



Research article

Isolation of stigmasterol from Kra Don (*Careya arborea* Roxb.) and bioactivities of its crude extracts against free radicals and human immunodeficiency virus

Sorawit Bapia^{a,†}, Nattawut Srisombata^a, Siriluk Ratanabunyong^b, Kiattawee Choowongkamon^b, Srunya Vajrodaya^a, Narong Wongkantrakorn^a, Sutsawat Duangsrirai^{a,*}

^a Phyto-chemodiversity and Ecology Research Unit, Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

^b Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

Article Info

Article history:

Received 9 January 2020

Revised 2 December 2020

Accepted 22 December 2020

Available online 31 January 2021

Keywords:

Antioxidant,

Anti-human immunodeficiency virus (HIV),

Careya arborea,

Stigmasterol

Abstract

Careya arborea Roxb. (family Lecythidaceae) has long been used as a medicinal plant for the treatment of many diseases. In this study, extracts were taken from the stem bark, wood and leaves using methanol and then investigated for antioxidant activities, such as 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity, nitric oxide radical scavenging activity (NO), superoxide radical scavenging activity (SOD), ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and total flavonoid content (TFC). For the antioxidant activities, the stem bark extract had the highest mean ($\pm$ SD) levels (623.96 ± 12.50 μ g/mL, 140.86 ± 2.63 μ g/mL, 152.38 ± 6.35 μ g/mL, 252.44 ± 4.87 mg Trolox equivalent/g and 128.19 ± 2.12 mg gallic acid equivalent/g, respectively) except for the total flavonoid content, for which the leaf extract produced the highest amount (30.62 ± 0.29 mg quercetin equivalent/g). However, regarding the anti-human immunodeficiency virus (HIV) activity using an HIV-1 reverse transcriptase inhibition assay, the stem bark and leaf extracts had a high inhibitory effect against HIV-1 reverse transcriptase with values of more than 100% of the relative inhibition for a comparable level of the standard drug (Nevirapine). The highest ranking of the antioxidant and anti-HIV activities of the fractions was the stem bark followed by the leaf and wood extracts, respectively. Stigmasterol was isolated from combined fraction number 3 of the lipophilic extract eluted from n-hexane-ethyl acetate (95:5) via column chromatography. The ¹H and ¹³C NMR data obtained were in accordance with previous reports. Future study will investigate further isolation of these bioactive compounds and evaluation of their bioactivities.

Introduction

In recent years, the destructive effects of free radicals and other oxidants have been recognized globally as a serious concern, as the

imbalance of reactive oxygen species (ROS) and oxidative stress in organisms can damage important cellular structures including proteins, lipids and nucleic acids (Pizzino et al., 2017). This is not the only impact following exposure by various endogenous systems as it can also impact different pathological states. For example, free radicals are responsible for causing several diseases including cancer,

[†] Equal contribution.

* Corresponding author.

E-mail address: fscissw@ku.ac.th (S. Duangsrirai)

cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol-induced liver disease, ulcerative colitis, aging and atherosclerosis (Alam et al., 2013). Therefore, investigating the potential sources of antioxidants is a key to improving the quality of life against cell degeneration from degenerative diseases.

HIV (human immunodeficiency virus) is a virus resulting in acquired immunodeficiency syndrome (AIDS). Since the virus first began spreading, approximately 37.9 million people have been infected as at 2018 (United Nations Programme on HIV and AIDS [UNAIDS], 2020). In addition, AIDS is the most important cause of mortality in low-income countries and one of the 10 leading causes of death worldwide (Sonar et al., 2017). Because HIV-1 is a retrovirus, the first process for viral propagation in host cells is reverse transcription. The enzyme HIV-1 reverse transcriptase (HIV-1 RT) is responsible for the transcription of viral RNA into DNA, which is later integrated into the host cell and carries information for the synthesis of new viral particles and this enzyme is one of the main targets for inhibiting the reproduction of HIV (Silprasit et al., 2011). Nowadays, the development of virus resistance to drugs is a continuing problem. Therefore, a new source for anti-viral drugs is necessary. Recently, natural products such as plant extracts seem to have become a major focus for anti-HIV drug discovery and development (Yang et al., 2012).

There are many plants in the forest that have been used for medicinal purposes in many regions for a long time. For example, in rural areas, many medicinal plants are used in the treatment of various diseases to cure the sickness by themselves because they are easy to be found in the local area and are well-known by practitioners of traditional medicine (Palombo, 2006). The secondary metabolites are useful compounds that can be produced under controlled conditions independent of climatic, environmental changes or soil conditions due to over pollution (Tiwari and Rana, 2015). Many plant extracts are thought to have anti-HIV properties (Behbahani, 2014). For example, the stem bark extract of *Hymenodictyon orixense* (Roxb.) Mabb. had a high relative inhibition against HIV-1 RT compared with nevirapine, an anti-HIV drug, as a positive control (Soun-udom et al., 2019). Srisombat et al. (2019) reported the methanolic extract from the stem bark of *Cratogeomys formosum* subsp. *pruniflorum* (Kurz) Gogelein also had, at least a similarly high level of anti-HIV-1 RT activity as the standard drug. The alkaloids extract from *Phyllanthus niruri* could decrease cytopathogenicity in MT-4 cells caused by HIV infection (Kim et al., 2011) and *Camellia sinensis* extract, which contain epicatechin gallate and other substances, expressed inhibitory effects on the HIV RT enzyme using radioactive assay (Nagao et al., 1996). In addition, the metabolites could also be used as natural precursors for the semi-synthesis of high potential compounds and this modification can also improve the already proven high levels of bioactivity, such as anti-viral activity, of several basic structures (Venditti et al., 2017). Therefore, plant metabolites have drawn a large amount of attention as a potential source for developing new drugs for the above-mentioned diseases.

Careya arborea Roxb. is a deciduous plant in the family Lecythidaceae that is distributed throughout many regions in South

Asia and South-east Asia. In Thailand, this plant is known as 'kra don' and can be found in deciduous dipterocarp forests as well as dry evergreen forests (Office of the Forest Herbarium, National Park, Wildlife and Plant Conservation Department, 2012). From previous studies concerning the ethnobotany and bioactivity of this plant, many parts of the plant have been used in many regions as ingredients for traditional medicine. (Karunamoorthi et al., 2012). For example, the whole plant can be used for healing snake bites (Bhandary and Chandrashekhar, 2000), while the stem bark is used to treat skin diseases (Malhotra and Moorthy, 1973). The leaf extract can inhibit the growth of *Leishmania donovani* (strain AG 83) by approximately 75% in 7 d (Mandal et al., 2006). The extract from the bark has also shown potent cytotoxicity against cancer cell lines, such as cancerous RD, HEp-2 and HeLa (Senthilkumar et al., 2007). Until now, there has been no report of phytochemical investigation concerning the antioxidant properties from wood and no report on the anti-HIV properties of this plant.

Therefore, the purpose of this study was to investigate the antioxidant and HIV-1 RT activities of three parts of this plant (stem bark, wood, leaf) using different assays and to isolate any potent compounds.

Materials and Methods

Plant material preparation

Careya arborea Roxb. stem bark, wood and leaves were collected from a deciduous dipterocarp forest in Chai Nat province, Thailand. The plant was identified to the species level by Mr. Sorawit Bapia, Department of Botany, Kasetsart University, Bangkok, Thailand. The dried plant materials and voucher specimens preserved for further reference (No. PCERU_CA004) were deposited at the Phyto-Chemodiversity and Ecology Research Unit, Department of Botany, Kasetsart University. Samples of the stem bark, wood and leaves of this plant were cleaned, air-dried at room temperature and separately ground into powder using an electric grinder.

Extraction and fractionation

The extract from the dried stem bark was obtained using methanol (7 d per extraction with three repetitions) at room temperature. The extracted methanol solution was filtered and evaporated in a rotary evaporator at a reduced pressure to provide a crude methanol extract, which was used for the bioactivities screening. Then, the crude methanol extract, which has the highest potential bioactivity levels, was re-extracted with a mixture of water and chloroform to separate it based on polarity into two layers, namely the lipophilic and hydrophilic solutions. The lipophilic solution, (the lower layer) was evaporated to give a crude lipophilic extract.

The crude lipophilic extract was fractionated using column chromatography with a wet column technique over a 0.2–0.5 mm Merck Si gel column and eluted with mixtures of n-hexane, n-hexane-ethyl acetate (95:5), n-hexane-ethyl acetate (9:1), n-hexane-ethyl

acetate (7:3), n-hexane-ethyl acetate (1:1), n-hexane-ethyl acetate (3:7), ethyl acetate, acetone and methanol. Collection of 500 mL of each fraction was carried out, and each fraction was evaporated in a rotary evaporator at reduced pressure until dry.

After fractionation, the thin-layer chromatography (TLC) technique was used in the investigation of the chemical profile of each fraction. TLC was performed on the surface of an aluminum plate, which was coated with a thin layer of silica gel. The fractions having similar chemical profiles were combined. Then, the plate was placed in a closed chamber with a mobile phase mixture organic solvent and the chemical profile was investigated under ultraviolet light at 254 and 365 nm wavelengths.

Isolation and elucidation of structure

The recrystallization technique involving column chromatography with a wet column technique over a Sephadex LH-20, and preparative thin-layer chromatography were performed using silica gel GF254 to isolate and purify the chemical compounds from the extract. Then, the structural elucidation of compounds was investigated using proton (^1H) and carbon-13 (^{13}C) heteronuclear data and comparisons were made with Cayme and Ragasa (2004). The ^1H and ^{13}C spectral data were obtained using nuclear magnetic resonance with chloroform- d (CDCl_3) as the solvent (Bruker Avance III HD 400 MHz NMR spectrometer; USA), in which the chemical shift values were reported in parts per million. In addition, the mass spectra of the compounds were determined using mass spectrometry (Bruker-microTOF-Q III; USA) at the Department of Chemistry and Scientific Equipment Center, Faculty of Science, Kasetsart University.

Antioxidant activities screening

2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity

Free radicals were evaluated using a 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity (DPPH) test as described by Khlift et al. (2013) and Gourine et al. (2010) with some modifications. Various concentrations of plant samples (31.25–2,000 $\mu\text{g/mL}$, 1 mL) were mixed with DPPH in methanol (0.1 mM, 1 mL), incubated for 30 min in darkness. Then the absorbance (A_{sample}) was measured at 517 nm against a blank sample (A_{control}). The free radical scavenging activity was calculated as the percentage inhibition using Equation 1:

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

The activity of plant samples was express in terms of the half-maximal inhibitory concentration (IC_{50}) to find the concentration of plant sample that could decrease the DPPH concentration to 50%.

Nitric oxide radical scavenging activity

To evaluated nitric oxide radical scavenging (NO), this assay used sodium nitroprusside to generate nitric oxide in solution; the interaction of nitric oxide with oxygen which produced nitrite ion was

detected using Griess reagent. Nitric oxide radical scavenging assay was evaluated using the method of Ferreres et al. (2017) with some modification. Various concentrations of plant sample (31.25–1,000 $\mu\text{g/mL}$, 0.5 mL) were mixed with sodium nitroprusside in phosphate buffer (0.1 M, pH 7.4, 0.5 mL) and incubated for 150 min in darkness. Then, the mixture was added with Griess reagent (1 mL) and the absorbance (A_{sample}) was measured at 560 nm against a blank sample (A_{control}). The free radical scavenging activity was calculated as the percentage inhibition using Equation 2:

$$\% \text{Inhibition} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (2)$$

The activity of plant samples was express in terms of the IC_{50} to find the concentration of plant samples that could inhibit the nitric oxide radical by 50%.

Superoxide radical scavenging activity

Superoxide anion scavenging (SOD) was evaluated using the method of Alam et al. (2013) with some modifications. Various concentrations of plant sample (31.25–1,000 $\mu\text{g/mL}$, 100 μL) were mixed with 0.3 mM Nitroblue tetrazolium in 16 mM Tris-HCl (200 μL), NADH solution (936 mM, 1,000 μL) and Tris-HCl (16 mM, pH 8.0, 50 μL), with reaction occurring when phenazine methosulfate (0.12 mM, 200 μL) was added into the mixture. Then, the absorbance (A_{sample}) was measured at 560 nm against a blank sample (A_{control}). The free radical scavenging activity was calculated as the percentage inhibition using Equation 3:

$$\% \text{Inhibition} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (3)$$

The activity of plant samples was expressed in terms of the IC_{50} to find the concentration of plant samples that could decrease the superoxide radical concentration to 50%.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay is a simple means of measuring antioxidant capacity. The FRAP assay was evaluated using a modified method of Iqbal et al. (2015) and Gourine et al. (2010). Acetate buffer (300 mM, 100 mL, pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl; 10 mL) and ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 20 mM, 10 mL) were mixed together for preparing fresh FRAP reagent. The plant sample (0.1 mg/mL, 0.15 mL) was mixed with fresh FRAP reagent (2.85 mL) and incubated for 30 min in darkness. The absorbance was measured at 593 nm against a blank sample. Trolox was used as the standard. The results were expressed as Trolox equivalent (TE) per gram of extract.

Total phenolic content

The total phenolic content (TPC) was evaluated using the method of Folin and Ciocalteu (1927) as described by Khlift et al. (2013) with some modification. The plant sample (1 mg/mL, 0.25 mL) was mixed

with Folin-Ciocalteu reagent (0.2 N, 1.25 mL) and incubated for 5 min at room temperature. Then sodium carbonate (75 g/L in water, 1 mL) was added and incubated for 1 hr. The absorbance was measured at 765 nm against a blank sample. Gallic acid was used as a standard. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

Total flavonoid content

The total flavonoid content (TFC) was evaluated using Dowd's method as described by Khlif et al. (2013) with some modifications. The plant sample (1 mg/mL, 0.8 mL) was added with aluminum trichloride (AlCl_3) in methanol (2% weight per volume, 0.8 mL) and incubated for 15 min at room temperature. The absorbance was measured at 415 nm against a blank sample consisting of extract and methanol. Quercetin acid was used as a standard. The results were expressed as quercetin equivalent (QE) per gram of extract.

Anti-HIV activity screening

HIV-1 reverse transcriptase inhibition assay

Anti-HIV1 RT activity was determined using the protocol in Silprasit et al. (2011) and Kanyara and Njagi (2005). All reagents were from the Reverse Transcriptase Assay Kit, (EnzChek; the Netherlands). The samples (RT sample) consisted of 5 μL of 25 nM/ μL purified HIV-1 RT and 5 μL of 50 $\mu\text{g/mL}$ of the extract and fractions, or 300 μM of the HIV-1 RT inhibitor Nevirapine (GPO, Thailand). All samples were mixed into each well of a 96-well fluorescence plate (Cortar; USA). Thereafter, 15 μL of primer and template polymerization buffer were added. The reactions were stopped by the addition of 2 μL of 0.2 M ethylenediaminetetraacetic acid (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 immediate addition of EDTA (RT_{background}), respectively. The inhibitory effect on HIV-1 RT was measured as 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. The results were compared based on the percentage of relative inhibition using Equation 4:

$$\% \text{Relative Inhibition} = \left[\frac{(\text{RT}_{\text{control}} - \text{RT}_{\text{background}}) - (\text{RT}_{\text{sample}} - \text{RT}_{\text{background}})}{A_{\text{control}}} \right] \times 100 \quad (4)$$

The percentages of relative inhibition of methanolic extracts were plotted on a graph to compare their inhibitory effects.

Statistical analysis

Each test in the antioxidant and anti-HIV1 RT assays had three replicates per extract or fraction. The results were expressed as mean $\pm$ SD ($n = 3$). The differences among the treatments were determined, using analysis of variance and a least significant difference test for the post-hoc comparison at 95% confidence interval. The analyses were performed using the R Statistical Programming Language (R Core Team, 2017).

Results and Discussion

Antioxidant activity screening

The antioxidant activities of the crude methanolic extracts of *C. arborea* were evaluated using six different assays to measure the potency of activity against free radicals as well as the amounts of chemical substances that were known to exhibit antioxidant activity. Evaluating the antioxidant activities using only 1–2 assays would not accurately represent the overall antioxidant potential of each plant because the antioxidant activity of plants can be influenced by many factors; thus, it is important to use a variety of antioxidant activity measurements to assess the various mechanisms of antioxidant action (Gan et al., 2010).

The free-radical scavenging activities of the stem bark, wood and leaf samples were evaluated using three different assays, as shown in Fig. 1A–C. The results were expressed in terms of the IC_{50} . DPPH radical scavenging activity, the activity against DPPH radicals of stem bark, wood and leaf extracts were $623.96 \pm 12.50 \mu\text{g/mL}$, $1,135.88 \pm 12.52 \mu\text{g/mL}$ and $551.25 \pm 0.81 \mu\text{g/mL}$, respectively. For nitric oxide radical scavenging activity, the activity levels against nitric oxide radicals of stem bark, wood and leaf extracts were $140.86 \pm 2.63 \mu\text{g/mL}$, $588.69 \pm 94.54 \mu\text{g/mL}$ and $379.79 \pm 55.91 \mu\text{g/mL}$, respectively. In terms of superoxide radical scavenging activity, the activity levels against superoxide radicals of stem bark wood were $152.38 \pm 6.35 \mu\text{g/mL}$, $931.37 \pm 1.49 \mu\text{g/mL}$ and $809.53 \pm 9.68 \mu\text{g/mL}$, respectively.

The ferric reducing antioxidant power assay, total phenolic content and total flavonoid content are shown in Fig. 1D–F. The results were calculated from the standard graph, which was linear over the calibration range with coefficient of determination (R^2) values of 0.9936, 0.9999 and 0.9870, respectively. The FRAP assay is dependent on the reduction of ferric ion into ferrous ion (Benzie and Strain, 1996). The extract from stem bark had higher activity than the extracts from wood and leaf ($252.44 \pm 4.87 \text{ mg TE/g}$, $118.20 \pm 0.13 \text{ mg TE/g}$ and $188.11 \pm 16.38 \text{ mg TE/g}$, respectively). The total phenolic content values of stem bark, wood and leaf extracts showed that bark ($128.19 \pm 2.12 \text{ mg GAE/g}$) had a higher amount of phenolic compounds than the wood and leaf extracts ($60.09 \pm 1.10 \text{ mg GAE/g}$ and $112.18 \pm 0.12 \text{ mg GAE/g}$, respectively). The total flavonoid content values in the leaf extract ($30.62 \pm 0.29 \text{ mg QE/g}$) had a higher content of phenolic compounds than stem bark and wood extracts ($2.03 \pm 0.05 \text{ mg QE/g}$ and $0.89 \pm 0.03 \text{ mg QE/g}$, respectively).

Many parts of *C. arborea* are known to be used for therapeutic properties, but little supporting scientific evidence exists, such as for antioxidant activity. In fact, there is no report on antioxidant research of the wood. Taking into account the results of all the antioxidant assays in the current study, the stem bark extract had the highest antioxidant activity. This was consistent with Senthilkumar et al. (2007) who evaluated antioxidant activity and cytotoxicity and they reported that the methanolic extract from the stem bark of *C. arborea* had strong antioxidant activity against many oxidants such as 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and DPPH free radicals, hydrogen peroxide, nitric oxide, the hydroxyl radical by deoxyribose and by p-NDA and the superoxide radical by alkaline DMSO and lipid peroxidation.

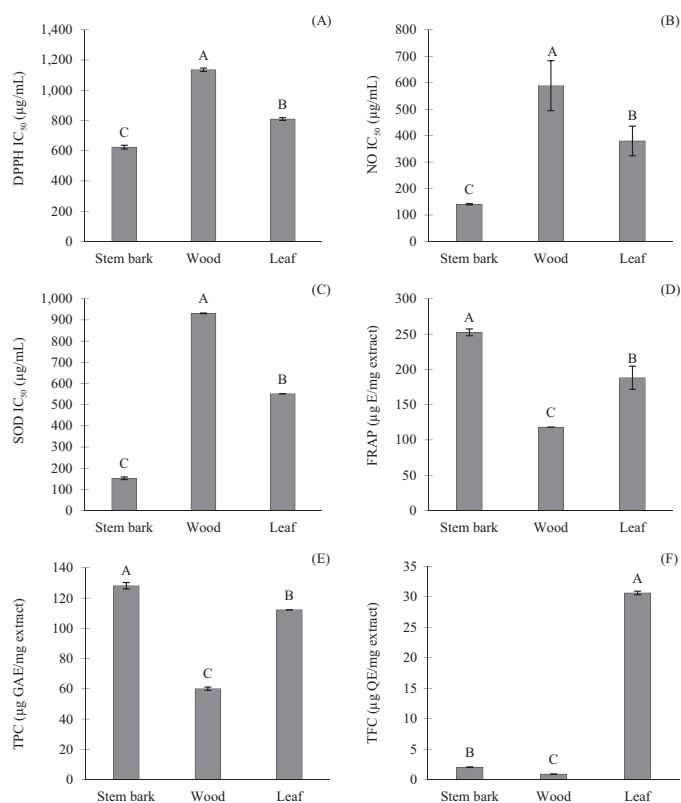


Fig. 1 Autoxidation activity screening of stem bark and wood extract of *C. arborea* in six assays: (A) 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity (DPPH); (B) nitric oxide radical scavenging activity (NO); (C) superoxide radical scavenging activity (SOD); (D) ferric reducing antioxidant power assay (FRAP); (E) total phenolic content (TPC); (F) total flavonoid content (TFC), where $n=3$, values are presented as mean $\pm$ SD and IC_{50} = the half-maximal inhibitory concentration. Different letters above bars indicate significant differences ($p < 0.05$) among the treatments.

The current results could also be observed in the absence of flavonoid content in the stem bark and wood extract. This was consistent with Kapoor et al. (1972), who investigated the apparent absence of alkaloids and flavonoids from stem bark.

Polyphenols are normally abundant in stem bark (Vazquez et al., 2008) and comprise a very important plant constituent because of their scavenging ability on free radicals due to their hydroxyl groups (Tosun et al., 2009). They also have high potential in many bioactivities. Most of these effects are believed to be due to antioxidant properties (Moure et al., 2001). An extract that has high amounts of phenolic and flavonoid compounds also shows high activity in terms of ferric reducing antioxidant power (Hwang et al., 2013; Khanum et al., 2014).

Anti-HIV activity screening

HIV type 1 (HIV-1) is the main type of AIDS infection worldwide and reverse transcription is an essential step for retroviral replication (Hu and Hughes, 2012). Most chemotherapeutic agents are directed to the HIV-1 RT, a key enzyme that plays an essential and multifunctional role in the replication of HIV-1 (Castro et al., 2006). A natural product

with the ability to inhibit HIV-1 RT would be an obvious therapeutic target for drug design in the future.

The extract from stem bark wood and leaf of *C. arborea* were utilized for evaluation of anti-HIV activity using an HIV-1 reverse transcriptase assay. At a final concentration of 10 μ g/mL, the results indicated that the relative inhibition levels of stem bark wood and leaf extracts of *C. arborea* were $143.33 \pm 2.21\%$, $74.52 \pm 4.45\%$ and $124.36 \pm 1.36\%$, respectively (Fig. 2). The relative inhibition of Nevirapine at a concentration of 300 μ M was $98.82 \pm 0.80\%$. These results suggested that the stem bark and leaf extracts had higher levels of HIV-1 RT activity than the standard drug.

This study was the first report concerning the HIV-1 RT enzyme of this plant species. Based on the report of (Silprasit et al., 2011), an extract should show relative inhibition of more than 80% to be considered having a strong inhibitory action against HIV-1 RT. Therefore, the stem bark and leaf extracts could be considered to exhibit high inhibition activity, while the wood extract could be considered to have moderate inhibitory activity for HIV-1 RT.

Some literature has reported that the high value of phenolic, flavonoids and the high antioxidant activities also imply high activity against HIV-1 reverse transcriptase (Eldeen et al., 2011) because the accumulation of ROSs has been linked to the simulation of HIV-1 developing into AIDS (Gil et al., 2003). Therefore, antioxidants can protect cells against the excessive free radicals that have been implicated in the causality of many diseases (Devasagayam et al., 2004).

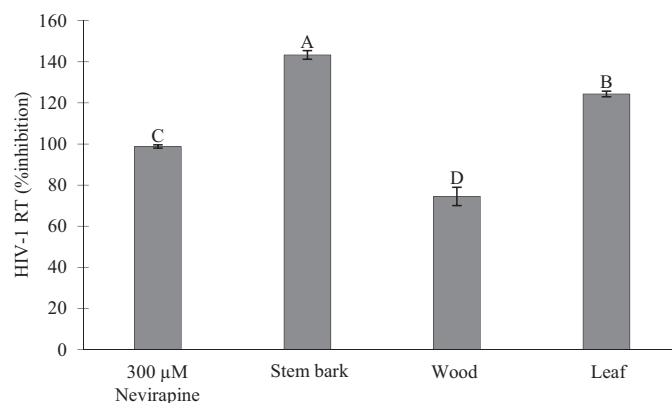


Fig. 2 Percentage of relative inhibition of anti-human immunodeficiency virus reverse transcriptase (HIV-1 RT) of stem bark and wood extract of *C. arborea* at 10 μ g/mL compared with standard drug (Nevirapine), where $n=3$ and values are mean $\pm$ SD. Different letters above bars indicate significant differences ($p < 0.05$) among the treatments.

Ranking of antioxidant and anti-HIV activities of *C. arborea* extract

For selecting the parts of *C. arborea* having the highest levels of bioactivity against free radicals and HIV, the overall results from six assays of antioxidant activities and anti-HIV activity of three parts were ranked according to the method described by Silva and Sirasa (2018). From the rankings (Table 1), the extract from the stem bark was ranked first in terms of overall antioxidant activities, though not for total flavonoid content. The stem bark extract was also ranked first in terms of HIV-1 reverse transcriptase activity.

Table 1 Ranking of stem bark, wood and leaf extracts of *C. arborea* in six assays ($n = 3$) of antioxidant activities and anti-human immunodeficiency virus reverse transcriptase activity

Plant part	Antioxidant activity						HIV-1 RT	Σ Rank	Ranking
	DPPH	NO	SOD	FRAP	TPC	TFC			
Stem bark	1	1	1	1	1	2	1	8	1
Wood	3	3	3	3	3	3	2	20	3
Leaf	2	2	2	2	2	1	1	12	2

DPPH = 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity; NO = nitric oxide radical scavenging activity; SOD = superoxide radical scavenging activity; FRAP = ferric reducing antioxidant power assay; TPC = total phenolic content; TFC = total flavonoid content; HIV-1-RT = anti-human immunodeficiency virus reverse transcriptase

The high levels of antioxidant activity might promote the reduction of the viral load in HIV-infection (Kapewangolo et al., 2013). Oxidative stress has been linked to the development of HIV and has also been implicated in the pathogenesis of HIV/AIDS, since the virus replicates in a highly oxidized environment (Valle et al., 2013). Antioxidants have been shown to reduce oxidative stress and in the process, may also contribute to a reduction in viral load (Allard et al., 1998). From the current results, it could be concluded that a plant part having a high level of polyphenol, such as the stem bark, can inhibit the HIV virus better than other parts.

Various pieces of related literature have reported that phenolic compounds, especially tannin, can inhibit the activity of HIV. Lu et al. (2004) studied the inhibition of tannin (a phenolic compound) from HIV-1 entry by targeting gp41. They concluded that tannin is a potent anti-HIV agent with many mechanisms, such as inhibiting HIV-1 replication, inhibiting HIV-1 mediated cell-fusion and interfering with gp41 six-helix bundle formation. The findings from the study of Lu et al. (2004) were also consistent with the current results that showed the stem bark extract contained a high amount of phenolic compounds as well as having substantial activity against HIV through the inhibition of HIV-1 RT activity. Therefore, the stem bark extract was subjected to further fractionation and isolation to investigate the bioactive compounds in more detail.

Extraction and fractionation

Stem bark extract was prepared again with the same method described above. Then, the extract was separated to get the lipophilic

extract. The lipophilic extract was chromatographed with mixtures of ethyl acetate-n-hexane. In total, 149 fractions were identified: 1–20 (n-hexane), 21–40 (n-hexane-ethyl acetate, 95:5), 41–60 (n-hexane-ethyl acetate, 9:1), 61–80 (n-hexane-ethyl acetate, 7:3), 81–100 (n-hexane-ethyl acetate, 1:1), 101–120 (n-hexane-ethyl acetate, 3:7), 121–130 (ethyl acetate), 131–142 (acetone) and 143–149 (methanol). The fractions with similar profiles were aggregated into 11 combined fractions as shown in Table 2 to isolate bioactive compounds further.

Isolation and elucidation of structure

The combined fraction number 3 (fraction numbers 38–39) was eluted with n-hexane-ethyl acetate (95:5) and yielded crystals, which were purified using the recrystallization technique with a mixture of chloroform-methanol and then, subsequently purified using Sephadex LH-20 eluted with methanol to produce compound 1 that exhibited blue fluorescence under ultraviolet light at 365 nm. The chemical structures were identified according to their atmospheric pressure chemical ionization mass spectrometry (APCI-MS) using the ^1H and ^{13}C NMR spectral data.

Compound 1 was isolated as a white crystalline solid. The ^1H -NMR spectrum of this compound showed protons combined with 29 carbon signals from the ^{13}C -NMR spectrum. The ^1H and ^{13}C NMR spectral data for this compound showed chemical shifts similar to those reported by Cayme and Ragasa (2004) as shown in Table 3, which might indicate that this compound was stigmasterol ($\text{C}_{29}\text{H}_{48}\text{O}$).

Table 2 Combined fraction code, number of fractions, weight and ratio of solvent system for column chromatography of each combined fraction for bark extracts of *C. arborea*

Combined fraction	Fraction number	Weight (g)	Solvent system
Fraction 1	1–25	1.6705	n-Hexane, n-hexane-ethyl acetate (95:5)
Fraction 2	26–35	1.2865	n-Hexane-ethyl acetate (95:5)
Fraction 3	36–43	2.1676	n-Hexane-ethyl acetate (95:5, 9:1)
Fraction 4	44–64	2.6342	n-Hexane-ethyl acetate (95:5, 9:1)
Fraction 5	65–74	0.8921	n-Hexane-ethyl acetate (7:3)
Fraction 6	75–80	0.9875	n-Hexane-ethyl acetate (7:3)
Fraction 7	81–88	1.0978	n-Hexane-ethyl acetate (1:1)
Fraction 8	89–104	0.9979	n-Hexane-ethyl acetate (1:1, 3:7)
Fraction 9	105–132	0.8859	n-Hexane-ethyl acetate (3:7), ethyl acetate, acetone
Fraction 10	133–142	4.6954	Acetone
Fraction 11	143–149	6.7381	Methanol

Table 3 ^1H spectral data (400 MHz, CDCl_3) and ^{13}C NMR spectral data (100 MHz, CDCl_3) of compound 1 and stigmasterol ($\text{C}_{29}\text{H}_{48}\text{O}$) (Cayme and Ragasa, 2004)

Position	^1H spectral data of stigmasterol	Compound 1	^{13}C NMR spectral data of stigmasterol	Compound 1
	δ ^1H (ppm)	δ ^1H (ppm)	δ ^{13}C (ppm)	δ ^{13}C (ppm)
1	1.08	1.08	37.2	37.3
	1.83	1.83		
2	1.49	1.49	31.6	31.6
	1.82	1.82		
3	3.53 (m)	3.52 (m)	71.8	71.8
4	2.24	2.24	42.27	42.3
	2.28	2.28		
5	-	-	140.7	140.7
6	5.35 (d, 4.7 Hz)	5.35 (d, 5.2 Hz)	121.7	121.7
7	1.53	1.53	31.9	31.9
	1.98	1.98		
8	1.46	1.46	31.9	31.9
9	0.94	0.94	50.1	50.1
10	-	-	36.5	36.5
11	1.45	1.45	21.06	21.08
	1.48	1.48		
12	1.15	1.15	39.7	39.7
	1.97	1.97		
13	-	-	42.19	42.2
14	1.00	1.00	56.8	56.9
15	1.06	1.06	24.4	24.4
	1.55	1.55		
16	1.27	1.27	28.9	28.9
	1.71	1.71		
17	1.13	1.13	55.9	55.9
18	0.70 (s, Me)	0.70 (s, Me)	12.04	12.05
19	1.01 (s, Me)	0.99 (s, Me)	19.4	19.4
20	2.04	2.04	40.5	40.5
21	1.02 (d, 6.8 Hz, Me)	1.01 (d, 7.5 Hz, Me)	21.09	21.1
22	5.15 (dd, 8.4, 15.1 Hz)	5.15 (dd, 8.6, 15.2 Hz)	138.3	138.3
23	5.02 (dd, 8.4, 15.1 Hz)	5.01 (dd, 8.6, 15.2 Hz)	129.2	129.2
24	1.53	1.53	51.2	51.2
25	1.44	1.44	31.9	31.9
26	0.84 (d, 6.4 Hz, Me)	0.84 (d, 6.4 Hz, Me)	21.2	21.2
27	0.83 (d, 6.1 Hz, Me)	0.83 (d, 6.0 Hz, Me)	18.97	19.0
28	1.15	1.15	25.4	25.4
29	0.80 (t, 6.0 Hz, Me)	0.79 (t, 7.0 Hz, Me)	12.3	12.3

NMR = nuclear magnetic resonance; ppm = parts per million

The mass spectrum of this compound was determined using APCI-MS. From the results, the base peak of this compound was observed at m/z 395.3648 (Fig. 3). Due to the atmospheric pressure chemical ionization, which is a soft ionization technique that affects the results shown in $[\text{M}+\text{H}^+-\text{H}_2\text{O}]$, there were fragments indicating a loss of one water molecule. This observation corresponded to the results of Kemmo et al. (2007), in which the main fragment of stigmasterol was m/z 395.4 $[\text{M}+\text{H}^+-\text{H}_2\text{O}]$. To confirm whether this compound was stigmasterol, the exact mass from the experiment (412.3675) was determined and was similar to the monoisotopic mass of stigmasterol (412.3705). Therefore, compound 1 was confirmed as stigmasterol (Fig. 4).

Stigmasterol is one type of phytosterol that can be found in various plants (Grace et al., 2016; Miras-Moreno et al., 2016; Scapinello

et al., 2018; Wang et al., 2018) and has been reported to have several interesting properties. For example, it can accelerate the oxidation of both methyl linoleate in solution and 1-palmitoyl-2-linoleoyl-phosphatidylcholine liposomal membranes in aqueous dispersions (Yoshida and Niki, 2003). Phytosterols, such as stigmasterol, may offer protection against the most common cancers, including colon, breast and prostate cancer (Awad and Fink, 2000). Stigmasterol showed chemopreventive activity in an experimental model of cancer, which might be linked to the oxidative stress of stigmasterol (Ali et al., 2015). Stigmasterol also controlled inflammatory features, such as ear skin oedema and neutrophilia, and reduced the serum levels of TNF α induced by topical application of tissue plasminogen activator (Antwi et al., 2018) and thus is anti-inflammatory (Columba-Palomares et al., 2018).

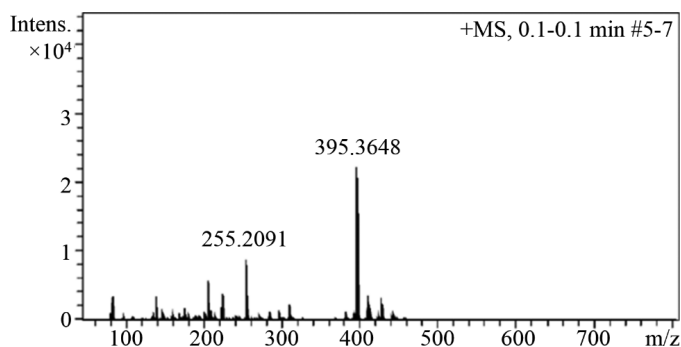


Fig. 3 Mass spectrum of stigmasterol

The anti-HIV activity of this compound has not been reported yet. However, there are still several chemical compounds, especially triterpenoids, which are the precursor to all steroids, that are known to exert anti-HIV activity. Several triterpenoids have exhibited antiretroviral activity with different mechanisms of action. For example, betulinic acid, which is a triterpenoid substance, disrupts viral fusion to the cells in a post-binding step through interaction with the viral glycoprotein gp41, while other substances disrupt the assembly and budding of the HIV-1 virus (Asres et al., 2005). Oleanolic acid and pomolic acid, which were isolated from *Rosa woodsii* and *Ternstroemia gymnanthera*, had anti-HIV activity and could inhibit uninfected H9 cell growth (Kashiwada et al., 1998). Nigranoic acid was also found to be a substance having high potential against HIV reverse transcriptase and in DNA polymerase assays (Sun et al., 1996).

In conclusion, the results from the current work suggested that the extract of *C. arborea* was a potential source for antioxidant and anti-HIV activities. Specifically, the extract from stem bark had the highest antioxidant and HIV-1 reverse transcriptase levels of inhibition activity compared to the wood and leaf extracts. Stigmasterol was isolated from the stem bark extract. Future investigation should study: phytochemical analysis, the isolation of other pure compounds from the stem bark extract of *C. arborea* and the bioactivities of stigmasterol and other pure compounds.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank Asst. Prof. Dr. Pakorn Wattana-Amorn from the Department of Chemistry, Kasetsart University, Bangkok, Thailand for his help in the NMR experiments. This research was supported under the Strengthening and Developing New Researcher Plan, in conformance with the Research and Innovation of Graduate Study Strategy of the National Research Council of Thailand (NRCT) in the 2018 fiscal year.

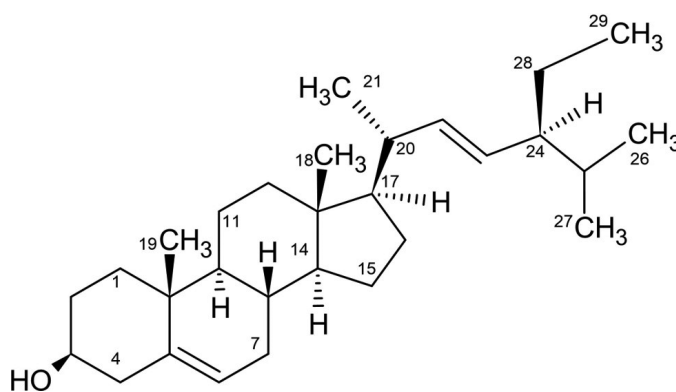


Fig. 4 Chemical structure of stigmasterol

References

- Alam, M.N., Bristi, N.J., Rafiquzzaman, M. 2013. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharm. J. 21: 143–152. doi.org/10.1016/j.jsps.2012.05.002
- Ali, H., Dixit, S., Ali, D., Alqahtani, S.M., Alkahtani, S., Alarifi, S. 2015. Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. Drug Des. Dev. Ther. 9: 2793–2800. doi.org/10.2147/DDDT.S83514
- Allard, J.P., Aghdassi, E., Chau, J., Tam, C., Kovacs, C.M., Salit, I.E., Walmsley, S.L. 1998. Effects of vitamin E and C supplementation on oxidative stress and viral load in HIV-infected subjects. AIDS 12: 1653–1659. doi.org/10.1097/00002030-199813000-00013
- Antwi, A.O., Obiri, D.D., Osafo, N., Essel, L.B., Forkuo, A.D., Atobiga, C. 2018. Stigmasterol alleviates cutaneous allergic responses in rodents. Biomed. Res. Int. 2018: 1–13. doi.org/10.1155/2018/3984068
- Asres, K., Seyoum, A., Veeresham, C., Bucar, F., Gibbons, S. 2005. Naturally derived anti-HIV agents. Phytother. Res. 19: 557–581. doi.org/10.1002/ptr.1629
- Awad, A.B., Fink, C.S. 2000. Phytosterols as anticancer dietary components: Evidence and mechanism of action. J. Nutr. 130: 2127–2130. doi.org/10.1093/jn/130.9.2127
- Behbahani, M. 2014. Anti-HIV-1 activity of eight monofloral Iranian honey types. PLoS One. 9: e108195. doi.org/10.1371/journal.pone.0108195
- Benzie, I.F.F., Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant Power”: The FRAP assay. Anal. Biochem. 239: 70–76. doi.org/10.1006/abio.1996.0292
- Bhandary, M.J., Chandrashekhara, K.R. 2000. Treatment for poisonous snake bites in the ethnomedicine of coastal Karnataka. J. Med. Aromat. Plants. 22: 505–510.
- Castro, H.C., Loureiro, N.I.V., Pujol-Luz, M., et al. 2006. HIV-1 reverse transcriptase: A therapeutic target in the spotlight. Curr. Med. Chem. 13: 313–324. doi.org/10.2174/092986706775476089
- Cayme, J.C., Ragasa, C.Y. 2004. Structure elucidation of β -stigmasterol and β -sitosterol from *Sesbania grandiflora* [Linn.] Pers. and β -carotene from *Heliotropium indicum* Linn. by NMR spectroscopy. KIMIKA 20: 5–12. doi.org/10.26534/kimika.v20i1.5-12
- Columba-Palomares, M.C., Villarreal, M.L., Marquina, S., Romero-Estrada, A., Rodríguez-López, V., Alvarez, A.Z., Alvarez, L. 2018. Antiproliferative and anti-inflammatory acyl glucosyl flavones from the leaves of *Bursera copallifera*. J. Mex. Chem. Soc. 62: 214–224. doi.org/10.29356/jmcs.v62i4.624

- Devasagayam, T.P.A., Tilak, J.C., Boloor, K.K., Sane, K.S., Ghaskadbi, S.S., Lele, R.D. 2004. Free radicals and antioxidants in human health: Current status and future prospects. *J. Assoc. Physicians India*. 52: 794–804.
- Eldeen, I.M.S., Seow, E.M., Abdullah, R., Sulaiman, S.F. 2011. *In vitro* antibacterial, antioxidant, total phenolic contents and anti-HIV-1 reverse transcriptase activities of extracts of seven *Phyllanthus* sp. *S. Afr. J. Bot.* 77: 75–79. doi.org/10.1016/j.sajb.2010.05.009
- Ferreres, F., Duangsrirai, S., Gomes, N.G.M., et al. 2017. Anti-inflammatory properties of the stem bark from the herbal drug *Vitex peduncularis* Wall. ex Schauer and characterization of its polyphenolic profile. *Food Chem. Toxicol.* 106: 8–16. doi.org/10.1016/j.fct.2017.05.020
- Folin, O., Ciocalteu, V. 1927. On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.* 73: 627–650.
- Gan, R.Y., Xu, X.R., Song, F.L., Kuang, L., Li, H.B. 2010. Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. *J. Med. Plants Res.* 4: 2438–2444. doi.org/10.5897/JMPR10.581
- Gil, L., Mart'inez, G., González, I., et al. 2003. Contribution to characterization of oxidative stress in HIV/AIDS patients. *Pharmacol. Res.* 47: 217–224. doi.org/10.1016/S1043-6618(02)00320-1
- Gourine, N., Yousfi, M., Bombarda, I., Nadjemi, B., Stocker, P., Gaydou, E.M. 2010. Antioxidant activities and chemical composition of essential oil of *Pistacia atlantica* from Algeria. *Ind. Crops Prod.* 31: 203–208. doi.org/10.1016/j.indcrop.2009.10.003
- Grace, M.H., Esposito, D., Timmers, M.A., Xiong, J., Yousef, G., Komarnsky, S., Lila, M.A. 2016. Chemical composition, antioxidant and anti-inflammatory properties of pistachio hull extracts. *Food Chem.* 210: 85–95. doi.org/10.1016/j.foodchem.2016.04.088
- Hu, W.S., Hughes, S.H. 2012. HIV-1 reverse transcription. *Cold Spring Harb. Perspect. Med.* 2: a006882. doi.org/10.1101/cshperspect.a006882
- Hwang, Y.J., Lee, E.J., Kim, H.R., Hwang, K.A. 2013. *In vitro* antioxidant and anticancer effects of solvent fractions from *Prunella vulgaris* var. *lilacina*. *BMC Complement. Med. Ther.* 13: 310. doi.org/10.1186/1472-6882-13-310
- Iqbal, E., Salim, K.A., Lim, L.B.L. 2015. Phytochemistry screening, total phenolics and antioxidant activities of bark and leaf extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam. *J. King Saud. Univ. Sci.* 27: 224–232. doi.org/10.1016/j.jksus.2015.02.003
- Kanyara, J.N., Njagi, E.N.M. 2005. Anti-HIV-1 activities in extracts from some medicinal plants as assessed in an *in vitro* biochemical HIV-1 reverse transcriptase assay. *Phytother. Res.* 19: 287–290. doi.org/10.1002/ptr.1536
- Kapewangolo, P., Hussein, A.A., Meyer, D. 2013. Inhibition of HIV-1 enzyme, antioxidant and anti-inflammatory activities of *Plectranthus barbatus*. *J. Ethnopharmacol.* 149: 184–190. doi.org/10.1016/j.jep.2013.06.019
- Kapoor, L.D., Srivastava, S.N., Singh, A., Kapoor, S.L., Shah, N.C. 1972. Survey of Indian plants for saponins, alkaloids and flavonoids. *Lloydia* 35: 288–295.
- Karunamoorthi, K., Jegajeevanram, K., Vijayalakshmi, J., Mengistie, E. 2012. Traditional medicinal plants: A source of phytotherapeutic modality in resource-constrained health care settings. *J. Evid. Base. Integr. Med.* 18: 67–74. doi.org/10.1177/2156587212460241
- Kashiwada, Y., Wang, H., Nagao, T., et al. 1998. Anti-AIDS agents. 30. anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *J. Nat. Prod.* 61: 1090–1095. doi.org/10.1021/np9800710
- Kemmo, S., Ollilainen, V., Lampi, A.M., Piironen, V. 2007. Determination of stigmaterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. *Food Chem.* 101: 1438–1445. doi.org/10.1016/j.foodchem.2006.03.052
- Khlift, D., Hayouni, E.A., Valentin, A., Cazaux, S. 2013. LC-MC analysis, anticancer, antioxidant and antimalarial activities of *Cynodon dactylon* L. extract. *Ind. Crops. Prod.* 45: 240–247.
- Khanum, R., Mazher, F., Jahangi, M. 2015. Antioxidant evaluations of polar and non-polar fractions of *Cajanus cajan* seed. *J. Med. Plant Res.* 9: 193–198. doi.org/10.5897/JMPR2014.5456
- Kim, S.G., Nam, H.G., Kim, J.H., Mun, S.Y. 2011. Optimal design of a four-zone simulated moving bed process for separation of homoharringtonine and harringtonine. *Can. J. Chem. Eng.* 89: 304–313. doi.org/10.1002/cjce.20401
- Lu, L., Liu, S.W., Jiang, S.B., Wu, S.G. 2004. Tannin inhibits HIV-1 entry by targeting gp41. *Acta Pharmacol. Sin.* 25: 213–218.
- Malhotra, S.K., Moorthy, S. 1973. Some useful medicinal plants of Chandrapur district. *Bull. Bot. Surv. India.* 15:13–21.
- Mandal, D., Panda, N., Kumar, S., Banerjee, S., Mandal, N.B., Sahu, N.P. 2006. A triterpenoid saponin possessing antileishmanial activity from the leaves of *Careya arborea*. *Phytochemistry.* 67: 183–190. doi.org/10.1016/j.phytochem.2005.10.028
- Miras-Moreno, B., Sabater-Jara, A.B., Pedreño, M.A., Almagro, L. 2016. Bioactivity of phytosterols and their production in plant in vitro cultures. *J. Agric. Food. Chem.* 64: 7049–7058. doi.org/10.1021/acs.jafc.6b02345
- Moure, A., Cruz, J.M., Franco, D., Dom-Inguez, J.M., Sineiro, J., Domínguez, H., Nunez, M.J., Parajo, J.C. 2001. Natural antioxidants from residual sources. *Food Chem.* 72: 145–171. doi.org/10.1016/S0308-8146(00)00223-5
- Nagao, Y., Sata, M., Suzuki, H., Tanikawa, K., Itoh, K., Kameyama, T. 1996. Effectiveness of glycyrrhizin for oral lichen planus in patients with chronic HCV infection. *J. Gastroenterol.* 31: 691–695. doi.org/10.1007/BF02347618
- Office of the Forest Herbarium, National Park, Wildlife and Plant Conservation Department. 2012. The selection of tree species for reforestation to prevent flooding guidebook. Religious Publishing. Bangkok, Thailand.
- Pizzino, G., Irrera, N., Cucinotta, M., et al. 2017. Oxidative stress: Harms and benefits for human health. *Oxid. Med. Cell. Longev.* 2017: 1–13. doi.org/10.1155/2017/8416763
- Palombo, E.A. 2006. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytother. Res.* 20: 717–724. doi.org/10.1002/ptr.1907
- R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/, 11 January 2021.
- Scapinello, J., Aguiar, G.P.S., Magro, C.D., Capelezzo, A.P., Niero, R., Magro, J.D., Oliveira, D., Oliveira, V. 2018. Extraction of bioactive compounds from *Philodendron bipinnatifidum* Schott ex Endl and encapsulation in PHBV by SEDS technique. *Ind. Crops Prod.* 125: 65–71.
- Senthilkumar, N., Badami, S., Cherian, M.M., Hariharapura, R.C. 2007. Potent *in vitro* cytotoxic and antioxidant activity of *Careya arborea* bark extracts. *Phytother. Res.* 21: 492–495. doi.org/10.1002/ptr.2118
- Silprasit, K., Thammaporn, R., Tecchasakul, S., Hannongbua, S., Choowongkorn, K. 2011. Simple and rapid of the enzyme kinetics of HIV-1 reverse transcriptase and anti-HIV-1 agents by a fluorescence based method. *J. Virol. Methods* 171: 381–387. doi.org/10.1016/j.jviromet.2010.12.001
- Silva, K.D.R.R., Sirasa, M.S.F. 2018. Antioxidant properties of selected fruit cultivars grown in Sri Lanka. *Food Chem.* 238: 203–208. doi.org/10.1016/j.foodchem.2016.08.102
- Sonar, V.P., Corona, A., Distinto, S., et al. 2017. Natural product-inspired esters and amides of ferulic and caffeic acid as dual inhibitors of HIV-1 reverse transcriptase. *Eur. J. Med. Chem.* 130: 248–260. doi.org/10.1016/j.ejmech.2017.02.054

- Soun-udom, M., Choowongkamon, K., Vajrodaya, S., Ratanabunyong, S., Suksungworn, R., Srisombat, N., Bapia, S., Duangsrisai, S. 2019. Antioxidant and anti-HIV properties and isolation of bioactive compound of *Hymenodictyon orixense* (Roxb.) Mabb. Agr. Nat. Resour. 53: 621–628. doi.org/10.34044/j.anres.2019.53.6.09
- Srisombat, N., Bapia, S., Ratanabunyong, S., Choowongkamon, K., Vajrodaya, S., Duangsrisai, S. 2019. Isolation of betulinic acid and antioxidant and anti-HIV-1 reverse transcriptase activity of *Cratoxylum formosum* subsp. *pruniflorum* (Kurz) gogelein extract. Agr. Nat. Resour. 53: 674–680. doi.org/10.34044/j.anres.2019.53.6.16
- Sun, H., Qiu, S., Lin, L., et al. 1996. Nigranoic acid, a triterpenoid from *Schisandra sphaerandra* that inhibits HIV-1 reverse transcriptase. J. Nat. Prod. 59: 525–527. doi.org/10.1021/np960149h
- Tiwari, R., Rana, C.S. 2015. Plant secondary metabolites: A review. Int. J. Res. Gen. Sci. 3: 661–670.
- Tosun, M., Ercisli, S., Sengul, M., Ozer, H., Polat, T., Ozturk, E. 2009. Antioxidant properties and total phenolic content of eight *Salvia* species from Turkey. Biol. Res. 42: 175–181. doi.org/10.4067/S0716-97602009000200005
- Valle, L.G.D., Hernández, R.G., Ávila, J.P. 2013. Oxidative stress associated to disease progression and toxicity during antiretroviral therapy in human immunodeficiency virus infection. J. Virol. Microbiol. 2013: 1–15. doi.org/10.5171/2013.279685
- United Nations Programme on HIV and AIDS (UNAIDS). 2020. Global HIV and aids statistics. https://www.avert.org/global-hiv-and-aids-statistics#footnote1_by7xpmp/, 31 December 2020.
- Vazquez, G., Fontenla, E., Santos, J., Freire, M.S., Gonzalez-Alvarez, J., Antorrena, G. 2008. Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. Ind. Crops Prod. 28: 279–285. doi.org/10.1016/j.indcrop.2008.03.003
- Venditti, A., Lorenzetti, L.M., Frezza, C., Serafini, M., Ornano, L., Bianco, A. 2017. Semi-synthesis of three precursors of carbocyclic nucleoside analogues. Chemistry 18: 101–105.
- Wang, M., Huang, W., Hu, Y., et al. 2018. Phytosterol profiles of common foods and estimated natural intake of different structures and forms in China. J. Agric. Food. Chem. 66: 2669–2676. doi.org/10.1021/acs.jafc.7b05009
- Yang, J., Li, L., Tan, S., et al. 2012. A natural theaflavins preparation inhibits HIV-1 infection by targeting the entry step: Potential applications for preventing HIV-1 infection. Fitoterapia 83: 348–355. doi.org/10.1016/j.fitote.2011.11.016
- Yoshida, Y., Niki, E. 2003. Antioxidant effect of phytosterol and its components. J. Nutr. Sci. Vitaminol. 49: 277–280. doi.org/10.3177/jnsv.49.277