Antiviral Activity of Polyphenols Extracts From *Daucus carota* against Herpes Simplex Virus type 1

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Abstract: Herpes Simplex Virus type 1 (HSV-1), is a common and recurrent human virus with no medical cure. Polyphenols, on the other hand, are known to exhibit very strong oxygen scavenging properties; therefore they are considered a very rich source of antioxidants.

In this research, the effect of extraction method using different solvents on the content of polyphenols extracted from the leaves and roots of *D.carota* was studied, and their relationship with antioxidant activity was investigated.

The examined extracts were tested for cytotoxicity on Vero cell line, with reference to IC_{50}, and other non-toxic concentrations of all the extracts. The antiviral activity against HSV-1 for all non-toxic concentrations of the extracts was determined using plaque reduction assay, which revealed that the inhibitory activity of the HSV-1 virus was dose dependent on the polyphenol content of the examined extracts. The MIC for all the extracts was also determined as well as the EC_{50} and the SI for all the extracts. Calculated SI values for the examined extracts showed promising SI on the aqueous extracts of the *D.carota* roots and leaves, and hence can be used as therapeutic medication for HSV-1.

Direct contact between the HSV-1 and the examined extracts in cell-free assay system showed different degrees of virucidal activity depending on the polyphenolic content of these extracts. In order to study other possible mode of action, Vero cells were treated with the examined extracts before, during, and after virus infection to give an insight on the interference of the extract in each step in the virus life cycle. Examined extracts exhibited the antiviral activity against HSV-1 via blocking of the virus attachment and penetration and inhibition of the early stage of viral replications.

Keywords: antiviral activity, HSV-1, polyphenols

Introduction

Polyphenol compounds from green tea, berries, olive oil, fruits, and vegetables have been linked recently with good health, due to the fact that they have a strong preventive role for chronic and inflammatory diseases, as they have redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin (Ciz et al., 2008; Gebicka and Banasiak, 2009). It is sort of commonly known now that the greater the human consumption of these compounds is, the better the health will be. Recently, however research has been conducted on the safe levels of consumption of these polyphenolic compounds, as it was reported in the American Chemical Society's Journal that that regardless the fact that the consumption of phytochemicals like polyphenols and flavonoids can boost the general health, and have plenty of beneficial biological effects, there are evidences that these precious values come on the expense of some risk. Hence, polyphenolic compounds can be very beneficial and can be very risky, and the solution is to use them with moderation as food supplements, and in specified doses if used as drug components.

The real value of Phenolic compounds is that they possess antioxidant activity which allows them to scavenge both active oxygen species and electrophiles, to inhibit nitrosation and to chelate metal ions, to have the potential for autoxidation and the capability to modulate certain cellular enzyme activities (Rice et al., 1995). Carrots are known for their richness in vitamin A, and they are also very rich in α- and β- carotenes (Urrea et al., 2011). Carotenes have been proved to possess antioxidant activity (Block 1994). There is little known information about phenolic compounds in carrots, their antioxidant properties, and their relation to antiviral activity against HSV-1.

HSV-1 on the other hand, is a DNA virus that causes fever blisters, and the primary symptoms of this virus infection are flu-like with fever, followed by the itching and finally those painful papules (Khan et al., 2005). Now the real problem with this virus is not about those painful papules, it is about how this virus remains in a latent state in the sensory neurons for a recurrent infection. This recurrent infection, or virus reactivation, is

usually triggered by stresses like radiation, and other related factors such as sunlight, menstruation and therapeutic irradiation (Collier and Oxford, 2000; Wagner et al., 2008). Hence, if the HSV-1 reactivation is triggered by stress which triggers the oxidative stress, then maybe using antioxidants can cause the oxidative stress to return to the balance state and inhibit the HSV-1 infection. The real problem with herpes is that there is no real cure for it, once a person has the virus, it remains in the body. The virus lies inactive in the nerve cells until something triggers it to become active again. Treatments however can relieve the symptoms, and decrease the pain. Treatments can also shorten healing time and decrease the total number of re-infection that is why antiviral agents from plants with new effective compounds exhibiting different modes of action against viral infections are urgently needed.

The objective of this work is to extract polyphenolic compounds from the leaves and roots of carrots using two different extraction methods to compare which combined method with plant part gives the maximum polyphenolic content, and which gives better antioxidant activity. The effect of all the extracts on the cell viability will be assayed, and then anti-HSV-1 virus activity screening will take place afterwards to check the effect of the extracts on virus infectivity. Afterwards, safe concentrations will be selected to determine their effect on each step of the virus life cycle.

Materials and Methods

Chemicals

Chemicals used for the study were from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

Plants

Carrot (*Daucus carota* L.) is a very common little plant with lacy, pinnately leaves. Fresh carrot plants were purchased from the local farm market with the roots and leaves, packed in paper bags and stored at suitable temperature prior to the analysis.

Extraction

Extractions were done for both roots and leaves of the carrots in two ways using acetone and water. Acetone extracts were prepared according to Donglin (2004) with modifications, by sampling 20g from each leaves and roots in 30ml of acetone and extracted for 60mins. The homogenate was centrifuged at 7000xg for 20mins. The supernatant was collected and the residue was added with 20ml of 80% acetone for two successive re-extraction. The collected supernatants were combined and the residue was discarded. Acetone was removed from the supernatant by evaporation under vacuum at 35°C, pigments and fatty acids were eliminated by two successive extractions with petroleum ether (2:1, v:v). The aqueous phase was collected as a crude extract.

Water extracts were prepared according to Kratchanova et al., (2010) with little modifications as follows: 10g of the plant powder (roots or leaves) was added to 400 ml water (90 ºC). Plant leaves were incubated for 30 min, whereas roots were incubated for 1 hour. The extract was centrifuged (7000 × *g)* and supernatants were used for further analysis. Both extracts were obtained from acetone and aqueous extracts subjected to lyophillization.

Determination of total phenolic content

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This reagent, based on the Slinkard and Singleton method (1977), and the early work of Singleton and Rossi (1965) is a colorimetric oxidation/reduction method for phenolic compounds. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. Briefly, a 20 µL of the diluted sample was added to 100 µL of Folin–Ciocalteu reagent. After 8 min, 300 µL of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 764 nm. The calibration curve was prepared with gallic acid solutions ranging from 0 to 500 mg/L, and the results are given as gallic acid equivalents (GAE).

Antioxidant Activity

Antioxidant activity was evaluated based on the method reported by Taga et al., (1984) of coupling the oxidation of β-carotene and linoleic acid. 6 mg of β-carotene was dissolved in 20ml chloroform. 3ml of the solution was added to 40µl linoleic acid and 400µl Tween 20. Rotary evaporator was then used under vacuum at 35°C to remove the chloroform. Oxygenated distilled water (100 ml) was added to the β-carotene emulsion and mixed well. 3 ml aliquot of the β-carotene emulsion and 0.2 ml of the diluted extract were placed in a test tube and mixed well. The tubes were immediately placed in a water bath and incubated at 50ºC. Oxidation of β-carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Sample absorbance was measured at 10, 20, 30, 40, 50, and 60 min after incubation. A control consisted of 0.2 ml distilled water, instead of the extract. The degradation rate of the extracts was calculated by first order kinetics:

Sample degradation rate = ln (a/b) ×1/t

where: ln = natural log; a = initial absorbance at time 0; b = absorbance at 10, 20, 30, 40, 50 and 60 min; t = time (min). Antioxidant activity (AA) was expressed as % inhibition relative to the control using the equation:
AA(%) =
(Degradation of control – Degradation of sample) x 100 / Degradation of control

Cells and Viruses

For the anti HSV-1 activity screening, African Green Monkey kidney cells (Vero), were grown in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml). Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. The HSV-1 was propagated in Vero cells, while stock viruses were prepared as previously described (Simões et al. 1999). After three cycles of freezing/thawing, the fluids were titrated on the basis of PFU count as previously described (Burleson et al. 1992) and stored at -80°C until use. The virus titer expressed by ($TCID_{50\%}$)was determined by cytopathic effect in cell culture.

Effect of acetone and water extracts of leaves and roots of *D.carota* on cell viability (cytotoxicity)

For each extract, 400mg of lyophilized powder was diluted in 200ml od dimethylsulfoxid (DMSO) and 800ml of strile phosphate buffer solution (PBS) to obtain a stock solution with concentration 400µg/ml. Two fold dilution were prepared in minimal essential medium (MEM) complemented with 2% fetal calf serum, sodium bicarbonate and penicillin / streptomycin / amphotericinb. Confluent Vero cell mono-layers were overlayed with the different dilutions of the extract and were incubated at 37°C with 5% CO_2 during five days. Toxicity was determined by observation of the morphology of the cells in comparison with the cell control without the extract. After five days of incubation, neutral crystal violet was added for confirmation of cell viability. The cell monolayer was examined by microscopic assessment of changes cell morphology or visible toxic effect (Ojo et al., 2009). The cells grown in the absence of extracts were used as 100% cell survival. The concentration at which the cell number was reduced to 50% is cytotoxic concentration CC_{50}(or inhibitory concentration IC_{50}) The concentration which had no or little effect on the cell number, maximum tolerated concentration or minimum inhibitory concentration (MIC) was also observed.

Viral plaque number reduction assay

Confluent cell monolayers in 24-well tissue culture plates were adsorbed with 100-200 PFU of HSV for 1 h at room temperature. Then, the infected cells were incubated with the different minimum inhibitory concentration of the examined extracts, 230µg/ml for water roots, 86.37µg/ml for water leaves, 72.6µg/ml for acetone roots, and 48.4µg/ml for acetone leaves. Infected cells were then overlaid with medium, containing 1.5% carboxymethylcellulose. After incubation for 5 days at 37°C in 5% CO2, infected cells were stained with 0.1% crystal violet, in 1% ethanol, for 15 min. Percentage of viral inhibition after treatment with the extracts was calculated as percentage inhibition compared with untreated viral infected cells control from triplicate experiments.

Virucidal Activity

It was tested by incubation of virus with aqueous and acetone extracts from the leaves, and root of *D.carota*, directly before inoculation of virus onto cells. Briefly, 6- well plate was cultivated with Vero cells, and 50µl of virus diluted to 100-200 PFU/ml was added to equal volume (V/V) of each extract at concentrations giving maximum viral inhibition to give total volume of 100µl. After 1hr of incubation with shaking at 37ºC, the mixture was diluted by 10 fold dilution in a way that still gave suitable count of viral particles. Then 100µl of the mixture were added to the cell monolayer sheet, and incubated with shaking for 1hr at 37ºC, and 3ml of overlay medium was added. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated (Schuhmacher *et al*., 2003).

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on Vero cells before HSV-1 adsorption (effect on pretreated cells)

It was tested by subjecting the extract to the monolayer sheet of cells for 2hr before virus inoculation. Briefly, 6-well plate was cultivated with Vero cells and non cytotoxic concentrations of all examined extracts were added to the monolayer sheet cells at concentrations giving high percentage of viral inhibition and incubated for 2hr at 37ºC. Extract was removed by washing the cells three successive times with media without supplements. Virus was diluted to 100-200 PFU/ml then 50µl of the virus stock was applied to the monolayer confluent sheet of cells, and then incubated with shaking for 1hr at 37ºC for virus adsorption. Un-adsorbed viral particles were removed by washing the cells sheet three successive times with medium without supplements, and 3ml of overlay medium was added to the cell monolayer sheet. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated (Zhang *et al*., 1995).

Effect of aqueous and acetone extracts from the leaves and roots of *D.carota* on HSV-1 during viral adsorption (effect on attachment and penetration)

Confluent cell monolayers, cultivated in 24-well plates, were infected with 100-200 PFU of HSV-1. Various non toxic concentrations of each examined extract was added into the cell monolayers and incubated for 1 h at room temperature during virus adsorption. After that, the inoculum was removed and infected cells were overlaid with overlay medium, and incubated at 37°C in 5% CO_2 for 5 days**.** The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 min. The 50% effective concentration was calculated and compared with the untreated viral infected cell controls.

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on virus replication

It was tested by post inoculation of extracts after HSV-1 virus application to cells. A 6-well plate was cultivated with Vero cells and incubated for 1-2 days at 37ºC for formation of confluent sheet. Virus was diluted to 100-200 PFU/ml then 50µl of the virus stock was applied to the monolayer confluent sheet of cells, and then incubated with shaking for 1hr at 37ºC. Un-adsorbed viral particles were removed by washing the cells sheet three successive times with medium without supplements. Non cytotoxic concentrations with high antiviral activity were applied at different time intervals after virus adsorption at 1, 4, 8, 24, and 48. After each time interval, 3ml of overlay medium was added to the cell monolayer sheet. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated (Amoros *et al*., 1994).

Results and Discussions

Determination of total polyphenolic content

Results in table (1) show a variation in the poyphenol content according to the type of extract used and the part of the plant. Some studies encourage the use of different extraction systems like ethanol (Djeridane et al., 2006), and methanol (Wojdylo et al, 2007) to extract polyphenols with antioxidant activities. Some other studies preferred the use of water extraction methods of polyphenols (Zheng and Wang, 2001; Katalinic et al., 2006; Dalva et al., 2009).

The obtained results in table (1) indicate that the total polyphenol content in acetone extracts for leaves and roots showing 387.2 mg/100g and 290.6 mg/100g are higher than the corresponding values in the aqueous extracts showing 345.5 mg/100g and 230 mg/100g The variation in values of polyphenol content between acetone and aqueous extracts maybe due to variation of polarity of polyphenols according to the type of solvent used (Wojcikowski et al., 2007). Also, the total polyphenol content of *D.carota* leaves had the higher amount showing 387.2 mg/100g, and 345.5 mg/100g in acetone and aqueous extracts compared with the content in roots showing 290.6 mg/100g, and 230 mg/100g in acetone and aqueous extracts respectively. These results show different values for the polyphenol content of the *D.carota* roots than those reported by Donglin, and Yasunori (2004) on the carrot's peel. This difference may be caused by the difference in the extraction methods and the tissue of the plant under study. Comparable results were also reported by Trouong et al., (2011) on the sweet potatoes.

Table 1: Polyphenol content of water and acetone extracts of leaves and roots of *D.carota*

Part of the plant	Polyphenol content in mg/100g	
	Water Extract	Acetone Extract
Leaves	345.5	387.2
Roots	230	290.6

Antioxidant Activity

Results in table (2) show that the antioxidant activities of polyphenols from acetone extracts are higher than those of the aqueous extracts for each of the leaves and roots of the *D.carota* showing 96.4% and 84% for leaves and roots respectively, whereas, the aqueous extract showed antioxidant activity of 92%, and 76% for leaves and roots respectively. Also, acetone and water extracts of the leaves of *D.carota* showed higher antioxidant activity than the corresponding values in the roots extracts showing 96.4%, and 92% for leaves and 84%, and 76% respectively. This same result was reported by Donglin et al., (2004), who stated that the choice of solvent used in the extraction may have a significant impact on the antioxidant properties.

Table 2: Antioxidant activity of water and acetone extracts of leaves and roots of *D.carota*

Part of the plant	Antioxidant activity (%)	
	Water Extract	Acetone Extract
Leaves	92	96.4
Roots	76	84

From our results, a certain relation between the amount of polyphenol in the extracts and their corresponding antioxidant activities has been identified. When polyphenols content increase, a noticeable increase in antioxidant activity happens; this is indicated by the fact that the highest amount of polyphenols detected in the acetone extract of the leaves, showing 387.2 µg/ml, gave the highest antioxidant activity of 96.4%. Same results were reported by Donglin, and Yasunori, (2004).

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on cell viability (cytotoxicity)

The evaluation of the effects of all examined extracts on the growth and morphology of the Vero cell line using the cytotoxic assay is shown in table (3) and figure (1) below. Two-fold dilutions for aqueous and acetone lyophilized extracts of the leaves and roots of *D.carota* were prepared. These dilutions were used for biological activity toward the viability of Vero cell line. Determination of cytotoxicity of the used extract is an important

factor in the evaluation of any antiviral substance, since the potent extract should be chosen for the antiviral activity with little or no effects on the metabolism of host cells. All the different dilutions of the aqueous extract of the roots of *D.carota* (figure 1C) had no apparent cytotoxic effect with IC_{50} of 506 µg/ml and cell-safe concentrations <=230µg/ml. The different dilutions of the aqueous extracts of the leaves (figure 1A) showed variant degrees of toxicity especially on higher concentrations (345.5 µg/ml, and 172.8 µg/ml), while the lower concentrations showed no cytopathic effect on the Vero cell line with IC_{50} of 129.5 µg/ml and cell-safe concentrations <=86.3 µg/ml.

On the other hand, all dilutions of acetone extracts of the roots (figure 1D) showed different degrees of the cytopathic effect especially on higher concentrations (290.6 µg/ml, and 145.3 µg/ml) , while the lower concentrations showed no cytopathic effect on the Vero cell line with IC_{50} of 108.9 µg/ml and cell-safe concentrations <=72.6 µg/ml. Finally, all dilutions of acetone extracts of the leaves (figure 1B) showed different degrees of the cytopathic effect especially on higher concentrations (387.2 µg/ml, 193.6, and 96.8 µg/ml) , while the lower concentrations showed no cytopathic effect on the Vero cell line with IC_{50} of 72.6 µg/ml and cell-safe concentrations <= 48.4 µg/ml.

Table 3: Cytopathic effect % of acetone and water extracts of the leaves and roots of *D.carota*

Dilution	Water		Acetone	
	Leaves	Roots	Leaves	Roots
1	90	22.5	100	82.1
½	76.1	8.2	92.4	64
¼	22	0	68.3	21.3
1/8	17.4	0	28.1	12.1
1/16	7.2	0	13.6	7.5
1/32	0	0	2.5	0

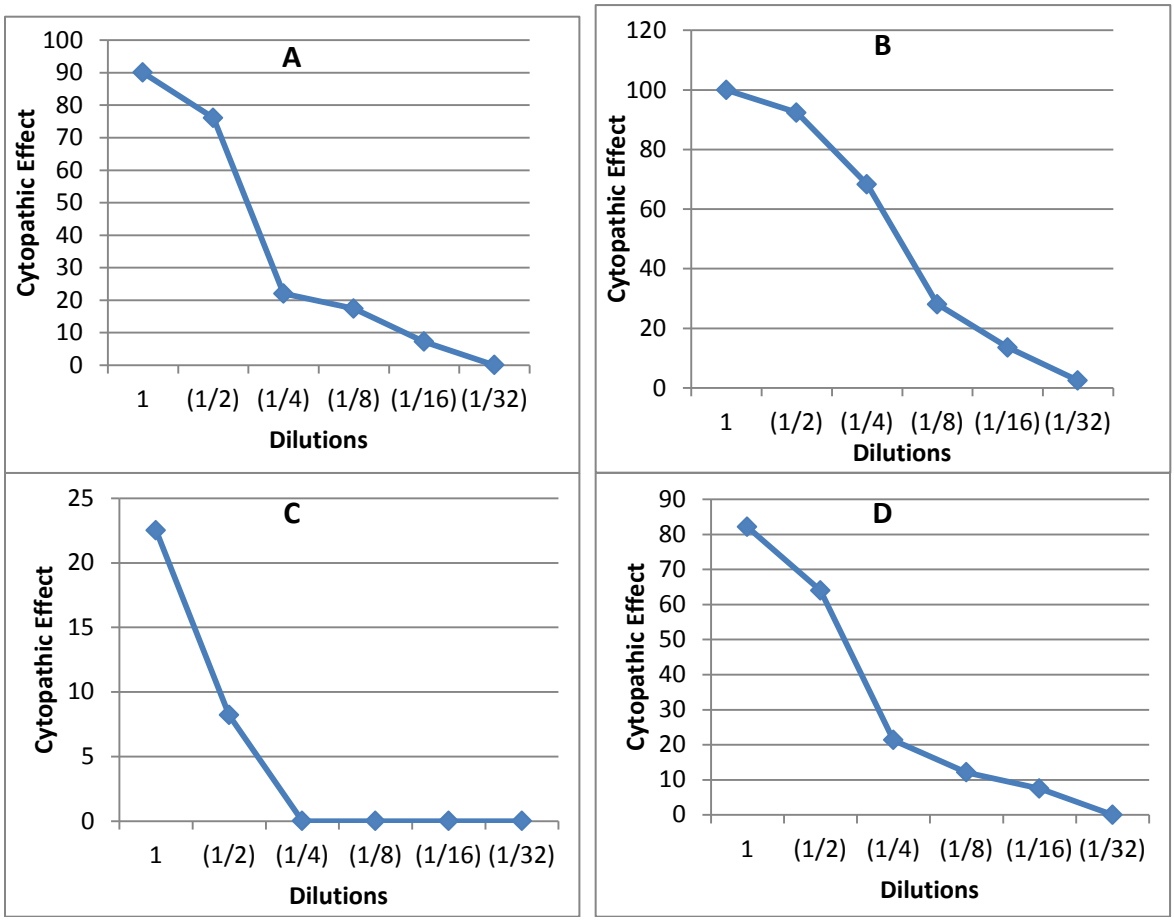

Figure 1: Cytopathic effect % of the water and acetone extracts of leaves and roots of *D.carota*. A: Water leaves; B: acetone leaves; C: water roots; and D: acetone roots.

Viral plaque number reduction assay

The experiment of the cytotoxic effect of the aqueous and acetone extracts from the leaves, and roots of *D.carota* extracts on the Vero cell line gave the safest concentrations, and those concentrations were used in the evaluation of the antiviral activity of the extracts in the following experiments to study the effect of the extract on the virus life cycle, and determine the EC_{50}, which is the 50% viral inhibitory concentration, and the selectivity index (SI) which is calculated as the IC_{50}/EC_{50} and finally, to choose the minimum inhibitory concentration (MIC) that is the concentration with no or little cytotoxicity and maximum antiviral activity.

The antiviral activity of the polyphenol extracts of leaves and roots of *D.carota* were assayed by plaques number reduction assay. Results in table (4) below show that the aqueous roots extract (figure 2C) started with a very strong antiviral activity of 95% at dilution 1, which is equivalent to concentration 230 µg/ml. This extract lost a considerable part of its antiviral ability when diluted to 1/2 giving antiviral activity of 72%, which started to drop gradually afterwards as the extract's dilutions increase, the extract have EC_{50} of 60.3 µg/ml, SI of 8.39, and MIC of 230 µg/ml, as this is the concentration giving the maximum antiviral activity for the minimum cell cytotoxicity.

As for, the antiviral activity of the aqueous leaves extract (figure 2A) started with a moderate antiviral activity of 70% at dilution 1/4, which is equivalent to concentration 86.3 µg/ml. This extract started to lose its antiviral activity gradually, the extract have EC_{50} of 39.1 µg/ml, SI of 3.3 and MIC of 86.3 µg/ml. The acetone extract of the roots (figure 2D), on the other hand started also with a moderate antiviral activity of 67% at dilution 1/4 which is equivalent to concentration 72.6 µg/ml. This antiviral activity almost disappeared at dilution 1/8 giving 39.2% antiviral activity, the extract has EC_{50} of 51.9 µg/ml, SI of2.09 and MIC of 72.6 µg/ml. Finally, the acetone extract of the leaves (figure 2B) started already with a moderate to low antiviral activity of 59% at dilution 1/8, which is equivalent to concentration 48.4 µg/ml. This extract started to drop further until it almost vanished, the extract has EC_{50} of 67.6 µg/ml, SI of 1.07 and MIC of 48.4 µg/ml.

Hence, it can be concluded from the above results that as the polyphenol content increases in the extract the antiviral activity increases as well.

As efficient SI values should have very little cytopathic effect on the cells, and very high antiviral effect on the virus, It can be concluded from the results that the aqueous extract of the roots has the most promising results as a therapeutic treatment for HSV-1, followed by the aqueous extract of the leaves, then the acetone extract of the roots, and finally the worst SI value was recorded by the acetone extract of the leaves. So, although the fact that acetone extract of the leaves has the greatest polyphenol content in all the used extracts, it has the worst SI because it requires to use a very low concentration of it in order to be safe on the cell, and consequently will have a low antiviral activity, which means a weak effect as a therapeutic agent. On the other hand, the aqueous extract of the roots had the least amount of polyphenol content in all the used extracts, but since almost all of its concentrations used were safe and consequently a high concentration can be used which will give a high antiviral activity and a strong effect as a therapeutic agent (figure 2`).

Table 4: Plaque reduction assay of each of the cell-safe dilutions of water and acetone extracts for leaves and roots of *D.carots*

	Water Leaves				Acetone Leaves			Water Roots						Acetone Roots			
Dilutions	1/4	1/8	1/16	1/32	1/8	1/16	1/32	1	1/2	1/4	1/8	1/16	1/32	1/4	1/8	1/16	1/32
% Inhibition	70	54.2	29.3	9.5	59	33.3	4.5	95	72	61.5	34.1	15.0	2.3	67	39.2	13.2	4.9

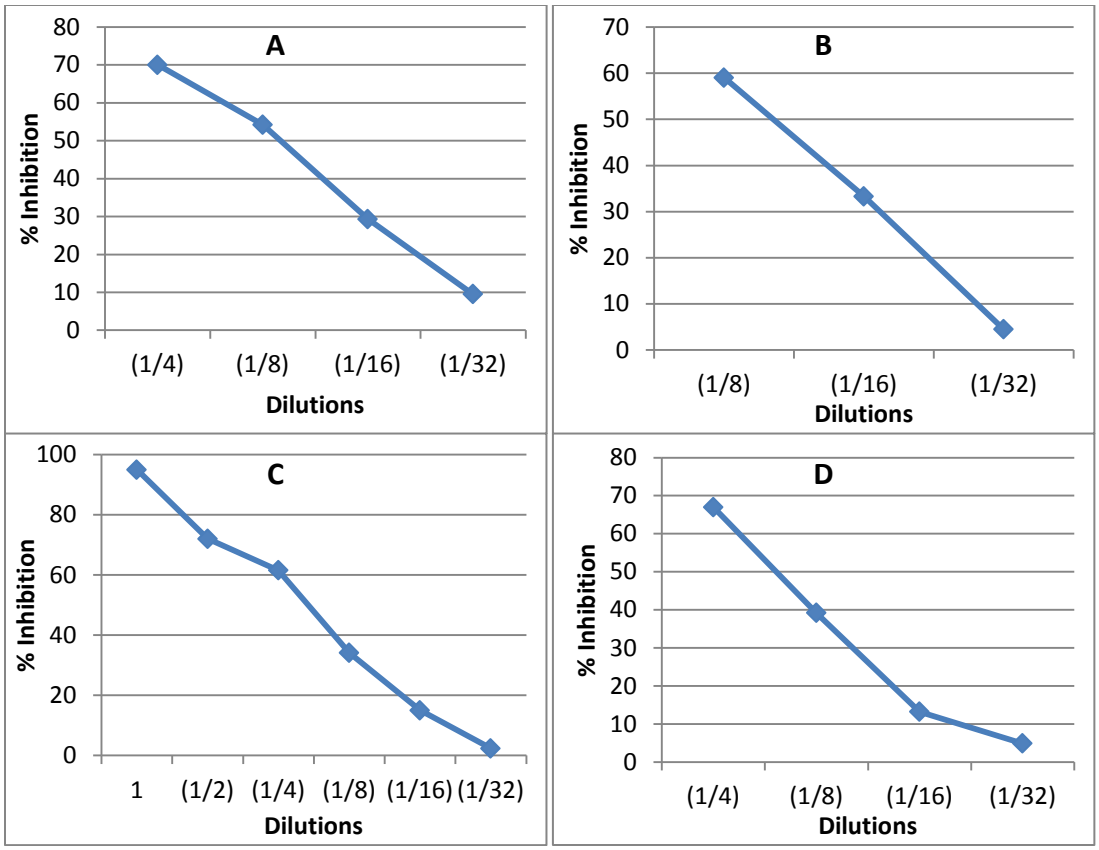

Figure 2: Plaque reduction assay of each of all cell-safe dilutions of water and acetone extracts for leaves and roots of *D.carots*. A: Water leaves; B: acetone leaves; C: water roots; and D: acetone roots.

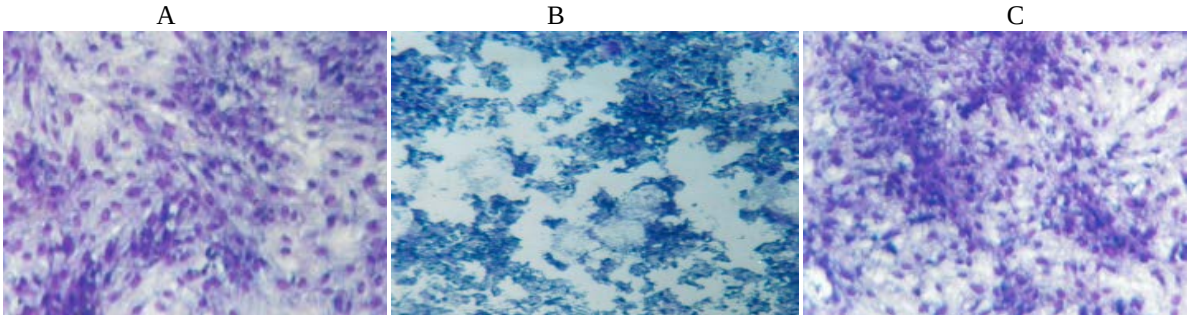

Figure 2`: Reduction of HSV-1 cytopathic effect using the acetone extract of *D.carota* leaves. A:control Vero cells; B:viral cytopathic effect; and C:Vero cells infected with HSV-1 in the presence of the aqueous extract of the *D.carota* roots

Virucidal Activity

After determination of toxic and safe concentrations of all aqueous and acetone extracts from the leaves, and roots of *D.carota* on Vero cell line, all MIC concentrations of all extracts were individually incubated with HSV-1 in cell free culture media for one hour. Results in table (5) and figure (3) below show that considerable amount of the virus inhibition was due to direct virus inactivation by all concentrations of the examined extracts, showing maximum virucidal activity of 98.2% at the aqueous root extract with concentration of 230 µg/ml, and 91.2% at the aqueous leaves extract with concentration 86.37 µg/ml, followed by 87% at the acetone root extract with concentration 72.6 µg/ml, and finally 82% at the acetone leaves extract with concentration 48.4 µg/ml. These results indicate that all the extracts examined showed different degrees of viral inactivation due to the direct contact with the virus particles. The degree of viral inhibition is directly correlated with the

concentration of the polyphenol of the extract. Those results come in accordance with Schuhmacher et al., (2003), Kratz et al., (2008), Raenu et al., (2010), and Danaher et al., (2011), who stated that the exposure of the cell free HSV-1 to black berry for 15mins in room temperature had a virucidal activity.

Table 5: Virucidal activity of selected extracts' concentrations

Concentration (µg/ml)	Water Leaves	Acetone Leaves	Water Roots	Acetone Roots
	86.37	48.4	230	72.6
% Inhibition	91.2	82	98.2	87

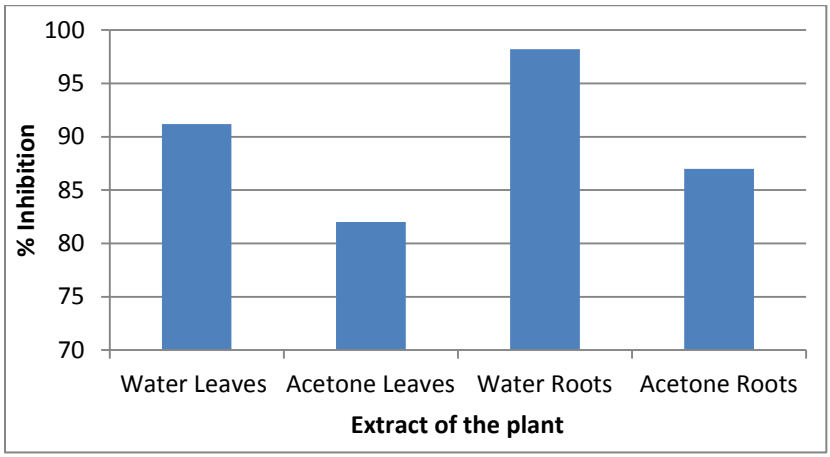

Figure 3: Virucidal activity of MIC concentrations

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on pretreated cells

In order to determine the mode of antiviral action, the examined *D.carota* extracts, were added to the Vero cells at different time intervals before, during, and after the virus adsorption.

All MIC concentrations of all extracts were individually added to the Vero cells for 1 hour and completely removed by washing the cells, before HSV-1 inoculation. Plaque reduction assay showed that all the extracts showed very low degree of virus inhibition as shown in table (6) and figure (4) below. It can be concluded from these results that pretreatment of cells of tissue culture with the examined *D.carota* extract before virus inoculation has no effect on virus infectivity. Comparable results were reported by Raenu et al.,(2011), who concluded their research stating that the extracts they used did not have any effect on the HSV-1 virus infectivity when used on the Vero cells before the virus inoculation.

Table 6: Plaque reduction assay before virus inoculation on selected extracts' concentrations

	D.carota Extract			
	Water leaves	Acetone leaves	Water roots	Acetone roots
%Inhibition	7.6	9.2	11.7	8.9

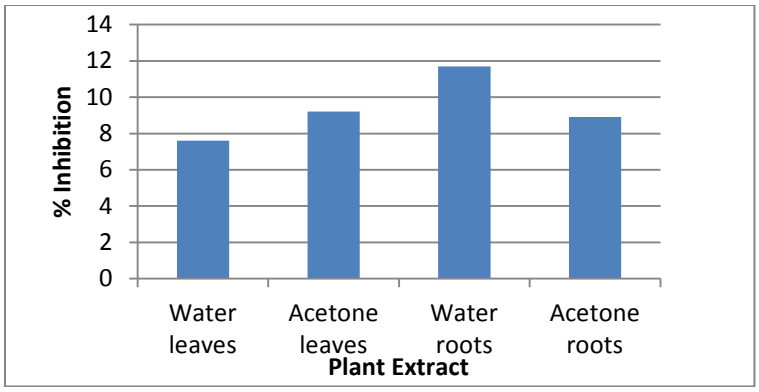

Figure 4: Plaque reduction assay before virus inoculation on selected extracts' concentrations

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on HSV-1 during viral adsorption (effect on attachment and penetration)

All MIC concentrations of all extracts were individually added at the same time of the HSV-1 inoculation. Results in table (7) and figure (5) below, show that the interference of the examined extracts on the viral adsorption was significantly observed at 230 µg/ml for aqueous roots extract, 86.37 µg/ml for aqueous leaves extract, 72.6 µg/ml for acetone roots extract, and 48.4 µg/ml for acetone leaves extract, giving 96.1%, 74.8%, 70.5%, and 64.3% respectively.

Those results indicate that all examined extracts inhibited the virus replication at different degrees according to the concentration via blockage of the adsorption of the virus either by the deactivation of binding of the surface glycoprotein of the viral envelope of virus particles to the cell receptor sites or inhibiting the viral cell fusion in the early replication stage and finally, preventing the initial stages of the viral reproduction. Same results were reported by Pujol et al., (2002), Kratz et al., (2008) and Xiang et al., (2011), who stated that the extracts they used directly inactivated HSV-1 particles, leading to the failure of early infection, including viral attachment and penetration (Arthanari et al., 2012).

Table 7: Antiviral activity of selected extracts' concentrations during viral adsorption

Concentration (µg/ml)	Water Leaves	Acetone Leaves	Water Roots	Acetone Roots
	86.37	48.4	230	72.6
% Inhibition	74.8	64.3	96.1	70.5

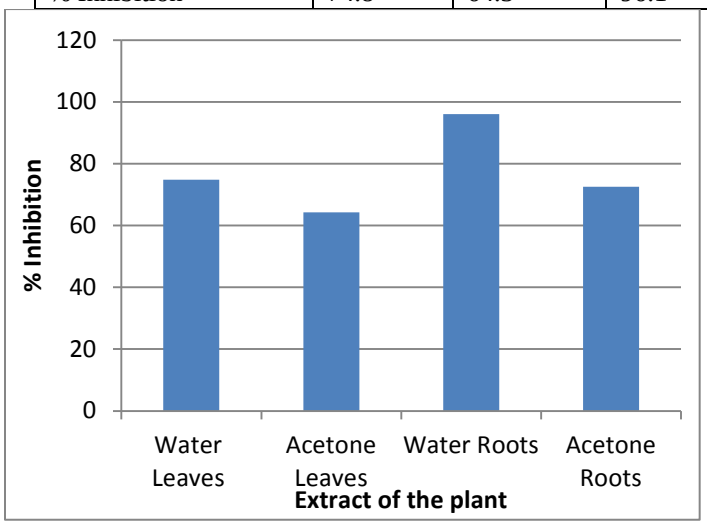

Figure 5: Antiviral activity of selected extracts' concentrations during viral adsorption

Effect of aqueous and acetone extracts from the leaves, and roots of *D.carota* on virus replication

The activity of all MIC concentrations of all examined extracts on viral replication was demonstrated at different time intervals (1, 4, 8, 24 and 48 hours) after virus inoculation. Results in table (8) and figure (6) below show that different extracts caused different degree of inhibition depending on the time of addition after virus inoculation. Also, treatment of Vero cells one to four hours after virus inoculation showed the highest virus inhibition of 95.5% for aqueous roots extract, 73% for aqueous leaves extract, 69% for acetone roots extract, and 61% for acetone leaves after the first hour and 61% for aqueous roots extract, 52% for aqueous leaves extract, 51% for acetone roots extract, and 49% for acetone leaves extract, after four hours of virus inoculation.

From the results, it can be concluded that, as the time interval increases, between the virus adsorption and addition of the extract, the inhibition of virus activity decreases significantly after four hours until it vanishes. These results indicate that the viral inhibition observed at the early hours just after the virus inoculation happens via the interference of the examined extract with the viral protein biosynthesis and transcription through the early phase of viral replication. Comparable results were reported by Sayed et al., (2012).

Table 8: Antiviral activity of selected extracts' concentrations at different time intervals after virus inoculation

Time after virus inoculation in hours	Water Leaves 86.37 µg/ml	Acetone Leaves 48.4 µg/ml	Water Roots 230 µg/ml	Acetone Roots 72.6 µg/ml
1	73	61	95.5	69
4	52	49	61	51
8	26	18	34	21
24	18	11	19	7
48	11	1.5	14	4

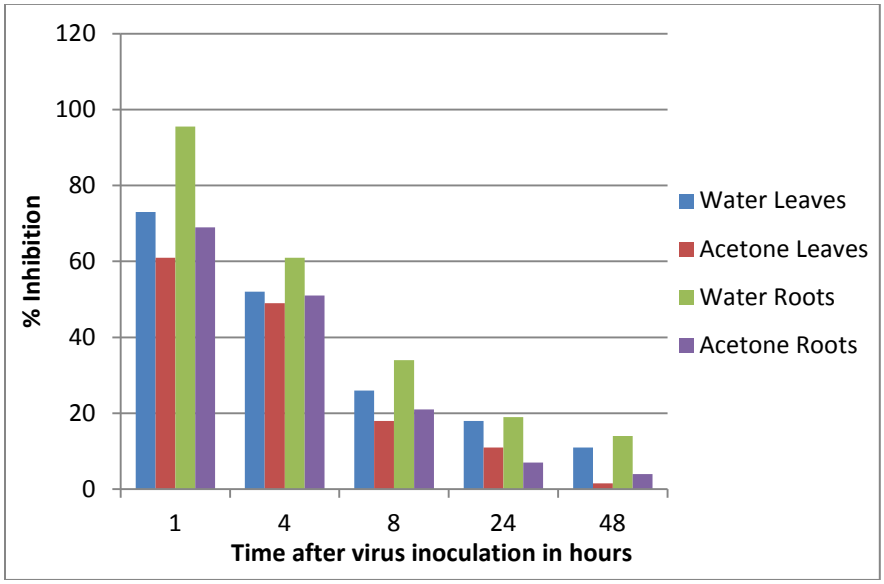

Figure 6: Antiviral activity of selected extracts' concentrations at different time intervals after virus inoculation

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