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SYNTHESIS OF SOME NOVEL FORMAZANS AS POTENTIAL ANTITUBERCULAR AGENTS

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ABSTRACT

Various substituted formazan derivatives have received considerable importance during last decade as they are covered with wide variety of biological and pharmacological activities and have a wide range of therapeutic importance. Based on this a series of new heterocyclic containing formazan derivatives are synthesized by the treatment of pyridine-4–carbaldehyde with phenylhydrazine in presence of natural acidic catalyst like lemon juice yields Schiff bases. This Schiff base is then treated with different diazonium salts in presence of pyridine to form a novel series of Formazan derivatives. The synthesized compounds were characterized by physical studies like solubility, melting point, TLC and subjected to spectral studies like IR, ^{1}H-NMR spectroscopy and Mass spectrometry. All the synthesized compounds were screened for biological activity like *In-vitro* anti-tubercular activity was performed by MicroplateAlamar Blue Assay method (MABA) by using Pyrazinamide, Ciprofloxcin and Streptomycin as reference standards. Results suggested that electron withdrawing groups like nitro, chloro, containing compounds shown potent anti-tubercular activity. Rest of compounds showed mild to moderate activity.

KEYWORDS: Formazans, Diazotization, Antitubercular activity.

INTRODUCTION

Formazans are characterized by intense colors, ranging from cherry red to a deep purplish black and contain the characteristic chain of atoms -N=N-C=N-NH-.[1,2] Formazans are generally solids of relatively low melting point in spite of large the size of the molecules.It is

obtained from reduction of tetrazolium salts. Tetrazolium salts are colourless or faintly yellow compounds and they are reduced to deeply coloured compounds known as formazans. The formazanmoiety is substituted with three phenyl groups at R, R', R" which is called 1, 3, 5-triphenylformazan.They are often particularly soluble in chloroform and acetone; in water the solubility appears to be negligible, the solvent being colored.

Therapeutic properties of formazan moiety include[3]:

- Antiviral &Anti-HIV,
- Anti-tubercular,
- Anti-oxidant,
- Anti-inflammatory,
- Analgesic,
- Antifertility,
- Antimicrobial,
- Anti-parkinsonism,
- Anti-proliferative.

Introduction of Antitubercular activity

Tuberculosis

Tuberculosis is a widespread bacterial infectious, disease caused by various strains Of mycobacteria, usually *mycobacterium tuberculosis.*[4] Tuberculosis typically attacks the lungs, but can also affect other parts of the body. It is spread through the air when people who have an active TB infection cough, sneeze, or otherwise transmit respiratory fluids through the air.

Signs and symptoms

General signs and symptoms includes Fever, Chills, Night sweats, Loss of appetite, Weight loss, Fatigue. Significant Nail clubbing may also occur[5].

Types of Tuberculosis

Based on the site of infection, it is classified into two types.

a) pulmonary tuberculosis.

b) Extrapulmonarytuberculosis.

a) pulmonary tuberculosis

It most commonly involves the lungs (in about 90% of cases).Tuberculosis may become a chronic illness and cause extensive scarring in the upper lobes of the lungs. The upper lung lobes are more frequently affected by tuberculosis than the lower ones.The reason for this difference is not entirely clear.It may be due either to better air flow, or to poor lymphdrainage within the upper lungs.Symptoms may include chest pain and a prolonged cough producing sputum.[6]

b) Extrapulmonary tuberculosis

Extrapulmonary TB occurs when tuberculosis develops outside of the lungs, causing other kinds of TB.These are collectively denoted as "extrapulmonary tuberculosis".[7] It occurs more commonly in immunosuppressed person and young children. In those with HIV, this occurs in more than 50% of cases.

Screening Methods

Various screening methods are available for evaluating the Antitubercular activity are.[8]

a. Drug Susceptibility Testing (DST)

In the 1950s, the first Drug Susceptibility Testing (DST) method for*m. tuberculosis*, which was a agar dilution method, involving the preparation of aconcentration series of drugs against *M. tuberculosis* complex in Lowenstein-Jensen medium, inoculation of the bacterial cultures on the slants, and reading of the inhibition of growth by drugs at different concentrations. The agar dilution tests permit to determine the Minimum Inhibitory Concentration (MIC), however, none of its worked outmodifications was repeatedly used over a longer period of time. Disadvantage is the high need of amounts of test compounds (20 mg/plate to test 1.000 mg/mL), which restricted itsuse to easily available test materials.DST developed a vast majority of following techniques are follows

i) Colorimetric method

A number of low-cost colorimetric DST assays using oxidation/reduction indicator dyes have been described, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 2,3,5-triphenyltetrazolium chloride (TTC), and2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT).MTT,XTT and TTC are tetrazolium salts that are reduced to purple formazan crystals in respiratory chain, with which, the growth/inhibition can be read visually; and the reduced form of these dyes can also be quantitated colorimetrically by measuring absorbance at 570 nm. However, these

tests have limitations; several compounds can interfere with the formazan production in the assay and give rise to false-negative results and provide an under estimation of activity. The following are *in-vitro* colorimetric screening methods for determining the anti-tubercular activity are

- Microplatealamar blue assay (MABA).
- Resazurinmicrotitre assay method (REMA).

ii) Flourometric testing

The Gold Standard of fluorometric tests is the automated system BACTEC MGIT 960™(Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) which was highly sensitive and specific in the detection of rifampicin-resistant TB, and has been evaluated extensively for DST of anti TB drugs, replacing the BACTEC 460™ system for this task. BACTEC MGIT 960™ platform contains a modified Middlebrook 7H9 broth with a fluorescence quenching-based oxygen sensor that detects the amount of oxygen consumption by growing microorganisms. This measure of growth kinetics in liquid culture will facilitate mycobacterial quantification and will be especially beneficial for evaluating bactericidal activity of new anti-tuberculosis drugs and their combinations.

iii) Flow cytometry

The modern flow cytometer analyzes and sorts cells or particles at rates up to 50000 per second. A broad range of flow cytometric applications for biotechnology includes applications in diagnostics and vaccine development, genomics, proteomics and protein engineering, drug discovery, reproductive biology, plant and marine biology, toxicology, and single molecule detection.

iv) HighThroughout Screening

High-throughput screening (HTS) is a method used in drug discovery and relevant to the fields of biology and chemistry. Using robotics, data processing and control software, liquid handling devices, and sensitive detectors, high-throughput screening allows a researcher to quickly conduct millions of chemical, genetic or pharmacological tests. The TAACF perform screens of chemical libraries against various biochemical target assays that have been modified, validated, and optimized for a high throughput format. *M. tuberculosis* targets selected byTAACF are as follows.

- *M. tuberculosis* Dihydrofolatereductase.

- *M, tuberculosis* Enoyl-ACP Reductase.
- *M, tuberculosis* Isocitratelyase-malate synthase.
- *M. tuberculosis* PantothenateSynthetase.
- *M. tuberculosis* FtsZ and tubulin.

v) Microfluidic testing

Microfluidics research has produced sophisticated nanotechnological techniques for sample processing, fluid handling and signal amplification and detection. Microfluidic antimicrobial plug-based assays provide the ability to reduce detection time by confining bacteria into nanoliter-sized plugs. This approach increases the effective concentration of the bacterium and allows released molecules to accumulate in the plug.

vi) Biosensing technologies

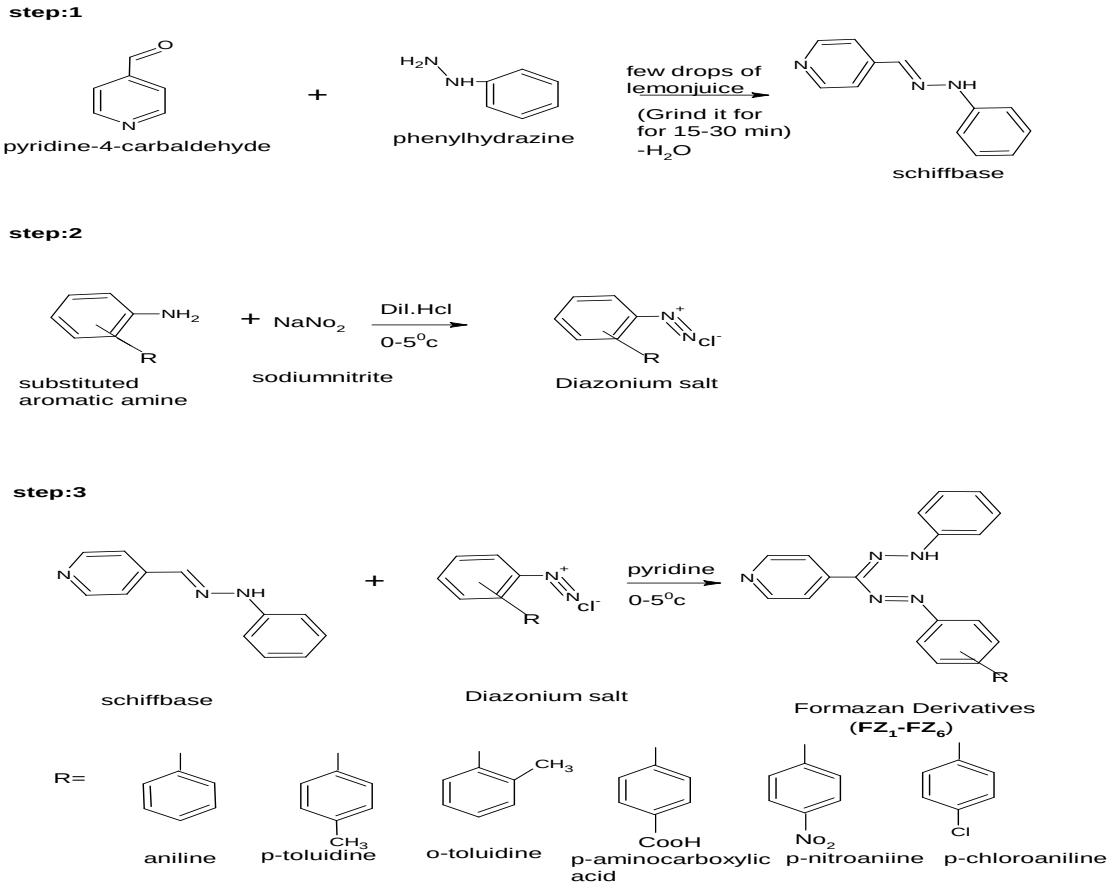
Current methods for DST of *M. tuberculosis* cannot provide results in real-time and most of these methods are centralized in large stationary laboratories because need complex instrumentation and highly qualified technical staff. An interesting alternative is the use of biosensors which are sensors that transduce bio-recognition processes via a physico-chemical transducer, with electronic and optical techniques as two major transducers. Biosensors have high sensitive and accuracy. This is because biomolecules often possess high affinity toward their targets and biological recognition is usually very selective.

MATERIALS AND METHODS

All the chemicals used were of analytical grade and purchased from SD Fine. Melting points of all the synthesized compounds were determined by open capillary tube method.The compounds are identified by TLC and spots was visualized using Uv Chamber.

Experimental work

Scheme



Procedure

General procedure for the Synthesis of Novel derivatives (1 to 3)

Step-1: Synthesis of Schiff base by GrindStone Technique

Take equimolar mixture of pyridine-4-carbaldehyde (0.01mol) and phenylhydrazine (0.01mol) into a motar . To above mixture add lemon juice (0.5 ml) and 5ml of water . The reaction mixture was grounded for 15-30 min. The complete reaction was monitored by TLC by using solvent system as chloroform : ethanol (9:1) ratio. After completion of reaction, add 25 ml of water and kept aside for few minutes. Product gets separated, filter it and wash it with water. The crude product was recrystallized by ethanol to obtain Corresponding Schiff base.

Melting point: 144^{0}C ; % Yield(w/v) : 91% ; R_fValue : 0.32 ; IR in cm-1:1492(N=CH).

Step-2: Diazotisation of Amines

Substituted aromatic amine (0.01 mol) dissolved in acetic acid (2 ml) and add 2ml of 0.5N hydrochloric acid. The solution was cooled to 0-5°C and sodium nitrite (0.015mmol)

dissolved in water was added drop wise within 10 min, thus yielded respective diazonium salts.

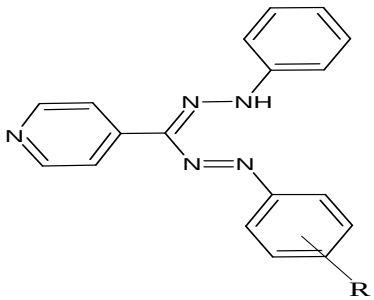
Step-3: Preparation of formazan Derivatives

Schiff base (0.01 mol) was dissolved in pyridine (5 ml) and cooled to 0-5°C. To this cold solution, diazotized amine solution was added drop wise with shaking within 10 min and solution left at room temperature for 3 hr and Solid separated out. Solid was filtered, washed with water and Recrystallized from ethanol to give corresponding formazan derivatives. Melting point : 140-220 ^{0}C ; Novel synthesized derivatives showed IR absorption at the region of Hetero C- H(Ar)stretching at 3850-3034 cm-1 , C-H(Ar)stretching at 3035-3066 cm-1 , -C=N-stretching at 1400-1494cm-1 , -N=N-stretching at 1543-1558 cm-1 , 2^{0} and3^{0} amines stretching at 3230- 3332 cm-1.In ^{1}H NMR spectra delta values of compounds were found in the range of 6.7-8.4for aromatic protons, 6.5-8.6 for CH=CH protons , 1.7-1.8 for methyl protons , and 14.2 forCOOH protons .δ ppm : 302.2-355.8.

RESULTS AND DISCUSSION

The selected compounds were screened for their anti-tubercular activity by microplatealamar blue assay (MABA) method. The effectof the synthesized compounds were tested with different concentrations (0.8,1.6,3.12,6.25,12.5,25,50,100µg/ml) against standards like pyrazinamide, ciprofloxacin, streptomycin and Strain used: M. tuberculosis (H37 RV strain).All selected derivatives shows resistance at lower drug concentrations (0.8, 1.6,3.12, 6.25µg/ml). The results were tabulated in table no. 4,selected synthesized compounds FZ_1 ,FZ_2,FZ_5 and FZ_6 showed highest anti-tubercular activity and among them FZ_1& FZ_2shown highest antitubercular activity at 1.6 µg/ml concentration when compared with the standard and exhibited> 90% inhibition at lower concentration.

Table 1: Physical characteracteristic data of formazan derivatives



COMPOUND NAME	MOLECULAR FORMULA	MOLECULAR WEIGHT	SOLUBILITY	COLOUR & STATE	MELTING POINT	% YIELD	Rf VALUE
FZ_1	$C_{18}H_{15}N_5$	301.3	Chloroform	Brickredcolour solid	140	46	0.81
FZ_2	$C_{19}H_{17}N_5$	315.3	Chloroform	Brickredcoloursolid	182	89	0.67
FZ_3	$C_{19}H_{17}N_5$	315.3	Chloroform	Yellow coloursolid	192	63.6	0.68
FZ_4	$C_{19}H_{15}N_5O_2$	345.3	Chloroform	Brick colour solid	210	85.2	0.15
FZ_5	$C_{18}H_{14}N_6O_2$	346.3	Chloroform	Brick colour solid	148	66.6	0.77
FZ_6	$C_{18}H_{14}N_6O_2$	355.79	Chloroform	Brick red colour solid	180	80	0.71

Table 2: Anti-TB activity of various formazan derivative against different concentrations

S.no	Sample	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	FZ_1	S	S	S	S	S	S	S	R
2	FZ_2	S	S	S	S	S	S	S	R
3	FZ_5	S	S	S	S	S	S	R	R
4	FZ_6	S	S	S	S	S	R	R	R

Table 3: Anti-TB activity of Standard drugs against different concentrations

S.no	Standard drugs	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	Pyrazinamide	S	S	S	S	S	S	R	R
2	Ciprofloxacin	S	S	S	S	S	R	R	R
3	Streptomycin	S	S	S	S	S	S	R	R

Table 4: Represents minimum inhibitory concentration of novel formazan derivatives when compared with standard

S.NO	COMPOUND	Minimum Inhibitory Concentration (MIC,µg/ml)
1.	FZ_1	1.6
2.	FZ_2	1.6
3.	FZ_5	3.12
4.	FZ_6	6.25
5.	PYZ	3.125
6.	CPF	6.25
7.	STR	3.125

From the above resultsMIC values of selected formazan derivatives by MABA methodHere pyrazinamide (PYZ), Ciprofloxacin (CPF) and Streptomycin (STR) are used asReference standards.

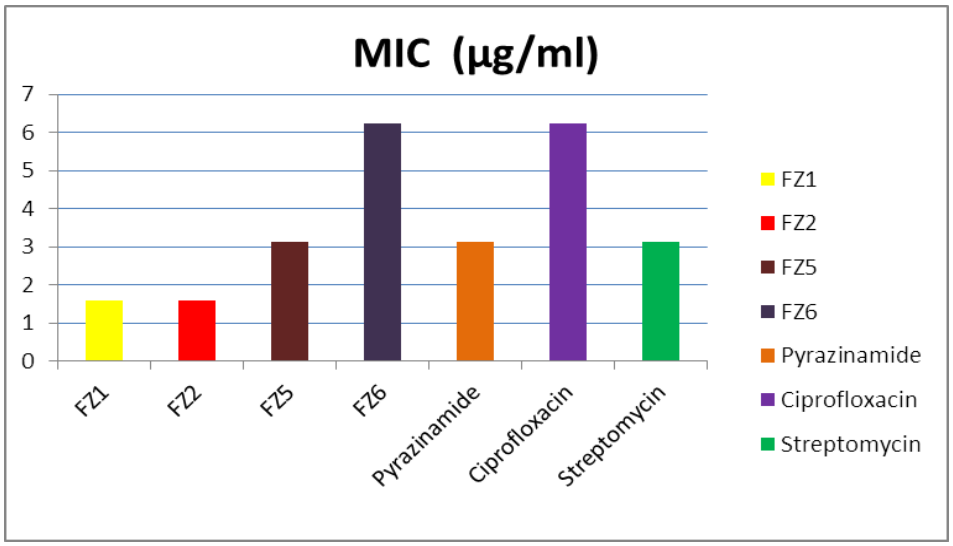


Figure 1.Graphical presentation of the minimum inhibitory concentrations of the selected formazan derivatives against Mycobacterium tuberculosis obtained through Microplate Alamar Blue Assay method.

CONCLUSION

The selected four derivatives (FZ_1,FZ_2,FZ_5,FZ_6) revealed that they were highly potent when compared to standard drugs like pyrazinamide, streptomycin and ciprofloxacin. Among them highest activity was shown by FZ_1 and FZ_2 at 1.6µg/ml concentration and exhibited > 90% inhibition at lower drug concentration.

The formazan moiety itself possesses various activities. Futurescope of this work is to develop novel formazan derivatives by changing various pharmacophore groups on basic nucleus which may result into more potent anti-tuberculosis activity than existing one and research can also be carried out further in order to know the relationship between structure and biological activities.

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