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RESEARCH ARTICLE

Cytotoxicity of *Carica papaya, Phyllanthus emblica, Syzygium aromaricum,* and *Citrus aurantifilia* leaf extract against African Green monkey epithelial (Vero) cells and *Aedes albopictus* (C6/36) cells

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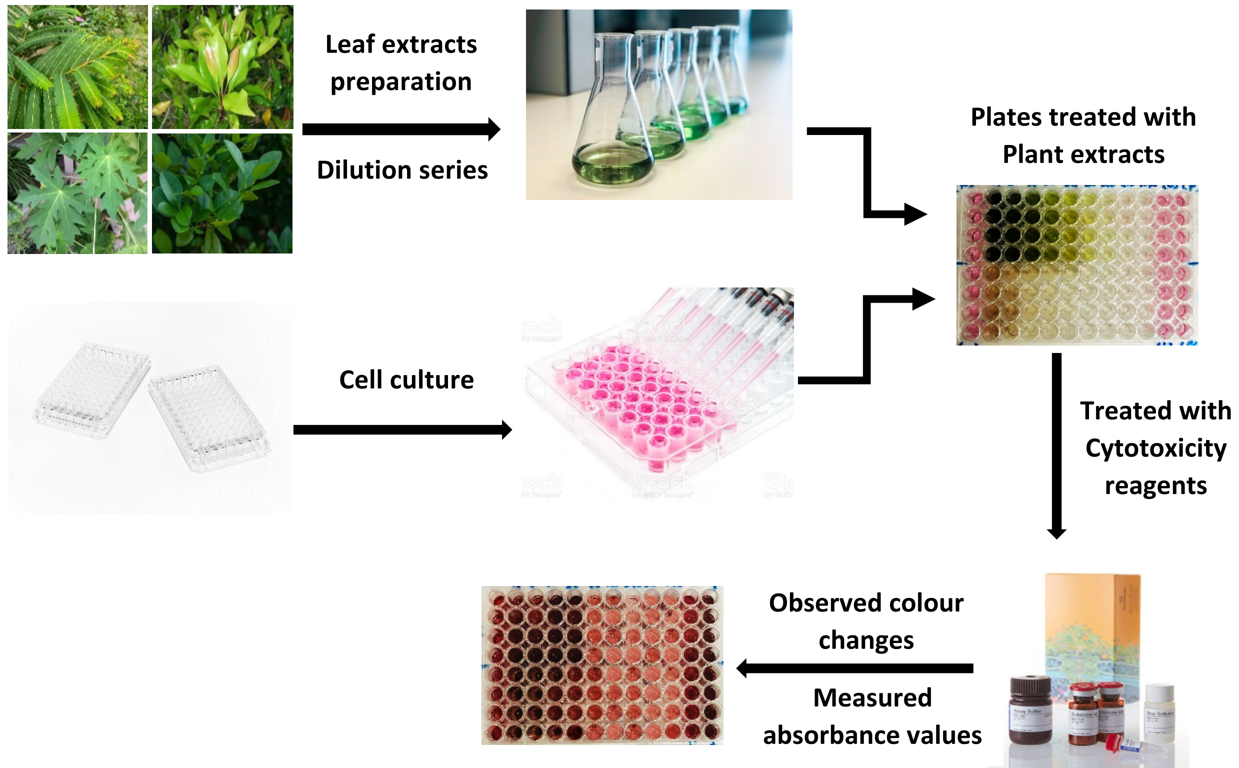

Highlights

- High concentrations of Nelli, Pawpaw, Clove & Lime leaf extracts were toxic to Vero and C6/36 cells.
- Cytotoxic data on Vero cells helps to select minimum toxic concentrations for antiviral testing.
- Cytotoxicity data on C6/36 cells helps to select optimum concentrations for anti-*Aedes* activity.

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Cytotoxicity of *Carica papaya, Phyllanthus emblica, Syzygium aromaricum,* and *Citrus aurantifilia* leaf extract against African Green monkey epithelial (Vero) cells and *Aedes albopictus* (C6/36) cells

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Abstract: Plant-based compounds are an important option in developing new drugs. Leaves of Nelli (*Phyllanthus emblica*), Pawpaw (*Carica papaya*), Clove (*Syzygium aromaticum*) and Lime (*Citrus aurantifolia*) consist of anti-microbial properties. This study aimed to identify the cytotoxicity of leaf extracts of Nelli, Pawpaw, Clove and Lime against Vero cells and *Aedes albopictus* C6/36 cells. CytoTox 96® is a colorimetric assay that quantitatively measures lactate dehydrogenase released upon cell lysis. Leaf extracts were prepared in two-fold dilution series. Two 96-well assay plates were prepared for each plant extract for Vero cells and C6/36 cells. The assay was set up with an analytical system based Negative control, Vehicle control and Positive control with four replicates. Plant extracts were added into the test wells at different concentrations and one plate was incubated for 5 h and the next plate was incubated for 24 h at 37 °C for Vero cells and 25 °C for C6/36 cells. Absorbance data were measured using a standard 96-well plate reader. The cytotoxicity-driven colour intensity and absorbance values decreased with the decreasing concentrations of leaf extracts. High concentrations of the leaf extracts had cytotoxicity properties against both Vero cells and C6/36 cells.

Keywords: Cytotoxicity; *Carica papaya (Pawpaw); Phyllanthus emblica (Nelli); Syzygium aromaticum (Clove); Citrus aurantifolia (Lime)*

INTRODUCTION

Natural products play a major role in the discovery of new drugs. During the drug screening process, plant-based natural products are tested for cytotoxicity (Abdel-Massih *et al*., 2010). Cytotoxicity studies are the first step in evaluating the toxicity of test substances for medicinal properties. Moreover, cytotoxicity testing allows researchers to investigate antimicrobial of plant extracts and compounds to detect selective activity against certain micro-organisms at an early stage. A product is considered cytotoxic if it interferes with the attachment of cells, morphology, and cell growth in *in-vitro* cell culture systems (Horvath, 1980). The mechanism of action of most toxic compounds causes alterations in the basic biochemical processes of all types of cells. It has been suggested that there is a link between *in vitro* and *in vivo* toxicity (McGaw *et al*., 2014). New medications undergo rigorous cytotoxicity testing with multiple experiments.

Toxicity is a complex process involving direct cellular damage, physiological impacts, inflammation and other systemic effects in humans and animals. Measuring systemic and physiological effects *in vitro* is difficult and thus most assays focus on cellular effects (Freshney, 2015). Many *in vitro* cytotoxicity experiments are done considering the financial and ethical aspects before moving to definite *in vivo* experiments. Moreover, *in vitro* tests are widely used in ethno-pharmacological research due to the ethical and financial constraints of using animal tissue or animals. *In vivo* cytotoxicity experiments also facilitate bioassay-guided isolation of "active compounds" or those responsible for any activity shown in a bioassay using the total extract. *In vitro* cytotoxicity assays also have the advantage of requiring a substantially less material for testing (Houghton *et al*., 2007). The cytotoxicity detail of an extract provides possibilities for further studies to identify suitable products that could be bioactive with high pharmacological activity with minimal toxicity. Like in the medicinal screening of natural products against different diseases, synergistic effects between different fractionized compounds in a plant extract may affect the activity or toxicity in relation to the drug or insecticide screening against viral infections like dengue.

Dengue endemic tropical world has been experiencing a dramatic increase in dengue cases in recent years. No antiviral treatments are available against dengue, and research on possible antiviral agents is necessary to combat the increasing dengue disease burden, especially in tropical and sub-tropical countries. Plant products have become the primary source of test material in developing antiviral drugs in traditional medical practices. The secondary metabolites of various medicinal plants contain a wide range of biologically active compounds with few side effects, bioavailability and less environmental toxicity. This research focused on the cytotoxic activity of four leaf extracts derived from fourplants used in Sri Lankan traditional medicine. The chances of finding active biological compounds are higher in plants already used for traditional medicinal purposes than in those selected

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randomly (Cordell, 1995; Unander *et al*., 1995; Betancur-Galvis *et al*., 2002;). *Phyllanthus emblica* (Nelli) *(*Alvarez, *et al*., 2009; Kamruzzaman and Hoq, 2016) and *Syzygium aromaticum* (Clove) have antiviral properties (Pulikottil and Nath, 2015; Kaur and Chandrul, 2017; Batiha *et al*., 2020). *Carica papaya (*Pawpaw*)* is used in Asia to treat dengue (Senthilvel *et al*., 2013; Sarala and Paknikar, 2014; Sharma *et al*., 2019) while *Citrus aurantifolia* (Lime) contains antimicrobial activity and a wide range of biologically active compounds (Enejoh *et al*., 2015; Aladekoyi *et al*., 2016; Ehsanfar *et al*., 2020; Kurniawati and Nastiti, 2020). These plant extracts have been commonly used in Ayurveda without an understanding of their toxicity. Here we tested these four plant leaf extracts for toxicity to decide on an optimal concentration for antiviral studies with minimum toxicity. Other studies have also reported dose-dependent cytotoxicity of different parts of the *C. papaya* extracts (El-Desouky*et al*., 2008; Joseph *et al*., 2015; Salim and Abu, 2018), *P. emblica* (Qi *et al*., 2013;), *S. aromaticum* (Prashar *et al*., 2006; Aisha *et al*., 2012) and *C. aurantifolia* (Dougnon*et al*., 2017; Şeker*et al*., 2021). It is necessary to examine the toxicity of the plant products on different types of cells including *Aedes albopictus,* C6/36 cells.

The objective of this study was to identify the *in vitro* cytotoxicity of *P. emblica, C. papaya, S. aromaticum, and C. aurantifolia* leaf extracts against normal African green monkey kidney epithelial (Vero) cells and *Ae. albopictus* (C6/36) cells. For that, we used the CytoTox 96® Non-Radioactive Cytotoxicity Assay, which is a colourimetric assay that quantitatively measures lactate dehydrogenase (LDH) released upon cell lysis. The colour intensity is proportionate to the rate of cell lysis.

MATERIALS AND METHODS

Preparation of leaf extracts

Phyllanthus emblica, C. papaya, S. aromaticum and *C. aurantifolia* leaves were collected from the Central Province of Sri Lanka. Leaves were thoroughly washed under running tap water to remove dust and other unwanted substances on the plants from their natural environment. Then the leaves were washed with 1% Clorox for 5 min and then washed with running water. Leaf blades were then blot dried using tissue papers. Primary veins were removed and cut into small pieces using sterile scalpel blades. Fresh leaf extract was ground using a laboratory mortar and pestle and crushed using the mechanical juice extractor (Philips, Malaysia) without adding water. Then the extract was filtered using a clean muslin cloth. The pure extract was mixed with a similar volume of normal saline and filtered using Whatman No 1 filter paper. The filtrate was prepared in two-fold dilutions from neat to 1/1024 in normal saline.

Culture of Vero cells

Normal African green monkey kidney epithelial (Vero) cells were taken from virus tanks stored in Liquid Nitrogen canisters under -196 °C at the Department of Microbiology, Faculty of Medicine, University of Peradeniya. These Vero cells were established and maintained in minimal essential media (MEM) (Gibco-life technologies) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (Invitrogen),1% Pen/Strep (Invitrogen) at 37 °C in 5% CO_2.

Culture of C6/36 cells

Aedes albopictus C636 cells were obtained from virus tanks stored in liquid nitrogen canisters under -196 °C at the Department of Microbiology, Faculty of Medicine, University of Peradeniya. C6/36 were established and maintained in minimal essential media (MEM) (Gibco-life technologies) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (Invitrogen), 1% Pen/Strep (Invitrogen) at 28 °C in 5% CO_2.

Cytotoxicity assay using Vero cells (Jinga *et al*., 2017; Promega, 2021)

Two 96-well assay plates were prepared for each plant extract separately for Vero cells, and the assay was set up with an analytical system based 1. Negative control - without Vero cells, 2. Vehicle control - untreated cells, 3. Positive control - lysis solution. Four replicate wells were used for each concentration and controls. Then 100 µl of leaf extracts were added to wells allocated for tests. For the vehicle control, 10% FBS containing cell culture medium was added instead of leaf extracts. Then one plate was incubated at 37 °C for 5 h (LDH has a half-life of approximately 9 h), and the other plate was incubated at 37 °C for 24 h. Positive control was prepared before 45 min of adding CytoTox96® reagent. Then 50 µl aliquots from all test and control wells were transferred to fresh 96 well plates. A volume of 50 µl of CytoTox96 reagent was added to each well of the newly set plates. Then the plate was covered with aluminium foil and incubated for 30 min at room temperature. After this, 50 µl of stop solution was added to all wells. Then the absorbance values for different wells were measured at 490nm using a plate reader (Labtech LT-4500, Singapore). Then the averages of the absorbance values were taken from four replicates to calculate the percentage cytotoxicity.

Cytotoxicity assay usingC6/36 cells

Two 96-well assay plates were prepared for each plant extract separately with C6/36 cells. The same procedure was carried out as mentioned for Vero cells with four replicate wells per concentration. One plate was incubated at 28 °C for 5 h and other plate was incubated at 28 °C for 24 h and the absorbance values for different wells were measured at 490 nm.

Calculation of percentage cytotoxicity

To obtain the corrected values, average background values of the culture medium (negative control) were subtracted from all values of experimental wells. Corrected values were used in the following formula to compute the percentage cytotoxicity.

$$\text{Percentage cytotoxicity} = \frac{\text{Experimental LDH Release (OD490)}}{\text{Maximum LDH Release (OD490)}} \times 100$$

Interpretation of percentage cytotoxicity

Test absorbance average = positive control absorbance average if the percentage cytotoxicity is 100.

If test absorbance average > positive control absorbance average, then the percentage cytotoxicity > 100.

Test absorbance average = negative control absorbance average if the percentage cytotoxicity is 0.

If test absorbance average < negative control absorbance average, then the percentage cytotoxicity is < 0.

Statistical analysis

To test the difference in the percentage cytotoxicity of the different leaf extracts between the two time periods (5 and 24 h), log transformed cytotoxicity percentage data were analyzed using paired *t* test with 95% confidence level.

RESULTS

The cytotoxicity-driven colour intensity (Figure 1) and absorbance values (Table 1) decreased with decreasing concentrations of *P. emblica, C. papaya, S. aromaticum* and *C. aurantifolia* leaf extracts in Vero cells.

The cytotoxicity-driven colour intensity (Figure 2) and absorbance values (Table 2) decreased with decreasing concentrations of *P. emblica, C. papaya, S. aromaticum* and *C. aurantifolia* leaf extracts against C6/36 cells.

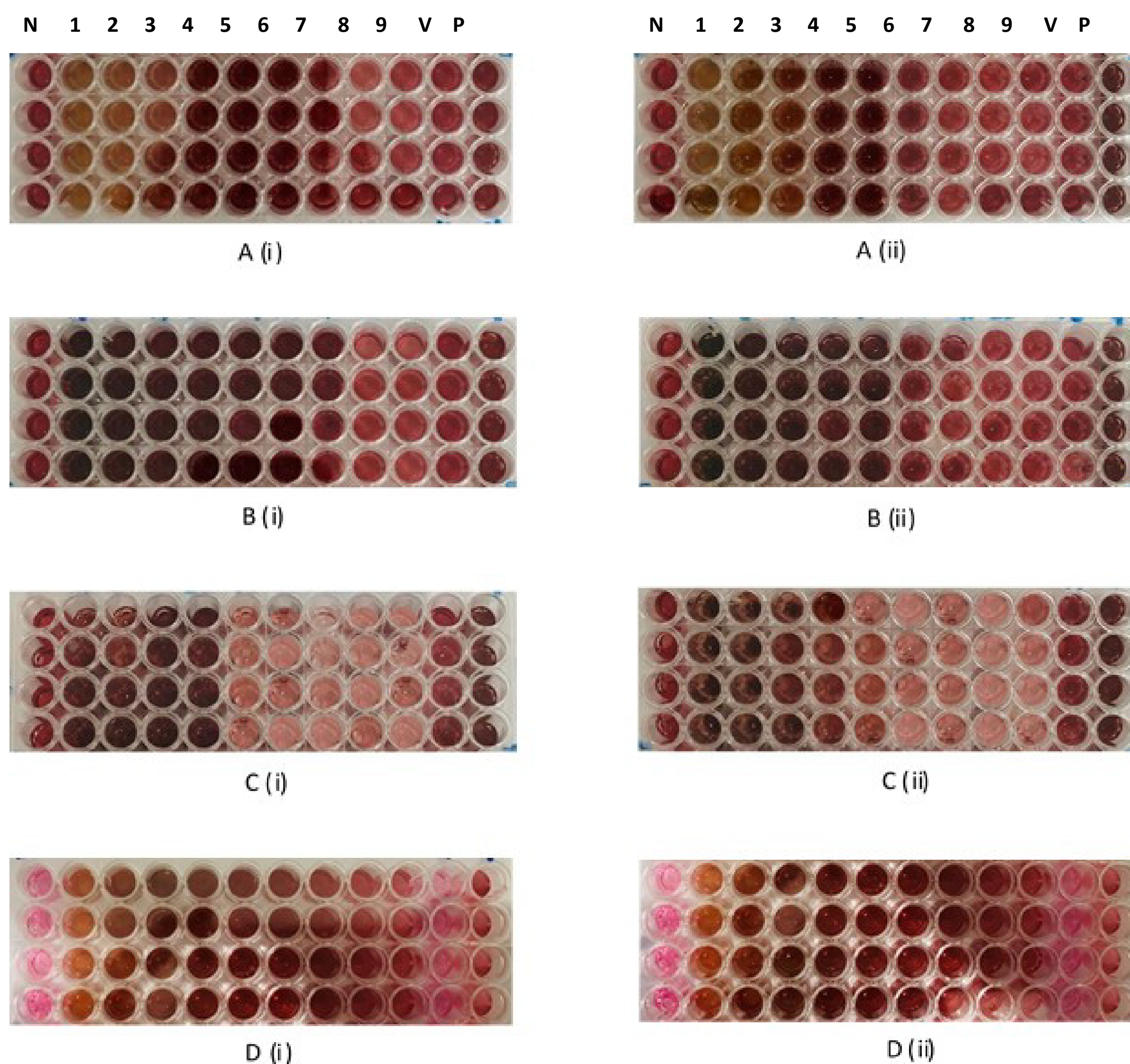

Figure 1: Cytotoxicity-driven colour intensity changes of wells with Vero cells at different concentrations of (A) *Phyllanthus emblica,* (B) *Carica papaya,* (C) *Sysygium aromaticum* and (D) *Citrus aurantifolia* leaf extracts after 5(i) and 24 h (ii) of incubation.

N – Negative Control; 1 – 1/4 concentration; 2 – 1/8 concentration; 3 – 1/16 concentration; 4 – 1/32, concentration; 5 – 1/64 concentration; 6 – 1/128 concentration; 7 – 1/256 concentration; 8 – 512 concentration; 9 – 1/1024 concentration; V – Vehicle control; P – Positive control.

Table 1: Percentage cytotoxicity of *Phyllanthus emblica, Carica papaya, Sysygium aromaticum* and *Citrus aurantifolia* leaf extracts against Vero cells after 5 and 24 h of incubation

Dilution	Cytotoxicity (%) of *Phyllanthus emblica* Incubation period		Cytotoxicity (%) of *Carica papaya* Incubation period		Cytotoxicity (%) of *Sysygium aromaticum* Incubation period		Cytotoxicity (%) of *Citrus aurantifolia* Incubation period	
	5 h	**24 h**	**5 h**	**24 h**	**5 h**	**24 h**	**5 h**	**24 h**
1/4	73.033	88.73	102.25	152.32	124.27	87.26	104.99	104.55
1/8	14.91	39.89	80.41	134.84	101.1	39.37	83.96	91.16
1/16	16.3	-31.07	84.90	130.59	77.73	20.95	75.33	53.26
1/32	6.82	-55.79	82.10	133.11	73.51	-16.33	82.96	45.83
1/64	7.89	-35.62	83.94	121.75	-26.32	-33.15	22.23	-17.61
1/128	-6.35	-30.92	56.13	76.93	-28.17	-44.42	35.56	-31.52
1/256	-13.85	-38.09	4.64	19.12	-22.72	-57.66	25.97	-34.47
1/512	-13.69	-21.97	1.89	2.67	-21.66	-70.73	11.26	-30.51
1/1024	-12.05	-46.23	-10.50	7.26	-21.61	-89.31	5.82	-25.73
Negative control	0	0	0	0	0	0	0	0
Positive control	100	100	100	100	100	100	100	100

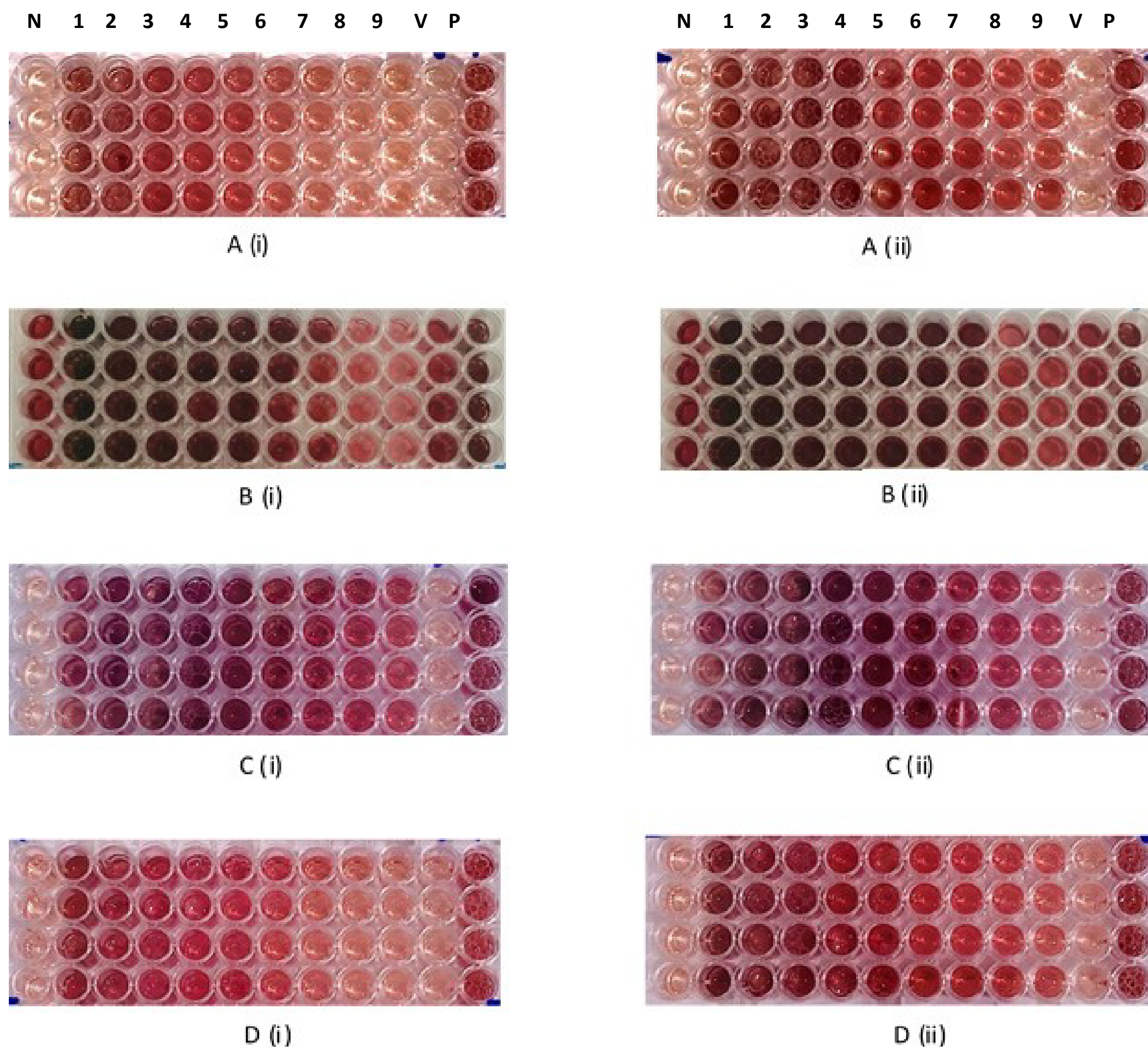

Figure 2: Cytotoxicity-driven colour intensity changes of wells with C6/36 cells at different concentrations of (A) *Phyllanthus emblica,* (B) *Carica papaya,* (C) *Sysygium aromaticum and* (D) *Citrus aurantifolia* leaf extracts after 5 (i) and 24 h (ii) of incubation.

N – Negative Control; 1 – 1/4 concentration; 2 – 1/8 concentration; 3 – 1/16 concentration; 4 – 1/32, concentration; 5 – 1/64 concentration; 6 – 1/128 concentration; 7 – 1/256 concentration; 8 – 512 concentration; 9 – 1/1024 concentration; V – Vehicle control; P – Positive control.

Table 2: Percentage cytotoxicity of *Phyllanthus emblica, Carica papaya, Sysygium aromatic* and *Citrus aurantifolia* leaf extracts against C6/36 cells after 5 and 24 h of incubation

Dilution	Cytotoxicity (%) of *Phyllanthus emblica* Incubation time		Cytotoxicity (%) of *Carica papaya* Incubation time		Cytotoxicity (%) of *Sysygium aromaticum* Incubation time		Cytotoxicity (%) of *Citrus aurantifolia* Incubation time	
	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h
1/4	99.62	101.17	85.55	233.77	134.26	233.39	99.42	119.10
1/8	99.20	99.82	38.81	126.85	122.83	164.99	80.34	96.59
1/16	98.37	99.23	33.13	90.64	93.89	111.48	50.09	63.43
1/32	97.44	90.11	33.11	52.22	65.61	64.77	36.46	42.04
1/64	89.58	56.89	31.11	36.54	60.02	47.41	12.23	31.42
1/128	54.60	36.02	27.36	22.86	40.90	31.25	7.12	20.14
1/256	36.40	24.32	18.09	20.20	37.07	24.23	7.44	9.21
1/512	25.58	18.64	14.37	15.49	19.09	17.87	-7.19	5.19
1/1024	17.61	12.72	11.71	12.94	20.63	11.64	-8.99	1.73
Negative control	0	0	0	0	0	0	0	0
Positive control	100	100	100	100	100	100	100	100

Table 3: Difference in the percentage cytotoxicity of the different leaf extracts between the two time periods (5 and 24 h) based on the paired *t* test results

Leaf extracts of	Difference in the percentage cytotoxicity between the two time periods (5 and 24 h) in Vero cells	Difference in the percentage cytotoxicity between the two time periods (5 and 24 h) in C6/36 cells
Phyllanthus emblica	$p = 0.022$*	$p = 0.029$*
Carica papaya	$p < 0.0001$*	$p = 0.052$
Sysygium aromaticum	$p = 0.013$*	$p = 0.569$
Citrus aurantifolia	$p = 0.006$*	$p < 0.0001$*

*There is a significant difference between 5 and 24 h incubation period ($p < 0.05$) in the percentage cytotoxicity and 24 h of exposure produced more cytotoxicity than 5 h exposure in Vero cells. The same was observed for *P. emblica* and *C. aurantifolia* leaf extracts in C6/36 cells. However, *C. papaya* and *S. aromaticum* leaf extracts did not exhibit significantly different percentage cytotoxicity between 5 and 24 h of exposure in C6/36 cells.

To assess the difference in the percentage cytotoxicity of the different leaf extracts between the two time periods (5 and 24 h), we conducted a paired *t* test with 95% confidence level. According to the Table 3 results, there were significant differences between 5 and 24 h incubation period for all four plant leaf extracts in Vero cells ($p< 0.05$). However, only *P. emblica* and *C. aurantifolia* leaf extracts showed significant differences between 5 and 24 h incubation period in C6/36 cells ($p< 0.05$). *C. papaya* and *S. aromaticum* did not exhibit significant differences between 5 and 24 h incubation period in C6/36 cells cells ($p> 0.05$).

DISCUSSION

The cytotoxicity assay used in the study quantitatively measured LDH, a stable cytosolic enzyme released upon cell lysis. LDH is measured with a 30-minute coupled enzymatic assay, which changes the tetrazolium salt (iodonitrotetrazolium violet, INT) into a red formazan compound. The colour formed is proportionate to the number of lysed cells. Measuring the LDH using tetrazolium salts in combination with diaphorase have been used widely for many years (Nachlas *et al*., 1960) for cytotoxicity testing. Variations in this method have been minimized to measure the cytotoxicity of extracts more accurately (Korzeniewski and Callewaert, 1983; Decker andLohmann-Matthes, 1988).

The substrate mix of this assay comprises lactate, NAD+, INT dye and diaphorase. When *P. emblica, C. papaya, S. aromaticum* and *C. aurantifolia* leaf extracts were added, the cell membranes of the Vero cells and C6/36 cells were lysed. Then the cells release the intercellular constituents with the LDH. Then lactate reacts with the released LDH and forms pyruvate by converting the NAD+ to NADH. This NADH is measured using the INT dye and diaphorase forming the Formazan, an orangish-red colour. The formazan production amount is proportional to the amount of LDH released.

In our experiment, one plate was incubated for 5 has LDH has a half-life of 9 h and the other plate was incubated for 24 h as the test exposure period was 24 h in antiviral activity testing experiments.Percentage cytotoxicity data of four plant extracts in dilutions of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024 are given for Vero

cells in Table 1 and C6/36 cells in Table 2. Colour intensity and absorbance values decreased with the *P. emblica, C. papaya, S. aromaticum and C. aurantifolia* leaf extract in decreasing concentrations. High concentrations of these leaf extracts had high LDH activity and cell lysis. High concentrations of *P. emblica, C. papaya, S. aromaticum,* and *C. aurantifolia* leaf extracts were cytotoxic to both cell types.

Taken the period of exposure, we were able to observe differences in the percentage cytotoxicity among different leaf extracts between the two time periods (5 and 24 h) in Vero and C6/36 cells. This difference may be that the cytotoxic activity increases with an increase in the duration of exposure as also demonstrated by a previous study (Ugartondo *et al*., 2008). *P. emblica, C. papaya, S. aromaticum,* and *C. aurantifolia* leaf extracts showed a dose and duration of exposure dependent cytotoxicity in Vero cells. Dose and duration of exposure dependent cytotoxicity was observed for *P. emblica* and *C. aurantifolia* leaf extracts in C6/36 cells too. Although, *C. papaya* and *S. aromaticum* leaf extracts exhibited different dose dependent cytotoxicity, these extracts did not exhibit duration of exposure dependent cytotoxicity between 5 and 24 h of exposure in C6/36 cells. The reason for the latter finding is not clear.

Cytotoxicity data of these leaf extracts in Vero cells helps select the minimum toxic concentrations for testing the antiviral activity of these extracts for the pre-clinical development of antiviral drugs. High concentrations of these leaf extracts were also cytotoxic to C6/36 cells. Cytotoxicity data of these leaf extracts in C6/36 cells helps to test the larvicidal, insecticidal and ovicidal activity against *Aedes* mosquitoes. These findings can be used to control *Aedes* mosquito density. Overall, the cytotoxicity experiments help understand the dose and duration of exposure-dependent cytotoxicity of *P. emblica, C. papaya, S. aromaticum* and *C. aurantifolia* leaf extracts to use the optimal concentrations in the antiviral and ovi/larvicidal testing.

CONCLUSIONS

High concentrations of *P. emblica, C. papaya, S. aromaticum,* and *C. aurantifolia* leaf extracts caused a high rate of cell lysis. High concentrations of these leaf extracts were cytotoxic to both Vero and C6/36 cells.

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DECLARATION OF CONFLICT OF INTEREST

The authors have no conflicts of interest regarding the publication of this paper.

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