In vitro virucidal activity of *Kyllinga nemoralis* aqueous extract against herpes simplex virus

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ABSTRACT

Kyllinga nemoralis is an ignored plant distributed in tropical, subtropical, and warm temperature regions around the world. *K. nemoralis* plant is rich with bioactive molecules exhibiting antiherpetic properties. This study determined the antiviral activities of *K. nemoralis* stem with leaves aqueous extract against herpes simplex virus type 1 (HSV-1). The stem with leaves of *K. nemoralis* plant was extracted using aqueous extraction method. The cytotoxicity of *K. nemoralis* extract was evaluated using a cell viability assay. Plaque reduction assays were carried out to estimate the antiviral activity of *K. nemoralis* extract against HSV-1. Antiviral studies consisted of post-treatment, pre-treatment, and virucidal assay. Cell viability assay revealed *K. nemoralis* stem with leaves aqueous extract is non-toxic toward Vero cells. *K. nemoralis* stem with leaves aqueous extract inhibited HSV-1 replication in Vero cells with 50% effective concentration (EC_{50}) = 0.004 mg/mL and selective index (SI) = 14 when given after adsorption to the cells. The EC_{50} against HSV-1 was 0.013 mg/mL and SI = 4.31 when cells were treated 24 h before virus infection. *K. nemoralis* stem with leaves aqueous extract exhibited direct virucidal activity against HSV-1 with EC_{50} = 0.0065 mg/mL and SI = 8.62. The results presented the potential of *K. nemoralis* as an anti-HSV-1 agent by the following modes: direct damage to viral particle, interruption of virus attachment, and suppression of viral replication cycle.

1. INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is classified under the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, and *Simplexvirus* genus [1]. HSV-1 is highly contagious and still endemic in various parts of the world [2]. Primary HSV-1 infection mainly affects the oral mucosal epithelium and is clinically manifested as herpes labialis or herpes gingivostomatitis [3]. The initial infection can become a latency stage, permanently affecting human hosts' peripheral nervous system [4]. Commercially available antiherpetic drugs have a narrow mechanism of action. For instance, nucleoside and nucleotide analogs only target the viral DNA replication stages; therefore, a complete prevention or cure is not achieved [5]. Hence, it is vital to conduct research on natural products to discover their potential as therapeutic agents against HSV-1 infection. Natural products have distinct structure and chemical compounds which could be an ideal source of new antiherpetic agents [6]. In recent times, bioactive compounds derived from natural products such as plants, microorganisms, marine organisms, fungi, and animal are being explored with great interest as potential antiherpetic agents [7]. Plant-derived bioactive

compounds have been shown to be able to elicit potent antiviral effects against HSV at multiple phases of viral replication including latency stage [8-11]. Besides that, natural botanical products are less cytotoxic toward mammalian cells, produce less undesired secondary effects, and are cheap in terms of production [12].

Kyllinga nemoralis, also known as whitehead spike sedge, poverty grass, white globose spike, or white *Kyllinga* is a monocotyledonous flowering graminoid plant generally similar to grasses, herbs, or rushes [13,14]. The medicinal values of *K. nemoralis* are often attributed to bioactive compounds in the plant. Various plant morphological sections and extracts contain secondary bioactive compounds and metabolites. The chemical compounds are usually identified through a phytochemical screening. A whole plant can be converted into several decoctions through a solvent mixing process to produce various aqueous, chloroform, ethanol, methanol, and hexane extracts [15]. The aerial divisions of *K. nemoralis* aqueous extract contain chemicals such as carbohydrates, phenolic compounds, protein, amino acids, flavonoids, and glycosides [16]. The whole *K. nemoralis* plant aqueous extract possesses potent secondary metabolites such as carbohydrates, saponin, anthocyanin, and coumarins [17]. A combination of roots and leaves sections of *K. nemoralis* has active chemical substances such as phenolic compounds, terpenoids, saponin, glycosides, triterpenoids, flavonoids, and essential oils [18]. The distillate of *K. nemoralis* ethanolic rhizome roots is positive for volatile oil, carbohydrates, and alkaloids [19].

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Polyphenols are secondary metabolites derived from plants, often distinguished by more than 1 hydroxyl (-OH) group on complex aromatic ring or benzene ring formation. Phenolic acids, tannins, and flavonoids are polyphenols that exhibit antiviral, antibacterial, hepatoprotective, and anti-tumor activities [20]. Phenolic compounds alter the conformation and function of HSV-1 envelope proteins, which further prevent viral fusion and penetration [12]. Furthermore, the phenolic compound also inhibits the expression of the viral gene [7]. Flavonoids are diphenyl propane-structured secondary metabolites found in plants. Flavonoids have been known to prevent microbes from invading and infecting hosts. Therefore, plants containing flavonoids are widely used as traditional therapy for treating various communicable diseases caused by microbes [21]. Research in the past has shown evidence of flavonoids exhibiting antiviral activity. Flavonoid compounds interrupt HSV-1 viral entry into host cells by directly inhibiting viral particles [22]. It is a vital discovery because the current antiviral drug, acyclovir (ACV), only inhibits viral DNA replication [23].

The antiviral activity of *K. nemoralis* extract against HSV-1 has been scarcely explored. However, other researchers have investigated this plant's broad curative properties. *K. nemoralis* ethanol extract exhibited intense larvicidal activity against three species of larvae that transmit dengue, malaria, and Japanese encephalitis; the exposure of larvae to plant extract resulted in pathological damages such as cell degradation and lysis [17]. Methanol extract of *K. nemoralis* exhibits antibacterial properties; it showed the highest inhibition against *Streptococcus pneumoniae* growth on the culture medium [24]. Another study reported the anthelminthic activity of the plant's root ethanol extract. These scientific research show that *K. nemoralis* can destroy several types of organisms. However, the antiviral effect of *K. nemoralis* extract has not been investigated. Therefore, in this present study, we explored the possible action of *K. nemoralis* stem with leaves aqueous extract against HSV-1 replication *in vitro*.

2. MATERIALS AND METHODS

2.1. Plant Material

The plants were collected in Kuala Nerus, Terengganu, Malaysia. The stem with leaf of *K. nemoralis* plants (500 g) was cleaned with water and then dried at 27°C or 80 F until it dried thoroughly. The plant parts were then oven-dried at 65–70°C for 48 h. After that, the plant parts were cut into small fragments and ground using electric food processor (Kenwood Ltd., Havant, United Kingdom). The coarse powder was preserved in air-tight container.

2.2. Extraction Method

For preparing aqueous extract of *K. nemoralis*, 100 g powder of the stem with leaf was mixed into deionized water with a ratio of powder to water of 10:1 according to a published method with some modifications [25]. The mixture was boiled for 2 h. The mixture was then allowed to cool down to room temperature before being filtered to collect the extract. After that, the extract was frozen at −20°C for 7 h. Then, freeze-dry technique was performed to lyophilize the extract.

2.3. Cells and Virus

The Vero cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 5% fetal bovine serum (FBS; JR Scientific, USA) and penicillin/streptomycin (100 IU) (Gibco-Life Technologies, USA). Clinical strain of HSV-1 propagation was done in Vero cells cultured in DMEM containing 5% FBS and

incubated at 37°C, 5% CO_2 until cytopathic was observed. After that, plaque assay has been used in determining the titer of virus stock.

2.4. Cytotoxicity Test

Colorimetric analysis in 96-well plate utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was carried out to determine the cytotoxicity level of *K. nemoralis* stem with leaves aqueous extract as previously described [26]. The 50% cytotoxicity concentration (CC_{50}) was characterized as the sample concentration that is able to reduce 50% of cell viability compared to the untreated cells. Briefly, monolayer of Vero cells was seeded in 96-well microplates (2.0×10^4 cells/well) and exposed with several concentrations of extract (31–1000 µg/mL) in triplicates. MTT was added into each well after 48 h of incubation. The plate was further incubated for at least 2 h and dimethyl sulfoxide was added to each well to dissolve the formazan crystals. Optical density of each well was measured using spectrophotometer at 540 nm and the dose-response curve was plotted using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

2.5. Post-Treatment of HSV-1-Infected Cells with Plant Extract

Antiviral activity of *K. nemoralis* stem with leaves aqueous extract against intracellular replication of HSV-1 was carried out by exposing the Vero cells (2.0×10^6 cells/well) with extract after adsorption with 50 plaque-forming unit (PFU) of virus for 2 h at 37°C to permit virus adsorption. The different concentrations of extract (1–31 µg/mL) were mixed with DMEM + methylcellulose immediately after the adsorption period and added into all test wells. The cells were then incubated for 48 h. After incubation, the cells were stained using crystal violet and plaques were counted according to published protocol [27].

2.6. Pre-Treatment of Plant Extract on HSV-1-Infected Cells

The assay was conducted as reported by a previously published method [28]. Initially, Vero cells (2.5×10^6 cells/well) were seeded into 24-well plate and incubated for 24 h. Cells were pre-treated with different concentrations of extract (1–31 µg/mL) for 24 h before being withdrawn and infected with 300 µL of virus at 50 PFU. The cells were incubated at 37°C in 5% CO_2 for 1 h 30 min to ensure the adsorption of virus into cells. After incubation, the cells were stained using crystal violet and plaques were counted. Selective index, SI (CC_{50}/effective concentration [EC_{50}] ratio, where EC_{50} is 50% EC_{50}), was used to evaluate *K. nemoralis* effectiveness as a prospective antiviral agent.

2.7. Direct Virucidal Assay

Extracellular anti-HSV-1 activity of *K. nemoralis* stem with leaves aqueous extract was determined by incubating 50 PFU HSV-1 suspension with equal volume of the various concentrations of extract (1–31 µg/mL) for 30 min at 37°C. Then, viral suspension was infected to the Vero cells for 1 h at 37°C. The cells were washed with phosphate-buffered saline to remove the unabsorbed viruses. Then, the cells were incubated at 37°C for 48 h. Antiviral activity was evaluated by viral plaques reduction assay [29].

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity of *K. nemoralis* Stem with Leaves Aqueous Extract

Cytotoxicity assay was first done ahead of antiviral screening to determine *K. nemoralis* stem with leaves aqueous extract concentration which is cytotoxic to 50% of cell population (CC_{50}). From this CC_{50}

value, lower concentrations which were least toxic or non-toxic to the cells were used in antiviral screening. To evaluate the non-toxic dose, Vero cells were treated with 2-fold serially diluted extract at concentrations ranging from 31 to 1000 µg/mL. In this assay, the CC_{50} value of *K. nemoralis* stem with leaves aqueous extract was determined at 56 µg/mL [Figure 1]. No signs of toxicity were remarked in Vero cells when the extract was added at concentrations ranging from 2 to 31 µg/mL. The cells exhibited a normal monolayer similar to the healthy control cells. No loss of monolayer, granulation, rounding, or dwindling of cells was seen. *K. nemoralis* stem with leaves aqueous extract CC_{50} was considered to be not in the range of cytotoxic compound since the value of CC_{50} of the extract was determined to be higher than minimum value for compound with active cytotoxicity. Any CC_{50} or IC_{50} (50% inhibitory concentration) value of a compound that was <4 µg/mL was considered to have active cytotoxic effect [30].

3.2. Antiviral Assays with *K. nemoralis* Against HSV-1

The extract was further evaluated for their ability to inhibit replication after infection of the cells with the virus (post-treatment), the effect of the extract as prophylactic agent in protecting the cell from virus adsorption and penetration (pre-treatment), and the ability of the extract to act directly against virus virion particle (virucidal). Plaque reduction assays were done to screen for anti-HSV-1 activity using *K. nemoralis* stem with leaves aqueous extract with different concentrations. Extract was added at the different stages of viral replication cycle: (i) treatment for 48 h after 2 h of post-adsorption, (ii) pre-treatment for 24 h after infection for studying, the prophylactic activity, and (iii) direct treatment to cell with virus suspension to evaluate direct virucidal effect.

Potency of some extracts can be assessed using SI. Antiviral agent can be considered to have high potential to be developed as antiviral drug if their SI value is more than 10 [31]. Comparison of SI for the extract between post-treatment, pre-treatment, and virucidal assay is shown in Table 1. SI for post-treatment for the extract showed high values compared to those obtained from pre-treatment and virucidal assay. Therefore, the post-treatment was more efficient in suppressing viral replication compared to pre-treatment and virucidal. Results from our study suggest that *K. nemoralis* stem with leaves aqueous extract shows strong *in vitro* antiviral activity against all stages of the HSV-1 replication cycle such as intracellular virus replication (SI = 14.0). On the other hand, *K. nemoralis* stem with leaves aqueous extract showed mild direct virucidal activity (SI = 8.62), while its prophylactic effect against HSV-1 replication was weak (SI = 4.31).

Post-treatment assay aims to investigate the intracellular HSV-1 inhibitory activity of *K. nemoralis* aqueous extract [32]. The highest plaque inhibition of 74% was observed in the post-treatment assay when HSV-1-infected Vero cells were treated with 31 µg/mL of *K. nemoralis* stem with leaves aqueous extract [Figure 2]. The effectiveness of the plant extract during post-treatment assay was attributed to its ability to inhibit intracellular virus replication upon entry into the cell. Viral gene replication of HSV-1 is a cascade event that involves the production of immediate-early, early, and late genes in sequential order [33]. In addition to the finding that *K. nemoralis* stem with leaves aqueous extracts block intracellular replication on viral entry into cells, the effectiveness of an antiviral agent during post-treatment step may indicate its ability to interfere with viral replication, DNA translation, assembly, and egress of progeny virions from cells [34]. Flavonoid subclasses, especially baicalin, fisetin, and quercetin, are classified as flavonols possessing anti-HSV-1 properties [35]. Flavonols such as quercetin have been found to be actively existing in *K. nemoralis*
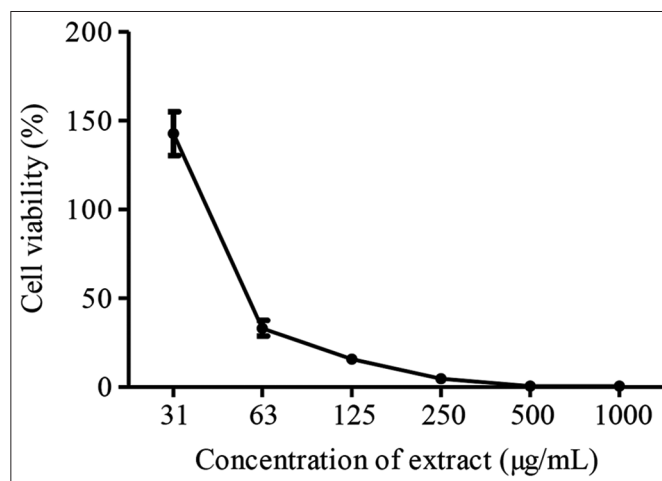

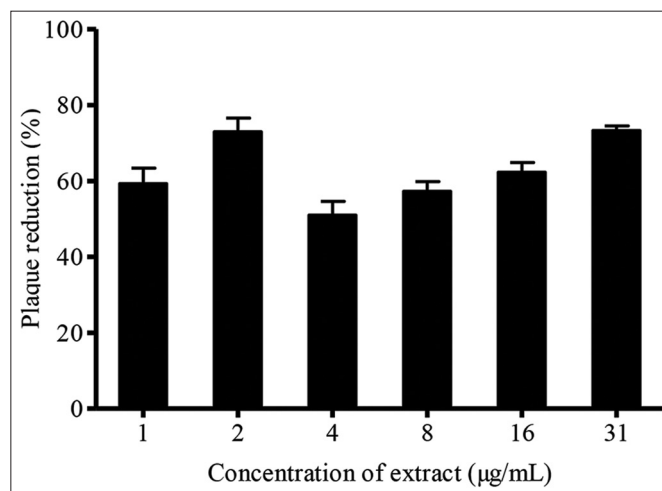**Figure 1:** Cytotoxicity analysis of *Kyllinga nemoralis* stem with leaves aqueous extract.

Figure 2: Effects of post-treatment of *Kyllinga nemoralis* stems with leaves aqueous extract on HSV-1 plaque reduction.

Table 1: CC_{50}, EC_{50}, and SI values of *Kyllinga nemoralis* stem with leaves aqueous extract in post-treatment, pre-treatment, and virucidal assay.

| Assay	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI (CC_{50}/EC_{50})
Post-treatment	56	4	14.0
Pre-treatment	56	13	4.31
Virucidal	56	6.5	8.62

plant extract [36]. This was further supported by the observation in a study in which quercetin compounds vigorously hampered HSV-1 infectivity principally during post-treatment step [37]. Hence, the anti-herpetic activity of *K. nemoralis* aqueous extract could be related to the presence of flavonol sub-compounds such as quercetin. Besides that, there are mounting evidence of plant-derived polyphenols manifesting anti-herpetic efficacy through synergistic interaction during post-treatment stage [38].

Pre-treatment assay evaluated the potential of *K. nemoralis* stem with leaves and roots aqueous extract to interact with host cells to stop viral adsorption into cells. The cells were pre-incubated with plant extract for 24 h to ensure sufficient absorption of respective decoction into cells.

In this assay, the highest plaque inhibition of 65% was examined when Vero cells were pre-treated with 1 µg/mL of *K. nemoralis* stem with leaves aqueous extract [Figure 3]. The EC_{50} of *K. nemoralis* stem with leaves aqueous extract in pre-treatment was higher, 13 µg/mL, than post-treatment, 4 µg/mL. The contrast in findings suggests that stem with leaves aqueous extract has impotent interactions with Vero cells. This ineffectual interactivity may have caused the weak prophylactic activity of stem with leaves extract on host cells. Furthermore, the higher EC_{50} corresponding to a lower SI of 4.31 shows that the extract is relatively toxic to cells when administered as pre-treatment. Therefore, this extract exhibits a weak direct protective effect on Vero cells. The 24 h incubation of cells extract might be inadequate to exert any protective effects on cells. However, this contradicts the above finding in which the extract is toxic to cells when given as pre-treatment, and more prolonged incubation would only contribute to more toxicity. Antiviral activity exhibited by extracts could be due to the inhibition of host cell glycoprotein receptors or prevention of HSV-1 virions binding to host cells. A study has shown that mutated cultured cells lacking glycosaminoglycans such as heparan sulfate reduced the binding efficiency of the virus to cells [39]. Therefore, the destruction of cellular receptors vital for membrane fusion could be the most plausible mechanism exerted by this extract.
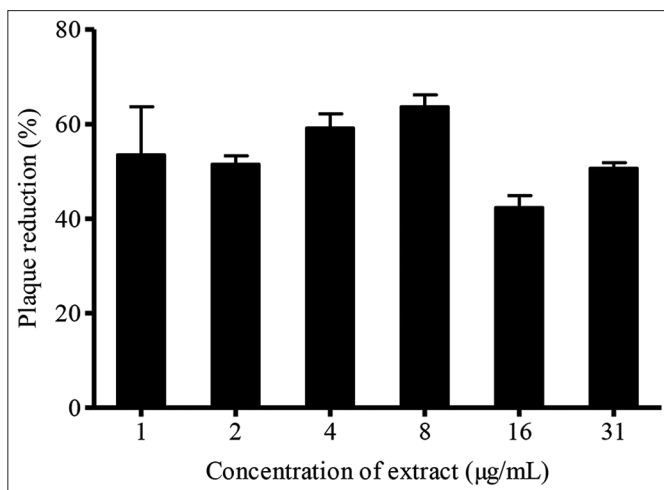
Figure 3: Effects of pre-treatment of *Kyllinga nemoralis* stems with leaves aqueous extract on HSV-1 plaque reduction.
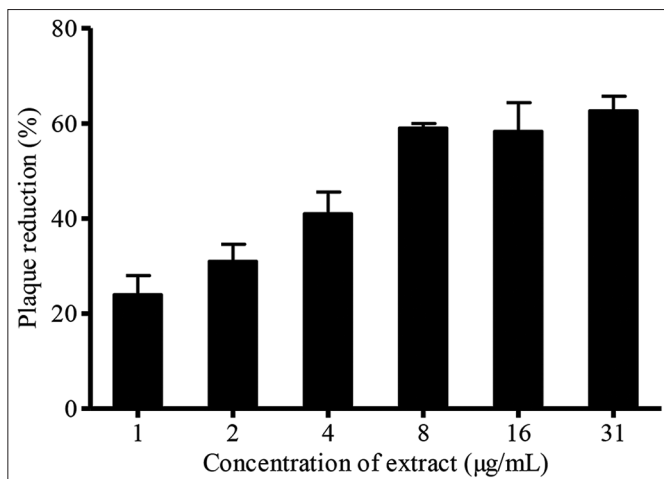
Figure 4: Effects of virucidal assay of *Kyllinga nemoralis* stems with leaves aqueous extract on HSV-1 plaque reduction.

Virucidal assay determined the direct virucidal effect of plant extract on HSV-1. In this assay, *K. nemoralis* stem with leaves aqueous extract inhibited 66% of virus plaque at 31 µg/mL. As for EC_{50}, *K. nemoralis* stem with leaves aqueous extract exhibited a lower value, 6.5 µg/mL [Figure 4]. This indicates that the extract can produce antiviral effects at a lower concentration within the safe cytotoxic span. The direct virucidal activity of *K. nemoralis* stem with leaves aqueous extract shows its ability to interact with and inhibit HSV-1 virions outside Vero cells [40]. An initial deactivation of HSV-1 particle could avert the establishment of primary viral infection [34]. In this study, the direct disruption of virus particles could be a target to suppress initial stages of infection such as virus adsorption, membrane fusion, and entry into host cells [41]. Apart from the whole virus destruction, *K. nemoralis* stem with leaves aqueous extract may have interfered with the viral envelope to conceal viral glycoproteins necessary for HSV-1 attachment or entry into cells. The virucidal effectiveness of natural compound during extract-virus interaction exhibits its potential to prevent virus attachment to host cells. At this stage of investigation, the specific viral glycoprotein/glycoproteins blocked by plant extract to mediate the anti-herpetic mechanism is yet to be identified. Nevertheless, the outcome of the present study confirms the virucidal potential of *K. nemoralis* stem with leaves aqueous extract which is most likely due to the suppression of HSV-1 surface glycoproteins and consequent inactivation of viral particles [42].

4. CONCLUSION

This study presented the potential of *K. nemoralis* aqueous extract as a new anti-HSV-1 agent. A more comprehensive study will be needed to explain the mechanisms involved and to determine whether the antiviral activity of *K. nemoralis* toward HSV-1 could be more potent when purified active compounds are used.

5. ACKNOWLEDGMENTS

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

The data presented in this study are not available on request from the corresponding author.

10. PUBLISHER'S NOTE

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