CHAPTER-3

Synthesis of oxadiazolothiones, triazolothiones and triazolothiadiazines
3.1. Theoretical

Oxadiazoles are five membered heterocycles possessing one oxygen atom and two nitrogen atoms. Oxadiazoles exist in different isomeric forms such as 1,2,4- (78), 1,2,5- (79), 1,2,3- (80) and 1,3,4-oxadiazoles (81). 1,3,4-Oxadiazole is derived from furan by substitution of two methylene groups (=CH) with two (-N=) of pyridine type nitrogens. Out of these isomers, 1,3,4- and 1,2,4- oxadiazoles are more widely studied due to their significant chemical and biological importance.

\[
\begin{array}{ccc}
\text{(78)} & \text{(79)} & \text{(80)} \\
\text{(81)} & & \\
\end{array}
\]

The presence of three nitrogen atoms in five membered ring compounds describes an important class of heterocycles, the triazoles. The two isomers of triazole are 1,2,3- (82) and 1,2,4-triazole (83).

\[
\begin{array}{cc}
\text{(82)} & \text{(83)} \\
\end{array}
\]

1,2,4-Triazole is very stable nucleus shown by different chemical reactions and it can be considered as aromatic, stabilized by resonance as shown below:

\[
\begin{array}{cc}
\text{HN} & \text{HN} \\
\text{HN} & \text{HN} \\
\text{HN} & \text{HN} \\
\end{array}
\]
Further, 4-amino-1,2,4-triazol-3-thiones were found to be useful synthon or tools for the synthesis of triazolothiadiazines\textsuperscript{1}. For the synthesis of condensed heterocyclic structures, the mercapto and amino groups are good nucleophilic centres.

Banday and Rauf\textsuperscript{2} reported the synthesis of 1,2,4-triazole derivatives (86) by the intermolecular cyclization of thiosemicarbazides (85) in alkaline medium. These thiosemicarbazides were prepared by the reaction of fatty acid hydrazides (22-25) and phenylisothiocyanate (84) in dry benzene.

\[\text{RCONHNH}_2 + \text{Ph-N=C=S} \xrightarrow{\text{dry benzene}, \text{reflux}} \text{RCONHNHCNSNHPh} \]

\[(22-25) \quad (84) \quad (85)\]

\[\text{R: CH}_2=\text{CH} (\text{CH}_2)_8, \]
\[\text{CH}_3(\text{CH}_2)_2 \text{CH}=\text{CH} (\text{CH}_2)_{7}, \]
\[\text{CH}_3(\text{CH}_2)_2 \text{CHOHCH}_2 \text{CH}=\text{CH} (\text{CH}_2)_{7}, \]
\[\text{CH}_3(\text{CH}_2)_4 \text{CH}=\text{CH} (\text{CH}_2)_2 \text{CHOH}(\text{CH}_2)_{7} \]

Padmavathi \textit{et al.}\textsuperscript{3} synthesized 1,3,4-oxadiazole derivatives (90, 91) by the reaction of acid hydrazides (87, 88) and carboxylic acids (89) in presence of phosphorus oxychloride. Further, these oxadiazole derivatives on reaction with excess hydrazine produce 3-(arylsulfonylmethyl)-5-phenyl-4H-1,2,4-triazol-4-amines (92) and 3-(arylmethanesulfonylmethyl)-5-phenyl-4H-1,2,4-triazol-4-amines (93).
Banday et al.\(^4\) reported the synthesis of 2-amino-1,3,4-oxadiazole (94) and 2-alkenyl-5-phenyl-1,3,4-oxadiazole (95) using cyanogens bromide and benzoyl chloride or benzoic acid as reagents, respectively.

Lotfi et al.\(^5\) reported the synthesis of 2,5-disubstituted-1,3,4-oxadiazoles (96) by electrocyclization of semicarbazone (97).
Deohate\(^6\) outlined the synthesis of 6,7-disubstituted-phenyl-3-pyridin-4-yl-5H-[1,2,4]-triazolo[3,4-b]-[1,3,4]-thiadiazines (100) by reacting 4-amino-3-mercapto-5-pyridin-4-yl-4H-[1,2,4]-triazole (98) with substituted benzoins (99) in presence of potassium hydroxide.

Singh and Singh\(^7\) described the synthesis of 6,7-diphenyl-3,5-disubstituted-s-triazolo[3,4-b][1,3,4]-thiadiazine (103) by the reaction of compound (102) with hydroxyl ketone in the presence of potassium hydroxide using triazole (101) as starting material.
Nitrogen, oxygen and sulfur containing compounds are the most common heterocycles which serve as the core component of a large number of biochemical materials which are essential to life such as nucleic acids. Oxadiazoles are used as support on which pharmacophores are placed to provide potent and selective medicines. During the past few years, considerable evidence has been accumulated that demonstrates the efficacy of 1,3,4-oxadiazoles including insecticidal, analgesic, diuretic, CNS depressant, antiviral, herbicidal, antihypertensive, pesticidal activities and these are cited in literature. Oxadiazoles also possess antitubercular, antimalarial, antileishmanial and anticancer activities.

The 1,2,4-triazole derivatives possess a broad spectrum of activity including antimalarial, anticancer and antitubercular and also having a wide range of therapeutic properties like analgesic, insecticidal, hypoglycemic, antiparasitic, herbicidal and plant growth activities. The 1,2,4-triazole nucleus is extensively used in medicines. The substituted 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivatives have been reported to possess antimicrobial activity mainly due to N-C-S linkage in the skeleton of triazolothiadiazine and also possess anticancer activity. These
biheterocyclic triazolothiadiazine derivatives also possess broad spectrum of pharmacological activities\textsuperscript{26,27}.

Zibotentan (104) and raltegravir (105) are two examples of compounds that are used in clinical medicine which contains 1,3,4-oxadiazole unit\textsuperscript{28}. Also, triazole moiety is present in many antifungal drugs such as fluconazole (106) and voriconazole (107).
3.2. Synthesis and Anticancer Activity of Long Chain Substituted 1,3,4-Oxadiazol-2-thione, 1,2,4-Triazol-3-thione and 1,2,4-Triazolo[3,4-b]-1,3,4-thiadiazine Derivatives

The literature survey reveals that there are many examples of triazole fused with pyridines, pyridazines, pyrimidines, pyrazines and triazines but triazolothiadiazines are not very common moieties. Earlier works showed that these heterocycles were synthesized from different substituted carboxylic acids and hydrazides (other than long chain alkenyl/hydroxyalkenyl hydrazides). Literature survey also reveals that minor change in the structure of 1,3,4-oxadiazoles, 1,2,4-triazoles and 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines can lead to quantitative and qualitative changes in biological activity. Recently, stearic acid (a saturated fatty acid) analogs having 1,3,4-oxadiazole, 1,2,4-triazole and 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole were reported as antidepressant and antimicrobial agents. Also, the usage of fatty acids shows an increasing trend in the treatment of neuropsychological disorders such as depression and schizophrenia. Despite this, some of the fatty acids have been found to play a regulatory role in tumor growth progression and were reported as effective anticancer agents. Keeping in view the significance, the aforementioned facts of long chain alkenyl/hydroxyalkenyl carboxylic acids as potential pharmacophores and in continuation of earlier research work in our laboratory on the synthesis of novel series of biologically active heterocyclic derivatives of fatty acids, the synthesis of three different novel series of biologically important heterocyclic fatty acid analogs described in this chapter.

"Research paper entitled ‘Synthesis and anticancer activity of long chain substituted 1,3,4-oxadiazol-2-thione, 1,2,4-triazol-3-thione and 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivatives’ is in press. (Aiman Almad, Varshney, H., Rauf, A., Sherwani, A., Owais, M., 2014. Arab. J. Chem., http://dx.doi.org/10.1016/j.arabjc.2014.01.015)"
After synthesis, all the newly synthesized compounds were characterized by different spectral techniques and further evaluated for in vitro anticancer activity against three different human cancer cell lines and peripheral blood mononuclear cells (PBMCs), normal human cells. The salient features of the procedure described in this chapter are taking short reaction time, not require elevated temperatures, the use of cheap reagents and easily available starting materials.

3.3. Results and discussion

3.3.1. Chemistry

Earlier in our laboratory it has been reported\(^4\) that some other 1,3,4-oxadiazoles substituted with fatty acid chain possess antibacterial activity. Due to the biological importance of heterocyclic fatty acid analogs, synthesis of target compounds (108-119) and evaluation of anticancer activity was reported in this chapter. All reactions are outlined in Scheme 4 and 5. The physicochemical parameters of all the newly synthesized compounds are tabulated in Table 7. Three novel series of oxadiazolthiones, (108-111); triazolthiones, (112-115) and triazolothiadizines, (116-119) were synthesized from the corresponding unsaturated/hydroxy-unsaturated fatty acid hydrazides, (22-25). These fatty acid hydrazides used as starting materials and were synthesized from the corresponding fatty acids\(^3\). The cyclization reaction of carbon disulfide with fatty acid hydrazides gave a novel series of 5-long chain alkenyl/hydroxyalkenyl-1,3,4-oxadiazol-2-thiones, (108-111). 4-Amino-5-long chain alkenyl/hydroxyalkenyl-1,2,4-triazol-3-thiones, (112-115) were synthesized by the reaction of carbon disulfide and fatty acid hydrazides on treatment with hydrazine hydrate. Furthermore, biheterocyclic derivatives of fatty acids i.e. 4-amino-5-long chain alkenyl/hydroxyalkenyl-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines, (116-119) were synthesized from corresponding compounds (112-115) and phenacyl bromide by ring closure reaction.
Scheme 4: Synthesis of 5-substituted-1,3-4-oxadiazol-2-thiones and 4-amino-5-substituted-1,2,4-triazol-3-thiones
**Scheme 5: Synthesis of 3-substituted-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines**

**Compound Codes**

- **112, 116**

- **113, 117**

- **114, 118**

- **115, 119**

R

---

**PhCOCH₂Br, EtOH, Reflux**
**Oxadiazolthiones, triazolthiones and triazolothiadiazines**

Table 7: Physico-chemical properties of all the newly synthesized compounds, (108-119)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound Code</th>
<th>R</th>
<th>Molecular Formula</th>
<th>Physical State</th>
<th>M.P. (°C)</th>
<th>% Yield</th>
<th>Molecular Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>108</td>
<td><img src="image1.png" alt="Image" /></td>
<td>C_{12}H_{20}N_{2}OS</td>
<td>White powder</td>
<td>86.88</td>
<td>90</td>
<td>240.157</td>
</tr>
<tr>
<td>2</td>
<td>112</td>
<td><img src="image2.png" alt="Image" /></td>
<td>C_{12}H_{20}N_{2}S</td>
<td>White powder</td>
<td>84.66</td>
<td>90</td>
<td>254.388</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td><img src="image3.png" alt="Image" /></td>
<td>C_{20}H_{20}N_{4}S</td>
<td>Brown sticky liquid</td>
<td>-</td>
<td>85</td>
<td>354.470</td>
</tr>
<tr>
<td>4</td>
<td>109</td>
<td><img src="image4.png" alt="Image" /></td>
<td>C_{19}H_{22}N_{2}OS</td>
<td>White powder</td>
<td>87.89</td>
<td>85</td>
<td>338.492</td>
</tr>
<tr>
<td>5</td>
<td>113</td>
<td><img src="image5.png" alt="Image" /></td>
<td>C_{19}H_{34}N_{4}OS</td>
<td>White powder</td>
<td>76.77</td>
<td>85</td>
<td>352.519</td>
</tr>
<tr>
<td>6</td>
<td>117</td>
<td><img src="image6.png" alt="Image" /></td>
<td>C_{27}H_{28}N_{4}S</td>
<td>Brown sticky liquid</td>
<td>-</td>
<td>62</td>
<td>452.631</td>
</tr>
<tr>
<td>7</td>
<td>110</td>
<td><img src="image7.png" alt="Image" /></td>
<td>C_{19}H_{32}O_{2}S</td>
<td>Yellow solid</td>
<td>76-78</td>
<td>80</td>
<td>354.431</td>
</tr>
<tr>
<td>8</td>
<td>114</td>
<td><img src="image8.png" alt="Image" /></td>
<td>C_{19}H_{36}N_{4}OS</td>
<td>Yellow solid</td>
<td>62-64</td>
<td>78</td>
<td>368.518</td>
</tr>
<tr>
<td>9</td>
<td>118</td>
<td><img src="image9.png" alt="Image" /></td>
<td>C_{27}H_{40}N_{4}OS</td>
<td>Brown sticky liquid</td>
<td>-</td>
<td>59</td>
<td>466.630</td>
</tr>
<tr>
<td>10</td>
<td>111</td>
<td><img src="image10.png" alt="Image" /></td>
<td>C_{19}H_{46}N_{2}O_{2}S</td>
<td>Yellow solid</td>
<td>75-76</td>
<td>84</td>
<td>354.431</td>
</tr>
<tr>
<td>11</td>
<td>115</td>
<td><img src="image11.png" alt="Image" /></td>
<td>C_{19}H_{38}N_{4}OS</td>
<td>Yellow solid</td>
<td>64-66</td>
<td>89</td>
<td>368.518</td>
</tr>
<tr>
<td>12</td>
<td>119</td>
<td><img src="image12.png" alt="Image" /></td>
<td>C_{27}H_{18}N_{4}OS</td>
<td>Brown sticky liquid</td>
<td>-</td>
<td>68</td>
<td>468.633</td>
</tr>
</tbody>
</table>

%Y: % Yield

M.P.: Melting point.

The 1,3,4-oxadiazole-2-thiones and 1,2,4-triazol-3-thiones may exist in thiol-thione tautomeric forms, but in solid state thione form dominates. Such observations are also [63]
reported in literature. Structure of compound (109) appeared in literature also, but without spectral data. All reactions were monitored by using TLC time by time. Products were purified by column chromatography. The structure of all the newly synthesized compounds was determined on the basis of their IR, $^1$H NMR, $^{13}$C NMR and mass spectral data. Characteristic $[M+Na]^{+}$ ion peaks were observed for all the compounds under study. The detailed spectral description for compounds (108, 112 and 116) is discussed below.

IR spectrum of compound 5-(dec-9'-enyl)-(3H)-1,3,4-oxadiazol-2-thione, (108) revealed characteristic bands at 3215 cm$^{-1}$ for N-H stretching, 2921 cm$^{-1}$ for C-H stretching and the detection of C=N stretching band at 1613 cm$^{-1}$, C=S stretching band at 1165 cm$^{-1}$ and C-O-C absorption band at 1054 cm$^{-1}$ for evidence of ring closure of 1,3,4-oxadiazol-2-thione ring. No peak was observed around 2600-2550 cm$^{-1}$ for thiol group, further confirmed the structure of compound (108). In the $^1$H NMR spectrum of compound (108), characteristic peak was observed for N-H proton at $\delta$ 10.30 as singlet, in addition to peaks of fatty acid chains. The $^{13}$C NMR characteristic peaks for compound (108) were observed at $\delta$ 166.8, 165.4, 123.0 and 122.3. Further evidence for the formation of (108) was obtained by recording the mass spectrum which showed the $[M+Na]^{+}$ ion peak at $m/z$ 262.880. Similarly, the structures of compounds (109-111) were confirmed from their spectral data given in experimental section (3.4.1).

The structure of compound 4-amino-5-(dec-9'-enyl)-1,2,4-triazol-3-thione, (112) was confirmed by the appearance of absorption bands at 3228 cm$^{-1}$ for N-H stretching, 2924 cm$^{-1}$ for C-H stretching, 1599 cm$^{-1}$ for C=N stretching and for C=S stretch, the absorption band appeared at 1186 cm$^{-1}$. No peak was observed around 2600-2550 cm$^{-1}$ for thiol group, further confirmed the thione structure of compound (112). The $^1$H NMR characteristic peaks were observed at $\delta$ 11.60 as singlet for N-H proton, $\delta$ 4.57 as broad singlet for NH$_2$ protons. The $^{13}$C NMR characteristic peaks for compound (112) were observed at $\delta$ 167.0, 160.5, 139.1 and 121.2. In addition, evidence for the formation of (112) was obtained by recording the mass spectrum which showed the $[M+Na]^{+}$ ion peak at $m/z$ 276.830. Similarly, the structures of compounds (113-115) were confirmed from their spectral data given in experimental section (3.4.1).
The structure of compound 3-(dec-9'-enyl)-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (116) was confirmed by the appearance of absorption bands at 2923 cm\(^{-1}\) for C-H stretching, 1604 cm\(^{-1}\) for C=\(\text{N}\) stretching, 1125 cm\(^{-1}\) for C-N stretching, 652 cm\(^{-1}\) for C-S-C. The disappearance of absorption peaks for N-H and C=S stretching was further confirmed the structure of compound (116). The \(^1\)H NMR characteristic peaks were observed at \(\delta\) 7.55-7.39 as multiplet for five aromatic protons, \(\delta\) 4.12 as singlet for two ring protons. The disappearance of \(^1\)H NMR peak for N-H proton further confirmed the structure of compound (116). The \(^13\)C NMR characteristic peaks for compound (116) were observed at \(\delta\) 168.1, 165.4, 160.8, 134.8, 132.9, 132.5, 131.1, 130.5, 124.8, 122.5, 115.4 and 36.2. Confirmation for the formation of (116) was also obtained by recording the mass spectrum which showed the [M+Na]\(^+\) ion peak at \(m/z\) 378.041. Similarly, the structures of compounds (117-119) were confirmed from their spectral data given in experimental section (3.4.1).

3.3.2. Biology

3.3.2.1. In vitro cytotoxicity evaluation

*In vitro* cytotoxicity of all the newly synthesized compounds (108-119) was measured by MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay against a panel of three different human cancer cell lines namely; human hepatocellular carcinoma (Hep3 B), human breast adenocarcinoma (MCF 7) and human cervical carcinoma (HeLa). These cell lines are procured from Cell Repository—National Centre for Cell Science, Pune (India). Normal human cells (PBMCs) were also used for the determination of cytotoxicity of synthesized compounds. The MTT assay is a colorimetric assay for measuring the cellular growth that reduces the tetrazolium yellow dye MTT, to its insoluble formazan (purple colour) by mitochondrial dehydrogenases of living cells. MTT is used to determine the cytotoxicity of potential drugs and other toxic compounds. The insoluble purple formazan product is dissolved into a coloured solution by the addition of a suitable solvent. At certain wavelength, the absorbance of this coloured solution can be measured. The potency of the drug in causing cell death can be concluded through the production of dose-response curves when the purple formazan produced by untreated control cells. Curves of dose-
Oxadiazolthiones, triazolthiones and triazolothiadiazines

dependent effects of (108-119) on cell viability of different human cancer cell lines (Hep3 B, MCF 7, HeLa) and normal human cells (PBMCs) are displayed in Figure 2. Doxorubicin and 5-fluorouracil were used as standard drugs. Experiment was performed in a triplicate. For each of the tested drug IC50 was calculated and the results are summarized in Table 8. Experiments revealed that there was substantial increase in cytotoxicity in cancer cell lines with increasing exposure to compound concentration i.e. showing low IC50 values and the in-house synthesized compounds were not showing marked effects on normal human cells (PBMCs) i.e. showing high IC50 values. None of the synthesized compound showed cytotoxicity to normal human cells (PBMCs). Present study showed that among the three human cancer cell lines tested, Hep3 B cells were found to be sensitive to all the tested compounds while HeLa and MCF 7 cells were found to be sensitive to some selected compounds. The obtained results were revealed that compound (108, 110, 111, 112, 114, 115, 116, 117, 118 and 119) showed remarkable inhibitory activities against different human cancer cell lines and were also comparable to the standard drugs.
Oxadiazolothiones, triazolothiones and triazolothiadiazines

**Figure: Cell viability assay for compounds 109, 110, and 111.**

- **Compound 109**
  - Hep3B
  - MCF 7
  - HeLa
  - PBMC

- **Compound 110**
  - Hep3B
  - MCF 7
  - HeLa
  - PBMC

- **Compound 111**
  - Hep3B
  - MCF 7
  - HeLa
  - PBMC

Concentration in µM

[67]
Oxadiazolthiones, triazolthiones and triazolothiadiazines

Compound 112

- Hep3B
- MCF 7
- HeLa
- PBMC

Concentration in µM

Compound 113

- Hep3B
- MCF 7
- HeLa
- PBMC

Concentration in µM

Compound 114

- Hep3B
- MCF 7
- HeLa
- PBMC

Concentration in µM
Oxadiazothiones, triazothiones and triazolothiadiazines

![Graphs showing cell viability against concentration for different compounds and cell lines](image_url)

[69]
Figure 2: Dose-response effect of all the synthesized compounds (108-119) on cell-viability of Hep3 B, MCF 7, HeLa and PBMCs. Data expressed here is mean±standard deviation of three independent experiments.
Oxadiazothiones, triazolothiones and triazolothiadiazines

Table 8: Anticancer data (IC₅₀ values in µM) of all the synthesized compounds and standard drugs against three different human cancer cell lines and normal human cells

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound codes</th>
<th>Hep3 B</th>
<th>MCF 7</th>
<th>HeLa</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>108</td>
<td>08.94±2.5</td>
<td>13.60±2.5</td>
<td>11.90±1.3</td>
<td>35.32±3.1</td>
</tr>
<tr>
<td>2</td>
<td>109</td>
<td>16.42±1.5</td>
<td>19.00±2.5</td>
<td>17.60±2.3</td>
<td>39.12±1.7</td>
</tr>
<tr>
<td>3</td>
<td>110</td>
<td>09.00±1.6</td>
<td>15.90±1.6</td>
<td>12.90±1.3</td>
<td>45.42±4.1</td>
</tr>
<tr>
<td>4</td>
<td>111</td>
<td>10.10±2.6</td>
<td>16.83±2.6</td>
<td>13.30±3.3</td>
<td>41.38±2.5</td>
</tr>
<tr>
<td>5</td>
<td>112</td>
<td>09.20±0.8</td>
<td>14.00±0.6</td>
<td>11.10±2.1</td>
<td>39.12±1.6</td>
</tr>
<tr>
<td>6</td>
<td>113</td>
<td>16.60±1.2</td>
<td>19.90±0.7</td>
<td>18.80±1.3</td>
<td>43.83±1.9</td>
</tr>
<tr>
<td>7</td>
<td>114</td>
<td>09.48±1.4</td>
<td>13.20±0.5</td>
<td>16.20±2.4</td>
<td>33.57±2.3</td>
</tr>
<tr>
<td>8</td>
<td>115</td>
<td>09.96±2.8</td>
<td>14.50±1.6</td>
<td>15.82±3.1</td>
<td>35.25±1.9</td>
</tr>
<tr>
<td>9</td>
<td>116</td>
<td>07.40±2.2</td>
<td>10.30±2.7</td>
<td>08.01±1.3</td>
<td>32.78±2.9</td>
</tr>
<tr>
<td>10</td>
<td>117</td>
<td>09.49±1.5</td>
<td>11.27±2.4</td>
<td>10.03±1.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>11</td>
<td>118</td>
<td>06.50±2.4</td>
<td>08.59±1.5</td>
<td>08.83±1.4</td>
<td>&gt;50</td>
</tr>
<tr>
<td>12</td>
<td>119</td>
<td>07.36±1.6</td>
<td>08.80±2.1</td>
<td>06.00±3.1</td>
<td>39.12±3.1</td>
</tr>
<tr>
<td>Doxo³</td>
<td>02.35±1.2</td>
<td>03.12±1.7</td>
<td>03.56±2.7</td>
<td>09.23±2.6</td>
<td></td>
</tr>
<tr>
<td>5-Fu³</td>
<td>03.54±2.1</td>
<td>04.12±2.3</td>
<td>02.78±2.6</td>
<td>08.91±1.9</td>
<td></td>
</tr>
</tbody>
</table>

Standard drugs used for reference: a: Doxorubicin, b: 5-Fluorouracil

3.3.3. Structure-activity relationship (SAR) studies

On the basis of structure-activity relationship, it could be concluded that 1,2,4-triazole fused with 1,3,4-thiadiazine ring were found to have better antitumor activity than those of 1,2,4-triazoles and 1,3,4-oxadiazoles. The structural activity study shows that anticancer activity may also dependent on the nature of alkenyl/hydroxyalkenyl fatty acid chain. From IC₅₀ values a number of correlations can be made. It is apparent from the IC₅₀ values that, all the tested compounds showed moderate to good cytotoxicity against different human cancer cell lines. For C₁₀ terminal alkenyl fatty acid chain residue which is substituted at 5-position of 1,3,4-oxadiazol-2-thione (compound, 108) and at 5-position of 1,2,4-triazol-3-thione (compound, 112) lead to remarkable increase in potency against human hepatocellular carcinoma cells (IC₅₀ [71])
value of 0.98±2.50 µM and 0.92±1.5 µM, respectively). Incorporation of 1,3,4-thiadiazine ring with 1,2,4-triazole ring having C10 terminal alkenyl fatty acid chain residue at 3-position of 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazine (compound, 116) lead to increase in cytotoxicity against all the three human cancer cell lines; IC50 values of 0.74±2.2 µM (against Hep3 B cells), 1.03±2.7 µM (against MCF 7 cells), 0.80±1.3 µM (against HeLa cells). Increase in carbon chain length (C17) of internal alkenyl fatty acid chain residue at 5-position of 1,3,4-oxadiazol-2-thione (compound, 109) and at 5-position of 1,2,4-triazol-3-thione (compound, 113) lead to high IC50 values i.e. IC50 value was above 16.42 µM against all the three tested human cancer cells. In case of 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazine ring system, even the chain length of the internal alkenyl substituent at 3-position (compound, 117) was increased (C17) still it displayed good antitumor activity against all the three cancer cell lines (IC50 value of 0.95±1.60 µM against Hep3 B cells, 1.17±2.4 µM against MCF 7 cells and 1.03±1.7 µM against HeLa cells), this may be due to the fused ring system. Presence of hydroxyl group on alkenyl fatty acid chain residue which is attached to 1,3,4-oxadiazol-2-thione at 5-position (compounds, 110 and 111) was responsible for increase in potency against hepatocellular carcinoma cells (IC50 values of 0.90±1.60 µM and 1.01±2.6 µM, respectively). Also, in case of 1,2,4-triazole, the presence of hydroxyl group on the alkenyl fatty acid chain residue at 5-position was responsible for increased cytotoxicity of compounds (114 and 115) against Hep3 B cells (IC50 value of 0.98±1.4 µM and 0.96±2.8 µM, respectively). For compounds (118 and 119), there may be two reasons for increased in potency against all the three cancer cell lines: the presence of hydroxyl group on the alkenyl fatty acid substituent and the other was the presence of fused ring system i.e. incorporation of thiadiazine with triazole ring (IC50 values of 0.65±2.4 µM against Hep3 B cells, 0.85±1.5 µM against MCF 7 cells, 0.85±1.4 µM against HeLa cells and 0.74±1.6 µM against Hep3 B cells, 0.80±2.1 µM against MCF 7 cells, 0.60±3.1 µM against HeLa cells, respectively). These initial findings lead to further derivatization of heterocyclic fatty acids.
3.3.4. Conclusion

In conclusion, the synthesis of novel heterocyclic fatty acid analogs and in vitro anticancer activity evaluation of synthesized compounds against different human cancer cell lines and PBMCs by MTT assay were described. Anticancer activity results revealed that the synthesized compounds were non-toxic to the normal human cells. The results of cytotoxic study also showed that, all compounds possess moderate to good activity but compounds (118 and 119) were the most promising cytotoxic agent with IC₅₀ values below 08.83±1.4 μM against all the three tested human cancer cells (Hep3 B, MCF 7, HeLa cells) may be due to the presence of hydroxyl group on fatty acid chain and fused ring system (triazolothiadiazine nucleus). From this study, it can be concluded that the potency of drugs depend on the nature of fatty acid chain and the heterocyclic ring system. From these studies, it is comprehensible that further derivatization of different heterocyclic analogs of fatty acids can be serve as new templates for antitumor chemotherapy and could be probably lead to more active molecules in the area of cancer chemotherapy.
3.4. Experimental

3.4.1. Chemistry

Physical and spectroscopic measurements

Anhydrous conditions were achieved by drying flasks and other equipments in the oven. Reagents were of commercial grade and were used without further purification. When required, solvents were dried and distilled before use. The source of olefinic and hydroxy-olefinic fatty acids, instrumental details of IR, NMR and mass spectrometry are already detailed in experimental section of chapter 1 (page number 19). Long chain alkenyl/hydroxyalkenyl hydrazide used as starting material which was synthesized from the corresponding fatty acids\(^9\). All the products were purified by column chromatography. Carbon disulfide, potassium hydroxide, phenacyl bromide, hydrazine hydrate were purchased from Merck, Mumbai, India. TLC was performed on glass plates with a layer of silica gel G (Merck, Mumbai, India, 0.55 mm thickness). Developing solvents used were the mixture of petroleum ether-diethyl ether-acetic acid (75:25:1; v/v).

**Synthesis of novel series of 5-long chain alkenyl/hydroxyalkenyl-1,3,4-oxadiazol-2-thiones, (108-111)**

A mixture of 0.01 mole of long chain alkenyl/hydroxyalkenyl hydrazide\(^9\) (22-25), 0.01 mole of potassium hydroxide and 10 mL of carbon disulfide were refluxed (80-90°C) in 50 mL ethanol for 8 hours. The reaction mixture was concentrated on water bath, then cooled to room temperature, acidified with dilute HCl at 0°C and the solid product was separated out. After that the product was filtered and washed with cold water. The solid products (108-111) were then air dried. Further, the products (108-111) were purified by silica gel column chromatography with petroleum ether and diethyl ether as eluent. The products were identified by spectral data.
The spectroscopic data for the synthesized compounds (108-111) are presented below:

5-(Dec-9'-enyl)-(3H)-1,3,4-oxadiazol-2-thione, (108)

IR (KBr, cm⁻¹): 3215 (N-H stretching), 2921 (C-H stretching), 1613 (C=N stretching), 1165 (C=S stretching), 1054 (C-O-C).

¹H NMR (CDCl₃, δ(H)): 10.30 (1H, s, NH), 5.81 (1H, tdd, J_H-C₂ = 6.48 Hz, J_H-N = 10.31 Hz, J_H-CH₂ = 17.24 Hz, CH₂=CH), 5.01 (1H, dd, J_H-c = 10.31 Hz, J_H-C₂ = 2.00 Hz, H₂(C=CH), 4.92 (1H, dd, J_H-C₂ = 16.91 Hz, J_H-C₂ = 2.00 Hz, H₂(C=CH), 2.69 (2H, t, J = 7.50 Hz, CH₂ a to ring), 2.01 (2H, m, CH₂=CH.CH₂), 1.74 (2H, m, CH₂ β to ring), 1.30 (10H, br.s, (CH₂)₅).

¹³C NMR (CDCl₃, δ(C)): 166.8, 165.4, 123.0, 122.3, 30.9, 30.8, 30.1, 29.9, 28.5, 27.1, 26.2, 24.2.


5-(Heptadec-8'-enyl)-(3H)-1,3,4-oxadiazol-2-thione, (109)

IR (KBr, cm⁻¹): 3218 (N-H stretching), 2925 (C-H stretching), 1615 (C=N stretching), 1166 (C=S stretching) 1059 (C-O-C).

¹H NMR (CDCl₃, δ(H)): 10.85 (1H, s, NH), 5.35 (2H, m, CH=CH), 2.69 (2H, t, J = 7.58 Hz, CH₂ a to ring), 2.01 (4H, m, CH₂CH=CHCH₂), 1.75 (2H, m, CH₂ β to ring), 1.31 (20H, br.s, (CH₂)₁₀), 0.88 (3H, dist.t, CH₃).

¹³C NMR (CDCl₃, δ(C)): 165.3, 163.2, 126.0, 125.8, 35.0, 33.9, 31.0, 29.4, 28.9, 28.7, 28.0, 27.3, 26.8, 25.8, 23.7, 23.4, 23.0, 22.0, 14.0.

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5-[(8'R, 11'R)-11'-Hydroxyheptadec-8'-enyl)-(3H)-1,3,4-oxadiazol-2-thione, (110)

IR (KBr, cm⁻¹): 3398 (O-H stretching), 3121 (N-H stretching), 2923 (C-H stretching), 1625 (C-N stretching), 1159 (C=S stretching), 1056 (C-O-C).

¹H NMR (CDCl₃, δH): 12.34 (1H, s, Na), 5.47 (2H, m, CH=CH), 3.68 (1H, m, CHOH), 2.68 (2H, t, J = 7.49 Hz, CH₂ α to ring), 2.28 (1H, m, CHOH), 2.04 (4H, m, CH₂CH=CHCH₂), 1.72 (2H, m, CH₂ β to ring), 1.28 (18H, br.s, (CH₂)₉), 0.88 (3H, dist.t, CH₃).

¹³C NMR (CDCl₃, δC): 168.3, 164.2, 133.2, 125.1, 72.0, 37.3, 36.6, 35.1, 31.8, 30.1, 29.7, 29.6, 29.1, 28.9, 27.2, 25.4, 24.0, 23.4, 14.2.


5-[(8'R, 11'Z)-8'-Hydroxyheptadec-11'-enyl)-(3H)-1,3,4-oxadiazol-2-thione, (111)

IR (KBr, cm⁻¹): 3353 (O-H stretching), 3159 (N-H stretching), 2921 (C-H stretching), 1619 (C-N stretching), 1152 (C=S stretching), 1051 (C-O-C).

¹H NMR (CDCl₃, δH): 11.56 (1H, s, NH), 5.37 (2H, m, CH=CH), 3.59 (1H, m, CHOH), 2.70 (2H, t, J = 7.50 Hz, CH₂ α to ring), 2.25 (1H, m, CHOH), 2.00 (4H, m, CH₂CH=CHCH₂), 1.72 (2H, m, CH₂ β to ring), 1.29 (18H, br.s, (CH₂)₉), 0.87 (3H, dist.t, CH₃).

¹³C NMR (CDCl₃, δC): 167.5, 165.8, 133.0, 125.9, 71.9, 37.1, 36.4, 35.1, 32.8, 31.4, 30.9, 29.9, 28.2, 27.6, 26.9, 25.2, 24.6, 23.1, 14.0.


Synthesis of novel series of 4-amino-5-long chain alkenyl/hydroxyalkenyl-1,2,4-triazol-3-thiones, (112-115)

To a solution of 0.01 mole of potassium hydroxide in 50 mL absolute ethanol, 0.01 mole of long chain alkenyl/hydroxyalkenyl hydrazide, (22-25) and 0.013 mole of
carbon disulfide was added. The reaction mixture was stirred for 8 hours at room
temperature. The reaction mixture was then diluted with 30 mL diethyl-ether and
stirred for additional 1 hour. The potassium salt without further purification was used
for the next step. 0.02 Mole of hydrazine hydrate in 20 mL water was gradually added
to the above potassium salt with constant stirring and then the reaction mixture was
refluxed for 4 hours. During refluxing, H$_2$S gas released and the reaction mixture
colour changed to light pink. The reaction mixture then cooled and acidified with
concentrated HCl. The white solid product was separated out which was then filtered
and washed with water. The solid products (112-115) were then air dried. The
products (112-115) were purified by silica gel column chromatography with
petroleum ether and diethyl ether as eluent. The products were identified by spectral
data.

The spectroscopic data for the synthesized compounds (112-115) are presented
below:

4-Amino-5-(dec-9'-enyl)-1,2,4-triazol-3-thione, (112)

IR (KBr, cm$^{-1}$): 3228 (N-H stretching), 2924 (C-H stretching), 1599 (C=O stretching),
1186 (C=S stretching).

$^{1}H$ NMR (CDCl$_3$, $\delta$): 11.60 (1H, s, NH), 5.79 (1H, tdd, $J_{H-H}$ = 6.70 Hz, $J_{H-NH}$ =
10.10 Hz, $J_{H-H}$ = 17.20 Hz, CH$_2$=CH$_2$), 5.01 (1H, dd, $J_{H-H}$ = 10.10 Hz, $J_{H-NH}$ = 1.30
Hz, H$_2$C=CH$_2$), 4.94 (1H, dd, $J_{H-H}$ = 17.20 Hz, $J_{H-NH}$ = 1.30 Hz, H$_2$C=CH$_2$), 4.57
(2H, br.s, NH$_2$), 2.74 (2H, t, $J$ = 7.50 Hz, CH$_2$ to ring), 2.02 (2H, m, CH$_2$=CH-CH$_2$),
1.70 (2H, m, CH$_2$ to ring), 1.29 (10H, br.s, (CH$_2$)$_5$).

$^{13}C$ NMR (CDCl$_3$, $\delta$): 167.0, 160.5, 139.1, 121.2, 33.7, 31.8, 29.5, 29.4, 28.8, 27.2,
26.1, 24.8.

4-Amino-5-[(8'Z)-heptadec-8'-eny1]-1,2,4-triazol-3-thione, (113)

**IR** (KBr, cm⁻¹): 3224 (N-H stretching), 2924 (C-H stretching), 1595 (C=N stretching), 1179 (C=S stretching).

**¹H NMR** (CDCl₃, δH): 11.32 (1H, s, NH), 5.35 (2H, m, CH=CH), 4.55 (2H, br.s, NH₂), 2.75 (2H, t, J = 7.50 Hz, CH₂ α to ring), 2.03 (4H, m, CH₂CH=CHCH₂), 1.68 (2H, m, CH₂ β to ring), 1.29 (20H, br.s, (CH₂)₁₀), 0.88 (3H, dist.t, CH₃).

**¹³C NMR** (CDCl₃, δC): 167.3, 165.6, 134.6, 122.1, 36.5, 34.1, 30.9, 29.2, 29.0, 28.9, 28.6, 27.4, 26.0, 25.9, 24.6, 24.1, 23.2, 22.4, 14.2.


4-Amino-5-[(8'R, 11'R)-11'-hydroxyheptadec-8'-eny1]-1,2,4-triazol-3-thione, (114)

**IR** (KBr, cm⁻¹): 3380 (O-H stretching), 3214 (N-H stretching), 2924 (C-H stretching), 1622 (C=N stretching), 1162 (C=S stretching).

**¹H NMR** (CDCl₃, δH): 12.17 (1H, s, NH), 5.41 (2H, m, CH=CH), 4.51 (2H, br.s, NH₂), 3.69 (1H, m, CHOH), 2.70 (2H, t, J = 7.51 Hz, CH₂ α to ring), 2.26 (1H, m, CHOH), 2.02 (4H, m, CH₂CH=CHCH₂), 1.67 (2H, m, CH₂ β to ring), 1.29 (18H, br.s, (CH₂)₉), 0.89 (3H, dist.t, CH₃).

**¹³C NMR** (CDCl₃, δC): 168.0, 163.2, 135.1, 121.9, 71.9, 37.3, 36.2, 34.9, 32.8, 30.1, 29.9, 29.6, 29.1, 28.2, 28.0, 27.8, 25.4, 24.0, 13.9.


4-Amino-5-[(8'R, 11'Z)-8'-hydroxyheptadec-11'-eny1]-1,2,4-triazol-3-thione, (115)

**IR** (KBr, cm⁻¹): 3376 (O-H stretching), 3217 (N-H stretching), 2921 (C-H stretching), 1619 (C=N stretching), 1164 (C=S stretching).
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$^1$H NMR (CDCl$_3$, $\delta$): 12.09 (1H, s, NH), 5.47 (2H, m, CH=CH$_2$), 4.56 (2H, br.s, NH$_2$), 3.68 (1H, m, CHO$_2$H), 2.72 (2H, t, $J$=7.50 Hz, CH$_2$ to ring), 2.27 (1H, m, CHO$_2$H), 2.04 (4H, m, CH$_2$CH=CHCH$_2$), 1.69 (2H, m, CH$_2$ to ring), 1.28 (18H, br.s, (CH$_2$)$_9$), 0.87 (3H, dist.t, CH$_3$).

$^{13}$C NMR (CDCl$_3$, $\delta$): 167.8, 164.4, 135.2, 122.5, 70.4, 37.2, 36.9, 34.0, 32.4, 30.1, 29.8, 29.5, 29.2, 28.7, 28.0, 27.6, 25.4, 23.5, 14.2.


Synthesis of novel series of 3-long chain alkenyl/hydroxyalkenyl-6-phenyl-7H-1,2,4-triazolo[3,4-b]1,3,4-thiadiazines, (116-119)

To a solution of 0.0025 mole of 4-amino-5-long chain alkenyl/hydroxyalkenyl-1,2,4-triazol-3-thiones, (112-115) in 15 mL absolute ethanol, 0.0025 mole of phenacyl bromide was added and the reaction mixture was refluxed at 90°C for 12 hours on oil bath. When all the triazole was consumed the reaction mixture was neutralized by ammonium hydroxide. The product was extracted with dichloromethane and washed with water. The organic layer dried over anhydrous sodium sulfate. The solvent was evaporated on water bath from the oily product (116-119). Further, the products (116-119) were purified by silica gel column chromatography with petroleum ether and diethyl ether as eluent. The products were identified by spectral data.

The spectroscopic data for the synthesized compounds (116-119) are presented below:

3-(Dec-9'-enyl)-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine, (116)

IR (KBr, cm$^{-1}$): 2923 (C-H stretching), 1604 (C=N stretching), 1125 (C-N stretching), 652 (C-S-C).

$^1$H NMR (CDCl$_3$, $\delta$): 7.55-7.39 (5H, m, ArH), 5.78 (1H, tdd, $J_{\text{H-N}} = 6.72$ Hz, $J_{\text{H-CH}} = 10.00$ Hz, $J_{\text{H-CH}} = 17.24$ Hz, CH$_2$=CH$_2$), 5.01 (1H, dd, $J_{\text{H-N}} = 10.12$ Hz, $J_{\text{H-CH}}$...
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4.12 (2H, s, CH₂ ring), 2.75 (2H, t, \( J = 7.50 \) Hz, CH₂ \( \alpha \) to ring), 2.04 (2H, m, CH₂=CH-CH₂), 1.69 (2H, m, CH₂ \( \beta \) to ring), 1.28 (10H, br.s, (CH₂)₅).

\(^{13}\)C NMR (CDCl₃, δc): 168.1, 165.4, 160.8, 134.8, 132.9, 132.5, 131.1, 130.5, 124.8, 122.5, 115.4, 36.2, 34.2, 33.9, 32.8, 32.1, 31.7, 30.9, 29.4, 25.0.


3-[(8'Z)-Heptadec-8'-eny1]-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine, (117)

IR (KBr, cm\(^{-1}\)): 2926 (C-H stretching), 1599 (C=N stretching), 1120 (C-N stretching), 660 (C-S-C).

\(^1\)H NMR (CDCl₃, δh): 7.62-7.40 (5H, m, ArH), 5.35 (2H, m, CH=CH), 4.09 (2H, s, CH₂ ring), 2.67 (2H, t, \( J = 7.54 \) Hz, CH₂ \( \alpha \) to ring), 2.03 (4H, m, CHF₂CH=CHCH₂), 1.64 (2H, m, CH₂ \( \beta \) to ring), 1.25 (20H, br.s, (CH₂)₁₀), 0.87 (3H, dist.t, CH₃).

\(^{13}\)C NMR (CDCl₃, δc): 168.3, 165.6, 160.5, 134.5, 132.0, 131.5, 130.8, 129.7, 125.0, 122.1, 35.5, 34.0, 32.5, 30.5, 29.0, 28.9, 28.4, 28.0, 27.2, 26.6, 25.5, 24.4, 24.0, 23.1, 21.4, 14.3.


3-[(8'Z,11'R)-11'-Hydrazyheptadec-8'-enyl]-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine, (118)

IR (KBr, cm\(^{-1}\)): 3360 (O-H stretching), 2922 (C-H stretching), 1613 (C=N stretching), 1115 (C-N stretching), 665 (C-S-C).

\(^1\)H NMR (CDCl₃, δh): 7.60-7.35 (5H, m, ArH), 5.40 (2H, m, CH=CH), 4.10 (2H, s, CH₂ ring), 3.62 (1H, m, CHOH), 2.72 (2H, t, \( J = 7.50 \) Hz, CH₂ \( \alpha \) to ring), 2.28 (1H, m,
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CHOH), 2.01 (4H, m, CH₂CH=CHCH₂), 1.68 (2H, m, CH₂β to ring), 1.29 (18H, br.s, (CH₂)₉), 0.87 (3H, dist.t, CH₃).

¹³C NMR (CDCl₃, δc): 167.0, 164.1, 163.8, 135.1, 132.7, 132.2, 131.4, 130.1, 123.4, 123.0, 121.9, 112.8, 36.3, 34.8, 33.9, 32.4, 30.3, 29.9, 29.5, 29.2, 28.7, 28.3, 27.8, 26.6, 24.7, 24.4, 14.2.


3-{(S'R, 11,Z)-8'-Hydroxyheptadec-11'-enyl}-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine, (119)

IR (KBr, cm⁻¹): 3382 (O-H stretching), 2924 (C-H stretching), 1615 (C=N stretching), 1122 (C-N stretching), 667 (C-S-C).

¹H NMR (CDCl₃, δh): 7.58-7.35 (5H, m, ArH), 5.37 (2H, m, CH=CII), 4.19 (2H, s, CH₂ ring), 3.64 (1H, m, CHOH), 2.69 (2H, t, J = 7.50 Hz, CH₂β to ring), 2.28 (1H, m, CH₂OH), 2.04 (4H, m, CH₂CH=CHCH₂), 1.65 (2H, m, CH₂β to ring), 1.28 (18H, br.s, (CH₂)₉), 0.87 (3H, dist.t, CH₃).

¹³C NMR (CDCl₃, δc): 167.8, 166.2, 164.4, 133.2, 132.6, 131.9, 131.5, 130.6, 129.6, 123.1, 122.5, 70.2, 37.1, 36.5, 32.8, 32.6, 30.3, 29.8, 29.4, 29.1, 28.5, 28.0, 27.8, 27.2, 25.8, 23.9, 14.1.


3.4.2. Biology

3.4.2.1. In vitro anticancer activity

3.4.2.1.1. Peripheral blood mononuclear cell isolation

Fresh blood (20–15 mL) was kindly provided by blood bank, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh. The blood sample was diluted with the same volume of phosphate buffer saline (PBS). After that, the diluted blood
sample was carefully layered on Ficoll-Histopaque (Sigma Aldrich, USA). The mixture was centrifuged under at 400×g for 30 minutes at 20-22°C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pelleted down with three volumes of PBS for twice and resuspended RPMI-1640 media (Sigma Aldrich, USA) with antibiotic and antymycotic solution (Sigma Aldrich, USA) 10%, v/v fetal calf serum (FCS) (Sigma Aldrich, USA). Cell counting was performed to determine the PBMC cell number with equal volume of trypan blue41,42.

3.4.2.1.2. MTT assay

The PBMCs/HeLa/Hep3 B/MCF 7 cell lines were maintained in RPMI 1640 (Sigma Aldrich) culture medium supplemented with 10% heat-inactivated fetal calf serum and antibiotic antimycotic solution (Sigma Aldrich). The cells were plated at a density of 5 × 10^3 cells per well in a 96-well plate, and cultured for 24 hours at 37°C. The cells were subsequently exposed to drugs. The plates were incubated for 48 hours, and cell proliferation was measured by adding 20 µL of MTT (Sigma Aldrich) dye (5 mg/mL in phosphate-buffered saline) per well. The plates were incubated for a further 4 hours at 37°C in a humidified chamber containing 5% CO2. Formazan crystals formed due to reduction of dye by viable cells in each well were dissolved in 150 µL dimethyl sulfoxide (DMSO), and absorbance was read at 570 nm. The absorption values were expressed as the cell viability (%), according to the control group as 100%. The concentration required for 50% inhibition of cell viability (IC50) was calculated using the software "Prism 3.0".
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