Antiviral effects of blackberry extract against herpes simplex virus type 1

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Objective. The aim of this study was to evaluate antiviral properties of blackberry extract against herpes simplex virus type 1 (HSV-1) in vitro.

Study design. HSV-infected oral epithelial (OKF6) cells and cell-free virus suspensions were treated with blackberry extract (2.24-1,400 μg/mL), and virus yield and infectivity were quantified by direct plaque assay.

Results. Blackberry extract ≥56 μg/mL inhibited HSV-1 replication in oral epithelial cells by ≥99% (P < .005). Concentrations ≥280 μg/mL were antiviral when the extract was added after virus adsorption and entry. Exposure of cell-free virus to ≥280 μg/mL blackberry extract for 15 minutes at room temperature was virucidal (P < .0002). The virucidal effects were not due to pH changes at concentrations up to 1,500 μg/mL.

Conclusions. Blackberry extract inhibited the early stages of HSV-1 replication and had potent virucidal activity. These properties suggest that this natural fruit extract could provide advantage as a topical prophylactic/therapeutic agent for HSV infections. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:e31-e35)

Herpes simplex virus (HSV) is an epitheliotropic virus that infects children and the majority of adults.1,2 Infection of epithelial cells (i.e., mucosa and skin) results in deenvelopment, rapid replication, and spread to adjacent cells and nerve cell endings. Uptake of virions by nerve endings permits the virus to travel retroaxonally to the nerve cell bodies within the trigeminal ganglion, whereupon a quiescent state is established.3,4 HSV DNA resides long-term within the neuronal nuclei in a state that permits periodic reactivation and recrudescence of progeny virus.5,6 The ability of the virus to hide within relatively immune-privileged sites makes elimination of this virus difficult.

Therapeutic options for HSV infections include the use of topical or systemic antiviral agents.7-11 Topical agents have the potential advantages of direct contact and disruption of various aspects of the virus life cycle, however most commercially available topical agents do not penetrate effectively and therefore have been shown to only have minor benefit in limiting lesion eruption and duration.11-14 Also, topical antivirals can be expensive to use, especially as they often require frequent and recurrent applications. As an alternative, naturally occurring antiviral biochemicals found in plants and fruits could be less costly and more efficacious to use. To date, extracts from seeds, plants, roots, and fruits have been shown to have antiviral activities.15-21 Of note, a large number of small molecules, such as phenolics, polyphenols, and flavonoids, found in fruits have been reported to be active antitherpetic agents.16

Previously, our laboratory has shown that blackberries, which are rich in phenolics, polyphenols, and flavonoids have antioxidant, antiinflammatory, and antiproliferative properties.22,23 However, to our knowledge, scientific studies on the antiviral properties of blackberry extract have not been reported. Because HSV infections remain a global health care issue, we sought to investigate the antiviral effects of the extract against this well recognized viral pathogen.

MATERIALS AND METHODS

Cell culture and virus infectivity assays

The immortalized keratinocyte cell line OKF6/hTERT-2 (OKF6), established by ectopic expression of the telomerase catalytic subunit (hTERT) in cells from normal oral mucosal epithelium, was obtained from Dr. James Rheinwald, Harvard Medical School.24 Vero cells were obtained from American Type Culture Collection (Rockville, MD). Cells were plated in 6-well tissue culture dishes at 3.75 and 6.25 × 10^5 cells/well,
respectively, in Ker-SFM media and incubated at 37°C in a humidified incubator with 5% CO₂. The following day, cells were inoculated with HSV-1 strain 17, obtained from N. Fraser of the Wistar Institute, Philadelphia, at a multiplicity of infection (MOI) of ≤0.05 and incubated at 37°C for 1 hour. After 2 rinses with phosphate-buffered saline solution (PBS), fresh medium was added and incubation at 37°C was continued overnight. Twenty-four hours after inoculation, virus was released by 3 freeze-thaw cycles, and virus yield was determined in duplicate by direct plaque assay on Vero cell monolayers as we have previously described.25 In the viral replication inhibitory assays, triplicate cultures were supplemented with blackberry extract at the times (during adsorption, after removal of virus inoculi, or throughout) and concentrations indicated. In the virucidal assays, cell-free virus suspensions (25,000-50,000 plaque-forming units [PFU]/mL) were incubated with blackberry extract at 37°C for 1 hour or room temperature for 15 minutes. Virucidal assays were terminated at the indicated times by dilution of reaction mixture to nonantiviral levels of blackberry extract (1:100 culture media). Virus infectivity was determined by the direct plaque assay and is presented as the number of remaining PFUs relative to control-treated virus suspensions.

Blackberry extract preparation

Hull blackberries (Rubus eubatus cv. “Hull”) were grown at Windstone Farms (Paris, Kentucky). Seeds and skin were removed with the use of a Langsenkamp type 161 Colossal Pulper, and the resultant purée was stored at −20°C. Ethanol extracts were obtained from the puree.22 Briefly, blackberry purée (10 g) was treated under sonication for 30 minutes with 25 mL extraction solvent of ethanol containing 0.01% HCl (v/v). The supernatants were collected after filtration and dried by rotary evaporation at 40°C. The dried extract was re-dissolved in deionized water and filtered through 20-25 µm filter paper and lyophilized to obtain dried ethanol extracts. Dried extracts was then redissolved in deionized water as a stock solution (140 mg/mL, pH 1.9-2.0) and stored at −80°C until use.

RESULTS

Blackberry extract has anti–HSV-1 properties

To determine if blackberry extract has antiviral properties, its ability to inhibit the HSV-1 replication cycle in oral epithelial cells was evaluated. To broadly evaluate possible antiviral activities, cultures were maintained in the presence of increasing concentrations of blackberry extract (0, 2.24, 11.2, 56, 280, and 1,400 µg/mL) throughout the entire replication cycle (i.e., adsorption, entry, and production of progeny). As shown in Fig. 1, blackberry extract at 56 µg/mL significantly reduced HSV-1 yield by >99% (P = .004), and progeny virus was not detectable in cultures treated with extract at concentrations ≥280 µg/mL. Notably, in all experiments, even at the highest concentrations, the extract was not toxic to the epithelial cells (data not shown).

Higher concentrations are required for antiviral effects when blackberry extract is added after HSV-1 entry

OKF6 cells were inoculated with virus (MOI 0.05), incubated at 37°C for 1 hour, and rinsed 2× with PBS before the addition of media supplemented with the indicated concentration of blackberry extract. Virus yield was assessed 24 hours after inoculation by titration on Vero cells after 3 freeze-thaw cycles. Although 56 µg/mL blackberry extract dramatically reduced virus yield when present throughout the entire replication cycle (Fig. 1), significant reductions in virus yield when provided after the 1 hour adsorption and entry stage required concentrations >280 µg/mL (Fig. 2). Similar results were obtained in experiments where Vero cells were infected with HSV-1 and then exposed to blackberry extract (data not shown).
Incubation with blackberry extract for 1 hour at 37°C inactivates HSV-1

The fact that blackberry extract at concentrations ≥56 μg/mL greatly (>99%) reduced virus yield when present throughout the inoculation and replication period but had no effect on yield at concentrations ≤280 ng/mL when added after virus entry (Fig. 2) indicated that blackberry extract may either inactivate the virus directly or block virus entry. To determine if blackberry extract inactivates HSV-1, the extract was added to cell-free virus suspensions at the indicated concentrations and incubated at 37°C for 1 hour to mimic the adsorption and entry phase. Blackberry extract–treated virus suspensions were diluted beyond the antiviral concentration (1:100) with culture medium, and then virus infectivity was quantified by direct plaque assay. In these experiments, even at the highest concentrations used the extract did not affect the number of viable epithelial cells (data not shown). As indicated in Fig. 3, the amount of infectious HSV-1 virus remaining after 1 hour of incubation at 37°C with 280 μg/mL blackberry extract was significantly reduced compared with control cultures. Taken together, these findings indicate a dose-dependent antiviral effect of blackberry extract and that this effect was most pronounced at the level of inactivation of cell-free virus.

Short-term incubation with blackberry extract at room temperature inactivates HSV-1

To further evaluate the effectiveness of blackberry extract on virus infectivity, HSV-1 suspensions were incubated with the indicated concentrations of extract at room temperature for 15 minutes to simulate intraoral use. HSV-1 infectivity was determined by direct plaque assay on Vero cell monolayers. Similarly to the preceding experiments, exposure of cell-free virus to ≥280 μg/mL blackberry extract for as little as 15 minutes significantly reduced HSV-1 infectivity (Fig. 4). Higher concentrations of blackberry extract were required, with ≥560 μg/mL blackberry extract eliminating ≥50% of infectious virus and concentrations ≥1,500 μg/mL blackberry extract reducing >90% of infectious virus. Because acidic pH is known to alter the infectivity of HSV-1, we quantified the pH of all of the concentrations tested. The diluted extract remained within neutral pH (i.e., >6.8), suggesting that the antiviral effect was more likely associated with the natural constituents within the blackberry extract than with pH.

DISCUSSION

Blackberries are a natural and abundant fruit that grow in the wild and as cultivars. As early as the 16th century, blackberry juice was used to treat infections of the mouth and eyes. The extract, juice, and leaves of blackberries are rich in polyphenols, which have anti-inflammatory and antibacterial properties.23,26 Here we further extended studies of blackberry extract by investigating its antiviral properties and mechanism of ac-
tation. These studies add to the growing body of evidence that blackberry extract may provide benefit to a large percentage of the world’s population by targeting a well recognized viral pathogen.

In the viral inhibition studies performed with blackberry extract, we found that there was near total inhibition of growth of HSV-1 in vitro at concentrations of >56 μg/mL. The antiviral effects were most noticeable when free virus was directly exposed to the blackberry extract (i.e., before the infection), and less effective once HSV-1 had adsorbed and entered epithelial cells (i.e., either OKF6 or Vero cells). 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. Specifically, the flavonoids galangin, quercetin, procyanidin, and pelargonidin, as well as procyanidin C-1, are found to be virucidal against HSV. The antiviral effect of these substances is greatest when used before virus adsorption, which is consistent with our findings. Blackberries are known to contain high amount of anthocyanins and ellagitannins; therefore, these polyphenols in blackberry extract are likely individual or synergist contributors to the antiviral effects observed.

In summary, our findings suggest that blackberry extract with its viral replication inhibitory and rapid virucidal activities could be an effective topical treatment and/or prophylactic agent for oral herpetic infections. These findings set the stage for future studies that would isolate and identify the bioactive anti-HSV molecules in blackberry extract, and determine whether the bioactive principle(s) in blackberry extract exhibit antiviral effects against additional HSV strains and serotypes (i.e., HSV-2) in preclinical and toxicity evaluations.

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