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Original article

Synthesis, characterization and antibacterial screening of some novel 1,2,4-triazine derivatives

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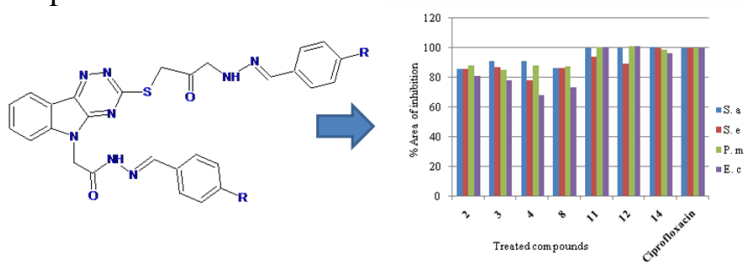[b] College of Medicine Aldawadmi Shaqra University, Kingdom of Saudi Arabia
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Graphical abstract

A new library of 1,2,4-triazine derivatives were synthesized and evaluated for antibacterial activity. The MIC was detected using the double dilution method. Selected compounds were subjected for MTT-assay using HepG2 cell line.

ABSTRACT

A new series of 1, 2, 4-triazine derivatives possessing indole nucleus were synthesized with an aim to explore their effect on *in vitro* growth of microorganisms causing microbial infection. In vitro antimicrobial activity was performed against S. aureus, S. epidermidis, P. mirabilis and E. coli using disk diffusion method. The MIC was detected using the double dilution method. The results were compared by calculating percent inhibition area/µg of the compounds with the standard drug "Ciprofloxacin". Selected compounds were evaluated for toxic effects using human hepatocellular carcinoma (HepG2) cell line by MTT-assay. Results revealed that some compounds of the series were found to exhibit better activity with less toxicity than Ciprofloxacin

1. Introduction

Microorganisms are almost everywhere in nature and infections caused by small pathogenic microorganisms could lead to illnesses even a fatal one [1]. The development of resistance to the currently available antimicrobial agents [2], compounded problems in the therapeutics such as multidrug-resistant gram-positive pathogens, methicillin-resistant *Staphylococci aureus* (MRSA), penicillin resistant *Streptococcus pneumoniae* (PRSP) and vancomycin-resistant *Enterrococci* (VRE) [3, 4]. Therefore, the search for new lead structures and chemical entities (NCEs) for the development of antimicrobial agents is an increasingly important problem in medicinal chemistry. Unfortunately, pharmaceutical companies are more and more leaving this area behind due to economic reasons [5]. Compounds containing the variety of heterocyclic nucleus has been long aimed by the researchers as an antimicrobial agent [6-15]. On the other hand 1,2,4-triazine derivatives have been reported to possess biological activities as anti-AIDS [16], anticancer [17], antimicrobial [18–25] antitumor [26, 27], anti-inflammatory and analgesic agents [28], cytotoxicity [29, 30], antileishmanial [31], antiproliferative [32-33], antinociceptive [34], anticytokine [35], cyclooxygenase-2 inhibitors [36], neuroprotective agents [37], antiviral [38] and antimalarial [39-40] activities. Besides, indole nucleus is found in a number of biologically active compounds such as anti-inflammatory and analgesic [41-45], antifungal [41,46], antimicrobial [47-48], insecticidal activity [41-49], anticancer [41, 50–53], 5-lipoxygenase inhibitors [54], anti-HIV [41, 55], antioxidant [56-57], antitubercular [41,58], antiviral [41], plant growth regulator [41], antidepressant, tranquillizing, anticonvulsant [31,49], cardiovascular [3, 50], antihypertensive [41], antihistaminic [61], opioid antagonist [62], photochemotherapeutic [63], antidiabetic [64], LXR receptor agonist [65], ACAT inhibitor [66] and many other activities. On the other hand hydrazones have been reported to possess antimicrobial [67], antitubercular [68-69], anticonvulsant [70], analgesic [71], anti-inflammatory [72-73] antiplatelet [74], anticancer [75-77], antifungal [78], antiviral [79], antitumoral [80-81], antibacterial [82] and antimalarial [83] activities. Therefore, the biological importance of 1,2,4-triazine derivatives, indole nucleus and hydrazone functionality prompted us to design and synthesize some novel moieties which have both the important nucleus and hydrazone functionality and it was believed that their combination will be important to enhance activity.

2. Results and discussion

2.1 Chemistry

Present study was undertaken to synthesize some novel 1,2,4-triazine derivatives. All the synthesized compounds were then subjected for their antimicrobial evaluation. The target compounds **1-14** were synthesized by four step procedure represented in Scheme 1. First step involves the formation of compound **A** by the reaction of isatin with thiosemicarbazide in presence of potassium bicarbonate. Subsequently, compound **A** on reaction with ethylchloroacetate in presence of potassium carbonate yield compound **B**. Compound **B** reacted with hydrazine hydrate in ethanol to yield compound **C.** Finally, compound **C** reacted with appropriate aldehyde to form hydrazone derivatives **(1-14)**. All the compounds in solid state showed sharp melting points and the elemental analysis was found to be in accordance with ± 0.3%. The compounds were stable and soluble in DMSO, methanol and chloroform. All the synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, and Mass spectroscopy. Characteristic IR bands provide significant indication for the formation of compounds **1-14**. The appearance of characteristic band at/or around 2260 cm^{-1} and 3250 cm^{-1} due to SH and NH respectively, provide strong information about the formation of compound **A**. The formation of compound **C** was confirmed by the absence of band at/or around 1695 cm^{-1} and 1685 cm^{-1} due to the presence of C=O and also by the presence of bands due to C-S and C-N at 662 cm^{-1} and 1105 cm^{-1}, respectively. The appearance of characteristic band at/or 3280 cm^{-1} and 3325 cm^{-1} due to NH and NH$_2$ respectively strongly favor the formation of compound **C**. The disappearance of bands at around 3325 cm^{-1} and appearance of bands at 672-679, 1120-1129 and 1603-1618 cm^{-1} due to C-S, C-N and C=O respectively confirms the formation of compounds **1-14.**

Further confirmation of all the synthesized compounds was done using ^{1}H NMR spectroscopic data. Appearance of a singlet at 3.33 ppm and 11.23 ppm due to SH and NH proton, respectively, provides strong confirmation of the compound **A.** Structure of compound **C** was confirmed by the absence of peak at 3.33 ppm and 11.23 ppm due to SH and NH proton and also by the appearance of a triplet and quartet at 1.82 ppm and 4.09 ppm due to CH$_2$ and CH$_3$ protons. Structure of compound **C** was confirmed by appearance of singlet at 11.09 ppm and 2.50 ppm due to NH and NH$_2$ respectively. Structural confirmation of compounds **1-14** was achieved by the absence of singlet at 11.09 ppm and 2.50 ppm due to NH and NH$_2$ respectively and also by the presence of singlet at 8.11-8.22 ppm due to CH=N proton. The ^{13}C NMR spectra of compounds **1-14** showed some prominent signals, such as signals in the range of 139.00-141.36 ppm, 142.10-143.69 ppm due to presence of CH=N which strongly confirms the formation of hydrazine and some more signals in the range (166.45-167.34) ppm, (169.04-172.77) ppm and (166.45-166.99) ppm due to the presence of C-S and C=O respectively and provide strong recommendation for the formation of these compounds **1-14**. Other peaks were also present in the NMR spectra of these compounds at their usual positions described in the experimental section.

2.2 Antibacterial activity

All the compounds **1-14** were screened for their *in vitro* antimicrobial activity using the Gram-positive and Gram-negative bacteria. Four different cultures, two each of Gram-negative (*Escherichia coli* and *Proteus mirabilis*) and Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis)* were treated with the synthesized compounds **1-14** using disk diffusion

method. The minimum inhibitory concentration (MIC) is defined as the lowest concentration (highest dilution) required to arrest the growth of bacteria and was determined by using different dilutions of the treatment compounds. The results were compared with positive control, the standard drug ciprofloxacin and negative control, the DMSO. The MIC was evaluated by macro-dilution test using standard inoculums of 10^{-5} CFL/mL. Serial dilutions of the test compounds, previously dissolved in dimethyl sulfoxide (DMSO), were prepared to final concentrations of 400, 200, 100, 50, 12.5, 6.25, 3.125 µg/mL. The dilutions were added to 24 hour old inoculums. The susceptibility of the bacteria to the test compounds was determined by the formation of an inhibitory zone after 18 h of incubation at 37 °C. The inhibition zones (mm) of each compound and the minimum inhibitory concentration are presented in Table 1 and Table 2, respectively. The zone of inhibition was measured at the minimum inhibitory concentration. To compare the antimicrobial effect of the compounds with that of the Ciprofloxacin, the inhibition zone and MIC were considered. We calculated the percent area of inhibition per microgram of compounds and standard which exhibited that compounds **11, 12** and **14** were found to possess better activity against all microorganisms while compound **8** posses similar activity. On the other hand compounds **2, 3** and **4** showed 80% resemblance with standard drug "Ciprofloxacin" (Fig.-2). The importance of such work lies in the possibility that the new compound might be more effective against bacteria for which a thorough investigation regarding the structural activity relationship, toxicity and the biological effects would be helpful in designing more potent antibacterial agents for therapeutic use.

2.3 In vitro cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [84-85] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [86-88].

In the present study, some newly synthesized 1,2,4-triazine derivatives were screened for their antimicrobial activity and then evaluated for their cytotoxicity against human hepatocellular carcinoma cell line (HepG2) to ensure their toxic effect. Ciprofloxacin was used as a reference drug in the experiment. A sub-confluent population of HepG2 cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.125-100 µmol/L. The cell viability (%) obtained with continuous exposure for 48 h are depicted in Fig. 3. The cytotoxicity of all the compounds was found to be concentration-dependent and results revealed that all the compounds including the reference drug "Ciprofloxacin" showed viability ranging from 95%-100% at the concentration range of 3.125 µmol/L and up to a concentration of 25 µmol/L all the compounds showed a viability of ≥90%. On increasing the concentration range up to 50 and 100 µmol/L the compounds did not show any remarkable toxicity against the HepG2 cell line. All the tested compounds showed a viability of ≥80 % at a concentration of 100 µmol/L as depicted in Fig. 3.

3. Conclusion

New library 1,2,4-triazine analogues was synthesized with few steps reactions to make cost effective chemicals that can be used as an antibacterial agent targeting the Gram positive (*S. aureus and S. epidermidis*) and Gram negative (*P. mirabilis and E. coli*) bacteria. Considering the individual drug activity of 1,2,4-triazine nucleus, indole moiety and hydrazone functionality the scheme was designed in such a way that the target compound will be having all these important structures. All the substituted 1,2,4-triazine analogues **1-14** containing indole moiety were screened for their antimicrobial activity against microbes discussed above. Several compounds showed excellent activity better than the standard drug in terms of zone of inhibition and MIC values. Among them, many compounds represented good activity while some compounds found to possess better activity than the standard with less cytotoxicity.

4. Experimental

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results were uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ± 0.4% of the theoretical values. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 MHz spectrometer using DMSO as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm.

4.1 General procedure for the synthesis of compound A

A mixture of isatin (10 mmol), thiosemicarbazide (10 mmol) and K_2CO_3 (15 mmol) in 50 mL of water was refluxed with stirring for 3 h. On cooling the mixture was filtered and precipitated by acidification with acetic acid. The solid was washed with water and dried to obtain as yellow solid. 5*H*-[1,2,4]triazino[5,6-b]indole-2-thiol (**A**): Yellow crystals, yield: 80%; IRν$_{max}$ 3250,

2560 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.234 (s, 1H, NH), 7.987 (d, 1H, CH-Ar), 7.429-7.788 (m, Ar-H), 3.332 (s, 1H, SH).

4.2 General procedure for the synthesis of compound *B*

A mixture of compound **A** (10 mmol), ethyl chloroacetate (10 mmol) in 50 mL of DMF was refluxed at 100 °C with stirring for 3 h. Progress of the reaction was monitored by using thin layer chromatographic plates. On cooling the mixture was filtered and precipitated washed with water and dried under vacuum. Ethyl {3-[(ethoxy carbonyl)sulfanyl]-5*H*-[1,2,4]triazino[5,6-b]indole-5-yl}acetate (B): Yellow crystals, yield: 80%; IRv$_{max}$ 1695, 1685, 1105, 662 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.488-7.833 (m, Ar-H), 8.351-8.377 (d, 1H, CH-Ar), 5.291 (s, 2H, N-CH$_2$), 4.985 (s, 2H, S-CH$_2$), 4.098 (q, 3H, CH$_2$), 1.826 (t, 3H, CH$_3$).

4.3 General procedure for the synthesis of compound *C*

A mixture of compound **B** (10 mmol), in ethyl alcohol (10 mL), hydrazine hydrate (10 mmol) was added and resulting mixture was heated to 80 °C for 10 h. The mixture was concentrated to obtain solid. The resulting solid was filtered out and washed with water. The recrystalization of the compound **C** was performed using ethyl alcohol which yielded white solid crystals. S-[5-(2-hydrazinyl-2-oxoethyl)-5*H*-[1,2,4]triazino[5,6-b]indol-3-yl] hydrazinecarbothiate (**C**): White crystals, yield 84%; IRv$_{max}$ 3325, 3320, 3285, 3280, 1698, 1690, 1118, 690 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.091 (s, 1H, NH), 7.198-7.747 (m, Ar-H),), 5.324 (s, 2H, N-CH$_2$), 5.008 (s, 2H, S-CH$_2$2.507 (s, 2H, NH$_2$).

4.4 General method for preparation of compound *1-14*

The mixture of compound **C** and substituted aldehyde in absolute ethanol was taken in a round bottom flask and a few drops of glacial acetic acid added and refluxed for 12-24 h. After cooling, the obtained product was filtered off and recrystallized from ethanol to yield the desired products.

N'-[(*E*)-phenylmethylidene]-2-[3-(phenylmethylidene sulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**1**): dark yellow crystals, Yield 90%; mp 202-204 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-2-chlorophenylmethylidene]-2-[3-(2-chlorophenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**2**): Yellow crystals, yield 90%; mp 208-210 °C; IRv$_{max}$ 3280, 3277, 1688, 1673, 1610, 1603, 1124, 675 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.83 (s, 1H, NH), 11.90 (s, 1H, NH), 8.212 (s, 1H, CH=N), 8.120 (s, 1H, CH=N), 7.898-8.041 (m, Ar-H), 7.738 (s, 1H, Ar-H), 7.724-7.734 (m, Ar-H), 7.319-7.484 (m, Ar-H), 5.616 (s, 2H, N-CH$_2$), 4.092 (s, 2H, S-CH$_2$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.16 (C=O), 167.24 (C=O), 166.96 (C-S), 166.25, 164.24, 146.83, 142.18 (CH=N), 141.35 (CH=N), 140.10, 139.23, 138.92, 131.30, 130.72, 129.31, 128.90, 128.73, 128.20, 123.42, 121.82, 117.64, 112.13, 40.69 (CH$_2$), 40.50 (CH$_2$); ESI-MS *m/z* [M+1]$^+$, 591.08 (calcd. 591.10); Anal. calc. for C$_{27}$H$_{22}$Cl$_2$N$_8$O$_2$S: C, 54.83; H, 3.41; N, 18.94; Found: C, 54.82; H, 3.43; N, 18.90.

N'-[(*E*)-4-Chlorophenylmethylidene]-2-[3-(4-chlorophenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**3**): Brown crystals, yield 90%; mp 206-208 °C; IRv$_{max}$ 3281, 3273, 1686, 1674, 1616, 1602, 1121, 677 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.84 (s, 1H, NH), 11.71 (s, 1H, NH), 8.222 (s, 1H, CH=N), 8.124 (s, 1H, CH=N), 7.898-7.947 (m, Ar-H), 7.488-7.494 (m, Ar-H), 7.313-7.481 (m, Ar-H), 5.539 (s, 2H, N-CH$_2$), 4.064 (s, 2H, S-CH$_2$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.10 (C=O); 167.28 (C=O), 166.94 (C-S), 166.20, 164.25, 146.89, 142.18 (CH=N), 141.33 (CH=N), 140.10, 139.25, 138.94, 131.30, 130.74, 129.39, 128.90, 128.77, 128.20, 123.45, 121.80, 117.60, 112.16, 40.73 (CH$_2$), 40.52 (CH$_2$); ESI-MS *m/z* [M+1]$^+$, 591.08 (calcd. 591.12); Anal. calcd. for C$_{27}$H$_{22}$Cl$_2$N$_8$O$_2$S: C, 54.83; H, 3.41; N, 18.94; Found: C, 54.84; H, 3.40; N, 18.91.

N'-[(*E*)-4-Methylphenylmethylidene]-2-[3-(4-methylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**4**): White crystals; yield 80%; mp 198-200 °C; IRv$_{max}$ 3289, 3270, 1681, 1672, 1613, 1605, 1124, 676 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.82 (s, 1H, NH), 11.83 (s, 1H, NH), 8.344-8.369 (d, 1H, Ar-H), 8.162 (s, 1H, CH=N), 8.105 (s, 1H, CH=N), 7.873 (s, 1H, CH-Ar), 7.733-7.793 (m, Ar-H), 7.305-7.612 (m, Ar-H), 6.754-6.862 (m, Ar-H), 5.541 (s, 2H, N-CH$_2$), 4.152 (2H, s, S-CH$_2$), 2.50 (1H, s, CH$_3$), 2.27 (1H, s, CH$_3$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.04 (C=O), 167.25 (C=O), 166.97 (C-S), 166.26, 164.26, 146.88, 142.10 (CH=N), 141.36 (CH=N), 140.12, 139.29, 138.99, 131.34, 130.78, 129.35, 128.91, 128.75, 128.26, 123.48, 121.85, 117.67, 112.10, 40.79 (CH$_2$), 24.32 (CH$_3$), 40.49 (CH$_2$), 24.37 (CH$_3$); ESI-MS *m/z* [M$^+$+1], 551.19 (calcd. 551.14); Anal. calcd. for C$_{29}$H$_{29}$N$_8$O$_2$S: C, 63.26; H, 4.76; N, 20.35; Found: C, 63.23; H, 4.75; N, 20.37.

N'-[(*E*)-2-Nitrophenylmethylidene]-2-[3-(2-nitrophenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**5**): White crystals; yield 95%; mp 202-204 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-3-Nitrophenylmethylidene]-2-[3-(3-nitrophenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**6**): White crystals; yield 96%; mp 202-204 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-4-Nitrophenylmethylidene]-2-[3-(4-nitrophenylmethylidenesulfanyl)-5-*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**7**): White crystals; yield 90%; mp 202-204 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-4-Methoxylphenylmethylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**8**): Creamy white crystals, yield 80%; mp 204-206 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-3,4-Dimethoxylphenylmethylidene]-2-[3-(3,4-dimethoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**9**): white crystals White crystals; yield 90%; mp 210-212 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-2-ethoxylphenylmethylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**10**): White crystals; yield 80%; mp 218-220 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*E*)-4-Ethoxylphenylmethylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**11**): White crystals; yield 80%; mp 218-220 °C; IRv_{max} 3284, 3274, 1686, 1673, 1609, 1608, 1126, 677 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.72 (s, 1H, NH), 11.80 (s, 1H, NH), 8.167 (s, 1H, CH=N), 8.113 (s, 1H, CH=N), 7.875-7.890 (m, Ar-H), 7.732-7.790 (m, Ar-H), 6.750-6.861 (m, Ar-H), 5.538 (s, 2H, N-CH$_2$), 4.150 (s, 2H, S-CH$_2$), 3.962 (q, 2H, CH$_2$), 3.952 (q, 2H, CH$_2$), 1.320 (t, 3H, CH$_3$), 1.341 (t, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.04 (C=O), 167.25 (C=O), 166.97 (C-S), 166.26, 164.26, 146.88, 142.10 (CH=N), 141.36 (CH=N), 140.12, 139.29, 138.99, 131.34, 130.78, 129.35, 128.91, 128.75, 128.26, 123.48, 121.85, 117.67, 112.10, 64.94 (CH$_2$), 64.22 (CH$_2$), 40.79 (CH$_2$), 40.49 (CH$_2$), 14.80 (CH$_3$), 14.71 (CH$_3$); ESI-MS m/z [M+1]$^+$, 611.21 (calcd. 611.22); Anal. calcd. for $C_{31}H_{30}N_8O_4S$: C, 60.97; H, 4.95; N, 18.35; Found: C, 60.90; H, 4.93; N, 18.33.

N'-[(*Z*)-2-Pyridin-2-yl-methylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**12**): Dark yellow crystals; yield 85%; mp 202-204 °C; IRv_{max} 3287, 3274, 1680, 1679, 1616, 1606, 1123, 679 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.73 (s, 1H, NH), 11.69 (s, 1H, NH), 8.631 (s, 1H, Ar-H), 8.530 (d, 1H, Ar-H), 8.154 (s, 1H, CH=N), 8.151 (s, 1H, CH=N), 8.317 (d, 1H, Ar-H), 7.459-7.755 (m, Ar-H), 5.656 (s, 2H, N-CH$_2$), 4.148 (s, 2H, S-CH$_2$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.20 (C=O), 167.24 (C=O), 166.94 (C-S), 166.26, 164.28, 146.86, 142.17 (CH=N), 141.34 (CH=N), 140.13, 139.25, 138.97, 131.33, 130.75, 129.30, 128.92, 128.74, 128.27, 123.49, 121.86, 117.65, 112.12, 40.77 (CH$_2$), 40.45 (CH$_2$); ESI-MS m/z [M$^+$+1], 525.15 (calcd. 525.14); Anal. calcd. for $C_{25}H_{20}N_{10}O_2S$: C, 57.24; H, 3.84; N, 26.70; Found: C, 57.26; H, 3.82; N, 26.71.

N'-[(*Z*)-4-Pyridin-2-yl-methylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**13**): Dark yellow crystals; yield 90%; mp 202-204 °C; for all the spectroscopic details please see the Supporting information.

N'-[(*Z*)-thiophen-2-yl-methylidene]-2-[3-(4-methoxylphenylmethylidenesulfanyl)-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl]acetohydrazide (**14**): Dark yellow crystals; yield 92%; mp 202-204 °C; IRv_{max} 3286, 3279, 1686, 1674, 1613, 1607, 1124, 679 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.339-8.365 (d, 1H, Ar-H), 8.111 (s, 1H, CH=N), 8.103 (s, 1H, CH=N), 7.469-7.860 (m, Ar-H), 6.565-6.972 (m, Ar-H), 5.555 (2H, s, N-CH$_2$), 4.153 (2H, s, S-CH$_2$); ^{13}C NMR (DMSO-d_6, 500 MHz): δ 169.04 (C=O), 167.25 (C=O), 166.97 (C-S), 166.26, 164.26, 146.88, 142.10 (CH=N), 141.36 (CH=N), 140.12, 139.29, 138.99, 131.34, 130.78, 129.35, 40.49 (CH$_2$), 128.91, 128.75, 128.26, 123.48, 121.85, 117.67, 112.10, 40.79 (CH$_2$); ESI-MS m/z [M+1]$^+$, 535.07 (calcd. 535.08); Anal. calcd. for $C_{23}H_{18}N_8O_2S_3$: C, 51.67; H, 3.39; N, 20.96; Found: C, 51.63; H, 3.41; N 20.98.

4.5 Anti-bacterial activity

Organism culture and *in vitro* screening for antibacterial activity was done by the disk diffusion method with minor modifications. *S. aureus, S. epidermidis, P. mirabilis*, and *E. coli* was sub cultured in nutrient agar medium and incubated for 18 h at 37 °C. The bacterial cells were suspended for incubation according to the McFarland protocol in saline solution to produce a suspension of about 10^5 CFU/mL. 10 mL of this suspension was mixed with 10 mL of sterile antibiotic agar at 40 °C and poured on to an agar plate in a laminar flow cabinet. Five paper disks (6.0 mm diameter) were fixed onto nutrient agar plate. One milligram of each test compound was dissolved in 100 mL DMSO to prepare stock solution. From the stock solution different dilutions of each test compound were prepared and poured over disk plate. Ciprofloxacin was used as a standard drug (positive control) and DMSO as negative control. The susceptibility of the bacteria to the test compounds was determined by the formation of an inhibitory zone after 18 h of incubation at 36 °C. Table 1 shows the zone of inhibition of the oxadiazoline derivatives (**1-14**). The results were compared with the positive control and the zone of inhibitions was measured at the minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) was evaluated by the macro-dilution test using standard inoculum of 10^5 CFL/mL. Serial dilutions of the test compounds, previously dissolved in dimethyl sulfoxide (DMSO) were prepared to final concentrations of 400, 200, 100, 50, 12.5, 6.25 and 3.125 µg/mL. To each tube was added 100 mL of 24 h old inoculums. The MIC, defined as the lowest concentration (highest dilution) required to arrest the growth of bacteria, which inhibits the visible growth after 18 h, was determined visually after incubation of 18 h at 37 °C, and the results are presented in

4.6 Cytotoxicity studies (MTT-assay)

Cell culture: The human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, at

37 ºC in a saturated humidity atmosphere containing 95% air/5% CO_2 [89]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

MTT assay: The MTT assay was carried out using HepG2 following the procedure discussed by Mosmann 1983, the assay was performed in triplicate and repeated thrice. Percent viability was defined as the relative absorbance of treated versus untreated control cells [90].

Conflict of interest

The authors have no conflict of interest

Acknowledgement

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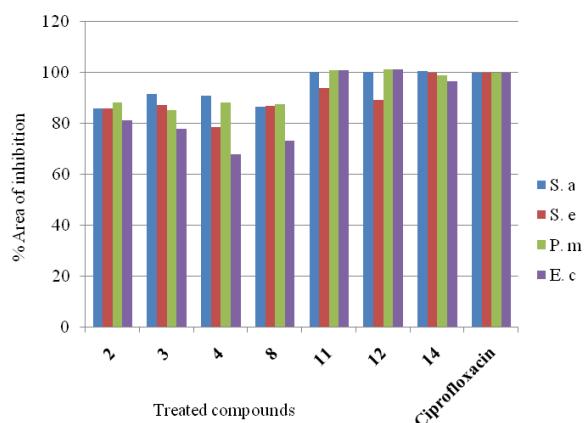
Fig. 2 The graph showing comparative percent area inhibition per µg of the compounds and the Ciprofloxacin against all microorganisms.
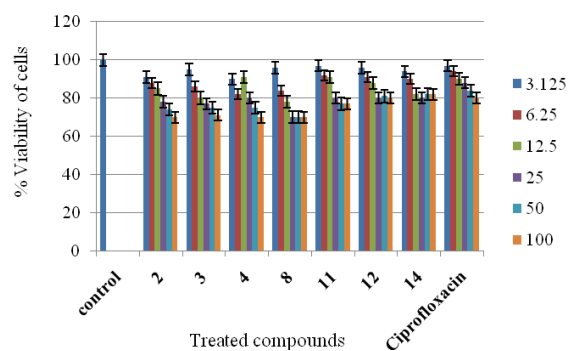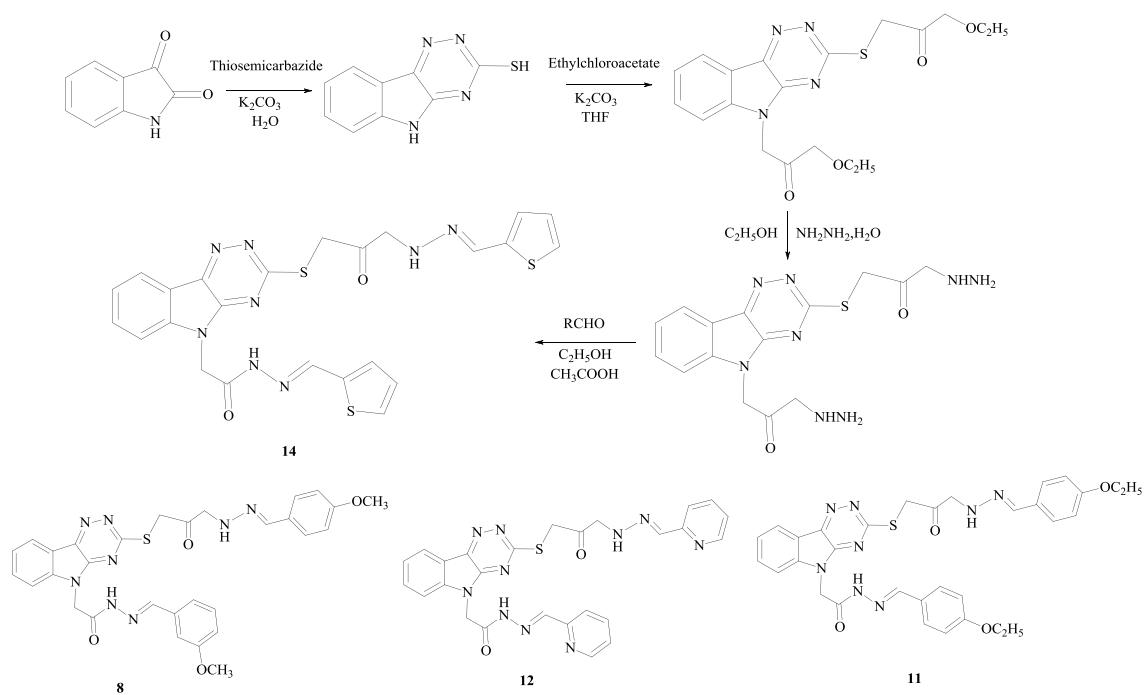

Fig. 3 Percent viability of cells after 48 h pre-treatment of HepG2 cell line with active compounds of the series and Ciprofloxacin by MTT assay.

Scheme 1 Schematic representation of the route adopted for the synthesis of **compounds 2-4, 8, 11, 12 and 14.**

Table 1 Inhibitory effects of compounds (**1-14**) on growth of microorganism by halo zone test. Ciprofloxacin used as standard drug and DMSO as negative control.

Compound	Gram positive			Gram negative	
	S. aureus	*S. epidermidis*		*P. mirabilis*	*E. Coli*
1	8.85±0.09	10.47±0.10		10.74±0.02	14.96±0.02
2	18.46±0.19	19.46±0.21		19.61±0.09	19.36±0.03
3	19.62±0.27	19.72±0.04		18.92±0.03	18.56±0.01
4	19.52±0.02	17.77±0.13		19.6±0.09	16.18±0.01
5	-	-		-	-
6	13.90±0.02	12.58±0.07		15.5±0.03	13.26±0.02
7	10.76±0.023	10.78±0.04		8.95±0.03	12.26±0.24
8	18.61±0.02	19.64±0.10		19.46±0.15	17.44±0.02
9	11.24±0.07	10.81±0.02		14.44±0.27	13.46±0.04
10	13.55±0.21	12.70±0.03		13.34±0.24	14.91±0.02
11	21.50±0.18	21.26±0.07		22.42±0.02	24.02±0.02
12	21.52±0.36	20.19±0.07		22.53±0.02	24.10±0.22
13	9.94±0.04	11.02±08		10.83±0.03	14.24±0.02
14	21.62±0.33	22.64±0.03		22.02±0.04	23.02±0.02
Ciprofloxacin	21.46 ±.31	22.64±.54		22.24±.30	23.82±.47

Table 2 Representing minimum inhibitory concentration (µg/mL) of 1,2,4-triazine derivatives (**1-14**). Ciprofloxacin was used as standard.

Compound	Gram positive			Gram negative	
	S. aureus	*S. epidermidis*		*P. mirabilis*	*E. Coli*
1	100	>100		>100	>100
2	12.5	6.25		12.5	25
3	12.5	6.25		12.5	25
4	12.5	6.25		12.5	25
5	-	-		-	-
6	>100	>100		50	50
7	100	>100		100	>100
8	6.25	3.125		6.25	12.5
9	100	100		25	100
10	100	100		12.5	50
11	6.25	3.125		6.25	12.5
12	6.25	3.125		6.25	12.5
13	100	100		100	50
14	6.25	12.5		6.25	12.5
Ciprofloxacin	6.25	3.125		6.25	12.5