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Synthesis, Reactions, and Biological Activity of Some Triazine Derivatives Containing Sulfa Drug Moieties[1]

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Abstract—Thienyl-triazine-sulphonamide conjugates were prepared from their precursor amines using triethyl orthoformate or ethyl chloroformate as cross coupling reagents. The progress of these reactions was investigated by spectral (IR, NMR, MS) and microanalytical techniques. The synthesized compounds were in vitro screened for antibacterial, antifungal, antioxidant, and anticancer activity. 4-[({[3-Mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imino}methyl)amino]-benzenesulfonamide turned out to be a powerful antibacterial agent, while all the compounds prepared were inactive against fungal species tested. 4-{[({8-Cyano-4-oxo-3-[2-(2-thienyl)vinyl]-4*H*,8*H*-[1,2,4]triazino[3,4-*b*][1,3,4]thiadiazin-7-yl}amino)(ethoxy)methyl]amino}benzenesulfonamide displayed in vitro promising cytotoxicity against Ehrlich ascites carcinoma cell line with concurrent attenuation of malonodinitrile and it was unique among other compounds being unable to increase glutathione S-transferase and reduced glutathione S-transferase activities. This compound exhibited also good antioxidant properties that together with its cytotoxicity nominated it for further investigation in cancer research.

Keywords: *1,2,4-triazine, triazinothiadiazine, triazinecarbonitrile, sulfa drugs, biological activity*

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INTRODUCTION

Virulence of human microbial pathogens, cancer (as body dysfunction syndrome in consequence of mutations), and evolution of multidrug resistant species are posing serious global problems. Impairment of drug efficacy leads to prolonged therapy and even high mortality rates, particularly, in the case of epidemic disieses and cancer metastasis [1]. Mutant bacteria are stimulated to excrete β-lactamases that are able to detoxify β-lactam antibiotics. Clavulanic acid (**I**) and tazobactam (**II**) (Scheme 1) are co-administered to neutralize these resistance vectors [2].

Intrinsic fungal resistance, primary and secondary, is well documented in fighting mycosis. Upregulation of drug target fungal enzymes and activation of efflux systems are amongst the common fungal mechanisms to bypass the drug toxicity [3, 4]. Drug efflux proteins, for instance, P-glycoproteins are resistance vectors in several cancer species. Captopril (**III**) [5] is a commercial P-gp inhibitor co-administered with anticancer antibiotics to avert cancer resistance. Another complication in cancer therapy is the oxidative stress of many anticancer drugs. Drug candidates of dual antioxidant potency, therefore, are highly recommended [6].

Merging of specific pharmacophores in single architecture is a promising strategy to struggle against pathogens resistance. Sulphonamides, thiophenes, and 1,2,4-triazines are relevant multifunctional pharmacophores. Thus, sulphonamides reported mainly as antibacterial agents [7, 8], are also functioning as insulin releasing stimulants [9] and anti-inflammatory [10] and cytotoxic [11] agents.

1,2,4-Triazines documented as anti-HIV, anti-H5N1 virus agents [12–14] are also antiproleferative [15, 16] agents acting on VEGFR tyrosine kinases [17] as well as anti-tuberculosis [18], anti-anxiety, anti-inflammatory agents[19, 20], potent antidepression and antianxiety agents [21], and anti-breast cancer candidates [22]. Finally, thiophenes are well known as inhibitors of BACE1 preventing β-amyloid plaques formation in Alzheimer's disease [23, 24] and *Plasmodium falciparum* differentiation [25], anti-HIV [26], antiproleferative [27, 28], anti-inflammatory [29–31], antibacterial [32], and antiprotozoal [33] agents.

In continuation to our program on triazine chemistry [34–38], this article describes the design and syn-

Abbreviations: BACE1, Beta-secretase 1; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EACC, Ehrlich ascites carcinoma cell line; GST, glutathione S-transferase; GST-Rd, reduced glutathione S-transferase; HIV, human immunodeficiency virus; MDR, multidrug resistance; P-gp, P-glycoproteins; SAR, structure-activity relationship; VEGFR, vascular endothelial growth factor receptor.

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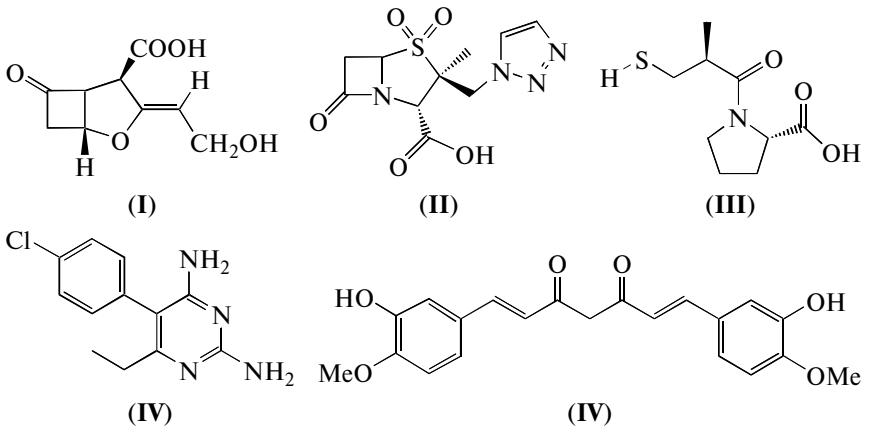
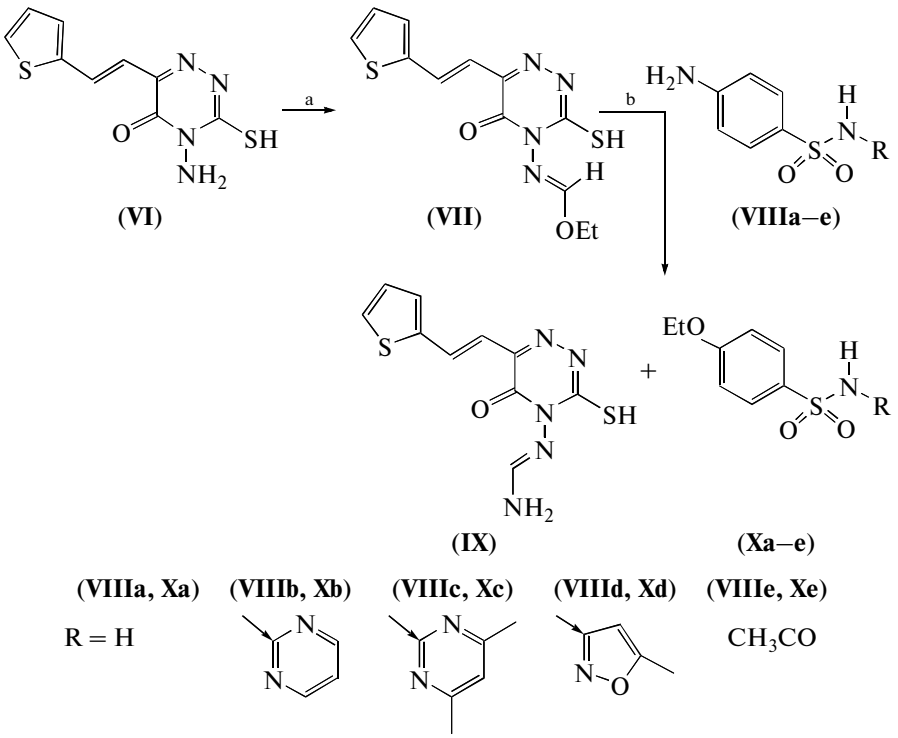


Scheme 1. Selected β-lactamase and P-gp inhibitors.



Scheme 2. Reagents and conditions: (a) $CH(OEt)_3$, AcOH, rfx. (92%); (b) AcOH, rfx. (64%).

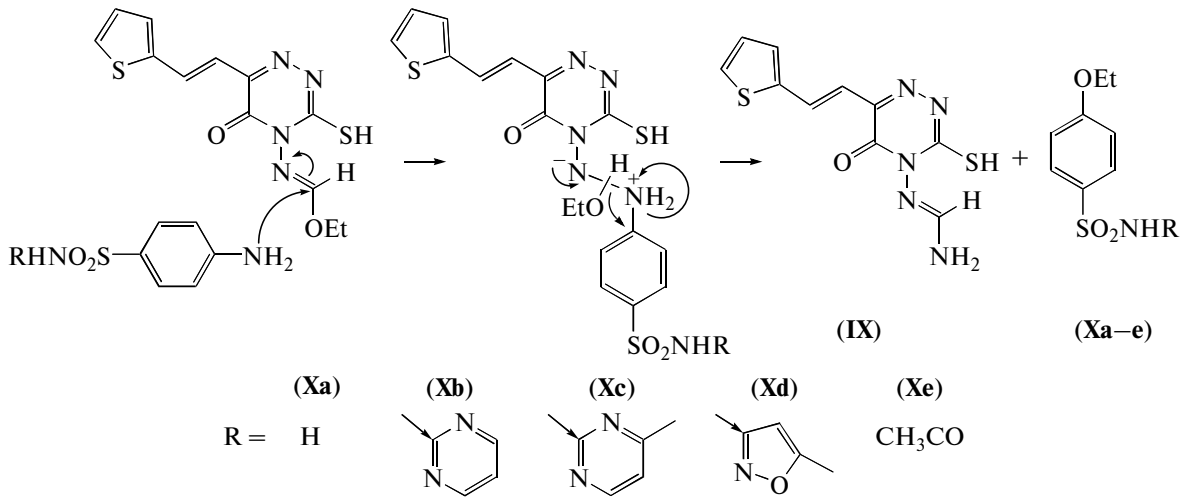
thesis of new thienyl-1,2,4-triazinyl sulphonamides as tripharmacophoric probes for screening their latent antimicrobial, antioxidant, and antiproliferative potencies—the way that might lead to discovering new pharmacophore lead structures for application and optimization.

RESULTS AND DISCUSSION

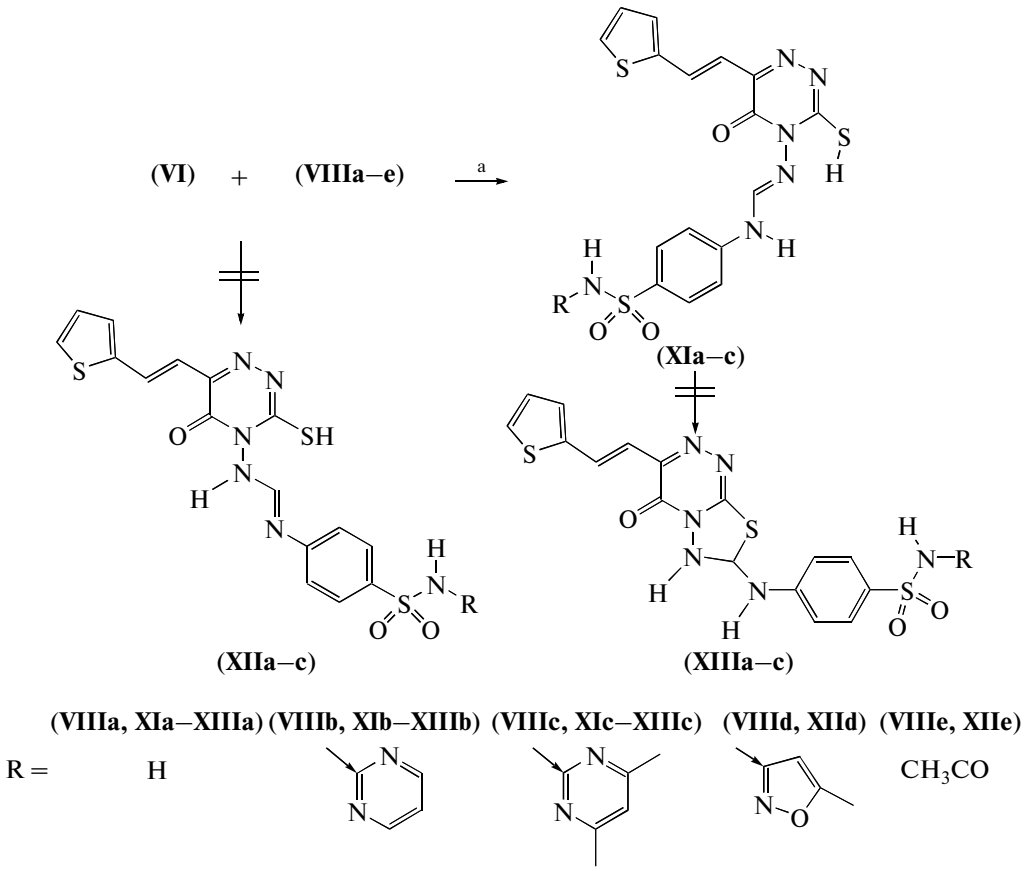
Chemistry

In earlier attempts, 4-amino-3-mercapto-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-5(4*H*)-one (**VI**) [13] was activated as formamidate (**VII**) by refluxing with triethyl orthoformate as cross coupling reagent in glacial acetic acid (Scheme 2). Compound (**VII**) showed ^{1}H NMR signals typical for ethyl group at δ 1.48 ppm, for CH_3 as triplet, and at δ 4.52 ppm for CH_2 as quartet. In ^{13}C NMR spectrum, imine carbon was observed at δ 150.80 ppm. The IR spectrum was free from NH_2 stretching vibration bands in the range 3300–3100 cm^{-1}.

Coupling of formamidate (**VII**) with available sulfa drug species (**VIIIa**–**e**) in refluxing AcOH led to transetherification of sulfa drugs yielding sulfonamides (**Xa**–**e**) and unique imine (**IX**). ^{1}H NMR spectrum of imine (**IX**) showed an NH_2 signal at δ



Scheme 3. Plausible mechanism for the formation of compound (**IX**).



Scheme 4. Reagents and conditions: $CH(OEt)_3$, AcOH, Ac_2O, rfx. (86% for (**Xa**); 74% for (**Xb**), and 68% for (**Xc**)).

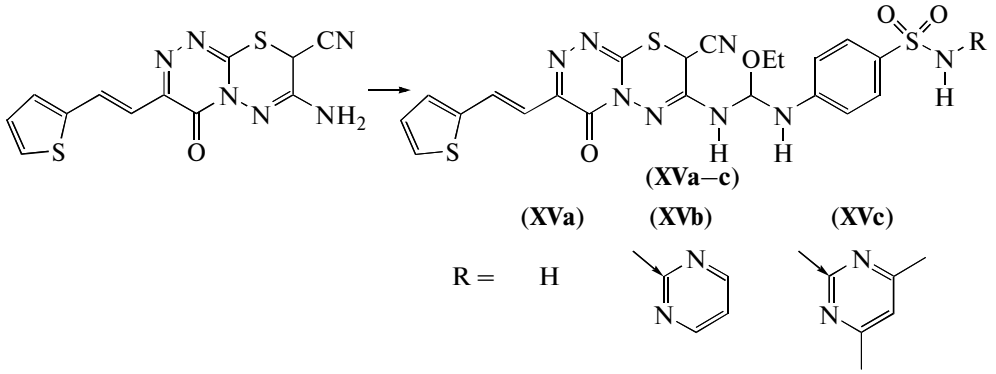
6.46 ppm and a thiol signal at δ 14.00 ppm. This high downfield shift might be attributed to intramolecular hydrogen bonding with the imine moiety in a five-membered ring structure. In ^{13}C NMR spectrum, the imine carbon was observed at δ 154.00 ppm.

A plausible mechanism for this transetherification is described in Scheme 3.

Attempts to activate amines (**VIIIa–e**) by triethyl orthoformate followed by coupling with triazole (**VI**) were unsuccessful. Then, attention was given to three-component one-pot coupling of triazole (**VI**), amine (**VIIIa–e**), and $CH(OEt)_3$ in AcOH containing several drops of Ac_2O. Three target compounds (**XIa–c**) were successfully obtained (Scheme 4).

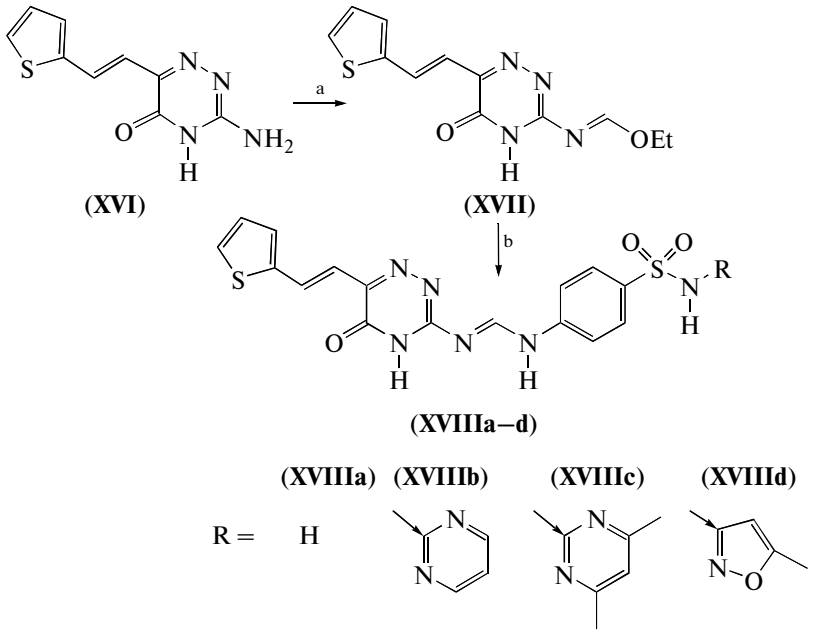
^{1}H NMR spectra of target compounds (**XIa–c**) showed a number of N-H signals equivalent to those observed in corresponding structures along with the olefinic protons in *s-trans* configuration according to high vicinal coupling value, $J \sim 16.2$ Hz. The presence of S-H signal at δ ~14.00 ppm excludes the bicylic structure (**XIII**) and the high chemical shift value supports structure (**XI**) instead of structure (**XII**) according to anticipated intramolecular hydrogen bond formation between the sulfohydryl group and the imine nitrogen in a five-membered ring structure, which is more favorable in structure (**XI**) than in structure (**XII**). The NH_2 and N-H stretching vibration bands in sulfonamide (**XIa**) were well distinguished and two N–H bands were also discriminated in case of derivatives (**XIb**) and (**XIc**). The C=O stretching bands were clearly seen in all conjugates and all these signals and bands gave supporting evidences for modular coupling of compound (**VI**) with sulfonamides (**VIIIa–c**).

Following the same procedure, compound (**XIV**) [38] was cross coupled with sulfonamides (**VIIIa–c**) affording conjugates (**XVa–c**) in excellent yields (Scheme 5).



Scheme 5. Reagents and conditions: (**VIIIa–e**), AcOH, Ac_2O, rfx. (77% for (**XIVa**), 82% for (**XIVb**), and 92% for (**XIVc**)).

The IR spectrum showed two NH bands at 3240 and 3150 cm^{-1} along with disappearance of the NH_2 band of precursor (**XIV**) reported at 3317 cm^{-1}. The C≡N stretching vibration band was observed around 2200 cm^{-1}. The CH_3CH_2 signals in ^{1}H NMR spectra appeared typically at δ 1.05–1.08 ppm for CH_3 and

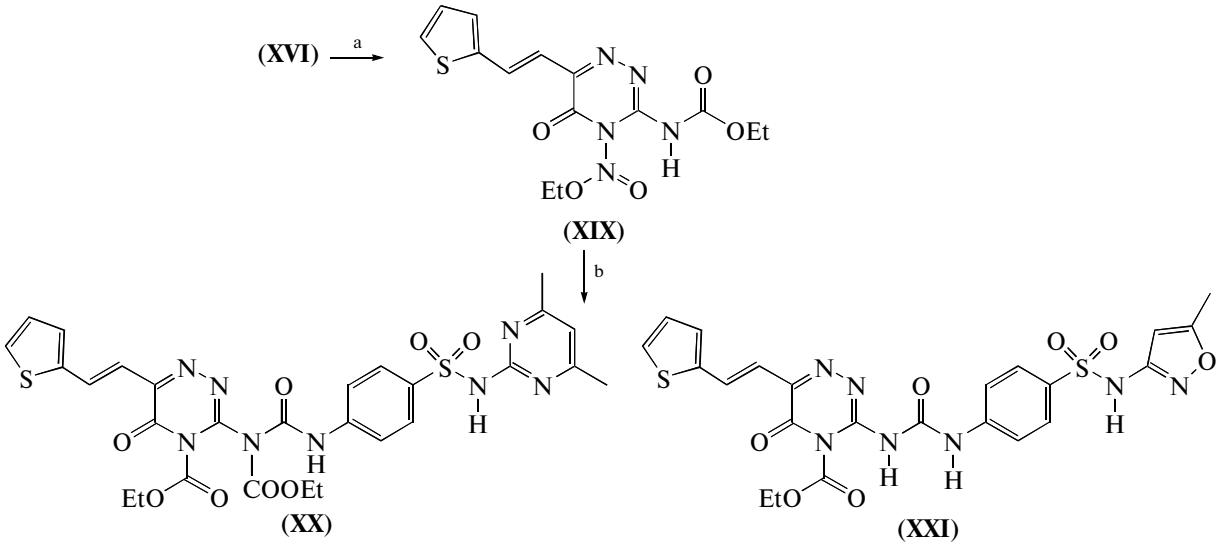


Scheme 6. Reagents and conditions: (a) $CH(OEt)_3$, AcOH, Ac_2O, rfx. (95%); (b) (**VIIIa–e**), AcOH, Ac_2O (72% for (**XVIIa**), 64% for (**XVIIb**), 80% for (**XVIIc**), and 54% for (**XVIId**)).

3.37–3.47 ppm for CH_2. The SO_2N–H signal was observed at δ ~10.20 ppm. The ^{13}C NMR spectrum supported the structure of compounds (**XVa–c**) through the appearance of signals at δ ~59.9, 14.9, and 114.2 ppm arising from CH_2, CH_3, and C≡N groups.

In a third attempt, compound (**XVI**) [39] was activated with $CH(OEt)_3$ affording imidoformite (**XVII**) in good yield as described for the synthesis of compound (**VII**). The NH_2 band at 3353 cm^{-1} disappeared upon activation and the appearance of CH_3CH_2 signals in ^1H NMR at δ 1.88 and 3.88 ppm were good evidences for triazole (**XVII**). Compound (**XVII**) was successfully coupled with amines (**VIIIa–c**) and (**VIIIe**) in AcOH containing several drops of Ac_2O to afford compounds (**XVIIIa–c**) andcompound (**XVIIId**) in very good yields (Scheme 6). IR spectra showed the number of NH_2/NH stretching vibration bands corresponding to those in sulfonamides (**XVIIIa–c**) and (**XVIIIe**). The C=O stretching vibration bands were also visible. The signals of ethyl group in ^1H NMR-spectrum disappeared upon coupling and the N–H signals were in good accordance with the proposed structures; the olefinic protons in *s-trans* configuration were good evidences for coupling of the sulfa drug species with triazole (**XVII**).

Unfortunately, coupling via one pot procedure was not sucsessful for sulfonamides (**XIa–c**). In the last investigation, ethyl chloroformate was used as alternative cross coupling reagent. Thus, treatment of compound (**XVI**) with ClCOOEt in pyridine followed by addition of the appropriate sulfa drugs (**VIIIc**) and (**VIIId**) afforded conjugates (**XX**) and (**XXI**) (Scheme 7).



Scheme 7. *Reagents and conditions:* (a) ClCOOEt, Pyr., rfx.; (b) (**VIIIc**) or (**VIIId**), rfx (83% for (**XIX**) and 89% for (**XX**)).

The IR spectrum of derivative (**XX**) showed bands at 3221 cm^{-1} for the two NH moieties and three bands within the range 1777–1665 cm^{-1} for three C=O groups. ^1H NMR spectrum showed signals at δ 1.02–1.21 and 3.42–4.12 ppm for the two C_2H_5 groups and a new signal at δ 10.13 ppm for SO_2NH. On the other hand, ^{13}C NMR spectrum displayed signals at 16.90, 17.12, and 18.07 ppm corresponding to four CH_3 groups, as well as two signals at δ 57.75 and 58.08 ppm for the ($2CH_2$) moieties.

Finally, the IR spectrum of isoxazole (**XXI**) displayed two N–H and two C=O stretching vibration bands within the ranges 3290–3192 cm^{-1} and 1742–1657 cm^{-1}, respectively. The ^1H NMR displayed signals for CH_3CH_2 group at δ 1.21 and 4.13 ppm and a new signal at δ 10.13 ppm related to SO_2NH. ^{13}C NMR spectrum displayed signals at δ 12.41 and 13.86 ppm for two CH_3 groups, at 61.34 ppm for CH_2, and at 95.06 ppm for C-4 of isoxazole, respectively.

Pharmacology

Twelve newly synthesized thieny-triazinyl-sulphonamide tripharmacophoric probes were screened in vitro as antimicrobial and cytotoxic agents and antioxidants. These compounds had common thienyl and sulphonamide pharmacophoric terminal domains and varied only by the middle azaheteroaryl pharmacophoric domain and the linker arm to the sulphonamide. Therefore, they were divided into three groups. **Group I** comprized derivatives (**XIa–c**) and (**XVIIIa–d**), where the azaheteroaryl was a 1,2,4-triazinyl moiety and the linker originated from $CH(OEt)_3$ as cross-coupler. **Group II** included compounds (**XVa–c**), where the azahetroaryl was presented by 1,2,4-triazi-

Results of antimicrobial screening against two Gram-negative, two Gram-positive, and two fungal species

	E. coli	*S. typhimurium*	*S. aureus*	*B. subtilis*	*A. flavus*	*C. albicans*
(XIa)	17^{42}*	16	14^{106}*	15	–	10
(XIb)	5	4^{93}*	15^{77}**	13	11^{146}*	–
(XIc)	10^{117}*	13	10^{124}*	11	9^{164}*	10
(XVa)	3^{139}*	4	5^{113}*	9	–	11
(XVb)	10^{126}*	12	12^{98}*	15	–	10
(XVc)	5	4	4	6	–	10
(XVIIIa)	5	5	5	5	–	–
(XVIIIb)	–	–	–	–	–	–
(XVIIIc)	5	5	4	4	–	–
(XVIIId)	15	17	9	12	–	–
(XX)	–	–	–	–	–	–
(XXI)	4	5	9	10	–	–
Ampicillin	22^{64}*	22	18^{86}*	18	–	–
Amphotericin B	–	–	–	–	17^{96}*	20

* Inhibition zone diameters (mm) were measured at concentration of 100 μg/mL, and the superscripts stand for some estimated MIC_{90} values.

nyl-[3,4-b]-1,3,4-thiadiazinyl, and that was the main difference from compounds of **Group I**. **Group III** included two compounds, (**XX**) and (**XXI**) and differed from **Group I** by the urea linker originated from ClCOOEt as cross-coupling reagent. In the following sections, the structure-activity relationship (SAR) is based on the classification mentioned above.

Antimicrobial Activity

The antimicrobial screening of the newly synthesized probes in **groups I–III** was performed in vitro using the modified Kirby–Bauer disc diffusion method [40]. Tested pathogenic microorganisms were *Escherichia coli* (ATCC 11775) and *Salmonella typhimurium* (CAICC 31) as Gram-negative bacteria, *Bacillus subtilis* and *Staphylococcus aureus* (ATCC 12600) as Gram-positive bacteria, and fungal species *Aspergillus flavus* and *Candida albicans* (ATCC 26555) (table).

Against Gram-negative species, compounds (**XIa**) and (**XVIIId**) from **group I** showed appreciable activities. Thus, either free sulphonamide in sulfonamide (**XIa**) or the methoxazolesulphonamide in acetamide derivative (**XVIIId**) were preferential over the pyrimidine ring in the same group or other structural variations in compounds of **groups II** and **III**. The activity index, i.e. the ratio of inhibition zone diameter to inhibition zone diameter of the positive control [41], was 0.77 for the sulfonamide derivative (**XIa**), which corresponded to 77% of the control in case of *E. coli*, and 0.72 or 72% of the control in the case of *S. typhimurium*. However, the MIC_{90} value for sulfonamide derivative (**XIa**) in the case of *E. coli* was 33% higher than that for ampicillin. For acetamide derivative (**XVIIId**), the activity index was 0.68 or 68% of the control activity in the case of *E.coli* and 0.77 for the activity index, which corresponded to 77% of the control activity, in the case of *S. typhimurium*.

Against Gram-positive species in the case of *S. aureus*, compound (**XIb**) from **group I** showed the largest values of inhibition zone diameter and activity index of 0.83 and 83% of ampicillin activity. The most important was its low MIC_{90} value, that is 11% higher than that of ampicillin. Despite the fact that the inhibition zone diameter for the sulfonamide derivative (**XVb**) was lower than that of another sulfonamide derivative (**XIa**), the MIC_{90} value for compound (**XVb**) was higher than that for compound (**XIa**). Thus, the MIC_{90} value of sulfonamide (**XVb**) was 14% less than that of ampicillin, while for sulfonamide (**XIa**) it was 23% less than the MIC_{90} value for the control. Against *B. subtilis*, compounds (**XIa**) and (**XVb**) were the most active and had similar activity index (0.83) corresponding to 83% of ampicillin activity.

Thus, the free sulphonamide moiety in sulfonamide (**XIa**) was a common feature in high antibacterial activity against both types of organisms, and the methoxazole ring in sulfonamide (**XVIIId**) promoted the activity against Gram negative species, while the free pyrimidine ring in derivative (**XVb**) augmented the activity against Gram-positive species.

In general, the compounds studied were not active against two fungal species tested. Only compounds (**XIb**, **c**) showed modest activity against *A. flavus* and MIC_{90} values were too low (50% lower than that of amphotericin B). The same activity was observed for sulfonamides (**XIa**, **c**) and (**XVa–c**) against *C. albicans*.

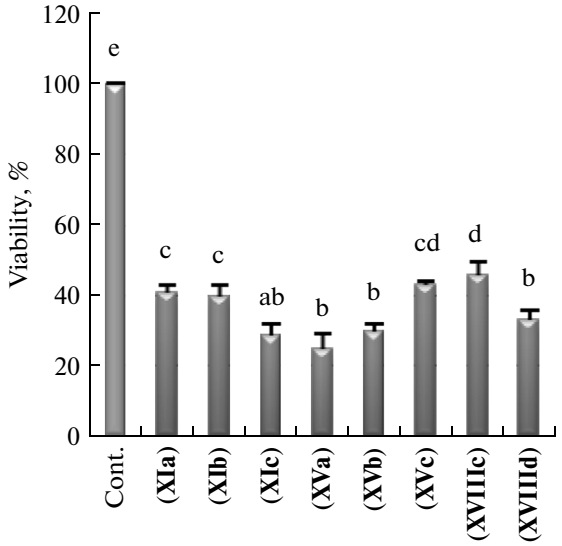


Fig. 1. Viability of Ehrlich ascites carcinoma cell line after treatments. Letters a–d above the bars stand for statistical variations at $P < 0.001$.

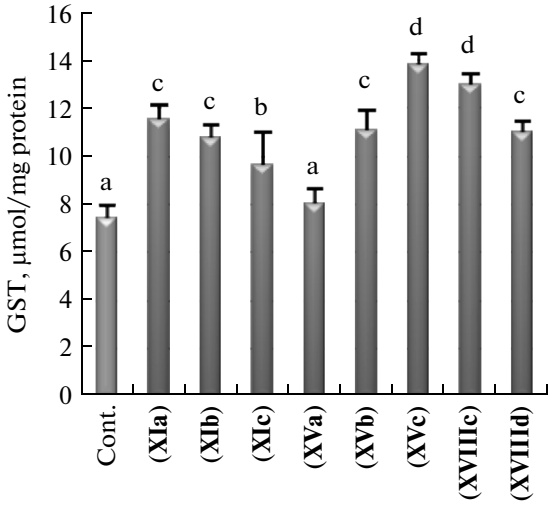


Fig. 2. Habig assay of glutathione S-transferase content in EACC after treatments. Letters a–d above the bars stand for statistical variations at $P < 0.001$.

Cytotoxicity. Cell Viability

Viability of EACC cell line in the presence of eight compounds was separately evaluated in vitro (Fig. 1). Results were expressed as viability percent according to trypan blue assay [42].

Viability in the presence of all compounds significantly differed from that of the control assigned 100%. Derivatives (**XIc**), (**XVa**), (**XVb**), and (**XVIIId**) (Fig. 1) were the most activeones. Mortality of cells in their presence fell within the range of 67–75% and insignificantly varied between each other. Compounds (**XIa**) and (**XIb**) were also effective, corresponding mortalities were 59 and 60%, respectively. Mortality of cells in the presence of sulfonamide (**XVIIId**) was the lowest one (54%). Variation of derivative (**XVIIIc**) with compounds (**XIa**), (**XIb**), and (**XVIIId**) was insignificant, therefore, it might be concluded that it has pseudo variation with these compounds. Thus, free sulphonamide, pyrimidine, and methaoxazole sulphonamides of compounds belonging to **groups I** and **II** could effectively attenuate viabilities of EACC cells.

Oxidative and Antioxidant Parameters in Carcinoma Cells

Glutathione S-transferases (GSTs) constitute a family of phase II isozymes that catalyze the conjugation of glutathione reduced form to xenobiotic substrates for the purpose of detoxification [43] and are implemented in cell signaling pathways involved in cell proliferation and cell death [44]. GSTs are observed to be overexpressed in many carcinomas and play an important role in cancer development and drug resistance [45]. The high levels of these enzymes in plasma from cancer patients is a marker of cancer and GSTs inhibitors are target anticancer drugs. For instance, pyrimethamine (**IV**) (Scheme 1) is known for a long time as a GST P1-1 inhibitor at a single-digit micromolar concentration range ($IC_{50} = 1$ μM) [46]. Curcumin (**V**) is also well known in this regard [47].

Concurrent effect of tested compounds on the GST level in EACC cell line was investigated according to Habig's assay [48]. The results (Fig. 2) revealed an augmentation in GST levels after treatment with all compounds except for sulfonamide (**XVa**), which insignificantly differed from the control. Compounds (**XVc**) and (**XVIIIc**) had the highest effect with insignificant variation between each other. Thus, the cytotoxic activity of sulfonamide (**XVa**), which was one of the most active derivatives in these investigations, was not accompanied by variations in GST levels in these cells.

In the case of assaying GST-Rd levels in EACC cell line in response to treatments with test compounds individually according to Beutler's procedure [49], all compounds except for (**XIc**) and (**XVa**) significantly differed from the control and led to elevation in GST-Rd levels. Again, only sulfonamide (**XVa**) had the advantage of being cytotoxic and neutrally affecting GST-Rd levels.

Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of membrane lipids in consequence of oxidative stress. MDA is involved in carcinogensis, where increased level of MDA is found in tumor tissues and plays a role in the early phases of tumor growth. Therefore, MDA is considered as a marker of abnormal lipid peroxidation and cancer [50, 51].

MDA levels in EACC cell line in consequence of individual treatments were determined according to

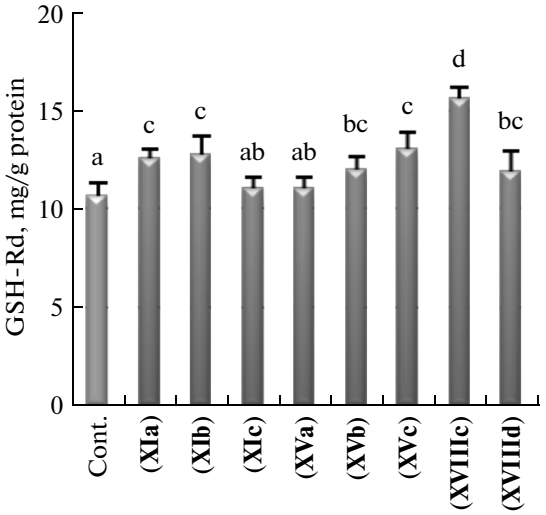


Fig. 3. Beutler assay of reduced glutathione content in EACC after treatments. Letters a–d above the bars stand for statistical variations at $P < 0.001$.

Esterbauer and Cheesman assay [52]. The results (Fig. 4) show that the cells treated with all compounds significantly differed from untreated cells and displayed decreased MDA levels. Compound (**XVIIIc**) was the most active attenuant of MDA levels (40%), while derivatives (**XVa**), (**XVb**), and (**XVIIId**) insignificantly differed from each other and were the weakest to reduce MDA levels (by 9–14%). Compound (**XIc**) significantly decreased MDA level (by 18.9%).

Clearly, the free terminal sulphonamide and the central [1,2,4]triazino[3,4-*b*][1,3,4]thiadiazine are essential structural motifs that evoke the activity of derivative (**XVa**) from **group II**.

Antioxidant Activity

Preliminary antioxidant efficiency of synthesized compounds was investigated by DPPH radical scavenging assay [53]. The free electron on the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from the antioxidant species. As shown in Fig. 5, compounds (**XIc**) and (**XVa**) were nearly similar in their antioxidant activities and were the best among other derivatives. They were 69% as active as the control. As soon as cytotoxic candidates of good antioxidant properties are highly required in cancer therapy to alleviate the oxidative stress of most anticancer drugs, compound (**XVa**) seems to be a potential target for further investigation in cancer related research.

EXPERIMENTAL

Chemistry

All chemicals were purchased from Sigma (NY, USA). Melting points were measured by a digital Electrothermal IA 9100 Series and are uncorrected. IR spectra were recorded on an ATR-Alpha FT-IR spectrophotometer in the range of ν values from 400 to 4000 cm^{-1}. ^1H and ^{13}C NMR spectra were recorded on a Brüker AC-300 instrument at 300 and 75 MHz, respectively, in DMSO-d_6 used as a solvent. Chemical shifts are expressed as δ (ppm) relative to TMS as internal standard. Mass spectra were recorded on a GCMS-QP 1000Ex Shimadzu spectrometer at the Microanalytical unit, Cairo University. Elemental analyses were performed in the Microanalytical Center, Cairo University. Pharmacological investigations were carried out in the Biochemistry Department, Faculty of Agriculture, Cairo University.

Ethyl[3-mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imidoformate (VII). A mixture of the triazine derivative (**VI**) (0.01 mol) and triethyl orthoformate (0.01 mol) in glacial acetic acid (25 mL) was stirred under reflux for 1 h, cooled to room temperature, and the precipitate formed was filtered and crystallized from EtOH to afford formamidate (**VII**) as yellow crystals. Yield 92%, mp 180–182°C. IR spectrum: 1660 ($C{=}O_{str}$), ($C{=}N_{str}$). ^1H NMR spectrum: 1.48 (t, 3H, *J* 7.2, CH_3), 4.52 (q, 2H, *J* 7.2, CH_2), 6.87 (d, 1H, *J* 16.2, $_{Thioph}CH{=}CH_{Triaz}$), 7.04 (dd, 1H, *J* 3.60,

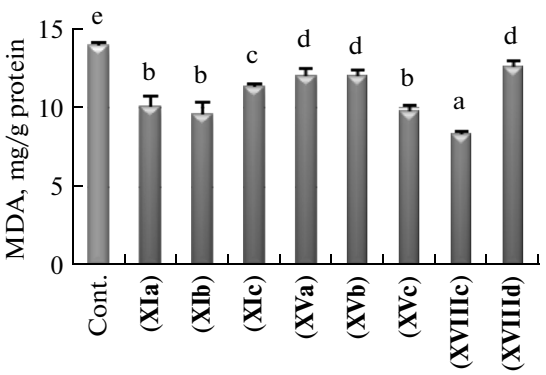


Fig. 4. Esterbauer and Cheesman assay of MDA formation in EACC after treatments. Letters a–d above the bars stand for statistical variations at $P < 0.001$.

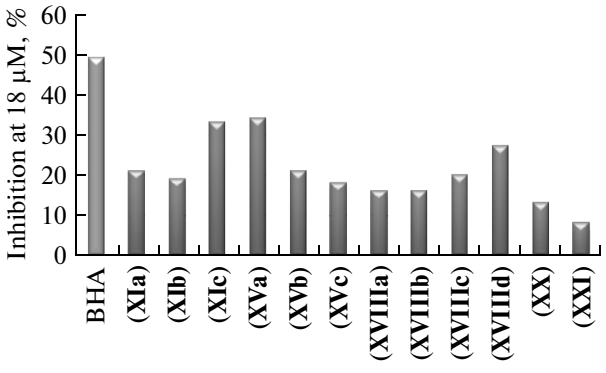


Fig. 5. DPPH scavenging assay.

4.80, H-3_{Thioph}), 7.19 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.35 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.59 (s, 1H, N=CH), 8.03 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 14.00 (s, 1H, S–H); ^{13}C spectrum: 14.33, 61.35 (C_2H_5), 127.8, 128.1, 128.3, 129.4, 131.2, 133.7, 141.0, 145.8, 157.8, 166.8 (10 C). Found: C 46.70, H, 3.86, N, 18.16%. Calcd.: $C_{12}H_{12}N_4O_2S_2$ (308.38): C 46.74, H, 3.92, N, 18.17%.

N'-[3-Mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imidoformamide (IX). A mixture of compound (**VII**) (0.01 mol) and a derivative of sulfa drugs (**VIIIa–e**) (0.01 mol) in glacial AcOH (25 mL) was stirred under reflux for 3 h and then cooled to room temperature. The precipitate was filtered and crystallized from EtOH to yield compound (**IX**) as yellow crystals. Yield 64%, mp 243–245°C. IR spectrum: 3292 ($NH_{2\ str}$), 1660 (C=O_{str}). ^{1}H NMR spectrum: 6.46 (s, 2H, NH_2), 6.87 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.02 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.21 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.35 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.60 (s, 1H, N=CH), 8.09 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 13.98 (s, 1H, S–H). ^{13}C NMR spectrum: 127.8, 128.1, 128.3, 129.1, 131.2, 133.6, 141.0, 145.9, 158.9, 166.7 (10 C). EI-MS (*m/z*, %): 279.00 (1.56), 252.00 (0.32), 241.00 (0.31), 182.00 (0.37), 122 (0.32), 108 (0.50), 92 (0.31), 65 (0.47). Found: C 43.21, H 3.24, N 25.10%. Calcd.: $C_{10}H_9N_5OS_2$ (279.34): C 43.00, H 3.25, N 25.07%.

*Synthesis of Sulfonamides (**XIa–c**). General Procedure*

A mixture of compound (**VI**) (0.01 mol), glacial AcOH (20 mL), Ac_2O (2 mL), $CH(OEt)_3$ (0.01 mol), and a derivative of the sulfa drugs (**VIIIa–e**) (0.01 mol) was stirred under reflux for 3 h and then cooled to room temperature. The precipitate was filtered at the pump and crystallized from EtOH.

4-[({[3-Mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imino}methyl)amino]-benzenesulfonamide (XIa). Yellow crystals. Yield 86%, mp 240–242°C. IR spectrum: 3292 (NH_{2str}), 3198 (NH_{str}), 1663 (C=O_{str}). ^{1}H NMR spectrum: 6.75 (s, 1H, N-H), 6.79 (d, 2H, *J* 1.8, H-2_{Ar}, H-6_{Ar}), 6.84 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.13 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.45 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.64 (s, 1H, N=CH), 7.93 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.99 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 8.02 (d, 2H, *J* 1.8 Hz, H-3_{Ar}, H-5_{Ar}), 11.02 (s, 2H, NH_2), 14.41 (s, 1H, S-H). ^{13}C NMR spectrum: 119.2, 119.8, 126.7, 127.1, 127.3, 128.0, 128.1, 129.1, 129.4, 131.1, 141.2, 141.4, 147.9, 167.1 (16 C). Found: C 44.19, H 3.24, N 19.35%. Calcd.: $C_{16}H_{14}N_6O_3S_3$ (434.52): C 44.23, H 3.25; N 19.34%.

4-[({[3-mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imino}methyl)amino]-*N*-pyrimidin-2-ylbenzenesulfonamide (XIb). Yellow crystals. Yield 74%, mp 228–230°C. IR specrtum: 3293–3195 (2N–H_{str}), 1663 (C=O_{str}). ^{1}H NMR spectrum: 6.00 (s, 1H, N-H.), 6.53 (d, 2H, *J* 1.8, H-2_{Ar}, H-6_{Ar}), 6.76 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 6.98 (d, 1H, *J* 1.8 Hz, H-4_{Pyrim}), 7.10 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.43 (d, 1H, *J* 3.6 Hz, H-4_{Thioph}), 7.62 (s, 1H, NC-H), 7.59 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.64 (d, 2H, *J* 1.8 Hz, H-5_{Pyrim}., H-6_{Pyrim}), 7.96 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 8.46 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 11.24 (s, 1H, SO_2N–H), 14.10 (s, 1H, S–H). ^{13}C NMR spectrum: 118.3, 122.0, 126.9, 127.0, 128.6, 129.1, 129.6, 131.4, 141.1, 141.6, 142.3, 148.1, 152.8, 155.1, 163.4, 165.4, 167.1 (20 C). Found: C 46.83, H 3.18. N, 21.79%. Calcd.: $C_{20}H_{16}N_8O_3S_3$ (512.59): C 46.86, H 3.15, N 21.86%.

N-(4,6-Dimethylpyrimidin-2-yl)-4-[({[3-mercapto-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazin-4(5*H*)-yl]imino}methyl)amino]benzenesulfonamide (XIc). Yellow crystals. Yield 68%, mp 220–222°C. IR spectrum: 3287–3190 (2 N–H_{str}), 1663 (C=O_{str}). ^{1}H NMR spectrum: 2.38 (s, 6H, 2 CH_3), 6.75 (s, 1H, N–H_{str}), 6.79 (d, 2H, *J* 1.8, H-2_{Ar}, H-6_{Ar}), 6.85 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.13 (s, 1H, H-5_{Pyrim}), 7.45 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.49 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.63 (s, 1H, NC–H), 7.68 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.93 (d, 1H, *J* 16.2 Hz, $_{Thioph}$C*H*=CH$_{Triaz}$), 8.02 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 11.02 (s, 1H, SO_2N–H), 14.41 (s, 1H, S–H). ^{13}C NMR spectrum: 15.95 (2 CH_3), 118.3, 122.0, 126.9, 127.1, 128.6, 129.1, 129.6, 131.4, 141.1, 141.6, 142.3, 148.1, 152.8, 155.1, 163.4, 165.4, 167.1 (20 C). Found: C 48.84, H 3.70, N, 20.69%. Calcd.: $C_{22}H_{20}N_8O_3S_3$ (540.64): C 48.87, H 3.73, N, 20.73%.

*Synthesis of Sulfonamides (**XVa–c**). General Procedure*

A mixture of compound (**IX**) (0.01 mol), glacial AcOH (20 mL), Ac_2O (2 mL), $CH(OEt)_3$ (0.01 mol), and a derivative of the sulfa drugs (**VIIIa–c**) (0.01 mol) was stirred under reflux for 3 h and then cooled to room temperature. The precipitate was filtered at the pump and crystallized from chloroform/benzene.

4-{[({8-Cyano-4-oxo-3-[2-(2-thienyl)vinyl]-4*H*,8*H*-[1,2,4]triazino[3,4-*b*][1,3,4]thiadiazin-7-yl}amino)(ethoxy)methyl]amino}benzenesulfonamide (XVa). Reddish crystals. Yield 77%, mp 229–231°C. IR spectrum: 3350 (NH_{2str}), 3176 (2 N–H_{str}), 2202 (C≡N_{str}), 1654 (C=O_{str}). ^{1}H NMR spectrum: 1.05 (t, 3H, *J* 7.2, CH_3), 2.77 (s, 1H, SC–H), 3.47 (q, 2H, *J* 7.2, CH_2), 4.93 (br. s, 1H, N-H), 6.90 (s, 2H, SO_2NH_2), 7.13 (d, 1H, *J* 14.4, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.15 (dd, 1H, *J* 1.2, 2.4, H-3_{Thioph}), 7.24 (s, 1H, EtOC*H*), 7.46 (d, 1H, *J* 1.8, H-4_{Thioph}), 7.63 (s, 1H, ArN–*H*), 7.75 (d, 2H, H-2_{Ar}, H-6_{Ar}), 7.82 (d, 2H, H-3_{Ar}, H-5_{Ar}), 7.84 (d, 1H, *J* 5.4, H-5_{Thioph}), 8.17 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$). ^{13}C NMR spectrum: 13.95 (CH_3), 28.07 (CN*C*H), 56.79 (CH_2), 98.38 (*C*HOEt), 114.3 (CN), 118.3, 119.2, 126.7, 127.1, 127.4, 128.0, 128.4, 129.4, 129.5, 131.1, 141.2, 141.5, 147.9, 167.1 (16 C). Found: C 46.29, H 3.72, N 20.60%. Calcd.: $C_{21}H_{20}N_8O_4S_3$ (544.63): C 46.31, H 3.70, N 20.57%.

4-{[({8-Cyano-4-oxo-3-[2-(2-thienyl)vinyl]-4*H*,8*H*-[1,2,4]triazino[3,4-*b*][1,3,4]thiadiazin-7-yl}amino)(ethoxy)methyl]amino}-*N*-pyrimidin-2-ylbenzenesulfonamide (XVb). Reddish crystals. Yield 82%, mp 216–218°C. IR spectrum: 3190 (2N–H_{str}), 2200 (C≡N_{str}), 1670 (C=O_{str}). ^{1}H NMR spectrum: 1.08 (t, 3H, *J* 7.2, CH_3), 2.75 (s, 1H, CHCN), 3.37 (q, 2H, *J* 7.2, CH_2), 4.91 (b, 1H, N-H), 7.11 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.13 (dd, 1H, *J* 1.2, 2.4, H-3_{Thioph}), 7.15 (s, 1H, EtOC*H*), 7.17 (s, 1H, PhN–*H*), 7.46 (d, 1H, *J* 1.8, H-4_{Thioph}), 7.63 (t, 1H, H–5_{Pyrim}), 7.71 (d, 2H, H-2_{Ar}, H-6_{Ar}), 7.88 (d, 2H, H-3_{Ar}, H-5_{Ar}), 7.91 (d, 1H, *J* 5.4, H-5_{Thioph}), 8.17 (d, 1H, *J* 16.2, $_{Thioph.}$CH=C*H*$_{Triaz}$), 8.48 (d, 2H, H-$4_{Pyrim.}$, H-6_{Pyrim}), 10.39 (s, 1H, SO_2N–H). ^{13}C NMR spectrum: 14.37 (CH_3), 27.98 (S*C*HCN), 57.53 (CH_2), 97.97 (*C*HOEt), 113.1 (C≡N), 118.3, 120.1, 126.6, 126.8, 127.5, 128.1, 128.6, 129.3, 129.7, 131.4, 132.7, 136.9, 139.8, 140.8, 141.4, 141.5, 141.6, 147.9, 167.3 (20 C). Found: C 48.17, H 3.55, N 22.42%. Calcd.: $C_{25}H_{22}N_{10}O_4S_3$ (622.70): C 48.22, H 3.56, N 22.49%.

4-{[({8-Cyano-4-oxo-3-[2-(2-thienyl)vinyl]-4*H*,8*H*-[1,2,4]triazino[3,4-*b*][1,3,4]thiadiazin-7-yl}amino)(ethoxy)methyl]amino}-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide (XVc). Reddish crystals. Yield 92%, mp 220–222°C. IR spectrum: 3222 (3 N–H_{str}), 2207 (C≡N_{str}), 1668 (C=O_{str}). ^{1}H NMR spectrum: 1.08 (t, 3H, *J* 7.2, CH_3), 2.05 (s, 6H, $2CH_3$), 2.75 (s, 1H, CH), 3.42 (q, 2H, *J* 7.2, CH_2), 3.78 (b, 1H, N-H), 7.10 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.13 (dd, 1H, *J* 1.2, 2.4, H-3_{Thioph}), 7.15 (s, 1H, EtOC*H*), 7.19 (s, 1H, N–H_{Ar}), 7.46 (d, 1H, *J* 1.8, H-4_{Thioph}), 7.64 (t, 1H, H–$5_{Pyrim.}$), 7.70 (d, 2H, H-2_{Ar}, H-6_{Ar}), 7.86 (d, 2H, H-3_{Ar}, H-5_{Ar}), 7.89 (d, 1H, *J* 5.4, H-5_{Thioph}), 8.17 (d, 1H, *J* 16.2 Hz, $_{Thioph}$C*H*=CH$_{Triaz}$), 10.20 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 13.90, 14.65 ($3CH_3$), 28.07 (SCH), 56.79 (CH_2), 98.16 (*C*HOEt), 113.10 (C≡N), 118.30, 119.20, 126.60, 126.80, 127.50, 128.10, 128.60, 129.30, 129.60, 131.40, 132.70, 136.90, 139.50, 140.80, 141.30, 141.4, 141.60, 147.80, 167.30 (20 C). Found: C 49.80, H 4.08, N 21.57%. Calcd.: $C_{27}H_{26}N_{10}O_4S_3$ (650.75): C 49.83, H 4.03, N 21.52%.

Ethyl{5-oxo-6-[2-(2-thienyl)vinyl]-4,5-dihydro-1,2,4-triazin-3-yl}imidoformate (XVII). A mixture of compound (**XVI**) (0.01 mol), glacial AcOH (20 mL), Ac_2O (2 mL), and $CH(OEt)_3$ (0.01 mol) was stirred under reflux for 3 h and then cooled to room temperature. The precipitate was filtered at the pump and crystallized from EtOH to afford imidoformate (**XVII**) as pale yellow crystals. Yield 95%, mp over 300°C. IR spectrum: 3176 (N–H_{str}), 1664 (C=O_{str}). ^{1}H NMR spectrum: 1.88 (t, 3H, *J* 7.2, CH_3), 3.88 (q, 2H, *J* 7.2, CH_2), 6.87 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.00 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.02 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.53 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.57 (s, 1H, NC–H), 8.00 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 12.20 (s, 1H, triazine CONH). Found: C 52.10, H 4.37, N, 20.25%. Calcd.: $C_{12}H_{12}N_4O_2S$ (276.31): C 52.16, H 4.38, N 20.28%.

Preparation of Compounds (XVIIIa–d). General Procedure

A solution of compound (**XVII**) (0.01mol) in a mixture of glacial AcOH (20 mL) and Ac_2O (2 mL) was treated under stirring with $CH(OEt)_3$ (0.01 mol) followed by the appropriate (**VIIIa–e**) derivative (0.01 mol). After being refluxed for 3 h, it was cooled to ambient temperature, the precipitate formed was filtered in vacuo and crystallized from benzene to afford compounds (**XVIIIa–d**) as yellow crystals.

4-{[({5-Oxo-6-[2-(2-thienyl)vinyl]-4,5-dihydro-1,2,4-triazin-3-yl}imino)methyl]amino}benzene-sulfonamide (XVIIIa). Yield 72%, mp over 300°C. IR spectrum: 3348–3090 (NH_2 $_{str}$, 2 N–H_{str}), 1662 (C=O_{str}). ^{1}H NMR spectrum: 6.72 (d, 1H, *J* 16.2 Hz, $_{Thioph}$CH=C*H*$_{Triaz}$), 6.85 (2s, 3H, SO_2NH_2 and N=CH), 7.07 (d, 2H, *J* 2.7, H-2_{Ar}, H-6_{Ar}), 7.08 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.09 (d, 2H, *J* 2.7, H-3_{Ar}, H-5_{Ar}), 7.12 (s, 1H, N–H_{Ar}), 7.29 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.53 (d, 1H, *J* 5.1 Hz, H-5_{Thioph}), 8.00 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 12.26 (s, 1H, H-4_{Triaz}). ^{13}C NMR spectrum: 118.3, 122.0, 126.9, 127.0, 128.0, 128.6, 129.6, 131.4, 141.4, 142.3, 148.1, 152.7, 155.1, 167.1 (16 C). Found: C 47.70, H 3.46, N 20.90%. Calcd.: $C_{16}H_{14}N_6O_3S_2$ (402.45): C 47.75, H 3.51, N 20.88%.

N-(4,6-Dimethylpyrimidin-2-yl)-4-{[({5-oxo-6-[2-(2-thienyl)vinyl]-4,5-dihydro-1,2,4-triazin-3-yl}imino)methyl]amino}benzenesulfonamide (XVIIIb). Yield 64%, mp over 300°C. IR spectrum: 3348–3101 (3 N–H_{str}), 1665 (C=O_{str}). ^{1}H NMR spectrum: 6.74 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 6.82 (s, 1H, N=CH), 7.00 (t, 1H, H–5_{Pyrim}), 7.09 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.12 (s, 1H, N–H_{Ar}), 7.40 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.59 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.70 (d, 2H, *J* 1.8, H-2_{Ar}, H-6_{Ar}), 7.86 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 8.11 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 8.48 (d, 2H, H-4_{Pyrim}, H-6_{Pyrim}), 10.29 (s, 1H, H-4_{Triaz}), 11.70 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 117.7, 118.5, 118.9, 126.4, 126.6, 127.5, 127.8, 127.9, 128.2, 128.8, 129.9, 130.1, 139.9, 141.5, 141.6, 152.7, 162.3 (20 C). Found: C 50.05, H 3.38, N, 23.24%. Calcd.: $C_{20}H_{16}N_8O_3S_2$ (480.52): C 49.99, H 3.36, N 23.32%.

N-(4,6-Dimethylpyrimidin-2-yl)-4-{[({5-oxo-6-[2-(2-thienyl)vinyl]-4,5-dihydro-1,2,4-triazin-3-yl}imino)methyl]amino}benzenesulfonamide (XVIIIc). Yield 80%, mp over 300°C. IR spectrum: 3348–3101 (2N–H_{str}), 1666 (C=O_{str}). ^{1}H NMR spectrum: 1.70 (s, 6H, 2 CH_3), 6.79 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 6.85 (s, 1H, N=CH), 7.03 (t, 1H, H–5_{Pyrim}), 7.11 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.12 (s, 1H, N–H_{Ar}), 7.38 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.59 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.71 (d, 2H, *J* 1.8, H-2_{Ar}, H-6_{Ar}), 7.89 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 8.15 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 10.28 (s, 1H, H–4_{Triaz}), 11.77 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 13.04, 13.05 ($2CH_3$), 118.4, 118.9, 126.4, 126.7, 127.4, 127.7, 127.9, 128.0, 128.4, 128.7, 129.7, 130.1, 139.7, 141.0, 141.5, 152.4, 167.3 (20 C). Found:

C 51.95, H 3.98, N 22.10%. Calcd.: $C_{22}H_{20}N_8O_3S_2$ (508.58): C 51.96, H 3.96, N 22.03%.

N-[(4-{[({5-Oxo-6-[2-(2-thienyl)vinyl]-4,5-dihydro-1,2,4-triazin-3-yl}imino)methyl]amino}-phenyl) sulfonyl]acetamide (XVIIId). Yield 54%, mp over 300°C. IR spectrum: 3348–3096 (2 N–H_{str}), 1661 (C=O_{str}). ^{1}H NMR spectrum: 1.90 (s, 3H, CH_3), 6.72 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 6.85 (s, 1H, N=CH), 7.07 (d, 2H, *J* 2.7, H-2_{Ar}, H-6_{Ar}), 7.08 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.09 (d, 2H, *J* 2.7, H-3_{Ar}, H-5_{Ar}), 7.1 (s, 1H, N–H_{Ar}), 7.29 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.53 (d, 1H, *J* 5.1, H-5_{Thioph}), 8.00 (d, 1H, *J* 16.2, $_{Thioph}$C*H*=CH$_{Triaz}$), 11.96 (s, 1H, H–4_{Triaz}), 12.26 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 13.05 (CH_3), 116.9, 118.3, 122.1, 126.4, 126.6, 127.0, 127.1, 128.0, 128.6, 129.7, 131.4, 141.5, 142.3, 148.1, 152.7, 167.2 (17 C). Found: C 48.63, H 3.60, N 18.87%. Calcd.: $C_{18}H_{16}N_6O_4S_2$ (444.49): C 48.64, H 3.63, N 18.91%.

Synthesis of Compounds (XX) and (XXI). General Procedure

$CH(OEt)_3$ (0.01 mol) was added to a solution of compound (**XVI**) (0.01 mol) in pyridine (20 mL) and the mixture was refluxed for 10 min, then a derivative of (**XVIIIa–e**) (0.01 mol) was added and reflux was continued for additional 2 h. The mixture was evaporated in vacuo and the residue was crystallized from benzene.

Ethyl3-[{[(4-{[(4,6-dimethylpyrimidin-2-yl)amino] sulfonyl}phenyl)amino]carbonyl}-(ethoxycarbonyl) amino]-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazine-4(5*H*)-carboxylate (XX). Orange crystals. Yield 83%, mp 218–220°C. IR spectrum: 3221 (2 N–H_{str}), 1777–1785 (2 C=$O_{str\ Carbam}$), 1665 (C=$O_{st\ Urea}$). ^{1}H NMR spectrum: 1.02 (t, 3H, *J* 7.2, CH_2CH_3), 1.21 (t, 3H, *J* 7.2, CH_2CH_3), 2.24 (s, 6H, 2 CH_3), 3.42 (q, 2H, *J* 7.2, CH_2CH_3), 4.12 (q, 2H, *J* 7.2, CH_2CH_3), 6.73 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.08 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.27 (s, 1H, H–5_{Pyrim}), 7.29 (d, 2H, *J* 3.6, H-2_{Ar}, H-6_{Ar}), 7.54 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.60 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 7.73 (d, 1H, *J* 5.1, H-5_{Thioph}), 7.99 (d, 1H, *J* 16.2 Hz, $_{Thioph}$C*H*=CH$_{Triaz}$), 8.75 (s, 1H, N-H), 10.13 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 16.90, 17.12, 18.07 (3 CH_3), 57.75, 58.08 (2 CH_2), 117.3, 118.5, 126.3, 126.6, 127.4, 127.8, 128.0, 128.4, 129.7, 130.2, 138.5, 139.8, 141.0, 141.6, 145.0, 147.1, 152.3, 152.6, 158.2 (23 C). Found: C 50.33, H 4.26, N 16.71%. Calcd.: $C_{28}H_{28}N_8O_8S_2$ (668.70): C 50.29, H 4.22, N, 16.76%.

Ethyl3-({[(4-{[(5-methylisoxazol-3-yl)amino]sulfonyl}phenyl)amino]carbonyl}amino)-5-oxo-6-[2-(2-thienyl)vinyl]-1,2,4-triazine-4(5*H*)-carboxylate (XXI). Orange crystals. Yield 89%, mp 214–216°C. IR spectrum: 3290–3192 (3 N–H_{str}), 1742 (C=$O_{str\ Carbam}$), 1657 (C=$O_{str\ Urea}$). ^{1}H NMR spectrum: 1.21 (t, 3H, *J* 7.2, $CH_{3isoxazole}$), 2.28 (t, 3H, CH_2CH_3), 4.13 (q, 2H, *J* 7.2,CH_2CH_3), 6.11 (s, 1H, N–H_{Ar}), 6.73 (d, 1H, *J* 16.2, $_{Thioph}$CH=C*H*$_{Triaz}$), 7.08 (dd, 1H, *J* 3.60, 4.80, H-3_{Thioph}), 7.29 (s, 1H, H–4_{Isoxaz}), 7.33 (d, 2H, *J* 3.6, H-2_{Ar}, H-6_{Ar}), 7.56 (d, 1H, *J* 3.6, H-4_{Thioph}), 7.61 (d, 2H, *J* 1.8, H-3_{Ar}, H-5_{Ar}), 7.74 (d, 1H, *J* 5.1 Hz, H-5_{Thioph}), 7.99 (d, 1H, *J* 16.2, $_{Thioph.}$C*H*=CH$_{Triaz}$), 8.82 (s, 1H, N-H), 10.13 (s, 1H, SO_2NH). ^{13}C NMR spectrum: 12.41, 13.86 (2 CH_3), 61.34 (CH_2), 95.06 (C-$5_{Isoxazole}$), 118.0, 122.6, 127.1, 128.0, 128.1, 128.4, 129.5, 129.7, 138.5, 140.2, 141.0, 141.1, 141.7, 145.3, 147.2, 152.8, 162.4 (19 C). Found: C 48.39, H 3.75, N, 17.11%. Calcd.: $C_{23}H_{21}N_7O_7S_2$ (571.59): C, 48.33, H 3.70, N 17.15%.

Pharmacology Antimicrobial Assay

Briefly, 100 μL of the tested bacteria and/or fungi were grown in 10 mL of fresh media until they reached approximate count of 10^8 cells/mL for bacteria and 10^5 cells/mL for fungi [54]. 100 μL of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Isolated colonies of each organism that might be playing a pathogenic role were selected from primary agar plates and tested for susceptibility by disc diffusion method [64, 65]. Disc diffusion method used for filamentous fungi testing was the approved standard method (M38-A) developed by National Committee for Clinical Laboratory Standards (NCCLS) [55] for evaluation of the susceptibilities of filamentous fungi to antifungal agents. Yeasts were evaluated according to the M44-P disc diffusion method.

Plates were inoculated with filamentous fungi *Aspergillus flavus*, Gram-postive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*, and yeast *Candida albicans*. Discs of sterilized whatman No. 1 filter paper (8.0 mm diameter) were saturated with 10.0 μL containing 100 μg/mL from each test compound in DMSO and left to dry. Discs were inserted in the assay plates and then incubated at 25°C for 48 h for *Aspergillus flavus*, at 35–37°C for 24–48 h for Gram-positive and Gram-negative bacteria, and at 30°C for 24–48 h for *Candida albicans*. After elapse of the incubation period, the inhibition zones surrounding the discs were measured with silipping calipers according to the NCCLS [56]. Antimicrobial activities are expressed as inhibition diameter zones (mm). Experiments were carried out in triplicates and the average zone of inhibition was calculated. Ampicillin and amphotericin B were used as positive control for antibacterial and antifungal comparisons respectively, while DMSO was used as negative control.

MIC_{90} Evaluation

The MIC_{90} values of compounds that showed significant antibacterial and/or antifungal activities were

determined by agar dilution method [55]. Positive controls were evaluated under the same conditions.

In vitro Cytotoxicity and Related Metabolic Disorders Assays

Experimental animals. Female Swiss albino mice weighing 25–30 g were obtained from the Toxicology Department at the Central Agricultural Pesticide Laboratory, Agriculture Research Center, and were housed at constant temperature (24 ± 2°C) with alternating 12 h light and dark cycles and fed with standard laboratory food along with water ad libitum. Animal care and handling was according to the guidelines set by the World Health Organization, Geneva, Switzerland, and was approved by the committee for animal care at Toxicology Department, Central Agricultural Pesticide Laboratory, Agriculture Research Center.

Tumor cells. EACC cell line purchased from the National Cancer Institute (NCI), Cairo University was injected in female Swiss albino mice through serial intraperitoneal inoculation at 7 or 8 days intervals in our laboratory in an ascites form.

In vitro Cytotoxicity Assay

In vitro cytotoxicity of the newly synthesized compounds was tested against EACC cells by trypan blue exclusion assay. EACC cell were obtained by needle aspiration of the ascitic fluid from preinoculated mice under aseptic conditions [57]. Tumor cell suspensions (2×10^6 cells per mL) were prepared in RPMI-1640 media supplemented with 10% fetal bovine serum and L-glutamine. Test compounds at 0.18, 0.36, and 0.56 mM concentrations in DMSO were incubated with 2 mL of cell suspension under CO_2 (5%) at 37°C overnight. The percentage of cell viabilities was evaluated by the trypan blue exclusion affinity.

$$\text{Cell viability (\%)} = (\text{Number of viable cells/total count of cells}) \times 100\%.$$

Determination of Oxidative and Antioxidant Parameters in Carcinoma Cells

Glutathione S-Transferase (GST) Activity

GST activity in carcinoma cells was measured spectrophotometrically according to Habig assay using 1-chloro-2,4-dinitrobenzene as electrophilic substrate that binds to glutathione (GSH) with the participation of the enzyme and forms a colored GSH complex, which absorbs at 340 nm. The activity of GST was expressed in terms of mmol/min/mg protein.

Reduced Glutathione Content

Reduced glutathione content (GSH-Rd) in carcinoma cells was evaluated according to Beutler method using commercial reduced glutathione kits (Biodiagnostic for diagnostic reagents: Dokki, Giza, Egypt). Determination of GSH is based on the reaction of DTNB (5,5-dithiobis(2-nitrobenzoic acid) with GSH to yield a yellow colored chromophore with a maximum absorbance at 412 nm. The amount of GSH present in the carcinoma cells calculated as mg/g protein.

Lipid Peroxidation (LPO) Level

Lipid peroxidation process is determined in carcinoma cells by the thiobarbituric acid (TBA) method, which estimates the malondialdehyde formation (MDA) according to Esterbauer and Cheesman.

A suspension of the carcinoma cells (250 μL) was added to 1.5 mL of 1% phosphoric acid (pH 2.0) and 1 mL of 0.6% of TBA in air-light tubes and placed in a boiling water bath for 25 min. After incubation, the sample was cooled to room temperature and MDA-TBA were extracted with 2.5 mL of butanol. The organic phase was separated by centrifugation at 2000 *g* for 5 min and absorption was measured at 532 nm. The concentration of MDA calculated using the absorbance coefficient of MDA-TBA complex ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$). Lipid peroxidation is expressed as nM MDA/mg protein.

Tissue Protein Assay

The total protein level of carcinoma cells was determined according to Bradford method [58] using coomassie brilliant blue G-250 reagent with bovine serum albumin as standard.

DPPH Assay for Antioxidant Activity Screening

The antioxidant activity of the synthesized compounds was determined by the DPPH free radical scavenging assay. One hundred microliter of each test compound at 100 mM in DMSO was mixed with 900 μL of 60 μM solution of DPPH in MeOH to reach final concentrations of 18 μM. The mixture was shaken vigorously and left in the dark for 60 min until stable absorption values at 517 nm were obtained. The radical scavenging activities (RSA) were calculated as a percentage of DPPH discoloration using the equation:

$$\%\text{RSA} = (A_{DPPH} - A_S)/A_{DPPH} \times 100\%,$$

where A_{DPPH} is the absorption of pure DPPH and A_S is the absorption after adding the sample.

Butylated hydroxyanisole (BHA) was used as positive control. Measurements were taken in triplicate and the results were averaged.

CONCLUSIONS

In this work, three-pharmacophoric probes arising from conjugation of thienyltriazines with sulphonamides via triethyl orthoformate and ethyl chloroformate as cross coupling reagents were synthesized and

their potentials as antimicrobial, cytotoxic, and antioxidants were screened in vitro. Compound (**XIa**) seems to be a powerful antibacterial agent, while the whole series was not active against considered fungal species. Derivative (**XVa**) showed interesting cytotoxic and antioxidant properties, which nominate it to be further investigated in cancer research.

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