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Research article

Isolation of betulinic acid and antioxidant and anti-HIV-1 reverse transcriptase activity of *Cratoxylum formosum* subsp. *pruniflorum* (Kurz) Gogelein extract

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Abstract

Cratoxylum formosum subsp. *pruniflorum* (Kurz) Gogelein has been used as a medicinal plant for the treatment of many diseases. *C. formosum* bark was macerated with methanol and partitioned using hexane and chloroform. The chloroform bark extract was fractionated using column chromatography. Five combined fractions were obtained (CFA–CFE) and then investigated for antioxidant activity based on the total phenolic content (TPC), total flavonoids content (TFC), ferric reducing antioxidant power (FRAP) and nitric oxide (NO) radical scavenging activity and also for anti-HIV-1 reverse transcriptase (RT) activity. CFE had the highest antioxidant activity, with values for TPC, TFC and FRAP of 44.84 ± 0.05 µg of gallic acid equivalents/g, 580.69 ± 0.78 µg of quercetin equivalents/g and 82.56 ± 0.08 µg of Trolox equivalents/g of dry weight extract, respectively, and CFB inhibited the nitric oxide radical at the lowest concentration (96.73 µg/mL). However, the methanolic bark extract, fractions CFC, CFD and CFE had high inhibitory effects against HIV-1 RT with values greater than 60% relative inhibition. CFE had the highest percentage relative inhibition among the fractions and was at a comparable level to a standard drug (Nevirapine). The ranking of the antioxidant and anti-HIV-1 RT activities of the fractions was CFE followed by CFD, CFC, CFB and CFA, respectively. Betulinic acid was isolated from fraction 52 of the chloroform extract that was eluted with chloroform-acetone (1:9, volume per volume). The ^{1}H and ^{13}C nuclear magnetic resonance data obtained were in accordance with the previous published data. There is a need for further isolation of bioactive compounds and evaluation of their antioxidant and anti-HIV activities.

Introduction

HIV is a major public problem in the world, according to the World Health Organization which reported 36.9 million people in 2015 living with HIV/AIDS worldwide. More recently, the severity of HIV has decreased, since antiretroviral drugs have helped patients to live longer and have an improved quality of life (World Health Organization, 2017). Nonetheless, the medicine is very expensive and it cannot completely cure the patient and supplies are scarce in developing countries (Souteyrand et al., 2008). Moreover, there have been reports that currently used drugs such as Nevirapine and TIBO derivatives

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can give rise to a resistant virus *in vitro* (De Clercq, 1996). Therefore, screening research for anti-HIV agents from natural sources is another option for the application of medicinal plants in the management of HIV infection or AIDS diseases (Kanyara and Njagi, 2005).

The medicinal properties provide an alternative use for plants that have been used by humans for a long time as food, building materials and clothing, as these plants have rich antioxidant properties that can inhibit the oxidation of organic molecules (Masuda et al., 2003), have low toxicity, complete biodegradability, availability from substitute sources, low cost (Yingngam et al., 2015) and may help reduce the risk of many diseases (Tunsaringkarn et al., 2012).

Cratoxylum formosum subsp. *pruniflorum* (Kurz) Gogelein, commonly known in Thai as 'tio-khon' is a tree of the family Hypericaceae and is widely distributed in dry deciduous dipterocarp forest in many southeast Asian countries (Boonnak et al., 2006) and China (Yingngam et al., 2014). In Thailand, there are six species of this genus (Smitinand, 2001). This plant has been used by local Thai people as a folk medicine for the treatment of diarrhea, internal bleeding, food poisoning (Anderson, 1986), liver cirrhosis (Fransworth and Bunyapraphatsara, 1992) as a diuretic and stomachic, to heal wounds, for its tonic effects and to reduce flatulence (Boonnak et al., 2006).

Previous phytochemical studies on *C. formosum* subsp. *pruniflorum* reported antioxidant activity of the ethanolic leaf extract (Yingngam et al., 2014), anti-inflammatory activity of phenolic compounds, xanthones, quinone, quercetin and its glycosides from leaves (Choi et al., 2012; Xiong et al., 2014), anticancer activity of ethanolic twig extract, hot water leaf extract and ethanolic leaf extract (Machana et al., 2012; Issara-Amphorn and T-Thienprasert, 2014) antibacterial and cytotoxic activities of xanthones, anthraquinones and qiunonoids from the roots and bark (Boonnak et al., 2006; Boonsri et al., 2006; Boonnak et al., 2007), antifungal and cytotoxic activity of gum extract (Thaweboon et al., 2014), anti-gastric ulceration of ethanolic leaf extract in rats (Sripanidkulchai et al., 2010) and anti-proliferation and antimigration activity of human breast cancer MCF-7 cells from leaf extract (Buranrat et al., 2017).

Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic triterpene (Fulda, 2009) and can be found in various plants including *C. arborescens*, *Syzigium Claviflorum*, and *Doliocarpus schottianus* (Fujioka et al., 1994; Reutrakul et al., 2006). This compound also has many types of biological activity, such as anti-HIV-1 (Reutrakul et al., 2006), anti-cancer (Fulda, 2009), anti-inflammatory (Costa et al., 2014), antibacterial (Woldemichael, et al., 2003) and antimalarial (Alakurtti et al., 2006). Consequently, the current study examined the activities of this compound.

There are records of preliminary screening of the antioxidant activity of some plants in dry deciduous dipterocarp forest such as *Xylia xylocarpa* (Roxb.) Taub., *Syzygium cumini* (L.) Skeels., *Mitragyna diversifolia* (Wall. ex G. Don) Havil and *C. formosum* subsp. *pruniflorum* which are well known in ethanobotany. Consequently, it can be expected that plants in this forest type have many secondary metabolite substances as a response to wildfire adaptation. *C. formosum* methanolic extract has reportedly had the highest total flavonoids contents, TFC and half maximal inhibitory concentration (IC_{50}) on nitric oxide (NO) radical scavenging than other plants (Table 1), which makes it interesting to test it for other biological activity. Therefore, the objective of this study was to extract, fractionate and determine the antioxidant and anti-HIV-1 RT activities of the crude extract and its fractionations.

Table 1 Ranking of antioxidant activity based on total phenolic content (TPC), total flavonoids content (TFC), ferric reducing antioxidant power (FRAP) and nitric oxide radical scavenging (NO) of selected plant species in dry deciduous dipterocarp forest

Plant	Antioxidant assay				SUM	Ranking
	TPC	TFC	FRAP	NO		
Xylia xylocarpa	2	4	1	2	9	2
Cratoxylum formosum	3	1	3	1	8	1
Syzygium cumini	1	3	2	4	10	3
Mitragyna diversifolia	4	2	4	3	13	4

Sample n = 3 per species.

Materials and Methods

Plant materials

Stem bark samples of *C. formosum* subsp. *pruniflorum* (Kurz) Gogelein were collected from a dry deciduous dipterocarp forest in Chainat province, Thailand, in August 2016. The plant material was identified to the species level. The dried plant materials were deposited as voucher specimens (No. PCERU_CF0002) at the Phyto-Chemodiversity and Ecology Research Unit, Department of Botany, Kasetsart University, Bangkok, Thailand.

Extraction and fractionation

Dried stem bark (2.5 kg) was ground into powder and extracted using 95% methanol (8 L, for 7 d) at room temperature in the dark. The methanolic solution (J.T. Baker; USA) was passed through filter paper and then evaporated using a rotary evaporator (Büchi Rotavapor R-205; Thailand) at a reduced pressure to give a solid methanolic extract. The methanolic extract was separated into two parts using water and hexane, and chloroform (J.T. Baker, USA), resulting in 35.50 g and 15.10 g of hexane (HEX) and chloroform (CHL), respectively.

Crude chloroform extract was isolated using wet column chromatography over a 0.2–0.5 mm silica gel (Merck; Germany) column (375 g, 5 cm × 90 cm) and eluted with the mixtures of solvents, consisting of hexane-chloroform (1:1), hexane-chloroform (3:7), hexane-chloroform (1:9), chloroform-acetone (9:1), chloroform-acetone (7:3), chloroform-acetone (1:1), chloroform-acetone (3:7), acetone and methanol. Samples (250 mL) of each of the following fractions were collected: Fractions 1–30 (hexane-chloroform, 1:1), 31–50 (hexane-chloroform, 3:7), 51–80 (hexane-chloroform, 1:9), 81–100 (chloroform-acetone, 9:1), 101–120 (chloroform-acetone, 7:3), 121–140 (chloroform-acetone, 1:1), 140–161 (chloroform-acetone, 3:7), 161–170 (acetone) and 171–180 (methanol). Chemical profiles were obtained by analysis of the fractions using thin-layer chromatography (TLC; silica gel 60 F_{254}; Merck; Germany).

Fractions with similar TLC profiles were combined into five fractions (CFA–CFE) for further testing of bioactivity.

TLC was used to group fractions having similar chemical profiles. TLC was performed on a sheet of aluminum plate, coated with a thin layer of silica gel. Fractions were spotted on the starting line of the plate. Then, the plate was run in a closed chamber with a mobile phase mixture of organic solvent and the chemical profile was investigated under ultra-violet light as wavelengths of 254 nm and 365 nm.

Antioxidant activities

Total phenolic content

The TPC of each combined fraction was determined using a modified assay from Khlifi et al. (2013). First, 0.25 mL at 500 µg/mL of each combined fraction was mixed with 1.25 mL of 2 N Folin-Ciocalteu reagent (Merck; USA) and incubated for 5 min. Then, 1 mL of 75 g/L sodium carbonate (Merck; Germany) was added to the mixture and incubated for 1 hr before absorbance measurement at 765 nm. A standard curve was prepared using gallic acid (Merck, USA) as a reference. Results were extrapolated as micrograms of gallic acid equivalents (GAE) per gram of dry weight extract.

Total flavonoids content

The TFC of each combined fraction was determined using a modified assay from Khlifi et al. (2013). A sample (0.8 mL) at 500 µg/mLof each fraction was mixed with 0.8 mL 2% aluminum trichloride (Univar; New Zealand) in methanol and incubated for 15 min before absorbance measurement at 415 nm. A standard curve was plotted using quercetin (Sigma-Aldrich; Germany) as a reference. The results were extrapolated as micrograms of quercetin equivalents per gram of dry weight extract.

Ferric reducing antioxidant power

The FRAP of each combined fraction was determined using a modified assay from Gourine et al. (2010). A sample (0.15 mL) at 500 µg/ml of each fraction was mixed with 2.85 mL of FRAP reagent (100 mL of 300 mM sodium acetate (Univar; New Zealand) buffer), 10 mL of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) (Fluka; Switzerland) in 40 mM HCl (Merck; Germany), 10 mL of 20 mM ferric chloride (Chem-supply; Australia) and incubated for 30 min before absorbance measurement at 593 nm. A standard curve was plotted using Trolox (Sigma-Aldrich; Germany) as a reference. The results were compared as micrograms of Trolox equivalents per gram of dry weight extract.

Nitric oxide radical scavenging

The NO radical scavenging of each combined fraction was determined using a modified assay from Ferreres et al. (2017). A sample (0.25 mL) of each fraction and 0.25 mL of the standard (L-ascorbic acid; Unilab; New Zealand) at various concentrations (0.1 µg/mL, 1, 10 µg/mL, 100 µg/mL, 500 µg/mL) were mixed with 1.25 mL of 10 mM sodium nitroprusside (Himedia; India) in phosphate buffer saline and incubated for 150 min at 25ºC before adding 1.5 mL of Griess reagent (1% weight per volume, w/v) sulfanilamide (Carlo Erba; France), 2% (w/v) H_3PO_4 (Macron Fine Chemicals; China) and 0.1% (w/v) naphthaylethylenediamine hydrochloride (AppliChem Panreac; Germany) and then measuring absorbance at 546 nm. The results were calculated as the half maximal inhibitory concentration (IC_{50}) according to Equation 1:

$$\% \text{ Inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample.

Anti-HIV-1 reverse transcriptase activity

The anti-HIV1 RT activity was determined, using the protocol in Silaprasit et al. (2011). All reagents were from the Reverse Transcriptase Assay Kit (EnzChek; the Netherlands). A total of 5 µL of 25 nM/µL purified HIV-1 RT and 5 µL of 50 µg/mL of the extract and fractions or 300 µM of the HIV-1 RT inhibitor Nevirapine (GPO; Thailand) and called the RT_{sample} were mixed into each well of a 96-well fluorescence plate (Cortar; USA). Thereafter, 15 µL of the primer and template polymerization buffer were added. The reactions were stopped by the addition of 2 µL of 0.2 M EDTA after incubation at 25°C for 30 min. The addition of 5 µL of Tris buffer (Merck; USA) was used in place of the samples as the control reaction ($RT_{control}$) and the background reaction was stopped by the addition EDTA immediately ($RT_{background}$), respectively. The inhibitory effect on HIV-1 RT was measured by the production of nucleic acid by adding 173 µL of picogreen in TE buffer (1:1500) and using a fluorescence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm, with the results being compared using the percentage of relative inhibition based on Equation 2 according to Kanyara and Njagi (2005) and Silaprasit et al. (2011):

$$\% \text{ Relative inhibition} = \{[(RT_{control} - RT_{background}) - (RT_{sample} - RT_{background})]/(RT_{control} - RT_{background})\} \times 100 \quad (2)$$

The percentage relative inhibition of the methanolic extract and five fractions were plotted to compare their inhibitory effects. Fractions providing greater than 60% relative inhibition of HIV-1 RT were selected and the bioactive pure compound was isolated and tested further for anti-HIV-1 RT activity.

Study design and statistical analysis

The antioxidant and anti-HIV1 RT assays involved three replicates per extract or fraction. The results were expressed as a mean value ± SD (n = 3). The differences among the treatments were determined using analysis of variance and a least significant difference test for *post hoc* comparison. Means differing at the $p < 0.05$ level were considered significantly different. The analyses were performed using the R Statistical Programming Language (R Core Team, 2017).

Betulinic acid isolation and structure elucidation

The combined fraction CFC was eluted with hexane-chloroform (1:9) solvent. The elution produced 15 mg of compound 1. The structural elucidation of compound 1 was carried out using proton (^{1}H), carbon-13 (^{13}C), heteronuclear data and comparison with the literature. The ^{1}H and ^{13}C data were obtained using nuclear magnetic resonance (Bruker Avance III HD 400 MHz nuclear magnetic resonance (NMR) spectrometer; USA) and the molecular weight of the compound was determined using mass spectrometry (Bruker/microtof-Q III; USA) at the Department of Chemistry and Scientific Equipment Center, Faculty of Science, Kasetsart University, Bangkok, Thailand. The chemical shift values were reported in parts per million.

Results and Discussion

Extraction and fractionation of stem bark material

The CHL extract was fractionated using the chromatography technique by mixing solvents from low polarity to high polarity. For further isolation of the bioactive compounds, the fractions with similar profiles were combined into 14 combined fractions, namely: 1, 1–18; 2, 19–22; 3, 23–37; 4, 38–50; 5, 51–66; 6, 67–81; 7, 82–86; 8, 87–111; 9, 112–114; 10, 115–128; 11, 129; 12, 130–160; 13, 161–170; and 14, 171–180. Then the 14 fractions were divided into five combined fractions (CFA–CFE, Table 2) for testing their biological activity based on their antioxidant and anti-HIV-1 reverse transcriptase activities.

Table 2 Combined fraction code, number of fractions and ratio of solvent system for column chromatography of each combined fraction for bark extracts of *Cratoxylum formosum* subsp. *pruniflorum*

Combined fractions code	Number of fractions	Ratio of solvent system (volume per volume)
CFA	1–30	Hexane-chloroform (1:1)
	31–37	Hexane-chloroform (3:7)
CFB	38–50	Hexane-chloroform (3:7)
CFC	51–80	Chloroform-acetone (1:9)
CFD	81–100	Chloroform-acetone (9:1)
	101–120	Chloroform-acetone (7:3)
	121–140	Chloroform-acetone (1:1)
	141–160	Chloroform-acetone (3:7)
CFE	161–170	Acetone
	171–180	Methanol

Antioxidant activities

Total phenolic content

The TPC differed significantly between the methanolic extract and the five fractions. The highest total phenolic content was in fraction CFE, followed by fractions CFD, CFC, CFB, the methanolic extract, and fraction CFA, respectively, compared with the standard curve for gallic acid (coefficient of determination, R^2 = 0.9987, Fig. 1A). The results were consistent with previous studies involving extracts of *Trifolium pretense* L. and of *C. formosum* leaves, which had a higher TPC in the methalonic extract than for the extracts with any other solvents (Esmaeili et al., 2015; Promraksa et al., 2015). More phenolic compounds were found in high polarity solvents than low polarity solvents because the hydroxyl group in the phenolic compounds creates polarity and allows the compounds to dissolve more readily in a high polarity solvent (Galanakis et al., 2013). Antioxidant activity has been shown to be correlated with the position or the number of hydroxyl group in phenolic compounds, where a greater number of hydroxyl groups in the compound is more likely to increase the antioxidant activity (Kuo et al., 2015; Tan and Lim, 2015). Therefore, extraction using a high polarity solvent can potentially increase the opportunity to obtain a compound with high anti-oxidant activity.

Total flavonoids content

The TFC also differed significantly between the methanolic extract and five fractions. CFE had the highest TFC (580.69 µg of QE/g; R^2 = 0.9932; Fig. 1B). The TFC values in the fractions had a similar pattern to the TPC, being higher in extracts from a high polarity solvent (Esmaeili et al., 2015; Promraksa et al., 2015). However, flavonoids can also be found in extract with a low polarity solvent, as reported in the extraction from leaves of *Vitex doniana* (Yakubu et al., 2014), where the highest TFC (390.00 mg/mL of quercetin) was in chloroform extract. This was because the basic structure of the flavonoids allows them to dissolve in a low polarity solution. However, when that structure is replaced with a hydroxyl group, it can dissolve in a high polarity solution, which was most likely the case with the extractions in the current study.

Ferric reducing antioxidant power

The methanolic extract and five fractions were significantly different in their FRAP values. Similar to TPC and TFC, the CFE fraction also had the highest FRAP value (82.56 ± 0.08 µg of TE/g of extract; R^2=0.8499; Fig. 1C) High polarity fractions have high amounts of phenolics and flavonoids and this was in agreement with previous reports by Hwang et al. (2013) and Khanum et al. (2015). Methanol extract of *Prunella vulgaris* var. lilacina aqua extract and *Cajanus cajan* seed had high antioxidant activity, which may have been due to the phenolic compound having the ability to donate hydrogen to Fe (III) (Al-Snafi, 2015; Edwards et al., 2015).

Nitric oxide radical scavenging

The NO radical scavenging was expressed as IC_{50} values, which refer to the concentration at which 50% inhibition of nitric oxide occurs. Different fractions had significantly different IC_{50} values. The CFB fraction inhibited The NO radical at the lowest concentration (96.73 µg/mL; R^2 = 0.9648; Fig. 1D), but this IC_{50} value was not significantly different from those of the fractions CFC, CFD, CFE (R^2 = 0.9866, 0.9864 and 0.9580, respectively). These results were consistent with previous study that showed that isolated compounds (as well as methanolic extractions and fractions) from the root of *Cratoxylum formosum* could inhibit NO production (Boonnak et al., 2006).

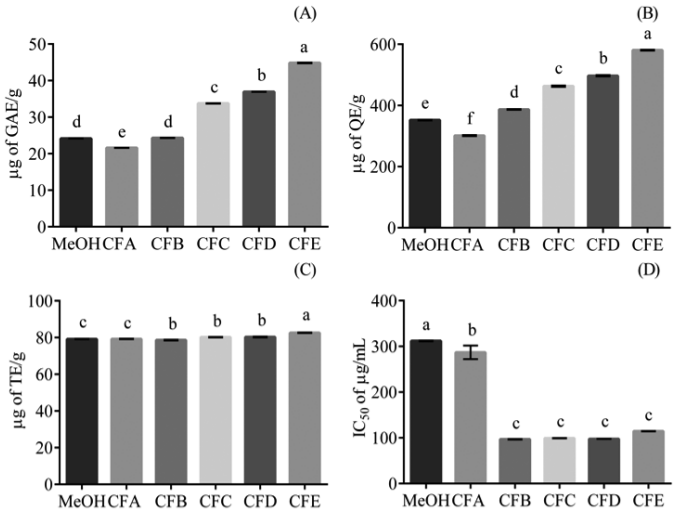

Fig. 1 Antioxidation activity of methanolic extract and combined fractions (details shown in Table 2) of *C. formosum* subsp. *pruniflorum* in four assays: (A) Total phenolic content; (B) Total flavonoids; (C) Ferric reducing antioxidant power; (D) Nitric oxide radical scavenging, where $n = 3$; error bars represent SD; different letters above bars indicate significant differences ($p < 0.05$) among the treatments

Anti-HIV-1 reverse transcriptase activity

The crude methanolic extract and five combined fractions had significantly different anti-HIV-1 RT activity. While most of the fractions had significantly lower percentage inhibition values than the positive control of the standard drug, Nevirapine, the CFE fraction had a higher value with significantly different percentage of inhibition than that of Nevirapine (Fig. 2). The results suggested that the CFE fraction had, at least, a similarly high level of anti-HIV-1 RT activity as the standard drug. This current study is the first to report on the anti-HIV-1 RT activity of this plant species. A previous study in a related species, *Cratoxylum arborescens*, also reported anti-HIV-1 RT activity in its crude methanolic extract and pure compounds (Reutrakul et al., 2006).

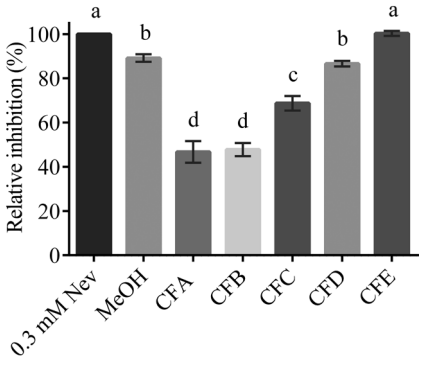

Fig. 2 Percentage relative inhibition of anti-HIV-1 RT from methanolic extract and combined fractions (details shown in Table 2) at final concentration of 10 µg/mL from bark extract of *C. formosum* subsp. *pruniflorum*, compared with the standard drug Nevirapine (NEV) where $n = 3$; error bars represent SD; different letters above bars indicate significant differences ($p<0.05$) among the treatments

Moreover, anti-HIV-1 RT activity has been found in extractions of other plant species, including hot/warm aqua extract of *Acacia mellifera* (Vahl) Beuth, *Maytenus buchanani* (Loes) Wilczer, *Maytenus senegalensis* (Lam.) Excell., *Rhus natalensis* (Kraus), *Melia azedarach*, *Vernonia jugalis* (Olive & Hiern) and *Prunus africana* (Hook. F.) Kalkm. (Kanyara and Njagi, 2005), in methanolic extract of *Phyllantus pulcher*, *P. urinaria*, *P. myrtifolius* (Eldeena et al., 2011), in hexane extract of *Cinnamomum loureiroi*, *Quercus infectoria*, *Plumbago indica* L., *Artocarpus heterophyllus* Lam. and *Acorus calamus* L. (Silaprasit et al., 2011), in aqua/ethanolic extract of *Hypoxis sobolifera* and in aqua extract of *Bulbine alooides* and *Leonotis leonurus* (Klosa et al., 2009). The diversity of plants that exhibit anti-HIV-1 RT activity suggests that medicinal plants remain a rich source of anti-HIV agents for future exploration.

Betulinic acid isolation and structural elucidation

The isolation and purification of the pure compounds focused on the combined fractions CFC, CFD and CFE, because they showed high level of antioxidant and anti HIV-1 RT activities (Table 3). From the CFC combined fraction (fraction 52) that was eluted with chloroform-acetone (1:9) and showed moderate anti-HIV-1 RT activity, a white crystal compound was isolated and purified using Sephadex LH-20 (Sigma-Aldrich; Germany) eluted with methanol and by comparing the ^{1}H and ^{13}C NMR data with previous reports as shown in Table 4 (Ressmann et al., 2017). The isolated compound was identified as betulinic acid with a molecular weight of 457.36 [$M+H^+$] (calculated MW = 456.7 g/mol) as shown in Fig. 3. Therefore, the biological activities of this compound will be further investigated.

Table 3 Ranking of five fractions (details shown in Table 2) of antioxidant activity based on total phenolic content (TPC), total flavonoids content (TFC), ferric reducing antioxidant power (FRAP) and nitric oxide radical scavenging (NO) and on anti-HIV-1 RT activity

Fraction	Antioxidant assay				HIV-1 RT	SUM	Ranking
	TPC	TFC	FRAP	NO			
CFA	5	5	5	5	5	30	5
CFB	5	4	4	1	4	22	4
CFC	3	3	3	3	3	18	3
CFD	2	2	2	2	2	11	2
CFE	1	1	1	4	1	10	1

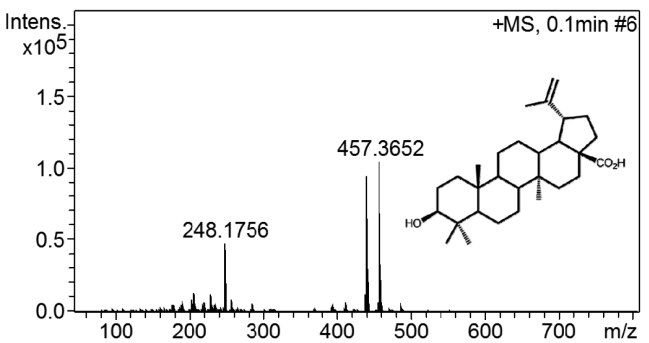

Fig. 3 Mass spectral data and structure of betulinic acid

Table 4 ^{1}H spectral data and ^{1}C nuclear magnetic resonance (NMR) spectral data (400 MHz) of compound 1 and compared betulinic acid (Ressmann et al., 2017)

^{1}H spectra data of betulinic acid	Compound 1	^{1}C NMR spectra data of betulinic acid	Compound 1
δ ^{1}H (ppm)	δ ^{1}H (ppm)	δ ^{13}C (ppm)	δ ^{13}C (ppm)
0.67	0.64	15.1	14.9
0.75	0.75	15.7	16.2
0.80	0.86	16.4	16.3
0.93	0.92	16.5	16.4
0.96	0.97	18.6	18.4
0.97	0.98	19.7	19.4
1.18	0.19	21.2	20.9
1.27	1.26	25.8	25.5
1.69	1.63	27.8	27.6
1.97	2.09	28.3	28.6
2.19	2.19	30.1	29.6
2.26	2.25	30.9	30.6
3.00	2.96	32.5	32.2
3.19	3.16	34.7	34.4
4.60	4.56	37.4	36.8
4.74	4.69	37.6	37.2
		38.7	38.7
		39.1	38.9
		39.2	39.3
		41.0	40.7
		42.8	42.5
		47.2	47.1
		49.6	49.0
		50.9	50.4
		55.7	55.4
		56.6	55.9
		79.4	77.3
		110.1	110.1
		150.8	150.8
		180.2	177.7

ppm = parts per million

Conflict of Interest

The authors declare that there are no conflicts of interest.

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