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HPLC fingerprint profile, *in-vitro* cytotoxicity and anti-herpes simplex virus activity of methanol extract from *Strophanthus hispidus* DC (Stem bark)

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ABSTRACT

Herpes simplex virus (HSV) infection is quite common in adults and neonates and is associated with oral mucocutaneous lesions and/or genital infections. Therefore, the aim of this present study was to determine the *in-vitro* cytotoxicity and anti-herpes simplex virus (1 and 2) activity of methanol extract of *Strophanthus hispidus* (*S. hispidus*) DC (Stem bark) and his HPLC fingerprint profile. The cytotoxicity test on Vero cells was performed by MTT assay and various concentrations of extract less than the maximum nontoxic concentration were used against Vero cells for anti-HSV-1 and ant-HSV-2 effects by plaque assay. The CC₅₀ values of methanolic extracts of *S. hispidus* (stem bark) and acyclovir against Vero cells were 94.46 µg/mL and 118.78 µg/mL, respectively. Whereas the EC₅₀ and selectivity index values of this extract against herpes simplex virus 1 and herpes simplex virus 2 strains were 36.60 µg/mL (SI=2.58) and 39.47 µg/mL (SI=2.39), respectively. HPLC finger-printing of this extract was developed and showed seven major compounds including ascorbic acid, quercetin, resorcinol and gallic acid represented in large amount. In conclusion, the methanolic extract of *Strophanthus hispidus* DC (stem bark) has good antiherpetic activity against HSV- 1 and HSV-2 but it is also highly toxic. Therefore, it will be important to separate toxic molecules from those responsible of the antiherpetic activity by bio guided fractionation procedures.

Introduction

For some time, many scientific researchers have been directed towards the discovery and development of new antiviral drugs because diseases caused by viral infections are one of the major causes of death in the world [1-8]. Several

deadly viral diseases have plagued humanity over the past decade such as COVID-19, human immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis virus subtype A, B, and C (HAV, HBV, HCV), influenza virus and so others [2,3,5]. However, there are really no safe and

effective antivirals that can fully combat and cure these viral infections. Some synthetic antivirals have been discovered that were effective against these viruses but unfortunately they have many side effects and also become less effective against new strains of resistant viruses [3,5,6]. Among these viral diseases, there is also herpes simplex virus (HSV) infection which is a DNA-containing enveloped virus, which brings commonly viral infections in humans causing a variety of diseases [7-9]. This viral infection is frequently found in adults and newborns; and is the primary cause of neonatal and sporadic encephalitis. In absence of treatment, the mortality rate associated with central herpes simplex virus infection is around 70% [7,10]. Some drugs have been discovered to combat herpes simplex virus infection such as acyclovir (ACV) and their analogues (valacyclovir VCV, famciclovir, and ganciclovir) [8-11]. However, faces to the emergence of resistant viral strains, these drugs seem increasingly ineffective because they have the same mechanism of action against the virus [12]. This is why it is important to look for other anti-herpes simplex virus infection drugs with high viral activity and less toxic.

In recent times, traditional medicinal plants and their metabolites have been a major asset in the discovery of new antiviral drugs [8-12]. The Congo Basin contains a diversity of plants used in traditional medicine and for other purposes [13-18]. One of them is the *S.s hispidus* DC which belongs to the Apocynaceae family. It is found all over Africa (D.R. Congo, Senegal, Ghana, Sudan, Uganda and Tanzania) in savannah and forests. The roots stem barks and leaves of *S. hispidus* are traditionally used in the treatment of Syphilis ulcers, skin diseases, leprosy, conjunctivitis, bony syphilis, guinea-worm sores, wounds, arthritis, stroke, heart failure, rheumatism, and like antidote to snakevenom and gonorrhoea [19-23]. Documented studies have shown that the stem bark of *S. hispidus* DC possess anti-ulcer, anti-diabetic, antibacterial, anti-lipidemic, and antioxidant activities which may be responsible for its therapeutic use in alternative medicine [19-23]. The goal of the present work was to investigate the antiviral effect of methanolic extract from *S. hispidus* DC against HSV-1 and his HPLC fingerprint profile.

Materials and methods

Plant collection

The stem barks of *S. hispidus* were collected in December 2018 from their natural habitats in Mayala village, Kongo Central (DRC). The collected plant materials were authenticated by INERA (Institute National d'Etudes et Recherches Agronomiques) Herbarium at Faculty of Science, University of Kinshasa. Unless stated otherwise, all the chemicals were purchased from Sigma (Deisenhofen, Germany).

Extraction procedure

Methanol extracts of *S. hispidus* (Stem barks) was obtained by Soxhlet extraction as previously described by **Mulula et al.** [19]. Extracts were concentrated on a rotary evaporator and the resulting residue stored at -20 °C until needed. Stock solutions of 20 mg/ml of *S. hispidus* (Stem barks) extract was prepared in 99% methanol and diluted as needed for different assays. Diluted extract solution was filtered and sterilized before use.

Thin-layer chromatography (TLC)

Silica gel thin-layer chromatography (TLC) was employed to estimate the approximate number of distinct chemical entities within each extract. Briefly, a small sample of the stock extract solutions were dissolved in 1 mL methanol and spotted on an in-house prepared 10 cm by 5 cm and 0.2 mm thick silica gel plate as previously described by **Mulula et al.** [19]. Developing solvent system utilized for the separation of constituents was ethyl acetate and petroleum ether in a 4:1 ratio. Iodine vapor visualization of resolved chromatographic bands and calculation of R_f values of constituent bands were performed as described in the literature [19].

HPLC finger-printing of extracts

The HPLC finger-printing of the methanol extracts was performed on Shimadzu LC-10 AT VP, Luna 5u C18 reverse-phase analytical column (250×4.6 mm) with binary gradient mode, SPD-M10A VP photo diode array detector (PDA), injection volume 20 µL, total flow 1 mL/min, column oven temperature 25°C and detection wavelength 280 nm. Fifty five milligrams per milliliter (55mg/mL) of extract were dissolved in 3 ml of methanol for the analysis. Ascorbic acid, gallic acid, resorcinol and quercetin were used as standard. Eluent A (acetonitrile); eluent B (0.1% phosphoric acid in water); gradient elution program was begun with 92% of solvent B and was held at this concentration for 0–25 min. This was followed by 78% of solvent

B for the next 25–40 min. Total run time was 40 min. gradient elution of standards: 92% of solvent B (0-5 min) and 78% of solvent B (5-20 min).

Phytochemical analyses

Phytochemical composition of each crude extracts was assessed using slight modifications of the methods described by Mulula et al. [20]

Total phenolic content by Folin-Ciocalteu method

Estimation of the total phenolic contents of the methanolic extract was performed using the Folin-Ciocalteu reagent with protocols published elsewhere [19]. Interpolation from the standard gallic acid curve provided total phenolic contents of samples in gallic acid equivalents (GAE). A triplicate test for each sample concentration presents each sample point as a Mean \pm SD.

Cells and viruses

Vero (African green monkey kidney) cells (ATCC CCL-81) were cultured and maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 50 μ g/ml gentamycin in an incubator set at 37°C, with 5% CO₂ and 95% relative humidity. HSV-1 and HSV-2 (clinical strain and sensitive to Acyclovir kindly provided by Virology Department of National Research Center) stocks were propagated in Vero cells and titrated. The virus was then stored at -20°C until use.

Cytotoxicity test

The evaluation of cytotoxicity of *Strophanthus hispidus* methanolic extract and acyclovir on Vero cells was carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT is reduced by mitochondrial dehydrogenases to the water insoluble pink compound formazan, depending on the viability of cells.

Hundred microliters of Vero cells were plated at an initial density of 1×10^4 cell/ml in a 96-well plate and treated with 100 μ L of various concentrations of *S. hispidus* methanolic extract (5, 10, 20, 40, 60, 90, 125 and 150 μ g/mL) or vehicle for 48 h. Twenty microliters of MTT (0.5 mg/mL) was added to the cells were then incubated with at 37°C for 4 h. The formed formazan crystals were extracted and dissolved using of dimethyl sulfoxide (DMSO). The absorbance after then was estimated at 560 nm. The resultant data were expressed as the percentage of

viable cells relative to untreated controls and were calculated by:

$$\% \text{ cell viability} = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100, \text{ where}$$

absorbance control is the absorbance of cells treated with DMSO 1% and absorbance sample is the absorbance of cells treated with test sample. The CC₅₀ is the concentration that is able to destroy 50% of the cells.

Antiviral activity assay

80% confluent cell monolayers seeded in 96-well plates were incubated with increasing non-cytotoxic concentration of *S. hispidus* methanolic extract. 1.09, 2.19, 4.38, 8.75, 17.5, 35, 70 and 90 μ g/mL of *S. hispidus* methanolic extract were used. Six wells at least were used for every concentration. After 2 h, the cell were infected with HSV-1 or HSV-2 at a multiplicity of infection of 0.001 pfu/cell, and re-incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The viral cytopathic effect (CPE) was examined daily using a light microscope. When CPE was observed in all virus control wells, the 50% inhibitory concentration (EC₅₀) of viral CPE was estimated in comparison to the virus control. Acyclovir (Sigma) at concentration of 1.09, 2.19, 4.38, 8.75, 17.5, 35, 70 and 90 μ g/mL served as the positive control.

Inhibition of HSV-1 and HSV-2 were estimated with plaque reduction assay. Vero cells were distributed in 24-well plates at a density of 10×10^4 cells. The plaque reduction assay was done in infecting cell monolayers with HSV-1 or HSV-2 at a MOI of 0.001 pfu/cell in presence of serial dilutions of the non-cytotoxic concentration of *S. hispidus* methanolic extract (1.09, 2.19, 4.38, 8.75, 17.5, 35, 70 and 90 μ g/mL) for 2 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was subsequently discarded from the wells, and the cells were washed with clean medium twice and covered again with a fresh medium containing 1.2% methylcellulose (Sigma) with *S. hispidus* methanolic extract at the same concentration. After incubation for 24 h at 37°C in humidified atmosphere with 5% CO₂, the supernatant was discarded, and the cells were fixed in 10% formalin in phosphate-buffered saline and then stained with 0.8% crystal violet in 50% ethanol and the viral plaques were counted microscopically at low power.

Data analysis

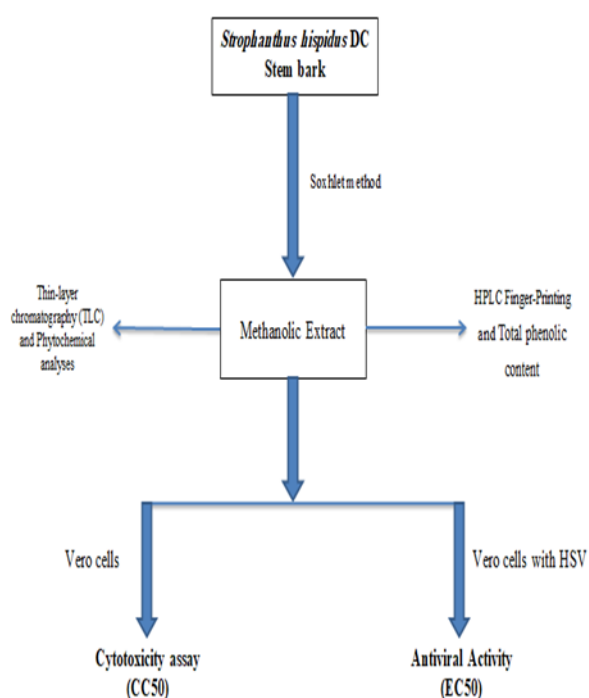
The 50% inhibitory concentration (CC₅₀) and 50% effective (EC₅₀) concentrations were calculated from concentration-effect curves after linear

regression analysis. The results represent the mean \pm standard error of the mean values of three different experiments.

Figure 1. *Strophanthus hispidus* plant.



Figure 2. Flow chart of the methodology.



Results and discussion

Thin-layer chromatography (TLC) and preliminary phytochemical screening

Thin-layer chromatography profiling of the extracts gives an impressive result that directing towards the presence of number of phytochemicals. Various phytochemicals give different Rf values in different solvent system. This variation in Rf values of the phytochemicals provides a very important clue in understanding their polarity and also helps in the selection of appropriate solvent system for separation of compounds by preparative-TLC. Compound showing high Rf value in less polar solvent system have low polarity and with less Rf

value have high polarity. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extracts can only be achieved by analyzing the Rf values of compounds in different solvent system.

Thin layer chromatography demonstrated that each plant extract comprise multiple chemical entities and is in line with the observed varied phytochemical compositions of the extract. The six observable TLC bands (spots) for *S. hispidus* (Stem bark) extract (**Table 1**) are on the lower scale of chemical entities present in the extract and the problem might be due to the lower separator efficiencies of the utilized chromatographic method. Nevertheless, Rf values in **table (1)** show that all chromatographic bands were reasonably well-resolved. These spots (6) indicated the presence of six major groups of phytochemical constituents in this methanolic extract.

Additional confirmatory evidence supportive of the TLC is provided by the phytochemical composition that denoted a multiplicity of functional groups embedded, potentially, in different chemical entities.

Results of chemical screening of methanolic extract of *S. hispidus* (stem bark) shown in **table (1)** revealed the presence of tannins in large amount, alkaloids, flavonoids, saponins, steroids and glycosides. The metabolites found in the methanolic extract of stem bark were identical to those in the methanolic and ethanolic extracts of leaves and roots of the same plant [20,22,23]. These phytochemical molecules are potentially bioactive.

Total phenolic content by Folin-Ciocalteu method

The total phenolic content in methanolic extract of *S. hispidus* (stem barks) was evaluated quantitatively according to the standard *in vitro* spectrophotometrical-based Folin-Ciocalteu colorimetric assay as described in material and methods section. Interpolation from the standard gallic acid curve provided total phenolic contents of samples in gallic acid equivalents (GAE).

The data shows that methanolic extract of *S. hispidus* (stem bark) is a polyphenol-rich plant whose total phenols exist in structurally distinct forms as tannins and flavonoids. Total phenolic content of methanolic extracts was 54.91g GAE/100 g.

HPLC finger-printing of methanolic extract of *Strophanthus hispidus* (Stem barks)

The HPLC finger printing of the extract was determined to identify the major peaks (compounds)

in the extract for the purposes of identification and quality control (**Figure 3**). HPLC profiles of methanolic extract of *S. hispidus* (stem bark) was analyzed for four phenolic compounds viz., ascorbic acid, gallic acid, resorcinol and quercetin.

All of these four phenolic compounds were present in the methanolic extract of *S. hispidus* (stem bark) with different retention times (RT) of 2.98 (Peak 1), 6.17 (Peak2), 10.95 (Peak 4), and 13.96(Peak 5) for ascorbic acid, gallic acid, resorcinol and quercetin respectively.

The HPLC chromatogram showed seven major peaks at the retention times (min.) of 3.11, 6.23, 10.44, 11.21, 14.12, 28.36, and 33.58 respectively at wavelength of 280 nm (**Figure 1**) and the major peak with a retention time (min.) of 6.2. The retention time of this peak corresponds to that of gallic acid (6.1 minutes) used as a standard in the same solvent system. These results are similar to the phytochemical screening which showed the presence of tannins in the large amount.

Cytotoxicity and antiherpetic test

Medicinal plants are a source of antiviral products with fewer side effects compared to antivirals from synthesis. The cytotoxicity test of methanolic extracts of *S. hispidus* (stem bark) on Vero cells was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the results are shown in **figure (4)**. The CC_{50} of methanolic extracts of *S. hispidus* (stem bark) against Vero cells was 94.46 $\mu\text{g/mL}$. **Ambali et al.** during their ethnobotanical studies on plants used for cancer treatment in Akinyele (Nigeria), found the cytotoxicity values on Vero cells of 47.54 $\mu\text{g/mL}$ and 35.78 $\mu\text{g/mL}$ for aerial part and root of *S. hispidus*, respectively [24]. These results show that the stem bark extract of *S. hispidus* are less toxic than those of roots and aerial part. Whereas, the cytotoxicity of acyclovir was 118.78 $\mu\text{g/mL}$.

Taking this EC_{50} (the concentration which causes 50% cytotoxic effect) value into account, only concentrations of *S. hispidus* methanolic extracts below or equal to 90 $\mu\text{g/mL}$ were used to assess antiherpetic activity.

Results of the antiviral activity and the cytopathic inhibitory assay of *S. hispidus* methanolic extracts against HSV-1 and HSV-2 were presented in **table (2)**, **figures 5 (a, b)**.

As illustrated in **table 2** and the **figures 5 (a, b)**, only non-cytotoxic concentrations of 70 $\mu\text{g/mL}$ and 90 $\mu\text{g/mL}$ completely inhibited the growth of the HSV-1 and HSV-2 viral strains. However, the 35 $\mu\text{g/mL}$ concentration partially inhibited the growth of the HSV-1 viral strain. The methanolic extract of *S. hispidus* shown antiherpetic activity against herpes simplex virus 1 and herpes simplex virus 2 strains with the EC_{50} of 36.60 $\mu\text{g/mL}$ and 39.47 $\mu\text{g/mL}$, respectively. The Selectivity index (SI) were 2.58 and 2.39, respectively. Whereas, the EC_{50} and selectivity index (SI) of acyclovir against HSV-1 and HSV-2 were 4.13 $\mu\text{g/mL}$ (SI=28.76) and 6.86 $\mu\text{g/mL}$ (SI=13.31), respectively.

It is important to emphasize that the EC_{50} values obtained for *Strophanthus hispidus* DC methanolic extracts against HSV-1 and HSV-2 clearly correlate with its traditional use [19-23]. In fact, the *S. hispidus* are traditionally used in the treatment of syphilis ulcers, skin diseases, leprosy, conjunctivitis, bony syphilis, guinea-worm sores, wounds, arthritis, stroke, heart failure, rheumatism, and like antidote to snakevenom and gonorrhoea [19-23]. The anti-herpetic activity of this extract may be attributed to astringent nature of the phenolic constituents including tannins, flavonoids, anthraquinones and other secondary metabolites such as terpenoids, alkaloids present in the extracts. Several secondary metabolites, including flavonoids, saponins, and tannins, have been reported to present antiviral activity [25,26].

Table 1. Thin layer chromatography (TLC) and phytochemical contents report of methanolic extracts of *Strophanthus hispidus* (Stem barks)

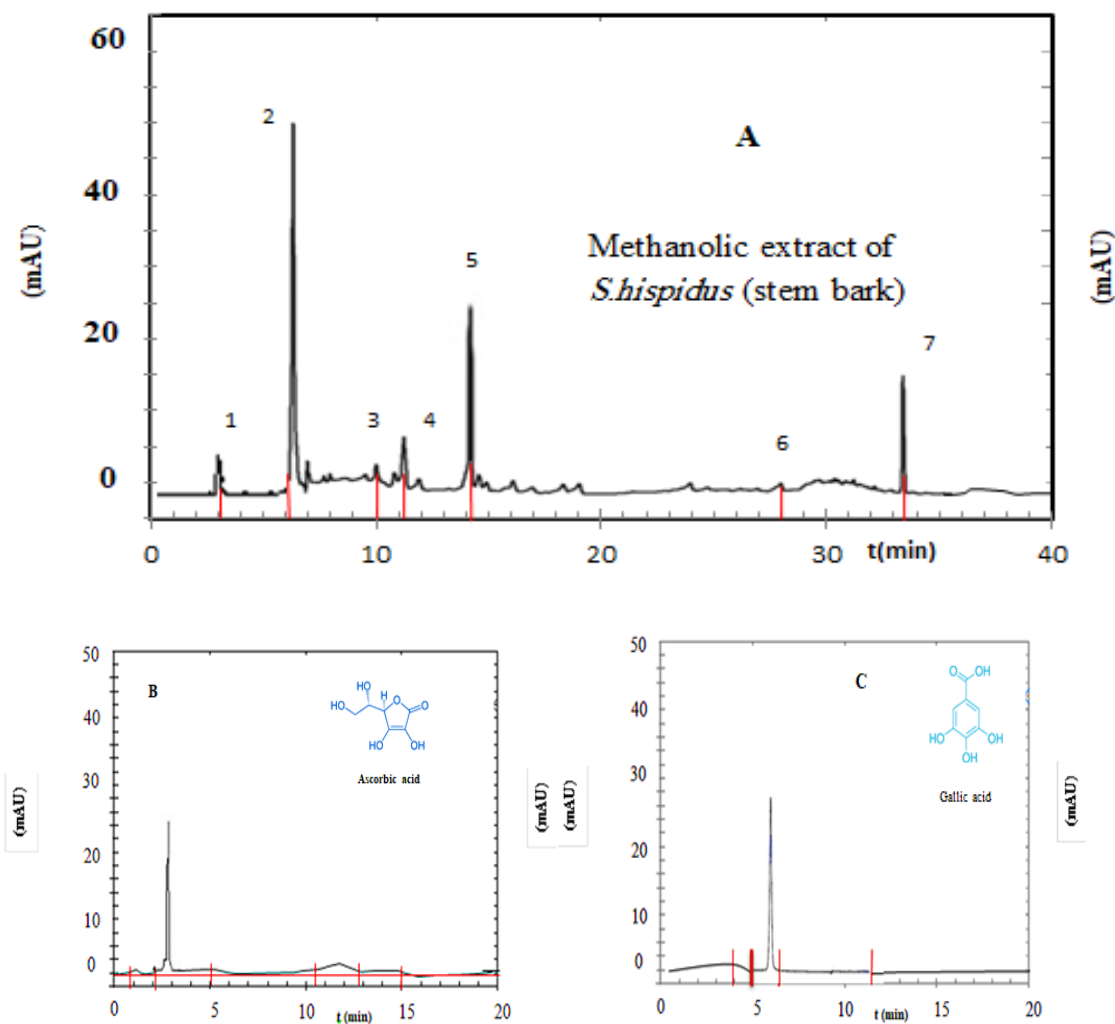
| Sample | TLC Result | | Phytochemicals screening | |
|---|--|--|--------------------------|-----|
| | Number of spots from TLC and Rf values | | | |
| Methanolic extract of <i>Strophanthus hispidus</i> (Stem barks) | Six(6) 0.07, 0.18, 0.34, 0.46, 0.57, 0.69 | | Alkaloids | + |
| | | | Flavonoids | + |
| | | | Tannins | +++ |
| | | | Anthraquinones | - |
| | | | Steroids | + |
| | | | Glycosides | + |
| | | | Saponins | + |

+: presence of secondary metabolite; +++: abundance presence of secondary metabolites; -: absence of secondary metabolite.

Table 2. Anti-herpetic activity of methanolic extracts of *Strophanthus hispidus* (Stem barks) against HSV-1 and HSV-2.

| Sample/Standard | Viral strains | Concentrations (µg/mL) | | | | | | | | EC ₅₀ (µg/mL) | SI (CC ₅₀ /EC ₅₀) |
|--|---------------|------------------------|------|------|------|------|----|----|----|--------------------------|--|
| | | 1.09 | 2.19 | 4.38 | 8.75 | 17.5 | 35 | 70 | 90 | | |
| Methanolic Extract <i>S. hispidus</i> Stem barks | HSV-1 | - | - | - | - | - | ± | + | + | 36.60 | 2.58 |
| | HSV-2 | - | - | - | - | - | - | + | + | 39.47 | 2.39 |
| Acyclovir | HSV-1 | - | - | + | + | + | + | + | + | 4.13 | 28.76 |
| | HSV-2 | - | - | - | + | + | + | + | + | 6.86 | 17.31 |

Figure 3. HPLC chromatogram (fingerprinting) (A) Methanol stem bark extract of *S. hispidus*, (B) Ascorbic acid, (C) Gallic acid; (D) Resorcinol; (E) Quercetine at λ 280 nm.



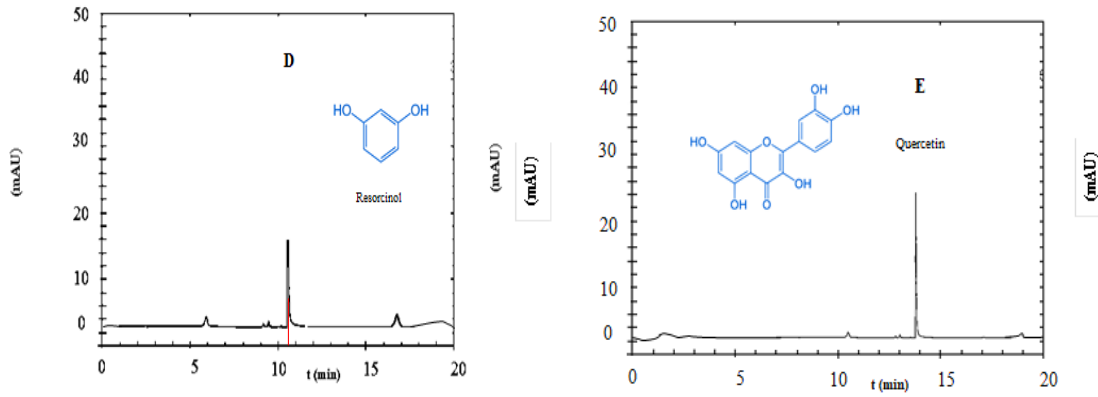


Figure 4. Concentration effect of *S. hispidus* methanolic extracts on Vero cells. The EC₅₀ was calculated using regression line. Data were expressed as mean ± SD from three independent experiments.

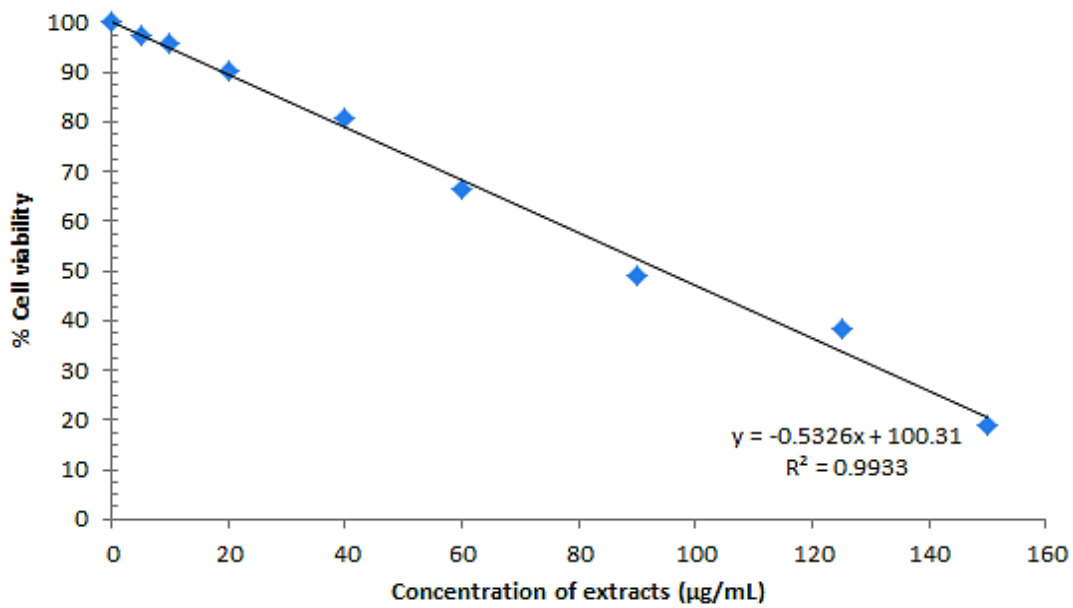


Figure 5a. Inhibition of plaque against HSV-1.

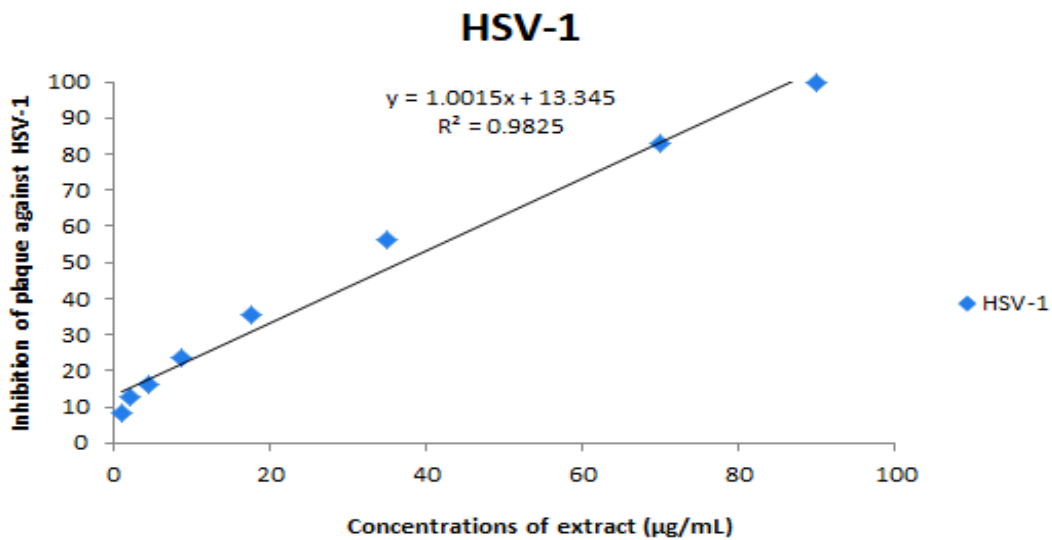
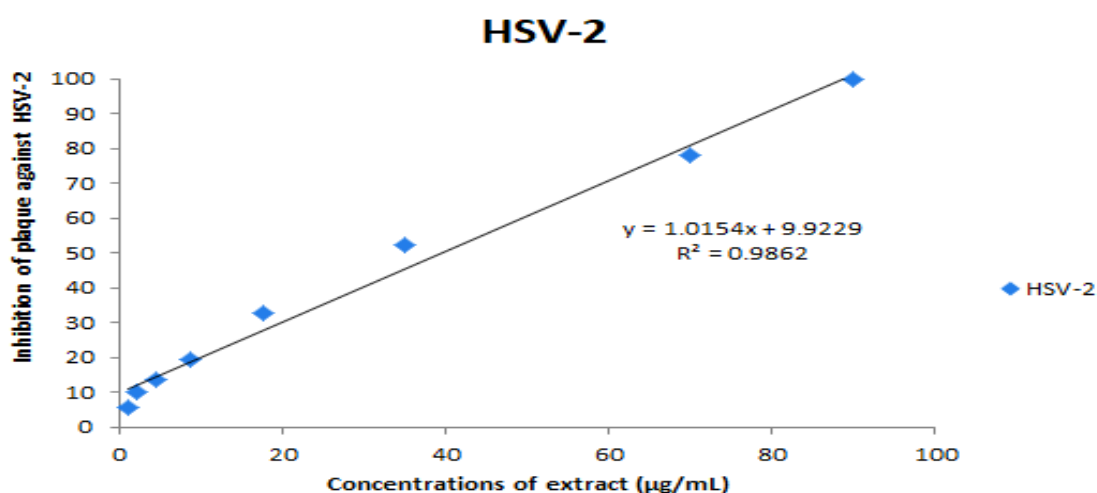


Figure 5b. Inhibition of plaque against HSV-2.

Conclusions

The observation of popular knowledge is the most common strategy for selecting plant species that may be potentially used to treat diseases. In conclusion, this study provides new scientific information about the methanolic extract of *S. hispidus* DC (stem bark), based on its antiherpetic activity against HSV-1 and HSV-2.

The methanolic extract of *Strophanthus hispidus* DC (stem bark) has good values of antiherpetic activity against herpes simplex virus 1 and herpes simplex virus 2 strains with the EC₅₀ of 36.60 µg/mL and 39.47 µg/mL, respectively. However, it is also highly toxic resulting in the Selectivity Index of 2.58 and 2.39 against HSV-1 and HSV-2, respectively. Since methanolic extract of stem bark from *S. hispidus* is a heterogeneous mixture of different chemical compound. Thus, it might be possible to separate toxic molecules from those responsible of the antiherpetic activity.

For these, further studies with purification fractions of the extract should be carried out. The results presented here justify further investigation of the extracts by bioguided fractionation procedures that hopefully will allow identifying active molecule(s) involved in anti-HSV-1 and anti-HSV-2 activities.

Conflict of interest

The authors declare that they have no conflict of interests.

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