European Review for Medical and Pharmacological Sciences 2016; 20: 2418-2424

Bilberry extracts are not created equal: the role of non anthocyanin fraction. Discovering the "dark side of the force" in a preliminary study

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Abstract. – OBJECTIVE: Several experimental studies and clinical trials support the potential of bilberry (*Vaccinium myrtillus* L) extracts in promoting eye health and circulation. Many active ingredients have been isolated from the berries and leaves of the bilberry plant. However, anthocyanins represent the most widely studied bioactive compounds in this plant.

PATIENTS AND METHODS: The aim of this registry, supplement study was to evaluate the effects of Mirtoselect® (standardized in 36% anthocyanins and obtained by an industrial extraction process that preserves the full range of the non-anthocyanin components, mainly natural sugars and polyphenols) in different types of retinal vasculopathies. In total, 140 patients with different types of retinopathy spontaneously decided to join one of the following groups: standard management (SM) only (n=38); SM associated with Mirtoselect® supplementation (n=47); SM associated with a generic bilberry extract supplementation (n=55). Retinal circulatory parameters and flow measurements of the retinal vessels were evaluated at the inclusion and after 6-months supplementation.

RESULTS: Overall, significant improvements in several retinal circulatory parameters such as retinal blood flow velocity, with respect to the values at inclusion, were observed in both supplementation groups, especially in Mirtoselect® supplementation group. However, at 6 months, inter-group comparison revealed a statistical advantage in all tested parameters for Mirtoselect® supplementation groups. No side effects or tolerability concerns were reported.

CONCLUSIONS: Our registry study suggests that Mirtoselect® supplementation could represent an effective and safe integrated approach for the treatment of different retinopathies.

Key Words:
Mirtoselect®, Anthocyanins, Retinopathy, Metabolomics, Bilberry, Vaccinium myrtillus, Phytoequivalent.

Introduction

Bilberry or European blueberry (*Vaccinium myrtillus* L.) belongs to the genus *Vaccinium*, which includes several small fruit species, like blueberries (e.g., *V. corymbosum*, *V. angustifolium*), cranberry (*V. macrocarpon*), and lingonberry (*V. vitis-idaea*). Bilberry grows wild in Europe and Asia, most abundantly in Northern and Eastern Europe, and at higher elevations in Southern Europe[1]. The bilberry is recognized by traditional medicine as an herbal remedy with interesting pharmacological activities. Besides its antioxidant and anti-inflammatory properties, current research efforts are mainly focused on bilberry-based strategies for the treatment of eyes and vascular disorders, and diabetes mellitus[2]. Several active ingredients have been isolated from the berries and leaves of the bilberry plant. These include flavonoids anthocyanosides, vitamins, sugars and pectins found in the berries, and quercetin, catechins, tannins, iridoids and phenolic acids found mainly in the leaves[2]. Among these ingredients, anthocyanins represent the most widely studied bioactive compounds in this plant. Anthocyanins (from the Greek *anthos* for flower and *kyanose* for blue) are flavonoids, water-soluble polyphenolic compounds, consisting of an anthocyanidin "core" with a sugar moiety attached (glucose, galactose, xylose, arabinose, or rhamnose) at various positions[3]. Anthocyanins vary also in the number and position of hydroxyl and methoxyl groups attached to the core of the anthocyanidin structure[3]. Therefore, although there are less than 20 naturally occurring anthocyanidins, there are many hundreds of different anthocyanins. Five main anthocyanidins are common in bilberry fruit extracts: cyanidin, delphinidin (these two compounds account for more than 60% of the total anthocyanin

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content), malvidin, peonidin, and petunidin. The glycoside forms of these anthocyanidins are mainly 3-glucosides, 3-galactosides and 3-arabinosides[4]. The amount of anthocyanins content in *Vaccinium myrtillus L.* berries generally ranges from 300 to 698 mg/100 g of fresh fruit. However, it varies with cultivar, growing conditions, and degree of ripeness of the berry[5].

According to the European Pharmacopoeia 8.0, the refined and standardized commercially dry extract of fresh bilberry fruit must contain 32.4 per cent to 39.6 percent of anthocyanins, assessed by High Performance Liquid Chromatography (HPLC) using specific cyanidin-3-glucoside chloride as absorbance reference value[6]. However, a decrease in anthocyanin content can occur when there has been an incorrect extract production and/or storage[7]. Recently, there have also been reported several cases of adulteration[8,9]. However, even among bilberry extracts standardized to guarantee a content of anthocyanins of 36%, significant differences exist in the manufacturing process, and hence in the composition of the non-anthocyanin fraction (mainly represented by natural sugars and organic acids).

In this study we aim at evaluating the biological relevance of this non-anthocyanin natural fraction of bilberry extracts, composed mainly of sugars and polyphenolic compounds, and accounting for the 64% of the total extract. To this purpose, we compared the well known protective effects on retinal circulation[10-13] of two typologies of standardized bilberry extracts obtained through different manufacturing processes: Mirtoselect® (standardized in 36% anthocyanins and obtained by extracting the full range of the non-anthocyanin components) and a generic bilberry extract standardized in 36% anthocyanins (produced by diluting a highly purified anthocyanins-rich extract with maltodextrins). In particular, we assessed retinal circulatory parameters and flow characteristics of the retinal vessels in subjects affected by retinal vasculopathy and supplemented with different standardized bilberry extracts.

Patients and Methods

Patients

This was an open-label, registry, supplement study (see[14-17], for a complete description of these studies), conducted in 140 individuals affected by different types of retinopathy (diabetics with non-proliferative retinopathy – level R1 and R2 in NHS-UK grading; diabetics with initial proliferative retinopathy – level R3 in NHS-UK grading; retinal post thrombosis; hypertensive retinopathy; glaucoma and ischemic retinopathy) not taking any vascular drugs for the retinal circulation in the 6 months before inclusion.

The retinal problems were further divided into a predominantly "hyperflow" retinopathy group that presented increased systolic and diastolic flow (patients with diabetic proliferative and non-proliferative retinopathy resulting in increased systolic and diastolic flows) and in a predominantly ischemic retinopathy group that presented decreased systolic and diastolic flow (patients with post-thrombotic, hypertensive, glaucoma and ischemic retinopathy, presenting a significant reduction in systolic and diastolic components at the central retinal artery).

Subjects who were pregnant, breastfeeding, or planning conception were excluded. Also, subjects with any degenerative eye disorder were excluded.

Cardiovascular examination indicated that patients had no significant systemic diseases (excluding the risk condition of diabetes mellitus and hypertension). Diabetics (not using insulin) and hypertensives (treated with single angiotensin-converting-enzyme inhibitors) were well compensated, with a body/mass index <25. All subjects were informed about the aim of the investigation and treatment procedure, according to the Declaration of Helsinki, and gave their consent.

Informed participants (n=140) freely decided to be enrolled in the following groups: standard management (SM) only (n=38); SM associated with Mirtoselect® supplementation (1 capsule/day, corresponding to 160 mg Mirtoselect®) (n=47); SM associated with generic bilberry extracts supplementation (1 capsule/day, corresponding to 160 mg of bilberry extract) (n=55). SM of retinopathies includes sodium chloride and caffeine restriction, physical exercise, regular habits recommendations (diet, sleep) and control of risk factors. The following parameters were evaluated before and at the end of the observational period: 1) edema; 2) capillary microaneurisms; 3) dot&blot retinal hemorrhage; 4) hard exudates; 5) cotton-wool spots (soft exudates); 6) arteriolar vasoconstriction; 7) arteriovenous crossing; 8) atherosclerosis, wall changes, copper wiring, silver wiring. Patient's visual acuity was assessed using the standard Snellen chart. The subjects were included if their Snellen score was at least 6/10. Eye flow, edema and blurring were al-

so evaluated and classified with an arbitrary scale (ranging from 0 to 3) based on clinical observation: 0 = normal; 1 = minimal alterations; 2 = important alterations; 3 = severe alterations.

Basic blood tests were also evaluated before and after the observational period.

Treatment Formulations

Mirtoselect® (Indena, Milan, Italy) is a bilberry dried extract formulation derived from *Vaccinium myrtillus* L. fresh frozen fruits.

A generic, commercially-available, bilberry extract, standardized at 36% anthocyanins, was purchased from the market.

These two bilberry extracts were formulated into similar appearance capsules, with the same content of anthocyanins (standardized in 36%, according to the European Pharmacopoeia 8.0). However, metabonomics data from nuclear magnetic resonances (NMR) and HPLC analyses (data not shown, under publication) revealed differences especially in sugar contents and maltodextrins, due to the use of these compounds as diluting agents in the generic bilberry extract. In fact, generic bilberry extract presented higher content of anthocyanidins (degradation products), fibers, maltodextrins and maltose, and lower content of fructose, glucose and organic acids.

Measurements of Ocular Blood Flow Velocity

High resolution color Doppler imaging (Preirus, Hitachi, Tokyo, Japan) was used to measure the peak systolic flow velocity and the diastolic flow velocity of the central retinal artery, as previously described[18]. In subjects with previous retinal thrombosis (diagnosed at least 6 months before the present study), the most affected eye was evaluated.

Statistical Analysis

A percentage of at least 25% female subjects were included in each group. Intra- and inter-group comparisons of numerical data were performed by Mann-Whitney U test and ANOVA test, respectively. Categorical data differences between groups were evaluated by Fisher's exact test. A p-value <0.05 was considered statistically significant. According to the previous evidence[19] on comparable groups of patients with retinopathy, at least 20 subjects were considered an adequate sample to define a difference in target outcomes at 6 months.

Results

The groups had similar demographics and clinical characteristics at inclusion (Table I).

Table II summarizes the main signs/symptoms associated with retinopathy observed before and at the end of the observational period. Overall, significant improvements in several retinal circulatory parameters, with respect to the values at inclusion, were observed in both supplementation groups, especially in Mirtoselect® supplementation group. On the other hand, no improvement was observed in the SM only group, except for edema. At 6 months, inter-group comparison revealed a statistical advantage in all parameters for Mirtoselect® supplementation groups compared both with the SM only group and with generic bilberry extract supplementation group. Objective evaluation using the Snellen chart showed no visual acuity improvement due to bilberry extract supplementations (Table III).

An analogue scale ranging from 0 to 3 was also established in order to evaluate objectively the clinical observations regarding flow, edema and blurring. Mirtoselect® supplementation exerted a beneficial

Table I. Details of subjects enrolled in the study.

	Standard Management	Standard Management + Mirtoselect®	Standard Management + generic bilberry extract
Diabetic non proliferative	10 (6)	12 (6)	9 (5)
Diabetic proliferative	7 (4)	8 (4)	11 (4)
Retinal post-thrombosis	6 (4)	8 (3)	12 (5)
Hypertensive retinopathy	7 (3)	10 (5)	11 (4)
Glaucoma	8 (5)	9 (4)	12 (6)
Total subjects	**38**	**47**	**55**
Age, years (mean ± SD)	44.5±2.2	44.3±1	44.1±2.2

SD: standard deviation

Table II. Evaluation of the main signs/symtoms associated with retinopathy observed before and at the end of the observational period.

	SM only (n=38)		SM+Mirtoselect® (n=47)		SM+generic bilberry extract (n=55)	
	inclusion	6 months	inclusion	6 months	inclusion	6 months
Edema	18	16+	19	12*#+	21	18+
Capillary microaneurisms	11	11	13	7*#	14	12
Dot&Blot retinal hemorrage	8	9	8	6*+	10	7*+
Hard exudates	7	7	9	2*#+	7	3*+
Soft exudates	8	9	11	4*#+	8	5
Arteriolar vasoconstriction	8	6	12	4*#+	11	6*+
Arteriovenous crossing	8	8	11	8*#+	10	9
Aterosclerosis	12	10	13	5*#+	12	8*+

SM = standard management. $^{*}p<0.0.5$ vs. SM; $^{\#}p<0.05$ vs. SM+generic bilberry extract; $^{+}p<0.05$ vs. inclusion

effect at 6 months in all the observed parameters, compared to both SM only and the generic bilberry extract supplementation, and also with respect to the values at the inclusion (Table III). Generic bilberry supplementation exerted a beneficial role in some parameters evaluated (Table III).

Non-invasive examinations also included high-resolution ultrasound color Doppler of the retina and retinal vessels. To this purpose, patients were further divided into two groups, hyperflow and ischemic, based on the flow pattern observed (Figure 1). Overall, supplementation with Mirtoselect® enhanced retinal blood flow. As shown in Table IV, systolic and diastolic flows significantly decreased in diabetic patients with proliferative and non-proliferative retinopathy (hyperflow group) supplemented with Mirtoselect®. On the other hand, Mirtoselect® supplementation induced significant systolic and diastolic flow reduction in patients with post-thrombotic, hypertensive, glaucoma and ischemic retinopathy (ischemic group) (Table IV). Neither safety and tolerability issues nor clinically relevant variations in the blood and physiological parameters were observed at inclusion and at the 6 months (data not shown). In diabetic subjects, fasting glucose and mean glycosylated hemoglobin at inclusion (103.3±8 and 6.85±0.5, respectively) and at the end of the study (105±7 and 6.75±0.6, respectively) resulted comparable. In patients with glaucoma, intraocular pressure (IOP) did not significantly change during the study (21.2±1.2 mmHg at inclusion vs. 21.1±2 mmHg at 6 months). Therefore, the improvement in retinopathy conditions observed in association with bilberry extract supplementation, particularly Mirtoselect® supplementation, seems to be independent from IOP and metabolic changes.

Discussion

This observational registry study provides further evidence on the efficacy of standardized bilberry extract in alleviating retinopathy-associated symptoms. Noteworthy, our study showed a remarkably different biological effect between Mirtoselect® (a naturally standardized bilberry extract, with 36% an-

Table III. Outcome of target test parameters investigated in this study.

	SM only		SM+Mirtoselect®		SM+generic bilberry extract	
	inclusion	6 months	inclusion	6 months	inclusion	6 months
Snellen Chart score	8/10	7/10	8/10	9/10	8/10	7/10
Flow (0-3 score)	2.2±0.2	2.1±0.2	2.2±0.2	1.8±0.2$^{*\#+}$	2.1±0.3	2.0±0.2
Edema (0-3 score)	1.7±0.5	1.7±0.1	1.8±0.5	1.1±0.3$^{*\#+}$	1.8±0.1	1.5±0.2^{*+}
Blurring (0-3 score)	2.1±0.2	2±0.2	2±0.4	1.2±0.3$^{*\#+}$	2.2±0.3	1.6±0.3^{*+}

Evaluation scale: 0: normal; 1: minimal alterations; 2: important alterations; 3: severe alterations Data are reported as mean ±standard deviation. $^{*}p<0.05$ vs. SM; $^{\#}p<0.05$ vs. SM+generic bilberry extract; $^{+}p<0.05$ vs. inclusion

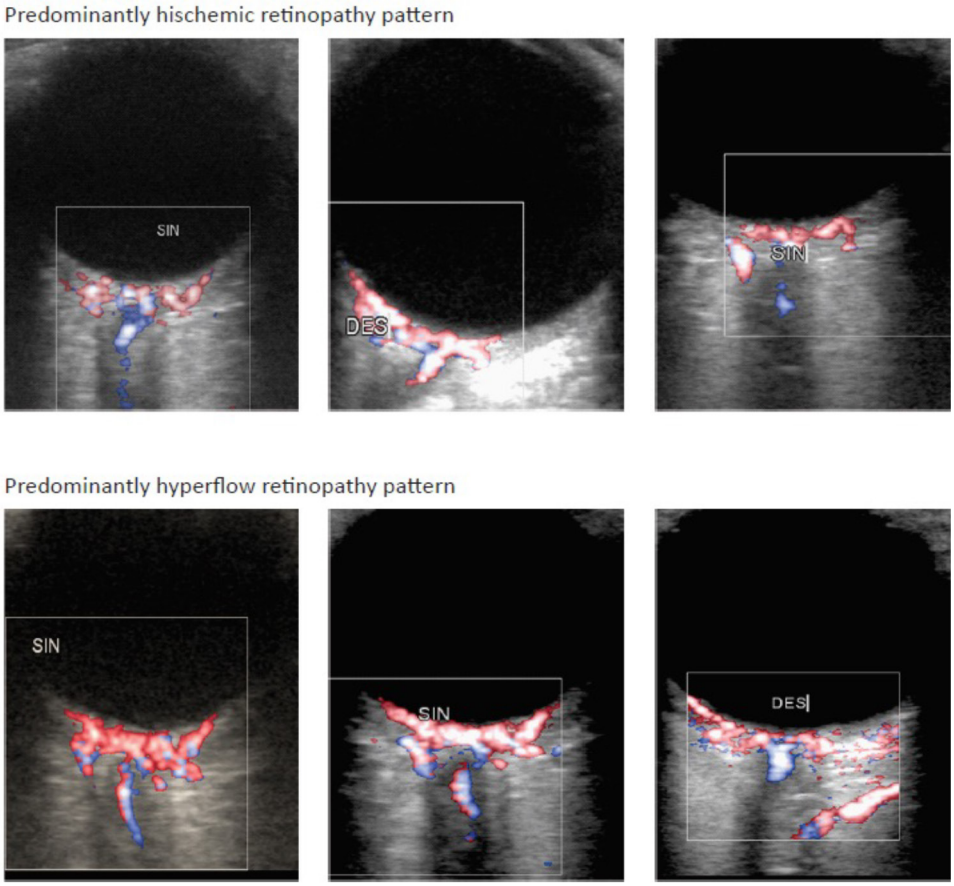

Figure 1. Orbital colour Doppler imaging. Left and right eyes color Doppler ultrasonography of the central retinal vein and artery at their emergence from the optic nerve showing predominantly hischemic retinopathy patterns in the top panel, and predominantly hyperflow retinopathy pattern in the bottom panel.

Table IV. Measurements of ocular blood flow velocity.

	SM only		SM+Mirtoselect®		SM+generic bilberry extract	
	inclusion	6 months	inclusion	6 months	inclusion	6 months
Hyperflow group	28±3/16±2	28±3/15±3	29±2/15±1	24±2/12±1*#+	28±2/17±2	28±3/16±2
Ischemic group	17±2/11±1	17±2/12±1	16±2/11±2	23±2/14±2*#+	17±2/10±1	19±2/11±1*+

Diastolic/systolic flow velocity. Data are expressed in cm/sec as mean ±standard deviation. *p<0.05 vs. SM; #p<0.05 vs. SM+generic bilberry extract; +p<0.05 vs. inclusion

thocyanins and the full range of non-anthocyanin components) and a commercially-available, generic bilberry extract (constituted by 36% anthocyanins with maltodextrins as carrier).

To achieve phytoequivalence, extract preparations must have the same metabolic profile, qualitatively and quantitatively[20]. However, identical chromatographic profiles may not assure true therapeutic equivalence. In our study, although, both Mirtoselect® and the generic bilberry extract contains 36% of anthocyanins, their "metabolic fingerprint" are different, especially in terms of sugars and maltodextrins composition, as evidenced by 1H-NMR-HPLC analysis. Moreover, compared to the generic bilberry extract, Mirtoselect® supplementation resulted in statistically significant and clinically relevant improvements in several retinal circulatory parameters. Therefore, commercially-available, generic bilberry extracts are not phytoequivalent to Mirtoselect®. We could

speculate that the remaining non-anthocyanin fraction of natural bilberry extract can explain these differences. In fact, the relevant (64% of the extract composition) non-anthocyanin component, resulted to be different in these two extracts, could markedly influence the biological effect or the gastrointestinal stability of the main bioactive anthocyanin component (accounting only for 36% of the composition).

The efficacy of most natural medicines lies in the synergy of diverse components rather than in a single compound. Many of the natural product extracts tested had yielded activities that later disappeared when the extracts were fractionated into individual chemical components[21]. Although our pilot study has a number of limitations (e.g., small size, non-homogeneous retinopathies), it is intriguing that the current specifications of the commercially-available bilberry extracts are not sufficient to explain by themselves the chemical and therapeutic differences we have observed. It is, therefore, necessary to develop some analytical methods capable of detecting all compounds simultaneously, thus providing a mean for standardizing and controlling the quality of herbal preparations based on the whole phytochemical composition, not just limited to some bioactive ingredients. Newly emerged metabolomic technologies such as 1H-NMR-HPLC, have the potential to define the phytoequivalence of extracts.

Conclusions

This preliminary registry, supplement study indicates that Mirtoselect® is effective in different types of retinal microangiopathy, although specific studies on selected retinal conditions are recommended. In fact, each type of retinopathy would need specific investigations to evaluate the long-term effect of Mirtoselect® also in regards to the prevention of the most severe possible complications (sudden loss of vision due to an acute vascular event, or progressive vision impairment). Moreover, chemical and therapeutic comparison with commercially-available, generic bilberry extract, highlights the need to apply modern analytical methods in defining the phytoequivalence of extracts.

Acknowledgements

We thank Sara Parodi, PhD, and Luca Giacomelli, PhD, who provided medical writing services, supported by Indena S.p.A.

Conflicts of interest

The authors declare no conflicts of interest.

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