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Research paper

Lignosus rhinocerus TM02® sclerotia extract inhibits dengue virus replication and Infection

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ABSTRACT

Lignosus rhinocerus (*L. rhinocerus*) is a rare in Southeast Asia and has been long used by the natives for various medicinal purposes. In the advent of the mushroom's successful cultivation, a novel cultivar, named *L.rhinocerus* TM02®, was produced and has been tested for anti-inflammatory, anti-proliferative, antioxidant, and other medicinal properties. However, the antiviral effects of TM02® cultivar have not been demonstrated. In this study, the antiviral properties of cold-water extract (CWE) from the sclerotium of *L.rhinocerus* TM02® cultivar were investigated, in inhibiting dengue serotype 2 infection in Vero cells. The authors observed a dose-dependent inhibition on dengue virus replication, particularly during the early stage of dengue infection. The extract also exhibited a significant virucidal effect and displayed a mild prophylactic effect. Hence, in the absence of anti-dengue treatment and limited dengue vaccination, these results indicate that TM02® CWE may serve as an alternative medicine in reducing dengue infection. Further studies are required to identify and verify the bioactive compounds of TM02® CWE that mediate the antiviral activity.

1. Introduction

Lignosus rhinocerus, also known as Tiger Milk mushroom, belongs to the *Polyporaceae* family. It is found in Southeast Asia, especially in Malaysia, South China, and certain parts of Australasia (Nallathamby et al., 2017). This mushroom has been traditionally consumed by the natives for more than 400 years for both health and medicinal purposes. However, the reported benefits of *L. rhinocerus* could not be substantiated due to the scarcity of the mushroom in the wild. A breakthrough in the cultivation method of the mushroom in 2009 (Tan, 2009) enabled large-scale productions of this mushroom. *L. rhinocerus* has been validated *in vitro* and *in vivo* to possess various medicinal properties such as improving cognitive function and neuritogenesis, immune modulation, anti-coagulation, anti-inflammatory, anti-asthmatic, anti-microbial, anti-obesity, anti-cancer, antiviral and antioxidant properties (Ellan et al., 2019; Nallathamby et al., 2017).

The cultivation technology has also led to the development of a novel cultivar, *L.rhinocerus* TM02®. Proprietary indoor cultivation method specifically enriches the sclerotium of TM02® with bioactive compounds (Yap et al., 2013). However, the antiviral activity of the *L. rhinocerus* TM02® cultivar is not clear.

Dengue (DENV) is the most rapidly spread mosquito-borne disease, affecting 96 million people and causing about 20,000 death annually worldwide (WHO, 2018). Dengue infection is prevalent in tropical and subtropical areas including Malaysia. In Malaysia, 108 698 dengue cases were recorded in 2014 and this amount is projected to increase exponentially in the coming years (Bujang et al., 2017). Currently, there is no antiviral treatment against dengue and the first dengue vaccine, CYD-TDV or Dengvaxia, has yet to be approved in Malaysia. This is due to several major limitations of this vaccine including moderate efficacy (about 50 %) against serotype 1 and 2 and varied efficacy by age at vaccination and by serostatus at baseline (WHO, 2018). Importantly, vaccination of seronegative individuals with Dengvaxia could instead lead to severe dengue. Thus, at present, Dengvaxia vaccination is only recommended on seropositive individuals (from previous exposure or infection) and individuals between 9–45 years old (WHO, 2018). Therefore, there is still a paucity of effective dengue treatment or prevention method.

Previously, the authors reported that several approved drugs or nutritional supplement exhibited anti-DENV effects and could

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potentially be re-purposed to treat dengue infection (Rothan et al., 2019, 2013). One of the drugs, mefenamic acid, is a non-steroidal anti-inflammatory drug that is clinically used for pain management, as a fever reducer, and headache reliever (Cimolai, 2013). Mefenamic acid also exhibits anti-cancer, antioxidant, and neuroprotective effects (Joo et al., 2006; Kovala-Demertzi et al., 2009; Shiiba et al., 2017; Woo et al., 2004). In this study, the antiviral property of *L. rhinocerus* TM02® cultivar to those of mefenamic acid was compared.

It was found that *L. rhinocerus* TM02® sclerotial cold-water extract (CWE) could reduce dengue virus replication in a dose-dependent manner, significantly inactivate viral particles, and prevent dengue infection in Vero cells.

2. Materials and methods

2.1. Preparation, extraction and fractionation of the sclerotial cold water extract of L. Rhinocerus TM02®

Freeze-dried sclerotial powder of *L. rhinocerus* (TM02®) (0.2 mm sieve) cultivar was a gift from Ligno Biotech Sdn Bhd (Balakong Jaya, Selangor, Malaysia,). The consistency and quality of the production batch were verified by microbial assays and chemical profiling via HPLC-MS/MS (Batch no.: PL/1107/020) as previously mentioned (Yap et al., 2018) and identified by the internal transcribed spacer (ITS) regions of their ribosomal RNA (Tan et al., 2010). The mushroom specimen voucher is deposited in the Royal Botanic Garden Kew, K(M) 177812. The preparation method for the cold-water extract (CWE) was adopted from our previous paper (Yap et al., 2018). In brief, the sclerotial powder was extracted by stirring in cold water at 4 °C for 24 h at a ratio of 1:20 (w/v). TM02® CWE obtained by filtration through Whatman No. 1 filter paper was then freeze-dried. The freeze-dried extract was prepared fresh in serum free DMEM prior to use.

2.2. Virus and cells

Dengue virus serotype 2 (DENV2), Malaysia M2 isolate (GenBank Taxonomy No.: 11,062) was propagated in C6/36 mosquito cells. The mosquito cells were maintained in Leibovitz's L-15 medium supplemented with 10 % fetal bovine serum (FBS) and cultured at room temperature. FBS concentration was reduced to 2% post infection and supernatant containing the virus were snap-frozen in liquid nitrogen and stored in −80 °C freezer. Viral titer was determined via plaque assay using Vero cells (African green monkey kidney cells) which were maintained in high glucose DMEM supplemented with 10 % FBS. The virus stock solution was serially diluted and used to infect Vero cells monolayer for one hour with gentle shaking after 10 min. The cells were layered with DMEM supplemented with 2% FBS and 0.5 % agarose following a washing step. After 72 h, the cells were fixed with 4% paraformaldehyde, washed with 1x phosphate-buffered solution, and stained with crystal violet. The number of plaques was counted to determine the viral titer.

2.3. Cell toxicity assay

Toxicity assay was carried out to determine the maximum non-toxic dose (MNTD) of the extract on Vero cells. Vero cells were seeded in 96-well plate in duplicates and incubated at 37 °C and 5% CO_2. After an overnight incubation, the cells were treated with TM02® CWE in concentrations ranging from 50 to 4000 μg/mL prepared in DMEM supplemented with 2% FBS. Cell viability was analyzed after 48 h using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacturer's protocol. The absorbance at 490 nm for each sample was corrected against no-cell blank and normalized against no treatment control.

2.4. Antiviral assay (post infection)

Vero cells were seeded in 96-well plate. After an overnight incubation, the cells were infected with dengue virus (MOI of 0.1) for one hour with gentle shaking every 20 min. The cells were then washed with serum free DMEM and cultured in maintenance medium (DMEM supplemented with 2% FBS) with or without TM02® CWE (50, 100 or 150 μg/mL). Medium containing 100 μM mefenamic acid (Sigma M4267) served as the positive control for virus inhibition. This concentration was chosen based on the authors previously published result where dengue protease activity was significantly inhibited at this concentration (Rothan et al., 2013). In separate experiments, after one hour of infection, the cells were washed and cultured in maintenance medium (DMEM + 2% FBS). The medium was then replaced with fresh maintenance medium containing 150 μg/mL of the extract at the indicated time points to determine the effective time point for dengue inhibition. After 48 h, cell viability was determined using Non-Radioactive Cell Proliferation assay (Promega, USA) as previously mentioned.

2.5. Virucidal and prophylaxis assays

Vero cells were seeded in 6-well plate and cultured overnight. Virucidal assay: 200 PFU of dengue virus was treated with 150 μg/mL TM02® CWE or 100 μM mefenamic acid for 15 min at 37 °C. Dengue virus without any treatment served as the negative control. Vero cells were infected with the treated viral inocula for one hour with gentle shaking every 10 min. Prophylaxis assay: This assay was performed based on a previously published article (Zandi et al., 2011). Briefly, the cells were treated with 150 μg/mL CWE or 100 μM mefenamic acid for 5 h at 37 °C in the cell culture incubator. Cells without any treatment served as the negative control. The cells were then infected with 200 PFU of dengue virus for one hour with gentle shaking every 10 min. For both assay: After infection, the cells were washed with serum free DMEM and layered with 0.5 % agarose prepared in DMEM supplemented with 2% FBS. Plaque formation was assessed 72 h post infection as previously described. The number of plaques was compared to the negative control and plotted as percent plaque reduction.

2.6. Statistical analyses

Samples were prepared in duplicate for each experiment and the experiments were performed at least three times. The mean and the standard deviation (SD)/standard error of the means (SEM) for the samples were plotted. Unpaired *t*-test with Welch's correction was used to test for statistical significance using GraphPad prism software (CA, USA) where p-values were denoted as follows: p-value <0.05 (*), <0.01 (**), <0.001 (***). Non-significant difference was denoted as n.s.

3. Results and discussion

In this study, the antiviral property of TM02® cold-water extract (CWE) against dengue virus serotype-2 (DENV2) in Vero cells was analyzed.

3.1. Cytotoxicity analysis

Cell viability assay was performed to determine the maximum non-toxic dose (MNTD) of TM02® CWE in Vero cells. Based on Fig. 1, the MNTD of TM02® CWE on Vero cells was 150 μg/mL where cell viability was 116.00 ± 10.15 % with a CC_{50} of 234.3 μM. Viability steadily declined at higher concentrations and plateaued at 2000 μg/mL. Toxicity of the extract may not be an issue *in vivo* as this extract demonstrated no toxicity in rodents with a none-observed-adverse-effect level (NOAEL) of more than 1000 mg/kg. At 1000 mg/kg, TM02® did not cause any anomaly in complete blood count, biochemistry clinical parameters and histopathological analyses of several organs (Lee et al.,

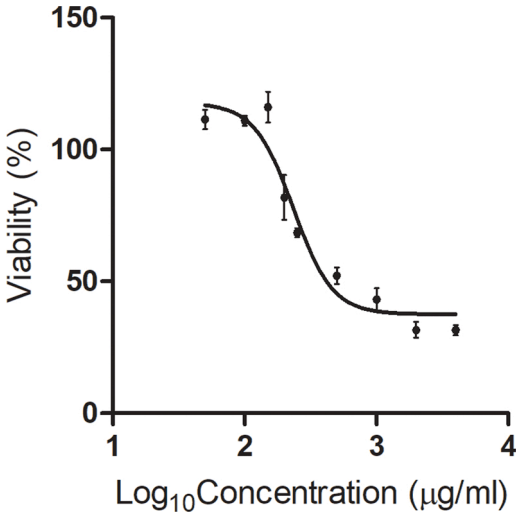

Fig. 1. TM02® CWE toxicity analysis on Vero cells.
Cells were incubated with different concentrations (0.05–4000 µg/ml) of TM02® CWE for 48 h. MTS assay was performed to determine cell viability. Viability was normalized to DMEM + 10 % FBS control (no extract). The mean and standard deviation (SD) of viability percentage at each concentration were plotted from duplicate sample from n > 3.

2011).

3.2. Antiviral effect

Vero cell viability post DENV2 infection with or without TM02® CWE treatment was used to assess the potential antiviral effect of the extract. Without TM02® CWE treatment, the viability of DENV2-infected cells (+DENV2) was 52.6 ± 8.2 % (Fig. 2a). Mefenamic acid treatment (+DENV2/+Mefe), serving as the positive control for dengue inhibition, limited DENV2-induced cell death, resulting in 89.1 ± 6.3 % cell viability (Fig. 2a). Treatment with TM02® CWE at low concentrations (25 and 50 µg /mL) did not rescue cell viability but higher TM02® CWE concentrations (100 and 150 µg /mL) significantly improved viability similar to mefenamic acid treatment (Fig. 2a). Time effective assay revealed that the extract primarily inhibited DENV at the early stage of infection where simultaneous incubation with DENV and 150 µg /mL TM02® CWE substantially rescued cell viability from 48 % (+DENV) to 88 % (Fig. 2b).

Previously, it was reported that 2500 µg/mL cold extract of wild-type *L.rhinocerus* could inhibit dengue by 36 % in plaque reduction assay (Fung and Tan, 2017) whereas the hot water extract exhibited an IC_{50} of 520 µg/mL (Nallathamby et al., 2017). However, these studies were inconclusive as several important information such as the cell lines used and the CC_{50} or MNTD of the extract was not reported. A recent paper compared the antiviral properties of several mushroom extracts and reported significant antiviral activities of *L.rhinocerus* especially in inhibiting DENV attachment and internalization (Ellan et al., 2019). However, it is unclear which cultivar was used in the published paper. In this study, the extract was prepared from TM02® cultivar, a novel cultivar of *L.rhinocerus*. The sclerotial powder of this strain contains higher levels of protein content and mineral when compared to the wild-type cultivar and exhibited better antioxidant and anti-cancer activities (Yap et al., 2013). Ellan et al. also employed a different extraction method to prepare the aqueous-soluble fraction. The cold-water extraction method used in the present study was performed by stirring the sclerotial powder in water for 24 h at 4 °C whereas Ellan et al. prepared their aqueous-soluble extract from the hexane-insoluble fraction (Ellan et al., 2019). Here, an improvement of cell viability post DENV2 infection at a much lower concentration than previous studies was observed potentially by inactivating viral particles or by blocking DENV2 entry into the cells as demonstrated by the time effective assay.

3.3. Virucidal and prophylactic effects and DENV NS2B-NS3 inhibition

A virucidal assay was performed to determine whether the extract could inactivate the viral particles since the most prominent antiviral effect was observed when the cells were simultaneously exposed to both DENV and TM02® CWE. Pre-incubation of DENV2 with mefenamic acid resulted in 30.75 % reduction in plaque formation. TM02® CWE showed comparable virucidal activity where plaque formation was reduced by 31.63 % at its MNTD (Fig. 3a and b).

Prophylaxis assay was performed to determine whether the extract could block virus attachment or internationalization and subsequently prevent virus infection. A five-hour pre-treatment with TM02® CWE resulted in a small but statistically significant plaque reduction (12 %) whereas pretreatment with mefenamic acid prevented plaque formation by 20 % (Fig. 3c). *L.rhinocerus* is typically consumed on a regular basis as a nutritional supplement which may correspond to a prolonged pre-treatment period. Therefore, regular *L.rhinocerus* extract consumption may provide a more significant prophylactic effect. TM02® CWE showed a slightly better DENV2 inhibition in virucidal assay than the viability and prophylaxis assays. Thus, viral particle inactivation could be the main antiviral mechanism for TM02® CWE. Pretreatment with the extract also limited plaque formation potentially indicating a minor inhibition on viral attachment and/or internalization.

In addition to these direct antiviral mechanisms, some fractions or active compounds from the extract also exhibited immunomodulatory and anti -inflammatory effects. Specifically, a water-soluble polysaccharide protein complex (PRW1) from the sclerotium of the mushroom can stimulate macrophages by elevating the production of pro-

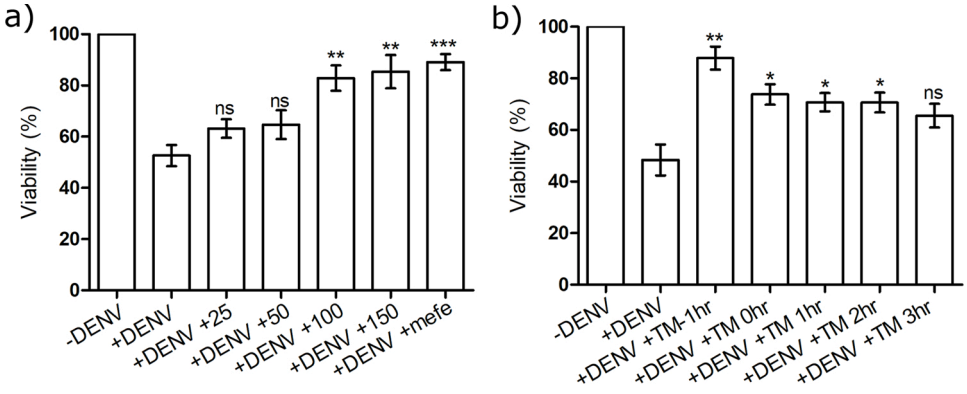

Fig. 2. TM02® CWE rescues cell survival post DENV2 infection.
a) Vero cells were infected with DENV2 in 96-well plate and then treated with TM02® CWE at the indicated concentration or 100 µM mefenamic acid for 48 h. b) Vero cells were infected with DENV2 in 96-well plate. Treatment with 150 µg/mL TM02® CWE were performed at the indicated time points. No infection (-DENV2) and infected Vero cells without any treatment (+DENV2) served as the negative and positive control of DENV infection, respectively. Viability was assessed using Promega MTS assay and each absorbance reading was corrected against the background reading and normalized to the positive control. Each bar represents the mean with SEM for duplicate samples from n = 4.

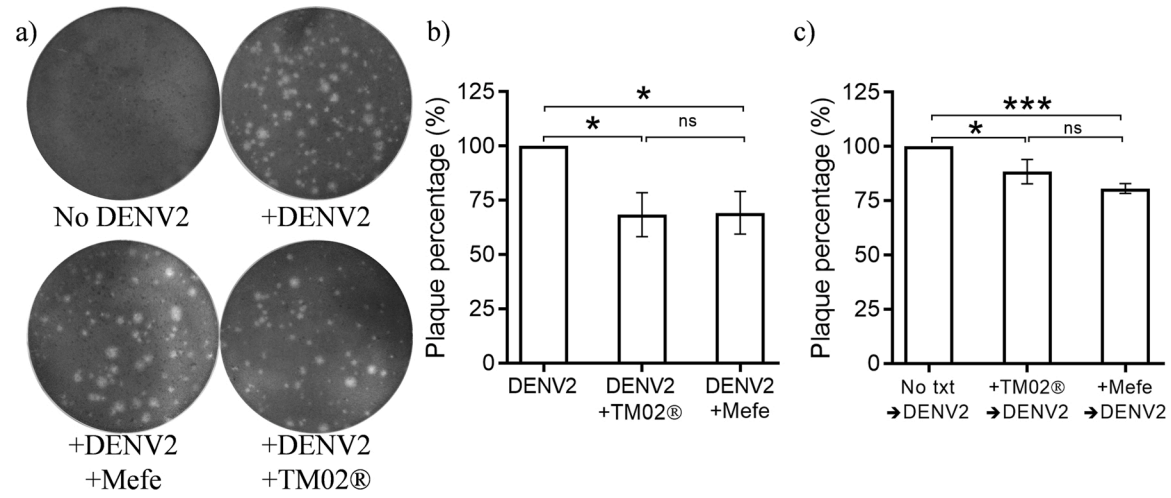

Fig. 3. TM02® CWE exhibits a significant virucidal effect but only a modest prophylactic effect.
a) Representative images of the plaque formation in virucidal assay. b) Plaque quantification in virucidal assay. Viral inoculum (200 PFU) was incubated with 150 μg/mL TM02® CWE or 100 μM mefenamic acid at 37 °C in the incubator for 15 min. Viral inoculum without any treatment was used as the negative control (DENV2) and mefenamic acid treatment served as the positive control for virus inhibition. c) Plaque quantification in prophylaxis assay. Vero monolayers were pretreated with the extract or mefenamic acid for 5 h, followed by (arrow) infection with 200 PFU DENV2. The number of plaques was counted and normalized to the infected cells without any prior treatment (No txt➔DENV). Each bar represents the mean with SD for duplicate samples from n = 3 (for b) and n = 4 (for c).

inflammatory cytokines such as interleukin (IL)-6, IL-12, granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Liu et al., 2016). IL-12 and IL-18 are important cytokines that are required for interferon activity against DENV (Fagundes et al., 2011). Additionally, proteomics analysis of TM02® identified a protein containing fve domain (Yap et al., 2015) which has been reported to enhance interferon-gamma expression and modulate immune response by inducing lymphocyte proliferation (Paaventhan et al., 2003). These findings suggested that bioactive compounds from the mushroom may also indirectly potentiate antiviral activity through cytokine/chemokine signaling especially via interferon activity which is essential for innate immune response against dengue (Aguirre et al., 2012). TM02® CWE has also been shown to exhibit anti-inflammatory effects particularly through the high-molecular weight (HMW) fraction of the CWE (Lee et al., 2014). In dengue patients, several inflammatory cytokines are down-regulated during the early stage of dengue fever but elevated as the disease progresses (Bozza et al., 2008; Rathakrishnan et al., 2012). Therefore, the immunostimulatory effects of PRW1 from the mushroom may be useful in boosting the immune system to prevent dengue infection and the progression of dengue fever into severe dengue whereas the anti-inflammatory effect by the HMW fraction may help in alleviating the “cytokine storm” in patients with severe dengue (Srikiatkhachorn et al., 2017). These indirect antiviral effects may work synergistically with the direct effect on DENV reported here to further augment anti-DENV activities of TM02® CWE.

4. Conclusion

In conclusion, the antiviral properties of *L. rhinocerus* TM02® cold-water extract against dengue virus serotype 2 (DENV2) was investigated in Vero cells. The extract significantly inhibited dengue virus replication, inactivated viral particles and slightly prevented viral infection. Further works are required to determine the bioactive compound(s) that mediate these effects.

Author contribution

Khazali A.S: Investigation, Writing-original draft, Methodology, Validation. **Nor Rashid N.:** Supervision, Conceptualisation and Writing-Review and Editing. **Fung S.Y**: Resources, Writing-Review and Editing. **Yusof R.**: Funding acquisition and Supervision

Credit authorship contribution statement

Ahmad Suhail Khazali: Conceptualisation, Writing-Original draft manuscript, Data analysis. **Nurshamimi Nor Rashid**:Conceptualisation, Data analysis, Reviewing and Editing.**Fung Shin Yee:**Herbal drug preparation, Reviewing and Editing. **Rohana Yusof:** Reviewing and Editing

Declaration of Competing Interest

The authors declare no conflict of interest.

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