ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Manaf ALMATAR

COMBINATION OF ANTI-TUBERCULOSIS DRUGS WITH POMEGRANATE JUICE AGAINST MULTIDRUG-RESISTANT TB AND EXAMINATION OF THE INFLUENCE OF THIS MIX ON RESISTANT AND EFFLUX PUMP GENES

DEPARTMENT OF BIOTECHNOLOGY

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Doctor of Philosophy by the board of jury on 27/07/2018.

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ABSTRACT

PhD THESIS

COMBINATION OF ANTI-TUBERCULOSIS DRUGS WITH POMEGRANATE JUICE AGAINST MULTIDRUG- RESISTANT TB AND EXAMINATION OF THE INFLUENCE OF THIS MIX ON RESISTANT AND EFFLUX PUMP GENES

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The aim of present study was to investigate the effect of fresh pomegranate juice (FPJ) on the antibacterial activity of anti-tuberculosis drugs (Rifampin (R) and Isoniazid (INH)) against MDR-TB clinical isolates. Four concentrations of fresh pomegranate juice (FPJ) (5%, 10%, 15%, and 20%) were evaluated in combination with R and INH at a dose range of (1.0 µg/ml), and (0.1µg/ml), respectively. Moreover, this study investigated individual phenolic compounds of FPJ by using high-performance liquid chromatography (HPLC). The total polyphenols (TP), total flavonoid (TF), total anthocyanins content (TAC), and the antioxidant capacity were also assessed in FPJ. Additionally,we evaluated the contribution of resistant genes associated with INH and R resistance plus six putative drug efflux pump genes to the drug resistance levels in MDR-TB clinical isolates. While the expression level of ***katG*** was down-regulated in 24/27 (88.88%) MDR-TB isolates, ***inhA***, ***oxyR-ahpC***, and ***rpoB*** appeared to be either overexpressed or up-regulated in 8/ 27 (29.62%), 4/27 (14.81 %), and 24/ 27 (88.88%), respectively in MDR-TB isolates. Moreover, the efflux pump genes ***drrA***, ***drrB***, ***efpA***, ***Rv2459***, ***Rv1634***, and ***Rv1250*** were overexpressed under INH/RIF plus FPJ stress

Key words: Fresh pomegranate juice (FPJ), Tuberculosis, Synergistic effect, Antimicrobial activity, New anti-tuberculosis drugs

ÖZ

DOKTORA TEZİ

ÇOKLU ILAÇ DİRENÇLI TÜBERKÜLOZA KARŞI ANTI-TÜBERKÜLOZ ILAÇLARI ILE BİRLİKTE NAR SUYUNUN KOMBINASYONU VE BU KARIŞIMIN DIRENÇ VE EFFLUX POMPA SISTEM GENLERINE ETKİSININ INCELENMESI

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ÇUKUROVA ÜNİVERSİTESİ
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Bu çalışmada amaç, taze nar suyunun (TNS=FPJ) tek başına ve şu ana kadar tedavide kullanılanılmakta olan antitüberküloz ilaçlarından Rifampin (R) ve Isoniazid (INH)' ile kombinasyonlarının MDR-TB klinik izolatları üzerine antibakteriyel aktivitesini araştırmaktır .Dört adet taze nar suyu konsantrasyonu ile ilaca dirençli izolatlara karşı sırasıyla; (FPJ) (% 5,% 10,% 15 ve% 20), R ve INH ile kombinasyon halinde (1.0 ug / ml) ve (0.1ug / ml) doz aralığında değerlendirilmiştir. Ayrıca, çalışmamızda yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak FPJ'nin tek tek fenolik bileşikleri araştırılarak; FPJ'de toplam polifenoller (TP), toplam flavonoid (TF), toplam antosiyanin içeriği (TAC) ve antioksidan kapasite de değerlendirilmiştir. Çalışmamızda, INH ve R direnci ile ilişkili dirençli genler ile altı efflux pompa genlerinin MDR-TB klinik izolatlarındaki ilaç direnci seviyelerine katkısı da değerlendirilmiştir. MDR-TB izolatlarında *KatG*'nin ekspresyon seviyesi 24/27 (%88.88) 'de azalırken; MDR-TB izolatlarında sırasıyla 8 /27 (% 29.62) ve 4/27 (% 14.81), *inhA, oksiR-ahpC* ve *rpoB* genleri 24 /27'de (% 88.88) yüksek düzeyde eksprese edilmiş veya artmıştır. Ayrıca, efflux pompa genleri *drrA, drrB, efpA, Rv2459, Rv1634 ve Rv1250*, INH/R ile FPJ kombinasyonu etkisinde, yüksek düzeyde eksprese edilerek MDR-TB izolatlarının genel direncine katkı gösterdiği görülmüştür.

Anahtar Kelimeler: Taze nar suyu (FPJ), Tüberküloz, Sinerjik etki, Antimikrobiyal aktivite, Yeni anti-tüberküloz ilaçlar

EXPANDED ABSTRACT

Mycobacteria are aerobic-to-microaerophilic, Grampositive, and non-sporulating members which belong to the Actinomycete family (Shinnick and Good, 1995). A well-known mycobacteria characteristic is that, during staining with arylmethane dyes, the bacteria appeared to resist destaining with the weak mineral acids or acidalcohol solutions that are normally used during the Gram stain. Therefore, mycobacteria are considered as Acid-Fast Bacillus (AFB). Acid-fastness is described as a unique but not exclusive property of mycobacteria. Mycobacteria are relatively slow-growing. While for mycobacteria have a generation time *in vitro* of approximately 18-24 hours, it is 20 minutes for several different types of bacteria. This slow growth is an impairment to timely diagnosis through culture-based methods. More than 20 mycobacterial species appeared to cause disease in humans, but *M. tuberculosis* is by far the most significant human pathogen. It is an obligate intracellular pathogen that belongs to the *M. tuberculosis* complex (MTBC). The MTBC also comprises subspecies *M. bovis, M. bovis* BCG (the vaccine strain), *M. microti and M. africanum* (Clinical and Institute, 2008), but *M. tuberculosis* causes almost all human TB by its unique pathophysiology

Considered as one of mankind's widespread, tuberculosis (TB) is infectious and deadly diseases. Millions of new cases are documented globally every year and approximately 30% of the global populace appeared to have potential TB infection. Specifically, tuberculosis is triggered by *Mycobacterium tuberculosis* (MTB) (Feng et al., 2010). World Health Organization (WHO) has reported in 2015 10.4 million cases of TB, equaling for every 100,000 people around 142 instances. An approximate 1.4 million mortalities, which are documented amid HIV- caused by TB, appeared to be negative individuals during 2015, with an additional 0.39 million deaths triggered by TB amid HIV-negative people. TB, described as one of the 10 prevalent causes of mortality globally, caused more mortalities than HIV/AIDS during 2015. In 2015, around 480,000

novel cases of multidrug-resistant TB (MDR-TB) and 100,000 individuals with rifampicin-resistant TB (RR-TB) were reported to be eligible for MDR-TB treatment. MDR/RR-TB caused the death of 250,000 during 2015. The majority of TB cases and deaths occurred in Asia. An approximate 52% of those eligible for MDR/RR-TB therapy were treated successfully, with 17% dying and 9% experiencing treatment failure. The level of treatment success for patients suffering XDR-TB did not exceed 26% (Organization, 2016). The 2015 annual tuberculosis report from WHO states that "in the absence of new tuberculosis drugs and regimens, it will not be easy to advance therapy results in the near future. Moreover, intensified investigations and development are considered as one of the three tenets of WHO Global Tuberculosis Strategy that will have essential role in enhancing the diminution in the occurrence of tuberculosis (Wallis et al., 2016). The development of new antimycobacterial drugs, which provide hope that future treatment regimens for drug-resistant TB will be more effective and more efficient than the current programs, appeared to be as one of the great achievements in TB management over the past 15 years

Considerably elevate levels of polyphenols are found in pomegranates (*Punica granatum Linn*) in contrast to alternative fruits and vegetables. Within Ayurvedic, Unani and Chinese oriental medicine practices, the pomegranates have been applied extensively. In folklore medicine, the plant has been used in the treatment of different ailments like ulcer, snakebite, and hepatic damage. Middle-Eastern and Mediterranean nations comprise the key areas of pomegranate growth and production (Jbir, Hasnaoui, Mars, Marrakchi, & Trifi, 2008; Melgarejo et al., 2009). Pomegranates are a significantly rich origin of punicalagin isomers, varied flavanols (catechins beingcatechins and epicatechin, and gallocatechins being gallocatechin and epigallocatechin), ellagic acid, anthocyanins (cyaniding 3, 5-di as well as 3-O-glucoside, delphinidin 3,5- di in addition to 3-O-glucoside, pelargonidin 3,5-di as well as 3-O-glucoside), etc (González-Molina et al., 2009; Kelawala and Ananthanarayan, 2004; Xu et al., 2005).

The global growth in multi-drug resistant *M. tuberculosis* and the imminence of tuberculosis epidemic demand unconventional therapeutic approaches to enhance the efficiency of existing drugs. *Punica granatum*, known as pomegranate, is a member of the Punicaceae family, broadly documented for its potency against a wide spectrum of bacterial pathogens, deserves further scrutiny in this respect. Within this scope, the aim of present study was to investigate the effect of fresh pomegranate juice (FPJ) on the antibacterial activity of anti-tuberculosis drugs (Rifampin (R) and Isoniazid (INH)) against MDR-TB clinical isolates. Resistance profiles of *M. tuberculosis* were determined by susceptibility test using BACTEC MGIT 960 system. Four concentrations of fresh pomegranate juice (FPJ) (5%, 10%, 15%, and 20%) were evaluated in combination with R and INH at a dose range of (1.0 µg/ml), and (0.1µg/ml), respectively against the drug-resistant isolates by the BACTEC MGIT 960 system. Moreover, this study investigated individual phenolic compounds of FPJ by using high-performance liquid chromatography (HPLC). The total polyphenols (TP), total flavonoid (TF), total anthocyanins content (TAC), and the antioxidant capacity were also assessed in FPJ. Synergistic effects were observed between R and INH with FPJ against all tested strains. However, combination therapy of R was more effective than INH one. Therefore, the combination of R and FPJ has been used against (27) MDR-TB clinical isolates. 5% of FPJ plus R (1.0 µg/ml) were found to suppress the growth of one isolates for first group (INH and R resistant), while, for second (SM, R and INH resistant) and third group (INH, EMB, R and SM resistant), 5% of FPJ demonstrated no synergistic impact with R. Moreover, 10% of FPJ and R (1.0 µg/ml) inhibited the bacterial growth of three isolates of first group and two and one for second and third group, respectively. Remarkably, 15% of FPJ plus R (1.0 µg/ml) appeared to inhibit the growth of MDR-TB isolates for all tested groups indicating a strong synergistic effect. Regarding H37RV, the complete inhibition of the bacterial growth was found to occur at 15% and 20% concentrations of FPJ only. Minimum inhibitory concentration (MIC) of FPJ for first group ranged from

(4%-13%), while for second group and third group were approximately between (10%-15%). Thus, FPJ at 15% inhibited 100% of bacteria for all tested isolates ($MIC_{100\%}$ =15%). Phenolic compounds identified in analyzed cultivars were shikimik cid, gallic acid, benzoic acid, syringic, folic acid, pelargonidin, naringin+ellagic acid, naringenin, chlorogenic acid, caffeic acid, catechin, myricetin, Kaempferol, Quercetin, cyanidin-3-glycoside, p-cummaric acid, ferulic acid, and rutin. Total phenolic (TP), total flavonoid (TF), and total anthocyanin (TA) content were 841.5 mg /L, 638.73 mg RE/L, and 47.43 mg/L, accordingly. Overall, FPJ displayed synergetic effect with R against MDR-TB clinical isolates, which might be credited to its high content of polyphenol and antioxidant capability.

Numerous investigations demonstrate efflux as a worldwide bacterial mode of action contributing to drug resistance and further the antibiotics activity subject to efflux can be improved by the combined usage of efflux inhibitors. Nevertheless, the efflux contribution to the overall antibiotic resistance levels of *M. tuberculosis* clinical isolates is poorly understood and still is ignored by many. Here, we evaluated the contribution of resistant genes associated with INH and R resistance plus six putative drug efflux pump genes to the drug resistance levels in MDR-TB clinical isolates. While the expression level of *katG* was down-regulated in 24/27 (88.88%) MDR-TB isolates, *inhA*, *oxyR-ahpC*, and *rpoB* appeared to be either overexpressed or up-regulated in 8/ 27 (29.62%), 4/27 (14.81 %), and 24/ 27 (88.88%), respectively in MDR-TB isolates. Moreover, the efflux pump genes *drrA*, *drrB*, *efpA*, *Rv2459*, *Rv1634*, and *Rv1250* were overexpressed under INH/RIF plus FPJ stress demonstrating the contribution of these efflux pumps to the overall resistance of MDR-TB isolates. These results displayed that the drug resistance levels of MDR-TB clinical isolates are a combination among drug efflux and the presence of target-gene mutations, a reality that is often disregarded by the specialists of tuberculosis in favor of the almost undoubted significance of drug target-gene mutations for the resistance in *M. tuberculosis*.

GENİŞLETİLMİŞ ÖZET

***Mycobacterium tuberculosis* özellikleri:** Bakteriyolojik özellikleri ve DNA benzerlikleri nedeniyle birbirleriyle yakın ilişkili türler, "kompleks" başlığı altında toplanmaktadırlar."*M. tuberculosis* kompleks" *M. tuberculosis, M. bovis, M. microti, M. africanum* ve *M. bovis* BCG' yi içermektedir.Klinik açıdan bakıldığında hastalık yapma potansiyeli ve halk sağlığı ile yakın ilişkisi nedeniyle *M. tuberculosis* cinsin en önemli üyesidir ve günümüzde insanlarda görülen tüberkülozun esas nedenidir. Çok az sayıda (% 1 - 2) olguda *M. bovis* ve *M. africanum* etken olarak saptanır. *M. microti* ise kemiriciler için patojen olup insanlarda hastalık yapmaz. *M. tuberculosis* kompleks dışındaki mikobakterilere "tüberküloz dışı mikobakteriler" veya "atipik mikobakteriler" denmektedir. Bunlar doğada, toprakta, suda bolca bulunurlar, insandan insana geçişleri çok enderdir ve çoğu patojen değildir.Bu grubun üyeleri ender olarak hastalık oluştururlar.

Mikobakterilerde Dışa Atim Pompaları Ve Ilaç Direnci: Tüberküloz tedavisinde kullanılan ilaçlar ikiye ayrılırlar: 1) Kabul edilebilir düzeydeki toksisite profili ile birlikte en efektif olan "birinci sıra" ilaçlar, 2) Genellikle daha az etkili, daha pahalı ve daha çok toksisitesi olan "ikinci sıra" ilaçlar. Birinci sınıfta bulunanlar; başta izoniazid olmak üzere, rifampin, etambutol, streptomisin ve pirazinamid (ve onun türevi morfozinamid)' dir. Tüberkülozlu hastaların büyük çoğunluğu bu ilaçlarla başarılı bir şekilde tedavi edilebilirler. İkinci sınıfta etionamid, paraminosalisilik asid, tiasetazon (amitiozon), sikloserin, siprofloksasin, ofloksasin ve streptomisin dışı aminoglikozidler olan viomisin, kapreomisin, kanamisin ve amikasin bulunur. Son 20 yılda AIDS olgularının artması ve bu hastalığın ileri döneminde olguların yaklaşık 1/3' ünde *Mycobacterium avium* kompleksi (MAC) bakterilere bağlı dissemine atipik tüberküloz enfeksiyonu gelişmesi yukarıdaki sınıflandırmaya "MAC türü mikobakterilere karşı kullanılan" ilaçlar sınıfının eklenmesini gerektirmiştir. MAC mikobakterileri yukarıda belirtilen birinci sınıftaki ilaçlara ve ikinci sınıftakilerin çoğuna dirençlidir. Bu

üçüncü sınıfta bulunan ilaçlar rifabutin, makrolid antibiyotikler olan klaritromisin ve azitromisin.fluorokinolon türevleri olan siprofloksasin ve ofloksasin, aminoglikozitlerden amikasin ve bir lepra ilacı olan klofazimin' dir.

M. tuberculosis'te MFS ailesine ait pompaları kodladığı düşünülen genlerin olduğu gösterilmiştir. Bu dışa atım pompalarının izoniazid, rifampin, tetrasiklin, eritromisin, kanamisin gibi tüberküloz ilaçlarına karşı oluşan dirençten sorumlu olduğu düşünülmektedir. SMR ailesi, sekonder tip dışa atım pompalarının en küçük ailesini oluşturmaktadır. Tetrafenil fosfoniyum, etidyum bromid, eritromisin, akriflavin, safarin O, pironin Y gibi bileşiklerin dışa atımından sorumlu tutulmaktadır. *M. tuberculosis*'te SMR ailesine ait Mmr dışa atım pompalarının varlığı gösterilmiştir. RND ailesi ise tüm canlı organizmalarda bulunmaktadır, fakat sadece gram negatif bakterilerde ilaç direnci ile ilişkilendirilmiştir. Bu tip dışa atım pompaları zar füzyon proteini ve dış zar proteininin yapısal organizasyonu ile dışa atımı gerçekleştirir. Eritromisin, novobiosin, sodyum dodesil sulfat gibi bileşikler bu pompa aracığıyla hücre dışına atıl maktadır. *M. tuberculosis* genomu bu aileye ait proteinleri kodlayan 13 gen bulundurmaktadır. Bu proteinler mikobakteri türlerine özgül olmalarından dolayı mikobakteri zar proteini olarak adlandırılmaktadır. ABC tipi dışa atım pompalarını kodlayan genler, *M. tuberculosis* genomunun %2,5'ini oluşturmaktadır. *M. tuberculosis*'de 37 tam veya tam olmayan ABC dışa atım pompası tanımlanmıştır ve bunlardan bazıları ilaç direnci ile ilişkilendirilmektedir

Tropik ve subtropik meyve olarak bilinen narın (*Punica granatum*L.), zengin besin değeri ve insan sağlığı üzerindeki olumlu etkilerinden dolayı günümüzde önemi giderek artmaktadır. Nar;eski çağlardan beri bilinmekte, taze olarak tüketilebildiği gibi, meyve suyuna, meyve suyu konsantresine, reçele, şaraba ve likör işlenebilen, çeşitli gıdalara renk verici ve tatlandırıcı olarak katılan ve içerdiği biyoaktif bileşenler sayesinde yüzyıllardan beri halk arasında uygulanan geleneksel tedavi yöntemlerinde kullanılan bir meyvedir. Bu bağlamda, uzun yıllar

süren bilimsel çalışmalar, narın insan vücudunu pozitif olarak etkileyen besin içerikleri ile yüklenmiş olduğunu ortaya çıkarmıştır.

Çalışmanın Amacı

1- Klinik örneklerden izole edilen çoklu ilaç dirençli TB izolatlarına karşı Rifampin ve Isoniazid ile taze nar suyunun antimikrobiyal aktivitesinin incelenmesi
2- Taze nar suyunun polifenolik profilinin değerlendirilmesi
3- Tedavi uygulandıktan sonra *rpoB* (R), *katG* (INH), *inhA* promotörü (INH) ve *oxyR-ahpC* bölgesinin açık/kapalı olduğunun kontrol edilmesi
4- Karışımın *efpA, Rv1250, Rv1634, Rv2459, drrA,* and *drrB* gibi efflux genleri üzerindeki etkisinin incelenmesi

M. tuberculosis 'in direnç profilleri BACTEC MGIT 960 sistemi kullanılarak duyarlılık testi ile belirlenmiştir. Dört adet taze nar suyu konsantrasyonu BACTEC MGIT 960 sistemi ile ilaca dirençli izolatlara karşı sırasıyla; (FPJ) (% 5,% 10,% 15 ve% 20), R ve INH ile kombinasyon halinde (1.0 ug / ml) ve (0.1ug / ml) doz aralığında değerlendirilmiştir. Ayrıca, çalışmamızda yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak FPJ'nin tek tek fenolik bileşikleri araştırılarak; FPJ'de toplam polifenoller (TP), toplam flavonoid (TF), toplam antosiyanin içeriği (TAC) ve antioksidan kapasite de değerlendirilmiştir.

Test edilen tüm suşlarda R ve INH ile FPJ arasındaki sinerjitik etki gözlenmiştir. Ancak; R+ FPJ'nin tedavi kombinasyonun INH+ FPJ’ den daha etkiliyeci olduğu görülmüştür. Bu sebeple, R ve FPJ kombinasyonu 27 MDR-TB klinik izolatına karşı denenmiştir. % 5 oranında R+ FPJ'nin (1.0 ug / ml), birinci gruptan (INH ve R dirençli) bir izolatın üremesini baskılarken, ikinci (SM, R ve INH dirençli) ve üçüncü grupta (INH, EMB, R ve SM dirençli), % 5’ lik FPJ, R ile sinerjik bir etki göstermemiştir. Ayrıca, % 10’ lik FPJ ve R (% 1.0 ug/ml), birinci gruptaki üç izolatın, ikinci ve üçüncü grup için sırasıyla iki ve bir izolatın bakteriyel üremesini inhibe etmiştir. Kayda değer şekilde %15 ‘lik FPJ ve R’ nin

(1.0 µg/ml) güçlü sinerjik etki gösteren tüm test gruplarında MDR-TB izolatlarının gelişimini inhibe ettiği görülmüştür. Referans suş olarak çalışmada kullanılan H37RV' ye bakıldığında, %15 ve %20' lik konsantrasyonlarında FPJ'nin tek başına tamamen bakteriyel üremeyi engellediği bulunmuştur. Birinci grup için FPJ' nin minimum inhibitör konsantrasyonu (MİK), % 4-% 13 iken ikinci grup ve üçüncü grup için % 10-% 15 arasında bulunmuştur. Böylece, %15' lik FPJ, test edilen tüm izolatlarda % 100 bakteriyi inhibe etmiştir. (MIC100% =% 15).

Nar suyunun HPLC analizi sonucunda tanımlanan fenolik bileşikler, gallik asit, benzoik asit, siringik, folik asit, pelargonidin, naringin + ellagic asit, naringenin, klorojenik asit, kafeik asit, kateşin, mirisetin, Kaempferol, Quercetin, siyanidin-3-glikozit, p-cummarik asit, ferulik asit ve rutin olduğu görülmüştür. Analiz sonucunda, Toplam fenolik (TP), toplam flavonoid (TF) ve toplam antosiyanin (TA) içeriği, 841.5 mg / L, 638.73 mg RE / L ve 47.43 mg / L olarak bulunmuştur. Genel olarak; FPJ'de gösterilen bu yüksek polifenol ve antioksidan potansiyeli MDR-TB klinik izolatlarına karşı R ile sinerjik etki göstermesinde etkili olduğunu düşündürmüştür.

Çalışmamızda, INH ve R direnci ile ilişkili dirençli genler ile altı efflux efflux pompa genlerinin MDR-TB klinik izolatlarındaki ilaç direnci seviyelerine katkısı da değerlendirilmiştir. MDR-TB izolatlarında *KatG*'nin ekspresyon seviyesi 24/27 (% 88.88) 'de azalırken; MDR-TB izolatlarında sırasıyla 8 /27 (% 29.62) ve 4/27 (% 14.81), *inhA, oksiR-ahpC* ve *rpoB* genleri 24 /27'de (% 88.88) yüksek düzeyde eksprese edilmiş veya artmıştır. Ayrıca, efflux pompa genleri *drrA, drrB, efpA, Rv2459, Rv1634* ve *Rv1250,* INH/R ile FPJ kombinasyonu etkisinde, yüksek düzeyde eksprese edilerek MDR-TB izolatlarının genel direncine katkı gösterdiği görülmüştür. Bu sonuçlar, MDR-TB klinik izolatlarında ilaç direnç seviyesinin ilaç atımı ve hedef gen mutasyonlarının varlığı arasında bir kombinasyon olduğu sonucunu göstermiştir. Aslında *M. tuberculosis* ilaç dirençliliğinde neredeyse şüphesiz önemi olduğu bu çalışmayla gösterilen hedef-gen mutasyonların uzmanlarca dikkate alınması ve çalışılması önem arz etmektedir.

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LIST OF SYMBOLS AND ABBREVIATONS

MTB	: *Mycobacterium tuberculosis*
MDR-TB	: Multidrug-resistant tuberculosis
°C	: Degree Celcius
WHO	: World Health Organization
RR-TB	: Rifampicin-resistant tuberculosis
TB	: Tuberculosis
HIV	: Human immunodeficiency
INH	: Isoniazid
EMB	: Ethambutol
PZA	: Pyrazinamide
R	: Rifampin/Rifampicin
CT	: Clinical trials
ATP	: Adenosine triphosphate
NSAIDs	: Aanti-inflammatory drugs
AMPs	: Antimicrobial peptides
P. granatum L	: Pomegranates fruits
FPJ	: Fresh pomegranate juice
TPC	: Total phenolic content
TF	: Total flavonoids
TA	: Total anthocyanins
qPCR	: Real-time quantitative PCR
ORF	: Open reading frame
ETH	: Ethionamide
XDR TB	: Extensively drug-resistant tuberculosis
HPLC	: High-performance liquid chromatography

1. INTRODUCTION

Considered as one of mankind's widespread, tuberculosis (TB) is infectious and deadly diseases. Millions of new cases are documented globally every year and approximately 30% of the global populace appeared to have potential TB infection. Specifically, tuberculosis is triggered by *Mycobacterium tuberculosis* (MTB) (Feng et al., 2010). World Health Organization (WHO) has reported in 2015 10.4 million cases of TB, equaling for every 100,000 people around 142 instances. An approximate 1.4 million mortalities, which are documented amid HIV- caused by TB, appeared to be negative individuals during 2015, with an additional 0.39 million deaths triggered by TB amid HIV-negative people. TB, described as one of the 10 prevalent causes of mortality globally, caused more mortalities than HIV/AIDS during 2015. In 2015, around 480,000 novel cases of multidrug-resistant TB (MDR-TB) and 100,000 individuals with rifampicin-resistant TB (RR-TB) were reported to be eligible for MDR-TB treatment. MDR/RR-TB caused the death of 250,000 during 2015. The majority of TB cases and deaths occurred in Asia. An approximate 52% of those eligible for MDR/RR-TB therapy were treated successfully, with 17% dying and 9% experiencing treatment failure. The level of treatment success for patients suffering XDR-TB did not exceed 26% (Organization, 2016). The 2015 annual tuberculosis report from WHO states that "in the absence of new tuberculosis drugs and regimens, it will not be easy to advance therapy results in the near future. Moreover, intensified investigations and development are considered as one of the three tenets of WHO Global Tuberculosis Strategy that will have essential role in enhancing the diminution in the occurrence of tuberculosis (Wallis et al., 2016). The development of new antimycobacterial drugs, which provide hope that future treatment regimens for drug-resistant TB will be more effective and more efficient than the current programs, appeared to be as one of the great achievements in TB management over the past 15 years

Considerably elevate levels of polyphenols are found in pomegranates (*Punica granatum Linn*) in contrast to alternative fruits and vegetables. Within Ayurvedic, Unani and Chinese oriental medicine practices, the pomegranates have been applied extensively. In folklore medicine, the plant has been used in the treatment of different ailments like ulcer, snakebite, and hepatic damage. Middle-Eastern and Mediterranean nations comprise the key areas of pomegranate growth and production (Jbir, Hasnaoui, Mars, Marrakchi, & Trifi, 2008; Melgarejo et al., 2009). Pomegranates are a significantly rich origin of punicalagin isomers, varied flavanols (catechins beingcatechins and epicatechin, and gallocatechins being gallocatechin and epigallocatechin), ellagic acid, anthocyanins (cyaniding 3, 5-di as well as 3-O-glucoside, delphinidin 3,5- di in addition to 3-O-glucoside, pelargonidin 3,5-di as well as 3-O-glucoside), etc (González-Molina et al., 2009; Kelawala and Ananthanarayan, 2004; Xu et al., 2005). The chemical formula of major identified compounds of pomegranate are documented in Table 1.1. Within this context, the aim of our study is

1- To examine the antimicrobial activity of rifampin (R) and isoniazid (INH) with fresh pomegranate juice (FPJ) against Multidrug-resistant TB clinical isolates.
2- To evaluate polyphenolic profile of fresh pomegranate juice (FPJ).
3- To check whether *rpoB* (R), *katG* (INH), the *inhA* promoter (INH), and *oxyR-ahpC* region (INH) are up regulated or down regulated after applying the treatment.
4- To examine the influence of this mix on efflux genes such as *efpA*, *Rv1250*, *Rv1634*, *Rv2459*, *drrA*, and *drrB*.

Table 1.1. Chemical formula of major identified compounds of pomegranate

Name of compounds	Chemical formula
Caffic acid	$C_9H_8O_4$
Caffeoylquinic acid	$C_{16}H_{18}O_9$
***p*-coumaric acid glucuronide**	$C_{15}H_{18}O_9$
Brevifolin carboxylic acid	$C_{13}H_8O_8$
Ellagic acid	$C_{14}H_6O_8$
Glucogallin	$C_{13}H_{16}O_{10}$
Eschweilenol-C	$C_{20}H_{16}O_{12}$
Quercetin-3-O-glucoside	$C_{21}H_{20}O_{12}$
Galloyl HHDP glucuronide Lagerstannin C	$C_{27}H_{22}O_{19}$
Punicalin	$C_{34}H_{22}O_{22}$
Digalloyl HHDP hex pedunculagin II	$C_{34}H_{26}O_{22}$
Gallic acid	$C_7H_6O_5$

1.1. Current treatment methods

The treatment method for TB, which aims to cure the patient, avert difficulties and death, avoid relapses, diminish the transmission potential to susceptible individuals, and restrict the emergence and spread of drug-resistant strains, requires the use of multiple medications. Therapy should encompass an intensive stage with aim to reduce the bacterial burden, followed by a consolidation stage for six months duration. Patients with extensive bone involvement or those with cerebral tuberculomas might require longer treatments (Organization and Initiative, 2010). During the previous ten years, tuberculosis diagnosis has undergone swift evolution. Culture remains the standard approach for diagnosis and drug-susceptibility testing, however, molecular diagnostic techniques appeared to become broadly attainable and enable preliminary assessment of drug susceptibility in addition to rapid diagnosis. Such methods, which might be expected to be effective for patients, facilitate prompt commencement of TB

treatment regimens. Preferably, the initial isolate for each patient has to be assessed in order to abolish baseline drug resistance for all patients who have a history of prior TB treatment or contact with an individual with drug-resistant isolates. The regular therapy regimen for drug-susceptible tuberculosis, includes an induction phase, consists of rifampin, isoniazid, and pyrazinamide. Ethambutol, which is added against unknown resistance to one of the three core agents, can be discontinued once susceptibility to isoniazid, rifampin, and pyrazinamide has been verified. The toxic effects of ethambutol is challenging in young children, therefore, this drug is frequently excluded if the source of infection is known to have drug-susceptible tuberculosis. The induction phase is succeeded by a consolidation phase that comprised of isoniazid and rifampin for an extra 4 months of therapy. The regular therapy regimen for drug-susceptible tuberculosis is described as a long course of therapy in contrast to the duration of treatment of other bacterial infectious diseases (Clark, 2010; Hayashi et al., 2011). The major challenges of the prolonged to success are managing drug noxiousness and ensuring that patients adhere the full treatment course. As the drug toxicity is considerable, thus, it has been reported that 3 to 13 percent of patients have hepatotoxicity (Saukkonen et al., 2012). A 15% occurrence of adverse drug reactions results in interruption or discontinuation of one or more of the medications for patients with drug-susceptible tuberculosis, a recent prospective cohort study reported (Lv et al., 2013). Of these undesirable reactions, 7.7% caused hospitalization, death or disability. Hepatotoxic effects, gastrointestinal disorders, allergic reactions, and arthralgias were considered as the most common adverse reactions. Overall, 16-49% of individuals did not complete their treatment (Volmink and Garner, 2007). Failure to complete therapy is due to several reasons including adverse drug reactions, treatment cost, stigma, and the belief of the patient that cure has been accomplished if the symptoms have resolved and bacteria is no longer existed in the sputum (Munro et al., 2007). Treatment support and direct-observation programs are useful in improving adherence but have not

entirely overcome these factors. There is more evidence to support endorsements for treatment of drug-susceptible tuberculosis than there is to support treatment recommendations for drug-resistant tuberculosis (Horsburgh Jr, Barry and Lange, 2015). The treatment success rate of drug-resistant TB is 50%, however, the outcome is far better for patients suffering drug-susceptible TB (85%) (Organization, 2013). MDR-TB is described to be as resistance to at least rifampicin and isoniazid. XDR-TB comprises MDR-TB instances with additional resistance to any fluoroquinolone and any of the injectable agents. In XDR-TB cases, the outcomes of the treatment are low (40% on average) particularly when the tuberculosis is maintained by isolates of MTB with a resistance pattern beyond XDR (Falzon et al., 2012; Migliori et al., 2012). Based on the outcomes of drug-susceptibility testing of the *M. tuberculosis* isolate from the patient, the primary treatment should be individually specified. Empirical regimens could be applied in case this information is not available and when the dug susceptibility results become accessible, the treatment regime has to be modified (Lange et al., 2014). The initial regimen, which has been recommended by WHO for MDR-TB treatment, includes four agents to which the patient isolate is sensitive in the induction phase, lasts 6 to 8 months.(Ahuja et al., 2012). It has been documented that an induction phase with more medications to which the patient's isolate is sensitive is related with improved results (Aung et al., 2014; Franke et al., 2015; Kuaban et al., 2015). The poorer antimycobacterial activity of these drugs needs more medication to be used as compared with rifampin, isoniazid, and pyrazinamide. Moreover, these drugs more toxic than those employed to treat drug-susceptible tuberculosis. The appropriate duration of MDR-TB treatment still requires to be defined.

The WHO suggests a minimum of 20 months (encompassing the induction phase), although this recommendation appeared to be based on a database which included few patients treated for shorter periods. An observational cohort research of a highly intensive susceptible 9-month regimen of seven medications for the

treatment of MDR-TB in Bangladesh showed a raised proportion of fruitful results, and the regimen is presently going through assessment within a potential, randomized, multicenter clinical trial (Aung et al., 2014).

The WHO recommended 20 months for the treatment of MDR-TB based on a database that comprised several patients who treated for shorter periods. Seven drugs, which have been used for nine months to treat MDR-TB in Bangladesh, displayed a high proportion of successful results, and the regimen is currently undergoing evaluation in a prospective, randomized, multicenter clinical trial. The 'Bangladesh regimen' and similar regimens, which have been used for the treatment of MDR-TB for 9 and 12 months, achieved an average cure level of 85 to 89% with minor relapses. However, until the outcomes of the randomized trial are accessible, it is not apparent whether these results could be generalized.

Patients suffering human immunodeficiency (HIV) infection who infected either by drug-sensitive or drug resistant tuberculosis should receive antiretroviral therapy (ART) while being given treatment for tuberculosis (Aung et al., 2014; Kuaban et al., 2015). Interactions between antiretroviral medications and tuberculosis agents are usually occurred and might necessitate dose adjustment or replacement of another drug (Semvua et al., 2015). Patients being given ART and tuberculosis treatment are at high risk of IRIS (the immune reconstitution inflammatory syndrome), cytokine storm syndrome and a sudden systemic inflammation (Müller et al., 2010).

1.2. Composition of the Pomegranate

Extracts of pomegranate (juice, seed oil and flower) are known for having strong in vitro as well as in vivo anti-inflammatory, antidiabetic, antitumour, and antioxidant effects (Aviram et al., 2008). Within traditional Chinese medicine, different extracts and preparations of pomegranate encompassing the fruit juice, root or bark as well as the dried peels have been employed in the treatment of various diseases. The preparations, which are edible, have been used as fresh juice,

jam, jelly and canned drinks among others, in the colouring and flavouring of products, as well as in therapeutic mixtures (Lei et al., 2007; Viuda-Martos et al., 2010). These pomegranate preparations comprise considerably raised levels of antioxidants compared with an alternative vegetable or fruit, and this includes polyphenols and flavonoids (Fadavi et al., 2005; Mousavinejad et al., 2009) (Figure1.1).

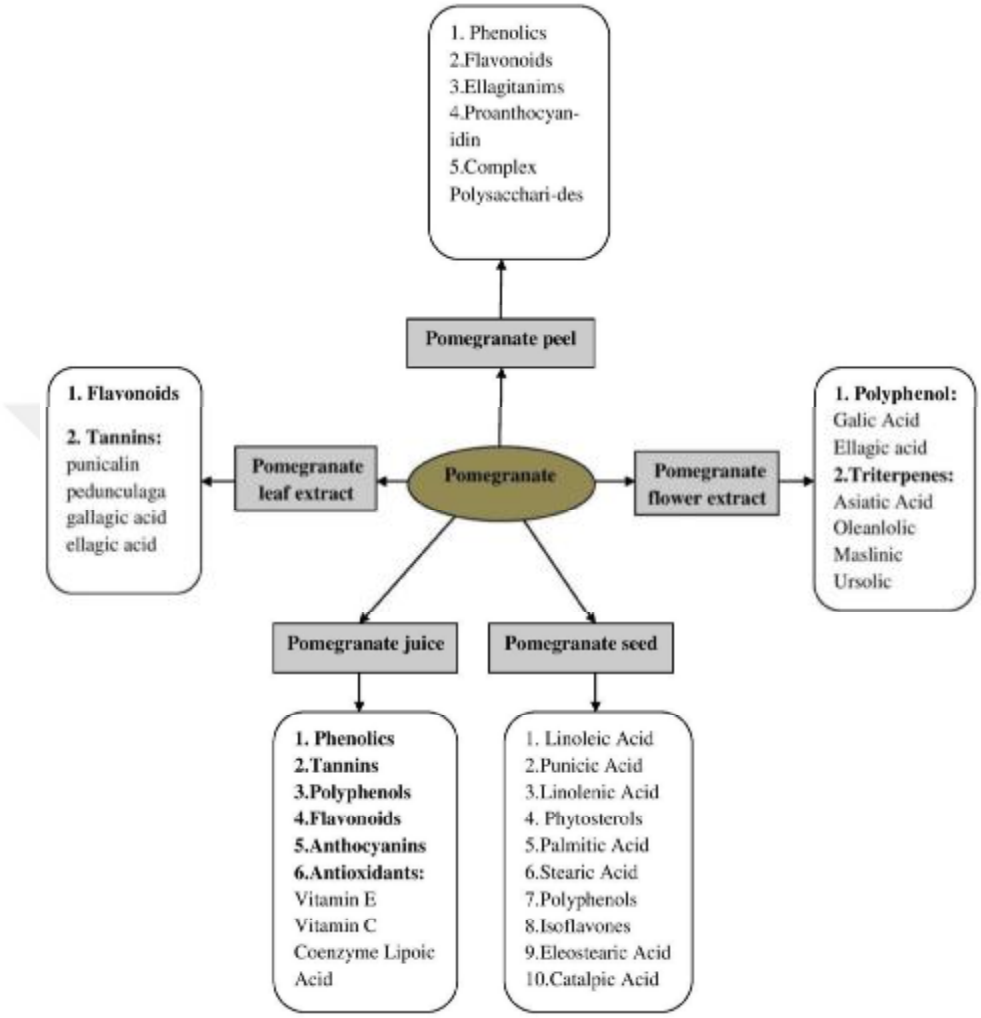

Figure.1.1. Chemical composition of pomegranate

2. LITERATURE REVIEW

2.1. Mycobacteria

Mycobacteria are aerobic-to-microaerophilic, Grampositive, and non-sporulating members which belong to the Actinomycete family (Shinnick and Good, 1995). A well-known mycobacteria characteristic is that, during staining with arylmethane dyes, the bacteria appeared to resist destaining with the weak mineral acids or acidalcohol solutions that are normally used during the Gram stain. Therefore, mycobacteria are considered as Acid-Fast Bacillus (AFB). Acid-fastness is described as a unique but not exclusive property of mycobacteria. Mycobacteria are relatively slow-growing. While for mycobacteria have a generation time *in vitro* of approximately 18-24 hours, it is 20 minutes for several different types of bacteria. This slow growth is an impairment to timely diagnosis through culture-based methods. More than 20 mycobacterial species appeared to cause disease in humans, but *M. tuberculosis* is by far the most significant human pathogen. It is an obligate intracellular pathogen that belongs to the *M. tuberculosis* complex (MTBC). The MTBC also comprises subspecies *M. bovis, M. bovis* BCG (the vaccine strain), *M. microti and M. africanum* (Clinical and Institute, 2008), but *M. tuberculosis* causes almost all human TB by its unique pathophysiology.

2.2. Relevant Pathophysiology

M. tuberculosis is transmitted when droplet nuclei is expelled by a patient with pulmonary TB into the air (usually by coughing), which are then inhaled by a susceptible person. There are three different outcomes to this inhalation. The best outcome is that the bacteria do not create infection or the initial infection is entirely eliminated by the host’s immune system. Alternatively, the bacteria are not completely eliminated but organized in a dormant state, called latent TB infection, which is routinely abbreviated as LTBI. LTBI is by definition asymptomatic, and roughly one-third of the world's population has been infected with *M. tuberculosis*

(Dye et al., 1999). The third potential outcome of the droplet nuclei inhalation is that persons with LTBI might progress to active TB. This progression happens anytime from immediately to decades after infection. Approximately 10% of persons with LTBI eventually progress to TB, but persons with an impaired immune response (e.g. HIV) increase the risk of progression to active TB (Gray and Cohn, 2013).

Pulmonary TB manifests classically by chronic, usually productive, coughing. Patients sometimes observe blood in their sputum (haemoptysis), which reflects offensive disease. Other common TB symptoms are night sweats, fever, inadvertent weight loss and fatigue. *M. tuberculosis* bacteria can escape from the lungs, thus, TB can also present outside of the lungs in any so-called extrapulmonary site, such as the central nervous system, bone, or lymph nodes (Zumla et al., 2013).

2.3. Necessity for new anti-tuberculosis medications and schemes

Current regimens for the treatment of drug-sensitive tuberculosis appeared to be high potent particularly when the patient adherence is ideal. However, when the realities of actual life tuberculosis programmatic conditions are considered outcomes are far from ideal (Shin and Kwon, 2015; AlMatar et al., 2017). The proposed treatment regimens by WHO's have diverse inherent problems for not only drug-susceptible but also drug-resistant tuberculosis, rendering the discovery of novel antituberculosis agents a priority for both clinical and public health (Nunn et al., 2011; EAST, 1973; EAST, 1974; WHO organization, 2010; Falzon et al., 2011; Sacks and Behrman, 2008). The first challenge of the treatment of drug-susceptible tuberculosis is long-lasting, which extends at least six months (Ginsberg and Spigelman, 2007). Moreover, the most potent first-line oral agents, which are isoniazid (INH) , ethambutol (EMB), pyrazinamide (PZA), and rifampin (R), have to be taken concurrently in the first 2 months of treatment , and two (INH and R) for a successive period of 4 months in the continuation phase, resulting in

the complications associated with the adherence of patients' to the treatment. These regimens, which are linked with patients' non-adherence and high death rates, can lead to establish incurable cases of drug-resistant tuberculosis (Arbex et al., 2010). Therefore, a priority for the development of new tuberculosis drugs has been for effective sterilising agents that can diminish the duration of treatment to 2 months or less, could reduce distribution cost and programme supervision and improve the adherence of patients (Conde and Silva, 2011). Moreover, drugs that would diminish not only the total duration of the treatment but also the frequency of drug intake would be considered as ideal agents. The life cycle studies of ***M. tuberculosis*** showed the ability of mycobacteria to develop a dormancy phenotype under both anaerobic circumstances and nutrient deprivation (Garton et al., 2002; Wayne and Hayes, 1996; Loebel et al., 1933; Shleeva et al., 2011). Persisters bacterial populations, which might last for a maximum of 100 days following the start of the treatment of antituberculosis treatment, need reviving factor for replication in order to prompt rapid duplication of the dormant bacilli. The reason for a long duration of therapy for tuberculosis is that these dormant bacteria appear to be tolerant to diverse antituberculosis agents. Therefore, novel regimens are required for an entire killing of the persisters, regardless of the development stage reached by the ***M. tuberculosis*** bacilli (Mukamolova et al., 2010; Zhang et al., 2012; Honeyborne et al., 2016).

Secondly, the growing global problem for both multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant TB (XDR-TB) required new antituberculosis agents to tackle this issue (Sullivan and Amor, 2016). Caused by ***M. tuberculosis*** bacilli resistant to at least INH and R, MDR-TB is currently widespread at the world level, with around 500,000 cases documented in 2015 (Nachega and Chaisson, 2003). XDR-TB, which is resistant to R, INH, plus any fluoroquinolone, and at least one of three injectable second-line drugs: amikacin, kanamycin, or capreomycin, has been recorded in 95 countries of the world. A combination of both second-line and third-line antituberculosis drugs, which are

considerably costlier, noxious and less efficient than standard treatment, is needed for patients suffering MDR-TB. Based on WHO's recommendations, second-line drugs are used for both MDR-TB or XDR-TB with a therapy duration of 18-24 months or longer (Prasad, 2010). These guidelines, which do not have the rigour of evidence according to the data from randomised controlled trials, are based on low-grade evidence, expert viewpoint, and little observational information. Implementations of such guidelines are dependent on not only the availability of drug-susceptibility testing, cost, doctors' preference, but also drug availability in developing countries. Therefore, there is an urgent need for new regimens to treat both MDR-TB and XDR-TB, which are shorter, more tolerable, more potent and have been evaluated under programmatic conditions (Abubakar et al., 2013, London et al., 2016). Thirdly, anti-tuberculosis medications, which have a great management problem in sub-Saharan Africa where the TB cases are mostly driven by the HIV epidemic, might interact with antiretroviral therapy (ART) (McIlleron et al., 2007).

Fourthly, new treatments are required for those with latent tuberculosis (LTBI) from drug-sensitive and drug-resistant strains of M tuberculosis before it might convert into active state. Based on WHO estimation, two billion people appeared to have LTBI and around 100 million people with LTBI will convert into active TB during their lifetime (Menzies et al., 2011; Sudre et al., 1992; Zumla et al., 2011). Although the occurrence of TB is comparatively low in the most developed countries, the LTBI reactivation in local and migrant societies account for a great TB burden (Yuen and Woo, 2002). The novel anti-tuberculosis medications with other intervention tactics, which play an essential role in order to improve the treatment of LBTI, are seen as the best way towards the eradication of TB. Thus, the novel anti-tuberculosis agents, which should have a good safety profile, be more potent than the existing agents, capable of reducing the treatment duration, be potent in the treatment of both MDR-TB and XDR-TB, be compatible with concomitant ARTs, and have no adverse activity with other tuberculosis

medications in the treatment regimen, are urgently needed. To treat LTBI, the medications should competently act against the diverse replications and physiological states of *M. tuberculosis*(Barry et al., 2009).

2.4. New anti-tuberculosis drugs

Currently, great efforts have been made in the development of novel anti-TB agents, which they are in various stages of the discovery, preclinical as well as clinical development (Figure 2.1). Therefore, the present TB medications are discussed (early stage, Phases I, II, III) and their activities as well as mode of action.

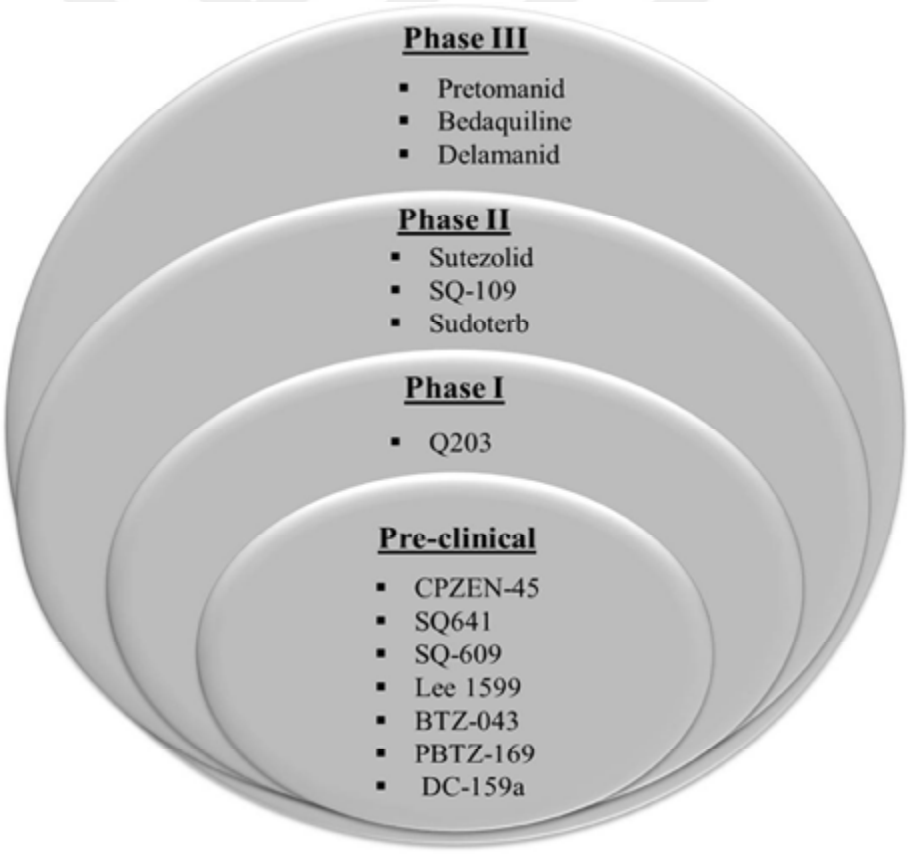

Figure 2.1. Anti-TB drugs at different stages which hold clinical promise

2.4.1. Bedaquiline

The diarylquinolones, approved to have bedaquiline under this classification, are the most advanced series of new anti-tuberculosis medications. Bedaquiline appears to be greatly selective and precisely hinders the activity of essential ATP synthase in both dormant and replicating mycobacteria but not in other eukaryotic or prokaryotic cells (Koul et al., 2007; Diacon et al., 2009). The rotor ring of the organism's F0F1 ATP synthase was the target due to the growth of drug-resistant mutants (Andries et al., 2005). *M. tuberculosis*, which survives in non-replicating state, is used a pool of ATP to retain an energized membrane manufactured by the synthase of F0F1 ATP (Watanabe et al., 2011). Therefore, bedaquiline is considered as a prime weapon to be used against dormant Mtb subpopulation. Bedaquiline, which is discovered during a phenotypic screen carried out by Janssen, displays great activity against both drug-sensitive and drug resistant isolates with MIC of 0.03 μg/mL and 0.12 μg/mL, respectively. Even though R and INH have a distinctive mode of action, bedaquiline is a potent inclusion to an MDR-TB therapy. Bedaquiline showed an outstanding activity in murine models. However, bedaquiline's effectiveness in mice has not been as distinct in humans, possibly due to restricted distribution in human granulomas (Lounis et al., 2008). Three clinical trials (CT), C208, C208-2 and C209, have been performed using bedaquiline against MDR-TB. Clinical trial C208, which is a phase II randomized investigation and double blinded placebo controlled, compared bedaquiline versus placebo when added to second-line anti-tuberculosis regimen in MDR-TB. Trials were conducted in two subsequent stages. An exploratory phase includes eight weeks bedaquiline treatment succeeded by standard therapy for MDR-TB followed by efficacy assessment stage which comprises twenty-four weeks bedaquiline therapy succeeded by standard therapy for MDR-TB up to 104 weeks (Diacon et al., 2009; Diacon et al., 2012a). Both phases were scrutinized separately. After the double-blinded phase was completed, patients were still given their treatment for MDR-TB. Microbiological efficacy,

pharmacokinetics, Safety and tolerability were checked after receiving last dose of bedaquiline. This trial has been performed twice with a larger sample of 161 patients including new countries and appraising clinical response at 24, 72 and 120 weeks. Time to alteration to a negative sputum and culture rate were investigated. C208-2 trail data has been released recently (Diacon et al., 2014). 205 patients with MDR-TB or XDR-TB were included in trial C209 where VIH+ patients with CD4 < 250 cells/µL were excluded. Bedaquiline dosage was given based on national guideline with a duration of 72-96 weeks and at least 12 months following culture conversion. A phase III trial included 600 patients with MDR-TB or pre-XDR-TB, smear sputum-verified, has been recently annulled (Fox and Menzies, 2013; Organization, 2015). Bedaquiline, well-known to have PK drug–drug interactions with the CYP3A4 inducer R, is a large lipophilic agent which is subject to the metabolism of CYP3A4. Bedaquiline, which has been associated with QT-prolongation, should be carefully given with other medications such as fluoroquinolones, macrolides, clofazimine, or medications that inhibit CYP3A4. Therefore, second generation diaryl quinolones that will enhance the physiochemical properties of drug and eliminate its cardiac liability are required. Great efforts have been made in order to develop more potent and less toxic analogs of beadquiline by which the co-crystal structure of bedaquiline bound to its active region within the ATP synthase (Preiss et al., 2015).

2.4.2. Delamanid

Delamanid, which is thought to mainly inhibit the synthesis of mycobacterial cell wall components including methoxy-mycolic and keto-mycolic acid, does not inhibit alpha-mycolic acid (Matsumoto et al., 2006; Lewis and Sloan, 2015). The clinical advantage for delamanid to be used only against mycobacterial infection might help prevent the generation of resistance as it has no activity against either gram-negative or gram-positive bacteria. Delamanid, described as a produg like pretomanid, needs metabolic activation for anti-

tuberculosis activity to be exerted. Reactive interferes in the bicyclic nitroimidazoles pathway might provide extra mode of action, encompassing disruption of cellular respiration (Mukherjee and Boshoff, 2011). The mycobacterial F420 coenzyme system is through to have a significant role in the activation of delamanid (Gurumurthy et al., 2012). Delamanid, which has MIC ranged from 0.006 to 0.024 g/mL, showed strong activity *in vitro* against standardized and clinical isolates of *M. tuberculosis*, with no antagonistic activity towards R, EMB, SM or INH, and no cross-resistance to these medications (Saliu et al., 2007; Sasaki et al., 2006). Regarding nontuberculous mycobacteria, delamanid displayed activity against *M. kansasii* and *M. bovis*, but not *M. abscessus*, *M. avium*, *M. fortuitum* or *M. chelonae*. *M. tuberculosis* might adapt intracellular sanctuary inside macrophages in order to avoid drug pressure. Delamanid appears to have similar intracellular bactericidal action of rifampicin (Matsumoto et al., 2006).

In vivo, delamanid, which was equally potent in immunocompromised mice, showed a dose-dependent reduction in *M. tuberculosis* colony counts. Medication doses associated with a diminution of 95% in CFU were 0.625 mg/kg, 40 mg/kg, 5 mg/kg, 3.5 mg/kg, and 160 mg/kg for delamanid, streptomycin, isoniazid, rifampicin, and ethambutol, respectively. A six-month a regimen of delamanid, rifampicin, and isoniazid was used to compare with standard treatment regimen of rifampicin, isoniazid, pyrazinamide, and ethambutol in a mouse model in order to assess the activity of delamanid. After six months, 0/6 mice within the delamanid division had traceable colonies of *M. tuberculosis*, versus 4/5 within the regular treatment group (Matsumoto et al., 2006). The MDR-end, A5343, TB-end trials will pay more attention on the employment of delamanid in combination therapy. The MDR-end trial, sponsored by the Korean centre for disease control, includes a regimen encompassing notonly delamanid and linezolid but also levofloxacin and Pyrazinamide. The A5343 trial considers linezolid and delamanid for the treatment of MDR-TB. The VTEU trial, sponsored by NIH

Division of Microbiology and Infectious Diseases, scrutinises the delamanid-encompassing injectable-free MDR-TB regimen against the care standard. Another study, sponsored by Otsuka Pharmaceuticals, commenced against MDR-TB in order to investigate the six-month safety, effectiveness and pharmacokinetic trial of delamanid within paediatric patients (Wishart et al., 2006).

2.4.3. Pretomanid

Pretomanid, which is presently in phase III clinical trials as division of a drug regimen comprising bedaquiline and linezolid, is a bicyclic nitroimidazofuran (Stover et al., 2000; Rivers and Mancera, 2008). Recently, a study has been commenced in order to assess safety, effectiveness and tolerability of pretomanid in combination with both bedaquiline and linezolid. The duration of this study was six months to treat patients with XDR-TB and intolerant or non-responsive MDR-TB. It has been documented that pretomanid appeared to be active against some drug-resistant and wild-type strains with no cross-resistance to other anti-tuberculosis medications. Moreover, pretomanid might be used for the treatment of LTBI as it displays an activity against non-replicating bacteria (Chetty et al., 2017). Bactericidal activity of pretomanid is exerted by inhibiting the biosynthesis of mycolic acid and by serving as a nitric oxide donor with consequent intracellular ATP depletion (Singh et al., 2008; Manjunatha et al., 2009). Furthermore, pretomanid exerts antimycobacterial activity against both replicating and nonreplicating mycobacteria and under aerobic and anaerobic conditions due to these disparate modes of action (Manjunatha et al., 2009). Pretomanid has produced promising findings from studies investigating pretomanid as part of a new TB regimen, including pretomanid, moxifloxacin, and pyrazinamide (PaMZ). However, there is no approval to be clinically used by any major regulatory agencies. Pretomanid, moxifloxacin, and pyrazinamide (PaMZ) regimen efficacy was first reported *in vivo*. Mice received PaMZ were healed more swiftly than mice treated by INH, R, and PZA regimen (Nuermberger et al., 2008). Consequently, a

randomized study was carried out in patients with treatment-naïve pulmonary TB by using number of novel anti-TB drugs including PaMZ regimen which has found to have the greatest between the new medication combinations, with its impact being comparable with INH, R, PZA, and EMB. Currently, the strongest experience with the PaMZ regimen and with the pretomanid has been provided by a phase 2 trial of bactericidal activity (Diacon et al., 2012b; Dawson et al., 2015b). In partly randomized clinical trial, 207 patients with pulmonary TB, drug-susceptible, smear-positive received for 8 weeks either PaMZ with pretomanid dosed at 100mg daily (Pa100MZ) or PaMZ with pretomanid dosed at 200 mg daily (Pa200MZ) or standard combination treatment with INH, R, PZA, and EMB. Twenty six patients suffering MDR-TB have received Pa200MZ regimen which showed potent anti-tuberculosis comparable with that attained within patients having drug-susceptible TB who treated by regular therapy. Regarding patients with drug-susceptible TB, Pa200MZ displayed strongest anti-tuberculosis activity, as compared with standard treatment. Furthermore, patients who treated by PaMZ regimens attained culture negative in liquid media more than those who treated by regular therapy. The final outcomes, which suggested a new regimen that might be potent for rapid treatment of MDR-TB, are promising, although substantial additional investigations are required (Dawson et al., 2015b). The PRACTECAL study, which was examined the use of various combinations of pretomanid, bedaquiline, moxifloxacin, linezolid and clofazimine for a 6 month period against MDR-TB, conducted by the Medecins Sans Frontiers (MSF) and received regulatory and ethics endorsement (Lessem, 2016). To date, pretomanid appeared to remain a promising new agent with the possibility to alternation the treatment approach of MDR-TB when used as an element of new therapy regimens (Dawson et al., 2015b; Diacon et al., 2015).

2.4.4. Q203

Q203, which is a new imidazopyridine that has displayed promise as a candidate drug for TB in preclinical studies, entered phase I clinical trials at the end of 2015, found to have activity in both anaerobic and aerobic conditions against not only intra-cellular and extracellular TB but also replicating and non-replicating bacteria (Lessem, 2016). Q203 showed strongly potent anti-tuberculosis *in vitro* against MDR-TB and XDR-TB at low concentration of drug. Regarding *in vivo* studies, Q203 was effective at does less than 1 mg per kg body weight (Pethe et al., 2013; Kang et al., 2014). The respiratory cytochrome bc1 complex considers as target to Q203 to inhibit ATP synthesis thus restricting energy conversion (Kang et al., 2014).

2.4.5. Sutezolid

Sutezolid is a linezolid analogue which exerts anti-tuberculosis activity by inhibiting the synthesis of proteins in bacteria (Kang et al., 2014). *In vitro*, Sutezolid appeared to be active against both *M. tuberculosis* and *M. avium*. Based on *in vitro* and *in vivo* studies, sutezolid showed a greater antimycobacterial activity and safety profile compared with linezolid. A dose of 600 mg of sutezolid given twice daily found to have greater whole-blood anti-tuberculosis activity as compared with linezolid dose (Cynamon et al., 1999; Wallis et al., 2011).

Sutezolid has been given to fifty patients having drug-susceptible TB at 600 mg doses twice daily or 1200 mg of doses once daily for two weeks (Zheng et al., 2013). Both dosing regimens, generally safe and well tolerated, resulted in easily detectable anti-tuberculosis activity in both sputum and blood. Sutezolid, which enters currently in phase II, is not ready yet to be used for patients and will need costly development with approval steps before it can assist to address tuberculosis (Wyskiel, 2016).

2.4.6. SQ109

SQ109, which was derived from the pharmacophore of ethambutol, is a 1,2-ethylenediamine (Protopopova et al., 2005). SQ109 displayed activity against wild-type *M. tuberculosis* as well as MDR-TB and XDR-TB in both *in vitro* and *in vivo* (Sacksteder et al., 2012). SQ109, which enters currently phase II trials, appeared to have favourable effects when used in combination with sutezolid and bedaquiline (Reddy et al., 2010; Reddy et al., 2012). Patients suffering pulmonary TB received variable doses up to 300 mg daily of SQ109 with or without rifampicin as co-administration for two weeks (Heinrich et al., 2015). Even though SQ109 was considered to be well tolerated and safe at the doses administered, moderate gastrointestinal complaints are the most frequently reported side effects for this drug. Plasma concentrations remained less than the MIC of SQ109 with no remarkable bactericidal impact of this medication either in combination with rifampicin or alone (Clain and Escalante, 2016a). SQ109, which has a distinct mode of action from ethambutol, exerts anti-tuberculosis activity by interfering with the assembly of cell wall through targeting the transport molecule MmpL3 (Sacksteder et al., 2012; Tahlan et al., 2012).

2.4.7. Sudoterb

Although the mechanism of action for this class of medications has not yet been established, Sudoterb (MIC_{90} 0.25 μg/mL) is used in combination with current anti-tuberculosis medication to remove *M. tuberculosis* from both lungs and spleens in less time than conventional treatment (Shakya et al., 2012). Figure 2.2 briefly demonstrated the possible action mechanisms of novel anti- TB agent. Besides that early stage drugs with their activities have been encapsulated in Table 2.

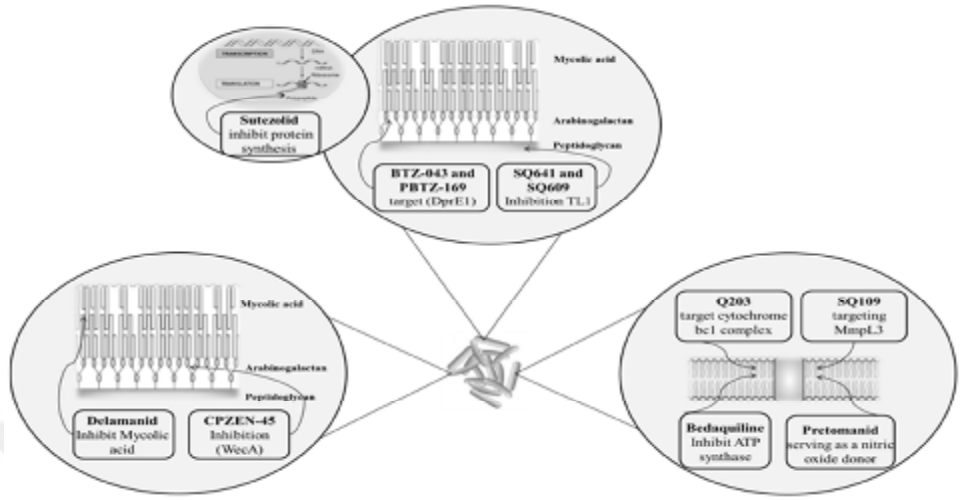

Figure 2.2. Displayed how novel anti-TB agents impact on bacterial cells survival

Table 2.1. Depicts pre-clinical TB agents with their activities and the mechanism of action

Drug Name	Activity	Mechanism of action	References
CPZEN-45	Have activity against both replicating and non-replicating bacteria and has demonstrated efficacy against both drug- sensitive and XDR-TB	Inhibition of decaprenyl-phosphate–GlcNAc-1-phosphate transferase	(Ishizaki et al., 2013; Salomon et al., 2013)
SQ641	Shows good activity against both *M. tuberculosis* (MIC = 1.0 μg/ml) and *M. avium* complex (MIC = 0.016 to 16 μg/ml) bacteria and kills *M. tuberculosis* faster than antitubercular drugs including INH and RIF. SQ641 possesses several disadvantages such as poor water solubility and oral absorption which limits its therapeutic efficacy (poor in vivo activity) against TB. SQ641 exhibited only 1.0–1.5 log CFU reduction in lungs.	Inhibits an enzyme present in *M. tuberculosis*, translocase I (TL1) that is essential for the biosynthesis of peptidoglycan (cell wall)	(Reddy et al., 2008; Koga et al., 2004; Bogatcheva et al., 2010; Yamaguchi et al., 1986)

Table 2.1. Continue

SQ-609	Showed >90% inhibition of intracellular bacterial growth at 4 μg/ml without any toxic effect. It is currently in pre-clinical trials and has *in vivo* activity against drug-sensitive and drug-resistant forms of TB.	Inhibition of bacterial cell wall synthesis	(Bogatcheva et al., 2011; Nikonenko et al., 2004)
Lee 1599	*In vivo*, Lee 1599 demonstrated excellent efficacy in a dose dependent manner. Treatment resulted in a 2.2 log CFU reduction in total lung tuberculosis burden versus the control group after 4 weeks of treatment at the highest dose (200 mg/kg, 3 days per week). In vitro, synergism between clindamycin and Lee 1599 (0.6μg/mL) was particularly notable, as clindamycin itself was inactive against all *M. tuberculosis strains* investigated (MIC≥ 200μg/mL).	possesses high selectivity for bacterial RNA and escapes efflux pump Rv1258c.	(Bruhn et al., 2015; Lee et al., 2014; Hoagland et al., 2016)
BTZ-043	BTZ-043 displayed in vitro bactericidal activity against *M. tuberculosis* H37Rv with MIC 0.001 μg/ml. BTZ043 has been found to be less efficient in murine models of chronic and acute TB due to its poor pharmacokinetic profile.	The target of BTZ043 in *M. tuberculosis* is the essential flavoenzyme DprE1, decaprenylphosphoryl-β-d-ribose 2′-epimerase. The flavoenzyme DprE1 catalyze the conversion of decaprenyl-phosphoryl-β-d ribose to decaprenyl-phosphoryl-β-d-arabinose, the important arabinose precursor for the synthesis of arabinogalactan and lipoarabinomannan, the critical components of mycobacterial cell wall.	(Makarov et al., 2009; Trefzer et al., 2010; Kumar et al., 2015)

Table 2.1. Continue

PBTZ-169	PTBZs were found to be more active in vitro than BTZ043 and displayed MIC values against *M. tuberculosis* H37Rv in the range 0.00019–0.00075 μg/ml. In the zebrafish model, it displayed an improved potency, safety and efficacy profile compared with BTZ-043 It has in vivo activity in murine models, and has additive effects with a number of anti-TB therapeutic drugs and a synergistic effect if taken with bedaquiline	The mode of action of PBTZ169 is the same as that of BTZ043, that is, DprE1. When compared with BTZ043, it offers better pharmacokinetic profile and shows higher affinity toward DprE1. Furthermore, PTBZ169 appears to be more stable than the other BTZs derivatives, probably because of the cyclohexyl moiety which protects it against nitroreductase attack.	(Makarov et al., 2014; Reddy et al., 2010; Chetty et al., 2017)

2.5. Host-directed therapies for TB

Health services providers across the world face great challenges to accomplishing optimal results from the current treatment of TB. Some of the challenges originate not only from the spread of MDR-TB and XDR-TB but also from the regimens for the TB treatment that are complex, damage to lung and other tissues, co-infection with HIV, and co-morbidity with non-communicable diseases like diabetes and immune reconstitution inflammatory syndrome. Another challenge is the time for TB medications to fully cure patients due to the existence of dormant *M. tuberculosis* bacilli which are commonly resistant to the drugs currently used for TB treatment. The current TB medications, which treat the disorder by targeting *M. tuberculosis* directly, can only suppress bacterial replication (Wallis and Hafner, 2015). The best way to tackle this problem is to develop new medications for the treatment of TB (Zumla et al., 2013; Zumla et al., 2015a). The host-directed therapies portfolio are aimed at decreasing the time required for the treatment, enhance the treatment results, and further diminish both long-term functional disability and lung pathology for both sensitive and resistant

to drugs (Zumla et al., 2015c; Oehlers et al., 2015). Some of these host-directed therapies (HDT), which are presently being established comprise cellular therapy, appeared to diminish tissue pathology, including that triggered by tuberculosis-(TB-) associated immune reconstruction inflammatory syndrome (TB-IRIS).

Cellular therapy, which utilises the bone marrow taken from the patient's own cells, may also include taking drugs usually used for the treatment of diseases such as peptic ulcers, diabetes, cancer, asthma, arthritis, and hypercholesterolemia. Other medications that are being repurposed with the ambition of turning them into host-directed therapies comprise micronutrients, vaccines, checkpoint inhibitors, and antimicrobial peptide inducers (Oehlers et al., 2015; Zumla et al., 2016).

Host-directed therapies act to be immune to the most challenges that pathogen-directed therapies have to cope with (Schwegmann and Brombacher, 2008). They have a way not only to boost the prevailing immune defences used to defeat *M. tuberculosis* but also have a way to induce new lines of defence. These types of therapies target host proteins in order to make it difficult to be mutated by bacteria in such a way that has a direct impact on withdrawing compound binding (Tobin, 2015). The aim of host-directed therapies is to minimize inflammation, damage caused by the disorder to end organs, restoration of damaged tissue and further assist other TB treatment to be more potent in eliminating the infection. Patients infected with both HIV and TB can minimise the risk linked with TB agents interacting with antiretroviral medications by using host-directed therapies. The combination of host-directed therapies with TB drug regimens appeared to reduce the risk of patients developing drug resistance and also eradicate the reinfection chances (Zumla et al., 2015b). In order to restrict the growth of *M. tuberculosis*, there is a need for complete responses of T-cell and the interferon-γ production. A great damage of tissue, pathology of pulmonary tissue, and protective anti-TB reactions in patients with tuberculosis have been noticed who are essentially infected by tumour necrosis factor (TNF)-α-mediated inflammation (O'Garra et al., 2013; Zumla et al., 2015c).

2.6. Repurposing drugs offer a promising TB treatment strategy

Several drugs with potential for repurposing as HDTs already have well-defined safety and pharmacokinetic profiles and are ready to progress to randomized, controlled clinical trials that evaluate their effectiveness in TB, TB–HIV co-infection and TB co-morbidity with other diseases (Zumla et al., 2015b). The great potential for repurposing offered by nonsteroidal anti-inflammatory drugs (NSAIDs) in the context of anti-TB therapy has been recognized relatively recently (Gold et al., 2012; Guzman et al., 2013; Ivanyi and Zumla, 2013). NSAIDs are a group of molecules from chemically diverse families that suppress inflammation by inhibiting the formation of prostaglandins, the mediators of inflammatory response. They achieve this by inhibiting cyclooxygenase enzymes, COX-1 and COX-2, involved in the synthesis of prostaglandins and other prostanoids. Classical NSAIDs block both enzymes without differentiation; however, newer COX-2 inhibitors are selective, and thus exhibit less gastric irritation as an adverse effect—increasing their popularity. There is significant evidence supporting the notion that some of these drugs also modulate immune responses via pathways independent of the cyclooxygenase– prostaglandin route (Lee and Wang, 1999; Neal et al., 1987).

Dexamethasone and prednisone, which have been used in a number of trials, are the standard-of-care for some severe forms of TB. They represent a relatively accessible and inexpensive approach to limiting inflammation, which can be a prime cause of morbidity and mortality. They have proved particularly effective in cases of TB meningitis (Schoeman et al., 1997; Thwaites et al., 2004; Prasad and Singh, 2008) and have been adopted as standard-of-care for TB pericarditis and meningitis (Hakim et al., 2000; Thwaites et al., 2009).

A recent meta-analysis of clinical trials using corticosteroids showed a 17% reduction in mortality across 41 clinical trials (Critchley et al., 2013). Thalidomide has been used in clinical pediatric trials of adjunctive therapy for TB meningitis, and the limitation of TNF production is thought to be key (Schoeman

et al., 2000; Schoeman et al., 2004). However, the extreme teratogenic properties of thalidomide make it particularly dangerous (Kim and Scialli, 2011),and other modulators of TNF-mediated pathologies in TB would be preferable. Moreover, CC-3052, a thalidomide analog, increased the effectiveness of isoniazid in a mouse model through reduced TNF production (Koo et al., 2011; Subbian et al., 2011). It was recently shown that activation of the IL-1β pathway with zileutona, a clinically used asthma drug, during TB pathogenesis triggers expression of cyclooxygenase 2 (COX-2) which leads to prostaglandin E2 (PGE2) releas) Mayer-Barber et al., 2014(This helps to ameliorate excessive type 1 IFN-driven hyper-inflammation in the lung, restoring organ function while reducing ***M. tuberculosis*** burden. Vitamin D has long been proposed to have important host-beneficial effects in TB, including immunomodulatory effects. In human macrophages, TLR2-mediated or INF-g-mediated activation of cyp27b1 leads to conversion of 25-hydroxyvitamin D3 (25(OH)D3) to bioactive 1,25-dihydroxyvitamin D3 (Liu et al., 2006; Krutzik et al., 2008; Fabri et al., 2011). In contrast to rodent models, the primary antimycobacterial effector function on TLR stimulation of this conversion event lies in the generation of antimicrobial peptides (Liu et al., 2006). IFN-g antimicrobial activity can be mediated through activation of CYP27B1. More recently, 1,25-dihydroxyvitaminD(1,25D) was shown to enhance IL-1b transcription in macrophages and induce antimicrobial peptide production in co-cultured lung epithelial cells (Verway et al., 2013). Finally, vitamin D has been shown to induce autophagy in cell culture via production of LL-37 (Yuk et al., 2009), thus linking to an additional host-protective process that might also contribute to bacterial control ***in vivo***. In addition to its antimicrobial effects on the host macrophage, subgroup analysis of a clinical trial showed that vitamin D as an adjunctive therapy may reduce hyperinflammatory responses and thus may contribute in multiple ways to improved outcomes (Coussens et al., 2012). The anti-diabetic drug metformin (which activates AMP-activated protein kinase) was shown to kill intracellular ***M. tuberculosis*** in monocytes and macrophages via

induction of mitochondrial reactive oxygen species (Singhal et al., 2014). Furthermore, metformin dampened the inflammatory response milieu in lungs of treated *M. tuberculosis* -infected mice while enhancing the efficacy of anti-TB drugs. Cellular therapy using infusions of autologous bone-marrow derived mesenchymal stromal cells into patients with drug-resistant TB is another avenue being pursued to treat *M. tuberculosis* –induced severe pulmonary inflammation, and regenerating anti-TB immune responses (Skrahin et al., 2014). Histone deacetylase inhibitors valproic acid (VPA) and vorinostat, both licensed for neurological indications, unveiled highly encouraging observations in the context of human immunodeficiency virus (HIV) infection. Both drugs perpetrated reactivation of latent viral reservoirs in CD4 T cells and increased virus susceptibility to immune attack as well as antiretroviral treatment, subsequently warranting their first large-scale clinical trial in HIV-infected individuals (Rasmussen et al., 2013). VPA also induces the up-regulation of major histocompatibility complex (MHC) class I antigen presentation, which may facilitate tailored CD8+ T cell activity against *M. tuberculosis*-infected cells (Khan et al., 2008). ABL tyrosine kinase inhibitor imatinib mesylate is known commercially as Gleevec. Previously, it had been shown that imatinib was able to inhibit poxvirus pathogenesis via limitation of actin motility of the cell-associated enveloped virions (Reeves et al., 2005). In mycobacterial infections with both *M .tuberculosis* and the closely related *M. marinum,* there appear to be both cell autonomous and cell nonautonomous beneficial effects of inhibition, with inhibition of ABL resulting in increased killing and promoting acidification of the mycobacterial phagosome (Napier et al., 2011; Bruns et al., 2012).

2.7. Aptamers and Antisense technology against TB

Nucleic acid aptamers are ligands developed by an *in vitro* screening method known as systematic evolution of ligands by exponential enrichment (SELEX) (Irvine et al., 1991; Stoltenburg et al., 2007). Because of their unique

three-dimensional structure, the aptamers are capable of binding targets with high affinity and exquisite selectivity. They are relatively cheap and possess low toxicity, making them ideal candidates in the development of therapeutic agents (Dupont et al., 2011). Short (30-mer) single-stranded DNA aptamers has been identified as a novel class of potent inhibitors of Mtb-AHAS through an ***in vitro*** DNA-SELEX method. Among all tested aptamers, two candidate aptamers (Mtb-Apt1 and Mtb-Apt6) demonstrated the greatest inhibitory potential against Mtb-AHAS activity with IC50 values in the low nanomolar range (28.94±0.002 and 22.35±0.001nM respectively) (Baig et al., 2015). Chen et al. (2007) (selected DNA aptamers using whole living bacteria as SELEX target. A single injection of 0.8 mg of NK2 aptamer decreased the amount of mycobacteria in MTB-infected mice, alleviated disease manifestations and prolonged the survival rate. Further studies revealed that all the aptamers obtained (Chen et al., 2007) possess high binding affinity, with the NK2 aptamer being found to be the most effective binder (Kd ¼ 31 nM) and being able to inhibit the invasion of MTB into macrophages (Chen et al., 2012; Chen et al., 2013). These aptamers are considered promising therapeutic agents and are also of interest for the development of molecular probes to study the mechanisms of bacterial invasion into cells. Another approach for obtaining anti-tuberculosis aptamers was developed by Shum et al. (2011). The bacterial enzyme polyphosphate kinase 2 (PPK2) was used as a SELEX target. PPK2 is a polyphosphate-dependent nucleotide diphosphate kinase that plays an important role in the synthesis of mycolic acid and other surface polysaccharides critical for bacterial survival during active growth. The highest target binding affinity (Kd ¼ 870 nM) was demonstrated for the quadruplex forming aptamer G9 and its shortened versions. The aptamer G9 inhibited the activity of the enzyme to 50% at concentration of 39.3 nM. This aptamer could be a potential therapeutic agent as soon as an efficient system for its cell delivery can be developed (Shum et al., 2011).

The first complete experimental confirmation of short antisense RNAs in mycobacteria was published in 2009, which revealed 5 trans-acting and 4 cis-acting siRNAs in MTB H37Rv in the context of pH and oxidative stress (Arnvig and Young, 2009). By the end of 2013, a total of more than 200 endogenous antisense RNAs were experimentally identified in various mycobacteria, including 70 in MTB (Arnvig et al., 2011; DiChiara et al., 2010; Tsai et al., 2013; Pelly et al., 2012; Warner et al., 2007; Miotto et al., 2012; Hartkoorn et al., 2012; Houghton et al., 2013; McGuire et al., 2012), 90 in *M. bovis* (DiChiara et al., 2010; Tsai et al., 2013; Golby et al., 2013) , 9 in *M. avium* (Ignatov et al., 2013), and 44 in *M. smegmatis* (Arnvig et al., 2011; DiChiara et al., 2010; Tsai et al., 2013; Li et al., 2013). From these recent studies, a stronger connection between mycobacterial pathogenesis and the levels of expression of the antisense RNAs have emerged but many new questions about their potential pathogenic and housekeeping functions remain to be answered. The lack of identification of an Hfq homolog in mycobacteria prevents the current approach of co-immunoprecipitation, making the study of the role of antisense RNAs all the more difficult in this genus (Arnvig et al., 2011). Pandey et al. (2011) have proposed an alternative protein, Rv2367, as a potential RNA chaperon in place of Hfq (Pandey et al., 2011), however, studies are ongoing in this direction to find a functionally-equivalent chaperon or to get around this issue (Li et al., 2013). Antisense RNAs can repress or modulate expression of target genes by a variety of mechanisms, including transcription interference, transcription attenuation, and degradation by endo- or exonucleases, or by blocking ribosome binding. When inducing transcription interference, the transcription of antisense RNA from one promoter hinders the RNA polymerase from either binding or extending the target gene transcript from the opposite strand (Andre et al., 2008; Callen et al., 2004).

2.8. Novel drug targets against *M. tuberculosis*

Many metabolic pathways, including cell wall synthesis, the energy production, and protein synthesis are emerging new drug targets. The rise of drug-resistant TB emphasises the urgency of the need for novel TB agents with new modes of action. Described as a tetramic acid substance obtained from *Melophlus sarassinorum*, Melophlin appears to have an anti-dormant mycobacterial activity. The derivatives of melophlins, which are A(1), B, G(2), H(3) and I(4), showed the ability to bind to the GTPase in Hela cells (Xu et al., 2006; Knoth et al., 2009). The *M. tuberculosis* dormancy is due to the nutrient starvation where the low production of Rv1026 leads to modify the permeability of cell wall and slowed *M. tuberculosis* growth. Therefore, the melophlin A targets the BCG1083 protein of putative exopolyphosphatase and the BCG1321c protein of diadenosine 5′,5‴-P 1,P 4-tetraphosphate phosphorylase (Chuang et al., 2015; Thayil et al., 2011; Arai et al., 2016). Griselimycin, which has been isolated from *Streptomyces*, has discarded due to its high toxicity. The cyclohexyl derivative of the griselimycin has showed to modify the metabolic pathway through the proline alkylation. In order to support the intermediate site of the DNA polymerase and sliding clamp, the cyclohexyl derivative appears to associate to the hydrophobic amid the clamp domains II and III (Holzgrabe, 2015). Of late, the anti-microbial activity of the griselimycin against Mycobacterium species has been revealed (Kling et al., 2015). The griselimycin effect on *M. tuberculosis* described the vital desirable features for the novel anti-TB agents. The griselimycin as well as its derivatives showed great activity against *M. tuberculosis* through hindering the DnaN as a new target. More studies on mode of action of the griselimycin and its derivatives are still required (Kling et al., 2015; Müller, 2015). Teixobactin has shown an action against some drug-resistant strains as well as gram positive pathogens. Teixobactin appears to inhibit cell wall biosynthesis through binding on lipid II and III as highly conserved motifs. Teixobactin, which inhibits the peptidoglycan synthesis without apparent effect on label integration into the cellular DNA, RNA and protein, has

shown lack of toxicity with does 100 µg/mL against mammalian HepG2 and NIH/3T3 cells. To date, teixobactin has the ability to kill ***M. tuberculosis*** and no ***M. tuberculosis*** strains resistant to teixobactin yet isolated (Ling et al., 2015). A promising strategy to prevent the growth of ***M. tuberculosis*** is provided by the combination of trimethoprime and sulfamethoxazole which reacts on different side of tetrahydrofolic acid necessary during the nucleic acid biosynthesis. Thus, sulfamethoxazole and trimethoprime combination can diminish the emergence of drug resistance (Liu et al., 2015; Pacaud et al., 1973)

2.9. Novel treatment regimens against *M. tuberculosis*

The early encounters with the new agents imply that it is improbable that a sole new medication will basically alter the entire course of drug-sensitive TB. It is more feasible that it will consist of novel mixtures of medications, which could encompass one or several new agents, which may offer novel paradigms within the control of drug-resistant illness (Clain and Escalante, 2016b). The OFLOTUB trial is contrasting the regular 6-month regimen with a regimen comprised of a 2-month rigorous stage employing gatifloxacin to replace ethambutol, succeeded by a 2-month stage of maintaining isoniazid, gatifloxacin and rifampicin (Merle et al., 2014; Merle et al., 2012; Smythe et al., 2013). The TRUNCATE-TB research, consisting of four two-month regimens and contrasting it with the regular DS-TB therapy, has of late acquired ethics and protocol endorsement. This trial was scheduled to begin at the conclusion of 2016. One arm comprises an elevated dose of RIF, linezolid, INH, ethambutol and PZA; the second arm comprises a similar treatment although replacing linezolid with clofazimine, the third arm employs rifapentine, linezolid, PZA and levofloxacin, and the fourth arm comprises linezolid, ethambutol, 0bedaquiline, PZA and INH (Lessem, 2016). REMox TB is contrasting regular 6-month treatment with two study regimens (2 months of moxifloxacin, riampicin, pyrazinamide and isoniazid succeeded by 2 months of rifampicin, moxifloxacin and isoniazid succeeded by a further 2 months of

rifampicin and moxifloxacin) (Clain and Escalante, 2016b). The STREAM-1 trial employs moxifloxacin combined with a number of the present anti-Tb drugs in addition to an injectable and clofazimine (Lessem, 2016). In contrast to moxifloxacin, levofloxacin has reduced QT extension influence. Presently, the Opti-Q research financed by the National Institute of Allergy and Infectious Diseases (NIAID) is attempting to establish the best levofloxacin dose against *M. tuberculosis* (Chetty et al., 2016). Clofazimine, a riminophezine analogue is a medication which has been repurposed for TB therapy. Initially an antileprotic medication, it is presently being employed concurrently with alternative anti-TB medications in different clinical trials–PRACTECAL, STREAM-I, STREAM-II and end TB research. Linezolid consists of a section of the NiX-TB trial which is presently within stage III clinical trials. This medication has become growingly essential in the therapy of MDR-TB as well as XDR-TB (Lessem, 2016). The most current research by Dawson et al. (2015b) highlights new viewpoints on what treatments may be planned using the newly accessible anti-TB medications. This novel stage IIb assessment contrasted the bactericidal operation of 8-week regimens encompassing prematonid and moxifloxacin (100 or 200 mg, subject to the arm) as well as pyrazinamide resistant the regular anti-TB treatment for addressing sputum smear positive individuals with drug-sensitive and drug-resistant TB (Dawson et al., 2015a; Sotgiu and Migliori, 2015).

2.10. Antimicrobial peptides as an alternative to anti-tuberculosis drugs with their Activity *in vitro* and *in vivo*

Oligo-N-substituted glycines (peptoids) are sequence-specific peptidomimetics that are based on a“biomimetic” peptide backbone identical to that of natural proteins but have their side chains attached to the amide nitrogen (Patch and Barron, 2002; Zuckermann et al., 1992). This structural difference makes them highly resistant to protease activity (Kirshenbaum et al., 1998; Miller et al., 1995). 1-$C1_{34mer}$ showed interesting tuberculocidal activity against *M.*

tuberculosis with MIC 6.6 µM. Peptoid 1-$C1_{34mer}$ is four residues long and cationic, with a 13-carbonaliphatictailonitsNterminus. The hydrophobictail of peptoid 1-$C1_{34mer}$ gives it a substantial surfactant character—as a monomer—and would be expected to interact strongly, and disruptively, with the waxy, hydrophobic, lipidrich outer layer of Mycobacterium, enabling better peptoid penetration of the bacterium, in a manner not seen in the case of unalkylated peptoid 1_{4mer}. In general, cationic surfactants are toxic to mammalian cells, as well as to bacteria, but this case, the strong self-association of this oligopeptoid enforced by its C13 tail must protect macrophages, which, unlike bacteria, are not substantially anionic (orhydrophobic, inthecase of ***M. tuberculosis***) on their outer membranes. Micellization of 1-$C1_{34mer}$ at its MIC would be fully expected to increase its anti-M. tuberculosis potency, since the labile micelles, after binding to the anionic/hydrophobic bacterial membrane, would dissociate, creating a high local concentration at the cell surface (Shai, 2002). The cell membrane of Mycobacterium differs greatly from that of typical gram-positive and gram-negative bacteria. The outer layer of Mycobacterium is comprised of lipid rich, hydrophobic layers of mycolic acid, and this aliphatic, hydrophobic wax barrier reduces the permeability of ***M. tuberculosis*** to the typical anti-TB drugs (Brennan and Nikaido, 1995; Johnson, 2007). The inefficient penetration of the drugs and/or the entrapment of drugs by the waxy envelope layer are, in part, the reasons for the higher resistance of Mycobacterium to antibiotics (Méndez-Samperio, 2008). The very slow metabolism of Mycobacterium also has a tendency to render most conventional drugs less effective because the latter compounds often work by impairing particular steps in the cell's metabolic cycle. Furthermore, it has been reported that azurophil granule proteins (AZP) were active against mycobacteria. 100 mg/ml of AZP has been observed to kill about 55% of ***M. tuberculosis*** after 24h of incubation, whereas, complete killing of ***M. smegmatis*** was observed in 50 mg/ml of AZP. Electron microscopic studies demonstrate that the AZP disintegrate

bacterial cell membrane resulting in killing of mycobacteria (Hancock and Sahl, 2006).

It is well established that a specific class of peptides derived from diverse gram-positive bacteria species—bacteriocins (Bcn)—exert activity against a wide range of bacteria. Bcn produced by lactic acid bacteria are categorized into three classes, and the mode of action of class I and IIa Bcn has been thoroughly studied(Chatterjee et al., 2005, Guinane et al., 2005). Bacteriocins (Bcn1-Bcn5) exhibited stronger antimycobacterial activity against ***M. tuberculosis*** with MIC 0.01–1 μg/ml (Sosunov et al., 2007). The main mechanism of action by which Bacteriocins (Bcn1-Bcn5) influence ***M. tuberculosis*** is due to the formation of pores in cell membranes leading to the cell death (Chatterjee et al., 2005; Guinane et al., 2005).

Antimicrobial peptides (AMPs) that assume an a-helical structure are widespread and abundant in nature. The selectivity, potency and safety of a-helical AMPs heavily depend on their physicochemical properties including their amphipathic nature, net charge, charge angle, overall hydrophobicity and conformational flexibility (Jiang et al., 2011). In addition, the use of D-conformation peptides is preferred because it has been shown that the L to D-amino acid substitution of AMPs confers resistance to the activity of proteolytic enzymes and therefore improves AMP biostability, without impairing antimicrobial activity (Hong et al., 1999; Grieco et al., 2013). Peptide D-V13K (D5) is a 26-residue amphipathic peptide consisting of all D-amino acid residues, which adopts an α-helical conformation in a hydrophobic environment and contains a hydrophilic and positively-charged valine with lysine at position 16 as a “specificity determinant”. Peptide D-V13K (D5), the most active analog against ***M. tuberculosis*** had a MIC value of 11.2 μM (35.2 μg/ml) against H37Rv strain and 15.6 μM (49 μg/ml) against the MDR strain. Peptide D1, which positively-charged lysine residue in the center of the non-polar face (position 13) as a “specificity determinant”, had similar activity as D5 against the MDR strain (57

μg/mL), a 9-fold improvement in hemolytic activity and a 7.4-fold better therapeutic index compared to D5 (Jiang et al., 2011). Moreover, D-LAK120-A and D-LAK120-HP13 showed the highest potency, as the mycobacteria were not detected in the wells at the concentration of 50 μM or above. In contrast, DLAK120- H and D-LAK120-AP13 appeared to be the least potent, as a considerable amount of mycobacteria could still be seen in the wells even at 100 μM. For the MDR-TB strain, although none of the D-AMPs managed to abolish the growth of bacteria even at the highest concentration used, D-LAK120 and D-LAK120- HP13 were the most effective D-AMPs against this strain, followed by D-LAK120-A and D-LAK120-H. For the XDR strain, D-LAK120 and D-LAK120-A were the most effective peptides. Again, none of the D-AMPs managed to eradicate the growth of XDR strain. In general, D-LAK120, D-LAK120-A and D-LAK120-HP13 were the three most potent D-AMPs against the clinical strains of ***M. tuberculosis in vitro*** (Lan et al., 2014). A series of synthetic a-helical peptides modified with hydrophobic amino acids have been evaluated for their antimycobacterial activities. The peptide (LLKK)2 modified with two methionine residues such as M(LLKK)2M was the most effective peptide against both susceptible (MTB) and drug-resistant (MDR-TB) ***M. tuberculosis*** with MIC 125mg L^{-1} and 62.5 mg L^{-1}, respectively (Khara et al., 2014).

Although the exact mechanism of action of AMP's is not known and can differ depending on the AMP, it is clear that the positively charged residues of the AMP are attracted to the negatively charged surface of the bacterial membrane. The non-polar face of the AMP must be incorporated into the lipid bilayer where peptide accumulation in the membrane results in increased permeability and loss of barrier function, leakage of cytoplasmic components and cell death (Jiang et al., 2011). D-AMPs also demonstrated the ability to break down the heavily clumped bacteria so that they appeared to be more dispersed and scattered inside the culture well. This unique phenomenon of D-AMPs may be explained by their amphipathic properties. Although the overall hydrophobicity of the different D-AMPs varies, all

of them are able to produce a ‘detergent- like effect’ by reducing the hydrophobic interaction between the cell walls of the bacteria and preventing cell clumping (Lan et al., 2014).

Human b-defensins (HBDs) have been reported and considered part of the immune system (Schutte et al., 2002). Many of these HBDs have been discovered using computational techniques applied to the human genome sequence (Schutte et al., 2002) and have been shown to exhibit a broad antimicrobial activity spectrum with a low susceptibility to generate bacterial resistance (Song et al., 2009; Ganz, 2002; Ganz, 2003). HBDs are cationic peptides containing three antiparallel b-strands stabilized by three conserved disulfide bridges (Klüver et al., 2005) that provide a compact small structure (Schneider et al., 2005). Some of them also possess an a-helix at the N-terminal region (Hoover et al., 2003). Similar to other antimicrobial peptides, the principal function of HBDs is to control bacterial growth by either affecting their surrounding membrane surface, as in the case of HBD2 and HBD3(Cutrona et al., 2015), or by acting into phagolysosomes (HBD1) (Schröder and Harder, 1999). Therefore, HBDs are endogenous antibiotics with broad bacteriocidal activity toward gram-positive and gram-negative bacteria that also inhibit the growth and development of fungi (Schneider et al., 2005; Schwaab et al., 2009), capsulated viruses and protozoa (Schneider et al., 2005; Schröder and Harder, 1999; Schwaab et al., 2009; Lehrer and Ganz, 2002; Mathews et al., 1999). It has been reported that HBD2 had antimicrobial activity against the ***M. tuberculosis*** strain H37Rv (1.5 μM) and the heterologous tandem peptide, HBD3-MHBD2, had the best minimal inhibitory concentration (MIC) value (2.7 μM) against a multidrug resistance strain (MDR) of ***M. tuberculosis***, demonstrating the feasibility of the use of HBDs against pathogenic ***M. tuberculosis*** reported to be resistant to commercial antibiotics (Corrales-Garcia et al., 2013). Santos et al. (2012) have been documented that Magainin-I analog peptide (MIAP) with 300 μg/ml showed an activity 2-fold higher than the native peptide magainin-I with MIC 1200 μg/ml against ***M. tuberculosis*** (Santos et al., 2012). It has been

suggested that MIAP interferes with H+ pumping by inhibiting the *M. tuberculosis* plasma membrane F1F0-H+ ATPase. The inhibition of this enzyme can be interpreted as an impaired ability to regulate the intracellular H+ concentration (pHi) of mycobacteria. Thus, the viability of mycobacteria is affected because the deregulation of pH is deleterious against bacteria, and strict pH control must be maintained for the proper function of vital proteins and to maintain virulence. The results obtained suggest that according to their orientation, AMPs, especially MIAP, have the ability to bind to the cytosolic and periplasmic sides of the mycobacterial plasma membrane. In addition, the results obtained indicate that AMPs may interact with the membranes of mycobacteria interfering to ATPase activity, specifically the F1F0-ATPase, which functions when the peptides are attached to the internal membrane side. Therefore, a combined effect of cell disrupting and inhibition of ATPase activity of MIAP cannot rule out (Santos et al., 2012).

Nisin A is the prototype lantibiotic and has safely been incorporated into a wide range of commercial products since its acceptance by the Food and Drug Administration (FDA) as a food additive in 1988. As with all lantibiotics, synthesis of the mature nisin peptide involves extensive post-translational modification resulting in the formation of the unusual amino acids lanthionine and β-methyllanthionine, as well as dehydrated amino acids. The bio-engineering of lantibiotics has been particularly useful with respect to elucidating the importance of specific residues and domains within the peptides. In particular, the N-terminal region responsible for binding lipid II in the cell wall of target cells was elucidated, as was the C-terminal region which inserts into the cell membrane to cause pore formation. Most noteworthy is the work focusing on the central hinge region which allows the aforementioned domains to move relative to one another. In contrast however, the generation of lantibiotics with enhanced features has been infrequently reported. Some successes have occurred, such as the generation of nisin variants with enhanced solubility at neutral pH or increased antimicrobial

activity against non-pathogenic strains. Interestingly it has been established that modification of the hinge region in nisin Z (a natural variant of nisin A which differs by only one amino acid) led to the identification of variants (N20K and M21K) with increased efficacy against gram-negative species, i.e., *Shigella*, *Pseudomonas* and *Salmonella*. The activities of nisin A and novel bioengineered hinge derivatives, nisin S, nisin T and nisin V have been evaluated. All variants displayed greater anti-mycobacterial activity than nisin A. Nisin S was the most potent variant against *M. tuberculosis*, *M. kansasii* and *M. avium*, retarding growth by a maximum of 29% when compared with nisin A (Carroll et al., 2010).

Protegrin-1 (PG-1) is present in porcine neutrophils, where may play a role in the non-oxidative activity of these cells. The mechanism by which protegrins kill bacteria is not clear, but the ability to disrupt microbial membranes is believed to be crucial (Martineau et al., 2007). PG-1 forms cation-selective channels in lipid membranes that contain bacterial lipopolysaccharide. The activities of two cysteine-rich AMPs, namely the 18-residue beta-sheet PG-1 from porcine leukocytes (Martineau et al., 2007) and the 36-residue beta-sheet epithelial beta-defensin-1 (HBD-1) from humans (Gennaro and Zanetti, 2000), against drug-susceptible or multidrug-resistant (MDR) *M. tuberculosis* strains have been assessed. PG-1 was significantly active against susceptible strain H37Rv, with reduction in the CFU numbers at 64 μg/ml (68.4%) and 128 μg/ml (96.7%), respectively. HBD-1 was also active against this organism, with a significant CFU reduction being observed at 128 μg/ml (49.9%). MDR Strain RM22 was more resistant than H37Rv to both peptides, a significant CFU decrease being observed only at 128 μg/ml of PG-1 (45.1%) (Hoover et al., 2001). PR-39, a proline-arginine-rich antibacterial peptide from porcine leucocytes, was found to be active against drug-susceptible as well as multi-drug-resistant (MDR) clinical isolates of *M. tuberculosis*. The activity of PR-39 was concentration dependent, with 80% growth inhibition of *M. tuberculosis* H37Rv at 50 mg/L. The MDR-TB strains

were less susceptible to PR-39, with 39 and 49% growth inhibition at 50 mg/L peptide, respectively (Fattorini et al., 2004).

Endogenous host defence peptides are well recognized components of the innate immunity and they have been suggested to have an important role in TB infections. Such peptides can inhibit microbial growth directly through a variety of membrane and non-membrane targets. The anti-infective activity of these three synthetic IDR peptides against ***M. tuberculosis*** in a murine model of progressive pulmonary TB using both drug sensitive and MDR-TB strains have been evaluated. A dose of 32 mg of each peptide in 100 mL dissolved in saline solution (1 mg/kg), which was administered intratracheally three times a week for 30 days. HH2 and 1018 IDR peptides lead to efficient suppression of the growth of tuberculosis bacilli and reduction of the pneumonic area in an experimental model of pulmonary TB after 15 and 30 days of treatment, although the peptide 1002 did not show significant decrease in bacillary loads. This occurred when mice were infected with either drug-sensitive or drug-resistant mycobacteria using a high dose model of infection in Balb/c mice treated with the given therapeutic regime (Linde et al., 2001). Furthermore, ***in vivo*** killing of ***M. tuberculosis*** induced by LLKKK18 (AMP)-loaded HA nanoparticles with 1.2-log reduction at 100 μM. C57BL/6 mice were infected with ***M. tuberculosis*** via the pulmonary route. After 3 months, five doses of the treatments were administered intratracheally every other day (Jena et al., 2012).

Lactoferrin is an iron-binding glycoprotein that is found in mucosal secretions and neutrophilic granules. It is also considered as a basic component of innate immunity, and has the potential to modulate host responses during MTB infection (Hancock and Sahl, 2006). Lactoferrin treated mice demonstrated a 1 $\log_{10}$ reduction after 3 weeks of oral administration. These favorable effects were evident even when the mice were treated one week post-infection, indicating that lactoferrin has the potential as a novel agent for the treatment of TB. Quantitative immunohistochemistry using multispectral imaging demonstrated that lung

inflammation was significantly reduced in both groups of lactoferrin treated mice, with decreased foamy macrophages, increased total lymphocytes, and increased numbers of CD4þ and CD8þ cells. ELISpot analysis showed that lactoferrin treated mice had increased numbers of CD4 þ IFN-gþ and IL-17 producing cells in the lung, cells that have protective functions during MTB infection. Lactoferrin alone did not alter the proliferation of MTB in either broth or macrophage culture, but enhanced IFN-g mediated MTB killing by macrophages in a nitric oxide dependent manner. Thus lactoferrin may be a novel therapeutic for the treatment of tuberculosis, and may be useful in infectious diseases to reduced immune-mediated tissue damage (Hilchie et al., 2013). H37Rv and MDR-infected mice treated three times per week with 32 µg/mouse of LL37 for 28 days demonstrated approximately 53% killing of *M. tuberculosis* and 45% of MDR. These results indicate that antimicrobial peptides might constitute a novel therapy against TB (Rivas-Santiago et al., 2013). Figure 2.3 shows the possible mechanism of actionof AMPs against TB *in vitro* and *in vivo*

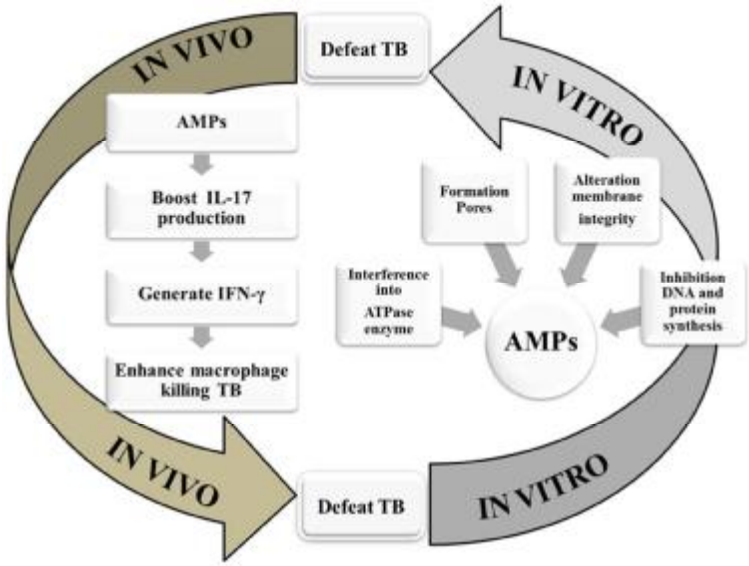

Figure 2.3. Displayed how AMPs impact on bacterial cells survival *in vivo* and *in vitro*

2.11. Efflux pumps in mycobacteria

Efflux pumps are found in almost all bacterial species and genes encoding this class of proteins can be located on chromosomes or plasmids. Bacterial efflux pumps are categorized into five families: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family. The RND superfamily which is only found in Gram-negative bacteria, however, efflux systems of the other four families: MFS, ABC, SMR and MATE are widely distributed in both Gram-positive and negative bacteria. Efflux pumps are either single-component transporters or multiple-component systems containing not only an inner membrane transporter, but also an outer membrane channel and a periplasmic adaptor protein, such as the RND type efflux pumps (Sun et al., 2014). Recentely, several mycobacterial drug efflux pumps have been recognized and characterized (Louw et al., 2009; Viveiros et al., 2003). Genes encoding ABC transporters include about 2.5% of the *M. tuberculosis* genome and at least 37 complete and incomplete ABC transporters have been recognized (Braibant et al, 2000). However, only a few of these transporters have been characterized and appeared to be involved in drug resistance. *M. tuberculosis* encompasses a putative doxorubicin-resistance operon, drrABC (Choudhuri et al., 2002). The *drrA* and *drrB* genes, which expressed in *M. smegmatis*, confer resistance to a broad range of antibiotics, such as tetracycline (TET), erythromycin, EMB, norfloxacin, SM and chloramphenicol. The resistant phenotype is reversed by using efflux pump inhibitors such as reserpine or verapamil (Choudhuri et al., 2002). Studies suggested that the main role of the Drr proteins of *M. tuberculosis* might be the lipids export to the peripheral of the cell and, in particular, *drrC* appears to be involved in the transport of phthiocerol dimycocerosates (Camacho et al., 2001). The *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon encodes an ABC transporter accountable for fluoroquinolone efflux when produced from a

multicopy plasmid. When overexpressed in *M. smegmatis*, this operon increases 8-fold and 2-fold the MIC of ciprofloxacin and MIC of norfloxacin, respectively. The level of resistance diminishes in the presence of reserpine, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil (Pasca et al., 2004).

The LfrA protein of *M. smegmatis*, which might be responsible for low-level resistance to fluoroquinolones, acridine and quaternary ammonium compounds, was the first efflux pump to be described in the genus Mycobacterium (Liu et al., 1996; Sander et al., 2000; Takiff et al., 1996). When overexpressed, *lfrA* appeared to play a significant role in the resistance to ciprofloxacin in *M. smegmatis*. However, in the absence of overexpression, *lfrA* have no effect on the susceptibility to fluoroquinolones. In fact, *lfrA* disruption diminished 8-fold the MICs of ethidium bromide (EtBr) and acriflavine and 2-fold the MICs of ciprofloxacin, doxorubicin and rhodamine, respectively (Li et al., 2004). This difference of results between EtBr and ciprofloxacin can be elucidated if EtBr is a better substrate for *lfrA* (Li et al., 2004).

The expression of *Rv1258c* enhanced in the presence of R and ofloxacin in clinical strain of *M. tuberculosis* (Siddiqi et al., 2004). Moreover, Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), reserpine and chlorpromazine appeared to reduce the MIC of TET in a *M. smegmatis* strain (Ramon-Garcia et al., 2006). Efflux activity of Tap protein from *M. fortuitum* is inhibited by CCCP and reserpine, a result compatible with the reduction of the MIC of TET in the presence of these compounds. Furthermore, CCCP, reserpine and chlorpromazine reduced the MIC of TET in a *M. smegmatis* (Ramon-Garcia et al., 2006).

It has been suggested that *Rv1634* is described as a new fluoroquinolone efflux transporter in *M. tuberculosis* (De Rossi et al., 2002). It was revealed that this gene diminished susceptibility to numerous fluoroquinolones when overexpressed in *M. smegmatis*. In addition, *Rv1634* seems to be involved in norfloxacin and ciprofloxacin efflux (De Rossi et al., 2002). It has been documented that the *P55* efflux pump has been associated with low-level

resistance to several drugs including TET, aminoglycosides and R (Ramon- Garcia et al., 2009; Silva et al., 2001). A recent investigation showed that *P55* plays significant role in at least three important processes: (i) it provides resistance to several drugs (including rifampin); (ii) it is part of the oxidative stress response; and (iii) it is required to maintain normal bacterial growth on both solid and liquid media (Ramon-Garcia et al., 2009).

The putative efflux protein EfpA of *M. tuberculosis* presents the transporter motifs characteristic of efflux-mediated antiseptic resistance gene *qacA* of *S. aureus*, including those related with proton antiporter function and those specific to drug transporters. It was shown that the expression of *efpA* increased in the presence of INH, indicating that the protein encoded by this gene transports molecules involved in mycolic acid synthesis (Wilson et al., 1999). Although the deletion of the *efpA* homologue in *M. smegmatis* increased 2-fold the susceptibility to EtBr, gentamicin and fluoroquinolones and 8-fold to acriflavine, it resulted in 4-fold diminished susceptibility to rifamycins and chloramphenicol and 2-fold reduced susceptibility to INH and erythromycin (Li et al., 2004). Moreover, this efpA deleted mutant grew more slowly than the wild-type strain, which could mean that its higher susceptibility may be because of impaired growth (Li et al., 2004). Therefore, since the deletion of *efpA* enhances susceptibility to EtBr, it is possible that EtBr is a substrate of this gene (Li et al., 2004).

The genome of *M. tuberculosis* contains several genes that code for putative transport proteins of the RND superfamily. These proteins have been designated MmpL (mycobacterial membrane proteins, large) and are thought to be involved in the transport of fatty acids (Tekaia et al, 1999). In *M. tuberculosis*, MmpL7 protein appeared to export phthiocerol dimycocerosate (PDIM) which is a lipid component of the outer membrane (Camacho et al., 2001). Upstream from the *mmpL7* gene is the *fadD28* gene, which encodes an acyl-CoA synthase probably associated in the release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. A strain with an insertion in *mmpL7* gene yields a

dimycocerosate (DIM) molecule that is retained in the cytoplasm or the cytoplasmic membrane. The production of MmpL7 protein in ***M. smegmatis*** increased 32- fold the MIC of INH. However, this phenotype is reversed if ***fadD28*** and ***mmpL7*** are expressed simultaneously, suggesting that DIM and INH compete for the same transporter of MmpL7 (Pasca et al., 2005).

2.12. Pomegranate (*Punica granatum* L)

The pomegranate tree, which typically grows 12−16 feet, has many spiny branches. It can be tremendously long-lived, as evidenced by trees at Versailles, France, known to be over 200 years old. The leaves are glossy and lance shaped. The bark of the tree turns gray as the tree ages. The flowers are large, red, white, or variegated, and have a tubular calyx that eventually forms the fruit . The ripe pomegranate fruit can be up to five inches wide with a deep red, leathery skin. It is grenade-shaped, and crowned by the pointed calyx. The fruit contains many seeds (arils) separated by white, membranous pericarp, and each is surrounded by small amounts of tart, red juice . Although pomegranate cultivated and naturalized since ancient times over the entire Mediterranean region, it is native of the Himalayas in northern India to Iran. The tree is also cultivated for its fruit in the drier regions of California and Arizona (Jurenka, 2008).

2.12.1. Botanical Classification

Botanical name : Punica granatum
Kingdom : Plantae (Angiosperms)
Order : Myrtales
Family : Lythraceae
Genus : Punica
Species : *P. granatum*

2.13. Medical application of pomegranate

The pomegranate fruit, which could be considered a functional food, has valuable compounds in different parts of the fruit that demonstrate functional and medicinal effects. These can act as antitumoral , as antioxidant or antihepatotoxic agents, and enhance cardiovascular health. They apeared to have antimicrobial, antiinflammatory, antiviral, antidiabetic properties, and they have ability to improve oral and skin health. They help prevent Alzheimer's disease and improve quality of sperm and erectile dysfunction in male patients. However, few well-controlled clinical trials have been accomplished and these effects have not been firmly established (Viuda-Martos et al., 2010). We agree that much deeper investigations into this rapidly growing field are required to evaluate the overall value and safety of pomegranate as an complete fruit or of different extracts originated from pomegranate components.

2.13.1. Antimicrobial activity

Medicinal plants, which represent a rich source of antimicrobial agents, are used medicinally in different countries and are a source of many effective and powerful medications. It has been documented that the fruit peel compound of pomegrannate displayed antimicrobial activity against *S. aureus* and *P. aeruginosa* (Burapadaja and Bunchoo, 1995; ALBARRI et al., 2017). Moreover, methanolic extract of peels appeared to have great activity against *Listeria monocytogenes*, *S. aureus*, *E. coli* and *Yersinia enterocolitica*. *In vitro* antibacterial activities of fruit peels and arils of pomegranate were assessed against food-related bacteria. The outcomes of this study demonstrated that all extracts of pomegranate contained high levels of phenolic compounds and showed strong antibacterial activity against all tested strains (Nuamsetti et al., 2012). Pericarp extract of pomegranate was found to have strong activity against the multiple resistance of *Salmonella typhi*. Aqueous extract of pomegranate was greatly effective against *E. coli* O157:H7 with MIC and minimal bactericidal concentration (MBC) values of 0.78 and 0.39

mg/ml, respectively (Voravuthikunchai et al., 2004). The extract of pomegranate can be used to manage the adherence of various microorganisms in the oral cavity. It has been reported that pomegranate extract showed a strong activity against streptococci strains, *S. mutans*, and *S. mitis* and *C. albicans* (Vasconcelos et al., 2006). In another study performed ***in vivo*** hydroalcoholic extract from pomegranate possess strong activity against dental plaque microorganisms and the number of CFU/ml was diminished by 84% as compared to the control group (11% reduce). These findings indicated that the hydroalcoholic extract might play a significant for dental plaque treatment (Menezes et al., 2006). Low extract concentration (1%, v/v) of pomegranate resulted in a delay in bacterial growth, whereas a higher concentration (1%, v/v) of pomegranate extract eradicated the bacterial growth. A 0.05% (v/v) concentration of pomegranate extract appeared to inhibit Staphylococcal enterotoxin (SE) production (Braga et al., 2005). Pomegranate sauce appeared to show remarkable activity against *E. coli* O157:H7 and *salmonella* spp (Değirmenci ve Var, 2017). Table 2.2 illustrates an outline of investigations which appraised the possible advantages of pomegranate as an antimicrobial agent

Table 2.2. *In vitro* Antibacterial Activity of Pomegranate

Pomegranate part	**Microorganism**	**Refrences**
Pomegranate fruit peel	*M. tuberculosis* and *Klebsiella pneumoniae.*	(Dey et al., 2015)
Fresh pomegranate juice (FPJ)	*Staphylococcus epidermidis.*	(Betanzos-Cabrera et al., 2015)
Methanolic extract of peels	*Listeria monocytogenes, S. aureus, E. coli , Yersinia enterocolitica, and Salmonella enteritidis*	(Al-Zoreky, 2009)
Pomegranate aril extracts	*Pseudomonas aeruginosa, S. aureus, Corynebacterium xerosis, E. coli, Enterococcus faecalis, and Micrococcus luteus*	(Nuamsetti et al., 2012)
Pomegranate sauce	*E. coli* O157:H7 and *salmonella* spp	(Değirmenci ve Var, 2017)

2.13.2. Anti-Inflammatory and analgesic effect

Non-steroidal anti-inflammatory medications, which exhibit adverse effect through the inhibition of arachidonic acid metabolism, are effective in the cure of inflammation. However, using natural products as an alternative medicine are very effective in the inflammation cure without any serious side effects on both the cyclo-oxygenase enzyme activity and arachidonic acid metabolism. The role of pomegranate in the decrease of inflammation by modulation of several physiological activities was confirmed. Anti-inflammatory and analgesic activities of fruit, flower, and leaves have been documented and the pre-treatment with the dried extracts produced important and dose dependent inhibition of edema (Bagri et al., 2010). The anti-inflammatory compounds were mostly reported from the seeds in which polyphenols and fatty acids were considered as the major anti-inflammatory elements. The extract of pomegranate from the cold pressed seed oil mainly consist of polyphenols and fatty acids which displayed about 31-44% inhibition of sheep cyclooxygenase and 69-81% inhibition of soybean lipooxygenase, whereas the extract from fermented juice exhibited 21-30% inhibition of soy bean lipooxygenase (Schubert et al., 1999).The polyphenols obtained from the extract of pomegranate in cold pressed seed oil were found to suppress inflammatory cell signalling in colon cancer cells (Adams et al., 2006). The extract of pomegranate showed anti-inflammatory activity through the NF-κB inhibition and Erk1/2 activation with reduction of NO and PGE2 synthesis in human intestinal Caco-2 cells. Furthermore, ellagic acid was able to reduce the activation of NF-κB via a mechanism independent of Iκ-Bα phosphorylation *In vitro* studies on antibacterial, anti-inflammatory and anti-allergic activities of standardized pomegranate rind extract (SPRE) containing 13% (w/w) ellagic acid have been carried out. The activity of anti-inflammatory of SPRE was assessed by determining the inhibition of nitric oxide (NO) production in murine macrophage-like RAW264.7 cells. SPRE displayed a strong inhibitory effect on the production of NO, with an IC- 50 of 10.7 l g/ml (Panichayupakaranant et al., 2010).

2.13.3. Anti-viral activity

Tannin from the pericarp is considered as an effective compound against hepatitis C and genital herpes virus (HSV-2) that kills virus and blocks its absorption to cells (Zhang et al., 1995; AlMatar et al., 2018). It has been documented that the acidity of juice and liquid extract solutions contribute to swift activity against influenza virus (Sundararajan et al., 2010).

2.13.4. Anti-fungal activity

Antifungal activities of pomegranate fruit have been evaluated. Based on the results, the greatest antibacterial activity was recognized against *S. aureus* and among fungi high activity was noticed against *Aspergillus niger* (Dahham et al., 2010). Pomegranate showed considerable antimicrobial activity against yeast cells of *Candida* (Anibal et al., 2013).

2.13.5. Anti-tumor activity

Cancer is described as multi-factorial disorders and several factors involve in this process. In this vista, pomegranates fruits, seed and peels, which are rich sources of antioxidants, illustrate cancer preventive role. The impacts of pomegranate extract (PE) were scrutinised on a mouse mammary cancer cell line WA4. It was observed that PE exhibited the inhibition of the WA4 cells proliferation in a time- and concentration dependent manner (Dai et al., 2010).

The efficiency of pomegranate fruit extract and diallyl sulphide alone and in combination was studied. Results demonstrated that diallyl sulphide and fruit extract alone delayed commencement and tumor occurrence by around 45% and around 55%, respectively, whereas their combination synergistically reduced tumor occurrence more potentially at low doses (Dai et al., 2010). Earlier investigators demonstrated that apoptotic cell numbers and the pro-apoptotic gene Bax expression were significantly increased, and anti-apoptotic gene Bcl-2 was reduced after pomegranate fruit extract treatment (Dikmen et al., 2011).

2.13.6. Wound healing activity of pomegranate fruit

Antioxidant immunity, epithelialisation and typical biochemical features comprise the familiar properties of the wound healing procedure which are prevalent in damaged tissues. The applications of pomegranate for wound healing are broadly and considerably efficient, as it is composed of the phytochemical elements available in the extract as saponins, triterpenes, tannins, alkaloids, flavonoids, and cardiac glycosides (Ismail et al., 2012). The healing impact of pomegranate husk extract (PHE) for intense second-degree burn wounds in rats has been explored. The animals were put into two divisions as subsequently: (1) deep second degree burn model (control) division, (2) burns model treated with 1% silver sulfadiazine (SSD) division, and (3) burns model treated with PHE division. Following 21 days, the average wound region of PHE therapy division (0.21 ± 0.07cm2) and SSD therapy division (1.15± 0.1cm2) were much smaller contrasted to the control division (2.42± 0.2cm2, $p < 0.01$) correspondingly. As shown by histological results, inflammatory cells disappeared noticeably and were exchanged by new granulation tissue by means of PHE therapy or SSD therapy, although the control group still exhibited extreme inflammatory cell infiltration. Therefore, PHE is a competent and conceivably appropriate therapeutic herb for the treating of second degree burns (Ma et al., 2015). The necessary period for entire healing of severe second degree burns in the absence of the administration of particular therapeutic agents may comprise an further three to six weeks, and these burns could result in scar tissue which may undergo hypertrophy and tighten on its own (Johnson and Richard, 2003). The *in vivo* wound healing assurance of an SPRE (standardised pomegranate peel extract) and its chief antioxidant ingredient, ellagic acid (EA, 13%w/w), were explored. The revelation was made that SPRE (5 and 2.5%) in addition to its equivalent quantity of EA (0.65 and 0.325%) elevated the tensile support of the incision wound by as much as 35.43 and 31.82% correspondingly. SPRE at 5 and 2.5% increased wound contraction of the excision wound in addition to the burn wound, though EA was effective merely at 0.65%

within the two wound models. Additionally, SPRE improved collagen synthesis by up to 21.83 mg/g and restricted the permeation of neutrophil dose autonomously, while EA was not efficient in raising collagen amassment and its restrictive influence on neutrophil permeation was less. Thus, the topical use of SPRE in addition to its marker compound EA swiftened dermal wound healing for the models of the rat incision, excision and burn wound (Mo et al., 2014). In a research concerning the healing ability of hydroalcoholic extract from pomegranate peel (Hayouni et al., 2011), besides ellagitannins being the key compounds (punicalagin A, punicalagin B, gallic acid and ellagic acid), some anthocyanidins were further traced within the hydroalcoholic extract (delphinidin-3, 5-diglucoside, quercitin-3, 40-O-diglucoside, pelargonidin-3-glucoside and quercitin-3, 40-glucoside). These compounds could also be available in SPRE and assist in the healing effectiveness of SPRE. Furthermore, the synergy influence is a widespread occurrence with herbal extracts, which are usually comprised of several compounds (Süntar et al., 2010; Tam et al., 2011; Wagner and Ulrich-Merzenich, 2009). A more elevated pharmacological effect from SPRE is credited to the interactions of the numerous compounds within it which reinforces its advantage above EA. Therefore, SPRE comprises a potential efficient phytopharmaceutical which enables wound healing and is better than the marker compound EA (Mo et al., 2014). An alternative research has scrutinised the impact of pomegranate seed extract (PSE) which is administrated orally on healing of surgically caused full thickness skin wounds. The rate of wound closure was considerably improved in rabbits treated with PSE. The average wound region documented on the 5th, 10th, 15th and 20th days was considerably reduced in the treated group as contrasted to controls. On the 20th day, contraction and epithelisation of the wound was reduced in the control rabbits as opposed to those administered with PSE. From the PSE group, all of the rabbits had entire collagen amassment, granulation tissue creation as well as re-epithelialisation in contrast to the control group. Furthermore, the raised levels of malondialehyde (MDA) and the reduced glutathione (GSH) level, glutathione-

peroxidase (GSH-Px) as well as catalase (CAT) functions within the control groups contrasted to the test groups could imply that PSE is a strong antioxidant (Kandemir et al., 2013). Oxidants have a significant function in healing of wounds (Pin et al., 2008; Sen and Roy, 2008), offering protection and signalling against microorganisms. Nonetheless, the oxidants require detoxifying so as to avoid any impairment to host cells. An antioxidant defense structure involves minimising (scavenging) or dismutation of O2- and/or HO2- and their protonated states. If the antioxidant defense structure is incapable of eliminating the oxidants, oxidative stress results from the change in homeostasis. Superoxide comprises the chief element of ROS. H2O2 and O2 can be swiftly dismutated by inducible and constitutive superoxide dismutases (SOD). Catalases to H2O and O2 may also detoxify H2O2 (Dalton et al., 1999; Shan et al., 2010). Within the wound healing procedure, different inflammatory cells such as neutrophils, endothelial cells, fibroblasts and macrophages generate superoxide. Functioning macrophages and neutrophils generate great quantities of superoxide and its derivatives through the phagocytic isoform of NADPH oxidases (Droge, 2002). Antioxidants are essential to sustain reduced levels of free radicals as well as reactive non-radical species originating from radicals. The enzymes Superoxide dismutases (SOD), catalase and glutathione are part of these, in addition to non-enzymatic compounds including vitamin E, glutathione, ascorbate and β-carotene (Droge, 2002). These antioxidants could function to reduce the oxidants due to the raised amount of protease operation during the initial stages of wound healing (Barrick et al., 1999; Rosenblat et al., 2006). The wound healing capability of ***Punica granatum L***. peels methanolic extract-based ointment has been analysed by Hayouni et al. (2011) in guinea pigs. On the 8th day, it was observed that the level of wound contraction was considerably swifter in the set administered with ***P. granatum*** ointment as opposed to the untreated group (control). On the 16th day, healing of 83.5% was displayed by the ***P. granatum*** extract-based ointment group, while the untreated group displayed healing of 43%. On an animal treated with ***P. granatum*** ointment,

no scars were visible as opposed to the untreated animals on the 20th day. In addition, the hydroxyproline, DNA, complete protein and tensile strength of animals administered with *P. granatum* ointment were considerably higher than those from the untreated group. The histological scrutiny disclosed that on the 4th day, areas illustrated raised cellular infiltration for animals given topical administration of the *P. granatum* ointment as opposed to control. On the 12th day, a well-progressed organization of granulation tissue and constant epithelialisation was noted in the treated group. On the 20th day, there was a complete thickness epidermal restoration which entirely encompassed the wound section (Hayouni et al., 2011).

The DNA and protein substance of granulation tissues signify the extents of cellular propagation and protein synthesis. Raised DNA and protein contents of the treated wounds imply that *P. granatum* ointment encourages cellular proliferation. However, it was recently determined that a strong dermal impact was observed in an aqueous extract of pomegranate peel through encouraging propagation of dermal fibroblast and collagen synthesis while reducing the key collagen-reducing enzyme within skin (matrix metalloproteinase-1), although it had no development-sustaining impact on keratinocyte (Aslam et al., 2006). The collagen/DNA ratio of the granulation tissues additionally implies that *P. granatum* ointment could raise the collagen synthesis of every cell. The synthesise collagen molecules are arranged on the wound area and become cross linked to create fibres. Wound potency is gained from the remodelling of collagen as well as creation of strong inter- and intra-molecular crosslinks. Wounds administered with *P. granatum* additionally illustrated a raised wound contraction level, resulting in a swift healing as affirmed by the reduced epithelialisation time frame when contrasted to untreated control wounds (Figure 2.4). Therefore, the prepared ointment could well be employed as a skin repair instrument with no danger to human wellbeing subject to these findings, as well as the fact that it has been

determined that pomegranate ex tracts have been employed in circumstances comparable to those employed by traditional medicine (Hayouni et al., 2011).

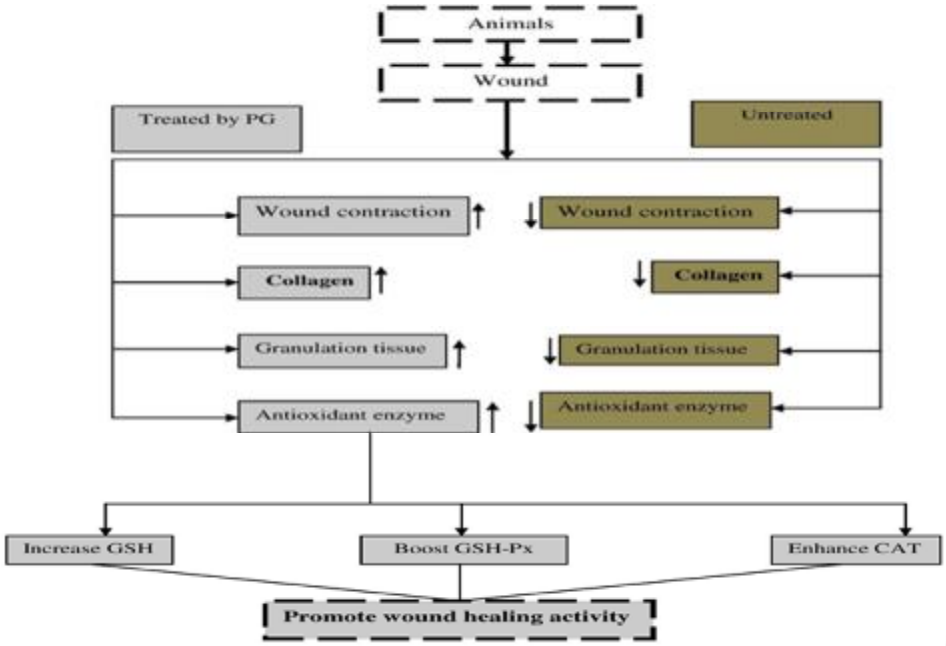

Figure 2.4. Mechanism of action of pomegranate in the treatment of Wound Healing

2.13.7. Neuroprotective effects of pomegranate fruit

Susceptibility to oxidative stress comprises a widespread pathological aspect of all neurodegenerative diseases (Butterfield and Kanski, 2001). Many of the chief proteins that are actually creating aggregates in these circumstances are oxidized (Aguzzi and O'connor, 2010; Morales et al., 2009), and for prion disease, oxidization of Met residues within PrP Helix3 comes before its obtaining of protease resistance (Canello et al., 2010). Additionally, brain lipids are oxidised within these and alternative brain diseases (Uttara et al., 2009; Haider et al., 2011), implying that lipid oxidisation could have a significant function in the pathogenesis of alternative brain conditions (Perluigi et al., 2012; Adibhatla and Hatcher, 2010; Reed, 2011). Compounds like acrolein and 4-oxo-2nonenal are generated by

oxidised phospholipids, and are mainly noxious to brain cells (Singh et al., 2010). Current evidence proposes that as well as autoimmune trajectories, the neurological impairment in demyelinating diseases like multiple sclerosis (MS) (Lassmann, 2008) may additionally be of neurodegenerative nature (Herz et al., 2010; Witte et al., 2009). Brain protein oxidisation was truly illustrated to take place in MS as well as experimental autoimmune encephalomyelitis, a well-scrutinised animal MS model. More significantly, lipids within MS plaques are oxidized, as illustrated by immunostaining with an antibody against oxidised phospholipids (Castegna et al., 2011).

EAE- induced mice administered with natural pomegranate seed oil (PSO) provided some decreasing in disease load; this positive effect was considerably raised when EAE mice were administered with Nano-PSO of particular magnitude nanodroplets at considerably reduced oil intensities. The significance of the nanodroplet size has been illustrated regarding their therapeutic effect since 30 nm droplets of PSO were inactive in this clinical setting as opposed to those approximately 200 nm size (Binyamin et al., 2015). The small droplets could actually yield to disturbance from bio-surfactants like bile salts at a varying rate than greater droplets (González-Sarrías et al., 2010; Johanningsmeier and Harris, 2011) which could stay undamaged for greater periods and consequently traverse the blood-brain barrier (BBB) (Zhang et al., 2013; Schubert and Schmidt, 1988). Pathological scrutiny disclosed that Nano-PSO treatment drastically decreased lipid oxidation and demyelination within the brains of infected animals, which are characteristic of this relentless neurological disease. It has therefore been suggested that new compounds of natural antioxidants like Nano-PSO could be contemplated in the treating of patients dealing with demyelinating conditions (Binyamin et al., 2015). From the mechanistic view, it has been illustrated that Nano-PSO may limit lipid oxidation as well as demyelination of EAE brains, while immune infiltrates are available within the central nervous system (CNS). These outcomes signify that although oxidised lipids might not be available in the primary activation of

immune cells, they could actually have a key function in the consequent demyelination, as implied formerly (Ljubisavljevic, 2016). Due to the neurotoxic nature of oxidised lipoproteins and their pro-inflammatory features, demyelination and axonal damage in MS could result from lipid peroxidation products (Ferretti and Bacchetti, 2011; Leitinger, 2008). Furthermore, the Nano-PSO activity, or possibly the particle size illustrated to be advantageous for EAE could be mainly restricted to the CNS, thus intruding on brain oxidation characteristics and demyelination, and less with infiltration inhibition. Due to the absence of noxiousness of these reagents, Nano-PSO could comprise a suitable option for people during the primary phases of demyelinating conditions like MS. During subsequent phases, Nano-PSO could be employed in addition to progressed MS treatments like Natalizumab (Tysabri), which minimises the movement of lymphocytes towards the CNS through binding to the α4 integrin very late antigen, or as an addition to alternative antioxidant compounds (Sellebjerg and Sørensen, 2015; Kizelsztein et al., 2009). As well as demyelinating conditions, oxidative stress additionally comprises a significant element of the majority of neurodegenerative diseases (Witte et al., 2009). The EO6 antibody displayed here to stain plaques within the EAE brains that have not been given therapy as it was illustrated to stain human MS plaques also responds considerably with brain slices from TgMHu2ME199K mice, a mouse model for genetic prion condition, implying that lipid oxidation could comprise an overall characteristic of neurodegeneration. More significantly, Nano-PSO was additionally beneficial for the avoidance and control of genetic prion state within the TgMHu2ME199K model, showing that reagents which can avoid lipid oxidation could be useful for a range of neurodegenerative conditions (Mizrahi et al., 2014).

A great quantity of natural antioxidants is commonly accessible within a good human diet. Many of them, like sulforaphane within broccoli, epigallocatechin gallate and curcumin within green tea were highlighted for their neuroprotective aspects within cells and assessed in suitable animal models.

Nevertheless, their *in vivo* action was hindered by the subpharmacological doses taken from food, their inefficient bioavailability to people, fast chemical degradation, as well as minimal circulation to various organs within the body, especially the CNS (Han et al., 2007; Hatcher et al., 2008; Choi et al., 2001). Thus, natural mixtures may be created as active drugs and could be efficient in prevention of conditions as well as abrogation of disease advancement. Furthermore, it has been illustrated for once that pre-dosing of pomegranate to rats may provide a considerable dose-dependent neuroprotective operation opposing cerebral I/R brain damage as well as DNA impairment through anti-inflammatory, antioxidant, anti-apoptotic as well as ATP-replenishing (Ahmed et al., 2014). Alternative research has targeted exploration of the constructive impact of antioxidants available in figs, pomegranates and dates on a number of neuroinflammatory markers within brain areas and blood plasma. In both hippocampus and cortex, considerable elevations in levels of Aβ1-40 and Aβ1-42 have been observed (Essa et al., 2015). The raised Aβ1-40 and Aβ1-42 amassment will be inclined to enhance oxidative stress liable for the advancement of neurodegeneration (Subash et al., 2015). Additionally, the extents of pro-inflammatory cytokines, especially IL-1β, TNF-α and IL-6 within the brains of trial animals were raised in APPsw (Tg2576) mice. The extents of IL-1β, TNF-α and IL-6 within the cerebral cortex as well as the hippocampus were reduced within Tg2576 mice provided with diets supplemented by dates, figs or pomegranates (Essa et al., 2015). IL-1β, an essential cytokine within the conduction of the complex immune reaction to injury and infection, was initially described as a peripheral immune cell mediator (Dinarello, 1996). Additionally, synthesis of this cytokine has been stated to occur within the brain through particular neurons and glial cells; and in various areas of the brain IL-1β receptors were discovered, with the greatest profusion within the hippocampus (Rothwell and Hopkins, 1995; Besedovsky and del Rey, 1996; Haas and Schauenstein, 1997). Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 have been stated to be

considerably high within the plasma or cerebrospinal liquid fluid of individuals with Alzheimer's (AD) (Fillit et al., 1991; Blum-Degena et al., 1995). Furthermore, a significant function of inflammation within AD is supported adequately through the inverse association amid anti-inflammatory medical treatment, and the onset and symptoms (Rogers et al., 1993; Breitner et al., 1994). The expression of IL-1β in various kinds of plaque in AD was documented by Griffin et al. (1995), signifying that an inflammatory reaction has a core function in the progression of plaque and dystrophic neurite creation. IL-6 was available in the primary phases of plaque creation and expression of this cytokine was associated with clinical dementia (Huell et al., 1995). In rat pheochromocytoa cells, IL-1β improves the cytotoxicity of Aβ-peptide (Fagarasan and Aisen, 1996). It has been reported that β-amyloid proteins in addition to interferon (IFN)-δ encourage microglia to produce neurotoxic TNF-α and reactive nitrogen intermediates, and these incidences may have a purpose in the pathogenesis of neural degeneration observed in AD and aging (Meda et al., 1995).

The structure of the reduction of IL-1β, TNF-α and IL-6 by pomegranates is indefinite, as its numerous active elements such as, catechin, caffeic acid, quercetin, ellagic acid, ascorbic acid, anthocyanins gallic acid, flavanoids, tannins, alkaloids, rutin and epigallocatechin gallate (EGCG), have multifunctional application, thus causing it to be pharmacologically complex. These present findings, in concurrence with prior reports, imply that the amounts of cytokine have indeed been reduced by the pomegranates in the diet (Shukla et al., 2008; Winand and Schneider, 2014; Neyrinck et al., 2013; Celik et al., 2013; de Oliveira et al., 2013; Asgary et al., 2014). Nonetheless, the antioxidant features of pomegranates have been adequately documented. These features encompass restriction of lipid per-oxidation and free radical scavenging in addition to an improvement of antioxidant status (Tezcan et al., 2009; Zhang et al., 2011; Jing et al., 2012) as well as neuroprotection (Hartman et al., 2006; Braidy et al., 2013; Rojanathammanee et al., 2013). Therefore, the figs, date palm fruits and

pomegranates demonstrated capable treatment possibilities for neurodegenerative conditions encompassing AD in Tg2576 AD mouse brain, and the safety systems could be associated with their antioxidant operations of phenolic elements (Loren et al., 2005).

During their *in vivo* research, Sarkaki and Rezaiei (2013) outlined that dispensation of PSO in extended cerebral ischemia results in a considerable enhancement on memory with criterion condition responses (CCRs) within Y-maze as well as step-through latency (STL) within two-way shuttle box. The outcomes illustrated that active as well as passive avoidance memories were significantly damaged in rats following cerebral hypoxia-ischemia (CHI) and occlusion of bilateral common carotid arteries (2CCAO) (Sarkaki and Rezaiei, 2013). It was stated by Gabizon et al. (2014) that dispensation of great PSO concentrations could defer the materialisation of neurodegenerative diseases including prion diseases in young transgenic mice (Tgs). Furthermore, reduced amounts of Nano-PSO considerably deferred the onset of disease in asymptomatic TgMHu2ME199Kmice and deferred the aggravation of disease in already unwell mice (Figure 2.5). Thus, PSO compounds could be impressive on neurodegenerative diseases (Gabizon et al., 2014).

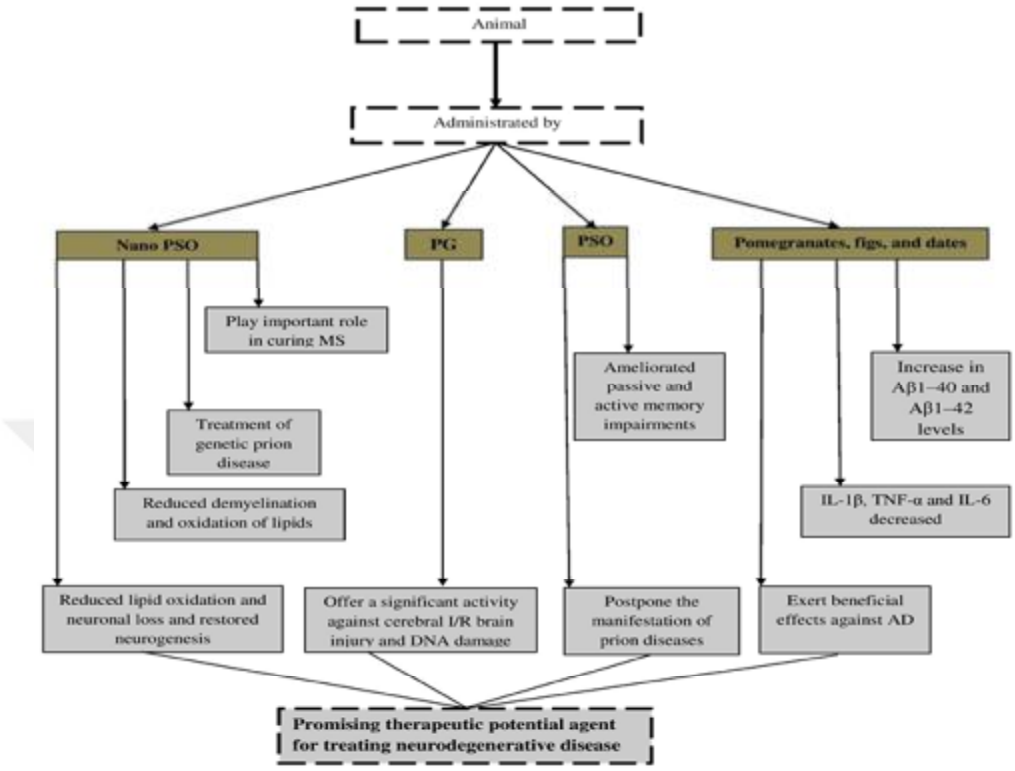

Figure 2.5. The proposed mechanism of action of pomegranate for treating neurodegenerative diseases

2.13.8. Antidiabetic and Antiobesity properties of pomegranate fruit

Obesity is the result of a discrepancy in energy, where energy absorption is more than consumption. The avoidance and treatment of this imbalance in energy necessitates lifestyle alterations regarding physical fitness as well as a raise consumption of natural foods which assist in expenditure of energy through encouraging thermogenesis and oxidation of fat (Hill and Peters, 2002; Hursel and Westerterp-Plantenga, 2010). Numerous natural products, which comprise crude extracts as well as individual compounds derive from plants may cause a reduction in body weight and avoid obesity caused by diet. The pomegranate and its elements is one fruit that is the subject of extensive research (Al-Muammar and Khan, 2012). Reports have presented the anti-obesity influence within OVX rats; the

combinations of 120mg/kg dried pomegranate concentrate powder (PCP) and Red Clover (RC) minimised intake of food and water contrasted to a single mixture of PCP and RC. Furthermore, the 120mg/kg combinations of PCP and RC resulted in raised urinary and fecal excretion as opposed to PCP or RC administration although not notably impacting intake of food and water. These outcomes signify that the inclusion of suitable quantities of PCP raised the restrictive impact in OVX-caused estrogen scarcity and associated obesity (Kang et al., 2015). These influences are credited to the improvement of motility within the digestive tract or diuretic influences, in addition to restriction of estrogen through augmentation of the satiating strength of digestive hormones, cholecystokinin (CCK) (Thammacharoen et al., 2007), as well as glucagon(Geary and Asarian, 2001). Raise fecal excretion was caused by raised digestive motility, which subsequently reduced the rats' body weight, comparable to the reduced body weight resulting from diuretics in rats (Snedeker and Hay, 2012; Verlander et al., 1998). The control of food intake and body weight involves estradiol which mostly alters the strength of feedback signals which manage size of meals (Asarian and Geary, 2006). The structure accorded more analysis indicates that the satiating power of exogenous and endogenous CCK is increased by estradiol (Thammacharoen et al., 2007). A comparable clarification could be pertinent to glucagon as estradiol amplifies the influence of glucagon antibodies and glucagon within OVX rats (Geary and Asarian, 2001). The lack of estradiol results in a provisional rise in intake of food and a maintained rise in body weight (Butera, 2010). This occurrence is clinically significant due to the reduction of levels of estradiol in menopausal females; significantly, postmenopausal females comprise a raised proportion of the obese populace (Lutz, 2011). Reduction of estrogen resulting from ovariectomy within rats caused rigorous increases in the consumption of food and water, although not in fecal and urinary excretion, and this enabled body fat deposition, particularly within the abdominal cavity as asserted by alternative investigators (Ateba et al., 2013). The fat collection or rise in fat deposition within

the body comprises a key feature of obesity, and a cellular hypertrophy seems to be the main means of extension for intra-abdominal adipose tissues within rats (Kim et al., 2013). Thus, a possible impact of RC-PCP combination raised the anti-obesity influence of RC within OVX rats (Kang et al., 2015). Furthermore, for male SD rats, dispensation of 150mg/kg pomegranate extract over eight weeks comprising 40% punicalagin adequately avoided high-fat diet (HFD)-caused obesity related amassment of cholesterol and cardiac triglyceride in addition to myocardial impairment. The AMP-activated protein kinase (AMPK) trajectory was simultaneously activated, which could be the reason for the avoidance of mitochondrial loss through upregulation of mitochondrial biogenesis as well as amelioration of oxidative pressure through improvement of stage II enzymes within the heats of HFD rats. In addition to the normalised expression of uncoupling proteins as well as mitochondrial dynamic regulators, pomegranate extract (PE) considerably avoided cardiac ATP loss caused by HF. Therefore, the key active element in PE, punicalagin, could alter mitochondria and stage II enzymes by means of AMPK trajectory to avoid cardiac metabolic disorders resulting from HFD (Cao et al., 2015; Kukidome et al., 2006; Tedesco et al., 2008). Green tea and pomegranate extracts are capable of safeguarding from ER stress caused by HFD within the skeletal muscles of mice, even though amassment of fat is not avoided. They additionally assuage oxidative pressure, activation of the ubiquitin-proteasome trajectory as well as upregulation of genes inferred in autophagy regulation. Each of these procedures are closely interrelated and may assist in the protective operation of green tea and pomegranate extracts opposing ER pressure and muscle protein degradation for obese individuals (Rodriguez et al., 2015). It was illustrated by Sadeghipour et al. (2014) that dispensation of hydro-ethanolic extract from *P. granatum* peel illustrated discernible antihyperlipidemic impacts in rats given a high lipid diet. Tri-glycerides, serum cholesterol, AP, AST, LDL and ALT were reduced by pomegranate extract, while serum HDL levels were raised in rats given a high lipid diet as opposed to rats

given saline. Additionally, liver impairment was assuaged by the extract, encompassing fatty change in hepatocyte, and sinusoid dilation (Sadeghipour et al., 2014). Addition of pomegranate vinegar (PV) raised phosphorylation of AMP-activated protein kinase (AMPK), resulting in alterations in mRNA expressions: increases in the case of hormone susceptible lipase and mitochondrial uncoupling protein 2 (UCP2), and reductions for sterol control element binding protein-1c (SREBP-1c) as well as peroxisome proliferator-activated receptor γ (PPARγ) within adipose tissues; within the liver, increases in the instance of PPARα and carnitine palmitoyltransferase-la (CPT-la) and reductions for SREBP-1c. Simultaneously, PV decreases reduction in plasma triglcerides, body mass, hepatic triglycerides and fat mass. PV therefore assuages adiposity by means of the coordinated management of AMPK, which results in the enhancement of lipolysis within adipose tissue as well as activation of oxidation of fatty acid within the liver (Ok et al., 2013). Gut microbiotain mice were altered in preference of bifidobacteria by the dispensation of a pomegranate peel extract (PPE) rich with Balb/c polyphenols. A reduced expression of key inflammatory expression within the colon as well as the visceral adipose tissue followed this prebiotic influence. As a result of the PPE therapy, the gut microbiota alterations were additionally followed by an enhancement of atherogenic markers like LDL-cholesterol within obesity caused by HF diet. Thus, the implication of these findings is that constructive health effects related to alteration of gut microbiota may be conveyed by PPE and could comprise a natural option in the avoidance of cardiovascular and obesity (Neyrinck et al., 2013). As documented by Vroegrijk et al. (2011), male C57B1 mice given 1g of pomegranate seed oil for every 100g of a HFD over a period of 12 weeks caused a reduced body weight, mirrored by a decreased fat mass in contrast to controls, although no variation was observed in lean mass of the two groups. The research affirmed prior investigation carried out by McFarlin et al. (2008) in the CD1 mouse strain who found a decreased insulin or fasting glucose strength in animals fed pomegranate seed oil, which was not seen in the study by

Vroegrijk et al. (2011). This could be due to the varying mouse strains and a contrary fat diet. Nonetheless, Vroegrijk et al. (2011) stated that inclusions of pomegranate seed oil augmented sensitivity in mice on HFD, somewhat validating the intolerance of ameliorated glucose determined by Hontecillas et al. (2009) who studied the anti-obesity effect of the pomegranate leaf in a mouse model of obesity resulting from HFD. These mice were treated with PLE (pomegranate leaf extracts) in doses of 800mg/kg over five weeks. The oral dispensation of PLE in doses of 800mg/kg reduced body weight and different proportions of adipose pad weight, in addition to the total cholesterol/HDL cholesterol ratio, levels of glucose and serum total cholesterol triacylglycerol. Furthermore, the PLE reduced the dyslipidemia of obesity as well as cardiovascular risk elements due to a reduction in the pad weight proportion of abdominal fat as contrasted to the control mice. Consumption of food was also reduced in obese mice administered with PLE, comparable to obese mice administered with sibutramine. Ellagic acid and tannic acid are credited with the activity (Lei et al., 2007).

Numerous researches have provided substantiation for control of body weight employing pomegranate flower extracts (PFEs). The impact of PFEs towards abnormal cardiac lipid metabolism was evaluated by Huang et al. (2005). As amassment of triacylglycerol and raised oxidation of fatty acid resulted in cardiac dysfunction of diabetic hearts, PFE500mg/kg was given to Zucker diabetic fatty rats; PFE comprises a traditional anti-diabetic treatment. The PFE was dispensed over six weeks, causing a rectified abnormal cardiac lipid metabolism through the stimulation of PPAR-a (peroxisome proliferator-activated receptor- a), reduced distributed lipids, as well as a restriction of cardiac uptake. PPARs are renowned as core lipid and carbohydrate metabolism modulators (Huang et al., 2005).

Obesity is indicative of the availability of fibrosis, which develops into progressed liver conditions. Non-alcoholic fatty liver conditions more free fatty acids and hypertriglycerides are available in the majority of patients who have the

metabolic syndrome and type-2 diabetes (Stefanovic-Racic et al., 2008). The ursolic acid and oleanolic acid available in PFEs comprise antihyperlipidemic features (Li et al., 2008; Liu, 1995; Liu, 2005). GA, available in PFEs, is known to rectify fatty liver and hyperlipidemia caused by HFD in mice (Jang et al., 2008). The influence of PFE towards hepatic lipid collection in Zucker diabetic fatty rats that had advanced liver conditions was explored by Xu et al. (2009).

Zucker diabetic fatty and male Zucker lean rats were administered PFE 500 mg/kg over six week, which caused a reduction in the hepatic triacylglycerol substance as well as fatty droplets, even though not altering the hepatic total cholesterol amount. The greatest effects were observed in Zucker diabetic fatty rats in comparison to Zucker lean rats (Xu et al., 2009).

Additional visceral adiposity inside the abdominal cavity is a risk element of the metabolic syndrome, insulin resistance and cardiovascular disease (Chung et al., 2006). While seeking natural origins of anti-obesity compounds, de Melo et al. (2010) appraised the influence of oleanolic acid usually available in the PFE, which displays a broad array of biochemical and pharmacologic effects. To oppose obesity, obese mice with induced HFD were administered with oleanolic acid 10mg/kg every day over 7 days, which caused considerable reductions in gain of body mass (11.11%,) as well as visceral abdominal fat (1.28-fold). Levels of blood glucose were additionally noted to reduce to the level of 31% (de Melo et al., 2010).

Research by Cerdá et al. (2003) scrutinised the impact of 6% punicalagin available in pomegranate juice (PJ) on female rats over 37 days. A consumption of 4800 mg .kg-1. d-1 had a reducing impact on the body mass and food intake of the animals. These findings clearly signify the string, constructive impact of PJ in restraining obesity as well as altering risk elements of heart disease for hyperlipidemic individuals(Cerdá et al., 2003) (Figure 2.6).

In the case of OLETF rats, a precise strain of rats applied in the exploration of type II diabetes with obesity, it was observed that these rats were

still reasonably lean when punicic acid (PA) was encompassed in the diet and the fat cells could go through automatic cell death when in contact with PA (Arao et al., 2004; Nishimura et al., 2002). Additionally, PA has been observed to decrease levels of fasting blood sugar within streptozotocin-induced (STZ) (Banihani et al., 2013) although not for Alloxan-induced type-II diabetic rats, and to avoid resistance to insulin and obesity caused by diet (Jelodar et al., 2007).

For mice given a high fat diet with or in the absence of 1% SPE over 12 weeks to bring about obesity and resistance to insulin, mice given PSO were observed to be leaner and have better insulin sensitivity compared to those not given PSO. The safety provided by PSO opposing diet-induced obesity and resistance to insulin was observed to be autonomous from differences in food ingestion or use of energy. Through use of hyperinsulinemic-euglycemic clamp technology, the lack of influence on liver insulin susceptibility and enhanced peripheral neurosensitivity resulting from PSO was illustrated (Vroegrijk et al., 2011).

PPAR (Peroxisome proliferator-activated receptor) γ is a nuclear receptor, mainly available within adipose tissues, managing the collection of fatty acids, glucose metabolism, in addition to molecules overwhelmed by several antidiabetic agents. PPARs were defined by Bassaganya-Riera et al. (2010) as molecular objectives for disorders and diseases related to diabetes, and highlighted the requirement for the seeking of natural PPAR γ agonistic medications. In contrast to the synthetic analogs, compounds originating from plants comprise enhanced safety elements and function by means of a number of molecular targets, therefore PA could potentially be employed to control levels of blood sugar and manage inflammation of the intestine. Additionally, PA has been demonstrated to enhance advancement of the immune system, assist in improving or sustaining the extents of CD4+ and CD8+T lymphocyte, raising immune reaction to viruses, as well as avoiding or ameliorating obesity and type II diabetes(Bassaganya-Riera, 2014,

Yuan et al., 2015). Therefore, due to its PPAR γ agonist features, PA could be advocated as a therapeutic and prophylactic molecule (Anusree et al., 2014).

It has been demonstrated for wild type CD-1 mice that dispensing PSO in addition to HFD could decrease the leptin intensity and enhance the concentration of adiponectin within the controls of HFD. The body mass, percentage weight gain, as well as insulin were illustrated to also be minimized by PSO. PSO may possibly reduce gains in mass through the trajectory arbitrated by insulin and leptin/adiponectin. Thus, intake of PSO could minimize the danger of type II diabetes, although danger of cardiovascular diseases (CVD) was not altered as energy consumption, biomarkers of systemic inflammation and cholesterol profile are not affected (McFarlin et al., 2008).

In the case of STZ-caused diabetes, the impact of PA and eleostearic acid (ESA) against oxidative pressure, abnormality in erythrocyte morphology and inflammatory problems was analysed by Saha and Ghosh, (2012). Intense antioxidant and anti-inflammatory activity against STZ-caused pressure, greatly reduced lipid per-oxidation, and returned amounts of SOD (superoxide dismutase), glutathione peroxidases (GPx) and catalase, in addition to reducing Nitric oxide (NO) and glutathione synthase (GSH) amounts within the erythrocyte lysate, blood and pancreas which were exhibited by both isomers. Furthermore, administration of Conjugated alpha linolenic acid (CLnA) overturned the STZ-induced changes, restored the tumor necrosis factor (TNF)-α as well as interleukin (IL)-6 levels within the blood and articulation of hepatic NF-kappa B (p65). Additionally, PSO has been observed to restrict the articulation of pro-inflammatory cytokines (like TNF- α, IL-6, IL-8, IL-23 and IL-12) by means of PPAR γ and δ alteration (Colombo et al., 2013). PA displayed anti-diabetic features through the increase of levels of serum insulin in male Sprague-Dawley rats with diabetes induced by nicotinamide and STZ; nonetheless, the raised levels of total cholesterol (TC), LDL, TAG an glucose were unaffected. Lipid peroxidation was not influenced by PA, although it could minimize the oxidative pressure caused by diabetes through

raising GPx levels. Therefore, within this type-II diabetes model, secretion of insulin could be raised by PA, although not impacting the levels of fasting glucose. This occurrence is credited to resistance to insulin by the researchers, as it has an essential role in the advancement of type-II diabetes as well as abnormal mitochondria operation (Jafri et al., 2000).

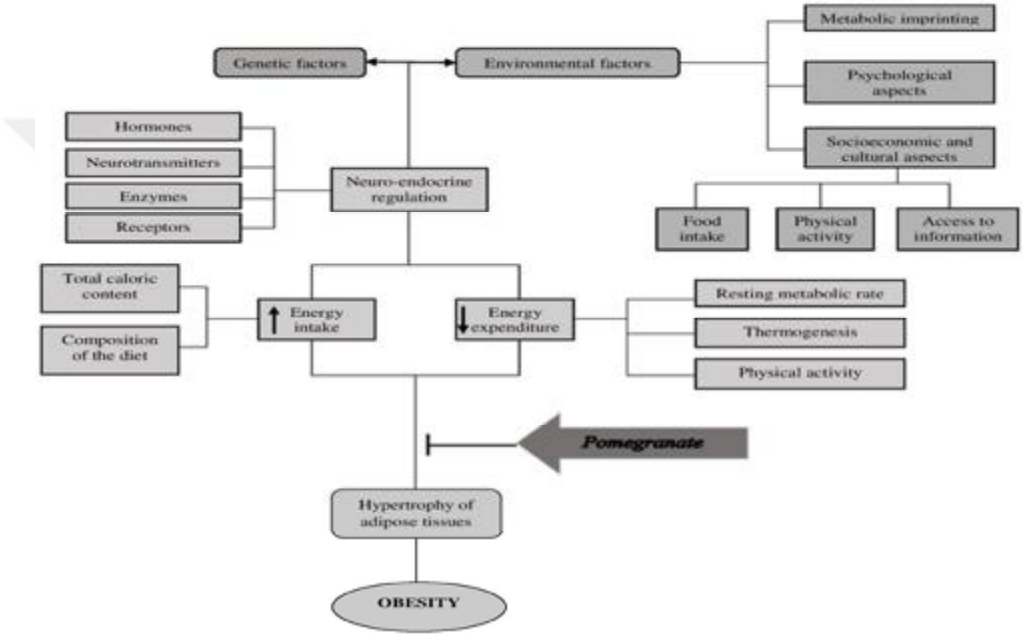

Figure 2.6. How does pomegranate reduce the obesity (Mechanism of action)

Jafri et al. (2000) stated that oral dispensation of a pomegranate flower aqueous-ethanolic extract comprised a considerable effect of reducing blood glucose in regular rats that were glucose-fed, hyperglycemic and alloxan-induced. The utmost impact of the extract was attained at 400mg/kg. Alternatively, It has been observed that the dispensation of 200mg/kg pomegranate peel extract regularized all the negative consequences caused by alloxan, a compound broadly employed in the inducing of diabetes mellitus as it raises the serum levels of α-amylase and glucose activity and the level of water intake as well as peroxidation

of lipids within cardiac, renal and hepatic tissues (Figure 2.7). The polyphenols are the chief compounds comprising anti-diabetic features, which could influence glycemia by means of various systems, encompassing the restriction of absorption of glucose within the gut or its absorption through peripheral tissues (Parmar and Kar, 2007; Scalbert et al., 2005). For a 10mg/kg diet of diacetylated anthocyanins administered to 8 week old Sprague Dawley rats orally, the hypoglycemic impact was noted when the origin of glucose was maltose, although not with glucose or sucrose (Matsui et al., 2002) . This implies that these influences are the result of the restriction of α-glucosidase within the mucosa of the gut. As highlighted by Gumieniczek et al. (2002), within test Diabetes Mellitus (DM), pulmonary oxidative stress happens as a result of a decrease in antioxidant enzyme functioning and a raise lipoperoxidation which is revealed through a reduction in superoxide dismutase (SOD) operation as well as a rise in catalase operation. Nonetheless a research by Ozansoy et al. (2005) illustrated that diabetes resulted in changes to the lung antioxidant enzyme aptitude with no alteration in levels of malondialdehyde (MDA). Though the manner in which rates of MDA were kept constant regardless of the decrease in catalase operation as well as enhancement of GSH-Px is not apparent, the authors clarify that there could be somewhat greater opposition to peroxidative pressure from the diabetic rat's lung. Additionally, they state that the raised in GSH-Px activity took place as a substitute for reduced catalase operation, depending on the actuality that detoxification of H2O2 is vitally reliant on the operation of GSH-Px (Ozansoy et al., 2005; Carrard et al., 2002).

As previously stated, protein oxidation may result from raised oxidative pressure in diabetes, consequently altering the protein carbonyl content (PCC). The translation of proteins into PCC derivatives takes place by immediate oxidation through reactive oxygen species (ROS) with the ultimate creation of oxidised amino acids (Stadtman and Levine, 2000). This oxidative protein alteration could influence a range of cellular activities, and the most appropriate indication of intracellular impairment comprises the PCC (Cukurova et al., 2012). Within a test

research, rats with diabetes induced by STZ were administered daily with 100µL pomegranate juice concentrate, thereby sustaining 2.8 µmol total polyphenols each day over ten weeks (Cukurova et al., 2012). They were contrasted with diabetic and control rats concerning oxidative stress indications, antioxidant enzyme operation as well as structural pulmonary disturbance. PCC, an additionally dependable indication of intracellular oxidative stress-reliant cellular impairment, in addition to sialic acid (SA) were evaluated, as well as the more regularly quantified signals of antioxidant enzyme function in diabetes, specifically SOD and reduced GSH. SA and PCC were raised, while SOD was reduced in diabetic rats, although GSH remained constant. In addition to reversing all these alterations PJ additionally reduced the histological changes within alveolar basal membrane as well as mononuclear inflammatory cell permeation resulting from these oxidative alterations (Cukurova et al., 2012). The immunohistochemical evaluation applied by this research disclosed that eNOS articulation within the lungs of diabetic rats was raised, which was subsequently reduced considerably following PJ administration. In an alternative section of research, SOD operation, circulation of eNOS and iNOS isoforms and lipid peroxidation were analysed in the lungs of diabetic rats (Hürdağ et al., 2008). Likewise, improved oxidative pressure and raised levels of NOS were invalidated through an alternative antioxidant, α-lipoic acid. The antioxidant features of pomegranate, as detailed previously, are assigned to its raised aptitude to scavenge for free radicals and to limit peroxidation of lipids. Suppression of NF-κB inactivation has been observed by its juice (Schubert et al., 2002). It is most likely in this circumstance that PJ administration decreases the raised eNOS expression by means of inactivation of NF-κB. Therefore, pomegranate and its elements could comprise considerable potential clinical employment in the avoidance of oxidative damage to the lungs during diabetes (Schubert et al., 2002). As well as the possible effect of employment of pomegranate as neuroprotective, antidiabetic, wound healing and anti-obesity

agent, Table 2.3 illustrates an outline of test studies which appraised the possible advantages of pomegranate as an antioxidant agent countering different diseases.

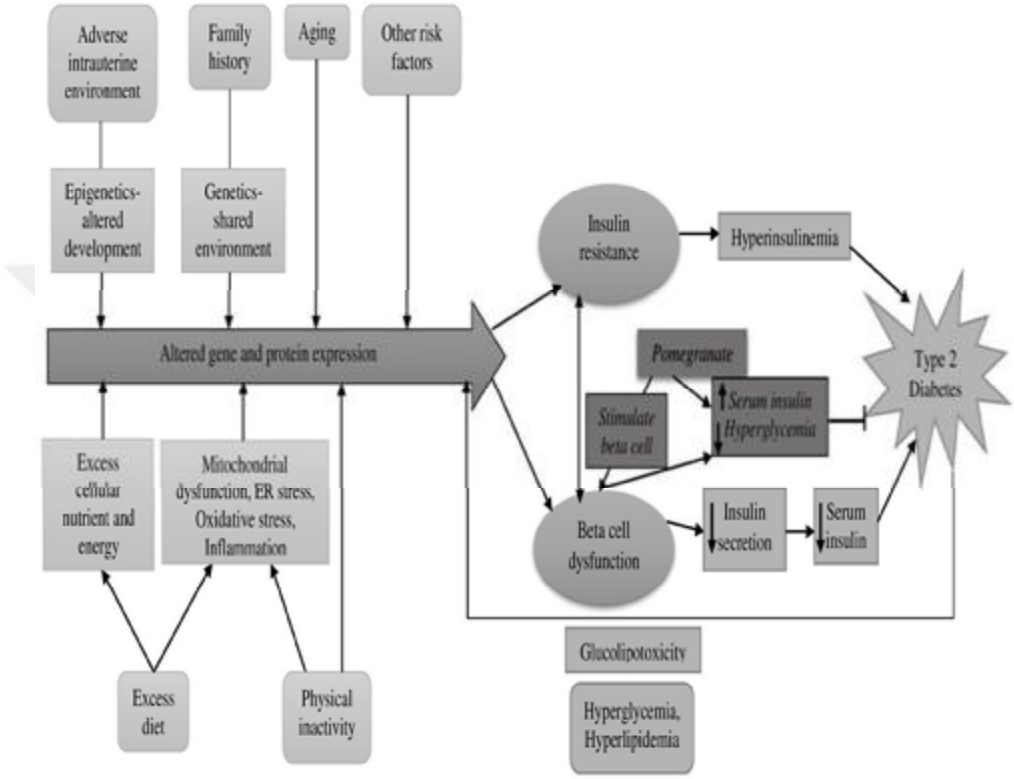

Figure 2.7. The model of how pomegranate acts against diabete

Table 2.3. Characteristics of experimental studies that evaluated the efficacy of Pomegranate in the prevention or treatment of various diseases

Disease	**In vivo/ vitro studies**	**Pomegranate dose, route, and duration of administration**	**Main result**	**References**
Acute liver injury	Male Sprague Dawley rats	Pomegranate was administered orally (800 mg/kg/day) over two weeks prior to induction of sepsis through CLP (cecal ligation and puncture).	Pomegranate enhanced survival and assuaged inflammatory reaction of the liver, possibly associated with down-regulation of mRNA articulation of toll like recptor-4, decreased nuclear translocation as well as DNA binding operation of proinflammatory transcription element NF-κB subunit p65, reduced mRNA as well as protein articulation for tumor necrosis factor-alpha and decrease in mRNA expression and myeloperoxidase activity. Pomegranate additionally reduced oxidative stress caused by CLP as mirrored by diminished content of malonialdehyde, and raised rates of glutathione and superoxide dismutase functioning. These outcomes substantiate the ant ioxidant and anti-inflammatory pomegranate effects	(Makled et al., 2016)

Table 2.3. Continue

			within acute liver damage caused by CLP mediated by restricting TLR4/NF-κB pathway, neutrophil infiltration and lipid peroxidation.	
Hypoxia and apoptosis in mouse placenta	Pregnant mice	Pregnant mice were treated daily with 250 μl of PJ	Oxidative stress and augmented apoptosis within mouse placentas was brought about by exposing pregnant mice to FiO2¼12%, as quantified by HSP90 as well as cleaved caspase 3, correspondingly. The HPX mice's cross-sectional surface regions of the placental junctional areas were greater than those of N-FR mice. Augmentation with PJ reduced oxidative stress caused by hypoxia in the junctional area and labyrinth, decreased the cross-sectional surface area from the junctional region, and reduced the level apoptosis within the labyrinth simultaneously raising apoptosis within the junctional area. Thus, augmentation of pomegranate juice within pregnant mice decreases oxidative stress caused by hypoxia while	(Chen et al., 2015)

Table 2.3. Continue

			differentially altering apoptosis within the two elements of the mouse placenta.	
Castration-resistant prostate cancer (CRPC)	Male PTEN−/− mice and PCa cell lines (22RV1 and LNCaP)	20 weeks administering (0.17 g/L in drinking water)	*In vitro*, the findings illustrated that pomegranate extracts (POM) (0-12µg/mL) decreased the generation of DHEA, DHT, pregnenolone, androsterone and androstenedione within both cell lines. Furthermore, in vivo information verifies this fact with a decrease in serum steroids established following 20 weeks of POM administration (0.17 g/L in drinking water). Corresponding to these findings, total prostate specific antigen (tPSA) and Western blotting of cell lysates established that PSA was considerably reduce by the POM dispensation. Remarkably, levels of AR and AKRIC3 were demonstrated to be higher in both cell lines, possibly as unconstructive feedback influence as a reaction to restriction of steroids. Generally, these findings offer mechanistic	(Ming et al., 2014)

Table 2.3. Continue

			verification to sustain the logic for current clinical reports detailing effectiveness of POM for CRPC patients.	
Gastric Inflammation	Male Wistar rat	Oral pre-treatment with three varying doses of extract (25, 50 and 100 mg/kg) administered over 15 days	A noteworthy protective impact was generated in rats by 50mg/kg of black fruit peel extract ($p<0.05$) with a preventive index comprising 65.87%. Alternative doses were considerably protective from gastric ulcer caused by ethanol in rats. Alternatively, north white peel had no impact (50mg/kg), demonstrating an ulcer index comprising 49.52 ± 1.99. Histopathological scrutiny of the ulcerated animals administered with white peel's stomach (50mg/kg) demonstrated serious erosion in the sub-mucosal edema, neutrophil infiltration and gastric mucosa. Thus, the antiulcer features of the methanol extracts from black peel, sour summer and north white peel (25, 50, 100 mg/kg) of pomegranate were	(Moghaddam et al., 2014)

Table 2.3. Continue

			probably applied through its raised antioxidant function.	
Bone Marrow Toxicity	Wistar albino rats	PMG was administered orally for seven days at 225 mg/kg	A reduction in counts of haemoglobin, hematocrit, white blood cell and platelet were observed within the methotrexate (MTX) division, although these alterations were avoided within the sets given Carvacrol (CARV) and PMG. Additionally, reduced cellularity of bone marrow was established in the sets given MTX, while the CARV and PMG sets comprised comparable cellularity to controls. Remarkably, within the MTX set oxidative stress rose but was eventually reduced in the rats given CARV and PMG antioxidants. PMG and CARV were established to be protective from oxidative bone marrow impairment caused by methotrexate. Employment of these antioxidants in addition to chemotherapeutics could assist in decreasing some	(Sen et al., 2014)

Table 2.3. Continue

			negative consequences of methotrexate.	
Hpatitis C virus	BALB/c mice andHuh7 cell line harboring HCV	A sole dose (1000 mg/kg per mouse) of PGN, PLN and EA was dispensed orally. Huh7 cells concealing HCV replicon were administered with growing concentrations of test compounds, PGN, PLN and EA (10–150 mM) and incubated additionally over 48h	The HCV NS3/4 protease operation in vitro was particularly obstructed by the pure compounds separated from the extract, including ellagic acid, punicalin and punicalagin. Structural evaluation employing a computational technique additionally illustrated the interaction of ligand molecules with the substrate and catalytic binding deposits of NS3/4A protease, resulting in restriction of the enzyme operation. Additionally, punicalin and punicalagin considerably decreased the replication of HCV within cell culture structure. More significantly, these compounds are accepted well ex vivo and no noted negative consequence level (NOAEL) was determined up to an acute dose of 5000mg/kg within BALB/c mice. Furthermore,	(Reddy et al., 2014)

Table 2.3. Continue

			pharmacokinetics research illustrated that the compounds are bioavailable. Administered together, this research offers a proof-of- concept technique for the possible employment of antiviral as well as non-toxic key ellagitannins from pomegranate in avoidance and management of complications caused by HCV.	

3. METHOD AND MATERIAL

3.1. Materials

3.1.1. Fresh pomegranate juice

Pomegranates fruits (*P. granatum* L.), called "Silifke Aşısı", have been collected from Adana market, Turkey in April 2017. The fruit has handpicked, washed, and stored at 4ºC. Fresh aril juice has used to confirm that the outcomes would be useful for a juice as consumed by humans. The fruits have been peeled out, and the arils have been crushed and then squeezed by using a household juicing appliance. The fresh pomegranate juice (FPJ) has been sterilized by using 0.45 μm and then 0.22 μm filters prior to use.

3.1.2. Clinical isolates of *M. tuberculosis*

Mycobacterial isolates in MGIT tubes have been examined under microscope in order to distinguish *M. tuberculosis* from nontuberculous mycobacteria. The presence of cording on Ziehl-Neelsen staining and formation of clumps with nonappearance of turbidity in the medium led to identify these isolates as *M. tuberculosis*. Reference strain (H37Rv) collected from National Tuberculosis Reference and Research Laboratory in Ankara, Turkey.

3.1.3. Antibiotics and chemicals

MGIT tubes and isoniazid (INH), rifampin (R), ethambutol (EMB), and streptomycin (SM) were purchased from *Z-DNA* company, Turkey. All solutions were prepared on the day of the experiment. Trizol was purchased from Thermo Fisher Scientific company, USA. Isopropanol and Chloroform were purchased from polar company, Turkey.

3.2. Methods

3.2.1. Drug susceptibility test using BACTEC MGIT 960 system

M. tuberculosis clinical isolates have been collected from Tropical Diseases Research and Application Center, Çukurova University, Turkey. *M. tuberculosis* clinical isolates were subjected to susceptibility testing with isoniazid (INH), rifampin (R), ethambutol (EMB), and streptomycin (SM) based on the recommend method using BACTEC MGIT 960 system (Figure 3.1) (Adami et al., 2017). The final concentration of INH, RF, EMB, and SM were 0.1 µg/ml, 1.0 µg/ml, 5.0 µg/ml, and 1.0 µg/ml, respectively. Subsequently, thirty MDR-TB isolates, which are resistant to INH, R, EMB, and SM, have been selected. Second drug susceptibility test has been manipulated to check the possible effect of different concentrations (5%, 10%, 15%, and 20%) of fresh pomegranate juice (FPJ) with R (1.0 µg/ml) and INH (0.1 µg/ml) with slightly modifications on the recommended method (Figure 3.2) (Adami et al., 2017). Furthermore, H37Rv sample has been tested with (5%, 10%, 15%, and 20%) of FPJ alone (without antimicrobial agents). pH has been measured after the addition of FPJ (5%, 10%, 15%, and 20%). MDR-TB samples have been divided into three groups. While first group was resistant to R and INH, second one showed resistance to SM, R, and INH resistant. And third one appeared to be resistant to INH, EMB, R, and SM. Firstly; we picked up one sample from each group of MDR-TB isolates in order to check the efficacy of each combination (R+FPJ, INH+FPJ, and R+INH plus FPJ). Subsequently, we used R plus FPJ combination to be examined against twenty seven MDR-TB isolates based on our findings in pilot study (Table 3.1). All tests were conducted twice.

Table 3.1. MDR-TB isolates based on their resistance profile

MDR-TB isolates (n=27)		
I, R (Resistant)	**S, I, R (Resistant)**	**S, I, R, E (Resistant)**
1	10	19
2	11	20
3	12	21
4	13	22
5	14	23
6	15	24
7	16	25
8	17	26
9	18	27

Figure 3.1. Displayed BACTEC MGIT 960 system (A), anti-TB agents used in the drug susceptibility test (B), and MGIT tubes (C)

3.2.1.1. Determination of minimum inhibitory concentrations (MICs) of fresh pomegranate juice

The MICs of FPJ have been determined based on the recommend method (Adami et al., 2017) by using BACTEC MGIT 960 system after applying RIF plus FPJ treatment against twenty seven MDR-TB clinical isolate. The MIC was

defined as the lowest FPJ concentration in combination of R that prevented the bacterial growth to exceed the break points which accurately distinguish between susceptibility and resistance of *M. tuberculosis*.

3.2.2. Total phenolic content of pomegranate juice

Total phenolic content (TPC) has been determined for pomegranate juice sample by the Folin-Ciocalteu colorimetric method with slight modifications (Fawole et al., 2012). Briefly, 50 µl of fresh pomegranate juice (FPJ) has been mixed with 3.9 ml of distilled water followed by the addition of 250 µl Folin–C. The mixture has been vortexed for (30) second and then 750 µl of sodium carbonate (2 %) has been added. The mixture has been allowed to stand for 120 minutes at room temperature in the dark before the absorbance has been measured by a spectrophotometer at 760 nm. Results have been expressed as gallic acid equivalents (GAE).

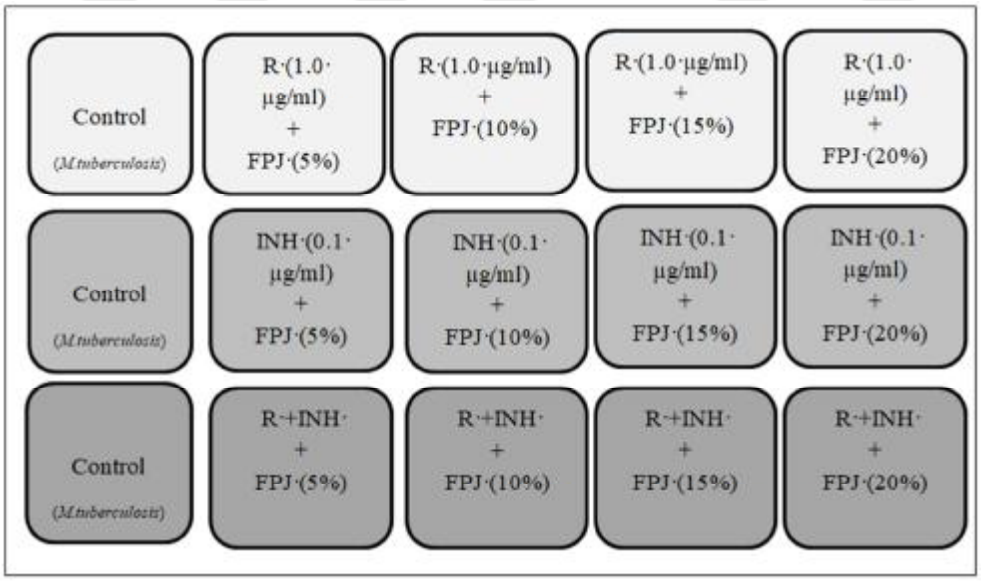

Figure 3.2. Illustrates the diagram of the second Drug susceptibility test (DST) with the concentrations of fresh pomegranate juice (FPJ), Rifampicin (R), and Isoniazid (INH)

3.2.3. Identification and quantification of phenolic compounds of pomegranate juice by HPLC analysis

Before injection, FPJ juice was centrifuged in an eppendorf tube (5 min at 5000 rpm) and the centrifuged supernatant was allowed to pass through a 0.45 µm filter. Injection volume was 50 µl. Separation was manipulated on a reverse-phase Inertsil ODS-3, C18, 5µ column. Column temperature was maintained at 40 ^{o}C with a flow rate of 1 mL/min. The chromatogram was scrutinized concurrently at 200, and 600 nm. The UV spectra of the different compounds were recorded with a diode array detector. Elution was composed by using solvent A (2.5%, v/v, solution of formic acid in water) and solvent B (2.5%, v/v, solution of formic acid in acetonitrile) at different ratios. The elution program of the solvent (B) was increased from 5 to 13% in 15 min, from 13 to 15% in 5 min, from 15 to 30% in 5 min, maintained for 3 min, from 30 to 45% in 4 min, maintained for 3 min, from 45 to 90% in 5 min, maintained for 5 min, and then returned to initial conditions in 5 min (Figure 3.3). Individual components were identified based on the comparison of the retention time with those of authentic standards (Mousavinejad et al., 2009).

Figure 3.3. High-performance liquid chromatography (HPLC)

3.2.4. Total flavonoids of pomegranate juice

Total flavonoids (TF) was determined by using aluminium trichloride method and rutin used as a reference compound to make the calibration curve (Zhishen et al., 1999). Briefly, 1 ml of diluted sample was separately mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 510 nm. The total flavonoids content was expressed as g Rut.100g-1ext.

3.2.5. Total anthocyanins of pomegranate juice

Total anthocyanins (TA) was determined based on Ozgen et al. (2008) method with slight modifications. The pH differential method was performed by using two buffer systems: potassium chloride buffer pH 1.0 (0.2 M) and sodium acetate buffer pH 4.5 (1 M) (Ozgen et al., 2008). After equilibration at room temperature for 15 min, the absorbance of two dilutions was read at 510 nm and 700 nm, and the total monomeric anthocyanins was calculated.

3.2.6. Antioxidant activity of pomegranate juice

The antioxidant activities of fresh pomegranate juice (FPJ) have been determined by previously recommended method carried out by Gil et al. (2000). In this method, the 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical has been used to determine the antioxidant activity of juices (Brand-Williams et al., 1995). 100 μL of FPJ, diluted in the ratio of 1:100 with methanol: water (6:4), was mixed with 2 mL of 0.1 mM DPPH in methanol. The mixture has incubated at room temperature for 30 min in the dark and then the absorbance of the mixture has been measured at 517 nm. Radical scavenging activity has been expressed as the inhibition percentage.

3.2.7. Data analysis

SPSS 16.0 has been used to perform One Way ANOVA, and the difference was considered to be statistically significant when $P < 0.05$.

3.3. Gene Expression

3.3.1. Reagents and Equipments for RNA extraction

- TRIzol Reagent
- RNase-free water
- Chloroform
- Isopropyl alcohol (2-Propanol)
- 75% Ethanol
- Sterile or RNase treated pipette tips, microcentrifuge tubes, and sterile Pasteur pipette
- Microcentrifuge
- Heat block

3.3.2. RNA extraction and reverse transcription

All *M. tuberculosis* strains were sub-cultured in MGIT tubes, and collected for RNA extraction. MDR-TB samples have been divided into three groups. While first group was resistant to R and INH, second one showed resistance to SM, R, and INH resistant. And third one appeared to be resistant to INH, EMB, R, and SM. For the twenty seven (27) MDR-TB isolates, R and INH were added to these cultures individually with FPJ at sub-inhibitory concentrations (half of the MIC), incubated at 37°C for 14 days, and collected for RNA extraction. Moreover, twenty seven (27) MDR-TB isolates were sub-cultured without neither drug nor FPJ inducement. H37Rv was used as reference strain and this strain has been sub-cultured by using by R and INH individually with FPJ at sub-inhibitory

concentrations (half of the MIC).Total bacterial RNA was isolated using Trizol reagent according to the manufacturer's instructions with some modifications as follow: 1.5 ml aliquot of the cell suspension was placed in sterile (1.5 ml) microcentrifuge tubes, centrifuge at 12,000 × g for 5 min to pellet the cells, the supernatant was poured off and 1 ml of TRIzol reagent was added to the tubes, cells were lysed by repetitive pipetting until there are no visible clumps, samples were incubated for 5 min to permit complete dissociation of the nucleoproteins complex, 0.2 mL of chloroform was added per 1 mL of TRIzol™ Reagent and samples were vortexed vigorously for 15 seconds and then incubated on ice for 15 min followed by centrifugation at 12,000 × g for 15 min to get phase separation, the aqueous phase was transferred to a fresh RNase-free microcentrifuge tubes by the tube at 45° and pipetting the solution out, 0.5 mL of isopropanol was added to the aqueous phase and then samples were incubated on ice for 10 min for RNA precipitation followed by centrifugation at 12,000 × g for 10 min with a removal of supernatant, pellet was washed with 1 ml 70% ethanol by flicking and samples were centrifuged at 75,00 × g for 10 min followed by decant the supernatant and air dry the pellet for 5–10 min, RNA pellet was dissolved in 40µl of RNase-Free H2O, then samples were incubated in heat block set at 60°C for 10 min, and finally samples were stored at –70°C. The ratio of A260nm/A280nm of the RNA was assessed using a NanoDrop™ Lite Spectrophotometer (Figure 3.4). RNA was reverse transcribed according to the manufacturer's recommendations (Figure 3.5) (Thermo Fisher Scientific Company, USA). 10 µL of 2X RT master mix(Table 3.2) was pipetted into individual tube. Afterward, the 2X RT master mix was placed on ice and mix gently. Next, 10 µL of RNA sample was added to each tube and mixed gently by pipetting up and down two times. Subsequently, tubes were immediately placed in a thermal cycler and the program was as follows: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The cDNA was stored at −20°C.

Figure 3.4. NanoDrop™ Spectrophotomete

To synthesize single-stranded cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kits:

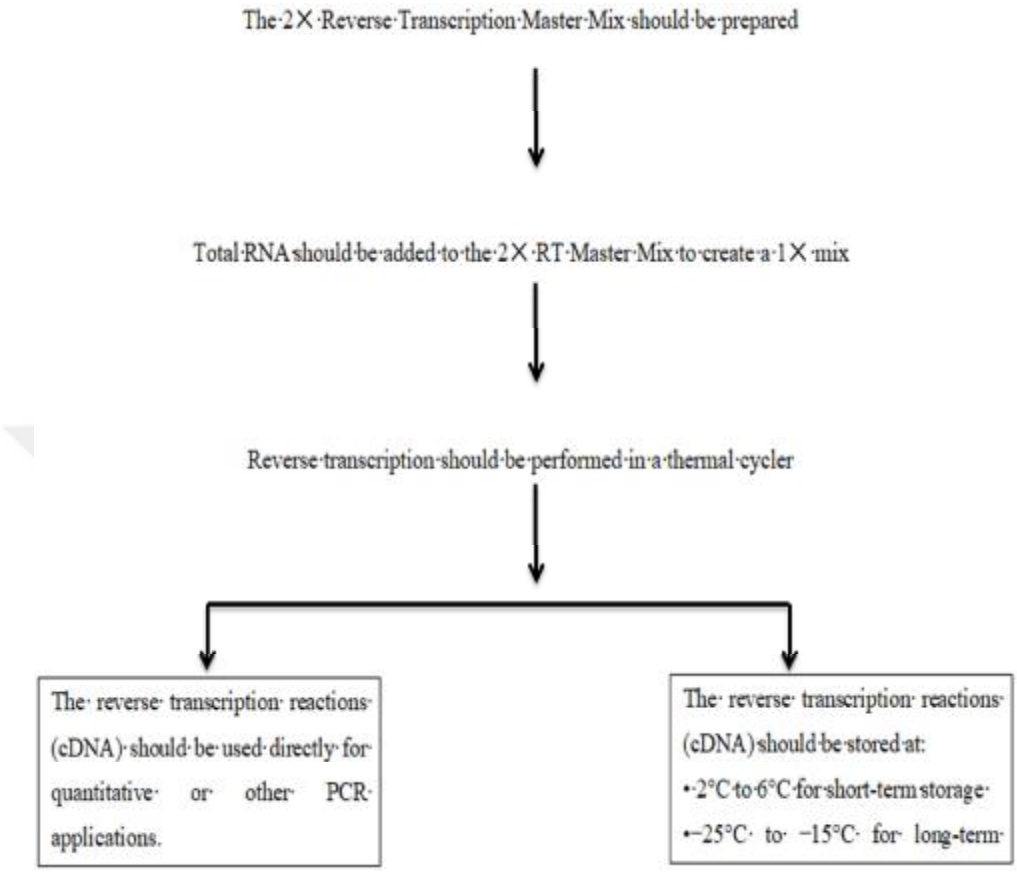

Figure 3.5. An overview of cDNA synthesis procedures

Table 3.2. Reaction mixture for the reverse transcription reactions (cDNA)

Component	**Volume**
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL
10X RT Random Primers	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
Nuclease-free H2O	4.2 µL
Total per reaction	10.0 µL

3.3.3. Real-time quantitative PCR (qPCR)

3.3.3.1. Materials and Equipments

- qPCR primers
- DNA template
- Barrier pipette tips
- Sterile, nuclease-free, DNA-free tubes for reaction mix setup
- Optical multi-well reaction plates and adhesive film covers
- Real-time thermal cycler
- Alternative normalization dye (e.g., fluorescein for BioRad instruments)

3.3.3.2. Quantification of gene expression using real-time quantitative PCR (qPCR)

The primers of the 11 genes are described in Table 3.3. The assay was performed using a qPCR kit (Promega Company, USA) in Applied Biosystems 7300 Real-Time PCR System (Figure 3.6). Briefly, each 0.2 mL tube contained 10 μL 2× qPCR mix, 0.5 μL each primer, 100 ng cDNA, and RNase-free water to a final volume of 20 μL. The thermal cycling conditions were as follows: 95°C for 10 min, then 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 1min, and the last step consisted of a melting curve analysis (65–95°C).

Figure 3.6. Applied Biosystems 7300 Real Time PCR System

The fold change in the genes expression under drug stress in twenty seven (27) MDR-TB isolates was calculated by $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (Livak and Schmittgen, 2001). When the expression of genes compared with non-exposed control genes displaying levels more than 1 were found to be increased; equal to or above four were found to be overexpressed (Livak and Schmittgen, 2001; DeMarco et al., 2007; Rodrigues et al., 2012; Bustin et al., 2010). The expression of genes of (27) MDR-TB isolates without drug inducement was calculated by $2^{-\Delta CT}$ method. The delta Ct values were derived by subtracting the CT value of housekeeping gene (*polA*) from the CT value obtained for each gene. The values of ΔΔCT were derived by subtracting the value of ΔCT obtained for each gene without drug stress from the value of ΔCT of genes induced by INH/RIF plus FPJ stress. polA,which is expressed at a stable level in the isolates and can used as an internal invariant control, is described as a housekeeping gene

Table 3.3. Primers used in this study for real-time quantitative PCR (qPCR)

Gene	**Primer**	**Sequence (5′→3′)**	**Amplicon size (bp)**
rpoB	F	ACCGACGACATCGACCACTT	450
	R	GTACGGCGTTTCGATGAACC	
katG	F	AATCGATGGGCTTCAAGACG	500
	R	CTCGTAGCCGTACAGGATCTCG	
inhA	F	TATGCTTCGATGGCCAAGGC	150
	R	CGCCACGAACCTGTTGACCG	
oxyR-ahpC	F	CGGCGATGCCGATAAATATG	101
	R	TCATCAAAGCGGACAATGCA	
efpA	F	CGCCCTACGGGAAACCAACAAAGA	226
	R	GCGGAACAAGTGGAACGGCACGAC	
Rv1250	F	GCAGCCTTGGATTTGGGCGGTGAT	133
	R	GGACAAGCTGAAGTTCCGGTCGTT	
RV1634	F	TCGATACCTACGTGCCGCTGTTCG	157
	R	GCTGCCACGACATGCCCGATAACT	
RV2459	F	CGTCGCCCTGATCGCATACA	213
	R	CAGGACATCACCACGAAGTAGACG	
drrA	F	TAGACATCGCGTGCGGATTGGT	147
	R	GCGTGGTCAACAACGTGGCAAT	
drrB	F	TCGCCAGCAACTTAGGGCAATACA	233
	R	TCCGATGACGTAGCCGCAAACTAG	
polA	F	GTCGTGGTTGGACCTTGGAGGG	181
	R	GCGTCCGTATCGTCGTCATCG	

4. RESULT AND DISCUSSION

4.1. Susceptibility testing

Mycobacterial isolates identified as *M. tuberculosis* have been subjected to drug susceptibility test against isoniazid (INH), rifampin (R), ethambutol (EMB), and streptomycin (SM). The concentrations of INH, R, EMB, and SM were 0.1 µg/ml, 1.0 µg/ml, 5.0 µg/ml, and 1.0 µg/ml, respectively (Adami et al., 2017). *M. tuberculosis* clinical isolates, which showed resistant to at least two drugs, including INH and R, are considered as MDR-TB (Nathanson et al., 2010; Organization, 2010). Whereas, XDR-TB is caused with additional resistance to a fluoroquinolone such as ciprofloxacin and one second-line injectable agent including amikacin or kanamycin (Kolyva and Karakousis, 2012).

4.2. Synergistic studies

Thirty (30) MDR-TB clinical isolates have been selected and divided into three groups based on their resistance profile. While first group showed resistance profile of R and INH, second one was resistant to SM, R, and INH resistant. And third one appeared to be resistant to INH, EMB, R, and SM **(** Figure 4.1).

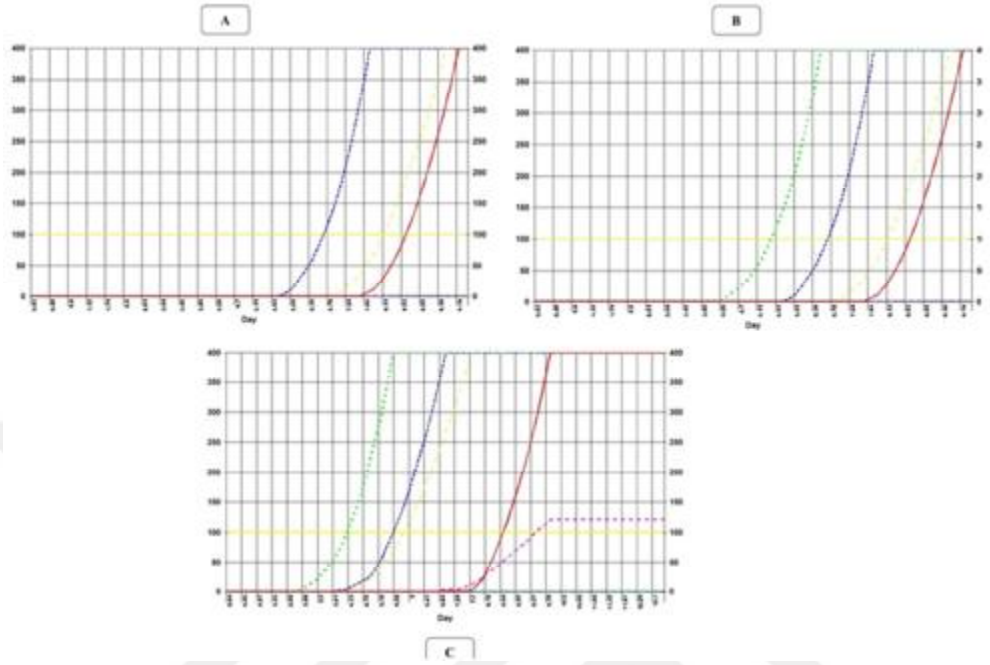

Figure 4.1. Displays MDR-TB groups used in this study. (A) Resistant to INH, R, (B) Resistant to SM, R, and INH, and (C) resistant to INH, EMB, R, and SM

One sample from each group of MDR-TB clinical isolates has been picked up in order to check the efficacy of each combination (R+FPJ, INH+FPJ, and R+INH plus FPJ). Four concentrations of FPJ (5%, 10%, 15%, and 20%) have been used. pH values ranged from 5.8- 6.8 which is considered as suitable values for TB growth. It has been reported that pH between 6.0-6.2 of 7H12 broth was within the optimal range for ***M. tuberculosis*** cultivation (Heifets, 2002).

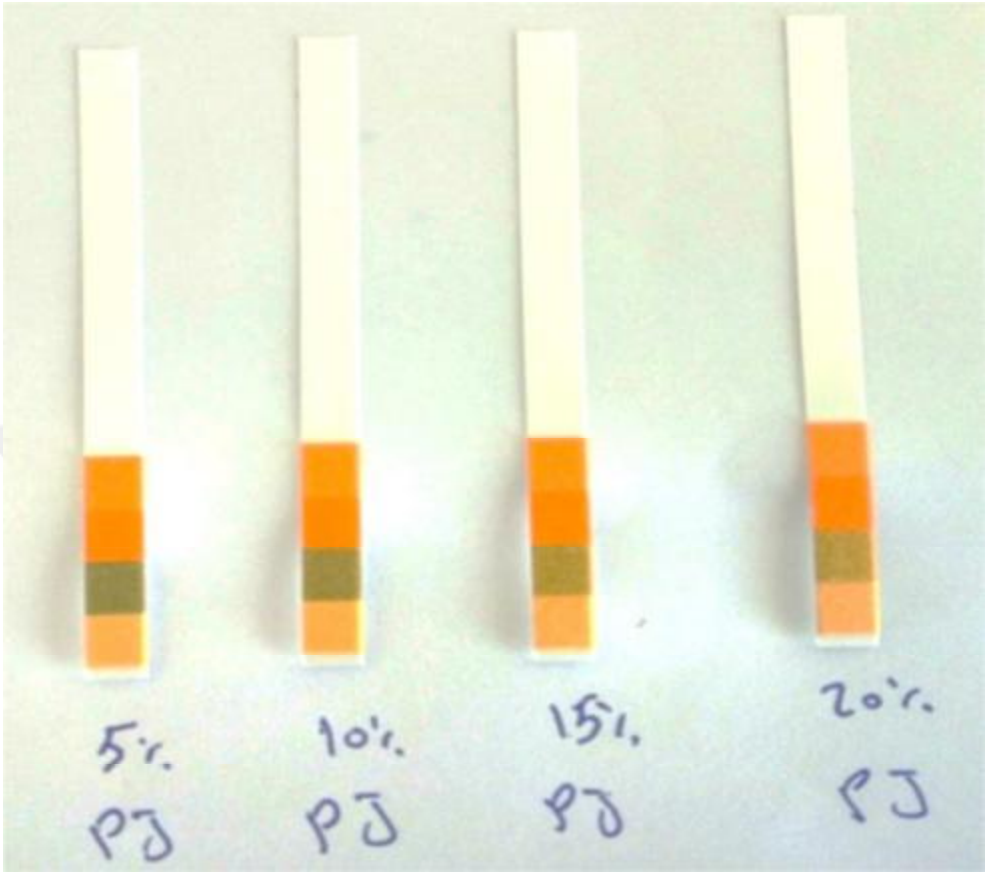

Figure 4.2. Displays the absolute pH values for the four concentrtions (5%, 10%, 15%, 20%) of FPJ

Additionally, ***M. tuberculosis*** efficiently multiplied and the pH quickly increased, in keeping with the knowledge that optimal growth of ***M. tuberculosis*** in liquid media was found between pH 5.8 and 6.7 (Vandal et al., 2009). Notably, both 15% and 20% concentrations of FPJ showed synergetic effect with R, INH, and R+INH with almost complete inhibition of the growth of all MDR-TB clinical isolates. However, 5% and 10% concentrations FPJ did not show remarkable effects with INH and R for second (SM, R, and INH resistant), and third group (INH, EMB, R, and SM resistant). Moreover, 5% concentration of FPJ has not displayed noticeable synergetic impact with INH for first group (R and INH

resistant). Although the bacterial inhibition of R+INH plus FPJ appeared to be slightly effective than RIF+FPJ and INH+FPJ, however, no much difference has been observed between R+INH plus FPJ and R+FPJ (Figure 4.3). Thus, we have carried out for the twenty seven (27) MDR-TB isolates by using R in combination of FPJ with three concentrations only (5%, 10%, and 15%).

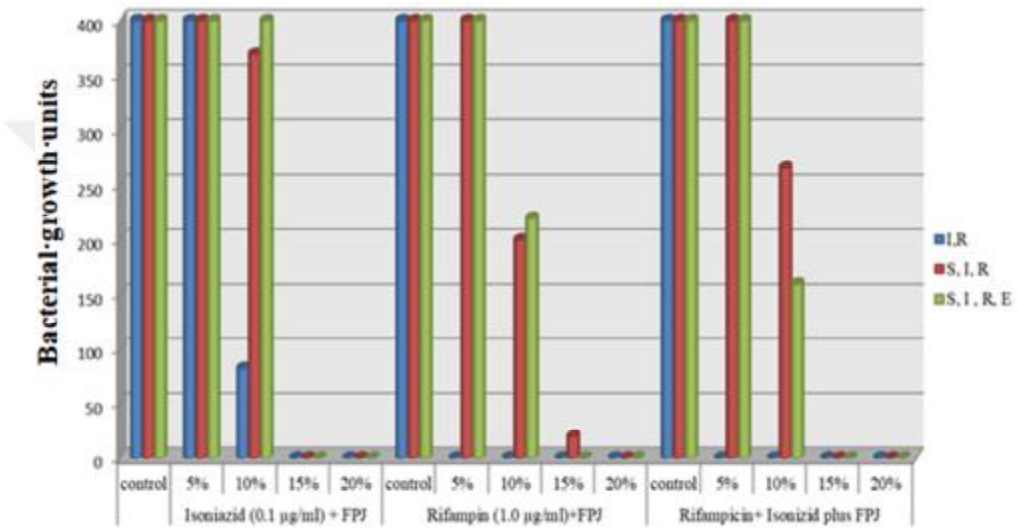

Figure 4.3. Shows the growth inhibition of MDR-TB isolates by FPJ + Rifampin (R), FPJ+ Isoniazid (INH), and FPJ plus Rifampin (R)+ Isoniazid (INH)

The *in vitro* bactericidal effect of combinations of two or three major anti-TB drugs has been investigated with complex patterns of response (Dickinson et al., 1977). Remarkably, in this study, the combination of INH and RIF appeared to be non-additive (Dickinson et al., 1977). However, in mice, INH displayed antagonism of R plus pyrazinamide activity (Almeida et al., 2009; Grosset et al., 1992). Moreover, the *in vitro* influence of the combination of R plus moxifloxacin against *M. tuberculosis* has been examined (Drusano et al., 2010). The combination has not exhibited a killing effect faster than monotherapies with R or moxifloxacin alone in log-phase cultures of susceptible micro-organisms. However, this

combination was antagonistic for the bactericidal activity in non-replicating bacteria. A response-surface model was used, known as the Greco model (Greco et al., 1990), to quantify the combined action of R and linezolid against *M. tuberculosis* in the hollow fiber infection model (Drusano et al., 2014). The experimental data showed that the combined action has a tendency to be hostile, even though the departure from additivity appeared to be insignificant (Drusano et al., 2014). Therefore, those published articles suggested that there is no simple and common pattern of combined action of TB agents.

An acidic culture model of *M. tuberculosis* has been used to examine drug activity against aerobic 5-day-old (A5) and hypoxic 5-, 12-, and 19-day-old (H5, H12, and H19, respectively) bacilli after 7, 14, and 21 days of exposure to ten drugs (Piccaro et al., 2013). Rifampin (R), which inhibits RNA synthesis, was the most active against all the examined four cell stages (A5, H5, H12 and H19) (Piccaro et al., 2013; Sacchettini et al., 2008). The observation that R competently eradicated A and H cells in acidic conditions is significant because R and pyrazinamide (Z), which considered as a drug active only at acidic pH, are in combination responsible for most of the activity of the treatment of TB (Mitchison and Fourie, 2010; Vandal et al., 2009; Zhang and Mitchison, 2003). Isoniazid (INH), which acts on mycolic acid synthesis, was bactericidal against A5 cells, but showed limited activity against H5 cells and no activity against H12 and H19 cells (Sacchettini et al., 2008; Piccaro et al., 2013). Thus, R appeared to be more effective than INH against *M. tuberculosis* grown in aerobic and hypoxic acidic conditions.

Twenty seven (27) MDR-TB isolates, divided into three groups based on their resistance profile as mentioned previously, have been examined in combination with R and FPJ. 5% of FPJ plus R (1.0 µg/ml) was effective to suppress the growth of one isolates for first group (INH and R resistant). However, for second (SM, R, and INH resistance) and third group (INH, EMB, R, and SM resistance), 5% of FPJ demonstrated no synergistic impact with R. Moreover, 10%

of FPJ and R (1.0 µg/ml) appeared to inhibit the bacterial growth of three isolates of first group and two and one for second and third group, respectively. Additionally, 15% of FPJ plus R (1.0 µg/ml) showed remarkable ability to inhibit the growth of MDR-TB clinical isolates for all tested groups indicating a strong synergistic effect (Figure 4.4 Figure 4.5 , Figure 4.6 and Table 4.1).

Table 4.1. Bacterial growth of MDR-TB clinical isolates when (FPJ and R) are combined

	MDR-TB clinical isolates (N=27)		
FPJ concentration	**I, R Mean**	**S, I, R Mean**	**S, I, R, E Mean**
Concentration 5%	1.78	2.00	2.00
Concentration 10%	1.33	1.67	1.78
Concentration 15%	0.00	0.00	0.00

I, R (Resistant to INH, and R), S, I, R (Resistant to INH, S, and R), and S, I, R, E (Resistant to INH, S, R, and EMB). Different means represent significant differences ($p < 0.05$) between the different concentrations of FPJ based on a one-way ANOVA.

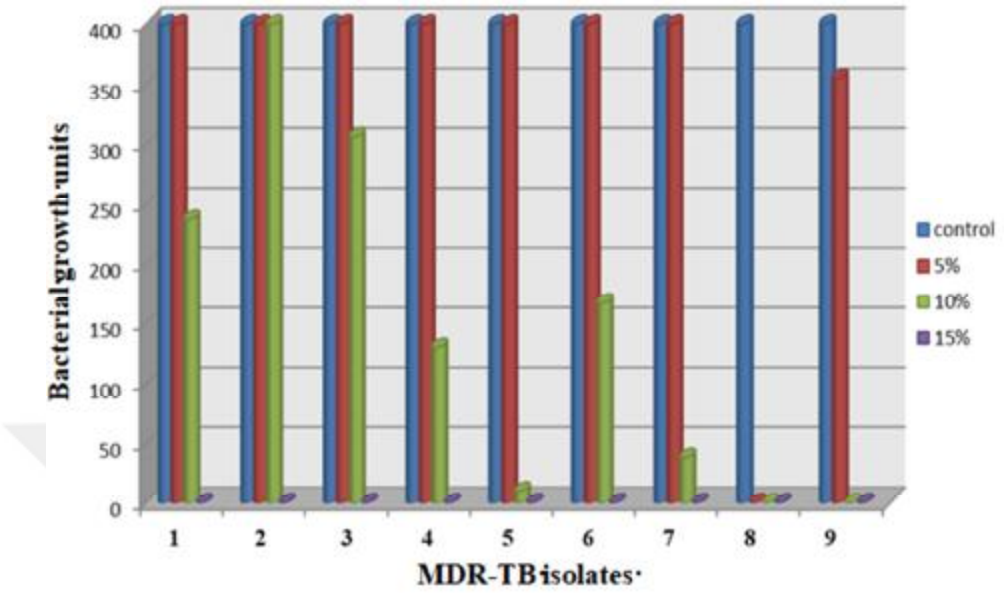

Figure 4.4. (a) Displays the bacterial growth of nine MDR-TB isolates with resistance to Isoniazid (INH) and Rifampin (R).

Regarding H37RV, the complete inhibition of the bacterial growth found to occur at 15% and 20% concentrations of FPJ (Figure 4.5). Minimum inhibitory concentration (MIC) of FPJ for first group ranged from (4%-13%), while for second group and third group were approximately between (10%-15%). Therefore, FPJ at 15% inhibited 100% of bacteria for all tested isolates ($MIC_{100\%}$ =15%) (Table 4.2). Betanzos-Cabrera et al. (2015) studied the antimicrobial activity of FPJ on *S. epidermidis* and reported that 20% of FPJ inhibited 100% of bacteria (Betanzos-Cabrera et al., 2015).

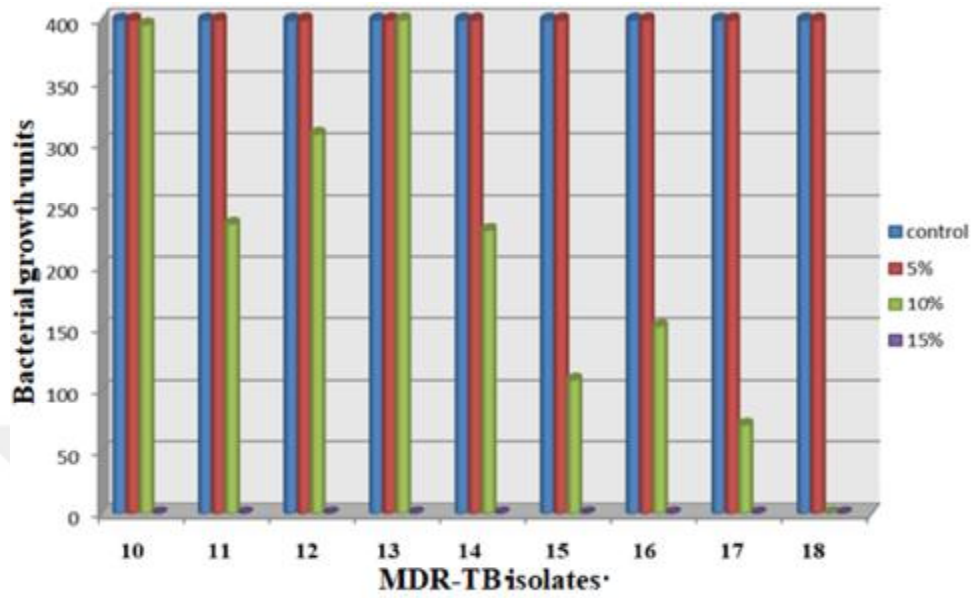

Figure 4.4. (b) Showed how MDR-TB isolates with resistance to Streptomycin (SM), Isoniazid (INH) and Rifampin responded to the combination (FPJ and R).

The pomegranate juice extract displayed remarkable inhibitory activity against *Streptococcus mutans* with a MIC (25 μg/μl) and a minimal bactericidal concentration (MBC) (40 μg/μl. However, it showed moderate inhibitory activity against *Rothia dentocariosa* with MIC and MBC (20 μg/μl) and (140 μg/μl), respectively (Ferrazzano et al., 2017).

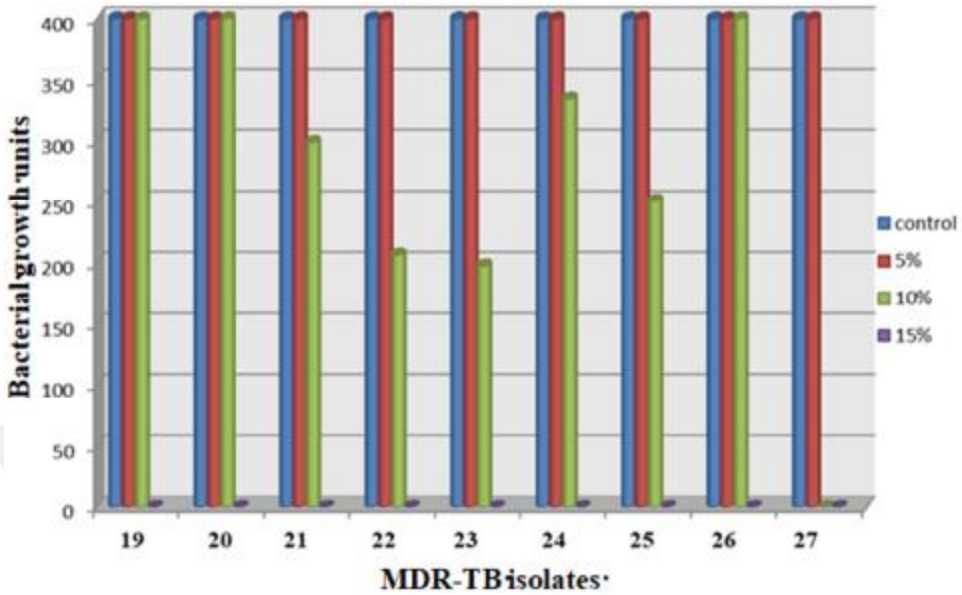

Figure 4.4 (c) The effect of (FPJ +R) on the MDR-TB isolates with resistance to Streptomycin (SM), Isoniazid (INH), Rifampin (R) and Ethambutol (EMB).

The antimicrobial activity of pomegranate juice is due to the existence of several compounds. Tannins, abundant in pomegranate, are described as toxic compounds for micro-organisim. The hydrophobic part of tannin is immersed in the non-polar inner region of the bacterial membrane which might cause variability of the membrane, and therefore disturbing the substrates transport into the cell (Viuda-Martos et al., 2010; Cristani et al., 2007).

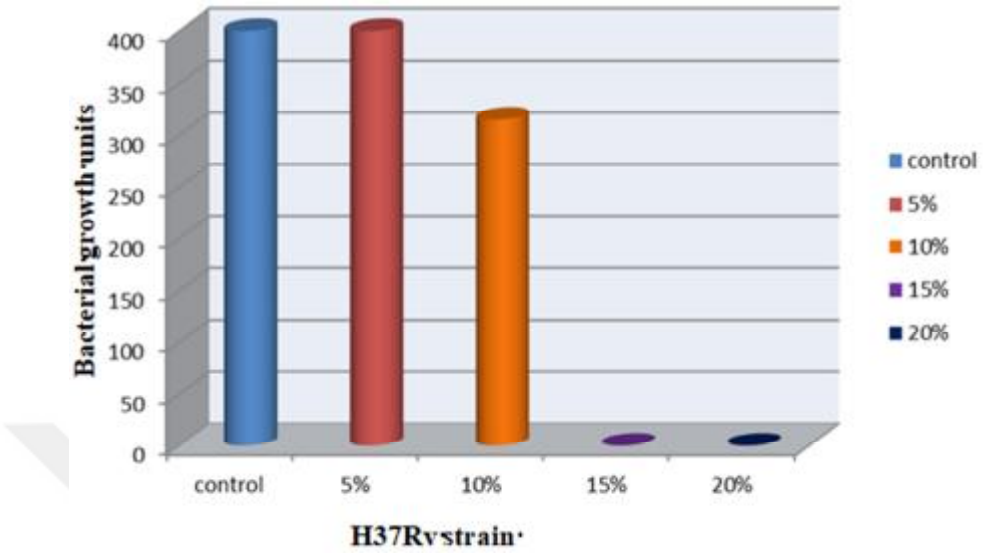

Figure 4.5. How FPJ influences the bacterial growth of H37RV reference strain.

It has been documented that the interaction of phenolic compounds with sulfhydryl groups or non-specific proteins leads to the loss of membrane function. Moreover, phenols appear to interfere with bacterial protein secretions or to render substrates unavailable to microorganisms (Naz et al., 2007; Seeram et al., 2008). Ahn et al. (1998) suggested that phenolic compounds in pomegranate, including gallic acid and ellagic acid, in addition to punicalagin as a major ellagitannin, may be responsible for the antimicrobial activity (Ahn et al., 1998). However, the antimicrobial activity might attribute to the other unidentified compounds.

Table 4.2. FPJ Interaction on the inhibitory effects of Rifampin against MDR-TB clinical isolates

Sample	Rifampin	MIC of FPJ%
I, R (Resistance)		
1	(1.0 µg/ml)	11%
2		11%
3		14%
4		11%
5		10%
6		12%
7		10%
8		4%
9		10%
S, I, R (Resistance)		
10	(1.0 µg/ml)	13%
11		15%
12		12%
13		15%
14		12%
15		11%
16		12%
17		10%
18		10%
S, I, R, E (Resistance)		
19	(1.0 µg/ml)	13%
20		11%
21		13%
22		13%
23		11%
24		12%
25		14%
26		13%
27		10%

4.3. Identification and quantification of phenolic compounds by HPLC analysis

Table 4.3 illustrates the concentrations of individual phenolic compounds recognized in FPJ. A total of 17 phenolic compounds, which are carboxylic acid such as benzoic acid, hydroxycinnamicacids such as p-cummaric acid, chlorogenic, caffeic and ferulic acids, hydroxybenzoic acids such as ellagic acid and gallic acid, O-methylated trihydroxybenzoic acid such as syringic acid, flavan-3-ols such as catechin, flavonols such as kaempferol, quercetin, naringenin and myricetin, anthocyanin such as cyanidin-3-glycoside and pelargonidin, glycosides such as rutin, folate such as folic acid, have been identified in the FPJ.

In our study, the concentrations of phenolic compounds were as follows: gallic acid (106.62 mg/L), benzoic acid (17.20 mg/L), syringic acid (1.14 mg/L), folic acid (8.35 mg/L), pelargonidin (140.43 mg/L), naringin+ellagic acid (4.01 mg/L), nringenin (0.25 mg/L), chlorogenic acid (19 mg/L), caffeic acid (6.29 mg/L), catechin (3.51 mg/L), myricetin (63.99 mg/L), Kaempferol (2.19 mg/L), quercetin (0.59 mg/L), cyanidin-3-glycoside (42.68 mg/L), p-cummaric acid (0.12 mg/L), ferulic acid (5.94 mg/L), and rutin (9.48 mg/L). The concentrations, reported in this work, represent only the free forms of phenolic acids due to no hydrolysis was used to the samples before proceed to the HPLC analysis.

It has been documented that the concentrations of phenolic compounds were as follow; gallic acid (12.42–88.51 mg/L), catechin (1.31–6.84 mg/L), epicatechin (1.14–13.88 mg/L), ellagic acid (23.43–95.02 mg/L), chlorogenic acid (0.9–2.63 mg/L), caffeic acid (0.16–1.64 mg/L), ferulic acid (0.41–4.78 mg/L), quercetin (0.60–5.61 mg/L), phloridzin (0.07– 0.62 mg/L), and rutin (0.68–3.12 mg/L) (Hmid et al., 2017). The presence of chlorogenic acid, gallic acid, O-coumaric acid, quercetin, and catechin has also been documented by Artik et al. (1998) in pomegranate juices. In the present study, the cultivar, called “Silifke Aşısı” grown in Turkey, contains high concentration of gallic acid (106.62 mg/L) compared to those cultivars (12.42–88.51 mg/L) and (2.6–34.1 mg/L) grown in

Morocco and Italy, respectively (Hmid et al., 2017; Russo et al., 2018). Therefore, this cultivar should have a valuable effect on human health as this compound appeared to have antioxidative, anti-apoptotic, cardioprotective, neuroprotective, and anticancer properties by influencing the ROS signaling networks (Kosuru et al., 2017). Gallate is induced the mitochondrial permeability transition pore (MPTP) and causes the damage of mitochondrial membrane. Moreover, gallate detaches the oxidative phosphorylation, which generates a state of energy insufficiency and provokes apoptosis or halting of anabolic reactions. The apoptosis-inducing feature is mostly utilized by the cancer researchers (Kosuru et al., 2017).

Table 4.3. Composition of Phenolic compounds of fresh pomegranate juices (FPJ) analyzed by HPLC.

Phenolic compounds	Concentrations mg/L
gallic acid	106.62
benzoic acid	17.208
syringic	1.14
folic acid	8.352
pelargonidin	140.436
naringin+ellagic acid	4.014
naringenin	0.258
chlorogenic acid	19.002
caffeic acid	6.294
catechin	3.51
myricetin	63.996
kaempferol	2.19
quercetin	0.594
cyanidin-3-glycoside	42.684
p-cummaric acid	0.126
ferulic acid	5.94
rutin	9.48

4.4. Total phenolic (TP), total flavonoid (TF), and total anthocyanin (TA) content

Total phenolic concentration of FPJ was 841.5 mg /L. This is in agreement with the findings of Akhavan et al. (2015) who showed that MTKA, known as an Iranian cultivar, had (827.0 ± 29.7) mg/L total phenolic content (Akhavan et al., 2015) . Moreover, Gómez-Caravaca et al. (2013) reported that total phenolic content ranged from 580.8 to 2551.3 mg/L of pomegranate juice (Gómez-Caravaca et al., 2013). Diverse amounts of total phenolic content are attributed to various factors such as cultivar, analytical methods, environmental conditions, and maturity stage (Fischer et al., 2011).

Total flavonoid was found to be 638.73 mg RE/L. Total flavonoid values of pomegranate cultivars grown in Morocco ranged from 14,446 to 56,989 mg RE/L (Hmid et al., 2017). It has been documented that the value of total flavonoid content obtained from pomegranate juice and apple juice were 174 mg/L and 92 mg/L, respectively. Therefore, it can be concluded that daily consumption of pomegranate juice appears to be potentially better than apple juice in amelioration of antioxidant function in the elderly (Guo et al., 2008).

Considered as water-soluble pigments responsible for the red–purple colours of many fruits including pomegranate juice, anthocyanins are well known for their antioxidant capability (Seeram et al., 2008). The total anthocyanin content of FPJ in the present study was 47.43 mg/L. While our results were lower than the results published for eighteen cultivars grown in Morocco (Hmid et al., 2017), however, the total anthocyanin content of one Tunisian pomegranate cultivar called ‘Mezzi 1’ was only 43.7 mg/L (Hasnaoui et al., 2011).

4.5. Antioxidant activities

The results for antioxidant activities were measured by using DPPH. Generally, the DPPH radical scavenging assay is used to assess the ability of antioxidant to scavenge free radicals. The degree of discoloration specifies the

scavenging potentials of the antioxidant extract. In this study, the antioxidant capacity of FPJ was 80.73%. The results displayed considerable antioxidant activity of juice sample. The antioxidant capacity of pomegranate juice depends on not only the cultivar and growing region but also fruit maturation and agricultural factors. The technology, which is used for juice processing, might affect antioxidant capability of pomegranate juice (Çam et al., 2009). Our results were higher than the findings published for pomegranate cultivars grown in Morocco and Iran, with DPPH values between 45.65 and 76.3%, and 18.8 and 71.3 %, respectively (Hmid et al., 2017; Akhavan et al., 2015).

4.6. Expression of resistant genes under INH/R plus FPJ stress in MDR-TB isolates

Twenty seven (27) MDR-TB isolates, divided into three groups based on their resistance profile, have been used for quantification of gene expression. While first group showed resistance profile of R and INH, second one was resistant to SM, R, and INH resistant. And third one appeared to be resistant to INH, EMB, R, and SM. The real-time quantitative PCR (qPCR) was used to quantify the *katG*, *inhA* and *oxyR-ahpC* transcripts of MDR-TB isolates and H37Rv. The cDNA concentrations of the transcripts were calculated by comparing the $2^{-\Delta\Delta CT}$ values of the tested cDNA samples with those of the external controls. While, the expression level of *katG* in twenty four (24) MDR isolates appeared to be down-regulated (Figure 4.6, Table 4.4), it appeared to be overexpressed in H37Rv isolate (Figure 4.6, Table 4.5). Although the expression level of *katG* was up-regulated in three MDR isolates, the level of *inhA* and *oxyR-ahpC* expression was either up-regulated or overexpressed, indicating that those isolates are resistant to INH. Resistance to isoniazid is typically caused by mutations in either *katG* or the *inhA* promoter (Niehaus et al., 2015). The overexpression of *inhA*, which is due to the up-regulation mutation in the promoter region of *inhA*, can cause resistance to INH by a titration mechanism (Banerjee et al., 1994; Bardou et al., 1998; Basso et al.,

1998; Dessen et al., 1995; Heym et al., 1999; Marrakchi et al., 2000). By using absolute quantification, independent experiment demonstrated that the average concentration of *katG* transcript expressed by MDR isolates was 0.524 fold, whereas the average concentration of *katG* transcript in H37Rv was 5.540 fold. Tudó et al. (2010) postulated that transcriptome patterns might reveal mechanisms of action that elucidate INH tolerance, such as alterations in expression levels for genes involved in drug efflux, change in target levels (*inhA*) or diminished *katG* expression (Tudó et al., 2010). It has been documented that the expression levels above 1 were considered to be increased; equal to or above four were considered to be overexpressed (Li et al., 2015a). Our results were in accordance with what has been found by Ando et al. (2011) who reported that *M. tuberculosis* isolates with mutations in the *furA–katG* intergenic region showed a decrease in the expression of *katG* and therefore conferred INH resistance. These results indicated that down-regulation of *katG* is a mode of action of INH resistance in *M. tuberculosis* and that mutations in the *furA–katG* intergenic region appear to play a significant role in this resistance mechanism. The down regulation of the expression level of *katG*, which indicates to the presence of mutations in the regulatory and structural regions of *katG*, appears to be associated with INH resistance in *M. tuberculosis*. Therefore, mutations in *katG* were found in 24 (88.8%) of the 27 MDR-TB isolates. Peng et al. (2016) documented that 90.0% of the INH-resistant strains harbored mutations in the *katG* coding region (Peng et al., 2016). The frequency of *katG* mutations was present in 92% of INH^r samples in Moldova and 89% of INH^r samples in India but only 44% of INH^r specimens in South Africa (Georghiou et al., 2016). Moreover, the prevalence of mutations in *katG* gene was 86–94% of strains isolated in Russia, Lithuania and Latvia (Bakonyte et al., 2003, Mokrousov et al., 2002, Tracevska et al., 2002), 60-87% in Brazil (Silva et al., 2003; Cardoso et al., 2004), about 65% in Australia, China and Kuwait (Zhang et al., 2005; Ahmad and Mokaddas, 2004; Lavender et al., 2005), 53% in the Netherlands(van

Soolingen et al., 2000), 46% in Spain (de Viedma et al., 2002) and 38% in Italy (Rindi et al., 2005).

Considered as a keystone of treatment since its introduction in 1952, isoniazid (INH) is one of the most effective first-line anti-TB agents in current use (Bernstein et al., 1952). However, the appearance of INH resistant (INH^r) *M. tuberculosis* is a grave threat to the TB control (Espinal et al., 2001). A considerable interest in identifying the molecular basis of INH resistance in clinical *M. tuberculosis* isolates has been devoted in order to develop both rapid diagnostic approaches to detect INH^r *M. tuberculosis* isolates and novel anti-TB medications (Ando et al., 2014). INH is a prodrug which activated by a catalase peroxidase enzyme (KatG), encoded by the *katG* gene (Zhang et al., 1992). Activated INH appears to block the essential mycolic acids synthesis by inhibiting the NADH dependent enoyl-acyl carrier protein reductase *inhA* (Vilchèze et al., 2006; Banerjee et al., 1994).

KatG is considered as a co-transcribed with its negative regulator *furA*, a gene encoding a ferric uptake regulation protein (Pym et al., 2001; Zahrt et al., 2001). *furA* deletion in *M. tuberculosis* results in *katG* overexpression and hypersusceptibility to INH, suggesting the activity of this drug, at least *in vitro*, is limited by the rate of its activation and not by the nature of its interaction with other cellular processes, such as the inhibition of mycolic acid synthesis. Thus, the design of INH analogues that are more efficiently activated by *katG* would therefore be a sagacious tactic for manufacturing a drug with enhanced antimycobacterial potency (Pym et al., 2001). Transcription of *M. tuberculosis katG* has occur from two different promoters: one upstream of the *furA* gene, which itself is upstream of *katG*, and second one directly upstream of *katG*, which is situated between *furA* and *katG* (Master et al., 2001). Furthermore, transcriptional analyses demonstrate that *katG* is regulated by the sigma factor *sigI* (Lee et al., 2012). *M. tuberculosis ΔsigI* had reduced the activity of catalase and was more resistant to INH than wild-type *M. tuberculosis in vitro* and *in vivo*.

Chemically-induced overexpression of ***sigI***, which improved the susceptibility of ***M. tuberculosis*** to INH by 2-fold, resulted in an increase level of the ***katG*** expression, suggesting a direct transcriptional effect (Lee et al., 2012).

A polypurine sequence has been found to act as the ***katG*** ribosome binding site in the ***furA–katG*** intergenic region complementary to 16SrRNA. Mutations altering complementarity to 16S rRNA diminished the transcription level. Therefore, the intergenic region plays a critical role in ***katG*** expression and single mutations in these polypurine sequences resulted in a decrease level of the expression of ***katG*** and conferred INH resistance in ***M. tuberculosis*** (Sala et al., 2008; Ando et al., 2011). The majority of clinical isolates, which are resistant to INH, harbors mutations in ***katG*** (Gagneux et al., 2006; Ramaswamy et al., 2003). A wide range of polymorphisms, including missense and nonsense mutations, insertions, deletions, truncation, and rarely, whole gene deletions, has been documented worldwide (Zhang et al., 1992; Heym et al., 1995; Heym and Cole, 1992). The ***katG*** resistance-conferring mutants were assayed for their capability to produce the INH-NADH adduct in the presence of peroxide, superoxide, and no exogenous oxidant. The location of residue plays a considerable role in determining INH-resistance mechanisms related with the activation of INH; however, various mutations at the same position can generate vastly different reactivities that are oxidant-specific. These data suggested that the presence of a second mechanism of action of INH-resistance that is not associated with the formation of the INH-NADH adduct (Cade et al., 2010). S315T is described as the most common mutation occurring in clinical isolates in ***katG*** (Ghiladi et al., 2004; Brossier et al., 2006; Cardoso et al., 2004; Guo et al., 2006; Zhang et al., 2005; Hazbón et al., 2006).

Assessment the activities of INH oxidase of ***M. tuberculosis*** might give valuable information regarding the resistance of INH. The activities of INH oxidase of ***katG*** mutants displayed considerable correlations with the degree of INH resistance. Other enzymatic activities of ***katG*** mutants, including peroxidase

and catalase activities, were also associated with the degree of INH resistance. INH^r clinical isolates have been associated with loss of the catalase and peroxidase activities of *katG*, resulting in high-level INH resistance. Mutations in *katG* can lead to a loss of fitness to the bacterium due to the enzymatic activities of peroxidase and catalase which are critical for *M. tuberculosis* defense against reactive oxygen species and virulence *in vivo* (Ando et al., 2010; Zhang and Yew, 2009; Ng et al., 2004; Heym et al., 1997; Pym et al., 2002). Moreover, it has been documented that *M. tuberculosis* strains with mutations in *katG* had significantly lower INH oxidase activities than the wild type, and each mutant displayed different levels of activity. *M. tuberculosis* isolates having mutations with comparatively low activities of INH oxidase exhibited high-level INH resistance (Ando et al., 2010).

InhA was overexpressed in two MDR isolates and up-regulated in three isolates from first group. However, the expression of *inhA* in four MDR isolates appeared to be down-regulated (Figure 4.5, Table 4.4). The reduced mRNA expression leads to the down-regulation of *inhA* which is probably due to the non-existence of mutation in the promoter region of *inhA*. Resistance to INH is typically caused by mutations in either *katG* or the *inhA* promoter (Niehaus et al., 2015). Therefore, the down regulation of the *katG* expression in these four MDR isolates confers their resistance to INH. Regarding the second group of MDR-TB isolates, *inhA* were overexpressed and up-regulated in three and five MDR isolates, respectively (Figure 4.6, Table 4.4). However, the expression level of *inhA* in one isolate from second group was down-regulated. Although the expression study revealed the *inhA* expression in MDR-TB isolates, which are resistant to INH, EMB, R, and SM, was up-regulated in four isolates when compared with that of H37Rv (Figure 4.6, Table 4.5), the level of expression of *inhA* in five MDR isolates was down-regulated under INH plus FPJ stress (Figure 4.6, Table 4.4). By using quantitative reverse transcription-PCR (RT-qPCR) to measure the expression levels of *inhA*, the average concentration of *inhA* transcript expressed by MDR

isolates was 3.567fold, whereas the average concentration of *inhA* transcript in H37Rv was 0.156 fold. The *inhA* gene displayed more than 8-fold up-regulation of expression in the INH-resistant *M. tuberculosis* strains at the 99% confidence interval. All resistant strains appeared to harbor a t-to-a mutation at position 8 (t-8a) in the fabG1 promoter region, which causes the up-regulation of *inhA*. All tested *M. tuberculosis* isolates, which have mutations in the *inhA* promoter region, had enhanced relative normalized levels of *inhA* expression compared to those in isolates without the mutation (de Welzen et al., 2017). The single C-15T mutation in the promoter region of *inhA* in *M. tuberculosis* results in the overexpression of *inhA* (increase *inhA* mRNA levels by 20-fold) and consequently 8-fold increase in INH and ETH MICs in *M. tuberculosis* (Machado et al., 2017). Therefore, the overexpression of *inhA* in eight MDR isolates indicates to the presence of mutations in the regulatory region of *inhA*. The prevalence of *inhA* mutations occurred in 8/27 (29.6%) MDR-TB isolates. It has been reported that 15.1% of the INH-resistant isolates carried mutations in the promoter region of *mabA-inhA* (Peng et al., 2016). Furthermore, the frequency of *inhA* mutations with or without a mutation of *katG* was 30.3% (Niehaus et al., 2015). Several recent reports, which have examined relative frequencies of *katG* and *inhA* promoter mutations in INH-resistant isolates, have shown considerable geographic variation, with 4% incidence of *inhA* in Poland, 22% in the Philippines, 0.8% (without *katG*) mutation in Ethiopia, and 9.6% in Chonqing, China (Jagielski et al., 2014; Abate et al., 2014; Wade and Zhang, 2004; Herrera et al., 2004). A study conducted in the Western and Eastern Capes of South Africa documented that nearly 40% of MDR-TB isolates harbored a mutation in the promoter region of *inhA* without concurrent *katG* mutation. However, there is significant genetic assortment of TB throughout South Africa and this precludes extrapolating results among provinces (Müller et al., 2011; Chihota et al., 2011).

Mutations in the *inhA* open reading frame (ORF) and promoter region are the major mechanism of resistance to ETH (Vilchèze et al., 2006; Vilchèze and

Jacobs, 2014). *M. tuberculosis* strains have the C-15T mutation and additionally have a mutation in the ORF of *inhA* contributing to their high-level resistance to INH, as previously reported (Machado et al., 2013). It has been documented that overexpression of *inhA* confers INH and ethionamide (ETH) resistance which led to the conclusion that *inhA* encoded the target of INH and ETH in *M. tuberculosis*, *M. smegmatis*, and *M. bovis* BCG (Larsen et al., 2002). Therefore, *inhA* is considered as the primary target of INH, ethionamide and triclosan (Banerjee et al., 1994; McMurry et al., 1999).

InhA is an essential gene; therefore, the occurrence of *inhA* mutations in the coding region is rare. About 15 mutations in *inhA* have been reported in INH-resistant isolates, even though two of them were also identified in INH sensitive isolates (Brossier et al., 2006; Morlock et al., 2003). While the frequency of *inhA* structural mutations was uncommon, the S94A mutation diminishes the binding affinity of the NADH cofactor and confers low-level resistance to INH (Blanchard, 1995). The S94A mutation, which was identified as the first mutation in *inhA* in *M. smegmatis*, has been found in INH-resistant clinical isolates of *M. tuberculosis* with no other mutation present in the *fabG-inhA* intergenic region or *katG*. This indicates that the mutation of S94A is sufficient to confer INH and ETH-resistant clinical isolates of *M. tuberculosis* (Brossier et al., 2006; Morlock et al., 2003).

Mutations in *inhA* and its promoter region have been determined in both INH- and ETH-resistant *M. tuberculosis* clinical isolates. However, no base pair deletions or insertions have been detected. The c-15t mutation in the *inhA* promoter region, which was overly represented in XDR-TB cases in South Africa, is found in up to 35% of INH-resistant and 55% of ETH-resistant clinical isolates, but never in INH- or ETH-sensitive strains. The occurrence of c-15t mutation was 30% of strains mono-resistant to INH, 48% of MDR-TB, and 85% of XDR-TB in a study conducted in South Africa, suggesting that this mutation could be a marker for XDR-TB(Müller et al., 2011). The combined mutations in the *inhA* promoter

region at positions (-8, -15, and -17) were detected in 92% of the XDR-TB versus 62% of the MDR-TB clinical isolates (Müller et al., 2011).

The mode of action by which the mutation of S94A leads to INH and ETH resistance has been well investigated. *inhA* is described as an NADH-dependent enoyl-ACP reductase, and the binding of the enoyl substrate to *inhA* is not disturbed by the mutation of S94A; however, the mutation leads to a five-fold increase in the K_M for the *inhA* cofactor NADH (Vilchèze et al., 2006). On the other hand, the capability of the INH-NAD adduct is markedly reduced for the inhibition of *inhA* (S94A), because the K_i and IC_{50} are 30 and 17 times higher, respectively, for the mutated protein. The reduction in the INH-NAD adduct is due to the loss of a water molecule and disruption of a hydrogen bonding network in the mutated protein, a comparison study of the crystal structures of *inhA* (S94A) to wild-type *inhA* revealed (Vilchèze et al., 2006). Moreover, it has been hypothesized that *inhA* interacts with FASII enzymes and this interaction is disturbed by the mutation of S94A, resulting in INH resistance (Dias et al., 2007).

The expression of *oxyR-ahpC* in the first group of MDR-TB isolates was up- regulated for three isolates. However, the *oxyR-ahpC* expression in six MDR isolates of the same group appeared to be down-regulated (Figure 4.6, Table 4.4). While the expression level of *oxyR-ahpC* in two isolates from the second group of MDR-TB was overexpressed and five isolates were up-regulated, it appeared to be down- regulated in two isolates of MDR-TB (Figure 4.6, Table 4.4). The study of gene expression profiling revealed that the expression level of *oxyR-ahpC* in five MDR isolates from the third group were up-regulated and overexpressed in two isolates under INH plus FPJ stress when compared with that of H37Rv (Figure 4.6, Table 4.5**)**. However, expression level of the *oxyR-ahpC* was found to be down-regulated in two isolates of the third group **(**Figure 4.6, Table 4.4**)**. Quantitative reverse transcription-PCR (RT-qPCR) was used to measure the expression levels of *oxyR-ahpC*. The average concentration of *oxyR-ahpC* transcript expressed by MDR isolates was 1.92 fold, whereas the average concentration of *oxyR-ahpC*

transcript in H37Rv was 0.104 fold. The reduction in the mRNA expression of *oxyR-ahpC* is probably due to the non-existence of mutations in the *oxyR-ahpC* intergenic region where the putative promoter of *ahpC* is located. Genetic and biochemical studies have revealed that mutations in the promoter region of *ahpC* result in overexpression of *ahpC* as a compensatory mechanism for the loss of catalase activity due to *katG* mutations (Kelley et al., 1997; Wilson et al., 1998). Thus, the overexpression of *oxyR-ahpC* intergenic region implies to the existence of mutations in the promoter of *ahpc*. The frequency of mutations in *oxyR-ahpC* intergenic region was found in 4/27 (14.81%) MDR-TB isolates. Of 166 INH-resistant strains, 27(16.3%) had mutations in the intergenic region of *oxyR-ahpC* (Peng et al., 2016). Jagielski et al. (2014) reported that the prevalence of mutations in the *oxyR-ahpC* regulatory region occurred in 10% of the MDR-TB isolates (Jagielski et al., 2014). Moreover, a study conducted in China showed that mutations in the *oxyR-ahpC* intergenic region occurred in 11.5% of the INH-resistant strains (Zhang et al., 2005). Mutations in the promoter of *ahpC* were detected in INH resistant *M. tuberculosis* with a deficient activity of *katG*, but not in INH susceptible isolates (Springer et al., 2001; Dhandayuthapani et al., 1996). However, mutations in the regulatory region of *oxyR-ahpC* have also been found in INH-susceptible *M. tuberculosis* strains, indicating that these mutations have slight influence on the isoniazid resistance phenotype (Hazbón et al., 2006). *OxyR*, which is described as a regulatory protein that is an oxidative-stress sensor and activator of gene transcription, controls the expression of *katG* and *ahpC* genes coding for the catalase-peroxidase (KatG) and alkyl hydroperoxidase (AhpC), respectively (Farr and Kogoma, 1991). *OxyR* is naturally inactive which confers increased susceptibility to INH, providing an elucidation to the high susceptibility of *M. tuberculosis* to INH (MIC of 0.05 mg/L). This inactivation is due to several frame-shift mutations and deletions in *M. tuberculosis* (Pagán-Ramos et al., 2006). The low incidence of mutations in the promoter region of *ahpC* might partly be elucidated by the fact that they have rarely been recognized in isolates already

harboring *katG* mutations (Hazbón et al., 2006; Cardoso et al., 2007; Luo et al., 2010; Sreevatsan et al., 1997). This is because the mutations in *katG* appear to preserve enough catalase activity and allow *in vivo* survival of *M. tuberculosis* in the absence of compensatory mutations of *ahpC* (Kelley et al., 1997).

Twenty four of MDR-TB isolates showed either overexpression or up-regulation of *rpoB* as compared to H37Rv. However, three MDR isolates appeared to reveal down regulation of *rpoB* (Figure 4.6, Table 4.4, Table 4.5). The average concentration of *rpoB* transcript expressed by MDR-TB and H37Rv were 41.169 fold and 0.133 fold, respectively. More than 95% of RIF-resistant strains comprise mutations in the *rpoB* gene of *M. tuberculosis* (Ramaswamy and Musser, 1998). An 81-bp fragment of *rpoB* encoding codons 507–533 (*E. coli* numbering system), identified as the rifampicin resistance determining region, carries most mutations conferring high-level resistance (Heep et al., 2001). Mutations at positions 531, 526 or 516 are considered as the most commonly mutations in *M. tuberculosis* clinical isolates (Musser, 1995; Brandis and Hughes, 2013; Qian et al., 2002). It has been reported that MDR –TB isolates displayed up-regulation of *rpoB* and *sigM* which are part of transcriptional mechanism (Chatterjee et al., 2013). Moreover, expression genes which are part of the *WhiB7* regulon appeared to be up-regulated in the *rpoB* gene with mutation at codon 526 of rifampicin resistant *M. tuberculosis* clinical isolates. These findings imply that the position of the *rpoB* gene mutation, as well as the strain genetic background play a vital role in the gene expression profile of *rpoB* mutants (Du Plessis, 2014). *WhiB7* expression, which is induced upon exposure to fatty acids and antibiotics such as erythromycin and tetracycline, plays a significant role in regulating gene expression by binding to region 4 of the housekeeping σ factor, σA (Morris et al., 2005; Burian et al., 2013). Therefore, *whiB7* was up-regulated in the *rpoB*526 mutant strains. A recent investigation demonstrated that MTS2823 sRNA has an important role in mediating the genes repression involved during exponential growth of *M. tuberculosis* (Arnvig et al., 2011). Even though the regulatory mode of action of

MTS2823 sRNA remains ambiguous, it is comparable to 6S RNA in *E. coli*, which binds directly to RNA polymerase (RNAp) (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2005). The interaction of sRNA-RNAp might be affected by the presence of mutations in *rpoB*, which may cause variance expression of genes under the control of regulatory sRNA. Alternatively, RNAp processes including transcription elongation and termination could also be influenced by the existence of *rpoB* mutations.

RpoB mutation at S531L confers no loss of *M. tuberculosis* fitness, and is often associated with additional compensatory mutations in the *rpoA* and *rpoC* genes that partially recover fitness costs (Mariam et al., 2004; Song et al., 2014; Brandis et al., 2012; Poon et al., 2005). Moreover, *rpoB* mutations appear to assist the bacterium to adapt and endure in harsh environments; however they can lead to an increased fitness cost when grown in a system without cellular stress such as antibiotic pressure and the host immune response (Du Plessis, 2014). The down regulation of *rpoB* in three MDR-TB isolates does not rule out the resistance of these isolates to rifampin. This might be due to a silent mutation that does not result in alteration of a single amino acid or may imply the presence of another resistance mechanism of action. Rifat et al. (2017) found that the H526D mutation is associated with reduced expression of the *rpoB* gene (Rifat et al., 2017). The up-regulation of *rpoB* indicates to the existence of mutations in this gene. The frequency of mutations in *rpoB* was found in 24/27 (88.8%) MDR-TB isolates. It has been documented that mutations were found in 37/43 (86%) of the rifampicin-resistant *M. tuberculosis* isolates (Chikaonda et al., 2017). A study conducted in China and Pakistan found that the prevalence of *rpoB* mutations in rifampicin-resistant *M. tuberculosis* were 85% and 81%, respectively (Yue et al., 2003; Khan et al., 2013).

Genetic alterations in the binding site of rifampin make mycobacteria and Gram-negative genetically resistant to rifampin and illustrate the primary genetic mode of drug resistance, accounting for almost all clinically significant instances

of rifampin resistance (Campbell et al., 2001; Vassylyev et al., 2007). Mycobacteria produce a remarkably complex cell envelope consist of phospholipid, mycolyl lipid, and other layers that are actively regulated and altered as part of the switch to dormancy or in response to metabolic stresses (Manganelli et al., 1999). Mutations in *rpoB* could possibly modify cellular function through modification of *M. tuberculosis* transcriptional responses and, thus, alter the structure, composition and integrity of the cell wall. Therefore, it is plausible that organism-wide modifications in cell wall lipids may appear after genetic acquisition of rifampin resistance. *M. tuberculosis rpoB* mutant isolates have a role in the alteration of the expression of several cell wall proteins and lipids (Bisson et al., 2012b; Du Preez and Loots, 2012; Lahiri et al., 2016).

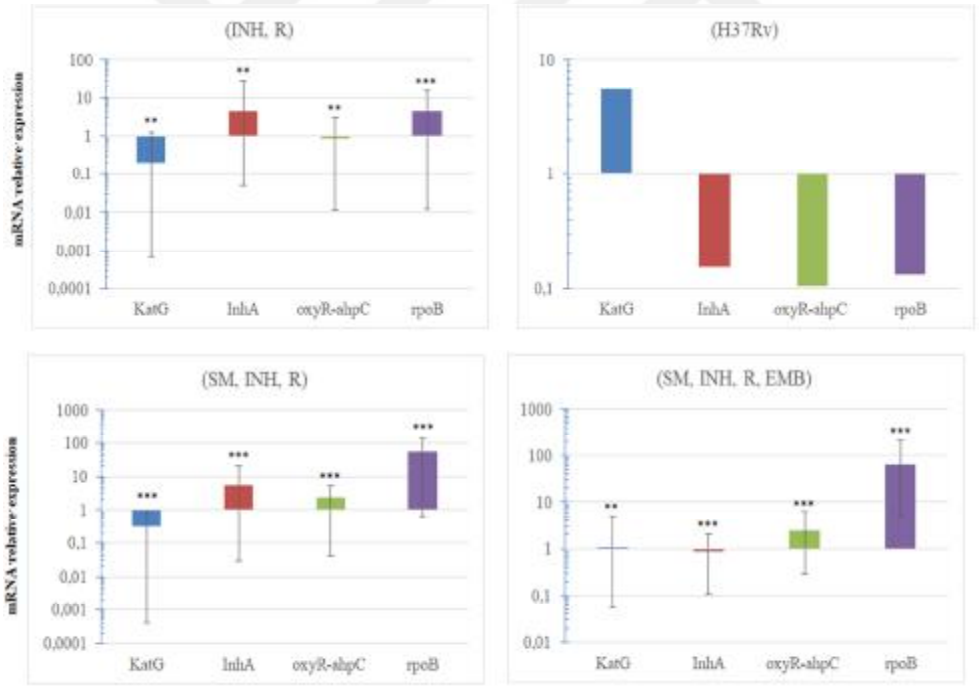

Figure 4.6. Quantification of the relative mRNA expression levels of a panel of resistant genes (*KatG*, *InhA*, *oxyR-ahpC*, *rpoB*) in MDR-TB clinical isolates. The results were considered significant when $*P < 0.05$; and highly significant when $**P < 0.01$ and $***P < 0.001$.

Table 4.4. Depicts differential expression levels of resistant genes (*KatG*, *InhA*, *oxyR-ahpC*, *rpoB*) under (INH+ FPJ and R + FPJ) stress in MDR-TB clinical isolates

			Number of MDR-TB isolates with various fold change ($2^{-\Delta\Delta CT}$ value, n = 9 for each group)				
			<1	>1	>2	>3	≥4
Stress condition	**MDR-TB group**	**Gene**					
INH/R + FPJ	**INH, R**	*KatG*	9	1	0	0	0
		InhA	4	3	0	0	2
		oxyR-ahpC	6	2	0	1	0
		rpoB	2	1	0	2	4
	SM, INH, R	*KatG*	9	0	0	0	0
		InhA	1	2	1	2	3
		oxyR-ahpC	2	3	1	1	2
		rpoB	1	0	0	1	7
	SM, INH, R , EMB	*KatG*	7	0	1	0	1
		InhA	5	3	1	0	0
		oxyR-ahpC	2	3	1	1	2
		rpoB	0	0	0	0	9

Table 4.5. The expression levels of efflux pump genes and resistant genes under (INH+ FPJ and R + FPJ) stress in H37Rv *M. tuberculosis*

		M. tuberculosis H37Rv isolate	
		down-regulation	**up-regulation**
Stress condition	**Gene**		
INH/R + FPJ	*KatG*	-	+
	InhA	+	-
	oxyR-ahpC	+	-
	rpoB	+	-
INH + FPJ	*efpA*	+	-
	drrA	+	-
	drrB	+	-
	RV1250	+	-
	RV1634	+	-
	RV2459	+	-
R + FPJ	*efpA*	+	-
	drrA	+	-
	drrB	+	-
	RV1250	+	-
	RV1634	+	-
	RV2459	+	-

4.7. Differential expression of efflux pump genes of *M. tuberculosis* in response to antituberculosis agents (INH or R) plus FPJ

Twenty seven (27) MDR-TB isolates classified based on their resistance profile and divided into three groups as mentioned previously have been used for quantification of the expression of efflux pump genes under INH plus FPJ stress. Twenty six (26) MDR isolates out of 27 had at least one gene that was either overexpressed or up-regulated under INH plus FPJ. In the first group where *M. tuberculosis* isolates showed complete resistance to INH and R, *efpA* was found to be overexpressed in six isolates and up-regulated in three of MDR-TB isolates when compared with that of H37Rv. While *drrA* was overexpressed in three isolates, it appeared to be up-regulated in two of MDR-TB isolates. *drrB and Rv1250* showed overexpression in one isolate and up-regulation in three and five MDR-TB isolates, respectively. Both *Rv1634 and Rv2459* displayed overexpression in one isolate, however, *Rv1634* and *Rv2459* were up-regulated in five and three isolates of MDR-TB, accordingly (Figure 4.7, Table 4.6). In the second group of MDR-TB isolates, which was resistant to SM, R, and INH, *efpA* was overexpressed in four isolates and up-regulated in three of MDR-TB isolates as compared to H37Rv. *drrA* was overexpressed in six isolates and up-regulated in two of MDR-TB isolates. The overexpression and the up-regulation of *drrB* gene occurred in one and three of MDR-TB isolates, respectively. Both *Rv1250* and *Rv2459* demonstrated overexpression in two MDR-TB isolates. However, *Rv1250* and *Rv2459* appeared to be up-regulated in four and five isolates of MDR-TB, accordingly. *Rv1634* showed overexpression in three MDR-TB isolates and up-regulation in four isolates of MDR-TB (Figure 4.7, Table 4.6). *efpA* showed overexpression in one isolates and up- regulation in three isolates of the third group of MDR- TB that displayed resistance to INH, EMB, R, and SM when compared to that of virulent H37Rv strain (Figure 4.7, Table 4.5). *drrA and drrB* were up-regulated in five and six isolates of MDR-TB, respectively. *Rv1250*, *Rv1634*, and

Rv2459 demonstrated overexpression in one isolate and up-regulation in five isolates of MDR-TB (Figure 4.7, Table 4.6).

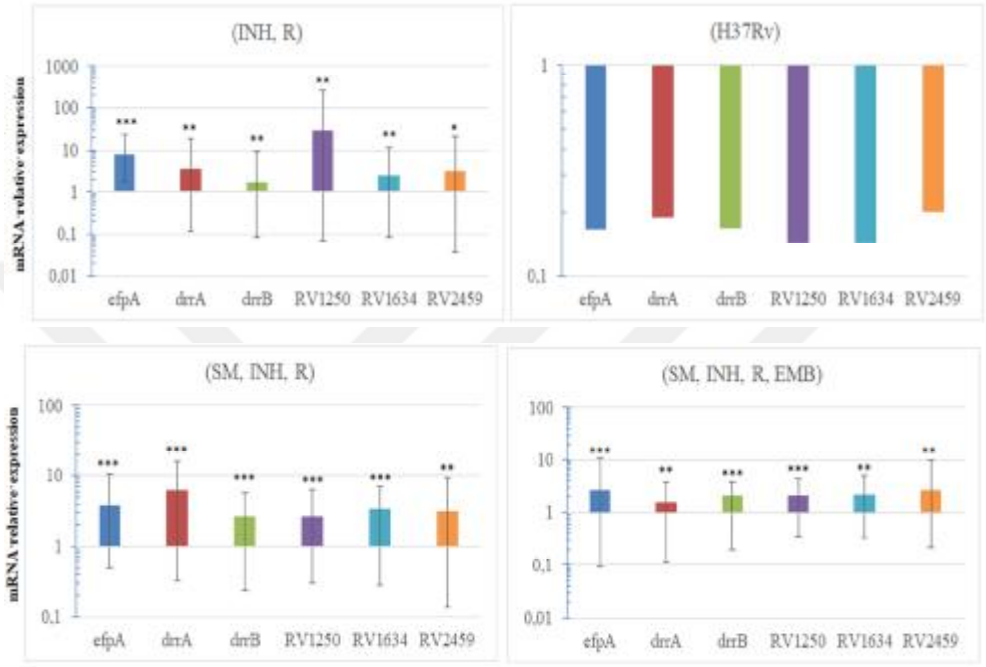

Figure 4.7. Expression levels of a panel of drug efflux pump genes of MDR-TB and *M. tuberculosis* H37Rv treated by INH plus FPJ. The results were considered significant when $*P < 0.05$; and highly significant when $**P < 0.01$ and $***P < 0.001$

Table 4.6. Shows variance expression levels of efflux pump genes treated by INH plus FPJ in MDR-TB clinical isolates

Stress condition	MDR-TB group	Gene	Number of MDR-TB isolates with various fold change ($2^{-\Delta\Delta CT}$ value, n = 9 for each group)				
			<1	>1	>2	>3	≥4
INH + FPJ	INH, R	*efpA*	0	2	1	0	6
		drrA	4	2	0	0	3
		drrB	5	3	0	0	1
		RV1250	3	5	0	0	1
		RV1634	2	5	1	0	1
		RV2459	5	2	1	0	1
	SM, INH, R	*efpA*	2	1	0	2	4
		drrA	1	1	0	1	6
		drrB	2	2	1	2	2
		RV1250	2	2	1	2	2
		RV1634	2	1	1	2	3
		RV2459	2	1	2	2	2
	SM, INH, R , EMB	*efpA*	1	4	3	0	1
		drrA	4	2	1	2	0
		drrB	3	1	1	4	0
		RV1250	3	5	0	0	1
		RV1634	3	2	1	2	1
		RV2459	3	1	3	1	1

Twenty six 26/27 of MDR-TB isolates treated by R plus FPJ had at least one gene that was either overexpressed or up-regulated. In the first group, *efpA* was overexpressed in six isolates and up-regulated in two MDR-TB isolates as compared to H37Rv (Figure 4.8, Table 4.5). *drrA*, *drrB*, and *Rv2459* showed overexpression in five isolates and up-regulation in two isolates of MDR-TB. *Rv1250* was overexpressed in four isolates and up-regulated in three isolates of MDR-TB (Figure 4.8, Table 4.7)Regarding the second group, *efpA*, *drrA*, *drrB*, *Rv1634*, and *Rv2459* were overexpressed in seven isolates and up-regulated in at least in one isolate of MDR-TB. *Rv1634* displayed overexpression in six isolates and up-regulation in two isolates of MDR-TB (Figure 4.8, Table 4.7). *efpA*, *drrB*, *Rv1634*, and *Rv1250* appeared to be overexpressed in all isolates of the third group of MDR-TB. *drrA* and *Rv2459* were overexpressed in eight isolates and up-regulated in one isolate of MDR-TB (Figure 4.8, Table 4.7).

To further validate these findings, the transcriptional profile of putative efflux pumps described in the previous studies has been analyzed as being associated with drug resistance phenotypes of *M. tuberculosis* (Viveiros et al., 2012, Black et al., 2014, da Silva et al., 2016, Li et al., 2015b, Garima et al., 2015, Li et al., 2015a). The contribution of the membrane transporters of *M. tuberculosis* for this enhanced efflux activity and resistance towards INH and R was corroborated by a general and marked overexpression of almost all efflux genes examined by RT-qPCR.

Machado et al. (2017) reported that the occurrence of important alterations in the expression levels of *efpA* in all MDR-TB strains upon exposure to INH/R except in the susceptible H37Rv strain (Machado et al., 2017). Moreover, the exposure to rifampicin resulted in increased expression of *mmr*, *mmpL7*, *p55*, *Rv1217c*, and *efpA* than those obtained for INH (Machado et al., 2016). In our study, we have observed that the overexpression of *rpoB* was associated with the overexpression of the most of examined efflux pump genes. This suggests that *rpoB* mutation may have analogous effects on specific gene up-regulation in *M.*

tuberculosis. It has been documented that the polyketide synthase gene cluster *ppsA-ppsE* and *drrA* was significantly up-regulated in *rpoB* mutant *M. tuberculosis* (Bisson et al., 2012a). The expression level of almost all tested efflux genes was either overexpressed or up-regulated under R plus FPJ. This might be due to the efficacy of this combination (R+FPJ) in acidic conditions against MDR-TB isolates than INH plus FPJ. It has been documented that the expression level of *efpA* enhanced in response to tetrahydrolipstatin, isoniazid, isoxyl, and thiolactomycin. Furthermore, the expression level of *efpA* was 4.5- and 4 -fold greater under INH stress, respectively, which was in accordance with the results in our study (Gupta et al., 2010, Fu, 2006, Betts et al., 2003). Rv2459 appeared to respond to INH stress, and the increased transcription of Rv2459 leads to enhanced resistance to EMB and INH in *M. tuberculosis* (Gupta et al., 2010; Campus, 2010). Rv1634 is described as a member of the main facilitator superfamily in *M. tuberculosis*, similar to many drug resistance efflux proteins, comprising DTDP-glucose dehydratase from *Streptomyces violaceoruber* (Cole et al., 1998; De Rossi et al., 2002; Rossi et al., 2005; Li et al., 2004). *Rv1634* has been found to diminish susceptibility to the numerous fluoroquinolones antibiotics when overexpressed in *M. smegmatis* and involve in norfloxacin and ciprofloxacin efflux. *Rv1634* showed up-regulation in Beijing strain after exposure to rifampin for 24 h. *Rv1250*, which is similar to *Rv3239C* from *M. tuberculosis*, is a probable drug transport integral membrane protein having 579 amino acid (aa) and highly similar to the tetracenomycin C protein from *Streptomyces glaucescen* . *Rv1250* was induced under INH stress in INH-resistant *M. tuberculosis* isolates (Narang et al., 2017). Li et al. (2015b) reported that rifampin-monoresistant isolates carried mutations in *rpoB* were either overexpressed or up- regulated in at least one of the following putative efflux pump genes: *efpA*, *drrA*, *drrB*, *Rv 1250*, *Rv1634*, and *Rv2459*. Furthermore, rifampin did not increase expression level of H37Rv for most of the examined efflux genes (Li et al., 2015b). Our findings are in agreement with what has been found by Li et al. (2015a) who reported that *drrA*, *drrB*, *efpA*, *jefA* (Rv2459), *Rv1634*, and *Rv1250*

were overexpressed under INH/R stress in MDR-TB isolates. However, Li et al. (2015a) documented that *drrA* was overexpressed in zero MDR-TB isolates induced by R, which was not consistent with our results (Li et al., 2015a). In our study, almost all MDR-TB isolates showed overexpression or up-regulation of *drrA*, suggesting that *drrA* was one of the elements for R resistance in *M. tuberculosis*. Pang et al. (2012) found that *drrA* may be responsible for low-level resistance to rifampin (Pang et al., 2012), which was consistent with our findings. *drrABC* is described as a putative doxorubicin-resistance operon in *M. tuberculosis* (Cole et al., 1998). *drrAB*, which expressed in *M. smegmatis*, conferred resistance to a wide array of clinically relevant drugs, such as EMB, SM, norfloxacin, erythromycin, tetracycline, and chloramphenicol (Choudhuri et al., 2002). The systematic increase in the expression of efflux pump gene after drug treatment might be elucidated by two possible modes of action: 1) increase in the expression level of genes is due to a mutation in the promoter region of the efflux pump genes, leading to constitutive gene expression; and 2) the overexpression of efflux pump genes is most probably a global response mediated by global regulators (Machado et al., 2016). It has been documented that the drug resistant clinical strains are more prompt to respond to the drugs through an efflux-mediated response. However, the susceptible H37Rv strain displays a less prompt detoxifying response to the antibiotics to which it was exposed. Altogether, the results obtained demonstrate that *M. tuberculosis* clinical isolates are primed to efflux poisonous compounds and displayed that efflux pump induction is being associated with the drug resistance pattern of *M. tuberculosis* strains, emphasizing the validity of the hypotheses that efflux contributes to the resistance level of these strains (Machado et al., 2017).

In H37Rv isolate, none of 6 genes were induced to overexpress by R and INH plus FPJ in our study, which was consistent with the results that none of 20 efflux genes were induced to overexpress by RIF and INH in 10 pan-sensitive isolates (Zhang et al., 2013). Additionally, Li et al. (2015a) reported that no

decreases in the MIC of INH and R in the presence of the efflux pump inhibitors Carbonyl cyanide m-chlorophenylhydrazone (CCCP) or reserpine in these isolates (Li et al., 2015a).

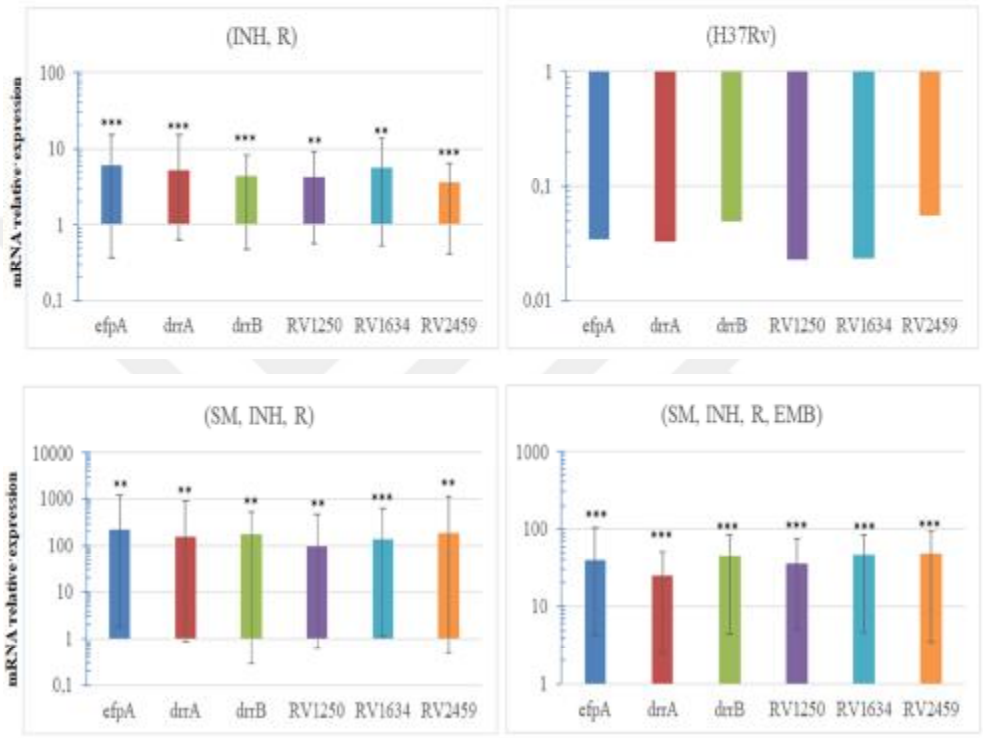

Figure 4.8. The level of expression of a panel of antibiotic efflux pump genes of MDR-TB and *M. tuberculosis* H37Rv treated by R plus FPJ and quantified by real-time RT–PCR. The results were considered significant when $*P < 0.05$; and highly significant when $**P < 0.01$ and $***P < 0.001$

Table 4.7. Relative expression levels of antibiotic efflux genes treated by R plus FPJ in MDR-TB clinical isolates

Stress condition	MDR-TB group	Gene	Number of MDR-TB isolates with various fold change ($2^{-\Delta\Delta CT}$ value, n = 9 for each group)				
			<1	>1	>2	>3	≥4
R + FPJ	INH, R	*efpA*	1	2	0	0	6
		drrA	2	1	1	0	5
		drrB	2	0	1	1	5
		RV1250	2	1	2	0	4
		RV1634	3	0	1	0	5
		RV2459	2	1	0	1	5
	SM, INH, R	*efpA*	0	1	1	0	7
		drrA	1	1	0	0	7
		drrB	1	1	0	0	7
		RV1250	1	1	1	0	6
		RV1634	0	2	0	0	7
		RV2459	2	0	0	0	7
	SM, INH, R , EMB	*efpA*	0	0	0	0	9
		drrA	0	0	1	0	8
		drrB	0	0	0	0	9
		RV1250	0	0	0	0	9
		RV1634	0	0	0	0	9
		RV2459	0	0	0	1	8

In this study, we scrutinized the expressional differences between MDR-TB isolates and H37Rv isolate without drug inducement. The expression levels of examined genes (*efpA*, *Rv1250*, *Rv1634*, *Rv2994*, *Rv2459*, *drrA*, and *drrB*) were significantly higher in MDR-TB isolates than in H37Rv isolate (Figure 4.9**)**, which was consistent with the findings of previous research carried out by Li and his co-worker (Li et al., 2015a). Moreover, 25/27 MDR-TB isolates showed at least up-regulation or overexpression at least one of these six genes. These results suggested that the efflux pumps role is undoubtedly not to pump out anti-TB agents but, rather, that they appear to regulate the intracellular levels of nutrients and co-factors. The differences between MDR-TB and H37Rv isolates imply that quantifying the expression levels of efflux pump genes of *M. tuberculosis* might be a new approach to diagnose resistant tuberculosis and decide whether the addition of efflux pump inhibitors to anti-TB agents would be potent to defeat resistant strains of *M. tuberculosis*.

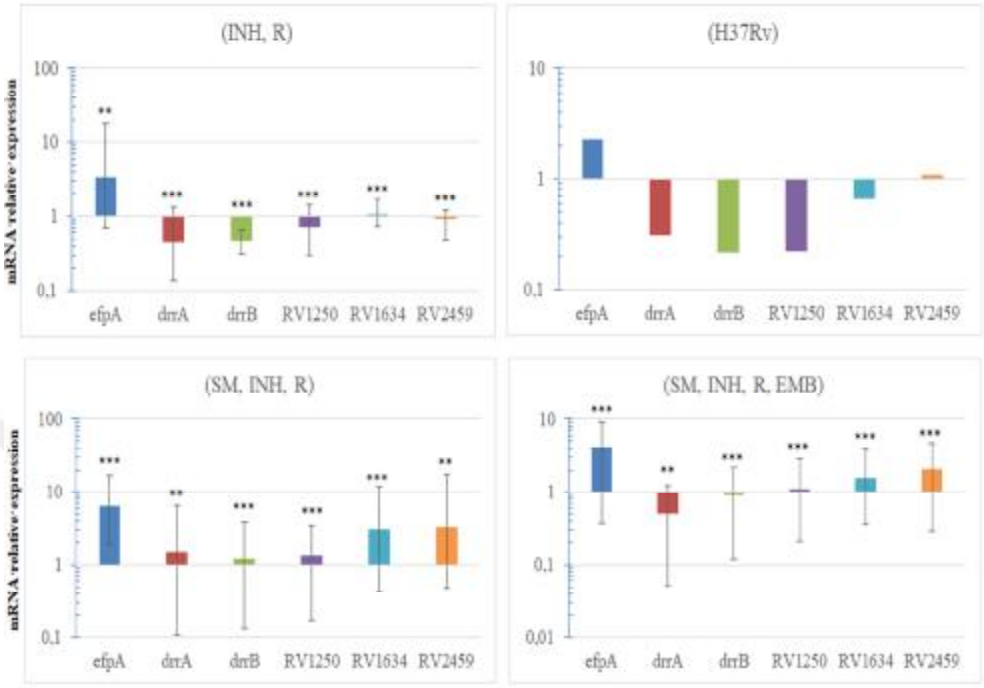

Figure 4.9. Relative fold change in the level of expression of a panel of antibiotic efflux pump genes of MDR-TB and *M. tuberculosis* H37Rv without inducement. The results were considered significant when $*P < 0.05$; and highly significant when $**P < 0.01$ and $***P < 0.001$.

5. CONCLUSION

5.1. Conclusion

Tuberculosis (TB) has endured as worldwide emergency ever since it was announced as such by the WHO, followed by a global growth in the incidence of MDR-TB in current years, revealed new opportunities and prompted the requirement for the formation of novel and more effective medications for TB therapy. The employment and innovation of a first line drug was a significant success in dealing with drug-sensitive TB. However, the robust character of the bacterium and its competence to form a dormant condition or create resistance has marked TB as a main cause of mortality due to infectious disease. Efflux pump is being involved in bacterial drug resistance which is considered as a major cause of concern in many pathogenic strains that have developed MDR phenotypes. MDR transporters, which have the ability to pump drugs out of the cell, help bacteria to escape conventional antibiotic therapies. Therefore, they form potentially precious targets in the search for novel inhibitors restoring the effectiveness of conventional therapy.

In addition to confirming the well-publicized high antioxidant ranking of such foods as blueberries, the researchers found that pomegranate arils also have antibacterial activity and might be used as medicine for humans. This diminishes the cost and the risk of drug consumption. Moreover, added value from the peels which could provide health benefits to humans and might be utilized in food preservation and pharmaceutical purposes.

This study aimed to investigate the effect of fresh pomegranate juice (FPJ) on the antibacterial activity of anti-tuberculosis drugs (Rifampin (R) and Isoniazid (INH)) against MDR-TB clinical isolates. Furthermore, this study scrutinized individual phenolic compounds of FPJ by using high-performance liquid chromatography (HPLC). The total polyphenols (TP), total flavonoid (TF), total anthocyanins content (TAC), and the antioxidant capacity were also evaluated in

FPJ. Synergistic effects were remarked between R and INH with FPJ against all tested strains. However, combination therapy of R was more effective than INH one. Therefore, the combination of R and FPJ has been used against twenty seven (27) MDR-TB clinical isolates. Additionally, we assessed the contribution of resistant genes associated with INH and R resistance plus six putative drug efflux pump genes to the drug resistance levels in MDR-TB clinical isolates.

Observations of this research imply that FPJ appeared to have a remarkable synergetic effect with rifampin (R) against MDR-TB clinical isolates. Total phenolic content (TPC), total flavonoid (TF), and total anthocyanin of FPJ were 841.5 mg /L, 638.73 mg RE/L, and 47.43 mg/L, respectively. These results highlighted that FPJ not only does not reduce the antibacterial activity of rifampin, but also enhance its effects.

katG was down-regulated in 24/27 (88.88%) MDR-TB isolates. *inhA*, *oxyR-ahpC*, and *rpoB* were either overexpressed or up-regulated in 8/ 27 (29.62%), 4/27 (14.81 %), and 24/ 27 (88.88%), respectively in MDR-TB isolates. Furthermore, the efflux pump genes *drrA*, *drrB*, *efpA*, *Rv2459*, *Rv1634*, and *Rv1250* were overexpressed under INH/RIF plus FPJ stress signifying the contribution of these efflux pumps to the overall resistance of MDR-TB isolates. This study shows us that the main mode of actions associated with antibiotic resistance in *M. tuberculosis* correlates mutations in target genes with improved efflux. The level of antibiotic resistance in *M. tuberculosis* is a combination among the existence of a mutation in the antibiotic target genes and a general stress response to the presence of toxic compounds that regulates the intracellular level of a drug.

5.2. Future work

Much of the evidence for antibacterial and antiviral activities of pomegranates against foodborne pathogens and other organisms that are responsible of infectious disease comes from *in vitro* cell based assays, demanding

further validation of *in vivo* efficiency through human clinical trials. Therefore, more controlled trials are necessary using different concentrations of pomegranate fruit extracts to verify its action upon antimicrobial *in vivo*. Within the limits of the present study, it may be concluded that the fresh pomegranate juice (FPJ) plus rifampin (R) are effective against MDR-TB clinical isolates. Further research is required to ascertain the actual benefits of pomegranate as a therapeutic and preventive agent for tuberculosis disease and to identify the specific active elements in FPJ that could be useful as antimicrobial agents and the safer dose that can be used for humans.

Additional efforts are necessary to clarify the real roles of drug efflux pumps in the drug resistance of *M. tuberculosis.* More efflux pump genes should be included in further coming studies in order to demonstrate a direct relationship among the resistance level of INH and/or R and the activation of specific genes.

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