KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

Viktorija Puzerytė

Phytochemical composition, *in vitro* antioxidant and biological activity of extracts from sea buckthorn (*Hippophea rhamnoides*) berry pomace and leaves

Master's Degree Final Project

Supervisor
Assoc. prof. Vaida Kitrytė

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Reviewer
Assoc. prof. Jonas Damašius

Project author
Viktorija Puzerytė

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Šaltalankių (*Hippophae rhamnoides* L.) uogų išspaudų ir lapų ekstraktų fitocheminė sudėtis, *in vitro* antioksidacinės savybės bei biologinis aktyvumas

Baigiamasis magistro projektas

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Vadovė

Doc. dr. Vaida Kitrytė

Recenzentas

Doc. dr. Jonas Damašius

Projekto autorė

Viktorija Puzerytė

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DECLARATION OF ACADEMIC INTEGRITY

4 June 2018

Kaunas

I confirm that the final project of mine, **Viktorija Puzerytė**, on the topic „ **Phytochemical composition*, in vitro* antioxidant and biological activity of extracts from sea buckthorn (*Hippophea rhamnoides*) berry pomace and leaves**" is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarised from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by law) have been paid to anyone for any contribution to this project.

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List of abbreviations, acronyms and symbols

Abbreviations	Full form
AAPH	2',2'-Azobis (2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
Ac	Acetone
CAA	Cellular antioxidant activity
CAE	Caffeic Acid Equivalent
DW	Dry Weight
EtOH	Ethanol
EtOH/H_2O (70/30 % v/v)	Hydroethanolic 70/30 % mixture
GAE	Gallic Acid Equivalent
GC-FID	Gas Chromatography – Flame Ionization Detector
He	Hexane
HORAC	Hydroxyl Radical Adverting Capacity
HOSC	Hydroxyl Radical Scavenging Capacity
Milli-Q water	Ultrapure water
ORAC	Oxygen Radical Adverting Capacity
PBS	Phosphate Buffer Saline
QE	Quercetin Equivalent
SFE-CO_2	Supercritical CO_2 extraction
SLE	Solid-liquid extraction
Sox	Soxhlet extraction
SPB	Sodium Phosphate Buff
TE	Trolox Equivalent
TEAC	Trolox Equivalent Antioxidant Capacity
TPC	Total Polyphenol Content
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC	Ultra Performance Liquid Chromatography

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Research field and area: Technological Sciences, Food Technology.

Keywords: Sea buckthorn (*Hippophae rhamnoides*), convectional extractions, high-pressure extractions, in vitro antioxidant activity, phytochemical composition, cytotoxicity, cellular antioxidant activity.

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SUMMARY

Sea buckthorn (*Hippophae rhamnoides L.*), widespread of the species genus, widely cultivated in Europe and Asia. Berry fruit of sea buckthorn has been utilized for juice and oil production including food additives to candies, jellies, jams, beverages and cosmetics and pharmaceuticals properties. Moreover, sea buckthorn leaves are used to produce leaf extracts, tea, tea powder or cosmetics. The aim of this study was to fractionate sea buckthorn pomace and leaves with the different polarity solvents, evaluate phytochemical composition, *in vitro* and *ex vivo* antioxidant activity of non – polar and polar constituents by using different assays.

In the present study sea buckthorn pomace and leaves were extracted different extractions: Soxhlet extraction, solid-liquid extraction (SLE), pressurized liquid extraction (PLE) using n-hexane, supercritical fluid extraction (SFE) using CO_2. Sea buckthorn pomace and leaves solid residues after fluid extraction with CO_2 has been applied for the solid-liquid (SLE) using four solvents of increasing polarity (acetone, ethanol, water, hydroethanolic 70/30 % mixture).

Total phenolic content using Folin-Ciocalteu's method, *in vitro* antioxidant activity was measured using $ABTS^{\bullet+}$, ORAC, HOSC, HORAC, also *ex vivo* cellular antioxidant (CAA) method. Solid residue antioxidant activity was measured with TPC and $ABTS^{\bullet+}$ scavenging capacity methods by approaching QUENCHER method. Non – polar sea buckthorn pomace and leaves SFE-CO_2 extracts oxidative stability in rapeseed oil was measured with Oxipres method. Selected extracts cytotoxicity activity after 1 h incubation and cellular antioxidant activity were estimated using human colon adenocarcinoma model Caco-2 cell line.

Phytochemical characterisation of chosen extracts was identified by UPLC-QTOF-MS, fatty acids composition was determined by GC-FID method.

Puzerytė, Viktorija. Šaltalankių (*Hippophae rhamnoides L.)* uogų išspaudų ir lapų ekstraktų fitocheminė sudėtis, *in vitro* antioksidacinės savybės bei biologinis aktyvumas. *Magistro* baigiamasis projektas / vadovė doc. dr. Vaida Kitrytė; Kauno technologijos universitetas. Cheminės technologijos fakultetas.

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Reikšminiai žodžiai: šaltalankiai (*Hippophae rhamnoides*), tradicinės ekstrakcijos, aukšto slėgio ekstrakcijos, in vitro antioksidacinis aktyvumas, fitocheminė sudėtis, citotoksiškumas, ląstelių antioksidacinis aktyvumas.

Kaunas, 2018. 102 p.

SANTRAUKA

Šaltalankiai (*Hippophae rhamnoides L.*) ir jų rūšys yra plačiai paplitusios Europoje ir Azijoje. Šaltalankių uogos naudojamos sulčių gamybai, aliejaus gamybai, maisto produktų gamybai-saldainiams, uogienėms, gėrimams, tai pat kosmetikoje ir farmacijoje. Šaltalankiu lapai naudojami kaip ekstraktai, geriamoji arbata, lapų milteliai, tai pat lapai naudojami kosmetikoje. Šio darbo tikslas buvo nustatyti šaltalankių uogų išspaudų ir lapų ne polinių ir polinių ekstraktų fitocheminę sudėtį, *in vitro* antioksidacines savybes bei biologinį aktyvumą.

Šio tyrimo metu šaltalankių uogų išspaudų ir lapų ekstraktai buvo išgauti naudojant tradicinę daugiapakopę soksleto ekstrakciją ir ekstrakciją organiniais tirpikliais, tai pat naudojant aukšto slėgio pagreitintą ekstrakciją organiniais tirpiklias ir superkritiną anglies dioksido ekstrakciją. Šaltalankių išspaudos ir lapai po superkritinės CO_2 ekstrakcijos buvo ekstrahuojami skirtingais organiniais tirpikliais (acetanu, etanoliu, vandeniu ir etanalio/vandens 70/30 % mišiniu).

Bendras fenolinių junginių kiekis šaltalankių išspaudų ir lapų ekstraktams buvo nustatytas Folin-Ciocalteu metodu. Skirtingų ekstraktų antioksidacinis aktyvumas buvo nustatytas naudojant $ABTS^{\bullet+}$, ORAC, HORAC ir HOSC metodus, taip pat antioksidacinis aktyvumas ląstelėse buvo įvertintas naudojant CAA metodą. Prieš ekstrakciją ir po ekstrakcijų skirtingais tirpikliais likusios kietos frakcijos bendras fenolinių junginių kiekis bei antioksidacinis aktyvumas tirtas Folin-Ciocalteu ir $ABTS^{\bullet+}$ metodais, pritaikius QUENCHER procedūrą. Oksidacinis stabilumas šaltalankių išspaudų ir lapų nepolinių ekstraktų buvo matuojamas Oksipres metodu. Citotoksiškumo tyrimuose su atrinktais šaltalankių išspaudų ir lapų ekstraktais po 1 val. inkubaciojos periodo parodė, kad ekstraktai buvo netoksiški Caco-2 ląstelėse.

Fitocheminė sudėtis šaltalankių išspaudų ir lapų ekstraktuose buvo nustatyta naudojant UPLC-QTOF-MS, riebalų sudėtis buvo nustatyta naudojant GC-FID metodą.

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) belongs to the Elaeagnaceae family. *H. rhamnoides* are widely spread in various locations in Europe and Asia [1]. Sea buckthorn berries are yellow-orange to red color fruits which are rich with bioactive compound such as vitamins (A, C, E, riboflavin, folic acid, and K), carotenoids (α, β, ð-carotene,and lycopene), sterols, fatty acids, tocopherols, tocotrienols, phenolic compounds, flavonoids, organic acids, amino acids [2, 3, 4]. Sea buckthorn berries products are wide spread on the market including oils, juices, and food additives to candies, jellies, jams, beverages and cosmetics and pharmaceuticals properties [5]. One of the main sea buckthorn berry fruit industrial processing examples are oil and juice production. However, pressing of juice produces high amounts of by-products (pomace), which currently are discarded as a waste or utilized rather inefficiently. Therefore, considerable amounts of nutrients are lost every year [6]. In addition, recent studies reported that compounds obtained from sea buckthorn are considered to be valuable drugs in the treatment of cancer, gastric ulcers, skin diseases, inflammatory diseases: thrombosis, reducing the risks of cardiovascular disease, diabetes, injuries of tendons and the ligaments [7]. Recently, another sea buckthorn berry fruit harvesting and processing by-product – leaves – were also considered for their antioxidant potential, correlated to flavonoids and phenolic acids derivatives. [4]. Sea buckthorn leaves are used to produce leaf extracts, tea, tea powder or cosmetics [8]. Accumulating evidence suggests that sea buckthorn leaves is a promising plant that could serve as a natural remedy for the reduction of cardiovascular disease risk and other health related problems like inflammatory diseases, diabetes, thrombosis and cancer [9].

The quantitative and qualitative composition of sea buckthorn pomace and leaves bioactive compounds can significantly change due to the plant subspecies, growing region, climate condition such as harvest time and extraction method. Isolation of target constituents is commonly accomplished by convectional (e.g., Soxhlet extraction, solid-liquid extraction) and, more recently, innovative (e.g. high-pressure extractions) fractionation techniques. However, it is widely reported that conventional extractions are time consuming, requires evaporation of the huge amount of solvent, and offers low extraction selectivity [10]. Common solvents that are used in these processes are hexane and acetone, which have limited applications in food industries due to the possible toxic effects and their residual amounts are strictly regulated by EU Directive 2009/32/EC. Therefore, various studies outline the potential of high pressure extraction methods, which are rapid in performance (e.g., pressurized liquid extraction), or utilizes food-grade non-toxic (so-called “green”) solvents and does not require solvent removal procedure after extraction (e.g., supercritical

fluid extraction with CO_2). In addition, high-pressure extraction under optimized conditions can offer high selectivity, thus is commonly incorporated into the complex multi-step biomass valorization schemes for target bioactive compounds isolation [11, 12].

The aim of this study was to investigate phytochemical composition, *in vitro* antioxidant capacity and biological activity of sea buckthorn pomace and leaves extracts, isolated using different conventional and high-pressure extraction techniques with different polarity solvents for these by-products valorization. The goals that has been set to fulfill these aims are the following:

1. To characterize sea buckthorn pomace and leaves by determining selected chemical composition parameters: lipid, protein, mineral, moisture and dry matter content.
2. To determine the efficiencies of conventional and high-pressure extraction techniques for non-polar and polar constituent isolation from sea-buckthorn pomace and leaves and to develop multi-step fractionation scheme for these by-products' valorization.
3. To determine fatty acid composition of various non-polar sea buckthorn pomace and leaves extracts by GC-FID analysis.
4. To evaluate phytochemical composition of the various non-polar and polar sea buckthorn pomace and leaves extracts by means of UPLC-QTOF-MS analysis.
5. To evaluate total phenolic content and *in vitro* radical scavenging properties of various sea buckthorn pomace and leaves non-polar and polar extracts, starting plant material and solid residues after different steps of extraction
6. To determine the effects of the selected non-polar sea buckthorn pomace and leaves extracts on the oxidative stability of edible oils.
7. To determine cytotoxic activity of the selected non-polar and polar sea buckthorn pomace and leaves extracts on Caco-2 cells.
8. To determine cellular antioxidant activity of the selected non-polar and polar sea buckthorn pomace and leaves extracts on Caco-2 cells.

1 LITERATURE REVIEW

1.1 Sea buckthorn: morphology and cultivation

Sea buckthorn (genus (*Hippophae rhamnoides L.*) belongs to the Elaeagnaceae family. *Hippophae rhamnoides* are widely spread in various locations in Europe and Asia. The classification of genus *Hippophae* has been modified over the years. Originally, it was constituted by only one species, namely *H. rhamnoides*, with 3 subspecies, namely *rhamnoides*, *salicifolia*, and *tibetana* [1]. *H. rhamnoides* was further divided into 9 subspecies: *carpatica*, *caucasica*, *fluviatilis*, *gyantsensis*, *mongolica*, *rhamnoides*, *sinensis*, *turkestanica*, and *yunnanensis* [13]. The differences between all these subspecies are mainly their size, shape, the number of main lateral veins in their leaves, and the leaves' quantity [1].

Wild sea buckthorn is widespread in Europe on rivers bank and coastal dunes along the Baltic Coasts of Finland, Poland, and Germany [13, 14, 15]. Also, these plants are spread along the Gulf of Bothnia in Sweden, and coastal regions of the United Kingdom as well as Asia. Most of *Hippophae* species are distributed around the northern region of China, throughout the Himalayan region, including India, Nepal, Bhutan, and in northern Pakistan and northern Afghanistan [15]. Sea buckthorn can grow in very poor soils including rivers bank, steep slopes, and acid and alkaline soils. Although, they can grow on hills and hillsides, valleys, along coastal regions and in islands. These plants are able to grow both in small and isolated space, as well as mixed with other species of shrubs or trees.

Sea buckthorn plants spread fast, into small forests: it takes on average of 3~5 years to grow. Usually it forms a shrub or a small tree with 3 – 4 meters of height sprouting several leaves, branches and systems of roots. Leaves are alternate, narrow and lanceolate, with a silver-gray color as depicted in Figure 2. The male bud consists of four to six apetalous flowers, which produce wind-distributed pollen whereas, the female bud usually contains one single apetalous flower with one ovary and one ovule. Moreover, a female plant produces soft, juicy and rich in oils, berry-like fruits 6–9 mm diameter. The ripe barriers are drupe-like colored in orange/red and have a single seed surrounded by a soft, fleshy outer tissue. Seeds are dark brown, glossy, ovoid to elliptical shape and 2.8–4.2 mm size [1, 7, 13].

Figure 1. The distribution of sea buckthorn plants in Europe an Asia

Figure 2. Sea buckthorn plant morphology

Hippophae is a specie, commonly found in Eurasia and have great economic potential due to it is several possible applications. Different anatomical parts of the plants can be used as nutritious food supplement of it is functional properties as well as pharmaceutical and cosmetic ingredients. Furthermore, these plants can sever as soil enhancers, be used as a source of energy, dried leaves can be useful for production of teas enriched with antioxidant activity and anti-obesity properties. In particular, a number of pharmacological activities such as cytoprotective, anti-stress, immunomodulatory, hepatoprotective, radioprotective, anti-atherogenic, anti-tumor, anti-microbial and tissue regeneration have been reported for various sea buckthorn [7].

1.2 Sea buckthorn domestic and industrial applications

1.2.1 Berry fruits and their products

Sea buckthorn (*H. rhamnoides L.*) is a unique plant currently being domesticated in several parts of the world. [16]. Sea buckthorn berries products are wide spread on the market including oils, juices, and food additives to candies, jellies, jams, beverages, and cosmetics and pharmaceuticals properties Figure 3 [5]. According Lu et al. (1992) sea buckthorn natural harvest yield is from 750 to 1500 kg/ha of berries [5]. In 2017 there was obtained high harvest yield in Lithuania, which was 2-3 t/ha. In comparison, 2016 harvest yield was 500 kg/ha.

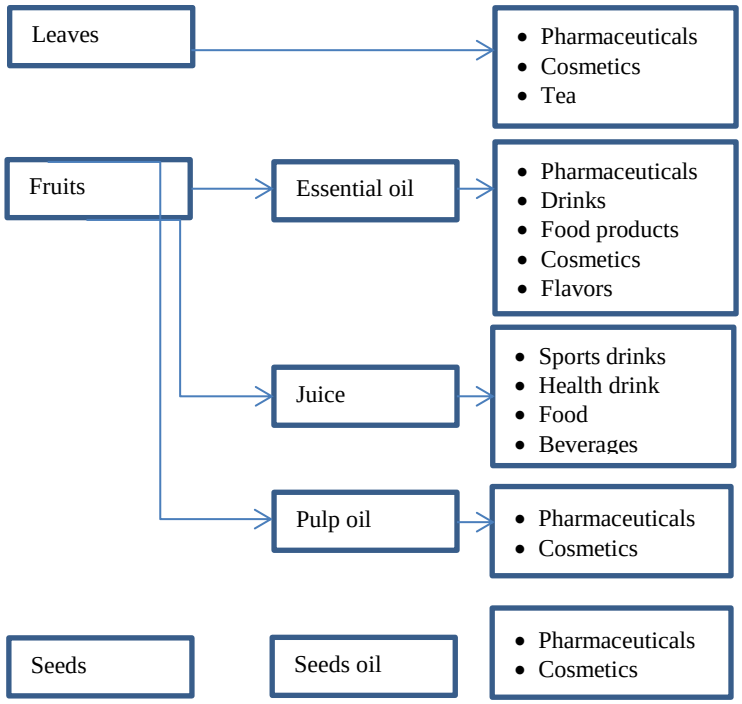


Figure 3. Potential uses of components from different parts of sea buckthorn

Berry fruits of *H. rhamnoides L*. has been traditionally utilized for juice and oil production. A schematic diagram of sea buckthorn berries juice processing is shown in Figure 4. First of all, fresh sea buckthorn berries fruits were collected and cleaned. Then cleaned and blanched berries were mechanically screw pressed. The juice with pulp oil centrifuged to separate clear juice from orange/red pulp oil and residual solids as sludge. According the fibrous cake with seed can be dried and separated into fibrous residue and seeds [17]. Moreover, sea buckthorn juice contains high concentrations of vitamin C [18]. There is information available on the concentration of other antioxidants such as tocopherols and tocotrienols, carotenoids, flavonoids and nutritionally important fatty acids. Due to their functional properties, and unique taste and flavor, sea buckthorn berry juice can be further processed to enrich candies, jellies, jam, alcoholic or non alcoholic beverages, or as flavoring of dairy productsas well as cosmetics products (Table 1) [5]. Also, sea buckthorn berries are used as a nutritional ingredient in baby food. Fruity drinks were among the earliest sea buckthorn products developed in China. However it is also popular enrich food and cosmetic products in USA, Canada Europe Germany, Poland and Scandinavian and Nordic countries [19].

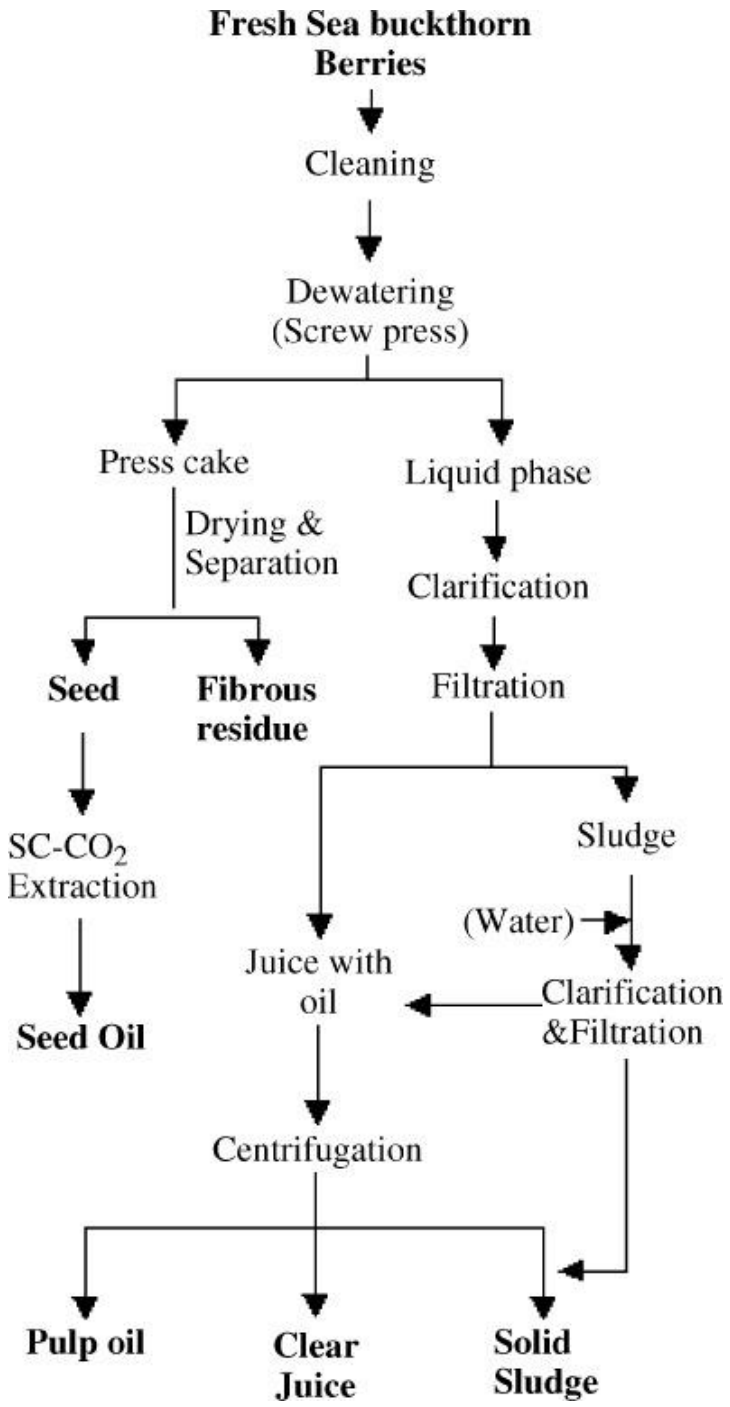


Figure 4. Integrated processing of fresh sea buckthorn berries for pulp oil and juice production [17]

In general, there are two different oil extractions of sea buckthorn: the pulp oil and the seed oil [20]. The mature seeds contain 8 - 20% of oil, the dried fruit pulp (flesh and peel) about 20 - 25% of oil, whereas the berries residue left after juice extraction about have 15 - 20% of oil [21]. Sea buckthorn fruit's oil contain rich unsaturated fatty acids, among them linoleic acid, and palmitoleic acid (up to 50%), specially it is high level of carotenoids [22] .

Table 1. Domestic and industrial application of sea buckthorn berries, oil, juice of food production

Country	Company name	Domestic products
Lithuania	"Sveikatos sauja" "Ekologinis Mindaugo Sakalausko ūkis" "Amberry saltalankiu ukis" "Vertas" "Valio gefilus" "Milzinu uoynas" "Karpaty botanica" "Karvelio imone" "Serksno medus"	• Sea buckthorn tea • Sea buckthorn oil • Sea buckthorn jam • Spices with sea buckthorn • Sea buckthorn juice • Milk products with sea buckthorn • Candies with sea buckthorn • Sea buckthorn powder • Honey with sea buckthorn
Latvia	"Medus veikals" "Lielauces Kliņģeris" "Lazdona" "Lakto" "Baltais" "Rudolfs bio" :Em-eukal" "AnevaJ"	• Sea buckthorn tea • Sea buckthorn oil • Cocoa butter with sea buckthorn oil • Honey with sea buckthorn oil • Lollipops with sea buckthorn • Rice cream with sea buckthorn • Milk product with sea buckthorn syrup • Buttermilk with sea buckthorn
China	"Bridgegap" "Zelang" "Qingdao Sunrise Biotechnology" "Xiamen Yiyu Biological" "Changzhou Greater Asia Pacific Trading" "Qinghai General Health Bio-Science"	• Sea buckthorn powder • Sea buckthorn Fruit Oil • Sea buckthorn beverage • Sea buckthorn juice • Super food
Canada	"Mont Echo Naturels" "Gourmet nature" "Hollow Reed Holistic" "Solberry"	• Sea buckthorn Oil • Sea buckthorn juice • Sea buckthorn jelly
India	"Minchy's Food Products" "Qtrove" "OrganicFacts"	• Sea buckthorn jam • CO_2 Critical Extracted - Sea Buckthorn Seed Oil • Sea buckthorn juice
United Kingdom	"BuyWholeFoodsOnline" "Raw Living Home" "Pearls of Samarkand"	• Dried sea buckthorn berries • Sea buckthorn juice powder • Sea buckthorn powder • Sea buckthorn juice
Romania	"CatargActiv"	• Sea buckthorn berries • Sea buckthorn berries juice
Armenia	"Noyan"	• Sea buckthorn juice
South Korean	"Samsung herb medicine co"	• Sea buckthorn fermented and extracted vinegar and herbal Extract juice
Russia	"Diveevo" "LLC "Jam Empire" "Specialist"	• Sea buckthorn juice • Sea buckthorn jam • Sea buckthorn oil
Germany	"Kräuterhaus Sanct "Bernhard" "Uwe Rolf GmbH Ostfriesischer" "Sandokan" "Alamy" "Pension Bradhering"	• Sea buckthorn juice • Sea buckthorn sweets • Sea buckthorn spirits • Sea buckthorn jam • Sea buckthorn jelly
Poland	"Ecological Shop Ekoflos" "Towicz" "EcaMedica" "Vitacymes" "BIO SOFA VIVIO" "Lyczek Herbaty" "Konopia Pharmacy- Hemp products"	• Sea buckthorn juice • Dried sea buckthorn fruits • Sea buckthorn jam • Sea buckthorn oil • Sea buckthorn tea • Sea buckthorn and hemp drink

Sea buckthorn berries uses for medical reasons were reported in Asia and Europe. First initiated medical tests in 1950's was reported in Russia [7]. The most important pharmacological functions of sea buckthorn's seeds and berries oil are: anti – inflammatory, antimicrobial [23], pain relief and the promotion of tissue regeneration. Moreover, this oil is recommended as a treatment for radiation damage, any kind of burns, duodenal ulcers, gastric ulcers, chilblains, skin ulcers caused by malnutrition and other skin problems [16]. Sea buckthorn is considered to be useful in treating tumours, stomach ulcers, skin diseases and arsenic poisoning [24]. Also, sea buckthorn berry's oil has been long used in Asia for treating skin conditions. Berries effectiveness is based on a combination of lipophilic compounds, working synergistically to protect and regenerate stressed skin cells and other protecting tissues [21]. More medical properties of sea buckthorn different anatomical parts are presented in Table 2.

Table 2. Medical properties of sea buckthorn different parts

Anatomical parts	**Medical properties**	**References**
Berries fruits	Gastric ulcers	[25]
	Skin disorders	[7, 26, 27, 28]
	Cardiovascular diseases	[29, 30, 31]
	Radiation-induced oxidative damage	[32]
	Wound healing	[33]
	Thrombosis and platelet aggregation	[34]
Seeds and berry oil	Dermatitis and thrombosis	[28, 35]
	Eczema, lupus erythematosus, chronic wounds, inflammatory diseases, erosion of the cervix and uterus, keratitis, trachoma, and conjunctivitis	[36]
	Anti-atherogenic effects, cardioprotective	[20, 21, 37]
	Antimicrobial	[38, 39]
	Antidiabetic	[40]
	Eye health	[36]

In region of Eastern Europe, sea-buckthorn berries are often used in homemade cosmetics. In particular, recipes for moisturizing lotions, dandruff control and hair-loss prevention are widely known and used in Russia [41]. There are known that sea-buckthorn oil has unique anti-ageing properties and, as a result, is becoming an important component of many facial creams manufactured in Asia and Europe. Berry fruits contain important antioxidants, including vitamins C and E. According to the University Maryland Medical Center, one form of vitamin E, in particular, α-tocopherol, reduced skin roughness, length of facial lines and the depth of wrinkles when applied topically. Also, sea buckthorn is source of vitamin C which is another antioxidant that helps maintain skin and hair health [42].

1.2.2 Leaves and their products

Sea buckthorn leaves are used to produce leaf extracts, tea, tea powder or cosmetics [8]. Sea buckthorn is considered to be useful in treating tumours, stomach ulcers, skin diseases and arsenic poisoning [24]. Accumulating evidence suggests that sea buckthorn is a promising plant that could serve as a natural remedy for the reduction of cardiovascular disease risk and other health related problems like inflammatory diseases, diabetes, thrombosis and cancer [9] it is shown in Table 4. Also leaves can be produced as green powder, which can be used as food additive or food supplement e.g. in bread, pills and capsules. Different regulations control the use for different purposes [43].

The leaves, either fresh or dried are rich in nutraceutical components [8]. Tea prepared from the dried leaves of sea buckthorn not only has a delicate fragrant and pleasant aroma but is also rich in calcium, potassium, magnesium, β-carotene and vitamin E. Sea buckthorn leaves tea supplementation suppressed body weight gain in a dose-dependent manner and significantly reduced visceral fat, plasma levels of leptin, triglyceride and total cholesterol, and alanine aminotransferase activity compared to high-fat-fed control mice studied for six weeks [4, 43]. Leaves tea are produced by some Europe countries, India and Canada it is shown in Table 3. Moreover, sea buckthorn leaf tea supplementation has potential anti-visceral obesity properties and antioxidant activity mediated by the regulation of lipid and antioxidant metabolism in high-fat diet-induced obese mice. Medical properties from sea buckthorn's different parts are shown in Table 4 [21, 22].

Table 3. Domestic and industrial application of sea buckthorn leaves

Country	Company name	Domestic products
Lithuania	"Gyduolis"	• Sea buckthorn leaves tea
Canada	"Gescina-The "Chemistry of Nature"	• Sea buckthorn leaves tea
India	"Smartcooky" "Indiamart"	• Under eye gel • Dry sea buckthorn leaves
Germany	"Zagori - Hippophaes" "Sandicca distributor"	• Sea buckthorn leaves tea

Table 4. Medical properties of sea buckthorn leaves

Anatomical part	Medical properties	References
Leaves	Rheumatoid arthritis	[44]
	Overweight, visceral fat, leptin, triglyceride, and total cholesterol	[43]
	Inflammation	[9, 30, 45]
	Hypoxia-induced cytotoxicity and DNA damage	[46]
	Cold-hypoxia-restrain	[47]
	Cytotoxicity	[48]
	Cardiovascular diseases	

Table 4 is shown that leaves has beneficial properties for *Rheumatoid arthritis*, overweight, cardiovascular diseases prevention as well as improving blood pressure and lowering cholesterol; preventing and controlling blood vessel diseases; and boosting immunity [4, 7, 18].

1.3 Bioactive compounds of sea buckthorn berries and pomace

1.3.1 Non – polar constituents

In general, sea buckthorn berries contain vitamin C, mineral elements, monosaccharides sugar, organic acids, free amino acids, fatty acids (saturated, unsaturated), carotenoids and vitamin E. Main constituents of sea buckthorn oils from seed, pulp and fruit residue oil after removing juice is shown in Table 5.

Table 5. Main constituents of sea buckthorn oils from seed, pulp and fruit residue oil [49]

	Concentration (mg/100 g)		
Ingredient	**Seed oil**	**Pulp oil**	**Fruit residue oil**
Vitamin E	207	171	300 – 600
Vitamin K	110 – 230	54 – 59	-
Carotenoids	30 – 250	300 – 870	1280 – 1860
Total acids	11	38	-
Total sterols	1094		-
Unsaturated fatty acids	87 %	67 %	70 %
Saturated fatty acids	13 %	33 %	30 %

Commonly sea-buckthorn oils can be obtained from two parts of the plant [50, 51]. Firstly, sea-buckthorn oils may be extracted in the process of mechanical cold pressing of seeds which contain up to 12.5 % of oil [2, 21, 52]. Secondly, the oil is obtained by extraction or in cold pressing of fruit pulp which contains 8–12 % oil. Finally, the obtained fractions are filtered [50, 52, 53, 54]. There are two types of oil differ significantly in terms of appearance and properties. Sea buckthorn seed oil is rich in the two unsaturated fatty acids, linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids. The proportions of the two fatty acids in seed oil are commonly 35–40 and 20–35%, respectively. Other major fatty acids in seeds are oleic (18:1 n-9, 15–00%), palmitic (16:0, 6–10%), stearic (18:0, <0.5%), and palmitoleic (1:16 <0.5%) acids [2, 55, 56, 57]. Another oil from sea buckthorn berries has the highest content of palmitoleic acid (omega-7) range from 15 to 40 %, which is higher than in sea-buckthorn seed oil [58, 59]. Moreover, sea buckthorn pulp oil is rich of palmitic, stearic, oleic fatty acid. Fatty acids composition of sea buckthorn is shown in Table 6. The oil from juicy berries obtained to be thick, dark orange or red orange oil [35, 59, 60]. In general, sea buckthorn seed oil and fruit oil differ significantly in terms of their content of active compounds [35, 61, 62, 63]. However, both oils contain a wide range of essential unsaturated fatty acids, in particular unique

palmitoleic acid (C16:1) which is highly valued in cosmetology. The composition of fatty acids with various properties ensures multidirectional effects of sea-buckthorn oil in different layers of epidermis. Also, a high content of saturated fatty acids (above 30 %) causes the oil to soften the epidermis as a functions to protect it and secure it against trans-epidermal water loss [64].

Table 6. Composition of fatty acids in sea buckthorn oil [19, 37, 50]

Common name	Numerical symbol	Content seed oil in %	Content pulp oil in %	Omega family
Palmitic acid	C16:0	6 – 10	15 – 40	
Stearic acid	C18:0	<0.5	15 – 50	
Palmitoleic acid	16:1	<0.5	15 – 40	7
Oleic acid	18:1	15 – 20	10 – 20	9
Linoleic acid	18:2	35 – 40	5 – 15	6
α-Linolenic acid	18:3	20 – 35	5 – 10	3

Both the seeds and the pulp oil are good sources of tocopherols. The total content of tocopherols and tocotrienols varies within the range 100–300 mg/kg in seeds and 10–150 mg/kg in fresh berries [2, 56, 65]. In the pulp oil α-tocopherol alone constitutes up to 90% of the total tocopherols and tocotrienols while both α- and γ-isomers (each representing 30–50% of total) are the major ones in seeds. α-, β-, and γ-Tocotrienols (amount individually of ∼0.5–5% of total tocopherols and tocotrienols) in pulp oil, whereas in seeds the β-isomer 2–8% clearly dominates accompanied by only trace amounts of α- and γ-isomers [2, 65]. [50]. Tocopherols and tocotrienols are also used to increase the shelf life and the stability of foods. α-tocopherols have shown better antioxidant activity than γ-tocopherols in oils and fats. Moreover, tocotrienols have potent antioxidant and anti-inflammatory properties that are superior to tocopherols in prevention and treatment against major chronic diseases properties (anti – cancer, anti-diabetic, anti-inflammatory, immuno-stimulatory and nephroprotective) [66].

Mainly, carotenoids exist in the pulp oil, giving the berries their beautiful yellow-orange color. The concentration in seeds is typically 1/20–1/5 of that in pulp oil [35]. β-carotene contain 15–55% of total carotenoids [2]. Even though α-carotene, γ-carotene, dihydroxy-β-carotene, lycopene, zeaxanthin and canthaxanthin have been reported to be the other carotenoids in sea buckthorn berries. The content of carotenoids in the berries are subject to extreme variation; differences up to 10-fold has been reported even within the same natural population and subspecies. Levels of β-carotene from 0.2 to 17 mg/100 g and total carotenoids (calculated as β-carotene) from 1 to 120 mg/100 g in fresh berries have been reported in the literature [2, 20, 65]. Vitamin A was found in the form of carotenoids. It provides regenerative and anti-wrinkle properties of the oil [67].

Carotenoids such as α- and β- have the added advantage of being able to be converted to Vitamin A helping preventing diseases like cancer, cardiovascular disease, osteoporosis and diabetes [68].

The sterol content in sea-buckthorn oils differ according to both raw materials and methods of oil isolation [35, 65]. Typical values are 1–2% in seed oil and 1–3% in oil from the soft parts. Sitosterol constitutes 60–70% of seed sterols and up to 80% of those in soft parts. Another major sterol representing 10–20% of seed sterols and 2–5% of sterols of the soft parts is isofucosterol [65]. In general, sterols, which strengthens the lipid barrier of the skin, protects from harmful substances of external origin and reduces the excessive water loss through the epidermis, thereby improving the skin elasticity and firmness. Also, sterols decrease cholesterol levels in blood [69]. Chemical composition of tocopherols, tocotrienols, carotenoids and sterols are shown in Table 7.

Table 7. Chemical composition of sea buckthorn seed and pulp oil (mg/100g) [21, 37, 61]

Compounds	Seed oil	Pulp oil
Tocopherol		
α-tocopherol	223.4	143.7
β-tocopherol	12.1	21.1
γ-tocopherol	177.4	11.1
δ-tocopherol	8.8	6.5
Tocotrienols		
β-tocotrienol	9.7	-
γ-tocotrienol	-	2.5
Carotenoids		
α-,β-,γ-carotene	10 – 50	350 – 520
Sterols		
Cholesterol	3.7	4.6
Campesterol	22.5	12.4
Stigmasterol	2.7	10.8
β-sitosterol	590 – 750	520 – 580

All four isomers of tocopherol was found in sea buckthorn seeds and pulp oil. Sea buckthorn seed oils is rich of all forms of tocopherols and also sterols, pulp oil is richer in carotenoids then in seeds oil. Both part of sea buckthorn berries show hight amount of oil. The oil from both part of the berries became important food additions in cancer therapy [20].

1.3.2 Polar constituents

Sea buckthorn berries are a great source of valuable polar compounds. They are usually orange-yellow to red color fruits and rich in flavonoids, organic acids, amino acids, micro and macronutrients [7]. Phenolic compounds can protect the human body from free radicals, whose formation is associated with the normal natural metabolism of aerobic cells. The activity of

flavonoids and phenols is mainly based on the structural relationship between different compounds of their chemical structure [70].

In general, total phenolic compounds can just be found in free fractions. They are classified into four categories, phenolic acids, flavones flavonoids – monoglycosides and flavonoid – diglycosides. There are reports of sea buckthorn's main phenolic compounds such as areisorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-glucosid, isorhamnetin-3-O-glucoside-7-O-rhamnoside, isorhamnetin-3-O-sophoroside-7-O-rhamnoside, kaempferol-3-O-sophoroside-7-O-rhamnoside, isorhamnetin, quercetin, kaempferol, gallic acid, ferulic acid, protocatechuic acid, epicathin and catechin. The content of phenolic compounds are presented in Table 8 [71, 72]

Table 8. Content of phenolic compounds of sea buckthorn [72]

Content	**Amount (mg/100g)**
Total phenolic acids	62.9
Gallic acid	19.8
Protocatechuic acid	39.3
Ferulic acid	3.76
Total flavones	**30.9**
Catechin	8.99
Epicathin	2.14
Quercetin	5.51
Kaempferol	1.23
Isorhamnetin	13.1
Total flavonoid-monoglycosides	**147**
Quercetin-3-O-rutinoside	32.9
Quercetin-3-O-glucoside	39.7
Isorhamnetin-3-O-rutinoside	58.6
Isorhamnetin-3-O-glucoside	15.5
Total flavonoid-diglycosides	**233**
Kaempferol-3-O-sophoroside-7-O-rhamnoside	45
Isorhamnetin-3-O-sophoroside-7-O-rhamnoside	39.7
Isorhamnetin-3-O-glucoside-7-O-rhamnoside	148
Total phenolics	**473**

Sea buckthorn berries are known for their high levels of vitamin C (from 360 to 2500 (mg/ 100g)) [37, 73, 74]. Vitamin C concentration in sea buckthorn depends on populations and subspecies [74]. Sea buckthorn is also rich in vitamins B1, B2, [37, 74].

The most common sugar components are glucose, fructose and xylose [73]. Glucose is a major sugar component in all sea buckthorn species. Both glucose and fructose vary widely from 0.6 – 24 (g/100ml berries juice) [73] accounting for around 90% of the total sugar content [73, 74].. The

presence of sugar alcohols mannitol, sorbitol, and xylitol at low levels has been observed [74]. The content of sugar in sea buckthorn depends of climate and subspecies of sea buckthorn berries [18]. Sugars and organic acids are the main soluble ingredients of sea buckthorn berries having a major effect on taste, fruit ripeness, or even present an index of consumer acceptance.

In particular, the berries of sea buckthorn contain organic acids mainly malic and quinic acids together constituting around 90% of all the fruit acids in different origins. There are large variations in concentrations of acids have been also reported amongst different origins. The highest concentrations of organic acid with a range of 3.5–9 g/100 ml in sea buckthorn berries [73].

A total of 18 out of 22 known amino acids have been found in sea buckthorn fruit, half of which (threonine, valine, methionine, leucine, lysine, trytophan, isoleucine, and phenylalanine) are essential since they play a critical role in several processes within our bodies such as energy production, building cells and muscles, fat loss, and mood and brain functions [73, 74]. Organoleptic assessment is greatly influenced by the relative and total amounts of sugars and acids in the sea buckthorn berries [75]. Amino acids stimulate protein synthesis primarily including the dose and composition of the amino acid mixture or protein [76, 77].

There are many mineral elements present in berries/juice of sea buckthorn. Potassium is the most abundant of all the elements investigated in berries or juice [73, 74].

Sea buckthorn berries are rich very important bioactive compounds. The assays for phenolic compounds are total phenolic content (TPC). The common assays for antioxidant activity *in vitro* are 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the oxygen radical absorbance capacity (ORAC). Cellular antioxidant activity (CAA) assay is used to quantify the antioxidant activity at the cell level *ex vivo* [39, 72]. The samples of cellular antioxidant activity are performing with concentration which is not cytotoxic [72]. The intensive of antioxidant activity depends of bioactive compounds, extraction methods and solvents. Some of antioxidant activity with different methods of sea buckthorn berries and pomace are shown in Table 9.

Table 9. Antioxidant activity with different methods and different extracts methods of sea buckthorn berries [72, 78], pomace [63].

Sample	**TPC (mg GAE/g)**	**DPPH (mg TE/g)**	**ORAC (μmol TE/g)**	**CAA (μmol QE/mol of phenolic)**
Berries	27 – 39	304		
Methanol extract	15 – 23	178	152	17 – 63
Pomace				
Water extract	70			
Ethanol extract	17			

Based on the nature of mechanism of interaction, antioxidant can be classified as primary, secondary, endogenous, exogenous, enzymatic, and non-enzymatic. Generally, antioxidants are the molecules that contain reactive hydroxyl groups, which may be phenolic or non-phenolic that includes ascorbic acid, tocopherol, polyphenols, and flavanoids. This definition is not rigid as deviation in classical definition, for example, ecdysteroids which although do not contain active hydroxyl groups but still possess antioxidant property and free-radicals cavenging tendency.

The Folin-Ciocalteu method has been used to measure total phenolics content in natural products. This method was improved by adding a higher proportion of molybdate and lithium sulfate to prevent precipitation, and this modification yielded higher sensitivity and reproducibility. The basic mechanism is an oxidation/reduction interaction contributed by the reducing properties of phenols, other non-phenolic reducing agents, and possibly metal chelators. The quantification basis of this method is the oxidizability of the phenolic compounds. The interferences with the "total phenols" measurement was contributed by non-phenolic antioxidants and reducing substances, such as ascorbic acid, glucose, fructose, and sulfites, that are common food additives or are naturally present in juices, fruits, and vegetables. Aromatic amino acids (tyrosine and tryptophan) and proteins [79].

The ORAC assay has been largely applied to the assessment of free radical scavenging capacity of human plasma, proteins, DNA, pure antioxidant compounds and antioxidant plant/food extracts [80]. The ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) [81].

To evaluate the antioxidative activity of specific compounds or extracts, the latter are allowed to react with a stable radical, 2,2-Diphenyil-picrylhydrazyl (DPPH °) in a methanol solution [82].

The original $ABTS^{\bullet+}$ scavenging assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. Addition of antioxidants to the pre-formed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction [83].

Although these chemical assays are widely used, they do not adequately reflect the real antioxidant activity of samples *in vivo*, not only because the activity is beyond estimation due to numerous factors,

such as metabolism, physiological conditions and bioavailability, but also due to the pathway of simply scavenging free radicals inhibiting the production of radicals or enhancing the level of endogenous antioxidants.

The cellular antioxidant assay (CAA) is a relatively new approach for the quantification of antioxidants under physiological conditions. This assay can reflect the cellular uptake efficiency of the tested samples, and as such, the cellular antioxidant levels may be influenced by digestion. A Caco-2 cell model has been reported to be a simple and useful system for investigating bioavailability of whole food phytochemicals by determining the cell uptake of the main compounds [84].

1.4 Bioactive compounds of sea buckthorn leaves

1.4.1 Non – polar constituents

Sea buckthorn fresh leaves are rich in carotenoids (20.33 – 24.57 mg/100g) and chlorophyll (98.8 mg/100g) [8]. Several lipophilic compounds, previously homologues of tocopherol (α-T, β-T and γ-T), PC-8 (plastochromanol-8) and β-carotene were identified in sea buckthorn leaves [85] The dominant compound is α-tocopherol, constituting 50.6–70.1 g/100g of total identified lipophilic compounds, while other tocopherol homologues (β and γ) were recorded at lower percentages (0.6–1.5 % and 0.7–1.8 %, respectively). PC-8 (plastochromanol-8) and β-carotene accounted for a significant quantitative proportion in the total identified lipophilic compounds (4.7–10.2 and 18.1–41.9 g/100g, respectively) [3, 85]. The concentration of lipofilic compounds depends on sea buckthorn leaves plants' sex (female and male) [85]. Total contents of tococpherols, PC-8(plastochromanol-8) and β-carotene of sea buckthorn leaves are presented in Table 10.

Table 10. Contents of tocochromanols, plastochromanol-8 and b-carotene in sea buckthorn leaves [3].

Plant sex	Tocopherol (mg/100g)			PC–8 (mg/100g)	β-carotene
	α-tocopherol	β-tocopherol	γ-tocopherol		
Female	35.41	2.43	1.51	1.79	20.33
Male	23.96	1.69	1.27	1.61	24.57

The composition of free fatty acids, esters, and alkanes, which amount to a total is 65.89 % of sea buckthorn leaves. The tree major free fatty acids are n-hexadecanoic acid (26 – 37%), oleic acid (6 – 9%), and tetradecanoic acid (~4%). Two major esters are dibutyl phthalate (7 – 14%), 8,11-octadecadienoic acid.

1.4.2 Polar constituents

Sea buckthorn's fresh leaves's content has a remarkable amount of nutrients and bioactive components, especially phenolics (1453 – 2218 mg/100g) [8, 19]. The polyphenolic compounds in the leaves are represented by flavonols, leucoanthocyanidins, (−) epicatechin, (+) gallocatechin, (−) epigallocatechin and gallic acid [7]. The leaves of sea buckthorn are rich in kaempferol-3-O-β-D-(6″-O-coumaryl) glycoside, 1-feruloyl-β-D-glucopyranoside, isorhamnetin-3-O-glucoside, quercetin-3-O-β-D glucopyranoside, quercetin-3-O-β-D-glucopyranosyl-7-O-α-L-rhamnopyranoside, and isorhamnetin-3-O-rutinoside [4, 36, 86] .Dried leaves still contained large quantities of bioactive compounds. The total amount of phenolic compounds depends on dried temperature.

Sea buckthorn leaves are very rich in phenolic acids [87]. Phenolic acids include two main groups namely, hydroxybenzoic acid and hydroxycinnamic acid derivatives with different number and position of hydroxylation and methoxylation in aromatic ring. The most common phenolic acids in sea buckthorn leaves are gallic acid, proto catechuic acid, p-hydroxy benzoic acid, vanillic acid, salicylic acid, cinnamic acid, p-coumaric acid, ferulic acid, caffiec acid. The amount of these phenolic acids are shown in Table 11 [87]. Phenolic acids are distributed as their free and bound forms in nature, more often bound forms occur as their esters and glycosides. Phenolic acids in plant materials are found as their free, esterified and glycosidic forms. The extractability of phenolic acids from plant tissues largely depends on their chemical nature, solvent polarity and extraction conditions.

The flavonoids are a very important bioactive compound in sea buckthorn. The most common flavonoids in sea buckthorn leaves are cateechin, rutin, quercetin, quercetin 3-galactoside, quercetin 3-O-β-d-glucopyranoside (isoquercetin), quercetin 3-methyl ether kaempferol, kaempferol 3-O-β-d-glucopiranoside (astragalin), isorhamnetin, isorhamnetin 3-O-β-d-glucopyranoside-7-O-α-l-rhamnoside and myricetin [36, 88, 89] .

Table 11. Content of phenolic acids in sea buckthorn leaves (mg/kg of dry matter)[87]

Hydroxybenzoic acid derivatives					**Hydroxycinnamic acid derivatives**				**Total**
Gallic acid	**Proto catechuic acid**	**p-hydroxy benzoic acid**	**Vanillic acid**	**Salicylic acid**	**Cinnamic acid**	**p-coumaric acid**	**Ferulic acid**	**Caffiec acid**	
4222	50	247	37	-	238	1	175	18	4988

Some of sea buckthorn leaves compounds have important biological activities like anticancer [90], antibacterial [39, 91], antifungal, antiviral, spasmolytic, hypoglycaemic, antihistaminic and radio-protective potential [19, 90, 92]. Some of these properties derive from the free radical-scavenging activities of flavonoids, that's the reason why they stimulated by the health benefits from their antioxidant [48] .

Sea buckthorn leaves also contain significant amounts of proteins (16 – 23g/ 100 g), amino acids (0.73g/ 100 g lysine, 0.13g/ 100 g methionine & cysteine) threonine, valine, methionine, and phenylalanine.[93]. Protein is one of the most important chemical components in sea buckthorn leaves which can be used as a source of unconventional protein for human food [62]. Sea buckthorn leaves' aqueous extract showed significant wound-healing activity, as assessed by the increase in the hydroxyproline and protein contents [94]. Furthermore, there are significant differences regarding the protein content of sea buckthorn leaves between male and female plants, or drying methods: leaves should be harvested from late July to early August to reach higher protein content [93].

Sea-buckthorn leaves are rich in minerals, but their levels depend on many factors, including genetic characteristics, climate, soil conditions, plant's maturity , and the time of harvesting [95]. The leaves' average mineral content was rich in K (116%), Mg (31%), Ca (94%), and Fe (574%).

Sea buckthorn leaves have very important non-polar and polar bioactive compounds. The leaves' most important compounds are phenolic compounds. An extract of sea buckthorn leaves showed potent antioxidant activity. Antioxidant activity with different methods of sea buckthorn leaves are shown in Table 12.

Table 12. Antioxidant activity with different methods and different extracts methods of sea buckthorn leaves [39, 48, 96]

Sample	**Polyphenols (mg GAE/g)**	**Flavonoids (mg/ CE/g)**	**ABTS (mg TE/g)**	**DPPH (mg TE/g)**	**FRAP (mg TE/g)**
Water extract	76 – 93	14 – 29	38 – 119	86 – 255	93 – 217
Ethanol 70 % v/v extract	28 – 60	47 – 66		194 – 353	219 – 277
Ethanol extract	65		166	175	171
Hexane extract	64			87	16

Antioxidant capacity of sea buckthorn leaves can be evaluated using *in vitr*o methods. Furthermore, sea buckthorn leaves extract can be used for *ex vivo* assay. Assays using living cells have proven to be very useful in the routine testing of various products, being fast, sensitive, reproducible, as well as producing reliable results in terms of the identification of biological and

antioxidant activity. Antioxidant activity *in vitro* and *ex vivo* assays is discussed more in 1.3.2 chapter.

1.5 Isolation of bioactive constituents by conventional and high-pressure extraction techniques

1.5.1 Conventional extraction methods

Bioactive compounds from plant materials can be extracted by various convectional extraction techniques. Most of these techniques are based on the extracting of different solvents in use and the application of heat and mixing. In order to obtain bioactive compounds from plants, the existing classical techniques are: 1) Soxhlet extraction method, 2) Solid liquid extraction method [11].

The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from several natural sources. It is used as a model for the comparison of new extraction technique. First, it was designed for isolate non-polar fraction using n-hexane, but now is using to isolate polar compounds too. Polar constituents extracted typically with acetone, methanol, ethanol, water and hydroethanolic mixtures. Soxhlet extraction method compared with other techniques consume a lot of time for samples preparation and a large amount of solvent is wasted [97].

Solid-liquid extraction (SLE) technique is widely used for the early purification of natural products from plant material. This technique has been used for many decades, but it takes a long extraction time. The common solvent to be used can be the same as the one in soxhlet extraction. The results of Soxhlet extraction with different conditions and parts of sea buckthorn plant are shown in Table 13.

Table 13. Soxhlet extraction methods of sea buckthorn

Plant material	Extraction method	Solvent	Condition	Yield, g/ 100 g	Ref.
Sea buckthorn berries	Soxhlet	n-hexane	4-5 h, 40 -50 °C	32 – 34	[98]
Sea buckthorn berries	Soxhlet	petroleum ether	70°C, 2h	23.92	[99]
Sea buckthorn pulp	Soxhlet	n-hexane	4-5 h, 40 -50 °C	22 - 23	[98]
Sea buckthorn seeds	Soxhlet	n-Hexane	10g of sample, 7h	12.1	[52]
Sea buckthorn leaves	Soxhlet	Ethanol/water 70/30	80°C, 6-10h, 100g of sample	24 – 32	[48]
Sea buckthorn leaves	SLE	Ethanol/water 70/30	25 °C, 24h, 1:5 (w/v)	23.27	[100]

The major challenges of conventional extraction are longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and

thermal decomposition of thermo labile compounds. Finally, these methods are cheap, which has favoured significantly their widespread use particularly both in industries and routine laboratories.

1.5.2 High-pressure extraction methods

Techniques are called high-pressure extraction techniques includes ultrasound assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and pressurized liquid extraction.

The concept of Pressurized liquid extraction (PLE) is the application of high pressure to remain solvent liquid beyond their boiling point. Automation techniques are the main reason for the greater development of PLE-based techniques along with the decreased extraction time and solvents requirement. PLE technique requires small amounts of solvents because of the combination of high pressure and temperatures which provides faster extraction. The higher extraction temperature can promote higher solubility by increasing solubility and mass transfer rate and, also decrease the viscosity and surface tension of solvents [53]. Comparing with conventional Soxhlet extraction and Solid liquid extraction, PLE were found to decrease extraction time consumption and solvent use [11, 53].

A basic SFE system contains the following parts: a tank of mobile phase, usually CO_2, a pump to pressurize the gas, co-solvent vessel and pump, an oven that contains the extraction vessel, a controller to maintain the high pressure inside the system and a trapping vessel. Usually different type of meters like flow meter, dry/wet gas meter could be attached to the system. The successful extraction of bioactive compounds from plant materials rely upon several parameters of SFE and most importantly these parameters are tunable. These parameters need to be precisely controlled for maximizing benefits from this technique. The major variables influencing the extraction efficiency are temperature, pressure, particle size and moisture content of feed material, time of extraction, flow rate of CO_2, and solvent-to-feed-ratio [53, 101, 102]. SFE technique are considered as “green techniques. Green technique include less hazardous chemical synthesis; designing safer chemicals, safe solvents auxiliaries, design for energy efficiency, use of renewable feedstock, reduce derivatives, catalysis, design to prevent degradation, atom economy, and time analysis for pollution prevention and inherently safer chemistry for the prevention of accident [11].

Due to the selectivity of CO_2 for non-polar components, co-solvents like ethanol, water, or mixtures,thereof, need to be added for the extraction of higher polarity compounds, such as polyphenols. This approach offers the possibility of obtaining fractions enriched in specific bioactive compounds [103]. Non-polar constituent samples usually were analyzed for fatty acids,

tocopherols, tocotrienols and carotenoids. Samples of high-pressure extraction methods are shown in Table 14.

Table 14. Different extraction methods and solvents of sea buckthorn pomace and leaves

Plant material	Extraction method	Solvent	Condition	Yield, g/100 g	Ref.
Sea buckthorn berries	SFE – CO_2		34.5 °C, 27.6MPa, 82 min, 17 L/h rate, 300g of sample	20.8	[104]
Sea buckthorn berries	SFE – CO_2		50 °C , 350 bars, 120 min, 0.4 L/min. rate, 500g of sample	20 - 21	[105]
Sea buckthorn seeds	SFE – CO_2		40 °C, 60MPa, 9h, 0.8 g/min rate 7g of sample	10.93	[52]
Sea buckthorn pomace	SFE – CO_2		60°C, 46 MPa, 6-7 h, 800g of sample	15.8	[106]
Sea buckthorn pomace	SFE – CO_2		60°C, 35 MPa, 180 min. 1.8 g/L rate, 150g of sample	14.6	[63]
	PLE	Ethanol	70°C, 10.3 MPa, 15min., 25g of sample	13.5	
Sea buckthorn seeds	SFE – CO_2		60°C, 35 MPa, 180 min. 1.8 g/L rate, 150g of sample	13.5	
	PLE	Ethanol	70°C, 10.3 MPa, 15min., 25g of sample	2.8	
Sea buckthorn leaves	PLE	Ethanol	60°C, 100 bar, 15min, 3g of sample	18-19	[39]
		Ethyl acetate	60°C, 100 bar, 15min, 3g of sample	9.5	

Extraction is very important in the isolation, identification of phenolic compounds. Supercritical fluid extraction are increasingly replacing organic solvents such as n-hexane, chloroform. SFE – CO_2 extraction it is low toxicaly, nonflammability and compability with procssed food stuff. Nowadays,

SFE – CO_2 has become method of choice, that's why its important to know optimal parameters of extraction. A lot of researchers tried to find optimal parameters of sea buckthorn berries. The effectiveness of supercritical carbon dioxide extraction and Soxhlet extraction with hexane was compared.

Pressurized liquid (PLE) extraction to obtain higher value fraction rich in phenolic compounds. Solid liqued extraction is common to use with polar solvents (ethanol, methano, water). However, SLE takes too much time for extraction then PLE, of this reason these days SLE method is not popular to use to isolate polar constituents.

2 MATERIALS AND METHODS

2.1 Plant material

Fresh sea buckthorn pomace and leaves were obtained from the local food factory 'Šliauterio ūkis' (Akmene, Lithuania). Samples were frozen (-18 °C) by the manufacturer directly sea buckthorn pomace after juice processing, transferred in the cooler bags, freeze-dried, ground by an ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.5 mm hole size sieve and kept in tightly closed, dry glass jars, in dark, well-ventilated place prior to the analysis.

2.2 Chemicals

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$, Sigma-Aldrich, Steinheim, Germany), 3,4,5-trihydroxybenzoic acid (gallic acid, 99%, Sigma-Aldrich, Steinheim, Germany), 2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoic acid (Fluorescein (FL), Fluka Analytical, Bornem, Belgium), 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%, Sigma-Aldrich, Steinheim, Germany), Folin & Ciocalteu's phenol reagent ((2M), Fluka Analytical, Bornem, Belgium), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), Na_2CO_3 (Sigma-Aldrich), H_2SO4, NaOH, H_3PO_4, (Sigma-Aldrich), HCl (35-38%, Chempur, Piekary Slaskie, Poland), squalene (99%, Supelco Analytical, Bellefonte, PA, USA), hydrogen peroxide (H_2O_2), caffeic acid ($C_9H_8O_4$), cobalt (II) fluoride tetrahydrate (CoF_2) and reagent used for sodium phosphate buffer solution (SPB), acetonitrile, methanol, dichlormetane, pentane, hexane (HPLC grade, Sigma-Aldrich Chemie, Steinheim, Germany), boron trifluoride (24% methanol solution, Acros organics, Geel, Belgium), microcrystalline cellulose (20 μm, Sigma-Aldrich, St. Louis, MO, USA), catalytic tablet (K_2SO_4, $CuSO_4$, Sigma-Aldrich), ASE filters (Glass Fiber_(X)_Cellulose, Dionex Corporation, Sunnyvale, CA,USA), diatomaceous earth (100 % SiO2, Dionex Corporation, Sunnyvale, CA, USA), cotton-wool (Bella-cotton, Poland), ethanol (96.3%, food grade, Stumbras, Kaunas, Lithuania), nitrogen liquid (AGA SIA, Riga, Latvia), carbon dioxide gases and nitrogen gases (99.9%, Gaschema, Jonava region, Lithuania). All solvents were of analytical and HPLC-grade grade. Chemicals used for cell-based assays were: 2',7'-dichlorofluorescin diacetate (DCFH-DA), quercetin (95 %) from Sigma-Aldrich (St. Quentin Fallavier, France) and EtOH (99 %) from Scharlau (Barcelona, Spain). Caco-2 cell culture media and supplements: namely glutamine, trypsin, RPMI 1640, PS (penicillin streptomycin) and fetal bovine serum (FBS) were

obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK) and PBS used for cells was acquired from Sigma-Aldrich (St. Louis, USA).

2.3 Cell lines and culture

Human Caco-2 cell line was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, Germany). This cell lines were cultured in RPMI-1640 medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and 1% penincilin-streptomycin (PS). The cell was incubated at 37 °C with 5% CO_2 in a humidified incubator and routinely grown as monolayer in 75 cm^2 culture flasks. The cell culture medium and supplements were purchased from Invitrogene (Gibco, Invitrogene Corporation, Paisley, UK). The cell line was split twice a week and the morphology and growth of the cells were monitored daily.

2.4 Determination of the selected chemical composition indices

2.4.1 Moisture content

To the heated, dry, constant weight glass with cap and rod, 5.000±0.002 g of sea buckthorn pomace (particle size 0.5 mm) and 5.000±0.002 g of sea buckthorn leaves (particle size 0.5 mm) were added and dried in the oven at 100-105°C for 3 hours, afterwards placed in a desiccator for 25 minutes and weighted on the analytical balances. The heating-weighting procedure afterwards was repeated every hour until variation between two weighting results was less that 0.005 g. Experiments were performed in duplicate. Moisture content was calculated using the formula below (g/100g).

$$x = \frac{(m_1 - m_2)*100}{m_1 - m}; g/100g \qquad (1)$$

m – glass with cap and rod weight g; m_1 – glass weight with sample before drying g; m_2 – glass weight with sample after drying, g.

2.4.2 Mineral content

3.000 ±0.002 g of sea buckthorn pomace (0.5 mm fraction) and 3.000±0.002 g of sea buckthorn leaves (particle size 0.5 mm) was added to dry, constant weight crucible, heated on electric hotplate and kept in muffle for ~16 hours at 600-650°C, afterwards placed in a desiccator for 25 minutes and weighted on the analytical balances. The heating-weighting procedure was repeated until variation between two weighting results was less that 0.005 g. Experiments were performed in duplicate. Ash (mineral) content, expressed as a percentage, is calculated by the following formula:

$$x = \frac{(m_2 - m)*100}{m_1 - m}; g/100g \qquad (2)$$

m – crucible weight, g; m_1 – crucible weight with sample, g; m_2 – crucible weight with burned sample, g.

2.4.3 Total nitrogen content by Kjeldahl method

To a Kjeldahl flask, 1.000 ±0.002 g of sea buckthorn pomace (0.5 mm fraction), and 1.000±0.002 g of sea buckthorn leaves (particle size 0.5 mm) 20 ml of 98 % conc. H_2SO_4 and catalyst tablet (3.5 g K_2SO_4 and 0.4 g $CuSO_4$) were added. Content was mineralized until solution in the flask became transparent (heating intensity 60%, time – 90 min) and distillated with automatic steam distillation system (3 s NaOH and 3 s H_3BO_4 filing parameters, distillation time 300 min, steam intensity 80%). Distillate was collected in flask, followed with the addition of Tashiro indicator and titrated with 0.01 N HCl until the colour change from light green to grey-violet. Experiments were performed in dublicate. Control sample (water) was prepared and analysed under the above described conditions. The nitrogen content, expressed as a percentage, was calculated using the following formula:

$$x = \frac{0.0014 * A}{m} * 100; g/100g \qquad (3)$$

A – 0.1N HCl amount, used for distillate titration, ml; m – sample weight, g; 0.0014 – nitrogen amount equivalent 1 ml 0.1 N HCl. Total nitrogen amount is calculated by multiplying the amount of nitrogen from the conversion factor 5.7.

2.5 Conventional extraction techniques

2.5.1 Soxhlet extraction

Soxhlet extraction was performed from 5.000±0.002 g of ground sea buckthorn pomace (0.5 mm) and 5.000±0.002 g of ground sea buckthorn leaves (particle size 0.5 mm), inserted into an inner tube (rolled up tightly in filter paper) of the in an automated Soxhlet extractor EZ100H apparatus (Behr Labor-Technik, Düsseldorf, Germany). Non-polar fractions were isolated using hexane (Sox-He), while residues (0.5 mm fraction) were further extracted with acetone (Sox-He-Ac) and ethanol(Sox-He-Ac-EtOH). The rate of extraction was 1 cycle per 5 min, total extraction time – 360 min (6 hours). Organic solvents were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Extract yields were determined gravimetrically (±0.001 g) and expressed as (g/100 g extract) and (g/100 g DW). Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. The solid residue was collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis. Experiments were performed in duplicate.

2.5.2 Solid-liquid extraction (SLE)

SLE was performed in a thermostatically controlled shaker from 5.000±0.002 g of sea buckthorn pomace (0.5 mm fraction) and 5.000±0.002 g of sea buckthorn leaves (particle size 0.5 mm) and 15.000±0.002 g sea buckthorn pomace and leaves residue after SFE-CO_2 (0.5 mm) and 150 mL of hexane or acetone or ethanol (solid: liquid ratio 1:30 w/v) at the following conditions: temperature 60°C (for hexane extraction) (SLE-He-1), 40°C (for acetone extraction) (SLE-He-Ac-1) 60°C (for ethanol extraction) (SLE-He-Ac-EtOH-1), time 360 min, residue after SFE-CO_2 was extracted with 150 mL of acetone or ethanol or water or ethanol/water 70/30 % v/v mixture (solid: liquid ratio 1:30 w/v) at the following conditions: temperature 40°C (for acetone extraction) (SLE-Ac-2), 60°C (for ethanol extraction) (SLE-Ac-EtOH-2), 60°C (for water extraction) (SLE-Ac-EtOH-H_2O-2), 60°C (for ethanol/water mixture extraction) (SLE-EtOH/H_2O-3)(SLE-Ac-EtOH/H_2O-2) time 360 min, 800 rpm, followed by the rapid cooling and centrifugation (9000 rpm, 10 min) and filtration. Organic solvents from the optically clear supernatants were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. SLE-He-1, SLE-He-Ac-1 and SLE-He-Ac-EtOH-1, SLE-Ac-2, SLE-Ac-EtOH-2, SLE-Ac-EtOH-H_2O-2, SLE-Ac-EtOH/H_2O-2, SLE-EtOH/H_2O-3 pomace and leaves extracts yield was determined gravimetrically (±0.001 g) and expressed as (g/100 g extract) and (g/100 g DW). The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis.

2.6 High-pressure extraction techniques

2.6.1 Supercritical CO_2 extraction (SFE-CO_2)

SFE-CO_2 was performed in a supercritical fluid extractor Helix extraction system (Applied Separation, Allentown, PA, USA) by modified procedure of Kraujalis and Venskutonis (2013)[107]. Each extraction was carried out using 90.000 ± 0.002 g of ground sea buckthorn pomace (0.5 mm) and 140.000 ± 0.002 g of ground sea buckthorn leaves (0.5 mm), which was placed in the 70 mm diameter and 95 mm length plastic basket with porous stainless steel filter at the bottom, to avoid particles clogging.The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min at standard state (PCO_2 = 100 kPa, TCO_2 = 20°C, ρCO_2 = 0.0018 g/ml). Extraction conditions were set as follows: extraction pressure 350 - 450 bar, temperature 60ºC, dynamic extraction time 360 min. The static extraction time was 30 min for two experiments (SFE-CO_2-1, SFE-CO_2-2). Additionaly experiment were concducted in

static/dynamic cycles, each interval being 30 minutes for a total 480 min (pomace) and 360 min (leaves). Extracts were collected to an opaque bottle and kept in a freezer until further analysis. Solid residues were kept dry, well-ventilated place prior to the further analysis. All extractions were performed in triplicates.

2.6.2 Pressurised liquid extraction (PLE)

PLE was performed in ASE-350 (Thermo Scientific Dionex, Sunnyvale, CA, USA) apparatus following modified procedure of Kraujalis et al. (2013) [108] from 5.000 ± 0.002 g of ground sea buckthorn pomace (0.5 mm) was mixed with 5.000 ± 0.002 g diatomaceous earth (1/1, w/w) and placed to 66 ml stainless-steel extraction cells, with two cellulose filters in the both ends to avoid particle release to the system. Additionally, in order to compare conventional and high-pressure extraction efficiencies, sea buckthorn pomace material was extracted with hexane under the following conditions: extraction temperature 60°C, time 15 min (5min x 3 cycles) (PLE-He-1), 30 min (10min x 3 cycles) (PLE-He-2), 45min (15min x 3 cycles) (PLE-He-3). The system pressure (103 bar or 10.3 MPa), pre-heating time (5 min, 10 min and 15 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extracts in the vials was kept constant for all PLE experiments. The yields of extracts were determined gravimetrically (±0.001 g) and expressed as g/100g DW, extracts were kept in brown glass bottles in the freezer prior to the analysis. All extractions were performed in duplicate.

2.7 *In vitro* antioxidant activity assessment of extracts and solid residues

For the in vitro antioxidant activity measurements in Folin-Ciocalteu's, $ABTS^{\bullet+}$, ORAC, HOSC, HORAC assays, various extracts after different steps of extraction were dissolved in methanol and further diluted with methanol to a final concentration from 100 µg/mL to 10000 µg/mL. Water-soluble fractions after hydrodistillation were dissolved in methanol to a final concentration from 100 µg/mL to 250 µg/mL.

Antioxidant capacity of starting plant material and solid residues after various steps of extraction was evaluated by QUENCHER method (Gökmen et al., 2009) [109]. As previously described by Kitrytė et al. (2014), stock mixtures were prepared with 0.2 mm fractions and microcrystalline cellulose at a concentration of 500 µg/mg. Final solid dillutions for analysis were prepared at concentrations of 0.5 µg/mg to 10000 µg/mg. All extractions were performed in duplicate.

2.7.1 Total phenolic content (TPC) by Folin-Ciocalteu's assay

Folin-Ciocalteu's assay was carried out by the procedure of Singleton, Orthofer and Lamueal-Raventós (1999), with some modifications. For the analysis, 150µL of sample (100-500µg/mL) or MeOH (blank) were mixed with 750µL Folin-Ciocalteu's reagent (2M), previously diluted with distilled water (1:9, v/v), and after 3 min of reaction, 600µL of Na2CO3 solution (75g/L), left in dark for 2 hours and absorbance was measured at 760 nm with with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, as previously described by Kitrytė et al. (2014) [110], 10 mg of sample (2-20µg/mg) or cellulose (blank) were mixed with 150µL of distilled water, 750µL Folin-Ciocalteu's, 600µL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm in the dark for 2 hours, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm with spectrometer. Gallic acid solutions (150µL) at various concentrations (0-80µg/mL) were used for calibration. The TPC of extracts and solid samples was expressed as gallic acid equivalents (mg GAE/g sample and mg GAE/ g DW) by means of dose-response curves for gallic acid. All sample dilutions and the blank, were analyzed at least in triplicates.

2.7.2 The $ABTS^{\bullet+}$ scavenging assay

The $ABTS^{\bullet+}$ assay was carried out by the method of Re at al. (1999) [83] with slight modifications. Firstly, phosphate buffered saline (PBS) solution (75 mmol/L; pH 7.4) was preperad by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of distilled water. The $ABTS^{\bullet+}$ solution was prepared by mixing 50 mL of $ABTS^{\bullet+}$ (2 mmol/L PBS) with 200 µL $K_2S_2O_8$ (70 mmol/L) and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. The working solution was prepared by diluting the $ABTS^{\bullet+}$ solution with PBS to obtain the absorbance of AU 0.700±0.010 at 734 nm. For the QUENCHER procedure, 10 mg of sample (2-5 µg/mg) or cellulose (blank) were mixed with 25 µL of MeOH and 1500 µL of working $ABTS^{\bullet+}$ radical solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 734 nm. Trolox solutions (25 µL) at various concentrations (0-1500 µmol/L MeOH) were used for calibration. ABTS of extracts and solid samples were calculated by means of dose-response curves for Trolox.

2.7.3 Oxygen radical absorbance capacity (ORAC) assay

ORAC of the samples was evaluated as described by Huang et al. (2002) modified for the FLx800 microplate fluorescent reader as described by Feliciano et al. (2009) [111]. In the 96-well black opaque microplates, 25 µL sample (0.6-50 µg/mg) was mixed with 150 µL of fluorescein solution

($3x10^{-4}$ mM) were added to each well. The microplate was put in an FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA) at 37°C, for 10 minutes. The reaction took place at the same temperature and was started with 25 μL of AAPH (153 mM), which was added through the injector linked to the reader to each well. The fluorescence emitted by the reduced form of FL was recorded every 1 minute at the emission wavelength of 530±25 nm and excitation wavelength of 485±20 nm, for a period of 40 min, controlled by software Gen5. Phosphate buffer saline (PBS), 75 mM, pH=7.4, was used to prepare the solutions of AAPH and FL and then used as a blank. Solutions of (5-40) μM of Trolox were prepared using the same PBS solution, and were used as control standards. All sample dilutions, the blank and Trolox concentrations, were analyzed at least in triplicates.

The ORAC values were calculated by a linear regression equation between the Trolox concentration and the net area under the FL decay curve (AUC), taking into account that the results of antioxidant capacity depend on sample concentration (Bolling et al., 2012). These results were expressed as (mg TE/g of extract) and (mg TE/g DW).

2.7.4 Hydroxyl Radical Adverting Capacity (HORAC)

Hydroxyl Radical Adverting Capacity assay was based on the method described by Ou et al. (2002) [112], with slight modifications, and adapted for the FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA), as described by Serra (2010). The hydroxyl radical was generated by a Co (II)-mediated Fenton-like reaction and, as in the ORAC assay, the fluorescence decay curve of FL was used to quantify the HORAC value. In a black 96-well microplate, 30 μL of appropriate sample (12-500 μg/mg) dilutions were added to 170 μL of FL ($9.28x10^{-8}$ M). Then, 40 μL of hydrogen peroxide (H_2O_2), 0.20 M, were added to each well of the microplate. Finally, the reaction was started by adding 60 μL of cobalt (II) fluoride (CoF_2), 1.15 mM, to the mixture previously placed in the microplate. Sodium phosphate buffer (SPB), 75 mM, pH=7.4, was used to prepare the solution of FL, H_2O_2 and CoF_2 were prepared with Milli-Q water. Caffeic acid was used as a standard, and (50-250) μM solutions in acetone:Milli-Q water (50:50 v/v) were used to create the calibration curve. Acetone:Milli-Q water (50:50 v/v) solution was used to prepare the samples and as a blank. The fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute during 60 minutes, at 37°C. The FLx800 fluorescence microplate reader was controlled by software Gen5 and was used with fluorescence filters for an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm. All samples were analyzed in triplicates.

The HORAC values were calculated based on the caffeic acid calibration curve and on the average of the net area under the FL decay curves (AUC), which presented a linear profile. The final results were expressed as (mg CAE/g of extract) and (mg CAE/g DW).

2.7.5 Hydroxyl Radical Scavenging Capacity (HOSC)

Hydroxyl Radical Scavenging Capacity assay was based on the method described by Moore et al. (2006) [113] and adapted for the FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA), as described by Serra et al. (2013). The hydroxyl radical was generated using a Fenton-like Fe (III)/ H_2O_2 reaction and, as in the ORAC and HORAC assays, the fluorescence decay curve of FL was used to quantify the HOSC value. In a black 96-well microplate, 30 μL of appropriate sample (0.6--50 μg/mg) dilutions, followed by 40 μL of H_2O_2 (0.20 M), were added to 170 μL of FL ($9.28x10^{-8}$ M). The reaction was started by adding 60 μL of iron (III) chloride ($FeCl_3$), 3.43 mM, to the wells of the microplate. SPB, 75 mM, pH=7.4, was used to prepare the solution of FL, and the solutions of H_2O_2 and $FeCl_3$ were prepared with Milli-Q water. Trolox was used as a standard, and (5-30) μM solutions in acetone:Milli-Q water (50:50 v/v) were used to perform the calibration curve. Acetone:Milli-Q water (50:50 v/v) solution was used to prepare the samples and as a blank. The fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute, during 60 minutes, at 37°C. The FLx800 fluorescence microplate reader was controlled by software Gen5 and was used with fluorescence filters for an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm. All samples were analyzed in duplicates, and the blank and the controls in triplicates.

The HOSC values were calculated by a linear regression equation between the Trolox concentration and the net area under the FL decay curve (AUC), taking in to account that the results of antioxidant capacity depend on sample concentration (Bolling et al., 2012). These results were expressed as (mg TE/g of extract) and (mg TE/g DW).

2.7.6 Measurement of oxidation induction period by Oxipres

The effect of *sea buckthorn* pomace and leaves extracts on the oxidative stability of commercial rapeseed oil was tested by instrumental Oxipres method (Trojáková et al., 1999; Basegmez et al. (2017) [114]. The samples were prepared by mixing rapeseed oil with 0.5%, 1%, 5% SFE-CO_2-2, (as control sample was used pure rapeseed oil). 5±0.002 g of prepared (or control) sample were placed in a reactor tube and thermostated at 110°C under oxygen atmosphere at 5 bar in Oxipres apparatus (Mikrolab, Aarhus, Denmark), which measures pressure changes due to the absorption of oxygen consumed for oil oxidation. The induction period (IP) was calculated as the

time after which the pressure began to decrease abruptly (its end was measured from the cross-section point of tangents of the first part and the subsequent part of the curve recording the pressure changes). Each measurement was done in duplicate.

2.8 *Ex vivo* cytotoxicity Assay

Cytotoxicity assays of the extracts were performed using confluent and non-differentiated Caco-2 cells. Caco-2 cells share some characteristics with crypt enterocytes, healthy cells that can be found in human small intestine and colon. This assay was performed as described by Serra (2010) [115], with some modifications. The cells were seeded into 96-well culture plates at a cellular density of 2×10^4 cell/well and were allowed to grow for 7 days until reaching confluency. The medium was changed every 2 days. Polar extracts samples were diluted in RPMI-1640 culture medium with 0.5% FBS and then added to the wells, except to the control cells which contained the culture medium alone. Non – polar extracts were dissolved in ethanol (stock solution) and then diluted in medium. The incubation carried out during 1 hours and the experiments were done in triplicates. After incubation the medium was removed, cells washed one time with warm PBS and 100 µL of the colorimetric reagent Cell Titter® aqueous one solution cell proliferation assay (MTS) according with manufacturer protocol. Viability was quantified by of the absorbance at 490 nm in a microplate reader Spectrophotometer Powerwave XS (BioTek Instruments, Winooski, VT, USA). The results were expressed as percentage (%) of cell viability relative to the control.

2.9 *Ex vivo* Cellular Antioxidant Activity (CAA)

In order to evaluate the cellular antioxidant activity of the sea buckthorn pomace and sea buckthorn leaves extracts, Caco-2 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and the medium was changed every 48 h. The experiments were performed using completely confluents cells (4 days of culture). Intracellular antioxidant activity of the different grape pomace extracts was evaluated following the formation of reactive oxygen species in Caco-2 cells after treatment with a chemical stress inducer (AAPH). The formation of intracellular reactive oxygen species was monitored using the fluorescent probe, DCFH-DA, as described by Wang and Joseph (1999) [116] and Serra et al. (2010) [115]. Briefly, confluent Caco-2 cells were washed with PBS twice. Then, cells were exposed with different concentrations of the different extracts dissolved in PBS (50 µL/well) and with 50 µM DCFH-DA (50 µL/well), to be later incubated during 1 hour at 37°C in a 5% CO_2 humidified atmosphere. Quercetin was used as standard. After the incubation time, the medium was removed and the APPH solution with a concentration of 0.6 mM was added (100

μL/well). Fluorescence was measured for each sample between 0 and 60 min in FLx800 BioTek fluorescence microplate reader. Cellular antioxidant activity of the extracts was quantified according to Wolfe and Liu (2007) [84] and expressed as (μmol QE/g of extract) and (mg QE/g DW). At least three experiments were performed in triplicate.

2.10 Phytohemical characterization of extracts

2.10.1 Fatty acid composition analysis by gas chromatography (GC-FID)

Fatty acid composition analysis was performed by the procedure of Moreda et al. (2001) [117]. For triglycerides esterification and free acids saponification, 0.500 ± 0.002 g extract (SFE-CO_2, Soxhlet-He, SLE-He, PLE-He-1) and 4 ml of methanolic NaOH (0.5 N) was poured into 50 ml round-bottomed flask and heated with condenser until disappearance of the fatty phase (5-10 min). After esterification, over the top of condenser 5 ml of 24% boron trifluoride/methanol complex was poured and boiled for 2 min., then cooled to room temperature. The sample was diluted with 5 ml n-hexane and the same amount of NaCl was added, well-shaken and left still until layers separated. The top hexane phase was collected with a Pasteur pipette and stored at 4ºC until analysis. For analysis, 100 μl of hexane phase was diluted with 900 μl pure GC-grade hexane. Analysis was carried out with gas chromatograph HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) using a flame ionization detector with a pole SPTM-2560 column (100 m long, 0,25 mm internal diameter the adsorbent layer of 0,20 μm (Supelco, Bellefonte, PA, USA). Oven temperature was programmed from 80°C to 240°C and increasing every 4°C/min. Injector temperature – 220°C and detector – 240°C. Injected amount of sample – 1μl. For compounds identification, a mixture of 37 fatty acids (SupelcoTM) were used as standards. Fatty acid methyl esters were identified by the retention time and the percentage of fatty acid composition was calculated comparing peak areas to the corresponding reference compounds.

2.10.2 Phytochemical characterization by UPLC/ESI-QTOF-MS

Phytochemical composition of sea buckthorn pomace and leaves Soxhlet extracts (Sox-He-1, Sox-He-Ac-EtOH-1), SFE-CO_2-2 extract at (45PMa, 60 °C, 360 min., 2.0 SL/min flow rate) parameters, SLE extracts (mechanical shaking with SLE-Ac-2, SLE-Ac-EtOH-2 SLE-Ac-EtOH-H_2O-2, SLE-EtOH/H_2O-3), were screened on an Acquity UPLC system (Waters, Milford, USA) equipped with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germant), binary solvent delivery system, an autosampler with a 10 μL sample loop, column manager, photodiode array (PDA) detector and an Acquity BEH C18 column (1.7 m. 50 x 2.1 mm, i.d.), as

previously described by Kraujalyte et al. (2013) [118] with following modifications. The mobile phase initially consisted of eluent A (0.4 v/v formic acid in ultra-pure water), followed by an increase from 0% to 100% of eluent B (acetonitrile) over 9 min. During the following 2 min, the amount of eluent B was maintained at 100 %, then in 1 min, column was equilibrated initial conditions, were re-introduced for 1 min. Separation of compounds was performed at 25°C; the column was equilibrated for 2 min before each run; the flow rate was 0.4 mL/min; extract concentrations 1 mg/ml; injection volume 1 µL. The effluent (monitored at 254 nm) from the PDA detector was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source. MS data was recorded in two runs in ESI negative and positive ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar. For the instrument control and data acquisition, the Compass 1.3 (HYStar 3.2 SR2) software was used. Preliminary peak identification was carried out by comparing accurate masses of compounds with hose reported in literature sources and free chemical databases (Metlin, Chempspider).

2.11 Statistical analysis

Mean values and standard deviations were calculated using MS Excel 2016. One-way analysis of the variance (ANOVA), followed by the Tukey's posthoc test to compare the means that showed significant variation ($p < 0.05$), were performed and calculated using GraphPad Prism 6.01 software (2012).

3 RESULTS AND DISCUSSIONS

3.1 Structure of the research work from crude plant material and residue after SFE-CO_2 from sea buckthorn pomace and leaves

Food processing wastes as pomace have long been discuss as a matter of treatment minimization and prevention due to their environmental influence. Berries pomace after juice or oil pressing still are source of bioactive compounds. Therefore, this research work from sea buckthorn pomace and leaves are very important. Research work from crude plant material consist of six main parts. The principal schemes are presented in Figure 5, Figure 6.

1. Chemical composition of sea buckthorn and leaves
2. Convectional (traditional) extraction methods (Soxhlet extraction method, SLE method), high-pressure extraction methods (PLE method, SFE-CO_2 method);
3. Assays used to identify antioxidant activity (TPC, $ABTS^{\bullet+}$, QUENCHER method, ORAC, HOSC, HORAC);
4. Assays used to evaluate chemical composition (UPLC/ESI-QTOF-MS) and identify fatty acids composition (GC-FID)
5. Ex vivo cytotoxicity assay of selected sea buckthorn pomace and leaves extracts
6. Ex vivo cellular antioxidant activity (CAA) assay of selected sea buckthorn pomace and leaves extracts

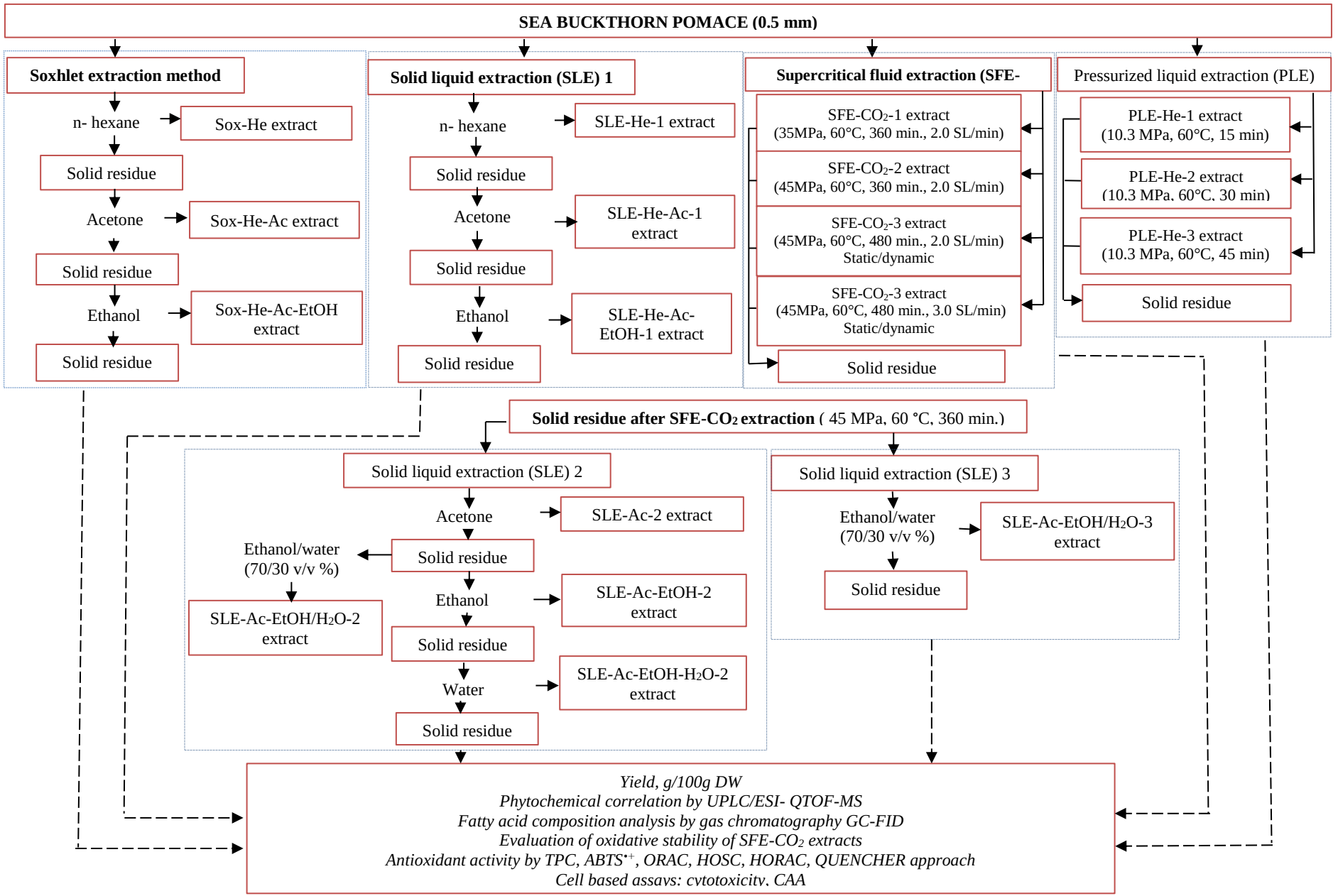


Figure 5. Scheme of sea buckthorn pomace research work

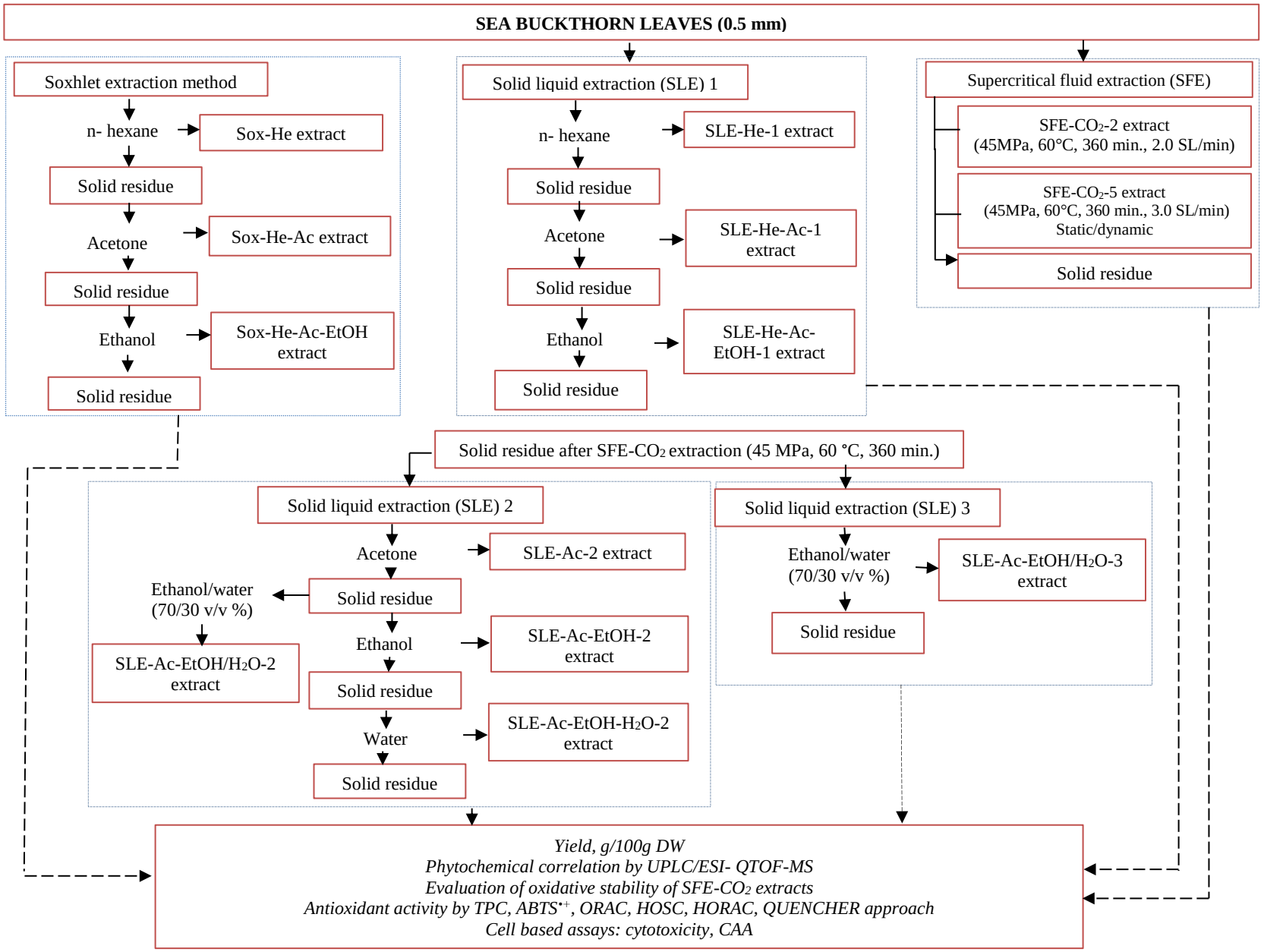


Figure 6. Scheme of sea buckthorn leaves research work

3.2 Chemical composition of sea buckthorn pomace and leaves

The graphs summarise the chemical composition of sea buckthorn pomace and leaves. The results of chemical analysis showed amount of sea buckthorn pomace dry mass (94 g/100g), fat content (23.43 g/100g), total nitrogen content (11.91 g/100g) and mineral substances (2.52 g/100g). The result obtained of sea buckthorn leaves showed amount of dry mass (88.68 g/100g), total nitrogen content (17.34 g/100g), mineral substance (4.54 g/100g). Chemical composition of sea buckthorn pomace and leaves are presented in Figure 7.

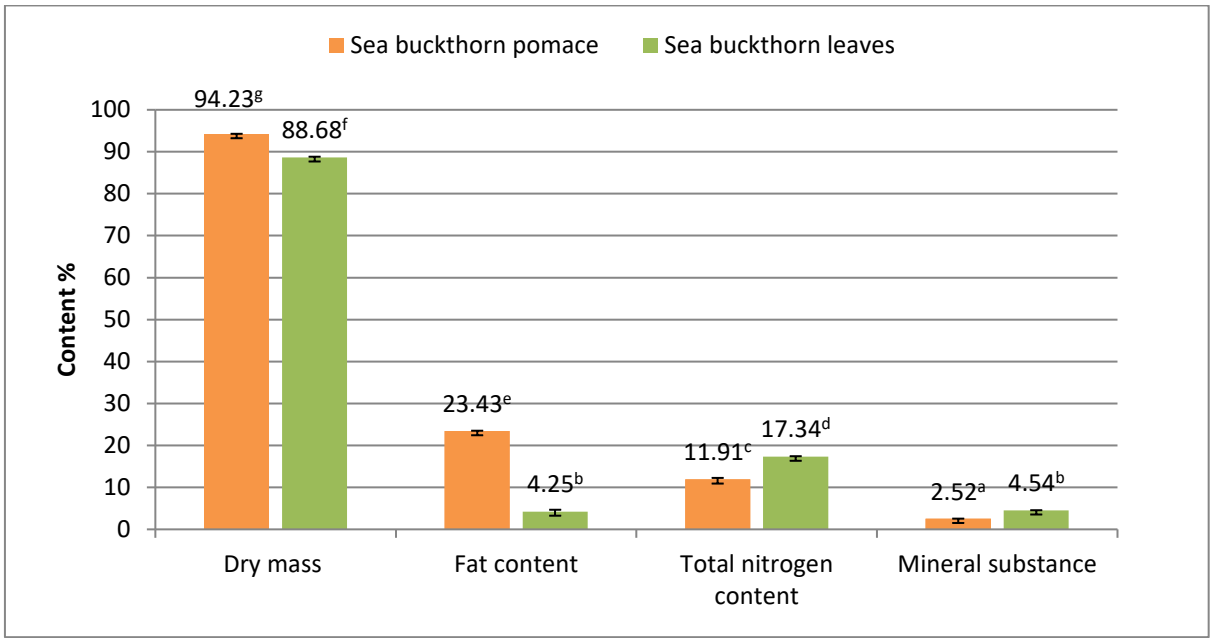


Figure 7. Chemical composition of sea buckthorn pomace and leaves

The results for chemical composition show that sea buckthorn pomace and leaves significant difference between dry mass, fat content, total nitrogen content and mineral substance. Sea buckthorn pomace presented 6% higher amount of dry mass, also high amount of fat content, 82 % higher than leaves fat content. Sea buckthorn leaves are rich of total nitrogen content, 31 % higher than it was obtained in sea buckthorn pomace. Mineral substance was 2 times less in sea buckthorn leaves. Comparing obtained results with the data reported in literature sources, similar results of sea buckthorn leaves were previously reported by Gradt et al., (2017) it was obtained that fat content is 2.87 – 4.41 %, protein concentration is 20.97 -24.03 % [119].

3.3 Comparison of conventional and high – pressure extracts efficiencies for non – polar and polar content isolation

In the present study sea buckthorn pomace and leaves were extracted applying high pressure extraction techniques, namely supercritical fluid extraction with CO_2 (SFE-CO_2) and pressurized liquid extraction (PLE) using n-hexane, and convectional (traditional) extraction methods namely, Soxhlet and solid-liquid extraction (SLE) method, using three solvents of increasing polarity (n-hexane, acetone, ethanol). Yields of different fractions obtained were expressed as grams per 100 gram of solid sample prior to the extraction and further recalculated as grams per 100 gram of DW starting (crude, unextracted plant material). The obtained results are presented in Table 15.

Hexane is a commonly used solvent to isolate lipid-soluble constituents from various matrixes phenolic compounds. Generally, it may be observed that the differences between the yields obtained with convectional and high-pressure extractions methods were not significant in the case of non-polar fraction isolation from sea buckthorn pomace, amounting 19.00 – 23.44 g/100g DW. Although sea buckthorn pomace yield of n-hexane fraction was found to be the highest with Soxhlet extraction (23.44 g/100g DW), however the extraction time was 24 times shorter with PLE-He (15 min. versus 360 min) (20.76 g/100 g) extraction then in other extraction methods. It may be observed that the difference between the yields obtained at the tested extraction parameters of SFE-CO_2 experiments were not remarkable (19.72 – 21.68 g/100g DW) (Table 14). However, using environment-friendly and food-grade solvent CO_2, as compared to hexane-extraction based techniques, and does not require solvent removal after extraction (according to Directive 2009/32/EC, maximum hexane residue limits in the extracted foodstuff can be 1mg/kg). The variable with effect on oil yield was extraction pressure, extraction flow rate and extraction time. SFE-CO_2 extraction yield with 35MPa was 6% lower when it was tested with 45 MPa and the same temperature, time and CO_2 flow rate. Sea buckthorn pomace yield was slightly higher when SFE-CO_2 was conducted in static/dynamic (30/30 min.) cycle mode (45MPa, 60℃, 480 min, 3.0 SL/min flow rate), yield was slightly lower changing flow rate, but SFE-CO_2 also was conducted in static/dynamic (30/30 min.) cycle mode (45MPa, 60℃, 480 min, 2.0 SL/min flow rate). The effect of extraction time was checked by determining the kinetics of the SFE-CO_2 extraction was conducted in static/dynamic (30/30 min.) cycle mode (SFE-CO_2-3, SFE-CO_2-4). The obtained data are presented in Figure 8, Figure 9, respectively. SFE-CO_2-3 (45MPa, 60 ℃, 480 min, 2.0 SL/min flow rate) extraction up to the half portion (57 %) of non-polar extract was obtained after 180 min of extraction, the major portion (87 %) of extract was obtained after 360 min, while addition 120 min contributed to remaining 13 % on non-polar extract. The similar tendency was with SFE-CO_2-4 (45MPa, 60 ℃, 480 min, 3.0 SL/min flow rate) extraction. The half of portion (54 %) of non-polar extract was

obtained after 180 min of extraction, the major portion (90 %) of extract was obtained after 360 min, while addition 120 min contributed to remaining 10 % on non-polar extract.

These findings suggest that supercritical fluid extraction with CO_2 conducted in static/dynamic (30/30 min.) cycle mode can offer low CO_2 consumption than SFE-CO_2 extraction using dynamic mode for all (360 min.) extraction. The static interval allowes the sea buckthorn pomace and leaves to soak to that the CO_2 can penetrate the matric and extract the oil. During the dynamic interval, CO_2 carrying sea buckthorn pomace and leaves extracts flowed out of the unit and into a pre-weighed collection flask, where the CO_2 was vented to a fume hood [120]. Based on these results, it is important to know CO_2 consumption[1] of each different SFE-CO_2 extraction. For example, CO_2 consumption of SFE-CO_2-1 and SFE-CO_2-2 extractions consumed the same amount of CO_2 (1.33) by for 360 min., extractions. Looking at the calculation of static/dynamic mode, it was obtained that SFE-CO_2-3 static/dynamic (45MPa, 60 ℃, 480 min, 2.0 SL/min flow rate) extraction used 1.5 times less CO_2 than SFE-CO_2-4 static/dynamic (45MPa, 60 ℃, 480 min, 3.0 SL/min flow rate) extraction and 1.5 times less CO_2 than SFE-CO_2-1 and SFE-CO_2-2. The kinetic results of the SFE-CO_2 static/dynamic extractions obtained that the major portion (90%) of extract was obtained after 360 min (180 dynamic min). Considering CO_2 consumption, it is clearly indicated that SFE-CO_2-3 static/dynamic (45MPa, 60 ℃, 480 min, 2.0 SL/min flow rate) extraction after 180 min consumed 2 times less CO_2 consumption than after 240 min, and SFE-CO_2-4 static/dynamic (45MPa, 60 ℃, 480 min, 3.0 SL/min flow rate) extraction after 180 min consumed 1.3 times less CO_2 consumption than after 240 min dynamic of extraction.

The analysis for lipophilic fraction yield of sea buckthorn leaves showed that there is a significant difference between hexane and CO_2-facilitated extraction methods, resulting of lipophilic fraction variations in the range of 2.22 – 4.25 g/100g DW. The highest yield of the non-polar fraction of sea buckthorn leaves were found with Soxhlet extraction method (4.25 g/100g DW), which is 19 % higher than with SLE-1 method. The variable with effect on oil yield was extraction pressure, extraction flow rate doing cycle (changing static and dynamic times). Two types of SFE-CO_2 extraction gave similar results (2.22 – 2.43 g/100g DW) (Table 14). The effect of extraction time was checked by determining the kinetics of the SFE-CO_2 extraction doing cycle (SFE-CO_2-5) (Figure 10). SFE-CO_2-5 static/dynamic cycle mode (45MPa, 60 ℃, 480 min, 3.0 SL/min flow rate) extraction up to the half portion (67 %) of non-polar extract was obtained after 180 min of extraction, the major portion (86 %) of extract was obtained after 240 min, while addition 120 min contributed to remaining 14 % on non-polar extract. The

[1] ρ_{CO2}=1.842 kg/m^3 (at normal temperature and pressure 20ºC, 1 atm)
For example, Flow rate: 2SL/min; t=360 min; 90 g plant material

1) Flow rate × density=2*1.842=3.684×10^{-3} kg/min
2) 360 × 3.684×10^{-3}= 1.33 kg (CO_2)
3) CO_2/plant material=1.33/0.09=14.8

results of this study have clearly indicated that CO_2 consumption of SFE-CO_2-5 static/dynamic cycle mode (45MPa, 60 ℃, 480 min, 2.0 SL/min flow rate) extraction after 120 min consumed 2 times less CO_2 consumption than after 180 min (0.99).

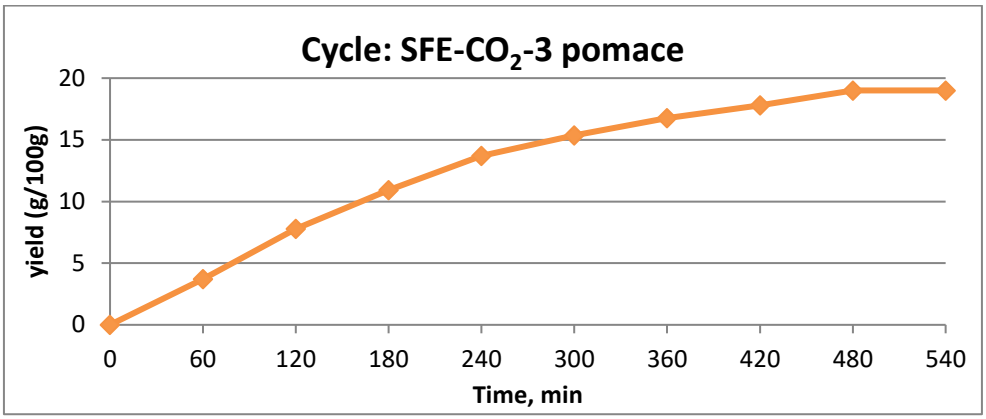


Figure 8. Sea buckthorn pomace SFE-CO_2-3 kinetic at (45 MPa 60 ℃, 480 min, 2.0 SL/min flow rate) parameters

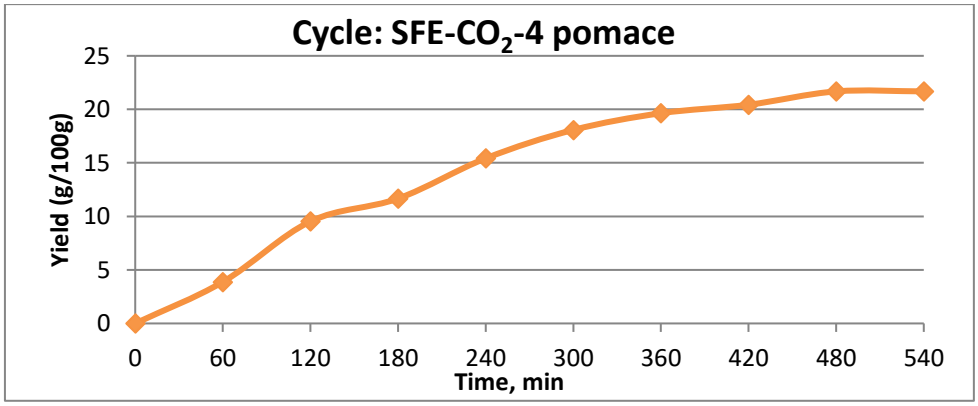


Figure 9. Sea buckthorn pomace SFE-CO_2-4 kinetic at (45 MPa 60 ℃, 480 min, 3.0 SL/min flow rate) parameters

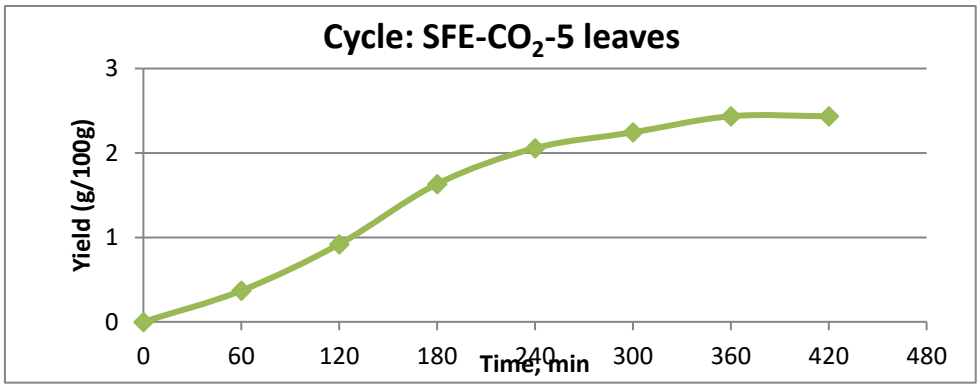


Figure 10. Sea buckthorn leaves SFE-CO_2-5 kinetic at (45 MPa 60 ℃, 480 min, 3.0 SL/min flow rate) parameters

Table 15. Non-polar and polar extracts of sea buckthorn pomace and leaves

Extraction method	Particle size, mm	Extraction parameters						Yield			
								Pomace		Leaves	
		Pressure, MPa	Temp.,° C	Time, min	Static	Dynamic	Flow rate, SL/min	g/100g extract	g/100g DW	g/100g extract	g/100g DW
High pressure extraction techniques											
SFE-CO_2 (from starting plant material)											
SFE-CO_2-1	0.5	35	60	360	-	-	2.0		19.72 ± 2.97^{c}		
SFE-CO_2-2	0.5	45	60	360	-	-	2.0		20.84 ± 1.40^{cd}		2.22 ± 0.03^{a}
SFE-CO_2-3	0.5	45	60	420	30×8	30×8	2.0		19.00 ± 1.18^{c}		
SFE-CO_2 -4	0.5	45	60	420	30×8	30×8	3.0		21.68 ± 0.81^{cd}		
SFE-CO_2 -5	0.5	45	60	360	30×6	30×6	3.0				2.43 ± 0.09^{ab}
PLE-He (from starting plant material)											
PLE-He-1	0.5	10.3	60	15					20.76 ± 0.10^{cd}		
PLE-He-2	0.5	10.3	60	30					20.68 ±0.06^{cd}		
PLE-He-3	0.5	10.3	60	45					20.44 ±0.38^{cd}		
Convectional extraction techniques											
Sequential Soxhlet extraction method (from starting plant material)											
Sox-He	0.5	0.1	70	360					23.44 ± 0.09^{d}		4.25 ± 0.07^{b}
Sox-He-Ac	0.5	0.1	60	360				7.70 ± 0.02^{c}	6.05 ± 0.24^{ab}	3.22 ± 0.15^{a}	2.92 ± 0.05^{ab}
Sox-He-Ac-EtOH	0.5	0.1	80	360				4.39 ± 0.05^{a}	3.19 ± 0.08^{a}	15.13 ± 0.11^{bc}	13.29 ± 0.04^{d}
Convectional extraction from SLE method (from starting plant material)											
SLE-He-1	0.5	0.1	60	360					21.54 ± 0.68^{cd}		3.45 ± 0.01^{ab}
SLE-He-Ac-1	0.5	0.1	40	360				10.91 ± 0.16^{e}	7.97 ± 0.12a^{b}	3.19 ± 0.09 ^{a}	2.81 ± 0.11^{ab}
SLE-He-Ac-EtOH-1	0.5	0.1	60	360				6.10 ± 0.21^{b}	3.70 ± 0.08^{a}	14.42 ± 0.28^{b}	11.32 ± 0.89^{cd}
SLE extraction from residue after SFE-CO_2 (45 MPa, 60 °C , 360 min 2.0 g/L rate)											
SLE-Ac-2	0.5	0.1	40	360				8.75 ± 0.36^{d}	7.06 ± 0.30^{b}	3.90 ± 0.9^{a}	3.11 ± 0.06^{ab}
SLE-Ac-EtOH-2	0.5	0.1	60	360				4.84 ± 0.16^{a}	3.47 ± 0.11^{a}	13.88 ± 0.17^{b}	10.39 ± 0.30^{c}
SLE-Ac-EtOH-H_2O-2	0.5	0.1	60	360				4.13 ± 0.20^{a}	2.93 ± 0.16^{a}	16.30 ± 1.41^{bc}	10.71 ± 0.99^{c}
SLE-Ac-EtOH/H_2O-2 (70/30 v/v %)	0.5	0.1	60	360				5.65 ± 0.42^{b}	4.01 ± 0.20^{a}	17.30 ± 0.34^{c}	16.71 ± 0.30^{e}
SLE-EtOH/H_2O-3 (70/30 v/v %)	0.5	0.1	60	360				11.56 ± 0.39^{e}	8.95 ± 0.41^{b}	24.93 ± 1.38^{d}	19.73 ± 1.10^{f}

Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$)

Since most of the studies on sea buckthorn leaves are focused on polar fraction isolation utilizing ethanol and/or hydroethanolic mixtures [48, 85], crude (unextracted) sea buckthorn pomace and leaves as well as residues after SFE-CO_2 were additionally treated with polar solvents employing two convectional extractions (Soxhlet and SLE). Acetone fraction yield was higher in sea buckthorn pomace (7.97 g/100g DW) with SLE-Ac-1 method than Soxhlet extraction. Soxhlet extraction and SLE-Ac-2 extraction for yields of acetone fraction did not show significant difference. Sea buckthorn pomace gave low amounts of soluble constituents after ethanol (3.19 – 3.70 g/100g DW) and water (2.93 g/100g DW) fractionation with no significant difference between extraction methods. As it is reported in Table 15, slightly higher amounts of polar constituents were isolated treating pomace residue after SFE-CO_2-2 with ethanol/water mixture (4.01-8.95 g/100g DW).

Looking at the results of another tested sea buckthorn by-product, low yield of acetone fraction was obtained from leaves (2.81 – 3.11 g/100g DW) with no significant differences between extractions methods tested. Meanwhile, ethanol and hydroethanolic mixture were the most effective solvents of sea buckthorn leaves fractionation, yielding 10.39 – 19.73 g/100g DW. The highest yield was obtained extracting residue after SFE-CO_2 with ethanol/water mixture (16.71 – 19.73 g/100g DW) under SLE-3 conditions (direct extraction of pomace avoiding the acetone extraction step). This yield was 15 % higher as compared to the SLE extraction scheme, which includes sample treatment with acetone prior to the fractionation with hydroethanolic mixture.

The total amount of sequential convectional (Soxhlet extraction, SLE methods) and SFE-CO_2 + SLE extractions with different polarity solvents (acetone, ethanol, ethanol/water 70/30 % v/v mixture and water) enable to isolate up to 72 % of lipophilic fraction and up to 38 % of polar fraction of sea buckthorn pomace (Figure 11). It may be observed that the difference between the total amount of non-polar and polar fractions yields obtained with different sequential extractions (29.79 – 34.3 g). The most effective sequential extraction is obtained in scheme-3 (34.3 g) from which 61 % of lipophilic fraction and 39% polar fraction. However, extraction in scheme-5 showed 13 % lower total yield from which 70 % lipophilic fraction and 30 % polar fraction, which is similar with sequential extraction in scheme-1 and extraction in scheme-4. It can be concluded that extraction in scheme-2, scheme-3, scheme-4 have higher polar fraction, because 18-23 % of polar fraction absorb acetone fraction.

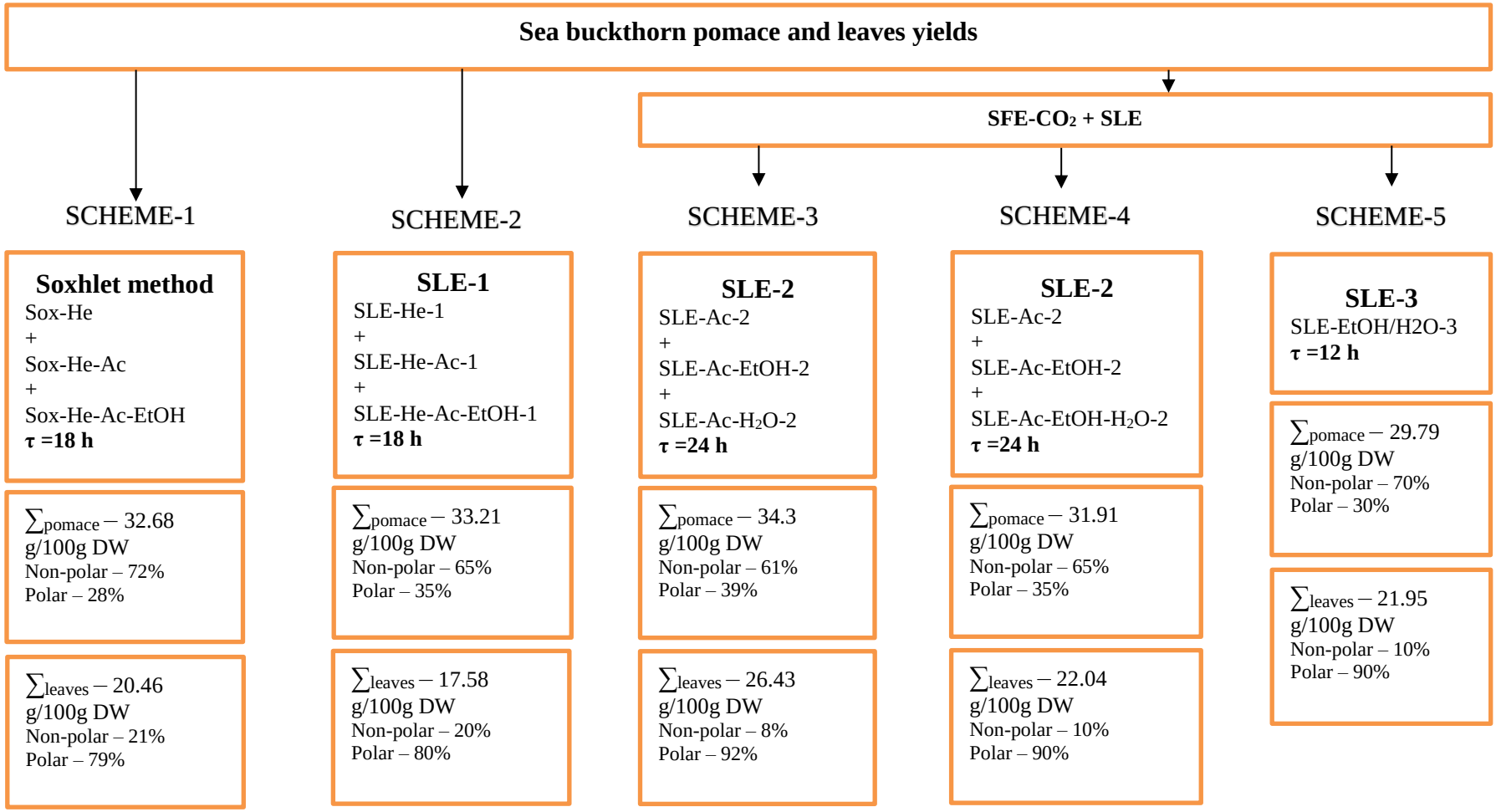


Figure 11. Sea buckthorn pomace and leaves different extractions influence of total extractable constituent

The total amount of sequential convectional (Soxhlet extraction, SLE methods) (scheme-1, scheme-2, respectively) and scheme-3, scheme-4, scheme-5 extractions with different polarity solvents (acetone, ethanol, ethanol/water 70/30 % v/v mixture and water) enable to isolate up to 21 % of lipophilic fraction and up to 92 % of polar fraction of sea buckthorn leaves (Figure 12). It can be seen, the difference between the total amount of non-polar and polar fractions yields extracted with different block sequential extractions (17.58 – 26.43 g). The highest yield was obtained with sequential extraction presented in scheme-3 (26.43 g) from which is 8 % of lipophilic fraction and 92 % polar fraction. However, extraction in scheme-2 showed 33 % lower yield than extraction in scheme-3 from which is 20 % of lipophilic fraction and 80 % of polar fraction. Total amounts of extraction which is presented in scheme-1, scheme-4, scheme-5 showed similar yield. It is highly probable that obtained results in sheme-3, scheme-4 and scheme-5 extractions have higher polar fraction, because 39 % absorb ethanol and 40 % absorb water, 75 % absorb ethanol/water mixture and 90% absorb ethanol/water mixture of polar fraction, respectively. The effect on total extraction time showed that extraction in scheme-3 takes 6 hours longer extraction time than extraction in scheme-1 or scheme-2, or scheme-4, and 12 hours longer than extraction in scheme-5. It can be concluded that the best extraction way including extraction yield is shown in scheme-5 and including extraction time is shown in scheme-3.

Comparing obtained results with the data reported in literature sources, similar results of sea buckthorn berries were previously showed by Mironov et al. (1980),the yield of sea buckthorn oil amounted 22-23 %, as achieved in Soxhlet extraction using n-hexane as solvent [98]. In the other studies, Soxhlet extraction of sea buckthorn berries with petroleum ether yielded 23.92 g/100g of lipophilic constituents [99]. Sajfrtová et al. (2010) reported that sea buckthorn seeds oil yield using Soxhlet extraction with n-hexane was 12.1 g/100g [52]. Similar data were also reported by Xu et al. (2008): SFE-CO_2 of whole sea buckthorn berries resulted in 20.8 g/100g of CO_2-soluble fraction [104]. V. Kitryte et al. (2017) indicated that sea buckthorn pomace and seeds yield using SFE-CO_2 were 14.6 g/100g and 13.5 g/100g, respectively [63]. Thus, comparing the data of non-polar fraction yields, results of our study correspond well with those reported in literature. Sea buckthorn pomace residue after SFE-CO_2 was extracted with PLE-EtOH, the yield was 13.4 g/100g [63], which is higher than it was found in this study. Differences in polar fraction amounts could be partially ascribed to the different nature and composition of pomaces tested.

Concerning another sea buckthorn by-product, similar yield results were reported by Kumar et al. (2011) for leaves, extracted by Soxhlet extraction at extraction temp., of 30 °C, 50 °C and 80 °C. The yield of leaves ethanol/water (70/30 % v/v) extracts under different experimental conditions

was 24 g/100 g, 27.25 g/100g and 31.65 g/100 g, respectively [48]. Yogendra Kumar et al. (2013) reported that sea buckthorn leaves were extracted with SLE method and the yield of ethanol/water (70/30 % v/v) extract was 23 g/100 g. In the other studies of sea buckthorn leaves PLE-EtOH extraction 18 – 19 g/100gof ethanol-soluble constituents were obtained [39]. Identification of phytochemicals in sea buckthorn pomace and leaves plants extracts.

3.4 Identification of phytochemicals in sea buckthorn pomace and leaves plants extracts

3.4.1 Fatty acid composition of non – polar sea buckthorn pomace and leaves extracts

Fatty acids composition of sea buckthorn pomace and leaves total lipids extracts obtained by SFE-CO_2-2, PLE-He-1, Sox-He and SLE-He-1, respectively were analysed by GC-FID and results are presented in Table 16 and Table 17, respectively.

The fatty acid composition of non-polar sea buckthorn pomace extracts contained from 31.38 to 41.27 % saturated fatty acids, from 33.63 to 42.20 % monounsaturated fatty acids and from 7.43 to 8.27 % polyunsaturated fatty acids from which linoleic acid is omega-6 fatty and α-linolenic acid is omega-3 fatty acids. The major fatty acids of sea buckthorn pomace non-polar extracts were palmitic acid (30.13 – 40.29 %), palmitoleic acid (16.50 – 24.02 %), oleic acid (17.11 – 19.53 %), linoleic acid (5.16 – 6.29 %), α-linolenic acid (1.63 – 2.32 %), stearic acid (0.69 – 1.00 %), myristic acid (0.24 – 0.26 %). It can be observed that some fatty acids have significant difference between extraction techniques. Palmitic acid is one of the dominant fatty acid in sea buckthorn pomace. The highest values of palmitic acid (40.29%) was found with SFE-CO_2 technique. Using SLE-He method it was 25 % less amount than using SFE-CO_2 technique. Sox-He and PLE-He-1 showed similar amount of palmitic acid ~ 34 %. Three major fatty acids – linolenic acid (21.4-25.2 %), oleic acid (20.7-26.5 %) and palmitic acid (12-14.4 %) – were detected in lipophilic fractions of sea buckthorn leaves.

The ratio of sea buckthorn pomace unsaturated/unsaturated (U/S) fatty acids is a significant factor of lipid content. The biggest U/S ratio is 1.40 in the lipid content extracted by SFE-CO_2-2 method. This can be explained by the fact that the higher amount (41.27 %) of saturated palmitic acid was determined in SFE-CO_2-2 non-polar extract and the fact why sea buckthorn oil was buttery texture. In the lipophilic fractions obtained by another methods U/S ratio is 1.18-1.32.

Table 16. Composition of fatty acids in sea buckthorn pomace

Common name	Numerical symbol	Sox- He	SLE-He-1	SFE-CO_2-2	PLE- He-1
Myristic acid	C14:0	0.25 ± 0.01^{a}	0.26 ± 0.01^{a}	0.24 ± 0.01^{a}	0.24 ± 0.01^{a}
Palmitic acid	C16:0	34.33 ± 2.02^{ab}	30.13 ± 2.91^{a}	40.29 ± 0.25^{b}	33.87 ± 0.09^{ab}
Palmitoleic acid	C16:1	22.68 ± 2.69^{b}	16.50 ± 1.04^{a}	24.015 ± 0.25^{ab}	19.49 ± 0.65^{ab}
Stearic acid	C18:0	0.96 ± 0.10^{ab}	1.00 ± 0.03^{b}	0.74 ± 0.03^{a}	0.95 ± 0.04^{ab}
Oleic acid	C18:1n9c	19.53 ± 1.20^{a}	17.14 ± 0.47^{a}	17.11 ± 0.25^{a}	18.42 ± 0.35^{a}
Linoleic acid	C18:2n6c	5.16 ± 0.27^{a}	5.30 ± 0.17^{a}	5.57 ± 0.09^{a}	6.29 ± 0.00^{b}
α-Linolenic acid	C18:3n3	2.27 ± 0.24^{a}	2.22 ± 0.19^{a}	1.82 ± 0.35^{a}	1.92 ± 0.15^{a}
∑ Saturated fatty acids		35.53^{b}	31.38^{a}	41.27^{c}	35.08^{b}
∑Monounsaturated		42.20^{c}	33.63^{a}	41.30^{c}	37.91^{b}
∑ Polyunsaturated		7.43^{a}	7.57^{a}	7.53^{a}	$8,.27^{b}$
Saturated/Unsaturated		1.40^{a}	1.31^{a}	1.18^{a}	1.32^{a}

Different superscript letters within the same line indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

Table 17. Composition of fatty acids in sea buckthorn leaves

Common name	Numerical symbol	Sox- He	SLE-He-1	SFE-CO_2-2
Palmitic acid	C16:0	12.19 ± 0.64^{a}	12.69 ± 0.06^{a}	14.40 ± 1.43^{b}
Oleic acid	C18:1n9c	26.47 ± 1.23^{b}	25.34 ± 0.49^{b}	20.74 ± 0.91^{a}
Linoleic acid	C18:2n6c	22.49 ± 1.46^{a}	21.38 ± 1.52^{a}	25.15 ± 1.23^{b}
∑ Saturated fatty acids		12.19^{a}	12.69^{a}	14.40^{b}
∑Monounsaturated		26.47^{b}	25.34^{b}	20.74^{a}
∑ Polyunsaturated		22.49^{a}	21.38^{a}	25.15^{b}
Saturated/Unsaturated		4.02^{c}	3.68^{b}	3.19^{a}

Different superscript letters within the same line indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

The most common saturated fatty acids in sea buckthorn oil include palmitic, stearic, myristic acids. They ensure high stability of the oil and it is stand to oxidation [121]. These findings suggest that sea buckthorn berries pomace are rich of monounsaturated fatty acids which is affect risk for cardiovascular disease and reducing cholesterol level [122]. Polyunsaturated fatty acids can lower the risk of heart attack, reduce the risk of cardiovascular disease [37].

In general, the fatty acids composition of sea buckthorn pulp and seed oil have been reported to be rich in palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid [19, 28, 37]. Similar results were obtained from pulp oil palmitic acid (15 – 40 %), palmitoleic acid (15 – 40 %), stearic acid (15 – 40 %), oleic acid (10 – 20 %), linoleic acid (5- 15 %), linolenic acid (5 – 10 %) [19]. According to these studies, sea buckthorn pulp oil content is close to research pomace oil content [37]. Based on the results, it can be found that sea buckthorn pomace consists of a sea buckthorn pulp.

3.4.2 Phytochemical composition of sea buckthorn pomace and leaves non – polar and polar extracts

Preliminary phytochemical composition of sea buckthorn pomace and leaves extracts was analyzed using UPLC/ESI-QTOF-MS. The conceivable compounds were identified in sea buckthorn pomace and leaves (Sox-He, Sox-Et, SFE-CO_2-2, SLE-Ac-2, SLE-Ac-EtOH-2, SLE-Ac-EtOH-H_2O-2, SLE- EtOH/H_2O-3) extracts by measuring their accurate mass and retention time. Table 18 shows the list of 15 compounds which were identified in sea buckthorn pomace and Table 19 shows the list of 17 compounds which were found in sea buckthorn leaves extracts. In UPLC–QTOF–MS analysis the compound **1** gave an m/z value of 133.0141 correlates with the molecular ion formula $C_4H_5O_5$ and identified as malic acid. The compound **2** gave an m/z value of 191.0560 corresponding to the molecular ion formula $C_7H_{11}O_6$, it was identified as quinic acid. Malic acid and quinic acid are dominating acids in sea buckthorn berries [123]. The peak **3** gave an *m/z* value of 383.1196 corresponding to the molecular ion formula $C_{14}H_{23}O_{12}$, it was identified as dihexoside by metlin. The compound **4** gave an *m/z* value of 169.0143 corresponding to the molecular ion formula $C_7H_5O_5$, it was identified as gallic acid, which is common phenolic acid in sea buckthorn [124]. Gallic acid and galic acid derivatives are natural products, it has a wide range of biological activities, including anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer activities [125] .The peak **5** was identified with *m/z* of 153.0192, fitting the molecular ion formula $C_7H_5O_4$, it was identified as protocatechuic acid. Protocatechuic acid is phenolic acid which is strong antioxidant and has anticarcinogenic effect [126]. The peak **6** had an *m/z* of 137.0240, corresponding to the molecular formula $C_7H_5O_3$, it was identified by sesamol. Sesamol is a phenolic compound with is one of the phenolic compounds which increase the oxidative stability of oil [127, 128]. The peaks **7**, **8** and **9** gave an *m/z* value of 301.0353, 461.1087 and 315.0511 corresponding to the molecular ion formula $C_{15}H_9O_7$, $C_{22}H_{21}O_{11}$ and $C_{16}H_{11}O_7$, respectively. It was identified as flavonoids quercetin, kaempferol and isorhamnetin, they and their glycosides are common flavonoids in sea buckthorn berries [29, 129, 130]. The peak **10** had an *m/z* of 194.0823, corresponding to the molecular formula $C_{10}H_{12}NO_3$, it was identified by N-methyl hippuric acid. The compounds **11**, **12**, **13**, **14** and **15** are fatty acids which is dominant in sea buckthorn berries [55, 131]. These fatty acids were identified in sea buckthorn pomace non-polar extracts before with GC-FID.

Table 18. Identification data of sea buckthorn pomace by UPLC-Q/TOF

No.	Compounds	UPLC-QTOF-MS			Conventional extractions						High pressure extraction
					Soxhlet	SLE					
		RT (min)	MS [M-H]$^-$ m/z	Formula [M-H]	Hexane	Ethanol	Acetone	Ethanol	H_2O	Ethanol/water (70/30% v/v)	SFE– CO_2
1	Malic acid[a]	0.3-0.4	133.0141	$C_4H_5O_5$		+		+		+	
2	Quinic acid[a]	0.3-0.4	191.0560	$C_7H_{11}O_6$		+			+	+	
3	Dihexoside derivatives[a]	0.3-0.4	383.1196	$C_{14}H_{23}O_{12}$		+			+		
4	Gallic acid[a]	0.9-1.0	169.0143	$C_7H_5O_5$		+	+	+		+	
5	Protocatechuic acid[a]	1.2-1.3	153.0192	$C_7H_5O_4$		+			+	+	
6	Sesamol[a]	1.6-1.7	137.0240	$C_7H_5O_3$				+			
7	Quercetin[a]	3.1-3.2	301.0353	$C_{15}H_9O_7$			+	+		+	
8	Kaempferol[a]	3.2-3.3	461.1087	$C_{22}H_{21}O_{11}$						+	
9	Isorhamnetin[a]	3.6-3.8	315.0511	$C_{16}H_{11}O_7$			+	+		+	
10	N-methyl hippuric acid[a]	5.2-5.3	194.0823	$C_{10}H_{12}NO_3$						+	
11	Linolenic Acid[a]	7.5-7.6	277.2174	$C_{18}H_{29}O_2$	+						+
12	Palmitoleic acid[a]	7.8-7.9	253.2173	$C_{16}H_{29}O_2$	+						+
13	Palmitic acid[a]	8.5-8.6	255.2335	$C_{16}H_{31}O_2$	+						+
14	Oleic Acid[a]	8.6-8.6	281.2490	$C_{18}H_{33}O_2$	+						+
15	α-Linoleic acid[a]	8.08.1	279.2328	$C_{18}H_{31}O_2$	+						+

[a] Confirmed by parent ion mass using free chemical databases (Chemspider, Metlin).

Sea buckthorn leaves compounds which was identified by UPLC-Q/TOF are presented in Table 19. The peak **16** was identified with *m/z* of 131.0462, fitting the molecular ion formula $C_4H_7N_2O_3$ was characterized as asparagine amino acid, which is continual in sea buckthorn berries and leaves [37]. The compound **17** gave an m/z value of 133.0141 correlates with the molecular ion formula $C_4H_5O_5$. The peak **18** had an *m/z* of 165.0405, corresponding to the molecular formula $C_6H_5N_4O_2$, it was identified by ribonic acid [132]. The peak **19** had an *m/z* of 179.0561, corresponding to the molecular formula $C_6H_{11}O_6$, it was identified by hexose. The compound **20** gave an m/z value of 191.0560 corresponding to the molecular ion formula $C_7H_{11}O_6$, it was identified as quinic acid. Quinic and malic acid were identified in sea buckthorn pomace extracts too. The peak **21** was identified with *m/z* of 341.1039, fitting the molecular ion formula $C_{10}H_9N_{14}O$ and identified as dihexoside. The peak **22** was showed with *m/z* of 191.0199, fitting the molecular ion formula C_6H_7O and identified as citric acid isomers [133]. The compound **23** gave an *m/z* value of 169.0143 corresponding to the molecular ion formula $C_7H_5O_5$, it was identified as gallic acid in sea buckthorn pomace and leaves extracts [124]. The peak **24** had an *m/z* of 633.0723, corresponding to the molecular formula $C_{27}H_{21}O_{18}$, it was identified by ellagnitanin [134]. The peak **25** had an *m/z* of 935.0787, corresponding to the molecular formula $C_{41}H_{27}O_{26}$, it was identified by ellagitannin. Their antioxidant and free radical scavenging, antimicrobial, anti-inflammatory, antimutagenic, and anticarcinogenic properties [135]. The peak **26** had an *m/z* of 300.9889, corresponding to the molecular formula $C_{14}H_5O_8$, it was identified by ellagic acid which is common to identify in sea buckthorn leaves [136]. The peak **27** had an *m/z* of 623.1620, corresponding to the molecular formula $C_{28}H_{31}O_{16}$, it was identified by disaccharide [137, 138]. The peak **28** had an *m/z* of 593.1304, corresponding to the molecular formula $C_{30}H_{25}O_{13}$, it was identified as glycosidic flavonoid and have anti-inflammatory, antioxidant, anticarcinogenic activities and inhibited body weight [139]. The peak **29** and **3** had an *m/z* of 327.2180, 194.0824 and 415.3219, corresponding to the molecular formula $C_{30}H_{25}O_{13}$, $C_{18}H_{31}O_5$ and $C_{27}H_{43}O_3$, respectively and they were not identified. The peak **30** had an *m/z* of 277.2174, corresponding to the molecular formula $C_{18}H_{29}O_2$, it was identified by α-linolenic acid. This fatty acid was identified in sea buckthorn leaves non-polar extracts before with GC-FID.

Table 19. Identification data of sea buckthorn leaves by UPLC-Q/TOF

No.	Compounds	UPLC-QTOF-MS			Conventional extractions						High pressure extraction
					Soxhlet extraction		SLE				
		RT (min)	MS $[M-H]^-$ m/z	Formula [M-H]	Hexane	Ethanol	Acetone	Ethanol	H_2O	Ethanol/water (70/30 v/v %)	SFE– CO_2
16	L-Asparagine[a]	0.3-0.4	131.0462	$C_4H_7N_2O_3$						+	
17	Malic acid[a]	0.3-0.4	133.0141	$C_4H_5O_5$		+		+		+	
18	Ribonic acid[a]	0.3-0.4	165.0405	$C_6H_5N_4O_2$						+	
19	Hexose[a]	0.3-0.4	179.0561	$C_6H_{11}O_6$						+	
20	Quinic acid[a]	0.3-0.4	191.0560	$C_7H_{11}O_6$		+		+		+	
21	Dihexoside[a]	0.3-0.4	341.1039	$C_{10}H_9N_{14}O$		+		+			
22	Citric acid isomer[a]	0.6-0.7	191.0199	C_6H_7O						+	
23	Gallic acid[a]	0.9-1.0	169.0143	$C_7H_5O_5$		+	+	+		+	
24	Ellagnitannin[a]	1.5-1.7	633.0723	$C_{27}H_{21}O_{18}$					+	+	
25	Ellagitannin[a]	1.5-1.7	935.0787	$C_{41}H_{27}O_{26}$					+	+	
26	Ellagic acid[a]	2.2-2.3	300.9889	$C_{14}H_5O_8$		+		+	+	+	
27	Disaccharide[a]	2.2-2.3	623.1620	$C_{28}H_{31}O_{16}$		+		+			
28	Glycosidic flavonoid[a]	3.1-3.3	593.1304	$C_{30}H_{25}O_{13}$		+		+		+	
29	[ni]	3.6-3.7	327.2180	$C_{18}H_{31}O_5$						+	
30	Linolenic acid[a]	7.5-7.6	277.2174	$C_{18}H_{29}O_2$	+		+	+			+
31	[ni]	10.0-10.1	415.3219	$C_{27}H_{43}O_3$	+						+

[a] Confirmed by parent ion mass using free chemical databases (Chemspider, Metlin).
[ni] not identified

Comparing obtained results with the data reported in literature sources, previously results by Xinjie Zhao et al., [140] reported UPLC Q-TOF MS results of sea buckthorn oil, but representative peak and identified compounds were different than represented in this study. Catalina S. Cuparencu et al., [141] were tested sea buckthorn berries puree, UPLC Q-TOF MS result detected some same compounds which were detected in this study. According to V. Kitryte et al., [63] UPLC Q-TOF MS results of sea buckthorn pomace and seeds representative peaks were detected in this study (1, 9, 17, 19, 22, 24) were the same.

3.5 *In vitro* antioxidant activity assessment of sea buckthorn pomace and leaves extracts and solid residues

3.5.1 Total phenolic content and $ABTS^{\bullet+}$ scavenging properties

The antioxidant activity of obtained extracts depends on phytochemical composition and bioactive compounds, especially polyphenolic compound content such as flavonoids and phenolic acids [142, 143]. In the present study sea buckthorn pomace and leaves total phenolic content (TPC) and synthetic radical $ABTS^{\bullet+}$ scavenging activity was determined fraction, obtained from: 1) SFE-CO_2 and PLE using n-hexane. 2) Convectional (traditional) extraction methods namely, Soxhlet extraction method and SLE, using three solvents of increasing polarity (n-hexane, acetone, ethanol, ethanol/water 70/30% v/v, water). The TPC and $TEAC_{ABTS}$ values, obtained for sea buckthorn pomace and leaves extracts are presented in Table 20 and Table 21, respectively.

The amount of TPC values in sea buckthorn pomace and leaves non-polar fraction ranged from 4.5 to 6.42 mg GAE/g DW and from 1.92 to 4.72 mg GAE/g DW, respectively. There is no significant difference between non-polar Sox-He and SLE-He-1 extracts of sea buckthorn pomace. The lowest value was obtained with PLE-He-1 extract which is 30 % lower than in Sox-He extract. Furthermore, sea buckthorn leaves total phenolic content of Sox-He and SLE-He-1 extracts did not show significant difference, but SFE-CO_2 extract value was 70 % lower than Sox-He-1 extract.

Polar fraction values of sea buckthorn pomace and leaves ranged from 2.27 to 20.58 mg GAE/g DW and from 3.94 to 56.75 mg GAE/g DW, respectively. As can be seen the amount of total phenolic content of sea buckthorn pomace decrease in the following order: SLE-EtOH/H_2O-3>SLE-Ac-2>Sox-He-Ac>Sox-He-Ac-EtOH>SLE-He-Ac-1>SLE-He-Ac-EtOH-1>SLE-Ac-EtOH-2>SLE-Ac-EtOH-H_2O-2. The highest values of sea buckthorn pomace extracts were obtained in acetone and ethanol/water 70/30 v/v % mixture. There is significant difference between all acetone extracts obtained with different extraction methods. While, SLE-Ac-EtOH-1, SLE-Ac-EtOH-2 extracts did not show significant different between extraction methods.

Table 20. Total phenolic content of sea buckthorn pomace and leaves extracts

Extraction method	Particle size, mm	Extraction parameters			TPC			
					Pomace		Leaves	
		Pressure, MPa	Temp.,°C	Time, min	mg GAE/g extract	mg GAE/g DW	mg GAE/g extract	mg GAE/g DW
High pressure extraction techniques								
SFE-CO_2 (from starting plant material)								
SFE-CO_2-2	0.5	45	60	360	25.67 ± 0.32^{a}	5.35 ± 0.06^{c}	89.08 ± 1.41^{a}	1.92 ± 0.03^{a}
PLE-He (from starting plant material)								
PLE-He-1	0.5	10.3	60	15	21.97 ± 0.61^{a}	4.51 ± 0.13^{b}	-	-
Convectional extraction techniques								
Sequential Soxhlet extraction method (from starting plant material)								
Sox-He	0.5	0.1	70	360	28.02 ± 0.48^{a}	6.42 ± 0.11^{d}	109.42 ± 2.54^{b}	4.72 ± 0.11^{b}
Sox-He-Ac	0.5	0.1	60	360	139.51 ± 1.13^{c}	8.44 ± 0.06^{f}	169.51 ± 2.32^{d}	4.93 ± 0.06^{b}
Sox-He-Ac-EtOH	0.5	0.1	80	360	239.42 ± 0.92^{h}	7.65 ± 0.02^{e}	249.27 ± 1.24^{g}	33.19 ± 0.16^{f}
Convectional extraction from SLE method (from starting plant material)								
SLE-He-1	0.5	0.1	60	360	28.65 ± 0.18^{a}	6.18 ± 0.04^{d}	90.14 ± 0.56^{a}	3.17 ± 0.02^{ab}
SLE-He-Ac-1	0.5	0.1	40	360	182.69 ± 7.89^{b}	6.51 ± 0.21^{c}	140.57 ± 0.65^{c}	3.94 ± 0.02^{ab}
SLE-He-Ac-EtOH-1	0.5	0.1	60	360	163.84 ± 2.14^{e}	6.35 ± 0.06^{c}	221.63 ± 5.51^{e}	25.19 ± 0.62^{d}
SLE extraction from residue after SFE-CO_2 (45 MPa, 60°C, 360 min 2.0 g/L rate)								
SLE-Ac-2	0.5	0.1	40	360	182.69 ± 7.89^{f}	12.89 ± 0.56^{g}	283.65 ± 5.24^{h}	10.91 ± 0.20^{c}
SLE-Ac-EtOH-2	0.5	0.1	60	360	163.84 ± 2.14^{d}	5.69 ± 0.07^{c}	331.49 ± 3.51^{j}	42.61 ± 0.45^{g}
SLE-Ac-EtOH-H_2O-2	0.5	0.1	60	360	78.17 ± 3.58^{b}	2.27 ± 0.11^{a}	214.18 ± 3.04^{e}	28.27 ± 0.40^{e}
SLE-EtOH/H_2O-3 (70/30 v/v %)	0.5	0.1	60	360	230.00 ± 3.39^{g}	20.58 ± 0.31^{h}	231.73 ± 8.02^{f}	56.75 ± 1.95^{h}

Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

As the same time, the amount of total phenolic content of sea buckthorn leaves decrease in the following order: SLE-EtOH/H_2O-3> SLE-Ac-EtOH-2> Sox-He-Ac-EtOH> SLE-Ac-EtOH-2> SLE-He-Ac-EtOH-1> SLE-Ac-2> Sox-He-Ac> SLE-He-Ac-1. As for pomace extracts the highest values of sea buckthorn leaves extracts were obtained ethanol, water and hydroethanolic mixture. There are significant different between all extraction methods using ethanol solvent, but there is no significant difference between Sox-Ac and SLE-Ac-1 extracts, but SLE-Ac-2 extract in TPC is 2 time higher than in others extracts using acetone solvent.

The $ABTS^{\bullet+}$ antioxidant capacity values in sea buckthorn pomace and leaves non-polar fraction ranged from 2.10 to 2.75 mg TE/g DW and from 1.16 to 1.88 mg TE/g DW, respectively. Non-polar extracts of sea buckthorn pomace and leaves did not show significant difference between different extraction methods tested.

The $ABTS^{\bullet+}$ antioxidant capacity values in sea buckthorn pomace and leaves polar fraction ranged from 15.18 to 30.48 mg TE/g DW and from 8.54 to 161.93 mg TE/g DW, respectively. As can be seen, the $ABTS^{\bullet+}$ scavenging capacity values of sea buckthorn pomace decrease in the following order: SLE-EtOH/H_2O-3>SLE-Ac-EtOH-H_2O-2>Sox-He-Ac-EtOH>SLE-He-Ac-EtOH-1>SLE-Ac-2>SLE-He-Ac-1>Sox-He-Ac>SLE-Ac-EtOH-2. In $ABTS^{\bullet+}$ system. ethanol/water 70/30 % v/v mixture and water extracts showed the highest antioxidant activity, while is well in agreement with TPC data. Acetone fraction extracts did not show significant difference between TEAC values in different extraction methods. Ethanol fraction values did not show significant difference between Sox-He-EtOH and SLE-He-Ac-EtOH-1 extracts, but SLE-Ac-EtOH-2 extract activity was 19 % lower than of others.

Meanwhile, the amount of $ABTS^{\bullet+}$ antioxidant capacity values of sea buckthorn leaves decrease in the following order: SLE-EtOH/H_2O-3>SLE-Ac-EtOH-H_2O-2>Sox-He-Ac-EtOH> SLE-Ac-EtOH-2>SLE-He-Ac-EtOH-1>SLE-Ac-2>Sox-He-Ac>SLE-He-Ac-1. Antioxidant activity values are higher with ethanol, ethanol/water 70/30 % v/v mixture and water extracts. Acetone fraction of sea buckthorn leaves extract did not show significant difference. The lowest activity of ethanol fraction was found for SLE-He-Ac-EtOH-1 extract.

The total phenolic content and $ABTS^{\bullet+}$ antioxidant activity obtained conduction sequential convectional (scheme-1 and 2) and SFE-CO_2 + SLE (scheme-3 and 4) extraction schemes with different polarity solvents (acetone, ethanol, ethanol/water 70/30 % v/v mixture and water) are presented in Figure 12 and Figure 13, respectively. The total phenolic content isolated from sea buckthorn pomace and leaves after sequential extractions are ranged from 19.04 to 26.2 mg GAE/g DW and from 32.3 to 83.71 mg GAE/g DW, respectively. The highest total phenolic content was

obtained in scheme-3 and scheme-4, from which 20 % values were from lipophilic fraction and 80% from polar fraction. Scheme-1, allowed to isolate 27 % lower phenolic content than other sequential methods, while scheme-2 obtained 13% lower content than others extraction methods. It can be concluded, that extractions in scheme-3 and scheme-4 can isolate higher values of total phenolic content, because 49 % of polar fraction absorb acetone fraction and 79 % of polar fraction absorb ethanol/water mixture fraction, respectively.

The highest total phenolic content of sea buckthorn leaves sequential extraction is obtained in scheme-3 extraction, from which 2 % of lipophilic fraction and 98% can be isolated from polar fraction. However, scheme-4 allowed to isolate 39 % lower phenolic content than it was obtained in scheme-3 extraction. It can be concluded that scheme-3 extraction has higher total phenolic content, because 68 % of polar fraction absorb ethanol and water fractions.

The $ABTS^{\bullet+}$ antioxidant capacity in sea buckthorn pomace and leaves sequential extraction methods are ranged from 33.25 to 59.61 mg TE/g DW and from 78.04 to 180.2 mg TE/g DW, respectively. The highest antioxidant activity of sequential extraction is obtained in scheme-3, from which is 5 % of lipophilic fraction activity and 95 % polar fraction activity of sea buckthorn pomace. At the same time, extraction in scheme-4 is allowed to isolate the lowest antioxidant activity. Scheme-1 and scheme-2 show similar amount of antioxidants activity (Figure 14).

The highest $ABTS^{\bullet+}$ antioxidant activity of sea buckthorn leaves sequential extractions are obtained in scheme-3 and sheme-4, from which is 1 % of lipophilic fraction activity and 99% polar fraction activity. However, extraction in sheme-1 has to isolate 43 % lower activity than extraction in scheme-3 and sheme-4, but non-polar and polar fractions can isolate similar amount of antioxidant activity.

Differences values of phenolic compounds and $ABTS^{\bullet+}$ scavenging activity can be because extractions, polarity of solvents which was used for extraction, environmental factors-light, temperature, time which could influence bioactive compounds activity in plants [144]. The highest amounts of phenolic compounds and the stronger $ABTS^{\bullet+}$ scavenging activity of sea buckthorn pomace and leaves are in the Solid liquid extraction method obtained extract with ethanol/water 70/30 v/v% mixture. Sequential extraction in scheme-3 showed the highest $ABTS^{\bullet+}$ scavenging activity of sea buckthorn and pomace, but this extraction is 6 hours longer than extraction in sheme-1, 2 and 12 hours longer than in scheme-4 extraction. Based on total phenolic content and antioxidant activity it can be concluded that the best extraction is shown in scheme-3.

Table 21. $ABTS^{\bullet+}$ scavenging activities assay of sea buckthorn pomace and leaves extracts

Extraction method	Particle size, mm	Extraction parameters			$ABTS^{\bullet+}$			
					Pomace		Leaves	
		Pressure, MPa	Temp.,°C	Time, min	mg GAE/g extract	mg GAE/g DW	mg GAE/g extract	mg GAE/g DW
High pressure extraction techniques								
SFE-CO_2 (from starting plant material)								
SFE-CO_2-2	0.5	45	60	360	13.33 ± 0.72^{a}	2.77 ± 0.15^{a}	53.44 ± 4.59^{a}	1.16 ± 0.10^{a}
PLE-He (from starting plant material)								
PLE-He-1	0.5	10.3	60	15	10.29 ± 0.46^{a}	2.10 ± 0.09^{a}	-	-
Convectional extraction techniques								
Sequential Soxhlet extraction method (from starting plant material)								
Sox-He	0.5	0.1	70	360	11.37 ± 0.51^{a}	2.75 ± 0.19^{a}	43.73 ± 1.44^{a}	1.88 ± 0.06^{a}
Sox-He-Ac	0.5	0.1	60	360	264.99 ± 15.86^{c}	15.94 ± 0.95^{b}	300.92 ± 9.82^{b}	8.91 ± 0.28^{b}
Sox-He-Ac-EtOH	0.5	0.1	80	360	589.88 ± 12.45^{h}	18.66 ± 0.39^{c}	607.14 ± 18.34^{c}	79.83 ± 2.43^{d}
Convectional extraction from SLE method (from starting plant material)								
SLE-He-1	0.5	0.1	60	360	12.56 ± 0.42^{a}	2.75 ± 0.09^{a}	51.54 ± 2.59^{a}	1.82 ± 0.09^{a}
SLE-He-Ac-1	0.5	0.1	40	360	218.19 ± 13.78^{b}	17.17 ± 1.09^{bc}	302.54 ± 6.27^{b}	8.45 ± 0.17^{b}
SLE-He-Ac-EtOH-1	0.5	0.1	60	360	495.38 ± 14.75^{g}	18.14 ± 0.54^{c}	603.61 ± 12.09^{c}	67.77 ± 1.36^{c}
SLE extraction from residue after SFE-CO_2 (45 MPa, 60 °C, 360 min 2.0 g/L rate)								
SLE-Ac-2	0.5	0.1	40	360	248.59 ± 7.07^{c}	17.55 ± 0.49^{bc}	292.54 ± 6.71^{b}	11.15 ± 0.25^{b}
SLE-Ac-EtOH-2	0.5	0.1	60	360	434.75 ± 9.75^{f}	15.18 ± 0.34^{b}	602.14 ± 13.29^{c}	78.02 ± 1.71^{cd}
SLE-Ac-EtOH-H_2O-2	0.5	0.1	60	360	291.11 ± 6.89^{d}	24.11 ± 0.56^{e}	679.76 ± 5.87^{d}	89.87 ± 0.77^{e}
SLE-EtOH/H_2O-3 (70/30 v/v %)	0.5	0.1	60	360	333.46 ± 17.89^{e}	30.48 ± 1.60^{f}	662.74 ± 3.88^{d}	161.93 ± 0.94^{f}

Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

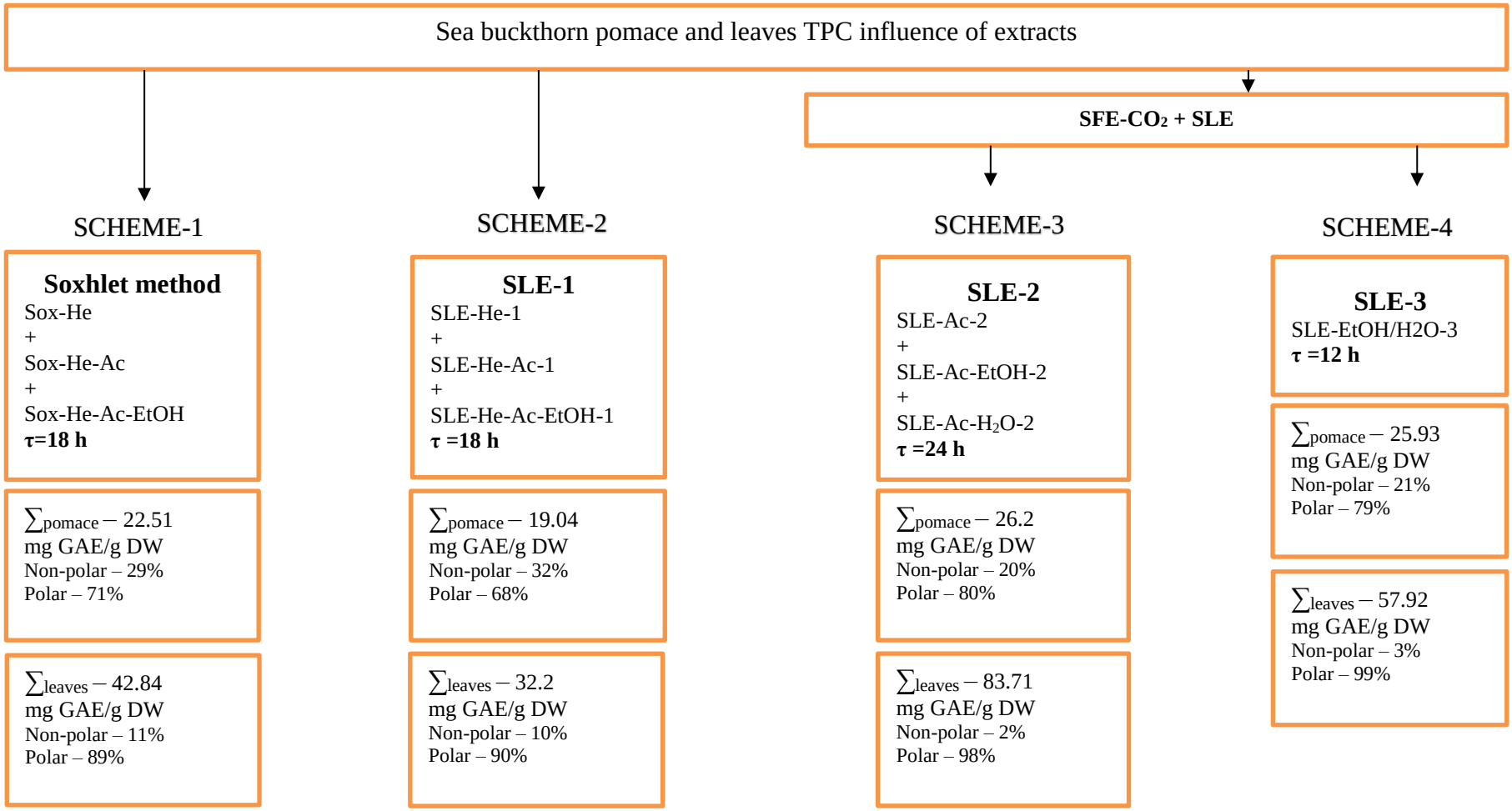


Figure 12. Sea buckthorn pomace and leaves different extractions influence of extract total phenolic content

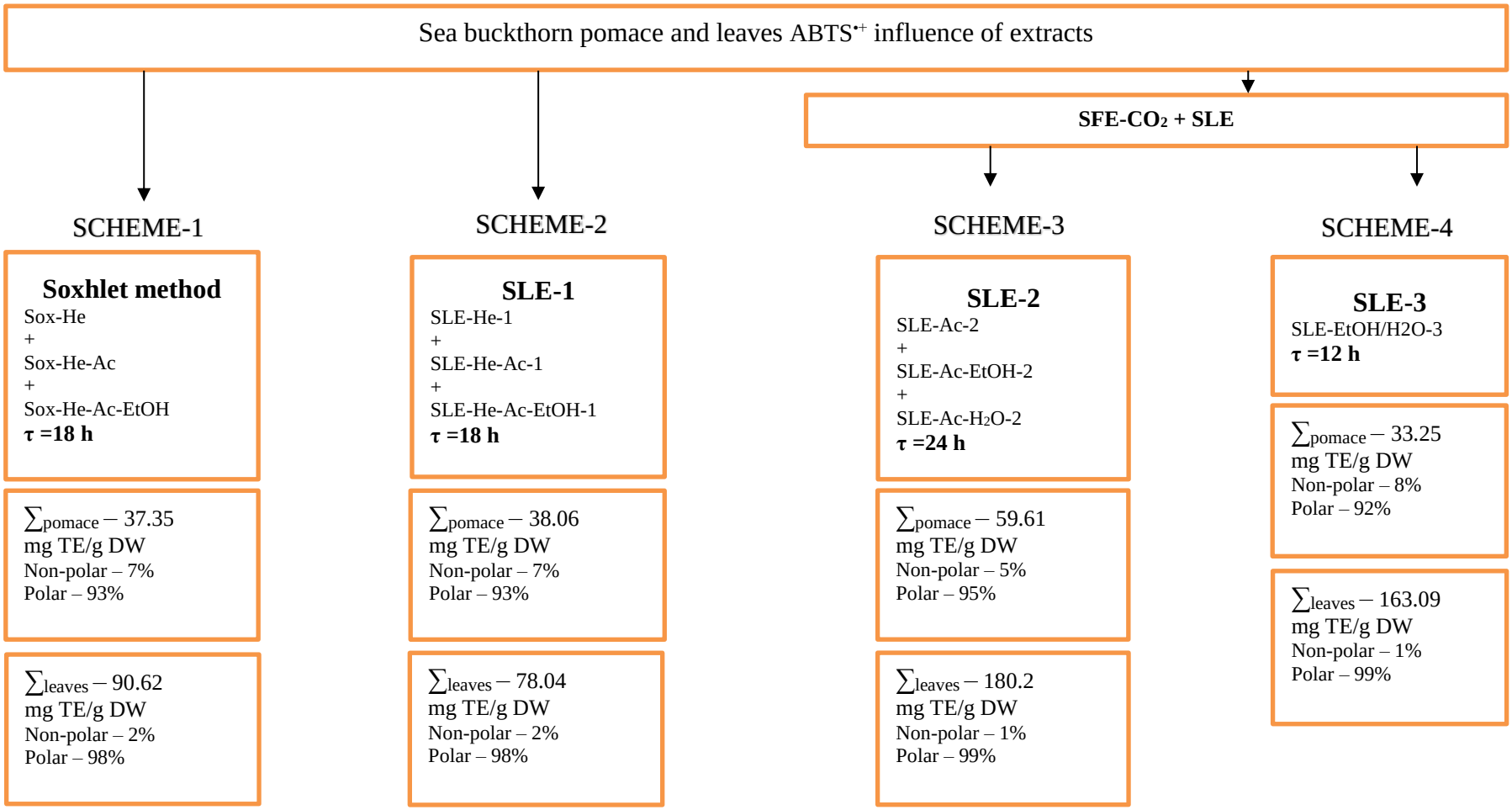


Figure 13. Sea buckthorn pomace and leaves different extractions influence of extract antioxidant activity

Comparing obtained results with the data reported in literature sources, similar results were previously showed by Alam Zeb et al., (2015) that TPC of sea buckthorn oils is 3.38 mg GAE/g DW [145]. Similar results were also obtained by previous studies conducted on different species of sea buckthorn berries: TPC of methanol extracts ranged from 8.62 to 14.17 mg GAE/g DW [146]. G. Korekar et al., (2011) reported that TPC values in methanol extracts of sea buckthorn is 40.56 mg GAE/g DW, in water extract 16.66 mg GAE/g DW which are much higher than in this study [147]. Water and ethanol/water 70/30 % v/v mixture of sea buckthorn leaves extract showed values 40.49 mg GAE/g DW and 56.28 mg GAE/g DW, respectively [86]. Korekar et al., (2011) reported that TPC values in methanol extracts of sea buckthorn leaves is 61.00 mg GAE/g DW, in water extract 32.49 mg GAE/g DW which is similar than it was obtained in this study data [147].

Upendra K. Sharma et al., (2008) reported that $ABTS^{\bullet+}$ values obtained for the extracts are ranged from 2.03 to 182.13 mg/g of sea buckthorn [148], which is similar with this study. Similar result were reported by Nitin at al.: water and ethanol/water 70/30 % v/v mixture of sea buckthorn leaves extract showed $ABTS^{\bullet+}$ scavenging activity 119.86 mg TE/g DW and 166.67 mg TE/g DW, respectively [86].

Although, obtained extracts show high values of TPC and strong $ABTS^{\bullet+}$ antioxidant activity, for this reason it is very important to measure antioxidant activity of plant material and solid residue after each extraction step of extraction. This would be achieved using the QUENCHER method which is based on the direct application of the free radicals on sample and bound active compounds [149]. Therefore, antioxidant activity was measured for solid fractions applying the QUENCHER method for the total phenolic compounds (TPC) and $ABTS^{\bullet+}$ scavenging assays. Results of sea buckthorn pomace and leaves crude plant and solid residue after SFE-CO_2-2 extraction are presented in Table 22.

Total phenolic content in crude plant material of sea buckthorn pomace and leaves values were 21.56 mg GAE/g DW and 58.79 mg GAE/g DW, respectively. Subject to the different solvents used for the extraction TPC residue of sea buckthorn pomace and leaves ranged from 3.20 to 18.30 mg GAE/g DW and from 5.33 to 57.55 mg GAE/g DW, respectively. Residues after non-polar fraction has the highest content in TPC with all extraction methods of sea buckthorn pomace. SLE-Ac-EtOH-2, SLE-Ac-H_2O-2 values did not show significant difference, than residue after non-polar extractions. For instance, residue after SLE-Ac-2, SLE-EtOH/H_2O-3 (70/30 % v/v) remarkably decreased total phenolic content 65 % and 85 %, respectively. The sum of total phenolic content of extract and solid residue is similar with starting plant material of sea buckthorn pomace in all extraction methods.

Residues after non-polar fraction and residue after acetone extraction have the highest content in TPC with all extraction methods of sea buckthorn leaves. Total phenolic content slightly decreased after SLE-He-EtOH-1, SLE-Ac-H_2O-2 extractions. However, TPC values remarkably decrease after SLE-Ac-EtOH-2, SLE-EtOH/H_2O-3 extractions 74% and 91%, respectively. The amount of total phenolic content of extract and residue after extraction is similar with crude plant material of sea buckthorn leaves in all extraction methods.

The $ABTS^{\bullet+}$ antioxidant capacity of sea buckthorn pomace and leaves crude plant material activity were 104.54 mg TE/g DW and 315.12 mg TE/g DW, respectively. In $ABTS^{\bullet+}$ scavenging system after non-polar extraction and polar extraction with different solvents plant material activity was from 64.94 to 97.30 mg TE/g DW and from 121.78 to 303.22 mg TE/g DW, respectively. Antioxidant capacity after non-polar extractions and after SLE-Ac-2, SLE-Ac-EtOH-2 extraction have the highest activity in sea buckthorn pomace. The antioxidant activity slightly decreased after SLE-Ac-H_2O-2, SLE-EtOH/H_2O-3 extractions. The amount of $ABTS^{\bullet+}$ antioxidant capacity of extract and residue after extraction is higher with SLE-Ac-2, SLE-Ac-EtOH-2 extractions 2-4 % than crude plant material, Sox-He, SFE-CO_2 amount of residue and extract were lower 15-20 % than sea buckthorn pomace crude plant material.

Antioxidant activity after non-polar extraction and extraction with acetone fraction showed the highest activity of sea buckthorn leaves residue. Residue activity after SLE-Ac-H_2O-2, SLE-EtOH/H_2O-3 extractions showed the lowest activity 47% and 61%, respectively. The amount of $ABTS^{\bullet+}$ antioxidant capacity of extract and residue after extraction is higher with SLE-Ac-2, SLE-Ac-EtOH-2 extractions 2-6 % than crude plant material, SLE-Ac-EtOH-H_2O-2 extract and residue activity amount were lower 19 % than sea buckthorn leaves crude plant material activity.

Consequently, residues after non-polar fraction has the highest activity in TPC and $ABTS^{\bullet+}$ scavenging assays, because there were not extracted phenolic compounds accordingly residue after ethanol, water and ethanol/water 70/30 % v/v mixture fractions showed the lowest total phenolic content and antioxidant activity in sea buckthorn pomace and leaves residue.

Table 22. Total phenolic and antioxidant activities of solid residue and crude plant materials of sea buckthorn pomace and leaves

Solid residues	TPC		$ABTS^{\bullet+}$	
	mg GAE/g sample	mg GAE/g DW	mg TE/g sample	mg TE/g DW
Crude plant materials				
Sea buckthorn pomace		21.56 ± 3.04		104.54 ± 4.17
Sea buckthorn leaves		58.79 ± 4.44		315.12 ± 11.27
Residue after non- polar constituent				
Sea buckthorn pomace				
Sox-He	21.37 ± 2.39^{c}	16.37 ± 1.83^{d}	124.40 ± 6.23^{bc}	97.30 ± 4.57^{d}
SFE-CO_2	21.79 ± 0.97^{c}	16.7 ± 0.71^{d}	113.57 ± 6.6^{b}	87.01 ± 5.07^{c}
Sea buckthorn leaves				
Sox-He	55.86 ± 5.50^{f}	53.49 ± 5.27^{e}	792.40 ± 54.25^{f}	273.98 ± 22.50^{e}
SFE-CO_2	58.85 ± 0.71^{f}	57.55 ± 0.96^{e}	622.17 ± 22.05^{e}	303.22 ± 10.84^{f}
Residue after polar constituent				
Sea buckthorn pomace				
Sox-He-Ac-EtOH	19.98 ± 1.16^{c}	13.46 ± 0.78^{c}	96.39 ± 5.40^{a}	64.94 ± 3.64^{a}
SLE-He-Ac-EtOH-1	20.69 ± 0.67^{c}	13.82 ± 0.44^{c}	124.62 ± 2.15^{bc}	83.23 ± 1.44^{c}
SLE-Ac-2	10.54 ± 0.81^{b}	7.60 ± 0.57^{b}	125.98 ± 2.31^{bc}	90.83 ± 1.66^{cd}
SLE-Ac-EtOH-2	24.67 ± 0.93^{d}	16.93 ± 0.64^{d}	133.64 ± 9.97^{bc}	91.71 ± 6.84^{cd}
SLE-Ac-H_2O-2	27.85 ± 0.29^{e}	18.30 ± 0.19^{d}	114.26 ± 8.04^{bc}	75.08 ± 5.28^{bc}
SLE-EtOH/H_2O-3 (70/30 v/v %)	4.55 ± 0.73^{a}	3.20 ± 0.51^{a}	101.92 ± 1.25^{a}	71.54 ± 0.88^{ab}
Sea buckthorn leaves				
Sox-He-EtOH	30.21 ± 1.74^{c}	24.03 ± 1.38^{c}	239.91 ± 6.09^{c}	200.71 ± 2.32^{c}
SLE-He-EtOH-1	39.18 ± 1.27^{d}	32.26 ± 1.05^{d}	365.57 ± 10.12^{d}	229.77 ± 8.38^{d}
SLE-Ac-2	51.14 ± 4.21^{ef}	48.04 ± 3.95^{e}	335.81 ± 6.22^{d}	310.20 ± 7.02^{f}
SLE-Ac-EtOH-2	18.91± 0.61^{b}	15.34 ± 0.50^{b}	346.65 ± 9.22^{d}	257.57 ± 3.55^{e}
SLE-Ac—EtOH-H_2O-2	47.26 ± 1.58^{e}	32.08 ± 1.07^{d}	85.77 ± 7.83^{a}	165.77 ± 9.37^{b}
SLE-EtOH/H_2O-3 (70/30 v/v %)	7.26 ± 0.33^{a}	5.33 ± 0.24^{a}	168.05 ± 12.92^{b}	121.78 ± 9.54^{a}

Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

The results of this study showed that residue fraction before and after extractions for sea buckthorn leaves have better antioxidant activity and phenolic content than sea buckthorn pomace. It can be because sea buckthorn leaves are rich source of flavonoids and sea buckthorn fruits have higher contents of vitamin C, E and carotenoids [150].

For the further antioxidant activity (ORAC, HOSC, HORAC) analysis was selected sea buckthorn pomace SFE-CO_2-2, SLE-Ac-2, SLE-Ac-EtOH, SLE-EtOH/H_2O 70/30 v/v % mixture extracts and sea buckthorn leaves SFE-CO_2-2, SLE-Ac-2, SLE-Ac-EtOH-2, SLE-EtOH/H_2O-3 70/30 v/v % mixture and water extracts, because these extracts showed higher antioxidant activity.

3.5.2 Oxygen and hydroxyl radical scavenging activity of sea buckthorn pomace and leaves

The normal redox state of biological tissues can be disturbed by highly reactive free radicals (molecules). Radicals derived from oxygen, termed reactive oxygen species (ROS), such as superoxide ion ($O2^{-\cdot}$), hydroxyl radicals ($HO^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$), are involved in the pathophysiology of aging and a lot of diseases, such as atherosclerosis, cardiovascular diseases and cancer [151, 152]. The antioxidant capacities of selected extracts were researched with Oxygen Radical Absorbance Capacity (ORAC)[1], Hydroxyl Radical Scavenging Capacity (HOSC)[2] and Hydroxyl Radical Adverting Capacity (HORAC)[2] assays. The results of ORAC and HOSC were reported as mg of Trolox equivalents per gram of extract or starting plant material (TE/g extract and mg TE/g DW). The results of HORAC were expressed as mg of caffeic acid equivalents (mg CAE/g of extract and mg CAE/g DW). For these analyses, 9 sea buckthorn by-product extracts, which showed higher values in TPC and $ABTS^{\bullet+}$ scavenging assays, were selected, namely:

- Sea buckthorn pomace extracts: SFE-CO_2-2 (45 MPa, 60 ºC, 360 min.), SLE-Ac-2 (0.1 MPa, 40 ºC, 360 min), SLE-Ac-EtOH-2 (0.1 MPa, 60 ºC, 360 min), SLE- EtOH/H_2O-3 (70/30 % v/v hydroethanolic mixture) (0.1 MPa, 60 ºC, 360 min);
- Sea buckthorn leaves extracts: SFE-CO_2-2 (45 MPa, 60 ºC, 360 min.), SLE-Ac-2 (0.1 MPa, 40 ºC, 360 min), SLE-Ac-EtOH-2 (0.1 MPa, 60 ºC, 360 min), SLE- EtOH/H_2O-3 (70/30 % v/v hydroethanolic mixture) (0.1 MPa, 60 ºC, 360 min) and SLE-Ac-EtOH-H_2O-2 (0.1 MPa, 60 ºC, 360 min)

The sea buckthorn pomace and leaves (mg TE/g extract and mg TE/g DW) $TEAC_{ORAC}$ values are presented in Figure 14 (A, B). Activity of selected extracts of radical scavenging capacity ranged in the interval of 166 – 1125g TE/g extract and 370 – 1511 mg TE/g extract for sea buckthorn pomace and leaves, respectively. When recalculated per gram of starting plant material, these values amounted 32 – 101 mg TE/g DW and 10.3 – 386.4 mg TE/g DW, respectively. For both sea-buckthorn by-products tested, the lowest activity was noticed for non-polar SFE-CO_2-2 and semi-polar SLE-Ac-2 extracts (14.2 – 32 mg TE/g DW) while the most active fractions were derived after sequential extractions with polar solvents – pure ethanol (37.8 – 182 mg TE/g DW) and water (147 mg TE/g DW) or hydroethanolic (100.7 – 3684 mg TE/g DW) mixture. The highest activity (mg TE/g DW) was obtained for sea buckthorn leaves SLE-EtOH/H_2O-3 extract (368.4 mg TE/g DW), which was by 50 % higher than of SLE-Ac-EtOH-2 from leaves and 73 % higher than of SLE-EtOH/H_2O-3 pomace extract.

[1] The analysis was prepared in IBET – Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal

Comparing obtained results with the data reported in literature sources, similar results were noticed by Ruixue Guo et, al (2017) that ORAC values of 4 sea buckthorn species extracted with etilacetate ranged from 266 to 369 μmol TE/g DW [72].

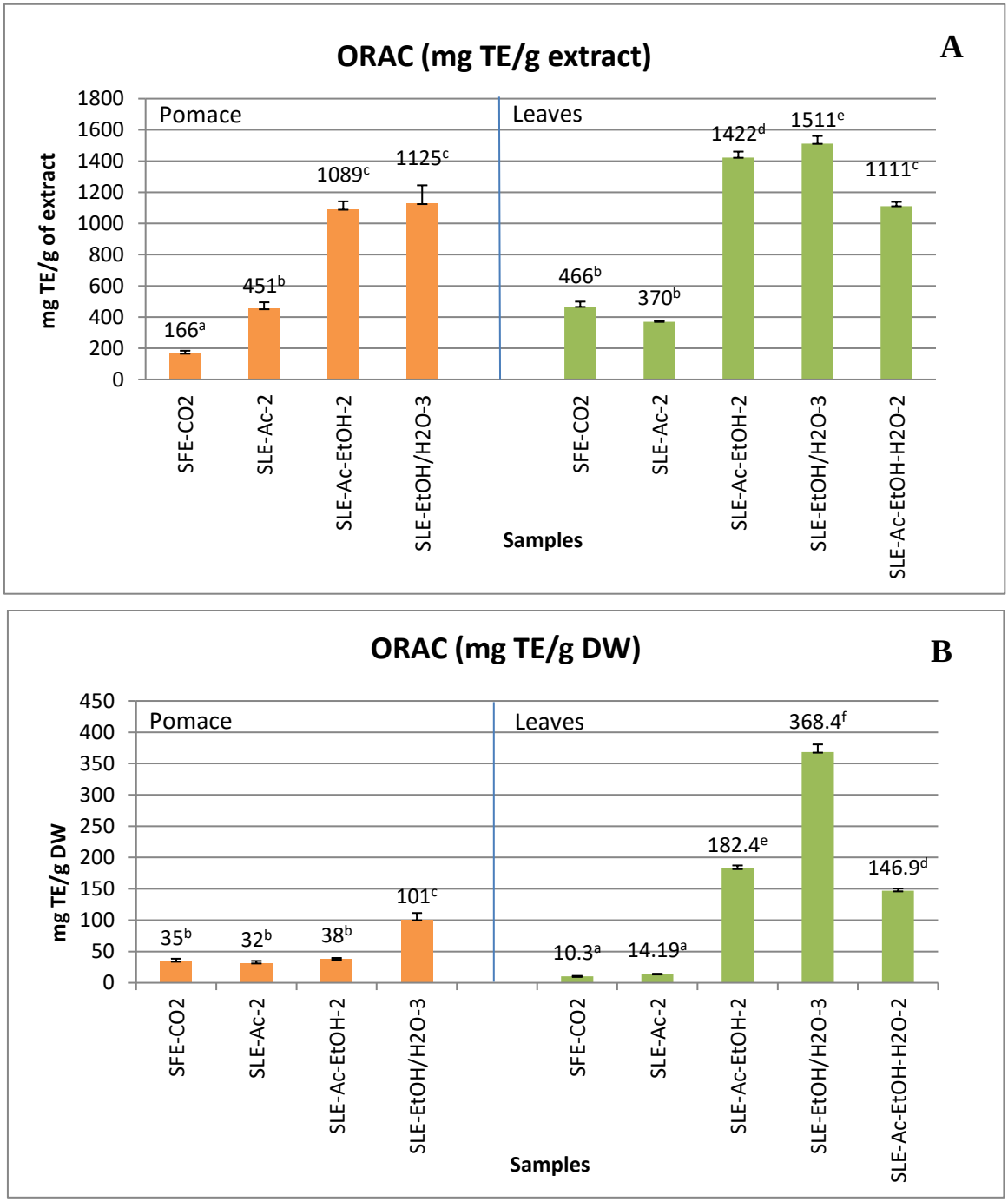
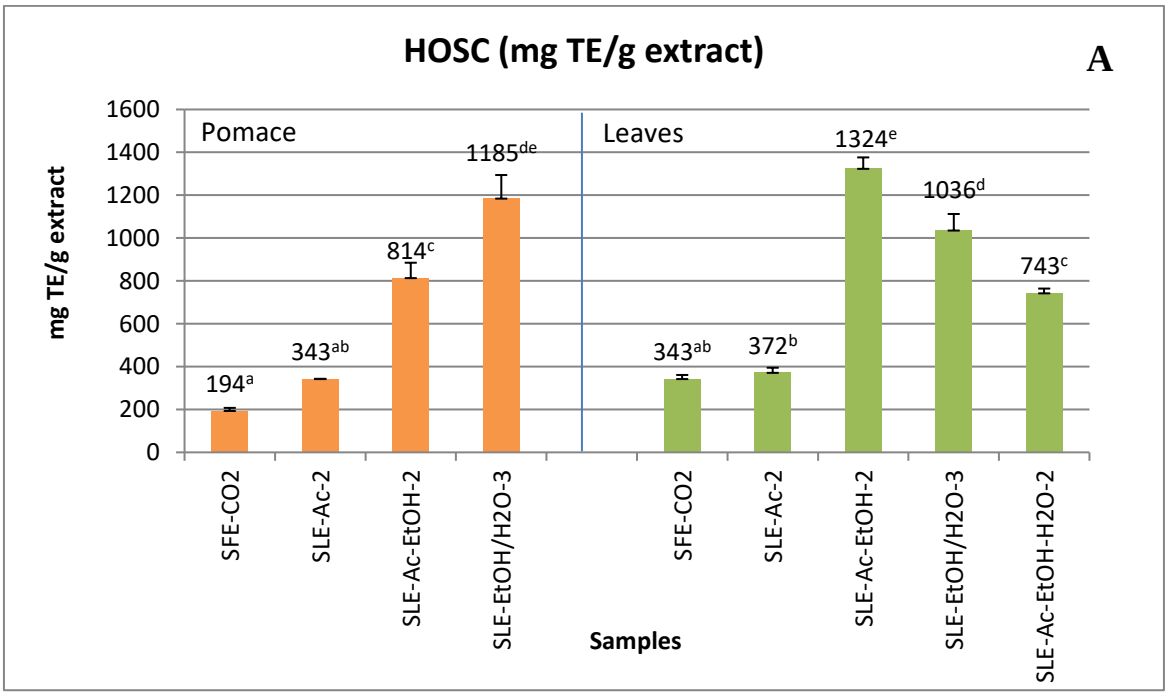


Figure 14. Oxygen radical absorbance capacity (ORAC) of sea buckthorn pomace and leaves expressed as (A) mg TE/g extract (B) mg TE/g DW

The hydroxyl radical (• OH) is extremely reactive with almost every type of biomolecules and is possibly the most reactive chemical species known. Hydroxyl radicals may serve as an excellent target to investigate dietary antioxidants for their potential to directly react with and quench free radicals and protect important biomolecules from radical-mediated damage.

The sea buckthorn pomace and leaves (mg TE/g extract and mg TE/g DW) results of HOSC assay are presented in Fig 15 (A, B). Selected extract hydroxyl radical scavenging capacity was measured in the interval of 194 – 1185 mg TE/g extract and 343 – 1323 mg TE/g extract for sea buckthorn pomace and leaves, respectively (or 24 – 106 mg TE/g DW and 7.6 – 252.5 mg TE/g DW, respectively). In agreement to ORAC assay results, he lowest $TEAC_{HOSC}$ values were calculated for CO_2 and acetone-derived fractions (7.6 – 40 mg TE/g DW) while polar extracts were by 62 – 97 %-fold more active. The highest activity (mg TE/g DW) was again shown for sea buckthorn leaves SLE-EtOH/H_2O-3 extract, which was by 58 % higher than corresponding SLE-EtOH/H_2O-3 pomace extract and by 9 – 33 %higher than other polar fractions of leaves after SLE with pure ethanol and water.



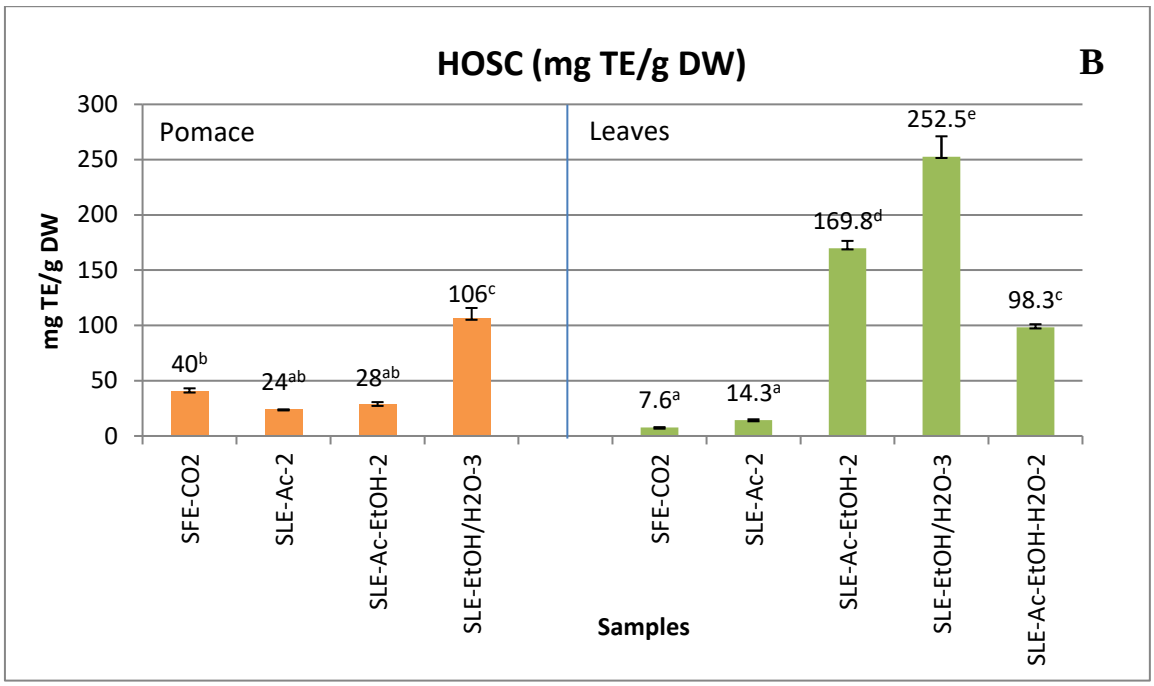


Figure 15. Hydroxyl radical scavenging (HOSC) of sea buckthorn pomace and leaves expressed as (A) mg TE/g extract (B) mg TE/g DW

The HORAC assay measures the ability of the antioxidant present to chelate Co (II) prior to the occurrence of Fenton reaction. Fig 16 (A, B) reports HORAC assay results for various Sea buckthorn pomace and leaves extracts, expressed as caffeic acid activity equivalents (mg CAE/g extract and mg CAE/g DW). As it can be seen from graphs, hydroxyl hydroxyl radical antioxidant capacity was ranging from 80 to 215 mg CAE/g extract and from 149 to426 mg TE/g extract for sea buckthorn pomace and leaves, respectively. When referred to one gram of crude (unextracted) plant material, the following values were obtained: 5.9 – 19.3 mg CAE/g DW for sea buckthorn pomace and 3.3 – 103.8 mg CAE/g DW for leaves. As for the results displayed in Fig.13-14, the lowest activity was reported for sea buckthorn pomace SFE-CO_2-2 and SLE-Ac-2 extracts (3.3 – 16.6 mg CAE/g DW. Also, these values significantly increased when polar fractions were analysed. Sea buckthorn leaves SLE-EtOH/H_2O-3 extract exerted the highest activity, followed by the SLE-Ac-EtOH-2 leave fraction (55% lower activity), SLE-Ac-EtOH-H_2O-2 leave fraction (75% lower activity) and SLE-EtOH/H_2O-3 pomace extract (81 % lower activity).

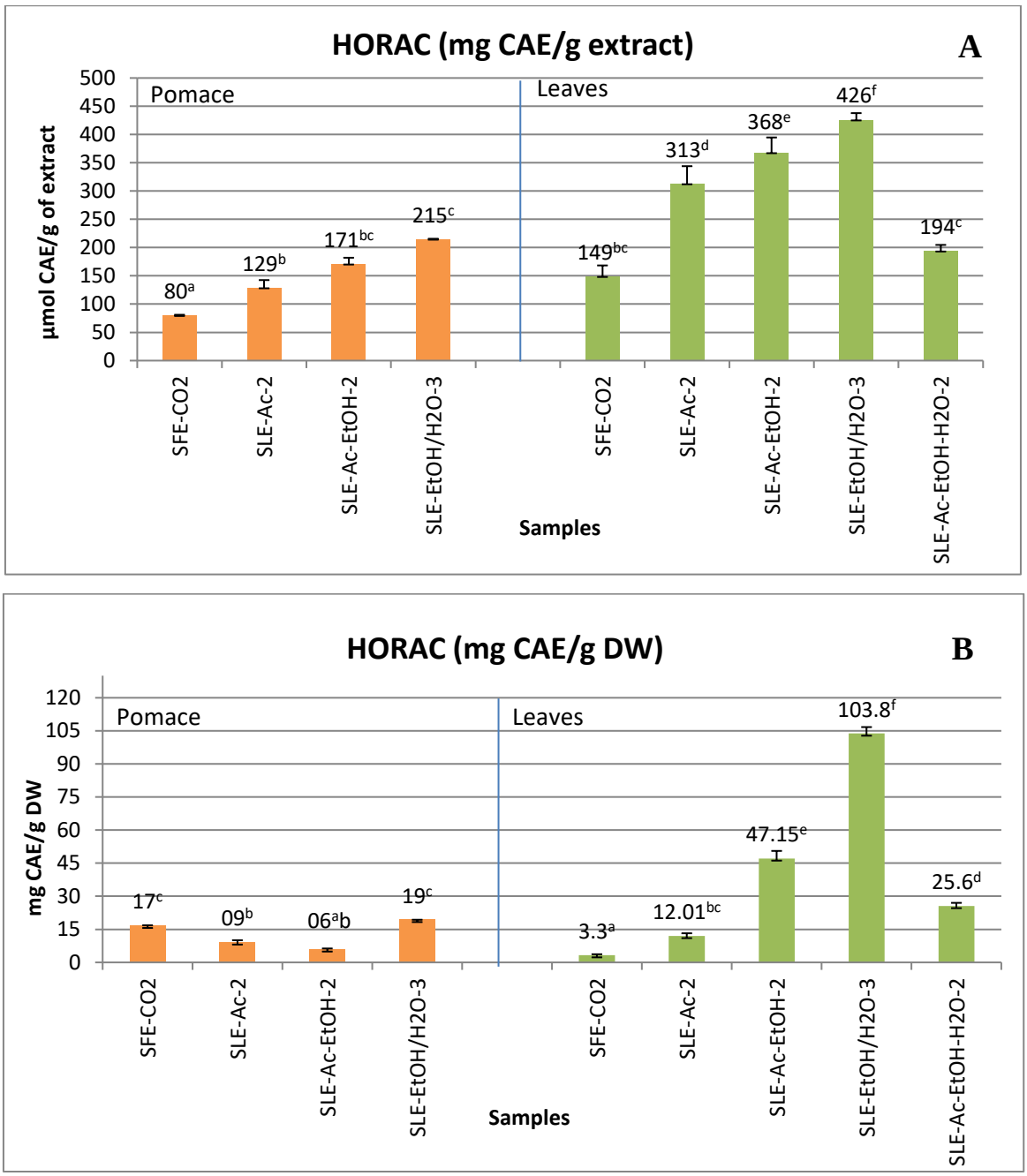


Figure 16. Hydroxyl radical antioxidant capacity (HORAC) of sea buckthorn pomace and leaves expressed as (A) mg CAE/g extract (B) mg CAE/g DW

Summarizing, in all antioxidant activity assays (TPC, ABTS, ORAC, HOSC, HORAC) the highest antioxidant activity was obtained for SLE-EtOH/H_2O-3 (70/30 % v/v) and SLE-Ac-EtOH-H_2O-2 extracts from sea buckthorn pomace and leaves. Accordingly, these fractions and SFE-CO_2-2 derived lipophilic extract were further utilized for cellular antioxidant activity assessment of sea buckthorn pomace and leaves.

3.6 Evaluation of oxidatvive stability of rapeseed oil with SFE-CO_2 extracts

Oxidative stability of rapeseed oil with added sea buckthorn pomace and leaves SFE-CO_2-2 extracts was analyzed using Oxipres method, where oxidation of rapeseed oil with SFE-CO_2-2 extracts (0.5 %, 1 %, 5 % w/w) or without (control sample) is accelerated by heating samples at 110 °C and pressurizing with oxygen at 0.5 MPa. The obtained results are presented in Figures 17 and 18, respectively, while calculated induction periods (IP) are reported in Table 23.

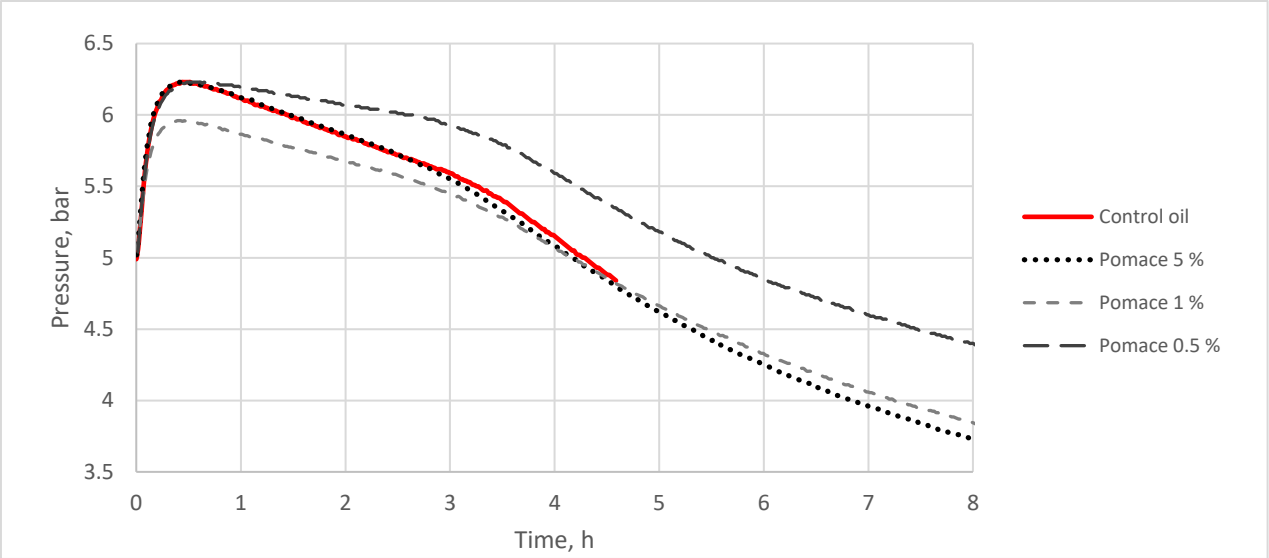


Figure 17. Effect of 0.5 %, 1 % and 5 % sea buckthorn pomace SFE-CO_2-2 extracts additives on rapeseed oil

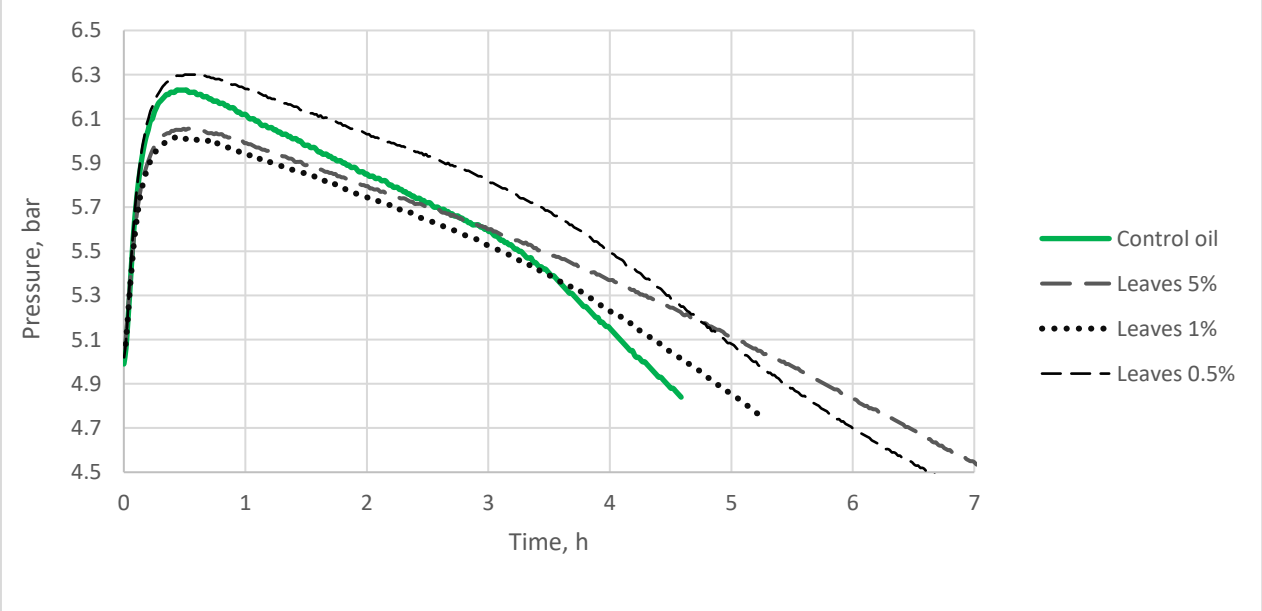


Figure 18. Effect of 0.5 %, 1 % and 5 % sea buckthorn leaves SFE-CO_2-2 extracts additives on rapeseed oil

Table 23. Sea buckthorn pomace and leaves SFE-CO_2-2 extracts evaluated in rapeseed oil oxidation test (Oxipres method)

Sample	Conc., %	Sea buckthorn pomace IP	Sea buckthorn leaves IP
Control	0	3.3 ± 0.01^{b}	3.3 ± 0.01^{a}
SFE-CO_2-2	0.5	3.12 ± 0.12^{ab}	3.88 ± 0.01^{b}
SFE-CO_2-2	1	2.96 ± 0.04^{a}	3.98 ± 0.01^{b}
SFE-CO_2-2	5	2.89 ± 0.04^{a}	4.95 ± 0.14^{c}

It may be observed that the induction period of rapeseed with sea buckthorn pomace SFE-CO_2-2 lipophilic extracts was decreasing from 3.12 to 2.89 hours with the increasing amount of extract added from 0.5% to 5% (w/w). Therefore, only lower than 0.5% concentrations of CO_2 derived pomace fraction could be used developing novel products (e.g. oils enriched with specific bioactive constituents or cosmetic products) in order not to induce significant decrease in oxidative stability of vegetable oil.

Looking at the results of other sea buckthorn by-product, in contrast to pomace addition, rapeseed oil stability was significantly increasing from 3.88 to 4.95 with the higher amount of leave extract used. At the highest concentration tested (5 % w/w), the induction period of sample was 1.5-fold higher as compared with raw rapeseed oil (control). Sea buckthorn leaves extracts with 1% and 0.5% concentrations did not show significant difference, but induction period was still longer up to 15% than of rapeseed oil without extract added.

3.7 *Ex vivo* cytotoxicity activity of the selected sea buckthorn pomace and leaves extracts

For cytotoxicity assessment, of 5 selected sea buckthorn pomace and leaves were tested, namely SFE-CO_2-2 and SLE-EtOH/H_2O-3 from sea buckthorn pomace, and SFE-CO_2-2, SLE-EtOH/H_2O-3 and SLE-Ac-EtOH-H_2O-2 from sea buckthorn leaves. The selection of polar fractions of interest was based on their high activity in TPC, ABTS, ORAC, HOSC, HORAC assays (e.g. SLE-EtOH-3, SLE-Ac-EtOH-H_2O-2 extracts showed the highest *in vitro* radical scavenging activity). SFE-CO_2-2 extracts were additionally selected to test the cytotoxicity of non-polar fractions due to their possible applications in food and cosmetics industries. Extracts cytotoxicity[2] was estimated using human colon adenocarcinoma model Caco-2 cell line after incubation at 37 °C for 1 hour. The short incubation period (1 h) was selected in order to avoid possible contamination of Caco-2 cells. Previously it was reported that due to prolonged contact between tested extracts and target cells, probability of contamination in increasing and could furtherlead to false results these

[2] The analysis was prepared in IBET – Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal

assays [153]. The results, presented in Figures 19-23, clearly show that both sea buckthorn pomace and leaves extracts did not induce cytotoxic effects in the range of tested concentrations for non-polar and polar extracts: 0.01 – 5 mg/ml, and 0.31 – 10 mg/ml, respectively.

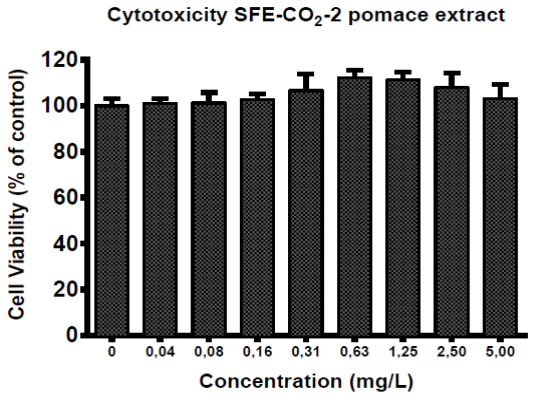


Figure 19. Cell cytotoxic analysis in Caco-2 cells treated for 1 h with different concentrations of sea buckthorn pomace non-polar extract. Results were expressed in terms of mean ± SD of three independent experiments. There were no significant difference between tested concentrations ($p < 0.05$).

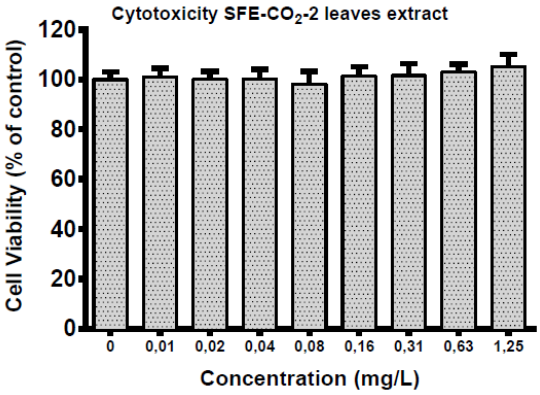


Figure 20. Cell cytotoxic analysis in Caco-2 cells treated for 1 h with different concentrations of sea buckthorn leaves non-polar extract. Results were expressed in terms of mean ± SD of three independent experiments. There were no significant difference between tested concentrations ($p < 0.05$).

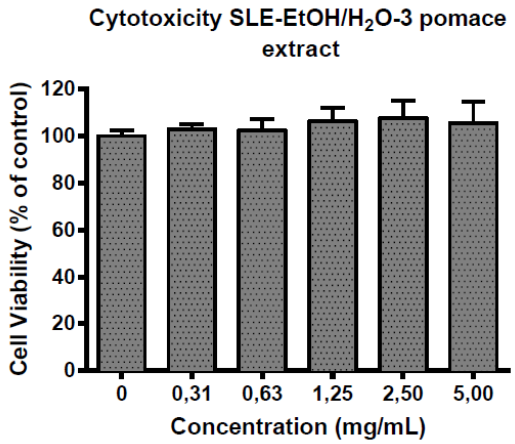


Figure 21. Cell cytotoxic analysis in Caco-2 cells treated for 1 h with different concentrations of sea buckthorn pomace SLE-EtOH/H_2O-3 extract. Results were expressed in terms of mean ± SD of three independent experiments. There were no significant difference between tested concentrations ($p < 0.05$).

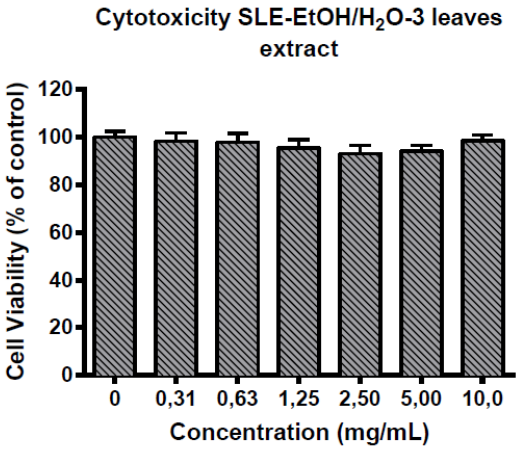


Figure 22. Cell cytotoxic analysis in Caco-2 cells treated for 1 h with different concentrations of sea buckthorn leaves SLE-EtOH/H_2O-3 extract. Results were expressed in terms of mean ± SD of three independent experiments. There were no significant difference between tested concentrations ($p < 0.05$).

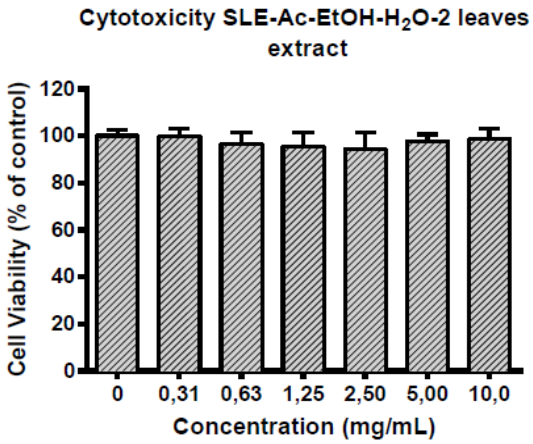


Figure 23. Cell cytotoxic analysis in Caco-2 cells treated for 1 h with different concentrations of sea buckthorn leaves SLE-Ac-EtOH-H_2O-2 extract. Results were expressed in terms of mean ± SD of three independent experiments. There were no significant difference between tested concentrations ($p < 0.05$)

There are some similar reports in literature concerning sea buckthorn berries and leaves extracts. Based on one of the studies describes four subspecies of Sea buckthorn (*H. rhamnoides* L. subsp. *sinensis* (Sinensis), *H. rhamnoides* L. subsp. *yunnanensis* (Yunnanensis), *H. rhamnoides* L. subsp. *mongolica* (Mongolica) and *H. rhamnoides* L. subsp. *turkestanica* (Turkestanica) with cytotoxic effects, using HepG2 cell line. The results shown that Sinensis and There are some similar reports in literature concerning the cytotoxicity assessment sea buckthorn berries and leaves extracts. For example, one of the studiesdescribed cytotoxic effects of four subspecies of Sea buckthorn (*H. rhamnoides* L. subsp. *sinensis* (Sinensis), *H. rhamnoides* L. subsp. *yunnanensis* (Yunnanensis), *H. rhamnoides* L. subsp. *mongolica* (Mongolica) and *H. rhamnoides* L. subsp. *turkestanica* (Turkestanica) on HepG2 cell line. These researchers found that half maximal cytotoxicity concentration (CC50) ranged from 8.31 to 16.8 mg/mL of sea buckthorn extracts in different subspecies. [72]. In another study, BHK-21 cell line sensitivity towards and sea buckthorn leaves aqueous and hydroalcoholic (ethanol/water 70/30 %) extracts was investigated. The cytoprotective activity was noticed with 250 μg/ml of each extract [86]. Furthermore, sea buckthorn leaves ethylacetate fraction was tested on PC-12 cells and extract had no cytotoxicity up to 20 μg/ml concentration.

To the best of our knowledge, this is the first report showing that both non-polar and polar extracts from sea buckthorn pomace and leaves are not cytotoxic to Caco-2 cells up to 10 mg/mL concentration.

3.8 Evaluation of the cellular antioxidant activity of selected sea buckthorn pomace and leaves extracts

The cellular antioxidant activity assay (CAA)[2] is used to quantify the antioxidant activity for selected extracts and dietary supplements at the cellular level [72]. There are two opportunities for compounds to exert their antioxidant effects in CAA assay. They can act at the cell membrane and break peroxyl radical chain reaction at the cell surface, or they can be taken up by the cell and react with ROS intracellularly [84]. Therefore, CAA assay is a valuable tool for measuring the antioxidant activity of antioxidants in cell culture [84]. It is an improvement over the traditional *in vitro* antioxidant activity assays (ORAC, HOSC, HORAC), because it mimics some of the cellular processes that occur *in vivo*.

The results from extract cytotoxic assessment showed that selected fraction () could further used for the cellular antioxidant activity assay. The results of CAA values of sea buckthorn pomace and leaves selected extracts were expressed as μmol quercetin equivalents per gram of extract of crude (unextracted) plant material (mg QE/g of extract and mg QE/g DW) and presented in Figures 24 and 25, respectively.

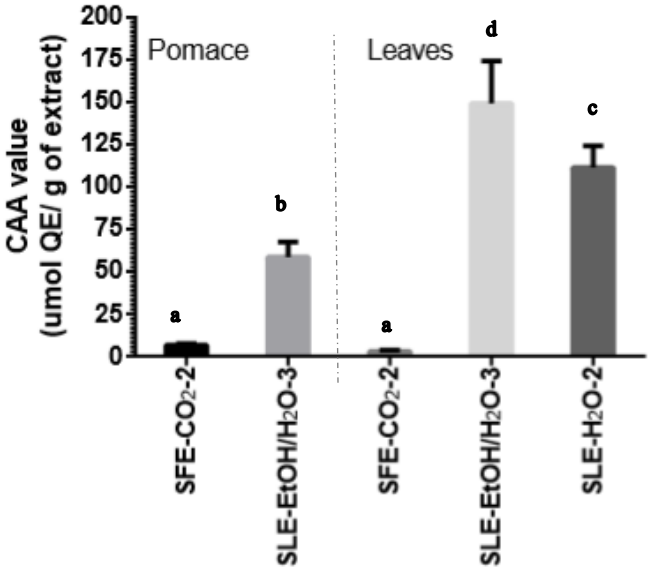


Figure 24. Cellular antioxidant activity (CAA) of selected sea buckthorn pomace and leaves extracts mean (μmol QE/mg of extract ± SD). Bars with different letters are significantly different ($p < 0.05$).

[2] The analysis was prepared in IBET – Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal

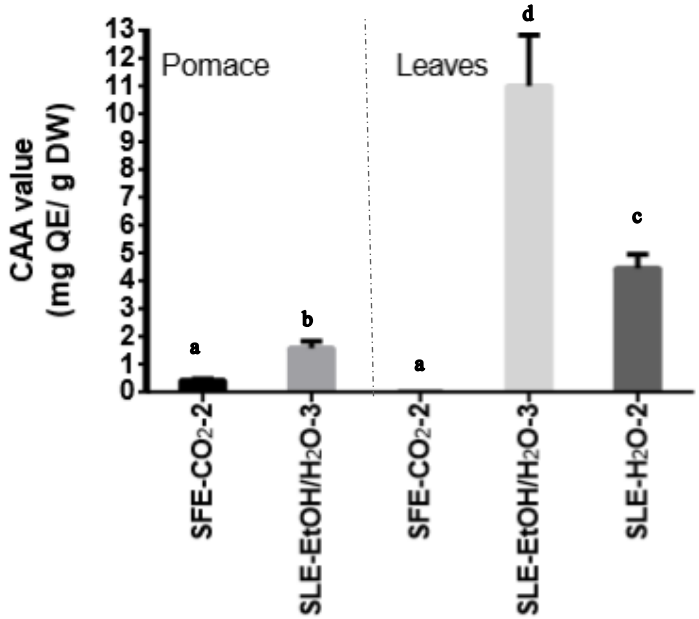


Figure 25. Cellular antioxidant activity (CAA) of selected sea buckthorn pomace and leaves extracts mean (mg QE/mg of DW ± SD). Bars with different letters are significantly different ($p < 0.05$).

Values of selected extracts cellular antioxidant activity were measured in the interval of 6.63 – 58.58 μmol QE/mg and 2.88 – 149.33 μmol QE/mg for sea buckthorn pomace and leaves, respectively (or 0.41 – 1.58 mg QE/g DW and 0.02 – 11 mg QE/g DW, respectively). The cellular antioxidant activity of various sea buckthorn by-product extracts increased in the following order: pomace extracts SFE-CO_2-2<SLE-EtOH/H_2O-3; leaves extracts SFE-CO_2-2<SLE-Ac-EtOH-H_2O-2<SLE-EtOH/H_2O-3 leaves. The same tendency was obtained with sea buckthorn pomace and leaves in ORAC assay, which was expected since both methods utilize peroxyl radical as the oxidation initiators. Moreover, there is no significant difference between non-polar extracts (mg QE/g DW). The high content of phenolic compounds at leaves extracts [4] can explain the higher antioxidant activity here observed, where sea buckthorn leaves SLE-EtOH/H_2O-3 (70/30 % v/v) extract got bigger CAA value. . Previous studies reported that sea buckthorn berries ethyl acetate fraction CAA values were between 11 and 197 μmol QE/100 g DW using HepG2 cell line [72]. CAA values presented in literature are similar as received in this research work with Caco-2 cell line.

From the literature data it is known that different phytochemicals exert different response in the CAA assay. For example, favonoids were likely well-absorbed by Caco-2 cells, while ascorbic acid, gallic acid, caffeic acid and catechin had less than 10% of the activity of quercetin in the CAA assay. Hydrophobic flavonoids may become deeply embedded in membranes, where they can influence membrane fluidity and break oxidative chain reactions. More polar compounds interact with membrane surfaces via hydrogen bonding, where they are able to protect membranes from

external and internal oxidative stresses [154]. The preliminary phytochemical composition analysis showed the presence of flavonoids in sea buckthorn pomace and leaves SLE-EtOH/H_2O-3 and SLE-Ac-EtOH-H_2O-2 extracts, which could be partially explained why these extracts have the highest activity in cellular antioxidant assay with Caco-2 cells.

This study shows that the cellular antioxidant activity assay may offer an additional advantage over the *in vitro* methods used to evaluate the antioxidant efficacy of pure phytochemical compounds, plant extracts, and dietary supplements, since cellular uptake, distribution, efficiency on protection against peroxyl radicals under physiological conditions and potential bioactivity of substances are taken into consideration.

CONCLUSIONS

1. The following chemical composition were determined for sea buckthorn by-products:
    - Sea buckthorn pomace: lipid content 23.4 g/100g, total nitrogen content 11.9 g/100g, mineral content 2.5 g/100g and dry mass 94.2 g/100g, TPC=21.6 mg GAE/g DW, $TEAC_{ABTS}$=104.5 mg TE/g DW;
    - Sea buckthorn leaves: lipid content 4.25 g/100g, total nitrogen content 17.34 g/100g, mineral content 4.54 g/100g and dry mass 88.68 g/100g TPC=58.79 mg GAE/g DW, $TEAC_{ABTS}$=315.12 mg TE/g DW.
2. Using convectional extraction (Soxhlet extraction, SLE) and high-pressure (SFE-CO_2, PLE-He) extraction techniques for sea buckthorn pomace and leaves valorization, non-polar constituent yields varied from 19.0 to 23.4 g/100g DW and from 2.2 to 4.3 g/100g DW, respectively, while 2.9-9.0 g/100g and 2.8-19.7 g/100g of polar fractions were obtained, respectively. On average, 21 g/100 g of lipophilic fraction was obtained with no significant differences between different extraction methods tested. The most effective extraction for polar constituent isolation was SLE using EtOH/H_2O (70/30% v/v) mixture. Proposed multi-step sea buckthorn by-product biorefining scheme consequently combines SFE-CO_2 and SLE-Ac-EtOH-H_2O extractions, allows to obtain 34.3 g/100g extractable constituents (65 % non-polar and 35% polar) from sea buckthorn pomace and 26.4 g/100g extractable constituents (8% non-polar and 92% polar) from sea buckthorn leaves and offers an advantage of using mainly food-grade solvents (CO_2, ethanol and water).
3. The fatty acid composition of non-polar sea buckthorn pomace extracts contained 31.4-41.3 % of saturated fatty acids, 33.6-42.2% of monounsaturated fatty acids and 7.4-8.3% of polyunsaturated fatty acids. The dominant fatty acids of sea buckthorn pomace are palmitic acid (30.1-40.3 %), palmitoleic acid (16.5-24.0 %), oleic acid (17.1-19.5 %), linoleic acid (5.2-6.3 %) and α-linolenic acid (1.6-2.3 %). The highest amounts of saturated fatty acids were present in SFE-CO_2 extraction, the highest amount of monounsaturated fatty acids was found after Sox-He and SFE-CO_2 extractions, while the highest content of polyunsaturated fatty acids was obtained with PLE-He extraction method. Three major fatty acids – linolenic acid (21.4-25.2 %), oleic acid (20.7-26.5 %) and palmitic acid (12-14.4 %) – were detected in lipophilic fractions of sea buckthorn leaves.
4. In non-polar extracts of sea buckthorn pomace and leaves 5 compounds were tentatively identified, mainly belonging to fatty acids group. In-semi polar and polar sea buckthorn pomace

and leaves extracts 26 compounds were tentatively identified, mainly belonging to chemical groups of flavonoids, organic acids and disaccharides.

5. Antioxidant capacity of non-polar and polar constituents of sea buckthorn by-products were in the following ranges:
   - Sea buckthorn pomace: total phenolic content and *in vitro* radical scavenging capacity of various sea buckthorn pomace extracts ranged from 2.27 to 20.58 mg GAE/g of pomace and from 15.18 to 106 mg TE/g and from 5.9 to 19.3 mg CAE/g of pomace. The highest activity was obtained for SLE-EtOH/H_2O-3 and SLE-Ac-2 extracts, while the lowest – for PLE-He-1, SFE-CO_2 and SLE-Ac-EtOH-2 fractions.
   - Sea buckthorn leaves: total phenolic content and *in vitro* radical scavenging capacity of various extracts ranged from 1.97 to 56.75 mg GAE/g and from 8.91 to 368 mg TE/g and from 12.01 to 103.8 mg CAE/g of leaves. SLE-EtOH/H_2O-3 extract showed the highest activity in all assays, while SFE-CO_2 was the least active fraction.
   - Proposed biorefining scheme of sea buckthorn pomace and leaves (SFE-CO_2 + SLE-Ac-EtOH-H_2O-2 extractions) allowed to obtain the highest TPC (26.2 mg GAE/g DW and 83.7 g GAE/g DW for pomace and leaves, respectively), and the strongest $ABTS^{\bullet+}$ scavenging properties (59.6 mg TE/g DW and 180.2 mg TE/g DW for pomace and leaves, respectively). Solid residues after the last step of consecutive valoraztion showed 85-91% lower TPC and 32-61% lower $TEAC_{ABTS}$ values, as compared to sea buckthorn pomace and leaves prior to the extractions.
6. The induction period of rapeseed oil with sea buckthorn pomace SFE-CO_2-2 lipophilic extracts was decreasing from 3.12 to 2.89 hours with the increasing amount of extract added from 0.5% to 5% (w/w). Therefore, only lower than 0.5% concentrations of CO_2 derived pomace fraction could be used developing novel products. Vice versa, rapeseed oil stability was significantly increasing with the addition of sea buckthorn leaves extract. At the highest concentration tested (5 % w/w), the induction period of sample (4.95 hours) was 1.5-fold higher as compared with raw rapeseed oil (control).
7. Selected sea buckthorn pomace (SFE-CO_2-2 and SLE-EtOH/H_2O-3) and leaves (SFE-CO_2-2, SLE-EtOH/H_2O-3 and SLE-Ac-EtOH-H_2O-2) extracts did not show cytotoxic effect on Caco-2 cell line after 1h incubation in the range of tested concentrations for non-polar and polar extracts: 0.01 – 5 mg/ml, and 0.31 – 10 mg/ml, respectively.

8. The results of cellular antioxidant activity on Caco-2 cells of sea buckthorn pomace (SFE-CO_2-2 and SLE-EtOH/H_2O-3) and leaves (SFE-CO_2-2, SLE-EtOH/H_2O-3 and SLE-Ac-EtOH-H_2O-2) selected extracts were 0.41 – 1.58 mg QE/g DW and 0.02 – 11 mg QE/g DW, respectively. The highest values were recorded for SLE-EtOH/H_2O-3 extracts, while the lowest cellular antioxidant activity was obtained for SFE-CO_2 extracts of sea buckthorn pomace and leaves. These results are well in agreement with *in vitro* antioxidant activity (TPC, $ABTS^{\bullet+}$, ORAC, HOSC, HORAC) assays.

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