SYNTHESIS AND ANTICANCER ACTIVITIES OF THIAZOLES, 1,3-THIAZINES, AND THIAZOLIDINE USING CHITOSAN-GRAFTED-POLY(VINYLPYRIDINE) AS BASIC CATALYST

Sobhi M. Gomha,¹ Sayed M. Riyadh,*¹,² Elmahdi A. Mahmmoud,¹ and Mahmoud M. Elaasser³

¹) Department of Chemistry, Faculty of Science, University of Cairo, Giza, 12613, Egypt
²) Department of Chemistry, Faculty of Science, Taibah University, Almadinah Almunawrah, 30002, Saudi Arabia
³) Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt

* Corresponding author: E-mail: riyadh1993@hotmail.com

Abstract – Three different series of ethylidenehydrazonothiazoles 5a-c, 6a-c, ethylidenehydrazono-1,3-thiazines 9a-i and ethylidenehydrazonothiazolidine 12 have been prepared via reactions of ethylidenethiosemicarbazide 3a or ethylidenethiocarbohydrazide 3b with α-halocarbonyl compounds 4a-c, acrylonitrile derivatives 7a-i, and dimethyl acetylenedicarboxylate 10, respectively. Different basic catalysts were used in these reactions such as, triethylamine, chitosan, and chitosan-grafted-poly(vinylpyridine) and the latter catalyst has precedence as environmentally friendly basic catalyst. Moreover, the selected newly synthesized products were evaluated for their anti-cancer activity against a colon carcinoma cell line (HCT-116) and liver carcinoma cell line HEPG2 and revealed promising activity especially 1,3-thiazines 9c-i.

Biocatalysis is one of the most important tools for green chemistry.¹ This technique is based on the use of natural renewable biological materials, such as enzymes² and polymers,³ that provide cleaner methodologies with high selectivity and energy-efficient operation under mild conditions in contrast to the traditional chemical catalysts. Chitosan, the naturally occurring polysaccharides, is a copolymer containing both glucosamine units and acetylglucosamine. It can be extracted from chitin by extensive
deacetylation under alkaline conditions (Figure 1). 4

Figure 1. Extraction of chitosan from chitin

Chitosan used as heterogeneous phase transfer basic biocatalyst in heterocyclic synthesis, such as enantioselective syntheses of asymmetric products with chiral center(s) 5 and Michael additions. 6-8 Chitosan was also used to support metal for the preparation of heterogeneous catalysts. 9 One of the main problem of using chitosan that it is highly hygroscopic, leading it to form gels, thereby it could not easily be recycled from the reaction mixture. To overcome this drawback, chitosan-grafted-poly-(vinylpyridine) 10 (Figure 2) has been used as a basic biocatalyst with the following advantages; a) it can be used in the form of beads which increase its catalytic activity, 9 b) easily recycled with the same catalytic efficiency, c) higher basic character due to presence of lone pairs of electrons on nitrogen atoms in pyridine rings. 10

Figure 2. Structure of grafted chitosan

Meanwhile, many heterocyclic compounds containing the S-C-N framework have extensive pharmaceutical applications. Hydrazonothiazoles have demonstrated anticancer activity 11 by inhibition of
histone acetyl transferases (Gcn5 HAT), enzymes are located in the nucleus that are responsible for regulation of gene expression and cooperate with activators to enhance transcription. Also, 2,4-disubstituted thiazoles have been reported as potent anticancer agents for different cell lines.\textsuperscript{12-14} Furthermore, 1,3-thiazines are an important group of S,N-containing heterocycles, which revealed selective antitumoral activity against leukemia cells.\textsuperscript{15,16} In view of these precedents and as a part of our research interest towards developing new routes for the synthesis of a variety of heterocyclic systems with promising biological and pharmacological activities,\textsuperscript{17,18} we present in this report an efficient synthesis of a new series of hydrazonothiazoles and 1,3-thiazines using chitosan-grafted-poly(vinylpyridine) as an eco-friendly biopolymeric basic catalyst. Also, we have examined the anticancer activity of these compounds against different cell lines.

Condensation of 3-acetyl-4,5-diphenyl-2-methyl-1\textsubscript{H}-pyrrole (1)\textsuperscript{19} with thiosemicarbazide (2\textsubscript{a}) or thiocarbohydrazide (2\textsubscript{b}) in absolute ethanol in the presence of catalytic amount of HCl led to formation of 1-[1-(2-methyl-4,5-diphenyl-1\textsubscript{H}-pyrrol-3-yl)ethylidene]thiosemicarbazide (3\textsubscript{a}) or 1-[1-(2-methyl-4,5-diphenyl-1\textsubscript{H}-pyrrol-3-yl)ethylidene]thiocarbohydrazide (3\textsubscript{b}), respectively as depicted in Scheme 1.

![Scheme 1. Synthesis of compounds 3a and 3b](image)

An easy way to differentiate between two geometric structures (syn, and anti) of products 3\textsubscript{a,b} could be achieved through NOE difference experiments. \textsuperscript{1}H NMR spectra of compounds 3\textsubscript{a,b} revealed, in each case, two singlet signals at $\delta = 1.56-1.76$ and $2.36-2.37$ ppm assignable to methyl group on C2 of pyrrole ring\textsuperscript{19} and another methyl group adjacent to hydrazone (Me-C=N-NH),\textsuperscript{20} respectively, in addition to NH signal of hydrazone group (C=N-NH) at $\delta = 10.82-10.87$ ppm (see experimental). Thus irradiating of NH proton at $\delta = 10.82-10.87$ ppm led to enhancement of methyl protons on C2 of pyrrole ring at $\delta = 1.56-1.76$ ppm suggesting (syn-) form while in the (anti-) form this irradiation did not affect such enhancement. In our case, irradiating of NH proton at $\delta = 10.82-10.87$ ppm did not affect enhancement on...
C2-methyl protons on pyrrole ring and led to enhancement of methyl protons adjacent to hydrazone group (Me-C=N-NH) at \( \delta = 2.36-2.37 \) ppm which confirmed the (anti-) form and excluded (syn-) form. Recently, it has been reported that, treatment of ethylidenethiosemicarbazide derivative with appropriate \( \alpha \)-haloketones afforded the respective thiazoles.\textsuperscript{21-25} However, the reactivity of ethylidenethiocarbonylhydrazide derivative towards \( \alpha \)-haloketones has not been reported. In the present study, we have investigated the reactions of either ethylidenethiosemicarbazide 3\textsubscript{a} or ethylidenethiocarbonylhydrazide 3\textsubscript{b} with \( \alpha \)-halocarbonyl compounds 4\textsubscript{a-c} in dioxane under microwave irradiation (6-10 min) in the presence of different basic catalyst such as triethylamine, chitosan, and chitosan-grafted-poly(vinylpyridine). These reactions afforded ethylidenehydrazonothiazoles 5\textsubscript{a-c} and ethylidenehydrazono-aminothiazoles 6\textsubscript{a-c} (Scheme 2). At the outset, the performance of different basic catalysts has been explored (Table 1).

**Scheme 2.** Synthesis of compounds 5\textsubscript{a-c} and 6\textsubscript{a-c}

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>R</th>
<th>Time (min)</th>
<th>TEA (%)</th>
<th>Chitosan (%)</th>
<th>g-Chitosan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textsubscript{a}</td>
<td>Me</td>
<td>6</td>
<td>62</td>
<td>73</td>
<td>86</td>
</tr>
<tr>
<td>5\textsubscript{b}</td>
<td>OEt</td>
<td>5</td>
<td>61</td>
<td>71</td>
<td>86</td>
</tr>
<tr>
<td>5\textsubscript{c}</td>
<td>PhNH</td>
<td>8</td>
<td>60</td>
<td>72</td>
<td>82</td>
</tr>
<tr>
<td>6\textsubscript{a}</td>
<td>Me</td>
<td>6</td>
<td>64</td>
<td>80</td>
<td>89</td>
</tr>
<tr>
<td>6\textsubscript{b}</td>
<td>OEt</td>
<td>6</td>
<td>54</td>
<td>78</td>
<td>88</td>
</tr>
<tr>
<td>6\textsubscript{c}</td>
<td>PhNH</td>
<td>10</td>
<td>63</td>
<td>77</td>
<td>85</td>
</tr>
</tbody>
</table>

We have observed that under the same reaction conditions the yields of the desired products 5\textsubscript{a-c} and 6\textsubscript{a-c} increase by changing triethylamine into chitosan. Moreover, using of grafted-chitosan as a basic catalyst has significant increasing effect on the product yields. Elemental analyses and spectral data \( ^1\text{H} \)}
NMR, $^{13}$C NMR, IR and MS) are compatible with the elucidated structures of the products. For example, $^1$H NMR spectra of compounds 5a-c and 6a-c revealed, in each case, two singlet signals at $\delta = 2.37-2.42$ and 2.58-2.67 ppm assignable to methyl group adjacent to hydrazone group (Me-C=N-NH)$_2$ and another methyl group on C4 of thiazole ring, respectively (see experimental). In addition, $^1$H NMR spectra of compounds 6a-c showed a characteristic broad signal at $\delta = 3.40-3.64$ ppm attributed to amino group which confirmed by absorption bands at $\nu = 3427-3255$ cm$^{-1}$ in IR spectra. The sequence of preparation of target compounds is preceded by displacement of hydrogen chloride followed by dehydrative cyclization to give the isolated products 5a-c and 6a-c.

The green protocol for the efficient synthesis of functionalized 1,3-thiazines was extended. Thus, reactions of ethylidenethiosemicarbazide 3a with different acrylonitrile derivatives such as, arylidenemalononitriles 7a-g, 2-cyano-N,3-diphenylacrylamide (7h), and 2-cyano-3-(4-chlorophenyl)-prop-2-enethioamide (7i), in dioxane under microwave irradiation (6-8 min) furnished the respective 1,3-thiazines 9a-i in agreement with literature reports concerning the reactions of thiosemicarbazides with substituted acrylonitriles (Scheme 3). In this study we have investigated the effect of nature of basic catalyst such as triethylamine, chitosan, and chitosan-grafted-poly(vinylpyridine) on the percent yields of isolated 1,3-thiazines (Table 2).

As shown in Table 2, grafted chitosan has precedence as basic catalyst for synthesis of 1,3-thiazines over chitosan and triethylamine under the same reaction conditions.
Table 2. Effect of nature of basic catalyst on the product yields 9a-i

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Ar</th>
<th>Y</th>
<th>Time (min)</th>
<th>(%) yield TEA</th>
<th>Chitosan</th>
<th>g-Chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a</td>
<td>C₆H₅</td>
<td>CN</td>
<td>5</td>
<td>63</td>
<td>75</td>
<td>87</td>
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<tr>
<td>9b</td>
<td>4-ClC₆H₄</td>
<td>CN</td>
<td>5</td>
<td>65</td>
<td>79</td>
<td>89</td>
</tr>
<tr>
<td>9c</td>
<td>4-MeC₆H₄</td>
<td>CN</td>
<td>6</td>
<td>60</td>
<td>72</td>
<td>82</td>
</tr>
<tr>
<td>9d</td>
<td>4-MeOC₆H₄</td>
<td>CN</td>
<td>6</td>
<td>60</td>
<td>73</td>
<td>84</td>
</tr>
<tr>
<td>9e</td>
<td>benzo[b][1,3]dioxol-2-yl</td>
<td>CN</td>
<td>8</td>
<td>62</td>
<td>76</td>
<td>85</td>
</tr>
<tr>
<td>9f</td>
<td>CH=CH-Ph</td>
<td>CN</td>
<td>8</td>
<td>63</td>
<td>77</td>
<td>84</td>
</tr>
<tr>
<td>9g</td>
<td>2-furyl</td>
<td>CN</td>
<td>6</td>
<td>65</td>
<td>75</td>
<td>88</td>
</tr>
<tr>
<td>9h</td>
<td>C₆H₅</td>
<td>CONHPh</td>
<td>8</td>
<td>60</td>
<td>69</td>
<td>80</td>
</tr>
<tr>
<td>9i</td>
<td>4-ClC₆H₄</td>
<td>CSNH₂</td>
<td>8</td>
<td>61</td>
<td>66</td>
<td>81</td>
</tr>
</tbody>
</table>

The elucidation for the structures of 1,3-thiazines 9a-i was based on spectroscopic data (IR, ¹H NMR, ¹³C NMR, MS) and elemental analyses. IR spectra of compounds 9a-g, 9h, and 9i revealed characteristic signals at ν = 2185-2220, 1643, and 1300 cm⁻¹ assignable to (C≡N), (C=O), and (C=S) groups, respectively. Also, ¹H NMR spectra of compounds 9a-i showed, in each case, two signals at δ = 3.62-3.80 and 4.55-5.35 ppm assignable to CH-thiazine ring and amino group on C4 of thiazine ring. The formation of 1,3-thiazines could be suggested by addition of thiol group in thiosemicarbazone moiety into activated double bond to give the non-isolable intermediates 8a-i. Intramolecular cyclization of intermediates 8a-i via addition of amino group into nitrile group afforded 6H-1,3-thiazine derivatives 9a-i.

The reactivity of ethylidenethiosemicarbazide 3a towards activated triple bond has also been investigated. Thus, treatment of ethylidenethiosemicarbazide 3a with dimethyl acetylenedicarboxylate (DMAD) (10) under the employed reaction conditions, using grafted chitosan as a basic catalyst, furnished the corresponding thiazolidin-4-one derivative 12 (Scheme 4). The structure of the isolated product was inferred from its elemental analysis and spectral data [IR and ¹H NMR]. Its IR spectrum showed absorption bands at ν = 3366, 3247 (2NH), 1703, 1684 (2C=O), and 1605 (C=N) cm⁻¹, its ¹H NMR spectrum revealed a pair of singlet signal at δ 11.19, 12.59 ppm (D₂O-exchangeable) assignable to (2NH) groups and another singlet signal at δ 6.61 ppm due to vinylic-H (C=CH). Revealing of the latter signal of vinylic-H excluded the isomeric structure of 1,3-thiazin-4-one 13. To account for the formation of product 12 we assumed that the reaction initially proceeded via addition of thiol group in thiosemicarbazone moiety into triple bond to give the non-isolable intermediate 11. Elimination of
methanol molecule from the latter intermediate afforded the isolated product 12 (cf. Scheme 4).

![Scheme 4. Synthesis of compound 12](image)

**Anti-cancer Activity**

The anticancer activity of some newly synthesized compounds was determined against a colon carcinoma cell line (HCT-116) and liver carcinoma cell line HEPG2, using doxorubicin as a reference drug. Data generated were used to plot a dose–response curve of which the concentration (μM) of test compounds required to kill 50% of cell population (IC₅₀) was determined. Cytotoxic activity was expressed as the mean IC₅₀ of three independent experiments (Table 3). The results showed that 1,3-thiazine derivatives with different aryl moieties at C6 have promising anticancer activity against HCT-116 and HEPG2 cell lines. Structure activity relationship was exemplified by substituting phenyl moiety of 9a (IC₅₀ = 2.67 and 8.85 μM) by electron donating group e.g. (4-Me and 4-OMe groups) affording 9c and 9d analogues (IC₅₀ = 0.48 & 1.41 and 0.24 & 1.52 μM, respectively), this substitution effectively increased the anticancer activity against both cell lines with IC₅₀ close to doxorubicin. On the other hand, substituting phenyl moiety of 9a by electron withdrawing group e.g. (4-Cl) affording compound 9b, diminished the activity (IC₅₀ = 1.33 & 3.32 μM). Similarly, different aryl or heterocyclic rings at C6 were replaced by phenyl moiety, to afford 9e-9i revealed positive impact on the anticancer activity. SAR of thiazoles 5a-c
and aminothiazoles 6a-c was explained by the order of inhibition activity for both cell lines
[ethoxycarbonyl (5b) > phenylcarboxamide (5c) > acetyl (5a)] and [phenylcarboxamide (6c) > acetyl (6a)
> ethoxycarbonyl (6b)] moieties on C5 of thiazole and aminothiazole ring, respectively (Table 3). Thiazolidin-4-one derivative 12 has weak activities against both cell lines.

Table 3. Cytotoxic activities of selected compounds against tumor cell lines
(HCT-116 and HEPG2-1)

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>IC₅₀ (µM)</th>
<th></th>
<th></th>
<th>Compound No.</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCT-116</td>
<td>HEPG2</td>
<td></td>
<td></td>
<td>HCT-116</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.79</td>
<td>0.72</td>
<td></td>
<td>Doxorubicin</td>
<td>0.79</td>
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<tr>
<td>(standard)</td>
<td></td>
<td></td>
<td></td>
<td>(standard)</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>11.87</td>
<td>14.51</td>
<td></td>
<td>9c</td>
<td>0.48</td>
</tr>
<tr>
<td>5b</td>
<td>1.38</td>
<td>1.57</td>
<td></td>
<td>9d</td>
<td>0.24</td>
</tr>
<tr>
<td>5c</td>
<td>6.89</td>
<td>11.70</td>
<td></td>
<td>9e</td>
<td>0.53</td>
</tr>
<tr>
<td>6a</td>
<td>4.40</td>
<td>5.91</td>
<td></td>
<td>9f</td>
<td>0.45</td>
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<tr>
<td>6b</td>
<td>46.30</td>
<td>75.69</td>
<td></td>
<td>9g</td>
<td>0.30</td>
</tr>
<tr>
<td>6c</td>
<td>2.33</td>
<td>1.17</td>
<td></td>
<td>9h</td>
<td>0.32</td>
</tr>
<tr>
<td>9a</td>
<td>2.67</td>
<td>8.85</td>
<td></td>
<td>9i</td>
<td>0.61</td>
</tr>
<tr>
<td>9b</td>
<td>1.33</td>
<td>3.32</td>
<td></td>
<td>12</td>
<td>18.40</td>
</tr>
</tbody>
</table>

CONCLUSION
We have developed a green technique for preparation of ethylidenehydrazonothiazoles, ethylidenehydrazono-1,3-thiazines and ethylidenehydrazonothiazolidine by using chitosan-grafted-poly-
(vinylpyridine) as environmentally friendly basic catalyst. The synthesized products exhibited high to
moderate anti-cancer activities.

EXPERIMENTAL
General
All melting points were determined on an electrothermal Gallenkamp apparatus and are uncorrected.
Solvents were generally distilled and dried by standard literature procedures prior to use. The IR spectra
were measured on a Pye-Unicam SP300 instrument in potassium bromide discs. The ¹H NMR and ¹³C
NMR spectra were recorded on a Varian Mercury VXR-300 spectrometer (300 MHz for ¹H NMR and 75
MHz for ¹³C NMR) and the chemical shifts were related to that of the solvent DMSO-δ₆. The mass
spectra were recorded on a GCMS-Q1000-EX Shimadzu and a GCMS 5988-A HP spectrometers, the ionizing voltage was 70 eV. Elemental analyses were carried out by the Microanalytical Center of Cairo University, Giza, Egypt. Microwave experiments were carried out using CEM Discover Labmate microwave apparatus (300 W with Chem. Driver Software). Antitumor activity was evaluated by the National Institute of Cancer, Biology Department, Cairo University, Egypt. 3-Acetyl-4,5-diphenyl-2-methyl-1H-pyrrole (1)\textsuperscript{19} was prepared following literature method.

**Synthesis of ethylenethiosemicarbazide 3a and ethylenethiocarbohydrazide 4a**

These compounds were prepared following the procedure described by Gomha et al.\textsuperscript{21}

**1-[1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene]thiosemicarbazide (3a)**

White solid (11.83 g, 68%); mp 248-250 °C (EtOH/dioxane); IR (KBr): ν 3406-3257 (2NH+NH\textsubscript{2}), 1590 (C=N) cm\textsuperscript{-1}; \textsuperscript{1}H-NMR (DMSO-\textit{d}\textsubscript{6}): δ = 1.76 (s, 3H, pyrrole-CH\textsubscript{3}), 2.36 (s, 3H, CH\textsubscript{3}-C=N-NH), 6.98-7.31 (m, 12H, Ar-H+NH\textsubscript{2}), 10.82 (s, 1H, NH), 11.50 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 348 (M\textsuperscript{+}, 46), 273 (100), 77 (24), 60 (43). *Anal. Calcd for C\textsubscript{20}H\textsubscript{20}N\textsubscript{4}S (348.14): C, 68.93; H, 5.79; N, 16.08; S, 9.20. Found: C, 69.11; H, 5.86; N, 16.23; S, 9.41%.*

**1-[1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene]thiocarbohydrazide (3b)**

Yellow solid (10.70 g, 59%); mp 180-182 °C (EtOH); IR (KBr): ν 3398-3198 (3NH+NH\textsubscript{2}), 1596 (C=N) cm\textsuperscript{-1}; \textsuperscript{1}H-NMR (DMSO-\textit{d}\textsubscript{6}): δ = 1.56 (s, 3H, pyrrole-CH\textsubscript{3}), 2.37 (s, 3H, CH\textsubscript{3}-C=N-NH), 4.21 (br, 2H, NH\textsubscript{2}), 7.07-7.54 (m, 10H, Ar-H), 10.87 (s, 1H, NH), 11.21 (s, 1H, NH), 11.55 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 363 (M\textsuperscript{+}, 40), 273 (100), 77 (53). *Anal. Calcd for C\textsubscript{20}H\textsubscript{21}N\textsubscript{5}S (363.15): C, 66.09; H, 5.82; N, 19.27; S, 8.82. Found: C, 66.18; H, 5.66; N, 19.33; S, 9.01%.*

**Reactions of 3a and 3b with α-halocarbonyl compounds**

**Method A**

To a solution of 3a or 3b (1 mmol) in dioxane (20 mL), containing triethylamine (0.1 g), was added 3-chloro-2,4-pentanedione (4a) or ethyl 2-chloro-3-oxobutanoate (4b) or N1-phenyl-2-chloro-3-oxobutanamide (4c) (1 mmol of each). After complete addition, the reaction mixture was irradiated by MW at 400 Watt in a closed Teflon vessel until all the starting material was consumed (6–10 min as monitored by TLC). The mixture was poured into ice/HCl mixture and the solid that precipitated was filtered off, washed with water, dried and finally crystallized from EtOH to give the respective products 5a-c and 6a-c.

**Method B**
To a solution of 3a or 3b (1 mmol) in dioxane (20 mL), containing chitosan (0.1 g), was added 3-chloro-2,4-pentanedione (4a) or ethyl 2-chloro-3-oxobutanoate (4b) or N1-phenyl-2-chloro-3-oxobutanamide (4c) (1 mmol of each). After complete addition, the reaction mixture was irradiated by MW at 400 Watt in a closed Teflon vessel until all the starting material was consumed (6–10 min as monitored by TLC). The hot solution was filtered off to remove chitosan and the mixture was poured into ice/HCl mixture. The solid that precipitated was filtered off, washed with water, dried and finally crystallized from EtOH to give the respective products 5a-c and 6a-c.

**Method C**

Same procedure in method B using grafted-chitosan (0.1 g) instead of chitosan.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-methylthiazole (5a).

Yellow solid; mp 210-212 °C; IR (KBr): ν 3318, 3295 (2NH), 1702 (C=O), 1606 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.74 (s, 3H, pyrrole-CH₃), 2.37 (s, 3H, CH₃-C=N-NH), 2.44 (s, 3H, COCH₃), 2.58 (s, 3H, thiazole-C₄-CH₃), 7.09-7.13 (m, 10H, Ar-H), 10.32 (s, 1H, NH-N=), 11.45 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ = 8.22 (thiazole-C₄-CH₃), 13.11 (CH₃-C=N-NH), 14.42 (pyrrole-CH₃), 31.54 (COCH₃), 116.21, 122.03, 122.68, 123.45, 125.98, 126.53, 127.75, 128.64, 129.22, 130.45, 132.65, 134.78, 137.57, 149.11, 152.53, 170.11 (Ar-Cs + C=N-NH), 186.22 (COCH₃) ppm; MS m/z (%): 428 (M⁺, 65), 273 (100), 258 (54), 77 (18). Anal. Calcd for C₂₅H₂₄N₄OS (428.17): C, 70.07; H, 5.64; N, 13.07; S, 7.39. Found: C, 70.11; H, 5.58; N, 13.28; S, 7.39%.

Ethyl 2-[2-{1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-methylthiazole-5-carboxylate (5b).

Yellow solid; mp 164-166 °C; IR (KBr): ν 3308, 3295 (2NH), 1708 (C=O), 1596 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.23 (t, 3H, J = 7 Hz, CH₃CH₂), 1.74 (s, 3H, pyrrole-CH₃), 2.39 (s, 3H, CH₃-C=N-NH), 2.61 (s, 3H, thiazole-C₄-CH₃), 4.20 (q, 2H, J = 7 Hz, CH₂CH₃), 7.09-7.37 (m, 10H, Ar-H), 10.34 (s, 1H, NH-N=), 11.47 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ = 8.11 (thiazole-C₄-CH₃), 13.08 (CH₃-C=N-NH), 13.56 (OCH₂-CH₃), 14.40 (pyrrole-CH₃), 58.11 (OCH₂CH₃), 116.28, 121.87, 122.62, 123.40, 125.88, 126.51, 127.54, 128.22, 129.31, 130.05, 132.62, 134.70, 137.61, 149.43, 152.21, 170.09 (Ar-Cs + C=N-NH), 185.92 (C=O) ppm; MS m/z (%): 458 (M⁺, 25), 273 (70), 113 (89), 84 (100). Anal. Calcd for C₂₆H₂₆N₄O₂S (458.18): C, 68.10; H, 5.71; N, 12.22; S, 6.99. Found: C, 68.18; H, 5.59; N, 12.38; S, 7.09%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-methyl-N-phenythiazole-
5-carboxamide (5c).

Yellow solid; mp 230-232 °C; IR (KBr): ν 3324-3212 (3NH), 1660 (C=O), 1595 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.76 (s, 3H, pyrrole-CH₃), 2.37 (s, 3H, CH₃-C=N-NH), 2.63 (s, 3H, thiazole-C4-CH3), 7.06-7.67 (m, 15H, Ar-H), 9.87 (s, 1H, NH), 10.28 (s, 1H, NH-N=), 11.31 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ = 8.82 (thiazole-C4-CH₃), 13.75 (CH₃-C=N-NH), 14.87 (pyrrole-CH₃), 116.21, 120.43, 122.11, 122.76, 123.07, 124.65, 125.62, 126.53, 126.89, 127.73, 128.64, 129.20, 130.41, 131.77, 132.35, 134.65, 137.32, 148.91, 151.83, 170.31 (Ar-Cs + C=N-NH), 176.65 (CONH) ppm; MS m/z (%): 505 (M⁺, 94), 231 (100), 118 (93), 77 (60). Anal. Calcd for C₃₀H₂₇N₅OS (505.19): C, 71.26; H, 5.38; N, 13.85; S, 6.34. Found: C, 71.33; H, 5.19; N, 14.02; S, 6.49%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-3-amino-4-methyl-5-acetyl-2,3-dihydrothiazole (6a).

Yellow solid; mp 224-226 °C; IR (KBr): ν 3427-3257 (NH+NH₂), 1692 (C=O), 1606 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.65 (s, 3H, pyrrole-CH₃), 2.42 (s, 3H, CH₃-C=N-NH), 2.67 (s, 3H, thiazole-C4-CH₃), 3.40 (s, 2H, NH₂), 7.11-7.34 (m, 10H, Ar-H), 11.46 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ 9.11 (thiazole-C4-CH₃), 13.34 (CH₃-C=N-NH), 13.92 (pyrrole-CH₃), 32.86 (COCH₃), 116.20, 121.93, 122.64, 123.03, 125.17, 126.03, 128.05, 128.64, 129.87, 131.45, 132.63, 134.78, 137.57, 149.11, 152.53, 158.11 (Ar-Cs + C=N-NH), 183.20 (COCH₃) ppm; MS m/z (%): 443 (M⁺, 35), 273 (100), 258 (42), 77 (30). Anal. Calcd for C₂₅H₂₅N₅OS (443.18): C, 67.69; H, 5.68; N, 15.79; S, 7.35. Found: C, 67.51; H, 5.42; N, 15.67; S, 7.35%.

Ethyl 2-[2-{1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-3-amino-4-methyl-2,3-dihydrothiazole-5-carboxylate (6b).

Yellow solid; mp 172-174 °C; IR (KBr): ν 3423-3255 (NH+NH₂), 1711 (C=O), 1603 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.20 (t, 3H, J = 7 Hz, CH₃CH₂), 1.74 (s, 3H, pyrrole-CH₃), 2.39 (s, 3H, CH₃-C=N-NH), 2.58 (s, 3H, thiazole-C4-CH₃), 3.51 (s, 2H, NH₂), 4.28 (q, 2H, J = 7 Hz, CH₂CH₃), 7.11-7.37 (m, 10H, Ar-H), 11.51 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ 9.23 (thiazole-C4-CH₃), 13.12 (CH₃-C=N-NH), 14.11 (pyrrole-CH₃), 14.74 (OCH₂-CH₃), 56.11 (OCH₂CH₃), 116.28, 120.82, 121.92, 123.40, 125.43, 126.90, 127.12, 128.82, 129.11, 130.95, 132.12, 134.62, 137.32, 149.41, 152.21, 158.29 (Ar-Cs + C=N-NH), 186.90 (C=O) ppm; MS m/z (%): 473 (M⁺, 23), 273 (45), 113 (50), 77 (100). Anal. Calcd for C₂₆H₂₇N₅O₃S (473.19): C, 65.94; H, 5.75; N, 14.79; S, 6.77. Found: C, 66.08; H, 5.62; N, 14.54; S, 6.62%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-3-amino-4-methyl-N-phenyl-2,3-
dihydrothiazole-5-carboxamide (6c).

Yellow solid; mp 236-238 °C; IR (KBr): ν 3409-3267 (2NH+NH₂), 1660 (C=O), 1600 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.77 (s, 3H, pyrrole-CH₃), 2.37 (s, 3H, CH₂-C=N-NH), 2.63 (s, 3H, thiazole-C₄-CH₃), 3.64 (s, 2H, NH₂), 7.12-7.64 (m, 15H, Ar-H), 9.87 (s, 1H, NH), 11.42 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ = 9.82 (thiazole-C₄-CH₃), 13.15 (CH₃-C=N-NH), 14.17 (pyrrole-CH₃), 116.20, 120.43, 121.71, 122.70, 123.14, 124.62, 125.32, 126.58, 126.82, 127.78, 128.20, 129.61, 130.44, 131.12, 132.88, 134.62, 137.30, 148.76, 151.81, 159.01 (Ar-Cs + C=N-NH), 177.05 (CONH) ppm; MS m/z (%): 520 (M⁺, 52), 231 (55), 118 (53), 77 (100). Anal. Calcd for C₃₀H₂₈N₆OS (520.20): C, 69.21; H, 5.42; N, 16.14; S, 6.16. Found: C, 69.33; H, 5.29; N, 16.02; S, 6.29%.

**Synthesis of 1,3-thiazine derivatives 9a-i**

**Method A**

To a solution of 1-[1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene]thiosemicarbazide (3a) (0.348 g, 1 mmol) in dry dioxane (20 mL), containing 0.1 g of triethylamine, was added acrylonitrile derivatives 7a-i (1 mmol of each). After complete addition, the reaction mixture was irradiated by MW at 400 Watt in a closed Teflon vessel until all the starting material was consumed (6–8 min as monitored by TLC). The mixture was poured into ice/HCl mixture and the precipitate was filtered, washed with MeOH, and crystallized from EtOH to give products 9a-i.

**Method B**

To a solution of 1-[1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene]thiosemicarbazide (3a) (0.348 g, 1 mmol) in dry dioxane (20 mL), containing 0.1 g of chitosan, was added acrylonitrile derivatives 7a-i (1 mmol of each). After complete addition, the reaction mixture was irradiated by MW at 400 Watt in a closed Teflon vessel until all the starting material was consumed (6–8 minutes as monitored by TLC). The hot solution was filtered off to remove chitosan then the mixture was poured into ice/HCl mixture and the precipitate was filtered, washed with MeOH, and recrystallized from EtOH to give products 9a-i.

**Method C**

Same procedure in method B using grafted-chitosan (0.1 g) instead of chitosan.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-phenyl-6H-1,3-thiazine-5-carbonitrile (9a).

Yellow solid; mp 188-190 °C; IR (KBr): ν 3423-3196 (NH₂+2NH), 2185 (C≡N), 1603 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.52 (s, 3H, pyrrole-CH₃), 2.41 (s, 3H, CH₂-C=N-NH), 3.65 (s, 1H, thiazine-H),
4.55 (s, br, 2H, NH₂), 7.07-7.53 (m, 15H, Ar-H), 10.32 (s, 1H, NH), 11.45 (s, 1H, pyrrole-NH) ppm; ¹³C-NMR (DMSO-d₆): δ = 13.15 (pyrrole-CH₃), 16.17 (CH₃-C=N-NH), 51.87 (C₆-thiazine), 110.31 (CN), 116.19, 118.13, 120.43, 120.92, 122.15, 123.91, 124.37, 125.34, 126.18, 126.93, 128.12, 128.88, 129.01, 131.55, 134.22, 139.11, 148.32, 158.44, 161.76, 162.15 (Ar-Cs + C=N-NH) ppm; MS m/z (%): 502 (M⁺, 45), 371 (72), 80 (100), 77 (47). Anal. Calcd for C₃₀H₂₆N₆S (502.19): C, 71.69; H, 5.21; N, 16.72; S, 6.38. Found: C, 71.51; H, 5.38; N, 16.61; S, 6.19%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(4-chlorophenyl)-6H-1,3-thiazine-5-carbonitrile (9b).

Yellow solid; mp 234-236 °C; IR (KBr): ν 3419-3192 (NH₂ +2NH), 2199 (C≡N), 1603 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.61 (s, 3H, pyrrole-CH₃), 2.42 (s, 3H, CH₃-C=N-NH), 3.78 (s, 1H, thiazine-H), 4.68 (s, br, 2H, NH₂), 7.09-7.58 (m, 14H, Ar-H), 10.35 (s, 1H, NH), 11.51 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 538 (M⁺+2, 32), 536 (M⁺, 88), 386 (100), 110 (71), 77 (40). Anal. Calcd for C₃₀H₂₅ClN₆S (536.15): C, 67.09; H, 4.69; N, 15.65; S, 5.97. Found: C, 66.91; H, 4.48; N, 15.72; S, 6.09%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(4-methylphenyl)-6H-1,3-thiazine-5-carbonitrile (9c).

Yellow solid; mp 210-212 °C; IR (KBr): ν 3407-3153 (NH₂+2NH), 2207 (C≡N), 1590 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.77 (s, 3H, pyrrole-CH₃), 2.24 (s, 3H, Ar-CH₃), 2.39 (s, 3H, CH₃-C=N-NH), 3.64 (s, 1H, thiazine-H), 4.61 (s, br, 2H, NH₂), 7.07-8.73 (m, 14H, Ar-H), 10.00 (s, 1H, NH), 11.50 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 516 (M⁺, 44), 514 (M⁺, 88), 386 (100), 110 (71), 77 (40). Anal. Calcd for C₃₁H₂₈N₆S (516.21): C, 72.07; H, 5.46; N, 16.27; S, 6.21. Found: C, 71.88; H, 5.35; N, 16.40; S, 6.08%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(4-methoxyphenyl)-6H-1,3-thiazine-5-carbonitrile (9d).

Yellow solid; mp 182-184 °C; IR (KBr): ν 3405-3151 (NH₂+2NH), 2220 (C≡N), 1588 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.76 (s, 3H, pyrrole-CH₃), 2.41 (s, 3H, Ar-CH₃), 3.78 (s, 1H, thiazine-H), 3.91 (s, 3H, OCH₃), 4.58 (s, br, 2H, NH₂), 7.06-8.40 (m, 14H, Ar-H), 10.00 (s, 1H, NH), 11.52 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 532 (M⁺, 44), 386 (60), 108 (45), 77 (100). Anal. Calcd for C₃₁H₂₈N₆O₃S (532.20): C, 69.90; H, 5.30; N, 15.78; S, 6.02. Found: C, 69.81; H, 5.45; N, 15.70; S, 6.11%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(benzo[b][1,3-dioxol-3-yl)-6H-1,3-thiazine-5-carbonitrile (9e).

Yellow solid; mp 154-156 °C; IR (KBr): ν 3412-3185 (NH₂+2NH), 2197 (C≡N), 1606 (C=N) cm⁻¹;
1H-NMR (DMSO-\(d_6\)): \(\delta = 1.51\) (s, 3H, pyrrole-CH\(_3\)), 2.40 (s, 3H, CH\(_3\)-C=N-NH), 3.62 (s, 1H, thiazine-H), 4.61 (s, br, 2H, NH\(_2\)), 5.97 (s, 2H, CH\(_2\)), 7.05-7.58 (m, 13H, Ar-H), 10.34 (s, 1H, NH), 11.48 (s, 1H, pyrrole-NH) ppm; MS \(m/z\) (%): 546 (M\(^+\), 26), 380 (35), 121 (60), 77 (100). *Anal.* Calcd for C\(_{31}\)H\(_{26}\)N\(_6\)O\(_2\)S (546.18): C, 68.11; H, 4.79; N, 15.37; S, 6.09%. 

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-styryl-6H-1,3-thiazine-5-carbonitrile (9f).

Yellow solid; mp 226-228 °C; IR (KBr): \(\nu\) 3428-3198 (NH\(_2\)+2NH), 2219 (C≡N), 1595 (C=N) cm\(^{-1}\);

1H-NMR (DMSO-\(d_6\)): \(\delta = 1.75\) (s, 3H, pyrrole-CH\(_3\)), 2.41 (s, 3H, CH\(_3\)-C=N-NH), 3.80 (s, 1H, thiazine-H), 4.59 (s, br, 2H, NH\(_2\)), 6.24 (d, 1H, \(J = 13\) Hz, CH=), 6.67 (d, 1H, \(J = 13\) Hz, CH=), 7.07-7.95 (m, 15H, Ar-H), 10.32 (s, 1H, NH), 11.51 (s, 1H, pyrrole-NH) ppm; MS \(m/z\) (%): 528 (M\(^+\), 45), 371 (72), 77 (100). *Anal.* Calcd for C\(_{32}\)H\(_{28}\)N\(_6\)S (528.21): C, 72.70; H, 5.34; N, 15.90; S, 6.07. Found: C, 72.53; H, 5.18; N, 16.01; S, 6.22%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(furan-2-yl)-6H-1,3-thiazine-5-carbonitrile (9g).

Yellow solid; mp 258-260 °C; IR (KBr): \(\nu\) 3418-3180 (NH\(_2\)+2NH), 2210 (C≡N), 1596 (C=N) cm\(^{-1}\);

1H-NMR (DMSO-\(d_6\)): \(\delta = 1.75\) (s, 3H, pyrrole-CH\(_3\)), 2.39 (s, 3H, CH\(_3\)-C=N-NH), 3.68 (s, 1H, thiazine-H), 5.35 (s, br, 2H, NH\(_2\)), 7.01-7.38 (m, 13H, Ar-H), 10.34 (s, 1H, NH), 11.48 (s, 1H, pyrrole-NH) ppm; MS \(m/z\) (%): 492 (M\(^+\), 26), 380 (35), 77 (100). *Anal.* Calcd for C\(_{28}\)H\(_{24}\)N\(_6\)OS (492.17): C, 68.12; H, 4.75; N, 17.11; S, 6.39%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-N,6-diphenyl-6H-1,3-thiazine-5-carboxamide (9h).

Yellow solid; mp 244-246 °C; IR (KBr): \(\nu\) 3406-3151 (NH\(_2\)+3NH), 1643 (CO), 1595 (C=N) cm\(^{-1}\);

1H-NMR (DMSO-\(d_6\)): \(\delta = 1.76\) (s, 3H, pyrrole-CH\(_3\)), 2.45 (s, 3H, CH\(_3\)-C=N-NH), 3.72 (s, 1H, thiazine-H), 4.68 (s, br, 2H, NH\(_2\)), 7.06-8.28 (m, 20H, Ar-H), 10.41 (s, 1H, NH), 11.51 (s, 1H, pyrrole-NH), 11.63 (s, 1H, NH) ppm; MS \(m/z\) (%): 596 (M\(^+\), 45), 386 (40), 77 (100). *Anal.* Calcd for C\(_{36}\)H\(_{32}\)N\(_6\)OS (596.24): C, 72.46; H, 5.41; N, 14.08; S, 5.37. Found: C, 72.61; H, 5.28; N, 14.12; S, 5.18%.

2-[2-{1-(2-Methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-amino-6-(4-chlorophenyl)-6H-1,3-thiazine-5-carbothioamide (9i).

Yellow solid; mp 216-218 °C; IR (KBr): \(\nu\) 3406-3152 (2NH\(_2\)+2NH), 1591 (C=N), 1300 (C=S) cm\(^{-1}\);

1H-NMR (DMSO-\(d_6\)): \(\delta = 1.76\) (s, 3H, pyrrole-CH\(_3\)), 2.49 (s, 3H, CH\(_3\)-C=N-NH), 3.66 (s, 1H, thiazine-H),
4.56-5.32 (s, br, 4H, 2NH₂), 7.06-8.22 (m, 14H, Ar-H), 10.00 (s, 1H, NH), 11.52 (s, 1H, pyrrole-NH) ppm; MS m/z (%): 572 (M⁺+2, 14), 570 (M⁺, 38), 386 (20), 110 (71), 77 (100). Anal. Calcd for C₃₀H₂₇ClN₆S₂ (536.15): C, 63.09; H, 4.76; N, 14.71; S, 11.23. Found: C, 62.91; H, 4.58; N, 14.52; S, 11.18%.

Synthesis of methyl 2-[2-{1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene}hydrazono]-4-oxothiazolidin-5-ylidene ethanoate (12).

To a solution of 1-[1-(2-methyl-4,5-diphenyl-1H-pyrrol-3-yl)ethylidene]thiosemicarbazide (3a) (0.348 g, 1 mmol) in dry dioxane (20 mL), contains 0.1 g of grafted chitosan, was added dimethyl acetylenedicarboxylate (0.142 g, 1 mmol). After complete addition reaction mixture was irradiated by MW at 400 Watt in a closed Teflon vessel until all the starting material was consumed (4 minutes as monitored by TLC). The hot solution was filtered to remove grafted chitosan and the precipitate was filtered, washed with MeOH, and recrystallized from EtOH to give product 12. Canary yellow solid (0.34 g, 74%); mp 300-302 °C; IR (KBr): v 3366, 3247 (2NH), 1703, 1684 (2C=O), 1605 (C=N) cm⁻¹; ¹H-NMR (DMSO-d₆): δ = 1.74 (s, 3H, pyrrole-CH₃), 2.35 (s, 3H, CH₃-C=N-NH), 3.78 (s, 3H, COOCH₃), 6.61 (s, 1H, C=CH), 7.05-7.33 (m, 10H, Ar-H), 11.49 (s, 1H, pyrrole-NH), 12.59 (s, 1H, NH) ppm; MS m/z (%): 458 (M⁺, 63), 284 (87), 135 (100), 77 (30). Anal. Calcd for C₂₅H₂₂N₄O₃S (458.14): C, 65.48; H, 4.84; N, 12.22; S, 6.99. Found: C, 65.21; H, 4.66; N, 12.12; S, 7.09%.

Evaluation of the antitumor activity using Viability assay:

Human colon carcinoma (HCT-116) and human hepatocellular carcinoma (HEPG2) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg/mL gentamycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary. The cells were grown as monolayers in growth RPMI-1640. The monolayers of 10⁴ cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37 °C in a humidified incubator with 5% CO₂. 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 tested sample in fresh maintenance medium and incubated at 37 °C. A control of untreated cells was made in the absence of tested sample. Positive controls containing Doxorubicin drug was also tested as reference drug for comparison. 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 590 nm using microplate reader (SunRise, 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 microplate reader as previously mentioned before and the percentage of viability was calculated as \[1-(\text{OD}_t/\text{OD}_c)\times100\%\] where \(\text{OD}_t\) is the mean optical density of wells treated with the tested sample and \(\text{OD}_c\) is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC\textsubscript{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

**REFERENCES**