Natural products produced by living organisms that showed 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.

Dengue virus causes dengue disease 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 inhabits, 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 products 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 potentials [3], diuretic [4], anti-allergic
Metabolites from *Euphorbia hirta* Linn. Authors et al

http://wjst.wu.ac.th

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, none has been reported about its efficacy against either dengue or the Dengue Hemorrhagic Fever (DMF). Tawa-tawa is a traditional herb and abundant in open grasslands that has been 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. The fact that there is no existing approved drug and vaccine yet 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 tawa-tawa herb.

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

**Materials and methods**

About 2.0 kg sample 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 50 % EtOH/MeOH solution. The extract was then filtered, concentrated *in vacuo*, and partitioned with EtOAc/H2O. A small portion (100 mL) of both organic (EtOAc or ethyl acetate) and water fractions were concentrated *in vacuo* and saved for 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 anti-dengue test. These samples (EtOAc, water layer, and tea) were sent to 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 via silica (SiO$_2$) gel column chromatography.

The structure elucidation of isolated compounds was done through Nuclear Magnetic Resonance (NMR) Spectroscopy. The $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR spectra were recorded in CDCl$_3$ ($\delta_H$ 7.24 ppm), CD$_3$OD ($\delta_H$ 3.30 ppm) and acetone-$d_6$ ($\delta_H$ 2.04 ppm) using 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 $\mu$g/mL) for 1 h. Vero cells were harvested and plated on muti-well plates at 2.5 $\times$ 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 by hexane/EtOAc solvent system to afford \textbf{iii-1} (49.0 mg), \textbf{iii-2} (9.0 mg), \textbf{iii-3} (11.0 mg), \textbf{iii-4} (2.0 mg), \textbf{iii-5} (10.0 mg), \textbf{iii-6} (30.0 mg), \textbf{iii-7} (2.0 mg), \textbf{iii-8} (4.0 mg), and \textbf{iii-9} (27.0 mg) as shown in Figure 3.

The first metabolite that was isolated is \textbf{iii-1}, a white compound that was partially soluble with hexane and has an $R_f$ value of 0.62 in TLC (20% EtOAc/hexane). The APCIMS spectrum of \textbf{iii-1} showed a protonated molecular ion signal at $m/z$ 425.3755, indicating its molecular formula as $C_{30}H_{48}O$. 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, eight singlet methyls were also observed in the $^1$H NMR spectrum of \textbf{iii-1}. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments support the foregoing discussion and establish the planar structure of \textbf{iii-1} depicted in Figure 3. The up and down orientation 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, \textit{iii-1} was identified as taraxerone.

Compound \textit{iii-2} that was eluted from the 30% EtOAc/hexane fraction is a white powder and has an \( R_f \) value of 0.43 in TLC (20% EtOAc/hexane). This metabolite was isolated via ODS (Yamazen Corp. Ultra Pack ODS-SM, 50 mm, Size B 26 × 300 mm) with MeOH/H\(_2\)O system. The APCIMS \( m/z \) found 409.3800 [M+H-H\(_2\)O]\(^+\), calcd for C\(_{30}\)H\(_{49}\): 409.3829 which is consistent with its molecular formula as C\(_{30}\)H\(_{50}\)O. The \( ^1\)H NMR spectrum of \textit{iii-2} showed signals for the six singlet methyl groups at \( \delta \) 0.76, 0.79, 0.83, 0.94, 0.97, and 1.03 ppm. It further displayed an isopropenyl side chain with signals at \( \delta \) 1.68 (3H, \textit{s}), 4.57 (1H, \textit{dd}, \( J = 2.1, 1.1 \) Hz) and 4.69 (1H, \textit{d}, \( J = 2.2 \) Hz) manifesting its relation with the lupane-type of triterpenoids. The oxygenated methine signal at 3.19 ppm (1H, \textit{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 discussions to establish its planar structure depicted in Figure 3. Literature search revealed that the \( ^1\)H and \( ^{13}\)C NMR data of \textit{iii-2} were identical to those of lupeol [20].

In the same manner described for \textit{iii-2}, \textit{iii-3} was also isolated from 30% EtOAc/hexane fraction by ODS. This colorless compound has an \( R_f \) value similar to \textit{iii-2} under same condition. 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. The \( ^1\)H NMR spectrum in CDCl\(_3\) showed a pair of very upfield doublets at 0.56 and 0.33 ppm (1H each, \textit{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, \textit{m}) and two singlet methyls at 1.68 (3H, \textit{s}) and 1.61 (3H, \textit{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 moiety of iii-3. Further 2D NMR (COSY, DEPT, HMQC, and HMBC) experiments also supported 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] (iii-4) was next isolated in similar fashion described for iii-2 and iii-3. It 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. The $^1$H NMR spectrum of iii-4 showed signals for the seven singlet methyl groups at $\delta$ 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].

Interestingly, the $^1$H and $^{13}$C NMR spectrum of iii-5 is almost similar to iii-1 except that an oxygenated proton appeared at 3.18 ppm indicating that iii-5 is a reduced form of iii-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 iii-1 and iii-5 in the $^{13}$C NMR experiments. This compound was obtained as white powder and has an $R_f$ value of 0.43 (TLC, 20 % EtOAc/hexane). The APCIMS $m/z$ found 409.3820 [M+H-H$_2$O]$^+$, calcd for C$_{30}$H$_{49}$: 409.3829, indicating the molecular formula as C$_{30}$H$_{50}$O. Compound iii-5 is two hydrogen atoms larger
than that of \textbf{iii-1}, which is consistent with the above discussions. Thus, \textbf{iii-5} is an alcohol derivative of \textbf{iii-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 up and down orientation 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.

Further purification of Fr B-5 over silica gel chromatography gave a white powder \textbf{iii-6}. It was partially soluble in methanol and has an $R_f$ value of 0.29 in TLC (20 % EtOAc/hexane). The APCIMS $m/z$ found 397.3803 [M+H-H$_2$O]$^+$, calcd for C$_{29}$H$_{49}$O: 397.3829 and consistent with its molecular formula as C$_{29}$H$_{49}$O. Compound \textbf{iii-6} has an alcoholic proton (3.53 ppm), an olefinic proton (5.36 ppm), two singlet methyls (0.68 and 1.01 ppm), three 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 very identical to those of the literature values, \textbf{iii-6} was elucidated as $\beta$-sitosterol [26, 27] as shown in Figure 3.

The authors then investigated the most polar fraction (100 % EtOAc). After a series of purifications (silica gel column and Preparative TLC), the extract afforded three flavonoid-type of compounds. Among these, \textbf{iii-7} was first identified as 3-O-arabinofuranoside of kaempferol and has an $R_f$ value of 0.6 in TLC (CHCl$_3$/EtOAc/MeOH 3:1:1). The ESIMS $m/z$ found 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$_{10}$. The aromatic region of the $^1$H NMR spectrum in acetone-$d_6$ showed an AA'XX' system at $\delta$ 8.04 (H-2' and H-6') and $\delta$ 7.01 (H-3' and H-5'). An AX system was also detected at $\delta$ 6.46 (H-8) and $\delta$ 6.24 (H-6) which is in accordance with a
Metabolites from *Euphorbia hirta* Linn. Authors et al

http://wjst.wu.ac.th

Since 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 moiety 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 at 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 had provided correlation to H-2' and H-6'. This NOE pattern was also observed for both iii-8 and iii-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 sugar moiety was further confirmed due to similar GC profiles of derived peracetate of authentic D-arabinose and those of natural product after acidic hydrolysis and subsequent acetylation of iii-7 with acetic anhydride in pyridine as shown in the Supplementary Data.

Compound iii-8 was next identified as 3-O-rhamnopyranoside of kaempferol and has an *R*$_f$ value of 0.5 in TLC (CHCl$_3$/EtOAc/MeOH 3:1:1). The ESIMS spectrum of iii-8 showed a protonated molecular ion signal at *m/z* 433.1103, indicating its molecular formula as C$_{21}$H$_{20}$O$_{10}$. 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
Metabolites from *Euphorbia hirta* Linn. Authors et al. 

http://wjst.wu.ac.th

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 quaternary carbons of the aglycone moiety. The sugar protons were then assigned by COSY and coupling constant analyses. The doublet methyl at 1.01 ppm ($J = 5.5$ Hz) in CD3OD is a typical 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 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 iii-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 iii-8 in CD$_3$OD were identical to the literature values reported by Fossen et al [29].

Lastly, iii-9 was identified as 3-O-rhamnopyranoside of quercetin and has an $R_f$ value of 0.4 in TLC (CHCl$_3$/EtOAc/MeOH 3:1:1). The ESIMS spectrum of iii-9 showed a protonated molecular ion signal at $m/z$ 449.1071, indicating its molecular formula as C$_{21}$H$_{20}$O$_{11}$. 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 moiety. The sugar protons were also assigned by COSY and coupling constant analyses. Further homodecoupling of δ
Metabolites from Euphorbia hirta Linn. Authors et al
http://wjst.wu.ac.th

0.90 methyl (H-6") had changed the signal pattern of the vicinal δ 3.42 proton (H-5") from multiplet to 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) was characteristic of a 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 iii-7 and iii-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 1H and 13C NMR spectra of iii-9 in CD3OD were identical to the literature values reported by Fossen et al [29].

Conclusion

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 dengue infected person. However, there are still many unknowns of 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 dengue virus (serotypes 1 and 2) on its preliminary test. It is therefore possible that the natural product
Metabolites from *Euphorbia hirta* Linn. Authors et al

http://wjst.wu.ac.th

responsible for neutralizing the virus is one of those nine isolated compounds. The anti-dengue assay and other biological tests of these pure compounds are recommended and currently being done by the authors.

**Acknowledgements**

We thank Dr. Filipinas F. Natividad of Research and Biotechnology Division, St. Luke’s Medical Center, Philippines for the anti-dengue assay by Plaque Reduction Neutralization Test (PRNT). The corresponding author is also grateful to MONBUKAGAKUSHO Japan for the financial support.
Figures

Against Dengue Virus Serotype 1

![Graph showing the effects of taw-tawa extracts on dengue serotype 1.](image)

**Figure 1** Effects of taw-tawa extracts on dengue serotype 1.
**Figure 2** Effects of tawa-tawa extracts on dengue serotype 2.
Figure 3 Secondary metabolites isolated from *Euphorbia hirta* Linn.