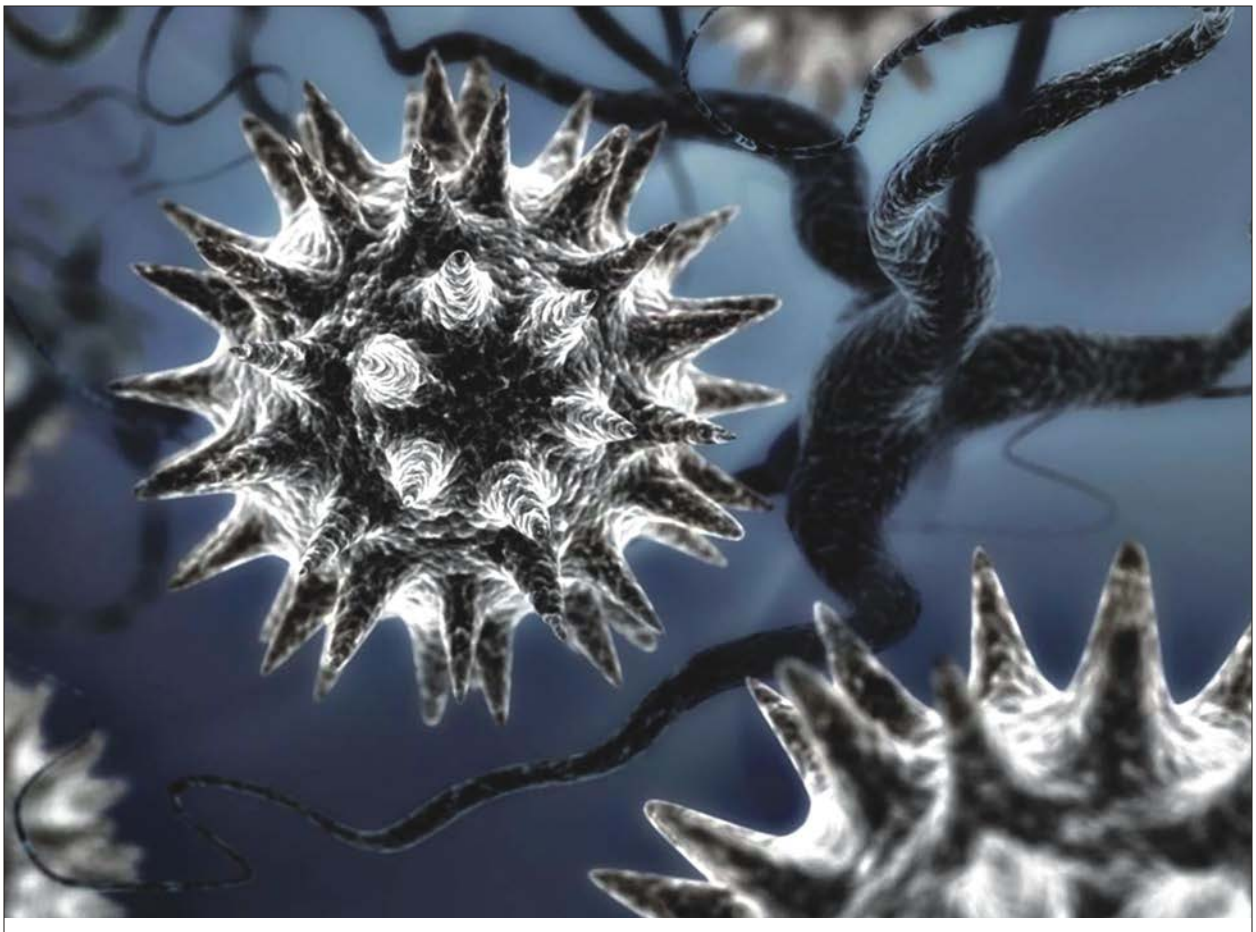

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Research Article
Pharmacological Evaluation of the *Hibiscus* Herbal Extract Against Herpes Simplex Virus-type 1 as an Antiviral Drug *in vitro*

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Abstract

Background and Objective: High prevalence of viral infections and constant emergence of resistant virus strains worldwide calls for an urgent need to develop therapeutic agents to improve the treatment of virus infection especially against Herpes Simplex Virus-type 1. The objective of this study was to conduct phytochemical screenings of the methanolic/aqueous Hibiscus extracts and evaluate their antiviral activity as well as their therapeutic and prophylactic activity. **Materials and Methods:** Identification and evaluation of phytochemical ingredients and essential components of the *Hibiscus* extracts through different chemical analytical methods were conducted. Antiviral activity of *Hibiscus* extracts was evaluated using plaque reduction assay of the viral cytopathic effect as well as cell viability and proliferation assays using MTT, to evaluate the extract feasibility as a potential therapeutic agent. Experiments like viral binding and adsorption, virucidal and time of addition assays were designed to investigate the mechanism of antiviral activity of *Hibiscus*. Moreover, viral kinetics and antigen expression were demonstrated by fluorescence microscopy. MS Excel 2010 was used to provide statistical analysis like average and standard deviation. **Results:** Phytochemical analysis succeeded in extracting major constituents of *Hibiscus* like polyphenols, flavonoids and anthocyanin. *Hibiscus* showed a potential activity of methanolic and aqueous extracts against HSV-1 as indicated by selectivity index values of 8.0 and 7.7 correspondingly. Mechanism of action, revealed that the extract not only prevented the virus particles from interacting with the Vero cells in the pre-treatment assay but also had a prophylactic effect blocking the replication of the virus causing prophylactic selectivity indices of 6.1 and 5.2 for methanolic and aqueous extracts correspondingly, confirming a maximum protection of Vero cells against HSV-1 attack. Antigen expression results showed that the effect of *Hibiscus* on inhibiting the HSV-1 virus after 5 h is much better than their effect 10 h post infection. **Conclusion:** Results demonstrated the potent and broad spectrum antiviral activity of *Hibiscus* due to the multiple components contained in the extract from active ingredients which cooperate together in synergetic manner and that all *Hibiscus* extracts tested were effective as virucidal and prophylactic agent, which makes *Hibiscus* a novel treatment against HSV-1.

Key words: Herpes simplex virus, *Hibiscus*, therapeutic agent, antiviral, herbal

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants and herbs have been used as remedies in traditional medicine for hundreds of years to treat all kinds of diseases[1,2] and recently pharmaceutical companies have been extracting these plants and developing therapeutic agents[3]. Synthetic drugs have shown many problems in the last decades like toxic side effects, sensitivity and developing drug resistance, which triggered the scientific community as well as the leader pharmaceutical companies to search for better sources like medicinal plants to replace synthetic drugs, with natural ones[4]. This discovery of effective, potent, inexpensive and safe antiviral agents to enable treatment of acute and chronic life-threatening viral infections from medicinal plants may secure the human being from drug resistant viruses[5] and boost the safety use of antiviral agents. Herpes Simplex Virus (HSV) is a very concerning virus as it causes infections that can be life threatening[6]. Recently HSV has been a popular research interest in many research communities studying the mechanisms of virus replication and potential antiviral agents[7]. Acyclovir (ACV) is widely used to treat HSV but the real problem is the development of drug resistant HSV strains usually in immune compromised patients[8]. Hence, the need for antiviral agents from medicinal plants with new mode of actions towards attacking the virus is a must.

Plants contain a special group of non-nutrient compounds called phytochemicals. Phytochemicals have been found to be preventive against many human degenerative diseases like coronary heart diseases[9], Alzheimer's disease, atherosclerosis cataracts and inflammation[10-11].

Hibiscus from the Malvaceae family is cultivated and naturalized in tropical and subtropical regions. The plant has been used in different countries as a medicinal substance. In folk medicine, the calyx extract of this plant is used for treatment of several illnesses, including high blood pressure, gastrointestinal disorders, hypercholesterolemia, liver diseases and fever[12]. This plant has also been shown to have anticancer effects in vitro against gastric cancer[13], hepatocellular cancer[12] and leukemia[14]. *Hibiscus* protocatechuic acid has also been shown to inhibit the carcinogenic effect of various chemicals in different tissues of rats, including the liver, oral cavity, colon, glandular stomach tissue, bladder and skin[15].

Ali *et al.*[15] also added that different strains of *Hibiscus* from different countries may differ in one or several of these constituents. Recently, trials to use the *Hibiscus* extract as an antibacterial agent have been conducted[16,17].

Aqueous *Hibiscus* extracts possess antioxidant activity[18,19] and antimicrobial properties with limited information available on their antiviral effects[20].

This study is aimed to find out the feasibility of using the *Hibiscus* aqueous and methanolic extracts as therapeutic agents in treating HSV-1. The study starts by investigating the phytochemistry of the major constituents of the methanolic/aqueous *Hibiscus* extracts and then the cytoxicity of both extracts against the Vero cell line. Afterwards, the antiviral activity of both extracts against HSV-1 is going to be studied, to find out the safest and best concentrations to use of the two extracts and finally the underlying mechanism of action of antiviral activity is studied as well as the antiviral kinetics.

MATERIALS AND METHODS

Study timeline: This study was conducted between January, 2016 and April, 2017

Plant material, chemicals and reagents: Dry herbal *Hibiscus* was purchased from a local market store. Acyclovir, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Dimethylsulphoxide (DMSO) were purchased from Sigma (St. Louis, USA). FITC-labeled anti-HSV-1 mouse monoclonal antibodies (Fluorescein isothiocyanate) as well as secondary rabbit polyclonal antibodies and DAPI (4', 6-diamidino-2-phenylindole) were purchased from ThermoFisher, USA. All the other reagents were of analytical grade.

Preparation of extracts: Various *Hibiscus* herbal formulations were collected from the market. Plant samples were dried at room temperature and finely ground with a hammer mill. Two extracts will be prepared from the plant samples, aqueous and methanol extracts. Each 10 g of powdered plant material was extracted by maceration overnight with 100 mL methanol/water at room temperature. After filtration, methanol/water was evaporated under reduced pressure till complete dryness and the crude extract of each extract was dissolved in dimethyl sulpheroxide (DMSO) to a final concentration of 100 mg mL^{-1}. All stocks were kept t-20°C for cytotoxicity testing procedures.

Total phenolic content determination: The total phenolic content of the *Hibiscus* freeze dried extract was determined using Folin-Ciocalteu method mentioned by Siriwoharn *et al.*[21].

Total flavonoids determination: Total flavonoids content of freeze-dried extract was determined using the method described by El Hariri *et al.*[22].

Total anthocyanin determination: Total anthocyanin content of freeze-dried extract was determined using the method described by Cesoniene *et al.*[23].

Cell culture and virus: Herpes simplex virus type 1 CATCC-733, African green monkey kidney cells (Vero) ATCCCCL-81 was obtained from American type culture collection. African Green Monkey kidney cells (Vero) were grown in modified Eagle's medium; Gibco, USA, supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (100 U mL^{-1}), streptomycin (100 µg mL^{-1}) and amphotericin B (25 µg mL^{-1}) (Gibco, USA). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The HSV-1 was propagated in Vero cells, while stock viruses were prepared as previously described in this study. After three cycles of freezing/thawing, the fluids were titrated on the basis of PFU count and stored at -80°C until use. Virus stock was prepared by infection of confluent monolayer Vero cells in 75 cm^2 culture flasks using DMEM medium with 2% FBS at 37°C in 5% CO_2. Virus titer was determined by cytopathic effect (CPE) of HSV-1 in Vero cells and was expressed as the 50% Tissue Culture Infective Dose ($TCID_{50}$) per mL. Virus stocks were propagated in Vero cells and used at a concentration of 10^{-6} $TCID_{50}$ in all *in vitro* experiments.

Cell viability (cell proliferation assay) of methanolic/aqueous extract of *Hibiscus* and acyclovir: Prior to the investigation of anti-HSV-1 activity, the cytotoxic effect (CPE) of the extract was determined. Briefly, Vero cells were seeded onto 96-well plates with a concentration of 1×10^5 cells/well with final volume of 100 µL per well. After incubation at 37°C with 5% CO_2 for 24 h, when the cell monolayer was confluent, the cell culture medium of cells aspirated and washed with PBS. Cells were incubated with 100 µL $well^{-1}$ of (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5) mg mL^{-1} concentrations of methanolic/aqueous extract (in triplicates) or with 2-fold serial dilutions from acyclovir stock (0.06 mg mL^{-1}) dissolved in DMSO solution and incubated at 37°C with 5% CO_2 for further 3 days. Negative control wells contained cells with maintenance medium (200 µL $well^{-1}$). Cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumbromide), (Sigma, USA), by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product and the procedure was followed as described earlier[24]. Briefly, the supernatants were removed from the wells and 50 µL of an MTT (Sigma, USA) solution (1 mg mL^{-1} in DMSO) was added to each well. The plates were incubated for 4 h at 37°C and placed on a shaker for 15 min and the absorbance were read on an enzyme-linked immunosorbent assay (ELISA) reader (STATA FAX 2100, USA) at 492 nm. Data were calculated as the percentage of toxicity using the following formula:

$$\text{Toxicity (\%)} = 100 - \frac{\text{At}}{\text{As}} \times 100 \quad (1)$$

where, At and As refer to the absorbance of the test substance and the solvent control, respectively[25]. The 50% cytotoxic concentration (CC_{50}) was defined as the cytotoxic concentration of the crude extract by regression analysis.

Antiviral activity by viral plaque formation number reduction assay: This assay followed the procedures previously described by Kodama *et al.*[26]. Vero cells (3×10^5 cells per well) were seeded onto 24-well culture plates and after 24 h, the cells were infected with 50 µL of HSV-1 diluted to 10^6 PFU mL^{-1}. After 1 h adsorption at 37°C, the plates were washed and overlaid with MEM plus 1.5% carboxymethylcellulose (Sigma) containing or not different concentrations of the *Hibiscus* at 0.5, 1, 1.5, 2, 2.5 and 3 mg mL^{-1} for methanolic extract and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 mg mL^{-1} for aqueous extracts. After 72 h, the cells were fixed and stained with crystal violet (Sigma) and viral plaque formation was counted. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral plaque formation when compared to untreated controls. Acyclovir (Sigma) was used as a positive control (10 µg mL^{-1}), since it completely inhibited the viral replication. Its stock solution was prepared in DMSO (Sigma) at the final concentration of 1000 µg mL^{-1}. Results were expressed as 50% cytotoxic (CC_{50}) and 50% inhibitory (IC_{50}) concentrations, respectively, in order to calculate the selectivity index ($SI = CC_{50}/IC_{50}$) of each extract[27]. The antiviral activity of *Hibiscus* was determined by the following formula[27]:

$$\text{Percentage inhibition} = 1 - \frac{\text{No. of plaques tested}}{\text{No. of plaques in control}} \times 100 \quad (2)$$

Time of addition assay

Inhibition of viral adsorption assay: Viral adsorption assay was conducted using the method of Zhang *et al.*[28].

Inhibition of viral replication assay: Viral replication assay was conducted using the method laid out by Amoros *et al*.[29].

Virucidal assay (direct inactivation assay): The method of Schuhmacher *et al*.[30] was followed for the virucidal assay. Selectivity Index (SI) ratio is calculated by dividing the concentration giving 50% cytotoxicity (CC_{50}) over the concentration giving 50% viral inhibition (IC_{50}).

Virus infectivity and antigen expression assay: HSV-1 infected Vero cells monolayers were treated with methanolic and aqueous *Hibiscus* extracts at their effective doses for different time intervals (0, 5 and 10 h) to study the effect of the extract on viral replication cycle. Plates were washed twice with phosphate buffered saline (PBS, pH 7.2) to remove the cell debris. The cells were then fixed with para-formaldehyde (4%) and blocked with 1% Bovine Serum Albumin (BSA) in 0.1% PBS-triton X100 solution. The cells were washed with PBS and then permeabilized with 0.1% triton X100 in PBS and incubated overnight with FITC-labeled anti-HSV-1 mouse monoclonal antibodies (ThermoFisher, USA). After washing with PBS, secondary rabbit polyclonal antibodies (ThermoFisher, USA) and DAPI (4',6-diamidino-2-phenylindole) were added and the cells were observed under epifluorescence microscope (AmScope 40X-1600X Widefield Epi-Fluorscent Binocular-FM580BA).

Statistical analysis: Data was collected for each experiment individually; means and standard deviation were computed using Microsoft Excel 2010. Microsoft excel was also used to draw the maps representing the data.

RESULTS

Phytochemical evaluation of *Hibiscus*: Phytochemistery of the methanolic/aqueous extracts of *Hibiscus* showed a higher value of the flavonoids, 12.5 mg g^{-1} in the methanolic extract than the aqueous extract which reported 4.5 mg g^{-1}. Also, the methanolic extract showed a higher value of anthocyanins 21.25 mg g^{-1} comparing to the aqueous extract which reported 19.3 mg g^{-1} and finally, the phenolics were higher in the methanolic extract showing 75.2 mg g^{-1} comparing to 64.5 mg g^{-1} recorded in the aqueous extract. These results indicated that the efficacy of both solvents water and methanol in extracting the active ingredients from *Hibiscus*.

Crude *Hibiscus* extract was selected to study its antiviral potential because using the crude extract over the fractionated extract has a beneficiary effect on antiviral activity, especially when the crude extract is composed of large mixture of polyphenols that could effectively increase the antiviral activity.

Effect of cytotoxicity of methanolic and aqueous *Hibiscus* extracts by MTT: To determine the cytotoxicity of the methanolic and aqueous extracts of *Hibiscus*, MTT assays were performed. For both the two extracts, the viability of Vero cells was maintained up till concentration 2 mg mL^{-1} for aqueous *Hibiscus* extract and up till concentration 1 mg mL^{-1} for methanolic extract. Viability of cells was dose dependent for both extracts. The highest concentration of extract that did not significantly decrease the number of viable cells relative to the control was used for all subsequent analysis.

No significant cytotoxicity was detected for methanolic extract of *Hibiscus* at concentrations up to 1 mg mL^{-1} in Vero cells and a 50% cytotoxic concentration (CC_{50})>3.2 mg mL^{-1} was obtained. The antiviral assays were performed at concentrations below or equal to 3 mg mL^{-1}. The percentage of cell viability for the methanolic extract of *Hibiscus* was found to be >95% at 1 mg mL^{-1} for all the extracts (Fig. 1).

On the other hand, the percentage of cell viability for the aqueous extract was found to be >90% at all concentrations tested up to 2.0 mg mL^{-1} and started to decline slightly afterwards reaching a CC_{50} of about 4.9 mg mL^{-1}. The anti-HSV-1 activity was therefore determined at concentration of 5 mg mL^{-1} or lower for the aqueous extracts (Fig. 1).

Anti HSV-1 assay of methanolic and aqueous *Hibiscus* extracts detected by plaque reduction assay: It has been shown that the plaque reduction assay is a powerful technique for evaluating the efficacy of potential antiviral agents. One set of the Vero cells were incubated with the methanolic extract of *Hibiscus* at concentrations 0.5, 1, 1.5, 2, 2.5 and 3 mg mL^{-1} and the other set were incubated with the aqueous extract at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg mL^{-1} and then estimated by cytopathoic effect in the plaque reduction assay. The results showed that the antiviral activity of *Hibiscus* extract is dose dependent, after addition of different concentrations of methanolic/aqueous extracts of *Hibiscus* on HSV-1 a significant reduction >50% in plaque number was observed for concentration of 405 μg mL^{-1} in case of methanolic extract and 625 μg mL^{-1} in case of the aqueous extract (Fig. 2). Results showed that using concentrations of both extracts exceeding IC_{50} caused a gradual increase in inhibition of the performing of cytopathic effect, suggesting the inhibition of HSV-1 replication in Vero cells upon the increase in concentration of each extract.

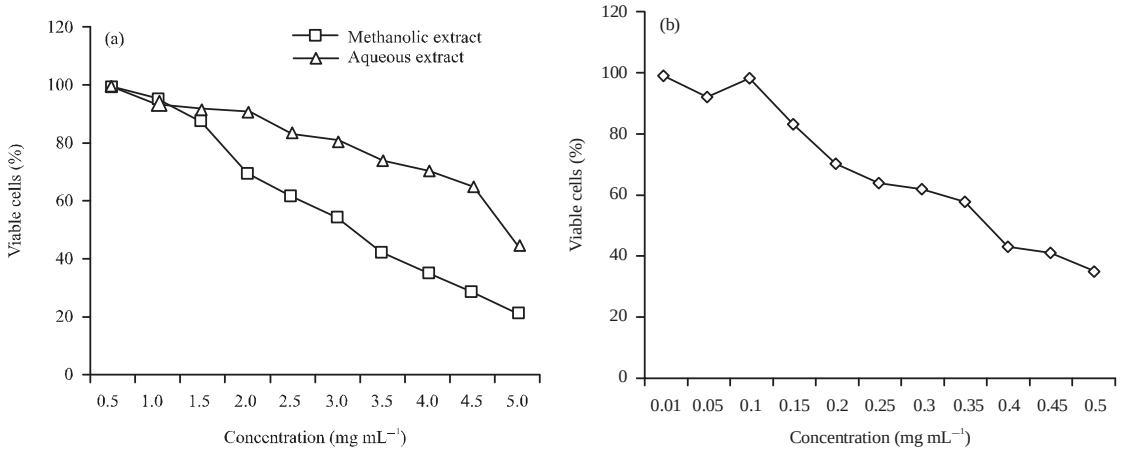

Fig. 1(a-b): Determination of cytotoxicity activity of (a) Methanolic and aqueous extracts of *Hibiscus* with CC_{50} of 3.2 and 4.9 mg mL^{-1}, respectively and (b) Acyclovir with CC_{50} of 0.37 mg mL^{-1}

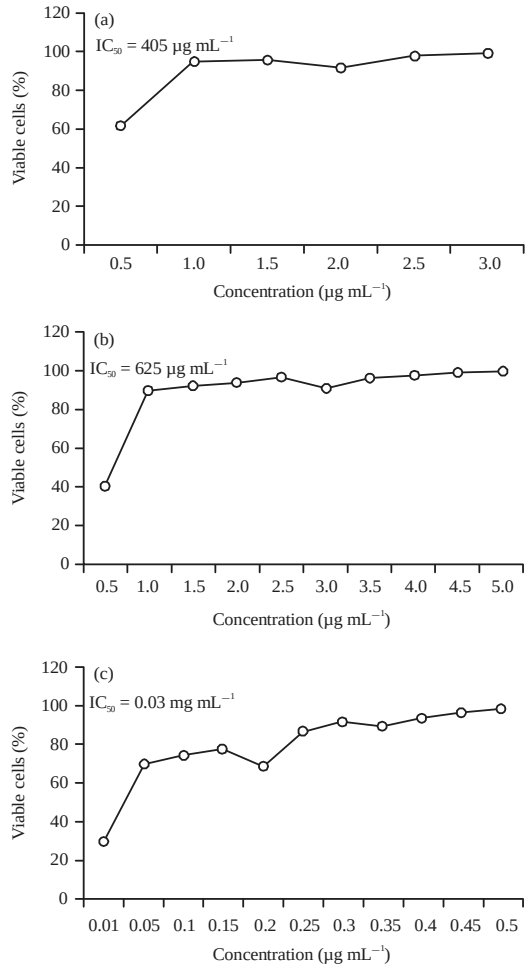

Fig. 2(a-c): Anti HSV-1 assay of (a) Methanolic extract, (b) Aqueous extract of *Hibiscus* and (c) Acyclovir by plaque reduction assay

The IC_{50} was calculated using regression line. Data were expressed as Mean±SD from three independent experiments

According to the findings, percentage of virus inhibition reached a peak at 1 mg mL^{-1} of methanolic extract recording 94.2% inhibition and then the inhibition was hardly increasing until reaching the 3 mg mL^{-1} of methanolic *Hibiscus* extract. As for the aqueous *Hibiscus* extract percentage inhibition made a big jump from 40% at 0.5 mg mL^{-1} to 90.7% at 1 mg mL^{-1} and afterwards the increase was very slow reaching the peak of 99.4% inhibition at 5 mg mL^{-1}.

As a result of this experiment and taking into consideration the antiviral activity of the extract together with the cytotoxicity results it seems that 1 mg mL^{-1} is the right choice to go forward with the rest of the experiments, because at this concentration both methanolic and aqueous extract possess a high antiviral activity against HSV-1. No cytotoxic effect was observed on cell multiplication at concentration that achieves antiviral activity. Anti HSV-1 assay using acyclovir was also conducted to present a baseline for validating the possibility of using *Hibiscus* extracts as antiviral agents.

Time of addition assay (Prophylactic effect): To determine whether the inhibitory activity of the methanolic and aqueous *Hibiscus* extracts occurred during the adsorption, corresponding to the attachment phase or post the infection corresponding to the replication phase of the virus, this experiment was carried out for the methanolic/aqueous *Hibiscus* extract where the treatment happened prior the infection and after the infection.

Results in Table 1 and Fig. 3 showed that both extracts exert great effect in the viral titer reduction observed when the treatment was performed pre and post to the infection. Virus Inhibition for the pre-treatment phase reached a

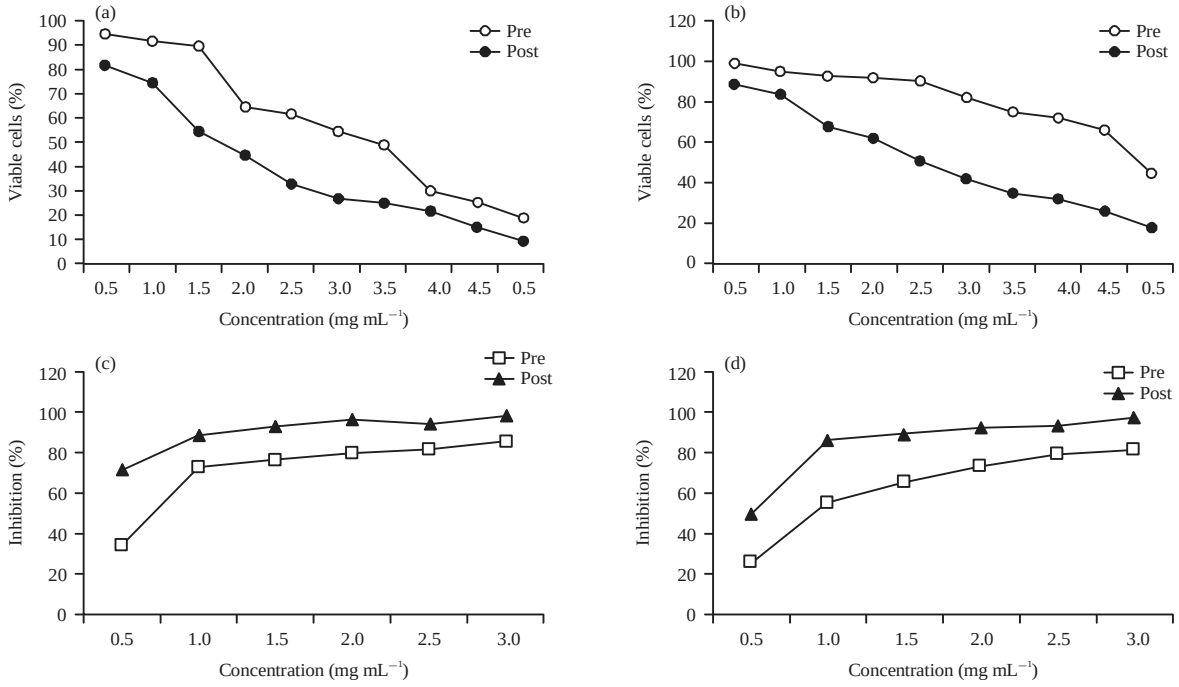

Fig. 3(a-d): Determination of cytotoxicity activity of pre-treatment and post-treatment for (a) Methanolic extract , (b) Aqueous extract of *Hibiscus* and Anti HSV-1 assay of pre-treatment and post-treatment for (c) Methanolic extract and (d) Aqueous extract of *Hibiscus*

Data were expressed as Mean±SD from three independent experiments

Table 1: Effect of the prophylactic activity due to application of the methanolic/aqueous *Hibiscus* extract on HSV-1 infected Vero cells

		Viral count (PFU mL^{-1})×10^6		Methanolic extract				Aqueous extract			
Extract	Initial pre viral count	Methanolic extract	Aqueous extract	Inhibition (%)	CC_{50} (mg mL^{-1})	IC_{50} (mg mL^{-1})	SI	Inhibition (%)	CC_{50} (mg mL^{-1})	IC_{50} (mg mL^{-1})	SI
Pre	3×10^6	0.80	0.92	63.2	3.4	0.70	4.9	55.4	4.7	0.98	4.7
Post		0.32	0.40	89.3	1.8	0.29	6.1	86.5	2.6	0.5	5.2

percentage inhibition of 63.2% at 1 mg mL^{-1} and 55.4% at 1 mg mL^{-1} for the methanolic and aqueous extracts respectively, which means that the *Hibiscus* extract blocked the delivery of the viral RNA into cells demonstrating that it worked at the level of entry of the virus. Treatment with the *Hibiscus* extract post the viral infection showed a max inhibition of 89.3% at 1 mg mL^{-1} and 86.5% at 1 mg mL^{-1} for methanolic and aqueous *Hibiscus* extracts respectively. These results suggested that the *Hibiscus* extract has a considerable effect on the HSV-1 virus in all stages of the infection but the strongest effect of the *Hibiscus* extract was actually on the early replication, then the attachment.

On the other hand, results revealed that the post-treatment of the Vero cells with the aqueous *Hibiscus* extract gave SI value of 5.2 whereas; the pre-treatment gave a lower SI value of 4.7. Whereas, the methanolic extract, when applied post infection gave SI value of 6.1 and when applied pre infection gave a value of 4.9 only which means that methanolic *Hibiscus* extract has a better potential as a therapeutic agent against HSV-1 than the aqueous one and that both extracts have stronger effect on HSV-1 when applied post-infection than pre-infection .

Virucidal activity of *Hibiscus* on HSV-1 (Direct virus inactivation assay): The neutralization and inhibitory effects of the methanolic/aqueous *Hibiscus* extract and the HSV-1 virus, the virucidal activity were tested, in cell free preparations, by incubating the methanolic/aqueous *Hibiscus* extract with the virus and leave it for 0, 15, 30, 45 and 60 min before applying on the Vero cells, Table 2, Fig. 4 and 5 showed that as time goes by, the virucidal activity of the *Hibiscus* gets considerably stronger ranging from 54.5% at time 0 with SI of 5.0, to 81.2% after 1 h of the mix with SI of 7.0, respectively in case of the aqueous extract and from 56.3% at time 0 with SI of 5.3, to 85% after 1 h of the mix with SI of 7.7, respectively in case of the methanolic extract.

Potential efficacy of virucidal versus prophylactic properties of *Hibiscus* on HSV-1: Results of the effect of each assay, virucidal (direct inactivation protocol, DIP), the inhibition in virus replication and the proper incubation time for both can be combined with the selectivity index of that protocol for *Hibiscus* extract were compared together to find the best protocol for antiviral activity. Summarized results of SI values show that *Hibiscus* extracts were effective as both virucidal and prophylactic agent giving high SI values of 7.7 as a virucidal when incubated for an hour with HSV-1, 6.1 for post treatment and 4.8 for pre-treatment in case of the methanolic extract. On the other hand aqueous extract showed also high SI values of 7.0 as virucidal when incubated for 1 h with HSV-1, 5.2 for post treatment and 4.7 for pre-treatment.

Virus infectivity and antigen expression assay: To confirm the time kinetics effect of *Hibiscus* extract on HSV-1 on viral antigen expression. Indirect immunoflurescence assay was carried out at zero time to serve as a baseline, at 5 h post infection, since this is time causing maximum inhibition of HSV-1 virus using methanolic and aqueous *Hibiscus* extracts and finally at 10 h post infection to check whether there is still inhibition of the virus or not. Results in Fig. 6 showed a characteristic pattern of small foci of fluorescent cells in Vero cells treated at 5 h post infection (Fig. 6d, e), revealing a considerable percentage of virus inhibition, suggesting that the drug inhibited the viral dissemination and acted as a prophylactic drug which can be used after the virus infection. This study revealed that H ibiscus extract has a good anti-HSV-1 activity, probably by inhibiting the early stage of multiplication (0-5 h) (Fig. 6d, e). Results also showed that virus foci were much larger in number when incubated for 10 h with HSV-1 (Fig. 6f, g) indicating that the virus started to ware out the Vero cells and *Hibiscus* extract are not as effective as they were 5 h post infection.

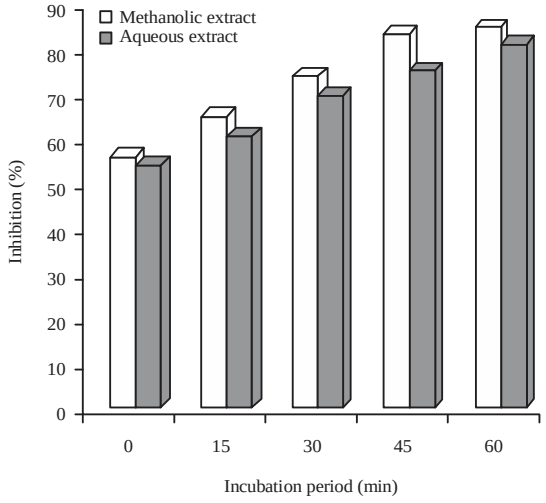

Fig. 4: Effect of the virucidal activity due to application of the methanolic/aqueous *Hibiscus* extract on HSV-1 infected Vero cells

DISCUSSION

Evaluating the medicinal value of the *Hibiscus* extract has been carried over to highlight the preliminary phytochemical analysis which revealed that *Hibiscus* extract contained anthocyanin, polyphenols and flavonoids which constitute the major phytochemical compounds. Results were comparable with these reported by Al-Hashimi[31] who investigated the antioxidant and antibacterial activities of *Hibiscus* as well as its chemical analysis which revealed some different results than those shown in this study. Difference in results may be due to the difference in the *Hibiscus* species used and isolation/extraction methods implemented.

Studying the effect of methanolic and aqueous *Hibiscus* extracts on cell viability by MTT revealed that methanolic and aqueous *Hibiscus* extracts showed a high viability on Vero cells. These results come in agreement with the study conducted by Baatartsogt *et al.*[32] on the antiviral effects of the aqueous *Hibiscus* tea extract on the avian influenza virus showed that there was no observed cytotoxicity for all tested concentrations of the extract and the cell viability reported

Table 2: Effect of the virucidal activity due to application of the methanolic/aqueous *Hibiscus* extract on HSV-1 infected Vero cells

Incubation period (min)	Initial viral count	Viral count (PFU mL^{-1}) $\times 10^6$ Methanolic extract	Aqueous extract	Methanolic extract Inhibition (%)	CC_{50} ($mg\ mL^{-1}$)	IC_{50} ($mg\ mL^{-1}$)	SI	Aqueous extract Inhibition (%)	CC_{50} ($mg\ mL^{-1}$)	IC_{50} ($mg\ mL^{-1}$)	SI
0	3×10^6	1.22	1.61	56.3	3.70	0.70	5.3	54.5	4.45	0.89	5.0
15		1.04	1.18	65.5	3.50	0.62	5.6	60.6	4.85	0.82	5.9
30		0.77	0.91	74.3	3.20	0.55	5.8	69.7	4.8	0.74	6.5
45		0.49	0.75	83.5	3.13	0.49	6.4	75.0	4.75	0.69	6.9
60		0.45	0.56	85.0	3.16	0.41	7.7	81.2	4.27	0.61	7.0

SI: Selectivity index, CC: Cytotoxic concentration

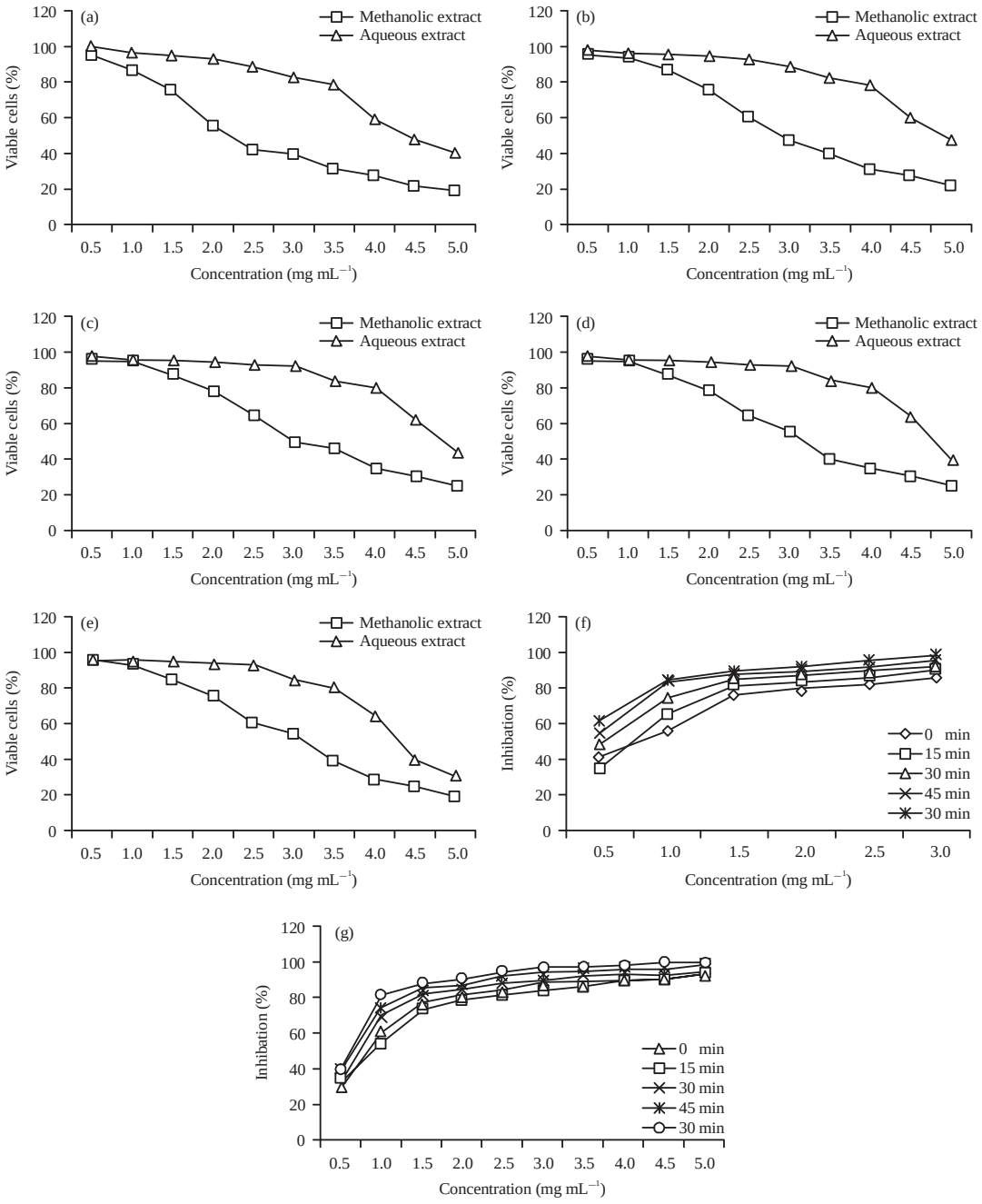

Fig. 5(a-g): Determination of cytotoxicity activity of methanolic and aqueous extracts of *Hibiscus* for Incubation period (a) 0 min, (b) 15 min, (c) 30 min, (d) 45 min, (e) 60 min and anti HSV-1 assay of 0, 15, 30, 45 and 60 min for (f) Methanolic extract and (g) Aqueous extract of *Hibiscus*

Data were expressed as Mean±SD from three independent experiments

was very high. Furthermore, Da-Costa-Rocha *et al.*[33] also stated that *Hibiscus* extract has an excellent safety and tolerability record.

Anti HSV-1 assay of methanolic and aqueous extracts of *Hibiscus* showed very promising results for both extracts as potential antiviral agents. In order to gain insight on the possible mechanism of action of *Hibiscus* extract on HSV-1, investigation has been conducted to detect the prophylactic response that *Hibiscus* extracts trigger to resist HSV-1. Results showed that percentage of inhibition of the HSV-1 post-treatment was relatively high. The percentage of inhibition observed in post-treatment may be due to the high

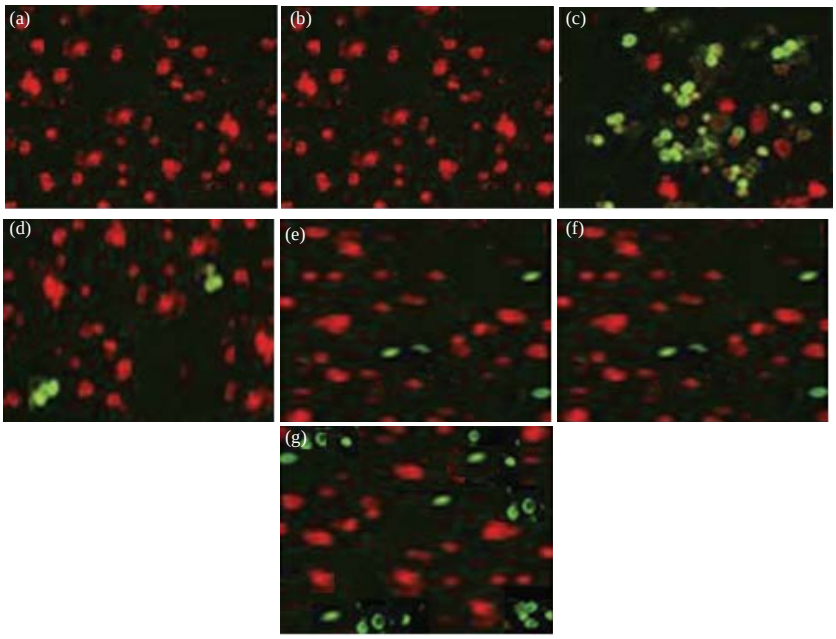

Fig. 6(a-g): Effect of *Hibiscus* extract on HSV-1 antigen expression (a) Cell control, (b) Virus control, (c) Infected cells, (d) Infected cells treated with methanolic *Hibiscus* extract at 5 h post infection, (e) Infected cells treated with aqueous *Hibiscus* extract at 5 h post infection, (f) Infected cells treated with methanolic *Hibiscus* extract at 10 h post infection and (g) Infected cells treated with aqueous *Hibiscus* extract at 10 h post infection

content of polyphnolics and flavonoids, which are considered the major constitutes of the *Hibiscus* extract and may play a role in preventing the virus binding to the host cell receptors and block or reduce the entry of the virus into the cell to protect the cell from the virus destruction and prevent the viral replication[34]. Also, the different active components in the extract may affect the different steps of the antiviral activity in synergetic manner.

Several mechanisms of antiviral activity have been proposed to offer some valuable insights for researchers to study the antiviral mechanism related to the *Hibiscus* extract consumption. Also, the reduction observed in the virus titer when the treatment was performed after the infection suggested that the *Hibiscus* extract can affect the replication stage. Some researchers reported that crude extract can inhibit viral replication[35]. Cheng *et al.*[36] found out that proanthocyanin A-1 inhibited viral attachment and penetration and affected the late stages of HSV-2 infection. These results come in agreement with Rebensburg *et al.*[37], who studied the antiviral activity of *Cistus incanus* extract against HIV virus and found out that Ci extract affect the early attachment phase of the HIV virus.

On the other hand, experiments conducted to determine the virucidal activity showed that a total reduction of virus titer due to mixing of the methanolic/aqueous *Hibiscus* extract with the virus and leaving it for 0, 15, 30, 45 and 60 min before applying on the Vero cells. This reduction may be due to the fact that both extract preparations contain polyphenols, flavonoids and anthocyanins which may play a role in the potency of antiviral activity.

Haslam[38] suggested that plant polyphenols exert a direct action on the viral particles, inhibiting the adsorption of the virus to the host cell receptors. Li *et al.*[39] reported that the viral capsid protein affected by polyphenolic content which may cause irreversible damage or reversible blocking of certain regions of the capsid protein. These results nominate the *Hibiscus* extract as a prophylactic therapeutic agent against the HSV-1 infection.

Results of the comparison between antiviral, prophylactic and virucidal assays for both extracts of *Hibiscus*, it can be concluded that the *Hibiscus* extract is a potent antiviral drug and its action is not related to the cytotoxicity of the host cells. Also, the *Hibiscus* extract was highly selective against HSV-1 and against the virally infected cells, so this extract can act as a virucidal agent as well as prophylactic agent.

These findings point out the importance of *Hibiscus* extract as a drug with the ability to interact with the viral envelope as well as its ability to interact with Vero cell surface in a potentially effective way.

All the previously mentioned SI values were considered high when compared with the selectivity index of acyclovir value of (0.37/0.03) 12.3 and keeping in mind the added value of using a natural substance instead of a chemical with potential side effects and probably a higher cytotoxicity rates.

Studying the time kinetics of *Hibiscus* extracts on HSV-1 showed that the *Hibiscus* extract inhibited the early stages of viral multiplication. Comparable results were reported by Bag *et al.*[40], who reported that using Mallotus peltatus extract on the HSV-1 infected Vero cells showed a characteristic pattern of small foci of fluorescent at different time interval (2-4 h post-infection) revealing that the antiviral activity of all *Hibiscus* extracts tested was effective during the different steps of the virus replication cycle and thus, *Hibiscus* extract may provide a novel treatment for HSV-1 infectivity.

CONCLUSION AND FUTURE RECOMMENDATIONS

This study demonstrated the potent and broadband antiviral activity of *Hibiscus* extracts *in vitro* and gave a strong evidence that *Hibiscus* extract can be is a potential therapeutic agent to treat the HSV-1 virus infection. Clinical studies that show the feasibility and safety profile of *Hibiscus in vivo* are strongly recommended to follow up this study in order to fully evaluate the *Hibiscus* extract for antiviral therapeutic applications. Future studies may also include identification of individual antiviral agents from *Hibiscus* extract, followed by an extensive analysis of its pharmacokinetic properties. Another point of research as an amendment to this current study would be to identify a single active compound from complex or simplified *Hibiscus* extract which may then lead to a novel antiviral agent as well as provide new therapeutic agents for Herpes infection and monitoring of pharmacological use as antiviral agents in clinical studies.

SIGNIFICANCE STATEMENTS

This study discovers the possible usage of *Hibiscus* extracts as a therapeutic agent for treating herpes simplex virus-type1 that can be beneficial for replacing Acyclovir that is being used now to treat herpes patients. This replacement is not about replacing a drug with another, it's about replacing a synthetic chemical compound with a natural product that does not initiate drug resistant varieties of the virus like what happens with Acyclovir. This study will help the researcher to uncover the potential use of *Hibiscus* extract as a real therapeutic agent and lighten the way to complete the research *in vivo*.

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