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RESERCH ARTICLE

Microwave Assisted Organic Synthesis of Novel 1, 2, 4-Triazolium Salts as Antimicrobial Agents

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ABSTRACT

Microwave assisted organic synthesis (MAOS) is emerging as a new tool in the organic synthesis. This approach is considered to be an important step towards green chemistry. This technique stands out as it is more eco-friendly compared to traditional heating methods in organic chemistry. Synthesis of new chemical entities is the most important step in drug discovery. The increasing problem of antimicrobial resistance has made it highly urgent to design and develop novel types of antimicrobial agents consisting different chemical structures of the traditional drugs. It is rational to investigate triazolium compounds as novel antimicrobial agents since triazole has shown a broad range of pharmacological activity. In view of this, a series of 1, 2, 4-triazolium disubstituted derivatives have been synthesized starting from Isoniazid. The structures were confirmed with the help of IR, NMR, and Mass spectroscopy. The compounds were tested for antibacterial, antifungal and anti-tubercular activity and have shown poor to moderate activity.

KEYWORDS

Green chemistry, 1, 2, 4-triazolium salts, anti-bacterial, anti-fungal, anti-tubercular

INTRODUCTION

Microwave Assisted Organic Synthesis (MAOS)

Microwave-assisted organic synthesis (MAOS) is emerging as a new tool in organic synthesis. This approach is considered to be an important step towards green chemistry.[1] This technique stands out as it is more eco-friendly compared to traditional heating methods in organic chemistry. Synthesis of new chemical entities is the most important step in drug discovery.

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MAOS offers a simple, clean, fast, efficient, and economical method for the synthesis of a large number of organic molecules.[2] It helps in achieving a highly accelerated rate of reaction, reduction in reaction time, high yield and quality product. This technique helps in the growth of green chemistry and helps in significant reduction of by-product, lower waste production, and a lower energy consumption by reducing the reaction time to fewer minutes. It has the ability to couple directly with reaction molecule and by using thermal conductivity leading to a rapid rise in the temperature.[3] Hence it is very important to extensively use this technique on laboratory scale since it can be very impactful in the fields of screening, combinatorial chemistry, medicinal chemistry and drug development.[4]

Rationale for Synthesis of Triazolium Salts

Azolium salts have attracted a huge amount of attention due to its application in several different fields. Azolium compounds have shown a wide range of pharmacological activities. Imidazolium and triazolium salts have been pursued for their bioactivity. A 1.2.4-triazolium salt (Figure 1) is composed of a cationic five-membered ring containing three nitrogens two of which are bonded to one another. The positively charged triazolium core is associated with a negatively charged counterion.[5]

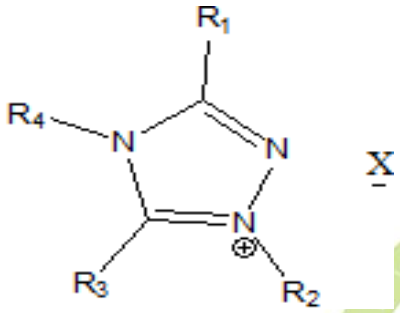


Figure 1: A triazolium salt

The increasing problem of antimicrobial resistance has made it highly urgent to design and develop novel types of antimicrobial agents consisting different chemical structures of the traditional drugs. Many pieces of research have focussed their attention on the triazole ring by as antimicrobial agents. It is rational to investigate triazolium compounds as novel antimicrobial agents since triazole has shown a broad range of pharmacological activity. So far, however, triazolium derivatives as antimicrobial agents have been rarely reported. Some reports in the literature showed that the addition of a positive charge group in the triazole derivatives is helpful to increase the water solubility and membrane permeability,[6,7] which could result in the enhancement of biological activities. In addition, many investigations could show that the introduction of variable aromatic and aliphatic substituents could sufficiently affect the biological activities of triazole derivatives.[8,9] Generally, the mode of action of quaternary ammonium compounds consists of their interaction with the cytoplasmic membrane of bacteria and the subsequent loss of permeability properties of the membrane.[10]

In view of this, a series of 1, 2, 4-triazolium disubstituted derivatives have been synthesized[11,12] starting from Isoniazid, and their antibacterial and antifungal activities were evaluated. Since triazolium derivatives of isoniazid are prepared they were also tested for anti-tubercular activity.

MATERIALS AND METHODS

Materials

The solvents such as ethanol, methanol, diethyl ether, chloroform, acetone, DMF, toluene were purchased from Loba chemicals, India.

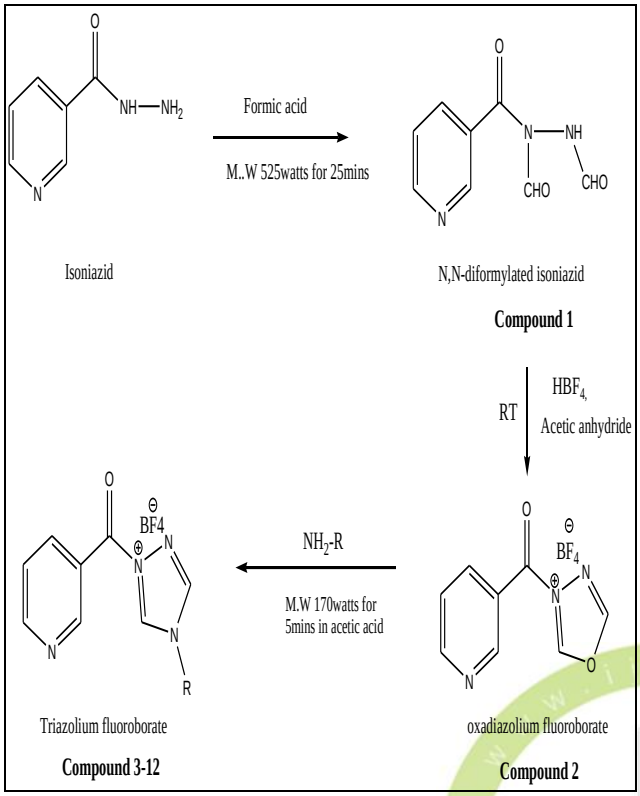
Isoniazid, formic acid, perchloric acid(70%), fluoroboric acid(40%), diethyl ether, acetonitrile, glacial acetic acid, 4-methoxy aniline, cyclohexylamine, 4-aminobenzoic acid, 2,4,6-trimethylaniline, 4-nitroaniline, 4-methylaniline, aniline, 4-bromoaniline, 2-chloroaniline, 4-hydroxyaniline, were purchased from Rajesh Chemicals, India. Acetic anhydride was purchased from Chemdyes Corporation, India.

Throughout this work, solvents were used after distillation. Melting points were determined by the open capillary method on a "Veego" VMP-I apparatus and are uncorrected. Microwave synthesis was carried out using Catalyst Microwave oven - CATA-2R, at the power level ranging from 1 to H at 85 to 850 watts. The purity of compounds was checked on silica G 60 F_{254} plates and visualized in UV chamber.

The IR spectra were recorded in the 4000-400 cm^{-1} range in SHIMADZU IR INFINITY by placing the sample directly on a probe. ^1H - NMR was recorded on BRUKER AVANCE II (400 MHz) spectrometer in $CDCl_3$ or DMSO-d_6 as solvent using trimethylsilane (TMS) as internal reference standard and values are expressed in δ ppm.

^1H-NMR and Mass spectra were recorded at Sophisticated Analytical Instrument Facility (SAIF). Biological activities were done at Central Drug Research Institute, Lucknow.

Scheme of Synthesis



Experimental Section

Step 1: Synthesis of N, N-diformyl isoniazid (Compound 1)

Isoniazid (10 gm, 0.07 moles) was taken in RBF and cooled down to 0˚C by keeping it in ice and salt mixture. The mixture of isoniazid and formic acid mixture was heated in the microwave at 595 watts (Power 5) for 25 mins. The reaction was monitored using TLC using solvent phase (Ethyl acetate: Hexane: Chloroform) (2:2:1). The compound obtained was washed with methanol (15-20 ml) to remove excess of starting material. The compound obtained was purified by recrystallization with ethanol.

Step 2: Synthesis of Isonicotinoyl 1,3,4-oxadiazolium fluoroborate (Compound 2)

The N, N –diformylisoniazid (1 gm, 0.00518 mol) is suspended in acetic anhydride 6 ml. The reaction vessel was kept so as to maintain the temperature below 30˚C. Perchloric acid (70 %) or Fluoroboric acid (40 %) (1.05 ml, 0.00598 mol) was added slowly dropwise while maintaining the temperature below 30˚C. The mixture was allowed to cool so as to separate oxadiazolium salt usually; otherwise ether is added for the separation. If salt is oily, then it is induced to solidify by scratching or triturating with ether. Then salt is filtered off and washed with ether. The reaction can be monitored using TLC [Ethyl acetate: hexane (2:1)]. The compound was recrystallized using ethanol as the solvent.

Step 3: Synthesis of triazolium fluoroborate (Compound 3-12)

Oxadiazolium salt (1 gm, 0.0038 mol) and amines (0.013 mol) were dissolved in 10 -15 ml of glacial acetic acid. Oxadiazolium salt and amine mixture in acetic acid was heated in microwave at 225 watts (Power 2) for 5-10 mins. Triazolium salt usually separated when the mixture was cooled; otherwise, ether was added to incipient turbidity. If oil separated, it was caused to solidify by trituration with ether. The reaction was monitored using TLC [Ethyl acetate: hexane (2:1)]. The compound was recrystallized using ethanol and water mixture (80:20).

Biological Activity

All derivatives of 1, 4-disubstituted-1, 2, 4-triazolium salts were screened for anti-bacterial, anti-tubercular and anti-fungal activities using following protocols:

Antibacterial Activity

1. Materials - ATCC (ATCC, Manassas, VA, USA) and Mueller–Hinton broth II (MHB II) (Becton Dickinson) were used as media.
2. Experimental procedure - Determination of MIC - The compounds were serially diluted utilizing 2-fold dilutions and bacteria were subsequently added to a final count of 10^4-10^5 CFU/ml. The 96-well plates were incubated at 37^0C for 18-24 h and the antimicrobial activity was determined by visual inspection. The MIC of the active compounds was determined and was defined as the lowest concentration of the compound that inhibited visible growth after 24 h.

Antitubercular Activity

1. Materials - All the chemicals such as sodium salt XTT [2,3-Bis-(2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carbxanilide], DMSO and rifampicin were purchased from Sigma-Aldrich, USA. Dubos medium (enrichment media) was purchased from DIFCO, USA. Compounds were dissolved in DMSO, this solution was used as stock solutions for further antitubercular testing. Microbial strains of *Mycobacterium tuberculosis* H37Ra was obtained from AstraZeneca, India. The stock culture was maintained at -80^{0}C and subcultured once in a liquid medium before inoculation into an experimental culture. Cultures were grown in dubos media. For antitubercular assay, M. phlei medium (minimal essential medium) was used. The composition of dubos medium is 0.5g potassium dihydrogen phosphate, 0.25g trisodium citrate, 60mg magnesium sulphate, 0.5g aspargine and 2mL glycerol in distilled water (100mL) followed by pH adjustment to 6.6.

2. Experimental procedure - *Mycobacterium tuberculosis* H37Ra were grown to logarithmic phase in a dubos or M. phlei medium. The stock culture was maintained in the refrigerator and sub-cultured once in the dubos medium before inoculation into experimental culture. Inhibitors solution were added into 96 well plate which is solubilized in 100% DMSO. Conc. was used according to need for primary 30, 10, 3 µg/mL and 100, 50, 25..... for dose response. 0.1% of OD culture was used for screening. For standardization of % inoculation, we put different (%) culture in media. Ranging from 0.1 to 0.5% and another set 0.5-2% culture in the media. We found that 0.1% is good enough for screening. Here we use both dubos as well as M. phlei medium. M. phlei shows the same result with dubos and not interferes in compound screening, so we decide is suitable for screening. 0.1% inoculated culture was added to each well of 96 well plate. Kept it into 37^{0}C incubator. Rifampicin was used for standard inhibitor. Cells in the presence of vehicle (DMSO) without inhibitor used as control. Media in the presence of a vehicle without cell used as blank. % inhibition was calculated by using the following formula -

% Inhibition = 100 - [(Comp OD - blank/ Control OD - blank) x 100]

All experiments were performed in triplicates.

Antifungal Activity

1. Materials - The media Brain Heart Infusion (BHI) broth used was provided by HIMEDIA.

2. Experimental procedure - Determination of MIC - 9 dilutions of each drug are done with BHI for MIC. In initial tube, 20 microliters of the drug were added into the 380 microliters of BHI broth. For dilutions, 200 microliters of BHI broth was added into the next 9 tubes separately. Then from the initial tube, 200 microliters were transferred to the first tube containing 200 microliters of BHI broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube, 200 microliters were transferred to the second tube to make 10^{-2} dilution. This serial dilution was repeated up to 10^{-9} dilution for each drug. From the maintained stock cultures of required organisms, 5 microliters were taken and added into 2mL of BHI broth. In each serially diluted tube, 200 microliters of above culture suspension were added. The tubes were incubated for 24 hrs and observed for turbidity.

RESULTS & DISCUSSION

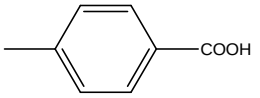
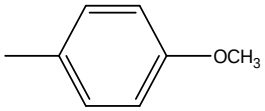
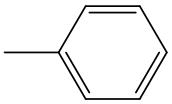
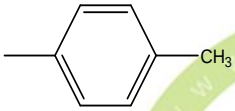
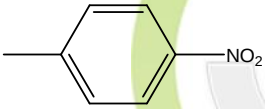
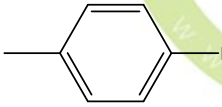
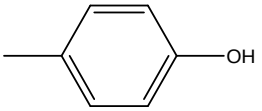
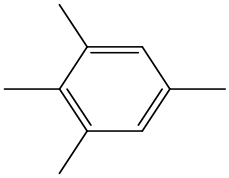
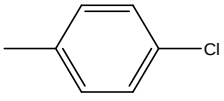
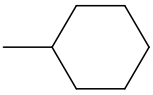
The structure, physical properties, yield, R_f values, Melting point are given in **table 1.**

Spectral Characteristics of the Synthesized Compounds:

1-Isonicotinoyl-4-(4-carboxyphenyl)-1,2,4-triazolium fluoroborate **(Compound 3)**

$C_{15}H_{11}N_4O_3BF_4$, **IR Assignments (cm^{-1}):** 3236.66 (O-H str.), 3005.20 (C-H Ar str.),

Table 1: Physicochemical Properties of Different Analogues

Compound code	Substituent -R	Yield (%)	Rf value	M.P.(°C)
3		55	0.58	207-210
4		40	0.49	>280 decomp.
5		35	0.55	276-280
6		39	0.64	>280 decomp.
7		87	0.66	190-194
8		48	0.63	259-261
9		39	0.56	>280 decomp.
10		41	0.60	>280 decomp.
11		51	0.65	>280 decomp.
12		47	0.52	>280 decomp.

1643.41(C=O str. Amide), 1600.97 (C=N str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 10.10 (1H, s, carboxylic acid), 8.92 (2H, d, Ar-Py), 8.1-8.08 (2H, s, imine), 7.8 (2H, d, Ar-Py), 7.69-7.66 (4H, m, Ar-Ph).

1-Isonicotinoyl-4-(4-methoxyphenyl)-1,2,4-triazolium fluoroborate **(Compound 4)**

$C_{15}H_{13}N_4O_2.BF_4$, **IR Assignments (cm^{-1}):** 3065 (C-H Ar str.), 1691.57 (C=O str. Amide), 1616 (C=N str.), 1080 (ether stretch), **MS:** M/z = 365.90 (M-1) $^+$(78%), -$C_{10}H_{10}BF_4N_3O_2^+$ m/z = 289 (30%).

1-Isonicotinoyl-4-phenyl-1,2,4-triazolium fluoroborate **(Compound 5)**

$C_{14}H_{11}N_4O.BF_4$, **IR Assignments (cm^{-1})**: 3051 (C-H Ar str.), 1691.57 (C=O str. Amide), 1600 (C=N str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 8.80-8.78 (2H, d, Ar-Py), 8.5-8.4 (2H, t, imine), 7.8 (2H, d, Ar-Py), 7.57-7.55 (2H, d, Ar-Ph), 7.29-7.27 (2H, d, Ar-Ph), 7.03-6.94 (1H, d, Ar-Ph).

1-Isonicotinoyl-4-(4-methylphenyl)-1,2,4-triazolium fluoroborate **(Compound 6)**

$C_{15}H_{13}N_4O.BF_4$, **IR Assignments (cm^{-1})**: 3043 (C-H Ar str.), 1691.57 (C=O str. Amide), 1612 (C=N str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 8.79-8.78 (2H, d, imine), 7.8 (4H, dd, Ar-Py), 7.45-7.43 (2H, d, Ar-Ph), 7.08-7.06 (2H, d, Ar-Py), 1.9 (3H, methyl).

1-Isonicotinoyl-4-(4-nitrophenyl)-1,2,4-triazolium fluoroborate **(Compound 7)**

$C_{14}H_{10}N_5O_3.BF_4$, **IR Assignments (cm^{-1})**: 3000 (C-H Ar str.), 1676.14 (C=O str. amide), 1597 (C=N str.), 1560 & 1346.31 (NO_2 str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 8.90-8.88 (2H, dd, Ar-Py), 8.19-8.16 (2H, s, imine), 7.8 (2H, d, Ar), 8.06-8.03 (2H, dd, Ar-Ph), 7.81-7.78 (2H, m, Ar-Ph).

1-Isonicotinoyl-4-(4-bromophenyl)-1,2,4-triazolium fluoroborate **(Compound 8)**

$C_{14}H_{10}BrN_4O.BF_4$, **IR Assignments (cm^{-1})**: 3000 (C-H Ar str.), 1664.57 (C=O str. amide), 1584 (C=N str.), 603.77 (C-Br str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 8.76-8.75 (2H, dd, imine), 7.8 (4H, dd, Ar-Py), 7.56-7.54 (2H, dd, Ar-Ph), 7.41-7.39 (2H, m, Ar-Ph).

1-Isonicotinoyl-4-(4-hydroxyphenyl)-1,2,4-triazolium fluoroborate **(Compound 9)**

$C_{14}H_{11}N_4O_2.BF_4$, **IR Assignments (cm^{-1})**: 3385 (O-H Ar str.), 1641.42 (C=O str. amide), 1614 (C=N str.), 1078 (C-O str.) **NMR Assignments (DMSO-d_6, δ, ppm):** 8.78-8.77 (2H, dd, imine), 8.19-8.16 (2H, s, imine), 7.8 (2H, d, Ar-Py), 7.34-7.32 (2H, dd, Ar-Ph), 7.15-7.14 (2H, dd, Ar-Ph).

1-Isonicotinoyl-4-(2,4,6-trimethylphenyl)-1,2,4-triazolium fluoroborate **(Compound 10)**

$C_{17}H_{17}N_4O.BF_4$, **IR Assignments (cm^{-1})**: 3000 (C-H Ar str.), 2922.16 (C-H aliphatic) 1641.42 (C=O str. amide), 1604 (C=N str.) **MS:** m/z = 381.5 $(M+1)^+$ (85 %), -$C_8H_6N_4O^+$m/e =174.054 (100 %).

1-Isonicotinoyl-4-(2-chlorophenyl)-1,2,4-triazolium fluoroborate **(Compound 11)**

$C_{14}H_{10}ClN_4O.BF_4$, **IR Assignments (cm^{-1})**: 3000 (C-H Ar str.), 2922.16 (C-H aliphatic) 1691.42 (C=O str.), 1604.77 (C=N str.), 759.95 (C-Cl str.) **MS**: m/z = 372.81 (M^+) (100%), -$C_6H_4NO^+$, m/e = 106.029 (88 %).

1-Isonicotinoyl-4-cyclohexyl-1,2,4-triazolium fluoroborate **(Compound 12)**

$C_{14}H_{17}N_4O.BF_4$, **IR Assignments (cm^{-1})**: 3090 (C-H Ar str.), 2924.09 (C-H aliphatic) 1708.93 (C=O str.), 1612.49 (C=N str.) **NMR Assignments (DMSO-d_6, δ, ppm) :** 8.74-8.71 (2H, dd, Ar), 8.2 (2H, s, imine), 8.0-7.8 (2H, d, Ar-Py), 2.9 (1H, q, -CH), 1.9 (2H,q, -CH_2), 1.7 (2H, q, -CH_2), 1.3-1.2 (6H, q, -CH_2).

The compounds were synthesized and characterized using IR, NMR and Mass spectroscopy and the structures were confirmed with the help of the above data. Hence this observation shows that microwave synthesis of the above compounds was highly energy efficient compared to the conventional methods involving several hours of reflux. This result satisfies the principles of green chemistry by

Table 2: MIC Values of Final Compounds (Antibacterial Activity)

	MIC µg/ml				
Compound Code	EC (***Escherichia coli*** - gram negative)	SA (***Staphylococcus aureus*** - gram positive)	KP (***Klebsiella pneumoniae*** -gram negative)	AB (***Acinetobacter bitumen*** - gram negative)	PA (***Pseudomonas aeruginosa*** - gram negative)
3	>64	>64	>64	>64	>64
4	>64	>64	>64	>64	>64
5	>64	>64	>64	>64	>64
6	>64	>64	>64	>64	>64
7	>64	>64	>64	>64	>64
8	>64	>64	>64	>64	>64
9	>64	>64	>64	>64	>64
10	>64	>64	>64	>64	>64
11	>64	>64	>64	>64	>64
12	>64	>64	>64	>64	>64
Levofloxacin	<0.5	<0.5	64	32	<0.5

using less energy. This microwave method also helps in safer reaction conditions.

Biological Evaluation

Antibacterial Activity

For antibacterial activity Gram-positive strain of *Staphylococcus aureus* and Gram-negative strain of *Escherichia coli, Klebsiella pneumonia, Acinetobacter baumannii,* and *Pseudomonas aeruginosa* were used and standard used was Levofloxacin. MIC of all the targeted compounds is shown in **Table 2.**

Levofloxacin was used as the standard and has MIC values of <0.5 µg/ml against *Escherichia coli,* <0.5 µg/ml against *Staphylococcus aureus,* 64 µg/ml against *Klebsiella pneumonia,* 32 µg/ml against *Acinetobacter baumannii* and <0.5 µg/ml against *Pseudomonas aeruginosa* as shown in Table 2. The Above results indicated that all synthesized compounds were found to be less active as compared to the standard

against Gram-ve bacteria & Gram +ve bacteria. All the synthesized compounds showed MIC values **>64 µg/ml.**

Antifungal Activity

For antifungal activity, *Candida albicans* were used and standard used was Fluconazole. MIC of all the targeted compounds is shown in **Table 3.**

Table 3: MIC Values of Final compounds (Antifungal Activity)

Compound Code	*Candida albicans* MIC (µg/ml)
3.	>125
4.	>125
5.	>125
6.	>125
7.	>125
8.	>125
9.	>125
10.	>125
11.	>125
12.	>125
Fluconazole	16µg/ml

Fluconazole was used as a standard and has a MIC value of **16 µg/ml** against ***Candida albicans*** as shown in **Table 3.** The above results indicated that all synthesized compounds were found to be less active as compared to the standard against ***Candida albicans*** with MIC value **>125 µg/ml**.

Antitubercular Activity

For antitubercular activity *Mycobacterium fortuitum, Mycobacterium chelonae, Mycobacterium abscessus, Mycobacterium tuberculosis* H37Rv strain was used and standard used was Isoniazid. MIC of all the targeted compounds is shown in **Table 4.**

Table 4: MIC Values of Final Compounds (Antitubercular Activity)

MIC (µg/ml)				
Code	***M.fortuitum***	***M. chelonae***	***M. abscessus***	***M.tb* H37Rv**
3.	>64	>64	>64	>64
4.	>64	>64	>64	>64
5.	>64	>64	>64	>64
6.	>64	>64	>64	>64
7.	>64	>64	>64	>64
8.	>64	>64	>64	>64
9.	>64	>64	>64	>64
10.	>64	>64	>64	>64
11.	>64	>64	>64	>64
12.	>64	>64	>64	>64
Isoniazid	0.03	0.03	0.03	0.03

Isoniazid was used as a standard and has a MIC value of **0.03 µg/ml** against ***Mycobacterium tuberculosis*** shown in Table 4. The above results indicated that all synthesized compounds were found to be less active as compared to the standard against ***Mycobacterium tuberculosis*** with MIC value **>64 µg/ml.**

CONCLUSIONS

Derivatives of 1, 2, 4-triazolium salts were characterized on the basis of IR, ^{1}H-NMR and Mass spectral data and the structures of the synthesized compounds were confirmed. Conventional heating and microwave irradiation method were compared in terms percent yield, reaction time. The results showed

that the microwave irradiation method was greener and eco-friendly than conventional heating. All compounds were screened for their *in vitro* antibacterial, anti-tubercular and antifungal activity. However, the results indicated that the synthesized compounds possess poor to moderate activity with reference to their respective standards.

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CONFLICT OF INTEREST

The authors confirm that this article's content has no conflicts of interest.

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