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Photochemistry and Photocytotoxicity of Alkaloids from *Goldenseal* (*Hydrastis canadensis L.)* 3. Effect on Human Lens and Retinal Pigment Epithelial Cells

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Abstract

The dried root or rhizome of *Goldenseal* (*Hydrastis canadensis* L.) contains several alkaloids including berberine, hydrastine, palmatine and lesser amounts of canadine and hydrastinine. Preparations derived from *Goldenseal* have been used to treat skin and eye ailments. Berberine, the major alkaloid in *Goldenseal* root powder, has been used in eye drops to treat trachoma, a disease characterized by keratoconjunctivitis. Berberine and palmatine are also present in extracts from *Berberis amurensis Ruprecht* (Berberidaceae) which are used to treat ocular disorders. We have previously shown that *Goldenseal* alkaloids are phototoxic to keratinocytes (*Chem Res Toxicol.* 14, 1529, 2001; *ibid* 19, 739, 2006) and now report their effect on human lens and retinal pigment epithelial cells. Human lens epithelial cells (HLE-B3) were severely damaged when incubated with berberine (25 μM) and exposed to UVA (5 J/cm^2). Under the same conditions palmatine was less phototoxic and hydrastine, canadine and hydrastinine were inactive. Moderate protection against berberine phototoxicity was afforded by the antioxidants ascorbate (2 mM) and N-acetylcysteine (5 mM). When exposed to UVA (5 J/cm^2) both berberine (10 μM) and palmatine (10 μM) caused mild DNA damage as determined by the alkaline Comet assay which measures single strand breaks. Berberine and palmatine are the only *Goldenseal* alkaloids with appreciable absorption above 400 nm. Because light at wavelengths below 400 nm is cut off by the anterior portion of the human eye only berberine and palmatine were tested for phototoxicity to human retinal pigment epithelial (hRPE) cells. Although berberine did damage hRPE cells when irradiated with visible light (λ>400 nm) approximately ten times higher concentrations were required to produce the same amount of damage as seen in lens cells. Palmatine was not phototoxic to hRPE cells. Neither berberine nor palmatine photodamaged RPE DNA. Infusions of *Goldenseal* are estimated to contain ~1 mM berberine while in tinctures the alkaloid concentration may be more than 10 times higher. Our findings show that eyewashes and lotions derived from *Goldenseal* or containing berberine must be used with caution when the eyes are exposed to bright sunlight but that oral preparations are not likely to cause ocular phototoxicity.

Keywords

Berberine; palmatine; hydrastine; canadine; hydrastinine; Goldenseal; phototoxicity; Comet assay; human lens cells; human retinal pigment epithelial cells

Introduction

The dried root or rhizome of *Goldenseal* (*Hydrastis canadensis* L.) contains several alkaloids including berberine, hydrastine, palmatine and lesser amounts of canadine and hydrastinine (1-4) (Figure 1,Table 1). Preparations derived from *Goldenseal* have been used to treat wounds and ulcers, as well as skin and eye ailments (1,5,6). Berberine, the major alkaloid in

Goldenseal root powder, has been used in eye drops (7) to treat trachoma, a disease characterized by keratoconjunctivitis (8,9). Berberine and palmatine are also present in extracts from *Berberis amurensis Ruprecht* (Berberidaceae) which are used to treat ocular disorders (10). In vitro experiments have shown that berberine and palmatine can inhibit lens aldolase reductase and these alkaloids have been proposed for the treatment of certain diabetic ocular complications, such as retinopathy and cataract (11,12).

We have previously reported that berberine and palmatine are phototoxic to keratinocytes when they are UVA irradiated (13,14). In addition, berberine and UVA caused DNA damage in keratinocytes. All of the *Goldenseal* alkaloids have UVA absorption (Figure 1) so their presence in eye drops raises the possibility of ocular phototoxicity (15). Eye irritation resulting from the ingestion of edible oil contaminated with argemone oil has indeed been attributed to the presence of berberine (16). We have therefore studied the UVA phototoxicity of berberine, hydrastine, palmatine, canadine and hydrastinine towards cultured lens cells. Berberine and palmatine also absorb above 400 nm. Because only wavelengths above 400 nm reach the adult retina (Figure 2) (17-19) we have also examined the effect of visible light irradiation (λ>400 nm) in the presence of berberine or palmatine on retinal pigment epithelial cells.

Materials and Methods

Berberine, palmatine, hydrastine and hydrastinine were obtained from Sigma Chemical Co. (St. Louis, MO). Canadine (tetrahydroberberine) was supplied by Midwest Research Institute (Kansas City, MO). All other chemicals were reagent grade or better.

Absorption Spectra

Absorption spectra were recorded using an HP diode array 8451 spectrophotometer (Hewlett Packard Co., Palo Alto, CA).

Cell culture

Human RPE cells (hRPE) were isolated from donor eyes using the method of Hu et al. (20) and cultured in Falcon flasks (75 cm^2) with F12 nutrient mixture supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 μg/ml gentamicin (Gibco, BRL Products, Rockville, MD) in an atmosphere of air containing 5% CO_2 at 37°C. The cells (passage number 15-17) were detached with trypsin-EDTA solution, diluted 1:3 - 1:4, and plated for subculture. The cells used in the present studies consisted of a pure culture of one cell line of hRPE in active growth status. The purity of the cell line was demonstrated by immunocytochemical methods: hRPE cells display S-100 and cytokeratin, while uveal melanocytes display S-100 antigen but not cytokeratin, and fibroblasts display neither of these proteins (21). The initial viability of the hRPE cells was 97.0±0.4% as estimated by trypan blue exclusion. Light microscopy indicated that the cells were amelanotic.

Human lens epithelial cells (HLE B-3) (22) were grown in Eagle Minimum Essential Medium (Sigma Chemical Co., St Louis MO) supplemented with 20% FBS, 2 mM L-glutamine and 50 μg/ml gentamicin and 20% FBS in an atmosphere of air containing 5% CO_2 at 37°C. Cells were fed twice a week and after attaining confluence were passaged using trypsin-EDTA.

Cell viability

HLE-B3 cells were grown to 90% confluency in 96-well dishes (Costar, Corning International, Corning, NY). The medium was replaced by sterile HBSS containing the alkaloids and incubated for 30 min at 37°C in the dark in a 95% air / 5% CO_2 atmosphere before exposure (15 min) to UVA radiation from 4 fluorescent PUVA lamps (Houvalite F20T12BL-HO, National Biological Corp., Twinsburg, OH) emitting radiation between 300 nm and 400 nm

with a maximum at 350 nm. Fluence was measured using a Goldilux UV meter equipped with a UVA probe (Oriel Instruments, Stratford, CT). After exposure the HBSS solution was replaced by MEM nutrient mixture supplemented with 2% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin and the cells were kept in the incubator overnight. An aliquot of the medium (20 μl) from each well was transferred to a new 96-well plate and assayed for LDH release (CytoTox 96 Aqueous Non-Radioactive Proliferation Assay; Promega Corp., Madison, WI) monitored at 492 nm using the Spectrafluor Plus (Tecan US, Research Triangle Park, NC) plate reader. The remaining medium was removed, the cells were washed (2×) with PBS, and cell viability measured after the addition of PBS/glucose with the MTS assay (CellTiter 96 Aqueous Non-Radioactive Proliferation Assay; Promega Corp., Madison, WI) monitored at 492 nm.

The hRPE cells were grown in 96-well dishes (Costar, Corning International, Corning, NY). The medium was replaced by sterile HBSS containing the alkaloids and incubated for 30 min at 37° C in the dark in a 95% air / 5% CO_2 atmosphere before exposure for 10 min to visible radiation from a 150 W floodlamp emitting light between 350 nm and 850 nm but filtered to remove wavelengths below 400 nm. Fluence was measured using a YSI-Kettering Model 65A Radiometer (Yellow Springs Instrument Co., Yellow Springs, OH). After exposure (10 min) the HBSS solution was removed and replaced with F12 nutrient mixture supplemented with 2% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin and the cells were kept in the incubator overnight. Cell damage was measured using the LDH and MTS assays as described above for the HLE-B3 cells.

DNA damage assay

DNA damage was assessed using the alkaline single gel (“comet”) assay (23) which measures single strand breaks. Briefly, hRPE or HLE-B3 cells grown to ~85% confluency were trypsinized then ~50,000 cells were suspended in 100 μl 1% (w/v) low melting point agarose in PBS, pH 7.4, at 37°C and immediately pipetted onto a frosted microscope slide that had been pre-coated with 1% (w/v) normal melting point agarose. Slides were allowed to cool at 4°C for ~10 minutes before a second layer of 100 μl of low melting point agarose was pipetted on top of the previous layer. Again slides were allowed to cool for ~10 minutes at 4°C. The slides were then immersed in a 10 μM solution of alkaloid dissolved in HBSS, incubated at room temperature for 30 min and then exposed to either UVA (5 J/cm^2) or visible irradiation (λ>400 nm) from a floodlamp (*vide supra*). During the visible irradiation the slides were kept on an icepack to prevent overheating. After exposure the slides were washed once in cold PBS and immediately put into lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, and 1% (v/v) Triton-X 100, NaOH to pH 10.0) at 4°C overnight. Following lysing slides were neutralized for 5 minutes in 0.4 M Tris-HCl, pH 7.5. Slides were then placed in a horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA pH 13 to unwind for 20 min before electrophoresis at 25 V constant for 40 minutes. The slides were washed 3 times for 5 minutes each with 0.4 M Tris-HCl, pH 7.5 and then placed in cold EtOH for a minimum of 30 minutes. Slides were dried, stained with 20 μg/ml of ethidium bromide (Sigma Chemical Co.) and 50 comets/slide were scored using Komet 5.0 (Kinetic Imaging Ltd., Liverpool UK).

Results

Absorption Spectroscopy

The absorption spectra of the alkaloids are shown in Figure 2. Because sunlight contains wavelengths as short as 290 nm all of the alkaloids could potentially absorb solar UVA radiation and cause photodamage when applied to the eye in an eyewash or lotion. However, canadine has weak absorption above 300 nm and is found in such small amounts (Table 1) that it is unlikely to act as a cellular photosensitizer. Although hydrastine also has minimal UVA absorption its concentration in *Goldenseal* preparations approaches that of berberine (Table 1)

and so it has the potential to cause phototoxicity. Hydrastinine has strong UVA absorption but is present in only trace amounts in *Goldenseal*. Because wavelengths below 400 nm are cut off when sunlight passes through the anterior segment of the eye (i.e. cornea and lens) (17, 19) only berberine and palmatine have the potential to damage the retina (Figure 2).

Phototoxicity

Lens Epithelial Cells—HLE-B3 **c**ells were exposed to the alkaloids, irradiated with UVA and cell viability measured using the MTS and LDH assays (Figure 3). No toxicity was observed when cells were exposed to UVA alone or to the alkaloids in the dark. However, when lens cells were UVA irradiated (5 J/cm^2) in the presence of 25 μM palmatine or berberine there was a significant decrease in cell viability (Figure 3). The remaining alkaloids were not phototoxic under these conditions. Berberine exhibited phototoxicity at 5 μM that increased with concentration up to 50 μM (Figure 4). In contrast palmatine was much less phototoxic requiring 50 μM to produce the same amount of damage as 5 μM berberine (Figure 4).

The lens and aqueous humor contain relatively high levels of antioxidants: 0.8-2 mM ascorbate (24-26) and 4-10 mM glutathione (27,28). To determine whether ascorbate would protect against berberine phototoxicity, lens cells were irradiated in the presence of 25 μM berberine and increasing concentrations of ascorbate. Significant protection was seen at 0.5 mM ascorbate which increased up to 2 mM. A further increase to 5 mM resulted in a loss of protection (Figure 5A). N-acetylcysteine at 5 mM partially protected against berberine phototoxicity (Figure 5B). At higher concentrations N-acetylcysteine was toxic in the dark (data not shown).

Both berberine and palmatine intercalate into DNA, where they undergo an increase in fluorescence quantum yield.(29,30) We have previously shown using the Comet assay that berberine in combination with UVA causes DNA damage in keratinocytes (14). The possibility that the alkaloids in this study could also damage lens DNA was therefore investigated by means of the Comet assay (23). As can be seen from Figure 6 berberine or palmatine (10 μM) in combination with UVA (5 J/cm^2) caused a modest increase in DNA damage as measured by the Comet assay Olive Tail moment.

Retinal Pigment Epithelial Cells

When light passes through the anterior segment of the eye wavelengths below 400 nm are removed (Figure 2) so that only visible light reaches the retina. Because berberine and palmatine are the only *Goldenseal* alkaloids with appreciable absorption above 400 nm (Figure 2) their phototoxicity towards hRPE cells was studied. While berberine was phototoxic to hRPE cells it caused much less damage than that seen in lens cells (Figure 7). Furthermore, palmatine (5-100 μM) did not photodamage hRPE cells (data not shown). Neither berberine nor palmatine damaged hRPE DNA either in the dark or when irradiated with visible light (data not shown).

Discussion

The MTS assay measures the activity of (mitochondrial) dehydrogenases present in the cell, while the LDH assay estimates the amount of cytosolic LDH released as a result of membrane damage and cell lysis. Nevertheless these two assays agreed well (Figures 3 and 4) and showed that, when UVA irradiated, berberine and to a lesser extent palmatine are phototoxic to lens cells. The IC_{50} for berberine lies between 5 and 10 μM while for palmatine the IC_{50} is >50 μM. The toxicological importance of these findings for the lens depends on a number of factors including the ability of the alkaloids to penetrate the cornea. Unfortunately there are no published data on the penetration of *Goldenseal* alkaloids into the eye (31). However, Medow and Greco have claimed (32) in a patent application that topically applied berberine, hydrastine

and canadine anethetize the cornea, and paralyze the sphincter muscle of the iris and ciliary body, sugestsing that these alkaloids do indeed penetrate into the aqueous humor. Additional factors influencing the ocular effect of the *Goldenseal* alkaloids are their relative concentrations in eye lotions and washes and their respective absorption spectra. We calculated the phototoxic potential of the individual alkaloids based on their concentrations in *Goldenseal* root powders and their absorption (Table 1). Berberine had the highest phototoxic potential followed by hydrastine and palmatine. However, the actual concentrations of the alkaloids in *Goldenseal* preparations are probably quite variable (33). Based on the data in Table 1 we estimate that an infusion derived from dissolving one teaspoon (~5 g) of *Goldenseal* powder in one pint of water would contain final concentrations of berberine and palmatine of ~1 mM and 400 μM respectively. The concentration of berberine and palmatine in tinctures, generally standardized contain to contain 8-10% alkaloids (33), must be much higher. At the recommended dilution for eyewashes the final concentration of berberine is probably of the order of 40 mM (33). Thus it appears that both alkaloids could be phototoxic when present in eye preparations. Both the lens and aqueous humor contain antioxidants present to protect the lens from light damage. The concentration of ascorbic acid in the lens is 1.2 mM (24) which may serve to partially protect it against berberine phototoxicity (Figure 5A).

In addition to being applied topically to the eye *Goldenseal* alkaloids could also reach the inner eye as a result of oral administration of the powder. Wang and coworkers (34) measured plasma levels of 1.8 μg/ml (~ 5 μM) berberine in rats receiving an intravenous injection of 3 mg/kg berberine in *Coptidis rhizoma* extract. *Coptidis rhizoma* is a traditional Chinese medicine containing both berberine and palmatine at approximately the same concentrations as *Goldenseal* (Table). They also found 350 ng/g (~ 1 μM) berberine in the hippocampus showing that berberine can cross the blood-brain barrier. Because of the high functional similarity of the blood-brain and blood-retina barriers (35) berberine could be present in the retinal pigment epithelium at a concentration comparable to that of the brain. The lens in the human eye is fed by the aqueous humor which is continuously produced by the ciliary body. This additional barrier can significantly lower the concentrations of alkaloids in close contact with the lens in comparison to their levels in the plasma. Nevertheless, Cui and coworkers have reported (36) that oral administration of berberine inhibits chemokine, protein and cell levels in the aqueous humor of rats treated with lipopolysaccharide.

A typical oral dose of *Goldenseal* powder is 125 mg 4 times daily (33) which equates to ~ 7 mg/kg for a 70 kg individual. Lu et al. found peak plasma levels of palmatine and berberine of 5 ng/ml (~15 nM) and 2.5 ng/ml respectively in rats receiving an oral dose of 4.38 g/kg *Coptidis rhizome* extract (37). Thus it is likely that the plasma levels of berberine or palmatine in humans after oral administration of *Goldenseal* powder would be too low to pose a phototoxic threat to the lens. Berberine and palmatine were much less phototoxic to RPE cells (Figure 7). In vivo RPE cells are protected from visible light damage by the presence of melanin granules. Because our cultured RPE were amelanotic they are probably more susceptible to the phototoxic effect of berberine and palmatine. Thus it seems unlikely that these alkaloids could damage RPE cells in vivo.

In summary our findings show that eyewashes and lotions derived from *Goldenseal* must be used with caution when the eyes are exposed to bright sunlight but that oral preparations are not likely to cause ocular phototoxicity.

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Abbreviations

FBS, fetal bovine serum; HBSS, Hank's buffered salt solution; hRPE, human retinal pigment epithelial; LDH, lactate dehydrogenase; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PBS, phosphate buffered saline; PBS/glucose, PBS containing 10mM glucose; UVA, 320-400 nm wavelengths.

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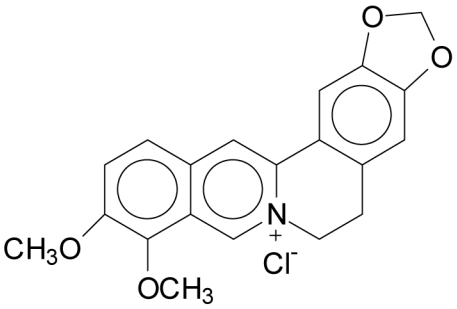
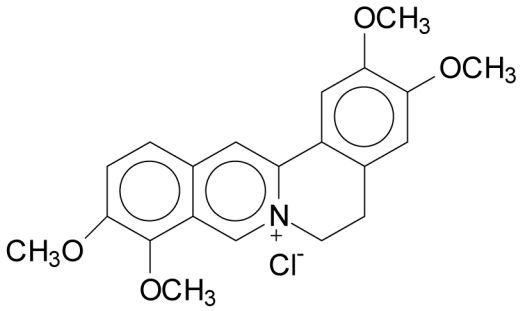


Berberine



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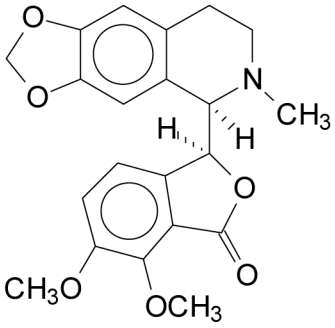
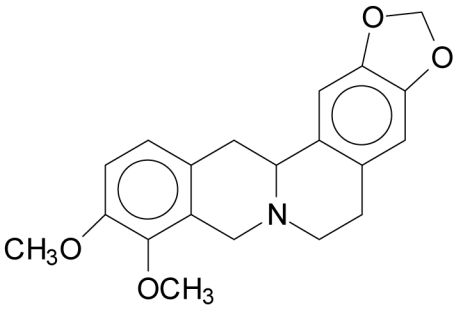
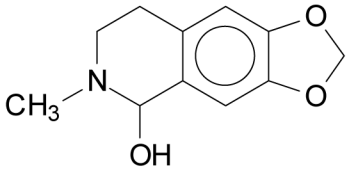


Hydrastine



Canadine
(Tetrahydroberberine)



Hydrastinine

Figure 1.
Structures of the *Goldenseal* alkaloids

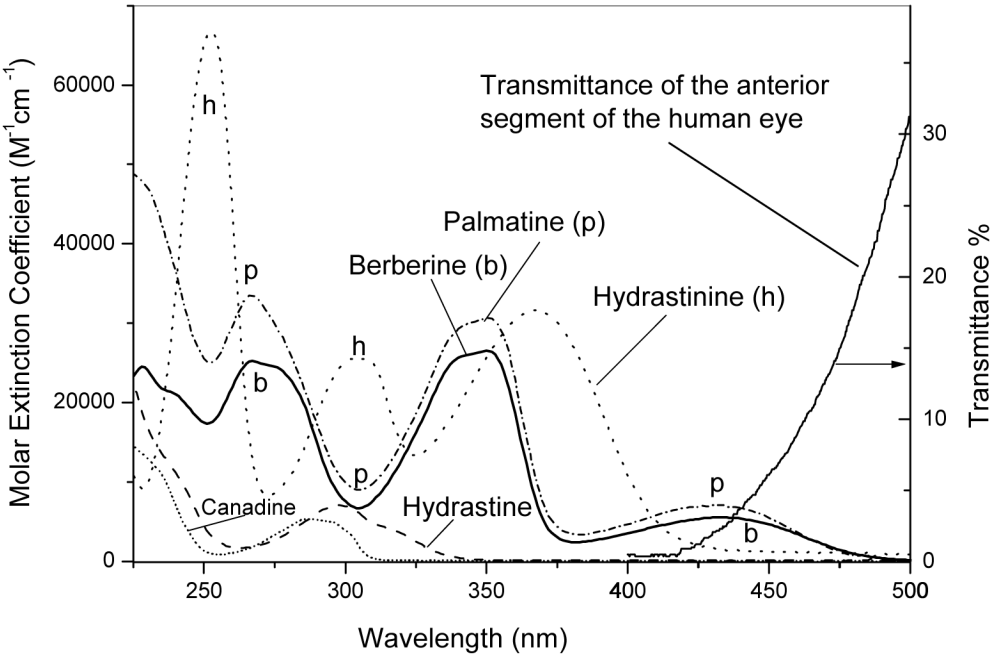


Figure 2.
Absorption spectra of the *Goldenseal* alkaloids in ethanol (modified from reference (13)) and the transmittance of the anterior segment of the human eye (modified from reference (19)).

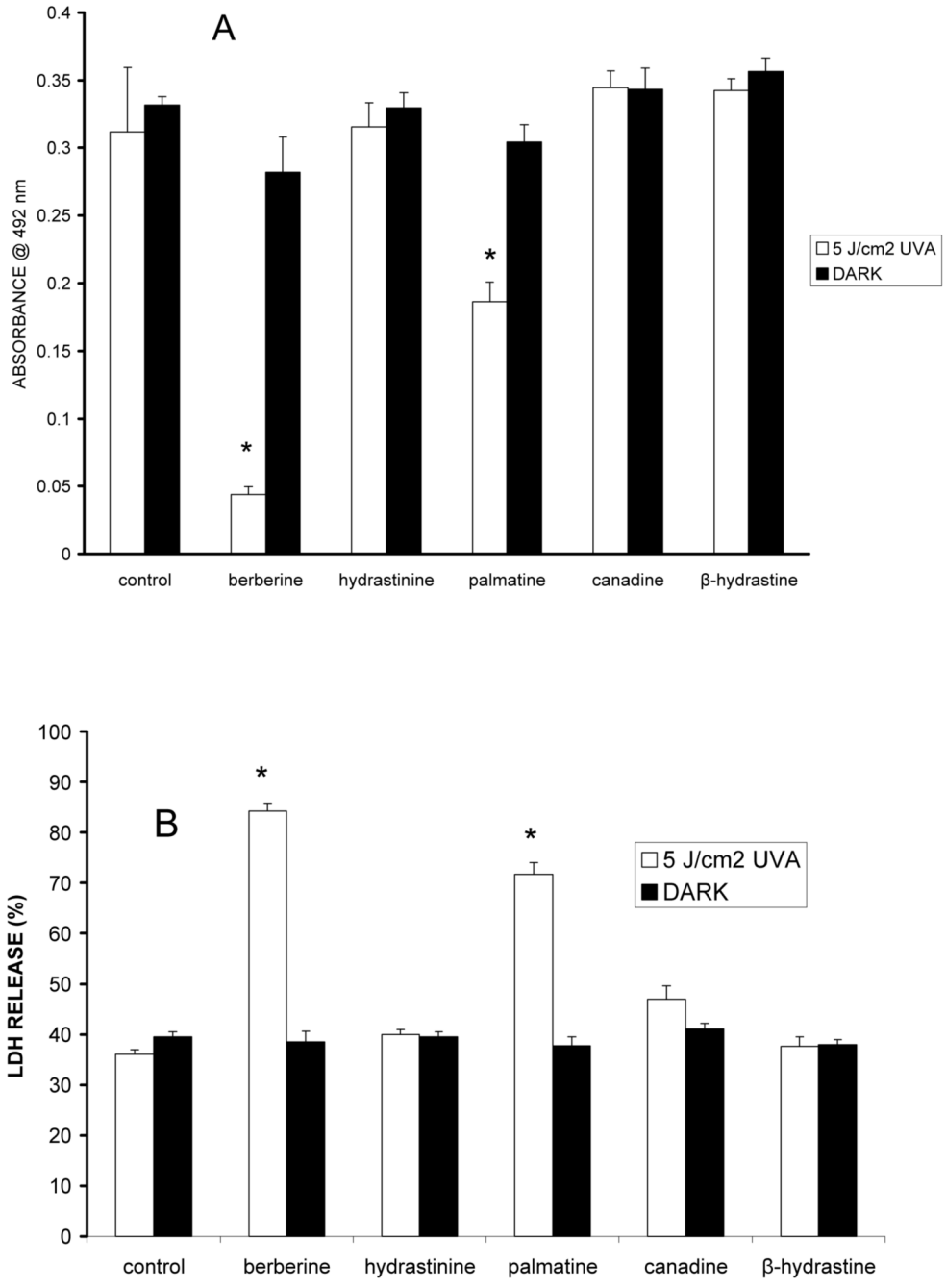
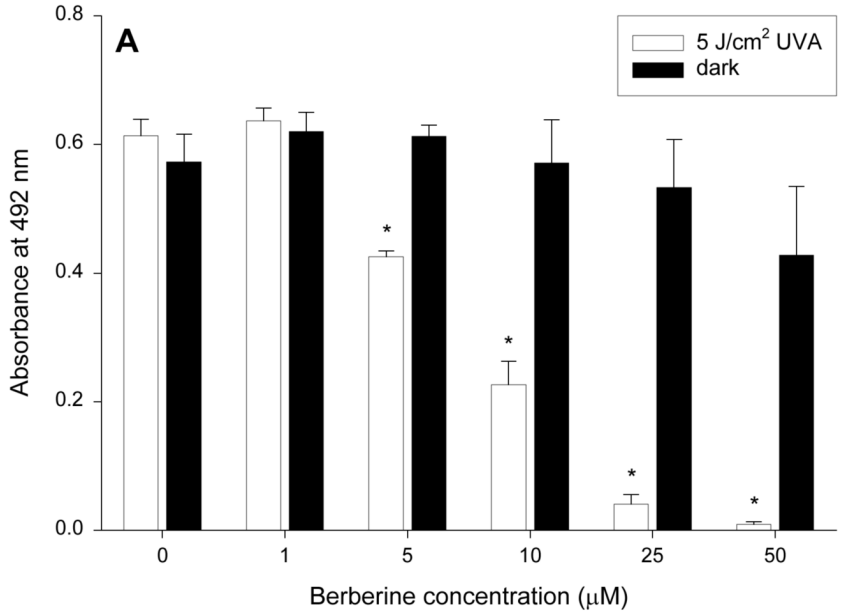
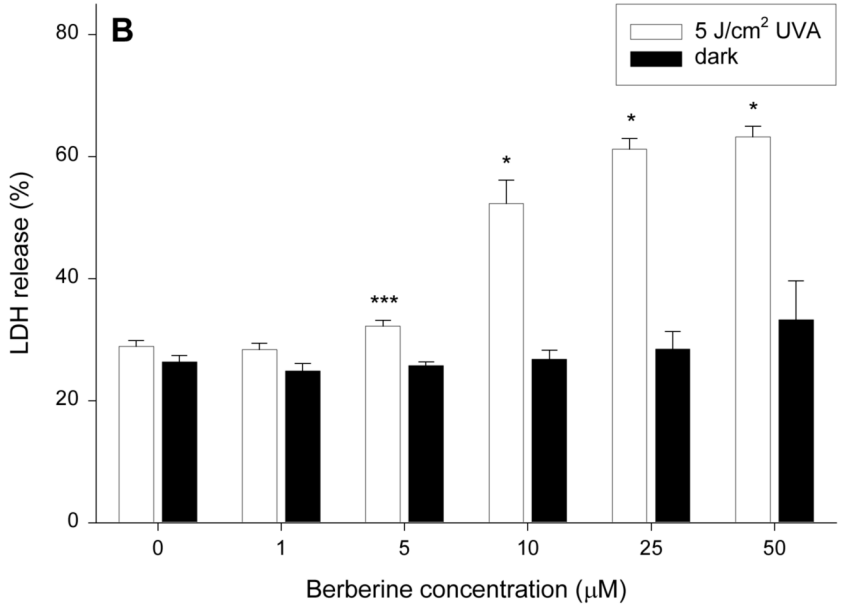


Figure 3.

The effect of *Goldenseal* alkaloids (25 μM) and UVA (5 J/cm^2) on the viability of HLE-B3 lens cells using the MTS (A) and LDH (B) assays. Values are the means ± S.E. (n = 8). * $p<0.001$ *vs.* control.





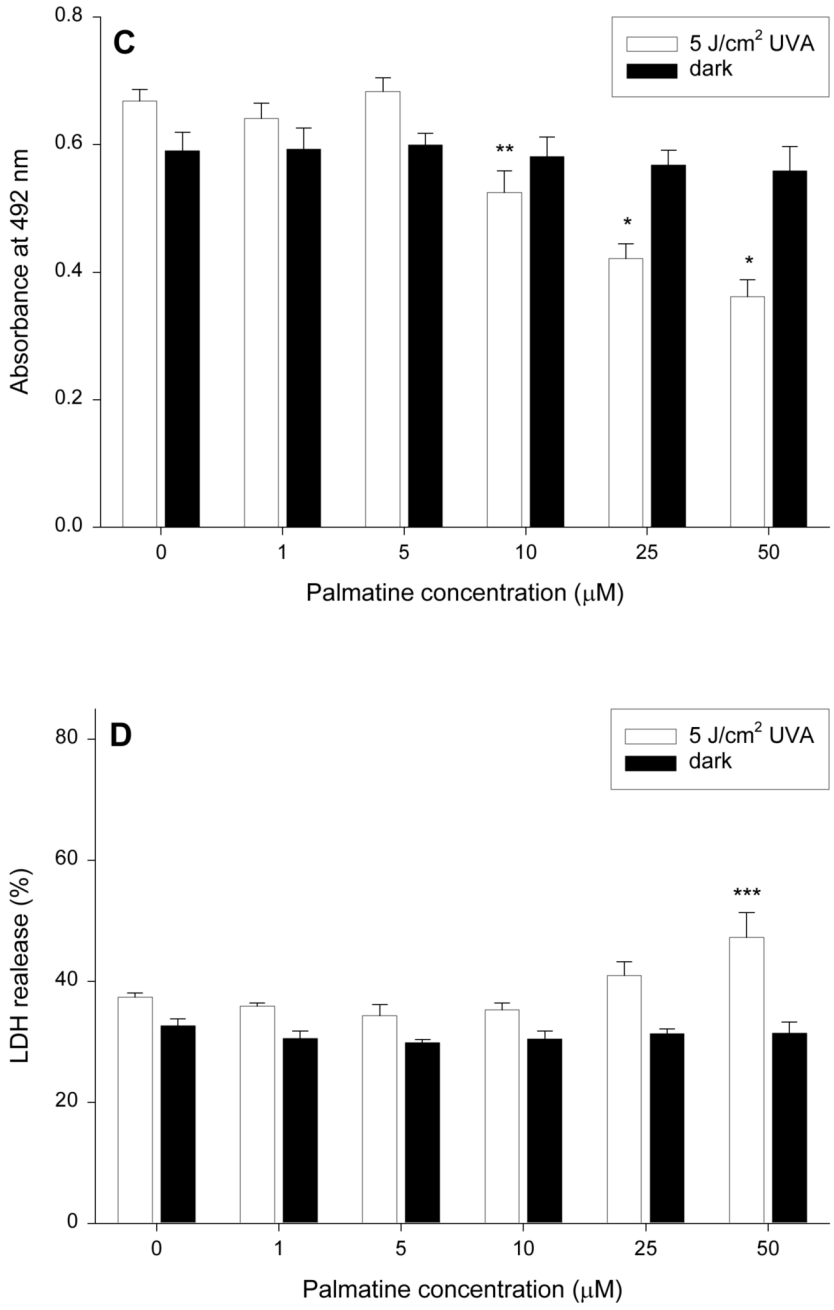


Figure 4.

The effect berberine or palmatine and UVA (5 J/cm^2) on the viability of HLE-B3 lens cells using the MTS (A and C) and LDH (B and D) assays. Values are the means ± S.E. (n = 8). * $p<0.001$, ** $p<0.005$, *** $p<0.05$ *vs.* control (0 berberine).

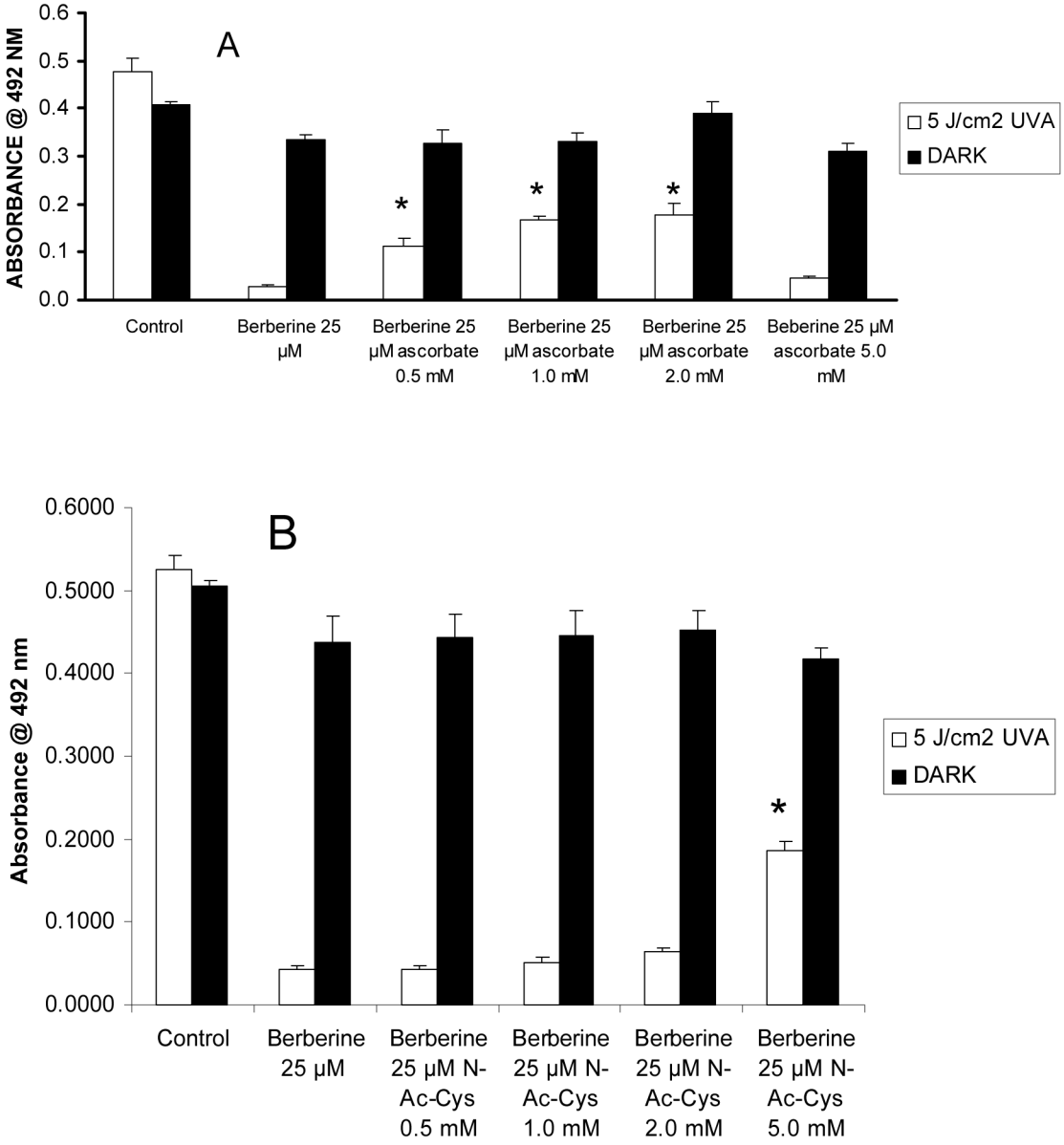


Figure 5.

The protective effect of (A) ascorbate or (B) N-acetylcysteine (N-Ac-Cys) on the viability of HLE-B3 lens cells exposed to berberine (25 µM)/UVA (5 J/cm^2) using the MTS assay. Values are the means ± S.E. (n = 8). * $p<0.001$ *vs.* berberine alone.

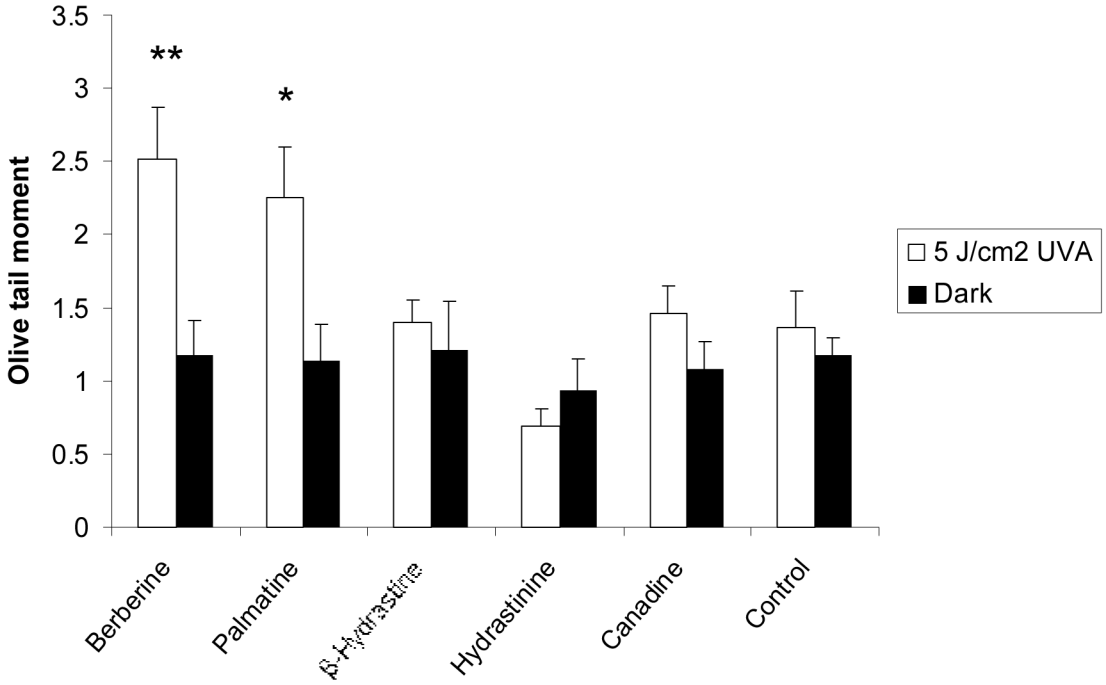


Figure 6.

Single strand DNA damage to HLE-B3 lens cells resulting from exposre to the *Goldenseal* alkaloids (10 μM) and UVA (5 J/cm^2). Results are expressed as the Olive tail moments. Values are the means ± S.E. (n = 50). * $p<0.05$, **$p<0.01$ *vs.* control.

The effect of Goldenseal alkaloids (10 μM) and UVA (5 J/cm^2) on the Olive tail moment of HLE-B3 lens cells. Values are the means ± S.E. (n = 50). * $p<0.05$, **$p<0.01$ *vs*. control.

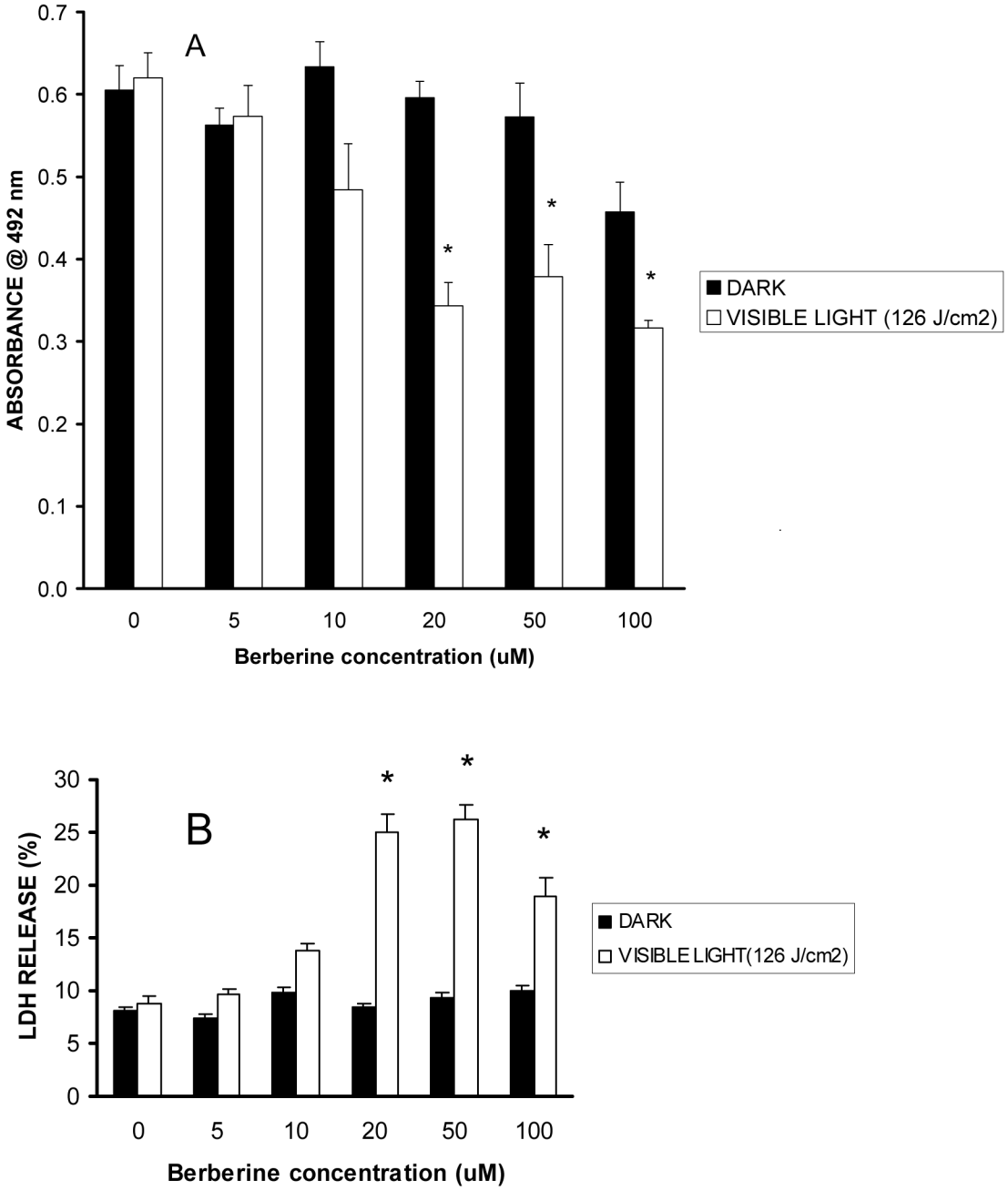


Figure 7.

The effect of berberine (25 μM) and visible light (>400 nm; 126 J/cm^2) on the viability of human RPE cells using the MTS assay. Values are the means ± S.E. (n = 8). * p<0.001 *vs.* control.

Table 1

Alkaloid Composition of *Goldenseal* and *Coptidis rhizoma* Root Powders

Alkaloid	% by weight[†]	% by weight[*]	% by weight[≠]	Phototoxic potential of *Goldenseal* Root Powder (% by weight[*] × integral 280-500 nm[§] × 10^{-6})
Berberine	2.52	1.9	3.51	3.66
Hydrastine	1.38	1.3	0.64	0.35
Palmatine	0.22	0.1	-	0.23
Canadine	0.04	0.06	-	0.0072
Hydrastinine	n.d.	trace	-	n.d.

n.d. not determined

[†] *Goldenseal* from reference (2)

[*] *Goldenseal* from reference (3)

[≠] *Coptidis rhizoma* from reference (38)

[§] Calculated from Figure 2.