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Impact of some cationic bichalcophene compounds on bacillus species

Sally M. Elmogya*, Mohamed A. Ismailb, Rabeay Y. Hassanc,d , Ashraf A. Elsayeda

[a]*Botany Department, Faculty of Science, Mansoura University, Elgomhouria St.,35516 Mansoura City, Egypt*

[b]*Chemistry Department, Faculty of Science, Mansoura University, Elgomhouria St.,35516 Mansoura City, Egypt*

[c]*Center for Materials Science, Zewail City of Science and Technology, 6th October City, 12588 Giza, Egypt*

dApplied Organic Chemistry Department, National Research Centre (NRC), Dokki, 12622 Giza, Egypt

**Corresponding author: Sally_Elmogy@yahoo.com, Phone: 01006167554*

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Abstract: Bacterial resistance to multiple antibiotics is a great health problem; hence different antibacterial agents need to be constantly discovered. The antimicrobial activities of the newly synthesized fluorophenyl-2,2′-bichalcophene derivatives were evaluated against Bacillus sp. The impact of these bichalcophenes on Bacillus sp. was studied at the molecular level via SDS-PAGE and RAPD-PCR methods. The investigated bichalcophenes are shown to have a potent suppressing effect on Bacillus sp. growth. Among the investigated bichalcophenes, fluorinated thiophene-furan derivatives MA-1115 and MA-1116 showed the highest activity against Bacillus sp. bacterial strain.Also it can be observed that, the bichalcophenes MA-11156, MA-1114, and MA-1113 exhibited a good antimicrobial activity against Bacillus sp., and were more higher in activity than that of the parent compounds MA-0944 (bithiophene derivative) and MA-0947 (bifuran derivative). The minimum inhibitory concentration (MIC) values for the two selected fluoroarylbichalcophenes MA-1115 and MA-1116 were 32 and 64 µM, respectively. Over a period of 7 days Bacillus sp. did not develop resistance to the two selected bichalcophenes at higher concentrations (2, 3x MIC in case of MA-1115 and 3x MIC in case of MA-1116). The biomarker assay detecting the protein changes based on SDS-PAGE profile showed that, there were six bands disappeared from some concentrations after treatment with 8 and 16 µM of MA-1115 and 16 and 32µM of MA-1116. On the other hand, three new bands appeared comparing to untreated bacteria. Also the genetic changes based on RAPD-PCR manipulation of compounds MA-1115 and MA-1116 manifested a polymorphic pattern when treated and untreated bacteria were compared. The impact of compounds MA-1115 and MA-1116 was obviously strong on genetic structure and protein of Bacillus sp. based on RAPD-PCR manipulation and SDS-PAGE profile, so these two bichalcophene compounds could be recommended for their application as effective antibacterial agents.

keywords: bacterial resistance, bichalcophenes, antimicrobial activity, *Bacillus* sp.

1.Introduction

Several antibiotics have been developed to control bacterial infections after penicillin discovery [1]. Using antibacterial agents along with improved sanitary conditions proposed that the war against pathogenic microorganisms had been earned. However, the resistance of bacteria to several antibiotics has developed and this has become a major health problem over the past few years [2]. The development rate of resistance is affected by several factors, including excessive use and abuse of antibiotics [3].

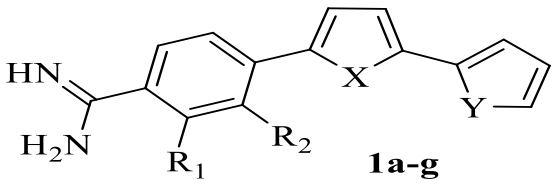
Pentamidines as an example of aromatic diamidines had been widely used against many human diseases [4, 5]. Renal and hepatic toxicities are associated with some of

furamidine derivatives, so they have been prevented from human trials [6]. Aza-furamidine derivatives were found to be more active than the furamidine itself [7, 8]. Cationic heterocyclic compounds containing thiophene and/or furan rings showed wide biological activities [9]. One bifuran derivative of a bichalcophene series was more effective than antibiotic vancomycin toward the infection of methicillin-resistant *Staphylococcus aureus* in mice [10]. To develop the pharmacological properties, a series of fluoroarylbichalcophenes have been synthesized (figure 1) as an approach to drug discovery and tested for their toxic effect on *S. typhimurium* TA1535 viability. It was found that, all the investigated fluorine–containing bichalcophenes exhibited a significant reduction in the *S. typhimurium* TA1535 viability at 50 and 100 µM. Moreover, these investigated compounds were found to act as potent antimutagenic [11] and anticancer agents [12].

Due to the promising antimicrobial effects of the tested fluorophenylbichalcophenes, this study reported herein was launched to examine their antibacterial effect against *Bacillus* sp.

Bacillus sp. is one of the most common Gram-positive bacteria that has a ubiquitous nature giving it great colonization ability as well as the ability of its spores to resist environmental changes as dry heat and certain chemical disinfectants for moderate's periods. *Bacillus* sp. would participate in food spoilage or causing food poisoning when ingested with food. Its source is not the food itself, but the humans who contaminate foods after processing [13].

Diffusion methods that are extensively used to investigate the antibacterial activity are used in our study. These assays are based on the use of discs as reservoirs containing the solutions of substances to be examined [14]. Also, the MIC was determined. The development of spontaneous resistance to flouroarylbichalcophenes was examined in order to investigate their stability as antimicrobial agents. Furthermore, the impact of flouroarylbichalcophenes on the tested pathogenic bacteria *Bacillus sp.* was performed at the molecular level.



1a-g

Code:	R_1	R_2	X	Y
1a (MA-0944)	H	CH	S	S
1b (MA-0947)	H	CH	O	O
1c (MA-1156)	F	CH	S	S
1d (MA-1115)	CH	F	O	S
1e (MA-1116)	F	CH	O	S
1f (MA-1113)	CH	F	O	O
1g (MA-1114)	F	CH	O	O

Fig. (1): Monocationic bichalcophene derivatives that have been tested for their antimicrobial activity. Chemical Names: **1a** (4-(2,2'-bithiophene-5-yl)benzamidine); **1b** (4-(2,2'-bifuran-5-yl)benzamidine); **1c** (4-(2,2′-bithiophen-5-yl)-2-fluorobenamidine); **1d** (2-fluoro-4-(5-(thiophen-2-yl)furan-2-yl)benzamidine); **1e** (3-fluoro-4-(5-(thiophen-2-yl)furan-2-yl)benzamidine); **1f** (4-(2,2′-bifuran-5-yl)-3-fluorobenzamidine) **1g** (4-(2,2′-bifuran-5-yl)-2-fluorobenzamidine).

2. Materials and methods

This study had been conducted at Cytology and Genetics Lab, Botany department, Faculty of science, Mansoura University from August 2017.

1. Tested compounds and bacteria

Two non-fluorinated parent compounds **1a**, **b** and five fluorophenylbichalcophenes **1c-g** (Figure 1) were available from previous studies [11, 15] and provided by Professor M.A. Ismail to be used throughout the present study. *Bacillus* sp. used in this study was obtained from Biotechnology and Genetic Engineering Unit, Mansoura University, Egypt. The strain was maintained in 50% glycerol stocks at -20 ºC. *Bacillus* sp. was sub-cultured at 37 ºC for 24 hr every month.

2. Antimicrobial susceptibility test of bichalcophenes

To evaluate the antibacterial activity of the

bichalcophene compounds **1a**-**1g** against Gram-positive *Bacillus* sp. bacteria, the disc agar diffusion method was used [14]. The tested compounds were liquefied at 10 mM concentration in DMSO. LB agar plates were inoculated separately with 10^7 CFU of bacterial culture and regularly spread on the whole surface of each plate [16]. The 5 mm diameter sterile discs were saturated with 10 µl of the tested bichalcophene compounds and placed on LB plates inoculated with bacterial culture. The plates were incubated for 24 hr at 37 ºC, after that inhibition zones were measured in millimeters and compared with a negative control 10% DMSO. Each assay in this test was done in three replicates.

3. Determination of MIC of the novel bichalcophene derivatives

The two bichalcophene derivatives MA-1115 and MA-1116 that showed the best results in antimicrobial susceptibility test were selected for the minimum inhibitory concentration (MIC) experiment. Results were determined according to a process described by Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards techniques CLSI/NCCLS [17]. Different concentrations (1-128 µM) of compounds MA-1115 and MA-1116 dissolved in DMSO were added independently to LB broth medium which was previously autoclaved. For the current assay, prepared culture of LB *Bacillus* sp. was used. Twenty µl *Bacillus* sp. seed culture having nearly 5×10^4 colony forming units (≈0.5 OD) was used as an inoculum for testing the bichalcophene derivatives. Broth alone and culture were incubated overnight at 37 ºC and measured at 600 nm optical density.

4. Detection of bichalcophenes-resistant variants (for MA-1115 and MA-1116)

In order to evaluate the development of spontaneous resistance in *Bacillus* sp. against two tested compounds MA-1115 and MA-1116, growth assays were performed in the presence or absence of each compound with different concentrations (1, 2 and 3 x MIC). [18]. The details have been described elsewhere [19].

Molecular studies

A- SDS-PAGE

Bacillus sp. samples treated with two bichalcophene derivatives MA-1115 and MA-1116 and the untreated sample (control) were cultured in LB broth media at 37 ^oC and 120 rpm for 24 hr. The bacterial cells were harvested by centrifugation at 10.000 rpm for 5 min. The pellets were homogenized in phosphate buffer (0.6 M, pH 6.8) using glass beads and FastPrep®-24 homogenizer and then centrifuged at 10.000 rpm for 5 min for protein isolation. Ten µl protein samples were boiled into 2X sample buffer (10 ml Distilled Water, 2.5 ml Tris HCl pH 6.8, 2 ml Glycerol, 4 ml of 10% SDS and 1 ml β- mercaptoethanol) for 2 min, cooled immediately on ice. Around 20 µl treated protein were loaded over acrylamide gel. Acrylamide gel was prepared according to [20] from two layers; 4% stacking gel on top of 12% separating gel. After electrophoresis at 100 V for 2 hr, gel was overnight stained in Commassie brilliant blue R250 and visualized by soaking in distaining solution on shaker for some hours. The gel was documented and analyzed using gel analyzer 3 program.

B- RAPD- PCR

Bacillus sp. was cultured in LB broth media provided with 8 µM, 16 µM concentrations of MA-1115 and 16 µM, 32 µM concentrations of MA-1116 and incubated at 37 ^oC for overnight. The genomic DNA was isolated from the bacterial pellets according to the instructions of I-genomic BYF DNA extraction Mini Kit (Intron, Korea). The purified DNA was used as a template for RAPD-PCR reaction using two primers RAPD5 (TTCGACCCAG) and RAPD6 (AAAGCTGCGG). The reaction mixture was adjusted with a total volume of 20 µl: 1 µl DNA template, 4 µl 5x master-mix buffer, 2 µl 1:10 RAPD-primers, 0.5 µl Taq DNA polymerase and 12.5 µl distillate water. The PCR program was: 94 ^oC for 3 min, 94 ^oC for 1 min, 30 ^oC for 30 sec, 72 ^oC for 1 min, and 72 ^oC for 5 min (40 cycles). The PCR products were detected on 1.0% agarose gel by gel documentation system (Nippon Genetics Company, Germany), followed by introducing to Gel Analyser3 program for analysis.

3. Results and Discussion

1. Antimicrobial susceptibility test of the tested bichalcophenes

The antimicrobial activity of tested bichalcophenes **1a-1g** at 10 mM concentration were measured as inhibition zones (mm) against the *Bacillus* sp. by disc diffusion method (Fig. 2). The clear zones were measured and compared to the standard recommendation of Clinical Laboratory Standard Institute (CLSI). The results indicated that different bichalcophenes derivatives had a wide range of antibacterial activity with different degrees of sensitivity of *Bacillus* sp. Growth inhibition was not observed around the control disc containing DMSO. MA-1115 and MA-1116 bichalcophenes showed the maximum antimicrobial activity with inhibition zone diameter 12 mm for both compounds. MA-1156, MA-1113 and MA1114 compounds have a considerable antimicrobial activity with inhibition zone diameter (9 mm, 10 mm, 8 mm) respectively. However the two parent bichalcophenes 944 and 947 exhibited the minimum antimicrobial activity with inhibition zone diameter 6 mm and 7 mm respectively (Table 1).

Table (1) : Effect of the bichalcophenes on *Bacillus* sp.

Tested Bichalcophenes 10 mM	Inhibition zone diameter (mm)
1a (MA-0944)	6
1b (MA-0947)	7
1c (MA-1156)	9
1d (MA-1115)	12
1e (MA-1116)	12
1f (MA-1113)	10
1g (MA-1114)	8

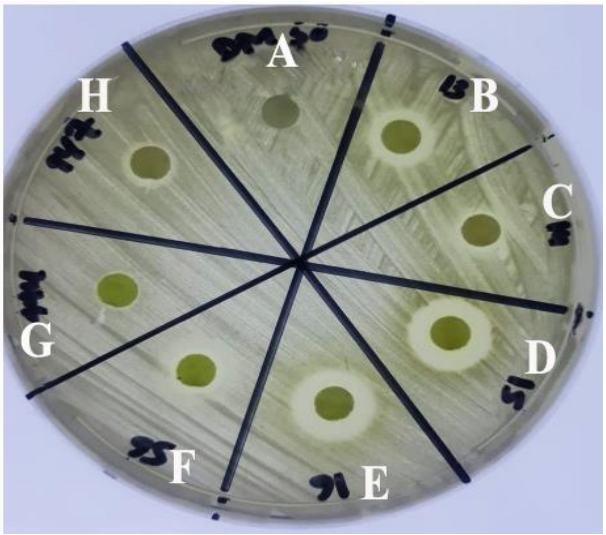


Fig. (2): Bichalcophenes susceptibility of *Bacillus* sp. on LBA medium incubated at 37 °C for 24 hrs. **A**: DMSO, **B**: 1f, **C**: 1g, **D**: 1d, **E**: 1e, **F**: 1c, **G**: 1a and **H**: 1b.

2. MIC determination of MA-1115 and MA-1116 bichalcophene derivatives

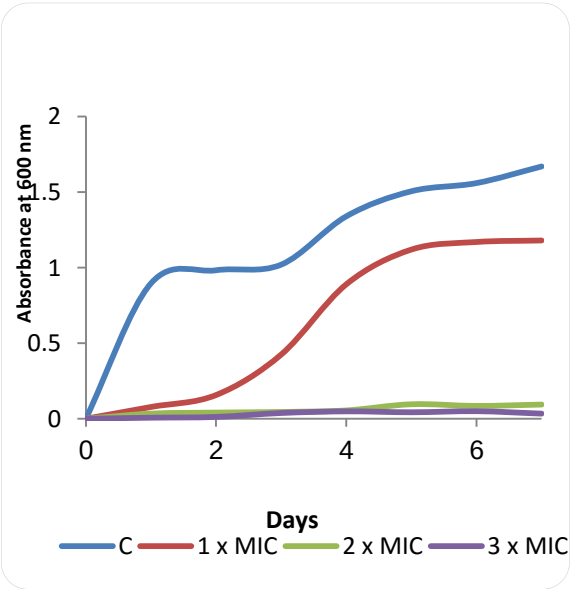
The MIC at which no growth was observed was taken as the MIC values (Table 2 and figure 3). It was found that MA-1115 had higher activity against *Bacillus* sp. than MA-1116 with an MIC value at 32 µM. Furthermore MA-1116 exhibited a potent inhibitory activity with MIC value 64 µM.

Table (2): MIC of MA-1115 and MA-1116 bichalcophene derivatives against *Bacillus* sp. Data was expressed as means of three independent replicates.

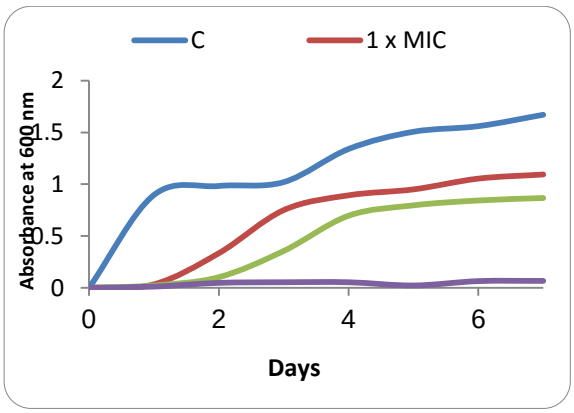
TestedBichalcophense	MIC values mM (mg/mL)
1d (MA-1115)	32 (10.76)
1e (MA-1116)	64 (21.23)

3. Detection of MA-1115 and MA-1116 bichalcophenes-resistant variants

On investigation of the development of resistant variants, MA-1115 was more effective than MA-1116 at preventing the growth of *Bacillus* sp. In contrast, MA-1115 was potent at preventing the bacterial growth at higher concentrations (2 and 3 × MIC) and cells failed to develop resistance over a period of seven days. Compound MA-1116 could prevent the bacterial growth at only one concentration (3 x MIC). However, the growth of *bacillus* sp. was detected at day 2 at the lowest concentrations in both compounds (Figure 3).



A



B

Fig. (3): Development resistance by *Bacillus* sp. in the presence of MA-1115 and MA-1116 (3, 2, and 1 × MIC) for 7 days. (**A**) MA-1115 Compound; (**B**) MA-1116 Compound

Abbreviation: MIC, minimum inhibitory concentration.

1. Effect of bichalcophenes derivatives on protein pattern of *Bacillus* sp.

Protein profile of *Bacillus* sp. treated with MA-1115 with concentrations (8µM, 16µM) and MA-1116 with concentrations (16µM, 32 µM) was documented in (Figure 4). The total number of protein bands recorded as 30 distributed as 21 monomorphic and 9 polymorphic bands. There were six bands disappeared from some concentrations after treatment (asterisk). Two bands with molecular weights 92.864 and 72.769 KDa disappeared from samples treated with 16 µM of MA-1115 and both concentrations of MA-1116. For MA-1115 treatment, two bands with molecular mass 125.045 and 21.987 KDa disappeared only with 8 µM, whereas another band with molecular mass 34.548 KDa disappeared in both concentrations 8 and 16 µM. For MA-1116 treatment, there was a band with molecular mass 30.548 disappeared from only sample treated with 32 µM.

New three bands with molecular mass (90.405, 79.76 and 36.946 KDa) were raised in the samples treated with both compounds (arrows). Two bands with molecular mass 90.405 and 79.76 KDa appeared in samples with both concentrations (16 and 32 µM) of MA-1116 besides 16 µM concentration of MA-1115. Another new band with molecular mass 36.946 KDa appeared only in samples treated with MA-1115.

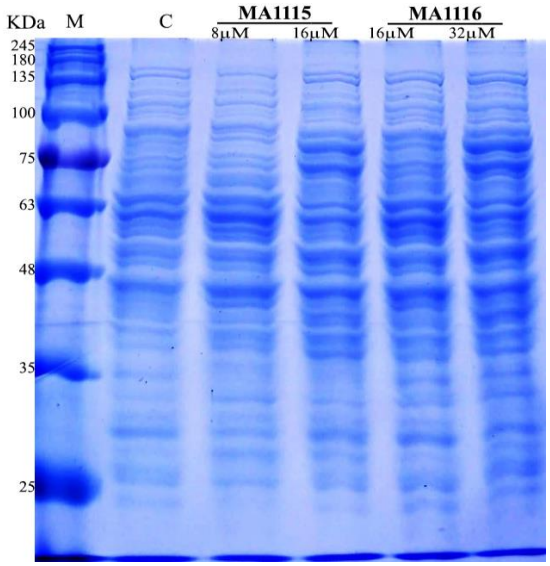
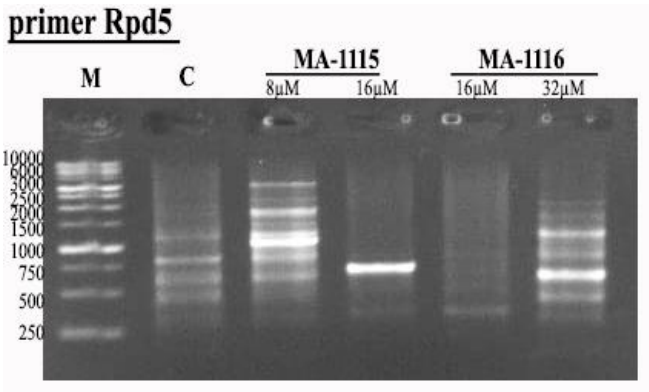


Fig. (4): Protein pattern of *Bacillus* sp. cultivated on LB broth media provided with MA-1115 (8, 16 µM) and MA-1116 (16, 32µM) M= marker, C= control or untreated bacteria, black arrow= new bands, asterisk = disappeared from some concentrations after treatment.

2. Effect of bichalcophenes derivatives on *Bacillus* sp. genomic DNA

The stability of the bacterial genomic DNA of *Bacillus* sp. after the treatment with the selected two monocationic fluoroarylbichalcophenes, MA-1115 with concentrations 8µM, 16µM and MA-1116 with concentrations 16µM, 32µM was evaluated using two RAPD-primers RPD5 and RPD6 (**Fig.** 5).

From DNA finger-print of *Bacillus* sp. after treatment, the total number of bands recorded as 11 bands distributed as 1 monomorphic, 6 polymorphic and 4 unique bands (black arrows) at RPD5 primer (Table 3). In case of RPD6 primer, the total number of bands recorded as 5 bands distributed as, 3 polymorphic and 2 monomorphic bands (Table 4).



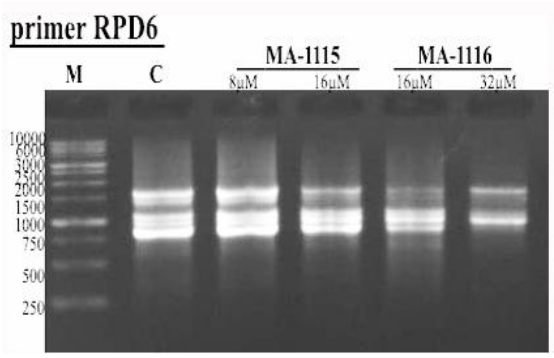


Fig. (5): DNA finger-print of *Bacillus* sp. using two RAPD-primers; a) RPD5, b) RPD6. (M): 1 Kb DNA ladder. bp= base pair, M=marker, C= control.

Table (3) : Analysis of the DNA finger-print of *Bacillus* sp. grown on LB media provided with MA-1115 (8, 16 µM) and MA-1116 (16µM, 32µM) using RPD5 primer.

Band no.	Mol.wt of bands	pattern	1	2	3	4	5
1	3947.782	Unique	-	+	-	-	-
2	2761.637	Polym.	-	+	-	-	+
3	2263.255	Unique	-	+	-	-	-
4	1914.482	Polym.	-	+	-	-	+
5	1466.062	Polym.	+	+	-	-	+
6	1274.255	Unique	-	+	-	-	-
7	928.43	Polym.	+	+	-	-	+
8	747.238	unique	-	-	+	-	-
9	649.476	Polym.	+	+	-	-	+
10	434.242	Polym.	+	+	-	-	+
11	312.128	Monom.	+	+	+	+	+

Table (4) : Analysis of the DNA finger-print of *Bacillus* sp. grown on LB media provided with MA-1115 (8, 16 µM) and MA-1116 (16µM, 32µM) using RPD6 primer.

Band no.	Mol. wt of bands	pattern	1	2	3	4	5
1	2160.537	Monom.	+	+	+	+	+
2	1753.75	Polym.	+	+	-	-	-
3	1335.986	Polym.	+	+	+	+	-
4	1171.354	Monom.	+	+	+	+	+
5	933.72	Polym.	+	+	+	+	-

Discussion

The rapid and extensive development of antibiotic resistance in bacteria is a serious world-wide health problem. Therefore, there must be continuous effort to develop novel antimicrobial agents or increase the efficacy of the antibiotics currently in use by reducing the development of resistance in bacteria.

In earlier studies, the inhibition action of the non-fluorinated (parent) compounds as bithiophene and `bifuran derivatives (1a and 1b) had been investigated [21]. bichalcophenes that contain furan and thiophene rings have wide biological activities [9, 22].

Then, five fluoroaryl bichalcophene derivatives **1a** (4-(2,2'-bithiophene-5-yl)benzamidine); **1b** (4-(2,2'-bifuran-5-yl)benzamidine); **1c** (4-(2,2′-bithiophen-5-yl)-2-fluorobenamidine); **1d** (2-fluoro-4-(5-(thiophen-2-yl)furan-2-yl)benzamidine); **1e** (3-fluoro-4-(5-(thiophen-2-yl)furan-2-yl)benzamidine); **1f** (4-(2,2′-bifuran-5-yl)-3-fluorobenzamidine) **1g** (4-(2,2′-bifuran-5-yl)-2-fluorobenzamidine) were synthesized to achieve better pharmacological properties, have maximum antimicrobial activity and lower toxicity than other biologically active amidines [11].

The biological and chemical properties of compounds can be altered due to the substitution of Fluorine and this can lead to developing a massive number of novel fluorinated drugs. The fluorine substituent high electronegativity can affects a molecule metabolism, distribution, and absorption by modifying the electron distribution in this molecule [23]. The presence of fluorine atom generally increases lipophilicity and therefore biological availability [24]. Fluorine-substitution inhibited the formation of the enamine epoxide in the pyridine moiety and deprived this molecule of mutagenicity of quinolone through microsomal inhibition [25]. In this study, all monocationic fluorinated bichalcophenes were more effective than their corresponding parent compounds in the inhibition activity against *Bacillus* sp. The inhibition zone diameter of corresponding mononitriles (MA-0944 and MA-0947) was 6 and 7 mm respectively. From our study, we noted that, the antibacterial activity against *Bacillus* sp. was enhanced due to the presence of electron-withdrawing substituent (F).

From the five types of fluoroarylbichalcophenes, only the thiophene-furan compounds, MA-1115 and MA-1116 recorded the highest antimicrobial activity with inhibition zone diameter 12 mm and MIC value

32 and 64 µM respectively against *Bacillus* sp. The only difference between MA-1115 and MA-1116 compounds is the substitution of F atom. We also noted that, the MIC is better enhanced when the fluorine atom is next to the bichalcophene moiety as in 1d compound than its positional isoster 1e compound in which the fluorine atom is next to the amidino group leading to improving the ability of compounds to inhibit the bacterial activity [26].

The observed inhibition trend indicates that 1d and 1e compounds are superior in inhibition than other flourinated bichalcophenes. Among these two compounds 1d is the most efficient inhibitor due to the F atom attachment to the phenyl ring increases its hydrophobicity and stabilizes the charge on the compound.

Due to poor solubility of our fluoroarylbichalcophene in water, DMSO was used as a solvent during the determination antimicrobial activities of these compounds. A DMSO control sample was performed against *Bacillus* sp. to ensure that the results obtained were reflective of the antimicrobial activity of the tested compounds without interference of the solvent DMSO. The results showed that *Bacillus* sp. had the ability to grow in presence of DMSO alone. On the other hand, if DMSO was used at higher concentrations, it can act as a hydrophobic stressor of the cell [27].

In order to monitor the spontaneous development of resistance, growth assays were performed in the presence of different concentrations of MA-1115 and MA-1116 for 7 days. These assays provided valuable tool to monitor the resistance development pattern [19]. During our study, we observed that, after 2 days of incubation, *Bacillus* sp. developed resistance to MA-1115 at the lowest concentration (1x MIC). On the other hand, the same bacteria could not develop resistance against MA-1116 at the highest concentration only (3 × MIC) after 7 days of incubation.

The biomarker assay detecting the protein changes based on SDS-PAGE profile and the genetic changes based on RAPD-PCR manipulation of MA-1115 and MA-1116 manifested a polymorphic pattern when comparing between the treated and untreated bacteria. This genetic changes represented in disappearance of some bands verify the capacity fluoroarylbichalcophenes compounds (MA-1115 and MA-1116) to generate some kind of mutation or genetic disorder or at least one nucleotide change (point mutation) which disturb the gene expression as well as the DNA and protein synthesis [28] [29].

Monocationic bichalcophenes were found to cause DNA degradation to bacteria in previous study [15]. The antimicrobial activity of these bichalcophenes could occur because the synthesis of protein and/or nucleic acids is inhibited [30].

Conclusion

From the obtained data, the thiophene/furan-containing bichalcophenes MA-1115 and MA-1116 among the tested monocationic bichalcophene compounds displayed the highest antimicrobial activity against *Bacillus* sp. that couldn't develop resistance to both compounds at higher concentrations after 7 days' incubation. The impact of bichalcophenes MA-1115 and MA-1116 was obviously strong on genetic structure and protein of *Bacillus* sp. based on RAPD-PCR manipulation and SDSPAGE profile.

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