Research Article

Isolation and Characterization of Antimycobacterial Compounds from Fruits of *Aegle marmelos* (L.) Correa

Suja KP[1], Leny Jose[2], Divya Lakshmanan[3], Anu G Vidya[4], Reshmi Nair RJ[5], R Ajay Kumar[6]

Abstract

Root extracts of *Aegle marmelos,* an evergreen tree indigenous to India, is an important ingredient in Ayurvedic preparations used for the treatment of pulmonary tuberculosis. Leaves of the tree are used as a remedy for respiratory ailments in folk medicine. The fruit is extensively used in the treatment of various bacterial diseases such as dysentery, diarrhea, etc. The present study was designed to screen organic solvent extracts of fruits of *A. Marmelos* for their inhibitory activity on *Mycobacterium tuberculosis (M. tuberculosis),* and to isolate and identify the compounds responsible for antimycobacterial activity. The minimum inhibitory concentration (MIC) of the extract and purified molecules on virulent laboratory strain *M. tuberculosis* H37Rv was determined by resazurin microtiter assay (REMA). Structural elucidation of the compounds was carried out employing NMR. Cytotoxicity of the compounds was evaluated against human monocyte THP1-derived macrophages by MTT assay. The hexane extract of *A. marmelos* fruits inhibited the growth of *M. tuberculosis* at an MIC of 50 µg/mL. Fractionation of the hexane extract by column chromatography led to the isolation of imperatorin, β-sitosterol, plumbagin, marmesin, marmin, and stigmasterol which individually inhibited the growth of *M. tuberculosis*. *In vitro* cytotoxicity assay revealed that the compounds are not cytotoxic at the concentrations that inhibited the growth of *M. tuberculosis*.

Keywords: Imperatorin, β-sitosterol, Plumbagin, Marmesin, Marmin, Stigmasterol

Abbreviations

MIC, Minimum Inhibitory Concentration; NMR, Nuclear Magnetic Resonance; THP1, Tamm-Horsfall Protein 1; MTT, Methylthiazoltetrazolium; HIV/AIDS, Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome; TLC, Thin Layer Chromatography; HPLC-High Performance Liquid Chromatography; DMSO, Dimethysulfoxide.

Introduction

Tuberculosis (TB) is more prevalent in the world today than at any other time in the human history. *Mycobacterium tuberculosis,* the pathogen responsible for TB, uses diverse strategies to survive in a variety of host lesions and to evade

[1,4,5]Natural Products Division, Corporate R&D Centre, HLL Lifecare Limited, Thiruvananthapuram 695017, Kerala, India.
[2,3,6]Mycobacterium Research Laboratory, Tropical Disease Biology Division, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, Kerala, India.

Correspondence: Dr. R Ajay Kumar, Tropical Disease Biology Division, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, Kerala, India.

E-mail Id: rakumar@rgcb.res.in

Orcid Id: http://orcid.org/0000-0002-1063-7158

How to cite this article: Suja KP, Jose L, Lakshmanan D et al. Isolation and Characterization of Antimycobacterial Compounds from Fruits of *Aegle marmelos* (L.) Correa. *J Commun Dis* 2017; 49(4): 32-38.

Digital Object Identifier (DOI): https://doi.org/10.24321/0019.5138.201732

ISSN: 0019-5138

immune surveillance. One-third of the world population is infected with the bacterium.[1] According to World Health Organization estimates, there were 10.4 million new TB cases and 1.4 million deaths in 2015.[2] Compared to many other diseases, the number of drugs available for the treatment of TB are very limited. Moreover, the emergence and spread of multidrug-resistant and extensively drug-resistant tuberculosis (MDR and XDR-TB), accelerated by the HIV/AIDS pandemic, is now posing a serious threat to global TB control programs.[3] The recent emergence of totally drug-resistant (TDR) TB is a warning of how *M. tuberculosis* can continuously evolve to become resistant to any drug used in therapy.[4] However, until effective vaccines or novel treatment methodologies are introduced, drugs will remain the mainstay of TB management. Thus there is an urgent need to discover and develop new anti-TB molecules, particularly to combat drug-resistant TB. New drugs that are cheap, effective, less toxic, and capable of bringing down the duration of treatment would greatly benefit global TB control programs.

Medicinal plants have been used for centuries all over the world by traditional medicinal systems such as Ayurveda of India for curing diseases. Plant-derived molecules are a source of novel therapeutics to treat TB.[5] India is one of the few countries which has an incredible wealth of medicinal plants and vast traditional knowledge on the use of herbal products to cure various diseases. A number of Indian plants have been shown to possess antimycobacterial activity.[6] In an earlier study from our laboratory, we have shown that ethyl p-methoxycinnamate (EPMC) isolated from *Kaempferia galanga* possesses inhibitory activity against clinical isolates of drug-resistant *M.tuberculosis in vitro*.[7]

Aegle marmelos (L.) Correa belongs to the family *Rutaceae* and is commonly known as bale tree in India and golden apple in English. It is used in Ayurveda for various ailments. Antioxidant, antidiabetic, anticancer, antihyperlipidemic, anti-inflammatory, antimicrobial, antispermatogenic effects of the crude extracts of this plant have been extensively studied.[8] Phytochemistry and medicinal uses of the fruit have been reviewed extensively.[9] The root is a major ingredient in many Ayurvedic preparations prescribed for the treatment of TB.[10] The crude extract of leaf of this plant is also proven to be inhibitory to *M. tuberculosis.*[11] The traditional Siddha medicine uses a confection called 'ilakam' which is made from the fruits of this tree for the treatment of TB.[12] In the present study, we evaluated the antimycobacterial activity of *A. marmelos* fruit extract and compounds isolated from it against virulent *M. tuberculosis*.

Materials and Methods

General

TLC plates (pre-coated silica gel 60 F_{254}), solvents (HPLC and analytical grade) and chemicals were purchased from Merck, India. ^{1}H and ^{13}C NMR spectra were recorded on AV500 NMR spectrometer (^{1}H) and 125 MHz (^{13}C). Chemical shifts were recorded in ppm (parts per million) in $CDCl_3$ with TMS as the internal reference.

Plant Material

Ripe fruits of *A. marmelos (L.) Corrêa,* commonly known as bale, were obtained from the Ayurvedic Research Institute, Thiruvananthapuram, Kerala, where they were identified and authenticated and the voucher specimen HLL/01/2013 was deposited at the Corporate R&D Centre, HLL Lifecare Limited.

Extraction and Isolation

Whole fruits were cleaned, dried under shade, and powdered, and 24 g of the powder was subjected to pressurized sequential extraction using accelerated solvent extractor (ASE 150, Dionex Inc., USA). The material was mixed with diatomaceous earth at 4:1 ratio and the mixture was placed into a sample cell (100 mL) and loaded onto the system. Extraction was performed under pressure (1500 psi) at 100°C with a flush volume of 60% using two static cycles and sequentially using HPLC grade n-hexane, chloroform and methanol. The process was repeated several times and 500 g of sample was extracted to get the required amount of extracts. The solvents were then evaporated in a rotary evaporator (Buchi, Switzerland). The dried extracts were weighed and dissolved in DMF to the desired concentrations and were assayed for antimycobacterial activity.

The hexane extract on further fractionation using silica gel column chromatography (60–120 mesh; 100 g) with n-hexane-ethyl acetate in gradient elution afforded 12 fractions (H1–H12). Six of these fractions were found to be active against *M. tuberculosis* H37Rv. These fractions were subjected to further purification using preparative TLC. The purified compounds were identified using spectroscopic techniques. The structures of the compounds were confirmed based on comparison of their spectral data with those reported in the literature.

Antimycobacterial Activity of *A. marmelos* Fruit extract and Compounds by Resazurin microtitre Assay (REMA)

Resazurin microtitre assay[13] was carried out to analyze the activity of the crude extract as well as the isolated compounds against *M. tuberculosis*. To prepare the inoculum, two 1 µL-loops of bacteria were suspended in 3 mL Middlebrook 7H9 medium (Difco) in sterile 5-mL glass vials containing glass beads. The bacterial suspension was homogenized using an ultrasound water-bath (Elmasonic, Germany) and it was then adjusted to a McFarland turbidity of 1.0 followed by 1:20 dilution in 7H9 medium. From

 ISSN: 0019-5138

this, 100 µl of the culture was added to 100 µl medium containing the extract in 96-well plates. The concentrations of the compound or the extracts were adjusted to get 100, 50, 25, 12.5, 6.25, 3.125 and 1.5 µg/mL by serial dilutions. The plates were incubated statically at 37˚C for 7 days. Growth controls consisted of cultures with DMF alone, and Rifampicin at 1 µg/mL served as positive control. Negative controls contained medium alone. After incubation, 30 µl of 0.02% resazurin (Sigma-Aldrich) solution in water was added and incubated again at 37˚C until the color of the dye changed from blue to pink in the control wells. The minimal inhibitory concentration (MIC) of the extract and the isolated compounds was defined as the lowest concentration that prevented color change of resazurin from blue to pink. All experiments were performed in triplicates.

Cytotoxicity Assay

Cytotoxicity of the compounds was evaluated against human THP1-derived macrophages by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay.[14] Stock solutions of the extracts for the viability assay were prepared in 100% DMSO so that the final concentration of DMSO in the medium did not exceed 0.1% (v/v). THP1 monocytes were seeded at a density of 40,000 cells per well and induced into macrophages using phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) at a final concentration of 20 ng/mL. Various concentrations of the extract and compounds in RPMI medium containing 10% foetal bovine serum (FBS) were added to the 96-well plate. Control wells did not contain the extract or any compound. The cells were incubated at 37˚C in the presence of 5% CO_2. After 24 h, 100 µL MTT (2.5 mg/mL stock) dissolved in RPMI was added and again incubated at 37˚C. After 2 h, 100 µL of lysis buffer (20% SDS in 50% DMF) was added and incubated at 37˚C. As a measure of cell viability, the conversion of MTT into a tetrazolium salt was determined by measuring the absorbance at 570 nm. Percentage viability of the cells was plotted against increasing concentrations of the compounds.

Results

Anti-mycobacterial activity of the Hexane Extract of *A. Marmelos* Fruit

Dried, powdered fruits were extracted with hexane, chloroform and methanol and the yields from the extracts were 3.0, 1.5 and 18.3%, respectively. The anti-mycobacterial activity of these extracts was evaluated against *M. tuberculosis* H37Rv by REMA. We found that the hexane extract and chloroform extract inhibited bacterial growth at 50 µg/mL and methanol extract at 100 µg/mL (Table 1). The hexane extract was selected for further studies.

Identification of Compounds in the Hexane Extract

The hexane extract was sequentially eluted with n-hexane, benzene followed by gradient mixtures of n-hexane-ethyl acetate successively to obtain 12 fractions (H1 to H12). The fraction H2 obtained from benzene separated as powder which was further purified and recrystallized with benzene – MeOH (90:1) into colorless needles (50 mg, mp 100–102˚C). R_f: 0.8 in benzene – EtOAc (9:1) system. This compound (H2) was identified as imperatorin (PubChem CID: 10212). The spectral values correlated with the reported values.[15]

[^{1}H-NMR (500 MHz, $CDCl_3$): 7.77 (d, 1H, J=9.5 Hz), 7.69 (d, 1H, J=2 Hz), 7.36(s, 1H), 6.81 (d, 1H, J=2 Hz), 6.37(d, 1H, J=9.5 Hz), 5.62 (t, 1H, J=7 Hz), 5.01(d, 2H, J=7 Hz), 1.74 (s, 3H), 1.72 (s, 3H)].

^{31}C NMR: 146.98, 107.07, 160.90, 115.05, 116.85, 113.5, 125.87, 148.97, 126.87, 140.13, 70.48, 120.11, 132.07, 18.43, 26.13.

The hexane extract on further fractionation using silica gel column chromatography (60–120 mesh; 100g) with n-hexane-ethyl acetate in gradient elution afforded eight fractions (H3 to H12). Fraction H3 eluted with n-hexane-ethyl acetate (9:1) gave two compounds, which on further purification with preparative TLC, yielded a white powder which was recrystallized with ethanol into white needles (120 mg) with mp 131°C. The R_f value of this compound is 0.7 (benzene-ethyl acetate; 9:1). This was identified as β-sitosterol (H2A) (PubChem CID: 222284) and the values matched with published reports.[16]

[^{1}H NMR (500 MHz, $CDCl_3$): 5.40 (1H, t, J=4.5Hz), 3.52 (1H, m), 1.01(3H, s), 0.93 (3H, d, J=6.5 Hz), 0.86 (3H, t, J=8.5 Hz), 0.83 (3H, d, J=4.5 Hz), 0.81 (3H, d, J=6.7 Hz)0.68 (3H).

^{31}C NMR: 37.59, 32.01, 72.17, 42.65, 141.11, 122.08, 32.25, 32.01, 50.47, 36.85, 21.42, 40.12, 42.65, 57.11, 26.38, 28.59, 56.39, 36.49, 19.12, 34.28, 26.38, 46.17, 23.40, 12.32, 29.48, 20.16, 19.74, 19.12, 12.32.

The other fraction was purified and recrystallized from MeOH-$CHCl_3$ as white solid. The yield was low (45 mg). This was identified as stigmasterol (PubChem CID: 5280794) (H2B) with an mp of 166–167˚C. The spectral values were comparable with published reports[16] and the values are given below.

[^{1}H-NMR (500 MHz, $CDCl_3$): 3.54 (tdd, 1H, J=6.5, 5, 4.5Hz) 5.35 (t, 1H, J=5Hz), 5.17(1H, m, J=8.5Hz), 5.04 (m, 1H) 0.84 (t, J=7Hz), 0.805 (d, 3H, J=6Hz) , 0.79 (d, 3H, J=6Hz) 0.69 (s, 3H) 1.02 (s, 3H)

^{31}C NMR: 37.25, 31.89, 71.81, 42.21, 140.75, 121.71, 31.88, 31.66, 50.15, 36.51, 21.21. 39.67, 42.30, 56.86, 24.36, 28.92, 55.95, 40.49, 21.07, 138.32, 129.27, 25.41, 12.04, 19.40, 18.97, 12.25].

On elution with 20% ethyl acetate, yellow needles were separated which were recrystallized from $CHCl_3$ (H3). The compound (60 mg) had an R_f value of 0.6 (Hexane-ethyl acetate, 1:1) and the spectral values are given below (mp 76–78°C). The compound was identified as plumbagin (PubChem CID: 10205).[17]

[^{1}H NMR (500 MHz, $CDCl_3$): 2.20 (s, 3H), 6.82 (m, 1H, J=1Hz), 7.27 (m, 1H, J=7.5 Hz) 7.64 (2H, dd, J=5.7 Hz), 11.984 (1H, s)].

^{13}C NMR: 184.80, 149.62, 136.11, 190.28, 161.18, 124.17, 135.46, 119.30, 132.07, 115.13, 16.52.

On further elution H4, H5, H6 were eluted. H5 was eluted at 50% ethyl acetate and it was further purified by preparative TLC in a solvent system of chloroform:methanol (9.5:0.5). This was recrystallized from chloroform:methanol with mp of 189°C and the yield was 10 mg. The compound was identified as marmesin and the spectral values matched with reported values[18] (PubChem CID: 334704).

^{1}H NMR (CDCl3, 500 MHz): 7.78 (1H, d, J=9.5 Hz), 7.40 (1H, s), 6.83 (1H, d), 6.39 (1H, d, J=9.5 Hz), 4.60 (2H, m, J=3.5 Hz), 3.33 (1H, t, J=5.5 Hz), 1.35 (3H, s), 1.28 (3H, s).

^{13}C NMR: 160.38, 148.33, 146.83, 144.34, 131.47, 126.01,116.52, 114.82, 106.82, 77.28, 72.48, 61.35, 24.56, 18.87

H6 was further purified by preparative TLC (benzene-ethyl acetate; 1:1) and the fraction was identified as marmin[19] (PubChem CID: 6450230). $C_{19}H_{26}O_5$ with mp of 124°C. The spectral values were comparable with reported values[19] and values are given below.

[^{1}H-NMR, 300MHz ($CDCl_3$): 6.25 (d,1H, J=9Hz) 7.64 (d, 1H, J=9.5Hz)), 7.37 (d, 1H, J=8.5Hz)), 6.86 (d, 1H, J=2Hz), 6.82 (d, 1H)), 4.61(q, 1H, J=7Hz), 5.38 (m, 1H), 2.34 (m, 2H, J=11Hz)), 2.03 (2H, s due –OH), 1.66 (m, 2H), 1.76 (s,3H), 1.30 (s, 3H), 1.25 (s, 3H).

The yield of marmin was relativelylow (6 mg).

Anti-TB Activity of the Compounds

Among the isolated compounds, plumbagin showed the highest anti-TB activity with an MIC value of 12.5 µg/mL followed by imperatorin, marmin and β-sitosterol (MIC: 25 µg/mL – for each). Stigmasterol and marmesin were active at a higher concentration of 100 µg/mL (Table 1).

Table 1.Inhibitory Activity of the Extract and Compounds Isolated from the Fruit of *A. marmelos* against *M. tuberculosis* H37Rv

No.	Extract/compound	MIC (µg/mL)
1	Hexane extract	50
2	Chloroform extract	50
3	Methanol extract	100
4	Plumbagin	12.5
5	Imperatorin	25
6	Stigmasterol	100
7	Marmesin	100
8	Marmin	25
9	β-sitosterol	25

Cytotoxicity Assay

The cytotoxicity of the purified compounds against THP1-derived macrophages was analyzed by MTT assay. Plumbagin was found to be cytotoxic to THP1-derived macrophages at concentrations above 25 µg/mL. β-sitosterol inhibited the growth of macrophages at 100 µg/mL. None of the other compounds showed cytotoxicity even at the highest concentration tested (100 µg/mL) (Fig. 1).

The cytotoxic effect of the isolated compounds in THP1-derived macrophage cell lines was analyzed by MTT assay after 24 h of treatment. The data is represented as percentage viability compared to control (untreated cells) Mean±SEM, n=3.

ISSN: 0019-5138

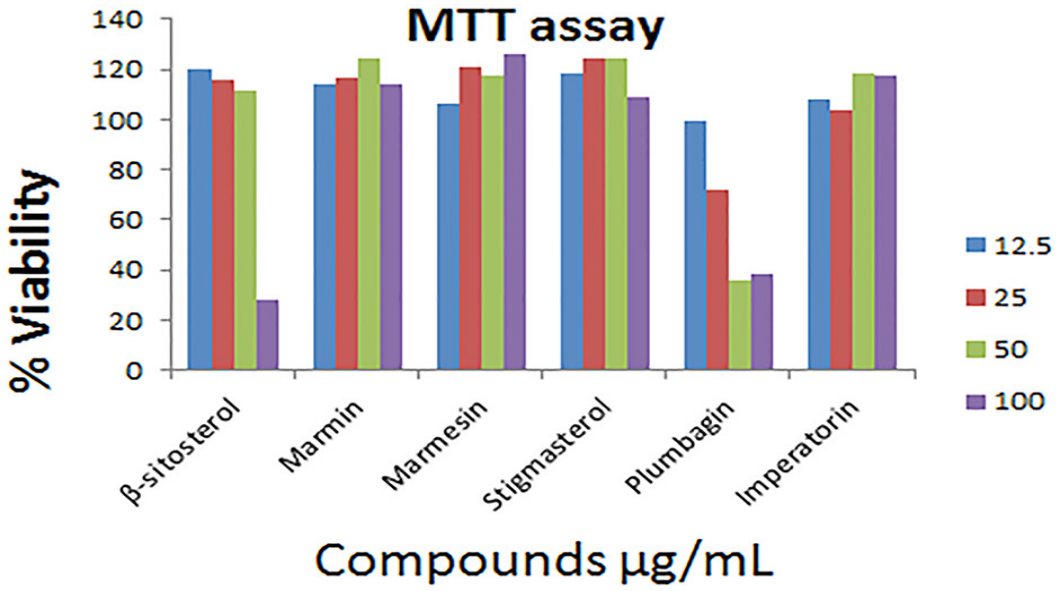

Figure 1.Determination of Cytotoxicity of the Purified Compounds by MTT Assay

Discussion

The plant kingdom can be considered as an important source of new drugs for the treatment of tuberculosis because of its enormous chemical diversity.[6] In an earlier study from our laboratory, we have shown that ethyl *p*-methoxycinnamate (EPMC) isolated from *Kaempferia galanga* inhibited *M. tuberculosis* H37Ra, H37Rv, drug-susceptible and multidrug-resistant clinical isolates *in vitro.*[7]

Traditionally, the ripe and unripe fruits, roots and leaves of *A. marmelos* have been used against various diseases. In Ayurveda, the ripe fruit has been used to treat chronic diarrhoea and dysentery, and also as a tonic for the heart and brain. The effectiveness of *A. marmelos* fruit in diarrhoea and dysentery has been mentioned in ancient Indian medical texts[20] and the British pharmacopoeia.[21] A decoction of the root has been used to treat melancholia, intermittent fevers and palpitation; the roots have mainly been used as an ingredient of the Ayurvedic concoction "Dashmula". The root extract of *A. marmelos* is also included in medications prescribed for TB treatment – Dashmuladhikashayam, Bilwamithradhikashayam.[10]

The leaf extracts of the plant were found to possess moderate activity against *M. tuberculosis* but the active principles were not isolated and identified.[22] However, there are very few reports on the anti-mycobacterial activities of the fruit extract of this plant. Recently, hexane extracts of the leaves were found to possess anti-TB activity at an MIC of 50 µg/mL.[11] In this study, for the first time, we report the inhibitory effect of the hexane extract of *A. marmelos* fruit on virulent *M. tuberculosis.* Six compounds responsible for the anti-mycobacterial activity were identified as imperatorin, β-sitosterol, stigmasterol, plumbagin, marmesin and marmin.

Plumbagin, a naphthaquinone derivative, is a well-known molecule which exerts anticancer, anti-proliferative and chemopreventive properties.[23,24] In addition, plumbagin possesses anti-inflammatory and growth-modulatory effects.[25] It shows inhibitory activity against the Gram-positive bacteria *S. aureus* at (MIC 1.56 µg/mL) and yeast *C. albicans* (MIC 0.78 µg/mL).[26] Several studies have already reported the anti-TB activity of plumbagin. Plumbagin isolated from *Plumbago zeylanica* has an MIC of 12.5 µg/mL.[27] From our assay, we found that the MIC of plumbagin isolated from *A. marmelos* also had an MIC of 12.5 µg/mL against *M. tuberculosis* H37Rv.

Imperatorin, a widely researched furanocoumarin possesses antibacterial activity against Gram-positive and negative bacteria including *S. aureus,* MRSA and *Shigella dysenteriae.*[28] On the contrary, imperatorin isolated from *Balsamo citruscamerunensis* when tested against *E. aerogenes , K. pneumonia , P. stuartii, P. aeruginosa* did not exhibit any inhibitory activity and was found to be active only against *E. coli* at a high concentration of 512 µg/mL.[29] It shows excellent anti-listerial activity with an MIC of 15.62 µg/mL against *L. monocytogenes.*[30] Imperatorin is also reported to inhibit HIV-1 replication.[31] The diverse biological activities of imperatorin have been reviewed in detail.[32] Though it shows cytotoxic effects on tumor cell lines like HL-60 (human leukemia), it is not cytotoxic to PBMCs.[33] It showed no activity at concentrations up to 1.9 mM against several species of rapidly growing mycobacteria, such as *M. abscesus, M. aurum, M. fortuitum, M. phlei* and *M. smegmatis.*[34] Our study showed that this compound is selectively inhibitory to slow growing *M. tuberculosis* at a concentration of 25 µg/mL.

Though the anti-allergic effect of marmin was studied for its effects on histamine release from rat mast cells induced

by histamine stimulants, the anti-mycobacterial activity of marmin and marmesin against virulent *M. tuberculosis* has not been reported so far.[35]

Stigmasterol isolated from the methanolic extract of *Aegle marmelos* leaves shows anti-bacterial activity against *S. aureus, E. coli, P. aeruginosa* and *S. thyphimurium* when tested by zone inhibition assay.[36] Stigmasterol has an MIC of 100 µg/mL[37] and 1:1 mixture of stigmasterol and sitosterol has an MIC of 12.5 µg/mL against *M. tuberculosis.*[38]

To our knowledge, this is the first study reporting the anti-tubercular activity of the hexane extract of the fruit of *A. marmelos* and we have identified six active principles in it which inhibited the growth of the virulent strain of *M. tuberculosis*. A recent study which analyzed the anti-TB activity of the n-butanol extract of *A. marmelos* fruit pulp has also discovered marmin as one of the active compounds against avirulent *M. tuberculosis* H37Ra and *M. bovis.*[39] None of the compounds from our study showed cytotoxicity to THP1-derived macrophages at the concentrations that inhibited *M. tuberculosis.*

Conclusions

The present study confirms the anti-mycobacterial activity of six molecules of *Aegle marmelos* fruit against the virulent strain of *M. tuberculosis* H37Rv *in vitro*, which corroborates the use of the fruit in the treatment of TB in Ayurveda, the traditional system of medicine in India.

Acknowledgments

SKP thanks Department of Science and Technology, New Delhi, Government of India, for financial support. RAK thanks Department of Biotechnology, New Delhi, and Government of India for financial support. LJ and DL thank Council of Industrial and Scientific Research (CSIR), New Delhi, for senior research fellowship. We thank Dr. Jayakumar, senior research officer, Government Ayurveda Research Institute, Poojappura, Thiruvananthapuram, for authenticating the plant. We gratefully acknowledge National Institute of Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram for carrying out the NMR analyses.

Conflict of Interest: None

References

1. Zumla A, Atun R, Maeurer M et al. Viewpoint: Scientific dogmas, paradoxes and mysteries of latent Mycobacterium tuberculosis infection. *Tropical Medicine & International Health* 2011; 16(1): 79-83.
2. World Health Organization, Global tuberculosis report, 2016. Geneva: *World Health Organization* 2016. http://www.int/tb/publication/global.
3. Getahun H, Gunneberg C, Granich R et al. HIV infection-associated tuberculosis: The epidemiology and the response. *Clinical Infectious Diseases*, 2010; 50(Supplement 3), S201-S207.
4. Velayati AA, Farnia P, Masjedi MR. Letter to Editor: The totally drug resistant tuberculosis (TDR-TB). *Int J Clin Exp Med* 2013; 6(4): 307-09.
5. Nguta JM, Appiah-Opong R, Nyarko AK et al. Current perspectives in drug discovery against tuberculosis from natural products. *International Journal of Mycobacteriology* 2015; 4(3): 165-83.
6. Gautam R, Saklani A, Jachak SM. Indian medicinal plants as a source of antimycobacterial agents. *Journal of Ethnopharmacology* 2007; 110(2): 200-34.
7. Lakshmanan D, Werngren J, Jose L et al. Ethyl p-methoxycinnamate isolated from a traditional anti-tuberculosis medicinal herb inhibits drug resistant strains of Mycobacterium tuberculosis in vitro. *Fitoterapia* 2011; 82(5): 757-61.
8. Rahman S, Parvin R. Therapeutic potential of Aegle marmelos (L.) – An overview. *Asian Pacific Journal of Tropical Disease* 2014; 4(1): 71-77.
9. Baliga MS, Bhat HP, Joseph N et al. Phytochemistry and medicinal uses of the bael fruit (Aegle marmelos Correa): A concise review. *Food Research International*, 2011; 44(7): 1768-75.
10. Sharma DR, Sharma DS. Sahasrayogam, re-edited. *Delhi: Chowkhambha Sanskrit Pratisthan* 2004.
11. Kaur R, Kaur H. Antitubercular activity and phytochemical screening of selected medicinal plants. *Orient J Chem* 2015; 31(1).
12. Ramachandran J. Herbs of Siddha medicine/The first 3D book on herbs. *Murugan PPatthipagam, Chennai, India* 2008; 156.
13. Palomino JC, Martin A, Camacho M et al. Resazurinmicrotiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy* 2002; 46(8): 2720-22.
14. Van de Loosdrecht AA, Beelen RHJ, Ossenkoppele GJ et al. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of immunological methods* 1994; 174(1-2): 311-20.
15. Ghosh K. A furocoumarin, Imperatorin isolated from Urenalobata L. (Malvaceae). *Molbank* 2004; 2004(1), M382.
16. Chaturvedula VSP, Prakash I. Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of Rubus suavissimus. *International Current Pharmaceutical Journal* 2012; 1(9): 239-42.
17. Raj G, Kurup R, Hussain AA. Distribution of naphthoquinones, plumbagin, droserone, and 5-O-methyl droserone in chitin-induced and uninduced Nepenthes khasiana: molecular events in prey capture.

Journal of Experimental Botany 2011; 62(15): 5429-36.
18. Chatterjee MA, Dutta CP, Bhattacharyya MS et al. The structure of marmin. *Tetrahedron Letters* 1967; 8(5): 471-73.
19. Jain M, Trivedi A, Mishra SH. TLC determination of marmesin, a biologically active marker from Feronia Limonia L. *American Journal of Plant Sciences* 2010; 1(01): 12.
20. Joshi PV, Patil RH, Maheshwari VL. In vitro antidiarrhoeal activity and toxicity profile of Aeglemarmelos Correa ex Roxb. dried fruit pulp. *Natural Product Radiance* 2009; 8(5): 498-502.
21. Chopra RN, Chopra IC. *Indigenous drugs of India*. *Academic Publishers* 1933.
22. Tawde KV, Gacche RN, Pund MM. Evaluation of selected Indian traditional folk medicinal plants against Mycobacterium tuberculosis with antioxidant and cytotoxicity study. *Asian Pacific Journal of Tropical Disease* 2012; 2 (Supplement 2): S685-S691.
23. Hazra B, Sarkar R, Bhattacharyya S et al. Synthesis of plumbagin derivatives and their inhibitory activities against Ehrlich ascites carcinoma in vivo and Leishmania donovani promastigotes in vitro. *Phytotherapy Research* 2002; 16(2): 133-37.
24. Srinivas P, Gopinath G, Banerji A et al. Plumbagin induces reactive oxygen species, which mediate apoptosis in human cervical cancer cells. *Molecular Carcinogenesis* 2004; 40(4): 201-11.
25. Checker R, Sharma D, Sandur SK et al. Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes. *International immunopharmacology* 2009; 9(7): 949-58.
26. Paiva SRD, Figueiredo MR, Aragão TV et al. Antimicrobial activity in vitro of plumbagin isolated from Plumbago species. *Memorias do Instituto Oswaldo Cruz* 2003; 98(7): 959-61.
27. Mossa JS, El-Feraly FS, Muhammad I. Antimycobacterial constituents from Juniperus procera, Ferula communis and Plumbago zeylanica and their in vitro synergistic activity with isonicotinic acid hydrazide. *Phytotherapy Research* 2004; 18(11): 934-37.
28. Rosselli S, Maggio A, Bellone G et al. Antibacterial and anticoagulant activities of coumarins isolated from the flowers of Magydaris tomentosa. *Planta medica* 2007; 73(02): 116-20.
29. Fouotsa H, Mbaveng AT, Mbazoa CD et al. Antibacterial constituents of three Cameroonian medicinal plants: Garcinia nobilis, Oricia suaveolens and Balsamocitrus camerunensis. *BMC Complementary and Alternative Medicine* 2013; 13(1): 81.
30. Rahman A, Na M, Kang SC. Antilisterial potential of imperatorin and limonin from Poncirus trifoliata Rafin. *Journal of Food Biochemistry* 2012; 36(2): 217-23.
31. Sancho R, Márquez N, Gómez-Gonzalo M et al. Imperatorin inhibits HIV-1 replication through an Sp1-dependent pathway. *Journal of Biological Chemistry* 2004; 279(36): 37349-59.
32. Ulazka B, Głowniak K, Sieniawska E. Imperatorin-pharmacological effects and possible implication in pharmacotherapy. *Curr Issues Pharm Med Sci* 2012; 25(1): 80-87.
33. Yang LL, Wang MC, Chen LG. Cytotoxic activity of coumarins from the fruits of Cnidium monnieri on leukemia cell lines. *Planta medica* 2003; 69(12): 1091-95.
34. Schinkovitz A, Gibbons S, Stavri M et al. Ostruthin: an antimycobacterial coumarin from the roots of Peucedanum ostruthium. *Planta medica* 2003; 69(04): 369-71.
35. Nugroho AE, Riyanto S, Sukari MA et al. Anti-allergic effects of Marmin, a coumarine isolated from Aegle marmelos Correa: In vitro study. *International Journal of Phytomedicine* 2011; 3(1): 84.
36. Edilu A, Adane L, Woyessa D. In vitro antibacterial activities of compounds isolated from roots of Cayluseaabyssinica. *Annals of Clinical Microbiology and Antimicrobials* 2015; 14(1): 15.
37. Nyila MA, Leonard CM, Hussein AA et al. Activity of South African medicinal plants against Listeria monocytogenes biofilms, and isolation of active compounds from Acacia karroo. *South African Journal of Botany* 2015; 78: 220-27.
38. Navarro-García VM, Luna-Herrera J, Rojas-Bribiesca MG et al. Antibacterial activity of Aristolochia brevipes against multidrug-resistant Mycobacterium tuberculosis. *Molecules* 2011; 16(9): 7357-64.
39. Chinchansure AA SNH, Arkile M, Sarkar D et al. Antimycobacterium activity of coumarins from pulp of *AegleMarmelos* (L.) Correa. *International Journal of Basic and Applied Chemical Sciences* 2015; 5 (3) (July-Sep): 39-44.

Date of Submission: 2017-10-23
Date of Acceptance: 2017-11-28