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Committee on Herbal Medicinal Products (HMPC)

Assessment report on *Vaccinium myrtillus* L., *fructus*

Based on Article 16d(1), Article 16f and Article 16h of Directive 2001/83/EC as amended (traditional use)

Draft

Herbal substance(s) (binomial scientific name of the plant, including plant part)	*Vaccinium myrtillus* L., fructus siccus *Vaccinium myrtillus* L., fructus recens
Herbal preparation(s)	*Vaccinium myrtillus* L.), fructus siccus - comminuted *Vaccinium myrtillus* (L.), fructus recens - dry extract; DER 153-76:1; extraction solvent methanol 70% v/v
Pharmaceutical forms	• Myrtilli fructus siccus Herbal substance or comminuted herbal substance as herbal tea for oral use or for decoction preparation for oromucosal use • Myrtilli fructus recens *Herbal preparation in solid or liquid forms for oral use*
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Note: This draft assessment report is published to support the public consultation of the draft European Union herbal monographon *Vaccinium myrtillus* L., fructus siccus *and Vaccinium myrtillus* L., fructus recens. It is a working document, not yet edited, and shall be further developed after the release for consultation of the monograph. Interested parties are welcome to submit comments to the HMPC secretariat, which will be taken into consideration but no 'overview of comments received during the public consultation' will be prepared on comments that will be received on this assessment report. The publication of this draft assessment report has been agreed to facilitate the understanding by Interested Parties of the assessment that has been carried out so far and led to the preparation of the draft monograph.

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1. Introduction

1.1. Description of the herbal substance(s), herbal preparation(s) or combinations thereof

- Herbal substances

Vaccinium myrtillus (L.), fructus siccus (bilberry fruit dried): dried ripe fruit of *V. myrtillus* (L) (Eur. Pharmacopoeia 8.0 a). It contains minimum 1.0% of tannins expressed as pyrogallol.

Vaccinium myrtillus (L.), fructus recens (bilberry fruit fresh): fresh or frozen ripe fruit of *V. myrtillus* (L.) (Eur. Pharmacopoeia 8.0 b). It contains minimum 0.30% of antocyanins, expressed as cyaniding 3-O-glucoside chloride (chrysanthemin) (dried drug)

Bilberry (*V. myrtillus* L.) is a species of a shrubby perennial plant of the heather family (Ericaceae) reaching from 15 to 60 cm in height. It has many common names, including blueberry. It is widespread in Asia, Europe and North America in the areas with a temperate and arctic climate. The flowers, blooming from April to June, are pollinated by insects. The time from pollination to full ripeness of the fruit is about two months. The plant occurs from lowlands to high mountain positions (even above the tree line) and plant prefers strongly acidic soil in the pine forests, other coniferous forests, oak woods, beech forests and moors. The fruits are the black berries with a bluish, waxy bloom (Frohne, 2006) with sweet and slightly astringent taste (European Pharmacopoeia 8.0 a and b).

Dried fruits are traditionally used in therapy of digestive disorders, particularly in diarrhoea. The traditional use and some not controlled studies from 1960 and 1970 suggested the potential benefits of bilberry preparations for improvement of night vision, but more recent well-designed studies did not show any advantage (Canter and Ernst 2004).

The following substances were found in bilberry fruits:

Polyphenols

Flavan-3-ols and Proanthocyanidins

The amount is oppositely correlated with the degree of fruit ripening. Epicatechin-dimer B-2, catechin-dimer B-3, catechin-epicatechin-dimer B-1, catechin-epicatechin-dimer B-4, and other unidentified dimers or oligomers of procyanidins were found (Hansel *et al*., 1994).

Anthocyanosides

Anthocyanic compounds are present in plant cells in the form of glycosides (antocyanins). There are about 400 known anthocyanic glycosides. The most important anthocyanins are the cyanidin glycosides, as they represent 50% of the pigment composition of fruits (Kong *et al.*, 2003). Anthocyanins are present in ripe fruits of *Vaccinium* species but the highest total anthocyanin content occurs in the bilberry (*V. myrtillus* L.) (Kalt *et al.* 1999).

Total anthocyanin amount ranges from 300 to 700 mg per 100 g in ripe fruits of V. *myrtillus* (Prior *et al.* 1998; Prior and Cao 2000). There is a great diversity of the total content of anthocyanins in bilberry collected in various geographical areas, from 19.3 to 38.7 mg/g dry weight (Lätti *et al.*, 2008). Moreover, in concentrated bilberry extracts the total content of anthocyanins may amount to 24% (Zhang *et al.* 2004). According to European Pharmacopoeia 8.0 the standardized dry extract of *V. myrtillus* contains 32.4 per cent to 39.6 percent of anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride.

The most of researchers investigating anthocyanin composition in bilberries have reported mainly 14 or 15 anthocyanins (Lätti *et al.*, 2008; Yue and Xu, 2008). Fourteen anthocyanins have been identified in bilberry fruit, juice, and extract (1 – delphinidin 3-galactoside; 2 – delphinidin 3-glucoside; 3 – cyanidin 3-galactoside; 4 – delphinidin-3-arabinoside; 5 – cyanidin 3-glucoside; 6 – petunidin 3-galactoside; 7 – cyanidin 3-arabinoside; 8 – petunidin 3-glucoside; 9 – peonidin 3-galactoside; 10 – petunidin 3-arabinoside; 11 – peonidin 3-glucoside; 12 – malvidin 3-galactoside; 13 – malvidin 3-glucoside; 14 – malvidin 3-arabinoside).

During ripening process, there is an increase in the quantity of anthocyanidins in fruits. Usually the highest content of anthocyanins is found in berries collected late summer, at the end of August and the beginning of September. The levels are approximately 1.5-fold those of July. Late harvest collection and location further north have an impact on anthocyanin content. The amount of anthocyanins in the fruit increases as they ripen (Hansel *et al*.,1994; Morazzoni and Bombardelli, 1996; Upton *et al.,* 2001). Burdulis *et al.* (2007) have shown in their experiments that in all samples of bilberry fruits, cyanidin was found in the highest quantities subsequently followed by delphinidin, petunidin, peonidin and malvidin. Only in fruits harvested in Sweden, malvidin was more abundant (1.5-fold) than delphinidin and petunidin. The amount of malvidin was almost the same throughout the picking (0.016-0.017 μg/ml) (Burdulis *et al.* 2007).

Anthocyanins are pigments highly soluble in water and polar solvents. They are unstable and are oxidized under the influence of various factors (pH, temperature, enzymes, UV radiation, SO_2, ascorbic acid, metal ions), resulting in colour change and degradation (Rivas-Gonzalo, 2003). Processing and storage at low temperatures can improve the stability of anthocyanins (Delgado-Vargas *et al.*, 2003).

There is a great diversity of the total content of anthocyanins in bilberry collected in various geographical areas, from 19.3 to 38.7 mg/g dry weight (Lätti *et al.*, 2008). Delphinidin and cyanidin derivatives dominated in these samples (Lätti *et al*. 2008; Prior and Cao 1999; Bilberry Fruit Extract. SUMMARY OF DATA FOR CHEMICAL SELECTION 84082-34-8; Available at: http://ntp-server.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Bilberry_508.pdf)

In the study of Yue and Xu (2008) the thermal stability and degradation of the anthocyanins derivatives conjugated with a variety of sugars (delphinidin, cyanidin, petunidin, peonidin and malvidin) at the heating temperature 80, 100, and 125^{o}C were alike. However, when the heating temperature was increased to 125^{o}C, degradation of each compound increased sharply, with half-life to be less than eight minutes (Yue and Xu 2008).

Bilberry anthocyanin content was studied by Kähkönen *et al.*, 2003. Individual compounds were identified and quantified using HPLC and HPLC/ESI–MS techniques. The total anthocyanin content in the phenolic extracts of bilberry was 6000 mg $kg^{-1}$of fresh weight. There were 15 dominant compounds in bilberry (monoglycosides of cyanidin, delphinidin, malvidin, peonidin and petunidin) (Kähkönen *et al.*, 2003).

Stability of anthocyanins is affected by several environmental factors, particularly by thermal treatment. Interestingly, some acylated anthocyanins have an unusual stability in neutral or weakly acidic solutions. Packaging can also speed up the non-enzymatic browning and reduce the concentration of anthocyanins. Especially packaging in atmosphere of high CO_2 free amino acids are released due to tissue damage of fresh fruits and react with anthocyanins. Data from the analysis of solid forms (dry extracts) lead to the conclusion that they are degraded through an increase in ambient temperature (Patras *et al.* 2010; Yamamoto *et al.* 2013).

Flavonoids

The average amount contained in 100 g of fruits is 14 mg of flavonoid glycosides (Hansel *et al*.,1994). Since June flavonoids concentration decreases as fruits ripen. There are: apigenin, luteolin, chrysoriol, kaempferol, hyperoside, quercetin, quercitrin, isorhamnetin, myricetin, laricitrin, syringetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucuronide, avicularine, quercetin-3-*O*-rhamnoside, quertine-3-*O*-galactoside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-xyloside, 3-[[4-O-(4-carboxy-3-hydroxy-3-methyl-1-oxobutyl)-6-deoxy-α-L-mannopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-Benzopyran-4-one, 3-[[4-O-(4-carboxy-3-hydroxy-3-methyl-1-oxobutyl)-6-deoxy-α-L-mannopyranosyl]oxy]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-Benzopyran-4-one, isorhamnetin-3-*O*-galactoside, myricetin-3-*O*-glucuronide, myricetin-3-*O*-glucoside, myricetin-3-*O*-xyloside, laricitrin-3-*O*-glucuronide, isorhamnetin-3-*O*-glucoside, syringetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, myricetin-3-*O*-galactoside, isorhamnetin-3-*O*-xyloside, quercetin-3-*O*-rutinoside, astragalin (Laaksonen *et al*. 2010;Spela *et al*. 2011; Su 2012; Bilberry Fruit Extract. SUMMARY OF DATA FOR CHEMICAL SELECTION 84082-34-8; Available at: http://ntp-server.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Bilberry_508.pdf).

Alkaloids

The quinolizidine alkaloid myrtine was found. However it is not explained precisely by the authors of the publication, whether it comes from the fruit or the leaves (Slosse and Hootelé, 1978).

Iridoides

Asperuloside and monotropeine are found in immature fruits, in ripe fruits they are no longer detectable (Friedrich and Schonert 1973).

Tannins

Condensed and hydrolyzable tannins. Dried ripe fruit contains minimum 1.0 per cent of tannins, expressed as pyrogallol (Eur. Pharmacopoeia 8.0 a).

Triterpenes

Oleanolic acid, ursolic acid (0.25%) (Szakiel et al. 2012)

Organic acids

Chlorogenic, ferulic, syringic, caffeic, p-coumaric acids (Clifford 1999; Brenneisen and Steinegger 1981; Määttä-Riihinen *et al*. 2004).

Vitamins

In fresh fruits: Vitamin C, $B_{1,}$ panthotenic acid, nicotinamide (Hansel *et al*.,1994). **Other substances**

Aliphatic alcohols, aldehydes, ketones, terpene derivatives. For the distinctive aroma of fruits shall be responsible: *trans*-hexenal, ethyl 3-methyl butyrate and ethyl 2-methylbutyrate (Garcia 2008).

- Herbal preparation(s)

V. myrtillus (L.), fructus dry extract prepared from fresh bilberry fruit; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins (BEM - see section 2.1.1. Information about products on the market in the EU/EEA Member States).

Extracts from bilberry are usually refined to the range of 34 to 36% anthocyanosides, which corresponds to a content of 25% anthocyanidins (aglycons).

Table 1. Anthocyanin composition in bilberry raw extract (after Kähkönen *et al*. 2003)

Anthocyanin	% of total anthocyanins

Dephinidin-3-glu	13.7
Cyanidin-3-gal	9.0
Cyanidin-3-glu	8.5
Cyanidin-3-ara	13.6
Petunidin-3-glu	6.0
Peonidin-3-gal	0.6
Peonidin-3-ara	1.0
Malvidin-3-glu	8.4
Delphinidin-3-gal	14.9
Delphinidin-3-ara[a]	15.3
Petunidin-3-gal[b]	2.1
Petunidin-3-ara[b]	1.3
Peonidin-3-glu[c]	0.1
Malvidin-3-gal[d]	3.1
Malvidin-3-ara[d]	2.4
Total	100.0

Dp, delphinidin, Cn, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; gal, galactoside; glu, glucoside; ara, arabinoside; rut, rutinoside.
[a–d]Corresponding standards not available; quantified as [a]Dp-3-glu, [b]Pt-3-glu, [c]Pn-3-gal,[d]Mv-3-glu.

The amount of anthocyanin in many commercial preparations substantially varies fluctuating between 2.0 - 200 mg/g (Prior and Cao 2000).

Extracts are mainly prepared from the fresh bilberry fruits by a suitable procedure using ethanol (96 per cent *V/V*) or methanol (minimum 60 per cent *V/V*) at 10 - 60 C, diluted with water, filtered and afterwards concentrated and refined usually by means of ion-exchange chromatographic techniques (Upton *et al.* 2001; European Pharmacopoeia 8.0 c).

According to the European Pharmacopoeia 8.0 the refined and standardized dry extract of fresh bilberry fruit contains 32.4 per cent to 39.6 percent of anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride. The appropriate chromatogram for the assay of refined and standardized fresh bilberry fruit dry extracts contains: 1. Delphinidin 3-*O*-galactoside chloride, 2. Myrtillin (delphinidin 3-*O*-glucoside chloride), 3. Cyanidin 3-*O*-galactoside chloride, 4. Delphinidin 3-*O*-arabinoside chloride, 5. Chrysanthemin (cyanidin 3-*O*-arabinoside chloride, 6. Petunidin 3-*O*-galactoside chloride, 7. Cyanidin 3-*O*-arabinoside chloride, petunidin 3-*O*-glucoside chloride, 9. Delphinidin chloride, 10. Peonidin 3-*O*-galactoside chloride, 11. Petunidin 3-*O*-arabinoside chloride, 12. Peonidin 3-*O*-glucoside chloride, 13. Malvidin 3-*O*-galactoside chloride, 14. Peonidin 3-*O*-arabinoside chloride, 15. Malvidin 3-*O*-glucoside chloride, 16. Cyanidin chloride, 17. Malvidin 3-*O*-arabinoside chloride, 18. Petunidin chloride, 19. Peonidin chloride, 20. Malvidin chloride.

- Combinations of herbal substance(s) and/or herbal preparation(s) including a description of vitamin(s) and/or mineral(s) as ingredients of traditional combination herbal medicinal products assessed, where applicable.

Not applicable.

1.2. Search and assessment methodology

Databases assessed up to September 2013:

Science Direct, PubMed, Embase, Medline, Academic Search Complete, Toxnet

Search terms: *Vaccinium myrtillus*, bilberry, anthocyanins

2. Data on medicinal use

2.1. Information about products on the market

2.1.1. Information about products on the market in the EU/EEA Member States

Dry bilberry fruit has been present as single active ingredient in 115 herbal teas on the German market for more than 30 years, traditionally used for unspecific acute diarrhoea, mild inflammation of the oropharyngeal mucosa.

Comminuted dry bilberry fruit has been on the Polish market as single active ingredient for more than 30 years and it is also registered as a herbal tea for unspecific acute diarrhoea in Austria.

A bilberry methanolic dry extract prepared from fresh fruit (DER 153-76:1; extraction solvent methanol 70% v/v) containing 36% anthocyanosides, corresponding to 25% anthocyanidins has been on the Italian market for more than 30 years, at least since 1984, being the active substance of three medicinal products, in soft and hard capsules and as a granulate for oral solution.

Information on medicinal products marketed in the EU/EEA

Table 2 Overview of data obtained from marketed medicinal products

Active substance	Indication	Pharmaceutical form Strength (where relevant) Posology Duration of use	Regulatory Status (date, Member State)
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted	Unspecific acute diarrhoea	Herbal tea (1 tea bag contains 2.0 g Myrtilli fructus siccus, comminuted) Posology: 2 tea bags per cup of tea. 4-6 cups daily (= 16-24 g Myrtilli fructus) Duration of use: 2 days	Since 07/2009 Registration as traditional herbal medicinal product according to Directive 2004/24 Austria
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted	a) Traditionally used as adjuvant in unspecific acute diarrhoea b) Unspecific acute diarrhoea, mild inflammation of the oropharyngeal mucosa (German Standard Marketing Authorisations)	Herbal tea (1 tea bag contains 2.0 g Myrtilli fructus siccus, comminuted) Posology: adults and adolescents over 12 years 1 cup of tea from 2 tea sachets 4-6 times daily Use in children up to 2 years is contraindicated, use in children (3 to 11 years)	More than 30 years (Date of TU registration:13/10/2010) Germany

Active substance	Indication	Pharmaceutical form Strength (where relevant) Posology Duration of use	Regulatory Status (date, Member State)
		is not recommended. Use during pregnancy and lactation is not recommended due to insufficient data, even if the use as medicinal product and food has not shown risks.	
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted	Traditionally used as adjuvant in unspecific acute diarrhoea	Herbal tea Posology: 1 tablespoon (about 4 g) bilberry fruit pour to 1 cup of cold water, bring to a boil and cook, cover for about 10 minutes and strain. Drink 2 - 4 times a day 1/2 - 1 cup of brew.	More than 30 years (Date of TU registration:16/12/2010) as traditional herbal medicinal product according to Directive 2004/24 Poland
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted	Traditionally used as adjuvant in unspecific acute diarrhoea	Herbal tea (1 tea bag contains 2.0 g Myrtilli fructus siccus, comminuted) Posology: Adults and children over 12 years: 2 sachets (4 g) fruit pour to a cup (200 ml) of boiling water, infuse under cover or in a thermos for 10-20 minutes. Drink 1 glass of infusion 2 - 3 times a day. Always use fresh brew.	More than 30 years (Date of TU registration:16/12/2010) as traditional herbal medicinal product according to Directive 2004/24 Poland
V. myrtillus L., anthocyanosidic extract (no further detail available)	No data available in database	Dragee (20 mg of extract) Posology: No data available in database	From 1976 until 1978 as traditional medicinal product Belgium (lack of authorised products on the market)
V. myrtillus L., fructus recens dry extract; DER 153-76:1; extraction	Conditions of capillary fragility	Soft capsules Posology: 180 mg 3 times daily	Since 27/05/1985 Italy

Active substance	Indication	Pharmaceutical form Strength (where relevant) Posology Duration of use	Regulatory Status (date, Member State)
solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins		daily or according to medical prescription	
V. myrtillus L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins	Symptoms of venous insufficiency; conditions of capillary fragility	Hard capsules Posology: Adults: 80 mg up to 4 times daily or 160 mg up to 2 times daily or according to medical prescription.	Since 20/12/1984 Italy
V. myrtillus L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins	Symptoms of venous insufficiency; conditions of capillary fragility	Granules for oral solution Posology: Adults: 80 mg up to 4 times daily or 160 mg up to 2 times daily or according to medical prescription.	Since 1/10/1994 Italy

This overview is not exhaustive. It is provided for information only and reflects the situation at the time when it was established.

Information on relevant combination medicinal products marketed in the EU/EEA

Belgium

Pharmaceutical form> containing 100 mg *V. myrtillus* L., anthocyanosidic extract (no further detail available) + 5 mg beta-carotene (not authorized).

Indication: Capillary fragility, CVI, visual problems related to circulatory problems

Posology: 3 to 6 x 100 mg a day

On the market from 1965 to 1991.

Italy

Soft capsules containing 70 mg V. myrtillus L, fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins + 40 mg d,l-alfa-Tocoferil-acetato + 10 mg beta-carotene

Indication: Conditions of capillary fragility

Posology: 4 to 6 capsules a day or according to medical prescription

On the market since 1993.

Information on other products marketed in the EU/EEA (where relevant)

Five single active ingredient herbal teas on the Polish market as food supplements (for unspecific acute diarrhoea).

2.1.2. Information on products on the market outside the EU/EEA

Not applicable.

2.2. Information on documented medicinal use and historical data from literature

The use of *V. myrtillus* L., fructus has been included in the following handbooks:

Table 3: Overview of historical data

Herbal preparation	Documented Use / Traditional Use	Pharmaceutical form Strength (where relevant) Posology Duration of use	Reference
Dried bilberry fruit	Oral use: for supportive treatment of acute non-specific diarrhoea Topical use: topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral use: 20 -60 g/day of the comminuted dry fruit Topical use: decoction 10% Duration of use: No restriction	Schilcher 1992
Dried bilberry fruit	For supportive treatment of acute non-specific diarrhoea Topical use Topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral use: 20 -60 g/day of the comminuted dry fruit Topical use: decoction 10% Duration of use: No restriction	Wichtl, 1994
Dried bilberry fruit	Traditionally used in the treatment of diarrhoea, dysentery, haemorrhoids, gastrointestinal inflammations, mouth infection, scurvy and urinary complaints	Oral use: 20 -60 g/day of the comminuted dry fruit Topical use: decoction 10% Duration of use: No restriction	Barnes *et al.* 2002
Dried bilberry fruit	Oral Use: For supportive treatment of acute non-specific diarrhoea Topical use: Topical	Oral use: 20 -60 g/day of the comminuted dry fruit	Commission E monographs 1990

Herbal preparation	Documented Use / Traditional Use	Pharmaceutical form Strength (where relevant) Posology Duration of use	Reference
	treatment of mild inflammation of the mucous membranes of the mouth and throat	Topical use: decoction 10% and equivalent preparations Duration of use: No restriction	
Fresh or dried bilberry fruit Extract containing 25 to 36% anthocyanosides (no further detail)	Oral use: For supportive treatment of acute non-specific diarrhoea Topical use: topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral use: 20 -60 g/day of the unprocessed fruit Extract containing 25 to 36% anthocyanosides (in tablets or capsules):60-160 mg 3 times daily Topical use:decoction 10% Duration of use: No restriction	Gruenwald *et al*. 2000
Dried bilberry fruit	For supportive treatment of acute non-specific diarrhoea Topical use Topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral Use: 20 -60 g/day External use: a 10% decoction of dried bilberry fruit Duration of use: No restriction	ESCOP Monographs, 2003
Dried bilberry fruit	Oral Use: For supportive treatment of acute non-specific diarrhoea Topical use	Oral Use: 20 -60 g/day Topical use: a 10% decoction of dried bilberry fruit in gastrointestinal inflammations, mouth infections Duration of use: No restriction	Frohne 2006
Dried bilberry fruit	Traditionally used for the treatment diarrhoea, dysentery, gastrointestinal inflammation, haemorrhoids, gastrointestinal inflammations, mouth infection,	Oral Use: 10-15 g at a time I diarrhoea in adults and in children A 10% decoction of dried bilberry fruit in gastrointestinal inflammations	Zanetti-Ripamonti 1940

Herbal preparation	Documented Use / Traditional Use	Pharmaceutical form Strength (where relevant) Posology Duration of use	Reference
Dried or fresh fruit Liquid extract 1:1 (no further detail) Concentrated extract from fresh fruit with specific anthocyanin content (no further detail)	Peripheral vascular disorders of various origins.Traditionally used for the treatment diarrhoea, dysentery, gastrointestinal inflammation, haemorrhoids	Oral use: Dried or fresh fruit as a deconction (no further detail) Tablets providing 50 – 120 anthocyanins/day, equivalent to 20 – 50 g of fresh fruit, Liquid extract: 3 – 6 ml/day Topical use: decoction or extract (no further detail) Duration of use: No restriction	Mills and Bone 2000
Extracts containing 25 – 36% anthocyanins Dried bilberry fruits	Peripheral vascular disorders of various origins.	160-480 mg/day in 2 to 3 divided doses (40-120 mg/ anthocyanins Decoctions of dried bilberry fruits (no further information) Duration of use: No restriction	Rotblatt and Ziment 2002
Dried or fresh fruit (as an infusion) Liquid extract 1:1 (no further detail)	Peripheral vascular disorders, haemorrhoids	Oral use: Liquid extract 1:1 (3 – 6 ml/day) Tablets providing 50 – 120 mg anthocyanins/day, equivalent to 20 – 50 g of fresh fruit Topical use (no further information): Infusions of dried or fresh fruit or extracts Duration of use: No restriction	Capasso *et al*., 2003

2.3. Overall conclusions on medicinal use

Table 4: Overview of evidence on period of medicinal use

Herbal preparation Pharmaceutical form	Indication	Strength Posology	Period of medicinal use
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted Herbal tea	Traditionally used as adjuvant in unspecific acute diarrhoea	Posology: adults and adolescents over 12 years 4.0 g of Comminuted herbal substance in a cup as a herbal tea 4-6 times daily Duration of use: 2 days (Austria) Use in children up to 2 years is contraindicated; use in children (3 to 11 years) is not recommended (Germany). Use during pregnancy and lactation is not recommended due to the lack of adequate data (Germany).	More than 30 years in Germany (German Standard Zulassung -Date of TU registration:13/10/2010) Since 07/2009 in Austria (Registration as traditional herbal medicinal product according to Directive 2004/24)
V. myrtillus L., fructus siccus (dry bilberry fruit), comminuted Herbal tea	Traditionally used as adjuvant in unspecific acute diarrhoea	Posology: 4 g of comminuted herbal substance as a decoction (10 min) in 200 ml of water. 100 – 200 ml of decoction 2 - 4 times daily. Adults and adolescents over 12 years: 4 g of comminuted herbal substance as an infusion in 200 ml of boiling water 20 min), 2 - 3 times daily.	More than 30 years in Poland (Date of TU registration:16/12/2010 as traditional herbal medicinal product according to Directive 2004/24)

Herbal preparation Pharmaceutical form	Indication	Strength Posology	Period of medicinal use
		Always use fresh brew.	
V. myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral use: for supportive treatment of acute non-specific diarrhoea	Acute non-specific diarrhoea Mild inflammation of the mucous membranes of the mouth and throat Oral use: 20 -60 g/day of the comminuted dry fruit Topical use: decoction 10%	Schilcher 1992
V. myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral use: For supportive treatment of acute non-specific diarrhoea Topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral use: 20 -60 g/day of the comminuted dry fruit Topical use: decoction 10%	Wichtl, 1994
V myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Traditionally used in the treatment of diarrhoea, dysentery, haemorrhoids, gastrointestinal inflammations, mouth infection, scurvy and urinary complaints	Oral use: 20 -60 g/day of the comminuted dry fruit as a decoction 10%	Barnes, Anderson Philipson *et al*. 2002
V. myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral Use: For supportive treatment of acute non-specific diarrhoea Topical use: Topical treatment of mild inflammation of the mucous membranes of the	20 -60 g/day of the comminuted dry fruit Topical use: decoction 10% and equivalent preparations	Commission E monographs 1990

Herbal preparation Pharmaceutical form	Indication	Strength Posology	Period of medicinal use
	mouth and throat		
V. myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral use: For supportive treatment of acute non-specific diarrhoea Topical use: topical treatment of mild inflammation of the mucous membranes of the mouth and throat	Oral use: 20 -60 g/day of the comminuted dry fruit Topical use:decoction 10%	Gruenwald *et al*. 2000
V myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral use: For supportive treatment of acute non-specific diarrhoea	Oral Use: 20 -60 g/day	ESCOP Monographs, 2003
V myrtillus L., fructus siccus (dry bilberry fruit) Herbal tea	Oral use: For supportive treatment of acute non-specific diarrhoea Topical use	Oral Use: 20 -60 g/day Topical use: a 10% decoction of dried bilberry fruit in mild inflammation of the mucous membranes of the mouth and throat	Frohne 2006
Standardized bilberry extract containing 36% of anthocyanins	For treatment of problems related to varicose veins, such as painful and heavy legs	Oral Use 320- 480 mg/day; equivalent preparations Duration of use: No restriction	ESCOP Monographs, 2003
V. myrtillus L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins (BEM) Solid dosage forms (soft capsules)	Conditions of capillary fragility	Posology: Soft capsules 180 mg 3 times daily daily or according to medical prescription	Since 20/12/1984 in Italy
V myrtillus L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36%	Symptoms of venous insufficiency; conditions of	Posology: Hard capsules and Granules for oral solution	Hard capsules: Since 20/12/1984 in Italy Granules for oral solution:

Herbal preparation Pharmaceutical form	Indication	Strength Posology	Period of medicinal use
anthocyanosides, corresponding to 25% anthocyanidins (BEM) Solid dosage forms (hard capsules and granules for oral solution)	capillary fragility	Posology: Adults: 80 mg up to 4 times daily or 160 mg up to 2 times daily or according to medical prescription.	Since 1/10/1994 in Italy

Long-standing medicinal use for at least 30 years within the European Community, is therefore demonstrated for the following preparations and indications:

1) *V. myrtillus* L., fructus siccus (dry bilberry fruit), whole or comminuted, as herbal tea for oral use as an adjuvant in unspecific acute diarrhoea. Traditional medicinal use of this preparation is substantiated by extensive bibliography and the presence on the German and Polish market for more than 30 years. The daily dose in adults and adolescents over 12 years ranges from 15 to 60 g, divided in 3-4 single dose of 5 to 15 g in 250 ml as a 10 minutes decoction. (In Poland it is used also as an infusion (for 10 -20 min under cover): 4 g in 200 ml of boiling water, 2 - 3 times daily)

2) *V. myrtillus* L., fructus siccus (dry bilberry fruit), whole or comminuted, as a decoction for oromocosal use for the topical treatment of mild inflammation of the mucous membranes of the mouth and throat. Traditional medicinal use of this preparation is substantiated by extensive bibliography and the presence on the German and Polish market for more than 30 years. It is used as a 10% decoction to rinse the mouth several times daily.

2) *V myrtillus* L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins (BEM), in solid dosage forms for oral use for the treatment of symptoms of venous insufficiency and conditions of capillary fragility. Traditional medicinal use of this preparation is substantiated by the presence of medicinal products since 1984 in Italy. Single dose: 80 -160 mg; Daily dose: 160- 540 mg

3. Non-Clinical Data

3.1. Overview of available pharmacological data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

3.1.1. Primary pharmacodynamics

Bilberry extract (BE)

Vasoactive properties

***In vitro* Experiments**

Bilberry extract

Effect of the *V. myrtillus* fresh fruits extract, DER 153-76:1, extraction solvent methanol 70% v/v containing 36% anthocyanosides (corresponding to 25% of anthocyanidines) (BEM) on the venous smooth muscles to contraction response to the 5-HT was investigated *in vitro* by Bettini *et al.* (1984a).

The study was performed on isolated thoracic vein calf preparations. *V. myrtillus* extract (25% of anthocyanosides) (25 – 100 μg/ml) alone caused a moderate decrease in tension as the response to the contractions induced by 5-HT (0.5 – 1 μg/ml). The effect was more pronounced after addition of ascorbic acid (1 – 4 μg/ml). The relaxation effect was nullified or highly decreased by the pre-treatment with indomethacin (1 μg/ml) or lysine acetylsalicylate (1 μg/ml) (Bettini *et al.* 1984a).

Contractility of the segments of internal thoracic vein calf preparations induced by barium chloride (50 μg/ml) was reduced by *V. myrtillus* extract concentration dependently (25, 50, 75, 100 μg/ml). Indomethacin (1 – 30 μg/ml) and lysine acetylsalicylate (1 – 30 μg/ml) reduced or completely suppressed the reduction of the venous muscle tone produced by BEM (Bettini *et al*. 1984 c).

In other experiment the influence of BEM on contractility of the smooth muscles of the calf splenic arteries segments induced by 5-HT was investigated. *V. myrtillus* extract (25, 50, 75, 100 μg/ml) alone caused a concentration dependent decrease in tension of the arterial muscles as the response to the contractions induced by 5-HT (0.2 μg/ml). The effect was potentiated after addition of ascorbic acid (1 – 4 μg/ml). Indomethacin (1 - 30 μg/ml) and lysine acetylsalicylate (1 – 30 μg/ml) decreased the reduction of the arterial muscle tone produced by BEM. The results obtained by the authors indicate that the mechanism of the vasodilatating effect of BEM on vascular muscles is based on the local synthesis of prostacyclin (Bettini *et al.* 1984 b).

Effect of the extract of BEM on contractility of the calf isolated coronary artery preparations was tested *in vitro* by Bettini *et al.* (1985a). Contractions induced by barium chloride (50 μg/ml) were concentration dependently mildly suppressed by BEM (25, 50, 75, 100 μg/ml). The effect was more pronounced after addition of ascorbic acid (1 – 4 μg/ml). Relaxation induced by BEM was concentration dependently reduced with indomethacin (1 – 30 μg/ml) or lysine acetylsalicylate (1 – 30 μg/ml) (Bettini *et al.* 1985a).

Bettini *et al.* (1985b) reported the influence of BEM on potentiation of activity of adrenaline on the isolated calf coronary vessels. Adrenaline (0.2 μg/ml) vasodilating activity was concentration dependently increased by BEM (25, 50, 75, 100 μg/ml) (Table 5).

Table 5. Mean percentage increase (± S.D.) in the response of the preparation of the calf coronary artery to adrenalin (0.2 μg/ml) in the presence of BEM (after Bettini *et al.* (1985b)

Concentration of the *V. myrtillus* extract (μg/ml)	**N**	**Mean percentage increase in the response to adrenalin ± S.D.**
25	20	38 ±1.7
50	20	77 ± 1.3
75	20	140 ± 2.1
100	20	193 ± 2.5

The potentiating effect was completely abolished in the presence of pyrogallol (50 μg/ml) a catechol-o-methyl transferase (COMT) inhibitor. The authors conclude, that the vasodilating mechanism of BEM results from COMT inhibition (Bettini *et al.* 1985b).

In another study Bettini *et al.* (1991) investigated contractile responses of the isolated calf coronary vessels to acetylcholine (ACh) and methylene blue. Experiments were carried out without removal of the endothelium. BEM (50 – 200 μg/ml) decreased both the tone and in higher concentrations the contractile response of the preparations to ACh (0.001 – 1 μg/ml). A small decrease of the tone was observed with BEM alone (50 – 200 μg/ml) and the effect was more marked with addition of the

ascorbic acid (100 – 300). Indomethacin (40 μg/ml) and lysine acetylsalicylate (40 μg/ml) reduced or completely suppressed the reduction of the venous muscle tone produced by BEM. Pre-treatment with methylene blue (24 μg/ml) resulted in the partial reduction of the vasodilatatory effect of BEM. The authors concluded, that the vasodilator effect of BEM is related to the release of prostaglandins and to a facilitation of the endothelium-derived relaxing factor (EDRF) release, as the methylene blue is known to block the release of EDRF (Bettini *et al.* 1991).

Continuing research of Bettini *et al.* (1993) found after use of BEM (5- 100 μg/ml) a significant increase in contractility of endothelium-deprived isolated calf coronary arteries induced by ACh administration (0.001 – 1 μg/ml). The potentiating effect was completely suppressed by methylene blue (24 μg/ml) or haemoglobin (0.015 – 0.020 μg/ml) which block the release of EDRF (Bettini *et al.* 1993).

Influence of fresh fruits BEM on the capillary fragility has been studied in the model of rats deprived of dietary flavonoids (Cristoni and Magistretti 1987). Wistar rats were fed for 3 weeks on the diet devoid of flavonoids. On depilated skin the capillary resistance - the lowest negative pressure on the skin that induces petechiae was defined by use of the vacuum gauge. Immediately after the BEM corresponding to 25% anthocyanidins was administered by intraperitoneal injection and the capillary fragility was estimated again after 2, 4 and 6 hours (Table 6).

Table 6. Activity of BEM on the capillary resistance of rats fed on a flavonoid-devoid diet. (after Cristoni and Magistretti 1987)

Substance	Dose mg/kg i.p.	No of animals	Capillary resistance in cm Hg (M± S.E.			
			Baseline	2 hours	4 hours	6 hours
Controls		12	15.83 ± 0.32	16.66 ± 0.45 (+5)	17.08 ± 0.58 (+8)	16.50 ± 0.42 (+4)
Bilberry extract	200	9	15.55 ±	25.44 ± 2.28 (**) (+64)	27.44 ± 2.04 (**) (+77)	25.55 ± 2.30 (**) (+64)

The direct vasorelaxating activity of a lyophilized dry BE (no further detail) was tested *in vitro* by Bell and Gochenaur (2006) on coronary arterial rings isolated from pigs. BE contained 15 different anthocyanins including cyanidin, peonidin, delphinidin, petunidin, and malvidin. The total anthocyanin composition was 12.1 g/100 g and total phenolics 35.7 g/100 g. BE produced dose- and endothelium-dependent vasorelaxation in isolated rings with endothelium (% maximal relaxation at 5 mg/l total anthocyanins: 59 ± 10). The authors tested the role of nitric oxide (NO) in these relaxations and found that such relaxation could be abolished by the application of 100 μM NO_2-L-arginine (*Nitric Oxide Complex* with *L-arginine*). These observations suggest that the endothelial NO system may be involved in the relaxation response of coronary arteries to the BE. At a concentration too low to directly alter coronary vascular tone, they did not alter coronary responses to endogenous or exogenous NO. However, this same low concentration (≅100 nM) had a considerable potential to prevent loss of endothelial-dependent relaxation caused by exposure of arteries to exogenous ROS (*Reactive Oxygen Species*) as pyrogallol. As anthocyanins are absorbed intact across the gastrointestinal tract and such concentration roughly reflects that seen in several studies to exist even in human plasma after oral consumption of these products they may have vasoprotective properties (Bell and Gochenaur 2006).

Mechanism of vasodilatation induced by bilberry anthocyanins was investigated by Ziberna *et al.* (2013). Vascular reactivity was assessed in thoracic aortic rings obtained from male Wistar rats. The endothelium was preserved in the rings. Pre-treatment of aortic rings with anti-sequence bilitranslocase antibodies targeting the endothelial plasma membrane carrier, that transports flavonoids, resulted in decrease of vasodilatation induced by cyanidin 3-glucoside and bilberry anthocyanins.

For experiments a purified methanolic BE (no further detail) was used. Anthocyanins composition was analyzed by HPLC-DAD method: 14.3% of delphinidin 3-galactoside, 14.0% of delphinidin 3-glucoside, 9.2% of cyanidin 3-galactoside, 12.1% of delphinidin 3-arabinoside, 10.1% of cyanidin 3-glucoside, 4.0% of petunidin 3-galactoside, 7.7% of cyanidin 3-arabinoside, 8.8% of petunidin 3- glucoside, 1.1% of peonidin 3-galactoside, 2.6% of petunidin 3-arabinoside, 3.7% of peonidin 3-glucoside, 2.5% of malvidin 3-galactoside; 0.5% of peonidin 3-arabinoside, 7.9% of malvidin 3-glucoside, 1.5% of malvidin 3-arabinoside. Concentration of total anthocyanins in the BE was expressed in mg/l as equivalents of cyanidin 3-glucoside (no further detail given). In vascular reactivity experiments concentration-relaxation curves to cyanidin 3-glucoside (1 nmol/l - 10 mmol/l) and bilberry anthocyanins (0.01 - 20 mg/l expressed as equivalents of cyanidin 3-glucoside) were constructed. Vasorelaxations were examined before the 30-min incubation in each of the tested solutions, and after incubation followed by 30-min equilibration periods to avoid ACh tolerance.

Pre-treatment of aortic rings with anti-sequence bilitranslocase antibodies targeting the endothelial plasma membrane carrier, that transports flavonoids, resulted in decrease of vasodilatation induced by cyanidin 3-glucoside and bilberry anthocyanins.

Anthocyanins are especially rich in bilberries, consisting on the average 80 - 90% of total phenolic compounds. Anthocyanins are substrates of bilitranslocase, suggesting that they greatly contribute to the endothelium-dependent vasodilatory effect. In presented experiments, observed vasodilatation activity was in the range from 10 nM on, thereby in the range of reported pharmacokinetic post-absorption plasma concentration (Ziberna *et al.* 2013).

Anti-inflammatory activity

In vitro Experiments

Bilberry extract

Triebel *et al.* (2012) studied the influence of a lyophilized BE (no further detail) and comprising anthocyanins on pro-inflammatory genes in IFN-γ/IL-1β/TNF-stimulated human colon epithelial cells (T84) by real-time polymerase chain reaction (qRT-PCR) and cytokine activity. Analysis of the extract showed the following composition (Table 7). Fifteen anthocyanins were detected in the BE by HPLC-DAD analysis, the most numerous being del-3-gal, del-3-glc, del-3-ara, cy-3-gal, and cy-3-glc, at concentrations (mg/g extract) which also shows the concentrations (µM) of the substances in 25 µg/ml BE extract (used in incubations with the cultured cells).

Table 7. Anthocyanin concentrations in the BE and corresponding initial concentrations in *in vitro* incubations with 25 µg/ml extract (after Triebel *et al.* 2012)

Anthocyanin	Concentration in BE[a] (mg/g)	Incubation concentration[b] (µM)
delphinidin-3-glc	46.8	2.5
Cyanidin-3-glc	46.2	2.6
delphinidin-3-ara	37.1	2.1
Cyanidin-3-gal	27.8	1.5

delphinidin-3-gal	27.2	1.5
petunidin-3-glc	18.6	1.0
Cyanidin-3-ara	18.2	1.1
Malvidin-3-glc	14.5	0.7
petunidin-3-gal	9.7	0.5
petunidin-3-ara	8.6	0.5
Peonidin-3-glc	8.0	0.4
Malvidin-3-ara	5.9	0.3
Malvidin-3-gal	5.0	0.3
Peonidin-3-gal	1.6	0.1
Peonidin-3-ara	0.9	0.1
Total amount	276.1	15.1

[a]According to the manufacturer's specifications.
[b]For an *in vitro* incubation with 25 µg/ml extract.

The authors studied the expression of inflammatory bowel diseases-associated pro-inflammatory marker genes (TNF-α, IP-10, IL-8) in the cultures of the human colon epithelial cells (T84) by quantitative real-time PCR. The cytotoxic effects of BE and the singular anthocyanins/anthocyanidins on T84 cells were determined using a resazurin reduction assay. Selected cytokines and chemokines were analyzed using the "Human Cytokine Array Panel A" antibody array.

After 4 and 24 h, 250 µg/ml BE reduced viability to 80 ± 5 and 60 ± 3% and 200 µM cyanidin reduced it to 83 ± 2 and 78 ± 3%, whereas 200 µM delphinidin reduced it to 79 ± 1 and 63 ± 2%, respectively. All investigated anthocyanins had only slight cytotoxic effects at 200 µM (cell viability > 95%) after 4 and 24 h of incubation.

Cytotoxic effects of the anthocyanidins were stronger than the corresponding anthocyanins declining in the order delphinidin > cyanidin > pelargonidin >peonidin > malvidin so that increasing with increases in hydroxylation.

BE significantly and dose-dependently inhibited expression of the pro-inflammatory marker genes TNF-α and IP-10 in CM stimulated T84 cells, at concentrations of 2.5 and 25 µg/ml, respectively.

Influence of chosen anthocyanins on expression of TNF-α, IL-8, IP-10 genes in T84 stimulated cells depended on both the aglycone and of sugar residues. IP-10 expression was significantly inhibited by cyanidin-3-ara, the most potent inhibitor, cyanidin-3-glc at 25 µM (the lowest concentration tested), and cyanidin-3-gal at 50 µM. Peonidin-3-*O*-glycosides were active concentration dependently only as glucose conjugates in the tested concentrations (25, 50, 100 µM). The investigation of activity of the corresponding anthocyanidins have shown that cyanidin, delphinidin, and petunidin significantly down-regulated IP-10 mRNA expression, but peonidin or malvidin did not (even at 100 µM). Cyanidin significantly reduced TNF-α transcript levels at ≥50 µM, but peonidin or malvidin did not have any effect. Pre-treatment with cyanidin (25 µM) and BE (25 µg/ml) completely inhibited synthesis of interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell alpha chemo-attractant (I-TAC), and soluble intercellular adhesion molecule 1 (sICAM-1).

Authors conclude that single anthocyanins from BE modulates inflammatory genes and protein secretion *in vitro* and thus may act as transcription-based inhibitors of the pro-inflammatory gene expression associated with inflammatory bowel diseases. Moreover, the anti-inflammatory activity of the investigated anthocyanins is strongly dependent on their aglycone structure and the attached sugar moieties (Triebel *et al.* 2012).

In the study of Song *et al.* (2010), a newly established human corneal limbal epithelial cell line (HCLEC) was investigated to study the effects of a BE on the cell growth, cell cycle and the expression of hyaluronic acid (HA) and glycosaminoglycans (GAGs) in corneal epithelial cells. A commercially available BE containing 25% total anthocyanins (no further detail) was used. The content of

anthocyanins present in BE was quantified by HPLC using cyanidin-3-O-glucoside as external standard. The cells were incubated with different concentrations of BE for 24 h and 48 h.

BE (10^{-5}M) promoted cell growth to about 120% compared with the control group ($p < 0.05$) after 24 h incubation while three concentrations (10^{-6}, $10^{-5}$and 10^{-4}m) were effective in increasing cell viability to 112.9%, 130.1% and 113.8%, respectively, ($p < 0.05$) after 48 h incubation. For the HA and GAGs assay, HCLEC cells were incubated with different concentrations of BE. The GAGs content in the supernatant of the cells increased significantly after incubation with BE for 48 h, but the increase was not dose-dependent. Two separate concentrations (10^{-7} and 10^{-4} M) significantly induced the secretion of GAGs ($p < 0.05$), while no significant changes were observed for the expression of hyaluronic acid. The results show that BE may be advantageous for the physiological recovery and homeostasis of corneal epithelial cells (Song *et al.* 2010).

Isolated compounds

***In vitro* Experiments**

Hou *et al.* (2005) investigated cyclooxygenase-2 (COX-2) inhibiting activity of five anthocyanidins. Only delphinidin and cyanidin inhibited lipopolysaccharide (LPS)-induced COX-2 expression in the culture of macrophage RAW264 cells. The significant dose dependent inhibition was present at the concentrations of 50, 75 and 100 μM, but peonidin and malvidin did not. Delphinidin was the most potent on mRNA and protein level. Delphinidin suppressed LPS-mediated COX-2 expression by blocking mitogen-activated protein kinase (MAPK) pathways with the attendant activation of nuclear factor-kB (NF-κB), CCAAT/enhancer-binding protein (C/EBPδ) and activator protein-1 (AP-1) (Hou *et al.* 2005).

Microcirculation injury

***In vivo* Experiments**

Bilberry extract

In another study Bertuglia *et al.* (1995) tested activity of the fresh fruits BEM (100 mg per day/kg p.o. for 2 and 4 weeks) in the microcirculation ischemia model. Ischemia was induced by clamping the hamster cheek pouch for 30 min with subsequent reperfusion also lasting 30 minutes. Changes in the microcirculation were visualized by fluorescence method. Ischemia and reperfusion were associated with increased number of leukocytes sticking to venules, decreased number of perfused capillaries and increased permeability.

After treatment there was a significant reduction in ischemic symptoms ($p<0.01$, Table 8) (Bertuglia *et al.* 1995).

Table 8. Number of sticking leukocytes (L per 100 μm venules), percentage of perfused capillary length (capillary perfusion), permeability increase (normalized grey levels) in Control (Con) and in hamsters treated with BEM for 2 (A) and 4 (B) weeks after ischemia reperfusion (Bertuglia *et al*. 1995).

	Con	A	B
L *n* per 100 μm ven	11.5 ± 1.8	5.5 ± 0.3*	4.5 ± 0.5*
Capillary perfusion	45 ± 12%	70 ± 7%*	88 ± 6%*
Grey levels			
Ischemia	0.35 ± 0.1	0.23 ± 0.10*	0.09 ± 0.06*†

Reperfusion	0.55 ± 0.07	0.35 ± 0.12*	0.19 ± 0.04*†

*p<0.01, compared to controls, †p<0.05 relative to bilberry A group.

Table 9: Overview of the main non-clinical data/conclusions

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
V. myrtillus fresh fruits extract (BEM) (25% of anthocyanosides)	25 - 100 μg/ml	*In vitro* Isolated thoracic vein calf preparation	Bettini *et al.* 1984 a	5-HT (0.5 – 1 μg/ml). induced contractions Moderately decreased by BEM. More pronounced effect addition of ascorbic acid (1 – 4 μg/ml). BEM induced relaxation nullified or decreased by pre-treatment with indomethacin (1 – 30 μg/ml) or lysine acetylsalicylate (1 – 30 μg/ml)
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	25, 50, 75, 100 μg/ml	*In vitro* Internal thoracic vein calf preparation	Bettini *et al.* 1984 c	Barium chloride (50 μg/ml) induced contractions dependently reduced by BEM. BEM induced muscle tone reduction decreased or suppressed by indomethacin (1 – 30 μg/ml) or lysine acetylsalicylate

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
				(1 – 30 μg/ml)
V. myrtillus fresh fruits extract (BM (corresponding to 25% of anthocyanosides)	25, 50, 75, 100 μg/ml	*In vitro* Smooth muscles of the calf splenic arteries segments	Bettini *et al.* 1984 b	5-HT (0.2 μg/ml). Effect potentiated after ascorbic acid addition (1 – 4 μg/ml). BEM produced reduction of the arterial muscle tone concentration dependently decreased with indomethacin (1 – 30 μg/ml) or lysine acetylsalicylate (1 – 30 μg/ml)
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	25, 50, 75, 100 μg/ml	*In vitro* Isolated calf coronary artery	Bettini *et al.* 1985 a	Barium chloride (50 μg/ml) induced contractions dependently mildly suppressed by BEM. More pronounced effect after addition of ascorbic acid (1 – 4 μg/ml). BEM induced relaxation concentration dependently reduced with indomethacin (1

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
				– 30 μg/ml) or lysine acetylsalicylate (1 – 30 μg/ml)
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	25, 50, 75, 100 μg/ml	*In vitro* Isolated calf coronary vessels	Bettini *et al.* 1985 b	Adrenaline (0.2 μg/ml) vasodilating activity concentration dependently increased by BEM
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	50, 100, 150, 200 μg/ml	*In vitro*	Bettini *et al.* 1991	Methylene blue (24 μg/ml) partially reduced vasodilator effect of BEM (50 -200 μg/ml). Indomethacin (40 μg/ml) and lysine acetylsalicylate (40 μg/ml) significantly reduced venous muscle tone produced by BEM.
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	10 – 50, 100 μg/ml	*In vitro*	Bettini *et al.* 1993	BEM increased the metacholine-induced relaxation of coronary arteries. This effect was inhibited by methylene blue and haemoglobin.

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	10, 25, 50, 100, 200 mg/kg	*In vivo*	Cristoni and Magistretti 1987	Significant antiulcer activity of BEM in different experimentall models of gastric ulcer (pyloric ligature, reserpine, phenylbutazone , acetic acid, restraint). The influence of the metabolism of the mucopolisaccharides is proposed as the mechanism of healing.
V. myrtillus lyophilized extract (containing 25% anthocyanins - no further detail)	25, 100 μg/ml	*In vitro*	Triebel *et al.* 2012	BE and comprising anthocyanins induced significant inhibition of the expression aand secretion of the inflammatory mediators (TNFα/ IP-10, I-AC, sICAM-1, GRO-α) in the stimulated human colon epithelial cells (T84).
V. myrtillus extract (containing 25% total anthocyanins - no further detail)	Concentrations equalized to the content of 10^{-9} – 10^{-4} M of cyanidin-3-*0*-glucoside	*In vitro*	Song *et al.* 2010	After 48^{h} of incubation BE (10^{-6}, 10^{-5}, 10^{-4} M) increased corneal cell viability . The

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
				number of cells decresed in G_o/G_1 phase and significantly increased in S and G_2/M phases after treatment of the high concentration of BE (10^{-4}). The expression of glycosaminoglycans after incubation for 48^h ($10^{-7,}$ 10^{-4} M) also significantly increased.
V. myrtillus fresh fruits extract (BEM) (corresponding to 25% of anthocyanosides)	100 mg/kg, oral	*In vivo*	Bertuglia *et al.*, 1995	BEM inhibited vasoconstriction and reduced the number of adhering leukocytes to venous vessel walls and inhibited permeability after capillary reperfusion with preservation of endothelium.
V. myrtillus lyophilized extract (12,1% of anthocyanins – 35,7% total phenolic costituents)	5 mg / L	*In vitro*	Bell and Gochenaur 2006	BE produced endothelium dependent relaxation of the isolated porcine coronary arterial rings

Herbal preparation tested	Strength Dosage Route of administration	Experimental model *In vivo*/ *In vitro*	Reference Year of publication	Main non-clinical conclusions
Purified methanolic *V. myrtillus* extract (no further detail - specific anthocyanins composition analyzed) Antocyanins expressed as equivalents of cyanidin -3-*0*-glucoside	0.01 – 20 mg / L	*In vitro*	Ziberna *et al.* 2013	Vasodilatation of isolated aortic rings induced by bilberry anthocyanins was inhibited by anti-sequence bilitranslocase antibodies targeting plasma membrane transporter.
Single substances: Different anthocyanidins	25 -100 µM of delphinidin for 30 min	*In vitro*	Hou *et al.* 2005	Out of five anthocyanidins only delphinidin and cyanidin inhibited COX_2 expression in LPS activated murine macrophage RAW264 cells, but pelargonidin, peonidin and malvidin did not induced any effect.

A large amount of data from preclinical studies on the beneficial effects of anthocyanins on the regeneration of rhodopsin exist. Such studies, among others, were carried out by Matsumoto *et al.* 2003; Tirupula *et al.* 2009; Yanamala *et al.* 2009. Circadian clock regulation of the pH in the retina of vertebrates shows the pH increase upon exposure to light.

It appeared that the pH of the retina is more alkaline during the day. This may be important because the Authors realized, that the pH of the environment for the various activities for anthocyanins plays an important role. Their concentration in the tissues of the eye is very low (Manach *et al.*, 2005; Ichiyanagi *et al.* 2004a, 2004b; Matsumoto *et al.* 2003).
As believed by Kalt *et al.* (2010) other phenolic flavonoids may play an important role in the survival of the cultured human retinal pigment epithelial cells and regeneration of rhodopsin. These are such as flavanone eriodictyol (Johnson *et al.* 2009, Maher and Haneken 2005, 2008) or baicalein, luteolin, galangin, fisetin and quercetin.

3.1.2 . Secondary Pharmacodynamics

Impact on lipid peroxidation

***In vitro* Experiments**

Bilberry extract

The comprehensive and extensive monograph by Upton *et al.* (2001) showed that an extract of bilberries protected microsomes from rat liver against oxidative damage and apolipoprotein B before brought about by UV radiation.

An anthocyanoside complex extract from *V. myrtillus* was tested for its ability to inhibit lipid peroxidation and to scavenge hydroxyl and superoxide radicals (Martín-Aragón *et al.* 1997; 1999). An antiperoxidative action of this *V. myrtillus* extract was assayed by the Fe 3^+ -ADP/NADPH method in rat liver microsomes. Superoxide anions were generated by preparing a mixture of hypoxanthine and xanthine oxidase. The results were expressed as percentage inhibition of cytochrome C reduction (Upton *et al.* 2001).

Antioxidant activity

In *in vitro* studies conducted by Cluzel *et al.*, (Cluzel *et al.* 1969; Cluzel *et al.* 1970) it was found that anthocyanins of *V. myrtillus* affected the activity of various enzymes of retina in the pig and in the rabbit (inhibiting the activity of phosphoglucomutase, and increasing the activity of lactate dehydrogenase, α-hydroxybutyrate dehydrogenase, 6-phosphogluconate dehydrogenase and α-glycerophosphate dehydrogenase). However, in these studies, the authors used a complex formulation consisting, beyond of an extract of bilberry, of other components, including beta-carotene. Therefore the significant effect of beta-carotene contained in the preparation in large quantities cannot be excluded.

It was found, that the extract scavenged superoxide anion and inhibited microsomal lipid peroxidation at all concentrations (25, 50 75 and 100 µg/ml) ($p<0.01$) and a 50% inhibition of rate of reaction was observed with a final concentration of 25 µg/ml. The anthocyanoside complex extract was able to inhibit lipid peroxidation (IC_{50} = 50.28 mg/ml) and to scavenge superoxide anion (IC_{50} < 25 mg/ml). The ability to remove hydroxyl radical exerted by this extract was detectable from 50 mg/ml of extract in the reaction mixture (Martin-Aragon 1998). According to Prior *et al.* (1998), comparison of the antioxidant capacity variety of *Vaccinium* species have shown high activity of *V. myrtillus* (Table 10):

Table 10. Antioxidant activity of *V. myrtillus* L. (after Prior *et al*. 1998).

***V. myrtillus* L.**					
Cultivar, state, and source	**ORAC ROO [a] µmol/g)**	**Anthocyanin [b] (mg/100 g)**	**Phenolics [c] (mg/100 g)**	**A/P [d] (mg/mg)**	**Ascorbate (mg/100 g)**
Bilberry	44.6 ± 2.3 (282.3)	299.6 ± 12.9	525.0 ± 5.0	0.571	1.3 ± 0.1

[a]Expressed as micromole Trolox equivalents per gram of fresh fruit. Oxygen radical absorbance capacity (ORAC ROO). Data in parentheses expressed per gram of dry matter. Bilberry was harvested on 7/2/97.
[b]Concentration based upon cyanidin-3-glucoside as standard.
[c]Concentration based upon gallic acid as standard.
[d]Anthocyanin/phenolics.
Bilberries were extracted with acetonitrile/acetic acid for the analysis of ORAC, total anthocyanins, and total phenolics.

In conclusion the increased maturity at harvest increased the ORAC, the anthocyanin, and the total phenolic content. A linear relationship existed between ORAC and anthocyanin (r_{xy}=0.77) or total phenolic (r_{xy} = 0.92) content (Prior *et al.* 1998).

Direct *in vitro* influence of the bilberry fruit extracts on the oxidative phosphorylation of isolated rat heart mitochondria was tested by Trumbeckaitè *et al.* (2013). For testing two types of extracts were used: the hydroethanolic extract (BEE) of the crushed plant material was prepared by maceration with 50% ethanol at room temperature (1:10, v/v), initially for 48 h and thereafter until exhaustion; the aqueous extract (BAE) was prepared using repercolation method (1:10, v/v). The obtained hydroethanolic extract was filtered and concentrated under vacuum (at 50^{o}C) and then subjected to freeze drying. Freeze dried bilberry powder was packed into a glass jar and dissolved before experiments. The levels of anthocyanidins, measured by use of HPLC, varied in the two extracts (see Table 11)

Table 11. Anthocyanidins in the two extracts tested by Trumbeckaitè *et al.* (2013).

Amount of anthocyanidins (ng/ml) in 1 µl) of bilberry fruit extracts						
Bilberry extract	**Delphinidin**	**Cyanidin**	**Petunidin**	**Peonidin**	**Malvidin**	**Total**
BAE	0.14±0.1	0.36±0.05	0.22±0.2	0.15±0.3	0.31±0.1	1.18±0.3
BEE	0.18±0.2	0.80±0.1	0.26±0.1	0.19±0.2	0.28±0.3	1.71±0.2

BAE, bilberry aqueous fruit extract; BEE, bilberry ethanolic fruit extract.

When measured the effects of BEs on complex I-dependent substrate pyruvate plus malate oxidation, mitochondrial respiratory rates only in the presence of 5–30 ml/1.5 ml of the BAE extract the mitochondrial state 3 respiration rate decreased from 33% to 61% ($p < 0.05$). BEE induced the decrease in state 3 respiration rate also starting from 5 ml/1.5 ml. High doses of BEE (15–30 ml/1.5 ml) induced a decrease in the state 3 respiration rate by 35%–56%, that is similar to BAE. In effect at higher concentrations, BAE and BEE induced significant uncoupling of oxidative phosphorylation and decrease in the state 3 respiration rate. The true mechanism of the diminishing of the state 3 respiration rate by BEs may be the inhibition of mitochondrial respiratory chain at complexes I and II.

Pure anthocyanins, the main components of used extracts, malvidin-3-glucoside, malvidin-3-galactoside, and cyanidin-3-galactoside, had no effect on oxidation of pyruvate plus malate. A statistically significant decrease in H_2O_2 production by mitochondria was found in the presence of bilberry fruit extracts. BAE at concentrations of 1.5 and 15 µl/1.5 ml clearly suppressed this process and caused a 46% and 62% reduction, respectively, in the H_2O_2 generation as compared with that in the absence of BAE.

Similar effects (reduction by 50%) were obtained by BEE (15 ml/1.5 ml), whereas lower amounts of BEE (1.5 ml/1.5 ml) suppressed H_2O_2 generation by 16%, that is, less than BAE.

The results revealed that the effect of BAE and BEE on mitochondrial function is bivalent: lower concentrations (they correspond to 6–9 mg/l of total anthocyanins) had no effect on mitochondria, whereas at high concentrations (they would correspond to 18–52 mg/l of total anthocyanins), the extracts caused an obvious decrease in the state 3 respiration, but the radical scavenging activity remained increased. The effects of BAE and BEE on mitochondria were dose dependent (Trumbeckaitè *et al.* 2013).

A BE from dried fruits containing anthocyanins (25.0%, w/w) (no further detail) reduced UVA-induced oxidative stress in keratinocytes (Svobodova *et al.* 2008). 1) In the first experiment keratinocytes grown in culture medium were pre-treated with the BE (5–100 mg/l) in serum free medium at 37°C for 1 h, irradiated and incubated in serum-free medium at 37°C for another 4 h. 2). In the second experiment keratinocytes were irradiated and after UVA exposure the BE (5–100 mg/l) in the serum-free medium was added to the cells for 4 h. The effect of extract in the concentration range of 1–250 mg/l, various UVA doses (10–40 J/cm^2) or combinations of the extract and UVA on keratinocytes cell viability was assessed after 4/24 h. Pre-treatment (1 h) or post-treatment (4 h) of keratinocytes with the BE resulted in attenuation of UVA-caused damage. Viability of the cells was determined photometrically. BE at the concentrations tested (1–250 mg/l) did not affect incorporation of water-soluble dyes into lysosomes, but decreased lactate dehydrogenase (LDH) activity in medium samples containing 100 and 250 mg/l of the extract after 24 h. The last finding evaluates activity a cytosolic enzyme, which reflects cell membrane integrity. Moreover application of the extract significantly reduced UVA-stimulated ROS (*Reactive Oxygen Species*) formation in keratinocytes: the maximal decline in ROS generation was at concentrations of 50 and 100 mg/l of the extract. Administration of BE also prevented/reduced UVA-caused peroxidation of membrane lipids: the maximal protection was observed in pre-treatment at a concentration of 50 mg/l (over 90%), post-treatment with the extract also markedly inhibited membrane lipid damage with maximum at concentrations of 25 and 50 mg/l (75–80%). The extract also induced depletion of intracellular GSH: pre-treatment with the extract significantly protected against UVA caused GSH depletion, especially at concentrations of 25 and 50 mg/l (55%). Post-treatment was the most effective in the concentration range of 50–100 mg/l (50%) (Svobodova *et al.* 2008).

In the other experiments of the same group HaCaT keratinocytes were used to assess the effects of pre-and post-treatment with BE phenolic fractions (5–50 mg/l) on keratinocyte damage induced UV radiation by a solar simulator (295–315 nm) (Svobodowa *et al.* 2009). For the assessment of UVB (photo) protective potency of phenolic fractions non-toxic concentrations (5, 10, 25 and 50 mg/l) of BE were used. BE efficiently reduced the extent of DNA breakage (especially at concentrations of 25 and 10 mg/l) together with caspase-3 and -9 activity. The effect of post-treatment on caspase-3 activity was similar for all concentrations tested. Pre-treatment of keratinocytes with BE also reduced caspase-9 activity. BE effect was concentration-dependent (maximal protection of 87%). BE was the most potent at a concentration of 5–10 mg/l (around 80%), which slightly decreased in higher concentrations.

Application of the extract before UVB exposure significantly prevented DNA fragmentation. BE effectiveness culminated at a concentration of 10 mg/l (70%) and at higher concentrations the protection diminished to 50% and 40% at a concentration of 25 mg/l.

The phenolic fraction of *Vaccinium myrtilli* berries significantly decreased generation of reactive oxygen and nitrogen species (RONS), of oxidizing lipids, proteins and DNA. Application of the BE (4 h) to non-irradiated HaCaT slightly reduced RONS generation compared to untreated non irradiated cells. The effectiveness of the extract showed 40% protection at the highest concentration. Supreme RONS elimination was found in post-treated cells. At concentrations of 25 and 50 mg/l phenolic fractions reduced RONS amount to control level.

The extract decreased IL-6 production in irradiated cells, when it was applied before UVB exposure. The effect of BE was concentration-dependent with maximal protection 35%. The maximal potency was found at the highest concentration approximately 33% (Svobodowa *et al.* 2009).

Antioxidant activity of bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) was examined at the cellular level in different cell lines: human colon cancer (Caco-2), human hepatocarcinoma (HepG2), human endothelial (EA.hy926) and rat vascular smooth muscle (A7r5). The

bilberry crude methanolic extract was further purified in order to obtain the anthocyanin fraction: [(crude BE: phenolic acids, proanthocyanidins, flavanols, flavonols) →(purified bilberry extract BE: anthocyanin fraction)]. Anthocyanins had intracellular antioxidant activity if applied at very low concentrations (<1 µg/l; nM range) (Table 12). Delphinidin and cyanidin glycosides were the predominant anthocyanins in BEs, whereas malvidin glycosides dominated in the blueberry extract (Bornsek *et al.* 2012).

Table 12. Half maximal effective concentrations (EC 50) of the extracts in 4 cell lines (after Bornsek *et al.* 2012)

Parameter	Cell line	Crude blueberry extract	Crude bilberry extract	Purified bilberry Extract
EC 50 (µg/l)	Caco-2	0.78 ± 0.15[a]	0.29 ± 0.02[b]	0.53 ± 0.04[ab]
	HepG2	0.88 ± 0.10[a]	0.59 ± 0.05[b]	0.63 ± 0.03[ab]
	Ea.hy926	0.17 ± 0.02[a]	0.22 ± 0.02[a]	0.59 ± 0.06[b]
	A7r5	5.99 ± 0.81[a]	0.36 ± 0.02[b]	1.38 ± 0.10[b]

Data were expressed as mean ± SEM, number of independent measurements was n = 6. Statistical analysis was performed using one-way ANOVA with post-Bonferroni test. Statistically significant differences ($p < 0.05$) are marked with letters ([a,b,c]) in the same row.

The effective concentrations achievable after oral administration (Mazza *et al.*, 2002; Felgines *et al.*, 2008) are in the range of plasma anthocyanin concentrations in the presented experiments showing cellular antioxidant activity at very low concentrations in different human cell lines. Such values in the range of 1nM are attained after consumption of ordinary servings of berries (McGhie and Walton 2007).

Cytoprotective effect of a fresh fruits BE against oxidative damage in primary cultures of rat hepatocytes was studied by Valentová *et al.* (2007). The BE analysed by HPLC contained 25.0% of total anthocyanins. Activity of BE against oxidative cell damage induced by tert-butyl hydroperoxide and allyl alcohol in primary cultures of rat hepatocytes was investigated. The hepatocyte monolayers were incubated with the tested extract for 4, 24 and 48 h and the viability of the cells was assessed by the MTT test. In the concentrations tested (100 and 500 µg/ml), no significant toxicity was registered.

The extract showed significant dose-dependent protective activity against oxidative damage in rat hepatocytes primary cultures induced by tert-butyl hydroperoxide and allyl alcohol. Maximum cytoprotection (58.16%) was noted in the culture pre-incubated with 500 µg/ml of the extract (Table 13) (Valentová *et al.* 2007).

Table 13. Protective effect of the bilberry extract on tert-butyl hydro peroxide induced damage of rat hepatocytes primary cultures (after Valentova *et al.* 2007)

	Non-treated	*t*BH	*t*BH + BE 500 µg/ml
MTT ($A_{540\ nm}$)	1.13 ± 0.01	0.22 ± 0.01[a]	0.71 ± 0.04[b]
LDH (µkat/l)	7.74 ± 0.25	29.8 ± 1.6[a]	18.7 ± 1.8[b]
TBARS (µmol/l)	1.29 ± 0.07	4.87 ± 0.15[a]	2.94 ± 0.4[b]

After 30 min of pre-incubation with the BE, the cell monolayers were treated with tert-butyl hydroperoxide (tBH, 0.5 mmol/l) during 1.5 h. Results are expressed as mean ± SD, n = 9.

[a] $P < 0.01$ vs. non-treated cells.

[b] $P < 0.01$ vs. tBH-treated cells.

Antiradical activity was evaluated spectrophotometrically as the ability of the tested substances to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The extract has shown scavenging activity; 50%

inhibition was achieved at 3.99 ± 0.14 µg/ml (IC_{50}). In the same experimental condition, IC_{50} of the synthetic analogue of vitamin E, trolox, was 2.15 ± 0.06 µg/ml (8.57 ± 0.25 µmol/l).

Estimation of the antioxidant activity of the BE was also investigated in the xanthine/XOD superoxide generating system. The BE scavenged the superoxide radical and its activity was equivalent to 108 ± 7.2 units of SOD per mg of extract. In the same system, trolox had an activity equivalent to 16.4 ± 0.19 units of SOD/mg (Valentova *et al*. 2007).

Ogawa *et al.* (2011) studied the lipid peroxidation and free radical scavenging activity of a BE (containing more than 25% anthocyanosides – no futher detail) in murine stomach tissue homogenates. BE in the concentration dependent range significantly induced decrease of activity of the lipid peroxide levels and revealed strong scavenging activity against superoxide and hydroxyl radicals (Table 14; Table 15).

Table 14. Superoxide anion radical scavenging activity of the bilberry extract and its main anthocyanidins (delphinidin, cyanidin and malvidin) (after Ogawa *et al.* 2011)

Compound	IC_{50}			
	µg/ml		µM	
Bilberry extract	1.2	(1.0-1.5)		
Delphinidin	1.2	(0.9-1.6)	3.5	(2.5-4.7)
Cyanidin	31.8	(21.8-50.9)	98.4	(67.5-157.8)
Malvidin	1.0	(0.7-1.4)	2.8	(2.0-3.8)
Trolox	130.8	(113.4-154.0)	522.4	(453.1-615.1)

IC_{50}, 50% inhibitory concentration. The parentheses show 95% confidence limits

Table 15. Hydroxyl radical scavenging activity of the bilberry extract and its main anthocyanidins (delphinidin, cyaniding and malvidin) (after Ogawa *et al*. 2011)

Compound	IC_{50}			
	µg/ml		µM	
Bilberry extract	116	(80-192)		
Delphinidin	237	(203-305)	0.7	(0.6-0.9)
Cyanidin	>323	>1.0		
Malvidin	>367	>1.0		
Trolox	325	(275-400)	1.3	(1.1-1.6)

IC_{50}, 50% inhibitory concentration. The parentheses show 95% confidence limits.

Isolated compounds

Different berry phenolics, *V myrtillus* included, and their antioxidant activity were investigated by Kähkönen *et al.* (2001). Extraction methods for berries and apples were examined to create phenolic extracts with high antioxidant activity. Evaluation of antioxidant activity was performed by auto-oxidazing methyl linoleate (40 °C, in the dark). The extraction method affected prominently both the phenolic composition and the antioxidant activity (Table 16). However, from the observation of the results obtained the effects cannot be clearly related with the content of phenolic of the individual subgroups (Kähkönen *et al.* 2001).

Table 16. Anthocyanin, flavonol, hydroxycinnamic acid (HCA), hydroxybenzoic acid (HBA), ellagitannin, flavanol and procyanidin, and total phenolic contents (data expressed as mg per 100 g of weight), and antioxidant activity (data expressed as inhibition percentage) of bilberry extracts produced using different extraction methods[a]) (after Kähkönen *et al.* (2001).

Extraction method	anthocyanin[b]	flavonol[c]	HCA[d]	HBA[e]	ellagitannin[f]	flavanol[g]	total phenolics[h]	inh % raw[i]	inh % after SPE[j]
methanol, 60%	2023 ± 6a	62 ± 4a	203 ±4ab	3.1 ± 0.2a	ND	13 ± 1a	3057 ± 55a	46 ± 10ab	93 ± 2ab
acetone, 70%	2387 ± 30b	54 ± 2a	228 ± 10a	3.9 ± 0.1a	ND	7.1 ± 0.2b	3343 ± 84a	55 ± 4a	96 ± 2b
H_2O	944 ± 30c	18 ± 1b	133 ± 4c	1.0 ± 0.0b	ND	8.0 ± 0.1b	1110 ± 21b	38 ± 1c	92 ± 4a
Refluxing	721 ± 20d	30 ± 1b	168 ± 1bc	5.8 ± 0.1c	ND	10 ± 1c	2925 ± 40a	48 ± 8bc	92 ± 4
Hexane	6.0 ± 0.4e	ND	0.2 ± 0.0d	ND	ND	ND	ND	6 ± 5d	NA

[a]Means (SD of duplicate assays. Values in the same column for each berry having the same letter are not significantly different at P < 0.05. ND, not detected. NA, not analyzed.
[b]Concentration based upon cyanidin-3-glucoside as standard.
[c]Concentration based upon rutin as standard.
[d]Concentration based upon chlorogenic acid as standard.
[e]Concentration based upon gallic acid as standard.
Concentration based as ellagic acid as standard.
[g]Concentration based as (+)-catechin as standard.
[h]Concentration based upon gallic acid as standard.
[i]Inhibition of methyl linoleate hydroperoxide formation after 72 h of incubation at the concentration of 500 ppm of dry raw extract.
[j]Inhibition of methyl linoleate hydroperoxide formation after 72 h of incubation at the concentration of 500 ppm of dry extract after sugar removal with SPE.

The antioxidant activity of phenolics (at concentrations of 1.4, 4.2, and 8.4 µg of purified extracts/ml of liposome sample) such as anthocyanins, ellagitannins, and proanthocyanidins from bilberry was studied by Viljanen *et al.* (2004) in a lactalbumin-liposome system. Phenolic profile of BE determined using an analytical HPLC method is shown in Table 17.

The extent of protein oxidation was measured by determining the loss of tryptophan fluorescence and formation of protein carbonyl compounds and that of lipid oxidation by conjugated diene hydroperoxides and hexanal analyses (Table 17).

Table 17. Inhibition of lipid and protein oxidation (after 6 days of oxidation) by bilberry phenolics incorporated into lactalbumin-lecithin liposomes (percent inhibition, Mean ± SD) (after Viljanen *et al.* 2004)

Bilberry extract	Conjugated diene hydroperoxides			Hexanal		
	1.4 µg/ml	**4.2 µg/ml**	**8.4**	**1.4 µg/ml**	**4.2 µg/ml**	**8.4 µg/ml**
	19.4 ± 2.6[b]	**-13.1 ± 0.3[c]**	**66.5 ± 1.5[a]**	**38.8 ± 0.5[b]**	**57.3 ± 3.4[a]**	**98.4 ± 0.1[a]**
	Tryptophan fluorescence			**Carbonyl gain**		
	22.9 ± 0.5[a]	**6.1 ± 0.2[a]**	**27.9 ± 0.6[b]**	**67.6 ± 0.1[b]**	**49.1 ± 0.1[a]**	**79.9 ± 0.3[a]**

[a]SD, standard deviation. Negative values indicate pro-oxidant activity. Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$).

Bilberry phenolics exhibited good overall antioxidant activity toward protein oxidation. The antioxidant effect toward lipid oxidation was more pronounced than the effect on protein oxidation (Table 18) (Viljanen *et al.* 2004).

Table 18. Phenolic profiles of bilberry extract (expressed as percent of total phenolics measured using HPLC; ND, not detected) (after Viljanen *et al.* 2004)

Extract	Anthocyanins	Flavonols[b]	OH-C[c]	OH-B[d]	ProAs[e]	ET[f]	EA[g]	mg phenolic compounds/ g dry weight
Bilberry	94.8	0.9	4.2	0.1	ND	ND	ND	572.8

Anthocyanins; amount based upon cyanidin-3-glucoside as standard.
[b](+)-Catechin as standard.
[c]Chlorogenic acid as standard.
[d]Gallic acid as standard.
[e]ProAs, proanthocyanidins; flavan-3-ol as standard
[f]ET, ellagitannins; ellagic acid as standard.
[g]EA, ellagic acid.

Antimicrobial activity

It has long been known that several phenolic substances such as flavonoids, phenolic acids, tannins and lignans have antimicrobial activity (Heinonen 2007). It is believed that it is the flavonoid anthocyanins component in *V. myrtillus* that exerts such an effect. The mechanism of antimicrobial activity may include antiadhesion activity, destruction of the cytoplasmic phospholipid bilayer of the cell wall in microbes, damage of the outer membrane with disintegration of the liposaccharide (LPS) layer by phenolics, tannins complexation of metal ions and inhibition of plasma coagulation by bacteria. Another mechanism is the inhibition of antibacterial multidrug resistance (MDR) and impairment of the efflux pump activity in bacteria (Puupponen-Pimiä *et al.* 2005a, 2005b, 2005c).

Bilberry extract

Rauha *et al.* (2000) evaluated the antimicrobial activity of a number of plants, including bilberry. To the *in vitro* studies, an aqueous solution of the dry extract prepared from the dry plant material (acetone/methanol 70% V/V – no further detail) was used to determine the diameter of the inhibition zones in the agar cultures of bacteria. Clear antimicrobial effect has been found for the BE (500 µg samples) against the *Micrococcus luteus* (inhibition zone (i.z.) of sample= 3 - 4 mm > i.z. of methanol and slight antimicrobial activity against *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli* and *Candida albicans* (i. z. of sample = 1 - 3 mm) > i.z. of methanol (Rauha *et al.* 2000).

The antimicrobial activity of many plants, including *V. myrtillus* extract (acetone-water 70:30 V/V; elution with MeOH) prepared from fresh frozen berries was screened against the human pathogenic microbial strains on agar plates to estimate their growth and adherence of the bacterial cells to a berry material. The BE (1 mg/ml) revealed the death of the culture of *Helicobacter pylori*, very strong inhibition of growth of *Bacillus cereus* and strong inhibition of growth of *Clostridium perfringens* and *Staphylococcus aureus* (Nohynek *et al.* 2006).

Binding of *Neisseria meningitidis* pili to *V. myrtillus* berries and juice polyphenolic fractions containing anthocyanins, proanthocyanidins and flavonols have been identified by Toivanen *et al.* (2009; 2011). Prevention of adhesion of pathogenic bacteria to host cell surfaces may constitute the protection from the activity of bacteria that use adhesins to colonize the host cells.

Activity of bilberry against Gram positive and Gram negative intestinal pathogens was examined in *in vitro* cultures of *Salmonella*, *Staphylococcus*, *Listeria* and *Lactobacillus* bacteria (Puupponen-Pimiä *et al.* 2005a; 2005b). The BEs (water/ethyl acetate/methanol) prepared from fresh frozen berries containing phenolic acids and fractions eluted with methanol (ellagitannins and anthocyanins) were tested. BE (2 mg/ml) inhibited the growth of *Staphylococcus aureus* for 12 and 24 hours (5 x 101 - 5 x 102) and *Salmonella enterica Typhimurium* (10- 5 x 101). BE fractions (10 mg/ml) exhibited stronger inhibition against *Staphylococcus aureus* (>5 x 104) for 12 and 24 hours compared with control. Stronger inhibition of growth was also seen against *Salmonella enterica Typhimurium* (5 x 102 - 5 x 103) compared with control.

Influence of various preparations of BE prepared from fresh berries, on trophozoites of *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum oocysts* was examined in *in vitro* experiments by Anthony *et al.* (2007, 2011). The water soluble extracts of bilberry containing polyphenols (167 µg/ml of gallic acid equivalents) killed 90.4 + 2.8% of *Giardia duodenalis trophozoites*. Increase of the spontaneous excystation of *Cryptosporidium parvum oocysts* observed after administration of the BE (equivalent to 213 µg/ml of the gallic acid). Because anthocyanins represent more than 70% of the polyphenols, it is believed that they are responsible for antiprotozoan activity of bilberry.

Bilberry juice

Similar experiments were conducted by Huttunen *et al.* (2011) who found through *in vitro* studies that the fraction (10 - 100 kDa), 9 mg/g of the bilberry juice inhibits the binding of *Streptococcus pneumoniae* to human bronchial cells (Calu-3) in the adhesion model. The adhesion inhibition of bilberry juice, consisting essentially of small amounts of phenolic compounds with a low molecular weight, was 52%. The test fractions were devoid of solvents and are water-soluble.

In contrary, juice fractions of *V. myrtillus* of the higher molecular weight with the dominance of anthocyanins, proanthocyanidins and flavonol glycosides exerted antiaggregation effect on the pairs of bacteria common in the pathology of the dental plaque in the oral cavity: *Streptococcus mutans* with *Fusobacterium nucleatum* or *Actinomyces naeslundi* (Riihinen *et al.* 2011).

Antineoplastic activity

Bilberry extract

Bilberry fruit extracts were screened to antineoplastic activity by *in vitro* tests to determine the ability to induce phase II detoxification of quinone reductase and inhibition of the induction of ornithine decarboxylase. Raw extracts containing anthocyanin and proanthocyanidin fractions showed little activity (Bomser *et al.* 1996).

Esselen *et al.* (2011) investigated the influence of BE on topoisomerases activity in a cell-free system and in human HT29 colon carcinoma cells. Topoisomerases I and II are targets of clinically used anti-cancer drugs. A BE containing 36% w/w anthocyanin was used. The major anthocyanins concentrations were established by HPLC mass spectrometric analysis. About 1.2 million HT29 cells were spread into Petri dishes (two Petri dishes for one concentration) and allowed to grow for 48 h. Topoisomerase protein was detected using rabbit polyclonal antibodies specific for either topoisomerase I, topoisomerase II a, or topoisomerase II b. The effect of BE on the growth of the cell line HT29 was determined using the sulforhodamine B assay. Incubation of HT29 cells with the extract up to 500 µg/ml for 72 h led to an inhibition of cell growth but without reaching an IC_{50} value. However, the extract potently inhibited the catalytic activity of topoisomerase I at a concentration of 25 µg/ml. Activity of topoisomerase I was completely suppressed by the extract at concentrations ≥50 mg/ml. The BE was found to inhibit the catalytic activity of topoisomerase II at concentrations exceeding 1 µg/ml. The catalytic activity of topoisomerase IIa was fully blocked at 5 µg/ml. Similar effects were

obtained with recombinant topoisomerase IIb. Pre and co-incubation of HT29 cells with BE (≥1 ≥g/ml) significantly suppressed (p <0.001) the strand-breaking effects of camptothecin. The extract was found to significantly diminish doxorubicin-mediated DNA strand breaks at concentrations ≥1 µg/ml (p<0.001). Authors concluded that anthocyanins show a preference for inhibition of topoisomerase II (Esselen *et al.* 2011).

Isolated compounds

Lamy *et al.* (2007) studied the activity of anthocyanidins (aglycons of anthocyanins) in prevention of migration of glioblastoma cells. Because the full resection of malignant glioblastomas is not possible due to their diffuse structure, the development of new projects for cancer therapy and prevention is very important. It was found that aglycons of the anthocyanins: cyanidin, delphinidin, and petunidin acts as potent glioma (U-87) cell migration inhibitors. The most potent was found delphinidin 3-*0*-glucoside (5, 10, 20 µM, (p<0.05, p<0.01) and 50 µM (p<0.001)), with significant differences versus control alone. Since the antocyanins cross the blood brain barrier and pass to the CNS in the concentration range of 192.2 ± 57.5 ng/g after administration of the single dose (Passamonti *et al.* 2003), the chronic intake can significantly inhibit the migration of glioblastoma cells and may affect the results of cancer treatment (Lamy *et al.* 2007).

***In vivo* Experiments**

Bilberry extract

Antiulcer activity

Antiulcer activity of a BE (corresponding to 25% anthocyanidins) was tested *in vivo* in Wistar rats in experimental models of pyloric ligature induced ulcers, ulcers induced with the use of reserpine, phenylbutazone, ulcers caused by restraint and a local application of acetic acid to the gastric mucosa (Cristoni and Magistretti 1987). The results of experiments were compared with the control groups and groups of rats receiving carbenoxolone and cimetidine. The results of the performed experiments were analyzed by the Mann-Whitney U test or the Dunnett t test (Table 19; Table 20; Table 21; Table 22; Table 23).

Table 19. Activity of *V. myrtillus* extract in pyloric ligature ulcer model in rats (after Cristoni and Magistretti 1987)

Substance	Dose mg/kg p.o.	No of animals	Mean index of ulceration Mean ± S.E.	% of Inhibition	No of stomachs not ulcerated
Controls	-	10	49.00 ± 15.35	-	0
Bilberry extract	10	12	40.50 ±11.49	-17	1
Controls	-	11	35.36 ± 9.10	-	2
Bilberry extract	25	13	18.85± 4.77	-47	0
	50	13	16.54 ± 3.71	-53	1
Controls	-	13	47.46 ± 10.37	-	1
Bilberry extract	100	13	12.46 ± 3.89 (**)	-74	3
Controls	-	14	44.50 ± 6.83	-	1
Carbenoxolone	50	12	28.16 ± 7.02 (*)	-37	0
	100	12	28.08 ± 5.93 (*)	-37	0
Controls	-	14	43.78 ± 6.99	-	1
Cimetidine	50	13	30.15 ± 7.17	-31	2

	100	16	31.43 ± 7.14	-28	1

Substances were given 50, 30, 25, 6 hours before and immediately after pyloric ligature
(*) p<0.05; (**) p<0.01; Mann Whitney U test.

Table 20. Activity of *V. myrtillus* extract in reserpine ulcer model in rats

(after Cristoni and Magistretti 1987)

Substance	Dose mg/kg p.o.	No of animals	Mean index of ulceration Mean ± S.E.	% of Inhibition	No of stomachs not ulcerated
Controls	-	18	19.56 ± 2.31	-	0
Bilberry extract	25	17	10.52 ± 1.41(**)	-46	0
	50	16	6.31 ± 1.35 (**)	-68	0
	100	14	3.71 ± 0.92 (**)	-81	2
Controls	-	12	21.25 ± 4.72	-	0
Carbenoxolone	25	13	8.88 ± 2.27 (**)	-58	0
	50	12	6.16 ± 0.99 (**)	-71	0
	100	12	1.42 ± 0.97 (**)	-93	
Controls	-	17	20.28 ± 3.39	-	2
Cimetidine	100	14	24.3 ± 6.78	+20	0

Substances were given once a day for 8 days; (**) p<0.01 (Mann-Whitney U test).

Table 21. Activity of *V. myrtillus* extract in phenylbutazone ulcer model in rats (after Cristoni and Magistretti 1987)

Substance	Dose mg/kg p.o.	No of animals	Mean index of ulceration Mean ± S.E.	% of Inhibition
Controls	-	20	0.42 ± 0.07	-
	-	33	3.39 ± 0.26	-
Bilberry extract	50	19	2.55 ± 0.16	-25
	100	17	1.88 ± 0.20 (*)	-45
	200	20	1.27 ± 0.23 (**)	-63
Controls Phenylbutazone	-	35	3.08 ± 0.28	-
Carbenoxolone	50	25	2.25 ± 0.31	-27
	100	10	1.49 ± 0.41 (*)	-52
	200	10	1.31 ± 0.24 (**)	-57
Controls Phenylbutazone	-	10	2.14 ± 0.39	-
Cimetidine	20	10	1.62 ± 0.37	-24
	40	10	1.09 ± 0.34 (*)	-49
	80	10	0.62 ± 0.20 (*)	-71

Substances were given twice a day for 4 days

(*) p<0.05; (**) p<0.01; Mann Whitney U test.

Table 22. Activity of *V. myrtillus* extract in restraint ulcer model in rats (after Cristoni and Magistretti 1987)

Substance	Dose mg/kg p.o.	No of animals	Number of ulcers (Mean ± S.E.) on days				
			0	1	3	6	9
Controls	-	8	3.75 ± 0.97	4.37 ± 0.65	3.50 ± 0.53	3.25 ± 0.45	2.75 ± 0.53
Bilberry extract	25	8	3.87 ± 0.83	3.75 ± 0.56	3.62 ± 0.50	2.00 ± 0.38 (*)	0.75 ± 0.25 (**)
	50	8	4.00 ± 0.65	2.87 ± 0.40	2.12 ±0.55	1.25 ±0.45 (**)	0 (**)
	100	8	3.75 ± 0.83	1.75 ± 0.80 (**)	1.12 ±0.40(**)	0.25± 0.25 (**)	0 (**)
Controls	-	8	3.87 ± 0.47	4.0 ± 0.27	5.37 ±0.56	4.25 ±0.25	1.87 ± 0.23
Carbenoxolone	50		3.87 ± 0.47	3.50 ±0.60	4.12 ± 0.55 (*)	3.37 ± 0.18 (*)	1.62 ± 0.37

Substances were given once a day.

(*) p<0.05; (**) p<0.01; Mann Whitney U test.

Table 23. Activity of *V. myrtillus* extract in acetic acid ulcer model in rats (after Cristoni and Magistretti 1987)

Substance	Dose mg/kg p.o.	Area of ulcers in mm^2 (Mean ± S.E.)			
		No of animals	6	No of animals	12
Controls	-	15	52.35 ± 2.25	20	13.12 ± 0.99
Bilberry extract	50	15	44.19 ±1.64 (**) (-16)	19	9.67 ±0.86 (-26)
	100	15	39.27 ± 2.08 (**) (-25)	20	7.62 ±0.63 (**) (-42)
Controls	-	16	60.86 ± 1.95	15	11.32 ± 0.64
Carbenoxolone	50	17	54.52 ± 1.56 (*) (-10)	15	8.40 ± 0.80 (**) (-26)
Controls	-	12	55.02 ±2.56	12	11.52 ±2.15
Carbenoxolone	100	10	35.60 ± 1.93 (**) (-35)	12	5.34 ± 0.78 (*) (-33)
Controls	-	12	50.97 ± 2.11	12	14.32 ± 1.66
Cimetidine	100	12	37.83 ± 2.88 (**) (-26)	11	9.58 ± 1.53 (*) (-33)

Smooth muscles contractility

In vivo Experiments

Isolated rat stomach

Influence of a BE (corresponding to 25% of anthocyanidins on the rat stomach muscles to stimulation of post-ganglionic fibres was studied *in vitro* by Bettini *et al.* (1986). The extract in concentration of 1-4 µg/ml enhanced contractility of the rat stomach muscles preparations

stimulated intramurally. The effect was potentiated by addition of an ascorbic acid (500 µg/ml). Ganglionic blockade with use of hexamethonium (20 µg/ml) partially decreased the facilitatory response (Table 24). Authors concluded that the BE enhances the liberation of ACh at the level of the postganglionic nerve endings.

Table 24. Mean percentage increase in the response of the preparation to transmural stimulation (after Bettini *et al.* 1986)

	A	B
Bilberry extract(4 µg/ml)	**140 ± 7**	**78 ± 2.25**
Ascorbic acid (500 µg/ml)	**45 ± 5**	**20 ± 1.5**
Bilberry extract(4 µg/ml) + Ascorbic acid (500 µg/ml)	**200 ± 15**	**100 3.5**

A) in standard Krebs solution; B) with hexamethonium (20 µg/ml); note that the percentage increase is greater in the absence of hexamethonium.

3.1.3 Safety Pharmacology

Platelet aggregation

Effects of BE (containing 36% of anthocyanosides) on platelet aggregation in humans was studied by initially by Botecchia *et al.* (1987), later by Pulliero *et al.* (1989) and by Morazzoni and Magistretti (1990) in rabbit and rats.

Bilberry extract

In preliminary *in vitro* studies Bottechia *et al.* (1987) showed 50% inhibition of the clot retraction at concentration of 75 µ/ml of the BE. Moreover, the platelet aggregation induced by ADP, collagen and arachidonic acid was inhibited in a concentration dependent manner (50, 100 and 150 µ/ml).

The researchers believe that BE stimulates the release of prostacyclin (PGI2), which has the effect of increasing the concentration of the intracellular cAMP or reducing the level of thromboxane A2 in platelets.

In experiments conducted by Morazzoni and Magistretti (1990) in rabbits and rats *in vitro* and *in vivo* activity, not only the activity of the BEM was tested, but also three principal anthocyanosides occurring in the extract. BE, as dipyridamole and aspirin inhibited platelet aggregation (Table 25). Both cyanidin 3-0-glucoside, delphinidin 3-0-glucoside and malvidin 3-0-glucoside added to rabbit plasma inhibited platelet aggregation induced by ADP, collagen and sodium arachidonate (Table 26).

Table 25. Inhibition of rabbit platelet aggregation by BEM, dipyridamole and aspirin (after Morazzoni and Magistretti 1990).

Aggregating agent	IC_{50} values* (mg/ml PRP or GFP**)		
	Bilberry extract	**Dipyridamole**	**Aspirin**
ADP	0.36 (0.89-0.14)	0.36 (0.64-0.20)	IC_{50} not obtainable (active only 2nd phase)
Collagen	0.32 (0.42-0.24)	0.22 (0.31-0.16)	0.04 (0.05-0.02)
Arachidonate	0.60 (0.84-0.43)	0.50 (0.77-0.32)	0.01 (0.01-0.009)
Arachidonate (GFP)	0.81 (1.14-0.58)	_	_

PRP (Platelet-rich plasma)

*IC_{50} values were determined after 3 min incubation of test compound (30 sec for dipyridamole) withy PRP or GFP at 37^0C. Each IC_{50} value and its confidential limits (in brackets) were calculated on four to eleven experiments performed with four to six concentrations of inhibitor.

[**]Gel-filtered platelets (GFP) were prepared using Tyrode's albumin buffer according to Tangen *et al.* 1971.

Tab 26. Inhibition of rabbit platelet aggregation by cyanide 3-0-glucosoide, malvidin 3-0-glucoside and delphinidin 3-0-glucoside (after Morazzoni and Magistretti 1990).

Aggregating agent	IC_{50}Values [*] (mg/ml PRP)			Bilberry extract	Reconstituted extract[**]
	Cyanidin 3-0-glucoside	Malvidin 3-0-glucoside	Delphinidin 3-0-glucoside		
ADP	0.39 (0.70-0.22)	0.43 (0.62-0.29)	0.36 (0.45-0.28)	0.36 (0.89-0.14)	0.51 (0.60-0.44)
Collagen	0.26 (0.53-0.13)	0.64 (1.10-0.38)	0.57 (0.73-0.45)	0.32 (0.42-0.24)	0.87 (1.01-0.74)
Arachidonate	0.42 (0.82-0.22)	0.68 (0.97-0.47)	0.55 (0.72-0.42)	0.60 (0.84-0.43)	0.73 (0.89-0.59)

PRP (Platelet-rich plasma)
[*]IC_{50} values were determined after 3 min incubation of test compound with PRP at 37°C
Each IC_{50} value and its confidential limits (in brackets were calculated on four to eleven experiments performed with four to six concentrations of inhibitor.
[**]Reconstituted extract: a mixture containing 38% of cyaniding 3-0-glucoside, malvidin 3-0-glucoside and delphinidin 3-0-glucoside in the same ratio as in the *V. myrtillus* extract

BEM significantly and dose dependently (5 – 400 mg/kg) induced in rats *in vivo* lengthening of **bleeding time**. The effect was independent from the influence on the coagulation system. Indeed hematocrit (44.9 ± 0.3; Control 44.7 ± 0.4), Cephotest (18.6 ± 0.7; Control 19.4 ± 0.9) and Normotest (26.7 ± 1.2; control 25.8 ± 1.1) values were normal after 2 hours after a single oral dose of the extract (400 mg/kg) (Table 27).

Table 27. Time course of the effects of single oral doses of BEM (100 mg/kg) on bleeding time in rats (after Morazzoni and Magistretti 1990)

Bleeding time (sec) (Mean ± S.E.)										
10 animals /group	**Before Mean ± S.E.**	**After 2h Mean ± S.E.**	**Before Mean ± S.E.**	**After 4h Mean ± S.E.**	**Before Mean ± S.E.**	**After 8h Mean ± S.E.**	**Before Mean ± S.E.**	**After 24h Mean ± S.E.**	**Before Mean ± S.E.**	**After 48h Mean ± S.E.**
Control	96±6	102±6	96±7	93±7	93±7	90±6	93±7	96±9	96±7	99±5
Bilberry extract 100 mg/kg	96±7	174±6[**]	99±8	159±11[**]	93±7	150±7[**]	96±6	144±7[**]	102±6	90±10
Aspirin Dipyridamol 50-25 mg/kg	105±7	177±8[**]	102±7	174±9[**]	90±6	171±10[**]	87±7	114±7[*]	105±8	105±8

[*]$p \leq 0.05$; [**]$p \leq 0.01$ with Student's test for paired data. The experiment was performed on groups of 10 rats for each time.

Similar to ticlopidine change in **platelet adhesiveness** was found in male mouse after single oral administration of BEM at the dose 400 mg/kg two hours before the test. In the treated group marked decrease in the number of the adhesive platelets was found compared to control values (Table 28)

Table 28. Effect of single oral dose of BEM and ticlopidine hydrochloride on male mouse platelet adhesiveness (after Morazzoni and Magistretti 1990).

	Dose mg/kg	No of animals	Adhesive platelets (%) Mean ± S.E.	% Change in adhesive platelets
Control	_	105	47.06 ± 1.39	_
Bilberry extract	400	20	25.30 ± 2.66**	-46
Ticlopidine hydrochloride	100	72	26.82 ± 1.82**	-43

BEM and ticlopidine hydrochloride were administered orally two hours before the test (**$p \leq 0.01$ with Dunnett's t test).

3.1.4 Pharmacodynamic interactions

Fuchikawa et al. (2006) studied *in vitro* the influence of the water/ethanol bilberry extract on the uptake estrone-3-sulfate by the organic anion-transporting polypeptide B (OATP-B) in the culture of human embryonic kidney (HEK293) cells. The bilberry extract potently inhibited estrone-3-sulfate uptake (about 75%) by the transporter protein OATP-B expressed in the intestine. This transporter plays a role in absorption of several drugs as glibenclamide, fexofenadine and pravastatin. So there is a possibility that the extract could reduce the absorption of these drugs and their therapeutic effectiveness.

Except this single report no animal studies of the bilberry fruit or extracts interactions were identified (Gardner and McGuffin 2013).

3.1.5 Conclusions

Available non-clinical data make plausible the traditional use of bilberry fruits and extracts for the treatment of diarrhoea and peripheral vascular disorders. The therapeutic effects observed in clinical trials may be explained by the results of preclinical pharmacological studies. The results of preclinical studies carried out both *in vitro* and *in vivo* fully confirm the expected clinical efficacy.

As dried ripe fruits of bilberry contain at least 1% of tannins, the traditional use of the dry fruit preparations in diarrhea and topically in mild inflammation of the mucosa is justified on the basis of their astringent properties.

Bilberry is also recommended for the treatment of vascular disorders. Many therapeutic proprieties of bilberry is ascribed to anthocyanin activity. This concerns both the relaxing action on blood vessels like arteries and veins, an improvement of capillaries after microcirculation injury and protective effects of the BE and anthocyanins against oxidative damage. Preclinical studies have also reported their antimicrobial, antinflammatory, antiplatelet and antineoplastic activity.

3.2. Overview of available pharmacokinetic data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

Absorption

Bilberry extract

In the study of Talavera *et al.* (2003) anthocyanins from BEM (88 µM/l) were injected directly into the stomach of anaesthetized rats for 30 min and their appearance in plasma was a measure of absorption from the stomach. The absorption of anthocyanins was very diverse (19 – 37%) and depended on their

structure. Of bilberrry anthocyanins the greatest absorption underwent delphinidin glycosides. After absorption from the stomach glycosides were rapidly excreted into the bile.

Other bioavailability studies in rats demonstrated that after oral administration of a single dose of 400 mg/kg of BEM (mixture of 15 anthocyanosides) they were quickly absorbed with Cmax = 2.5 µg/ml at the plasma peak Tmax value of 15 min (absorption rate constant = 0.13 /min). After 2 hours after administration no detectable concentrations were observed. Despite the low bioavailability of BEM the concentration of 2 – 3 µg/ml in plasma obtained after administration of a single dose can guarantee sufficient biological effects. There is a lot of evidence that anthocyanidins are enabled *in vitro* at concentrations of about 2 - 7 µg/ml (Morazzoni *et al.* 1991, after Morazzoni and Bombardelli 1996).

Isolated compounds

Anthocyanins, present a low bioavailability as evaluated by urinary excretion, appear very quickly after ingestion in systemic circulation (peak plasma concentrations appear after 1.5 h). Several data show that only less than 1% of the consumed anthocyanins can be detected in the plasma and urine (Felgines *et al.* 2005; Kay *et al.* 2005; Nielsen *et al.* 2003). Intact anthocyanidin glycosides have been detected in plasma indicating that cyanidin-glycosides are absorbed from the digestive tract into the blood stream in their intact glycosylated forms, as the only flavonoids which occur as glycosides in plasma. Indeed, anthocyanins are mainly absorbed as intact glycosides from the stomach and also from the upper part of the small intestine (McGhie *et al.*, 2007; Matuschek *et al.*, 2006; Passamonti *et al.*, 2003; Nurmi *et al.* 2009). Anthocyanins absorption through the mucosa of the gastrointestinal tract was evaluated in tests carried out *in vivo* (in situ perfusion model). It was found that 10-22% the anthocyanins administered is absorbed in the small intestine.
This high level of anthocyanins absorption was comparable to the studies of Felgines *et al.* (2006) (about 20% in the stomach and 7% in the small intestine). It can generally be assumed on the basis of these results that about 10% total the anthocyanins administered can be absorbed from the stomach and small intestine (Talavera, *et al.* 2004).

Anthocyanin specific transporter is the bilitranslocase, a membrane carrier in the liver responsible for the transport of the dye bromosulphophtalein. This transporter is located on luminal side in the apical domain of epithelial cells of the stomach and intestines. Experimental simulation of antocyanins absorption with the use of intestinal epithelial Caco-2 cells demonstrated high retention of anthocyanins in the cells (up to 60%) but complete lack of their diffusion across the basolateral (serosal) side (Steinert *et al.* 2008).

This phenomenon may explain why the bioavailability of anthocyanins is so low. When the anthocyanins come into contact with the bacterial microflora of the colon, rapid deglycosylation occurs with conversion to phenolic metabolites (Fleschhut *et al.* 2006; Keppler and Humpf 2005; Kemperman *et al.* 2010; Nurmi *et al.* 2009; Selma *et al.* 2009). Most anthocyanins do not appear to undergo extensive metabolism of the parent glycosides to glucuronic, sulfo- or methyl- derivatives (Mc Ghie and Walton 2007; Ichiyanagi *et al.*, 2004a, 2004b).

Distribution

Bilberry extract

Lietti and Forni (1976) studied the tissue distribution of anthocyanidins after the administration to rats of the extract of *V. myrtillus* equivalent to 25% of anthocyanidins dissolved in saline in doses of 25 mg/kg i.p. or 20 - 40 mg/kg i.v. Bilberry anthocyanidins were rapidly distributed in the tissues especially to skin and kidney as compared to plasma (Table 29). The calculated estimated volume of distribution in the rat as extrapolated from the plots was 22 ml, which corresponds approximately to the sum of the plasma plus the interstitial fluid volume.

Table 29. Tissue distribution of anthocyanidins in the rat 1 h after i.p. administration of 200 mg of *V. myrtillus* anthocyanosides (equivalent to 25% of anthocyanidins). Mean ± SE of five animals per group (after Lietti and Forni 1976).

Anthocyanidins (μg/g tissue)		Tissue/plasma ratio
Plasma	25.6 ± 1.9	-
Heart	13.6 ± 2.6	0.55
Kidneys	79.0 ± 9.8	3.18
Liver	21.0 ± 4.5	0.84
Lungs	12.2 ±0.0	0.49
Plasma	19.0 ± 1.3	-
Skin	27.4 ± 1.8	1.48

Anthocyanidins seem to have longer persistence in the skin compared with the plasma (Table 29). This long-term persistence of anthocyanidin in the skin may be associated with the observed increase in capillary resistance, once they are not found in detectable concentrations in the blood. (Table 30 adapted from Lietti and Forni 1976).

Table 30. Relationship between plasma and skin level of anthocyanidins and pharmacological activity of *V. myrtillus* anthocyanosides (200 mg/kg i.p.) on rat capillary resistance. Mean ± SE of five rats per group (after Lietti and Forni 1976)

Time after treatment (min)	Anthocyanidins		Percent increase of capillary resistance
	Plasma (μg/ml)	Skin (μg/ml)	
15	43.6 ± 0.4	21.3 ± 2.2	17.3
120	7.5 ±o.4	17.4 ± 1.5	34.0
240	3.1 ± 0.1	15.1 ±1.8	38.7

The distribution of bilberry anthocyanidins was tested in mice (Sakakibara *et al.* 2009). After single oral administration of 100 mg/kg of BE (obtained by use of 80% ethanol acidified with hydrochloric acid) with the total concentration of anthocyanins 67.3 μmol/100 mg of the extract the total plasma concentration has attained a maximum of 1.18 ± 0.3 μM after 15 min and afterwards rapidly decreased almost to basal levels after 120 min (Table 31).

Table 31. Time-dependent changes of plasma anthocyanins after administration of bilberry extracts (after Sakakibara *et al.* 2009)

Peak No	Anthocyanin	Administration amounts (μmol/100 mg extracts/kg body weight)	nM				
			Times after administration of bilberry extracts (min)				
			0	15	30	60	120
1	Delphinidin 3-galactoside	10.2	n.d.	175(154-211)	149(115-214)	27(n.d.-61)	n.d.
2	Delphinidin-3-glucoside	8.9	n.d.	110(94-133)	108(53-70)	18(n.d.-36)	6(n.d.-17)
3	Cyanidin-3-galactoside	8.3	n.d.	179(156-219)	149(98-138)	33(n.d.-54)	n.d.
4	Delphinidin-3-arabinoside	7.8	n.d.	114(94-142)	110(89-138)	24(n.d.-50)	n.d
5	Cyanidin-3-glucoside	6.8	n.d.	128(95-185)	124(81-180)	19(n.d.-50)	n.d.
6	Petunidin-3-galactoside	2.4	n.d.	48(42-53)	36(19-60)	n.d.	n.d.
7	Cyaniding-3-arabinoside	5.7	n.d.	45(38- 54)	45(36-57)	17(n.d.-28)	n.d.
8	Petunidin3-glucoside	4.6	n.d.	57(52-64)	43(n.d.-79)	n.d.	n.d.
9	Peonidin-3-galactoside	0.8	n.d.	29(14-55)	19(n.d.-43)	n.d.	n.d.

Peak No.		Administration	nM				
10	Petunidin-3-arabinoside	1.7	n.d.	21(18-23)	10(n.d.-29)	n.d.	n.d.
11,12	Malvidin-3-galactoside/peonidin-3-glucoside	4.3	n.d.	145(104-222)	113(81-153)	52(37-60)	8(n.d. - 25)
13,14	Malvidin-3-glucoside/peonidin-3-arabinoside	4.7	n.d.	115(101-140)	104(76-137)	37(27-47)	5(n.d.-15)
15	Malvidin-3-arabinoside	1.1	n.d.	9(n.d.-14)	6(n.d.-17)	n.d.	n.d.
Total		67.3	n.d.	1,177(1,000-1,514)	1,013(762-1,481)	227(135-386)	19(n.d.-42)

[a]Values are indicated as the mean (min-max) of plasma anthocyanins, n.d., under the detection limit in this study (<15 nM).

When mice received a diet containing 0.5% BE for 2 weeks, anthocyanins were detected in the liver, kidney testes and lung. Malvidin-3-glucoside and -3-galactoside were the prevailing anthocyanins (Table 32). Anthocyanins were not detectable in spleen, thymus, heart, muscle, brain, white fat and eye balls. After the long intake of BEs, the levels of anthocyanins are maintained in the liver, kidney, testes, and the lung (Sakakibara *et al.* 2009).

Table 32. Anthocyanin concentrations in tissues of mice fed a 0.5% bilberry diet for 2 weeks (after Sakakibara *et al.* 2009)

	Pmol/g wet wt. of each tissue				
				Methoxy anthocyanins	
	Σ anthocyanins	Del-G[b]	Cy-G[b]	Pet-G[b]	Mal-G/Peo-G[b]
Plasma (nM)	153(n.d.-258)	42(n.d.-80)	26(n.d.-80)	n.d.	84(n.d.-178)
Liver	173(65-605)	34(n.d.-178)	23(n.d.-159)	8(n.d.-49)	108(42-218)
Kidney	114(n.d.-207)	25(n.d.-178)	n.d.	n.d.	88(n.d.-146)
Testes	148.5	n.d.	n.d.	n.d.	88(n.d.-146)
Lung	116.0	34.0	11.5	n.d.	70.5

[a]Values are indicated as the mean (min-max) of 10 mice, n.d., under the detection limit in this study as follows: <15 nM in plasma, <10 pmol/g tissues.

[b]Del-G, delphinidin glycosides (peak no. 1 + 2 + 4); Cy-G, cyaniding glycosides (peak no. 3 + 5 + 7); Pet-G, petunidin glycosides (peak no. 6 + 8 + 10); Mal-G/Peo-G, malvidin glycosides + peonidin glycosides (peak no. 9 + 11 + 12 + 13 + 14 + 15). Peak numbers refer to Table 26.

Binding of anthocyanins to plasma albumin

Isolated compounds

Cahyana and Gordon (2013) have found that the effect of structure of anthocyanins (pelargonidin, cyanidin, delphinidin, malvidin) on the affinity to human plasma albumins was pH-dependent. Electrostatic binding of anthocyanins to albumins is favoured at pH 7.4. The more polar glucosides showed stronger binding, while methylation, which reduced the polarity of the anthocyanins, decreased the association. Hydroxyl substituents and glycosylation of anthocyanins decreased the affinity for binding to at lower pH (especially pH 4), but increased the strength of binding at pH 7.4. In contrast, methylation of a hydroxyl group enhanced the binding at acidic pH, whilst this substitution reduced the strength of binding at pH 7.4 (Fossen *et al.* 1998).

The above experiments have shown that the most common anthocyanins in plasma and tissues were malvidin glycosides, and in second place followed by peonidin glycosides. These and other anthocyanins are converted to methoxyl or glucosyl substituents with subsequent result on the

interaction with serum albumin. Since the pH at inflammatory sites is acidic it can determine their beneficial health effects here in this place (Cahyana and Gordon 2013).

Elimination

Isolated compounds

Elimination of anthocyanidins proceeds quite fast regardless of the route of administration (Lietti and Forni 1976). After 4 hours, approximately 20% of the administered dose was eliminated in the urine. After 24 hours, 15% of the intravenous dose and 18% of the administered via the intraperitoneal route was excreted in the bile.

Anthocyanins are readily reactive compounds and therefore are easily deteriorating or reacting with the other ingredients in the mixtures to form colourless or brown compounds. After the passage through the gastrointestinal tract after oral administration they are exposed to different pH and temperature conditions and to different chemical substances. Their different molecular forms are in dynamic equilibrium. Most of the anthocyanins are transformed in the colon to phenolic acids by bacteria (Fleschhut *et al.*, 2005; Aura *et al.*, 2005; Keppler & Humpf, 2005). The enzymatic reactions include ring cleavage, hydrolysis of glycosides, glucuronides, amides and esters, and reduction, decarboxylation, demethylation and hydroxylation (Aura 2008; Dall'Asta *et al.* 2012; Kay *et al.* 2005; Kay 2006; Manach *et al.* 2005; Williamson and Manach 2005). On the other hand, anthocyanins given to patients with an ileostomy are accumulating mostly unchanged in the bag (85%), giving little evidence of metabolism after gastrointestinal transfer up to ileum (Kahle *et al.* 2006). Known mammalian metabolites of anthocyanidins are presented in the Table 33 (after Williamson and Clifford 2010).

Table 33. Known mammalian metabolites of anthocyanidins (after Williamson and Clifford 2010).

The expected B-ring fragments for the common anthocyanidins and their known mammalian metabolites			
Anthocyanidin	**Initial B-ring fragmentation product**	**Known mammalian metabolites**	
		Human	**Animal**
Pelargonidin	4-Hydroxybenzoic acid	Benzoic acid-4-*0*-sulphate 4-Hydroxybenzoyl-Gly	
Cyanidin	3,4-Dihydroxybenzoic (protocatechuic) acid	3-Methoxy-4-hydroxybenzoic (vanilic) acid Protocatechuic acid conjugates in Caco-2-cells	Methylated, glucuronidated or Gly conjugated metabolites including vaniloyl-Gly
Delphinidin	3,4,5,-Trihydrobenzoic (gallic) acid	3-*0*-methylgallic acid 4-*0*-methylgallic acid 3,4-*0*-dimethylgallic acid	Pyrogallol Pyrogallol-1-*0*-β-D-glucuronide 4-*0*-methylgallic acid-3-*0*-sulphate 2-*0*-methylpyrogallol-1-*0* β-D glucuronide 2-*0*-methylpyrogallol 4-*0*-methylgallic acid
Peonidin	3-Methoxy-4-hydroxybenzoic (vanilic) acid	Vanilic acid-4-*0*-sulphate or 4-*0*- β-D-glucuronide	
Petunidin	3-Methoxy-4,5,-dihydroxybenzoic acid		
Malvidin	3,5,-Dimethoxy-4-hydroxybenzoic (syringic) acid		

The metabolites of anthocyanins most likely are the C6, C1-dihydro acids. Microbial metabolites may be much more efficiently absorbed than the parent compounds, because they are there in high concentrations (similar to mM) and the surface of absorption from the colon mucosa is large.

In vitro studies with anaerobic human microflora demonstrated that protocatechuic acid is the most probable main degradation product of anthocyanins (Galvano *et al.* 2008). Also, studies in humans with the administration of orange juice and administration of cyanidin-3-O-glucoside confirmed that protocatechuic acid is the predominant unconjugated metabolite of anthocyanins (Vitaglione *et al.* 2007).

The oral intake by volunteers of oats added to a purée of bilberries (glycosides of delphinidin, accompanied by small amounts of malvidin, peonidin and petunidin glycosides) and lingonberries (cyanidin glycosides) (Ek *et al.* 2006) resulted in urinary excretion of 3-methoxy-4-hydroxyphenylacetic (homovanilic) and vanilic acid and a low amounts of syringic acid (Ichyanagi *et al.* 2004a, 2004b; Katsube *et al.* 2003). Urinary excretion of these acids was maximal at 4–6 h (Nurmi *et al.* 2009).

3.3. Overview of available toxicological data regarding the herbal substance(s)/herbal preparation(s) and constituents thereof

3.3.1. Single dose toxicity

Herbal preparations

The acute oral toxicity of BE was studied in mice and rats and no signs of toxicity were observed at doses significantly higher than 2000 mg/kg.

Oral administration of a single dose of BEM (no further details) (3000 mg/kg, equivalent to 1.08 g/kg anthocyanins) to dogs, besides a clear darkening stool and urine, did not result in adverse reactions (Eandi, Data on file 1987; quoted after Morazzoni and Bombardelli 1996). This symptom explicitly testified of absorption and elimination of the preparation by the kidney.
Dosage used, however, broadly exceeds the human exposure (usually around 5 - 10 mg / kg). The above studies suggest that the extract used is practically non-toxic.

3.3.2. Repeated dose toxicity

Subacute

No toxic effects were noted in guinea pigs received daily BEM for 2 weeks and rats for 6 weeks at doses up to 43 mg/kg (Pourat *et al.* 1967, quoted in Upton 2001).

Similarly no abnormalities were found in rats receiving BEM for 4 weeks up to 36 mg/kg i.v. daily or in dogs treated for 13 weeks 12 mg/kg daily. There was, however, a dark blue colour of the urine, skin, eyes, and, in certain cases, liver, kidney and the ovaries (Eandi, Data on file 1987, quoted in Morazzoni and Bombardelli 1996).

Both oral daily administration of the BEM to rats for 6 months (125 – 500 mg/kg) and to dogs (80-320 mg/kg) did not have a toxic effect or resulted in adverse reactions (Eandi, Data on file 1987, quoted by Morazzoni and Bombardelli 1996). There were no variations of haematological or biochemical parameters. The autopsy also showed no morphological changes.

Chronic Toxicity

No 2-year toxicity studies of BE were identified in the available literature.

Toxicological studies of anthocyanins are limited and are made using extracts from the various fruits. Human population is naturally affected by exposure to anthocyanins as regularly eating fruits and vegetables.

3.3.3. Genotoxicity

Mutagenicity

Weak mutagenic activity in the Ames test was observed after application of the extract of *V. myrtillus* (Schimmer *et al.* 1994). However, the results refer to the extract of the leaves of the plant and not to fruit preparations.

Antimutagenicity

In the study of Malaveille *et al.*, (1996) cyanidin chloride inhibited hepatic S-9-mediated mutagenicity in the *Salmonella typhimurium* TA98 heterocyclic amines, (for 10 and 45% with 2 and 6 µg respectively) by 2-amino-3 ,8-dimethyl-imidazo [4,5-f] quinoxaline (MeIQx) and (for 5, 10, 20 and 50% with 0.2, 0.6, 2 and 6 µg respectively) 2-amino-1-methyl-6-phenyl-[4,5-b] pyridine (PhIP).

On the contrary, the cyanidin, malvidin, and delphinidin were inactive in prevention of the S-9 mediated mutagenicity in *S. typhimurium* TA98 of the heterocyclic amines, 2-amino-3-methylimidazo [4,5-f] Quinoline (IQ) and 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P72) from cooked food (Edenharder *et al.*, 1993).

3.3.4. Carcinogenicity

Laboratory and clinical studies provide strong evidence that increased intake of berries which have among others a high content of anthocyanins, may contribute to a reduced risk of certain cancers, especially colorectal cancer (Brown *et al.* 2012; Stoner *et al.* 2008; Wang and Stoner 2008).

3.3.5. Reproductive and developmental toxicity

In several non controlled studies with the participation of more than 200 women in pregnancy receiving BE equivalent to 57 – 173 mg/kg/day anthocyanins for 60 to 102 days for venous complaints, including haemorrhoids, no adverse effects were registered (quoted after review of Morazzoni and Bombardelli 1996).

In the study of Pourrat *et al.* (1967) anthocyanin glycosides from the currants, blueberries and elderberries, when given in doses of 1.5, 3, or 9 g/kg for 3 successive generations did not induce teratogenic activity in rats, mice and rabbits (http://www.inchem.org/documents/jecfa/jecmono/v17je05.htm).

Bhargava (1990) reported inhibition of malvidin chloride of spermatogenesis in langur monkeys receiving 50 mg/kg for 60 days. Testicular and epididymal mass was diminished and the disappearance of Leydig cells was seen. The level of total RNA protein, sialic acid, the acid/alkaline phosphatase in the testes was reduced as the amount of cholesterol in epididymides.

3.3.6. Local tolerance

No data available

3.3.7. Other special studies

No data available

3.3.8. Conclusions

Toxicological studies are limited.

Tests on reproductive toxicity and genotoxicity were performed almost 50 years ago and are not in accordance with the current standards.

Adequate tests on toxicity, genotoxicity and carcinogenicity have not been performed. No reason of concern is arisen from data of human consumption.

3.4. Overall conclusions on non-clinical data

Non-clinical data on vasoactive influence, microcirculation, anti-inflammatory and antioxidant activity of BE fully support the traditional use to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.

Results from relevant experimental studies are very limited, but the antimicrobial, astringent and antihadesive properties of the tannins present in the herbal substance can explain the traditional uses of the dried fruit as a decoction for symptomatic treatment of mild diarrhoea and for symptomatic treatment of minor inflammations of the oral mucosa.

Pharmacokinetic studies show that anthocyanin glycosides are rapidly absorbed from the stomach after ingestion and they enter the central compartment after first pass through the liver. In the liver undergo methylation and glucuronidation reactions and some of the metabolites are transported to the bile. Anthocyanin glycosides which are not absorbed from the stomach move into the jejunum, and are absorbed to systemic circulation. Anthocyanins that get the colon are exposed to a microbial transformation with production of phenolic compounds, later they are degraded to aldehydes and phenolic acids.

As there is no valid information on reproductive and developmental toxicity the use during pregnancy and lactation cannot be recommended.

Toxicological studies are limited and were performed using extracts from the various fruits.

Adequate tests on reproductive toxicity, genotoxicity and carcinogenicity have not been performed.

Oral administration and oromucosal use of decoction of dried bilberry fruit can be regarded as safe at traditionally used doses and adequate duration of use.

4. Clinical Data

4.1. Clinical pharmacology

4.1.1. Overview of pharmacodynamic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

Impact on lipid peroxidation

Bilberry extract

***In vitro* Experiments**

Laplaud *et al.* (1997) tested *in vitro* an aqueous extract of *V. myrtillus* on human low density lipoproteins. They found, that the extract, which contained 74.2±4.9 mg/g of total polyphenols with a proportion of catechin of 17.3±3.3% caused potent protective action on LDL particles during *in vitro* copper-mediated oxidation. Trace amounts of *V. myrtillus* aqueous extract (15 to 20 µg/ml) induced

statistically significant changes in the oxidation behaviour of low density lipoproteins (LDL). Observed effects included: 1) Prolongation of the lag-phase of conjugated diene production ($P < 0.01$); 2) Reduction in the formation of lipoperoxides and of thiobarbituric acid-reactive substances up to 7 hours and especially between 1 and 5 hours ($P < 0.01$); 3) Inhibition of modification in the net negative charge of LDL. The antioxidant effect of the extract can be compared to the corresponding action of ascorbic acid or butylated hydroxytoluene in the protection of LDL particles from oxidative stress.

Platelet aggregation

Bilberry extract

Clinical Studies

In clinical study Puliero *et al.* (1989) 30 volunteers of both sexes (mean age 45 years) were involved. They were divided into three groups, of which received orally for 60 days : A) BEM 480 mg/day in 3 divided doses; B) Ascorbic acid 3 g in three divided doses, C) BEM 480 mg/day in 3 divided doses + ascorbic acid 3 g in three divided doses. Blood samples were taken before treatment and after 30, 60 and 120 days after the beginning of treatment. The combination of BEM and ascorbic acid concentration dependently reduced the platelet aggregation induced by either collagen (2-4 µ/ml) or ADP (0.5, 1, 2, and 3 µM), the combination was more active than each product separately. The values of platelet aggregation returned to baseline in the treated groups in 120 days after beginning of the treatment.

4.1.2. Overview of pharmacokinetic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

Elimination

Isolated compounds

***In vitro* Experiments**

In vitro studies with anaerobic human microflora demonstrated that protocatechuic acid is one of the most probable main degradation products of anthocyanins (Galvano *et al.* 2008). Also, studies in humans with the administration of orange juice and administration of cyanidin-3-O-glucoside confirmed that protocatechuic acid is the predominant unconjugated metabolite of anthocyanins (Vitaglione *et al.* 2007).

Clinical Studies

The oral intake of oats added to a purée of bilberries by volunteers (glycosides of delphinidin, accompanied by small amounts of malvidin, peonidin and petunidin glycosides) and lingonberries (cyanidin glycosides) (Ek *et al.* 2006) resulted in urinary excretion of 3-methoxy-4-hydroxyphenylacetic (homovanilic) and vanilic acid and a low amounts of syringic acid (Ichyanagi *et al.* 2004a, 2004 b; Katsube *et al.* 2003). Urinary excretion of these acids was maximal at 4–6 h (Nurmi *et al.* 2009).

4.2. Clinical Efficacy

4.2.1. Dose response studies

No data available

4.2.2. Clinical studies (case studies and clinical trials)

Clinical studies on humans

Retinopathy						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Perossini *et al.* 1987 Diabetic and hypertensive retinopathy	Randomized, double blind, parallel with placebo group. After 1 month all placebo patients whose retinopathy symptoms were unchanged, worsened or only slightly improved continued the trial for an additional month receiving BE. Patients originally receiving *verum* did not continue the study. **Endpoints:** Patients underwent ophthalmologic examination and fluoro angiographic evaluation (before admission to the study and after 30 days; up to 60 days for patients from placebo group). Blood pressure, heart rate, blood glucose levels, glycosylated haemoglobin were also recorded at the same time. **Duration**: 1 month	Bilberry fruit extract (BEM) Quantity 160 mg Plant to extract ratio 100:1 containing 25% anthocyanidins	BEM (160 mg, 2 times daily) No. of patients enrolled: 40 No. of patients completed the trial: 36Drop out: 4 **Age**: 19-78 years (mean: 59.5) **Sex**: Male and female Of the 36 subjects ending the trial, 33 had diabetes and 3 had arterial hypertension. **Inclusion criteria**: Diabetic or hypertensive retinopathy **Exclusion criteria**: Advanced or irreversible damage to the retina (stage IV), severe metabolic, hyperproteinemia, uncontrolled diabetes, or liver damage, patients with severe or malignant hypertension, glaucoma,	At baseline, 28 subjects had opthalmoscopically detectable retinal abnormalities, 13 in the *verum* group and 15 in the placebo group. After the placebo-controlled study, 10 of the patients taking bilberry were improved, none showed any change in the placebo group. Also at baseline, after a fluoro angiographic exam, 17 patients taking bilberry and 18 taking placebo were found to have abnormalities. In the bilberry group, 13 revealed improvement, whereas in the placebo group only one patient showed improvement, 14 had no change in their condition, and 3 showed worsening. After the initial one-month study, patients originally taking placebo were given bilberry for an additional month. After this treatment, 12 of the 15 patients with	Student t - Test Post hoc Scheffé test. Non parametric Friedman test Despite the information of the use of statistical tests in the Method Section no statistics was reported in the Results section and Discussion !	Diabetic or/and hypertensive outpatients (n=14) with vascular retinopathy underwent treatment with bilberry fruit extract (160 mg, 2 times daily) or placebo (n=20) for one month in randomized, double blind study. At the end of the month the patients received placebo were treated with *verum* for one other month. Ophtalmoscopic and fluoro angiographic findings obtained before and after treatments revealed an improvement ranging from 77 to 90% of *verum* patients.

				opthalmoscopic abnormalities demonstrated an improvement, and 17 of the 18 with fluoro angiographic abnormalities showed improvement. Ophtalmoscopic and fluoro angiographic findings obtained before and after treatments revealed an improvement ranging from 77 to 90% of *verum* patients. No side effects or adverse drug reactions were recorded. No essential changes in blood pressure, blood glucose levels or glycosylated haemoglobin were found.		
Retinopathy						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Repossi *et al.* 1987 Early diabetic or hypertensive retinopathy	Parallel with placebo group. **End points:** All patients were examined by fluoro angiography before the trial and after 12 months. Opthalmoscopic evaluations were conducted every three months. Hard exudation was used as an	Bilberry fruit extract (BEM). Quantity 160 mg Plant to extract ratio 100:1, containing 25% anthocyanidins	BEM (160 mg, 2 times daily) **No. of patients enrolled**: 40 **No. of patients completed the trial**: 40 **Age** = not given **Sex** = not given **Inclusion criteria:** Diabetic patients with retinopathy in a relatively initial phase, showing at the back pole some hard exudates	Of patients showing hard exudates in the back pole, 50% improved, 30% percent remained the same, and 20% worsened with treatment with anthocyanosides. In the placebo group, 20% improved, 45% remained the same, and 35% worsened. Of patients showing circinate deposits of the hard exudates,	No statistics reported	Improvements were observed in 50% (vs. 20% in control group). Patients with exudate deposits improved in 15% of the cases (vs. 10% control group). A lower percentage of patients (10% vs. 15%) with hard exudates worsened. The results obtained with

	index of alteration of capillary permeability to evaluate the integrity of the hematoretinal barrier. **Duration**: 1 year		distributed in circinate form, but not involving the macular region. **Exclusion criteria:** Patients with hard exudates affecting the macular region of the eye were eliminated.	15% improved, 60% remained the same, and 25% worsened with treatment with anthocyanosides. In the placebo group, 10% improved, 50% remained the same, and 40% worsened. **Side effects** No short- or long-term side effects.		the group of patients treated with anthocyanosides for a period of 12 months are significant. The results point out that the highest efficacy is gained with very early diagnosis and immediate therapy. However, the sample was not randomized; there is no description of blinding; and there is no description of withdrawals or dropouts.

Retinopathy						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Boniface and Robert 1996 Diabetic retinopathy	Open trial **Endpoints**: Change in protein biosynthesis activity in connective tissue after treatment. **Duration**: 2 months Samples of gingiva tissue were taken before and after treatment. Samples were incubated with radio-active labelled amino acids. The measure of radioactivity from	Bilberry fruit extract 100 mg capsules Twelve adult diabetics were treated with 600 mg anthocyanosides twice daily for two months. **Dosage**: 3 x2 capsules 100 mg per day	**No. of patients enrolled**: 12 **No. of patients completed the trial**: 12 **Age**: not given **Inclusion criteria**: Diabetic patients with retinopathy **Exclusion criteria**: not known	The use of radio-labelled amino acid ^{3}H-proline and ^{14}C-glucosamine, show significant decrease of biosynthetic activity of connective tissue (reduction of synthesis of collagen, urea and glycoproteins).	Student t-Test	**Comment**: Retinopathy is a serious complication of diabetes, resulting from the overproduction of abnormal proteins produced when the organism attempts to repair damaged capillaries. Anthocyanins appear to prevent this damage to blood vessels and also might prevent production of abnormal proteins.

	different connective tissue extracts can show a changed protein biosynthesis activity.					

Retinopathy						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Kim *et al.* 2008 Prospective clinical trial of clinical manifestations of symptoms after 12 months of administration of anthocyanosides to patients with non proliferative diabetic retinopathy.	*V. myrtillus* extract Prospective multicenter clinical trial **Endpoints**: **Primary**: corrected visual acuity and contrast sensitivity which were checked at 2 months following the beginning of treatment. **Secondary**: The number of hard exudates, microaneurysms, leaking points and the changes of foveal thickness. These were examined at the beginning of 6 months after and 12 months after treatment. **Duration** : 12 months	*V. myrtillus* extract (no further detail) (170 mg/capsule, **Dosage**: 3 capsules 170 mg per day	No. of patients enrolled: 88 No. of patients completed the trial: 88 **Age** = not given **Sex** = not given **Inclusion criteria**: Diabetic patients with retinopathy in a relatively initial phase, showing at the back pole some hard exudates distributed in circinate form, but not involving the macular region. **Exclusion criteria**: Patients with hard exudates affecting the	Corrected visual acuity showed no significant changes during 12 months. Contrast sensitivity improved gradually, especially in 12, 16 cycles per degree. There was no statistically significant change in the numbers of hard exudates, microaneurysms and leaking points. Foveal thickness was maintained and there was no aggravation of macular oedema.	No statistics reported (Korean language, English abstract)	There was marked improvement of contrast sensitivity in patients with NPDR after 12 months of administration of *V. myrtillus* extract (510 mg daily). Visual acuity and macular oedema were maintained without deterioration.

			macular region of the eye were eliminated.			

Retinopathy						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Scharer and Ober, 1981 Diabetic retinopathy	Open, parallel study with placebo group. **End points:** Two patients with haemorrhages due to anticoagulants, 4 with arterial sclerosis with haemorrhages of the retina, 20 with diabetic retinopathy (Keith Wagner Stages II and III). **Duration**: 4 weeks	Bilberry fruit extract (no further detail) + 5 mg beta-carotene 1 coated tablet contains 100 mg anthocyanidins from bilberry and 5.0 mg beta-carotene Two 100 mg capsules, 3 times daily (600 mg of the BE + 30 mg of beta-carotene daily).	Bilberry fruit extract (200 mg, 3 times daily) No. of patients enrolled: 33 No. of patients completed the trial: 31 **Age** = range 34 – 78 years **Sex** = not given **Inclusion criteria:** Diabetic patients with retinopathy. **Exclusion criteria:** not known	Reduced vascular permeability during treatment. Remission of changes of retinal vessels and prevention of alterations in the visual field. Patients with diabetic retinopathy showed marked improvement in permeability and a reduced tendency to haemorrhage when treated with bilberry fruit extract	No statistics reported	The sample was not randomized; there is no description of blinding; and there is no description of withdrawals or dropouts

Night vision						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Vannini 1986 Night time vision in healthy subjects	Non randomized Placebo controlled study Double blind Duration =2 hours	Bilberry fruit extract (BEM) Contain 36% anthocyanins (equivalent to 25% by weight of anthocyanidins)	BEM (240 mg) No. of patients enrolled: 40 No. of patients completed the trial: 40	Improved pupillary photo motor response, most evident 2 hours after administration. Decreased total pupillary contraction time	ANOVA ?	Significant improvement of the pupillary muscle contraction in reduced light conditions in healthy young subjects.

	Acute treatment Single dose	Single dose (240 mg)	Age = mean 25.5 years Inclusion criteria: Healthy subjects Exclusion criteria: not known	(p<0.05) Increased pupillary contraction (p<0.05)		

Night vision and contrast sensitivity						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Jayle and Aubert 1964 **Endpoints**: Improvement of night vision and contrast sensitivity	Randomized Controlled Trial Parallel Blinding not reported **Acute** treatment 400 mg (n = 18) **Long term treatment** (7 days) = 2 tablets twice daily (400 mg of the extract + 20 mg of beta-carotene) (n = 19)	*V. myrtillus* fructus extract (no further detail) (1 tablet = 100 mg *V. myrtillus* extract + 5 mg of beta-carotene)	Bilberry fruit extract (200 mg, 3 times daily) No. of patients enrolled: 40 No. of patients completed the trial: 37 Age = mean 32 years Sex = not given **Inclusion criteria**: Healthy young subjects, normal vision **Exclusion criteria**: not known Age = range 34 – 78 years Sex = not given	Dark-adaptation threshold at 8 intervals up to 30 min: Lower thresholds & mean threshold at 4h (3.99 vs. 2.55Ul) (p < 0.05), NSD at 24h or day 8 Visual field in high mesopic light: larger at 4h (25.14' vs 24.1') (p < 0.05), NSD at 24h or day 8 Frequency of appearance & density of central scotoma in high mesopic light: reduced at 4h, 24h, & day 8 (p = ?) Size and density of central scotoma in low mesopic light: smaller at 4h (p ?) NSD at 24h and day 8; density reduced at 4h, 24h & day 8 (p = ?) Light recognition thresholds for	Chi-square test. ANOVA	There was a statistically significant improvement in threshold light levels (statistical test not stated) in thresholds at 4 hours but not at 24 hours or in the long-term treatment group at 8 days. Visual field in high mesopic light was increased. Frequency of appearance and density of central scotoma in high mesopic light was reduced at 4 h.

				cinematic objects: a) basic shape b) precise identification: more correct responses at 4h (& 24h ?) (p = ?). NSD at 8 days.		

Night vision and contrast sensitivity						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Muth *et al.* 2000 Improvement of night vision and contrast sensitivity	Randomized Double –blind, placebo-controlled, crossover. **Endpoint** The objective of this study was to examine the impact of bilberry on night visual acuity (VA) and night contrast sensitivity (CS). **Duration**: 21 days 3 weeks active treatment 11 month washout period 3 weeks active treatment 2 For the VA measurement, the light level used was in the scotopic region, below cone threshold. testing was conducted in a target luminance of 0.005 candelas/meter^2 (cd/m^2). Subjects were dark adapted in complete darkness for a minimum of 30 minutes. Order of testing: night VA first and night CS second.	Bilberry fruit extract not specified containing 25% anthocyanosides. Capsule = 160 mg of BE. Placebo: magnesium aspartate and colouring.	Bilberry fruit extract (160 mg , 3 times daily=480 mg) for 21 days No. of patients enrolled:15 No. of patients completed the trial: 15 *Verum* = 7 subjects Placebo = 8 subjects Age = range 25 – 47 years Sex = male **Inclusion criteria**: Visual acuity correctable to 20/20 or better **Exclusion criteria**: not known	The trial failed to find an effect of BE treatment on night visual acuity (VA) (F [2/28] = 1.8, p > 0.15) or night contrast sensitivity (CS) (F [2/28] =1.0, p > 0.35). The last night CS measurement during active treatment and the last night CS measurement during the placebo treatment did not reveal any difference in night CS (F[2/28] = 0.8, p> 0.45).	The baseline night VA and night CS = the median of the three pre-treatment measurements. The last night VA and night CS measurements taken during the active and placebo treatments were examined for each subject. ANOVA: (1) average night VA; (2) average night CS; (3) last night VA; and (4) last night CS. Non-parametric McNemar's test: improvement on both active and placebo treatment, no improvement on either active or placebo treatment, improvement on active treatment only, improvement	Comment: This survey sheds question the claim that the bilberry treatment in the form of accessible recommended doses, improves night VA or night CS.

					on placebo treatment only.	

Night vision and contrast sensitivity						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Zadok *et al.* 1999 Treatment with anthocyanosides in a multiple oral dose to improve night vision in normal individuals Full field scotopic retinal threshold (SRT) Mesopic contrast sensitivity (MCS) Dark-adaptation rate(DAR) SRT, MCS and DAR tests are directly related to the ability to identify field targets at night. Tests were performed 3 – 5 hours after drug application.	Randomized Double-blind Placebo-controlled cross-over study **Duration**: 4 days (1st active treatment) **Washout**: 2 weeks 4 days (2nd active treatment) **Washout**: 2 weeks 4 days (3rd active treatment)	Tablets containing 12 mg of anthocyanosides and 2 mg beta-carotene Experimental group (n = 6) 1st active treatment: Group 1 (12 mg) Group 2 (24 mg) Group 3 Placebo 2nd active treatment: Group 1 (24 mg)Group 2 Placebo Group 3 (12 mg) 3rd active treatment: Group 1 Placebo Group 2 (12 mg) Group 3 (24 mg)	Tablets containing 12 mg of anthocyanosides and 2 mg beta-carotene Multiple oral administrations of 12 and 24 mg anthocyanosides given twice daily. No. of patients enrolled: 18 No. of patients completed the trial: 18 Age = range 21 – 30 years (mean= 26 ± 2.1 years) Sex = male Inclusion criteria: healthy, young male subjects Visual acuity correctable to 20/25 or better (mean20/22.5 ± 2.0) Exclusion criteria: not known	No significant effects was found on any test: Full field scotopic retinal threshold (SRT) (p = 0.12) Mesopic contrast sensitivity (MCS) (p = 0.73) Dark-adaptation rate(DAR) (p = 0.86)	ANOVA (within subjects repeated measures analysis of variance) P<0.05	**Comment** A controlled randomized clinical study showed no benefit of the effect of anthocyanosides on night vision, even with multiple-dose, indicating that claims of improvement of night vision should be seen with caution. However, this study measured the effect of 4 days of treatment, and perhaps a longer period of administration should be carried out.

Night vision and contrast sensitivity						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality	Comments

	of study				score e.g. Jadad score	
Levy and Glovinsky 1998 Treatment with anthocyanosides in a multiple oral dose to improve night vision in normal individuals Full field scotopic retinal threshold (SRT) Mesopic contrast sensitivity (MCS) Dark-adaptation rate (DAR). SRT, MCS and DAR tests are directly related to the ability to identify field targets at night. Tests were performedimmediately after the drug administration and 4, 8, and 24 h after.	Randomized Double-blind Placebo-controlled cross-over study **Duration**: 24 hours Week 0 active treatment **Washou**t: 2 weeks Week 2 active treatment) **Washout**: 2 weeks Week 4 active treatment **Washout**: 2 weeks Week 6 active treatment	Tablets containing 12 mg of anthocyanosides and 2 mg beta-carotene Experimental group (n = 4) **Week 0 active treatment**: Group 1 (12 mg) Group 2 (24 mg) Group 3 (36 mg) Group 4 Placebo **Week 2 active treatment**: Group 1 (12 mg) Group 2 36 mg Group 3 Placebo Group 4 (12 mg) **Week 4 active treatment:** Group 1 (36 mg) Group 2 Placebo Group 3 (12 mg) Group 4 (24 mg) **Week 6 active treatment** Group 1 Placebo Group 2 (12 mg) Group 3 (24 mg) Group 4 (36 mg)	Tablets containing 12 mg of anthocyanosides and 2 mg beta-carotene Multiple oral administrations of 12 and 24 mg anthocyanosides given twice daily. No. of patients enrolled: 18 No. of patients completed the trial: 16 Age = range 21–30 years (mean= 26 ± 2.1 years) Sex = male Inclusion criteria: healthy, young male subjects Visual acuity correctable to 20/25 or better (mean20/22.5 ± 2.5) 13 subjects were emmetropic, and 3 had ametropia with -2.25D to + 2 D, the greatest cylinder being 1.25 D in one case. Exclusion criteria: not known	No significant effects was found on any of the three night vision tests (full field scotopic retinal threshold (SRT), mesopic contrast sensitivity (MCS), dark-adaptation rate (DAR) during the 24 h following a single oral administration of 12, 24 or 36 mg of anthocyanosides.	ANOVA (within subjects repeated measures analysis of variance) Spearman rank correlation between repeated test results. P<0.05	**Comment** A controlled randomized clinical study showed no benefit of the effect of anthocyanosides on night vision, even with multiple-dose, indicating that claims of improvement of night vision should be seen with caution. However, this study measured the effect of 4 days of treatment, and perhaps a longer period of administration should be carried out.

Visual acuity and contrast vision						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex,inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Mayser and Wilhelm 2001 (Poster) Improvement of visual acuity and contrast vision in healthy subjects Visual acuity and contrast vision were measured at baseline and at the end of each 4-week treatment period.	Randomized Double-blind Placebo-controlled cross-over study **Duration**: 12 weeks 4 Weeks first active treatment Washout: 4 weeks 4 weeks second active treatment (crossover)	Bilberry fruit extract not specified containing 25% anthocyanosides. Capsule = 160 mg of BE (40 mg of anthocyanosides)	Bilberry fruit extract Capsule = 160 mg (40 mg anthocyanosides) daily for 4 weeks Placebo: 160 mg lactic sugar Washout: 4 weeks No. of patients enrolled: 119 No. of patients completed the trial: 119 Age = range 21 – 30 years (mean= 26 ± 2.1 years) Sex = male Inclusion criteria: healthy, young male subjects	Not significantly different between groups: contrast sensitivity, visual acuity, final dark-adaptation threshold (mean of last 4 readings), dark-adaptation rate, time to rod-cone break.	No statistics reported ANOVA ?	Contrast vision and visual acuity improved over the four test points for both groups but were not significantly different between groups. A sub-analysis of 30 matched pairs of subjects with similar dark-adaptation threshold at the first visit also showed no significant difference between groups after treatment. Nor were there significant differences between groups in dark-adaptation rate or time to reach the cone–rod break.

Non Randomized Studies Visual acuity and contrast vision						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Jayle *et al.* 1965 Improvement of visual acuity and contrast vision in healthy subjects The global light threshold of the retina and at thresholds at 10 discrete points were determined before and then 1 hour and 2 hours after a single 600 mg dose of BE.	Non randomized Parallel Placebo controlled Acute single treatment: 600 mg: **Duration**: 2 hours (acute single treatment) Determination of the light threshold at: 1. Before treatment 2. After 1 hour 3. After 2 hours	Bilberry fruit extract (no further detail) 600 mg	Bilberry fruit extract (no further detail) 600 mg No. of patients enrolled: 60 No. of patients completed the trial: 60 Age = young (?) Sex = male Inclusion criteria: healthy, young male subjects with healthy vision Exclusion criteria: no data			

Non Randomized Studies Reduced Light Conditions						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Alfieri and Sole 1966 The effect of anthocyanosides on adaptation of electroretinogram on red light in healthy patients.	Non randomized Double-blind Placebo controlled *Verum*: 6 patients Placebo: 6 patients	Anthocyanosides not specified + beta-carotene. Dose: 2.88 g Oral administration (per-lingual)	Anthocyanosides not specified + beta-carotene. No. of patients enrolled: 12 No. of patients completed the	Time to point α ~9 min in treated subjects was reduced to ~ 6.5 min. The mean time to point α was maintained between 1-hour and 3-	ANOVA	Comment: Administration of anthocyanosides in very high dose facilitates retinal adaptation in

Red filter Wratten Kodak No 26 was used to aid separation of phototopic (b1) and scototopic (b2) traces.	Duration: Single acute administration Recording of electroretinograms after 1 and 3 hours after administration of anthocyanosides		trial: 12 *Verum* = 6 subjects Placebo = 6 Inclusion criteria: The wakeful patients with the diameter of the pupil remaining within the limit of 6 – 8 mm. Exclusion criteria: not known	hour post tests with no significant differences between two tests. at 1h and 3h ($p<0.01$) ANOVA showed significant differences between the results of electroretinogram *verum* and placebo group: 6.5 min versus 9.0 min [$F=11.55$]. Post-treatment electroretinogram values of b2/b1were higher at each time point during the 15 minutes of dark adaptation monitoring.		accordance with the opinion of the authors possibly as a result of the prompt regeneration of rhodopsin.

Non Randomized Studies **Absolute visual threshold (the complex formulation)**						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Gloria and Perla (1966) Effects of anthocyanosides on the absolute visual threshold	Non randomized Open study A single administration of anthocyanosides + beta-carotene. The absolute visual threshold was recorded in 5 patients with the method of constant stimuli. Duration: Single administration	Anthocyanosides from "ophtalmological preparation for improving vision" + beta- carotene Doses: not known	Anthocyanosides not specified + beta-carotene. No. of patients enrolled: 5 No. of patients completed the trial: 5 *Verum* = 5 subjects Inclusion criteria: not known Exclusion criteria: not known	An improvement was registered in the absolute threshold of about 2 log. units in 4 patients.	No statistic reported	

Non Randomized Studies Myopia						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Contestabile *et al.* 1991 Electrophysiological response in myopic patients after prolonged treatment with high dosage of bilberry anthocyanosides.	Open study Not controlled study No control group Variations of the critical central retinal fusion frequency with recording of electroretinograms (Filter Kodak Wratten) before and after therapy Duration: 90 days	*V. myrtillus* extract (no further detail) 1 capsule = 160 mg of anthocyanosides Oral administration Dosage: 2 capsules daily for 90 days	*V. myrtillus* extract Oral administration Dosage: 2 capsules daily for 90 days No. of patients enrolled: 26 No. of patients completed the trial: 26 (total 52 eyes) *Verum*=26 subjects Inclusion criteria: Myopia 1st group=34 eyes (6 Dioptres) 2nd group=18 eyes (18 >6 Dioptres)	The 90 days treatment resulted in significant improvement of the scotopic function in all patients ($p<0.01$) and an improvement of photopic function in the group of subjects suffering from myopia of light and medium degree (central fusion frequency: $p<0.005$; $p<0.01$).	Student T-test Accepted $p<0.05$	Prolonged 90 days treatment with BE in myopic patients resulted in improvement in scotopic and photopic function.

Non Randomized Studies Reduced Light Conditions						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score. e.g. Jadad score	Comments
Magnasco and Zingirian 1966 Anthocyanosides impact on diversity in the visual field of	Non Randomized Controlled placebo Crossover study Static	*V myrtillus* extract (no further detail) Dose: not known	No. of patients enrolled: 16 No. of patients completed the trial: 16	Pre-post improvements with *verum* in all subjects: Reduced threshold in foveal and	No data	A significant lowering of visual threshold or enlargement of the visual plateau was

normal subjects adapted to mesopic light.	perimetry to plot variations in retinal threshold at a series of points in the visual field of 16 normal subjects adapted to mesopic light was used. Acute, single treatment. Subjects were tested at baseline and 4 hours after treatment. Duration: ? Washout period: ? Placebo and active treatment were given on separate days.		Inclusion criteria: Healthy subjects Differential mesopic retinal threshold at points 0–180 to the meridian. Exclusion criteria: not known	perifoveal regions (n = 3) ~20% ($p < 0.01$), enlarged mesopic plateau without change in level, (n= 1) 10^0 nasally, 4^0 temporally. Global elevation of mesopic plateau, (n = 6) 20–30% increase in sensitivity. Increased sensitivity of the central-pericentral plateau, (n = 6) 20–50% and enlarged plateau 3–13. nasally & 4–10. temporally. Non-significant in pre-post measures with placebo.		seen in all subjects 4 hours after active treatment. The largest effect was observed in the pericentral area.

Non Randomized Studies Reduced Light Conditions (the complex formulation)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Belleoud *et al.* 1967 Change of the scotopic light threshold after 30 minutes of dark adaptation measured before and after a night flight.	Non Randomized Double blind Controlled placebo. Scotopic light threshold after 30 minutes of dark adaptation was measured before and after a night flight. After pre-testing and 4 hours before take-	Bilberry fruit extract (no further detail)+ 5 mg beta-carotene 1 coated tablet contains 100 mg of anthocyanidins from bilberry and 5.0 mg beta-carotene Four100 mg capsules, (400 mg of the BE + 20 mg of beta-carotene daily).	No. of patients enrolled: 40 No. of patients completed the trial: 40 Inclusion criteria: Healthy subjects 20 jet pilots 20 helicopter pilots An entry requirement for personnel in these squadrons was	At pre-test 13 of the pilots in the experimental group had scotopic thresholds categorized as excellent (1.12 B/hm^2) and showed only small reductions in threshold at post test. The seven pilots with mediocre scotopic thresholds at pre-test showed	Statistics (?)	Experimental subjects reported an improvement in their ability to adapt to reduced light. All experimental subjects (including controls) reported a reduction in after-image effects caused by dazzling

	off, subjects were given either two *verum* tablets or placebo. 90 minutes before flight they received two additional tablets. Night flight lasted 1-2 hours (post tests after landing). Number of the flight hours: 1 – 5.5		that their night vision threshold should be 1.12 B/hm^2 (10 Cd/m^2) is the currently recognized value) or less but this threshold varies with fatigue. Exclusion criteria: not known Dosage: 4 Difrarel tablets	significant improvements at post-test with thresholds falling to 1.12 B/hm^2 or less with an average reduction of 0.30 B/hm^2 (0.18–0.67 B/hm^2). All experimental subjects, including 5 who had flown for more than 4 hours that day, reported a reduction in visual fatigue and a slight feeling of euphoria, effects not reported by control subjects. No changes in controls with initial low thresholds. Personal reports: reduced and shorter-lived post-dazzling after-images, reduced visual fatigue, improved dark adaptation.		

Non Randomized Studies Reduced Light Conditions (the complex formulation)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Sala *et al.* 1979 Improvement of night vision in railway night workers with normal	Non Randomized Double blind Controlled placebo. Probably parallel design ? Duration: 7 days ? Dark	Anthocyanosides (Source: *V myrtillus*?)	Anthocyanosides (Source: *V myrtillus*?) Daily dose: 300 mg/day No. of patients enrolled: 46 No. of patients completed the trial: 46	Dark-adaptation curve (ERG), initial and final thresholds were reduced after treatment at 3 and 7 days ($p < 0.05$). Time to point α: non-significant	Statistics ? ANOVA $P<0.05$	In night time working railway workers the dark – adaptation was significantly improved after 3 and 7 days of administration of anthocyanosides.

vision.	adaptation Mesopic light threshold Recovery speed after dazzling Photochromatic interval for violet Photochromatic threshold for violet light		Inclusion criteria: Healthy subjects. railway night workers with normal vision. *Verum*: 3 days (N = 24) *Verum*: 7 days (N = 20) Placebo: N = 20	Mesopic light threshold: non-significant Recovery speed after dazzling: non-significant Photochromatic interval for violet: non-significant Photochromatic threshold for violet light: lowered (Significance ?)		

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Coget and Merlen 1968 Determination of the impact of *V. myrtillus* anthocyanosides on the fragility of blood vessels	Open observational study Performed on 27 patients with symptoms of capillary fragility. Duration: 2 months Primary endpoints: Reduction of the number of petechiae on skin after treatment with anthocyanosides of *V. myrtillus*. They are the result of minor haemorrhage (tiny hemorrhagic spots < 3 mm). Prior to administration of the drug in	Bilberry fruit extract 1 tabl = 20 mg anthocyanosides	Dose: 4 – 6 tabl/daily 1 tabl = 20 mg anthocyanosides Therapy: 10-15 days in a month, 4 – 6 tablets daily. No. of patients enrolled: 27 No. of patients completed the trial: 27 Inclusion criteria: petechiae on skin No control group Age: 19 – 72 years Sex: 1 males	Reduction of the number of petechiae on skin after treatment with anthocyanosides.	Nonparametric subjective evaluation	According to the study the use of anthocyanosides for two months induced positive effect and reduced the number and size of petechiae. This assessment, however, was made subjectively, without a control group, and no statistical validation.

	patients induced formation of the control petechiae by the pressure of the piston (30 mg Hg for 60 seconds on the skin. Then, they were compared with the size and intensity after 2 months of therapy.		and 26 females			

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e. e.g. Jadad score	Comments
Mian 1977 Reduction of symptoms of ulcerative dermatitis due to post thrombophlebitis	Open Study Non controlled study On the base of the prior observations that indicated the formation of complexes of anthocyanosides and some phospholipids, investigated the changes brought about by the local and the systemic administration of anthocyanosides: 1) The foreign body granuloma formation 2) the composition of the protein fractions in	*V. myrtillus* extract (BEM)	*V. myrtillus* extract (BEM) Dose: 240 mg/day No. of patients enrolled: 15 No. of patients completed the trial: 15 Inclusion criteria: symptoms of ulcerative dermatitis due to post thrombophlebitis	BEM administration reduced the protein content of the exudate produced by venous occlusion and stasis. In the examined patients there was a substantial increase in the number of the newly formed capillaries and collagen fibrils produced by anthocyanosides.	No data	The biochemical and histochemical data show that the BEM protect the capillary walls with a double mechanism: 1. Increase of the endothelial barrier by stabilizing the membrane phospholipids 2. Increase of the biosynthesis of acid mucopolysaccharides of the total matrix, by restoring mucopolysaccharidic precapillary sheath. This latter effect could explain the substantial increase in the newly formed capillaries and collagen fibrils produced by anthocyanosides.

	exudate from the capillaries the granulation tissue, growing on the varicose veins leg ulcers. Duration: 10 days					

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Gatta. 1988 Improvement of symptoms of venous insufficiency	Single blind Placebo controlled study Duration: 30 days Prospective reduction of symptoms: 1.Oedema 2.Sensations of pressure 3. Parestesia 4. Cramp-like pain	*V. myrtillus* extract (BEM) Tablet = 80 mg	*V. myrtillus* extract Tablet = 160 mg Dosage: 80 mg 3 x2 per day (total 480 mg daily) No. of patients enrolled: 60 No. of patients completed the trial: 60 Inclusion criteria: venous insufficiency Placebo control group Age: mean 44 years	As a result of administration of BEM for 30 days in all patients was achieved a significant ($p<0.01$) reduction in the intensity of the symptoms: 1.Oedema 2.Sensations of pressure 3.Parestesia 4.Cramp-like pain	ANOVA (?)	In all patients was achieved a significant reduction in the intensity of the symptoms of venous insufficiency after treatment with BEM.

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments

Allegra *et al.* 1982 Decrease of symptoms of oedema, paresthesia and in patients with Raynaud's disease.	Double blind Placebo controlled study Prospective decrease of symptoms of oedema, paresthesia and pain while increasing joint mobility in patients with Raynaud's disease. Duration: 30 days	*V. myrtillus* extract (BEM)	*V. myrtillus* extract Dosage: 480 mg/day No. of patients enrolled: 47 No. of patients completed the trial: 47 Inclusion criteria: peripheral vascular disorder Placebo control group	In treated patients the decrease of oedema, paresthesia, and pain was observed while increasing joint mobility.	Statistics ?	In patients receiving BEM the decrease of symptoms of peripheral venous insufficiency was observed.

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency induced by pregnancy)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Grismondi 1981 Improvement of symptoms of peripheral venous insufficiency induced by pregnancy	Estimation of the reduction of symptoms of burning, itching, heaviness of legs and pain, reduction of diurnal and nocturnal cramps and oedema before, and at 30, 60 and 90 day of treatment. Duration: 60 – 90 days	*V. myrtillus* extract (BEM) containing 25% of anthocyanosides Tablet = 80 mg	*V. myrtillus extract* (BEM) Tablet = 80 mg Dosage: 320 mg daily (4 tablets) started in 6th month of pregnancy No. of patients enrolled: 54 No. of patients completed the trial: 54 Inclusion criteria: peripheral vascular disorder	Improvements in burning and itching ($p<0.001$), heaviness ($p<0.001$), and pain ($p<0.001$) were observed in Patients receiving BEM, as well as in diurnal and nocturnal cramps ($p<0.01$), and a reduction in oedema and in capillary fragility ($p<0.001$).	Student T-test for independent groups	In 54 pregnant patients BEM administration significantly reduced symptoms of peripheral venous insufficiency.

Non Randomized Studies Treatment of disorders of the circulatory system (e.g. polyneuritis and altered microcirculation)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Pennarola *et al.* 1980 Improvement of peripheral circulation disturbances induced by toxicity of industrial adhesives.	No control group No placebo group Examination of ungual capillary bed by capillaroscopy and by use of pletysmographic and thermographic techniques. Duration: 60 days.	*V. myrtillus* extract (BEM) containing 25% of anthocyanosides Tablet = 80 mg	*V. myrtillus* extract (BEM) Tablet = 80 mg Dosage: 80 mg daily for 60 days No. of patients enrolled: 15 No. of patients completed the trial: 15 Sex: female Age: 18 – 34 years Inclusion criteria: polyneuritis induced by industrial adhesives Exclusion criteria: no data	Treatment with BEM significantly improved microcirculatory disturbances ascertained with capillary, thermographic and plethysmographic methods.	No data	After administration of BEM for 60 days, 15 patients with polyneuritis arising out of professional contact with adhesives significant clinical improvement was observed.

Randomized Studies Treatment of disorders of the circulatory system (e.g. complications and side effects of the contraceptive intrauterine devices use)						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Cerutti *et al*. 1984 Reduction of side effects of the contraceptive	*Verum* group Control group Prevention of "minor side effects" of the	*V. myrtillus* extract (BEM) containing 25% of anthocyanosides	*V. myrtillus* extract (BEM) Tablet = 80 mg Dosage: 320	Using in the *verum* group during six months BEM decreased the occurrence of "minor side	No data	In BEM users decreased incidents of spotting and hyper-poly-menorrhea were observed

intrauterine devices	copper IUD contraception as spotting and hyper-poly-menorrhea. Duration: 6 months	Tablet = 80 mg	mg daily for 6 months No. of patients enrolled: 96 No. of patients completed the trial: 96 *Verum* group: n = 48 Control group: n = 48 Sex: female Age: 19 – 48 years Inclusion criteria: contraceptive intrauterine devices use Exclusion criteria: no data	effects "of copper IUD contraception as spotting and hyper-poly-menorrhea.		as side effects of contraceptive intrauterine devices

Controlled study **Dry eye syndrome (DES) (keratoconjunctivitis sicca)**						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Anderson *et al.* 2011 Reduction of the dry eye syndrome (DES) (keratoconjunctivitis sicca)	Placebo controlled study Patients with self-reported symptoms of dry eye were assessed at baseline by the Ocular Surface Disease Index (OSDI), TBUT, and Schirmer's test. Pre-test and post-test reported	Bilberry (extract ?) Capsules 80 mg	Bilberry (extract ?) Capsules 80 mg Dosage: 80 mg orally twice daily for 30 days No. of patients enrolled: 22 No. of patients completed the trial: 19 *Verum* group: n = 13	Mean OSDI baseline for bilberry patients was 22.8, SD ± 611.0 with post-treatment values of 13.7, SD ± 68.5 (p 0.01). Baseline for placebo OSDI was 27.9, SD ± 626.8 with post-treatment values of 19.6, SD ±	Mann- Whitney 2-tailed test.	Bilberry OSDI group showed a statistically significant improvement (p , 0.01)

	symptoms, tears break-up and increased tear production. Duration: 30 days		Placebo group: n = 6 Sex: 14 males, 8 females Age: 23 – 59 years (mean = 29.9 years) Inclusion criteria: Patients with self-reported symptoms of dry eye Exclusion criteria: no data	620.0 (p 5 ns). Bilberry baseline measures were TBUT 5.6, SD ± 61.0 and Schirmer 16.0, SD ± 611.2 with post-treatment values of TBUT 7.4, SD ± 63.4 and Schirmer 12.2, SD ± 610.0. Placebo patients' baseline was TBUT 10.1, SD ± 64.9 and Schirmer 10.4, SD ± 611.4 with post-treatment of TBUT 8.8, SD ± 62.7 and Schirmer 10.8, SD ± 69.8.		

Normal tension glaucoma						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Shim *et al.* 2012 Treatment with BE on visual function in patients with normal tension glaucoma.	Retrospective open study with a chart review from Outpatients from the Department Ophtalmology with previous 12-month follow-up. *Verum* group Control group Duration: mean 24.8 ± 10.4 months (range: 12 -59 months)	*V. myrtillus* extract (no further detail), capsule = 60 mg (anthocyanins?)	*V. myrtillus* extract, capsule = 60 mg (anthocyanins ?) Dosage 60 mg orally twice daily No. of patients enrolled: 229 No. of patients completed the trial: 229 Sex: both sexes (no data) *Verum* group: n = 132 Control group: n = 97 Age: 20 – 89	After *V. myrtillus* extract treatment: 1.BCVA (mean) for all eyes improved from 0.16 (–0.34) to 0.11 (–0.18) log MAR units (P = 0.008) 2. HVF mean deviation improved from - 6.44 (–7.05) to -5.34 (–6.42) (P = 0.001). The results	ANOVA Paired t-tests p<0.05	The results of the long treatment with the *V. myrtillus* extract demonstrated that the final BCVA was significantly affected by systemic treatment (p< 0.001).

	Humphrey Visual Field (HVF) test, logarithm of the minimal angle of resolution best-corrected visual acuity (log MAR BCVA), intraocular pressure, blood pressure, and fasting blood glucose were determined before and after treatment.		years (mean = 52.9 ± 13.7 years) **Inclusion criteria**: Patients with a diagnosis of NTG, defined as optic disc abnormalities consistent with glaucomatous optic neuropathy with or without visual field loss. 1. Intraocular pressure (IOP) of 21-mm Hg or less without topical hypotensive therapy 2. 20 years or older; 3. logarithm of the minimal angle of resolution best-corrected visual acuity (log MAR BCVA) 0.70 or better; 4. no history of amblyopia; 5. no history of ocular or neurologic disease or surgery; 6. mental and physical capacity to perform the tests **Exclusion criteria**: 1. active ocular disease; 2. use of other ocular medications; 3. ocular surgery; 4. use of other similar systemic medications	were not affected by different demographics between the groups (age P =0.402, diabetes mellitus P =0.114 and hypertension P =0.357). No ocular or systemic side effects were noted.		

Modulation of inflammatory markers in subjects at increased risk of CVD						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Karlsen *et al.* 2010 The effect of bilberry juice supplementation on biomarkers of inflammation (NF-κB) in men and women with elevated levels of at least one CVD risk factor.	Randomized parallel controlled trial *Verum* = bilberry juice Control = water Endpoint: change in the plasma concentration of C-reactive protein (CRP), interleukin (IL)-6, IL-15, and monokine induced by INF-γ (MIG) and TNF-α. Duration: 4 weeks	*V. myrtillus* juice prepared by steam processing of fresh fruits, no sugar additives added.	*V. myrtillus* juice prepared by steam processing of fresh fruits, no sugar additives added. Dosage: 330 ml diluted to 1 l with water daily No. of patients enrolled: 62 No. of patients completed the trial: 62 *Verum* group: n = 31 (21 men, 10 women) Control group: n = 31 (25 men, 7 women) Age: Men 30 – 70 years; women 45 – 70 years Inclusion criteria: subjects with elevated risk of CVD if at least one of the criteria was fulfilled: 1. systolic blood pressure (BP) between 135 and 160 mmHg; 2. diastolic BP between 90 and 100 mmHg; 3. low-density lipoprotein (LDL) cholesterol ≥ 3.4 mmol/l; 4. total/high-density lipoprotein (HDL) cholesterol ratio > 4;	After 4 weeks administration of bilberry juice the values for CRP were significantly decreased (p= 0.027); as for IL15 (p = 0.037); for IL15 (p = 0.008) and for MIG (p = 0.047). The values for the TNF-α were significantly increased (p = 0.017). Significant increase of the concentrations of quercetin and p-coumaric acid in the bilberry group as compared with the water group (p = 0.029 and p = 0.016, respectively) was found.	Student T-test Mann-Whitney non-parametric test (for non-normally distributed data)	The four weeks lasting increased intake of polyphenols from bilberry juice induced modulation of NF-kB inflammatory markers in subject at increased risk of CVD. It resulted in significant decreases in plasma concentrations of C-reactive protein (CRP), interleukin (IL)-6, IL-15, and monokine induced by INF-c (MIG). Unexpected increase of the concentration of the tumour nuclear factor-α (TNF-α) was found. The *V. myrtillus*polyphenols may modulate the inflammation processes.

			5. elevated haematocrit (≥ 0.40 from women or ≥ 0.42 for men); 6. smoking a minimum of three cigarettes daily. **Exclusion criteria**: 1. chronic diseases - impaired renal function, diabetes mellitus, CVD, liver, gastrointestinal disease or cancer within the last 5 years; 2. use of lipid-lowering drugs, diuretics, or hormone replacement therapy for women; 3. subjects with a BMI > 31, whose alcohol consumption was [3 units/day for men or >1 unit/day for women and subjects who had donated blood within the last 6 months.			

Reduction of inflammation in subjects with metabolic syndrome						
Study Aim of the study	Design Primary endpoint(s) / objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, nclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Kolehmainen *et al.* 2012 The influence of *V. myrtillus* on inflammation and gene expression profile in	Randomized controlled trial	*V. myrtillus* fresh and dried fruits	*V. myrtillus* fresh and dried fruits. **Dosage**: Equivalent dose of 400 g fresh bilberries comprising of 200 g of mashed bilberries and 40 g of dried	After 8-weeks treatment with bilberry an inflammation score was significantly different between the groups (p =0.024). In transcriptomics analyses (3	The normality of distributions of the study variables was tested with the Kolmogorov–Smirnov test with Lilliefors' significance correction. Paired	Long term (8-weeks) administration of *V. myrtillus* results in reduction of low-grade inflammation showing decreased cardiometabolic risk.

peripheral blood mononuclear cells in subjects with metabolic syndrome.	**Duration**: 16 weeks (4-week run-in, 8-week dietary intervention, and 4-week recovery periods).		bilberries (eq. 200 g of fresh bilberries). The anthocyanin content of the mashed and dried berries were 524 and 832 mg/100 g, respectively, and the flavonol contents were 12 and 31 mg/100 g, respectively (expressed as the weight of the aglycone moiety of these flavonoid glycosides). Administration for 8 weeks. No. of patients enrolled: 34 No. of patients completed the trial: 27 **Verum** group: n = 15 (5men, 10 women) **Control** group: n = 12 (3 men, 9 women) **Age**: Men 30 – 70 years; women 45 – 70 years **Inclusion criteria**: overweight (BMI 26–39 kg/m^2), and two of the following: 1. elevated fasting plasma glucose in the absence of diabetes (5.6–6.9 mmol/l), 2. abnormal fasting serum lipids (triglycerides ≥1.7 mmol/l; 3. HDL cholesterol<1.0 mmol/l (males) or <1.3 mmol/l (females)); 4. waist circumference >102 cm (males) or >88 cm	subjects with improved oral glucose tolerance test in the bilberry group), Toll-like receptor signaling, cytoplasmic ribosomal proteins, and B-cell receptor signaling pathways were differently regulated. QPCR analyses (n =13 and 11 in the bilberry and control groups, respectively) showed decreased expression of MMD and CCR2 transcripts associated with monocyte and macrophage function associated genes. The bilberry supplementation tended to decrease serum high-sensitivity C-reactive protein, IL-6, IL-12, and LPS concentrations. No differences between the groups were found in body weight, glucose, or lipid metabolism,	samples t-test was used for comparing the measurements. Non normally distributed variables were analyzed using Wilcoxon nonparametric test for paired comparisons or by Mann–Whitney test to compare the results between the groups. Correlation analyses were done using Pearson's method. $P < 0.05$	

			(females); 5. blood pressure ≥130/85 mmHg **Exclusion criteria**: subjects with lipid lowering medications were excluded from the trial.			

Reduction of damaged protein biomarkers in diabetic overweight subjects						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Campbell *et al.* 2012 Reduction of the markers of oxidative stress in diabetic overweight subjects.	Pilot open study No control group Alterations of the activity of oxidised protein markers in the blood plasma of overweight/obese type 2 diabetic in comparison to pre-administration values. Determination of the three different oxidised protein markers in plasma samples: protein-pyrroles, protein-nitrotyrosine (3-NT) and oxidised LDL **Duration**: 3 weeks	Bilberry extract (BEM) containing 36% (w/w) anthocyanins formulated in gelatin capsules.	BEM **Dosage**: 1.4 g daily for 3 weeks No. of patients enrolled: 11 No. of patients completed the trial: 11 **Sex**: male Age: no data **Inclusion criteria**: overweight (BMI >25). Type 2 diabetes controlled by diet alone	Administration of BEM for 3 weeks produced significant changes in the levels of: 1. Pyrroles (nmol/mg protein - 9.3% of change; $p<0.05$); 2. Protein-3 NT (U/µg NT-BSA – 9.1% of change; $p<0.05$); 3. Oxidised LDL (U/l) – 14.4% of change; $p<0.05$)	T-test for paired samples $P<0.05$	Three weeks of administration of BEM resulted in a significant reduction in three sensitive markers of oxidative stress in the plasma of overweight/obese type 2 diabetics.

Ulcerative colitis treatment						
Study Aim of the study	Design Primary endpoint(s)/ objective(s) Duration of study	Herbal preparation Pharmaceutical form	Posology Study population (n°, age, sex, inclusion criteria), drop out	Outcomes (primary and secondary)	Statistical analysis (e.g. ITT yes/no, C.i.95%) Quality score e.g. Jadad score	Comments
Biedermann *et al.* 2013 Treatment of the mild to moderate ulcerative colitis (UC) **Screening:** 1 week Full medical history, physical examinations, lab screen CAI score(Clinical Activity Index); Mayo Score, Short Inflammatory Bowel Disease Questionnaire (SIBDQ) The treatment period was 6 weeks In the meantime visits at week: 3, 5, 7 and final follow-up between weeks 9 and 11.	Open pilot trial **Primary endpoint**: Achievement of remission (CAI<4) at the end of treatment. **Secondary endpoints**: Response (reduction of CAI ≥ 3 or remission), 2 point improvement of endoscopic Mayo Score, improvement of SIBDQ and improvement of CAI Duration: 6 weeks **Determination during study**: 1.Fecal calprotectin 2. Sigmoidoscopy with biopsy (twice – at 1 week and 7 week) 3.Biopsies from all patients from the rectum and sigmoid colon	Dried sieved bilberries (56.63%) and concentrated bilberry juice (25.9%)	Dried sieved bilberries (56.63%) and concentrated bilberry juice (25.9%). **Dosage**: daily dose of 160 corresponding to 95 g dry weight (corresponding to an amount of around 600 g of fresh fruit, assuming a water content in fresh bilberries of 80–85%)(average anthocyanin dose of 840 mg No. of patients enrolled: 13 No. of patients completed the trial: 13 Sex: male **Age**: 18 – 55 years **Inclusion criteria**: 1. Established diagnosis of UC for at least 6 month 2. Mild to moderate disease activity (clinical activity index (CAI): 4–8) 4. A stable use of medication: with 5-ASA, thiopurines, therapeutic antibodies and of corticosteroids for at least 3 months and 4 weeks prior to inclusion, respectively **Exclusion criteria**: 1.An intake of drugs or natural products	After the 6 week treatment interval 63.4% of patients achieved remission, the primary endpoint, while 90.9% of patients showed a response. In all patients a decrease in total Mayo score was detected (mean: 6.5 and 3.6 at screening and week 7, respectively; $p < 0.001$). Fecal calprotectin levels significantly decreased during the treatment phase (baseline: mean 778 µg/g, range 192–1790 µg/g; end of treatment: mean 305 µg/g, range <30–1586 µg/g; $p = 0.049$), including 4 patients achieving undetectable levels at end of treatment. The decrease of endoscopic Mayo score and histologic Riley index confirmed the beneficial	Wilcoxon rank-sum test Friedman-test or the paired t-test P <0.05	The study resulted in promising therapeutic potential of a bilberry preparation anthcyanins rich in ulcerative colitis in humans. However randomised controlled trials are expected !

			including bilberries, grapefruit and quinine containing drinks with known interference with CYP3A or CYP2D6 in the last two weeks prior to inclusion 2.HIV, Hepatitis B and C infection, abscesses, elevation of CRP over 100 mg/l, known intolerance to anthocyanin or related substances, or a simultaneous participation in another clinical trial within the last 30 days prior to inclusion	effect. However, an increase of calprotectin levels and disease activity was observed after cessation of bilberry intake. No serious adverse events were observed.		

4.3. Clinical studies in special populations (e.g. elderly and children)

No data available

4.4. Overall conclusions on clinical pharmacology and efficacy

Bilberry fruits are traditionally used for diarrhoea, dyspepsia and in the conditions of increased fragility of blood vessels and chronic venous insufficiency. Bilberry intake is claimed to produce improvement of microcirculation and lymphatic drainage improvement.

Anecdotal explanations start back to World War 2, when British pilots supposedly ate bilberry jam before the night flights. This information was not confirmed however, and is likely to mask the real reason for military success-the secret of radar technology (Kramer 2004).

Numerous clinical trials have been carried out since the early 1960s, involving the treatment of vascular fragility and improvement of vision. Until today are not known the modern-controlled, randomized clinical trials that would have confirmed the benefits of using products containing *Vaccinium myrtillus* in people with good vision. Such studies have been carried out just 50 years ago, with well-known methodological limitations (Camire 2002).

The systematic review of clinical studies dedicated to improving vision in conditions of reduced light (Canter and Ernst 2004) does not provide clear conclusions. Of the 12 studies with placebo, five studies were randomized. Unfortunately, four of these recent studies gave negative results. The only positive study (Jayle and Aubert 1964) showed an increase in the area of the visual field. In this study patients received, however, a complex product containing BE and beta-carotene.

In most discussed publications the age of the patients varied greatly from young to advanced age. It is known, however, that normal night vision begins to decline in middle age. In addition, it has not been mentioned anywhere at what time of day research was carried out.

It is recommended to conduct rigorous clinical trials in the face of criticism that discredited the former clinical studies devoted to the night vision (Canter and Ernst 2004).

The problem of stability of anthocyanins is essential, as improper storage leads to their degradation. It is especially relevant when carrying out long, ongoing clinical trials.

5. Clinical Safety/Pharmacovigilance

5.1. Overview of toxicological/safety data from clinical trials in humans

No data available

5.2. Patient exposure

Bilberry is often used in self-treatment. Recent epidemiological data from Scandinavia estimate that it is the most commonly used herbal product co-used every day with conventional drugs in Norwegian general practice. Almost 40% of patients on anticoagulants used garlic and bilberry. The patients were mostly female, above of 70 years with chronic diseases. Only 50% of patients informed physician on using herbal products together with other treatment (Djuv *et al.* 2013).

5.3. Adverse events and serious adverse events and deaths

No cases of death were reported.

5.4. Laboratory findings

Very limited data are available.

5.5. Safety in special populations and situations

5.5.1. Use in children and adolescents

Particular use in children has not been reported. Therefore, the use in children up to 6 years is not recommended.

According to the instruction of the package leaflet of the products marketed in Poland and in Germany the use in children of comminuted dry bilberry fruit is not recommended.

In Germany the use of in children up to 2 years is contraindicated.

5.5.2. Contraindications

None reported

5.5.3. Special Warnings and precautions for use

When used to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances, if there is inflammation of the skin, thrombophlebitis or subcutaneous

induration, severe pain, ulcers, sudden swelling of one or both legs, cardiac or renal insufficiency, a doctor should be consulted.

5.5.4. Drug interactions and other forms of interaction

Peris *et al.* (2008) described the case of the patient (age and sex not stated) after the 6-month follow-up. They observed intensification of the oral anticoagulant action expressed by an increased value of the international normalized ratio (INR 16.6, the correct values in patients not treated 0.8 - 1.2). Unfortunately, dosages, duration of treatments, reaction onset and therapeutic indication were not stated.

Paoletti *et al.* (2011) reported the case of the reduction to 1.6 of INR (time to onset 4 days) in 80 years old woman, who was receiving long-term oral anticoagulant therapy with warfarin (27.5 mg/week for 1.5 year) and received bilberry juice (200 ml/day, indication not reported). Herbal drug was withdrawn, warfarin dosage increased to (32.5mg/w) and patient completely recovered. According to the Naranjo adverse drug reaction (ADR) probability scale, authors classified the probability that an adverse event is related to drug therapy (possible value of 4). In this case, the reduction activity of warfarin is surprising, since bilberry anthocyanosides, having antiplatelet properties should increase the risk of bleeding with the concomitant use of an anticoagulant therapy. The mechanism of such a reciprocal interaction is unclear.

Aktas *et al.* (2011) published a case history of the 77 year old man with hypertension, who consumed for 5 years large amounts of bilberries. The patient for 6 years suffered from hypertension, and because it was found recently that he has an atrial fibrillation and a year before he had a stroke, he received an anticoagulant treatment. In emergency he sought medical assistance because of rectal bleeding and dizziness, which occurred 16 days after introduction of warfarin therapy.

His prothrombin time (PT) was 110.5 seconds, INR = 15.0, and the activated partial thromboplastin time (APTT) 76.4 seconds. He was given intravenous plasma, but the next day he returned with abundant haematuria and dizziness. His INR was 6.24, and the prothrombin time (PT) was 55.7 seconds. So again he was the subject of further hospitalization.

Despite the presented anecdotal cases it has not been possible to prove a genuine threat of interactions with anticoagulants and antiplatelet agents at the recommended dose of bilberry preparations.

5.5.5. Fertility, pregnancy and lactation

No adverse effects were registered in more than 200 women receiving BEM equivalent to 57 – 320 mg/kg/day anthocyanins for 60 to 102 days during pregnancy for venous complaints, including haemorrhoids in several non controlled studies (quoted after review of Morazzoni and Bombardelli 1996).

However the safe use of BE and BEM in pregnancy and lactation has not been adequately investigated and established. In the absence of sufficient data, the use during pregnancy and lactation is not recommended.

No concern has arisen about any malformation in humans, following the consumption of dried bilberry fruit.

No fertility data available.

5.5.6. Overdose

No cases of overdose have been reported

5.5.7. Effects on ability to drive or operate machinery or impairment of mental ability

No data available.

5.5.8. Safety in other special situations

Not applicable

5.6. Overall conclusions on clinical safety

Some anecdotal reports presented in section 5.4.4. do not provide real threat to confirm interactions with anticoagulants and antiplatelet drugs at the recommended doses of bilberry. However, some attention is needed in patients treated with antiplatelet agents or anticoagulants.

6. Overall conclusions

The results of clinical data available for bilberry fruit preparations are not considered sufficient to support a well-established use indication.

The systematic review, conducted in 2004 by Canter and Ernst, of clinical trials designed to improve vision in conditions of poor light with preparations of bilberry fruits did not provide clear conclusions. Out of 12 studies with placebo, only five studies were randomized. Unfortunately, four of these recent studies gave negative results. The only positive study (Jayle and Aubert 1964) showed an increase in the area of the visual field. In this study patients received, however, a complex product containing BE and beta-carotene.

Several clinical studies on disorders of the circulatory system (e.g. altered microcirculation and peripheral venous insufficiency) were performed about 30 - 40 years ago and were not randomized. Hence, despite the conviction of the beneficial effects of the anthocyanins therapy in cardiovascular diseases, the medicinal use of bilberry preparations is based on tradition.

All the requirements for TU (self-medication character, specified strength/posology, appropriate route of administration, period of traditional use, plausibility and safety) are met.

The traditional medicinal use of bilberry dried fruit and bilberry fruit extract has been documented in several medicinal handbooks with indications consistent with the existing pertinent pharmacological experiments performed *in vitro* and *in vivo* and it is substantiated by the presence of medicinal products on the European market.

The experimental toxicological data are limited, but given the history of long-term and present use in humans, also in food, there are no safety concerns for the oral or oromucosal use of decoctions from dried bilberry fruit and the oral use of the bilberry dry extract from fresh fruit.

Long-standing traditional medicinal use within the European community for at least 30 years according to Directive 2004/24/EC is therefore considered fulfilled for the following preparations and indications:

1) *V. myrtillus* L., fructus siccus (dry bilberry fruit), whole or comminuted

a) Traditional herbal medicinal product for symptomatic treatment of mild diarrhoea. Daily dose in adults and adolescents over 12 years as an herbal tea for oral use: 15 to 60 g, divided

in 3-4 single dose of 5 to 15 g in 250 ml as a 10 minutes decoction. Therapeutic area for browse search with TU indications: gastrointestinal disorders.

b) Traditional herbal medicinal product for symptomatic treatment of minor inflammations of the oral mucosa. It is used as a 10% decoction for oromocosal use to rinse the mouth several times daily. Therapeutic area for browse search with TU indications: mouth and throat disorders.

2) *V. myrtillus* L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins, in solid dosage forms for oral use the following indications:

a) Traditional herbal medicinal product to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.

b) Traditional herbal medicinal product to relieve symptoms of cutaneous capillary fragility.

Single dose: 80 -180 mg; Daily dose: up to 160 - 540 mg.

Therapeutic area for browse search with TU indications: venous circulatory disorders.

Whole or comminuted dried bilberry fruit

As a general precaution related to the therapeutic indication “for symptomatic treatment of mild diarrhoea”, the product information should include a warning text advising the patient to consult a doctor or a qualified health care practitioner if the symptoms worsen or persist longer than 3 days during the use of the product. In case of the oromucosal use “for symptomatic treatment of minor inflammations of the oral mucosa” the warning should refer to 1 week.

Bilberry fruit cannot be recommended for oral use in children under 12 years of age due to lack of adequate data.

No concern has arisen about any malformation in humans, following the consumption of dried bilberry fruit. They can be used during pregnancy and lactation if clinically needed. No data on fertility is available.

Fresh bilberry fruit dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins

The recommended duration of use of the fresh bilberry fruit dry extract “to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances” or “to relieve symptoms of cutaneous capillary fragility” is 4 weeks. However, if the symptoms persist for more than 2 weeks during the use of the medicinal product, a doctor or a qualified health care practitioner should be consulted.

The following warnings are included in section 4.4. of the monograph on bilberry dry extract: “If there is inflammation of the skin, thrombophlebitis or subcutaneous induration, severe pain, ulcers, sudden swelling of one or both legs, cardiac or renal insufficiency, a doctor should be consulted.

In the event of inadequate or unsatisfactory symptomatic response within 2 weeks, a doctor should be consulted as oedema may have alternative causes.”

Bilberry dry extract cannot be recommended for oral use in children and adolescents under 18 years of age due to lack of sufficient safety data.

Safety during pregnancy and lactation has not been established. In the absence of sufficient data, the use during pregnancy and lactation is not recommended. No fertility data is available.

On the basis of the available information anthocyanins are considered by the HMPC as contributing to the activity of the *V. myrtillus L.*, fructus recens dry extract (DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins) and therefore they might be used as active markers. Improper storage leads to their degradation.

A Community list entry is not supported due to lack of adequate data on genotoxicity.

Annex

List of references