CHAPTER-II, SECTION-2

Development of stilbene derivatives as potent tyrosinase inhibitors
2.2.1. Introduction

Tyrosinase, an enzyme catalyzing the rate-limiting step in the biosynthetic pathway of melanin pigments, is widely distributed in nature.\textsuperscript{1} The enzyme participates in several important reactions of host defence, wound healing and sclerotization in insects and other arthropods.\textsuperscript{1} Tyrosinase has also been found to be responsible for undesired enzymatic browning of farm products, such as bruised or cut fruits and vegetables, which subsequently leads to a significant decrease in their nutritional and market values.\textsuperscript{2} In view of these different functions ascribed to tyrosinase, it seems that studies aimed at the design and development of novel tyrosinase inhibitors and a comprehensive understanding about their mode of action can prove very useful for understanding many life processes. Such studies besides providing sufficient insight into mechanism underlying the regulation of skin pigmentation in mammals, are expected to help in a great way in search for proper cure to many dermatological disorders in mammals, design of alternative insect control agents and products useful to food technology and food processing. It is in this context that designing of novel tyrosinase inhibitors is receiving a considerable attention from food and animal scientists.\textsuperscript{3}

Realization about the importance of tyrosinase inhibitors for their impact on many physiological aspects especially in mammals and insects, has initiated an intense research activity aimed at designing of novel tyrosinase inhibitors and exploration of their mode of action. As an outcome of these studies, a huge library of tyrosinase inhibitors has been discovered from natural sources or synthesized in the laboratories. However, various limitations have been reported about the use of these currently known tyrosinase inhibitors. While potentially active agents, such as kojic acid and arbutin, are yet to be demonstrated clinically efficient,\textsuperscript{4} others are associated with disadvantages like high cytotoxicity, insufficient penetrating power, low activity and low stability. It is in this context that the use of traditional skin whitening products like hydroquinone, corticosteroids and mercury containing products has been prohibited, because these compounds have been found to be potentially mitogenic on account of their cytotoxicity to melanocytes.\textsuperscript{4,5}

In light of these reports, currently there is a great demand for the development of natural product inspired skin whitening agents, which are free from harmful side
effects. In this regard, phenolic compounds whose activity has been attributed to their structural resemblance to L-DOPA and tyrosine (the natural substrates of tyrosinase),\(^6\) seem to be very potent agents.

2.2.2. Present work

The present work is an outcome of our investigations aimed at designing of natural product inspired potent tyrosinase inhibitors that are free from side effects. From our literature survey exercise in this regard, we concluded that though exhaustive studies have been carried out on tyrosinase inhibition by stilbene moieties possessing hydroxyl groups, the mechanism behind their activity is yet to be fully understood.

For a molecular level understanding of the inhibitory action of stilbenoid moieties, we explored the structure-murine tyrosinase inhibition activity relation for variedly substituted stilbene compounds using resveratrol (1) as a positive control. Since the already established SARs for this class of inhibitors indicate that the trans-olefin structure in the parent stilbene skeleton is essential for tyrosinase inhibition,\(^7\) we restricted our studies to only trans derivatives of stilbene compounds with a diverse substitution pattern on and around aromatic rings and olefinic bond.

2.2.3. Experimental Section

2.2.3.1. Synthesis

Synthesis of stilbene derivatives

A library of twenty two stilbene derivatives was synthesized in the present study. Wittig reaction between substituted aromatic aldehydes and Wittig-salts derived from simple/substituted benzyl chlorides (Scheme 1) was employed for the synthesis of compounds 2 to 16 (Table 1).

![Scheme 1: Synthesis of stilbenes using Wittig reaction.](image-url)
Base catalyzed condensation reaction of substituted benzaldehydes with phenyl acetic acid, phenyl acetaldehyde and phenyl methylacetate in presence of pyridine/acetic anhydride or sodium methoxide/methanol in order to achieve the substitution on olefinic bond (Scheme 2), was used for the synthesis of compounds 17 to 21 (Table 2).

**Scheme 2: Base catalyzed synthesis of stilbenes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Melting point (°C)ᵃ</th>
<th>Yield (%)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>4-OCH₃</td>
<td>3′, 4′-OCH₃</td>
<td>COOH</td>
<td>170-171</td>
<td>35</td>
</tr>
<tr>
<td>18</td>
<td>4-OCH₃</td>
<td>4′-OCOCH₃</td>
<td>COOH</td>
<td>149-150</td>
<td>37</td>
</tr>
<tr>
<td>19</td>
<td>4-OCH₃</td>
<td>3′-OCH₃, 4′-OH</td>
<td>COOH</td>
<td>82-83</td>
<td>46</td>
</tr>
<tr>
<td>20</td>
<td>4-OCH₃</td>
<td>3′-OCH₃, 4′-OH</td>
<td>COOCH₃</td>
<td>80-81</td>
<td>44</td>
</tr>
<tr>
<td>21</td>
<td>H</td>
<td>4′-OCH₃</td>
<td>CHO</td>
<td>114-115</td>
<td>43</td>
</tr>
</tbody>
</table>

**Table 1: Stilbenes synthesized using Wittig reaction.**
ᵃMelting points are uncorrected.
ᵇIsolated yields after chromatographic purification.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Melting point (°C)ᵃ</th>
<th>Yield (%)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>H</td>
<td>H</td>
<td></td>
<td>116-117</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>4-F</td>
<td>H</td>
<td></td>
<td>126-127</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>4-Cl</td>
<td>H</td>
<td></td>
<td>130-131</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>4-Br</td>
<td>H</td>
<td></td>
<td>128-129</td>
<td>84</td>
</tr>
<tr>
<td>6</td>
<td>4-NO₂</td>
<td>H</td>
<td></td>
<td>142-143</td>
<td>81</td>
</tr>
<tr>
<td>7</td>
<td>4-CN</td>
<td>H</td>
<td></td>
<td>138-139</td>
<td>86</td>
</tr>
<tr>
<td>8</td>
<td>4-OCOCH₃</td>
<td>H</td>
<td></td>
<td>149-150</td>
<td>81</td>
</tr>
<tr>
<td>9</td>
<td>4-OH</td>
<td>H</td>
<td></td>
<td>189-190</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>4-OCH₃</td>
<td>H</td>
<td></td>
<td>135-136</td>
<td>86</td>
</tr>
<tr>
<td>11</td>
<td>4-F</td>
<td>4′-OCH₃</td>
<td></td>
<td>147-148</td>
<td>82</td>
</tr>
<tr>
<td>12</td>
<td>3-OCH₃, 4-Br</td>
<td>H</td>
<td></td>
<td>135-136</td>
<td>86</td>
</tr>
<tr>
<td>13</td>
<td>3,5-F</td>
<td>H</td>
<td></td>
<td>132-133</td>
<td>85</td>
</tr>
<tr>
<td>14</td>
<td>4-OCH₃</td>
<td>3′, 4′, 5′-OCH₃</td>
<td></td>
<td>159-160</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>2,3,4,5,6-F</td>
<td>H</td>
<td></td>
<td>139-140</td>
<td>84</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>naphthalene</td>
<td></td>
<td>132-133</td>
<td>86</td>
</tr>
</tbody>
</table>

**Table 2: Stilbenes synthesized through catalysis**
ᵃMelting points are uncorrected.
ᵇIsolated yields after chromatographic purification.
Base (piperidine/pyridine) catalyzed condensation of 4-hydroxyphenylacetonitrile with 4-hydroxybenzaldehyde and nicotinaldehyde respectively at 115 °C (Scheme 3), was followed for the synthesis of 22 and 23 (Table 3).

![Scheme 3: Base catalyzed condensation for synthesis of stilbenes](image)

**Table 3: Stilbenes synthesized through base catalyzed condensation**

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>R</th>
<th>Melting point (°C)(^a)</th>
<th>Yield (%)^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>CH</td>
<td>OH</td>
<td>248-249</td>
<td>35</td>
</tr>
<tr>
<td>23</td>
<td>N</td>
<td>H</td>
<td>68-69</td>
<td>37</td>
</tr>
</tbody>
</table>

\(^a\)Melting points are uncorrected.
\(^b\)Yields are isolated yields

**General procedure for the synthesis of compounds 2-16 (Scheme 1)**

To a solution of benzyl chloride (0.126 g, 1.00 mmol) in dry toluene (7 mL), was added triphenylphosphine (0.313 g, 1.20 mmol) at ambient temperature. The reaction mixture was allowed to reflux for 18 h under N\(_2\) atmosphere; during this time the Wittig salt was precipitated out, filtered and used for further reaction. To a solution of Wittig salt (0.08 g, 0.20 mmol) in CH\(_2\)Cl\(_2\), was added NaOH solution (0.01 g, 0.25 mmol, 3 mL water) at ambient temperature. The solution turned orange red indicating the formation of Wittig ylide. To this solution were added benzaldehyde (0.018 g, 0.16 mmol) and the reaction mixture allowed to stir at ambient temperature for 3 h. The crude product formed was extracted with CH\(_2\)Cl\(_2\), washed with brine and evaporated to dryness. After usual column chromatography (hexane: EtOAc; 9:1), the product obtained was a mixture of cis and trans-isomer, isolated in 87% yield. The above mixture was dissolved in hexane containing catalytic amount of iodine and allowed to reflux for 1 h. The reaction mixture was cooled down to ambient temperature, washed with Na\(_2\)S\(_2\)O\(_5\) solution and the organic layer evaporated to give pure trans-isomer in quantitative yield.
General procedure for the synthesis of compounds 17-18 (Scheme 2)
A mixture of 4-methoxyphenylacetic acid (0.2 g, 12.00 mmol), 2,3-dimethoxybenzaldehyde (0.2 g, 12.00 mmol) and Et₃N (1 mL) in Ac₂O (5 mL), was heated at 140 °C for 12 h. After completion of the reaction (monitored by TLC), the reaction mixture was brought to ambient temperature and evaporated to dryness. The residue was diluted with aqueous NaOH for saponification. The solution was then acidified with AcOH and extracted with CH₂Cl₂ (2 × 50 mL). The combined organic layers were dried over Na₂SO₄ and evaporated under vacuum. The crude product was purified by column chromatography (hexane: EtOAc; 6:4) in 35% yield.

General procedure for the synthesis of compounds 19-21 (Scheme 2)
A mixture of 4-methoxyphenylacetic acid (0.2 g, 1.20 mmol) and 4-hydroxy-3-methoxybenzaldehyde (0.915 g, 6.00 mmol) was charged in THF (15 mL). A solution of NaOMe (0.648 g, 12 mmol) in MeOH (25 mL) was added to the above mixture. The reaction mixture was allowed to stir at ambient temperature for 6 h. After completion of the reaction (monitored by TLC), the reaction mixture was neutralized with 0.1 N HCl, extracted with EtOAc (2 × 50 mL), dried over Na₂SO₄ and evaporated under vacuum. Finally the product was purified by column chromatography (hexane: EtOAc; 7:3) in 46% yield.

General procedure for the synthesis of compounds 22-23 (Scheme 3)
A mixture of 4-hydroxyphenylacetonitrile (0.3 g, 2.20 mmol), 4-hydroxybenzaldehyde (0.274 g, 2.20 mmol) and catalytic amount of dry piperidine, in 5 mL of dry pyridine, was allowed to reflux at 120 °C for 48 h. After completion of the reaction (monitored by TLC), the reaction mixture was neutralized with 2 N HCl, poured into ice cold water and extracted with EtOAc (2 × 50 mL), dried over Na₂SO₄ and evaporated under vacuum. Finally column chromatography was performed to purify the compound (hexane: EtOAc; 6:4) giving 45% yield.
2.2.3.2. Spectral data

(E)-1,2-Diphenylethene (2):

\[
\text{\(E\)}-1,2\text{-Diphenylethene (2):} \\
\text{\(1H\) NMR (200 MHz, CDCl\textsubscript{3}):} \ \delta 7.12 (s, 2H), 7.30-7.45 (m, 6H), 7.72 (m, 4H).
\]

Mass (ESI-MS): 180.9 (M\textsuperscript{+} + H).

C, H analysis for C\textsubscript{14}H\textsubscript{12}:
Calculated C, 93.29; H, 6.71. Found C, 93.27; H, 6.76.

(E)-1-Fluoro-4-styrylbenzene (3):

\[
(E)-1\text{-Fluoro-4-styrylbenzene (3):} \\
\text{\(1H\) NMR (200 MHz, CDCl\textsubscript{3}):} \ \delta 7.03-7.09 (m, 4H), 7.26-7.37 (m, 3H), 7.46-7.52 (m, 4H).
\]

Mass (ESI-MS): 198 (M\textsuperscript{+}).

C, H analysis for C\textsubscript{14}H\textsubscript{11}F:
Calculated C, 84.82; H, 5.59. Found C, 84.88; H, 5.57.

(E)-1-Chloro-4-styrylbenzene (4):

\[
(E)-1\text{-Chloro-4-styrylbenzene (4):} \\
\text{\(1H\) NMR (200 MHz, CDCl\textsubscript{3}):} \ \delta 7.08-7.13 (m, 2H), 7.48-7.53 (m, 9H).
\]

Mass (ESI-MS): 254 (M\textsuperscript{+} + H).

C, H analysis for C\textsubscript{14}H\textsubscript{11}Cl:
Calculated C, 78.32; H, 5.16. Found C, 78.30; H, 5.18.

(E)-1-Bromo-4-styrylbenzene (5):

\[
(E)-1\text{-Bromo-4-styrylbenzene (5):} \\
\text{\(1H\) NMR (200 MHz, CDCl\textsubscript{3}):} \ \delta 7.04 (d, 1H, J = 13.96 Hz), 7.13 (d, 1H, J = 16.22 Hz), 7.24-7.31 (m, 3H), 7.37 (d, 2H, J = 7.80 Hz), 7.50-7.54 (m, 4H).
\]
Mass (ESI-MS): 260 (M$^+$ + H).

C, H analysis for C$_{14}$H$_{11}$Br:
Calculated C, 64.89; H, 4.28. Found C, 64.85; H, 4.23.

$(E)$-1-Nitro-4-styrylbenzene (6):

\[
\begin{align*}
&\text{O}_2\text{N}-
\end{align*}
\]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.14 (d, 1H, $J = 16.40$ Hz), 7.28 (d, 1H, $J = 14.41$ Hz), 7.32-7.43 (m, 3H), 7.56 (d, 2H, $J = 7.21$ Hz), 7.64 (d, 2H, $J = 8.80$ Hz), 8.23 (d, 2H, $J = 8.82$ Hz).

Mass (ESI-MS): 224.7 (M$^+$ - H).

C, H, N analysis for C$_{14}$H$_{11}$NO$_2$:
Calculated C, 74.65; H, 4.92; N, 6.22. Found C, 74.68; H, 4.96; N, 6.26.

$(E)$-4-Styrylbenzonitrile (7):

\[
\begin{align*}
&\text{NC}-
\end{align*}
\]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.26 (d, 1H, $J = 16.16$ Hz), 7.29 (d, 1H, $J = 16.44$ Hz), 7.31-7.39 (m, 3H), 7.52-7.67 (m, 6H).

Mass (ESI-MS): 228 (M$^+$ + Na).

C, H, N analysis for C$_{15}$H$_{11}$N:
Calculated C, 87.77; H, 5.40; N, 6.82. Found C, 87.72; H, 5.48; N, 6.83.

$(E)$-4-Styrylphenyl acetate (8):

\[
\begin{align*}
&\text{H}_3\text{COOC-}
\end{align*}
\]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 2.31 (s, 3H), 7.09-7.14 (m, 4H), 7.33-7.38 (m, 3H), 7.52-7.57 (m, 4H).

Mass (ESI-MS): 261 (M$^+$ + Na).

C, H analysis for C$_{16}$H$_{14}$O$_2$:
Calculated C, 80.65; H, 5.92. Found C, 80.62; H, 5.96.
(E)-4-Styrylphenol (9):

![Structure of (E)-4-Styrylphenol]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 6.86 (d, 2H, $J = 7.32$ Hz), 7.04 (d, 2H, $J = 16.16$ Hz), 7.34-7.54 (m, 7H).

Mass (ESI-MS): 194.7 (M$^+$ - H).

C, H analysis for C$_{14}$H$_{12}$O: Calculated C, 85.68; H, 6.16. Found C, 85.70; H, 6.19.

(E)-1-Methoxy-4-styrylbenzene (10):

![Structure of (E)-1-Methoxy-4-styrylbenzene]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.84 (s, 3H), 6.91 (d, 1H, $J = 16.07$ Hz), 7.03 (d, 1H, $J = 16.14$ Hz), 7.31-7.36 (m, 3H), 7.45-7.51 (m, 4H).

Mass (ESI-MS): 210.9 (M$^+$ + H).

C, H analysis for C$_{15}$H$_{14}$O: Calculated C, 85.68; H, 6.71. Found C, 85.65; H, 6.76.

(E)-1-Fluoro-4-(4-methoxystyryl)benzene (11):

![Structure of (E)-1-Fluoro-4-(4-methoxystyryl)benzene]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.84 (s, 3H), 6.99-7.04 (m, 6H), 7.46-7.52 (m, 4H).

Mass (ESI-MS): 228 (M$^+$).

C, H analysis for C$_{15}$H$_{13}$FO: Calculated C, 78.93; H, 5.74. Found C, 78.97; H, 5.75.
(E)-2-Bromo-1-methoxy-4-styrylbenzene (12):  

\[
\begin{align*}
\text{H}_3\text{CO} &\quad \text{Br} \\
\text{H}_\text{C-C} &\quad \text{H}_\text{C-C}
\end{align*}
\]

\( ^1\text{H} \text{NMR (200 MHz, CDCl}_3\text{):}\) \(\delta 3.95 \text{ (s, 3H), 6.92 (d, 1H, } J = 8.54 \text{ Hz), 7.02 (m, 2H), 7.29-7.44 (m, 5H), 7.78 (d, 1H, } J = 7.07 \text{ Hz), 8.43 (s, 1H).}\)

Mass (ESI-MS): \(289.17 \text{ (M}^+\text{).}\)

C, H analysis for 
\(\text{C}_{15}\text{H}_{13}\text{BrO:}\) Calculated C, 62.30; H, 4.53. Found C, 62.35; H, 4.52.

(E)-1,3-Difluoro-2-styrylbenzene (13):

\[\begin{align*}
\text{F} &\quad \text{F} \\
\text{H}_\text{C-C} &\quad \text{H}_\text{C-C}
\end{align*}\]

\( ^1\text{H} \text{NMR (200 MHz, CDCl}_3\text{):}\) \(\delta 7.45-7.53 \text{ (m, 5H), 7.62-7.72 (m, 5H).}\)

Mass (ESI-MS): \(216 \text{ (M}^+\text{).}\)

C, H analysis for 
\(\text{C}_{14}\text{H}_{10}\text{F}_2:\) Calculated C, 77.77; H, 4.66. Found: C, 77.72; H, 4.68.

(E)-1,2,3-Trimethoxy-5-(4-methoxystyryl)benzene (14):

\[\begin{align*}
\text{H}_3\text{CO} &\quad \text{OCH}_3 \\
\text{OCH}_3 &\quad \text{OCH}_3 \\
\text{H}_\text{C-C} &\quad \text{OCH}_3 \\
\text{H}_\text{C-C} &\quad \text{OCH}_3
\end{align*}\]

\( ^1\text{H} \text{NMR (200 MHz, CDCl}_3\text{):}\) \(\delta 3.84 \text{ (s, 3H), 3.91 (s, 9H), 6.72 (s, 2H), 6.90 (m, 4H), 7.45 (d, 2H, } J = 8.70 \text{ Hz).}\)

Mass (ESI-MS): \(301 \text{ (M}^+\text{ + H).}\)

C, H analysis for 
\(\text{C}_{18}\text{H}_{20}\text{O}_4:\) Calculated C, 71.98; H, 6.71. Found C, 71.92; H, 6.73.
(E)-1,2,3,4,5-Pentafluoro-6-styrylbenzene (15):

![Chemical structure](image)

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 6.99-7.04 (m, 2H), 7.40-7.46 (m, 3H), 7.50-7.57 (m, 2H).

Mass (ESI-MS): 292.7 (M$^+$ + Na).

C, H analysis for C$_{14}$H$_7$F$_5$:


(E)-9-Styrylanthracene (16):

![Chemical structure](image)

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 6.96 (d, 1H, $J = 16.17$ Hz), 7.02 (d, 1H, $J = 16.22$ Hz), 7.43-7.52 (m, 6H), 7.69-7.74 (m, 2H), 7.96-8.01 (m, 3H), 8.39-8.44 (m, 3H).

Mass (ESI-MS): 280 (M$^+$).

C, H analysis for C$_{22}$H$_{16}$:

Calculated C, 94.25; H, 5.75. Found C, 94.22; H, 5.78.

(E)-3-(3,4-Dimethoxyphenyl)-2-(4-methoxyphenyl)acrylic acid (17):

![Chemical structure](image)

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.81 (s, 3H), 3.85 (s, 3H), 3.91 (s, 3H), 6.35 (d, 1H, $J = 7.75$ Hz), 6.67-7.05 (m, 3H), 7.15 (d, 2H, $J = 8.48$ Hz), 7.46 (d, 1H, $J = 8.56$ Hz), 7.85 (s, 1H).
Mass (ESI-MS): 337 (M⁺ + Na).

C, H analysis for C₁₈H₁₈O₅: Calculated C, 68.78; H, 5.77. Found: C, 68.77; H, 5.78.

(E)-3-(4-Acetoxyphenyl)-2-(4-methoxyphenyl)acrylic acid (18):

![Chemical Structure](image)

¹H NMR (200 MHz, CDCl₃): δ 2.24 (s, 3H), 3.83 (s, 3H), 6.83-6.98 (m, 5H), 7.14-7.21 (m, 3H), 7.85 (s, 1H).

Mass (ESI-MS): 335 (M⁺ + Na).


(E)-3-(4-Hydroxy-3-methoxyphenyl)-2-(4-methoxyphenyl)acrylic acid (19):

![Chemical Structure](image)

¹H NMR (200 MHz, CDCl₃): δ 3.83 (s, 3H), 3.95 (s, 3H), 6.89 (m, 3H), 7.00 (d, 2H, J = 8.13 Hz), 7.43 (d, 2H, J = 8.66 Hz), 7.87 (s, 1H).

Mass (ESI-MS): 301 (M⁺ + H).

(E)-Methyl-3-(4-hydroxy-3-methoxyphenyl)-2-(4-methoxyphenyl)acrylate (20):

\[
\begin{align*}
&\text{\textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3})}: \quad \delta 3.49 (s, 3H), 3.97 (s, 6H), 7.05 (d, 2H, J = 7.48 Hz), 7.43-7.49 (m, 6H). \\
&\text{Mass (ESI-MS)}: \quad 314 (M\textsuperscript{+}). \\
&\text{C, H analysis for } \text{C}_{18}\text{H}_{18}\text{O}_{5}: \quad \text{Calculated C, 68.78; H, 5.77. Found C, 68.74; H, 5.78.}
\end{align*}
\]

(E)-3-(4-Methoxyphenyl)-2-phenylpropenal (21):

\[
\begin{align*}
&\text{\textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3})}: \quad \delta 3.79 (s, 3H), 6.75 (d, 2H, J = 8.84 Hz), 7.15-7.44 (m, 8H), 9.73 (s, 1H). \\
&\text{Mass (ESI-MS)}: \quad 261 (M\textsuperscript{+} + Na). \\
&\text{C, H analysis for } \text{C}_{16}\text{H}_{14}\text{O}_{2}: \quad \text{Calculated C, 80.65; H, 5.92. Found C, 80.68; H, 5.96.}
\end{align*}
\]

(E)-2,3-bis(4-Hydroxyphenyl)acrylonitrile (22):

\[
\begin{align*}
&\text{\textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3})}: \quad \delta 5.32 (\text{broad s, } 2 \times \text{OH}), 6.76 (d, 2H, J = 7.07 Hz), 6.83 (d, 2H, J = 7.02 Hz), 7.57-7.75 (m, 4H), 8.04 (s, 1H).
\end{align*}
\]
C, H, N analysis for C\textsubscript{15}H\textsubscript{11}NO\textsubscript{2}: Calculated C, 75.94; H, 4.67; N, 5.90. Found C, 75.95; H, 4.65; N, 5.94.

(E)-2-(4-Hydroxyphenyl)-3-(pyridin-3-yl)acrylonitrile (23):

\[
\begin{align*}
\text{HO} & \quad \text{CN} \\
\text{N} & \quad \text{CN}
\end{align*}
\]

\(\text{\textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3})}:\) \(\delta\) 5.32 (broad s, OH), 6.90 (m, 3H), 7.46 (s, 1H), 7.49 (d, 2H, \(J = 8.71\) Hz), 7.75 (d, 2H, \(J = 8.71\) Hz), 8.26 (s, 1H).

Mass (ESI-MS): 245 (M+ + Na).
C, H, N analysis for C\textsubscript{14}H\textsubscript{10}N\textsubscript{2}O: Calculated C, 75.66; H, 4.54; N, 12.60. Found C, 75.67; H, 4.52; N, 12.62.

2.2.3. 3. Biological experiments

All the synthesized compounds were screened for their tyrosinase inhibitory activity using resveratrol (1) (3,5,4´-Trihydroxy-trans-stilbene) as the positive control.

Materials and methods

L-3,4-Dihydroxyphenylalanine (L-DOPA), resveratrol (3,5,4´-Trihydroxy-trans-stilbene) and other chemical reagents were purchased from Aldrich Chemical Co.

The inhibitory effect of all synthesised stilbenoid derivatives on murine tyrosinase activity was evaluated using L-DOPA as substrate. Tyrosinase was prepared from murine B16 melanoma cells (Riken Cell Bank, Tsukuba, Japan). The cells were lysed by incubating at 4 °C for 1 h in lysis buffer (10 mM Tris HCL, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 10 µg/mL leupeptin). The lysates were centrifuged at 50,000 x g for 30 min to obtain the supernatant as a source of tyrosinase. The reaction mixture contained 50 mM phosphate buffer, pH 6.8, 0.05% L-DOPA and the supernatant (tyrosinase). After incubation in the absence or presence of various stilbene derivatives (with varying concentrations ranging from 1
µg/mL to 100 µg/mL) at 37 ºC for 20 min, dopachrome was monitored by measuring absorbance at wavelength 492 nm by using a Molecular Devices microplate reader. Finally the IC$_{50}$ values were calculated using Microsoft Office Excel.

2.2.4. Results

The results from our investigations aimed to explore the inhibitory effects of the stilbene compounds on the murine tyrosinase activity are presented in table 4. In these investigations, the inhibitory effects of all the compounds screened for the said activity were evaluated at varying concentrations (10, 30 and 100 µg/mL). As is evident from the data, all the screened compounds showed inhibitory effect on murine tyrosinase activity.

![Table 4: Effect of stilbenoid derivatives on murine tyrosinase activity](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition for tyrosinase in µg/mL concentration</th>
<th>IC$_{50}$ value (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% inhibition for tyrosinase in µg/mL concentration</td>
<td>IC$_{50}$ value (µg/mL)</td>
</tr>
<tr>
<td>(2)</td>
<td>6.49 12.48 28.66</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(3)</td>
<td>41.00 67.15 99.28</td>
<td>15.73</td>
</tr>
<tr>
<td>(4)</td>
<td>16.64 27.34 65.96</td>
<td>68.62</td>
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<tr>
<td>(5)</td>
<td>13.08 26.65 63.81</td>
<td>71.63</td>
</tr>
<tr>
<td>(6)</td>
<td>14.94 23.75 60.47</td>
<td>81.41</td>
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<tr>
<td>(7)</td>
<td>49.04 62.28 81.36</td>
<td>10.24</td>
</tr>
<tr>
<td>(8)</td>
<td>10.53 6.82 11.12</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(9)</td>
<td>57.27 63.02 63.60</td>
<td>3.41</td>
</tr>
<tr>
<td>(10)</td>
<td>4.64 15.09 55.51</td>
<td>90.88</td>
</tr>
<tr>
<td>(11)</td>
<td>50.05 55.01 68.43</td>
<td>11.41</td>
</tr>
<tr>
<td>(12)</td>
<td>38.83 71.33 100</td>
<td>16.24</td>
</tr>
<tr>
<td>(13)</td>
<td>9.83 22.41 56.76</td>
<td>85.10</td>
</tr>
<tr>
<td>(14)</td>
<td>2.12 5.69 52.69</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(15)</td>
<td>5.94 8.35 29.24</td>
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<tr>
<td>(16)</td>
<td>17.07 28.64 100.00</td>
<td>46.60</td>
</tr>
<tr>
<td>(17)</td>
<td>10.20 1.95 0</td>
<td>NT</td>
</tr>
<tr>
<td>(18)</td>
<td>4.04 0.23 0</td>
<td>NT</td>
</tr>
<tr>
<td>(19)</td>
<td>2.24 0.69 0</td>
<td>NT</td>
</tr>
<tr>
<td>(20)</td>
<td>1.44 1.48 10.64</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(21)</td>
<td>16.55 21.68 76.83</td>
<td>65.70</td>
</tr>
<tr>
<td>(22)</td>
<td>90.94 94.33 94.43</td>
<td>1.20</td>
</tr>
<tr>
<td>(23)</td>
<td>34.06 40.18 57.50</td>
<td>60.26</td>
</tr>
<tr>
<td>(1)</td>
<td>80.74 88.68 94.02</td>
<td>2.46</td>
</tr>
</tbody>
</table>

a) Measurement of tyrosinase activity was performed as described in materials and methods.

b) Less than 10% inhibition at the concentration of 100µg/mL. NT, not tested.

c) Resveratrol (I) was used as a positive control.
However, among all the compounds we screened, compounds 3, 7, 9, 11, 12, 16 and 22 showed remarkably significant tyrosinase inhibitory activity at investigated range of concentrations. Compounds 7, 9 and 22 proved to be very effective for tyrosinase inhibition in comparison to 1. Compound 22 was found to have a strong inhibitory effect on murine tyrosinase activity (IC$_{50} = 1.20$ µg/mL) even higher that observed for the standard, 1 (IC$_{50} = 2.46$ µg/mL). Since the % inhibition by 1, 7, 9 and 22 was more than 50% at 10 µg/mL, the dose response study for these compounds was also carried at 1 µg/mL and the recorded observations are presented as table 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition for tyrosinase at 1 µg/mL concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.49</td>
</tr>
<tr>
<td>7</td>
<td>18.17</td>
</tr>
<tr>
<td>9</td>
<td>24.34</td>
</tr>
<tr>
<td>22</td>
<td>40.46</td>
</tr>
</tbody>
</table>

Table 5: Effect of stilbenoid derivatives at lower concentrations on murine tyrosinase activity

We observed that compound 22 exhibits maximum inhibitory effects, with 40.46% inhibition at 1 µg/mL, 90.94% at 10 µg/mL, 94.33% at 30 µg/mL and 94.43% at 100 µg/mL and an IC$_{50}$ value of 1.20 µg/mL on murine tyrosinase activity (Figure 1). Resveratrol (1) showed similar levels of inhibitory effects on the enzyme activity with 41.49% inhibition at 1 µg/mL, 80.74% at 10 µg/mL, 88.68% at 30 µg/mL and 94.02% at 100 µg/mL and an IC$_{50}$ value of 2.46 µg/mL (Figure 1).

![Figure 1: Dose-dependent inhibitory effects on murine tyrosinase by compounds 1 and 22. Effects on tyrosinase activity by the samples as a function of concentration are represented as inhibition %, mean ± S.E. of three independent tests.](image)
Thus it seems that compound 22 exhibits a 2-fold stronger inhibitory effect on murine tyrosinase activity than the standard (Table 4). In the investigated series of compounds, 3, 4, 5, 7, 9, 11, 12, 16, 21 and 23 were less active than 22, while 6 and 13 were found to be least active. Compounds 2, 8, 10, 14, 15, 17-20 exerted little or no inhibitory effect compared to the impact of rest of the investigated compounds on tyrosinase activity.

2.2.5. Discussion

In the series of stilbene moieties possessing only electron withdrawing groups, we found that their IC$_{50}$ values vary in the order of 7 (IC$_{50} = 10.24 \mu g/mL$) > 3 (IC$_{50} = 15.73 \mu g/mL$) > 4 (IC$_{50} = 68.62 \mu g/mL$) > 5 (IC$_{50} = 71.63 \mu g/mL$) > 6 (IC$_{50} = 81.41 \mu g/mL$) > 8 (IC$_{50} > 100 \mu g/mL$). In view of these observations, we therefore infer that inhibitory potency of these substituents varies in the order of CN > F > Cl > Br > NO$_2$ > OCOCH$_3$. All these compounds significantly inhibited the activity of murine tyrosinase but were less active in comparison to the standard (1). Their inhibitory effect was also compared with compounds 2, 9, 10 and 14. We observed that while stilbene without any substitution (2) is least effective with an IC$_{50}$ value of >100 \mu g/mL, compound 9 possessing hydroxy group proved to be very potent with an IC$_{50}$ value of 3.41 \mu g/mL. These results indicate that presence of phenolic hydroxy group in aromatic ring is very effective for tyrosinase inhibitory activity in stilbene derivatives. Since the IC$_{50}$ values were lower for hydroxyl (OH) and cyano (CN) group, we synthesized compound 22 (containing both groups) and evaluated its tyrosinase inhibitory activity. In our investigations, we found that compound 22 shows a stronger suppressive action on the enzyme tyrosinase with IC$_{50}$ value of 1.20 \mu g/mL, in comparison to the standard for which IC$_{50}$ value of 2.46 \mu g/mL was observed under similar conditions. To assess the efficacy of compound 22 against other tyrosinase inhibitors, its effect was compared with the well-known tyrosinase inhibitors viz., oxyresveratrol (IC$_{50} = 12.8 \mu g/mL$)\textsuperscript{1} and gnetol (IC$_{50} = 1.098 \mu g/mL$)\textsuperscript{8} which are often used as standard inhibitors for murine tyrosinase activity. Interestingly, the tyrosinase inhibitory effect we observed for 22 was significantly stronger than that observed for oxyresveratol and almost equivalent to what has been reported for gnetol. This proves that compared to three hydroxyl groups in stilbene
skeleton (as in resveratrol), a cyano group together with two hydroxyl groups lead to better tyrosinase inhibition (as observed for compound 22).

Activity studies for compound 23 indicate that pyridine ring reduces the inhibitory power to a great extent. This is evident from IC\textsubscript{50} value of this compound, which is equal to 60.26 µg/mL. The results for compound 16 (IC\textsubscript{50} = 46.60 µg/mL) indicate that increase in the aromatic character of the stilbene moiety increased its inhibitory potential.

Attachment of methoxy groups to the basic skeleton has been found to reduce the inhibitory activity.\textsuperscript{7} This is well attested by our observations for compounds 10 (IC\textsubscript{50} = 90.88 µg/mL) and 14 (IC\textsubscript{50} > 100 µg/mL) carrying one and four methoxy groups respectively. To ascertain the role of such groupings, we introduced electron withdrawing groups to methoxy/acetoxy substituted stilbenes and evaluated their inhibitory potential. Results obtained were quite interesting for compounds 11, 12 and 21 possessing F, Br and an aldehydic (-CHO) group with IC\textsubscript{50} values of 1.41 µg/mL, 6.24 µg/mL and 65.70 µg/mL respectively. As is clear from data related to compounds 17-20, COOH/COOCH\textsubscript{3} groups showed no effect over the inhibitory effect of stilbenes. Even the introduction of hydroxyl group to these moieties (compounds 19 and 20) was observed to have no significant effect on their inhibitory potential. With an increase in number of electron withdrawing groups in the basic skeleton, we observed a subsequent decrease in the inhibitory power of these compounds. This is clearly visible from results recorded for compounds 3 (IC\textsubscript{50} = 15.73 µg/mL), 13 (IC\textsubscript{50} = 85.10 µg/mL) and 15 (IC\textsubscript{50} > 100.24 µg/mL) possessing one, two and six fluorine atoms respectively.

From our observations recorded in the present study, we conclude that the presence of hydroxyl group on phenyl rings is important for the inhibition of tyrosinase activity and apart from the hydroxyl groups; electron withdrawing groups play an important role in influencing the tyrosinase inhibitory activity of stilbenes. Moreover, electron withdrawing and hydroxyl groups jointly exert a synergistic effect in the basic structure, thereby markedly influencing its tyrosinase inhibitory activity. Similarly the addition of electron withdrawing groups to stilbene moieties containing methoxy groups enhances their tyrosinase inhibitory activity. Our results also prove that aldehydic (-CHO) group is more effective than acidic (-COOH) group in enhancing
the tyrosinase inhibitory activity. The reason for lesser impact of -COOH than that of -CHO group in the stilbene moiety may be the higher steric hinderance due to the former that reduces its ability to bind with the enzyme. Further, on the basis of our observations, we argue that presence of halogens and increased aromatic character appreciably affects the tyrosinase inhibition activity of stilbenes. In summary, the results presented in the current section indicate that the variety of trans-stilbene moieties synthesized for the present work, exhibit excellent tyrosinase inhibition activity and hence posses a good potential for their use as pharmacological or cosmetic agents.

2.2.6. Conclusion
The results presented in current section are presaged to provide useful clues for the design and development of new, safe and effective tyrosinase inhibitors. However, some more concrete studies with a human clinical point of view are required to lay down a fool proof set of rules to be followed for the design of tyrosinase inhibitors with desired characteristics.
2.2.7. References


$^1$H NMR spectrum of compound 22
ESI-MS spectrum of compound 22
$^{1}$H NMR spectrum of compound 18
ESI-MS spectrum of compound 18