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SYNTHESIS AND STUDY OF ANTITUBERCULAR ACTIVITY OF PLUMBAGIN ANALOGS

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ABSTRACT

Tuberculosis has been a serious public health problem for a long time. With the advent of anti-tubercular antibiotics in the late 1940s, the battle against tuberculosis seemed to have been won but the multi-drug resistance; re-infection and latent infection have become the major cause of concern for the treatment of tuberculosis all over the world. In the present study derivatives of Plumbagin, obtained from *Plumbago zeylanica* (Family-*Plumbaginaceae*) have been synthesized. Out of the various analogs synthesized, the antitubercular activity of compound 1,2 and 3 was evaluated using standard $H_{37}Rv$ and S,H,R and E sensitive *M. tuberculosis* strains using LRF assay method. Compound 1 showed strongest activity against both standard $H_{37}Rv$ and S,H,R and E sensitive *M. tuberculosis* strains as compared to standard Rifampicin. The other compounds are proved to be more active against standard $H_{37}Rv$ and S,H,R and E sensitive *M.tuberculosis* strain as compared to Rifampicin.

KEYWORDS: **Plumbagin, *Streptomycin(S), Isoniazid(H), Rifampicin(R),Ethambutol(R)***

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INTRODUCTION

Tuberculosis is one of the major killer diseases of modern times. Tuberculosis is defined as an airborne bacterial disease that generally affects the lungs of human body, though it can also affects the kidney, spine and brain[1]. The causative organism of tuberculosis is *Mycobacterium tuberculosis*. Other related bacteria such as *Mycobacterium bovis*, *Mycobacterium smegmatis or Mycobacterium africanum*, can occasionally cause tuberculosis[2]. In India, Tuberculosis continues to remain one of the most pressing health problems. India is the highest tuberculosis burden country in the world, accounting for one fifth of the global incidence - an estimated 2.0 - 2.5 million cases annually. In 2011, approximately 8.7 million new cases of tuberculosis and 1.4 million people die from tuberculosis each year worldwide.[2]

The various forms of tuberculosis include[3]:

1. *Multidrug-resistant tuberculosis (MDR-TB) is caused by organisms that are resistant to the most effective anti-tubercular drugs (isoniazid and rifampicin).* Multidrug-resistant tuberculosis results from either infection with organisms which are already drug-resistant or may develop in the course of a patient's treatment.[4]
2. *Extensively drug-resistant tuberculosis (XDR-TB) is a form of tuberculosis caused by organisms that are resistant to isoniazid and rifampicin (i.e. MDR-TB) as well as any fluoroquinolone and any of the second–line anti-tubercular injectable drugs (amikacin, kanamycin or capreomycin).*
3. *People with advanced HIV infection are vulnerable to a wide range of infections and malignancies that are called 'opportunistic infections'. These infections are called 'opportunistic' because they take advantage offered by a weakened immune system. Tuberculosis is one such HIV-related opportunistic infection.* A person that has both HIV and active tuberculosis is said to have an AIDS defining illness.[5]

The multi-drug resistance, reinfection and latent infection have become the major cause of concern for the treatment of tuberculosis in all over the world. This together with the problem of the interactions of the current tuberculosis drugs with the antiretroviral drugs taken by HIV positive people, means that there is an urgent need for new tuberculosis drugs.

Plumbagin is a naphthoquinone analog (2-methyl-5-hydroxy-1,4-naphtoquinone) inhibits the menaquinone pathway which is essential for the growth of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Esters of 5-hydroxyl group of Plumbagin have been reported to have activity against Mycobacterium species.[6]

MATERIALS AND METHODS

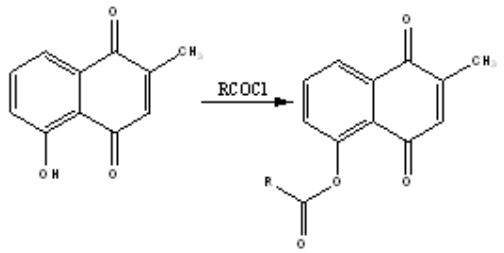
Materials

Plumbagin was purchased from Sigma-Aldrich, Germany. Ortho-toluoyl chloride, Meta-toluoyl chloride, Para-toluoyl chloride, 1-Naphtholyl chloride, 2-Naphtholyl chloride, Hexanoyl chloride and Pyrazinamide were purchased from Sigma-Aldrich, Germany. Isoniazid and Ethionamide were gift samples from Themis medicare. The uncorrected melting points were taken in open glass capillaries. The IR spectra were recorded on a Cary 630 *FTIR* Spectrometer. The 1H NMR spectra have been recorded on Brucer 400 Avance Fourier Transform Spectrometer operating at 400 mega hertz in deutirated dimethylsulfoxide (DMSO-d6) with all shifts referred to internal tetramethylsilane (TMS). The mass spectra were recorded on a LCMS Agilent Technology model 6520. All other reagents used in synthesis as well as analysis were of synthetic grade and analytical grade respectively.

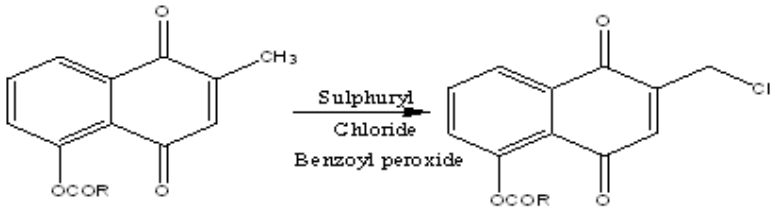
Experimental

Plumbagin derivatives have been synthesized as per the following scheme given below-

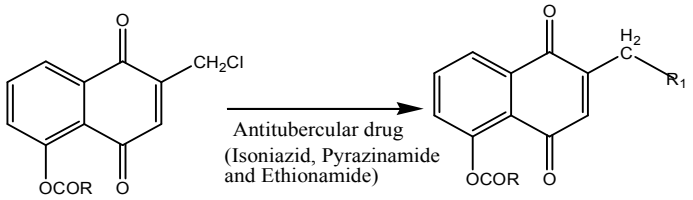
a. Treatment with Acyl chloride



b. Chlorination of Methyl group of Plumbagin



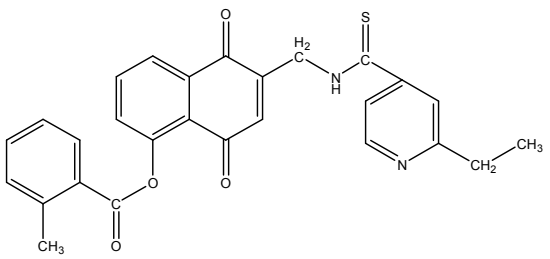
c. Condensation with Anti-tubercular drugs



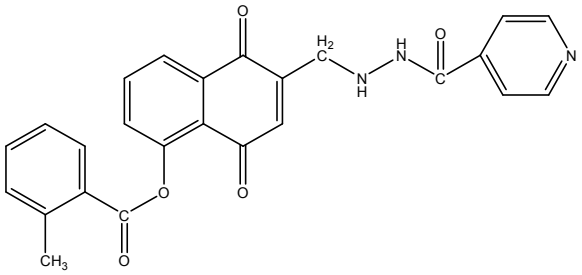
Where R = Ortho-toluoylchloride, Meta-toluoylchloride, Para-toluoylchloride

R_1= Ethionamide, Isoniazid, Pyrazinamide

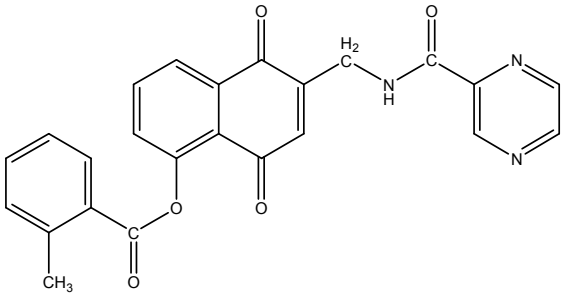
Compound 1
Ortho-toluoylchloride-Ethionamide complex



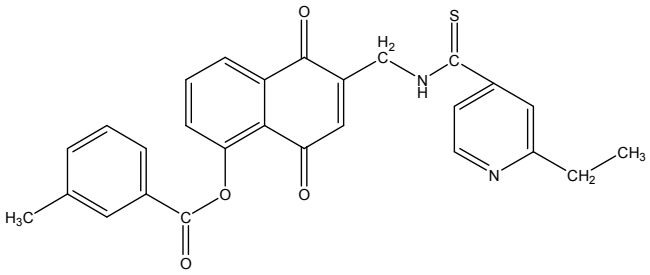
Compound 2
Ortho-toluoylchloride-Isoniazid complex



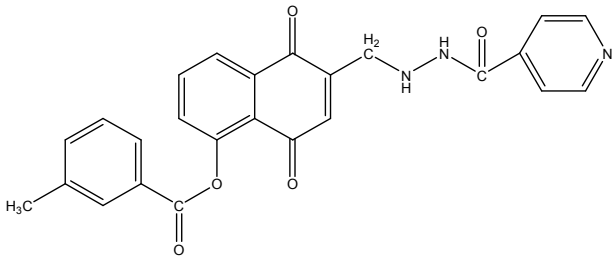
Compound 3
Ortho-toluoylchloride-Pyrazinamide complex



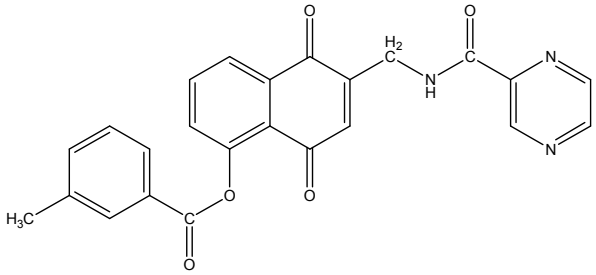
Compound 4
Meta-toluoylchloride-Ethionamide complex



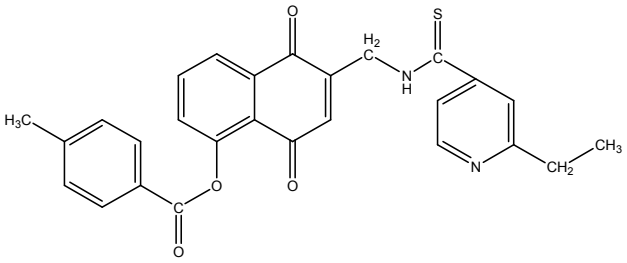
Compound 5
Meta-toluoylchloride-Isoniazid complex



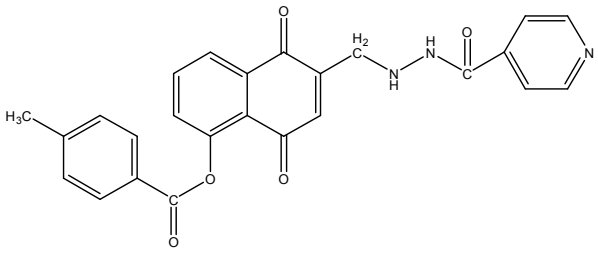
Compound 6
Meta-toluoylchloride-Pyrazinamide complex



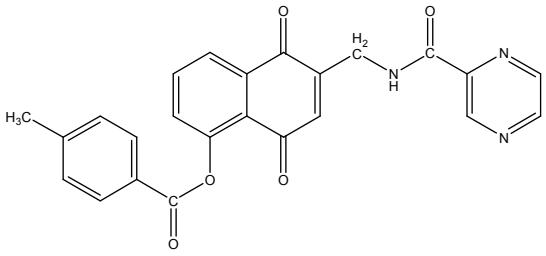
Compound 7
Para-toluoylchloride-Ethionamide complex



Compound 8
Para-toluoylchloride-Isoniazid complex



Compound 9
Para-toluoylchloride-Pyrazinamide complex



The steps involved in synthesis of plumbagin derivatives included:

Step1. Plumbagin was dissolved in dichloromethane and pyridine at 0°C. Solution was stirred for approximate 5 minutes. To this solution, added acid chlorides dropwise to the reaction mixture at 0°C. Reaction mixture again stirred for 3 hrs. at room temperature. After 3 hrs, reaction mixture was diluted with dichloromethane and washed with water and brine solution. Products were separated using separating funnel. Oily product obtained.

Step2. Taken step 1 product dissolved it in ethanol. To this solution, sulfuryl chloride and benzoyl peroxide were added. Stirred for 2 hrs at room temperature and dried it in oven. Step3. Dissolved step 2 product in ethanol and added anti-tubercular drug. Refluxed for 3 hrs and dried it in an oven.

Compound1

2-(2'-thylisonicotinoylthiamido)-methyl-5-(*o*-toluoyl)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.55 (Silica Gel G, n-hexane: EtOAc, 10:2); IR (cm^{-1}) 3331, 2992, 1760,1639, 1540 ; (400 MHz, DMSO-d6), H ppm: 8.21-8.02 (1H, d, J = 11.2 Hz), 8.07-8.06 (1H, t, J= 4),7.87-7.85 (1H, d, J= 8), 7.43-7.41 (1H, t, J= 8), 7.23-7.13(1H, d, J=7.7 Hz), 7.01 (1H, s), 3.75-3.69 (3H, q, J=3.18 Hz), 1.28-1.24 (3H, d, J = 2.28 Hz), , m⁄z 471(M+).

Compound 2

2-(2'-Isonicotinoylhydrazino)-methyl-5-(*o*-toluoyl)-oxy-1,4-naphthoquinone

This was synthesized using the above scheme. R_f 0.51 (Silica Gel G, hexane: EtOAc, 10:2); IR $(cm)^{-1}$ 3075, 2985, 1687, 1605, 1192 (400 MHz, DMSO-d6), H ppm 8.06-7.97(1H, d, J = 5.64 Hz), 7.72-7.70 (2H,d, J= 8),7.55-7.46 (1H, m, J = 7.02Hz), 7.44-7.38 (3H, t, J= 24), 7.23-7.21 (3H, t, J= 8), 7.20-7.14 (2H, d, J= 24), 3.75-3.73(2H,d, J=0.096 Hz), 1.06(3H, s); m⁄z 442(M+).

Compound 3

2-(Pyrazinecarboxamido)-methyl-5-(*o*-toluyol)-oxy-1,4-naphthoquinone (55)This was synthesized using the above scheme. R_f 0.46 (Silica Gel G, hexane: EtOAc, 10:2); IR $(cm)^{-1}$ 3417, 2691, 1760, 1678, 1579, 1175; (400 MHz, DMSO-d6), H ppm: 9.15 (1H, s), 8.77-8.69 (2H, d, J = 4.86 Hz), 7.76-7.48 (2H, d, J=16.6), 7.46-7.42 (2H, d, J= 16), 7.40-7.38 (3H, t, J= 8), 7.26-7.24 (3H, t, J= 8), 7.20-7.07 (2H, d, J= 52), 3.58 (3H, s), 2.47(3H, m); m⁄z 428(M+).

Compound 4

2-(2'-Ethylisonicotinoylthiamido)-methyl-5-(*m*-toluoyl)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.53 (Silica Gel G, n-hexane: EtOAc, 10:2); IR (cm^{-1}) 3298, 2985, 1698, 1492 ; (400 MHz, DMSO-d6), H ppm: 8.62-8.59(2H, m, J=1.2), 8.10-8.06 (2H, t, J = 2.1 Hz), 7.91-7.89 (2H, d, J=), 7.70-7.68 (2H, d, J=), 7.46-7.44 (2H, d, J=)7.10-7.08 (1H, d, J = 1.2 Hz), 6.95 (1H, s), 3.03-2.98 (2H, J = 3 Hz); m⁄z 471(M+).

Compound 5

2-(2'-Isonicotinoylhydrazino)-methyl-5-(*m*-toluoyl)-oxy-1,4-naphthoquinone

This was synthesized using above scheme. R_f 0.49 (Silica Gel G, hexane: EtOAc, 10:2); IR (cm^{-1}) 3331, 3033, 1663, 1540, 1499, 1212 (400 MHz, DMSO-d6), H ppm: 8.82–8.79(3H, t, J=2.7 Hz), 7.91-7.89 (2H, d, J= 8), 7.84-7.83 (2H, d, J= 4), 7.46-7.44 (2H, d, J= 8), 3.73-3.68 (2H, q, J = 3.18 Hz), 2.47 (1H, s); m⁄z 442(M+).

Compound 6

2-(Pyrazinecarboxamido)-methyl-5-(*m*-toluyol)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.44 (Silica Gel G, hexane: EtOAc, 10:2); IR (cm^{-1}) 3425, 2928, 1760, 1674, 1587, 1452, 1218; (400 MHz, DMSO-d6), H ppm: 9.15(1H, s), 8.82-8.82 (2H, m, J = 0.36 Hz), 8.63-8.61 (3H, t, J= 8), 7.92-7.90 (2H, d, J= 8), 7.83-7.79 (2H, d, J= 16), 7.69-7.64 (2H, d, J= 20), 6.96 (1H, s), 2.46-2.32 (2H, q , J = 8.7 Hz); m⁄z 428(M+).

Compound 7

2-(2'-Ethylisonicotinoylthiamido)-methyl-5-(*p*-toluoyl)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.54 (Silica Gel G, n-hexane: EtOAc, 10:2); IR (cm^{-1}) 3391, 2967, 1663, 1491, 1175 ; (400 MHz, DMSO-d6), H ppm: 8.77–8.76(1H, m, J=0.78Hz), 7.48–7.44 (3H, t, J = 2.28 Hz), 7.27-7.25 (3H, t, J = 1.2 Hz), 7.08 (1H, s), 2.46 (3H, m); m⁄z 471(M+).

Compound 8

2-(2'-Isonicotinoylhydrazino)-methyl-5-(*p*-toluoyl)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.50 (Silica Gel G, hexane: EtOAc, 10:2); IR (cm^{-1}) 2993, 1700, 1678, 1498, 1058 (400 MHz, DMSO-d6), H ppm: 9.06-9.02(2H, m, J=2.7 Hz), 7.57-7.55 (3H, t, J = 1.62 Hz), 7.90-7.80 (3H, t, J= 40) 2.46-2.33 (2H, m, J = 8.22 Hz); m⁄z 442(M+).

Compound 9

2-(Pyrazinecarboxamido)-methyl-5-(*p*-toluyol)-oxy-1,4-naphthoquinone This was synthesized using above scheme. R_f 0.44 (Silica Gel G, hexane: EtOAc, 10:2); IR (cm^{-1}) 3419, 2878,1702, 1655, 1423, 1259, 1024; (400 MHz, DMSO-d6), H ppm: 9.15(1H, s), 8.82–8.81 (2H, m, J = 0.3Hz), 7.91-7.89 (3H, t, J= 8), 7.83-7.80 (3H, t, J = 1.8 Hz), 6.98 (1H, s), 2.46 (3H, s); m/z 428(M+).

Mycobacterial growth inhibitory assay

Luciferase Reporter Phage (LRF) Assay

Standard strain $H_{37}Rv$, a clinical sensitive *M.tuberculosis* strain and a clinical isolate S,H, R and E sensitive *M.tuberculosis* strain were grown in Middlebrook 7H complete medium with and without extracts of samples for 3 days at 37°C. Luciferase Reporter Phage Assay was done using concentrations of 50 and 100 µg/ml of samples. Rifampicin was included as an assay control and DMSO as the solvent control. LRP phage AETRC21 was added and the samples were incubated for four hours. Equal volume of the cell phage mixture was mixed with 0.3Mm D-Luciferin in 0.05M sodium citrate buffer of pH 4.5 and light output was immediately measured as RLU (Relative light units) in the uminometer at 10 seconds integration. Compounds exhibiting a reduction of 50% or more in RLU in the test vials compared to that of the control were considered to have anti mycobacterial activity. These LRP assays offer an elegant means of detecting viable mycobacteria and provide a rapid tool for drug susceptibility screening.

RESULTS AND DISCUSSION

Plumbagin analogs and its ester were synthesized. Plumbagin has been referred to possess antitubercular activity. Accordingly, various newer analogs have been synthesized and tested for their antitubercular activity. Compounds were synthesized by treating Plumbagin with ortho-toluoylchloride, meta-toluoylchloride and para-toluoylchloride. Further the synthesized Plumbagin derivatives were condensed with Antitubercular drugs-Isoniazid, Pyrazinamide and Ethionamide. IR spectra of synthesized compounds exhibit a band in the region IR spectra confirmed the formation of product. Further, formations of compounds were confirmed by Proton NMR and Mass Spectra. Computational studies (calculating logP values from ChemOffice 2004, logP value directly related to biological activity) revealed that among synthesized compounds ortho-toluoylchloride derivatives of Plumbagin possess best Antitubercular activity. Thus, ortho-toluoylchloride derivatives of Plumbagin synthesized by condensation of Isoniazid, Pyrazinamide and Ethionamide were sent to National Institute for Research in Tuberculosis (ICMR), Chennai for screening of Antitubercular activity. The compounds were screened in both standard $H_{37}Rv$ and clinical isolate S,H,R and E sensitive *M.tuberculosis* strain with taken Rifampicin as a standard drug. Compounds showed better Antitubercular activity as compared to Rifampicin against standard $H_{37}Rv$ *M.tuberculosis* strain while Compounds showed alone best Antitubercular activity as compared to Rifampicin against clinical isolate S,H,R and E sensitive *M.tuberculosis* strain. Out of various compounds, compounds 1(ortho-toluoylchloride-Ethionamide analog of Plumbagin) was found to be most effective in clinical isolate:S,H,R and E sensitive *M.tuberculosis*. (Table 1) and (Table 2).

Table 1

Percentage reductions in relative light units (RLU) by 50µg/ml against $H_{37}Rv$ Standard and Clinical isolate: S,H,R&E sensitive Mycobacterium tuberculosis

Compound code	$H_{37}Rv$ Standard	Clinical isolate: S,H,R&E sensitive
1	62.44	96.80
2	95.05	91.01
3	0	34.96
Rifampicin (2 µg/ml)	97.54	16.93

Table 2
Percentage reductions in relative light units (RLU) by 100µg/ml against $H_{37}Rv$ Standard and Clinical isolate: S,H,R&E sensitive Mycobacterium tuberculosis

Compound code	$H_{37}Rv$ Standard	Clinical isolate: S,H,R&E sensitive
1	98.04	98.92
2	97.56	94.59
3	0	47.21
Rifampicin (2 µg/ml)	97.54	16.93

CONCLUSION

Based on the computational studies, all the synthesized compounds must possess Antitubercular activity. The Antitubercular activity showed that compounds can cure tuberculosis against standard $H_{37}Rv$ *M.tuberculosis* strain but compounds 1 (approx. 98% reduction) have more potential to cure tuberculosis against clinical isolate S,H,R and E sensitive *M.tuberculosis* strain as compared to Rifampicin (approx 16% reduction). Thus, compounds will be beneficial for multidrug resistant tuberculosis (MDR TB) and extensively drug-resistant tuberculosis (XDR TB).

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