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SYNTHETIC APPROACH TO NOVEL PYRIMIDO-TRIAZINES WITH ANTIMICROBIAL ACTIVITY

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***Key word**: Antibacterial,antifungal,cytotoxicity, pyrimidine,triazine.*

Abstract – In an effort to discover novel antimicrobial compounds we describe here the synthesis of various series of traizine analogues including 2,5-diamino-5-(4-chlorophenyl) diazenyl)-6-chloropyrimidine (**1**) and 2,5 diamino-5-(4-bromophenyl) diazenyl)-6 chloropyrimidine (**2**) with carbon disulphide in the presence of sodiumhydroxide as basic medium afforded unexpectedly sodium 6-amino-2-(4-chlorophenyl)-8-chloro-1,2dihydropyrimido [4,5-e][1,2,4] triazine-3-thiolate (**3**) and sodium 6-amino-2-(4-bromophenyl)-8-chloro-1,2-dihydropyrimido [4,5-e][1,2,4] triazine-3-thiolate (**4**). IR, NMR (^{1}H and ^{13}C) and HSQC spectra were used to identify analogues structure. The newly synthesized compounds were screened in vitro for their antibacterial, antifungal activities and human red blood cells (RBCs) cytotoxicity. The result showed that only compound (**3**) has a significant inhibitory activity against the selected Gram positive and negative bacterial strains and the most common fungalpathogens including filamentous and yeasts form. This synthesized compound had no toxic effect against human RBCs. This work indicated that compound (**3**) has a promising role to treat and manage pathogenic bacterial and fungal infections with no toxicity.

INTRODUCTION

Many of nitrogen heterocyclic compounds have therapeutic properties. Pyrimidine, which being an integral part of nucleic acid bases for DNA and RNA, is one of these synthesized compounds (Kumar and Narasimhan, 2018). These compounds play a vital role as bactericide, fungicide, vermicide, insecticide, anticancer and antiviral agents, in addition to, their anti-inflammatory activity (Holla *et al*., 2008). Many studies were done on design and synthesis novel pyrimidine derivatives that possess a significant antimicrobial activity for treating bacterial and fungal infections (Mallikarjunaswamy *et al.*, 2017). The target actions of pyrimidine analogues in inhibiting and eliminating pathogenic bacterial and fungal growth and infections associated with alteration in bioactive the physiochemical microbial cell structure. For instant, microbial cellmembrane is a first barrier that separates pivotal cytoplasmic contain from outside surrounding environmental effects (Bush, 2012; Epand *et al*., 2016). Studies suggested that pyrimidine analogues may causea disruption in the cell membrane as a result of increasing in permeability, leaking or casing a change in surface charge of this membranes by its physicochemical characteristicand eventually microbial cell death (Abhay *et al*., 2016; Banerjee *et al*., 2016). The impact of synthesis new series of azo-pyrimidines derivatives with active antimicrobial activity have been increased such the treating of 6-chloro 2,4-diamino pyrimidine with various amines (Al-Masoudi *et al*., 2014). In recent years, many of pathogenic fungi and bacterial strains have emerged as serious human pathogens and causing life-threatening infections of humans due to developing multidrug-resistant to commonly used antimicrobial drugs (Fisher *et al*., 2018; Jatana *et al*., 2018). Based on that, the need for novel approaches for synthesis novel antimicrobial agents is becoming more important, particularly with the dramatic expanding of microbial community and the rise in nosocomial infections.

We report here an approach for synthesis novel series of pyrimidines substituted amino and thio groups that could have a significant impact in treating and managing bacterial and fungal infections.

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MATERIAL AND METHODS

Physical Instrumentation

Melting points are uncorrected and were measured on a büchi melting point apparatus B-545. NMR data were obtained on 400 and 600 MHz (^{1}H) and 150.91 MHz (^{13}C) spectrometers (Avance III, Bruker, Germany) with TMS as internal standard and on the ä scale in ppm. Heteronnuclear assignments were verified by ^{1}H, ^{13}C and HSQC NMR experiments. Analytical silica gal TLC plates 60 F254 were purchased from Merck. All reagents were obtained from commercial suppliers and used without further purification.

Synthesis

General Procedure for the Preparation of Pyrimido–triazine (3 and 4)

A solution of azopyrimidine (80 mmol) in ethanol (70 mL) was treated with sodium hydroxide (3.2 g, 80 mmol) in water (10 mL) whilst cooling and stirring carbon disulphide (6.08 g, 80 mmol) then added gradually with continuous cooling over 1 hr and maintained the temperature below 10°C. The precipitate of the pyrimido-triazine was filtered off, washed with ethanol and dried (Liu and Wang, 1998).

Sodium6-amino-2-(4-chlorophenyl)-8-chloro-1,2-dihydropyrimido[4,5, e][1,2,4triazine-3-thiolate (3). This compound was prepared from 2,6-Diamino-4-chloro-5-p chlorophenylazopyrimidine (1).
Yield: 358.1mg (92%), m.p.> 360°C dec., R_f = 0.15.- FT-IR (neat, cm^{-1}): $l_{max.}$ 3421-2978 (N-H), 1446 (C.....N), 1006(CS), 864 (C-S), 1041 (C=S), $^{-1}$H NMR-([D_6]DMSO): ä = 10.05, 9.25 (2xbr s, 2H, NH_2), 8.11 (s, 1H, NH), 7.80-7.73 (2xd, 4H, J = 8.5 Hz, H-Ar). - ^{13}C NMR ([D_6]DMSO): ä = 164.4 (C(8a)), 160.6 (C-S), 155.2 $(C(2)_{pyrimid.})$, 152.1 (C(4)-Cl), 134.8 (C(1'), 133.4 $(C(3',5')_{arom.})$,129.5 $(C(2',6')_{arom})$, 123.3 (C(4a)), 119.2 $(C(4')_{arom})$ – $C_{11}H_7Cl_2N_6NaS.H_2O$(367.18): calcd. C 35.98, H2.47, N 22.89. found C 35.77, H 2.34, N 22.97.

Sodium6-amino-2-(4-bromophenyl)-8-chloro-1,2-dihydropyrimido[4,5,-e][1,2,4]triazine-3-thiolate (4). This compound was prepared from the reaction of 2,6-Diamino-4-chloro-5-p-bromophenyl azopyrimidine (2) with carbon disulphide.

Yield: 335.6 mg (89%), m.p.> 360°C dec., R_f = 0.15. - FT-IR (neat, cm^{-1}): $l_{max.}$ 3448-2974 (N-H) , 1450 (CN), 1006(CS),864 (C-S),1064 (C=S), $^{-1}$H NMR-([D_6]DMSO): ä = 9.25, 8.15 (2xbr s., 2H, NH_2) , 7.74-7.69 (2xd, 4H, J = 8.6 Hz, H-Ar), 7.31 (s, 1H, NH). ^{13}C NMR ([D_6]DMSO): ä = 165.2 (C(8a)), 161.7 (C-S), 156.4 $(C(2)_{pyrimd})$, 151.8 (C(4)-Cl), 132.7 $(C(1')_{arom.})$,129.1 $(C(3',4',5')_{arom})$, 123.8 (C(4a)), 122.7 $(C(2',6')_{arom})$ – $C_{11}H_7BrClN_6NaS.H_2O$(411.64): calcd. C 32.10, H 2.20, N 20.42. found C 33.21, H 2.14, N 20.26.

Microorganisms

Details of pathogenic bacteria and fungi that used in this study are in Table 1. All microbial samples were provided by the Department of Biology, College of Science, University of Basrah, Iraq. Bacterial strains were routinely maintainedandcultured on sterile either Nutrient Agar (Oxoid) plates and/or Nutrient Broth tubes. All fungi were grown and maintained on slopes or on Petri dishes of Potato Dextrose Agar (PDA) (Difco) 2.4% containing 2% (w/v) agar). Media was autoclaved at 121°C for 15 min prior to use. Microorganisms are incubated at 37°C prior susceptibility test.

Table 1. Details of microorganisms that used in this study.

Microorganism	Details
Staphylococcus aureus	Bacteria (Gm+)
Staphylococcus sciuri	Bacteria (Gm+)
Bacillus cereus	Bacteria (Gm+)
Escherichia coli	Bacteria (Gm-)
Klebsiella pneumonia	Bacteria (Gm-)
MDR *Pseudomonas aeruginosa*	Bacteria (Gm-)
Aspergillus niger	Filamentous fungi
Aspergillus flavus	Filamentous fungi
Aspergillus fumigatus	Filamentous fungi
Aspergillus terrus	Filamentous fungi
Candida albicans	Yeast
Candida tropicalis	Yeast

Antimicrobial Activity of Synthetic Compounds

Primary Screening for Antibacterial Activity

The antibacterial activity of the prepared pyrimido-triazinecompounds (3 and 4) was evaluated against six selected pathogenic bacterial strains. For antibacterial assay, stock solution 100 mg/mlof titled compounds was done by dissolving in Dimethyl Sulfoxide (DMSO). After inoculation of bacterial isolates on Mueller Hinton agar Petri dishes via spreading at concentration of 10^8 Colony Forming Unit (CFU)/mL (0.5 McFarland turbidity standards). Well diffusion method on agar plates was used testing antimicrobial activity. Briefly, wells with diameter 6 mm were made by using a sterile cork borer. Next, a 50 µL of each tested compound were

pipettedgently into the wells. Plates were incubated at 37°C for 24 hr and then mean of inhibition zones (IZ), triplicates each, were measured in millimetres (mm) (Padhi and Tayung, 2015). The starting compounds (1 and 2) were also examined following the pervious steps.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal = Concentration (MBC) Determination

For this experiment, only compound (**3**) was tested as exhibited abroad-spectrum activity. To determine the MIC of synthesized compound **3**, serial concentrations of tested compounds in DMSO were prepared 75, 50, 25, 10, 5, 4, 3, 2 and 1 mg/mL. The bacterial inoculum at 10^8CFU/mL was seeded on Mueller Hinton agar plates and well diffusion technique was used as described previously. Plates were incubated for 24 hr at 37"C. After incubation period, the MIC was presented as the minimum concentration that prevent visible bacterial growth, while the MBC was indicated to the lowest compound concentration resulting in no growth or microbial death on the agar plate that resulting in the inability to re-culture bacteria.

Primary Screening for Antifungal Activity

The antifungal activity of all the title compounds was primary screened in vitro using the well diffusion method as described previously on PDA plates. For fungal species refreshment, fungal isolates were subcultured on sterile PDA plates and incubated at 37 °C for 72 hr. After fungal spore suspensions were prepared at $1x10^6$ cell /mL, a 150 mL was vaccinated on fresh PDA plates by spreading using a sterile L-shape. Following making four holes 6 mm in diameter per a plate by a sterile cork borer, then were flooded by 50 mLof compounds stock solutions with three replicates each. The plates were incubated at 37 °C for 72 hr. The inhibitory activity of tested compounds against fungal growth was observed by measured the IZ in mm.

MIC Determination of Antifungal Compounds

only synthesized compound (**3**) were assessed their MIC mg/mL in triplicate sets against six opportunistic fungal pathogens including filamentous and yeasts by well diffusion technique. Various concentrations of tested compounds in DMSO were prepared (75, 50, 25, 10, 5, 4, 3, 2, 1 mg/mL) as described previously. Fresh PDA plates were inoculated with spore suspension by spreading, then four holes were made by cork borer, followed by adding 50 µL of certain concentrations. The plates were incubated for 24 – 72 hr at 37 °C. At the end of the incubation period, MIC values were recorded as the lowest concentration of the substance that gave no visible fungal growth.

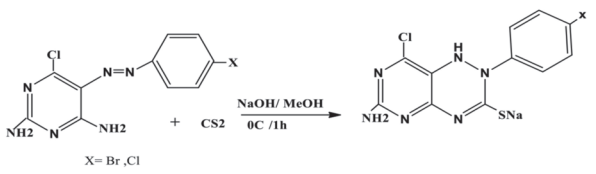
Cytotoxicity Assay

The cytotoxicity was determined by testing the haemolytic activity of the synthesized compounds, the concentration that leading to 50% or more lysis of human red blood cells (RBCs) is considered cytotoxic. A haemolytic method was developed, one ml of freshly obtained RBCs was placed in heparinized tubes to avoid coagulation. After gently mixing, was diluted via adding to a sterile glass test tube containing 20 mL of sterile phosphate buffer saline PBS solution (PBS) (0.8% NaCl; 0.02 % KCl; 0.115 % Na_2HPO_4; 0.02% KH_2PO_4; pH was adjusted to 7.4). Besides that, a negative and positive control tubes contacting PBS only and tap water respectively were prepared. Next, range of synthesized compounds concentrations were set up in PBS (100, 200, 300, 400 mg/mL), then a 100 µL was added to 2 mL of diluted RBCs. Tested tubes were incubated at 37°C for 1 and 24 hr. The degree of haemolysis was determined comparing with the controls visually in triplicates to be represented as (-) = no-lysis; (+) = 25% lysis; (++) = 50% lysis; and (+++) = complete RBCs lysis).

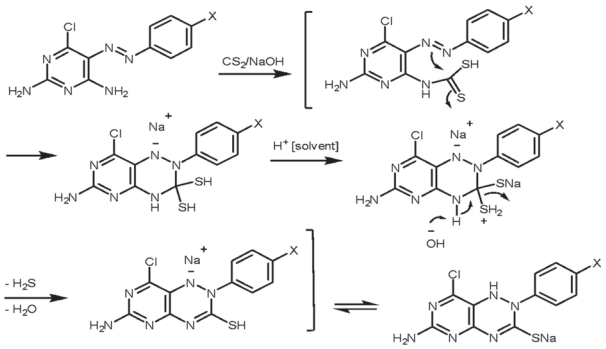
RESULTS AND DISCUSSION

Chemistry

Recently, azopyrimidines (1, 2) (Al-Masoudi *et al*., 2014) was prepared and selected here as a starting material for the synthesis of new pyrimido-trizines by the treatment of various azopyrimidine analogues with CS_2 in basic media in the present of methanol as solvent at 0°C for 1 hr, leading to new derivatives (3, 4) in good to moderate yield (92% and 89%) thus, might due to the steric factor (Scheme 1), which could be explained via the following mechanism as shown in (Scheme 2).



Scheme 1: Synthesis of pyrimidotriazine analogues.



Scheme 2: Mechanism formation of pyrimidotrizine analogues.

The structures of (3, 4) were established by ^{1}H, ^{13}C NMR and IR spectra. In^{1} H NMR spectra amino at (C-2) of pyrimidine backbone appeared almost at the same regions as broad singlets at δ = (10.05-8.15) ppm. The (NH) group of the analogue (3, 4) showed δ = (8.11 - 7.31) ppm respectively. The ^{1}H NMR spectra showed rather similar patterns for phenyl in (3, 4) compounds. The aromatic and the aliphatic protons were fully analogue (c.f. Experimental section). In the ^{13}C NMR spectra of (3, 4), C-8a of new analogues scaffold resonated at the region δ = 165.2 -164.4 pmm, respectively. C-S appeared at the regions δ = 161.7 -160.5 ppm, respectively, C-Cl of the pyrimidine appeared at the regions δ = 156.4 - 155.2 ppm, respectively. The aromatic carbon atoms C- 2',6' and C- 3',5' of the new analogues (3,4) were resonated at the regions δ = 129.5-122.7 ppm and ä = 133.4-129.1 ppm, while the C-4a appeared at the regions ä = 123.8-123.3 ppm, respectively. In FT-IR spectral data, two compounds (3, 4) show a sharp bond between (1450 - 1446) cm^{-1} which attributed to íC=N stretching vibration which lie between íC-N and íC=N in (1250-1350) cm^{-1} and (1640-1690) cm^{-1} .Abroad peak at (2908 - 3352) cm^{-1} in the spectrum of all the compounds assigned to the presence of water. This broad peak was also coupled with μ NH_2. All ^{1}H and ^{13}C are presented (Figures 1 to 8).

Antibacterial Activity

In the current study, four compounds including two

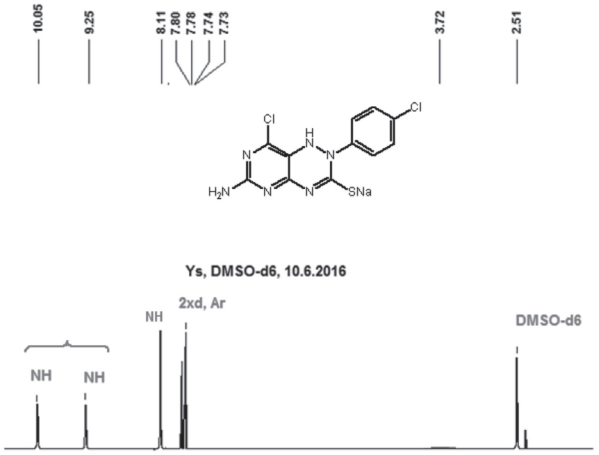


Fig. 1. ^{1}H NMR spectrum of **3**.

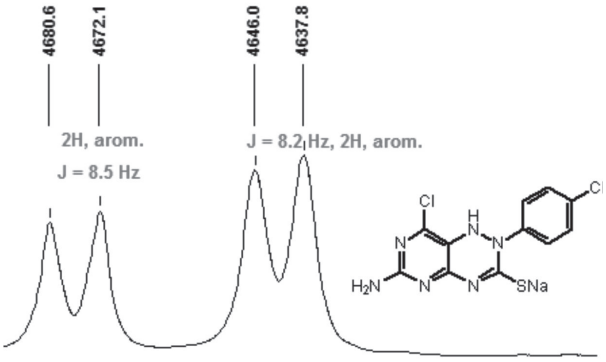


Fig 2. Expansion of ^{1}H NMR spectrum of **3**.

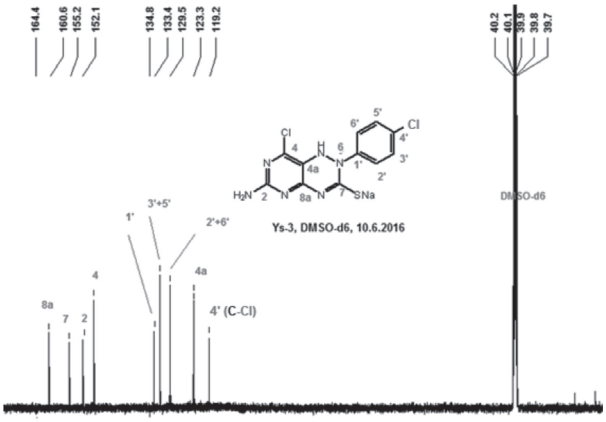


Fig. 3. ^{13}C NMR spectrum of **3.**

Table 2. Primary screening for antibacterial activity of tested compounds.

Chemical	Inhibition Zone (IZ) (mm))					
	E. coli	*S. aureus*	*P. aeruginosa*	*S. sciuri*	*K. pneumonia*	*B. cereus*
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	15	40	25	40	30	45
4	**-**	**20**	**-**	**25**	**-**	**20**

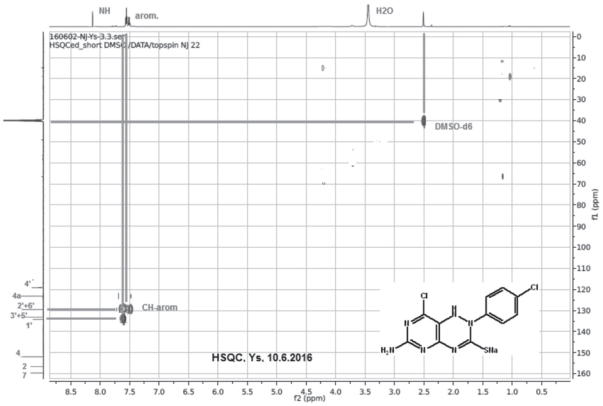


Fig. 4. HSQC NMR spectrum of **3**.

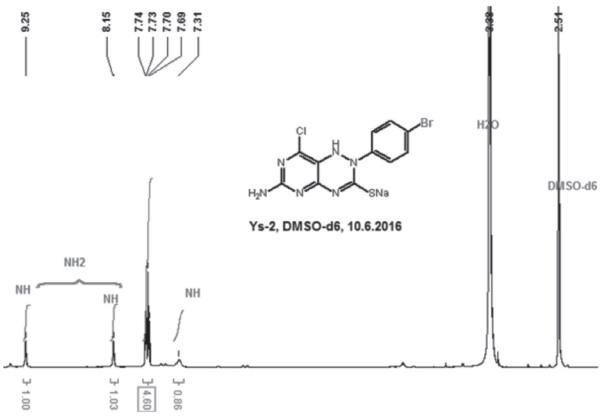


Fig. 5. ^{1}H NMR spectrum of **4**.

starters 1 and 2 and two synthesized compounds **3** and **4** were tested for bioactivities against the selected bacteria. Using the well diffusion method on Mueller Hinton Agar plates, the result in (Table 2) and (Figure 9) showed that the compound 3 exhibits a unique antimicrobial activity against pathogenic bacteria in comparing with compound 4, 1 and 2. For this reason,we have selected compound 3 for extra investigations because of many bacteria have developed resistance against renowned antibiotics (Otzen *et al.*, 2004). The title compound 3 has a broad-spectrum effect. The inhibitory activity in Gm+ including *Staphylococcus aureus*, *S.sciuri* and *Bacillus cereus*, (IZ 40, 40 and 45 mm) respectively, was more than Gm- bacteria including MDR *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (IZ between 15 – 30 mm) respectively (Figure 9). The activity of *pyrimidotrizine* (**3**) may be attributed to the presence of chlorine group that possess the bioactivity feature against bacteria. Active agent can induce damage to the cytoplasmic membrane by

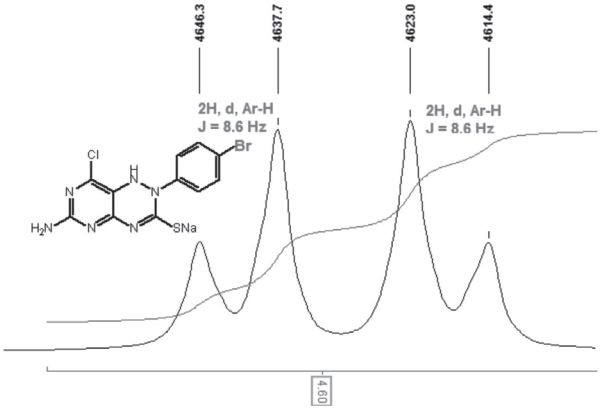


Fig. 6. Expansion of ^{1}H NMR spectrum of **4**.

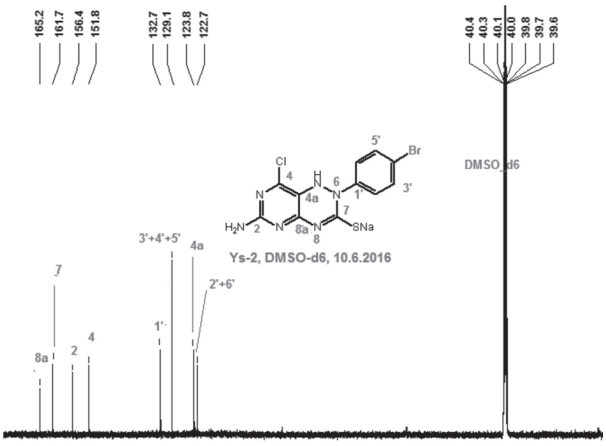


Fig. 7. ^{13}C NMR spectrum of **4**.

Table 3. The result of MIC and MBC values of compound (**3**) antibacterial activity.

Isolates	Tested compound Concentration mg/ml									
	1	2	3	4	5	10	25	50	MIC	MBC
	Inhibition Zone (IZ in mm)									
E. coli	0	0	15	20	12	12	14	16	2	3
S. aureus	10	10	10	15	15	14	15	16	<1	4
S. sciuri	5	10	10	15	20	15	15	15	<1	4
K. pneumonia	0	0	0	0	9	13	14	15	4	5
p. aeruginosa	0	0	0	0	0	0	15	18	10	25
B. cereus	10	10	13	15	18	20	28	30	<1	4

action upon the membrane potentials, bound enzymes or permeability and this lead to leakage potassium ion, and other cellular components and eventually eliminating microbial infections (Banerjee *et al.*, 2016). Compound **4** had just a narrow-spectrum in the effect against the Gm+ bacteria (IZ = 20 – 25 mm) and no inhibitory feature was noticed against Gm- bacteria. It is obvious that the difference in action against Gm+ bacteria may be due to the differences of cell wall structure between Gm+ and Gm- bacteria.

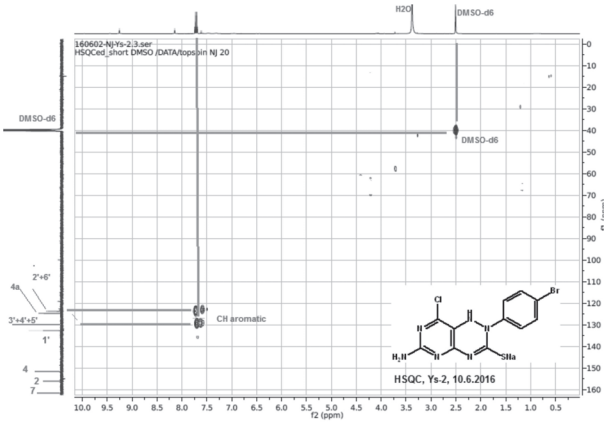


Fig. 8. HSQC NMR spectrum of **4**.

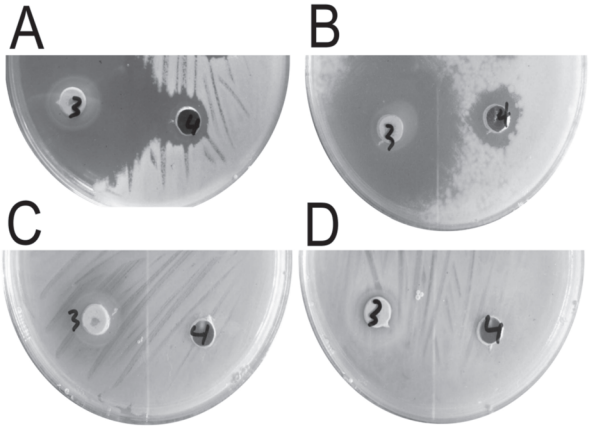


Fig. 9. Primary screening for antibacterial activity of synthetic compounds against Gm+ and Gm- bacteria. (**A**) *Staphylococcus aureus* (Gm+), (**B**) *Bacillus subtilus* (Gm+), (**C**) *Escherichia coli* (Gm-), and (**D**) *Pseudomonasaeruginosa* (Gm-).

The MIC values for antibacterial activity of compound 3 were presented in Table 3. Selected compound 3 inhibited all the tested Gm+ pathogenic bacteria *Staphylococcus* strains and *B. cereus*was less than 1 mg/mL, while it was 2, 4 and 10 mg/mL for the Gm- strains, *E. coli, K. pneumonia* and MDR *P. aeruginosa* respectively. The activity group of compounds 3 and 4 may associated with the presence of thio group comparing with azopyrimidines of compound 1 and 2. Sulphur may act as growth factor and bacteria require it in very little amount. Other reports also indicate antimicrobial activity of Sulphur containing compounds (Kim *et al.*, 2006). Furthermore, antimicrobial results of this study mimic the findings of (Fathalla *et al*., 2013). The compound **3** performedan inhibitory activity against MDR *P. aeruginosa*. *P. aeruginosa* is well known as a multidrug-resistant pathogen for commonly used antibiotics (Lister *et al.*, 2009).

Antifungal Activity

The result of testing antifungal activity of selected synthetic compounds showed in Table 4. The highest IZ was on *Aspergillus niger* (45 mm) followed *A. terreus, A. fumigatus* and *A. flavus* 40, 35 and 25 mm respectively. Yeasts represented by two *Candida* species were exhibited high sensitivity to compound **3**, *C. tropicalis* and *C. albicans* IZs were 35 and 33 mm respectively. Besides on these result, compound **3** tends to have a unique fungal inhibitory with bleaching effects on pigmented pathogenic fungi (Fig. 10). The colour of most tested *Aspergillus* isolates were turned from black (*A. niger*), yellow-green (*A.flavus*) and blue-green or dark turquoise (*A. fumigatus*) to white. We recommend further future study to understand the mechanism of compound **3** affection on fungi. The activity group of compound **3** may due to pyrimidine group is abroad spectrum fungicides (Abhay *et al*., 2016).

The MIC of compound **3** that inhibited all fungal

Table 4. Primary screening for antifungal activity of the prepared compounds.

Compound	Inhibition Zone (IZ/mm)					
	A. flavus	*A. fumigatus*	*A. niger*	*A. terreus*	*C. albicans*	*C. tropicalis*
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	25	35	45	40	33	35
4	**-**	**-**	**-**	**-**	**-**	**-**

growth of six opportunistic pathogens three of *Aspergillus* and two *Candida* species was less than 3 mg/mL (Table 5 and Figure 11). The remarkable antimicrobial activity of synthesized compound 3 against pathogenic bacteria and fungi associated with the presence of chlorine that act as electron-windrowing group at position play a key role in antimicrobial activity against both bacterial and fungal pathogens. The control and management of fungal pathogens during infections is a serious problematic in field of medicine due to the ability of fungi resist commonly used drugs and bosses eukaryotic structure similar to human cells (Xie *et al*., 2014). We have design a novel antifungal drug that has a significant inhibition effects on fungi with low toxicity.

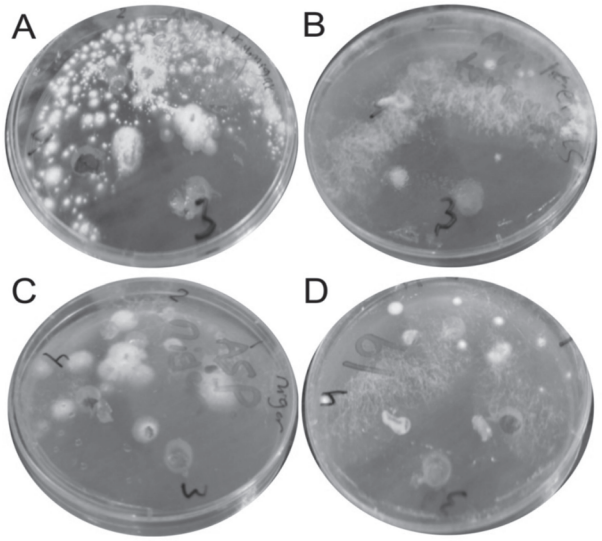


Fig. 10. Primary screening for testing antifungal activity of synthetic compounds against selected pathogenic fungi. **(A)** *Aspergillus fumigatus*, **(B)** *Aspergillus terreus*, **(C)** *Aspergillus niger*and **(E)** *Candida albicans*.

Cytotoxicity assay

The cytotoxicity of selected synthetic compounds 3 and 4 was tested on human RBCs in vitro. Measuring the level of compound cytotoxicity based on hydrolysis of RBCs. The result showed in Table 6 and Figure 12 presented that compound *3* was non-toxic after 1 hr and 24 hr of incubation at 37°C. However, *pyrimidotrizine* (4) was toxic to human RBCs at concentration >200 mg/mL.

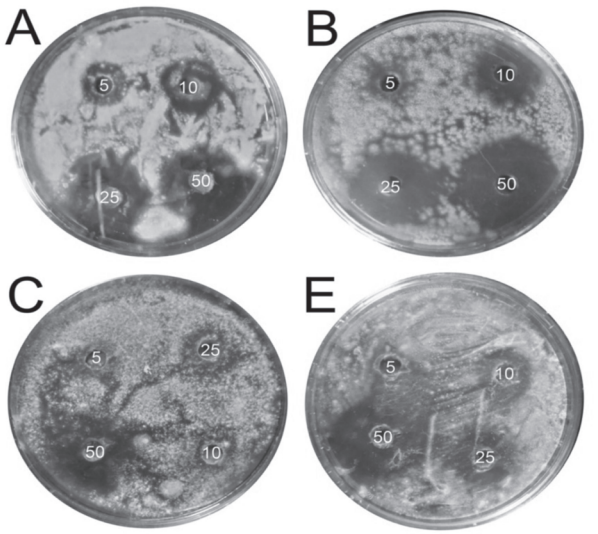


Fig. 11. Minimum inhibitory concentrations (MIC mg/ml) for pathogenic fungal isolates of selected compound **4. (A)** *Aspergillus fumigatus*, **(B)** *A. niger*, **(C)** *A. flavus*and **(E)** *Candida albicans*.

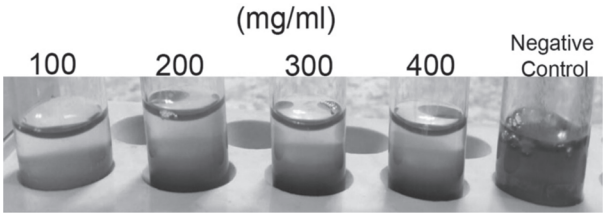


Fig. 12. Cytotoxicity test of selected compound **3** in vitro against human red blood cells.

Table 5. MIC and MBC values of compound (4) antifungal activity.

Isolates	Tested compound Concentration (mg/mL)									
	1	2	3	4	5	10	25	50	MIC	MBC
	Inhibition Zone (IZ in mm)									
A. flavus	0	0	0	10	10	15	20	25	3	4
A. fumigatus	0	0	0	15	15	20	30	30	3	4
A. niger	0	0	0	20	20	25	35	40	3	4
A. terrus	0	0	0	20	10	15	20	25	3	4
C. albicans	0	0	0	15	15	20	30	30	3	4
C. tropical	0	0	0	10	5	15	20	30	3	4

Table 6. Human Red Blood Cells (RBC) cytotoxicity assay in vitro for synthetic compounds.

Tested compound	Concentration (mg/mL)	Incubation period	
		1 hr	24 hr
3	100	-	-
	200	-	-
	300	-	-
	400	-	-
4	100	-	-
	200	+	+
	300	+	+
	400	++	++

CONCLUSION

This work demonstrates the successful synthesis of pyrimidine analogue that has a significant inhibitory activity against pathogenic bacteria and fungi with no toxicity effects against human RBSc. This compound could be considered as an optimistic antimicrobial agent to control infections.

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