)
+++++ (>0.64)	-----(>0.42)

Key Efflux

Control (0.31)	
+ (0.32)	- (0.30)
++ (0.33)	-- (0.29)
+++ (0.34)	--- (0.28)
++++ (0.35)	---- (0.27)
+++++ (>0.36)	-----(>0.26)

Key Biofilm Disruption

Control (0.87)	
+ (0.88 -0.89)	- (0.86-0.84)
++ (0.90-0.91)	-- (0.83-0.81)
+++ (0.92-0.93)	--- (0.80-0.78)
++++ (0.94-0.95)	---- (0.77-0.75)
+++++ (>0.96)	-----(>0.74)

Key Adhesion

Control (0.40)	
+ (0.41)	- (0.39-0.38)
++ (0.42)	-- (0.37-0.36)
+++ (0.43)	--- (0.35-0.34)
++++ (0.44)	---- (0.33-0.32)
+++++ (>0.44)	-----(>0.32)

Key Biofilm

Control (0.45)	
+ (0.46)	- (0.44-0.42)
++ (0.47)	-- (0.41-0.39)
+++ (0.48)	--- (0.38-0.36)
++++ (0.49)	---- (0.35-0.33)
+++++ (>0.50)	-----(>0.32)

Table 5: Correlation table of selected FTC against Ms wt

A

Resistant-Breaking (Selected FTC)						
Ms Wt	Control	Sor (3X) (RB-89)	Mal (3X) (RB-69)	Sor+Try + Malt (RB-73.5)	Myo+Mal (1.5X) (RB-50.5)	Ado+Cel+Inu (RB-50)
Cell and colony morphology	long rods, pink	long rods, pink	long rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.16	+++++	-----	-	--	----
Efflux assay	0.1	0	---	+	+++	+
Adhesion	0.69	-----	-----	----	+++++	-----
Adhesion Disruption	1.07	++++	-----	-	+	-----
Biofilm	1.03	0	--	-----	+++++	-----
Biofilm Disruption	1.49	---	-----	-----	---	-----

B

Resistant-Inducing (Selected FTC)						
Ms Wt	Control	Lac (3X) (RI-123.5)	Sia+Ado (RI-91.5)	Try (3X) (RI-82.5)	Raf (3X) (RI-73.5)	Ado+Xyl+Raf (RI-69.5)
Cell and colony morphology	long rods, pink	long rods, pink	long rods, pink	long rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.16	+++++	--	---	---	--
Efflux assay	0.1	+++	+++	0	++	+
Adhesion	0.69	----	--	-----	-----	-----
Adhesion Disruption	1.07	++++	+++++	-----	-----	-----
Biofilm	1.03	-	++++	-----	-----	-----
Biofilm Disruption	1.49	----	+	---	---	-----

Assay Key tables of the selected triple combinations against Ms (WT)

Key Accumulation

Control (0.16)	
+ (0.17)	- (0.15-0.14)
++ (0.18)	-- (0.13-0.12)
+++ (0.29-0.20)	--- (0.11-0.10)
++++ (0.21-0.22)	---- (0.09-0.08)
+++++ (>0.23)	-----(<0.07)

Key Adhesion Disruption

Control (1.07)	
+ (1.08 -1,09)	- (1.06-1.04)
++ (1.10-1.11)	-- (1.03-1.01)
+++ (1.12-1.13)	--- (1.00-0,98)
++++ (1.14- 1.15)	---- (0..97-0.95)
+++++ (>1.15)	-----(<0.95)

Key Efflux

Control (0.10)	
+ (0.11)	- (0.09)
++ (0.12)	-- (0.08)
+++ (0.13)	--- (0.07)
++++ (0.14)	---- (0.06)
+++++ (>0.15)	-----(<0.05)

Key Biofilm Disruption

Control (1.49)	
+ (0.50)	- (0.48-0.47)
++ (0.51)	-- (0.46-0.45)
+++ (0.52)	--- (0.44-0.43)
++++ (0.53)	---- (0.42-0.41)
+++++ (>0.53)	-----(<0.41)

Key Adhesion

Control (0.69)	
+ (0.70)	- (0.68-0.66)
++ (0.71)	-- (0.65-0.63)
+++ (0.72)	--- (0.62-0.60)
++++ (0.73)	---- (0.59-0.57)
+++++ (>0.73)	-----(<0.57)

Key Biofilm

Control (1.03)	
+ (1.04 -1,05)	- (1.02-1.01)
++ (1.06-1.07)	-- (1.00-0.99)
+++ (1.08-1.09)	--- (0.98-0.97)
++++ (1.10-1.11)	---- (0.96-0.95)
+++++ (>1.11)	-----(<0.95)

Table 6: Correlation table of selected FTC against eMsA

A

Resistant-Breaking (Selected FTC)						
eMsA	Control	Sor (3X) (RB-89)	Mal (3X) (RB-69)	Sor+Try + Malt (RB-73.5)	Myo+Mal (1.5X) (RB-50.5)	Ado+Cel+Inu (RB-50)
Cell and colony morphology	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.15	+	+++++	+	++	+++++
Efflux assay	0.6	-	+++++	+++	+++++	+++++
Adhesion	0.21	+++++	++++	+++	+++	++
Adhesion Disruption	0.82	--	-----	-----	+++	-----
Biofilm	0.54	-----	-----	-----	-----	-----
Biofilm Disruption	0.67	+++	-----	---	--	---

B

Resistant-Inducing (Selected FTC)						
eMsA	Control	Lac (3X) (RI-123.5)	Sia+Ado (RI-91.5)	Try (3X) (RI-82.5)	Raf (3X) (RI-73.5)	Ado+Xyl+Raf (RI-69.5)
Cell and colony morphology	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.15	0	+	+	-	++
Efflux assay	0.6	++++	+++++	---	--	+++++
Adhesion	0.21	+++++	++++	--	----	----
Adhesion Disruption	0.82	-----	---	-----	-----	-----
Biofilm	0.54	-----	-----	-----	-----	-----
Biofilm Disruption	0.67	-	++++	-----	-----	-----

Assay key tables of the selected triple combinations against eMsA

Key Accumulation

Control (0.15)	
+ (0.16-0.17)	- (0.14)
++ (0.18-0.19)	-- (0.13)
+++ (0.20-0.22)	--- (0.12)
++++ (0.23-0.24)	---- (0.11)
+++++ (>0.25)	-----(<0.10)

Key Adhesion Disruption

Control (0.82)	
+ (0.83)	- (0.81-0.79)
++ (0.84)	-- (0.78-0.76)
+++ (0.85)	--- (0.75-0.73)
++++ (0.86)	---- (0.72-0.70)
+++++ (>0.86)	-----(<0.70)

Key Efflux

Control (0.60)	
+ (0.61-0.62)	- (0.59-0.58)
++ (0.63-0.64)	-- (0.57-0.56)
+++ (0.65-0.66)	--- (0.55-0.54)
++++ (0.67-0.68)	---- (0.53-0.52)
+++++ (>0.69)	-----(<0.51)

Key Biofilm Disruption

Control (0.67)	
+ (0.68-0.70)	- (0.66-0.64)
++ (0.71-0.73)	-- (0.63-0.61)
+++ (0.74-0.76)	--- (0.60-0.58)
++++ (0.77-0.79)	---- (0.57-0.55)
+++++ (>0.79)	-----(<0.55)

Key Adhesion

Control (0.21)	
+ (0.22-0.23)	- (0.20-0.19)
++ (0.24-0.25)	-- (0.18-0.17)
+++ (0.26-0.27)	--- (0.16-0.15)
++++ (0.28-0.29)	---- (0.14-0.13)
+++++ (>0.29)	-----(<0.13)

Key Biofilm

Control (0.54)	
+ (0.55)	- (0.53-0.51)
++ (0.56)	-- (0.50-0.48)
+++ (0.57)	--- (0.47-0.45)
++++ (0.58)	---- (0.44-0.42)
+++++ (>0.58)	-----(<0..42)

Table 7: Correlation table of selected FTC against eMsB

A

Resistant-Breaking (Selected FTC)						
eMsB	Control	Sor (3X) (RB-89)	Mal (3X) (RB-69)	Sor+Try + Malt (RB-73.5)	Myo+Mal (1.5X) (RB-50.5)	Ado+Cel+Inu (RB-50)
Cell and colony morphology	short rods, pink	cocci, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.4	+	--	++++	+++	+
Efflux assay	0.36	++++	---	+	+	-
Adhesion	0.22	-	---	-	--	---
Adhesion Disruption	0.38	++++	-----	-----	-----	-----
Biofilm	0.79	-----	-----	-----	-----	-----
Biofilm Disruption	0.44	--	----	--	++	-----

B

Resistant-Inducing (Selected FTC)						
Ms Wt	Control	Lac (3X) (RI-123.5)	Sia+Ado (RI-91.5)	Try (3X) (RI-82.5)	Raf (3X) (RI-73.5)	Ado+Xyl+Raf (RI-69.5)
Cell and colony morphology	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink	short rods, pink
Accumulation assay	0.4	+	++	++	----	+++++
Efflux assay	0.36	-	-	0	+	++
Adhesion	0.22	--	---	----	----	----
Adhesion Disruption	0.38	-----	---	-	++	-
Biofilm	0.79	-----	-----	-----	-----	-----
Biofilm Disruption	0.44	---	-	-----	----	-----

Assay key tables of the selected triple combinations against eMsB

Key Accumulation

Control (0.40)	
+ (0.41 -0.43)	- (0.39-0.37)
++ (0.44-0.46)	-- (0.36-0.34)
+++ (0.47-0.49)	--- (0.32-0.30)
++++ (0.50-0.54)	---- (0.29-0.27)
+++++ (>0.55)	----- (<0.26)

Key Adhesion

Control (0.22)	
+ (0.23)	- (0.21-0.19)
++ (0.24)	-- (0.18-0.16)
+++ (0.25)	--- (0.15-0.13)
++++ (0.26)	---- (0.12-0.10)
+++++ (>0.26)	----- (<0.10)

Key Efflux

Control (0.36)	
+ (0.37 -0.38)	- (0.35)
++ (0.39-0.40)	-- (0.34)
+++ (0.41-0.42)	--- (0.33)
++++ (0.43-0.44)	---- (0.32)
+++++ (>0.45)	----- (<0.31)

Key Biofilm Disruption

Control (0.44)	
+ (0.45)	- (0.43-0.41)
++ (0.46)	-- (0.40-0.38)
+++ (0.47)	--- (0.37-0.35)
++++ (0.48)	---- (0.34-0.32)
+++++ (>0.48)	----- (<0.32)

Key Adhesion Disruption

Control (0.38)	
+ (0.39 -0.40)	- (0.36-0.35)
++ (0.41-0.42)	-- (0.34-0.33)
+++ (0.43-0.44)	--- (0.32-0.31)
++++ (0.45-0.46)	---- (0.30-0.29)
+++++ (>0.46)	----- (<0.29)

Key Biofilm

Control (0.79)	
+ (0.80)	- (0.78-0.77)
++ (0.81)	-- (0.76-0.75)
+++ (0.82)	--- (0.74-0.73)
++++ (0.83)	---- (0.72-0.71)
+++++ (>0.83)	----- (<0.71)

4.4 RESULTS ON THE EXPRESSION PATTERNS OF STRESS RESPONSE GENES IN TREATED CELLS

The effect of the compounds on some selected stress response genes was analyzed. The top two Resistant-breaking (Sor(3X) and Mal(3X)) and Resistant-inducing (Lac(3X) and Ado+Xyl (1.5X)) from the list of triple compounds were used for this assay.

Six (6) different stress phenotypes were analyzed with specific genes for this project. The six stress phenotypes are Toxins and Anti-toxin System (VAPB and VAPC), Nutritional and Growth Regulation (RELA and USPC), RNA Polymerase attachment and Housekeeping Genes (2758 and SIGMA), Antibiotic and Efflux Inhibition (MSHB and ACR), Virulence (LSR), and Cytoskeletal Regulation (END).

Table 8: List of stress response genes with their function

GENE (*Mycobacterium smegmatis*)	**COMPONENT**
VAPB	Non-cognate antitoxin to neutralize VapC
LSR2	Global gene regulator that encodes major virulence factors, such as the ESX secretion systems
VAPC	Toxins that inhibit the growth of the bacteria
MSHB	Isoniazid and Ethionamide resistance
USPC	Universal stress response gene that regulates mycobacterial growth
2758	Initiation factors that promote the attachment of RNA polymerase
SIGMA	Promote the attachment of RNA polymerase

	(Housekeeping Gene)
ACR	Involved in Efflux pump formation
RELA	Synthesizes guanosine tetraphosphate (ppGpp) (p)ppGpp in nutrient-deprived conditions.
END	Regulates Actin cytoskeleton complex

The effect of the compounds on some selected stress response genes was analyzed. The top two Resistant-breaking (Sor(3X) and Mal(3X)) and Resistant-inducing (Lac(3X) and Ado+Xyl (1.5X) and triple compounds were used for this assay. Six (6) different stress phenotypes were analyzed with specific genes for this project. The six stress phenotypes are; Toxins and Anti-toxin System (VAPB and VAPC), Nutritional and Growth Regulation (RELA and USPC), RNA Polymerase attachment and Housekeeping Genes (2758 and SIGMA), Antibiotic and Efflux Inhibition (MSHB and ACR), Virulence (LSR), and Cytoskeletal Regulation (END).

In eMsA treated with mal (3X) had an increased expression of VAPB. Mal (3X) is inducing resistance because VapB is an antitoxin and an increase in the expression of it implies the bacteria want to survive. Sia+Ado (1.5X), also caused an increase in expression in eMsB. Not much expression was observed across the other treatments (Fig 4.27A). There was an increase in the expression of this gene across the treatments for three organisms. LSR encodes virulence in the organism hence increase in the expression of this gene in the treatments means an increase in resistance inducing (Fig 4.27B).

There was increased expression of the VAPC gene in the presence of Lac(3X) for all three organisms (Figure 4.27C). VAPC encodes for toxins in the organism, increased expression of this gene will lead to an increase in toxicity in the organism.

There is an increase in expression of the MSHB gene mostly in eMsA and eMsB in the presence of Sor(3X), Mal(3X), and Sia+Ado(1.5X) treatments. There was a low expression of the gene in the wt (Fig 4.28A). This gene is responsible for the resistance to isoniazid and ethionamide. The increased expression of these genes in the mutant strains was not surprising since they are more resistant compared to the wt. The presence of the compounds seems to increase this expression.

There was an increase in expression of the END gene in the presence of Sor(3X), Mal(3X), and Sia+Ado(1.5X) treatments (Figure 4.28B). There was not much difference observed for the other treatment. Generally, not much change was observed in the USPC gene across the treatments for the organism except for Lac(3X) which increased the expression of the gene in eMsB (Fig 4.28C). USPC is a universal gene that regulates mycobacterial growth hence increase in expression of it suggests the drug might induce resistance.

Sor(3X), Lac(3X), and Sia+Ado(1.5X) increased the expression of the ACR gene in eMsA (Fig 4.29B). An increase in expression of this gene will increase efflux activity making organisms more resistant. Sor(3X) and Sia+Ado(1.5X) increased the expression of RelA in eMsA (Fig 4.29C). RelA is upregulated when the bacteria are deprived of nutrients, increased expression of this gene by this compound implies it increases resistance.

The summary table of the expression analysis shows a lot of increase in resistant breaking genes which correlates with most of the results obtained for the interaction and phenotypic assays (Table 4.22).

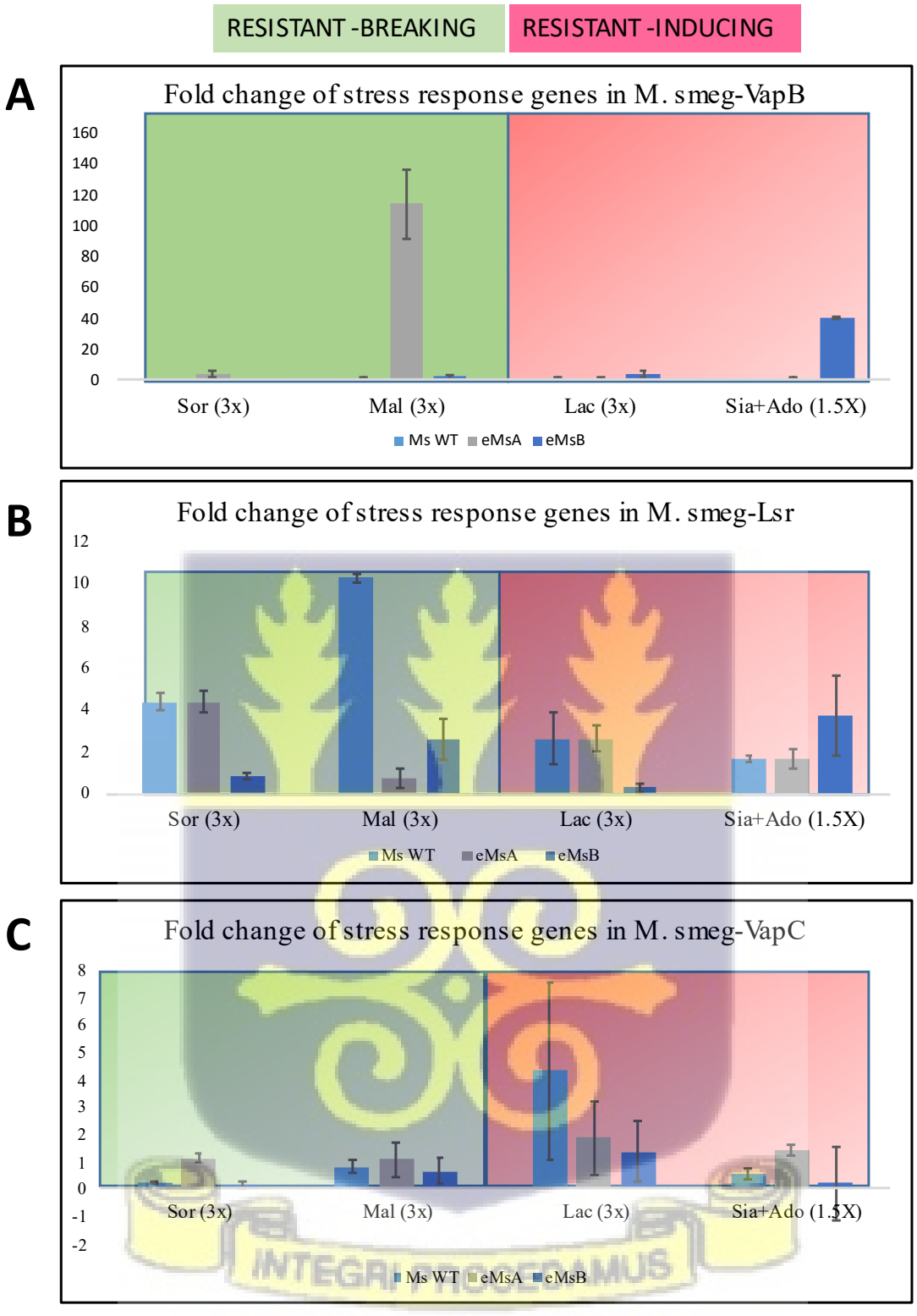


Figure 4.27: Expression analysis of vapB, lsr and vapC genes in Ms wt, eMsA and eMsB. *RNA from the treated cells was extracted and used for RT-qPCR. The Applied Biosystems real-time machine was programmed using the thermocycling protocol as stated in the Luna® Universal One-Step RT-qPCR Kit.*

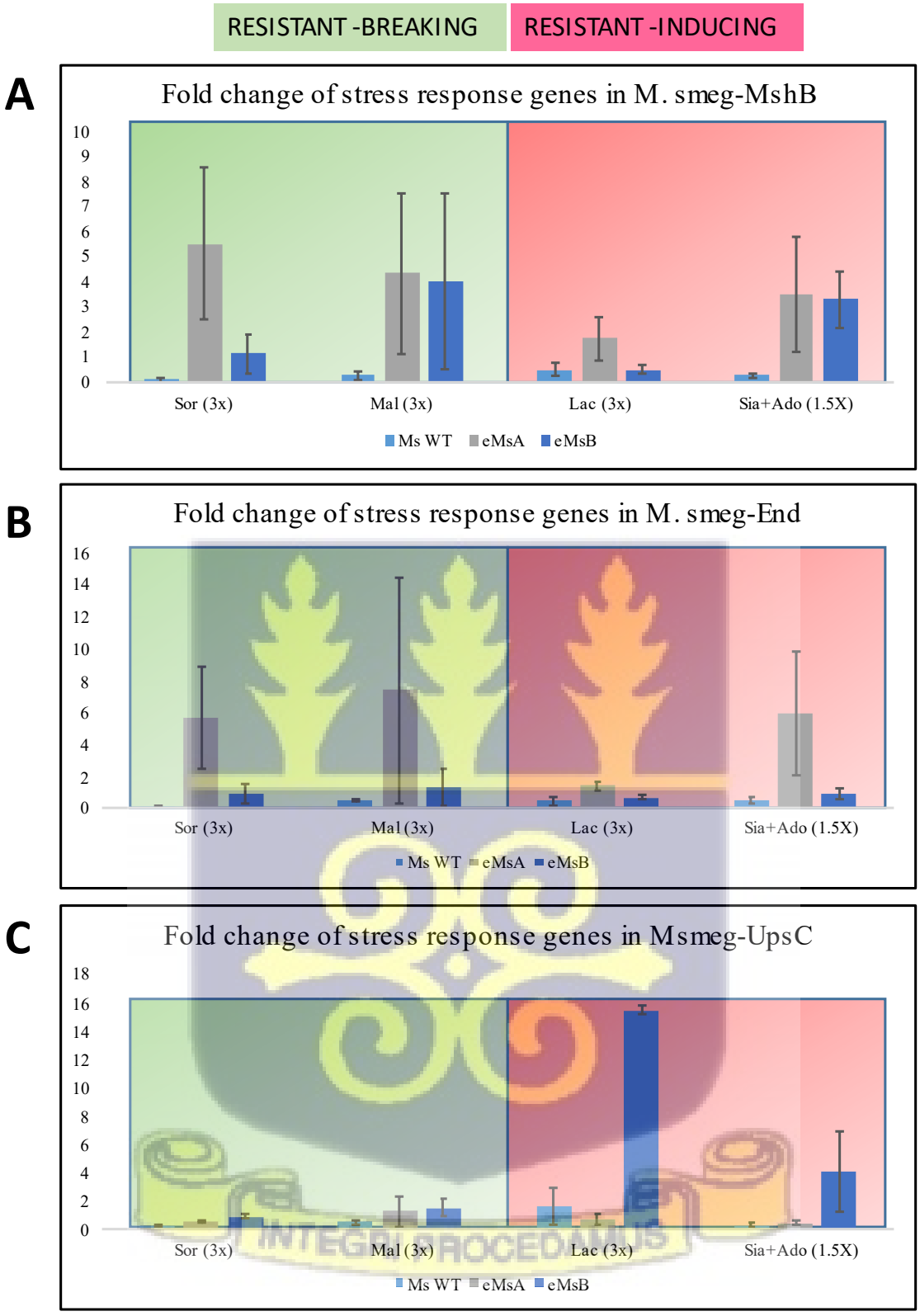
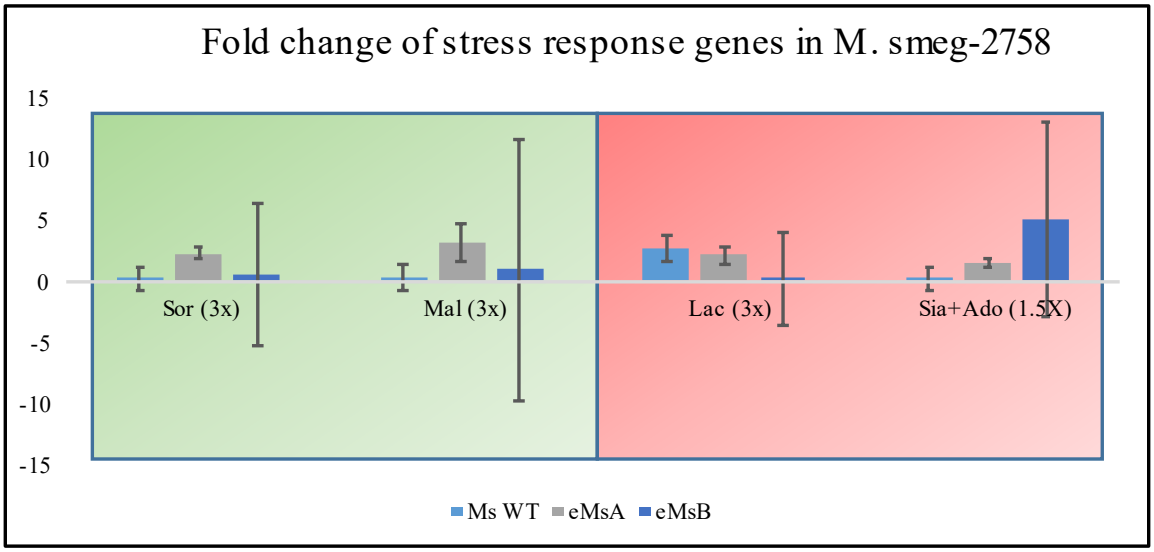


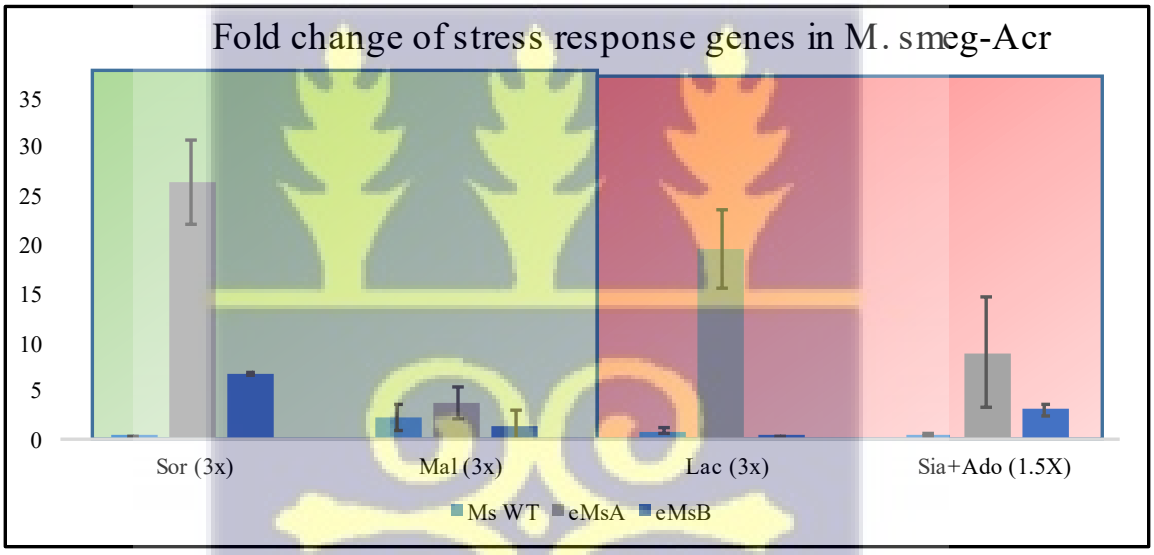
Figure 4.28: Expression analysis of mshB, end, and uspC genes in Ms wt, eMsA, and eMsB. *RNA from the treated cells was extracted and used for RT-qPCR. The Applied Biosystems real-time machine was programmed using the thermocycling protocol as stated in the Luna® Universal One-Step RT-qPCR Kit.*

RESISTANT -BREAKING | RESISTANT -INDUCING

A



B



C

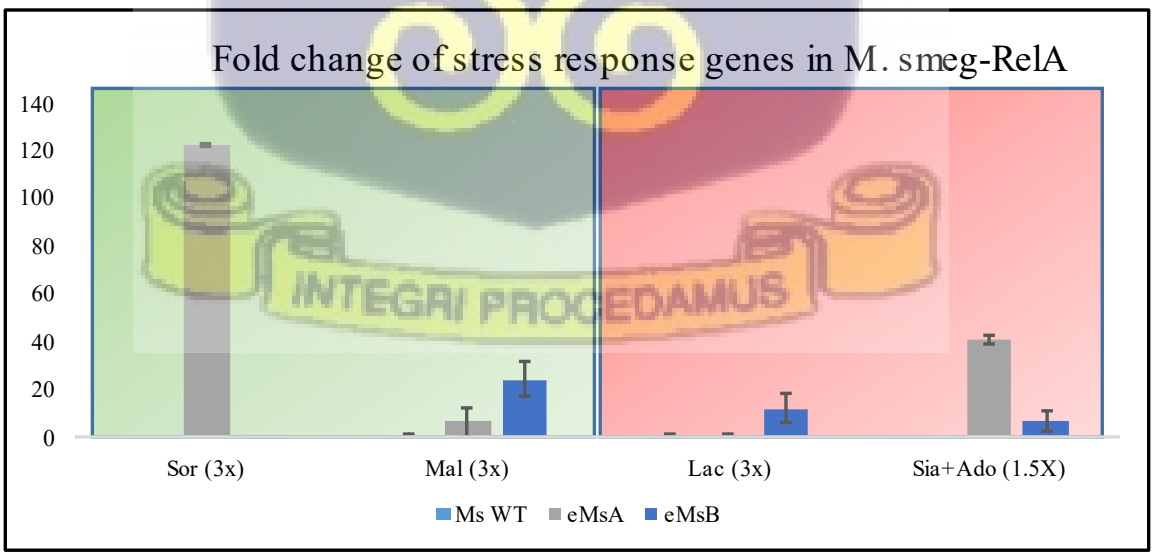


Figure 4.29: Ex Expression analysis of 2758, acr, and relA genes in Ms wt, eMsA, and eMsB. *RNA from the treated cells was extracted and used for RT-qPCR. The Applied Biosystems real-time machine was programmed using the thermocycling protocol as stated in the Luna® Universal One-Step RT-qPCR Kit.*

A summary of the effect of the various treatments on the three strains of *Mycobacteria* used (Ms wt, eMsA, and eMsB) are summarized in the table below:

Table 9: Summary of expression analysis of stress response genes

Functional Class	Genes	General Expression Levels	Antibiotic Effect
1. Toxins and Anti-toxin System	1. VAPB (Anti-toxin)	Decreased	Resistant-breaking
	2. VAPC (Toxin)	Incraesed	Resistant-breaking
2. Nutritional and Growth Regulation	3. RELA	Decreased	Resistant-breaking
	4. USPC	Decreased	Resistant-breaking
3. RNA Polymerase attachment	5. 2758	Decreased	Resistant-breaking
4. Antibiotic and Efflux Inhibition	7. MSHB	Increased	Resistant-inducing
	8. ACR	Decreased	Resistant-breaking
5. Encoding Virulence	9. LSR	Increased	Resistant-inducing
6. Cytoskeletal Regulation	10. END	Increased	Resistant-breaking

CHAPTER FIVE

DISCUSSION

The pace of novel antibiotic development has never been able to match the rate of bacterial evolution which has led to an increase in antibiotic resistance (Fair & Tor, 2014). With limited antibiotic mechanisms of action which are subject to the constant evolution of bacteria, defeating antibiotic resistance seems like an impossible reality (Fair & Tor, 2014). New antibiotic therapeutic strategies are being sought every day to decrease the effect and emergence of antibiotic resistance. One such strategy is the usage of antibiotic adjuvants. Antibiotic adjuvants are compounds with no or little antibiotic effect but are used to improve antibiotic efficacy and impede resistance (Wright, 2016). Most antibiotic adjuvants work in synergy with antibiotics to increase antibiotic efficacy by either acting on the pathogen or the host (Douafer et al., 2019). This strategy will increase the potency of antibiotics, revive already existing antibiotics against resistant microbes, and complement the development of novel antibiotics (Worthington & Melander, 2012; Wright, 2016; Yang et al., 2017).

The potential of 17 compounds to work as antibiotic adjuvants were analyzed using interaction and phenotypic assays against M. smegmatis wt and two mutant strains (eMsA and eMsB). The antibiotic-compound interaction assays were extended to other organisms, Escherichia coli (wt and RecA mutant), Candida albicans, and Saccharomyces cerevisiae (Table S13 and S14). The compounds had significant changes in the fungi strains, but subtle changes were observed with the E. coli strains. We proceeded with M. smegmatis strains because they presented more interesting patterns and robust effects, observed especially with the mutant strains.

Most of the compounds were able to make significant changes to the antibiotic susceptibility profile of these organisms through the interaction assay. The compounds were grouped into structural combinations based on their structure and diversity. The goal was to increase the effect

that was observed but this was not seen as the structural combination did not yield any significant changes as expected. Another set of triple combinations was generated based on the activities of the single compounds. this set of functional triple combinations was more robust and significantly increased the activities of the antibiotics against our test strains, especially the mutant strains. The compounds were classified as either resistant breaking or resistant inducing based on their effect on the antibiotics.

The top five resistant-breaking single compounds were inulin, cellobiose, raffinose, rhamnose, and lactose (Table 4.3) while the top five resistant-inducing compounds were maltose, sorbose, rhamnose, salicin, and lactose (Table 4.4). Some of the compounds had a dual effect where they acted as resistant-breaking with some antibiotics and induced resistance in other antibiotics (lactose and rhamnose).

Against Ms wt, the effect of inulin correlated with the accumulation assay but did not correlate with the efflux assay. It also correlated with adhesion inhibition and disruption but did not correlate with the biofilm assays. In the presence of inulin, the cells were shorter than the control and had pale pink staining. Previous studies have shown the antioxidant and prebiotic properties of inulin and its ability to complement some antibiotics against organisms like lactobacilli (James, 2014; Kareem et al., 2014; Nooshkam et al., 2019), this correlates with the observed result against M. smegmatis. The effect obtained for cellobiose correlated with the accumulation, efflux, adhesion inhibition, and disruption assays but did not correlate with the biofilm assays. In the presence of cellobiose, the cell morphology was short but maintained its pink coloration. Previous work by Kulakovskaya E. et. Al proved that cellobiose possessed membrane-damaging activities against Filobasidiella neoformans and Candida tropicalis (Kulakovskaya et al., 2014), this property might have played a role in complementing the effect we observed with cellobiose. Raffinose, like the other two resistant-breaking, had short cells and pink staining of rods. It correlated with both efflux

and accumulation assays and disruption of both adhesion and biofilm assays. Strangely, when incubated with Ms wt to inhibit adhesion and biofilm, it does not correlate contrary to what was observed by Kim H. and colleagues where they found raffinose binding to LecA and inhibiting biofilm formation in Pseudomonas aeruginosa (Kim et al., 2016). In the presence of rhamnose, the cells are short and pink which correlates with its resistant-breaking effect but does not correlate with the other phenotypic assays (accumulation, efflux, adhesion, and biofilm). This observation for rhamnose was contrary to what was previously observed, where rhamnose inhibits biofilm formation in P. aeruginosa by binding to PA14, a biofilm regulator (Fu et al., 2019). Lactose, one of the top five resistant breaking compounds correlates with both efflux and accumulation assays. It does not correlate with the other phenotypic assays (inhibition and disruption of adhesion and biofilm). Consistent with the other resistant breaking compounds, the cells were short and pink in coloration. Most bacteria organisms have been found to possess lactose-breaking enzymes, lactase or Beta-galactosidase, and lactose dehydrogenase and as such can make use of lactose (Ibrahim et al., 2021; Mehrad et al., 2015). Hence the presence of this metabolite can be used as a source of energy for the bacteria which regulates the bacteria's metabolism (Ammar et al., 2018). The effect of sorbose, contrary to the resistant breaking compounds did not correlate with most of the phenotypic assays except the biofilm inhibition and disruption. The cell morphology had pale staining but was long. Sorbose has previously been characterized as a carbon and energy source for some bacteria like E. coli (Lehmacher & Bockemühl, 2007; Soemphol et al., 2007) but its effects in M. smegmatis are yet to be determined and it was observed to inhibit biofilm formation. Salicin's data correlated with just biofilm inhibition and did not correlate with the other phenotypic assays. The cell's morphology was shorter than the control's (Table 4.15). Previous works have shown the presence of salicin to de-repress multiple antibiotic operons (B. R. Singh, 2014; Zhang et al., 2022) and increase the sensitivity of microbe to antibiotics, which correlated with the observations of salicin being resistant inducing.

Against eMsA, cellobiose correlated with efflux and biofilm inhibition but did not correlate with accumulation, biofilm disruption, adhesion inhibition, and disruption. Inulin had a similar correlation pattern to cellobiose, correlated with both efflux and biofilm inhibition like cellobiose but did correlate with accumulation, adhesion inhibition, and biofilm disruption. Raffinose correlated with both accumulation and efflux assays but did not correlate with the other phenotypic compounds (inhibition and disruption of adhesion and biofilm). Rhamnose correlated with efflux and biofilm inhibition but did not correlate with the other phenotypic assays (accumulation, biofilm disruption, adhesion inhibition, and disruption). Lactose correlated with both accumulation and biofilm but did not correlate with any of the other phenotypic assays (inhibition and disruption of adhesion and biofilm). Sorbose correlated with adhesion inhibition and biofilm disruption but did not correlate with both accumulation, efflux, adhesion disruption, and biofilm inhibition assays. Salicin likewise correlated with just two of the phenotypic interactions, biofilm disruption, and adhesion inhibition just like sorbose (Table 4.16).

Inulin correlated with most of the phenotypic assays (5/6) against eMsB. Aside from adhesion inhibition that did not correlate with, it correlated with the other assays (accumulation, efflux, adhesion disruption, biofilm disruption, and inhibition). Cellobiose, like inulin, correlated with 5/6 phenotypic assays, accumulation, efflux, adhesion inhibition, biofilm inhibition, and disruption. It did not correlate with adhesion disruption. Raffinose also correlated with 4/6 of the phenotypic assays. Aside from accumulation, and adhesion disruption, it correlated with all the other phenotypic assays. Rhamnose, being the least resistant breaking compound among the top 5 had the least correlations (3/6). It correlated with just accumulation and biofilm inhibition and disruption. Sorbose surprisingly did not correlate with any of the phenotypic assays against eMsB. Salicin had a similar correlation pattern like sorbose, affecting just one phenotypic assay, adhesion inhibition (Table 4.17).

It can be observed that all the resistant breaking compounds had a consistent and increased correlation against eMsB compared to the other strains. The resistant-inducing compounds did not correlate with most of the phenotypic assays across the three strains.

There was much correlation observed in the selected triple combination against Ms wt and two mutant strains (eMsA and eMsB). For the resistant breaking group, against Ms wt, Sor (3X) maintained the pink coloration of the cells and correlated with accumulation assay and biofilm disruption. It did not correlate with efflux, adhesion disruption, and biofilm inhibition. Mal (3X) also maintained the normal morphology of cells (pink and long rods) and correlated with both adhesion and biofilm inhibition and disruption. It did not correlate with both accumulation and adhesion. Sor+Try+Malt treated cells had pink but short rods. Aside from not correlating with efflux, it correlated with all the other phenotypic assays (5/6). Myo+Mal (1.5X) correlated with just 2/6 phenotypic assays even though is resistant-breaking. Ado+Cel+Inu treated cells were short but maintained the pink coloration. It correlated with almost all the phenotypic assays (5/6). Aside from accumulation assay that it did not correlate to, it did correlate with efflux, inhibition, and disruption of adhesion and biofilm. For resistant-inducing compounds, Lac(3X) treated cells maintained the long and pink rods but correlated with just adhesion disruption. It did not correlate with efflux and accumulation, adhesion inhibition, and both biofilm inhibition and disruption. Sia+Ado (1.5X) treated cells had long and pink rods and correlated with 3/6 phenotypic assays. It correlated with accumulation, adhesion disruption, and biofilm adhesion assay. Try (3X) treated cells had long and pink rods like the control but did not correlate to all the phenotypic assays except the accumulation assay. Raf (3X) had pink and short rods and just like Try(3X) correlated with just accumulation assay. It did not correlate with efflux, inhibition and disruption adhesion, and efflux. Ado+Xyl+Raf had short and pink rods and correlated with just accumulation assay. It did not correlate with the other phenotypic assays (Table 4.18). There was much correlation between

the phenotypic assays and interaction assay of the resistant breaking compounds compared to the resistant inducing compounds.

Against eMsA, Sor(3X) correlated with accumulation, adhesion disruption, biofilm inhibition, and disruption. It did not correlate with efflux, adhesion inhibition, and biofilm disruption. The cells had short and pink stained cells similar to the control. Mal(3X) similar to the effect observed against Ms wt, correlated significantly with 5/6 phenotypic assays- accumulation and efflux, adhesion disruption, biofilm inhibition, and disruption. Sor+Try+Malt also had short and pink staining rods and correlated with 5/6 phenotypic assays. Aside from adhesion inhibition its resistant breaking property correlated with all the other phenotypic assays. Myo+Mal (1.5X) correlated with 4/6 phenotypic assays and also maintained the pink and short rods similar to the control. It correlated with accumulation, efflux, biofilm inhibition, and adhesion assays. Ado+Cel+Inu correlated with 5/6 phenotypic assays while maintaining the cell's classical short and pink rod shape. It correlated significantly with accumulation and efflux assays. It correlated significantly with adhesion disruption and biofilm inhibition and disruption. For resistant-inducing compounds against eMsA, Lac (3X) correlated with 1/6 phenotypic assays- adhesion inhibition assay, all the other assays did not correlate with it. Sia+Ado did not correlate with most of the phenotypic assays, it was only correlated with adhesion inhibition and biofilm disruption. Try (3X) had short and pink rod-like cells and correlated with only efflux assay, it did not correlate with the other phenotypic assays. Raf(3X) treated cells maintained the cellular morphology compared to the control but correlated with just accumulation and efflux assays. It did not correlate with either inhibition and disruption of adhesion and biofilm assays. Ado+Xyl+Raf just like the other resistant-inducing compounds maintained the short and pink rods but did not correlate with any of the other phenotypic assays.

Most of the resistant breaking compounds were consistent and correlated with their interaction assays similar to the results obtained with wt (Table 4.19).

For eMsB, Sor(3X) treated were short and pink and correlated with 5/6 phenotypic assays- accumulation, adhesion, efflux, inhibition, and disruption of biofilm assay. Mal (3X) treated cells had short rods with pink staining cells. It correlated with all phenotypic assays except efflux and accumulation it did not correlate with. Sor+Try+Malt treated cells had short and pink cells and correlated with all the phenotypic assays, significantly with accumulation, adhesion disruption, and biofilm inhibition. Myo+Mal (1.5X) correlated with most of the phenotypic assays (5/6), aside from biofilm disruption it correlated with all the other phenotypic assays. Ado+Cel+Inu treatment also had short cells with pink coloration and correlated with 5/6 phenotypic assays. Aside from efflux assay, it correlated with all the other phenotypic assays, most significantly with adhesion disruption, biofilm inhibition, and disruption. For resistant inducing compounds, Lac (3X) short and pink rods. This treatment did not correlate with any of the phenotypic assays except the efflux assay. Sia+Ado (1.5X) treated cells had short and pink rods and correlated with just one of the phenotypic assays- efflux assay just like Lac (3X). Try (3X) also had pink and short rods and did not correlate with any of the phenotypic assays. Raf (3X) had a short pink rod and correlated with accumulation and adhesion disruption assays. Ado+Xyl+Raf also had short and pink rods. Like the other resistant-breaking compounds, this treatment did not correlate with any of the phenotypic assays (Table 4.20).

Most of the resistant breaking compounds had consistent correlation results with the phenotypic assays while the resistant inducing compounds did not correlate much with the phenotypic assays.

Most of the compounds had a significant effect during the phenotypic assays. The cell and colony morphology were changed in the presence of most of the compounds. The compounds also affected

accumulation, efflux, adhesion, and biofilm assays. Some of the compound's interaction assays correlated with their phenotypic assay showing their consistent effect on different assay fronts.

To understand the molecular effect of the compounds, stress response genes targeting different functional classes were investigated. Some of the resistant-breaking and resistant-inducing compounds correlated with the expression analysis observed. The resistant-breaking compounds used in the expression analysis increased the expression of some stress response genes such as *lsr*, which encodes for virulence. It also increases the expression of *mshb* which encodes for efflux activity, this contradicts its resistant breaking activity. Lac (3X) induces the expression of *vapC* which encodes for toxins in the cell, this effect contradicts its resistant inducing property.

CHAPTER 6

CONCLUSION

In this study, we sought to determine the effect of seventeen compounds in potentiating selected antibiotics against Mycobacterium smegmatis wild-type (WT) and two multi-drug resistant strains, Erythromycin-resistant Mycobacterium smegmatis A & B. Synergistic interaction assays were done between modulating compounds and selected antibiotics to improve the efficacy of the antibiotics. Significant changes were observed with an increase and decrease in the efficacy of some of the antibiotics. Some of the inactive antibiotics got revived in the presence of these modulating compounds. Most of the inactive antibiotics got revived against mutant strains which were interesting. The compounds were grouped into structural and functional triple combinations based on their structure and activity in the initial assay respectively. The functional triple combination proved more robust compared to the structural combination based on the number of classes of antibiotics affected, the number of revived antibiotics, and the quantum of antibiotic change. All seventeen compounds were then also classified into resistant breaking and resistant inducing compounds based on whether they were increasing or decreasing the activity of the antibiotics respectively.

To determine the consistency of the activities observed, phenotypic assays (colony morphology, accumulation, efflux, adhesion, and biofilm) were performed using the single and some selected triple combination compounds. Most of the resistant breaking compounds correlated with the phenotypic assays' contrary to the resistant inducing compounds which did not correlate much with the phenotypic assays. SOR (3X) and MAL (3X) were the top resistant-breaking candidates which led to the activation of fifteen (15) antibiotics and the top two (2) that induced resistance (LAC (3X) and SIA+ADO (1.5X)) led to the loss of efficacy of twelve (12) antibiotics.

Analysis of the expression some stress response genes in the presence of the top two resistant breaking and resistant inducing compounds revealed that some of the resistant-breaking compounds induce the expression of *vapC* genes that responsible for toxicity in the organism, which correlates with its resistant-breaking property. It also increases the expression of virulence genes, that is *lsr* which is contrary to its property. The resistant-inducing compounds did not change much the expression profile of the genes.

FUTURE PERSPECTIVE

In future perspectives, more mechanistic studies can be used to determine the mechanism of action of the top hit compounds. RNA sequencing can also be employed to understand the entire genes affected in the presence of the top hit compounds. The genetic targets can serve as therapeutic targets to screen for new antimicrobial compounds.

Additionally, a ranking of the 17 compounds should be made based on the performance of the compounds in the phenotypic screening alone. Those that show strong phenotypic hallmarks for increasing or decreasing antibiotic resistance can be tested to confirm such effects as well as gene expression. This will be an interesting approach that is alternative to the approach adopted in this study based on direct measurement of changes to antibiotic resistance first.

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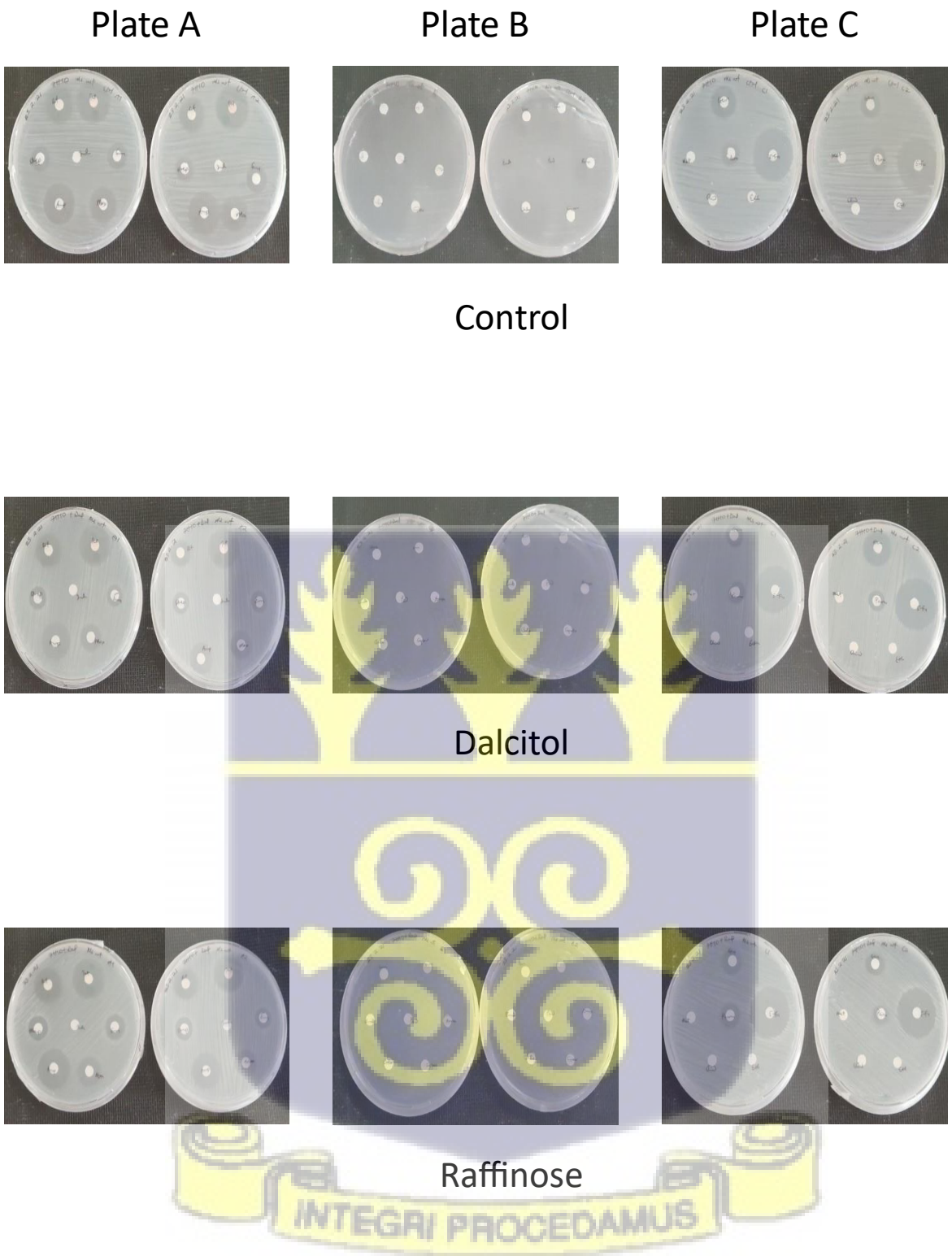
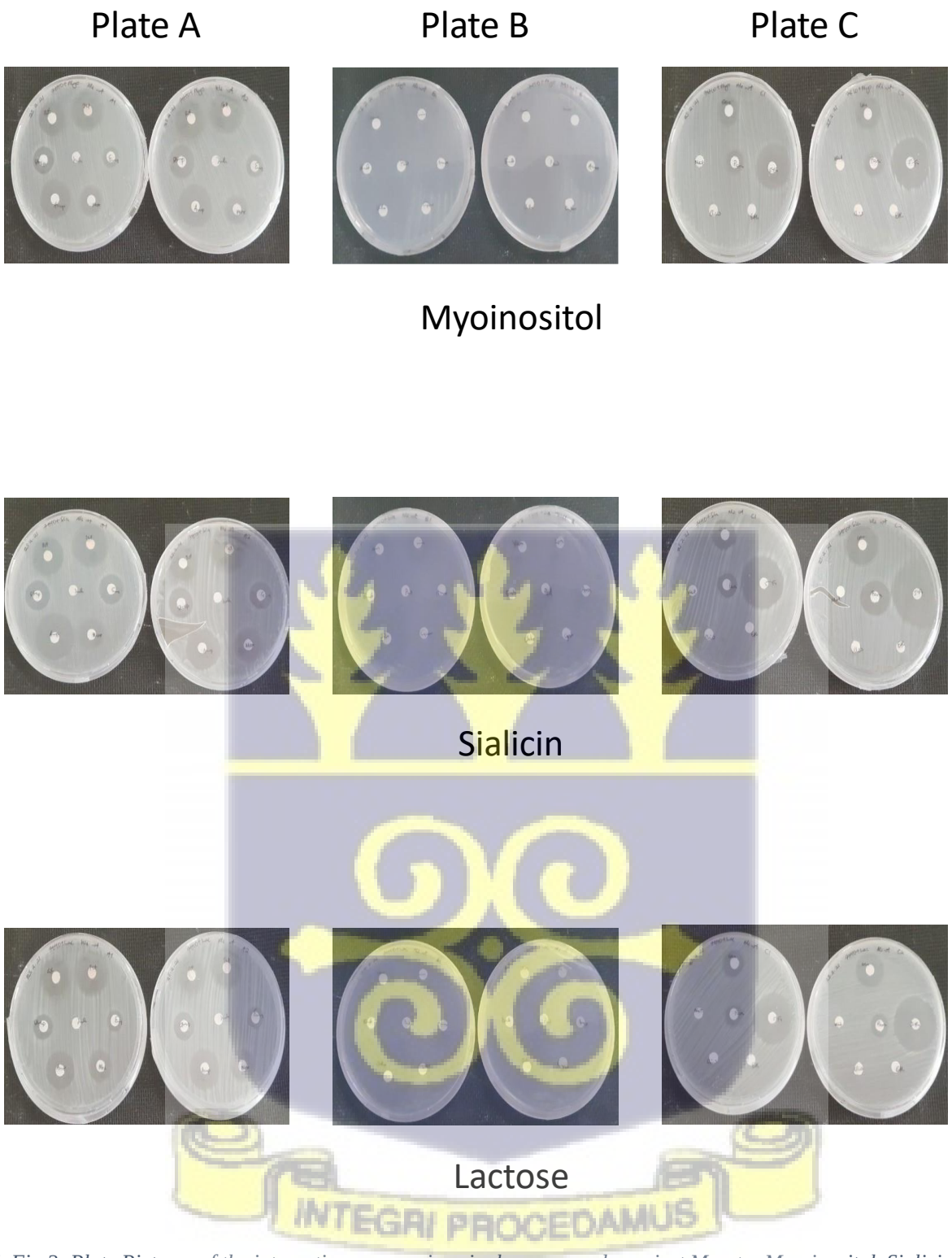
APPENDIX

List of Supplementary Figures

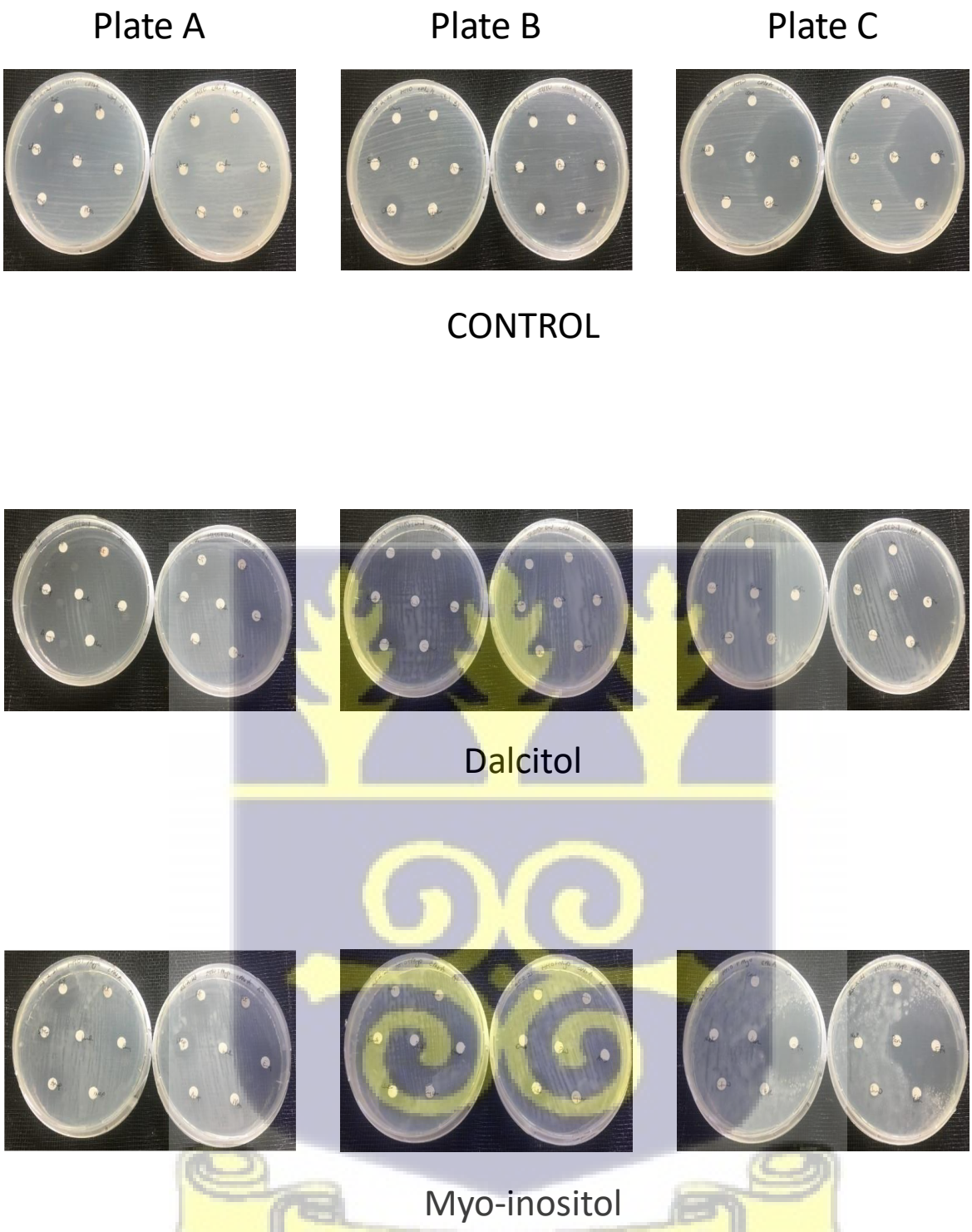
List of Supplementary Tables



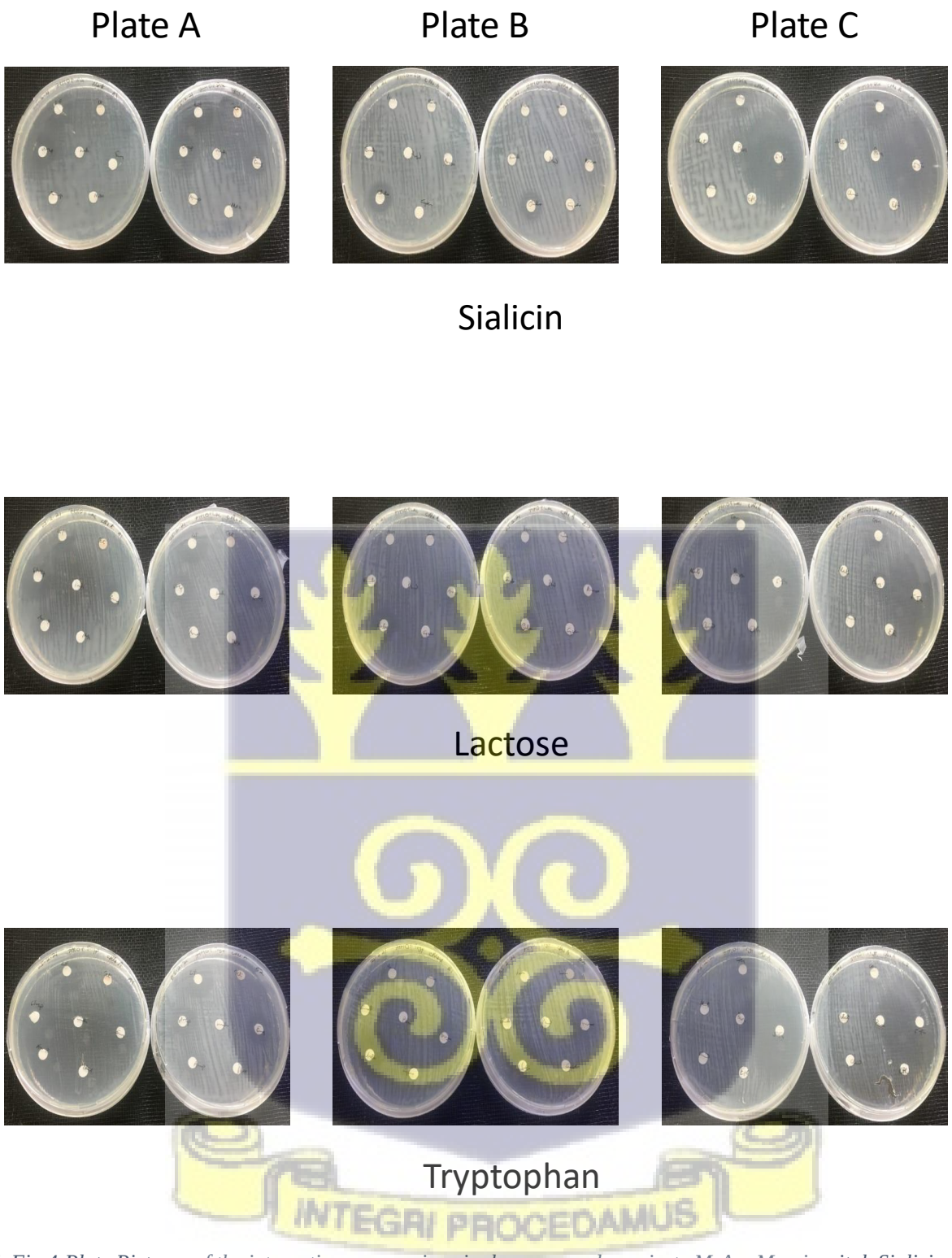
S. Fig 1: Plate Pictures of the interaction assay using single compounds against Ms wt – Control, Dalcitol, and Raffinose



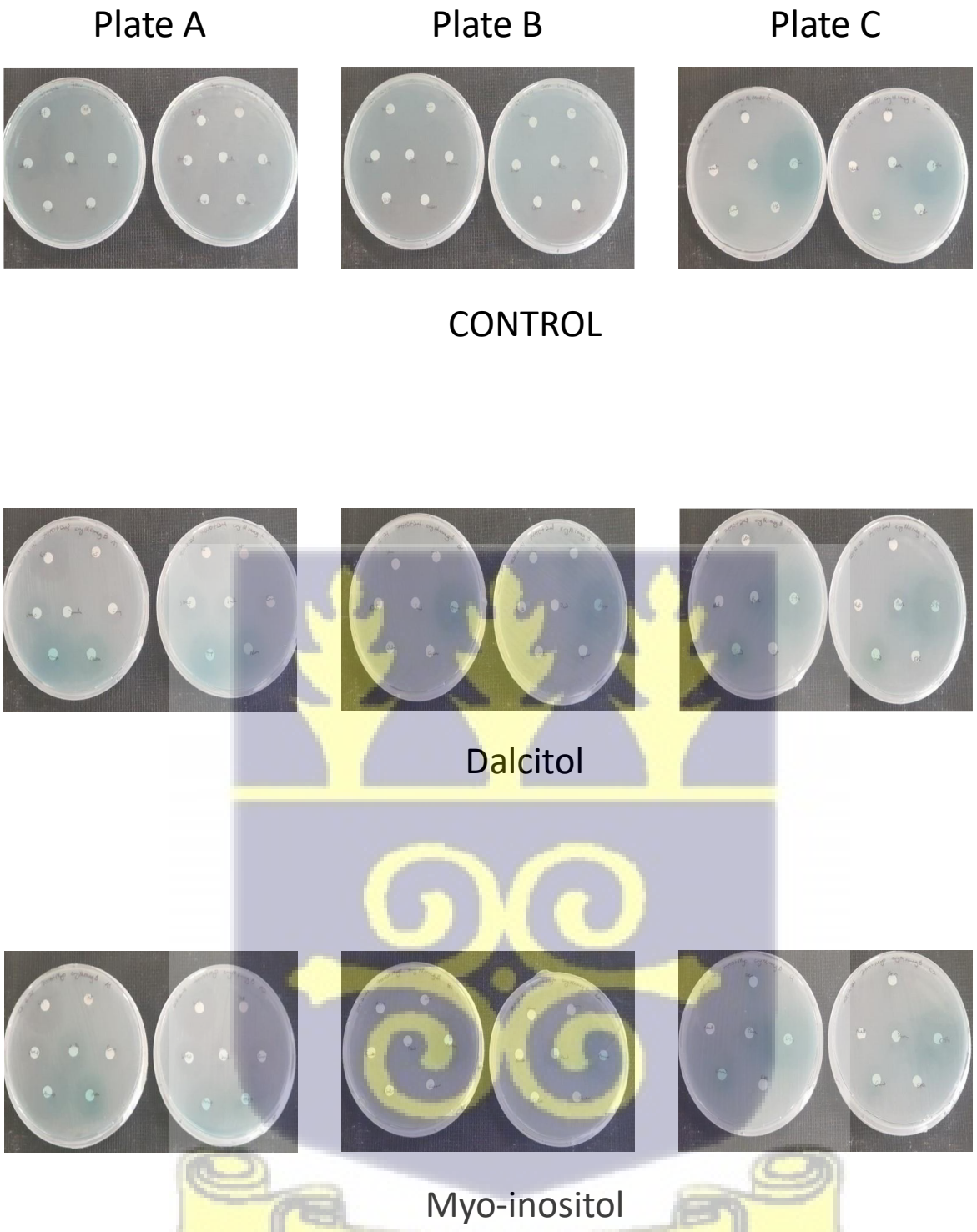
S. Fig 2: Plate Pictures of the interaction assay using single compounds against Ms wt – Myo-inositol, Sialicin, and Lactose



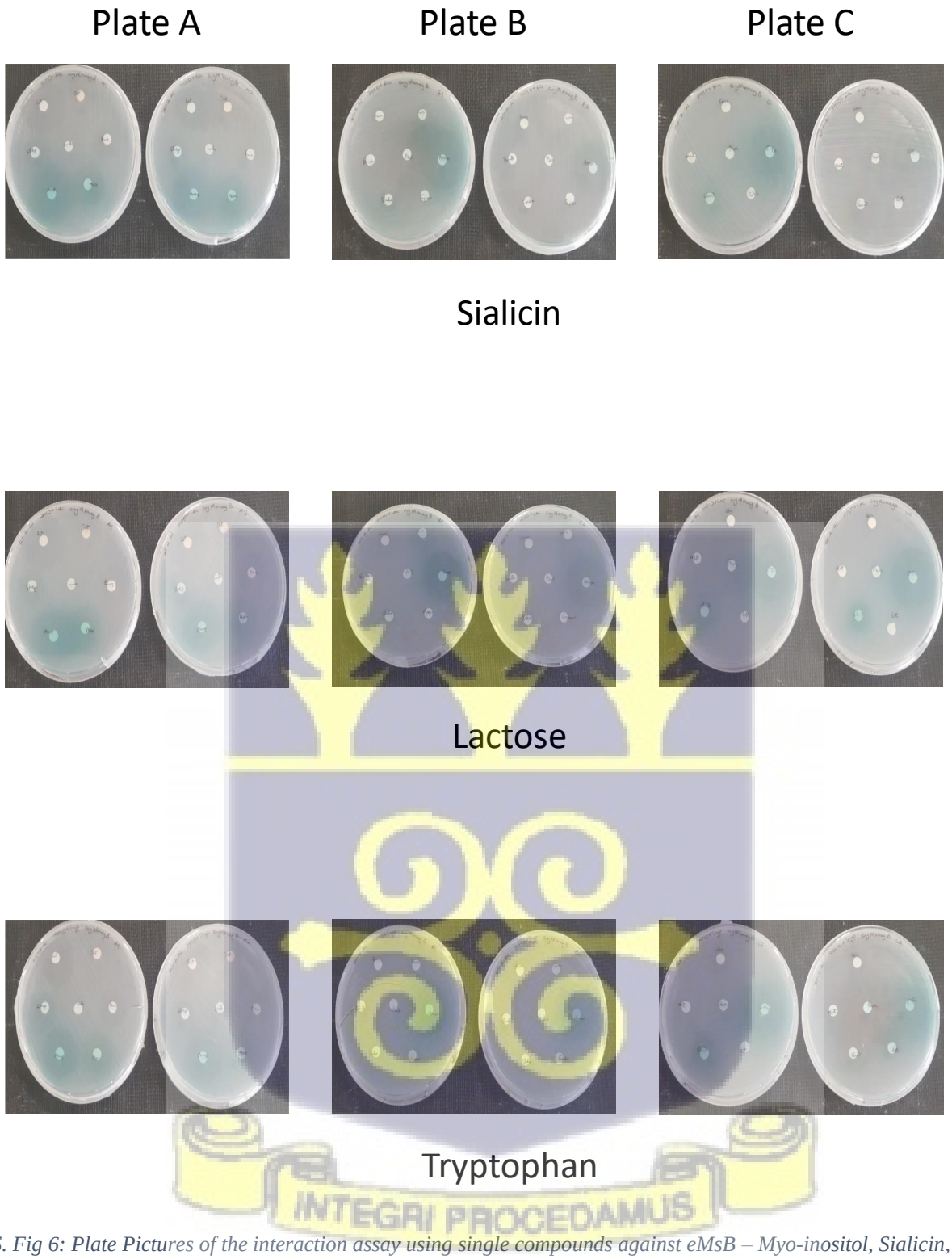
S. Fig 3: Plate Pictures of the interaction assay using single compounds against eMsA– Control, Dalcitol, and Raffinose



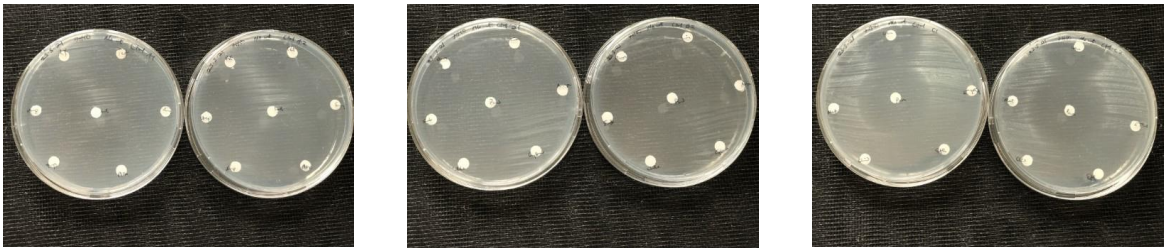
S. Fig 4:Plate Pictures of the interaction assay using single compounds against eMsA – Myo-inositol, Sialicin, and Lactose



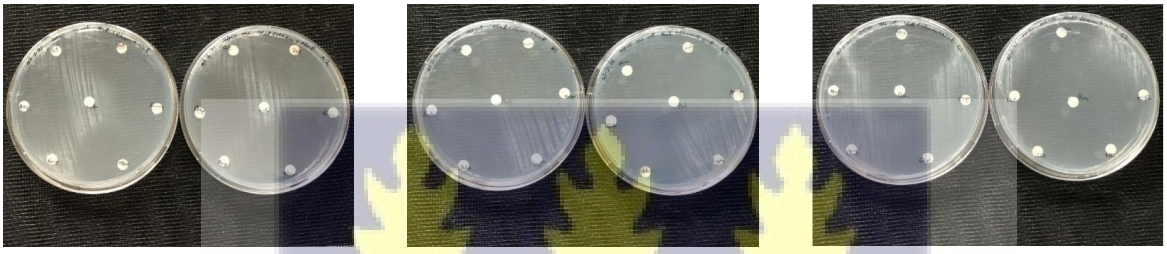
S. Fig 5:Plate Pictures of the interaction assay using single compounds against eMsB – Control, Dalcitol, and Raffinose



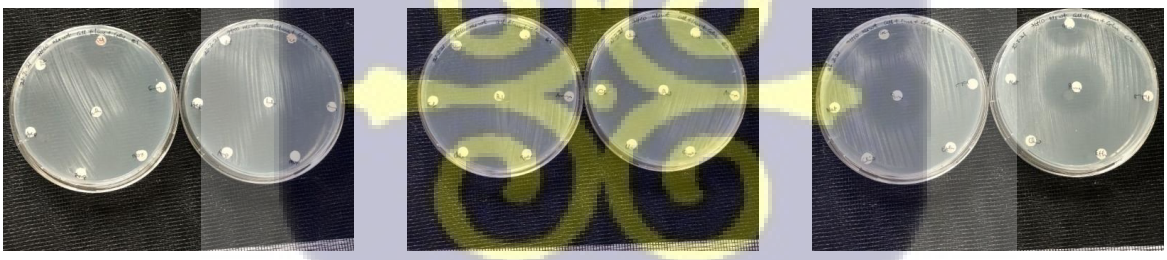
S. Fig 6: Plate Pictures of the interaction assay using single compounds against eMsB – Myo-inositol, Sialicin, and Lactose



Control

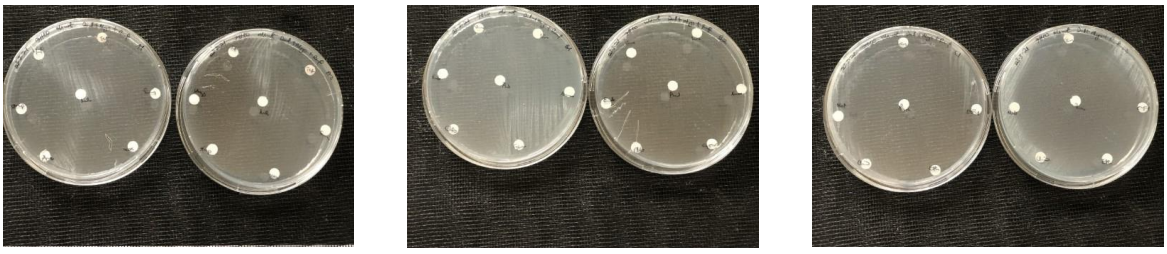


Mal+Rham+NaP

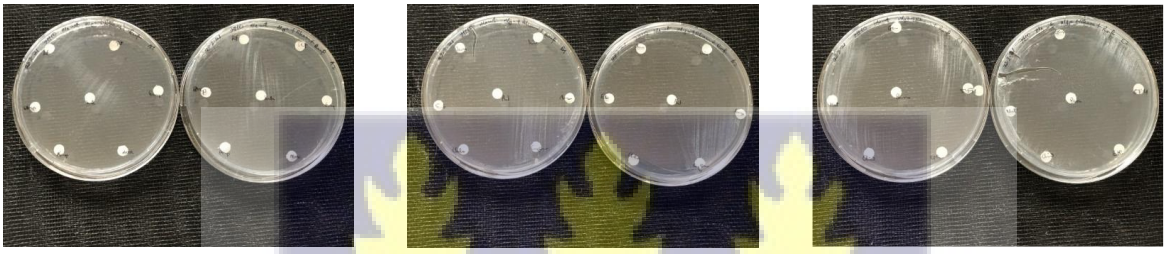


Cell+Inu+Glu

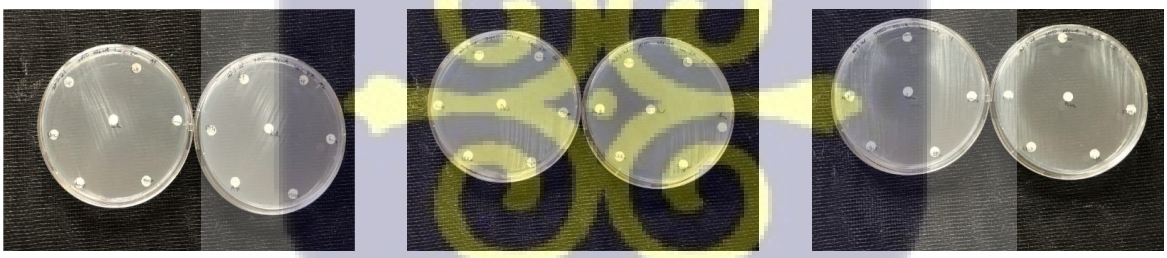
S. Fig 7: Plate Pictures of the interaction assay using triple combinations against Ms wt – Control. Mal+Rham+NaP and Cell+Inu+Glu



Dal+Myo+Raf

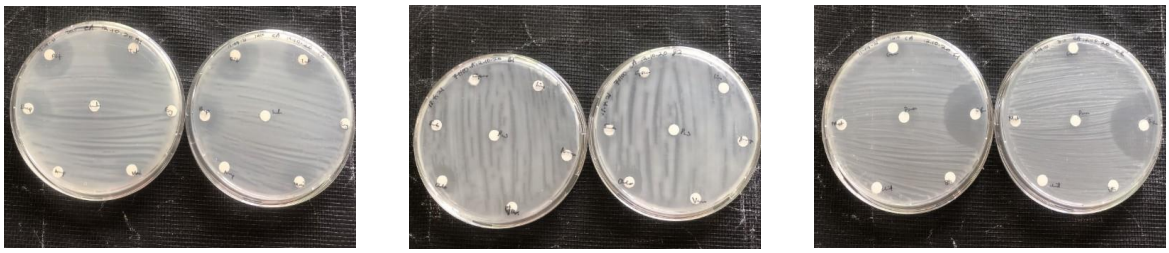


Myo+Rha+Raf

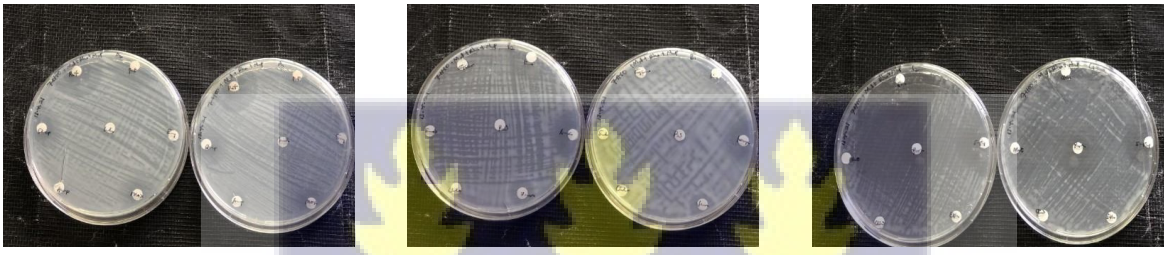


Lac (3x)

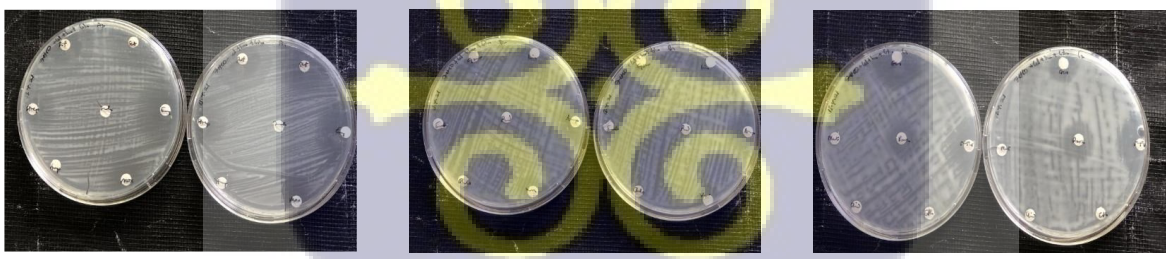
S. Fig 8:Plate Pictures of the interaction assay using triple combinations against Ms wt – Dal+Myo+Raf, Myo+Rha+Raf, and Lac (3X)



Control

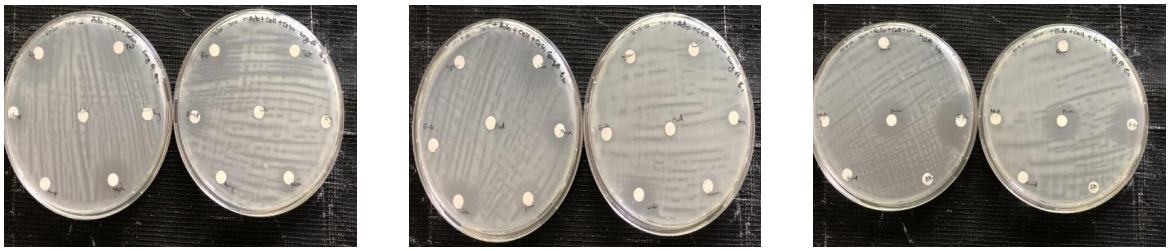


Mal+Rham+NaP

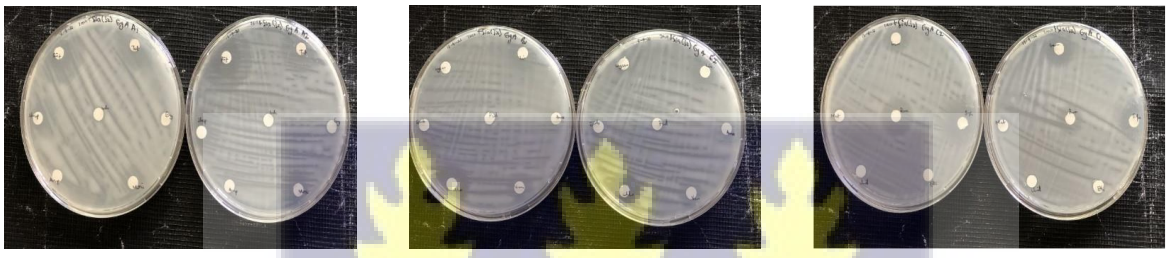


Cell+Inu+Glu

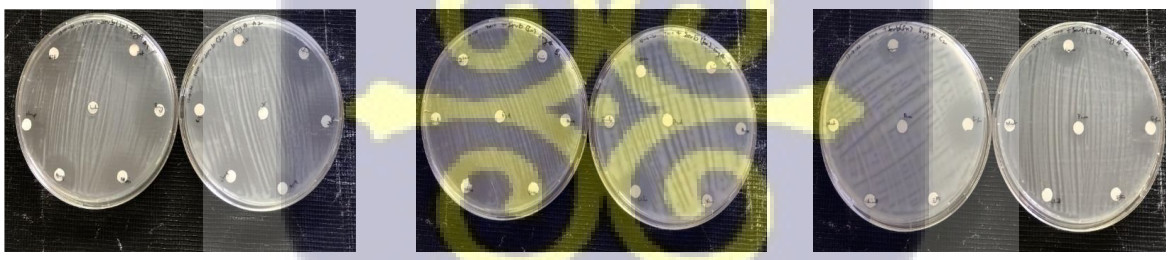
S. Fig 9: Plate Pictures of the interaction assay using triple combinations against eMsA – Control. Mal+Rham+Nap and Cell+Inu+Glu



Ado+Cel+Glu

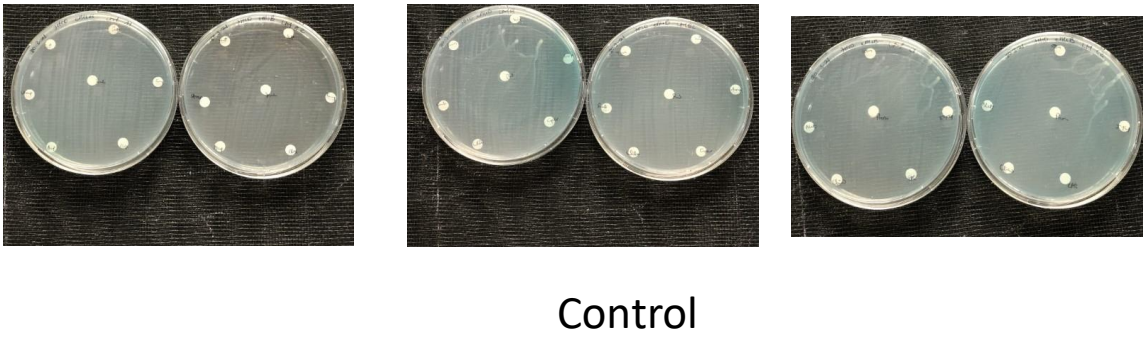


Sia (3X)

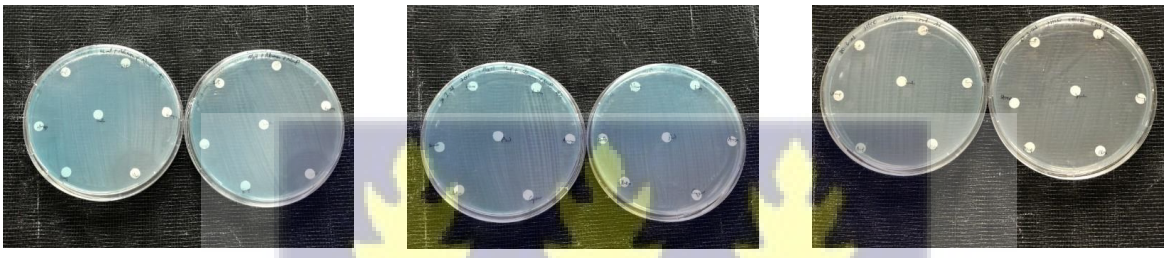


Sorb (3X)

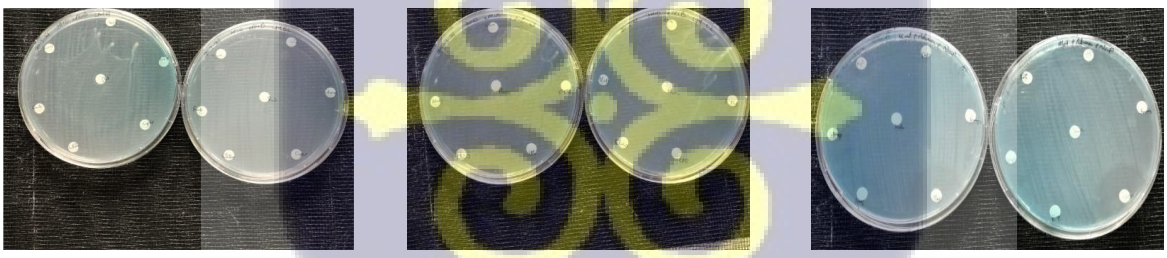
S. Fig 10: Plate Pictures of the interaction assay using triple combinations against eMsA – Ado+Cell+Glu, Sia (3X), and Sorb (3X)



Control

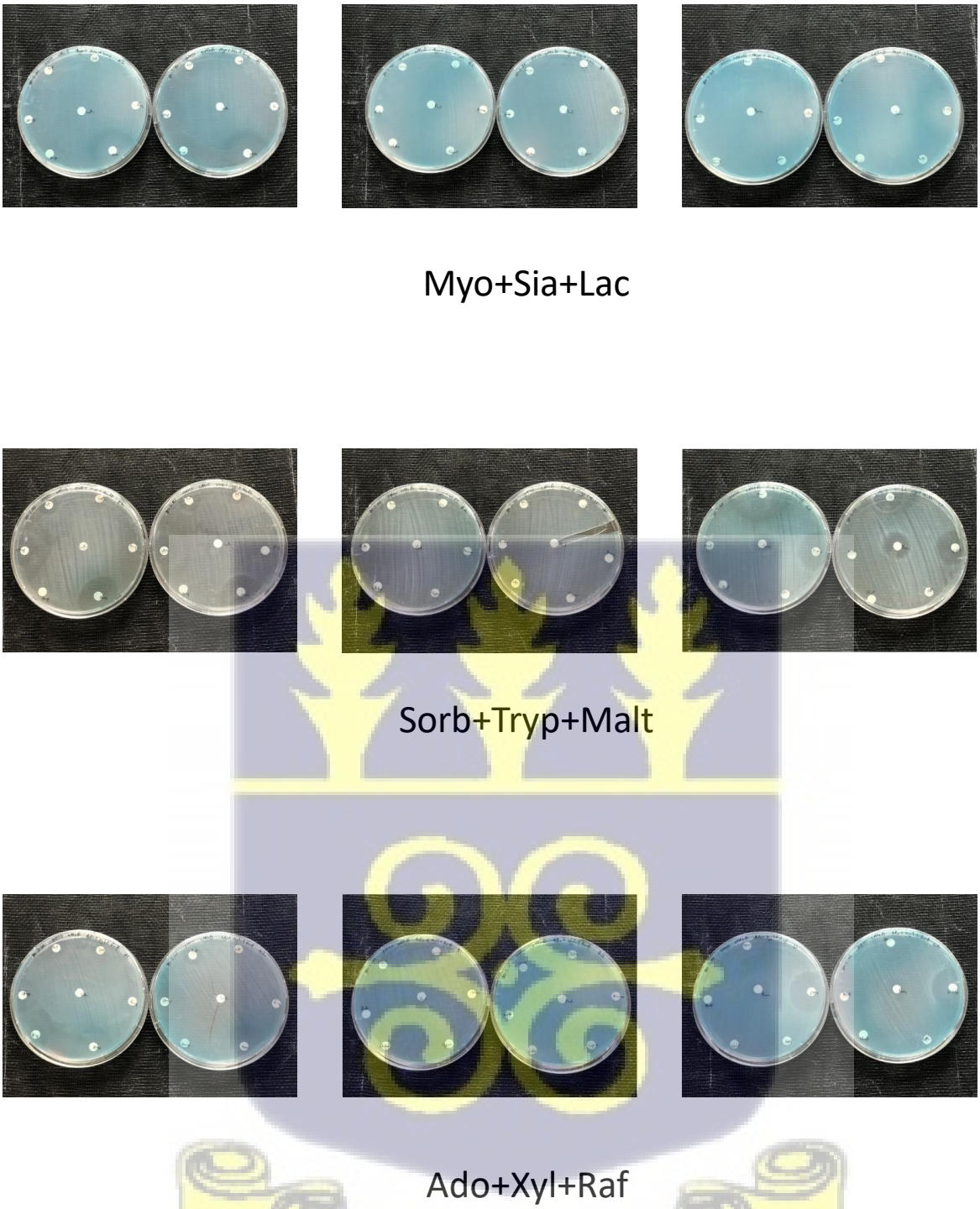


Mal+Rham+NaP



Cell+Inu+Glu

S. Fig 11: Plate Pictures of the interaction assay using triple combinations against eMsB – Control. Mal+Rham+Nap and Cell+Inu+Glu



S. Fig 12: Plate Pictures of the interaction assay using triple combinations against eMsB – Myo+Sia+Lac, Sorb+Tryp+Malt, and Ado+Xyl+Raf

Table S 1: Interaction assay of single compounds and antibiotics against Ms wt:

	Interaction assay of single compounds and antibiotics against Ms wt																	
Antibiotics (ug)	Ctrl	Dalcitol	Myo-inositol	Sialicin	Lactose	Sorbose	Tryptophane	Malonic Acid	Rhamnose	Sodium Pyruvate	Maltose	Adonitol	Xylose	Raffinose	Cellobiose	Inulin	Gluconic Acid	Galactose
Amp 40	20	17	22	23	19	17	19	18	20	20	18	24	22	20	19	19	20	19
Amx 40	27	14	27	12	13	25	15	11	13	11	11	14	15	15	26	14	27	25
Van 40	21	21	20	17	18	13	16	13	17	17	11	20	15	18	29	13	19	15
INH 10	14	12	0	0	8	0	0	16	10	11	0	0	0	9	0	11	13	12
Emb 10	43	40	46	40	43	42	43	44	45	40	33	48	41	39	46	40	44	43
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	19	14	14	17	15	13	16	13	15	15	14	17	12	14	14	14	12	14
Rif 10	17	16	20	20	19	17	17	17	18	19	16	18	19	18	17	16	15	17
Lin 5	23	18	25	15	16	20	15	16	17	15	12	16	16	16	30	15	21	15
Tet 20	20	21	20	20	21	19	18	19	18	18	15	19	19	21	18	18	14	19
Chlo 40	32	26	30	28	29	30	25	27	0	29	21	30	31	30	31	14	34	35
Ery 40	13	11	11	28	10	10	12	11	13	14	10	30	31	11	11	10	34	35
Strep 30	16	11	16	15	13	12	12	11	13	14	11	15	15	12	12	11	11	11
Cyser 20	0	0	0	0	0	0	0	0	8	0	0	0	0	0	9	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	15	16	16	18	16	12	16	13	13	13	13	14	15	12	20	13	13	10
Para 20	12	11	14	16	11	10	16	13	10	12	10	12	13	12	12	11	11	10
5-fu 1	26	26	24	30	27	23	30	13	27	26	28	26	27	26	24	24	25	25
Clind 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S 2: Interaction assay of single compounds and antibiotics against eMsA

	Interaction assay of single compounds and antibiotics against eMsA																	
Antibiotics (ug)	Ctrl	Dalcitol	Myo-inositol	Sialicin	Lactose	Sorbose	Tryptophane	Malonic Acid	Rhamnose	Sodium Pyruvate	Maltose	Adonitol	Xylose	Raffinose	Cellobiose	Inulin	Gluconic Acid	Galactose
Amp 40	0	0	12	0	0	12	0	14	10	0	0	0	13	0	13	0	10	0
Amx 40	0	0	15	0	15	0	12	0	0	12	0	0	0	0	14	0	0	15
Van 40	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	15	0	0
INH 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Emb 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rif 10	21	19	21	19	0	17	19	18	18	18	23	21	19	18	21	20	21	19
Lin 5	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0
Tet 20	0	0	0	0	0	0	0	0	0	0	20	0	0	11	0	0	0	7
Chlo 40	18	19	17	15	16	16	21	18	15	17	16	15	16	17	16	15	15	23
Ery 40	0	10	0	0	11	0	12	0	0	11	13	0	0	0	14	0	0	15
Strep 30	19	19	14	25	18	21	18	19	14	27	23	13	17	25	15	19	15	15
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	0	8	14	23	15	20	15	15	12	20	22	0	14	12	10	16	0	10
Para 20	12	10	14	15	12	0	11	12	13	10	0	12	13	15	10	15	18	15
5-fu 1	49	39	42	51	47	32	46	43	58	42	47	44	45	48	51	48	53	44
Clind 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0	11	0	0	11	0	10	0

Table S 3: Interaction assay of single compounds and antibiotics against eMsB

	Interaction assay of single compounds and antibiotics against eMsB																	
Antibiotics (ug)	Control	Dalcitol	Myo-inositol	Sialicin	Lactose	Sorbose	Tryptophane	Malonic Acid	Rhamnose	Sodium Pyruvate	Maltose	Adonitol	Xylose	Raffinose	Cellobiose	Inulin	Gluconic Acid	Galactose
Amp 40	0	15	0	0	14	0	0	0	15	0	16	11	11	15	11	16	14	0
Amx 40	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	13	0	0
Van 40	0	9	0	0	0	0	0	0	10	0	0	0	0	10	0	11	0	0
INH 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Emb 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	12	11	6	15	14	12	15	9	12	14	11	12	11	0	12	13	14	14
Rif 10	13	11	11	12	12	8	5	13	13	11	9	11	10	12	10	13	12	12
Lin 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tet 20	10	10	12	9	14	0	8	0	9	9	8	10	12	13	9	12	0	0
Chlo 40	0	0	0	0	11	0	11	12	13	0	0	13	0	8	11	11	0	0
Ery 40	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Strep 30	15	12	14	16	11	13	7	10	13	14	9	13	13	9	9	11	15	15
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	17	17	15	15	12	13	12	14	15	16	13	16	13	16	14	15	16	15
Para 20	10	10	13	10	9	9	8	9	0	11	0	10	9	10	14	9	11	10
5-fu 1	24	23	11	20	24	22	21	21	20	24	23	23	20	21	20	19	23	24
Clind 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S 4: Interaction assay of functional triple combination compounds and antibiotics against Ms wt (1)

	Interaction assay of functional triple combination compounds and antibiotics against Ms wt (1)											
Antibiotics	Control	Mal+Rham+NaP	Cell+Inu+Glu	Myo+Sia+Lac	Sorb+Tryp+Malt	Ado+Xyl+Raf	Dal+Myo+Raf	Myo+Rham+Raf	Lac 3X	Malt+Raf+Gal	Sorb+Mal+Glu	Lac+Tryp+Mal
Amp 40	9	0	9	0	8	8	9	10	0	10	8	8
Amx 40	15	16	9	16	25	17	0	15	0	17	14	0
Van 40	19	20	15	8	11	17	16	15	8	16	18	18
INH 20	0	0	0	0	0	0	0	0	0	0	0	0
Emb 10	49	43	45	51	44	47	51	46	48	52	46	43
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	27	40	41	38	39	37	37	41	39	46	32	40
Rif 10	8	12	12	9	11	10	13	8	0	8	24	9
Lin 5	39	31	34	42	40	38	34	48	42	38	47	29
Tet 20	21	28	29	36	37	35	39	40	39	37	29	43
Chlo 40	37	31	32	32	37	33	37	42	33	32	34	33
Ery 40	24	32	31	32	37	29	32	48	45	36	25	37
Strep 30	40	33	45	45	48	50	46	49	51	47	34	49
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	31	35	29	36	31	36	48	40	31	38	41	34
Para 20	39	55	36	43	33	52	65	55	44	41	50	39
5-fu 1	11	15	29	8	8	0	8	13	0	0	8	0
Clind 40	0	0	0	0	0	0	0	0	0	0	0	0
Eth 40	27	38	36	33	30	36	26	30	33	33	26	36

Table S 5: Interaction assay of functional triple combination compounds and antibiotics against Ms wt (2)

	Interaction assay of functional triple combination compounds and antibiotics against Ms wt (2)											
Antibiotics	Ado+Raf+Cello	Rham+Inu (1.5X)	Lac+Tryp+NaP	Xyl+Glu (1.5X)	Sia+Ado (1.5X)	Malt+Cel (1.5X)	Glu (3X)	Sia+NaP+Raf	Tryp+Malt+Raf	Sorb+Rham+Malt	Myo+Mal (1.5X)	Myo (3X)
Amp 40	10	0	0	0	0	0	0	8	9	0	0	9
Amx 40	0	0	20	0	0	12	0	12	28	13	10	11
Van 40	14	16	12	10	11	13	14	13	0	13	13	13
INH 20	0	0	0	0	0	0	0	0	0	0	0	0
Emb 10	52	52	48	49	47	49	51	46	52	53	50	47
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	44	37	37	31	29	30	42	30	38	38	38	32
Rif 10	9	15	0	0	0	11	13	8	0	8	9	11
Lin 5	30	29	26	24	22	27	36	30	36	38	26	34
Tet 20	40	34	34	29	29	30	32	34	30	42	37	39
Chlo 40	38	33	32	32	32	39	30	33	28	31	27	39
Ery 40	42	25	26	31	33	33	29	33	31	35	32	32
Strep 30	49	47	31	39	43	37	44	45	49	41	39	48
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	15	0	15	0	0
Gen 10	30	41	32	28	35	43	37	28	38	33	28	58
Para 20	35	43	49	38	53	40	40	44	48	40	40	50
5-fu 1	8	9	10	10	12	0	11	10	10	0	15	0
Clind 40	0	0	0	0	0	0	0	20	0	18	0	0
Eth 40	30	24	27	30	39	30	30	29	35	26	25	38

Table S 6: Interaction assay of functional triple combination compounds and antibiotics against Ms wt (3)

	Interaction assay of functional triple combination compounds and antibiotics against Ms wt (3)										
Antibiotics	Mal (3X)	Inu (3X)	Ado+Cel+Glu	Sia (3X)	Sorb (3X)	Tryp (3X)	Rham (3X)	Malt (3X)	Xyl (3X)	Raf (3X)	Cello (3X)
Amp 40	12	10	12	0	9	0	10	0	9	0	7
Amx 40	11	11	19	17	15	0	17	0	10	10	18
Van 40	13	13	15	13	10	13	15	13	7	10	15
INH 20	0	0	0	0	0	0	0	0	0	0	0
Emb 10	43	47	51	51	48	48	53	56	45	51	51
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	45	39	35	39	36	41	36	38	37	47	26
Rif 10	12	0	11	11	0	0	0	8	0	0	10
Lin 5	29	39	37	37	37	38	38	36	34	25	37
Tet 20	35	36	29	34	39	38	34	31	32	39	19
Chlo 40	33	33	42	31	39	39	34	31	33	31	38
Ery 40	33	28	31	28	37	31	30	29	27	26	27
Strep 30	47	49	42	47	46	46	50	51	44	45	41
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	11	0	0	0	0	0	0	0	15
Gen 10	30	38	38	33	53	30	42	30	25	34	47
Para 20	30	41	45	36	40	37	43	31	27	37	49
5-fu 1	0	11	0	11	10	0	10	0	0	0	16
Clind 40	0	0	0	0	0	0	0	0	0	0	0
Eth 40	25	31	29	28	28	29	27	28	26	31	21

Table S 7: Interaction assay of functional triple combination compounds and antibiotics against eMsA (1)

	Interaction assay of functional triple combination compounds and antibiotics against eMsA (1)											
Antibiotics	Control	Mal+Rham+NaP	Cell+Inu+Glu	Myo+Sia+Lac	Sorb+Tryp+Malt	Ado+Xyl+Raf	Dal+Myo+Raf	Myo+Rham+Raf	Lac 3X	Malt+Raf+Gal	Sorb+Mal+Glu	Lac+Tryp+Mal
Amp 40	0	0	0	0	0	0	0	0	0	0	0	0
Amx 20	0	17	12	0	0	0	0	12	12	0	14	0
Van 20	0	13	0	13	15	0	0	11	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	16	15	14	21	23	11	15	15	12	19	10	15
Rif 5	24	26	30	26	26	18	31	25	12	25	26	19
Lin 10	0	0	0	0	0	0	0	0	0	0	0	0
Tet 30	19	16	21	12	25	0	0	0	0	21	0	0
Chlo 30	16	0	0	25	26	24	18	18	23	18	0	15
Ery 20	0	0	30	0	15	0	0	0	0	0	0	18
Strep 15	23	20	20	22	13	10	13	11	13	19	17	13
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	16	30	22	13	16	14	19	10	0	0	27	13
Para 20	14	16	15	20	15	16	21	15	16	14	12	0
5-fu 1	32	33	28	22	20	10	25	21	18	26	37	30
Clind 40	0	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	0	0	0	0	0	0	0	0

Table S 8: Interaction assay of functional triple combination compounds and antibiotics against eMsA (2)

	Interaction assay of functional triple combination compounds and antibiotics against eMsA (2)										
Antibiotics	Ado+Raf+Cello	Rham+Inu (1.5X)	Dal (3X)	Lac+Tryp+NaP	Xyl+Glu (1.5X)	Sia+Ado (1.5X)	Malt+Cel (1.5X)	Glu (3X)	Sia+NaP+Raf	Tryp+Malt+Raf	Sorb+Rham+Malt
Amp 40	0	0	0	0	0	0	0	15	0	0	0
Amx 20	0	0	0	0	19	0	14	0	14	0	0
Van 20	13	14	0	0	10	0	0	0	0	0	15
INH 40	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	0	11	15	10	11	16	0	15	14	9	13
Rif 5	22	28	21	29	28	15	16	25	17	20	18
Lin 10	0	0	0	0	0	0	0	0	0	0	0
Tet 30	20	31	0	19	18	0	0	31	0	0	0
Chlo 30	16	17	16	13	0	0	0	20	0	15	15
Ery 20	21	0	0	22	21	0	23	24	18	21	0
Strep 15	12	15	20	19	15	16	15	25	13	13	15
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	16	18	25	10	35	15	32	16	16	0	0
Para 20	11	15	0	10	15	0	0	16	13	15	11
5-fu 1	33	46	33	33	33	33	38	0	24	24	36
Clind 40	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	0	0	0	0	0	0	0

Table S 9: Interaction assay of functional triple combination compounds and antibiotics against eMsA (3)

	Interaction assay of functional triple combination compounds and antibiotics against eMsA (3)												
Antibiotics	Myo+Mal (1.5X)	Myo (3X)	Mal (3X)	Inu (3X)	Ado+Cel+Glu	Sia (3X)	Sorb (3X)	Tryp (3X)	Rham (3X)	Malt (3X)	Xyl (3X)	Raf (3X)	Cello (3X)
Amp 40	0	0	0	0	0	0	14	0	12	0	0	0	9
Amx 20	0	0	0	0	0	0	13	0	11	13	0	0	0
Van 20	0	0	16	0	0	11	0	0	0	0	12	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	13	15	12	16	16	10	21	25	21	15	13	17	0
Rif 5	19	20	24	0	19	28	21	12	32	23	29	19	16
Lin 10	0	0	0	0	0	15	0	0	0	0	0	0	0
Tet 30	0	17	21	21	0	20	19	0	22	30	15	0	0
Chlo 30	14	14	20	20	16	18	20	16	24	21	20	15	20
Ery 20	0	20	0	0	0	0	25	0	0	0	0	0	0
Strep 15	16	22	15	21	12	14	14	21	20	16	13	14	18
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	9	21	13	16	9	12	15	17	17	10	11	22	18
Para 20	10	20	12	20	17	18	0	12	15	15	15	14	14
5-fu 1	9	0	36	27	22	14	21	22	26	22	19	13	25
Clind 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S 10: Interaction assay of functional triple combination compounds and antibiotics against eMsB (1)

Interaction assay of functional triple combination compounds and antibiotics against eMsB (1)												
Antibiotics	Control	Mal+Rham+NaP	Cell+Inu+Glu	Myo+Sia+Lac	Sorb+Tryp+Malt	Ado+Xyl+Raf	Dal+Myo+Raf	Myo+Rham+Raf	Lac 3X	Malt+Raf+Gal	Sorb+Mal+Glu	Lac+Tryp+Mal
Amp 40	0	0	0	0	0	0	0	0	0	0	0	0
Amx 20	0	0	0	0	0	0	0	0	0	0	0	0
Van 20	0	0	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	28	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	24.5	26	22.5	29.5	28	24.5	18	18.5	25.5	18	27.5	20.5
Rif 5	16.5	13.5	17	14.5	14.5	14.5	14	12.5	14.5	15	15.5	13.5
Lin 10	0	0	0	0	0	0	0	0	0	0	0	0
Tet 30	0	0	0	10	13	0	0	0	0	0	0	0
Chlo 30	0	0	0	0	0	0	0	0	0	0	20	12
Ery 20	0	0	0	0	0	0	0	0	0	0	0	0
Strep 15	21.5	25	16.5	25.5	19.5	21.5	17.5	18.5	18.5	0	13.5	21
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0	0
Gen 10	15	24.5	21	27	39	25.5	20.5	25.5	25	26	20	24.5
Para 20	12	12	13.5	13.5	15.5	11.5	17	17	0	0	0	17.5
5-fu 1	28	34.5	25.5	35	26	28.5	24.5	28	34.5	28	30	24
Clind 40	0	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	0	0	0	0	0	0	0	0

Table S 11: Interaction assay of functional triple combination compounds and antibiotics against eMsB (2)

Interaction assay of functional triple combination compounds and antibiotics against eMsB (2)											
Antibiotics	Ado+Raf+Cel	Rham+Inu (1.5X)	Dal (3X)	Lac+Tryp+NaP	Xyl+Glu (1.5X)	Sia+Ado (1.5X)	Malt+Cel (1.5X)	Glu (3X)	Sia+NaP+Raf	Tryp+Malt+Raf	Sorb+Rham+Malt
Amp 40	0	12	8.5	0	0	0	0	0	0	0	0
Amx 20	0	0	0	0	0	0	0	0	0	25.5	0
Van 20	0	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	21	21.5	26.5	25.5	24	26.5	23.5	15	24	26	27
Rif 5	15.5	16.5	18.5	14.5	15	28	17	14.5	14.5	12.5	14
Lin 10	0	0	0	0	0	0	0	0	0	0	0
Tet 30	11.5	0	11.5	12	11.5	12	9.5	0	0	0	0
Chlo 30	0	0	0	0	0	0	16.5	0	0	26.5	0
Ery 20	0	0	0	0	0	0	0	0	0	0	0
Strep 15	25	25	21	24	24	23	15	14	18.5	16	24
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	19.5	34	26	27	21.5	30.5	17.5	37.5	25.5	22.5	22
Para 20	11	15.5	10.5	12.5	14	22.5	18.5	13.5	15	11	0
5-fu 1	21.5	25.5	33	30.5	23.5	29	25.5	16	27.5	35	31
Clind 40	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	0	0	0	0	0	0	0

Table S 12: Interaction assay of functional triple combination compounds and antibiotics against eMsB (3)

Interaction assay of functional triple combination compounds and antibiotics against eMsB (3)													
Antibiotics	Myo+Mal (1.5X)	Myo (3X)	Mal (3X)	Inu (3X)	Ado+Cel+Glu	Sia (3X)	Sorb (3X)	Tryp (3X)	Rham (3X)	Malt (3X)	Xyl (3X)	Raf (3X)	Cello (3X)
Amp 40	11	0	0	0	0	0	0	0	0	0	0	0	0
Amx 20	11.5	0	0	0	0	0	0	13	0	0	0	0	0
Van 20	0	0	18	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	31	28	29.5	28	27	24.5	29	31	27	23	25.5	25.5	21
Rif 5	16	19.5	17	21	12.5	13	16.5	16.5	14	13	17	15.5	13
Lin 10	0	0	0	0	0	0	0	0	0	0	0	0	0
Tet 30	10	0	12	0	9	0	9	0	0	0	0	0	0
Chlo 30	11	13	17	19	0	0	0	15	0	0	0	0	0
Ery 20	0	0	0	0	0	0	0	0	0	0	0	0	0
Strep 15	23	25.5	22	26	13.5	16.5	15	21.5	24	18.5	24	14.5	16
Cyser 20	0	0	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	11	0	0	0	0	0	0	0	0
Gen 10	23	25	19.5	24.5	17	25.5	36	26	16.5	19	17	16	26
Para 20	20.5	10	10	15	13	11	9	9	0	12	16	11.5	11.5
5-fu 1	31	28.5	39	35.5	31	39	37	39	30	28	31	33	28
Clind 40	0	0	0	0	0	0	0	0	0	0	0	0	0
Eth 40	0	0	0	0	11	0	0	0	0	0	0	0	0

Table S 13: Interaction assay of structural triple combination compounds and antibiotics against Ms wt

	Interaction assay of structural triple combination compounds and antibiotics against Ms wt										
Antibiotics	Control	GLU+MAL+MYO	RAF+ADO+RHA	MAL+NaP+GLU	CEL+GLU+TRY	DAL+ADO+TRY	SOR+RHA+XYL	MYO+SIA+TRY	DAL+ADO+GLU	RAF+CEL+INU	TRY+GLU+GAL
Amp 40	20.5	19		24.5	21	22.5	20.5	22	20.5	22	19
Amx 40	25.5	11	18	11	19	12	12	13	13	12	16
Van 40	21.5	17	15.5	13	14	17	20	20	15	11	15
INH 10	0	0	0	0	0	0	0	0	0	0	0
Emb 10	41	43	41	39.5	37.5	40	43	40	44.5	36.5	41
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	26	19	16	15.5	16	14.5	16	14.5	16.5	16.5	17.5
Rif 10	15	16	17.5	21	20	18	15.5	17	19	16.5	19.5
Lin 5	18	13.5	15	13	14.5	14	12	11	11.5	15	12.5
Tet 20	25.5	15.5	13	13.5	18.5	14.5	14.5	14	16	16	18
Chlo 40	25	23	27	25	26	22	28	20	32.5	21	26
Ery 40	22	11	14	14	13	10.5	10.5	12.5	11.5	22	14
Strep 30	27	20	17.5	20	15	22	21	12.5	16.5	23.5	17
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	15.5	12	14	12.5	11.5	11.5	14.5	13	17	10.5	13
Para 20	13.5	11	10.5	9.5	9.5	9.5	10.5	11	11	9.5	12
5-fu 1	29	36	31.5	32.5	32.5	33	32	33.5	37.5	32	32
Clind 10	10.5	0	0	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0

Table S 14: Interaction assay of structural triple combination compounds and antibiotics against eMsA

	Interaction assay of structural triple combination compounds and antibiotics against eMsA										
Antibiotics	Control	GLU+MAL+MYO	RAF+ADO+RHA	MAL+NaP+GLU	CEL+GLU+TRY	DAL+ADO+TRY	SOR+RHA+XYL	MYO+SIA+TRY	DAL+ADO+GLU	RAF+CEL+INU	TRY+GLU+GAL
Amp 40	0	15	0	14	14.5	0	15	0	11	15	17
Amx 40	0	0	0	0	0	0	15	16.5	0	0	0
Van 40	0	0	0	0	0	0	0	0	0	0	0
INH 10	0	0	0	0	0	0	0	0	0	13.5	0
Emb 10	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	19	14	13.5	0	15	12	15.5	16.5	9	20	24
Rif 10	17.5	15	13.5	18	17.5	15	13	13	16	13.5	11
Lin 5	0	0	0	0	0	0	0	0	0	0	0
Tet 20	0	0	0	15	10	0	0	0	0	0	0
Chlo 40	16.5	20	14.5	0	14.5	23.5	16	18.5	14.5	15.5	22.5
Ery 40	0	0	0	0	0	0	0	0	0	0	0
Strep 30	17	20.5	18	17	13.5	17.5	17	13	18	15.5	17
Cyser 20	0	0	0	0	0	0	0	0	12	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	17	22	27.5	17.5	17	20	17	15.5	21.5	20	19.5
Para 20	9	10	0	13	0	0	0	15	8	20	10.5
5-fu 1	43	42.5	35	29	33.5	37	39.5	38	39	36.5	0
Clind 10	0	0	0	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0

Table S 15: Interaction assay of structural triple combination compounds and antibiotics against eMsB

	Interaction assay of structural triple combination compounds and antibiotics against eMsB										
Antibiotics	Control	GLU+MAL+MYO	RAF+ADO+RHA	MAL+NaP+GLU	CEL+GLU+TRY	DAL+ADO+TRY	SOR+RHA+XYL	MYO+SIA+TRY	DAL+ADO+GLU	RAF+CEL+INU	TRY+GLU+GAL
Amp 40		0	0	0	0	0	0	0	0	0	0
Amx 40	0	0	0	0	0	0	0	0	0	0	0
Van 40	0	0	0	0	0	0	0	0	0	0	0
INH 10	0	0	0	0	0	0	0	0	0	0	0
Emb 10	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 0.5	17	11	14.5	18	19	15	13.5	18	14	18	0
Rif 10	28.5	25	21	27.5	29	28	29.5	10.5	9	11	29.5
Lin 5	0	0	0	0	0	0	0	0	0	0	0
Tet 20	0	0	0	0	0	0	0	0	0	0	0
Chlo 40	20	20	15	19	18.5	15	18	12	0	0	19
Ery 40	0	0	0	0	0	0	0	0	0	0	0
Strep 30	11.5	10	11.5	10.5	11	10.5	13.5	10.5	9	11	16.5
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	16.5	20	12	14	12	14	15.5	12	11.5	12	13
Para 20	0	0	0	0	0	9.5	0	0	0	0	0
5-fu 1	41	44	37	40	36	36	38	39	37	39	36.5
Clind 10	0	0	0	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0	0	0	0


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Table S 16: Interaction assay of single compounds and antibiotics against E. coli wt

Interaction assay of single compounds and antibiotics against E. coli wt																		
Antibiotic	Control	Dalcitol	Myo-inositol	Lactose	Inulin	Sorbose	Tryptophane	Malonic Acid	Rhamnose	Sodium Pyruvate	Maltose	Adonitol	Xylose	Raffinose	Sialicin	Cellobiose	Galactose	Gluconic acid
Amp40	29	16	23.5	24	28	23.5	28.5	26	29	27	27	31	25	27	25.5	29.5	26.5	26
Amx 20	19	11	24	23	20	23	21	21	23	22	20	20	22.5	20	23.5	22	23.5	23
Van 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pzd40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	20	20	18.5	19	19	20	19	18	21	18	19	20	21	21	21.5	22	19	19.5
Rif 5	13	9	12.5	13	10	10	11	12	12	8	10	9	10.5	12	11.5	13	13	11
Lin 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tet 30	28	28	23.5	24	21	23	21	23	26	20.5	22	21	23	23	26	24.5	23	21
Chlo 30	27	27	20	22	26	23.5	24	27	25	21	22	25	25	21	25.5	22.5	25	11.5
Ery 20	13	16	13.5	11	14	12.5	9	11	10	10	12	9	13	12	13	12.5	11.5	11.5
Strep 15	22	10	19.5	23	19	20	20.5	19	18	20	19	21	21	22	18	11	19	20
Cyser20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Met30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gen10	12	13	8.5	10	12	8.5	8.5	14	12	11	10	10	10	14	10.5	7	10	8
Para20	18	12	11	12	12	11.5	22	12	11.5	10	12	15	12.5	11	15	14	14.5	12
5-Fu 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clind10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S 17: Interaction assay of structural triple combination compounds and antibiotics against E. coli wt

Interaction assay of structural triple combination compounds and antibiotics against E. coli wt											
Antibiotics	Control	GLU+MAL+MYO	RAF+ADO+RHA	MAL+NaP+GLU	CEL+GLU+TRY	DAL+ADO+TRY	SORB+RHA+XYL	MYO+SIA+TRY	DAL +ADO+GLU	RAF+CEL+INU	TRY+GLU+GAL
Amp 40	28	27	27	29	15	24.5	29	28	31	28	31
Amx 20	22	23	22	23	23	23.5	22	21	26	24	22
Van 20	0	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	19	21	15	20	22.5	19	21	19	22	21	22
Rif 5	12	13	12	15.5	13	11	11	13	13	13	13
Lin 10	0	0	0	0	0	0	0	0	0	0	0
Tet 30	23	22	21	20	23.5	21.5	24	21	26	24	17
Chlo 30	25	23	23	24	27	27	26	26	28	23	27
Ery 20	15	13	7	6	11	11.5	9	11	13	12	11
Strep 15	13	16	12	15	14	14	14	14	16	15	17
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	13	19	13	12.5	17.5	16.5	16	15	16	16	15
Para 20	16	15	14	11	16.5	16	16	16	16	15	15
5-fu 1	0	0	0	0	0	0	0	0	0	0	0
Clind 10	0	0	0	0	0	0	0	0	0	0	0

Table S 18: Interaction assay of structural triple combination compounds and antibiotics against E. coli recB

Interaction assay of structural triple combination compounds and antibiotics against E. coli recB											
Antibiotics	Control	GLU+MAL+MYO	DAL+ADO+GLU	SOR+RHA+XYL	CEL+GLU+TRY	MYO+SIA+TRY	DAL+ADO+TRY	MAL+NaP+GLU	TRY+GLU+GAL	RAF+CEL+INU	RAF+ADO+RHA
Amp 40	26	28	25	22	25	26.5	27	24.5	30	25.5	28.5
Amx 20	22	20.5	19	19	22	21	25	24	26	21	20.5
Van 20	0	0	0	0	0	0	0	0	0	0	0
INH 40	0	0	0	0	0	0	0	0	0	0	0
Emb 50	0	0	0	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0	0	0	0
Moxi 1	20	20	20	22.5	19.5	20	20	18.5	20.5	20	20
Rif 5	11	10	10.5	9.5	11	12	9	10	16	11.5	11.5
Lin 10	0	9	0	0	0	0	0	0	0	0	0
Tet 30	23.5	22.5	20.5	23.5	23	23.5	24	22.5	22.5	22.5	24
Chlo 30	22.5	22	21.5	23.5	24.5	25.5	27	22	24.5	25.5	25
Ery 20	10.5	10	12	11	10.5	13	10.5	11	10	7	10.5
Strep 15	11.5	13	12	12.5	7	8	13.5	12.5	15	14	14
Cyser 20	0	0	0	0	0	0	0	0	0	0	0
Met 30	0	0	0	0	0	0	0	0	0	0	0
Gen 10	13	13	10.5	13.5	13.5	16	14	12	12.5	15	14.5
Para 20	12	13	11	12	15	13.5	14	13	12.5	14	12.5
5-fu 1	0	0	0	0	0	0	0	0	0	0	0
Clind 10	0	0	0	0	0	0	0	0	0	0	0

Table S 19: Interaction assay of single compounds and antibiotics against Candida albicans

Interaction assay of single compounds and antibiotics against *Candida albicans*																		
Antifungal	Control	Dalcitol	Myoinositol	Salicin	Lactose	Tryptophane	Sorbose	Malonic acid	Rhamnose	Sodium pyruvate	Maltose	Adonitol	Xylose	Raffinose	Cellobiose	Inulin	Gluconic acid	Galactose
Cycloheximide	0	14	0	0	17	0	11	0	0	0	0	0	0	10	0	0	0	0
Amphotericin B	10	16	15	11	11	11	12	10	9	9	10	8	9	12	10	12	7	7
Benzoic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5-Fluorouracil	12	13	9	8	16	14	15	14	12	0	0	8	5	16	9	11	0	4
Fluconazole	15	17	19	20	19	0	0	16	13	11	0	11	0	6	10	15	0	20
Sertraline	12	17	11	10	20	13	15	14	12	10	10	12	13	10	14	12	10	9
Griseofulvin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paramomycin	11	12	10	12	15	12	11	9	10	10	8	10	10	8	12	11	8	7

Table S 20: Interaction assay of single compounds and antibiotics against Saccharomyces cerevisiae

Interaction assay of single compounds and antibiotics against *Saccharomyces cerevisiae*																		
Antifungal	Control	Dalcitol	Myoinositol	Salicin	Lactose	Tryptophane	Sorbose	Malonic acid	Rhamnose	Sodium pyruvate	Maltose	Adonitol	Xylose	Raffinose	Cellobiose	Inulin	Gluconic acid	Galactose
Cycloheximide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amphotericin B	9	9	9	8	9	9	10	10	8	7	8	7	10	11	11	8	8	7
Benzoic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5-Fluorouracil	12	0	0	0	6	0	9	14	0	0	0	0	6	10	0	0	7	0
Fluconazole	13	18	23	18	18	13	33	16	14	11	19	12	21	20	14	15	14	17
Sertraline	10	9	12	10	6	11	11	14	9	9	10	9	11	11	11	10	11	10
Griseofulvin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paramomycin	11	10	13	11	14	11	13	9	10	11	11	11	13	14	13	8	11	11

Table S 21: Interaction assay of structural triple combination compounds and antibiotics against Candida albicans

Interaction assay of structural triple combination compounds and antibiotics against Candida albicans											
Antifungals	Control	Ref+Cel+Inu	Dul+Ado+Glu	Sor+Rha+Xyl	Raf+Ado+Rha	Cel+Glu+Try	Glu+Mal+Myo	Try+Glu+Gal	Mal+NaP+Glu	Myo+Sia+Try	Dul+Ado+Try
CyH (20)	0	0	0	0	0	0	0	0	0	0	0
AmpB (10)	11.5	13.5	14	13.5	12	14.5	14	11	15	13	11.5
Ben (10)	0	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	12	14.5	15	12.5	15.5	14.5	11.5	19	19	22.5	9
Flu (10)	23.5	36	36	44	39	35	39	36	38	40	34.5
Set (100)	9.5	11	11	10.5	8	11.5	14.5	11.5	11.5	10	8.5
Para (20)	0	0	0	0	0	0	0	0	0	0	0

Table S 22: Interaction assay of structural triple combination compounds and antibiotics against Saccharomyces cerevisiae

Interaction assay of structural triple combination compounds and antibiotics against *Saccharomyces cerevisiae*											
Antifungals	Control	Ref+Cel+Inu	Dul+Ado+Glu	Sor+Rha+Xyl	Raf+Ado+Rha	Cel+Glu+Try	Glu+Mal+Myo	Try+Glu+Gal	Mal+NaP+Glu	Myo+Sia+Try	Dul+Ado+Try
CyH (20)	0	0	0	0	0	0	0	0	0	0	0
AmpB (10)	9.5	13	12	11.5	14	11	13	9.5	12.5	11	10.5
Ben (10)	0	8	7.5	7	8	0	7.5	0	7	8	0
5-Fu (1)	13.5	12.5	12	14.5	11.5	13	14	9.5	13.5	14.5	13
Flu (10)	20	44	37	44	41	39	37	37	36	38	36
Set (100)	7	9.5	0	7.5	11.5	0	0	7.5	0	0	0
Para (20)	0	0	0	0	0	0	0	0	0	0	0

Table S 23: Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (1)

Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (1)												
Antifungals	Control	Mal+NaP+ Rham	Cell+Inu+Glu	Sorb+Tryp+Malt	Ado+Xyl+Raf	Dal+Myo+Raf	Myo+Rham+Raf	Lac 3X	Malt+Raf+Gal	Sorb+Mal+Glu	Lac+Tryp+Mal	Ado+Raf+Cell
CyH (20)	0	0	0	0	0	0	0	0	0	0	0	0
AmpB (10)	10	9.5	13	9.5	11	10	9	10	12	11	13	9.5
Ben (10)	0	0	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0	0	0
Flu (10)	22.5	17.5	18.5	19.5	23	15.5	22	23	23.5	27.5	25	24.5
Set (100)	10	10.5	19	15	20	22.5	10	7.5	11.5	15.5	13	11
Para (20)	0	0	0	0	0	0	0	0	0	10.5	0	0

Table S 24: Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (2)

Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (2)										
Antifungals	Control	Rham+Inu 1.5X	Dal 3X	Lac+Tryp+NaP	Xyl+Glu 1.5X	Sia+Ado 1.5X	Malt+ Cell 1.5X	Glu 3X	Sia+NaP+Raf	Tryp+Malt+Raf
CyH (20)	0	0	0	0	0	0	0	0	0	0
AmpB (10)	10	9	7	10	12	6.5	9	6.5	11.5	8.5
Ben (10)	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0
Flu (10)	22.5	13.5	12	15.5	15.5	14.5	13	0	21	18.5
Set (100)	10	10.5	0	8	12	9.5	0	10.5	10	11.5
Para (20)	0	0	0	0	0	0	8.5	0	0	0

Table S 25: Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (3)

Interaction assay of functional triple combination compounds and antibiotics against Candida albicans (3)											
Antifungals	Control	Sorb+Rham+Malt	Myo+Mal 1.5X	Inu 3X	Ado+Cell+Glu	Sia 3X	Sorb 3X	Malt 3X	Xyl 3X	Raf 3X	Cell 3X
CyH (20)	0	0	0	0	0	0	0	0	0	0	0
AmpB (10)	10	8	9	7	9	9	10	9	9	11.5	9
Ben (10)	0	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0	0
Flu (10)	22.5	17	18.5	0	13	0	0	16	22	20	21
Set (100)	10	9	0	11	11	8.5	13.5	0	0	0	9
Para (20)	0	0	0	0	0	0	0	9	8	9	0

Table S 26: Interaction assay of functional triple combination compounds and antibiotics against Saccharomyces cerevisiae (1)

Interaction assay of functional triple combination compounds and antibiotics against *Saccharomyces cerevisiae* (1)										
Antifungals	Control	Mal+NaP+ Rha	Cell+Inu+Glu	Myo+Sia+Lac	Sorb+Tryp+Malt	Ado+Xyl+Raf	Myo+Rham+Raf	Lac 3X	Malt+Raf+Gal	Sorb+Mal+Glu
CyH (20)	0	0	0	0	0	0	0	0	11	10
AmpB (10)	8.5	13	10	12	11.5	11	10.5	11	10.5	7
Ben (10)	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0
Flu (10)	16.5	24	11.5	19.5	8.5	19	26.5	24.5	10	7
Set (100)	9	17	16.5	16	18.5	21	13	14.5	15	10
Para (20)	0	0	0	0	0	0	0	0	0	0

Table S 27: Interaction assay of functional triple combination compounds and antibiotics against Saccharomyces cerevisiae (2)

Interaction assay of functional triple combination compounds and antibiotics against *Saccharomyces cerevisiae* (2)											
Antifungals	Control	Lac+Tryp+Mal	Ado+Raf+Cell	Rham+Inu 1.5X	Dal 3X	Lac+Tryp+NaP	Xyl+Glu 1.5X	Sia+Ado 1.5X	Glu 3X	Sia+NaP+Raf	Tryp+Malt+Raf
CyH (20)	0	11	0	0	0	0	0	0	0	0	0
AmpB (10)	8.5	13	12	12	11	11	10.5	11	8	7.5	8.5
Ben (10)	0	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0	0
Flu (10)	16.5	18	0	23	21	22	20	22	0	16.5	16.5
Set (100)	9	17.5	10	17	16.5	17.5	11.5	13.5	10	9.5	10
Para (20)	0	0	0	0	0	0	0	0	0	0	0

Table S 28: Interaction assay of functional triple combination compounds and antibiotics against Saccharomyces cerevisiae (3)

Interaction assay of functional triple combination compounds and antibiotics against *Saccharomyces cerevisiae* (3)											
Antifungals	Control	Sorb+Rham+Malt	Myo+Mal 1.5X	Myo 3X	Ado+Cell+Glu	Mal 3X	Sorb 3X	Malt 3X	Xyl 3X	Raf 3X	Cell 3X
CyH (20)	0	0	0	0	0	0	0	0	0	0	0
AmpB (10)	8.5	10.5	4	11	13	12.5	14	8	8	8	12.5
Ben (10)	0	0	0	0	0	0	0	0	0	0	0
5-Fu (1)	0	0	0	0	0	0	0	0	0	0	0
Flu (10)	16.5	19	20	12	18.5	16.5	11.5	19	14.5	15	17
Set (100)	9	12.5	13.5	15	25	15	25	8.5	8	8	10
Para (20)	0	0	0	0	0	0	0	0	0	0	0

Table S 29: RNA Extraction Table for Ms wt

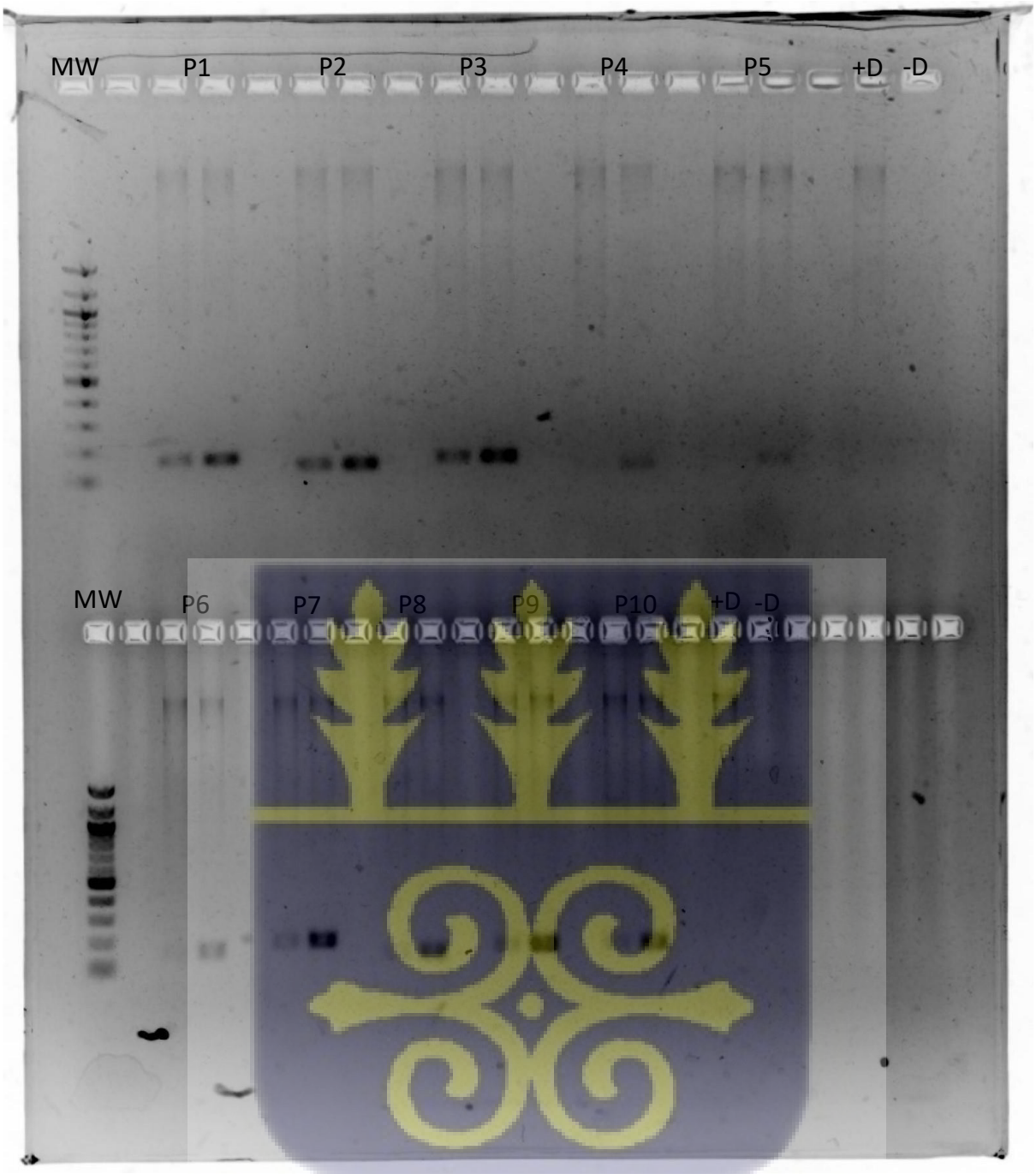
RNA Extraction Table for Ms wt							
Condition	Treatment	Replicates	OD	weight of cells (mg)	RNA yield (ng/ul)	260/280	260/230
Resistant-breaking	Sorb (3x)	A	0.665	6442	103.2	2.07	0.15
		B	0.715	5681	890.7	2.08	0.09
		C	0.708	7233	687	1.59	0.18
	Mal (3x)	A	0.676	9671	114.2	2.15	0.12
		B	0.708	6347	160.3	2.09	0.18
		C	0.614	7818	741.1	1.71	0.59
Resistant-inducing	Lac (3x)	A	0.626	5636	122	2.01	0.14
		B	0.574	6620	131.9	2.11	0.22
		C	0.584	7467	182.3	2.08	0.42
	S+A (1.5X)	A	0.696	7876	110.4	2.03	0.32
		B	0.673	8903	65	2.12	0.15
		C	0.69	5387	2525.4	1.65	0.41
Unchanged	Unchanged	A	0.67	7030	87	2.36	0.17
		B	0.54	6698	303.7	1.97	0.49
		C	0.493	6990	1799.3	1.99	0.2

Table S 30: RNA Extraction Table for eMsA

RNA Extraction Table for eMsA							
Condition	Treatment	Replicates	OD	weight of cells (mg)	mRNA yield (ng/ul)	260/280	260/230
Resistant-breaking	Sorb (3x)	A	1.79	188.6	221.01	1.94	1.01
		B	1.66	327	296.7	1.93	1.81
		C	1.56	335.6	54.3	1.92	0.84
	Mal (3x)	A	1.285	389.1	54.4	2.08	0.48
		B	1.74	368.7	145.7	1.88	0.51
		C	1.87	102.9	150.8	1.92	0.8
Resistant-inducing	Lac (3x)	A	1.56	7.8	103.4	1.9	1.1
		B	2.03	201.3	15.7	2.07	0.23
		C	174	332	94.9	1.89	1.34
	S+A (1.5X)	A	1.66	360.4	598.2	2.07	1.08
		B	1.77	203.2	2264	2.01	1.49
		C	1.08	166.1	472.4	1.99	0.49
Unchanged	Unchanged	A	1.75	269.1	1793.2	2.04	1.22
		B	2.21	302.3	2005	1.65	0.99
		C	1.5	437.3	740.2	1.98	1.4

Table S 31: RNA Extraction Table for eMsB

RNA Extraction Table for eMsB							
Condition	Treatment	Replicates	OD	weight of cells (mg)	mRNA yield (ng/ul)	260/280	260/230
Resistant-breaking	Sorb (3x)	A	1.96	534	18382	1.69	0.92
		B	1.83	502	137.9	1.94	0.41
		C	1.885	464	791.1	1.91	0.28
	Mal (3x)	A	1.285	427	1150	1.9	0.14
		B	1.725	486	924.3	1.93	1.71
		C	1.945	605	2424.1	1.96	0.54
Resistant-inducing	Lac (3x)	A	2.055	634	104.6	1.87	0.19
		B	1.84	547	2242.6	1.79	1
		C	1.78	522	78.4	1.28	0.39
	S+A (1.5X)	A	1.625	358	1065.5	1.9	1.62
		B	1.885	576	2263.4	1.96	1.68
		C	1.485	563	1581	1.92	1.46
Unchanged	Unchanged	A	1.635	429	54.5	1.74	0.12
		B	1.72	506	1227.7	1.96	0.99
		C	1.72	353	1909.6	1.97	1.45



S. Fig 13: PCR Primer Efficiency Control. MW= Ladder, P1=vapB , P2=lsr2 , P3=vapC , P4=mshB , P5=end , P6=uspC , P7=2758 , P8=sigma , P9=acr , P10=relA , +D= only DNA, -D= no DNA