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Synthesis and characterization of pectin derivative with antitumor property against Caco-2 colon cancer cells

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ABSTRACT

New pectin derivative (Pec-MA) was obtained in specific reaction conditions. The presence of maleoyl groups in Pec-MA structure was confirmed by ^{1}H NMR and FTIR spectroscopy. The substitution degree of Pec-MA (DS = 24%) was determined by ^{1}H NMR. The properties of Pec-MA were investigated through WAXS, TGA/DTG, SEM and zeta potential techniques. The Pec-MA presented amorphous characteristics and higher-thermal stability compared to raw pectin (Pec). In addition, considerable morphological differences between Pec-MA and Pec were observed by SEM. The cytotoxic effect on the Caco-2 cells showed that the Pec-MA significantly inhibited the growth of colon cancer cells whereas the Pec-MA does not show any cytotoxic effect on the VERO healthy cells. This result opens new perspectives for the manufacture of biomaterials based on Pec with anti-tumor properties.

1. Introduction

Pectins (Pec) are anionic polysaccharides extracted from cell walls present in most plants. They consist primarily of (1 → 4)-linked α-D-galacturonyl units occasionally interrupted by (1 → 2)-linked α-L-rhamnopyranosyl residues (Racovita, Vasiliu, Popa, & Luca, 2009). Pectins are used as gelling and thickening agents and also present application in drug delivery systems, due to their excellent biocompatibility properties and good response to the pH. Furthermore, it is believed that Pec help to reduce cholesterol levels in blood, aid the reduction of glucose uptake, facilitate the excretion of toxins and divalent metals in urine, and they have anti-tumor qualities (Ogonczyk, Siek, & Garstecki, 2011; Rosenbohm, Lundt, Christensen, & Young, 2003).

Derivatives of Pec methacrylated (Souto-Maior, Reis, Pedreiro, & Cavalcanti, 2010), amidated (Mishra, Datt, Pal, & Banthia, 2008), thiolated (Perera, Hombach, & Bernkop-Schnurch, 2010), and sulfated (Cipriani et al., 2009) which have already been obtained and studied. Among these, the methacrylated derivative of Pec receives greater attention since it can be polymerized and then used to prepare biodegradable hydrogels for application in biomaterial field (Oh, Lee, & Park, 2009). The modification of biopolymers with methacrylate agents aims to obtain derivatives containing vinyl groups. The vinyl sites enable the chemical cross-linking and subsequent formation of hydrogels by covalent cross-links between chains of changed polymers (Maior, Reis, Muniz, & Cavalcanti, 2008). Studies allowed to develop materials based on biopolymers that are susceptible to enzymatic degradation by bacteria in the colon (Reis, Guilherme, Cavalcanti, Rubira, & Muniz, 2006) as well as to achieve methacrylated derivatives based on the Pec using maleic anhydride (MA) as agent of change. When compared with more commonly used methacrylated derivatives, the maleoyl derivatives can attract most interest due to their good biocompatibility and high reactivity of MA (Huang, Wang, & Luo, 2010). The MA is a low acquisitive-valued material and generally used in the production of unsaturated polyesters, which in turn are used in resins, composite materials, biomedical devices and release devices (DiCiccio A.M., & Coates G.W.; Yao et al., 2011). Furthermore, copolymers containing modified products of MA have been considered as versatile materials which can enable

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new applications in various fields of the industry. Styrene-maleic anhydride copolymer that presents anti-tumor activity is a good example (Karakus, Yenidunya, Zengin, & Polat, 2011).

Cancer continues to pose significant health problems worldwide. Despite medical advances over the last decade, further increase in understanding genetics of cancer and the application of novel drug therapy is going on. Approximately 782,000 people are diagnosed with colon cancer annually (Boca et al., 2011). Late diagnosis and difficult accessibility make the available therapies ineffective, leading to small success rates in beating the disease. Conventional treatments of colon cancer occur by surgical ablation, with chemotherapy and/or radiotherapy used as an adjunctive treatment. However, it is known that the conventional treatment is not selective to the cancerous cells and can also cause injury to the healthy cells. As the conventional approaches are not always effective against the disease, the development of new therapeutic methods is essential in improving the success rates in the colon cancer treatment (Scolaro et al., 2006; Takahara, Rosenzweig, Frederick, & Lippard, 1995). Conventional chemotherapeutic agents such as alkylating agents or anti-metabolites, although decreasing the tumor size, often fail to eradicate them and prevent their recurrence. Therefore, it is crucial to develop new substances that inhibit tumor growth selectively, without affecting healthy cells. So, it is interesting that such materials present properties to induce apoptosis in the cancer cells (Yallapu, Jaggi, & Chauhan, 2012).

Thus, the aim of this study was to obtain a new derivative based on pectin (Pec) with the potential to be applied in the manufacture of biomedical products such as hydrogels and scaffolds, among others. The new unsaturated derivative of pectin (Pec-MA) obtained contains ester bonds and carboxyl group terminals and was characterized by the ^{1}H NMR, FTIR, WAXS, TGA/DTG, SEM, and zeta potential techniques. Additionally, the cytotoxicity effects of Pec and Pec-MA on the human colon cancer cells (Caco-2 cells) and healthy VERO cells were evaluated, providing experimental support for the development of a new biomaterial based on Pec-MA with potential tumor combination therapy.

2. Materials and methods

2.1. Materials

Pectin (Pec) and dialysis tubes with 32 mm in diameter were purchased from Sigma-Aldrich (Brazil). Maleic anhydride (MA) was purchased from Vetec (Brazil). Other reactants such as N,N-dimethylformamide (DMF) and acetone were also utilized in this work and were of analytical importance. All reactants were used as received without some further purification step.

VERO (African green monkey kidney) cells and Caco-2 cell line, originated from a human colonic adenocarcinoma, were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco®, Grand Island, NY, USA). The samples were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco®) and 50 μg ml^{-1} gentamycin in an incubator set at 37 °C, 5% CO_2 and 95% relative humidity. The cells were expanded when monolayer reached confluence after 3 ± 1 day. After reaching 80% confluence, cells were digested by using Trypsin/EDTA solution (0.25% trypsin-Gibco®, and 1 mmol l^{-1} EDTA).

2.2. Synthesis of pectin derivative (Pec-MA)

The synthesis of pectin maleate (Pec-MA) was performed according to procedure previously published (Hamcerencu, Desbrieres, Popa, Khoukh, & Riess, 2007) with modifications. The Pec and AM samples were previously dried in vacuum for 12 h at 40 °C. So, the dried Pec (1.0 g) was dissolved in DMF (10 ml) while 3.0 g of maleic anhydride (MA) were dissolved in DMF (15 ml). Both solutions were maintained under stirring at room temperature for 12 h. Then, the MA-solution was dropped slowly into Pec-solution, under stirring at room temperature. The reaction was subjected to heating and maintained at 70 °C under stirring for 24 h. Finally, the resulting product was precipitated in acetone (200 ml), separated by filtration, re-dissolved in distilled water and placed in cellulose tubes for dialysis. The dialysis was performed against deionized water at pH 6.0 by four days, changing the buffer twice daily, and then the material was frozen and lyophilized at −55 °C by 72 h. The final product obtained was labeled as Pec-MA.

2.3. FTIR measurements

FTIR spectra were recorded using a Fourier transform infrared spectrophotometer (Shimadzu Scientific Instruments, Model 8300, Japan), operating from 4000 to 500 cm^{-1} at resolution of 4 cm^{-1}. FTIR spectra were obtained from KBr-based pellets. The pellets were prepared with 3 mg of the sample in 100 mg of KBr.

2.4. NMR measurements

^{1}H NMR spectra were performed on a Varian Mercury Plus 300 BB NMR spectrometer, operating at 300.06 MHz for ^{1}H frequency. For the acquisition of ^{1}H NMR spectra, 5.0 mg of Pec or Pec-MA were dissolved in 1.0 ml of D_2O. ^{1}H NMR spectra were acquired at 80 °C and the main acquisition parameters were as follows: pulse of 45°, recycle delay of 10 s, and acquisition of 128 transients.

The substitution degree (DS) of Pec-MA was determined through of the ratio between the areas of the signals: (i) due to the vinyl hydrogen [H7] at 6.60 ppm, (ii) due to the hydrogen of [H4] at 4.44 ppm. The DS was obtained from the equation below:

$$DS = ([H7])/([H4]) \times 100\% \quad (1)$$

2.5. Scanning electron microscopy (SEM)

Dry samples of Pec and Pec-MA were investigated through scanning electron microscopy (SEM) images (Shimadzu, model SS 550). The surfaces of samples were sputter coated with a thin layer of gold for SEM visualization. The SEM images were taken by applying an electron accelerating voltage of 10–12 kV.

2.6. Thermogravimetric analysis

Thermogravimetric analyses (TGA) of Pec and Pec-MA samples were carried out on a thermogravimetric analyzer (Shimadzu, modelo TG-50) at a rate of 10 °C min^{-1} under nitrogen atmosphere with N_2 flowing at 50 ml min^{-1} and at temperature ranging from 25 to 800 °C.

2.7. Measures of zeta potential and hydrodynamic diameter of polymer coils

Considering the polymer coils as spherical, the average hydrodynamic diameter (D_h) was obtained using a Zetasizer Nano ZS with He–Ne ($\lambda = 633$ nm) laser coupled, at a fixed angle of 173°. The measures of Zeta Potential (ZP) were performed in the same equipment using capillary cell with electrodes. The measures were performed with samples (Pec or Pec-MA particles at 1.0 mg ml^{-1}) in phosphate buffer solution at pH 7.0. The measures were done in triplicates.

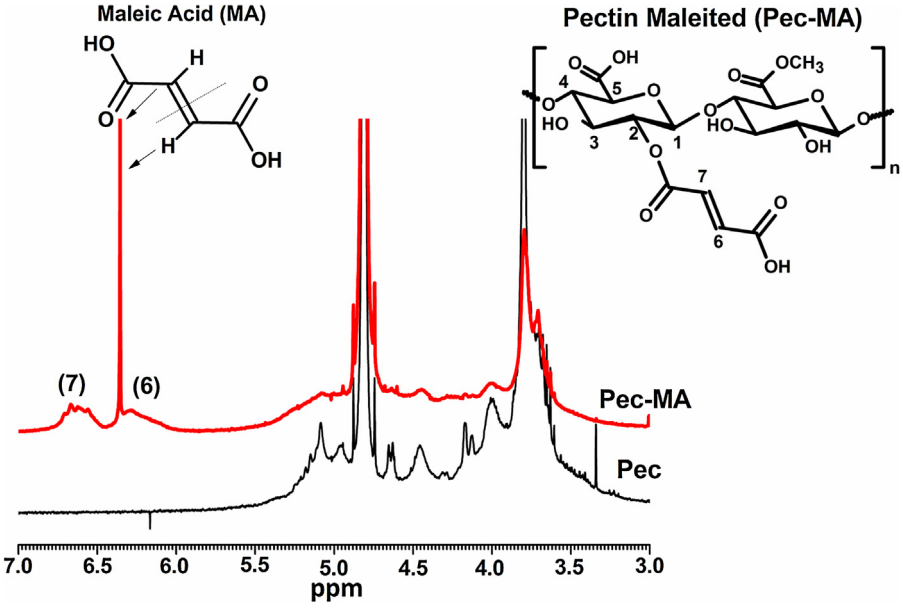
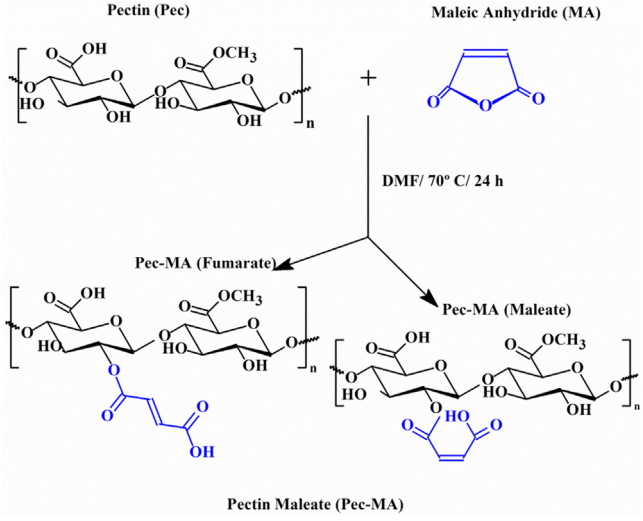


Fig. 1. ^{1}H NMR spectra of PEC and Pec-MA obtained in D_2O at 80 °C.

2.8. Cell viability assays

The cytotoxicity activities of Pec and Pec-MA against Vero and Caco-2 cells were determined through sulforhodamine assays (Fajardo et al., 2013; Skehan et al., 1990). The Vero and Caco-2 cells were seeded in 24 and 96 well tissue plates (TPP—Techno Plastic Products, Trasadingen, Switzerland) at a density of 2.5×10^5 and 8×10^5 cell ml^{-1} in 100 μl medium for 24 h in the CO_2 incubator, respectively. The samples were dissolved in water and after 8 h were added to the medium at various concentrations. After incubation for 48 h, the cell monolayers were washed with 100 μl phosphate buffered saline (PBS) fixed with trichloroacetic acid and stained for 30 min with 0.4% (w/v) sulforhodamine B (SRB-Sigma Chemical Co., St. Louis, MO, USA) dissolved in 1% acetic acid. The dye was removed by four washes with 1% acetic acid. Protein-bound dye was extracted with 10 mM unbuffered Tris-base solution [tris (hydroxymethyl)aminomethane] for determining the sample's optical density in a computer-interfaced, 96-well microtiter plate reader (Power Wave XS, BIO-TEK®, Winooski, VT, USA).



Scheme 1. Synthetic reaction route of Pec with maleic anhydride (MA).

3. Results and discussion

3.1. Characterization of Pec-MA

The maleic anhydride (MA) is susceptible to nucleophilic attack of hydroxyl or amino groups (Martins, de Oliveira, Pereira, Rubira, & Muniz, 2012), which are often present in the structures of polysaccharides. On the other hand, the hydroxyl groups present in the structures of polysaccharides are susceptible to esterification reactions. In general, primary hydroxyl groups (bonded to C6) or those situated in the equatorial position (bonded to C2) are the most reactive (Carey, 2000, Chapter 3). In this way, the esterification reaction between the MA and Pec is represented with detail in Scheme 1.

3.1.1. H^1 NMR measurements

Pec-MA having maleate groups was synthesized by esterification reactions with the MA, according to Scheme 1. The modification reaction starts by the nucleophilic attack on carbonyl of MA by hydroxyl groups present in the biopolymer leading to the formation of maleic acid. The vinyl group of maleic acid can lead to the formation of *trans* fumarate and *cis* maleate isomers (Scheme 1) (de Melo, da Silva, Santana, & Airoldi, 2009).

Fig. 1 shows the ^{1}H NMR spectra of Pec and Pec-MA. The signal at 3.78 ppm was attributed to the hydrogen atoms of esterified methoxyl groups of galacturonic acid units (—$COOCH_3$), or AGalMe. On the other hand, the signals at 3.70, 3.98, and 4.44 ppm were assigned to the hydrogen atoms H2, H3, and H4, respectively (Fig. 1) (Morris et al., 2002; Mukhiddinov, Khalikov, Abdusamiev, & Avloev, 2000; Rosenbohm et al., 2003; Tamaki, Konishi, Fukuta, & Tako, 2008; Winning, Viereck, Norgaard, Larsen, & Engelsen, 2007). The hydrogen atoms H1 that are present in the galacturonic acid units (AGal) and AGalMe units occur at 5.06–5.16 and 4.97–4.92 ppm (Fig. 1) (Renard & Jarvis, 1999). The hydrogen atoms H5 that are present in the AGal units appears between 4.5–4.7 ppm and the same occurs at 4.9–5.1 ppm in the AGalMe units (de Souza et al., 2009).

The presence of vinylic carbons in the Pec-MA structure was evidenced by the appearance of two new peaks at 6.30 and 6.60 ppm (Fig. 1). The peak at 6.30 ppm was assigned to vinyl

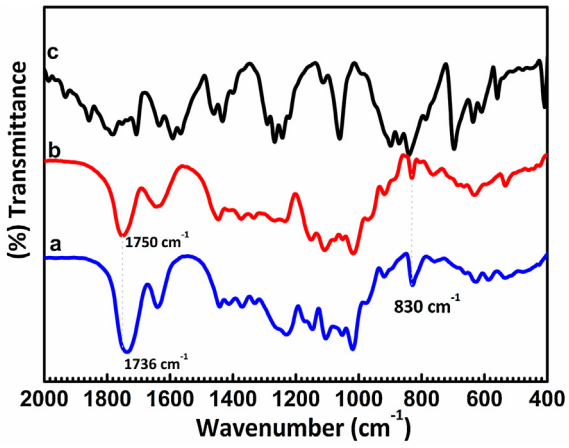


Fig. 2. FTIR spectra of Pec-MA (a), Pec (b), and MA (c).

hydrogen H6 adjacent to the carboxylic acid group, while the peak at 6.60 ppm was attributed to the vinyl hydrogen H7, which is adjacent to the ester group. The peak at 6.35 ppm was assigned to the presence of residual maleic acid (Hamcerencu et al., 2007). The Pec-MA presented DS of 24%, which was determined by ^{1}H NMR spectroscopy. Maleic acid has an axis of symmetry, showing a single peak for the vinyl hydrogen at 6.35 ppm. This peak did not affect the area under the peak at 6.60 ppm, selected to determine the DS. Hamcerencu et al. (2007) obtained a xanthan gum derivative with DS of 11%, using MA as modification agent and DMF as solvent at 70 °C for 24 h. The low DS occurred due to the complex structure of xanthan gum, which enables the low mobility of polysaccharide chains and hence decreases the reactivity of the sample in such reaction medium. However, the considerable high DS value obtained in the Pec-MA, compared to xanthan gum derivative, was attributed to the low molar mass of Pec.

3.1.2. FTIR spectroscopy

The incorporation of MA in the Pec structure was confirmed by FTIR spectroscopy (Fig. 2). In the FTIR spectra of Pec, Pec-MA, and MA, discrete changes as the shift of broadband at 1750 cm^{-1} (Fig. 2b)–1736 cm^{-1} (Fig. 2a) were observed. This effect was attributed to existence of conjugated esters on Pec-MA structure, confirming the presence of vinyl carbons (C=C) in the Pec-MA. The incorporation of MA in Pec structure increased the proportion of —COOH groups. Therefore, increase of area in the Pec-MA FTIR spectrum related to the signal (at 1736 cm^{-1}) assigned to the stretch of —COOH groups compared to the FTIR spectrum of raw Pec (peak 1, Fig. 3a and b) was observed (Follmann et al., 2012; Martins et al., 2013). The band at 830 cm^{-1} in the Pec-MA FTIR spectrum, related to the deformation of —COOH groups out of the plane, also presented increasing intensity compared to the FTIR spectrum of raw Pec (Fig. 3c). The intensity of area increased from 2.13 in the Pec FTIR spectrum to 8.79 in the Pec-MA FTIR spectrum (Fig. 3c). However, it was observed that the relative area of vibration the linkages —COO^{-} decreases after the modification process (peak 2, Fig. 3a and b). The occurrence of this fact was attributed to the possible formation of dimers that result from the interactions of hydrogen bonds between the carboxyl groups present in the chains of Pec-MA (Karakus et al., 2011). Moreover, the results presented in this section show that most of the carboxylic groups present in Pec-MA chains are not ionized. The FTIR spectral changes were significant, confirming the obtainment of Pec-MA derivative.

Table 1
Average size of Pec and Pec-MA particles.

Samples	Diameter (nm)	Zeta potential (mV)
Pec	463 ± 16	−13.0 ± 0.5
Pec-MA	91 ± 19	−22.8 ± 1.3

3.1.3. WAXS analysis

Fig. 4a shows the WAXS profiles of Pec, Pec-MA, and MA. The broad diffraction peaks with low intensity in the range (2θ) from 15° to 40° characterizes the low crystallinity of Pec and Pec-MA samples (Fig. 4a). However, the WAXS profile of MA and Pec-MA presents a narrow diffraction peak of high intensity at 2θ = 31.6° that was attributed to ordered regions formed by H-bonds among Pec-MA/Pec-MA chain segments (Fig. 4b). It is suggested that the Pec-MA chain segments present organized regions, where the formation of hydrogen bonds between the hydroxyl groups (—OH) and carbonyl (C=O) are favored (Fig. 4) (de Melo et al., 2009; Karakus et al., 2011).

3.1.4. Diameter of polymer coils, zeta potential and SEM analysis

The Zetasizer Nano ZS with He–Ne (λ = 633 nm) laser at fixed angle of 173° allowed to determinate the average hydrodynamic diameter of Pec and Pec-MA, considering the coils as spherical. The average diameter of Pec-MA coils is lower than the Pec (Table 1). Additionally, the zeta potential (ZP) measurements, performed in same equipment, showed that the density of negative charges on the Pec-MA surface was greater as compared to the surface of raw Pec (Table 1). The higher negative charge density in Pec-MA structure occurs due to insertion of carboxylic groups in Pec chains, a fact that confirmed the structural modification of Pec molecules.

The structural modification of Pec chains also was confirmed through analysis of SEM images, since the significant changes in the surface morphology of samples Pec and Pec-MA were observed (Fig. 5). According to the SEM images, the raw Pec presented a roughened surface whereas the Pec-MA derivative showed a smooth surface (Fig. 5).

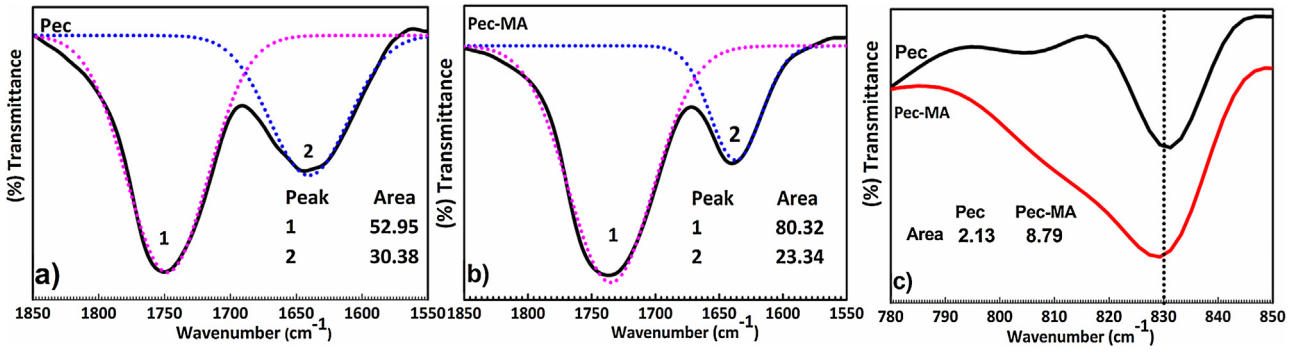


Fig. 3. Relative to the absorption area (COOH/COO^{-}) Pec (a), Pec-MA (b), and COOH absorption area relating to deformation outside the plane of Pec and Pec-MA (c).

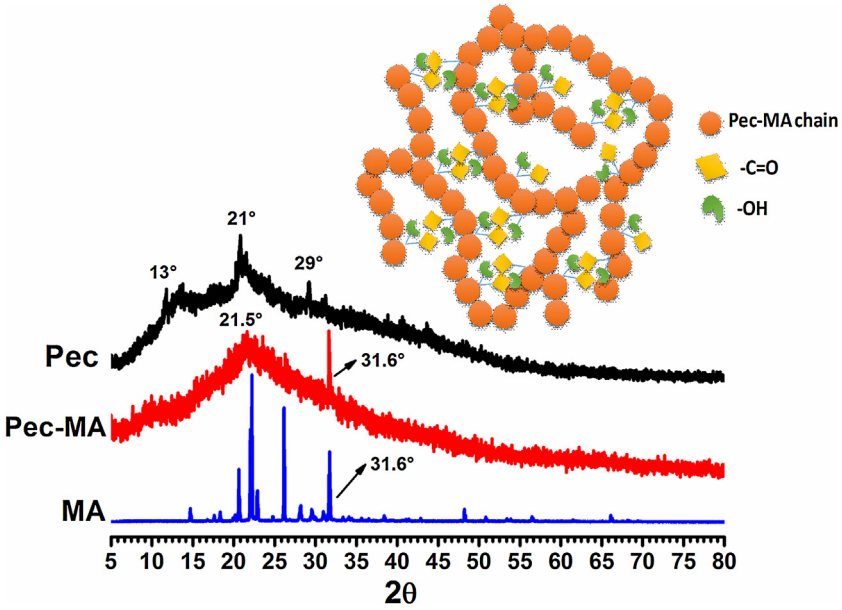


Fig. 4. WAXS profile of Pec, Pec-MA and MA (a); Probable structure of Pec-MA chains organized (b).

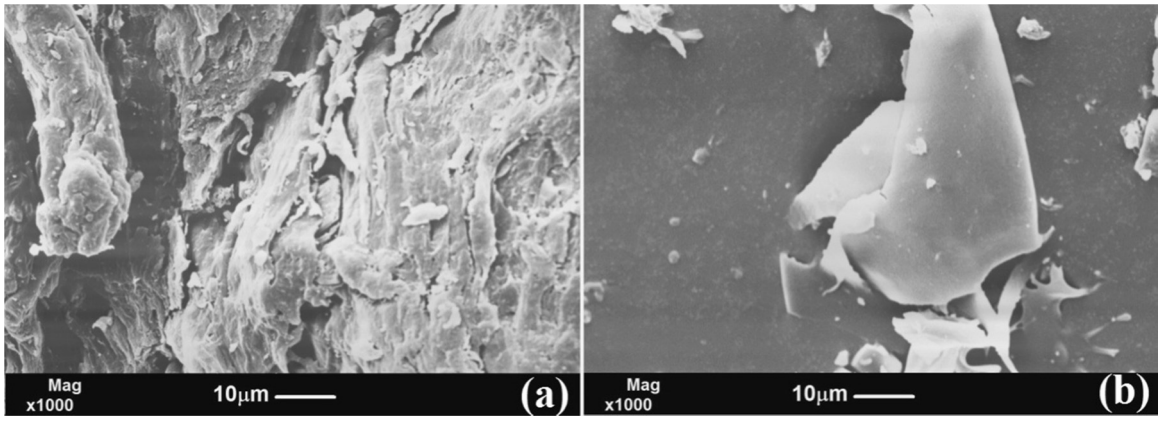


Fig. 5. SEM images of Pec (a) and Pec-MA (b).

3.1.5. *Thermal stability (TGA/DTG analysis)*

Fig. 6 shows the TGA/DTG curves of Pec and Pec-MA samples. The TGA/DTG curves show two pronounced mass loss that were attributed to different thermal events, being the first event related to the loss of water and volatile compounds. The second event of mass loss was attributed to degradation of the samples, which occurred in the temperature range of 220–320 °C. The samples Pec and Pec-MA showed intense degradation at temperatures of 250 and 268 °C, respectively (Fig. 6). The higher thermal stability of Pec-MA compared to Pec was assigned to the modification process. According to literature, the presence of MA in polysaccharide structures improves the stability of the obtained derivative (DiCiccio & Coates, 2011). On the other hand, it was observed that the degradation of Pec-MA starts at lower temperature, related to Pec sample. This can be attributed to the greater number of ester bonds in the Pec-MA structure, a fact resulting from the modification process of Pec. The not ordered regions of Pec-MA initiate the degradation at temperature lower than Pec but the degradation of ordered regions will initiate at temperature higher than the Pec. This explains the higher stability of Pec-MA compared to Pec.

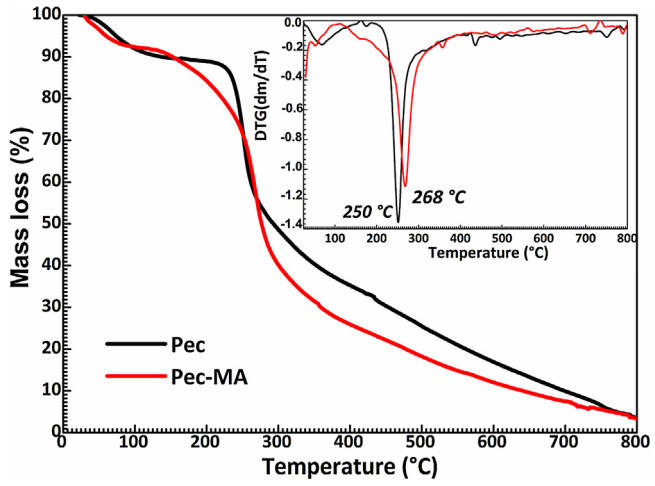


Fig. 6. TGA/DTG curves of Pec and Pec-MA.

3.2. *Cytotoxicity assays*

One objective of this study was to develop a new material that could be used effectively in the treatment of colon cancer. For this, the cytotoxic effects of Pec and Pec-MA systems against human colon cancer cells (Caco-2 cells) and against healthy cells of an

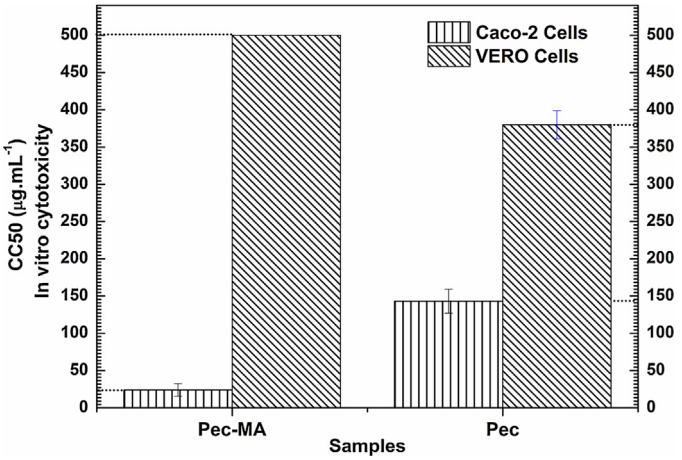


Fig. 7. Cytotoxicity effect of Pec and Pec-MA samples on the cell viability of Caco-2 colon cancer cells and healthy VERO cells. Error bars represent the standard deviation of three measurements.

African green monkey (VERO cells) were investigated. Fig. 6 shows the cytotoxic effect of Pec and Pec-MA samples on the Caco-2 and VERO cells after incubation of 48 h. It was verified that the Pec-MA acted more destructively against Caco-2 colon cancer cells but not against healthy VERO cells (Fig. 6). The half cytotoxicity concentration (CC_{50}) was calculated for both cases in the two samples (Pec and Pec-MA). Cell viability assays indicated that the Pec-MA system presented high cytotoxic effects on the Caco-2 cancer cells since the CC_{50} was 25 $\mu g\,ml^{-1}$ (Fig. 7). On the other hand, Pec-MA showed lower cytotoxicity effects on the healthy VERO cells, resulting in CC_{50} of 500 $\mu g\,ml^{-1}$ (Fig. 7). So, the Pec-MA was much more cytotoxic against Caco-2 cells since that the CC_{50} of Pec-MA on the healthy VERO cells was 20 times higher as compared to CC_{50} for Caco-2 cells.

By comparison, the Pec sample presented CC_{50} value of 140 $\mu g\,ml^{-1}$ and 380 $\mu g\,ml^{-1}$ against Caco-2 cells and healthy Vero cells, respectively (Fig. 7). These results demonstrated that the Pec-MA system is much more efficient than the Pec sample in inhibiting the growth of tumor cells of colon cancer, whereas the sample Pec-MA did not show cytotoxic effects on healthy VERO cells (Fig. 7). Thus, the MA insertion in Pec chain segments contributed to an increase of the cytotoxic effects on cancer cells, potentiating the antitumor activity on the Caco-2 cells and, at the same time, decreased significantly the cytotoxic effect against VERO cells. The results of this work showed that the new unsaturated derivative of Pec can be a promising material for use in the treatment of colon cancer. Also, the Pec-MA derivative can be used in the synthesis of hydrogels, employed as devices of controlled drug release, and also in the development of biomaterials with application in the biomedical and pharmaceutical field.

4. Conclusions

New unsaturated derivative of pectin (Pec-MA) was obtained through the esterification reaction of pectin (Pec) with maleic anhydride (MA) under specific conditions. The substitution degree (DS) of Pec-MA derivative was 24% being the DS determined by ^{1}H NMR. The Pec-MA was further characterized through FTIR spectroscopy, WAXS, TGA/DTG, SEM, and zeta potential techniques. The results showed that the Pec-MA has organized regions and high thermal stability as compared to raw Pec. The surface of Pec-MA presented higher density of negative charge due to the presence of higher amount of carboxylic groups as compared to raw Pec. Finally, cytotoxicity assays revealed that the Pec-MA system was more efficient in inhibiting the growth of Caco-2 colon cancer cells relation to raw Pec. On the other hand, the Pec-MA showed good biocompatibility against healthy VERO cells whereas Pec presented considerable cytotoxicity. The promising results presented here open perspectives for *in vivo* testing of the materials developed in this work.

Acknowledgments

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