Chapter-IV

Experimental
A. Synthesis

General procedure for the synthesis of 1-Benzyl-4-(2,4,6-trimethoxy-phenyl)-1,2,3,6-tetrahydro-pyridine (I): Step I [1, 2]

Equimolar quantity (0.005 mole) corresponding to 0.89 ml of n-benzyl piperidone was added with constant stirring to a solution of 0.84 g of 1,3,5-trimethoxy benzene in glacial acetic acid while maintaining the temperature at 20 °C. To the resulting viscous brown solution, 10 ml of concentrated HCl was added. Alternatively, HCl gas was bubbled at a rapid rate through this mixture for 2-3 h. After stirring for 7-8 h, the reaction mixture turned into a clear pink solution. It was then heated on an oil bath maintained at 80-90 °C for 6-7 h, as shown in Figure 2. The solution was then concentrated by removing glacial acetic acid. To the concentrate, crushed ice was added and contents were mixed with cold distilled water. The cold solution was then filtered to remove white precipitates of the unreacted trimethoxy benzene (approximately equivalent to 0.0002 mole). To the remaining filtrate, 100 g ice was added and the pH was adjusted to 10-11 by drop wise addition of 50% w/v NaOH aqueous solution while maintaining conditions below 10 °C. The pinkish white precipitate separated out from the solution was filtered and washed repeatedly with ice cold water.

The dried crude product was purified by dissolving the precipitate in boiling petroleum ether (60-80 °C) and the solution was filtered hot through an activated charcoal-bed. The resulting solution was stored in refrigerator till the cream colored purified product separated out from it. Further, the product so obtained was recrystallized with acetone: water mixture to obtain pale pink needle shaped crystals of intermediate (I).
Figure 2: Synthesis of 1-Benzyl-4-(2,4,6-trimethoxy-phenyl)-1,2,3,6-tetrahydro-pyridine(I). Reaction conditions: i) Glacial acetic acid, HCl, 20°C, stir 7-10 h; ii) Heat at 80-90°C for 6-7 h.
General procedure for the synthesis of 1-[3-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-hydroxy-4,6-dimethoxy-phenyl]-ethanone (II): Step II [1, 3]

To a cold solution of 1.2 g of intermediate-(I) (0.003 mole) in 15 ml of dichloromethane, 4.2 ml of boron trifluoride diethyl etherate (0.065 mole) was added drop wise with constant stirring under anhydrous conditions. After 6-8 h of constant stirring on an ice bath at a temperature of 0-5 °C, the reaction mixture turned buff colored. To this, 4.8 ml of acetic anhydride (0.049 mole) was added drop wise, as shown in Figure 3. The solution was then stirred for 48 h in the dark at room temperature and in anhydrous conditions. After 48 h, the reaction mixture was concentrated by removing dichloromethane and poured into crushed ice. It was then neutralized with 20% w/v Na₂CO₃ aqueous solution. The brown solid precipitate was washed with water, filtered and dried. The dried crude product was purified by recrystallization using methanol: water mixture to obtain yellow colored needle shaped pure intermediate (II).

**Figure 3**: Synthesis of 1-[3-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-hydroxy-4,6-dimethoxy-phenyl]-ethanone (II).

Reaction conditions: i) BF₃·O(C₂H₅)₂, 0-5 °C, stir 6-8 h; ii) (CH₃CO)₂O, stir for 48 h at rt, dark, anhydrous condition.
General procedure for the synthesis of 1-[3-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-hydroxy-4,6-dimethoxy-phenyl]-3-(substituted phenyl)-propenone or chalcones: Step III [3, 4]

Further the purified intermediate-(II), 0.367 g (0.001 mole) was dissolved in 5 ml of 20% w/v KOH in ethanol solution. To this solution, substituted aldehyde (0.0012 mole) was added and the reaction mixture was stirred at room temperature under anhydrous conditions, as shown in Figure 4. After the completion of the reaction, the mixture was poured drop wise with constant stirring into crushed ice and the precipitate so obtained was filtered and washed several times with cold water. The crude product obtained was then purified by recrystallization using different mixtures organic-aqueous solutions. Further purification was done by column chromatography using silica gel (100-200 mesh) and chloroform:methanol as mobile phase in gradient elution upto 1.5 % v/v methanol to obtain pure products, SNJ 1-30, as shown in Table 1.

![Diagram](image)

**Figure 4**: Synthesis of 1-[3-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-hydroxy-4,6-dimethoxy-phenyl]-3-(substituted phenyl)-propenone (SNJ 1-30).

Reaction conditions: i) 20 % w/v KOH in ethanol, rt, 1-12 h, anhydrous conditions.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Code</th>
<th>Substituted aldehydes (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SNJ 1</td>
<td>2-chlorobenzaldehyde</td>
</tr>
<tr>
<td>2.</td>
<td>SNJ 2</td>
<td>4-chlorobenzaldehyde</td>
</tr>
<tr>
<td>3.</td>
<td>SNJ 3</td>
<td>3-chlorobenzaldehyde</td>
</tr>
<tr>
<td>4.</td>
<td>SNJ 4</td>
<td>2-bromobenzaldehyde</td>
</tr>
<tr>
<td>5.</td>
<td>SNJ 5</td>
<td>4-bromobenzaldehyde</td>
</tr>
<tr>
<td>6.</td>
<td>SNJ 6</td>
<td>3-bromobenzaldehyde</td>
</tr>
</tbody>
</table>
7. **SNJ 7** 2-fluorobenzaldehyde

8. **SNJ 8** 4-fluorobenzaldehyde

9. **SNJ 9** 4-methylbenzaldehyde

10. **SNJ 10** 4-dimethylaminobenzaldehyde

11. **SNJ 11** 2-methoxybenzaldehyde

12. **SNJ 12** 4-methoxybenzaldehyde

13. **SNJ 13** 4-ethoxybenzaldehyde
14. **SNJ 14**  3,4-dimethoxybenzaldehyde

![3,4-dimethoxybenzaldehyde]

15. **SNJ 15**  2,4,5-trimethoxybenzaldehyde

![2,4,5-trimethoxybenzaldehyde]

16. **SNJ 16**  2,4-dichlorobenzaldehyde

![2,4-dichlorobenzaldehyde]

17. **SNJ 17**  2-nitrobenzaldehyde

![2-nitrobenzaldehyde]

18. **SNJ 18**  3-nitrobenzaldehyde

![3-nitrobenzaldehyde]

19. **SNJ 19**  3-phenylprop-2-enal

(cinnamaldehyde)

![3-phenylprop-2-enal]
20. **SNJ 20** 4-Formylbenzonitrile

21. **SNJ 21** Pyrrole-2-carboxaldehyde

22. **SNJ 22** Furan-2-carboxaldehyde

23. **SNJ 23** 4-(2-Pyridyl)benzaldehyde

24. **SNJ 24** Naphthalene-2-carbaldehyde

25. **SNJ 25** Anthracene-9-carbaldehyde
26. **SNJ 26**  Piperidine-1-carbaldehyde

![Piperidine-1-carbaldehyde](image1)

27. **SNJ 27**  Pyridine-4-carbaldehyde

![Pyridine-4-carbaldehyde](image2)

28. **SNJ 28**  Thiophene-2-carbaldehyde

![Thiophene-2-carbaldehyde](image3)

29. **SNJ 29**  Indole-3-carbaldehyde

![Indole-3-carbaldehyde](image4)

30. **SNJ 30**  5-methoxy indole-3-carbaldehyde

![5-methoxy indole-3-carbaldehyde](image5)
General procedure for the synthesis of 7-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-(substituted-benzylidene)-4,6-dimethoxy-benzofuran-3-one or aurone: Step IV. [1]

Substituted chalcone (0.001 mole) was dissolved in 20 ml of 20% w/v KOH in methanol solution till a clear solution was obtained and maintained on an ice bath at 5-10°C with constant stirring. To this solution, 2 ml of 30% w/w hydrogen peroxide (H₂O₂) solution was added drop wise and the mixture was then continuously stirred for 6-8 h while maintaining the reaction condition at 15-20°C in a closed reaction vessel, as shown in Figure 5. After the completion of reaction, the precipitate was filtered and washed several times with cold water. The crude product so obtained was then subjected to purification by column chromatography on silica gel (100-200 mesh) using chloroform: methanol as mobile phase in a gradient elution upto 1% v/v of methanol. Further, the product obtained from column chromatography was recrystallized using solvent mixtures consisting of different organic and aqueous solvents to obtain the final compounds or aurones (SNJ 31-44), as shown in Table 2.

**Figure 5**: Synthesis of 7-(1-Benzyl-1,2,3,6-tetrahydro-pyridin-4-yl)-2-(substituted-benzylidene)-4,6-dimethoxy-benzofuran-3-one (SNJ 31-44). Reaction conditions: i) 20 % KOH in methanol, 30 % H₂O₂ at 5-10°C followed by stirring at 15-20°C for 6-8 h.
Table 2: Various substituted chalcones used for synthesis of aurones (SNJ 31-44)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Code</th>
<th>Chalcones substituted with</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SNJ 31</td>
<td>Br H H H H</td>
</tr>
<tr>
<td>2.</td>
<td>SNJ 32</td>
<td>H H Br H H</td>
</tr>
<tr>
<td>3.</td>
<td>SNJ 33</td>
<td>H Br H H H</td>
</tr>
<tr>
<td>4.</td>
<td>SNJ 34</td>
<td>Cl H H H H</td>
</tr>
<tr>
<td>5.</td>
<td>SNJ 35</td>
<td>H H Cl H H</td>
</tr>
<tr>
<td>6.</td>
<td>SNJ 36</td>
<td>F H H H H</td>
</tr>
<tr>
<td>7.</td>
<td>SNJ 37</td>
<td>H H F H H</td>
</tr>
<tr>
<td>8.</td>
<td>SNJ 38</td>
<td>OCH₃ H H H H</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>9.</td>
<td><strong>SNJ 39</strong></td>
<td>H</td>
</tr>
<tr>
<td>10.</td>
<td><strong>SNJ 40</strong></td>
<td>H</td>
</tr>
<tr>
<td>11.</td>
<td><strong>SNJ 41</strong></td>
<td>H</td>
</tr>
<tr>
<td>12.</td>
<td><strong>SNJ 42</strong></td>
<td>OCH₃</td>
</tr>
<tr>
<td>13.</td>
<td><strong>SNJ 43</strong></td>
<td>H</td>
</tr>
<tr>
<td>14.</td>
<td><strong>SNJ 44</strong></td>
<td>H</td>
</tr>
</tbody>
</table>
B. Characterization

The melting point of all the synthesized compounds was measured using melting point apparatus and was found uncorrected.

Spectral analysis

The UV-visible spectra for the test compounds were recorded at different concentrations using methanol as solvent. Determination of wavelength of maximum absorbance ($\lambda_{\text{max}}$), molar absorption coefficient ($\epsilon$) at the $\lambda_{\text{max}}$ and other UV characteristics was done.

The infrared spectra for the test compounds was obtained using KBr disc. The mass spectra for the test compounds were obtained by dissolving the compounds either in acetonitrile or in methanol as solvent.

The $^1$HNMR and $^{13}$CNMR spectra for the test compounds were obtained using either deuterated-dimethyl sulfoxide (DMSO-$d_6$) or deuterated-chloroform (CDCl$_3$) as solvents. 2D-NMR analysis was carried out for one of the test compounds and CDCl$_3$ was used as the solvent.
C. **Physicochemical parameters**

**Determination of lipophilicity**

Lipophilicity of the test compounds was determined using shake flask method and was expressed in terms of distribution coefficient $(\log D)_{7.4}$ value [5]. Equal volumes of $n$-octanol as the organic phase and phosphate buffer (pH 7.4) as the aqueous phase were mixed and the resulting biphasic solution was pre-saturated for 24 h. To this biphasic solution system, test compounds previously dissolved in DMSO were added. The solution was then allowed to shake on an orbital shaker at 800 rpm for 24 h. After the completion of shaking, the solution was allowed to stand till the two phases were distinctly separated. The concentration of the test compound before and after shaking was determined using spectrophotometer. The distribution coefficient at pH 7.4, $(\log D)_{7.4}$ value was calculated as the logarithm of the ratio of concentration of analyte in the $n$-octanol phase to the concentration in the buffer phase.

**Determination of electrochemical oxidation potential**

The determination of electrochemical oxidation potential for the test compounds was carried out using cyclic voltammeter. Experiments were performed using glassy carbon (GE) as working electrode, silver-silver chloride (Ag-AgCl) as reference electrode and platinum wire as counter electrode [6]. Tetrabutylammonium perchlorate was used as the supporting electrolyte at 0.01 M concentration using dimethyl sulfoxide as solvent. The stock solution of test compounds and standard compounds namely ascorbic acid and quercetin was prepared in DMSO. The voltammogram was recorded using a test concentration of 500 µM in the electrolyte solution. The potential of the resulting solution was scanned positively (reverse scan) at different scan rates of 500, 250, 100, 75, 50, 25 and 10 mV sec$^{-1}$. 
References


Biological activity
A. In vitro Studies

Anti-oxidant activity

Oxidative metabolism is an integral regulatory mechanism for the normal functioning and survival of the cell. Oxidative changes and metabolic activities lead to the generation of free radicals such as ROS, RNS and RSS that are counteracted within the system by the various anti-oxidant defence mechanisms [1]. However, an internal imbalance between the production and neutralisation of free radicals leads to the development of oxidative stress conditions that are associated with a number of diseases/disorders such as cancer, Parkinsonism, ulcerative colitis, alcohol induced hepatotoxicity, atherosclerosis, immunological an neuronal diseases, Alzheimer’s, premature aging and many other [2]. It is noteworthy to mention the role of anti-oxidants that act as protective agents against the harmful effects of free radicals. They significantly delay or inhibit the oxidation of an oxidizable substrate by acting as preventive oxidants, reducing agents, chain breaking anti-oxidants, singlet oxygen quenchers, as metal chelators, by synergism with other anti-oxidants and finally as inhibitors of pro-oxidative enzymes [3]. The anti-oxidant potential of a substance could be determined by using different anti-oxidant assays that are further classified into various categories based on the type of reaction, enzyme or non-enzyme based, cell-based or cell-free based and a few others.

Neutralization of free radicals is mostly based on the two important types of reactions namely hydrogen atom transfer (HAT) and single electron transfer (SET). HAT-based methods measure the ability of an anti-oxidant (AH) to quench free radicals by hydrogen donation. In general, the scavenging of peroxyl radicals by phenolic anti-oxidants is considered biologically more relevant [4].
The HAT mechanisms of anti-oxidant action where the hydrogen atom (H) of a phenol (Ar–OH) is transferred to a ROO• radical could be summarized as below,

$$\text{ROO}^- + \text{AH}/\text{ArOH} \rightarrow \text{ROOH} + \text{A}^-/\text{ArO}^-$$

where, the aryloxy radical (ArO•) formed from the reaction of an anti-oxidant phenol with peroxyl radical is stabilized by resonance. The AH and ArOH species denote the protected biomolecules and phenolic anti-oxidants, respectively. Anti-oxidants need to react faster with free radicals than the biomolecules so as to protect the latter from oxidation [4].

On the contrary, SET-based methods are based on the ability of a potential anti-oxidant to transfer one electron to reduce any compound including metals, carbonyls, and radicals [5].

In most of the SET-based assays, a suitable redox-potential probe is employed such as a fluorescent or colored probe (oxidizing agent) that could be estimated spectrophotometrically by observing the changes in color when reduced, instead of peroxyl radical [4]. 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS•+)/Trolox-equivalent anti-oxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are two such widely used decolorisation assays owing to their simple, rapid, sensitive and reproducible procedures [6].

Although, the TEAC and DPPH assays are usually classified as SET reactions, these two indicator radicals might get neutralized either by direct reduction through electron transfers or by radical quenching through H atom transfer [5].

**DPPH- free radical scavenging assay**

DPPH is one of the few nitrogen radicals that is stabilized by the delocalization of spare electron. It bears a deep purple color with a characteristic absorption band near 517 nm UV spectrum, as shown in Figure 6. The color of the radical could be lost mostly based
Synthesis of novel tetrahydropyridinyl chalcones and aurones as useful therapeutic agents

Chapter IV

on a SET reaction, while HAT mechanism is only a minor reaction pathway [5]. This method is simple, accurate, sensitive, reproducible and economical however, steric accessibility of the radical site is a major limitation of the reaction. Therefore, small molecules that have better access to the radical site may show higher anti-oxidant capacity using this test [5, 7].

![Figure 6: DPPH free radical scavenging by an anti-oxidant compound](image)

**Procedure**

DPPH assay was performed in a 96 well plate. Serial dilutions of the methanolic stock solution of the test compounds and standard anti-oxidants were carried out to obtain concentration the range of 3.9-1000 µM. Quercetin and ascorbic acid were used as the standards. To 100 µl of this solution, 100 µl of 200 µM DPPH methanolic solution was added. The experiment was done in triplicate and the plate was then incubated for 30 min in the dark and later the absorbance was measured at 517 nm using Elisa reader. The percentage DPPH scavenging was calculated using the formula, (Control Absorbance – Test Absorbance)*100/ (Control Absorbance). The anti-oxidant activity of the test compounds was expressed in terms of concentration required for 50 percentage inhibition (IC$_{50}$).
**ABTS\(^+\) radical scavenging assay**

ABTS\(^+\) is one of the stable radicals that is not found in the human body. This assay is employed widely for determining the anti-oxidant activity, as the radical is soluble both in aqueous and organic solvents. Further, it is not affected by the ionic strength and could be used to determine anti-oxidant capacities of both lipophilic and hydrophilic extracts and body fluids. The reduction of the ABTS\(^+\) to ABTS by an anti-oxidant leads to the decolorisation of the blue green chromophore of the ABTS\(^+\) that could be detected spectrophotometrically at 415 and 645 nm, though the peak wavelength of 734 nm is predominantly preferred owing to less interference from plant pigment, Figure 7 [2, 5].

**Procedure:**

The assay was performed in a 96 well plate. A 2 mM ABTS\(^+\) working solution was prepared in phosphate buffer. To this, 200 μl of aqueous potassium persulfate (70 mM) solution was added and solution was incubated for 24 h in the dark at room temperature to develop a blue-green color. To 30 μl ABTS\(^+\) working solution, 50 μl of different concentrations (3.9-1000 μM) of the test sample solution and 170 μl of phosphate buffer (pH-7.4) were added in a well. The plate was then incubated in the dark at room temperature for 10 min. After the incubation, absorbance was measured at 690 nm using Elisa reader. Quercetin and ascorbic acid were used as the standards. The percentage scavenging of ABTS\(^+\) was calculated using the formula, (Control Absorbance – Test Absorbance)*100/ (Control Absorbance) and the anti-oxidant activity was expressed in terms of IC\(_{50}\) value.
Figure 7: ABTS \(^\cdot\) radical scavenging by an anti-oxidant compound
**Anti-cancer activity**

**Cell viability**

Screening of compounds using cell-based assays has been widely used to determine their cytotoxic effects on the cellular components and functions. Determination of cell viability is one of the parameters that gives an indication of the cytotoxic nature of test compounds and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is most commonly employed for this purpose. MTT being positively charged, has the ability to penetrate into viable cells. This cell-based assay calorimetrically estimates the extent of reduction of the yellow colored MTT by the mitochondrial succinate dehydrogenase in metabolically active cells based on electron transfer by NADH. The reduced formazan product being insoluble in nature accumulates inside the cells as well as gets deposited near the cell surface and in the culture medium. It is then solubilized using one of the solubilizing agents such as acidified isopropanol, DMSO, DMF and SDS. The extent of cell viability is indicated by the extent purple colored formazan formation that is measured spectrophotometrically at 570 nm or even at 630 nm, as shown in Figure 8. The use of this assay is often limited by the interference from reducing agents and by non-enzymatic reduction by compounds such as ascorbic acid, reduced glutathione, coenzyme A, and dithiothreitol [8].

![Figure 8: Reaction involved in MTT assay](image-url)
Procedure

Cytotoxicity screening for the test compounds was performed using MTT assay [9]. Cell lines representing breast carcinoma such as MCF-7, MDAMB-231 and T47D, hepatocellular carcinoma such as HepG2 and normal cells such as Vero cells were procured from National Centre for Cell Science, Pune and maintained in our tissue culture lab.

Cells were routinely grown in the 25 cm² tissue culture flasks with perforated screw-caps containing culture medium composed of DMEM supplemented with 8 % FBS and 50 μg ml⁻¹ gentamycin sulphate. The flasks were incubated at 37 ⁰C with 5% CO₂ humidified air inside CO₂ incubator. Approximately 5 x 10⁵ cells/ 100 μl of medium per well were seeded in 96 well plates and left overnight for the attachment. Desired concentrations of the test compounds in the range of 6.25-200 μM was obtained by serial dilution of their DMSO stock solutions using culture medium. Doxorubicin at (0.1-10 μM) was used as the standard and DMSO at 0.1 percent v/v in culture media was used as the solvent control. After 48 h of incubation, the media was removed carefully from the wells and the cells were then incubated with MTT reagent (100 μl of 1 mg ml⁻¹ solution in phosphate buffer) for 4 h at 37 ⁰C. The yellow formazan crystals so produced were solubilised by the addition of 100 μl DMSO. The plate was then placed on a micro vibrator for 5 min and absorbance was recorded at 540 nm using ELISA reader. The percentage cytotoxicity was calculated from the determination of cell viability using the formula, (Control Absorbance – Test Absorbance)*100/ (Control Absorbance) and the cytotoxic activity of the test compounds was expressed in terms of IC₅₀ value.
Cell staining

Apoptosis or programmed cell death is one of the hallmarks of cancer pathogenesis. Cells that undergo apoptosis show characteristic features such as membrane blebbing, chromatin condensation, nuclear shrinkage and fragmentation, cytoplasmic constriction, loss of cell volume, and formation of apoptotic bodies. Morphological examination of cells using fluorescent light microscopy with differential stains using dyes such as acridine orange (AO)/ethidium bromide (EB) is one of the methods of choice for the identification of cell death mechanism. AO/EB is a dual stain comprising of DNA binding fluorescent dyes. AO could permeate across the cells with unbroken cell membrane and make the nuclei appear green, while EB could only stain cells that have lost cell membrane integrity. Therefore, after AO/EB staining the viable cells tend to show uniform green color; early apoptotic cells appear bright green to yellow or show green yellow fragments with condensed nucleus or fragmented chromatin; late apoptotic cells are stained orange or display orange fragments representing the condensed and fragmented chromatin and necrotic cells show orange to red color fluorescent nuclei without any chromatin fragmentation [10, 11].

Procedure:

Exponentially growing MCF-7 cells were harvested and approximately $1 \times 10^6$ or $5 \times 10^4$ cells per well were seeded into 6-well or 12-well plate, respectively. The cells were incubated and allowed to attach overnight or for 24 h. They were then treated with the test compound at concentration close to their $IC_{50}$ and incubated at $37^\circ C$ in CO$_2$ incubator. Doxorubicin at 1µM was used as the standard and DMSO at 0.1 percent v/v was used as the control. After 24 h of treatment, the media was removed carefully followed by washing of the wells with PBS. The cells were then fixed using ice-cold ethanol for 20 min, after which the ethanol was removed and further the well was
washed with PBS. To each well, 300 µL of a previously prepared dual stain solution comprising of 20 µg mL⁻¹ AO and 30 µg mL⁻¹ EB in PBS was added and incubated for 20 min at 37°C. After incubation, the unbound stain was removed by washing with PBS. Both the treated and the untreated cells were then examined under fluorescence microscope at excitation wavelength of 450–490 nm [12].

**Cell cycle analysis**

The eukaryotic cell cycle is broadly divided into two basic parts namely mitosis and interphase. As the process of mitosis and cytokinesis last only about for an hour, consequently nearly 95% of the cell cycle time is spent in the interphase or the period between mitosis. During interphase, the chromosomes get de-condensed and are distributed throughout the nucleus and the cell grows at a steady rate throughout. Based on the timing of DNA synthesis, the eukaryotic cell cycle is divided into four different phases. The M phase corresponds to mitosis and is usually followed by cytokinesis. The G1 phase corresponds to the interval between mitosis and initiation of DNA replication. Here, the cell is metabolically active and continuously grows however, DNA replication does not take place at this time. It is followed by the S phase (synthesis) during which DNA replication occurs and enters the G2 phase during which the cell growth continues. Further, the protein synthesis also takes place in order to prepare the cell for mitosis. Progression between these phases of the cell cycle is controlled by a conserved regulatory apparatus, which not only coordinates the different events of the cell cycle but also links the cell cycle with extracellular signals that control cell proliferation [13]. The deregulation of the cell cycle has been well documented as a characteristic feature commonly found in several types of cancers [14].
Procedure:
Approximately $1 \times 10^6$ cells per well of MCF-7 cells were seeded in a 6-well plate and incubated for 24 h for adherence. The cells were then treated with the test compounds at concentration near to their IC$_{50}$ value and allowed to incubate for 24 h. Doxorubicin at 1 µM was used as the standard and DMSO at 0.1 percent v/v was used as the control. After 24h, the culture medium was removed from the wells and the cells were washed with PBS. The cells were then trypsinized, suspended in PBS and the suspension was centrifuged to obtain cell pellet. The pellet so obtained was fixed using 70% v/v ice cold ethanol and kept at -20°C for 24 h. Further, it was washed with PBS and an isotonic PI reagent solution (25 µg mL$^{-1}$ propidium iodide, 0.03% of NP-40 and 40 µg mL$^{-1}$ RNase A) was added. The cells were then incubated for 20 min in the dark. The stained cells were then analysed using Accuri C6 flow cytometer, BD Biosciences (San Jose, CA, USA) using BD AccuriTM C6 software [12].

B. In vivo studies

Anti-cancer activity

Animals
All experiments performed on the animals were done in accordance with the guidelines from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. A prior permission was sought from the Institutional Animal Ethics Committee for conducting the study (clearance certificate No. IAEC-KMC/21/2012). Female Swiss albino mice and Sprague-Dawley rats were procured from an inbred colony at the Central Animal Research Facility (CARF), Manipal University and maintained under controlled conditions of temperature (23±2°C), humidity (50 ±5%) and light (12 h of light and dark, each respectively). They were provided with the standard diet consisting of food pellets and water ad libitum.
Acute toxicity

The test dose for the two compounds namely SNJ 13 and 15, that showed promising results in vitro studies was determined as per OECD-425 guidelines for testing of chemicals. The compounds were administered at a dose of 1000 mg kg⁻¹ p.o. to fasted Swiss albino female mice. The animals were then observed continuously for the first 4 h and then daily for 14 days to record any changes in their morphological, behavioural, and neurological patterns, toxicity and mortality. As per the guidelines, a dose corresponding to 1/10th of the limit dose, i.e., 100 mg kg⁻¹ body weight was then selected for both the compounds for in vivo studies.

DMBA-induced mammary tumor model

The in vivo anti-cancer activity of the test compounds was studied using 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor model in Sprague-Dawley female rats. DMBA is a polycyclic aromatic hydrocarbon and an environmental carcinogen that is used to promote tumors in laboratory animals. It causes up-regulation of the cellular cytosolic receptor, the aryl hydrocarbon receptor (AhR) [15]. Upon ligand activation, the AhR translocates into the nucleus and associates with the cofactor AhR nuclear translocation protein (ARNT). This activated AhR/ARNT complex then binds to specific DNA recognition sites upstream of AhR responsive genes and induces gene transcription. The early steps in tumorigenesis involves AhR-dependent up-regulation of cytochrome P450 enzymes, which metabolize DMBA into a mutagenic epoxide intermediate that readily forms DNA adducts. These adducts are associated with DNA mutations and malignant transformations.

The susceptibility of the mammary gland to DMBA-induced carcinogenesis is strongly age-dependent, and the effect is maximum when it is administered at the age of sexual maturity (approximately 45 and 60 days old animals). Active organogenesis and high
rate of glandular epithelium proliferation in the gland are the characteristics of this period and results in higher DMBA activation [16].

Human breast cancer is a heterogeneous disease with respect to pathology, biochemistry and etiology[17]. However, the rat mammary gland is also believed to have a terminal duct-lobular unit (TDLU) and comprises of a terminal duct and lobules similar to that of human breast. This model is widely employed as it bears close resemblance to the histopathological changes and hyperplastic progression of breast cancer from premalignant to malignant lesions originating from TDLU in humans [17].

**Procedure:**

The study was performed on female Sprague–Dawley rats that were not older than 6-7 weeks in age. They were then divided into two groups namely a normal control (Group I) consisting of animals with normal mammary gland development and a DMBA-induced mammary tumor (Group II) that received a total DMBA dose of 30 mg kg$^{-1}$ body weight p.o. dissolved in sesame oil. All animals were maintained under controlled conditions and the Group II was routinely monitored by physical examination for the development of mammary tumors. After 60 days of DMBA administration, the first sign of palpable tumor appeared in the Group II animals. However, after a period of 85 days, stable tumors were developed in the animals, and they were then divided into DMBA control (Group II), and different treatment groups (Group III-VI) with a minimum of six animals in each group, as shown in Table 3. Test compounds were prepared as suspension in 0.25% w/v CMC, and administered at a dose of 100 mg kg$^{-1}$ body weight, p.o. Quercetin at 100 mg kg$^{-1}$ body weight, p.o. and doxorubicin at 2 mg kg$^{-1}$ body weight, i.p. were used as standards [18, 19]. All the treatments groups received respective drugs daily for a period of 3 weeks (21 days), except for doxorubicin, which was administered only once a week. At the end of the third week,
the study was terminated in all groups. Animals were weighed, and blood was collected from the retro-orbital plexus. They were then sacrificed, and vital organs such as heart, liver, spleen, and kidney, together with mammary tumors, were collected and weighed. Mammary tumors were measured using vernier callipers, and tumor volume was calculated using the formula \([\text{length} \times \text{width}^2]/2\). Tissue samples were stored in 10% v/v formaldehyde solution for histopathological observations.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Treatments</th>
<th>Dosage regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>Vehicle (0.25% w/v CMC), single dose/day, oral</td>
</tr>
<tr>
<td>II</td>
<td>DMBA control</td>
<td>Vehicle (0.25% w/v CMC), single dose/day, oral</td>
</tr>
<tr>
<td>III</td>
<td>SNJ 13</td>
<td>100 mg kg(^{-1}), single dose/day, oral</td>
</tr>
<tr>
<td>IV</td>
<td>SNJ 15</td>
<td>100 mg kg(^{-1}), single dose/day, oral</td>
</tr>
<tr>
<td>V</td>
<td>Quercetin</td>
<td>100 mg kg(^{-1}), single dose/day, oral</td>
</tr>
<tr>
<td>VI</td>
<td>Doxorubicin</td>
<td>2 mg kg(^{-1}), single dose/week, intra-peritoneal</td>
</tr>
</tbody>
</table>
References


aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1.


