Synthesis and screening of Clerodane Diterpene analogues from 16 hydroxycleroda 3,13(14)-Zdien 15,16- olide for potential anti-mycobacterial activity

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Additional Declarations:
Table 1 to 8 are available in the Supplementary Files section.

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Abstract:

In recent years, Clerodane diterpenes, a class of bioactive compounds, have come into the spotlight due to their amazing bioactivities. The "Indian Mast Tree" is a common name for a tall evergreen tree scientifically known as *Polyalthia longifolia var. pendula.* Three novel clerodane diterpene analogues were obtained by synthesizing 16-hydroxycleroda-3,13(14)-Zdien-15,16-olide (Lactone) with primary amines. Anti-tubercular activity was determined using Microplate Alamar Blue Assay. Among all the synthesised compounds from methanolic extract of seeds, results clearly showed that compounds 3 and 5 have significant anti tb activity with an MIC of 1.56 µg/ml against the *Mycobacterium tuberculosis MTB H37Rv* bacilli strain than the gold standard drugs pyrazinamide (3.125 µg/ml), ciprofloxacin (3.125 µg/ml), streptomycin (6.25 µg/ml) and rifampicin (6.25 µg/ml). Antimicrobial activity was also carried out by agar well diffusion method and its minimum inhibitory concentrations (MIC) were determined against Gram +ve (*Staphylococcus aureus)* and Gram -ve (*Escherichia coli)* bacteria. Compound 5 exhibited significant antibacterial activity with Zone of inhibition of 10.8 mm with Gram +ve and 7.95 mm with Gram -ve bacteria at a conc of 50 µg/ml respectively. Furthermore, Compound 5 showed MIC at 5 µg/ml with both the bacterial strains. While tested for anti-fungal activity, Compound 5 showed Zone of inhibition of 10.8mm at conc. of 50 µg/ml. In the current investigation, three novel heterocycles (compounds 3-5) of the diterpenoid (compound 1) were prepared, in high yield, using one-pot, efficient approach. Further studies on *in vivo,* cytotoxic tests, mechanism of action and synthesis of a series of lactones will be used to determine the structural activity relationship of bioactive diterpenoid 1.

Introduction: Clerodane diterpenes, are a large group of secondary metabolites that are widely distributed in plants, fungi, bacteria, and marine sponges. During the last 25 years, over 1300 diterpenoids and nor-diterpenoids with the clerodane carbon skeleton have been isolated [1]. Structurally, the clerodane diterpenes are bicyclic. The basic skeleton is split into two fragments: a fused-ring decaline fraction (C-1-C-10) and a six-carbon side chain at C-9 (C-11-C-16, with C-16 attached at C-13, i.e., 3-methylpentyl). The remaining four carbons (C-17-C-20) are bonded at C-8, C-4, C-5, and C-9, respectively, in the decalin system [1] (Fig 1).

These natural neo-clerodanes have been classified into seven different groups on the basis two fragments, the C-11–C-16 moiety and the decalin moiety [1]. Approximately 25% of clerodanes have a cis ring fusion, and the remaining 75% have a trans ring fusion and may have other stereochemical conformations. In addition, it is susceptible to the migration of methyl and hydride groups [1,2]. The expressive chemical diversity of clerodanes enables the discovery of broad biological activities and guarantees an improvement in the profile concerning efficacy/safety [1,2]. Clerodanes have several therapeutic applications, such as columbin, which has anti-inflammatory and anticancer activity, 6-a-hydroxy-clerode-3,13-dien-15,16-olide, for the treatment of infectious sleeping sickness, and salvinorin A, which entered phase 1 clinical trials for its possible application in the treatment of drug addiction and neuropsychiatric disorders [3].

An incisive knowledge on the chemistry and pharmacological activities of clerodane diterpenes continues to grow rapidly. Clerodane diterpenoids have been found in hundreds of plant species from a number of different families. Several genera from the Verbenaceae and Lamiaceae families have been identified as rich sources of neoclerodane diterpenoids. Clerodane diterpenes identified in several plant species, totaling 54 substances registered and distributed in *Casearia, Polyalthia, Ajuga, Salvia, Croton, Dodonaea, Scutellaria, Gymnosperma, Tessmannia, Excoecaria* and *Nepeta* [10]. Thus, the predominant genres in the research are *Casearia* and *Ajuga*. They exhibit a diverse spectrum of biological activities, such as cytotoxicity [4, 5], immunomodulation [6], anti feedant effects [7], and anti-inflammatory properties [8]. In addition, the most described biological activities for clerodams were anti-cancer, anti-inflammatory, and antimicrobial [10]. The biological and chemical attributes of clerodanes have generated a great deal of interest in the synthesis of these compounds [9].

These diterpenoids, can also frequently be discovered in the *Polyalthia* genus [11-14]. *Polyalthia longifolia var. pendula* is the scientific name for the high evergreen tree that is often commonly referred to as the "Indian Mast Tree." The aqueous plant decoction and the alcoholic extract of the plant have been utilised as medicinal therapies for the treatment of skin problems, helminthiasis, pyrexia, diabetes, and hypertension [15]. It has been demonstrated that this plant produces a variety of distinct kinds of diterpenoids, some of which have cytotoxic [16, 17], antibacterial [18, 19], antifungal [20], and anti-inflammatory [21] activities.

We have chosen to extract diterpenoids from the plant *Polyalthia longifolia var. Pendula*. Lactones with a clerodane skeleton are very interesting because many of them have biological activity. As a continuation of our research on the Mannich bases of 8-hydroxyquinoline and in an effort to determine the structure-activity relationship between the two, 16 hydroxycleroda 3, 13(14)-Zdien 15, 16-olide (Lactone) was subjected to a two-component Mannich reaction in which it was treated with primary amines (Hydrazine hydrate and Hydroxylamine Hcl). This treatment resulted in the formation of a new Ý-lactone rather than a Mannich base. Based on this finding and the fact that numerous primary amines were permitted to remain at room temperature, a reaction was carried out that, utilising a single pot and using no catalyst, produced two more novel lactones [22] in high yield. Like many other classes of compounds, lactones are extraordinarily varied in their chemical structure and biological activity. Additionally, lactones are used as precursors in the production of numerous other chemical substances. Therefore, the purpose of this research is to examine the potential anti-tubercular and antibacterial activity of 16 hydroxycleroda 3,13(14)-Zdien 15,16-olide (Lactone) and its three novel heterocycles against *Mycobacterium tuberculosis,* Gram-positive and Gram-negative pathogens such as *Streptococcus aureus* and *Escherichia coli*.

2. Results and Discussion:

2.1. Chemistry

Using the techniques described earlier [23], the 16-hydroxycleroda 3,13(14) Zdien15,16 olide (1) and the 16-Oxoecleroda-3, 13 (14) E-diene-15 oic acid (2) were successfully extracted from the seeds of methanolic extract of *Polyalthia longifolia var. pendula (Linn*), respectively. The diterpenoid (1), when reacted in one pot with primary amines such as hydrazine hydrate and hydroxylamine HCl, produced three novel amino substituted heterocycles. These reactions took place in 100% ethanol, without any catalyst at room temperature. (Fig 2). Lactones were successfully synthesised under solid phase condition in two hours. The mechanism involves the in-situ formation of a reactive intermediate-A, in which hydroxyl group of acid attacks intra-molecularly with the preparation of six membered heterocycles (Fig 3). Through ^{1}H, ^{13}C, HMBC, COSY NMR spectral data, the structures of all the synthesised lactones have been determined.

Compound 1 and Compound 2 were obtained as a white crystalline powder and Oily yellow gum, m.p. 100-101°C & 168-170˚C and it was examined on TLC using 20% EtoAc: Hexane as solvent phase and R_f value was found to be 0.6. The spectral results indicate that there were specific signature signals that we had identified. Following an extensive review of the relevant literature, we compared compounds 1 and 2 with previous reported compounds [16]. In ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm) and ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm), the diterpene structure was confirmed by the presence of nine cyclic protons at δ$_H$ 1.45 (2H, m, H1), 2.04 (2H, m, H2), 1.15 (1H, m, H6), 1.467 (2H, m, H7), 1.452 (1H, m, H8), 1.45 (2H, m, H10). Also, the NMR spectral data exhibited four methyl signatures at δ$_H$ 0.82 (3H, d, J=5.1Hz, H17), 1.60 (3H, s, H18), 1.01 (3H, s, H19), 0.78 (3H, s, H20), with carbon signals at δ$_C$ 16.0 (C17), 18.1 (C18), 19.9 (C19), 18.2 (C20), and two methylene protons at δ$_H$ 5.19 (1H, m, H3), 5.83 (1H, s, H14) and 6.02 (1H, s, H16) with carbon signal at δ$_C$ 120.4 (C3), and for compound 2 we have identified the open cleavage of lactone into acid and aldehyde groups, and this was confirmed by analyzing NMR spectral data. The aldehyde proton was observed at H 9.49 (1H, s, H16, 1-CHO), while the equivalent carbonyl carbon signal was observed at C 196.5 (C16) and remaining signals which were present in decaline moiety are same. From the data provided above, we could validate that both compounds, namely compounds 1 and 2, are clerodane diterpenes. In figures and tables, we included all our spectral data in comparison with the previously reported compound [16].

Compound 3 is a novel semisynthetic heterocyclic derivative obtained as a white crystalline powder; m.p. 195-197˚C and it was examined on TLC using 30% EtoAc: Hexane as solvent phase and R_f value was found to be 0.5 in hexane: ethyl acetate. IR spectrum (in KBr) showed bands at 3575(N-H stretching), 2958(0-H stretching), 1676(C=O), 1603(C=C) (supp.data). ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm) (supp.data) and ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm) (supp.data), the diterpene structure was confirmed by the presence of ten cyclic protons at δ$_H$ 1.43 (2H, m, H1), 2.05 (2H, m, H2), 5.22(1H, m, H3), 1.27-37 (1H, m, H6), 1.41 (2H, m, H7), 1.40 (1H, m, H8), 1.38 (2H, m, H10), and the ethylene bridge was confirmed by δ$_H$ 1.50-1.52 (1H, m, H11), 2.50 (2H, m, H12), with corresponding carbon signals at δ$_C$ 36.3 (C11), 19.8 (C12), respectively. Also, the NMR spectral data exhibited 4 methyl signatures at δ$_H$ 0.78 (3H, d, J=5.1Hz, H17), 1.64 (3H, s, H18), 1.03 (3H, s, H19), 0.86 (3H, s, H20), with carbon signals at δ$_C$ 16.1 (C17), 18.3 (C18), 20.6(C19), 19.6 (C20), respectively, and two methylene protons at

δ_H 5.22 (1H, m, H3), and 6.71 (1H, s, H14), with carbon signal at δ_C 120.2 (C3) and 149.5 (C14), respectively. The formation of pyridazine ring was confirmed with -OH, was noticed at δ_H 11.20 (1H, s, H15), with corresponding carbonyl carbon signal at δ_C 161.5 (C15) and proton at 16th position was also noticed at δ_H 7.65 (1H, s, H16) with corresponding carbonyl carbon signal at δ_C 139.3 (C16). The HMBC (supp.data) and INEPT (supp.data) correlation spectrum has provided more insight into the structural features of the compound (Fig 4). The following are the correlations were obtained in HMBC correlation spectra. The protons resonating at δ 7.65 showed correlation with carbons resonating at δ 19.84, the protons resonating at δ 11.20 showed correlation with carbons resonating at δ 149.5 and the protons resonating at δ 6.71 showed correlation with carbons resonating at δ 19.84. And for **Compound 4** we observed the methylene proton at H-3 is reduced and the proton signal is 1.39 with carbon signals at δ_C 31.4 (C3) and one methylene proton at δ_H 6.62 (1H, s, H14), with carbon signal at 124.25 (C14), respectively, including an -OH, was also noticed at δ_H 12.84 (1H, s, H15), with corresponding carbonyl carbon signal at δ_C 141.51 (C15) and -H at 16 th position proton adjacent to nitrogen was also noticed at δ_H 7.76 (1H, s, H16) with corresponding carbonyl carbon signal at δ_C 138.19 (C16).

From the above data we confirmed that pyridazine was formed and it undergoes keto-enol tautomerism. Hence, compound 3 and 4 were Novel semisynthetic heterocycles derived from compound 1. **Compound 5** obtained as a white crystalline powder; m.p. 190-205˚C and it was examined on TLC using 40% EtoAc: Hexane as solvent phase and R_f value was found to be 0.5. IR spectrum (in KBr) showed bands at 3266 (N-H stretching), 2958 (0-H stretching), 1681(C=O), 1641(C=C) (supp.data). ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm) (supp.data) and ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm) (supp.data), the diterpene structure was confirmed by the presence of ten cyclic protons at δ_H 1.38 (2H, m, H1), 1.46 (2H, m, H2), 1.42 (1H, m, H6), 1.43-1.46 (2H, m, H7), 1.63 (1H, m, H8), 1.0 (2H, m, H10), and the ethylene bridge was confirmed by δ_H 1.50-1.52 (1H, m, H11), 1.99 (2H, m, H12), with corresponding carbon signals at δ_C 36.19 (C11), 32.28 (C12), respectively. Also, the NMR spectral data exhibited 4 methyl signatures at δ_H 1.01 (3H, d, J=5.1Hz, H17), 1.54 (3H, s, H18), 1.03 (3H, s, H19), 0.83 (3H, s, H20), with carbon signals at δ_C 16.0 (C17), 18.1 (C18), 19.9(C19), 18.2 (C20), respectively, and two methylene protons at δ_H 5.14 (1H, m, H3), and 6.05 (1H, s, H14), with carbon signal at δ_C 120.79 (C3) and 149.56 (C14), respectively. The formation of oxazine ring was confirmed with -OH, noticed at δ_H 11.425 (1H, s, H15), with corresponding carbonyl carbon signal at δ_C 170.59 (C15) and -H at 16th position noticed at δ_H 8.27 (1H, s, H16) with corresponding carbonyl carbon signal at δ_C 152.27 (C16). The HMBC (supp.data) correlation spectrum has provided more insight into the structural features of the compound. The correlations obtained in HMBC correlation spectra were shown in Fig 2. The protons resonating at δ 6.05 showed correlation with carbons resonating at δ 32.28, the protons resonating at δ 8.27 showed correlation with carbons resonating at δ 32.28 and the protons resonating at δ 11.42 showed correlation with carbons resonating at δ 149.5. From the above data we confirmed that formation of oxazine ring and there were no changes at decaline moiety when compared with the standard compound 1. Complete spectral data and Physico chemical constants were provided in Table I and Table II and supplementary data.

2.2 Antibacterial Activity:

The agar well diffusion method was used to evaluate antibacterial activity and its MIC were determined. Among the synthesized compounds (Compounds 1-5) from seeds of methanolic extract of *Polyalthia longifolia var pendula,* Compound 5 exhibited significant antibacterial activity with Zone of inhibition of 10.8 mm with Gram +ve and 7.95 mm with Gram -ve bacteria at a conc of 50 µg/ml respectively. The results were presented in the Table III and Fig 5.

2.3 Determination of MIC for antibacterial activity (Broth dilution method):

Among the synthesized compounds (Compounds 1-5) from seeds of methanolic extract of *Polyalthia longifolia var pendula*. Compound 1, 2 & 5 showed potent anti-bacterial activity against Gram +ve and Gram –ve organisms with an MIC of 5 µg/mL which is equal to standard Rifampicin MIC of 5 µg/mL. The compounds 3 & 4 showed MIC of 10 µg/ml. The results are shown in Table IV.

2.3 Antifungal activity:

The antifungal activity of the seeds of the methanolic extract of *Polyalthia longifolia var. pendula* was evaluated using the agar well diffusion method. Among all the synthesised compounds (3-5), Compound 5 showed Zone of inhibition of 10.8mm against *Candida albicans* when compared with standard fluconazole with Zone of inhibition of 12.5mm at conc. of 50 µg/ml. The results were presented in the Table V and Fig 6.

2.4 In Vitro antitubercular activity screening:

All the synthesized compounds (3-5) from seeds of methanolic extract of *Polyalthia longifolia var pendula* were tested for anti-tubercular activity by MABA assay. Compounds 3 & 5 exhibited potent activity at a MIC of 1.56 µg/ml by Microplate Alamar Blue Assay (MABA) against the *Mycobacterium tuberculosis* MTB H37Rv bacilli strain when compared with standard drugs (Pyrazinamide (MIC of 3.125 µg/ml), Ciprofloxacin (MIC of 3.125

µg/ml), Streptomycin (MIC of 6.25 µg/ml) and Rifampicin (MIC of 6.25 µg/ml)). The results were shown in Table VI represented in Fig 7 & 8 They also shown significant antibacterial activity against gram +ve (*Staphylococcus aureus (*NCIM 2122*)*) and gram -ve (*Escherichia coli* (NCIM 2137)) bacteria at 50µg/mL indicating good selectivity against *Mycobacterium tuberculosis*. The comparatively extended therapeutic period of tuberculosis will deeply affect the gut and other areas of our body's microbial ecology. As a result, it is always sought after to find specific anti-mycobacterial medicines such as INH, which has almost minimal action against other microorganisms. The anti-tubercular results clearly confirm that potency is increased with the groups containing the lactone moiety along with the ester containing groups. Potent anti TB molecules obtained by the MABA assay are further confirmed by undergoing MTT Assay and Axenic assay. The results are shown in Table VII and Table VIII represented in Fig 9.

2.5 SAR for antitubercular activity:

Based on the spectral data, we have found that the heterocycles obtained from 16-hydroxy cleroda 3,13(14)-dien15,16-olide i.e., compound 3 and 5 shown potent anti-tubercular activity than compound 1 and the anti-tubercular results clearly confirm that potency increased by addition of nitrogen at para and -OH at meta position at C-13 position and there were no changes observed at decalin moiety as shown in Fig 10.

3. Experimental work:

3.1 General:
        Everything utilised in this experiment is of reagent grade. The reactions were monitored with silica gel TLC using appropriate mobile phase. Visualisation of the spots was done either with help of UV, iodine, and acid spray. Melting points were observed using EZMELT 120 (Stanford Research Systems, USA) and are uncorrected. IR spectrum data is acquired using Bruker ALPHA-T FTIR instrument using KBr pellet technique. NMR spectrum data was acquired on Bruker- 400 MHz system in appropriate deuterated solvent using TMS as internal standard. Elemental analysis studies were conducted using Carlo Erba elemental analyzer. ESIMS mass spectral measurements were done using Agilent 6410 QQQ MS equipment.

3.2 Synthesis of heterocycles from 16-hydroxy cleroda 3,13(14)-dien15,16-olide*:*
        A mixture of 1.0 eq (0.3 mM) of 16-hydroxy cleroda 3,13(14)-dien15,16-olide (Lactone) and 2.5 eq (0.75 mM) of hydrazine hydrate (Compound 5 & 6), 1 eq (0.3 mM) of hydroxylamine Hcl (Compound 7) in ethanol (5 ml) were kept at room temperature. Thin layer chromatography (silica gel, Merck 60, F254, 0.2 mm) was used to monitor progress of the reaction. After the reaction is finished, the ethanol is evaporated, and then ethyl acetate and water were used to extract the product. The ethyl acetate layer is then concentrated and purified by using column chromatography (silica gel, 100-200 mesh) by sequential elution of 2.5L of n-hexane, ethyl acetate and methanol each. We found a pale-yellow crystalline powder at 5% EtoAc: hex fraction (Compound 6). Three new crystal compounds were purified by the process of recrystallization using hexane, and then they were dried. The newly synthesised lactones were new moieties, and its structures was deduced through the application of various spectrum techniques like IR, 1D and 2DNMR, MASS Spectral data.

3.3 Antibacterial activity:

Agar well-diffusion method was followed to determine the antimicrobial activity against gram positive (*Staphylococcus aureus (NCIM 2122)*) and gram-negative bacteria (*Escherichia coli (NCIM 2137)*). All the compounds (Compounds 1-5) were weighed accordingly and 1mg/ml stock solutions were prepared using DMSO as a vehicle and Rifampicin was used as a standard drug. Into the sterilized agar medium, 100 µl of bacterial suspension (*E. coli & S. aureus)* was added. The inoculated agar medium was poured into sterilized petri plate and after 15 minutes, wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. All isolated compounds, control and Rifampicin; each of 100 µl quantity were added into the bores using micropipette. The petri plate was incubated at 37ºC up to 24 hrs to determine the zone of inhibition.

3.4 Determination of minimum inhibitory concentration (MIC) for antibacterial activity:
All compounds (Compounds 1-5) from seeds of methanolic extract of *Polyalthia longifolia var pendula* were examined for their antibiotic susceptibility to assess MIC (Minimum inhibitory concentration). According to the Clinical and Laboratory Standards Institute, the nutrient broth dilution method was employed (formerly known as National Committee for Clinical Laboratory Standards, 2000). The turbidity of bacterial suspensions can be adjusted using Mc Farland standards to ensure that the quantity of bacteria falls within a predetermined range for microbiological testing. Once the soup had been sterilised, it was put into 9-ml sterilised boiling tubes. The bacteria Escherichia coli (NCIM 2137) and Staphylococcus aureus (SA) were then added to each broth solution

(NCIM 2122). We used sterile sealers to cover the tops of boiling tubes and incubated them at 37$^{\text{O}}$C for 24 hours with 1 ml of diluted produced chemicals and 0.5 ml of a bacterial suspension. Rifampicin was used as a standard medication to determine the MIC, along with positive and negative controls. The absence of turbidity in the solution implies that no growth has occurred at that concentration. The minimal inhibitory concentration (MIC) of a test substance was determined to be the concentration at which no observable growth occurred when the drug was tested on that bacterial species. All our experiments were repeated triplicate to ensure accuracy.

3.5 AntiFungal activity: The agar well diffusion method, described earlier, was utilised to determine whether any of the compounds possessed antifungal activity. All compounds (Compounds 1-5) from seeds of methanolic extract of *Polyalthia longifolia var pendula* were examined to see whether they were able to inhibit the growth of *Candida albicans* in vitro (NCIM 3102). The agar well diffusion method was utilised for the purpose of determining whether the substances possessed antifungal properties. After accurately weighing each component, stock solutions of 1 mg/ml were produced in DMSO, which served as the medium for the experiment. The preparation of fluconazole, which is the standard medicine, was carried out in the same manner. One hundred microliters of a fungal suspension containing *Candida albicans* were added to the sterilised Sabouraud's medium. After waiting 15 minutes, bores were created in the solidified Sabouraud's agar medium that was placed in a petri dish that had been previously sterilised. By using a sterile cork borer, we drilled holes in each of these plates measuring 10 millimetres in diameter and approximately 2 centimetres apart. A total of 100 µl of each of the separated compounds, the control, and the fluconazole solution were each applied to the petri plate. In order to establish the zone of inhibition, the petri dish was kept at a temperature of 26$^{\text{O}}$C for a period of forty-eight hours.

3.6 In Vitro antitubercular activity screening:
        The synthesized compounds were tested for anti TB activity (according to NCCL standards 1985) against *Mycobacterium tuberculosis H37Rv* by Micro-plate Alamar Blue Assay (MABA). The procedure involves the following steps: Growth on Lowenstein Jensen (LJ) medium was suspended in sterile Middlebrook 7H9 broth with 0.2 % glycerol and 10 % OADC (oleate-albumin dextrose-catalase) enrichment, and a 1:20 dilution was used as the inoculum for MABA. All the deceptions were done with the right safety hoods. To reduce evaporation of medium in the test wells during incubation, 200 µl of sterile deionized water was added to all outer perimeter wells of a sterile 96 well plate. The 96 well plate was filled with 100 µl of Middlebrook 7H9 broth, and compounds were serially diluted on the plate. The final drug concentrations tested were 25, 12.5, 6.25, 3.125, 1.6 and 0.8 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for ten days. After this, 25 µl of a freshly made mixture of Alamar Blue reagent and 10% tween 80 was put on the plate, and it was put in an incubator for 24 hours. Bacterial growth was read as a lack of pink coloration in the well, and vice versa for blue. The minimum effective concentration (MIC) was determined to be the lowest concentration of medication that stopped the blue to pink color shift.

3.7 Antitubercular screening of potent compounds by MTT assay and Axenic assay

3.7.1. MTT Assay

   The MTT Assay (cell proliferation Assay) was used to test the top 2 compounds (Compound 3 & 5) against *Mycobacterium tuberculosis* MTB H37Ra as mentioned in Table 7 and Figure 6. These compounds had shown strong anti-TB activity (MIC 1.56 g/mL) in the MABA assay (MTCC 300). In this method, the right amount of Sauton media was mixed with the right amount of cell suspension. In 25 ml McCartney bottles, 10 ml of the above solution was added. Then, a sample of the test was put into each bottle (in duplicates). The standard drugs (pyrazinamide, ciprofloxacin, streptomycin Rifampicin) are then put in their own bottles and set apart from the control bottles (which only have cells and Sauton, but no inhibitor). These were kept in a 37$^{\text{O}}$C oven for 10 days, 1 ml of culture from each of the 10day culture bottles was taken and put in a different bottle. 5 mg/ml of yellow MTT (di methyl thiazolyl diphenyl tetrazolium bromide) dye was added to 200 l of 1 ml cell culture, and the mixture was kept at 37$^{\text{O}}$C for 4 hours. The crystals that had formed were then dissolved by adding 1 ml of lysis buffer, which was 50% formamide and 20% SDS (sodium dodecyl sulphate) to each tube. The spectrophotometric reading for the purple solution that was made after an overnight incubation was 570 nm. By making MTT formazan, the increase in absorbance shows that the number of cells has grown.

3.7.2. AXENIC Assay

        Top 2 potent compounds (Compound 3 & 5) by MABA assay were further evaluated by undergoing Axenic culture method on MTB H37Ra (MTCC 300) as mentioned in Table 8 and Figure 6. In this method, calculated amount of cell suspension was added to the required amount of sauton media. 10 ml of the above solution was added in 25 ml of McCartney bottles. Then test sample was added into the respective bottles (in duplicates),

control bottles (only cells plus sauton, without inhibitor) and then standard bottles (Isoniazid and Rifampicin). These are incubated for 10 days at 37$^\text{O}$C and OD was taken at 540 nm.

$$\% \text{ Inhibition} = (\text{control-test})/\text{control}*100$$

4. Conclusion:

In the present study, a simple, one pot and efficient method was developed serendipitously for the preparation of three novel heterocycles (compounds 3-5) of the diterpenoid (compound 1), in high yield. All the synthesized lactones, parent diterpenoid (compound 1) along with related diterpenoid (compound 2) were evaluated for the disease tuberculosis using MABA assay against *Mycobacterium tuberculosis*. Based on these studies, it was found that compound 1 and its six membered heterocycles (Compound 3 & 5) displayed potent mycobacterial activity. Compound 3 & 5 showed two times antimycobacterial activity than the standard antibiotics (Pyrazinamide, Ciprofloxacin, Streptomycin and Rifampicin). They also showed good antimicrobial activities. It is very promising and motivating as compound 1 abundantly found in seeds of the plant, which can be developed into an effective, safe compounds. In order to determine the structural activity relationship between the bioactive diterpenoid compound 1 and its analogues, further research will be carried out on *in vivo* and cytotoxic tests, the mechanism of action, and the synthesis of a series of subsequent lactones.

5. Acknowledgments

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Figure:
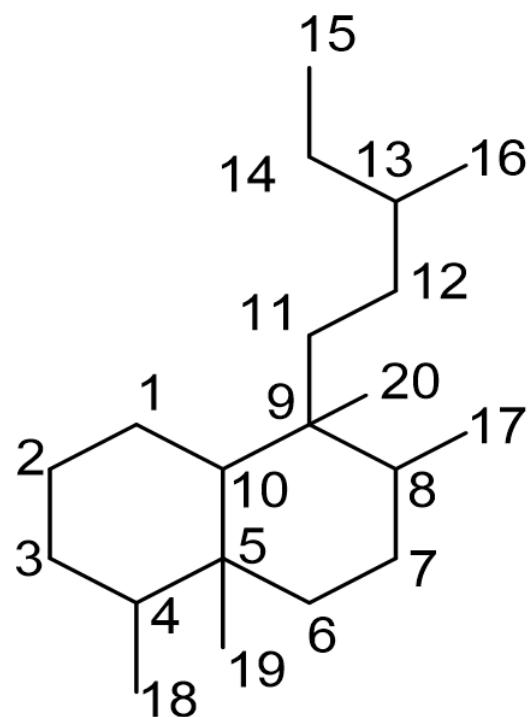
Fig 1: Basic skeleton of clerodane diterpene

16hydroxycleroda3,
13(14) Zdien15,16
olide
1

INTERMEDIATE 1

OXIME
R= -NH$_2$, OH

AMINE
3,4,5

3. R$_1$= N, R$_2$= N, R$_3$= OH   4. 3rd position C-CH$_2$
5. R$_1$= O, R$_2$= N, R$_3$= OH
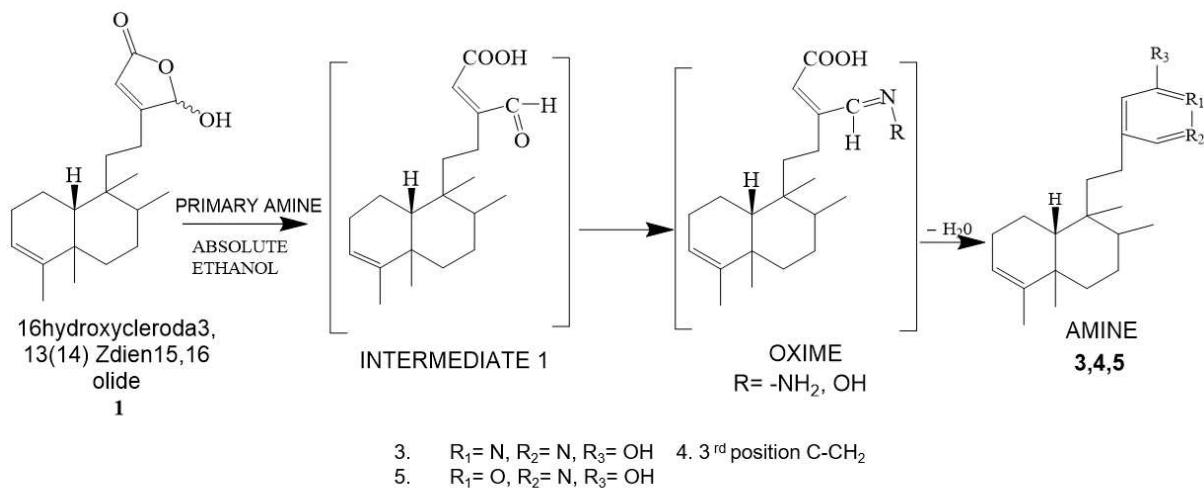
Fig 2 The synthetic route for the preparation of lactones (3-5) from the diterpenoid

16-hydroxycleroda3,13(14)
Zdien15,16 olide
1

16-Oxoecleroda-3, 13 (14)
E-diene-15 oic acid
2

5-(2-((8a*R*)-1,2,4,5-tetramethyl-
1,2,3,4,4a,7,8,8a-octahydronaphthalen-
1-yl)ethyl)pyridazin-3-ol
3

5-(2-((8a*S*)-1,2,4a,5-tetramethyl
decahydronaphthalen-1-yl)
ethyl)pyridazin-3-ol
4

4-(2-((8a*R*)-1,2,4,5-tetramethyl-
1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)
ethyl)-6*H*-1,2-oxazin-6-ol
5
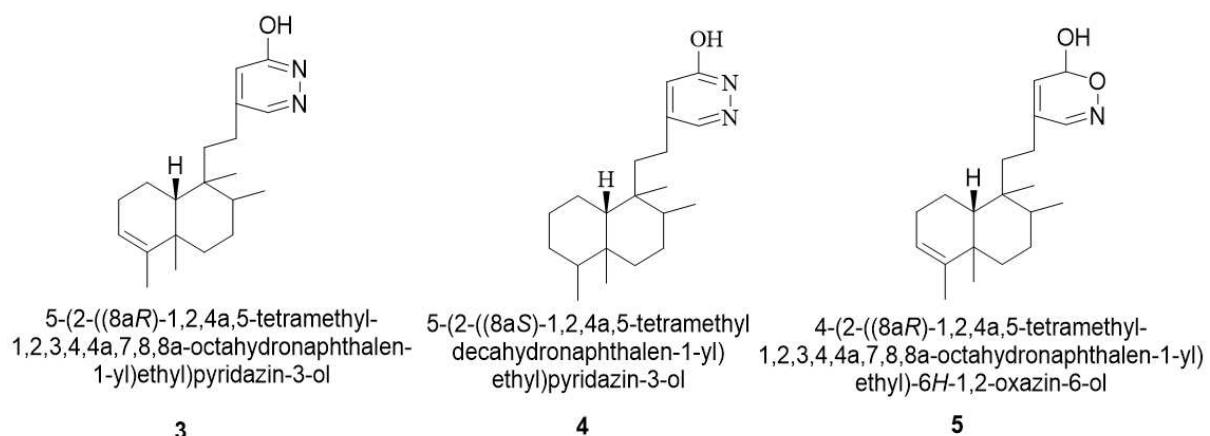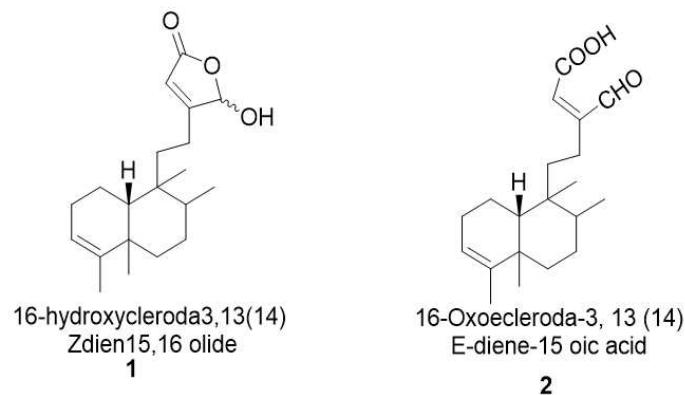
Fig 3: Isolated 16-hydroxycleroda3,13(14) Zdien15,16 olide (1), 16-oxo-xleroda-3, 13(14) E-dien-15 oic acid (2) and synthesized amino lactone derivatives (3-5)

Compound 3
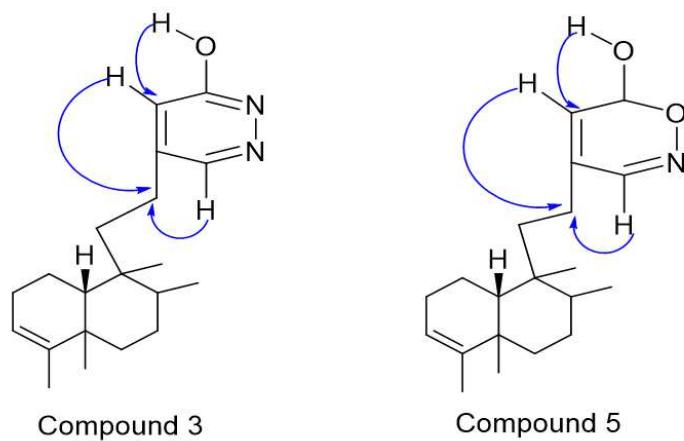
Compound 5

Fig 4: HMBC correlations for compounds 3 & 5

ANTI BACTERIAL ACTIVITY

Legend: Gram +Ve 100µg/ml Gram +Ve 50µg/ml Gram -Ve 100µg/ml Gram -Ve 50µg/ml
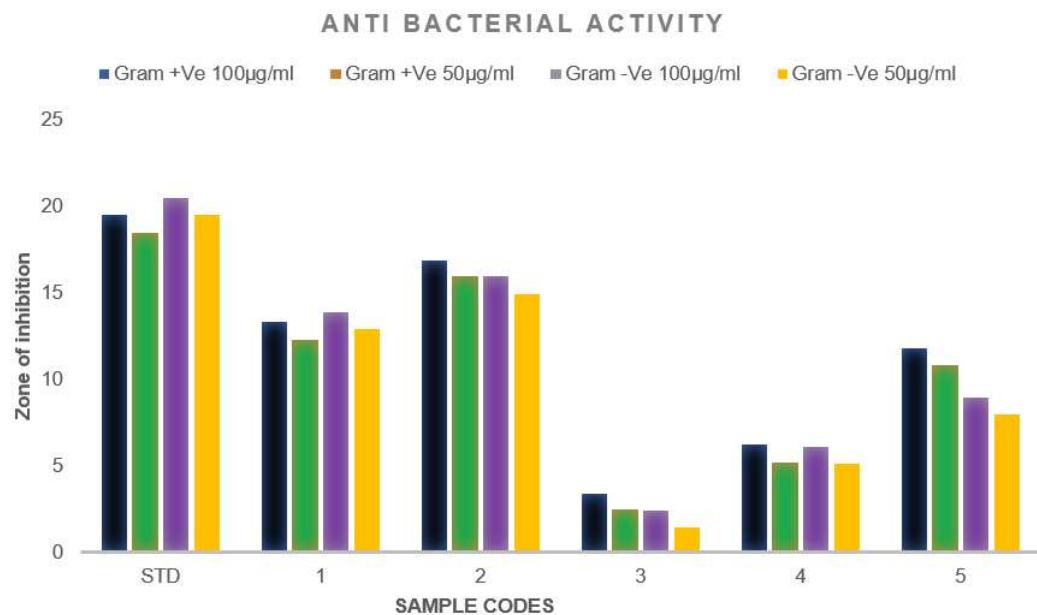
Fig 5: Zone of inhibition for anti-bacterial activity for isolated compounds

ANTIFUNGAL ACTIVITY

Legend: MIC (µg/ml) 50µg/ml MIC (µg/ml) 100µg/ml
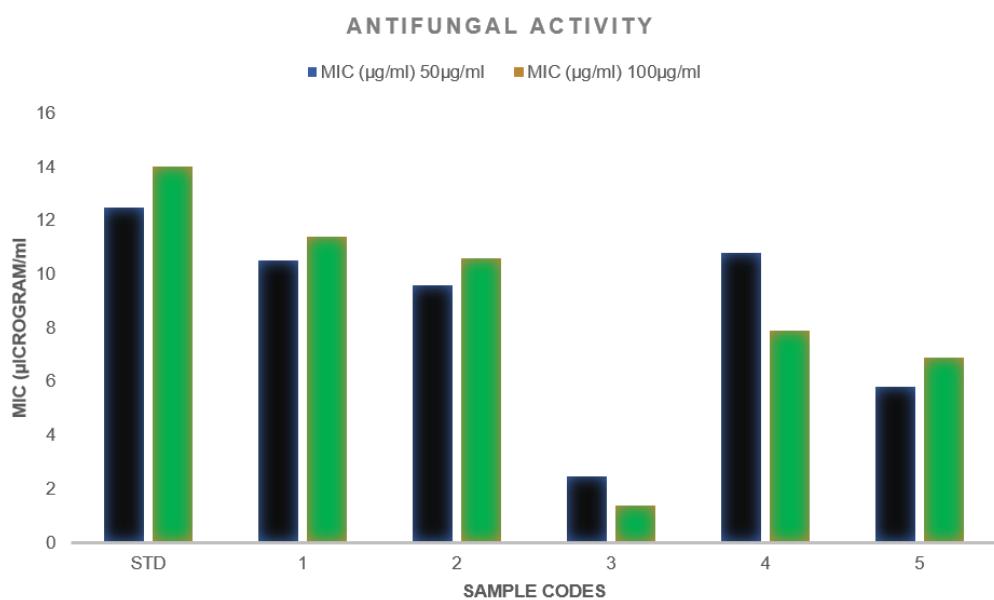
**Fig 6: Zone of inhibition of anti-Fungal activity for isolated compounds**
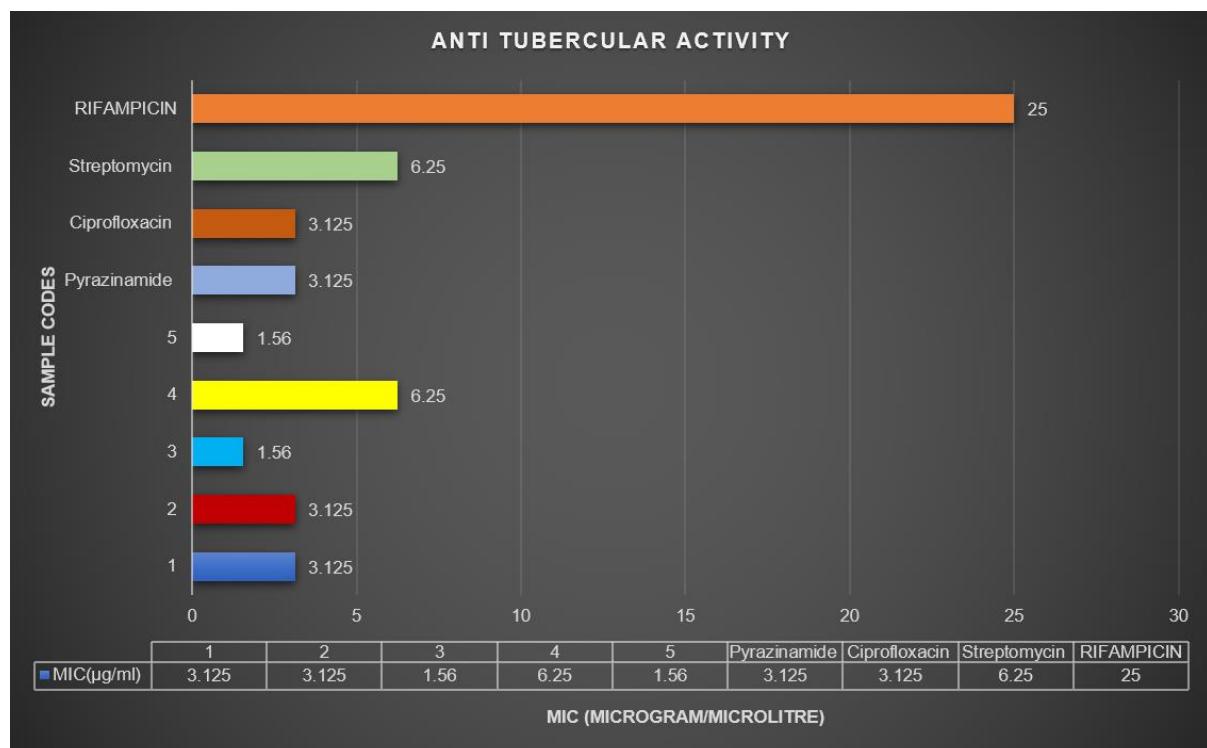
Fig7: MIC of anti-tubercular for isolated compounds
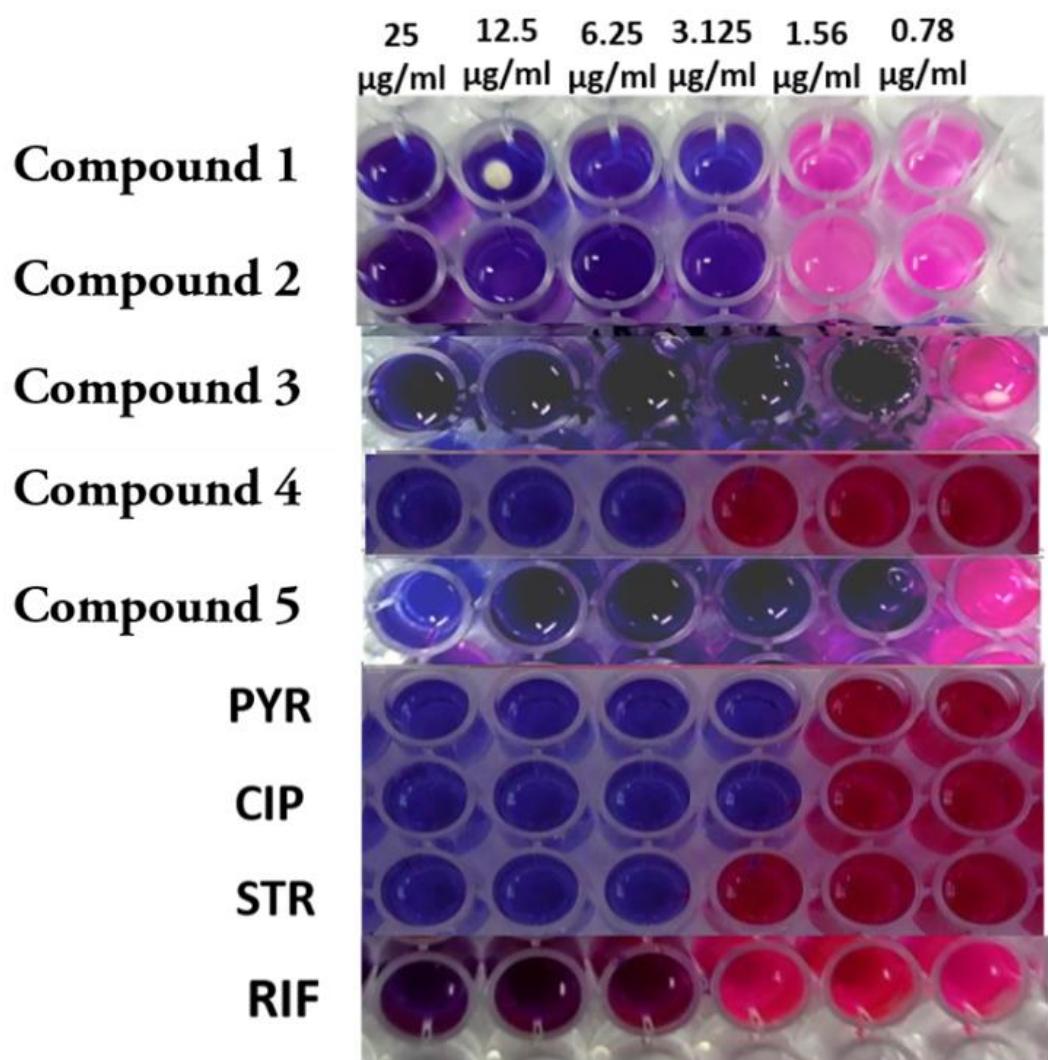
Fig 8: MABA assay for Isolated compounds against MTB H37Rv
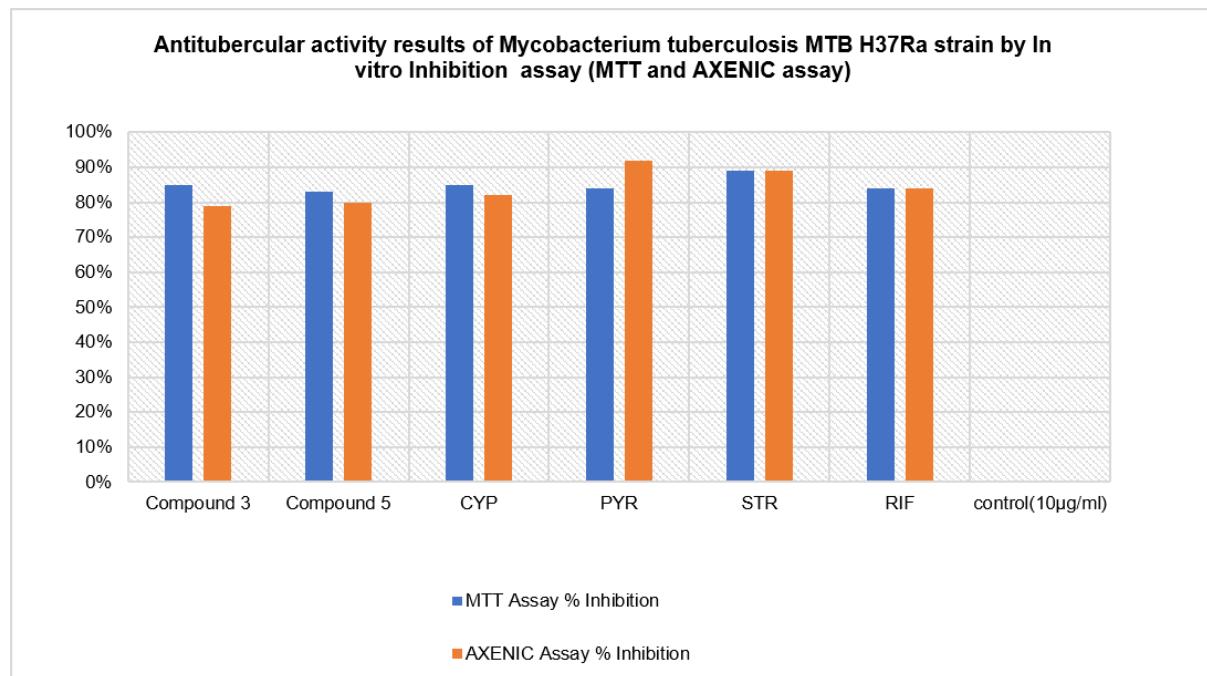
Fig 9: Antitubercular activity results of *Mycobacterium tuberculosis* MTB H37Ra strain by *In vitro* Inhibition assay (MTT & Axenic Assay)


Substitution of C13 with Pyridazine and Oxazine increases TB activity
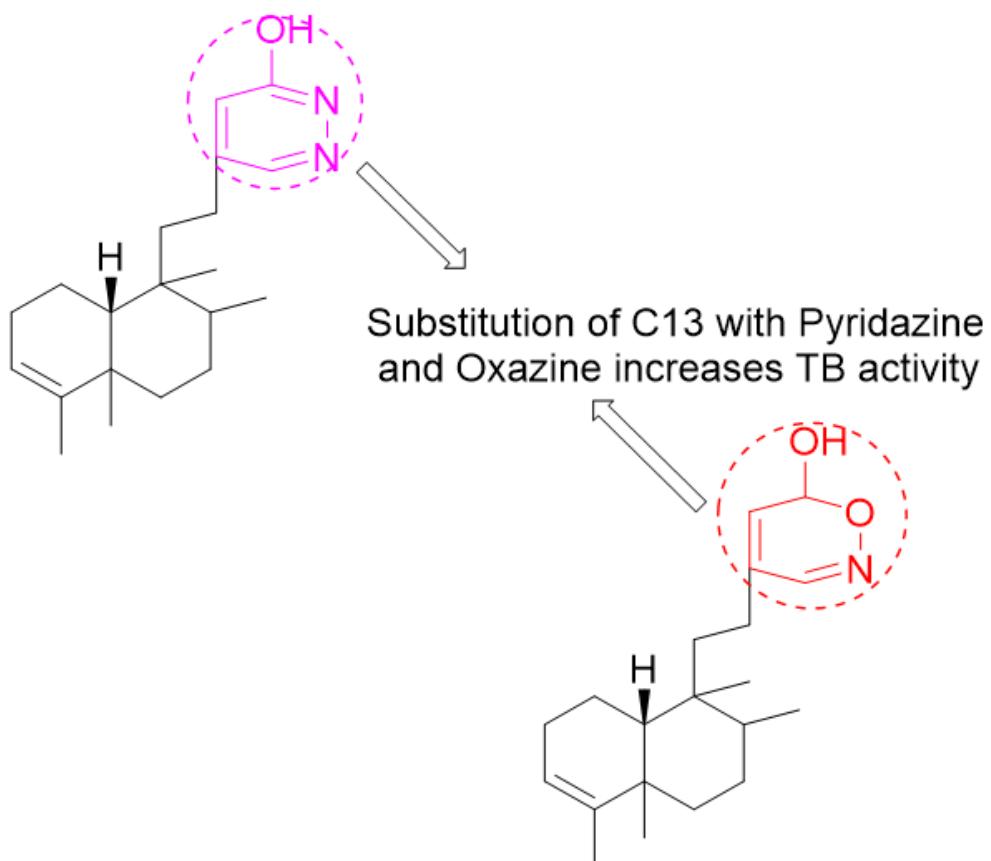
Fig 10: SAR observed for anti-TB activity in novel heterocyclic compounds from 16α-hydroxycleroda3,13(14) Z dien15,16 olide

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableI.docx
- TableII.docx
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- TABLEIV.docx
- TABLEV.docx
- TABLEVI.docx
- TABLEVII.docx
- TABLEVIII.docx
- supplementarydata.docx