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Original article

Novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates from biologically active 1-aryl-2-hydrazinoimidazolines: Synthesis, crystal structure and antiproliferative activity

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Abstract

The 1-aryl-2-hydrazinoimidazolines (**2a–h**) were directly obtained from appropriate 1-aryl-2-methylthioimidazolines (**1a–h**) by condensation reaction with hydrazine hydrate. Antimicrobial activities of two 1-aryl-2-hydrazinoimidazolines (**2b** and **2e**) are presented. Their chemical structures were confirmed by IR, ^{1}H NMR, EI-MS and elemental analysis. The susceptibility of Gram-positive and Gram-negative bacterial strains, mould and yeast-like fungi strains to synthesized compounds and the MIC values against two reference strains of bacteria were determined. The strongest antibacterial activity for compound **2b** in relation to reference Gram-negative *Escherichia coli* ATCC 25922 bacterial strain with minimal inhibitory concentration (MIC) value of 3.91 μg mL^{-1} was found. Compound **2b** showed superior activity (MIC) to ampicillin and comparable to chloramphenicol. A novel compound **2e** was found to be effective against *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 at concentrations of 7.81 μg mL^{-1} and 15.62 μg mL^{-1}, respectively. Compound **2e** revealed antibacterial activity against *E. coli* ATCC 25922, superior to ampicillin and inferior to chloramphenicol. Against *S. aureus* ATCC 25923 strain tested, compound **2e** demonstrated MIC value inferior to ampicillin and chloramphenicol.

Moreover, the synthesis, crystal structure and antiproliferative activity of novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates (**3a–f** and **3g–j**) are presented. These compounds were obtained from 1-aryl-2-hydrazinoimidazolines (**2a–f**) by the addition and cyclization reactions with fumaric acid esters. Molecular structures of these compounds were confirmed by elemental analysis, IR, ^{1}H NMR, ^{13}C NMR, EI-MS and by X-ray crystallography (for **3g**).

The tested imidazotriazines **3e**, **3i** and **3j** exhibited anticancer activities towards the following cancer cells: LS180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701, uterus cancer cells), and T47D (ECACC 85102201, human breast carcinoma cells). Compounds **3i** and **3j** having comparable GI values (above 50%) towards uterus cancer cell line (SiHa) at both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}) were found to be the most effective against this cancer cell line; their GI factors were 53%, 51% and 62%, 55%, respectively, in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}). Furthermore, the distinctly marked lower cytotoxicity of tested imidazotriazines **3i** and **3j** against normal cell lines (HSF, human skin fibroblast cells and Vero African Green Monkey Kidney cells, GMK clone) and almost 2-times higher against the majority of cancer cell lines was confirmed.

Keywords: Imidazolines; Hydrazino group; Antibacterial activity; In vitro study; MIC; Imidazo[2,1-*c*][1,2,4]triazines; Crystal structure; Antiproliferative activity

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1. Introduction

Imidazoline(4,5-dihydroimidazole) and its derivatives occupy a pivotal position in modern heterocyclic chemistry. Many of them show pharmacological activity as ligands of the imidazoline receptor. Imidazoline system is the structural element of many drugs possessing different pharmacological activities. The following adrenergic imidazoline derivatives are applicable in medicine: naphazoline, xylometazoline, oxymetazoline, fenoxazoline, and tetryzoline. Others derivatives such as tolazoline and phentolamine are used as α-adrenolytics, cifenline as antiarrhythmic, clonidine as hypotensive and antazoline as antihistaminic [1,2].

Moreover, from the literature data, it follows that depending on the type of substituent, derivatives of imidazoline may also show antimicrobial properties [3–5]. The definite derivatives of imidazoline, aromatic diimidazolines related to pentamidine, were proved to be active against AIDS related opportunistic pathogens, such as *Candida albicans* and *Candida neoformans* [3].

Intercalating antineoplastic bisantrene, therapeutically used against adult acute non-lymphotic leukaemia, is structurally based on the 2-hydrazino-Δ^2-imidazoline heterocyclic system. Some antibacterial hydrazine derivatives are applied as antituberculostatic drugs (thioacetazone, isoniazid, ftivazid, verazid, furilazone) [1].

Compounds containing the 1,2,4-triazine ring are found in natural sources and many of them showed important biological activities. For example azaribine – antiviral drug is structurally based on the 1,2,4-triazine heterocyclic system [2]. Condensed 1,2,4-triazines found application as pharmaceuticals, herbicides, pesticides and dyes [6–10]. Pyrrolo[2,1-*f*][1,2,4]triazines showed an interesting broad spectrum of antiproliferative activity and a pronounced in vitro growth inhibitory activity against leukaemic cell lines (comparable to that of 9-deazaadenosine), whilst pyrrolo[2,1-*c*][1,2,4]triazines demonstrated inhibitory effects on the growth of a wide range of cancer cells generally at 10^{-5} M level and, in some cases, even at micromolar concentrations [11,12]. Pyrazolo[5,1-*c*][1,2,4]triazines exhibited antitumour and antifungal activities [13,14]. It is noteworthy that many potential anticancer and antiviral drugs have been modeled on them [15–18].

Previous studies concerning the synthesis of imidazo[2,1-*c*][1,2,4]triazin-4(4*H*)-ones [19–22] carried out in the Department of Synthesis and Technology of Drugs have disclosed some compounds with various aryl substituents at the 8-position, and with benzyl, substituted benzyl, methoxycarbonylmethyl or hydroxyl substituents at the 3-position. These compounds have revealed a significant antinociceptive activity on the central nervous system in behavioral animal tests, and a low acute toxicity (LD_{50} in the range from over 1100 to over 2000 mg kg^{-1} i.p.). On the other hand, the definite derivative of imidazo[2,1-*c*][1,2,4]triazine, viz. that with 4-chlorophenyl substituent at the 8-position and with hydroxycarbonylmethyl substituent at the 3-position, showed a significant activity against all Gram-negative bacterial strains tested [23].

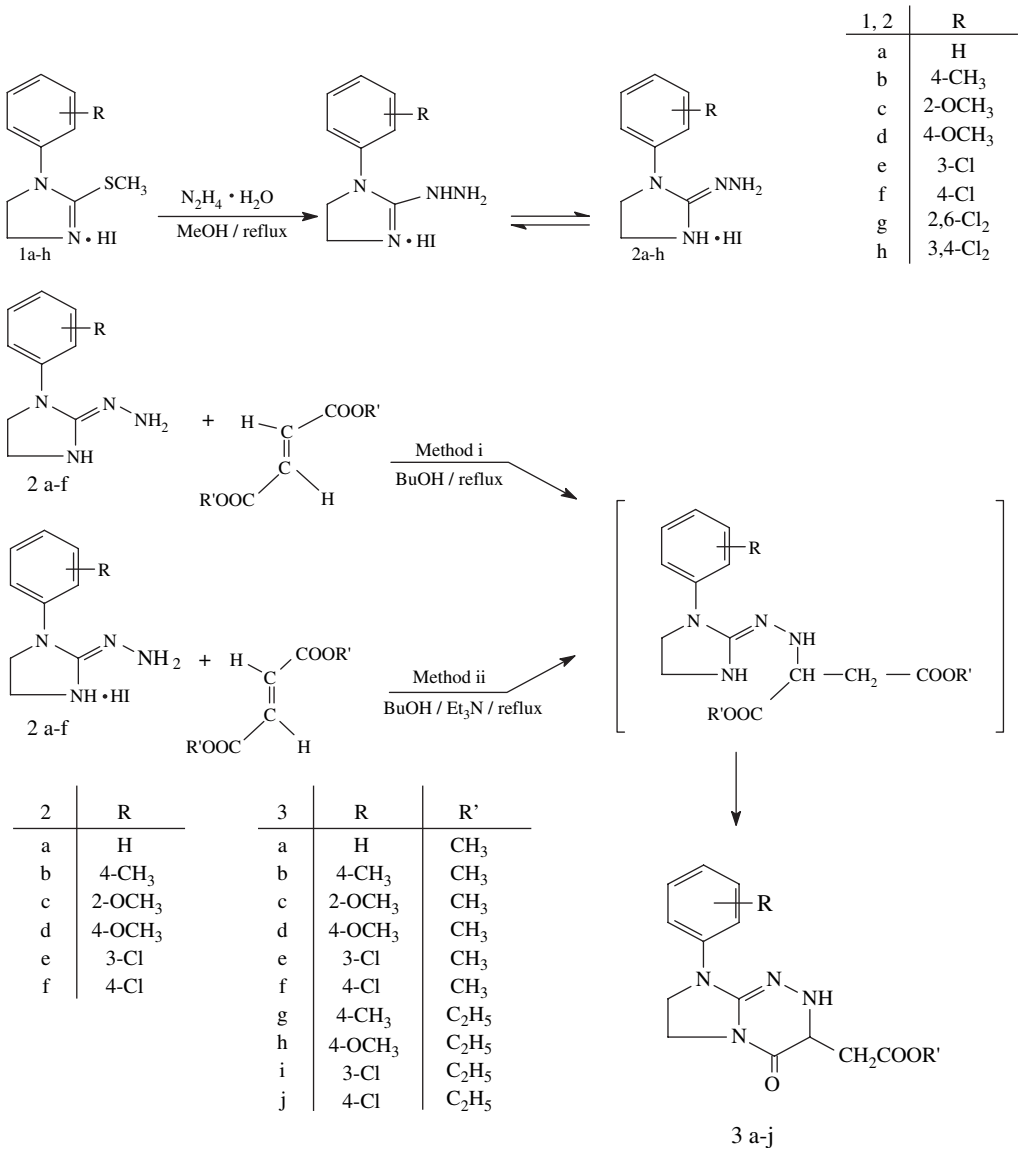
Prompted by these reports, and in continuation of search for bioactive molecules, it seemed worthwhile to synthesize some newer congeners of 1-aryl-2-hydrazinoimidazoline and novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates. This paper presents the preparation of six new 1-aryl-2-hydrazinoimidazolines. Two of them (**2b** and **2e**) proved active as antimicrobials. Furthermore, we report herein the synthesis of 10 novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates and the cytotoxic activity of three representatives of this group (**3e**, **3i**, and **3j**).

2. Chemical part

The synthetic pathway for compounds described is illustrated in Scheme 1.

Biologically active 1-aryl-2-methylthioimidazolines (**1a–h**) as the starting materials were prepared by patent pending according to Sztanke et al. and previous paper [22]. Treatment of compounds **1a–h** with hydrazine hydrate afforded the corresponding 1-aryl-2-hydrazinoimidazolines of type **2a–h**. These compounds were obtained by earlier described method [24]. Compounds **2b** and **2f** are known [24] but the other ones such as **2a**, **2c–e**, **2g**, and **2h** are new and their synthesis, biological activities, physicochemical and spectral data have not been described in the literature as yet. NMR spectral characteristic of compounds **2a–h** revealed in their ^{1}H NMR spectra signals of the aromatic protons in the range 6.92–7.74 ppm and signals of both the H-4 and H-5 methylene protons in the range 3.81–4.51 ppm; new signals derived from hydrazine structure appeared between 4.62–5.26 ppm (–NH*N*H_2) and in the range 7.65–8.71 ppm (–*N*H*N*H_2), integrating for two protons and one proton, respectively (controlled by exchange with D_2O). Methoxy groups in the 2- and 4-position of the phenyl ring (compounds **2c** and **2d**) were observed as characteristic singlets at 3.83 and 3.84 ppm. Besides compounds **2a–h** gave molecular ion peaks which were consistent with their molecular formulae. The elemental analyses of synthesized compounds are consistent with the assigned structures.

New derivatives of methyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (**3a–f**) were afforded by the reaction of appropriate 1-aryl-2-hydrazinoimidazolines (1-aryl-2-hydrazonoimidazolidines) with dimethyl fumarate, whereas new derivatives of ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (**3g–j**) were obtained from respective 1-aryl-2-hydrazonoimidazolidines on reacting with diethyl fumarate. The reaction of 1-aryl-2-hydrazinoimidazoline (1-aryl-2-hydrazonoimidazolidine) derivatives with fumaric acid esters has not been described in the literature as yet, and this reaction represents a novel synthetic route for preparing the polynitrogenated bicyclic imidazo[2,1-*c*][1,2,4]triazine system. The synthetic pathway in the synthesis of 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetic acid ester derivatives may be useful in view of biological interest in these class of compounds. The above-mentioned reaction can be carried out



1, 2	R
a	H
b	4-CH_3
c	2-OCH_3
d	4-OCH_3
e	3-Cl
f	4-Cl
g	2,6-Cl_2
h	3,4-Cl_2

2	R
a	H
b	4-CH_3
c	2-OCH_3
d	4-OCH_3
e	3-Cl
f	4-Cl

3	R	R’
a	H	CH_3
b	4-CH_3	CH_3
c	2-OCH_3	CH_3
d	4-OCH_3	CH_3
e	3-Cl	CH_3
f	4-Cl	CH_3
g	4-CH_3	C_2H_5
h	4-OCH_3	C_2H_5
i	3-Cl	C_2H_5
j	4-Cl	C_2H_5

Scheme 1. Synthetic route to obtained compounds.

starting both from free base (method i) or hydroiodide in the triethylamine presence (method ii) with comparable yields. The reaction was carried out by refluxing the appropriate 1-aryl-2-hydrazinoimidazoline with fumaric acid esters in alcoholic medium for 6–7 h. The reaction conditions were established experimentally. The course of the reaction includes the formation of intermediate chain derivatives (dimethyl succinates and diethyl succinates) as a result of the addition reaction. These ones may cyclize to the imidazo[2,1-*c*][1,2,4]triazine system of the **3a–j** type (methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate derivatives) as illustrated in Scheme 1, or to the imidazo [2,1-*c*][1,2,4]triazole system (with liberation of methyl or ethyl acetate). Based on the spectral data results the concurrent course of the cyclization reaction was excluded. The scrutiny of ^{1}H NMR, ^{13}C NMR, EI-MS and IR spectra confirms that under the reaction conditions, the formation of the bicyclic imidazo[2,1-*c*][1,2,4]triazine ring system is accompanied with the liberation of methanol (**3a–f**) or ethanol (**3g–j**). Finally, the structure of **3g** was confirmed by X-ray crystallography. Perspective view of the molecule **3g** with atom numbering is shown in Fig. 1. In the ^{1}H NMR spectra of compounds **3a–f** signals derived from the ester group

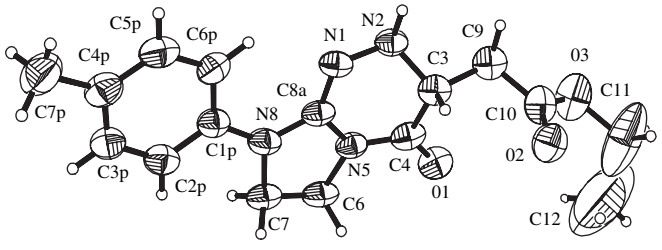


Fig. 1. Structure of molecule **3g**. Bond distances within the heterocyclic ring system are N1–N2 1.442(5); N2–C3 1.453(6); C3–C4 1.508(7); C4–N5 1.342(6); N5–C6 1.448(6); C6–C7 1.501(7); C7–N8 1.484(6); C8a–N8 1.367(6); N1–C8a 1.278(5); C8a–N5 1.401(6); C4–O1 1.235(6) Å.

protons ($-OCH_3$) were observed at 3.61 ppm, integrating for three protons, whereas in the ^{13}C NMR spectra, the signals belonging to the same group were recorded at 52.5 ppm. In the ^1H NMR spectra of these compounds, additional signals derived from two methylene protons of the ester group, connected with the triazine–CH (triazine–CH–CH_2–$COOCH_3$), were observed as two double doublet for each non-equivalent proton at ca. 2.61 ppm with a coupling constant of $^2J \sim 16.3$ Hz and $^3J \sim 6.5$ Hz and at ca. 2.76 ppm with a coupling constant of $^2J \sim 16.3$ Hz and $^3J \sim 5.7$ Hz, integrating for one proton and one proton, respectively. Additionally in the ^{13}C NMR spectra, the secondary carbon signal derived from the methylene group at the 3-position in [1,2,4]triazine ring resonated at 33.8 (**3a**) and 33.7 ppm (**3e** and **3f**). In the ^1H NMR spectra of compounds **3a–f**, the signals derived from triazine–NH group at the 2-position were observed as a doublet with a coupling constant of $J \sim 3.4$ Hz and resonated between 6.04 and 6.3 ppm, integrating for one proton (exchangeable with D_2O). The multiplet in the range 3.74–3.98 ppm confirmed the presence of four protons derived from ethylene formation ($-CH_2-CH_2-$) of the imidazolidine ring and one proton derived from the triazine–CH. The ^{13}C NMR chemical shift values of the C-7 and C-6 carbon atoms confirmed unequal character of both methylene groups as well (ca 45.5 ppm for C-7 and 39.9 ppm for C-6). In the ^{13}C NMR spectra of imidazotriazines **3a**, **3e**, and **3f** the carbon signals derived from the exocyclic ester group ($-COOCH_3$) were recorded in the range of 171.1–171.6 (exocyclic-C=O) and 52.5 ppm ($-CH_3$), whereas the triazine–C-4 (triazine–C=O) and triazine–C-3 (asymmetric carbon atom) signals were found at about 167.9 and 54.0 ppm, respectively.

In the IR spectra of compounds **3b** and **3f** the presence of absorption bands at 1683 (**3f**) and 1689 cm^{-1} (**3b**), ascribable to the triazine–C=O group confirmed as well the formation of cyclic products. These compounds also exhibited characteristic absorption bands at about 1740 cm^{-1}, denoting the presence of the exocyclic ester-C=O group.

In the ^1H NMR spectra of compounds **3g–j** additional signals derived from the ester group protons were observed at about 4.06–4.1 ($-OCH_2CH_3$) and 1.19 ppm ($-OCH_2CH_3$), integrating for two protons and three protons, respectively. In the ^{13}C NMR spectra, the signals belonging to the secondary and primary carbons of the same ester group were recorded in the region of 60.2 and 14.0 ppm, respectively. Signals derived from two methylene protons of side chain in 3-position of the triazine ring (triazine–CH–CH_2–$COOC_2H_5$) were observed as two double doublet for each non-equivalent proton at ca. 2.60 ppm with a coupling constant of $^2J \sim 16.4$ Hz and $^3J \sim 6.3$ Hz and at ca. 2.75 ppm with a coupling constant of $^2J \sim 16.3$ Hz and $^3J \sim 5.83$ Hz, integrating for one proton and one proton, respectively. Additionally, in the ^{13}C NMR spectra of compounds **3g–j** the secondary carbon signal derived from the methylene group at the 3-position in the triazine ring was observed at 33.0 ppm. In the ^1H NMR spectra of compounds **3g–j**, the signals derived from the triazine–NH group at the 2-position were observed as a doublet with a coupling constant of $J \sim 3.4$ Hz and resonated between 6.03 and 6.19 ppm, integrating for one proton (exchangeable with D_2O). The multiplet in the range 3.61–4.02 ppm confirmed the presence of four protons derived from ethylene formation ($-CH_2-CH_2-$) of the imidazolidine ring and one proton from triazine–CH. The ^{13}C NMR chemical shift values of the C-7 and C-6 carbon atoms confirmed unequal character of both methylene groups as well (ca. 44.7 ppm for C-7 and 39.0 ppm for C-6). In the ^{13}C NMR spectra of compounds **3g**, **3h**, and **3j** the carbon signals derived from the exocyclic ester group ($-COOCH_2CH_3$) were recorded in the range of 170.2–170.3 (exocyclic-C=O), 60.2 ($-CH_2CH_3$) and 14.0 ppm ($-CH_2CH_3$), whereas the triazine–C-4 and triazine–C-3 signals were observed at about 167.1 and 53.2 ppm, respectively.

In the IR spectra of compounds **3g–j** the presence of absorption bands in the range 1684–1692 cm^{-1} confirmed the formation of cyclic products, whereas the presence of absorption bands at about 1741 cm^{-1} was characteristic for the ester-C=O group derived from the side chain at the 3-position of the triazine ring.

3. Pharmacology

3.1. 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) [25–27] and the disc-diffusion method by Kirby–Bauer [28–30].

The in vitro activities of the two compounds obtained against pathogenic bacteria, yeast-like fungi and moulds were compared. The following microorganisms were used *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae* (Gram-positive bacteria), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* (Gram-negative bacteria), *C. albicans*, *Aspergillus* spp. The majority of strains under study were clinical isolates, identified with conventional morphological and biochemical methods. The microdilution method for the estimation of MIC values (the lowest concentration of compounds required to inhibit the growth of the tested microorganism) was applied to evaluate the antibacterial activity. In this method two reference strains of bacteria – *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 – were included in this study. The antibacterial potency of the compounds under conditions was compared with the activities of topical antibacterial drugs – ampicillin and chloramphenicol.

3.2. Antiproliferative activity studies

The newly synthesized compounds **3e**, **3i** and **3j** were evaluated for their antiproliferative activities towards human tumour cell lines derived from various human cancer types (colon, breast, uterus): LS 180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701 uterus cancer cells), and T47D (ECACC 85102201,

human breast carcinoma cells); besides these two 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).

4. Results and discussion

The antimicrobial activities of obtained compounds against bacterial, moulds and yeast-like fungi strains and the MIC values against two reference strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922) were tested by using the disc-diffusion method and the microdilution assay. The results from experiments were compared with those of ampicillin and chloramphenicol as references for antibacterial agents. The MIC values of compounds tested and both standard drugs are listed in Table 1.

Two compounds tested (**2b** and **2e**) in the present study were found to have highly significant antibacterial activities against the microorganisms listed in Table 1 but no antifungal activities. The examined compounds were also inactive against moulds and yeast-like fungi. 1-Aryl-2-hydrazinoimidazolines (**2b** and **2e**) showed good activity in relation to reference bacterial strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922). Compound **2e** was effective against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at concentrations of 7.81 μg mL^{-1} and 15.62 μg mL^{-1}, respectively. Its antibacterial potency against *E. coli* ATCC 25922 was 1.6-fold higher than that of ampicillin and 2-fold lower than that of chloramphenicol. Against *S. aureus* ATCC 25923 strain, compound **2e** demonstrated MIC values ranging from 1.2- to 4-fold higher than those of ampicillin and chloramphenicol, respectively. Moreover, compound **2e** was active against *P. aeruginosa*, *Pr. vulgaris* at concentrations of 100 μg mL^{-1} and 200 μg mL^{-1} and against *S. epidermidis* at a concentration of 200 μg mL^{-1} in the disc-diffusion assay. Compound **2b** was active against *E. coli* ATCC 25922 at a concentration of 3.91 μg mL^{-1} and inactive against *S. aureus* ATCC 25923. It is noteworthy that compound **2b** was generally active against all Gram-negative bacterial strains tested (*E. coli*, *P. aeruginosa*, *Pr. vulgaris*, *K. pneumoniae*, *En. aerogenes*) and inactive against all Gram-positive bacterial strains, which were examined. Compound **2b** was found to be over 3-fold more potent against *E. coli* ATCC 25922 to ampicillin and equipotent to chloramphenicol.

As a result compound **2e** was found to exhibit the most potent in vitro antibacterial activity with MIC value of 7.81 μg mL^{-1} against *S. aureus* ATCC 25923 and may be considered promising for the development of new antibacterial agents.

Compound **2b** was found to exhibit the most potent in vitro anti-Gram-negative activity with MIC value of 3.91 μg mL^{-1} against *E. coli* ATCC 25922. Compound **2b**, more potent to ampicillin and equipotent to chloramphenicol, was generally active against all Gram-negative bacterial strains tested and may be considered promising for the development of selective anti-Gram-negative antibacterial agents.

Imidazotriazines **3e**, **3i** and **3j** were evaluated for their anticancer activities. Results for each test compound are reported as the growth inhibition percentage of tested cells in comparison to the untreated cells. Compounds which reduced the growth are passed on for evaluation towards three human cancer and two normal cell lines. These compounds were found to be active and they exhibited a different level of anticancer properties. According to the data listed in Table 2, compounds **3i** and **3j** having comparative GI values (above 50%) towards uterus cancer cell line (SiHa) at both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}) were found to be the most effective against this cancer line; their GI factors were 53%, 51% and 62%, 55%, respectively. Compound **3i** was about twice more potent towards human breast carcinoma cells (T47D) and insignificantly less potent against human colon adenocarcinoma cells (LS180) in comparison with **3j**. These compounds are the ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate derivatives and they differ only in location of a chloro substituent at *para* (**3j**) and *meta* (**3i**) position of phenyl ring. Compound **3e**, i.e. methyl 2-[4-oxo-8-(3-chlorophenyl)-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl]acetate, was active against the colon, breast and uterus carcinoma lines (LS180, T47D, SiHa), especially at a concentration higher than 10 μg mL^{-1}. According to the data listed in Table 2, compound **3e** had comparative potential to reduce the growth of three above-mentioned cancer lines. The most sensitive cell line was the colon carcinoma cell line LS180 against which compound **3e** showed GI value of 44%. The remaining uterus

Table 1
Antibacterial activity expressed as MIC (μg mL^{-1}) of tested compounds

Microorganisms/code	Compound		Standard drugs	
	2b	**2e**	A	C
Escherichia coli ATCC 25922	3.91	7.81	12.5	3.91
Staphylococcus aureus ATCC 25923	NT	15.62	12.5	3.91

NT = not tested due to inactivity in the agar-well diffusion assay. Standard drugs: A – ampicillin; C – chloramphenicol.

Table 2
Inhibition of in vitro normal and tumour cells' growth by imidazotriazines **3e**, **3i**, and **3j**

Cell line	Cytotoxicity (GI in %)					
	3e		**3i**		**3j**	
	I	II	I	II	I	II
Normal cell lines						
HSF	12	17	19	20	12	16
GMK	20	35	20	25	20	20
Cancer cell lines						
LS180	30	44	30	35	32	43
SiHa	30	40	53	62	51	55
T47D	24	38	24	44	10	29

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 85102201) – human breast carcinoma cells; I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

and breast cancer cell lines (SiHa, T47D) showed similar susceptibility levels to this compound, especially at a higher concentration. Compounds **3e** and **3i** showed similar potentials to reduce the growth of breast cancer line (T47D) at both examined concentrations, while **3e** and **3j** demonstrated similar inhibitory effects on the growth of colon cancer line (LS180). Compound **3i** was less potent against this cancer cell line in comparison with **3e** and **3j**, but only at a higher concentration. Besides, the distinctly marked lower cytotoxicity of compounds **3i** and **3j** against normal cell lines (HSF, human skin fibroblast cells and Vero African Green Monkey Kidney cells, GMK clone) and almost 2-times higher against the majority of cancer cell lines was ascertained (Figs. 2–6). Taking into consideration the growth inhibition comparative study results concerning the influence of tested compounds on cancer and normal cell lines it can be expected the selective action of examined compounds. Also the anticancer activity of tested compounds seemed to be dose-dependent.

In conclusion two tested imidazo[2,1-*c*][1,2,4]triazine derivatives (**3i** and **3j**) were found to be most effective in vitro against uterus cancer cell line in both examined concentrations. These compounds demonstrate antiproliferative properties justifying further investigation as potential anticancer agents. Further studies are in progress to define the important mechanisms of action of the above-mentioned compounds.

5. Experimental protocols

5.1. Chemical and crystallographic analyses

Chemicals (hydrazine hydrate, dimethyl fumarate and diethyl fumarate) were purchased from Merck 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. ^{1}H NMR spectra for compounds **2a**–**h** were recorded on a Tesla BS-567A 100 MHz spectrometer in DMSO-d_6 (**2a**, **2c**–**e**, **2g**, and **2h**), $CDCl_3$ (**2b**) or $(CD_3)_2CO$ (**2f**). NMR spectra (^{1}H and ^{13}C) of the other ones were recorded on a Bruker 300 MHz spectrometer in DMSO-d_6 with TMS as an external standard

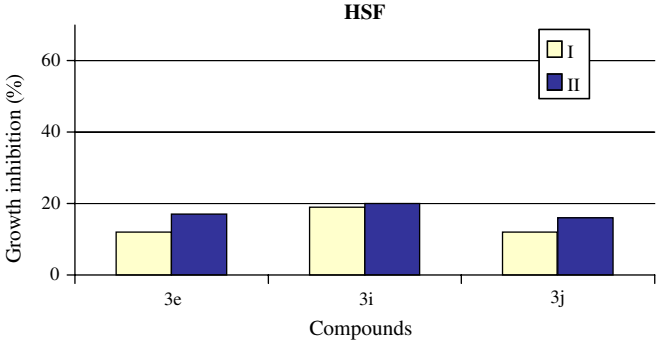


Fig. 2. Cytotoxicity of tested imidazotriazines **3e**, **3i**, and **3j** in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}) towards human skin fibroblast (HSF) cell line expressed as GI (%); I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

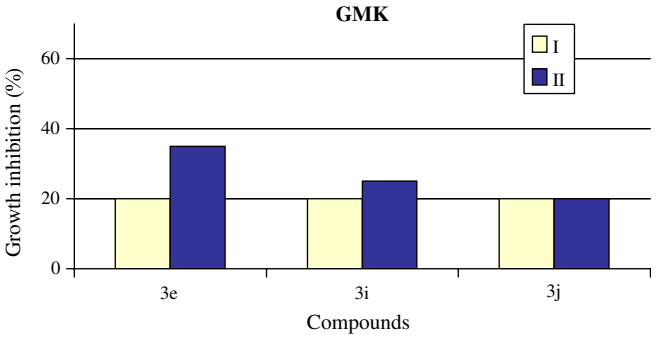


Fig. 3. Cytotoxicity of tested imidazotriazines **3e**, **3i**, and **3j** in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}) towards African Green Monkey Kidney (GMK) cell line expressed as GI (%); I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

at 295 K. Mass spectroscopic analyses for compounds **2a**–**h** were performed on AMD-402 mass spectrometer for molecular ion peaks. EI-MS spectra for compounds **3a**, **3d**–**h**, and **3j** were recorded at 70 eV on a Trace DSQ mass spectrometer. Diffraction data for **3g** were measured at 295 K on a KM4 diffractometer using variable scan speed in the ω–2θ scan mode and graphite monochromated Cu Kα radiation ($\lambda = 1.54178$ Å). A single crystal of dimensions $0.37 \times 0.37 \times 0.26$ mm was used. 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. Elemental analyses were performed on a Perkin–Elmer analyzer and were in range of ±0.4% for each element analyzed (C, H, N, Cl, I).

*5.1.1. Synthesis of 1-aryl-2-hydrazinoimidazoline hydroiodides (**2a**–**h**) (general procedure)*

Appropriate 1-aryl-2-methylthioimidazoline hydroiodide (0.1 mol) and hydrazine hydrate (0.11 mol) were heated under reflux in 80 mL of methanol for 30 h. The solvent was removed by evaporation and upon cooling oily residue was

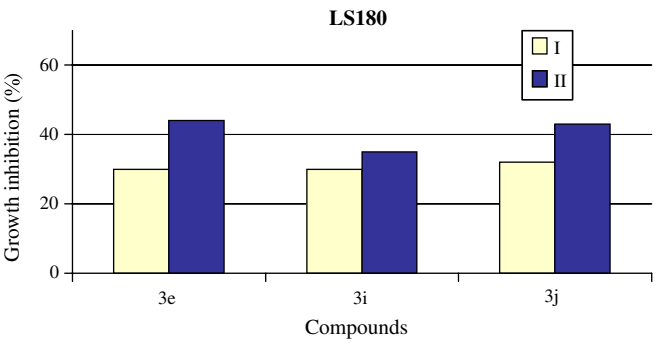


Fig. 4. Inhibition of in vitro human Caucasian colon adenocarcinoma cell line (LS180) growth (expressed as GI percentage) by tested imidazotriazines **3e**, **3i**, and **3j** in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}); I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

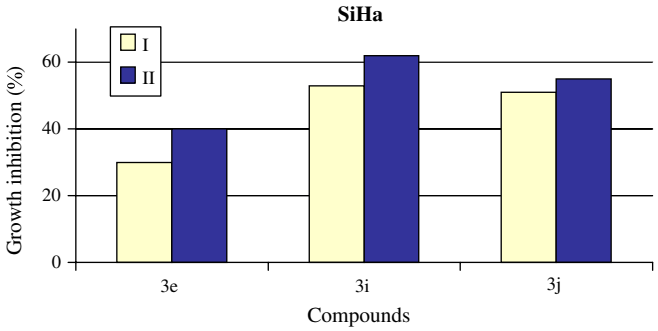


Fig. 5. Inhibition of in vitro human uterus carcinoma cell line (SiHa) growth (expressed as GI percentage) by tested imidazotriazines **3e**, **3i**, and **3j** in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}); I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

triturated with ethanol. The crude product was separated and crystallized from propan-2-ol or DMF.

*5.1.1.1. 1-Phenyl-2-hydrazinoimidazoline hydroiodide (**2a**).* Recrystallization from propan-2-ol, yield 71%, m.p. 139–140 °C. Analysis (calc/found%) for $C_9H_{13}IN_4$: C: 35.54/35.7, H: 4.31/4.3, I: 41.73/41.6, N: 18.42/18.5; IR (KBr) (ν, cm^{-1}): 1510 (N^+H), 1571 (C=N), 3460–3285 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.81–4.24 (m, 4H, $2CH_2$), 5.1 (s, 2H, NH_2), 6.92–7.62 (m, 5H, $CH_{arom.}$), 8.25 (s, 1H, NH), 9.06 (s, 1H, N^+H); *m/z*: 304[M^+].

*5.1.1.2. 1-(4-Methylphenyl)-2-hydrazinoimidazoline hydroiodide (**2b**).* Recrystallization from propan-2-ol, yield 76%, m.p. 65–68 °C. Analysis (calc/found%) for $C_{10}H_{15}IN_4$: C: 37.75/37.9, H: 4.75/4.8, I: 39.89/39.7, N: 17.61/17.7; IR (KBr) (ν, cm^{-1}): 1515 (N^+H), 1572 (C=N), 3466–3284 ($NH + NH_2$); ^1H NMR (δ, ppm, $CDCl_3$, TMS): 2.33 (s, 3H, CH_3), 3.90–4.15 (m, 4H, $2CH_2$), 4.62 (s, 2H, NH_2), 7.12–7.38 (m, 4H, $CH_{arom.}$), 7.66 (s, 1H, NH), 8.05 (s, 1H, N^+H); *m/z*: 318[M^+].

*5.1.1.3. 1-(2-Methoxyphenyl)-2-hydrazinoimidazoline hydroiodide (**2c**).* Recrystallization from propan-2-ol, yield 69%, m.p. 188–189 °C. Analysis (calc/found %) for $C_{10}H_{15}IN_4O$: C: 35.94/35.8, H: 4.52/4.4, I: 37.98/37.8, N: 16.77/16.9; IR (KBr) (ν, cm^{-1}): 1512 (N^+H), 1579 (C=N), 3431–3280 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.83 (s, 3H, OCH_3), 3.88–4.12 (m, 4H, $2CH_2$), 5.22 (s, 2H, NH_2), 7.18–7.65 (m, 4H, $CH_{arom.}$), 8.52 (s, 1H, NH), 9.21 (s, 1H, N^+H); *m/z*: 334[M^+].

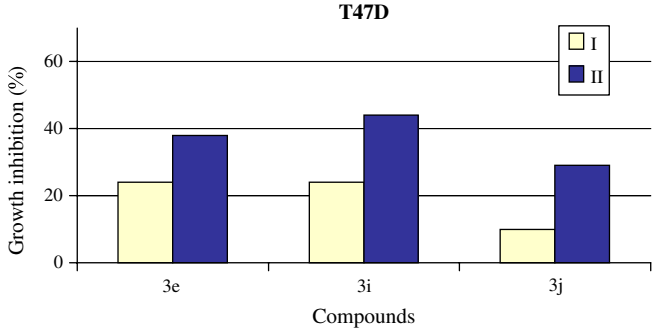


Fig. 6. Inhibition of in vitro human breast carcinoma cell line (T47D) growth (expressed as GI percentage) by tested imidazotriazines **3e**, **3i**, and **3j** in both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}); I – concentration of 10 μg mL^{-1} and II – concentration of 50 μg mL^{-1}.

*5.1.1.4. 1-(4-Methoxyphenyl)-2-hydrazinoimidazoline hydroiodide (**2d**).* Recrystallization from propan-2-ol, yield 73%, m.p. 194–196 °C. Analysis (calc/found%) for $C_{10}H_{15}IN_4O$: C: 35.94/36.1, H: 4.52/4.6, I: 37.98/37.9, N: 16.77/16.7; IR (KBr) (ν, cm^{-1}): 1511 (N^+H), 1577 (C=N), 3426–3278 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.84 (s, 3H, OCH_3), 3.96–4.17 (m, 4H, $2CH_2$), 5.12 (s, 2H, NH_2), 6.94–7.35 (m, 4H, $CH_{arom.}$), 8.14 (s, 1H, NH), 9.1 (s, 1H, N^+H); *m/z*: 334[M^+].

*5.1.1.5. 1-(3-Chlorophenyl)-2-hydrazinoimidazoline hydroiodide (**2e**).* Recrystallization from propan-2-ol, yield 62%, m.p. 55–56 °C. Analysis (calc/found%) for $C_9H_{12}ClIN_4$: C: 31.93/31.8, H: 3.57/3.6, Cl: 10.47/10.5, I: 37.48/37.4, N: 16.55/16.6; IR (KBr) (ν, cm^{-1}): 1509 (N^+H), 1571 (C=N), 3460–3315 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.95–4.22 (m, 4H, $2CH_2$), 5.22 (s, 2H, NH_2), 7.25–7.53 (m, 4H, $CH_{arom.}$), 8.65 (s, 1H, NH), 9.26 (s, 1H, N^+H); *m/z*: 338[M^+].

*5.1.1.6. 1-(4-Chlorophenyl)-2-hydrazinoimidazoline hydroiodide (**2f**).* Recrystallization from propan-2-ol, yield 72%, m.p. 164–166 °C. Analysis (calc/found%) for $C_9H_{12}ClIN_4$: C: 31.93/32.0, H: 3.57/3.5, Cl: 10.47/10.4, I: 37.48/37.5, N: 16.55/16.5; IR (KBr) (ν, cm^{-1}): 1504 (N^+H), 1571 (C=N), 3456–3313 ($NH + NH_2$); ^1H NMR (δ, ppm, $(CD_3)_2CO$, TMS): 3.92–4.51 (m, 4H, $2CH_2$), 4.86 (s, 2H, NH_2), 7.47–7.74 (m, 4H, $CH_{arom.}$), 8.61 (s, 1H, NH), 9.30 (s, 1H, N^+H); *m/z*: 338[M^+].

*5.1.1.7. 1-(2,6-Dichlorophenyl)-2-hydrazinoimidazoline hydroiodide (**2g**).* Recrystallization from propan-2-ol, yield 65%, m.p. 242–244 °C. Analysis (calc/found%) for $C_9H_{11}Cl_2IN_4$: C: 28.98/28.8, H: 2.97/2.9, Cl: 19.01/18.9, I: 34.02/34.1, N: 15.02/15.1; IR (KBr) (ν, cm^{-1}): 1508 (N^+H), 1583 (C=N), 3452–3305 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.88–4.12 (m, 4H, $2CH_2$), 5.24 (s, 2H, NH_2), 7.18–7.65 (m, 3H, $CH_{arom.}$), 8.55 (s, 1H, NH), 9.23 (s, 1H, N^+H); *m/z*: 373[M^+].

*5.1.1.8. 1-(3,4-Dichlorophenyl)-2-hydrazinoimidazoline hydroiodide (**2h**).* Recrystallization from propan-2-ol, yield 68%, m.p. 126–128 °C. Analysis (calc/found%) for $C_9H_{11}Cl_2IN_4$: C: 28.98/28.9, H: 2.97/2.9, Cl: 19.01/19.1, I: 34.02/33.9, N: 15.02/14.9; IR (KBr) (ν, cm^{-1}): 1505 (N^+H), 1581 (C=N), 3447–3302 ($NH + NH_2$); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 3.88–4.2 (m, 4H, $2CH_2$), 5.26 (s, 2H, NH_2), 7.14–7.50 (m, 3H, $CH_{arom.}$), 8.71 (s, 1H, NH), 9.25 (s, 1H, N^+H); *m/z*: 373[M^+].

5.1.2. Synthesis of methyl 2-(4-oxo-8-aryl-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)acetate (method i) (general procedure)

Free base of 1-aryl-2-hydrazinoimidazoline (0.05 mol) was dissolved in 80 mL of *n*-butanol. Dimethyl fumarate of 0.05 mol (7.21 g) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was refrigerated overnight and the precipitation yielded was collected and purified by crystallization from DMF/methanol mixture.

5.1.3. Synthesis of methyl 2-(4-oxo-8-aryl-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)acetate (method ii) (general procedure)

Dimethyl fumarate of 0.05 mol (7.21 g) was added to the suspension of appropriate 1-aryl-2-hydrazinoimidazoline hydroiodide (0.05 mol) in 80 mL of *n*-butanol. The mixture was stirred and triethylamine (5 mL) was added. The reaction was carried out under reflux for 6 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF/methanol mixture.

*5.1.3.1. Methyl 2-(4-oxo-8-phenyl-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)acetate (**3a**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 63% (method i), 57% (method ii), m.p. 162–164 °C. Analysis (calc/found%) for $C_{14}H_{16}N_4O_3$: C: 58.33/58.45, H: 5.59/5.63, N: 19.43/19.38; ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.62 (dd, $^2J = 16.32$ Hz, $^3J = 6.42$ Hz, 1H, $-CH$COOCH$_3$), 2.74 (dd, $^2J = 16.32$ Hz, $^3J = 5.76$ Hz, 1H, $-CH$COOCH$_3$), 3.61 (s, 3H, CO$-OCH_3$), 3.74–3.97 (m, 4H, 2CH$_2$ + 1H, CH), 6.14 (d, $J = 3.45$ Hz, 1H, NH), 6.95–7.65 (m, 5H, CH$_{arom.}$); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 33.8 ($-$CH$_2$), 39.9 (imidazolidine-C-6), 45.5 (imidazolidine-C-7), 52.5 ($-$CO$-OCH_3$), 54.0 (triazine–C-3), ar C: [117.5 (CH), 121.9 (2CH), 129.5 (2CH), 141.4 (C)], 143.5 (C-8a), 167.9 (triazine–C-4), 171.1 (exocyclic-C=O); EI-MS [70 eV, *m/z* (%)]: 289 (5.1), 288 (M$^+$, 30.9), 286 (1.4), 229 (1.7), 228 (7.7), 227 (4.0), 217 (1.4), 216 (17.0), 215 (100.0), 213 (2.4), 212 (1.4), 188 (2.4), 187 (2.4), 172 (1.5), 171 (2.1), 160 (4.3), 159 (1.9), 158 (2.1), 147 (4.6), 146 (1.9), 145 (4.1), 144 (1.7), 132 (1.6), 131 (2.5), 130 (1.4), 128 (1.9), 119 (6.5), 118 (9.3), 117 (5.6), 107 (3.1), 106 (6.5), 105 (3.7), 104 (8.3), 103 (1.7), 92 (2.2), 91 (6.3), 90 (1.4), 86 (5.0), 78 (2.7), 77 (22.7), 70 (7.8), 69 (1.5), 65 (4.9), 59 (1.4), 55 (7.9), 51 (4.4), 44 (1.8), 43 (2.8), 42 (4.8), 41 (1.7).

*5.1.3.2. Methyl 2-[4-oxo-8-(4-methylphenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3b**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 64% (method i), 55% (method ii), m.p. 133–135 °C. Analysis (calc/found%) for $C_{15}H_{18}N_4O_3$: C: 59.59/59.42, H: 6.00/5.94, N: 18.53/18.42; IR (KBr) (ν, cm^{-1}): 1738 (ester–C=O), 1689 (triazine–C=O); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.25 (s, 3H, CH$_3$), 2.61 (dd, $^2J = 16.30$ Hz, $^3J = 6.50$ Hz, 1H, $-CH$COOCH$_3$), 2.75 (dd, $^2J = 16.30$ Hz, $^3J = 5.70$ Hz, 1H, $-CH$COOCH$_3$), 3.61 (s, 3H, CO$-OCH_3$), 3.72–3.98 (m, 4H, 2CH$_2$ + 1H, CH), 6.04 (d, $J = 3.50$ Hz, 1H, NH), 6.94–7.52 (m, 4H, CH$_{arom.}$).

*5.1.3.3. Methyl 2-[4-oxo-8-(2-methoxyphenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3c**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 59% (method i), 52.5% (method ii), m.p. 97–99 °C. Analysis (calc/found%) for $C_{15}H_{18}N_4O_4$: C: 56.60/56.72, H: 5.70/5.73, N: 17.60/17.68; ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.62 (dd, $^2J = 16.30$ Hz, $^3J = 6.50$ Hz, 1H, $-CH$COOCH$_3$), 2.80 (dd, $^2J = 16.30$ Hz, $^3J = 5.50$ Hz, 1H, $-CH$COOCH$_3$), 3.60 (s, 3H, CO$-OCH_3$), 3.74–3.97 (m, 4H, 2CH$_2$ + 1H, CH + 3H, OCH$_3$), 6.05 (d, $J = 3.50$ Hz, 1H, NH), 6.96–7.55 (m, 4H, CH$_{arom.}$).

*5.1.3.4. Methyl 2-[4-oxo-8-(4-methoxyphenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3d**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 67% (method i), 62% (method ii), m.p. 157–159 °C. Analysis (calc/found%) for $C_{15}H_{18}N_4O_4$: C: 56.60/56.52, H: 5.70/5.63, N: 17.60/17.57; ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.60 (dd, $^2J = 16.30$ Hz, $^3J = 6.48$ Hz, 1H, $-CH$COOCH$_3$), 2.74 (dd, $^2J = 16.30$ Hz, $^3J = 5.50$ Hz, 1H, $-CH$COOCH$_3$), 3.61 (s, 3H, CO$-OCH_3$), 3.74–3.96 (m, 4H, 2CH$_2$ + 1H, CH + 3H, OCH$_3$), 6.05 (d, $J = 3.51$ Hz, 1H, NH), 6.94–7.54 (m, 4H, CH$_{arom.}$); EI-MS [70 eV, *m/z* (%)]: 319 (5.4), 318 (M$^+$, 31.5), 259 (1.5), 258 (6.4), 257 (3.5), 247 (1.6), 246 (15.5), 245 (100.0), 243 (4.5), 230 (2.6), 217 (1.4), 203 (2.5), 202 (1.4), 190 (2.4), 177 (2.8), 176 (1.7), 175 (4.4), 174 (2.4), 162 (1.5), 161 (1.5), 160 (1.4), 159 (2.3), 149 (2.5), 148 (5.7), 147 (6.0), 146 (1.5), 136 (3.6), 135 (2.4), 134 (4.5), 133 (5.9), 132 (2.3), 129 (3.4), 122 (6.4), 121 (3.5), 120 (7.3), 119 (1.6), 108 (2.3), 107 (2.3), 105 (2.6), 101 (2.7), 92 (3.6), 78 (2.1), 77 (4.1), 70 (2.8), 65 (2.0), 64 (1.7), 55 (4.9), 43 (2.4), 42 (2.7), 41 (1.5).

*5.1.3.5. Methyl 2-[4-oxo-8-(3-chlorophenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3e**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 61% (method i), 56% (method ii), m.p. 175–176 °C. Analysis (calc/found%) for $C_{14}H_{15}ClN_4O_3$: C: 52.10/52.23, H: 4.68/4.73, Cl: 10.99/11.04, N: 17.36/17.29; ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.61 (dd, $^2J = 16.30$ Hz, $^3J = 6.39$ Hz, 1H, $-CH$COOCH$_3$), 2.78 (dd, $^2J = 16.30$ Hz, $^3J = 5.79$ Hz, 1H, $-CH$COOCH$_3$), 3.61 (s, 3H, CO$-OCH_3$), 3.74–3.98 (m, 4H, 2CH$_2$ + 1H, CH), 6.30 (d, $J = 3.36$ Hz, 1H, NH), 6.99–7.98 (m, 4H, CH$_{arom.}$); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 33.7 ($-$CH$_2$), 39.9 (imidazolidine-C-6), 45.5 (imidazolidine-C-7), 52.5 ($-$COOCH_3), 54.0 (triazine–C-3), ar C: [115.6 (CH), 117.1 (CH), 121.5 (CH), 131.1 (CH), 134.1 (C), 142.6 (C)], 143.2 (C-8a), 167.8 (triazine–C-4), 171.6 (exocyclic-C=O); EI-MS [70 eV, *m/z* (%)]: 324 (9.8), 323 (6.1), 322 (M$^+$, 31.3), 264 (4.5), 263 (4.2), 262 (12.9), 261 (5.1), 252 (6.2), 251 (41.2), 250 (20.9), 249

(100.0), 247 (4.2), 214 (3.6), 194 (3.6), 181 (5.3), 179 (3.0), 159 (4.1), 158 (4.2), 154 (3.2), 153 (4.7), 152 (6.8), 151 (3.2), 140 (6.9), 139 (3.2), 138 (7.8), 131 (2.7), 125 (7.2), 118 (3.2), 117 (5.1), 113 (6.5), 112 (3.6), 111 (21.0), 104 (3.0), 103 (2.6), 99 (4.8), 90 (3.6), 86 (2.8), 85 (3.5), 77 (3.7), 76 (2.6), 75 (9.3), 74 (2.6), 70 (14.4), 69 (3.3), 59 (2.7), 55 (13.4), 44 (3.0), 43 (4.8), 42 (7.4), 41 (2.8).

*5.1.3.6. Methyl 2-[4-oxo-8-(4-chlorophenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3f**).* Recrystallization from dimethylformamide/methanol mixture (1:3), yield 61% (method i), 58% (method ii), m.p. 173–174 °C. Analysis (calc/found%) for $C_{14}H_{15}ClN_4O_3$: C: 52.10/51.99, H: 4.68/4.63, Cl: 10.99/10.89, N: 17.36/17.44; IR (KBr) (ν, cm^{-1}): 1742 (ester –C=O), 1683 (triazine–C=O); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.60 (dd, $^2J = 16.20$ Hz, $^3J = 6.60$ Hz, 1H, $-CH$COOCH$_3$), 2.75 (dd, $^2J = 16.20$ Hz, $^3J = 5.70$ Hz, 1H, $-CH$COOCH$_3$), 3.61 (s, 3H, CO–OCH_3), 3.74–3.95 (m, 4H, $2CH_2 + 1H$, CH), 6.20 (d, $J = 3.39$ Hz, 1H, NH), 7.38–7.66 (m, 4H, $CH_{arom.}$); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 33.7 ($-CH_2$), 39.9 (imidazolidine-C-6), 45.6 (imidazolidine-C-7), 52.5 ($-COOCH_3$), 54.0 (triazine–C-3), 119.0 (2CH), 125.5 (C), 129.3 (2CH), 140.2 (C)], 143.3 (C-8a), 167.9 (triazine–C-4), 171.6 (exocyclic-C=O); EI-MS [70 eV, *m/z* (%)]: 324 (6.9), 323 (4.2), 322 (M^+, 23.4), 320 (1.7), 264 (3.2), 263 (3.2), 262 (9.6), 261 (4.3), 252 (4.5), 251 (32.4), 250 (15.8), 249 (100.0), 247 (2.8), 214 (2.2), 197 (2.0), 194 (2.0), 187 (1.9), 186 (2.9), 181 (3.4), 179 (1.9), 165 (1.7), 159 (2.3), 158 (2.2), 154 (2.5), 153 (3.9), 152 (6.1), 151 (4.1), 140 (6.0), 139 (3.0), 138 (6.6), 131 (2.2), 126 (1.8), 125 (8.0), 118 (1.8), 117 (3.5), 113 (4.2), 112 (1.8), 111 (13.8), 103 (1.9), 99 (3.7), 90 (2.5), 86 (2.4), 85 (2.3), 75 (5.0), 70 (8.1), 55 (7.9), 44 (2.0), 43 (3.4), 42 (5.8), 41 (2.0).

5.1.4. Synthesis of ethyl 2-(4-oxo-8-aryl-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)acetate (method i) (general procedure)

Free base of 1-aryl-2-hydrazinoimidazoline (0.05 mol) was dissolved in 80 mL of *n*-butanol. Diethyl fumarate of 0.05 mol (8.61 g) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was refrigerated overnight and the precipitation yielded was collected and purified by crystallization from DMF/methanol mixture.

5.1.5. Synthesis of ethyl 2-(4-oxo-8-aryl-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)acetate (method ii) (general procedure)

Diethyl fumarate of 0.05 mol (8.61 g) was added to the suspension of appropriate 1-aryl-2-hydrazinoimidazoline hydroiodide (0.05 mol) in 80 mL of *n*-butanol. The mixture was stirred and triethylamine (5 mL) was added. The reaction was carried out under reflux for 6 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF/methanol mixture.

*5.1.5.1. Ethyl 2-[4-oxo-8-(4-methylphenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3g**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 66.4% (method i), 57.5% (method ii), m.p. 142–144 °C. Analysis (calc/found%) for $C_{16}H_{20}N_4O_3$: C: 60.75/60.87, H: 6.37/6.41, N: 17.71/17.76; IR (KBr) (ν, cm^{-1}): 1739 (ester –C=O), 1690 (triazine–C=O); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 1.19 (t, $J = 7.11$ Hz, 3H, $-OCH_2CH_3$), 2.25 (s, 3H, CH_3), 2.61 (dd, $^2J = 16.40$ Hz, $^3J = 6.30$ Hz, 1H, $-CH$COOC$_2$H$_5$), 2.74 (dd, $^2J = 16.40$ Hz, $^3J = 5.85$ Hz, 1H, $-CH$COOC$_2$H$_5$), 3.61–3.94 (m, 4H, $2CH_2 + 1H$, CH), 4.09 (q, $J = 7.11$ Hz, 2H, $-OCH_2CH_3$), 6.09 (d, $J = 3.5$ Hz, 1H, NH), 7.12–7.54 (m, 4H, $CH_{arom.}$); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 14.0 ($-CH_2CH_3$), 20.2 ($-CH_3$), 33.0 ($-CH_2$), 39.0 (imidazolidine-C-6), 44.6 (imidazolidine-C-7), 53.2 (triazine–C-3), 60.2 ($-CH_2CH_3$), ar C: [116.6 (2CH), 129.0 (C), 129.8 (2CH), 138.1 (C)], 142.6 (C-8a), 167.1 (triazine–C-4), 170.3 (exocyclic-C=O); EI-MS [70 eV, *m/z* (%)]: 317 (3.6), 316 (M^+, 21.2), 302 (6.1), 243 (2.1), 242 (8.9), 241 (5.3), 230 (13.3), 229 (100.0), 227 (2.3), 202 (1.8), 201 (1.8), 186 (1.3), 185 (1.5), 174 (2.4), 172 (1.4), 161 (4.3), 160 (1.8), 159 (3.6), 158 (1.8), 145 (1.6), 133 (2.9), 132 (4.7), 131 (5.5), 130 (1.4), 121 (1.2), 120 (5.0), 119 (3.4), 118 (7.5), 117 (3.1), 115 (2.0), 114 (1.4), 106 (1.9), 105 (2.5), 104 (1.9), 94 (1.3), 92 (2.0), 91 (12.9), 90 (1.9), 89 (1.9), 86 (1.2), 79 (1.6), 78 (1.4), 77 (3.0), 70 (3.5), 65 (4.2), 55 (4.6), 44 (1.4), 43 (1.8), 42 (3.1), 41 (1.3). Crystal data for **3g**: $C_{16}H_{20}N_4O_3$, FW = 316.36, trigonal, space group $R\bar{3}$, $a = b = c = 13.561(2)$ Å, $\alpha = \beta = \gamma = 93.7(1)°$, $V = 2475.0(4)$ Å^3, $Z = 6$, $d_{calc} = 1.274$ g cm^{-3}, μ(Cu Kα) = 0.741 mm^{-1}. In the θ range 3.28–80°, 10,487 reflections were collected of which 3615 were unique ($R_{int} = 0.09$; $R_\sigma = 0.1$). The structure was solved by direct methods using SHELXS-97 [31] program and refined by full-matrix least-squares on F^2 using SHELXL-97 [32] program. The non-hydrogen atoms were refined with anisotropic displacement parameters. H-atom bonded to the N2 atom was located in difference electron density map. Other H-atom positions were calculated from the geometry. H-atoms were given isotropic factors of 1.2 U_{eq} of the bonded C-atoms; the C–H bond 'riding' model was used in the refinement. The intermolecular hydrogen bond N2–H···N2($-y$, $-z$, $-x$) links molecules around the $\bar{3}$ axis forming molecular hexamer. These hexameric units interact through weak van der Waals forces only, which cause high values of displacement parameters and poor diffraction. Final discrepancy factors are $R1 = 0.0712$, $wR2 = 0.1855$ for 1061 reflections with $I > 2\sigma(I)$, and $R1 = 0.2400$, $wR2 = 0.2739$ for all data, $S = 0.913$.. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as CCDC No. 275240. Copies of the data can be obtained on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; e-

mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

*5.1.5.2. Ethyl 2-[4-oxo-8-(4-methoxyphenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3h**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 62.4% (method i), 58.5% (method ii), m.p. 133–134 °C. Analysis (calc/found%) for $C_{16}H_{20}N_4O_4$: C: 57.82/57.77, H: 6.07/6.12, N: 16.86/16.94; IR (KBr) (ν, cm^{-1}): 1740 (ester –C=O), 1692 (triazine–C=O); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 1.19 (t, $J = 7.11$ Hz, 3H, $-OCH_2CH_3$), 2.59 (dd, $^2J = 16.30$ Hz, $^3J = 6.33$ Hz, 1H, $-CHCOOC_2H_5$), 2.75 (dd, $^2J = 16.30$ Hz, $^3J = 5.80$ Hz, 1H, $-CHCOOC_2H_5$), 3.72–3.96 (m, 4H, $2CH_2 + 1H$, $CH + 3H$, OCH_3), 4.07 (q, $J = 7.11$ Hz, 2H, $-OCH_2CH_3$), 6.03 (d, $J = 3.54$ Hz, 1H, NH), 6.92–7.58 (m, 4H, $CH_{arom.}$); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 14.0 ($-CH_2CH_3$), 33.0 ($-CH_2$), 39.0 (imidazolidine-C-6), 44.9 (imidazolidine-C-7), 53.2 (triazine–C-3), 55.2 ($-CH_3O$), 60.2 ($-CH_2CH_3$), ar C: [113.9 (2CH), 118.2 (2CH), 134.0 (C), 153.9 (C)], 142.8 (C-8a), 167.1 (triazine–C-4), 170.3 (exocyclic-C=O); EI-MS [70 eV, *m/z* (%)]: 333 (3.4), 332 (M^+, 19.7), 318 (4.3), 259 (2.2), 258 (9.5), 257 (5.6), 247 (1.6), 246 (15.2), 245 (100.0), 243 (4.7), 230 (2.1), 217 (1.4), 203 (1.9), 201 (1.3), 190 (2.1), 177 (3.0), 176 (1.6), 175 (3.4), 174 (2.1), 162 (1.4), 161 (1.4), 160 (1.2), 159 (1.9), 149 (2.0), 148 (4.9), 147 (5.3), 146 (1.4), 136 (3.5), 135 (2.4), 134 (4.3), 133 (5.3), 132 (2.0), 129 (2.9), 122 (5.9), 121 (3.2), 120 (6.5), 119 (1.4), 108 (1.9), 107 (2.0), 105 (2.3), 101 (2.2), 92 (3.0), 78 (1.6), 77 (3.2), 70 (2.3), 65 (1.6), 64 (1.3), 55 (4.3), 43 (1.5), 42 (2.3).

*5.1.5.3. Ethyl 2-[4-oxo-8-(3-chlorophenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3i**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 60.3% (method i), 57.5% (method ii), m.p. 104–105 °C. Analysis (calc/found%) for $C_{15}H_{17}ClN_4O_3$: C: 53.50/53.64, H: 5.09/5.14, Cl: 10.53/10.49, N: 16.64/16.72; IR (KBr) (ν, cm^{-1}): 1743 (ester –C=O), 1685 (triazine–C=O); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 1.19 (t, $J = 7.11$ Hz, 3H, $-OCH_2CH_3$), 2.60 (dd, $^2J = 16.40$ Hz, $^3J = 6.41$ Hz, 1H, $-CHCOOC_2H_5$), 2.78 (dd, $^2J = 16.30$ Hz, $^3J = 5.82$ Hz, 1H, $-CHCOOC_2H_5$), 3.80–4.02 (m, 4H, $2CH_2 + 1H$, CH), 4.10 (q, $J = 7.11$ Hz, 2H, $-OCH_2CH_3$), 6.20 (d, $J = 3.40$ Hz, 1H, NH), 7.01–7.99 (m, 4H, $CH_{arom.}$).

*5.1.5.4. Ethyl 2-[4-oxo-8-(4-chlorophenyl)-2H-3,4,6,7-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]acetate (**3j**).* Recrystallization from dimethylformamide/methanol (1:3) mixture, yield 64.2% (method i), 57.3% (method ii), m.p. 153–154 °C. Analysis (calc/found%) for $C_{15}H_{17}ClN_4O_3$: C: 53.50/53.55, H: 5.09/5.13, Cl: 10.53/10.59, N: 16.64/16.59; IR (KBr) (ν, cm^{-1}): 1743 (ester –C=O), 1684 (triazine–C=O); ^1H NMR (δ, ppm, DMSO-d_6, TMS): 1.19 (t, $J = 7.11$ Hz, 3H, $-OCH_2CH_3$), 2.60 (dd, $^2J = 16.40$ Hz, $^3J = 6.30$ Hz, 1H, $-CHCOOC_2H_5$), 2.75 (dd, $^2J = 16.40$ Hz, $^3J = 5.85$ Hz, 1H, $-CHCOOC_2H_5$), 3.79–3.98 (m, 4H, $2CH_2 + 1H$, CH), 4.06 (q, $J = 7.11$ Hz, 2H, $-OCH_2CH_3$), 6.19 (d, $J = 3.42$ Hz, 1H, NH), 7.38–7.68 (m, 4H, $CH_{arom.}$). ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 14.1 ($-CH_2CH_3$), 33.0 ($-CH_2$), 39.1 (imidazolidine-C-6), 44.6 (imidazolidine-C-7), 53.2 (triazine–C-3), 60.3 ($-CH_2CH_3$), ar C: [121.1 (2CH), 127.9 (C), 129.5 (2CH), 138.5 (C)], 142.7 (C-8a), 167.1 (triazine–C-4), 170.2 (exocyclic-CO); EI-MS [70 eV, *m/z* (%)]: 338 (7.9), 337 (5.3), 336 (M^+, 25.7), 264 (6.0), 263 (5.9), 262 (17.6), 261 (8.1), 252 (6.0), 251 (40.1), 250 (21.2), 249 (100.0), 247 (4.3), 222 (2.1), 221 (2.6), 214 (2.6), 197 (2.7), 194 (2.6), 187 (2.5), 186 (3.5), 185 (2.2), 181 (5.1), 180 (2.1), 179 (2.5), 159 (2.2), 158 (2.1), 154 (2.7), 153 (4.5), 152 (7.0), 151 (5.0), 142 (2.0), 140 (6.9), 139 (3.8), 138 (8.0), 131 (2.5), 126 (2.1), 125 (10.0), 117 (3.7), 113 (4.5), 112 (2.7), 111 (18.3), 99 (4.1), 97 (2.3), 90 (2.3), 77 (2.1), 75 (6.2), 71 (2.2), 70 (10.2), 55 (8.5), 43 (2.1), 42 (4.1).

5.2. Microbiology

5.2.1. Disc-diffusion assay

Assay of antimicrobial activity in vitro: the synthesized compounds 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 tested microorganisms were isolated from clinical specimens of the Laboratory of Medical Microbiology Department, Medical University of Lublin. The assayed collection included the following microorganisms: *S. epidermidis*, *St. pyogenes*, *St. agalactiae* (Gram-positive bacteria), *P. aeruginosa*, *Pr. vulgaris*, *K. pneumoniae*, *En. aerogenes* (Gram-negative bacteria), *C. albicans*, *Aspergillus* spp. Besides, two reference strains of bacteria – *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included in these studies.

In the disc-diffusion method, sterile paper discs (ϕ 5 mm) impregnated with dissolved dimethyl sulfoxide (DMSO) compound at concentrations of 100 $\mu g\,mL^{-1}$ and 200 $\mu g\,mL^{-1}$ 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*, *S. epidermidis*, *St. pyogenes*, *St. agalactiae*, *E. coli*, *P. aeruginosa*, *Pr. vulgaris*, *K. pneumoniae*, *En. aerogenes*, 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/24 h for the microorganism cultures. After incubation, the growth inhibition zones around the discs were observed indicating that the examined compound inhibits the growth of microorganism [27–29]. Each assay in this experiment was repeated three times.

Ampicillin was used as a standard drug. Dimethyl sulfoxide was used as solved control. Results were interpreted in terms of the diameter of the inhibition zone and are shown in Table 3.

Table 3
Antimicrobial activities of evaluated compounds against the tested bacterial and fungal isolates using the disc-diffusion method

Microorganisms	Compound				Standard
	2b		**2e**		
	I	II	I	II	
Escherichia coli (ATCC)	+++	+++	++	+++	++
Pseudomonas aeruginosa	+++	+++	+	++	−
Proteus vulgaris	++	+++	+	++	−
Klebsiella pneumoniae	++	+++	−	−	−
Enterobacter aerogenes	++	+++	−	−	−
Staphylococcus aureus (ATCC)	−	−	+	++	++
Staphylococcus epidermidis	−	−	−	+	+
Streptococcus pyogenes	−	−	−	−	++
Streptococcus agalactiae	−	−	−	−	++
Candida albicans	−	−	−	−	−
Aspergillus spp.	−	−	−	−	−

Zones of growth inhibition: −, +, ++, +++; −: 0–10 mm (R, resistant); +: 11–16 mm (I, intermediate susceptible); ++: 17–25 mm (S, susceptible); +++: >25 mm (S, susceptible); standard: ampicillin; I − concentration of 100 μg mL^{-1} and II − concentration of 200 μg mL^{-1}.

5.2.2. Microdilution assays

The minimal inhibitory concentration (MIC) values for all compounds tested, defined as the lowest concentration of the compound preventing the visible growth, were determined by using microdilution broth method according to NCCLS standards [25]. The inocula of microorganisms were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The test compounds dissolved in dimethyl sulfoxide (DMSO) were first diluted to the highest concentration (500 μg mL^{-1}) to be tested. Then serial 2-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 reference bacterial strains: *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 towards two compounds determined in the disc-diffusion assay. Ampicillin and chloramphenicol were used as standard drugs for comparison in the antibacterial study. Control experiments using dimethyl sulfoxide were done for antibacterial 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.

5.2.3. Inhibition of tumour cell growth assay

Compounds **3e**, **3i**, and **3j** were tested towards in vitro three cancer cell lines: human Caucasian colon adenocarcinoma (LS180), human uterus cancer (SiHa), and human breast carcinoma (T47D) (Figs. 4–6). Besides two normal cell lines: human skin fibroblasts (HSF) and Vero (Green Monkey Kidney cells) were 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 compounds were then added at both examined concentrations (10 μg mL^{-1} and 50 μg mL^{-1}) and culture incubated for 72 h.

End-point determinations were made with 5-bromo-2′-deoxy-uridine (BrdU) [33–36] labeling and detection kit III (Roche) on Elisa reader (BIO-TEC Instruments USA).

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

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