European Journal of Medicinal Chemistry 63 (2013) 185–195

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, antimicrobial, antiquorum-sensing, antitumor and cytotoxic activities of new series of fused [1,3,4]thiadiazoles

Nadia S. El-Gohary [a,*], Mona I. Shaaban [b]

[a] Department of Medicinal Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
[b] Department of Microbiology, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

ARTICLE INFO

Article history:
Received 1 October 2012
Received in revised form
4 February 2013
Accepted 9 February 2013
Available online 18 February 2013

Keywords:
Synthesis
Fused [1,3,4]thiadiazoles
Antimicrobial activity
Antiquorum-sensing activity
Antitumor activity
Cytotoxic activity

ABSTRACT

New series of [1,3,4]thiadiazolo[3,2-*a*]pyrimidines, benzo[*h*][1,3,4]thiadiazolo[2,3-*b*]quinazolines, benzothiadiazolotriazocine, and imidazo[2,1-*b*][1,3,4]thiadiazoles have been synthesized and characterized by analytical and spectrometrical methods (IR, MS, ^{1}H and ^{13}C NMR). Twenty of the synthesized compounds were screened for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. They were found to be either moderately active, slightly active or inactive against the tested microorganisms. The antifungal activity of these compounds were also tested against *Candida albicans*, *Aspergillus fumigatus* 293 and *Aspergillus flavus* 3375. Compounds **3g**, **h** showed potent antifungal activity against *C. albicans*. In addition, the same compounds were tested for antiquorum-sensing activity against *Chromobacterium violacium* ATCC 12472, where compounds **3b**,**c**, **3f**–**h**, **6b**–**d**, **9**, **10** and **12** demonstrated acceptable activity. Compounds **3d**, **9** and **10** were screened for antitumor activity at National Cancer Institute, USA. The *in vitro* cytotoxic activity of eighteen of the synthesized compounds was studied by brine shrimp lethality bioassay, and results indicated that compounds **6c**, **13**, **3h**, **6d** and **3d** have the highest cytotoxic activity.

1. Introduction

The global rise of antimicrobial resistance, combined with the rapid rate of microbial evolution, and the slower development of novel antibiotics, underscores the urgent need for innovative therapeutics. Development of new antimicrobial or antipathogenic agents that act upon new microbial targets is a necessity [1]. In view of the fact that quorum-sensing (QS) is involved in microbial pathogenesis, research efforts have focused recently upon developing antipathogenic agents to control bacterial diseases by inhibiting quorum sensing [2]. QS is a term that describes an environmental sensing system that allows bacteria to monitor their own population density which contributes significantly to the development and size of the biofilm. Many gram negative bacteria use *N*-acyl-homoserine lactones (acyl HSLs) as quorum-sensing signal molecules. The signal molecules aid in biofilm formation which in turn confer various properties of pathogenicity including drug resistance [3]. Antiquorum-sensing agents are viewed as blockers of bacterial pathogenicity rather than as antimicrobials and therefore their use is highly attractive, particularly with respect to the emergence of multi-antibiotic resistant bacteria [4].

Also, cancer is one of the most insidious and feared diseases. It is a group of diseases characterized by the uncontrolled growth of abnormal cells which have the ability to spread throughout the body [5]. Therefore, development of novel chemotherapeutic agents is an important and challenging task for the medicinal chemists. Hence, many research programs are directed toward the design and synthesis of new drugs for their chemotherapeutic usage.

During recent years there have been intense investigations on fused thiadiazole systems. Literature survey revealed that [1,3,4]thiadiazolo[3,2-*a*]pyrimidine nucleus is associated with diverse pharmacodynamic and chemotherapeutic activities [6–15], including antimicrobial [9,10,14,15] and antitumor activities [6–9]. Moreover, many imidazo[2,1-*b*][1,3,4]thiadiazole compounds are known to possess interesting pharmacological properties, including antibacterial [16–20], antifungal [21], antimicrobial [22–24] and antitumor activities [25,26]. Also, they exhibited cyclooxygenase-2 inhibitory activity [27]. Considering these published data and our previous interests on fused thiadiazole systems [7,8], and in searching for new compounds of potent antimicrobial, antiquorum-sensing and antitumor activities, it seemed interesting to synthesize new compounds incorporating these heterocyclic systems to explore their antimicrobial, antiquorum-sensing and antitumor activities.

* Corresponding author. Tel.: +20 10 00326839; fax: +20 50 2247496.
E-mail address: dr.nadiaelgohary@yahoo.com (N.S. El-Gohary).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.02.010

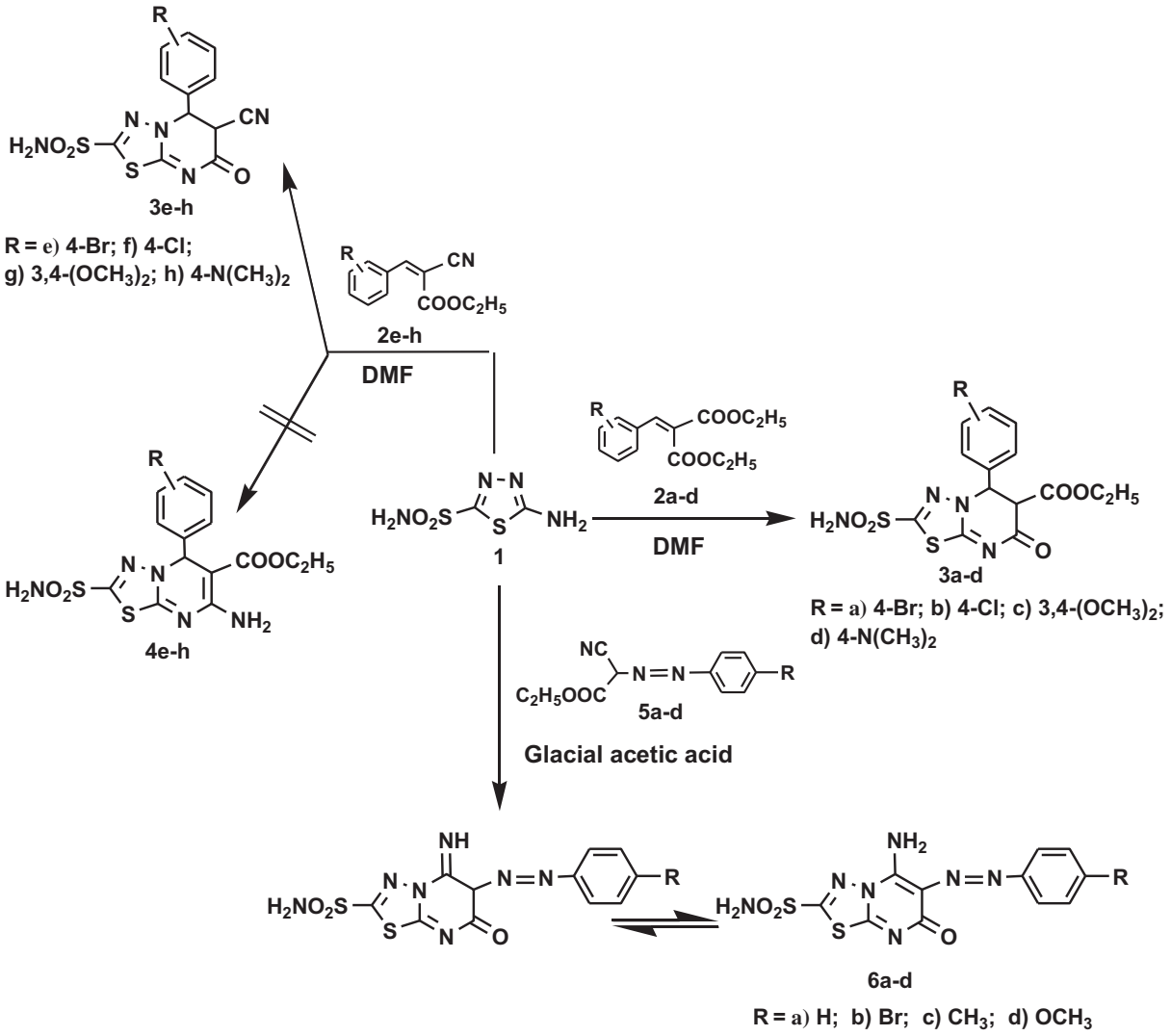
2. Results and discussion

2.1. Chemistry

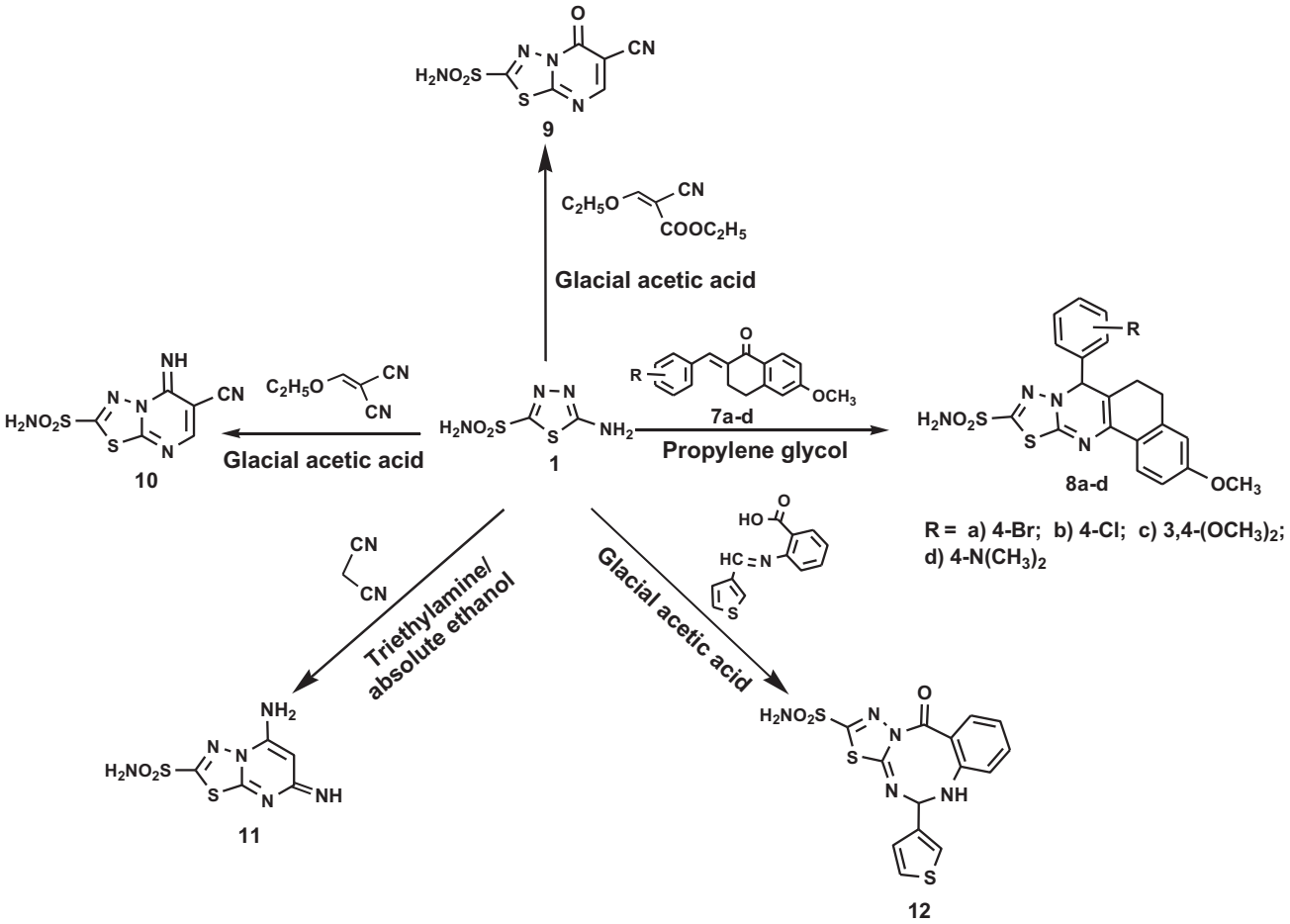
A general approach to synthesize the designed compounds is outlined in Schemes 1–3. 2-Amino-1,3,4-thiadiazole-5-sulfonamide (**1**) was used as a key starting material in this study for syntheses of other fused heterocycles. Amine **1** was synthesized *via* acid hydrolysis of 2-acetamido-1,3,4-thiadiazole-5-sulfonamide [28]. Cyclocondensation of amine **1** with benzylidene derivatives of diethyl malonate **2a**–**d** [29] afforded ethyl 5,6-dihydro-7-oxo-5-(substituted)phenyl-2-sulfamoyl-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-6-carboxylates (**3a**–**d**) (Scheme 1). When amine **1** was allowed to react with ethyl benzylidenecyanoacetates **2e**–**h** [29] in refluxing *N*,*N*-dimethylformamide, the two possible structures, **3e**–**h** (as reported in literature [30–32] and **4e**–**h** (as reported in literature [33]) could be proposed for the reaction product (Scheme 1). Interestingly, this reaction gave only one product, **3e**–**h** as confirmed by spectral data. The IR spectra of the products showed bands corresponding to nitrile group and no bands for carbonyl ester group. In addition, ^{1}H NMR spectra did not exhibit the triplet-quartet pattern characteristic for the ethyl protons, thus structures **3e**–**h** could be ruled out. Moreover, 5-amino-7-oxo-6-[4-(substituted)phenyldiazenyl]-5*H*, 7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-2-sulfonamide derivatives **6a**–**d** were obtained *via* reaction of amine **1** with ethyl 2-cyano-2-[4-(substituted)phenyldiazenyl]acetates (**5a**–**d**) [34] in refluxing glacial acetic acid (Scheme 1).

Interaction of equimolar amounts of amine **1** with benzylidenetetralones **7a**–**d** [35] in refluxing propylene glycol gave the benzo[*h*][1,3,4]thiadiazolo[2,3-*b*]quinazoline derivatives **8a**–**d** in 45–55% yield (Scheme 2). When, compound **1** was allowed to react with ethyl 2-(ethoxymethylenecyano)acetate or 2-(ethoxymethylene)malononitrile in refluxing glacial acetic acid, compounds **9** and **10**, respectively, were obtained (Scheme 2). In addition, reaction of **1** with malononitrile in absolute ethanol and in the presence of catalytic amount of triethylamine afforded 5-amino-7-imino-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-2-sulfonamide (**11**) (Scheme 2). Moreover, the synthesis of benzo[*g*][1,3,4]thiadiazolo[3,2-*a*][1,3,5]triazocine-2-sulfonamide derivative **12** was achieved through reaction of **1** with 2-[(thiophen-3-yl)methyleneamino]benzoic acid in refluxing glacial acetic acid (Scheme 2).

The reaction of 2-aminothiadiazole **1** with 3-chloropropionyl chloride or ethyl chloroacetate in refluxing glacial acetic acid afforded 6,7-dihydro-7-oxo-5*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-2-sulfonamide (**13**) and 5,6-dihydro-6-oxoimidazo[2,1-*b*]



Scheme 1. Synthesis of compounds **3a**–**h** and **6a**–**d**.

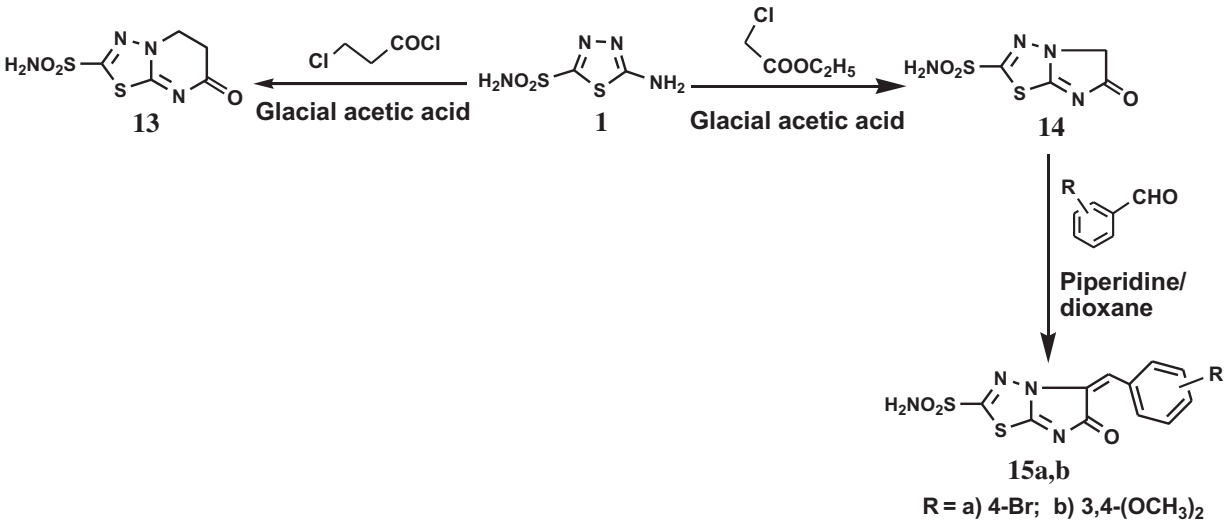


Scheme 2. Synthesis of compounds **8a**–**d** and **9**–**12**.

[1,3,4]thiadiazole-2-sulfonamide (**14**), respectively (Scheme 3). When imidazo[2,1-*b*][1,3,4]thiadiazole **14** was heated under reflux with benzaldehyde derivatives in dioxane and in the presence of catalytic amount of piperidine, 5-(substituted benzylidene)imidazo[2,1-*b*][1,3,4]thiadiazoles **15a**,**b** were obtained (Scheme 3).

2.2. Antimicrobial and antiquorum-sensing screening

Twenty of the synthesized compounds; namely, **3b**–**d**, **3f**–**h**, **6a**–**d**, **8a**, **b**, **9**–**14**, and **15a**,**b** were screened for *in vitro* antimicrobial activity against two species of gram-positive bacteria, *Staphylococcus aureus* & *Bacillus cereus* and one gram-negative



Scheme 3. Synthesis of compounds **13**,**14** and **15a**,**b**.

bacteria, *Escherichia coli* [36]. Antifungal assay against *Candida albicans*, *Aspergillus fumigatus* 293 and *Aspergillus flavus* 3372 was also performed [37]. The antimicrobial screening results were determined by measuring the average diameter of the inhibition zones, expressed in millimeters (mm). As shown in the results (Table 1), all tested compounds exhibited varying degree of inhibitory effect on the growth of different tested microbial strains. Compound **3g** showed promising activity toward *B. cereus*. Compounds **9** and **13** showed excellent antibacterial activity against *Staphyllococcus aureus*, while compounds **3g** and **6b** showed moderate activity against the same microorganism. Furthermore, compounds **6a** and **13** demonstrated moderate activity against *E. coli*. The rest of the tested compounds showed either weaker activity or were completely inactive against the selected microorganisms. Regarding the antifungal activity, compounds **3g**,**h** demonstrated strong antifungal activity against *C. albicans*, compounds **3d**, **6c**, **8a** and **13** exhibited moderate activity toward the same microorganism. In addition, compounds **15b** showed interesting antifungal activity against *Aspergillus fumigatus*. These observations may promote a further development of fused [1,3,4]thiadiazoles and may lead to compounds with better pharmacological profile than the standard antibacterial and antifungal drugs.

The same compounds were tested for antiquorum-sensing activity against *Chromobacterium violaceum* ATCC 12472 [38]. The QS system of *Chromobacterium violaceum* was used for this assay. QS in this wild type strain of bacteria produces violacein (a purple pigment) in response to autoinducer molecules known as acyl HSLs [39,40]. Thus, drugs that inhibit acyl HSL-mediated QS activity in *Chromobacterium violaceum* will prevent the production of this purple pigment. Screening results for their ability to inhibit QS regulated violacein production against *Chromobacterium violaceum* (based on measuring the radius of pigment inhibition in mm) are presented in Table 1 and indicated that compounds **3b**, **c**, **3f**–**h**, **6b**–**d**, **9**, **10** and **12** have antiquorum-sensing activity.

2.2.1. Structure–activity relationship (SAR) studies

- Concerning series **3a**–**h**, compounds possessing an electron-donating substituent on the 5-phenyl moiety produce enhanced antibacterial and antifungal activities over those substituted with electron-withdrawing groups (compare **3c**,**d** and **3b** & **3g**,**h** and **3f**); on the other hand, the antiquorum-sensing activity was decreased. In addition, the presence of 6-cyano group improved the antibacterial and antifungal activities over those analogs with 6-ethoxycarbonyl group (compare **3g**,**h** and **3c**,**d**).
- Regarding series **6a**–**d**, the presence of para substituent regardless of its type on the phenyl ring of the 6-phenyldiazenyl moiety increased the activity against *Staphylococcus aureus*, also the antifungal and antiquorum-sensing activities were improved. On the other hand, the activity against *B. cereus* and *E. coli* was decreased (compare **6b**–**d** and **6a**).
- Taking into account the structure of imidazothiadiazoles **14** and **15a**,**b**, it is assumed that the presence of 5-benzylidene moiety substituted with electron-donating group improved the antibacterial activity against *Staphylococcus aureus* and *B. cereus*. Furthermore, it enhanced the antifungal activity against *Aspergillus fumigatus* and *Aspergillus flavus*, but decreased the activity against *C. albicans* (compare **15b** versus **14** and **15a**).

2.3. Preliminary in vitro antitumor screening

Out of the newly synthesized compounds, three derivatives **3d**, **9** and **10** were selected by the National Cancer Institute (NCI) *in vitro* disease-oriented human cells screening panel assay. A single dose (10 μM) of the test compounds were used in the full NCI 60 cell lines panel assay which includes nine tumor subpanels; namely, leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancer cells [41–44]. The data reported as mean-graph of the percent growth of the treated cells,

Table 1
Antimicrobial and antiquorum-sensing activities of compounds **3**–**15**.[a,b,c]

Comp. No	Inhibition zone diameter (mm)						
	E. coli	*B. cereus*	*S. aureus*	*C. albicans*	*A. fumigatus*	*A. flavus*	*Ch. violaceum*
3b	–	4	3	–	–	–	11
3c	–	7	8	4	–	–	8
3d	–	5	5	10	4	–	–
3f	–	–	–	8	6	–	11
3g	–	9	10	24	8	–	8
3h	–	6	7	32	8	–	6
6a	10	7	7	4	–	–	–
6b	–	5	9	6	4	–	12
6c	4	3	8	12	6	5	12
6d	5	3	8	6	–	5	11
8a	–	–	–	14	6	–	–
8b	–	–	–	–	–	–	–
9	5	2	18	4	–	–	10
10	–	–	–	–	–	–	11
11	–	–	–	–	–	–	–
12	–	–	–	–	–	–	6
13	11	4	16	12	8	10	–
14	–	–	–	5	–	–	–
15a	–	2	2	8	–	–	–
15b	–	4	4	4	16	7	–
Ampicillin	23	3.5	14				
Fluconazole				22	–	–	

E. coli: *Escherichia coli*; *B. cereus*: *Bacillus cereus*; *S. aureus*: *Staphylococcus aureus*; *C. albicans*: *Candida albicans*; *A. fumigatus*: *Aspergillus fumigatus*; *A. flavus*: *Aspergillus flavus*; *Ch. violaceum*: *Chromobacterium violaceum*.

[a] Sample concentration: 5 mg/mL, Sample volume 100 μl/well.

[b] Results are calculated after subtraction of DMSO activity.

[c] Not active (–, inhibition zone<2 mm); weak activity (2–8 mm); moderate activity (9–15 mm); strong activity (>15 mm).

Table 2
Percentage growth inhibition (GI %) of *in vitro* subpanel tumor cell lines at 10 μM concentration of compounds **3d**, **9** and **10**.

Subpanel tumor cell lines	% Growth inhibition (GI %)[a]		
	3d	**9**	**10**
Leukemia			
CCRF-CEM	–	–	–
HL-60(TB)	21.0	18.4	29.3
K-562	–	–	–
MOLT-4	–	–	–
RPMI-8226	–	–	–
SR	10.3	–	–
Non-small lung cancer			
A549/ATCC	–	–	–
EKVX	12.2	–	–
HOP-62	–	–	10.7
HOP-92	51.2	12.3	20.3
NCI-H226	–	–	–
NCI-H23	–	–	–
NCI-H460	–	–	–
NCI-H522	19.3	–	–
Colon cancer			
COLO 205	–	–	–
HCC-2998	–	–	–
HCT-116	–	–	–
HCT-15	–	–	–
HT29	–	–	–
KM 12	–	–	–
SW-620	–	–	–
CNS cancer			
SF-268	–	–	–
SF-295	–	–	–
SF-539	–	–	–
SNB-19	–	–	–
SNB-75	10.1	10.3	–
Melanoma			
LOX IMVI	11.7	10.7	15.1
MALME-3M	14.9	28.0	–
M14	–	–	–
MDA-MB-435	21.3	–	–
SK-MEL-2	–	–	–
SK-MEL-28	–	–	–
SK-MEL-5	–	–	–
UACC-257	–	–	–
UACC-62	13.7	–	–
Ovarian cancer			
IGORV1	–	–	–
OVCAR-3	–	–	–
OVCAR-4	–	–	–
OVCAR-5	–	–	–
OVCAR-8	–	–	–
NCI/ADR-RES	–	–	–
SK–OV-3	–	–	–
Renal cancer			
786-0	–	–	–
A498	16.5	34.2	14.9
ACHN	–	–	–
CAKI-1	21.1	–	11.5
RXF 393	–	–	–
SN12C	–	–	–
TK-10	–	–	–
UO-31	14.7	10.5	–
Prostate cancer			
PC-3	11.4	–	–
DU-145	–	–	–
Breast cancer			
MCF-7	10.2	–	–
MDA-MB-231/ATCC	34.6	–	12.0
HS 578T	18.5	12.8	–
BT-549	–	–	–
T-47D	–	–	–
MDA-MB-468	–	–	–

[a] –, GI < 10%.

and presented as percentage growth inhibition (GI%). The obtained results of the tested compounds **3d**, **9** and **10** (Table 2), showed distinctive potential pattern of selectivity, as well as broad-spectrum antitumor activity.

Concerning the activity toward individual cell lines; the three tested compounds showed selective activity against leukemia (HL-60(TB)) cell line, non-small lung cancer (HOP-92) cell line, melanoma (LOX IMVI) cell line and renal cancer (A498) cell line. With regard to broad-spectrum antitumor activity; close examination of the data presented in Table 2, revealed that compound **3d** is the most active member in this study, showing effectiveness toward numerous cell lines belonging to different tumor subpanels.

2.4. Cytotoxicity testing using brine shrimp lethality bioassay

Cytotoxicity *via* brine shrimp lethality test was studied in order to reveal new antimicrobial and anticancer compounds [45]. Toxicity to brine shrimps has a good correlation with antitumor, pesticidal [46] and antitrypanosomal activities [47] in man. The brine shrimp larvae (*Artemia salina*) responds similarly to the corresponding mammalian systems [48] since the DNA-dependent RNA polymerases of *A. salina* have been shown to be similar to the mammalian type [49]. This test is not only used for predicting cytotoxicity, but also it is used to predict antitumor, antibacterial and pesticidal activities [50]. Thus, it is possible to evaluate the cytotoxicity of compounds using brine shrimp lethality bioassay rather than the more tedious *in vitro* and *in vivo* antitumor assays.

Eighteen of the synthesized compounds; namely, **3b**–**d**, **3f**–**h**, **6a**–**d**, **8b**,**c**, **9**, **10**, **12**, **13** and **15a**,**b** were screened for cytotoxic activity against brine shrimp larvae (nauplii) adopting the microplate assay, method of Meyer *et al.* [51], and using 5-flurouracil as a reference drug.

Cytotoxic activity of the tested compounds was determined by measuring the median lethal concentration; LC_{50} (concentration that kills 50% of brine shrimp nauplii) expressed in μg/mL. LC_{90} (90% mortality) values were also determined to establish the therapeutic index. The results were shown in Table 3 and indicated that compounds **6c**, **13**, **3h**, **6d** and **3d** have good cytotoxic activity against brine shrimp nauplii in comparison with 5-flurouracil with LC_{50} values of 392.46, 398.72, 399.68, 403.87 and 404.85 μg/mL, respectively, which represent high cytotoxicity and hence may predict antitumor and antimicrobial potential of those compounds. The rest of the tested compounds showed weaker activity, whereas compounds **3b**, **15a**, **3g** and **3f** are the least cytotoxic analogs in this study with LC_{50} values of 508.13, 551.26, 598.08 and 659.63 μg/mL, respectively.

2.4.1. Structure–activity relationship (SAR) studies

- Concerning series **3a**–**h**, compounds possessing an electron-donating substituent on the 5-phenyl moiety showed enhanced cytotoxic activity over those substituted with electron-withdrawing groups (compare **3c**,**d** and **3b** & **3g**,**h** and **3f**). In addition, the presence of 4-dimethylamino substituent on the 5-phenyl moiety improved the activity over that of the 3,4-dimethoxy substituent (compare **3d** and **3c** & **3h** and **3g**).
- Regarding series **6a**–**d**, the presence of electron-donating substituent at the para position of the phenyl ring of the 6-phenyldiazenyl moiety increased the cytotoxic activity against brine shrimp larvae (compare **6c**, **d** and **6a**, **b**).
- Taking into account the series of benzothiadiazoloquinazolines **8a**–**d**, it is assumed that the presence of 5-phenyl moiety substituted with electron-withdrawing group increased the cytotoxic activity (compare **8b** and **8c**).

Table 3
Cytotoxic activity of compounds **3**–**10**, **12**, **13** and **15** using brine shrimp lethality bioassay.[a]

Comp. No.	Conc. (μg/mL)	LogC	Number of dead nauplii	% Mortality	LC_{50} (μg/mL)	LC_{90} (μg/mL)
3b	1000	3	25	83.3	508.13	914.63
	500	2.698	21	70.0		
	100	2	16	53.3		
	10	1	11	36.6		
3c	1000	3	28	93.3	442.47	796.46
	500	2.698	25	83.3		
	100	2	21	70.0		
	10	1	15	50.0		
3d	1000	3	30	100.0	404.85	728.74
	500	2.698	28	93.3		
	100	2	25	83.3		
	10	1	20	66.6		
3f	1000	3	20	66.6	659.63	1187.33
	500	2.698	15	50.0		
	100	2	11	36.6		
	10	1	7	23.3		
3g	1000	3	21	70.0	598.08	1076.55
	500	2.698	18	60.0		
	100	2	15	50.0		
	10	1	11	36.6		
3h	1000	3	30	100.0	399.68	719.42
	500	2.698	29	96.6		
	100	2	26	86.6		
	10	1	21	70.0		
6a	1000	3	29	96.6	419.46	755.03
	500	2.698	27	90.0		
	100	2	24	80.0		
	10	1	20	66.6		
6b	1000	3	27	90.0	459.55	827.20
	500	2.698	24	80.0		
	100	2	20	66.6		
	10	1	14	46.6		
6c	1000	3	30	100.0	392.46	706.43
	500	2.698	30	100.0		
	100	2	29	96.6		
	10	1	27	90.0		
6d	1000	3	30	100.0	403.87	726.97
	500	2.698	28	93.3		
	100	2	26	86.6		
	10	1	22	73.3		
8b	1000	3	29	96.6	438.21	788.78
	500	2.698	24	80.0		
	100	2	20	66.6		
	10	1	15	50.0		
8c	1000	3	28	93.3	455.37	819.67
	500	2.698	23	76.6		
	100	2	19	63.3		
	10	1	14	46.6		
9	1000	3	29	96.6	432.15	777.87
	500	2.698	25	83.3		
	100	2	21	70.0		
	10	1	15	50.0		
10	1000	3	29	96.6	419.11	754.40
	500	2.698	27	90.0		
	100	2	24	80.0		
	10	1	22	73.3		
12	1000	3	26	86.6	477.09	858.77
	500	2.698	23	76.6		
	100	2	20	66.6		
	10	1	14	46.6		
13	1000	3	30	100.0	398.72	717.70
	500	2.698	29	96.6		
	100	2	27	90.0		
	10	1	21	70.0		
15a	1000	3	24	80.0	551.26	992.28
	500	2.698	18	60.0		
	100	2	12	40.0		
	10	1	7	23.3		
15b	1000	3	29	96.6	425.17	765.30
	500	2.698	26	86.6		
	100	2	23	76.6		
	10	1	19	63.3		

Table 3 (*continued*)

Comp. No.	Conc. (μg/mL)	LogC	Number of dead nauplii	% Mortality	LC_{50} (μg/mL)	LC_{90} (μg/mL)
5-Fu	1000	3	30	100.0	409.16	736.49
	500	2.698	27	90.0		
	100	2	25	83.3		
	10	1	19	63.3		
Control	–	–	0	0.0	–	–

LC_{90}, compound concentration required to kill 90% of brine shrimp nauplii.
[a] LC_{50}, compound concentration required to kill 50% of brine shrimp nauplii.

- For the imidazothiadiazoles **15a**,**b**, it is clear that the presence of 5-benzylidene moiety substituted with electron-donating group enhanced the cytotoxic activity against brine shrimp larvae (compare **15b** and **15a**).

3. Conclusion

3.1. From the results of antimicrobial and cytotoxicity testing we can conclude that

- The unsubstituted thiadiazolopyrimidin-7-one nucleus enhances the antibacterial activity against *Staphylococcus aureus*, antifungal activity against *C. albicans*, and cytotoxic activity against brine shrimp larvae (compound **13**).
- The thiadiazolopyrimidine nucleus possessing dimethylamino substituent at the para position of the 5-phenyl moiety improved antifungal activity against *C. albicans* as well as cytotoxic activity against brine shrimp larvae (compound **3h**). On the other hand, the presence of chloro substituent at the para position of the 5-phenyl moiety decreased the antibacterial, antifungal, and cytotoxic activities (compounds **3b** and **3f**).
- The thiadiazolopyrimidine nucleus possessing methyl substituent at the para position of 6-phenyldiazenyl moiety increased the antifungal activity against *C. albicans* as well as cytotoxic activity (compounds **6c**).
- The imidazothiadiazole nucleus possessing 5-benzylidene moiety substituted with electron-donating group increased antibacterial activity against *B. cereus* & *Staphylococcus aureus*, antifungal activity against *Aspergillus fumigatus* 293 & *Aspergillus flavus*, and cytotoxic activity (compounds **15b**). On the other hand, electron-withdrawing substituent on 5-benzylidene moiety reduced the antibacterial and antifungal activities against the same microorganisms, as well as cytotoxic activity (compounds **15a**).

4. Experimental

4.1. Chemistry

All melting points (°C) were recorded on Fisher-Johns melting point apparatus and are uncorrected. The infrared spectra were recorded in KBr disc using a Unicam SP 1000 IR spectrometer (ν in cm^{-1}) at Faculty of Science, Mansoura University. Nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were obtained on 300 MHz FT-NMR spectrometer at Faculty of Science, Cairo University. The chemical shifts are expressed in δ ppm using tetramethylsilane (TMS) as internal reference and DMSO-d_6 as solvent. Mass spectra were recorded on JEOL JMS-600H spectrometer using electron impact technique at 70 eV at Microanalytical Unit, Cairo University. Microanalyses (C, H, N) were performed at Microanalytical Unit, Cairo University, and were in agreement with the proposed structures. Reaction times were monitored using TLC plates, Silica gel 60 F_{254} precoated (E. Merck) and the spots were visualized by UV. Chloroform: methanol (9:1) was adopted as elution solvent. Compound **1** was prepared as reported [28], compounds **2a**–**h** were prepared according to the literature method [29], compounds **5a**–**d** were prepared adopting the published method [34], compounds **7a**–**d** were prepared following the literature procedure [35].

*4.1.1. General procedure for the preparation of 6-(ethoxycarbonyl or cyano)-5,6-dihydro-7-oxo-5-(substituted phenyl)-[1,3,4]thiadiazolo [3,2-a]pyrimidine-2-sulfonamides (**3a**–**h**)*

A mixture of 2-amino-1,3,4-thiadiazole-5-sulfonamide (**1**) (0.45 g, 0.0025 mol) and the appropriate benzylidene derivative of diethyl malonate **2a**–**d** or ethyl cyanoacetate **2e**–**h** (0.0025 mol) in DMF (10 mL) was heated under reflux for 18–20 h. The reaction mixture was cooled and poured onto ice-water (50 mL). The precipitated solid was collected by filtration, dried and crystallized from ethanol/water (3:1).

*4.1.1.1. Ethyl 5-(4-bromophenyl)-5,6-dihydro-7-oxo-2-sulfamoyl-[1,3,4]thiadiazolo[3,2-a]pyrimidine-6-carboxylate (**3a**).* Yield 45%, m.p. 197–199 °C. IR spectrum (KBr, ν, cm^{-1}): 3265, 3192 (NH_2), 1750 ($\underline{COO}C_2H_5$), 1628 (C=O). MS *m/z* (%): 462 (0.22, $M^+ + 1$), 64 (100.00). Anal. Calcd for $C_{14}H_{13}BrN_4O_5S_2$ (461.31): C 36.45, H 2.84, N 12.15. Found: C 36.67, H 2.55, N 11.87.

*4.1.1.2. Ethyl 5-(4-chlorophenyl)-5,6-dihydro-7-oxo-2-sulfamoyl-[1,3,4]thiadiazolo[3,2-a]pyrimidine-6-carboxylate (**3b**).* Yield 56%, m.p. 187–189 °C. ^1H NMR spectrum: (DMSO-d_6, δ ppm): 1.30 (t, 3H, $CH_2\underline{CH_3}$), 3.86 (d, 1H, C_6–H), 4.28–4.35 (q, 2H, $\underline{CH_2}CH_3$), 5.52 (d, 1H, C_5–H), 7.68 (d, 2H, Ar–H), 8.06 (d, 2H, Ar–H), 8.41 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 13.0, 53.2, 54.6, 61.1, 128.7 (2C), 130.1 (2C), 133.4, 135.2, 157.8, 161.1, 168.7, 169.2. MS *m/z* (%): 419 (10.10, $M^+ + 2$), 418 (14.00, $M^+ + 1$), 417 (16.10, M^+), 101 (100.00). Anal. Calcd for $C_{14}H_{13}ClN_4O_5S_2$ (416.86): C 40.34, H 3.14, N 13.44. Found: C 40.67, H 3.47, N 13.19.

*4.1.1.3. Ethyl 5,6-dihydro-5-(3,4-dimethoxyphenyl)-7-oxo-2-sulfamoyl-[1,3,4]thiadiazolo[3,2-a]pyrimidine-6-carboxylate (**3c**).* Yield 45%, m.p. 238–240 °C. ^1H NMR spectrum: (DMSO-d_6, δ ppm): 1.29 (t, 3H, $CH_2\underline{CH_3}$), 3.80 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.26–4.34 (m, 3H, $\underline{CH_2}CH_3$, C_6–H), 5.45 (d, 1H, C_5–H), 7.17–7.78 (m, 3H, Ar–H), 8.31 (s, 2H, NH_2). MS *m/z* (%): 442 (20.98, M^+), 151 (100.00). Anal. Calcd for $C_{16}H_{18}N_4O_7S_2$ (442.47): C 43.43, H 4.10, N 12.66. Found: C 43.75, H 4.38, N 12.37.

*4.1.1.4. Ethyl 5,6-dihydro-5-(4-dimethylaminophenyl)-7-oxo-2-sulfamoyl-[1,3,4]thiadiazolo[3,2-a]pyrimidine-6-carboxylate (**3d**).* Yield 65%, m.p. 165–166 °C. IR spectrum (KBr, ν, cm^{-1}): 3183 (NH_2), 1742 ($\underline{COO}C_2H_5$), 1635 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 1.28 (t, 3H, $CH_2\underline{CH_3}$), 3.08 (s, 6H, $2CH_3$), 3.82 (d, 1H, C_6–H), 4.23–4.29 (q, 2H, $\underline{CH_2}CH_3$), 5.47 (d, 1H, C_5-H), 6.83 (d, 2H, Ar–H), 7.94 (d, 2H, Ar–H), 8.01 (s, 2H, NH_2). Anal. Calcd for

$C_{16}H_{19}N_5O_5S_2$ (425.48): C 45.17, H 4.50, N 16.46. Found: C 45.40, H 4.85, N 16.14.

*4.1.1.5. 5-(4-Bromophenyl)-6-cyano-5,6-dihydro-7-oxo-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**3e**).* Yield 65%, m.p. 173–174 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3430, 3327 (NH_2), 2212 (C≡N), 1640 (C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 4.16 (d, 1H, C_6–H), 5.06 (d, 1H, C_5–H), 7.28–7.76 (m, 6H, Ar–H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 38.6, 46.2, 116.4, 122.3, 128.9 (2C), 130.6 (2C), 139.8, 151.9, 162.3, 169.7. Anal. Calcd for $C_{12}H_8BrN_5O_3S_2$ (414.26): C 34.79, H 1.95, N 16.91. Found: C 34.45, H 2.23, N 16.69.

*4.1.1.6. 5-(4-Chlorophenyl)-6-cyano-5,6-dihydro-7-oxo-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**3f**).* Yield 70%, m.p. 179–180 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3376, 3318 (NH_2), 2220 (C≡N), 1637 (C=O). MS *m/z* (%): 371 (0.40, M^++2), 370 (0.51, M^++1), 369 (0.59, M^+), 344 (100.00). Anal. Calcd for $C_{12}H_8ClN_5O_3S_2$ (368.98): C 38.97, H 2.18, N 18.94. Found: C 39.25, H 1.93, N 18.72.

*4.1.1.7. 6-Cyano-5,6-dihydro-5-(3,4-dimethoxyphenyl)-7-oxo-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**3g**).* Yield 60%, m.p. 191–192 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3376, 3318 (NH_2), 2220 (C≡N), 1662 (C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.31 (d, 1H, C_6-H), 5.65 (d, 1H, C_5–H), 7.02–7.81 (m, 3H, Ar–H), 9.20 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 38.6, 46.0, 55.5, 55.7, 111.6, 116.8, 118.7, 120.6, 136.5, 138.2, 148.4, 148.8, 160.7, 169.2. MS *m/z* (%): 395 (41.80, M^+), 119 (100.00). Anal. Calcd for $C_{14}H_{13}N_5O_5S_2$ (395.41): C 42.53, H 3.31, N 17.71. Found: C 42.36, H 3.57, N 17.98.

*4.1.1.8. 6-Cyano-5,6-dihydro-5-(4-dimethylaminophenyl)-7-oxo-[1,3,4]thiadiazolo[3,2-a]- pyrimidine-2-sulfonamide (**3h**).* Yield 75%, m.p. 177–178 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3374 (NH_2), 2210 (C≡N), 1662 (C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.82 (s, 3H, CH_3), 2.97 (s, 3H, CH_3), 4.21 (d, 1H, C_6–H), 5.09 (d, 1H, C_5–H), 6.64–7.51 (m, 4H, Ar–H), 8.46 (s, 2H, NH_2). Anal. Calcd for $C_{14}H_{14}N_6O_3S_2$ (378.40): C 44.43, H 3.73, N 22.21. Found: C 44.67, H 4.11, N 21.96.

*4.1.2. General procedure for the preparation of 5-amino-7-oxo-6-[4-(substituted)phenyldiazenyl]-5H, 7H-[1,3,4]thiadiazolo[3,2-a] pyrimidine-2-sulfonamides (**6a**–**d**)*

A mixture of amine **1** (0.45 g, 0.0025 mol) and the appropriate ethyl 2-cyano-2-[4-(substituted)phenyldiazenyl]acetate **5a**–**d** (0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 14–16 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, dried and crystallized from acetic acid/water (2:1).

*4.1.2.1. 5-Amino-7-oxo-6-phenyldiazenyl-5H, 7H-[1,3,4]thiadiazolo [3,2-a]pyrimidine-2-sulfonamide (**6a**).* Yield 75%, m.p. 237–238 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3300, 3205 ($2NH_2$), 1687 (C=O). MS *m/z* (%): 351 (0.29, M^+), 43 (100.00). Anal. Calcd for $C_{11}H_9N_7O_3S_2$ (351.36): C 37.60, H 2.58, N 27.90. Found: C 37.35, H 2.37, N 27.63.

*4.1.2.2. 5-Amino-6-[(4-bromophenyl)diazenyl]-7-oxo-5H, 7H-[1,3,4] thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**6b**).* Yield 60%, m.p. 238–240 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3289, 3203 ($2NH_2$), 1689 (C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 3.10 (s, 2H, C_5–NH_2), 7.25–7.73 (m, 6H, Ar–H, SO_2NH_2). MS *m/z* (%): 431 (5.28, M^+ + 1), 91 (100.00). Anal. Calcd for $C_{11}H_8BrN_7O_3S_2$ (430.26): C 30.71, H 1.87, N 22.79. Found: C 30.43, H 1.59, N 23.12.

*4.1.2.3. 5-Amino-6-[(4-methylphenyl)diazenyl]-7-oxo-5H, 7H-[1,3,4] thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**6c**).* Yield 65%, m.p. 175–177 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.18 (s, 3H, CH_3), 3.19 (s, 2H, C_5–NH_2), 6.40–7.65 (m, 6H, Ar–H, SO_2NH_2). MS *m/z* (%): 365 (1.92, M^+), 117 (100.00). Anal. Calcd for $C_{12}H_{11}N_7O_3S_2$ (365.39): C 39.44, H 3.03, N 26.83. Found: C 39.77, H 2.85, N 26.56.

*4.1.2.4. 5-Amino-6-[(4-methoxyphenyl)diazenyl]-7-oxo-5H, 7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**6d**).* Yield 70%, m.p. 200–202 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.99 (s, 2H, C_5–NH_2), 3.81 (s, 3H, OCH_3), 6.78–7.83 (m, 6H, Ar–H, SO_2NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 55.1, 87.8, 113.7 (2C), 120.5 (2C), 132.4, 144.2, 154.9, 167.7, 168.8, 169.6. MS *m/z* (%): 381 (0.16, M^+), 60 (100.00). Anal. Calcd for $C_{12}H_{11}N_7O_4S_2$ (381.39): C 37.79, H 2.91, N 25.71. Found: C 37.51, H 3.23, N 25.49.

*4.1.3. General procedure for the preparation of 6,7-dihydro-9-methoxy-5-(substituted phenyl)-5H-benzo[h][1,3,4]thiadiazolo[2,3-b]quinazoline-2-sulfonamides (**8a**–**d**)*

A mixture of 2-amino-1,3,4-thiadiazole-5-sulfonamide **(1)** (0.45 g, 0.0025 mol) and the appropriate benzylidenetetralone derivative **7a**–**d** (0.0025 mol) in propylene glycol (10 mL) was heated at 200–220 °C for 10–12 h. The mixture was cooled and diluted with water (50 mL) with vigorous stirring. The separated solid was collected by filtration, washed with water, dried and crystallized from ethanol/water (2:1).

*4.1.3.1. 5-(4-Bromophenyl)-6,7-dihydro-9-methoxy-5H-benzo[h][1,3,4]thiadiazolo[2,3-b]quinazoline-2-sulfonamide (**8a**).* Yield 50%, m.p. 226–227 °C. IR spectrum (KBr, *ν*, cm^{-1}): broad band at 3427 (NH_2). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.65-3.10 (m, 4H, $2CH_2$), 3.85 (s, 3H, OCH_3), 4.18 (s, 1H, C_5–H), 7.46–7.70 (m, 9H, Ar–H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 22.6, 28.5, 51.6, 55.8, 112.1, 112.3, 112.8, 117.9, 119.8, 126.5, 128.6 (2C), 130.7 (2C), 132.3, 139.2, 140.7, 156.4, 157.7, 161.3. MS *m/z* (%): 506 (0.09, M^++1), 505 (0.01, M^+), 504 (0.22, M^+ – 1), 77 (100.00). Anal. Calcd for $C_{20}H_{17}BrN_4O_3S_2$ (505.41): C 47.53, H 3.39, N 11.09. Found: C 47.75, H 3.33, N 11.37.

*4.1.3.2. 5-(4-Chlorophenyl)-6,7-dihydro-9-methoxy-5H-benzo[h][1,3,4]thiadiazolo[2,3-b] quinazoline-2-sulfonamide (**8b**).* Yield 55%, m.p. 211–212 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.56–2.84 (m, 4H, $2CH_2$), 3.83 (s, 3H, OCH_3), 4.11 (s, 1H, C_5–H), 7.19–7.49 (m, 9H, Ar–H, NH_2). MS *m/z* (%): 461 (4.29, M^+), 460 (4.72, M^+ – 1), 59 (100.00). Anal. Calcd for $C_{20}H_{17}ClN_4O_3S_2$ (460.96): C 52.11, H 3.72, N 12.15. Found: C 51.85, H 3.53, N 11.83.

*4.1.3.3. 6,7-Dihydro-9-methoxy-5-(3,4-dimethoxyphenyl)-5H-benzo[h][1,3,4]thiadiazolo[2,3-b]quinazoline-2-sulfonamide (**8c**).* Yield 45%, m.p. 145–146 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.72–3.11 (m, 4H, $2CH_2$), 3.85–3.98 (m, 10H, $3OCH_3$, C_5–H), 6.51–7.15 (m, 8H, Ar–H, NH_2). MS *m/z* (%): 488 (9.67, M^++2), 487 (16.67, M^+ + 1), 165 (100.00). Anal. Calcd for $C_{22}H_{22}N_4O_5S_2$ (486.56): C 54.31, H 4.56, N 11.51. Found: C 54.65, H 4.33, N 11.67.

*4.1.3.4. 6,7-Dihydro-9-methoxy-5-(4-dimethylaminophenyl)-5H-benzo[h][1,3,4]thiadiazolo[2,3-b]quinazoline-2-sulfonamide (**8d**).* Yield 48%, m.p. 152–153 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.65–2.92 (m, 4H, $2CH_2$), 2.99 (s, 3H, CH_3), 3.04 (s, 3H, CH_3), 3.42 (s, 1H, C_5–H), 3.88 (s, 3H, OCH_3), 6.60–7.53 (m, 7H, Ar–H), 7.82 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 22.7, 28.5, 40.6 (2C), 51.3, 55.6, 111.7, 111.9, 112.3, 112.8 (2C), 118.1, 125.8, 126.3 (2C), 131.5, 132.1, 138.8, 146.7, 153.4, 158.2, 160.6. MS *m/z* (%): 470 (0.24, M^++1), 469 (0.39, M^+), 101 (100.00). Anal. Calcd for $C_{22}H_{23}N_5O_3S_2$ (469.58): C 56.27, H 4.94, N 14.91. Found: C 56.55, H 4.67, N 14.73.

*4.1.4. Preparation of 6-cyano-5-oxo-5H-[1,3,4]thiadiazolo[3,2-a] pyrimidine-2-sulfonamide (**9**)*

A mixture of amine **1** (0.45 g, 0.0025 mol) and ethyl 2-(ethoxymethylene)cyanoacetate (0.423 g, 0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 16 h. The reaction

mixture was cooled and the precipitated solid was collected by filtration, dried and crystallized from acetic acid/water (3:1).

Yield 72%, m.p. 280–282 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3327, 3203 (NH_2), 2220 (C≡N), 1680 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 8.29 (s, 2H, NH_2), 8.84 (s, 1H, C_7–H). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 104.7, 116.2, 153.6, 157.9, 161.2, 168.7. MS *m/z* (%): 258 (3.60, $M^+ + 1$), 257 (6.10, M^+), 117 (100.00). Anal. Calcd for $C_6H_3N_5O_3S_2$ (257.25): C 28.01, H 1.18, N 27.22. Found: C 28.32, H 1.35, N 27.51.

4.1.5. Preparation of 6-cyano-5-imino-5H-[1,3,4]thiadiazolo[3,2-a] pyrimidine-2-sulfonamide (**10**)

A mixture of amine **1** (0.45 g, 0.0025 mol) and 2-(ethoxymethylene)malononitrile (0.305 g, 0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 20 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, dried and crystallized from ethanol.

Yield 68%, m.p. 228–230 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3429, 3327 (NH_2, NH), 2200 (C≡N). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 4.16 (s, 1H, NH), 7.25 (s, 2H, NH_2), 8.13 (s, 1H, C_7-H). MS *m/z* (%): 257 (1.10, $M^+ + 1$), 256 (7.10, M^+), 64 (100.00). Anal. Calcd for $C_6H_4N_6O_2S_2$ (256.26): C 28.12, H 1.57, N 32.79. Found: C 27.93, H 1.73, N 32.98.

4.1.6. Preparation of 5-amino-7-imino-7H-[1,3,4]thiadiazolo[3,2-a] pyrimidine-2-sulfonamide (**11**)

A mixture of amine **1** (0.45 g, 0.0025 mol), malononitrile (0.165 g, 0.0025 mol) and triethylamine (1 mL) in absolute ethanol (15 mL) was heated under reflux for 18 h. The reaction mixture was concentrated, cooled and the precipitated solid was collected by filtration, dried and crystallized from ethanol/water (3:1).

Yield 65%, m.p. >300 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3407, 3332, 3245, 3175 ($2NH_2$, NH). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 4.10 (s, 2H, C_5–NH_2), 7.29 (s, 1H, C_6–H), 7.47 (s, 2H, SO_2NH_2), 8.03 (s, 1H, NH). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 85.4, 157.2, 160.7, 163.5, 168.1. MS *m/z* (%): 247 (0.12, $M^+ + 1$), 246 (0.14, M^+), 64 (100.00). Anal. Calcd for $C_5H_6N_6O_2S_2$ (246.27): C 24.39, H 2.46, N 34.13. Found: C 24.46, H 2.62, N 33.89.

4.1.7. Preparation of 11-oxo-5-(thiophen-3-yl)-5H, 6H-benzo[g] [1,3,4]thiadiazolo[3,2-a][1,3,5]triazocine-2-sulfonamide (**12**)

A mixture of amine **1** (0.45 g, 0.0025 mol) and 2-[(thiophen-3-yl)methyleneamino]benzoic acid (0.578 g, 0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 20 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, dried and crystallized from acetic acid.

Yield 75%, m.p. 160–162 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3445, 3387 (NH_2), 3329 (NH), 1688 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 2.93 (s, 1H, C_5–H), 7.00–8.52 (m, 9H, Ar–H, NH_2), 11.20 (s, 1H, NH). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 81.1, 115.1, 116.8, 117.4, 120.7, 127.9, 128.2, 129.9, 130.4, 138.2, 146.0, 153.2, 154.6, 168.9. MS *m/z* (%): 395 (0.02, $M^+ + 2$), 394 (0.01, $M^+ + 1$), 393 (0.02, M^+), 179 (100.00). Anal. Calcd for $C_{14}H_{11}N_5O_3S_3$ (393.46): C 42.74, H 2.82, N 17.80. Found: C 42.85, H 2.97, N 17.59.

4.1.8. Preparation of 6,7-dihydro-7-oxo-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**13**)

A mixture of amine **1** (0.45 g, 0.0025 mole) and 3-chloropropionyl chloride (0.317 g, 0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 15 h. The solvent was concentrated and the precipitated solid was collected by filtration, dried and crystallized from ethanol.

Yield 72%, 257–258 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3139, 3200 (NH_2), 1652 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 2.84 (t, 2H, CH_2), 3.01 (t, 2H, CH_2), 8.20 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 32.8, 40.3, 161.1, 164.3, 169.2. MS *m/z* (%): 395 (0.02, $M^+ + 2$), 394 (0.01, $M^+ + 1$), 393 (0.02, M^+), 179 (100.00). MS *m/z* (%): 234 (0.05, M^+), 85 (100.00). Anal. Calcd for $C_5H_6N_4O_3S_2$ (234.26): C 25.64, H 2.58, N 23.92. Found: C 25.43, H 2.75, N 23.64.

4.1.9. Preparation of 5,6-dihydro-6-oxoimidazo[2,1-b][1,3,4] thiadiazole-2-sulfonamide (**14**)

A mixture of amine **1** (0.45 g, 0.0025 mole) and ethyl chloroacetate (0.306 g, 0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 15 h. The solvent was concentrated and the precipitated solid was collected by filtration, dried and crystallized from ethanol.

Yield 56%, m.p. 268–270 °C. IR spectrum (KBr, *ν*, cm^{-1}): 3303, 3186 (NH_2), 1679 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 2.88 (s, 2H, CH_2), 8.17 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 44.7, 157.6, 162.1, 167.9. MS *m/z* (%): 221 (0.23, $M^+ + 1$), 220 (0.10, M^+), 117 (100.00). Anal. Calcd for $C_4H_4N_4O_3S_2$ (220.23): C 21.81, H 1.83, N 25.44. Found: C 21.95, H 2.11, N 25.36.

4.1.10. Preparation of 5,6-dihydro-6-oxo-5-(substituted benzylidene) imidazo[2,1-b][1,3,4]thiadiazole-2-sulfonamide (**15a**,**b**)

A mixture of compound **14** (0.55 g, 0.0025 mole), the appropriate benzaldehyde (0.0025 mol) and a catalytic amount of piperidine (0.3 mL) in dioxane (10 mL) was heated under reflux for 8–10 h. The solvent was concentrated and the precipitated solid was collected by filtration, dried and crystallized from ethanol.

*4.1.10.1. 5-(4-Bromobenzylidene)-5,6-dihydro-6-oxoimidazo[2,1-b] [1,3,4]thiadiazole-2-sulfonamide (**15a**).* Yield 50%, m.p. 210–211 °C. ^1H NMR spectrum: (DMSO-d_6, δ ppm): 7.31–7.65 (m, 7H, Ar–H, C=CH, NH_2). MS *m/z* (%): 385 (0.15, $M^+ - 2$), 84 (100.00). Anal. Calcd for $C_{11}H_7BrN_4O_3S_2$ (387.23): C 34.12, H 1.82, N 14.47. Found: C 34.36, H 1.97, N 14.71.

*4.1.10.2. 5,6-Dihydro-5-(3,4-dimethoxybenzylidene)-6-oxoimidazo [2,1-b][1,3,4]thiadiazole-2-sulfonamide (**15b**).* Yield 65%, m.p. 177–179 °C. ^1H NMR spectrum: (DMSO-d_6, δ ppm): 3.76 (s, 6H, $2OCH_3$), 6.64–7.20 (m, 3H, Ar–H), 7.35 (s, 1H, C=CH), 7.95 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 55.6, 55.7, 112.2, 114.6, 118.5, 127.4, 129.2, 148.3, 148.5, 151.8, 157.3, 162.5, 169.8. MS *m/z* (%): 368 (12.30, M^+), 238 (100.00). Anal. Calcd for $C_{13}H_{12}N_4O_5S_2$ (368.39): C 42.38, H 3.28, N 15.21. Found: C 42.51, H 3.43, N 14.93.

4.2. Biological testing

4.2.1. Antimicrobial and antiquorum-sensing activities

Some of the prepared compounds, **3b**–**d**, **3f**–**h**, **6a**–**d**, **8a**,**b**, **9**–**14** and **15a**,**b** were screened for antibacterial activity against *E. coli*, *Staphylococcus aureus* and *B. cereus* in (Luria–Bertani agar media) [36] and antifungal activity against *C. albicans* in (Saboured's agar), *Aspergillus fumigatus* 293 and *Aspergillus flavus* 3375 in (glucose minimal media) [37]. In addition, their antipathogenic potential was checked by examining the antiquorum-sensing activity against *Chromobacterium violaceum* ATCC 12472 in (Luria–Bertani agar media) [38].

E. coli, *B. cereus*, *Staphylococcus aureus* and *C. albicans* were obtained from the culture collection of the Department of Microbiology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Filamentous fungi; *Aspergillus fumigatus* 293 and *Aspergillus flavus* 3375 were kindly provided from Keller Lab, UW, USA. *Chromobacterium violaceum* ATCC 12472 was kindly provided from Prof. Bob Mclean, Department of Biology, Texas State University, USA. All bacterial strains were propagated in Luria–Bertani (LB) agar media (Merck, Germany). *C. albicans* was grown in Saboured's media (Merck, Germany) and filamentous fungi were cultivated in glucose

minimal media (Merck, Germany). Ampicillin was obtained from EPICO company; Fluconazole was obtained from Pfizer company.

4.2.1.1. Antibacterial screening. All the bacterial strains were propagated in Luria-Bertani (LB) broth (1% peptone, 0.5% yeast extract, 0.5% NaCl) and solidified with 1.5% agar. Melted Muller Hinton agar (50 mL) at 50 °C were seeded with 50 μl of 1×10^6 CFU/mL of 18 h culture of tested microorganisms. The inoculated agar was mixed and poured into 15-cm-diameter plates to solidify. Wells were made in agar using cork borer. Tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL. Aliquots each of (100 μl) of each compound were applied into the wells, DMSO was also included as a negative control, and ampicillin in a concentration of 5 mg/mL was used as a reference antibacterial agent. The compounds were allowed to diffuse for 2 h at 4 °C and incubated at 37 °C for 24 h [36]. Inhibition zones were measured using Vernier caliper and the activity of the tested compounds was estimated in comparison to ampicillin (Table 1). The inhibition zone diameter of DMSO was subtracted from the antibacterial activity of tested compounds.

4.2.1.2. Antifungal screening. Saboured's media (50 mL) was inoculated with 50 μl of 1×10^6 CFU/mL of 24 h culture of *C. albicans*. For filamentous fungi, glucose minimal media (50 mL) was inoculated with 50 μl of 1×10^6 CFU/mL of *Aspergilli*. Wells were made in agar using cork borer. The tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL and 100 μl of test solution was applied into the wells. The standard antifungal drug (fluconazole) was also added at the same concentration to each plate. In addition, DMSO (control solvent) was added to each plate. Plates were incubated at 37 °C for 48 h for *C. albicans* and *Aspergillus fumigatus* and at 30 °C for 48 h for *Aspergillus flavus* [37]. Antifungal activity of the tested compounds was determined by measuring the diameter of the inhibition zone (Table 1). The inhibition zone diameter of DMSO was subtracted from the antifungal activity of tested compounds.

4.2.1.3. Antiquorum-sensing screening. Cultures were prepared by growing *Chromobacterium violaceum* ATCC 12472 in Luria Bertani (LB) broth and incubated for 16–18 h in an orbital incubator running at 28 °C and 150 rpm. Cultures were then adjusted to 0.5 McFarland standard (Ca. 1×10^6 CFU/mL). *Chromobacterium violaceum* was inoculated (100 μl/plate) in 50 mL LB agar and solidified. Wells were made in LB agar media using cork borer. The tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL and 100 μl of test solution was applied into the wells. DMSO (control solvent) was added to each plate. Plates were incubated at 30 °C for 48 h to check the inhibition of pigment production around the wells. Bacterial growth inhibition would result in a clear halo around the disc, while a positive quorum sensing inhibition is exhibited by a turbid halo harboring pigmentless bacterial cells of *Chromobacterium violaceum* ATCC 12472 monitor strain [38]. Bacterial growth inhibition by the tested compounds was measured as radius (r_1) in mm, while both growth and pigment inhibition was measured as radius (r_2) in mm. The pigment inhibition (QS inhibition) was determined by subtracting bacterial growth inhibition (r_1) from the total radius (r_2); thus, QS inhibition $= (r_2 - r_1)$ in mm (Table 1).

4.2.2. Antitumor screening

Under sterile conditions, cell lines were grown in RPMI 1640 media (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Biocell, CA, USA). The concentrations of the compounds ranging from 0.01 to 100 μM were prepared in phosphate buffer saline. Each compound was initially solubilized in DMSO, however, each final dilution contained less than 1% DMSO. Solutions of different concentrations (0.2 mL) were pipetted into separate wells of 96-multiwell microtiter plate, duplicate wells were prepared for each individual dose. Cell culture (1.8 mL) containing a cell population of 6×10^4 cells/mL was pipetted into each well. Controls containing only phosphate buffer saline and DMSO at identical dilutions, were also prepared in the same manner. The cultures were incubated with the compounds for 48 h in a humidified incubator at 37 °C and in atmosphere of 5% CO_2. After 48 h, cells in each well were diluted 10 times with saline and counted by using a coulter counter [41–44]. The data reported as mean-graph of the percent growth of the treated cells, and presented as percentage growth inhibition (GI%) (Table 2).

4.2.3. Cytotoxicity testing using brine shrimp lethality bioassay

A. salina Leach (brine shrimp eggs) are readily available as fish food in pet shops. Artificial "sea water" is prepared by dissolving sea salt in distilled water (40 g/liter) supplemented with dried yeast (6 mg/L). Brine shrimp eggs are hatched in artificial sea water during 48 h incubation in a warm room (22–29 °C), providing large numbers of larvae (nauplii). Brine shrimp larvae (nauplii) are collected with a Pasteur pipette after attracting the organisms to one side of the vessel with a light source. Nauplii are separated from the eggs by pipetting them 2–3 times in small beakers containing sea water. The test samples were made up to 1 mg/mL in artificial sea water (water insoluble compounds are dissolved in 50 μl DMSO prior to adding sea water). Serial dilutions (1000, 500, 100, 10 μg/mL) were added into separate wells of 96-multiwell microtiter plate in triplicate in 100 μl sea water. A suspension of nauplii containing 30 brine shrimp larvae in 100 μl sea water was added to each well with the help of a Pasteur pipette and the covered microwell plate was incubated at 22–29 °C for 24 h. The plate was then examined under a binocular microscope and the number of dead (non-mobile) nauplii in each well was counted. Methanol (100 μl) was then added to each well and after 15 min the total number of shrimps in each well was counted [51]. Plotting of log concentration (logC) versus % mortality for all tested samples showed an approximate linear correlation and the values of LC_{50} and LC_{90} were calculated by using Microsoft Excel XP (Table 3). All values were compared with the standard cytotoxic agent, 5-flurouracil with LC_{50} and LC_{90} values of 409.16 and 736.49 μg/mL, respectively.

Acknowledgments

The author would like to express her sincere thanks to Dr. Mona I. Shaaban, Microbiology Department, Faculty of Pharmacy, Mansoura University, Egypt, for carrying out the antimicrobial and antiquorom-sensing screening. Thanks to Mr. Ahmed Abbas, a technician at Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Egypt, for carrying out the cytotoxicity testing. Thanks also to the staff members of the Department of Health and Human Services, National Cancer Institute (NCI), Bethesda, Maryland, USA for performing the antitumor testing of the newly synthesized compounds.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.02.010.

References

[1] A. Coates, Y. Hu, R. Bax, C. Page, The future challenges facing the development of new antimicrobial drugs, Nat. Rev. Drug Discov. 1 (2002) 895–910.
[2] A. Adonizio, K. Kong, K. Mathee, Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by south Florida plant extracts, Antimicrob. Agents Chemother. 52 (2008) 198–203.

[3] D. Anbazhagan, M. Mansor, G.O.S. Yan, M.Y. Md Yusof, H. Hassan, S.D. Sekaran, Detection of quorum sensing signal molecules and identification of an autoinducer synthase gene among biofilm forming clinical isolates of *Acinetobacter* spp, PLoS ONE 7 (7) (2012) e36696. http://dx.doi.org/10.1371/journal.pone.0036696.

[4] A.L. Adonizio, K. Downum, B.C. Bennett, K. Mathee, Antiquorum sensing activity of medicinal plants in southern Florida, J. Ethnopharmacol 105 (2006) 427–435.

[5] C.M. Bagi, Summary-Cancer cell metastasis session, J. Musculoskeletal Neuron Interact 2 (2002) 579–580.

[6] A.T. Taher, H.H. Georgey, H.I. El-Subbagh, Novel 1,3,4-heterodiazole analogues: synthesis and *in vitro* antitumor activity, Eur. J. Med. Chem. 47 (2012) 445–451.

[7] N.S. El-Sayed, E.R. El-Bendary, S.M. El-Ashry, M.M. El-Kerdawy, Synthesis and antitumor activity of new sulfonamide derivatives of thiadiazolo[3,2-*a*]pyrimidines, Eur. J. Med. Chem. 46 (2011) 3714–3720.

[8] M.B. El-Ashmaway, M.A. El-Sherbeny, N.S. El-Sayed, Synthesis, *in vitro* antitumor activity and DNA-binding affinity of novel thiadiazolopyrimidine and thiadiazoloquinazoline derivatives, Mansoura J. Pharm. Sci. 26 (1) (2010) 60–68.

[9] M.A. Mahran, M.A. El-Sherbeny, A.M.A. El-Obaid, F.A. Badria, Heterocyclic systems containing pyrimidine nucleus as potential antimicrobial and antitumor agents, Alexandria J. Pharm. Sci. 12 (1998) 39–44.

[10] Y. Tokunaga, S. Ito, Y. Kojima, S. Maeno, N. Sawai, Y. Sasao, Preparation of 2-phenylsulfinyl-5*H*-1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5-one derivatives as agricultural and horticultural fungicides, Jpn. Kokai Tokkyo Koho JP Patent 01 (83) (1989) 084.

[11] S. Herrling, 5-Carboxy-7*H*-1,3,4-thiadiazolo[3,2-*a*]pyrimidin-7-ones as immune enhancers, Ger. Offen. Patent 2 (712) (1978) 932.

[12] M. Suiko, K. Maekawa, Synthesis and antitumor activity of 2-alkanesulfinyl (or alkanesulfonyl)-7-methyl-5*H*-1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5-ones, Agric. Biol. Chem. 41 (1977) 2047–2053.

[13] K. Maekawa, M. Mizumitsu, Thiadiazolo[3,2-*a*]pyrimidinone derivatives, Jpn. Kokai Patent 77 (118) (1977) 494.

[14] R.A. Coburn, R.A. Glennon, Z.F. Chmielewicz, Mesoionic purinone analogues. 7. *In vitro* antibacterial activity of mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidine-5,7-diones, J. Med. Chem. 17 (1974) 1025–1027.

[15] R.A. Coburn, R.A. Glennon, Mesoionic purinone analogues. VI. Synthesis and *in vitro* antibacterial properties of mesoionic thiazolo[3,2-*a*]pyrimidine-5,7-diones and mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidine-5,7-diones, J. Pharm. Sci. 62 (1973) 1785–1789.

[16] K.F.M. Atta, O.O.M. Farahat, A.Z.A. Ahmed, M.G. Marei, Synthesis and antibacterial activities of novel imidazo[2,1-*b*][1,3,4]thiadiazoles, Molecules 16 (2011) 5496–5506.

[17] A. Jakhar, J.K. Makrandi, Synthesis and antibacterial properties of some novel 2- substituted-6-(4-methyl-6-substituted cinnoline-3-yl)imidazo[2,1-*b*][1,3,4]thiadiazoles, Indian J. Chem. 49B (2010) 1547–1551.

[18] G.Q. Hu, L.L. Hou, S.Q. Xie, W.L. Huang, H.B. Zhang, Synthesis and antibacterial activity of imidazothiadiazoles and heterocyclic-amine Mannich-base hydrochloride, Yao Xue Xue Bao 34 (2008) 926–929.

[19] A.K. Gadad, C.S. Mahajanshetti, S. Nimbalkar, A. Raichurkar, Synthesis and antibacterial activity of some 5-guanylhydrazone/thiocyanato-6-arylimidazo [2,1-*b*][1,3,4]thiadiazole-2-sulfonamide derivatives, Eur. J. Med. Chem. 35 (2000) 853–857.

[20] A.K. Gadad, I.M. Khazi, C.S. Mahajanshetti, Synthesis of 2-alkyl-6-arylimidazo [2,1-*b*][1,3,4]thiadiazoles for antibacterial activity, Indian J. Heterocycl. Chem. 2 (1992) 125–128.

[21] C.S. Andotra, T.C. Langer, A. Kotha, Synthesis and antifungal activity of some substituted 1,3,4-thiadiazolo[3,2-*a*]-*s*-triazin-5-phenyl-7-thiones and imidazo [2,1-*b*]-1,3,4-thiadiazol-5-ones, J. Indian Chem. Soc. 74 (1997) 125–127.

[22] A. Kaur, R. Kumar, U. Kalidhar, Synthesis, spectral studies and biological activity of some novel biphenyl imidazo[2,1-*b*][1,3,4]thiadiazole derivatives, Res. J. Pharm. Biol. Chem. Sci. 3 (2012) 1084–1096.

[23] S.G. Alegaon, K.R. Alagawadi, Synthesis, characterization and antimicrobial activity evaluation of new imidazo[2,1-*b*][1,3,4]thiadiazole derivatives, Eur. J. Chem. 2 (2011) 94–99.

[24] J. Mohan, A. Rathee, Condensed bridgehead nitrogen heterocyclic systems: facile synthesis and antimicrobial activity of imidazo[2,1-*b*][1,3,4]thiadiazoles, Indian J. Heterocycl. Chem. 17 (2007) 101–102.

[25] M.N. Noolvi, H.M. Patel, N. Singh, A.K. Gadad, S.S. Cameotra, A. Badiger, Synthesis and anticancer evaluation of novel 2-cyclopropylimidazo[2,1-*b*][1,3,4] thiadiazole derivatives, Eur. J. Med. Chem. 46 (2011) 4411–4418.

[26] T. Nalan, A. Gursouy, Synthesis and anticancer evaluation of some new hydrazone derivatives of 2,6-dimethylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbohydrazide, Eur. J. Med. Chem. 38 (2003) 781–786.

[27] A.K. Gadad, M.B. Palkar, K. Anand, M.N. Noolvi, T.S. Boreddyc, J. Wagwadec, Synthesis and biological evaluation of 2-trifluoromethyl/sulfonamido-5,6-diaryl substituted imidazo[2,1-*b*][1,3,4]thiadiazoles: a novel class of cyclooxygenase-2 inhibitors, Bioorg. Med. Chem. 16 (2008) 276–283.

[28] V. Petrow, O. Stephenson, A.J. Thomas, A.M. Wild, Preparation and hydrolysis of some derivatives of 1,3,4-thiadiazole, J. Chem. Soc. (1958) 1508–1512.

[29] I. Meskini, L. Toupet, M. Daoudi, A. Kerbal, B. Bennani, P.H. Dixneuf, Z.H. Chohan, A.C.L. Leite, T.B. Hadda, An efficient protocol for accessing ß-amino dicarbonyl compounds through aza-Michael reaction, J. Braz. Chem. Soc. 21 (2010) S1–S18.

[30] S.A. Abdel-Mohsen, Heterocycles derived from 5-(2-aminothiazol-4-yl)-8-hydroxyquinoline: synthesis and antimicrobial activity, J. Chin. Chem. Soc. 50 (2003) 1085–1092.

[31] A.A. Hassanien, A.E. Amrb, S.A.S. Ghozlan, Utility of (*p*-sulfonamidophenyl) azomalononitrile, and ethyl acetoacetate in synthesis of fused azole and azines derivatives II, J. Chin. Chem. Soc. 47 (2000) 1273–1278.

[32] J. Quiroga, M. Alvarado, B. Insuasty, R. Mereno, Synthesis of 5-cyanopyrazolo [3,4-*b*]pyridines in the reaction of 5-amino-3-methyl-1-phenylpyrazole with arylidene derivatives of malononitrile and ethyl cyanoacetate, J. Heterocyclic Chem. 36 (1999) 1311–1316.

[33] D.K. Sain, B. Thadhaney, A. Joshi, N. Hussain, G.L. Talesara, Synthesis, characterization and biological evaluation of some heterocyclic compounds containing ethoxyphthalimide moiety *via* key intermediate 6-chloro-1,3-benzothiazole-2-amine, Indian J. Chem. 49B (2010) 818–825.

[34] K.M. Al Zaydi, R.M. Borik, Microwave assisted condensation reactions of 2-aryl-hydrazonopropanals with nucleophilic reagents and dimethyl acetylenedicarboxylate, Molecules 12 (2007) 2061–2079.

[35] S.W. Yee, C. Simons, Synthesis and CYP24 inhibitory activity of 2-substituted-3,4-dihydro-2*H*-naphthalen-1-one (tetralone) derivatives, Bioorg. Med. Chem. Lett. 14 (2004) 5651–5654.

[36] R.D. Pearson, R.T. Steigbigel, H.T. Davis, S.W. Chapmann, Method for reliable determination of minimal lethal antibiotic concentrations, Antimicrob. Agents Chemother. 18 (1980) 699–708.

[37] R.J. Holt, Laboratory tests of antifungal drugs, J. Clin. Pathol. 28 (1975) 767–774.

[38] K.H. McClean, M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S. Stewart, P. Williams, Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acyl homoserine lactones, Microbiology 143 (1997) 3703–3711.

[39] R. McClean, L.S. Pierson, C. Fuqua, A simple screening protocol for the identification of quorum signal antagonists, J. Microbiol. Methods 58 (2004) 351–360.

[40] W. Cha, D.A. Vattem, V. Maitin, M.B. Barnes, R.J. Mclean, Bioassays of quorum sensing compounds using *Agrobacterium tumefaciens* and *Chromobacterium violaceum*, Methods Mol. Biol. 692 (2011) 3–19.

[41] P. Skahan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.R. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxic assay for anticancer drug screening, J. Natl. Cancer Inst. 82 (1990) 1107–1112.

[42] M.R. Grever, S.A. Schepartz, B.A. Chabner, The National Cancer Institute: cancer drug discovery and development program, Semin. Oncol. 19 (1992) 622–638.

[43] A. Monks, D. Scudiero, P. Skehan, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines, J. Natl. Cancer Inst. 83 (1991) 757–766.

[44] M.R. Boyd, K.D. Paull, Some practical considerations and applications of the national cancer institute *in vitro* anticancer drug discovery screen, Drug Dev. Res. 34 (1995) 91–109.

[45] J.B. Harborne, Phytochemical Methods, third ed., Chapman and Hall, London, 1998, ISBN 0-412-57260-5, 1–302.

[46] J.L. Mclaughlin, Crown-gall tumors in potato discs and brine shrimp lethality: two simple bioassays for higher plant screening and fractionation, in: K. Hostettmann (Ed.), Methods in Plant Biochemistry, Academic Press, London, 1991.

[47] C.L. Zani, P.G. Chaves, R. Queiroz, N.M. Mendes, A.B. De Oliveira, J.E. Cardoso, M.G. Anjos, T.S.M. Grandi, Brine shrimp lethality assay as prescreening system for anti-*Trypanosoma cruzi*, Phytomedicine 2 (1995) 47–50.

[48] P.N. Solis, C.W. Wright, M.M. Anderson, M.P. Gupta, J.D. Phillipson, A microwell cytotoxicity assay using *Artemia salina*, Planta Med. 59 (1993) 250–252.

[49] H.C. Birndorf, J. D'Alossio, J.C. Bagshaw, DNA-dependant RNA-polymerases from *Artemia* embryos. Characterization of polymerases I and II from nauplius larvae, Dev. Biol. 45 (1975) 34–43.

[50] C. Sanchez, M. Gupta, M. Vasquez, de Noriega, G. Montenegro, Bioassay with *Artemia* to predict antibacterial and pharmacologic activity, Rev. Med. Panama 18 (1993) 62–69.

[51] B. Mayer, N. Ferrigni, J. Putnam, L. Jacobsen, D. Nichols, J. Mclaughlin, Brine shrimp: a convenient general bioassay for active plant constituents, Planta Med. 45 (1982) 31–34.