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The polysaccharide and low molecular weight components of *Opuntia ficus indica* cladodes: Structure and skin repairing properties

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ABSTRACT

The *Opuntia ficus-indica* multiple properties are reflected in the increasing interest of chemists in the identification of its natural components having pharmaceutical and/or cosmetical applications. Here we report the structural elucidation of *Opuntia ficus-indica* mucilage that highlighted the presence of components differing for their chemical nature and the molecular weight distribution. The high molecular weight components were identified as a linear galactan polymer and a highly branched xyloarabinan. The low molecular weight components were identified as lactic acid, D-mannitol, piscidic, eucomic and 2-hydroxy-4-(4′-hydroxyphenyl)-butanoic acids. A wound healing assay was performed in order to test the cicatrizing properties of the various components, highlighting the ability of these latter to fasten dermal regeneration using a simplified *in vitro* cellular model based on a scratched keratinocytes monolayer. The results showed that the whole *Opuntia* mucilage and the low molecular weight components are active in the wound repair.

1. Introduction

In traditional medicine, herbs and plants containing mucilage, polysaccharides and starch are widely employed in relieving skin epithelium wound and mucosal irritation (Trombetta et al., 2006). Nowadays, skin wound-healing and repairing effects have been demonstrated for some of traditionally used herbs, such as *Calendula officinalis* (Parente et al., 2012), *Malva silvestris* (Pirbalouti, Azizi, Koohpayeh, & Hamedi, 2010), *Typha latifolia* (Gescher & Deters, 2011). The beneficial effects in promoting skin repair have been observed on various phases of wound-healing such as in bioadhesive ability, immunomodulation, cell–cell and cell-matrix interactions and collagen synthesis (Gescher & Deters, 2011; Parente et al., 2012; Pirbalouti et al., 2010).

The prickly pear cactus *Opuntia ficus-indica* (L.) is a tropical and subtropical plant able to grow in arid climates, such as the Mediterranean and Central America regions. It has recently received an ever increasing attention from researchers worldwide for its multivalent pharmaceutical and cosmetical potential (Kaur, Kaur, & Sharma, 2012; Rizza, Frasca, Nicholls, Puglia, & Cardile, 2012; Trombetta et al., 2006). Cladodes are particularly rich in carbohydrate-containing polymers, known as mucilage, made up of several sugar residues such as arabinose, galactose, rhamnose, xylose and galacturonic acid (Sepúlveda, Sáenz, Aliaga, & Aceituno, 2007). It has been previously reported that polysaccharides from *Opuntia* plants can be used as mucoprotective agents due to their capability to form a molecular network and to retain huge amount of water. Thus, they can act as a protective layer on mucosal surfaces accelerating the re-epithelization of dermal wound (Galati, Monforte, Tripodo, d'Aquino, & Mondello, 2001; Galati, Pergolizzi, Miceli, Monforte, & Tripodo, 2002; Kaur et al., 2012). In this context, Trombetta et al. (2006) demonstrated that

Abbreviations: OPF, *Opuntia* polysaccharide fraction; ORE, *Opuntia* Rough extract; OSMF, *Opuntia* small molecular weight fraction.

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topical application of polysaccharides from *Opuntia* cladodes on skin lesions in rats can accelerate the re-epithelization by affecting cell-matrix interactions and by modulating laminin laying, owing to their peculiar hygroscopic, rheological and viscoelastic properties. Moreover, polysaccharides isolated from *Opuntia* cladodes have highlighted an interesting and remarkable bio-adhesive effect on mucosal surface in some *in vitro* cell models (Rizza et al., 2012). However, previous studies have been focused only on the purified polysaccharide fraction from *Opuntia* cladodes thus no information has been reported to date about the whole extract and other non-carbohydrate components. Thus, to the best of our knowledge, very little is known about the structure-activity relationship between above described skin wound-healing effects and the cladode mucilage molecular composition.

Here we report the complete structural characterization of the polysaccharide and non-polysaccharide low molecular weight components from *Opuntia ficus-indica* (L.) (referred to here on as *Opuntia*) cladodes mucilage. The repairing activity of the isolated components was also evaluated by an innovative cell model mimicking *in vivo* conditions.

2. Materials and methods

2.1. Samples preparation

Opuntia cladode extract was supplied by BIONAP srl (OPUNTIA BIOCOMPLEX SH, Italy). *Opuntia* cladode juice was extracted by a mechanically press system and lyophilized to obtain a powder containing *Opuntia* mucilage (ORE). Thereafter, a weighted amount of ORE was dissolved in water (0.5% w/w) and purified by ultrafiltration system, equipped with a semi-permeable PES (polyethersulfone) membrane (KMS HFK^{TM} – 131, Koch, USA) with a cut-off of 10 kDa. This process gave two fractions containing small molecular components (OSMF) and high molecular weight polysaccharides (OPF) respectively.

2.2. Chemical analyses on the Opuntia polysaccharide fraction (OPF)

Determination of the sugar residues by gas-liquid chromatography/mass spectrometry (GLC–MS) analysis was carried out as previously described (De Castro, Parrilli, Holst, & Molinaro, 2010; Leontein & Lönngren, 1978). Monosaccharides were detected as acetylated *O*-methyl glycoside derivatives. After methanolysis (1.25 M HCl/MeOH, 85 °C, 24 h) and acetylation with acetic anhydride in pyridine (85 °C, 30 min), the sample was analyzed by GLC–MS. The absolute configuration of sugar residues has been established by GLC–MS analysis of the acetylated *O*-(+)-2-octyl glycosides derivatives and comparison with authentic standards. Linkage analysis was performed by methylation of the complete saccharide portion as described elsewhere (Ciucanu & Kerek, 1984; De Castro et al., 2010); the sample was methylated with iodomethane, hydrolyzed with trifluoroacetic acid (2 M, 100 °C, 2 h), carbonyl-reduced with sodium tetradeuteroborate ($NaBD_4$), acetylated with acetic anhydride and pyridine, and analyzed by GLC–MS. An aliquot of OPF was treated for 48 h with α-amylase from *Aspergillus oryzae* (Sigma-Aldrich A6211) and dialyzed exhaustively against distilled water. The digested product was collected, lyophilized and underwent above mentioned chemical analyses and further analyzed by NMR spectroscopy. An aliquot of the OPF was also treated with hydrofluoric acid (HF) (40%) for 48 h at 4 °C and dialyzed. The product was then purified by gel-filtration chromatography and the fractions obtained were analyzed by NMR spectroscopy.

2.3. Purification and separation of the Opuntia small molecules fraction (OSMF)

20 mg of the lyophilized OSMF were injected into a 1.5 × 120 cm Bio-Gel P-2 Extra Fine packed column (the useful fractionation range for carbohydrate and small peptide separations is 100–1800 Da, according to Bio-Rad). Gel filtration chromatography was run at room temperature with a flow rate of 8 cm/hr, using distilled and degassed water as eluent. The four fractions of 1.8 mL were collected, lyophilized and analyzed by NMR spectroscopy. The column was calibrated using Bio-Rad's Gel Filtration Standards having known relative molecular weights ranging from 600 kDa to 1 kDa.

2.4. NMR spectroscopy analysis

1D and 2D ^{1}H NMR spectra were recorded in D_2O at 300 K at pD = 7 with a Bruker 600 DRX spectrometer equipped with a cryo-probe. The spectra were calibrated with internal acetone ($\delta H = 2.225$ ppm; $\delta C = 31.45$ ppm). Total correlation spectroscopy (TOCSY) experiments were performed with spinlock times of 100 ms by using data sets (t1 × t2) of 4096 × 256 points. Rotating frame Overhauser enhancement spectroscopy (ROESY) and Nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed by using data sets (t1 × t2) of 4096 × 256 points with mixing times between 100 and 400 ms, acquiring 16 scans. Double-quantum-filtered phase sensitive correlation spectroscopy (DQF-COSY) experiments were executed by using data sets of 4096 × 512 points. The data matrix in all the homonuclear experiments was zero-filled in both dimensions to give a matrix of 4 K × 2 K points and was resolution-enhanced in both dimensions by a cosine-bell function before Fourier transformation. Coupling constants were determined by 2D phase-sensitive DQF-COSY (Piantini, Sorensen, & Ernst, 1982; Rance et al., 1983), Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were executed in ^{1}H-detection mode by single-quantum coherence with proton decoupling in the ^{13}C domain using data sets of 2048 × 256 points. HSQC was carried out using sensitivity improvement and in the phase-sensitive mode using Echo/Antiecho gradient selection, with multiplicity editing during selection step (States, Haberkorn, & Ruben, 1982). HMBC was optimized on long range coupling constants, with low-pass *J*-filter to suppress one-bond correlations, using gradient pulses for selection. HMBC was optimized for a 6 Hz coupling constant. The data matrix in all the heteronuclear experiments was extended to 2048 × 1024 points by using forward linear prediction extrapolation (Stern, Li, & Hoch, 2002).

2.5. Cell culture and samples preparations

A spontaneously transformed non-tumorigenic human keratinocyte cell line (HaCat cells) was used for *in vitro* scratch-wound-healing assay as reported by D'Agostino et al. (2015).

The lyophilized samples highlighted a water content of about 18% w/w for OPF and 17% w/w for both OSMF and ORE. The effect of these samples on wound closure rate was evaluated, incubating the scratched monolayer with OPF, ORE, OSMF at a final concentration of 0.1% w/v directly in the medium. Medium pH and osmolality (7,2–7,4 and 300 mosm) containing the diverse substances were verified to ensure physiological conditions.

2.6. Hydrodynamical characterization of Opuntia samples

The chromatographic analyses of *Opuntia* samples were performed using the SEC–TDA equipment by Viscotek (Lab Service Analytica, Italy) as previously described (La Gatta, De Rosa,