Original Article

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In vitro Testing of Anti-HIV and Antioxidative Activities of *Argyreia nervosa* (Burm.f) Bojor Leaves

V. Sareedenchai[1], C. Wiwat[2], P. Wongsinkongman[3] and N. Soonthornchareonnon[4]*

[1] *Department of Pharmacognosy, Faculty of Pharmacy, Srinakarinviroj University, Pathumthani, Thailand.*

[2] *Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.*

[3] *Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand*

[4] *Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.*

Abstract

An 80% ethanolic extract of *Argyreia nervosa* (Burm.f) Bojor (Convolvulaceae) leaves was studied by chromatographic techniques. Three compounds: 1-hexacosanol ($C_{26}H_{54}O$), scopoletin and ethyl caffeate were isolated. The biological activities of ethanolic extracts were investigated for anti-HIV and free radical scavenging activities. From the results of preliminary screening, the ethanolic extract inhibited in syncytium reduction assay for 80.45% at the concentration of 200 µg/ml. Further investigation for anti-HIV activities was carried out with HIV reverse transcriptase (RT) and HIV protease (PR). Anti-HIV RT activity was studied by radiometric method and anti-HIV PR was studied by spectrophotometry. The ethanolic extract could not inhibit HIV RT and HIV PR, but three compounds could inhibit HIV PR. 1-Hexacosanol, scopoletin, and ethyl caffeate at concentrations of 200 µg/ml could inhibit HIV PR by 78.71%, 43.35% and 43.15%, respectively. The free radical scavenging activity was studied using 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The ethanolic extract showed free radical scavenging activity with an EC_{50} of 21.43 µg/ml. 1-Hexacosanol and scopoletin were inactive, whereas ethyl caffeate was almost twice as active as butylated hydroxyanisol (BHA).

Keyword: *Argyreia nervosa*, HIV reverse transcriptase, HIV protease, Antioxidant, DPPH

INTRODUCTION

Argyreia nervosa (Burm.f) Bojor, belongs to family Convolvulaceae, commonly known as Bai-rabad, is a woody climber distributed throughout tropical region. It is a popular Indian medicinal plant, which has long been used in traditional Ayurvedic Indian medicine for various diseases. Seeds are used for the treatment of anorexia, diabetes, and various skin disease, while roots are reported to be tonic, aphrodisiac, bitter, diuretic, and used for the treatment of rheumatoid, gonorrhea, chronic ulcer and diseases of nervous system. Leaves are used as an antiphlogistic, emollient, poultices of wounds, externally for skin disease, gleet, gonorrhea and chronic ulcers [1-5]. In Thailand, leaves are externally used for skin disease. Previous studies on bioactivities of *A. nervosa* have been reported. The alcoholic extract of seeds showed effect on cardiovascular system in dog and cat. The extract also showed antispasmodic activity on the isolated guinea pig ileum[3-4]. Roots showed antimicrobial, analgesic, anti-inflammatory, anticonvulsant, and nootropic activities[3-5]. Whole aerial part exhibited antipyretic activity in experimental animal models[6]. Leaf showed aphrodisiac activity as evidenced by an increase in mounting behavior of mice. The alcoholic extract of the leaves revealed antibacterial activity against *Staphylococcus aureus*[4]. Ointment formulation of water extract of the leaves exhibited significant anti-inflammatory activity against carrageenan-induced

***Corresponding author:** Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. 447 Sri Ayuthaya Road, Rajthevi, Bankok 10400, Thailand.
E-mail: noppamas.sup@mahidol.ac.th Tel.: +662644-8677 ext. 5527; Fax: +662644-8701

rat paw edema[7]. The petroleum ether extract of the leaves yielded 1-tricontanol, epifriedelinol acetate, epifriedelinol and β-sitosterol. Extraction of leaves with methanol led to the isolation of flavonoids, quercetin, kaemperol, kaemperol-3-*O*-1-rhamnopyranoside and flavone glycosides[3].

Recently, a number of anti-HIV agents have been isolated from natural products[7-11]. Some plant extracts, especially from the families of Convolvulaceae, Euphorbiaceae, and Zingiberaceae, had satisfied anti-HIV-1 activities[12]. From the preliminary screening for anti-HIV activity of Thai medicinal plants, ethanolic extract of *A. nervosa* leaves was found to inhibit syncytium reduction assay with an EC_{50} of 11.87 µg/ml[12]. Even through its potency was weaker than azidothymidine (AZT) used as a positive control, it is interesting to study its active compounds and inhibitory mechanism on HIV life cycle. The aims of this study were to screen antioxidant and anti-HIV activities of alcoholic extract of *A. nervosa* and isolate its chemical constituents.

MATERIALS AND METHODS

Plant material

The plant was collected from Sirikit Park, Bangkok. Species were identified by comparison with the authentic specimens at the Royal Forestry Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Voucher specimens were deposited at Herbarium of Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. The leaves of plant were washed, sliced into pieces, and dried in a hot air oven at 60° C.

Extraction and Isolation

The powdered, dried leaves of *A. nervosa* (600 g), were macerated with 80% ethanol for 3 days and then filtrated. The extract was concentrated under reduced pressure and fractionated by quick column chromatography using hexane : dichloromethane : methanol as eluents to yield 7 fractions (A, B, C, D, E, F and G). By using flash column chromatography, compounds **1, 2** were separated from fraction F. Compound **3** was separated from fraction C. The structures of compounds **1** and **3** were identified by spectroscopic techniques. Compound **2** TLC fingerprint was compared with that of reference standard, scopoletin using co-chromatography.

Assay of anti HIV activity

1. Syncytium assay[13]

The ability of extracts to inhibit syncytium formation of 1A2 cells derived from CEM-SS (human T-cell lymphoblastic leukemia) infected with ΔTat/Rev MC99 virus, which is incapable of replicating in peripheral blood mononuclear cells and integrating into host genome by reverting to wild type was determined in duplicate in 96-well tissue culture plates. Cells were treated with 10 µl/ml polybrene, and then incubated with ΔTat/Rev MC99 in order to obtain a virus titer of 100-200 syncytium forming unit (SFU)/50 µl. The 1A2 cells were suspended ($5x10^5$ cells/ml) and seeded at $5x10^4$ cells/100 µl/well that contained 50 µl of medium (RPMI 1640 plus 10% fetal calf serum and 1 µg/ml gentamicin) and 50 µl of 2-fold dilutions of lyophilized extracts dissolved in medium, in triplicate. Azidothymidine (AZT) was used as positive control. Negative controls included cells that contained neither the azidothymidine nor virus, uninfected cells treated with the corresponding dilutions of extract only and virus-infected cells. After incubation for 3 days at 37^0C under an atmosphere of 5% CO_2, the numbers of syncytia were counted under a light microscope. Anti-HIV-1 activity is expressed as the concentration of the plant extract that inhibits syncytium formation by 50% of infected cell control (EC_{50}).

2. HIV-1 reverses transcriptase inhibitor assay[14]

The assay was performed using the radiometric method. The detection and quantification of synthesized DNA were determined as a parameter to reverses transcriptase activity followed by counting radioactivity with liquid scintillation analyzer.

A reaction mixture (100 µl) contains 1M Tris-HCl (pH 8.0), 1M $Mg(OAc)_2$, 2M KCl, 2M dithiothreitol (DTT), 2.5% Nonidet

P-40, deionized distilled water (DDW), bovine serum albumin, (rA)n-$(dT)_{12-18}$ as template-primer, [methyl-^{3}H] dTTP as substrate, 1 unit of HIV-1 reverse transcriptase and 10 µl of the plant extract which was dissolved in distilled water or dimethyl sulfoxide (DMSO) (complete system + compound). The mixture was incubated at 37^{0}C for 1 hr. Two sets of negative control reactions were done under the same condition: (i) without the plant extracts (complete system + RT) and (ii) the mixture consisted of solvent but the reverse transcriptase was omitted (complete system – RT). Doxorubicin was used instead of plant extract as the positive control.

The reaction was terminated with heating the reaction mixture 100^{0}C in 10 min. Each of the terminated reaction mixture was then spot quenched reaction mixture on Whatman DEAE-cellulose (DE-81) paper disc. The disc was dried and washed with 3% cold Na_2HPO_4, DDW and absolute ethanol (10 ml, each), respectively. The paper disc was dried at room temperature and immersed in the vial containing 5 ml scintillation fluid. The amount of the polymerized product formed was determined by counting radioactivity with liquid scintillation analyzer (TRI-CARB 2100 TR, Packard Instrument Company). Each sample was performed in triplicate.

The inhibition ratio (% IR) of the tested plant extracts was calculated by the inhibition of incorporation ^{3}H-dTTP into the newly synthesized DNA strand on the presence of plant extracts as follows:

$$\% \text{ IR} = \left[\frac{1 - \text{CPM}_{(\text{complete system+compound})} - \text{CPM}_{(\text{complete system-RT})}}{\text{CPM}_{(\text{complete system+RT})} - \text{CPM}_{(\text{complete system-RT})}} \times 100 \right]$$

Where % IR is percent of inhibition ratio
CPM is count per minute

3. HIV-1 protease inhibitor assay[15]

HIV-1 protease processed viral polyproteins into function enzyme and structure proteins, thereby facilitating maturation and infectivity of the virion particles. The amino acid sequences were cleavaged by HIV protease at Phe-Pro, Pro-Tyr and Leu-Phe. A reaction mixture containing oligo-peptide substrate (*p*-nitrophenylalanine), HIV-1 protease and sample (inhibitor) was incubated at 37^{0}C. The cleavage site was analyzed by spectrophotometer detecting at 280 nm.

A reaction mixture (40 µl) contained 50 mM NaOAc, 200 mM NaCl, 5 mM dithiothreitol (DTT), 10 % glycerol, deionized distilled water (DDW), substrate, HIV-1 protease and 200 µg/ml of the plant extract which was dissolved in distilled water or dimethyl sulfoxide (DMSO). The mixture was incubated at 37^{0}C for 2 hr. Two sets of negative control reaction and positive control reaction were done under the same condition without the plant extracts. Pepstatin A was used instead of plant extract as the positive control.

The reaction was terminated by adding 2.5 µl of 10% trifluoroacetic acid (TFA). Each sample was performed in duplicate. Quantitative determination of cleavage protein was carried out using high performance liquid chromatography (HPLC) under the following condition:

Column	Hypersil BDS C18, particle size: 5 µm, 250´406 mm
Solvent	A: 0.1% TFA in H_2O B: 0.1% TFA in H_2O and acetonitrile (1:3)
Gradient	10-40%
Time	15 min
Flow rate	1.0 ml/min
Detection	280 nm

$$\%\text{Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

A was a relative peak area of the hydrolysate.

Assay of free radical scavenging activity

DPPH assay[16]

The reaction mixture consisted of 1 ml of 200 µg/ml sample and 1 ml of 60×10^{-6} M 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (in ethanol). Each sample was performed triplicately in each test and

prepared to six different concentrations with two-fold serial dilution. After incubated for 30 minutes at room temperature, the absorbance was measured at 520 nm. % Inhibition and EC_{50} were calculated using the following equation:

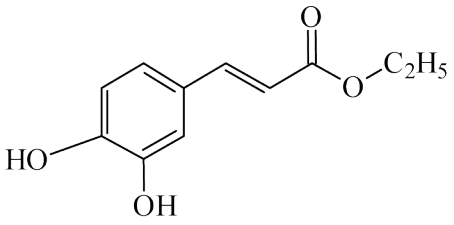
$$\% \text{ Inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

A was absorbance at 520 nm.

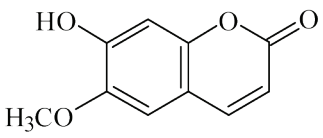
Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as positive controls. Determination of EC_{50} was obtained by linear regression analysis.

RESULTS AND DISCUSSION

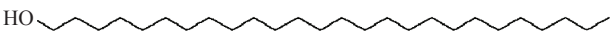
The dried leaves of *A. nervosa* (600 g) were macerated with 80%ethanol for 3 day and filtrated. The filtrate was evaporated in vacuo to obtain an alcoholic extract (66 g). The extract was subjected to silica gel (quick column chromatography) elute with hexane : dichloromethane : methanol, successively, to give 7 fractions (A, B, C, D, E, F, and G). The fraction F was further fractionated by silica gel (flash column chromatography), gave compounds **1** and **2**. The compound **3** was further fractionated from fraction C by silica gel (flash column chromatography). The compounds **1** (ethyl caffeate) and **3** [1-hexacosanol ($C_{26}H_{54}O$)] identified structure by spectroscopic techniques. Compound **2** (scopoletin) was compared with a reference standard, scopoletin by TLC fingerprints (co-chromatography). Ethyl caffeate and scopoletin were previously reported by Agarwal and Rastogi[17]. Compound **3** was identified as 1-hexacosanol by spectroscopic (IR, MS, ^{1}H-NMR and ^{13}C-NMR) (Figure 1).



Ethyl caffeate (**1**)



Scopoletin (**2**)



1-Hexacosanol (**3**)

Figure 1. Chemical structures of ethyl caffeate, scopoletin and 1-hexacosanol (C26H54O)

The crude extract of *A. nervosa* showed anti-HIV activity of syncytium reduction assay over 80% and presented an EC_{50} of 11.87 µg/ml. But the crude extract could not inhibit in HIV-1 reverse transcriptase and HIV-1 protease (Table 1). The crude extract of *A. nervosa* might inhibit another stage of HIV life cycle. In contrary with the isolated compounds, 1-hexacosanol and fraction C could inhibit enzyme HIV-1 protease 78.71 and 82.45%, respectively. Thus, other compounds in the ethanolic extract might be synergistic or might interfere the anti-HIV protease activity with the isolated compound.

Table 1. Percent Inhibition against HIV-1 reverse transcriptase and HIV-1 protease of the ethanolic extract of *A. nervosa*, its main fractions and the isolated compounds at the concentration of 200 μg/ml

Sample	% Inhibition	
	HIV-1 reverse transcriptase	HIV-1 protease
Ethanolic extract	6.94	9.08
Fraction A	5.48	NI
Fraction B	NI	NI
Fraction C	NI	82.45
Fraction D	NI	15.66
Fraction E	NI	NI
Fraction F	8.57	20.23
Fraction G	NI	NI
1-Hexacosanol	NI	78.71
Scopoletin	NI	43.35
Ethyl caffeate	24.99	43.15
Adriamycin	99.97	-
Pepstatin	-	97.18

NI = Not inhibit

Ethanolic extract of *A. nervosa* showed potent activity with an EC_{50} of 21.43 ug/ml. Free radical scavenging activity of each extractive fractions and isolated compounds were present in Table 2. 1-hexacosanol and scopoletin could not scavenge free radicals. Ethyl caffeate showed potent free radical scavenging activity (EC_{50} of 1.52 ug/ml.), which was higher than the positive control BHA. (EC_{50} of 3.15 μg/ml.) (Figure 2). The free radical scavenging activity of the extract was correlated with that of fraction F which provided ethyl caffeate. Ethyl caffeate presented the inhibition of free radical in electro spin resonance method[17].

Table 2. EC_{50} of free radical scavenging activity of main fractions and isolated compounds of *A. nervosa*

Sample*	EC_{50} (μg/ml)
Ethanolic extract	21.43
Fraction A	NI
Fraction B	NI
Fraction C	NI
Fraction D	NI
Fraction E	NI
Fraction F	7.63
Fraction G	23.57
1-Hexacosanol	NI
Scopoletin	151.53
Ethyl caffeate	1.52
BHA	3.15
BHT	9.84

NI = Not inhibit

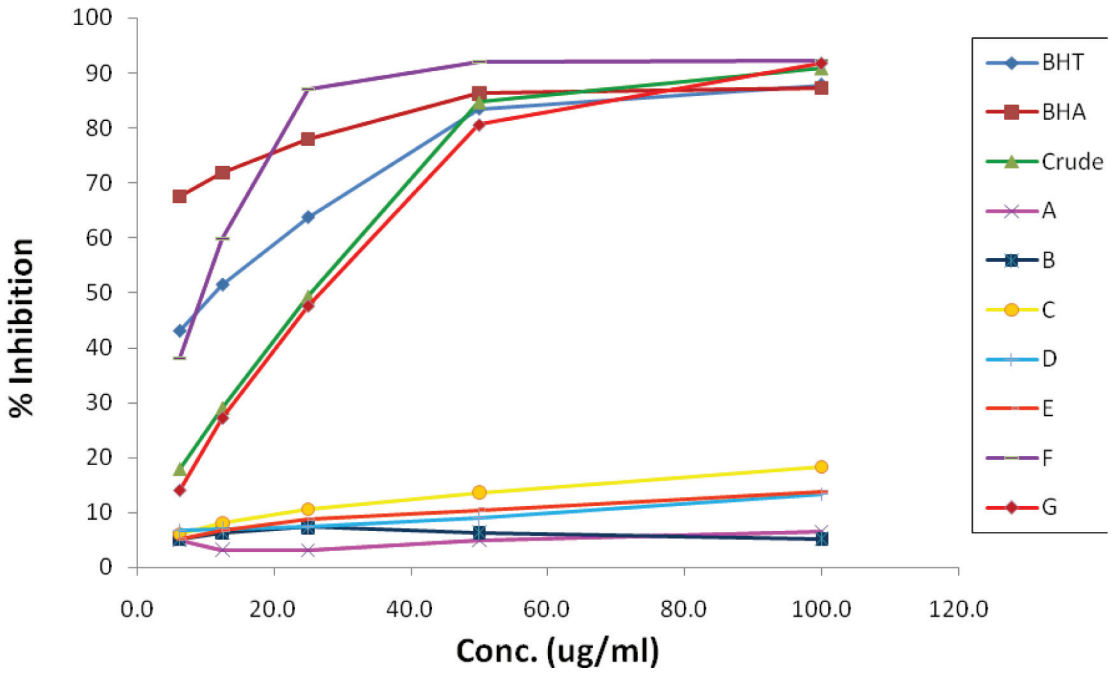


Figure 2. Concentration-response curves of free radical scavenging activity of the main fractions of *A. nervosa* and positive control (BHT and BHA).

CONCLUSION

This study has indicated that *A. nervosa* contain ethyl caffeate **(1)**, scopoletin **(2)** and 1-hexacosanol **(3).** The antioxidant activity of crude extract showed an EC_{50} of 21.43 µg/ml. Ethyl caffeate showed potent free radical scavenging activity (EC_{50} 1.52 µg/ml.), which was higher than BHA (EC_{50} 3.15 µg/ml.). Ethyl caffeate is the major component in ethanolic extract of *A. nervosa* that responsible for high free radical scavenging activity in DPPH assay.

The anti-HIV activity of syncytium reduction assay has an EC_{50} of 11.87 µg/ml. 1-hexacosanol could inhibit HIV-1 protease enzyme for 78.71% with unknown mechanism. Further studies may point to separation of other active compounds and study on the specific mechanism of anti-HIV activity.

REFERENCES

1. Jayaweera DMA. Medicinal Plants (Indigeneous & Exotic) used in Ceylon. Parts III. Columbo: The International Science Council of Sri Lanka, 1980.
2. Kapoor LD. Handbook of Ayurvedic Medicinal Plants. Boca Raton: CRC Press Inc., 2000.
3. Ashutosh M, Kumar AA, Ranjan PA. A literature review on *Argyreia speciosa* (Burm.f.) Bojer. *IJRAP* 2011;2(5):1501-4.
4. Galani VJ, Patel BG, Patel NB. *Argyreia speciosa* (Linn.f.) Sweet: A comprehensive review. *Pharmacogn Rev* 2010;4 (8):172-8.
5. Padhi M, Mahapatra S, Panda J, *et al.* Traditional uses and phytopharmacological aspects of *Argyreia nervosa*. *J Adv Pharm Res* 2013;4(1):23-32.
6. Jeet K, Tomar S, Thakur N. Antipyretic activity of whole aerial part from *Argyreia nervosa*. *Int J Pharm Pharm Sci* 2012; 4(4):76-7.
7. Modi Ashish J, Khadabadi SS, Farooqui IA, *et al.* Anti-inflammatory activity of leaves of *Argyreia nervosa* in carrageenan-induced paw edema in rats. *Phcog J* 2010;2(8):229-32.
8. Ines TK, Takeshi N, Hattori M. Screening of various plant extracts used in ayurvedic medicine for inhibitory effects on Human Immunodeficiency Virus Type 1 (HIV-1) protease. *Phytother Res* 1995;9:180-4.

9. Min W, Stephen B, Lai-Yeap F, *et al.* Screening of New Zealand plant extracts for inhibitory activity against HIV-1 protease. *Phytother Res* 1996;10: 589-95.
10. Vlietinck AJ, Bruyne TD, Apars S, *et al.* Plant-derived leading compounds for chemotherapy of Human Immunodeficiency Virus (HIV) infection. *Planta Med* 1998;64:97-109.
11. Gesa M, Anthony DW, Gabriele MK. HIV reverse transcriptase inhibitor of natural product original. *Planta Med* 1999;65:493-503.
12. Takashi Y, Hiroaki T, Koji S, *et al.* Screening of Thai plants for anti-HIV-1 activity. *Nat Med* 1997;51(6):541-6.
13. Thepouyporn A, Yoosook C, Chuakul W, *et al.* Purification and characterization of anti-HIV-1 protein from *Canna indica* L. leaves. *Southeast Asian J TropMed Public Health* 2012;43(5):1153-60.
14. Tan TG, Pezzuto MJ, Kinghorn DA. Screening of natural products as HIV-1 and HIV-2 reverse transcriptase (RT) inhibitor. In Chu CK, Culter HG. (Eds.). Natural Products as Antiviral Agents. New York: Plenum Press, 1992.
15. Kinchington D, Schinazi RF. Antiviral Methods and Protocols. New Jersey: Humana Press, 2000.
16. Yamazaki K, Hashimoto A, Kokusenya Y, *et al.* Electrochemical method for estimating the antioxidative effect of methanol extracts of crude drugs. *Chem Pharm Bull* 1994;42:1663-5.
17. Agarwal SK, Rastogi RP. Ergometrine and other constituents of *Argyreia speciosa. Indian J Pharmacol* 1974; 36:118-9.
18. Urizzi P, Souchard JP, Manje MC, *et al.* ESR antioxidative activity of phenolic acids and esters. 2nd International Electronic Conference on Synthetic Organic Chemistry, 1998.