

Evaluation of cytotoxicity and antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract

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ABSTRACT

This present study aimed to investigate the potential stems and leaves methanolic extract of *Kyllinga nemoralis* as an antiviral agent against Herpes Simplex Virus Type 1 (HSV-1). The leaves and stems of *K. nemoralis* methanolic extract were assessed in the cytotoxicity test using the MTT assays. The plaque reduction assay was evaluated in the post-treatment assay, pre-treatment assay, virucidal assay, time-addition test, and time-removal test. Cytotoxicity screening of stems and leaves of *K. nemoralis* against Vero cells shows that the cytotoxicity concentration of 50% of cell viability (CC_{50}) values was 0.75 mg/mL, which indicates that *K. nemoralis* is non-cytotoxic and safe. The antiviral assay provides a Selective Index (SI) value (CC_{50}/EC_{50}) to determine the good SI for antiviral activity. In post-treatment, the SI value for this extract against HSV-1 was 8.33. In pre-treatment, the SI value for this extract was 21.43. In the virucidal test, the SI value for this extract was 24.19 mg/mL. In the time addition assay, the plaque inhibition reached 43% when the extract was added at 2 h.p.i, and it dropped to 15% at 10 h.p.i. In the time removal assay, the plaque inhibition reached 50% after the extract was removed at 2 h.p.i., and it increased to 87% at 24 h.p.i. This study showed that *K. nemoralis* has potential as a drug used to fight herpes infections.

1. INTRODUCTION

Herpes simplex virus (HSV) is a virus from the Herpesviridae family, subfamily Alphaherpesvirinae and genus Simplex virus. This double-stranded DNA virus infects various host tissues and can cause a latent infection if not completely eliminated. This latent infection occurs based on the virus's type and the host's sensitivity [1]. HSV-1 infection is primarily associated with orolabial ulceration and is transmitted primarily through nonsexual contact during childhood following the disappearance of maternal antibodies in the 1st year of life [2]. The mucosal epithelia are the most prevalent infection site, including labial herpes keratitis, gingivostomatitis, and genital herpes. After an acute infection, a chronic condition can emerge when the infection spreads from the mucosal epithelia to other tissues and heals slowly. The worst effect caused by HSV-1 will occur in immunocompromised individuals, which include newly born newborns, transplant patients, or HIV patients who lack susceptibility, protracted toxicity, and prophylaxis and immunosuppressive medication regimens [3].

ACV-resistant infection occurs in immunocompromised patients due to the prolonged use of antiviral treatment [4]. The HSV resistance

mechanisms to ACV have been identified based on the following: (a) reduced viral TK-producing, (b) complete lack of viral TK activity, and (c) changed the DNA polymerase and viral TK protein. Consistent with the above mechanisms of action, viral mutations conferring ACV resistance are found in the viral DNA pol enzyme (UL30) and activating/phosphorylating genes (TK, UL23 kinase). Due to the addition or deletion of nucleotides in long homopolymeric runs of Gs and Cs, incomplete or deficient enzymes occurred due to viral mutations in the TK gene. In approximately 95% of cases, the isolation of ACV-resistant HSV has a TK-deficient phenotype [5].

Kyllinga nemoralis is known as whitehead spike sedge, white water sedge, *Kyllinga*, or poverty grass, and belongs to the Cyperaceae family. The morphology of the *K. nemoralis* plant has several parts, including the rhizomes, root, stem, leaves, fruit, and inflorescence. According to Rajagopal *et al.* [6], the *K. nemoralis* plant can treat several disorders and diseases, such as fever, diarrhea, cough, bronchitis, and fistula, and it is reported as an anti-helminth, anti-malaria, and hepatoprotectant agent. It is used in Malaysia to cure diarrhea, in India to treat stomach and intestinal disorders, in China to treat dysentery, and in Polynesia to treat joint pain and rheumatic problems [7]. Each part of this plant contains medicinal properties, such as the leaves of *K. nemoralis* having essential oils (terpenes α -cyperone, α -humulene, and β -selinene), saponins, terpenoids, and phenolic composition that can relieve malarial chills, skin disorders, and thirst due to fever. The leaves can also treat diabetes, fever, hepatopathy, splenopathy, and tumors [8].

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K. nemoralis methanolic extract was found to have antioxidant effects due to a significant number of total flavonoids, phenols, tannins, and flavonols. This effect can react with free radicals such as DPPH, superoxide radicals, hydroxyl radicals, and hydrogen peroxide radicals. Besides that, the presence of saponin, terpenoid, and steroids in the plant extract may be responsible for its radical scavenging capacity and antimicrobial action towards *Staphylococcus aureus* and multidrug-resistant *S. aureus* [9]. This work aims to evaluate the potential effect of *K. nemoralis* leaves and stems extract against HSV-1 replication *in vitro*. Our previous study on the anti-HSV-1 of *K. nemoralis* aqueous leaves and stems extract shows that the extract is most effective in pre-treatment assay and is an in-depth study to see the effect of the extract on HSV-1 gene expression [10]. However, the antiviral effect of this plant against other viruses has not been investigated, despite its folkloric use. This present study aimed to determine the ability of *K. nemoralis* leaves and stems methanolic extract to act as an antiviral agent in the HSV-1 replication cycle.

2. MATERIALS AND METHODS

2.1. Plant Materials and Extraction

Fresh leaves and stems part of *K. nemoralis* plants were dried at 60°C. The dried leaves and stems of *K. nemoralis* plants were cut into smaller pieces and blended to form a powder. The powder (300 g) was soaked in methanol solvent (500 mL) for 3 days. Due to its high polarity, which could result in high extraction yields, methanol is chosen in this study as extraction solvent. By utilizing a rotary vacuum evaporator, the solvent was evaporated under reduced pressure immediately after the extract was filtered. Finally, the extract was blow-dried to yield 30 g of extract. The extract was then kept in a refrigerator at 4°C until use for further analysis [11].

2.2. Cell Lines and Growth Conditions

To evaluate cytotoxicity, virus replication, and antiviral efficacy, Vero cells from the American Type Culture Collection CCL-81 were used. Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin (10,000 IU/mL) throughout the experiment. The clinical isolate strain of HSV-1 used in this study was a generous gift from the Faculty of Science and Technology at Universiti Kebangsaan Malaysia. In brief, Vero cells were used to propagate the virus, which was then harvested and tested for virus titer using the plaque-forming assay [12].

2.3. Cytotoxicity Test

According to Mohamad Ripim *et al.* [13], the cytotoxicity assay was conducted to determine the cytotoxic concentration (CC_{50}) of *K. nemoralis* extract in 96-well plates, the cells were planted at 2×10^5 per well. The extracts after serial two-fold dilutions were added to the wells in triplicate with several concentration (0.002–1 mg/mL). Cells were further incubated at 37°C in 5% CO_2 atmosphere for 48 h. Cell viability was measured using the MTT solution. The absorbance was measured at 540 nm wavelength using a 96-well plate reader. The graph of cell populations versus extract concentrations was used to determine the percentage of cell viability and CC_{50} value.

2.4. Antiviral Activity

Antiviral assays consisted of pre-treatment, post-treatment, virucidal, time-of-addition, and time-removal assays.

2.4.1. Pre-treatment assay

The 12 well plates were seeded with 2.0×10^5 cells/well of Vero cells and cultured overnight to allow cell adhesion. Before being infected with 50 plaque forming unit (PFU) HSV-1, cells were pre-treated with six concentrations of extracts (1, 0.5, 0.25, 0.125, 0.063, 0.031 mg/mL) for 24 h. DMEM+methyl cellulose was added after the adsorption period and incubated for 48 h. Cells were stained with crystal violet after incubation, and plaques were counted. Infected cells without treatment were used as the negative control, whereas infected cells treated with 5 M acyclovir (ACV) were used as the positive control. Cells were stained with crystal violet after the incubation period, and the plaque reduction percentage (%) was calculated according to Moses *et al.* [14]. The effectiveness of the *K. nemoralis* extracts as a potential antiviral drug was evaluated using the Selective Index, SI (CC_{50}/EC_{50} ratio).

$$\frac{\text{No.plaque}_{\text{infected non-treated}} - \text{No.plaque}_{\text{infected with treatment}}}{\text{No.plaque}_{\text{infected non-treated}}} \times 100$$

2.4.2. Post-treatment assay

The 12 well plates were seeded with 2.0×10^5 cells/well of Vero cells and cultured overnight in 5% CO_2 , 37°C incubator. Cells were infected with 50 PFU HSV-1 and were incubated for 2 h to allow virus adsorption. Six concentrations of extracts (1, 0.5, 0.25, 0.125, 0.063, and 0.031 mg/mL) were mixed with DMEM+methyl cellulose and were added into all of the test wells after the adsorption time. Then, cells were cultured for 48 h. Cells were stained with crystal violet after the incubation period, and plaque reduction percentage (%) was calculated using the procedure in 2.4.1.

2.4.3. Virucidal assay

The 12 well plates were seeded with 2.0×10^5 cells/well of Vero cells and cultured overnight in 5% CO_2 , 37°C incubator. Next, 300 μ L of HSV-1 at 50 PFU were exposed to 100 μ L of six concentrations of extract (1, 0.5, 0.25, 0.125, 0.063, 0.031 mg/mL) for 30 min at 37°C. The 100 μ L of extract+ 300 μ L of the virus with 400 μ L of 1% of DMEM+methyl cellulose were added into the well plate and incubated for 48 h. Cells were stained with crystal violet after the incubation period, and plaque reduction percentage (%) was calculated using the procedure in 2.4.1.

2.4.4. Time-of-addition assay

The 12 well plates were seeded with 2.0×10^5 cells/well of Vero cells and cultured overnight in 5% CO_2 , 37°C incubator. After that, Vero cells were infected with HSV-1 (50 PFU) for 2 h at 37°C. At 2, 4, 6, 8, and 10-time post-infection (h.p.i.), 0.015 mg/mL of *K. nemoralis* methanolic extract was added to the infected cells, followed by the addition of 1% methyl cellulose. The plate was incubated for 48 h in 5% CO_2 , 37°C incubator. Treatment with ACV was added on infected cells as a positive control, while infection without treatment was performed on infected cells as a negative control. After incubation, cells were stained with crystal violet solution (0.4%, w/v) and incubated at room temperature for 30 min. Plaque reduction percentage (%) was counted using a formula stated in 2.4.1.

2.4.5. Time removal assay

The 12 well plates were seeded with 2.0×10^5 cells/well of Vero cells and cultured overnight in a 5% CO_2 , 37°C incubator. The media was discarded and washed with PBS solution. Then, Vero cells were infected with 50 PFU of HSV-1 for 2 h at 37°C. Then, 0.015 mg/mL of *K. nemoralis* methanolic extract was administered to the infected cells. At 2, 4, 6, 8, 16, 18, 20, 22, and 24 h.p.i., cells treated with the

K. nemoralis methanolic extract were removed and overlaid with 1% methyl cellulose. The plate was cultured for 48 h in the 5% CO₂, 37°C. After incubation, cells were stained with crystal violet solution (0.4%, w/v) and incubated at room temperature for 30 min. Plaque reduction percentage (%) was counted using a formula stated in 2.4.1.

2.5. Statistical Analysis

The statistical software used was Prism 6 (GraphPad software, CA, USA). Means and standard errors of the means were used to express values. Values of EC₅₀ or CC₅₀ were determined by non-linear regression. Using Student's t-test (Microsoft Excel), the significance difference was determined as $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *K. nemoralis* Leaves and Stems Methanolic Extract was Non-Toxic to the Vero Cells

The cytotoxicity test was completed to define the CC₅₀ value for *K. nemoralis* methanolic extract to determine the safe range for the next antiviral tests. *K. nemoralis* provided a lower concentration of CC₅₀ in the population of Vero cells. This low cytotoxic value is enough to get the safe range to be the antiviral agent against HSV-1 infection. The extract was considered non-toxic when the cells maintained their viability at least 70% after 48 h of treatment [15], and the CC₅₀ value was more than 4 µg/mL [16]. Thus, the antiviral concentration for screening must be less than the determined CC₅₀ concentration. The CC₅₀ value of the leaves and stem of *K. nemoralis* methanolic extract was detected at 0.75 mg/mL [Figure 1].

The cytotoxicity assessments were aimed to define the concentration limit of the extract to be safely used for the antiviral tests. The extract should provide minimal toxicity against the cells as following the prerogative. In the basic cytotoxicity tests, the cell surface or membrane that was treated with the extract will change and turn irreversible, causing decreased dye uptake and binding. These changes make the living cells difficult to differentiate between dead or damaged cells [17].

3.2. Antiviral Screening

In the plaque reduction assays, six concentrations of *K. nemoralis* leaves and stems methanolic extract (1, 0.5, 0.25, 0.125, 0.063, and 0.031 mg/mL) were used in the post-treatment, pre-treatment, and virucidal assay to reduce the formation of the viral plaques. For the post-treatment assay, different concentrations of the extract were added to the cells after the cells were infected with the HSV-1. This assay was used to know their effect on virus replication, while in the pre-treatment assay; the cells were treated for 24 h with several concentrations of the extract before infections with the HSV-1 [18]. The pre-treatment assay was used to determine the ability of the extract to attach to the cells and intrude with their glycoprotein receptor on their cell membrane or hinder the binding of the HSV-1 to the cell surface [19]. For the virucidal assay, this assay was used to evaluate the extracellular virucidal activity of the extract. The different concentrations of extract were incubated with the virus to determine their reaction to the virus and residual infectivity [20,21].

Effective concentrations (EC₅₀) were observed at 50% of plaque reduction, and the SI value of the extract can be obtained based on EC₅₀ value and CC₅₀ value [Table 1]. This SI value was important because it indicates the safe value of plant extract for antiviral activity. A high value of SI indicated a large safety margin between beneficial and

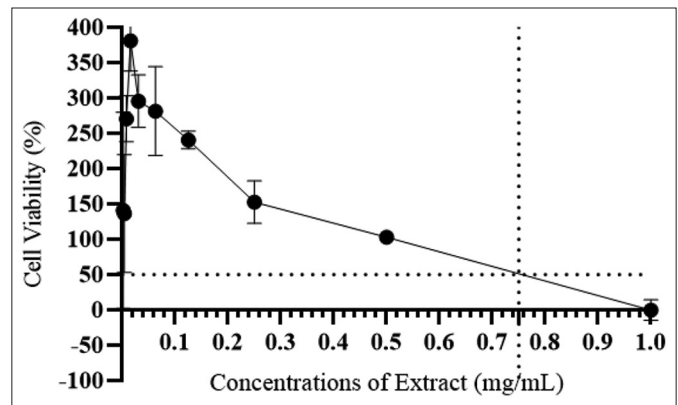


Figure 1: Cytotoxicity analysis of *Kyllinga nemoralis* leaves and stems methanolic extract.

Table 1: CC₅₀, EC₅₀, and SI values of *Kyllinga nemoralis* leaves and stems methanolic extract in post-treatment, pre-treatment, and virucidal assay.

Assays	CC ₅₀ (mg/mL)	EC ₅₀ (mg/mL)	SI (CC ₅₀ /EC ₅₀)
Pre-treatment	0.75	0.035	21.43
Post-treatment	0.75	0.09	8.33
Virucidal	0.75	0.031	24.19

CC₅₀: 50% cytotoxicity concentrations, EC₅₀: Half maximal effective concentration, SI: Selectivity index.

toxic doses [22]. As in the previous study, the antiviral activity with an SI value of more than 10 can be considered to possess a high potential for an antiviral agent [14]. The antiviral activity of *K. nemoralis* methanolic extract anti-HSV-1 activity depends on the concentration and is time-dependent.

3.2.1. Pre-treatment successfully increases the antiviral activity of *K. nemoralis* leaves and stems methanolic extract

The percentage of viral plaque reduction versus concentration of leaves and stems of *K. nemoralis* methanolic extract shows that the EC₅₀ value was observed at 0.035 mg/mL [Figure 2]. The SI value was 21.43 [Table 1]. In pre-treatment, the *K. nemoralis* leaves and stems methanolic extract were added to the cells, and then the pre-treated cells were exposed to the HSV-1. This test aimed to determine the extract's ability to modify the target cell membrane integrity or its effect on the capability of cell receptor molecules such as heparin sulfate to bind to HSV-1 complementary ligands that affect the capability of the HSV-1 to invade the cell at the time of the assay [23]. The finding indicates that the leaves and stems of *K. nemoralis* were a success in the pre-treatment assay as it shows the increase in the antiviral activity towards HSV-1. According to the calculated SI value, the leaves and stems of *K. nemoralis* methanolic extract show a high value compared to the root of the extract, probably due to phytochemical compounds in the leaves and stems of the extract.

In the previous study, Pucot and Demayo [24] described that the leaves of *K. nemoralis* are composed of phenolics, humulene, α -amyrin, β -caryophyllene, β -amyrin, and other phytochemicals. It has been demonstrated that one of these compounds, a phenolic compound, has an antiviral action against HSV. The action of phenolic compounds is depending on the presence of a significant number of carboxylic groups, and the mechanism of action of these compounds through inhibition of virus absorption into the cells [25]. Thus, the finding shows that the leaves and stems of *K. nemoralis* methanolic extract

have a high ability to bind to the Vero cells and inhibit the binding of HSV-1 to the cell surface of the Vero cells compared to the root of *K. nemoralis* methanolic extract. Based on the finding, the plaque formation started to decrease after adding the extract from lower to high concentrations (0.031 mg/mL to 1.000 mg/mL). This shows that the antiviral activity of the extract requires different concentrations of extract, possibly due to different amounts of antiviral compounds such as phenolic for the leaves and stems part of *K. nemoralis* and flavonoid compound for the root of *K. nemoralis* [26].

3.2.2. The leaves and stems of *K. nemoralis* methanolic extracts give mild protection to Vero cells after HSV-1 infection

The percentage of viral plaque reduction versus the concentration of leaves and stems of *K. nemoralis* methanolic extract shows that the EC₅₀ value was observed at 0.09 mg/mL [Figure 3]. The SI value was determined at 8.33 [Table 1].

In post-treatment, the cells infected with the virus were given *K. nemoralis* methanolic extract treatment. This test was carried out to determine if the activity of the extracts inhibits the viral replication cycle, which can downstream the entry process of HSV-1. The inhibition can occur at any point in the viral replication cycle, including viral gene transcription, during the egression or envelopment process [27]. As a result, the leaves and stems of *K. nemoralis* methanolic extract show a SI value of 8.33. When the SI value is higher than 10, it gives an idea to consider a good SI about the effect of extract concentration on selectively killing the virus [28]. Therefore, the *K. nemoralis* methanolic extract is considered quite a good antiviral agent that exerts anti-HSV-1 activity. This is a possibility due to the presence of the antiviral substance in *K. nemoralis* methanolic extract, which is a consequence of the fact that the extract possesses flavonoids, triterpenoids, and glycosides compounds [29]. In the plaque reduction assay, the flavonoid was confirmed to have anti-herpetic activity by inhibiting the reverse transcriptase. Besides that, the triterpenes in the extract were the source of compounds against HSV-1 by inhibiting viral capsid protein synthesis. The plaque formation of the virus also decreased after adding the extract from lower to higher concentrations to the infected cells. This shows that the antiviral activity of the extract relies on the different concentrations of extract, possibly due to the different amounts of antiviral substances such as flavonoids

in the *K. nemoralis* methanolic extract. When the extract concentration was high, the antiviral activity of the extract became high and could kill HSV-1 in the infected Vero cells.

3.2.3. *K. nemoralis* leaves and stems methanolic extract provide extracellular anti-HSV-1 activity

For the virucidal test, the test was done in one replicate. The *K. nemoralis* leaves and stems methanolic extract caused 50% plaque reduction at 0.031 mg/mL [Figure 4]. The SI value was 24.19 [Table 1]. In the virucidal test, the *K. nemoralis* methanolic extract was directly exposed to HSV-1 for half an hour, and the extract was directly affecting the virus without affecting the cells. According to the calculated SI values, the *K. nemoralis* methanolic extract provides extracellular anti-HSV-1 activity. By destroying the viral genome, entering the HSV-1 virion, or injuring the extracellular viral particles' protein coats, this extract will inactivate and target the extracellular viral particles, reducing their viral infectivity [30]. The result from the virucidal assay shows that the stem and leaves of the extract towards HSV-1 provide an SI value of more than 10 compared to the root of this extract. This shows that the

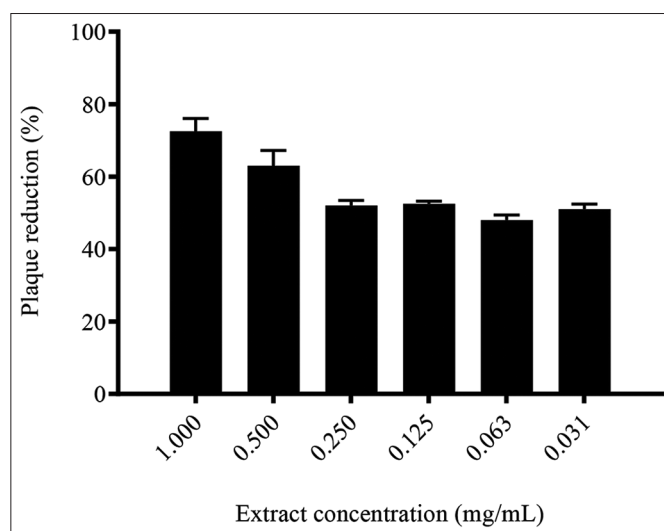


Figure 3: Antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract against HSV-1 through post-treatment assay.

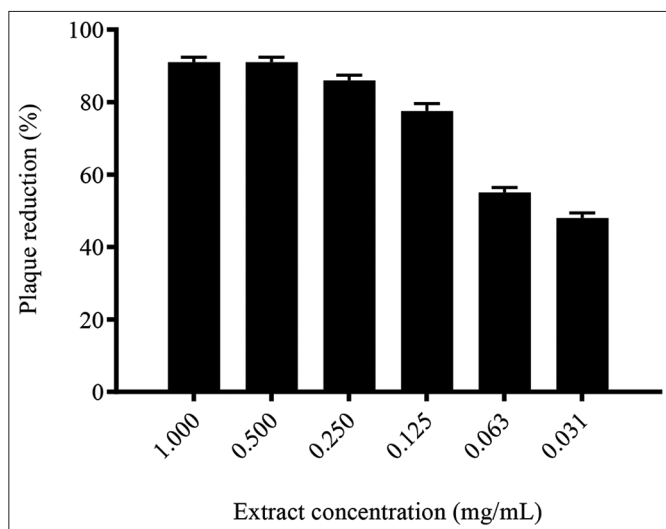


Figure 2: Antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract HSV-1 through pre-treatment assay.

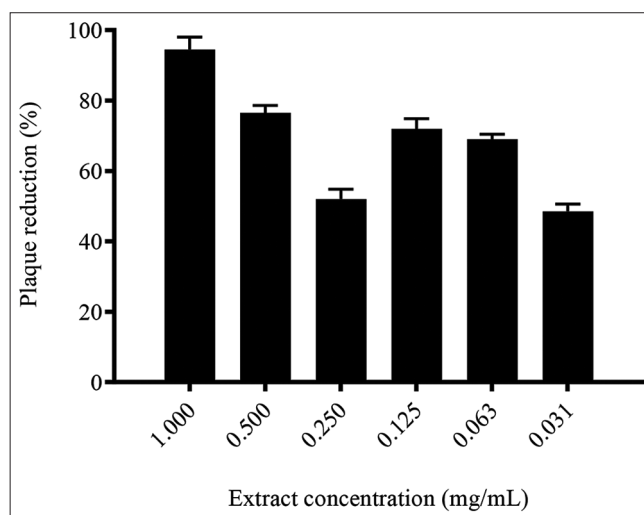


Figure 4: Antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract against HSV-1 through virucidal assay.

extract was strong enough to inactivate the extracellular component of HSV-1. It demonstrates a virucidal effect by directly rupturing the virus particles' outer surface membrane because it has antiviral properties such as phenolics that prevent the virus from entering cells. Thus, it has the specialty of administering resistant viruses to the currently used antiviral agents due to this unique membrane-targeting activity as well as the *K. nemoralis* methanolic extract exhibited virucidal activity and directly damaged the HSV-1 particles.

3.2.4. *K. nemoralis* leaves and stems methanolic extract inhibits all phases of viral replication in time-dependent assays

Time-of-addition was performed to determine the effect of *K. nemoralis* methanolic extract when applied at various times, starting from the time of infection and at 2-h intervals until 10 h.p.i., using the time-of-addition assay. Due to the *K. nemoralis* methanolic extract prevented the attachment and penetration of HSV-1 that was present in the early stage of the viral replication cycle of HSV-1, the percentages of plaque inhibition began to decrease when the extract was added from 2 h.p.i. until 10 h.p.i. Following the extract treatment at 2-h intervals, the viral plaque is reduced by around 43% at 2 h.p.i. and begins to decline to 15% at 10 h.p.i. [Figure 5].

Time removal assay was aimed at assessing the possibility of the extract inhibiting attachment and penetration at the early phase of HSV-1 replication and altering or lowering the infectivity level in HSV-1 progeny that was released from the infected cells during the late stages of virus replication [31]. During the whole duration of the experiment, continuous treatment with *K. nemoralis* methanolic extract was found to reduce plaque development. For the part of the leaves and stems of *K. nemoralis* methanolic extract, the plaque inhibition reached 50% when the extract was removed after 2 h.p.i. and it increased to 87% at 24 h.p.i. Time removal assay is used to assess the effectiveness of plaque inhibition from the early stage of viral replication, 2 h.p.i., to the late stages of viral replication, 24 h.p.i. Due to the ability of the extract to prevent attachment, penetration, and early stages of viral replication, the percentages of plaque inhibition began to increase from 2 h.p.i. After removing the treatment at 2-h intervals, approximately 50% of virus plaque reduction occurs at 2 h.p.i. and starts to increase to 87% at 24 h.p.i. [Figure 6]. However, the treatment was effective during the late phase of viral replication, 24 h.p.i., as the progeny was released from the infected cells. The extract was removed at a different time from the virus-infected cell at 2, 4, 6, 8, 16, 18, 20, 22, and 24 h.p.i. in the time removal assay.

Viruses infect their host cells to create progeny virus particles through the viral life cycle's sequential steps, which include viral attachment, entry, penetration, and post-entry processes. The replication of HSV-1 was completed within 10–12 h [32]. In the replication cycle, HSV-1 encodes around 80 proteins that are produced in a regulated cascade, and these proteins are separated into three categories: immediate-early (IE), early (E), and late (L). These HSV-1 genes were transcribed by the cellular RNA polymerase II (RNA Pol II). Four of the five IE proteins, ICP4, ICP0, ICP27, and ICP22, are involved in viral transcription regulation [33]. Therefore, time addition and time removal assays were done to determine the antiviral activity of the extract for HSV-1 at different replication phases, as seen in their impact on plaque inhibition. Time addition assay was used to determine which viral life cycle stages are inhibited by the extract [32]. This inhibition will prevent the virus attachment and penetration present during the early stage of viral replication. In the time addition assay, the extract was added to the infected cells at a different interval time from 2 h.p.i. (immediate early phase) until 10 h.p.i. (early phase) [34]. Based

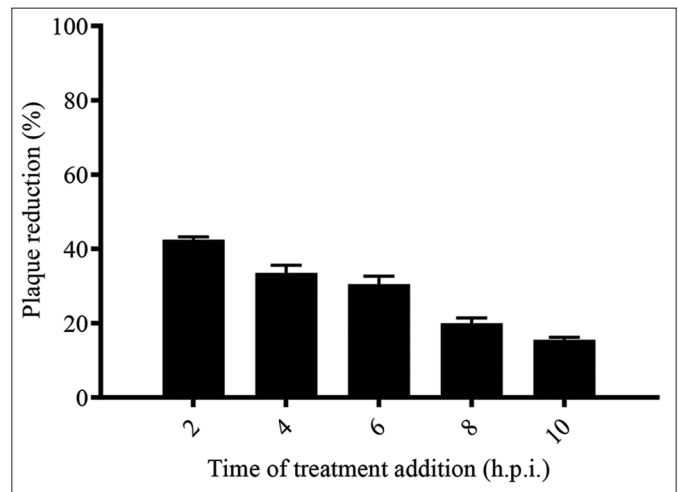


Figure 5: Antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract against HSV-1 through time-of-addition assay.

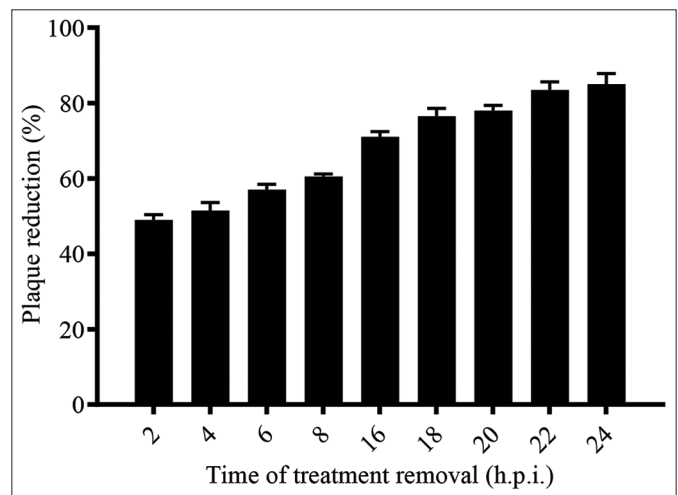


Figure 6: Antiviral activity of *Kyllinga nemoralis* leaves and stems methanolic extract against HSV-1 through time removal assay.

on the time addition assay finding, the maximum inhibitory impact was achieved when the extract was administered as soon as possible following the invasion. The plaque inhibition reached 43% when the extract was added after 2 h.p.i., and it dropped to 15% at 10 h.p.i. This result shows that the maximum extract activity occurs at 2 h.p.i. and is decreased when delayed in the treatment. This indicates that the extract inhibited the IE proteins that were produced in a regulated cascade during HSV replication. IE expression was induced within 1–2 h of infection by VP16, a virion protein [35].

K. nemoralis methanolic extract activity decreased after delayed in given the treatment until 10 h.p.i. because the IE expression started to peak at 3–4 h, and the next phase for the initial protein is at 4–7 h.p.i. This is preliminary evidence that the mechanism of *K. nemoralis* methanolic extract disrupts the initial cycle of viral replication. Furthermore, the antiviral activity of *K. nemoralis* methanolic extract can be seen in the immediate early and early gene cycles and begins to decline when delayed in giving the extract. This study confirms that the IE protein is a target for the antiviral action of the *K. nemoralis* leaves and stems methanolic extract.

4. CONCLUSION

The present study revealed that *K. nemoralis* had variable effects on HSV-1 replication in Vero cells, which had potent antiviral activity effects. The findings suggest that *K. nemoralis* crude extract contains antiviral active compounds and could be a potential antiviral agent. Although plaque reduction assays exhibited promising antiviral properties of *K. nemoralis* extract, further studies are required to explore the underlying mechanisms that contribute to its antiviral activity. In the future, a more thorough investigation will be required to clarify the inhibition processes involved and to confirm whether, when the purified active components are used, the inhibitory effect of *K. nemoralis* could be completely expressed to reveal more potent antiviral activity.

5. ACKNOWLEDGMENTS

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the 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

All the data is available with the authors and shall be provided upon request.

10. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

11. PUBLISHER'S NOTE

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