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IN VITRO BIOCHEMICAL ASSESSMENT OF ANTIOXIDANT POTENTIAL OF AN AYURVEDIC HERBAL EYE DROPS FOR DRY EYE SYNDROME

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ABSTRACT

Dry Eye Syndrome is a leading cause of ocular discomfort affecting millions of people. Dry Eye conditions are a spectrum of disorders with varied aetiology ranging from mild eyestrain to very severe dry eyes associated with visual complications. Dry eye is a condition produced by the inadequate interrelation between lachrymal film and ocular surface epithelium, and is caused by quantitative and qualitative deficits in one or both of them. Ocular surface conditions may result from the abnormalities in one or more of the tear film components, ocular or systemic diseases, various drugs and even environment factors. Oxidative stress is involved in many surface ocular diseases including dry eye syndrome (DES). The anterior eye segment and mainly the cornea are directly affected by the hazards from potential oxidative damage evoked by air pollution, radiation, chemicals as evident by proven role of oxidative stress in the pathogenesis surface ocular disease. Further certain scientific studies have demonstrated beneficial effects and protective role of topical and oral antioxidants in the management of surface lesions like dry eye disease. Along with measures such as tear preservation, use of tear stimulants, tear substitutes and control of infection, the advocacy of topical and oral antioxidant agents also forms an important component in the management of dry eye syndrome (DES). Owing to its importance, the biochemical assessment of antioxidant potential of the Ayurvedic herbal eye drops formulated for dry eye syndrome was performed. The plant based ingredients of the eye drop possess promising leads for their antioxidant, anti-inflammatory; anti-microbial potential. The study encompasses biochemical antioxidant assays viz. Nitric oxide radical scavenging assay, ABTS radical scavenging assay, DPPH radical scavenging assay and Ferric reducing antioxidant potential (FRAP) assay. These studies revealed significant antioxidant potential of herbal eye drops, possibly compliment to the management of dry eye syndrome and marinating the surface ocular health.

KEYWORDS: antioxidant activity, Ayurvedic herbal eye drop, dry eye syndrome.

INTRODUCTION

Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolality of the tear film and inflammation of the ocular surface. Based on the pathophysiology of tear film formation the classification of DES suggested by Holly and Lemp comprise Aqueous Tear Deficiency (ATD); Lacrimal surfactant (mucin) deficiency; Lipid layer abnormality; Impaired lid function or blinking; and Epitheliopathy.[1,2] The Latin phrase "kerato-conjunctivitissicca" indicates dryness and inflammation of the cornea and conjunctiva. Ayurvedic literatures describe DES as *shushkakshipaka, parishuskha-netra, ativishuskha--netra, asrusravarahita-netra* and *asnigdha-netra* indicative of dryness of eye due to deficiency in tear film components.[3] There are many conditions which cause dryness of the eyes such as hypo function of lacrimal glands, mucin deficiency, conjunctival scarring etc.[4] Dry eye syndrome is the most common eye disease, affecting 5 - 6% of the population.[5,6,7] Further it became a significant public health problem distributed among 10% of the adult population and 18% of the elderly population.[8]

Contemporary management strategies for Dry eye syndrome pose certain limitations. Management strategies of DES include mainly supplementation of tear preferably substitutes containing methylcellulose or carboxy-methylcellulose or identical substances which are viscous in nature**.** Preservatives used in formulations are known to cause dry eyes. All these drugs do not have any effect on basic path physiology and they provide only symptomatic relief.[9] The tear stimulants such as cholinergic drugs increases the tear production from lacrimal gland by stimulating secretions, but not been used in clinical practice. Tear Preservation can be done by occluding the puncta or minimizing evaporation, But is useful as short-term measure to assess the effect of occluding puncta before resorting to permanent measures. All these drugs do not have any effect on basic path physiology and they provide only symptomatic relief.[10] Further Topical antibiotics and corticosteroids are sometimes used to treat secondary infections and inflammation. But discontinuation of antibiotics, steroids and all preservative-containing eye drops is mandatory for relief of symptoms and progressively improving the tear film and ocular surface. In view of the above, there is an urgent need to evolve safe and effective management approach to tackle symptoms, infections and underlying pathology.

Protective role of antioxidant supplementation in the maintenance ocular surface health: Oxidative stress in the cornea influenced by several environmental factors such as air pollution, radiation, chemicals etc. leads to changes in corneal optical properties and decrease in visual acuity or even vision loss. The antioxidant agents help in suppressing the damages due to oxidative stress hand assist in restoring the corneal health.**[11]** The surface lesions and corneal diseases, associated with oxidative stress leads to corneal aging, b corneal inflammation In acute corneal inflammation the Reactive oxygen species (ROS) are highly involved. Studies on the oxidative reactions in tears of patients with dry eye disease confirmed a marked increase of inflammatory activity in the tear film of patients suffering from dry eye. These reactions lead to severe damage of the eye. Free radicals and inflammation may be involved in the pathogenesis or in the self-propagation of the dry eye disease**.** The antioxidant therapy with superoxide dismutase and dimethylthiourea are employed for the healing of corneal ulcers evoked by sodium hydroxide. Topical antioxidant therapy found effective in reducing the inflammatory corneal reaction.[12-18]

Further, the Antioxidant supplements such as vitamin C and vitamin E, probably have an important role in reducing the oxidative damage produced by nitric oxide and other free radicals and improving the ocular surface milieu.[19]

Need for development of Inclusive therapeutic strategies: in view of challenges of different conventional agents to manage dry eye disease. It is imperative to develop such topical dosage form which could offer safe, effective and comprehensive management. Ayurvedic literatures record more than fifty ophthalmic plant drugs and about forty metals minerals having diversified pharmacological actions on visual system and adnexa of the eye.[20,21,22] Adding to the references from Ayurvedic texts,few clinical studies on medicinal plants such as *Yastimadhu* (*Glyeyrrhiza glabra*) and *Daruharidra* (*Berberis aristata*) also substantiate the usefulness of these plants in surface inflammatory ocular lesions such as dry eye syndrome. A study intervention comprising of topical and internal use of *Daruharidra* (*Berberis aristata*) has shown significant improvement in subjective parameters like dryness, redness, photophobia etc. in DES. [23,24,25] Pharmacological actions such as *caksusya* (conducive to vision), *netrya* (conducive to adnexa of eye), *netraruja-hara* (analgesic ophthalmic action), netra-*sodhahara* (anti-inflammatory action) *netrakanduhara* (anti allergic action), *vrana-ropana* (wound healing effect),anti-bacterial actions are attributed to these drugs.[26,27] *Yastimadhu* (*Glyeyrrhiza glabra*) has shown notable anti-inflammatory action attributed to cortisone-like substance present in this plant that helps reduction of inflammation.[28] It is evident that the combination of ingredients viz. *Yastimadhu* (*Glyeyrrhiza glabra*), Daruharidra (*Berberis aristata*) may play a significant role in restoring the functions of tear film ,prevention of ulceration and related checking inflammatory process, infection and contributory to the comprehensive management of DES.

The medicinal plant ingredients rationally chosen in formulating the eye drops for dry eye disease viz. *Glycyrrhiza glabra* and *Berberis aristita* possess antioxidant, antimicrobial activity backed by scientific evidence. Methanolic extract of *Berberis aristita* has shown DPPH free radical Scavenging Activity expressed in % inhibition with L Ascorbic acid as standard showing IC50 9.6µg/ml and that of extract was 33.31µg/ml. Hydrogen peroxide radical scavenging activity was comparable to standard IC50 for L Ascorbic acid is 54.23µg/ml and that of *B. aristita* is 60.6µg/ml. Similarly, reducing power of plant extract at different concentration was comparable with L-Ascorbic Acid. The Antimicrobial screening revealed activity against *Candida albicans, Salmonella typhii, Pseudomonas aeruginosa and Escherichia coli* [29] In an experimental study, the antioxidant assay of methanaloic extract of *Glycyrrhiza glabra* confirmed the potent antioxidant activity.[30]

The eye drop is formulated with these two plant ingredients and developed complying quality standards and other safety parameters.[31,32,33] Ocular toxicity and safety studies revealed The eye drops did not cause irritation to ocular mucous membrane of eyes of rabbits such as opacity ulceration of cornea; congestion, swelling, moderate circumcorneal hyperemia; or injection, haemorrhage, gross destruction of iris; redness

and other signs of inflammation of palpebral and bulbar conjunctivae; chemosis of lids etc. and no evident signs of toxicity were observed.[34, 35]

MATERIALS AND METHODS

Objective: Reactive oxygen species (ROS) are highly involved in the pathogenesis of dry eye disease and topical and oral antioxidant therapy has shown a protective role in preventing the damage. In view of this It is imperative to explore the antioxidant potential of eye drop, as the suppression of Oxidative stress plays an important role in dry eye syndrome besides the management of symptoms due to deficient tear film components. The study aims at *In vitro* biochemical antioxidant screening comprising of Nitric oxide radical scavenging assay, ABTS radical scavenging assay, DPPH radical scavenging assay and Ferric reducing antioxidant potential (FRAP) assay.[36-41]

Determination of antioxidant potential was done adopting the following In *vitro* biochemical assays.

1. **Inhibition of Nitric oxide radical:** Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions. This was measured by the Griess reaction (Green *et al*., 1982; Marcocci *et al*., 1994). The reaction mixture (300µl) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and eye drops and the reference compound in different concentrations (10, 25, 50, 75 and 100 µg) were incubated at 25°C for 150 min. Each 30 min, 50µl of the incubated sample was removed and 50µl of the Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) were added. The absorbance of the chromophore formed was measured at 546 nm on ELISA plate reader (Bio-Tek). All the tests were performed in triplicate and the results averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples. Ascorbic acid served as a positive control compound.

2. **ABTS radical cation decolourisation assay**: In this assay, the oxidant is generated by persulfate oxidation of 2, 2,-azinobis (3-ethylbenzoline-6-sulfonic acid)-($ABTS^{2-}$) as described by Re *et al*., (1999). ABTS radical cation ($ABTS^{+}$) are produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixtures were allowed to stand in dark at room temperature for 12-16 hr before use. After 16hr, this solution was diluted with ethanol until the absorbance reaches 0.7 ± 0.02 at 734 nm. For the study, 100µl of eye drops (120µg/ml) were added to 200µl of ABTS solution. The absorbance was read at 745nm and the percentage inhibition calculated.

3. **Inhibition of DPPH radical:** The free radical scavenging activity of eye drops was measured by 1; 1-diphenyl-2-picryl-hydrazil (DPPH) uses the method of Blois (1958) and Gomez-Alonso *et al*., (2003). 0.1 mM solution of DPPH in methanol was prepared and 100 µl of this solution was added to 100 µl of eye drops and the reference compound (50, 100, 150, 200 and 250 µg). After 30 min, absorbance was measured at 517 nm. Butylated Hydroxy Anisole (BHA) was used as the reference material. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

4. **Reducing power/Ferric reducing antioxidant potential (FRAP) assay:** The reducing power of eye drops was determined according to the method of Oyaizu (1986). 100 µl of eye drops were mixed with phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50ºC for 20 min. A portion of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000g for 10 min. The upper layer of the solution (100 µl) was mixed with distilled water (50µl) and Ferric chloride ($FeCl_3$) (100 µl, 0.1%) and the absorbance was measured at 700 nm. Butylated Hydroxy Toluene (BHT) was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

RESULTS AND DISCUSSION

In vitro biochemical antioxidant assays such as Nitric oxide radical scavenging assay, ABTS radical scavenging assay, DPPH radical scavenging assay and Ferric reducing antioxidant potential (FRAP) assay have confirmed the antioxidant potential of the herbal eye drop. The Inhibitory Concentration (IC_{50}) obtained from the standard graphs of various assays are expressed in terms of their standard compounds (Table-1) (Figure-1, Figure-2, Figure.3, Figure 4).

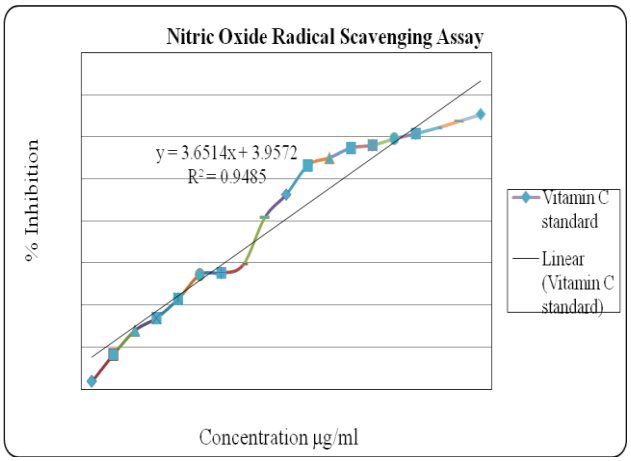

Figure 1: Nitric oxide radical scavenging assay obtained with vitamin C standard.

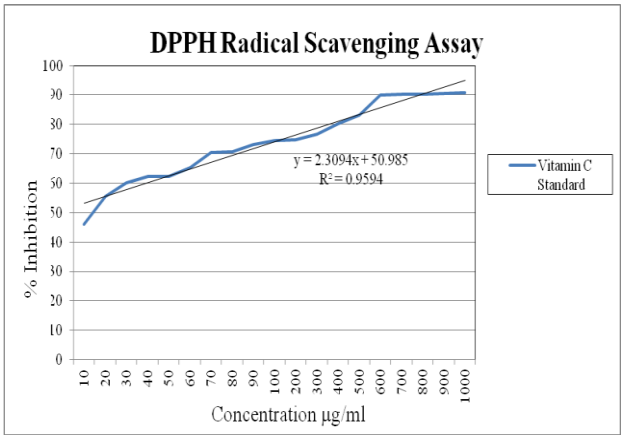

Figure 2. DPPH radical scavenging standard assay obtained with vitamin C standard.

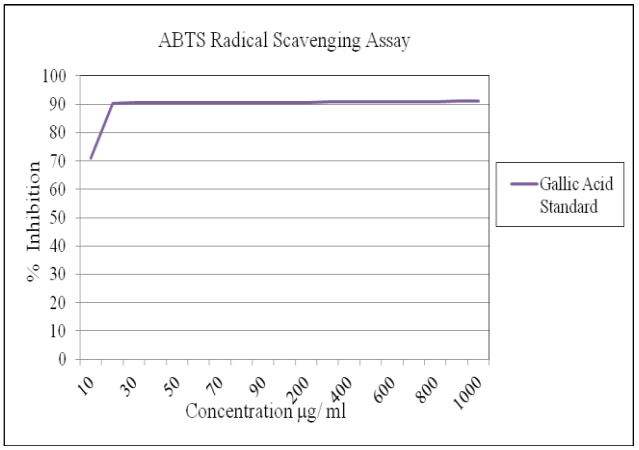

Figure 4: ABTS radical scavenging assay with gallic acid standard.

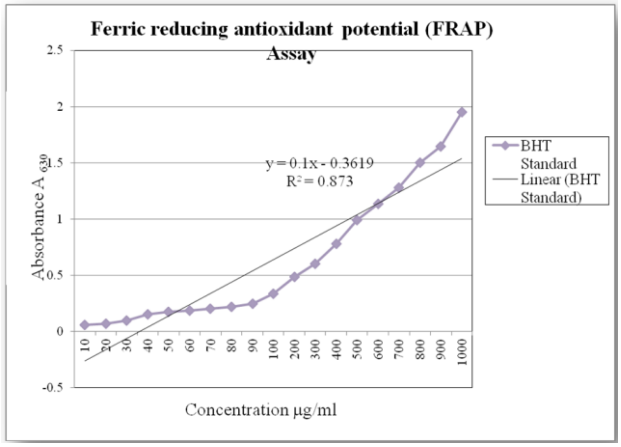

Figure 3: FRAP standard assay obtained with BHT standard.

Table 1: Inhibitory Concentration (IC_{50}) obtained from the standard graphs of various assays are expressed in terms of their standard compounds.

Sl. No.	Results	Sample volume of eye drops solution 120µg/ml	IC_{50} values equivalent to defend Standards
1.	Nitric Oxide Radical Scavenging Assay	100µl	632.99 µg/ml of Vitamin C Standard
2.	ABTS Radical Scavenging Assay	100µl	527.70 µg/ml of Gallic Acid Standard
3.	DPPH Radical Scavenging Assay	100µl	719.32 µg/ml of Vitamin C Standard
4.	Ferric reducing antioxidant potential (FRAP) Assay	100µl	276.69 µg BHT/ml

CONCLUSION

Despite progress in determining the etiology and pathogenesis of dry eye syndrome, current knowledge remains inadequate, and no preventive strategies have been found. The present-day management strategy of dry eye syndrome though clinically effective, poses certain limitations Moreover, the most common therapy for dry eye syndrome, artificial tears, provides only temporary and incomplete symptomatic relief. Hence, identification of modifiable risk factors for dry eye syndrome may suggest avenues for investigation of novel preventive and treatment measures.[42] In this scenario, it is becomes essential to translate some promising leads for management of dry eye syndrome from Ayurvedic literatures into, safe, effective and quality assured dosage forms to improve patient's compliance. In addition to the management of symptoms related to deficiency of tear components, prevention of damage due oxidative stress, arrest of further progress and control of infection also forms an vital component in dry eye disease. The close relationship between ocular surface epithelia and the preocular tear film ensures ocular

surface health. As such, dysfunctional protective elements that lead to ocular surface and tear disorders are heterogeneous, effective therapeutic strategies are the need of hour to tackle tear disorders attributed with diverse factors. The ocular toxicity studies of standardized herbal eye drops revealed its safety on topical ophthalmic use. Further the antioxidant and anti-microbial property may contribute to effective symptom management and extenuation of basic pathology linked with tears component deficiency. The eye drops developed rationally taking potential leads from codified Ayurvedic texts probably contribute by offering comprehensive management for dry eye syndrome.

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