Approaches to relieve the burden of cataract blindness through natural antioxidants: use of Ashwagandha (Withania somnifera)*

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Cataract is the major cause of blindness the world over. Efforts to ease the cataract burden will be of great social and health economic benefit. Oxidative stress is known to be a major factor in age-related cataract of the eye lens. Regular systemic intake of antioxidant vitamins appears to retard the progression of cataract. These are beyond the reach of people in developing countries, who could, however, be encouraged to use antioxidant plant products that form part of their diet and traditional health practices. Ashwagandha (extract of the plant Withania somnifera) is one such product used in traditional medicine. We have studied the antioxidant, cytoprotective and related properties of Ashwagandha here, and find it to be excellent in these respects. It is also able to retard the formation of ‘cold cataract’ in vitro, suggesting that Ashwagandha could well act as a cataracto-static agent.

AGE-related cataract is the leading cause of blindness and visual impairment worldwide. Effective surgical procedures are available for treatment, but besides the requirement of highly trained personnel, the problem of post-operative complications, cost of surgery and high number of people requiring surgery pose a substantial economic burden. In India alone, there are about 9.5 million people who are cataract-blind, and at least twice as many suffering from various stages of development of this sight-threatening condition.

Oxidative stress, either as the primary event or secondary to risk factors like ageing and smoking, is one of the predominant factors that leads to cataract. A major mode of damage to lens proteins involves oxidative reactions. For this reason, the possible role of antioxidants in delaying the onset or progression of age-related cataract has gained considerable interest. Endogenous defence mechanisms which protect the lens against oxidative damage include compounds like glutathione, ascorbate and antioxidant enzymes like catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase and related ones. But with increasing age, the levels of these protective enzymes are known to decline in the human eye. Supplementation with antioxidants thus appears to be an attractive possibility to delay the onset of age-related cataract. It has been estimated that a delay in cataract formation of approximately 10 years would reduce the cataract surgical burden by perhaps 45%.

Most research on nutrition and cataract has been regarding vitamins (vitamins A, C and E) and several studies have found their intake to be associated with a reduced risk of cataract. Supplementation of antioxidants on a regular basis is beyond the economic reach of people in the developing world, who form the major fraction of cataract-affected across the globe. However, they include a range of plant material, rich in antioxidants and micro-nutrients, in their diet and health practices. Hence, we have studied the role of certain plant extracts that are

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easily available, affordable, traditionally more acceptable and which form a part of the daily diet of many people, e.g. tea and greens. Earlier work from our laboratory had shown that extracts of tea (green and black) and Ginkgo biloba display excellent antioxidant activities, and also exhibit cataractostatic ability in animals in which oxidative cataract was induced through selenite. In this connection, we have chosen to investigate the antioxidant properties of extracts of Withania somnifera (Ashwagandha) of the Indian pharmacopoeia, which finds immense importance in Indian ayurveda.

W. somnifera is a dicotyledonous plant belonging to the family Solanaceae. Its roots have been compared with ginseng roots, and the plant is thus also known as the Indian Ginseng. Ayurvedic medicine includes W. somnifera in the class of herbs called ‘adaptogens’ or ‘vitalizers’. These relatively innocuous agents cause adaptive reactions to disease and appear to produce a state of non-specific increased resistance to adverse effects of biological agents. The medicinal properties of Ashwagandha have been attributed to its chemical constituents, mainly alkaloids and steroid lactones (primarily of the withanolide class). A detailed monograph on this plant describing the extensive characterization of its chemical and medicinal aspects has recently been published. Found and used in Africa, the Arab world and in South Asia, the W. somnifera extract (WSE), is used for a variety of illnesses such as asthma, rheumatic pain, inflammation of joints, nervous disorders, epilepsy, as a uterine sedative, antispassmodic, sedative and hypnotic, and against eye diseases.

W. somnifera has been reported to have a wide range of biological activities. It shows antimicrobial activity, anti-tumour and radiosensitizing effects, anti-inflammatory, immunomodulatory, antistress adaptogenic activity, anti-convulsive, hemopoetic, and rejuvenating properties. Recent literature suggests its efficacy as a cardioprotective agent, inhibitor of drug-induced urotoxicity, enhancer of white blood cell and platelet counts, an agent that enhances immunoprotection, cytokine production and stem cell proliferation, and an antioxidant.

In light of the interesting possibilities and applications of W. somnifera, we have monitored its antioxidant and cytoprotective abilities. We have worked with a sample of the root powder of Ashwagandha obtained from Kotakkal Arya Vaidya Sala, Kerala, India. Stock solution of the root powder (100 mg/ml) was prepared in water and the soluble part was used to determine the antioxidant activity as well as for protein cross-linking studies. For experiments with cell lines, solutions were made in sterile phosphate buffered saline (PBS) and filtered using a 0.2 μm filter.

The azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was performed based on the procedure of Miller et al. Briefly, when ABTS is incubated with a peroxidase (such as metmyoglobin) and H₂O₂, the relatively long-lived radical cation ABTS⁺⁺ is formed. In the presence of an antioxidant, the absorption of this radical cation (at 734 nm) is quenched. In a typical experiment, ABTS (30 μl, 5 mM), 50 μl metmyoglobin (50 μM), and 820 μl phosphate buffer (50 mM, pH 7.4; of which 10 μl is replaced with WSE (100 mg/ml stock) when the sample is being investigated) are mixed, and the reaction is initiated by the addition of 100 μl of H₂O₂ (1 mM). The absorbance at 734 nm is measured as a function of time at 5 min intervals for 30 min.

The electron spin resonance (ESR) method of trapping free radicals was also used to study the antioxidant property of WSE. The free radicals (superoxide or O₂⁻) were produced using the xanthine/xanthine oxidase system that produces O₂⁻ under metal-free conditions and dihydroxyaminopentaacetate (DEAPAC). The radicals were captured in a stable and conveniently measurable manner using the ‘spin trap’ DMPO (5,5-dimethyl-1-pyrroline-1-oxide), which produces long-lived and stable free radicals that can be measured and quantified, using an X-band ESR spectrometer. The reaction mixture consisted of 0.1 mM xanthine, 50 mM DMPO, 0.1 mM DEAPAC, 0.1 mM FeCl₃; EDTA, 100 U/ml catalase and 0.1 U/ml xanthine oxidase. Experiments were done at ambient temperature, with a 100 kHz modulation of the magnetic field, 10 mW microwave power and 0.25 G modulation amplitude.

The protein bovine pancreatic ribonuclease A, or RNAase A, (1 mg/ml) was irradiated in the presence of the photosensitizer molecule bis(hydroxypropyl)naphthaldimide (also called the Photo-Fenton reagent), which generates hydroxyl radicals upon irradiation at 366 nm. The reagent was adsorbed on controlled pore glass beads and suspended in the protein solution. The mixture was taken in a fluorescence cuvette, placed in the sample compartment of a Hitachi F2500 spectrofluorimeter, and irradiated at 366 nm for 90 min by tuning the excitation monochromator to this wavelength, with the excitation slit wide open. The light intensity falling on the sample has been earlier estimated to be about 0.5 mW/cm² (10¹⁵ photons incident/s). The ·OH so produced generates oxidative crosslinks in the protein, monitored by SDS–PAGE. Inhibition of the formation of high molecular weight products by the extract (WSE) was assayed by adding known amounts of the substance to the reaction mixture before irradiation. An identical procedure was adopted for the photosensitized oxidation of RNAase A, using riboflavin which, upon irradiation at 445 nm, yields singlet oxygen in high quantum yield. The damage to the protein, and protection by the extract, was monitored by SDS–PAGE.

Peroxynitrite was generated by reacting ice-cold solutions of NaNO₂ (0.6 M) with H₂O₂ (0.7 M) in acidic medium (0.6 M HCl) and rapidly quenched using NaOH (1.5 M). The reaction mixture was stored at –20°C, and the ONOO⁻ concentrated in the upper yellow layer was
collected. Its concentration was measured using a molar extinction coefficient of 1670 M⁻¹ cm⁻¹ at 302 nm. The test protein, bovine α-crystallin (1 mg/ml), was incubated with 1 mM peroxynitrite with or without 10 mM bicarbonate, and a series of increasing concentrations of the plant extract. Protein cross-linking was studied by SDS-PAGE.

DNA damage was evaluated by single-cell gel electrophoresis, also called the comet assay. Cultured HLE cells were trypsinized to obtain a single-cell suspension. Cells were irradiated for 1 h with broad-band visible light in the presence of 25 μM riboflavin, with or without WSE. The cell suspension was then mixed with low melting agarose (2%, in Ca- and Mg-free PBS), layered onto frosted glass slides and allowed to gel. Prepared slides were placed in lysis solution (154 mM NaCl, 10 mM EDTA and 0.5% N-laurylsarcosine, pH 7.0) for three minutes. Slides were then washed in distilled water for five minutes. Electrophoresis was carried out in buffer containing 25 mM NaCl and 1 mM EDTA at 7 V/cm² for three minutes. Finally, the slides were washed and stained with ethidium bromide (50 μg/ml). DNA damage was visualized using a fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. DNA migration, nuclear diameter and tail intensity were analysed.

The cytoprotective ability of W. somnifera extract was studied using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay. This assay is based on the ability of living cells to reduce MTT to form formazan products, which can be quantified at 540 nm by the intensity of blue colour. The rabbit corneal keratocyte cell line, SIRC, which was procured from the National Centre for Cell Sciences, Pune, India or HLE cells (1 x 10⁵ cells per well in 0.5 ml medium) were seeded in a 24-well tissue culture plate and incubated at 37°C, 5% CO₂. After 22 h of incubation, when cells were about 75% confluent, oxidative stress was induced using riboflavin (20 μM) as the photosensitizer, in the presence or absence of WSE. After exposing the cells to stress, they were incubated in fresh medium for 18 h at 37°C and 5% CO₂, after which 50 μl of 5 mg/ml MTT in PBS was added to each well. After 4 h of incubation at 37°C, the medium was removed and 500 μl of 40 mM HCl/isopropanol was added to stop MTT reduction. The blue colour developed was read at 540 nm.

The effect of WSE on the phase-separation temperature of lens cytoplasm was studied according to Hiroaka et al. using bovine lens homogenate in the presence of sucrose (0.9 M), since sucrose is known to bring up the lower critical solution temperature of liquid mixtures. Whole lenses were dissected out, cut into small pieces, homogenized at 37°C and centrifuged at 20,000 rpm for 1 h to remove membranes. The resulting homogenate was used to determine the phase-separation temperature. A stock solution of WSE was prepared in 0.1 M phosphate buffer, pH 7.0 and one part of this stock solution was mixed with five parts of fish lens homogenate to yield a final extract concentrations of 5 and 20 mg/ml. In the control, the extract was replaced with buffer. The sample tube was placed in a fluorescence spectrophotometer (Hitachi F2500) with a temperature-controlled circulating water bath, and the scatter at 600/600 nm was recorded over the range of +20 to -10°C. The transition from transparent to opaque was observed as a sharp increase in scatter, which occurred over a narrow temperature range. Similarly, the effect of WSE on thermally-induced self-aggregation of lens proteins was also studied. Scatter was recorded over the range of 25 to 75°C.

The free-radical scavenging activity of the root powder of W. somnifera has been reported earlier. Bhattacharya et al. studied the antioxidant activity of the glycowithinolides of W. somnifera, where the effect of intraperito-

**Figure 1.** ABTS antioxidant assay of WSE. a, WSE is an effective antioxidant. Formation of ABTS⁺ in the absence (●) and presence of 1 mg/ml WSE (○). b, WSE effectively scavenges superoxide. Assay of the ability of various concentrations of WSE in quenching superoxide radicals, measured by ESR spin-trapping.
neal injections of glycowithanolides on concentrations of antioxidant enzymes in brain frontal cortex and striatum of adult male Wistar rats was studied. We extend the antioxidant analysis of both GBE and Ashwagandha here, using the ABTS assay and EPR spectroscopy. As shown in Figure 1a, addition of WSE (1 mg/ml) led to a rapid drop in the ABTS absorption at 734 nm within minutes, showing it to be an efficient antioxidant. We then looked into the ability of WSE to quench individual oxydradicals. Figure 1b shows the EPR line intensity due to superoxide radicals with increasing concentrations of WSE.

Figure 2 shows the electrophoresis pattern of test proteins subjected to oxidative stress by either riboflavin or the Photo-Fenton reagent or peroxynitrite. WSE is able to effectively inhibit oxidative damage induced by the Photo-Fenton reagent, which generates hydroxyl radicals. When riboflavin was used as the sensitizer, WSE was able to quench $^1$O$_2$ and protect the protein from damage. WSE was also able to effectively inhibit peroxynitrite-mediated covalent modifications in the test protein, bovine $\alpha$-crystallin.

Figure 3 shows the ability of the test compounds to protect oxidative strand-breaks in the nuclear DNA in intact human lens epithelial cells. When single cells are electrophoresed, the intact DNA in the nuclei is seen as a compact disk or a ‘moon’; when strand-break and thus chain-scission occur by oxidative damage, it is visualized as a trailing ‘comet’ in the electrophoreogram. Addition of WSE is seen to inhibit the damage to DNA, reduce the tail and restore the ‘moon’.

The ability of WSE to enter cells and protect the nuclear DNA led us to study the cytoprotective properties of the extract. We subjected HLE or SIRC to riboflavin-induced stress in the absence and presence of WSE, and assayed the viability of cells using the MTT metabolism method. Figure 4a shows that the test compound protects HLE cells from permanent damage and keeps them viable. Figure 4b shows that this protective ability holds well even for cells of corneal origin, SIRC.

The extract is able to permeate cell membranes and exert its action on the nuclear DNA and cytoplasmic components, as the comet and MTT assays reveal. The steroidal lactones of $W$. somnifera appear to have useful biological properties in this regard. Though glycowithanolides are believed to be responsible for the antioxidant property, the chemistry needs to be studied in detail.

We next investigated the effect of the addition of WSE on the ‘cold cataract’ phenomenon (Figure 5). Solubility studies have shown that the lens proteins (essentially $\gamma$-crystallin) readily phase-separate, causing the phenomenon of cold cataract$^{3,36}$. The phenomenon is reversible, and the proteins go back into solution upon warming. Bovine

Figure 2. SDS–PAGE of oxidized proteins. a, WSE inhibits the photo-oxidation of proteins. RNAse A was irradiated at 445 nm in the presence of riboflavin (20 $\mu$M and WSE 0.1 mg/ml), and damage to the proteins was monitored by SDS–PAGE. b, To a 1 mg/ml solution of the protein RNAse A was added a suspension of the Photo-Fenton reagent bis(hydroperoxy)napthaldimide, and irradiated as described in the text. The protein was analysed after the reaction using SDS–PAGE. Lane 1 (from left), Control protein solution; Lane 2, Protein + Photo-Fenton reagent irradiated for 30 min; Lane 3, Protein + Photo-Fenton reagent + WSE (2 mg/ml) irradiated for 30 min. c, WSE inhibits peroxynitrite-mediated damage to $\alpha$-crystallin. Peroxynitrite (PON) used at 1 nM concentration, with or without added 10 mM bicarbonate, and reacted for 15 min with protein of 1 mg/ml concentration, in each case. SDS–PAGE profiles: Lane 1 (from left), Control protein; Lane 2, Protein + PON; Lane 3, Protein + PON + bicarbonate; Lane 4, Protein + PON + 2 mg/ml WSE, and Lane 5, Protein + PON + bicarbonate + 2 mg/ml WSE.
lens homogenate was found to dramatically increase light scattering when it was cooled to –6°C. When WSE was added to the homogenate to a final concentration of 5 mg/ml, this precipitation temperature was lowered to –7°C, and 20 mg/ml did not cause its precipitation even up to –10°C.

We then looked at the effect of the extracts on the high-temperature precipitation of lens homogenate (Figure 5). In the absence of any additive, the scattering of light sharply increased around 70°C for bovine lens proteins. Addition of WSE (5 and 20 mg/ml) did not inhibit this precipitation, but in fact advanced it to lower temperatures. Addition of 5 mg/ml WSE lowered the phase-transition temperature ($T_c$) by 0.5°C, while 20 mg/ml dropped it further to 60°C. Therefore, WSE could prevent only the ‘cold cataract’ phenomenon. This can be explained since neither of these phenomena involves any oxidative or other covalent chemical modifications.

Since some herbal extracts, e.g. hypericin from St. John’s wort, used as drugs and health supplements have been demonstrated to have inherent photosensitizer properties, we monitored the photodynamic properties of WSE. This was done by irradiating a 1 mg/ml solution of RNase A in the presence of the added extract (0.5 mg/ml) for 120 min (at 365 nm for Ginkgo biloba extract and 320 nm for Ashwagandha, wavelengths where the protein does not absorb but the extract does). Covalent damage to the protein was monitored by gel electrophoresis. No modifications were seen in the gel, indicating that WSE is not phototoxic, i.e., it does not act as a sensitizer producing any reactive species upon excitation.

Though Ginkgo biloba extract could prevent selenite-induced cataract in rat pup which is an oxidative model of cataractogenesis, the effect of WSE on an animal model of cataract is yet to be determined. Addressing the issue of toxicology and safety of W. somnifera doses to humans, a preliminary toxicity study of W. somnifera using total alkaloids from the roots showed an LD$_{50}$ of 465 mg/kg body wt. in rats and 432 mg/kg body wt. in mice.

Russo et al. investigated the free-radical scavenging capacity of methanolic extracts of W. somnifera and the effect on DNA cleavage induced by H$_2$O$_2$ UV-photolysis, and whether this extract was capable of reducing the hydrogen peroxide-induced cytotoxicity and DNA damage in human non-immortalized fibroblasts. The extract showed a dose-dependent free-radical scavenging capacity and a protective effect on DNA cleavage. These results were confirmed by a significant protective effect on H$_2$O$_2$-induced cytotoxicity and DNA damage in human non-immortalized fibroblasts. The antioxidant, antimicrobial and cytoprotective properties of WSE make it

Figure 3. WSE protects nuclear DNA from strand breakage. Comet assay of strand-break in DNA of human lens epithelial cells. Damage was effected by the photodynamic action of added 25 μM riboflavin, and cell nuclei visualized in the single-cell gel electrophoresis with ethidium bromide fluorescence. a. Untreated cells; b. Cells treated with riboflavin; and c. Cells treated with riboflavin in the presence of 0.2 mg/ml WSE.
Figure 4. a, WSE protects human epithelial cells from riboflavin-induced photodamage, followed by the MTT assay. b, WSE protects rabbit corneal keratocyte cells from riboflavin-mediated damage, monitored by the MTT assay.

an attractive drug of choice to be tried in ocular pharmacology. These antioxidant effects of *W. somnifera* may explain, at least in part, the reported anti-stress, immunomodulatory, cognition-facilitating, anti-inflammatory and anti-aging effects produced by them in experimental animals and in clinical situations, and may justify further investigation of their other beneficial biological properties.

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Interatomic contacts in viral capsids*

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Spherical viral capsids possess icosahedral symmetry and are made of a large number of densely-packed protein subunits, each consisting of thousands of atoms. Similarly, a large number of close interactions contribute to packing in crystals of virus particles. A computational method, based on the representation of the three-dimensional shape of the subunits in the icosahedral asymmetric unit as a binary map for the fast evaluation of all short interatomic contacts between subunits within the capsid as well as between particles in the crystal lattice is presented. This method might be useful in the examination of the spatial relations of three-dimensional objects. Its application to seshanba mosaic virus reveals subunit packing dominated by polar interactions, in consistence with observed properties.

The principles of helical and spherical virus architecture were enunciated forty years ago by Casper and Klug. These principles have been revisited and reviewed in

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