Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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**To cite this article:** Bui Huu Tai, Nguyen Duy Nhut, Nguyen Xuan Nhiem, Tran Hong Quang, Nguyen Thi Thanh Ngan, Bui Thi Thuy Luyen, Tran Thu Huong, Jennifer Wilson, John A. Beutler, Ninh Khac Ban, Nguyen Manh Cuong & Young Ho Kim (2011) An evaluation of the RNase H inhibitory effects of Vietnamese medicinal plant extracts and natural compounds, Pharmaceutical Biology, 49:10, 1046-1051, DOI: 10.3109/13880209.2011.563316

**To link to this article:** https://doi.org/10.3109/13880209.2011.563316

Published online: 20 May 2011.

*Pharmaceutical Biology*, 2011; 49(10): 1046–1051

ISSN 1388-0209 print/ISSN 1744-5116 online
DOI: 10.3109/13880209.2011.563316

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**RESEARCH ARTICLE**

# An evaluation of the RNase H inhibitory effects of Vietnamese medicinal plant extracts and natural compounds

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**Abstract**

*Context*: Acquired immune deficiency syndrome (AIDS) is a severe pandemic disease especially prevalent in poor and developing countries. Thus, developing specific, potent antiviral drugs that restrain infection by human immunodeficiency virus type 1 (HIV-1), a major cause of AIDS, remains an urgent priority.

*Objective*: This study evaluated 32 extracts and 23 compounds from Vietnamese medicinal plants for their inhibitory effects against HIV-1 ribonuclease H (RNase H) and their role in reversing the cytopathic effects of HIV.

*Materials and methods*: The plants were air-dried and extracted in different solvent systems to produce plant extracts. Natural compounds were obtained as previously published. Samples were screened for RNase H inhibition followed by a cytopathic assay. Data were analyzed using the Microsoft Excel.

*Results and discussion*: At 50 µg/mL, 11 plant extracts and five compounds inhibited over 90% of RNase H enzymatic activity. Methanol extracts from *Phyllanthus reticulatus* and *Aglaia aphanamixis* leaves inhibited RNase H activity by 99 and 98%, respectively, whereas four extracts showed modest protection against the cytopathic effects of HIV.

*Conclusion*: The screening results demonstrated that the butanol (BuOH) extract of *Celastrus orbiculata* leaves, methanol (MeOH) extracts of *Glycosmis stenocarpa* stems, *Eurya ciliata* leaves, and especially *P. reticulatus* leaves showed potential RNase H inhibition and protection against the viral cytopathic effects of HIV-1. Further chemical investigations should be carried out to find the active components of these extracts and compounds as potential anti-HIV drug candidates.

**Keywords:** HIV, RNase H, cytopathic, antivirus, Vietnamese medicinal plants, organic compounds

## Introduction

Human immunodeficiency virus (HIV) is a pathogenic retrovirus that can cause acquired immune deficiency syndrome (AIDS). After the first cases were identified in the USA in 1981, AIDS spread rapidly, becoming a pandemic disease. The Joint United Nations Programme on HIV/AIDS, UNAIDS, estimated that 33.4 million people suffer from HIV/AIDS worldwide. In Asia, the reported national HIV prevalence is highest in the southeastern countries, although the epidemic trends vary among them. While its prevalence in Cambodia, Myanmar, and Thailand has recently declined, it is increasing in Indonesia and Vietnam. Between 2000 and 2005, the estimated number of HIV sufferers in Vietnam is more than doubled, from 120,000 to 260,000 people (UNAIDS, 2009). Currently, HIV/AIDS is commonly treated with highly active antiretroviral therapy (HAART) (Richman et al., 2009; Broder, 2010). However, the available

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*(Received 01 November 2010; revised 31 January 2011; accepted 11 February 2011)*

antiretroviral drugs have fallen short of expectations in many ways, including the need for life-long therapy, the ultimate role of pre-exposure prophylaxis (PrEP), cardiometabolic side effects and other toxicities of long-term therapy, the emergence of drug-resistance and viral genetic diversity, and the continued pace of new HIV-1 infections in many parts of the world (Broder, 2010). Moreover, the rapid development of strains resistant to drugs and the high cost of drugs make them inaccessible to many people in developing countries (Rukunga et al., 2002).

HIV reverse transcriptase (RT) is important in the HIV life cycle and is one of the most reliable targets of potential anti-AIDS chemotherapy (Hassan Khan & Ather, 2007). This multifunctional enzyme not only exhibits reverse transcription RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP), but also inherent ribonuclease H (RNase H) activity (Herschhorn & Hizi, 2010). The DNA polymerization and RNase H functions are responsible for converting viral genomic RNA into proviral double-stranded DNA (Herschhorn & Hizi, 2010). Inhibiting RT function interferes with HIV production. RT inhibitors are classified into two broad groups with different inhibitory mechanisms: nucleoside analogs and non-nucleoside inhibitors (Cruchaga et al., 2007). Although both classes are useful therapeutically, their use in treating AIDS patients is limited because of the emergence of viral cross-resistance and cellular toxicity. Thus, developing specific, potent antiviral drugs to restrain HIV-1 infection remains an urgent priority.

Herein, we investigated the inhibitory effects of 32 extracts and 23 compounds from Vietnamese medicinal plants for their inhibitory effects against HIV-1 RNase H and their role in reversing the cytopathic effects of HIV.

## Material and methods

### Preparation of plant samples

Plants were collected in different geographical zones throughout Vietnam and were identified by Prof. Ngo Van Trai, at the National Institute of Medicinal Materials (NIMM), Ministry of Health, Hanoi, Vietnam (Table 1). Voucher specimens were deposited in the NIMM herbarium. The samples were dried in the shade and ground into a powder. Then, 20 g of each sample were extracted three times ultrasonically in 100 mL of different solvents at room temperature and filtered. The filtered solutions were combined and vacuum-dried to produce the extracts. Additionally, compounds from several plants, including the plant-based synthetic compound, indirubin-3′-oxime, were obtained as described previously (Cuong et al., 2006, 2010b; Cuong & Tuan, 2006; Nhut et al., 2007). Plant extracts and natural products were dissolved in dimethyl sulfoxide (DMSO) for the bioassays.

### Preparation of oligonucleotides

The oligonucleotides 5′-GAU CUG AGC CUG GGA GCU-fluorescein-3′ and 5′-dabcyl-AGC TCC CAG GCT CAG ATC-3′ were synthesized and provided as the annealed RNA/DNA hybrid by TriLink Biotechnologies (San Diego, CA). The oligonucleotides 5′-$(rA)_{22}$-fluorescein-3′ and 5′-dabcyl-$(dT)_{22}$-3′ were products of Dharmacon (Lafayette, CO) and Midland Certified Reagent Co. (Midland, TX), respectively. The hybrid heteroduplex was formed by mixing 5′-$(rA)_{22}$-fluorescein-3′ and 5′-dabcyl-$(dT)_{22}$-3′ dissolved in 50 mM Tris, pH 8.0, containing 60 mM KCl, in a ratio of 1:1.2 followed by heating at 90°C for 5 min and slow cooling to room temperature. Aliquots of stock hybrid heteroduplex were stored at –20°C until use. Recombinant wild-type p66/p51 HIV-1 RT was overexpressed and purified as described (Fletcher et al., 1996).

### Spectroscopic measurements

Details of the RNase H FRET assay have been previously described (Parniak et al., 2003). Inhibition assays in 96-well microplates were carried out using a SpectraMax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). Assays in 384-well microplates were performed using a Victor$^2$V multilabel plate reader (Perkin-Elmer Life Sciences, Boston, MA).

### Microplate assay of RNase H activity

The assays were conducted in a total volume of 100 μL containing 50 mM Tris, pH 8.0, 60 mM KCl, and 5 mM $MgCl_2$, with final concentrations of 0.25 μM RNA/DNA hybrid and 1.0 nM recombinant p66/p51 HIV-1 RT. Stock solutions of the RNA/DNA hybrid and HIV-1 RT were diluted appropriately immediately before use. Reactions were initiated by adding HIV-1 RT and ran for 30 min at 37°C. Reactions were quenched by the addition of 50 μL of 0.5 M EDTA at pH 8.0. The fluorescence intensity of each well was assessed using excitation and emission wavelengths of 490 and 528 nm, respectively, with the cutoff filter set to 515 nm. To assess the effect of the inhibitors, 1 μL of inhibitor in DMSO was added to the microplate well before adding the substrate and RT solutions (Parniak et al., 2003).

### HIV-1 cytopathic assay

Extracts were dissolved in DMSO at 20 mg/mL, and diluted 1:200 into the assay plates, yielding a final top concentration of 100 μg/mL, with eight 2-fold dilutions to a low dose of 0.78 μg/mL. Samples were tested in triplicate dose–response format using HIV-$1_{RF}$ in CEM-SS cells by a previously published method (Weislow et al., 1989). In brief, these are microtiter assays, which quantitate the ability of a compound to inhibit HIV-1-induced cell killing via syncytium formation. Cytoprotection and compound cytotoxicity are measured with the CellTiter 96 Reagent (Promega,

Table 1. RNase H inhibitory and HIV-1 cytopathic effects of Vietnamese medicinal plant extracts.

| Botanical family/plant species | Codes | Part used[a] | Extract | RNase H inhibition (% at 50 μg/mL) | HIV-1 $EC_{50}$ (μg/mL) | HIV-1 $IC_{50}$ (μg/mL) |
|---|---|---|---|---|---|---|
| Acanthaceae | | | | | | |
| *Peristrophe roxburghiana* Bremek. | VHKC-0013 | L | Water | 6 | — | — |
| *Strobilanthes cusia* (Nees) O. Ktze. | VHKC-0016 | L | $CH_2Cl_2$ | 67 | — | — |
| | VHKC-0017 | L | EtOAc | 92 | n.p. | 17.1 |
| | VHKC-0018 | L | BuOH | 42 | — | — |
| Annonaceae | | | | | | |
| *Fissistigma polyanthoides* (DC.) Merr. | VHKC-0052 | L | MeOH | 95 | n.p. | 17.8 |
| | VHKC-0053 | SB | MeOH | 95 | n.p. | 28.1 |
| *Goniothalamus gracilipes* Ban. | VHKC-0039 | L | *n*-Hexane | 56 | — | — |
| | VHKC-0040 | L | $CHCl_3$ | 79 | n.p. | 3.4 |
| | VHKC-0041 | L | EtOH | 97 | n.p. | 3.5 |
| *Goniothalamus tamirensis* Pierre ex & Gagn. | VHKC-0036 | L | *n*-Hexane | 38 | — | — |
| | VHKC-0037 | L | $CHCl_3$ | 66 | — | — |
| | VHKC-0038 | L | EtOH | 47 | — | — |
| *Goniothalamus vietnamensis* Ban. | VHKC-0050 | L | MeOH | 81 | n.p. | 11.7 |
| | VHKC-0051 | R | MeOH | 17 | — | — |
| Apocynaceae | | | | | | |
| *Bousingonia mekongense* Pierre ex Pl. | VHKC-0048 | WP | *n*-Hexane | 17 | — | — |
| | VHKC-0049 | WP | $CHCl_3$ | 97 | n.p. | 3.9 |
| Araliaceae | | | | | | |
| *Panax stipuleanatus* Tsai & Feng. | VHKC-0044 | Rh | MeOH | 31 | — | — |
| *Schefflera leucantha* Vig. | VHKC-0045 | L | $CH_2Cl_2$ | 66 | — | — |
| Buddlejaceae | | | | | | |
| *Buddleja officinalis* Maxim. | VHKC-0011 | F | Water | 32 | — | — |
| | VHKC-0012 | F | MeOH | 2 | — | — |
| | VHKC-0058 | F | EtOAc | 63 | — | — |
| Celastraceae | | | | | | |
| *Celastrus orbiculata* Thunb. | VHKC-0042 | L | EtOAc | 89 | n.p. | 33.0 |
| | VHKC-0043 | L | BuOH | 78 | 44.9 | >100 |
| Cucurbitaceae | | | | | | |
| *Trichosanthes kirilowii* Maxim. | VHKC-0046 | R | $CH_2Cl_2$ | 13 | — | — |
| | VHKC-0047 | R | Water | 8 | — | — |
| Euphorbiaceae | | | | | | |
| *Phyllanthus reticulates* Poir. | VHKC-0055 | L | MeOH | 99 | 5.6 | 6.3 |
| | VHKC-0056 | S | MeOH | 96 | n.p. | 20.8 |
| Meliaceae | | | | | | |
| *Aglaia aphanamixis* Pellegr. | VHKC-0063 | L | MeOH | 98 | n.p. | 8.9 |
| Rutaceae | | | | | | |
| *Glycosmis stenocarpa* (Drake) Tan. | VHKC-0057 | S | MeOH | 77 | 7.1 | 16.3 |
| Theaceae | | | | | | |
| *Camellia sinensis* (L.) O.Ktze | VHKC-0019 | L | EtOAC | 90 | n.p. | 2.1 |
| *Eurya annamensis* Gagn. | VHKC-0062 | L | MeOH | 93 | n.p. | 7.6 |
| *Eurya ciliata* Merr. | VHKC-0054 | L | MeOH | 96 | 12.1 | 15.9 |

[a]F: flowers, L: leaves, R: root, Rh: rhizome, S: stem, SB: stem bark, WP: whole plant.
n.p.: No protection from the cytopathic effect of the virus (inactive).
(—) Not tested.

Madison, WI) 6 days after infection. This reagent contains the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), and the electron-coupling agent phenazine ethosulfate in a colorless stable solution, which upon reduction by viable cells forms a colored solution with absorbance at 490 nm. Antiviral and toxicity data are reported as the concentration of compound required to inhibit 50% virus-induced cell killing (50% effective concentration [$EC_{50}$]) and the concentration of compound required to reduce cell viability by 50% ($IC_{50}$). All data are derived from triplicate tests with the variation of the mean averaging 10%.

### Statistical analysis

Statistical analysis was performed using Microsoft Excel. The data are the mean of triplicate experiments.

## Results and discussion

The ability of the 32 plant extracts to inhibit RNase H is summarized in Table 1. Of the samples examined, the *Aglaia aphanamixis* Pellegr. (Meliaceae), *Bousingonia mekongense* Pierre ex Pl. (Apocynaceae), *Camellia sinensis* (L.) O.Ktze, *Eurya annamensis* Gagn., *Eurya ciliata* Merr. (Theaceae), *Fissistigma polyanthoides* (DC.) Merr., *Goniothalamus gracilipes* Ban. (Annonaceae), and *Phyllanthus reticulatus* Poir. (Euphorbiaceae) extracts had over 90% inhibitory effect on RNase H at a concentration of 50 μg/mL. The methanol extracts of *P. reticulatus* leaves and stems inhibited RNase H the most, by 99 and 96%, respectively. The remaining samples exhibited moderate but significant inhibitory activity of 56–89%. As far as our knowledge, with the exception of *C. sinensis*, none of plant extracts in this study have been reported as an anti-HIV agent using RNase H inhibition or cytopathic assay. Neither *A. aphanamixis* nor *G. gracilipes* have been reported previously in phytochemical or biological studies. *E. ciliata* and *E. annamensis* contain triterpene fatty acid esters and flavonoids, including apigenin, chrysoeriol, and quercitrin, which exhibit considerable monoamine oxygenase (MAO) inhibitory activity (Cuong et al., 2006; Cuong & Tuan, 2006). Chrysoeriol from *E. ciliata* was also found to enhance the proliferation and differentiation of osteoblastic MC3T3-E1 cells (Tai et al., 2009). From the leaves and twigs of *B. mekongense*, scopoletin, ursolic acid, oleanolic acid, and α- and β-amyrin were isolated (Cuong et al., 2005). In folk remedies, *P. reticulatus* is used for a variety of ailments, including smallpox, syphilis, asthma, diarrhea, and gum bleeding. It is also claimed to have antidiabetic activity in tribal areas (Kumar et al., 2008). *Phyllanthus* species have been found to contain bioactive alkaloids, flavonoids, lignan, phenol, and terpenes (Lam et al., 2007). Previous reports have demonstrated its antiplasmodial activity (Omulokoli et al., 1997) and antidiabetic activity (Kumar et al., 2008). However, there are no reports on its inhibitory effect against RNase H.

Table 2. RNase H inhibitory effect of some natural compounds.

| Botanical family/plant species | Codes | Product name | RNase H inhibition (%) |
|---|---|---|---|
| Acanthaceae | | | |
| *Strobilanthes cusia* (Nees) O. Ktze. | VHKC-0028 | Indirubin-3′-oxime | 82 |
| | VHKC-0014 | Indigo naturalis | 67 |
| Apocynaceae | | | |
| *Bousingonia mekongense* Pierre ex Pl. | VHKC-0020 | Ursolic acid | 49 |
| Buddlejaceae | | | |
| *Buddleja officinalis* Maxim. | VHKC-0033 | Linarin | 2 |
| Gelidiaceae | | | |
| *Gelidiella acerosa* (Forsskal) Feldmann & Hamel | VHKC-0009 | Sulfated galactan | 91 |
| Gracilariaceae | | | |
| *Gracilaria asiatica* Zhang & Xia. | VHKC-0010 | Sulfated galactan | 21 |
| *Gracilaria bailiniae* Zhang & Xia. | VHKC-0008 | Sulfated galactan | 29 |
| *Gracilaria firma* Chang & Xia. | VHKC-0004 | Sulfated galactan | 26 |
| *Gracilaria fisheri* Xia & Abbott. | VHKC-0003 | Sulfated galactan | 77 |
| *Gracilaria tenuistipitata* Zhang & Xia. | VHKC-0006 | Sulfated galactan | 21 |
| Phaeophyceae | | | |
| *Sargassum polycystum* Agardh. | VHKC-0059 | Fucoidan | 67 |
| | VHKC-0060 | Fucoidan | 92 |
| | VHKC-0061 | Fucoidan | 97 |
| Rutaceae | | | |
| *Glycosmis petelotii* Guill. | VHKC-0021 | Glypetelotine | 6 |
| *Glycosmis stenocarpa* (Drake) Tan. | VHKC-0026 | 1-Hydroxy-3-methyl carbazole | 49 |
| | VHKC-0027 | Murrayafoline-A | 42 |
| Sargassaceae | | | |
| *Sargassum kuetzingii* Setchell. | VHKC-0001 | Fucoidan | 96 |
| *Sargassum xuanmaii* Mai. | VHKC-0007 | Fucoidan | 85 |
| Theaceae | | | |
| *Eurya cilliata* Merr. | VHKC-0022 | Chrysoeriol | 49 |
| | VHKC-0023 | Quercitrin | 31 |
| *Camellia sinensis* (L.) O.Ktze | VHKC-0025 | Epigallocatechin-3-gallate | 93 |
| Ulvaceae | | | |
| *Ulva fenestrata* Postels & Ruprecht. | VHKC-0005 | Sulfated rhamnan | 49 |
| *Ulva reticulata* Forssk. | VHKC-0002 | Sulfated rhamnan | 32 |

For further evaluation, plant extracts that inhibited RNase H by >75% in the enzymatic assay were tested in a cellular model of HIV-1 cytopathicity. Dose–response curves were constructed for each sample at eight concentrations ranging from 0.78 to 100 μg/mL to obtain their $EC_{50}$ and $IC_{50}$ values. Of the 16 extracts tested, only four showed significant protection against the viral cytopathic effect. These were the BuOH extract of *Celastrus orbiculata* leaves, and the MeOH extracts of *Glycosmis stenocarpa* stems, *E. ciliata* leaves, and *P. reticulatus* leaves (Table 1).

Additionally, 23 compounds from several plants including the plant-based synthetic compound (indirubin-3′-oxime) from previous works were also tested in the enzymatic assay. At the concentration of 50 μg/mL, almost half of these samples showed potent inhibition of activity with a range of 49–97% (Table 2). Of these, several anionic polysaccharide compounds, *epi*-gallocatechin-3-gallate (EGCG), and indirubin-3′-oxime exhibited higher effect with inhibition values over 80% at the tested concentration. EGCG is the most abundant catechin in green tea, and is also a potent antioxidant that may have therapeutic properties for many disorders including cancer (Katiyar et al., 2007). There has been research investigating the benefit of EGCG from green tea in the treatment of HIV infection. It has been shown to reduce plaques related to AIDS-related dementia as well as to block glycoprotein 120 (gp120). The fucoidan compounds isolated from the seaweeds *Sargassum polycystum*, and *Sargassum kuetzingii* also had high RNase H inhibitory activity. Generally, fucoidans were isolated from ground wet or frozen seaweed by either cold (20–25°C) or hot (60–70°C) extraction with 0.4% HCl, hydrophobic chromatography, and precipitation in 60–80% ethanol. Nowadays, pharmaceutical research has been done on fucoidans, which are now being marketed as nutraceuticals and food supplements. Other reports indicate that fucoidan can induce apoptosis in human lymphoma cell lines and inhibit hyperplasia in rabbits (Aisa et al., 2004).

Finally, the active compound indirubin-3′-oxime is considered a member of a new compound class for treating cancer, particularly leukemia and other immunological diseases (Kagialis-Girard et al., 2007). Indirubin-3′-oxime was synthesized with a condensation reaction between hydroxylamine and indirubin, an indole-type alkaloid that could be easily isolated from the leaves of several plants, such as *Polygonum tinctorium* (Polygonaceae), *Isatis indigotica* (Brassicaceae), *Indigofera suffruticosa* (Fabaceae), *Indigofera tinctoria* (Fabaceae), and *Strobilanthes cusia* (Acanthaceae) (Cuong et al., 2010a,b). Recently, indirubin-3′-oxime was found to induce cell cycle arrest and apoptosis in Hep-2 human laryngeal carcinoma cells (Kameswaran and Ramanibai, 2009). In the effects on RNase H activity, indirubin-3′-oxime exhibited inhibition of 82% at the concentration of 50 μg/mL. Based on this result, indirubin-3′-oxime and its derivatives may have potential as cancer and antiviral disease drugs.

## Conclusion

Fifty-five plant samples including extracts, organic compounds, and a plant-based synthetic compound from Vietnamese medicinal plants were screened for their inhibitory effects against HIV-1 RT RNase H activity. At 50 μg/mL, the methanol extracts *P. reticulatus* and *A. aphanamixis* leaves demonstrated the strongest inhibitory activities, of 99 and 98%, respectively. Second, the $CHCl_3$ extract of *B. mekongense* whole plant, the EtOAc extract of *C. sinensis* leaves, MeOH extract of *E. annamensis* leaves and *E. ciliata* leaves, EtOH extract of *G. gracilipes* leaves, MeOH extracts of *F. polyanthoides* leaves and stem barks also showed highly inhibitory effects against RNase H activity with inhibition values >90%. Natural compounds, including EGCG from *C. sinensis*, fucoidans from seaweeds *S. kuetzingii*, *S. polycystum*, sulfated galactan from *Gelidiella acerosa*, and the synthetic compound, indirubin-3′-oxime, inhibited RNase H activity with the inhibition values over 90%. Four of the plant extracts including the BuOH extract of *C. orbiculata* leaves, the MeOH extracts of *G. stenocarpa* stems, *E. ciliata* leaves, and especially *P. reticulatus* leaves showed potential inhibition of RNase H and protection against the viral cytopathic effect of the HIV-1. Further chemical investigations should be carried out to find the active components of these extracts and compounds as potential anti-HIV drug candidates.

## Acknowledgements

We would like to thank the Ministry of Science and Technology, Vietnam, for financial support in form of the Vietnam–Korea International Collaboration Project (No. 30/823/2007/HD-NDT). This work was supported partly by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815), Republic of Korea. This research was also supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

## Declaration of interest

The authors report no conflicts of interest.

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