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NEW DIBROMOINDOLIZINE DERIVATIVES AS PROMISING ANTI-TB AGENTS WITH THEIR CYTOTOXICITY AND SAR STUDIES

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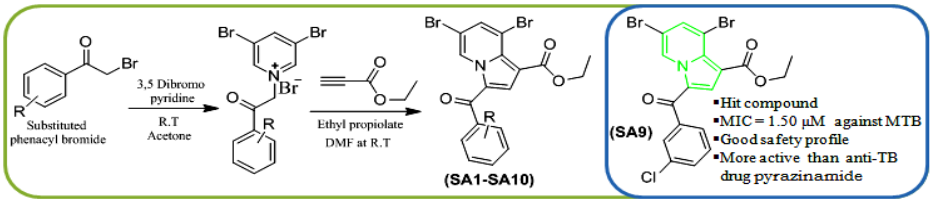
ABSTRACT

With the intent to discover new anti-TB agents, dibromoindolizine derivatives were synthesized **(SA1-SA10)**. All the newly synthesized compounds were screened for their in vitro anti-tubercular activity, while the most active compounds were subjected to a cytotoxicity assay on Vero cell lines. From the overall data, it was found that compound (**SA9**) (MIC =1.50 µM) and (**SA3**) (MIC = 2.75 µM) were found to be more active than the standard drug pyrazinamide (MIC = 2.90 µM). It wasalso interesting to note that compounds (**SA9**) and (**SA3**) which showed remarkable anti-TBactivity were also found to exhibit the best safety profile against Vero cells indicating good selectivity profile. Hence further structuraloptimization of these hit candidates may lead to the discovery of more potent anti-TBagents. These data will help in developing and discovering morepotent and safer anti-TB agents with enhanced property.

KEYWORDS: Indolizine, Anti-TB, cytotoxicity, SAR studies.

1. INTRODUCTION

For thousands of years, tuberculosis (TB) has co-evolved with humans.[1,2] Having passed through so many generations, TB remains a major infectious disease among the world's population, as up to one quarter (~25%) is still infected.[3,4] Even though 90 years of vaccines and 60 years of chemotherapy have been used to combat TB disease, the disease kills 1.3 million people every year.[5] This is due to the TB bacteria can survive in air for almost four hours, so people nearby may become infected by inhaling the bacteria.[4,6] In recent times, TB progress has been reversed by more than a decade due to Covid-19 pandemic, according to the WHO report of 2021. As a result of this, TB mortality has increased for the first time in over a decade.[5] Today, the hardest part about fighting tuberculosis is that the TB-bacteria are becoming resistant to nearly all drugs, resulting in multidrug-resistant-TB (MDR-TB) and extensively drug-resistant disease- TB (XDR-TB).[4,6] Also, the current treatment regime involves numerous drugs taken over a period of 6 to 7 months, resulting in high toxicity and side effects in patients.[4,6] A third serious issue is the synergy between TB and HIV, which increases the risk of re-infection with drug-sensitive or drug-resistant strains. It has been reported that HIV/AIDS was present in 8.6% of all TB cases, making them most likely to die from TB.[6] The probability of developing active TB is approximately 30 times higher for people with HIV who are co-infected with MTB.[7] The global number of deaths officially classified as caused by TB (1.3 million) in 2020 was almost double the number caused by HIV/AIDS (0.68 million).[5] Considering these problems and the threat of drug-resistant TB, it is imperative to intensify research efforts to develop newer and safer anti-TB drugs that are less toxic, reduce the intake of multiple drugs, shorten therapy duration, and enhance mechanism of action in order to minimize the risk of drug resistance.



In natural occurring compounds as well as in synthetic drugs, nitrogen-containing heterocyclic compounds are frequently found. They have received considerable attention because of their potential utility in medicinal chemistry. Indolizine is one of these nitrogen compounds that have wide applications in medicinal chemistry. Indolizines are significant to medicinal chemists because of their role in drug design and the challenges associated with developing indolizines with well-defined substitution patterns. Indolizines have been synthesized using a number of new and attractive methodologies, and a wide range of compounds with potential biological activity have been synthesized.[8, 9] Indolizines have seen significant advances in synthesis, as well as many new methodologies beyond the traditional Tschitschibabin reactions. Indolizines are synthesized throughvarious methods, including the Tschitschibabin reaction, cycloadditions (1,3-dipolar cycloadditions), intramolecularcyclisations with acetic anhydride, formation of C3-C4 bonds, formation of C1-C9 bonds, and formation of C8-C9. In many cyclization reactions, metal catalysts such as copper, platinum, silver, gold, and palladium are used. Nevertheless, there are methodologies that start with nitrogen-containing substrates, such as pyridine, quinoline, and isoquinoline, as well as pyrrole derivatives. Recent syntheses have utilized newer and more environmentally friendly technologies, including microwave heating, reactions on polymeric supports, and aqueous reactions. Tandem reaction mixtures, involving multicomponents, are also being developed. Indolizines have been the subject of recent synthetic and medicinal chemistry studies, and this area is likely to be researched in the future.[8,9,10]

Indolizine, belongs to one of the most promising bio-active compound belonging to a class of bridgehead-N-fused heterocyclic. Indolizine is biostere for indole which is widely common in large number of natural products, pharmaceuticals and approved commercial drugs. **Figure – 1** represents some selected indolizinenatural compounds such as (-)-Swainsonine (2), (+)-momomorine (3), ipalbidine(4), Harmicine (5), polygonatine (6), Juliflorine (7). Indolizine containing compounds have pronounced diverse biological properties and also employed in material science.[11-15] Further, with the growing interest in bioactive indolizine molecules, we expect that clinical trials of these highly potent inhibitory compounds aiming for commercial approval will become more likely in the future.

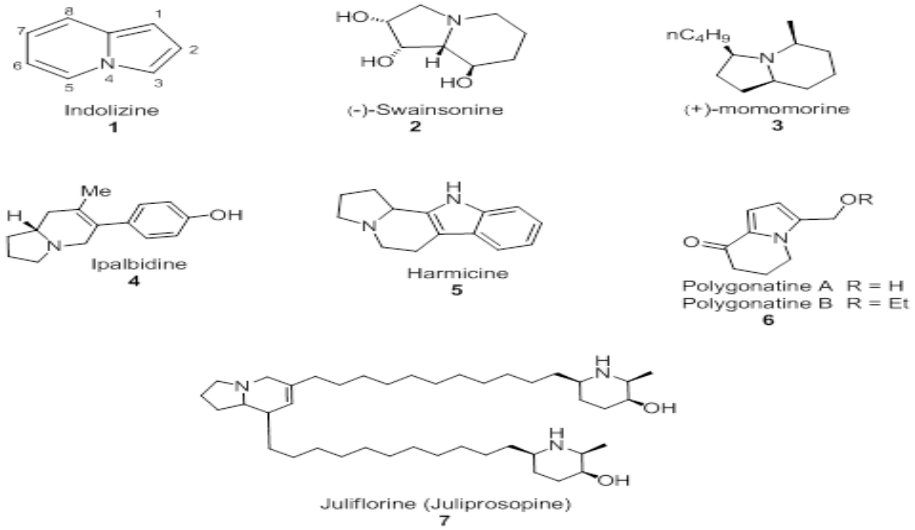


Figure 1: Structure of selected indolizines natural products.

In literature many indolizine based compounds have been discovered for their varied biological activity, out of which some compounds have known to exhibit potent activity. Herein we have identified some potent indolizine based bio-active compounds exhibiting potent activity such asindolizine derivative **(8)** having 2-pyridyl amide moiety **(Figure 2)** was synthesized and discovered as potent inhibitor for tumor regression with Gli LUC S12 IC_{50} value of 4.2 μM.[16] Inhibitions of tubulin polymerization and HL60 cell growth assays were reported using the synthesized indolizine compounds **(9)** and **(10)** (Figure 2).[17] A series of 3-cyclopropylcarbonyl-indolizines **(11)** (Figure 2) were prepared and investigated for their antiproliferative and epidermal growth factor receptor (EGFR) kinase inhibitory activity against Hep-G2 cell line (the human hepatocellular liver carcinoma) using MTT technique.[18] The synthesized benzoyl-indolizine derivatives **(12)** (Figure 2) were established as potent human farnesyltransferase (FTase) inhibitors.[19] The phenothiazine-based indolizine derivatives **(13)** and **(14)(Figure 2)** were synthesized by [3+2] cycloadditionreaction and then tested for their anti-proliferative activity against 60 cell lines and as microtubule-targeting agents.[20]

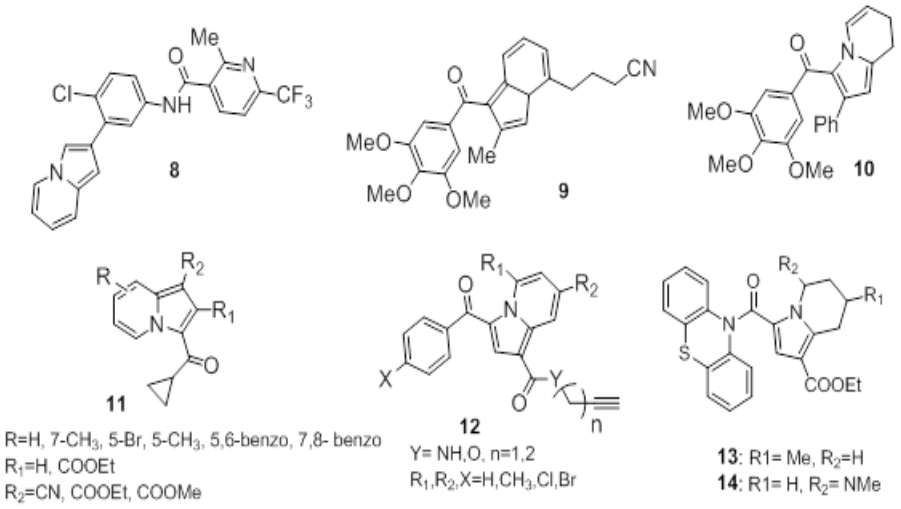


Figure 2: Structure of some potent indolizines derivatives 8-14.

The pyridoindolizineconjugates**(15)(Figure 3)** were reported as potent anticancer agents.[21] A series of 3-benzoyl-1-dimethoxytriazinyl-indolizine derivatives **(16)** (Figure 3) were synthesized and examined for their cellular growth in vitro and tubulin polymerization inhibition.[22] A number of phenanthroline-based indolizine ester derivatives **(17)** and **(18)** (Figure 3) were synthesized followed by studying their anticancer activity against 60 human tumor cell lines.[23] The antitumor activity of a number of the synthesized indolizinederivatives **(19)** and **(20)(Figure 3)** was evaluated using sulphorhodamine-B assay against three cancer cell lines [hepatoma (Hep3B), lung adenocarcinoma (A549) and breast (MCF7)] and normal fibroblast cells.[24] The synthesized indolizine-based phenothiazines**(21a–d)(Figure 3)** were reported to be highly efficient inhibitors of farnesyltransferase in vitro to identify potent antitumor agents.[25]

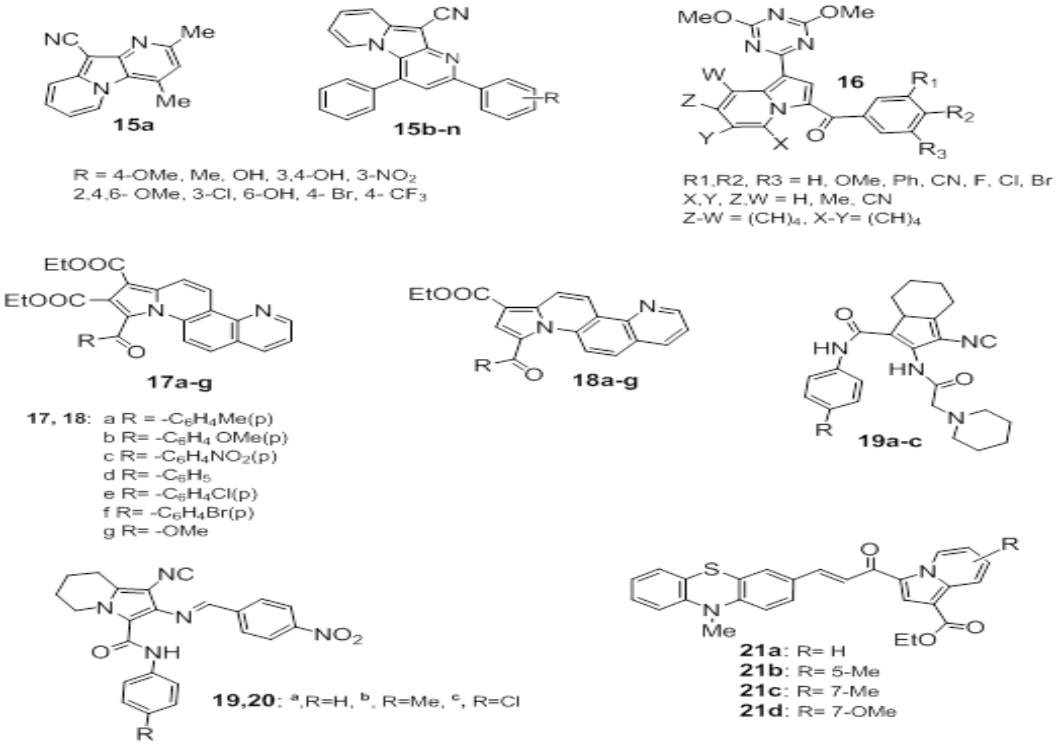


Figure 3: Structure of some potent indolizines derivatives 15-21.

The antiproliferative potentials of the prepared indolizine derivatives **(22-24)(Figure 4)** against tumor cell lines were screened.[26] Several indolizine scaffolds were constructed **(25-30) (Figure 4)** and their anticancer activity were tested.[27] The galactoside-based indolizine derivatives **(31a-e) (Figure 4)** were synthesized and evaluated as inhibitors against several human galectins using methyl β-D-galactopyranoside as a reference.[28]

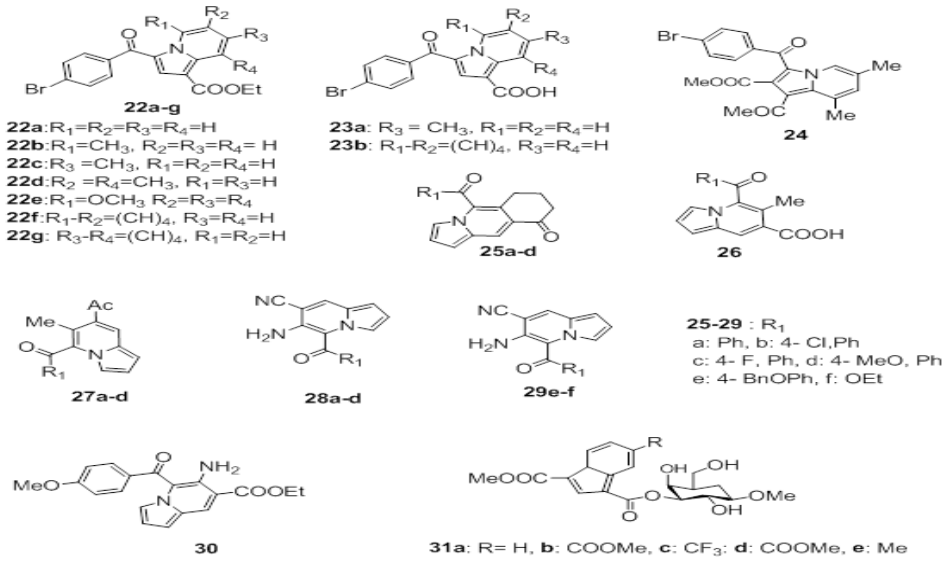


Figure 4: Structures of indolizines derivatives 22-31.

A large number of indolizine derivatives **(32-33) (Figure 5)** were prepared by 3+2 cycloaddition method and were reported as inhibitors of the bromodomain-containing proteins (BRD7 / BRD9), where BRD7 and BRD9 had biological roles in transcription and pathogenesis.[29] Substituted indolizine derivatives **(34)** and **(35)(Figure 5)** were prepared and their antitubulin and cytotoxic effects were examined.[30] Synthesis of a number of indolizine derivatives **(36) (Figure 5)** was carried out and screened for their in vitro anticancer activity against human cervix cancer cell line SiHa using adriamycin as reference.[31] The photophysical studies of the synthesized indolizine structures **(37) (Figure 5)** revealed that they had good application in the sensing of volatile organic compounds (VOCs) such as acetic acid and propionic acid derivatives which are biomarkers present in the exhaled breath of cancer patients.[32] Several indolizine-based chalcone hybrids **(38) (Figure 5)** were synthesized and their anticancer activity was conducted.[33]

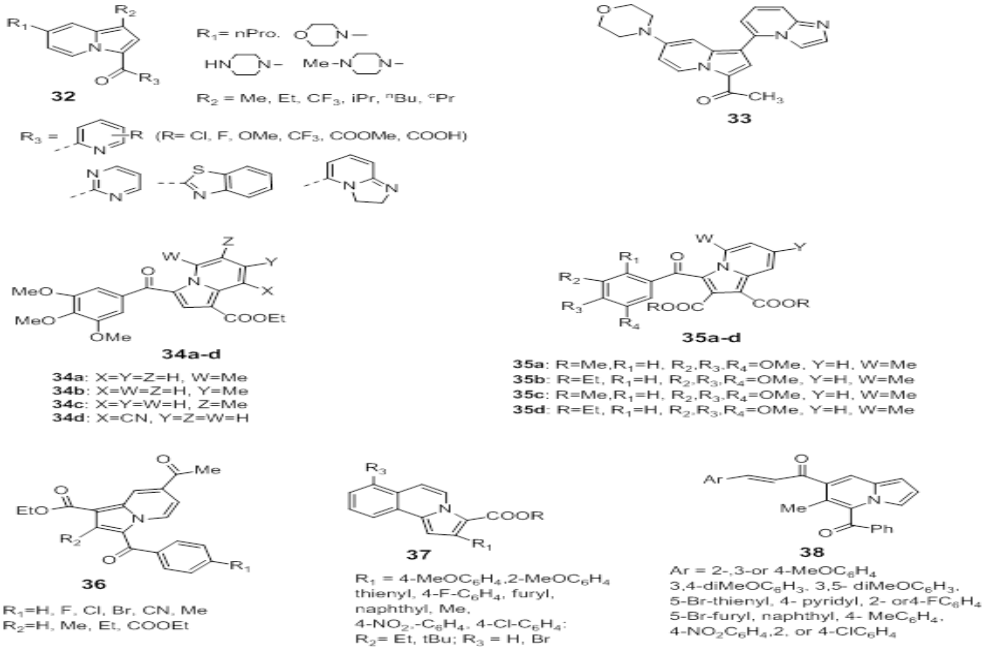


Figure 5: Structure of indolizines derivatives 32-38.

Several indolizine derivatives **39-42(Figure 6)** were synthesized and their in vitro antimycobacterial activities were screened against M. tuberculosis H37Rv (MTB) strain. Interestingly, the maximum inhibition activity was detected for compound **42**(MIC 7.6 μM) (R= 4-F) which displayed 7.6 and 4.7 times more powerful than ethambutol and ciprofloxacin reference drugs, respectively.[34] Next, the indolizineheterocycles**43-45 (Figure 6)** were synthesized and evaluated for their antimycobacterial effects. Particularly, compound **44d** displayed a powerful inhibition against M. tuberculosis (MIC 12.50 μM) and it had antimycobacterial activity

equal to that of anti-TB drug Ethambutol (MIC 12.50 μM).[35] Synthesis of the indolizine derivatives **46a,b** and **47(Figure 6)** was conducted and their Mycobacterium tuberculosis H37Rv growth and InhA inhibitory activities were evaluated. The compounds exhibited moderate activity.[36]

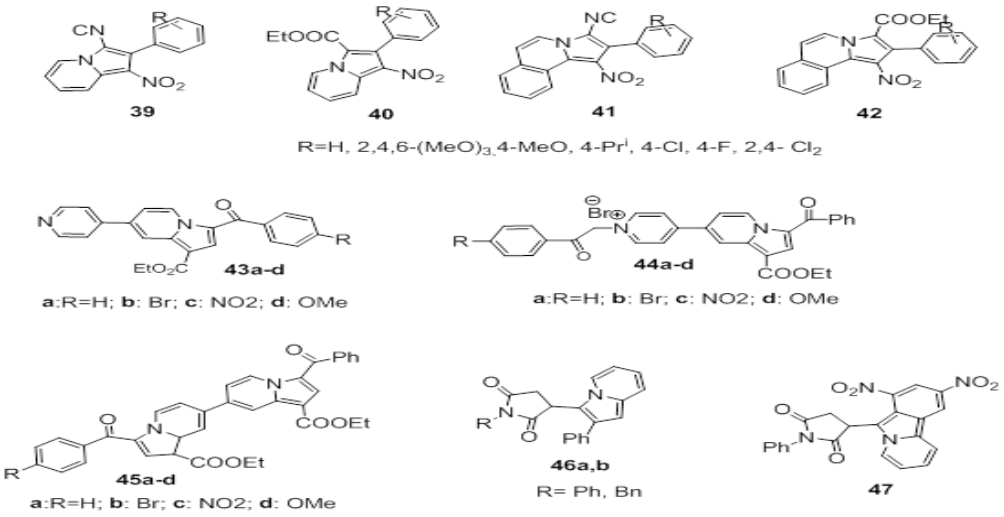


Figure 6: Structure of indolizines derivatives 39-47 exhibiting potent anti-TB activity.

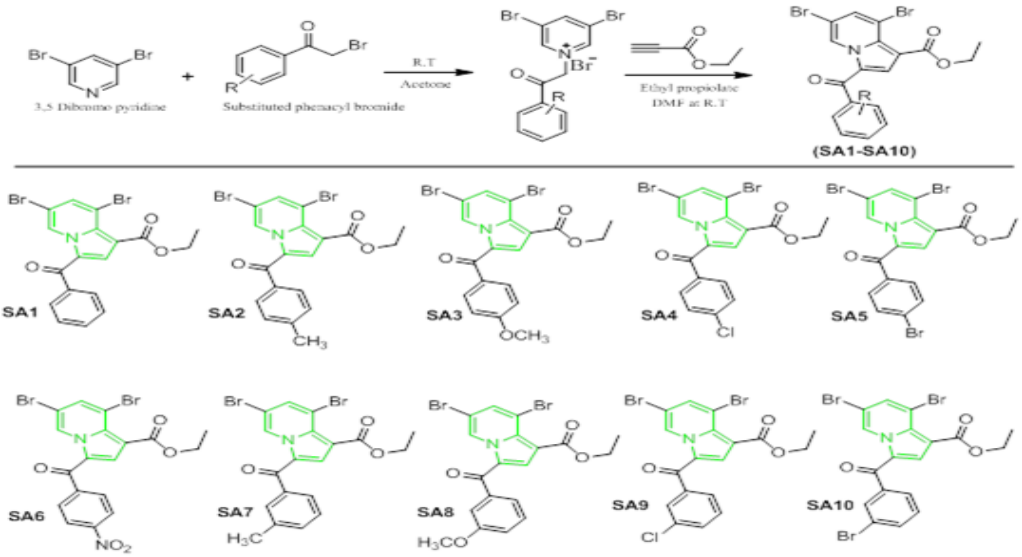
Consequently, considering the medicinal significance of indolizine and their promising anti-TB effects, we developed new dibromoindolizine derivatives (**SA1-SA10**) to investigate their anti-TB properties. In addition to lowering the barriers to discovery of new antitubercular agents, these various antitubercular compounds will encourage the pharmaceutical industry to join the fight against TB.

2. RESULTS AND DISCUSSION

2.1. Chemistry

2.1.1. Synthetic method to prepare dibromoindolizine derivatives (SA1-SA10)

3,5dibromopyridine (0.001 mol) is taken in a dry RB flask and anhydrous acetone (10 ml) was added. Then to it substituted phenacyl bromide (0.001 mol) was charged and stirred for 30 minutes at normal room temperature. Solid was obtained which filtered and under vacuum to get quartenary salt.[37] The obtained salt (0.001 mol) is taken in a dried RB flask. To it add dry DMF and ethylpropiolate (0.0001mol) and stir. Later after 5 minutes add K_2CO3(0.0001mol), and stir for 30 minutes in room temperature. The progress of the reaction was monitored by TLC. After completion, the reaction mass was poured into container which contain crushed ice and filtered on the vacuum pump. The crude compound was purified by column chromatography using hexane:ethylacetate as an eluent. The remaining indolizine derivatives were synthesized by using the same protocol. The series of reaction carried out have been represented in **Scheme-1.**



Scheme-1: Synthetic route to prepare new dibromoindolizine (SA1-SA10)derivatives.

2.2. Biological studies

All the title compounds (**SA1–SA10**) prepared herein were screened for their in vitro anti-TB activity against $MTBH_{37}Rv$ (ATCC-27294) strain, using the Microplate Alamar Blue Assay (MABA).[38] The active compounds were also tested for their cytotoxicity against *Vero* cells by MTT assay.[39]

2.2.1. Antitubercular activity

All the compounds (**SA1–SA10**) were initially screened at a single concentration of 25 µM against $MTBH_{37}Rv$ (ATCC-27294) strain in BACTEC 12 B medium, using microplatealamar blue assay (MABA). Compounds exhibiting ≥ 90% inhibition in the initial screening were tested at below 25µM to determine the actual minimum inhibitory concentration (MIC). A majority of the tested compounds were found to be effective against TB, as shown in (**Table 1)**. The activity of the compound was compared with standard front line anti-TB drug pyrazinamide (MIC = 2.90 µM). The data clearly indicates that the tested compounds exhibited moderate to excellent anti-TB activity with MICs in the range 19.30 – 1.50µM. Out of ten compounds, six compounds exhibited noteworthy activity with MICs below 20µM. In the primary screening, six compounds (**SA1**,**SA3**,**SA4, SA6, SA9** and**SA10)** displayed 91–97% inhibition at 25µMconcentration against $MTBH_{37}Rv$ strain. Whereas compound (**SA2), (SA5),** (**SA7)** and **(SA8)** showed only 88, 75, 66 and 71% inhibition at concentration 25µM respectively, hence they were not evaluated further. Among total synthesized, compound (**SA9)** and **(SA3)** exhibited significant activity with MIC 1.50 and 2.75µM respectively. The second line of activity was observed by two compounds (**SA6)** and (**SA10)**exhibiting MIC of 6.25 and 13.00µM respectively. The next line of activity was observed by compound (**SA1)** and **(SA4)** with MIC of 16.50 and 19.30µM respectively. Whereas, compound (**SA2), (SA5),** (**SA7)** and **(SA8)**were found to be inactive against $MTBH_{37}Rv$ strains. From the overall data, it was found that compound (**SA9)** (MIC = **1.50**µM) and (**SA3)** (MIC = **2.75**µM) were found to be more active than the standard anti-TB drug pyrazinamide (MIC = 2.90 µM). Compound (**SA9)** was found to be nearly 2 folds more active than the standard drug pyrazinamide. Whereas compound (**SA3)** was found to be more than1 fold more active than the standard drug pyrazinamide.

Having identified a good number of active antimycobacterialcompounds (**SA1**,**SA3**,**SA4**,**SA6, SA9** and**SA10)** the next step was to examine their toxicity against the Vero cell line, at a concentration 10 times their actual MIC value. From the results (**Table 1**), it is clear that the tested compounds exhibited moderate to low levels of cytotoxicity with a percentage survival of the Vero cells in the range of 46 to 81%. Among the total six compounds, five compounds were found to be non-toxic against Vero cells, suggesting great potential for their *in vitro* use as antitubercular agents. Whereas, compound (**SA4)** was found to be slightly toxic with 46%survival of Vero cells. Among the tested compounds, the highest cytotoxicity/safety profile against *Vero* cells were observed by (**SA9)** with a percentage survival of 81%. The second line of activity was observed by compounds (**SA3)**and **(SA6)** with a percentage survival of 77 and 70% respectively. Whereas, compounds(**SA10)** and **(SA1)** exhibited moderate safety profile with 63 and 57% survival of Vero cells respectively. It was observed that compounds which showed good anti-TB activity were also found to exhibit good safety profile against Vero cell line. It was also interesting to note that compounds (**SA9)** and (**SA3)** which showed remarkable anti-TB activity was found to exhibit the best safety profile against Vero cells. Hence further structural modification/optimisation of these hit candidates may lead to the discovery of powerful anti-TB agents with better anti-TB properties. These data will help in developing or producing more potent and safer anti-TB agents with enhanced property. (**Fig.7)** gives the comparison between the % survivals of Vero cells at concentration of the compound 10 times than their actual MIC value (µg/mL). Compound is considered toxic if it causes over 50% inhibition of Vero cells at concentration 10 fold higher than its MIC.[40]

Table 1: In-vitro antitubercular screening against *M.tb* $H_{37}Rv$ and cytotoxicity assay against Vero cells.

Compound code	% Inhibition at concentration 25 µM	MIC[a] (µM)	% Survival of Vero cells at conc. (10 X MIC) [b]
(SA1)	93	16.50	57
(SA2)	88	N.D	N.D
(SA3)	96	**2.75**	**77**
(SA4)	91	19.30	46
(SA5)	75	N.D	N.D
(SA6)	95	6.25	70
(SA7)	66	N.D	N.D
(SA8)	71	N.D	N.D
(SA9)	97	**1.50**	**81**
(SA10)	92	13.00	63
Pyrazinamide	99	2.90	88

[a]Minimum inhibitory concentration against $H_{37}Rv$ strain of MTB (µM).

[b]Compound is considered toxic if it causes over 50% inhibition of Vero cells at concentration 10 fold higher than its MIC.
[c]ND, not determined.

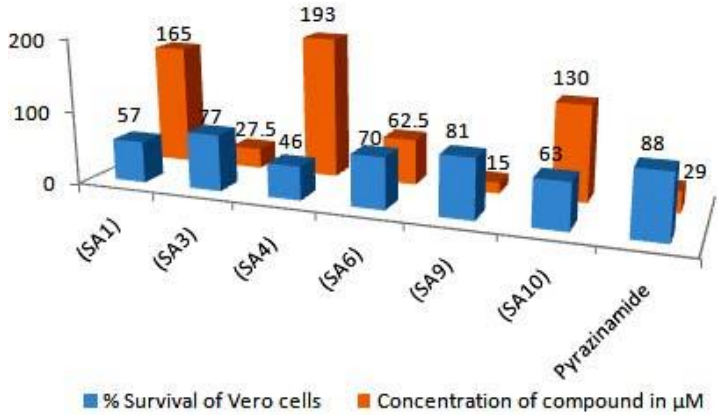


Fig. 7: Comparison between percent survival of Vero cells at concentration of the compound 10 times than their actual MIC value (µM).

3. Experimental

3.1. Instrumentation

All solvents and chemicals purchased did not require further purification. DMSO-D6was used to record ^{1}H(proton) and ^{13}C(carbon) NMR(Nuclear Magnetic Resonance) using Jeol (400 MHz)spectroscopy.With a Nicolet 5700 FT-IR spectrometer, KBr discs were used to obtain the IR spectra. Mass spectra of the conjugates were obtained with the Shimadzu GCM- SQP2010S mass spectrometer.

3.2. General procedure to prepare dibromoindolizine derivatives (SA1-SA10)

3,5dibromopyridine (0.001 mol) is taken in a dry RB flask and anhydrous acetone (10 ml) was added. Then to it substituted phenacyl bromide (0.001 mol) was charged and stirred for 30 minutes at normal room temperature. Solid was obtained which filtered and under vacuum to get quartenary salt. The obtained salt (0.001 mol) is taken in a dried RB flask. To it add dry DMF and ethylpropiolate (0.0001mol) and stir. Later after 5 minutes add K_2CO_3(0.0001mol), and stir for 30 minutes in room temperature. The progress of the reaction was monitored by TLC. After completion, the reaction mass was poured into container which contain crushed ice and filtered on the vacuum pump. The crude compound was purified by column chromatography using hexane:ethylacetate as an eluent. The remaining indolizine derivatives were synthesized by using the same protocol. The series of reaction carried out have been represented in **Scheme-1**.

3.2.1. Compound characterization

Ethyl 3-benzoyl-6,8-dibromoindolizine-1-carboxylate **(SA1)**: light yellow solid; mp: 211–213^{o}C; ^{1}H-NMR (400 MHz, DMSO-D6) δ 1.35 (t, J = 7.0 Hz, 3H), 4.32-4.38 (m, 2H), 7.01-7.85 (m, 8H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.2, 61.2, 113.3, 115.4, 117.0, 117.5, 122.8, 123.0, 128.4, 130.7, 133.6, 135.2, 136.2, 145.3, 152.7, 163.4; GC-MS: 451 $[M]^{+}$

Ethyl 6,8-dibromo-3-(4-methylbenzoyl)indolizine-1-carboxylate **(SA2)**: light yellow solid; mp: 217–218^{o}C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.32 (t, J = 7.0 Hz, 3H), 2.63 (s, 3H), 4.22-4.26 (m, 2H), 7.12-7.94 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.1, 21.2, 59.1, 114.1, 115.5, 117.2, 117.6, 121.7, 123.4, 128.6, 130.6, 133.1, 135.3, 136.5, 145.5, 152.3, 163.1; GC-MS: 465 $[M]^{+}$.

Ethyl 6,8-dibromo-3-(4-methoxybenzoyl)indolizine-1-carboxylate **(SA3)**: light yellow solid; mp: 215–216^{o}C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.38 (t, J = 7.0 Hz, 3H), 3.91 (s, 3H), 4.34-4.40 (m, 2H), 7.01-7.03 (m, 2H), 7.70-7.71 (m, 1H), 7.79-7.85 (m, 3H), 10.10 (s, 1H); ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.3, 55.6, 61.0, 113.9, 115.6, 117.1, 117.2, 122.2, 123.0, 127.1, 129.3, 131.2, 131.5, 131.7, 135.2, 136.2, 145.3, 152.7, 163.4; GC-MS: 481 $[M]^{+}$

Ethyl 6,8-dibromo-3-(4-chlorobenzoyl)indolizine-1-carboxylate **(SA4)**: light yellow solid; mp: 224–225^{o}C; ^{1}H-NMR (400 MHz, DMSO-D6) δ 1.30 (t, J = 7.0 Hz, 3H), 4.24-4.32 (m, 2H), 7.11-7.69 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.4, 61.5, 113.1, 115.3, 116.1, 116.6, 121.6, 122.5, 126.6, 129.6, 132.4, 134.1, 135.5, 142.1, 150.2, 161.2; GC-MS: 485 $[M]^{+}$

Ethyl 6,8-dibromo-3-(4-bromobenzoyl)indolizine-1-carboxylate **(SA5)**: light yellow solid; mp: 226–227^{o}C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.33 (t, J = 7.0 Hz, 3H), 4.21-4.27 (m, 2H), 7.21-7.77 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.6, 61.6, 113.2, 115.2, 116.0, 116.7, 121.5, 122.4, 126.4, 129.9, 132.2, 134.3, 135.4, 142.3, 150.3, 161.3; GC-MS: 529 $[M]^{+}$

Ethyl 6,8-dibromo-3-(4-nitrobenzoyl)indolizine-1-carboxylate **(SA6)**: light yellow solid; mp: 233–235^{o}C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.32 (t, J = 7.0 Hz, 3H), 4.19-4.24 (m, 2H), 7.31-7.82 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.3, 61.4, 113.2, 115.4,

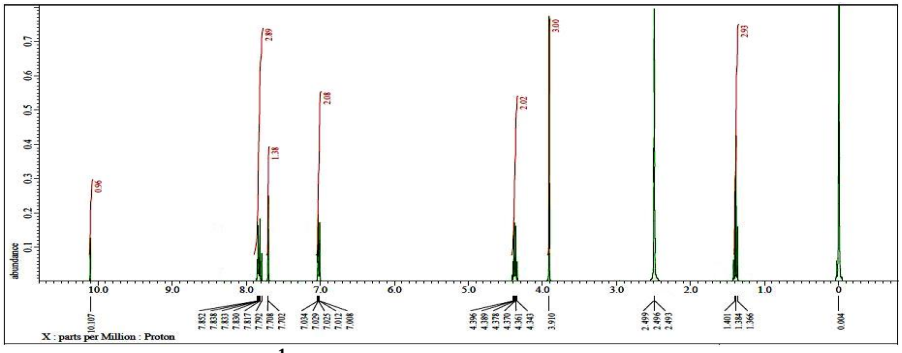
116.0, 116.7, 121.8, 122.7, 126.5, 129.7, 132.2, 134.0, 135.4, 142.2, 150.3, 161.8; GC-MS: 496 $[M]^+$

Ethyl 6,8-dibromo-3-(3-methylbenzoyl)indolizine-1-carboxylate **(SA7)**: light yellow solid; mp: 238–239°C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.33 (t, J = 7.0 Hz, 3H), 2.65 (s, 3H), 4.21-4.26 (m, 2H), 7.14-7.88 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.4, 21.1, 59.6, 114.7, 115.4, 117.5, 117.9, 121.8, 123.5, 128.7, 130.8, 133.5, 135.2, 136.4, 145.6, 152.5, 163.2; GC-MS: 465 $[M]^+$

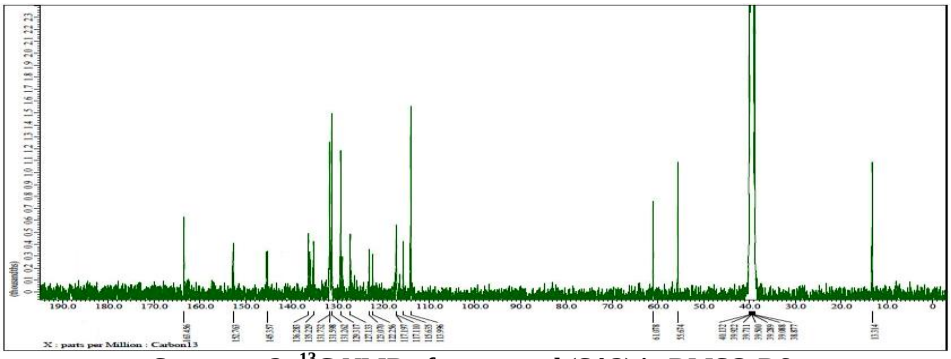
Ethyl 6,8-dibromo-3-(3-methoxybenzoyl)indolizine-1-carboxylate **(SA8)**: light yellow solid; mp: 244–245°C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.37 (t, J = 7.0 Hz, 3H), 3.89 (s, 3H), 4.32-4.36 (m, 2H), 7.05-7.08 (m, 2H), 7.71-7.72 (m, 1H), 7.80-7.86 (m, 3H), 10.01-10.02 (m, 1H); ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.2, 55.8, 61.1, 113.4, 115.3, 117.0, 117.4, 122.5, 123.1, 127.3, 129.5, 131.5, 131.9, 132.6, 135.3, 136.4, 145.7, 147.1, 152.8, 163.1; GC-MS: 481 $[M]^+$

Ethyl 6,8-dibromo-3-(3-chlorobenzoyl)indolizine-1-carboxylate **(SA9)**: light yellow solid; mp: 248–249°C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.33 (t, J = 7.0 Hz, 3H), 4.23-4.30 (m, 2H), 7.12-7.70 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.2, 61.6, 113.2, 115.4, 116.4, 116.5, 121.7, 122.4, 126.2, 129.1, 132.5, 134.7, 135.4, 142.0, 150.1, 161.4; GC-MS: 485 $[M]^+$

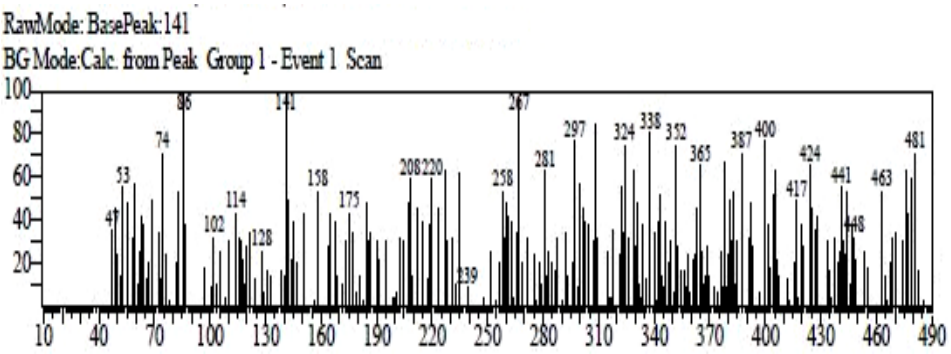
Ethyl 6,8-dibromo-3-(3-bromobenzoyl)indolizine-1-carboxylate **(SA10)**: light yellow solid; mp: 255–257°C;^{1}H-NMR (400 MHz, DMSO-D6) δ 1.30 (t, J = 7.0 Hz, 3H), 4.23-4.26 (m, 2H), 7.23-7.80 (m, 7H), ^{13}C-NMR (100 MHz, DMSO-D6) δ 13.2, 61.3, 113.1, 115.0, 116.4, 116.8, 121.2, 122.6, 126.6, 129.3, 132.1, 134.4, 135.3, 142.6, 150.2, 161.2; GC-MS: 529 $[M]^+$



Spectrum 1: ^{1}H NMR of compound (SA3) in DMSO-D6.



Spectrum 2: ^{13}C NMR of compound (SA3) in DMSO-D6.



Spectrum 3: Mass spectrum of compound (SA3).

4. CONCLUSION

From the overall data, it was found that compound (SA9) (MIC =1.50 µM) and (SA3) (MIC = 2.75 µM) were found to be more active than the standard anti-TB drug pyrazinamide (MIC = 2.90 µM). Compound (SA9) was found to be nearly 2 folds more active than the standard drug pyrazinamide. Whereas compound (SA3) was found to be more than 1 fold more active than the standard drug pyrazinamide. It was also interesting to note that compounds (SA9) and (SA3) which showed remarkable anti-TB activity was found to exhibit the best safety profile against Vero cells. Hence further structural optimization of these hit candidates may lead to the discovery of powerful anti-TB agents with better anti-TB properties. These data will help in developing or producing more potent and safer anti-TB agents with enhanced property.

5. Biological assay

5.1. Antitubercular assay

All the compounds were first screened at a minimum inhibition concentration of 25µg/mL against *M. tuberculosis* $H_{37}Rv$ (ATCC-27294) in BACTEC 12 B medium using the MicroplateAlamar Blue Assay (MABA). Compounds exhibiting < 90% inhibition in the primary screening were not evaluated further, while compounds exhibiting ≥ 90% inhibition were re-tested against $MtbH_{37}Rv$ at lower concentrations in order to determine the actual minimum inhibitory concentration (MIC) in the MABA. The experiment was carried out in triplicate using a 96-well plate, to each well100µL of Middle brook 7H9 broth was added and a serial dilution of compounds was made directly on the plate. Initially, 200µL of the sterile deionized water was added to all outer perimeter wells of sterile 96-well plate to minimize evaporation of medium in the test wells during incubation. Pyrazinamide was included as a positive drug control. The test compounds were diluted by two-fold serial dilution method. The plates were covered and sealed with para film and incubated at 37º C for 5 days. After this, 25 µL of freshly prepared1:1 mixture of Alma Blue reagent and 10% tween 80 were added to the plate and incubated for 24 h. The blue color in the wells indicated the inhibition of bacterial growth while the pink color was scored as growth. Further, the minimum concentration of the compound required to inhibit the bacterial growth was determined.

5.2. Assay for *in vitro* cytotoxicity against *vero* cells

The cytotoxicity of the most active compounds was evaluated using *Vero* cells. The *Vero* cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 2 mMNa_2CO_3 supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were incubated at 37^{o}C under 5% CO_2 and 95% air in a humidified atmosphere until confluent and then diluted with phosphate-buffered saline to obtain 10^6 cells per mL. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The medium was removed and replaced by 180 mL of fresh medium containing the test compound at a concentration 10 fold its actual MIC value. After incubation at 37^{o}C for 72 h, the medium was removed and the monolayer was washed twice with 100 µL of warm Hanks' balanced salt solution (HBSS). One hundred microliters of warm medium and 20 µL of freshly made MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenylmethasulfazone] (100 : 20) (Promega) were added to each well, the plates were incubated for 3 h, and the absorbance was determined at 560 nm using a microplate reader. The percentage of cell survival was calculated after considering the control wells (cells incubated in DMSO-containing medium).

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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