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Biochemical and biophysical characterization of water-soluble pectin from *Opuntia ficus-indica* and its potential cytotoxic activity

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ABSTRACT

This work aims to fill the gap in the present knowledge about the structure of pectin from *Opuntia ficus-indica*. The water-soluble pectin (WSP) fraction, extracted with the Microwave Assisted Extraction (MAE), was further deproteinated (dWSP) and analyzed through several biophysical and biochemical techniques. HPSEC, light scattering and FTIR data showed that dWSP is low methylated high molecular weight pectin. The biochemical structure of dWSP, after methanolysis, silylation, carboxyl reduction showed that dWSP belongs to rhamnogalacturonan I class. Then, dWSP was heat-modified (HM) to obtain small-molecular weight deproteinated fraction (HM-dWSP). Both species, dWSP and HM-dWSP, were tested in LAN5 and NIH 3T3 model cells to study their biological effect. Results indicated that both dWSP and HM-dWSP exerted cytotoxic activity affecting selectively LAN5 cancer cells, without any effect on NIH 3T3 normal cells.

1. Introduction

An important non-cellulosic matrix polysaccharides in plants is pectin (Tanczos et al., 2003). It is a complex polysaccharide composed of α-1,4-linked D-galacturonic acid (GalA) backbone and segments consisting of alternating sequences of α-(1,2)-linked L-rhamnosyl and α-1,4-linked D-galacturonosyl residues ramified with side chains composed mainly of arabinose, mannose and galactose (de Vries et al., 1983; Niaounakis, 2013). The carboxyl groups of the constituent galacturonic acid molecules are esterified to varying extents, and pectins are mainly described as high- or low-methoxyl (HMP and LMP respectively), the latter having less than 50% of possible carboxyl groups esterified (Judd and Truswell, 1982). Pectin can be classified according to their molecular weight (Mw) into pectin with high Mw or low Mw (Zhang et al., 2015).

Pectin has lubricating and cementing functions. It is degraded during attack by plant pathogens and oligogalacturonides function as elicitor in the host-pathogen interaction (Albersheim et al., 1981). Commercial pectin is extracted from citrus, apple, or other higher plants, and is used as a stabilizer, thickener, gelling agent, emulsifier, and drug vehicle in the food and pharmaceutical industries (Wicker et al., 2014). Applications of pectin in cancer therapy, antitumor activity of modified pectin and its application as an excipient for antitumor drugs have been reported (Zhang et al., 2015). Pectin and other sources of dietary fibers are associated with gastrointestinal health, glucose tolerance, lipid digestion and weight management (Dikeman and Fahey, 2006). Many researches are carried out on the effects of what is called 'heat-modified' (HM) pectin or 'pectin oligosaccharides' (POS), in which the native molecules have been broken down into smaller fragments that, in theory, can be absorbed by our organism. Action mechanisms against cancer are still unclear but evidence suggests that small pectin fragments can bind to the carbohydrate recognition domain on the pro-metastatic protein galectin-3 (Gal3) and may block its interactions with other proteins and peptides, inhibiting Gal3 ability to promote cell adhesion and migration, and to prevent apoptosis. This raises the possibility to use HM pectin and POS as potentially safe, non-toxic approach for preventing or reducing carcinogenesis (Maxwell et al., 2012). The modified commercial pectin was mainly used to explore its bioactivity. It is possible that other plant sources, in combination with alternative extraction procedures, may

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Table 1
Monosaccharides composition detected as alditol-acetates and their relative concentrations.

Alditol	Retention time (min)	Relative Concentration (%)	Significant ions (*m/z*)
Neutral sugars			
Rhamnitol	18.90	06.20	115 > 99 > 129 > 159 > 70 > 145 > 201 > 217
Arabitol	19.25	15.07	129 > 171 > 115 > 99 > 157 > 87
Xylitol	19.74	02.89	129 > 171 > 115 > 157
Glucitol	26.93	11.40	115 > 145 > 103 > 128 > 187 > 170 > 217
Galactitol	27.18	23.10	115 > 139 > 187 > 157 > 103 > 259 > 217
Uronics acids			
Galacturonic acid[a]	/	41.25	/

[a] *The content of GalA (41.25%) has been calculated from the following equation derived from calibration curve of GalA in 3-phenylphenol microassay*. GalA % = (DO – 0.037)/(0.092).

afford more functional and bioactive products.

Despite enormous efforts that have been made in the search for novel drugs and treatments, cancer continues to be a major public health problem. Moreover, the emergence of resistance to cancer chemotherapy often prevents complete remission. Researchers have thus turned to natural products mainly from plant origin to circumvent bacterial resistance. Pectin and pH-modified or heat-modified (HM) pectin have demonstrated chemo-preventive and antitumor activities against some aggressive and recurrent cancers. In the present work, the deproteinated chemical structures of water soluble pectin (dWSP) fraction from *Opuntia ficus-indica*, a widespread plant in the Mediterranean area, was determined by biochemical and biophysical methods. Furthermore, to the best of our knowledge, for the first time the effect of dWSP and its heat-modified derivative (HM-dWSP) were tested on human neuroblastoma LAN5 and normal fibroblast NIH 3T3 model cells. We report that both molecular species are able to cause significantly slowdown of the cellular grow rate only in cancer cells without affecting normal ones.

2. Results and discussion

2.1. WSP deproteination

To avoid any interference during chemical derivatization for biochemical and structural analysis of the extracted WSP, a deproteination step seems to be of great importance. Proteins were removed using a modified Sevag method (York et al., 1986). Samples were scanned in the 240–400 nm wavelength range. A great decrease in protein concentration was observed after protein removal procedure, pointed out by the reduction of the absorption at 260–280 nm. Purification of the crude polysaccharide by Sevag method is based on the precipitation of protein with chloroform and *n*-butanol (Sevag et al., 1938).

In WSP the proteins total amount is 0.66% and it reduces to 0.063% after the Sevag deproteination steps. This means that the deproteination extent is about 90.50%. A loss of pectin is observed if Sevag deproteination is repeated many times. In fact, proteins cannot be completely eliminated because they are tightly connected to some pectic sites (Liu et al., 2008; Xie et al., 2011), particularly to the low molecular pectic fraction (Goycoolea and Cárdenas, 2003).

2.2. Molecular weight and size analysis

HPSEC analysis of WSP was already done in previous work (Lefsih Khalef et al., 2017). It is constituted of high molecular weight pectin appeared in the elution volume between 11 and 16 mL, while a smaller molecular weight proteinaceous fraction eluted between 18 and 20 mL. This last fraction was eliminated by Sevag deproteination step. The DLS measurements were done to check the effect of heat treatment on the degradation of dWSP into oligosaccharides. The electric field correlation functions, measured on dWSP before and after applying the degradation protocol (heat treatment), were analyzed by cumulant method (Koppel, 1972). The values of the hydrodynamic radius and polydispersity index for dWSP were 114 nm and 0.59, while those of HM-dWSP were 44 nm and 0.58 (Supplementary Information Fig. S1). This is an evidence that the heat treatment at 120 °C/20min reduced of about three times the average size of pectin molecules without changing the polydispersity index.

2.3. Monosaccharides composition of dWSP

Five standard alditol-acetates, prepared in our laboratory, were employed to ensure accurate identification of the monosaccharide's GC peaks observed during the analysis of dWSP samples. GC retention times (RT) of the alditol standards are provided as Supplementary Information (Fig. S2).

GC chromatogram stemming from dWSP, and retention times of detected alditol-acetates are displayed in Table 1. Six different acetylated alditols have been detected, rhamnitol, arabinitol, xylitol, glucitol and galactitol with relative contents of 6.20%, 15.07%, 2.89%, 11.40% and 23.10%, respectively.

It is well known that the decarboxylation of GalA, under reductive conditions, gives arabinose (Avigad et al., 1961; Conrad, 1931). So, we can say that the presence of arabinose is mainly stemming from the decarboxylation of GalA. This suggestion is confirmed by both TMS-derivatization and PMAA as we can see later.

Generally, there are very few studies showing that glucose (Glc) is part of the pectin structure. Zhang et al. (2013) have reported glucose as the second most abundant component of apple pectin (28.7%), which was ascribed to remnant soluble sugar that was not completely removed during the processing of pectin. However, Nunes et al. (2012) have shown the occurrence of GalA substituted by glucose, and Glc-β-(1-4)–Glc as structural features occurring in pectic polysaccharides, which could account for the glucose found in the composition of pectic material. Furthermore, glucose content might also be from non-pectic polysaccharides that were also extracted. These findings were probably due to the intensive binding of some proteins to polysaccharides and the existence of glycoprotein or proteoglycan. These binding proteins couldn't be easily eliminated by Sevag method (Chen et al., 2012).

The mass spectra of the analyzed carbohydrates were dominated by fragment ions at *m/z* 73, 117, 147, 204, 217, 361, 437, and 451. The RT were constant at the GC conditions with the derivatization procedures performed in this study. Fragments are typically produced by cleavage of the alditol chain (primary fragments) or by elimination of ions (secondary fragments) such as acetoxy group (*m/z*59), acetic acid (*m/z*60), formaldehyde (*m/z* 30) and ketene (*m/z* 42) (Biermann and McGinnis, 1988). The interpretation of the mass fragmentation for glucitol-hexaacetate, mannitol-hexaacetate and galactitol-hexaacetate is complex because they are stereoisomers having similar fragmentations. Significant ions such as *m/z* 145 (cleavage between C-2 and C-3), *m/z* 218 (cleavage between C-3 and C-4), *m/z* 289 (cleavage between C-4 and C-5) and *m/z* 361 (cleavage between C-5 and C-6) indicate presence of glucitol-hexaacetate.