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Synthesis of Some Novel Pyrazolo [3, 4-*e*] [1, 2, 4] Triazines As Potential Antimicrobial Agents

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ABSTRACT

Triazine backbone is present in a large number of bioactive substances and is known for their pharmacological activities like antimicrobial, antitumour and antiviral activity. Pyrazoles are another class of heterocyclic compounds associated with significant biological activities like anti-inflammatory, anticonvulsant and antimicrobial activity. In this view, it was proposed to synthesize some novel pyrazolo[3,4-*e*][1,2,4]triazines from 3,7-dimethyl pyrazolo[4,3-*e*] oxadiazine. In the current investigation, ethyl acetoacetate was condensed with hydrazine hydrate in ethanol to give 3-methylpyrazol-5-one. The pyrazole derivative was brominated by using bromine in acetic acid as brominating agent to give 4, 4- dibromo-3-methylpyrazol-5-one. This compound was then cyclized with acetic anhydride and hydrazine hydrate to get 3, 7-dimethyl pyrazolo[4,3-*e*]oxadiazine. This compound on further reaction with different substituted amines generates 4-(aryl/heteroaryl)-3,7-dimethyl-pyrazolo [3,4-e][1,2,4]triazines. The synthesized compounds were screened for their antibacterial and antifungal activity against *S. aureus* strain of Gram positive, *E. coli* the strain of Gram negative and *A. niger* strain of fungus by standard methods. The results suggest that the compounds ***4a*** and ***4c*** exhibited significant antifungal activity. Gentamycin, ciprofloxacin, cefotaxime and amphotericine B were used as standard drugs. The structures of the synthesized compounds were confirmed using analytical and spectral techniques (IR, ^{1}H NMR and Mass spectral data).

Keywords: 3-methylpyrazol-5-one, 3,7-dimethylpyrazolo[4,3-*e*] oxadiazine, pyrazolo [3,4-*e*] [1,2,4]triazine, antibacterial activity, antifungal activity.

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INTRODUCTION

Infectious diseases caused by bacteria and fungi affect millions of people worldwide, and in the United States alone cause a disease burden of more than $20 billion annually. Concerted and systematic programs to discover and develop new antibiotics and antifungals have been driven to a considerable extent by the development of resistance by these organisms to the drugs commonly used against them. As antimicrobial resistance increases into a major public health problem, the race will intensify between microbes and novel drug discovery and development efforts. Infectious diseases are as old as life itself. They have certainly played a major part in shaping human history, not only because of the decimating effects of the various plagues through the centuries, but also because of the intense efforts made to find cures for them, thus advancing medical science[1]. Efficient syntheses of some novel potent compounds are the target to overcome problem of multi-drug resistant (MDR) bacteria and fungi resulting from the widespread use and misuse of classical anti-microbial agents. For these reasons, our present work has been carried out to synthesize efficient compounds to get desired antimicrobial activity.

Staphylococcus aureus is the most common species of *staphylococcus* to cause Staph infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections[2]. Methicillin-resistant *S. aureus*, abbreviated MRSA is one of a number of greatly feared strains of *S. aureus* which have become resistant to most β-lactam antibiotics. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections. Each year, some 500,000 patients in American hospitals contract a staphylococcal infection[3].

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains

are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia[4].

Aspergillus niger can cause a serious lung disease, aspergillosis. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane[5].

Recently, there is a widespread interest in the design and synthesis of novel pyrazolo [3, 4-*e*] [1, 2, 4] triazine derivatives because of their potential biological activities associated with their skeleton[6].

Heterocyclic chemistry forms the basis of organic chemistry research worldwide. In particular, heterocyclic structure serves the basis of many pharmaceutical, agrochemical and veterinary products. Among the wide variety of nitrogen heterocycles that have been explored for developing pharmaceutically important molecules, triazines have played an important role in medicinal chemistry[7].

Triazine is the heterocyclic six member compounds, analogous to the six-membered benzene ring but with three carbons replaced by nitrogens. The three isomers of triazine are distinguished from each other by the positions of their nitrogen atoms, and are referred to as 1,2,3-triazine, 1,2,4-triazine, and 1,3,5-triazine.1,2,4-Triazine and its fused derivatives are important as the basic framework for a variety of pharmaceuticals and agrochemicals[8]. Pyrazolo-1,2,4-triazines have received considerable attention due to their association with variety of biological applications. Many derivatives of the system show a triazines moiety show interesting properties great number of biological activities such as antidepressant[9], antitumour[10], antiparasitic[11], antifungal[12], antiallergic[13], anti-inflammatory[14], analgesic[15], antiviral[16] and antimalarial[17] activity. Polycyclic compounds containing triazine moiety act as good antibiotics and herbicides etc.

In the present investigation, some of the novel pyrazolotriazine derivatives were synthesized and evaluated for their antibacterial and antifungal activity.

MATERIALS AND METHODS

Materials:

The chemicals and reagents used in the project work were of AR and LR grade, procured from Astron chemicals, Ahmedabad; Loba chemie private Limited, Mumbai; Krishna chemical Industry, Vadodara and Merck private Limited, Mumbai.

Analytical techniques:

All the melting points were determined in open capillary tubes and are uncorrected. The purity of compounds was checked by TLC on silica gel G coated glass plates and spots were located by iodine vapors. In some cases, TLC GF-254 was used and spots were visualized under UV light.

Instruments:

The IR spectra of synthesized compounds were recorded in the range of 4000 – 400 cm^{-1}on FTIR DRS 8400, Shimadzu. ^{1}H NMR spectra were recorded on Bruker Advance II 500MHz FT-NMR spectrophotometer (TOPSPIN 1.3 version) in DMSO solvent and TMS as internal standard. Mass spectra were recorded on 2010 EV LCMS Shimadzu instrument at 70eV. Elemental analyses were performed on a Carlo Erba EA 1108 elemental analyzer.

METHODS:

Synthesis of 3-methylpyrazol-5-one[18] (1)

To ethyl acetoacetate (0.05mol, 6.5g, 6.34mL) in a conical flask, a solution of hydrazine hydrate (99%) (0.05mol, 2.5g, 2.45mL) in absolute ethanol was added slowly with constant stirring. The temperature of the reaction mixture was not allowed to rise above 60 ^{0}C. On further stirring for one hour at room temperature, the reaction mixture was cooled in the ice bath to complete the crystallization, filtered and the product was washed with ice cold ethanol. The product was recrystallized using rectified spirit. Yield: 90%, m.p.: 220-222^{0}C (Reported: 222^{0}C); R_f Value: 0.30; IR(KBr cm^{-1}): 3341(N-H str.), 2928(C-H str.), 1681(C=O str.) 1622(C=N str.), 1542(C=C str.), 1364(CH_3 str.), 1080(C-N str.).

Synthesis of 4, 4-dibromo-3-methylpyrazol-5-one[18] (2)

To a solution of 3-methylpyrazol-5-one (40mmol, 3.92g) in acetic acid (20 mL) the solution of bromine (10mL) in cold glacial acetic acid (25 mL) was added drop wise by keeping the reaction mixture in cold water and then refluxed at 60^{0}C for four hours. The contents were poured into ice to isolate the product. A light orange residue was separated out and washed with cold water. Yield: 70%, m.p.: 126-129^{0}C (Reported:128^{0}C); Rf Value: 0.78; KBr cm^{-1}): 3325(N-H str.), 2925(C-H str.) 1679(C=O str.) 1624(C=N str.), 1379(CH_3 str.), 1080(C-N str.) 650(C-Br str.).

Synthesis of 3, 7-dimethyl pyrazolo[4,3-*e*]oxadiazine (3)

4, 4-Dibromo-3-methylpyrazolo-5-one (25mmol, 6.4g) was dissolved in acetic anhydride (97%) (25mmol, 2.55g, 2.36mL) and hydrazine hydrate (99%) was added drop wise. The reaction mixture was refluxed at 80 ^{0}C for six hours. A light yellow compound was obtained by pouring into ice cold water. It was filtered, washed with water and recrystallized with ethanol. Yield: 74%, m.p.: 148-151^{0}C; Rf Value: 0.42; IR(KBr cm^{-1}): 1624(C=N str.), 1379(CH_3 str.), 1261,

1175 (C-O-C str.).

General procedure for synthesis of 4-(Aryl/heteroaryl)-3,7-dimethyl-pyrazolo[3,4-*e*] [1,2,4]triazine (4a-4c)

A mixture of 3,7-dimethylpyrazolo[4,3-*e*]oxadiazine (0.01mol,1.5g) and aromatic amines (0.01mol) in ethanol(10 mL) were refluxed for six hours on water bath and poured into ice to isolate the product. It was recrystallized from rectified spirit.

Synthesis of (4-(3,7-dimethyl-4*H*-pyrazolo[3,4-*e*][1,2,4]triazin-4-yl)-3-nitrophenyl) (phenyl)methanone (4a)

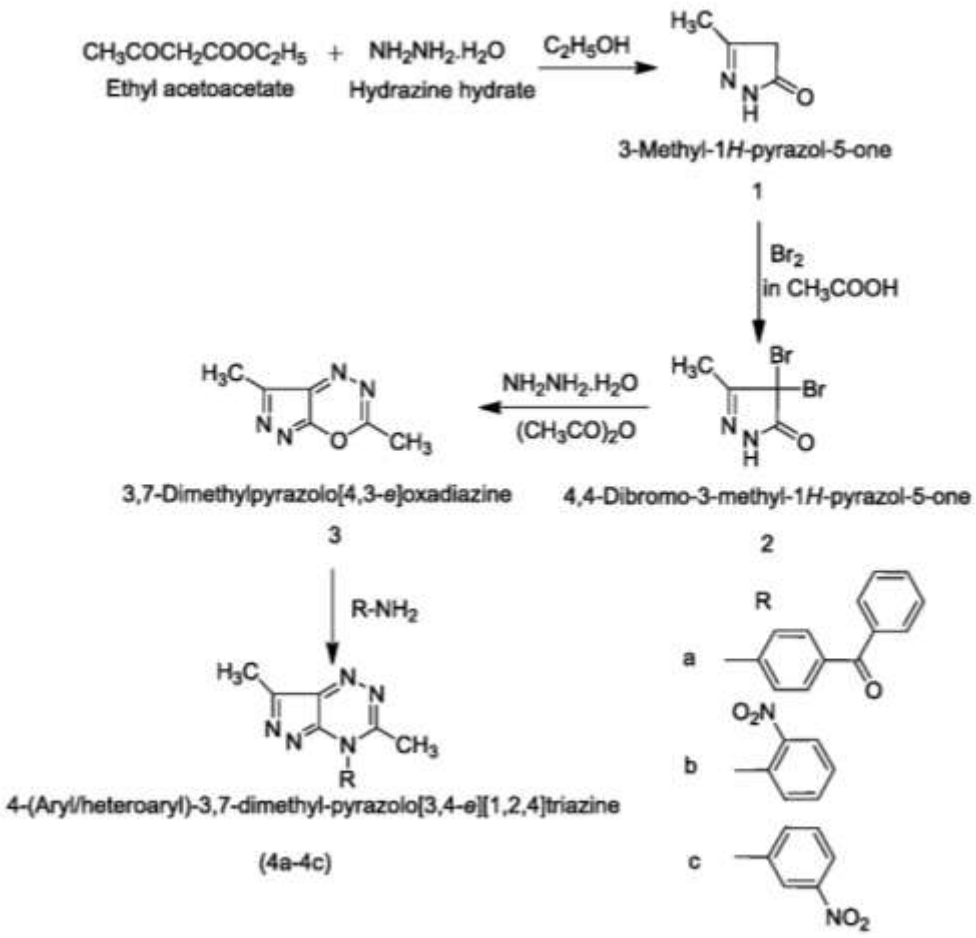
A mixture of 3,7-dimethylpyrazolo[4,3-*e*]oxadiazine (0.01mol,1.5g) and 4-amino-3-nitro benzophenone(0.01mol, 2.42g) in ethanol(10 mL) was refluxed for six hours on water bath and poured into ice to isolate the product. It was recrystallized from rectified spirit. Yield: 82%, m.p.: 170-172^{0}C; Rf Value: 0.75; IR(KBr cm^{-1}): 1681 (C=O str.), 1622 (C=N str.), 1532 (C=C str.), 1498, 1354 (NO_2 str.), 1372(CH_3 str.),1085 (C-N str.); ^{1}H NMR (500 MHz, DMSO) δ ppm: 3.34(s, 3H, Ar-CH_3), 3.43(s, 3H, Ar-CH_3), 7.55-7.70(m, 5H, Ar-H), 7.14-7.16(d, a-1H, Ar-H)(ortho coupling), 7.84-7.86(dd, b-1H, Ar-H)(ortho-meta coupling),8.352-8.356 (d, c-1H, Ar-H,(meta coupling) ; m/z: 375.1(M^{+1}); Anal: Calcd for $C_{19}H_{14}N_6O_3$: C, 60.96; H, 3.72; N, 22.45; O, 12.82. Found: C, 60.89; H, 3.72; N, 22.41; O, 12.76.

Synthesis of 3, 7-dimethyl-4-(2-nitrophenyl)-4*H*-pyrazolo[3,4-*e*][1,2,4]triazine (4b)

A mixture of 3,7-dimethylpyrazolo[4,3-*e*]oxadiazine (0.01mol,1.5g) and 2-nitro aniline (0.01mol, 1.38g) in ethanol(10 mL) was refluxed for six hours on water bath and poured into ice to isolate the product. It was recrystallized from rectified spirit. Yield: 74%, m.p.: 164-166^{0}C; Rf Value: 0.72; IR(KBr cm^{-1}): 1620 (C=N str.), 1542(C=C str.), 1496, 1342 (NO_2 str.), 1365(CH_3 str.), 1081(C-N str.); m/z: 269.4 (M^{+1}); Anal: Calcd for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10; O, 11.84. Found: C, 53.29; H, 3.67; N, 31.08; O, 11.62.

Synthesis of 3,7-dimethyl-4-(3-nitrophenyl)-4*H*-pyrazolo[3,4-*e*][1,2,4]triazine (4c)

A mixture of 3,7-dimethylpyrazolo[4,3-*e*]oxadiazine (0.01mol,1.5g) and 3-nitro aniline (0.01mol,1.38g) in ethanol(10 mL) was refluxed for six hours on water bath and poured into ice to isolate the product. It was recrystallized from rectified spirit. . Yield: 72%, m.p.: 168-170^{0}C; Rf Value: 0.71; IR(KBr cm^{-1}): 1620 (C=N str.), 1512(C=C str.), 1492, 1337 (NO_2 str.), 1361(CH_3 str.), 1082(C-N str.); Anal: Calcd for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10; O, 11.84. Found: C, 53.28; H, 3.70; N, 31.07; O, 11.78.



Scheme: Synthesis of 4-(Aryl/heteroaryl)-3, 7-dimethyl-pyrazolo[3,4-*e*][1,2,4]triazines

BIOLOGICAL STUDIES

Antibacterial and antifungal activity by cup plate agar diffusion method

All the newly synthesized compounds have been evaluated for their in vitro for their antimicrobial activity. The antimicrobial activities are carried out against Gram positive bacteria viz., *Staphylococcus aureus* (ATCC 25923) and Gram negative bacteria viz., *Escherichia coli* (ATCC 25922) and antifungal activity towards *Aspergillus niger* (ATCC 16404) at a concentration of 100 µg/mL. The biological activities of synthesized compounds were compared with standard drugs viz., gentamycin, ciprofloxacin, cefotaxime for antibacterial activity and antifungal activity was compared with amphotericine B.

RESULTS AND DISCUSSION

In pyrazolo[3,4-*e*]triazine derivatives**(*4a-4c*)** showed characteristic band in the frequency region of NO_2 str. in the range of 1498-1492 cm^{-1} and 1352-1337 cm^{-1}. The ^1H NMR spectra of compounds were studied in DMSO. Aryl protons of compound ***4a*** resonate at around 7.55-7.70

ppm as multiplet, The aryl proton ***a*** was found to appear as doublet at 7.14-7.16 due to ortho coupling. While the aryl proton ***b*** was found to appear as doublet of doublets at 7.84-7.86 due to ortho-meta coupling and the aryl proton ***c*** was found to appear as doublet at 8.352-8.356 due to meta coupling. The proton of methyl substitutents attached to aryl rings were found to appear as singlet at 3.3-3.4 ppm. Further, Mass spectrum of compound ***4a*** showed a (M^{+1}) peak at m/z 375.1 corresponding to its molecular formula, $C_{19}H_{14}N_6O_3$. While mass spectrum of compound ***4b*** showed a (M^{+1}) peak at m/z 269.4 corresponding to its molecular formula, $C_{12}H_{10}N_6O_2$.

All the compounds were screened for their antibacterial activity by Cup-Plate Method using different strain like *E. coli and S. aureus* 100µg/mL. Gentamycin, ciprofloxacin and cefotaxime were taken as the standard drugs for antibacterial activity. Amphotericine B was taken as the standard drugs for antifungal activity. The compound ***4b*** (R= 2-nitro phenyl) and ***4c*** (R= 3-nitro phenyl) showed good antibacterial activity. The compound ***4a*** (R= 3-nitrophenyl) (phenyl) methanone moiety) and ***4c*** (R= 3-nitro phenyl) exhibited significant antifungal activity.

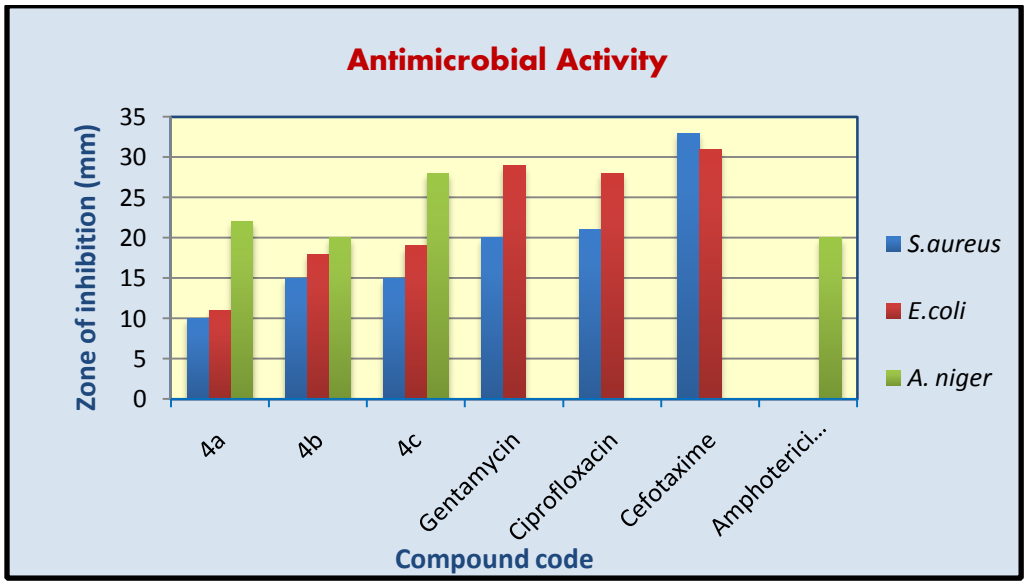


Figure 1: Antimicrobial activity of synthesized compounds 100 µg/mL concentration

Table 1: Antimicrobial activity of synthesized compounds at 100 µg/mL concentration

Compound Code	Zone of Inhibition (mm)		
	***S. aureus*(Gm +ve)**	***E. coli*(Gm -ve)**	***A. niger*(Fungi)**
4a	10	11	22
4b	15	18	20
4c	15	19	28
Gentamycin	20	19	-
Ciprofloxacin	21	28	-
Cefotaxime	33	31	-
Amphotericine B	-	-	20

CONCLUSION

This presented research work as carried out to synthesize, purity, characterize the various derivatives of pyrazolo[3,4-*e*][1,2,4]triazine and all the synthesized compounds are evaluated for their antibacterial and antifungal activity.

Among all synthesized compound, the compounds ***4b*** and ***4c*** showed comparable antibacterial activity with gentamycin against *E. coli*. The compounds ***4a*** and ***4c*** showed significant antifungal activity against *A. niger* than the rest of compounds.

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