JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3891-3896

In vitro Anti-viral Activity of Orange Methanolic Peel Extract

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(Received: 02 August 2014; accepted: 12 September 2014)

The study aimed to investigate antiviral properties of orange peel methanolic extract against herpes simplex virus type 1 and 2 (HSV-1 and 2) 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 orange peel methanolic extract at 100μg/ml provided 68.5% inhibition against HSV-1. In addition, orange methanolic extract at 100 μg/ml provided 72.8% inhibition against HSV-2. The extract showed 100% inhibition against HSV-1 and 2 at 200, 250 and 300 μg/ml. These properties suggest that this orange peel could provide advantage as a topical prophylactic/therapeutic agent for herpes infections.

Keywords: Antiviral; Orange peel, Herpes simplex virus.

Citrus fruits, which belong to the family of rutaceae are one of the main fruit tree crops grown throughout the world. Orange (*Citrus sinensis*) is the major fruit in this group accounting for about 70% of citrus output (Okwi and Emenike 2006).Citrus fruits are well endowed with a variety of phytonutrients which are vital in both; health promotion and disease prevention (Okwu 2005).

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).

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Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), also known as human herpesvirus 1 and 2 (HHV-1 and HHV-2), are two members of the herpesvirus family, Herpesviridae, that infect humans (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). One valuable source for new treatments is the abundance of molecules from natural products that have been shown to possess antiviral properties 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).

Our study was performed to investigate the anti-herpes viruses 1 and 2 effects of orange peel methanolic 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 methanol. Extract was filtered using filter to remove insoluble particles. The extract was lyophilized with a freeze-dryer-cryodo. While, the methanol was removed under reduced pressure to obtain a semisolid mass of methanolic extract of citrus peel. Both extracts were then stored in -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 uL of 5% Sodium nitrite ($NaNO_2$).Six min later, after addition of 180uL 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 preincubated 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 or 2 (10 TCID50), incubated at 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 to 2 ug/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 types were 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 are expressed as the mean ± standard deviation from at least three separate experiments.

RESULTS AND DISCUSSION

In orange, the flavonoids have strong inherent ability to modify the body's reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory and anti-microbial activity (Okwu 2005). Also, flavonoids are plant water-soluble super antioxidants, which prevent oxidative cell damage and have strong anti-cancer activity and inhibit all stages carcinogensis (Okwu 2004). In vitro, flavonoids display anti-proliferative effect on various human neoplasic cell lines as observed in myeloid and lymphoid leukemia cells (Larcocca *et al*., 2008).

Table 1 shows the flavonoids and total polyphenolic contents of citrus peel. Flavonoids content in methanolic extract was 83.6 µg/mg quercetin equivalents of flavonoids/mg extract. The total polyphenolic content was 125.9 µg/ mg gallic acid equivalent of polyphenols/mg methanolic 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 orange 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 methanol extracts 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 815 µg/ml.

The present study was carried out to test the antiviral activity of orange peel extracts against

Table 1. Total phenolics and flavonoids contents of orange peel.

Conditions	Total phenolics[a]	Total flavonoid[b]
Orange peel methanolic extract	125.9±4.3	83.6±1.8

(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 orange peel

Orange peel	Cell Cytopathic Effect (CPE)	
	HSV-1	HSV-2
100 µg/ml	+	+
150 µg/ml	+	+
200 µg/ml	-	-
250 µg/ml	-	-
300 µg/ml	-	-

herpes simplex virus types-1 and 2 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 or anti-HSV-2 at high concentration, 200 µg/ml. In this manner, the cytopathic effect was showed at 150 µg/ml.

Plaque inhibition assay was carried out to determine the IC_{50}. As shown in Figure 2, orange peel extract at 100 µg/ml provided 68.5% inhibition against HSV-1. Both extracts showed 100% inhibition against HSV-1 at 200, 250 and 300 µg/ml. 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**.**

The *in vitro* anti-HSV-2 activity of the orange peel extract was investigated (Figure 3). The HSV-2 strain was more sensitive to the samples. According to the present results, orange peel extract at 100 µg/ml provided 72.8% inhibition against HSV-2. The extract showed 100% inhibition against HSV-2 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 *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;

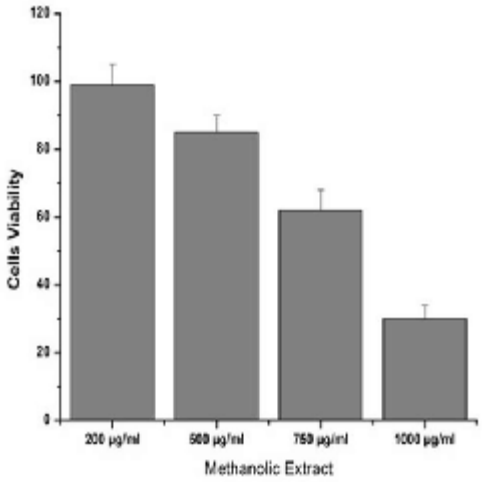

Fig. 1. The cytotoxic effect of orange peel extract.

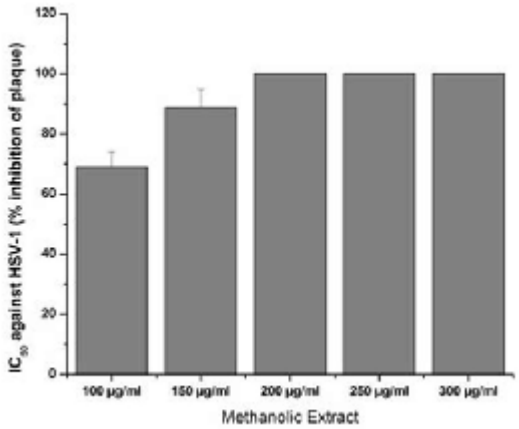

Fig. 2. The anti-herpes virus 1 activity of orange peel extract

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.

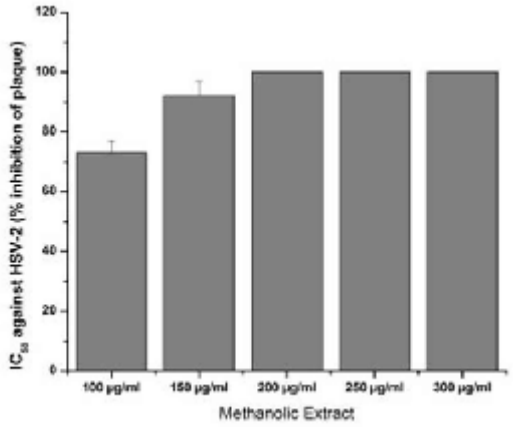

Fig. 3. The anti-herpes virus 2 activity of orange peel extract.

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.

Jassim and Naji, 2003) and several works is in progress to identify plants and their active components having anti-herpes virus activity.

In the viral inhibition studies performed with orange peel extracts, we found that there was total inhibition of growth of HSV-1 and 2 *in vitro* at concentrations of 150 ìg/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, orange peel extracts emerges as a potential candidate in the development of effective antiviral drugs against HSV-1 and HSV-2, 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.

ACKNOWLEDGMENTS

The authors would like to extend their sincere appreciations to the Deanship of Scientific Research at king Saud University for its funding this Research group NO. (RG -1435-198).

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