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ANTIVIRAL ACTIVITY OF PHYSTA®, STANDARDISED WATER EXTRACT OF *EURYCOMA LONGIFOLIA* AGAINST SARS-COV-2: AN *IN VITRO* STUDY

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ABSTRACT

Eurycoma longifolia Jack (Simaroubaceae) root extract is known to exhibit anti-inflammatory, antiviral and immunomodulatory activities. Therefore, E. longifolia, through a multimodal approach could potentially be used in COVID-19 management. However, to date there has been no investigation into the antiviral activity of E. longifolia root extract against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The objective of the study is to investigate the antiviral activity of Physta® (a standardised water extract of E. longifolia) against SARS-CoV-2. A mitochondrial metabolic activity assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to determine the cytotoxicity of the Physta® extract in Vero cells, with concentrations ranging from 1.95 µg/mL to 1,000 µg/mL. Physta® was tested for antiviral activity at six different concentrations, ranging from 3.12 µg/mL to 50 µg/mL. The half maximal cytotoxic concentration (CC_{50}) value of Physta® against Vero cells was estimated at 1,117 µg/mL and the maximum non-toxic dose (MNTD) value was estimated at 60 µg/mL. Physta® inhibited SARS-CoV-2 replication in a dose-dependent manner, and the half maximal inhibition concentration (IC_{50}) was estimated to be 36.3 µg/mL. This study has demonstrated the antiviral activity of Physta® against SARS-CoV-2. Future evaluations in animal and clinical settings should be conducted to determine whether Physta® can be used alone or in combination with other antiviral agents to alleviate COVID-19.

Keywords: Physta®, *Eurycoma longifolia*, COVID-19, SARS-CoV-2, Anti-viral

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INTRODUCTION

The first known case of coronavirus 2019 disease (COVID-19) which was caused by the severe acute respiratory syndrome corononavirus-2 (SARS-CoV-2) was detected in Wuhan, China in December 2019, and by March 2020, the World Health Organization (WHO) declared the COVID-19 outbreak as a global pandemic (World Health Organization 2020). SARS-CoV-2 belongs to the genus *Betacoronavirus* and shares high genetic similarity to two other highly pathogenic coronaviruses, SARS-CoV and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) (Chen *et al*. 2021). Changes in the genetic sequence of the virus caused the emergence of numerous SARS-CoV-2 variants. Among those, up to 15 March 2023, five variants were reported as variants of concerns (VOC) by WHO: Alpha, Beta, Delta Gamma and Omicron (parent lineage) (World Health Organization 2023). WHO has granted emergency use listing (EUL) for more than 10 vaccines (Chavda *et al*. 2023). Even though vaccination is considered the best method to control the pandemic, the acceptance level of vaccines is still low. Based on a systematic review done in the year 2020 (Wang *et al*. 2022) the global acceptance level of COVID-19 vaccines was only 67.8%. However, although a survey from 23,000 respondents in 23 countries found that vaccine acceptance was 79.1% in 2022, an increase from 75.2% in 2021, unfortunately, the authors also reported an increase in vaccine hesitancy by 1.0%–21.1% in various countries (Lazarus *et al*. 2023). Vaccine hesitancy is driven by a complex multitude of factors. Inherently, in some individuals, vaccines can cause mild to severe adverse events, including allergic and anaphylactic reactions, thrombosis, myocarditis, Bell's palsy, transient myelitis, Guillen-Barre syndrome and recurrence of Herpes-Zoster (Lamprinou *et al*. 2023).

To manage the low vaccine acceptance rate, countries with a long history of traditional medicine use, such as India and China, have explored utilising traditional medicine to treat COVID-19. Recently, Thailand regulatory authority has approved the use of an Asian herb Andrographis paniculate to treat early symptoms and manage the severity of COVID-19 (Yearsley 2021). A review on four Malaysian herbs, *Nigella sativa*, *Vernonia amygdalina*, *Azadirachta indica* and *Eurycoma longifolia*, reported their potential as complementary medicine in the management of COVID-19 as the herbs had demonstrated antiviral, anti-inflammatory and immunomodulatory effects in past studies (Lim, Teh and Tan 2021).

Eurycoma longifolia Jack or locally known as *Tongkat Ali*, is a popular herbal tropical plant native to South-East Asian countries. Decoction of the roots of *E. longifolia* are consumed among the Malay community as a tonic for sexual enhancement (Gimlette 1939) and to regain energy after childbirth (Burkill 1996). The plant extract, especially the root extract, has demonstrated antimalaria, antipyretic, anti-diabetic, aphrodisiac and antimicrobial activities (Bhat and Karim 2010). Clinical studies have shown that the supplementation of *E. longifolia* extract is able to restore testosterone levels in both men and women (Henkel *et al*. 2014; George and Henkel 2014; Chinnappan *et al*. 2021). Apart from validating the traditional use of *E. longifolia,* studies were done to explore other benefits of this plant extract. A clinical study done in the Japanese population with low levels of immunity suggested that supplementation of *E. longifolia* improved comprehensive immunity among the subjects (George *et al.* 2016). Recent research shows *E. longifolia* was able to inhibit the replication of dengue virus (DENV) (George *et al.* 2019), possibly due to the presence of various quassinoids in the root of *E. longifolia* (He *et al*. 2023). This study aims to investigate the *in vitro* antiviral activity of water extracts of *E. longifolia* roots against SARS-CoV-2, as the extracts demonstrated antiviral activity in previous studies.

MATERIALS AND METHODS

Plant Extract

The *E. longifolia* extract was supplied by Biotropics Malaysia Berhad. It is manufactured under Good Manufacturing Practice (GMP) and is commercially available under the trade name Physta®. Physta® is standardised based on the Malaysia Standards for *E. longifolia* water extract MS 24089:2011, where the specification is 0.8%–1.5% eurycomanone, with not less than 22% of total protein, 30.0% polysaccharide and 40.0% glycosaponin. Physta® from batch number TA2106051 was used in the study and based on the certificate of analysis, it contained 1.24% of eurycomanone, 29.1% protein, 35.3% polysaccharide and 48.5% glycosaponins. The Physta® extract was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mg/mL stock solution. The stock solution was stored at −20°C until further use. The working solution was prepared by diluting the stock solution in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) and filter-sterilised using a 0.2 µm pore size syringe filter (Millipore, MA, USA) right before each experiment.

Vero cells (African green monkey kidney) Vero E6 (ATCC CRL-1587; passage number 25) were used to propagate and evaluate the antiviral activity of SARS-CoV-2. The Vero cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS). The cells were maintained at 37°C with 5% CO_2. The SARS-CoV-2 strain (MY.TIDREC/6-3/Vero/2020) (Wuhan variant) used in the study was isolated from an anonymous patient sample and confirmed by whole genome sequencing at the Biosafety Level 3 Laboratory at the Tropical Infection Diseases Research and Education Centre (TIDREC), Universiti Malaya, Malaysia. Virus titres were determined by plaque assay and expressed as plaque forming unit (PFU)/mL. The virus stock was stored at −80°C until further use.

Cytotoxicity Assay

A mitochondrial metabolic activity assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to determine the cytotoxicity of the Physta® extracts in Vero cells. Physta® stock was prepared at 100 mg/mL and further diluted in culture medium to obtain concentrations ranging from 1.95 µg/mL to 1,000 µg/mL. Vero cells were seeded at 1×10^4 in a 96-well microplate and treated with 10 different concentrations of the Physta® extract in triplicates. The treated cells were incubated for 3 days at 37°C, followed by the addition of 20 µL of MTS solution (Promega, WI, USA) to each well. The optical density (OD) of treated and non-treated wells cells were read by using a plate reader 490 nm–700 nm wavelength filter (TECAN, Mannendorf, Switzerland). The cytotoxicity of the extract was calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) along with its dose-response curve plotting.

Antiviral Screening of Physta® Extract against SARS-CoV-2

Approximately 100 µL containing approximately 50 PFU SARS-CoV-2 was added to an equal volume of diluted Physta® solution. The final concentration of the Physta® solution in the mixture was 0, 3.12, 6.25, 12.5, 25 and 50 µg/mL, respectively. The mixture was then added to confluent Vero cell monolayers for virus pre-adsorption for 1 h at 37°C. Thereafter, the mixture was removed and the monolayers were rinsed twice with sterile phosphate buffer saline (PBS) to eliminate the unabsorbed virus. DMEM containing

0.9% carboxymethylcellulose, 2% FBS and Physta® extract (final concentration of 0, 3.12, 6.25, 12.5, 25 or 50 µg/mL, respectively) was used as the plaque medium overlay. The plates were incubated for 72 h at 37°C and 5% CO_2, after which the plates were fixed in 4% formaldehyde and stained with 0.5% crystal violet solution to visualise the virus plaque formation. The virus titres were determined from the plaque count and expressed as PFU/mL. The antiviral activity of Physta® was calculated by the following equation:

$$\text{Percentage of inhibition} = [(\text{number of plaques in control} - \text{number of plaques in treatment})/\text{number of plaques in control}] \times 100\%$$

RESULTS

Cytotoxic Activity of Physta®

The cytotoxicity of Physta® extract in Vero cells was evaluated using the MTS reagent. The related half maximal cytotoxic concentration (CC_{50}) and maximum non-toxic dose (MNTD) values for 72 h were calculated using GraphPad Prism, version 5.0 (GraphPad Software Inc., San Diego, CA) (Table 1 and Figure 1). Cell viability was more than 50% at the highest tested concentration of 1,000 µg/mL. The CC_{50} value of Physta® against Vero cells was estimated at 1,117 µg/mL and the maximum non-toxic dose MNTD value was estimated at 60 µg/mL. According to the CC50 and MNTD value, the safe dose (50 µg/mL) was chosen for antiviral screening against SARS-CoV-2.

Table 1: Cytotoxicity activity of Physta® extract on Vero cells.

Samples	CC_{50} (µg/mL)	MNTD (µg/mL)
Physta® extract	1,117	60

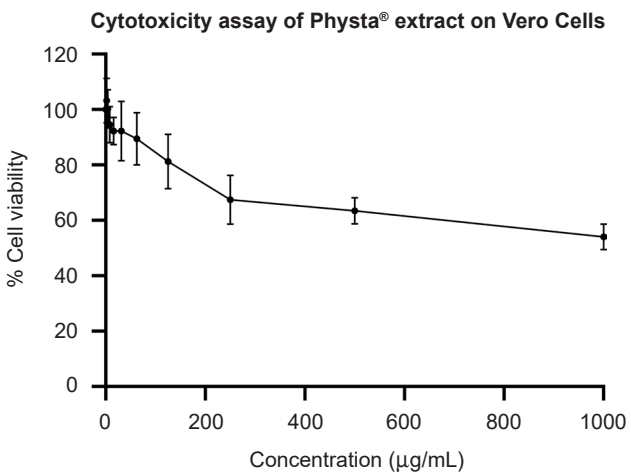

Figure 1: Cytotoxicity of the Physta® extract on Vero cells at various concentrations. The CC_{50} value calculated using GraphPad Prism was 1,117 µg/mL.

Antiviral Screening of Physta® Extract against SARS-CoV-2

The antiviral activity of Physta® extract was evaluated against SARS-CoV-2 using virus plaque forming reduction assay. The results showed Physta® inhibited SAR-CoV-2 replication in a dose dependent manner. At 50 µg/mL Physta® extract, the crystal violet-stained monolayer was lighter than the controls, plaques were visible but not clear enough to be counted (Figure 2A). The results at this concentration were excluded from subsequent analysis. The half maximal inhibition concentration (IC_{50}) was estimated to be 36.3 µg/mL (Figure 2B). The selectivity index (SI) was calculated as the ratio of CC50 to IC_{50} with SI at 30.7 (Table 2).

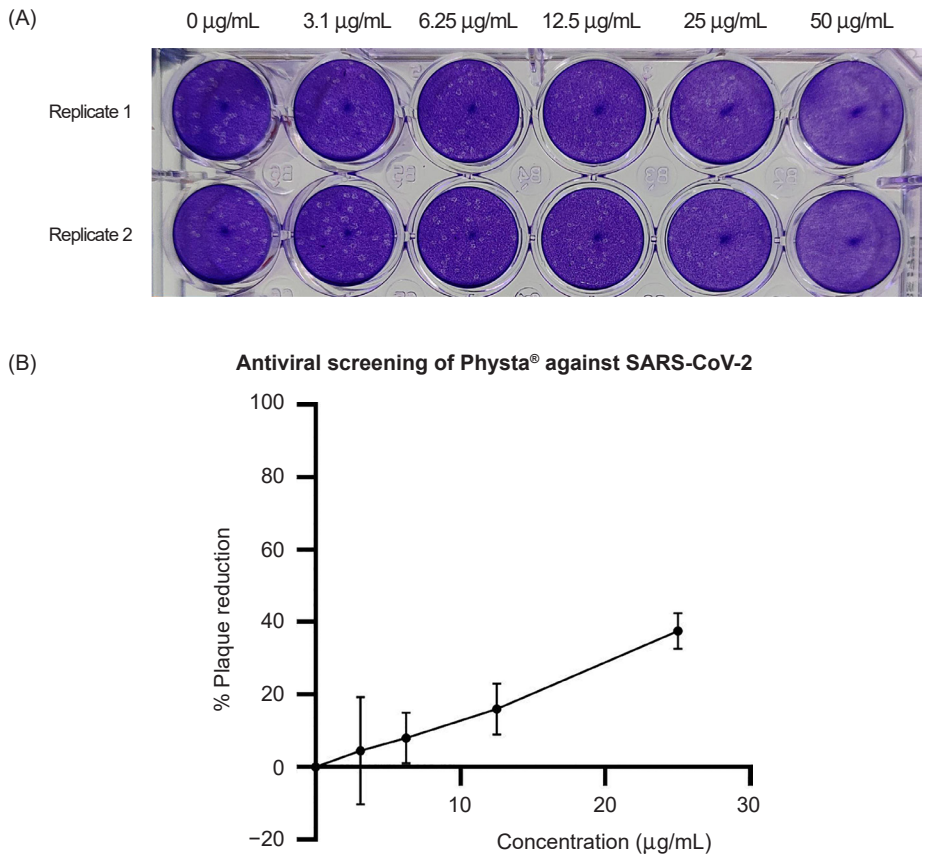

Figure 2: (A) SARS-CoV-2 plaques photo. The photo shows a dose-depending effect of Physta® extract against SARS-CoV-2, concentration of Physta® from left to right: 0 µg/mL, 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL and 50 µg/mL. Rows 1 and 2 representing replicates 1 and 2, respectively. The plaques are visible but not clear to be counted at 50 µg/mL. (B) Physta® extract was tested against SARS-CoV-2 using plaque assay and inhibited the virus with IC_{50} = 36.3 µg/mL.

Table 2: Antiviral screening of Physta® extract against SARS-CoV-2.

Samples	IC_{50} (µg/mL)	SI
Physta® extract	36.3	30.7

DISCUSSION

E. longifolia roots have been found to contain phytochemical compounds in the form of alkaloids, quassinoids, flavonoids, saponins and tannins (Khanijo and Jiraungkoorskul 2016). In this study, the Physta®, *E. longifolia* standardised water extract of roots exhibited antiviral activity against SAR-CoV-2 with an IC_{50} value of 36.3 µg/mL. Eurycomalactone, eurycomanone, dihydroeurycomanone are some of the quassionoids previously isolated from *E. longifolia* (Khanijo and Jiraungkoorskul 2016). Recently a study was done to evaluate the antiviral activity of eurycomalactone and eurycomanone. It was found that both quassinoids have evinced strong antiviral activity against human coronavirus OC43 (HCoV-OC43) and SARS-CoV-2, with IC_{50} values in the range of 0.32 µM–0.51 µM (Choonong *et al*. 2022). The Physta®, *E. longifolia* extract used in the study contains 1.24% eurycomanone and this could have contributed to the anti-SARS-CoV-2 activity observed in the study.

SARS-CoV-2 has four main structural proteins: the spikes (S), the membrane (M), the envelope (E) and the nucleocapsid proteins (N). The main function of the N proteins is to regulate survival of the virus in the host cell by modulating the host cellular machinery *in vitro* (Fehr and Perlman 2015). Based on published data (Fadya *et al*. 2022), generally *E. longifolia* roots contain 53% saponins. According to the manufacturer of Physta®, the *E. longifolia* extract contains 48.5% of gylcosaponins. Saponins are able to inhibit viral capsid protein synthesis which is core in N-type protein and DNA replication (Anand *et al*. 2021). By reducing capsid protein synthase, the saponin is able to inhibit cellular attachment, entry, adsorption and penetration of the virus into the host cell (Anand *et al*. 2021). The saponins could be another contributor to the anti-SARS-CoV-2 activity of Physta®.

In efforts to curb the pandemic, plants with known antiviral and or anti-inflammatory activities were heavily screened for antiviral activity against SARS-CoV-2. In a recent study, a plaque assay was used to evaluate 122 Thai medicinal plant extracts (Kanjanasirirat *et al*. 2020). Plants such as *Boesenbergia rotunda*, *Andrographis paniculate* and *Zingiber officinale* were found to inhibit the replication of SAR-CoV-2 and had IC_{50} values in the range of 4 ug/mL–69 µg/mL. Comparing these values, this study showed that Physta® had good inhibitory activity against SAR-CoV-2 with IC_{50} of 36.3 µg/mL with low toxicity in Vero cells with a cytotoxicity concentration (CC_{50}) of 1,117 µg/mL. It shows Physta® is able to inhibit the virus without harming the cells as reflected by the high SI of 30.7. The safety of Physta® has also been demonstrated in toxicity studies, whereby based on acute and sub-acute studies the no observed adverse effect level (NOAEL) was more than 1,000 mg/kg (Choudhary *et al*. 2012).

When SAR-CoV-2 enters the human body, the innate immune response will release cytokines and interferon to activate the adaptive immune response. Delayed production of cytokines and interferon may lead to inflammation and the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukins IL-6 and IL-8 will be higher than normal. On the contrary, the number of immune cells such as B-cells, T-cells and natural killer cells will be low (Wong 2021). The anti-inflammatory property of *E. longifolia*

has been published whereby bioactive compounds extracted from *E. longifolia* including eurycomalactone, 1-4-1-5β-dihydroklaieanone and 1-3-2-1 dehydroeurycomanone demonstrated potent NF-κB inhibitory effects (Tran *et al*. 2014). Additionally, phenolic components isolated from the roots of *E. longifolia* were reported to significantly decrease the expression of IL-6 in lipopolysaccharide-stimulated raw macrophage cell lines (Ruan *et al*. 2019). A clinical study conducted in Japan demonstrated supplementation of *E. longifolia* can increase T-cells especially $CD4^+$ in the middle-aged population (George *et al*. 2016). The ability of *E. longifolia* in managing inflammation by suppressing pro-inflammatory cytokines and increasing the T-cells can further support use of *E. longifolia* in managing COVID-19. The content of Physta® is characterised based on four main compounds i.e. eurycomanone, total protein, polysaccharides and glycosaponins. In the study, the total extract (Physta®) was used and thus it is currently not clear which compound is responsible for the antiviral activity observed. Further studies on characterising the antiviral activity of fractions high in each compound would shed light on the antiviral mechanisms of the Physta® extract. Apart from focusing on the antiviral activity of a specific compound or fraction of Physta®, further studies should be carried out to confirm the antiviral activity of Physta® against various concentrations of SARS-CoV-2, incubation times and different assay methods, such as the cytopathic effect assay. These parameters were not covered in the current study and can be considered as limitations of the study.

CONCLUSION

The study found that the Physta® standardised water extract of *E. longifolia* roots possessed antiviral activity against SAR-CoV-2 and low cytotoxicity against Vero cells with a SI of 30.7. The extract has high potential to be used as antiviral agent. Further studies are needed to elucidate the mechanism of action of the extract and the compounds responsible for the antiviral activity. Evaluation in animal and clinical settings in future should be conducted so Physta® can be used with or without other antiviral agents in alleviating COVID-19.

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Conflict of Interests

SMC and AG are employees of Biotropics Malaysia Berhad. The authors declare that they have no conflicts of interest.

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