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Citrus sinensis Peel Extract Induced In vitro Effects on Herpes Simplex Virus

Hamed A. El-Serehy[1,2]*, Khaled A. Al-Rasheid[1], Fahad Al-Misned[1] and Golam Mortuza[1]

[1]Zoology Department, College of Science, King Saud University, Riyadh 1145, Saudi Arabia.
[2]Port Said University, Faculty of Science, Marine Science Department, Port Said, Egypt.

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Herpes simplex virus (HSV) primarily causes infections of the oral–facial, ocular or genital mucosa with high worldwide prevalence. The aim of this study is to evaluate antiviral properties of *Citrus sinensis* peel aqueous extract against herpes simplex virus type 1 (HSV-1) in vitro. HSV-infected Vero cells and cell-free virus suspensions were treated with orange peel extracts, and virus yield and infectivity were quantified by direct plaque assay. The results of the present study showed that C. *sinensis* peel aqueous extract, at 150 µg/ml provided 71.3% inhibition of plaque of HSV-1. The aquous extract showed 100% inhibition against HSV-1 at 200, 250 and 300 µg/ml. These properties suggest that this C. *sinensis* peel could provide advantage as a topical prophylactic/therapeutic agent for herpes infections.

Key word: Orange peel, Herpes simplex virus; Antiviral.

Citrus fruits contain various bioflavonoids. Naringin and hesperidin, glycosylated citrus flavonoids, are two major bioflavonoids identified in tangerine-peel extract (Bok *et al*., 1999). Citrus industry by-products, if utilized optimally could be major sources of phenolic compounds as the peels, in particular, have been found to contain higher amounts of total phenolics compared to the edible portions. Citrus peels are waste materials, obtained after extraction of juice from citrus fruit. Methanolic extract of citrus peel is known to have different antioxidative compounds (Abdel Moneim, 2013; Green *et al*., 2013; Xu *et al*., 2008; Ziaur, 2006).

Herpes simplex virus 1 (HSV-1) belong to diverse family of *Herpesviridae*, causing oral herpes lesions (HSV-1), genital lesions (HSV-2), meningitis, and encephalitis (Hong *et al*., 2014). Primary infection within the genital tract, followed by an established latency phase give rise to life-long infection in humans (Ge *et al*., 2013).

Treatment of herpes infection is thus cause of major concern owing to the difficulty in eliminating it from the ganglion, high cost of treatment, increasing drug resistance, and association with HIV-1.The standard therapy for management of HSV infections is based on nucleoside analogues that target the viral DNA polymerase. These include acyclovir, penciclovir and their derivates, valacyclovir, and famciclovir (Donalisio *et al*., 2013). Due to the drug resistance, there is a great demand for the development of new antiviral drugs with novel mode of action. In this context, natural products are very important source of anti-HSV agents and several extracts and pure compounds from natural products have been reported to exert an anti-HSV activity (Cecilio *et al*., 2013).

* To whom all correspondence should be addressed.
Tel.: 00966-14675753; Fax: 00966-14678514;
E-mail: hel_serehy@yahoo.com

Herein, the present work was performed to investigate the anti-herpes viruses 1 effects of C. *sinensis* peel aqueous extract.

METHODS

Viruses and cell lines

Vero cells were grown in Dulbecco-modified Eagle's Minimum Essential Medium (DMEM; Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco®, Brazil) and gentamicin (80 µg/ml). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The Herpes Simplex Virus type 1 and 2 were propagated in Vero cells, titrated on the basis of plaque forming units (PFU) count by plaque assay as previously described (Burleson *et al*., 1992) and stored at "80 °C until the experiments.

Plant Material

Citrus sinensis (*C. sinensis*) fruits were collected from market of East Cairo, Egypt in the months of February-March, 2013. The plant material was authenticated on the basis of taxonomic characters and by direct comparison with the herbarium specimens that available at the herbarium of the Botany Department.

Extraction

Fresh fruit peels of *C. sinensis* were taken and grounded, and about 500 g of the plant material was consecutively macerated for three days in water.

Extracts were filtered to remove insoluble particles. The extract was lyophilized with a freeze-dryer-cryodo and then stored at -20 °C until used.

Determination of total phenols

The total polyphenolic contents (TPC) were measured using Folin-Ciocalteau reagent based on the oxidation of polyphenols to a blue colored complex with an absorbance maximum of 750 nm. Calibration curve was prepared using gallic acid as standard for TPC which was measured as mg gallic acid equivalents (GAE) per milliliter of the sample (µg/ ml).

Determination of flavonoid content

For the assessment of flavonoids, a colorimetric method was used. Briefly, 1.50 ml of the deionized water was added to 0.25 ml of the sample and then 90 µl of 5% Sodium nitrite ($NaNO_2$).Six minutes later, after addition of 180 µl of 10% $AlCl_3$, mixture was allowed to stand for another 5 min before mixing 0.6 ml of 1M NaOH. By adding deionized water and mixing well, final volume was made up to 3 ml. The absorbance was measured at a fixed wavelength 510 nm. Calibration curve was prepared using quercetin as standard for total flavonoids which was measured as mg quercetin equivalents (QE) per milliliter of the sample (µg/ ml).

Cytotoxicity assay

Confluent Vero cells were exposed to different orange peel concentrations (1-5000 µg/ ml) for 72 h. After incubation, cell viability was assessed by a MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay (Mosmann, 1983). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced cell viability by 50% when compared to untreated controls.

Antiviral activity assay

The 96-well plates containing confluent cell monolayers were pre-incubated for 1 h with increasing non-cytotoxic concentration of orange peel. Six wells were used for each concentration. Afterwards, the cell were infected with HSV-1 (10 TCID50), incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and observed daily for cell cytopathic effect (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration, as described previously. Acyclovir at concentration of 0.05- 2 µg / ml served as the positive control.

Viral plaque number reduction assay

This assay followed the procedures previously described (Silva *et al*., 2010), with minor modifications. Approximately 100 PFU of HSV was adsorbed for 1 h at 37 °C on confluent cells and overlaid with MEM plus 1.5% carboxymethylcellulose (CMC, Sigma®, St. Louis, MO, USA) either in the presence or the absence of different concentrations of orange peel. After 72 h, the cells were fixed and stained with naphthol blue-black (Sigma) and plaques were 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. Results were expressed as CC_{50} and IC_{50} values in order to calculate the selectivity index ($SI = CC_{50}/IC_{50}$) of each sample (Cos *et al*., 2006).

Statistical analysis

Data were expressed as the mean ± standard deviation from at least three separate experiments.

RESULTS AND DISCUSSION

Table 1 shows the flavonoids and total polyphenolic contents of citrus peel. Flavonoids content in aqueous was 67.3 µg/mg quercetin equivalents of flavonoids/mg extract, respectively. The total polyphenolic content was 98.6 µg/ mg gallic acid equivalent of polyphenols/mg aqueous extract.

The type of solvent used for extraction is important for both quantification and classification of phenolic compounds occurring in plants and obtaining pure compounds for their analysis (Sultana *et al*., 2009). Various solvents generally used for extraction of different phenolic compounds include water, ethanol-water or acetone-water. For extraction of catechins, methanol-water or ethanol-water have been used and phenolic acids have been extracted with acetone-water, dimethylformamide-water. Meanwhile, methanol containing hydrochloric acid has been used for extraction of condensed tannins.

The differences in the extract yields from the tested plant materials in the present analysis might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants (Hsu *et al*., 2006). The amount of the antioxidant components that can be extracted from a plant material is mainly affected by the vigor of the extraction procedure, which may probably vary from sample to sample. Amongst other contributing factors, efficiency of the extracting solvent to dissolve endogenous compounds might also be very important (Sultana *et al*., 2007).

Examination of the cytotoxicity of C. *sinensis* peel extracts were performed in the range of concentrations up to 5000 µg/ml. The maximum non-cytotoxic concentrations were read individually from the obtained survival curves.

Cytotoxicity of orange peel aqueous extract was evaluated in cultured Vero cells by the MTT assay. According to the results of this experiment (Figure 1), the aqueous extract of orange peel has cytotoxicity at 586 µg/ml.

The present study was carried out to test the antiviral activity of C. *sinensis* peel extracts against herpes simplex virus types-1 using cytopathic inhibitory assay (Table 2). On the basis of cytopathic effect (CPE) of the virus-infected confluent monolayer of Vero cells, orange peel extracts showed strong antiviral activity against HSV-1 at high concentration, 200 µg/ml. In this manner, the cytopathic effect was showed at 100 µg/ml.

Plaque inhibition assay was carried out to determine the IC_{50}. As shown in Figure 2, C. *sinensis* peel aqueous extract at 150 µg/ml provided 71.3% inhibition of plaque of HSV-1 and orange peel methanolic extract at 100 µg/ml provided 68.5% inhibition against HSV-1. The extract showed 100% inhibition against HSV-1 at 200, 250 and 300 µg/ml.

Several herbal medicinal products are potential sources of functional foods and have various bioactivities like immunomodulatory and antitumor functions. Although the development of anti-herpetic agents from herbal source is less explored probably because there are a very few specific viral targets for small natural molecules to interact with herpes. However, several studies showed that anthraquinones (Sydiskis *et al*., 1991), polysaccharides (Marchetti *et al*., 1996), triterpenes and saponins (Simoes *et al*., 1999), and polyphenols (Chattopadhyay and Naik, 2007; Kuo

Table 1. Total phenolics and flavonoids contents of C. *sinensis* peel extract

Conditions	Total phenolics[a]	Total flavonoid[b]
Aqueous extract	98.6±2.7	67.3±3.2

(a) Flavonoids are expressed as µg/ mg quercetin equivalents of flavonoids/ml juice. (b) Total phenolics are expressed as µg/ mg gallic acid equivalent of polyphenols/ml juice. Data are represented as mean ± SEM of two independent experiments each performed in duplicate.

Table 2. The antiviral activity of C. *sinensis* peel extract

Peel extract	Cell Cytopathic Effect (CPE)
100 µg/ml	+
150 µg/ml	+
200 µg/ml	-
250 µg/ml	-
300 µg/ml	-

et al., 2002) isolated from several plants inhibit the replication of herpes viruses. A large number of plant-derived and synthetic anti-herpes virus agents have also been described (Ikeda *et al*., 2000; Jassim and Naji, 2003) and several works is in progress to identify plants and their active components having anti-herpes virus activity.

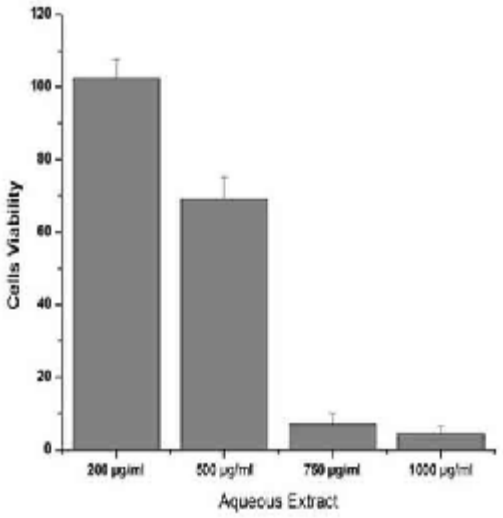

Fig. 1. The cytotoxic effect of C. *sinensis* peel extract
CC_{50} is the concentration of the 50% cytotoxic effect. Data are represented as Mean ± SEM of two independent experiments each performed in triplicate

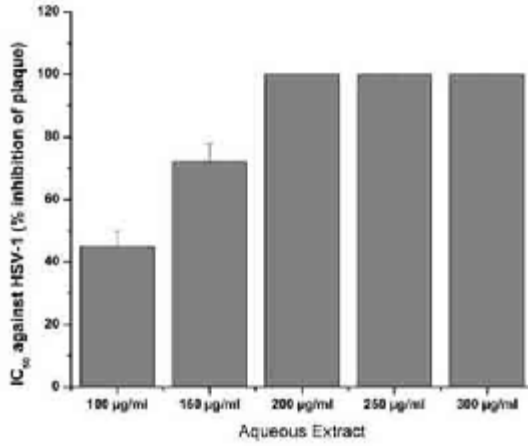

Fig. 2. The anti-herpes virus 1 activity of orange peel extract
IC_{50} is the concentration of the sample required to inhibit 50% virus-induced CPE. Data are represented as Mean ± SEM of two independent experiments each performed in triplicate

In the viral inhibition studies performed with C. *sinensis* peel extracts, we found that there was total inhibition of growth of HSV-1 *in vitro* at concentrations of 150 µg/ml. This indicates that the extract has strong antiviral effects that interfere with adsorption or entry into host cells and some intracellular activity. Although the minimum inhibitory concentrations were defined, it remains to be determined whether the main inhibitory effect is due to impairment of viral proteins involved in host cell receptor binding, adsorption, and/or penetration of virions.

Polyphenols derived from plants have been shown to have antiviral activity (Cushnie and Lamb, 2005; Khan *et al*., 2005). Specifically, the flavonoids galangin, quercetin, procyanidin, and pelargonidin, as well as procyanidin C-1, are found to be virucidal against HSV (Danaher *et al*., 2011; Shahat *et al*., 2002). The antiviral effect of these substances is greatest when used before virus adsorption (Schnitzler *et al*., 2010; Shahat *et al*., 2002), which is consistent with our findings. Orange peel is known to contain high amount of anthocyanins and ellagitannins; therefore, these polyphenols in orange peel extract are likely individual or synergist contributors to the antiviral effects observed.

In conclusion, C. *sinensis* peel extract emerges as a potential candidate in the development of effective antiviral drugs against HSV-1, although these findings set the stage for future studies that would isolate and identify the bioactive anti-HSV molecules in orange peel extract. In addition, further in-depth studies are needed to provide an insight into the mechanism involved and the identification of responsible target.

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