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Ronald Bartzatt
*University of Nebraska at Omaha*

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# Potency and Properties of Hydrazide Compounds That Inhibit the Growth of *Mycobacterium tuberculosis*

**Ronald Bartzatt**[1*]

[1]*Durham Science Center, University of Nebraska, 6001 Dodge Street, Omaha, Nebraska 68182, USA.*

***Author's contribution***

*The sole author designed, analyzed and interpreted and prepared the manuscript.*

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## ABSTRACT

**Aims:** To examine the properties of hydrazide compounds shown to inhibit *Mycobacterium tuberculosis*. To identify properties that affect efficiency of bacterial inhibition.
**Study Design:** Utilizing data from previous studies of compounds that inhibit *Mycobacterium tuberculosis*, then statistical and pattern recognition methods are applied to identify interrelationships.
**Place and Duration of Study:** Department of Chemistry, Durham Science Center, University of Nebraska at Omaha, from January 2016 to July 2016.
**Methodology:** Interrelationships of pharmacological properties were identified by use of various pattern recognition techniques, such as hierarchical cluster analysis and path analysis. Molecular properties and descriptors for all compounds were determined, with additional characteristics such as structure scaffolding and functional group position was accomplished. Statistical analysis, including Pearson r correlation, Mann-Whitney test, one-way ANOVA, Kruskal-Wallis, and descriptive statistics were determined. Multiple regression analysis of molecular property values allows prediction of similar compounds. Determination of any numerical outliers was accomplished by applying Grubb's test.

**Corresponding author: E-mail: rbartzatt@unomaha.edu;*

**Results:** *Mycobacterium tuberculosis* inhibiting compounds contained either an aromatic ring or were non-aromatic structures (no ring). There was weak negative correlation of MIC to formula weight. The average formula weight, polar surface area, and Log P, is 183.55 grams/mole, 63.70 $A^2$, and 0.768, respectively. Values of MIC ranged from 14.7 µg/mL to 100 µg/mL. Extent of bacterial inhibition was similar between aromatics to non-aromatics. No outliers were identified by Grubb's test for all values of MIC taken together. Path analysis showed polar surface area to have most effect on MIC.

**Conclusion:** The measured level of growth inhibition MIC, showed strong positive relationship to polar surface area, number of hydroxyl and amine groups, oxygen and nitrogen atoms. Two aromatic compounds having a pyridine ring were found to be most similar to isoniazid. Aromatic and non-aromatic compounds showed similar levels of bacterial inhibition overall.

*Keywords: Tuberculosis; TB; hydrazides; Mycobacterium tuberculosis.*

## ABBREVIATIONS

*PSA: Polar surface area; FW: Formula weight; nOHNH: Number of hydroxyl and amine groups; nON: Number of oxygen and nitrogen atoms; MIC: Minimum inhibitory concentration; MDR-TB: Multi-drug resistant tuberculosis; XDR-TB: Extensively drug resistant tuberculosis; CNS: Central nervous system; TDR-TB: Totally drug-resistant tuberculosis.*

## 1. INTRODUCTION

The clinical treatment and control of tuberculosis disease is significantly threatened with the appearance of multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) [1]. The appropriate use of second-line drugs is required to treat cases of MDR-TB, and to inhibit the proliferation of XDR-TB [1]. The World Health Organization has been surveilling the rate of resistant tuberculosis in numerous countries, and an estimate of over four million cases of MDR-TB is thought to emerge in 2006 [1]. The tuberculosis bacterial infection of the central nervous system (CNS) accounts for about 1% of all cases of tuberculosis [2]. The CNS case of tuberculosis is highly devastating, causing high mortality and neurological damage [2]. Measurement of this extra pulmonary infection within the United States showed that 5% to 10% of these cases involved the CNS [2]. CNS tuberculosis exists primarily as tuberculous meningitis, and less as encephalitis, intracranial tuberculoma, or tuberculous brain abscess [2].

Treatment of CNS associated tuberculosis infection requires a minimum of 10 months of treatment [3]. Early treatment of CNS tuberculosis is vital and dramatically improves patient outcome [4]. In incidences of tuberculosis meningitis there is a thick gelatinous exudate in the proximity of basal cisterns, brainstem, and cerebellum [2,4]. Treatment of tuberculosis meningitis is usually followed with treatment with isoniazid, rifampicin, pyrazinamide, and ethambutol for a period of two months, then isoniazid and rifampicin only for an additional ten months [5]. The addition of aspirin in treatment has been shown to improve mortality rates by inhibiting complicating infarctions [6]. Children, especially those less than 1 year of age, are particularly at risk for CNS tuberculosis [7].

Totally drug-resistant tuberculosis (TDR-TB) have been identified in Italy, Iran, India, and South Africa [8]. TDR-TB is described as resistance to all first-line and second-line drugs utilized in clinical treatment [8]. Extra pulmonary tuberculosis can coexist simultaneously with pulmonary tuberculosis [9]. It is the high lipid content of the bacterial membrane that contributes to the difficulty of treating tuberculosis [10]. Patients having latent tuberculosis infection are not considered to be contagious to others, but 10% of these cases will move on to develop active tuberculosis infection [11]. Various symptoms of CNS involved tuberculosis infection are the following: brain abscesses, encephalopathy, encephalitis, and arteritis [12].

With threats such as multi-drug resistant, extensively drug resistant, and totally drug resistant tuberculosis, the investigation and development of new clinical drugs are an urgent necessity to confront these forms of tuberculosis. Novel structure scaffolding of compounds have been evaluated for bacterial inhibition and potential use as clinical drugs, have been shown to be effective for inhibiting tuberculosis

[13,14,15]. The design and testing of novel molecular structures has been shown to be effective inhibitors of other bacterial infections such as *Staphylococcus aureus* [16,17,18], and *Escherichia coli* [19,20,21,22]. Clearly, the investigation of new molecular structures have benefitted the identification of powerful inhibitors of bacterial infection. Novel analytical methods to identify functional groups have accompanied the generation of novel hydrazide-type drug designs that assist in synthesis, quality control, and assay [23]. This study examines the contribution of drug molecular properties to the effectiveness of their inhibition of *Mycobacterium tuberculosis*.

## 2. METHODOLOGY

### 2.1 Properties and Molecular Modeling

Molecular properties and descriptors of all compounds were determined by utilizing Molinspiration (Molinspiration Cheminformatics, Nova ulica 61, SK-0026 Slovensky Grob, Slovak Republic) http://www.molinspiration.com/cgi-bin/properties. Additional characteristics was accomplished through the use of ACD ChemSketch Modeling v. 12.01 (Advanced Chemistry Development, 110 Yonge Street, Toronto Ontario, M5C 1T4 Canada, http://www.molinspiration.com/services/search.html).

### 2.2 Pattern Recognition and Multivariate Analysis

Numerical properties can demonstrate underlying patterns and relationships which can be identified by use of various pattern recognition techniques. Hierarchical cluster analysis cluster analysis was performed by KyPlot version 2.0 beta 15 (copyright Koichi Yoshioka 1997-2001). Various statistical tests were performed by PAST version 2.06 (copyright Oyvind Hammer, D.A.T. Harper 1999-2008). Path analysis was determined by utilzing OpenStat (copyright William Miller, September 11, 2008).

### 2.3 Various Statistical Analysis of Data

Statistical analysis, including Pearson r correlation and descriptive statistics were accomplished by Microsoft EXCEL v.14.0.6112.5000 (EXCEL Professional plus 2010). Multiple regression analysis of molecular property values was accomplished by GraphPad Instat version 3.00 (GraphPad Software, Inc., San Diego, California USA; www.graphpad.com). Determination of any numerical outliers was accomplished by applying Grubb's test (also known as extreme studentized deviate) http://www.graphpad.com/quickcalcs/index.cfm. Mann-Whitney test, Kruskal-Wallis test, one-way ANOVA, F and T test, were determined by PAST version 2.06.

## 3. RESULTS AND DISCUSSION

### 3.1 Structure and Properties

The investigation of novel drugs for the treatment of tuberculosis is a vitally important endeavor due to the burden of the disease worldwide and the appearance of multi-drug resistant, extensively drug resistant, and totally drug resistant tuberculosis [1,8]. In this study, a group of novel hydrazide compounds have been compared to the first-line drug isoniazid for efficiency of *Mycobacterium tuberculosis* inhibition. The effectiveness of inhibition is measured by in vitro method, with effectiveness measured as MIC for comparison.

The structures of all compounds tested in vitro and evaluated [13,14,15], are presented in Fig. 1, for comparison to the first-line drug isoniazid (compound 14) and other hydrazide compounds. Characteristic of all the compounds shown is the hydrazide group ($-C(=O)-NHNH_2$) that is a substituent of the molecular structures. Compounds 1, 2, 3, 4, 8, 9, 10, 13, and 14 (isoniazid) have aromatic ring (contain conjugated planar ring systems), with compounds 8, 13, and 14 being heterocyclic compounds having a nitrogen atom substitution within the ring (see Fig. 1).

Compounds 5, 6, 7, and 11 have aliphatic chains (chain of $–CH_2-$), albeit compound 5 has a terminal bromine atom. Aliphatic groups are nonpolar and hydrophobic, with hydrophobicity increasing with increasing number of C atoms in the hydrocarbon chain. Hydrophobicity reduces aqueous solubility and increases lipophilic character. More lipophilic character is generally viewed as increasing the ability of a drug to penetrate the membrane bilayer. Each of these compounds have the hydrazide group ($-C(=O)-NHNH_2$). Compounds 1, 2, 4, 9, and 10 have substituents covalently bonded to an aromatic ring: methyl-, methyl-, chloro-, 2-methylpropyl-, and acetate, respectively. Compounds 12 has two hydrazide groups. All other compounds have only a single hydrazide group.

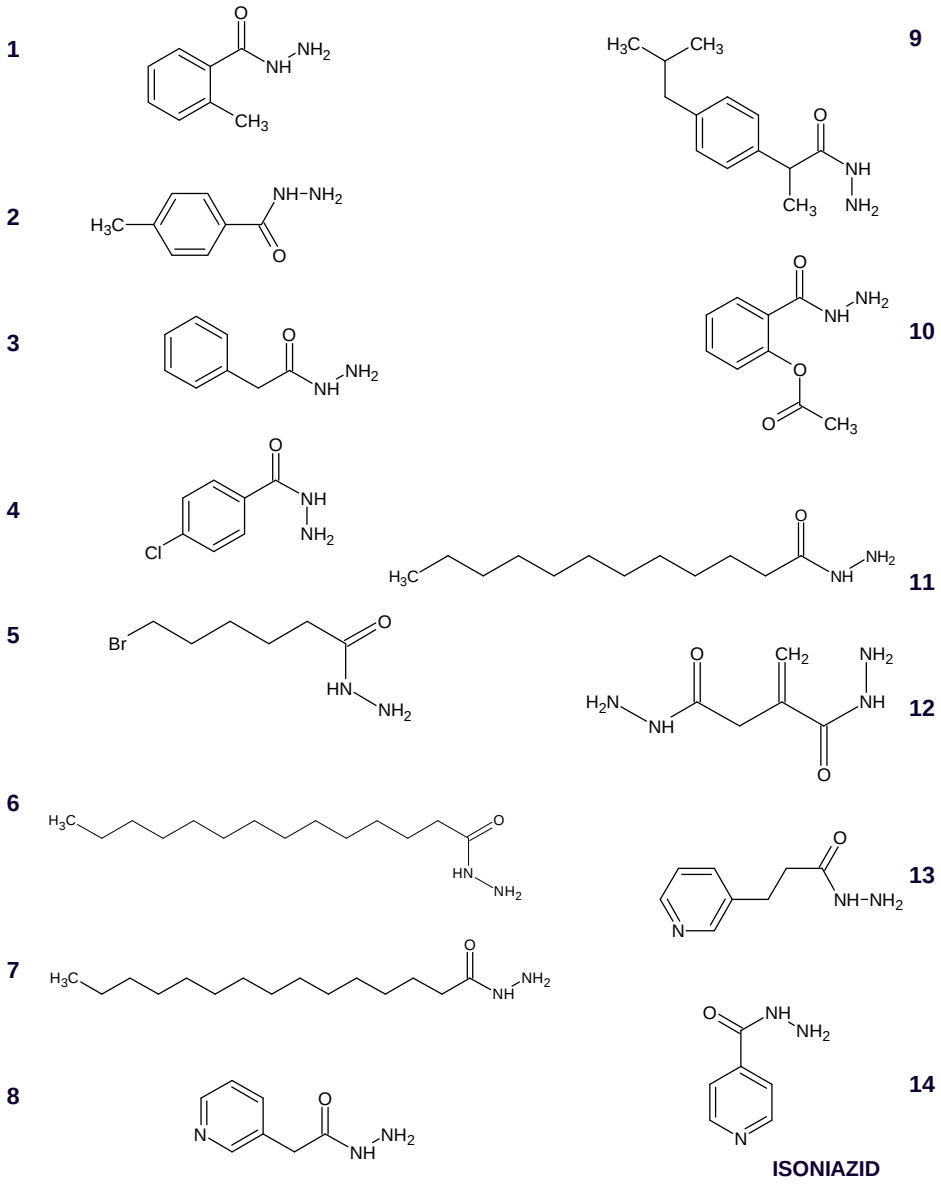


**Fig. 1. Compounds previously shown to inhibit the growth of *Mycobacterium tuberculosis* [13,14,15]. All compounds possess the hydrazide group (-C(=O)NHNH$_2$). Compounds 1, 2, 3, 4, 8, 9, 10, 13, and 14 are aromatic organic compounds. Compounds 5, 6, 7, 11, and 12 are non-aromatic organic compounds. Compound 12 bears two hydrazide functional groups**

The comparison of aromatic and nonaromatic molecular scaffolding for delivery of the hydrazide group will elucidate the effects upon the molecular properties of the drug, such as: Log P, polar surface area, formula weight, number of hydroxyl, and number of amine groups.

In turn, these aspects of the molecular structure have been shown to influence the drug likeness (i.e. a qualitative concept for how drug like a compound in respect to factors like bioavailability). Various criteria have been developed to screen a potential drug for drug likeness, such as the rule of five, which states that poor absorption or permeation is more likely for a drug candidate when there are more than [24]: 1) Five hydrogen bond donors; 2) Ten hydrogen bond acceptors; 3) The molecular weight is greater than 500; and 4) Calculated Log

P (CLogP) is greater than 5. Zero violations of the rule of five indicates favorable drug likeness, with favorable absorption or permeation [24]. Shown in Table 1, compound 12 demonstrates only one violation of the rule of five, however, all other compounds demonstrate zero violations. This outcome indicates that these aromatic and nonaromatic compounds will have favorable absorption and membrane permeation.

The molecular properties and MIC (compound concentration when only 50% of the bacteria in culture are viable) of all compounds are presented for comparison in Table 1. A multivariate table permits the pattern recognition of underlying relationships as well as statistical analysis. For all these hydrazide compounds the average MIC, formula weight, Log P, and polar surface area are 46.15 µgram/mL, 183.55, 0.767, and 63.70 Angstroms$^2$, respectively. Notably, only compound 12 has one violation of the rule of five, whereas, all the others have zero violations, indicating favorable drug likeness and favorable drug absorption.

The addition of a second hydrazide group (-C(=O)-NHNH$_2$) onto the scaffold for compound 12 increased (doubled) the number of hydrogen bond donors (-NH$_n$, -OH) and hydrogen bond acceptors (oxygen and nitrogen atoms).

These 14 compounds have some level of similarity in molecular properties but vary in values of MIC. For example, applying the Grubb's test for detecting outliers [25], no outliers were found in numerical values of Log P, formula weight, and MIC. Compound 12 is an outlier for values of polar surface area, number of –OH and –NH$_n$, as well as number of oxygen & nitrogen atoms. Interestingly, the sum of five of the molecular properties (excluding MIC and rule of five) show similar means for these 14 compounds, a result calculated by one-way ANOVA analysis ($P$ = 1.0) [25].

All 14 compounds have been tested by in vitro methods for the magnitude of growth inhibition of *Mycobacterium tuberculosis* [13,14,15]. The individual MIC values for each compounds is presented in Fig. 2 bar graph for comparison to isoniazid and other compounds. The overall mean for the MIC values obtained from aromatic compounds is 47.66 µg/mL, which is very close to the average MIC for nonaromatic compounds to be 43.43 µg/mL. The overall values of all MIC values taken together have no outlier in numerical values, determined by Grubb's test [25].

Interestingly, there appears to be no advantage overall in MIC values of either type, aromatic or nonaromatic, compared to the other. Although the MIC values vary widely individually, from 14.7 µg/mL (isoniazid) to 100 µg/mL (9 and 10), when comparing aromatic and nonaromatic group values of MIC, the F and T test and one-way ANOVA show that the two group means of MIC are equal ($P$ =.82 and P= .82, respectively). In addition, the two group values in MIC have equal medians by the Mann-Whitney test ($P$ = .68) and Kruskal-Wallis test ($P$ = .64) [25].

Therefore, the overall effectiveness of these aromatic compounds is considered statistically to be essentially equal overall to the nonaromatic compounds. However, clearly there are substantial improvements in bacterial inhibition, indicated by MIC values, from individual drug scaffolding and design. For example, clearly for aromatic compounds the drugs 14, 8, 3, and 13 have a substantially lower MIC than compounds 9 and 10. Likewise, for MIC values for nonaromatic compounds, drugs 11, 6, and 7 have substantially smaller MIC than for compound 12 (see Fig. 2).

This study comparing aromatic scaffolding and non-aromatic scaffolding clarifies structural features important for potential clinical application. Even though the aromatic type compounds are overall statistically equal to nonaromatic compounds, it is still very easy to see structure substituents, size in formula weight, Log P, and polar surface area can still be selected to increase effectiveness in bacterial inhibition and smaller MIC.

For example, in the aromatic group of compounds the three lowest MIC occur for compounds 8, 13, and 14. All three of these compounds possess a single pyridine ring with the hydrazide group covalently bonded. Compounds 8 and 13 have a single methylene bridge and 2 methylene bridge groups, respectively. Compounds 8 and 13 are two members of a homologous series having a constant unit of –CH$_2$- [26]. For many series of compounds lengthening by adding –CH$_2$- to a chain produces an increase in pharmacological effects [26]. Although compound 13 has greater formula weight than that of compound 8, the value of MIC does not increase with an increase in formula weight.

**Table 1. Molecular properties and MIC for compounds**

| Compound | MIC (microgram per milliliter) | Formula weight | Log P | Polar surface area (Angstroms$^2$) | Number of –OH and -$NH_n$ | Number of oxygen and nitrogen atoms | Violations of rule of 5 |
|---|---|---|---|---|---|---|---|
| 1 | 47.1 | 150.2 | 0.721 | 55.121 | 3 | 3 | 0 |
| 2 | 38.6 | 150.2 | 0.769 | 55.121 | 3 | 3 | 0 |
| 3 | 28 | 150.2 | -0.226 | 55.121 | 3 | 3 | 0 |
| 4 | 47.1 | 170.6 | 0.998 | 55.121 | 3 | 3 | 0 |
| 5 | 47.1 | 209.1 | 0.258 | 55.121 | 3 | 3 | 0 |
| 6 | 26.7 | 242.41 | 4.46 | 55.121 | 3 | 3 | 0 |
| 7 | 26.7 | 256.434 | 4.97 | 55.121 | 3 | 3 | 0 |
| 8 | 26.7 | 151.17 | -1.463 | 68.013 | 3 | 4 | 0 |
| 9 | 100 | 220.32 | 1.874 | 55.121 | 3 | 3 | 0 |
| 10 | 100 | 194.19 | 0.372 | 81.426 | 3 | 5 | 0 |
| 11 | 16.7 | 214.33 | 3.449 | 55.121 | 3 | 3 | 0 |
| 12 | 100 | 158.16 | -3.52 | 110.24 | 6 | 6 | 1 |
| 13 | 26.7 | 165.2 | -0.945 | 68.013 | 3 | 4 | 0 |
| 14 isoniazid | 14.7 | 137.14 | -0.969 | 68.013 | 3 | 4 | 0 |

*$A^2$ = Angstroms$^2$*

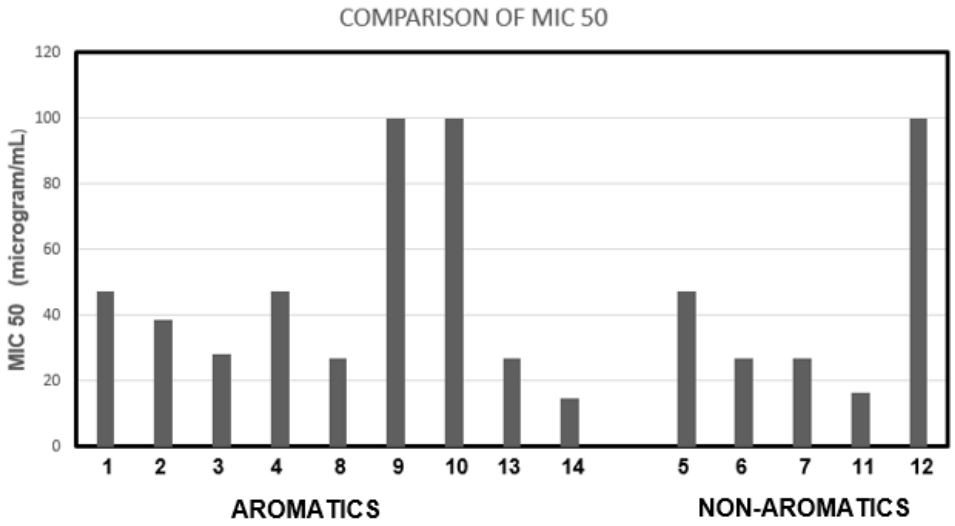


**Fig. 2. Comparison of MIC for compounds 1 to 14 (see Fig. 1). Aromatic compounds have at least one aromatic ring. Non-aromatics possess no aromatic ring. Statistical analysis is administered to compare results of aromatic and non-aromatic**

## 3.2 Numerical Analysis of Properties

Pattern recognition is the process of classifying multivariate data into objects or groups based on key properties. There are two classification methods in pattern recognition: supervised and unsupervised classification [25]. The supervised classification of input data into the pattern recognition method uses supervised learning algorithms that create classifiers based on training data. The classifier then accepts input data and will assign the object to appropriate group [25]. The unsupervised classification method works by locating hidden structures in unlabeled data by segmentation or clustering techniques.

Hierarchical cluster analysis is a method of cluster analysis which seeks to build a hierarchy of clusters [25]. Objects are classified into clusters containing members having highest similarity based on properties collected into a multivariate table (see Table 1). Hierarchical cluster analysis of Table 1 properties, produces

the vertical dendrogram results in Fig. 3. Conditions of cluster analysis are single-linkage clustering (similarity of two clusters is based on the similarity of their members) and Euclidean distance (straight-line distance between two points) [25].

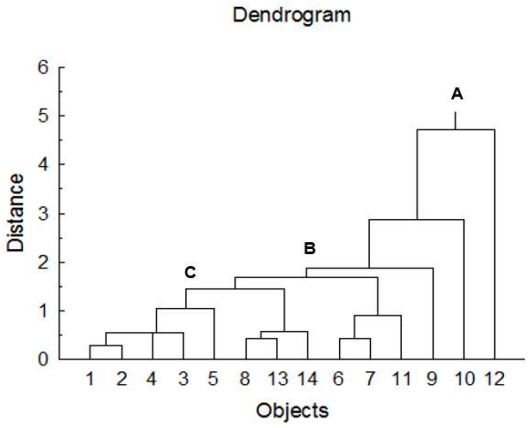


**Fig. 3. Vertical dendrogram showing results of hierarchical cluster analysis of molecular properties shown in Table 1. Note initial node A is divided to distinguish compound 12 from the all the remaining compounds. Node B encompasses all other compounds save for compound 12 and 10 that are distinguished. Within node B the clusters show compounds 6, 7, and 11 are most similar (with compound 9 distinct). Node C encompasses cluster 8, 13, and 14 to be most similar; with cluster 1, 2, 4, 3, and 5 to be most similar**

Beginning with node A containing all compounds, they are distinguished into compound 12 (separate from all others), compound 10, and node B. Under node B, there is further distinction into compound 9 and cluster of compounds 6, 7, and 11 (most similar). Under node C further distinction and classification by similarity shows compounds 8, 13, and 14 (most similar). Clustering continues with most similar compounds 1, 2 (most similar), 4, 3, and 5. This method of numerical analysis shows, based on property values, which compounds are most similar and potentially equal in activity [26].

Path analysis is an extension of multiple regression. Its aim is to provide estimates of the magnitude and significance of hypothesized causal connections between sets of properties [27]. Path analysis of properties in Table 1 produced causal connections to MIC presented in Fig. 4. By this analysis, clearly the polar surface area property has highest causal connection to MIC, having path coefficient of 109.144. Interestingly, the lowest value at -85.214 is number of oxygen & nitrogen atoms. The second to lowest is number of –OH & -$NH_n$ groups at -29.572. Path analysis can reveal the possible causal connections of properties, which in turn can indicate causality to MIC.

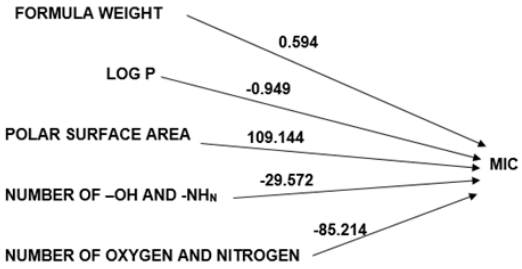


**Fig. 4. Path analysis of molecular properties and their association to MIC values, to determine the most influential property. Polar surface area is clearly shown to have greatest influence to MIC with path coefficient of 109.144. Properties quantifying oxygen atoms, nitrogen atoms, hydroxyl groups, and amine groups are clearly of substantially lesser influence (smaller path coefficients)**

### 3.2.1 Multiple regression analysis for prediction

Multiple regression is a statistical tool that allows you to examine how multiple independent variables are related to a dependent variable. Once you have identified how these multiple variables relate to your dependent variable, you can take information about all of the independent variables and use it to make much more powerful and accurate predictions concerning these variables. It is used when we want to predict the value of a variable based on the value of two or more other variables [25].

To form a model for prediction and elucidation of relations among variables, multiple regress is performed utilizing properties in Table 1. The result is shown in equation (1) where: FW is formula weight, Log P is partition coefficient, PSA is polar surface area, nOHNH is number of –OH & -$NH_n$ groups, and nON is number of oxygen & nitrogen atoms.

$$FW = 98.658 + 18.363(\text{Log P}) + 8.473(\text{PSA}) - 36.858(\text{nOHNH}) - 98.129(\text{nON}) \quad (1)$$

For equation (1), the $R^2$ value is 0.7759, indicating 77.59% of the variance in formula

weight is explained by the model. In this model, the constant 98.658 and Log P make the most significant contribution to the model. Here the conclusion of the multiple regression is the relationship of various properties to the formula weight.

Utilizing equation (1), it would be possible to predict favorable values of pharmacological important properties for perspective drug candidates. The design of new potential drugs can be enhanced by targeting the characteristic molecular structure of previously successful compounds. The design of additional tuberculostatic drugs is highly important, the appearance of multiple multi-drug resistant tuberculosis and extensively drug resistant tuberculosis pose a genuine threat to social health.

Tuberculosis is a serious health threat and is thought to cause up to 1.5 million deaths annually [28]. The emergence of drug-resistant strains of tuberculosis and with the HIV co-infection the incidence of this disease has increased [28]. Almost one-third of the world's population is infected with *Mycobacterium tuberculosis* [29,30]. Annually, up to 8.8 million patients are newly diagnosed with an active infection of tuberculosis [30]. The current first-line drugs for treatment (isoniazid, rifampin, pyrazinamide, ethambutol) must be taken for up to 6 months to achieve high level of cure rates (>95%) [30]. Clearly, the investigation and pursuit of new drugs is vital for the clinical treatment of tuberculosis.

## 4. CONCLUSION

Compounds shown to be effective in inhibiting growth of *Mycobacterium tuberculosis* include those that are aromatic (contain conjugated planar ring systems) and non-aromatic. Ranges in important properties of these compounds such as Log P and polar surface area are broad, from -3.52 to 4.97 and 55.12 $A^2$ to 110.24 $A^2$, respectively. Ranges of MIC are from 14.7 µg/mL for isoniazid to 100 µg/mL for compounds 9 and 10 (aromatic compounds). Ranges of MIC for aromatic and non-aromatic compounds actually shows equal group means by F and T test ($P$ = .82) and one-way ANOVA ($P$= .82). Hierarchical cluster analysis showed compounds 8 and 13 are most similar to isoniazid. Path analysis of properties showed polar surface area to be most direct effect on MIC. Multiple regression analysis indicated that Log P makes the greatest contribution within the equation model. This study identifies the molecular properties and structural features of compounds that enable the effective inhibition of *Mycobacterium tuberculosis*.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

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