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Full Length Research Paper

Anti-HIV-1 reverse transcriptase activities of hexane extracts from some Asian medicinal plants

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Numerous herbal remedies against viral infections, including HIV-1, have been used as a part of Asian traditional medicine. However, the viral protein targets for inhibition by these medications are less well identified. HIV-1 reverse transcriptase is one of the targets, since high-efficiency inhibitors such as non-nucleoside HIV-1 reverse transcriptase inhibitor have been successfully used. In this work, hexane extracts of Asian medicinal plants were screened against HIV-1 reverse transcriptase. The results showed that the crude extracts from plants *Cinnamomum loureiroi* (stem bark), *Quercus infectoria* (fruit), *Plumbago indica* L. (root), *Artocarpus heterophyllus* Lam. (seed), *Ocimum sanctum* L. (leaves), *Allium sativum* L. (bulb) and *Acorus calamus* L. (rhizomes) showed strong HIV-1 reverse transcriptase inhibitory effects. The efficiency of anti-HIV-1RT activity was reported as 50% inhibitory concentrations (IC_{50}). This showed that the hexane crude extracts from *A. calamus* L. and *A. heterophyllus* Lam. contained potent activity against HIV-1 RT, with IC_{50} of 32.96 ± 3.17 and 34.69 ± 2.41 µg/ml, respectively.

Key words: Anti-HIV agents, HIV-1 reverse transcriptase, inhibitor screening, PicoGreen.

INTRODUCTION

Infection by the HIV-1 virus causes AIDS, a dramatically epidemic disease throughout the entire world, especially in Africa and Southeast Asia. The virus can live in infected people without symptoms, and an infected carrier can transfer the virus to other people, for example, the transmission of mother to child.

Because HIV-1 is a retrovirus, reverse transcription is the first process for viral propagation in host cells. HIV-1 reverse transcriptase (HIV-1 RT) is responsible for transcription of viral RNA into DNA, which is later integrated into the host cell and carries the information for the synthesis of new viral particles. This enzyme is one of the main targets for inhibiting the reproduction of HIV.

Today, there are two known types of HIV-1 RT inhibitor. The first is nucleoside HIV-1 RT inhibitor (NRTI), a nucleoside analog that resembles the polymerase substrate. The binding of these inhibitors to catalytic sites causes an interruption of HIV-1 RT function. However, the nucleoside-like shape of NRTI can inhibit human mitochondrial polymerase as well; thus NRTI have highly toxic side effects. The second type of inhibitor is non-nucleoside HIV-1 RT inhibitor (NNRTI). Because of their hydrophobic property, these inhibitors bind to the HIV-1 RT hydrophobic pocket (allosteric site) which is far away from the catalytic position. NNRTI show very high selectivity, and bind specifically to HIV-1 RT only (Shen et al., 2003; Masuda et al., 2004; de Béthune, 2010).

Long-term treatment with commercial drugs can cause the propagation of drug-resistant strains of HIV-1. Searching for new inhibitors to overcome this problem is an area of innovative research. The biodiversity of plants

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Abbreviations: HIV-1 RT, HIV-1 reverse transcriptase; **NNRTI,** non-nucleoside reverse transcriptase inhibitor; **NRTI,** nucleoside reverse transcriptase inhibitor.

makes them a promising source of new drugs. The long history of folklore medicines demonstrates the potential of plants as sources of lead compounds. Numerous anti-HIV agents have been discovered from plants: for example, calanolide A, which is among a series of coumarins originating from *Calophyllum lanigerum* Miq. var. *austrocoriaceum* (Fuller et al., 1994); swertiabisxanthone-1 from *Swertia macrosperma* (Zhou et al., 1989); swertipunicoside from *Swertia punicea* (Cordell et al., 1994); swertifrancheside from *Swertia franchetiana* (Wang et al., 1994); and new glucopyranosides and four flavonoids discovered from the stem-bark of *Juglans mandshurica* (Min et al., 2000). In order to identify the mechanism of these plant extracts, we decided to perform testing of some Asian medicinal plants for inhibition of HIV-1 RT. Because of the hydrophobic property of most HIV-1 RT inhibitors, hexane extraction was used for recovery of aromatic and hydrophobic compounds (Eufemia et al., 2002; Souchon et al., 2002; Szolar et al., 2002; Aisha et al., 2009). To our knowledge, this preliminary study represents the first attempt to search for a hydrophobic HIV-1 RT inhibitor that can be obtained from plants. Inhibitory assays were performed by the fluorescence method; the IC_{50} indicated that some traditional Asian medicinal plants contain highly effective compounds.

MATERIALS AND METHODS

Plant materials

All plant materials were collected from commercial markets and herbal stores in Bangkok, Thailand. The plant parts tested were chosen based on their traditional medicinal use in Asia; the details are shown in Table 1.

Plant extract preparation

Fresh and dried plant materials were extracted successively with hexane. The fresh materials were dried in a hot-air oven overnight at 55°C. All dried materials were cut into small pieces and ground into powder. 10 mg of each material was extracted with 180 ml of hexane in a Soxhlet extractor for 8 h. The hexane extracts were dried under vacuum using a rotary evaporator at 50°C. 1 ml of DMSO was added to dissolve the crude compounds, which were then diluted to 16 mg/ml in 50% DMSO-containing buffer. Samples were kept at 4°C until required.

HIV-1 reverse transcriptase inhibition assay

The inhibition assay by fluorescence method followed Silprasit et al. (2011). To measure HIV-1 RT activity with inhibitors, 2 µl of 30 ng/µl purified recombinant HIV-1 RT was added into each well of a 96-well plate; then 2 µl of 16 mg/ml of each plant extract sample was added into the wells for sample testing reaction (RTSample, final concentration of 4 mg/ml). The addition of 2 µl of Tris buffer instead of plant extract sample served as the control reaction (RTControl). The blank reaction (RTBackground) was prepared by adding 5 µl of 0.2 M EDTA, and the HIV-1 RT inhibitor nevirapine was added instead of plant extract sample for the positive control reaction. The plate was gently mixed before adding 4 µl of primer/template polymerization buffer into all wells. Reactions were started by incubation at 37°C for 10 min, and then stopped by the addition of 5 µl of 0.2 M EDTA. Three independent experiments were performed. The fluorescence of the terminated reactions was measured using a fluorescence microplate reader with an excitation wavelength of 502 nm and an emission wavelength of 523 nm. The inhibitory effect on HIV-1 RT activity was compared by the percent of relative inhibition, which was calculated using the following Equation (1) (Silprasit et al., 2011; Kanyara and Njagi, 2005):

$$\% \text{ Relation Inhibition} = \frac{[(RT_{Control} - RT_{Background}) - (RT_{Sample} - RT_{Background})]}{[(RT_{Control} - RT_{Background})]} \times 100 \quad (1)$$

The percentages of relative inhibition of all plant samples were plotted to compare their inhibitory effects. Crude extracts which inhibited the enzyme by more than 80% relative inhibition were selected for further testing.

Fifty percent inhibitory concentration (IC_{50}) determination

2 µl of each crude extract was diluted serially twofold. Then, 2 µl of 30 ng/µl purified HIV-1 RT was added and mixed. Then 4 µl of the template/primer polymerization buffer was added into each well. The mixtures were incubated at 37°C for 10 min. The reactions were stopped with 5 µl of 200 mM EDTA, and the samples were immediately incubated on ice for 30 min. Activity was determined by the PicoGreen fluorometric method. Nonlinear regression dose-response curves were plotted against percent inhibition and log inhibitor concentration, and 50% inhibitions (IC_{50}) were calculated using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The plants tested have been used in traditional medicine as anti-microorganism agents and as treatments for infection. The 40 samples collected were members of various plant families; only certain parts of each plant were used. The extraction yields of all plant extracts are shown in Table 1. Various amounts of crude compounds were extracted by hexane extraction. All crude extracts were completely dissolved in DMSO and kept as stock solutions.

HIV-1 reverse transcriptase inhibitory assay

Testing of plant extracts for anti-HIV-1 RT was carried out by our previously developed method for RT inhibition assay (Silprasit et al., 2011). The inhibition results for all 40 selected plant extracts are shown in Figure 1. At 4 mg/ml, crude extracts from *Cinnamomum loureiroi*, *Quercus infectoria*, *Plumbago indica* L., *Artocarpus heterophyllus* Lam., *Ocimum sanctum* L., *Allium sativum* L. and *Acorus calamus* L. showed a strong inhibitory effect against HIV-1 RT (>80% relative inhibition). At the

Table 1. Selected Thai medicinal plants, the parts used, and hexane extraction yields.

S/N	Scientific name	Family	Part used	Yield (mg/ml)
1	*Azadirachta indica*	Meliaceae	Leaves	375.9
2	*Rhinacanthus nasutus*	Acanthaceae	Aerial part	420.7
3	*Brucea amarissima*	Simaroubaceae	Fruit	136
4	*Cinnamomum loureiroi*	Lauraceae	Stem bark	145.8
5	*Quercus infectoria*	Fagaceae	Fruit	67.4
6	*Saccharum officinarum*	Poaceae (Gramineae)	Stem	370.6
7	*Myristica fragrans*	Myristicaceae	Stem	290.9
9	*Curcuma longa*	Zingiberaceae	Rhizomes	455
10	*Lawsonia inermis*	Lythraceae	Aerial part	419.5
11	*Strychnos nux-vomica*	Strychnaceae	Seed	236.4
12	*Curcuma xanthorrhiza*	Zingiberaceae	Rhizomes	734.1
13	*Terminalia bellirica*	Combretaceae	Fruit	47.6
14	*Sapium indicum*	Euphorbiaceae	Fruit	269.3
15	*Terminalia chebula*	Combretaceae	Fruit	1,513.9
16	*Salacia chinensis*	Celastraceae	Stem	218
17	*Mallotus philippensis*	Bixaceae	Flower	207.5
20	*Spondias pinnata*	Anacardiaceae	Fruit	948.6
21	*Maclura cochinchinensis*	Moraceae	Stem	562.5
22	*Pentace burmanica* Kurz	Tiliaceae	Stem bark	237
23	*Acacia catechu*	Leguminosae-Mimosoideae	Resin	442.5
24	*Caesalpinia sappan*	Caesalpiniaceae	Stem	143.2
25	*Diospyros mollis* Griff.	Ebenaceae	Stem	572.1
26	*Garcinia mangostana* L.	Guttiferae	Fruit bark	949.4
27	*Citrus hystrix*	Rutaceae	Fruit bark	687.9
28	*Mammea siamensis* T. Anders.	Guttiferae	Flower	562.5
29	*Euphorbia hirta* L.	Euphorbiaceae	Whole plant	596
30	*Vitex negundo* L.	Verbenaceae	Root	455.8
31	*Plumbago indica* L.	Plumbaginaceae	Root	251.1
32	*Dracaena loureiri*	Agavaceae	Stem	603.6
33	*Hibiscus sabdariffa* L.	Dracaenaceae	Flower	961.2
34	*Moringa oleifera* Lam.	Moringaceae	Seed	1,415.9
35	*Artocarpus heterophyllus* Lam.	Moraceae	Seed	171.3
36	*Ocimum sanctum* L.	Labiatae	Leaves	519.1
37	*Ocimum basilicum* L.	Labiatae	Leaves	364.8
38	*Allium sativum* L.	Alliaceae	Bulb	572.3
39	*Punica granatum* L.	Punicaceae	Fruit bark	885.2
40	*Acorus calamus* L.	Araceae	Rhizomes	283.9

same concentration, the extracts from *Lawsonia inermis*, *Sapium indicum*, *Vitex negundo* L. and *Dracaena loureiri* had moderate inhibitory activity (50 to 80% relative inhibition). The rest of the extracts showed weak HIV-1 RT inhibitory activity (<50% relative inhibition). Crude extracts which showed relative inhibition of more than 80% were studied further. The activity of these extracts was confirmed by determination of HIV-1 RT inhibition with serial dilution (final concentrations were 0.91 to 0.0036 mg/ml). Figure 2 shows the increased inhibitory effect related to higher crude compound concentration.

IC_{50} determination

The HIV-1 RT inhibition efficiency of hexane crude extracts was indicated by 50% inhibitory concentrations (IC_{50}). Dose-response curves are frequently used to determine IC_{50} (Budihas et al., 2005; Odriozola et al., 2003; Di and Kerns, 2006). HIV-1 RT activity was measured using different sample concentrations which were diluted in a 96-well plate. Dose-response curves using nonlinear regression were plotted in terms of percent inhibition and log inhibitor concentration

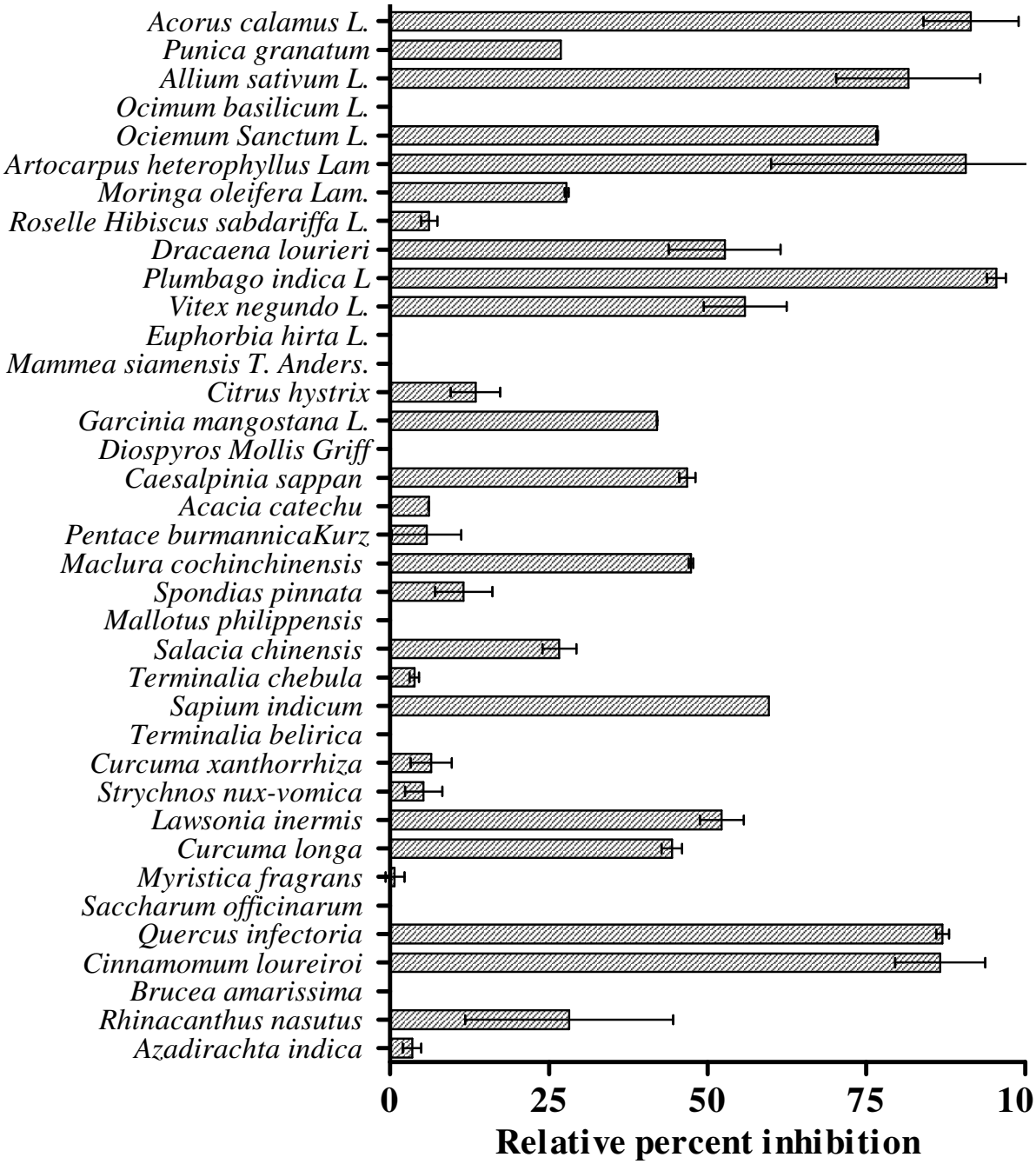

Figure 1. Percentage of HIV-1 RT inhibition of hexane extracts from 40 Thai medicinal plants using fluoroscopic assay (n = 3). Error bar represents the standard deviation. The final concentration of the extracts used was 4 mg/ml.

(Figure 3). The 50% inhibitory concentrations were then calculated (Table 2). The hexane crude extracts from *A. calamus* L. and *A. heterophyllus* Lam. showed strong activity against HIV-1 RT, with IC_{50} of 32.96 ± 3.17 and 34.69 ± 2.41 µg/ml, respectively; while the IC_{50} of *Q. infectoria*, *A. sativum* L., *O. sanctum* L., *C. loureiroi* and *P. indica* L. were 56.08 ± 8.71, 64.08 ± 1.09, 72.22 ± 6.04, 84.58 ± 5.01 and 146.50 ± 3.03 µg/ml, respectively.

DISCUSSION

In this research, plants containing potential antiviral activity were collected and tested for HIV-1 RT inhibition. The strongest inhibitory action against HIV-1 RT was found in seven plants: *C. loureiroi* (stem bark), *Q. infectoria* (fruit), *P. indica* L. (root), *A. heterophyllus* Lam. (seed), *O. sanctum* L. (leaves), *A. sativum* L. (bulb) and

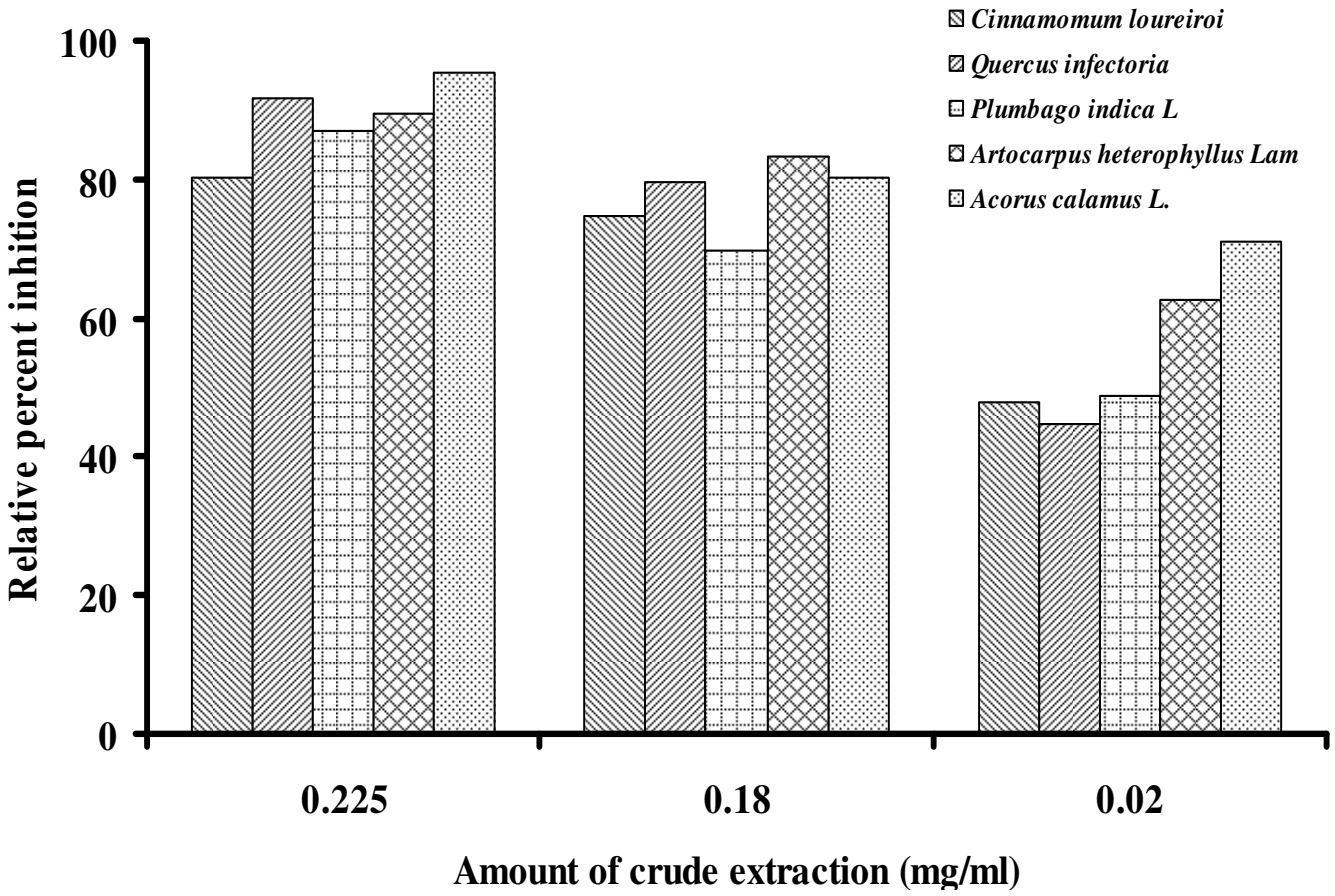

Figure 2. Increased inhibitory effect was related to concentration of crude compounds: inhibition of HIV-1 RT activity versus concentration of extracts from *Acorus calamus*, *Artocarpus heterophyllus* Lam., *Quercus infectoria*, *Allium sativum* L., *Ocimum sanctum* L., *Cinnamomum loureiroi* and *Plumbago indica* L.

A. calamus L. (rhizomes). The results showed that these plants contained anti-HIV properties, which was in accordance with previous reports in which the plants *A. calamus* L. and *P. indica* L. exhibited potent antiviral activity against the Herpes simplex viruses HSV-1 and HSV-2 (Elaya Raja et al., 2009; Akanitapichat et al., 2002). *A. sativum* was reported to be effective against HIV infection by inhibiting virus replication (Harris et al., 2001), specifically by interfering with viral reverse transcriptase activity. *O. sanctum* L. was found to demonstrate antibacterial, antifungal and antiviral activity (Prakash and Gupta, 2005), this report also showed that the plant possessed an anti-HIV property through inhibition of viral reverse transcriptase activity. Furthermore, *A. heterophyllus* Lam. has never been studied for anti-HIV activity. Two species of the same *Artocarpus* genus, *Artocarpus gomezianus* and *Artocarpus reticulates*, have reportedly demonstrated anti-HIV activity (Jagtap and Bapat, 2010). This is similar to *Q. infectoria*, which also has never been studied for anti-HIV activity. However, three plants in the *Quercus* genus – *Quercus myrsinifolia*, *Quercus stenophylla* and *Quercus pedunculata* – were shown to inhibit HIV reverse transcriptase and HIV cell growth (Vermani and Garg, 2002). The present research also confirmed the anti-HIV reverse transcriptase activity of another *Quercus* species, *Q. infectoria*. The *Ocimum* genus has also been reported to possess anti-HIV properties. An extract of *Ocimum gratissimum* caused a reduction in HIV-1 RT activity (Ayisi and Nyadedzor, 2003). This research reported that an additional plant from the *Ocimum* genus, *O. sanctum* L., can inhibit HIV-1 reverse transcriptase activity.

Conclusion

In conclusion, from the testing of hexane extracts from 40 traditional Asian medicinal plants, seven of the extracts showed a high HIV-1RT inhibitory effect. This strong inhibitory effect was confirmed by their IC_{50}. Further investigations should be undertaken to identify the active agent responsible for this inhibiting effect, which could be

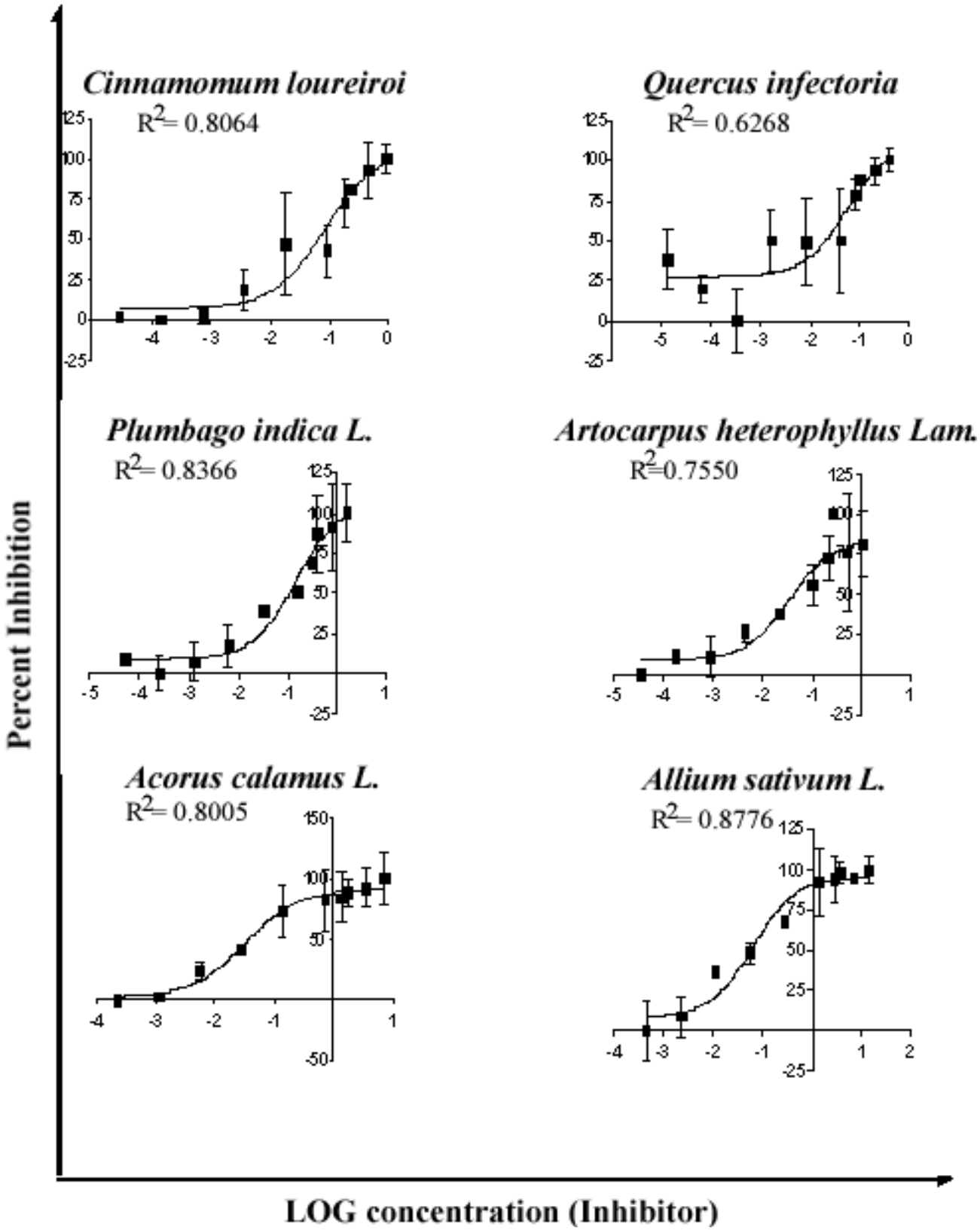

Figure 3. Nonlinear regression dose-response plot determining IC_{50} values of some plant extracts; curve and IC_{50} were automatically determined by a computer program. Values were calculated using the following equation: Y = bottom + (top – bottom)/(1+10^((logEC50-X)), where X is the logarithm of concentration and Y is the response. Y increases in a sigmoid shape.

Table 2. Fifty percent inhibitory concentrations (IC_{50}) of selected hexane plant extracts against HIV-1 RT using fluoroscopic assay.

Sample	Part used	IC50 (µg/ml)
Cinnamomum loureiroi	Stem bark	84.58 ± 5.01
Quercus infectoria	Fruit	56.08 ± 8.71
Plumbago indica L.	Root	146.50 ± 3.03
Artocarpus heterophyllus Lam.	Seed	34.69 ± 2.41
Ocimum sanctum L.	Leaves	72.22 ± 6.04
Allium sativum L.	Bulb	64.08 ± 1.09
Acorus calamus L.	Rhizomes	32.96 ± 3.17

Values are expressed as mean ± SD (n = 3).

developed as a new lead compound. Further experiments on purification of this compound would also be necessary.

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