Inhibitory Activities of Methanol Extracts of *Andrographis paniculata* and *Ocimum sanctum* Against Dengue-1 Virus

Anna Pick Kiong LING, Bee Fong KHOO, Ching Hua SEAH, Kar Yue FOO, Rock Kee CHEAH, Soi Moi CHYE, and Rhun Yian KOH

***Abstract*—**Dengue viruses are mosquito-borne members under Flaviviridae family that cause dengue fever and its associated complications such as dengue shock syndrome and dengue haemorrhagic fever. This study focused on determining the inhibitory effect of methanolic extract of *Andrographis paniculata* and *Ocimum sanctum* towards DENV-1 using HepG2 cells. The maximum non-toxic dose (MNTD) of extract and median tissue culture infective dose (TCID50) of DENV-1 towards HepG2 cells were determined prior to antiviral assay. The antiviral activity was determined via degree of inhibition based on cytopathic effects (CPE), cells viability using MTT assay as well as plaque inhibition assay. Studies showed that HepG2 cells treated with *O. sanctum* extract at MNTD and ½MNTD possess inhibitory effects towards DENV-1. Significant level of DENV-1 inhibition based on CPE was observed in HepG2 cells treated with MNTD of *A. paniculata*. Nevertheless, significant inhibition was not reflected in cell viability and plaque inhibition assay, indicated that viral replication was not inhibited.

***Keywords*—** *Andrograhis paniculata*, antiviral, dengue, *Ocimum sanctum*

I. Introduction

DENGUE viruses (DENV) are categorized under Flaviviridae family, in which they carried single stranded RNA in its genome. DENV are transmitted via mosquito bite by several mosquito species within the genus Aedes, especially *Aedes aegypti* [1]. Once the viruses get into human body, it will be able to cause dengue fever which characterised by symptoms of headache, fever, joint paint, muscle pain, and skin rash. In some cases, life-threatening associated complications of the disease such as dengue haemorrhagic fever and dengue shock syndrome would be developed, resulting in bleeding, blood plasma leakage, and extremely low blood pressure [2]. The virus has four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. Usually, infection with one of the serotype will give life-long immunity to that particular serotype and short-term immunity to the other serotypes. However, severe complications might occur if the person is to be infected by different serotypes subsequently.

Dengue has become a global problem since many years ago. Every year, approximately 20 million cases of severe dengue diseases were reported in endemic countries such as South America, Southeast Asia and so on [3]. Many efforts have been initiated to develop an effective drug or vaccine to fight against dengue virus. Nevertheless, there is still no proven vaccine that can protect one against dengue virus infection as developing a vaccine that can provide immunity equally to all serotypes is one of the greatest challenge faced by the researchers. Scientists have attempted to combat dengue fever by developing genetically modified mosquitos that pass their genetic faults to their offspring and kill them at the larval stage of the life cycle [4]. Even though this method can reduce the mosquito population and limit the spread of dengue, it has a disadvantage of leaving great impact to the environment as eliminating the entire species of mosquito would lead to severe consequence to the animals that feed exclusively on it. Another favourable strategy is to develop a live vaccine [5]. Attenuation was obtained by repeated passage of dengue virus in cell culture as well as modern technique using genetic manipulation [6]. However, this approach has the difficulty in finding the optimum attenuation of the candidate vaccine strains, as the condition of virus attenuation in vitro does not appear to be predictive of attenuation in vivo [7].

In view of that, traditional medicines have been recommended and used widely in many countries. For example, Brazilian used cat's claw herb to treat dengue and Filipinos use 'tawa-tawa' herbs and sweet potato tops juice to increase the platelets counts. As in Malaysia, different types of natural medicines such as *Andrographis paniculata* and *Ocimum sanctum* are used. *O. sanctum*, also known as 'holy basil', is a valuable plant in curing and preventing disease such as cough, fever, ulcer etc. [8]. It is also believed to have antiviral properties as it has been commonly used in folk medicine to treat viral infection diseases especially dengue fever [9]. On the other hand, *A. paniculata* is a well-known plant that has been used in traditional Asian medicine for centuries and animal studies have shown that its extracts are biologically active. This plant contains compounds such as lactones, diterpenoids, diterpene glycosides, flavonoids and

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flavonoid glycosides [10]. Andrographolide, a diterpenoid lactone is one of the major compounds of *A. paniculata* and it was reported to have antiviral, anti-inflammatory and anticancer properties [11]-[13]. Besides, methanolic extract of *A. paniculata* was reported to be able to reduce the cytopathic effect of dengue 1 virus-infected Vero E06 cells, which were kidney epithelial cells derived from African Green Monkey [14]. Nevertheless, dengue viral replication has been reported to happen mostly in liver cells and causes hepatomegaly [15]. Hence, this study aimed to focus on the inhibitory effect of methanolic extract of *O.sanctum* and *A. paniculata* towards DENV-1 using human liver (HepG2) cell culture model.

II. Materials And Methods

A. Preparation, Extraction and Characterisation of Extracts

The methanol extract of *O. sanctum* and *A. paniculata* was prepared and characterised by Tang et al. [14]. The extracts were characterised to contain 88.6 ± 21.4% and 24.3 ± 3.0% of total flavonoids content, respectively. In preparation of the stock solution of *O. sanctum* and *A. paniculata*, 0.015g of the extract was weighted and dissolved in 220 µL and 350 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA), accordingly. The solution was further diluted with fresh culture medium until the desired concentration for the cytotoxicity and antiviral studies was achieved. Finally, the solution was filtered through 0.20 µm pore filter (Minisart, Germany) prior to addition into the cells.

B. Cell Culture

The HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, UK) with 10% Fetal Bovine Serum (FBS) (GIBCO, South America), penicillin (100 units/mL) and streptomycin (100 µg/mL) (GIBCO, South America). The cultures were incubated at 37oC with 5% CO_2 and allowed to grow until reaching 70%-100%.

C. Determination of Maximum Non-toxic Dose (MNTD)

MNTD was performed to determine the maximum concentration of methanolic extract that would not kill the cells. Firstly, 5 X 10^3 cells/well was seeded into 96-well flat-bottom plate (Corning, USA) and incubated at 37^{o}C with 5% CO_2 for 1-2 days until approximately 70% confluent. Then, two-fold serially diluted extract with the concentrations ranging from 0 to 1000 µg/mL were prepared and added into the cells. After incubated for 48 hours, 10µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt solution was added into each well. The plate was then further incubated for 4 hours. Following that, the solution was removed and 100 µL of DMSO (Sigma Aldrich, USA) was added into each well and mixed well. The absorbance reading was measured using microplate reader (Dynex,USA) at 570nm. Based on the absorbance reading obtained, the percentage of cell cytotoxicity was calculated. In order to determine the MNTD of the extract, a graph of percentage of cytotoxicity against extract concentration was plotted.

D. Determination of Median Tissue Culture Infective Dose ($TCID_{50}$)

The DENV-1 virus used in this study was kindly provided by Prof Shamala of Unviersiti Malaya. To determine $TCID_{50}$ of the virus stock, 5×10^3 cells/well was seeded into 96-well plate and incubated at 37°C with 5% CO_2 until 70% confluent. Then, 20 µL of two-fold serially diluted DENV-1 were added after the removal of used medium from the wells and the plate was further incubated in 5% CO_2 humidified incubator at 37^{o}C. The plate was gently shaken every 15 minutes up to 3 hours to maximise the viral adsorption to the cells. Then, 20 µL of fresh medium was added and the plate was incubated for 10 days. After 10 days post infection, cytophatic effects (CPE) of the cells were observed under inverted microscope (Motic AE31, USA) and the percentage of cell viability was determined through MTT assay.

E. Determination of Antiviral Activity

The procedures of antiviral assay was initiated by seeding 5×10^3 cells/well into 96-well plate and incubated at 37°C with 5% CO_2 until 70% confluent. After that, the used medium was removed and 100 µL extract at its MNTD or half MNTD was added. The plate was incubated for 1 hour and 20 µL of DENV-1 was then added to the wells. The plate was gently shaken for every 15 minutes up to 3 hours to maximise the viral adsorption to the cells. After 3 hours, 20 µL of fresh medium was added into each well and the plate was further incubated for 10 days. After 10 days, the degree of inhibition was determined based on the grading system through observing the CPE. The grading of CPE was carried out as described by Kudi and Myint [9], in which '++++' denoted total inhibition, '+++' denoted 75% inhibition, '++' denoted 50% inhibition, '+' denoted 25% inhibition, and '-' denoted no inhibition. In addition, the potency of the extracts on DENV-1 inhibition was also determined using MTT assay as described earlier. The percentage of cell viability in each treatment was then calculated.

F. Plaque Inhibition Assay

Plaque inhibition assay was also used to determine anti-DENV-1 activity of the extract. The process was initiated by plating 5 x 10^4cells/well into 24-well plate (Corning, USA) and incubated for 24 hours at 37^{o}C with 5% CO_2. At 70% confluency, medium was removed and plant extract at the concentration of ½MNTD was added. After incubating for 2 hours, 200µL of DENV-1 from virus stock was added. The plate was further incubated to allow virus adsorption for 3 hours at 37^{o}C with intermittent shaking every 15 minutes. Following that, 1% of methyl cellulose (Sigma Aldrich, USA) dissolved in 2X DMEM medium (with 20% FBS) was added and the plate was incubated for 10 days. The antiviral assay was conducted with the positive (cells treated with virus only) and negative (untreated cells) controls.

To visualise the plaque formation, the medium was carefully removed and the plaques were fixed with 3.7% formaldehyde (Friendemann Schmidt, Australia). After 30 minutes, 1% crystal violet solution (Sigma Aldrich, China)

was added. . The number of plaques formed was counted and the Plaque Forming Unit (PFU) per mL was determined.

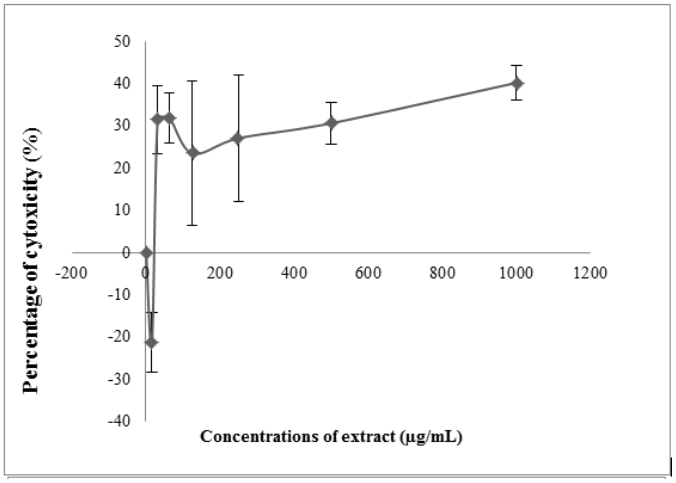
G.Statistical Analysis

The experiments were performed with five replicates and data was presented as mean ± standard deviation. One Way Analysis of Variance (ANOVA) was performed followed by post hoc Duncan Multiple Range Test (DMRT) using SPSS software to determine significant differences (p<0.05) between means.

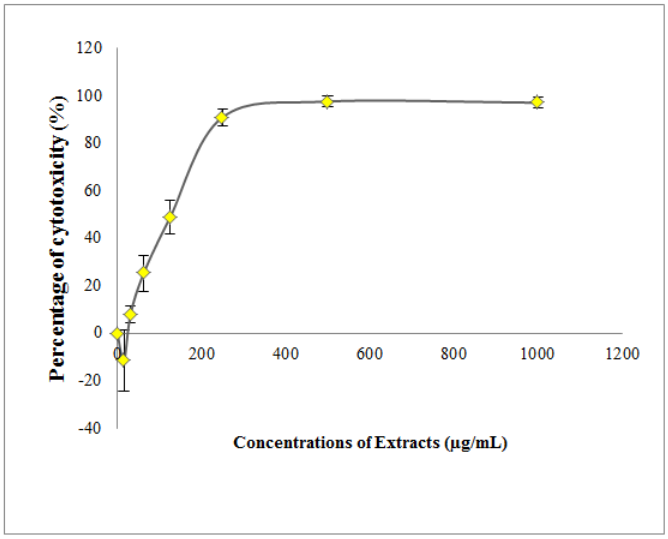
III. Results And Discussion

A. Determination of maximum non-toxic dose (MNTD)

MNTD was carried out to determine the maximum concentration of methanolic extract, which could be non-toxic to the cells. As shown in Fig. 1A and 1B, at the lowest tested concentration (15.625µg/mL) of both extracts, negative value was recorded in the percentage of cytotoxicity. This was followed by a rapid increase in cytotoxicity when the concentration of extracts was doubled. Based on the graphs constructed, the MNTD attained was 23.44 and 20.00 µg/mL for *O. santucm* and *A. paniculata*, respectively. The results obtained also exhibited that extract at concentrations below MNTD value did not show any cytotoxicty. In contrast, it enhanced the cells growth. This could be explained by the anti-oxidant properties of the flavonoid presented in the methanolic extracts. Flavonoid can protect the cells by forming bond with free radicals which able to cause cells abnormalities and cells death, converting them into a more stable form and less reactive molecules [16]. On the other hand, another possible route that the flavonoid acts on to protect the cells is through interaction with various enzyme systems. An event called lipid peroxidation, which resulting in cell damage could happen when free radicals species are in the presence of iron. Flavonoid compound could stop this dreadful event from happening as it is known to chelate iron, thereby removing a vital factor for the development of free radicals [17]. However, despite low concentration of flavonoid can protect the cells from free radicals, high concentration of flavonoid could generate reactive oxygen species by autoxidation and redox-cycling, inducing cell apoptosis as well as DNA damage [18]. This could possibly explain the reason why the percentage of cytotoxicity increased as the concentration of extract increased in this study. According to Tang et al. [14], the MNTD of methanolic extract of *O. sanctum* and *A. paniculata* on Vero E6 cells was 0.10 and 0.05 mg/mL, respectively. However, the MNTD value obtained from this study was 0.023 mg/mL for *O. sanctum* and 0.020 mg/mL for *A. paniculata*, values that were much lower than those reported in Vero E6 cells. This could indicate that both extracts are more cytotoxic towards HepG2 cells as compared to Vero E6 cells.



(A)



(B)

Fig. 1 The percentage of cytotoxicity of methanolic extract of (A) *Ocimum sanctum* and (B) *Andrographis paniculata* on HepG2 cells after incubated in vitro for 48 hours.

B. Determination of Median Tissue Culture Infective Dose ($TCID_{50}$)

$TCID_{50}$ was carried out to determine the concentration of DENV-1 that would induce cytopathologic change in 50% of the inoculated HepG-2 cells. The study was conducted by determining the percentage of cell viability after 10 days of infection with DENV-1. The cells treated with undiluted virus stock showed various form of CPE. However, significant CPE could not be observed when the cells were treated with two-fold diluted virus stock. From the observation, it was clearly shown that DENV-1 infected cells would undergo structural changes, whereby several types of CPE included syncytia and blebbing could be seen under the microscope. Syncytia formation refers to the condition of viral protein that mediate fusion of the infected cell with neighboring cells, resulting in multinucleated enlarged cells. On the other hand, blebbing refers to the small detached remnants of apoptotic bodies in the cell plasma membrane. In addition, upon infecting the HepG2 cells with DENV-1, cell lysis could also be seen as opposed to the epithelial-like normal HepG2 cells that contain

a clearly demarcated cell membranes.

To further confirm the findings based on CPE, the percentage of cell viability for each two-fold serially diluted virus stock was also determined through MTT assay. From the results obtained, both studies recorded similar observation, in which $TCID_{50}$ of the virus stock could not be determined as at two-fold dilution, percentage of cell viability as high as 99% was achieved (Table 1). This could be due to the low amount of DENV-1 present in the virus stock. Furthermore, the thawing process could also have some negative impacts on the stability of the viruses. As such, for further antiviral assay, initial virus stock without any dilution was used.

TABLE I
THE PERCENTAGE OF CELL VIABILITY OF HEPG-2 CELLS UPON INFECTION WITH DIFFERENT DILUTIONS OF DENV-1 STOCK CULTURE AFTER INCUBATED *IN VITRO* FOR 10 DAYS.

Dilutions (times)	Cell viability (%)
0	70.2 ± 6.6*
2	99.4 ± 0.2
4	109.1 ± 11.7
8	110.3 ± 12.7
16	107.2 ± 8.0
Negative control (untreated cells)	100 ± 0.0

The data shown are mean ± S.D. '*' denotes significant difference at $P<0.05$, compared to the negative control using ANOVA followed by post hoc Duncan Multiple Range Test (DMRT), performed by SPSS software.

C. Determination of Antiviral Activity of O. sanctum

Antiviral assay is a vital step to test the ability of the plant extract to inhibit the virus replication in the cell culture. In the present study, antiviral assay was performed by grading the inhibition effects of plant extract on CPE as well as the percentage of cell viability. Studies revealed that DENV-1-infected HepG2 cells treated with *O. sanctum* at both MNTD and ½MNTD brought about 75% of CPE inhibition (+++) as compared to less than 50% inhibition (+) in the positive control (Table 2).

TABLE II
ANTIVIRAL ASSAY UPON TREATING THE DENV1-INFECTED HEPG-2 CELLS WITH METHANOL EXTRACT OF *OCIMUM SANCTUM*

Treatments	Degree of inhibition	Cell viability (%)
Negative control (untreated cells)	++++	100.00 ± 0.00*
Positive control (cells infected with DENV-1)	+	49.59 ± 3.50
MNTD + virus	+++	64.29 ± 5.95*
½ MNTD+ virus	+++	68.67 ± 11.36*

++++ 100% inhibition, +++ 75% inhibition, ++ 50% inhibition, + <50% inhibition, - no inhibition
The data shown are mean ± S.D. '*' denotes significant difference at $P<0.05$, compared to the negative control within the same column using ANOVA followed by post hoc Duncan Multiple Range Test (DMRT), performed by SPSS software.

These findings were further proven through the determination of percentage of cell viability. Through MTT assay, the negative control (uninfected cells) and positive control (HepG-2 cells infected with virus) recorded a total of 100% and 49.59% of cell viability, respectively. However, upon treating the DENV-1 infected cells with O. sanctum at MNTD and ½MNTD, an increase in the percentage of cell viability was observed. For these two treatments, the percentage of cell viability recorded was 64.29% and 68.67%, respectively. These values were significantly different from the positive control, which indicated that methanol extract of *O. sanctum* did possess DENV-1 inhibitory activity.

The anti-dengue properties of *O. sanctum* extract was further examined using plaque inhibition assay and the results were shown in Table 3. A minimum amount of plaques were seen in both untreated and ½MNTD-treated cells. Plaques were formed in both DENV-1 infected cells and ½MNTD treated cells, showing empty spaces between the cells. The *O. sanctum* extract at ½MNTD showed significant plaque inhibition compared to the positive control. With the presence of ½MNTD, it only showed a total of 157.5 ± 67.6 PFU/mL, which was much lower than 1020.0 ± 271.0 PFU/mL recorded in the positive control. However, it was higher than both negative control and ½MNTD control, which were 6.3 ± 7.5 and 58.8 ± 45.2 PFU/mL, respectively.

TABLE III
PLAQUE FORMING UNIT PER SAMPLE (PFU/ML) IN DENV1-IFECTED HEPG2 CELLS TREATED WITH *O. SANCTUM* AND *A. PANICULATA*

Treatments	*O. sanctum*	*A. paniculata*
Negative control (untreated cells)	6.3 ± 7.5*	23.3 ± 27.5*
Positive control (cells infected with DENV-1)	1020.0 ± 271.0	78.3 ± 2.9
½ MNTD	58.8 ± 45.2*	13.3 ± 10.4*
½ MNTD+ virus	157.5± 67.6*	48.3± 2.9

The data shown are mean ± S.D. '*' denotes significant difference at $P<0.05$, compared to the negative control within the same column using ANOVA followed by post hoc Duncan Multiple Range Test (DMRT), performed by SPSS software.

The antiviral assays used in this study showed that methanol extract of *O. sanctum* possess inhibitory properties towards DENV-1. This could be related to the flavonoids present in the extract. *O. sanctum* has been found to content flavonoids such as orientin and vicenin. According to previous study, orientin extracted from *Trollius chinensis* Bunge possessed antiviral activity against para-influenza type 3 virus [19]. Besides, result from previous study also shown that vicenin extracted from *Urtica circularis* possessed significant anti-inflammatory activity [20]. Furthermore, both orientin and vicenin also known to have radical scavenging activity [21]. There are several pathways that could be the mode of action of anti-DENV1 by *O. sanctum*. Dengue virus E glycoprotein protein has been reported to play an important role in cell membrane attachment [22]. Hence, there is possibility that the *O. sanctum* extract blocked the E protein of DENV-1, preventing it from entering the cells. Apart from that, antiviral could have happened when the reverse transcriptase of virus was affected. Previous study proved that

flavonoid compound could cause some inhibitory effects on the reverse transcriptase of the virus [23]. As such, it is possible that the flavonoid compounds in methanol extract of *O. sanctum* could have interfered the reverse transcriptase of DENV-1. Zandi et al. [24] suggested that fisetin, a flavonol that possess anti-DENV2 property did not affect DENV-2 binding to the cells but rather affected the DENV genome copy number. Thus, the possible route of inhibition could be via interfering DENV genome copy number by binding directly to virus RNA, forming flavonoid-RNA complex [25].

D. Determination of Antiviral Activity of A. paniculata

In vitro antiviral assay was also conducted in order to evaluate the anti-dengue properties of methanolic extract of *A. paniculata*. Based on the CPE denoted by degree of inhibition in Table 4, the negative control recorded a total inhibition without showing any sign of CPE whereas for positive control, cells showed a total inhibition of less than 50%. Furthermore, the degree of inhibition for both MNTD+virus and ½MNTD+virus was 50% and less than 50%, respectively. These data revealed that methanolic extract of *A. paniculata* at MNTD could possess the anti-DENV1 property. Nevertheless, insignificant findings were obtained when the DENV-1 infected cells were subjected to cell viability assay. Based on the results presented in Table 4, the negative control cells which were not infected with DENV-1, showed 100% of cell viability. In contrast, the positive control that referred to infected cells recorded cell viability of 61.3±5.1%. The cells viability for treatment using MNTD and ½MNTD was 77.1± 20.9% and 71.9± 15.5%, respectively. Although the inclusion of extract either at MNTD or ½MNTD managed to increase the cell viability of the cells as compared to the positive control, no significant difference was recorded at $p < 0.05$.

TABLE IV
ANTIVIRAL ASSAY UPON TREATING THE DENV1-INFECTED HEPG-2 CELLS WITH METHANOL EXTRACT OF *ANDROGRAPHIS PANICULATA*

Treatments	Degree of inhibition	Cell viability (%)
Negative control (untreated cells)	++++	100.0± 0.0*
Positive control (cells infected with DENV-1)	+	61.3±5.1
MNTD + virus	++	77.1± 20.9
½ MNTD+ virus	+	71.9± 15.5

++++ 100% inhibition, +++ 75% inhibition, ++ 50% inhibition, + <50% inhibition, - no inhibition
The data shown are mean ± S.D. '*' denotes significant difference at $P<0.05$, compared to the negative control within the same column using ANOVA followed by post hoc Duncan Multiple Range Test (DMRT), performed by SPSS software.

As for plaque inhibition assay, the PFU for negative control was as low as 23.3 while the PFU value for positive control (virus alone) and cells treated with ½MNTD alone was 78.3 and 13.3, accordingly (Table 3). A very low amount of plaques were seen in the negative control while a large number of plaques were formed in the positive control. Nevertheless, when DENV1-infected cells were treated with methanol extract of *A. paniculata* at ½MNTD, PFU was reduced to 48.3. However, this value did not show any significant difference from the positive control at $p<0.05$. This could be due to the fact that extract at ½MNTD was unable to inhibit the viral replication in HepG2 cells effectively. The variation in the findings based on CPE, cell viability and plaque inhibition assay could be due to the fact that *A. paniculata* extract was able to inhibit the CPE, but it was unable to inhibit the virus replication in HepG2 cells. The RNA structure of the DENV genome helps to modulate viral replication [26]. Thus, it could be concluded that the compounds presence in the methanol extract of *A. paniculata* could not destroyed the RNA structure of the DENV genome and hence did not significantly affect DENV replication.

Various flavonoid compounds have been reported to possess antiviral properties. Nevertheless, none of the flavonoid compounds found in *A. paniculate* such as 7-O-methylwogonin, apigenin, onysilin and 3,4-dicaffeoylquinic acid has been reported to possess anti-DENV1 properties. Thus, it is postulated that the anti-DENV-1 activities determined in this study could be due to the presence of diterpneoid compounds. Diterpenoid is well known compound to treat some virus and bacterial infection. In this plant extract, it contains the major diterpenoid known as andrographolide [27]-[29]. Andrographolide had been reported to possess anti-HIV activity when the aqueous extract of this plant was used to treat HIV infected H9 cell line [30]. In addition, this plant extract also contain, neoandrographolide and 14-deoxy- 11,12-didehydroandrographolide which were reported to inhibit herpes simplex virus 1 (HSV-1) [31]. Furthermore, the *A. paniculata* ethanol extract and andrographolide inhibited the expression of Epstein-Barr virus (EBV) lytic proteins during the viral lytic cycle in P3HR1 cells, an oral lymphoma cell line latently infected by EBV. Not only that, this component was found to inhibit HIV by interfering with HIV-induced cell fusion and with HIV's binding to the cell [32]. There is a possibility that a similar mechanism may apply to this plant extract towards DENV-1. In dengue infection, DENV-1 releases its E protein which plays an important role in cell membrane adhesion. Andrographile may bind or block the E protein and cause failure of membrane adhesion by DENV-1 [33].

IV. CONCLUSIONS

In conclusion, methanolic extract of *O. sanctum* exhibited antiviral properties towards DENV-1 through inhibition of CPE formation as well as viral replication. Meanwhile, the methanolic extract of *A. paniculata* at its MNTD possess anti-dengue 1 inhibitory activity only through inhibition of CPE formation rather than viral replication. Further investigation should be conducted to figure out the potential compounds presented in these two extracts that lead to its anti-DENV1 properties. Furthermore, studies should be done extensively to determine the mode of antiviral actions of *O. sanctum* and *A. paniculata* to provide more insight into inhibition of dengue virus.

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