Original Article

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Effect of Thai Medicinal Plant Extracts against Dengue Virus *in vitro*

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Abstract

Dengue hemorrhagic fever is one of the major public health problems in Thailand. The disease caused by the mosquito-borne dengue virus (DENV). DENV is the member of the *Flavivirus* genus, *Flaviviridae* family. There are four genetically related but distinct serotypes DENV 1, 2, 3, and 4. It is transmitted to people through the bite of mosquitoes *Aedes aegypti* and *Aedes albopictus*. It has been reported that dengue virus infects 50 to 100 million people annually. However, there is neither vaccine nor effective antiviral drugs to treat dengue virus infection. Consequently, the development of antiviral drugs licensed for treatment of patients remains an urgent need to prevent dengue fatalities. Many studies have been conducted for exploring the antiviral activity of chemical compounds against DENV. Among these compounds, some are small molecules that can inhibit specific steps of viral intracellular replication or effect at viral proteins. But none of these compounds have been approved to be used in human. Thailand has many traditional medicinal plants that have been reported on strong antiviral activity and some of them have already been used to treat people who were infected with viruses. Therefore, the aim of this study is to investigate the *in vitro* anti-dengue activity from Thai medicinal plants. In this present study, ten medicinal plants were collected from Siri Ruckhachati Natural Park, Salaya campus, Mahidol University. These plants were extracted with dichloromethane ethanol and subsequently tested for their anti-dengue type 2 activities in Vero cell by MTT method. The results showed that the ethanol extracts of *Rhizophora apiculata* Blume., *Flagellaria indica* Linn. and *Cladogynos orientalis* Zipp. at a concentration of 12.5 µg/ml exhibited inhibitory activity on DENV-2 with 56.14%, 45.52% and 34.85%, respectively. Moreover, *Houttuynia cordata* Thunb. exhibited inhibitory activity against DENV-2 with 35.99% at a concentration of 1.56 µg/ml. In addition, *Cladogynos orientals* Zipp., *Piper retrofractum* Vahl. and *Rhizophora apiculata* Blume. exhibited an inactivated viral particle activity with 52.9%, 84.93% and 41.5% at a concentration of 100 µg/ml. This study indicated that *Rhizophora apiculata* Blume., *Flagellaria indica* Linn., *Cladogynos orientalis* Zipp. and *Houttuynia cordata* Thunb. have a significant potential effect on the dengue virus *in vitro*. These medicinal plants could potentially be sources for developing the anti-DENV drug.

Key words: Dengue virus type 2, Thai medicinal plants, Anti-dengue activity

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INTRODUCTION

Dengue hemorrhagic fever is one of the most important emerging tropical diseases in the 21st century[1]. In the latter part of the 20th century, globalization and rapid urbanization of many developing tropical countries produced increased transmission and hyperendemicity of the disease[2]. World Health Organization (WHO) estimates that there are as many as 50 million cases of dengue infection worldwide and global warming provide a significant selective advantage for dengue infection spreading into new areas[3]. Dengue infection is caused by dengue virus (DENV). DENV belongs to the genus *Flavivirus* in the family *Flaviviridae*. There are four distinct serotypes of DENV: 1, 2, 3, and 4. The virion contains a positive-sense, single-stranded RNA molecule of approximately 11 kb in length, inserted in an icosahedral nucleocapsid and surrounded by a lipid envelope-covered with peplomers. The viral genome is translated into three structural proteins, capsid (C), pre-membrane (prM), envelope (E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5)[3,4]. DENV is transmitted principally by *Aedes aegypti* mosquito. Other mosquito such as *Ae. albopictus* and *Ae. polynesiensis* can also transmit epidemic dengue, but less efficiently[5]. Clinical manifestation of DENV infection range from subclinical to a self-limited fever and rash (dengue fever (DF)) to a severe and sometimes deadly illness characterized by capillary leakage, thrombocytopenia and hypovolemic shock (dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS))[6].

At present, no specific treatments for DENV infection are clinically available. Control of DENV by safe, low-cost and long-lasting vaccination has not been established. Several types of antiviral agent have been sought intensively, including inhibitors against viral replication, posttranslational processing of viral proteins and E protein functions such as membrane fusion and viral attachment[7]. A number of antiviral compounds, i.e. ribavirin, mycophenolic acid, 7-deaza-2'-c-methyl-adenosine and 6-O-butanoyl castanospermine have shown their efficacy in inhibiting DENV replication *in vitro*[8]. The only available treatment is supportive therapy. Therefore, it is necessary to find new alternative antiviral compounds against DENV[9]. Plants have long been used as a source of medicine from ancient time to today all over the world[10]. Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection by inhibiting the replication cycle of various types of DNA and RNA virus[11]. However, very little is known about potential of plants against dengue virus. The objective of the present study is to investigate the *in vitro* inhibitory activity of Thai medicinal plants toward DENV2 infection in Vero cells.

MATERIALS AND METHODS

Preparation of plant extracts

All medicinal plants were collected from Siri-Ruckhachati Natural Park, Salaya campus, Mahidol University. The whole plants were dried under shade and grinded to powder. Plants powder were soaked in dichloromethane at 4°C for 18 hours by the ratio of plant: solvent at 1:3. The crude extracts were filtered through Whatman filter paper No.1 and evaporated in water bath at 80°C until dry. The dried extracts were weighed and stored at -20°C until use. The residual from dichloromethane extraction was subsequently extracted again with 70% ethanol at 4°C for 18 hours by the ratio of plant: solvent 1:3. The crude extracts were filtered through Whatman filter paper No.1 and evaporated in water bath at 80°C until dry. The dried extracts were weighed and stored at -20°C until use.

Cell culture and virus

C6/36 mosquito cell line, African green monkey kidney epithelial cells (Vero cell) and Dengue virus type 2 strain 16681 were kindly provided by Dr. Sutee Yoksan (Center for Vaccine Development, Institute for Molecular Bioscience, Mahidol University). The C6/36 mosquito cell line was grown

as monolayer at 28ºC in minimum essential medium (MEM) supplement with 1% non-essential amino acids and 10% fetal bovine serum (FBS). The Vero cell was grown as monolayer at 37ºC with 5% CO_2 in minimum essential medium (MEM) supplement with 1% glutamine and 10% fetal bovine serum (FBS). Dengue virus type 2 was propagated in C6/36 cell line for 7 days at 28ºC in the absence of CO_2. Culture supernatant was harvested and centrifuged at 3000g for 15 min. The supernatant was collected and stored at-80ºC as virus stock until use.

Antiviral assay

Vero cells were seeded at $1x10^5$ cells/ml per well in 96-well plate and exposed to DENV2. After that, the various non-cytotoxic concentrations of crude extracts were added and incubated for 5 day at 37ºC with 5% CO_2. After incubation, 20 µl of 3-(4,5-dimethylthaiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution were added to each well. The plate was further incubated for 2-4 hours. Then, a constant volume of medium (150 µl) from each well was removed and solubilized the formazan crystals by adding 130 µl of the acidified Triton X-100 and isopropanol solution to each well. The formazan crystal was completely dissolved by gently shaking for 15 mins. The absorbance value at wavelength 595 nm was measures by a microplate reader 680 (Bio-Rad). The activity of crude extracts were determined as percent inhibition which calculated by the following formula.

$$\% \text{ Inhibition} = [(OD_T)_V\text{-}(OD_C)_V/(OD_C)_M\text{-}(OD_C)_V]x100\ (\%)$$

Where

$(OD_T)_V = A_{595}$ in virus infected cells with test compounds

$(OD_C)_V = A_{595}$ in virus infected cells without test compounds

$(OD_C)_M = A_{595}$ the mock infected control

Cytotoxicity assay

Vero cells in growth medium were seeded at $1x10^5$ cells/ml per well in 96-well microtiter plate and allowed to adhere for 24 hours at 37ºC in 5% CO_2. Then, the medium was removed from the wells, cells were treated with 100 µl 2-fold serial dilution of crude extracts. In control, only 100 µl of medium was added to the cells. After 72 hours, 20 µl of MTT solution were added to each well and the plates were further incubated for 2-4 hours. After incubation, MTT solution was removed without disturbing the cell and 100 µl of dimethy sulfoxide (DMSO) was added to each well to stop reaction. Subsequently, the absorbance value was measured by a microplate reader 680 (Bio-Rad) at wavelength 595 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of each extract that reduced the absorbance of treated cell to 50% when compared with the cell control.

Virucidal assay

A virus suspension of DENV-2 containing approximately 80-100 PFU was incubated with an equal volume of diluents with or without 0.1 mg/ml crude extracts for 1.5 h at 37ºC before infection. Then 200 µl of treated solutions were added on Vero cells monolayer to determine residual infectivity. After 1.5 h of adsorption at 37ºC, cells were washed twice with PBS. Subsequently, overlay medium was added to each well. Plates were incubated at 37ºC with 5% CO_2 for 7 days. Percent inhibition was calculated from the reduction of plaque number.

RESULTS AND DISCUSSIONS

Herbal medicines are potential source for the development of new antiviral drugs, since they can be selected on the basis of their ethnomedicinal use, for example, against infection[12]. These plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources are of interested as possible sources to control viral infection[13].

In this study, a total sixteen extracts from ten plants species were examined for their antiviral activity against DENV2. The antiviral activity and cytotoxicity of plant extracts were determined using MTT method by measuring the absorbance

value at wavelength 595 nm. This assay has several advantages: it is easy to perform, the evaluations are objective, it can be automated using a personal computer and the toxicity evaluation can be made in parallel with antiviral activity evaluation[11]. The anti-DENV2 activity of each extracts was shown in Table 1. At a concentration of 12.5 µg/ml, ethanol extracts of *Rhizophora apiculata* Blume., *Piper retrofractum* Vahl., *Flagellaria indica* Linn. and *Cladogynos orientalis* Zipp. exhibited the inhibitory activity against DENV2 with 56.14%, 53.53%, 45.52% and 34.85% inhibition, respectively. At the same concentration, dichloromethane extract of *Piper retrofractum* Vahl. showed the inhibitory activity against on DENV2 with 32.06%. While, ethanol extract of *Houttuynia cordata* Thunb. exhibited inhibitory activity on DENV2 with 35.99% at a concentration of 1.56 µg/ml. On the other hand, dichloromethane and ethanol extracts of *Acacia catechu* Willd., *Piper sarmentosum* Roxb., *Cassia tora* Linn., *Phyllanthus urinaria* Linn. and *Ricinus communis* Linn. did not exhibit inhibitory activity against DENV2 in Vero cell.

From previous experiment, it was found that ethanol extracts of *Rhizophora apiculata* Blume., *Piper retrofractum* Vahl., *Cladogynos orientalis* Zipp., *Houttuynia cordata* Thunb., *Flagellaria indica* Linn. and dichloromethane of *Piper retrofractum* Vahl. showed good antiviral activity against DENV2. Then, the cytotoxicity of these extracts were further evaluated by MTT method to determine the non-toxic concentration in Vero cell. Evaluation of cytotoxicity is an important part of the assessment of the potential antiviral agent since the beneficial extracts should be selective for virus-specific processes with little or no effects on metabolism of host cells[14]. The results of the cytotoxicity evaluation of the tested extracts were shown in Table 2. The 50% cytotoxic concentration (CC_{50}) of ethanol extracts of *Rhizophora apiculata* Blume. and *Piper retrofractum* Vahl. were 625 µg/ml. And, the CC_{50} of ethanol extracts of *Cladogynos orientalis* Zipp., *Flagellaria indica* Linn. and *Houttuynia cordata* Thunb. were 312 µg/ml. Whereas, the CC_{50} of dichloromethane extract of *Piper retrofractum* Vahl. was 156.25 µg/ml.

Table 1. Antiviral activity against DENV2 determined by MTT method

Plant	Concentration (µg/ml)	% Inhibition	
		Dichloromethane	Ethanol
Acacia catechu	12.5	None	None
Cassia tora	12.5	None	None
Cladogynos orientalis	12.5	-	34.85
Flagellaria indica	12.5	-	45.52
Houttuynia cordata	1.56	-	35.99
Phyllanthus urinaria	12.5	None	None
Piper retrofractum	12.5	32.06	53.53
Piper sarmentosum	12.5	None	None
Ricinus communis	12.5	None	None
Rhizophora apiculata	12.5	-	56.14

-: Not tested; None : No activity

Table 2. Cytotoxicity of Thai medicinal plants extracts on Vero cells[a]

Plant	Extract	CC_{50} (µg/ml)
Cladogynos orientalis	Ethanol	312.5
Flagellaria indica	Ethanol	312.5
Houttuynia cordata	Ethanol	312.5
Piper retrofractum	Ethanol	625
	Dichloromethane	156.25
Rhizophora apiculata	Ethanol	625

[a] Monolayers of cells exposed to MEM alone were used as a control

Table 3. Virucidal activity of ethanol extracts of Thai medicinal plants against DENV2

Plant	Concentration (µg/ml)	% Inhibition
Cladogynos orientalis	100	52.92
Piper retrofractum	100	84.93
Rhizophora apiculata	100	41.5

The ethanol extracts of *Cladogynos orientalis*, *Piper retrofractum* and *Rhizophora apiculata* were selected for determining their ability to inactivate viral particles before adsorption on Vero cells. Result in Table 3 showed that the viral particles were inactivated by the ethanol extracts of *Cladogynos orientalis, Piper retrofractum,* and *Rhizophora apiculata* with 52.9%, 84.93% and 41.5% at concentration of 100 µg/ml, respectively.

Taken together, these results indicated that *Rhizophora apiculata* Blume., *Piper retrofractum* Vahl., *Flagellaria indica* Linn., *Cladogynos orientalis* Zipp., and *Houttuynia cordata* Thunb. exhibited inhibitory activity against DENV2 in Vero cell. Moreover, *Cladogynos orientals* Zipp., *Piper retrofractum* Vahl. and *Rhizophora apiculata* Blume. also had a potential to inactivate DENV2. However, the possible other mechanisms of their antiviral activity have not studied yet.

CONCLUSION

The *in vitro* study demonstrated that *Rhizophora apiculata* Blume., *Piper retrofractum* Vahl, *Flagellaria indica* Linn. *Cladogynos orientalis* Zipp. and *Houttuynia cordata* Thunb. could be a potential sources of novel anti-dengue compounds. However, the active compounds have not yet been identified. Further studies are needed to investigate the exact mechanism of their antiviral action, purification and characterization of their active compounds.

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