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Studies on Polyphenols from Sea Buckthorn Berries and Pomegranate Peels Ethanol Extracts. Recovery, Bioactivities and Encapsulation into Polymers

A. Moschona[1], K. Rouptsiou[1], S. Theodoridou[2] and M. Liakopoulou-Kyriakides[1]

[1]Faculty of Chemical Engineering, Aristotle University of Thessaloniki - 54124, Greece.
[2]Blood Bank Center, Hippokration Hospital of Thessaloniki - 54642, Greece.

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***Corresponding Author Email:** markyr@eng.auth.gr

INTRODUCTION:

It is known that the consumption of fruit, vegetables and other plant products is beneficial for our health due to the presence of several bioactive compounds. Most of these compounds are aromatic secondary metabolites, which are synthesized in plants during their normal growth and under stressful circumstances [1]. They possess several activities, such as antioxidant, antibacterial, antifungal etc. [2-7]. In general, the by-products of many fruits represent a source of sugars, minerals, organic acids, anthocyanins and phenolic compounds. The last two categories are known for their important biological activities, including antioxidant, antibacterial, cardio-protective, anti-carcinogenic and anti-mutagenic effects [8-10]. Synthetic antioxidants were used in the food, pharmaceutical and cosmetics industry, over the past decades, in order to maintain the stability and quality of each product, over a longer period of time, though toxicity and some other side effects that observed, led researchers towards recovery and isolation of bioactive compounds from natural resources [11].

Hippophae rhamnoides L. commonly known as sea buckthorn is a plant producing yellow-orange berries with bioactive constituents up to 190 [12] including polyphenols, numerous carotenoids and vitamin C [13-15]. The most common among them are ferulic acid, p-coumaric acid, ellagic acid, quercetin and kaempferol [13].

Phenolic compounds play a quite important role in food science and technology. They can be used as natural food colorants or as antioxidants, protecting specific ingredients that are sensitive to oxidative changes, such as unsaturated fatty acids, but also as antimicrobial agents [16]. Nowadays, the interest of scientists, however, focuses on the extraction process and on the selective separation of phenolic compounds from various natural sources. Additionally, the recovery of natural high added value compounds from wastes has gained increasing importance nowadays, due to the growing threat of infectious diseases and carcinogenicity of synthetic compounds. Natural products continue to provide unlimited opportunities for finding new drugs [17]. In previous studies, we have reported on valorization of wine wastes and recovery of bioactive phenolic compounds [18]. The large amounts of by-products obtained as a result of processing pomegranates is also of concern due to the major environmental impact induced by their disposal. Nowadays, improvements in process engineering have made the valorization of wastes interesting, than simple management of them. The new aspects concerning the exploitation of wastes on the recovery of high added value products have gained significant interest, not only due to economically benefits, but also as good and cheap source of bioactive compounds, with wide range of activities in humans.

Phenolic compounds are unstable and sensitive under various conditions used in food processing, such as light, pH, and thermal stress. It has been also mentioned that phenolic compounds can lose their biological activities during food processing and storage [19, 20]. Therefore, alternatives for their protection and in order to retain their stability and their biological activities should be examined. Their encapsulation into polymers or liposomes offers many advantages and is preferred in cases of their further use in food and pharmaceutical industries.

The encapsulation of bioactive ingredients, has become one of the most promising research fields and it is designed for the protection of these compounds against any undesirable interactions with the environment, enhance their stability and ensure that the taste and odour of the food will remain unchanged. Encapsulation is a promising way to ensure the bioavailability of the trapped component and to increase the effectiveness of "functional foods" [21, 22].

Biodegradable natural and synthetic polymers have been extensively used as delivery systems of several bioactive components, such as carotenoids, vitamins, lipids, peptides and others. The advantages are numerous as polymeric nanoparticles protect bioactive compounds from degradation, improve their solubility and promote controlled drug release and targeting [23-26].

Thus, in this study, sea buckthorn berries and pomegranate peels (*Wonderful* cultivar) were investigated towards an integrated study for isolating phenolic compounds with high biological impact. Sea buckthorn (*Hippophae rhamnoides* L.) known as “super fruit” contains several beneficial ingredients [27]. On the other hand, pomegranate (*Punica granatum* L.) is one of the oldest edible fruit, well known for its nutritional and medicinal value from ancient times [28]. It is consumed transformed into fresh juices, beverages and jams [29, 30], resulting also in high amounts of wastes. Specifically, during the industrial processing of pomegranates about 50% of the fruit, mostly seeds and peels, are disposed as residues [31].

Therefore, the aim of this work was firstly the optimization of the extraction of phenolic compounds from dried sea buckhorn berries and pomegranate peels, using Response Surface Methodology (RSM). Total phenolic content (TPC) and total flavonoids (TF) were firstly determined. In addition, antioxidant and antibacterial activity against Gram- positive and negative strains were studied and also, their effect on human platelet aggregation, in *in vitro* experiments. Analysis and identification of the major phenolic compounds of the extracts using reference compounds and/or their MS (LC/MS analysis) was conducted. GC-MS analysis was used for the identification of the fatty acids contained in sea buckthorn extracts. Encapsulation of the extracted phenolics, from both samples, within copolymers of alginate, polyethylene glycol (PEG) and chitosan was attempted and optimized, in order to maximize the encapsulation efficiency and to minimize the size of the beads. Kinetics of release experiments was also conducted and the functionality of the released phenolics was evaluated in terms of the antioxidant activity.

Furthermore, the study aims to examine whether the same or similar protocol could be applied for the recovery of the different phenolic compounds existed in these two samples, considering both maximum yield and biological activity. Processing of the extracts to increase stability over the time that could eventually be used in food and pharmaceutical industries is also described.

MATERIALS AND METHODS:

Plant material

Dried sea buckthorn berries were purchased from a local market in Thessaloniki and milled in a commercial blender (i.d. ≤1 mm). Fresh pomegranates (*Wonderful* variety) were also purchased from a local market in Thessaloniki, Greece. Pomegranate seeds were manually separated and removed and only skins and peels were collected. Peels were washed carefully to remove any adhering materials, dried at ambient temperature in a desiccator and milled in a commercial blender (i.d. ≤1 mm).

Optimization of extraction

The extraction was carried out using a sonication bath (General sonic, 41 kHz, 320 W, thermostatically adjustable). Dried sea buckthorn berries and pomegranate peels were extracted with a certain volume of ethanol, as a solvent. Methanol and ethanol found to extract TPC at about the same concentrations and the latter was used in our experiments, as the most appropriate solvent, according to Food and Drug Administration. The optimization of the extraction process was done using RSM, in order to obtain the maximum amount of TPC with the maximum antioxidant activity. The influence of three parameters, specifically the pH (2.0 to 7.0), the temperature (35, 45 and 60 °C) of the extraction and the dry weight ratio of sample to solvent volume (1/3, 1/5, 1/10, 1/15, 1/20 g/mL) were investigated. The extract thus obtained was then centrifuged (4500 rpm, 10 min), stored in the refrigerator (-20°C) and further analysed. The Minitab® 17.1 statistical suite (Minitab Inc.

Pennsylvania, USA) was used to graphically analyse the data.

Total phenolic content (TPC)

The content of total phenolic compounds, in all extracts, was determined using the Folin–Ciocalteu colorimetric method [32]. Gallic acid was used as a reference standard and the results (TPC) were expressed as mg of gallic acid per g of dry mass weight.

Determination of total flavonoid content

Determination of the total flavonoids in the extracts was also carried out using the aluminum chloride colorimetric method [33]. Briefly, equal amounts of extract and 2% w/v ethanol solution of $AlCl_3$ were mixed and incubated for 1 h at room temperature. The absorbance was measured at 420 nm and the total flavonoid content was expressed as mg of quercetin per g of dry mass weight.

Determination of antioxidant activity

1. DPPH radical scavenging activity

The DPPH assay was estimated using the 2, 2-diphenyl-1-picrilhydrazyl (DPPH) method with modifications [34]. This method is based on the evaluation of the free-radical scavenging capacity. The DPPH solution (0.1 g/L in ethanol) was prepared daily, stored in a flask covered with aluminum foil and kept in the dark at 4 °C between measurements. Radical scavenging activity was determined by the decolorization of the DPPH solution and was expressed as the inhibition percentage (**Eq. 1**).

$$\% \text{ Inhibition} = [(A_{DPPH} - A_{Extr})/A_{DPPH}] * 100 \quad (1)$$

Where A_{DPPH} is the absorbance value of the DPPH blank sample and A_{Extr} is the absorbance value of the test solution.

2. $ABTS^{\bullet+}$ radical cation assay

The second method applied here is based on the measurement of the $ABTS^{\bullet+}$ radical cation decolorization assay [33]. ABTS radical cation ($ABTS^{\bullet 1}$) was produced by reacting ABTS stock solution (7 mM in water) with potassium persulfate (140 mM) at final concentration of 2.45 mM. This mixture was allowed to stand in the dark at room temperature for 16-18 h before use. The aforementioned mixture was diluted with ethanol, for the study of antioxidant activity of extracts, to an absorbance of 1.0 at 735 nm, at room temperature. The percentage inhibition of absorbance at 734 nm is calculated according to **Eq. 2**.

$$\% \text{ Inhibition} = [(A_{ABTS\bullet+} - A_{Extr})/A_{ABTS\bullet+}] * 100 \quad (2)$$

Where $A_{ABTS\bullet+}$ is the absorbance value of the $ABTS^{\bullet+}$ blank sample and A_{Extr} is the absorbance value of the test solution.

***In vitro* platelet aggregation experiments**

Platelets were obtained from venous blood of healthy donors (males and females). Volunteers, aged from 25-45 years old had not taken any medication within the last 15 days. The blood was immediately mixed with 3.8% sodium citrate solution and after that was centrifuged at 1000 rpm for 10 min to yield platelet rich plasma (PRP). The remainder recentrifuged at 4000 rpm for 10 min to obtain platelet poor plasma (PPP). Three platelet aggregation inducers were used collagen (5 µg/mL), ADP (20 µM) and ristocetin (0.5 mg/mL). Platelet aggregation experiments were performed, according to the method of Born and Cross [35], by a conventional photometric technique in a four-channel aggregometer (Carat TX4 Platelet Aggregometer), at 37 °C. The aggregation was determined by recording the increase of light transmission and the calibration was performed using PPP [36]. The results are expressed as antiplatelet activity % according to **Eq. 3**.

Antiplatelet Activity % = (maximum aggregation of collagen - maximum aggregation of sample)/ maximum aggregation of collagen*100% (**3**)

Antibacterial activity

The antibacterial activity of the extracts was evaluated against the gram negative bacteria *Escherichia coli* (BL21 [DE3]) and the gram positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) and *Bacillus cereus* (ATCC 11778) photometrically. Strains were completely grown in Luria-Bertani nutrient medium at 37 °C, for 24 h, before being used for antimicrobial tests. The growth of each microorganism in the presence of the extracts was evaluated. Absorption measurement was performed at a wavelength of 600 nm and the results were calculated according to **Eq. 4**. Since methanol was used here, as a solvent, it was also tested under the same conditions and has been subtracted from the calculation of each sample. IC_{50} (Inhibitory Concentration 50%) was also determined.

$$\% \text{ Antimicrobial activity} = [1 - [(A_{Sample} - A_{PC}) / (A_{NC} - A_{PC})]] * 100 \quad (4)$$

Where: A_{PC}: absorption of the positive control (nutrient and extract), t=0 min, and A_{NC}: absorption of the negative control (nutrient and microorganism).

Analysis and identification of major phenolic compounds

The ethanol extracts obtained from pomegranate peels and sea buckthorn berries were analyzed by HPLC and ESI-mass spectrometry. Analysis was carried out using a Thermo Finnigan Spectra HPLC system (San Jose, California) model UV 6000 LP, equipped with EZChromeElite software, Version 3.1.7., four Q-Grad pumps, a diode array detector (DAD) and a Grace Smart RP C-18 column (250x4.6 mm i.d.; 5 µm particle size). The injection volume was 20 µL and the wavelengths used were 280, 365 and 450 nm. The mobile phases were 2% (v/v) acetic acid in milli-Q water as eluent A and 100% acetonitrile as eluent B and a gradient program was set as follows: from 0 min, 100% A; 4 min, 85% A/15% B; 20 min, 60% A/40% B; 40 min, 45% A/55% B. The flow rate was 1 mL/min and the total run time was 40 min.

Electrospray ionization mass spectrometry (ESI-MS) experiments, for the identification of specific phenolic compounds, were performed using a Thermo Fisher Scientific (Bremen, Germany) model LTQ Orbit rap Discovery MS. The experiments were run using a standard ESI source operating in a positive and negative ionization mode. The source voltage was 3.7 kV and the MS worked with a 300 °C heated capillary.

Identification of fatty acids

The extraction and identification of fatty acids of the sea buckthorn berries was carried out following the methodology proposed by Folch et al. [37]. The method consisted in the extraction, for 20 min, of fatty acids from the sample with a mixture of chloroform and methanol (2:1, v/v). After the acids were extracted, centrifuged and received, FAMEs (fatty acid methyl esters) were prepared according to IUPAC 1987 procedure ([38]. The analysis of FAMEs was performed on a gas chromatograph (Shimadzu) equipped with flame ionization detector (260 °C) and a CP-Sil 88 capillary column (chrompack) with a length of 50 m, 0.25 mm i.d., 0.2 mm film thickness. The oven temperature was raised from 140 °C in the first 3 min, increasing up to 170 °C with a rate of 18 °C/min over a period of 8 min, after which the temperature was increased to 205 °C with a rate of 2 °C/min and maintained during 17 min. Gas pressure was 200 kPa. FAMEs were identified by comparing their retention times with those obtained from the analysis of standard mixture of known fatty acids, using OpenChrom System.

Encapsulation of phenolic compounds into biopolymers

The method proposed by Pasparakis and Bouropoulos [39], with some modifications made by Moschona and Liakopoulou-Kyriakides [40], was used for the encapsulation of phenolic compounds extracted from sea buckthorn berries and pomegranate peels extracts into the biopolymers. Three cases were examined, the first one for the encapsulation using alginate alone, the other one using alginate-PEG and the third one using alginate-chitosan as wall materials.

Specifically, in the first procedure, appropriate concentration of sodium alginate solution in water and calcium chloride water solutions were prepared in two separate vessels. Calcium chloride solution adjusted to pH 2 by the addition of acetic acid. Subsequently, appropriate amount of the plant extract was added into the sodium alginate solution, and the mixture was allowed to stir for 30 min. Then, sodium alginate-extract solution was drop wise added into the calcium chloride solution, through a fine 21-gauge stainless steel needle, while the distance between the edge of the needle and the surface of the calcium chloride solution was approximately 5 cm. The formed beads were allowed to stay in the gelling medium for 20 min, at room temperature under mild agitation, and then separated from the solution through vacuum filtration and washed with water. Concerning the alginate-chitosan system, similar procedure was applied as above, with chitosan addition to calcium chloride solution under magnetic stirring, till homogenization, followed by the drop wise addition of sodium alginate solution.

Accordingly, for the encapsulation of phenolic extracts using alginate and PEG, an alginate-PEG solution (using the appropriate ratio) was prepared by dissolving the appropriate amounts of sodium alginate and PEG in water. Subsequently, the calculated amount of the extract was added into the aforementioned solution and the mixture was allowed to stir for 30 min. Calcium chloride solution was also prepared by dissolving the appropriate amounts of it in water, respectively. The calcium–alginate/PEG beads were prepared by drop wise-addition of alginate/PEG-extract solution into the calcium chloride solution through a fine 21-gauge stainless steel needle. The same procedure, as above, followed to obtain the beads.

In all cases, the amount of the entrapped phenolic compounds into the beads was calculated, as described previously, according to the **Eq. 5**:

$$\textbf{Encapsulation efficiency (\%)} = [(TPC_i - TPC_d)/TPC_i] \times 100\% \quad (5)$$

Where, TPC_i is the initial phenolic compounds dissolved in the encapsulation solution and TPC_d is the phenolic compounds measured in the gelling medium after the procedure of encapsulation.

Optimization of the encapsulation, for the alginate-chitosan system with the most promising results was performed. Thus, the interactive effects of the sodium alginate concentration (0.5-3.0 % w/v), the 2nd polymer, chitosan solution concentration (0.5-3.0 % w/v) and the calcium chloride concentration (1.0–2.0 % w/v) on the encapsulation efficiency (%) and the size of the obtained beads (μm), as response variables, were studied. The levels of the variables were chosen after a series of preliminary experiments. Experiments were performed using a face-centered central composite statistical design for the study of the three independent variables. The Minitab® 17.1 statistical suite (Minitab Inc. Pennsylvania, USA) was also used to graphically analyze the data.

Release studies

Weighed amounts of beads were suspended in several release solutions, each time, in order to succeed maximum release of the encapsulated phenolic compounds. The solvents tested here, were (a) ethanol solution (70%), at room temperature (25 °C), (b) ethanol solution (70%), at 40 °C, (c) 0.2 M sodium citrate solution, pH 4.0 and (d) 0.1M hydrochloric acid solution, pH 1.0. Tests were performed using 1 gr of beads, which was added to a 5-fold volume of the respective release solution, followed by magnetic stirring for 48 hours. Samples of supernatant were taken after predetermined time intervals and the total phenolic content and the antioxidant activity were determined. In these experiments, the effect of time on the release of phenolic compounds from the beads was also studied.

Stability of encapsulated phenolic compounds during storage

In order to evaluate the stability of the total phenolic content and of their antioxidant activity, samples of the beads were stored for 20 days at 40 °C. Samples were analyzed on the days 1, 5, 10, 15 and 20 during storage. All samples were protected from light until analysis. Each sample was dissolved in the best release solution and the total phenolic content, as well as their antioxidant activity were determined.

Data analysis

All samples were prepared in triplicate and the results are expressed as mean ± standard deviation (SD) values (n=3). Statistical analysis was carried out using the statistic part of MINITAB™ statistical software (17.1.0.0) and all data were considered statistical significant at $p<0.05$.

RESULTS AND DISCUSSION:

Optimization of extraction and determination of total phenolic compounds, flavonoids and antioxidant activity

Minitab® 17.1 was used to implement the RSM. Specifically, twenty experiments were performed using a face-centered central composite statistical design for the study of the three independent variables as done previously [40] aiming in deriving the same or close results for their recovery. The second order polynomial model of response for three quantitative factors was fitted and used to construct the surface plots for sea buckthorn berries, as shown in Figures 1 and 2.

As can be seen from Figure 1, the recovery of phenolic compounds is not significantly affected by the ratio of the solid to solvent ratio, although, it shows to decrease by increasing the pH value. From Figure 2, the surface plots of the antiradical activity of the extract showed that the temperature does significantly affect the activity, at the solid to solvent ratio of 1/5. Therefore, it was observed that the maximum extraction of phenolic compounds (14.6±1.54 mg/g), and the maximum antioxidant activity (DPPH method 94±2.12%) of sea buckthorn berries, was observed at solid to solvent ratio of 1/5, 45 °C temperature and pH value equal to 2.0. Under these conditions, the content of flavonoids of the extract was found to be 5.2±0.24 mg/g. In addition, the antioxidant activity of the extract was also determined for comparative reasons using ABTS method and found to be 97±3.00%. Korekar et al. [41] found that in several studied populations of sea buckthorn the total phenolic content was ranged from 9.64 to 10.70 mg/g.

The same experimental procedure for the optimization of extraction was followed also for the pomegranate peels (*data not shown*), and the results showed that the maximum extraction of phenolic compounds (119±2.91 mg/g), and the maximum antioxidant activity (DPPH method 92±1.54% and ABTS method 94±1.76%), was observed at solid to solvent ratio of 1/5, pH value equal to 2.0 and at lower temperature (35 °C), compared to sea buckthorn. Fawole et al. [42] have reported a higher TPC value at 189 mg/g for the same cultivar. In addition, the content of flavonoids of the extract was found to be 60±3.25 mg/g. The amount of TPC of pomegranate peels extracts is about 2-fold higher than that of its juice, similarly to other reports [43,44].

Regarding the two methods applied for measuring the antioxidant capacity of samples, no significant differences were found and showed a good correlation with each other, with a *Pearson*

correlation coefficient of 0.996. The slightly better results appeared against the $ABTS^{\bullet+}$ radical, can be explained since the DPPH has lower sensitivity than the $ABTS^{\bullet+}$ [45, 46].

Determination of antibacterial activity

The antibacterial activity of the two extracts examined at four different concentrations of phenolic compounds in the range between 150-600 μg/mL, and the inhibition of growth of each microorganism (%), in the presence of the extract, was measured, according to equation 3. It was observed that the extracts inhibited the growth of all four strains tested here with the highest inhibition observed for *B. subtilis* with IC_{50} values calculated to 95 and 62 μg/mL for the sea buckthorn and the pomegranate peels extract, respectively.

The observed antibacterial activity of sea buckthorn berries comes in accordance with previously reported results ([47]. Specifically, *E. coli* showed higher resistance for both extracts, followed by *S. aureus,* whereas, stronger inhibitory effect was observed against *B. cereus* and *B. subtilis*. The inhibition was generally stronger with the increase in the concentration of the extracts. The differences in susceptibility of Gram-positive and negative bacteria, have also, previously been reported [48]. This can be explained because for Gram-positive bacteria, antibacterial agents can easily destroy the bacterial cell wall, whereas outer membrane of Gram-negative bacteria provides a hydrophilic surface to block the external hydrophobic substances, resulting in a stronger resistance to antibacterial compounds [47, 49].

***In vitro* platelet aggregation experiments**

Figure 3 (a, b and c) depicts the effect of different TPC of sea buckthorn extract on platelet aggregation induced by collagen, ADP and ristocetin, respectively. The curve corresponding to "*control*" depicts to the maximum aggregation of platelets caused by the three inducers, while the curve corresponding to "*hip*" represents the platelet inhibition caused by the sea buckthorn extract. The concentration of each extract was calculated according to their TPC. It is found that the extract prevented significantly platelets from ristocetin/ADP/collagen-induced aggregation, with IC_{50} values 0.05, 0.8 and 0.9 mg of phenolics/g, respectively. Similar effect appears from the pomegranate peels extract on human platelet aggregation. The IC_{50} values for pomegranate peels estimated at 0.016, 0.018, 0.021 mg/g for ristocetin, ADP, collagen, respectively. The higher activity of the extracts using ristocetin and ADP than collagen, as platelet activators could be explained since collagen is a more powerful agent, compared to ADP, which is considered as mild factor [50].

Our previous experiments showed that isolated phenolic compounds and specifically ellagic acid, kaempferol, ferulic acid and quercetin, present a dose dependent anti-platelet activity [51]. Thus, the antiplatelet activity of the extracts tested here, could be attributed to the above compounds contained in the extracts with the contribution of all other co-present phenolic compounds not to be ignored.

Identification of the major phenolic compounds by HPLC and ESI-MS

The HPLC analysis of pomegranate peels extract recorded at 365 and 450 nm (Figure 4a and 4b) showed the presence of a derivative of caffeic acid, coumaric acid, catechin, epicatechin and b-carotene. As it can be concluded from this figure, b-carotene can be easily recovered from pomegranate peels extract. This is also an important finding regarding its production. b-Carotene, as a precursor of vitamin A, has significant biological activities, as reported previously [52-54], in addition to its colour, which makes it still very attractive in the food, pharmaceutical and cosmetic industries.

The HPLC analysis of the sea buckthorn berries extract at 280 nm and at 365, respectively (*data not shown*) revealed the presence of gallic acid, ellagic acid, myricetin, isorhamnetin rhamnoside, quercetin and kaempferol.

Their identification was based on their retention times, UV adsorption spectra of peaks and MS data with those of reference standards. Under negative mode ellagic acid (M-H 301), isorhamnetin rhamnoside (M-H 623), caffeic acid derivative (M-H 299), coumaric acid (M-H 163) and b-carotene (M-H 536) showed a higher signal, while under positive mode Gallic acid (M-H 171), myricetin (M-H 319), quercetin (M-H 303), kaempferol (M-H 287), catechin (M-H 291) and epicatechin (M-H 291) detected.

Identification of fatty acids using GC-MS

Total lipid extract obtained from whole sea buckthorn berries was analyzed by gas chromatography-mass spectrometry (GC-MS), as described previously. Palmitoleic acid (C-16:1), stearic acid (C-18:0) and vaccenic acid (C-18:1) were each present at 5±0.2, 3±0.2 and 3±0.4% of the total fatty acids and are in accordance with previous reports [55]. Oleic acid, C-18:1, (20±1.5%), a-linoleic acid, C-18:2, (32±1.4%), linoleic acid, C-18:3, (25±1.8%) and palmitic acid C-16:0, (12±0.5%) were detected in high amounts as has also been reported [56].

It has been mentioned that ω-6: ω-3 ratio may be a stronger predictor of inflammation than either fatty acid alone [57]. A higher ω-6: ω-3 ratio has been

related to higher levels of pro-inflammatory markers (IL-6, IL-1 receptor antagonist, TNF-α, and CRP) and lower levels of anti-inflammatory markers (IL-10 and transforming growth factor β). In addition, the Western diet contains a very high (15:1) ω-6: ω-3 fatty acid ratio, which has been linked with cancer, cardiovascular, inflammatory and autoimmune diseases [58]). Therefore, the low ω-6: ω-3 ratio (1.28) in sea buckthorn berries may contribute to beneficial health effects.

Optimization of encapsulation

Encapsulation of the bio-active phenolic compounds into polymers, extracted from the sea buckthorn berries and pomegranate peels extract, was studied. In order to find the best polymeric system for the maximum encapsulation of phenolic compounds, alginate, chitosan and PEG were firstly, examined at three different concentrations using the extrusion method as described above. This method was preferred as a mild one, in order to phenolic compounds retain their biological activities and avoid any degradation [59].

The effect of the polymers concentrations, on the encapsulation efficiency, of phenolic compounds, is shown in Table 1 and as it can be seen, the encapsulation efficiency using alginate and chitosan, as wall materials, is higher than using alginate or alginate and PEG.

Based on these results subsequently, a series of optimization experiments was performed, using Minitab® 17.1 and the RSM, in order to find the appropriate concentration of alginate, chitosan and calcium chloride that lead to the maximum encapsulation efficiency of the phenolic compounds with the minimum size of the formed beads as well. The three independent variables which were tested are the sodium alginate concentration (0.5-3.0 % w/v), the chitosan solution concentration (0.5-3.0 % w/v) and the calcium chloride concentration (1.0 – 2.0 % w/v) and the effect of them on the encapsulation efficiency and on the size of beads, are shown in Fig. 5a and 5b.

As it can be seen from this Figure, the increase of chitosan concentration, while reducing the concentration of alginate, results in increased encapsulation efficiency. The above results regarding the effect of chitosan concentration on the encapsulation efficiency of phenolic compounds are similar to other reports [60]. Furthermore, Figure 5b, shows that the two factors affecting the most the size of the beads are sodium alginate and calcium chloride concentrations, followed by chitosan concentration. From the response surface of the microcapsule size can be concluded that sodium alginate and chitosan concentrations have negative correlation, while calcium chloride concentration has positive correlation with the size of the beads. Concluding, the polymers at concentration of 1.36% w/v for alginate and 1.84% w/v for chitosan and calcium chloride at a concentration of 1.66% w/v were found more efficient, giving 95.8±2.2% encapsulation efficiency of the extracts. Under these optimum conditions, the diameter of the spherical shaped beads was approximately 740±7.6 μm.

Alginate-chitosan was preferred, for the encapsulation of phenolic compounds extracted from pomegranate peels, concluding in encapsulation efficiency close to 100%. Alginate was preferred as a natural polymer, while its use has valuable advantages in encapsulation techniques, such as resistance to stomach acidity, controlled release in the intestinal media and biocompatibility of the polymer [61-63]. Both alginate and chitosan are excellent biocompatible, non-toxic and bio-absorbable polymers for application in drug delivery systems and tissue engineering scaffolds [64-67]. Also, chitosan increases the capsule strength, flexibility, and biocompatibility [68, 69] and the storage stability of microcapsules, supporting that encapsulation offers chemical stability and antioxidant activity of the encapsulated phenolic compounds.

Kinetics of release experiments

The phenolic compounds release profile was investigated after placing appropriate amount of beads in several mild release media and then measuring the total phenolic content, as described previously. In Figure 6, it is presented the release profile of sea buckthorn extract beads using several release solutions for 48 h. This figure indicates that the release of phenolic compounds from loaded alginate-chitosan beads occurred in a controlled manner, while most of the phenolic compounds (40.5%) were released in the first 8 h, whereafter a steady state is reached and about 54.5% of the phenolic compounds were released. The incomplete and prolonged release of alginate-chitosan beads may be attributed to higher retention characteristics due to the alginate–chitosan membrane and the possible interaction between chitosan and phenolic compounds [70, 39, 71]. The same experimental procedure was followed for the release of encapsulated phenolic compounds of the pomegranate peels extract, the results of which showed that approximately 62.3% of the phenolic compounds were released in the first 8 hours.

Antioxidant activity of the released phenolic compounds is of great importance as well. The results showed that besides the incomplete release, phenolic compounds retained antioxidant activity at

high values 68±2.7 % and 74±2.0 % for sea buckthorn berries and pomegranate peels extracts, respectively.

It was found that the best release of phenolic compounds for both extracts was observed with HCl solution (pH 1.0) indicating their possible use in pharmaceutical applications, where the drug should release in the stomach (pH 1.0).

Storage stability

The total phenolic content and their antioxidant stability of the obtained beads were evaluated during 20 days of storage at 40 °C and the results are presented in Table 2. As it can be seen, the phenolic content and the antioxidant activity had no significant changes during the first 5 days, while after 10 days both slightly decreased (2.5%). By the end of the 20 days storage of micro beads a 5.5% and 11.8% decrease of TPC and antioxidant activity, respectively, was recorded, while the shape of the beads showed no significant visual changes. Similar decrease of TPC and of their antioxidant activity during storage, at 40°C has also been reported previously [72, 73].

Table 1: Results of encapsulation efficiency of phenolic compounds from sea buckthorn berries at different concentrations of alginate, chitosan and PEG, as wall materials for the formation of the beads.

Experiment	Polymers	Polymers Concentration (% w/v)	% Encapsulation efficiency
1	Alginate	4	29.0 ± 1.0
2	Alginate PEG	1 3	0.0 ± 0.0
3	Alginate PEG	2 2	91.0 ± 3.2
4	Alginate PEG	3 1	90.5 ± 3.0
5	Alginate Chitosan	1 3	95.8 ± 2.5
6	Alginate Chitosan	2 2	95.0 ± 2.2
7	Alginate Chitosan	3 1	92.0 ± 3.0

Table 2: TPC and antioxidant activity of encapsulated phenolic compounds, determined during 20 days of storage at 40 °C.

	Days				
	1	**5**	**10**	**15**	**20**
TPC (mg/g)	7.96±0.97	7.91±0.98	7.76±0.79	7.59±0.76	7.52±0.78
Antioxidant activity (%)	68±2.7	67±2.0	65±1.9	61±2.0	60±1.9

Fig. 1: Surface plots of TPC (mg/g) of sea buckthorn berries extract, as a function of pH, temperature (˚C) and solid to solvent ratio (g/mL).

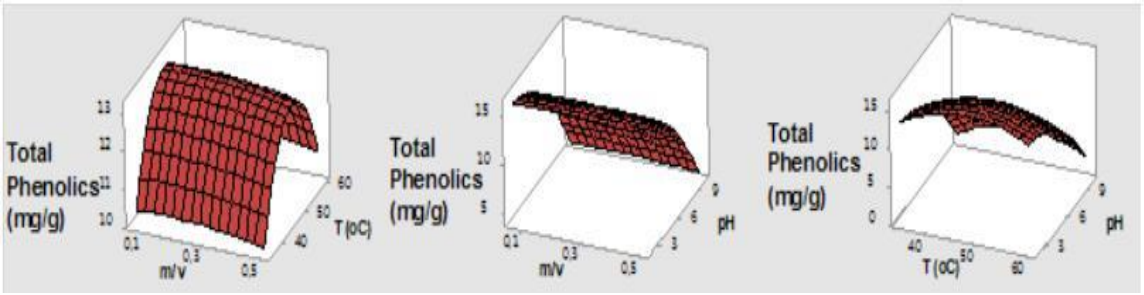


Fig. 2: Surface plots of antiradical activity (%) of sea buckthorn berries extracts, as a function of pH, temperature (˚C) and solid to solvent ratio (g/mL).

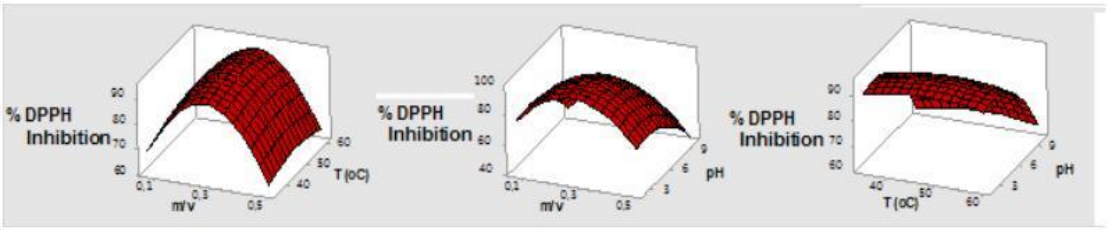
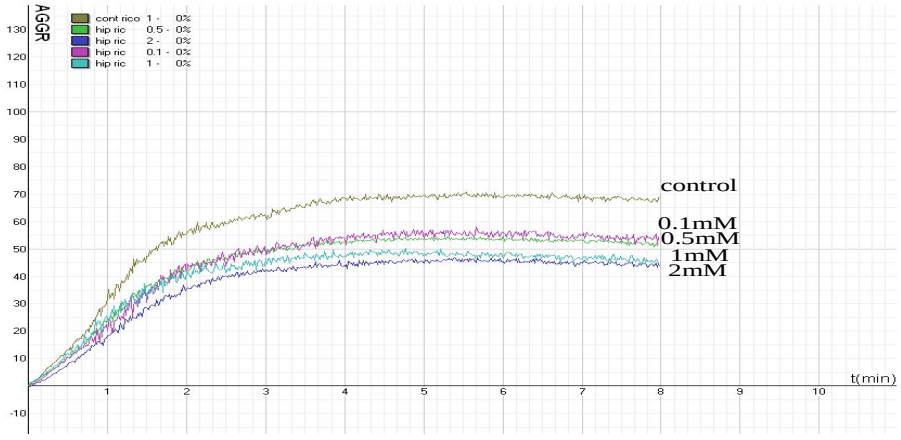
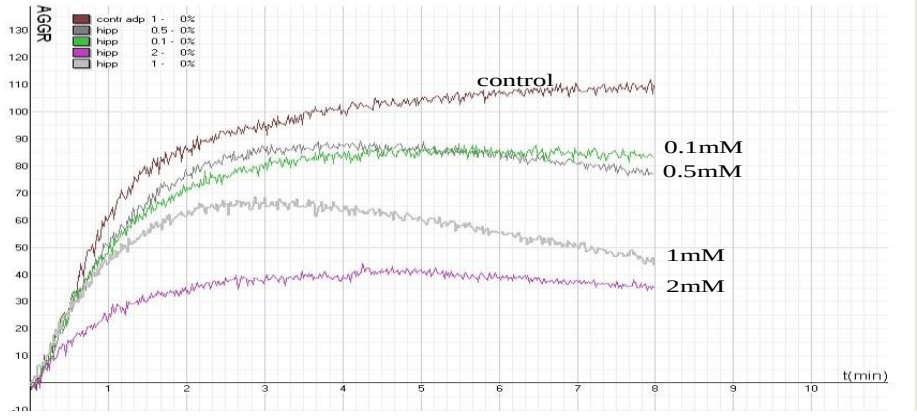


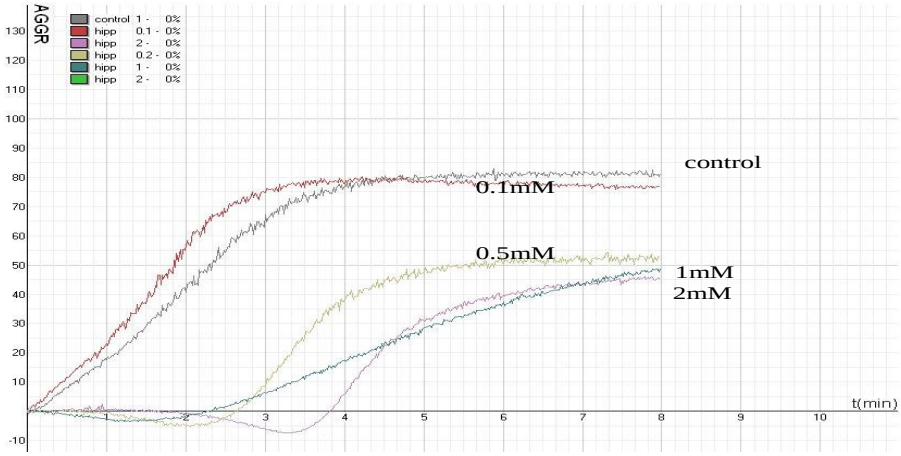
Fig. 3: Effect of sea buckthorn berries extract on human platelet aggregation induced (a) by ristocetin, (b) by ADP and (c) by collagen at different concentrations, *in vitro*.



(a)



(b)



(c)

Fig. 4: HPLC analysis of pomegranate peels extract (a) at 360 nm and (b) at 450 nm. Peak identities: (1) caffeic acid derivative, (2) coumaric acid, (3) catechin, (4) epicatechin, (5) ellagic acid and (6) b-carotene.

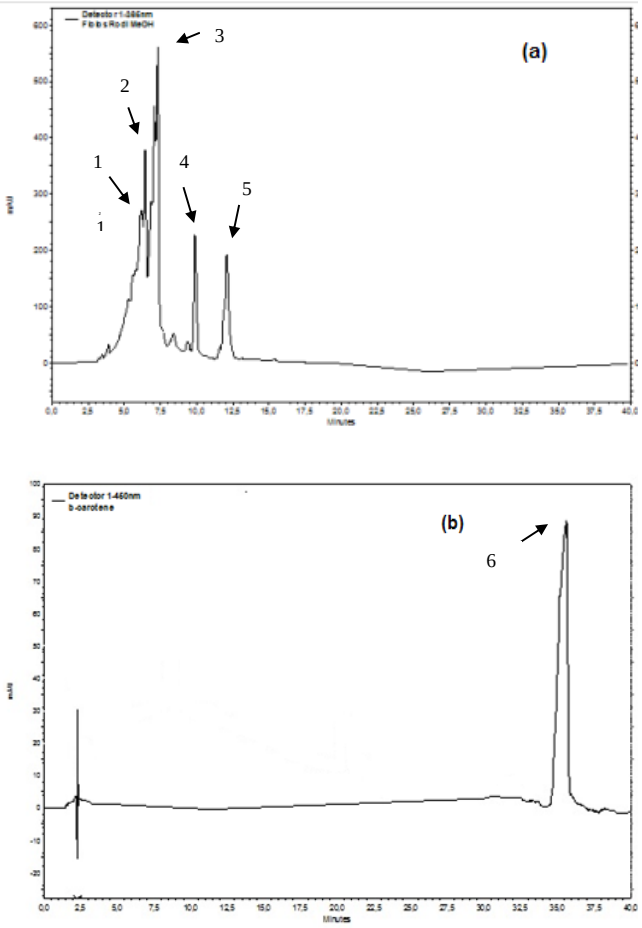
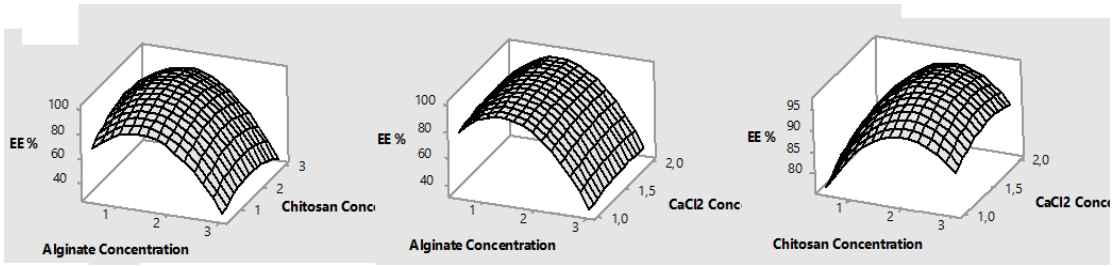
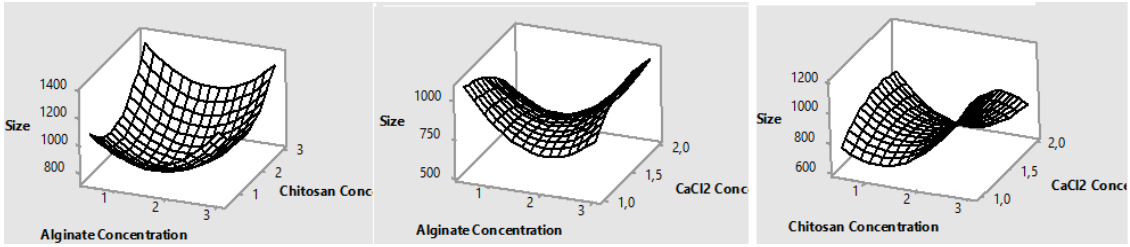


Fig. 5: (a) Surface plots of encapsulation efficiency of phenolic compounds (%), as a function of sodium alginate, chitosan and calcium chloride concentrations (% w/v) and (b) Surface plots of particle size (µm) of the encapsulated phenolic compounds, as a function of sodium alginate, chitosan and calcium chloride concentrations (% w/v).

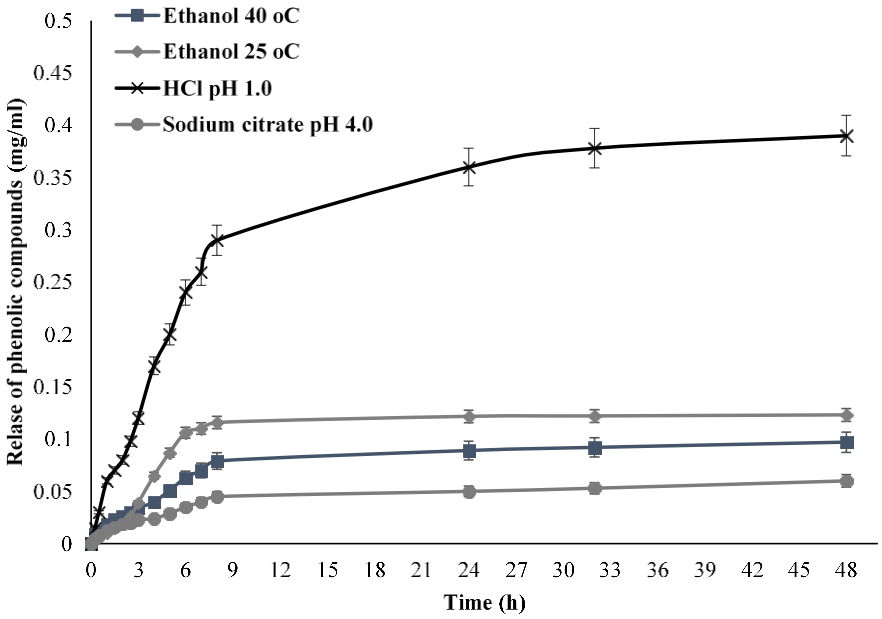


(a)



(b)

Fig. 6: Release kinetics of phenolic compounds, from sea buckthorn extract, from alginate-chitosan beads in several solutions.



CONCLUSIONS:

TPC of pomegranate peels extract is about 10 times more than that of sea buckthorn berries and 2 times more than its juice. Interesting, the antibacterial profile of both extracts and the results obtained from their inhibitory effect on human platelet aggregation were similar, with the stronger effect presented by pomegranate peels extract in both cases. Optimization studies in extraction of phenolic compounds, examining the same three parameters, revealed that these could be obtained by a similar procedure with pomegranate at lower temperature (35 °C) than sea buckthorn (45 °C). Aiming at a general method applied for the recovery of most phenolic compounds from plant resources (fruits, vegetables and possibly wastes) our findings are encouraging. Furthermore, encapsulation of the extracts in alginate-chitosan copolymers seems so far a good formulation method for these bioactive compounds. Our study is complementary of other previous reports on multifunctionality of sea buckthorn berries and therefore of its health benefits. In parallel, the advantages of recovering bioactive phenolic compounds from pomegranate peels, is of great importance as potential source of food supplements.

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DECLARATION OF INTEREST:

Declarations of interest: none

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