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Original article

Synthesis of some new S-triazine based chalcones and their derivatives as potent antimicrobial agents

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ABSTRACT

Base catalysed condensation of ketone **5** with different aldehydes give chalcones, 2.4-bis-(phenylamino)-6-[4′-{3″-(4‴-substituted phenyl/2‴-furanyl/2‴-thienyl)-2″-propenon-1″-yl}phenylamino]-s-thriazines **6a–e**. These chalcones on cyclization with hydrazine hydrate in the presence of glacial acetic acid, guanidine nitrate in the presence of alkali and malononitrile in the presence of ammonium acetate give the corresponding acetylpyrazolines **7a–e**, aminopyrimidines **8a–e** and cyanopyridines **9a–e** respectively. The products **6a–e**, **7a–e**, **8a–e** and **9a–e** were fully characterized by spectroscopic and elemental analysis and also tested for antibacterial activity.

1. Introduction

The s-triazine based chalcones and their derivatives demonstrate a range of biological activities and in general have been studied extensively because of their wide range of biological activity [1–14]. They are found to be effective as local anaesthetics [1], antibacterial [2,3], antimalarial [4–6], antiprotozoal [7,8] antitubercular [9], anticancer [10,11] and antifungal agents [12,13]. These diverse properties of chalcones have prompted us to synthesize them in order to study their biological activities.

The study of pyrazoline derivatives has been a developing field within the realm of heterocyclic chemistry for the past several decades because of their ready accessibility through synthesis, wide range of chemical reactivity and broad spectrum of biological activity [14–21]. Pyrazoline derivatives have been found to be bactericidal [14,15] fungicidal [10,16,17] and insecticidal agents [18,19]. A survey of more recent literature reveals that some pyrazoline derivatives possess cerebroprotective properties [20] and antidepressant activity [21,22].

Pyrimidines play a vital role in many biological processes since their ring system is present in several vitamins, coenzymes and nucleic acids. Synthetic members of this group are also important as chemotherapeutic agents [23,24]. Recently much interest has focused on the synthesis of pyrimidines possessing fungicidal [25], herbicidal [26], antidepressant [27] and antitumor properties [28,29]. Finally, within the context of our biological studies, cyanopyridines have attracted considerable attention as they posses antibacterial [30], antitubercular [31], antifungal [32] and analgesic [33] activities.

In continuation of work on some novel s-triazine based chalcones [34,35] and their derivatives, we herein, report the reaction of 2,4-bis-(phenylamino)-6-(4′-acetylphenylamino)-s-triazine **5** with different aldehydes to form chalcones **6a–e** and their subsequent conversion to products **7a–e**, **8a–e** and **9a–e** possessing pyrazoline, pyrimidine and cyanopyridine components.

2. Results and discussion

2.1. Chemistry

Compound **5** was prepared by the condensation of cyanuric chloride **1** and aniline **2** at 0–5° to form **3**, which reacts further with aniline at room temperature to form **4.** This, in turn, on

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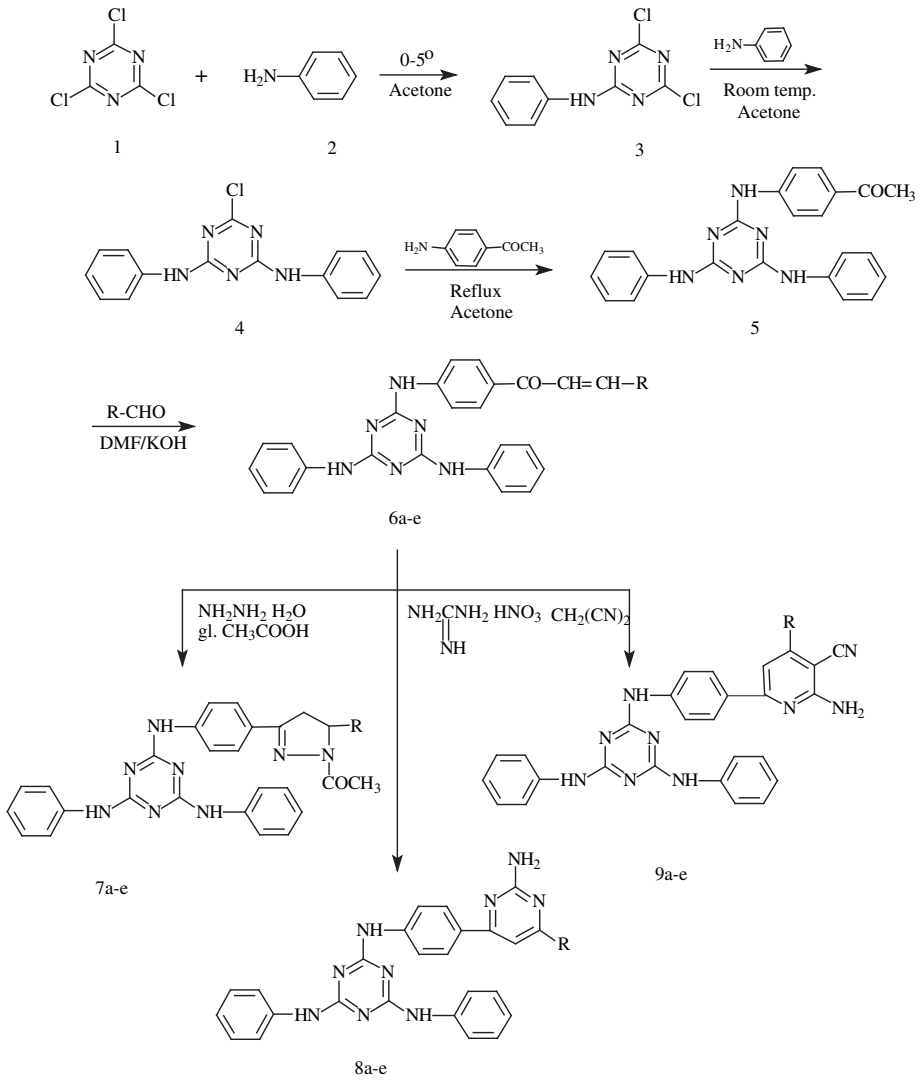

Scheme 1. Synthetic procedure for preparation of title compounds.

treatment with 4-aminoacetophenone, gives compound **5** (Scheme 1). Subsequent condensation of ketone **5** with various aldehydes led to compounds **6a–e**. Chalcones **6a–e** were cyclised with hydrazine hydrate in the presence of glacial acetic acid, guanidine nitrate in the presence of alkali and malononitrile in the presence of ammonium acetate to form acetylpyrazolines **7a–e,** aminopyrimidines **8a–e** and cyanopyridines **9a–e** respectively (Scheme 1).

The structures of the synthesized compounds were assigned on the basis of elemental analysis (Table 1), IR and ^{1}H NMR (Table 2). The IR spectrum of compound **6e** shows the characteristic band at 1647 cm^{-1} due to the –C═O group, the IR spectrum of compound **7e** shows the characteristic band at 1573 cm^{-1} due to the –C═N group and the IR spectrum of compounds **8e** and **9e** shows the characteristic bands in the region of 3300–3400 cm^{-1} which indicate the presence of a primary amine. There are no absorptions in the region of 1600–1700 cm^{-1} in IR spectra of compound **8e** and **9e**, indicating the absence of a–C═O group in these structures. The ^{1}H NMR spectrum of compound **6e** showed doublet of –CO–CH═ at δ 6.90 ppm and doublet of Ar–CH═ at δ 8.05 ppm, which confirm the presence of chalcone moiety. The ^{1}H NMR spectrum of compound **7e** showed singlet of –$COCH_3$ at δ 2.42 ppm. Compounds **8e** and **9e** showed singlet between δ 5.00–6.00 ppm due to –NH_2 group. The aromatic cluster of compounds also support the synthesis of compounds.

2.2. Antibacterial activity

All synthesized compounds were screened for their antibacterial activity by using the disc-diffusion method, TLC- bioautographic and microdilution methods against a panel of selected Gram-positive and Gram-negative bacteria. DMSO was used as a solvent while Streptomycin and Ampicillin were used as a control.

The results of antibacterial activity of titled compounds by disc-diffusion method are presented in Table 3 as inhibition zones of tested compounds at 10 μg/disc. The molecular weights of the newly synthesized triazines and their derivatives varied from 478.55 (**6c**) to 592.61 (**9a**) and were significantly higher than that of Ampicillin (403.45) and different to Streptomycin (581.60). To eliminate this difference, the μg/ml concentrations were converted in μmol/ml and results are also presented in Table 3a as inhibition zones gave by 1 μmol/disc.

It can be seen that the most sensitive bacteria among Gram-positive in disc-diffusion method is *Micrococcus flavus*. Compounds **6c**, **7a**, **7c**, **8b**, **8c**, **8d**, **8e**, **9a**, **9b**, **9d** and **9e** showed lower activity against this bacteria than Streptomycin but higher than Ampicillin

Table 1
The physicochemical data of compounds **6a–e**, **7a–e**, **8a–e** and **9a–e**.

Compd No.	R	M.P. °C	Yield %	Molecular formula	Elemental analysis (Found/calcd) %		
					C	H	N
6a	4-Nitrophenyl	149–150	78	$C_{30}H_{23}N_7O_3$	(68.04 68.05)	(4.38 4.35)	(18.51 18.53)
6b	4-Chlorophenyl	181–182	82	$C_{30}H_{23}N_6OCl$	(69.43 69.43)	(4.47 4.44)	(16.19 16.20)
6c	2-Furanyl	131–133	76	$C_{28}H_{22}N_6O_2$	(70.87 70.89)	(4.67 4.64)	(17.71 17.72)
6d	2-Thienyl	111–113	70	$C_{28}H_{22}N_6OS$	(68.55 68.57)	(4.52 4.49)	(17.13 17.14)
6e	2-Methoxyphenyl	173–175	68	$C_{31}H_{26}N_6O_2$	(72.36 72.37)	(5.09 5.06)	(16.33 16.34)
7a	4-Nitrophenyl	154.5–155	64	$C_{32}H_{27}N_9O_3$	(65.63 65.64)	(4.65 4.62)	(21.53 21.54)
7b	4-Chlorophenyl	185–186	67	$C_{32}H_{27}N_8OCl$	(66.84 66.84)	(4.73 4.70)	(19.48 19.50)
7c	2-Furanyl	148–149	58	$C_{30}H_{26}N_8O_2$	(67.91 67.92)	(4.94 4.91)	(21.12 21.13)
7d	2-Thienyl	148–150	62	$C_{30}H_{26}N_8OS$	(65.92 65.93)	(4.79 4.76)	(20.50 20.51)
7e	2-Methoxyphenyl	178–180	64	$C_{33}H_{30}N_8O_2$	(69.46 69.47)	(5.30 5.27)	(19.64 19.65)
8a	4-Nitrophenyl	154–155	68	$C_{31}H_{24}N_{10}O_2$	(65.48 65.49)	(4.25 4.23)	(24.63 24.65)
8b	4-Chlorophenyl	153.5–154	61	$C_{31}H_{24}N_9Cl$	(66.72 66.73)	(4.33 4.30)	(22.59 22.60)
8c	2-Furanyl	133–135	59	$C_{29}H_{23}N_9O$	(67.82 67.84)	(4.51 4.48)	(24.55 24.56)
8d	2-Thienyl	147.5–147	58	$C_{29}H_{23}N_9S$	(65.77 65.78)	(4.38 4.36)	(23.80 23.82)
8e	2-Methoxyphenyl	146–148	66	$C_{32}H_{27}N_9O$	(69.42 69.44)	(4.92 4.88)	(22.77 22.78)
9a	4-Nitrophenyl	163–164	64	$C_{33}H_{24}N_{10}O_2$	(66.88 66.89)	(4.08 4.05)	(23.64 23.65)
9b	4-Chlorophenyl	114–115	66	$C_{33}H_{24}N_9Cl$	(68.10 68.10)	(4.16 4.13)	(21.66 21.67)
9c	2-Furanyl	134–136	61	$C_{31}H_{23}N_9O$	(69.26 69.27)	(4.31 4.28)	(23.45 23.46)
9d	2-Thienyl	163.5–164	63	$C_{31}H_{23}N_9S$	(67.25 67.27)	(4.19 4.17)	(22.77 22.78)
9e	2-Methoxyphenyl	113–115	69	$C_{34}H_{27}N_9O$	(70.70 70.71)	(4.71 4.68)	(21.82 21.84)

except of **8c** and **8e** which possessed the same inhibition zones. Compounds **8b** and **9c** inhibited *Staphylococcus. aureus* with lower inhibition zones than Streptomycin and with slightly higher activity than Ampicillin, while all tested compounds are not active against *Bacillus cereus*. In case of Gram-negative bacteria *Escherichia coli* is inhibited by **6a**, **6c**, **8c**, **9b** and **9e** with lower activity than Streptomycin but better than Ampicillin. *Pseudomonas aeruginosa* is not inhibited by standard drugs used, but compounds **6c** and **6e** showed inhibition zones. Also, *Listeria monocytogenes* is inhibited by **9e** while standard drugs were not effective against this bacteria. All the tested compounds are not active against *Salmonella typhimurium* and *Enterobacter cloacae*. In general compounds **8b–9e** showed the best activity against Gram-positive bacteria, being more active than ampicillin while compounds **9b** and **9e** exhibited almost the same activity against both Gram-positive and Gram-negative bacteria. Moreover, compound **9e** is active on *L. monocytogenes* while no other compounds possessed any activity against this bacteria. It can be noticed that all tested compounds showed lower activity than Streptomycin but higher than Ampicillin.

The results of TLC-bioautographic method are presented in Table 4. All the compounds tested showed activity against all the bacteria used. Streptomycin was also active against all the bacteria tested, while there is no activity observed for Ampicillin in this method. It can be seen again that the most sensitive bacteria among Gram-positive in this method, like in previous one, is *M. flavus*. All compounds tested exhibited good ability to inhibit this bacteria, even much better than antibiotic, used as reference drug, except of **6a** which showed the same activity with Streptomycin. It was found that compounds **6c**, **6e**, **7b**, **7c**, **8b**, **8c**, **8d** and **8e** are active against *S. aureus*. It should be noted that compounds **6c**, **6e**, **7b**, **7c** and **8c** are more active than Streptomycin, while compound **8b** exhibited the same activity with the reference drug. Compounds **8d** and **8e** are the less potent. In the case of another Gram-positive bacteria, *B. cereus*, compounds **7a**, **8b** and **8d** are more active, **7b** and **7d -**equipotent and **6c** and **6e -**slightly less active than standard drug. Among Gram-negative bacteria the most sensitive bacteria are *S. typhimurium* and *En. cloacae*. All the compounds tested are more active against *L. monocytogenes* than Streptomycin with exception of **6a** and **6b**. The most active compound in this case is **8a**, followed by **7a**, **7b** and **8e**. All the compounds, except of **8a**, **8b** and **8e** are also more active than Streptomycin and against *En. cloacae*. The best activity was found for compound **7d**, followed by **6a**, **7a–7c**, as well as **9a**. In the case of *S. typhimurium* compounds **6b**, **7a**, **7c** and **8d** possessed activity better than Streptomycin with the most potent compound being **7a.** Compounds **7b**, **8b** and **9c–9e** are less active than the reference drug, while **6a** and **9b** are equipotent to it. It could be noticed that *E. coli* is a less sensitive bacteria compared with ones previously mentioned. Thus, only five compounds **6c**, **8b**, **8c** and **9b**, and **9d** are active against this bacteria. Compounds **6c**, **8b**, **8c** and **9b**, showed the same inhibition zones as Streptomycin while **9d** is more potent against *E. coli*. The most resistant bacteria is *P. aeruginosa* being sensitive only to **9a** and **9b** which are more potent than Streptomycin. In general compounds **9a–9e** possessed better activity against Gram-negative bacteria, however only compound **9b** is active against all the Gram-negative bacteria tested. From another side, compounds **8a–8e** are the most active against Gram-positive bacteria. In general, active compounds tested by this method are more potent against Gram-negative than Gram-positive bacteria, showing higher inhibition zones than Streptomycin.

The results of antibacterial activity obtained by microdilution method are presented in Table 5. The kind of the exerted antibacterial activity was investigated by determining the minimal bactericidal concentrations (MBCs). The experimental data (values in brackets) presented in Table 4 show that all derivatives **6a–9e** possess bacteriostatic properties, being MBCs twofold or more higher than the corresponding MICs.

All the compounds tested showed MIC ranging from 4.5 to 78.0 μmol/ml × 10^{-2} and MBC ranging from 9.0 to 78.0 μmol/ml × 10^{-2}. Only compound **7c**, did not show MBC against *E.colacae*, *S. typhimurium* and *L. monocytogenes*. Standard drugs are also active against all the bacteria tested and show MIC ranging from 4.3 to 25.8 μmol/ml × 10^{-2} for Streptomycin and 24.8–74.4 μmol/ml × 10^{-2} for Amplicillin. MBC for the antibiotics are 8.6–51.6 μmol/ml × 10^{-2} and 37.2–124.0 μmol/ml × 10^{-2}, respectively.

The most sensitive bacteria is *B. cereus* with very low MIC and MBC, while *L. monocytogenes* is the most resistant one with high MIC and MBC. From the group **6a–e**, compound **6e** exhibited the best antibacterial activity (MIC of 13.4–26.8 μmol/ml × 10^{-2} and MBC of 26.8–53.6 μmol/ml × 10^{-2}). The worst activity is obtained for compound **6c** (MIC of 21.1–42.2 μmol/ml × 10^{-2} and MBC of 42.2–84.4 μmol/ml × 10^{-2}). The highest antibacterial activity in group **7a–e**, was observed for compound **7a** (MIC of 8.5–68.4 μmol/ml × 10^{-2} and MBC of 17.1–68.4 μmol/ml × 10^{-2}), while **7c** showed the lowest activity with MIC of 37.8–75.6 μmol/ml × 10^{-2} and MBC

Table 2
IR and ^{1}H NMR spectra of synthesized compounds.

Compd No.	IR	^{1}H NMR ($CDCl_3$) δ ppm δ C (CDCl3)
6a	1643 (C=O), 1597 (–CH=CH–, str.), 1338 (C–N), 811 (C–N, s-triazine).	6.90 (d, 1H, –CO–CH=), 7.15–7.80 (m, 18 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH=). 119, 120.6, 122.3, 123.9, 126.3, 128.4, 128.9, 129.8, 129.8, 130.4, 139.7, 140.1, 141.4, 145.3, 147.95, 163.9, 164.1, 187.1
6b	1646 (C=O), 1593 (–CH=CH–, str.), 1336 (C–N), 809 (C–N, s-triazine), 786 (C–Cl).	6.92 (d, 1H, –CO–CH=), 7.10–7.75 (m, 18 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH=). 118.9, 120.5,122.3, 122.9, 128.4, 128.9, 129.6, 130.5, 130.7, 133.9, 134.8, 141.5, 145, 163.9, 164, 187.3
6c	1649 (C=O), 1592 (–CH=CH–, st.), 1342 (C–N), 810 (C–N, s-triazine).	6.89 (d, 1H, –CO–CH=), 7.15–7.85 (m, 17 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH=). 112.98, 116.4, 118.9, 119.1, 120.5, 122.9, 126.2, 129.6, 130.8, 136.2, 139.7, 139.9, 144.9, 145.9, 151.3, 163.96, 164.1, 186.8
6d	1650 (C=O), 1591 (–CH=CH–, str.), 1344 (C–N), 811 (C–N, s-triazine).	6.91 (d, 1H, –CO–CH=), 7.10–7.85 (m, 17 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH=). 119.1, 120.5, 122.3, 128.4, 128.7, 129.4, 130.0, 130.8, 132.4, 135.8, 139.7, 139.9, 144.9, 163.98, 164.1, 186.9
6e*	1647 (C=O), 1595 (–CH=CH–, str.), 1340 (C–N), 812 (C–N, s-triazine).	3.95 (s, 3H, $-OCH_3$), 6.90 (d, 1H, –CO–CH=), 7.10–7.80 (m, 18 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH=). 114.2, 120.3, 122.1, 127.3, 128.2, 129.2, 130.4, 130.9, 139.5, 142.7, 160.95, 163.7, 187.
7a	1650 ($-COCH_3$), 1575 (C=N), 806 (C–N, s-triazine).	2.42 (s, 3H, $-COCH_3$), 3.15 (dD, 1H, C_4-H_A), 3.65 (dd, 1H, C_4-H_B), 5.60 (dd, 1H, –CH–CH_2), 6.90–7.80 (m, 18 Ar–H and 3NH). 118.9, 119.7, 120.5, 122.3, 123.9, 125.3, 127,1, 128.4, 128.9, 130.3, 139.7, 144.8, 146.7, 149.9, 154.1.
7b	1656 ($-COCH_3$), 1577 (C=N), 806 (C–N, s-triazine), 768 (C–Cl).	2.42 (s, 3H, $-COCH_3$), 3.10 (dD, 1H, C_4-H_A), 3.60 (dd, 1H, C_4-H_B), 5.62 (dd, 1H, –CH–CH_2), 6.90–7.80 (m, 18 Ar–H and 3NH). 118.9, 119.6, 120.4, 122.2, 124.2, 127.0, 127.5, 128.4, 128.6, 131.6, 139.8, 141.5, 142.2, 154.1, 163.9, 164.0, 167.2
7c	1654 ($-COCH_3$), 1576 (C=N), 806 (C–N, s-triazine).	2.42 (s, 3H, $-COCH_3$), 3.14 (dD, 1H, C_4-H_A), 3.66 (dd, 1H, C_4-H_B), 5.58 (dd, 1H, –CH–CH_2), 6.90–7.80 (m, 17 Ar–H and 3NH). 21.7, 39.9, 40.1, 106.8, 110.4, 119.7, 120.4, 122.2, 124.2, 127.0, 128.4, 139.8, 142.2, 142.3, 152.9, 154.1, 163.9, 164.1, 167.2.
7d	1651 ($-COCH_3$), 1572 (C=N), 806 (C–N, s-triazine).	2.42 (s, 3H, $-COCH_3$), 3.13 (dD, 1H, C_4-H_A), 3.62 (dd, 1H, C_4-H_B), 5.65 (dd, 1H, –CH–CH_2), 6.90–7.80 (m, 17 Ar–H and 3NH). 21.6, 40.1, 41.7, 119.9, 120.4, 122,2, 124.2, 124.5, 124.9, 126.7, 127.1, 128.4, 128.7, 131.6, 131.7, 139.8, 142.2, 145,0, 154.3, 163.9, 164.1, 166.9, 167.2.
7e*	1653 ($-COCH_3$), 1573 (C=N), 806 (C–N, s-triazine).	2.42 (s, 3H, $-COCH_3$), 3.15 (dD, 1H, C_4-H_A), 3.65 (dd, 1H, C_4-H_B), 3.80 (s, 3H, $-OCH_3$), 5.60 (dd, 1H, –CH–CH_2), 6.90–7.80 (m, 18 Ar–H and 3NH). 113.7, 119.5, 120.2,122.0, 124.2, 126.6, 126.8, 128.2, 134.4, 139.5, 141.9, 153.9, 158.2, 163.2, 163.8, 166.9
8a	3398 ($-NH_2$), 1575 (C=N), 806 (C–N, s-triazine).	5.15 (s, 2H, $-NH_2$), 6.95–8.15 (m, 19 Ar–H and 3NH). 102.0, 118.8, 120.5, 122.2, 123.1, 127.4, 128.1, 128.3, 128.5, 128.8, 129.1, 129.8, 130.2, 139.6, 139.7, 139.8, 142.6, 143.6, 144.7, 144.8, 145.8, 152.9, 162.1, 163.9, 164.0, 165.1, 196.3, 196.5
8b	3398 ($-NH_2$), 1575 (C=N), 806 (C–N, s-triazine), 770 (C–Cl).	5.12 (s, 2H, $-NH_2$), 6.90–8.10 (m, 19 Ar–H and 3NH). 101, 118.8, 119.5, 120.2,120.4, 122.0, 122.2, 127.2, 128.0, 128.2, 128.4, 128.6, 128.7, 128.8, 128.9, 129.4, 130.3, 139.5, 139.7, 163.8, 164.0, 196.8
8c	3398 ($-NH_2$), 1575 (C=N), 806 (C–N, s-triazine).	5.05 (s, 2H, – NH_2), 6.90–8.15 (m, 18 Ar–H and 3NH). 110.4, 110.5, 113.0, 116.4, 118.8, 119.0, 120.3, 120.5, 122.2, 128.3, 128.5, 129.0, 129.2, 129.5, 130.7, 139.6, 139.7, 139.8, 144.1, 145.9 144.8, 145.9, 151.2, 163.9, 164.0, 186.8
8d	3398 ($-NH_2$), 1575 (C=N), 806 (C–N, s-triazine).	5.14 (s, 2H, – NH_2), 6.85–8.20 (m, 18 Ar–H and 3NH). 118.8, 118.8, 119.0, 119.4, 119.5, 120.4, 122.1, 122.2, 127.1, 128.3, 128.6, 128.8, 128.9, 129.0, 129.3, 130.0, 132.3, 139.6, 139.7, 139.8, 163.9, 164,.0, 186.8
8e*	3398 ($-NH_2$), 1575 (C=N), 806 (C–N, s-triazine).	3.85 (s, 3H, $-OCH_3$), 5.10 (s, 2H, – NH_2), 6.90–8.15 (m, 19 Ar–H and 3NH). 114.0, 120.6, 120.7, 122.3, 128.2, 139.9, 140.1, 140.2, 140.3, 164.2
9a	3396 ($-NH_2$), 2205 **(C≡N)** 803 (C–N, s-triazine).	5.20 (s, 2H, – NH_2), 6.90–8.25 (m, 19 Ar–H and 3NH).
9b	3386 ($-NH_2$), 2210 **(C≡N)** 805 (C–N, s-triazine), 776 (C–Cl).	5.26 (s, 2H, – NH_2), 6.90–8.20 (m, 19 Ar–H and 3NH). 112.9, 113.2, 113.8, 118.8, 118.9, 119.5, 119.7, 119.8, 120.3, 120.4, 120.5, 121.9, 122.0, 122.2, 127.6, 128.2, 128.5, 128.6, 128.8, 130.0, 130.1, 131.1, 132.8, 133.6, 136.8, 139.5, 139.6, 139.7, 163.8, 163.9, 175.2, 194.4
9c	3392 ($-NH_2$), 2209 **(C≡N)** 801 (C–N, s-triazine).	5.22 (s, 2H, – NH_2), 6.85–8.15 (m, 18 Ar–H and 3NH). 108.2, 110.6, 112.7, 113.1, 113.8, 114.1, 118.9, 120.5, 122.2, 128.2, 128.5, 128.8, 128.9, 129.0, 139.5, 139.6, 143.1, 144.4, 145.2, 150.9, 163.8, 163.9, 175.2, 194.0
9d	3399 ($-NH_2$), 2204 **(C≡N)** 804 (C–N, s-triazine).	5.25 (s, 2H, – NH_2), 6.85–8.18 (m, 18 Ar–H and 3NH).
9e	3396 ($-NH_2$), 2200 **(C≡N)** 806 (C–N, s-triazine).	3.95 (s, 3H, $-OCH_3$), 5.28 (s, 2H, – NH_2), 6.90–8.20 (m, 19 Ar–H and 3NH). 108.1, 114.0, 114.3, 117.3, 119.4, 120.2, 122.0, 127.4, 128.2, 129.1, 129.6, 130.6, 139. 6, 154.0, 158.0, 160.2, 160.7, 163.8, 163.9.

of 75.6 μmol/ml × 10^{-2}. In group **8a–e**, compound **8b** possessed inhibitory activity of 8.95–35.8 μmol/ml × 10^{-2} and bactericidal of 17.9–71.6 μmol/ml × 10^{-2}, while **8c** inhibited bacterial growth with higher MIC 19.5–78.0 μmol/ml × 10^{-2} and showed bactericidal effect at 39.0–78.0 μmol/ml × 10^{-2}. Compound **9d** possessed the best antibacterial activity in group **9a–e** with MIC 4.5–18.0 μmol/ml × 10^{-2} and MBC of 9.0–36.0 μmol/ml × 10^{-2}, while **9b** showed the lowest activity with MIC 17.2–68.8 μmol/ml × 10^{-2} and MBC of 34.4–68.8 μmol/ml × 10^{-2}.

Among all the compounds tested, **9d** exhibited the best antibacterial activity against B.cereus, with very low MIC and not antibacterial activity, with very low MIC.

It is clear that all synthesized compounds are more active against Gram-positive bacteria than Gram-negative bacteria in this method.

It can be seen that the growth of tested bacteria responded differently to the compounds tested, which indicates that different components may have different modes of action or that the metabolism of some bacteria is able to better overcome the effect of the agents or adapt to it. Gram-negative bacteria are in general more resistant than Gram-positive [36].

The inhibition zones obtained from TLC-bioautographic method are higher than in disc-diffusion method. More compounds are active in TLC-bioautographic method than in disc-diffusion and a great number of compounds showed inhibition in

Table 3
Antibacterial activity of compounds **6a–e**, **7a–e**, **8a–e** and **9a–e** (conc. 10 μg/disc) tested by disc-diffusion method.

Comp No.	R	Antibacterial activity							
		Zone of Inhibition in mm							
		B.c	*M.f*	*S.a*	*E.c*	*P.a*	*En.c*	*S.t*	*L.m*
6a	4-Nitrophenyl	–	–	–	6 ± 0.6	–	–	–	–
6b	4-Chlorophenyl	–	–	–	–	–	–	–	–
6c	2-Furanyl	–	8 ± 0.6	–	8 ± 0.3	6 ± 0.6	–	–	–
6d	2-Thienyl	–	–	–	–	–	–	–	–
6e	2-Methoxyphenyl	–	–	–	–	6 ± 0.6	–	–	–
7a	4-Nitrophenyl	–	10 ± 0.6	–	–	–	–	–	–
7b	4-Chlorophenyl	–	–	–	–	–	–	–	–
7c	2-Furanyl	–	10 ± 0.6	–	–	–	–	–	–
7d	2-Thienyl	–	–	–	–	–	–	–	–
7e	2-Methoxyphenyl	–	–	–	–	–	–	–	–
8a	4-Nitrophenyl	–	–	–	–	–	–	–	–
8b	4-Chlorophenyl	–	9 ± 0.6	7 ± 0.6	–	–	–	–	–
8c	2-Furanyl	–	6 ± 0.6	–	10 ± 1	–	–	–	–
8d	2-Thienyl	–	6 ± 0.6	–	–	–	–	–	–
8e	2-Methoxyphenyl	–	6 ± 0.3	–	–	–	–	–	–
9a	4-Nitrophenyl	–	6 ± 0.3	–	–	–	–	–	–
9b	4-Chlorophenyl	–	7 ± 1	–	7 ± 1	–	–	–	–
9c	2-Furanyl	–	–	6 ± 0.6	–	–	–	–	–
9d	2-Thienyl	–	7 ± 0.6	–	–	–	–	–	–
9e	2-Methoxyphenyl	–	10 ± 1	–	7 ± 0.6	–	–	–	6 ± 0.3
Standard Drugs	Streptomycin	18 ± 1	20 ± 1	13 ± 1	10 ± 0.6	–	12 ± 0.6	–	–
	Ampicillin	10 ± 1	8 ± 0.6	8 ± 0.3	6 ± 0.3	–	6 ± 0.6	–	–

– no inhibition; *B.c – Bacillus cereus*, *M.f – Micrococcus flavus*, *S.a – Staphylococcus aureus*, *E.c – Escherichia coli*, *P.a – Pseudomonas aeruginosa*, *En.c – Enterobacter cloacae*, *S.t – Salmonella typhimurium*, *L. m – Listeria monocytogenes*.

this method. The reason of this could be explained by different hydrophilic and diffusion capacity of different compounds through the agar medium used in disc-diffusion method [37–39]. Compounds with higher hydrophilic characteristics possess better diffusion capacity through the medium and, on the contrary, low water solubility limits diffusion through the medium. Therefore, specificity and levels of activity is related to the type of functional group, but also associated with hydrogen-bonding parameters in all cases.

Microdilution method, carried out in microtitre trays, has the advantage of lower workloads for a larger number of replicates and the use of small volumes of the test substance and growth medium. In addition, the results are more informative than in other methods and could be expressed as quantitative data.

As regards to the relationships between the structure and the detected antibacterial activity, the inhibitory effect appears to be dependent on the substituent of chalcone. It seems, that for chalcones **6a–6e** and its pyrazoline (**7a–7e**), pyrimidine (**8a–8e**) and cyanopyridine (**9a–9e**) derivatives the introduction of different substituents (R) play different role in antibacterial activity. It is interesting to point out that for chalcones and its pyrazoline and pyrimidine derivatives the presence of substituted benzene ring (**6**, **7**, **8a**, **8b**, **8e**) are mostly endowed with higher activity with respect to five-member heterocyclic rings (**6**, **7**, **8c**, **8d**). For example, for **6a-6e** group the most active is derivative with 2-methoxyphenyl substituent, for **7a–7e** – 4-nitrophenyl- (only in case of Gram-positive bacteria) while for **8a–8e** -the most active is derivative with 4-chlorophenyl substituent in aromatic ring. In the contrary, for the antibacterial activity of cyanopyridines important is substitution with 5-member-ring heterocycles. Thus, the best activity in this series was observed for 2-thienyl derivative followed by 2-furanyl one.

2.3. QSAR study

A QSAR study was conducted only on the antibacterial activities obtained by the microdilution method, because all the compounds had quantitative values for MIC and MBC. As the range of MIC and MBC values was very narrow (around one log unit), the QSAR models derived in the present study should be used with care for prediction and design of new compounds. The models goodness of fit was assessed by r^2, while their predictive ability was evaluated by leave-one-out cross-validation and assessed by q^2. Only models with $r^2 > 0.6$ and $q^2 > 0.3$ were considered as significant.

Table 3a
Antimicrobial activity of the studied compounds presented as a inhibition of 1 μmol/disc presented as inhibition zones (mm).

Compound	Antimicrobial activity presented in mm							
	B.c	*M.f*	*S.a*	*E.c*	*P.a*	*En.c*	*S.t*	*L.m*
6a	–	–	–	0.03	–	–	–	–
6b	–	–	–	–	–	–	–	–
6c	–	0.04	–	0.04	0.02	–	–	–
6d	–	–	–	–	–	–	–	–
6e	–	–	–	–	0.03	–	–	–
7a	–	0.04	–	–	–	–	–	–
7b	–	–	–	–	–	–	–	–
7c	–	0.05	–	–	–	–	–	–
7d	–	–	–	–	–	–	–	–
7e	–	–	–	–	–	–	–	–
8a	–	–	–	–	–	–	–	–
8b	–	0.05	0.04	–	–	–	–	–
8c	–	0.03	–	0.05	–	–	–	–
8d	–	0.04	–	–	–	–	–	–
8e	–	0.03	–	–	–	–	–	–
9a	–	0.04	–	–	–	–	–	–
9b	–	0.04	–	0.04	–	–	–	–
9c	–	–	0.03	–	–	–	–	–
9d	–	0.04	–	–	–	–	–	–
9e	–	0.06	–	0.04	–	–	–	0.03
Streptomycin	0.1	0.1	0.08	0.06	–	0.07	–	–
Ampicillin	0.04	0.03	0.03	0.02	–	0.02	–	–

– no inhibition; *B.c – Bacillus cereus*, *M.f – Micrococcus flavus*, *S.a – Staphylococcus aureus*, *E.c – Escherichia coli*, *P.a – Pseudomonas aeruginosa*, *En.c – Enterobacter cloacae*, *S.t – Salmonella typhimurium*, *L. m – Listeria monocytogenes*.

Table 4
Antibacterial activity of compounds **6a–e**, **7a–e**, **8a–e** and **9a–e** tested with TLC method and calculated as inhibition of 1 μmol/spot presented as inhibition zone (mm).

Comp No.	R	Antibacterial activity							
		Zone of Inhibition in mm							
		B.c	*M.f*	*S.a*	*E.c*	*P.a*	*En.c*	*S.t*	*L.m*
6a	4-Nitrophenyl	–	0.05	–	–	–	0.09	0.08	0.07
6b	4-Chlorophenyl	–	0.07	–	–	–	0.08	0.09	0.07
6c	2-Furanyl	0.07	0.07	0.08	0.08	–	0.08	–	0.08
6d	2-Thienyl	–	–	–	–	–	0.08	–	0.08
6e	2-Methoxyphenyl	0.06	–	0.07	–	–	0.08	–	0.08
7a	4-Nitrophenyl	0.1	0.07	–	–	–	0.09	0.1	0.09
7b	4-Chlorophenyl	0.08	–	0.08	–	–	0.09	0.07	0.09
7c	2-Furanyl	–	–	0.07	–	–	0.09	0.09	0.08
7d	2-Thienyl	0.08	–	–	–	–	0.1	–	0.08
7e	2-Methoxyphenyl	–	–	–	–	–	0.08	–	0.08
8a	4-Nitrophenyl	–	0.07	–	–	–	–	–	0.1
8b	4-Chlorophenyl	0.09	0.07	0.04	0.08	–	–	0.07	0.08
8c	2-Furanyl	–	0.07	0.06	0.08	–	0.08	–	0.08
8d	2-Thienyl	0.09	0.08	0.03	–	–	0.08	0.09	0.08
8e	2-Methoxyphenyl	–	0.06	0.03	–	–	–	–	0.09
9a	4-Nitrophenyl	–	0.07	–	–	0.09	0.09	–	0.1
9b	4-Chlorophenyl	–	0.07	–	0.09	0.09	0.08	0.08	0.08
9c	2-Furanyl	–	–	–	–	–	0.09	0.07	0.08
9d	2-Thienyl	–	0.08	–	0.06	–	0.08	0.07	0.09
9e	2-Methoxyphenyl	–	0.06	–	–	–	0.08	0.07	0.09
Standard drugs	Streptomycin	0.08	0.05	0.04	0.06	0.08	0.07	0.08	0.08
	Ampicillin	–	–	–	–	–	–	–	–

– no inhibition; *B.c – Bacillus cereus*; *M.f – Micrococcus flavus*; *S.a – Staphylococcus aureus*, *E.c – Escherichia coli*, *P.a – Pseudomonas aeruginosa*, *En.c – Enterobacter cloacae*, *S.t – Salmonella typhimurium*, *L. m – Listeria monocytogenes.*

2.3.1. Free-Wilson models

In the present study Free-Wilson models were generated using nine discontinuous descriptors accounting for the presence or absence of a substituent or a main structure in a molecule. Genetic algorithm (*GA*) was applied preliminary to select the most important descriptors, followed by multiple linear regression (MLR) on the selected variables. Among the 16 models obtained (8 for MIC and 8 for MBC) only one showed a moderate explained and predictive ability. This was the model, including MIC against *S. aureus* and it is given by the following equation:

$$\mathrm{pMIC}(S.aureus) = 0.110 * 2 - \text{thienyl} + 0.315 * 2 - \text{methoxyphenyl} - 0.308 * \text{chalcone} - 0.417 * \text{acetylpyrazoline} + 3.717$$

$$n = 20 r^2 = 0.637\ \mathrm{SEE} = 0.181\ F = 6.152\ p < 0.01\ q^2 = 0.303\ \mathrm{cvRSS} = 0.884$$

The chalcone and acetylpyrazoline main structures have negative contributions in the antimicrobial activity against *S. aureus*.

Table 5
Minimal inhibitory (MIC μmol/ml × 10^{-2}) and bactericidal concentration (MBC μmol/ml × 10^{-2}) of compounds **6a–e**, **7a–e**, **8a–e** and **9a–e** tested in microdilution method.

Comp No.	R	Antibacterial activity							
		Zone of Inhibition in mm							
		B.c MIC MBC	*M.f* MIC MBC	*S.a* MIC MBC	*E.c* MIC MBC	*P.a* MIC MBC	*En.c* MIC MBC	*S.t* MIC MBC	*L.m* MIC MBC
6a	4-Nitrophenyl	18.9 37.8	18.9 37.8	37.8 75.6	18.9 37.8	37.8 75.6	18.9 37.8	37.8 75.6	37.8 75.6
6b	4-Chlorophenyl	19.3 38.6	19.3 38.6	38.6 77.2	77.2 77.2	38.6 77.2	19.3 38.6	38.6 77.2	38.6 77.2
6c	2-Furanyl	21.1 42.2	42.2 84.4	42.2 84.4	21.1 42.2	21.1 42.2	21.1 42.2	21.1 42.2	42.2 84.4
6d	2-Thienyl	20.4 40.8	20.4 40.8	40.8 81.6	40.8 81.6	40.8 81.6	20.4 40.8	40.8 81.6	40.8 81.6
6e	2-Methoxyphenyl	13.4 26.8	26.8 53.6	13.4 26.8	13.4 26.8	26.8 53.6	26.8 53.6	26.8 53.6	26.8 53.6
7a	4-Nitrophenyl	8.5 17.1	17.1 34.2	17.1 34.2	17.1 34.2	68.4 68.4	68.4 68.4	68.4 68.4	68.4 68.4
7b	4-Chlorophenyl	69.6 69.6	69.6 69.6	69.6 69.6	34.8 69.6	69.6 69.6	69.6 69.6	34.8 69.6	69.6 69.6
7c	2-Furanyl	37.8 75.6	37.8 75.6	75.6 75.6	37.8 75.6	75.6 75.6	75.6 –	75.6 –	75.6 –
7d	2-Thienyl	18.8 37.6	18.8 37.6	37.6 73.2	18.8 37.6	37.6 73.2	73.2 73.2	37.6 73.2	73.2 73.2
7e	2-Methoxyphenyl	17.5 35.0	17.5 35.0	35.0 70.0	35.0 70.0	35.0 70.0	17.5 35.0	35.0 70.0	70.0 70.0
8a	4-Nitrophenyl	17.6 35.2	35.2 70.4	17.6 35.2	17.6 35.2	35.2 70.4	17.6 35.2	35.2 70.4	70.4 70.4
8b	4-Chlorophenyl	8.95 17.9	17.9 35.8	17.9 35.8	8.95 17.9	17.9 35.8	8.95 17.9	17.9 35.8	35.8 71.6
8c	2-Furanyl	19.5 39.0	19.5 39.0	19.5 39.0	19.5 39.0	78.0 78.0	19.5 39.0	19.5 39.0	78.0 78.0
8d	2-Thienyl	18.9 37.8	18.9 37.8	18.9 37.8	18.9 37.8	75.6 75.6	18.9 37.8	18.9 37.8	75.6 75.6
8e	2-Methoxyphenyl	9.05 18.1	9.05 18.1	9.05 18.1	18.1 36.2	18.1 36.2	18.1 36.2	18.1 36.2	72.4 72.4
9a	4-Nitrophenyl	16.9 33.8	33.8 67.6	33.8 67.6	33.8 67.6	67.6 67.6	16.9 33.8	67.6 67.6	67.6 67.6
9b	4-Chlorophenyl	17.2 34.4	17.2 34.4	17.2 34.4	17.2 34.4	68.8 68.8	17.2 34.4	17.2 34.4	68.8 68.8
9c	2-Furanyl	9.3 18.6	9.3 18.6	18.6 37.2	9.3 18.6	37.2 74.4	18.6 37.2	18.6 37.2	37.2 74.4
9d	2-Thienyl	4.5 9.0	4.5 9.0	9.0 18.0	4.5 9.0	9.0 18.0	9.0 18.0	4.5 9.0	18.0 36.0
9e	2-Methoxyphenyl	8.65 17.3	8.65 17.3	17.3 34.6	17.3 34.6	34.6 69.2	17.3 34.6	17.3 34.6	34.6 69.2
Standard drugs	Streptomycin	4.3 8.6	8.6 17.2	17.2 34.4	17.2 34.4	17.2 34.4	17.2 34.4	17.2 34.4	25.8 51.6
	Ampicillin	24.8 37.2	24.8 37.2	24.8 37.2	37.2 49.2	74.4 124.0	37.2 49.2	24.8 49.2	37.2 74.4

– no inhibition; *B.c – Bacillus cereus*, *M.f – Micrococcus flavus*, *S.a – Staphylococcus aureus*, *E.c – Escherichia coli*, *P.a – Pseudomonas aeruginosa*, *En.c – Enterobacter cloacae*, *S.t – Salmonella typhimurium*, *L.m – Listeria monocytogenes.*

Among the substitutents, 2-methoxyphenyl and 2-thienyl works positively against *S. aureus*.

2.3.2. LFER models

Continuous descriptors accounting for the molecular shape, electronic properties, polarity, lipophilicity and other molecular properties were selected by *GA* and included in the QSAR models for antimicrobial activity of the investigated compounds based on the linear free energy relationship (LFER). Only models with $r^2 > 0.6$ and $q^2 > 0.3$ were considered as significant. These were models describing the antimicrobial activities against *B. cereus*, *M. flavus*, *S. aureus*, *En. cloacae* and *L. monocytogenes* (Tables 2 and 3). Molecular shape descriptors, like ovality of molecule, surface, volume, refractivity or parahor, take part in these models. Electronic properties, like the polarity indices Qv and Qs, dipole moments, polarizabilities and specific polarizabilities are important against *B. cereus*, *M. flavus*, *S. aureus* and *En. cloacae*. Lipophilic compounds are more active against *B. cereus*, *S. aureus* and *En. cloacae*. Compounds with more rings in their structure are more active against *S. aureus* and less active against *En. cloacae*. Compounds with less hydrogen-bond acceptors are more active against *M. flavus* and *S. aureus*, while more hydrogen-bond acceptors work well against *B. cereus*. The nitro group in 4-nitrophenyl brings two more hydrogen-bond acceptors. Hydrogen-bond donors are important for the antimicrobial activity against *En. cloacae*. Compounds **6a–e** and **7a–e** can form three hydrogen bonds, in which they act as donors, while compounds **8a–e** and **9a–e** have an amino group and can form two additional hydrogen bonds. Flexible compounds with many free rotatable bonds have high activity against *S. aureus*.

2.3.2.1. Models for MIC. QSAR models with good explained and predictive ability regarding MIC were obtained for *B. cereus*, *S. aureus*, *En. cloacae* and *L. monocytogenes* (Table 2). Oval molecules with small surface, high polarity and many hydrogen-bond acceptors are good inhibitors of *B. cereus* growth. Compounds with low polarity, high lipophilicity, many rings and few hydrogen-bond acceptors work well against *S. aureus*. Lipophilic molecules with big surface, low polarity, few rings and many hydrogen-bond donors are good inhibitors of *En. cloacae*. Bulky lipophilic molecules have high inhibitory activity against *L. monocytogenes*.

2.3.2.2. Models for MBC. QSAR models with $q^2 > 0.3$ regarding MBC were obtained only for *B. cereus*, *M. flavus* and *S. aureus*. Oval, lipophilic molecules with small surface and low polarity have bactericidal activity against *B. cereus*. Similarly, oval molecules with small surface, low polarity and few hydrogen-bond acceptors are bactericidal against *M. flavus*. The bactericidal activity against *S. aureus* has the same structural requirements as its inhibitory activity. Compounds with wide surface, low polarity, many rings and few hydrogen-bond acceptors have high MBC values.

Generally, the QSAR study on the compounds investigated here shows that lipophilic molecules with small surface and low polarity have good antimicrobial activities. However, the great variety of descriptors involved in the models are indicative for the absence of a uniform mechanism of antimicrobial action against the different bacteria.

3. Conclusion

According to obtained results from disc-diffusion and TLC-bioautographic methods it can be concluded that compounds **8a–8e** showed better antibacterial activity against Gram-positive bacteria. Compounds **9a–9e** exhibited higher activity against Gram-negative bacteria in both methods. All the compounds tested, **6a–9e** are more active against Gram-positive bacteria than Gram-negative bacteria in microdilution method. Compound **9d** exhibited the best antibacterial activity against all the bacteria tested with very low MIC and MBC, much lower than Ampicillin and almost 1.5 times lower in most cases than Streptomycin.

4. Experimental

4.1. Chemistry

All melting points were determined in an open capillary and are uncorrected. Elemental analyses were performed on a Carlo Erba 1108 machine Quoted values are ±0.4% of the theoretical ones.

IR spectra were recorded on Perkin–Elmer 237 spectrophotometer and the reported wave numbers are given in cm^{-1}. ^1H NMR spectra, were recorded in $CDCl_3$ solution on a Bruker Avance DPX 200 MHz spectrometer Chemical shifts are reported as δ (ppm) relative to TMS as internal standard.

The progress of the reactions as well as purity of compounds was monitored by thin layer chromatography with F_{254} silica-gel precoated sheets (Merck, Darmstadt, Germany) using benzene/ethanol 8/2 as eluent; UV light was used for detection.

*4.1.1. 2-Phenylamino-4,6-dichloro-s-triazine (**3**)*

Aniline **2** (0.93 g,0.01 mole) was added slowly to cyanuric chloride **1** (1.845 g,0.01 mole) in acetone (30 ml) with constant stirring for 4 h at 0–5 °C. Sodium carbonate solution (10%) was added to neutralize HCl evolved during the reaction. Finally, the contents were poured into crushed ice. The solid separated, was filtered, washed with water, dried and recrystallized from ethanol to give compound **3**. M.p 196 °C, yield 86%. IR: 690 (C–Cl), 774 (C–Cl), 1357 (C–N), 805 (C–N, *s*-triazine). 1H NMR ($CDCl_3$) δ ppm: 7.20–7.80 (m, 5 Ar–H and 1 NH).

*4.1.2. 2,4-bis-(phenylamino)-6-chloro-s-triazine (**4**)*

Aniline **2** (0.93 g, 0.01 mole) was added slowly to compound **3** (2.41 g, 0.01 mole) in acetone (35 ml) with constant stirring for 4 h at room temperature. Sodium carbonate solution (10%) was added to neutralize HCl evolved during the reaction. Finally, the contents were poured into crushed ice. The solid separated, was filtered, washed with water, dried and recrystallized from ethanol to give compound **4**. M.p 179 °C, yield 80%. IR (KBr) cm^{-1}: 772 (C–Cl), 1359 (C–N), 805 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 7.20–7.80 (m, 10 Ar–H and 2 NH).

*4.1.3. 2,4-bis-(phenylamino)-6-(4′-acetylphenylamino) s-triazine (**5**)*

4-Aminoacetophenone (1.35 g, 0.01 mole) and compound **4** (2.975 g, 0.01 mole) were dissolved in acetone (40 ml). The reaction mixture was refluxed for 6 h. Periodically sodium carbonate was added to neutralize HCl evolved during the reaction. Finally, the reaction mixture was cooled and poured into crushed ice. The solid separated, was filtered, washed with water, dried and recrystallized from alcohol to give compound **5**. M.p 218 °C, yield 75%. IR (KBr) cm^{-1}: 1662 (C═O), 1355 (C–N), 805 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 2.6 (s, 3H, $-COCH_3$), 7.0–7.95 (m, 14 Ar–H and 3 NH).

*4.1.4. 2,4-bis-(phenylamino)-6-[4′-{3″-(4‴-methoxyphenyl)-2″-propenon-1″-yl}-phenylamino]-s-triazine (**6e**)*

Compound **5** (3.96 g, 0.01 mole) was dissolved in DMF (30 ml) and 4-methoxybenzaldehyde (1.36 g, 0.01 mole) was added with constant stirring at room temperature. Then KOH solution (40 wt%) was added to reaction mixture which was stirred for 24 h at room temperature. Finally the reaction mixture was poured into crushed ice and neutralized with HCl. The product separated out, was

filtered, washed with water, dried and recrystallized from ethanol to give compound **6e**. Mp. 174 °C, yield 68%. IR (KBr) cm^{-1}: 1647 (C═O), 1595 (–CH═CH–, str.), 1340 (C–N), 812 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 3.95 (s, 3H, $–OCH_3$), 6.90 (d, 1H, –CO–CH═, $J = 15$ Hz), 7.15–7.80 (m, 18 Ar–H and 3NH), 8.05 (d, 1H, Ar–CH═, $J = 15$ Hz).

Similarly the remaining compounds **6a–d** were prepared.

*4.1.5. 2, 4-bis-(phenylamino)-6-[4′-{5″-(4‴-methoxyphenyl)-1″-acetylpyrazolin-3 ″-yl}-phenylamino]-s-triazine (compound **7e**)*

A mixture of compound **6e** (5.14 g, 0.01 mole) and hydrazine hydrate (0.5 g, 0.01 mole) in dioxane (25 ml) in presence of glacial acetic acid (15 ml) was refluxed for 6 h. The reaction mixture after cooling was poured into crushed ice and the product separated out was filtered, washed with water, dried and recrystallized from ethanol to give compound **7e**. Mp. 180 °C, yield 64%. IR (KBr) cm^{-1}: 1650 ($–COCH_3$), 1573 (C═N), 806 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 2.42 (s, 3H, $–COCH_3$), 3.15 (dd, 1H, $C_4–H_A$, $J_{AB} = 13.68$ Hz, $J_{Ax} = 4.60$ Hz), 3.65 (dd, 1H, $C_4–H_B$, $J_{AB} = 13.68$ Hz, $J_{Bx} = 6.68$ Hz), 3.80 (s, 3H, $–OCH_3$), 5.60 (dd, 1H, –CH–CH_2, $J_{Ax} = 4.60$ Hz, $J_{Bx} = 6.68$ Hz), 6.90–7.80 (m, 18 Ar–H and 3NH).

Similarly the remaining compounds **7a–d** were prepared.

*4.1.6. 2, 4-bis-(phenylamino)-6-[4′-{2″-amino-6″-(4‴-methoxyphenyl)-pyrimidine-4″-yl}-phenylamino]-s-triazine (**8e**)*

A mixture of compound **6e** (5.14 g, 0.01 mole) in alcohol (25 ml), guanidine nitrate (1.22 g, 0.01 mole) and 1–2 ml of 40% KOH solution was refluxed for 10 h. The reaction mixture was then cooled, was poured into crushed ice and the product separated out was filtered, washed with water, dried and recrystallized from ethanol to give compound **8e**. Mp. 147 °C, yield 66%. IR (KBr) cm^{-1}: 3398 ($–NH_2$), 1575 (C═N), 806 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 3.85 (s, 3H, $–OCH_3$), 5.10 (s, 2H, $–NH_2$), 6.90–8.15 (m, 19 Ar–H and 3NH).

Similarly the remaining compounds **8a–d** were prepared.

*4.1.7. 2, 4-bis-(phenylamino)-6-[4′-{2″-amino-3″-cyano-4″-(4‴-methoxyphenyl)-pyridine-5″-yl}-phenylamino]s-triazine (**9e**)*

A mixture of **6e** (5.14 g, 0.01 mole) in alcohol (40 ml), malononitrile (0.66 g, 0.01 mole) and ammonium acetate (1.54 g, 0.02 mole) was refluxed for 8 h. After cooling the reaction mixture was poured into crushed ice. The product separated out was filtered, washed with water, dried and recrystallized from ethanol to give compound **9e.** Mp. 115 °C, yield 69%. IR (KBr) cm^{-1}: 3396 ($–NH_2$), 2200 (C≡N), 806 (C–N, s-triazine). ^1H NMR ($CDCl_3$) δ ppm: 3.95 (s, 3H, $–OCH_3$), 5.28 (s, 2H, $–NH_2$), 6.90–8.20 (m, 18 Ar–H and 3NH).

Similarly the remaining compounds **9a–d** were prepared.

4.2. Microbiology

The following Gram-negative bacteria were used: *E. coli* (ATCC 35210), *P. aeruginosa* (ATCC 27853), *S. typhimurium* (ATCC 13311), *En. cloacae* (human isolate) and the following Gram-positive bacteria: *B. cereus* (clinical isolate), *M. flavus* (ATCC 10240), and *S. aureus* (ATCC 6538). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

The antibacterial assays were carried out by the disc-diffusion [40] and microdilution method [41–43] in order to determine the antibacterial activity of components against the human pathogenic bacteria.

The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 cfu/ml. The inocula were prepared daily and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

4.2.1. Disc-diffusion test

Compounds were investigated by the disc-diffusion using 4 mm filter discs. Bacteria were cultured overnight at 28 °C in LB medium and then adjusted with sterile saline to a concentration of 1.0×10^5 cfu/ml. The suspension was added to the top of agar (6 ml) and dissolved in Petri dishes (2 ml/agar plate) with solid peptone agar (Institute of Immunology and Virology, Torlak, Belgrade, Serbia). Filter discs with components (10.0 μg/disc) were placed on agar plates. After 24 h of incubation at 28 °C the diameter of the growth inhibition zones was measured. Streptomycin and Amplicillin were used as a positive control, and 10 μl was applied to the discs from stock solution (1 mg/ml). All tests were done in duplicate; replicates were done for each compound.

4.2.2. Bioautographic assay test

Ten microliters (10 μg/spot) of each sample were applied on TLC plates (Kieselgel 60 F254, Merck, Art. 5721) and sprayed with freshly prepared bacterial (1.0×10^5 cfu/ml) in nutrient broth (Tryptic Soy Broth; Biolife Italiana S.r.l., Milano-Italia). The plates were incubated for 18 h at 37 °C and then sprayed with aqueous sol. 3% of *p*-iodonitrotetrazolium violet [2-(4-iodophenyl)-3-(4-nitrphenyl)-5-phenyltetrazolium chloride; Sigma], stored for another 3 h and sprayed with 70% EtOH to stop bacterial and fungal growth. Streptomycin and Amplicillin were used as a positive control, and 10 μl was applied to TLC plates from stock solution (1 mg/ml). White inhibition zones on a pinkish background were indicative of antimicrobial activity of tested extracts. The widths of these zones (mm) are the measure of efficiency [44] .The activity was evaluated as the diameters of the inhibition zones with standard errors.

4.2.3. Microdilution test

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 cfu/ml. Compounds to be investigated were dissolved in broth LB medium (100 μl) with bacterial inoculum (1.0×10^4 cfu per well) to achieve the desired concentrations (5.0–30.0 μg/ml). The microplates were incubated for 24 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation of 2 μl into microtitre plates containing 100 μl of broth per well and further incubation for 72 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. Streptomycin and Amplicillin were used as a positive control (1 mg/ml DMSO). Two replicates were done for each compound.

4.3. QSAR study

4.3.1. Antimicrobial activity presentation

For the purpose of the QSAR study the antimicrobial activities obtained by microdilution method (MIC and MBC) were presented in *p*-values (negative log values). The range of both MIC and MBC was very narrow (less than one log unit).

4.3.2. Molecular descriptors

Two types of descriptors were used in the present study: continuous and discontinuous. The continuous descriptors were

generated by ACD/Labs 9.00 [45] and MDL QSAR 2.2 software [46]. Descriptors relating to molecular shape (*Ovality*, surface *MolSurf*, volume *MolVol*, refractivity *MolRef*, *Parahor*), electronic properties and polarity (polarity indices *Qs* and *Qv*, sum of absolute values of the charges on each atom of molecule *ABSQ*, sum of absolute values of the charges on the nitrogen and oxygen atoms in molecule *ABSQon*, dipole moment *D*, the largest positive charge on hydrogen atom *MaxHp*, the largest negative charge over the atoms in molecule *MaxNeg*, the largest positive charge over the atoms in molecule *MaxQp*, polarizability *Pol*, specific polarizabilities *SpcPol* (*Pol*/molecular weight), polar surface area *PSA*), lipophilicity ($logP_{ACD}$ and $logP_{MDL}$), and general molecular properties (number of rings in molecule *nrings*, number of graph circuits *ncirc*, Kappa flexibility index *phia*, number of hydrogen-bond acceptors *numHBa* and donors *numHBd*, free rotatable bonds *FRB*, Lepinski's *rule of 5*) were calculated and used in LFER models to relate the molecular structure of the compounds to their antimicrobial activities.

Discontinuous descriptors were generated to express the presence of a certain substituent or main structure in the molecule. These descriptors take 1 when the substituent is present and 0 when it is absent. The set of studied compounds consists of four main structures (chalcone, acetylpyrazoline, aminopyrimidine and cyanopyridine) and five substitutents (4-nitrophenyl, 4-chlorophenyl, 2-furanyl, 2-thienyl and 2-methoxyphenyl). Thus, nine discontinuous descriptors were generated and took part in Free-Wilson models.

4.3.3. Variable selection

Genetic algorithm (*GA*) was used as a variable selection procedure implemented in MDL QSAR 2.2 software [46]. *GA* allows one to select a subset of the most significant predictors using two evolutionary operations: random mutation and genetic recombination (crossover). The algorithm was applied with uniform crossover, one-point mutation and adjusted r^2 used as a fitness function. The regression equations were generated on the basis of the selected variables by multiple linear regression (*MLR*). The *GA* was used in both type QSAR models – Free-Wilson and LFER.

4.3.4. Multiple linear regression

The selected by *GA* variables were included in the QSAR models as independent variables. The antimicrobial activities of the compounds were the dependent variables. The matrices were processed by multiple linear regression (*MLR*) using MDL QSAR option *All possible subsets regressions*. The models were assessed by the explained variance r^2, standard error of estimate *SEE*, *F*-ratio and *p*-value. Leave-one-out cross-validation (*LOO-CV*) was applied and the predictive ability of the models was assessed by cross-validated q^2 and cross-validated residual sum of squares *cvRSS*. The best models in terms of r^2 with up to 5 variables were considered.

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