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Short communication

Synthesis, determination of the lipophilicity, anticancer and antimicrobial properties of some fused 1,2,4-triazole derivatives

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Abstract

3-Unsubstituted and 3-substituted-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazoles (**1**−**14**) were designed and obtained from biologically active 1-aryl-2-hydrazonoimidazolidines by cyclocondensation reaction with triethyl orthoformates (**1**−**4**), phenoxyacetic acid derivatives (**5**−**13**) and carbon disulfide (**14**), respectively. Their chemical structures were confirmed by IR, ^{1}H NMR, ^{13}C NMR, MS spectra and elemental analysis. In the high performance liquid chromatographic series of experiments, fourteen synthesized compounds (**1**−**14**) were chromatographed on octadecyl silica adsorbent and their lipophilicity parameter (log k_W) was determined using various aqueous systems: mixture of water and organic modifiers (methanol − MeOH, acetonitrile − MeCN or dioxane − DX). Compounds **7** and **12** were evaluated for their cytotoxic activity against three cancer cell lines: human Caucasian colon adenocarcinoma cell line − LS180 (ECACC 87021202), human uterus carcinoma cell line − SiHa (ECACC 85060701) and human breast carcinoma cell line − T47D (ECACC 85102201). Compound **12** was found to be the most effective in vitro against human colon adenocarcinoma cell line (LS180). Moreover, the distinctly marked lower cytotoxicity of compounds **7** and **12** against the normal cell line − human skin fibroblasts (HSF) and almost several-fold higher against the examined cancer cell lines was ascertained. The cytotoxic effect of imidazotriazole **7** was noticed on DNA structure of breast cancer cell line (T47D) by using the comet assay. Compound **7** in concentration of 29.3 μM was found to possess efficiency for DNA strand breakage. In particular, this led to cutting of the DNA strands and formation of small fragments of DNA − two higher and one lighter in comparison with control DNA. Moreover, significant viability decreases in the human leukaemic RPMI 8226 cells treated with different concentrations of imidazotriazoles **8**−**12** were observed, suggesting their antiproliferative properties. Besides, three tested compounds (**9**, **13**, **14**) revealed significant antimicrobial activities with MIC values in the range of 30.9−44.0 μM. Compound **13** showed superior antibacterial activity to ampicillin and chloramphenicol in vitro, whereas **14** displayed superior antifungal activity to miconazole.

Keywords: Imidazo[2,1-*c*][1,2,4]triazoles; Structure elucidation; Lipophilicity; Cytotoxicity; Anticancer activity studies; The comet assay; A novel DNA strand breaking agent; Human colon adenocarcinoma cells (LS180); Human uterus cancer cells (SiHa); Human breast cancer cells (T47D); Human cervix epitheloid carcinoma cells (HeLa); Human leukaemic RPMI 8226 cells; Antibacterial activity; Antimycotic activity

1. Introduction

There exist a number of [1,2,4]triazoles having a wide range of pharmacological activities. The following [1,2,4]triazole derivatives are applied in medicine: alprazolam (tranquilizer), estazolam (hypnotic, sedative, tranquilizer), rilmazafon

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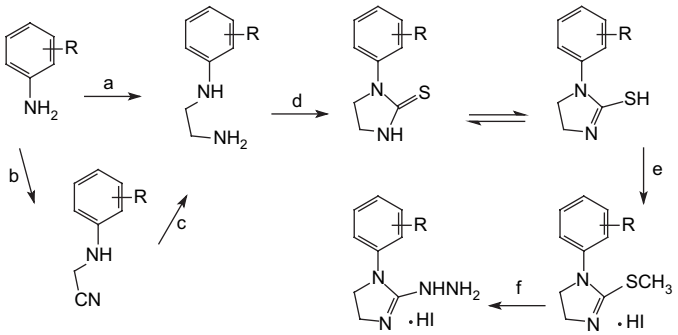
(hypnotic, anxiolytic, used in the case of neurotic insomnia), benatradin (diuretic), trapidil (hypotensive), trazodon (antidepressant, anxiolytic), etoperidone (antidepressant), nefazodone (antidepressant, 5-HT_2 A-antagonist), anastrozole, letrozole, vorozole (antineoplastics, nonsteroidal competitive aromatase inhibitors), ribavirin (the potent antiviral N-nucleoside), fluconazole, itraconazole, terconazole (the powerful azole antifungal agents) [1].

It follows from the literature survey that, depending on the type of substituent, the derivatives of [1,2,4]triazole have a high potential for biological activity, possessing a wide range of antimicrobial [2–5] and antitumour [6–9] properties. The other ones show also anti-inflammatory [10], antihypertensive [11], anticonvulsant and antiviral [12], analgesic [13] activities. Our previous studies concerning bridgehead nitrogen-heterocyclic compounds obtained by fusion of the 4,5-dihydroimidazole and [1,2,4]triazole nuclei, have identified one compound containing the methylthio group at position 3 and with a 4-methylphenyl substituent at position 7 (e.g., 7-(4-methylphenyl)-3-methylthio-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole) with a significant antibacterial activity. This heterobicycle was strongly active against *Staphylococcus aureus* ATCC 25923, with a MIC value of 31.7 μM and showed superior antibacterial activity to ampicillin [14]. On the other hand, a comprehensive literature search showed that some derivatives having the same heterocyclic skeleton, e.g., 7-aryl-5-methyl-3-thioloimidazo[2,1-*c*][1,2,4]triazol-6-ones have been reported as possible antifungal agents. These bioactive compounds exhibited fungicidal action almost equivalent to that of Mancozeb (Dithane M-45) at 1000 ppm concentration and inhibited the growth of *Aspergillus niger* and *Fusarium oxysporum* by more than 48 and 47%, respectively, even at 10 ppm concentration [15].

Prompted by these reports, and in continuation of our search for bioactive molecules [14,16–19] and imidazo-fused heterocycles revealing promising anticancer activities [20,21], it seemed worthwhile to synthesize some novel biheterocyclic derivatives containing the dihydroimidazole and [1,2,4]triazole nuclei as possible compounds of high biological potency. We report herein the synthesis, determination of the lipophilicity of 3-unsubstituted and 3-substituted-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole derivatives and testing results of possible antitumour and antimicrobial active 3-phenoxymethyl and 3-thiolo-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*]-[1,2,4]triazole derivatives.

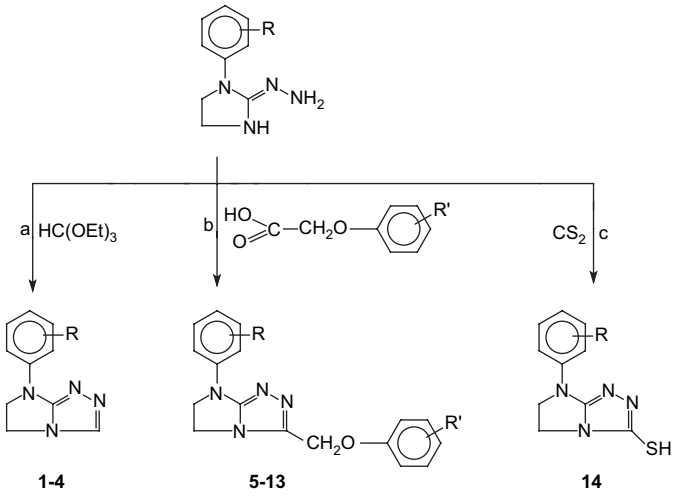
2. Chemistry

The starting biologically active 1-aryl-2-hydrazonoimidazolidines (1-aryl-2-hydrazinoimidazolines), used in this study were prepared by patent pending according to Sztanke from 1-arylimidazolidine-2-thiones by a previously reported method [16,19]. The four-step synthetic pathway for the preparation of starting hydrazones was achieved by a sequence of reactions starting from the respective anilines and is outlined in Scheme 1.



Scheme 1. Synthetic pathway for the preparation of starting 1-aryl-2-hydrazinoimidazolines. Reagents and conditions: (a) aziridine, $AlCl_3$, dry toluene, (b) HCHO, $Na_2S_2O_5$, NaCN, water, reflux; (c) H_2, NiRa, MeOH–NH_3, 100 °C; (d) CS_2, xylene, rt, 20 min, reflux, 7 h; (e) CH_3I–MeOH, rt, 48 h, reflux, 6 h; (f) hydrazine hydrate–MeOH, reflux, 24 h.

Thus in the first step, commercially available anilines were converted into *N*-arylethylenediamines by the Lehmann method [22] or by the classical Knoevenagel and Mercklin method with its further Takeda modification [23,24]. Their further condensation with carbon disulfide in the xylene medium led to the formation of intermediates – dithiocarbaminic acid derivatives, which could easily be cyclized in boiling solvent under reaction conditions to 1-arylimidazolidine-2-thiones (1-aryl-2-mercaptoimidazolines) with concomitant loss of hydrogen sulfide molecule. Because of existence of thiol–thione tautomerism, the alkylation of respective thiols with one equivalent methyl iodide was possible and afforded 1-aryl-2-methylthioimidazolines in 75–85% yields [14], these in turn were refluxed with hydrazine hydrate to obtain 1-aryl-2-hydrazinoimidazolines in good yields (65–76%). Finally these compounds were used as the starting materials in the synthesis of reported herein imidazotriazole derivatives (**1**–**14**) (Scheme 2).



Scheme 2. Synthetic route to obtain final imidazotriazoles of the type **1**–**14**. Conditions: (a) DMF, reflux, 6 h; (b) DMF, reflux, 6 h, NaOH (6%); (c) MeOH, NaOH–water, rt, 30 min, reflux (water bath), 14 h. R and R′ as shown in Table 1.

Cyclocondensation of 1-aryl-2-hydrazonoimidazolidines with triethyl orthoformate or derivatives of phenoxyacetic acid in refluxing DMF afforded the corresponding 3-unsubstituted (**1**–**4**) or 3-phenoxymethyl (**5**–**13**) 7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazoles, after a ring closure with concomitant loss of ethanol or hydrogen oxide molecule, respectively. Also, boiling of 1-(4-chlorophenyl)-2-hydrazonoimidazolidine with CS_2 in aqueous KOH resulted in 7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole-3-thiol (**14**) in 76.1% yield with concomitant loss of hydrogen sulfide molecule.

The majority of final products were obtained as previously described [17–19], with the exception of compounds **4**, **10**–**12** whose synthesis has not been described in the literature as yet. These compounds were generated according to the synthetic method described by Sztanke et al. [17,18] with its further modification. Thus, the highest yields for final imidazotriazole derivatives were achieved, when the reaction could be carried out starting from the free bases of 1-aryl-2-hydrazonoimidazolidine, instead of their hydroiodide salts. In this way improved yield values for compounds **1**–**3**, **5**–**9** and **13**–**14** were obtained. The physicochemical, infrared (IR) data and ^{1}H NMR, ^{13}C NMR resonances and mass spectroscopic analyses (EIMS) of the compounds synthesized are presented in Section 7.

A general synthesis of final imidazo[2,1-*c*][1,2,4]triazole derivatives is shown in Scheme 2.

In view of continuous and widespread interest in the design and the synthesis of novel heterocyclic derivatives containing a [1,2,4]triazole moiety, the synthetic approach leading to the formation of biheterocyclic dihydroimidazo[2,1-*c*][1,2,4]triazole derivatives, described by us, might be considered as a useful method for the preparation of these biologically active compounds because of the affordability of the starting materials, good yields obtained and straightforward product isolation.

NMR spectral characteristic of derivatives of the biheterocyclic, polynitrogenated dihydroimidazotriazole system (**1**–**5**, **10**–**14**), revealed in their ^{1}H NMR spectra the two double-doublet signals of the H5 and H6 at ca. 4.34 and 4.58 ppm, with the coupling constants of $J \sim 7.3$ Hz, $J' \sim 6.1$ Hz, respectively, or multiplet signals in the range of 3.77–4.55 ppm in the spectra of compounds **6**–**9**. The difference between chemical shifts of both signals can suggest an important difference in the acidity of hydrogen atoms, which was confirmed by the chemical shift values of respective carbon atoms in their ^{13}C NMR spectra (ca. 39.8 ppm for the C-5 and 53.3 ppm for the C-6). Apart from the signals of aromatic hydrogen atoms observed at aromatic region (6.97–7.65 ppm), the two additional singlet signals: first from the methylene protons derived from the phenoxymethylene formation (–C*H*$_2$OPh) and second from the triazole-C*H* were observed at ca. 5.26 ppm in the ^{1}H NMR spectra of compounds of the type **5**–**13** and at about 8.18 ppm in the ^{1}H NMR spectra of compounds **1**–**4**, integrating for two protons and one proton, respectively. The signals belonging to the methylene secondary carbon atoms derived from the phenoxymethylene formation were seen at 60.8, 60.9, 60.8, 60.9, 60.8, 60.9, 61.7, 61.9 ppm in the ^{13}C NMR spectra of compounds **6**–**13**, respectively. Also, both quaternary triazole-C-3 and C-7a carbon atoms signals were recorded at ca. 138.1 ppm and 159.3 ppm, respectively, in their ^{13}C NMR spectra.

The IR spectra confirmed the structure of the compounds investigated. The examined compounds can be recognized by the presence of specific absorption bands of the C=N moiety in the range of 1607–1619 cm^{-1}. The absence of any N–H characteristic bands in the shorter wavelength region (ca. 3400 cm^{-1}) suggests their cyclic structure.

EI mass spectra (EIMS) of compounds **1**–**5** and **8**–**12** provided their molecular ion peaks at *m*/*z*: 186, 216, 220, 254, 292, 340, 354, 360, 374 and 394, respectively, with intensities that varied from 4.5 to 100% confirming their molecular weights. The molecular ion for compounds **3**, **4**, **8**–**12** was calculated according to ^{35}Cl isotope. The data are presented in Section 5.

3. Lipophilicity results

In the high performance liquid chromatographic (HPLC) series of experiments, fourteen derivatives of 3-(un)substituted-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole (Table 1) were chromatographed on octadecyl silica adsorbent (SUPELCOSIL™ LC-18) using various aqueous systems: mixtures of water and organic modifiers (methanol – MeOH, acetonitrile – MeCN or dioxane – DX). Diethylamine (DEA) and phosphate buffer (pH = 3.5) were added to mobile phase (eluent containing 0.05 ML^{-1} DEA in organic modifier (MeOH, MeCN or DX) – buffered mobile phase).

The investigated compounds, such as heterocyclic bases, e.g., alkaloids are difficult for chromatographic analysis.

Since the investigated compounds appear in solutions as ionized and unionized forms, they are difficult for chromatographic separation. Mobile phase pH is a major factor for the separation of analytes with acid–base properties [25–29].

Retention models of ionizable solutes in liquid chromatography with a function of pH and solvent composition were described by Soczewiński [30,31] and reviewed by Schoenmakers and co-workers [32]. The theory of RP retention for ionic compounds with a function of pH assumes that a given solute exists in ionized and non-ionized forms and interacts by two different modes with chromatographic system components.

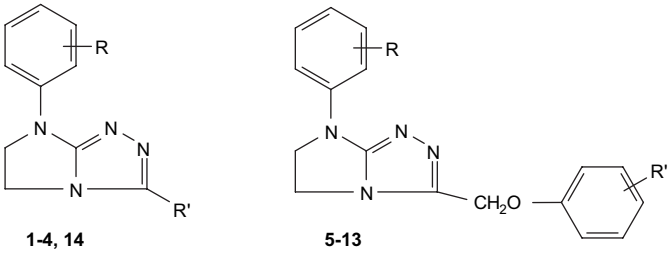
Basic analytes can interact with silanol groups of silica matrix, which can remain underivatised on bonded RP stationary phases [33]. Silica is also the most widely used material in chromatography. Silica supports are still superior to other supports in terms of efficiency, rigidity and performance.

Protonated basic compounds can interact with residual silanol groups of the stationary phase, as shown in the equation:

$$XH^+ + SiO^-Na^+ \leftrightarrow Na^+ + SiO^-XH^+$$

Thus, besides the reversed phase retention mechanism an ion-exchange retention mechanism also occurs, which often

Table 1
Chemical structures of 3-(un)substituted-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazoles whose lipophilicities were determined



No.	Compound	R	R′	pK_{a1}	pK_{a2}
1	7-Phenyl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	H	H	5.00	3.02
2	7-(4-Methoxylphenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4] triazole	4-OCH_3	H	3.07	−4.16
3	7-(4-Chlorophenyl)- 5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4] triazole	4-Cl	H	2.97	–
4	7-(3,4-Dichlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	3,4-Cl_2	H	2.91	–
5	3-Phenoxymethyl-7-phenyl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	H	H	2.95	2.03
6	3-(4-Chlorophenoxymethyl)-7-(4-methoxyphenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-OCH_3	4-Cl	2.97	−4.34
7	3-[(2-Methyl-4-chloro)phenoxymethyl]-7-phenyl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	H	2-CH_3; 4-Cl	2.94	2.03
8	3-(4-Chlorophenoxymethyl)-7-(4-methylphenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-CH_3	4-Cl	2.95	−4.30
9	3-[(2-Methyl-4-chloro)phenoxymethyl]-7-(4-methylphenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-CH_3	2-CH_3; 4-Cl	2.96	−4.28
10	3-(4-Chlorophenoxymethyl-7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-Cl	4-Cl	2.87	–
11	3-[(2-Methyl-4-chloro)phenoxymethyl]-7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-Cl	2-CH_3; 4-Cl	2.88	–
12	3-(2,4-Dichlorophenoxymethyl)-7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	4-Cl	2,4-Cl_2	2.85	–
13	3-(2,4-Dichlorophenoxymethyl)-7-(3,4-dichlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole	3,4-Cl_2	2,4-Cl_2	2.79	–
14	7-(4-Chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole-3-thiol	4-Cl	SH	2.74	–

results in asymmetry of peaks, irreproducible retention and worse separation.

Strong silanophilic interactions that can occur with these analytes and the adsorbent surface can lead to poor peak shape. In systems containing solely mixtures of water and organic modifier the peaks of chromatographed derivatives were tailing and asymmetric. Silanol interaction can be reduced, e.g., in chromatography of basic compounds by addition of a more basic compound, which interacts more strongly with residual silanols, allowing the less basic compound to interact solely with the alkyl ligand of the stationary phase [34]. Silanol interaction can be also reduced by using mobile phase of a high pH (>7.0), when the ionization of some determined basic compounds is suppressed [35]. Diethylamine (DEA) was added as silanol blocker to the eluent. To protect the stationary phases, buffered mobile phases were applied.

The pK_a values of investigated compounds have been evaluated by Pallas 3.1.1.2 software [36], and these have been used to choose optimal composition of mobile phase. The structures and pK_a values of the above mentioned compounds are shown in Table 1. In the structure of investigated compounds, there are basic nitrogen atoms in positions 4 and 7 of heterobicycle for which the pK_{a1} values are in the range of 2.74−5.00. The pK_{a2} values of the compounds lie between −4.34 and 3.02. The pK_a values indicate that the analytes are very weakly basic. The pH values of used mobile phases after addition of diethylamine and acetate buffer at pH = 3.5 were higher than the $pK_{a\ basic\ analyte} + 1.5$ (lies between 7.5 and 10) so that the basic investigated compounds were completely unionized. In addition, diethylamine caused blocking of residual silanol groups. Owing to application of ion suppression of analytes and blocking of free silanols the peak shape of chromatographed derivatives are improved.

The experimental data obtained for reversed phase HPLC systems: the intercepts ($\log k_W$), slopes (S), regression coefficients (r), F statistic and standard errors (s) calculated for the 95% confidence level are presented in Table 2. The $\log k_W$ value is widely known as a lipophilicity parameter and is often used for QSAR studies. The $\log k_W$ values of the compounds increase with addition of non-polar groups for reversed phase chromatographic systems, for example, the addition of non-polar substituents, e.g., −Cl [37]. Comparison of the $\log k_W$ values for the investigated derivatives, in general, indicates that the highest lipophilicity was observ ed for compounds with 4-Cl and 3,4-Cl_2 substituents. Compound **13** had the highest $\log k_W$ values in aqueous systems with methanol and dioxane. The influence was by the −Cl substituent at R and the values of $\log k_W$ increased generally in all RP chromatographic systems (**5**−**13**) in the order R = 4-Cl and R′ = −H < R = 4-Cl and R′ = 4-Cl < R = 4-Cl and R′ = 2-CH_3, 4-Cl < R = 4-Cl and R′ = 2,4-Cl_2 < R = 3,4-Cl_2 and R′ = 2,4-Cl_2.

Fig. 1 presents the correlation between $\log k_W$ values, determined in dioxane−water system, and $\log P$ values calculated by using Pallas 3.1.1.2 software [36]. The values of $\log P$ of the solutes **8**, **10**−**13** increased generally with additional substituent (4-CH_3 or 4-Cl), especially with R = 3,4-Cl_2 and R′ = 2,4-Cl_2. The solutes **8**, **10**−**13** had $\log P$ values higher with additional substituent (−CH_3 or −Cl), corresponding with their higher values of $\log k_W$.

Attempts to correlate the chromatographic lipophilicity parameters with $\log P$ values calculated with some computer programs failed; apparently, the structures of the compounds

Table 2
Terms of the equation $\log k = \log k_W - S\varphi$ for the compounds investigated on an octadecyl silica column (LC-18) with aqueous mobile phases

No.	$\log k_W$	S	r	F	s	n
DX–H_2O–20% buffer pH 3.5 containing 0.05 M L^{-1} DEA						
1	1.16 ± 0.09	4.43 ± 0.36	0.9900	148.11	0.1041	5
2	1.22 ± 0.08	4.67 ± 0.33	0.9927	204.56	0.0934	5
3	1.89 ± 0.09	4.88 ± 0.31	0.9922	252.76	0.1198	6
4	2.49 ± 0.08	5.57 ± 0.25	0.9970	495.38	0.0792	5
5	1.78 ± 0.11	4.43 ± 0.46	0.9894	92.96	0.1233	4
6	1.52 ± 0.13	6.81 ± 0.98	0.9898	48.11	0.1060	3
7	1.17 ± 0.09	4.43 ± 0.37	0.9900	146.24	0.1050	5
8	3.55 ± 0.20	6.48 ± 0.49	0.9971	173.58	0.0696	5
9	1.10 ± 0.05	4.45 ± 0.23	0.9987	385.37	0.0320	3
10	3.41 ± 0.19	5.84 ± 0.36	0.9944	264.33	0.1137	5
11	3.87 ± 0.21	6.40 ± 0.41	0.9939	245.10	0.1294	5
12	4.15 ± 0.21	6.67 ± 0.41	0.9945	269.28	0.1285	5
13	4.61 ± 0.16	6.39 ± 0.25	0.9978	667.43	0.0596	5
14	−0.09 ± 0.07	3.60 ± 0.30	0.9896	141.86	0.0865	5
MeCN–H_2O–20% buffer pH 3.5 containing 0.05 M L^{-1} DEA						
1	1.37 ± 0.10	4.78 ± 0.41	0.9891	135.67	0.0987	5
2	1.56 ± 0.11	5.54 ± 0.44	0.9906	157.68	0.1062	5
3	2.70 ± 0.17	8.01 ± 0.66	0.9900	148.41	0.1585	5
4	2.11 ± 0.27	4.52 ± 0.67	0.9785	45.04	0.1993	4
5	1.37 ± 0.11	4.78 ± 0.42	0.9888	131.41	0.1005	5
6	1.54 ± 0.13	5.37 ± 0.50	0.9873	116.23	0.1201	5
7	1.36 ± 0.11	4.74 ± 0.43	0.9880	122.93	0.1029	5
8	3.04 ± 0.26	5.10 ± 0.57	0.9879	81.22	0.1266	5
9	1.00 ± 0.16	3.87 ± 0.61	0.9644	36.05	0.1891	4
10	3.23 ± 0.23	5.26 ± 0.47	0.9921	124.34	0.1125	4
11	3.70 ± 0.21	5.81 ± 0.43	0.9945	181.42	0.1029	4
12	3.75 ± 0.25	5.81 ± 0.53	0.9918	120.22	0.1264	4
13	3.32 ± 0.25	3.76 ± 0.35	0.9873	116.14	0.0551	5
14	–	–	–	–	–	–
MeOH–H_2O–20% buffer pH 3.5 containing 0.05 M L^{-1} DEA						
1	1.62 ± 0.10	3.42 ± 0.23	0.9910	219.31	0.1115	6
2	1.87 ± 0.13	3.93 ± 0.28	0.9876	197.26	0.1481	7
3	2.19 ± 0.09	3.71 ± 0.17	0.9968	469.18	0.0542	5
4	2.92 ± 0.08	4.38 ± 0.15	0.9983	860.76	0.0472	5
5	1.62 ± 0.08	3.42 ± 0.19	0.9927	340.18	0.0982	7
6	1.88 ± 0.12	3.96 ± 0.28	0.9879	202.49	0.1471	7
7	1.62 ± 0.08	3.43 ± 0.19	0.9926	331.89	0.0995	7
8	4.27 ± 0.19	5.37 ± 0.26	0.9965	422.80	0.0826	5
9	1.58 ± 0.03	3.21 ± 0.07	0.9991	2112.1	0.0292	6
10	4.38 ± 0.19	5.49 ± 0.27	0.9964	416.66	0.0851	5
11	4.61 ± 0.20	5.56 ± 0.27	0.9977	439.00	0.0594	4
12	4.85 ± 0.16	5.72 ± 0.21	0.9986	722.13	0.0476	4
13	6.00 ± 0.16	6.77 ± 0.23	0.9983	874.52	0.0362	5
14	–	–	–	–	–	–

investigated are too complex for the programs available. The computer program [36] cannot discern different positions: *ortho*, *meta* and *para* of −Cl substituent. The solutes **7** and **9**, belonging to the examined 3,7-disubstituted imidazotriazoles, which have log *P* relatively much higher than the corresponding log k_W can be given as example. The solutes **1**–**4**, **14** lacking $-CH_2OPh$ formation at R′ have the lowest values of log *P* and the values of log *P* corresponding with their small values of log k_W. The regression equation for compounds **1**–**14** was $\log P = 0.6146 \log k_W + 2.382$ and the correlation coefficient was found to be $r = 0.6707$.

The lipophilicity of the compounds imperceptibly increases with the $-OCH_3$ group in reversed phases systems (relative value of *k* is 1.1 for 30% MeCN [37]), e.g., solute **6**.

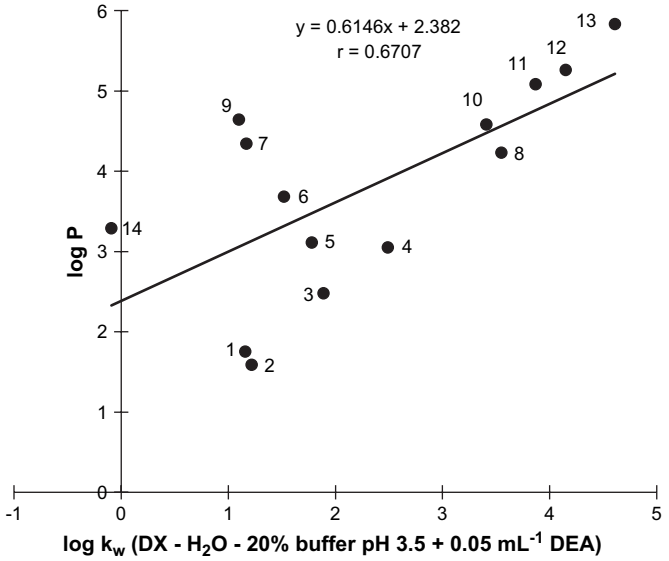


Fig. 1. Correlation between log k_W values for reversed mobile phases (dioxane–water − 20% buffer pH 3.5 containing 0.05 M L^{-1} DEA) on the octadecyl silica column (LC-18) and log *P* values calculated by using Pallas 3.11.2 software.

The high values of *r* ($r > 0.9644$) prove the excellent fit between experimental data and the logarithmic equation. The values of the *F* statistic are always higher than the critical value *F*, thus proving additionally a good fit of the data with the examined model.

4. Pharmacology

4.1. Antitumour screening studies

The newly synthesized compounds **7**, **12** were evaluated for their anticancer activity towards human tumour cell lines derived from various human cancer types (colon, breast, uterus): LS180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701, uterus cancer cells), T47D (ECACC 85102201, human breast carcinoma cells). Furthermore, both normal cell lines were included in the cytotoxicity study: HSF (human skin fibroblast) cells − primary cell line and Vero (ECACC 88020401, African Green Monkey Kidney cells, GMK clone).

We have also investigated the changes in survival of human leukaemic RPMI 8226 cells and HeLa cancer cells, induced by the designed imidazotriazole derivatives (**1**–**3**, **5**–**12**) in order to choose the compounds having promising antiproliferative properties. Their influence on normal human skin fibroblast (HSF) cells was also determined.

4.2. Antimicrobial studies

Determination of the in vitro antimicrobial activity of the compounds tested was performed using the microdilution method, according to the National Committee for Clinical

Laboratory Standards (NCCLS) [38–40] and the disc-diffusion method by Kirby–Bauer [40–43].

The in vitro activities of three examined compounds (**9**, **13**, **14**) against pathogenic bacteria, yeast-like fungi and moulds were compared. The following microorganisms were used: *S. aureus* ATCC 25923, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae* (Gram-positive bacteria), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* (Gram-negative bacteria), *Candida albicans* ATCC 10231 (yeast-like fungus), *Aspergillus* spp. (moulds). The majority of strains under study were clinical isolates, identified with conventional morphological and biochemical methods.

The microdilution method for estimation of MIC values (the lowest concentration of compounds required to inhibit the growth of the tested microorganism) was applied to evaluate the antimicrobial activity. In this method the two reference strains of bacteria – *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and one reference fungal strain – *C. albicans* ATCC 10231 were included in this study. The antibacterial (**9**, **13**) and antifungal (**14**) potencies of the compounds were compared with the activities of topical antibacterial (ampicillin, chloramphenicol) and antifungal (miconazole) drugs.

5. Results and discussion

5.1. Antitumour activity studies

Compounds **7** and **12** were evaluated for their anticancer activity. Results for each test agent are reported as the growth inhibition percentage of treated cells when compared to the untreated control ones. Compounds, which reduced the growth, are passed on for evaluation towards three human cancer and two normal cell lines. Compounds **7** and **12** were found to be active. These compounds were proved to exhibit different levels of anticancer properties. According to the results listed in Table 3, compound **12** was found to be the most active against the human Caucasian colon adenocarcinoma cell line (LS180), reaching comparative growth inhibition values (48 and 54%) for each tested concentration: 25.3 μM (10 μg mL^{-1}) and 126.7 μM (50 μg mL^{-1}), respectively. Moreover, it's distinctly marked lower cytotoxicity towards normal cell lines, especially towards human skin fibroblasts (HSF) and almost seven-fold higher against LS180 cancer cells in a concentration of 25.3 μM (10 μg mL^{-1}) was ascertained. This compound was also found to be above five-fold higher cytotoxic against LS180 cancer cell line than towards normal cell line – HSF in a concentration of 126.7 μM (50 μg mL^{-1}). Compound **12** was about 1.7 times and 1.4 times more active against human Caucasian colon adenocarcinoma cell line (LS180) than against human breast carcinoma cell line (T47D) and uterus cancer cell line (SiHa), respectively, at a single concentration of 25.3 μM (10 μg mL^{-1}). On the contrary, both, human colon adenocarcinoma cell line (LS180) and human breast cancer cell line (T47D) showed similar susceptibility levels to this compound, particularly when it was applied in higher concentration. Similarly, compound **7** exhibited 35 and 39% growth inhibition, respectively, in both tested concentrations against human colon adenocarcinoma cell line (LS180), whereas **12** showed similar inhibitory effect on SiHa cancer cell line. Its growth inhibition values were found to be 35 and 41%, respectively, at both examined concentrations: 25.3 μM (10 μg mL^{-1}) and 126.7 μM (50 μg mL^{-1}). Compound **12**, in comparison to **7**, was more potent against uterus cancer cells (SiHa) in both tested concentrations and against human breast carcinoma cell line T47D in higher examined concentration. Compound **7** was the most active against human Caucasian colon adenocarcinoma cell line (LS180) at both examined concentrations: 29.3 μM (10 μg mL^{-1}) and 146.7 μM (50 μg mL^{-1}) and against human breast carcinoma cell line T47D at a concentration of 146.7 μM (50 μg mL^{-1}). Both examined heterobicycles (**7**, **12**) are the 3-phenoxymethyl-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole derivatives and these molecules differ from in the presence of a methyl (**7**) or a chloro (**12**) substituent in *ortho* position of the phenoxymethylene formation and in the location of a chloro substituent at *para* position of the phenyl ring (**12**) or its lack in the case of compound **7**. Compounds **7** and **12** showed different log k_W values of 1.17 and 4.15, respectively, in dioxane–water system. It was found that the introduction of a chloro substituent to the phenyl ring, and the replacement of a methyl substituent by a chloro substituent at the 2-position of the phenyl ring, that of the phenoxymethylene formation in the case of compound **12** led to significant increase in lipophilicity (log $k_W = 4.15$ in aqueous system with dioxane) and influenced in increase of anticancer activity. Moreover, the distinctly marked lower cytotoxicity of derivatives **7** and **12** against normal cell lines (human skin fibroblast cells – HSF and Vero African Green Monkey Kidney cells – GMK clone) and almost several-fold higher against the majority of cancer cell lines was ascertained (Table 3 and Figs. 2–6). Taking into consideration the

Table 3
Inhibition of in vitro normal and cancer cells growth by imidazotriazoles **7** and **12**

Cell line	Cytotoxicity (growth inhibition in %)			
	7		**12**	
	I	II	I	II
Normal cell lines				
HSF	4	8	7	10
GMK	20	20	20	28
Cancer cell lines				
LS180	35	39	48	54
SiHa	30	30	35	41
T47D	25	40	28	48

HSF – human skin fibroblast cells – primary cell line; Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells); LS180 (ECACC 87021202) – human Caucasian colon adenocarcinoma cells; SiHa (ECACC 85060701) – uterus cancer cells; T47D (ECACC 86012201) – human breast carcinoma cells; I – concentration of 10 μg mL^{-1}, which correspond to concentration of 29.3 μM for compound **7** and 25.3 μM for compound **12**; II – concentration of 50 μg mL^{-1}, which correspond to concentration of 146.7 μM for compound **7** and 126.7 μM for compound **12**.

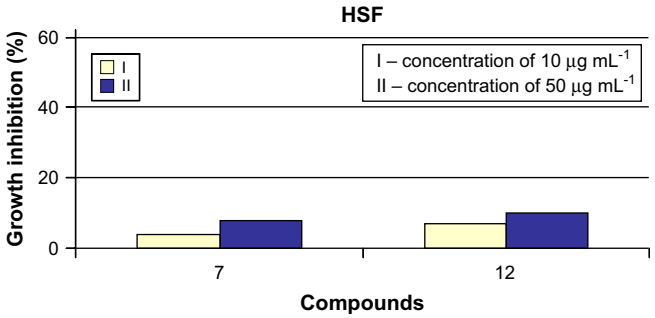


Fig. 2. Cytotoxicity of tested imidazotriazoles **7** and **12** in both examined concentrations (10 and 50 μg mL^{-1}) towards human skin fibroblast (HSF) cell line expressed as the growth inhibition (in %).

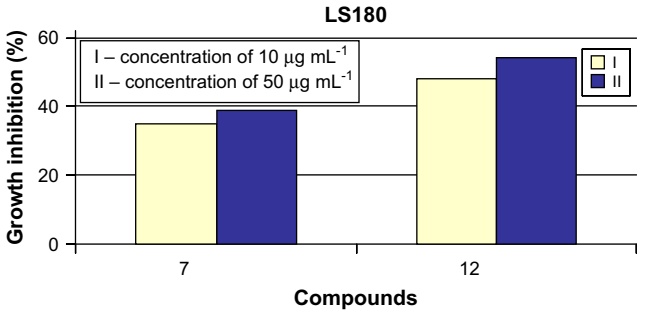


Fig. 4. Inhibition of in vitro human Caucasian colon adenocarcinoma cell line (LS180) growth (expressed as growth inhibition percentage) by tested imidazotriazoles **7** and **12** in both examined concentrations (10 and 50 μg mL^{-1}).

growth inhibition comparative study results concerning the influence of the tested compounds on cancer and normal cell lines, the selective action of the examined compounds can be expected .

It has been reported that compounds having [1,2,4]triazole moieties appear to be very effective inhibitors of many enzymes, for instance glycolytic, Krebs cycle enzymes in terms for preventing digestive tract cancer. It is known that [1,2,4]triazole moieties interact strongly with the heme iron and aromatic substituents in the active site of aromatase. Three drugs – nonsteroidal, competitive aromatase inhibitors – anastrozole, letrozole and vorozole (belonging to the [1,2,4]triazole derivatives) have been approved by the American food and drug administration (FDA) for first-line treatment of postmenopausal women with hormone-receptor positive locally advanced or metastatic breast cancer [7].

It can be speculated that the tested compounds possessing a dihydroimidazo[2,1-*c*][1,2,4]triazole scaffold inhibit the LS180 and SiHa cancer cell lines by possibly interacting with important metabolic enzymes.

Compound **12** was found to be the most effective in vitro against human colon adenocarcinoma cell line LS180 in both examined concentrations. The tested compound demonstrated antiproliferative properties those suggest its further investigation as a potential anticancer agent. Further studies are in progress to define the important mechanisms of action of the above mentioned compound.

Compound **7** affected the superoxide dismutase (SOD) gene expression in two cancer cells: LS180 and T47D (Fig. 7). Furthermore, the effect of the tested heterobicycle **7** was noticed on DNA structure of the human breast cancer cell line (T47D) by using the comet assay, which is shown in Photo 1. This sensitive and rapid microelectrophoretic technique, also called the single cell gel assay (SCG) and microgel electrophoresis (MGE), was introduced by Östling and Johanson for the direct visualization of DNA damage in individual mammalian cells. DNA was isolated from cell cultures after incubation with compound **7** by phenol–chloroform method. DNA damage in tested cancer cell lines after incubation with imidazotriazole **7** in a concentration of 29.3 μM (10 μg mL^{-1}) was analysed using the field gel electrophoresis. This compound was found to be effective in inducing DNA damage. This led to cutting of the DNA strands in tested cell lines and formation of small fragments of DNA – two higher and one lighter in comparison to control DNA molecules (Fig. 8). The effect of the examined imidazotriazole of the type **7** seemed to be similar to nuclease function. In conclusion compound **7** was found to possess the efficiency for DNA strand breakage of cancer cell lines such as the cytotoxic antibiotic – bleomycin [44], isolated from *Streptomyces verticillus* and effective in combination therapies against certain types of skin cancer, testicular carcinoma and lymphomas. DNA strand breakage caused by compound **7** can have dramatic effects on higher-order chromatin structure because of

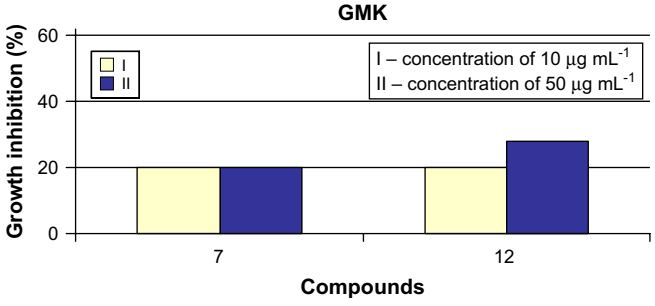


Fig. 3. Cytotoxicity of tested imidazotriazoles **7** and **12** in both examined concentrations (10 and 50 μg mL^{-1}) towards African Green Monkey Kidney cell line (GMK) expressed as growth inhibition (in %).

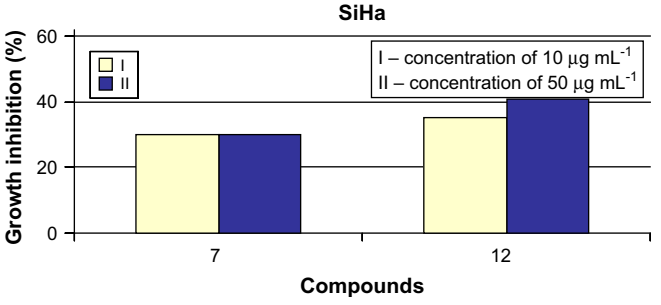


Fig. 5. Inhibition of in vitro human uterus carcinoma cell line growth (SiHa) (expressed as growth inhibition percentage) by tested imidazotriazoles **7** and **12** in both examined concentrations (10 and 50 μg mL^{-1}).

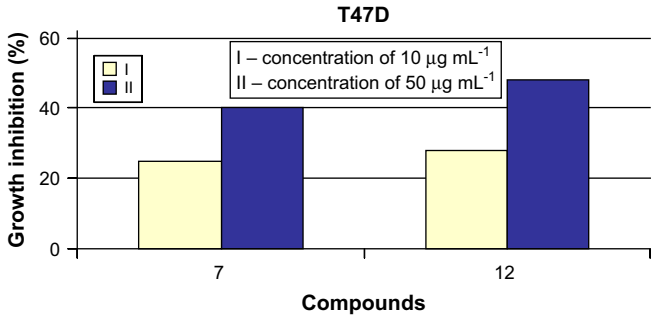


Fig. 6. Inhibition of in vitro human breast carcinoma cell line growth (T47D) (expressed as growth inhibition in %) by tested imidazotriazoles **7** and **12** in both examined concentrations (10 and 50 μg mL^{-1}).

its supercoiling and tight packaging within the nucleus. Taking into account the above mentioned biological activity, compound **7** may be promising for the development of novel agents that induce the DNA strand breakage.

In the next series of experiments, we have also investigated the action of the designed imidazo[2,1-*c*][1,2,4]triazoles (**1**–**3**, **5**–**12**) on the viability of human leukaemic RPMI 8226 cells and HeLa cancer cells in order to select compounds having promising antiproliferative activities. The complete set of data obtained in the MTT viability assay revealed that the leukaemia cell line RPMI 8226 was relatively more sensitive to five of 11 tested compounds (**8**–**12**) than was the other human cervix cancer cell line – HeLa (Table 4). The highest viability decreases (to 46, 32 and 31%, respectively, after exposure for 24 h) in leukaemic RPMI 8226 cells treated with 1, 50 and 100 μM heterobicycle **11** were observed. Also compound **10** in concentrations of 1, 50 and 100 μM caused significant viability decreases (to 60.1, 34.5 and 34%, respectively), in human leukaemic RPMI 8226 cells. The remaining compounds **8**, **9** and **12** in concentrations of 50 and 100 μM were proved to evoke viability decreases to 24.4–39.9% in leukaemic RPMI 8226 cells. On the contrary, about two to three-fold lower viability decreases were produced in HeLa cancer cells treated with the same concentrations of these compounds. However, heterobicycles **1**–**3**, **5**–**6** in three different concentrations of the compound solutions (1, 50, 100 μM) nearly did not change the viability of human leukaemic RPMI 8226 cells. Simultaneously, slight viability changes seemed to be mainly concentration-independent (Table 4). Furthermore,

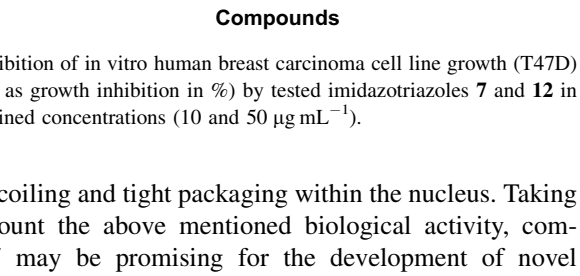


Photo 1a. Control cell of breast cancer line (T47D) (the comet assay).

compounds **1**–**3**, **5** and **12** were found to be more toxic against HeLa cancer cell line than towards normal HSF cells. Substance **6** revealed similar level of susceptibility towards both HeLa and HSF cells.

The majority of the investigated compounds revealed low toxicity towards the examined normal cell line – HSF cells. Only compound **10**, having a log k_W value of 3.41 in water system with dioxane, in a concentration of 100 μM caused significant decrease (to 16%) in viability of HSF cells.

All the compounds with the best cytotoxicity against leukaemic RPMI 8226 cells (**8**–**12**) possess the lipophilic weak-electron-withdrawing chloro substituent and the lipophilic weak electron-donating methyl group at the 4-position of the phenyl ring and simultaneously 2-CH_3; 4-Cl, 2,4-Cl_2 and 4-Cl substituents at the second phenyl ring, that of the phenoxymethylene moiety. These compounds, with exception of **9** showed relatively the highest log k_W values in the range of 3.41–4.15 (Table 2). Amongst those, the most potent was **11**, e.g., 3[(2-methyl-4-chloro)phenoxymethyl]-7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole, having a log k_W value of 3.87 in aqueous system with dioxane. This compound applied in a concentration of 1 μM caused the highest viability decrease (to 46.4%) in human leukaemic RPMI 8226 cells. The presence of both substituted phenyl rings: first at the C-3 (that of the phenoxymethylene formation) and second at the N-7 seemed to be an important factor that influenced the antitumour potency

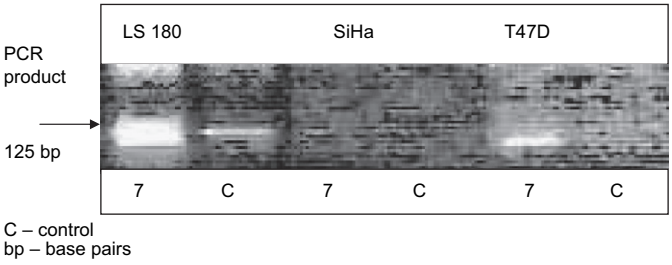


Fig. 7. SOD gene expression in cancer cell lines after incubation with tested imidazotriazole **7** in a concentration of 29.3 μM (10 μg mL^{-1}). Electrophoretogram on 1% agarose gel.

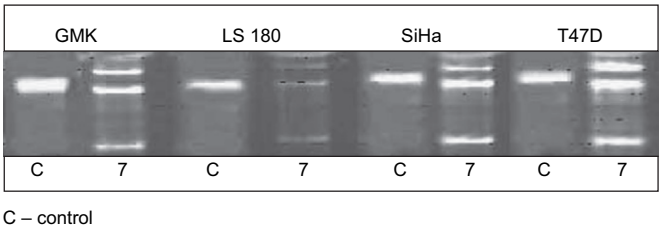


Fig. 8. DNA damage in tested cell lines after incubation with compound **7** in a concentration of 29.3 μM (10 μg mL^{-1}). Cells, prepared as agarose plugs, were lysed and subjected to pulsed field gel electrophoresis through a 1% agarose gel.

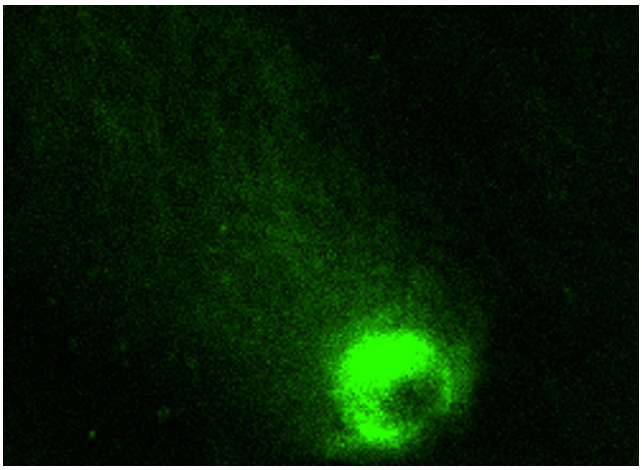


Photo 1b. DNA molecules damage in breast cancer cells (T47D) after incubation with compound **7** in a concentration of 10 μg mL^{-1} (the comet assay).

against that human hematopoietic cancer cell line. Thus tested imidazotriazoles unsubstituted in the 3-position of heterobicycle (**1**–**3**) and those possessing unsubstituted or disubstituted phenyl ring, that of the phenoxymethylene formation, and simultaneously having unsubstituted the second phenyl ring (**5**, **7**) were found to be inactive against leukaemic RPMI 8226 cells. All these compounds had relatively lower log k_W values in the range of 1.16–2.49 (Table 2). Also derivative **6** from this imidazotriazole scaffold, having a log k_W value of 1.52, and substituted at the N-7 by a 4-methoxyphenyl group and containing a chloro substituent at the 4-position of the second phenyl ring, that of the phenoxymethylene moiety, did not change the viability of leukaemic RPMI 8226 cells. The replacement of a 4-chloro and a 4-methyl group of an aromatic phenyl ring at the 7-position of heterobicycle in compounds **11** and **9** by a proton like in the case of compound **7** resulted in the complete loss of activity against human leukaemic RPMI 8226 cells.

Taking into consideration the influence of the following investigated compounds (**8**–**9** and **11**–**12**) on human leukaemic RPMI 8226 cells and normal cell line – human skin fibroblasts (HSF), the selective action of the examined derivatives can be expected. Thus, these ones may be used as a basis for the design of novel antiproliferative agents.

5.2. Antimicrobial action

Three imidazotriazoles of the type **9**, **13**, **14** have been evaluated for their general pharmacological activities and all these compounds have been found to possess positive antimicrobial activity (Tables 5 and 6). Two of three tested compounds (**9**, **13**) were found to have highly significant antibacterial activity but no antifungal potency. These compounds were also inactive against moulds. Compound **9**, e.g., 3-[(2-methyl-4-chloro)phenoxymethyl]-7-(4-methylphenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole, having a log k_W value of 1.10 in aqueous system with dioxane, showed good activity against *S. aureus* ATCC 25923 with a MIC value of 44.0 μM. Its antibacterial potency was found to be 1.2 to 3.6-fold lower than that of

Table 4
Percentage of viable normal human skin fibroblasts (HSF) and cancer cell lines (RPMI and HeLa) following 24 h treatment with the tested concentrations of imidazotriazoles **1**–**3**, **5**–**12**

Comp.	Concentration in μM	Cell viability in normal HSF cells	Cell viability in RPMI cancer cells	Cell viability in HeLa cancer cells
1	1	97.6 ± 2.8	99.3 ± 4.3	73.2 ± 4.6
	50	89.5 ± 3.3	101.6 ± 4.8	75.4 ± 9.8
	100	85.6 ± 3.2	97.6 ± 3.8	72.8 ± 5.7
2	1	104.7 ± 4.4	101.5 ± 6.5	93.0 ± 12.3
	50	95.2 ± 1.6	96.5 ± 5.5	91.7 ± 11.0
	100	89.3 ± 4.9	91.3 ± 7.5	80.6 ± 5.3
3	1	101.0 ± 4.7	103.6 ± 7.3	77.9 ± 7.0
	50	90.9 ± 2.7	96.9 ± 5.0	67.6 ± 2.8
	100	90.7 ± 1.9	87.5 ± 6.1	68.4 ± 2.7
5	1	91.7 ± 3.7	104.3 ± 5.1	85.3 ± 7.0
	50	87.6 ± 2.4	99.0 ± 5.1	71.1 ± 5.1
	100	83.0 ± 1.7	100.9 ± 4.0	70.3 ± 6.1
6	1	80.3 ± 1.3	99.7 ± 5.7	81.5 ± 7.2
	50	67.1 ± 1.5	98.4 ± 7.6	72.1 ± 6.5
	100	64.6 ± 3.0	94.9 ± 8.5	68.9 ± 6.0
7	1	79.2 ± 5.3	112.2 ± 4.8	65.6 ± 8.0
	50	76.8 ± 4.5	113.9 ± 4.9	75.1 ± 3.9
	100	72.5 ± 3.7	108.4 ± 4.3	75.9 ± 5.2
8	1	88.1 ± 4.6	104.0 ± 2.9	89.0 ± 6.1
	50	68.4 ± 4.2	39.5 ± 1.7	78.3 ± 6.1
	100	64.7 ± 3.7	39.9 ± 2.2	77.9 ± 5.2
9	1	74.9 ± 5.9	85.5 ± 3.7	95.1 ± 4.2
	50	56.9 ± 5.3	27.7 ± 1.0	75.2 ± 4.2
	100	62.1 ± 3.8	25.4 ± 1.5	75.6 ± 2.2
10	1	88.7 ± 2.6	60.1 ± 3.3	63.2 ± 2.3
	50	63.8 ± 5.3	34.5 ± 1.1	56.3 ± 2.8
	100	16.2 ± 7.2	34.0 ± 1.0	52.4 ± 1.3
11	1	79.5 ± 4.6	46.4 ± 2.4	92.1 ± 4.5
	50	74.0 ± 5.0	32.4 ± 1.5	71.8 ± 3.8
	100	71.0 ± 5.1	31.1 ± 2.0	71.0 ± 3.7
12	1	83.0 ± 4.9	98.4 ± 5.6	68.4 ± 7.9
	50	83.9 ± 11.9	24.4 ± 1.2	59.9 ± 4.2
	100	86.8 ± 14.2	24.5 ± 1.7	56.3 ± 4.9

HSF – human skin fibroblast cells – primary cell line; RPMI 8226 (ECACC 87012702) – human peripheral blood myeloma; HeLa (ECACC 93021013) – human Negroid cervix epitheloid carcinoma.

ampicillin and chloramphenicol, respectively. Moreover, compound **9** was found to inhibit the growth of *S. epidermidis* in concentrations of 281.8 μM (100 μg mL^{-1}) and 563.6 μM (200 μg mL^{-1}) in the disc-diffusion assay. Compound **13**, i.e., 3-(2,4-dichlorophenoxymethyl)-7-(3,4-dichlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole, with the highest lipophilicity (log $k_W = 4.61$ in aqueous system with dioxane) was active against *P. aeruginosa* ATCC 27853 with MIC results at 36.3 μM. It's antibacterial potency was superior to that of ampicillin and chloramphenicol as observed in Table 6. This compound was also found to exhibit activity against *Pr. vulgaris* at concentrations of 232.5 μM (100 μg mL^{-1}) and 465.0 μM (200 μg mL^{-1}) and *K. pneumoniae*, *En. aerogenes* at concentration of 465.0 μM (200 μg mL^{-1}) in the disc-diffusion assay.

Although, the structural changes of compounds **9** and **13**, significantly altered the log k_W values as observed for **9** (log $k_W = 1.10$) and **13** (log $k_W = 4.61$), however, these ones showed little effect on the antibacterial activity with MIC values of 44.0 and 36.3 μM, respectively. Simultaneously,

Table 5
Antimicrobial activities of three synthesized compounds against the tested bacterial and fungal isolates using disc-diffusion method

Microorganism	Compound and inhibition zone (mm)							
	9		**13**		**14**		A	M
	I	II	I	II	I	II		
Escherichia coli ATCC 25922	−	−	−	−	−	−	++	−
Pseudomonas aeruginosa ATCC 27853	−	−	+	++	−	−	+	−
Proteus vulgaris	−	−	+	++	−	−	−	−
Klebsiella pneumoniae	−	−	−	+	−	−	−	−
Enterobacter aerogenes	−	−	−	+	−	−	−	−
Staphylococcus aureus ATCC 25923	+	++	−	−	−	−	++	−
Staphylococcus epidermidis	+	++	−	−	−	−	+	−
Streptococcus pyogenes	−	−	−	−	−	−	++	−
Streptococcus agalactiae	−	−	−	−	−	−	++	−
Candida albicans ATCC 10231	−	−	−	−	++	++	−	++
Aspergillus spp.	−	−	−	−	−	−	−	−

Results were interpreted in terms of the diameter of the inhibition zone: (−): 0–10 mm (R, resistant); (+): 11–16 mm (I, intermediate); (++): 17–25 mm (S, susceptible); I − concentration of 100 μg mL^{-1}, which correspond to concentration range of 232.5–395.7 μM depending on molecular weight of the examined compound; II − concentration of 200 μg mL^{-1}, which correspond to concentration range of 465.0–791.3 μM depending on molecular weight of the examined compound; Standards: A − ampicillin in concentration of 572.4 μM (200 μg mL^{-1}); M − miconazole in concentration of 480.6 μM (200 μg mL^{-1}).

higher lipophilicity of **13** could possible be a qualitative explanation of changes in the antibacterial specificity of this compound in relation to **9** as observed in Tables 5 and 6.

Heterobicycle **14**, i.e., 7-(4-chlorophenyl)-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole-3-thiol, having the lowest value of log k_W (−0.09), revealed highly significant antifungal activity and was found to be completely inactive against all the examined bacterial and mould strains in the disc-diffusion method (Table 5). Full details of the method of testing have been described elsewhere [45]. In the present study in the microdilution assay imidazotriazole of the type **14** demonstrated good antifungal activity against *C. albicans* ATCC 10231 with a MIC value of 30.9 μM. Its antifungal potency was 1.2-fold higher than that of miconazole. An introduction of the sulfanyl group in the position 3 to the dihydroimidazotriazole system as in **14** significantly altered the log k_W value in aqueous system with dioxane. Imidazotriazoles **14** (R = 4-Cl, R′ = SH, log k_W = −0.09) and **3** (R = 4-Cl, R′ = H, log k_W = 1.89), which have different log k_W values can be given as example. Simultaneously, for compound **14** antifungal activity superior to miconazole was observed. Probably, the significant decrease in lipophilicity and an increasing percentage of sulfur element could be a qualitative explanation of changes in antimicrobial specificity of this compound in relation to **9**, **13** as observed in Tables 4 and 5. It is of interest that the literature search revealed that some derivatives having the same heterocyclic skeleton − e.g., 7-aryl-5-methyl-3-thiolo-imidazo[2,1-*c*][1,2,4]triazol-6-ones have been reported as possible antifungal drugs [15].

Table 6
Antimicrobial activity expressed as MIC (μM) of the tested imidazotriazoles **9**, **13**, **14**

Microorganism code	Compound			Standard		
	9	**13**	**14**	A	C	M
Staphylococcus aureus ATCC 25923	44.0			35.8	12.1	
Pseudomonas aeruginosa ATCC 27853		36.3		>357.7	>386.8	
Candida albicans ATCC 10231			30.9			37.5

Standards: A − ampicillin; C − chloramphenicol; M − miconazole.

As a result, compound **9** was found to exhibit the most potent in vitro antibacterial activity with a MIC value of 44.0 μM against *S. aureus* ATCC 25923 and may be considered promising for the development of new antibacterial agents.

Compound **13** was found to express the most potent in vitro anti Gram-negative activity with a MIC value of 36.3 μM against *P. aeruginosa* ATCC 27853. This compound was generally active against the majority of Gram-negative bacterial strains examined, with the exception of *E. coli* ATCC 25922, and therefore may be considered promising for the development of selective anti Gram-negative antibacterial agents.

As a result compound **14** demonstrated good antifungal activity against *C. albicans* ATCC 10231 with a MIC value of 30.9 μM. This compound showed superior antifungal activity to miconazole. Taking into account its antifungal effectiveness and lack of activity against all the examined Gram-positive and Gram-negative bacterial strains and moulds, this compound may be considered promising for the development of new antifungal agents.

6. Conclusion

In this report, an easy and useful method to synthesize antitumour and antimicrobial active imidazotriazole aryl derivatives containing the phenoxymethylene (**7–9**, **11–13**) formation or the sulfanyl group (**14**) has been presented. The identified analogues, in particular 3-phenoxymethyl-7-aryl-5*H*-6,7-dihydroimidazo[2,1-*c*][1,2,4]triazole derivatives (**7** and **12**), demonstrated antiproliferative and apoptotic properties justifying their further investigation as potential anticancer agents. Moreover, compound **7** was found to exhibit efficiency for DNA strand breakage and may be promising for the development of novel antitumour agents that induce the DNA strand breakage. Also significant viability decreases in human leukaemic RPMI 8226 cells treated with different concentrations of compounds **8–12** were observed, suggesting their antiproliferative properties. Compounds **9**, **13** can serve as novel templates for bacterial infection chemotherapy, whereas **14** may be potential candidate for new antifungal agents. Further optimization of these identified chemical leads can possibly lead to more active molecules. Since all the examined compounds (**7–14**) are showing promising results, studies to establish their in vivo efficacy and safety are being planned for their further development. It may be concluded that the fusion of 4,5-dihydroimidazole and [1,2,4]triazole nuclei in the case

of the examined bicycles (**7**–**14**) might result in bioactive molecules of high potency, particularly if the substituents are designed with optimum toxophoric requirements.

7. Experimental protocols

7.1. Instrumentations and general materials

Chemicals (methyl iodide, hydrazine hydrate, triethyl orthoformate, phenoxyacetic acid derivatives and carbon disulfide) were purchased from Merck (Darmstadt, Germany) as 'synthesis grade' and used without further purification. Melting points (m.p.) were determined on a Boetius apparatus and are given uncorrected. The IR spectra were measured as potassium bromide pellets using a Perkin-Elmer 1725X spectrometer. NMR spectra (^{1}H and ^{13}C) of the compounds synthesized were recorded on a Bruker 300 MHz spectrometer in hexadeuterodimethyl sulfoxide (DMSO-d_6) with TMS as an external standard at 295 K. Mass spectroscopic analyses for compounds **1**–**5**, **8**–**12** were recorded at 70 eV on a Trace DSQ mass spectrometer (Thermo Finnigan) for molecular ion peaks. The molecular ion for the compounds **3**, **4**, **8**–**12** was calculated according to ^{35}Cl isotope. Thin-layer chromatography was carried out on commercial Merck SiO_2 60 F_{254} plates having fluorescence indicator; the spots were visualized with UV light $\lambda = 254$ nm, and by spraying with a 2% ethanol solution of ninhydrin or charging reagent.

In the high performance liquid chromatographic (HPLC) series of experiments, fourteen derivatives of the type **1**–**14** were chromatographed on octadecyl silica adsorbent (SUPELCOSIL™ LC-18) using various aqueous systems: mixture of water and organic modifiers (methanol – MeOH, acetonitrile – MeCN or dioxane – DX). Diethylamine (DEA) and phosphate buffer (pH = 3.5) were added to mobile phase (eluent containing 0.05 M L^{-1} DEA in organic modifier (MeOH, MeCN or DX) – buffered mobile phase).

Elemental analyses were performed on a Perkin-Elmer analyzer and were in the range of ±0.4% for each element analyzed (C, H, N, Cl).

*7.1.1. General procedure for synthesis of 7-aryl-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazoles (**1**–**4**)*

A mixture containing 1-aryl-2-hydrazonoimidazolidine (0.03 mol) and triethyl orthoformate (0.03 mol) in 100 mL of DMF was heated under reflux for 6 h. The amount of the collected EtOH was a measure of the reaction course. Then the reaction mixture was concentrated to ca. 30 mL and cooled overnight. The precipitate yielded was filtered off, washed three times with cold methanol. After collection and purification by recrystallization from MeOH–DMF mixture or from DMF, the compounds **1**–**4** were obtained in 60.3–74.4% yields.

*7.1.1.1. 7-Phenyl-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**1**).* Recrystallized from MeOH–DMF mixture, yield 68.3%, m.p. 238–241 °C; IR (KBr) (ν, cm^{-1}): 1608 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 4.35 (dd, $J = 6.7$ Hz, $J' = 5.8$ Hz, 2H, CH_2), 4.54 (dd, $J = 6.8$ Hz, $J' = 5.8$ Hz, 2H, CH_2), 6.97–7.59 (m, 5H, ar-H), 8.19 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): 39.7 (imidazolidine-C-5), 52.9 (imidazolidine-C-6), 140.2 (triazole-C-3), 159.0 (C-7a), ar C: 114.1, 120.0, 128.6, 136.0; EIMS [70 eV, *m/z* (%)]: 187 (19.6), 186 (M$^+$, 100.0), 184 (82.0), 171 (3.3), 170 (2.5), 160 (10.1), 159 (15.5), 158 (37.6), 157 (2.1), 144 (1.5), 143 (2.2), 132 (4.3), 131 (13.5), 130 (13.8), 129 (2.7), 119 (1.6), 118 (10.8), 117 (31.9), 116 (5.9), 109 (7.0), 106 (1.5), 105 (9.2), 104 (38.9), 102 (57.4), 95 (1.9), 93 (4.8), 92 (2.3), 91 (11.8), 90 (4.3), 89 (1.5), 84 (4.0), 83 (14.8), 82 (2.4), 79 (1.8), 78 (6.9), 77 (53.7), 76 (37.2), 75 (8.0), 74 (4.6), 70 (1.4), 65 (4.0), 64 (3.2), 63 (4.6), 56 (3.2), 55 (7.1), 54 (3.1), 53 (2.3), 52 (4.1), 51 (13.4), 49 (6.7).

*7.1.1.2. 7-(4-Methoxyphenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**2**).* Recrystallized from MeOH–DMF mixture, yield 60.3%, m.p. 265–267 °C; IR (KBr) (ν, cm^{-1}): 1610 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 3.73 (s, 3H, OCH_3), 4.25 (dd, $J = 6.8$ Hz, $J' = 5.7$ Hz, 2H, CH_2), 4.52 (dd, $J = 6.8$ Hz, $J' = 5.7$ Hz, 2H, CH_2), 6.99 (d, 2H, ar-H), 7.42 (d, 2H, ar-H), 8.15 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): 39.7 (imidazolidine-C-5), 53.4 (imidazolidine-C-6), 55.1 (CH_3O), 135.9 (triazole-C-3), 159.3 (C-7a), ar C: 114.3, 115.3, 134.1, 153.4; EIMS [70 eV, *m/z* (%)]: 217 (18.0), 216 (M$^+$, 100.0), 215 (19.1), 202 (7.9), 201 (64.2), 189 (2.6), 188 (16.9), 175 (3.0), 174 (6.1), 173 (31.1), 161 (2.8), 158 (2.3), 147 (7.1), 146 (5.4), 145 (4.3), 134 (8.5), 133 (13.1), 132 (6.1), 121 (6.0), 120 (10.0), 119 (5.2), 118 (7.5), 117 (3.6), 109 (2.2), 108 (6.9), 107 (8.0), 106 (3.3), 105 (4.7), 104 (7.0), 103 (13.5), 102 (3.9), 95 (5.8), 92 (8.8), 91 (4.6), 90 (16.0), 83 (2.8), 80 (2.5), 79 (3.3), 78 (5.8), 77 (11.0), 76 (5.6), 75 (3.2), 66 (3.2), 65 (5.3), 64 (7.8), 63 (8.3), 55 (2.6), 53 (2.3), 52 (2.4), 51 (2.9).

*7.1.1.3. 7-(4-Chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**3**).* Recrystallized from MeOH–DMF mixture, yield 74.4%, m.p. 242–244 °C; IR (KBr) (ν, cm^{-1}): 1611 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 4.29 (dd, $J = 6.5$ Hz, $J' = 5.8$ Hz, 2H, CH_2), 4.52 (dd, $J = 6.5$ Hz, $J' = 5.7$ Hz, 2H, CH_2), 7.41 (d, 2H, ar-H), 7.53 (d, 2H, ar-H), 8.21 (s, 1H, CH); ^{13}C NMR (DMSO-d_6): 39.7 (imidazolidine-C-5), 53.1 (imidazolidine-C-6), 139.1 (triazole-C-3), 158.8 (C-7a), ar C: 115.7, 123.9, 128.5, 136.2; EIMS [70 eV, *m/z* (%)]: 223 (3.8), 222 (31.8), 221 (23.3), 220 (M$^+$, 100.0), 219 (35.9), 207 (1.2), 205 (1.4), 204 (1.3), 196 (3.1), 195 (3.9), 194 (18.2), 193 (9.3), 192 (26.5), 191 (1.1), 185 (4.3), 184 (5.0), 167 (2.3), 166 (3.3), 165 (5.0), 164 (5.1), 159 (1.2), 158 (5.5), 157 (8.8), 156 (2.5), 154 (1.7), 153 (5.6), 152 (5.0), 151 (15.2), 141 (2.0), 140 (8.3), 139 (14.8), 138 (23.0), 137 (32.5), 136 (1.2), 131 (3.6), 130 (3.6), 129 (1.5), 128 (1.0), 127 (2.3), 126 (1.1), 125 (6.6), 124 (1.6), 117 (3.4), 116 (2.4), 113 (6.6), 112 (2.3), 111 (20.2), 110 (2.4), 109 (3.8), 104 (1.4), 103 (4.4), 102 (25.6), 101 (2.4), 100 (1.9), 99 (2.7), 95 (2.6), 92 (2.8), 91 (1.9), 90 (2.7), 89 (2.6), 85 (2.1), 84 (4.2), 83 (13.7), 82 (1.1), 78 (1.4), 77 (4.9), 76 (8.4), 75 (24.7), 74 (6.0), 73 (2.9), 70 (1.5), 65

(1.1), 64 (2.2), 63 (5.2), 62 (2.5), 61 (1.5), 56 (5.1), 55 (9.4), 54 (3.1), 53 (2.3), 52 (2.6), 51 (9.7), 50 (11.7), 49 (1.0), 43 (1.1), 42 (1.7), 41 (1.9), 40 (1.3), 39 (3.6), 38 (2.8), 37 (2.1).

*7.1.1.4. 7-(3,4-Dichlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**4**).* Recrystallized from DMF, yield 65.5%, m.p. 199–202 °C; IR (KBr) (ν, cm^{-1}): 1609 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 4.29 (dd, $J = 7.4$ Hz, $J' = 6.2$ Hz, 2H, CH_2), 4.55 (dd, $J = 7.5$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 7.18–7.95 (m, 3H, ar-H), 8.16 (1H, CH); ^{13}C NMR (DMSO-d_6): 39.7 (imidazolidine-C-5), 53.2 (imidazolidine-C-6), 140.0 (triazole-C-3), 158.4 (C-7a), ar C: 114.3, 115.6, 121.6, 130.4, 131.3, 136.4; EIMS [70 eV, *m/z* (%)]: 258 (10.6), 257 (13.0), 256 (64.0), 255 (44.3), 254 (M^+, 100.0), 253 (57.0), 230 (8.3), 229 (5.9), 228 (23.8), 227 (8.8), 226 (25.0), 219 (6.5), 218 (7.0), 200 (4.2), 198 (4.8), 192 (8.5), 191 (8.0), 187 (9.3), 186 (5.1), 185 (13.8), 175 (5.5), 174 (11.8), 173 (21.2), 172 (18.1), 171 (31.4), 159 (4.4), 147 (10.3), 145 (16.9), 138 (6.4), 137 (4.0), 136 (15.8), 135 (4.0), 111 (10.3), 110 (11.0), 109 (34.2), 101 (4.4), 100 (21.0), 99 (5.8), 97 (4.9), 96 (4.2), 95 (5.9), 85 (4.6), 84 (8.0), 83 (16.0), 76 (4.1), 75 (13.5), 74 (13.2), 73 (7.2), 66 (4.4), 55 (9.1).

*7.1.2. General procedure for synthesis of the 3-phenoxymethyl-7-aryl-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole derivatives (**5–13**)*

The appropriate derivative of 1-aryl-2-hydrazonoimidazolidine (0.05 mol) and adequate phenoxyacetic acid derivative (0.055 mol) were refluxed in 80 cm^3 of DMF for 6 h. Then the excess of solvent was distilled under reduced pressure. The product was obtained by neutralizing the content with aqueous sodium hydroxide (6%) after adding cold water, and finally purified by recrystallization from DMF, *i*PrOH, EtOH or DMF–MeOH mixture in the proportion indicated.

*7.1.2.1. 3-Phenoxymethyl-7-phenyl-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**5**).* Recrystallized from DMF, yield 68.2%, m.p. 247–250 °C; IR (KBr) (ν, cm^{-1}): 1608 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 4.33 (dd, $J = 7.8$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 4.59 (dd, $J = 7.7$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 5.33 (s, 2H, OCH_2), 7.08–7.59 (m, 10H, ar-H); EIMS [70 eV, *m/z* (%)]: 292 (M^+, 4.5), 187 (11.9), 186 (100.0), 185 (55.7), 171 (1.7), 160 (4.8), 159 (7.4), 158 (15.9), 157 (2.0), 132 (1.9), 131 (6.0), 130 (6.1), 129 (1.8), 118 (5.1), 117 (14.7), 116 (2.6), 109 (4.3), 105 (6.2), 104 (28.0), 103 (41.0), 97 (2.6), 95 (2.6), 93 (3.6), 91 (7.1), 90 (2.1), 84 (3.0), 83 (9.3), 82 (2.3), 81 (1.9), 78 (3.3), 77 (26.1), 76 (15.2), 75 (3.3), 74 (1.9), 73 (1.8), 71 (1.9), 70 (1.7), 69 (3.5), 65 (2.6), 64 (1.9), 63 (2.6), 57 (2.3), 56 (2.0), 55 (5.5), 54 (2.1), 52 (2.5), 51 (8.5), 50 (4.1), 43 (2.2), 41 (2.1), 39 (2.7).

*7.1.2.2. 3-(4-Chlorophenoxymethyl)-7-(4-methoxyphenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**6**).* Recrystallized from DMF, yield 59.2%, m.p. 230–233 °C; IR (KBr) (ν, cm^{-1}): 1610 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 3.80 (s, 3H, OCH_3), 4.02–4.40 (m, 4H, $2CH_2$), 5.20 (s, 2H, OCH_2), 7.10–7.40 (m, 8H, ar-H); ^{13}C NMR (DMSO-d_6): 39.8 (imidazolidine-C-5), 53.5 (imidazolidine-C-6), 55.2 (CH_3O), 60.8 (CH_2O), 136.1 (triazole-C-3), 156.4 (C-7a), ar C: 114.1, 115.8, 128.5, 130.6, 136.5, 143.7, 157.2, 157.8.

*7.1.2.3. 3-[(2-Methyl-4-chloro)phenoxymethyl]-7-phenyl-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**7**).* Recrystallized from DMF, yield 44.2%, m.p. 248–251 °C; IR (KBr) (ν, cm^{-1}): 1612 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 2.27 (s, 3H, CH_3), 4.25–4.55 (m, 4H, $2CH_2$), 5.20 (s, 2H, OCH_2), 7.08–7.55 (m, 8H, ar-H); ^{13}C NMR (DMSO-d_6): 15.4 (CH_3), 39.9 (imidazolidine-C-5), 53.4 (imidazolidine-C-6), 60.9 (CH_2O), 138.4 (triazole-C-3), 159.6 (C-7a), ar C: 113.6, 114.2, 121.6, 124.6, 125.3, 127.9, 129.8, 142.6, 154.5.

*7.1.2.4. 3-(4-Chlorophenoxymethyl)-7-(4-methylphenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**8**).* Recrystallized from *i*PrOH, yield 59.3%, m.p. 273–276 °C; IR (KBr) (ν, cm^{-1}): 1607 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 2.30 (s, 3H, CH_3), 4.20–4.45 (m, 4H, $2CH_2$), 5.19 (s, 2H, OCH_2), 7.08–7.41 (m, 8H, ar-H); ^{13}C NMR (DMSO-d_6): 19.8 (CH_3), 39.8 (imidazolidine-C-5), 53.3 (imidazolidine-C-6), 60.8 (CH_2O), 137.7 (triazole-C-3), 159.9 (C-7a), ar C: 114.2, 116.6, 125.1, 129.0, 129.1, 129.2, 143.6, 156.3; EIMS [70 eV, *m/z* (%)]: 342 (2.8), 341 (2.0), 340 (M^+, 8.5), 215 (1.4), 214 (17.5), 213 (100.0), 211 (1.8), 185 (5.3), 184 (2.5), 183 (2.0), 171 (5.4), 170 (39.5), 169 (15.0), 158 (4.1), 157 (15.8), 155 (1.1), 153 (2.1), 145 (1.3), 144 (3.5), 143 (4.1), 142 (1.9), 131 (1.3), 130 (1.3), 129 (1.4), 128 (1.5), 127 (3.0), 119 (1.3), 118 (5.1), 117 (17.0), 116 (11.5), 115 (1.6), 111 (2.4), 105 (1.1), 101 (2.5), 99 (7.4), 92 (1.3), 91 (12.0), 90 (6.9), 89 (5.6), 77 (2.1), 75 (2.2), 73 (2.5), 68 (1.0), 67 (1.1), 66 (3.3), 65 (6.4), 64 (2.2), 63 (3.0), 54 (1.2), 41 (1.8).

*7.1.2.5. 3-[(2-Methyl-4-chloro)phenoxymethyl]-7-(4-methylphenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**9**).* Recrystallized from EtOH, yield 43.5%, m.p. 245–248 °C; IR (KBr) (ν, cm^{-1}): 1609 (C=N); ^{1}H NMR (DMSO-d_6, 300 MHz): 2.11 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 3.77–4.10 (m, 4H, $2CH_2$), 5.18 (s, 2H, OCH_2), 6.92–7.40 (m, 7H, ar-H); ^{13}C NMR (DMSO-d_6): 15.3 (CH_3), 19.8 (CH_3), 39.8 (imidazolidine-C-5), 53.3 (imidazolidine-C-6), 60.9 (CH_2O), 137.6 (triazole-C-3), 160.0 (C-7a), ar C: 113.6, 114.2, 124.6, 126.1, 128.5, 129.1, 129.8, 143.7, 154.4; EIMS [70 eV, *m/z* (%)]: 356 (1.5), 355 (1.2), 354 (M^+, 4.7), 215 (1.5), 214 (18.2), 213 (100.0), 211 (1.7), 186 (0.8), 185 (5.1), 184 (2.3), 183 (1.8), 172 (0.7), 171 (4.7), 170 (36.5), 169 (12.9), 159 (0.9), 158 (3.7), 157 (13.4), 155 (1.1), 145 (1.0), 144 (2.9), 143 (4.2), 142 (2.1), 141 (3.6), 131 (1.1), 130 (0.8), 125 (0.8), 120 (1.0), 119 (1.1), 118 (3.9), 117 (13.1), 116 (8.5), 115 (1.5), 113 (1.2), 107 (0.8), 105 (1.0), 104 (0.7), 92 (1.0), 91 (10.3), 90 (5.9), 89 (5.2), 78 (1.9), 77 (9.3), 68 (0.8), 67 (0.8), 66 (1.6), 65 (4.9), 63 (1.4), 51 (1.4), 41 (1.4).

*7.1.2.6. 3-(4-Chlorophenoxymethyl)-7-(4-chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole (**10**).* Recrystallized from DMF–MeOH mixture (2:1), yield 69.7%, m.p. 244–246

°C; IR (KBr) (ν, cm^{-1}): 1610 (C=N); ^1H NMR (DMSO-d_6, 300 MHz): 4.33 (dd, $J = 7.8$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 4.59 (dd, $J = 7.7$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 5.33 (s, 2H, OCH_2), 7.08–7.59 (m, 8H, ar-H); ^{13}C NMR (DMSO-d_6): 39.9 (imidazolidine-C-5), 53.5 (imidazolidine-C-6), 60.8 (CH_2O), 138.9 (triazole-C-3), 159.6 (C-7a), ar C: 115.9, 116.7, 124.1, 125.1, 128.7, 129.1, 144.0, 156.4; EIMS [70 eV, *m/z* (%)]: 362 (3.6), 361 (1.3), 360 (M^+, 5.8), 236 (4.0), 235 (30.1), 234 (13.9), 233 (100.0), 198 (1.7), 179 (4.8), 178 (2.1), 177 (16.1), 171 (8.7), 170 (73.9), 169 (30.4), 164 (2.3), 162 (2.4), 155 (2.4), 143 (7.8), 142 (3.0), 141 (1.4), 140 (3.6), 139 (9.1), 138 (4.6), 137 (26.1), 129 (2.1), 128 (2.5), 127 (5.7), 113 (4.4), 111 (14.1), 102 (9.0), 101 (4.1), 100 (1.2), 99 (10.0), 85 (1.4), 77 (1.6), 76 (3.2), 75 (11.2), 74 (1.4), 73 (4.3), 66 (5.2), 65 (1.4), 64 (1.6), 63 (3.7), 54 (2.4), 51 (2.2), 50 (2.2), 42 (3.1), 41 (9.1), 40 (1.3), 39 (2.3).

7.1.2.7. 3[-(2-Methyl-4-chloro)phenoxymethyl]-7-(4-chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole ***(11)***. Recrystallized from DMF–MeOH mixture (3:1), yield 66.4%, m.p. 246–247 °C; IR (KBr) (ν, cm^{-1}): 1608 (C=N); ^1H NMR (DMSO-d_6, 300 MHz): 2.19 (s, 3H, CH_3), 4.31 (dd, $J = 7.5$ Hz, $J' = 6.2$ Hz, 2H, CH_2), 4.57 (dd, $J = 7.5$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 5.20 (s, 2H, OCH_2), 7.12–7.55 (m, 7H, ar-H); ^{13}C NMR (DMSO-d_6): 15.6 (CH_3), 40.0 (imidazolidine-C-5), 53.5 (imidazolidine-C-6), 60.9 (CH_2O), 138.9 (triazole-C-3), 159.7 (C-7a), ar C: 113.5, 115.9, 124.1, 124.6, 126.2, 128.5, 128.7, 129.9, 141.1, 154.4; EIMS [70 eV, *m/z* (%)]: 376 (3.5), 374 (M^+, 5.5), 236 (4.8), 235 (30.6), 234 (15.4), 233 (100.0), 231 (1.7), 198 (1.7), 179 (4.4), 178 (1.9), 177 (13.9), 171 (8.7), 170 (72.5), 169 (27.1), 168 (2.0), 164 (1.9), 155 (2.5), 143 (8.7), 142 (3.8), 141 (6.4), 140 (2.9), 139 (9.0), 138 (5.5), 137 (24.4), 125 (2.7), 115 (1.6), 113 (4.7), 112 (1.6), 111 (9.9), 102 (8.7), 97 (2.8), 89 (2.7), 85 (1.6), 78 (3.5), 77 (17.6), 76 (2.9), 75 (7.7), 69 (2.0), 67 (1.9), 66 (9.0), 65 (8.1), 63 (2.8), 54 (4.0), 52 (1.7), 51 (5.4), 50 (2.0), 42 (2.8), 41 (8.3), 40 (4.2), 39 (4.7).

7.1.2.8. 3-(2,4-Dichlorophenoxymethyl)-7-(4-chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole ***(12)***. Recrystallized from DMF–MeOH mixture (3:1), yield 62.5%, m.p. 235–236 °C; IR (KBr) (ν, cm^{-1}): 1609 (C=N); ^1H NMR (DMSO-d_6, 300 MHz): 4.35 (dd, $J = 7.5$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 4.60 (dd, $J = 7.6$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 5.33 (s, 2H, OCH_2), 7.37–7.60 (m, 7H, ar-H); ^{13}C NMR (DMSO-d_6): 40.1 (imidazolidine-C-5), 53.5 (imidazolidine-C-6), 61.7 (CH_2O), 138.8 (triazole-C-3), 159.8 (C-7a), ar C: 115.9, 122.7, 124.2, 125.4, 128.0, 128.7, 129.3, 143.6, 151.9; EIMS [70 eV, *m/z* (%)]: 398 (1.5), 396 (4.4), 394 (M^+, 4.8), 236 (4.6), 235 (34.1), 234 (16.4), 233 (100.0), 198 (1.9), 179 (5.0), 178 (2.3), 177 (16.2), 171 (9.1), 170 (70.7), 169 (30.1), 165 (1.7), 164 (3.3), 163 (3.2), 162 (2.3), 161 (4.3), 155 (2.5), 143 (8.0), 142 (2.7), 140 (2.9), 139 (9.4), 138 (7.3), 137 (27.0), 135 (5.1), 133 (8.2), 113 (4.2), 112 (1.8), 111 (13.3), 109 (2.2), 102 (9.4), 99 (1.4), 85 (2.2), 77 (1.8), 76 (3.2), 75 (10.3), 74 (2.1), 73 (3.8), 67 (1.6), 66 (4.4), 63 (4.6), 62 (1.5), 54 (2.7), 51 (2.2), 50 (2.0), 42 (3.2), 41 (9.4), 39 (1.8).

7.1.2.9. 3-(2,4-Dichlorophenoxymethyl)-7-(3,4-dichlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole ***(13)***. Recrystallized from DMF, yield 44.7%, m.p. 228–230 °C; IR (KBr) (ν, cm^{-1}): 1611 (C=N); ^1H NMR (DMSO-d_6, 300 MHz): 4.38 (dd, $J = 7.5$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 4.65 (dd, $J = 7.6$ Hz, $J' = 6.3$ Hz, 2H, CH_2), 5.35 (s, 2H, OCH_2), 7.40–7.65 (m, 6H, ar-H); ^{13}C NMR (DMSO-d_6): 39.9 (imidazolidine-C-5), 53.1 (imidazolidine-C-6), 61.9 (CH_2O), 138.3 (triazole-C-3), 158.8 (C-7a), ar C: 114.6, 117.9, 122.0, 123.7, 126.6, 130.6, 131.1, 132.0, 139.5, 142.5, 146.9, 156.4.

7.1.3. Synthesis of 7-(4-chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole-3-thiol ***(14)***

1-(4-Chlorophenyl)-2-hydrazonoimidazolidine (0.01 mol) was dissolved in 30 mL of methanol. Sodium hydroxide (0.02 mol) dissolved in small amount of water and 20 mL of methanol was added. Then carbon disulfide (0.02 mol) in 30 mL of methanol was added dropwise at room temperature. The reaction mixture was stirred for 30 min at this temperature and then heated on the water bath as long as hydrogen sulfide was evolved (about 14 h). The residue was acidified and final product was obtained. The precipitate was filtered off, washed two times with water and finally purified by recrystallization from propan-2-ol.

7.1.3.1. 7-(4-Chlorophenyl)-5H-6,7-dihydroimidazo[2,1-c][1,2,4]triazole-3-thiol ***(14)***. Recrystallized from *i*PrOH, yield 76.1%, m.p. 294–297 °C; IR (KBr) (ν, cm^{-1}): 1619 (C=N); 2560 (SH); ^1H NMR (DMSO-d_6, 300 MHz): 3.80 (s, 1H, SH), 4.55 (dd, $J = 7.5$ Hz, $J' = 6.2$ Hz, dd, 2H, CH_2), 4.70 (dd, $J = 7.5$ Hz, $J' = 6.2$ Hz, dd, 2H, CH_2), 7.27–7.66 (m, 4H, ar-H); ^{13}C NMR (DMSO-d_6): 39.5 (imidazolidine-C-5), 53.0 (imidazolidine-C-6), 141.0 (triazole-C-3), 158.6 (C-7a), ar C: 115.5, 123.7, 128.8, 136.0.

7.2. HPLC experimental

HPLC analysis was performed using liquid chromatograph LC-10V AT_{VP} Shimadzu equipped with SUPELCOSIL™ LC-18 150 × 4.6 mm column (Supelco, Bellefonte, PA, USA) $d_p = 5$ μm, UV–vis SPD-10AV_{VP} Simadzu detector and Rheodyne 20 μL injector. Detection was at 254 nm. All chromatographic measurements were carried out at 22 ± 1 °C with eluent flow rate of 1.0 mL min^{-1}. Acetonitrile, methanol and dioxane of chromatographic quality, diethylamine were from Merck (Darmstadt, Germany). The pH of phosphate buffer used in experiments in 0.01 M L^{-1} concentrations were measured in aqueous solutions. Chromatograms were registered for using the program Shimadzu CLASS-VP.

Statistical analyses of the data were carried out with STATISTICA for Windows (Polish version, StatSoft, Inc. (1997), 2300 East 14 th Street, Tulsa, USA).

7.3. Biological assays

7.3.1. Inhibition of tumour cell growth assay

Compounds **7**, **12** were selected for screening towards in vitro three human cancer cell lines: human Caucasian colon adenocarcinoma (LS180), human uterus cancer (SiHa), human breast carcinoma (T47D). Besides two normal cell lines: human skin fibroblasts (HSF) and Vero (Green Monkey Kidney cells) were also included in the cytotoxicity study.

In the current protocol each cell line was inoculated at 10^4 cells per mL density and preincubated on a microtiter plate. Test agents were then added at double examined concentrations (10 and 50 μg mL^{-1}) and culture incubated for 72 h. End-point determinations were made with 5-bromo-2′-deoxy-uridine (BrdU) [46–49] labeling on Elisa reader (BIOTEC Instruments USA).

The growth percentage was evaluated spectrophotometrically versus untreated controls with used cell viability of growth assay. Results for each spectrophotometrical measure were noticed as percent of growth inhibition (Table 2). All experiments were done in triplicates. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

7.3.2. Detection of induction DNA damage in the tested cell lines after incubation with imidazotriazole 7

Following treatment of heterocycle **7** with 29.3 μM, cells were washed two times with phosphate buffered saline (PBS). In the next step the DNA was isolated by using the lysing buffer and proteolytic enzyme – proteinase K [50]. After purification, DNA samples were spread over 1% agarose gel with addition of propidium iodide. Electrophoresis was performed at 120 V for 1.5 h at the room temperature. All experiments were repeated three times. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

7.3.3. Evaluation of DNA damage

The evaluation of DNA damage was determined by using the comet assay [51]. DNA damage was quantified as an increase in tail moment, an indicator of damage that is proportional to the number of strand breaks per cell. Tail moment is the product of the percent of fluorescence in the tail and the distance between the means of the head and tail fluorescence distributions. All experiments were done in triplicates. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

7.3.4. Action of novel imidazotriazoles (1–3, 5–12) on the viability of RPMI 8226 and HeLa cancer cells

Human peripheral blood myeloma – RPMI 8226 (ECACC 87012702) derived from the peripheral blood of a 61-year-old male with multiple myeloma, is a recognized model for multiple myelomas [52,53]. Human Negroid cervix epitheloid carcinoma – HeLa (ECACC 93021013) is derived from a cervical carcinoma from a 31-year-old Negro. Both the above mentioned cancer cells were obtained from the European Collection of Cell Cultures (ECACC). Normal cell line – human skin fibroblasts (HSF) were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 100 μg mL^{-1} of streptomycin, 100 U mL^{-1} of penicillin in plastic tissue culture flasks (Nunc, Denmark).

Cells were maintained in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine and 10 μg mL^{-1} gentamycin and 1 mM Na pyruvate. Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2. These ones were passaged three times weakly and maintained at a density of 6×10^5 (leukaemia RPMI 8226), 3×10^5 (HeLa) and 1×10^5 cells (HSF) per mL. Cells used in the experiment were in logarithmic growth phase. The medium used for experiment had the same constituents as that used for cell passage, unless otherwise indicated.

MTT reduction cell viability assay was performed using cells cultured in 96-well plates. Compounds **1–3**, **5–12** were dissolved in DMSO prior to dilution into the biological assay. The effect of the examined concentrations (1, 50 and 100 μM) of heterocycles **1–3**, **5–12** on the cell viability was estimated by a colorimetric assay MTT based (the succinate dehydrogenase inhibition, SDI test) described by Takenouchi and Munekata [54]. The metabolic activity was measured in cell populations via incubation with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) that was reduced into a coloured, water-insoluble formazan salt by viable cells. Additions were made to the culture medium for 24 h. Cells were washed two times with HEPES-buffered incubation medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5.5 mM glucose and 20 mM HEPES, pH 7.4), and incubated for 45 min at 37 °C in HBM containing MTT (0.5 mg mL^{-1}). After this period, the HBM was carefully removed and the blue formazan product was solubilized in 300 μL of 100% DMSO. The absorbance of each well was read in an ELISA microplate reader at 570 nm. The obtained results were presented as percentage of cell viability in comparison to control. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland.

7.3.5. Antimicrobial activity studies

7.3.5.1. Disc-diffusion assay. Assay of antimicrobial activity in vitro: the synthesized compounds (**9**, **13**, **14**) were tested for their antimicrobial (antibacterial and antifungal) activities by disc-diffusion method by Kirby-Bauer, using Mueller-Hinton medium for bacteria and the same medium with 4% glucose for fungi. The majority of test microorganisms were obtained from clinical specimens of the Laboratory of Medical Microbiology Department, Medical University of Lublin. The assayed collection included the following microorganisms: *S. epidermidis*, *S. pyogenes*, *S. agalactiae* (Gram-positive bacteria), *Pr. vulgaris*, *K. pneumoniae*, *En. aerogenes* (Gram-negative bacteria), *and Aspergillus* spp. Moreover, two reference strains of bacteria – *S. aureus* ATCC 25923, *P. aeruginosa*

ATCC 27853 and one of fungi – *C. albicans* ATCC 10231 were included in these studies.

In the disc-diffusion method, sterile paper discs (ϕ 5 mm) impregnated with dissolved in dimethyl sulfoxide (DMSO) compound at concentrations of 100 μg mL^{-1} and 200 μg mL^{-1} were used. Discs containing DMSO were used as control. The microorganism cultures were spread over the following appropriate media: Mueller-Hinton agar for *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and Sabouraud agar for the yeast-like fungi (*C. albicans*) and for the moulds (*Aspergillus* spp.) in Petri dishes. Then, the paper discs impregnated with the solutions of the compound tested were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 35 °C for 24 h for the microorganism cultures. After incubation, the growth of inhibition zones around the discs were observed indicating that the examined compound inhibits the growth of microorganism [40–43]. Each assay in this experiment was repeated three times.

Ampicillin, chloramphenicol and miconazole were used as standard drugs. Dimethyl sulfoxide was used as a solvent control. Results were interpreted in terms of the diameter of the inhibition zones and are shown in Table 4.

7.3.5.2. Microdilution assays. The minimal inhibitory concentration (MIC) values for compounds tested (**9**, **13**, **14**), defined as the lowest concentration of the compound preventing the visible growth, were determined by using microdilution broth method according to NCCLS standards [38]. The inocula of microorganisms (10^6 c.f.u. mL^{-1}) were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The test compound dissolved in dimethyl sulfoxide (DMSO) was first diluted to the highest concentration (500 μg mL^{-1}) to be tested. Then serial two-fold dilutions were made in concentration ranges from 1.95 to 500 μg mL^{-1} in 10 mL sterile tubes. A prepared suspension of the standard microorganisms was added to each dilution in a 1:1 ratio. Growth (or its lack) of microorganisms was determined visually after incubation for 24 h at 37 °C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC.

The minimal inhibitory concentration (MIC) values were studied for two reference bacterial (*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853) and one fungal (*C. albicans* ATCC 10231) strains towards compounds **9**, **13**, **14** determined in the disc-diffusion assay. In this case, ampicillin, chloramphenicol and miconazole were used as standard drugs for comparison in the antimicrobial study. Control experiments using dimethyl sulfoxide were done for antimicrobial activity studies. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Medical Microbiology, Medical University, Lublin, Poland.

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