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Antiviral activity of a standardized root water extract of *Eurycoma longifolia* (Physta®) against dengue virus

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Abstract. The aim of this study was to investigate the antiviral property of *Eurycoma longifolia* Jack (EL) against dengue virus. A propriety standardized extract of *Eurycoma longifolia* Jack (Physta®) was tested for anti-viral activity after viral adsorption in Vero cell line. Viral yield was measured by qRT-PCR in four serotypes of dengue virus. The antiviral activity was further investigated in an *in vivo* AG129 mouse model for dengue inhibitory candidates. 100 mg/kg EL extract was fed twice daily and challenged with a lethal dose of (~$1x10^5$ PFU per mouse) of DENV-2 over a period of six days. Antiviral activity with IC_{50} of 33.84, 33.55, 58.35 and 119 µg/ml for DENV-1, DENV-2, DENV-3 and DENV-4 serotypes respectively was observed. The selectivity index (SI) values determined as the ratio of cytotoxic concentration (CC_{50}) to inhibitory concentration (IC_{50}) was the lowest for DENV-2 at 28.9. The dengue virus (DENV) replication measured by qRT-PCR showed a reduction of 100% for DENV-1, DENV-2, DENV-3 and 80% for DENV-4 at day 2 of exposure. In the *in vivo* AG129 mouse model, a lower weight reduction, 30% lower viral load and 12% higher platelet in the extract group compared to the control was observed at day 6. The extract of *E. longifolia* has potential anti-dengue properties with improving trends in platelet counts. *E. longifolia* supplementation is potentially a two-pronged approach in treating dengue fever.

INTRODUCTION

Dengue infection is a serious viral disease that is endemic in tropical and subtropical areas of the world (Gubler, 2006; CDC, 2010). The dengue virus, from the *Flaviviridae* family, rapidly spreads through mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. This mosquito-borne disease poses a threat to at least 2.5 billion people of tropical and subtropical regions (Kyle & Harris, 2008). Dengue virus (DENV) is an enveloped virus with four known genotypes (DENV-1, DENV-2, DENV-3 and DENV-4). The DENV-2 is currently the most lethal (Goel *et al.*, 2004). Infection with the virus can either be silent or mild febrile leading to dengue fever (DF) or more severe to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The latter two are often caused by a subsequent secondary infection by other serotypes of the virus. With half of the world's population residing in the tropics, an estimated 50 million cases of dengue fever (DF) annually which could lead to fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) can be expected

(Gibbons & Vaughn, 2002). Dengue poses a threat of global scale. With the current dengue epidemic distributed mostly in urban and suburban areas, it has become a focus of international public health awareness. The difficulty to control the outbreak in highly populated area in the cities makes the epidemic more lethal. The incidence of dengue in Malaysia has increased from 44.3 cases per 100,000 population in 1999 to 181 cases per 100,000 in 2007, an increase of almost six times (Lie *et al.*, 2016; Mia *et al.*, 2013). The high incidence of dengue with its' accompanied fatalities, is a huge financial burden to the country's' healthcare fund. As to date, no effective anti-dengue treatment is available although several clinical studies are underway (Marimuthu & Ravinder, 2016). A vaccine by the brand name Dengvaxia developed by Sanofi Pharmaceuticals became commercially available in the Philippines and Indonesia but is still threatened to be recalled as it made the children sick (Villar *et al.*, 2015). The vaccine was however not fully effective and may worsen conditions in first timers of dengue infection (Dans *et al.*, 2018). The current treatment are analgesics, fluid balance, electrolytes, blood clotting parameters and platelet replacement (Goel *et al.*, 2004). Therefore, finding an antiviral is an urgent need for this disease.

Herbals and traditional medicine have been reported to possess antiviral properties. Several plants and bioflavonoids have reported activities against DENV virus *in-vitro* (Kadir *et al.*, 2013; Chan *et al.*, 2004). A popular plant among the natives of Malaysia, the root of Tongkat Ali (*Eurycoma longifolia*) has been traditionally used for the treatment of malaria and fever (Bhat & Karim, 2010). Subsequent research validated this use by the antiplasmodial effects of the plant (Chan *et al.*, 2004; Kuo *et al.*, 2004). Anti-microbial effects (Kong *et al.*, 2014) and inhibition of tumor promoter-induced Epstein-Barr virus activation was also reported (Jiwajinda *et al.*, 2002). An increase in platelet was observed with *E. longifolia* supplementation in humans (George *et al.*, 2016). Viremia correlates with the severity of the disease hence a treatment that can reduce the level of viral load could possibly reduce the severity of the disease. In this study we investigate the role of *E. longifolia* against different stages of all four genotypes of DENV *in vitro* replication. The efficacy of the extract *in vivo* would additionally provide a greater understanding of its' mechanism in treating dengue fever holistically. The AG129 mouse model can be used to investigate dengue inhibitory candidates as it allows for infection by the four serotypes of dengue virus (DENV), whereby replication in relevant cell type is supported and the tissue types are comparable to human infection (Williams *et al.*, 2009). It allows for antibody-mediated protection and DENV infection is enhanced. The relevance of AG129 mice model is that it develops acute, lethal and infection is disseminated with viral loads which are systemic. This is typically characteristic of illness caused by dengue infection (Zellweger & Shresta, 2014. The D2Y98P is a Dengue virus (DENV) strain serotype 2 isolated in 1998 from a DENV-infected patient in Singapore which is transmitted only through mosquito cells (Tan *et al.*, 2010). The benefits of using the D2Y98P strain is its' ability to induce a virulent phenotype in AG129 (type I/II interferon receptor-negative) mice with low viral load, without the need for mouse-adaptation. The resulting viral replication and dissemination manifests itself clinically typical for dengue infection. A lower dose challenge of D2Y98P can be used to cause lethal disease within 10 days, when administered subcutaneously (Tan *et al.*, 2011). The objective of this study was to evaluate the *E. longifolia* for anti-viral properties against dengue virus in an *in-vitro* and *in vivo* mouse AG129 model.

MATERIAL AND METHODS

Plant extract

The plant extract was a standardized water extract of *E. longifolia* trademarked Physta® and manufactured under GMP, which was provided by Biotropics Malaysia

Berhad, Selangor, Malaysia. The extract was lyophilized and MilliQ water was used to dissolve the extract. The stock solution was stored at -20°C. The stock solution was diluted using cell culture medium and sterilized with 0.2 micron pore size syringe filter (Millipore, MA, USA) right before each experiment.

Cell cultures

The C6/36 mosquito cell line derived from *Aedes albopictus* was used for the propagation of all DENV isolates used in the investigation. Vero (African green monkey kidney) cell line was used for the evaluation of antiviral activity. The cell lines were maintained and propagated in Eagle's minimal essential medium (EMEM) (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA). The C6/36 and Vero cells were incubated at 28°C and 37°C in the presence of 3% and 5% CO_2 respectively. At the time of virus inoculation and antiviral assays, the concentration of FBS was reduced to 2%. Four different clinical DENV isolates representing the four serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) were used in this study. Cell lines and virus were provided by Virology laboratory of the Tropical Infectious Disease Research and Education Center, Faculty of Medicine, University of Malaya (Kuala Lumpur, Malaysia) and genotyped using full genome sequencing method by the same group. All the four clinical isolates were propagated in C6/36 cell line. After titration of the virus isolates, viral stocks were stored at -80°C for further use.

***In vitro* cytotoxicity assay**

An MTS assay was performed to determine the toxicity of the extract against cells. The confluent Vero cells were treated by different concentrations of the extract in triplicates in a 96-well microplate. The treated cells were incubated for 2 days, at 37°C followed by the addition of 15 µl of MTS solution (Promega, WI, USA) to each well. The microplate was incubated at 37°C for 4 hours. Then, 100 µl of the solubilization/ stopping solution was added to each well. The optical density (OD) of all wells including non-treated cells were read calorimetrically by a plate reader using 570 nm wavelength filter (TECAN, Mannendorf, Switzerland). Cytotoxicity of the extract was calculated using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA) along with its dose-response curve plotting.

Antiviral activity assays

In order to determine the effects of the extract against Dengue viruses (DENVs), *in vitro* confluent monolayers of Vero cell line were infected with multiplicity of infection (MOI) 0.1 of each DENV serotypes followed by virus adsorption for 1 hour at 37°C. The infected monolayer was rinsed twice with sterile PBS to eliminate the unabsorbed virus and supplemented with 2% FBS containing Eagle's Minimum Essential Medium (EMEM) (Gibco, NY, USA) with different concentrations of the extract. Later, the plates were incubated at 37°C for 2 days in the presence of 5% CO_2. DENVs yield was then evaluated by quantitative RT-PCR.

Quantitative RT-PCR

Each step of qRT-PCR was carried out in a final volume of 20 µl containing 5 µl of diluted RNA, 1 µl of probe/primer mix, 10 µl of real time master mix and 4 µl of nuclease-free water (genesig standard kit). Quantitative PCR measurement was performed using StepOnePlus real time PCR system (Applied Biosystems, USA) according to the manufacturer's protocol. Raw data was analyzed with StepOne™ Software v2.2.1 to determine baseline and threshold for computed tomography (CT). The percentages of DENV yield inhibition were obtained by comparing against untreated controls maintained in parallel. Data from triplicate experiments were plotted using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA.)

***In vivo* study of the efficacy of extract against DENV**

This study tested the efficacy of the extract in 6-8 week old female AG129 mice in

accordance with the guidelines set by the Noble Life Sciences (NLS) Animal Care and Use Committee, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), in accordance with the Public Health Service Policy, U.S. Dept. of Agriculture (USDA).

The AG129 mice were obtained from B&K Universal and challenge with Dengue virus (DENV) in a laboratory. Mice randomly assigned into Groups 1 and 2 were challenged with a lethal dose (~1x10^5 PFU per mouse) of Dengue virus serotype 2 (strain D2Y98P) via the subcutaneous (SC) route (0.200 ml) and were kept under *ad libitum* feeding conditions with 12 hr cycles of light and darkness. Each group had 6 mice (Martinez-Gutierrez *et al.*, 2014). Either test compound or PBS was administered to both groups via oral gavage (0.100 ml) twice daily (every 12 hours) for a total of 6 days post-challenge starting 1 hour post challenge. Both groups were monitored for weight loss, morbidity, and mortality for 15 days post-challenge. Survival and health of each mouse are evaluated once a day using a scoring system of 1-7 described in Table 1 (Tang *et al.*, 2016).

Mice displaying severe illness as determined by >20% weight loss, a health score of 5 or above, extreme lethargy, and/or paralysis were euthanized. Mice in both groups were test bled via retro orbital route on Day 6. One hundred microliters (0.1 ml) of whole blood from each sample was assessed for platelet counts. The remaining portion of each sample was processed for serum assessed for viral load via immunoplaque assay.

Table 1. Severity scores for test animals

Score	Abbreviation	Description
1	H	Healthy
2	SR	Slightly Ruffled
3	R	Ruffled
4	S	Sick
5	VS	Very Sick
6	E	Euthanize
7	D	Found Deceased

Statistical analysis

The half maximal cytotoxicity concentration (CC_{50}) and half maximal inhibitory concentration (IC_{50}) were used as the main parameters in this investigation. Selectivity index value (SI) was determined as the ratio of CC_{50}/IC_{50} of the compound. GraphPad PRISM for Windows, version 5 (GraphPad Software Inc., San Diego, CA, 2005) was used for all statistical analyses.

RESULTS

Cytotoxic activity of the extract

Cytotoxicity of the extract on Vero cells were evaluated using the MTS assay. The related half maximal cytotoxic concentration at 50% (CC_{50}) values for day 4 were calculated using Graph Pad Prism 5 (GraphPad Software Inc., San Diego, CA) (Figure 1a). The CC_{50} value for day 4 was 968.5 µg/ml. The maximum non-toxic (MNTD) dose was 480.1 ug/ml.

***In vitro* effect of *E. longifolia* extract on DENV replication**

Virus yield reduction assay using the specific qRT-PCR for all 4 DENV serotypes to evaluate the *in vitro* anti-dengue activity of the extract, confirmed antiviral effects by virus yield reduction based on the measurement of DENVs RNA copy number after 2 days post-treatment (Figure 1b). The extract exhibited a dose-dependent inhibition effects against all 4 DENV genotypes replication in Vero cells with a half maximal inhibition concentration (IC_{50}) values presented in Table 2. It was demonstrated that the extract showed significant antiviral activity against different genotypes of DENV. The SI values determined as the ratio of cytotoxic concentration (CC_{50}) to inhibitory concentration (IC_{50}) was the highest for DENV2 at 28.9.

***In vivo* effect of *E. longifolia* extract against DENV infected mice**

All mice receiving the extract succumbed to infection with a mean time to death of

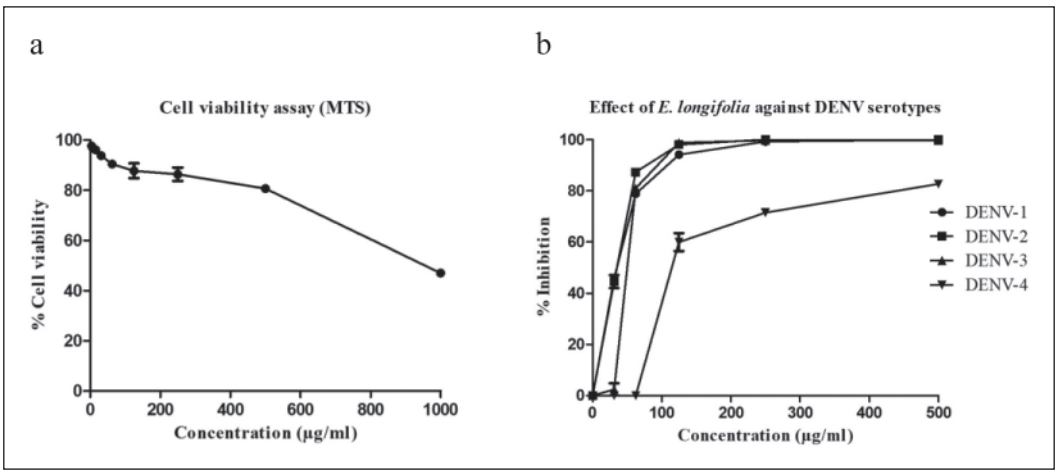

Figure 1a. Cytotoxicity of EL extract against Vero cells. The MNTD of the extract was 500 µg/ml whereby more than 90% of treated cells were viable. b. Anti-dengue virus activity of the extract evaluated by virus yield reduction assay using q-RT-PCR which measured respective DENV RNA copy numbers. The percentages DENV yield inhibitions were obtained by comparing against untreated controls maintained in parallel. Data from triplicate experiments were plotted using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA.).

Table 2. IC_{50} values of EL extract against DENV serotypes

Virus serotype	Antiviral activity, IC_{50} (µg/ml)
DENV-1	33.84
DENV-2	33.55
DENV-3	58.35
DENV-4	119

Days 6-7 post infection (Figure 2a). Similarly, all mice receiving PBS were dead by Day 7 post infection. Therefore, euthanasia was not carried out. Health and weight loss data support these findings in that greater morbidity and mortality and higher weight loss amounts were associated with mouse death (Figure 2b, 2c). However, the mice receiving the extract were found to have a slightly lower viral load at Day 6 post infection than the mice receiving PBS alone (Figure 2d). The analysis of blood platelet demonstrated that the PBS treated mice had a slightly lower platelet count than the test compound treated mice (Figure 2e).

DISCUSSION

Anti-dengue effects of medicinal plants have been reviewed by Kadir *et al.*, 2013. In the current study water extract of *E. longifolia* demonstrated anti-viral property observed with upto 100% inhibition of viral RNA replication in a manner which was dose dependent potentially via the inhibition of RNA polymerase. The lowest IC_{50} was for strain DENV-2 with an IC_{50}=33.55 µg/ml). Different compounds will have dengue inhibitory effect targeting on the different phases of the virus life cycle either through prevention of host cells infection, during the maturation of the virus, viral RNA synthesis, or the production of viral particles. The *E. longifolia* extract appears to inhibit DENV at the level of viral RNA synthesis. Macrophages and monocytes are attacked and infected during a dengue virus infection (Martina *et al.*, 2009). The dengue virus spreads via the infected macrophages and monocytes that pass through the lymphatic system. The dengue virus subsequently

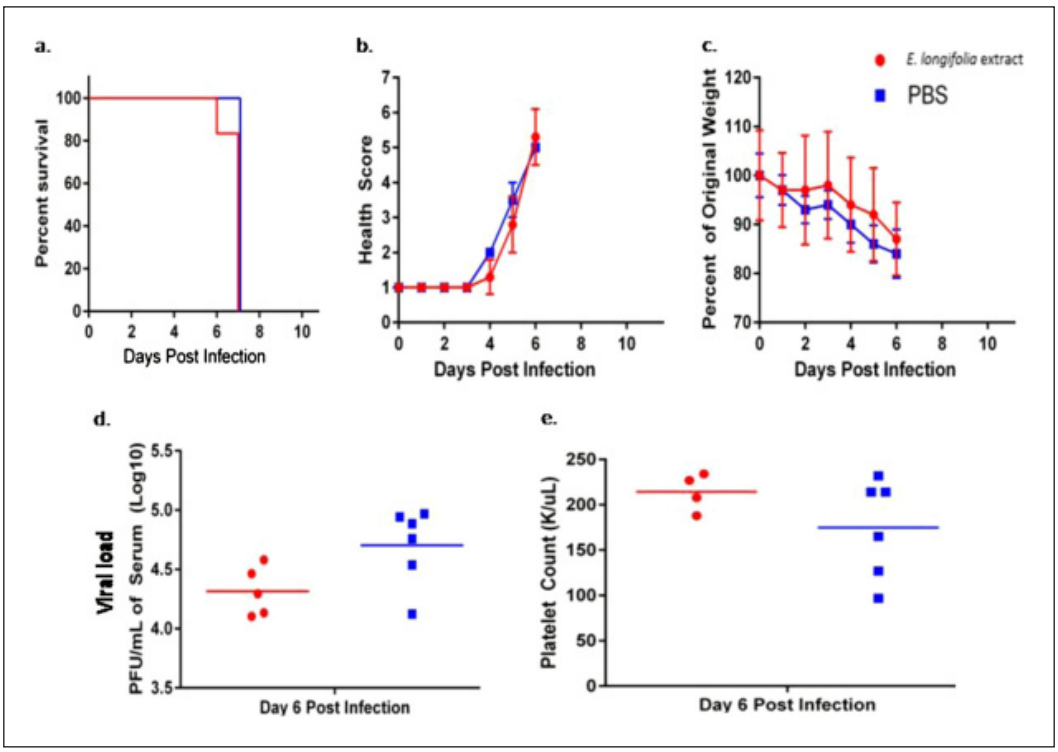

Figure 2. The status of mice post infection of DENV with regards to a. survivals rate, b. health score, c. occurrence of weight loss, d. viral load and e. platelet count.

infects other cells in the lymph nodes, bone marrow and macrophages in both the spleen and liver including monocytes in the blood. This results in viremia with a high degree of dengue virus in the bloodstream. The anti-viral effect in this study was confirmed *in vivo* with a 30% less viral load in supplemented animals. The *E. longifolia* could then be a potential candidate to prevent the further transmission of the virus thus minimizing disease severity, provided the concentration is sufficient to induce such an effect. This study reports for the first time an herbal tested in the mouse AG129 model for anti-dengue activity. The extract demonstrated lesser weight reduction in treated mice, 30% lower viral load, 12% higher platelet for treated mice and a higher health score. A lesser weight reduction (depicting better health) which relates to the better health score could translate to a better wellbeing in humans. The *E. longifolia* was proven for improvement in the quality of life and its' safe use demonstrated in several studies (Tambi *et al.*, 2006; Ismail *et al.*, 2012; Udani *et al.*, 2014). This however, would have to be confirmed in a dengue clinical study. According to Li *et al.* (2013) the acceptable daily intake (ADI) of up to 1.2 g/adult/day was determined for *E. longifolia* extract, calculated based on a 4-week subacute and 13-week subchronic exposure of upto 2 g/kg b.w. per day.

In this study, the *in vitro* effects are also replicated *in vivo*. In addition platelets counts increased. In the study by George *et al.* (2016), lymphocyte, T-cells and naïve T-cells (which contribute to adaptive immunity) improved significantly in subjects that were supplemented with *E. longifolia* extract. An increasing trend in platelets was also observed. The innate and adaptive immunity of the immune system is important in helping the patient recover from dengue infection (Diamond, 2003). In

an adaptive immune response to dengue infection, B cells produces antibodies IgM and IgG which specifically recognizes and neutralizes the dengue viral particles. Also, killer T cells is another adaptive immune response that recognizes and kills the dengue infected cells. The innate immune response on the other hand, responds by helping the antibodies and white blood cells remove the virus. The innate and adaptive immune system hand in hand neutralizes dengue infection to cause the patient to recovers from dengue fever. This attribute of *E. longifolia* may additionally aid in the quicker recovery from dengue infection which was observed in the *in vivo* study, where the Health Score of the animal was slightly of a better trend than the animals which were not supplemented. Thrombocytopenia during dengue fever and especially dengue hemorrhagic fever results in lower platelet counts in patients (Mourao *et al*., 2007). Supplementation with *E. longifolia* also raised platelet counts *in vivo* in this study and clinically (George *et al*., 2016) making it a potential treatment for dengue fever and the more severe dengue hemorrhagic fever.

Another popular plant used for the treatment of dengue is the leaves of *Carica papaya*. This has been clinically investigated in four clinical trials where an increase in platelet was observed (Charan *et al*., 2016). Anti-dengue activities of the extracts from *C. papaya* by using bioinformatics tools reported flavonoid quercetin of *C. papaya* with highest binding energy against dengue virus NS2B-NS3 protease (Senthilvel *et al*., 2013). However, in another study the antiviral activity of quercetin decreased by more than 67% in the presence of 50 ug/ml extract whereas in this study, a decrease of 80% could be seen at the same concentration. In another study, methanolic extracts, containing triterpenoids and flavonoids, showed cytotoxic effects (CC_{50} = 0.6156 mg ml^{-1}), whereas a chloroform extract, rich in alkaloids, tannin and saponin, was non-cytotoxic (CC_{50} = >1 mg ml^{-1}) to LLC-MK2 cells and it showed moderate inhibitory activity (EC_{50} = >1 mg ml^{-1}) against DENV2 with a selectivity index value of ± >1 (Joseph *et al*., 2015). In comparison, the CC_{50} of *E. longifolia* extract was 968.5 µg/ml demonstrating a high level of safety and DENV-2 viral inhibitory activity at IC_{50}= 33.55 µg/ml and a selectivity index of 28.9 exhibiting anti-viral activity with good selectivity index values. In addition, the *E. longifolia* has been reported to stimulate immune cells by increasing T-cells and lymphocytes (George *et al*., 2016) and natural killer cells (Muhamad *et al*., 2015) that could reduce symptoms of dengue fever. The *E. longifolia* is also an antioxidant that could protect against free radical damage arising from the disease which would improve quality of life of patients (Christapher *et al*., 2013).

Though an improving trend was observed with *E. longifolia*, it did not reach statistical difference between groups in the *in vivo* study. Using a lethal viral dose in the AG129 mouse model with a small number of animals inadvertently resulted in the mice dying at the end of the study. It would be beneficial to study the effect of the treatment in a non-lethal dose to establish the efficacy of *E. longifolia* as a broad range treatment solution to dengue fever.

The root water extract of *E. longifolia* demonstrated anti-viral effects against 4 serotypes of dengue virus potentially having a broad range activity against a mutative virus potssibly via the inhibition of the mRNA polymerase as synthesis of new virus was inhibited upto 100% in three of the dengue serotypes. Upon infection in a mice model, the extract was able to protect the mice observed by a lesser weight reduction, 30% lower viral load, 12% higher platelet and a higher health score. Hence, a treatment of this extract in human patients infected with the dengue virus, could provide a multi-pronged approach to dengue treatment, via antiviral effects (thus potentially lowering the intensity of the infection), while concurrently increasing platelet counts and enhancing the quality of life, health and recovery of dengue patients.

In the future, the extract which has proven safety and efficacy in humans from previous clinical studies could be investigated further to establish the efficacy of the extract as a treatment or adjuvant therapy for dengue fever in humans.

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