**Immunoregulatory and anti-HIV-1 enzyme activities of antioxidant components from lotus (*Nelumbo nucifera* Gaertn.) rhizome**

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**Abbreviations used:**

- AZT-TP, azidothymidine triphosphate
- BHA, butylated hydroxyanisole
- FDA, Food and Drug Administration
- FTIR, Fourier-transform IR
- GAPDH, glyceraldehyde-3-phosphate dehydrogenase
- IFN-γ, interferon γ
- IL-2, interleukin-2
- IN, integrase
- IOD, inhibition of delay
- IPTG, isopropyl β-D-thiogalactoside
- LTR, long terminal repeat
- NK, natural killer
- PR, protease
- RT, reverse transcriptase
- Th1, T-helper 1
- TNF-α, tumour necrosis factor α
- AZT-TP, azidothymidine triphosphate
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**INTRODUCTION**

*Nelumbo nucifera* Gaertn. is an aquatic deciduous perennial plant native to Asia, New Guinea, Queensland and the Northern Territory. It has been used in the Orient as food and a medicinal herb for over 1500 years. It has astringent, cardiotonic, febrifuge, hypotensive, tonic and vasodilating properties [1]. Previous studies focused on the seeds [2], leaves [3] and rhizome knots [4] of *N. nucifera*. Studies on rhizomes, the main edible part of lotus, were rare [5]. Lotus rhizome is one of the daily vegetables for many people all over the world. It is worth investigating active components in lotus rhizomes.

AIDS caused by HIV is still one of the lethal diseases. From the latest report of the WHO (World Health Organization) (December 2009), 33.4 million people were newly infected with HIV-1 in 2008 and there were 2.0 million AIDS-related deaths (http://www.unaids.org). There is still no cure for AIDS.

There is evidence that free radical damage contributes to many chronic health problems such as cardiovascular disease, cancer, inflammatory diseases and AIDS [6]. Inflammation occurring in these diseases is believed to be accompanied by the production of free radicals [7]. Oxidative stress plays a significant role in the pathogenesis of HIV-1/AIDS by promoting lymphocyte cell death [8]. Cytokines are critical regulatory factors and play important roles in the immune system. One theory of immune regulation involves homoeostasis between Th1 (T-helper 1) and Th2 activity. The capacity of the immune system to trigger primary Th1 or Th2 CD4+ T-cell responses depends on both the differential patterns of cytokines. Th1 cells drive the type-1 pathway (‘cellular immunity’) to fight viruses and other intracellular pathogens, and to eliminate cancerous cells. Th2 cells drive the type-2 pathway (‘humoral immunity’) and up-regulate antibody production to fight extracellular organisms [9]. Secretion of IFNγ (interferon γ) and IL-2 (interleukin-2) by CD4+ Th1 lymphocytes [as well as by CD8+ T-cells and NK (natural killer) cells] triggers
Macrophage activation and a potent response against intracellular pathogens, whereas Th2 cells typically produce IL-4, IL-6 and IL-10 driving humoral immunity such as Ig production and isotype switch from IgG to IgE in cells [10]. Many reports indicate an immune pathway shift from Th1 to Th2 after infection by HIV-1 [11]. All these studies indicate a close relationship among oxidant damage, immune imbalance and AIDS which suggest that antioxidants might play an important role in the treatment of AIDS.

Natural products constitute an important source of drugs. Polyphenols, especially phenolic acids, flavanols and flavonols, have been considered as active components in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases or diabetes [12]. Epidemiological and *in vitro* studies strongly suggest that foods containing phytochemical antioxidants have potentially protective effects against many diseases, including cancer, diabetes and cardiovascular diseases [13]. Natural antioxidant products with complex effects may help reduce oxidative damage and maintain immunological balance in AIDS-infected people. Some catechin derivatives have been known to be inhibitors of HIV-1 RT (reverse transcriptase) for over 20 years [14]. Some investigations indicate that catechin derivatives can inhibit HIV-1 replication with multiple mechanisms of action including inhibiting HIV-1 entry into target cells by blocking HIV-1 envelope glycoprotein-mediated membrane fusion [15]. However, few studies have been focused on the effects of catechins on HIV-1 PR (protease) and IN (integrate).

The aim of this study was to isolate potential natural antioxidant components from lotus rhizomes. Their effects on HIV-1 key enzymes and the regulation of main cytokines were studied.

**MATERIALS AND METHODS**

**Reagents**

BHA (butylated hydroxyanisole), TBA (thiobarbituric acid), L-ascorbic acid, Folin–Ciocalteau reagent, AZT-TP (azidothymidine triphosphate) and peptatin A were obtained from Sigma. AAPH [2,2-azobis-(2-amidinopropane) dihydrochloride] was purchased from Wako. Raltegravir and indinavir. The mice were housed under normal laboratory conditions (25 ± 2 °C, 12 h light/12 h dark) with free access to standard rodent chow and water. The animal experiments were performed in compliance with guidelines for animal research laid down by Nankai University.

**Sample preparation**

Lotus (*N. nucifera* Gaertn.) rhizomes called ‘Piaohua’ which were produced in Tianjin, China were bought from a local market in October. The lotus rhizomes were cleaned thoroughly with water to remove residual compost, cut into small pieces and crushed. The juice was decanted and centrifuged at 2000 g/min for 10 min to remove the precipitate. The supernatant was condensed and freeze-dried to form the crude extract named L.

The macroporous resin named NKA was used for chromatography of the crude extract L. NKA is composed of cross-linked polystyrene. It has no polarity. The bead size ranges from 0.3 to 1.0 mm, the surface area is 570–590 m²/g and the average pore diameter is 200–220 Å (1 Å = 0.1 nm). L was dissolved (500 mg/ml) in distilled water. The solution was kept at 4 °C for 12 h after adding 200 μl of 0.1 M phosphate buffer (pH 7.4). The supernatant was then transferred another clean beaker and then 200 μl of ethanol was added. The resulting solution was kept at 4 °C for 12 h. The precipitate was collected by centrifugation at 10,000 g for 10 min at 4 °C. The supernatant was discarded, and the precipitate was redissolved in 200 μl of distilled water.

**Assay for inhibition of erythrocyte haemolysis**

Erythrocyte haemolysis was mediated by peroxyl radicals in the assay system [16]. The percentage of haemolysis was calculated using the equation (1 − A/B) × 100%. L-Ascorbic acid was used as a positive control.

**Assay of lipid peroxidation using kidney homogenates**

BHA was used as a positive control. The inhibition ratio was calculated using the following equation: inhibition ratio
(%)) = (A - A1)/A × 100%, where A is the absorbance of the control, and A1 is the absorbance of the test sample [17].

Assay of HIV-1 RT inhibitory activity
Samples were tested for RT inhibitory activity against a purified recombinant HIV-1 RT (Merck) using the cell-free RT colorimetric kit (Roche Diagnostics). The screening assay for inhibitors was performed as described in the protocol included with the kit [18]. The standard RT inhibitor AZT-TP was used as a positive control.

Inhibition rate (%) = (A0 - A)/A0 × 100%,

where A0 is the absorbance of the system with RT but without any sample; A is the absorbance of the system with RT and sample; A0 is the absorbance of the system without RT and sample.

Assay of HIV-1 PR inhibitory activity
Recombinant HIV-1 PR of Escherichia coli origin was tested [19] in this assay. Pepstatin A exhibited higher PR inhibition and stability than IDV (indinavir) and RTV (ritonavir) in our previous studies. It was also a positive control in many studies that focused on PR inhibitor screening [20,21]. In this assay, the blank was the culture without IPTG (isopropyl β-D-thiogalactoside), the positive control was pepstatin A and the negative control was the culture with IPTG but without any samples.

The growth curves are shown as y = k × ln x + b

where A490 and x is the incubation time.

Growth decrease rate (%) = (Ks - K0)/(K0 - K0) × 100%

where K0 is the slope of the growth curve of bacteria with IPTG but without sample, Ks is the slope of the growth curve of bacteria with IPTG and sample and K0 is the slope of the growth curve of bacteria without IPTG and sample.

Assay for HIV-1 IN inhibitory activity
Two site-directed mutageneses F185K and C280S were induced into the full-length IN to increase its solubility by overlapping PCR [22]. The upstream fragment of IN was amplified by Primer 1 and Primer 2, whereas the downstream fragment was amplified by Primer 2 and Primer 3. The full-length IN was amplified by Primer 1 and Primer 2 with upstream and downstream fragments: P1: 5'-CAGCATATGGATATAGATAGCCCAAGATGGA-3'; P2: 5'-ATAGGAATCC(EcoR I)TTTACTATCCATCTGCTACTGCGGACACGACGTGTGATAC(Origami)-TGTTTACTCTGCTCTCCGACACGGGAGGAATCATG-3'; P3: 5'-CCCAAATGAC-3'; P4: 5'-CCCAAATGAC-3'.

The full-length IN1-288 (F185K/C280S) was ligated into pET-28a (Novagen) and then transformed into E. coli BL21 (DE3). Recombinant His-tag HIV-1 IN was purified by Ni-NTA (Ni2+-nitrilotriacetate), then freeze-dried and stored at −70°C.

The LTR (long terminal repeat) sequence was cloned into pUC19 to make the substrate plasmid pLTR. The reaction system contained 20 mM Tris/HCl (pH 8.0), 2 mM MnCl2, 5 mM 2-mercaptoethanol, 10 pmol of HIV-1 IN and 200 ng pLTR. The mixture was incubated at 37°C for 30 min and tested by using electrophoresis. The results were analysed by Labworks Image Acquisition and Analysis Software Version 4.5.00.0.

LTR-P1: 5'-CAGTGGATCC(EcoR I)GACTG(3'-process site)-TGGAGGGCTAAATTGGTC-3'; LTR-P2: 5'-CGAGGATTC(Bam H I)GACTG(3'-process site)CTAGAGATTTCCAC-3'. The positive control was Raltegravir. The negative control was Tris/HCl. The blank was the mixture without enzyme.

Inhibitory rate (%) = (I - I0)/(I0 - I0) × 100%

where I is the IOD (inhibition of delay) of (ccDNA + IDN + ocDNA)/IDNA with sample, I0 is the IOD of (ccDNA + IDN + ocDNA)/IDNA with enzyme but without any sample and I0 is the IOD of (ccDNA + IDN + ocDNA)/IDNA without enzyme and sample.

Raltegravir, a diketoacid derivative, is the unique anti-HIV-1 IN drug approved by the U.S. FDA (Food and Drug Administration). Diketoacid derivatives also exhibit some 3'-process activity besides strand transfer inhibitory activity, and have been used as positive controls in many 3'-process assays [23,24]. In the present study, a standard 3'-process inhibitor, salicylhydroxide, was also used as a positive control to confirm the feasibility of this model [25].

Real-time PCR
A total of 20 mice were divided randomly into four groups. Control mice (group A) were treated with 0.9% (w/w) NaCl by injection. L2f-2 mice (group B) were treated with L2f-2 (50 mg/kg of body weight). L2f-3 mice (group C) were treated with L2f-3 (50 mg/kg). LB2 mice (group D) were treated with LB2 (50 mg/ml). All mice were injected once a day for 7 days. On the eighth day, all mice received an intraperitoneal injection of purified LPS (lipopolysaccharide; derived from E. coli O55:B5; Sigma) at a dose of 3 mg/kg of body weight. All mice were killed 12 h later and the total splenic RNA was isolated by using the TRIZol® kit. Ascitic macrophages of mice in group D were collected and the total RNA was isolated by using the TRIZol® kit.

For reverse transcription, 1 μg of total RNA was heated at 65°C for 5 min. Reverse transcription was conducted at 37°C for 30 min. The reaction mixture was composed of 1–5 μg of total RNA, 0.5 mM dNTPs, 50 ng of oligo (dT)16 primer, reverse transcription buffer and 3 units of RT. RT was inactivated by heating at 95°C for 5 min.

The 25 μl Real-time PCR system contained 1 μl of upstream primer (500 pmol), 1 μl of downstream primer (500 pmol), 1 μl
Table 1 Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Product</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>Upstream 5′-TGAACTTGGACCTCTGCG-3′ Downstream 5′-AGGGCTTTGGAGATGATGC-3′</td>
<td>220</td>
</tr>
<tr>
<td>IL-4</td>
<td>Upstream 5′-TCCTGCTCTTCTTCTCG-3′ Downstream 5′-ATGCTCTTTAGGCTTTCC-3′</td>
<td>537</td>
</tr>
<tr>
<td>IL-6</td>
<td>Upstream 5′-TTCTTGGGACTGATGCTG-3′ Downstream 5′-CTGGCTTTGTCTTTCTTGTT-3′</td>
<td>380</td>
</tr>
<tr>
<td>IL-10</td>
<td>Upstream 5′-ACCAAGCCACAAAGCAG-3′ Downstream 5′-GGAGTGCGTATGAGCTATG-3′</td>
<td>249</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Upstream 5′-TGAGACAATGAACGCTAC-3′ Downstream 5′-TTCACATCTATGCCACT-3′</td>
<td>142</td>
</tr>
<tr>
<td>TNFα</td>
<td>Upstream 5′-CTGTTGAGGAAATGGGTGTT-3′ Downstream 5′-CAGGGAAGATGTCGAAAAGGTC-3′</td>
<td>384</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Upstream 5′-TCAACGGCACAGTCAAGG-3′ Downstream 5′-ACCAGTGGATGCAGGGAT-3′</td>
<td>470</td>
</tr>
</tbody>
</table>

Figure 1 Structures of L2f-2 (a) and L2f-3 (b)

of cDNA, 7 μl of double-distilled water and 10 μl of 2 × SYBR Green Realtime PCR Master Mix. The following thermocycler programme was used for real-time PCR: 1 min preincubation at 95 °C, followed by 40 cycles of incubation at 94 °C for 15 s, 55 °C for 15 s, 72 °C for 45 s. After amplification, melt curves were tested in these reactions every 0.5 °C. The primers of IL-2, IL-4, IL-6, IL-10, IFNγ, TNFα (tumour necrosis factor α) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) are shown in Table 1.

The \( 2^{-\Delta \Delta C_t} \) method [26] was used to analyse the results. GAPDH was the internal control. For the \( \Delta \Delta C_t \) calculation to be valid, the amplification efficiencies of the target and reference must be approximately the same. Amplification of cDNA synthesized from different amounts of RNA. The efficiency of amplification of the target gene (IL-2, IL-4, IL-6, IL-10, IFNγ, TNFα) and internal control (GAPDH) was examined using real-time PCR. \( \Delta C_t \) \( (C_{\text{target gene}} - C_{\text{GAPDH}}) \) was calculated for each cDNA dilution. The results were fit using least-squares linear regression analysis \( (n=3) \). If the absolute value of the slope is close to zero, the efficiencies of the target and reference genes are similar, and the \( \Delta \Delta C_t \) calculation for the relative quantification of target may be used.

Statistical analysis of data
All experiments were run in triplicate. The average value and S.D. were calculated. To determine whether there were any differences between the activities of samples, one-way ANOVA was applied to the results followed by the Tukey test. \( P < 0.05 \) was considered to represent a statistically significant difference.

RESULTS

Isolation of L2f-2, L2f-3 and LB2 from lotus rhizomes
L2f-2 and L2f-3 were well separated on HPLC. MS analyses showed that the \( m/z [M-H]^- \) of L2f-2 was 305.1 when tested with El-MS (electron ionization MS). From the results it can be concluded that its molecular mass was 306.1 Da with a molecular formula of \( C_{15}H_{14}O_7 \). According to its \( ^1H \) NMR spectrum, it can be concluded that L2f-2 was gallocatechin (Figure 1a). L2f-3 was identified as catechin based on the results of HPLC, LC-ESI-MS/MS (liquid chromatography electrospray ionization tandem
Table 2  Inhibitory activities of samples on erythrocyte haemolysis and lipid peroxidation

Values are means ± S.D. (n = 3). Different superscripts indicate statistically significant difference (P < 0.05) in inhibitory rate between fractions at the same concentration when the results were analysed by ANOVA followed by the Tukey test.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Sample</th>
<th>Inhibition of erythrocyte haemolysis (%)</th>
<th>Inhibition of lipid peroxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>L</td>
<td>65.62 ± 1.05</td>
<td>52.62 ± 2.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>21.27 ± 2.10</td>
<td>27.22 ± 2.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LB2</td>
<td>72.26 ± 0.81</td>
<td>83.25 ± 1.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>L</td>
<td>25.12 ± 1.85</td>
<td>15.97 ± 1.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>61.78 ± 3.55</td>
<td>93.77 ± 0.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>L2f</td>
<td>70.47 ± 2.11</td>
<td>80.21 ± 1.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>L2f-2</td>
<td>82.35 ± 1.27</td>
<td>94.20 ± 0.58&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>L2f-3</td>
<td>91.69 ± 0.69</td>
<td>96.38 ± 0.67&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>Positive control</td>
<td>90.53 ± 1.47</td>
<td>84.40 ± 0.72&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>13.40 ± 1.98</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 2  FTIR spectrum (a) and anti-HIV-1 RT activity (b) of LB2

MS and IR spectroscopy (Figure 1b) [27,28]. The yields of L2f-2, L2f-3 and LB2 were 0.65%, 0.39% and 2.20% respectively from crude extract L.

L2f-2 (gallocatechin): trans-2-(3,5,7-trihydroxyphenyl)-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol freeze-dried powder (20 mg). HPLC Rt 5.4 min. <sup>1</sup>H-NMR (600 MHz, DMSO-d<sub>6</sub>) 2.34 (dd, 1H, J = 7.8, 7.8 Hz), 2.59 (dd, 1H, J = 4.8, 4.2 Hz), 3.77 (dd, 1H, J = 7.2, 7.2 Hz), 4.42 (d, 1H, J = 7.2 Hz), 5.69 (s, 1H), 5.88 (s, 1H), 6.24 (s, 3H); LRMS (EI) 306 [M<sup>+</sup>].L2f-3 (catechin): trans-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1 [2H]-benzopyran-3,5,7-triol freeze-dried powder (10 mg). HPLC Rt 12.1 min. [α]<sub>22</sub> D-42.0 (c 0.1, acetone). <sup>1</sup>H-NMR (600 MHz, DMSO-d<sub>6</sub>) 2.34 (dd, 1H, J = 8.4, 7.8 Hz), 2.64 (dd, 1H, J = 4.8, 5.4 Hz), 3.80 (dd, 1H, J = 7.2, 7.2 Hz), 4.47 (d, 1H, J = 7.2 Hz), 5.69 (s, 1H), 5.88 (s, 1H), 6.58–6.71 (m, 3H); FTIR (potassium bromide) 1032, 1147, 1468, 1522, 1627 and 3397 cm<sup>−1</sup>. The yields of L2f-2, L2f-3 and LB2 were 0.65%, 0.39% and 2.20% respectively from crude extract L.

LB2 was a polysaccharide–protein complex with a molecular mass of 18.8 kDa. It was composed of mannose, rhamnose, glucose, galactose and xylose with a molar ratio 2:8:7:8:1. LB2 was identified as a polysaccharide sulfate containing α/β-pyranoid and α-furanoid monose by FTIR (Figure 2a). The wide peak at 3384 cm<sup>−1</sup> represented the vibrations of O–H, while peaks at 2900 and 1398 cm<sup>−1</sup> represented the stretching and angular vibrations of C–H which indicated that LB2 was a polysaccharide. The vibrations at 1680 and 1633 cm<sup>−1</sup> were due to C=O bonds. Peaks from 990 to 1200 cm<sup>−1</sup> and at 835 cm<sup>−1</sup> were vibrations of α/β-pyranoid, while peak at 802 cm<sup>−1</sup> was the vibration of α-furanose. The vibrations at 618 cm<sup>−1</sup> indicated that there were sulfurous acid ester linkages in this polysaccharide. The peak at 518 cm<sup>−1</sup> represented the vibrations of C-(CH<sub>2</sub>)<sub>n</sub>- (n ≥ 4).

Antioxidant activities of components from lotus rhizomes

The antioxidant activities of the extraction increased along with the purification. L2f-2, L2f-3 and LB2 showed strong antioxidant potencies (Table 2).
Figure 3  HIV-1 IN assay
(a) 3′-Processing activity of HIV-1 IN on substrate plasmid pLTR (1, 30 pmol IN; 2, 0 pmol IN; 3, 300 pmol IN; 4, 3 pmol IN). (b) The effect of different concentrations of LB2 on the 3′-processing activity of HIV-1 IN (1, blank/without IN; 2, negative control/without LB2; 3, 312.5 nM LB2; 4, 625 nM LB2; 5, 1.25 μM LB2; 6, 2.5 μM LB2; 7, 5 μM LB2; 8, 10 μM LB2; 9, 20 μM LB2). (c) Inhibitory rate (%) of raltegravir and salicylhydrazide on HIV-1 IN.

Table 3  Inhibitory effects of L2f-2, L2f-3, LB and pepstatin A on HIV-1 PR
Values are means ± S.D. (n = 3). Different superscripts indicate statistically significant differences (P < 0.05) in inhibitory rate between fractions when the results are analysed by ANOVA followed by the Tukey test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose of sample...</th>
<th>Inhibition of HIV-1 PR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70 μg/ml</td>
<td>140 μg/ml</td>
</tr>
<tr>
<td>L2f-2</td>
<td>17.61 ± 1.33a</td>
<td>–</td>
</tr>
<tr>
<td>L2f-3</td>
<td>15.67 ± 1.92d</td>
<td>–</td>
</tr>
<tr>
<td>LB2</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>7.36 ± 1.28b</td>
<td>11.46 ± 2.01c</td>
</tr>
</tbody>
</table>

HIV-1 RT inhibitory activity
L2f-2 and L2f-3 inhibited RT efficiently with the IC_{50} values of 10.41 and 9.58 μM (results not shown), while the IC_{50} value of the standard RT inhibitor AZT-TP was 0.082 μM. LB2 showed RT inhibitory activity with the IC_{50} value as 33.70 μM (Figure 2b). The inhibitory rate of LB2 was concentration-dependent.

HIV-1 PR inhibitory activity
L2f-2 and L2f-3 showed higher PR inhibitory rates than pepstatin A. LB2 did not exhibit any inhibitory activity on PR in this assay (Table 3).

HIV-1 IN inhibitory activity
IN exhibited 3′-processing activity on pLTR through cutting ccDNA into IDNA. IN showed processing activity at 3 pmol and plasmid degraded after dealing with 300 pmol of IN. So 30 pmol was chosen as the most appropriate IN concentration (Figure 3a).

LB2 showed the highest IN inhibitory activity with an IC_{50} value of 5.28 μM and its inhibitory rate was concentration-dependent (Figure 3b). The IC_{50} values of L2f-2 and L2f-3 were 412.45 and 432.40 μM (results not shown), while the IC_{50} values of standard IN inhibitors Raltegravir and salicylhydrazide were 686.17 and 145.86 μM respectively (Figure 3c).

Immunoregulation of HIV-1-related cytokines
The absolute values of the slopes for IL-2, IL-6 and IL-10 (Figure 4a) were 0.072, 0.062 and 0.020 respectively, while the absolute values of the slopes for IL-4, TNFα and IFNγ (Figure 4b) were 0.065, 0.060 and 0.093 respectively. For all the genes analysed, the absolute values of the slopes were close to 0, which indicated that the amplification efficiency of the target genes were in accordance with GAPDH, and the ΔΔCt calculation for the relative quantification of target could be used.

Both L2f-2 and L2f-3 up-regulated the transcription of IL-2 and IL-6 but down-regulated the expression of IL-10. The effect of L2f-2 on TNFα was different from that of L2f-3. L2f-2 up-regulated the expression of TNFα, while L2f-3 exhibited the
opposite effect. LB2 increased mRNA levels of IL-2, IL-4 and IL-10 but decreased that of TNFα (Table 4).

Table 4 Effects of L2f-2, L2f-3 and LB2 on the expression of target genes (n = 5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene</th>
<th>$\Delta C_t^{*}$</th>
<th>$\Delta \Delta C_t$</th>
<th>$2^{-\Delta \Delta C_t}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2f-2</td>
<td>IL-2</td>
<td>2.37 ± 0.07</td>
<td>-2.11</td>
<td>4.317</td>
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<tr>
<td></td>
<td>IL-4</td>
<td>6.66 ± 0.99</td>
<td>-0.08</td>
<td>1.059</td>
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<tr>
<td></td>
<td>IL-6</td>
<td>2.33 ± 0.03</td>
<td>-1.52</td>
<td>2.868</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>9.75 ± 1.58</td>
<td>1.17</td>
<td>0.444</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>6.59 ± 0.99</td>
<td>0.23</td>
<td>0.853</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>3.99 ± 1.07</td>
<td>-0.99</td>
<td>1.986</td>
</tr>
<tr>
<td>L2f-3</td>
<td>IL-2</td>
<td>2.81 ± 0.08</td>
<td>-1.67</td>
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<td></td>
<td>IL-4</td>
<td>6.73 ± 1.89</td>
<td>-0.01</td>
<td>1.007</td>
</tr>
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<td></td>
<td>IL-6</td>
<td>2.80 ± 0.17</td>
<td>-1.05</td>
<td>2.071</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>9.65 ± 0.14</td>
<td>1.07</td>
<td>0.476</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>6.01 ± 0.65</td>
<td>-0.35</td>
<td>1.272</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>5.79 ± 0.19</td>
<td>0.55</td>
<td>0.574</td>
</tr>
<tr>
<td>LB2</td>
<td>IL-2</td>
<td>6.15 ± 0.21</td>
<td>-0.67</td>
<td>1.591</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>10.85 ± 2.20</td>
<td>-1.22</td>
<td>2.329</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>4.53 ± 0.87</td>
<td>0.07</td>
<td>0.953</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>5.23 ± 0.77</td>
<td>-0.79</td>
<td>1.729</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>9.16 ± 2.05</td>
<td>0.47</td>
<td>0.720</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>6.20 ± 1.52</td>
<td>0.65</td>
<td>0.637</td>
</tr>
</tbody>
</table>

$\Delta C_t = C_{target gene} - C_{GAPDH}$ (amplification from the same cDNA).
$\Delta \Delta C_t = \Delta C_t_{treat group} - \Delta C_t_{control group}$.

The inhibitory activities towards key HIV-1 enzymes were studied for their RT inhibition. Epicatechin gallate from *Camellia sinensis* was reported to show RT inhibitory activity with an IC$_{50}$ value of $2.3 \times 10^{-2} - 4.6 \times 10^{-2}$ μM [14]. Geraniin from *Phyllanthus amarus* could exhibit antiviral activities against HIV-1.

DISCUSSION

Purification of the antioxidant components from *N. nucifera*

In traditional methods, an array of organic agents such as chloroform, ethyl acetate and diethyl ether must be used to purify natural products with a low molecular mass from crude samples [29]. All these solvents are harmful. Recently, natural product extraction focused on ‘water-extraction’ [30]. In the present study, an improved method was developed to purify active compounds using safer solvents such as water and ethanol.

The most extensively used synthetic antioxidants, such as BHA, and BHT (butylated hydroxytoluene), have restricted use in food and have been suspected to cause liver damage and carcinogenesis [31]. The natural products L2f-2, L2f-3 and LB2 exhibited good water-solubility. They could be absorbed easily by the human body that makes them more valuable than chemosynthetic oleophilic antioxidants.

Most researches focused on the antioxidant potency of *N. nucifera* [4], while few reports studied the antioxidant components purified from the plant [32,33]. In the present study, the antioxidant components catechin and gallocatechin from lotus rhizome were first purified by a quick and simple method. The yields of catechin and gallocatechin in lotus rhizomes extracted in the present study were 0.024% and 0.037%, which were 10-fold higher than those reported by other researchers [34]. Lotus rhizomes could be the main catechin source and the basic anti-aging food for human consumption. The antioxidative activity of green tea-derived polyphenols known as catechins has been extensively studied [35]. In contrast, researches on catechins from lotus rhizome were not intensive. Fewer papers focused on the purification and identification of catechins from lotus rhizomes. Many antioxidant polysaccharides have been found in fungi [36]. These polysaccharides were composed mainly of glucose [37]. A polysaccharide–protein complex named LB2 from lotus rhizomes was composed mainly of galactose and rhamnose in accordance with the results from other reports on lotus [38].
also inhibit RT with an IC\textsubscript{50} value of 1.9 μM. Its RT inhibitory activity was 1000 times higher than the standard RT inhibitor named AZT-TP [40]. It has been reported that the hydroxide groups in ring B of flavones contributed to their RT inhibitory activities [41]. These natural catechin-derived compounds could also inhibit the mutant RT (A17) that has a higher potency than most synthetic inhibitors [42]. L2f-2 and L2f-3 were catechins from \textit{N. nucifera} Gaertn. They potently inhibited RT.

On the contrary, research into the effect of catechin on PR and IN were rare. IN is an ideal target for anti-HIV therapy. But the agents against IN were developed so slowly and with only one drug against it named Raltegravir approved by FDA in 32 anti-HIV drugs. Proper screening models were important for drug development. In the present study, a novel 3’-process model was established for IN inhibition screening.

Polysaccharide–protein complex LB2 showed very strong inhibitory activities not only on RT but also on IN. LB2 might not be absorbed easily because it is a macromolecular complex. In the present study, the intracellular concentration of LB2 achieved might be too low to inhibit PR. Therefore, developing a new screening model for macromolecular samples will be our next target. It is valuable that anti-HIV potencies of the natural antioxidant components from \textit{N. nucifera} Gaertn. were investigated for their inhibition of RT, PR and IN.

**Immunoregulation of HIV-1 related cytokines**

IL-2 is necessary during T-cell development in the thymus for the maturation of a unique subset of T-cells termed regulatory T-cells (T-regs). It facilitates the production of lgs made by B-cells and induces the differentiation and proliferation of NK cells [43]. L2f-2 (gallocatechin) markedly increased the expression level of IL-2 (over 4-fold increase over control group) indicating that it acted as an activator of IL-2 in immunoreaction. L2f-3 (catechin) increased the level of IL-2 3-fold. In terms of host response to a foreign pathogen, IL-6 has been shown, in mice, to be required for resistance against the bacterium \textit{Streptococcus pneumoniae} [44]. Treatment with L2f-2 brought about a 2.9-fold augmentation in the level of IL-6. L2f-2 (gallocatechin) was more active than L2f-3 (catechin) which affected 2.1-fold elevation. IL-10 down regulates the expression of Th1 cytokines, MHC class II antigens and co-stimulatory molecules in macrophages. It is capable of inhibiting the synthesis of pro-inflammatory cytokines such as IL-2 made by Type 1 Th cells. IL-10 also displays potent abilities to suppress the antigen presentation capacity of antigen presenting cells. It is released by cytotoxic T-cells to inhibit the actions of NK cells during the immune response to viral infection [45]. TNF\textalpha is another affinitive cytokine to HIV-1. The expressive level of TNF\textalpha in immunocytes could be up-regulated after HIV-1 infection [46]. L2f-3 and LB2 might inhibit HIV-1 by down-regulating TNF\textalpha directly. LB2 exhibited positive regulation on IL-2, IL-4 and IL-10. It could improve the inhibitory state of cytokines in HIV-1 sufferers and balance the immune system.

**Co-existence of inhibitory activity towards HIV enzymes and antioxidant activity in lotus rhizome components**

Oxidative stress plays a significant role in the pathogenesis of HIV-1/AIDS, by promoting death of lymphocytes [8]. Accretion of immunodeficiency in HIV-infected individuals with micronutrient deficiency increases the risk for secondary infections as well as accelerates the onset of chronic AIDS. Supplementation with antioxidants offers a safe and economic treatment to slow the progression of HIV infection [47]. But the direct relationship between antioxidant and anti-HIV-1 activities needs further investigation. In the present study, antioxidant activity was used to screen and select natural compounds from lotus rhizomes. It was found that these antioxidant compounds could inhibit HIV-1 enzymes directly. With the presence of antioxidant activity, these natural compounds from lotus rhizomes may have a better potential for AIDS treatment and adjuvant therapy.

**Conclusions**

In the present study, natural products from \textit{N. nucifera} rhizomes were examined for their potencies to counter oxidation, inhibit key HIV-1 enzymes and affect immune regulation. Most chemically synthetic HIV-1 inhibitors could not deal with the mutant virus. Natural products with multiple anti-HIV-1 effects may circumvent this disadvantage. Therefore, the present investigation may be important to anti-HIV-1 drug development and application of natural products in HIV-1 therapy.

**AUTHOR CONTRIBUTION**

Yun Jiang and Fang Liua conceived the project and designed the experiments; Ning Li was responsible for compound extraction and identification; Zhaokun Liu and Changrong Wang performed the HIV-1 RT and PR assays; Yun Jiang conducted the other experiments described in this paper; Fang Liua, Tzi Bun Ng and Wentao Qiao supervised the scientific work; Yun Jiang wrote the manuscript and Tzi Bun Ng revised it.

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**REFERENCES**


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