ภาคผนวก 3 : ผลการวิจัยดีพิมพ์ เรื่อง Anti-HIV-integrase activity of medicinal plants used as self medication by AIDS patients.
Anti-HIV-1 integrase activity of medicinal plants used as self medication by AIDS patients

Supinya Tewtrakul¹, Sanan Subhadhirasakul² and Sopa Kummee³

Abstract
Tewtrakul, S., Subhadhirasakul, S., Kummee, S
Anti-HIV-1 integrase activity of medicinal plants used as self medication by AIDS patients

The extracts of selected medicinal plants used as self medication by AIDS patients were investigated for their inhibitory activities against HIV-1 integrase (HIV-1 IN) using the multiplate integration assay (MIA). Of these, the water extract of Eclipta prostrata (whole plant) exhibited the most potent inhibitory activity with an IC₅₀ value of 4.8 µg/ml, followed by the methanol extract of Eclipta prostrata (whole plant, IC₅₀ = 21.1 µg/ml), the water extract of Barleria lupulina (stem, IC₅₀ = 26.4 µg/ml), the chloroform extract of Barleria lupulina (stem, IC₅₀ = 33.0 µg/ml), the methanol extract of Barleria lupulina (stem, IC₅₀ = 38.2 µg/ml) and the chloroform extract of Piper betle (leaf, IC₅₀ = 39.3 µg/ml), respectively.

Key words: HIV-1 integrase, self medication, AIDS patients

¹Ph.D.(Pharmaceutical Sciences), Asst. Prof., ²Ph.D.(Pharmaceutical Sciences), Assoc. Prof., ³M.Sc.(Microbiology), Scientist, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand.
Corresponding e-mail: supinyat@yahoo.com
Received, 19 September 2005 Accepted, 3 December 2005
The effectiveness of combination therapy for HIV infection with drugs targeting protease and reverse transcriptase has recently been acknowledged. However, issues of patient compliance, drug toxicity, the emergence of multidrug-resistant phenotypes, and the presence of persistent reservoir of virus replication have launched the need to develop alternative therapeutic approaches utilizing other drug targets in the viral replication cycle. One of the essential enzymes in HIV-1 life cycle is the integrase. HIV-1 integrase (HIV-1 IN) integrates transcribed double strand DNA into the host chromosome (Kat and Skalka, 1994). Fusion of viral DNA with host chromosome subsequently triggers immunodeficiency that make patients susceptible to fatal opportunistic infections. Eleven Thai medicinal plants were studied for their inhibitory activities against HIV-1 IN. Most of them have been used in the primary health care project in Thailand and by AIDS patients. They are *Zingiber zerumbet* (rhizome), *Boesenbergia pandurata* (rhizome), *Piper chaba* (fruit), *Eclipta prostrata* (whole plant), *Barleria lupulina* (leaf, stem), *Acanthus ilicifolius* (leaf and stem), *Alpinia galanga* (rhizome), *Piper betle* (leaf), *Spilanthes acmella* (whole plant), and *Coccinia grandis* (leaf). Previously, we reported HIV-1 protease (HIV-1 PR) inhibitory effects and antifungal activities against opportunistic fungal pathogens of these Thai plants (Tewtrakul et al., 2003; Phongpaichit et al., 2005).

The aim of the present study was therefore to investigate HIV-1 IN inhibitory effects of these Thai medicinal plants used as self medication for AIDS treatment.

**Materials and Methods**

**Plant materials and preparation of extracts**

The plants were collected at the botanical garden of Prince of Songkla University and some areas in Songkhla province, Thailand, and were identified by Assoc. Prof. Dr. Sanan Subhadhirasakul. The voucher specimens are deposited at the Herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Ten grams of each dried plant were extracted successively by maceration for 1 week (3 times) with 200 ml of chloroform and methanol. After that, the marc left from methanol extraction was extracted with boiling water 200 ml for 3 hrs (3 times). The solvents were removed under reduced pressure to give chloroform-, methanol- and water extracts, respectively. The extracts were dissolved in 50% DMSO for bioassay.

**Enzymes and chemicals**

HIV-1 IN protein was expressed in *Escherichia coli*, purified according to the method described in the previous paper (Jenkins et al., 1996) and stored in -80°C before use.
Assay for HIV-1 IN inhibitory activity

**Oligonucleotide substrates**

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at -25°C before use. The sequence of biontillated LTR donor DNA and its unlabelled complement were 5’-biotin-ACCCTTTAGTCA GGTGGAAAATCTCTAGGAGT-3’ (LTR-D1) and 3’-GAAAATCATGTCACACTTCTTGTAGAG TCGTCA-5’ (LTR-D2), respectively; while those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3’-labelled complement were 5’TGACCAAGGGCTAATCTCAGGTC ACCCAGGATTA GTGA-5’ (TS-2), respectively.

**Multiplate integration assay (MIA)**

**Annealing of the substrate DNA**

Firstly, LTR-D1 and LTR-D2, TS-1 and TS-2 were mixed separately and then the final solution was diluted to a concentration of 2 pmol/ml, while the later one was made to 5 pmol/ml using a buffer solution containing 10 mM Tris-HCl (pH 8.0), 1mM EDTA and 100 mM KCl. Both solutions were then heated at 85°C for 15 min in an incubator. After heating, each solution was gradually cooled to room temperature and stored at -20°C until use.

**Pretreatment of the multiplex**

A 96-well plate was coated with 50 μl of a streptavidin solution containing 40 μg/ml streptavidin, 90 mM NaCO3 and 10 mM KCl. After discarding streptavidin coating solution, the coated plate was washed with 300 μl of sterile water twice and phosphate buffer saline (PBS, 300 μl) twice. The blocking buffer (300 μl) containing 1% skim milk in PBS was added into each well and the plate was kept at room temperature for 30 min. After discarding the blocking buffer, each well was washed with PBS solution (300 μl) four times and then the PBS solution was completely removed. A biontillated LTR donor DNA (50 μl) solution containing 10 mM Tris-HCl (pH 8.0), 1mM NaCl and 40 fmol/ml of LTR donor DNA was added into each well and the plate was shaken well, centrifuged and kept at room temperature for 60 min. After discarding the LTR donor solution, the microplate was washed with PBS solution four times and then each well was filled with 300 μl of PBS solution. Just before the integration reaction, the PBS solution of each well was discarded and rinsed with 300 μl of distilled water four times, and then the distilled water was removed completely.

**Integration reaction**

The integration reaction was evaluated according to the method previously described (Tewtrakul et al., 2001). A mixture (45 μl) composed of 12 μl of IN buffer [containing 150 mM 3-(N-morpholino)propane sulfonic acid, pH 7.2 (MOPS), 75 mM MnCl2, 5 mM dithiothreitol (DTT), 25% glycerol and 500 μg/ml bovine serum albumin], 1 μl of 5 pmol/ml digoxigenin-labelled target DNA and 32 μl of sterilized water were added into each well of a 96-well plate. Subsequently, 6 μl of sample solution and 9 μl of 1/5 dilution of integrase enzyme was added to the plate and incubated at 37°C for 80 min. After wells were washed with PBS 4 times, 100 μl of 500 mU/ml alkaline phosphatase (AP) labelled anti-digoxigenin antibody were added and incubated at 37°C for 1 hr. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS 4 times and with PBS 4 times. Then, AP buffer (150 μl) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl2 and 10 mM p-nitrophenyl phosphate was added to each well and incubated at 37°C for 1 hr. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control composed of a reaction mixture, 50% DMSO and an integrase enzyme, and a blank composed of buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA-2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme were employed. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control.

% Inhibition against HIV-1 IN

= [(OD control - OD sample)/OD control] x 100

*OD = absorbance detected from each well
Statistics

For statistical analysis, the results of anti-HIV-1 IN were expressed as mean ± S.D of four determinations. The IC_{50} values were calculated using the Microsoft Excel program.

Results and Discussion

Chloroform-, MeOH- and water extracts of Thai medicinal plants widely used in the primary health care project of Thailand, were prepared and tested for their HIV-1 IN inhibitory activities. As shown in Table 1, Table 2 and Figure 1, the results indicated that the water- and MeOH extracts of Eclipta prostrata (whole plant) strongly inhibited HIV-1 IN activity with IC_{50} values of 4.8 and 21.1 µg/ml, respectively, followed by the water extract of Barleria lupulina (IC_{50} = 26.4 µg/ml), the CHCl_{3} extract of B. lupulina (IC_{50} = 33.0 µg/ml), the MeOH extract of B. lupulina (IC_{50} = 38.2 µg/ml), the CHCl_{3} extract of P. betel (IC_{50} = 39.3 µg/ml) and the water extract of Coccinia glandis (IC_{50} = 44.6 µg/ml). This is the first report indicating that E. prostrata possesses active compounds having anti-HIV-1 IN activity. Previously, we reported HIV-1 PR inhibitory activities of these Thai plants (Tewtrakul et al., 2003). The result indicated that Boesenbergia pandurata extract exhibited the most potent activity against HIV-1 PR, whereas Eclipta prostrata possessed only weak activity. However, E. prostrata significantly inhibited HIV-1 IN activity in the present study. The result may imply that this plant shows selective inhibition of HIV-1 IN but not of HIV-1 PR. Regarding the chemical constituents of E. prostrata, it has been reported to contain coumestans (Wagner et al., 1986; Yahara et al., 1997), triterpenoid glycosides (Singh and Bhargava, 1992), thiophene derivatives (Yahara et al., 1997), triterpenoid saponins (Zhao et al., 2001), flavonoids (Sheih and Tsai, 1985) and wederolactone (Samiulla et al., 2003). Previous reports indicated that E. prostrata possessed various biological activities including anti-inflammatory (Kobori et al., 2004), immunomodulatory effect on T-lymphocytes (Liu et al., 2001), antimicrobial activity (Wiert et al., 2004) and hepatoprotective activities (Han et al., 1998). Therefore, the present result on anti-HIV-1 IN activity provides support for the beneficial effects of using this plant in AIDS treatment. The isolation of active principles against HIV-1 IN from E. prostrata is now in progress.

Table 1. HIV-1 integrase inhibitory activities of some Thai medicinal plants used as self medication for AIDS treatment at concentration of 100 µg/ml.

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Family</th>
<th>Part used</th>
<th>CHCl_{3} extract</th>
<th>MeOH extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Zingiber zerumbet L.</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>53.6±3.60</td>
<td>38.3±6.91</td>
<td>15.0±4.55</td>
</tr>
<tr>
<td>2. Boesenbergia pandurata</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>51.4±3.40</td>
<td>28.7±6.53</td>
<td>13.0±7.43</td>
</tr>
<tr>
<td>3. Alpinia galanga L.</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>-5.14±5.90</td>
<td>55.7±4.17</td>
<td>56.0±5.66</td>
</tr>
<tr>
<td>4. Piper chaba Hunt.</td>
<td>Piperaceae</td>
<td>Fruit</td>
<td>28.0±2.49</td>
<td>23.8±2.61</td>
<td>3.7±0.21</td>
</tr>
<tr>
<td>5. Piper betel L.</td>
<td>Piperaceae</td>
<td>Leaf</td>
<td>76.3±0.86</td>
<td>49.8±0.47</td>
<td>36.4±0.97</td>
</tr>
<tr>
<td>6. Eclipta prostrata L.</td>
<td>Compositae</td>
<td>Whole-plant</td>
<td>25.7±2.31</td>
<td>91.5±0.36</td>
<td>101.0±0.20</td>
</tr>
<tr>
<td>7. Spilanthes acmella L.</td>
<td>Compositae</td>
<td>Whole-plant</td>
<td>40.5±2.28</td>
<td>2.9±0.16</td>
<td>19.2±5.33</td>
</tr>
<tr>
<td>8. Barleria lupulina Lindl.</td>
<td>Acanthaceae</td>
<td>Leaf</td>
<td>29.1±3.49</td>
<td>61.6±0.78</td>
<td>60.6±3.48</td>
</tr>
<tr>
<td>9. Barleria lupulina Lindl.</td>
<td>Acanthaceae</td>
<td>Stem</td>
<td>72.5±0.88</td>
<td>83.6±0.56</td>
<td>83.9±0.59</td>
</tr>
<tr>
<td>10. Acanthus ilicifolius L.</td>
<td>Acanthaceae</td>
<td>Leaf and stem</td>
<td>37.9±5.46</td>
<td>45.6±2.85</td>
<td>40.6±1.93</td>
</tr>
<tr>
<td>11. Murraya paniculata L.</td>
<td>Rutaceae</td>
<td>Leaf</td>
<td>29.1±1.02</td>
<td>12.9±1.58</td>
<td>10.1±4.83</td>
</tr>
<tr>
<td>12. Coccinia glandis L.</td>
<td>Cucurbitaceae</td>
<td>Leaf</td>
<td>9.6±1.41</td>
<td>14.6±3.09</td>
<td>75.6±0.39</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± S.D (n = 4).
Table 2. The IC\textsubscript{50} against HIV-1 IN of active plant extracts used as self medication by AIDS patients

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Family</th>
<th>Part used</th>
<th>% Inhibition at various concentrations</th>
<th>IC\textsubscript{50} (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Piper betle</em> L. (CHCl\textsubscript{3})</td>
<td>Piperaceae</td>
<td>Leaf</td>
<td>10.25±5.28 43.07±3.37 76.32±0.68 91.58±0.36</td>
<td>39.3</td>
</tr>
<tr>
<td>2. <em>Eclipta prostrata</em> L. (MeOH)</td>
<td>Compositae</td>
<td>Whole-plant</td>
<td>21.65±4.80 71.24±2.22 91.58±0.36</td>
<td>21.1</td>
</tr>
<tr>
<td>3. <em>Eclipta prostrata</em> L. (Water)</td>
<td>Compositae</td>
<td>Whole-plant</td>
<td>37.50±2.54 67.59±1.09 85.68±0.43 101.06±0.20</td>
<td>4.8</td>
</tr>
<tr>
<td>4. <em>Barleria lupulina</em> Lindl. Acanthaceae (Water)</td>
<td>Stem</td>
<td></td>
<td>24.06±2.23 54.57±1.06 83.93±0.59</td>
<td>26.4</td>
</tr>
<tr>
<td>5. <em>Barleria lupulina</em> Lindl. Acanthaceae (MeOH)</td>
<td>Stem</td>
<td></td>
<td>0.20±2.22 43.58±4.56 83.69±0.56</td>
<td>38.2</td>
</tr>
<tr>
<td>6. <em>Barleria lupulina</em> Lindl. Acanthaceae (CHCl\textsubscript{3})</td>
<td>Stem</td>
<td></td>
<td>24.84±4.81 48.75±2.21 72.58±0.88</td>
<td>33.0</td>
</tr>
<tr>
<td>7. <em>Coccinia glandis</em> L. (Water)</td>
<td>Cucurbitaceae</td>
<td>Leaf</td>
<td>6.43±7.95 35.32±5.15 75.62±0.39</td>
<td>44.6</td>
</tr>
</tbody>
</table>

Suramin, a positive control 43.62±1.62 88.45±2.43 98.42±0.64 99.89±0.45 3.2

The results are expressed as mean ± S.D (n = 4)

Figure 1. Dose concentration dependence against HIV-1 IN activity of some plants used as self medication by AIDS patients. BL (*Barleria lupulina*), PB (*Piper betle*), EP (*Eclipta prostrata*), CG (*Coccinia glandis*), C (Chloroform extract), M (Methanol extract) and W (Water extract).

Acknowledgements

The authors are grateful to the Thai Government Budget for the grant and to Dr. Robert Craigie for providing of HIV-1 integrase enzyme. We also thank the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, for providing laboratory facilities.

References


Anti-HIV-1 integrase activity of medicinal plants