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# DETERMINATION OF EFFICACY OF ISOXYL, A MYCOLIC ACID INHIBITOR, IN VITRO AGAINST MYCOBACTERIUM.TUBERCULOSIS STRAINS

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## Abstract

*The enzymes involved in the biosynthetic pathways of the critical components including mycolic acids offer attractive and selective targets for the developments of novel anti-mycobacterial agents. Isoxyl (ISO), a mycolic acid inhibitor, is an old drug, which was used for the treatment of tuberculosis was evaluated. Determination of Minimum Inhibitory Concentration (MIC) pattern of clinical isolates of Mycobacterium tuberculosis (M. tuberculosis) to mycolic acid inhibitors namely ISO, Isoniazid (INH) and Ethionamide (ETH) by agar and broth dilution Method was done. Also the Minimum bactericidal concentrations were evaluated. Total 40 MDR and 20 susceptible strains of M tuberculosis were tested. The result of the MIC studies showed that ISO is capable of inhibiting the growth of M. tuberculosis in a range of 1-20µg/ml. Inhibitory activity of ISO was higher than activity of ETH in solid media. Amongst three antituberculosis drugs, INH showed highest bactericidal activity against M. tuberculosis strains followed by ETH. While ISO exhibited lowest bactericidal activity. Amongst, three drugs tested, ISO shows highest MBC/MIC ratio with lowest bactericidal activity. ISO showed significantly lower bactericidal activity against MDR strains than susceptible strains of M. tuberculosis. MBC/ MIC ratio of ISO was similar to MDR and susceptible strains of M. tuberculosis. Overall study implies that ISO may be suitable for the treatment of Tuberculosis, particularly multi-drug resistant kind.*

**Keywords**

MDR TB, Isoxyl, Mycolic Acid Inhibitor

## 1. Introduction

Developing new antituberculosis drugs is an expensive exercise and tuberculosis (TB) is not a disease of rich nations. Some development projects are underway, but more are needed. TB still remains a neglected disease in relation to drug development (Chopra, Meena, & Singh, 2003). The needs, challenges and recent advances towards development of novel chemical molecules against TB have been reviewed recently (Lienhardt, 2012).

Approximately 2 billion people of the world's population are latently infected with *Mycobacterium tuberculosis (M. tuberculosis)* and are at risk of reactivation to active disease (Diel et al., 2013). Even though an inexpensive and effective quadruple drug therapy regimen

comprising Isoniazid (INH), Rifampicin (RF), Ethambutol (EMB) and Pyrazinamide (PZ) was introduced 40 years ago, TB continues to spread in every corner of the globe (Raviglione et al., 2012).

The current treatment regimen has several drawbacks, including prolonged treatment time to completely eradicate the bacteria. This increases the opportunity for development of resistant strains of *M. tuberculosis* documented in almost every country where the disease is prevalent. These obstacles, in addition to an increasing prevalence of MDR, XDR and currently TDR strains, call for an urgent need to search for and develop novel agents against TB. Pulmonary TB remains a major health hazard in Asia, Africa and the Western Pacific region, despite its sharp decline in the Western world since the beginning of the 20th century (Mitra, 2012).

A number of approaches are considered to identify targets for novel anti-mycobacterial agents. They range from biochemical studies of essential pathways to the use of genome scale tools such as transposon mutagenesis, proteomics and transcript mapping on micro arrays. In combination with modern approaches, such as structure based drug design and combinatorial chemistry will lead to the development of new drugs that are not only active against drug resistant TB but shorten the chemotherapy schedule. Several targets have been identified and combination of high throughput screening and rational structure based design is being used to identify lead molecule (Chopra et al., 2003). Current chemotherapy for TB relies on Mycobacteria specific drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall polymer. (Balows et al., 1975)

These critical components of the cell envelope have been the subject of intense research for a number of years because of the fact that enzymes involved in their biosynthetic pathways including mycolic acids, offer attractive and selective targets for the developments of novel anti- mycobacterial agents (Brennan, 2002).

There is an urgent need to develop new effective antituberculosis compounds that can increase the permeability of the Mycobacterial cell wall by inhibiting the synthesis of cell wall components and enhance the activity of conventional drugs as a result of increased penetration of these latter agents to susceptible internal targets (David et al., 1988). This enhancement of antimicrobial activity theoretically affords the use of lower concentration of antibiotics associated with toxicity (Matlola, Steel, & Anderson, 2001). As drug development is a long

and expensive process, it becomes predominant to re-examine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's (Winder, 1982). Some authors demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary TB of various degree of difficulty (Tischer, 1966; Urbancik, 1970). While some concluded that INH and ISO were more effective than monotherapy with either drug (Schmid, 1970). The NCDDG group led by DR Patrick Brennan recently evaluated this drug and found it to be effective against MDR strains of *M. tuberculosis*. ISO, a thiourea (thiocarlide, 4, 4 –diisoamyloxythiocarbanilide) demonstrated potent activity against standard strain of *M. tuberculosis* (Phetsuksiri et al., 1999).It had been noted that it strongly inhibited mycolic acid synthesis in *M. bovis* (Winder, 1982).

Hence there was a thought to do more work on this compound, as it is an old drug and have proven its efficacy. Present study was conducted with the objective of determining the efficacy of ISO in vitro against *M. tuberculosis* strains.

## 2.Material and methods

### 2.1 Mycobacterial Strains

Total 60 Clinical isolates of *M. tuberculosis* were collected from Department of Microbiology of P.D. Hinduja Hospital and Medical Research Centre, Mumbai. The clinical isolates were defined as *M. tuberculosis* according to their growth rates, pigmentation properties of colonies, susceptibility to para-nitro benzoic acid, semi-quantitative catalase test, nitrate reduction test and niacin accumulation tests (Vestal, 1975). Drug susceptibility pattern of 60 clinical isolates of *M. tuberculosis* was determined by Bactec460TB system by modified Proportion Method (Siddiqui, S. H., Libonati, J.P. and Middlebrook G, 1981).

### 2.2 Determination of Minimum Inhibitory Concentration using Agar dilution method

Minimum Inhibitory Concentration (MIC) pattern of clinical isolates of M. tuberculosis to mycolic acid inhibitors was ascertained by agar dilution method. (Hawkins, et al., 1991). Total 40 MDR and 20 susceptible strains of M. tuberculosis along with standard strain of M. tuberculosis H37Rv were included in the study. Mycolic acid synthesis inhibitors studied were ISO (Cayman Chemical Co., U.S. A.), INH (Lupin Pharmaceuticals, India) and Ethionamide (ETH) ( Lupin Pharmaceuticals, India). Sterile Middlebrook 7H10 agar with Oleic acid,

Dextrose and Catalase (OADC) supplement (HiMedia Laboratories Pvt Ltd, India) was used for this method. Serial two fold dilutions of individual drug were prepared in sterile water for injection after dissolving it in suitable diluents. 1ml of test drug solution (20X) was mixed with 19 ml of sterile molten Middlebrook 7H11 medium with OADC supplements (HiMedia Laboratories Pvt Ltd, India) and poured in sterile petri plates. Media was cooled and agar was set. Test strains were inoculated in sterile Dubos broth with glucose and albumin supplement with 0.05% Tween 80 (HiMedia Laboratories Pvt Ltd, India) and incubated at 37◦ C for 7 to 10 days. Cultures were shaken daily on vortex mixture for 30 seconds. The cultures were adjusted to an optical density of 0.1 at 540 nm and then diluted 10 fold in 0.1% Tween 80 containing normal saline. Bacterial suspension in 5-microlt quantities was spotted on agar plates containing various drug concentrations. The control plates, containing no drug were also inoculated along with it.

Plates were incubated at 37◦ C for 14 days. The MIC was defined as the minimum concentrations of drugs that completely inhibited the growth of the test organism or allowed growth of not more than five colonies.

Following drugs concentrations (µg/ml) were used for the test:

- ISO: 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40 µg/ml
- INH: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µg/ml
- ETH: 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40 and 80 µg/ml

**2.3 Determination of Minimum Inhibitory Concentration using Broth dilution method**

Determination of MIC pattern of clinical isolates of *M. tuberculosis* to mycolic acid inhibitors by carried out using broth dilution method (Cruikshank, Duguid, & Swain, 1968; Tomioka, Saito, Fujii, Sato, & Hidaka, 1993).

MIC pattern of 40 MDR and 20 susceptible strains of *M. tuberculosis* along with standard strain of *M. tuberculosis* H37Rv were determined to mycolic acid synthesis inhibitors. Sterile Dubos broth with glucose and albumin supplement was used in the test. Serial two-fold dilution of individual drug was prepared in sterile Dubos broth with glucose and albumin supplement after dissolving it in suitable diluents. It was dispensed in 4.5-ml quantities in sterile tubes. Required drug concentration in 10 X concentrations was put in 0.5 ml quantity in medium to obtain desired concentration. Each of the drug containing tube and drug free tube

were inoculated with 0.1 ml. of test culture adjusted to McFarland No. 1 turbidity. The original inoculum size of each test strain was determined by appropriately diluting the culture and inoculating 0.1 ml of culture on sterile LJM slants. A tube containing the highest concentration of the drug without any culture was also incubated as a drug control to check if the drug agent contributed to the turbidity of the medium. Drug free tube inoculated with test culture was kept as positive control. Medium control was also kept. Inoculated and un-inoculated tubes were incubated at 37ºC for 15 days. Results were obtained by observing the tubes for inhibition of growth which was judged by a lack of opacity in tube.

The MICs were defined as the minimum concentrations of drugs that completely inhibited the growth of the test organism.

Following drugs concentrations (µg/ml) were used in the study:

- ISO: 0.035, 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40, 80 and 160 µg/ml
- INH: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µg/ml
- ETH: 0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40, and 80 µg/ml

**2.4 Determination of Minimum Bactericidal Concentration (MBC)**

MBC pattern of 40 MDR strains and 20 susceptible strains of *M. tuberculosis* along with standard strain of *M. tuberculosis* H37Rv was determined to ISO, INH and ETH by broth dilution method (Heifets, 1991; Reddy, V. M., Nadadhur, G., Daneluzzi, D., Dimora, V. and Gangadharam, P.R.J. 1995).

After 15 days of incubation for MIC determination of the drug, drug concentrations higher than or same as those used to determine the MIC was selected. Those drug containing tubes showing no turbidity were included in the study and made dilutions of drug concentrations to $10^{-3}$ to $10^{-4}$ in 10 ml quantity.

These dilutions were inoculated on sterile LJM slants in 0.1 ml quantity in duplicate. Further slants were incubated at $37^0$C for three weeks. Numbers of colony forming units (CFU) were counted on LJM. Number of CFUs developed after incubation was compared with the number of CFU's of originally inoculated culture. The lowest concentration of the drug that killed 99.9% of organisms was considered the MBC of that drug.

Following drugs concentrations (µg/ml) were used in the study:

- ISO: 0.07,0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40, 80 and 160 µg/ml

- INH: 0.003,0.006,0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µg/ml
- ETH: 0.035,0.07,0.15, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, 40 and 80 µg/ml

## 3. Results

**Table 1:** *MIC pattern of Mycolic acid inhibitors to MDR strains of M. tuberculosis in solid and liquid media*

| Serial No. | Mycolic acid inhibitors | Geometric mean (GM) MIC (µg/ml) | | MIC Range ( µg/ml) | |
|---|---|---|---|---|---|
| | | Solid media | Liquid media | Solid media | Liquid media |
| 1 | INH | 1.21 | 0.29 | 0.2 to 3.2 | 0.05 to 0.8 |
| 2 | ETH | 15.94 | 2.51 | 2.5 to 40 | 0.3 to 5.0 |
| 3 | ISO | 5.76 | 1.62 | 1.2 to 20 | 0.3 to 5.0 |

On solid media, Inhibitory activity of ISO was 4.76 times lower than activity of INH, while it was 2.77 times higher than activity of ETH. MIC range was narrower for ISO than ETH. In liquid media inhibitory activity of ISO was 5.59 times lower than the activity of INH, while it was 1.55 times higher than activity of ETH.MIC rage was similar for ISO and ETH. [Table 1]

**Table 2:** *MIC pattern of Mycolic acid inhibitors to MDR strains of M .tuberculosis in solid and liquid media in combination*

| Group | Mycolic acid inhibitors | Geometric mean MIC (µg/ml) | | (+/-)SD | | P value | | Remark |
|---|---|---|---|---|---|---|---|---|
| | | Solid media | Liquid media | Solid media | Liquid media | Solid media | Liquid media | |
| 1 | INH ISO | 1.21<br>5.76 | 0.29<br>1.62 | 0.92<br>3.65 | 0.23<br>1.07 | 3.00E-10 | 2.07E-09 | Difference is significant |
| 2 | ISO ETH | 5.76<br>15.94 | 1.62<br>2.51 | 3.65<br>11.98 | 1.07<br>1.78 | 2.99E-07 | 0.002 | Difference is significant |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 | INH ETH | 1.21<br>15.94 | 0.29<br>2.51 | 0.92<br>11.98 | 0.23<br>1.78 | 8.60E-10 | 3.90E-10 | Difference is significant |

Difference between MIC values between INH and ISO; ISO and ETH; INH and ETH to MDR strains of *M .tuberculosis* in solid and liquid media in combination were statistically significant. [Table 2]

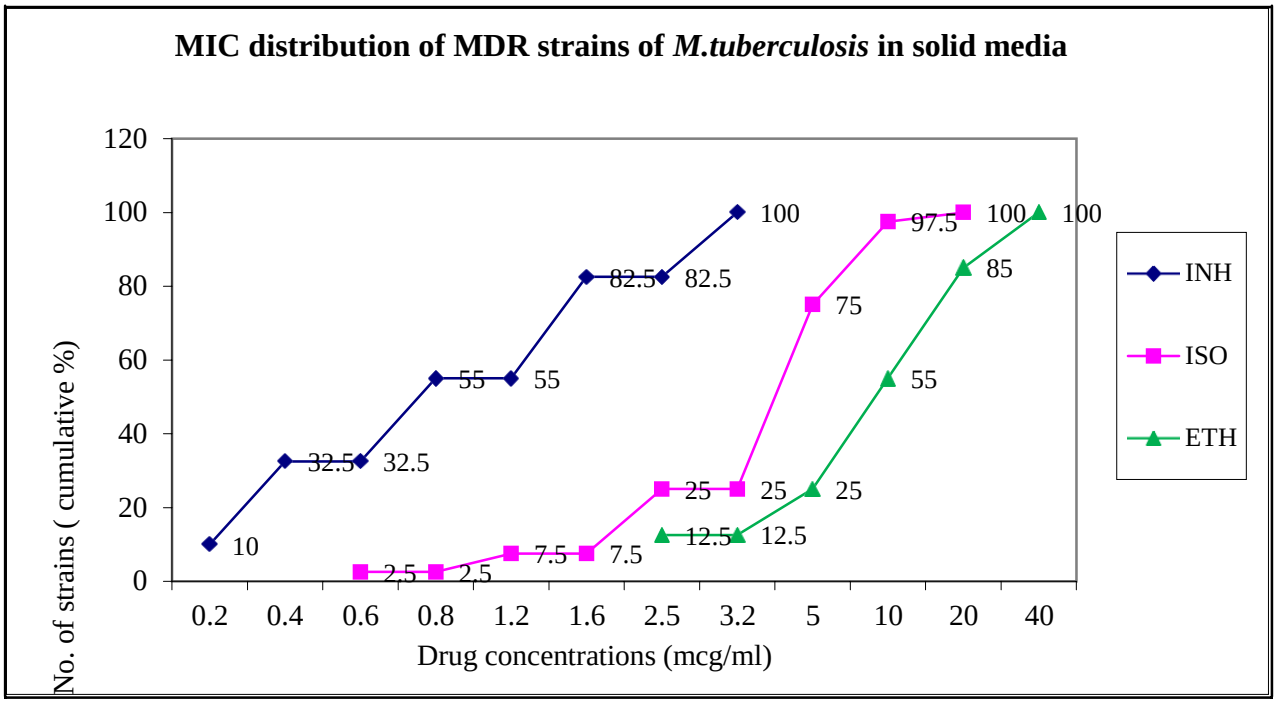


**Figure 1:** *MIC distribution of MDR strains of M.tuberculosis in solid media*

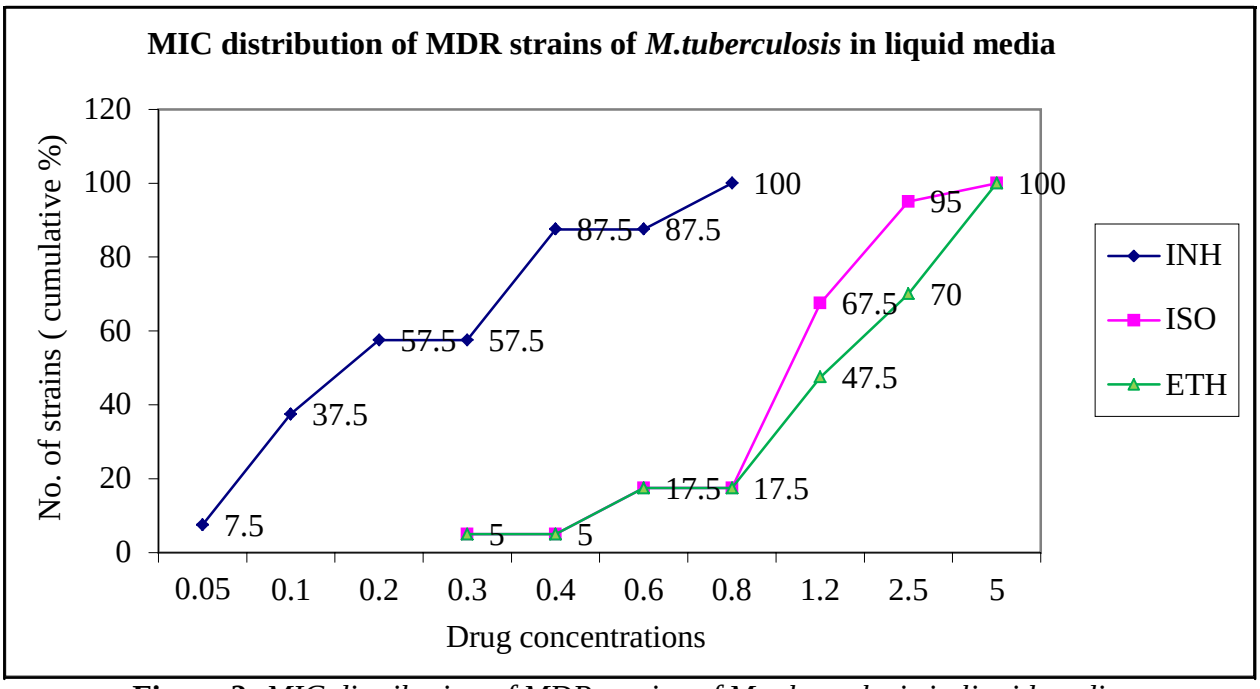


**Figure 2:** *MIC distribution of MDR strains of M.tuberculosis in liquid media*

**Table 3:** *MIC pattern of Mycolic acid inhibitors to susceptible strains of M. tuberculosis in solid and liquid media*

| Serial No. | Mycolic acid inhibitors | Geometric mean MIC (µg/ml) | | MIC Range ( µg/ml) | |
|---|---|---|---|---|---|
| | | Solid media | Liquid media | Solid media | Liquid media |
| 1 | INH | 0.08 | 0.02 | 0.025 to 0.1 | 0.012 to 0.05 |
| 2 | ETH | 3.5 | 0.54 | 1.2 to 10 | 0.3 to 2.5 |
| 3 | ISO | 1.07 | 0.50 | 0.3 to 2.5 | 0.3 to 2.5 |

In solid media inhibitory activity of ISO was13.38 times lower than the activity of INH, while it was 3.27 times higher than activity of ETH.MIC rage was narrower for ISO than ETH. In liquid media, Inhibitory activity of ISO was 25 times lower than activity of INH, while it was

1.08 times lower than activity of ETH. MIC range was similar for ISO than ETH. [Table 3]

**Table 4:** *MIC pattern of Mycolic acid inhibitors to susceptible strains of M. tuberculosis in solid and liquid media in combination*

| Group | Mycolic acid inhibitors | Geometric mean MIC (µg/ml) | | (+/-)SD | | P value | | Remark |
|---|---|---|---|---|---|---|---|---|
| | | Solid media | Liquid media | Solid media | Liquid media | Solid media | Liquid media | |
| 1 | INH<br>ISO | 0.8<br>1.07 | 0.02<br>0.50 | 0.1777<br>0.3830 | 0.01<br>0.52 | 2.8E-14 | 0.00038 | Difference is significant |
| 2 | ISO<br>ETH | 0.08<br>3.5 | 0.50<br>0.54 | 0.1777<br>0.2728 | 0.52<br>0.51 | 1.30E-25 | 0.604 | Difference is significant |
| 3 | INH<br>ETH | 1.07<br>3.5 | 0.02<br>0.54 | 0.3830<br>0.2728 | 0.01<br>0.51 | 4.11E-07 | .00017 | Difference is significant |

Difference between MIC values between INH and ISO; ISO and ETH; INH and ETH to susceptible strains of *M .tuberculosis* in solid and liquid media in combination was statistically significant. [Table 4]

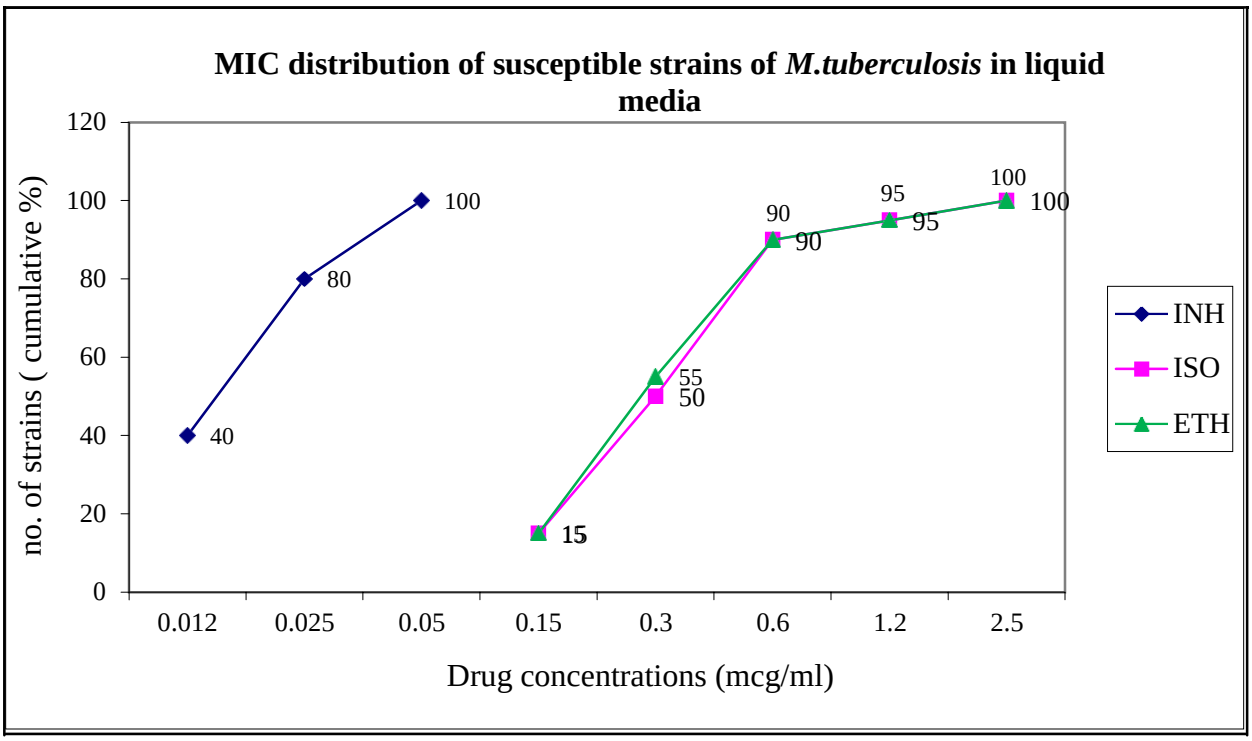


**Figure 3:** *MIC distribution of susceptible strains of M.tuberculosis in liquid media*

**Table 5:** *Comparison of MIC of Mycolic acid inhibitors to MDR and susceptible strains of M. tuberculosis in solid media*

| Drugs | Strains | GM MIC (µg/ml) | Ratio | (+/-)SD | P value | Remark |
|---|---|---|---|---|---|---|
| INH | Susceptible | 0.08 | 15.13 | 0.03 | 6.56E-07 | Difference is significant |
| | MDR | 1.21 | | 0.92 | | |
| ISO | Susceptible | 1.07 | 3.39 | 1.11 | 3.50E-07 | Difference is significant |
| | MDR | 5.76 | | 3.65 | | |
| ETH | Susceptible | 3.50 | 4.55 | 2.46 | 1.68E-05 | Difference is significant |
| | MDR | 15.94 | | 11.98 | | |

MIC ratio of ISO was lowest when compared for susceptible and MDR strains followed by ETH and INH in solid media. The difference between the MIC for each compound was statistically significant. [Table 5]

**Table 6:** *Comparison of MIC of Mycolic acid inhibitors to MDR and susceptible strains of M. tuberculosis in liquid media*

| Drugs | Strains | GM MIC (µg/ml) | Ratio | (+/-)SD | P value | Remark |
|---|---|---|---|---|---|---|
| INH | Susceptible | 0.02 | 14.5 | 0.014 | 1.97E-06 | Difference is significant |
| | MDR | 0.29 | | 0.233 | | |
| ISO | Susceptible | 0.50 | 3.24 | 0.52 | 3.14E-05 | Difference is significant |
| | MDR | 1.62 | | 1.066 | | |
| ETH | Susceptible | 0.54 | 4.65 | 0.511 | 6.21E-06 | Difference is significant |
| | MDR | 2.51 | | 1.776 | | |

MIC ratio of ISO was lowest when compared for susceptible and MDR strains followed by ETH and INH in liquid media. The difference between the MIC for each compound was statistically significant. [Table 6]

**Table 7:** *Comparison of activity of INH, ISO and ETH in solid and liquid media*

| Drugs | Medium | GM MIC (µg/ml) | Ratio | (+/-)SD | P value | Remark |
|---|---|---|---|---|---|---|
| INH | Broth | 0.20 | 4.1 | 0.23 | 1.21E-06 | Difference is significant |
| | Agar | 0.82 | | 0.92 | | |
| ISO | Broth | 1.24 | 3.35 | 1.06 | 4.88E-08 | Difference is significant |
| | Agar | 4.15 | | 3.76 | | |
| ETH | Broth | 1.83 | 6.37 | 1.74 | 9.96E-10 | Difference is significant |
| | Agar | 11.66 | | 11.44 | | |

MIC ratio of ISO was lowest when compared in solid and liquid medium to *M. tuberculosis* strains followed by INH and ETH. The difference between the MIC for each compound was statistically significant. [Table 7]

**Table 8:** *MBC pattern Mycolic acid inhibitors to MDR strains of M. tuberculosis*

| Sr. No. | Mycolic acid inhibitors | Geometric mean MBC (µg/ml) | MBC Range (µg/ml) |
|---|---|---|---|
| 1 | INH | 0.36 | 0.1 to 1.6 |
| 2 | ETH | 6.41 | 1.2 to 10 |
| 3 | ISO | 18.63 | 5.0 to 40 |

Bactericidal activity of ISO was 51.75 times lower than the activity of INH and 2.9 times lower than the activity of ETH.MBC range was wider for ISO than ETH. [Table 8]

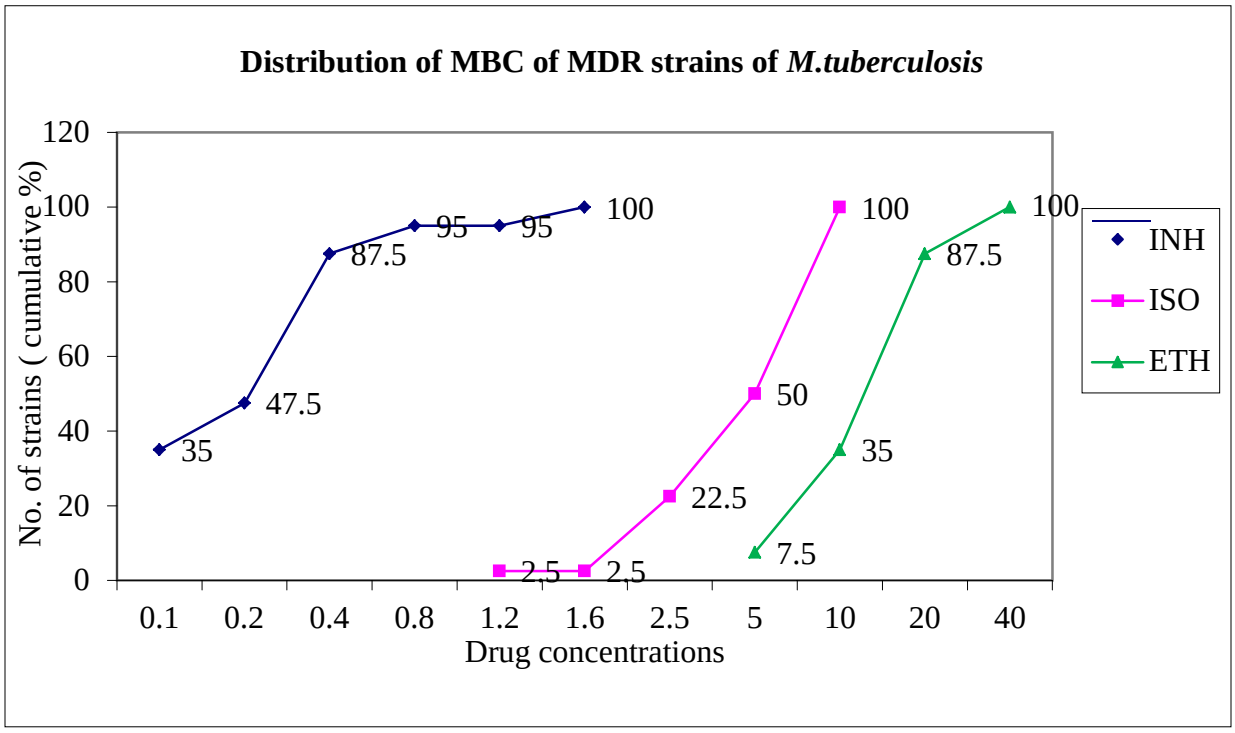


**Figure 4:** *Distribution of MBC of MDR strains of M. tuberculosis*

**Table 9:** *MBC pattern mycolic acid inhibitors to MDR strains of M. tuberculosisin combination*

| Group | Mycolic acid inhibitors | Geometric mean MBC (µg/ml) | (+/-) SD | P value | Remark |
|---|---|---|---|---|---|
| 1 | INH | 0.36 | 0.35 | 9.58E-15 | Difference is significant |
| | ISO | 18.63 | 9.74 | | |
| 2 | ISO | 18.63 | 9.74 | 4.30E-09 | Difference is significant |
| | ETH | 6.41 | 3.14 | | |
| 3 | INH | 0.36 | 0.35 | 8.42E-15 | Difference is significant |
| | ETH | 6.41 | 3.14 | | |

Difference between MBC values between INH and ISO; ISO and ETH; INH and ETH to MDR strains of *M .tuberculosis* in combination was statistically significant. [Table 9]

**Table 10:** *MBC pattern mycolic acid inhibitors to susceptible strains of M. tuberculosis*

| Serial No. | Mycolic acid inhibitors | Geometric mean MBC(µg/ml) | MBC Range (µg/ml) |
|---|---|---|---|
| 1 | INH | 0.03 | 0.1 to 1.6 |
| 2 | ETH | 2.26 | 1.2 to 10 |
| 3 | ISO | 6.40 | 5.0 to 40 |

Bactericidal activity of ISO was 213 times lower than the activity of INH and 2.83 times lower than the activity of ETH.MBC range was wider for ISO than ETH [Table 10]

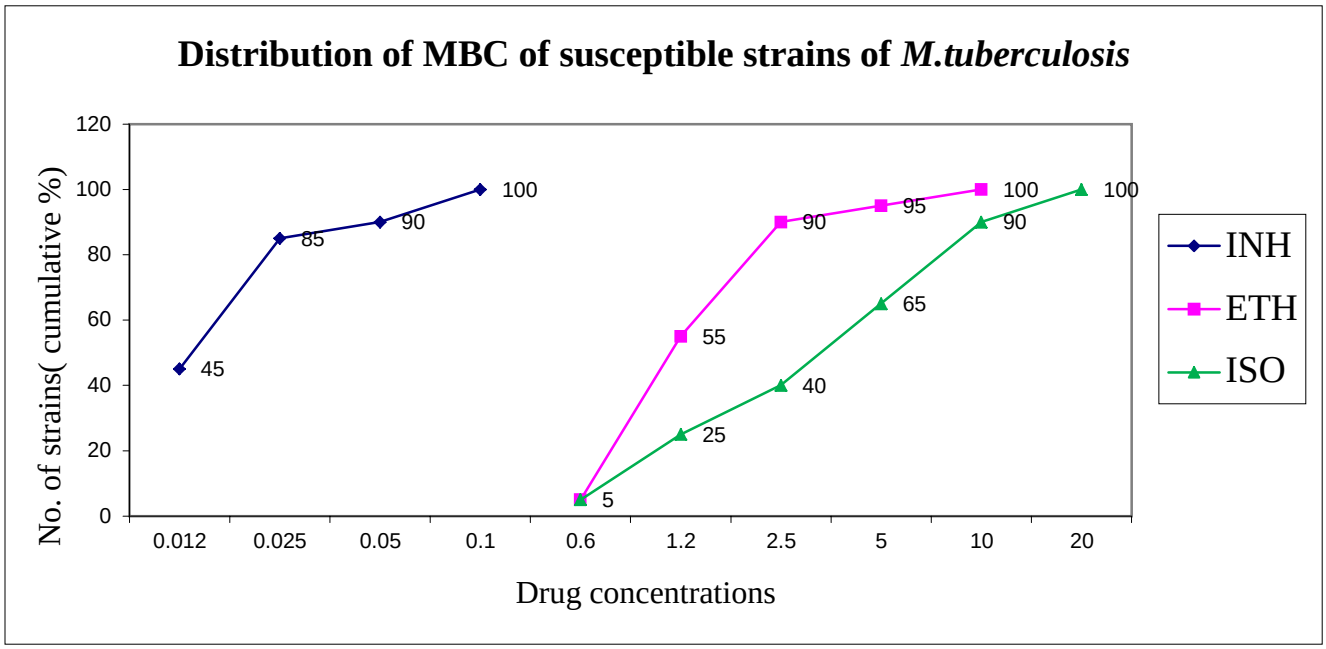


**Figure 5:** *Distribution of MBC of susceptible strains of M. tuberculosis*

**Table 11:** *MBC pattern mycolic acid inhibitors to susceptible strains of M. tuberculosis in combination*

| Group | Mycolic acid inhibitors | Geometric mean MBC | (+/-)SD | P value | Remark |
|---|---|---|---|---|---|
| 1 | INH | 0.03 | 0.03 | 9.24E-05 | Difference is significant |
| | ISO | 6.40 | 5.77 | | |
| 2 | ISO | 6.40 | 5.77 | 0.011 | Difference is significant |
| | ETH | 2.26 | 2.08 | | |
| 3 | INH | 0.03 | 0.03 | 0.000126 | Difference is significant |
| | ETH | 2.26 | 2.08 | | |

Difference between MIC values between INH and ISO; ISO and ETH; INH and ETH to susceptible strains of M .tuberculosis in solid and liquid media in combination was statistically significant. [Table 11]

**Table 12:** *MIC/MBC Ratio*

| Serial no. | Mycolic acid inhibitors | MDR strains | Susceptible strains |
|---|---|---|---|
| 1 | INH | 1.24 | 1.5 |
| 2 | ETH | 2.55 | 4.18 |
| 3 | ISO | 11.5 | 11.85 |

Highest MIC/MBC ratio was shown by ISO followed by ETH and the INH [Table 12]

**Table 13:** *Comparison of MBC of ISO against MDR and susceptible (s)strains of M.tuberculosis*

| Drugs | Strains | Mean | Ratio | (+/-)SD | P value | Remark |
|---|---|---|---|---|---|---|
| INH | S | 0.03 | 12 | 0.03 | 8.44E-05 | Difference is significant |
| | MDR | 0.36 | | 0.35 | | |
| ISO | S | 6.40 | 2.9 | 5.77 | 3.05E-06 | Difference is significant |
| | MDR | 18.63 | | 9.74 | | |
| ETH | S | 2.26 | 2.84 | 2.08 | 1.63E-06 | Difference is significant |
| | MDR | 6.41 | | 3.14 | | |

MBC ratio of ETH was lowest when compared for susceptible and MDR strains followed by ISO and then INH in liquid media. The difference between the MBC for each compound was statistically significant. [Table 13]

## 4. Discussion

MIC is the method used for evaluation of the antimicrobial activity of conventional and experimental agents. The results obtained by these techniques can provide information on the inhibitory (bacteriostatic) activity of an agent if the test is performed in a qualitative manner

and if inactivation of a drug in the medium is minimal, allowing the obtained MIC values to be reasonably compared with the pharmacokinetic data (Andrews, 2001).

In present study, total 40 MDR and 20 susceptible strains of *M. tuberculosis* were processed to determine in vitro inhibitory activity of ISO, INH and ETH in solid and liquid media. Values obtained in the present study for MIC's of INH and ETH in solid and liquid media for MDR and susceptible strains of *M tuberculosis* are comparable with various studies (Heifets, 1991).

We reported potent anti-mycobacterial activity of ISO against all MDR and susceptible strains of *M. tuberculosis*. They were susceptible in the range of 1 to 20 µg/ml**.** MIC values for susceptible strains were significantly lower than MDR strains of *M. tuberculosis* in solid media. Many authors in the past have shown powerful inhibitory action of ISO on human species of tubercle bacilli in vitro with minimum inhibitory concentration varying from 0.15 to 1.2 µg/ml in various media (Robinson, & Hunter, 1966).Another study showed MIC range of INH resistant strains to be 0.25 to 2 mg/ml for ISO and 1.25 to 10 µg/ml for ETH (Debarber, A. E., Mdluli, K., Basman, M. Bekkar, L. and Barry, C.E. (2000).

While in recent study it was reported that, ISO showed anti-mycobacterial activity against all strains of *M. tuberculosis* resistant to major first-line drugs and they were susceptible in the range of 1 to 10 mg/ml (Phetsuksiri et al., 1999). Another study showed MIC range of INH resistant strains to be 0.25 to 2 mg/ml for ISO and 1.25 to 10 µg/ml for ETH (Debarber, A. E., Mdluli, K., Basman, M. Bekkar, L. and Barry, C.E, 2000).We have reported the MIC range of ISO from 0.3 to 20 µg /ml

Our data particularly the MICs found in either in Dubos broth or 7H10 agar plates were in agreement with other author's data on MICs found in other types of liquid and solid media (Lord, 1966; Phetsuksiri et al., 1999). Some authors reported MIC range of ISO from 3.75 to 40

µg/ml, they lie within the value of attainable serum concentrations (Lord, 1966). These variations in the MIC range against strains of *M. tuberculosis* might be due to the geographical variations in the strains under study. The important point here is that ISO has potent antimycobacterial activity against MDR strains of *M. tuberculosis*.

We have reported the MIC range of ISO from 0.3 to 20 µg /ml in solid media, while in liquid media MIC range was 0.3to 5 µg/ml for MDR and susceptible strains of *M. tuberculosis.*

ISO was significantly more active on susceptible strains than MDR strains of *M. tuberculosis* in solid and liquid media .These variations in MIC values for ISO in solid media and liquid media could be due the use of different methodologies to determine MIC and the type of media used for it. The inhibitory action of ISO on MDR and susceptible strains of *M. tuberculosis* in liquid media seems to be significantly higher than in solid media. The difference between broth and agar determined MICs was related to the bactericidal potency of the drug in the type of medium used. This difference was minimal for ISO and larger for ETH, with INH in the intermediate position.

Some studies suggest that it is more favorable to use solid media when testing ISO. A fluid medium is not convenient for the examination of sensitivity because of a great asymmetry of the distribution of the MIC (Lord, 1966). Our study reported MIC of ISO for *M. tuberculosis* H37Rv strain to be 1.5µg/ml. While MIC value of ISO for *M. tuberculosis* H37Rv strain was in the range of 1.0 to 2.5 µg/ml compared to published values of 0.02 to 0.2 µg/ml to INH & 5 to 10 mg/ml for ETH (Lord, 1966).

Our study showed that inhibitory activity of ISO was higher than activity of ETH in solid media for MDR and susceptible strains of *M. tuberculosis*. While it was almost similar to the activity of ETH in liquid media for susceptible strains of *M. tuberculosis*. No cross resistance was observed between ISO and ETH for strains of *M. tuberculosis.* While it is reported that cross resistance between Thiacetazone, ISO and ETH exist but not in 100 % of the cases (Gallen, 1970). With regard to the cross resistance between ISO and ETH study in the past, shows there seems to be a difference between strains with primary resistance and strains with acquired resistance to these drugs. None of the strains with primary resistance showed cross-resistance, while strains with acquired resistance to ETH after treatment with this drug, showed cross- resistance to ISO that was not administered (Meissner & Stottmeier, 1965). Experiments have shown that the tuberculostatic activity of ISO clearly exceeds that of PAS and is comparable with that of ETH (Hekking & De Voogd, 1970).

ISO, one of the second line drugs proved to be very useful in the treatment of pulmonary TB, in the past, particularly as a substitute for PAS, when later was not tolerated for some reason. In the case of resistance to one or several first line drugs, too, it is advisable to use ISO in combination with other second line drugs. The tuberculostatic effect of ISO slightly

exceeds that of PAS, while its toxicity is less than that of PAS (Hekking & De Voogd, 1970).

The therapeutically efficacy of ISO, both in vitro and in vivo, has been clearly demonstrated by some studies (Robinson & Hunter, 1966).Thus the results of the MIC studies show that ISO is capable of inhibiting the growth of various Mycobacteria within a narrow range of low concentrations. The suggestion is that ISO may be suitable for the treatment of TB, particularly multi-drug resistant kind.

In our study we tested total 40 MDR and 20 susceptible strains of *M. tuberculosis* to determine MBC of INH, ETH and ISO. Amongst three antituberculosis drugs, INH showed highest bactericidal activity against *M. tuberculosis* strains followed by ETH. While, ISO showed lowest bactericidal activity. ISO was found to be significantly more bactericidal to susceptible strains than MDR strains of *M. tuberculosis* like INH and ETH. By describing the results of the MIC and MBC determinations, categorization of a drug is avoided as bactericidal and bacteriostatic. It can be assumed that it is most likely that the drug whose MBC is within the concentration achievable in blood or tissue, will produce a killing effect against the bacteria actively multiplying in vivo.

Still, such a drug may not be able to kill some parts of the bacterial populations in vivo, particularly the bacteria in a semi-dormant or dormant state or multiplying within macrophages. On the other hand, it is difficult to imagine that the drug whose MBC is very much greater than its achievable concentration would produce any substantial killing effect in vivo. Conclusions, about the bactericidal potency of certain drugs, derived from in vivo observations, sometimes contradict this logic. In the literature on tuberculosis, the sterilizing activity of a drug in vivo (in patients or in mice) is often interpreted as its bactericidal activity. The sterilizing activity of a drug measures the duration of treatment and is therefore crucial in the design of short -course regimen .The early bactericidal activity of drug has, however, little bearing on its use in therapy, except perhaps as an indication of the period during which a patient may be considered to be infectious to others.

It can be concluded that despite similarities in mode of action of INH, ETH and ISO, they showed different activity in vitro, especially the bactericidal activity. The difference between broth and agar determined MICs was related to the bactericidal potency of the drug. This variation could be due the use of different methodologies to determine MIC.

The MBC/MIC ratio is the usual standard for expression of bactericidal potency. This

ratio can be used to estimate the probability of a bactericidal effect upon the patient's strain if the degree its susceptibility has been expressed as the MIC (Mitchison, 1979).

The high bactericidal activity of INH was suggested in several publications (Middlebrook, 1952). Though the results usually have not been described in MBC values or in MBC /MIC ratios, many authors have reported that INH kills most of the bacterial population at very low concentrations, equivalent to the MIC or only slightly higher. Same observations were made in the present study. ETH has often been considered primarily a bacteriostatic drug due to its low sterilizing activity in vivo, though tuberculocidal concentrations can be reached with doses applicable to animals and man (Offen, 1988). MBC /MIC ratios for ETH were found to be in the range of 3 to 4.5 in the present study. Similar observations were made in other studies (Offen, 1988). These data confirms that ETH can produce a bactericidal effect (99%) killing in concentrations close to those attainable in vivo, especially when higher dosages are administered. Therefore, despite the relatively low sterilizing activity of ETH *in vivo*, this drug should not be labelled as bacteriostatic only.

MBC /MIC ratio was found to be high for ISO indicating low bactericidal activity of this drug. This ratio was similar for susceptible and MDR strains of *M. tuberculosis*. These data show that ISO cannot produce 99% killing in concentrations close to those attainable in vivo, especially when lower doses are administered. These findings can be correlated by past clinical studies, in which ISO was found to be more bacteriostatic than bactericidal drug. One study reported ISO as one of the second line drugs, proved to be useful in the treatment of pulmonary TB, particularly as a substitute for PAS, when later was not tolerated for some reason (Hekking & Voogd, 1970)**.** It was advisable in the case of resistance to one or several first line drugs, too, to use ISO in combination with other second line drugs.

## 5. Conclusion

This study reports potent anti-mycobacterial activity of ISO against MDR and susceptible strains of *M. tuberculosis*. They were susceptible in the range of 1 to 20 µg/ml and 0.3 to 5 µg/ml in solid and liquid medium respectively.. There was significant difference in MICs of MDR strains and susceptible strains of *M. tuberculosis* in solid and liquid media. Amongst three antituberculosis drugs, INH showed highest bactericidal activity against *M. tuberculosis* strains followed by ETH. While ISO showed lowest bactericidal activity. ISO

showed significantly lower bactericidal activity against MDR strains than susceptible strains of *M. tuberculosis.*

.Following conclusions can be drawn from these studies. Inhibitory activity of ISO is better than ETH in solid media and it is far better for susceptible strains in comparison to ETH. Inhibitory activity of ISO was similar to ETH for susceptible strains of *M. tuberculosis* in liquid media. No cross-resistance between ISO, INH and ETH was observed. Though inhibitory activity of ISO seems to be comparable to ETH, it is more of a bacteriostatic drug than bactericidal. ISO showed significantly lower bactericidal activity against MDR strains than susceptible strains of *M. tuberculosis.* .Despite similarities in mode of action of INH, ETH and ISO, they showed different activity in vitro, especially the bactericidal activity.

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