2. SYNTHESIS AND ANTICANCER ACTIVITY OF CHALCONES DERIVED FROM VANILLIN AND ISOVANILLIN

2.1 INTRODUCTION

Chalcones (Fig. 2.1) are phenyl styryl ketones in which the two aromatic rings are joined together by a three carbon α, β-unsaturated carbonyl linker. The term chalcone was coined by Kostanecki and Tambor. The first known example of chalcone is carthamin. It was isolated from the flowers of \textit{Carthamus tinctorius} (safflower) by Kmetaka and Perkin as red needles with green iridescence. Chalcones are considered to be precursors for flavanoids and isoflavanoids and belong to largest class of plant secondary metabolites. Several naturally occurring chalcones have been isolated from \textit{Leguminosae}, \textit{Asteraceae} and \textit{Moraceae} families. Chalcones can be detected using Wilson’s test where they give a pink colouration with concentrated sulphuric acid. Biosynthesis of chalcone and other flavanoid derivatives has been elaborated. Plants which are rich in chalcones have been used in traditional medicine for treating various disorders.

Though chalcones can be synthesised using contradistinct methods, Claisen Schmidt condensation using aromatic aldehydes/ketones is the most widely used method. Many condensing agents like hydrochloric acid, amino acids, perchloric acid and boron trifluoride can be used but alkali is the most used condensing agent due to better yields. Chalcones can also be synthesised using Heck-type reaction. Treatment of α, β-epoxy aryl ketones with Vilsmeier reagent yields chalcones. Chalcones can also be prepared by Friedel Crafts reaction of
polyhydroxy phenols with cinnamoyl chloride in the presence of aluminium chloride catalyst.\textsuperscript{10}

\[
\begin{align*}
\beta & \\
\alpha & 
\end{align*}
\]

Figure 2.1 Chalcone

The ultraviolet (UV) spectrum of chalcones exhibits two distinct maxima at 280 nm and 340 nm. Infrared (IR) spectrum of chalcones contain band in the region of 1665-1685 cm\textsuperscript{-1} due to $\alpha$, $\beta$ - unsaturated carbonyl group. But the characterisation of chalcones is preferably done using NMR spectroscopy, where resonance signals of H\textsubscript{\alpha} and H\textsubscript{\beta} protons appear as doublets in the range of $\delta$ 6.7-7.4 and $\delta$ 7.3-7.7 ppm respectively.\textsuperscript{11} From the coupling constant (J), the stereochemistry of chalcone can be ascertained. The \textit{trans} isomer (J =12-24 Hz) is thermodynamically more stable and it is formed substantially. Carbon signals observed in the range of $\delta$ 185-200, $\delta$ 116-129 and $\delta$ 137-145 ppm in the \textsuperscript{13}C NMR spectrum of chalcones correspond to carbonyl carbon, $\alpha$ and $\beta$ carbon atoms with respect to carbonyl carbon respectively.

Chalcones form the core skeleton of many biologically active compounds and they can be structurally modified to various heterocyclic compounds such as pyrazolines, cyanopyridines, flavonoids, pyrazoles, oxazoles, iso-oxazoles, pyrimidines, etc. Chalcones and their derivatives exhibit wide range of biological activities including anticancer,\textsuperscript{12} antidiabetic,\textsuperscript{13} antihypertensive,\textsuperscript{14} antiretroviral,\textsuperscript{15} anti-inflammatory,\textsuperscript{16} antifilarial,\textsuperscript{17} antimalarial,\textsuperscript{18} antioxidant,\textsuperscript{19} antifungal,\textsuperscript{20}
antibacterial, antitubercular, antispasmodic, antileishmanial and antiplatelet activities.

2.2 LITERATURE REVIEW

Some of the recent works published in the area of synthesis and anticancer activity of chalcones are briefly described below. Chalcones and flavanol derivatives have been synthesised and screened for their anticancer activity on human colorectal carcinoma cell line HCT116. Trans chalcone and licochalcone A have been screened for their cytotoxicity on breast cancer cell line (MCF-7) and normal mouse fibroblast cell line (3T3). The mechanism of action of these chalcones has been studied and these compounds are known to cause cell cycle arrest in G1 phase and also induce apoptosis. Novel chalcones from tetralones and 5-/6-indolecarboxaldehydes have been shown to induce dose dependant apoptosis on lung cancer cell line (A549) and cause cell cycle arrest at G2/M phase. Chalcone-benzoxaborole hybrid molecules have been synthesised and tested on three cancer cell lines (SKOV-3, MDA-MB-231 and HCT116) and two normal cell lines (MCF-10A and WI-38). A series of heteroaromatic chalcones have been evaluated for their cytotoxic activity and topoisomerase inhibitory effect on T47D and MDA-MB-468 cell lines. Novel triazole/azide chalcone derivatives were synthesised and screened for their anticancer activity on HeLa, RKO-AS45-1 and Wi-26 VA4 cell lines. Several chalcone-1,2,3-triazole-azole hybrids were synthesised and screened against three cancer cell lines SK-N-SH, EC-109 and MGC-803. A new library of monomer and dimer derivatives of dihydroartemisinin (DHA) containing substituted chalcones as a linker were synthesised and investigated for their cytotoxicity in human cancer cell lines HL-
Synthesis and Anticancer Activity of Chalcones Derived From Vanillin and Isovanillin

60 (leukemia), MIA PaCa-2 (pancreatic cancer), PC-3 (prostate cancer), LS 180 (colon cancer) and HePG2 (hepatocellular carcinoma). Chalcone and piperazine hybrid molecules containing substituted chalcones have been synthesised and screened on a panel of representative cell lines. A review on pharmacological screening of chalcones by Batovska and Todorova reveal that chalcones, apart from exhibiting excellent biological activities, show reasonable plasma concentration without causing toxicity.

Though there are numerous reports on anticancer activity of chalcones, studies on chalcones derived from vanillin, isovanillin and related compounds have been scanty and hence part of the work described in this thesis was undertaken with a view to focus on such chalcones particularly their synthesis, characterisation and biological screening for determination of their anticancer potential.

2.3 RESULTS AND DISCUSSION

Chalcones derived from vanillin (Fig. 2.3, 5a-5f) can be dubbed as mimics of curcumin (Fig. 2.4) due to structural similarities such as presence of two phenyl rings, α, β-unsaturated carbonyl unit and m-methoxy and p-hydroxy substituents in aromatic ring (ring B).

![Figure 2.2 Vanillin and related compounds](image)

<table>
<thead>
<tr>
<th>Formula</th>
<th>Substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>R₁ = -OCH₃; R₂ = -OH</td>
</tr>
<tr>
<td>2b</td>
<td>R₁ = -OH; R₂ = -OCH₃</td>
</tr>
<tr>
<td>2c</td>
<td>R₁ = R₂ = -OCH₃</td>
</tr>
<tr>
<td>2d</td>
<td>R₁ = -OCH₃; R₂ = -OCH₂COOH</td>
</tr>
<tr>
<td>2e</td>
<td>R₁ = -OCH₃; R₂ = -OTHP</td>
</tr>
<tr>
<td>2f</td>
<td>R₁ = -OTHP; R₂ = -OCH₃</td>
</tr>
</tbody>
</table>

Figure 2.2 Vanillin and related compounds
Synthesis and Anticancer Activity of Chalcones Derived From Vanillin and Isovanillin

Vanillin \(2a\) was chosen as the starting material. The hydroxyl group of vanillin was protected as tetrahydropyranyl (THP) ether \(2e\) using 3, 4-dihydro-2H-pyran (Scheme 2.1) in dichloromethane (DCM) solvent in the presence of pyridinium \(p\)-toluenesulphonate (PPTS) catalyst as described in the literature.\(^{36}\) The product was characterised using FTIR and NMR spectroscopy.
Scheme 2.1 Tetrahydropyranylation of vanillin

The THP ether of vanillin 2e was then condensed with differently substituted acetophenones in the presence of aqueous NaOH\(^{39}\) to yield the chalcones 3a-3f (Scheme 2.2) which are not reported in the literature.

Scheme 2.2 Claisen Schmidt condensation of THP ether of vanillin with differently substituted acetophenones

Chalcones 3a-3f were purified by crystallisation or column chromatography and characterised by FTIR and NMR spectroscopic techniques.
Deprotection of the THP group in 3a-3f was accomplished by treating them with pTSA in methanol at room temperature\(^{37}\) (Scheme 2.3) to yield chalcones 5a-5f with free hydroxyl group.

![Scheme 2.3 Deprotection of THP ether of chalcones](image)

The products were purified by crystallisation or column chromatography and thoroughly characterised using IR and NMR spectroscopy.

UV spectrum of a representative chalcone 5f shown in Fig. 2.5 exhibited bands at 355 nm and 256 nm which correspond to Band I and Band II (due to ring B and ring A of chalcones (Fig. 2.3))
Figure 2.5 UV spectrum of chalcone 5f (prepared from vanillin and 2,4-dichloro acetophenone) in acetonitrile
The purity of the chalcone 5f was assessed using HPLC (Fig. 2.6) and found to be >99%.

Figure 2.6 HPLC chromatogram of chalcone 5f (prepared from vanillin and 2,4-dichloro acetophenone)
1H NMR (Fig. 2.7) and 13C NMR (Fig. 2.8) spectra of a representative chalcone 5f are presented and discussed below.

Figure 2.7 1H NMR spectrum of chalcone 5f (prepared from vanillin and 2,4-dichloro acetophenone) in CDCl3 (400 MHz)
Fig. 2.7 displays the $^1$H NMR spectrum of chalcone 5f synthesised from vanillin and 2,4-dichloroacetophenone. The presence of methoxy group is identified from a sharp singlet at $\delta$ 3.86 ppm, while the two doublets at $\delta$ 6.87 ppm and $\delta$ 7.29 ppm correspond to vinylic protons which are $\alpha$ and $\beta$ with respect to carbonyl carbon respectively. The chalcone formed was found to be trans isomer as observed from coupling constant values ($J = 16$ Hz). The signals due to the three protons present in ring A (acetophenone ring) of chalcones appeared in the range of $\delta$ 7.27-7.40 ppm due to deshielding and the signals due to the remaining protons in ring B (vanillin ring) were observed in the range of $\delta$ 6.87-7.04 ppm due to shielding.
Figure 2.8 $^{13}$C NMR spectrum of chalcone 5f (prepared from vanillin and 2,4-dichloro acetophenone) in CDCl$_3$ (100 MHz)
In the $^{13}$C NMR spectrum (Fig. 2.8), the signals due to carbonyl carbon, the twelve aromatic carbons, two conjugated vinylic carbons and the methoxy carbon were observed at $\delta$ 193 ppm, $\delta$ 123-148 ppm, $\delta$ 110 ppm, $\delta$ 115 ppm and $\delta$ 56 ppm respectively.

Chalcones 3a-3f and 5a-5f were screened on A549, MCF-7 and MIA PaCa-2 using MTT assay and the results are tabulated in Table 2.1.

(b) A few chalcones were synthesised from isovanillin 2b (3-hydroxy-4-methoxy benzaldehyde) and veratraldehyde 2c (3,4-dimethoxybenzaldehyde) with differently substituted acetophenones (Schemes 2.4 & 2.5 respectively).

Scheme 2.4 Synthesis of chalcones from isovanillin
Synthesis and Anticancer Activity of Chalcones Derived From Vanillin and Isovanillin

Scheme 2.5 Synthesis of chalcones from veratraldehyde

(c) Vanillin 2a was converted into its aryloxyacetic acid 2d using standard procedure\textsuperscript{38} and the product was condensed with acetophenone to yield the corresponding chalcone 7 using the literature report\textsuperscript{39} (Scheme 2.6).

Scheme 2.6 Synthesis of chalcone from vanillin aryloxyacetic acid

(d) In order to study the effect of ring swapping, chalcones 10 and 11 were prepared from acetovanillone 9a (4'-hydroxy-3'-methoxy acetophenone) (Fig. 2.9) and 4-chloro and 2,4-dichloro substituted benzaldehydes (Scheme 2.7). The THP ethers of the acetovanillone based chalcones are not reported in the literature.
Figure 2.9 Acetovanillone and its THP ether

Figure 2.10 Acetovanillone based chalcones

Scheme 2.7 Synthesis of chalcones from acetovanillone

*In vitro* cytotoxicity of all the synthesised chalcones were determined using MTT assay on a panel of representative cell lines (A549, MCF-7 and MIA PaCa-2) and the results are given in Table 2.1 and Table 2.2.
Table 2.1 Cytotoxicity studies (MTT assay) of chalcones derived from (i) THP ether of vanillin and differently substituted acetophenones (3a-3f), (ii) THP ether of isovanillin and differently substituted acetophenones (4a-4d), (iii) vanillin and differently substituted acetophenones (5a-5f), (iv) isovanillin and differently substituted acetophenones (6a-6d), (v) aryloxyacetic acid of vanillin and acetophenone (7), (vi) veratraldehyde and differently substituted acetophenones (8a-8c), curcumin, cisplatin and 5-fluorouracil (positive controls)

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>IC50 values (µM)</th>
<th>A549</th>
<th>MCF-7</th>
<th>MIA PaCa-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>22.5±1.5</td>
<td>15.4±2.00</td>
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<td></td>
</tr>
<tr>
<td>3b</td>
<td>38.5±2.18</td>
<td>27.9±0.6</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3c</td>
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<td>9.6±0.38</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>17.9±0.6</td>
<td>10.4±1.39</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3e</td>
<td>17.4±0.3</td>
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<td>NA</td>
<td></td>
</tr>
<tr>
<td>3f</td>
<td>42.6±1.08</td>
<td>8.2±0.39</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>20.1±6.50</td>
<td>40±2.29</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>16.7±0.44</td>
<td>19.0±2.92</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4c</td>
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<tr>
<td>4d</td>
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<td>39.7±3.95</td>
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<td></td>
</tr>
<tr>
<td>5a</td>
<td>18.8±3.18</td>
<td>36.4±4.40</td>
<td>25.1±2.4</td>
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</tr>
<tr>
<td>5b</td>
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<td>&gt;100</td>
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<tr>
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</tr>
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<td>5d</td>
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<td>6a</td>
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<td>7</td>
<td>42.4±4.11</td>
<td>46.8±0.37</td>
<td>&gt;100</td>
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</tr>
<tr>
<td>8a</td>
<td>25.8±1.50</td>
<td>37.8±0.26</td>
<td>&gt;100</td>
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</tr>
<tr>
<td>8b</td>
<td>28.8±0.88</td>
<td>40.7±0.26</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>8c</td>
<td>17.6±1.2</td>
<td>17.8±0.5</td>
<td>18.6±1.5</td>
<td></td>
</tr>
<tr>
<td>curcumin</td>
<td>60.2±2.6</td>
<td>32.2±1.9</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>cisplatin</td>
<td>21±1.6</td>
<td>17±2.21</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td>-</td>
<td>-</td>
<td>38.8±0.8</td>
<td></td>
</tr>
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</table>
Table 2.2 Cytotoxicity studies (MTT assay) of chalcones derived from (i) THP ether of acetovanillilone (4’-hydroxy-3’-methoxy acetophenone) and 4-chlorobenzaldehyde (10a), (ii) THP ether of acetovanillone and 2, 4-dichlorobenzaldehyde (10b), (iii) acetovanillone and 4-chlorobenzaldehyde (11a) and (iv) acetovanillone and 2,4-dichlorobenzaldehyde (11b)

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>IC$_{50}$ values (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>10a</td>
<td>8.17±0.13</td>
</tr>
<tr>
<td>10b</td>
<td>10.86±2.56</td>
</tr>
<tr>
<td>11a</td>
<td>10.7±2.70</td>
</tr>
<tr>
<td>11b</td>
<td>8.9±0.5</td>
</tr>
</tbody>
</table>

From the results obtained, it is clear that when the hydroxyl group of chalcone 5a (Fig. 2.3) is protected as aryloxyacetic acid (Table 2.1, 7; Fig. 2.3) or methoxy (Table 2.1, 8a; Fig. 2.3), there is a decrease in cytotoxicity. It is also observed that the presence of electron releasing substituent i.e., methoxy substituent (Table 2.1, 5b; Fig. 2.3) in ring A (acetophenone ring) led to decrease in activity on all the three tested cell lines whereas presence of electron withdrawing substituents like nitro and fluoro (Table 2.1, 5c and 5d respectively; Fig. 2.3) resulted in enhanced activity in MCF-7. Interestingly, the same groups led to reduced activity in A549 and MIA PaCa-2 cell lines. In contrast, the presence of –Cl (Table 2.1, 5e; Fig. 2.3) increased the activity on all the three cell lines. Hence dichloro substituted chalcone 5f was synthesised, which showed increased cytotoxic effect as expected. Chalcone 5f was found to be the most potent chalcone among the chalcones tested on MIA PaCa-2 cell line with least
observed IC₅₀ value of 5.4±0.7 μM. Anticipating a similar trend, two chalcones 6c & 6d were prepared from isovanillin and 4-chloro and 2,4-dichloro acetophenones (Table 2.1; Fig. 2.3). As expected, these chalcones exhibited better potency than unsubstituted ones (Table 2.1, 6a; Fig. 2.3). Hence it can be concluded that introduction of a –Cl (chloro) substituent results in improved cytotoxicity. The cytotoxicity studies on ring swapped chalcones 10 and 11 (Table 2.2; Fig. 2.8) did not follow any particular trend warranting further investigation.

The most potent chalcone (Table 2.1, 5f) was subjected to further biological studies to gain insight into mechanism of action.
EFFECT ON CELLULAR MORPHOLOGY

Morphological changes on MIA PaCa-2 cells were identified on treatment with chalcone 5f. Cells were treated with 5f at IC_{50} concentration (5.4µM) and 2 X IC_{50} concentration (10.8 µM). After 24 h of treatment, cell shrinkage was observed along with the development of bubble like blebs on the membrane. These characteristic morphological features are indicative of cytotoxicity. **Fig. 2.11** shows the morphology of treated cells when observed under bright field 100X.

![Morphology of MIA PaCa-2 cells treated with chalcone 5f for 24 h as observed under bright field](image)

**Figure 2.11** Morphology of MIA PaCa-2 cells treated with chalcone 5f for 24 h as observed under bright field
Cell migration is a key property of cancer metastasis and inflammation. Hence, chalcone 5f was evaluated for its effect on the cell migration on MIA PaCa-2 cells by *in vitro* scratch assay. The cells were treated with increasing concentrations (1μM, 2μM, 3μM and 4μM) of 5f for 24 h. Positive control (10% FBS) was maintained simultaneously. After 24 h, a progressive inhibition in cell migration was observed with successive increase in the concentrations of 5f when compared to the control (10% FBS). Fig. 2.12 shows the progressive cell migration of MIA PaCa-2 cells on treatment with compound 5f. Inhibition of cell migration was found to dose dependent as shown in Fig. 2.10 with a slight decrease in wound closure with increasing concentration of chalcone 5f.

Figure 2.12 Images showing cell migration of MIA PaCa-2 cells on treatment with increasing concentrations of chalcone 5f
ANALYSIS OF MODE OF CELL DEATH BY AO/EB STAINING

To assess the type of cell death induced by chalcones in MIA PaCa-2 cells, the morphological changes after double staining cells with AO and EB were investigated. These dyes emit different shades of fluorescence after intercalation in DNA and can be used to detect nuclear changes that characterise apoptosis. Chalcone 5f was tested on MIA PaCa-2 cells and observed for changes in morphology after 24 h. Fluorescence microscopic analysis of treated cells revealed characteristic features of apoptosis such as chromatin condensation, nuclear fragmentation, alteration in the size and shape of cells. **Fig. 2.13** shows the fluorescence microscopy images of MIA PaCa-2 cells treated with 5.4 µM and 10.8 µM concentrations of chalcone 5f. Treatment with 10.8 µM concentration resulted in visibly higher number of apoptotic cells suggesting a dose dependent relationship.

![Fluorescence microscopy images](image-url)

**Figure 2.13** Fluorescence microscopy images of MIA PaCa-2 cells treated with chalcone 5f
CELL CYCLE ANALYSIS

MIA PaCa-2 cells were treated with IC$_{50}$ concentration of chalcone 5f for 24 h. Cell cycle analysis was performed using BD C6 flow cytometer. On treatment with IC$_{50}$ concentration of 5f, an increase in the percentage of cells in the G2/M phase of the cell cycle was observed. Percentage of cells in the G2/M phase of the cell cycle was found to increase to 29.08% as compared to 12.7% in case of vehicle control, implying that treated cells tend to accumulate in the G2/M phase. On treatment with 2 X IC$_{50}$ concentration, the percentage of cells in G2/M phase was found to increase further to 59.3%. These findings are suggestive that chalcone 5f exerts cytotoxicity in MIA PaCa-2 cell line by G2-M checkpoint arrest. Fig. 2.14 illustrates the distribution of cells in various stages of the cell cycle.

![Cell Cycle Analysis](image)

Figure 2.14 Distribution of MIA PaCa-2 cells in various stages of the cell cycle on treatment with chalcone 5f at IC$_{50}$ concentration and 2 X IC$_{50}$ concentration.
Thus from the biological assays, it was observed that the most potent chalcone **5f** induced cell morphological changes which were indicative of apoptosis. Dose dependant progressive inhibition of cell migration was observed. Fluorescence microscopic analysis revealed characteristic apoptotic features. From cell cycle analysis, it was observed that the chalcone **5f** induced cytotoxicity in MIA PaCa-2 cells by G2/M checkpoint arrest.

### 2.4 CONCLUSION

In this study, 28 chalcones were synthesised from vanillin, isovanillin, veratraldehyde and aryloxyacetic acid of vanillin, all of them were thoroughly characterised and screened for their cytotoxic effect on three cancer cell lines (A549, MCF-7 and MIA PaCa-2). The results indicated significant cytotoxicity against the cancer cell lines used. Out of the compounds tested, compound **5f** was found to be most potent on MIA PaCa-2 cell line with an IC_{50} value of 5.4µM. It was found to affect cell morphology of the MIA PaCa-2 cell line, inhibit cell migration and induce apoptosis in the treated cells. Cell cycle analysis suggested the induction of apoptosis by G2/M checkpoint arrest. Further study is in progress to establish these classes of compounds as potential anticancer drugs.
2.5 EXPERIMENTAL

2.5.1 Materials - chemistry

Vanillin LR grade was procured from Rankem Chemicals and was used without further purification. 3,4-dihydro-2H-pyran was purchased from Spectrochem Private Limited and used after drying and distillation. Acetophenone and substituted acetophenones were bought from Merck and used after distillation. All solvents were procured from Avra Synthesis Private Limited and used after drying and distillation. Paratoluensulphonic acid monohydrate (pTSA) 99% and Pyridinium p-toluensulphonate (PPTS) 98% were procured from Sigma-Aldrich and used without further purification. Isovanillin was procured from Himedia Laboratories Private Limited. Veratraldehyde was purchased from Sisco Research Laboratories Private Limited. Chloroacetic acid 99% was bought from Sigma-Aldrich. Silica gel (60-120, 100-200, 230-400) used for column chromatography were bought from Merck. TLC silica gel 60G F254 plates used for thin layer chromatography were purchased from Merck, Germany.

Melting points were recorded on Veego (WMP-DS) capillary melting point apparatus and are uncorrected. UV spectrum was recorded in Schimadzu UV-visible double beam spectrophotometer by dissolving 1 mg of sample in 10 ml of acetonitrile. HPLC was recorded in Schimadzu Nexera X2 using ODS column (250 mm long; 3.5 μm particle size) and the measurements were made at 254 nm. Acetonitrile-water system was used as the mobile phase and the flow rate was set to 0.6 ml / min. Photodiode array detector was used. FTIR spectra were recorded in KBr pellet using Bruker FTIR Alpha spectrometer in the range 500-4000 cm⁻¹. ¹H NMR and ¹³C NMR were run on Bruker 400 MHz and 100 MHz.
spectrometers respectively using TMS (tetramethylsilane) as internal standard in CDCl₃ and DMSO-d₆ solvents. Chemical shift and coupling constant values are given in δ (ppm) and Hz respectively.

2.5.2 Procedure for preparation of tetrahydropyranyl ether of vanillin

A mixture of vanillin (1.52 g, 10 mmol) and 3,4-dihydro-2H-pyran (1.26 g, 15 mmol) in 10 ml of dichloromethane was stirred at room temperature in the presence of PPTS (0.25 g, 1 mmol) for 3-4 hours and left overnight at room temperature. After monitoring progress of the reaction by TLC, reaction mixture was washed with dilute NaOH (0.1 N) to remove unreacted vanillin. The organic layer was washed with cold water till the washings are free from alkali, dried over anhydrous sodium sulphate and the solvent was removed under vacuum. The solid obtained was dried and weighed.

Similar procedure was followed to prepare tetrahydropyranyl ethers of isovanillin (2f) and acetovanillone (9b).

2.5.3 Procedure for preparation of chalcone from tetrahydropyranyl ether of vanillin and acetophenone

A mixture of tetrahydropyranyl ether of vanillin (0.236 g, 1 mmol) and acetophenone (0.120 g, 1 mmol) was stirred in methanol (5 ml) at room temperature in the presence of NaOH (60 mg, 1.5 mmol) for 3 hours and left overnight at room temperature. Solvent was removed under vacuum and the reaction mixture was poured into ice-cold water and neutralised with dil. HCl (2N). The yellow solid separated was filtered, washed thoroughly with water, dried and weighed. The crude product was recrystallised from ethanol.
Similar procedure was adopted for synthesising chalcones from tetrahydropyranyl ether of vanillin (2e) with differently substituted acetophenones and for preparing chalcones from tetrahydropyranyl ether of isovanillin (2f) with differently substituted acetophenones and tetrahydropyranyl ether of aceto vanillinone (9b) with 4-chloro and 2,4-dichloro benzaldehydes.

2.5.4 Procedure for deprotection of tetrahydropyranyl ether of chalcones

(3a-3f, 4a-4d, 10a&10b)

The tetrahydropyranyl ether of individual chalcones (1 mmol) were dissolved in methanol (5 ml) and stirred at room temperature with catalytic quantity of pTSA (0.1 mmol) for about 30 minutes. The completion of reactions were confirmed by TLC and solvent was removed under vacuum to obtain deprotected chalcones which were washed with water, dried and weighed.

2.5.5 Procedure for preparation of aryloxyacetic acid from vanillin

(2d)

A mixture of vanillin (1.63g, 10.7 mmol), ethyl bromoacetate (2.16 g, 12.8 mmol), potassium carbonate (1.78g, 12.8 mmol) and N, N-diethylformamide (7.5 ml) was stirred and heated to 50-55°C for 35 minutes. Then 10% aqueous sodium hydroxide (10 ml) and water (3 ml) were added and the mixture was stirred in steam bath for an hour. The solution was cooled to room temperature and then acidified with 4N HCl. The solid that separated was filtered, washed thoroughly with water, dried and weighed. The crude solid was recrystallised from methanol. Melting point of the solid was found to be 177-178°C which exactly matched with the literature report.
2.5.6 Procedure for preparation of chalcone from veratraldehyde and acetophenone (8a)

A mixture of veratraldehyde (1.66 g, 10 mmol) and acetophenone (1.2 g, 10 mmol) was stirred in 10 ml of ethanol. To this mixture, was added NaOH (0.512 g, 12.8 mmol) in about 40 ml of water dropwise with constant stirring in ice-cold condition. The reaction mixture was then stirred at room temperature for about 3 hours and left overnight at room temperature. The solvent was then removed under vacuum and the solid formed was filtered, washed with ice-cold water, dried and weighed. The crude solid was recrystallised from hexane : ethyl acetate. Similar procedure was followed to synthesise chalcones 8b and 8c.

2.5.7 Spectral data of chalcones

(E)-3-(3-methoxy-4-(((tetrahydro-2H-pyran-2-yl)oxy)phenyl)1-phenylprop-2-en-1-one (3a)

Yellow solid (80%); mp 81-83 °C; IR (KBr) ν<sub>max</sub> (cm<sup>-1</sup>): 3056 (Ar C-H), 1651 (C=O), 1589 (Ar C-C), 1506 (C-H bending), 1260 (C-O);<sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz), δ (ppm): 1.6-2 (m, 6H, H_2', H_3', H_4'), 3.6-3.8 (m, 2H, H_5'), 3.94 (s, 3H, -OCH<sub>3</sub>), 5.49 (t, 1H, H_1'), 7.16 (d, 1H, J= 8.4 Hz, Ar H_5), 7.17 (d, 1H, J = 2 Hz, Ar H_2), 7.22 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H_6), 7.39 (d, 1H, J = 15.6 Hz, =CH(α)), 7.51 (d, 2H, J = 8 Hz, Ar H_3' & Ar H_5'), 7.58 (m, 1H, Ar H_4'), 7.75 (d, 1H, J = 15.6 Hz, =CH(β)), 8.01 (d, 2H, J = 8.8 Hz, H_2' & Ar H_6');<sup>13</sup>C NMR
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(E)-3-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (3b)

Yellow solid (85%); mp >90 °C (d.c.); IR (KBr) νmax (cm⁻¹): 1654 (-C=O), 1601 (Ar C-C), 1459 (C-H bending), 1257 (C-O); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 1.2-2.3 (m, 6H, H₂”, H₃”, H₄”), 3.6-3.9 (m, 2H, H₅”), 3.89 (s, 3H, -OCH₃), 3.92 (s, 3H, -OCH₃), 5.48 (t, 1H, H₁”), 6.98 (d, 2H, J = 8.4 Hz, Ar H₃’ & Ar H₅’), 7.08-7.26 (m, 3H, Ar H₂, Ar H₃ & Ar H₆), 7.41 (d, 1H, J = 15.6 Hz, =CH(α)), 7.74 (d, 1H, J = 15.6 Hz, =CH(β)), 8.03 (d, 2H, J = 8.4 Hz, Ar H₂’ & Ar H₆’); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 188.85, 150.23, 148.59, 144.16, 131.31, 130.75, 129.28, 125.57, 122.52, 120.20, 116.99, 113.80, 111.57, 97.18, 62.19, 56.22, 55.49, 30.21, 25.15, 18.71.
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(E)-1-(4-fluorophenyl)-3-(3-methoxy-4-((tetrahydro-2H-pyran-2-yloxy)phenyl)prop-2-en-1-one (3c)

Yellow solid (82%); mp 101-103 °C; IR (KBr) νmax (cm⁻¹): 1651 (-C=O), 1599 (Ar C-C), 1262 (C-O), 1033 (Ar-F); ¹H NMR (CDCl₃, 400 MHz), δ (ppm):
1.55-2.06 (m, 6H, H₂", H₃", H₄"), 3.54-3.57 (m, 1H, H₅"), 3.8 (s, 3H, -OCH₃), 3.84-3.9 (m, 1H, H₅"), 5.42 (t, 1H, H₁"), 7.08 (d, 1H, J = 8.4 Hz, Ar H₅), 7.09 (d, 2H, J = 8.8 Hz, Ar H₅' & Ar H₅"), 7.12 (d, 1H, J = 2 Hz, Ar H₂), 7.14 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H₆), 7.29 (d, 1H, J = 15.6 Hz, =CH(α)), 7.69 (d, 1H, J = 15.6 Hz, =CH(β)), 7.97 (d, 2H, J = 8.8 Hz, Ar H₂' & Ar H₆'); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 188.95, 150.26, 148.91, 145.26, 134.79, 131.06, 130.97, 128.91, 122.78, 119.87, 116.92, 115.79, 111.58, 97.15, 62.19, 56.24, 30.19, 25.14, 18.68.

(E)-3-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)-1-(4-nitrophenyl)prop-2-en-1-one (3d)

Yellow solid (78%); mp 126-128 °C; IR (KBr) νmax (cm⁻¹): 1659 (-C=O), 1580 (Ar C-C), 1529 & 1351 (-NO₂), 1262 (C-O); ¹H NMR (CDCl₃, 400 MHz),
δ (ppm): 1.5 – 1.9 (m, 6H, H₂″, H₃″, H₄″), 3.54-3.90 (m, 2H, H₅″), 3.86 (s, 3H, -OCH₃), 5.44 (t, 1H, H₁″), 7.10 (d, 1H, J = 1.6 Hz, Ar H₂), 7.11 (d, 1H, J = 8.4 Hz, Ar H₅), 7.16 (dd, 1H, Ar H₆, J = 8.4 Hz, J' = 1.6 Hz), 7.26 (d, 1H, J = 15.6 Hz, =CH), 7.71(d, 1H, J = 15.6 Hz, =CH), 8.06 (d, 2H, J = 8.8 Hz, Ar H₂’ & Ar H₆’), 8.29 (d, 2H, J = 8.8 Hz, Ar H₂’ & Ar H₆’); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 189.12, 150.31, 149.95, 149.48, 147.07, 143.44, 129.35, 128.35, 123.83, 123.29, 119.56, 116.82, 111.65, 97.11, 62.20, 56.27, 30.16, 25.11, 18.64.

(E)-3-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)-1-(4-chlorophenyl)prop-2-en-1-one (3e)

Yellow gummy solid (60%); IR (KBr) νmax (cm⁻¹): 1639 (-C=O), 1565 (Ar C-C), 1278 (C-O), 1046 (Ar-Cl); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 1.5-2.16 (m, 6H, H₂″, H₃″, H₄″), 3.54-3.65 (m, 1H, H₅″), 3.85 (s, 3H, -OCH₃), 5.82 (t, 1H, H₁″), 7.08 (d, 1H, J = 8.4 Hz, Ar H₅), 7.02 (d, 2H, J = 8.8 Hz, Ar H₂’ & Ar H₆’), 7.18 (d, 1H, J = 2 Hz, Ar H₂), 7.22 (dd, 1H, J = 8.4 Hz, J’ = 2 Hz, Ar H₆), 7.29 (d, 1H, J = 15.6 Hz, =CH), 7.68 (d, 1H, J = 15.2 Hz, =CH), 7.85 (d, 2H, J = 8.8 Hz, Ar H₂’ & Ar H₆’); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 189.8, 149.6, 147.8, 145.26, 134.79, 131.06, 130.97, 128.91, 122.78, 119.87, 116.92, 115.79, 111.58, 102.15, 63.19, 56.24, 30.19, 25.14, 20.68.
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(E)-1-(2,4-dichlorophenyl)-3-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)prop-2-en-1-one (3f)

Yellow solid (78%); mp 134-135 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1641 (-C=O), 1270 (C-O), 1034 (Ar-Cl); \(^1\)H NMR (CDCl\(_3\), 400 MHz), \( \delta \) (ppm): 1.6-1.98 (m, 6H, H\(_2\), H\(_3\), H\(_4\)), 3.62-3.95 (m, 2H, H\(_5\)), 3.9 (s, 3H, -OCH\(_3\)), 5.51 (t, 1H, H\(_1\)), 7.40 (d, 1H, \( J = 16 \text{ Hz} \), =CH(\( \beta \))), 6.99 (d, 1H, \( J = 16 \text{ Hz} \), =CH(\( \alpha \))), 7.3-7.5 (m, 3H, Ar H\(_3\), H\(_5\), H\(_6\)), 7.22 (dd, 1H, \( J = 8.4 \text{ Hz} \), \( J' = 2 \text{ Hz} \) Ar H\(_6\)), 7.35 (d, 1H, \( J = 2 \text{ Hz} \), Ar H\(_2\)), 6.92 (d, 1H, \( J = 8.4 \text{ Hz} \), Ar H\(_3\)); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz), \( \delta \) (ppm): 192.80, 150.26, 149.28, 147.06, 137.66, 136.65, 132.28, 130.28, 130.15, 128.27, 127.24, 124.28, 123.20, 116.76, 111.33, 97.07, 62.17, 56.14, 30.14, 25.11, 18.63.

(E)-3-(4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)-1-phenylprop-2-en-1-one (4a)

Yellow solid (86%); mp 126-128 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1658 (-C=O), 1587 (Ar C-C), 1265 (C-O); \(^1\)H NMR (CDCl\(_3\), 400 MHz), \( \delta \) (ppm): 1.58-2.13 (m, 6H, H\(_2\), H\(_3\), H\(_4\)), 3.62-3.67 (m, 2H, H\(_5\)), 3.9 (s, 3H, -OCH\(_3\)), 5.46 (t, 1H, H\(_1\)).
6.92 (d, 1H, J = 8.4 Hz, Ar H_5), 7.28 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H_6), 7.37 (d, 1H, J = 15.6 Hz, =CH(α)), 7.46 (d, 1H, J = 2 Hz, Ar H_2), 7.49 (m, 2H, Ar H_3' & Ar H_5'), 7.57 (m, 1H, Ar H_4'), 7.75 (d, 1H, J = 15.6 Hz, =CH(β)), 8.00 (d, 2H, J = 8.8 Hz, Ar H_2' & Ar H_6'); ^{13}C NMR (CDCl_3, 100 MHz), δ (ppm): 190.68, 152.68, 146.54, 145.01, 138.53, 132.50, 128.55, 128.45, 127.95, 124.47, 120.17, 116.96, 112.03, 97.66, 62.23, 56.08, 30.34, 25.22, 18.78.

(E)-3-(4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (4b)

Yellow solid (88%); mp 122-124 °C (d.c.); IR (KBr) ν_{max} (cm^{-1}): 1655 (-C=O), 1603 (Ar C-C), 1264 (C-O); ^{1}H NMR (CDCl_3, 400 MHz), δ (ppm): 1.49-2.1 (m, 6H, H_2'', H_3'', H_4''), 3.53-3.6 & 3.89-3.96 (m, 2H, H_5''), 3.79 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 5.38 (t, 1H, H_1''), 6.83 (d, 1H, J = 8.4 Hz, Ar H_3), 6.89 (d, 2H, J = 8.8 Hz, Ar H_3' & Ar H_5'), 7.19 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H_6), 7.31 (d, 1H, J = 15.6 Hz, =CH(α)), 7.38 (d, 1H, J = 2 Hz, Ar H_2), 7.66 (d, 1H, J = 15.6 Hz, =CH(β)), 7.94 (d, 2H, J = 8.8 Hz, Ar H_2' & Ar H_6'); ^{13}C NMR (CDCl_3, 100 MHz), δ (ppm): 187.78, 162.22, 151.44, 145.43, 143.08, 130.31, 129.71, 127.08, 123.30, 118.82, 112.74, 110.95, 96.64, 61.21, 55.03, 54.44, 29.31, 24.19, 17.77.
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(E)-1-(4-chlorophenyl)-3-(4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)prop-2-en-1-one (4c)

Yellow gummy solid (76%); mp 122-124 °C; IR (KBr) ν_max (cm⁻¹): 1654 (-C=O), 1592 (Ar C-C), 1260 (C-O), 1028 (Ar-Cl); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 1.6-2.1 (m, 6H, H₂", H₃", H₄"), 3.6-3.7 & 3.96-4.05 (m, 2H, H₅"), 3.9 (s, 3H, -OCH₃), 5.47 (t, 1H, H₁"'), 6.92 (d, 1H, J = 8.8 Hz, Ar H₅), 7.27 (dd, 1H, J = 8.8 Hz, J' = 2 Hz, Ar H₆), 7.32 (d, 1H, J = 15.6 Hz, =CH(α)), 7.46 (d, 2H, J = 8.4 Hz, Ar H₂' & Ar H₆'), 7.47 (d, 1H, J = 2 Hz, Ar H₂), 7.75 (d, 1H, J = 15.6 Hz, =CH(β)), 7.95 (d, 2H, J = 8.4 Hz, Ar H₃' & Ar H₅'); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 189.62, 152.81, 146.44, 145.69, 138.98, 136.68, 129.87, 128.85, 127.67, 124.80, 119.44, 116.73, 111.97, 97.63, 62.24, 56.01, 30.25, 25.14, 18.65.

(E)-1-(2,4-dichlorophenyl)-3-(4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)prop-2-en-1-one (4d)
Yellow solid (72%); mp 120-122 °C; IR (KBr) ν_max (cm⁻¹): 1629 (-C=O), 1511 (Ar C-C), 1263 (C-O), 1028 (Ar-Cl); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 1.6-2.1 (m, 6H, H₂′, H₃′, H₄′), 3.61-3.69 & 3.96-4.04 (m, 2H, H₅″), 3.92 (s, 3H, -OCH₃), 5.44 (t, 1H, J = 8.4 Hz, Ar H₁), 6.92 (d, 1H, J = 15.6 Hz, =CH(α)), 7.22 (dd, 1H, J = 8.4 Hz, J′ = 2 Hz Ar H₆), 7.35 (d, 1H, J = 2 Hz, Ar H₂), 7.39 (d, 1H, J = 8 Hz, Ar H₆′), 7.40 (d, 1H, J = 15.6 Hz, =CH(β)), 7.35-7.50 (m, 2H, Ar H₃′ & Ar H₅′); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 189.62, 152.81, 146.44, 145.69, 138.98, 136.68, 129.87, 128.85, 127.67, 124.80, 119.44, 116.73, 97.63, 62.24, 56.01, 30.25, 25.14, 18.65.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (5a)

Yellow solid (78%); mp 92-94 °C; IR (KBr) ν_max (cm⁻¹): 3454 (-OH, H-bonded), 1690 (-C=O), 1550 (Ar C-C); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 3.77 (s, 3H, -OCH₃), 6.53 (d, 1H, J = 8 Hz, Ar H₃), 7.03 (d, 1H, J = 2 Hz , Ar H₂), 7.04 (dd, 1H, J = 8 Hz, J′ = 2 Hz, Ar H₆), 7.23 (d, 1H, J = 14.8 Hz, =CH(α)), 7.41-7.51 (m, 3H, Ar H₁′, Ar H₄′ & Ar H₅′), 7.68 (d, 1H, J = 15.2 Hz, =CH(β)), 7.93 (d, 2H, J = 8.4 Hz, Ar H₂′ & Ar H₆′); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 193.02, 161.39, 151.97, 149.51, 140.38, 133.53, 129.70, 129.37, 127.51, 123.31, 118.79, 116.02, 111.41, 56.13.
(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (5b)

Yellow solid (81%); mp 148-151 °C; IR (KBr) νmax (cm⁻¹): 3411 (-OH, H-bonded), 1653 (-C=O), 1277(C-O); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 3.89 (s, 3H, -OCH₃), 3.97 (s, 3H, -OCH₃), 6.95 (d, 1H, J = 8 Hz, Ar H₅), 6.99 (d, 2H, J = 8.8 Hz, Ar H₃' & Ar H₅'), 7.13 (d, 1H, J = 1.6 Hz, Ar H₂), 7.22 (dd, 1H, J = 8 Hz, J' = 1.6 Hz, Ar H₆), 7.39 (d, 1H, J = 15.6 Hz, =CH(α)), 7.74 (d, 1H, J = 15.6 Hz, =CH(β)), 8.03 (d, 2H, J = 8.8 Hz, Ar H₂' & Ar H₆'); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 188.84, 148.28, 146.78, 144.33, 131.35, 130.72, 128.34, 127.69, 123.14, 119.60, 114.84, 113.79, 110.04, 56.03, 55.50.

(E)-1-(4-fluorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (5c)

Pale yellow solid (76%); mp 90-92 °C; ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 3.97 (s, 3H, -OCH₃), 5.95 (bs, 1H, -OH), 6.96 (d, 1H, J = 8 Hz, Ar H₅), 7.2 (m, 2H, Ar H₂ & Ar H₆), 7.34 (d, 1H, J = 15.6 Hz, =CH(α)), 7.75 (d, 1H, J = 15.6 Hz, =CH(β)), 8.05 (m, 4H, Ar H₂', Ar H₃', Ar H₅' & Ar H₆'); ¹³C NMR (CDCl₃,
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100 MHz), δ (ppm): 189.11, 148.55, 146.98, 145.58, 131.18, 131.08, 127.52, 123.56, 119.44, 115.92, 115.71, 115.06, 110.23, 56.19.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-nitrophenyl)prop-2-en-1-one (5d)

Orange solid (70%); mp 170-174 °C; ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 3.98 (s, 3H, -OCH₃), 5.98 (bs, 1H, -OH), 6.98 (d, 1H, J = 8 Hz, Ar H₅), 7.28 (m, 2H, Ar H₂ & Ar H₆), 7.31 (d, 1H, J = 15.6 Hz, =CH(α)), 7.78 (d, 1H, J = 15.6 Hz, =CH(β)), 8.22 (d, 2H, J = 7.6 Hz, Ar H₂' & Ar H₆'), 8.35 (d, 2H, J = 7.6 Hz, Ar H₃' & Ar H₅'); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 189, 148, 147, 145, 131, 130, 127, 123, 119, 115.5, 115, 114, 110, 56.

(E)-1-(4-chlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (5e)

Yellow solid (64%); mp 195-196 °C; IR (KBr) ν max (cm⁻¹): 3429 (-OH, H-bonded), 1645(-C=O), 1513 (Ar C-C), 1275(C-O), 1032(Ar-Cl); ¹H NMR (CDCl₃, 400 MHz), δ (ppm): 3.98 (s, 3H, -OCH₃), 6.05 (s, 1H, -OH), 6.98 (d, 1H,
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J = 8 Hz, Ar H$_5$), 7.14 (d, 1H, J = 1.6 Hz, Ar H$_2$), 7.23 (dd, 1H, J = 8 Hz, J' = 1.6 Hz, Ar H$_6$), 7.34 (d, 1H, J = 15.6 Hz, =CH(α)), 7.48 (d, 2H, J = 8.8 Hz, Ar H$_3'$ & Ar H$_5'$), 7.77 (d, 1H, J = 15.6 Hz, =CH(β)), 7.97 (d, 2H, J = 8.4 Hz, Ar H$_2'$ & Ar H$_6'$); $^{13}$C NMR (CDCl$_3$, 100 MHz), δ (ppm): 189.31, 148.53, 146.86, 145.77, 138.96, 136.79, 129.85, 128.88, 127.30, 123.52, 119.18, 114.95, 110.12, 56.06.

(E)-1-(2,4-dichlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (5f)

Yellow solid (74%); mp 66-68 °C; IR (KBr) $\nu_{max}$ (cm$^{-1}$): 3397 (-OH, H-bonded), 1642 (-C=O), 1585 (Ar C-C), 1290 (C-O), 1031 (Ar-Cl); $^1$H NMR (CDCl$_3$, 400 MHz), δ (ppm): 3.86 (s, 3H, -OCH$_3$), 5.97 (s, 1H, -OH), 6.86 (d, 1H, J = 8 Hz, Ar H$_5$), 6.87 (d, 1H, J = 16 Hz, =CH), 7.04 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H$_6$), 7.27 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H$_3'$), 7.29 (d, 1H, J = 16 Hz, =CH), 7.33 (d, 1H, J = 8.4 Hz, Ar H$_6'$), 7.40 (d, 1H, J = 2 Hz, Ar H$_3'$); $^{13}$C NMR (CDCl$_3$, 100 MHz), δ (ppm): 192.82, 148.85, 147.28, 146.91, 137.68, 136.63, 132.25, 130.26, 130.14, 127.24, 126.80, 124.05, 123.73, 114.94, 109.82, 56.01.

(E)-3-(3-hydroxy-4-methoxyphenyl)-1-phenylprop-2-en-1-one (6a)
Pale yellow solid (85%); mp 92-94 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 3431 (-OH, H-bonded), 3060 (Ar C-H), 1650 (-C=O), 1270 (C-O); $^1$H NMR (CDCl$_3$, 400 MHz), $\delta$ (ppm): 3.9 (s, 3H, -OCH$_3$), 6.86 (d, 1H, $J$ = 8 Hz, Ar H$_6$), 7.08 (d, 1H, $J$ = 8 Hz, Ar H$_3$), 7.22 (bs, 1H, Ar H$_2$), 7.36 (d, 1H, $J$ = 15.6 Hz, =CH(α)), 7.48-7.52 (m, 2H, Ar H$_3'$ & Ar H$_5'$), 7.57-7.6 (m, 1H, Ar H$_4'$), 7.71 (d, 1H, $J$ = 15.6 Hz, =CH(β)), 7.98 (d, 2H, $J$ = 7.6 Hz, Ar H$_2'$ & Ar H$_6'$); $^{13}$C NMR (CDCl$_3$, 100 MHz), $\delta$ (ppm): 191.58, 150.50, 148.04, 145.95, 138.49, 132.77, 128.68, 128.53, 128.41, 121.73, 119.89, 114.21, 110.89, 55.88.

(E)-3-(3-hydroxy-4-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (6b)

Yellow solid (86%); mp 150-152 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 3461 (-OH, H-bonded), 3010 (Ar C-H), 1655(-C=O), 1459 (Ar C-C), 1277 (C-O); $^1$H NMR (CDCl$_3$, 400 MHz), $\delta$ (ppm): 3.91 (s, 3H, -OCH$_3$), 3.92 (s, 3H, -OCH$_3$), 7.00 (d, 1H, $J$ = 8.4 Hz, Ar H$_3$), 7.07 (d, 2H, $J$ = 8.8 Hz, Ar H$_3'$ & Ar H$_5'$), 7.21 (dd, 1H, $J$ = 8 Hz, $J'$ = 2 Hz, Ar H$_6$), 7.25 (d, 1H, $J$ = 2 Hz, Ar H$_2$), 7.57 (d, 1H, $J$ = 15.6 Hz, =CH(α)), 7.69 (d, 1H, $J$ = 15.6 Hz, =CH(β)), 8.09 (d, 2H, $J$ = 8.8 Hz, Ar H$_2'$ & Ar H$_6'$); $^{13}$C NMR (CDCl$_3$, 100 MHz), $\delta$ (ppm): 191.17, 165.33, 151.86, 146.13, 132.22, 132.04, 129.53, 123.56, 120.40, 115.16, 115.08, 112.62, 56.47, 56.16.
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(E)-1-(4-chlorophenyl)-3-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one (6c)

Pale yellow solid (75%); mp 195-196 °C; IR (KBr) ν<sub>max</sub> (cm<sup>-1</sup>): 3531 (-OH, H-bonded), 1650 (-C=O), 1513 (Ar C-C), 1264 (C-O), 1032(Ar-Cl); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz), δ (ppm): 3.88 (s, 3H, -OCH<sub>3</sub>), 5.63 (s, 1H, -OH), 6.81 (d, 1H, J = 8.4 Hz, Ar H<sub>6</sub>), 7.07 (d, 1H, J = 8.4 Hz, Ar H<sub>5</sub>), 7.21 (d, 1H, J = 2 Hz, Ar H<sub>2</sub>), 7.27 (d, 1H, J = 15.6 Hz, =CH(α)), 7.4 (d, 2H, J = 8.8 Hz, Ar H<sub>3</sub>’ & Ar H<sub>5</sub>’), 7.67 (d, 1H, J = 15.6 Hz, =CH(β)), 7.88 (d, 2H, J = 8.4 Hz, Ar H<sub>2</sub>’ & Ar H<sub>6</sub>’);

<sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz), δ (ppm): 189.15, 148.99, 145.97, 145.30, 139.01, 136.76, 129.84, 128.89, 128.39, 122.92, 119.70, 113.00, 110.60, 56.07.

(E)-1-(2)-4-dichlorophenyl 3-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one (6d)

Pale yellow solid (72%); mp 92-94 °C; IR (KBr) ν<sub>max</sub> (cm<sup>-1</sup>): 3417 (-OH, H-bonded), 1643 (-C=O), 1270 (C-O); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz), δ (ppm): 3.87 (s, 3H, -OCH<sub>3</sub>), 5.60 (bs, 1H, -OH), 6.79 (1H, d, J = 8 Hz, H<sub>5</sub>), 6.89 (1H, d, J = 16 Hz, =CH(α)), 7.00 (1H, dd, J = 8.4 Hz, J’ = 2 Hz, H<sub>6</sub>), 7.12 (1H, d, J = 2 Hz, H<sub>2</sub>), 7.27 (1H, dd, J = 8 Hz, J’ = 2 Hz, H<sub>5</sub>’), 7.31 (1H, d, J = 16 Hz, =CH(β)), 7.35
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(1H, d, J = 8 Hz, H₆'), 7.41 (1H, d, J = 2 Hz, H₃'); ¹³C NMR (CDCl₃, 100 MHz), δ (ppm): 192.58, 149.24, 146.66, 145.99, 137.70, 136.72, 132.33, 130.36, 130.17, 127.91, 127.24, 124.21, 122.92, 113.31, 110.60, 56.06.

(E)-2-(2-methoxy-4-(3-oxo-3-phenylprop-1-en-1-yl)phenoxy)acetic acid (7)

Off-white solid (76%); mp 148-149 °C; IR (KBr) ν_max (cm⁻¹): 3377 (-OH), 1740 (-C=O , acid), 1640 (-C=O), 1262 (C=O); ¹H NMR (DMSO-d₆, 400 MHz), δ (ppm): 3.86 (s, 3H, -OCH₃), 4.48 (s, 2H, -O-CH₂-COOH ), 6.83 (d, 1H, J = 8 Hz, Ar H₅), 7.32 (dd, 1H, J = 8 Hz, J' = 1.6 Hz, Ar H₆), 7.50 (d, 1H, J = 1.6 Hz, Ar H₂), 7.57 (m, 2H, Ar H₃'& Ar H₅'), 7.67 (m, 1H, Ar H₄'), 7.68 (d, 1H, J = 16 Hz, =CH(α)), 7.78 (d, 1H, J = 16 Hz, =CH(β)), 8.12 (m, 2H, Ar H₂' & Ar H₆'); ¹³C NMR (DMSO-d₆, 100 MHz), δ (ppm): 189.03, 169.96, 149.74, 148.99, 144.40, 137.79, 132.91, 128.70, 128.41, 127.89, 123.57, 119.77, 112.59, 111.19, 65.02, 55.77.

(E)-3-(3,4-dimethoxyphenyl)-1-phenylprop-2-en-1-one (8a)
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Yield (80%); mp 68-69 °C; IR (KBr) ν<sub>max</sub> (cm<sup>-1</sup>): 3059 (Ar C-H), 1659 (-C=O), 1591 (Ar C-C), 1260 (C-O); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz), δ (ppm): 3.94 (s, 6H, -OCH<sub>3</sub>), 6.9 (d, 1H, J = 8.4 Hz, Ar H<sub>5</sub>), 7.16 (d, 1H, J = 2 Hz, Ar H<sub>2</sub>), 7.23 (dd, 1H, J = 8.2 Hz, J' = 1.8 Hz, Ar H<sub>6</sub>), 7.39 (d, 1H, J = 15.6 Hz, =CH(α)), 7.5 (m, 2H, Ar H<sub>3</sub>' & Ar H<sub>5</sub>'), 7.58 (m, 1H, Ar H<sub>2</sub>'), 7.76 (d, 1H, J = 15.6 Hz, =CH(β)), 8.01 (dd, 2H, J = 8.4 Hz, J' = 1.2 Hz, Ar H<sub>2</sub>' & Ar H<sub>6</sub>'); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz), δ (ppm): 190.63, 151.49, 149.30, 145.03, 138.50, 132.56, 128.57, 128.43, 127.90, 123.18, 120.11, 111.19, 110.19, 56.

(E)-3-(3,4-dimethoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (8b)

Yellow solid (81%); mp 201-203 °C; IR (KBr) ν<sub>max</sub> (cm<sup>-1</sup>): 3230 (-OH), 1635 (-C=O), 1602 (Ar C-C), 1263 (C-O); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz), δ (ppm): 3.84 (s, 6H, -OCH<sub>3</sub>), 6.9 (d, 2H, J = 8.4 Hz, Ar H<sub>3</sub>' & Ar H<sub>5</sub>'), 7.01 (d, 1H, J = 8 Hz, Ar H<sub>5</sub>), 7.36 (dd, 1H, J = 8 Hz, J' = 1.6 Hz, Ar H<sub>6</sub>), 7.52 (d, 1H, J = 1.6 Hz, Ar H<sub>2</sub>), 7.64 (d, 1H, J = 15.6 Hz, =CH(α)), 7.8 (d, 1H, J = 15.6 Hz, =CH(β)), 8.08 (d, 2H, J = 8.4 Hz, H<sub>2</sub>' & Ar H<sub>6</sub>'); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz), δ (ppm): 187.02, 161.97, 150.96, 148.95, 143.19, 131.04, 129.31, 127.66, 123.62, 119.58, 115.27, 111.46, 110.51, 55.65, 55.51.
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(E)-1-(4-Chlorophenyl)-3-(3, 4-dimethoxyphenyl)prop-2-en-1-one (8c)

Yellow solid (78%); **mp** 103-105 °C; **IR** (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1658 (-C=O), 1591 (Ar C-C), 1271 (C-O), 1030 (Ar-Cl); **\(^1\)H NMR** (CDCl\(_3\), 400 MHz), \( \delta \) (ppm):
3.94 (s, 6H, -OCH\(_3\)), 6.9 (d, 2H, J = 8.8 Hz, Ar H\(_3\)' & Ar H\(_5\)''), 7.01 (d, 1H, J = 8 Hz, Ar H\(_5\)), 7.36 (dd, 1H, J = 8.2 Hz, J' = 1.8 Hz, Ar H\(_6\)), 7.52 (d, 1H, J = 1.6 Hz, Ar H\(_2\)), 7.64 (d, 1H, J = 15.6 Hz, =CH(α)), 7.8 (d, 1H, J = 15.6 Hz, =CH(β)), 8.08 (d, 2H, J = 8.4 Hz, H\(_2\)' & Ar H\(_6\)''). **\(^{13}\)C NMR** (CDCl\(_3\), 100 MHz), \( \delta \) (ppm): 189.28, 151.64, 149.30, 145.57, 138.97, 136.78, 129.86, 128.89, 127.68, 123.34, 119.46, 111.15, 56.03, 55.99.

(E)-3-(4-chlorophenyl)-1-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)prop-2-en-1-one (10a)

Pale yellow solid (80%); **mp** 194-196 °C; **IR** (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1653 (-C=O), 1607 (Ar C-C), 1258 (C-O); **\(^1\)H NMR** (CDCl\(_3\), 400 MHz), \( \delta \) (ppm): 1.64-1.76 & 1.92-2.12 (m, 6H, H\(_2\)'', H\(_3\)'', H\(_4\)''), 3.63-3.68 & 3.91-4.02 (m, 2H, H\(_5\)''), 3.97 (s, 3H, -OCH\(_3\)), 5.58 (t, 1H, H\(_1\)''), 7.23 (dd, 1H, J = 7.2 Hz, J' = 2 Hz, Ar H\(_6\)'').
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7.41 (d, 2H, J = 8.8 Hz, Ar H3 & Ar H5), 7.53 (d, 1H, J = 15.6 Hz, =CH(α)), 7.59 (d, 2H, J = 8.4 Hz, Ar H2 & Ar H6), 7.64 (d, 1H, J = 6.8 Hz, Ar H5'), 7.65 (d, 1H, J = 1.6 Hz, Ar H2'), 7.77 (d, 1H, J = 15.6 Hz, =CH(β)); 13C NMR (CDCl3, 100 MHz), δ (ppm): 188.52, 150.85, 150.22, 142.47, 136.19, 133.61, 132.07, 129.51, 129.20, 122.80, 122.27, 115.44, 111.79, 96.98, 62.15, 56.22, 30.14, 25.11, 18.58.

(E)-3-(2,4-dichlorophenyl)-1-(3-methoxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)prop-2-en-1-one (10b)

Pale yellow solid (82%); mp 106-109 °C; 1H NMR (CDCl3, 400 MHz), δ (ppm): 3.88 (s, 3H, -OCH3), 5.49 (t, 1H, -OH), 7.14 (d, 1H, J = 8 Hz, H5'), 7.23 (dd, 1H, J = 8 Hz, J' = 2 Hz, H6'), 7.4 (d, 1H, J = 2 Hz, Ar H5'), 7.41 (d, 1H, J = 15.6 Hz, =CH(α)), 7.5 (d, 1H, J = 2 Hz), 7.54 (dd, 1H, J = 8.4 Hz, J' = 2 Hz, Ar H3), 7.61 (d, 1H, J = 8.4 Hz, Ar H6), 8.01 (d, 1H, J = 15.6 Hz, =CH(β)); 13C NMR (CDCl3, 100 MHz), δ (ppm): 188.47, 150.93, 150.17, 138.55, 136.24, 135.96, 132.08, 131.75, 130.10, 128.51, 127.53, 124.90, 122.97, 115.32, 111.76, 96.93, 62.17, 56.20, 30.12, 25.09, 18.57.

(E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (11a)
Yellow solid (85%); mp 153-155 °C; \(^1\)H NMR (CDCl\(_3\), 400 MHz), δ (ppm): 3.99 (s, 3H, -OCH\(_3\)), 6.16 (bs, 1H, -OH), 6.99 (d, 1H, J = 8.4 Hz, Ar H\(_5\)'), 7.39 (d, 2H, J = 8 Hz, Ar H\(_3\) & Ar H\(_5\)), 7.52 (d, 1H, J = 15.6 Hz, =CH(α)), 7.57 (d, 2H, J = 8 Hz, Ar H\(_2\) & Ar H\(_6\)), 7.64 (m, 2H, Ar H\(_2\)' & Ar H\(_6\)'), 7.74 (d, 1H, J = 15.6 Hz, =CH(β)); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz), δ (ppm): 188.39, 150.67, 147.09, 142.60, 136.35, 133.73, 131.02, 129.65, 129.35, 123.88, 122.23, 113.96, 110.61, 56.31.

(E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (11b)

Yellow solid (82%); mp 192-194 °C (d.c.); \(^1\)H NMR (CDCl\(_3\), 400 MHz), δ (ppm): 3.92 (s, 3H, -OCH\(_3\)), 6.09 (bs, 1H, -OH), 6.92 (d, 1H, J = 7.2 Hz, Ar H\(_3\)'), 7.19-7.24 (m, 1H, Ar H\(_6\)'), 7.41 (m, 1H, Ar H\(_5\)), 7.42 (d, 1H, J = 15.6 Hz, =CH(α)), 7.56-7.62 (m, 3H, Ar H\(_2\)', Ar H\(_3\) & Ar H\(_6\)), 8.01 (d, 1H, J = 15.6 Hz, =CH(β)); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz), δ (ppm): 188.32, 150.78, 147.10, 138.64, 136.39, 132.23, 130.78, 130.25, 128.64, 127.67, 124.96, 124.04, 113.99, 110.69, 56.31.
2.5.8 Materials – Biology

Growth medium Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS), Acridine Orange (AO) and Ethidium Bromide (EB) were purchased from Himedia Laboratories. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and propidium iodide (PI) were purchased from Sigma. Dimethyl sulphoxide (DMSO) (spectroscopic grade) was purchased from Spectrochem and 5-FU (5-fluorouracil) was purchased from United Biotech. Cisplatin was purchased from Cipla.

2.5.9 Cell viability assay (MTT assay)

The cells were cultured in DMEM media containing 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. In total, 20,000 cells were seeded in each well (in 96-well plates) containing 200µl of DMEM medium. After 24h, different test concentrations of the chalcones were added, and again after 24 h of treatment, 20µl per well of MTT (5 mg/ml; stock solution, Sigma) was added. The plates were incubated at 37°C for additional 4 h. The medium was discarded and the formazan, which formed in the cells, was dissolved with 200 µl of DMSO. The intensity of colour production was measured at 570nm and 695nm in a spectrophotometer. IC₅₀ values were calculated using Microsoft Excel for semilog curve fitting with regression analysis.

2.5.10 Morphological changes

5 × 10⁵ were seeded in six-well plates and incubated in DMEM at 37°C in an atmosphere of 5% CO₂ for 24 h. Cells were treated with different concentrations of the most potent chalcone. After incubation for 24 h, cellular
morbidity was observed using phase contrast microscopy (Optica view). Images were captured at a magnification of 100x.

2.5.11 *In vitro* scratch assay (Cell migration assay)

3×10⁵ cells were seeded in six-well plates and incubated overnight at 37°C in an atmosphere of 5% CO₂ for 24 h. Following treatment with mitomycin C (5mg/ml) for 2 h, a scratch was induced using a sterile pipette tip. The cells were treated with increasing concentrations (1, 2, 3 and 4 μM) of the most potent chalcone for 24 h. Cells treated with 10% FBS were maintained as positive control. Follow incubation, the migration of cells into the marked region was observed and rate of migration was calculated using the formula

\[
\text{Percentage of migration} = \frac{(\text{Width at 0 h} - \text{Width at 24 h})}{\text{Width at 0 h}} \times 100
\]

The average data point was used to calculate the percentage migration. Respective photomicrograph was recorded and a graphical representation was made using the data obtained.

2.5.12 Acridine orange (AO) / Ethidium bromide (EB) staining

1 × 10⁵ cells were seeded on 12 well tissue culture plates and incubated overnight at 37°C in an atmosphere containing 5% CO₂. Once the cells reached 80% confluency, the cells were treated with IC₅₀ concentration of the most potent chalcone as well as 2 × IC₅₀ concentration for 24 h. Cells treated with 0.1% DMSO were maintained as vehicle control and cells treated with 5-fluorouracil (500 μg/ml) as positive control. Following treatment, cells were detached, pelleted and resuspended in complete media. 20 μl of cell suspension was treated with 10 μl of AO (1 mg/ml) and 10 μl of EB (1 mg/ml) and loaded onto a clean glass slide
and analysed under a fluorescence microscope. The cells were divided into four categories as live cells (green nucleus), early apoptotic cells (bright green nucleus with condensed or fragmented chromatin), late apoptotic cells (orange-stained nuclei with chromatin condensation or fragmented) and necrotic cells (uniform orange-stained cell nuclei).

2.5.13 Cell cycle analysis

Analysis of inhibition of cell cycle progression on MIA PaCa-2 cells treated with most potent chalcone and vehicle control was determined using flow cytometry. Using hypo PI method, the DNA of the cells was stained with PI, and the percentage of cells in each phase of the cell cycle was determined. Briefly, 4×10^5 cells were seeded in 35mm plate and incubated for 24 h for attachment. Following this, the cells were treated with IC_{50} and 2X IC_{50} concentrations of the most potent chalcone. The cells were maintained at 37°C with 5% CO_2 for 24 h. At the end of this incubation period, the cells were scraped, collected and centrifuged at 1500 rpm for about 5 min. Following this, 1ml of hypotonic solution was added and the cells were vortexed vigorously. Samples were analyzed on BD C6 Accuri flow cytometer with 10,000 events acquired for each sample.
2.6 REFERENCES


