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Research Article
An Efficient Synthesis of 1,2,4-triazine-6-one Derivatives and Their *in vitro* Anticancer Activity

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Abstract
Triazines and its derivatives have attracted considerable attention as cancer chemopreventive agents and also as cancer therapeutics. Many of its derivatives inhibit the growth of human cancer cell lines by triggering apoptosis. With this background, we planned to synthesis a series of triazine derivatives to assess their anti proliferation efficacy on human cancer cell lines. So, 2-(amino) thioxo-3-phenyl-1,2,5,6-tetrahydro-1,2,4-triazine-6-one was prepared via the reaction of (1) N- benzoyl glycine with (2) thiosemicarbazide under fusion at 130°C. Acetylation and (3) alkylation of compound with acetic anhydride and ethyl chloroacetate yielded the corresponding (4) N-acetamide derivative and (5) ethyl N-aminoacetate derivative, respectively. Fused triazo [2,1-a]-1,2,4-triazine-8-ones ($6_{a,b}$) were prepared from reaction of compound (3) with ω-bromomethyl aryl ketones in presence of fused sodium acetate. Acetylation of compound (6) with acetic anhydride yielded the corresponding N-acetyl derivatives ($7_{a,b}$). The cytotoxic activities of the 1,2,4-triazine- 6-one derivatives were studied on the tumor cell lines, human colon carcinoma (HCT-116) and human hepatocellular carcinoma cells (HepG-2) using the MTT viability test. The results showed that the investigated compound (6_b) had a significantly greater cytotoxic effect compared to that of the other compounds.

Key words: N-benzoyl glycine, acetylation, triazine, anticancer activity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many organic based cytotoxic agents have been discovered and they are extensively applied for treatment of cancer. Triazine derivatives have occupied a unique position in medicinal chemistry, so, triazine derivatives have attracted considerable pharmaceutical interest due to their antitumor (Jean-Claud *et al*., 1999; Gibson *et al*., 1984; Pilch *et al*., 1995; Smith *et al*., 1990; Unsalan and Rollas, 2007), anticonvulsant (Kumar *et al*., 1983) and antileukemic (Katsoulas *et al*., 2005; Seiter *et al*., 2002), activities and cytotoxic effects (Manolov *et al*., 2006). Among the compound having good antimicrobial properties (Dawane *et al*., 2010b), s-triazine derivatives constitute an important class of compounds possessing diverse pharmacological activities including broadly active triazine compounds. Also, triazine has been used to form many types of functional groups other than amines and used as protecting groups in natural product. Sztanke *et al*. (2008) reported the synthesis, structure elucidation and identification of antitumour properties of novel fused 1,2,4-triazine aryl derivatives, So, the synthetic strategy of the compounds is outlined in (Scheme 1). Synthesis of some new substituted 1,2,4-triazine-6-one derivatives was carried out by the fusion of N-benzoyl glycine with thiosemicarbazide. Cytotoxicity screen of the synthesized compounds were evaluated.

MATERIALS AND METHODS

Melting points were uncorrected and determined in an open capillary tube. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 500 MHz Jeol multinuclear NMR spectrometer; chemical shifts were referenced to Tetra Methyl Silane (TMS) as internal standard. The IR spectra were recorded on FTIR Shimadzu spectrometer. The mass spectra were recorded on E-Shimadzu-GC-MS spectrometer. Elemental analyses were performed on a Carlo Erba 106 Perkin-Elmer model 240 analyzer2-(Amino)thioxo-3-phenyl-1,2,5,6-tetrahydro-1,2,4-triazine-6-one 3:N-benzoyl glycine 1 (0.01 mole) was taken in 100 mL RBF and 0.01 mole of thiosemicarbazide 2 was fused on a hot-plate at 130-140°C for 1-1.5 h. The reaction mixture was also added to 30 mL of methanol and then the mixture is refluxed for about 3 h. The resultant solid obtained after cooling was filtered off, washed with cooled ethanol, dried and recrystallized from a acetic acid to give 3 as colorless crystals: Yield: 62%, m.p. 280°C. IR(KBr): = 3321, 3104 (NH_2), 3209 (NH), 1721 (C=O), 1645 (C=N), 1605, 1599 (C=C), 1366 (C=S) cm^{-1}. ^{1}H-NMR: d = 3.81 (s, ^{2}H. NCH_2CO), 4.40 (s, ^{2}H, NH_2), 7.46-7.89 (m, 5H, Ar-H), 9.00 (s, ^{1}H, NH) ppm. ^{13}C-NMR (DMSO-d_6): 168.32 (C=S), 166.75 (C=O), 151.02 (-C-(N_2), 134.43, 131.79, 128.80, 127.81(C-aromatic), 35.32 (N-CH_2-) ppm. MS: m/z (%) =235 (M^++1, 13.20), 234 (M^+, 37.35). Anal. Calcd. for $C_{10}H_{10}N_4OS$: C, 51.28; H, 4.27; N, 23.93; S, 13.68. Found: C, 51.19; H, 4.13; N, 23.73; S, 13.56.

N-(1-Acetyl-3-phenyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine-3-ylthioxo)-acetamide 4: A solution of 3 (0.01 mole) in acetic anhydride (25 mL) was heated under reflux for 2 h. The reaction mixture was cooled and poured into ice-water. The resultant solid was filtered off, washed with water, dried and recrystallized from ethanol to give 4 as pale yellow crystals: Yield: 63%, m.p. 250°C. IR(KBr): = 3235 (NH), 1701-1687 (br.CO), 1626 (C=N), 1605, 1585 (C=C), 1351 (C=S) cm^{-1}. ^{1}H-NMR: δ = 1.83 (s, 3H, CH_3), 1.84 (s, 3H, CH_3), 4.10 (s, 2H, NCH_2CO), 7.38-7.91 (m, 5H, Ar-H), 9.69 (s, 1H, NH) ppm. ^{13}C-NMR (DMSO-d6) 169.99 (C=S), 168.44, 166.84, 166.15 (C=O), 150.31 (C-(N_2), 134.30, 131.86, 128.17, 127.86 (C-aromatic), 35.60 (N-CH_2), 22.89 (CH_3), 20.92 (CH_3) ppm. MS: m/z(%) = 319 (M^++1, 13.50), 318 (M^+, 47.20), 277 (7.50), 276 (65.20), 235 (20.13), 234 (71.25). Anal. Calcd. for $C_{14}H_{14}N_4O_3S$: C, 52.83; H, 4.40; N, 17.61; S, 10.06. Found: C, 52.67; H, 4.23; N, 17.53; S, 9.98.

Ethyl-N-(3-phenyl-6-oxo-1,2,5,6-tetrahydro-1,2,4-triazine-3-ylthioxo)-amino-acetate 5: A mixture of 3 (0.01 mole) ethyl chloroacetate (0.01 mole) and fused sodium acetate (0.03 mole) in methanol (50 mL) was heated under reflux for 4 h. The reaction mixture was cooled and poured into water. The formed product was collected by filtration, washed with hot water, dried and recrystallized from ethanol to give 5 as colorless crystals: Yield: 66%, m.p. 200°C. IR(KBr): δ = 3214 (NH), 1765, 1698 (C=O), 1625 (C=N), 1605, 1587 (C=C), 1379 (C=S), 1055, 1022 (C-O) cm^{-1}. ^{1}H-NMR: δ = 1.17 (t, 3H, CH_3), 2.65 (s, 2H, $NHCH_2CO$), 3.37 (s, 2H, NCH_2CO), 4.09 (q, 2H, OCH_2), 7.46-7.92 (m, 5H, Ar-H), 9.12 (s, 1H, NH), 10.01 (s, 1H, NH) ppm. ^{13}C-NMR (DMSO-d_6): 169.29 (C=S), 168.33, 166.96 (C=O), 156.09 (-N=C-N), 134.44, 131.79, 128.51, 127.81 (C-aromatic), 61.43(OCH_2), 39.34 (NCH_2CO), 33.70 ($NHCH_2CO$), 14.42 (CH_3) ppm. MS: m/z (%) = 321 (M^++, 780), 320 (M^+ 21.20). Anal. Calcd. for $C_{14}H_{16}N_4O_3S$: C, 52.50; H, 5.00; N, 17.50; S, 10.00. Found: C, 52.39; H, 4.89; N, 17.39; S, 9.89.

1-Aryl-4-thioxo-5-phenyl-triazino[2,1-a]-7,8-dihydro-1,2,4-triazine-8-ones 6a,b: A mixture of 3 (0.01 mole), ω-bromomethyl aryl ketones (namely, phenacyl bromide and 4-chlorophenacyl bromide) (0.01 mole) and fused sodium acetate (0.03 mole) in glacial acetic acid (50 mL) was heated under reflux for 4 h. The reaction mixture was cooled and poured in to water. The obtained product was filtered off, washed with water, dried and recrystallized from ethanol to give 6.

1,5-Diphenyl-4-thioxo-triazino[2,1-a]-7,8-dihydro-1, 2,4-triazine-8-ones (6a) as pale yellow crystals, yield 71%, m.p.160°C. IR(KBr): = 3225 (NH), 1713 (C=O), 1632 (C=N), 1605, 1587 (C=C), 1378 (C=S) cm^{-1}. ^{1}H-NMR: δ = 3.52 (s, 2H, NCH_2CO), 7.45-8.01 (m, 11H, Ar-H and H-triazine), 10.23 (s, 1H, NH) ppm. ^{13}C-NMR (DMSO-d_6): 194.31(C=S), 166.93 (C=O), 157.30 (N = C-N), 136.02, 134.44, 134.33, 134.00, 131.87, 129.24, 128.81, 128.72, 127.87, 127.81 (C-aromatic), 36.07 (N-CH_2CO) ppm. MS: m/z (%) = 335 (M^++1, 8.90), 334 (M^+, 18.32). Anal. Calcd. for $C_{18}H_{14}N_4OS$: C, 64.67; H, 4.19; N, 16.76; S, 9.58. Found: C, 64.53; H, 4.09; N, 16.57; S, 9.49.

1-(4-Chlorophenyl)-4-thioxo-5-phenyl-triazino[2,1-a]-7, 8-dihydro-1,2,4-triazine-8-ones (6_b) as pale yellow crystals, yield 71%, m.p.171°C. IR(KBr): = 3214 (NH), 1705 (C=O),1605, 1585 (C=C), 1625 (C=N), 1379 (C=S) cm^{-1}. 1H-NMR: δ = 3.35 (s, 2H, N-CH_2CO), 7.44-8.04(m, 10H, Ar-H and H-triazine), 10.20 (s, 1H, NH) ppm. ^{13}C-NMR (DMSO-d_6): 193.41 (C=S), 166.96 (C=O), 156.36 (N = C-N), 139.08, 134.74, 134.32, 133.41, 131.93, 131.08, 130.73, 129.33, 128.75, 127.8 (C-aromatic and C-triazine), 36.15 (N-CH_2CO) ppm. MS: m/z (%) = 370 (M^++2, 7.31), 368 (M^+, 22.50). Anal. Calcd. for $C_{18}H_{13}ClN_4OS$: C, 58.69; H, 3.53; N, 15.22; S, 8.69. Found: C, 58.58; H, 3.48; N, 15.13; S, 8.59.

1-Aryl-3-acetyl-4-thioxo-5-phenyl-triazino[2,1-a]-7,8-dihydro-1,2,4-triazine-8-ones ($7_{a,b}$): A solution of $6_{a,b}$ in acetic anhydride (20 mL) was heated under reflux for 2 h. The reaction mixture was cooled and poured into ice-water. The resulting solid was filtered off, washed with water, dried and recrystallized from benzene to give 7.

1,5-Diphenyl-3-acetyl-4-thioxo-triazino[2,1-a]-7,8-dihydro-1,2,4-triazine-8-ones (7a) as pale yellow crystals, yield 63%, m.p.125°C. IR(KBr): = 1720-1698 (br.C=O), 1625 (C=N), 1605, 1585 (C=C), 1378 (C=S) cm^{-1}. 1H-NMR: δ = 2.20(s, 3H, $COCH_3$), 3.52 (s, 2H, NCH_2CO), 7.45-8.11 (m, 11H, Ar-H and H-triazine) ppm. ^{13}C-NMR (DMSO-d_6): 194.41(C=S), 167.20, 166.91 (C=O), 156.30 (N = C-N), 136.11, 134.42, 134.31, 134.01, 131.67, 129.21, 128.80, 128.71, 127.83, 127.78, 127.69 (C-aromatic and C-triazine), 35.50 (N-CH_2CO), 21.81 (CH_3) ppm. MS: m/z (%) = 377 (M^++1, 13.20), 376(M^+, 47.20). Anal. Calcd. for $C_{20}H_{16}N_4O_2S$: C, 63.83; H, 4.25; N, 14.89; S, 8.51. Found: C, 63.71; H, 4.17; N, 14.78; S, 8.35.

1-(4-Chlorophenyl)-3-acetyl-4-thioxo-5-phenyl-triazino [2, 1-a]-7,8-dihydro-1,2,4-triazine (7_b) as pale yellow crystals, yield 67%, m.p.131°C. IR(KBr): = 1705-1689 (br.C=O), 1627 (C=N), 1605, 1583 (C=C), 1378 (C=S) cm^{-1}. ^{1}H-NMR: δ = 2.20 (s, 3H, $COCH_3$), 3.56 (s, 2H, NCH_2CO), 7.41-8.03 (m, 10H, Ar-H and H-triazine) ppm. ^{13}C-NMR (DMSO-d_6): 194.20 (C=S), 167.30, 166.95 (C=O), 156.33 (N=C-N), 139.02, 134.72, 134.33, 134.31, 133.39, 131.91, 131.11, 130.71, 129.31, 128.72, 127.85 (C-aromatic and C-triazine), 36.13 (N-CH_2CO), 22.30 (CH_3) ppm. MS: m/z (%) = 412 (M^++1, 11.30), 410 (M^+, 34.31). Anal. Calcd. for $C_{20}H_{15}N_4ClO_2S$: C, 58.54; H, 3.66; N, 13.66; S, 7.80. Found: C, 58.45; H, 3.44; N, 13.55; S, 7.67.

In vitro studies

Cell lines: Human colon carcinoma (HCT-116) cells and human hepatocellular carcinoma (HepG-2) cells were obtained from the American type culture collection ATCC, Rockvill, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg mL^{-1} gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

Cytotoxic assay of 1,2,4-triazine derivatives: The cells were grown as monolayers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg mL^{-1} gentamycin. The monolayers of 10000 cells adhered at the bottom of the wells in a 96 well micro titer plate incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 μL from different dilutions of the test sample in fresh maintenance medium and incubated at 37°C. A control of untreated cells was made in the absence of the test sample. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and read the absorbance at 490 nm using ELISA reader (Sun Rise, TECAN, Inc., USA) after well mixing. The absorbance values from untreated cells were considered as 100% proliferation.

The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as:

$$1-\frac{ODt}{ODc}\times 100\%$$

Where:

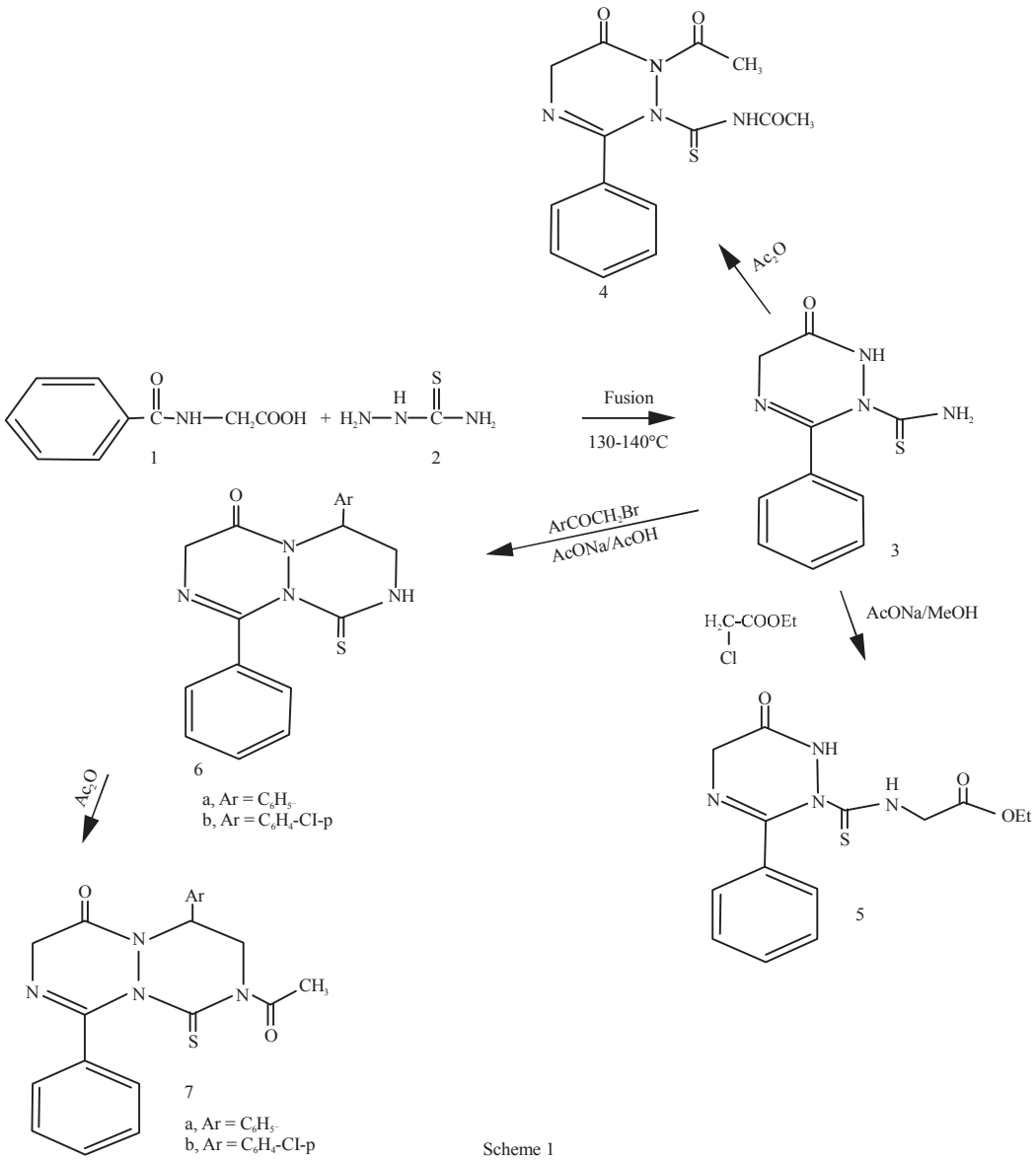
ODt = Mean optical density of wells treated with the test sample

ODc = Mean optical density of untreated cells

The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effect in 50% of inactivated cells, was estimated from graphic plots.

RESULTS AND DISCUSSION

As part of this study program, and in extension of our work on the development of friendly environmental methodologies for the preparation of biologically active compound (Kamble *et al*., 2007; Dawane *et al*., 2009, 2010a), herein we report an efficient synthesis of 1,2,4-triazine-6-one derivatives. The fusion of (1) N-benzoyl glycine and (2) thiosemicarbazide at 120-130°C as reaction without solvent to afford the corresponding (3) 2-(amino) thioxo -3-phenyl-1,2,5,6-tetrahydro-1,2,4-triazine-6-one. Acetylation (Mohamed *et al*., 2006; Chen *et al*., 2001) of (3) with acetic anhydride under reflux gave the corresponding N-(1-acetyl-3-phenyl-6-oxo-1,2,4-triazine-3-ylthioxo) acetamide (4). Treatment (El-Sakka *et al*., 2009) of compound (3) with ethyl chloroacetate in the presence of fused sodium acetate in methanol yielded the corresponding ethyl-N-(3-phenyl-6-oxo-1,2,4-triazine-2-yl thioxo) amino acetate (5). The reaction (El-Deen, 1998; Shelke *et al*., 2010) of 2-(amino)thioxo-3-phenyl-1,2,5,6-tetrahydro-1,2,4-triazine-6 -one (3) with ω-bromomethyl aryl ketones (namely, phenacyl bromide and 4-chlorophenacyl bromide) in the presence of fused sodium acetate in acetic acid yielded the 1-aryl-4-thioxo-5-phenyl-triazino [2,1-a]-7,8-dihydro-1,2, 4-triazine-8-ones ($6_{a,b}$). Acetylation of compound (6) with acetic anhydride under reflux led to the formation of 1-aryl-3-acetyl-4-thioxo-5-phenyl triazino [2,1-a]-7,8-dihydro-1,2,4-triazine-8-ones ($7_{a,b}$).



Scheme 1

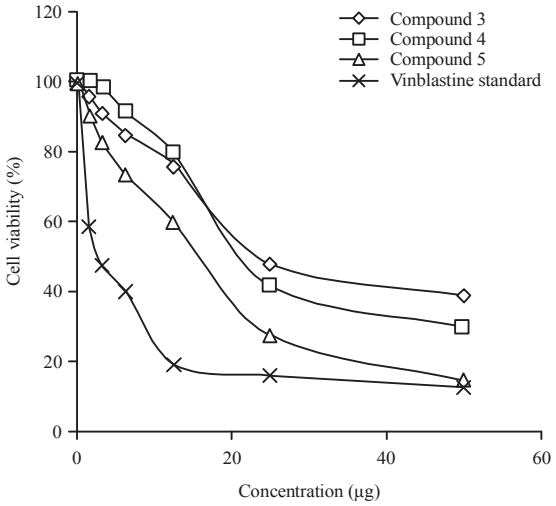


Fig. 1: Inhibitory activities against HCT-116 cell lines

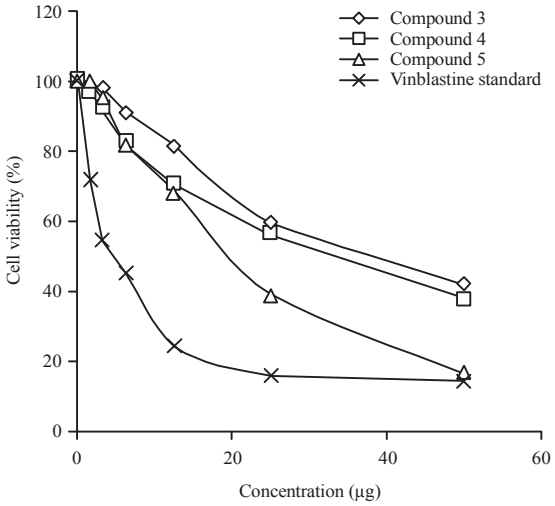


Fig. 3: Inhibitory activities against HepG-2 cell lines

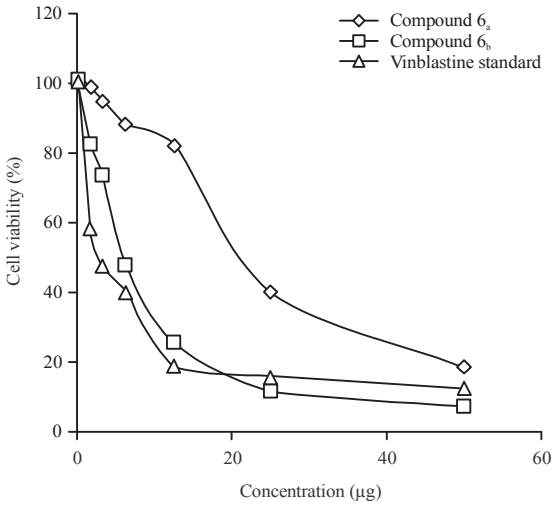


Fig. 2: Inhibitory activities against HCT-116 cell lines

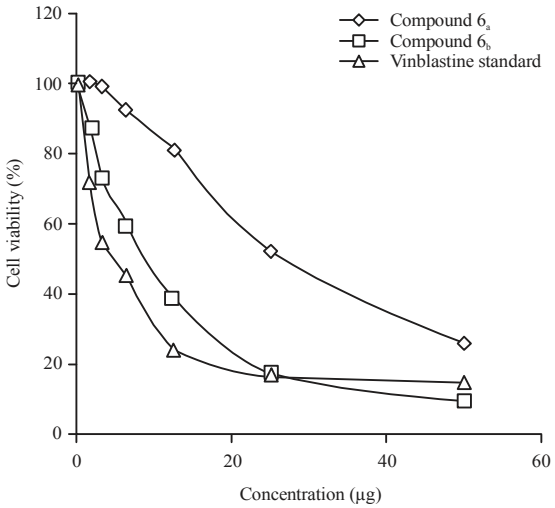


Fig. 4: Inhibitory activities against HepG-2 cell lines

Anticancer activity of new 1,2,4-triazine derivatives: In this study, the cytotoxic and antitumor activities of the synthesized 1,2,4-triazine derivatives (3-6_b) were tested against the human cancer cell lines, representing colon and liver cancer according to method of Mosmann (1983) and Gangadevi and Muthumary (2007). The inhibitory activities against human colon carcinoma cells (HCT-116) and Hepatocellular carcinoma cells (HepG-2) was detected by using different concentrations of the samples (50, 25, 12.5, 6.25, 3.125 and 1.56 µg) and the cell viability (%) was determined by colorimetric method. The drug Vinblastine was used as standard.

The result of 50% inhibitory concentration (IC_{50}) of the (HCT-1160) cell line was calculated from (Table 1, Fig. 1 and 2).

Table 1: Evaluation of cytotoxicity of 1,2,4-triazine derivatives against HCT-116

	Cell viability (%)					
Samples concentration (µg)	3	4	5	6_a	6_b	Vinblastine standard
50	38.66	29.84	14.54	18.34	6.81	12.16
25	47.58	41.68	27.38	39.82	11.72	15.54
12.5	75.76	79.47	59.82	81.58	25.34	18.92
6.25	84.64	91.32	73.16	87.86	47.23	39.86
3.125	90.82	98.16	82.97	94.59	73.58	47.30
1.56	95.38	100.00	90.06	98.87	81.92	58.11
0.00	100.00	100.00	100.00	100.00	100.00	100.00

The results of 50% inhibitory concentration (IC_{50}) of the HepG-2 cell line was calculated from (Table 2, Fig. 3 and 4). The result of 50% inhibitory concenttration (IC_{50} data are summarized in Table 3.

Table 2: Evaluation of cytotoxicity of 1 2,4-triazine derivatives against HepG-2

Samples concentration (μg)	Cell viability (%) 3	4	5	6_a	6_b	Vinblastine standard
50	41.97	38.12	16.81	24.93	8.76	14.38
25	59.83	56.54	39.24	51.82	17.34	16.13
12.5	81.42	70.96	68.46	80.94	38.52	24.25
6.25	91.08	82.35	82.07	92.56	59.38	45.13
3.125	97.63	91.74	95.42	98.93	72.95	55.00
1.56	99.12	97.28	100.00	100.00	87.31	72.13
0.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 3: IC_{50} (μM) values of the synthesized compounds after 72 h continuous exposure of tumor cell lines

Compound No.	3	4	5	6_a	6_b	Vinblastine standard
Tumor type/cell line						
HCT-116	33.9	22.2	16.3	22.0	5.92	2.38
HepG-2	38.8	33.9	20.4	26.7	9.06	4.60

The IC_{50} value is the concentration that induces 50% growth inhibition compared with untreated control cells.

- HCT-116: Human colon carcinoma cell lines
- HepG-2: Human hepatocellular carcinoma cell lines

The IC_{50} values of compound 6_b on the five cancer cell lines. In comparison with standard antitumor drug vinblastine, the pharmacological results showed that some compounds displayed weak to moderate and high levels of antitumor activities. compound 6_b was found to be active against HCT-116 and HepG-2 cell lines, while another compounds 3, 4, 5 and 6_a were observed to be weak active against HCT-116 and HepG-2.

Compound 6_b had increased anticancer activity than compound 6a (decreased anticancer activity) against HCT-116 and HepG-2 cell lines because compound 6_b contains chlorine atom.

CONCLUSION

A new series of 1,2,4-triazine derivatives were prepared in good yield. The structures of these compounds were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR, MS and elemental analysis. Antitumor activities of synthesized compounds were evaluated on human colon and liver cancer cell lines. As a result of the cell culture studies, all of the compounds have shown anticancer activity for colon and liver cancer cells. In conclusion, novel 1,2,4-triazine derivatives might be potentially useful in the field of cancer treatment. Finally, the new 1-(ρ-chlorophenyl)-4-thioxo-5-phenyl-triazino [2,1-a]-7,8-dihydro-1,2,4-triazine -8- one (6_b) can be suggested as potent candidates for colon and liver cancer drug.

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