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Antimicrobial, Genotoxic and Acute Toxic Effects of Newly Synthesized *N*-Pyridine Schiff Base Derivatives

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ABSTRACT

Nine *N*-pyridine Schiff bases (1-9) that were synthesized by reacting different *ortho*-, *meta*- and *ortho-meta*-substituted salicylaldehyde and 3,5-substituted aminopyridine were evaluated for their antimicrobial activity. The acute *in vivo* toxicity and genotoxic activity of these compounds were also tested. All of the compounds exhibited antimicrobial activity against Gram-positive (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus* and *S. epidermidis*) and Gram-negative bacteria (*Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Proteus vulgaris* and *Salmonella typhimurium*) and against yeasts (*Candida albicans, C. glabrata, C. utilis, Pichia membranafaciens* and *Rhodotorula rubra*). The minimum inhibitory concentrations (MICs) of the compounds ranged from 0.95 to 1000 µg/ml. For the yeasts tested, the MICs ranged from 0.97 to 250 µg/ml. Compounds 1-6, which were substituted with CH_3, NO_2, Br and Cl, showed especially significant inhibitory activity against *C. albicans*, and the MICs of these compounds ranged from 0.97 to 7.81 µg/ml. An *in vivo* brine shrimp (*Artemia salina*) acute toxicity assay was used to determine the LD_{50} values of the test compounds. The LD_{50} (24 h) values of these compounds ranged from 31.64 to 94.9 µg/ml. Additionally, according to *umu*-test results, none of the tested compounds showed a genotoxic effect over a range from 10 to 5000 µg/ml.

INTRODUCTION

Schiff bases play important roles in the development of biochemistry. Schiff base derivatives are also very useful biochemical materials that display a broad range of biological activities, including antifungal, antibacterial (Pandeya *et al.*, 1999), antimalarial, antiproliferative, anti-inflammatory, antiviral, antipyretic (Dhar and Taploo 1982; Przybylski *et al.* 2009), antitumor (Mladenora *et al.* 2002; Walsh *et al.*, 1996) and antioxidant (Thangadurai *et al.* 2002) activities. Similarly, pyridine derivatives have been of great interest because of their roles in natural and synthetic organic chemistry. Many products that contain a pyridine subunit are biologically active and can exhibit antimicrobial (Sharma *et al.*, 2009; Patel and Shaikh 2010) and antituberculosis (Revanasiddappa *et al.*, 2010) activities. The acid dissociation constants of biologically active Schiff bases have also been reported to elucidate structure-reactivity relationships (Öğretir *et al.*, 2006).

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Based on the above-mentioned properties of Schiff bases and pyridine derivatives, we report here some of the biological activities of newly synthesized aminopyridine Schiff base derivatives (1-9). These compounds were screened for antimicrobial activity, acute toxicity and genotoxicity.

MATERIALS AND METHODS

Newly synthesized aminopyridine Schiff base derivatives

Nine *N*-pyridine Schiff bases (Table 1) were synthesized without the need base catalyst (Scheme 1) by Assoc. Prof. Dr. Hakan Dal, Department of Chemistry, Anadolu University, Eskisehir-Turkey. Stock solutions of compounds were prepared in dimethyl sulphoxide (DMSO; 2000 µg/ml) and stored dark, until use.

Antimicrobial activity

Indicator test strains such as *Bacillus cereus* NRRL-3711, *Citrobacter freundii* NRRL-2643, *Enterobacter aerogenes* NRRL-3567, *Proteus vulgaris* NRRL-123, *Salmonella typhimurium* NRRL-4420, *Staphylococcus epidermidis* NRRL-4377, *Candida albicans* NRRL Y-12983, *Candida glabrata* NRRL Y-1431,

Candida utilis NRRL Y-900, *Pichia membranafaciens* NRRL-2026 and *Rhodotorula rubra* NRRL-2505 were kindly obtained from United States Department of Agriculture, Agricultural Research Service (NRRL, Peoria, Illinois, USA).

Other test strains namely *Escherichia coli* LMG 8223 (ATCC 25922), *Listeria monocytogenes* LMG 13305, *Staphylococcus aureus* LMG 8224 (ATCC 25923) were purchased from Belgian Co-ordinated Collections of Microorganisms (BCMM, Gent, Belgium). MIC of the synthesized compounds were determined by the broth micro dilution method according to the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 2004). Two-fold serial dilutions of compounds were prepared with sterile distilled water to achieve a decreasing concentration range of 1000 to 0.97 µg/ml.

DMSO alone was used as negative control under the same conditions for each microorganism. Control antibiotics (Gentamicin sulphate and Amphotericin B) were dissolved in sterile distilled water and their serial dilutions were prepared. Each well (contained 100 µl test compound) was inoculated with 100 µl of the bacterial suspension (suspended in double-strength of Muller Hinton broth) at a density of 10^6 cell/ml and of the yeast suspension (suspended in double-strength of sabouroud dextrose broth) at a density of 10^5 cell/ml. The microtiter plates inoculated with bacteria were incubated for 24 h at 35°C, and the yeast cultures were incubated at 30°C for 48 h. The lowest concentration of the compounds that prevented visible growth of tested microorganism was treated as the MIC. The minimum bactericidal/fungicidal concentrations (MBCs/MFCs) were determined by sub-culturing 10 µl from each negative well and from the positive growth control samples. The MBC/MFC was defined as the lowest concentration yielding negative subcultures. All compounds were examined in duplicate in two separate experiments.

Acute toxicity

In vivo brine shrimp (*Artemia salina*) toxicity assay was used to determine cytotoxicity levels of the test compounds (McLaughlin *et al.* 1991). Brine shrimp eggs were hatched in artificial sea water prepared by dissolving 38 g of commercial sea salt in one liter of distilled water. After a 24 h incubation period at 25-26 ^oC, the larvae were attracted one side of the vessel with a light source and collected with a pipette. Test compounds were dissolved in DMSO and diluted with artificial sea water. Four concentrations (1000, 100, 10 and 1 µg/ml) of compounds were used in this assay. Test compounds of respective concentrations were added to Petri dishes (5 cm diameter). To each Petri dishes, 10 brine shrimp larvae were added and incubated 24 h at 25-26 ^oC. Petri dishes were filled with sea water and DMSO only, serving as a negative control. Survivors were counted using a magnifying glass. The experiments were repeated three times. Mortality data was transformed by Probit analysis in a Finney computer program (SPSS 17.0) to determine the lethal concentration to half of the test organisms (LD_{50}; in µg/ml) value.

Genotoxicity

In this study, genotoxicity of our test compounds were determined by SOS-*umu* test system. The bacteria strains used in the experiment: *Salmonella typhimurium*, NM2009 and NM3009 strains were kindly provided by Dr. Yoshimitsu Oda (Osaka Prefectural Institue of Public Health, Osaka, Japan). The test strains used in the *umu*-test are constructed by introducing a vector plasmid pACYC184 carrying only O-AT gene (NM2009) and both O-AT and NR genes (NM3009), into the parental strain *S. typhimurium* TA 1535/pSK1002 harboring umuC'- 'lacZ fusion gene (Oda *et al.,* 1992; Oda *et al.,* 1993; Oda *et al.,* 1995) The genetic markers of these tester strains have been described elsewhere (Quillardet and Hofsung 1985). Frozen permanent stocks of the test strains were prepared from overnight cultures to which DMSO was added as a cryoprotective agent. The cultures were stored at -70 ^oC and the genetic markers of the strains were checked before mutagenicity performance tests according to Ames *et al*. (1973).

The determination of cytotoxic effect: After incubating the chemical used in the *umu*-test with a bacteria culture, the dosages whose bacteria density is readable at optic density 600 (OD_{600}) and that decrease the density according to control are defined as cytotoxic. We determined the cytotoxic dosage of amide derivatives of benzothiazole in the same way just before starting the experiment (Oda *et al.,* 1995).

The determination of genotoxic effect: the *umu*-test was preformed as recommended by Oda *et al*. (1985) Overnight cultures of bacterial strains were prepared in Luria broth medium (1% Bacto tryptone, 0.5 % Bacto yeast extract and 1% NaCl) including ampicillin (25 µg/ml) and chloramphenicol (5 µg/ml). The overnight culture was diluted 50-fold with TGA medium (1% Bacto tryptone, 0.5% NaCl, 0.2% glucose and 20 µg/ml ampicillin) and was incubated at 37 ^oC until the absorbance at 600 nm of the bacterial culture reached 0.25-0.30. The number of viable bacteria was determined as described by Oda *et al*. (1985). That a concentration of 5-6 $x10^{10}$ cells/ml will correspond to 0.25-0.30 absorbance unit (OD_{600}). The bacterial culture obtained, was divided into 2 ml fractions, nine in total. Then, 0.9 ml of this culture was transferred into a sterile tube and 30 µl of Schiff bases were added (five doses and five different derivatives), Figure 1-3. After 5 hours of incubation at 37^oC with shaking, the bacterial density was determined by measuring A_{600}.

The β-galactosidase (β-gal) activity was measured by the method of Miller (1972). The bacterial cultures were divided into 0.2 ml of fractions and were diluted by adding 1.8 ml of Z buffer (16.1g $Na_2HPO_4.7H_2O$, 5.5 g $NaH_2PO_4.H_2O$, 0.75 g KCl, 0.25 g $MgSO_4.7H_2O$ and 2.7 ml of β-mercaptoethanol/l of distilled water, pH=7). The bacterial cell permeability to the chromogenic substrate was attained by adding two drops of chloroform and 50 µl of 0.1 % SDS and mixing. The enzyme reaction was initiated by the addition of 0.2 ml of 4 mg/ml ONPG solution. The reaction was terminated when color development reached a spectroscopically observable level by the addition of 1ml of 1 M Na_2CO_3 solution and the absorbencies were measured at 420 and

550 nm against a blank which consisted of TGA medium of bacterial culture. The level of enzyme activity in units was calculated according to the equation:

B-gal activity (U) = 1000 x (A_{420}-1.75xA_{550} / t. v. A_{600})

where **t** is the reaction time in minutes and **v** is the volume of culture used in the assay (ml).

At least a 2- fold or greater than 2-fold increase in β-gal activity above the control level was accepted as positive result (Oda *et al.*, 1985).

RESULTS AND DISCUSSION

Antimicrobial potential of the compounds

Nine *N*-pyridine Schiff bases were evaluated for their antimicrobial activity against Gram-positive and Gram-negative bacteria and against yeasts by the micro dilution method. The MIC and minimum bactericidal or fungicidal concentrations (MBCs/MFCs, µg/ml) were determined for each compound. The antimicrobial activity test results of these compounds are listed in Table 1. All of the compounds inhibited the growth of the tested microorganisms at varying concentrations. From the results in Table 1, we conclude that the test compounds have moderate to high antimicrobial activity.

The MICs of the compounds ranged from 0.95 to 1000 µg/ml. However, the compounds were effective against the tested yeasts at relatively lower MIC values than against the tested bacteria. While the MIC values of the test compounds against bacteria ranged from 1.95 to 1000 µg/ml, the MBC values ranged from 7.85 to 1000 µg/ml. Meanwhile, the MIC and MFC values of the test compounds against the tested yeasts were 0.97−250 µg/ml and 1.95−500 µg/ml, respectively. When compared with the reference drug Amphotericin B, compounds 1-6 exhibited considerable antifungal activity against *C. albicans*. Notably, compounds 1 (MIC = 0.97 µg/ml) and 4 (MIC = 1.95 µg/ml) were found to display the most promising antifungal activity against *C. albicans*.

Compound 1 bears a CH_3 moiety at position R2 of the pyridyl ring, and compound 4 bears CH_3 and NO_2 moieties at positions R2 and R3. Compounds 1, 5, 6, 8 and 9 were more active against Gram-negative bacteria (such as *E. aerogenes*, *E. coli* and *P. vulgaris*) than against Gram-positive species. However, only compound 6 (substituted with Br and CH_3) was more active for inhibiting *E. coli* than the reference antibacterial drug gentamicin.

Acute toxicity of the compounds

Evaluation of the toxicology and genotoxicity of any newly synthesized chemicals intended for use in antimicrobial chemotherapy is a crucial part of their assessment for potential hazards (Rodeiro *et al.*, 2006). In our study, an *A. salina* acute toxicity test was used to determine the LD_{50} values of the test compounds.

This test has been widely used for screening the toxicity of natural and synthetic organic compounds (Atta-Ur–Rahman *et al.*, 2001) because *A. salina* is highly sensitive to a variety of chemical substances (Latha *et al*, 2007). It has also been shown that *A. salina* toxicity test results correlate with rodent and human acute oral toxicity data (Calleja and Persoone, 1992; Parra *et al.*, 2001). The activity of each compound in brine shrimp lethality is listed in Table 2.

The LD_{50} (24 h) values of the test compounds ranged from 31.64 to 94.9 µg/ml. The highest LD_{50} value was obtained with compound 8, which was followed by compounds 9 and 7. The LD_{50} values of compounds 1-6 were 4- to 9-fold higher than their MIC values against *C. albicans*. From these data, it may be concluded that these compounds have potential for use as anticandidal agents.

Genotoxic potential of the compounds

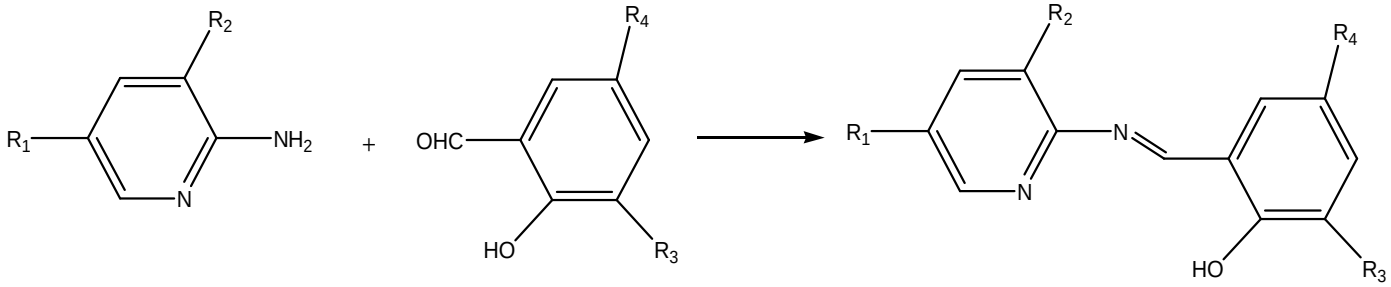
Genotoxicity testing is a crucial step for assessing the safety of newly synthesized compounds intended for use in humans. To determine the genotoxic potential of *N*-pyridine Schiff base derivatives, the *umu*-test system was performed as recommended by Oda *et al*. (1985). The *umu*-test is a quick screening test for evaluating the genotoxic effect of a large number of chemicals (Bagci, 1989).

Figure 1 shows the relative β-gal activity of 4-nitroquinolin 1-oxide (4NQO), a mutagen used as a positive control in the *umu*-test with *S. typhimurium* strains NM 2009 and NM 3009. As shown in Figures 2 and 3, none of the test compounds increased the β-gal activity of the either test strain by more than twofold. In other words, none of the compounds reacted with the macromolecular DNA of the *S. typhimurium* NM 2009 and NM 3009 strains to increase their DNA repair enzyme activity. On the basis of these results, it appears that none of the test compounds are defined as positive genotoxic agents at the tested concentration levels (10−5000 µg/ml).

CONCLUSIONS

The increasing resistance of human pathogens to current antimicrobial agents is a significant problem in modern healthcare. Resistance against antimicrobial agents develops quickly, even against synthetic compounds that bacteria have never previously encountered (Tsiodras *et al.*, 2001). Therefore, there is a real need for the development of new types of antimicrobial agents to treat patients infected with multidrug-resistant bacteria and fungi (Khan, et. al., 2009).

In this study, newly synthesized aminopyridine Schiff base derivatives were evaluated for their antimicrobial activity, acute toxicity and genotoxicity. The results suggest that compounds 1-6, substituted with CH_3, NO_2, Br and Cl, have promising anticandidal activity. Compound 1 (R2 substituted with CH_3), which has the simplest structure, is the most active agent against *C. albicans*. Furthermore, these compounds were neither toxic nor genotoxic at antimicrobially effective concentrations. In conclusion, these compounds have promising biological activities that warrant further assessment of their other biological activities and in vivo effects.



Scheme 1. General synthesis of compounds 1-9.

Table. 1: Nine *N*-pyridine Schiff bases tested for their biological activity in this study.

Compound No	Compound Code	R_1	R_2	R_3	R_4	Compound Name
1	701-3*	H	CH_3	H	H	2-[2-aza-2-(3-methyl-(2pyridly))vinyl] phenol
2	711-3*	H	CH_3	NO_2	H	2-[2-aza-2-(3-methyl-(2-pyridly))vinyl]-6-nitrophenol
3	721-3*	H	CH_3	H	NO_2	2-[2-aza-2-(3-methyl-(2-pyridly))vinyl]-4-nitrophenol
4	731-3*	H	CH_3	NO_2	NO_2	2-[2-aza-2-(3-methyl-(2-pyridly))vinyl]-4,6-dinitrophenol
5	742-5	CH_3	H	H	Cl	2-[2-aza-2-(5-methyl-(2-pyridly))vinyl]-4-chlorophenol
6	743-5	CH_3	H	H	Br	2-[2-aza-2-(5-methyl-(2-pyridly))vinyl]-4-bromophenol
7	350	Cl	H	H	H	2-[2-aza-2-(5-chloro-(2-pyridly))vinyl]phenol
8	352	Cl	H	H	Cl	2-[2-aza-2-(3- chloro-(2-pyridly))vinyl]-4-chlorophenol
9	353	Cl	H	H	Br	2-[2-aza-2-(5-chloro-(2-pyridly))vinyl]-4-bromophenol

*Öğretir *et al*., 2006.

Table 2. MIC, MBC/MFC (in brackets) and LD_{50} values of test compounds.

Microorganisms	MIC and MBC/MFC of test compounds and control antibiotics (µg/ml)										
	1	2	3	4	5	6	7	8	9	Gentamicin	Amphotericin B
B. cereus	125 (500)	500 (1000)	500 (1000)	250 (500)	500 (1000)	500 (1000)	500 (>1000)	250 (>1000)	250 (>1000)	2.50 (5.0)	NT
C. freundii	250 (500)	500 (1000)	500 (1000)	500 (1000)	500 (1000)	500 (1000)	125 (250)	125 (250)	250 (500)	1.25 (5.0)	NT
E. aerogenes	125 (500)	500 (1000)	500 (1000)	500 (1000)	125 (1000)	250 (1000)	500 (1000)	62.5 (500)	125 (500)	0.62 (5)	NT
E. coli	62.5 (125)	500 (1000)	500 (1000)	500 (1000)	250 (500)	1.95 (7.81)	500 (500)	250 (>1000)	500 (>1000)	2.5 (5.0)	NT
L. monocytogenes	125 (500)	500 (1000)	500 (1000)	500 (1000)	125 (500)	250 (500)	>1000 (>1000)	250 (>1000)	125 (>1000)	5.0 (5.0)	NT
P. vulgaris	62.5 (1000)	500 (1000)	500 (1000)	500 (1000)	62.5 (500)	125 (500)	500 (500)	500 (>1000)	500 (>1000)	1.25 (5.0)	NT
S. aureus	500 (1000)	1000 (>1000)	1000 (>1000)	500 (1000)	500 (1000)	250 (500)	1000 (>1000)	500 (>1000)	500 (>1000)	2.50 (20.0)	NT
S. epidermidis	500 (1000)	500 (1000)	500 (1000)	500 (1000)	500 (1000)	500 (1000)	125 (500)	250 (1000)	250 (1000)	5 (20)	NT
C. albicans	0.97 (1.95)	3.90 (7.81)	3.90 (7.81)	1.95 (3.90)	3.90 (7.81)	7.81 (31.25)	125 (250)	31.25 (31.25)	31.25 (31.25)	NT	0.31 (0.63)
C. glabrata	125 (250)	62.5 (125)	62.5 (125)	125 (250)	125 (250)	125 (250)	250 (500)	62.5 (125)	125 (250)	NT	0.16 (0.31)
C. utilis	62.5 (125)	125 (500)	125 (500)	250 (500)	1.95 (3.90)	3.90 (7.81)	31.25 (31.25)	15.63 (31.25)	15.63 (31.25)	NT	0.16 (0.31)
P. membranifaciens	250 (500)	125 (500)	125 (500)	0.97 (1.95)	250 (500)	250 (500)	62.5 (125)	31.25 (62.5)	62.5 (125)	NT	0.31 (0.63)
R. rubra	62.5 (250)	125 (250)	125 (250)	62.5 (250)	62.5 (250)	31.25 (250)	31.25 (125)	62.5 (125)	15.63 (62.5)	NT	0.16 (0.31)
LD_{50} values	69.02	36.71	33.81	33.41	36.30	31.64	78.27	94.90	83.82		

NT; not tested

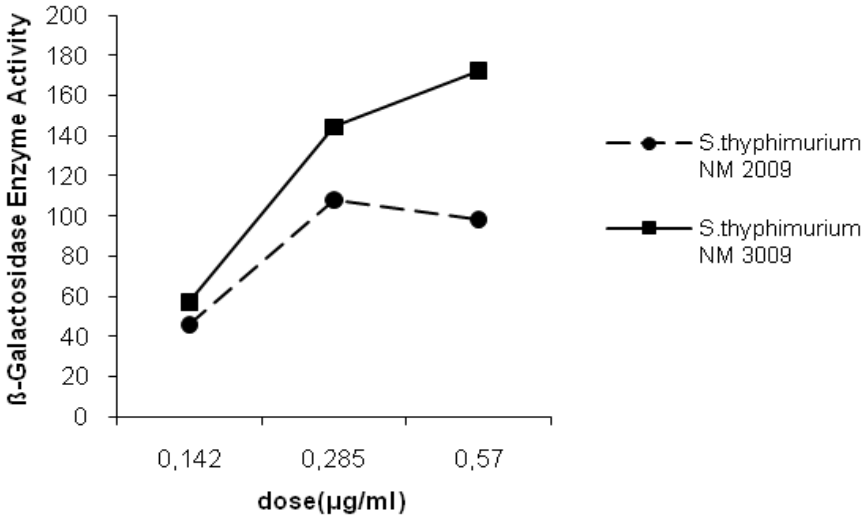


Fig 1. Relative *β*-galactosidase activity in the *umu*-test using 4NQO as a positive control mutagen with *S. typhimurium* strains NM 2009 and NM 3009.

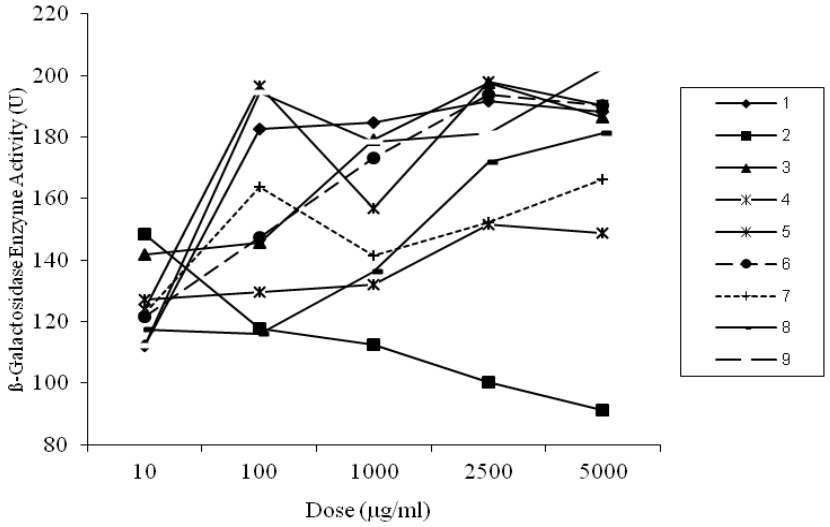


Fig 2. Relative *β*-galactosidase activity of tested Schiff base derivatives in the *umu*-test with *S. typhimurium* strain NM 2009.

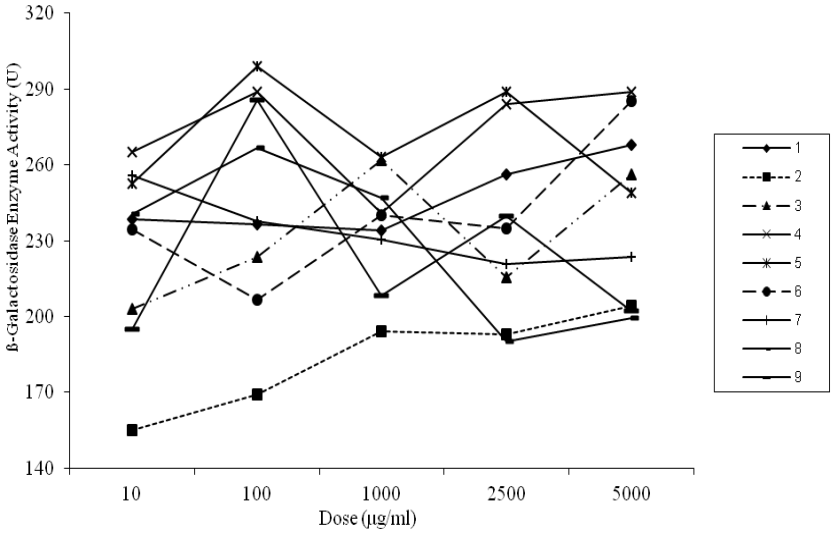


Fig 3. Relative *β*-galactosidase activity of tested Schiff base derivatives in the *umu*-test with *S. typhimurium* strain NM 3009.

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