The effect of a natural, standardized bilberry extract (Mirtoselect®) in dry eye: a randomized, double blinded, placebo-controlled trial

A. RIVA1, S. TOGNI1, F. FRANCESCHI1, S. KAWADA2, Y. INABA2, R. EGGENHOFFNER3, L. GIACOMELLI3

1Indena SpA, Milan, Italy
2Indena SpA, Tokyo, Japan
3Department of Surgical Sciences and Integrated Diagnostics, School of Medicine, Genoa University, Genoa, Italy

Abstract. – OBJECTIVE: Dry eye, a chronic disease of lachrymal fluid and corneo-conjunctival epithelium, could significantly impact visual function, affects quality of life and work productivity. Beside several conventional treatments, nutritional supplements based on bilberry extract have been identified as effective contributors to eye health. Here, we aim at investigating the bioavailability of a standardized bilberry extract, its ability to alleviate dry eye symptoms and its antioxidant potential.

MATERIALS AND METHODS: Either bilberry dried standardized extract derived from Vaccinium myrtillus L. fresh frozen fruits (Mirtoselect®) or a highly purified anthocyanin-rich extract, devoid of the non-anthocyanin component and supported on maltodextrins, were each orally administered to 5 male rats. Blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes after treatment, processed and analyzed by UV spectrophotometric method. In a parallel analysis, 22 otherwise healthy subjects suffering from dry eye symptoms were enrolled randomly assigned to receive the more bioavailable bilberry extract or placebo. Ophthalmological and clinical examinations including Schirmer’s test, pupil constriction, diacron-reactive oxygen metabolites (d-ROMs) test and biological antioxidant potential (BAP) test were performed at inclusion and after the 4-week study period.

RESULTS: The area under the curve of plasmatic levels of anthocyanosides in rats resulted 202.3±24.23 µg·min/ml for Mirtoselect® and 130.9±4.93 µg·min/ml for the highly purified anthocyanin-rich bilberry extract, notwithstanding the fact that the highly purified anthocyanin-rich extract group received an anthocyanins dosage much higher than the Mirtoselect® group (354 mg/Kg in anthocyanosides vs. 136 mg/Kg in anthocyanosides). 21 subjects, 11 subjects in the bilberry extract (Mirtoselect®) group and 10 subjects in the placebo group completed the clinical study. Schirmer’s test values indicating the volume of tear secretion were significantly improved in the bilberry extract group (p=0.019), whereas no significant changes were observed in the placebo group. A subset analysis revealed that Mirtoselect® could be more effective in subjects with higher tendency of dry eye. In terms of antioxidant potential, the bilberry extract produced significant improvement of BAP (p=0.003) and an increase of modified BAP/d-ROMs ratio, an indicator of overall balance between antioxidant potential and oxidative stress.

CONCLUSIONS: Our results suggested that natural, standardized bilberry extract (Mirtoselect®) is a natural more bioavailable delivery form anthocyanins, suggesting a strong matrix effect exerted by the non-anthocyanin component. Furthermore, it can improve tear secretion and plasmatic antioxidant potential in subjects suffering from DED symptoms.

Key Words: 
Dry eye, Bilberry extract, Bioavailability, Tear secretion, Antioxidant potential.

Introduction

According to the definition proposed by the International Dry Eye Workshop (DEWS) in 2007, dry eye is “a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage of the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface”1
Dry eye disease (DED) represents a common clinical problem, with a prevalence ranging from 5% to 35% at various ages, and around 5 million of Americans aged more than 50 years suffering from this condition\(^1\). European and Asian populations have a similar prevalence; a large population-based study in Japan revealed that prevalence of mild to severe, clinically diagnosed DED in men and women were 12.5% and 21.6%, respectively\(^2\). There are several recognized risk factors for the development of DED, including aging, female gender, hormonal changes, dietary imbalances (omega-3 essential fatty acids or vitamin A deficiencies), metabolic or immunological disorders and viral infections\(^3-7\). Also, one of the major factors involved in the onset of dry eye is thought to be the use of visual display terminal (VDT) at work\(^8\). However, recent technical innovations have made display terminals smaller and less expensive: notebook PCs, tablet terminals, handy terminals such as smartphones and portable video game consoles are rapidly spreading. As a result, eyes are increasingly strained also at home; therefore, the number of DED patients is expected to further increase. Dry eye could significantly impact visual function, which in turn affects quality of life and work productivity. The primary goals of DED treatment are relief of symptoms, improvement of visual acuity and patients’ comfort and restoration of normal ocular surface and tear film; pharmacological and non-pharmacological strategies for DED range from dietary and lifestyle modifications, artificial or autologous tear substitutes, punctual plugs, and topical and/or systemic anti-inflammatory medications to surgery\(^9\). However, correct management should take into account patient’s preference and must maintain a balance between efficacy and safety. Beside conventional treatments, nutritional supplements have been reported to be effective contributors to eye health\(^10\). Among supplement foods, bilberry, a type of shrub in Ericaceae family, which grows in Northern and Central Europe, is currently investigated for its antioxidant properties, capillary vessels protection and treatment of ocular disorders\(^11\). In particular, dietary supplementation with bilberry extract alone or combined with other ingredients safely improved subjective symptoms of computer eye strain\(^12\) or asthenopia\(^13\). So far, no direct evaluation has been made on the effect of bilberry in alleviating DED symptoms. Despite the most important pharmacologically active component in bilberry extract is anthocyanoside flavonoids (anthocyanins), several other active constituents such as vitamins, sugars, pectins and tannins can influence their biological effects\(^14\). In this study, we compared two different oral formulations for their bioavailability in rats: natural bilberry extract with standardized anthocyanins and full range of non-anthocyanin components (Mirtoselect\(^{®}\)), and highly purified anthocyanin-rich extract supported on maltodextrins. The preparation which resulted more bioavailable was then investigated in a randomized study in humans for its ability to alleviate dry eye symptoms and improve antioxidant potential in subjects with DED symptoms.

**Materials and Methods**

**In vivo Pharmacokinetic Study**

Male Sprague-Dawley rats (190-220 g) were maintained at 23±1°C under a controlled 12-hour light/dark cycle with free access to tap water and standard food for 3 days before the experiment. After 16-hour starvation, the rats were randomly assigned into two groups of 5 animals each. Anthocyanosides extracts, namely bilberry dried extract derived from Vaccinium myrtillus L. fresh frozen fruits (Mirtoselect\(^{®}\), Indena, Milan, Italy), containing 36% anthocyanins and matching the full phytochemical profile of the whole fruit, and a highly purified anthocyanin-rich extract, containing 89% anthocyanins and devoid of the non anthocyanin fraction, were orally administrated at 400 mg/kg body weight dissolved in water. Under ether anesthesia, blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes after treatment. The blood samples (0.5 ml) were centrifuged with 10% trichloroacetic acid (0.5 ml) at 5800 rpm for 5 minutes. Supernatants were collected. Pellets were resuspended in 5% trichloroacetic acid (1 ml) and centrifuged at 5800 rpm for 5 minutes. Supernatants were collected, gathered and analyzed by UV spectrophotometric method at 530 nm.

**Subjects and Clinical Study Design**

This was a randomized, double-blinded, placebo-controlled, parallel group comparison study. Otherwise healthy subjects who had fulfilled the inclusion criteria without presenting any of the exclusion criteria and agreed on voluntary participation at a pre-study explanation meeting were selected.

Inclusion criteria were:
- healthy subjects aged from 30 to 60 years, suffering from visual fatigue or eye strain;
- subjects who daily use video game consoles or a PC, or operate VDT for at least 4 hours per day;
- subjects who have total corrected visual acuity of at least 1.0 for both eyes, if a refractive error exists, it should be properly corrected;
- subjects who do not regularly use pharmaceuticals or health foods that are effective for eye strain.

Exclusion criteria were:
- subjects who have a treatment history of cardiovascular failure or cardiac infarction;
- subjects who are being treated or taking medications due to chronic diseases;
- subjects who are allergic to food related to the investigational product or pharmaceuticals;
- subjects who are using contact lenses;
- subjects who have eye diseases, entropium palpebrae, or entropium ciliarum;
- subjects who smoke;
- subjects who have poor eating habits;
- women during pregnancy and breast-feeding, or women of childbearing potential.

The study was conducted in compliance with the Declaration of Helsinki (adopted in 1964, revised in 2008) and received Ethic approval by the Seishin-kai Medical Association, Inc., Takara Clinic (Chairman, Tsuyoshi Takara). The study coordination and interpretation were conducted in Italy. All subjects signed a written informed consent before entering into the study. Participants were randomly assigned to either dietary supplement or placebo. The supplement finished dosage form was formulated in tablets, each consisting of 80 mg of Mirtoselect® (the bilberry standardized extract resulting more bioavailable in the animal study) and 170 mg of standard excipients, whereas placebo comprised excipients only. Participants received 2 tablets of Mirtoselect® (corresponding to 160 mg Mirtoselect®) daily or placebo for 4 weeks.

To reproduce the conditions of visual load by VDT use, participants were instructed to play on a video game console for 45 minutes before examination.

Ophthalmological and clinical examinations were performed at inclusion and after the 4-week study period at Ario Nishi-ari Eye Clinic (Tokyo, Japan), and included:
- Schirmer’s test to evaluate the volume of tear secretion was carried out by using ColorBar strips™ (Eagle Vision, Inc., Memphis, TN, USA). In details, the strips were placed on the lower eyelids and left for 5 minutes under spontaneous blinking; the length of the moistened part measured by a calibrated scale on the strip indicated the tear flow. A subset analysis was also performed by classifying participants as subjects with severe eye dry symptoms (Schirmer’s test ≤ 10 mm) and subjects with mild symptoms (Schirmer’s test > 10 mm).
- Pupil constriction was measured before and after the visual load by using TriIRIS® C9000 (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and the amount of change was used as an indicator of eye strain.
- Oxidative stress and antioxidant potential were evaluated by diacron-reactive oxygen metabolites (d-ROMs) test and biological antioxidant potential (BAP) test respectively, through free radical analytical system (FRAS) (Wismerll, Co., Ltd., Tokyo, Japan). d-ROMS test measures the concentration of blood hydroperoxide (ROOH). BAP test measures the inhibition of oxidation through electron transfer to active oxygen in plasma. In addition, modified BAP/d-ROMs ratio (as BAP/d-ROM/7.541) was calculated for comprehensive evaluation of the antioxidant potential of the body.

Statistical Analysis

Intragroup and intergroup comparisons were performed by using paired t-test and unpaired t-test, respectively. Multiple comparisons were performed by using ANOVA test with post-hoc Bonferroni correction. Statistical analysis was performed by IBM SPSS 18.0 software (SPSS Inc. Chicago, IL, USA). p-value <0.05 was considered significant.

Results

In vivo Pharmacokinetic Study

As shown in Figure 1, the plasma anthocyanosides level in male rat that received 400 mg/kg Mirtoselect® bilberry extract (136 mg/Kg in anthocyanosides) was much higher than that of rats that received 400 mg/kg of the highly purified anthocyanins-rich fraction (354 mg/Kg in anthocyanosides); the areas under the curve (AUC) were 202.34±24.23 vs 130.93±4.93 µg·min/ml, respectively. The relative bioavailability of Mirtoselect® compared to the highly purified anthocyanins-rich fraction, expressed as the ratio of the dosage adjusted-AUC values, resulted 4.

Clinical Study

Overall 22 subjects (8 men and 14 women) at the mean age of 45.5 ± 7.3 years were enrolled in the
study. As one participant dropped out during the study period for personal reasons, the final analysis set included 21 participants: 11 subjects (3 men and 8 women, 43.5 ± 5.1 years old) in the bilberry extract group and 10 subjects (5 men and 5 women, 48.8 ± 8.3 years old) in the placebo group.

**Schirmer’s Test**

The results of Schirmer’s test before and after 4-week study period for right and left eye and the means of both eyes are shown in Table I. Significant improvements in the mean values for right eye ($p = 0.009$) and both eyes ($p = 0.019$) as well as a beneficial tendency for left eye ($p = 0.062$) were observed in the group treated with bilberry extract. In the placebo group, the mean values of Schirmer’s test increased compared to the baseline for right eye ($p = 0.361$), left eye ($p = 0.118$) and both eyes ($p = 0.177$) but the differences were not significant. In addition, mean improvement rates from baseline to 4-week were higher in the bilberry extract group than in the placebo group for right eye, left eye and both eyes, with a statistically significant difference for the right eye ($p = 0.049$). Given that the results of Schirmer’s test at inclusion ranged from 5 to 35 mm for both eyes with a wide individual variation, we performed a subset analysis to examine whether the symptom severity influenced the effects of the investigational products. Participants were classified in subjects with severe symptoms of dry eye (dry condition) and subjects with mild symptoms (wet condition). Regarding right eye, the improvement rate among subjects with severe symptoms was higher in the bilberry extract group than in placebo group ($p = 0.015$). Among subjects with mild symptoms, no significant difference was observed between the bilberry extract group and the placebo group ($p = 0.131$). In the bilberry group, the improvement rate in participants with dry condition was significantly higher than that of participants with wet condition ($p = 0.028$). In the placebo group, no significant difference was recorded between the dry condition and the wet condition ($p = 0.516$). Similar results were obtained for the left eye; improvement rate for subjects with severe symptoms tended to be higher in the bilberry extract group than in the placebo group ($p = 0.067$). Among participants with mild symptoms, there was no significant difference between the bilberry extract group and the placebo group ($p = 0.986$). In the bilberry group, improvement rate of the participants with severe condition was significantly higher than that of participants with the wet condition ($p = 0.023$). No significant difference between the dry condition and the wet condition was observed in the placebo group ($p = 0.770$).

**Pupil Constriction**

No significant differences were observed in terms of pupil constriction measurements between before and after visual load (data not shown).

**Oxidative Stress and Antioxidant Potential**

Figures 2-4 show the results of d-ROMs test, BAP test and the modified BAP/d-ROMs ratio, respectively. In the bilberry extract group, only BAP significantly increased ($p = 0.003$) during the study period; d-ROMs ($p = 0.152$) and modified BAP/d-ROMs ($p = 0.187$) ratio did not significantly change. In the placebo group, d-ROMs increased significantly ($p = 0.013$); BAP increased ($p = 0.141$) while modified BAP/d-ROMs ratio decreased ($p = 0.293$), but these changes were not statistically significant.

**Discussion**

The aim of this study was to investigate the ability of bilberry extract to alleviate dry eye symptoms and its antioxidant potential. To identify oral bilberry extract formulations with high bioavailability, we firstly compared two preparations: natural bilberry extract with standardized anthocya-

<table>
<thead>
<tr>
<th></th>
<th>Bilberry extract (Mirtoselect®)</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inclusion</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Right eye (mm)</td>
<td>11.4±5.1</td>
<td>19.5±7.3**</td>
</tr>
<tr>
<td>Left eye (mm)</td>
<td>14.0±9.5</td>
<td>20.0±11.5</td>
</tr>
<tr>
<td>Both eyes (mm)</td>
<td>12.7±6.3</td>
<td>19.7±8.6*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation. *$p <0.05$ vs. inclusion; **$p <0.01$ vs. inclusion.
nins (Mirtoselect®) obtained by extracting the full range of the non-anthocyanin components, and a highly purified anthocyanin-rich fraction. In a previous study, these two extracts were already evaluated for their biological effects in a variety of retinopathy conditions, and Mirtoselect® supplementation resulted superior than a generic bilberry extract (obtained by the dilution of an anthocyanins-rich fraction with maltodextrins) in ameliorating retinal circulatory parameters. The authors speculated that the non-anthocyanin fraction of Mirtoselect®, mainly composed of naturally present sugars and organic acids poorly represented in the generic bilberry extract (as evidenced by 1H-NMR-HPLC analysis), can explain this different biological effect. It is well described in the literature that the natural matrix of a standardized extract, apparently considered a non-active fraction, has on the contrary an important role in the onset and the modulation of the biological effects of an extract. To ensure their maximum effectiveness, commercial bil-

Figure 1. Plasma levels of anthocyanosides after oral administration of two different bilberry extract formulations.

Figure 2. Assessment of the plasma diacron-reactive oxygen metabolites (d-ROMs); *p<0.05.
Bilberry extracts should be carefully characterized for purity, analytical composition in active and non-active ingredients, and pharmacokinetic properties. The results here described showed that anthocyanosides are four times more available when orally administrated in rat as natural bilberry extract (Mirtoselect®) than as an enriched fraction. This is of particular relevance in the clinical setting, as several reconstructed bilberry based ingredients, titrated to 36% anthocyanins, are in reality obtained through the dilution of highly purified anthocyanosides with maltodextrins, thus completing losing the non-anthocyanins natural component. The increased pharmacokinetic properties of standardized bilberry extracts could be, on the contrary, ascribed to their whole components, as the non-anthocyanin fraction could contribute to enhancing the plasma content of anthocyanins besides exerting itself a biological effect. Similarly, in a recent study on Coptidis Rhizoma extract, the Authors indicated that proteinaceous nanoparticles contained in the whole extract were crucial for the biological activities of berberine (the major active compound), because they acted as natural drug carriers that increased berberine absorption. Previous *in vivo* studies...
documented that the plasma peak concentration of anthocyanins was 1.2 \( \mu \text{M} \) at 15 minutes after oral administration of 400 mg/kg bilberry extract (153.2 mg/kg as anthocyanins)\(^{19}\). In addition, the bioavailability of anthocyanins in those rats was 0.93%\(^{19}\). 30.8% and 13.4% of anthocyanins were recovered in urine and bile, respectively, during the first 4 hours following the intravenous administration of bilberry extract\(^{19}\). The bilberry extract, which showed the highest bioavailability in \textit{in vivo} pharmacokinetic studies, was then tested for its therapeutic relevance in DED. The standardized bilberry extracts (Mirtoselect\(^{6}\)) produced beneficial effects on the volume of tear secretion, an important parameter to evaluate DED symptoms measured by Schirmer’s test. As the right eye and mean value of both eyes in the bilberry extract group showed significant increase, it could be concluded that Mirtoselect\(^{6}\) administration significantly improved the tear flow parameter, differently from placebo. In the subset analysis, participants with higher tendency of dry eye showed greater improvement rate in Schirmer’s test in the bilberry extract group than placebo group. Consistently, in the bilberry extract group, participants with severe symptoms of dry eye showed greater improvement rate than those with mild symptoms. Therefore, although further and larger investigations are needed, Mirtoselect\(^{6}\) supplementation could be more effective in subjects with a higher tendency of dry eye. Bilberry extract also seemed to exert positive effects as antioxidant agent. In the bilberry extract group, no significant change was observed in d-ROMs, an indicator of oxidative stress, while BAP, an indicator of antioxidant potential, significantly increased during the study period. Modified BAP/d-ROMs ratio, an indicator of overall balance between antioxidant potential and oxidative stress, increased in the bilberry extract group (contrary to the placebo group), indicating that Mirtoselect\(^{6}\) could exert a protective action against reactive oxygen metabolites. The results of our study support the outcomes of previous \textit{in vitro}20, \textit{in vivo}\(^{21}\) and clinical studies\(^{22}\) on a potential relationship between dry eye and reactive oxygen species\(^{23}\).

**Conclusions**

The findings of our study indicated that natural, standardized bilberry extract (Mirtoselect\(^{6}\)) may improve tear secretion and enhance antioxidant potential. In particular, here we confirmed the association between increased antioxidant potential and anthocyanins, the main active components of bilberry extract. We also showed some pharmacokinetic properties, and consequently therapeutic effects, of bilberry extracts may be the result of the synergistic combination of anthocyanins with the other constituents, naturally present in the bilberry fruit. However, we are aware that our study lacks a complete pharmacokinetic profile of bilberry extracts, as well as the mechanism of action of bilberry extract on dry eye deserves to be deeply investigated in further, larger studies. As the volume of tear secretion measured in our study is only one aspect of dry eye, other indicators of symptoms such as tear film breakup time should also be examined.

**Acknowledgments**

We thank Sara Parodi, PhD, who provided medical writing services, supported by internal funds.

**Conflict of interest**

AR, ST, FF are employees of Indena S.p.A. LG is a consultant of Indena S.p.A.

**References**


Bilberry extract in dry eye


15) Savino G, Prabhawasat P, Koijima T, Grieterich M, Espa-


