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Original Research Article

Biological Activities and Crystal Structure Determination of *trans, trans*-Cyclohexane-1,2,4,5-tetrol Monohydrate from *Pseuduvaria phuyensis* (R.M.K. Saunders)

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ABSTRACT

Pseuduvaria phuyensis (Annonaceae), was collected from Kanchanaburi Province, western Thailand. The study aimed to evaluate the antibacterial, anti-HIV-1RT, anti-syncytium (MC99+1A2), and cytotoxic potentials of the crude hexane, ethyl-acetate, and methanol extracts of *P. phuyensis* leaf-twigs and stems, all in comparison with standard drugs. Also, the isolation and characterization of a compound from one of the active extracts as well as its antibacterial and cytotoxicity activities were carried out. The MIC and MBC values of selected bacterial strains ranged from 3.125 – 200 and 6.25 – 200 mg/mL, respectively for all the extracts, while the isolated compound was not active. The anti-HIV-1 RT and the anti-syncytium assay of the hexane and ethyl acetate extracts revealed they were very active, with % inhibitions of 90.50 and 89.36, respectively. The best inhibitory concentration at 50% (IC_{50}) value was 44.33 µM as exhibited by the hexane extract of the stems of the reverse transcriptase assay, while, the best effective concentration at 50% (EC_{50}) value was 10.4 µM (TI>4.83) as displayed by the ethyl acetate extract of the stems. The cytotoxicity study showed that the hexane extract of the stems displayed high cytotoxicity with ED_{50} value at 13.07 µg/mL against the FaDu cancer cell line, whil that of the isolated compound was not active. The structure of the isolated compound was characterized and elucidated as *trans, trans*-cyclohexane-1, 2, 4, 5-tetrol monohydrate using spectroscopic technique and X-ray crystallography analysis.

Keywords: *Psuduvaria phuyensis*, *Annonaceae*, Polyoxygenated Cyclohexane, Antibacterial, Anti-HIV-1 Virus, Cytotoxicity Activity

Introduction

The Annonaceae family is one of largest flowering plant families consisting of approximately 2,500 species in 110 genera.[1] The family is composed of small trees, shrubs, and climbers found mainly in tropical and boreotropic areas.[2]

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There are more than 385 species in Brazil, approximately 51 genera, and more than 950 species found in Asia and Australia, while 40 genera with about 450 species are found in Africa and Madagascar, and 30 genera with 740 species are found in the American continent.[3] In Thailand, there are about 39 genera containing approximately 300 species.[4] Traditionally, several plants of the Annonaceae family are used in folk medicine such as, *Annona muricata* (*A .muricata)* is used in the treatment of various diseases, especially cancer, and parasitic infections. Also, *Annona cherimola* (*A .cherimola)* seeds are used as an insecticide, while a decoction of *Annona reticulata* (*A. reticulata)* is used in the treatment of malaria and syphilis. The leaf of *Annona squamosa (A .squamosa)* are also used as vermicide, against tumors, insect bites, and skin problems.[5,6] Phytochemistry research has revealed that the plants of the Annonaceae family are rich in many types of compounds, such as; alkaloids,[7] acetogenins,[8] flavonoids,[9] and other phenolic compounds.[10] *Psuduvaria phuyensis*) *P. phuyensis*((Figure 1) is a member of the *Psuduvaria* genus that belongs to the Annonaceae family.[11] *P. phuyensis* is a small tree)6 m (with pendent, unisexual flowers, the petals are in two whorls, with the larger inner whorl forming a mitriform dome above the reproductive organs.[12] Presently,

there are no research reports on the chemical composition and biological activity of *P. phuyensis* .Therefore, this research was undertaken. The findings from this research will serve as a useful baseline for other researchers to expand on other aspects of research into the plant.

Materials and Methods

General experimental procedures
Column chromatography) CC (was carried out using silica gel 60 H from E .Merck .70-230 mesh ASTM, cat .No .7734 and No.7736 .The thin-layer chromatography)TLC (technique was carried out on silica gel 60 PF_{254}-coated aluminum sheets and spots were identified under ultraviolet light .Infrared spectra)IR (were recorded on KBr pellets using a Shimadzu 8900 FT-IR spectrophotometer .Melting points were recorded on a Büchi 322 micro melting point apparatus and are uncorrected .The mass spectra were recorded on a Thermo Finnigan Polaris Q mass spectrometer at 70 eV)probe (and EIMS was measured by a Brüker Esquire apparatus .The X-ray absorption spectroscopy was recorded on a Bruker D8 QUEST CMOS PHOTON II .^{1}H)500 MHz(, ^{13}C)125 MHz(, and 2D NMR spectra were recorded on a Brüker AV-500 spectrometer in deuterated methanol)CD_3OD (solution and TMS was used as the internal standard.

Extraction and isolation procedures
The air-dried milled mixed leaf and twigs of *P. phuyensis* (0.9 kg) were extracted with hexane (5.5 liters × 9 days × 3 times), ethyl acetate (4 liters × 12 days × 4 times), and methanol (4.5 liters × 9 days × 3 times) to afford crude hexane extract (9.8 g), crude ethyl acetate extract (45.51 g) and crude methanol extract (53.31 g), respectively. Furthermore, the air-dried powdered stems of *P. phuyensis* (3.9 kg) were extracted with hexane (19 liters × 9 days × 3 times), ethyl acetate (19 liters × 12 days × 4 times), and methanol (17.5 liters × 9 days × 3 times) to afford crude hexane extract (14.24 g), crude ethyl acetate extract (62.01 g) and crude methanol extract (161.42 g), respectively. Each extract was tested for its antibacterial, anti-HIV-1 virus, and anticancer activities (Table 2). Furthermore, the crude methanol extract of *P. phuyensis* stems (quantity) was separated by column chromatography on silica gel eluted with a gradient system between hexane, ethyl acetate, and methanol to give ten fractions (A1-A10). Mixed fraction of A7 and A8 (4.92 g) were re-chromatographed to give eight sub-fractions (B1-B8). Fraction B7 (1.52 g) was recrystallized with ethanol to afford colorless needle crystals (0.5 g)

Plant and cells lines materials
The leaf and twigs and stems of *P. phuyensis* (BKF.140574) (Figure 1) were collected at Thong Pha Phum, Sangkhlaburi District, Kanchanaburi Province, Thailand, 14º15'10"N98 º40'30"E. Alt.450 m., in 25th November 2020. The plant materials were identified by Dr. Narong Nantasean, a botanist at the Department of Chemistry, Faculty of Science and Center of Innovation in Chemistry, Mahidol University, Rama VI Road, Bangkok 10400, Thailand. KKU-M213 (human cholangiocarcinoma) cells were kindly provided by Dr. Banchob Sripa from the Liver Fluke and Cholangiocarcinoma Research Center, Department of Pathology, Faculty of Medicine, Khon Kean University. MMNK1 (human cholangiocyte) cells[13] were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). FaDu (human hypopharyngeal carcinoma), HT29 (human colorectal adenocarcinoma), MDA-MB-231 (human mammary gland/breast adenocarcinoma), SH-SY5Y (human neuroblastoma), A549 (human lung carcinoma), and HepG2 (human hepatocellular carcinoma) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Bacterial strains
In vitro, antibacterial activity was carried out against nine strains)*S. aureus* ATCC 25923 DMST 8840, *E .aerogenes* ATCC13048 DMST 8841, *E .coli* O157 :H7 DMST 12743, *E .coli* Entertoxigenic, ETEC DMST 30543, *E .coli* Enteropathogenic, EPEC DMST 30546, *S. typhimurium* ATCC 13311 DMST 562, *S .flexneri* DMST 4423, *P. mirabilis* DMST 8212, *V .cholera* nonO1/nonO139 DMST 2873). Chloramphenicol was used as a positive control.

Figure 1: Leaf, Twigs, and Stems of *P. phuyensis*

Minimum inhibitory concentration (MIC)
The samples were dissolved in 10% DMSO to the concentrations of 200 mg/mL (crude extracts), 100 mg/mL (crude extracts), 2 mg/mL (isolated compounds) and 1 mg/mL (chloramphenicol). The extracts were diluted to final concentrations of 6.25 mg/mL, 3.125 mg/mL, 0.065 mg/mL, and 0.0325 mg/mL. The final concentration of *S. aureus*, *E. aerogenes*, *E. coli* 0157: H7, *E. coli* (ETEC), *E. coli* (EPEC), *S. typhimurium*, *S. flexneri*, *P. mirabilis*, and *V. cholera* in Mueller Hinton Broth (MHB) was 1×10^6 cfu/mL, 50 µL/well (Mcfarland standard No. 0.5) in a 96-well plate, and they were then mixed into the samples (50 µL/well). The plates were incubated at 37°C for 24 hours, and the growth of the organisms was observed by the color change of resazurin (1 mg/mL, 20 µL/well). No color change is desired for the prevention of microbial growth.[14]

Minimum bactericidal concentration (MBC)
The MBC assay was performed for samples that did not show any visible growth and were subsequently sub-cultured onto nutrient agar plates. These plates were incubated at 37°C for 24 hours. MBC was only used for the lowest concentration of the bacteria that did not retrieve 2 single colonies.[14]

HIVs assay
Anti-HIV1-RT)Reverse transcriptase assay)
Anti-HIV1-RT testing of the extracts of *P. phuyensis* and isolated compound were carried out at the Service Centre of the Department of Physiology and Microbiology, Mahidol University, Thailand .The anti-HIV1-RT activities were performed by testing RT inhibition.[15,16,17] The extracts were diluted to give 20 mg/mL of 100% dimethyl sulfoxide (DMSO) after the removal of tannin by polyvinylpyrrolidone (PVP). The final volume was 200 µg/mL in 10% DMSO, and Nevirapine, 2 µg/mL was used as a positive control. The HIV-1RT (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong) kit was used. The 96-well plate (100 U/µL, 4 µL/well) was filled with samples (2 µL/well), and then 2.5 µg/µL of poly-A and 0.125 µg/mL of oligo dT16 primer were added to 4 µL/well and incubated at 37°C for 20 mins. The reaction was affixed by 0.2 M EDTA (2 µL/well) and incubated at 4°C for 15 mins. The signal of fluorescence was measured at an emission wavelength of 535 nm and excitation wavelength of 480 nm after Pico green dissolved in TE buffer (1:2000) was put in (volume 200 µL/well). The results were evaluated as a percentage of inhibition.[15]

Cell-based assay for anti-HIV-1
The syncytium assays of the extracts of *P. phuyensis* and the isolated compound were carried out in triplicate using ΔTat/revMC99 virus and 1A2 cell system[18,19] starting at the final concentrations of 3.9–125 µg/mL or higher. Virus control and cell control wells contained neither the extracts nor the virus; cytotoxicity control wells containing cells with the extracts and positive control, i.e., azidothymidine (AZT), were included. The result was shown as 50% effective concentration (EC_{50}). Cytotoxicity of the extracts was also carried out, in parallel and in duplicate, using a colorimetric XTT assay. The result was indicated as the concentration that inhibited 50% formazan formation in uninfected cells (IC_{50}). The therapeutic index (TI) was calculated using the equation: $TI=IC_{50}/EC_{50}$.

Cytotoxicity assay
Cytotoxicity activity of the extracts of *P. phuyensis* and the isolated compound was investigated using the standard Sulforhodamine B (SRB) assay. Ellipticine was used as a positive control.[20,21] The concentrations of the samples were 0.16–20 µg/mL in 0.5% DMSO. The cancer cell lines were employed, including human intrahepatic

cholangiocarcinoma (KKU-M213), human pharyngeal squamous carcinoma (FaDu), human colorectal adenocarcinoma (HT-29), human mammary gland/breast adenocarcinoma (MDA-MB-231), human lung carcinoma (A 549), human neuroblastoma (SH-SY5Y), highly differentiated immortalized human cholangiocyte cell line (MMNK-1), and human liver cancer cell line (Hep G2). MEM (minimum essential medium with Earles salt and L-glutamine) in 10% FBS was used for culturing the cell lines. The cell lines were kept at a temperature of 37 °C for 72 hours at 5% CO_2 in the air, and 100% relative humidity, followed by stabilizing with 20% trichloroacetic acid at 4 °C for 60 minutes and then stained for 30 minutes by 0.4% SRB in 1% acetic acid at room temperature. The unbound dye was cleaned with 1% acetic acid, while the already-dried stain was mixed with 10 mM Tris base with pH = 10. The absorbance was measured at 510 nm on a microplate reader, and the 50% effective dose (ED_{50}) was calculated. Ellipticine was used as a positive control.

X-Ray crystallographic analysis of the isolated compound
The isolated compound was recrystallized with ethanol and the X-Ray crystallographic data were shown in Table 1.

Results and Discussion

Isolation and purification of leaf, twigs, and stem of *P. phuyensis* were found in one compound in the methanol extract. Structural elucidation of the isolated compound using spectroscopic techniques, such as ^{1}H, ^{13}C-NMR, and DEPT techniques. Furthermore, the X-ray analysis,[22,23] and bioactivities test, including antibacterial, anti-HIV, and cytotoxicity tests were performed.

Structural characterization of isolated compound
Colorless needles)EtOH(m.p. = 137–139 °C (lit. 131–132 °C).[24] IR (KBr) ν_{max} 3300 (OH(, 2967, 2899, 1076, 1044 cm^{-1} .(^{1}H)500 MHz, CD_3OD (NMR, δ_H :4.86 (4H, s, OH), 3.72)1H, m, H-1), 3.72)1H, m, H-2), 3.72)1H, m, H-4), 3.72)1H, m, H-5), 1.83)2H, m, H-3), 1.83)2H, m, H-6) .^{13}C)125 MHz, CD_3OD(NMR, δ_C :70.24) C-1, 2, 4, 5(, 34.43) C-3, 6 .(EI-MS: *m/z* (%) 167.23]M+H$^+$[ )5.21(, 166.23(6.17), 149.16)15.83(, 129.18)7.78(, 112.11)1.03(, 95.06)34.29(, 94.11)1.92(, 67.04)42.43(, 54.99(100) .The proposed mass fragmentation of the isolated compounds is shown in Figure 3. The X-ray crystallographic data of the isolated compounds have been documented in the Cambridge Crystallographic Data Centre as CCDC number 2127134.[25] From the spectral and X-ray crystallographic data, and the comparison of its spectral data with those in literature,[26] the isolated compound could be identified as *trans, trans*-cyclohexane- 1,2,4,5-tetrol monohydrate. The chemical structure and X-ray structure, and the X-Ray crystallographic data of isolated compound are shown in Figure 2, and Table 1.

Anti-bacterial activity
The crude ethyl acetate extract of the leaf and twigs of *P. phuyensis* showed the best activity against MIC/MBC at 6.25/6.25 mg/mL while, the crude methanol extract of the leaf and twigs of *P. phuyensis* and the crude hexane extract of stems of *P. phuyensis* showed the best MIC/MBC value against *S. typhimurium* at 6.25/6.25 mg/mL respectively. Furthermore, the crude ethyl acetate extract of the stems of *P. phuyensis* showed the best activity against MIC/MBC at 3.125/6.25 mg/mL while, the crude methanol extract of the stems of *P. phuyensis* offered the best MIC/MBC value against *E. coli* (ETEC) at 6.25/6.25 mg/mL. The isolated compound was not active versus 7 bacterial strains (Table 2). Although extracts from *P. phuyensis* can inhibit or kill 7 bacterial cells, especially strains *E. coli* (ETEC), *S. typhimurium*, and *P. mirabilis*, but were considered to have weak activity compared to Chloramphenicol as a standard comparator in this experiment. In addition, the experimental results showed that the isolated compounds did not inhibit or kill all 7 strains of bacterial cells. In the crude extract, there may be other compounds in very small amounts that can inhibit or kill bacteria cells but are lost during the purification process.

Table 1: X-Ray Crystallographic Data of the Isolated Compound

	Isolated compound
Formula	$C_6H_{14}O_5$
Molecular Weight	166.17
Space group	C2
Cell parameters (Å, deg)	
a	10.6841(4) Å
b	7.3563(2) Å
c	5.2038(2) Å
α	0.71073 Å
β	103.9070
γ	
V (Å^3)	397.01(2)
Z	2
T(K)	296
CCDC	2127134

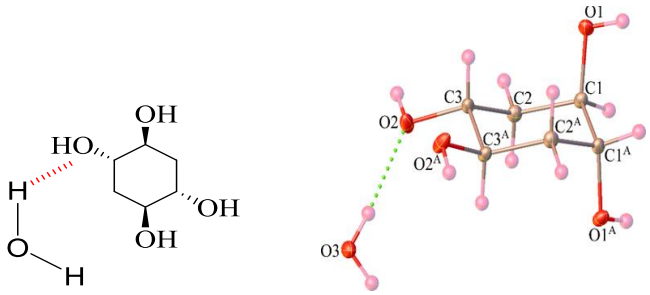

Figure 2: Structure and ORTEP Drawing of Isolated Compound from X-ray Crystallographic Analysis

Anti-HIV-1RT activity
The hexane and ethyl acetate extracts of the leaf and twigs of *P. phuyensis* showed the highest inhibition (>70 %, VA, very active) of HIV-1RT, while the methanol extract was inactive at the same concentration. The hexane and ethyl acetate extracts of the stems of *P. phuyensis* displayed moderate and weak inhibitions of HIV-1RT respectively, while the methanol extract remained inactive, all at the same concentration (Table 2). The anti-syncytium (MC99+1A2) activity of *P. phuyensis* was found in all of the extracts. Furthermore, the hexane extract of the stems of *P. phuyensis* showed the best IC_{50} and EC_{50} values at 44.33 and 13.86, respectively (Table 3). The isolated compound was not tested in this experiment since this compound was found in the methanol extract, but the extract was inactive against the anti-HIV-1RT virus, further testing of this bioactivity was not required. Although all the extracts of *P. phuyensis* were reduced to 50% syncytium formation (MC99+1A2) by ΔTat/RevMC99 virus in 1A2 cells, they were not as active as the azidothymidine (AZT) that was used as the standard.

Cytotoxicity
The hexane extracts of the leaf and twigs of *P. phuyensis* showed cytotoxicity against SH-SY5Y cell lines at the ED_{50} values of 16.08 μg/mL while, the hexane extract of the stems of *P. phuyensis* showed cytotoxicity against 5 cancer cell lines, especially, FaDu, A549 and, MNN-K1 cell lines at the ED_{50} values of 13.07, 14.45 and, 14.23 μg/mL, respectively (Table 4). Although the hexane extracts of the stems of *P. phuyensis* showed cytotoxicity against six cancer cell lines, they were not as good as ellipticine which was used as the standard compound in this experiment.

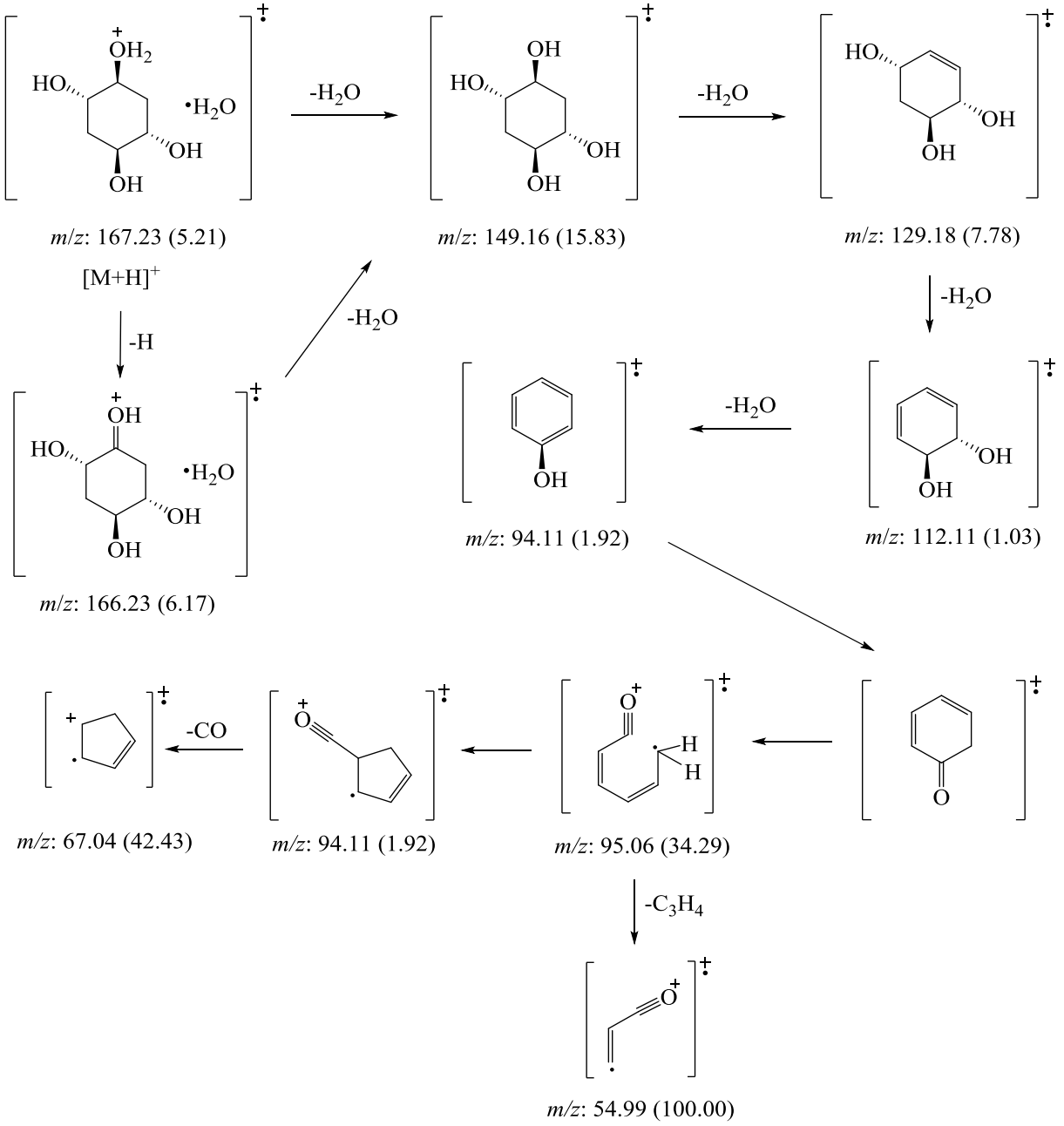

Figure 3: Proposed Mass Fragmentation of the Isolated Compound

Conclusion

The phytochemical investigation of the leaf, twigs, and stems of *P. phuyensis* were founded one compound and identified as *trans, trans*-cyclohexane-1,2,4,5-tetrol monohydrate. The crude ethyl extract of leaf and twigs *of P. phuyensis* showed the best antibacterial activity with *E. coli* (ETEC) and *S. typhimurium* and showed very active in anti-HIV-1RT. Moreover, the crude hexane extract was also against 6 cancer cell lines. Interestingly, the isolated compound is an important starting material in the total synthesis of biologically active compounds such as aminocyclitols and its analog.[26]

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 2: The MIC and MBC of Crude Extracts of *P. phuyensis* and Isolated Compounds in Comparison with Chloramphenicol Against Selected Bacterial Strains

Type	Extracts/ compound/ standard drug	Concentration of MIC/MBC (mg/mL)								
		S. aureus	*E. aerogenes*	*E. coli* O157:H7	*E. coli* (ETEC)	*E. coli* (EPEC)	*S. typhimurium*	*S. flexneri*	*P. mirabilis*	*V. cholerae*
Leaf	Hexane	NA	NA	NA	NA	NA	100/200	NA	100/200	NA
and	EtOAc	12.5/25	25/50	200/200	6.25/6.25	NA	6.25/6.25	NA	12.5/50	25/25
Twigs	MeOH	12.5/25	NA	25/25	NA	NA	6.25/25	200/200	12.5/50	25/25
Stems	Hexane	NA	NA	NA	NA	NA	6.25/6.25	NA	6.25/100	NA
	EtOAc	NA	NA	NA -	6.25/6.25	NA	6.25/25	NA	3.125/6.25	12.5/50
	MeOH	NA	NA	25/100	6.25/6.25	NA	25/200	25/50	50/100	100/100
	Isolated compound	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Chloramphenicol (control)	<0.03/0.5	0.0625/0.25	<0.03/0.25	<0.03/0.25	<0.03/1	<0.03/0.0625	<0.03/0.25	<0.03/0.03	<0.03/1

NA = Not active

Table 3: Anti-HIV-1 RT, Anti-syncytium)MC99+1A2) of Crude Extracts of *P. phuyensis*

Crude extracts	Anti-HIV-1 RT[a] (% inhibition)		Anti-syncytium (MC99+1A2)[b] (μM)				Azidothymidine (AZT)		
			IC_{50}	EC_{50}	TI[c]	Activity	IC_{50}	EC_{50}	TI[c]
Hexane extract (Leaf and Twig)	90.51	VA	49.83	22.20	2.24	Active	$>10^{-8}$	5.86×10^{-9}	>1.706
thyl acetate extract (Leaf and Twig)	89.36	VA	63.80	24.07	2.65	Active	$>10^{-8}$	5.86×10^{-9}	>1.706
Methanol extract (Leaf and Twig)	-13.37	I	166.60	117.63	1.42	Active	$>10^{-8}$	5.86×10^{-9}	>1.706
Hexane extract (Stems)	60.73	MA	44.33	13.86	3.20	Active	$>10^{-8}$	5.86×10^{-9}	>1.706
Ethyl acetate extract (Stems)	36.62	WA	50.22	10.4	4.83	Active	$>10^{-8}$	5.86×10^{-9}	>1.706
Methanol extract (Stems)	-14.13	I	142.11	123.9	1.15	Active	$>10^{-8}$	5.86×10^{-9}	>1.706

[a]Anti-HIV-1RT activity expressed as %inhibition at 200 μg/mL :very active) VA = (>70 %inhibition, moderately active) MA = (50 %to 69 % inhibition, weakly active)WA = (30 %to 50 %inhibition and inactive)I = (<30 %inhibition ; For determination of IC_{50} in the HIV-1 RT assay, the coefficients of determination, R^2 , were 0.98 −0.99 in all assays for 50 %end point.
[b]Anti-syncytium)MC99+1A2 (EC_{50} = dose of compound that reduced 50 %syncytium formation by ΔTat/RevMC99 virus in 1A2 cells .AZT, averaged from three experiments, EC_{50} 3.95×10^{-3} μM; [c]TI, Therapeutic Index :IC_{50}/EC_{50}

Table 4: Cytotoxicity Study of Crude Extracts and Isolated Compounds of *P. phuyensis*

	Cytotoxicity ED_{50} (μg/mL)[d]							
	KKU-M213	FaDu	HT-29	MDA-MB-231	A549	SH-SY5Y	MNN-K1	Hep G2
Hexane extract (Leaf and Twig)	NA	NA	NA	NA	NA	NA	NA	NA
Ethyl acetate extract (Leaf and Twig)	NA	NA	NA	NA	NA	NA	NA	NA
Methanol extract (Leaf and Twig)	NA	NA	NA	NA	NA	NA	NA	NA
Hexane extract (Stems)	18.60	13.07	NA	18.79	14.45	19.79	14.23	NA
Ethyl acetate extract (Stems)	NA	19.92	NA	NA	NA	NA	NA	19.32
Methanol extract (Stems)	NA	NA	NA	NA	NA	NA	NA	NA
Isolated compound	NA	NA	NA	NA	NA	NA	NA	NA
Ellipticine (control)	0.59	0.54	0.60	0.62	0.45	0.53	0.42	0.52

[d]ED_{50} less than 20 μg/mL were considered active for extracts and ED_{50} less than 4 μg/mL were considered active for pure compounds .Cancer cell lines :KKU-M213)Human cholangiocarcinoma (FaDu)Human squamous cell carcinoma (HT-29)Human colon adenocarcinoma (MDA-MB-231)Human mammary gland/breast adenocarcinoma (A549)Human lung adenocarcinoma (SH-SY5Y) Human neuroblastoma (MNN-K1) highly differentiated immortalized human cholangiocyte cell line (Hep G2)Human hepatocellular carcinoma(, NA = Not active

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