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Synthesis and Assessment of Antitubercular and Antimicrobial Activity of Some Novel Triazolo and Tetrazolo-Fused 1, 3, 4-Oxadiazole Molecules Containing Pyrazine Moiety

R Das[1]. G Shilakari Asthana[1]. KA Suri[2]. DK Mehta[1]. A Asthana[1]

[1]*M.M. College of Pharmacy, Maharishi Markandeshwar University, Mullana, Ambala, HR, India-133207*

[2]*Indian Institute of Integerative Medicine (IIIM), Jammu Tawi, J&K, India*

Abstract

The present work was aimed to synthesize and determine antitubercular and antimicrobial potential of some novel triazole and tetrazole fused 1, 3, 4-oxadiazole compounds from pyrazinoic acid as precursor, which is a well established antitubercular agent.

Pyrazinoic acid **1** was esterified first **2** followed by hydrazinolysis to produce hydrazide **3** which was refluxed with CS_2 to obtain 2-sulfanyl-5-pyrazyl-1, 3, 4-oxadiazole **4**. This was then further hydrazinolysed to obtain compound **5**.Cyclisation of compound **5** afforded compound **6** and **7**. Structures of newly synthesized compound were characterized and authenticated by TLC, IR, ^{1}H NMR and Mass spectrometry. Antibacterial and antifungal activities of all the synthesized compounds were assessed against various strains of bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and of fungi such as *Aspergillus niger* and *Candida albicans* and also compared with reference drugs (Amoxicillin and Miconazole). Further antitubercular potential of the synthesized compounds was determined against *Mycobacterium* tuberculosis H37Rv strain.

All oxadiazoles were synthesized in good yields. Among all the synthesized compounds, compound **5** displayed promising antibacterial, antifungal and antitubercular activity profile and would be an effective candidate for tuberculosis therapy.

Key words: Antimicrobial, Antitubercular activity, Oxadiazole, Triazole, Tetrazole

INTRODUCTION

Microbial infections remain the chief cause of mortality worldwide. Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is one of the main killers of people all over the world. The major hurdles with existing therapy are the lengthy regimen and appearance of multi drug resistant (MDR) and extensively drug resistant (XDR) strains of *M.tuberculosis.* As a result, there is a burning demand for a new class of antimicrobial agent with a different manner of action and it led medicinal chemists to investigate a wide range of chemical structures. In pursuit of this goal, our research efforts herein have been directed towards the discovery of new chemical entities that are effective antimicrobial and antitubercular agents [1].

The synthesis of fused heterocycles has attracted immense interest in heterocyclic chemistry as the blend of bioactive heterosystems has proved to be very attractive and valuable for the design of new molecular skeleton of prospective drugs with varying pharmacological profiles. Designing highly efficient chemical reaction sequences which provide molecules containing utmost intricacy and structural diversity with interesting bioactivities in minimum number of synthetic steps is the major challenge of the modern synthetic chemistry [1].

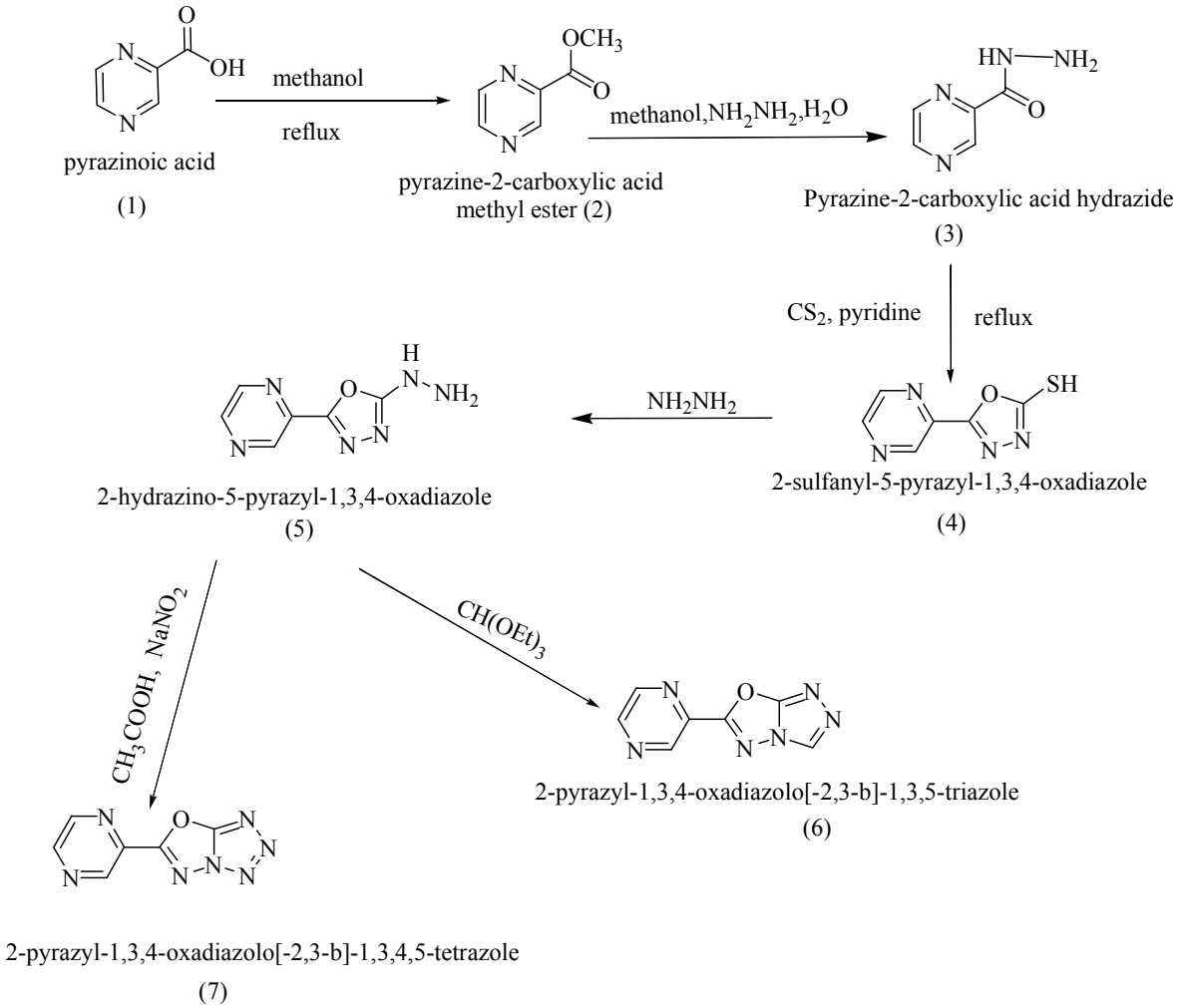
We have found that the triazoles and fused triazoles are multipurpose classes of fused heterocycles that are of substantial interest because of the diverse range of their biological properties and the potent pharmacological activities [2, 3].

Also it was found that tetrazoles and condensed tetrazoles possess diverse range of effective pharmacological activities [4-6].

The oxadiazoles skeleton is present in a variety of biologically active compounds and have played important role in medicinal chemistry, such as anti-inflammatory, anticonvulsant, antifungal, antibacterial and many more [7-15]. These compounds also have been reported to have significant antitubercular activity [16-18].

Hence on the basis of the above findings it can be summarized that the triazoles, tetrazoles and 1, 3, 4-oxadiazoles are privileged structures, which attracted considerable attention in the designing of pharmacologically dynamic molecules and combining them in one molecule framework it is anticipated to endow biologically active molecule with distinguishing features.

Still, after thorough literature survey it was observed that there were only limited research work devoted to the synthesis of triazolo-fused 1, 3, 4-oxadiazole and tetrazolo-fused 1, 3, 4-oxadiazole molecules. Accordingly here we report the synthesis and antitubercular and antimicrobial activity of some novel triazolo-fused 1, 3, 4-oxadiazole and tetrazolo-fused 1, 3, 4-oxadiazole molecules containing pyrazine moiety.



Scheme 1 Synthesis of 2-pyrazyl-1, 3, 4-oxadiazolo [-2,3-b]-1,3,5-triazole and 2-pyrazyl-1,3,4-oxadiazolo[-2,3-b]-1,3,4,5-tetrazole

MATERIAL AND METHODS

Experimental

Reagents: *Starting* materials, reagents and solvents were purchased from Sigma-Aldrich, USA and Merck, Germany and were used further without any purification.

Equipments: The progress of reaction was confirmed by using thin layer chromatography (TLC) method on silica gel plates (Merck, Germany) of 3x15 cm coated with silica gel G. The UV lamp (254nm) was used for TLC visualization of spots and R_f values were determined. Melting points were determined on digital melting point apparatus (Flora; Perfit, India) and were uncorrected. Elemental analysis (C, H, N) was conducted using Carlo Erba analyzer model 1108. The IR spectra (in NaCl prism) were recorded on Schimadzu FT-IR spectrophotometer using Nujol method. The ^{1}H NMR spectra were recorded (in DMSO) on a BRUKER AVANCE-400 MHz spectrometer using TMS as an internal standard, chemical shift values were expressed in ppm. The spin multiplicities are indicated by the symbols, s (singlet), d (doublet), t (triplet), m (multiplet) and br (broad). Mass spectra (ESI, 70ev) were recorded on Water, Q-TOF Micromass (LC-MS).

Methodology

The scheme for the synthesis of 2-pyrazyl-1, 3, 4-oxadiazolo [-2,3-b]-1,3,5-triazole and 2-pyrazyl-1,3,4-oxadiazolo[-2,3-b]-1,3,4,5-tetrazole from pyrazinoic acid is shown in Scheme 1.

*Synthesis and spectral data of compounds **2-7***:

*Pyrazine-2-carboxylic acid methyl ester **(2):*** Pyrazinoic acid (0.1mol), 60ml methanol and conc. H_2SO_4 (1.4ml) were refluxed for 7hrs. The progress of reaction was monitored via TLC till single spot. The mixture was then cooled and poured in crushed ice, made alkaline followed by extraction with ether. Ether layers were evaporated and kept aside to obtain shiny pale brownish crystals of compound **2** in good yield. Recrystallisation was done using methanol.

*2-pyrazylhydrazide **(3):*** In a round bottom flask mixture of ester **2** and hydrazine hydrate in 1:1 portion and 30 ml of ethanol were taken and refluxed for 4-6 hrs [19]. The progress of reaction was monitored via TLC till single spot. Excess of ethanol was removed by distillation. Product **3** separates out upon cooling in good yield which was filtered and dried. Recrystallisation was carried out with methanol.

*2-sulfanyl-5-pyrazyl-1, 3, 4-oxadiazole **(4):*** Aryl hydrazide 3 (0.001mol) was dissolved in aqueous solution of KOH

(0.001 mol) with constant stirring. The mixture was added to 13 ml of methanol to get a clear solution. CS_2 (0.3ml, 0.001mol) was then added and the mixture was refluxed for 6hrs [19]. The progress of reaction was monitored via TLC till single spot. It was concentrated on a water bath and the resulting solution was poured on ice water and filtered. The filtrate was acidified with dil. HCl. The product thus obtained was filtered, washed with cold water and dried. Recrystallisation was done using methanol to give compound **4**.

Obtained in 72% as light-brownish powder ; mp (°C): 180-182; IR (Nujol, cm^{-1}): 3001(Ar-C-H str.), 1475.27 (C=C str), 1615.50 (C=N), 1076.27 (C-O-C str in mjudc oxadiazole), 2720.45(C-SH str); ^{1}H NMR (DMSO) δ (ppm): 8.63-8.81(m,3H,CH of pyrazine) 3.35(s, ^{1}H,Ar-C-SH); Ms (m/z): 180(M^+), other fragments peak are 101,68; Anal. Calcd. for $C_6H_4N_4OS$ C, 39.99; H, 2.24; N, 31.09, Found: C, 39.85; H, 2.29; N, 31.02.

*2-hydrazino-5-pyrazyl-1, 3, 4-oxadiazole (**5**)*: The compound **5** was synthesized by refluxing a mixture of compound **4** (0.01mol) and 6ml hydrazine hydrate in methanol (35ml) for 6hrs. The progress of reaction was monitored via TLC till single spot. Then the mixture was allowed to cool and was concentrated. Crystals of **5** obtained were recrystallized with ethanol.

Obtained in 67% as yellow crystals; mp (°C): 176-178; IR (Nujol, cm^{-1}): 2995(Ar-C-H str.), 1614.25 (C=N), 1066.42 (C-O-C str in oxadiazole), 1495(C=C str), 3445.25(NH str); ^{1}H NMR (DMSO) δ (ppm): 8.61-8.73(m,3H,CH of pyrazine), 3.37(s,2H, NH_2), 4.31(s,1H, NH); Ms (m/z): 178 (M^+), other fragments peak are 162, 99; Anal. Calcd for $C_6H_6N_6O$ C, 40.45; H, 3.39; N, 47.17, Found: C, 40.41; H, 3.43; N, 47.12.

*2-pyrazyl-1, 3, 4-oxadiazolo [-2, 3-b]-1, 3, 5-triazole (**6**)*: To a mixture of compound **5** (0.015mol) and triethylorthoformate (3ml) in methanol (20ml) few drops of acetic acid were added. The mixture was refluxed for 4hrs and then allowed to cool and the solvent was evaporated. The progress of reaction was monitored via TLC till single spot. The solid product **6** thus obtained was recrystallized from acetic acid.

Obtained in 62% as brownish crystals; mp (°C): 162-163; IR (Nujol, cm^{-1}): 3015(Ar-C-H str.), 1465.40 (C=C str), 1612.56 (C=N), 1082.11 (C-O-C str in oxadiazole); ^{1}H NMR (DMSO) δ (ppm): 8.69-8.82(m,3H,CH of pyrazine), 8.34(s, 1H,CH-triazole) ; Ms (m/z): 188(M^+), other fragments peak are 109, 83; Anal. Calcd. for $C_7H_4N_6OC$, 44.69; H, 2.14; N, 44.67, Found: C, 44.73; H, 2.18; N, 44.71

*2-pyrazyl-1,3,4-oxadiazolo[-2,3-b]-1,3,4,5-tetrazole (**7**)*: Sodium nitrite solution (14ml,0.01mol) was added drop wise to a solution of compound **6** (0.01mol) in acetic acid (25ml) at 0ºC with occasional stirring for 2hrs. The progress of reaction was monitored via TLC till single spot. The resulting solid **7** was filtered and recrystallized from ethanol.

Obtained in 65% as yellowish crystals; mp (°C): 158-159; IR (Nujol, cm^{-1}): 2951(Ar-C-H str.)1600.99 (C=N), 1052.21 (C-O-C str in oxadiazole), 1457 (C=C str), 3405.47(NH str), 1544(N=N); 1H NMR (DMSO) δ (ppm): 8.63-8.75(m,3H,CH of pyrazine), 4.71(s, 1H,CH-tetrazole), 2.1(s,1H, NH); Ms (m/z): 191 (M^+), other fragments peak are 112, 86; Anal. Calcd. for $C_6H_5N_7OC$, 37.70; H, 2.64; N, 51.29, Found: C, 37.73; H, 2.69; N, 51.33

Antimicrobial Evaluation

In vitro anti-bacterial activity

The various test microorganisms *Bacillus subtilis* (MTCC121), *Staphylococcus aureus* (MTCC737), *Escherichia coli* (MTCC1687) and *Pseudomonas aeruginosa* (MTCC1688) were obtained from the Department of Microbiology, MM Institute of Medical Sciences and Research, MMU, Ambala, India. Cultures of the test bacteria were grown on Nutrient agar (NA) slant and incubated at 37 °C for 1-3 days, depending upon the growth period of bacteria.

Evaluation technique

The antibacterial evaluation was carried out by using agar cup-plate method [20, 21]. Each test compound (50 mg) was dissolved in DMSO (50 ml, 1000 µg /ml), which was used as sample solution. Sample size for all the compounds was fixed at 0.1 ml. Ampicillin was used as reference drugs and DMSO as a negative control. Zones of inhibition produced by each compound were measured in mm and the averages based on triplicate measurements were recorded.

In vitro anti-fungal activity

The clinical isolates of *Aspergillus niger* (MTCC281) and *Candida albicans* (MTCC183) were obtained from the Department of Microbiology, MM Institute of Medical Sciences and Research, MMU, Ambala, India. The isolate was subcultured on Sabouraud Dextrose Agar (Hi-Media) at 37°C for 48-72 hours.

Evaluation technique

The compounds were evaluated for their *in vitro* anti-fungal activity against pathogenic fungi using cup plate method with Sabouraud's dextrose agar media. Different concentrations of compounds in DMSO were added into each labeled well (DMSO, solvent control) and incubated at 37°C for 72 hours. Experiment was carried in triplicate. Miconazole was used as standard drug. The zone of inhibition was determined by measuring the diameter of the zone.

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was calculated using a method as reported in literature [20, 21]. Nutrient agar was prepared, sterilized, and cooled to 45°C with gentle shaking. It was inoculated with microorganism culture before pouring into the sterilized petri dishes. Two fold diluted solutions of the synthesized compounds (6.5, 12.5, 25, 50, 100, 200, 400, 800, and 1600 µg/ml) and reference drugs were used. The plates were incubated at 30-35°C for 24-48 hours. MIC values were determined at the end of the incubation period.

Anti-tubercular activity

Drug susceptibility of MIC of the test compounds against *M. tuberculosis* H37Rv (MTCC200) were performed by Lowenstein-Jensen (L.J) Agar (MIC) method [22], where a stock solution of 2000 µg /ml concentration of each compound was prepared in DMSO. Then primary 500, 250, 125 and secondary 50, 25, 12.5, 6.250, 3.125, 1.3625 µg

/ml dilutions of each test compounds were added liquid. Growth of bacilli was seen after 12 days, 22 days and finally 28 days of incubation. The concentration at which no development of colonies occurred or <26 colonies was taken as MIC concentration of test compound. The strain *M.tuberculosis* H37Rv (MTCC200) was tested with drug isoniazide and rifampicin as standard drugs.

RESULT AND DISCUSSION

Chemistry

The structures of all the newly synthesized 1, 3, 4-oxadiazoles **(4, 5, 6, 7)** were characterized and confirmed by chromatographic and spectroscopic (IR, ^{1}H NMR, and Mass spectrometry) methods. Both analytical and spectral data of all the synthesized compounds were in full agreement with the proposed structures. Scheme **1** shows the synthetic strategy to obtain the target compounds. The physical and molecular data are shown in Table **1.** The structures assigned to **5,6 and 7** were supported by IR spectra showing absorption bands between 1052.21 cm^{-1} - 1082.11 cm^{-1} due to C-O-C bending in oxadiazole. Stretching vibration due to C=N and Ar(C=C) were observed at 1612.56 -1614.25 cm^{-1} and 1455.35 - 1495.11cm^{-1}, respectively. Band at 2975-3015cm^{-1} was appeared due to Ar C-H stretching. The ^{1}H NMR of these compounds shows the presence of 3H, multiplet at 8.61-8.82 ppm due to Pyrazine , a singlet at 8.34ppm is due to presence of (1H,CH-triazole) group in (**6**) and singlet at 4.71ppm for (1H,CH-tetrazole) group in (**7**). All the other aliphatic and aromatic protons were observed within the expected regions. Mass spectra also supported the structures of the synthesized compounds

Biological activity

The antimicrobial activity of the synthesized compounds was carried out by agar cup plate method and the average diameter of zone of inhibition (mm) and MIC (μg/ml) were recorded in comparison with standard drugs.

All the synthesized compounds were screened for their *in vitro* antibacterial activity against gram negative (*P.aeruginosa, E.coli*), and gram positive (*S.aureus, B.subtilis*) bacteria taking Amoxicillin as a standard drug while *in vitro* antifungal activity against two fungal strains *C.albicans* and *A. niger* taking Miconazole as a standard drug.

The results of zone of inhibition studies as shown in Table **2** showed that the compound **4** and **5** have greater antibacterial potential than other compounds against the gram positive (*B.subtilis* and *S.aureus*) and gram negative (*E.coli* and *P.aeruginosa)* bacterias. Similarly antifungal activity of compound **4** and **5** displayed higher zone of inhibition as compared to other compounds against *C.albicans* and *A.niger*. The antimicrobial (antibacterial and antifungal) potential of the synthesized compounds were further confirmed by determination of MIC of all the compounds. The MIC is defined as the minimum concentration of compounds required to completely inhibit the bacterial growth. It was clearly observed from the results as shown in Table **3** and Fig. **1** and Fig. **2** that the compound **4** and **5** showed greater antibacterial and antifungal potential against gram positive and gram negative bacterias as compared to other synthesized compounds. Other oxadiazole containing compounds have shown fair to medium antibacterial and antifungal activities. No inhibitory effect was observed for DMSO.

Table 1 Physical and molecular properties of synthesized 1, 3, 4-oxadiazoles

Compound	Molecular Formula	Molecular Weight	M.P(°C)	Colour	Solubility	R_f value
4	$C_6H_4N_4SO$	180	180	Yellow Crystals	DMSO, MeOH, $CHCl_3$	0.31
5	$C_6H_6N_6O$	1 79	176	Yellow Crystals	DMSO, MeOH, $CHCl_3$	0.18
6	$C_7H_4N_6O$	189	162	Brownish Crystals	DMSO, MeOH, $CHCl_3$	0.22
7	$C_6H_3N_7O$	190	158	Yellowish Crystals	DMSO, MeOH, $CHCl_3$	0.29

Table 2 Antibacterial and Antifungal activity of synthesized 1, 3, 4 oxadiazoles

Compound	**Zone of inhibition** in mm against microorganisms					
	G +ve bacteria		G -ve bacteria		Fungal species	
	B.subtilis	*S.aureus*	*E.coli*	*P.aeruginosa*	*C.albicans*	*A.niger*
4	10	10	13	11	10	10
5	22	24	21	22	21	22
6	21	23	19	21	20	21
7	21	24	17	20	19	18
Amoxicillin	29	31	30	28	ND	ND
Miconazole	ND	ND	ND	ND	26	28
Control	NZ	NZ	NZ	NZ	NZ	NZ

ND: Not Determined
NZ: No Zone of Inhibition

Table 3 Antimicrobial activity of the synthesized 1, 3, 4-oxadiazoles (MIC's in µg/ml*)

Compound	MIC (µg/ml)					
	G +ve bacteria		G -ve bacteria		Fungal species	
	B.subtilis	*S.aureus*	*E.coli*	*P.aeruginosa*	*C.albicans*	*A.niger*
4	200	400	100	800	100	200
5	200	200	50	400	50	50
6	200	200	50	400	50	50
7	200	200	50	400	50	100
Control	-	-	-	-	-	-
Amoxicillin	50	100	12.5	200	ND	ND
Miconazole	ND	ND	ND	ND	25	12.5

*MIC- Minimum inhibitory concentration
ND: Not Determined

All the synthesized compounds were screened for their *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (MTCC200) by Lowenstein-Jensen (L.J.) Agar (MIC) method using isoniazid and rifampicin as standard drugs. Results as shown in Table **4** depicted that compound **5** with MIC value 6.25 µg/ml displayed significant antitubercular activity against the strain *Mycobacterium tuberculosis* H37Rv. Although the synthesized compounds specifically **5** displayed potent antitubercular activity but is still lesser active as compared to standard drugs(isoniazid and rifampicin).

Table 4 Anti Tuberculosis activity against *Mycobacterium tuberculosis* H37Rv (MTCC200)

Compounds	MIC* µg/ml
4	>100
5	6.25
6	50
7	50
Rifampicin	0.25
Isoniazid	0.20

*MIC- Minimum inhibitory concentration

An introspection of the active compound revealed that different structural features of the compounds have more influence on biological activity. Introduction of N=C-NH-NH_2 linkage at one side of 1, 3, 4-oxadiazole nucleus (as in compound **5**) is found to significantly increase the activity of the compound. Further cyclization of compound **5** resulted in introduction of triazole (as in compound **6**) and tetrazole (as in compound **7**) moieties fused to the 1, 3, 4-oxadiazole nucleus which increased the complexity of the whole molecule and decreased biological activity of the compounds.
Compound 2-hydrazino-5-pyrazyl-1, 3, 4-oxadiazole **5** was found to be promising lead molecule for further synthetic and biological exploration.

CONCLUSION

The present research work reports the successful synthesis of various fused -1, 3, 4-oxadiazole compounds and assessment of their antimicrobial and anti-tuberculosis activity. It was observed that the promising antimicrobials have proved to be better antitubercular agent. Particularly, compound **5** due to its better activity against *M.tuberculosis* H37Rv strain is the best choice for the preparation of new compounds in order to further improve antimicrobial and antitubercular activity in future.

Fig. 1 Minimum inhibitory concentrations (MIC) of synthesized 1, 3, 4-oxadiazoles against bacterial strains

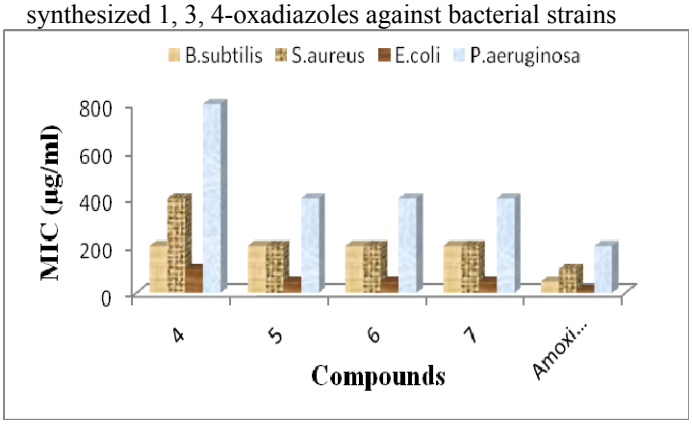
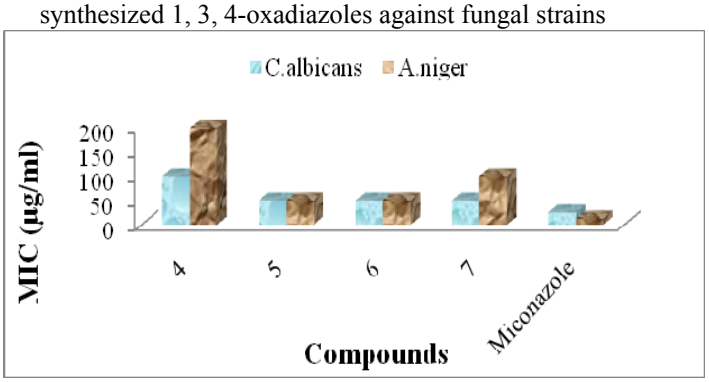


Fig. 2 Minimum inhibitory concentrations (MIC) of synthesized 1, 3, 4-oxadiazoles against fungal strains



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CONFLICT OF INTEREST

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

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