Universidade de Lisboa

Faculdade de Farmácia

Methoxylated flavones from *Citrus sp.* as resistance modifying compounds

Maria Teresa Pena Wemans

Mestrado Integrado em Ciências Farmacêuticas

2019

Universidade de Lisboa
Faculdade de Farmácia

Methoxylated flavones from *Citrus sp.* as resistance modifying compounds

Maria Teresa Pena Wemans

Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

Orientador: Professor Doutor Franz Bucar

Co-Orientador: Doutora Maria José Umbelino Ferreira, Professora Associada com Agregação

2019

This work is part of the Erasmus+ program and was accomplished in the Department of Pharmacognosy, Institute of Pharmaceutical Sciences of University of Graz, Austria, under the guidance of Professor Doctor Franz Bucar (Institute of Pharmaceutical Sciences, University of Graz, Graz, Austria).

Resumo

A Tuberculose permanece uma das principais causas de morte por doença infeciosa, afetando uma em quarto pessoas globalmente. Mais recentemente, a disseminação de novas formas multirresistentes de *Mycobacterium tuberculosis* mostrou poder tornar esta doença numa das piores epidemias do nosso tempo, sendo as opções terapêuticas diminutas e ineficientes. Esta resistência, intrínseca ou adquirida, pode envolver bombas de efluxo de antibióticos, mutações genéticas que evitam a atividade antibiótica ou mudanças na parede celular.

Este trabalho teve como foco a atividade inibitória de bombas de efluxo por certas polimetoxiflavonas , nomeadamente a 3,5,6,7,8,3′,4′-heptametoxiflavona, extraída da casca de *Citrus reticulata* Blanco.

As cascas de *C. reticulata* possuem uma das maiores concentrações de polimetoxiflavonas do género *Citrus*. Usando o aparelho extrator de Soxhlet e diclorometano como solvente, obtiveram-se 6.1g de extrato a partir de 293.9g de casca, originando um total de 0.0137g de 3,5,6,7,8,3′,4′-heptametoxiflavona pura. Depois de purificada através de HPLC semi-prep, em combinação com técnicas de cromatografia em coluna, a estrutura da heptametoxiflavona foi confirmada por NMR.

Foram determinados os valores da Concentração Minima Inibitória (MIC) da 3,5,6,7,8,3′,4′-heptametoxiflavona pura, do extrato de diclorometano de casca de *C. reticulata* e das frações 3 e 6 da primeira coluna cromatográfica realizada, usando-se *Mycobacterium smegmatis* como modelo para *Mycobacterium tuberculosis*. Realizaram-se ainda ensaios de modulação com rifampicina e brometo de etídio com a mesma cultura de *M. smegmatis*.

Três das quatro amostras testadas mostraram atividade moduladora significativa, reduzindo o valor de MIC da rifampicina 4 a 8 vezes. O modulador mais potente foi a fração 3, incrementando a suscetibilidade da cultura de *M. smegmatis* 8 vezes em relação ao brometo de etídio (MF=8). A 3,5,6,7,8,3′,4′-heptametoxiflavona pura foi a segunda amostra mais potente, com um aumento de suscetibilidade de *M. smegmatis* em relação ao brometo de etídio mais baixo do que em relação à rifampicina (MF= 2 e MF=8, respetivamente). Concluímos que esta polimetoxiflavona representa um

potencial muito interessante como ferramenta de otimização da terapêutica anti-tuberculótica com rifampicina.

Abstract

Tuberculosis remains one of the main causes of death due to an infectious disease, affecting one in four people globally. More recently, the spreading of multi-drug resistant *Mycobacterium tuberculosis* strains has threatened to become one of the worst epidemics of our time, with therapeutic options getting slimmer and less effective.

This resistance, acquired or intrinsic, acts through activation of efflux pumps that expel antibiotics, genetic mutations to avoid their activity or changes in cell wall.

This research focused on the efflux pump inhibitory activity of some polimethoxyflavones, mainly 3,5,6,7,8,3′,4′-heptamethoxyflavone, extracted from *Citrus reticulata* Blanco peel.

C. reticulata peels have shown to possess one of the highest counts of olimethoxyflavones of the genus *Citrus*. 6.1g of dichloromethane extract of mandarin orange peel, obtained by Soxhlet apparatus from 293.9g of peel, yielded 0.0137g of pure 3,5,6,7,8,3′,4′-heptamethoxyflavone.

3,5,6,7,8,3′,4′-heptamethoxyflavone was purified successfully through semi-preparative HPLC combined with column-chromatography techniques. The structure was confirmed through NMR.

Minimum inhibitory concentration (MIC) values of the pure 3,5,6,7,8,3′,4′-heptamethoxyflavone, dichloromethane extract and fractions 3 and 6 from the chromatographic fractionation process were determined using *Mycobacterium smegmatis* as a model of *Mycobacterium tuberculosis*. Modulation assays with rifampicin and etydium bromide were performed using the same *M. smegmatis* culture.

Three of the four samples tested had significant modulating activities by reducing the MIC value of rifampicin (MF_{RIF}) 4 to 8-fold. The most potent modulator was the fraction 3, as it increased the susceptibility of *M. smegmatis* towards EtBr and RIF remarkably (MF = 8). Pure 3,5,6,7,8,3′,4′-heptamethoxyflavone was the second most potent sample tested, with an increased susceptibility of *M. smegmatis* to EtBr lower

than that observed towards RIF (MF=2 an MF_{RIF}=8, respectively), showing a very interesting potential to optimize the therapeutic activity of one of the main drugs used in Tuberculosis treatment.

Keywords: *Citrus reticulata* Blanco, Polimethoxyflavones, Efflux pump inhibitors, Tuberculosis

Acknowledgements

Agradeço à minha família e aos meus amigos por me terem aturado nestes meses e nestes anos.

Um agradecimento especial à Rita e à Samantha, minhas companheiras de luta. Obrigada pelos risos e pelos abraços e pelas cusquices que fizeram da minha vida académica uma experiência de amizade inesquecível.

Ich danke Bettina und Emilia, fur das wichtigste Freundschaft in einem fernen Land.

Abbreviations

PMF - polimethoxylated flavones

OMTs - O-methyltransferases

CHS - chalcone synthase

PGE_2 - prostaglandin E_2

N_2O - nitric oxide

HMF - 3,5,6,7,8,3',4' heptamethoxyflavone

TB - Tuberculosis

UN – United Nations

LTBI – Latent Tuberculosis Infected

BCG – *Bacillus* Calmette-Guérin

WHO – World Health Organization

MDR-TB – Multidrug resistant Tuberculosis

XDR-TB – Extensively drug resistant Tuberculosis

EP – Efflux Pump

EPI – Efflux Pump Inhibitor

ABC - ATP-binding cassettes

MFS - Major Facilitator Superfamily

MATE - Multidrug and Toxic Efflux

SMR - Small Multidrug Resistance family

RND - Resistance-Nodulation Division

TLC - Thin Layer Chromatography

LC-PDA-ESI/MS - Liquid Chromatography- Photodiode Array Detection-Electrospray Ionization- Mass Spectrometry

HPLC – High-Performance Liquid Chromatography

DCC - Dry Column Chromatography

MIC - Minimal inhibitory concentration

DMSO - Dimethylsulfoxide

CFU – Colony-Forming Units

CBA – Clindamycin Blood Agar

MHB – Mueller-Hinton Broth

INH - Isoniazid

MTT - 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

RIF - Rifampicin

EtBr - Ethidium bromide

MF – Modulation Factor

Table of Contents

Figure Index

Table Index

Equation Index

Introduction

1. Methoxylated Flavones

a) Chemical Structure and Origins

Flavonoids are a large and chemically diverse family of secondary metabolites. The basic structure of all flavonoids is a 15-carbon phenylpropanoid core (C6-C3-C6 system) (**figure 1**), which is arranged into two aromatic rings (A and B) linked by a heterocyclic pyran ring (C). They can be found in all vascular plants and some mosses, taking the form of different subclasses. Some of the most prominent classes are chalcones, flavonols, flavandiols, anthocyanins, proanthocyanidins or condensed tannins, and flavones. A seventh group is found in some species, the aurones[1].

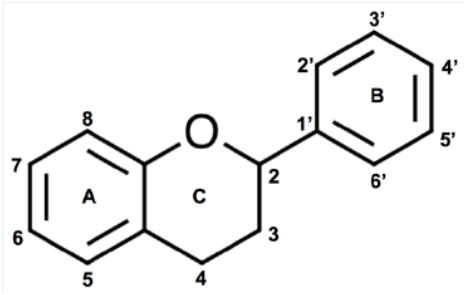

Figure 1: Flavonoid core

Flavones are one of the most interesting classes of flavonoids, in regards to their activity, bioavailability and metabolism in the human body. More than 500 distinct flavone compounds have been identified[2] and this number is expected to rise as more studies on activity and distribution are published. They differ from other flavonoids in having a double bond in C2-C3 and no substitution in the C3 position. However the similarities between flavones and flavanols, they don't share a biosynthetic pathway and both derive from different branches of the flavonoid biosynthetic network[3]. Diversity in the flavone sub-class is achieved by numerous combinations of substituents in the basic structure, which render the new compound different chemical properties and interaction possibilities. Conjugation with saccharides makes the molecule more hydrophilic, i.e. C-glycosylated vitexines and C- and O-glycosylated saponarines[4]. Methylations and prenylations make the flavone more lipophilic and so the polimethoxylated flavones (PMF), the subject of this study, are highly substituted, highly lipophilic molecules.

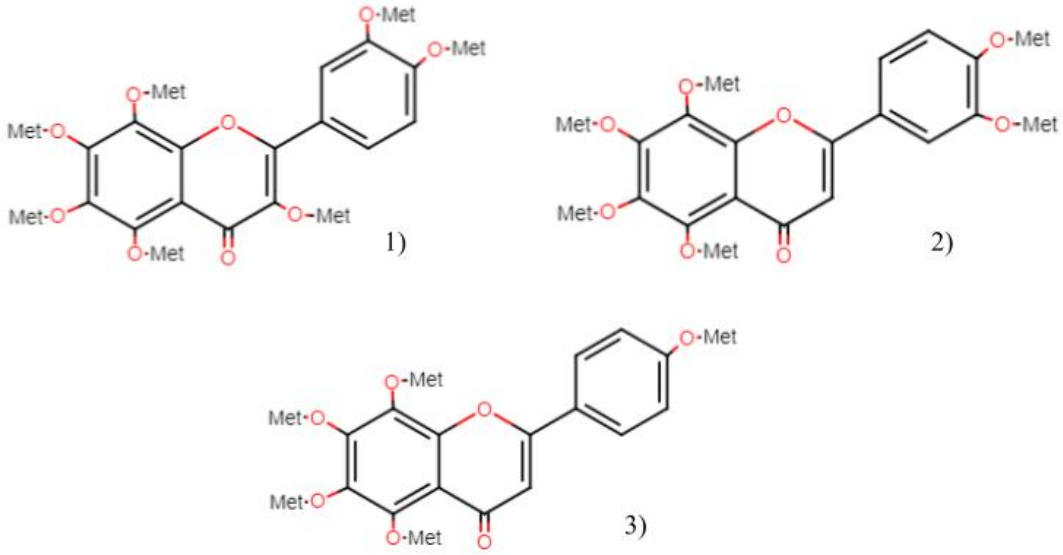

Figure 2: Polimethoxyflavones: **1-** 3,5,6,7,8,3',4' Heptamethoxyflavone, **2-** Nobiletin, **3-** Tangeretin

b) Biosynthesis

In contrast to the activity and metabolism in humans, biosynthesis of flavonoids *in vitro* is one of the most studied among secondary metabolites.

The common diphenyl-propanone (C6-C3-C6) is assembled in the endoplasmic reticulum and the final flavonoid is then transported to different cell compartments[5]. The A ring of the flavone is formed from resorcinol or phloroglucinol, which is synthesized via the acetate pathway and requires 3 x malonyl-CoA as a substract[6], has the characteristic 5' and 7' hydroxylation[7]. The B- and C- rings are formed from p-coumaryol-CoA, which derives from the lengthy studied shikimate and phenylpropanoid pathways. These two structures are condensed by chalcone synthase (CHS) and chalcone isomerase provides the stereospecific cyclization of the molecule, forming the flavanone[8,9].

After the flavanone core is formed, groups of enzymes, mostly hydroxylases, reductases and isomerases, change this structure to form the different flavonoid subclasses[3,8]. Transferases modify the basic flavonoid by adding sugars, methyl or acyl groups, thus changing its physiological activity by altering solubility, reactivity and interactions[10,11]. Some flavones are methylated by O-methyltransferases (OMTs), which transfer methyl groups from S-adenosyl-L-methionine to the hydroxyl groups of flavones, thus creating Polimethoxyflavones[12].

c) Activity

Flavonoids identified in citrus fruits are flavones, flavonols, flavanones, flavans and anthocyanins, only present in grapefruit**.** They are responsible for flower and fruit coloration. 7-O-glycosylflavanones are the most abundant flavonoids in citrus fruits[13], but highly methoxylated flavones present the highest biological activity[14]. Flavanone derivatives are specific for every variety, which renders them useful markers of adulteration in commercial juices[15,16].

The physiological role of mehtoxylated flavones in the peel varies from each species of *Citrus*, ranging from antimicrobial activity, protecting the fruit from various bacterial and fungal infections, to UV protection, being strong absorbents of radiation[17].

The potential biological activity of *Citrus* flavonoids is impressive. They show antioxidant activity, the flavonoids acting as radical scavengers attributed to their hydrogen-donating ability, antimicrobial activity in the form of extract, especially anti-fungal and anti-bacterial[18].

Polimethoxyflavones in *Citrus* peel extracts have shown promising inhibitory ability of the production of pro-inflammatory mediators, prostaglandin E_2 (PGE_2) and nitric oxide (N_2O), especially nobiletin (**2**)[19–21]. They are one of the most promising anti-cancer activity chemical groups, with various works presented on human neuroblastoma SH-SY5Y cells, HL-60 cell lines, human colon and prostate cancer cells, tumor necrosis factor, all showing a consistent inhibitory or anti-proliferative pattern[22–24].

The antimicrobial activity of polimethoxyflavones also ranges from anti-fungal to anti-bacterial. One study attributed nobiletin (**2**), sinensetin (**4**) and heptamethoxyflavone (**1**) from *Citrus reticulata* 100% inhibition of *Penicillium digitatum*, 72% of *Phytophthora citrophthora* and 47% of the *Geotrichum sp.*[25]. Another study, testing nobiletin (**2**) and tangerentin (**3**) against *Pseudomonas aeruginosa* and *P. fluorescens in vitro*, showed destroyed bacterial cell structure and induced plasmolysis after exposure to the polimethoxyflavones, pointing to a mechanism based on interference with the permeability of cell membranes as the most likely antibacterial action[26].

One particular study showed anti-mycobacterial activity of *C. sinensis* extracts against several strains of *Mycobacterium tuberculosis*. Tested against drug sensitive, isoniazid-

resistant and ethambutol-resistant strains, peel extract of *C. sinensis* showed MIC values of 200 µg/mL, 25 µg/mL and 50 µg/mL respectively. Although the MIC values were much higher when compared to the standard four anti-TB drugs, there is no way to dismiss a possible contribution to treatment of TB[27].

Another very interesting activity of polimethoxyflavones is their inhibitory effect on P-glycoprotein, involved in multidrug-resistance and cancer. This is a transmembrane protein that acts in the efflux of therapeutic drugs from the cell, thus constituting a very damaging form of drug-resistance. A study was conducted using an orange juice solution and various polimethoxyflavones, showing that heptamethoxyflavone (**1**) , tangeretin (**3**) and nobiletin (**2**) all increased the steady-state uptake of a substrate of P-gp, [3H]vinblastine, by Caco-2 cells in a concentration-dependent manner[28]. Another study using talinolol, a b-blocker P-gp substract, showed that tangeretin (**3**), 3,5,6,7,8,3',4' heptamethoxyflavone (**1**) and nobiletin (**2**) had significant effects on inhibition of the P-gp[29].

2. Citrus Family

a) Classification

Mandarin orange peels contain one of the highest amounts of polimethoxyflavones in the *Citrus* genus[30], hence why they were chosen as a source for this study.

The *Citrus* genus is a group of trees and shrubs belonging to the *Rutaceae* family and *Aurantioideae* sub-family, characterized by the strong smells. There has been a wide debate over the origins of the *Citrus* genus (genomics, nature) and most recently it has been discovered through genomic research that a common *Citrus* ancestor might have appeared in the late Miocene period, 6 to 8 million years ago[31–33]. Even more debated is the question of which *Citrus* fruits are purest and eldest[34,35]. Nowadays, after analyzing genetic data from most *Citrus* species, it is believed that all of the existent trees and shrubs derive from the cross-breeding, either human or natural, of 4 pure species: *C. reticulata* Blanco, *Citrus maxima* (Burm.) Merr., *Citrus medica* L. *and Citrus japonica* Thunb[36,37].

Citrus reticulata and *Citrus maxima* have the most descendants in terms of species, originating *Citrus sinensis* (L.) Osbeck, *Citrus x aurantium* L., *Citrus clementina*, *Citrus paradisi* Macfad and *Citrus limon* (L.) Osbeck. *Citrus reticulata*, the subject in

this thesis, has one of the highest degrees of relatedness among different cultivars of the plant[38] making it one of the purest species in the *Citrus* genus.

The *Citrus* genus is now widely distributed across the world and is the second largest fruit crop, second only to grape. In the United States alone in 2017, 6.13 million tons of citrus were produced[39]. In 2016, 125 million tons of citrus fruits were produced worldwide, of which 66 million were oranges and 33 million tangerines. 23.5 million tons of citrus were destined for processing, approximately 1/5th of the world production[40,41].

When processing citrus for juices and concentrates, there can be two byproducts that amount to 50-55% of total of the weight of processed fruit[42]: the peel and the molasses. The molasses is created by pressing the peel and separating the liquor from the solid components which is then evaporated until it has about 40.0º Brix (40 g of sugar in 100g of the solution). Due to the concentration and pressing processes, many secondary metabolites produced by citrus fruits are present in high concentration in the molasses[43,44]. Both the peel and the molasses can be sold to originate cattle feed and molasses may also be distilled into citrus alcohol in the beverage industry[45]. The need for industrial scale disposal of these materials makes the case for research and re-utilization even more appealing.

b) *Citrus reticulata* Blanco

C. reticulata is a small tree that with thorns on the trunk and branches, broad or slender lanceolate dark green leaves with rounded teeth and narrowingly-winged petioles. Although usually smaller than *C. sinensis*, dimensions vary with cultivar as does the spread. The flowers are small, white, with 5 petals.

The fruits are oblate and not spherical like *Citrus sinensis*. The peel is thin and loose with very little mesocarp, bright-orange or red-orange when ripe, very easily detached from the interior. It is textured, with indentations all around and with shine. The seeds are small, ovate with a sharp tip and green on the inside.

The species is hermaphrodite and is pollinated by aptomitic or with the help of insects. It can grow and reproduce in very acidic or very basic soil but not in the shade.

It was introduced from Southeast Asia in Europe in the early 1800's and from there to the US in 1850's. It is a symbol of good fortune and abundance in China.

Known cultivars of *C. reticulata* included Clementine, Dancy, Fina, Imperial, Emperor, Nova and Owari[46], but with the development of new varieties, the cultivars can now fall into 3 classes: Class I, Mandarin (Emperor, Changsa, Le-dar, Oneco, Willow-leaf); Class II, Tangerine (Clementine, Dancy, Cleopatra, Sunburst, Ponkan, Robinson); Class III, Satsuma (Wase, Owari, Kara)[47].

c) Composition

Citrus fruits are very rich in secondary metabolites. In fact, ever since the discovery of glucoside derivatives in Citrus[46], the presence of different flavonoids in tissues of different fruits has contributed immensely to the difficult taxonomy of the *Citrus* genus[48], especially in the establishment of species that were seen as only derivatives (*C. aurantium* VS *C. sinensis*, for example). The *Citrus* genus is known for the amounts of carotenoids, flavonoids and pectin it produces. All parts of citrus fruits have been used for extraction of flavonoids, the juice being the easiest way to access the general appearance of flavonoid content in fruits. Juiced *C. reticulata Blanco* from different varieties has shown the highest concentrations of hesperedin (**5**), narirutin (**6**), didymin (**7**) and sinensetin (**4**)[49]. The most prominent secondary metabolite family to appear in the peel of citrus fruits is flavonoids, with great variation as to which flavonoid is present within the family. *C. reticulata* has the highest flavonoid and carotenoid content in the peel[50], as well as o-coumaric acid. Although studies in citrus characterization and isolation of constituents are not leveled in the slightest, one of these counts up to 1,3 kg of polymethoxylated flavones for 1000 kg of 65º Brix orange molasses, including 0,31 kg of nobiletin (**2**), 0,11kg of 3,5,6,7,8,3',4' heptamethoxyflavone (**1**) and 0,04kg of tangeretin (**3**)[51].

d) Variations in polimethoxyflavone levels

The high variation of polimethoxyflavone concentrations within the different tangerine varieties makes the selection of the byproducts and varieties even harder. One study attributes the molasses of Dancy tangerine variety a 10-fold higher amount of PMF compared with the molasses prepared from other two tangerine varieties (Sunburst and Clementine) and orange varieties[30] but the concentration of 3,5,6,7,8,3',4'

heptamethoxyflavone (**1**) is much higher in the Clementine variety than the other two. Choosing a variety for isolation of a certain compound could be very beneficial, but without extended studies on the impact of storage and processing on the yield of polimethoxyflavones, the effort of choosing the right variety has limited results[52].

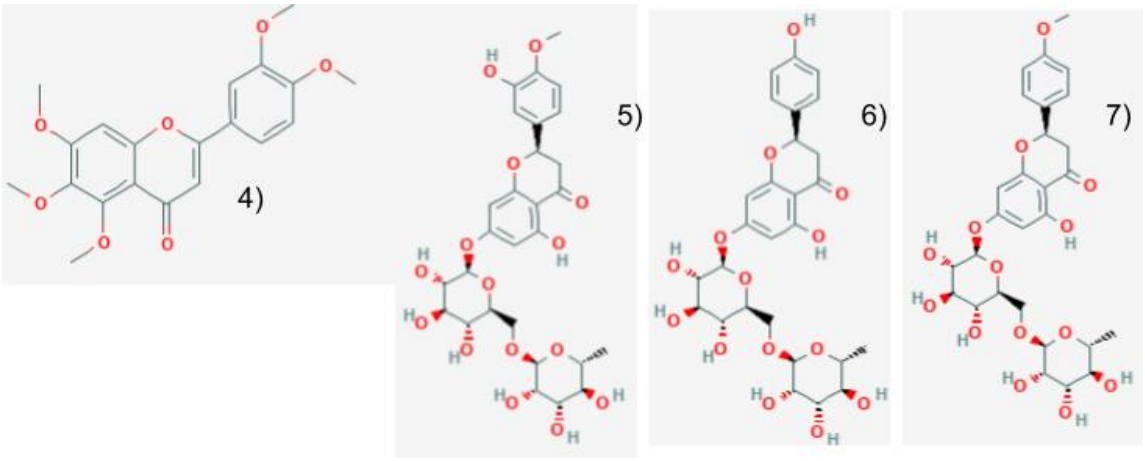

Figure 3: Polimethoxyflavones: **4**– Sinensetin, **5**– Hesperedin, **6**– Narirutin, **7**– Didymin (all structures adapted from PubChem.ncbi.nlm.nih.gov).

The developmental stage of the fruit is also of the highest importance and one which falls even further from the current analysis of *Citrus*, with most studies not even referring to the maturity stage of the material. The maximum concentrations of different polimethoxyflavones vary from 0 to 100 days after anthesis. According to research done with *Citrus Aurantium*, the ideal time to extract nobiletin (**2**), tangeretin (**3**) and sinensetin (**4**) would be the late maturity stages of the fruit (about 70 days), the 3,5,6,7,8,3',4' heptamethoxyflavone (**1**) would be 50 days and the quercetogenin would be 90[13]. Other types of citrus fruits have shown an inversion of these numbers, with 3,5,6,7,8,3',4' heptamethoxyflavone (**1**) and quercetogenin appearing only after a sharp decrease on the levels of the other polimethoxyflavones[53]. This may be related to the possible role of the HMF (**1**) against infection and it has been proven that this particular PMF is present in highest concentrations in the peel of citrus fruits precisely for this reason. However, the later appearance of HMF (**1**) would fall in line with the synthetic route of the polimethoxyflavones, in which nobiletin (2), tangeretin (3) and sinensetin are necessary to the formation of 3,5,6,7,8,3',4' heptamethoxyflavone (1) and quercetogenin.

3. Tuberculosis

a) General

Infection of *Mycobacterium tuberculosis* has been one of the biggest health and safety threats. It is estimated that ¼ of the world population is infected with the bacteria, having come to 1.6 million deaths in 2017, of which 300.000 were HIV positive as well[54]. Recent data has shown that the epidemics of tuberculosis (TB) have slowed its pace, in great part due to the efforts of vaccination and oral therapy and an improvement in diagnostics technology. TB remains associated with poverty and poor socio-economical circumstances, having been made a top priority in the UN member states in the past year[55].

The BCG vaccine is a powerful tool in the combat of child tuberculosis, decreasing infection by 30% and potentially offering some protection through adulthood[56,57]. The neonatal vaccination program has been ongoing in high burden countries for two decades and there is no doubt it has contributed to the attenuation of the epidemic of TB and leprosy. However, recent shortages of supply in high risk areas around the world have shown that a lenience in the programs will bring back high mortality rates in children[58].

Along with the active disease patients, there is a higher, dangerous source of infection in the form of Latent Tuberculosis Infection (LTBI) and highly under-diagnosed incipient and subclinical tuberculosis that can help propagate the infection[59,60]. Adding to this, there has been dissemination of various forms of Multidrug-Resistant TB (MDR-TB), which represents mycobacteria resistant to at least two first-line drugs (Rifampicin and Isoniazid) and Extensively Drug-Resintant TB (XDR-TB), resistant to all of the first-line therapeutic options[61].

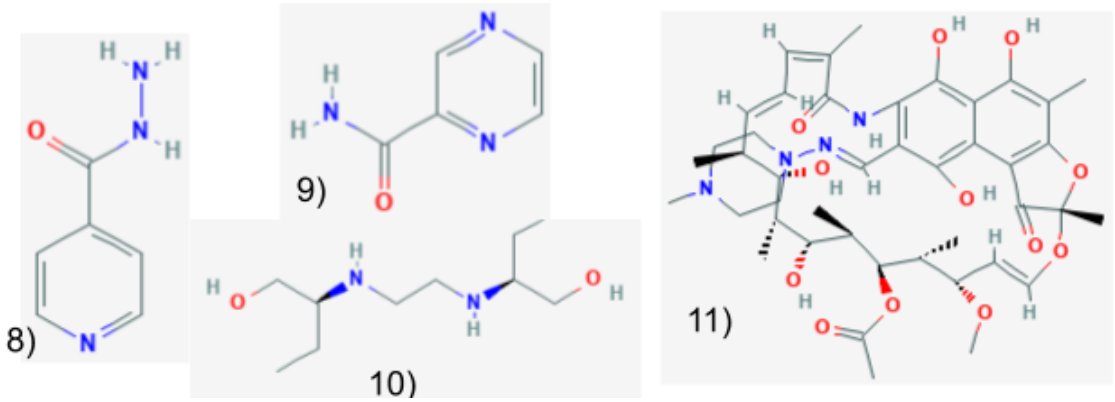

Figure 4: first-line TB treatment: 8- Isoniazid, 9- pyrazinamide, 10- ethambutol, 11- rifampicin (all structures adapted from PubChem.ncbi.nlm.nih.gov).

The lack of therapeutic alternatives, along with accentuated resistances to the four first-line drugs, makes it a top priority to discover and invest in new pharmaceutical technology[62].

b) Mechanisms of drug resistance in *M. tuberculosis*

Intrinsic Resistance - The majority of antibiotics are not successful chemotherapy agents against *M. tuberculosis* mostly due to its intrinsic resistance[63]. The low permeability of the cell envelope of the bacillus makes it very hard for the antibiotic agent to penetrate. Although they are classified as Gram-positive bacteria, the walls of *M. tuberculosis* are very thick and multilayered, thus creating a structure similar to the periplasm of Gram-negative bacteria[64] and a very efficient barrier. *M. tuberculosis* is also naturally resistant to lincosamides and macrolides through alterations in their 50s ribosomal subunit, resistant to fuorquinolones by means of a pentapeptide repeat protein called MfpA that resembles the DNA coil and thus avoiding the antibiotic from attaching to the real DNA strand[65].

One of the main resistance mechanisms present in *M. Tuberculosis* and other mycobacteria is using efflux pumps (EP) to expel the antibiotics that got into the cell. These efflux pumps are mostly used for non-related purposes like transport of nutrients, waste or signalling molecules out of the cell, but can also play a powerful role in resistance to antibiotics. Some transporters respond to transcription regulators activated by antibiotics, thus creating a very specialized form of resistance[66]. They can be classified into five major families present in mycobacteria: ATP-binding cassettes (ABC), Major Facilitator Superfamily (MFS), Multidrug and Toxic Efflux (MATE),

Small Multidrug Resistance family (SMR) and Resistance-Nodulation Division (RND)[67]. These differ in regards to energy usage and the substrate they carry out of the cell. There are approximately 26 efflux pumps indentified in various species of mycobacteria, most of them present in multiple strains of *M. Tuberculosis*, *M. Smegmatis* and *M. Bovis.*

All of the 4 first-line antibiotics used in treatment of TB infections are substrate of more than one efflux pump (see table 1).

Isoniazid	Rv1747[68] (ABC); PstB[69] (ABC); IniBAC[70] (Membrane protein); MmpL7[71] (RND) EfpA[72] (MFS) Rv1258c[73] (MFS - Tap homolog)
Rifampicin	PstB[69] (ABC); P55[74] (MFS); Rv1258c[75] (MFS - Tap homolog);
Pyrazinamide	Rv0191, Rv3756c, Rv3008, and Rv1667c[76]
Ethambutol	DrrA–DrrB[77] (ABC); PstB[78] (ABC); Rv1258c[79] (MFS - Tap homolog)

Table 1: 4 major first-line anti-TB treatment drugs with Efflux Pumps present in *M. Tuberculosis* and respective families.

Acquired Resistance - Acquired resistance appears through chromosomal mutations in response to selective pressure from antibiotic use[80]. These mutations can lead to conformational changes in the antibiotic target, a change in the antibiotic activation mechanism or an increased ability to counter the antibiotic effect.

c) Current Treatment of TB

The treatment scheme at the moment for active tuberculosis infections consists of a 2 month period on a quadruple therapy: rifampicin (**11),** isoniazid (**8**), pyrazinamide (**9**) and ethambutol (**10**); followed by at least four months of a double isoniazid+rifampicin therapeutic scheme. Current WHO guidelines for isoniazid-resistant patients propose using levofloxacin in replacement for 6 months. It is also recommended for concomitant HIV patients to start anti-retroviral therapy as early as possible[81]. The two new drugs bedaquiline ($C_{32}H_{31}BrN_2O_2$) and delamanid ($C_{25}H_{25}F_3N_4O_6$) have shown to be very effective in patients with multidrug-resistant infections and in preventive regimes for LTBI patients in shorter therapy schemes (4 months instead of 6+). Delamanid is the

new drug of choice for children under 6 years old with rifampicin-resistant tuberculosis. Data increasingly shows that these two drugs can be co-administered for optimum therapeutic value without any safety concerns.

Another therapeutic innovation based on repurposed drugs, such as linezolid and clofazimine and third generation fluoroquinolones, has been associated with improved treatment outcomes.

d) Rifampicin-resistance

Rifampicin (**11**) is one of the two main drugs for combating TB. The mechanism of action against M. tuberculosis consists of binding to the β-subunit of the RNA polymerase, inhibiting the elongation of mRNA[82]. In about 96% of M. tuberculosis isolates resistant to rifampicin (**11**), there are mutations in the rpoB gene, which codes for the β-subunit, causing conformational changes and compromising the affinity of the drug[83–85]. This resistance mechanism is not total. A large number of RNA polymerases with various degrees of sensitivity to rifampicin (**11**) have been found[86].

The efflux of rifampicin (**11**) through the various efflux pumps described above is more and more a major resistance factor.

It is proved that these mutations are facilitated when there is a mismanagement of treatment, such as single-drug treatment and inconsistent administration, or transmission of the resistant strains themselves once the resistance is acquired[87].

If rifampicin (**11**) can increase even more its powerful bactericidal potential, there could be a diminished number of resistant strains, given that curing the patient in the first attempted treatment is one of the most effective ways to deal with the rise of resistance due to sub-optimal concentrations.

e) New approaches to rifampicin treatment

New attempts to maximize the effect of rifampicin (**11**) are of major importance to combat TB. These include improving the poor bioavailability of rifampicin (**11**) by means of herbal modulation[88], liposphere carriers for pulmonary or other site-specific effects[89,90].

However, the most promising new target of MDG-TB treatment is efflux-inhibition[91,92]. polimethoxyflavones have a very high potential to inhibit P-gp in other cells. Nobiletin (**2**) has already showed powerful inhibitory activity in *M. smegmatis* culture and other polimethoxyflavones may also be active against the same culture.

f) Mycobacterium smegmatis as a model for M. tuberculosis

M. smegmatis has proved to be a very useful surrogate when investigating *M. tuberculosis* inhibitors *in vitro*, especially for its ease of use, fast growth, and low risk for the investigator performing the tests[93]. However, compared with *Mycobacterium bovis* BCG it is evidentially inferior, with one study showing that 50% of active compounds against *M. tuberculosis* were not detected, compared to 21% with *M. bovis*.In the same study it was determined by genomic comparison that approximately 30% of *M. tuberculosis* proteins lack orthologues in *M. smegmatis* compared to 3% in *M. bovis* BCG[94], which can have a big impact in inhibiting mechanisms.

However all this, *M. smegmatis* has proven to be superior model when it comes to study of efflux pumps. Recent studies have uncovered substrates and new efflux pumps in *M. smegmatis*[95,96], adding to the already existing list of genes encoding putative efflux pumps in its genome, proven this to be a good model for the research of new efflux pump inhibitors (EPI).

However more appropriate BCG models may be, the slow growth and higher risk of contamination favoured the utilization of *M. smegmatis* in this research.

Project goals

This project was executed under the European Comission's Erasmus+ agreement between the University of Lisbon and the Karl-Franzens University in Graz.

The main goal of this project was to extract and purify different polimethoxylated flavones from dried mandarin orange peels – *C. reticulata* - and test their antimicrobial activity by evaluating the Minimum Inhibitory Concentration (MIC) and also their potential to modulate therapeutic activity of rifampicin (**11**) and ethidium bromide by means of a modulation assay using *M. smegmatis* as a model of *M. tuberculosis*.

All experiments were conducted between February and April 2019.

Materials and Methods

1. Plant materials, Reagents and Solvents

Mandarin Orange peels were collected from various sources and from various cultivars available in the Graz region's markets.

Methanol ≥ 99.9% (4627.3), n-hexane ≥ 95%, (3907.3), ethyl acetate ≥ 95% (CP42.6), formic acid ≥98% (4724.1), ethanol ≥ 96% (T171.2), polyethyleneglycol 4000 (0156.1), dichloromethane, toluene, ethyl formiate and Natural Product reagent A (9920.2) were obtained from ROTH®.

For the HPLC, acetonitrile ≥ 99.95% (83639.320, VWR Chemicals), formic acid ≥ 99.95% (56302-1L-GL-F, Fluka® Analytical) and methanol ≥ 99.9% (20864.320, VWR Chemicals) were used.
Deionized water was obtained by PureLab, Prima system.
Ultrapure water was produced by an EASYPure RF, Barnstead, purification system.

2. General Experimental Procedures (TLC, LC-PDA-ESI/MS, HPLC)

a) TLC

The analytical thin layer chromatographies (TLC) were performed on precoated silica gel F254 plates (SiO_2, Merck 1.05554.0001) and visualized under UV light (λ254 nm and λ366 nm). Different mobile phases were used and optimized throughout the procedures: the crude extract, and fractions from the first columns were applied in a toluene:ethyl formiate:formic acid (5:4:1) solution, the fractions from the heptamethoxyflavone (1) and nobiletin isomer separation columns in a hexane:ethylacetate (1:1) and the tangeretin isomer separation column fractions in a hexane:ethylacetate (2:1) solution.

b) LC-PDA-ESI/MS

The liquid chromatography-photodiode array detection-electrospray ionization-mass spectrometry (LC-PDA-ESI/MS) analysis were performed with an UltiMate 3000 system, equipped with an Ultimate 3000RS pump, an Ultimate 3000 RS Diode Array Detector, an Ultimate 3000 RS Auto sampler and an Ultimate 3000 RS Column compartment, interfaced with a Thermo Scientific LTQ XL mass spectrometer, with an

ESI ion source temperature of 300ºC and capillary temperature of 350ºC. A Zorbax SB-C_{18} rapid resolution HD, Agilent Technologies (2.1 X 100 nm, 1.8 µm) column was used. The injection volume was 2 µL for the crude sample and 1 µL for the fraction samples.

For the positive polarity, was used a source voltage of 5.00 kV and a source current of 100.00 µA with a capillary voltage of 35.00 V. For the negative polarity, was used a source voltage of 3.50 kV and a source current of 100.00 µA with a capillary voltage of -17.00 V. Only the crude sample was analyzed through negative polarity. The tube lens voltage was the same for positive and negative polarity (110.00 V). In the ion source nitrogen was used as being the Sheath Gas and Aux Gas respectively with the flow rate of 40.00 and 10.00 arb.

Elution was carried out at a flow rate of 0.250 mL/min using a solvent system gradient of MeCN/ H_20 + 0.1% HCOOH. The system started at 30% MeCN and was linearly increased to 100% MeCN. After 10min, the system was brought back to the 30% MeCN. The temperature of the column was kept at 35ºC and post-column at 25ºC. The autosampler was at 10ºC; the elute was monitored by PDA at 190-500nm detection as well as ESI-MS at 50-2000 Da (full scan) followed by three data-dependent fragmentations of the most intensive precursor ions (MS^2-MS^4).

Data acquisition was performed with Xcalibur™ Software.

c) HPLC

The HPLC analysis were performed using a Dionex UltiMate 3000 system, equipped with an Ultimate 3000RS pump, an Ultimate 3000 RS Diode Array Detector, an Ultimate 3000 RS Auto sampler and an Ultimate 3000 RS Column compartment. The column used was a ZORBAX Eclipse Plus C-18, Rapid Resolution HT 2.1 x 100 mm, 1.8 µm (Agilent; P.N. 959764-902) and data acquisition and interpretation was performed with Chromeleon7™ software.

3. Extraction

The mandarin orange peels were dried and grind beforehand. The extraction was made with a Soxhlet apparatus. Extraction of crude by Soxhlet is considered one of the finest

methods of acquiring heat-resistant bioactive substances from natural solid and dried sources.

a) Soxhlet Method

The extract was obtained using 800 mL of dichloromethane for 154,8g (first extraction) and 139,1g (second extraction) of mandarin orange and the process ran for approximately 6 hours. The sample was tested in TLC against nobiletin, tangeretin, hesperidin and naringenin reference samples.

4. Isolation

Prior to the isolation procedure, a sample from the diclorometh ane extract was applied on an LC-PDA-ESI/MS with a 10mg/mL concentration in acetonitrile, using as a mobile phase a 0,1% solution of formic acid in water and acetonitrile. Nobiletin, tangeretin, hesperidin and naringenin reference samples were also applied.

a) Dry Column Chromatography

The extract was fractionated by dry column chromatography (DCC) using a 25 mm column. An initial 2g of the sample extracted were applied, using 20g of Silica 60 (0.063-0.200 nm). The mobile phase consisted of dichloromethane and ethyl acetate (1:1). 40 fractions were recovered. A second column was initiated after, using 3.2g of the extract and 20g of Silica 60 and another 40 fractions were recovered. The separation was accompanied by TLC analysis of the fractions.

Fractions from columns 1 (fractions 2, 5, 7, 9, 11, 12, 13, 20) and 2 (fractions 8, 11, 16, 20, 23, 28, 33, 40) were chosen based on their TLC profiles and run on LC-PDA-ESI/MS with a 1mg/mL concentration in methanol.

Based on the LC-PDA-ESI/MS results, the fractions from columns 1 and 2 were combined according to the similarities and interest for this research into three samples: one with fractions that contained the heptamethoxyflavone (**1**), a second with the ones that contained an unknown nobiletin isomer and a third with those that contained a tangeretin isomer.

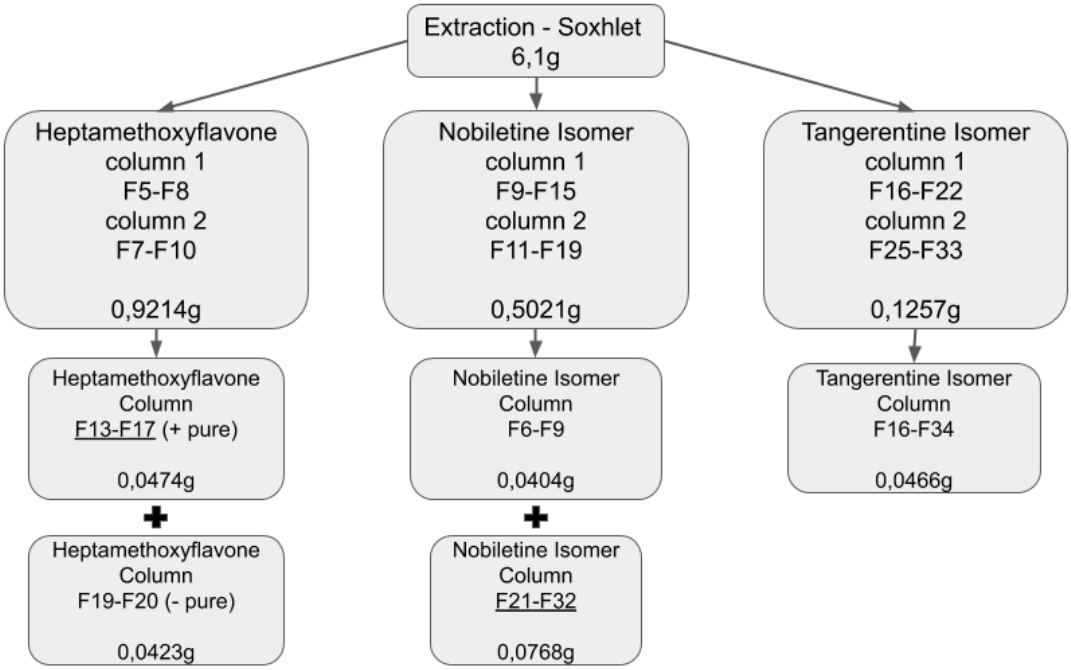

Table 2: Separation and purification scheme. Underlined are the samples applied to Semi-preparative HPLC.

5. Purification

The three main study samples were applied in DCC for further fractionation using a 25mm column. 0.9214g of the heptamethoxyflavone sample was applied on 10.7g of silica 60; 0.5021g of the nobiletin isomer was applied on 6.1g of Silica 60; 0.257g of the tangeretin isomer was applied on 3g of Silica 60. All the samples were combined with 3ml of the mobile phase before being applied to the column. The silica was prepared using a wet-packing method. The process was followed closely by TLC and cross-referenced with the previous results from the TLCs and HPLC. 40 fractions were recovered of each of the columns and combined based on their TLC profiles.

a) Semi-Preparative HPLC

The purified fractions of each compound were combined and applied to the Shimazu® Semi-Preparative HPLC.

Previously, an analytical HPLC procedure was performed using a Merck/Hitachi LaChrom® D-7000 HPLC System. For finding the ideal isocratic solvent system, a Lichrospher® 100 RP18 (5.0 µm) LichroCart ® (250 x 4.0 mm) was used. Elution was carried out at a flow rate of 0.640 mL/min using different isocratic systems for 30

minutes. The temperature of the oven was kept at 25 ºC and the elute was monitored between 200 nm and 400 nm. Data acquisition was performed with D-7000 HSM Software. These parameters were used in the semi-preparative HPLC. The separation was carried out by a High-Performance Semi-preparative System, equipped with a DGU-20A5R Degassing unit, a LC-20AT Pump, a SIL-10AF Auto sampler, a CBM-20A Communication module, a CTO-20AC Column oven, a SPD-M20A UV detector and a FRC-10A fraction collector, SHIMADZU. For isolation procedures, a Luna® 100 C18 (10 µm, 250 x 10 mm) was used. Elution was accomplished at a flow rate of 2.5 mL/min using an isocratic solvent system of 50% of acetonitrile and 50% of water for 45 minutes. The temperature of the oven was kept at 25 ºC and elute was monitored at 260 and 280 nm. Injection volumes varied from 50 to 300 µl (50µL in the first reading and 200/300µL for collecting the peaks).

Data acquisition was performed with LabSolutions® Software.

6. Minimal inhibitory concentration (MIC) assay

MIC-assay, a microbroth dilution method, serves to evaluate the antimicrobial profile of the plant substrates. The MIC is defined as the lowest concentration (mg/l) preventing bacterial growth under certain *in vitro* conditions[97].

The MIC-assay was performed in flat-bottomed 96 well plates whereas each sample was analyzed in duplicate including a control antibiotic (isoniazid (**8**)). A positive control (column 11) and a negative sterile control (column 12) were included in each plate. The assay was carried out with Mueller-Hinton Broth (MHB) as a growth medium.

a) Sample preparation

The start concentration for crude extracts was set to 512 mg/l, for fractions to 256 mg/l and for pure compounds to 128 mg/l. In order to obtain the correct start concentrations and the corresponding volume of dimethylsulphoxide (DMSO), the following formula was used:

[c] x 4 x V = n / 125 = factor
Factor x 0.146 = Minimum sample weight [mg]
Weight [mg] / factor = ml DMSO

Equation 1: c = start concentration [mg/L]; V = end volume in MHB [1ml]; 4 = dilution factor

b) Preparing bacterial inoculum for *M. smegmatis*

For the MIC and modulation assays, a bacterial inoculum with 5 x 10^5 Colony Forming Units (CFU)/ml is required. The mycobacterial strain *M. smegmatis* was grown on Lindamycin Blood Agar (CBA) for 3 days at 37°C. The bacterial culture was suspended in 2 ml of 0.9% NaCl solution, mixed with a sterile loop and slightly vortexed. Initially, a 10^8 suspension was prepared by mixing 100-300 µl of the culture suspension with 10 ml of 0.9% NaCl solution to conform to the turbidity of McFarland 0.5 standard solution. 1ml of the 10^8 suspension was diluted with 9 ml of 0.9 % NaCl solution to obtain a 10^7 CFU/ml solution. The end concentration of 5 x 10^5 CFU/ml was achieved by mixing 19 ml of 0.9% NaCl solution with 1 ml of 10^7 suspension. All dilution steps were carried out in sterile tubes (Sterilin, England).

Pipetting Scheme

- 125 µl aliquot of the control antibiotic (INH used for *M. smegmatis*) was dissolved in 875 µl MHB
- 50 µl of sample were added to 350 µl MHB
- 125 µl MHB were placed in each well from column 1-11
- 125 µl of the test and antibiotic solutions were added in column 1 in duplicate (rows A and B for the 3rd fraction of the DCC, C and D for the 6th fraction of the DCC and E and F for pure HMF)
- Serial dilution of column 1-10: each column was mixed by transferring 125 µl of solution from one column into the next one. Column 10 was transferred directly into column 12 – the sterile control of the extracts.
- 125 µl of 5 x 10^5 CFU/ml inoculum was added into column 1-11
- Incubation of test plates in a plastic container with moistened paper towel at 37°C for 72 hours for *M. smegmatis*

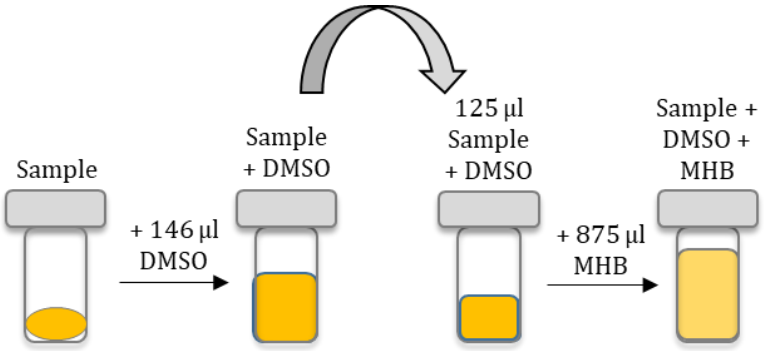

Figure 5: Pipetting scheme for the preparation of the *M. Smegmatis* inoculum

c) Evaluation of *M. smegmatis*

For the assessment of cell viability the determination of the MIC was carried out using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich), a purple tetrazolium salt which indicates bacterial growth by a visible color change from yellow to blue of metabolic active cells[98].

After incubating, the 96- well plates for 72 hours, 20 µl of MTT solution (5mg/ml) was added to each well and the test plates kept in the incubator for 30 min at 37° C.

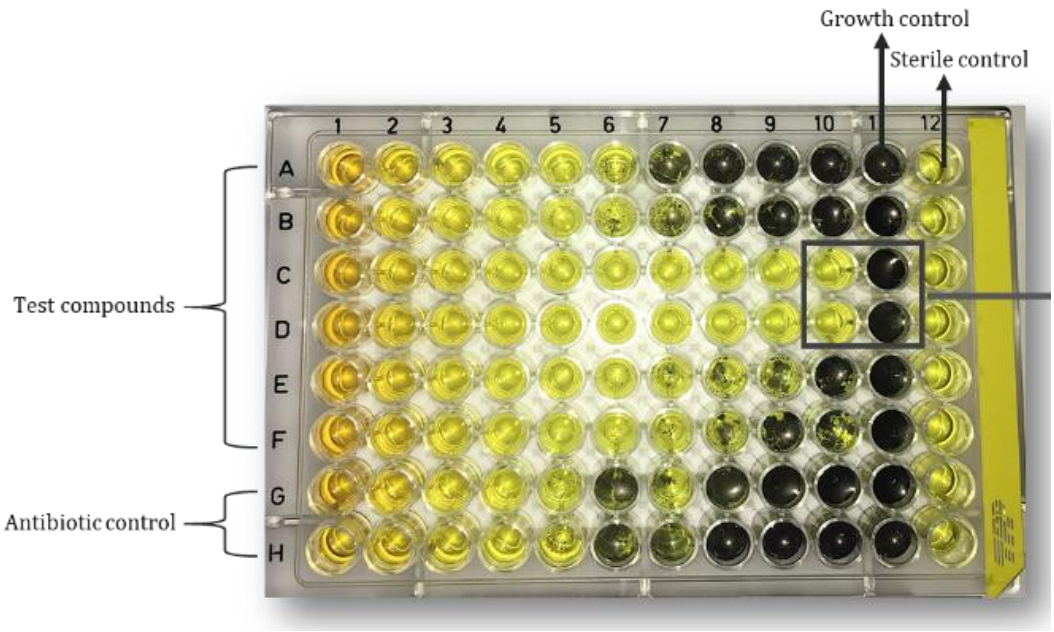

Figure 6: Incubation well-plate. Wells A+B= Fraction 1; C+D= Fraction 2; E+F= pure HMF; G+H= Isoniazid control. Row 11= Growth control; Row 12= Sterile control.

7. Modulation Assay

The modulation assay, a microbroth dilution method, is performed in order to investigate plant compounds for their synergistic effects with ethidium bromide (EtBr) and the antituberculotic drug rifampicin (RIF). The assay was carried out in the same manner as MIC assay but with one set-up: the antibiotic is serially diluted while the concentration of the modulator (plant compound) is kept constant. For screening purposes EtBr, a fluorescent dye and well-known substrate for efflux pumps, was used. The compounds were tested at sub-inhibitory concentrations corresponding to a quarter of their MICs (= [c] as modulator). All substrates were dissolved in DMSO calculated according Equation 1 and diluted in Mueller–Hinton broth using 96-well microtiter plates.

a) Sample preparation

Based on the MIC values of the compounds, the start concentration was calculated using the same formula as mentioned above for MIC-assay but with a modified end volume: V=3 ml. Preparing the bacterial inoculum of *M. smegmatis* was accomplished in the same manner as described for MIC-assay in Figure 4.

Pipetting scheme using a constant modulator concentration

- 125 µl aliquot of AB was dissolved in 875 µl MHB
- 75 µl of sample were added to 1725 µl MHB (= modulator solution)
- 125 µl of MHB were placed in column 11 and row G and H
- 125 µl of modulator solution were added in duplicate in column 1 of rows A-F
- diluting the remaining modulator solution 1:1 with MHB (+ 1550 µl MHB)
- 125 µl of the diluted modulator solution were added in the respective columns 2-10
- 125 µl of AB solution (EtBr) were added in all wells of column 1
- serial dilution of column 1-10: each column was thoroughly mixed and 125 µl of solution was transferred into the next column, column 10 was transferred into column 12 acting as sterile control of the extracts
- 125 µl of 5 x 10^5 CFU/ml inoculum was added into columns 1-11
- incubating the plates in a plastic container with moistened paper towels at 37°C for 72 hours for *M. smegmatis*

b) Evaluation

The analysis of modulation assay was carried out in the same way as for MIC Assay (figure 4) using MTT solution for *M. smegmatis*. Finally, the modulation factor was determined according to the following equation:

$$MF = [MIC \text{ of } AB] / [MIC \text{ of modulator} + AB]$$

Equation 2: Calculating the Modulating Factor.
MIC= Minimum Inhibitory Concentration

In case of a MF ≥ two, the modulating compound causes a potentiating effect on the antimicrobial activity of EtBr or the antibiotic RIF by inducing a reduction in their MICs.

Results

1. Crude extraction

The 154.8g and 139.1g of mandarin orange peels applied to the Soxhlet apparatus yielded 6.1g of crude dichloromethane extract.

2. Analysis of the crude by LC-PDA-ESI/MS and TLC with reference compounds

A TLC was performed on precoated silica and with a mobile phase of toluene:ethyl formiate:formic acid (5:4:1) solution. The dichloromethane extract is compared with references of nobiletin (**2**) and tangeretin (**3**). In fig. 5 there is also the comparison with the final pure obtained at the end of this research.

An analysis of the LC chromatograms allowed for a better characterization of the extracting result and provided a better understanding of the steps ahead. In the chromatograms and the corresponding MS fragments, it was clear that the isolation of the heptamethoxyflavone (**1**) and nobiletin (**2**) were possible through a simple separation process. The crude extract contained numerous isomers of the hexa and penta- methoxylated flavone that were also targeted for isolation.

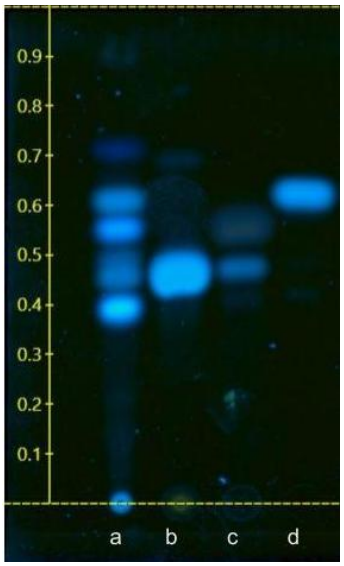

Figure 7: TLC performed on precoated sílica with a toluene:ethyl formiate:formic acid (5:4:1) solution, visualized through a λ366 nm UV light: a) Dichloromethane extract compared with references: b) nobiletin, c) tangeretin, d) final pure HMF obtained in this process

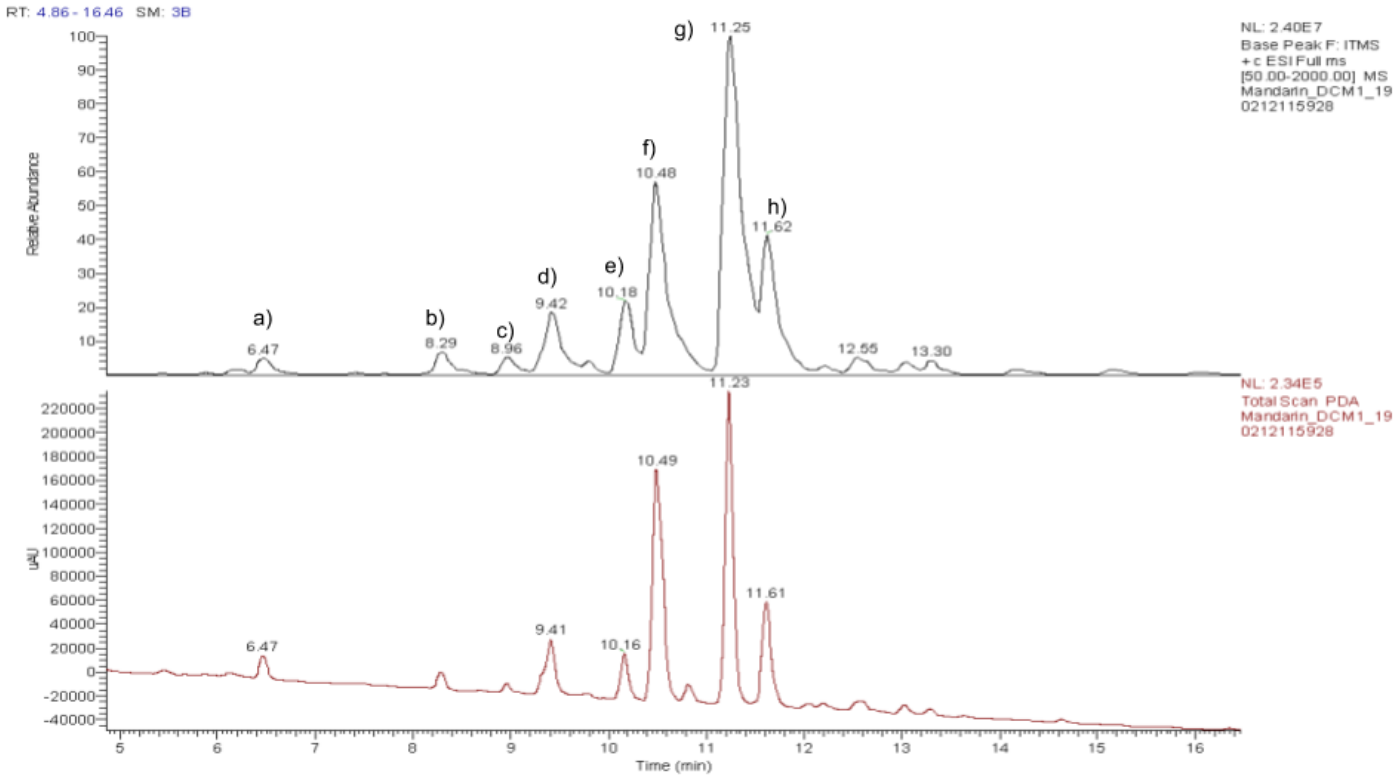

Figure 8: Base Peak and total scan chromatograms of crude mandarin orange peel extract: a) hexamethoxy-hidroxy-flavone; b) tangeretin isomer; c) hexamethoxyflavone not nobiletin; d) pentamethoxyflavone not tangeretin; e) hexamethoxyflavone not nobiletin; f) nobiletin; g) heptamethoxyflavone; h) tangeretin

Retention Time (min)	**[M]** [g/mol]	**$[M+H]^+$ (and major fragments)** [m/z]	**MS^2/MS^3** [m/z]	**Possible identity**
6,47		419	MS^2 [419]= 404, 389	Hexamethoxy-hydroxy-flavone
8,29	372	343 (373)	MS^2 [373]= 343, 378 MS^3 [343] = 315	Isomer of tangeretin
8,96	402	403 (373)	MS^2 [373]= 358, 312	Hexamethoxyflavone, not nobiletin (would have higher retention time)
9,42	372	373 (343)	MS^2 [373]= 358, 312	Pentamethoxyflavone, not tangeretin

10,18	402	403 (373)	MS^2 [403]= 373, 388	Hexamethoxyflavone, not nobiletin, and some fragments
10,50	402	403 (373)	MS^2 [403]= 373, 388	Nobiletin (**2**) (-OMet)
11,23	432	433 (403)	MS^2 [433]= 418, 403	3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**)
11,62	372	343 (373)	MS^2 [343]= 328, 315	Tangeretin (**3**) (last -OMet group is very labile and can fragment upon ionization)

Table 3: Analysis of LC-PDA-ESI/MS chromatograms

3. Analysis of selected fractions by LC-PDA-ESI/MS and TLC and further separation

After the separation process through a Silica gel column, selected fractions were applied on the LC-PDA-ESI/MS and compared with the pattern samples of nobiletin (**2**) and tangeretin (**3**). The fractions were grouped into three possible isolation targets, according to the results of the LC analysis and MS fragmentation. These isolation targets were set as: nobiletin isomer; tangeretin isomer; heptamethoxyflavone.

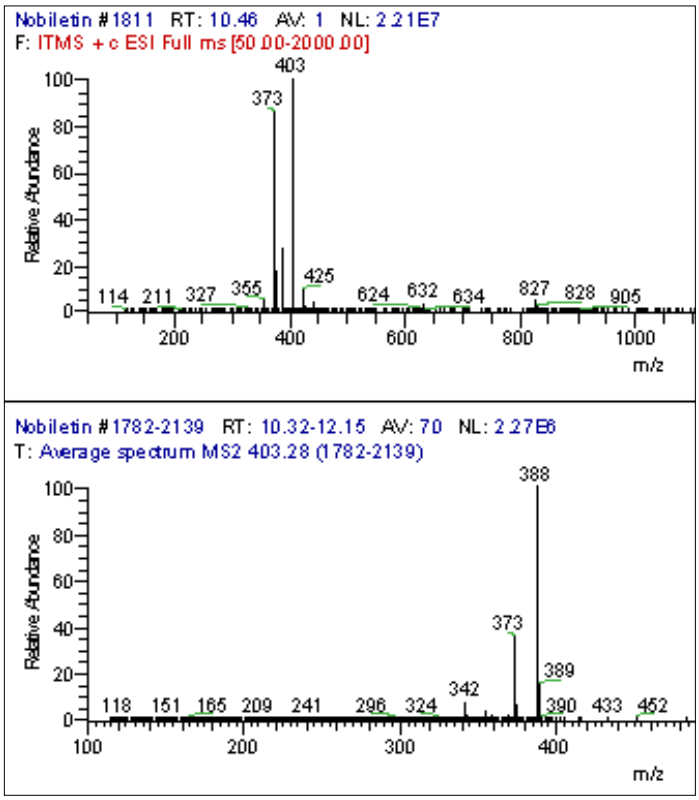

Figure 10: a) $[M+H]^+$ and MS^2 Chromatogram from the pattern sample Nobiletin. Rt= 10.46min

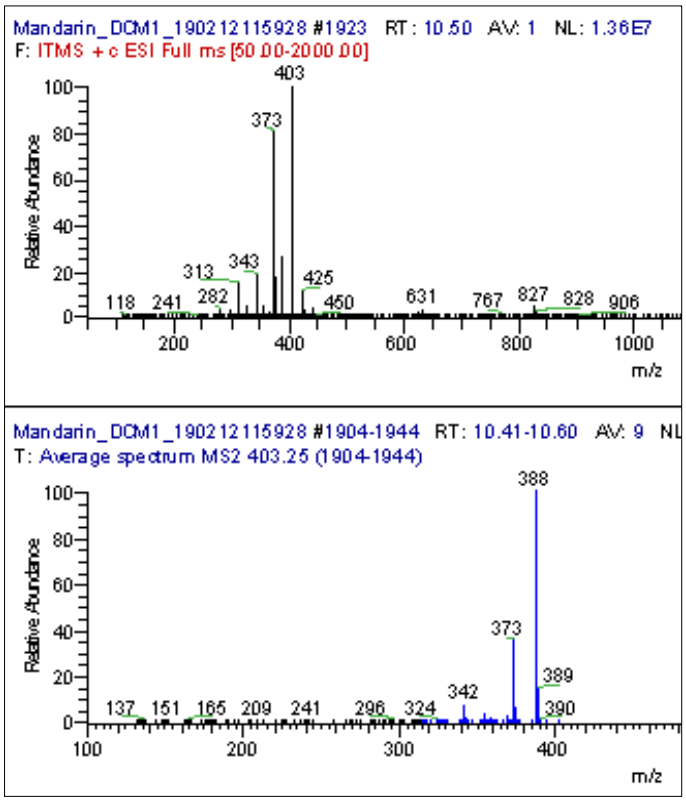

Figure 9: b) $[M+H]^+$ and MS^2 Chromatogram from the dichloromethane extract of mandarin orange. Rt= 10.50min

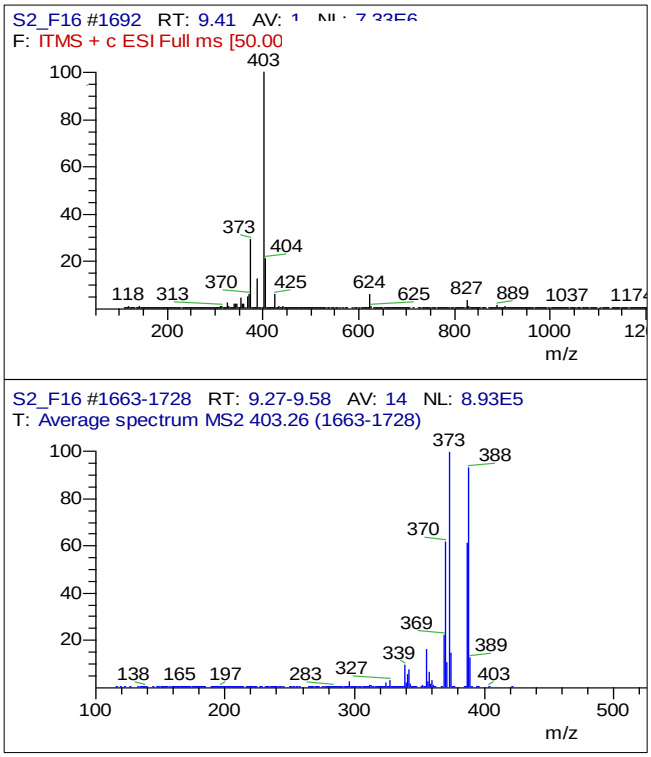

Figure 11: c) $[M+H]^+$ and MS^2 Chromatogram from the tangeretin isomer fraction. Rt=9.41min

a) Nobiletin

Analysis of Nobiletin Full MS and MS^2, comparing the dichloromethane extract compound (b) with the reference nobiletin (**2**) sample (a). Bellow (c) is the $[M+H]^+$ and MS^2 Chromatograms for the nobiletin isomer used for further separation. Chromatogram corresponds to fraction 16 of the second DCC separation. There is a 0.86min Rt shift from the first dichloromethane extract analysis.

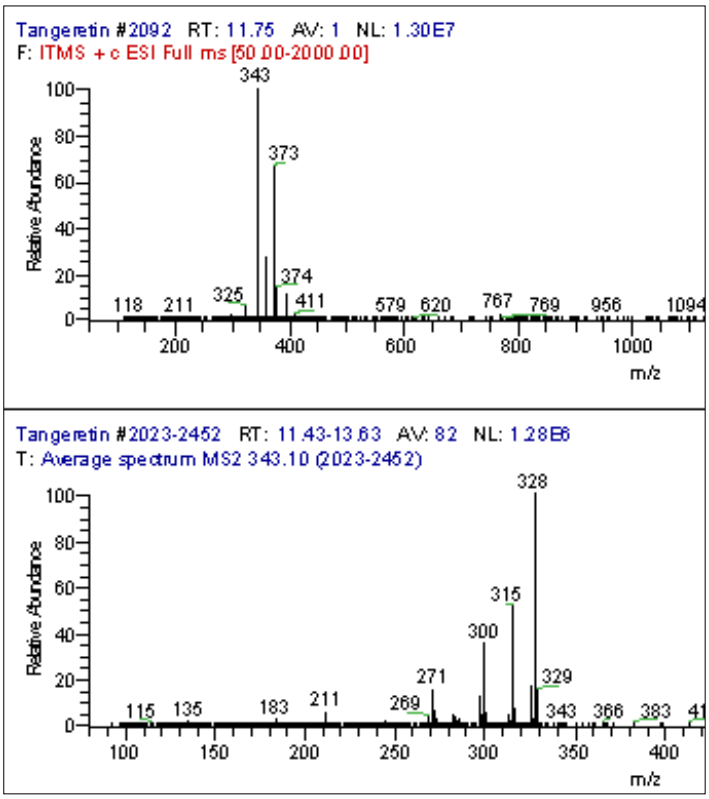

Figure 13: d) $[M+H]^+$ and MS^2 Chromatogram from the pattern sample of tangeretin. Rt= 11.75min

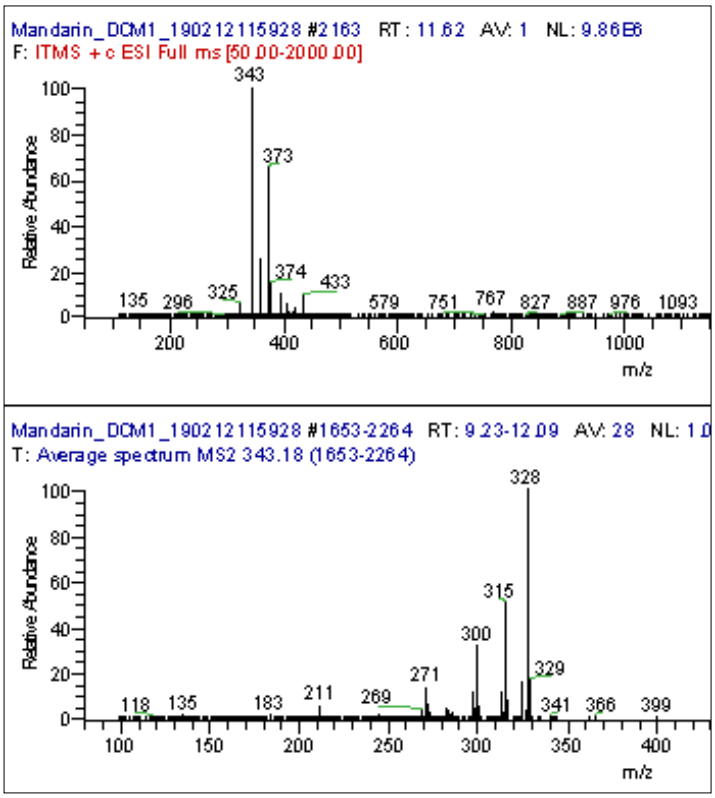

Figure 12: e) $[M+H]^+$ and MS^2 Chromatogram from the dichloromethane extract of mandarin orange. Rt= 11.62min

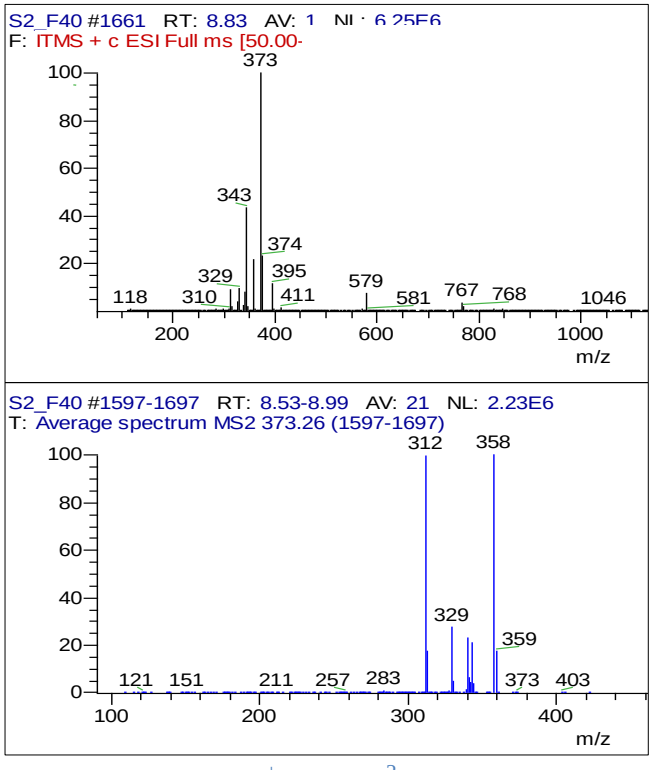

Figure 14: f) $[M+H]^+$ and MS^2 Chromatogram from the tangeretin isomer fraction. Rt=8.83min

b) Tangeretin

Analysis of Tangeretin Full MS and MS^2, comparing the dichloromethane extract compound (e) with the reference tangeretin (**3**) sample (d). Bellow (f) is the $[M+H]^+$ and MS^2 Chromatogram of the tangeretin isomer fractions of the second DCC separation. There was a 0.86min Rt shift between this analysis and the initial dichloromethane extract analysis.

After this distribution, the fractions of

heptamethoxyflavone, nobiletin and tangeretin isomers were applied through DCC columns again, or further isolation of their constituents. After these columns, fractions were selected according to their purity and HPLC results and samples of each group were laid for Semi-Preparative HPLC.

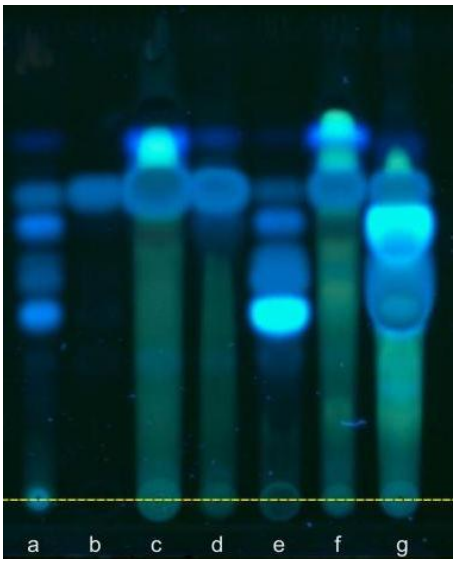

Figure 15: TLC of various fraction groups after the second DCC performed on precoated silica with a toluene:ethyl formiate:formic acid (5:4:1) solution, visualized through a λ366 nm UV light – a) dichloromethane extract; b) pure HMF; c) HMF fractions 13-17 and d) 19-20; e) Tangeretin isomer fraction 16-34; f) Nobiletin isomer fraction 6-9 and g) 21-32

4. Purification Results (amount and proportions)

From the Semi-Preparative HPLC process, only the heptamethoxyflavone sample produced an entirely pure and isolated compound. The nobiletin and tangeretin isomers could not be isolated or identified through the Semi-Preparative HPLC analysis and subsequent HPLC analysis due to their very low yield and contamination. From the sample of nobiletin isomer F21-F32 it was collected a fair amount of pure nobiletin.

Mandarin Orange Peel mass	293.9g
Amount of extract obtained through 2x Soxhlet apparatus	6.1g
Amount of fraction 13-17 containing pure heptamethoxyflavone post-columns	0.0474g

Amount of fraction 13-17 applied in Semi-Preparative HPLC analysis	0.030g
Amount of pure heptamethoxyflavone obtained through Semi-Preparative HPLC	0.0137g (Would be 0.0216g had we analyzed all 0.0474g)
Amount of nobiletin isomer fraction 21-34 applied through Semi-Preparative HPLC	0.0125g
Amount of pure nobiletin obtained through Semi-Preparative HPLC	0.0045g

Table 4: Semi-preparative HPLC results and yield

5. ^{1}H NMR and ^{13}C NMR Results and structure identification of heptamethoxyflavone ($CDCL_3$)

Position	**δ ^{13}C [ppm]**	**δ ^{1}H [ppm]**
2	153.1	
3	140.8	
3-OCH_3	59.9	3.89s
4	173.9	
4a	115.1	
5	148.2	
5-OCH_3	62.3	3.98s
6	143.2	
6-OCH_3	61.6	3.95s
7	151.3	
7-OCH_3	61.7	4.10s
8	137.8	
8-OCH_3	62.0	4.01s
8a	146.7	
1’	123.5	
2’	111.0	7.81 (d, J= 2.0)

3’	148.8	
3’-OCH_3	55.9	3.970s
4’	151.1	
4’-OCH_3	56.0	3.975s
5’	110.9	7.01 (d, J= 8.5)
6’	121.9	7.84 (dd, J= 8.5; 2.0)

Table 5: ^{1}H-NMR and 13C-NMR results of heptamethoxyflavone

The pure heptamethoxyflavone was dissolved in $CDCl_3$ and analyzed through Nuclear Magnetic Ressonance (NMR). The existence of a doublet in the H-NMR data in 5’ and a doublet of doublets in 6’ confirm that the structure could only be 3,3',4',5,6,7,8-Heptamethoxyflavone (**figure 17**).

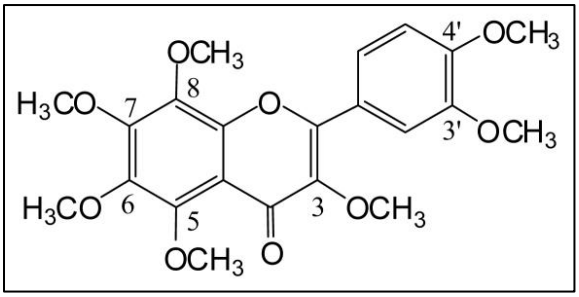

Figure 16: Confirmed structure for the heptamethoxyflavone

6. MICs and Modulation Factors

Mandarine	**MIC [mg/l]**	**[c] as modulator * [mg/L]**	**MF_{EtBr}**	**MF_{RIF}**
Extract	256	64	2	**4**
H1	128	32	2	**8**
F1 (3rd fraction)	64	4	**8**	**8**
F2 (6th fraction)	256	64	**1**	2

Table 6: MICs and modulation factors of the compounds for *M. smegmatis* mc^2 155

H1= pure HMF sample; F1= 3rd DCC fraction; F2= 6th DCC fraction

Minimum inhibitory concentration (MIC) of isoniazid (INH) = 4 mg/l, ethidium bromide (EtBr) = 8 mg/L and MIC of rifampicin (RIF) = 32 mg/L. Modulation factor (MF) = MIC (EtBr)/MIC (EtBr + modulator); *n* = 4

Within the antimicrobial screening on *M. smegmatis*, the 3rd fraction of the DCC proved to be active (MIC = 64 mg/l) against the mycobacterial strain. Corresponding to a

quarter of their MICs (*), all compounds were evaluated for their modulating potential on the antimicrobial activity of EtBr and rifampicin (a firstline anti-tuberculotic drug).

Except the 6th fraction of the DCC, all compounds had significant modulating activities by reducing the MIC of rifampicin (MF_{RIF}) to a 4 to 8-fold. The 3rd fraction appeared as the most potent modulator as it increased the susceptibility of *M. smegmatis* towards EtBr and RIF remarkably (MF = 8).

Discussion and Conclusion

This project was done using dried peels of commercial tangerines and clementines, of which the ripening stage at the picking moment is unknown. Most commercial citrus fruits in Austria come from Spain, Turkey or South Africa, covering long distances in space and in time. Industries normally pick these fruits very early so they can reach the destination without rotting. We can assume the fruits were at an early stage of ripening when picked, and so the peels may not have had the ideal amounts of PMF's for the project.

This project did not have the set goal of characterizing a certain cultivar and so a mix of different fruits has been used. As certain PMF's occur in high amounts in different cultivars, it would have been useful to signal those and optimize the extraction of PMF's in this project.

The dry-column chromatography for the isolation process proved to be a very time-consuming strategy, with high consumption of organic solvents and relatively low yield and resolution and the semi-preparative HPLC used for purification could only provide satisfactory results with the fractions of pure 3,3',4',5,6,7,8-heptamethoxyflavone. The mixture of nobiletin isomer yielded very low results and the tangeretin isomer mixture didn't have enough definition to provide a pure compound.

The isolation process of PMF's has been the subject of some debate in the community. There have been numerous attempts to develop a more efficient process of separating constituents of the extract.

The structure characterization of the pure 3,3',4',5,6,7,8-heptamethoxyflavone obtained through the various separations processes was made through ^{1}H and ^{13}C NMR. There were two possible structures for the heptamethoxyflavone ($C_{22}H_{24}O_9$) - 3,3',4',5,6,7,8-heptamethoxyflavone (**figure 17**) and 5,6,7,8,3',4',5'-heptamethoxyflavone (**figure 18**). Analysis of the NMR data confirmed that the structure was in fact the one described in literature as the flavonoid found in *Citrus* family species.

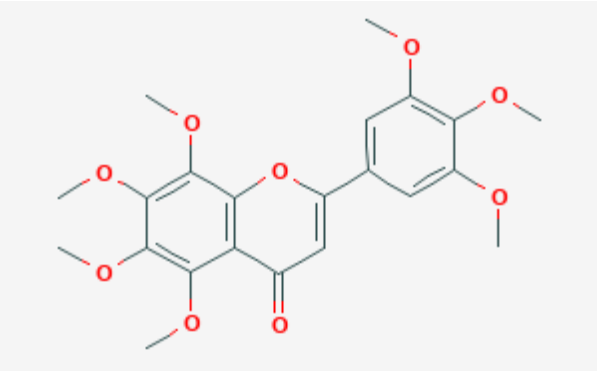

Figure 17: 5,6,7,8,3',4',5'-Heptamethoxyflavone

There was not enough time to test all the samples and apply them to the semi-preparative HPLC for isolation. For this reason, two of the early fractions taken from the dry-column chromatography were used in de microbiological assays to serve as comparison between the pure HMF and the extract with a poorer concentration of polimethoxyflavones – the 3rd and 6th fractions.

These fractions had impressive MIC values (3rd fraction) and modulation results (6th fraction). This can be due to an incorrect dry-column chromatography, in which the polimethoxyflavones had lower affinity for the stationary phase than expected, or due to other less polar components in the extract (non-polimethoxyflavones or isomers of polimethoxyflavones) that were eluted first.

The comparison with the unknown components in F1 (3rd fraction) and F2 (6th fraction) doesn't diminish the amazing antimicrobial activity of pure HMF (=128) and its modulating capacity with rifampicin (=8).

Given these results, further development of the project would be needed to clear some questions and optimize the processes, specially the isolation and purifying phase which seem to be the areas where most improvement can be made.

Bibliography

1. Winkel-Shirley B. *Update on Flavonoid Biosynthesis A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology 1*. Vol 126.; 2001.

2. Martens S, Mithöfer A. Flavones and flavone synthases. *Phytochemistry*. 2005;66:2399-2407. doi:10.1016/j.phytochem.2005.07.013

3. Falcone Ferreyra ML, Rius SP, Casati P, Hellmann HA, de Estudios Fotosintéticos Bioquímicos C. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front Plant Sci*. 2012;3(222). doi:10.3389/fpls.2012.00222

4. Jiang N, Doseff AI, Grotewold E. Flavones: From Biosynthesis to Health Benefits. *Plants*. 2016;5(2):27. doi:10.3390/plants5020027

5. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: Location and functional significance. *Plant Sci*. 2012;196:67-76. doi:https://doi.org/10.1016/j.plantsci.2012.07.014

6. Tohge T, de Souza LP, Fernie AR. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. *J Exp Bot*. 2017;68(15):4013-4028. doi:10.1093/jxb/erx177

7. Croft KD. The Chemistry and Biological Effects of Flavonoids and Phenolic Acids *A*. Vol 854.; 1998.

8. Martens S, Preuß A, Matern U. Multifunctional flavonoid dioxygenases: Flavonol and anthocyanin biosynthesis in Arabidopsis thaliana L. *Phytochemistry*. 2010;71:1040-1049. doi:10.1016/j.phytochem.2010.04.016

9. Saito K, Yonekura-Sakakibara K, Nakabayashi R, et al. The flavonoid biosynthetic pathway in Arabidopsis: Structural and genetic diversity. *Plant Physiol Biochem*. 2013;72(February):21-34. doi:10.1016/j.plaphy.2013.02.001

10. Bowles D, Isayenkova J, Lim E-K, Poppenberger B, Kutchan T, Dixon R. Glycosyltransferases: managers of small molecules. *Curr Opin Plant Biol*. 2005;8:254-263. doi:10.1016/j.pbi.2005.03.007

11. Ferrer J-L, Austin MB, Stewart C, Noel JP. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol Biochem*. 2008;46:356-370. doi:10.1016/j.plaphy.2007.12.009

12. David AB, Gang R. Methoxylated flavones: occurrence, importance, biosynthesis. *Phytochem Rev*. 2015. doi:10.1007/s11101-015-9426-0

13. Antonio J, Rio D, Arcas MC, Benavente O, Sabater F, Ortuño A. *Changes of Polymethoxylated Flavones Levels during Development of Citrus Aurantium (Cv. Sevillano) Fruits*. Vol 64.; 1998.

14. Benavente-García O, Castillo JN, Marin FR, Ortuñ A, Río JA Del. *Uses and Properties of Citrus Flavonoids*. Vol 45.; 1997.

15. Mouly PP, Arzouyan CR, Gaydou EM, Estienne JM. Differentiation of citrus

juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides. *J Agric Food Chem*. 1994;42(1):70-79. doi:10.1021/jf00037a011

16. Ooghe WC, Detavernier CM. Detection of the Addition of Citrus reticulata and Hybrids to Citrus sinensis by Flavonoids. *J Agric Food Chem*. 1997;45(5):1633-1637. doi:10.1021/jf9606262

17. Tripoli E, Guardia L, Giammanco S, Majo D Di, Giammanco M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chem*. 2007;(104):466-479. doi:10.1016/j.foodchem.2006.11.054

18. Favela-Hernández J, González-Santiago O, Ramírez-Cabrera M, Esquivel-Ferriño P, Camacho-Corona M. Chemistry and Pharmacology of Citrus sinensis. *Molecules*. 2016;21(2):247. doi:10.3390/molecules21020247

19. Ho S-C, Lin C-C. Investigation of Heat Treating Conditions for Enhancing the Anti-Inflammatory Activity of Citrus Fruit (Citrus reticulata) Peels. *J Agric Food Chem*. 2008;56(17):7976-7982. doi:10.1021/jf801434c

20. Huang Y-S, Ho S-C. Polymethoxy flavones are responsible for the anti-inflammatory activity of citrus fruit peel. *Food Chem*. 2010;119(3):868-873. doi:https://doi.org/10.1016/j.foodchem.2009.09.092

21. Yoshigai E, Machida T, Okuyama T, et al. Citrus nobiletin suppresses inducible nitric oxide synthase gene expression in interleukin-1β-treated hepatocytes. *Biochem Biophys Res Commun*. 2013;439(1):54-59. doi:https://doi.org/10.1016/j.bbrc.2013.08.029

22. Li S, Pan M-H, Lai C-S, Lo C-Y, Dushenkov S, Ho C-T. Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. *Bioorg Med Chem*. 2007;15(10):3381-3389. doi:https://doi.org/10.1016/j.bmc.2007.03.021

23. Manthey JA, Guthrie N. Antiproliferative Activities of Citrus Flavonoids against Six Human Cancer Cell Lines. *J Agric Food Chem*. 2002;50(21):5837-5843. doi:10.1021/jf020121d

24. Lai C-S, Li S, Miyauchi Y, Suzawa M, Ho C-T, Pan M-H. Potent anti-cancer effects of citrus peel flavonoids in human prostate xenograft tumors. *Food Funct*. 2013;4(6):944-949. doi:10.1039/C3FO60037H

25. Del Río JA, Arcas MC, Benavente-García O, Ortuño A. Citrus Polymethoxylated Flavones Can Confer Resistance against Phytophthora citrophthora, Penicillium digitatum, and Geotrichum Species. *J Agric Food Chem*. 1998;46(10):4423-4428. doi:10.1021/jf980229m

26. Yao X, Zhu X, Pan S, et al. Antimicrobial activity of nobiletin and tangeretin against Pseudomonas. *Food Chem*. 2012;132(4):1883-1890. doi:https://doi.org/10.1016/j.foodchem.2011.12.021

27. Camacho-Corona M del R, Ramírez-Cabrera MA, Santiago OG-, Garza-González E, Palacios I de P, Luna-Herrera J. Activity against drug resistant-tuberculosis strains of plants used in Mexican traditional medicine to treat

tuberculosis and other respiratory diseases. *Phyther Res*. 2008;22(1):82-85. doi:10.1002/ptr.2269

28. Takanaga H, Ohnishi A, Yamada S, et al. Polymethoxylated Flavones in Orange Juice Are Inhibitors of P-glycoprotein but Not Cytochrome P450 3A4. *J Pharmacol Exp Ther*. 2000;293(1):230 LP - 236. http://jpet.aspetjournals.org/content/293/1/230.abstract.

29. Mertens-Talcott SU, De Castro WV, Manthey JA, Derendorf H, Butterweck V. Polymethoxylated Flavones and Other Phenolic Derivates from Citrus in Their Inhibitory Effects on P-Glycoprotein-Mediated Transport of Talinolol in Caco-2 Cells. *J Agric Food Chem*. 2007;55(7):2563-2568. doi:10.1021/jf063138v

30. Manthey JA, Grohmann K. Phenols in Citrus Peel Byproducts. Concentrations of Hydroxycinnamates and Polymethoxylated Flavones in Citrus Peel Molasses. *J Agric Food Chem*. 2001;49:3268-3273. doi:10.1021/jf010011r

31. Scora RW. On the History and Origin of Citrus. *Bull Torrey Bot Club*. 1975;102(6):369-375. doi:10.2307/2484763

32. Ramana KVR, Govindarajan VS, Ranganna S, Kefford JF. Citrus fruits — varieties, chemistry, technology, and quality evaluation. Part I: Varieties, production, handling, and storage. *C R C Crit Rev Food Sci Nutr*. 1981;15(4):353-431. doi:10.1080/10408398109527321

33. Gmitter FG, Hu X. The possible role of Yunnan, China, in the origin of contemporary citrus species (rutaceae). *Econ Bot*. 1990;44(2):267-277. doi:10.1007/BF02860491

34. Barrett HC, Rhodes AM. A Numerical Taxonomic Study of Affinity Relationships in Cultivated Citrus and Its Close Relatives. *Syst Bot*. 1976;1(2):105-136. doi:10.2307/2418763

35. Swingle WT, Webber HJ. The botany of citrus and its wild relatives of the orange subfamily (family Rutaceae, subfamily Aurantioideae). 1943:iv, 129-474, 953-1021 p. file://catalog.hathitrust.org/Record/002003866.

36. Herrero R, Asíns MJ, Carbonell EA, Navarro L. Genetic diversity in the orange subfamily Aurantioideae. I. Intraspecies and intragenus genetic variability. *Theor Appl Genet*. 1996;92(5):599-609. doi:10.1007/BF00224564

37. Nicolosi E, Deng ZN, Gentile A, La Malfa S, Continella G, Tribulato E. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theor Appl Genet*. 2000;100(8):1155-1166. doi:10.1007/s001220051419

38. Albert Wu G, Terol J, Ibanez V, et al. Genomics of the origin and evolution of Citrus. *Nature*. 2018;(554):311-316. doi:10.1038/nature25447

39. USDA, National Agricultural Statistics Service. Citrus Fruits 2018 Summary. 2018;(August):35.

40. Forsyth J, Damiani J. CITRUS FRUITS | Types on the Market. *Encycl Food Sci Nutr*. January 2003:1329-1335. doi:10.1016/B0-12-227055-X/00240-6

41. FAO. Citrus Fruit fresh and processed. *CcpCi/St/2016, FAO*. 2016:47. doi:10.1016/j.jmgm.2005.11.005

42. Kesterson JW, Hendrickson R. Utilization of Citrus by-products. *Econ Bot*. 1958;12(2):164-185. doi:10.1007/BF02862771

43. Manthey JA. Fractionation of Orange Peel Phenols in Ultrafiltered Molasses and Mass Balance Studies of Their Antioxidant Levels. *Jouranl Agric Food Chem*. 2004;52:7586-7592. doi:10.1021/jf049083j

44. Manthey JA, Grohmann K. Concentrations of Hesperidin and Other Orange Peel Flavonoids in Citrus Processing Byproducts. *J Agric Food Chem*. 1996;44:811-814.

45. Berk Z. Citrus Fruit Processing. 1st ed. Academic Press; 2016.

46. Mabberley DJ. A Classification for Edible Citrus (Rutaceae). Vol 7.; 1997.

47. Morton J. Fruits of Warm Climates.; 1987. doi:10.1016/j.bbadis.2011.12.011

48. Kefford JF.The chemical Constituents of Citrus fruits. Vol 9.; 1960.

49. Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C. Flavonoid Composition of Citrus Juices. Vol 12.; 2007.

50. Wang Y-C, Chuang Y-C, Hsu H-W. The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chem*. 2007;(106):277-284. doi:10.1016/j.foodchem.2007.05.086

51. Manthey JA, Grohmann K. Phenols in Citrus Peel Byproducts. Concentrations of Hydroxycinnamates and Polymethoxylated Flavones in Citrus Peel Molasses. *J Agric Food Chem*. 2001;(49):3268-3273. doi:10.1021/jf010011r

52. Ledesma-Escobar CA, Luque De Castro M. Towards a comprehensive exploitation of citrus. *Trends Food Sci Technol*. 2014;(39):63-75. doi:10.1016/j.tifs.2014.07.002

53. Ortuño AM, Arcas MC, Benavente-García O, Del Río JA. Evolution of Polymethoxy ¯avones during Development of Tangelo Nova Fruits.; 1999.

54. World Health Organization. *WHO | Global Tuberculosis Report 2018*.; 2018.

55. Reid M, Goosby E, Kevany S. Leveraging health diplomacy to end the tuberculosis epidemic. *Lancet Glob Heal*. 2019;7(5):e561-e562. doi:10.1016/S2214-109X(19)30058-0

56. Furin J, Cox H, Pai M. Seminar Tuberculosis. *www.thelancet.com*. 2019;393:1642-1656. doi:10.1016/S0140-6736(19)30308-3

57. World Health Organization. BCG vaccine: WHO position paper, February 2018 Recommendations. *Vaccine*. 2018;36:3408-3410. doi:10.1016/j.vaccine.2018.03.009

58. du Preez K, Seddon JA, Schaaf HS, et al. *Global Shortages of BCG Vaccine and Tuberculous Meningitis in Children*. Vol 7.; 2019. doi:10.1016/S2214-

109X(18)30474-1

59. Flynn JL, Chan J. *Immunology of Tuberculosis*.; 2001.

60. Drain PK, Bajema KL, Dowdy D, et al. *Incipient and Subclinical Tuberculosis: A Clinical Review of Early Stages and Progression of Infection*. Vol 31.; 2018.

61. WHO | Drug-resistant TB: XDR-TB FAQ. *WHO*. 2018. https://www.who.int/tb/areas-of-work/drug-resistant-tb/xdr-tb-faq/en/#.XcSH_y_sAzY.mendeley. Accessed November 7, 2019.

62. Dookie N, Rambaran S, Padayatchi N, Mahomed S, Naidoo K. Evolution of drug resistance in Mycobacterium tuberculosis: a review on the molecular determinants of resistance and implications for personalized care. *J Antimicrob Chemother*. 2018;73(5):1138-1151. doi:10.1093/jac/dkx506

63. Morris RP, Nguyen L, Gatfield J, et al. Ancestral antibiotic resistance in Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A*. 2005;102(34):12200-12205. doi:10.1073/pnas.0505446102

64. Plitzko M, Engelhardt H, Hoffmann C, Leis A, Niederweis M. Disclosure of the mycobacterial outer membrane : Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc Natl Acad Sci U S A*. 2008;105(10):3963-3967.

65. Hegde SS, Vetting MW, Roderick SL, et al. A Fluoroquinolone Resistance Protein from Mycobacterium tuberculosis That Mimics DNA. *Science (80-)*. 2005;308(5727):1480 LP - 1483. doi:10.1126/science.1110699

66. Kanji A, Hasan R, Hasan Z. Efflux pump as alternate mechanism for drug resistance in Mycobacterium tuberculosis. *Indian J Tuberc*. 2019;66(1):20-25. doi:https://doi.org/10.1016/j.ijtb.2018.07.008

67. da Silva PEA, Von Groll A, Martin A, Palomino JC. Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. *FEMS Immunol Med Microbiol*. 2011;63(1):1-9. doi:10.1111/j.1574-695X.2011.00831.x

68. Braibant M, Gilot P, Content J. The ATP binding cassette (ABC) transport systems of Mycobacterium tuberculosis. *FEMS Microbiol Rev*. 2000;24(4):449-467. doi:10.1111/j.1574-6976.2000.tb00550.x

69. Chakraborti PK, Bhatt K, Banerjee SK, Misra P. Role of an ABC Importer in Mycobacterial Drug Resistance. *Biosci Rep*. 1999;19(4):293-300. doi:10.1023/A:1020598324663

70. Alland D, Steyn AJ, Weisbrod T, Aldrich K, Jacobs Jr WR. Characterization of the Mycobacterium tuberculosis iniBAC promoter, a promoter that responds to cell wall biosynthesis inhibition. *J Bacteriol*. 2000;182(7):1802-1811. doi:10.1128/jb.182.7.1802-1811.2000

71. Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G. mmpL7 gene of Mycobacterium tuberculosis is responsible for isoniazid efflux in Mycobacterium smegmatis. *Antimicrob Agents Chemother*. 2005;49(11):4775-4777.

doi:10.1128/AAC.49.11.4775-4777.2005

72. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the Mycobacterium tuberculosis complex. *Infect Genet Evol*. 2012;12(4):695-700. doi:https://doi.org/10.1016/j.meegid.2011.08.009

73. Liu J, Shi W, Zhang S, et al. Mutations in Efflux Pump Rv1258c (Tap) Cause Resistance to Pyrazinamide, Isoniazid, and Streptomycin in M. tuberculosis. *Front Microbiol*. 2019;10:216. doi:10.3389/fmicb.2019.00216

74. Ramón-García S, Martín C, Thompson CJ, Aínsa JA. Role of the Mycobacterium tuberculosis P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother*. 2009;53(9):3675-3682. doi:10.1128/AAC.00550-09

75. Adams KN, Verma AK, Gopalaswamy R, et al. Macrophage-induced rifampin tolerance across Mycobacterium tuberculosis lineages is Rv1258c-dependent. *bioRxiv*. January 2018:383109. doi:10.1101/383109

76. Zhang Y, Zhang J, Cui P, Zhang Y, Zhang W. Identification of Novel Efflux Proteins Rv0191, Rv3756c, Rv3008, and Rv1667c Involved in Pyrazinamide Resistance in Mycobacterium tuberculosis *Antimicrob Agents Chemother*. 2017;61(8):e00940-17. doi:10.1128/AAC.00940-17

77. Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drrA and drrB of Mycobacterium tuberculosis. *Biochem J*. 2002;367(1):279-285. doi:10.1042/bj20020615

78. Gupta AK, Chauhan DS, Srivastava K, et al. Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. *J Commun Dis*. 2006;38(3):246.

79. Ramón-García S, Mick V, Dainese E, et al. Functional and Genetic Characterization of the Tap Efflux Pump in Mycobacterium bovis BCG. *Antimicrob Agents Chemother*. 2012;56(4):2074 LP - 2083. doi:10.1128/AAC.05946-11

80. Nguyen L. Antibiotic resistance mechanisms in M. tuberculosis: an update. *Arch Toxicol*. 2016;90(7):1585-1604. doi:10.1007/s00204-016-1727-6

81. Organization WH. WHO consolidated guidelines on drug-resistant tuberculosis treatment. 2019.

82. Campbell EA, Korzheva N, Mustaev A, et al. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*. 2001;104(6):901-912. doi:10.1016/S0092-8674(01)00286-0

83. Palomino JC, Martin A. Drug Resistance Mechanisms in Mycobacterium tuberculosis. *Antibiot (Basel, Switzerland)*. 2014;3(3):317-340.

doi:10.3390/antibiotics3030317

84. Blanchard JS. Molecular Mechanisms of Drug Resistance in *Mycobacterium tuberculosis*. Vol 65.; 1996.

85. Telenti A, Imboden P, Marchesi F, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet (London, England)*. 1993;341(8846):647-650. doi:10.1016/0140-6736(93)90417-f

86. Wehrli W. Rifampin: Mechanisms of Action and Resistance. *Clin Infect Dis*. 1983;5(Supplement_3):S407-S411. doi:10.1093/clinids/5.Supplement_3.S407

87. Seung KJ, Keshavjee S, Rich ML. Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. *Cold Spring Harb Perspect Med*. 2015;5(9):a017863-a017863. doi:10.1101/cshperspect.a017863

88. Sachin BS, Sharma SC, Sethi S, et al. Herbal modulation of drug bioavailability: enhancement of rifampicin levels in plasma by herbal products and a flavonoid glycoside derived from Cuminum cyminum. *Phyther Res*. 2007;21(2):157-163. doi:10.1002/ptr.2046

89. D. F. BAIN A. SMITH DLM. Modulation of rifampicin release from spray-dried microspheres using combinations of poly-(DL-lactide). *J Microencapsul*. 1999;16(3):369-385. doi:10.1080/026520499289086

90. Singh C, Koduri LVSK, Dhawale V, et al. Potential of aerosolized rifampicin lipospheres for modulation of pulmonary pharmacokinetics and bio-distribution. *Int J Pharm*. 2015;495(2):627-632. doi:10.1016/J.IJPHARM.2015.09.036

91. te Brake LHM, de Knegt GJ, de Steenwinkel JE, et al. The Role of Efflux Pumps in Tuberculosis Treatment and Their Promise as a Target in Drug Development: Unraveling the Black Box. *Annu Rev Pharmacol Toxicol*. 2018;58(1):271-291. doi:10.1146/annurev-pharmtox-010617-052438

92. Pule CM, Sampson SL, Warren RM, et al. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *J Antimicrob Chemother*. 2015;71(1):17-26. doi:10.1093/jac/dkv316

93. Shiloh MU, DiGiuseppe Champion PA. To catch a killer. What can mycobacterial models teach us about Mycobacterium tuberculosis pathogenesis? *Curr Opin Microbiol*. 2010;13(1):86-92. doi:https://doi.org/10.1016/j.mib.2009.11.006

94. Altaf M, Miller CH, Bellows DS, O'Toole R. Evaluation of the Mycobacterium smegmatis and BCG models for the discovery of Mycobacterium tuberculosis inhibitors. *Tuberculosis*. 2010;90(6):333-337. doi:https://doi.org/10.1016/j.tube.2010.09.002

95. Bansal A, Mallik D, Kar D, Ghosh AS. Identification of a multidrug efflux pump in Mycobacterium smegmatis. *FEMS Microbiol Lett*. 2016;363(13). doi:10.1093/femsle/fnw128

96. Li X-Z, Zhang L, Nikaido H. Efflux Pump-Mediated Intrinsic Drug Resistance in Mycobacterium smegmatis. *Antimicrob Agents Chemother*. 2004;48:2415-2423.

doi:10.1128/AAC.48.7.2415-2423.2004

97. Kahlmeter G, Brown DFJ, Goldstein FW, et al. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *J Antimicrob Chemother*. 2003;52(2):145-148. doi:10.1093/jac/dkg312

98. Stockert JC, Horobin RW, Colombo LL, Blázquez-Castro A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta Histochem*. 2018;120(3):159-167. doi:https://doi.org/10.1016/j.acthis.2018.02.005