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ORIGINAL ARTICLE

Synthesis and characterization of some tetrazoles and their prospective for aerobic micro-fouling mitigation

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Abstract Two series of tetrazole derivatives of the type *N*-(1*H*-tetrazol-5-yl)-1-(aryl)methanimine **(101–106)** and 1-(4-alkoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine **(107–111)** were synthesized and characterized via conventional tools of analysis (elemental analysis, FT-IR and ^{1}H NMR spectroscopy). These two synthesized series were biologically evaluated for their potentials against some microbial biofilm causing strains (micro bio-foulants). Biological activities were evaluated by MIC values and cell viability percentages of them. In case of compounds **(107–111)**, **107** was the most potent antimicrobial one, where its MIC values were 10.666667 μg/ml; 12.82222 μg/ml and 21.43666 μg/ml for *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* respectively, whereas compound **106,** (of group **101–106),** MIC values were 16 μg/ml for all the tested microorganisms. Viability assay showed that **107** activity percentages were 96.99456%, 92.32886% and 89.09558% against Gm +ve bacteria, Gm −ve bacteria and yeast respectively, whereas **106** activity percentages were 95.255569%, 90.204675% and 86.710956% against *Gm +ve* bacteria, *Gm −ve* bacteria and yeast respectively. Two antimicrobial mode of actions were proposed and discussed depending on the two evaluated tetrazole groups.

1. Introduction

Heterocyclic compounds are generally wide-known as multifunctional antimicrobial agents (Omardien et al., 2018). Tetrazoles are an important class of heterocyclic compounds which possess wide spectrum of biological properties (Kumar et al., 2014). They have attracted much attention because of their unique structure and applications as antihypertensive, antialergic, antibiotic and anticonvulsant agents (Myznikov et al.,

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2007; Schocken et al., 1989; Butter, 1984; Mavromoustakos et al., 1999; Mekni and Bakloiti, 2008; Toney et al., 1998; Tamura et al., 1998; Lim et al., 2007). They are a class of synthetic organic heterocyclic compound, consisting of a 5-member ring of four nitrogen and one carbon atom (Nessim et al., 2018). From literature survey, it was found that tetrazole derivatives possess very interesting pharmacological and biological properties and are reported to exhibit variety of biological activities like antibacterial, antifungal and anticonvulsant, analgesic, anti-inflammatory, anti-tubercular activity and anticancer activity (Kumar et al., 2014).

Similarly, 1,5 disubstituted tetrazoles have long been known for their pharmaceutical activity as stimulants or depressants on the central nervous system and are reported to show oral antidiabetic and antithrombotic and antimicrobial properties (Mohite et al., 2009; Modarresi and Nasrollahzadeh, 2009). The development of tetrazole derivatives has been largely associated with wide scale of applications of these classes of compounds in medicine, biochemistry agriculture and large number of medicinally important tetrazole heterocyclic incorporated drugs approved by FDA.

Tetrazoles and its derivatives have been reported to possess antinociceptive anti-inflammatory antimicrobial and anticonvulsant properties. Benzotriazole moiety is a versatile lead molecule in pharmaceutical development and more favorable pharmacokinetic profile, wide range of biological activities (Maria Dorathi Anu et al., 2013). Accordingly, synthesis of tetrazole derivatives is obviously an important task in modern medicinal chemistry (Varadaraji et al., 2010).

Facet-attached microbial communities are known as biofilm. Growth of these biofilms can promote micro-biofouling or microbial fouling (Di Pippo et al., 2018). Biofouling could be defined as "the process in which prokaryotic and eukaryotic organisms adhere to solid surfaces immersed in water". Biofouling is wide-known to be responsible of different problems via extra fuel and maintenance costs in water- related industries. Microbial films and macrobial fauna as barnacles, mussels, and tunicates which accumulate on ships increase drag forces and surface corrosion, thereby causing biofouling dilemma (Crisp, 1974; Haderlie, 1974). Nowadays, biofouling is regarded as critical issue compromising environmental problems such as membrane water and wastewater treatment (Dadrasnia et al., 2017). Different microbial strains are involved in micro biofouling process such as *Staphylococcus aureus* (Parsek and Greenberg, 2005; Bixler and Bhushann, 2011); *Escherichia coli* (Pohl et al., 2015; Wood et al., 2016) and *Candida albicans* (Fernandes et al., 2016). In our work, two series of tetrazole derivatives were synthesized, characterized and evaluated as per their anti-microbial activity.

2. Experimental

2.1. Raw materials

All chemicals and solvents purchased from international chemical companies

(a) Alkyl halides, tetrabutylammonium bromide and potassium hydroxide (Merck), benzaldehyde, p-hydroxybenzaldehyde, p-tolualdehyde, p-anisaldehyde, and 1*H*-tetrazol-5-amine (Sigma-Aldrich) and p-chlorobenzaldehyde and p-nitrobenzaldehyde (Fluka). They were of analytical grade and used without further purification.
(b) Ethanol (absolute), benzene and tetrahydrofuran (Sigma-Aldrich).

2.2. Synthesis

2.2.1. Preparation of N-(1H-tetrazol-5-yl)-1-(aryl) methanimine (101–106)

A solution of 1*H*-tetrazol-5-amine (0.010 mol) and aromatic aldehydes (0.010 mol) (1–6) in ethanol was refluxed for 2 h in water bath Scheme 1. The resultant solution was cooled; the solid was filtered off and recrystallized from petroleum ether. Compounds **101–106** were obtained as crystalline mass (Kumar et al., 2014).

2.2.2. Preparation of 1-(4-alkoxyphenyl)-N-(1H-tetrazol-5-yl) methanimine (107–111)

These five compounds were synthesized in two steps, Scheme 2:

(a) Step 1: preparation of 4-alkoxybenzaldehyde:
As illustrated (Nessim et al., 2015), the five 4-alkoxybenzaldehydes were prepared via the reaction of 4-hydroxybenzaldehydes with five different alkyl bromides (C_4H_9Br, $C_6H_{13}Br$, $C_8H_{17}Br$, $C_{10}H_{21}Br$ and $C_{12}H_{25}Br$).
(b) Step 2: Synthesis of (**107–111**):
As discussed in Scheme 1 (Kumar et al., 2014), the five 4-alkoxybenzaldehydes reacted with 1*H*-tetrazol-5-amine to form compounds (**107–111**).

2.2.3. Preparation of stock standard solutions for biological assay

For each of the tested tetrazoles groups [(**107–111**) & (**101–106**)], stock standard solutions were freshly prepared and stored at 4 °C (Chemiasoft, 2011). Samples were dissolved in DMF that is widely known to have no antimicrobial activity against different microorganisms and is considered as negative control (Cooper, 1972).

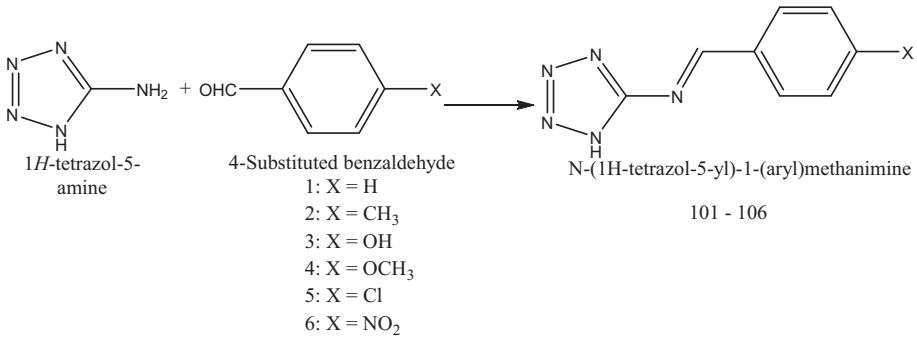
2.2.4. Model Organisms

Identified bacterial isolates of *Staphylococcus aureus, Escherichia coli and Candida albicans* were provided from Cairo MIRCEN (MIRCEN).

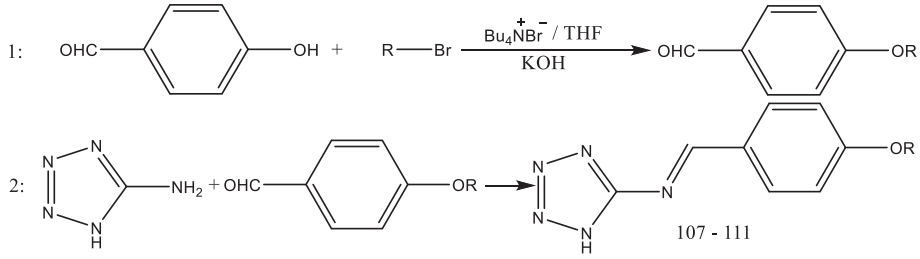
2.2.5. Antimicrobial susceptibility test methods

(A) Agar assay

Under sterilized conditions, for each of the synthesized tetrazole derivatives, agar-agar media (Balouiri et al., 2015) were freshly prepared and left till warmness and then seeded with 24 h grown bacterial suspensions (1–2 × 10^8 CFU/mL per McFarland standard), vortexed and poured onto Petri plates (4 mm height agar layer). Agar plates were then kept till dryness and stored at 4 °C for two hours. One millimeter wells were cork borered onto agar plates, inoculated with 100 µl of each of the test samples and incubated at 37 °C ± 2 for 48 h. Each experiment was done in triplicate (Parekh and Chanda,



Scheme 1 Preparation of *N*-(1*H*-tetrazol-5-yl)-1-(aryl)methanimine (**101–106**). **101**: 1-phenyl-*N*-(1*H*-tetrazol-5-yl)methanimine. **102**: *N*-(1*H*-tetrazol-5-yl)-1-(p-tolyl)methanimine. **103**: 4-((1*H*-tetrazol-5-yl)imino)methyl)phenol. **104**: 1-(4-methoxyphenyl)-*N*-(1*H*-tetrazol-5-yl) methanimine. **105**: 1-(4-chlorophenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine. **106**: 1-(4-nitrophenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine.



Scheme 2 Synthesis of compounds (**107–111**). **107**: 1-(4-butoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine. **108**: 1-(4-hexylkoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine. **109**: 1-(4-octylkoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine. **110**: 1-(4-decylkoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine. **111**: 1-(4-dodecylkoxyphenyl)-*N*-(1*H*-tetrazol-5-yl)methanimine.

2007). DMF was used as a negative control. Inhibition zones were measured, and activity indices were calculated according to Dut Jasuja et al. equation (Jasuja et al., 2014) [Eq. (1)].

$$Activity\ index = Inhibition\ zone\ of\ the\ sample\ (mm)/ zone\ of\ the\ standard\ (mm)$$

(B) Micro plate assay:

Recently, the 96-well Microtiter plate for MICs evaluation is commonly applied as time saver, a significant reducer of sample numbers experiment cost and place occupied, thus improving the repeatability of the results (Zhang et al., 2006). In a sterilized area, microbial suspensions were freshly cultured in suitable broth media (bacteria/yeast). About 0.125 µl of microbial cell suspension of 10^7 CFU/ml count were adjusted at 550 nm absorbance. Blank broth media were inoculated in the wells. Different sample concentrations were applied from 64 to 0.5 µg/ml via 10-fold serial dilutions. A volume of approximately 180 µl of a sample concentration is then injected to each well. The next step is to inoculate about 10 µl microbial suspension into each well. Some wells are specified to solvent, blank sample (without microbes), blank broth and untreated microbial suspensions. The 96-well micro plates were then shaker- incubated for 6–8 h at incubation temperature. Coloring agent (0.4% Resazurin) was applied to each of the 96 wells and the micro plates were incubated for two hours to accomplish dye reaction (Coffey and Anderson, 2014).

2.2.6. Cell cytotoxicity assay

Cell cytotoxicity assay is designed for screening the activity of a certain compound cellular effects such as cell proliferation or in the form of direct cytotoxic effects expressed by cell death. The metabolic activity of viable cells is commonly used as the basis for absorbance assay. Cell viable count is "a measure of the number of cells that are viable (i.e. alive and capable of growth) in a given area or volume". One use of viable cell count is to estimate cytotoxicity. Continuous UV–Vis spectroscopic measurements of a treated microbial suspension optical density in correlation to a 100% live microbial could be employed to estimate viable cell numbers and percentage (Park et al., 2012; Mageswari and Subramanian, 2012). Microbial suspensions were prepared, adjusted to 0.5 O.D. McFarland standards (1–2 × 10^8 CFU/mL) and sterilized. For each test time interval, bacterial suspensions were centrifuged at 10,000 r.p.m. for 10 min (Sigma Centrifuge Model 3 K 300), and the supernatants were removed. A process of three subsequent washing s of the remaining pellets were carried out, suspended in sterilized deionized water and well-vortexed (Germany International Corp Vortex, Model VM-300). For each time interval, 24 mL of each test agent were injected and mixed with 6 mL of the bacterial suspension and their optical densities were measured (ATI Unicam 8625 UV/Vis Spectrophotometer). The 0-time untreated remaining bacterial suspensions were used as 100% live cells and optical density reading was measured and plate counted in CFU/mL. Eq. (2) (Park et al., 2012). Experiment was terminated after 48 hrs. of exposure.

$$Cell\ Viability\ Percentage = \left\{\frac{Absorbance\ of\ test\ suspension}{Absorbance\ of\ 100\%\ live\ microbial\ suspension}\right\}(\%)$$

3. Results and discussion

3.1. Characterization of synthesized compounds

3.1.1. Elemental analysis

Data obtained in Table 1 showed that the calculated percentages of the elements coincides with the observed ones.

3.1.2. Infra-red spectroscopy

See Table 2.

3.1.3. Proton NMR spectroscopy of compounds (*101–111*)

101: C═CH at 9.38 singlet, 7.82 doublet, 7.55 doublet, 7.28 triplet and NH at 6.28 ppm singlet.

102: C═CH at 9.22 singlet, 7.68 doublet, 7.16 doublet, NH 6.32 singlet and CH_3 Protons at 2.60 ppm singlet.

103: OH at 9.98, C═CH at 9.18 singlet, 7.68 doublet, 7.02 doublet and NH at 6.33 ppm singlet.

104: C═CH at 9.36 singlet, 7.95 doublet, 7.56 doublet, NH 6.36 singlet and OCH_3 proton at 3.26 ppm singlet.

105: C═CH at 10.01 singlet, 7.95 doublet, 7.56 doublet and NH at 6.36 ppm singlet.

106: C═CH at 9.43 singlet, 8.40 doublet, 8.16 doublet and NH at 6.36 ppm singlet.

107: C═CH at 9.423 singlet, 7.826 doublet, 7.684 doublet, NH at 6.414 singlet, (e) CH_2 at 4.328 triplet, CH_2 Protons at 1.748 multiplet, CH_3 protons at 0.848 triplet.

108: C═CH at 9.246 singlet, 7.825 doublet, 7.721 doublet, NH at 6.402 singlet, CH_2 at 4.312 triplet, CH_2 1.738 multiplet, and CH_3 at 0.842 triplet.

109: C═CH at 9.864 singlet, 7.855 doublet, 7.113 doublet, NH at 6.441 singlet, CH_2 at 4.063 triplet, CH_2 at 1.728 multiplet, and CH_3 at 0.857 triplet.

110: C═CH 9.285 singlet, 7.675 doublet, 7.482 doublet, NH at 6.421 singlet, CH_2 at 4.214 triplet, CH_2 at 1.728 multiplet and CH_3 at 0.828 triplet.

111: C═CH at 9.114 singlet, 7.848 doublet, 7.763 doublet, NH at 6.460 singlet, CH_2 at 4.062 triplet, CH_2 at 1.715 multiplet and CH_3 at 0.830 triplet.

3.2. Biological assays

3.2.1. Activity index

Screening the antibacterial activity of the synthesized tetrazoles, using diffusion techniques (Fig. 1A and B), revealed that they apparently exhibited antibacterial activities according to their main substituted groups together with the main skeleton activity. It is worth to mention that *Gram-+ve* bacteria showed more susceptibility towards the tested tetrazoles than *Gram −ve* bacteria and yeast. The composition of their microbial cell wall is considered to be a detrimental criterion influencing their response towards the introduced agents. *Gram +ve* bacterial cell wall main component is a thick layer of peptidoglycan, whereas peptidoglycan layer in *Gram −ve* bacteria are enclosed between an inner cytoplasmic layer and an outer membrane. In yeast, cell wall is made of polysaccharides, proteins, lipids and chitin that make its cell wall much thicker. So, yeast is more anti-microbially resistive than *Gram −ve* and *Gram +ve* bacteria respectively (Salton and Kim, 1996). For tetrazoles, **(107–111)**, compound **107** revealed the highest activity index (Fig. 1A), whereas of the tetrazoles, **(101–106)**, compound **106** was the most potent one (Fig. 1B).

3.2.2. Micro plate evaluation

Results of MIC (μg/ml) of the synthesized tetrazoles against *Staphylococcus aureus* were observed to be lower than *Escherichia coli* and *Candida albicans* respectively. Compound **107** seemed to be more toxic against tested microbes, where low concentration was needed to inhibit microbial growth (MIC value = 10.66666667 μg/ml) (Figs. 2 and 3A). While a minimum concentration of compound **106** (16 μg/ml) was fair enough to inhibit the growth of all the tested organisms (Figs. 2 and 3B).

3.2.3. Cell viability and cytotoxicity assay

Cell Viability percentage can indirectly express sample activity and its cytotoxicity. Screening of cell viability (%) of

Table 1 Elemental analysis of compounds (**101–111**).

Compound	C %		H %		N %		Cl		Melting Point °C	Ref.
	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.		
101	55.48	55.21	4.07	4.16	40.44	40.58	–	–	210–12	Kumar et al. (2014)
102	57.74	57.52	4.85	4.95	37.41	37.52	–	–	236–39	Kumar et al. (2014)
103	50.79	50.71	3.73	3.84	37.02	36.98	–	–	130–33	
104	53.20	53.22	4.46	4.53	34.47	34.36	–	–	225–27	
105	46.28	46.18	2.91	2.96	33.73	33.81	17.07	17.05	256–56	Kumar et al. (2014)
106	65.34	65.26	7.44	7.56	27.21	27.18	–	–	248–50	
107	62.70	62.81	7.37	7.28	24.37	24.25	–	–	246–49	
108	64.73	64.80	7.99	7.86	22.20	22.25	–	–	240–42	
109	66.44	66.39	8.51	8.58	20.39	20.36	–	–	232–35	
110	67.89	67.77	8.95	9.01	18.85	18.90	–	–	228–31	
111	69.14	69.21	9.33	9.29	17.53	17.49	–	–	220–22	

Table 2 Infra-Red bands for compounds **101–111** (ν Cm^{-1}).

Cpd.	cm^{-1}						
	NH	C=N	$CH_{Aromatic}$	$CH_{Aliphatic}$	N=N	C–O–C	C=C
101	3394	1552	3087	2959	1451	–	1603
102	3386	1611	3199	2929	1449	–	1561
103	3327	1511	3195	2965	1451	–	1594
104	3383	1604	3199	2931	1451	–	1646
105	3383	1607	3199	2931	1450	–	1642
106	3254	1525	3071	2951	1525	–	1612
107	3391	1449	3200	2938	1449	1297	1654
108	3362	1511	3123	2952	1462	1263	1612
109	3389	1510	3125	2925	1466	1248	1605
110	3412	1510	3200	2926	1449	1258	1647
111	3413	1507	3203	2931	1454	1263	1645

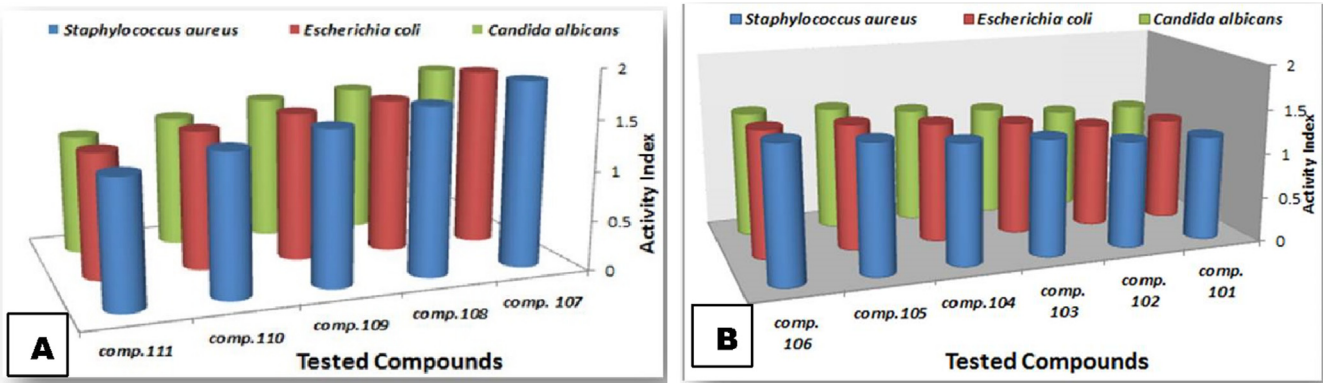


Fig. 1 Activity index of the tested groups (A) & (B) against *Staphylococcus aureus*, *Escherichia coli* & *Candida albicans*. (Standard deviation ± 0.02).

Staphylococcus aureus, Escherichia coli and *Candida albicans* exposed to the two tetrazoles series, **(101–106) and (107–111)**, are illustrated in Fig. 4A and B. Of the tetrazoles series **(107–111)** compound **107** showed highest activity, (average activity percentage = 93.282623%)**,** whereas the highest average activity percentage one of series **(101–106)** belonged to compound **106** (90.723733%).

3.2.4. Antibacterial mode of action

Yet, a little is published discussing tetrazoles antimicrobial mechanism. Few suggestions were reported.

In the present work, the two evaluated tetrazoles series are suggested to have two logical modes of actions.

- Considering series **(107–111)**, alkyl chain length seems to control their antimicrobial mechanism. Precisely, the alkyl chain length affects their trend which may be attributed to molecules lipophilicity enhancing the attacking process into microbial cell membranes leading to deterioration of its transmembrane signaling leading cytotoxic effects and death. So as the chain length decrease the efficiency increase (Gao et al., 1999).
- In series **(101–106)**, another electrostatic mode of action is proposed. Efficacy increases with the positively charged tetrazole ring bearing electron-withdrawing group (Suzen et al., 2006). Then, electrostatic interaction with negatively charged microbial cell membranes forms reactive oxygen species. Reactive oxygen species are the by-products of an oxidative stress resulted from the electrostatic interaction.
- Generally, antimicrobial action of an agent involves inhibition of enzymes involved in cell wall biosynthesis, nucleic acid (DNA) metabolism and protein synthesis responsible for membrane structure. Microbes have their protection mechanisms against external oxidative stresses (OS) expressed in altered enzymatic activities. The generally accepted aromatic tetrazoles mode of action entails inhibition of ergosterol in the microbial membrane. Recently, proposed mechanisms of antimicrobial mode of action depend mainly on the hypothesis of electron transfer, reactive oxygen species and oxidative stress that is considered as unifying process (Kovacic and Abadjian, 2017).

Tetrazoles act as free radical scavengers. For a certain extent, their antioxidant potential depends on substituted terminal groups and structure conjugation (Chand et al., 2018). Bacterial responses to tetrazoles oxidative stress are expressed as the generation of reactive oxygen species (ROS) leading to the oxidative damage to cellular proteins. In addition, utilization of electron transfer processes (ET) could also be responsible of different physiological alterations (Kovacic and

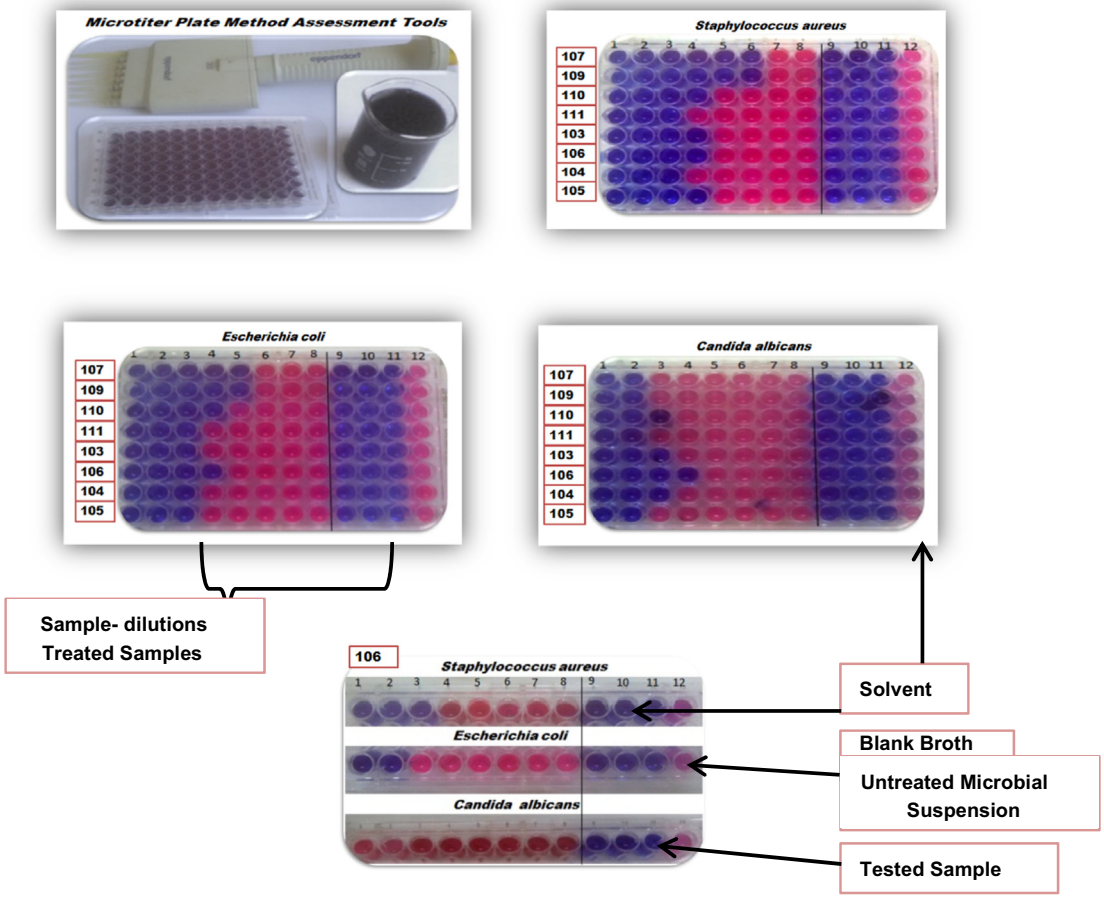


Fig. 2 Micro plate MIC evaluation of *Staphylococcus aureus*, *Escherichia coli* & *Candida albicans* treated with tested tetrazoles.

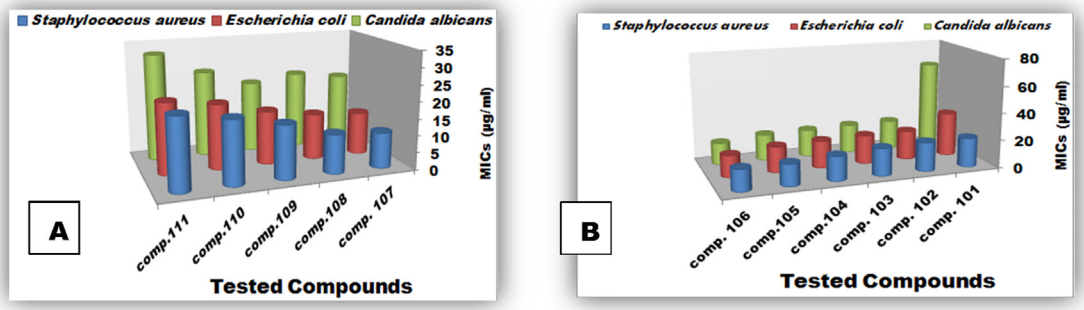


Fig. 3 MICs (µg/ml) of *Staphylococcus aureus, Escherichia coli & Candida albicans* treated with tested tetrazoles groups (A) & (B). (Standard deviation ± 0.02).

Abadjian, 2017; Kashmiri and Mankar, 2014; Kovacic and Somanathan, 2010; McDonnell and Denver Russell, 1999).

4. Conclusion

- Tetrazoles of the two tested series [(**107–111**) and (**101–106**)] were in vitro synthesized and evaluated for their anti-micro biofouling activity.
- Compound **107** of the tested group, (**107–111**), showed the highest efficacy. It possessed 96.99456%, 92.32886% and 89.09558% antimicrobial activity percentages against Gm +ve bacteria, Gm −ve bacteria and yeast respectively.
- On the other hand, compound 106 of group, (**101–106**), was the most potent tetrazole. Its antimicrobial activity percentages were 95.25557% against Gram positive bacteria, 90.20468% against Gram negative bacteria and

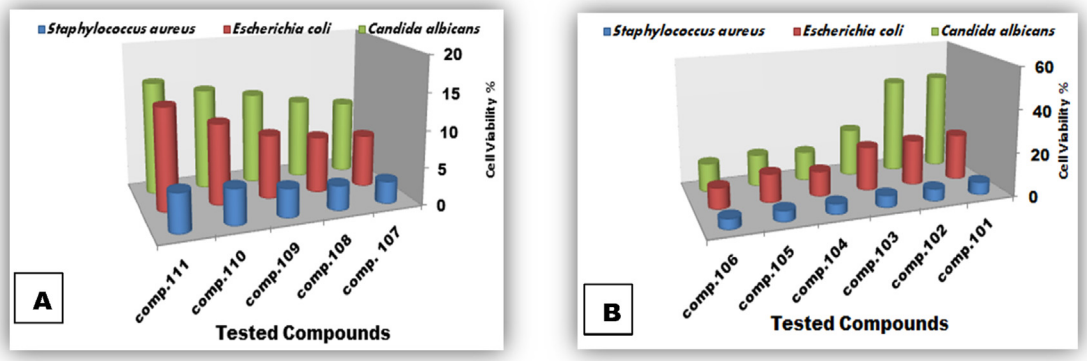


Fig. 4 Cell viability % of *Staphylococcus aureus, Escherichia coli & Candida albicans* treated with tested tetrazoles (A) & (B) after 48 h of exposure.

86.71096% against tested yeast. It is worth to mention that all the tested tetrazoles proved their promising anti-micro biofouling activity.

- For series (**107–111**), average activity percentage was in the range between 88.56631% and 93.282623%, whereas in series (**101–106**), ranged between 75.895098% and 90.723733%.
- Their modes of actions suggestions were articulated on two mechanisms. One depends on alkyl chain length (**107–111**) while the other (**101–106**) is an electrostatic mechanism. The type of mechanism is related to the nature of the tested tetrazoles.
- The hypothesis of ET-ROS-OS theory is a subsequent process of the two proposed mechanisms expressing microbial inhibition and cytotoxicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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