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THE HEALTH EFFECTS OF SEA BUCKTHORN BERRIES AND OIL

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ABSTRACT

Sea buckthorn (*Hippophaë*) berries are ingredients of the Chinese traditional medicine. In addition to China, they are nowadays cultivated for food in several European countries, Russia, Canada, the USA, and Japan. Sea buckthorn berries are a rich source of flavonoids, mainly flavonol glycosides and proanthocyanidins. Depending on the genetic background, growth conditions, and ripeness of the berries, vitamin C concentrations up to over 1 g/100 ml juice, have been reported. Sea buckthorn berries contain inositols and methyl inositols, components of messenger molecules in humans. Sea buckthorn seed oil is rich in essential α-linolenic and linoleic acids, whereas the most abundant fatty acids in the berry oil are palmitoleic, palmitic and oleic acids. Other potentially beneficial lipophilic compounds of sea buckthorn seeds and berries include carotenoids, phytosterols, tocopherols and tocotrienols.

The effects of sea buckthorn fractions on inflammation, platelet aggregation, oxidation injuries, the liver, skin and mucosa, among others, have been reported. The aim of the thesis work was to investigate the health effects of sea buckthorn berries and oil in humans. The physiological effects of sea buckthorn berries, berry components, and oil have mostly been studied *in vitro* and in animal models, leaving a demand for more clinical trials.

In the first randomized, placebo-controlled trial of this thesis healthy adults consumed 28 g/day of sea buckthorn berries for three months. The main objective was to investigate the effects on the common cold. In addition, effects on other infections, inflammation and circulating lipid markers associated with cardiovascular disease risk were studied. In the second randomized, placebo-controlled trial participants reporting dry eye symptoms consumed 2 g/day of sea buckthorn oil from the seeds and berries for three months. The effects on symptoms and clinical signs of dry eye were monitored. In addition, the effects on circulating markers of inflammation and liver functions were analyzed.

Sea buckthorn berries did not affect the common cold or other infections in healthy adults. However, a decrease in serum C-reactive protein was detected, indicating effects on inflammation. Fasting concentrations of serum flavonols, typical to sea buckthorn berry, increased without affecting the circulating total, HDL, LDL cholesterol, or triacylglycerol concentrations.

Tear film hyperosmolarity and activation of inflammation at the ocular surface are among the core mechanisms of dry eye. Combined sea buckthorn berry and seed oil attenuated the rise in tear film osmolarity taking place during the cold season. It also positively affected some of the dry eye symptoms. Based on the tear film fatty acid analysis, the effects were not mediated through direct incorporation of sea buckthorn oil fatty acids to tear film lipids. It is likely that the fatty acids, carotenoids, tocopherols and tocotrienols of sea buckthorn oil affected the inflammation of the ocular surface, lacrimal and/or meibomian glands. The effects on the differentiation of meibomian gland cells are also possible. Sea buckthorn oil did not affect the serum concentrations of inflammation markers or liver enzymes investigated.

In conclusion, this thesis work suggests positive effects of sea buckthorn berries and oil on inflammation and dry eye, respectively, in humans.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AI	atherogenic index
ALAT	alanine aminotransferase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ASAT	aspartate aminotransferase
BHT	butylated hydroxytoluene
bw	body weight
cAMP	cyclic adenosine mono phosphate
CI	confidence interval
COX	cyclo-oxygenase
CRP	C-reactive protein
DFO	deferoxamine
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picry hydrazyl
eNOS	endothelial constitutive nitric oxide synthase
extr.	extract
FID	flame ionization detector
GPx	glutathione peroxidase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSH-Rd	glutathione reducatase
GT	γ-glutamyl aminotransferase
HDL	high density lipoprotein
hex	hexane
HPLC	high performance liquid chromatography
i.g.	intragastric
i.p.	intraperitonial
IU	international unit
i.v.	intravenous
ICAM-1	intracellular adhesion molecule-1
IgE	immunoglobulin E
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IRF-1	interferon regulatory factor-1
LDH	lactate dehydrogenase
LDL	low-density lipoprotein

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LOX	lipoxygenase
LOX-1	lectinlike low density lipoprotein receptor-1
MAPK	mitogen activated protein kinase
MDA	malonialdehyde
mOSDI	modified Ocular Surface Disease Index
NF-kB	nuclear factor-kB
NL	neutral lipid
NO	nitric oxide
NOAEL	No Observed Adverse Effect Level
OEA	oleoylethanolamide
ox-LDL	oxidized low density lipoprotein
PAE	N-palmitoyl ethanolamide
PBMC	peripheral blood mononuclear cell
PG	propylene glycol
PKA	protein kinase A, cAMP-dependent protein kinase
PL	phospholipid
PPARγ	peroxisome proliferator-activated receptor γ
REA	retinol activity equivalent
RNS	reactive nitrogen species
ROO·	peroxyl radical
ROS	reactive oxygen species
RR	relative risk
sb	sea buckthorn
ssp.	subspecies
SCORAD	SCORing Atopic Dermatitis
SNP	sodium nitroprusside
SOD	superoxide dismutase
SS	Sjögren's syndrome
STZ	streptozotocin
TAG	triacylglycerol
t-BOOH	*tert*-butyl hydroperoxide
TBARS	thiobarbituric acid reactive substances
TBUT	tear film break-up time
TNF-α	tumor necrosis factor-α

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LIST OF ORIGINAL PUBLICATIONS

1. Larmo P, Alin J, Salminen E, Kallio H & Tahvonen R (2008) Effects of sea buckthorn berries on infections and inflammation: a double-blind, randomized, placebo-controlled trial. **Eur J Clin Nutr 62, 1123-1130**

2. Larmo P, Yang B, Hurme S, Alin J, Kallio H, Salminen E & Tahvonen R (2009) Effect of a low dose of sea buckthorn berries on circulating concentrations of cholesterol, triacylglycerols, and flavonols in healthy adults. **Eur J Nutr, 48, 277-282**

3. Larmo P, Järvinen R, Setälä N, Yang B, Viitanen M, Engblom J, Tahvonen R, Kallio H (2010) Oral sea buckthorn oil attenuates tear film osmolarity and symptoms in individuals with dry eye**. J Nutr 140: 1462–1468**

4. Järvinen R, Larmo P, Setälä N, Yang B, Engblom J, Viitanen M, Kallio H. Effects of oral sea buckthorn oil on tear film fatty acids in individuals with dry eye. **Cornea. Accepted manuscript**

5. Larmo P, Järvinen R, Setälä N, Venojärvi M, Yang B, Viitanen M, Alanko H, Kallio H. Effects of sea buckthorn (*Hippophaë rhamnoides*) oil on aminotransferases and biomarkers of inflammation. **Submitted to J Food Sci**

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1 INTRODUCTION

The seven species of the small genus *Hippophaë*, family Elaeagnaceae, are called sea buckthorn. *Hippophaë rhamnoides* is geographically the most widely distributed species [204]. It is also the most variable one having eight subspecies (ssp.), including the commercially important ssp. *sinensis* (Chinese subspecies), ssp. *rhamnoides* (European) and ssp. *mongolica* (Russian) [204, 238]. The other rarer species grow in high altitudinal areas in China, Tibet and Nepal [204]. In addition to the naturally growing species and subspecies of sea buckthorn, several commercial varieties and cultivars exist [36, 204, 238].

Sea buckthorn has been used in Eastern traditional medicine for centuries. In the Chinese Pharmacopeia, sea buckthorn berries are prescribed for relieving cough and for promoting digestion and blood circulation [39]. In Central Nepal, where only few people have access to modern medicine, sea buckthorn is among the medicinal plants with the widest spectra of use indications [217]. These include cough, diarrhoea, menstrual, and stomach disorders [217]. Nowadays it is known that sea buckthorn berries and leaves are rich in bioactive compounds, and their health effects are studied scientifically. A considerable number of studies have been carried out in China, Russia, and other Asian countries and are not published in English.

Despite their use in traditional medicine, sea buckthorn berries are food rather than drugs. In Europe they are consumed as juices, jams and food ingredients in domestic cooking and by the food industry. Sea buckthorn fractions, including oils and flavonoids are used as dietary supplements and ingredients in cosmetics. Although the definitions of food and drug may ovelap, supplements are in general classified as food in the European Union [19, 71]. A supplement may be defined as medicine if it has a modifying effect on physiological funtions [71]. If it contributes to the maintanence of healthy tissues and organs it is considered to be a food ingredient [71, 218]. Sometimes the difference is vague and similar preparations can be sold under the food and drug laws [71]. When evaluating the health effects of foods, less dramatic effects that develop during a longer period compared to those induced by drugs can be expected. Allergic reactions and food intolerances excluded, serious side effects caused by foods are rare. The physiological effects of foods are commonly mediated via the activity of several compounds.

In the literature review of this thesis the health effects of sea buckthorn berries and oil are reviewed and an introduction to the sea buckthorn components potentially beneficial for humans is presented. This work covers only the publications available in English. A review including articles in Chinese and Russian, concerning the health effects of lipophilic sea buckthorn compounds has been written by Yang in 2001 [230]. Only sea buckthorn berry and berry/seed oils are discussed in this thesis, whereas the literature concerning other parts of the plant are outside the scope of this work.

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The original research presented in this thesis comprises two clinical trials of randomized, double-blind, placebo- controlled, parallel design. One focuses on the effects of whole sea buckthorn berries, and the other on the sea buckthorn oil. In the whole berry trial, the effects of sea buckthorn on the common cold and inflammation are of main interest. The common cold is a mild upper respiratory illness, which usually is of short duration, and cures without medical intervention. Due to the high incidence, however, it causes considerable costs and bacterial complications may occur. As the common cold is caused by numerous viruses that have varying pathogenetic mechanisms, there is no universal treatment or medical prevention for it [80].

The oil trial focuses on dry eye. Dry eye is a common condition, reported to affect even up to over 30% of people aged 50 years or more [2, 159]. It causes symptoms of discomfort and is associated with ocular inflammation and hyperosmolarity of the tear film protecting the ocular surface. The two main types of dry eye, which often interlink and fortify each other, are the aqueous-deficient and evaporative dry eye. In the aqueous-deficient form the lacrimal secretion of tears is reduced. In the evaporative dry eye the evaporation of the aqueous tear film from the ocular surface is excessive, which may be due to abnormalities in the outermost lipid layer of the tear film [1, 159]. In addition to their individual main focuses, both trials investigate the effects of sea buckthorn on the risk factors associated with cardiovascular diseases and type 2 diabetes.

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2 REVIEW OF THE LITERATURE

2.1 COMPOUNDS OF SEA BUCKTHORN BERRIES ASSOCIATED WITH POTENTIAL HEALTH EFFECTS

2.1.1 Flavonoids, phenolic acids and lignans

Sea buckthorn berries are rich sources of flavonoids and phenolic acids: phenolic secondary metabolites of plants that participate in the defence against ultraviolet radiation, insects and pathogens. Accordingly, their synthesis is affected by these stimuli and they are generally enriched in the outer parts of fruits, berries and vegetables [122]. The main flavonoid classes in the sea buckthorn berries are flavonols and proanthocyanidins (condensed tannins), while smaller amounts of flavanols and phenolic acids are present [174] (Figure 1). Sea buckthorn does not contain anthocyanins typical for red and blue berries, and only very minor amounts of ellagitannins (hydrolysable tannins) have been detected [107]. Like most biological compounds, the type and amount of phenolics in sea buckthorn berry vary depending on the origin, year of harvest, ripeness, processing and storage [36, 59, 238].

The most abundant sea buckthorn flavonols are isorhamnetin (3'-methyl quercetin) and quercetin, which are mostly present as their 3-glycosides or 3, 7-diglycosides [174, 176, 238]. The presence of kaempferol in *H. rhamnoides* and other sea buckthorn species has been reported [36, 167], though it is not always detected [77, 118]. Myricetin is seldom found [118]. The most common sugar residue in the 7-position of the flavonol aglycone is rhamnose, whereas the 3-substituent is most often glucose, rutinose or sophorose [174, 176, 238]. In the commercially most important subspecies *H. rhamnoides* ssp. *sinensis*, ssp. *rhamnoides* and ssp. *mongolica*, the total content of flavonol glycosides varies between the range of 27 - 130 mg/100 g of fresh berries [238]. The highest levels were found in a Finnish cultivar Raisa (H. *rhamnoides* spp. *rhamnoides* X *H. rhamnoides* spp. *caucasica*) and the lowest in a Russian cultivar Vitaminaya (*H. rhamnoides* spp. *mongolica*). Pressed and pasteurized juice of *H. rhamnoides* cv. Hergo had a flavonol glycoside content of 36 mg/100 ml, and additionally contained isorhamnetin aglycone at a level of 0.14 mg/100 ml. Use of pectinolytic enzymes in juice manufacturing did not affect the total content of flavonol glycosides, but reduced the amount of isorhamnetin aglycone [174].

Compared to other foodstuffs, the content of isorhamnetin, the main flavonol aglycone in most sea buckthorn subspecies, is exceptionally high in sea buckthorn berries [214]. Values of 17 - 66 mg isorhamnetin aglycone/100 g fresh weight have been reported by Määttä-Riihinen et al. [118, 167] and approximately 30 - 35 mg/100 g by Yang et al. [238]. In the berries most of the aglycone is present as glycosides. The only foods having an isorhamnetin content above 10 mg/100g in the U.S. Department of Agriculture Database are

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dill (44 mg/100 g) and dried parsley (331 mg/100 g) [214]. The levels of quercetin aglycones in the study of the main commercial subspecies by Yang et al. [238] are ≈ 5 - 10 mg/100 g, whereas others have reported contents of 6 - 17 mg/100 g fresh weight [77, 118, 167]. In sea buckthorn and other natural sources, most of the quercetin aglycones are attached to sugar moieties. These values are higher compared to those found in most apple species, and comparable to the levels found in kale [214], both considered as good sources of quercetin. For onions, somewhat higher values of 7 - 33 mg/100 g are reported [214].

According to Rösch et al. sea buckthorn juice contains small amounts of catechin (1.9 - 2.6 mg/100 ml) and epicatechin (0.3 - 0.5 mg/100 ml) which belong to the flavanol group of flavonoids [174]. Considerably smaller concentrations have been reported as well [118]. The presence of monomeric (+)-gallocatechin and (-)-epigallocatechin in the pomace of cv. Hergo was reported by Rösch et al. [175].

Proanthocyanidins (condensed tannins) consist of flavanol subunits. The ones consisting of only (epi)catechin subunits are called procyanidins, whereas those consisting of (epi)gallocatechins are called prodelphinidins. The subunits are commonly linked through a C4→C8 or C4→C6 bond (B type). In the A-type proanthocyanidins there is an additional ether bond between C2→C7 [177]. A-type proanthocyanidins are present in cranberry (and other *Vaccinium* species), and are suggested to be responsible for the protective effects of cranberry against urinary tract infections [87, 169]. According to Rösch et al. [177], the composition of proanthocyanidins in sea buckthorn pomace is exceptional because of the high proportion of prodelphinidins, as commonly in foodstuffs the proanthocyanidins are exclusively procyanidins or mixtures of both. Määttä-Riihinen et al. [118] found almost equal amounts of procyanidins and prodelphinidins in *H. rhamnoides* berries.

The amounts of proanthocyanidins reported for sea buckthorn vary greatly in the literature. This is partly because of differences in genetic background and processing. Differences in the analysis methods may affect this as well, and the analysis is often described as challenging [86, 118, 174]. In cv. Hergo the total amount of proanthocyanidins is comparable to that of flavonol glycosides: 35 - 57 mg/100 ml juice [174]. Considerably higher (276 mg/100 g fresh weight) values were reported for berries grown in Canada (species undefined), and lower (0.1-1 mg/100 g fresh weight) for Finnish (*H. rhamnoides*) berries [86, 118]. In general, fruits, berries, beans, certain cereals and nuts, as well as wine and beer are good sources of proanthycyanidins. Among the richest berry sources are blueberries (180 - 332 mg/100 g), cranberries (419 mg/100g), chokeberries (664 mg/100 g), black currants (148 mg/100 g) and strawberries (145 mg/100 g) [70].

Proanthocyanidins together with ascorbic acid (in part due to its high concentration) account for the majority of the antioxidant activity of sea

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buckthorn juice *in vitro*. Isorhamnetin and its major glycosides are not as good radical scavengers as the other flavonoids and phenolic acids in the juice [174]. However, *in vitro* trials suggest other potentially important effects [78]. Depending on the choice of analysis method higher antioxidativity of isorhamnetin compared to ascorbic acid has been reported as well [155].

Sea buckthorn juice contains low concentrations of phenolic acids: gallic acid 0.15 – 0.26 mg/100 ml and protocatechuic acid 0.21 - 0.29 mg/100 ml (cv. Hergo) [174]. Also the presence of other hydroxybenzoic acid derivates, as well as hydroxycinnamic acids, has been indicated, several of them as esters or glycosides [9]. The amount of ellagitannins (hydrolysable tannins), rare in most foodstuffs but present in some berries, is low in sea buckthorn (1 mg/100 g fresh berries) [107].

The total contents of lignans secoisolariresinol and matairesinol in the *H. rhamnoides* ssp. *sinensis*, ssp. *rhamnoides* and ssp. *mongolica* berries vary between 8 to 139 μg/100 g fresh berries and 51 to 319 μg/100 g dry berries. These amounts are comparable to those reported for cloudberry, raspberry, black and red currant. However, they are lower compared to the levels found in strawberry (*Fragaria ananassa)* and berries of the *Vaccinium* genus, where secoisolariciresinol contents of up to 1510 μg/100 g have been reported. Linseeds are among the richest sources and contain lignans up to 400 000 μg/100 g of the dry mass [234].

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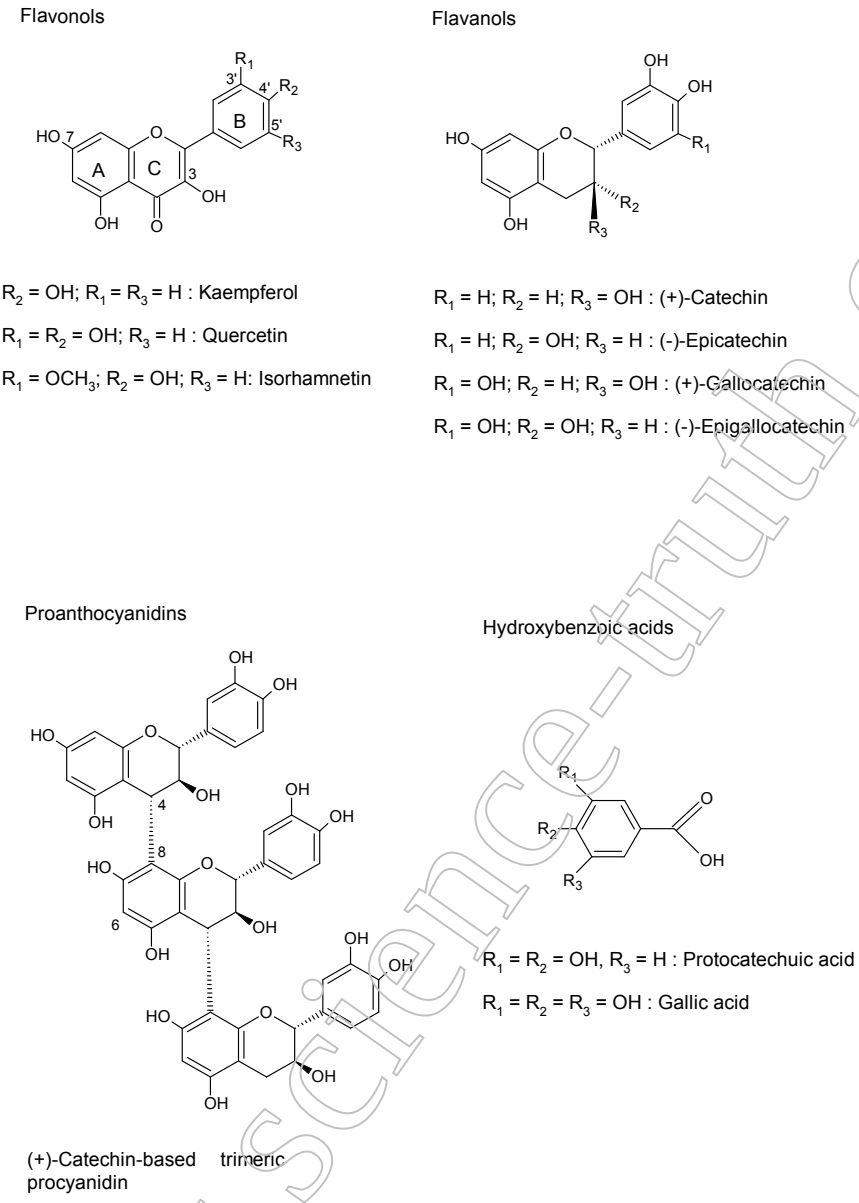

Figure 1. Main phenolic compounds of sea buckthorn berry

In vitro studies suggest antioxidantive, anti-inflammatory, antimicrobial and anti-proliferative effects of flavonoids found in sea buckthorn berries [23, 61, 78, 134, 166, 174]. Epidemiological data indicates that flavonoids may have beneficial effects on the risk and development of cardiovascular diseases, whereas the evidence concerning cancer is less consistent [11]. Intervention trials in humans indicate that quercetin may affect markers of carcinogenesis and antioxidant biomarkers in plasma. On the other hand, in several trials no effects were observed. The positive effects of proanthocyanidins and their

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monomeric subunits, flavanols, in human interventions include increase of plasma antioxidant activity, decrease of platelet aggregation, reduction of plasma low-density lipoprotein (LDL) cholesterol level and a decrease in susceptibility to oxidation [224]. The human intestinal bacteria can convert plant lignans into the enterolignans enterolactone and enterodiol, which can act as the agonists or antagonists of estrogen. Conclusions from epiodemiologic studies concerning lignans are similar to those made for flavonoids: lignans seem to have beneficial effects on the risk factors of cardiovascular diseases, while the effects on cancer are more debatable [11].

The effects of flavonoids *in vivo* cannot be predicted easily based on their concentrations in foods or their effects observed *in vitro*. As reviewed by Manach et al. [122], this is affected by the efficiency and site of absorption, activity of the metabolites and rate of elimination. It is likely that most of the polyphenol glycosides are not hydrolyzed in the stomach. In the small intestine only aglycones and some glucosides are absorbed, flavonol glucosides even more efficiently than the aglycones. Flavonoids linked to rhamnose must reach the colon, where they are hydrolyzed by the microflora before being absorbed. Trimers or larger oligomers of proanthocyanidins are likely not degraded in the stomach or absorbed in the small intestine in their native form. Due to their poor efficiency of absorption they may affect the gastrointestinal tract locally. Alternatively, the effects are caused by the phenolic acids produced through degradation and metabolism of larger phenolic compounds by the intestinal microbes. Manach [122] points out that as most of the other antioxidants are absorbed before the colon, the local actions of flavonoids may be of special importance for the health of the gastrointestinal tract.

After the absorption of flavonoids, three main types of conjugation take place: methylation (mainly in the liver and kidneys, but likely also in the intestine), sulfation (mainly in the liver) and glucuronidation (mainly in the enterocytes and liver) [122]. Lehtonen et al. [112] investigated the excretion and glucuronidation of sea buckthorn berry flavonols after ingestion of 300 g frozen berries as a breakfast. Post-prandially only glucuronides of isorhamnetin and kaempferol, but no flavonol glycosides or quercetin-glucoronides were detected in the plasma. The flavonoid metabolites are excreted through the urinary or the biliary route [122]. After a sea buckthorn meal both isorhamnetin glucuronides and isorhamnetin-3-glucoside, and quercetin-3-glucoside and -glucuronide were present in urine. In feces, glycosides of isorhamnetin, quercetin and kaempferol were observed. The levels of flavonols in the plasma returned to the baseline levels by 8 h after the sea buckthorn berry meal [112].

In the blood the flavonoid metabolites are bound to plasma proteins, especially albumin. Even though flavonoids may protect LDL against oxidation, only a small fraction of them is associated with LDL in the blood, and the protective effect is thought to take place at the interphase of the lipophilic and

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hydrophilic phases. Little is known about the accumulation of flavonoids in specific organs, but aortic endothelial cells and the brain have shown regional selectivity for certain flavonoids. In the organs further metabolism may take place, and the tissular metabolites may differ from the ones in the blood making the extrapolation of *in vitro* results to potential *in vivo* effects even more difficult [122].

2.1.2 Vitamin C

Sea buckthorn berry is among the richest food sources of vitamin C (Figure 2) [18], even though the genetic background, harvesting date [101, 167], growth conditions [208], storage and processing [75] greatly affect its concentrations and oxidation state. In general, the vitamin C content decreases during the ripening of the berries [101, 167]. Levels as high as 13 g/l of juice were reported in Chinese *H. rhamnoides* ssp. *sinensis* berries, and values around 10 g/l were found for several samples of this subspecies. As averages of several samples from different locations and harvesting times, the vitamin C contents of *H. rhamoides* ssp. *sinensis*, ssp. *rhamoides* and ssp. *mongolica* were 8.6, 1.7 and 0.5 g/l of berry juice, respectively [101]. In *H. rhamnoides* juice made of berries cultivated in Canada, average values of about 1.7 g vitamin C/l of juice were observed [18]. This resembles the concentrations found in Russian ssp. *mongolica* varieties grown in Finland (0.3- 1.3 g/l juice) [208], and in German *H. rhamnoides* cultivars (1.8-3.7 g/kg fresh berries) [167]. The vitamin C content in the ssp. *sinensis* berries is comparable to that reported for rose hip berries (roughly 12.5 g/kg), and several times higher than those in orange (roughly 0.5 g/kg), apple (0.1 g/kg) and black currant (1.2 g/kg) [135].

The physiological functions of vitamin C derive from its strong antioxidant activity and its action as an essential cofactor for several enzymes. It may regulate the expression of certain genes, affect the adhesion of monocytes to endothelium and the aggregation of platelets and leucocytes, among other functions [114]. Vitamin C supplementation may have beneficial effects on the common cold in certain subpopulations [81], and it is associated with lower levels of inflammatory markers in the circulation [22, 146].

Figure 2. Ascorbic acid and dehydroascobic acid

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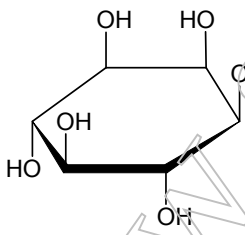
2.1.3 Inositols

Inositols (Figure 3) and their derivatives are essential messenger molecules in cells. *myo*-Inositol is present in cell membranes as phosphatidylinositols. Phosphatidylinositols can, due to extracellular stimuli, be enzymatically cleaved to form inositol phosphates and 1,2-diacylglycerol, both of which are involved in the intracellular signal transduction [97, 220]. Inositol hexaphosphate is a strong antioxidant and neurotransmitter. As reviewed by Vucenik et al. [220], inositol hexaphosphate and its less phosphorylated forms may have anti-cancer potential by affecting immune functions, inflammation, cell differentiation and apoptosis, among others. In plants, inositols are thought to act as cryoprotectants or regulators of osmotic stress, as their levels are increased under cold or dry conditions [100].

The presence of (-)-2-*O*-methyl-L-*chiro*-inositol (L-quebrachitol), and trace amounts of *chiro*-inositol and *myo*-inositol in the sugar fraction of sea buckthorn berries have recently been reported by Yang [237] and Kallio et al. [100]. The amount of L-quebrachitol was highest in the ssp. *sinensis*, where mean concentration of 0.8 mg/100 ml of juice was observed. Concentrations of 0.3 mg/100 ml and 0.2 mg/100 ml in the ssp. *rhamnoides* and ssp. *mongolica* juices, respectively, were observed [237]. Quebrachitol, having physiological effects similar to those with other inositols, had not been reported in edible fruits previously. Quebrachitol has antioxidant and free radical scavenging properties, which are likely to contribute the cytoprotective effects observed *in vitro* and in animals [47, 141]. In a mouse study concerning gastroprotection, the lowest doses tested (12.5 mg/kg and 25 mg/kg) were the most effective [47, 141].

myo - inositol



2-*O*-methyl-*chiro*-inositol (L-quebrachitol)

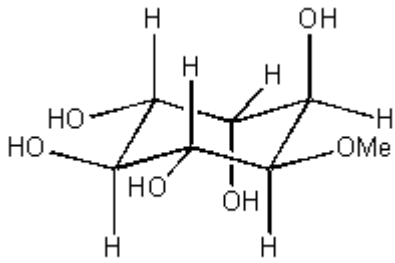


Figure 3. *myo*-inositol and 2-*O*-methyl-L-*chiro*-inositol (L-quebrachitol)

2.1.4 Berry and seed oils

The proportion of seeds is approximately 4 - 9% of the fresh weight of sea buckthorn berries. The oil content of the seeds is reported to be approximately 11% in ssp. *rhamnoides*, 13% in ssp. *mongolica* and 7 - 10% in ssp. *sinensis* [102, 196, 236]. The proportion of oil in the soft part was 2.8% of fresh weight in ssp.

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rhamnoides and 1.7% in ssp. *sinensis* [236]. In a whole fresh berry, including the seeds, the oil content was between 2.1 - 4.1% of fresh weight in ssp. *sinensis*, 3.5% in ssp. *rhamnoides* and 5.9% in ssp. *mongolica* [102, 236].

2.1.4.1 *Triacylglycerols, glycerophospholipids and fatty acids*

Triacyglycerols form the main lipid class in the seed and the soft part oils, and comprise approximately 80% of the oil from whole berries of ssp. *mongolica* and ssp. *sinensis* [102]. The proportion of glycerophospholips in the oil from whole berries is approximately 3 - 6% [102]. According to a study by Kallio et al. [102], the proportions of lipid classes in seed oil clearly differ between subspecies. The proportion of triacylglycerols varied from 86% in ssp. *sinensis* to 66% in ssp. *mongolica*, whereas glycerophospholipids comprised approximately 10 and 8% of the seed oils in ssp. *sinensis* and ssp. *mongolica*, respectively [102].

The fatty acids of the seed and soft part oil differ greatly. Due to the dominance of the pulp and peels in a whole berry, the composition of the oil from the whole berry resembles that of the soft part oil. Main fatty acids in the oil from the soft parts of the berry are palmitoleic (27 - 33%), palmitic (27 - 28%), oleic (17%), linoleic (9 - 13%), vaccenic (8 - 9%) and α-linolenic (3 - 7%) acids ([102, 196, 236], numerical values for ssp. *rhamnoides* and ssp. *sinensis*, according to the report by Yang & Kallio [236]). In the seed oils of all the tree major commercial subspecies the main fatty acids quite regularly are linoleic (39 - 41%), α-linolenic (27 - 31%), oleic (17 - 19%), palmitic (7 - 9%), stearic (3%) and vaccenic acids (2 - 3%).

In humans, α-linolenic and linoleic acids are precursors of other long-chain n-3 and n-6 fatty acids. They in turn are precursors for eicosanoids and other local hormones modulating inflammation and secretory and cardiovascular functions. As a not always accurate generalization, the effects of n-6 derived eicosanoids are commonly described as pro-inflammatory, whereas the effects of n-3 derived eicosanoids are considered antiinflammatory or neutral [31, 32, 97, 188]. Conversions of the 18-carbon n-3/n-6 fatty acids to their derivates of longer chains and a higher degree of unsaturation involve a series of desaturaturases and elongases shared by the n-3 and n-6-families (Figure 4). Competition between the fatty acids of n-3 and n-6 families for the same enzymes in these conversions makes the n-3/n-6 ratio in the diet important [31, 32, 97, 188]. Additionally, the n-3 fatty acids may also affect the expression of inflammatory genes and cytokine production [31, 32].

Western diets commonly contain n-6 and n-3 fatty acids in the ratio of 10:1 or higher, which is undesirably high according to most authorities. The current recommendations for the n-6/n-3 ratio in the diet vary from less than 4:1 to 10:1 [97]. Sea buckthorn seed and berry oils are considered to be of particular nutritional interest because their n-6/n-3 ratio of <2:1 is low, compared to most other vegetable oils [51].

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Recently, the role of fatty acid amides (endocannabinoids) in modulating inflammation has been extensively studied. This group of endogenous signalling lipids involves both saturated and unsaturated fatty acids, among them palmitic acid in N-palmitoyl ethanolamide (PAE), and oleic acid in oleoylethanolamide (OEA). PAE has anti-inflammatory effects via mechanisms that are not completely known so far. The inhibiting effect on cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression have been suggested [162].

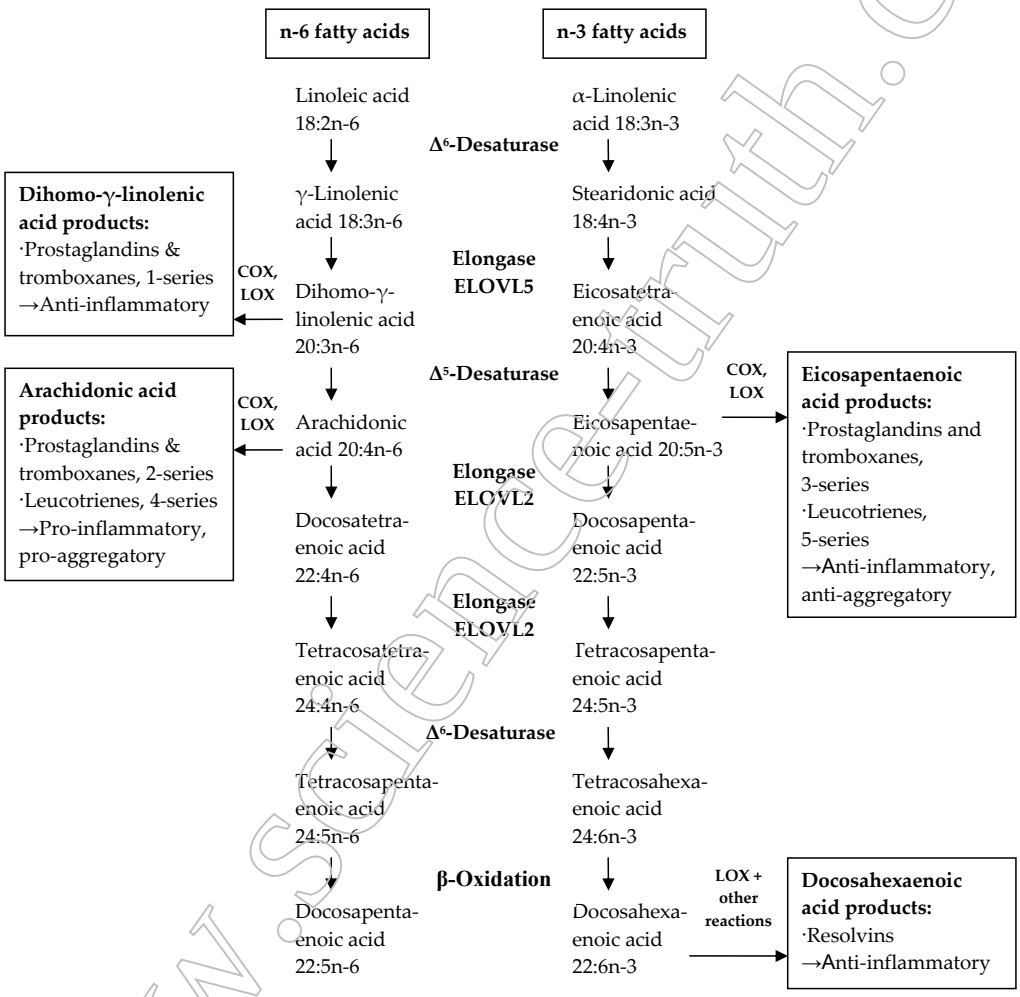

Figure 4. Synthesis of long-chain n-3 and n-6 fatty acids from α-linolenic and linoleic acids, respectively, and a simplified overview of the messangers derived from them in humans. Modified from [188] and [178]. COX = cyclo-oxygenase, LOX = lipoxygenase

2.1.4.2 *Vitamin E*

Vitamin E is a collective name for tocopherols (having a saturated phytol side chain attached to the chromanol structure) and tocotrienols (having an

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unsaturated side chain) [211]. Both sea buckthorn berry and seed oils contain tocopherols and tocotrienols, the amounts and proportions of which are dependent on the genetic origin, growth conditions and ripeness of the berries [6, 101, 102, 196]. The seeds of *H. rhamnoides* ssp. *mongolica* (average 250 mg tocols/ kg seeds) and ssp. *rhamnoides* (290 mg/kg) seem to be better sources of tocopherols and tocotrienols, compared to those of spp. *sinensis* (130 mg/kg). The major forms of vitamin E in the seeds are α-and γ-tocopherols [101, 196] (Figure 5), which represent typically 40 - 50% and 20 - 40%, respectively, of the total contents of tocols [101]. γ-Tocotrienol was the only tocotrienol detected in the choloform-methanol extracted sea buckthorn seed oils by St George & Cenkowski [196], whereas Kallio et al. [101] report the presence of β-tocotrienol in seed oils. According to Kallio et al. [101] the tocopherol and tocotrienol contents of chloroform-methanol-extracted seed oil is typically 100 - 300 mg/100 g in ssp. *rhamnoides* and ssp. *sinensis* from various growth conditions and harvest times, in accordance with the levels reported for ssp. *sinensis* seeds from a Canadian harvest (220 - 260 mg/100 g oil) by St George & Cenkowski [196].

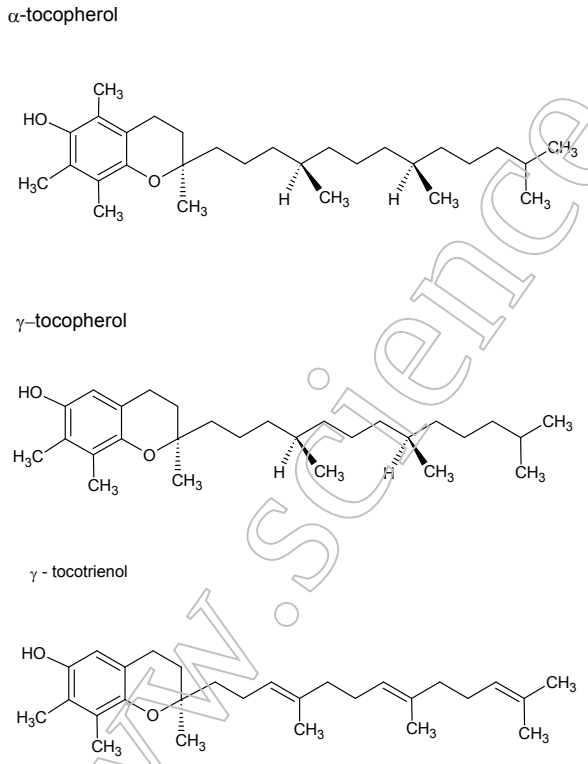

Figure 5. Main tocopherols of sea buckthorn seed oil and an example of seed oil tocotrienols found in smaller amounts

When the berries were compared in the study by Kallio et al. [101], the spp. *sinensis* was clearly the best source of vitamin E. Average concentrations of

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tocols in samples from several locations were: ssp. *sinensis* 120 mg/kg fresh berries, spp. *rhamnoides* 40 mg/kg berries, and ssp. *mongolica* 50 mg/kg berries. α-Tocopherol was the main form representing 70 - 80% of the total tocopherols and tocotrienols. The berry oil extracted by chloroform-methanol from ssp. *sinensis* contained typically 400 - 700 mg tocopherols and tocotrienols/100 g, whereas the tocol content in ssp. *rhamnoides* and ssp. *mongolica* oils was 100 - 200 mg/100g. Somewhat lower values were reported by St George & Cenkowski [196] for the ssp. *sinensis* berries grown in Canada (340 - 490 mg/100 g oil, mainly α-tocopherol). In the Canadian study, the oils for the tocol analyses were extracted using chloroform-methanol [196]. Both Kallio et al. [101] and St George & Cenkowski [196] specify β-tocotrienol as the major tocotrienol in the soft part of the ssp. *sinensis* berries. In ssp. *rhamnoides* and ssp. *mongolica* the main tocotrienol is γ-tocotrienol [101]. Due to the distribution of oil between the seeds and soft part of the sea buckthorn berry, the proportion of tocopherols and tocotrienos originating from the seeds and the soft part varied between 3 - 13% to 77 - 97%, respectively [101].

In dietary reference values and recommendations, α-tocopherol is the only tocol included in the definition of vitamin E [55, 211], and it is not recommended to include other forms by using conversion factors [211]. In the case of synthetic α-tocopherol, a conversion has to be made to compensate for the stereochemical differences from the natural form [211]. Also the food composition databases commonly state 1 mg vitamin E as equating 1 mg of α-tocopherol [135]. Sea buckthorn berries of the ssp. *sinensis* especially can be considered as a good source of vitamin E. The α-tocopherol contents in the ssp. *sinensis* berry samples with the highest concentrations [101] are comparable to the levels in hazel nuts (15 mg/100 g) and rapeseed oil (19 mg/100 g), commonly considered as good sources [135].

Antioxidant activity is the major function of vitamin E in the body. It is transported in the plasma lipoproteins and is found in membranes and organs rich in lipids, where it protects the unsaturated fatty acids from oxidation [211]. α-Tocopherol has been described to participate in the regulation of cell proliferation of the vascular smooth muscle cells, regulation of protein kinase C and the inhibition of phospholipase A_2. Epidemiological studies and some, but not all, of the interventions suggest positive effects of α-tocopherol on degenerative diseases such as cardiovascular diseases, Alzheimer's disease and cancer [211]. Conflicting effects on infection risk have been observed [82, 83, 84, 125]. Even though not included in the nutritional definition of vitamin E, and not as effective an antioxidant as α-tocopherol, other tocopherols may be beneficial as well [211]. γ-Tocopherol has a stronger anti-inflammatory effect compared to α-tocopherol *in vitro*. It inhibites COX-2 activity in macrophages and human epithelial cells and reduces the expression of iNOS in macrophages, whereas the α-tocopherol is ineffective or less effective [94].

Even if the amounts of tocotrienols in sea buckthorn oils are smaller compared to those of tocopherols, they may have specific physiological effects, as

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reviewed recently by Colombo [42]. Tocotrienols have been observed to inhibit cholesterol biosynthesis in animal cells by suppressing the enzymes involved. Indications of lowering effects on blood cholesterol in humans have been reported. Tocotrienols have *in vitro* antiproliferative and apoptotic activities in normal and cancer cells. α-Tocotrienol has been reported to demonstrate preventive effects against neurodegeneration. This has not been observed with α-tocopherol [42].

2.1.4.3 *Carotenoids*

The sea buckthorn pulp oil is especially rich in carotenoids (Figure 6), which give the berry its orange color. In general, the concentrations of carotenoids increase during ripening [5, 167, 196], but the effect of the genetic origin seems to be even stronger than that of the harvest year or time [5]. The total carotenoids in the pulp and seed oils of *H. rhamnoides* ssp. *sinensis* berries cultivated in Canada varied between 489 - 818 mg/100 g and 24 - 28 mg/ 100 g, respectively (lipids extracted using chloroform-methanol) [196]. Somewhat lower levels of 69 - 342 mg/100 g oil were reported for *H. rhamnoides, H. salicifolia* and *H. tibetana* berry oils collected from the Himalayas (lipids extracted using chloroform-methanol) [168]. According to Raffo et al. [167] zeaxanthin, β-carotene and β-cryptoxanthin are the main carotenoids in the three *H. rhamnoides* cultivars studied, where they were found at quantities of 5 - 17 mg/100 g fresh weight of berries (lipid extraction of carotenoid analyses using tetrahydrofuran-methanol and petroleum ether). Zeaxanthin was the main carotenoid in cultivars Askola and Hergo (82 - 91% of total), whereas in Leikora the proportions of zeaxanthin (49 - 57%) and β-carotene (33-39%) were more close to each other. The main carotenoids repoted in the three *H. rhamnoides* cultivars analysed by Andersson et al. [5] were zeaxanthin, β-carotene, β-cryptoxanthin, lutein, lycopene, and γ-carotene (1.5 - 18.5 mg/100 g fresh weight, lipid extraction using ethanol-hexane).

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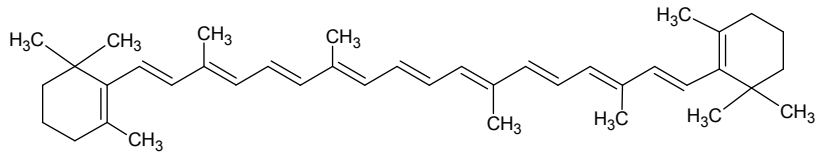
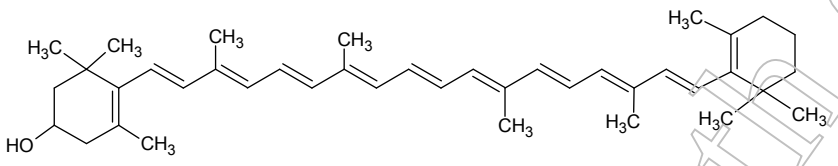
All-trans-β-carotene



β - cryptoxanthin



zeaxanthin

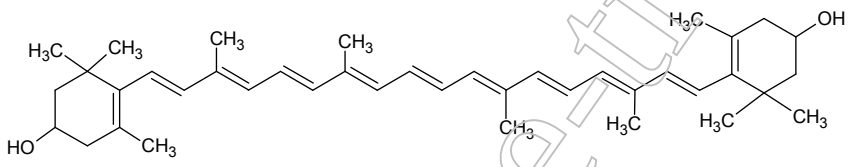


Figure 6. Main carotenoids of sea buckthorn berries

Sea buckthorn berries and oils are rich sources of carotenoids. According to rough estimates in the database of the National Institute for Health and Welfare, among the best food sources are rose hip berries (11 mg/100 g), watermelon (5 mg/100 g), carrots (11 mg/100g), sweet potato (9 mg/100 g) and sweet red pepper (6 mg/100g) [135]. For a carotenoid to have a provitamin A activity it must have at least one nonoxygenated β-ionone ring. Therefore, of the major carotenoids in sea buckthorn, zeaxanthin, lutein and lycopene are not precursors of vitamin A, instead they have other potential mechanisms of effect. For carotenoids, there is currently no recommended daily intake. The recommended dietary allowances of vitamin A are expressed as retinol activity equivalent (RAE), where 1 μg RAE = 12 μg all-*trans* β-carotene from foods or 2 μg all-*trans* β-carotene from supplements (oil supplements, a highly absorbable form) [180].

Epidemiological studies suggest that fruits and vegetables may be beneficial for reducing the risk of cardiovascular diseases and cancer. However, as reviewed by Ross [180], the evidence for the effects of certain individual components, including carotenoids is less consistent. In smokers and asbestos workers, supplementation with β-carotene showed even a negative effect on the cancer incidence, though, unlike vitamin A, carotenoids even at high doses are not considered toxic as such [180]. Both pro- and nonvitamin A carotenoids

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have modulatory effects on inflammation and immune fuctions *in vitro*, and may protect against age related macular degeneration [180, 183]. Carotenoids are important as antioxidants. As inflammatory reactions produce oxidant molecules, including nitric oxide (NO), peroxide and peroxynitrite, antioxidant action may be one of the mechanisms involved in the immunomodulation by carotenoids [180]. Intake of β-carotene seems to be negatively associated with the inflammatory marker C-reactive protein (CRP) in middle-aged and older women [221]. β-Carotene is successfully used for the treatment of certain photosensitivity disorders, and the positive effects of vitamin A (systemic and topical) on the treatment of skin disorders like cystic acne and psoriasis are well known [180].

2.1.4.4 Phytosterols

The total sterol content of the *H. rhamnoides* ssp. *rhamnoides* and spp. *sinensis* seed oils was reported by Yang et al. [233] to be 1.2 - 2.3%. Total sterol content in the oils from the soft part of the ssp. *rhamnoides* and *sinensis* berries varied between 1.0 - 2.9%. Sitosterol constituted approximately 60 – 80 % of the sterols in all the oils extracted using chloroform-methanol [233]. St. George & Cenkowski [196] used the same method as Yang et al. [233] to analyse sterols in the seeds and berries of *H. rhamnoides* ssp. *sinensis* cultivated in Canada. A total content of 0.7 – 1.0% and 0.5 – 0.7% sterols in the berry and seed oils, respectively was found. The main sterol was always β-sitosterol (Figure 7).

Phytosterols and phytosterol esters have a serum cholesterol lowering effect, most likely because they can interfere with the absorption of cholesterol [108]. β-Sitosterol has anti-inflammatory effects in human aortic endothelial cells *in vitro* [117]. As discussed by Loizou et al. [117] it may also have a cardioprotective effect due its activity as an antioxidant.

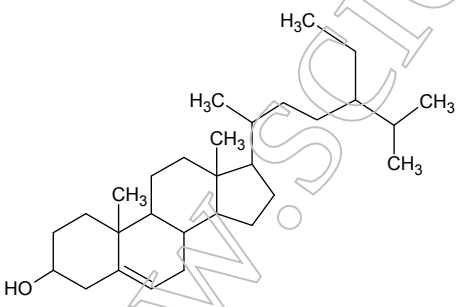

Figure 7. β-Sitosterol

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2.2 THE EFFECTS OF SEA BUCKTHORN BERRIES AND OIL ON THE HEALTH

2.2.1 Antioxidative and cytoprotective effects

2.2.1.1 In vitro cytoprotection by antioxidant activity

Oxidative or nitrosative stress, the imbalance in the equilibrium status between prooxidant/antioxidant systems in cells is considered to contribute to the pathogenesis of many human diseases including cancer, cardiovascular diseases, and rheumatic disorders, among others [24, 207]. Reactive radical oxygen (superoxide anion, $O_2^{\cdot-}$; hydroxyl radical, $HO^{\cdot}$; peroxyl radical, $ROO^{\cdot}$) and non-radical oxygen (hydrogen peroxide, H_2O_2; singlet oxygen $^1O_2$) and nitrogen (NO) species are produced in normal cellular processes, such as mitochondrial electron transport systems and cell signalling [207]. An excess of reactive oxygen (ROS) or nitrogen (RNS) species damage nucleic acids, lipids, proteins and carbohydrates, and ultimately interfere with the normal functions of cells and tissues [24, 207].

ROS are necessary for the microbicidal activity of immune cells and components of the signalling generated by cytokines. The activation of phagocytes by proinflammatory mediators or bacteria leads to the production of large quantities of superoxide anion radical [24, 207]. There is a delicate balance between the immune system and ROS. Even though immune cells produce significant amounts of ROS, they have a high proportion of polyunsaturated fatty acids in their cell membranes and therefore are especially sensitive to external ROS [24].

Cells have efficient enzymatic (superoxide dismutase, SOD; glutathione peroxidase, GPx; catalase) and nonenzymatic (glutathione, GSH; vitamin E in cell membranes) protective antioxidative mechanisms [24, 207]. In plasma, the concentrations of the above mentioned compounds are lower, and the role of vitamin C and proteins in antioxidative defence is considered to be more important [207]. In LDL, the oxidised form of which is considered highly atherogenic, the primary antioxidant is vitamin E [207]. Diet is important in providing the antioxidant components to the body [207]. Several sea buckthorn berry and oil components have antioxidant activity by their ability to scavenge free radicals, donate electrons and/or chelate metal ions catalyzing oxidation reactions.

Many chemicals including nicotine, heavy metals and other pollutants have detrimental effects on cells and tissues by inducing the generation of reactive oxygen species and increased oxidative/nitrosative stress [199, 225, 228]. Also the cytotoxic effects of ionizing radiation and hypoxia are thought to be largely mediated by free radicals and oxidative damage [193]. The effects of sea buckthorn berry fractions and oil against cytotoxicity by oxidizing chemicals,

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ionizing radiation, pollutants and heavy metals have been investigated in several *in vitro* (Table 1) and animal studies (Table 2).

The *in vitro* studies have mostly concerned cells of the immune system and have been carried out using ethanol/alcohol extracts of *H. rhamnoides* berries. Even if most studies do not report the chemical composition of the preparation used, the alcohol extracts are likely enriched with flavonoids, sugars and acids. Sea buckthorn *in vitro* trials indicate cytoprotective activity against chemicals and radiation due to the increased antioxidant protection of the cells (Table 1). The reviewed studies included control groups and report using statistical methods, even if for some individual assays results of the statisctis were not always presented.

Geetha et al. [63] investigated the effects of ethanol extract of sea buckthorn berries against nitrosative stress induced by sodium nitroprusside (SNP) in murine macrophages. SNP dissolves to water and realeases NO, which in the presence of superoxides forms higly reactive peroxynitrite radical. SNP at a concentration of 500 μg/ml generated NO at levels that were cytotoxic to the cells (decreased viability), increased the concentrations of reactive oxygen species, decreased the levels of intracellular antioxidants, induced mitochondrial and nuclear damage, and reduced phagocytosis by the macrophages. The addition of sea buckthorn berry extract to a level of 500 μg/ml medium significantly reduced all of the above mentioned negative effects of SNP. Compared to the sea buckthorn leaf extract, the berry extract was found to be more effective in protecting against SNP-induced cytotoxicity. The authors suggest this may be due to a greater proportion of lipid soluble antioxidants in the berry extract. However, the compositions of the extracts were not presented. Both fruits and leaves were extracted using Soxhlet extraction, which due to its long duration and high temperature may cause the loss of bioactive compounds. The berries used as a raw material in the extraction were of the species *H. rhamnoides*, collected from the Himalayas.

Recently, Geetha's group [62] investigated the effects of sea buckthorn berry pulp flavonoids against *tert*-butyl hydroperoxide (t-BOOH)-induced cytotoxicity in rat lymphocytes. t-BOOH is an organic hydroperoxide pro-oxidant that in the presence of Fe^{2+} generates butoxyl radicals. A commercial sea buckthorn flavonoid preparation, the detailed composition of which was not presented, was used. As in the group's previous study with macrophages [63], the increased oxidative stress lead to reduced cell viability, increased production of free radicals, increased lipid peroxidation, DNA strand breaks and apoptosis. The flavonol preparation at level of 100 μg/ml medium significantly restricted all of the above mentioned effects of t-BOOH. A negative control was included, and the effects of individual antioxidants vitamin C, vitamin E, iron chelator deferoxamine (DFO) and butylated hydroxytoluene (BHT) were investigated as well. Compared to the individual compounds, the sea buckthorn flavonoids demonstrated a wider spectrum of protective effects. The protective effect of vitamin E and BHT against

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cytotoxicity and free radical production was only small or nonexistent, whereas sea buckthorn flanoids, vitamin C and DFO all had marked effects. DFO, vitamin C and BHT did not protect against the lipid peroxidation effect, which was restricted by sea buckthorn flavonoids and vitamin E.

Kumar et al. [109] investigated the effects of a sea buckthorn berry alcoholic extract named RH-3 against ionizing radiation and t-BOOH in mice and calf thymocytes. Gamma-radiation used in the study generates reactive oxygen species. It can also directly cause the degradation of cellular macromolecules, including DNA. Berries for the RH-3 extract were of the *H. rhamnoides* species collected from the Himalayas. Although UV-Vis spectroscopic and high performance liquid chromatographic (HPLC) profiles of the extract were presented, the compounds were not identified or quantified. In isolated thymocytes both gamma-radiation and t-BOOH treatment caused strand breaks of DNA, which was restricted in a dose dependent manner by RH-3 preparation. The optimal doses in preventing the DNA strand breaks were 100 - 120 µg of RH-3/ml medium. At doses ≥ 150 µg/ml pre- or post- irradiation, RH-3 induced an appreciable increase in the compaction of the chromatin. This was associated with less strand breaks of DNA and greater resistance to gamma-radiation. At lower concentrations of <100 µg RH-3/ml medium the compaction was reversible, whereas high concentrations of ≥ 150 µg/ml induced irreversible compaction (concentration of 120 µg/ml not studied for reversibility of the compaction) [109].

In vivo, it is likely that the RH-3 concentrations would be lower and irreversible compaction may not take place. As reviewed by Kumar [109], histones and some other agents like polyamine, may protect DNA from strand breaks by modulating the chromatin organization. Though reversible condensation may be radioprotective, irreversible condensation may lead to cell death *in vivo*. However, as such, without radiation treatment no effects of RH-3 on the cell viability were observed at concentrations from 20 to 200 µg/ml. A mild free radical scavenging activity of RH-3 was observed. The authors suggest that *in vivo*, sea buckthorn flavonoids may affect chromatin organization and protect the cells against oxidative stress, and by both mechanisms protect tissues against radiation [109]. Throughout the study, a control group was included in the assays, but results of the statistical analyses were not always presented. Both pre- and post-radiation effects of RH-3 were investigated. However, it was not always clear for the reader which results concerned the post- and which the pre-radiation studies.

Also Shukla et al. [193] report on the radioprotective effects of ethanol extract of sea buckthorn berries in murine thymocytes. *H. rhamnoides* berries from the Himalayas were used for manufacturing the extract named REC-1001. The total polyphenol content of REC-1001 was 68% w/w. Kaempferol, isorhamnetin and quercetin were identified but not quantified. REC-1001 concentrations from 10 µg/ml to up to 500 µg/ml median were used in the experiments of this study. REC-1001 decreased the radiation-induced DNA damage in all the

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assays used to evaluate this. The highest concentrations (250 - 500 µg/ml mediun) gave the best protective effects. REC-1001 also dose-dependently scavenged the hydroxyl radicals induced by radiation, scavenged chemically induced superoxide anions and reduced Fe^{3+} to Fe^{2+}. It also totally stabilized the generation of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals already at 25 µg/ml, which was the lowest concentration of REC-1001 tested. The authors suggest that antioxidant activity is focal in the radioprotection by REC-1001.

Agrawala & Adhikari [3] investigated the effects of RH-3 against radiation induced cytotoxicity in human U87 cancer cells. RH-3 concentrations from 2.5 to 10 µg/ml medium were used in the assays. The chemical composition of the preparation was not defined but the extract was reported to be standardized. RH-3 alone without radiation did not affect the metabolic activity of U87 cells, suggesting it was nontoxic at these concentrations. The greatest effect against the loss of cell viability due to ionizing radiation was observed when the sea buckthorn extract was added to the medium 15 min before the radiation at concentrations of 7.5 and 10 µg/ml medium. At concentrations of 5 - 7.5 µg/ml, pretreatment of the medium with RH-3 restricted the increase of cellular and mitochondrial (7.5 µg/ml) free radicals after radiation. However, at the level of 10 µg/ml, an increase in mitochondrial free radicals was observed. Pretreatment with the extract at concentrations of 5 µg/ml medium or higher was efficient in preventing the apoptotic effects of radiation. The authors conclude that the antioxidant action of RH-3 prevents cellular and mitochondrial free radical production, which likely contributes to its ability to inhibit cytotoxicity and apoptosis induced by radiation.

2.2.1.2 *Animal studies on cytoprotection and antioxidant action*

Animal studies focusing on the protective effects of sea buckthorn via antioxidant mechanisms have concerned the effects of water and ethanol extracts, juice and seed oil (Table 2). As was the case for the *in vitro* studies, information concerning the composition of the fraction used is most often not comprehensive. All studies report positive findings, but in some cases the doses used for the effect are fairly high. The dose of 500 mg of dried sea buckthorn extract/kg body weight/day used in the arsenic prevention studies by Gupta et al. [73, 74] would mean a dose of 30 g/ day for a person weighing 60 kg.

Mice studies by Goel et al. [65, 66] support the indications of radioprotection by sea buckthorn observed *in vitro*. The ethanol extract coded as RH-3 was used in both trials of Goel's group. An HPLC chromatogram of the flavonoids in RH-3 is presented, but the compounds were not identified or quantified in the articles. In the first study [65], the number of mice was 56 or less depending on the effects investigated. Untreated controls were included but results of statistical analyses were not presented for all assays. A dose of 30 mg RH-3/kg body weight admistered 30 minutes before lethal gamma-irradiation had the greatest enhancing effect on the survival rate of mice 30 days post-radiation. In

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the 30 mg/kg group 82% of the mice survived, whereas in the negative control group all the mice were killed. RH-3 had a protective effect against changes in hematological parameters induced by the radiation, and it showed *in vitro* antioxidant activity by several mechanisms [65].

In the second study by Goel et al. [66], 12 mice per assay were included and statistical comparisons between the treatment and control groups were presented. RH-3 was admistered intraperitoneally 30 minutes before radiation. It was not clear from the article which were the doses used for the experiments. However, RH-3 was reported to increase the number of surviving crypts in the jejunum, and reduce the number of apoptotic bodies in the crypts. The gastrointestinal tract is extremely sensitive to radiation, and the crypts are essential for the renewal of the intestinal epithelium.

High level of arsenic in the drinking water is a problem in some developing countries. The intake of contaminated water may cause cancer, and skin and vascular lesions. Chronic exposure by different routes has also been associated with diabetes, hypertension, and atherosclerosis. The detrimental effects are thought to be mediated by increased oxidative stress induced by arsenic [73, 74]. Gupta et al. [73, 74] investigated the effects of three water and ethanol extracts of sea buckthorn berry against arsenic toxicity in mice in two studies. In both they used the fruits of *H. rhamnoides* from the Himalayas. The extraction methods were described, but the chemical compositions of the products were not analyzed.

In the first trial, 20 mice were exposed to arsenic in the drinking water for three months [74]. After that, the mice were orally given water or ethanol extracts of sea buckthorn for 10 days, or they did not receive any treatment. Additionally, five mice were treated as a control group, which received neither arsenic nor sea buckthorn during the course of the whole trial. The dose of the sea buckthorn extracts was 500 mg/kg body weight per day. After three months and ten days, the mice were killed and the effects of the arsenic and sea buckthorn were evaluated by analyzing biochemical markers from the blood and tissues.

In the second trial [73] of 25 animals, the mice were orally given the sea buckthorn extracts at a dose of 500 mg/kg body weight/ day for two weeks before and during the three week arsenic exposure. Control groups getting no arsenic or sea buckthorn, and getting arsenic only, were included. After the treatments, the animals were sacrificed and blood and tissue samples were collected. The studies indicate the protective effects of sea buckthorn against arsenic induced oxidative stress, disturbances in hematological variables, heam synthesis and liver functions. Only minor changes in the tissue concentrations of arsenic were observed. The authors conclude that the protective effect of sea buckthorn is likely not due to the chelation of arsenic, but rather because of protection against oxidative damage. The water extract prepared under room temperature was the most effective in both of Gupta's studies.

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As opposed to the arsenic studies, the protection against lead induced neurotoxicity by sea buckthorn juice in mice was suggested to result from the juice chelating the heavy metal [228]. Antioxidant activity was observed as well. Xu et al. [228] admistered sea buckthorn berry juice to mice orally via a stomach tube before and during exposure to lead for 25 days. The 20% or 40% juice was given at a dose of 1 or 0.1 ml/10 g body weight/ day. A total of 50 mice, including the control group, were included in the study. The *H. rhamnoides* berries for the juice were collected from the Huchun area of Jilin province in China, but the composition information of the juice was not provided. The sea buckthorn juice protected the mice against the lead acetate induced impairment in cognitive functions, rise of biomarkers of oxidation and decrease of several neurotransmitters. The effects of 40% juice were wider compared to those of the 20% juice.

Cerebral edema is a serious complication of injuries in the central nervous system, including stroke, high-altitude illness and head injury. All of these conditions are associated with tissue hypoxia, which in turn is suggested to induce vascular leakage in the brain leading to edema [164]. Superoxide generation can be increased in response to both high and low levels of oxygen *in vivo*, and in spite of the seeming contradiction, hypoxia induces the generation of reactive oxygen and nitrogen species [123, 164]. These in turn may affect the vascular permeability under hypoxia.

Purushothaman et al. [164] investigated the effects of sea buckthorn seed oil on hypobaric hypoxia in rats. Up to 56 rats, depending on the experiment, were included. CO_2-extracted seed oil from Himalayan origin *H. rhamnoides* berries was used. The chemical composition of the particular oil used in this study was not analysed, instead the compositional information based on literature was presented. Initially doses of 1.5 to 5 ml/kg body weight were admistered orally using gastric canula 12 h before exposure to hypobaric hypoxia. The dose of 2.5 ml/kg was found to be most effective and used in most experiments. Pretreatment with sea buckthorn oil protected the rats against hypoxia induced transvascular leakage in the brain. It restricted the rise in biomarkers of oxidation and free radicals and improved the hypoxic tolearance. Both positive and negative control groups were included in this study.

Sulfur dioxide (SO_2) is an air pollutant that is absorbed through the respiratory tract and distributed to tissues, where it can induce oxidative stress [181, 225]. Two mice studies by Ruan et al. [181] (a total of 84 animals) and Wu et al. [225] (60 mice), suggest that the intake of sea buckthorn seed oil may protect against the negative effects of SO_2. Ruan et al. used CO_2-extracted seed oil from wild Chinese *H. rhamoides* berries and reported the vitamin E and carotenoid content of the oil. Wu et al. did not report the method of oil extraction, chemical composition of the oil or the origin of the berries.

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Ruan et al. [181] intraperitoneally injected the mice with 2 - 8 ml of oil/kg body weight for three days before SO_2 inhalation for five days. It was not clearly specified in the article whether the sea buckthorn admistration was continued during the SO_2 inhalation. Wu et al. [225] used the same range of doses. They started the treatment three days before the SO_2 inhalation for seven days. The oil was given to the mouse throughout the study. Ruan et al. [181] report a modest protective effect of sea buckthorn seed oil against SO_2 induced changes in organ/body ratio and chromosome damage in bone marrow. Wu et al. [225] found that sea buckthorn seed oil prevented the SO_2 induced increase in the oxidation marker thiobarbituric acid reactive substances (TBARS) in the lungs and protected against SO_2 induced changes in the glutathione activities in the lungs.

Süleyman et al. [199] studied the effects of combined sea buckthorn juice and hexane extract against nicotine induced oxidative stress in rats. The extract was prepared from Turkish *H. rhamnoides* berries, and admistered to the rats orally by a stomach tube at 1 ml/kg body weight/ day for three weeks. The chemical composition of the berry preparation was not reported. A total of 32 rats were included to the four groups getting only nicotine, nicotine and sea buckthorn, nicotine and vitamin E, and a control group getting neither nicotine nor sea buckthorn for three weeks. Statistical analyses were performed. Sea buckthorn extract restricted the nicotine induced decrease in blood antioxidants and rise in biomarkers of oxidation. Contrary to vitamin E, sea buckthorn also restricted the nicotine induced decrease of superoxide dismutase activity in erythrocytes.

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Table 1. Cyto- and radioprotective properties sea buckthorn berry/berry fractions; *in vitro* studies [1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
SNP-induced cytotoxicity (nitrosative stress) in murine magrophages, sb before & during exposure	Ethanol extract	500 µg/ml mediun	Protection against: cytotoxicity (viable cells), production of free radicals, drop of antioxidant status, mitocondrial and nuclei damage	Cytoprotection by antioxidativity and radical scavenging	[63]
t-BOOH induced cytotoxicity (oxidative stress) in rat lymphocytes, sb before & during exposure	Pulp flavonoids	100 µg/ml mediun	Protection against: cytotoxicity (viable cells), production of free radicals, drop of antioxidant status Restriction of apoptosis Restriction of DNA breaks	Cytoprotection by antioxidativity and radical scavenging	[62]
Radiation and chemical oxidant (t-BOOH) induced damage in thymocytes, effect of sb	Alcohol extract (RH-3)	greatest effects: antiox >500 µg/ml DNA protection ≈100-120 µg/ml mediun	Protection against: cytotoxicity (reduced DNA strand breaks), free radicals, Too high dose: irreversible condensation of chromatin compaction (neg), Low enough dose: reversible (pos)	Antioxidativity + chromatin organization →DNA protection	[109]
Radiation induced damage in murine thymocytes, effect of sb	Ethanol extract (REC-1001)	greatest effects: 250 µg/ml mediun (radical reduction: 25 µg/ml)	Protection against: cytotoxicity (reduced DNA damage), free radicals	Antioxidativity + free radical scavanging → radioprotection	[193]
Radiation induced damage in U87 cells (human cancer cells), sb before exposure	Alcohol extract (RH-3)	most effective: depending on the assay 5 µg/ml medium and above	Protection against: cytotoxicity (viable cells), production of free radicals. Prevention of radiation-induced depletion in mitochondrial membrane potential and apoptotic changes	Antioxidativity preventing cellular and mitochondrial generation of free radicals	[3]

[1] Abbreviations: sb = sea buckthorn; SNP = sodium nitroprusside; t-BOOH = *tert*-butyl hydroperoxide

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Table 2. Cyto-, radio- and neuroprotective properties and protection against pollutants by sea buckthorn berry/berry fractions; animal models[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Arsenic toxicity in mice, effects of oral sb after the exposure	Water and ethanol extracts	500 mg/kg bw for 10 d	Protection against: oxidative stress, changes in hematological variables and liver enzymes	No chelation of arsenic, effect via antioxidativity against arsenic induced oxidative stress	[74]
Arsenic toxicity in mice, effects of oral sb before and during exposure	Water and ethanol extracts	500 mg/kg bw for 3 wk	Protection against: oxidative stress, and disturbance of heam synthesis pathway	No chelation of arsenic, rather antioxidativity against arsenic induced oxidative stress	[73]
Damage of SO_2 inhalation in mice, effects of sb i.p. before exposure	Seed oil (CO_2 extr.)	2-8 ml/kg bw for 3 d	Protection against: changes in organ/body ratio and chromosome damage in bone marrow	Authors hypothese protection against oxidative damage	[181]
Damage of SO_2 inhalation in mice, effects of sb i.p. before and during exposure	Seed oil	2-8 ml/kg bw for 10 d	Protection against: rise in biomarkers of oxidation and depletion of antioxidant in lungs	Antioxidativity against SO_2 induced oxidative stress	[225]
Nicotine induced oxidative stress in rats, effects of i.g. sb during nicotine exposure	Juice & hex extract	1 ml/kg bw for 3 wk	Protection against: decrease in antioxidants and rise in biomarkers of oxidation	Antioxidativity	[199]
Lethal whole body gamma-radiation in mice, effect of sb i.p. before radiation	Alcohol extr. (RH-3)	30 mg/kg bw i.p. 30 min prior	Better survival rate, *in vitro* antioxidativity, protection of hematological parameters	Antioxidativity, immunostimulation	[65]
Lethal whole body gamma-radiation in mice, effect of sb i.p. before radiation	Alcohol extr. (RH-3)	i.p. 30 min prior	Increase in surviving crypts in jejunum and villi cellularity suggesting radioprotection	Protection against loss of cellularity of crypts and villi	[66]
Lead induced memory impairment & neuronal damage in mice, sb orally before & during exposure to lead	Juice	*0.1 or 1ml/10 g bw 20/40% juice 25 d	Protection against: impairment in cognitive tests, increase of oxidation biomarkers, decrease in several neurotransmitters	Authors hypothese chelation, accelerated excretion of lead and antioxidativity	[228]
Hypobaric hypoxia induced cerebral vascular injury in rats, sb i.g. before exposure	Seed oil (CO_2 extr.)	2.5 ml/kg bw	Protection against: transvascular leakage in brain, increase in markers of oxidation and free radicals, increase in stress hormones	Antioxidativity against hypoxia induced oxidative stress	[164]

[1] Abbreviations: bw = body weight; d = day; extr. = extract; hex = hexane; i.g = intragastric; i.p = intraperitonial; sb = sea buckthorn
*conflicting information in the article

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2.2.2 Inflammation and immunomodulation

Oxidation reactions are involved in inflammation. Antioxidants are important in protecting cells of the immune system and modulating inflammation [15, 24] (Tables 1 and 2). A few studies especially aimed at investigating the effects of sea buckthorn berry fractions on inflammation and immunity have been carried out (Table 3). Outside the scope of this review, several articles have been published concerning the immunomodulative effects of sea buckthorn leaves.

Depending on the situation, either boosting or inhibiting the immune response is desirable. The anti-inflammatory effects of flavonoids and other food components are considered beneficial, as chronic low grade inflammation contributes to the pathogenesis of cardiovascular diseases, asthma and rheumatoid arthritis, among others [15, 154]. However, for the defence against microbial infections, potent inflammatory reactions and a competent immune system are essential, and excess suppression of inflammation is undesirable [195].

The immunomodulation studies have focused on effects of sea buckthorn flavonoids and flavonoid rich fractions. Both stimulation [128] and attenuation [23, 89] of inflammation has been reported depending on the study design and target cells. All studies included control group(s), and the results were analysed using statical methods.

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Table 3. Effects of sea buckthorn berry/berry fractions on inflammation and the immune system; *in vitro* studies and animal models[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Chromium(IV) induced oxidative damage in mice lympocytes, antioxidative and immunomodulatory effects of sb (*in vitro*)	Ethanol extract	500 µg/ml	Inhibition of free radical production, apoptosis, DNA fragmentation, restoration of antioxidant status. Arrestment of chromium(IV) induced inhibition of lymphocyte proliferation	Cytoprotective and immunomodulatory effects due to antioxidant activity	[64]
Effects of sb on cellular immunity in laying hens (*in vitro* investigation)	Ethanol extract*	50-600 µg/ml	Enhance of magrophage membrane function, dual effects on circulating phagocytes (dose), high sb concentration: inhibition of leucocyte proliferation	Authors speculate effect of vitamin C and antioxidativity, and synergy/antagonism of of active ingredients	[48]
Effects of sb on cAMP/PKA pathway (related to aging, inflammation) in rats	Fruit oil (crushing & centri-fuging)	0.72-4.5 g/kg bw/d for 180 d	Raising of serum cAMP in aged rats, raised cAMP concentration and PKA activity in hepatic tissue → resistance of age-related changes	Second messanger cAMP regulates antiinflammatory properties and is reduced in aging → antiinflammation, anti-senility by sb	[89]
Immunomodulatory action of sb in human PBMC and in mouse magrophages (*in vitro*)	Berry flavonoids	100-500 µg/ml	Stimulation of IL-6 & TNF-α production, increased phosphorylation of IkB, increased translocation of NF-kB (=activation of NF-kB)	Activation of NF-kB target genes → induction of immune responses and possibly benefial effects againts microbial infection	[128]
Anti-iflammatory actions of sb in cancer cell lines (*in vitro*)	Berry juice	25 µl/ml	Inhibition of TNF-induced expression of COX-2 in cancer cell line, inhibition of NF-kB activation	Authors discuss additive and synergestic effects of mixture of components in juice	[23]

[1] Abbreviations: bw = body weight; cAMP = cyclic adenosine mono phosphate; COX-2 = cyclo-oxygenase-2; NF-kB = nuclear factor -kB; PBMC= peripheral blood mononuclear cells; PKA = protein kinase A, cAMP-dependent protein kinase; TNF = tumour necrosis factor; sb = sea buckthorn

* It was not totally clear from the article whether the berries or some other part of the sea buckthorn plant was extracted

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An i*n vitro* study by Geetha et al. [64] concerning the ethanol extracts of sea buckthorn suggests positive effects in immune cells exposed to oxidative stress. The study was carried out using the ethanol extracts of leaves and berries of *H. rhamnoides* collected from the Indian Himalayas. The chemical composition of the extracts was not presented. At a dose of 500 µg/ml medium the leaf and berry extracts inhibited the chromium(IV) induced production of free radicals, apoptosis, fragmentation of DNA and drop of antioxidant status in rat lymphocytes. Both sea buckthorn fractions restricted the inhibition of lymphocyte proliferation caused by chromium(IV). The leaf extract had a stronger cytoprotective and antioxidative effect compared to the berry extract. The authors conclude that sea buckthorn extracts have immunomodulating activity, at least in part due to their antioxidativity.

Dorhoi et al. [48] studied the effects of herbal extracts on cellular immunity *in vitro* using samples from laying hens. From the study report it was not totally clear whether berries or some other part of *H. rhamnoides* was used to gain the ethanol extract used. The compositions of the extracts were not presented. Concentrations of sea buckthorn extract from 50 – 600 µg/ml medium were used. Sea buckthorn extract at a concentration of 50 µg/ml medium enhanced the membrane functions of the hen magrophages. At doses of 200 - 400 µg/ml medium sea buckthorn increased the activity of circulating phagocytes, whereas with higher or lower doses the effect was opposing. At concentrations of 400 µg/ml medium, sea buckthorn also inhibited leukocyte proliferation in another experiment of the study. Even though the composition of the sea buckthorn extract was not analysed, the authors speculate on the role of vitamin C and antioxidant activity [48].

Hu et al. [89] suggest the anti-inflammation and anti-senility effects of sea buckthorn berry oil via the modulation of cyclic adenosine mono phosphate (cAMP) production. cAMP is a second messenger that transmits signals from hormones and other extracellular transmitters in intracellular pathways, including those related to inflammation. cAMP's effects are mediated by its effects on the activity of the cAMP-dependent protein kinase (PKA). The elevation of intracellular cAMP is associated with the inhibition of lymphocyte activation. Compounds that can induce cAMP elevation have been shown to be immunosuppressive and anti-inflammatory. cAMP is commonly reduced in ageing [89].

Hu et al. [89] admistered sea buckthorn oil at doses of 0.72 - 4.5 g/kg body weight/ day to old rats for 180 days. Berry oil from Chinese sea buckthorn, extracted by crushing and centrifuging was used. The species of *Hippophaë* or details of the extraction method were not defined. The three main fatty acids of the oils were reported to be palmitic acid, palmitoleic acid and oleic acid. Sea buckthorn oil was found to raise the serum levels of cAMP and increase the cAMP concentration and PKA activity in the hepatic tissues of rats. The authors hypothesise this could contribute to the anti-inflammatory and anti-

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senility effects of sea buckthorn oil. For significant effects, a dose of 1.8 g sea buckthorn oil/kg body weight was required.

Nuclear factor-κB (NF-κB) is a transcription factor that regulates the expression of several inflammatory genes [15]. In unstimulated cells it locates in the cytoplasm bound to IκBα and IκBβ proteins. When stimulation occurs, IκB is phosphorylated by specific kinases, leading to degradation of IκB by proteosomes. Consequently NF-κB moves into nucleus, binds to promoter region of the target genes for inflammatory proteins (cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and adhesion molecules, among others), and induces their expression. NF-κB is activated by several stimuli, including certain cytokines, oxidants, viruses, antigens and ultra violet-radiation [15].

Mishra et al. [128] studied the *in vitro* immunomodulatory activity of sea buckthorn berry flavonoids in human and mouse cells. The flavonoids were obtained from a Chinese company but other information concerning the origin or processing of the berries was not reported. The total polyphenolic content of the preparation was 310 mg/ml as a gallic acid equivalent. Flavonoid concentrations from 100 to 500 µg/ml medium were used depending on the assay. At this concentration range no cytotoxic effects by the flavonoids were observed. The flavonoids, at a dose of 200 µg/ml medium, induced the activation of pro-inflammatory transcription factor NF-κB in human peripheral blood mononuclear cells (PBMC). At concentrations of 100 - 200 µg/ml medium, the flavonoids increased the production of IL-6 and TNF-α in stimulated (stimulation with lipopolysaccharide) and in nonstimulated PBMC. The sea buckthorn flavonoids did not affect the production of NO in the stimulated or nonstimulated mouse magrophages. The authors conclude that the activation of NF-κB target genes by sea buckthorn flavonoids may enhance immune responses and be beneficial against microbial infections.

Boivin et al. [23] investigated the *in vitro* effects of 13 berry juices on inflammation and cell proliferation in human cancer cells. The sea buckthorn berry juice tested was prepared from *H. rhamnoides* cultivar Sunny, using a domestic juice extractor. The chemical composition of the sea buckthorn juice or other juices was not reported. Sea buckthorn juice at a concentration of 25 µg/ml medium was among the berry juices that significantly inhibited the TNF-induced activativation of COX-2 expression, and the activation of NF-κB in human prostatic adenocarcinoma cells. Compared to most other juices, the anti-inflammatory actitivity of sea buckthorn was strong. Only gooseberry (in two of the assays) and blackberry (in one of the assays) showed a stronger effect.

Of the individual flavonols present in sea buckthorn, quercetin, kaempferol, and isorhamnetin inhibit the activation NF-κB in activated macropahes *in vitro* [78]. As antioxidants have been shown to block the activation of kinases responsible for IκB phosphorylation reactive oxygen species may be intermediary in this pathway. It is known that oxidative stress can fortify inflammation [15]. The reported opposing effects of the sea buckthorn on inflammation and NF-kB

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emphasize the influence of the chosen fraction, dose, target cells or animals, and other methods of investigation on the outcome of the study.

2.2.3 Proliferation, apoptosis and cancer

Cancer is a consequence of genetic damage leading to unregulated proliferation and suppressed apoptosis of cells [147, 195]. The effects of sea buckthorn berry components on these processes have been investigated frequently *in vitro*, but less *in vivo* (Table 4). Both types of studies indicate the positive effects of sea buckthorn berry, and the flavonoid rich ethanol extract in particular. Several interrelated mechanisms are suggested and the possibility of the synergetic effects of the components is stressed.

Although ethanol extracts and flavonoids are most often used as the test material in the cancer and tumorigenesis trials (Table 4), they may not be the most potent fractions. In human colon cancer cells in particular, the effect of the ethyl acetate fraction, rich in both ursolic acid and polyphenols, was more potent in inhibiting growth of cancer cells than the ethanol-water extract, richer in polyphenols and poorer in ursolic acid [69]. Ursolic acid (Figure 8) is a pentacyclic triterpene reported to have anticarcinogenic activity. Ursolic acid may inhibit the NF-κB dependent gene expression and thus reduce inflammation [183], a mechanism observed for flavonoids as well.

Carcinogenesis and chronic inflammation share common mechanisms. Inflammation-induced reactive oxygen and nitrogen species can damage DNA and other cellular components, and contribute to the formation of malignant cells [24, 110, 195]. Excessive expression or abnormal activity of proinflammatory contributors like iNOS, COX-2 [110, 195], proinflammatory cytokines and prostaglandins, can promote tumorigenesis by stimulating cell proliferation and inhibiting apoptosis among other mechanisms [110]. Anti-inflammatory agents are considered to be potential chemopreventives [110, 183, 195]. Due to the focal role of NF-κB in the regulation of inflammation [15], restricting its activation has been among the targets of recent chemoprevention studies [110, 195].

Grey et al. [69] evaluated the levels of ursolic acid in 24 cultivars and advanced selections of sea buckthorn berries. Three of them, having low (*H. rhamnoides* 'Bhi 10941'), medium (*H. rhamnoides* 'Bhi 10726') and high (*H. rhamnoides* 'Podaruk Sadu') contents of usolic acid were used for the proliferation experiments. Initially berry extracts were prepared using several different solvents. For further tests only the most potent fractions produced by ethyl acetate and two different ethanol-water extractions were chosen. Sea buckthorn extract concentrations from <0.1% to 2% v/v in the medium were used. The ursolic acid rich ethyl acetate fraction had the strongest antiproliferative effect in the colon cancer cells, whereas the ethanol-water extract had the strongest antiproliferative effect in the liver cancer cells. For the ethyl acetate fraction, an apoptosis inducing effect was observed as well. The

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study was carefully conducted and the results were expressed in relation to the control. The contents of the main phenolics and ursolic acid in the extracts were reported.

Ursolic acid

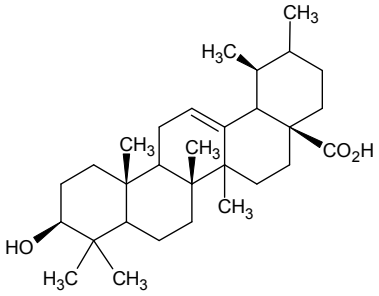

Figure 8. Ursolic acid

In the study by Boivin et al. [23], sea buckthorn juice (*H. rhamnoides* cv. Sunny) was among the berry juices that strongly inhibited the proliferation of human intestinal, breast, prostate and stomach cancer cells *in vitro* at a level of 50 juice µl/ml medium. For prostate and breast cancer cells the effect was dose dependent at concentrations from 10 to 50 µl/ml medium. As reviewed in the previous chapter, the anti-inflammatory effect of sea buckthorn juice was observed in this study as well. All the 13 berry samples tested had antioxidant activity. The antioxidativity did not, however, correlate with the antiprolifative effects.

Olsson et al. [147] stress the potential of the synergestic effects of vitamin C and other berry components in preventing the proliferation of colon and breast cancer cells. They studied the effects of ten fruit and berry fractions *in vitro*. The sea buckthorn sample was *H. rhamnoides* species of unknown origin from the Balsgård assortment, extracted with ethanol-water. An advantage compared to most other studies, was the detailed reporting of the chemical compositions of the fruit and berry extracts used [147]. Sea buckthorn was among the strongest inhibitors of cancer cell proliferation at the highest concentrations tested (0.25 and 0.5 % of plant dry material/ total weight of the test medium). Across the berries and fruits of the study, there was an inverse relationship between the vitamin C content and breast and liver cancer cell proliferation. At the second highest concentration (0.25 %) of fruit/berry extracts tested, there was an inverse association between lutein and β-carotene content and the proliferation of the breast cancer cells. The authors point out that the levels of the bioactive plant compounds in this study were within the range that can be found in human tissues.

Teng et al. [206] used isolated sea buckthorn isorhamnetin in their study, aiming to investigate its efficacy against human hepatocellular carcinoma cells.

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From the article it was not completely clear whether sea buckthorn berry or some other part of *H. rhamoides*, like the leaves were used for the isolation of isorhamnetin. Concentrations of 25 - 300 μg isorhamnetin/ml medium were used. It was found that isorhamnetin could permeate into the cancer cells and reduce their viability in a dose- and time-dependent manner. Isorhamnetin at a level of 50 μg/ml induced the fragmentation and condensation of chromatin in the cells. Chromosomal condensation is thought to preceed the formation of apoptotic bodies. When the amount of apoptotic cells was investigated using flow cytometry, signs of increased apoptosis induced by isorhamnetin was observed. However, the authors point out that the concentrations used in their study were high compared to the levels observed *in vivo*.

Padmavathi et al. [151] studied chemoprevention by sea buckthorn in mice. Berries of *H. rhamnoides* ssp. Turkestanica were extracted using ethanol to produce the fraction given by oral gavage to the mice. Doses from 150 to 300 mg/kg body weight were given for 2 - 6 weeks, depending on the experiment. The mice were admistered the berry extracts before the chemical induction of skin or forestomach papillomagenesis. In the skin papillomagenesis test, the intake of sea buckthorn extract was continued during the first weeks of induction of papillomas. Additionally, the effect of sea buckthorn on liver enzymes was investigated by giving the mice the sea buckthorn extract for 14 days at a dose of 150 or 300 mg/kg body weight. A total of 45 - 60 mice including a control group were included. Sea buckthorn extract inhibited the incidence of skin and forestomach tumours induced by 7,12-dimethyl-benzathracene and benzo(a)pyrene, respectively. The liver phase II enzymes catalyse the conjugation of xenobiotics to another molecule. This may change the activity of the xenobiotic and change it into an excretable form. The ethanolic extract of sea buckthorn induced the activity of phase II and antioxidant enzymes in the liver. It also induced the transcription factor interferon regulatory factor-1 (IRF-1), which has been shown to possess growth-inhibitory and antioncogenic effects [151].

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Table 4. Effects of sea buckthorn berry/berry fractions on cancer cells and induced tumorigenesis; *in vitro* studies and animal models[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects on cancer cell proliferation by sb *in vitro* and possible mechanism, 5 cancer cell lines	Berry juice	10 -50 µl/ml medium	Inhibition of proliferation of intestinal, breast, prostate and stomach cancer cells, Inhibition of TNF-induced expression of COX-2 in cancer cell line, inhibition of NF-kB activation	Authors discuss additive and synergestic effects of mixture of components in juice. No correlation between antioxidativity and anti-proliferative activity	[23]
Effects of different sb extracts on human colon and liver cancer cells and possible mechanisms *in vitro*	Berry extracts using several solvents	<0.1%-2% (v/v) medium	Inhibition of proliferation of both cancer cell lines. Ethanol-water (polyphenols) & ethyl acetate (ursolic acid+polyphenols) extracts had the strongest effects.	Ethyl acetate fr: both anti-proferative & increased apoptosis Ethanol-water fr: antiproliferative. Authors stress potential of synergetic effects and ursolic acid	[69]
Effects of sb on colon cancer and breast cancer cell lines *in vitro*	Ethanol-water extr.	0.025-0.5% of dry material/ weight of medium	Inhibition of proliferation of both cell lines, correlation of antiproliferation with the level of vitamin C and some carotenoids at certain levels (also other berries tested)	Authors stress the possibility of the synergetic effects of vitamin C and other components in berry extracts	[147]
Cytotoxic effects of sb isorhamnetin on human hepatocellular carcinoma cells, *in vitro*	Isorhamnetin aglyc. isolated from sb*	25 - 300 µg/ml medium	Cytotoxicity against cancer cells (loss of viability), permeation of isorhamnetin into cells, fragmentation & condensation of chromatin in cells, signs of increased apoptosis	Potential for apoptosis inducing drug, however authors point out the limited isorhamnetin concentrations *in vivo*	[206]
Chemoprevention by sb in mouse, effects on induced skin and forestomach papillomagenesis and possible mechanism, sb by oral gavage before and during exposure	Ethanol extract	150-300 mg/kg bw for 2-6 wk	Decreased incidence of skin and forestomach tumours, induction of phase II xenobiotic metabolizing enzymes, antioxidant enzymes, induction of transcription factor IRF-1 in the liver	Decrease in carcinogen induced tumorigenesis by up-regulation of phase II and antioxidant enzymes and the induction of IRF-1	[151]

[1] Abbreviations: bw = body weight; COX-2 = cyclo-oxygenase-2, extr. = extract; IRF-1 = interferon regulatory factor-1, transcription factor having cell growth-inhibitory and antioncogenic effects; NF-kB = nuclear factor-kB; TNF = tumour necrosis factor; sb = sea buckthorn; wk = week

* It was not totally clear from the article whether berries or some other parts (e.g. leaves) of sb were used as starting material

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2.2.4 Antimicrobial properties

The antimicrobial effects of berries, sea buckthorn among them, have been investigated extensively by Puupponen-Pimiä et al. [142, 165, 166]. As summarized in Table 5, sea buckthorn berry and/or the phenolic fractions of berries and seeds have *in vitro* anti-bacterial activity against several bacteria, including strains of human pathogenic *Salmonella enterica, Escherichia coli* and *Staphylococcus aureus*. No effects on the growth of probiotic bacteria were observed [142, 165, 166]. The suggested antibacterial mechanisms of berry compounds include disruption of the outer membrane of bacterial cell walls (gram negative bacteria) and mutagenic effects on bacterial DNA [142, 166].

Compared to the individual flavones, flavonols, flavanones, anthocyanidins and phenolic acids tested by Puupponen-Pimiä et al. [166], the inhibition of bacteria by berry extracts was stronger. This suggests effects of complex compounds, such as ellagitannins (hydrolysable tannins) or proanthocyanidins (condensed tannins), and/or synergy between several berry components [166]. Though showing antimicrobial activity, sea buckthorn was among the weakest berry inhibitors of bacterial growth *in vitro*, whereas cloudberry and raspberry consistently showed high efficacy [142, 165, 166]. This may be due their high content of ellagitannins, present in sea buckthorn only in trace amounts [107, 142].

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Table 5. Antimicrobial properties of sea buckthorn berry/berry fractions; *in vitro* studies[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects of sb (and other berries) on probiotic and other intestinal bacteria	Phenolic extract (Acetone-water)	0.8-7.0 mg /50 µl agar well or 1 mg/ml	Agar diffusion test: antimicrobial against *E. coli* CM871 and *Enterococcus faecalis.* Liquid culture: antimicrobial against *S. enterica*, *E.coli* 50 and *E. coli* CM871 No effect on *Lactobacillus* or *Bifidobactrium*	Authors discuss mutagenic activity on bacterial DNA & the disruptive effect on the outer membrane of gram neg. bacteria. Synergy of compounds	[166]
Effects of sb (and other berries) on intestinal pathogens	Phenolic extract & whole berry powder	Extract: 1 mg/ml medium Whole berry: 2-10 mg/ml medium	Whole berry: antimicrobial against *S. enterica* sv. Typhimurium, *S. aureus* Extract: antimicrobial against *S. aureus* but not *Salmonella* strains. No effects on *Listeria* or *S. enterica* sv. Infantilis	Different mechanisms of action for gram+ *S.aureus* compared to gram- *S.enterica*	[165]
Effects of sb (and other berries) on human pathogens	Phenolic extract (Acetone-water)	1 mg/ml medium	Antimicrobial against *Bacillus cereus* & *Clostridum perfringens*. No effect on *Campylobacter jejuni* or *Candida albicans*	Bacterial growth inhibition by sb weak compared to cloudberry and raspberry. They are rich in ellagitannins, likely focal for the effect	[142]
Effects of sb on *Listeria monocytogenes* and *Yersenia enterocolitica*	Aqueous extract of seeds	500-1000 ppm medium	100 % inhibition of growth at concentrations of 750 ppm for *L. monocytogenes* and 1000 ppm for *Y. enterocolitica*, respectively.	The composition of the seed water extract was not presented in detail, but was stated to contain phenolics, likely contributing to the effect	[34]
Effects of sb on *Bacillus cereus, B. coagulans, B. subtilis, L. monogytogenes* and *Y. enterocolitica*	Different solvent extracts of seeds	up to ≈ 800 ppm medium	Methanolic extract most effective in preventing bacteria growth, had the highest antioxidant activity, and highest content of phenolic compounds.	Phenolics in the extract are likely to cause the antimicorbial effect. Choroform extract had the least phenolics and was least effective	[136]

[1] Abbreviations: *E.coli* 50 = *Escherichia coli* 50; *E.coli* CM871= DNA-repair deficient *Escherichia coli*; *S.enterica* = *Salmonella enterica* SH-5014; *S.aureus* = *Staphylococcus aureus* E-70045; sb = sea buckthorn

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2.2.5 Wounds, skin and mucosa

Results from animal studies indicate that oral and topical sea buckthorn seed oil and topical sea buckthorn flavonoids can significantly promote the healing of wounds (Table 6) [72, 216]. Both sea buckthorn oil and flavonoids increased the antioxidant levels in the wound and reduced the levels of reactive oxygen species and/or markers of oxidation [72, 216]. The effects on inflammation were suggested as well. Inflammation is needed for healing of the wound, to prevent infections in it, and to induce the proliferation phase of the healing. However, excessive and long lasting inflammation is detrimental and leads to tissue damage [216].

Upadhyay et al. [216] investigated the effects of CO_2-extracted seed oil on burn wounds in rats. The wild *H. rhamoides* berries for the extraction of the oil were collected from the Western Himalayas. Even though the study was otherwise carefully conducted and reported, the composition of the particular oil used in this study was not analysed. Instead, for the composition of the oil the reader was referred to a previous article published two years earlier. An intial screening for the optimal dose and route of admistration was carried out by giving the rats oral doses of 1.0 - 5.0 ml oil/kg body weight for 7 days after creating burn wounds. The second group of rats was treated with applying topically doses of 100 - 400 µl oil/wound for 7 days after creating the wounds. Based on this, a combination of oral oil (2.5 ml oil/kg body weight/day) and topical oil (200 µl oil/wound/day) was used for the experiments (admistration of oil for 7 days after generation of the burn wound). In addition to the sea buckthorn group, positive and negative control groups were included (6 - 8 rats/group).

Co-admistration of topical and oral sea buckthorn oil was found to enhance wound healing as indicated, among others, by increased wound contraction and increased amount of hydroxyproline (component of collagen) in the tissue. The sea buckthorn oil treated wounds showed reduced oedema or no oedema, indicating attenuation of the inflammation. Up-regulated expression of the vascular endothelial growth factor (VEGF) indicated the induction of angiogenesis by the oil. Histopathological findings supported the positive effects of sea buckthorn oil on the regeneration of tissue in the wounds. Sea buckthorn oil reduced the amount of ROS and increased the amount of reduced glutathione in the wound granulation tissue. The authors suggest that the improved wound healing induced by sea buckthorn oil may be due to the antioxidant activity as one of the mechanisms involved [216].

Topical sea buckthorn berry flavonoids had similar positive effects on dermal wounds in the study of Gupta et al. [72]. *H. rhamnoides* flavonoids obtained from a Chinese company were mixed in propylene glycol base at concentration of 2%. The total polyphenolic content of the flavonoid preparation was reported, but no other details of the extraction methdods, origin of berries or chemical composition were presented. A total of 24 rats were divided into

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control and experimental animals. In the sea buckthorn group, 20 µl of the flavonoid rich base were applied topically to the wounded area for 7 - 16 days after the creation of the wounds. The sea buckthorn flavonoids were found to promote the wound healing as indicated by improved wound contraction, faster epithelialization and increased levels of hydroxyproline and hexosamine (indicating collagen production and stabilization) in the wound tissue. Also the antioxidant status in the wound tissue was increased by sea buckthorn flavonoids and the levels of lipid peroxides were lower compared to the control. The histopathological evaluation of the wounds showed a greater rate of tissue regeneration compared to the control. Vitamin C was among the antioxidants, the levels of which were greater in the sea buckthorn group compared to that of control. This may be due to presence of vitamin C in the sea buckthorn extract or the flavonoids may aid its preservation. Vitamin C is essential for collagen synthesis [72].

A clinical, randomized, placebo-controlled study by Yang et al. [231] (Table 6) indicates that CO_2-extracted sea buckthorn seed and pulp oil may beneficially affect atopic dermatitis characterized by dry and itchy skin and inflamed eczema lesions. Atopic dermatitis arises from possibly genetic disturbances in the epidermal function and/or immune system. Due to the essential role of long-chain n-3 and n-6 polyunsaturated fatty acids as eicosanoid precursors in the regulation of inflammation, they are hypothesized to be involved in the development of atopy. Atopic patients may have a deficiency in Δ^6-desaturase, the enzyme converting α-linolenic acid and linoleic acid to stearidonic and γ-linolenic acids, respectively (Figure 4). In addition, polyunsaturated fatty acids are components of sphingolipids contributing to the water barrier properties of the epidermis [231].

A total of 78 women and men were randomized to Yang's study [231]. Forty-nine of them completed and were included in the statistical analyses. Supercritical CO_2-extraction of *H. rhamnoides* seeds and combined berry flesh and peels were used for production of the study oils. Paraffin oil was used as a placebo. The fatty acid compositions of the oils were reported in the article. The participants daily took 5 g of sea buckthorn seed oil, sea buckthorn soft part oil or a placebo for four months. During the intervention, the severity of the atopic dermatitis was evaluated by the SCORing Atopic Dermatitis (SCORAD) index, and blood samples were collected for the determination of plasma fatty acids, lipid and immunoglobulin E (IgE) levels.

The atopic dermatitis symptoms at the end of the intervention were significantly milder in the pulp oil and placebo (paraffin oil) groups compared to the baseline. The seed oil group had a trend towards milder symptoms as well, but the changes were not significant. The proportion of α-linolenic acid increased significantly in the plasma neutral lipids in the seed oil group from baseline to the end. There was an almost significant increase in the proportions of α-linolenic, linoleic and eicosapentaenoic acids in the plasma phospholipids. The increases of α-linolenic acid in both lipid classes in the seed oil group were

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significantly positively correlated with the improvement of symptoms. The changes of plasma fatty acid proportions in the pulp oil group (increase of palmitoleic acid and decrease of pentadecanoid acid in phospholipids) did not correlate with the symptoms. There were no changes in the levels of plasma IgE [231]. The authors suggest that the lack of significance for the symptom effects in the seed oil group may be due to the small number of participants and mild symptoms at baseline. As the fatty acids of the pulp oil did not correlate with the symptoms, it is likely that other components contributed to the positive effects, and the anti-inflammatory potential of plant sterols was pointed out by the authors. The changes in the paraffin oil group indicate a significant placebo effect [231].

In their subsequent study, Yang et al. [232] investigated the effects of sea buckthorn seed and pulp oils on the fatty acid composition of skin glycerophospholipids of patients with atopic dermatitis. A randomized, parallel, placebo-controlled, double-blind design was used in the trial of 22 volunteers. The same oils and doses were used as in their study reviewed above [231]. The oil intervention lasted for four months. Skin biopsies for the analysis of fatty acid composition were taken at baseline and at the end of the intervention. Neither the seed nor the pulp oil induced significant changes in the glycerophospholipids of the skin [232] (Table 6). In the seed oil group, a trend towards increased proportion of eicosapentaenoic acid and decreased proportion of palmitic acid in the skin glycerophospholipids was observed. Additionally, the seed oil induced a significant increase in plasma linoleic acid. In the placebo group consuming paraffin oil, a nonsignificant ($0.05 < P < 0.1$) increase in the plasma concentrations of stearic and linoleic acids was observed. The authors concluded that the fatty acid composition of the skin glycerophospholipids is not easily affected by short-term dietary supplementation [232].

Two clinical studies by Yang and Erkkola [229] indicate the beneficial effects of sea buckthorn oil on the symptoms of vaginal inflammation and Sjögren's syndrome, an inflammatory autoimmune disease causing dryness, pain and inflammation in the mucous membranes. These trials had a low number of participants (five cases in the vaginal inflammation study and 25 in the Sjögren's syndrome study), but gave positive indications to further studies. These studies were carried out using a strandardized CO_2-extracted *H. rhamnoides* oil containing both pulp and seed oils.

In the vaginal inflammation study, the five women (35 – 79 years of age) participating had a history of vaginal inflammation symptoms for several years and had not benefited from earlier hormone replacement therapy or local corticoids. They consumed daily 3 g of sea buckthorn oil orally for 12 weeks. Three of the women, having the most severe symptoms at baseline, reported clear improvement of the vaginal inflammation symptoms. One participant reported a slight improvement and one participant did not report changes. The

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estrogen levels were not affected by the intervention. Due to the small number of participants, statistical analyses were not performed [229].

In the double-blind study concentrating on Sjögren's syndrome [229] 25 women (37 - 66 years of age) were randomized to receive sea buckthorn or a placebo (fractionated coconut oil) in a cross-over manner. The supplementation for each oil lasted for three months at a dose of 3 g/day. The symptoms of Sjögren's syndrome were evaluated using a visual analogue scale and by verbal description at the beginning of the study, at the end of the first intervention period, and at the end of the second intervention period. The participants reported an improvement of symptoms in both sea buckthorn and placebo groups from baseline to the end of the intervention. Clear differences between the sea buckthorn and placebo groups were seen in the percentage of participants reporting improvement of overall symptoms of Sjögren's syndrome after the first three months of the study. A significantly higher percentage of participants reported improvement in overall symptoms in the sea buckthorn group compared to the placebo. Among the individual symptoms positively affected by sea buckthorn were dryness of eyes and mouth (results of statistical analyses for individual symptoms not reported). When both of the three month intervention periods were included, a significantly greater proportion of the participants in the sea buckthorn group reported improvement in the symptoms of the genital tract, including itching and burning among others.

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Table 6. Effects of sea buckthorn berry/berry fractions on skin, wounds and mucosa; animal models and clinical trials[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects of sb on burn wounds in rats	Seed oil (CO_2 extr.)	2.5 ml/kg bw/d orally & 200 µl/d topically for 7 d after wounding	Augmentation of wound healing: increased wound contraction, hydroxyproline (collagen), hexosamine*, DNA & total protein, improvement in histopatholigical findings, decrease of oxidative stress (GSH↑, ROS↓). No adverse effects up to 10 ml/kg bw. No signs of sub-acute toxicity	Attenuation of inflammation, increase of angiogenesis, increased collagen, antioxidative properties	[216]
Effects of sb on dermal wound healing in rats	Flavonoids	1% flavoids in 20 µl PG base/d topically for 7-16 d, after wounding	Promotion of wound healing: improved wound contraction, faster epithelialization, increase in hydroxyproline and hexosamine*, improvements in histopathological findings, decrease of oxidative stress (GSH↑, catalase↑, vitaminC↑, lipid peroxides↓)	Antioxidative properties, contribution of vitamin C to collagen synthesis	[72]
Effect of sb on atopic dermatitis, clinical trial	Seed and pulp oils (CO_2 extr.)	5 g/d for 4 mo orally	Improvement of dermatitis in pulp oil and paraffin (placebo) group. Seed oil: 18:3n-3 ↑ in plasma NL, pulp oil: 16:1n-7 ↑, 15:0 ↓ in plasma PL & NL. Seed oil: positive correlation between symptom improvement and plasma 18:3n-3 in NL & PL. No effects on plasma IgE	Seed oil: effect of 18:3n-3 on 20:5n-3 and eicosanoids. Not only fatty acids important. Effects of pulp oil: sterols, anti-inflammatory; carotenoids: vitamin A, antioxidativity	[231]
Effect of sb on the fatty acids of glycero-phospholids in patients with atopic dermatitis	Seed and pulp oils (CO_2 extr.)	5 g/d for 4 mo orally	Seed oil: almost significant increase of 22:5n-3 & decrease of 16:0 in skin. Pulp oil: almost significant increase of 18:0 in skin. Paraffin placebo: almost sign. increase in 18:0, 18:2n-6. In plasma glycerophospholipids of the seed oil group: significant increase in 18:2n-6	Skin phosholipids are not easily affected by short term dietary supplementation	[232]
Effect of sb on chronic vaginal inflammation, clinical trial	Combined seed+pulp oil (CO_2)	3 g/d for 12 wk orally (Case-study)	Clear symptom improvement reported by 3 patients of 5, one reported slight improvement. No side effects reported	Effect did not take place through increase in circulating estrogen levels	[229]
Effect of sb on Sjögren's syndrome (SS), clinical trial	Combined seed+pulp oil (CO_2 extr.)	3 g/d for 3 mo	Symptom improvements in sb and placebo. Sb benefits over placebo: greater % of particants had overall improvement in first 3 mo, greater % had improved symptoms of genital tract mucosa	Mechanisms were not studied. SS is an inflammatory autoimmune disease, abnormalities in PUFA metabolism are reported	[229]

[1] Abbreviations: bw = body weight; extr. = extract; d = day; GSH= reduced glutatione; mo = month; NL= neutral lipids; PG = propylene glycol; PL= phospholipids; ROS= reactive oxygen species; sb = sea buckthorn; SS = Sjögren's syndrome; wk = week

*contributing to stabilization of collagen

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2.2.6 Gastric ulcer

Preventive and curative effects of sea buckthorn oils against gastric ulcers have been reported in rats (Table 7) [198, 227]. Süleyman et al. [198] investigated the effects of hexane extract from *H. rhamnoides* berries given orally by gavage before the induction of gastric ulcers by stress or by indomethacin. The origin of the berries, exact method of extraction, or the chemical composition of the sea buckthorn extract was not reported in the article. The rats in the sea buckthorn group were given 1 ml extract/kg body weight as a single dose. Positive and negative control groups were included in the experiments (n = 6 - 8 rats/group). The sea buckthorn oil admistration significantly reduced the number and size of ulcerative areas when the ulcer was induced either chemically or by stress. Stress and oxygen radicals contribute to the pathogenesis of gastric ulcers. The authors suggest that the hexane soluble components of sea buckthorn berry, including vitamin E and carotenoids, have cumulative antioxidantive effects that contribute to the prevention of mucosal injury.

Xing et al. [227] admistered CO_2-extracted seed and pulp oils of wild *H. rhamnoides* berries collected from Romania to rats to investigate the effects on gastric ulcers. The fatty acid composition and sitosterol, carotenoid, tocotrienol and tocopherol content of the oils were reported in the article. Daily doses of 3.5 or 7.0 ml oil/kg body weight were orally admistered to the rats for 7 - 12 days before or after the induction of gastric ulcers. Positive and negative control groups were included (n = 10 rats/group). Four methods for the induction of ulcers were used: water immersion stress, reserpine, pylorus ligation (sea buckthorn oils given before induction of ulcers) and acetic acid (sea buckthorn oils given after induction). In all experiments both seed and pulp oils had a positive effect against ulcer formation. In the case of water immersion and reserpine induction, a higher dose of 7 ml/kg was needed for significant effect. Significant protective and curative effects were observed at a dose of 3.5 ml/kg in the pylorus-ligation and acetic acid experiments, respectively. As both sea buckthorn seed and pulp oils were effective, it is likely that fatty acids are not the only components contributing to the effects. The authors suggest it is the inhibition of lipid peroxidation in gastric mucosa by the sterols, carotenoids and tocols in the oils. Sea buckthorn oil has been shown to promote wound healing in the skin [72, 216], and similar mechanisms may be important in the healing of the gastric mucosa as well.

Helicobacter pylori infection is associated with chronic inflammation, accumulation of reactive oxygen species, oxidative damage to gastric mucosa and increased risk of gastric ulcer [203]. Ethanol extracts of sea buckthorn leaves were reported to inhibit the growth of *H. pylori in vitro* [115], but this effect has not been reported for sea buckthorn berry or berry fractions in the journals published in English. Nohynek et al. [142] reported strong *in vitro H. pylori* inhibition by phenolic extracts of cloudberry, bilberry, black currant, raspberry and strawberry.

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Table 7. Effects of sea buckthorn berry/berry fractions on gastric ulcers; animal models[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effect of sb on gastric ulcer induced by stress and by indomethacin in rats	Hexane extract	1 ml/kg bw orally (gavage) prior to inducion of ulcer	The number and size of ulcerative areas were reduced significantly in both stress and indomethacin induced ulceration	Stress and oxygen free radicals contribute to the pathogenesis of ulcers. Authors suggest cumulative effect of vitamins as antioxidants and necessities for metabolic events to cause the effect	[198]
Effect of sb on gastric ulcer induced by stress, reserpine, pylorus ligation or acetic acid in rats	Seed oil and pulp oils (CO_2 extr.)	3.5 ml/kg bw/d and 7 ml/kg bw/d prior to/ after inducion of ulcer for 7 or 12 d, orally	Both oils reduced ulcer index and inhibited ulcer formation in all forms of inducion, including acetic acid inducion where the oil was given after inducion	Seed and pulp oils have both protective and curative effects. Both oils effective → fatty acids likely not the only important components, also sterols and carotenoids may affect, possibly the inhibition of lipid peroxidation in gastric mucosa	[227]

Table 8. Effects of sea buckthorn berry/berry fractions on hepatoxicity and liver fibrosis; an animal model and a clinical trial[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects of sb against carbon tetrachloride (CCl_4) -induced hepatotoxicity in mice	Seed oil	0.26-2.6 mg/kg bw orally for 8 wk after intoxication with CCl_4	Protection against: elevation of ALAT, ASAT, ALP, TAG, cholesterol in serum, MDA in the liver; decrease of SOD, catalase, GSH-Px, GSH-Rd, GSH in the liver. Protection against histopathological changes	Liver injury by hepatotoxins is largely caused by ROS, which damage the cells and may cause cell death. Antioxidative protection by sb is suggested (carotenoids, tochopherols), unsaturated fatty acids likely affect the changes in TAG and cholesterol	[88]
Effects of sb on chirrotic patients, clinical trial	SB extract, the solvent and plant part was not reported	15 g/d orally for 6 months	Presentation of the results somewhat ambigious concerning some markers. However, the authors conclude that the normalization rate of ALAT and ASAT enhanced, and serum levels of collagen, among others was reduced suggesting restrain of collagen synthesis in the liver	Authors discuss the role of retinyl esters and retinoic acid receptors in keeping the collagen producing and fat storing hepatic stellate cells in their inactivated state	[60]

[1] Abbreviations: ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; ALP = alkaline phosphatase; bw = body weight; d = day; extr. = extract; GSH= glutathione; GSH-Px= glutathione peroxidase; GSH-Rd= glutathione reducatase; MDA = malonialdehyde; SOD=superoxide dismutase; TAG=triacylglycerol; TNF-α= tumour necrosis factor-α; ROS= reactive oxygen species; sb = sea buckthorn

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2.2.7 Hepatotoxicity and liver fibrosis

Excessive alcohol consumption, virus infections, alterations in lipid and carbohydrate metabolism and xenobiotics, among others, induce liver damage. Reactive oxygen species are focal in the initiation and progression of the damages in all of the above-mentioned etiologies [131]. According to Hsu et al. [88] sea buckthorn seed oil has convincing potential against hepatotoxicity induced by carbon tetrachloride (CCl_4) in mice (Table 8). Cytochrome P-450, a phase I enzyme in the xenobiotic metabolism, catalyses the transformation of CCl_4 to trichloromethyl radical ($CCl_3\cdot$) and trichloromethyl peroxyl radical ($CCl_3O_2\cdot$), which can initiate peroxidation of membrane lipids, and ultimately lead to cell necrosis.

In Hsu's [88] study, which was otherwise conducted and reported very carefully, the information concerning the study oil was quite cursory. They report using commercial *H. rhamnoides* seed oil containing mainly linoleic and oleic acid. It was given orally for eight weeks at doses of 0.26 - 2.6 mg/kg body weight after exposing the the mice with CCl_4. Olive oil was used as a normal control and silymarin (a flavonolignan complex extract from *Silybum marianum*) was used as a positive control. Sea buckthorn oil protected the mice against the CCl_4-induced elevation of serum alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase, and triacylglycerols. It restricted the CCl_4-induced rise of body and liver weight which were considered as signs of hypertrophy of the liver tissue. The effects of sea buckthorn oil on hepatotoxicity were not dose-dependent, and a dose of 0.26 mg/kg bw/day was chosen to be the optimal one, equating a dose of only 15.6 mg/day for a person of 60 kg. Sea buckthorn oil significantly protected against a decrease of glutathione, superoxide dismutase, glutathione peroxidase and glutathione reductase in the liver, and the rise of malonialdehyde (a biomarker of lipid peroxidation) concentration, indicating protective effects due to enhanced antioxidative protection. [88].

Gao et al. [60] (Table 8) investigated the effects of sea buckthorn extract in 50 patients having liver fibrosis. The authors conclude that sea buckthorn may be a drug candidate for the prevention and treatment of liver fibrosis, and encourage further clinical trials on the topic. However, they did not specify which part of the plant was used for the preparation of sea buckthorn extract investigated, which was the extraction solvent used, and what was the composition of the extract. Better normalization rates of the serum ALAT and ASAT activities were reported for the sea buckthorn group compared to the control. However, the baseline values of ALAT differed greatly between the groups, being more than two times higher in the control group. A reduction of collagen production markers in serum was reported. The results concerning some of the other markers, including serum TNF-α and IL-6 were vaguely presented.

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2.2.8 Risk factors associated with cardiovascular diseases and diabetes

2.2.8.1 Endothelium

Several studies indicate that sea buckthorn beneficially affects the risk factors associated with cardiovascular diseases and diabetes (Tables 9 and 10). Bao & Lou [12] investigated the potential of sea buckthorn flavonoids against endothelial damage by oxidized low density lipoprotein (ox-LDL) *in vitro*. Endothelial dysfunction caused by ox-LDL contributes to pathogenesis of atherosclerosis. Ox-LDL induces changes in the secretory activities, consumes the antioxidant capacity and decreases NO synthesis in the endothelium. Synthesis of NO is critical to the normal function of endothelial cells. It mediates relaxation of smooth muscle and inhibits platelet activation [12, 13]. Though NO can react with superoxide to form peroxynitrite capable of LDL oxidation [207], it can also quench superoxide depedending on the environment [12, 13, 207].

Ox-LDL activates lectin like ox-LDL receptor-1 (LOX-1) in endothelial cells. Activation of LOX-1 leads to the activation of intracellular pathways including protein and tyrosine kinases and mitogen activated protein kinases (MAPK). These pathways contribute to the activation, dysfunction and apoptosis of endothelial cells. NF-κB is among the transcription factors whose activation is mediated by the MAPK cascades. Endothelial constitutive nitric oxide synthase (eNOS), whose expression is reduced due to LOX-1 activation is among the target genes of NF-κB [12, 13]. Ox-LDL decreases the levels of superoxide dismutase responsible for maintaining physiological levels of superoxide and increases the levels of reactive oxygen species making the cells vulnerable to oxidative damage [12, 13].

Bao & Lou [12] studied the potential of *H. rhamnoides* berry flavonoids against ox-LDL injuries in endothelial cells *in vitro*. The main components of the flavonoid preparation were quercetin and isorhamnetin. Concentrations of 9.38 - 37.5 µg sea buckthorn flavonoids/ml medium were used for the experiments of the study. ox-LDL induced endothelial cell deaths and secretion disorders (increase in the release of lactate dehydrogenase (LDH) and a reduced concentration of NO). It induced the production of superoxide, suppressed the content of superoxide dismutase, inhibited the expression of eNOS and increased the expression of LOX-1 in the endothelial cell culture. Sea buckthorn flavonols added to the medium before treatment with ox-LDL had a preventive effect against all of the above mentioned effects of ox-LDL. For the most protective effects, a concentration of 18.75 µl flavonoids/ml was required. Quercetin and isorhamentin as single components had similar protective potential as the sea buckthorn flavonoid mixture. In general, the effects of quercetin were more pronounced than those of isorhamnetin. As the authors note, reasonably high concentrations of flavonoids were used in the trial. However, the viability of the cells was not affected [12].

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Regardless of the impressive *in vitro* effects of sea buckthorn flavonoids on endothelial cells by Bao & Lou [12], the flavonoid rich sea buckthorn juice failed to show effects on intracellular adhesion molecule-1 (ICAM-1) in a randomized double-blind placebo-controlled clinical study by Eccleston et al. [53]. ICAM-1 is involved in the endothelial recruitment of monocytes into areas of inflammation. In the vascular walls, the infiltrated monocytes can internalize and scavenge oxidized LDL and be transformed to lipid rich foam cells, contributing to development of atherosclerotic plaque. During the study, the healthy male participants (a total of 30 men) daily consumed either 300 ml of commercial sea buckthorn juice or placebo juice for eight weeks. Blood samples for analyses of risk factors for cardiovascular disease were collected at the beginning and end of the intervention [53].

2.2.8.2 *Platelet aggregation and thrombosis*

The effects of sea buckthorn on platelet aggregation and thrombosis have been studied *in vitro*, in animals [37] and in clinical trials [53, 96] (Tables 9 and 10). The animal and *in vitro* study by Cheng et al. [37] concentrated on the effects of sea buckthorn flavonoids extracted from *H. rhamnoides* berries by methanol and ethyl acetate. The chemical composition of the extract was not presented in the paper. In the thrombosis aggregation experiment, the mice were given 100 – 300 μg flavonoids/kg body weight intravenously 15 min before inducing the formation of thrombus by photochemical reaction between intravenously injected rose bengal and green light irradiation. Aspirin was given to the mice in the positive control group. The number of mice was 6 - 8 /group. The flavonoid treatment at a dose of 300 μg /kg significantly prolonged the thrombotic occlusion time. The effect was similar to that of 10 mg/kg aspirin.

In Cheng's study [37], the anti-aggregation potential of sea buckthorn was investigated *in vitro* using flavonoid concentractions of 0.3 - 3.0 μg/ml medium. The flavonoids prevented platelet aggregation induced by collagen, but not those by adenosine diphosphate (ADP) or arachidonic acid. The authors suggest that sea buckthorn flavonoids inhibit platelet aggregation by suppressing the release of arachidonic acid from cell membrane phospholipids. Stimulation of collagen receptors leads to the activation of tyrosine kinase, which in turn leads to an increase in intracellular calcium and the activation of phospholipase A_2 [37]. Arachidonic acid released from the phospholipids by phospholipase A_2 is a precursor for thromboxanes A_2 and B_2 having vasoconstricting and platelet activating effects [188].

Johansson et al. [96] observed a platelet aggregation inhibitive effect of CO_2-extracted sea buckthorn oil in their cross-over study in healthy normolipidemic men. The duration of the two randomized treatment periods was four weeks and they were separated by a wash-out period of four to eight weeks. During their sea buckthorn period the participants consumed daily the combined sea buckthorn seed and pulp oil from *H. rhamnoides* berries at a dose of 5 g/day. During their placebo period, they took 5 g of fractionated coconut oil rich in

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medium chain fatty acids. The fatty acid compositions of the oils were reported in the article. A total of 12 volunteers participated in the study. The combined sea buckthorn seed and pulp oil decreased the rate and maximum of ADP induced platelet aggregation compared to the placebo. There was no effect seen on plasma lipids, fatty acid composition of plasma and platelet phospholipids or on platelet aggregation induced by arachidonic acid [96].

Sea buckthorn berry juice (300 ml/day for eight weeks) did not affect platelet aggregation induced by any of the aggregation agonists tested (ADP, collagen, arachidonic acid) in the clinical placebo-controlled, double-blind, randomized, parallel study by Eccleston et al. [53] (Table 10 & Chapter 2.2.8.1: more details of the study). Even though a small decrease in platelet aggregation over the study period was observed, this was evident in both sea buckthorn and placebo, and the groups did not differ significantly. The blood aggregation assays were conducted at the beginning and end of the intervention period.

2.2.8.3 Circulating lipids

The effects of sea buckthorn on circulating lipids have been investigated in several clinical trials and in an animal study (Tables 9 and 10). Basu et al. [17] investigated the antiatherogenic effects of sea buckthorn seed oil in rabbits. CO_2-extracted seed oil from the wild *H. rhamnoides* berries from Western Himalayas was orally admistered to rabbits at a dose of 1 ml/ day. The composition of the particular oil in question was not reported. Instead the authors cite a previous article. One group of rabbits received the seed oil for 18 days with a normal diet. Another group received the oil for 30 days in combination with a high-cholesterol diet after an initial period with a high cholesterol diet. Control groups getting only the high cholesterol diet and normal diet were included in the trial as well. The number of animals was five per group.

In the study by Basu et al. [17], sea buckthorn oil induced desirable changes in the lipid markers in the rabbits fed a normal diet in combination with sea buckthorn oil. In this group there was a decrease in plasma LDL cholesterol, atherogenic index [(total cholesterol - HDL cholesterol)/HDL cholesterol)] and LDL/HDL ratio. Additionally, a rise in HDL cholesterol and the ratio of HDL cholesterol/total cholesterol was observed. These changes were significant from the baseline. However, it was not completely clear from the article, whether the effect was significant as compared to the control group. A significant vasorelaxant effect was observed [17]. In the rabbits getting the cholesterol rich diet, normalization of plasma LDL cholesterol in the sea buckthorn group was better compared to the placebo. Compared to the beginning of the seed oil intervention, several other lipid markers were positively affected as well. Sea buckthorn oil induced a significant vasorelaxant effect in the cholesterol-fed animals. The authors suggest that vasorelaxation may be due to the modification of NO production. The positive effects on plasma cholesterol levels may be caused by the phytosterols of the oil [17].

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The effects of sea buckthorn oil on plasma cholesterol and triacyglycerols have been less consistent in clinical trials (Table 10). In their study concerning the effects of combined sea buckthorn seed and pulp oil (5 g/day for four weeks) Johansson et al. [96] did not detected effects on plasma triaglycerols and total, HDL or LDL cholesterol in normolipidemic men (study described in more detail in Chapter 2.2.8.2). Yang et al. [231] investigated the effects of sea buckthorn seed and pulp oils (5 g/day for four months) on atopic dermatitis. In the pulp oil group there was a significant increase in the plasma HDL cholesterol concentration from the baseline to the end of the intervention. Total or LDL cholesterol or triacylglycerols were not affected [231] (study described in more detail in Chapter 2.2.5). Sea buckthorn juice (300 ml/day for eight weeks) caused a nonsignificant increase in serum HDL cholesterol and triacylglycerols, but did not affect levels of LDL cholesterol in healthy men [53]. Also a nonsignificant increase in resistance to LDL oxidation was found in the study by Eccleston et al. [53] (study described in more detail in Chapter 2.2.8.1).

Suomela et al. [201] studied the effects of sea buckthorn nonglycosidic flavonols on markers of cardiovascular disease risk. Flavonols of sea buckthorn berry pulp (origin not defined) were extracted with ethanol-water. The oil was removed from the extract with hexane. The sugars were removed using water extraction. A total of 14 healthy males with slightly elevated total cholesterol levels were randomized to the double-blind, placebo controlled cross-over study. During the sea buckthorn period of four weeks the participants daily consumed 400 mg of the sea buckthorn extract containing 78 mg of nonglycosidic flavonols with oatmeal porrige. The main flavonols present were isorhamnetin, quercetin and kaempferol. The intake of flavonols did not affect the serum and plasma levels of oxidized LDL, total, HDL or LDL cholesterol, CRP or homocysteine [201].

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Table 9. Effects of sea buckthorn berry/berry fractions on the risk factors associated with cardiovascular disease; *in vitro* studies and animal models[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects of sb on ox-LDL induced injuries on endothelial cells, *in vitro*	Flavonoids	9.38-37.5 μg/ml medium pretreatment	ox-LDL induced cell death and secretion disorders (NO ↓, LDH ↑), superoxide production, suppression of SOD, inhibition of eNOS expression, increase of LOX-1 expression. Sb had preventive effect on all of the above mentioned changes	Protection againts cell deaths by antioxidativity and modulation of eNOS and LOX-1 expression. Contribution of quercetin and isorhamnetin	[12]
Effects of sb on thrombosis in mice, and on platelet aggregation *in vitro*	Flavonoids	100/300 μg/ kg bw, i.v. prior to tromb. induction; aggregation 0.3-3 μg/ml	Prolongation of thrombotic occlusion time, inhibition of platelet aggreation induced by collagen, not when induced by 20:4n-6 or ADP	Prevention of thrombosis likely due to the inhibition of platelet aggregation (effects on activity of tyrosine kinase)	[37]
Effects of sb on risk factors of atherogenesis in rabbits (body weight 2.5 ± 1.0 kg)	Seed oil (CO_2-extr.)	1 ml/d for 18 d (normal diet group) 1 ml/d for 30 d (cholestrol-rich diet)	Normal diet group: plasma LDL-C ↓, AI ↓, LDL/HDL ↓, HDL-C ↑, HDL-C/total-C ↑, vasorelaxation ↑. Sb after cholesterol rich diet: vasorelaxation ↑, LDL-C ↓	Vasorelaxation: possibility of modification of NO synthesis →relaxation of endothelium. Effect of phytosterols on cholesterol	[17]
Effects of sb on hypertension in sucrose-fed rats	Flavonoids (EtOH-extr.) from seed residues	50-150 mg/kg bw/d i.g. for 8 wk after & during high sucrose diet	Suppression of: elevated hypertension, hyperinsulinemia, dyslipidemia and insulin sensitivity caused by high sucrose. Increase of angiotensin II level in plasma comparable to that caused by irbesartan*	Antihypertensive effect by improving insulin sensitivity and blocking angiotensin II receptor on the cell surface	[152]
Effects of sb on serum glucose, lipids and antioxidants in diabetic rats (STZ induced type 1)	Aqueous extract of seed residues	400 mg/kg bw/d for 4 wk orally by gastric intubation	Reduction of serum glucose, TAG and NO in diabetic rats, increse of serum SOD activity and glutathione levels	Hypoglycemic effect, but no significant increase in insulin levels; mechanism not clear, antioxidativity may have a role	[240]

[1] Abbreviations: AI = atherogenic index= (total cholesterol -HDL cholesterol)/HDL cholesterol; ADP = adenosine-5'-phosphate; bw = body weight; d = day; eNOS = endothelial constitutive nitric oxide synthase; i.g. = intragastric; i.v. = intravenously; LDH = lactate dehydrogenase; LOX-1 = lectinlike low density lipoprotein receptor-1; NO = nitric oxide; ox-LDL= oxidized low-density lipoprotein; sb = sea buckthorn; SOD = superoxide dismutase; STZ= streptozotocin; wk = week; *an angiotensin II receptor antagonist used for the treatment of hypertension

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Table 10. Effects of sea buckthorn berry/berry fractions on the risk factors associated with cardiovascular disease; clinical trials[1]

Main interest, method	Berry part	Studied dose of sb	Main result	Suggested mechanisms	Reference
Effects of sb on risk factors of cardiovascular disease in healthy normolipidemic men	Combined seed and pulp oil (CO_2 extr.)	5 g/d orally for 4 wk	No effects on fatty acid composition of plasma or platelet phospholipids or on plasma lipids. Decrease of rate and maximum of ADP induced platelet aggregation, but not on that induced by 20:4n-6 as compared to placebo	Decreasing effect on blood clotting. Mechanism unclear as the eicosanoid sensitive process was affected without changes in fatty acids	[96]
Effects of sb on risk factors of cardiovascular disease in healthy men	Juice	300 ml/d orally for 8 wk	No effects on plasma total or LDL cholesterol, platelet aggregation, or ICAM-1. Nonsignificant increase in plasma HDL cholesterol, TAG and resistance of LDL to oxidation	Mechanism for HDL effect not established, TAG increase likely due to added sugar, combination of antioxidants may affect LDL ox	[53]
Effects sb on risk factors of cardiovascular diseases in healthy men with elevated cholesterol	Flavonol extract (flavonol aglycones)	400 mg/d (78 mg flavonol aglycones) orally for 4 wk	No effects on circulating ox-LDL, CRP, homocysteine, plasma antioxidant potential, lipids or paraoxonase activity. Sb oil seemed to increase the absorption of flavonols (nonsignificant)	No changes on the cardiovascular risk markers in spite of the significant increase of plasma flavonol concentration	[201]
Effect of sb on atopic dermatitis (see Table 6)	Seed and pulp oils (CO_2 extr.)	5 g/d for 4 mo orally	Pulp oil: increase in the concentration of HDL cholesterol. No effects on total or LDL cholesterol or triacylglycerols	Phytosterols in the plant oil may have contributed to the increase of HDL cholesterol	[231]

[1] Abbreviations: CRP = C-reactive protein; d = day; ICAM-1= intercellular adhesion molecule-1; mo = month; ox = oxidation; sb = sea buckthorn; wk = week

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2.2.8.4 *Hypertension and blood glucose*

Pang et al. [152] (Table 9) investigated the effects of sea buckthorn flavonoids from seed residues on hypertension, reduced insulin sensitivity and other undesirable changes induced by the large intake of sugar in rats. The seed flavonoid extracts were prepared from *H. rhamnoides* berries collected in Mongolia. The composition of the extract was not presented in the paper. A total of 66 rats were used in the trial including three sea buckthorn groups with different doses (50 – 150 mg flavonoids/ kg body weight/ day and positive and negative control groups. The rats were fed for six weeks a sucrose rich diet and after that supplemented with sea buckthorn flavonoids while still under the sucrose diet for another eight weeks. The sea buckthorn flavonoids induced a lowering of hypertension induced by sucrose, recovery of insulin sensitivity, and decrease in insulin levels [152].

In Pang's study [152] the sucrose feeding increased the levels of blood pressure rising angiotensin II hormone in the heart and kidneys. The levels of angiotensin II in the blood were unaffected. In the flavonoid-supplemented group, the levels of angiotensin II in the plasma increased, while the levels in the heart and kidneys were unaffected. With the highest dose of sea buckthorn flavonoids, the increase in the plasma was similar to that of ibertasan, an angiotensin II receptor antagonist used for the treatment of hypertension. The levels of angiotensin II in the heart and kidneys were not affected by ibesartan either. The above results indicate that sea buckthorn flavonoids may block angiotensin II receptors on the cell surface and keep it in the plasma, and by this mechanism reduce the blood pressure [152]. Angiotensin II preventing drugs may enhance insulin sensitivity, though the mechanism of the effect is still under investigation. Attenuative effects on inflammation and oxidative stress, among others, have been suggested. Angiotensin II receptor blockers have been found to inhibit the enzymes promoting oxidative stress [92].

Zhang et al. [240] (Table 9) studied the effects of water extract from sea buckthorn seed residue in diabetic rats. The *H. rhamnoides* ssp. *sinensis* seeds were obtained from berries collected from Inner Mongolia. The total flavonoid content of the hot-water extracted residue was 1.13%. The total content of carbohydrates was 3.52%. A total of 48 rats were included in the study. In 36 of them type 1 diabetes was induced by the admistration of oxygen radical producing streptozotocin. Four groups of 12 rats were monitored: a normal control group, a diabetic control group, a diabetic positive control group and a diabetic sea buckthorn group. The sea buckthorn group was orally supplemented with 400 mg of seed residue/ kg body weight daily for four weeks. Sea buckthorn extract had a hypoclycaemic effect in the diabetic rats. The effect was comparable to that of the positive control drug glibenclamide. Unlike glibenclamide, the sea buckthorn extract did not raise the insulin levels of the diabetic rats, indicating that the sea buckthorn effect was not due to the stimulation of insulin secretion. The seed extract induced a reduction of serum triacylglycerols as well. A restoration of the antioxidant systems in the diabetic

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rats by sea buckthorn was observed. The authors suggest that sea buckthorn extract may be useful in preventing diabetes complications by antioxidant and triacylglycerol lowering mechanisms [240] .

In Johansson's study [96] (see Table 10 and Chapter 2.2.8.2 for more details of the study protocol) concerning the effects of sea buckthorn oils in healthy men (5 g/day for four weeks), the fasting blood glucose levels were slightly elevated during the supplemention with both the placebo oil and the sea buckthorn oil. The authors suggest that this could be explained by the increased fat intake during the intervention.

2.2.9 Safety

According to Tulsawani [212], the No Observed Adverse Effect Level (NOAEL) of water extract of sea buckthorn berry in rats administered by gavage for 90 d is 100 mg/kg body weight/ day. The extract used in their study was prepared with *H. rhamnoides* berries collected from the Indian Himalayas. The total phenolic content was 50.5 mg/g as a gallic acid equivalent. No mortality or changes in the general behavior of the animals (15 mice in four groups, admistered 0 - 500 mg extract/kg/day) were observed, even at the highest doses used. Sea buckthorn extract in any of the doses did not induce significant changes to the mean body weight, to the organ/body weight ratio, or to the large variety of histological, hematological and biochemical parameters monitored in the rats as compared to the control group. However, intakes of 250 and 500 mg/kg induced an elevation in plasma glucose. The author suggests that the rise in plasma glucose was due to the sugars in the water extract. Even though the glucose elevation was restored within two weeks after the end of the sea buckthorn treatment, a NOEL level of 100 mg/kg/day for long term supplementation was decided.

Chawla et al. [35] reported a maximum tolerable dose of 200 mg/kg body weight/day for flavonoid rich ethanol extract of sea buckthorn berries admistered intraperitoneally as a single dose for mice. Doses above 200 mg/kg induced mortality. At a dose of 212 mg/kg 50% of the animals died within 72 h. The extract used by Chawla et al. [35] was prepared from *H. rhamnoides* berries collected from the Western Himalayas.

Goel et al. [65] (Table 2) investigated the acute toxicity of alcoholic sea buckthorn berry extract RH-3, which was administered intraperitonially to mice as single doses of different sizes. For two days after the intake of RH-3, effects on survival, behavior, neuromuscular co-ordination and the respiratory tract were monitored, among others. Detailed results of the individual assays were not presented, but the authors state that single doses of up to 40 mg/kg body weight were tolerated and the only apparent adverse effect was drowsyness for 3 - 5 minutes after intake. Doses of 45 mg/kg body weight or higher caused increased mortality in a dose dependent manner.

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As a part of their study concerning the effects of CO_2-extracted sea buckthorn seed oil on burn wounds, Upadhyay et al. [216] (Table 6) investigated the toxicity of the oil. In the acute oral toxicity test, the rats were given single doses of 2.5 - 10 ml sea buckthorn seed oil/kg body weight and symptoms of toxicity (mortality, signs of severe toxic symptoms) were monitored for 14 days. In the sub-acute oral toxicity test, sea buckthorn seed oil was daily admistered to the rats at doses of 2.5 or 5.0 ml/kg body weight for 14 days. In addition, the 2.5 ml/kg dose chosen for the wound healing experiments was fed for a group of rats for 28 days to monitor the signs of any sub-acute toxicity. For the evaluation of sub-acute toxicity, organ/body ratios and several biochemical and hematological parameters were analysed. No signs of acute toxicity were observed at any of the doses tested, and the results of the biochemical and hematological analyses did not significantly differ between the treatment and control groups (six rats per group).

In many of the health effect studies, the composition of the extract or other product used is not reported. In some cases even the method of extraction or the solvents used are described in a cursory manner only. In studies where the compositions of the ethanol/water extracts or flavonoid preparations have been reported, the amounts of the alledged active components have varied greatly [69, 193, 201, 240]. Therefore it is not easy to compare the results of the safety evaluations between studies. Flavonoids from different sources have been associated with effects on the metabolism of certain drugs, and drug-flavonoid interactions should be considered before supplementing people who have medication [163]. Otherwise, the *in vivo* effects of flavonoids are generally considered as positive, and supplementation of foods with quercetin to reach the levels of 200 – 500 mg quercetin/day is suggested to be safe [79].

Any undesired effects due to sea buckthorn interventions in clinical studies have been rare. Ten of the 30 healthy male participants recruited to the study of Eccleston et al. [53] dropped out because of gastro-intestinal upset and diarrhea during the intervention with sea buckthorn or placebo juice. The drop-outs were both from the sea buckthorn juice group and from the placebo juice group [53]. The acidity of the juices consumed at doses of 300 ml/day might have contributed to this side effect. Eccleston et al. also report an increase in fasting blood triacylglycerols during the intervention with sea buckthorn juice (300 ml/day for eight weeks). This effects, however, was not statistically significant. The authors suggest that the sugars of the juice may have contributed to this. Johansson et al. [96] found that sea buckthorn seed and pulp oil at 5 g/day for four weeks induced a small (+0.3 mmol/l) but statistically significant rise in fasting blood glucose in healthy men. The rise was evident in the placebo group as well (+0.25 mmol/l) and was explained by increased fat intake during the study [96].

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2.3 SUMMARY

Sea buckthorn flavonoids and alcohol extracts are among the berry fractions most intensively studied for their physiological effects (Table 11). The chemical composition of the fractions is reported in a minority of the articles only. Alcohol extracts most likely are enriched with flavonoids, other phenolics, sugars and vitamin C. It is probable that the composition of the flavonoid preparations is similar, since most authors do not specify whether the flavonoid fraction was purified to remove the other components. A few studies report using water extracts of berries or seeds. The phenolic content of the water extracts is lower compared to alcohol or alcohol-water extracts [69], but they likely are more abundant in the more polar components.

In vitro and animal studies show that sea buckthorn berry flavonoids as well as alcohol and water extracts have strong antioxidant activity. This contributes to their potential to protect cells against cytotoxicity and modulate inflammation (Table 11). The dual effects of sea buckthorn flavonoids on inflammation have been reported. Mishra et al. [128] observed *in vitro* the proinflammatory effects by sea buckthorn flavonoids in human peripheral mononuclear cells. This may be a desirable effect in the defence against microbial infections. Others have reported reduced inflammation by sea buckthorn flavonoids, alcohol extracts and juice *in vivo* and *in vitro* [12, 13, 23, 89]. This effect can be considered positive, as low grade chronic inflammation contributes to the pathogenesis of several diseases. The effects of sea buckthorn flavonoids on the immune system, inflammation, and the functions of the endothelium, seem to be mediated at least in part by modulating the activation of transcription factor NF-kB and the expression of inflammatory genes [12, 13].

In vitro studies and one animal study [151], respectively, suggest the inhibition of cancer cells and protection against induced tumorigenesis by sea buckthorn alcohol extracts (Table 11). In the animal study, the up-regulating effect of the fairly high dose of sea buckthorn alcoholic extract on the xenobiotic metabolizing enzymes was observed. There are indications of wound healing promotion by topical sea buckthorn flavonoids *in vivo* [72], due to antioxidant activity and enhanced collagen production. A mice study suggests the protective effect of sea buckthorn flavonoids against thrombotic events [37]. Flavonoids from sea buckthorn seeds restricted the sucrose-induced hypertension in rats [152]. Water extract from sea buckthorn seeds had a hypoglyglycemic effect in diabetic rats [240].

Despite the promising results *in vitro* and in animals, a clinical study involving healthy men with slightly elevated blood cholesterol levels did not result in significant changes by sea buckthorn flavonols on plasma lipids, CRP or other markers of cardiovascular disease risk [201]. The intake of flavonols taken was fairly low, and the level of CRP, among others, was within the reference range at baseline.

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The effects of sea buckthorn juice (Table 11) have been investigated in one clinical trial aiming to study the effects on the risk markers of cardiovascular disease [53]. No clear positive changes in the healthy men were observed, although there was a nonsignificant rise in plasma HDL and a nonsignificant small increase in the lag phase of LDL oxidation. As in the clinical flavonoid study [201], the number of participants was fairly low in the juice study as well, and prestudy power calculations were not presented. An animal study indicates the antioxidant activity of sea buckthorn juice against nicotine induced oxidative stress [199]. *In vitro* inhibition of cancer cells and the attenuation of inflammation by sea buckthorn juice has been reported [23]. Whereas the alcohol extracts are enriched with phenolics, sea buckthorn juice may additionally contain even up to >3% of oil [209], depending on the processing and origin of the berries.

The effects of sea buckthorn oil, especially sea buckthorn seed oil, have been studied intensively (Table 11). A majority of the studies, where the method of extraction was specified used CO_2-extracted oil. Compared to the hexane-extracted oil the concentrations of tocols, carotenoids and phytosterols are higher in the oils extracted with supercritical CO_2 [10]. In three clinical studies a combination of sea buckthorn seed and pulp oils have been used [96, 229].

In animals, the positive effects of sea buckthorn oils against SO_2-induced cytotoxicity and the detrimental effects of hypobaric hypoxia were observed [164, 181, 225]. Antioxidant activity most likely contributes to these effects. The beneficial effects of sea buckthorn seed oil in the treatment of wounds (a combination of oral and topical intake) were observed in a carefully conducted animal study [216]. In clinical investigations using doses several times lower, the positive effects of sea buckthorn on the skin and mucosa were shown [229, 231].

The positive effects of sea buckthorn seed and pulp oils against gastric ulcers were observed in two animal studies [198, 227]. One thorough animal study [88] suggests the protective effects of sea buckthorn seed oil against hepatotoxicity using a fairly modest dose of 0.26 mg oil/kg body weight/day. Antioxidant action is likely an important mechanism in both of these effects.

Concerning the effects of sea buckthorn oil on the risk factors of cardiovascular diseases and diabetes, several positive changes in lipid parameters were induced in rabbits [17]. In clinical studies, the inhibition of platelet aggregation and an increase in plasma HDL cholesterol levels have been observed with doses lower than those of the rabbit study [96, 231]. However, several parameters were also unaffected by sea buckthorn oils in the clinical studies by Yang et al. [231] and Johansson et al. [96]. In both studies the mean baseline levels of the measured risk markers were within or only slightly different from the reference values at baseline. A mild elevation of the serum glucose was observed in one study during the supplementation with sea buckthorn oil and placebo oil [96]. This was suggested to be because of increased fat intake during the study.

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Table 11. Summary of studies investigating the physiological effects of sea buckthorn berries and oil [1]

	Berry fraction					
Antioxidativity, cytoprotection, action against toxic substances	**Alcohol extr.**	**Flavonoids**	**Water extr.**	**Juice**	**Seed oil**	**Berry oil**
In vitro studies	+	+				
Animal studies	+		+	+	+	+
Inflammation and immunomodulation						
In vitro studies	+	+		+		
Animal studies						+
Clinical studies		+/-				
Proliferation and apoptosis, mechanisms related to cancer						
In vitro studies	+ [2]	+		+		
Animal studies	+					
Antimicrobial properties						
In vitro studies		+	+	+ [3]		
Wounds, skin and mucosa						
Animal studies		+			+	
Clinical studies					+ [4]	+ [4]
Gastric ulcers						
Animal studies					+	+
Hepatotoxicity and liver fibrosis						
Animal studies					+	
Clinical studies	One clinical study reporting positive results. Berry fraction not specified.					
Endothelium						
In vitro studies		+				
Clinical studies				+/-		
Platelet aggregation and thrombosis						
Animal studies		+				
Clinical studies				+/-	+ [5]	+ [5]
Circulating lipids						
Animal studies			+ (seed extr.)		+	
Clinical studies		+/-		+/-	+/-	+ & +/-
Hypertension						
Animal studies		+ (seed extr.)				
Blood glucose, insulin sensitivity						
Animal studies		+ (seed extr.)	+ (seed extr.)			
Clinical studies					mild- [5, 6]	mild- [5, 6]

[1] + = study/studies indicating positive effects by sea buckthorn; +/- = study/studies indicating no effects by sea buckthorn; - = study/studies indicating negative effects by sea buckthorn

[2] Positive effects also by ethanol acetate extract

[3] Whole berry powder

[4] Effect also by combined sea buckthorn seed and pulp oil

[5] Combined sea buckthorn seed and pulp oil

[6] Increase of blood glucose during both sea buckthorn oil and placebo oil interventions

In conclusion, the potential of the flavonoid rich fractions of sea buckthorn to reduce oxidative stress, protect against cytotoxicity and modulate inflammation has been clearly shown *in vitro*. These mechanisms contribute to the beneficial effects observed in animal studies, regarding the risk factors of cardiovascular

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diseases and protection against toxic subtances, among others. So far only a few fairly small clinical studies using sea buckthorn flavonoid rich fractions or juice have been carried out. Animal studies clearly show the antioxidant potential of sea buckthorn oils, and indicate anti-inflammatory and antioxidative effects. The benefits of sea buckthorn oils on wound healing, gastric ulcers and hepatotoxicity, shown in carefully conducted animal studies, deserve further investigation. The fairly few clinical studies conducted have found the beneficial effects of sea buckthorn oils on the skin and mucous membranes. The effects on the risk factors of cardiovascular diseases have been less consistent in humans, however. There is room for more, larger scale human investigations that concentrate on people having elevated levels of risk markers.

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3 AIMS OF THE STUDIES

The overall aim of the research project was to investigate the health effects of sea buckthorn berries and oil in humans. The project consists of two clinical trials with different target populations and aims, one focusing on the whole berries and one on the oil.

The primary objective of the Clinical Trial 1 was to study the effects of sea buckthorn berries on the common cold, other infections, and on inflammation in healthy adults. It was hypothesized that the bioactive compounds of the berry may modulate immunity and reduce the risk of infections and duration of symptoms. The main outcome measures of the Clinical Trial 1 were the number and duration of common cold cases. The second aim was to investigate the effects of the berries on the circulating lipid markers associated with the risk of cardiovascular diseases, and on circulating flavonols, expected to be among the berry compounds affecting in humans.

The primary objective of the Clinical Trial 2 was to study the effects of sea buckthorn oil on dry eye and the possible mechanisms of effect. The hypothesis was that the antioxidant and anti-inflammatory compounds of combined sea buckthorn seed and pulp oil would beneficially affect the symptoms and clinical markers of dry eye. The main outcome measures were tear film osmolarity, tear film break-up time, tear secretion, and dry eye symptoms evaluated using a dry eye symptom questionnaire. The second objective was to investigate the effects of sea buckthorn oil on circulating inflammatory markers and aminotransferases associated with the risk of cardiovascular diseases and type 2 diabetes.

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4 PARTICIPANTS, MATERIALS AND METHODS

4.1 CLINICAL TRIAL 1: EFFECTS OF SEA BUCKTHORN BERRIES ON INFECTIONS AND INFLAMMATION (I), AND ON CIRCULATING LIPID MARKERS AND FLAVONOLS (II)

4.1.1 Study design and participants (I-II)

The study was of double-blind, randomized, parallel design. The study protocol was supported by the by the Ethics Committee of the Hospital District of Southwest Finland. A total of 254 women and men of 19 to 50 years of age were included, after their informed written consent to the study procedures. Half of the participants were randomized to receive sea buckthorn and half to receive a placebo. Exclusion criteria were: 1) a chronic disease, 2) continuous medication affecting the immune system, 3) a body mass index of less than 18 or more than 30 kg/m^2, 4) an influenza vaccination taken during the last 6 months, 5) unwillingness to restrict the use of nutrient supplements, sea buckthorn products and certain probiotic products during the trial, and 6) if it was not possible for the candidate to store the study product as intended.

The intervention lasted for three months during a period from January 2005 to May 2005. During this time, the participants daily consumed 28 g of sea buckthorn or a placebo puree, and kept a logbook record concerning their symptoms of common cold, digestive tract infections and urinary tract infections. Questions concerning the compliance of the trial protocol and the medication used were answered each day as well. The participants were asked to give a nasal swab and blood samples when they felt they had a case of the common cold. In addition, a study visit at the beginning and end of the intervention was scheduled for the collection of blood samples and background information.

4.1.2 Study products (I-II)

The sea buckthorn product was a frozen puree of *Hippophaë rhamnoides* ssp. *mongolica* cv. Prozcharachnya berries. Also the seeds of the berries had been ground to facilitate the absorption of the seed components in the digestive system. In addition to the berries (96% w/w) the puree contained a sweetener solution and other additives. The placebo puree was similar in appearance, taste and smell and consisted of water, fructose, bread crumbs and additives. The participants were allowed to take the puree any time of the day, with or away from a meal, as a single or divided dose.

The daily dose of sea buckthorn puree contained 16.7 mg flavonol glycosides, approximately 9.0 mg/day calculated as aglycones. Glycosides of isorhamnetin were the most abundant. The flavonol glycosides as mg/28 g of puree were:

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isorhamnetin 3-O-glucoside-7-O-rhamnoside 5.8 ± 0.7, quercetin 3-O-rutinoside 1.5 ± 0.9, quercetin 3-O-glucoside 1.6 ± 0.4, isorhamnetin 3-O-rutinoside 5.1 ± 0.8, isorhamnetin 3-O-glucoside 2.4 ± 0.4 and kaempferol 3-O-rutinoside 0.3 ± 0.4 (tentative identification).

The flavonol glycosides and vitamin C content of the sea buckthorn puree were analyzed using HPLC-UV methods [99, 209, 238]. The vitamin E content was analyzed with a HPLC-fluorescence method [102]. The oil content was measured gravimetrically [209]. The fatty acids were analyzed as methyl esters [4] by gas chromatography [231].

4.1.3 Number and duration of the common cold and other infections (I)

The analysis of infections was based on the self-assessment of the participants. In the symptom logbooks, typical symptoms of the common cold, digestive tract infections and urinary tract infections were listed, with a severity scale from 0 = none to 3 = severe symptoms. The participants were asked to report a symptom only if it, in their assessment, was caused by an infection. A participant was considered to have a case of the common cold/digestive tract or urinary tract infection if he/she reported at least one symptom at a severity of ≥ 1 for at least one day. For the duration of the infection, each day with ≥ 1 symptoms was included. The symptom logbook was not validated.

4.1.4 C-reactive protein (I), lipid markers and flavonols (II)

A blood sample after a 12 h fast was taken at the beginning and end of the intervention for the analyses of CRP, lipid markers and flavonols.

Serum CRP concentrations were measured with a high sensitivity particle-enhanced immunoturbidometric assay using Roche tina-quant reagents (Roche Diagnostics, GmbH, Mannheim, Germany) and a fully automated analyzer Roche Modular P800 (Roche Diagnostics, GmbH, Mannheim, Germany). The serum total and HDL cholesterol, and triacylglycerol concentrations were measured by standard enzymatic methods using Roche Diagnostics reagents (Roche Diagnostics, GmbH, Mannheim, Germany) with a fully automated analyzer Roche Modular P800 (Roche Diagnostics, GmbH, Mannheim, Germany). All of the above-mentioned analyses were carried out at TYKSLAB (Turku, Finland). The LDL cholesterol was calculated using the Friedewald formula [57].

The concentrations of flavonols quercetin, kaempferol and isorhamnetin in the plasma were analysed using an HPLC fluorescence method [54, 201] after an enzymatic hydrolysis of flavonol conjugates to their aglycones [85, 201]. The *Helix pomatia* preparation (β-glucuronidase, type HP-2, Sigma, Saint Louis, MO, USA) used for the hydrolyses was contaminated with flavonols [33] and therefore purified using active charcoal prior to the analyses [124]. For

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quantitative analysis internal standard rhamnetin (Extrasynthese, Genay, France) was added to each sample.

4.1.5 Statistical analyses (I-II)

The sample size for the study was estimated according to the assumed number and duration of common cold cases. Fifty percent of the participants in the placebo group were assumed to have one case of the common cold during the study [80]. With a sample size of 120 participants per group, the study would have a power of 85% to detect a difference of 20 percentage units in the incidence of cases between the treatment groups if a χ^2 test was used (two-sided tests, 0.05 significance level, 10% assumed drop-out rate). If the symptoms lasted for 5 - 7 days [16], a difference of two days in the common cold duration would be detected with a sample size of 130 participants per group (standard deviation = 5 days, power ≈ 85%, two-sided tests, 0.05 significance level, 10% drop-out).

The statistical analyses of the logbook data were carried out using generalized linear models (SAS software, version 9.1.3 SP2, GENMOD procedure; SAS Institute Inc., NC). In all statistical models, a logarithmic link function was used to convert the estimates of the parameters into relative risks (RR; unadjusted, no covariates were used). The estimated RR for infections or the duration of symptoms were calculated always comparing the sea buckthorn group to the placebo group, i.e. RR significantly less than 1 would denote a beneficial effect of sea buckthorn. The values of the symptom duration were distributed abnormally. Accordingly, data concerning duration is expressed as median (range).

Due to the abnormal distribution of the CRP and flavonol data, they were analysed using rank analysis of covariance with the baseline measurement as a covariate (SAS software, version 9.1.3 SP2, SAS Institute Inc., NC). Data on the CRP and flavonol concentrations are expressed as median (range) and median (quartiles), respectively. To minimize the interference of acute infections on the CRP results, additional analyses excluding the participants with CRP values ≥ 10 mg/l [154] were carried out. The analyses of total, HDL and LDL cholesterol and triacylglycerols were carried out using linear models (MIXED procedure, adjusted for baseline concentration and other covariates). Fisher's exact test was used to compare the number of common cold samples taken in treatment groups (same software as above).

Two sets of analyses were always performed: one including all randomized participants (compliant and noncompliant), and one including only the compliant participants. Unless otherwise noted, the presented results concern the analyses of all participants. Two-sided significance tests and significance levels of 0.05 were used throughout.

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4.2 CLINICAL TRIAL 2: EFFECTS OF SEA BUCKTHORN OIL ON DRY EYE (III-IV) AND ON CIRCULATING AMINOTRANSFERASES AND BIOMARKERS OF INFLAMMATION (V)

4.2.1 Study design and participants (III-V)

A total of 100 women and men between 20 to 75 years of age were randomized to this double-blind, placebo-controlled, parallel trial. They gave their written informed consent to the study procedures, which were approved by the Ethics Committee of the Hospital District of Southwest Finland. The inclusion criterion was an experience of dry eye symptoms. The exclusion criteria were severe illness, pregnancy or breastfeeding, smoking, and regular use of strongly anticholinergic drugs. Fifty-two participants were randomized to the sea buckthorn group and 48 the placebo group. Eighty-six participants completed the study.

The intervention period lasted for three months from autumn 2008 to winter 2009. During this period the participants consumed 2 g of sea buckthorn or placebo oil daily in the form of 2 capsules twice/day with a meal. The participants attended a study visit at the beginning of the intervention, after one month, and at three months when the intervention ended. In addition, a post-check one to two months after taking the last study capsules was scheduled.

4.2.2 Study products (III-V)

The sea buckthorn capsules contained both sea buckthorn berry and seed oil. The combined oil was manufactured by Aromtech Ltd (Tornio, Finland) using supercritical carbon dioxide extraction. The most abundant fatty acids in the oil were: 16:1n-7 (346 ± 48 mg/daily dose of 2 g), 16:0 (338 ± 47 mg), 18:2n-6 (245 ± 34 mg), 18:3n-3 (149 ± 21 mg), 18:1n-9 (316 ± 45 mg), and 18:1n-7 (108 ± 15 mg). The α- and γ-tocopherol contents were 6.0 ± 0.4 mg/2 g and 0.8 ± 0.1 mg/2 g, respectively. The total carotenoid content was 1.8 ± 0.4 mg/2 g oil. The placebo oil consisted of triacylglycerols of medium-chain fatty acids isolated from coconut and palm kernels: 8:0 (884 ± 11 mg in the daily dose), 10:0 (733 ± 4 mg), 12:0 (1 ± 0 mg), and 14:0 (2 ± 0 mg). The placebo oil contained neither carotenoids nor γ-tocopherol, and only 0.2 ± 0.0 mg α-tocopherol per 2 g of oil. The fatty acids were analysed as methyl esters by gas chromatography [4, 232]. Tocopherols and carotenoids were analyzed using HPLC-UV/Visible methods.

4.2.3 Clinical tests and symptoms of dry eye (III)

At each study visit several dry eye tests were performed. The tear film osmolarity (mOsm/l) was measured using an electrochemical osmolarity meter (Tearlab, Ocusense Inc., San Diego, CA). The stability of the tear film was measured as tear film break-up time (TBUT: seconds until breakup of

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fluorescein tear film). Tear secretion was analyzed using the Schirmer test without anesthesia (length of wetting the Schirmer paper after 5 min).

During the intervention period the participants daily kept a logbook record on their dry eye symptoms. In the logbook, typical symptoms were listed and the participants were asked to estimate the severity of each symptom using a 4-point scale from 0 = none to 3 = severe. In addition, they were asked to record whether they had taken the study capsules, worn contact lenses, or used eye drops or other treatments for dry eye symptoms. At each study visit the symptoms were evaluated using a modified version of the validated dry eye symptom questionnaire the Ocular Surface Disease Index (mOSDI) [150, 185]. The symptom logbook and the mOSDI questionnaire were not validated.

4.2.4 Fatty acids of the tear film (IV)

To investigate the potential mechanisms of the effect of oil on dry eye, the fatty acid composition of the participants' tear film was analysed. Samples for the fatty acid analyses were collected at each study visit using a Schirmer paper (5 min tear flow from both eyes). Precautions were taken not to contaminate the sample with lipids from other sources. Participants' tear film fatty acids were analyzed using the *in situ* boron trifluoride methylation and gas chromatographic method described by Joffre et al. [95] with some minor modifications. The fatty acid methyl esters were analyzed using a Perkin-Elmer AutoSystem gas chromatograph (Norwalk, CT) equipped with a flame ionization detector (FID), and DB-1 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent Technologies Inc., Folsom, CA). To confirm the peak identities, some samples were also analysed by gas chromatography mass spectrometry (Shimadzu GC-MS QP5000 instrument, Shimadzu, Kyoto, Japan; DB-1 capillary column identical to that used with FID).

The identification of most fatty acids was based on comparisons of the retention times and mass spectra of sample analytes to those of commercial reference compounds. According to mass spectra [comparisons to the Wiley mass spectral database (Shimadzu, Kyoto, Japan)] and retention times relative to the known peaks, five compounds for which a reference standard could not be found, were tentatively identified. The Schirmer paper was found to contain fatty acids that were also present in the tear samples. However, for most compounds the amounts were small compared to those in the tear samples. The paper also contained an unidentified peak that was not found in the tear samples. The area of this peak was used to calculate a correction factor allowing us to deduct the effect of the Schirmer paper from the tear sample analyses. As the proportions of the blank Schirmer paper gas chromatographic signals were not entirely constant from paper to paper this produced extra variation in the results.

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4.2.5 Inflammatory markers and aminotransferases (V)

Relating to the risk of cardiovascular diseases and type 2 diabetes, the circulating levels of inflammatory markers and aminotransferases were analysed. At each study visit, blood samples were taken from the participants for the analyses of inflammatory cytokines IL-6 and TNF-α, CRP, and ALAT, ASAT and γ-glutamyl aminotransferase (GT). ALAT, ASAT and GT in lithium heparin plasma were analysed with photometric methods on a Modular P800 automatic analyzer (Roche Diagnostics GmbH, Mannheim, Germany). CRP in serum was analyzed with an immunonephelometric assay on a Siemens Dade Behring BN II Nephelometer analyzer (Siemens Healthcare Diagnostics, Inc., Siemens Aktiengesellschaft, Munich, Germany). These analyses were carried out by TYKSLAB, Turku Finland. TNF-α and IL-6 were analysed simultaneously from serum samples with a Milliplex Human Serum Adipokine Panel B kit (Millipore, Billerica, MA, Usa) according to the manufactures instructions, using the Bio-Rad Bio-Plex 200 System (Espoo, Finland).

4.2.6 Statistical analyses (III-V)

The sample size was estimated prior to the study for observing a mean difference of ≥ 4 points in the mOSDI scores between the treatment groups. With a sample size of 37 participants/group the trial would have a power of ≈ 80% to detect this difference at the end of the intervention (assumed standard deviation = 6 points, two-sided tests, 0.05 significance level). The drop-out rate was assumed to be 25% or less. Accordingly, a total of 100 participants were recruited.

For the clinical tests of dry eye and mOSDI, changes from baseline values were used as dependent variables in the statistical analyses. The variables of change were analysed with a two-way analysis of variance (ANOVA) with a repeated measure term included in the model (SAS MIXED procedure). Baseline values of clinical tests and mOSDI, age, contact lens wear and sex were considered as potential covariates in the model. Only significant covariates ($P < 0.05$) and those with significant interactions with other variables in the model ($P < 0.05$) were included in the final model. The group-change interaction was included in order to calculate the estimates of changes. The results were adjusted for multiplicity using Bonferroni correction. Values in the text are means ± standard deviation.

For each individual symptom in the logbook, the proportion of days with dry eye symptoms was calculated (number of days when the participant had the particular symptom/number of intervention days). This symptom day ratio was also calculated for overall non-specified eye symptoms. The symptom sum was calculated by summing the daily intervention period intensity scores. The proportions of participants having a symptom score of 0, 1, 2, or 3 as their maximum symptom in each group was calculated and the differences between the groups were tested using the Cochran-Mantel-Hanzel test. The differences

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the between groups of ratio and the sum of symptom days were estimated from an ANOVA model and from an analysis of covariance model (ANCOVA) using the SAS MIXED procedure. Age and use of contact lenses were introduced as covariates in the ANCOVA model. In addition, the symptom day ratio and symptom sum analyses were carried out separately for subgroups of participants above and below 45 years of age, and for contact lens wearers and those not wearing contact lenses. The logbook analyses were carried out by Statfinn Ltd (Turku, Finland). Values in the text are mean ± standard deviation or n (%) (maximum symptoms).

The proportions of fatty acids were used as dependent variables in the statistical analyses of the tear film fatty acids. Variables of change were analyzed with a general linear model with repeated measures term included (SAS MIXED procedure). The baseline proportion of the fatty acid in question, age, contact lens wear, gender and baseline clinical dry eye test results (tear film osmolarity, tear film break-up time and Schirmer test) were considered as potential covariates in the model. Only significant covariates ($P < 0.05$) and those with significant interactions with other parameters in the model ($P < 0.05$) were included in the final statistical model. The results were adjusted for multiplicity using the Bonferroni correction. The values in the text are mean ± standard deviation.

Due to the abnormal distribution of aminotransferase activities and inflammatory marker concentrations, they are expressed as median (quartiles). The values at the end of the intervention (three months) of the sea buckthorn and placebo groups were compared using a rank analysis of covariate with the baseline measurement as a covariate using SAS software version 9.2 (SAS Institute Inc., NC). For the analyses of inflammatory markers, participants having CRP values above 10 mg/l, indicating acute infection or inflammation [154] were excluded from the statistical analyses to minimize the effect of acute infections on the results of the group comparisons.

The primary data analyses were done including all randomized participants (compliant and noncompliant). Unless otherwise noted, the presented results concern these analyses. In addition, analyses including only participants who consumed the study capsules for at least 80% of the days during the intervention period (compliant participants) were conducted. Two-sided tests, significance levels of 0.05 and SAS software version 9.2 (SAS Institute Inc., NC) were used throughout.

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5 RESULTS AND DISCUSSION

5.1 EFFECTS OF SEA BUCKTHORN BERRIES ON INFECTIONS AND INFLAMMATION (I)

5.1.1 Common cold and other infections (I)

Sea buckthorn did not affect the risk [RR = 1.15, 95% confidence interval (CI) = 0.90 - 1.48] or duration (RR = 1.05, 95% CI = 0.87 – 1.27) of common cold infections. Neither were there effects on digestive tract infections: RR (95% CI) for the number and duration of infections, respectively, were 1.06 (0.67 – 1.68) and 1.08 (0.82 – 1.43). The number of reported urinary tract infections was small (6 cases in the sea buckthorn group, 7 cases in the placebo group), and therefore the results can be considered as indicative at the most. The groups did not differ when all participants were included in the analyses. When only the compliant participants were included, the statistical analyses suggested a significant difference for the number of urinary tract infections (RR = 0.43, 95% CI 0.20 – 0.94) but not for the duration (RR = 0.60, 95% CI 0.28 – 1.29).

The reasonably low dose of sea buckthorn berries realistic for long term everyday consumption was chosen for this trial. It approximates the dosage of dried berries (3 - 9 g) prescribed in the Chinese Pharmacopeia [39]. Compared to the recommended or reported average daily intakes, flavonols (approximately 9 mg/day calculated as aglycones) were the most affected of the potentially beneficial compounds. The estimated average Finnish daily intake of flavonols as aglycones is 5.4 mg [149], which is reasonably low compared to other Western countries [122]. The vitamin C content of the product was 15.6 mg/day which is about 21% of the recommended daily intake, and 13-17% of the average intake for adults in Finland [55, 153]. The amount of α-tocopherol in the daily dose was about 11-14% of the recommended daily intake and 11-15 % of the average reported real intake in Finland [55, 153]. The amount of ß-carotene in the daily dose equates to 31 retinol equivalents, which is only 3 - 4% of both the recommended and average daily intake of Finns [55, 153]. The amount of oil of in the product was only about 1 g/day. The amounts of the above-mentioned biologically active compounds and oil in the study puree were within the range reported in sea buckthorn berries in earlier studies, but far from the highest levels observed [174, 209, 235, 238].

As flavonoids were among the compounds most affected by the intervention it was expected that they would have an effect. In addition, as the whole berry was used, synergistic activity of the compounds was expected [126]. *In vitro* and animal models have shown the antiviral effects of several flavonoids, flavonols among them, against a wide range of viruses [134]. The viruses affected by flavonoids include influenza A virus [40], and picornaviruses [43, 91] that typically cause the common cold. In a cohort study, an inverse

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association between the consumption of wine, especially red wine known to be rich in flavonoids, and risk of the common cold was observed [205]. Extracts of Echinacea plant, containing flavonoids among others, are widely used for treating and preventing respiratory tract infections. According to a meta-analysis, some Echinacea herb preparations might be effective in shortening the duration and reliving the symptoms of the common cold [116].

Ascorbic acid and dehydroascorbic acid exhibit antiviral activity against several viruses with different structures and replication strategies [58]. The effects of vitamin C on the common cold have been extensively studied. According to a large review concerning interventions with vitamin C in doses of ≥ 200 mg/day, vitamin C supplementation does not affect the risk of catching a common cold in the general population. However, in the subgroup of people under heavy physical stress, vitamin C reduced the incidence of the common cold [81]. Prophylactic consumption of vitamin C had a significant beneficial effect on the duration and severity of the common cold. However, the practical significance of this result was questioned. If taken after the onset of symptoms, the pooled estimates of the trials did not show significant benefits on the duration or the severity of symptoms [81].

Like for vitamin C, results concerning the effect of vitamin E on the common cold have been inconsistent. The protective effect in nursing home residents (200 IU/day for one year) [125] and in older men (50 mg/day for 4 years) [83] have been detected. However, also the negative effects of vitamin E on the common cold among older people have been reported [82, 84], and the effect is modified by smoking status and dwelling place [82, 83, 84]. In the same cohort of the Alpha-Tocopherol Beta-Carotene study, where the effects of vitamin E were investigated, β-carotene supplementation had no effect on the common cold [83], but an interaction between vitamin E and β-carotene supplementation was detected [82]. The participants in the current study were healthy adults, who exercised moderately and very few smoked. On the grounds of their eating habit information, it is likely that the participants obtained at least reasonable intakes of flavonoids and vitamins in their normal diet.

As discussed in the literature review, the antibacterial properties of phenolic berry extracts, including sea buckthorn berry, have been reported *in vitro* [166]. Sea buckthorn berry extract was among the weakest inhibitors, but it still exhibited activity against the gram-negative bacteria tested, including the pathogenic species [166]. Cranberry juice has well-documented beneficial effects on urinary tract infections [93, 169]. Consuming juices in general, especially those made of berries is inversely associated with the risk of recurrence of urinary tract infection [106]. The effect of cranberry is thought to take place by two possible mechanisms: its components prevent the adhesion of the uropathogenic *Escherichia coli* bacteria to the uroepithelial cells, and/or it may affect the intestinal bacteria and promote those strains that are less adherent [93, 169]. Fructose and A-type proanthocyanidins, unique to the

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Vaccinium berries are the components that prevent the *E. coli* adhesins necessary for its adhesion to the receptors of the uroepithelial cells [87, 169]. A mild activity of certain B-type proanthycyanidins was detected *in vitro*, but not *in vivo* [87].

In this study, the definition of infections was based on self-assessment by the participants, a method that is frequently used in common cold studies [25, 111, 213]. The clinical diagnosis of the common cold is in most cases simple, and can reliably be made by adult patients themselves [80]. The common cold can be considered in large part subjective [76], since even with modern diagnostic methods 20 - 30 % of cold cases remain without a proven viral cause [80]. Randomization was used to distribute the bias and noise potentially introduced by self-assessment of symptoms equally between groups. Regardless, they still interfere with the detection of a true signal [76]. The same applies for the urinary tract infections and digestive tract infections. The recommended practice for the diagnosis of urinary tract infections in Finland states that only the non-complicated cystitis in women can be diagnosed based on symptoms, without bacterial culture analyses from the urine [202]. The small number of reported urinary tract infections during the study has to be taken into consideration as well. Therefore, the results concerning this infection type are indicative only.

5.1.2 Concentrations of C-reactive protein (I)

There was a small but significant reduction of serum CRP levels in the sea buckthorn group compared to the placebo ($P = 0.04$) during the intervention. This reduction was evident also when only the compliant participants were included ($P = 0.03$), and when the ≥10 mg/l values were excluded (all participants $P = 0.049$, compliant participants $P = 0.02$).

The acute-phase response is a non-specific systemic response of endothermic animals to most forms of tissue damage, infection, inflammation, and malignant tumours. It is characterized by fever, somnolence and metabolic alterations. In acute-phase response, the synthesis of so-called acute-phase proteins, including CRP, is rapidly up-regulated. The synthesis mainly takes place in the liver and is induced by cytokines originating at the site of the local detrimental events [157, 190]. All the biological functions of CRP are not fully understood [157]. It is known to bind to phospholipid constituents of damaged cells and microbes and to native and modified plasma lipoproteins. It activates the complement and induces inflammatory cytokines [157, 190]. CRP concentration in general population is stable. It reflects ongoing inflammation and tissue damage accurately compared to most other analysable markers [157].

CRP is considered an independent predictor of increased coronary risk. Its levels are increased in type 2 diabetes. The proinflammatory state contributes to the pathogenesis of both cardiovascular disease and type 2 diabetes that

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often develop side by side [154, 157, 161, 170, 171, 172, 189]. Serum values ≥ 10 mg/l are considered to reflect an acute ongoing infection or inflammation. From the cardiovascular health point-of-view, the cutpoints for the serum concentrations of CRP are: < 1 mg/l low risk, 1 - 3 mg/l average risk and >3 mg/l high risk [154]. It is possible that CRP is not just a marker of risk, but contributes to the pathogenesis of cardiovascular diseases by exacerbating inflammation and tissue damage. However, the question of the possible causal link is still under investigation [46, 157].

An inverse association between diets rich in plant based food and circulating concentrations of markers of inflammation, including CRP, has been reported [137, 222]. Oliveira et al. [146] found a negative association between blood CRP and the intake of fruits, vegetables, vitamin C, vitamin E and fibre in men only. Interventions with a 1000 mg/day dose of vitamin C for 2 months reduced CRP levels among healthy non-smokers, who had baseline levels indicating elevated cardiovascular risk (≥1 mg/l), but not in those with low baseline levels [22]. In the same trial, vitamin E (800 IU/day) had no effects. Wu et al. [226] found vitamin E supplementation (500 mg/day for 6 weeks) ineffective for reducing CRP in patients with type 2 diabetes. Of the carotenoids β-carotene seems to be negatively associated with CRP in middle-aged and older women [221].

As discussed in the literature review, anti-inflammatory actions of flavonoids and sea buckthorn flavonoid rich fractions have been well-documented *in vitro*. In U.S. adults, the levels of serum CRP were inversely associated with dietary flavonoid intake [143]. Both positive and inconclusive results have been reported from flavonoid interventions. A blackcurrant and orange juice supplementation (250 ml + 250 ml/day for 28 days) decreased serum CRP in patients with peripheral arterial disease [45]. Anthocyanins from elderberry (500 mg/day for 12 weeks) did not affect serum CRP or other markers of inflammation in postmenopausal women. No effects were observed on the markers of liver or kidney functions either [44].

5.2 EFFECTS OF SEA BUCKTHORN BERRIES ON CIRCULATING LIPID MARKERS AND FLAVONOLS (II)

5.2.1 Total, HDL and LDL cholesterol and triacylglycerols (II)

An increased serum total or LDL cholesterol and triacylglycerols, and decreased HDL cholesterol are risk factors for cardiovascular diseases [68]. The current guidelines on cardiovascular disease prevention in clinical practice recommend target values of fasting total cholesterol <5 mmol/l and LDL cholesterol <3 mmol/l in people having only few other risk factors for cardiovascular diseases. For those at high risk, the target values are lower. No specific targets for HDL cholesterol or triacylglycerols are given, but it is pointed out that HDL cholesterol <1 mmol/l in men and <1.2 mmol/l in women

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and fasting triacylglycerols >1.7 mmol/l are markers of increased risk [68]. In this study the intake of sea buckthorn berries did not affect the levels of total, HDL, and LDL cholesterol or triacylglycerols in circulation.

An inverse association between the intake of quercetin and circulating total and LDL cholesterol levels in Japanese women has been reported [8]. Oral quercetin lowered the serum cholesterol and phospholipids in mice, and had a nonsignificant reducing effect on the serum triacylglycerols. Quercetin reduced the activity and mRNA levels of enzymes involved in the hepatic fatty acid synthesis, suggesting the inhibitory effects on lipogenesis [144]. Of the flavonoids typical for sea buckthorn, kaempferol inhibited cholesterol biosynthesis in hepatic cancer cells (HepG2) and in breast cancer cells (MCF-7) [103]. In rats fed with cholesterol rich diet, oral isorhamnetin and quercetin decreased the serum total cholesterol. In rats getting a cholesterol-free diet, the total serum cholesterol tended to be lowered by isorhamnetin, but the effect was not significant. In rats getting the cholesterol-free diet the level of serum triacylglycerols in the quercetin group was significantly higher compared to the control. Isorhamnetin and quercetin had a lowering effect on the liver total cholesterol, and isorhamnetin reduced the liver triacylglycerols [90].

The amounts of flavonols used in the above mentioned trials were higher compared to those in the daily sea buckthorn dose of our study. As reviewed in the literature part of the thesis (Chapter 2.2.8.3) the clinical sea buckthorn juice and flavonol interventions have not been beneficial in reducing the circulating cholesterol or triacylglycerol levels, whereas more positive results have been observed for sea buckthorn oil and in animal trials.

5.2.2 Quercetin, kaempferol, isorhamnetin and their correlation with CRP (II)

Consuming a low dose of sea buckthorn berries significantly increased the plasma concentrations of quercetin ($P = 0.03$) and isorhamnetin ($P < 0.01$) compared to the placebo. There was a trend towards higher kampferol levels as well, but the effect was not significant ($P = 0.07$). The changes in flavonols did not correlate with the changes in CRP.

Sea buckthorn berry is among the richest sources of isorhamnetin (mostly present as glycosides) in foodstuffs [167, 214, 238] (Chapter 2.1.1). Though isorhamnetin in large concentrations is not very common in foods, part of the dietary quercetin can be methylated in the liver to form isorhamnetin [121, 133]. Methylation is extensive in rats [130], but likely of less importance in humans [121]. The methylation rate is affected by other components of the diet [49].

The fact that there was no association between the plasma flavonols and CRP indicates that the sea buckthorn effect on CRP was probably caused by a synergy of several compounds, instead of being due to the flavonols alone. A

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large proportion of sea buckthorn berry flavonoids are proanthyanidins, which were not analysed in this study. As reviewed in the first part of the thesis, they and several other components of sea buckthorn berry have been suggested to have immunomodulatory effects.

5.3 EFFECTS OF SEA BUCKTHORN OIL ON DRY EYE AND POTENTIAL MECHANISMS OF EFFECT (III-V)

5.3.1 Clinical tests and symptoms of dry eye (III)

There was an increase in the tear film osmolarity from baseline to the end of the intervention in both treatment groups. The increase was significantly less in the sea buckthorn group (all participants: P = 0.04; compliant participants: P = 0.02). Changes in TBUT, Schirmer or mOSDI did not differ between the groups.

According to the symptom logbooks, the maximum intensities of redness were significantly lower in the sea buckthorn group as compared to the placebo when all participants were included (P = 0.04). Compared to the placebo the proportion of participants reporting the highest redness scores of 3 during the intervention was 6% in the sea buckthorn group and 36% in the placebo group. The difference between the groups was not significant when only the compliant participants were included (P = 0.11), even though the trend was the same. The maximum intensities of burning were significantly lower in the sea buckthorn group in the compliant participants (P = 0.04), where 12% of the participants in the sea buckthorn group and 32%, in the placebo group reported 3 as their highest symptom score. The group difference was not significant when all participants were included (P = 0.05), though again the trend was towards lower maximums in the sea buckthorn group.

Other individual symptoms did not differ between the groups. There was a significant difference in the proportion of days recorded as overall eye symptom days, without specification of the symptom, in the subgroup of contact lens wearers. This proportion was smaller in the sea buckthorn group when all participants were included (P = 0.049) but not when only the compliant participants were included (P = 0.19).

Previous studies indicate that oral intake of n-6 γ-linolenic acid alone, or in combination with linoleic acid, and/or n-3 fatty acids may be beneficial for dry eye [7, 14, 104, 119, 127]. The effects of fatty acids are likely to be mediated through alleviating the ocular inflammation contributing to dry eye and associated with both aqueous deficient and evaporative dry eye [1]. The positive effects n-6 fatty acids may be due to the increased production of anti-inflammatory and tear production stimulating eicosanoid prostaglandin E1 from dihomo-γ-linolenic acid (20:3n-6) [7, 160]. Beneficial effects of antioxidants on dry eye have been reported as well [21]. This is likely to be due

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to the reduction of oxidative damage, which may activate inflammation [21, 156]. As the inclusion criterion in this trial was just the experience of dry eye symptoms, both dry eye types were represented. Based on the baseline values of the clinical tests, the dry eye of the participants was not very severe. The dry eye definition includes the aspect of symptoms of discomfort [1]. It is known that the association between dry eye symptoms and clinical dry eye tests is poor [138].

Tear film hyperosmolarity is a focal factor in dry eye, and common to its different forms. Sea buckthorn oil attenuated the increase in osmolarity taking place during the cold season. From the start to the end of the intervention, the temperature in Turku dropped considerably contributing to low air humidity indoors and outdoors. Low relative humidity increases tear evaporation rate [215], and dry eye symptoms are more common during periods when indoor heating systems are used [132]. A positive effect of sea buckthorn oil on typical symptoms of dry eye, redness (all partipants) and burning (compliant participants), was observed as well.

5.3.2 Fatty acids of the tear film (IV)

Monounsaturated and saturated branched-chain iso- and anteiso-fatty acids were the most abundant fatty acids in the tear film, each constituting about or just below 40% of all fatty acids at baseline. The proportions of straight-chain saturated and polyunsaturated fatty acids accounted for ≈ 15% and ≈ 7% of the baseline fatty acids, respectively. The most abundant individual compounds were oleic, vaccenic and palmitic acids. Changes in the proportions of individual fatty acids or groups of fatty acids during the intervention did not differ between the sea buckthorn and placebo groups (branched-chain fatty acids $P = 0.49$, saturated $P = 0.59$, monounsaturated $P = 0.53$, polyunsaturated $P = 0.16$).

Meibomian glands at the margins of the eyelids secrete meibum. Meibum lipids contribute to the lipids of the tear film and are essential for its normal function [27, 158]. Cholesteryl and wax esters are the main lipid classes of meibum, together in somewhat equal proportions representing about 60% of total lipids. Chain lengths of the fatty acid moieties in cholesteryl esters vary from 18 to over 30 carbons, most of them being monounsaturated or saturated. The most common wax ester fatty acids are 18:1, 18:2 and 18:0. Both cholesteryl and wax esters contain a large proportion of branched-chain fatty acids [26, 27, 28, 29, 30, 139, 191]. Even though the amount of polar lipids is small compared to the non-polar lipids, they are thought to be essential as an amphiphilic sub-layer between the aqueous tear film and the outermost non-polar lipid sub-layer. Recently, Butovich et al. [30] identified very long chain (*O*-acyl)-ω-hydroxy fatty acids in human meibum. They are negatively charged and are suggested to be the lipid group responsible for the amphiphilic sub-layer and possibly be affected in evaporative dry eye [27, 30].

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Meibomian gland dysfunction is a condition associated with evaporative dry eye and posterior blepharitis [1, 50]. The meibum lipids from meibomian gland dysfunction patients have a lower proportion of straight-chain saturated and a higher proportion of branched-chain saturated fatty acids compared to healthy people [95]. Blepharitis patients with ocular damage similar to that of dry eye had a lower proportion of phosphatidylethanolamine and sphingomyelin among meibum polar lipids compared to those blepharitis patients without dry eye signs [192]. Also the amount of carotenoids in meibum is reduced in meibomian gland dysfunction and older age [148]. The tear film lipid layer is thinner in people experiencing dry eye symptoms compared to those with no symptoms [20].

De novo fatty acid synthesis in meibomian glands has been observed in animals [52, 105]. Results from a mouse feeding trial indicate that dietary intake is not the major source of the monounsaturated fatty acids of the eyelid lipids [129]. The effects of dietary n-3 and n-6 fatty acids on the fatty acids of the phospholipids in the lacrimal glands have been reported, however. These fatty acid changes were associated with the effects on inflammation and dry eye [219].

Only few human intervention trials investigating the effects of oil intake on the fatty acid composition of the meibum or tear film have been carried out. Only one [120], was found, stating that supplementation with 6 g/day flaxseed oil rich in n-3 fatty acids for one year in patients with meibomian gland dysfunction resulted in improvements of symptoms and some of the clinical parameters. A decrease in the proportion of saturated fatty acids in the meibum of the flaxseed group was also reported. However, it was unclear from the article whether the change was statistically significant. The fatty acid compositions or the changes were not presented. A case-study by Sullivan et al. [200] found differences in the meibum polar lipid profiles of Sjögren's syndrome patients according to their n-3 fatty acid intake from their habitual diet. Sjögren's syndrome is an autoimmune disease that is associated with an increased risk of dry eye [1].

No effects on the fatty acid composition of the tear film were seen in this study. It is possible the benefits of sea buckthorn oil were mediated through effects on ocular inflammation. As the inclusion criterion in this trial was the experience of dry eye symptoms without further clinical tests, different dry eye types were represented. The meibum fatty acid composition of individuals with aqueous deficient dry eye is similar to that of healthy people [95]. Agonistic effects of sea buckthorn oil fatty acids on the peroxisome proliferator-activated receptor γ (PPARγ) may be possible [140]. PPARγ is a transcription factor regulating the lipid metabolism. It is activated by polyunsaturated fatty acids, including linoleic acid, which increases lipid production by sebaceous cells and is needed for their differentiation *in vitro* [179]. Nien et al. [140], based on their study on mice, suggests that PPAR ligands, including n-3 and n-6 fatty acids, may positively influence dry eye by stimulating meibocyte differentiation, and reducing inflammation and meibomian gland lymphatic infiltration.

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5.4 EFFECTS OF SEA BUCKTHORN OIL ON INFLAMMATORY MARKERS AND AMINOTRANSFERASES (V)

5.4.1 Concentrations of cytokines and C-reactive protein (V)

The circulating concentrations of IL-6 and TNF-α at baseline were not elevated as compared to the earlier trials [137, 173, 239], and the levels were not affected by supplementation with sea buckthorn oil (IL-6, P = 0.37; TNF-α, P = 0.97). The median (quartiles) serum concentrations of CRP at baseline were low [154] in both groups [1.0 (0.4; 1.5) mg/l in the sea buckthorn group; 1.1 (0.5; 2.1) mg/l in the placebo group]. The sea buckthorn oil supplementation did not affect the levels (P = 0.97).

Dry eye is associated with increased levels of IL-6 and/or TNF-α in tears [239]. However, the inflammatory markers in the blood are commonly not elevated [210, 239], with the exception of dry eye patients with Sjögren's syndrome [239]. Also, the comparatively low baseline concentrations of the participants in our trial suggest that the inflammation associated with dry eye is local, and not reflected in the serum markers of inflammation.

Production of CRP is stimulated by pro-inflammatory IL-6 and TNF-α, and all three inflammatory markers are associated with the risk of cardiovascular diseases [98, 154, 161, 170, 171, 172, 173, 189]. Consumption of whole sea buckthorn berries reduced the serum CRP in healthy adults in Clinical Trial 1. Even though the seeds of the sea buckthorn oil puree were crushed to make the seed oil available as well, the amount of oil in the daily dose of sea buckthorn in Trial 1 was only 1 g. Based on the results of Clinical Trial 2, it is likely that sea buckthorn oil, at least not alone, was not responsible for the observed decrease in CRP in Trial 1.

5.4.2 Aminotransferases (V)

Supplementation with sea buckthorn oil did not affect the activities of the plasma ALAT (P = 0.23), ASAT (P = 0.47) or GT (P = 0.21), the median levels of which were within the reference range throughout the study [182, 197].

Elevated activity of ALAT, ASAT and GT are indicators of liver diseases [41]. In a study concerning the US population, the elevation of aminotransferases could not, in most cases, be explained by alcohol consumption, viral infections or iron overload. Instead, the higher activities were associated with obesity and other risk factors of cardiovascular diseases and type 2 diabetes. This indicates they are markers of non-alcoholic fatty liver, the hepatic component of the metabolic syndrome [41]. Of the aminonotransferases ALAT has the strongest correlation with liver fat [223] and its increased concentration in the circulation is associated with a greater risk of type 2 diabetes and coronary heart disease events [41, 186, 187].

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A one year supplementation with n-3 fatty acids (2g/day) reduced the serum levels of aminotransferases and TNF-α, and improved the fatty liver in non-alcoholic fatty liver patients [194]. Vitamin E supplementation (800 IU/day) of almost two years improved the histological features of non-alcoholic steatohepatitis and reduced the serum ALAT and ASAT values in humans [184]. Consuming berry meals equalling with 163 g of fresh northern berries per day for five months lowered the ALAT values of slightly overweight women [113]. An ethanol extract of sea buckthorn berries and oil with a composition similar to the one used in this trial, were among the meals of the berry group. Results suggesting the hepatoprotective effects of sea buckthorn seed oil and an undefined sea buckthorn extract have been published [60, 88] as described in more detail in the literature review of this thesis (Chapter 2.2.7, Table 8).

Reductions of CRP concentrations and aminotransferase activities even within the current reference values are likely to be beneficial [38, 56, 67, 154, 172]. Despite the encouraging previous investigations, sea buckthorn oil was inefficient in this trial. In most previous trials indicating the beneficial effects of plant oils or sea buckthorn on aminotransferases and/or markers of inflammation, participants or animals susceptible to cardiovascular or liver diseases or type 2 diabetes have been used. Most often the doses have been higher as well.

5.5 SUMMARY

In the present study, the health effects of sea buckthorn berry and oil were investigated in two double-blind, placebo-controlled randomized clinical trials. The main interest of the berry trial was on infections and inflammation, whereas the oil trial concentrated on the dry eye effects. In addition, the effects on markers associated with cardiovascular and type 2 diabetes risk were investigated in both trials.

In the berry trial, the study product was frozen sea buckthorn berry puree, where also the seeds of the berries were ground fine. The daily berry dose of 28 g contained 1 g oil, mostly form the soft parts of the berry. Compared with the average daily intakes of Finns, the intervention had the greatest effects on the intake of flavonols (167% of the average daily intake in the daily dose of berry puree [149]) and vitamin C (13 - 17% of the average daily intake [153]), whereas the intake of lipophilic components was less affected.

In the oil study, combined sea buckthorn seed and berry oil extracted using supercritical CO_2 was used. The daily dose was 2 g in the form of four capsules. The α-tocopherol and α-linolenic acid contents in the daily dose of oil were 58 - 82% and 6-9%, respectively, of the average daily intakes of Finns [153]. The daily amounts of carotenoids were similar in the two study products: 1.7 mg/day in the berry puree and 1.8 mg/day in the oil. However,

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the bioavailability of carotenoids may have been better from the oil because of higher rate of absorption [145, 180].

In the berry trial, no effects on the risk or duration of the common cold or digestive tract infections were observed in healthy adults having healthy diets and lifestyles. Complementary statistical analyses suggested positive effects on urinary tract infections, but due to the small number of cases and the lack of confirmation of diagnosis by bacterial cultures, the results can be considered as indicative and hypothesis generating only. However, the low dose of only 28 g/day for 3 months resulted in a small, but statistically significant reduction in the levels serum CRP. As CRP is a focal inflammatory marker associated with the increased risk of cardiovascular diseases and type 2 diabetes, this result is interesting.

Consuming sea buckthorn berries increased the plasma concentrations of the flavonols isorhamnetin and quercetin, but did not affect the circulating total, LDL or HDL cholesterol or triacylglycerol levels in the participants, having baseline levels within the recommended range. The lack of correlation between plasma flavonols and CRP changes suggests that the CRP effects were not caused, at least solely, by the sea buckthorn flavonols, but more likely by the synergetic effects of different berry components.

In the oil trial, sea buckthorn oil (2 g/day for 3 months) was found to attenuate the increase of tear film osmolarity taking place during the cold season when the air is dry indoors and outdoors. Also the maximum severity of typical dry eye symptoms of redness (significant when all participants were included) and burning (significant in compliant participants) were lower in the sea buckthorn group. In the subgroup of contact lens wearers the proportion of days with overall, non-specified eye symptoms was smaller compared to the placebo (significant when all participants were included).

Oral intake of sea buckthorn oil did not affect the tear film fatty acid profile indicating the positive effects of the oil were not mediated by direct incorporation of fatty acids into the tear film. Involvement of also other compounds in the oil is likely, as an association between reduced meibum carotenoids and dry eye has been reported. The possible mechanisms of effect include modulation of the inflammatory reactions of dry eye and the effects on meibocyte differentiation or lipid excretion.

Sea buckthorn oil did not affect the levels of circulating CRP, IL-6 and TNF-α and aminotransferases, associated with the risk of cardiovascular diseases and diabetes in the participants having non-elevated median baseline levels. The lack of effect on the systemic markers of inflammation does not rule out the possibility of modulation of the local ocular inflammation associated with dry eye. Based on this, the decreasing effect of sea buckthorn berries on serum CRP was likely caused by the less lipophilic components of the berry, or their synergy with other compounds.

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6 CONCLUSIONS

Animal and *in vitro* studies clearly show the antioxidant effects of sea buckthorn berry fractions. Antioxidant action and modulation of inflammation are thought to be focal in mediating the beneficial effects observed in animals: protection against cytotoxic agents (oils and polar fractions), tumorigenesis (alcohol extract), wound healing (oils and flavonoids), gastric ulcers (oils), hepatotoxicity (oils) and risk factors of cardiovascular diseases (oils and polar fractions). Only a few clinical studies concerning the health effects of sea buckthorn berries and oil have been conducted. The positive indications of sea buckthorn oils on the skin and mucosa have been observed in humans. Clinical study observations on the prevention of platelet aggregation and the elevation of blood HDL cholesterol due to sea buckthorn oils have been published.

In the clinical studies of this thesis, the physiological effects of sea buckthorn berries and oil were investigated. It was observed that a modest intake of 28 g of sea buckthorn berry puree/day for 3 months mildly, but significantly reduced the serum levels of the inflammatory marker CRP in healthy adults. The puree did not affect the risk of a common cold or digestive tract infections. The circulating concentrations of isorhamnetin and quercetin were significantly elevated, but there were no effects on the plasma cholesterol or triacylglycerols.

In the second clinical study of this thesis, the intake of 2 g of CO_2-extracted sea buckthorn oil/day for 3 months significantly attenuated the rise of tear film osmolarity taking place during the cold season in participants with dry eye. The beneficial effects on dry eye symptoms were observed as well. The symptoms of redness and burning of eyes were milder in the participants of the sea buckthorn group compared to the placebo. Sea buckthorn oil did not affect the fatty acid composition of the tear film, indicating the effects may have been mediated via the modulation of inflammation rather than the incorporation of fatty acids from oral oil to the tear film. No effects on the circulating biomarkers of inflammation or aminotranferases were observed.

These results suggest the positive effects of sea buckthorn berries and oil on inflammation and dry eye, respectively, in humans. The positive findings concerning the physiological effects of sea buckthorn berry and oils in animals and in humans deserve further investigation. There is especially a need for more large-scale clinical studies recruiting participants with special conditions or risk factors related to inflammation and oxidative stress.

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