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Dissolution kinetics of polyphenol bearing calcium pectate hydrogels in simulated gastric or intestinal media and their anti-carcinogenic capacities

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ABSTRACT

Cherry laurel is an underutilized agricultural resource with a significant content of polyphenolic compounds that potentially may demonstrate antioxidant and anti-carcinogenic activities. In this study, the preparation of calcium pectate gels bearing cherry laurel polyphenols was aimed and their dissolution behaviors in simulated gastric and intestinal media were tested. In addition, the released fractions were collected and tested in cell culture media in order to determine the anti-proliferative characteristics. Cherry laurel polyphenol extracts were mixed with pectin dispersions (1:1) and calcium pectate gels were prepared by the extrusion dripping method. The freeze dried gel samples were analyzed for morphological characteristics (SEM) and dissolution kinetics in simulated gastrointestinal media. The rate of release in gastric medium was substantial (≥94.9%) in all cases and dissolved fractions demonstrated significant anti-proliferative activity (approx. 85% inhibition for 55K06 and 61K04 extracts) against MCT 116 colon carcinoma cells even after 20× dilution. The findings were discussed with emphasis on their relevance to pectin-polyphenol interactions in dissolution behavior of the gels in simulated gastrointestinal media, and also to the preparation of functional lyophilizates that can be utilized in functional foods and pharmaceuticals.

1. Introduction

Pectin is a major heteropolysaccharide found in plant cell walls, mainly composed of linearly linked galacturonic acid residues. In industrial applications, the degree of methyl esterification determines the gelation characteristics of pectin products. In the case of high methoxyl pectin (HMP) products, elevated solid material (i.e., sugar) content and low pH are necessary for gelation. Negatively charged carboxyl groups of low methoxyl pectin form cross-links with calcium ions which induces the ionotropic gelation of pectin (Sriamornsak, Thirawong, & Puttipipatkhachorn, 2005). LMPs can be gelled in media with lesser amounts of solids (%), consequently their utilization in calorie reduction is quite practical. LMPs are generally manufactured from HMPs based on acid, alkali or enzymatic treatments, whereas genuine sources of LMPs are relatively limited (Iglesias & Lozano, 2004). Sunflower heads are the major by-products of sunflower oil processing industry. Shi, Chang, Schwarz, Wiesenborn, and Shih (1996) developed an optimized methodology to isolate low methoxy pectin (LMP) from de-seeded sunflower heads. This product can safely be utilized as a fat replacer in food formulations and de-seeded heads contain 15–25% pectin by weight (Shi et al., 1996).

Pectin products have been widely utilized as drug carriers, ingredients in various controlled and sustained drug release formulations (Kyomugasho et al., 2016; Wong, Colombo, & Sonvico, 2011). Since pectins are generally resistant to digestion by human gastrointestinal enzymes but degraded by colonic microflora (Liu, Fishman, Kost, & Hicks, 2003), pectin based delivery systems have the potential to protect the bioactive compounds during the GI transit, while providing controlled release at targeted tissues. Tablets coated with a pectin film as well as pectin based pellets or microparticles are commonly used to deliver drugs to the colon (Wakerly, Fell, Attwood, & Parkins, 1996; Wong et al., 2011). Similarly, pectin products are being utilized in functional food formulations, for example, in the encapsulation of bioactive ingredients in order to improve the encapsulation efficiency and protect the bioactive materials (Drusch, 2007; Sansone et al., 2011).

In the intact plant cells, phenolic compounds are located

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primarily within the vacuoles which prevent their interactions with cell wall components (i.e., cellulose, pectin, hemicellulose etc). However, during the process of eating, rupturing of cell walls enables the interactions between the two moieties (i.e., phenolics and cell wall components) (Phan et al., 2015). The strength of interactions could affect the bioavailability of phenolics due to the varying rates of release in the gastrointestinal tract. According to Phan et al. (2015), for example, significant amounts of polyphenols can be bound to cellulose (0.4–1.4 g/g cellulose). Consequently, the behavior of polyphenol-carbohydrate complexes in the gastrointestinal tract needs to be clarified. As reviewed by Bordenave Hamaker & Ferruzzi (2014), rheological characteristics and digestibility of carbohydrate dispersions may be affected by the presence of phenolic compounds, while extractability of phenolics and their bioavailability are sensitive to the interactions with macromolecules including pectins. Furthermore, coexistence of apple phenolics and pectin were demonstrated to enhance the biological activity of the fruit (Aprikian et al., 2003). The adsorption characteristics of phenolic compounds to cell walls materials were dependent on the size of pores in cell walls and phenolic compounds and also their affinity to the cell wall materials (Bindon, Madani, Pendleton, Smith, & Kennedy, 2014). The coexistence of multiple phenolics in plant materials could affect both the extent of adsorption on cell walls and the extractability of the adsorbed materials (Bindon et al., 2014). This also could imply that absorption of phenolics in the human body may be clearly affected by noncovalent forces. According to Renard, Baron, Guyot, and Drilleau (2001), adsorption of polyphenols to apple cell wall carbohydrates was mostly governed by weak bonds such as H-bonds and possibly hydrophobic interactions, whereas Yuksel, Avci, and Erdem (2010) demonstrated that non-covalent hydrophobic interactions occurred between polyphenols and other food ingredients. Since polyphenols are small molecules, they might also be trapped into the porous network of larger molecules (Jakobek, 2015). Polymerized polyphenols could be encapsulated in hydrophobic pockets of macromolecules (Le Bourvellec & Renard, 2012). Pectin-rich fractions of blueberry could both protect anthocyanins in the body and promote colonic health (Lin, Fischer, & Wicker, 2016). These findings imply the utilization of pectin based matrices in the effective delivery of phenolic compounds. Due to the presence of an inert porous network, calcium pectate beads were shown to lead to the diffusion controlled release of active materials (Sriamornsak and Nunthanid, 1998; Aydin & Akbuğa, 1996).

Cherry laurel (*Laurocerasus officinalis* Roem, syn: *Prunus laurocerasus* L.) is a member of Rosaceae family, which is native to western Asia and also grown over the Balkans. Due to its edible fruits, it is commonly cultivated in northern Turkey (i.e., Black Sea region) as a native fruit crop (Islam, 2002) and also in Bulgaria, Serbia, Iran and some of the Mediterranean countries (Kolayli, Küçük, Duran, Candan & Dinçer et al., 2003). It can be classified to be an evergreen shrub or small tree (Sulusoglu, 2011; Çalışır & Aydın, 2004). The phenolic compound profile of cherry laurel has been partly elucidated and anti-oxidative properties of cherry laurel phenolics have been investigated (Alasalvar, Al-Farsi, & Shahidi, 2005; Karabegovic et al., 2014, 2013; Karahalil & Şahin, 2013; Kolayli et al., 2003). Recently, 14 different phenolic compounds were identified in cherry laurel, about half of which were demonstrated to exist in this fruit for the first time (Bayrambaş, 2016).

In this study, we have manufactured lyophilized forms of cherry laurel polyphenol bearing calcium pectate gel beads. The morphological characteristics of lyophilized beads as well as their dissolution kinetics in simulated gastrointestinal fluids were determined. The dissolved fractions were collected and their antiproliferative capacities against HCT 116 cells were studied. We discuss the findings in relevance to the utilization of functional lyophilizates in food formulations.

2. Materials and methods

2.1. Materials

All chemicals including simulated gastric and intestinal fluids were obtained from Sigma-Aldrich Corp and used without further purification.

2.2. Preparation of sunflower head pectin

Sunflower (*Helianthus annuus* L.) heads were collected from a local farm in Tekirdağ, Turkey. Pectin isolation was based on the optimized protocols described in Shi et al. (1996). Sunflower heads (SFH) were vacuum dried down to a moisture level of <8%. Immediately afterwards, dried SFH was ground to approx. 12 mm using a laboratory grinder. After grinding, powdered SFH was treated with an alkali solution (pH 7.5, 16 °C) for 25 min. The solvent to solute ratio was 28:1. Afterwards, the mixture was filtered and the filter cake was diluted with a sodium hexametaphosphate solution (1:25, 75 °C, pH 3.5, 1 h), while the medium pH was adjusted with 1 M phosphoric acid. The mixture was precipitated using 1 M nitric acid (5 °C, 1:5 acid solution:filtrate ratio) while the mixture was kept stirred (1 h). Once again, the precipitate was collected and mixed with 0.1 M nitric acid solution at a precipitate:solvent ratio of 1:2 for 20 min. This operation was repeated twice. Thus formed pectin gel was washed with an ethanolic solution (700 ml ethanol L^{-1}) 6 times at a gel to solvent ratio of 1:2. This process lowered the mineral content of pectin, enhanced the color attributes and removed acid which in turn increased the sample pH. After washing, the gel was dried in vacuum oven (55 °C, 16 h). Finally, dried products were ground using a laboratory grinder and sifted through 2 mm screens (Shi et al., 1996). Pectin samples were kept frozen at −20 °C until further use. In order to ensure the absence of proteins in the pectin samples, Kjeldahl and Lowry analyses were carried out for 10 different pectin samples, and proteins were not detected.

2.3. Fruit materials and the extraction of cherry laurel polyphenols

The fruit materials were harvested from three different cherry laurel varieties (55K06, 61K04 and 61K05) grown at Black Sea Region of Turkey, by the Black Sea Agricultural Research Institute Directorate of Samsun. 55K06 was harvested at an altitude of 30 m at Samsun province, Turkey. 61K04 and 61K05 were picked at Trabzon province near Araklı Yalıboyu, at an altitude of 10 and 40 m, respectively. Fruits were sampled when they reached full ripeness in September 2014. Approximately 20 kg of cherry laurel fruits were picked and kept at −20 °C immediately after the harvest and until further laboratory use.

40 g cherry laurel samples were mixed with 400 ml methanol and incubated for 15 min in an ultrasonic water bath kept at 65 °C (Karabegovic et al., 2014). Methanol insoluble materials were separated by filtration (i.e., 0.45 μm PTFE membrane filter). Using a rotary evaporator system (40 °C), methanol soluble phenolics were concentrated and methanol was removed from the samples. Concentrated polyphenol samples were transferred to amber glass containers and kept refrigerated (4 °C) until further use.

2.4. Sample preparation

Cherry laurel polyphenol bearing calcium pectate gels were prepared by the extrusion dripping method. 1% sunflower head pectin dispersions were prepared in high quality water. Three dilutions of cherry laurel extracts from the 3 varieties (55K06, 61K04 and 61K05) were mixed with pectin dispersions (1:1) in order to generate mixtures with a pectin concentration of 0.5% and cherry laurel extract concentrations between 5 and 50%. Using an automatic titrator, 2 ml of pectin-polyphenol mix was dropped into 100 ml $CaCl_2$ solution (0.2 M) at a rate of 1 ml per min. This mixture was kept stirred on a magnetic stirrer for 15 min. Immediately afterwards, beads were filtered and absorbance of the supernatant solutions were measured using 0.45 μm syringe filters, when necessary. The maximum absorbance wavelength was separately determined for all samples. Free (i.e., non-encapsulated) material content in the solution phase was calculated based on the absorbance values and consequently, encapsulation efficiency (%) was determined. Pectin beads were lyophilized (−54 °C at 1.28×10^{-2} mbar) using a Teknosem Toros TRS 2/2V Freeze Drier (İstanbul, Turkey) and kept frozen at −20 °C until further use.

2.5. Determination of encapsulation efficiency (%)

Immediately after the completion of extrusion dripping (Section 2.4), polyphenol bearing beads were gently filtered from the suspension. The remaining aqueous portion was scanned for its UV–Vis absorbance spectrum and the absorbance maximum (480 nm in $CaCl_2$ solutions) was determined as the detection wavelength. A reference curve was prepared using the diluted cherry laurel polyphenol extracts and based on the sample absorbance, polyphenol concentration in the supernatant was determined in comparison to the references. The extent of encapsulation was calculated based on the subtraction of non-encapsulated material in the supernatant from the polyphenol concentration in the properly diluted stock (51× dilution in 0.2 M $CaCl_2$ solution).

2.6. RP-HPLC analysis of cherry laurel polyphenols

The HPLC analyses of the extracts were performed on an Shimadzu LC-20AD HPLC system (Shimadzu Scientific Instruments) consisting of a pump, thermostated column compartment and photon diode array (PDA, SPD-M20A Model) detector used under previously described conditions and procedures with slight modifications (Gülseren & Corredig, 2013). Ascentis C18 Column, (4.6 mm ID × 250 mm, Supelco) with a particle diameter of 5 μm was used in the analysis. The mobile phase was composed of a mixture of water:glacial acetic acid (98:2,v/v %) (elution buffer A) and methanol (elution buffer B). Phenolic compound analysis was carried out at room temperature (25 °C) at a flow rate of 1 ml min^{-1}, and with PDA detection. The concentration of phenolic compounds in the extracts were calculated as mg phenolic compounds $g^{-1}$of fresh fruit.

2.7. Folin-Ciocalteu assay

Total amount of phenolic compounds in the cherry laurel samples in terms of gallic acid equivalency (GAE) were analyzed based on the Folin-Ciocalteu assay (Singleton, Timberlake, & Lea, 1978). Gallic acid was utilized as the reference compound and a variety of gallic acid concentrations were employed to prepare a standard curve. 1 ml gallic acid solution or sample was mixed with 45 ml water and 1 ml Folin-Ciocalteu reagent was immediately added to this mixture. Three minutes later, 3 ml Na_2CO_3 solution (3%) was added and the mixture was completed to 50 ml. The final mixture was kept stirred at room temperature and absorbance values were measured (720 nm, UV-1601 spectrophotometer, Shimadzu, Kyoto, Japan). Based on the calibration curve, gallic acid equivalency (GAE) values of the samples were determined (mg GAE per kg fresh fruit).

2.8. Scanning electron microscopy (SEM)

Freeze dried samples were coated with gold under argon plasma and visualized using a Bio-SEM instrument (JEOL-5000 NeoScope) at BİNATAM, İstanbul, Turkey. The accelerating voltage was 10 kV. Magnification range of the instrument was 10X-40000X without lens change. Vacuum mode was high, since secondary electron imaging detector was utilized. Finally, a sample stage travel range of 35 mm × 35 mm was used.

2.9. Dissolution kinetics in simulated gastrointestinal media and the extent of swelling

Dissolution kinetics were studied using PTWS 310 Dissolution Testing Instrument (PharmaTest, Hainburg, Germany). In order to monitor dissolution, 0.2 g of lyophilized sample was dispersed in 250 ml simulated gastric or intestinal fluids (SGF or SIF) without enzymes. The simulated media was brought to 37 °C prior to the addition of lyophilized samples. The stirring rate was 100 rpm. For SGF and SIF media, pedal and basket attachments were utilized, respectively. Approx. 3 ml samples were periodically removed from the dissolution chambers and tested for the rate of release from calcium pectate beads. The extent of release was calculated based on the reference curves of cherry laurel juices diluted in SGF or SIF media. The different pH values of SIF and SGF media alter the absorption values. In order to ensure the accuracy in the measurements, firstly wavelength scans were carried out for reference samples diluted in SIF and SGF media, and the corresponding absorption maxima for both sets of samples were chosen (520 and 400 nm for SGF and SIF media, respectively).

Gels were removed from the SIF medium after 24 h in the dissolution tester (37 °C, 100 rpm), blotted dry with tissue paper and weighed. The extent of swelling (%) was calculated from the mass measurements of wet and dry gel samples (Gunasekaran, Xiao, & Ould Eleya, 2006).

In the case of SIF medium analyses, an additional enzymatic treatment was also carried out in the presence of pectinase at an enzyme to substrate ratio of 1:500. The pectinase used here was purchased from Sigma-Aldrich Corp (P4716).

2.10. Cell culture

All work based on cell culture and cellular viability was carried out at GEMHAM, Marmara University, İstanbul, Turkey. Firstly, HCT 116 colon carcinoma cells were grown on McCoy's medium (10% FBS, 1% P/S and 1% L-glutamine). When a pre-determined cell concentration range was reached, the number of viable cells were determined using trypan blue and the cells were inoculated in petri dishes (60 mm) at a cellular concentration of 3×10^6. In order to facilitate adhesion, the cells were incubated for 24 h (37 °C, 5% CO_2). In the following day, the samples were added to the medium and upon the completion of a 24 h incubation period (5% CO_2), the influence of each treatment on cellular viability was determined based on the MTT assay.

2.11. MTT viability assay

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay is a colorimetric assay that is widely utilized in the evaluation of cytotoxic properties of bioactive compounds (Mosmann, 1983). In this assay, the number of viable cells after an anti-cancer treatment is determined based on the formation of purple formazan crystals by the enzymes in viable cells. Immediately after 24 h incubation with the treatment samples, media were removed from the petri dishes and fresh media (3 ml) that contained 100 μl MTT was added. The cells were kept incubated with MTT for another 2–4 h (37 °C, 5% CO_2). Once again, media were removed and 1 ml solubilizing solution was added to the petri dishes (5 min incubation at room temperature). Immediately afterwards, absorbance values (590 nm) were compared for treatments and a control sample in order to determine the extent of viability using a UV/Vis Multiwell Reader (96 well-plates and 50 μl/well). % Cellular viability was based on the absorbance values:

$$\%\,\text{Viability} = [(\text{Sample absorbance} - \text{blank})/(\text{control absorbance} - \text{blank})] \times 100$$

2.12. Statistical analysis

Data were presented as the means ± standard deviations from at least triplicate experiments in all cases. The differences between samples were also evaluated based on statistical significance ($p < 0.05$). Statistical significance was determined based on ANOVA tests.

3. Results and discussion

3.1. Encapsulation efficiency and particle morphology

Using an appropriate protocol, cherry laurel phenolic extracts were isolated from 3 different varieties (Bayrambaş, 2016). Three dilutions of each extract (100%, 50% and 10% stock) were prepared and mixed with pectin dispersions in order to prepare 9 different polyphenol bearing gel bead systems. Composition and coding of all samples were presented on Table 1. These samples were utilized in SEM imaging and dissolution measurements and coded as A1-A9, whereas the lyophilizate with no polyphenols was named as sample A (i.e., control sample).

First of all, pectin-polyphenol mixtures were dropped into $CaCl_2$ solutions and calcium pectate beads were prepared. In order to determine the encapsulation efficiency of the beads (Table 2), the supernatant solution was collected and analyzed for polyphenol content using UV–Vis spectrophotometry (480 nm). Based on the absorbance measurements, the encapsulation efficiency (%) values were calculated. In all cases, encapsulation efficiency was 94.9% or higher. Again in all cases, when 100% polyphenol stock was encapsulated, the pectin matrix was saturated and there was a small portion of polyphenols that remained in the solution phase (i.e., not encapsulated). At lower polyphenol concentrations, encapsulation efficiency clearly increased with decreasing polyphenol:pectin ratios and was 100% for all samples at the lowest stock concentration (5%). Gallic acid equivalency (GAE) values of the fresh fruits from all 3 varieties was in the order of 4 g kg^{-1} (Bayrambaş, 2016), whereas considering the amount of polyphenol loading in the current samples and the pectin concentrations utilized, the saturation concentration in the gels corresponded to a range of 0.2–0.4 mg GAE per mg pectin in all cases. However, there were slight differences between the behaviors of concentrates from the three varieties.

The findings of Bayrambaş (2016) were briefly summarized on Table 3. Chlorogenic acid was previously shown to be the major phenolic compound in cherry laurel varieties (Alasalvar et al., 2005). Similarly in 2 of the 3 varieties studied here, it was shown to be the major phenolic compound. In addition, a variety of other phenolic compounds (approx. 13) were detected in the extracts (Bayrambaş, 2016). The extraction methodology had a bearing on the phenolic profiles of the cherry laurel varieties, since the GAE values were relatively comparable (Table 3).

Immediately after the preparation of pectin beads, the supernatants were removed with filtration and the samples were freeze

Table 1
Sample coding of SEM and dissolution samples. Sample A denotes the reference calcium pectate gel sample with no polyphenols.

Cherry Laurel Type	Code	Pectin Concentration, %	Cherry Laurel Extract Concentration, %
	A	0.5	0
55K06	A1	0.5	50
	A2	0.5	25
	A3	0.5	5
61K04	A4	0.5	50
	A5	0.5	25
	A6	0.5	5
61K05	A7	0.5	50
	A8	0.5	25
	A9	0.5	5

Table 2
Encapsulation efficiency (%) of calcium pectate gels immediately prior to freeze drying. Three replicate samples were studied and relative standard deviation was less than 1% in all cases.

Cherry Laurel Type	Code	Encapsulation Efficiency (%)
55K06	A1	96.6
	A2	100
	A3	100
61K04	A4	94.9
	A5	97.5
	A6	100
61K05	A7	96.5
	A8	100
	A9	100

Table 3
Polyphenol composition (RP-HPLC) and gallic acid equivalency values of the cherry laurel extracts. Standard deviation values were <5% of the sample mean in all cases.

Measured variable	55K06	61K04	61K05
Chlorogenic acid (mg kg^{-1})	1604	51	852.8
Other phenolic compounds (mg kg^{-1})	1361.1	212.4	1284.7
GAE (mg kg^{-1})	4305	3738	4217

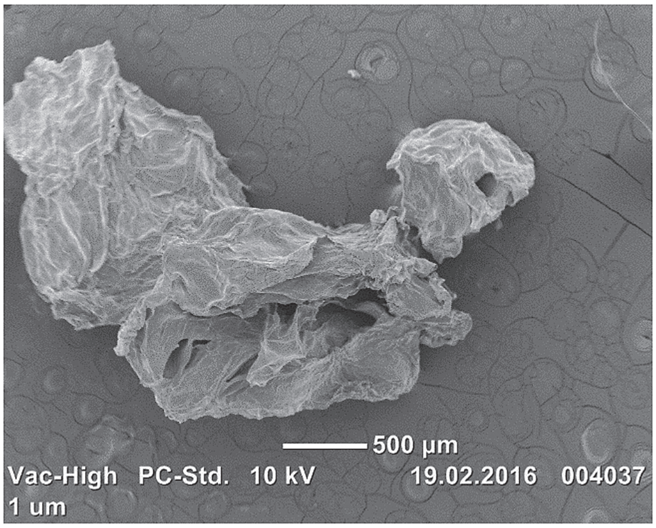

Fig. 1. SEM image for the reference calcium pectate gel prepared in the absence of any polyphenolic compounds.

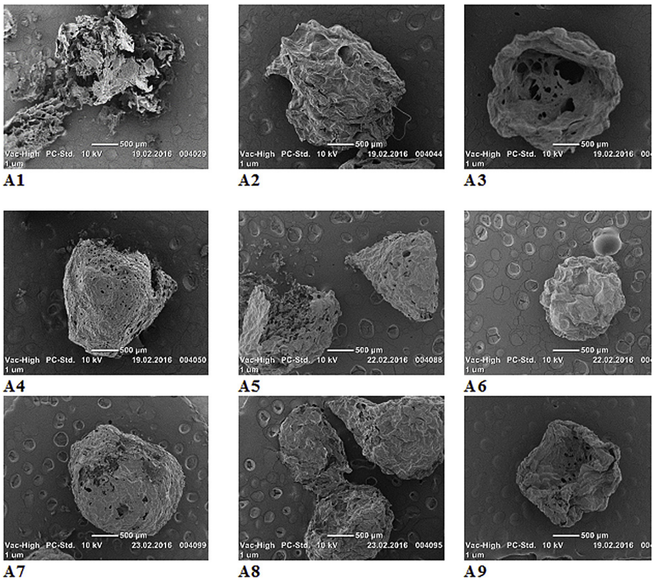

Fig. 2. A-I. SEM images for the calcium pectate gel containing polyphenolic compounds from cherry laurel varieties. The corresponding sample coding is indicated on Table 1.

Table 4
Dissolution kinetics of polyphenol bearing calcium pectate gels in simulated gastric medium. All measurements were carried out in triplicate and the relative standard deviation values were <3% of the sample mean in all cases.

Sample	t (min)	50% Stock % Dissolution	25% Stock % Dissolution	5% Stock % Dissolution
55K06	15	25.1	20	2.2
55K06	30	58.2	23.3	2.8
55K06	60	58.4	23.1	2.6
55K06	1440	58.4	23.1	3
61K04	15	34.2	23.3	ND
61K04	30	51.3	37.7	ND
61K04	60	51.3	37.5	ND
61K04	1440	51.6	37.1	ND
61K05	15	35.2	11.7	1.2
61K05	30	57.5	23.5	5.1
61K05	60	57.7	22.9	4.9
61K05	1440	57.7	23.5	5.1

Table 5
Dissolution kinetics of polyphenol bearing calcium pectate gels in simulated intestinal medium. All measurements were carried out in triplicate and the standard deviation values were <5% of the sample mean in all cases.

Sample	t (min)	50% Stock % Dissolution	25% Stock % Dissolution	5% Stock % Dissolution
55K06	10	0.7	ND	ND
55K06	20	1.7	ND	ND
55K06	30	0.7	ND	ND
55K06	40	2	ND	ND
55K06	1440	2	ND	ND
61K04	10	4.2	ND	ND
61K04	20	32.7	6.8	ND
61K04	30	94.3	65	53.6
61K04	40	96	64.1	71.7
61K04	1440	95	65	64.9
61K05	10	ND	ND	ND
61K05	20	ND	ND	ND
61K05	30	ND	ND	ND
61K05	40	ND	ND	ND
61K05	1440	ND	ND	ND

dried to investigate the particle morphology (Figs. 1 and 2) and dissolution characteristics (Tables 4 and 5). The morphology of calcium pectate beads (0.5% pectin, control sample with no polyphenols) was shown on Fig. 1. The particle surface was fairly smooth with a minimal amount of cracks and other surface defects. Although there were small holes in the center of particles, the particles were mostly intact. The SEM images for the polyphenol bearing samples (A1-A9) were shown on Fig. 2. In all cases, increased loading of phenolic compounds in gel beads were observed to visibly reduce the mechanical strength of the particles.

Since the pectin content was uniform among the samples and similar encapsulation efficiencies were measured (Table 1), certain phenolic compounds possibly affected the interactions within calcium pectate network. There are variations in phenolic compound profiles of the cherry laurel varieties, and based on the visual evaluation of the micrographs, 61K04 and 61K05 samples were thought to have lesser open pores than 55K06 samples. 55K06 sample had the highest GAE value and the chlorogenic acid concentration (approx. 88% higher than 61K05 and many times higher than 61K04), whereas both the GAE value and chlorogenic acid concentration of 61K04 were the lowest among all three samples (Bayrambaş, 2016).

It can be further anticipated that the variations in the morphology of the lyophilizates could influence the dissolution characteristics of encapsulated phenolic compounds from the pectin matrices. Therefore, dissolution kinetics of these samples were also investigated.

3.2. Dissolution kinetics in simulated gastrointestinal media

Lyophilizates (A1-A9) were dispersed in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) and the rate of phenolic compound dissolution was monitored (Tables 4 and 5). In SGF medium, the rate of dissolution was high and within 30 min of dispersing the lyophilizates, maximum phenolic compound concentration was achieved in SGF (Table 4). The extent of dissolution increased with the increased phenolic concentration which both increased the concentration gradient between the two phases and reduced the mechanical strength of the freeze-dried beads. In all cases, however, complete dissolution of the phenolic compounds was not possible which could mean that a certain amount of phenolics remained to be bound to the pectin molecules. Only in the case of 5% 61K04, no significant extent of dissolution was observed at the lowest polyphenol concentration (5% stock), possibly since the mechanical strength of the system was considerably higher and the polyphenol concentration was lower compared to the other samples. It must be stressed, however, that these experiments were carried out in the absence of enzymes. With enzyme addition, further dissolution of phenolic compounds can be expected. At low pH, the electrical charge on the pectin molecules is low (i.e., SGF medium), which in turn causes the pre-gelation of pectin in the form of a weak gel. Consequently, the ability of the pectin matrix to inhibit the release of active molecules could be limited (Ahrabi, Madsen, Dyrstad, Sande, & Graffner, 2000).

In the case of SIF dissolution, the rate of phenolic compound release was very limited (Table 5). For 55K06 and 61K05 extracts, 2% or less was released from the pectin matrices. Although the original mechanical properties of the lyophilizates were relatively uniform throughout every single sample and prior to both SIF and SGF experiments, much lower extents of release were observed during SIF studies. However, a substantial extent of release took place from the 61K04 samples (Table 5). These findings could once again be related to the differences in phenolic substance profiles and mineral contents of the three varieties and the influence of electrostatic interactions on the release characteristics of 61K04.

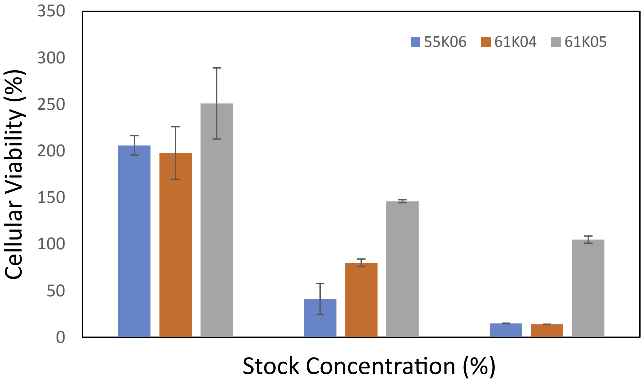

Fig. 3. Cellular viability (%) of HCT-116 cells as determined by the MTT assay (590 nm). The dissolved fractions from the SGF tests were utilized in the viability assays in order to determine their anti-proliferative capacities. Series were plotted in the order of increasing stock concentration (5%, 25%, and 50%) from left to right.

Possibly due to the relative importance of its phenolics on pectin binding, 61K04 restored a rigid mechanical structure ad SGF conditions, whereas at SIF conditions, repulsive electrostatic interactions might have reduced the capabilities of encapsulation. In the case of milk proteins, for example, electrostatic binding of polyphenols was found to be a major interaction mechanism (Xu & Chen, 2011). It must be stressed, however, that the pH values of all the samples were quite similar (pH 4.8–4.9) which in turn implied the relative importance of other compositional attributes.

A simple swelling protocol was established (Section 2.9) and the extent of swelling in SIF medium was calculated. The extent of swelling was approx. 107% after 1440 min (i.e., 24 h), while no significant extent of polyphenol release was observed. This meant that although the extent of dissolution and/or swelling could alter the release kinetics in SGF medium, the results of the SIF experiments were unaffected.

Furthermore, when a pectinase treatment was carried out in the SIF medium for 24 h, there were no significant changes in the polyphenol release or swelling characteristics of the lyophilizates (data not shown). Previously Holloway, Tasman-Jones, and Maher (1983) demonstrated that the extent of pectin degradation was minimal in the small intestine, whereas most pectin molecules were degraded in the large intestine. Consequently, with an appropriate gastric coating, these assemblies will be suitable for colonic delivery as well.

Based on these interactions, the current lyophilizates can be considered to release a certain portion of their phenolic content in gastric medium, whereas upon the progress of digestive processes, a lesser amount of remaining phenols can be expected to be released in the intestinal system. The phenolic compound profiles of the extracts seemed to have a bearing on the electrostatic binding characteristics as well. The dissolved fractions were collected and utilized in cell culture studies in order to probe their anti-proliferative activity against cancer cells. Due to the relative stability in the intestinal media, such lyophilizates could be delivered to colon once their gastric stability is ensured.

3.3. *Anti-proliferative activity of dissolved fractions*

The antioxidant activity of polyphenols could determine their anti-carcinogenic characteristics (Fiuza et al., 2004). Consequently, polyphenol rich fruit extract systems can be anticipated to demonstrate significant anti-carcinogenic activity. In this study, we have analyzed the anti-proliferative activity of cherry laurel extracts in cell culture experiments.

The dissolved fractions from SGF dissolution tests were collected and utilized in cell culture assays based on HCT 116 human colon carcinoma cells. The selection of SGF samples was based on the higher extent of release from the pectin matrices in SGF media (Table 3) compared to similar samples in SIF media. The SGF samples were incubated with HCT 116 cells (24 h) and the extent of cellular viability was determined via the MTT assay. Due to the high anti-proliferative potential of the extracts, cellular viability was largely impaired (data not shown). Consequently 5%, 25% and 50% (i.e., diluted) polyphenol stocks (see Table 1) were utilized in dissolution tests and using their dissolved fractions, MTT assay was carried out (Fig. 3).

Since the dissolved fractions were more dilute compared to the original extracts, their influence on the reduction of cellular viability was limited (Fig. 3). When the fractions generated from 5% stocks were administered, the cellular viability of the treated cells increased in all cases to approximately 206, 198 or 251% for 55K06, 61K04 and 61K05 samples, respectively. Along with a certain portion of dissolved polyphenols, these extracts possibly also contained varying amounts of minerals (i.e., calcium), sugars and sugar alcohols (i.e., glucose, fructose, sorbitol etc) (Alasalvar et al., 2005), and pectin. The presence of such ingredients possibly demonstrated a protective role against HCT 116 cells. As the polyphenol concentration of the original samples (i.e., lyophilizates) increased to 25% and 50%, the viability values steadily decreased in all cases. The extent of viability reduction was considerably higher in 55K06 and 61K04 samples compared to 61K05 counterparts. In the cases of 55K06 and 61K04 samples, the cellular viability was as low as 15 and 14%, respectively, after the treatment of HCT 116 cells with the SGF fractions of 50% stocks. The differences in viability might be attributed primarily to the differences in phenolic compound profiles of the extracts (Bayrambaş, 2016).

4. Conclusions

Both as a designed delivery system or a natural assembly, pectin-polyphenol interactions have the potential to influence the bioactivity of phenolic compounds. In this study, we have manufactured and lyophilized polyphenol bearing calcium pectate gels. These functional lyophilizates could offer pH tunable delivery with rapid release characteristics in the gastric or colon environments based on the utilization of appropriate coatings. We have effectively shown that these lyophilizates significantly lowered the viability of HCT 116 human colon carcinoma cells. Currently, biochemical investigations on the elucidation of anti-proliferative mechanisms are underway at our labs. In the future studies, we will make an attempt to enhance the suitability of the current lyophilizates to colonic delivery.

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