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Research article

The phytomedicine *Echinacea Purpurea* contains light dependent and light-independent antiviral activities

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Abstract

Antiviral activities have been demonstrated in many medicinal plant extracts, and in some cases these activities were light dependent, due to the presence of photosensitizers. Certain preparations of *Echinacea purpurea* were shown recently to contain virucidal activities against membrane containing viruses, such as influenza viruses, but significantly less activity against rhinoviruses, the common cold viruses, in conventional assay conditions. We therefore investigated the possibility that optimum antiviral activity might require light, and that furthermore, under the conditions resembling those of normal oral consumption, ie. brief exposure to relatively high concentrations of *E. purpurea* extracts, additional antiviral activities could be manifest. Light dependent activity was revealed by exposing viruses to dilute extracts of *E.purpurea* in the presence of visible light in the 400 – 550 nm range (blue part of the spectrum), but in addition, at high concentrations resembling normal consumption of the extract, a light-independent activity was observed against rhinovirus as well as influenza virus and herpes simplex virus. At the lower *E. purpurea* concentrations optimal activity required continuous exposure to light in the presence of the virus, suggesting the involvement of short-lived radicals such as singlet oxygen. These results provide further evidence to support the use of standardized *E. purpurea* extracts, at recommended doses, as potent virucidal agents in the treatment of colds and other respiratory virus infections.

Introduction

Potent antiviral activities have been demonstrated in various extracts derived from medicinal plants, or phytomedicines [1-4]. Some of these activities are due to photosensitizers, which are compounds whose bioactivities are dependent upon light, or are enhanced by light, of specific wavelengths. The absorption of photons by such compounds often results in production of singlet oxygen or other radicals, which are responsible for their antiviral actions, although the basic mechanisms have seldom been elucidated [1].

There are several reasons why the influence of light is important. Many medicinal plant extracts, especially in tropical regions, require sunlight, which is rich in long wave ultraviolet (UVA), as an adjunct to traditional therapy [5]. Furthermore, the utilization of this property has been applied recently in the form of photodynamic therapy for virus infections [6-9].

However, when plant extracts are analyzed for antiviral activities by conventional laboratory assays, the presence of photosensitizers could confuse the interpretation of the results, and could lead to false or inconsistent conclusions, depending on whether the assays were carried out in a dark incubator or in the presence of variable ambient light. Consequently some potentially valuable antiviral extracts or compounds could be missed entirely if they were not significantly active in the absence of ambient light [1]. In addition some extracts may contain photocytotoxic compounds, which are only observed under conditions of light exposure [10].

Certain standardized extracts of *Echinacea purpurea*, which are recommended for the treatment of colds, 'flu, and other respiratory infections, have been shown, in ambient light, to contain components active against membrane containing viruses [11, 12]. Traditionally, such extracts have been used for additional applications in various diseases [13], although the accessory use of light was not documented. However the normal oral consumption of Echinacea extracts does not necessarily involve deliberate exposure of the extract, or of the oral and nasal mucosa, to light. Therefore we decided to re-evaluate the antiviral activities of standardized *Echinacea purpurea* extract against several viruses, under conditions resembling normal consumption, and in the presence of measured doses of ambient, visible, or UVA light.

Experimental

Materials and Methods

***Echinacea* sources:** The test materials included standardized commercial products of *Echinacea purpurea*

(L.) Moench, consisting of ethanol extracts of aerial parts and roots. Authentication and chemical characterization of these extracts has been described in our comprehensive report [14].Samples of these products were stored by Dr. JT Arnason in the University of Ottawa Biology department. Chemical analysis by standard HPLC techniques revealed the following composition of marker compounds, in µg/mL [14]: caffeic acid, 0; caftaric acid, 264; chlorogenic acid, 40; cichoric acid, 314; cynarin, 0; echinacoside, 7; alkylamide PID 8/9, 36; polysaccharides, not detected.
No endotoxin was detected, as determined by means of a commercial assay kit from Lonza Walkersville Inc., MD(lower limit of detection 0.1 unit/mL). In this text, *Echinacea purpurea* is abbereviated to EP.

Cell lines & Viruses

All cell lines (Vero cells; MDCK canine kidney cells; H-1 sub clone of HeLa cells; A549 human lung epithelial cells; all acquired originally from American Type Culture Collection, Rockville, MD) were passaged regularly in Dulbecco MEM (DMEM), in cell culture flasks, supplemented with 5-10% fetal bovine serum (Invitrogen, Ontario), at 36° C in a 5% CO_2 atmosphere. No antibiotics or antimycotic agents were used. The following viruses were used: human influenza strains H1N1 (A1/Denver/1/57), H3N2 (A/Victoria/3/75), and B (B/Hong Kong/5/72) (all from BC Centre for Disease Control, Vancouver), were propagated in MDCK cells; HSV (herpes simplex virus type 1, BC-CDC), was propagated in Vero cells; rhinovirus types 1A and 14 (RV 1A and RV 14, from ATCC), were propagated in H-1 cells; Adenovirus types 3 and 11 (ATCC), in A549 cells. All the stock viruses were prepared as clarified cell-free supernatants, with titers ranging from $10^6$to 2 x10^8 pfu (plaque-forming units) per mL.

Antiviral Activity

Cytopathic (CPE) endpoint assays, from which MIC_{100} values were calculated, were carried out as described before [14], with modifications to allow variable light exposures, as follows:The extracts, in quadruplicate 200 µL aliquots in DMEM, were serially diluted two-fold across the rows of an empty 96-well tray, in medium. A known amount of virus was added to each well and allowed to interact with the extract for the appropriate time, at a temperature of 22^0C, either in the dark (trays covered in aluminum foil), or exposed to measured doses of light from the sources indicated in the individual experiments. These incubations were carried out either in an environmental chamber fitted with lamps, or within the biosafety cabinet with calibrated fluorescent lamps on. Following the incubation period, the mixtures were transferred to another tray of cells from which the medium had been aspirated. These trays were then incubated in a normal CO_2 incubator (without light) until viral CPE were complete in control wells containing untreated virus. Some wells contained cells not exposed to virus. The MIC_{100} was the maximum dilution at which CPE was completely inhibited by the extract. In most assays the replicate rows gave identical end-points; when two-fold differences (or occasional four-fold differences) were encountered, arithmetic means and standard deviations were calculated by means of the Prism version 7 program.Plaque assays with replicate agarose overlays in MEM were conducted for serially diluted virus-EP reactions (in light or dark), with the appropriate virus-cell line combination.
The recommended dose of *Echinaceapurpurea* (EP) for oral consumption is equivalent to approximately 1:10 dilution of the extract in water, followed by "mouthwash" and swallowing. To mimic this dose, EP was diluted to a final concentration of 1: 10 in DMEM, together with the appropriate amount of virus, and incubated at ambient temperature (22°C), with or without light exposure, for various times. The mixtures were then immediately diluted in excess DMEM and serially diluted, or frozen at -70°C followed by assay for viral TCD or pfu.

Light measurements

The following light sources were used: domestic cool white fluorescent GE or Sylvania lamps, of the kind used in most bio-safety cabinets, provided a source of visible light, which was predominantly in the range 470-590 nm, with peak emission around 580 nm (according to commercial specifications). GE BLB lamps (black-light-blue, emission range 315-400 nm, peak 360 nm) provided the long-wave UV light, UVA. In some experiments ambient light in various parts of the laboratory was measured and used as a visible light source.
Incident radiation (irradiance, in watts/m^2) was measured by a model IL-1350 radiometer (International Light Technologies, Peabody Mass.). In some cases a VWR International dual range light meter (cat #62334-944) was used to measure illuminance in lumens/m^2. These values were converted to watts/m^2 [14, 15]. Incident UVA radiation was measured by means of a UVX digital radiometer and a UVX36 sensor (UVP, San Gabriel, CA). The plastic material of the assay plates was completely permeable to UVA, but not to lower wavelengths. Standard photometric nomenclature and measurements are used in this report [15].

Results and Discussion

Results

Light requirement

We reported previously that EP (*Echinacea purpurea*) extracts possessed potent antiviral activity against several membrane containing viruses including HSV-1, influenza viruses, and respiratory syncytial virus [16]. However, we observed that whenever EP, at concentrations of 160 µg/mL or less (1:100 or higher dilutions of extract) was incubated with any of these viruses in the dark, as for example within a standard cell culture CO_2 incubator, there was substantially less antiviral activity.

We therefore compared the relative antiviral activity at several different EP concentrations in the presence and absence of light. Figure 1 shows the result for HSV-1. At the lowest concentration of EP tested, 0.8 µg/mL, there was substantial inactivation of the virus in light (fluorescent light), but very little inactivation in the dark. However with increasing EP concentrations antiviral activity increased, such that the gap between light and dark activities decreased.

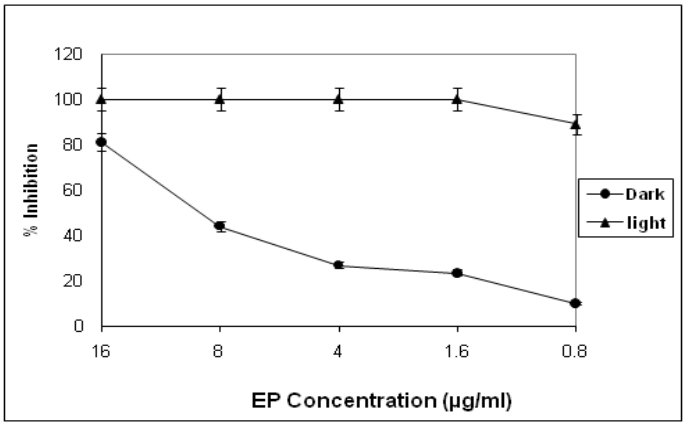

Figure 1. Anti-HSV activity in light and dark.
Aliquots of HSV-1 were incubated with various concentrations of EP for 30 min in the presence or absence of visible light (fluorescent lamps, incident radiation = 2 watts/m^2), and assayed for pfu, in comparison to untreated virus. Triangles, plus light; circles, minus light (reaction trays covered in aluminum foil).

Effect of type and dosage of light

To determine the type of light required for optimal activation of this antiviral activity, we compared the anti-HSV MIC's in the dark, or in the presence of measured doses of UVA, fluorescent light, and ambient light. Table 1 shows the result for EP, and for comparison the known UVA-activated phytochemical α terthienyl (αT). Since the peak absorption of αT is 360 nm [17], then UVA lamps provide maximal activation, although the fluorescent lamps, which emit mostly in the range of 450 – 580 nm, also include some overlap in absorption at 360 nm. In contrast EP is activated the most by the visible range of light, although the presence of significant activity in UVA light indicates that the active wavelengths are in the blue range, between 360 and 450 nm. Thus the active compound/s in EP are chemically different from trithiophenes such as alpha-tertthienyl, even though *Echinacea* is in the same plant family.

The effect of time of light exposure was analyzed by means of reactions between EP (100 µg/mL) and HSV-1 in visible light. Table 2 shows the result. The MIC$_{100}$ was relatively high in the dark, as expected, but with increasing light exposure time, it was reduced. A similar MIC value was obtained when the reaction was carried out in PBS (phosphate buffered saline), in place of medium, indicating that components of medium did not play a role in the photo-reactions.

Table 1. Light - Activated Antiviral Activity (HSV, MIC$_{100}$, µg/mL)

Treatment	Fluorescent light[1]	Ambient light	UVA[2]	Dark[3]
Echinacea purpurea (EP)	0.55 ±0.36	0.203 ±0.14	8.78 ±0.63	200 ±0
α terthienyl	1.42 ±0.55	1.42 ±0.55	0.38 ±0.26	> 167

[1]fluorescent lamps, peak emission at 580nm.
[2]long wave ultraviolet, 315-400 nm peak emission at 360 nm
[3]incubation trays enclosed in aluminum foil
Light doses measured by photometers (as described in Materials and Methods). Exposure times were adjusted to give equivalent fluences of 2 Kjoules.

Table 2. Time of light exposure[1]

Treatment	MIC100 (in µg/mL HSV-1)
Dark, 60 min	140.0 ±40.0
+ light 10 min	22.5 ±12.6
+ light 30 min	1.73 ±0.93
+ light 60 min	0.24 ±0.09
+ light 60 min (PBS)[2]	0.25 ±0.12

[1]Fluorescent lamp in biosafety cabinet, incident dosage = 775 mwatts/m^2
[2] PBS in place of standard medium (DMEM)

The effect of light dosage was measured by incubating HSV-EP mixtures for 60 min at different locations within the laboratory, accompanied by photometer measurements. These results are shown, as reciprocals of MIC$_{100}$ values, in Figure 2. The photo-activation effect of increasing doses of light are clearly evident, although a dose of 1336 mwatts/m^2 was evidently optimal.

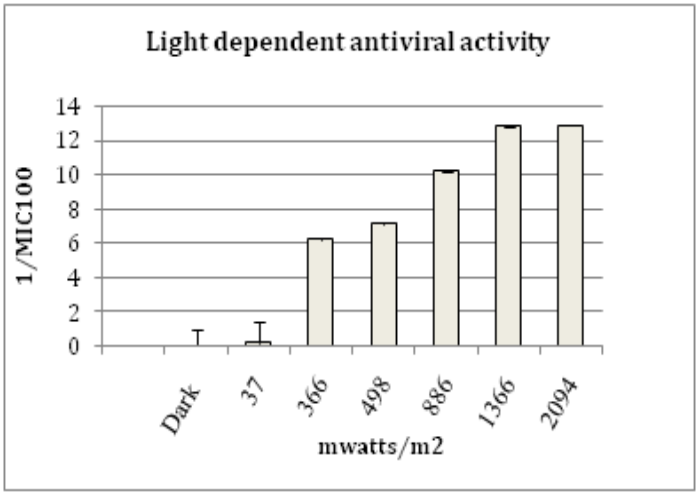

Figure 2. Effect of light dose.
Aliquots of HSV-1, in 6-well trays, were incubated with EP (final concentration 160 µg/mL) in the presence of ambient light in different parts of the laboratory. The amount of incident light at each location was measured by means of a photometer, as explained in the Materials and Methods. Residual virus was measured by cpe-endpoint dilution (to give MIC$_{100}$ values).

High concentration (real life consumption) of EP in light and dark

The dependence of antiviral activity on visible light at relatively low concentrations of EP was clear. However the data in Figure 1 suggested that at high concentrations of EP (similar to recommended doses for consumption), antiviral activity might be equally good in light and dark, and could conceivably be significant for viruses without membranes, such as rhinovirus.

Accordingly we evaluated anti-rhinovirus activity in high EP concentration (1.6 mg/mL; 1:10 dilution of extract) for short exposure times to mimic real life oral consumption. Under these conditions (Table 3) rhinovirus, the common-cold virus, was readily inactivated by more than 5 $\log_{10}$ TCD in light and dark for as little as 5 min exposure time.

Table 3. "High dose" EP (recommended oral consumption)

Treatment	Rhinovirus titer[1], Dark[2]	Rhinovirus titer[1], Light[3]
Ethanol control 30 min	4.8×10^7	4.8×10^7
EP 5 min	12.5×10^1	5×10^1
EP 30 min	12.5×10^1	12.5×10^1

[1]TCD/mL

[2] trays covered in aluminum foil

[3] Light exposure to fluorescent lamp was 775 mwatts/m^2

Table 4 shows the results of a comparison between the four viruses, HSV-1, influenza virus, RV-1A, and adenovirus-3, for 5 min exposures to high concentration EP (1.6 mg/mL) in ambient light. Approximately 5 $\log_{10}$ inactivation was attained for HSV-1, influenza, and RV-1A, as well as influenza H3N2 and influenza B (data not shown), but in contrast there was no effect on Ad-3 (or Ad-11, not shown).

When the different uninfected cell lines were exposed for 5-30 min to 1: 10 EP they showed no signs of cytotoxicity, according to microscopic examination and crystal violet staining, in agreement with our previous observations [16].

Table 4. "High dose" EP-different viruses

Virus	Virus + ethanol (6.5%)	Virus +EP	Log decrease
HSV-1	3.12×10^5	<5	> 4.7
influenza	2.36 x 105	0	> 5.4
RV-1A	7.0×10^5	10	4.84
Ad-3	2.4×10^6	2.4×10^6	0

Exposure times, 5 min at 775 mwatts/m^2

Titers in TCD/mL except influenza, pfu/mL

Pre-irradiation of EP with light

Since EP contained some photoactive antiviral components, we investigated the effect of pre-irradiation, i.e. pre-exposure, of different concentrations of EP for various times to light or dark, followed by normal infection of cells by the virus in light or dark. The results are shown in Figure 3. The black circles indicate the percent of inhibition of influenza pfu under optimal conditions, i.e. 30 min pre-exposure to light followed by 30 min incubation with virus in light. Complete inhibition was observed at all EP concentrations down to 10 μg/mL, below which inhibition was partial. At the other extreme, pre-exposure and virus incubation steps completely in the dark (black squares), 100% inhibition was achieved only at the highest concentration tested, 160 μg/mL (equivalent to the recommended oral dose; in agreement with the results shown in Table 4). At lower concentrations successively less inhibition was observed, down to zero inhibition at 5 μg/mL. The other lines in Figure 3 show the effects of shorter pre-exposures to light, and also illustrate the positive effects of short pre-exposures (1 – 30 min) to light followed by incubation in the dark. In other words a short pre-exposure to light followed by incubation in the dark gave rise to substantial antiviral activity, but not as effectively as incubation with virus in light.

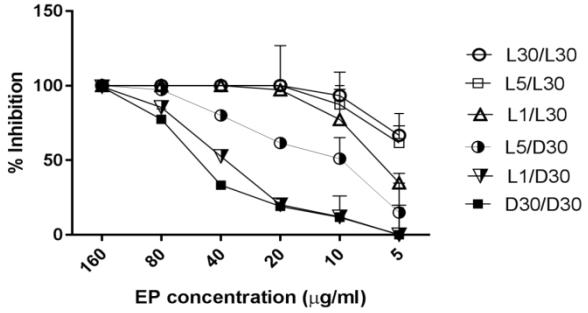

Figure 3. Effect of pre-exposure of Influenza H1N1 to EP in light or dark.

Serial dilutions of EP were made in multiple 96-well trays. Aliquots of influenza virus H1N1 were added at various time points in the presence or absence of light (775 mwatts/m2), using various combinations of light and dark exposure, as indicated in the side bar, and the reaction mixtures inoculated into aspirated monolayers of MDCK cells, followed by assaying for viral pfu.

Discussion

Echinacea purpurea (EP) is recommended for the treatment of cold and 'flu symptoms, and our previous studies on this herbal medicine revealed multiple bioactivities that could help to explain its efficacy. Thus in addition to its potent antiviral activity against HSV-1, influenza viruses, and respiratory syncytial virus (RSV), [11, 16], EP also showed bactericidal activities against certain respiratory bacteria and anti-inflammatory activities, as manifest by its ability to reduce substantially the cytokine stimulating properties of several viruses and bacteria [18]. However the mechanisms of action and the nature of the active ingredients have not been reported. Furthermore EP was shown to inhibit the hemagglutinating activity of influenza viruses, which suggests an interference with the penetration of these viruses into cells [12]. Such an event would explain why the antiviral effect of EP is only observed as a direct effect on the viruses, rather than an indirect intracellular effect.

The studies reported here confirmed that EP, in common with many other phytochemical compounds and extracts [1, 17], requires visible light for maximum antiviral activity at lower EP concentrations. Nevertheless, at EP concentrations recommended for normal consumption, i. e. 1: 10 diluted extract in water together with brief exposure to the oral mucosa (mouth rinse), followed by swallowing, rhinoviru was also effectively inactivated by more than 5 $\log_{10}$ infectious viruses, as were HSV and influenza, regardless of the presence of light. This indicates the presence of two or more distinct antiviral activities, light-dependent and light-independent activities.

The experiments reported in Figure 3 substantiated the efficacy of high concentrations of EP against influenza virus in the absence of light, although at lower concentrations antiviral activity could be enhanced considerably by light, and also to some extent by pre-exposure of the EP to light before incubating the EP with virus. However this activity might not be sufficiently stable to permit optimal antiviral activity during subsequent incubation in the dark. Such instability could reflect the involvement of singlet oxygen or other short-lived radicals, formed as a result of the active compound/s reacting with light of the appropriate wavelength [1, 9].

No cytotoxicity was observed in the earlier and current studies of EP at high concentrations. Thus a short exposure of the oral mucosa or throat to the recommended dose of EP should not be a safety issue [19].

Nevertheless, even at high concentrations, EP displayed no activity against adenovirus type 3 or type 11, in light or dark. This supports the concept of specificity of the reactions between EP constituents and certain susceptible viruses. We have shown previously that the different bioactivities attributed to *Echinacea* extracts are not restricted to a single constituent, and furthermore the anti-bacterial activities did not correlate with anti-cytokine or antiviral activities [20, 21].

Conclusions

In conclusion, standardized ethanol extracts of *E. purpurea* aerial parts and roots, contain a variety of different bioactivities that are beneficial for the control of cold and 'flu symptoms, including direct antiviral effects against several viruses incriminated in the genesis of these symptoms, and more importantly these activities are manifest under the conditions of normal consumption of the herbal extract, regardless of the ambient light exposure. Nevertheless, the presence of photo-sensitizers in standardized extracts of EP could also make them useful in photodynamic therapy [9].

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