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CHEMICAL CHARACTERIZATION AND PHARMACOLOGICAL INVESTIGATION OF BENZIMIDAZOLYL DERIVATIVES

Mayure Vijay kumar [1]*, V.Sravanthi [1], Donthineni Kalyan[2] & S.Srinivasa Rao[3]

[1]Department of pharmaceutical chemistry, Maheswara college of pharmaceutical sciences, Patancheru, Hyderabad, Telengana, INDIA

[2] Vikas college of pharmaceutical sciences, Suryapet, Nalgonda, Telengana, INDIA

[3] Pulla Reddy institute of pharmacy, Hyderabad, Telengana, INDIA

*Corresponding Author Email: mayurepharma688@gmail.com

ABSTRACT

*Emergence of 'multi-drug resistant' (MDR) TB of which over 0.4 million cases are occurring globally every year, is threatening the whole future of current anti-tubercular chemotherapy. The present work is undertaken with the following objectives. The substituted 1-(2-amino-4-substituted [1,3,5] triazino[1,2-a] benzimidazol-3(4H)-yl) ethanones (**KL 13-24**) and (**K 3,4**) were further converted in to final derivatives i.e. ureas (**KL 25-36**) and (**K 5,6**) by refluxing in alcohol for 6 h with potassium cyanate in the presence of Conc. HCl. The synthesized compounds were characterized by TLC, Melting point, IR, NMR and Mass spectral data. Anti-TB susceptibility testing was performed in black, clear bottomed 96-well micro plates in order to minimize background fluorescence. Initial drug dilution was prepared in dimethyl sulfoxide and subsequent two fold dilutions were performed in 0.1 ml of 7H12 media in the micro plates. The main objective of the present investigation is to explore newer molecules with potent biological activities such as antitubercular activity of newly synthesized compounds.*

KEY WORDS

Benzimidazolyl triazine Tuberculosis, TLC, Melting point, IR, NMR and Mass spectral data, antitubercular activity.

INTRODUCTION

Many important biochemical compounds and drugs of natural origin contain heterocyclic ring structures. Among these eg: Carbohydrates, essential amino acids, vitamins, alkaloids, glycosides etc. the presence of heterocyclic structures in such diverse types of compounds is strongly indicative of the diverse types of the pharmacological activity and recognition of this is reflected in efforts to find useful synthetic drugs. At the same time pharmaceutical chemistry being a specialized science depends on their chemical (inorganic, organic, analytical, physical and colloidal chemistry.) and also on medico biological (pharmacology, physiology, biological chemistry) disciplines. Tuberculosis is a chronic granulomatous disease and a major health problem in developing countries. About 1/3rd of the world's population is infected with *Mycobacterium tuberculosis*. As per WHO estimates, 9 million people globally develop active TB and 1.7 million die of it annually. In India, it is estimated that nearly 2 million people develop active disease every year and about 0.5 million die from it. While lately, the increase in the TB cases rate associated with HIV infection has been halted in USA, no such trend is apparent in India. Emergence of 'multi-drug resistant' (MDR) TB of which over 0.4 million cases are occurring globally every year, is threatening the whole future of current anti-tubercular chemotherapy.

MATERIALS & METHODS

Preparation of (1H)-benzimidazol-2-yl-guanidine (BG):

Into a 100 ml round bottomed flask introduced a mixture of o-phenylene diamine (2 gm, 0.02 mol), dicyanodiamidine (1.68 gm, 0.02 mol), water (30 ml) and Conc. HCl (4 ml) were introduced into a 500 ml round bottom flask and heated under reflux for 2 h. After cooling the reaction mixture was filtered and the filtrate was neutralized with dilute ammonia solution with continuous stirring. The separated solid product was collected by filtration and dried over night at 40 ^{o}C. The dried product was recrystallized from hot water and the yield was 3.5 gm (73 %), melting point was 256-258 ^{o}C [1].

Preparation of 4-(2-chlorophenyl)-3, 4-dihydro [1,3,5] triazino [1,2-*a*] benzimidazol-2-amine (KL-1):

Into a 100 ml round bottomed flask introduced a mixture of 2-guanidino benzimidazole (1.75 gm, 0.01 mol) **(BG),** 2-chloro benzaldehyde (0.01 mol) and ethanol (25 ml) was stirred for 10 min in a round bottomed flask to dissolve the solids. A solution of piperidine (1 ml) was added drop by drop into above mixture and heated under reflux for 4 h, cooled the reaction mixture and the solid product thus separated was collected by filtration, dried over night. The dried product was recrystallised from DMF, the yield was 60 % and melting point was 296-298 ^{o}C. The remaining derivatives of triazino benzimidazole (**KL 2-12**) were prepared by following the above mentioned procedure[2]. The percentage yield was between 60 to 70 % and the physicochemical data was given in the **Table-I**.

Preparation of 3,4-dihydro[1,3,5]triazino[1,2-*a*]benzimidazol-2-amine (K-1):

Into a 100 ml round bottomed flask introduced a mixture of 2-guanidino benzimidazole (1.75 gm, 0.01 mol) **(BG),** formaldehyde (0.01 mol) and ethanol (25 ml) were stirred for 10 min in a round bottomed flask to dissolve the solids. A solution of piperidine (1 ml) was added drop by drop into above mixture and heated under reflux for 4 h. Cooled the reaction mixture and the solid product thus separated was collected by filtration, dried over night and was recrystallised from DMF, the yield was 60 % and melting point was 286-88 ^{o}C.

Preparation of 4, 4-dimethyl-3, 4-dihydro [1, 3, 5] triazino [1, 2-*a*] benzimidazol-2-amine (K-2): Into a 100 ml round bottomed flask introduced a mixture of 2-guanidino benzimidazole (1.75 gm, 0.01 mol) **(BG),** acetone (25 ml) and were stirred for 10 min in a round bottomed flask to dissolve the solids. A solution of piperidine (1 ml) was added drop by drop into above mixture and heated under reflux for 8 h. Cooled the reaction mixture and solvent was removed by evaporation and the solid product thus separated was collected, dried over night and was recrystallised from DMF [3-6], the yield was 60 %, melting point was 272-274 ^{o}C and the physicochemical data was given in the **Table-II**.

Preparation of 1-(2-amino-4-(2-chlorophenyl) [1,3,5] triazino[1,2-*a*] benzimidazol -3(4*H*)-yl) ethanone (KL 13):

Into a 100 ml round bottomed flask introduced 4-(2-Chlorophenyl)-3,4-dihydro [1,3,5] triazino [1,2-*a*] benzimidazole-2-amine (**KL 1**) (1.75 gms, 0.01 mol) and was dissolved in glacial acetic acid (15 ml) with mild warming and then water (4 ml) was added with constant stirring and cooling. The reaction mixture was left overnight in the refrigerator. The separated solid product was collected by filtration and dried. The percentage yield was 50 %, melting point was 172-174 ^{o}C. The remaining acetyl triazine derivatives of this series (**KL 14-24**) were prepared by following the above mentioned procedure [7-9]. The percentage yield was between 50 to 60 % and physical characteristic data was given in the **Table-III**.

ANTI-TUBERCULAR ACTIVITY:

All the compounds synthesized in the present investigation were screened for their anti-tubercular activity by subjecting the compounds to standard procedures. The Anti-tubercular activity was evaluated against bacterial strain *M. tuberculosis* H37Rv by MABA method .The agar proportion susceptibility method is labor intensive, and results may take up to 2 months often making the result clinically irrelevant. Commercially available systems such as the BACTEC system and the newer Mycobacteria growth indicator tubes and the E test are simple and rapid but expensive, making them impractical for use in developing countries. Oxidation-reduction dyes, e.g., tetrazoliums have been used to obtain drug susceptibility measurements for bacteria

including mycobacteria. Yajko reported as a result of tests with clinical isolates, a good correlation between the proportion technique and a broth method with Alamar Blue, an ovel proprietary, resazurin based oxidation-reduction indicator which delivered colorimetric MICs for *M. tuberculosis* isolates in 14 days. A micro plate version of the Alamar Blue assay (MABA) with modified medium composition, reaction time and temperature, and inoculums preparation was evaluated as a high throughput screen by comparing the MICs of 30 antimicrobial agents for *M.tuberculosis* $H_{37}Ra$ and $H_{37}Rv$ obtained by MABA to the MICs obtained with the BACTEC 460 system [10-12].

Principles of the Procedure

The large number of inorganic salts in this medium provides substances essential for the growth of mycobacterium. Sodium citrate, when converted to citric acid, serves to hold certain inorganic cations in solution. The albumin acts as a protective agent by binding free fatty acids, which may be toxic to *Mycobacterium* species. In the enriched medium, the albumin is heat treated to remove lipase, which may release fatty acids from polysorbate 80;7 catalase destroys toxic peroxides that may be present in the medium; dextrose is an energy source; and sodium chloride provides essential electrolytes. Supplementation with glycerol or polysorbate 80 enhances the growth of mycobacterium.

Composition of growth medium (Difco™ Middlebrook 7H9 Broth):

The composition of the culture medium (Difco™ Middlebrook 7H9 Broth) used for the anti mycobacterial testing contains the following ingredients in quantities approximately for 900 ml.

Ammonium Sulfate	0.56g
L-Glutamic Acid	0.5 4g
Sodium Citrate	0.11g
Pyridoxine	1.1 mg
Biotin	0.7 mg
Disodium Phosphate	2.8 g
Monopotassium Phosphate	1.3 g
Ferric Ammonium Citrate	0.24 g
Magnesium Sulfate	0.25 g
Calcium Chloride	0.3 mg
Zinc Sulfate	1.5 mg
Copper Sulfate	1.3 mg

Bacterial strain *M Tuberculosis H37Rv* ATCC (American Type Culture Collection), inoculums was grown on 100 ml of Middle brook 7H9 broth (Difco, Detroit Mich.) supplemented with 0.2% (v/v) glycerol, 10% (v/v) OADC (Oleic acid, albumin, dextrose, catalase, Difco) and 0.5% (v/v) Tween 80. The complete medium referred to as 7H9GC-T80.

Micro Plate Alamar Blue Assay (MABA)

Anti TB susceptibility testing was performed in black, clear bottomed, 96 well micro plates in order to minimize background fluorescence. Initial drug dilution was prepared in dimethyl sulfoxide and subsequent two fold dilutions were performed in 0.1 ml of 7H12 media in the micro plates. The H_{37} Rv was diluted in 7H9 media to reach approximately $2X10^5$ cfu/ml and 0.1 ml was added to wells. Wells containing compounds only were used to detect auto fluorescence of the compounds. Plates were incubated at 37 °C. At day 7 of incubation, 20 µl of Almar Blue solution and 12.5 ml of 20 % Tween 80 were added to all the wells and the plates were reincubated at 37 °C for 24 h. Development of blue color in the well was interpreted as no bacterial growth and pink color was scored as growth. The MIC

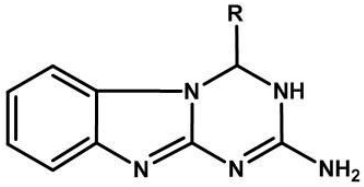
was defined as lowest drug concentration which prevented the color change from blue to pink. The results of the anti-tubercular activity were tabulated in the **Table No VII.**

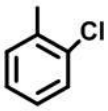
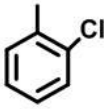
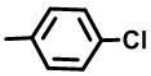
RESULTS & DISCUSSION

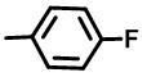
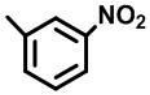
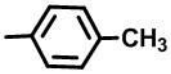
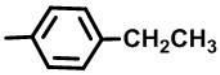
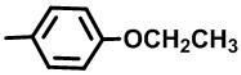
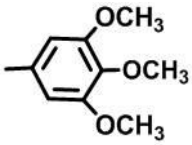
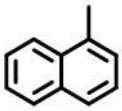
Preparation of 4, 4-dimethyl-3, 4-dihydro [1, 3, 5] triazino [1, 2- *a*] benzimidazol-2-amine (K-2): Into a 100 ml round bottomed flask introduced a mixture of 2-guanidino benzimidazole (1.75 gm, 0.01 mol) **(BG),** acetone (25 ml) and were stirred for 10 min in a round bottomed flask to dissolve the solids. A solution of piperidine (1 ml) was added drop by drop into above mixture and heated under reflux for 8 h. Cooled the reaction mixture and solvent was removed by evaporation and the solid product thus separated was collected, dried over night and was recrystallised from DMF, the yield was 60 %, melting point was 272-274 °C and the physicochemical data was given in the **Table-II**.

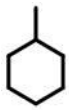
Preparation of 1-(2-amino-4-(2-chlorophenyl) [1,3,5] triazino[1,2-*a*] benzimidazol -3(4*H*)-yl) ethanone (KL 13): Into a 100 ml round bottomed flask introduced 4-(2-Chlorophenyl)-3,4-dihydro [1,3,5] triazino [1,2-*a*] benzimidazole-2-amine (**KL 1**) (1.75 gms, 0.01 mol) and was dissolved in glacial acetic acid (15 ml) with mild warming and then water (4 ml) was added with constant stirring and cooling. The reaction mixture was left overnight in the refrigerator. The separated solid product was collected by filtration and dried. The percentage yield was 50 %, melting point was 172-174 °C. The remaining acetyl triazine derivatives of this series (**KL 14-24**) were prepared by following the above mentioned procedure. The percentage yield was between 50 to 60 % and physical characteristic data was given in the **Table-III**.

Table-I: Physical characterization data of synthesized compounds (KL 1-12).



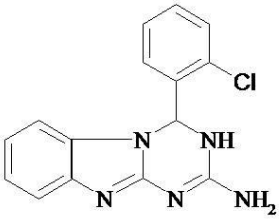
S.No	Compound Code	R	Molecular Formula	Molecular Weight	Melting Point (°C)
1	KL-1		$C_{15}H_{12}ClN_5$	297	296-98
2	KL-2		$C_{15}H_{12}ClN_5$	297	216-18
3	KL-3		$C_{15}H_{12}ClN_5$	297	250-52

4	KL-4		$C_{15}H_{12}FN_5$	281	240-42
5	KL-5		$C_{15}H_{12}N_6O_2$	308	240-42
6	KL-6		$C_{16}H_{15}N_5$	277	288-90
7	KL-7		$C_{17}H_{17}N_5$	291	270-72
8	KL-8		$C_{17}H_{17}N_5O$	307	280-82
9	KL-9		$C_{18}H_{19}N_5O_3$	353	280-82
10	KL-10		$C_{14}H_{12}N_6$	264	270-72
11	KL-11		$C_{19}H_{15}N_5$	313	290-92

12	KL-12		$C_{15}H_{19}N_5$	269	278-80

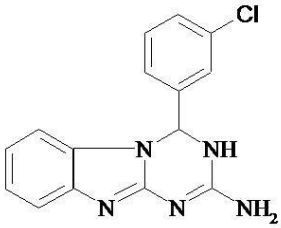
Structures of synthesized compound (KL1 - KL12)

Compound (KL-1)



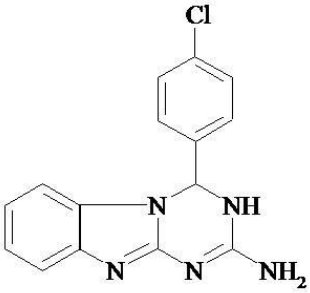
4-(2-Chlorophenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-2)



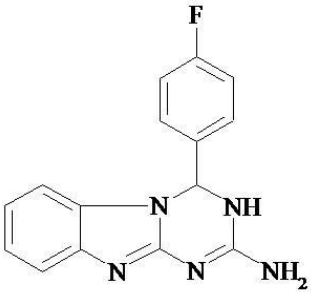
4-(3-Chlorophenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-3)



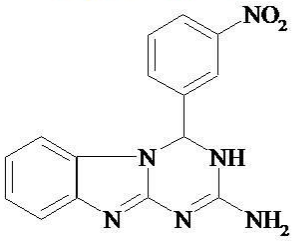
4-(4-Chlorophenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-4)



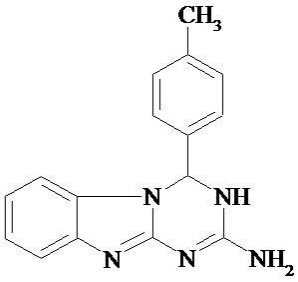
4-(4-Fluorophenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-5)



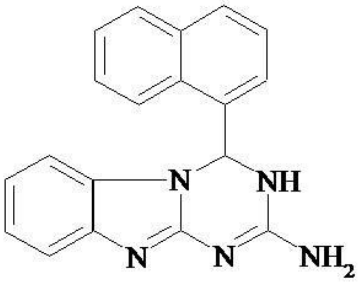
4-(3-Nitrophenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-6)



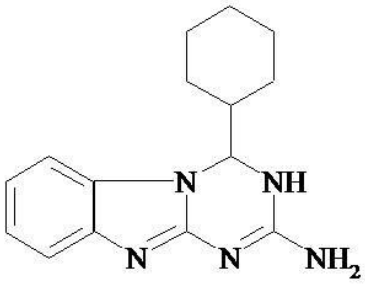
4-(4-Methylphenyl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-11)



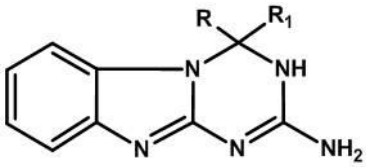
4-(Naphthalen-1-yl)-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

Compound (KL-12)



4-Cyclohexyl-3,4-dihydro[1,3,5] triazino[1,2-*a*] benzimidazol-2-amine

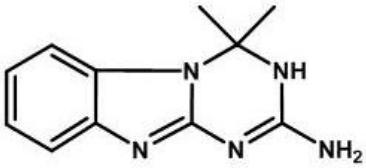
Table-II: Physical characterization data of synthesized compounds (K 1, 2).



S.No	Compound Code	R	R_1	Molecular Formula	Molecular Weight	Melting Point (°C)
1	K-1	CH_3	CH_3	$C_{11}H_{13}N_5$	215	272-74
2	K-2	H	H	$C_9H_9N_5$	187	286-88

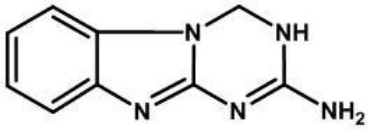
Structures of synthesized compound (K1 - K2)

Compound (K1)



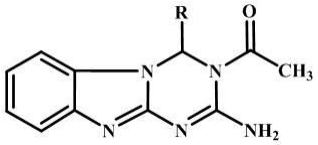
4,4-Dimethyl-3,4-dihydro[1,3,5]triazino [1,2-*a*] benzimidazol-2-amine

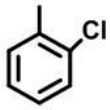
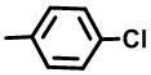
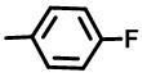
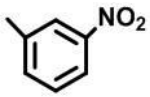
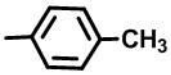
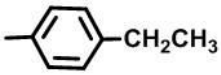
Compound (K2)

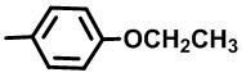
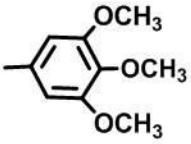
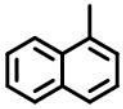
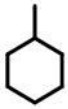


4,4-Dimethyl-3,4-dihydro[1,3,5]triazino [1,2-*a*] benzimidazol-2-amine

Table-III: Physical characterization data of synthesized compounds (KL 13-24).



S.No	Compound Code	R	Molecular Formula	Molecular Weight	Melting Point (°C)
1	KL-13		$C_{17}H_{14}ClN_5O$	339	172-74
2	KL-14		$C_{17}H_{14}ClN_5O$	339	144-46
3	KL-15		$C_{17}H_{14}ClN_5O$	339	214-16
4	KL-16		$C_{17}H_{14}FN_5O$	323	142-44
5	KL-17		$C_{17}H_{14}N_6O_3$	350	170-72
6	KL-18		$C_{18}H_{17}N_5O$	319	150-52
7	KL-19		$C_{19}H_{19}N_5O$	333	140-42

8	KL-20		$C_{19}H_{19}N_5O_2$	349	138-40
9	KL-21		$C_{20}H_{21}N_5O_4$	395	180-82
10	KL-22		$C_{16}H_{14}N_6O$	306	164-66
11	KL-23		$C_{21}H_{17}N_5O$	355	200-02
12	KL-24		$C_{17}H_{21}N_5O$	311	220-22

All the synthesized compounds herein (**K 5-6)** and **(KL 26-36**) were screened for their anti tubercular activity. The anti tubercular activity was carried out against *M.tuberculosis* H_{37} RV by micro plate alamar blue assay. The MIC values for the *in vitro* anti bacterial studies of the compounds are presented in **table No VII**. The data for the anti tubercular activity screening revealed that all the compounds showed activity at 100 µg/ml. The compounds **KL-27**, and **KL-31** exhibited very good activity against *Mycobacterium tuberculosis* strain to the level of 6.25 µg/ml, where as the compounds **K-5, KL-26, KL-28, KL-29, KL-30, KL-34** and **KL-36** good shown activity at 25 µg/ml and the compound **K-32** moderate activity at 50 µg/ml, and the compounds **K-6** and **KL-33** exhibited poor activity i.e., they only active at the concentration 100 µg/ml.

However the data of the anti tubercular activity screening revealed that the compounds **KL-27** and **KL-31** exhibited very good activity against *Mycobacterium tuberculosis* strain to the level of 6.25 µg/ml respectively. These compounds were found to contain chloro and ethyl groups as substituents at 4th position of benzimidazole triazine nucleus which is attached to the one of the urea amine group. Whereas the compounds **K-5, KL-26, KL-28, KL-29, KL-30, KL-34** and **KL-36** exhibited good activity against *Mycobacterium tuberculosis* strain to the level of 25

µg/ml respectively. The good anti antitubercular activity of the tested compounds may be due to the presence of electron withdrawing groups such as Cl (**KL-26, 27**), F (**KL-28**), NO_2(**KL-29**) which is attached at 4th position of the benzimidazolo triazine ring system and electron donating group like alkyl group (**KL-30, 31**) attached at the fourth position of the benzimidazolo triazine ring system.

TABLE No VII: Anti tubercular activity of synthesized compounds

Compound Code	Concentration (µg\ml)									
	100	50	25	12.5	6.25	3.125	1.6	0.8	0.4	0.2
K-5	**S**	**S**	**S**	R	R	R	R	R	R	R
K-6	S	R	R	R	R	R	R	R	R	R
KL-26	S	S	**S**	R	R	R	R	R	R	R
KL-27	S	S	**S**	**S**	**S**	R	R	R	R	R
KL-28	S	S	**S**	R	R	R	R	R	R	R
KL-29	S	S	**S**	R	R	R	R	R	R	R
KL-30	S	S	**S**	R	R	R	R	R	R	R
KL-31	S	S	**S**	**S**	**S**	R	R	R	R	R
KL-32	S	S	R	R	R	R	R	R	R	R
KL-33	S	R	R	R	R	R	R	R	R	R
KL-34	S	S	**S**	R	R	R	R	R	R	R
KL-36	S	S	**S**	R	R	R	R	R	R	R

S-Sensitive R-Resistant

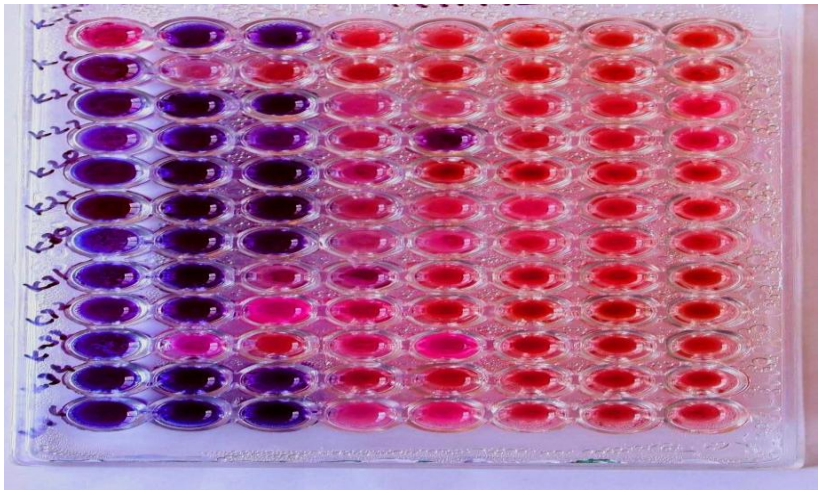


Figure 1: Anti tubercular activity of synthesised compounds

CONCLUSION

The present work, which has undertaken is bonafied, for the synthesis of new 1-[4-(substituted)-3, 4-dihydro [1, 3, 5] triazino [1, 2-a] benzimidazol-2-yl] ureas as possible potent inhibitors of the soluble epoxide hydrolase. In this view we have made an attempt in viewing the literature on Triazines and ureas derivatives for their medicinal significance with the help of chemical abstract, journals and internet sites. The literature review and survey was carried out from 1960 to 2012 related to ureas and traizines derivatives as anti-tubercular agents and as as potent

inhibitors of the soluble epoxide hydrolase . In the light of above, for the synthesis of 1-[4-(substituted)-3,4-dihydro[1,3,5] triazino[1,2-a] benzimidazol-2-yl] ureas were established on literature survey. Around 14 new compounds were synthesized, with the standard chemicals and procedures. The synthesized compounds were tested for their preliminary tests, physical constants and TLC. The structure of the final compounds was confirmed by HNMR, LC-MS analysis. The selected 12 synthesised compounds were screened for their activity.

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***Corresponding Author:**
Mayure Vijay kumar
E-mail: mayurepharma688@gmail.com
Department of pharmaceutical chemistry,
Maheswara college of pharmaceutical sciences,
Patancheru, Hyderabad,
Telengana , INDIA