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Isolation and Structure Elucidation of Potential Anti-Dengue Metabolites from Tawa-tawa (*Euphorbia hirta* Linn.)

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Abstract

Many natural products found in plants exhibit a variety of biological activities including antibiotic, antitumor, and antiviral activities that have been utilized in the medicinal field to cure millions of people from serious diseases. *Euphorbia hirta* Linn. herb, a folkloric medicinal plant in the Philippines against dengue is reportedly effective against the dengue virus. This deadly and painful mosquito-born disease continuously affects millions of people worldwide for there are no commercially developed drugs or vaccines available. From the viewpoint of overcoming the dengue disease, the authors investigated the chemical components of *Euphorbia hirta* Linn. Preliminary anti-dengue assay by Plaque Reduction Neutralization Test (PRNT) revealed that the ethyl acetate layer was the most active fraction and significantly reduced the plaque forming capacity of the dengue virus serotypes 1 and 2 relative to the control. Further purification of the active ethyl acetate fraction afforded 6 known triterpene and 3 flavonoid compounds. The planar structures of these molecules were established by ESIMS and NMR spectroscopic analyses.

Keywords: Tawa-tawa, nuclear magnetic resonance, structure elucidation, chromatography, dengue

Introduction

Natural products produced by living organisms that show biological action have been developed as powerful drugs to combat diseases and to save the lives of millions. Newman and Cragg [1] further demonstrated that natural products were still a significant source of new drugs especially in the anticancer therapeutic areas. These facts have led many scientists all over the world to explore useful and fascinating natural products from living things such as plants, animals and microorganisms.

The dengue virus causes dengue fever which is also known as break-bone fever because of the severity of pain that will be experienced by the infected person. In tropical areas where *Aedes aegypti* mosquito live, this disease is very serious and responsible for more than 50 million annual cases of dengue infection worldwide. This will sometimes lead to dengue haemorrhagic fever (DHF), a potentially fatal complication. The World Health Organization (WHO) reported that around 2.5 billion people (*i.e.*, two fifths of the world's population) are now at risk from dengue.

One such potential natural product source is *Euphorbia hirta* Linn., locally known as tawa-tawa or gatas-gatas in the Philippines. The Philippines has plenty and diverse medicinal plants that people are using especially in rural areas for curing common diseases. The skyrocketing prices of over-the-counter drugs have further made the herbal plants not only as an alternative medicine but a popular choice both against seasonal and year-round diseases. The tawa-tawa plant is relatively easy to use: the plant is

harvested whole at its flowering stage, boiled in water for about 15 min and then the decoction is given to the patient as tea [2]. Several scientific investigations have reported the biological activities of *Euphorbia hirta* Linn. These include antibacterial and toxicological potential [3], diuretic [4], anti-allergic property [5], anthelmintic [6], angiotensin converting enzyme (ACE) inhibitor [7], behavioral and neurotropic effects [8], antidiarrhoeic [9], antifungal [10], antimalarial [11], antioxidant, anti-proliferative, bronchodilatory activity [12], antidiabetic [13], cytotoxic activity on HEP-2 cells [14], cartilage degeneration in arthritic rats [15], anti-inflammatory [16], anti-tumor [17], and anti-viral activity [18]. However, no one has yet reported its efficacy against either dengue or the Dengue Hemorrhagic Fever (DHF). Tawa-tawa is a traditional herb and abundant in open grasslands that is claimed to be effective against dengue. However, there is no direct evidence yet to support this claim. Thus, this study attempts to isolate the secondary metabolites and evaluate the effectiveness of tawa-tawa extracts against the dengue virus. Because there is no existing approved drug and vaccine against dengue, the results that will be generated by this study may provide an excellent breakthrough and reference for further anti-dengue and other anti-microbial drug development of the tawa-tawa herb.

Although, the authors were not able to isolate novel compounds, the purification afforded 6 known triterpenes and 3 known flavonoids from *Euphorbia hirta* Linn. by Thin Layer Chromatography (TLC) and anti-dengue assay-guided fractionation.

Materials and methods

About 2.0 kg of *Euphorbia hirta* Linn. herb were collected, cut and air-dried. The dried plant was then soaked for 48 h in 6.0 L of a 50 % EtOH/MeOH solution. The extract was then filtered, concentrated *in vacuo*, and partitioned with EtOAc/H_2O. A small portion (100 mL) of both organic (EtOAc or ethyl acetate) and water fractions were concentrated *in vacuo* and saved for the anti-viral test. A decoction was also prepared as tea by boiling *ca*. 20 g of dried sample with 300 mL water for 15 min. The tea sample was allowed to cool, filtered, concentrated *in vacuo* and stored for the anti-dengue test. These samples (EtOAc, water layer, and tea) were sent to the Biotechnology and Research Division of St. Luke's Medical Center, Philippines for its anti-dengue assay. Purification was then focused on the active fraction to further isolate its secondary metabolites by silica (SiO_2) gel column chromatography. The active organic layer was then divided into two fractions, Fr A (eluted by 30 % hexane/EtOAc) and Fr B (eluted by 100 % EtOAc). Further SiO_2 gel chromatography of fraction A, afforded **1**, Fr A1 and Fr A2. Compound **1** is white, partially soluble in hexane and has an R_f value of 0.62 in TLC (20 % EtOAc/hexane). The APCIMS spectrum of **1** showed a protonated molecular ion signal at *m/z* 425.3755, indicating its molecular formula as $C_{30}H_{48}O$. Purification of Fr A1 that then followed using ODS (MeOH/H_2O) (Yamazen Corp. Ultra Pack ODS-SM, 50 μm, Size B 26 × 300 μm) yielded **2**, **3** and **4**. Compound **2** is white powder and has an R_f value of 0.43 in TLC (20 % EtOAc/hexane). The APCIMS spectrum showed *m/z* 409.3800 $[M+H-H_2O]^+$, calcd for $C_{30}H_{49}$: 409.3829 which is consistent with its molecular formula as $C_{30}H_{50}O$. Colorless compound **3** has an R_f value similar to **2** under the same conditions. The APCIMS spectrum exhibited a protonated molecular ion peak at *m/z* 427.3928 which is consistent with the molecular formula $C_{30}H_{50}O$. Compound **4** has an R_f value of 0.43 in TLC (20 % EtOAc/hexane) and showed a protonated molecular ion peak at *m/z* 413.3760 which corresponds to the molecular formula $C_{29}H_{48}O$. Further purification of Fr A2 via SiO_2 gel chromatography also gave compounds **5** and **6**. Compound **5** was obtained as white powder and has an R_f value of 0.43 (TLC, 20 % EtOAc/hexane). The APCIMS spectrum showed *m/z* 409.3820 $[M+H-H_2O]^+$, calcd for $C_{30}H_{49}$: 409.3829, indicating the molecular formula as $C_{30}H_{50}O$. Compound **6** was isolated as white powder, partially soluble in methanol and has an R_f value of 0.29 in TLC (20 % EtOAc/hexane). The APCIMS spectrum showed *m/z* 397.3803 $[M+H-H_2O]^+$, calcd for $C_{29}H_{49}$: 397.3829 and consistent with its molecular formula as $C_{29}H_{50}O$. Silica gel column chromatography of the polar Fr B followed by preparative TLC employing 100 % EtOAc developing solvent afforded the flavonoids **7**, **8** and **9**. Compound **7** has an R_f value of 0.6 in TLC ($CHCl_3$/EtOAc/MeOH 3:1:1). The ESIMS spectrum showed *m/z* 441.0780 $[M+Na]^+$, calcd for $C_{20}H_{18}O_{10}Na$: 441.0792 which is consistent with the molecular formula $C_{20}H_{18}O_{10}$. Compound **8** has an R_f value of 0.5 in TLC ($CHCl_3$/EtOAc/MeOH 3:1:1) and the ESIMS spectrum showed a protonated molecular ion signal at *m/z* 433.1103, indicating its molecular formula as $C_{21}H_{20}O_{10}$. Compound **9** has an

R_f value of 0.4 in TLC ($CHCl_3$/EtOAc/MeOH 3:1:1). The ESIMS spectrum of **9** showed a protonated molecular ion signal at *m/z* 449.1071, indicating its molecular formula as $C_{21}H_{20}O_{11}$.

The structure elucidation of the isolated compounds was done through Nuclear Magnetic Resonance (NMR) Spectroscopy. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in $CDCl_3$ (δ_H 7.24 ppm), CD_3OD (δ_H 3.30 ppm) and acetone-d_6 (δ_H 2.04 ppm) using a JEOL JNM-ECA500 spectrometer. Other structural determination techniques like Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Multiple Bond Coherence (HMBC) and Nuclear Overhauser Effect Spectroscopy (NOESY) were also performed following standard protocols. Splitting patterns are designated as *s* (singlet), *d* (doublet), *dd* (doublet of doublet), *t* (triplet), *q* (quartet), *m* (multiplet), and *br* (broad). The HRESI/MS spectrum was obtained by a HITACHI NanoFrontier LD spectrometer. Measurements of Infrared (IR) spectra were performed with a HORIBA FT-720 spectrometer. DENV1 99St-012 (160EIAu) and DENV2 were independently pre-incubated with the test extracts of varying concentrations (100, 50, 25, and 12.5 μg/mL) for 1 h. Vero cells were harvested and plated on muti-well plates at 2.5 × 105 cells/mL. Plates were infected for 3 h, after which plates were overlaid with semi-solid media (2× MEM with 1.5 % methylcellulose) and were incubated at 37 °C, 5 % CO_2 for 5 days. Cells on plates were fixed with 10 % formaldehyde and stained with 1 % crystal violet. Plaques were then counted and viral inhibition was determined relative to the controls.

Results and discussion

Interestingly, the EtOAc fraction showed the strongest anti-dengue activity as shown in **Figures 1** and **2**. The EtOAc fraction significantly reduced (85 %) the plaque forming capacity of dengue virus serotype 1 from ~1400 to ~200 PFU (**Figure 1**). Consistently, the EtOAc fraction also showed remarkable effects on dengue virus serotype 2 (~90 % reduction) (**Figure 2**). This means that the tawa-tawa extract can neutralize the dengue virus and the compound/s responsible for it is/are found in the organic layer. Because of these promising initial *in vitro* anti-viral test results, purification was focused on the said fraction. The remaining organic or ethyl acetate (EtOAc) fraction was then concentrated *in vacuo* and the residue was sequentially partitioned over silica gel column chromatography to afford **1** (49.0 mg), **2** (9.0 mg), **3** (11.0 mg), **4** (2.0 mg), **5** (10.0 mg), **6** (30.0 mg), **7** (2.0 mg), **8** (4.0 mg), and **9** (27.0 mg) as shown in **Figure 3**.

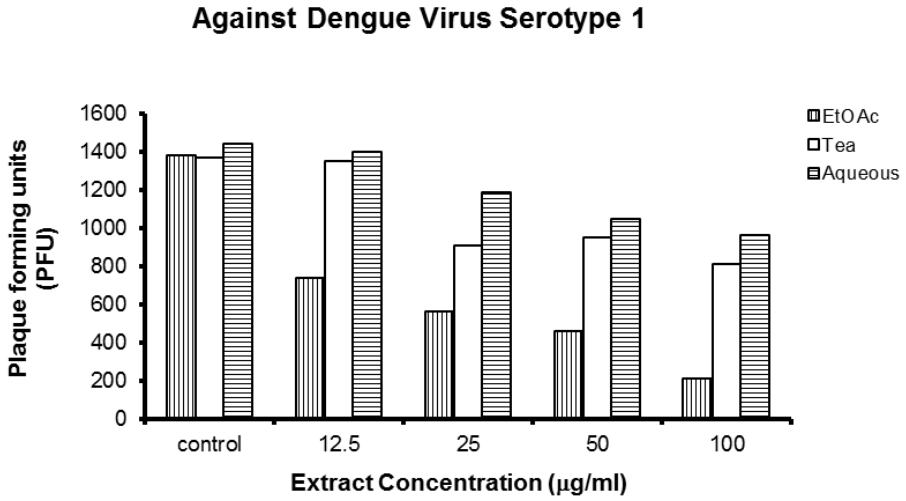

Figure 1 Effects of tawa-tawa etracts on dengue serotype 1.

The first metabolite that was isolated is **1**. The appearance of a double doublet proton signal at 5.49 ppm (^{1}H NMR) and carbon resonances (^{13}C NMR) at 217.6, 157.6, and 117.2 ppm suggested the presence of a ketone and an olefinic functionality. Moreover, 8 singlet methyls were also observed in the ^{1}H NMR spectrum of **1**. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments support the foregoing discussion and establish the planar structure of **1** depicted in **Figure 3**. The stereochemistry of these methyl groups were confirmed by NOE experiments as shown in the Supplementary Data. The ^{1}H and ^{13}C NMR data of this pentacyclic triterpene were identical to those of the literature values [19]. Thus, **1** was identified as taraxerone.

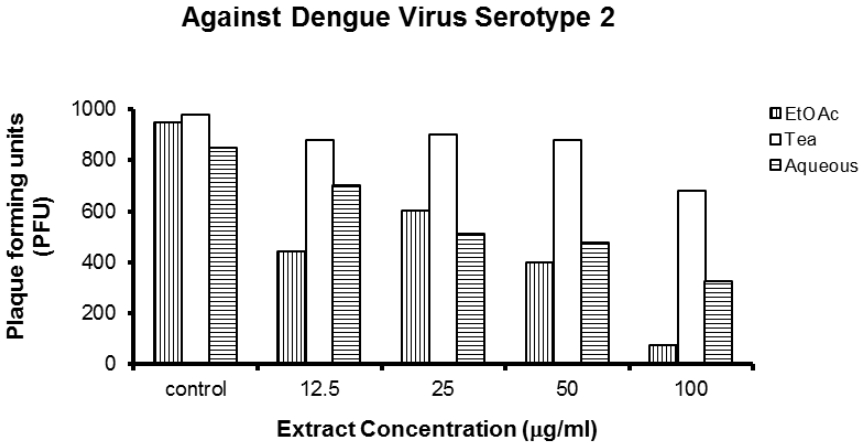

Figure 2 Effects of tawa-tawa extracts on dengue serotype 2.

The ^{1}H NMR spectrum of **2** showed signals for the 6 singlet methyl groups at δ 0.76, 0.79, 0.83, 0.94, 0.97, and 1.03 ppm. It further displayed an isopropenyl side chain with signals at δ 1.68 (3H, *s*), 4.57 (1H, *dd*, *J* = 2.2, 1.1 Hz) and 4.69 (1H, *d*, *J* = 2.2 Hz) manifesting its relationship with the lupane-type of triterpenoids. The oxygenated methine signal at 3.19 ppm (1H, *dd*, *J* = 11.4, 4.8 Hz) as well as its carbon resonance at 79.0 ppm indicated the presence of an alcohol moiety. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments also supported the above conclusions to establish its planar structure depicted in **Figure 3**. A literature search revealed that the ^{1}H and ^{13}C NMR data of **2** were identical to those of lupeol [20].

The ^{1}H NMR spectrum of **3** in $CDCl_3$ showed a pair of very upfield doublets at 0.56 and 0.33 ppm (1H each, *d* both, *J* = 4.2 Hz), which corresponds to the cyclopropyl methylene group of a cycloartane-type of triterpene. The slightly upfield olefinic proton signal at 5.10 ppm (1H, *m*) and 2 singlet methyls at 1.68 (3H, *s*) and 1.61 (3H, *s*) ppm was characterized by the terminal dimethylvinyl group in the side chain. The oxygenated methine signal at 3.28 ppm (1H, *m*) as well as its carbon resonance at 78.9 ppm indicated the presence of an alcohol moeity of **3**. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments also support the foregoing discussions to establish its planar structure depicted in **Figure 3**. The ^{1}H and ^{13}C NMR data were identical to those of cycloartenol [21].

A 28-nor oleanane derivative [22] (**4**) was next isolated in a similar fashion described for **2** and **3**. The ^{1}H NMR spectrum of **4** showed signals for the 7 singlet methyl groups at δ 0.79, 0.80, 0.91, 0.96, 1.00, 1.01, and 1.07 ppm which indicated a triterpene type of compound. It further showed an olefinic proton at 5.13 ppm (1H, *dd*, *J* = 4.4, 4.4 Hz) and an alcoholic proton at 3.23 ppm (1H, *dd*, *J* = 10.0, 5.0 Hz). HMQC result suggested that these protons were connected to 124.4 and 79.1 ppm carbons, respectively for the olefinic and oxygenated protons. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments also supported the foregoing discussions and elucidated its planar structure as shown in **Figure 3**. This compound is a C-17 decarboxylated derivative of oleanolic acid [23,24].

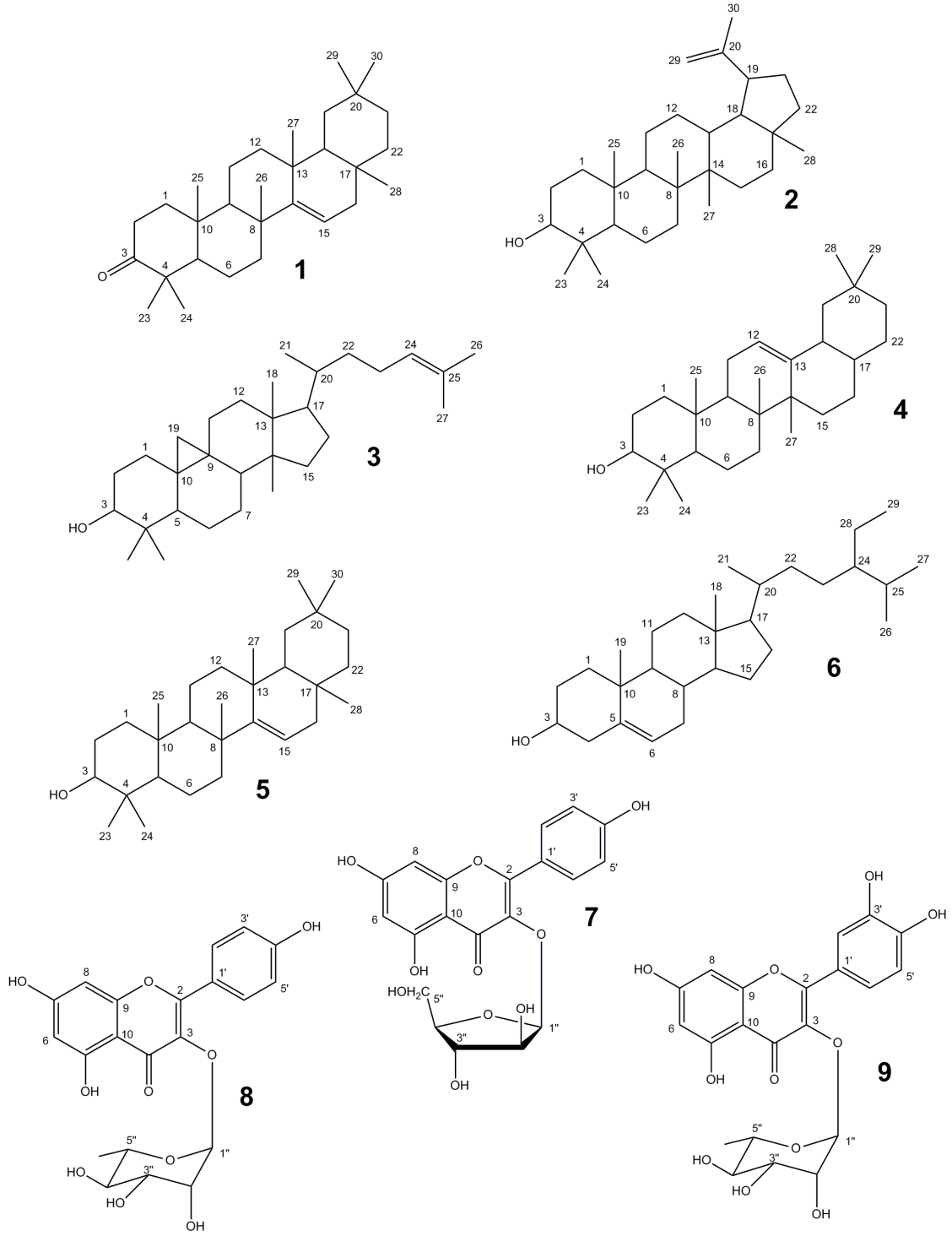

Figure 3 Secondary metabolites isolated from *Euphorbia hirta* Linn.

Interestingly, the ^{1}H and ^{13}C NMR spectrum of **5** is almost identical to **1** except that an oxygenated proton appeared at 3.18 ppm indicating that **5** is a reduced form of **1**. This was corroborated by the presence of a carbonyl ketone and an oxy-carbon signals at 217.55 and 79.07 ppm, respectively for **1** and **5** in the ^{13}C NMR experiments. Compound **5** is 2 hydrogen atoms larger than that of **1**, which is consistent with the above discussions. Thus, **5** is an alcohol derivative of **1**. Further 2D NMR (COSY, DEPT, HMQC and HMBC) experiments also supported the foregoing discussions to establish its planar structure depicted in **Figure 3** while the stereochemistry of these methyl groups were confirmed by NOE experiments as shown in the Supplementary Data. The ^{1}H and ^{13}C NMR data were identical to those of the literature values [19,25] and found to be taraxerol.

Compound **6** has an alcoholic proton (3.53 ppm), an olefinic proton (5.36 ppm), 2 singlet methyls (0.68 and 1.01 ppm), 3 doublet methyls (0.81, 0.84, and 0.92 ppm), and one triplet methyl group at 0.85 ppm which are indications of a triterpene type. Since the ^{1}H and ^{13}C NMR data were identical to those of the literature values, **6** was elucidated as β-sitosterol [26,27] as shown in **Figure 3**.

We then investigated the most polar fraction (100 % EtOAc). After a series of purifications (silica gel column and Preparative TLC), the extract afforded 3 flavonoid-type of compounds. Among them, **7** was first identified as 3-*O*-arabinofuranoside of kaempferol. The aromatic region of the ^{1}H NMR spectrum in acetone-d_6 showed an AA'XX' system at δ 8.04 (H-2' and H-6') and δ 7.01 (H-3' and H-5'). An AX system was also detected at δ 6.46 (H-8) and δ 6.24 (H-6) which is in accordance with a kaempferol derivative (**Figure 3**). Since the HMBC spectrum showed correlation signals such as H-2'/C-4', H-2'/C-2, H-6'/C-4', H-6'/C-2, H-3'/C-1', H-5'/C-1', H-8/C-6, and H-6/C-10, it then established the connectivity of the aglycone moiety. The sugar protons were assigned by COSY analyses. The downfield shift of C-4" at 89.8 ppm suggested the presence of a furanose sugar according to Bock and Pedersen [28]. The NOE correlations detected at H-2" (4.26 ppm) of the sugar moeity to H-1"(5.42 ppm) further indicated that the sugar unit was a β-D-arabinofuranosyl as shown in the Supplementary Data. Moreover, the presence of an HMBC correlation from an anomeric proton δ 5.42 (H-1") to 89.8 ppm (C-4") also corroborated the existence of a furan ring. Though, H-1" did not show an HMBC correlation to C-3 to establish the sugar-aglycone linkage, irradiation of this proton by NOE did provide correlation to H-2' and H-6'. This NOE pattern was also observed for both **8** and **9** that have C-1"-C-3 linkages. Subsequently, when H-2' and H-6' were irradiated, NOE correlations were detected as well for H-1", H-3', and H-5' to confirm the C-1"-C-3 bridge. The presence of arabinofuranosyl as a sugar moiety was further confirmed due to similar GC profiles of derived peracetate of authentic D-arabinose and those of the natural product after acidic hydrolysis and subsequent acetylation of **7** with acetic anhydride in pyridine as shown in the Supplementary Data.

Compound **8** was next identified as 3-*O*-rhamnopyranoside of kaempferol. The aromatic region of the ^{1}H NMR spectrum in acetone-d_6 showed an AA'XX' system at δ 7.85 (H-2' and H-6') and δ 7.01 (H-3' and H-5'). An AX system was also detected at δ 6.47 (H-8) and 6.27 (H-6) which is in accordance with a kaempferol derivative (**Figure 3**). The HMBC spectrum showed correlation signals at H-2'/C-2, H-2'/C-4', H-6'/C-2, H-6'/C-4', H-3'/C-1', H-3'/C-4', H-5'/C-1', H-5'/C-4', H-8/C-6, H-8/C-10, H-8/C-9, H-8/C-7, H-6/C-8, H-6/C-10, and H-6/C-7 which established the assignment of the quaternary carbons of the aglycone moeity. The sugar protons were then assigned by COSY and coupling constant analyses. The doublet methyl at 1.01 ppm (J = 5.5 Hz) in CD_3OD is typical for rhamnose H_3-6" methyl protons [29]. The HMBC correlation at δ 5.53 (H-1") to 71.3 ppm (C-5") also supported the above discussions (Supplementary Data). Thus, the sugar unit was identified as β-L-rhamnopyranosyl. Fortunately, the crosspeak in the HMBC experiment at δ 5.53 (H-1") to 135.7 ppm (C-3) between the anomeric rhamnosyl proton and C-3 of the aglycone was very clear, an indication that the sugar part was linked to the C-3 position of aglycone. Similar with **7**, NOE correlations were also detected at H-2'/H-1" and H-6'/H-1" to corroborate the discussions above. Furthermore, the ^{1}H NMR spectra of **8** in CD_3OD were identical to the literature values reported by Fossen *et al*. [29].

Lastly, **9** was identified as 3-*O*-rhamnopyranoside of quercetin. The aromatic region of the ^{1}H NMR spectrum in acetone-d_6 showed an ABX system at δ 7.38 (H-6'), 7.50 (H-2'), and 6.96 (H-5'), and a 2H AX system at δ 6.46 (H-8) and 6.25 (H-6) which is in accordance with a quercetin-type (**Figure 3**). The

HMBC spectrum showed correlation signals at H-6'/C-2', H-6'/C-3', H-6'/C-2, H-2'/C-1', H-2'/C-4', H-2'/C-3', H-2'/C-2, H-5'/C-2', H-5'/C-1', H-5'/C-4', H-5'/C-3', H-8/C-6, H-8/C-10, H-8/C-9, H-8/C-7, and H-8/C-4 which established the assignment of quaternary carbons of the aglycone moeity. The sugar protons were also assigned by COSY and coupling constant analyses. Further homodecoupling of δ 0.90 methyl (H_3-6") changed the signal pattern of the vicinal δ 3.42 proton (H-5") from a multiplet to a doublet (J = 9.5 Hz). Since H-4" showed coupling constants of 9.5 and 9.3 Hz, respectively for H-5" and H-3", therefore, the axial configurations of H-3", H-4", and H-5" oxymethines were established. The doublet methyl at 0.90 ppm (J = 6.1 Hz) is characteristic of rhamnose H_3-6" protons and the HMBC correlation for δ 5.51 (H-1") to 71.3 ppm (C-5") also corroborated the above discussions. Thus, the sugar unit was identified as β-L-rhamnopyranosyl. The crosspeak in HMBC experiment at δ 5.51 (H-1") to 135.7 ppm (C-3) between the anomeric rhamnosyl proton and C-3 of the aglycone indicated that the sugar part was linked to the C-3 position of aglycone (Supplementary Data). Similar to both **7** and **8**, NOE correlations were also detected at H-2'/H-1" and H-6'/H-1" to corroborate the C-1"-C-3 linkage. Furthermore, the ^{1}H and ^{13}C NMR spectra of **9** in CD_3OD were identical to the literature values reported by Fossen *et al*. [29].

Conclusions

The result of this study supports the folkloric claim that *Euphorbia hirta* Linn. is effective against dengue. Both the aqueous and tea samples reduced the plaque forming capacity of the virus to some degree. This perhaps validates the common practice of giving decoction and aqueous extract of the herb to a dengue infected person. However, there are still many unknowns as to how these natural products interplay and could affect other important biological processes in the human body. Unexpectedly, the crude ethyl acetate layer of *Euphorbita hirta* Linn. showed the most potent antiviral activity against the dengue virus (serotypes 1 and 2) on its preliminary test. It is therefore possible that the natural product/s responsible for neutralizing the virus is/are one of the 9 isolated compounds. The anti-dengue assay and other biological tests of these pure compounds are recommended and are currently being done by the authors.

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