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Anti-HIV-1 activity of phenolic compounds isolated from *Diospyros lotus* fruits

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Abstract

Phenolic compounds represent an important natural source of antiretrovirals for AIDS therapy due to their significant anti-HIV-1 activity and low toxicity. In our search for potent anti-HIV-1 agents from plants, phenolic compounds isolated from methanol (70%) extract of *Diospyros lotus* fruits were tested for anti-HIV-1 activity. Seven compounds, ellagic acid, methyl gallate, gallic acid, myricetin-3-O-β-glucuronide, myricetin-3-O-α-rhamnoside, myricetin and quercetin were identified by different spectroscopic methods (UV, ^{1}H-NMR, ^{13}C-NMR and MS). Gallic acid was the most active compound against HIV-1 with Therapeutic Index (TI) value of >32.84 and the other compounds were less potent active. *Diospyros lotus* fruits could provide a chemical reservoir of anti-HIV agents.

Keywords: *Diospyros lotus*; flavonoids; cytotoxicity; anti-HIV-1 activity

Introduction

The use of ethnomedicines to manage HIV/AIDS has recently gained public interest. Plants and other natural products present a large repertoire from which to isolate novel anti-HIV active compounds. Acquired immunodeficiency syndrome (AIDS) is a clinical syndrome that is the result of infection with human immunodeficiency virus (HIV), which causes profound immuno-suppression. HIV-1 is the cause of the world epidemic and is mostly commonly referred as HIV. It is a highly variable virus, which mutates readily. There are many different strains of HIV-1, which can be classified according to groups and subtypes; there are two groups, M and O. Within group M, there are currently known to be at least ten genetically distinct subtypes of HIV-1. These are subtypes A to J. In addition, Group O contains another distinct group of heterogeneous viruses. HIV begins its infection of a susceptible host cell by binding to the CD4 receptor on the host cell. CD4 is present of the surface of many

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lymphocytes, which are a critical part of the body's immune system. It is now known that a co-receptor is needed for HIV to enter the cell. Following fusing of the virus with the host cell, HIV enters the cell. The genetic material of the virus, which is RNA, is released and undergoes reverse transcription into DNA. An enzyme in HIV called reverse transcription is necessary to catalyse this conversion of viral RNA into DNA. Once the viral DNA is integrated into the genetic material of the host, it is possible that HIV may persist in a latent state for many years. This ability of HIV to persist in certain latently infected cells is the major barrier to eradication or cure of HIV. For this reason, based on current knowledge, patients must remain on anti-viral therapy for life (De Clercq, 1995). The introduction of highly active antiretroviral therapy has dramatically improved survival and quality of life for HIV patients. Despite the effectiveness of HAART in controlling HIV-1 replication, the emergence of drug-resistant viruses in infected patients and the severe side effects caused by the currently used drug regimen necessitate continued search for new inhibitors for HIV-1 (Veljko et al., 2007). Several reviews on the natural products for chemotherapy of HIV infection have been published earlier (Matthee et al., 1999, Jung et al., 2000, Yang et al., 2001, Cos et al., 2004). As a part of our screening program to investigate antiviral activity from plants, *Diospyros Lotus* L. is a tree native to middle east and south Asia especially from China and Japan (Hedrick 1972). In traditional medicine, *D. lotus* fruits was used as a sedative, antitussive, antiseptic, antidiabetic, antitumor, astringent, laxative, nutritive and as a febrifuge (Simmons, 1972, Chopra et al., 1986, Ebrahimzadeh et al., 2008), in addition, *D. lotus* fruits are used to treat diarrhea, dry coughs and hypertension (Bown, 1995). Previous phytochemical studies of *D. lotus* revealed the presence of some fatty acids and non volatile acids (Ahmet and Kadioglu, 1998), terpenes (Khasan et al., 1976) and Naphthoquinones (Yoshihira et al., 1971) in the fruits. The present work deals with the investigation of phenolic compounds isolated from *D. lotus* fruits as anti-HIV agents.

Materials and methods

Plant material

The ripe fruits of *Diospyros lotus* L. were collected from the Agricultural Research Centre, Giza, Egypt in April 2011 during flowering and identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereez Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen No. 2457 is deposited in the herbarium of Agricultural Research Centre, Giza, Egypt.

Chemicals

AZT (3'-azido-3'-deoxythymidine) was purchased from Sigma. All phenolic compounds were dissolved in DMSO. AZT was dissolved in RPMI-1640 and stored at -20. HEPES (N-2 (2-Hydroxyothyl) piperazine-N'-(2-ethanesufonic acid), MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMF (N, N'- Dimethyl formamine), Penicillin, Streptomycin sulfate, Glutamine were purchased from Sigma; 2-ME (2-Mercaptoethanol) was purchased from Bio-Rad. RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco.

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Cells and virus

C8166 cells and HIV-1_{IIIB} were kindly donated by Medical Research Council, AIDS Regent Project. The cells were maintained at 37 in 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivating FBS (Gibco). HIV-1_{IIIB} was prepared from the supernatants of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by Reed and Muench method. Virus stocks were stored in small aliquots at -70.

Extraction and isolation

The ripe fruits of *D. lotus* (500 g) were extracted with methanol 70% several times until exhaustion. The extract was concentrated under reduced pressure to give 100 g. the extract (100 g) was dissolved in 500 ml of distilled water and fractionated with N-hexane, dichloromethane and butanol, respectively. Butanol fraction (12.5 g) was subjected to polyamide column chromatography (300 g) and the column was eluted with H_2O to MeOH gradually. Eighty four fractions were collected. The fractions that showed similar Paper Chromatography (PC) in Butanol-Acetic acid-Water 4:1:5 (BAW) and 15% acetic acid were combined to give 4 fractions (I, II, III, and IV). Fraction I (800 mg) was subjected to sephadex LH-20 column which eluted with methanol (50%) to give compound 1 (ellagic acid). Fraction II (720 mg) was eluted with was subjected to sephadex LH-20 column eluted with water:methanol (70:30) to give compound 2 (methyl gallate) and compound 3 (gallic acid). Fraction III (650 mg) was subjected to Preparative Paper Chromatography (PPC), two dark purple bands appeared under UV light, changed with ammonia to yellow colour, each band was cutted off and eluted with MeOH (80%) and then purified on sephadex column eluted with methanol (50%) to give compound 4 (myricetin-3-O-β-glucuronide) and compound 5 (myricetin-3-O-α-rhamnoside), Fraction IV (350 mg) was subjected to sephadex LH-20 column which eluted with methanol 20% to give compound 6 (myricetin) and compound 7 (quercetin).

Cytotoxicity assay

The cellular toxicity of compounds on C8166 cells was assessed by MTT colorimetric assay. Briefly, 100µl of 4×10^5 cells were plated into 96-well plates, 100 µl of various concentrations of compounds was added and incubated at 37°C in a humidifed atmosphere of 5% CO_2 for 72 h. Discard 100 µl supernatant, MTT reagent was added and incubated for 4 h, 100µl 50% DMF-15% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 570 nm/630 nm. 50% cytotoxicity concentration (CC_{50}) was calculated (Zhang et al. 2010).

Inhibition of syncytia formation

The effect of compounds on acute HIV-1 infectivity was measured by the syncytia formation assay. In the presence or absence of various concentrations of samples, 4×10^4 C8166 cells were infected with HIV-1 at a multiplicity of infection (MOI) of 0.15, and cultured in 96-well plates at 37°C in 5% CO_2 for 3 days. AZT was used as a positive control. At 3 days post-infection, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well of 96-well plates under an inverted microsc-

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ope (100×). The inhibitory percentage of syncytia formation was calculated by the percentage of syncytia number in sample-treated culture compared to that in infected control culture. 50% effective concentration (EC_{50}) were calculated (Wang et al. 2008). According to the method described by Reed Muench, 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) was determined from dose–response curve. Therapeutic index (TI) of anti-HIV active is CC_{50}/EC_{50}

1. Cell viability (% of control) = $(OD_{test}-OD_{blk})/(OD_{ctrl} - OD_{blk})\times100$
2. CPE inhibition(%) = $(1-CPE_{test} /CPE_{ctrl})\times100$

Results

Phytochemical studies

Phytochemical analysis of *D. lotus* fruits methanol 70% extract results in seven phenolic compounds were isolated and identified as ellagic acid (1) and two hydrolysable tannins, methyl ester of gallic acid (2) and gallic acid (3) (Foo, 1993, Nawwar *et al.* 1994), two flavonol glycosides, myricetin-3-O-β-glucuronide (4) and myricetin-3-O-α-rhamnoside (5) (Markham 1982, Harborne and Mabry 1982) and two flavonol aglycones, myricetin (6) and quercetin (7) (Mabry *et al.* 1970). The structures (Figure 1) of the pure isolated compounds were established by means of UV, NMR and MS. Complete acid hydrolysis of flavonol glycosides (myricetin-3-O-β-glucuronide (4) and myricetin-3-O-α-rhamnoside (5)) was carried out for 2 h in methanol at 100 ^{o}C using 2N HCl, to give myricetin as flavonol aglycone and glucuronic and rhamnose as sugar moieties, respectively.

Ellagic acid (1), 100 mg: white amorphous powder. 1H-NMR (DMSO-d6,400 MHz): d 7.44 (2H, s, H-4,9). 13C-NMR (DMSO-d6, 100 MHz): d 158.8 (5,10-CO),147.8 (C 3,8), 139.3 (C-2,7), 136.1 (C-1a,6a), 112 (C-4b,9b), 110.2 (C-4,9), 107.3 (4a,9a).

Methyl gallate (2), 50 mg: White amorphous powder. 1H-NMR (DMSOd6, 400 MHz): d 6.94 (2H, s, H-2,6), 3.73 (3H, s, -OCH3). 13C-NMR (DMSO-d6, 100 MHz): d 166.8 (-COO), 146 (C-3,5), 138.9 (C-4), 119.8 (C-1), 109 (C-2,6), 52 (-OCH3).

Gallic acid (3), 45 mg: White amorphous powder. 1H-NMR (DMSO-d6, 400 MHz): d 7.15 (2H, s, H-2,6). 13C-NMR (DMSO-d6, 100 MHz): d 167.2 (-COOH), 145 (C-3,5), 137.7 (C-4), 121 (C-1), 109.1 (C-2,6).

Myricetin 3-*O*-β-glucuronide(4), 40 mg: Yellow amorphous powder.. ^{1}H-NMR (MeOD, 400 MHz): d 7.42 (2H, s, H-2',6'), 6.45 (1H, d, *J* =1.2 Hz, H-8), 6.22 (1H, d, *J* = 1.2 Hz, H-6), 5.47 (1H, d, *J* = 7.5 Hz, H-1"). (+) ESI-MS: m/z 495 $[M+H]^{+}$.

Myricetin 3-*O*-α-rhamnoside (5), 100 mg : Yellow amorphous powder. ^{1}H-NMR (DMSO-d_6, 400 MHz): d 6.85 (2H, s, H-2',6'), 6.35 (1H, d, *J* = 1.2 Hz, H-8), 6.15 (1H, d, *J* = 1.2 Hz, H-6), 5.15 (1H, d, H-1"), 0.9 (1H, d, *J* = 6 Hz, CH_3-rhamnosyl). ^{13}C-NMR (DMSO-d_6, 100 MHz): d 178 (C-4), 165.8 (C-7), 162.6 (C-5), 158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5(C-3), 137.1 (C-4'), 123.3 (C-1'),108.8 (C-2',6'), 104.7 (C-10), 102.8 (C-1"), 99.5 (C-8), 94.6 (C-6), 72 (C-5"), 71.8 (C-3"),71 (C-2"), 70.5 (C-4"), 18 (CH_3-rhamnosyl).

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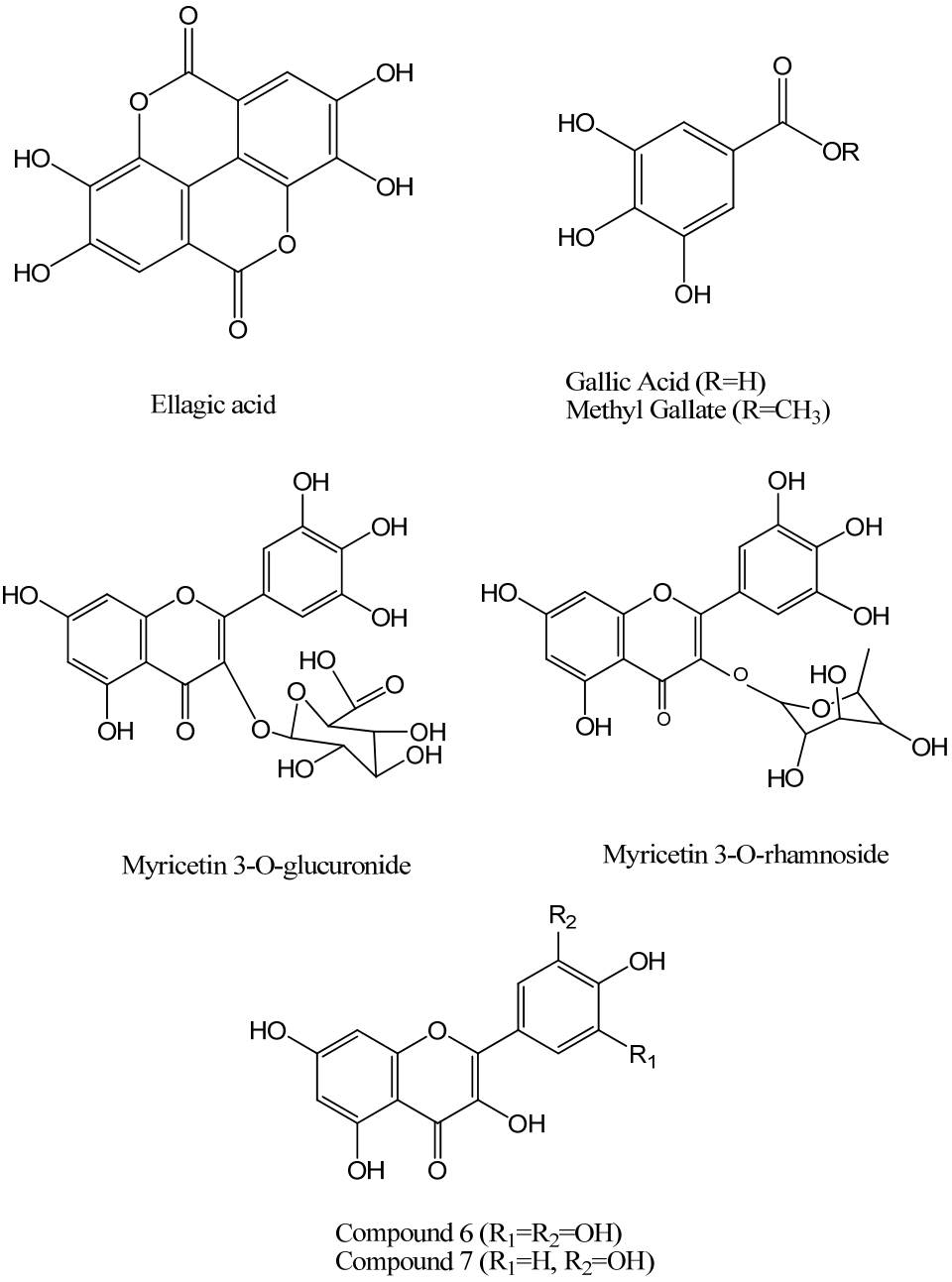

Figure 1. Chemical structures of isolated compounds.

Myricetin (6), 25 mg : Yellow powder. ^{1}H-NMR (MeOD, 400 MHz): d 7.35 (2H, s, H-2',6'), 6.43 (1H, d, *J* = 1.5 Hz, H-8), 6.18 (1H, d, *J* = 1.5 Hz, H-6). ^{13}C-NMR (MeOD, 100 MHz): d 177.5 (C-4), 165.8 (C-7), 162.6 (C-5),158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5 (C-3), 137.1 (C-4'), 123.3 (C-1'), 108.8 (C-2',6'), 104.7 (C-10), 99.5 (C-8), 94.6 (C-6). EI-MS: (+) ESI-MS: *m/z* 319$[M+H]^{+}$.

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Table 1. The summary of cytotoxicity and anti-HIV-1 activities of phenolic compounds.

Compounds	CC_{50} (μg/ml)	EC_{50} (μg/ml)	Therapeutic Index (TI)
1	35.84	12.32	2.9
2	>200	20.53	>9.74
3	>200	6.09	>32.84
4	>200	16.51	>12.11
5	>200	14.15	>14.13
6	>200	28.37	>7.05
7	>200	42.55	>4.70
AZT	3056	0.00234	1305983

Quercetin (7), 20 mg: Yellow powder. ^{1}**H-NMR (DMSO-d$_6$, 400 MHz):** d 7.74 (1H, d, *J* =8, 2 Hz, H-2'), 7.55 (1H, d, *J* =2 Hz, H-6'), 6.92 (1H, d, *J* = 8 Hz, H-5'), 6.42 (1H, d, *J* =1.2 Hz, H-8), 6.15 (1H, d, *J* =1.2 Hz, H-6). (+) ESI-MS: *m/z* 303$[M+H]^+$.

Anti-HIV-1 and cytotoxicity activities

All identified compounds were tested for their cytotoxicity (Table 1))and anti-HIV-1 (table 2) activities. For positive control, the marketed drug azido-thymidine (AZT) was also tested as a reference according to the same methods. The activity data were described as 50% cytotoxicity concentration (CC_{50}), 50% effective concentration (EC50%), and therapeutic index (TI), the ratio of CC_{50}/EC_{50}). Compound 2 to 7 (CC_{50} > 200 μg/ml) showed less toxic to C8166 cells comparison to compound 1 (CC_{50} = 35.84 μg/ml) (Table 2). Compound 3 (gallic acid) inhibited HIV-1_{IIIB} replication with EC_{50} value of 6.09 μg/ml and TI value of > 32.84, higher than any other compounds.

Discussion

The methanolic extract of *D. lotus* fruits was shown by two dimensional paper chromatography to contain a mixture of phenolic compounds. Seven phenolic compounds were isolated and purified by phytochemical methods. Compounds 1-3, white amorphous powder, showed chromatographic properties amd colour reactions (positive $FeCl_3$,and KIO_3 tests) indication of galloyl esters. Compounds 4, 5 showed dark purple spots under UV light which changed with ammonia to yellow and the hydrolytic of flavonoid –O-glycoside gives a myricetin as an aglycone and and sugar moieties, glucuronic acid and rhamnose, respectively. Compounds 6, 7 showed yellow spots under UV light which did not change with ammonia vapour. The identification of the isolated compounds was confirmed by co-chromatography with authentic samples, UV, NMR and MS. The spectral data of the isolated compounds were in agreement with the literature data.

Cytotoxicity of the phenolic compounds was carried out by using MMT colorimetric methods. The results showed that phenolic compounds isolated from the methanolic extract of *D. lotus* fruits were minimal toxic and of potent drug ability. Compound 1 (Ellagic acid) had a greater cytotoxic effect, it was significantly different from that the other phenolic compounds assayed and all the other compounds were less toxic. The anti-HIV-1 activity assay was performed by synctia formation. The seven phenolic compounds showed a good

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Table 2. Cytotoxicity of the phenolic compounds.

Compound (No.)	Concentration (µg/ml)	Cell viability±SD	CC_{50} (µg/ml)
1	200	98.86±6.28	35.84
	40	44.9±1.83	
	8	119.62±3.45	
	1.6	108.37±1.24	
	0.32	107.45±3.35	
	0.064	104.94±3.14	
2	200	70.46±2.08	>200
	40	120.58±2.47	
	8	121.4±8	
	1.6	106.79±0.44	
	0.32	102.7±5.49	
	0.064	103.48±4.13	
3	200	61.86±8.23	>200
	40	56.74±3.44	
	8	79.15±3.11	
	1.6	101.2±8.64	
	0.32	95.04±7.17	
	0.064	103.28±6.5	
4	200	63.17±1.06	>200
	40	114.85±4.71	
	8	108.61±7.55	
	1.6	97.62±8.45	
	0.32	95.56±4.2	
	0.064	93.18±7.95	
5	200	69.56±9.04	>200
	40	92.53±7.38	
	8	130.67±4.29	
	1.6	109.17±5.24	
	0.32	98.37±8.6	
	0.064	98.37±8.74	
6	200	77.82±3.23	>200
	40	76.84±6.13	
	8	105.46±3.52	
	1.6	97.9±2.86	
	0.32	93.79±5.26	
7	200	73.38±14.14	>200
	40	100.92±5.53	
	8	132.9±2.85	
	1.6	104.76±3.4	
	0.32	84.92±4.2	
AZT	4000	43.4±1.96	3056
	800	82.9±8.28	
	160	96.55±15.86	
	32	80±6.19	
	6.4	87.31±15.35	
	1.28	91.08±4.98	

anti-HIV-1 activity and compound 3 (gallic acid), a simple tannin compound was the most active and its TI value was the highest. These results are in agreement with that tannins inhibit HIV-1 entry by targeting gp41 (Collins et al., 1997), since tannin is a non-uniform polyphenolic compound and significant inhibit p24 production confirming that tannin indeed inhibits HIV-1 replication, as indicated by reduction of p24 antigen production and also tannins inhibited fusion of HIV-1_{IIIB}-infected of H9 cells with uninfected MT-2 cells and so tannin is a potent inhibitor of HIV-1 replication by targeting the viral proteins that mediate the late steps of HIV replication (Lu et al., 2004) and this suggest that *D. lotus* fruits phenolic comp-

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Table 3. Anti-HIV activity of the *phenolic compounds* in C8166 cell.

Compound (No.)	Concentration (µg/ml)	Cell viability±SD	CC_{50} (µg/ml)
1	200	100±0	12.32
	40	100±0	
	8	31.66±9.42	
2	200	100±0	20.53
	40	65.2±0.94	
	8	28.53±4.1	
3	200	100±0	6.09
	40	94.98±0.54	
	8	55.8±5.72	
	1.6	21.63±5.51	
4	200	100±0	16.51
	40	88.09±3.56	
	8	18.81±7.06	
5	200	100±0	14.15
	40	100±0	
	8	22.57±4.83	
6	200	100±0	28.37
	40	57.56±9.16	
	8	22.15±6.13	
7	200	100±0	4.48
	40	95.61±2.37	
	8	66.46±1.96	
	1.6	20.69±8.89	
AZT (ng/ml)	200	100±100	2.34
	40	91.62±100	
	8	85.34±89.53	
	1.6	30.89±44.5	

ounds could be a reservoir of anti-HIV agents.

In this study, we extracted *D. lotus* fruits with methanol 70% and the phytochemical analysis of the methanolic extract led to the isolation and identification of seven phenolic compounds, ellagic acid, methyl gallate, gallic acid, myricetin-3-O-β-glucuronide, myricetin-3-O-α-rhamnoside, myricetin and quercetin and these compounds were evaluated for their anti-HIV-1 activity, gallic acid was the most active compound against HIV-1 with Therapeutic Index (TI) value of > 32.84 and the other compounds were less potent active. *Diospyros lotus* fruits could provide a chemical reservoir of anti-HIV agents.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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