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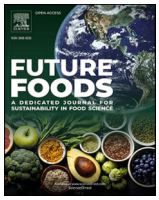

Optimization of ultrasound assisted extraction of the Sea buckthorn leaves, characterization of the phenolic compounds, and determination of bioactive properties of the extracts

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ABSTRACT

Sea buckthorn leaves are known to be rich in antioxidants and phenolic compounds. However, non-proper processing methods can result in a decrease of the beneficial properties. To address this, response surface methodology (RSM) was used in the modelling and optimizing of the ultrasound assisted extraction of the sea buckthorn leaves to obtain high yields of total phenolics content in the extract. The alike obtained extracts were subjected under investigations of bioactivities such as the antioxidant activities (FRAP and ORAC), antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* and anti-inflammatory characteristics. The results indicated that the extracts obtained under the optimal process conditions of 30 min, continuous pulse, and solid to liquid ratio of 0.2 showed antioxidant activity, and antibacterial activity against both Gram+ and Gram– bacteria at a moderate level. The extracts are rich in polyphenolic compounds, such as ellagic acid and flavonoids. The *in vitro* anti-inflammatory tests with THP-1 promonocyte model indicated that the sea buckthorn leaf extract obtained by ultrasound assisted extraction has powerful anti-inflammatory properties. These results prove that the ultrasound assisted extraction of sea buckthorn leaves provides a good source of phenolic compounds that have versatile bioactive properties and can generally be regarded as health promoting food compounds.

1. Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a deciduous shrub, which is acknowledged for its nutritional and medicinal properties and grows native in the cold-temperate regions of Europe and Asia (Hyvönen, 1996; Rousi, 1971). While it is mostly known for its orange-yellow berries with one of the highest nutrient contents of all the wild berries, sea buckthorn is also used for soil, water, antidesertification, and wild-life preservation purposes because of their strong roots and nitrogen fixation properties (Yang et al., 2005). For the commercial berry production, a common harvesting technique in Europe is to cut entire branches with berries using various ways (Janceva et al., 2022; Tkacz et al., 2021). Thus, during harvesting and post-harvest management of berries, the sea buckthorn leaves form a notable by-product (Bośko et al., 2024) that can be used e.g., for tea. Besides, the leaves are known to be rich in antioxidants, vitamins (A, C, and E) and flavonoids (Kumar et al., 2011; Kwon et al., 2017). In addition, leaves have been shown to possess beneficial antioxidant, antimicrobial, and immunomodulatory properties (Ganju et al., 2005; Upadhyay et al., 2010). The chronic silent inflammation and visceral obesity are major causative factors in several widespread diseases connected to western diet and lifestyle e.g., distorted hepatic and lipid metabolism, type 2 diabetes, hypertension, vascular diseases, arthritis, and older age memory diseases.

Although the health effects of sea buckthorn berries have been studied a lot, the in vivo studies with animal models on leaf extracts are still scarce (Ganju et al., 2005; Kwon et al., 2017; Lee et al., 2011). In these studies, the leaf extracts have showed significant health promoting effects (for reviews, see: (Kania-Dobrowolska et al., 2023; Ma et al., 2022; Shah et al., 2021)).

Lee et al. added for 6 weeks 1 % or 5 % (w/w) of roasted, dried sea buckthorn leaf powder to high fat feeds in obese mouse model (Lee et al., 2011). The leaf powder suppressed weight gain and visceral fat accumulation in dose dependent manner and lowered levels of leptin,

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triglycerides and total cholesterol as compared to high fat fed control mice. Also, biomarkers of hepatic and antioxidant metabolism were significantly normalized. In a later study by Kwon et al., sea buckthorn leaf extracts (from 80 % EtOH – extraction) and a glycoside extract were added to diet in a mouse obesity model (Kwon et al., 2017). Both leaf extract and flavonoid glycoside extracts were included 0.04 % or 1.8 % for (w/w) from 12 weeks to high fat diet of C57BL/6j mice. Both extracts suppressed lipogenesis and adiposity while increasing the energy expenditure and alleviated hepatic steatosis, decreased plasma inflammatory cytokine levels improved dyslipidemia and insulin sensitivity. Thus, sea buckthorn leaves can be a valuable health promoting part of the human diet as well.

To fully utilize the anti-inflammatory potential of leaves as raw material of health promoting products a scalable extraction solution based on water-ethanol extraction was tested *in vitro* in the present study.

It has been shown that flavonoid glycosides content in the berries is dependent on the environment: the total contents of flavonol di- and triglycosides in both varieties were higher in the north than in the south, whereas total flavonol monoglycoside content behaved vice versa ($p < 0.05$) (Zheng et al., 2016). It has been shown that polyphenols in the leaves were higher in the beginning of the 7-week harvest period. Growth location, namely north-south, comparison revealed fatty acids and sugars as discriminatory metabolites (Pariyani et al., 2020). Suvanto et al. further showed that the sea buckthorn leaves are a rich source of ellagitannins by investigating the concentration of ten different compound in different leaves (Suvanto et al., 2018). The total concentrations reached 42.5 to 109.1 mg g^{-1} DW and a new compound, Hippophaenin C, was detected for the first time (Suvanto et al., 2018).

Phytosterols and flavonols in leaves were investigated by Hellström et al. and the authors concluded that the leaves are rich sources of both compounds, which in turn have potential health promoting benefits (Hellström et al., 2015). The collection time of the leaves had an influence on the total availability of both compounds, phytosterols had the highest concentration in September where the berries are already fully ripened, while the flavonol concentration was its highest in early July, when the fruits were evolving (Hellström et al., 2015).

The concentration of bioactive compounds in the sea buckthorn leaves are not only determined by the collection time, but also by the processing method, as the high processing temperature might decrease the functionality of the extracts (Pap et al., 2021). The total phenolic contents might be affected, as for example in the berry fruit extract a total phenolic content reduction of 85–89 % was observed when temperature of 60 °C was applied for 5–20 min. This further increased up to 95 % when the temperature was increased to 100 °C for 25 min (Ursache et al., 2017).

For this reason, the aim of this work was to i) extract phenolic compounds from sea buckthorn leaves using ultrasound assisted extraction with a cooling loop to avoid overheating, ii) optimize the extraction using response surface methodology, iii) determine the biofunctionality of the extract obtained at the optimum process parameters such as the antioxidant capacity, antibacterial capacity using both Gram positive and Gram negative bacterial strains, and anti-inflammation properties.

2. Materials and methods

2.1. Collection of raw materials

Sea buckthorn leaves (var. Raisa) were collected in Nurmela, Finland, in September 2016. The leaves were stored in −20 °C until further use.

2.2. Extraction and design of experiments

The ultrasound assisted extraction of the phenolic compounds was carried out with Hielscher UP 400 St equipment (Hilscher Ultrasonics, Germany), where the samples were cooled at +8 °C. The tests were based on the design of experiments with a software MODDE 12.1 (Umetrics, Sweden), using Central Composite Face (CCF) design and quadratic model. The experiments were based on three factors: the solid to liquid ratio (0.1 to 0.2), pulse (10 % to 100 %) and extraction time (10 to 30 min), and the amplitude was fixed at 70 %. The experimental design included all together seventeen runs, with three replicates at the centre point to validate the model.The experiments were carried out in random order to avoid systematic error. The measured responses were the total phenolic contents measured after centrifugation of the extracts with 4000 *g* for 10 min at room temperature. The design is summarized in Table 1. From the analyses results an equation was derived including the significant terms while the non-significant terms was removed in the model refining stage. In our case these were the quadratic and the interaction terms. The centre point conditions are marked with a grey background in the table. and the established optimum conditions with bold letters and numbers. The optimum process parameters were determined using the optimizer function of the software and two additional tests were carried out at the optimum conditions to verify the fit of the model. As a last step, the experiment was carried out at large scale (100 g sample+500 g 80 % EtOH) to deliver samples for the biofunction tests. The ethanol was evaporated using Rotavapor (R-100, Finland).

The original leaf biomass was extracted twice with the obtained optimum conditions to obtain parallel samples A and B and to see the potential differences caused by the biomass itself. These samples were then subjected to the antibacterial analyses, antioxidant activity, total phenolic content, anti-inflammatory activity, and phenolic and other UV absorbing compounds determination to give further information about their beneficial properties.

2.3. Total dissolved solids (TDS)

Total dissolved solids (TDS) is one way to measure the extraction yield and was determined by overnight oven-drying of the extracts at 105 °C. After drying samples were placed into a desiccator to cool down and consequently weighted before TDS was calculated.

2.4. Antibacterial analyses, antioxidant activity, and total phenolic content

Extracts were analyzed for their total phenolic content by the Folin-Ciocalteu and Prussian blue methods. Ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) methods were used to evaluate the antioxidant activities. A Varioskan Flash multimode reader (Thermo Scientific, Thermo Electron Co., Waltham, MA, USA) in 96-well format was used for all the analyses. All reagents were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany unless otherwise stated.

2.4.1 The Folin-Ciocalteu method (Ainsworth and Gillespie, 2007; Singleton et al., 1999; Singleton and Rossi, 1965) was used for the measurement of the total phenolic content, which often correlates well with antioxidant activities. Samples were mixed with Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 20 % Na_2CO_3 (Merck KGaA, Darmstadt, Germany). After 30 min incubation at 40 °C in the dark, the absorbance was measured at 750 nm. The standard curve was prepared using gallic acid (0, 25, 100, 250, and 500 mg/L; stock solution 5 g/L (29.4 mM)). The results are expressed as gallic acid equivalents per gram extract dry weight (mg GAE /g).

2.4.2 Prussian blue assay for total phenolic content (TPC) is based on the ability of phenolic compounds to reduce chelated hexacyanoferrate (III) ion into hexacyanoferrate(II) ion, which reacts with Fe^{3+} to form a Prussian blue color. The method has been previously reported (Margraf et al., 2015). In brief, the samples were pipetted in technical triplicates and at least three dilutions into a microplate with 0.50 mM $FeCl_3 \bullet 6H_2O$ diluted in 0.01 M HCl. The reaction was initiated by injecting an aliquot

Table 1
Design matrix of the experiments. The extract obtained with conditions indicated in bold text was analyzed further.

Experimental runs		Factors			Response		
Exp no	Run order	S:L (g/L)	Pulse (%)	Time (min)	Observed TPC (mg GAE/L)	Predicted TPC (mg GAE/L)	Difference in %
1	12	0.1	10	10	905.2	831.75	8.1
2	5	0.2	10	10	1532.0	1604.3	4.7
3	15	0.1	100	10	1132.2	1193.0	5.4
4	2	0.2	100	10	2005.3	1965.6	2.0
5	17	0.1	10	30	1008.2	959.6	4.8
6	11	0.2	10	30	1713.0	1732.1	1.1
7	6	0.1	100	30	1289.5	1320.9	2.4
8	**16**	**0.2**	**100**	**30**	**2112.8**	**2093.4**	**0.9**
9	9	0.1	55	20	1043.4	1076.3	3.2
10	4	0.2	55	20	1878.0	1848.8	1.6
11	7	0.15	10	20	1056.9	N.A.	N.A.
12	1	0.15	100	20	1706.9	1643.2	3.7
13	8	0.15	55	10	1391.8	1398.7	0.5
14	14	0.15	55	30	1482.0	1526.5	3.0
15	3	0.15	55	20	1483.2	1462.6	1.4
16	13	0.15	55	20	1453.6	1462.6	0.6
17	10	0.15	55	20	1444.8	1462.6	1.2

TPC- total phenolics content.
N.A. - experiment no 11 was detected as outlier experimental run, therefore no predicted values existed for it.

of 0.50 mM $K_3[Fe(CN)_6]$ solution to the microplate and the plate was shortly shaken before incubating in dark. Absorbance was measured at 725 nm after 15 min using a microplate reader (Varioskan Flash, Thermo Scientific). The results are expressed as mg gallic acid equivalents (GAE) per extract dry weight (mg GAE / g).

2.4.3 The FRAP method is based on single electron transfer mechanism and measures the ability of antioxidants to reduce ferric Fe(III) to ferrous Fe(II) ions (Benzie and Strain, 1996). Four different dilutions of the ethanol-water extracts with three replicates for each were analysed as previously described (Fidelis et al., 2023). The reaction mixture contained 25 μM extract, 20 mM $FeCl_3$•6 H_2O and 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in 300 mM acetate buffer, pH 3.6. Reaction was started by addition of FRAP solution (acetate buffer, $FeCl_3$•6 H_2O, and TPTZ in proportion of 4:1:1). The plate was incubated at room temperature for 15 min, and the formation of a ferrous-tripyridyltriazine complex was measured by absorbance at 593 nm at 0 min of incubation and 15 min of incubation. A standard curve (0, 50, 200, 500, and 1000 μM) was prepared using $FeSO_4$•7 H_2O. L(+)-ascorbic acid (150 μM and 800 μM; VWR Chemicals) was used as a positive control. The results are expressed as μmol/g (extract dry weight) Fe(II) equivalents.

2.4.4 The ORAC method measures the ability of potential antioxidants to prevent peroxyl radicals from harming a fluorescent fluorescein molecule. The assay was modified from that of previously reported (Huang et al., 2002; Prior et al., 2003). Ethanol-water extracts of the leave samples with two technical replicates were measured with a series of five dilutions (1:1–1:320) as previously described (Fidelis et al., 2023). Additional dilutions were done when necessary. The reaction mixture contained the sample (50 μL) in 75 mM phosphate buffer, pH 7.5 (Merck), 150 μL of 0.0816 μM fluorescein (final concentration 0.054 μM) and 25 μL of 0.153 mM 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The reaction was started by addition of AAPH, and the 96-well plate transferred into the plate reader kept at 37 °C. Fluorescence was measured every minute for 40 min every 2 min. A standard curve was made with 50, 25, 12.5, and 6.25 μM of Trolox ((±)−6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). Finally, the results are expressed as Trolox equivalents (TE; μmol/g (extract dry weight)).

2.4.5 Antibacterial analyses were executed with a microplate method with bioluminescent indicator strains *Staphylococcus aureus* RN4220+pAT19 and *Escherichia coli* K12+pcGLS11 (Vesterlund et al., 2004) was used to study the antibacterial activity of the extracts. These strains have been constructed to produce a constant luminescent light signal and antibacterial effects can be observed as a loss of emitted light signal intensity. The storage, cultivation, and test protocol has been previously described (Jyske et al., 2023). In brief, the strains were stored at −80 °C and pre-cultivated approximately 16 h at 30 °C (for *E. coli*) and 37 °C (for *S. aureus*) in lysogeny agar plates (tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L; and agar 15 g/L). The LA plates were supplemented with 10 % (v/v) sterile filtered phosphate buffer (PB) (1 M, pH 7.0) and 100 μg/mL of ampicillin for *E. coli* and with 5 μg/mL erythromycin for *S. aureus*. Biosensor stocks were prepared by inoculating a single colony of bacteria in lysogeny broth with same supplements as plates. Stocks were cultivated for approximately 16 h at 300 rpm shaking at 30 °C (*E. coli*) and 37 °C (*S. aureus*). The sample extracts were evaporated dry, dissolved to small amount of DMSO and diluted with water to obtain contents of 0.25; 0.5 and 1 mg/mL of the sample extracts per microplate well with maximum of 1 % of DMSO per well. The sample concentrations, DMSO control, and positive (ethanol) and negative (double-distilled water) controls were pipetted in triplicates into opaque white polystyrene microplates with same volume of bacterial inoculation. The produced luminescent light signal was then measured using a Varioskan Flash Multilabel device (Thermo Fischer Scientific, Thermo Electron Co., Waltham, MA, USA) once every 5 min for 60 min at room temperature, and the plate was briefly shaken before every measurement. The results are expressed as inhibition% (Tienaho et al., 2015). Error bars represent the standard deviations between the sample triplicates. The incubation time 50 min was chosen to be the most descriptive time point to compare differences and content of 1 mg/mL of each extract.

2.5. Method for phenolic and other UV absorbing compounds by HPLC-DAD

For the determination and quantitation of phenolic and other UV-absorbing semi-polar compounds by liquid chromatography, the sample extracts (2 mL) were evaporated to dryness with a stream of nitrogen and redissolved in 1 mL methanol-water (4:1 v/v). Samples were filtered and analysed by Agilent 1100-series high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD) (Agilent, Santa Clara, CA, USA) as described before (Kahala et al., 2024). Compounds were separated on Phenomenex Kinetex® C18 (150 × 3.0 mm; 5 μm i.d.; 100 Å) column at 35 °C. The mobile phase consisted of 0.05 M phosphate buffer (A) at pH 2.4 and methanol (B) with the following gradient: 5 − 60 % B in 45 min; 60−98 % B in 10 min, 98−100 % in 10 min, and at 100 % for 20 min. The flow rate was 0.6 mL/min. The chromatograms were obtained at 245, 280 and 350 nm wavelengths and

UV spectra were recorded at 190−600 nm. Based on the in-house UV spectral libraries and combined with the retention times of reference compounds, some analytes were identified, while others were only putatively identified. Quantitation of the main components was done at 280 nm by gallic acid and ferulic acid at 350 nm (Sigma-Aldrich, Burlington, MA, USA). Flavonoids and ellagic acids, were identified as such by their typical UV-spectra and quantitated as quercetin and ellagic acid (Sigma-Aldrich) at 350 nm, respectively.

2.6. Methods for anti-inflammatory activity

2.6.1. Reagents and solutions for leukocyte experiments

Stock solution of 20 mM luminol (5-amino-2,3-dihydro-1,4-pthalazinedione; ICN Biomedicals, Aurora, OH, USA) was made in 0.2 M sodium borate buffer (pH 9.0). Solution was warmed up to 50 °C and shaken until the crystals were dissolved. Zymosan powder (zymosan A from yeast *Saccharomyces cerevisiae*; Sigma-Aldrich) was autoclaved (120 °C for 20 min) in phosphate buffered saline (PBS, pH 7.4) in concentration of 10 mg/mL, washed three times with centrifugation (10 min at 1000 *g*) in HBSS buffer (Hank's balanced salt solution, without phenol red; pH 7.4) and finally a 10 mg/mL solution of zymosan was made in HBSS buffer. The zymosan was opsonized with fresh human serum sample from a healthy adult volunteer and by incubating the zymosan with serum in water bath for 30 min at 37 °C with continuous gentle mixing. The opsonization mixture consisted of 50 % serum and of 50 % (v/v) zymosan solution in HBSS (5 mg/mL). The opsonized zymosan (OZ) was then washed three times with HBSS buffer as described above and suspended in HBSS (5 mg/mL) and divided into 1–2 mL aliquots and stored at −80 °C. Lipopolysaccharide (LPS) from *E. coli* 055:B5 (LPS; Sigma-Aldrich) and horseradish peroxidase (HRP; Merck 10,814,407,001 Roche, Germany) were dissolved in HBSS buffer to obtain 2.5 mg/mL and 800 U/mL solutions, respectively. LPS stock solution was divided into aliquots and stored at −22 °C. HRP solution HRP was prepared weekly by dissolving into PBS (pH 7.4) and was kept at +4 °C maximum five days.

2.6.2. THP-1 cell culturing

THP-1 human promonocytes leukemic cell line (Leibniz Institute, DMSZ German Collection of Micro-organisms and Cell Cultures) was cultured in RPMI 1640-GlutaMAX™ medium (Gibco® Invitrogen™, USA) supplemented with 10 % of heat-inactivated (30 min at 56 °C) fetal bovine serum (FBS, Gibco), 10 mM HEPES (BioWhittaker, Lonza), 1 mM sodium pyruvate (Gibco), 2.0 g/L [+]D-glucose (Gibco), 0.5 mM 2-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), 50 U/mL penicillin and 50 μg/mL of streptomycin (Gibco). Cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. Medium was changed every 3–4 days and cells were sub-cultured with resuspension of 2×10^5 viable cells/mL and the density of viable cells was not allowed to rise higher than 9×10^5 cells/mL. The viable cell concentration was counted by staining with 0.4 % trypan blue solution and counted by microscope and Bürker chamber or with Countess II FL cell counter (Invitrogen).

2.6.3. Sample preparation

The sample was in a liquid form suspension (EtOH% 80). The ethanol was removed by pouring the suspension as thin layers into Petri dishes and EtOH was evaporated overnight in a hood at RT. The suspension was centrifuged by 4500 *g* for 20 min in order to remove undissolved material. The supernatant was finally put through a 0.20 μm filter to remove microparticles. A sample was taken from permeate and dry matter content was measured from that by drying in oven at 98 °C for 1 d and weighting. After determining the dry weight, the pH of the permeate was adjusted to physiological 7.4 by adding 1 N NaOH or HCl. Then HBSS-buffer (pH 7.4) was added to obtain extract concentration of 15 mg/mL according to the known dry weight. The buffered sample was stored in aliquots at −22 °C. The anti-inflammatory activity was calculated according to the dry weight taken before the pH adjustment to avoid error due to added salts.

2.6.4. THP-1 cell activity

Kinetic measurements of THP-1 cell respiratory burst (RB) activity were conducted with a microplate reader (Hidex Sense, Turku, Finland) using white 96-well flat bottom non treated microtiter plates (Nunc 36,105, Roskilde, Denmark) as previously described (Tompa et al., 2011). Cells in passages 10–40 after thawing were used. The samples are put into microtiter wells in 50 μL and the plate is warmed to 37 °C in water bath. One bottle of ~18 mL (around 7×10^5 cells/mL) is washed once by centrifuging at 300 *g* for 5 min and gently suspended into 11.7 mL of gel-HBSS (HBSS buffer supplemented with 0.1 % w/v gelatin, gelatin A from swine skin, Sigma-Aldrich) at room temperature (RT) to obtain a concentration of about 1×10^6 cells/mL.

Cells (100 μL) were transferred into wells of a white flat bottom microtiter plate together with 50 μL of the sample fractions. The THP-1 cells (around 10^6/mL; 10^5 cells per well) were incubated in microtiter plate (in thermal chamber) with different concentrations of extracts at 37 °C for 15 min. Then part of the cells was primed to inflammatory state cells with LPS solution, and the incubation was continued for 35 min at 37 °C and part was kept unprimed. The final LPS concentration in well was 10 μg/mL. Part of the cells was not primed and HBSS was added instead of LPS. After altogether 50 min preincubation with extracts the plate was quickly put into luminometer and kept in luminometer measuring chamber for 5 min to stabilize the sample temperature to 37 °C. Then the reaction was started by promptly adding opsonized zymosan together with luminol and HRP in 50 μL volume.

Leukemic promonocytes have different properties from native peripheral blood monocytes. Since THP-1 cells do not express myeloperoxidase, HRP was added as a substitute to obtain a CL emission with sufficient intensity for reliable measurements. Luminol enhanced CL emission from leukocytes is solely dependent on peroxidase activity.

During the RB reaction the final concentration of HRP was 25 U/mL, luminol 0.5 mM and opsonized zymosan 250 mg/mL. The chemiluminescence (CL) signal (relative light units, rlu) was monitored for 90 min at 2.33 min intervals (integrated for 0.5 s) to obtain kinetic curves. The CL peak was regarded as the CL value of the sample representing the highest reaction activity. The areas of the CL curves were calculated and regarded as measures of total radical production during the RB. The efficacies of extracts were expressed as IC50-values i.e., concentrates which inhibit 50 % of the CL peak emissions or areas obtained from cells without extracts in same plate. In dose response curves the concentrations of extracts are those during the incubation with cells and LPS/HBSS.

2.7. Statistical analysis

A standard two-tailed *t*-test was used for antioxidant, antibacterial and total phenolic composition to determine the statistical significance of the differences between two parallel extract replicates obtained at the optimal conditions. Comparisons of IC_{50} values between activities of primed and non-primed cells THP-1 cells were made using the paired Student's *t*-test with Microsoft Excel program.

3. Results and discussion

3.1. Determination of optimum processing parameters

To establish a statistical model between the factors and the response, the total phenolics content was measured from all seventeen samples. The factors, the observed and predicted total phenolic conent as a response are described in Table 1. The differences in between the observed and predicted value was also calculated in included in the Table 1, indicating plus or minus values.

The model was fitted with multiple linear regression (MLR), and the analyses phase carried out which identified one outlier (run number 11).

To improve the reliability of the model, this run 11 was removed from the analyses which in turn showed the better values of R^2, R^2adj. and Q^2 to 0.984, 0.980 and 0.965, respectively, while the reproducibility was 0.996 and model validity 0.494.

Analyses of variance (ANOVA) were also carried out next and it revealed that the model is statistically good ($p = 0.000$) and there is no lack of fit in the model ($p = 0.133$). Based on this information, the model is viable to predict the optimum processing parameters that the optimizer function of the software allows to perform.

Besides, the analyses of coefficients showed that the only important terms in the function of the total phenolic contents were the major terms of S:L ratio, pulse and time, and the connection can be described by the following first order equation:

$$Y = 386.27x_1 + 180.63x_2 + 63.91x_3 + 1462.5 \quad (1)$$

where x_1 is the solid to liquid ratio, x_2 is the pulse, x_3 is the time of the extraction.

As the last step of the response surface methodology, the optimal process parameters to achieve the highest total phenolic content is determined by the optimization function. The results indicated that the solid:liquid of 1:5 (0.2 in the experimental design), the pulse of 100 % intensity and 30 min extraction time are the optimum parameters of the set up. The results are illustrated in Fig. 1 on the response surface plot. The numerical values are also indicated that at the optimum point, the % difference between the predicted and the observed values is 0.9 %.

Using these parameters, sample A and B were prepared, the achieved total phenolic contents were 2612.9 ± 27.1 and 2503.8 ± 20.2 mg/L, which are higher values then in the modelling phase. This phenomenon might be caused by the test runs being carried out at a different scale at the end of the experimental design.

3.1.1. Phenolic and other UV absorbing compounds by HPLC-DAD

The main peaks, intensity-wise (mAU), in the 280 nm chromatograms were gallic acid (RT 3.1 min), unknown compounds at 11.4 and 50.8 min, ellagic acids and various flavonoids. The latter two groups were the dominant peaks in 350 nm chromatogram. There was also a broad peak at 280 nm at retention time range 10–25 min with a number of small undistinguished peaks which are typical to poly- and oligomeric phenolics (Fig. 2a). Examples of the UV-spectra of peaks in the chromatograms are presented in Fig. 2b.

The retention time of ellagic acid standard was 28.05 min, but besides this, three other peaks showing similar UV-spectrum was present, at retention times 27.35, 27.74 and 28.56 min. However, they accounted only 19, 12 and 15 % of the sum of ellagic acids, respectively. The total amount of ellagic acid was 9.32–13.16 mg g^{-1} in extract DW. (Table 2).

Thirteen peaks were showing typical UV-spectrum of flavonols, like isorhamnetin, kaempferol and quercetin, and these were quantitated as quercetin at 350 nm (Fig. 2a). Their sum amount was 7.63–10.66 mg g^{-1} in extract DW (Table 2) and the results in biomass DW mg g^{-1} were similar to those of (Hellström et al., 2015).

Catechins and proanthocyanidins has been reported in sea buckthorn leaves (Raudone et al., 2021; Wang et al., 2022; Zheng et al., 2019), but due to the interfering compounds, they could not be identified or detected with our analytical setup.

Of the other compounds, gallic acid was present at 0.53–0.80 and free ferulic acid at 0.18–0.29 mg g^{-1} in extract DW.

Gallic acid has been reported in sea buckthorn leaves (Criste et al., 2020; Raudone et al., 2021; Wang et al., 2022). Our results as in biomass DW mg g^{-1} were well in line with Raudone et al., 2021 but higher, as also in case of ferulic acid, than those of Criste et al. (Criste et al., 2020).

3.2. Antioxidant and antibacterial analyses

The results of the analysis for total phenolics using Folin-Ciocalteu and Prussian blue methodologies, along with antioxidant and antibacterial assessments, are summarized in Table 3. Extract A exhibited superior activity in the Folin-Ciocalteu, Prussian blue, and FRAP assays, whereas extract B demonstrated higher efficacy in ORAC and antibacterial evaluations. The difference of the parallel extract samples is also seen in the phenolic composition characterized by HPLC-DAD in Table 2 and is likely caused by heterogeneity in the original extractable biomass. Tkacz et al. reported that the total phenolic content (TPC) in sea buckthorn leaves varies depending on the cultivar, ranging from 1095.45 ± 6.56 to 9280.13 ± 38.2 mg/100 g DM (Tkacz et al., 2021). Notably, the TPC results obtained in our study using the Folin-Ciocalteu test were slightly higher. This observation is consistent with previous findings, as Folin-Ciocalteu results often surpass those obtained via chromatography due to the presence of interfering substances such as ascorbic acid, monomeric sugars, and aromatic amines (Lester et al., 2012). The Prussian blue test has been proposed as a viable alternative to the Folin-Ciocalteu method due to its rapidity and simplicity (Margraf et al.,

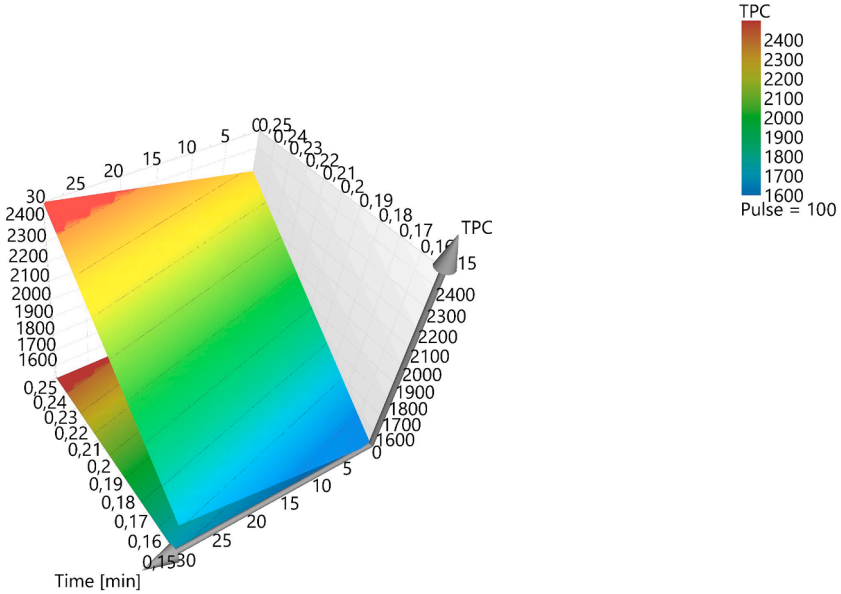

Fig. 1. Response surface plot from the optimization of the sea buckthorn leaves ultrasound assested extraction.

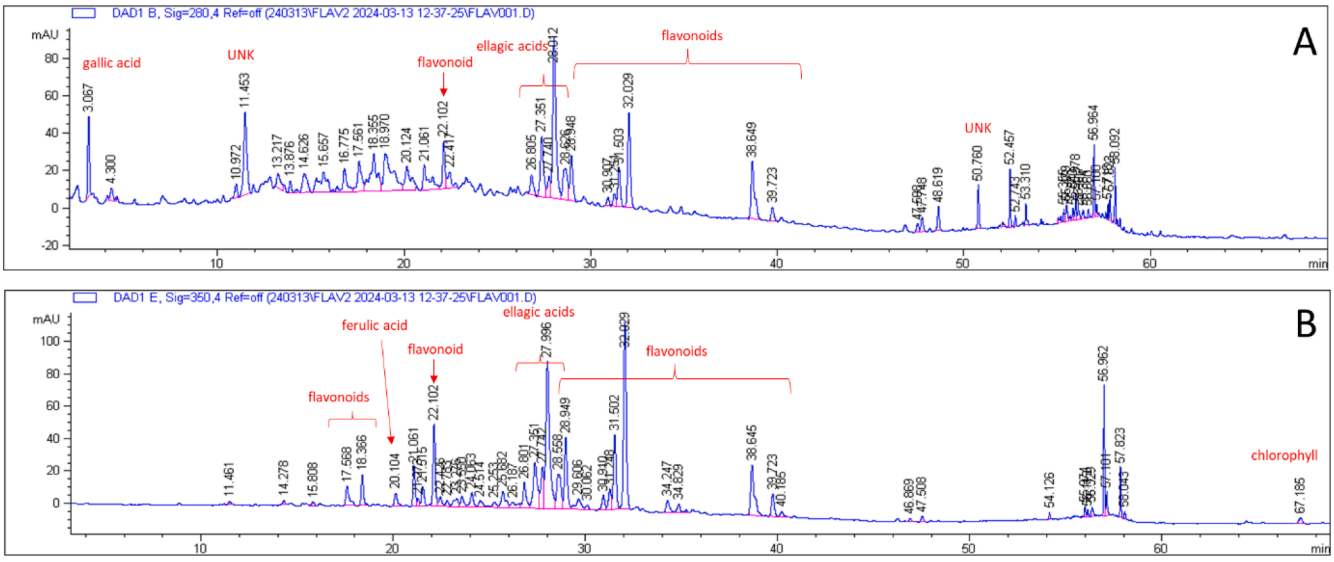

Fig. 2a. Example of the HPLC chromatogram of sample extract B at the wavelengths of 280 nm (A) and 350 nm (B). Marked compounds are those presented in Table 2. Carotenoids and chlorophylls elute after 54 min.

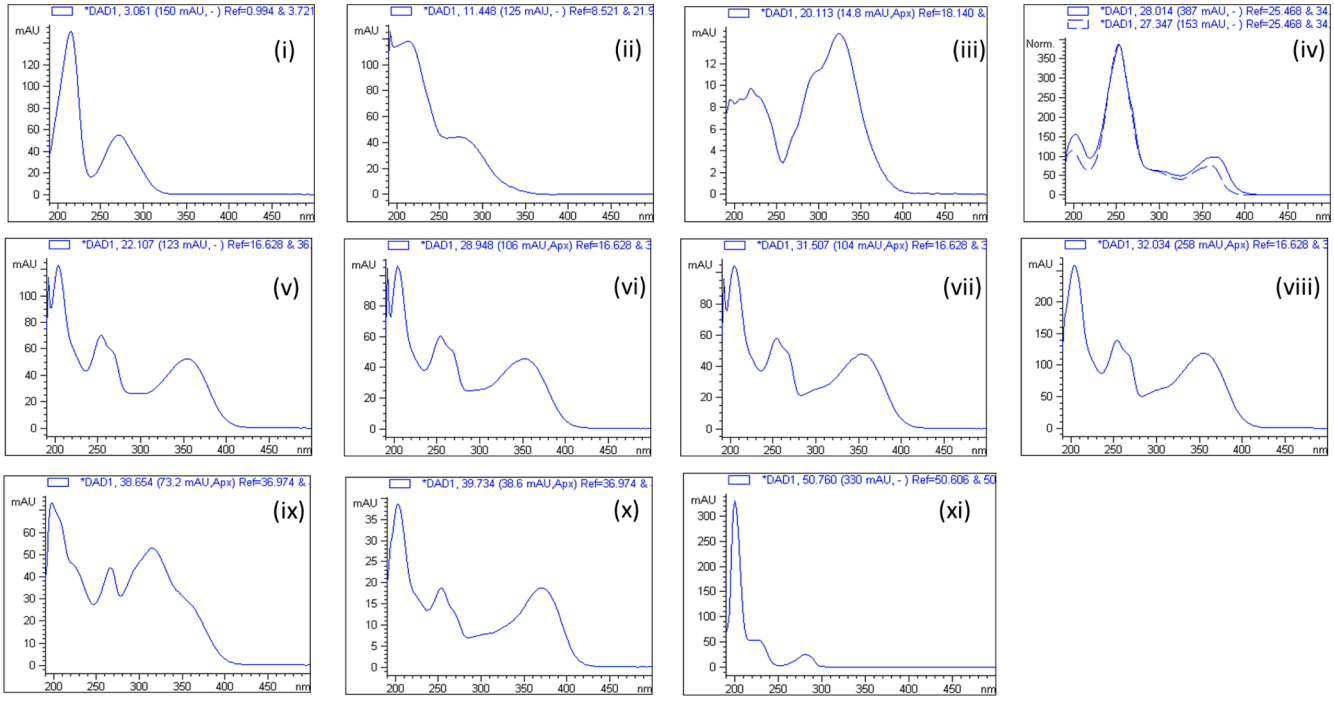

Fig. 2b. Examples of the UV-spectra of selected peaks HPLC chromatograms in Fig. 2a. Compounds: (i) gallic acid, (ii) unknown I, (iii) ferulic acid, (iv) ellagic acids, (v - x) flavonoids and (xi) unknown II.

2015). Studies have shown that the Prussian blue test correlates well with Folin-Ciocalteu results, albeit yielding slightly lower values (Pap et al., 2021), likely owing to its enhanced selectivity towards phenolic compounds. Both the Prussian blue and FRAP methods are based on the reduction reaction of iron complexes, which could explain the observed correlation between their outcomes. TPC and FRAP results also correlate well with the identified phenolic compound content (Table 2) by being generally more abundant in extract A. The obtained TPC results are slightly higher while ORAC results are very well in line with previously published results (Pap et al., 2021). ORAC results are also highly correlated with results obtained using microwave-assisted ethanol extraction of sea buckthorn leaf extracts (Ćulina et al., 2024). Difference in TPC is likely caused by both the solvent change and the ultrasound assistance used in the extraction of the present study. ORAC active substances have also previously been found to be less susceptible to the changes in biomass handling (Tienaho et al., 2024). Previous research has also indicated a strong correlation between the phenolic content of plant extracts and their antioxidant activity as assessed by various assays (Liaudanskas et al., 2014).

The Gram-positive *Staphylococcus aureus* strain displayed marginally higher susceptibility to the sea buckthorn leaf extracts utilized in this investigation. However, no statistically significant variance was observed in the inhibition percentages of leaf extracts against *S. aureus* (leaf extract A: 61.9 ± 1.0 %; leaf extract B: 62.1 ± 3.6 %) compared to

Table 2
Phenolic compounds of the parallel extract samples A and B by HPLC-DAD. Quantitation of gallic, ferulic and ellagic acid was done using authentic reference compounds, The two unknowns (UNK) at 280 nm were quantitated as gallic acid equivalents (GAE). Flavonoids were quantitated as quercetin equivalents. Results are shown in averages of three replicates ± standard deviation per g of extract and biomass (raw material) dry weight (DW).

RT (min)	Compound	Sample A /extract DW mg g^{-1}	Sample A /biomass DW mg g^{-1}	Sample B /extract DW mg g^{-1}	Sample B /biomass DW mg g^{-1}
280 nm					
3.1	Gallic acid	0.80 ± 0.11	0.37 ± 0.05	0.53 ± 0.01	0.25 ± 0
11.5	UNK a (as GAE)	1.71 ± 0.47	0.79 ± 0.22	1.05 ± 0.04	0.49 ± 0.02
50.8	UNK b (as GAE)	0.26 ± 0.04	0.12 ± 0.02	0.17 ± 0.01	0.08 ± 0
350 nm					
17.6	Flavonoid	0.40 ± 0.02	0.18 ± 0.01	0.31 ± 0	0.14 ± 0
17.6	Flavonoid	0.47 ± 0.04	0.22 ± 0.02	0.33 ± 0	0.15 ± 0
20.1	Ferulic acid	0.29 ± 0.04	0.13 ± 0.02	0.18 ± 0	0.08 ± 0
22.1	Flavonoid	1.28 ± 0.14	0.59 ± 0.06	0.87 ± 0	0.40 ± 0
27.4	Ellagic acid	2.49 ± 0.27	1.14 ± 0.12	1.75 ± 0.02	0.81 ± 0.01
27.7	Ellagic acid	1.63 ± 0.19	0.75 ± 0.09	1.13 ± 0.01	0.53 ± 0
28.0	**Ellagic acid**	**7.01 ± 0.80**	**3.22 ± 0.37**	**5.01 ± 0.02**	2.33 ± 0.01
28.6	Ellagic acid	2.02 ± 0.23	0.93 ± 0.11	1.43 ± 0.01	0.67 ± 0
29.0	Flavonoid	1.25 ± 0.15	0.57 ± 0.07	0.89 ± 0	0.41 ± 0
29.6	Flavonoid	0.14 ± 0.04	0.06 ± 0.02	0.25 ± 0.04	0.12 ± 0.02
30.6	Flavonoid	0.28 ± 0.05	0.13 ± 0.02	0.18 ± 0.01	0.08 ± 0
31.3	Flavonoid	0.36 ± 0.06	0.17 ± 0.03	0.24 ± 0.01	0.11 ± 0
31.5	Flavonoid	1.30 ± 0.18	0.60 ± 0.08	0.90 ± 0.01	0.42 ± 0
32.0	Flavonoid	3.11 ± 0.42	1.43 ± 0.19	2.19 ± 0.02	1.02 ± 0.01
34.3	Flavonoid	0.27 ± 0.03	0.12 ± 0.01	0.19 ± 0.01	0.09 ± 0
34.8	Flavonoid	0.16 ± 0.01	0.07 ± 0	0.14 ± 0.02	0.07 ± 0.01
38.6	Flavonoid	1.23 ± 0.14	0.56 ± 0.06	0.85 ± 0.01	0.40 ± 0
39.7	Flavonoid	0.40 ± 0.04	0.18 ± 0.02	0.29 ± 0	0.13 ± 0
	Sum Ellagic acids 350 nm	13.16 ± 1.49	6.04 ± 0.68	9.32 ± 0.03	4.34 ± 0.01
	Sum Flavonoids 350 nm	10.66 ± 1.30	4.89 ± 0.60	7.63 ± 0.08	3.55 ± 0.04

Table 3
The TPC with Folin-Ciocalteu (FC) and Prussian blue (PB) methods, antioxidant results with FRAP and ORAC methods, as well as bacterial inhibition% against *E. coli* and *S. aureus* for the parallel extract samples A and B. Results are shown in averages of at least three replicates ± standard deviation per g of extract and biomass (raw material) dry weight (DW). The coefficient of variation (CV%) indicates the ratio of the standard deviation to the mean. Significant differences between extracts A and B are indicated with asterisks: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

	TPC/FC (mg GAE/g)	TPC/PB (mg GAE/g)	FRAP (μM Fe (II) eq./g)	ORAC (μM TE/g)	*E. coli* (inh%)	*S. aureus* (inh%)
Sample A /extract DW	126.4 ± 4.6 CV% = 3.6	62.3 ± 3.3 CV% = 5.3	1099.8 ± 41.5 CV% = 3.8	2448.1 ± 81.4 CV% = 3.3	47.9 ± 1.3 CV% = 2.6	61.9 ± 1.0 CV% = 1.7
Sample A /biomass DW	58.0 ± 2.1 CV% = 3.6	28.6 ± 1.5 CV% = 5.3	504.9 ± 19.1 CV% = 3.8	1123.9 ± 37.4 CV% = 3.3	22.0 ± 0.6 CV% = 2.6	28.4 ± 0.5 CV% = 1.7
Sample B /extract DW	99.7 ± 2.3 CV% = 2.3	60.4 ± 5.7 CV% = 9.5	872.2 ± 46.9 CV% = 5.4	2950.5 ± 160 CV% = 5.4	60 ± 2.3 CV% = 3.9	62.1 ± 3.6 CV% = 5.8
Sample B /biomass DW	46.4 ± 1.1 CV% = 2.3	28.1 ± 2.7 CV% = 9.5	405.7 ± 21.8 CV% = 5.4	1372.6 ± 74.4 CV% = 5.4	27.9 ± 1.1 CV% = 3.9	28.9 ± 2.7 CV% = 5.8
Significant differences	**	_	***	*	**	_

leaf extract B against *E. coli* (60 ± 2.3 %). Notably, the inhibition exhibited by leaf extract A against *E. coli* was significantly lower (47.9 ± 1.3 %). Gram-positive bacteria lack a lipopolysaccharide outer layer in their cell walls, potentially rendering them more susceptible to various antibacterial agents (Kunnas et al., 2024). Michel et al. employed accelerated solvent extraction with organic solvents such as ethanol and ethyl acetate for sea buckthorn leaves (Michel et al., 2012). The resultant crude ethanol extracts displayed inhibition percentages within a comparable range to those observed in our study per extract DW: 42 % ± 1 against *E. coli* and 72 % ± 3 against *S. aureus*. These findings parallel the trend observed in our study, with Gram-positive strain *S. aureus* exhibiting greater sensitivity to the extracts. In a study by (Upadhyay et al., 2010), the agar diffusion assay demonstrated the effectiveness of sea buckthorn leaf extract against *Bacillus cereus* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative), *S. aureus*, and *Enterococcus faecalis* (Gram-positive). The authors attributed this efficacy to phenolic constituents in the extract, particularly quercetin derivatives. In contrast, one study (Pirvu et al., 2014) reported only weak activity of sea buckthorn leaf extract (P3), prepared in a 20 % propylene glycol solution, against *S. aureus* ATCC 25,923 and no activity against *E. coli* ATCC 8739. However, it is noteworthy that the choice of solvent can significantly influence activity. Propylene glycol is known to extract aroma compounds, essences, and fragrances, and it possesses inherent antibacterial properties (Kinnunen and Koskela, 1991; Nalawade et al., 2015; Vehapi and Özçimen, 2021). Nevertheless, some studies have reported that propylene glycol exerts bacteriostatic rather than bactericidal effects, with bacteriostatic effects requiring concentrations exceeding 25 vol-% (Shia et al., 2021).

3.3. Anti-inflammatory activity

In this study, the ethanol-water extract from sea buckthorn leaves had an inhibitory effect on phagocytosis receptor mediated RB activation of both normal state and inflammatory promonocytes. The activation of THP-1 cells was measured based on their oxygen radical production – both as peak reaction activity for maximum reaction rate (Max CL) and as total radical production.

The inhibitory effect on RB response was stronger on normal state (without priming with LPS) cells than on cells made inflammatory with LPS treatment. This is seen as significantly ($p < 0.0004$) lower IC50-concentrations (sample concentration that inhibit 50 % of the activity) in non-primed cells (max reaction rate 11.67 ± 3.51 mg/L and total radicals 12.55 ± 4.04 mg/L, $n = 9$) than in primed cells (18.17 ± 6.53 mg/L and 18.83 ± 5.33 mg/L, respectively, $n = 9$).

In small concentrations ranging from 0.65 to 1.96 mg/L the difference was most clear (see Fig. 3A and B). In LPS treated cells the leave extract increased the respiratory burst activity (in 0.65 mg/L, $p < 0.005$) whereas in non-primed cells the extract inhibited the respiratory burst

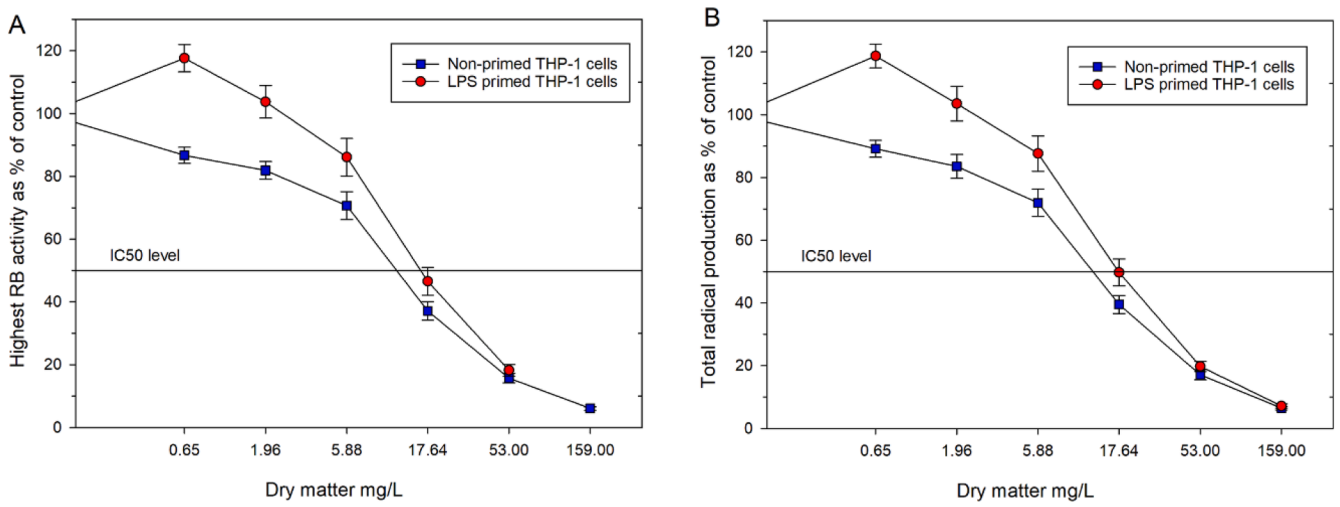

Fig. 3. Effects of water ethanol extract made from sea buckthorn leaf on peak respiratory burst activity (A) and on total radical production measured as area sum (B) of human THP-1 promonocytes. The "non primed" represent the effect on "normal state" promonocytes and "primed" to cells made inflammatory with *E. coli* LPS (10 mg/L). The RB response to opsonized zymosan was measured as luminol enhanced chemiluminescence for 90 min. The points represent the mean of nine independent experiments ($n = 9$) and the error bars represent the standard error of the mean (S.E.M).

already in smallest concentration then 0.65 mg/L ($p < 0.001$) and the inhibition was stronger when concentration increased. In inflammatory cells the inhibitory effect was dominant in concentrations higher than 5.88 mg/L. The dose responses were almost identical in maximum reaction rate and in total radical production (see Fig. 3A and B).

The difference in the effects on non-primed and LPS-primed cells could be explained e.g., by lectin polysaccharides in extract which could enhance the effect of LPS-treatment and cell activity by binding e.g., to toll like receptors in cell membrane.

These results with THP-1 promonocytes are essentially similar with earlier published studies where the experimental design was exactly similar except that the leukocyte cells were primed native fresh human peripheral blood neutrophils. In fresh sea buckthorn leave hot water extracts the IC50 concentrations were in average around 125 mg/L (Marnila et al., 2014) and extracts made from the same batches leaves by refluxing with 100 mL of 99.6 % ethanol, the IC50 concentrations varied from 41 to 62 mg/L (Marnila et al. 2015). These were lower activities than the present results with THP-1 cells. This emphasizes efficiency of the extraction method in present study and suggests that the relatively long storage time of the leaves in this study was not deleterious for the anti-inflammatory compounds.

The anti-inflammatory mechanisms of sea buckthorn leave extracts are according to current knowledge related to regulation of the free radicals and cytokines by phagocytic cells (Kania-Dobrowolska et al., 2023). In a cell model the flavonoids of sea buckthorn berries had anti-inflammatory effects on LPS stimulated RAW264.7 macrophages (Jiang et al., 2017). In the murine RAW 264.7 macrophage model, 70 % methanolic extract from sea buckthorn leaves from Himalaya inhibited nitric oxide radical production of LPS stimulated macrophages c.a. 50 % with 50 mg/L concentration (Padwad et al., 2006). Also the ethanol and methanol extracts from sea buckthorn leaves had a strong anti-inflammatory effects on LPS treated mouse peritoneal macrophages (Tanwar et al., 2018). THP-1 promonocytes, native granulocytes and macrophage cell lines share a similar respiratory burst activity which is triggered with similar stimuli and mediated via the same receptors (Lilius and Marnila, 1992). Since these receptors provide an essential link between humoral and cellular immune systems by functioning as key molecules for phagocytosis, clearance of immune complexes, adhesion and triggering the release of inflammatory mediators e.g., IL-1β and TNF-α. Chronic overactivity of this system contribute to development of metainflammation related widespread diseases such as neuroinflammation or type-2 diabetes.

Meta-inflammation is etiologically related to obesity, adiposity, hepatic steatosis, insulin resistance and vascular diseases. Thus, sea buckthorn leaf tea and extracts containing the phenolic compounds could be part of healthy diet to prevent and alleviate these wide-spread diseases.

4. Conclusions

Sea buckthorn leaves were extracted in ultrasound assisted extraction with 80 % ethanol-water solution using optimized process parameters by response surface methodology. The optimization results showed that the process can be described by first order equation. The resulting extract were studied to determine the phenolics composition and other UV absorbing compounds by HPLC-DAD. Based on the results, the extracts were rich in flavonoids and ellagic acid.

Optimized extraction conditions were proved to yield potent antioxidant, antibacterial and anti-inflammatory extracts with high total phenolic contents.

To the best of our knowledge there are not this far published studies where the different water and ethanol extraction methods were systematically compared with regard to the bioactivities of leave extracts. Thus, although a lot of research is done on health promoting effects of sea buckthorn berries there is still scarcely knowledge how sea buckthorn leaves, which are an under-utilized side stream of the sea buckthorn berry production, should be processed, extracted, and formulated. The results of this study present one scalable extraction solution to fully utilize the potential of leaves as raw material of health promoting products. The antimicrobial potential can be utilized by food industry as safe preservatives. Antimicrobial leaf compounds may also have beneficial effect on human intestinal microbiota. Antioxidant activity can also be utilized as food preservation. Antioxidative and anti-inflammatory activities may help to address the very real public health issues in providing nutritional solutions for improving health. It is also expected that identification of the active compounds in further studies will offer the opportunity for developing novel pharmaceutical products in the future as well as health promoting food products.

The new knowledge generated will create new opportunities for the sea buckthorn leaves and developing novel nutritional and therapeutic applications for the prevention/treatment of life-style related health problems.

Ethical statement

The authors confirm that the study did not involve experimentation on human or animal subjects.

CRediT authorship contribution statement

Nora Pap: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Pertti Marnila:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Juha-Matti Pihlava:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jenni Tienaho:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Data availability

Data will be made available on request.

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