ORIGINAL ARTICLE

Antiviral Effect of Crude Aqueous Extracts from Ten Philippine Medicinal Plants against Zika Virus

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ABSTRACT

Objective. This study aimed to determine the antiviral activity of ten Philippine medicinal plants against Zika virus (ZIKV).

Methods. Lyophilized aqueous plant extracts were used for cell cytotoxicity and virus inhibition assays. The therapeutic index was computed from the 50% cytotoxic concentration (CC50) and 50% effective concentration (EC50) values. Plant metabolites were also identified using mass spectroscopy. An *in-silico* screening of these metabolites was done using ZIKV enzymes and the Axl protein in human microglial cells as target proteins, followed by the ranking of binding energy scores to generate a hypothesis on the possible mechanism of antiviral action.

Results. The plants that demonstrated the highest therapeutic index were *Momordica charantia*, *Psidium guajava*, *Vitex negundo*, and *Blumea balsamifera*. The majority of the metabolites present in the aqueous extracts were saponin, terpenes and terpenoids, and anthocyanin. Further, *in-silico* docking results showed a higher binding affinity for viral replication proteins compared to the viral envelope protein.

Conclusion. The crude aqueous extracts of *M. charantia*, *P. guajava*, *V. negundo*, and *B. balsamifera* were the most potent candidate antiviral therapies against ZIKV among the ten plants tested. Meanwhile, the *in-silico* results suggested that the metabolites possibly employ an intracellular mechanism for the observed antiviral activity.

Key Words: Zika virus, herbal medicine, virus inhibition, Momordica charantia, Psidium guajava

INTRODUCTION

Zika virus (ZIKV), a mosquito-borne flavivirus transmitted by *Aedes* spp., causes a disease with symptoms that include mild fever, skin rash, conjunctivitis, muscle and joint pain, malaise, and headache lasting for about two to seven days.[1] The symptoms of ZIKV infection appear to be similar to those of other arbovirus infections such as dengue and infected people usually recover. However, in pregnant women, infection with Zika virus has been found to cause congenital brain abnormalities in their infants after exposure to the virus *in utero*.[1]

Currently, there are no specific treatments for ZIKV infection apart from bed rest and medications for pain and fever. There is also no vaccine available.[1] Since viral infections are self-limiting leading either to immune clearance or host death,[2] most are clinically handled through the management of symptoms.[3] However, some herbal medicines have perceived antiviral effects based on folkloric claims. Previous studies have also shown that using the leaves of *Psidium guajava* results in an increase in platelet count after dengue

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infection,[4] while the roots and fruits of *Momordica charantia* have shown inhibitory effects on dengue virus serotype 1 (DENV-1).[4] Thus, these studies serve as a basis for further conducting studies on the antiviral effects of medicinal plants.

In the Philippines, the Department of Health (DOH) endorses ten herbal medicines[5] that have clinically-proven medicinal value. They are as follows:

- *Senna alata* is also known as ringworm bush. As an herbal remedy, it is used against tinea infections, insect bites, ringworms, eczema, scabies and pruritus.[5] Its leaves, flowers, stem, and root barks were previously shown to have broad-spectrum antimicrobial activity.[6] Currently, no studies on its antiviral properties are reported.
- *Momordica charantia* or bitter gourd has compounds in its fruits and seeds that exhibit antidiabetic properties.[7] It also shows anticancer activity with its fruits exhibiting inhibition of breast cancer growth[7] while its leaves show anti-metastatic activity against prostate cancer.[8] DOH also endorses this plant for its folkloric use against hemorrhoids, coughs, burns, and scalds.[5] Pongthanapisith *et al.*[9] found that it has inhibitory activity against the H1N1, H3N2, and H5N1 subtypes of Influenza A. One of its proteins, MAP30, has inhibitory effects against HIV-1[10] while its roots and fruits were shown to inhibit dengue virus.[4]
- *Allium sativum* or garlic, which is claimed to reduce blood cholesterol levels is commonly used for hypertension.[5] Nwokocha and colleagues demonstrated the hypotensive effect of aqueous garlic clove extracts on both normotensive and hypertensive rats.[11] As an herbal remedy, it is also used as an antibacterial, anti-inflammatory and anticancer treatment.[5] As for its antiviral activities, studies have reported an *in vitro* activity against Influenza A and B, cytomegalovirus, rhinovirus, HIV, herpes simplex virus 1 and 2, viral pneumonia, and rotavirus.[12]
- *Psidium guajava,* also known as guava is traditionally used as an antidiabetic medicine,[5] and its leaves have been shown to lower blood glucose levels *in vivo.*[13] Its leaves also have inhibitory activities against H1N1[14] and simian rotavirus[15]. Other folkloric uses include use as an antiseptic, anti-inflammatory, antispasmodic, antioxidant, hepatoprotective, anti-allergy, antimicrobial, antiplasmodial, anti-cough and antigenotoxic treatment.[5]
- *Vitex negundo* also known as 5-leaved chaste tree is traditionally used to treat coughs, colds, and fever.[5] It is also commonly used to treat a variety of diseases including diarrhea[5] since its leaves exhibit antimicrobial activity against bacteria such as *Vibrio cholera* and *Escherichia coli.*[16] Other folkloric uses include use as a treatment for asthma, pharyngitis, rheumatism, dyspepsia, and boils.[5] Meanwhile, Kothandan and Swaminathan[17] evaluated its leaves' antiviral activity and found that while it could inhibit Asian strains of the chikungunya virus, it had a low selectivity index.
- *Combretum indicum* or Chinese honeysuckle is an herbal medicine whose leaves have anti-diarrheal properties.[18] Traditionally, it is used against intestinal parasites.[5] However, there are no reports on its antiviral activity.
- *Blumea balsamifera* or blue camphor is used in the Philippines as an herbal treatment for kidney stones.[5] Montealegre *et al.* found that its leaves favor the formation of small crystals, beneficial to kidney stone formers.[19] Folkloric uses include treatment for wounds, cuts, rheumatism, diarrhea, spasms, colds, coughs, and hypertension.[5] Currently, no literature is available on its antiviral activities.
- *Ehretia microphylla is* also called wild tea. Chandrappa *et al.* found that its stem extract has anti-inflammatory activity *in vitro.*[20] Traditionally, it is used as an herbal remedy for eczema, scabies, and pruritus.[5] It is also used as an antispasmodic and anti-diarrheal treatment, disinfectant during childbirth, and mouthwash for tooth decay.[21] However, its antiviral properties are not widely reported.
- *Peperomia pellucida* stem and leaf extracts are used to treat gout and arthritis.[22] On the other hand, its antiviral activity has not yet been established.
- *Clinopodium douglasii* or peppermint leaves were found to have anti-asthmatic effect when combined with oregano.[23] As an herbal remedy, it is used as a pain reliever as well as a treatment against rheumatism, gout, coughs, colds, and insect bites.[5] However, its antiviral activities have not been reported.

Since little is known about the antiviral activities of most of these plants, this study aimed to determine their antiviral potential against the Zika virus. Further, ZIKV being an emerging threat to healthcare and a significant public health concern prompted the World Health Organization to advocate for the rapid development of treatment against ZIKV infections.[24] Direct-acting antivirals were considered as a good option for treating ZIKV by repurposing already available drugs to address the ZIKV problem efficiently.[24] In the Philippines, we have these ten medicinal plants that are already being used for their various health benefits and can serve as a good starting point in the search for cheaper, plant-based alternatives to synthetic, pharmaceutical drugs.[25] This is important in the country since these plants are widely available and accessible especially in the provinces. Meanwhile, the *in-silico* part of this study aimed to find a possible mechanism for the metabolites in the crude aqueous extracts of the plants that exhibited antiviral activity.

MATERIALS AND METHODS

Herbal Plant Crude Aqueous Extracts

Fresh leaves of *Vitex negundo, Psidium guajava, Ehretia microphylla,* and *Blumea balsamifera* were collected from Tanauan, Batangas while those of *Momordica charantia,*

Peperomia pellucida, and *Clinopodium douglasii* were collected from Sta. Rosa, Nueva Ecija. *Senna alata* leaves were collected from Morong, Bataan, while *Combretum indicum* leaves came from General Santos City, South Cotabato. *Allium sativum* cloves were collected from Ilocos Norte. Plant parts were washed with distilled water and air-dried before extraction. One hundred grams of cloves for *A. sativum* and dried leaves for all other plants were cut and soaked in 1L of distilled water at 100˚C for fifteen minutes. The resulting extracts were filtered through a muslin cloth to separate it from the plant parts before lyophilization. The lyophilized extracts were stored at 4˚C until reconstitution. For the cytotoxicity test and virus inhibition assay, the extracts were reconstituted in Dulbecco's Modified Eagle Medium (DMEM), clarified by centrifugation, then filtered and stored at -20˚C until needed. For the screening of metabolites using mass spectroscopy, the extracts were reconstituted in HPLC-grade water before filtration.

Cells and virus

Vero cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Laboratories, Grand Island, NY, US), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, UT, US), 2mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM non-essential amino acids (Gibco, Life Technologies, Grand Island, NY), 20 mg/L 7.5% $NaHCo_3$, 100 U/mL penicillin (Gibco Laboratories, Grand Island, NY, US) and 100 µg/mL streptomycin (Gibco Laboratories, Grand Island, NY, US). Cells were maintained at 37˚C with 5% CO_2.

ZIKV strain MR766 was used for all virus inhibition assays, and was provided by Dr. Day-Yu Chao (NCHU, Taiwan). ZIKV stocks were propagated by infecting Vero cells in DMEM with 2% FBS for 7 days, at a multiplicity of infection (MOI) of 1.0. Cell supernatants were harvested, clarified by centrifugation and stored in aliquots at -80˚C until use. Virus titers were determined by plaque assay on Vero cells (ATCC).

Cytotoxicity test

Cell cytotoxicity was evaluated using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay following the protocol from American Type Culture Collection (2011)[26] with some modifications. The cells were diluted to a density of 1.5 x 10^5 cells/mL and 100 µL was seeded onto a 96-well plate. After 12 hours, 100 µL of serially diluted extracts was added onto each well. Cells with medium (DMEM) only served as the positive control, while wells with media only served as the negative control. Each plant extract was serially diluted in triplicates. The plates were incubated for 4 days before the addition of 100 µL of MTT reagent (0.5 mg/mL). The plates were then incubated in the dark at 37˚C and 5% CO_2 for 4 hours followed by the addition of 100 µL of DMSO. The plates were incubated again in the dark for 2 hours before reading the absorbance at 570 nm.

Virus Inhibition Assay

To test for virus inhibition using plaque assay, the protocol of Baer & Kehn Hall[27] was followed with modifications. Vero cells at a density of 2.5 x 10^5 cells/mL were seeded onto 12-well plates. Each of the plant extracts with a starting concentration between 0.0625 mg/mL and 1 mg/mL was serially diluted two-fold prior to incubation with ZIKV MR766 at 37˚C with 5% CO_2 for 1 hour. Vero cells containing DMEM with 2% FBS served as the negative control. After 1-hour incubation, 1% methylcellulose was used as an overlay medium, and plates were incubated at 37˚C with 5% CO_2 for 4 days. Cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet. Manual counting of the plaques was performed. All tests were done in duplicate.

Determination of CC50, EC50, and Therapeutic Index

The % cell viability of Vero cells after treatment with serially diluted plant extracts was calculated as:

$$\frac{\text{Abs (treated)} - \text{Abs (negative control)}}{\text{Abs (positive control)} - \text{Abs (negative control)}}$$

Logarithmic regression was used to determine the extract concentration at which only 50% of cells were viable. This concentration was considered the plant's CC50.

The potency of each plant extract concentration used was calculated as:

$$\frac{\text{\# of plaques (treated)}}{\text{\# of plaques (control)}} \times 100$$

Exponential regression was used to determine the extract concentration at which there was 50% potency. This concentration was considered the plant's EC50. The therapeutic index was calculated as the ratio of:

$$\frac{\text{CC50}}{\text{EC50}}$$

Identification of Plant Metabolites via Mass Spectroscopy

To identify the specific metabolites present in the crude aqueous extract of the top antiviral candidates, mass spectroscopy was performed. The lyophilized plant extracts were dissolved in liquid chromatography-mass spectrometry (LC-MS) grade water up to a final concentration of 0.5 mg/mL and then filtered using a 0.2µm syringe filter. Separation was performed in a 2.1 x 100 mm 1.8µm C18 column at 40˚C. The mobile phase A contained 0.1% formic acid in water, while mobile phase B contained 0.1% formic acid in acetonitrile. Initial conditions were kept for one minute before using a linear gradient program of

$$\frac{\text{5\% phase B}}{\text{95\% phase A}} \text{ to } \frac{\text{95\% phase B}}{\text{5\% phase A}}$$

for 10 minutes at a flow rate of 0.5 mL/min. For mass spectroscopy, the Waters UNIFI Scientific Information System v1.8.1.073 software was used. The parameters used were as follows: capillary voltage of 1.0 kV (ESI^+), source temperature at 120°C, desolvation temperature at 550°C, cone voltage of 40 V, cone gas flow at 40L/h, desolvation gas flow at 950 L/h, scan range from 50 to 1,200 m/z, scan time of 0.150s, and a collision energy ramp from 15 to 50 eV (high energy). Leucine enkephalin was used as a reference compound for mass correction.

In silico Docking of Plant Metabolites

Six candidate enzymes contributing to ZIKV cell entry and infection namely, ZIKV helicase (PDB ID: 5JRZ),[28] protease (PDB ID: 5H4I),[29] methyltransferase (PDB ID: 5KQR),[30] RNA-dependent RNA polymerase (PDB ID: 5U04),[31]and envelope protein (PDB ID: 5JHM)[32] as well as the human Axl protein (PDB ID: 5U6B; Gajiwala & Grodsky)[33] were used for docking based on the work of Byler et al.[34] The structures were downloaded from the protein data bank (www.PDB.org). Their active sites were determined by observing the protein in Chimera where they were prepared for docking via the procedure outlined by Lang.[35] AutoDock Tools v1.5.6 was used to create the docking grid box.

The metabolites identified using mass spectroscopy were searched against databases of Chem Spider and the Traditional Chinese Systems Pharmacology Database (TCMSP) for therapeutic potentials. Only ligands with available 3D structures were downloaded and prepared for docking using AutoDock Tools v1.5.6. A configuration file containing the docking grid dimensions was used for the actual docking experiment, which was performed in AutoDock Vina. Virtual screening was performed to generate the predicted free energy of binding for each metabolite. The metabolites were ranked according to their binding energies and tabulated for each plant.

RESULTS

Determination of the CC50 and EC50 of the Ten Plants

The MTT assay measures cell viability via the yellow MTT reagent that turns into purple-colored formazan when reduced by the dehydrogenase enzymes of metabolically active cells.[26] Based on the CC50 data (Figure 1), the plant that was most cytotoxic to Vero cells was *Ehretia microphylla* (0.2768 mg/mL), while the least cytotoxic plant was *Allium sativum* (9.91 mg/mL).

Meanwhile, the plaque assay measures viral concentration by counting the number of discrete plaques visible on the plate. These plaques are indicative of cellular dead zones due to viral infection.[27] This assay can be used to measure a treatment's potency by observing its plaque reduction capacity. Based on the EC50 values (Figure 2), the plant with the greatest antiviral effect was *Psidium guajava* (0.1575 mg/mL) while *A. sativum* (6.997 mg/mL) had the least antiviral effect.

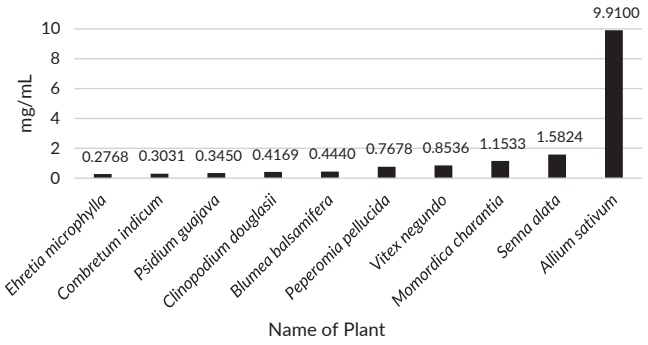

Figure 1. CC50 of the ten Philippine medicinal plants against ZIKV MR766.

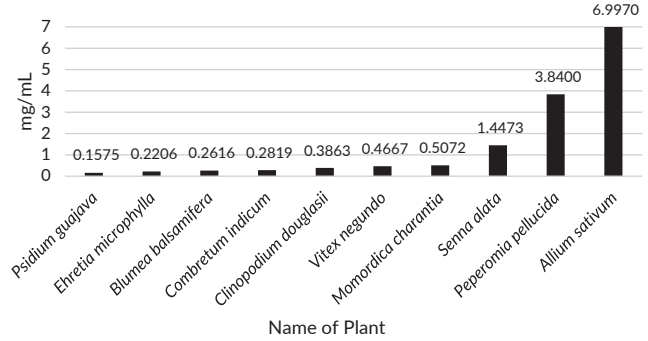

Figure 2. EC50 of the ten Philippine medicinal plants against ZIKV MR766.

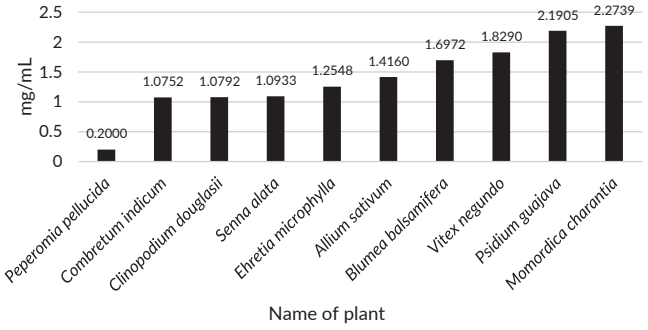

Figure 3. Therapuetic index of the ten Philippine medicinal plants against ZIKV MR766.

Determination of the Antiviral Candidates using Therapeutic Index

Based on the ratio of the CC50 and EC50 values, the therapeutic indices were derived. A ranking of the plants' therapeutic indices (Figure 3) show that the four plants namely, *Momordica charantia, Psidium guajava*, *Vitex negundo,* and *Blumea balsamifera,* are antiviral candidates.

Identification of Metabolites from Crude Aqueous Extracts using Mass Spectroscopy

This study found that saponins, terpenes, terpenoids, and anthocyanin were most commonly detected in the aqueous extracts. A comprehensive list and identity of the metabolites identified via mass spectroscopy are shown in Table 1. The metabolites with the highest binding affinity for each of the top four plants include saikosaponin D in *M. charantia,* benzoyl paeniflorin in *P. guajava*, macrophylloside D in *V. negundo,* and cyanidin 3-5 diglucoside in *B. balsamifera.*

In silico Docking of Metabolites to Proteins Involved in ZIKV Infection

The plants that were determined to have good ZIKV inhibitory activity underwent metabolite identification and *in silico* docking to generate a hypothesis on a possible mechanism of action for the observed antiviral effects. Data on the metabolite binding energies are shown in Table 2. The binding energies generated are expressed in kcal/mol and are measures of the strength of the interaction between the metabolite and the target protein. Binding energies of the top metabolites to the replication proteins ranged from -7 to -9.5 kcal/mol compared to the range of -5.5 to -7.4 kcal/mol for the viral envelope protein. Meanwhile, the binding affinity to the human Axl receptor ranged from -7.1 to -9.3 kcal/mol for the top metabolites that bound to it.

DISCUSSION

Determination of the CC50 and EC50 of the Ten Plants

A high CC50 value is desired for potential drug leads since this means that it requires a high dosage before the treatment becomes cytotoxic. Meanwhile, a low EC50 value is ideal for potential drug leads because only a low concentration of the antiviral agent is needed to inhibit the virus. While the CC50 and EC50 values may suggest that *E. microphylla* is a poor antiviral candidate due to high cytotoxicity and that *P. guajava* has the best antiviral activity among the plants analyzed, this is not accurate. Therefore, the ratio between the two was used as an indicator of selectivity.

Table 1. Metabolites identified using mass spectroscopy

Name of Plant	Metabolite	Observed mass (Da)	Observed m/z	Observed RT (min)
M. charantia	Saikosaponin D	780.4676757	803.4568965	7.540168762
	Esculentoside B	664.3831034	687.3723243	8.502177238
	Ganoderic acid Y	454.3443668	477.3335877	8.337738037
	Methyl euscaphate	502.3668356	525.3560565	9.341717720
	Cimiside F	650.4028926	673.3921134	5.725144863
	Esculentoside A	826.4370781	849.4262989	7.599523067
	Pomolic acid	472.3554303	495.3446511	10.12463284
	Ganoderic acid U	472.3560403	495.3452611	10.91231728
P. guajava	Benzoylpaeniflorin	600.1809998	601.1882762	6.502140999
V. negundo	Chrysanthemin	448.1001575	449.1074339	3.429554224
	Cimcifugic acid B1	448.1002838	449.1075603	3.984580994
	Macrophylloside D	558.1953234	581.1845442	3.015325069
	5, 7, 2', 5' – Tetrahydroxyflavone	286.0477752	287.0550516	4.642369270
	Mudanpioside E	526.1693374	549.1585582	3.860057831
	Cimiside F	650.4028926	673.3921134	5.725144863
	Chrysanthemin	448.1001575	449.1074339	3.429554224
	Tinoside	520.1944627	543.1836835	5.497520447
	8-epi Loganic acid	376.1362061	399.1254270	1.985986829
	Dihydrobrusatol 1	522.2096901	545.1989110	3.901344061
	Andrographatoside	498.2826021	521.2718229	6.256365299
B. balsamifera	Cyanidin 3,5-diglucoside	610.1532610	633.1424819	3.752222300
	Retinol	286.2291979	287.2364743	10.30376053
	Bruceine F	428.1676404	451.1568612	6.139420033
	Delphinidin	302.0421133	303.0493897	3.748351574
	Schizonepetoside A	330.1674309	353.1566518	3.874173641
	Arnicolide D	332.1594595	333.1667359	6.835141182
	6,7-Dehydroartemisinic acid	232.1458154	233.1530919	7.690526962
	Cylindrene	232.1454386	233.1527151	6.930004120
	Kirenol	338.2448700	361.2340909	8.367994308
	Nigakilactone K	406.1962836	407.2035600	7.499149799

Table 2. Top metabolite binding energies (in kcal/mol) for six key proteins in ZIKV infection for *M. Charantia*, *P. Guajava*, *V. Negundo* and *B. Balsamifera*

Plant	Methyltransferase (replication protein)	Helicase (replication protein)	Protease (replication protein)	Polymerase (replication protein)	Envelope Protein	AXL
M. charantia	Saikosaponin D -9.3	Saikosaponin D -9.5	Saikosaponin D -8.4	Saikosaponin D -9.1	Saikosaponin D -7.4	Saikosaponin D -9
	Esculentoside B -8.9	Esculentoside A -8.6	Cimiside F -7.7	Cimiside F -9	Cimiside F -7.2	Pomolic acid -8
	Ganoderic acid Y -8.6	Cimiside F -8.5	Methyl euscaphate -7.7	Esculentoside A -8.4	Pomolic acid -6.6	Ganoderic acid U -8
	Methyl euscaphate -8.3	Esculentoside B -8.4	Ganoderic acid Y -7.6	Ganoderic acid Y -8	Esculentoside A -6.1	Cimiside F -8
	Cimiside F -8.1	Pomolic acid -8.1	Pomolic acid -7.3	Methyl euscaphate -7.9	Esculentoside B -6.1	Ganoderic acid Y -8
P. guajava	Benzoyloxy-paeniflorin -9.8	Benzoyloxy-paeniflorin -8.2	Benzoyloxy-paeniflorin -8.3	Benzoyloxy-paeniflorin -8.8	Benzoyloxy-paeniflorin -6.6	Benzoyloxy-paeniflorin -9.3
V. negundo	Chrysanthemin -9	Macrophylloside D -9.1	Macrophylloside D -8.7	Cimiside F -9	Cimiside F -7.2	Cimiside F -8.4
	Cimicifugic acid B1 -9	Cimiside F -8.5	Mudanpioside E -8	Macrophylloside D -8.1	Tinoside -5.9	Macrophylloside D -8.3
	Macrophylloside D -8.8	Chrysanthemin -8.3	Cimiside F -7.7	Dihydrobrusatol1 -8	5,7,2',5'-Tetra-hydroxy-flavone -5.8	Tinoside -7.9
	5,7,2',5'-Tetra-hydroxy-flavone -8.1	Mudanpioside E -8.1	Chrysanthemin -7.7	Tinoside -7.9	Andrographatoside -5.8	Mudanpioside E -7.9
	Mudanpioside E -8.2	Tinoside -8	8-epi-Loganic acid -7.7	Andrographatoside -7.5	Cimicifugic acid B1 -5.8	Chrysanthemin -7.8
B. balsamifera	Cyanidin 3,5-diglucoside -8.6	Cyanidin 3,5-diglucoside -9.2	Cyanidin 3,5-diglucoside -8.1	Cyanidin 3,5-diglucoside -7.5	Cyanidin 3,5-diglucoside -6.4	Cyanidin 3,5-diglucoside -8
	Retinol -7.9	Bruceine F -8.6	Arnicolide D -7.9	Delphinidin -7.3	Bruceine F -5.6	Delphinidin -7.6
	Bruceine F -7.8	Delphinidin -7.7	Delphinidin -7.5	Bruceine F -7.3	Delphinidin -5.6	Arnicolide D -7.2
	Delphinidin -7.8	Schizonepetoside A -7.5	6,7-Dehydro-artemisinic acid -7.1	Schizonepetoside A -7.2	Arnicolide D -5.5	Bruceine F -7.1
	Schizonepetoside A -7.8	Retinol -7.1	Cylindrene -7.1	Nigakilactone K -7	Kirenol -5.5	Kirenol -7.1

Determination of the Antiviral Candidates using Therapeutic Index

The therapeutic index is a more reliable measure of the plant's antiviral potential compared to CC50 or EC50 alone. A high therapeutic index is indicative of a plant's potential as a good candidate against the virus since this means that it has a relatively high CC50 value compared to its EC50 value. *Momordica charantia, Psidium guajava*, and *Vitex negundo,* three of the four antiviral candidates found in this study, already have established antiviral activities based on the literature. *M. charantia* is known to have antiviral activity against Influenza A[9], HIV-1[10] and dengue[4], while *P. guajava* has been shown to inhibit H1N1[14] and simian rotavirus.[15] *Vitex negundo*, on the other hand, has antiviral activity against chikungunya virus but was also shown to have a low selectivity index.[17] The results of the present study support the claim that these three plants are potential antiviral agents and may therefore serve as rich sources of metabolites with high antiviral activities. This study also identified a new antiviral candidate, *B. balsamifera.* Meanwhile, *Allium sativum* had the poorest antiviral activity among the ten plants based on EC50 despite having an established antiviral activity against other viruses like Influenza A and B, cytomegalovirus, rhinovirus, HIV, herpes simplex virus 1 and 2, viral pneumonia, and rotavirus.[12] Despite having a therapeutic index of 1.416, which was fifth out of the ten plants, it qualifies as a poor antiviral candidate against ZIKV because it requires a very high dose before exhibiting antiviral activity. Meanwhile, the plants that ranked sixth to ninth namely, *Ehretia microphylla, Senna alata, Clinopodium douglasii* and *Combretum indicum,*

had therapeutic indices close to 1.0. Although they may have antiviral potential, they may not be good candidates since their effective concentration (EC50) against ZIKV is also close to its cytotoxic concentration. *Peperomia pellucida* showed the lowest therapeutic index (0.2). A value of less than 1.0 means that its cytotoxic properties exceed its antiviral properties hence a poor antiviral candidate.

Identification of Metabolites from Crude Aqueous Extracts using Mass Spectroscopy

According to Cowan,[36] the analysis of plants and their components usually begins by exploring plants already used by local healers. For extractions, crude aqueous or alcohol extracts are commonly used for the first screening, and then other organic extraction methods follow[36] should the plant show promising potential. The goal in extraction procedures is to separate the soluble active components of the plant using selective solvents.[37] Since solvents have varying polarities, the use of a particular solvent would result in a distinct metabolite profile. It was found that in aqueous extracts, active components present include anthocyanins, tannins, saponins, and terpenoids as well as starches, polypeptides, and lectins.[36] This is consistent with the results found in this study.

As for the metabolites with the highest binding affinity for each plant, three out of the four already have known antiviral activities. Saikosaponin D found in *M. charantia* in this study was isolated from *Bupleurum falcatum* L. by Ushio & Abe,[38] and was found to have direct inactivating effects on both the measles virus and herpes simplex virus. Benzoyl paeoniflorin found in *P. guajava* is also present in *Paeonia delavayi* root extracts and has been proven to have antiviral activity against influenza virus.[39] The observed antiviral activity was even higher than that of oseltamivir, an anti-flu drug currently in the market.[39] Meanwhile, cyanidin 3-5, diglucoside from *B. balsamifera* is one of the main anthocyanins found in elders, and these plants are known to have compounds with antiviral activity.[40] It is also highly antioxidant, and is known to be a potent anticancer agent.[40] These metabolites are good candidates for further studies since the literature has established that they possess antiviral activities while the results of this study show that they are present in plants with antiviral activity and they exhibit high binding affinity to viral proteins *in silico*.

In silico Docking of Metabolites to Proteins Involved in ZIKV Infection

The binding energy is a measure of the change in Gibb's free energy,[41] so more negative values are desired since this is also reflective of a metabolites' higher binding affinity to the protein. The binding affinities of the plant metabolites to the viral replication proteins were generally higher compared to that of the viral envelope protein which suggests that the mechanism of viral inhibition is more likely to be via binding with intracellular viral replication proteins than via extracellular binding to the ZIKV envelope protein. Further, the results showed that the metabolites may have antiviral potential via binding with viral replication proteins. Meertens et al. identified the Axl as the protein that binds ZIKV in human microglia, thus mediating infection.[42] The high binding affinity to the Axl protein suggests that if tested on human glial cells, it is possible that the plant metabolites from *M. charantia, P. guajava, V. negundo,* and *B. balsamifera* can inhibit ZIKV by binding to the Axl protein and thus preventing host cell attachment.

CONCLUSION

The results of the study show that *M. charantia, P. guajava, V. negundo,* and *B. balsamifera* are the best candidates against ZIKV among the ten plants. Although *E. microphylla* has high antiviral activity, it is not a good candidate because of its high cytotoxic property. Similarly, *A. sativum* would also not make a good candidate despite being the least cytotoxic because it also has the weakest antiviral activity. Lastly, the *in-silico* findings of the study suggest that intracellular binding with viral replication proteins may be a likely mechanism for the plant metabolites in ZIKV inhibition. It is recommended to further study the anti-ZIKV effects of the top four plants as well as the mechanism behind the viral inhibition by the plant metabolites that exhibited the highest binding affinities *in silico.* Since only crude extracts were used in this study, it is recommended to explore the isolation of specific metabolites in future experiments. Additionally, the metabolites identified in this study are limited to those present in the aqueous extracts of the plants. For further studies, the use of other solvents is recommended since this may uncover other metabolites with potential antiviral activity.

Statement of Authorship

All authors participated in data collection and analysis, and approved the final version submitted.

Author Disclosure

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