# Pectins from *Aloe Vera*: Extraction and Production of Gels for Regenerative Medicine

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## INTRODUCTION

*Aloe Vera* (*Aloe barbadensis Miller*) belongs to the Liliace family and it is mainly used for medical, cosmetic, pharmaceutical and food purposes. In the last decade, the modern science is validating the healing properties of the constituents found in *A. Vera*. Nowadays, it is well known that *A. Vera* pulp, as component of ointments and lotions, has several important therapeutic properties for wound healing,[1] anti-inflammatory treatments,[1] thermal injury healing,[2] and immunomodulation against infection.[3]

Aloe-based products for internal use are promising also for the treatment of coughs, diabetes, cancer, immune-system deficits, and many other conditions.[4–6] *A. Vera* gel is also extensively employed in the food industry because of its regenerating and anti-inflammatory properties.[7]

*A. Vera* has elongated and pointed leaves consisting primarily of two parts, an outer green rind that includes the vascular bundles, and the inner pulp. The *A. Vera* pulp is a hydrocolloid mainly composed of 98.5% of water and of 1.5% of several components such as vitamins, minerals, enzymes, organic acids, monosaccharides (including glucomannan, galactose, xylose, arabinose), and polysaccharides, such as celluloses, hemicelluloses, and pectins, which compose the cell wall.[4]

Pectins are biocompatible and versatile polysaccharides (Figure 1) recently used in pharmaceutical applications as drug delivery systems[8,9] and in biomedical field as coatings of different biomaterials.[10–13]

Pectin from the cell walls of *A. Vera* shows specific characteristics that can be considered appealing for biomedical applications,

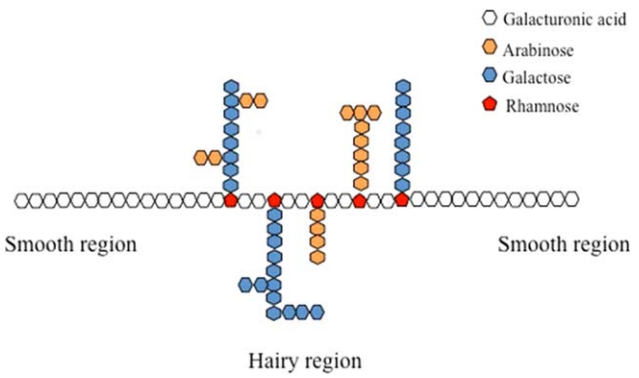


**Figure 1.** Selected regions of pectins: a backbone of 1,4-linked α-D-galacturonic acid, which represents the smooth region and contains a variable number of methyl ester groups and a hairy region consists of rhamnogalacturonan-I and rhamnogalacturonan-II, branched polysaccharides containing blocks of neutral monosaccharidic units, galactose, and rhamnose.[51] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

such as the great amount of rhamnose, at least two times higher than that in commercially available pectins, the high content of galacturonic acid (approximately 90% of the whole molecule) with a low Degree of Esterification (DE < 50%).[14] The branched rhamnose-rich regions of pectin chains seem to improve the molecular interactions between cells and the polysaccharide, while the branched hairy regions promote the formation of entangled structures, which may enhance the immobilization of healing agents.[10] Furthermore, the high content of galacturonic acid and the low DE promote the availability of $COO^-$ groups, involved in the gelling process.[15]

Pectin characteristics change in relation to the plant species from which it is extracted, however, pectins with different proprieties can derive from the same plant and even from the same cell wall.[16] In addition, the extraction processes can modify the pectin characteristics, and in particular the molecular weight: extractions at room temperature result in pectin with high molecular weight but low yield, whereas extractions at higher temperatures (over 80° C) lead to a lower molecular weight pectins, but with an increase in yield.[14]

Recently, the studies of our group were focused on different uses of pectin with the aim to obtain scaffolds, injectable gels, microspheres, and other systems in the perspective to use this interesting polysaccharide for regenerative medicine applications.[17–19] The gained experience and knowledge allowed studying the optimization of the extraction process of the Aloe pectin.

Pectin can be extracted from different sources (i.e. oranges, apples, sunflowers, pumpkins) with different methods, including the use of chelating agents, acids (like HCl) at high temperatures to increase the yield, bases (as NaOH) that, however, cause extensive degradation, or specific expensive enzymes (including arabinase, galactanase, polygalacturonase, and rhamnogalatturonase).[20–23]

The extraction procedure was developed according to the protocols found in the literature for the extraction of pectin from different sources.[20,22,24,25] This procedure was optimized by introducing a microwave pretreatment, to preserve the quality of the extracted pectin in terms of molecular mass and intrinsic viscosity,[26] and a pulp treatment with sodium citrate as a chelating agent, to limit the decrease of molecular weight of the extracts when operating in biocompatible conditions.[27] The extraction of Aloe pectins with the chelating agent ethylenediaminetetraacetic acid (EDTA), able to bind to the calcium ions of pectin chains, thus promoting pectin dissolution in water, was found to be highly efficient with a yield higher than 50% (w/w), with a galacturonic acid content of 70% (w/w).[14] However, EDTA has some limitations because of its cytotoxicity for biomedical application purposes.

In this work, for the first time at the best of our knowledge, pectin extraction from *A. Vera* was optimized to prepare A. vera pectin gels and assess their cytocompatibility.

## EXPERIMENTAL

Fresh leaves of *A. Vera,* kindly provided by Farmacia Ambreck (Milano, Italy), came from a controlled growing condition plantation in Israel. The *A. Vera* leaves had a pH of 4.65, with a loss on drying of 4.2 g/100 g and a content of moulds and yeast less than 10 per 1 g. The leaves were purchased at Aloe Vera House in the Moshav of Ein Yahav. The green rind of the fresh leaves was removed and the clear pulp was washed in distilled water for 30 minutes (10% (w/v)) to remove the anthraquinone-rich sap.

### Swelling and Stability of *A. Vera* Pulp

The clear pulp was characterized by evaluating the water uptake and weight loss after freeze-drying for 24 h at −50°C (LIO-5P, 5pascal srl). The swelling rate of the freeze-dried was investigated upon immersion of the samples ($n = 3$) in 15 mL of Phosphate Buffered Saline (PBS, P4417 Sigma Aldrich) up to 24 h at 37°C. At each time-point, the samples were wiped with filter paper to remove the excess liquid and weighed.

The water uptake (WU%) was determined according to the following equation:

$$\mathrm{WU\%} = \frac{W_t - W_0}{W_0} \times 100 \quad (1)$$

where $W_t$ is the wet weight at the time point t and $W_0$ is the initial weight of the samples.

The stability (WL%) of freeze-dried samples was determined by measuring the weight loss up to 14 days in PBS, accordingly to the following equation:

$$\mathrm{WL\%} = \frac{W_0 - W_f}{W_0} \times 100 \quad (2)$$

where $W_f$ is the final dry weight and $W_0$ is the initial weight of the samples.

### Ruthenium Red Assay

Samples of the inner gel from three leaves were stained with ruthenium red (84 071-5G, Sigma, Italy) to selectively detect pectin distribution within the cell walls. Transverse and longitudinal sections obtained from the pulp were immersed in 0.2% (w/v) ruthenium red solution (1 g of aloe gel in 10 mL of

ruthenium red solution) for 15 min at room temperature, keeping the solution under stirring to allow the penetration of the dye. Then, the samples were washed in distilled water and observed under a stereomicroscope (Nikon SMZ1000, WD 70, Japan). The presence of pectin was detected by red coloring of the pulp.

### Extraction of Aloe Pectin

**E1 Process.** Briefly, the first process (E1) started with a separation with ethanol (02860 Sigma Aldrich) of Alcohol Insoluble Residues (AIRs) which include pectin. To this aim, the inner gel of Aloe was sliced and treated in 90% (v/v) boiling ethanol, at 70°C for 30 min [20% ($w_{pulp}/v_{ethanol}$)]. The residue was then washed for 10 min in 50 mL of ethanol [96% (v/v)]. After cooling, the residue was separated from the extract by filtration on sintered glass funnel. AIRs (1 g) were dispersed in 30 mL of 50 m*M* HCl (35328 Sigma, pH = 2.9) and treated at 80°C for 1 h. The cooled extract was separated from the residue by filtration on sintered glass funnel. The pH of the extract was adjusted to 5.5 with 1*M* NaOH (S90637-369 Sigma). The product was then extensively dialyzed against deionized water at 4°C using a membrane with a molecular cut of 3500 Da (Spectra/Por® Dialysis membrane, Spectrumlabs), which allowed to remove the salts present in the solution and to retain a pectin- rich solution inside the membrane Finally, the freeze-drying of the final product was performed, as described before.

**E2 Extraction.** E2 followed the procedure for E1 extraction, but the isolation process was changed from freeze-drying to precipitation into isopropyl alcohol [96% (v/v), 278475 Sigma] and then the obtained product was centrifuged (ALC, 4236 Centrifuge, 2000 rpm for 1 minute).

**E3 Extraction.** E3 procedure followed that of E2 extraction, but a microwave pretreatment was added. Pulp samples were immersed in deionized water and heated at 400 W for 10 min in a microwave oven (Clatronic MWG 737) before the AIRs separation. To improve protein solubility, the volume of ethanol for separation of AIRs was varied from 90% (v/v) to 70% (v/v).

**E4 Extraction.** E4 procedure followed that of E3 extraction, but, instead of using HCl solution, the AIRs were dispersed in a 50 m*M* sodium citrate solution (71404 Sigma) (0.5% w/v) and treated at 70°C for 2 h. In these conditions, the dialysis time was extended from 12 to 24 h to allow the removal of the salts.

### Analysis of Extracted Pectin

**Pectin Yield.** The yield of the AIRs after ethanol treatment, compared to the fresh pulp weight, was estimated by the following equation:

$$\text{AIR yield} = (g_{(\text{dry AIRs})}/g_{(\text{fresh pulp})})100 \quad (3)$$

The yield of the extracted pectins from the dry AIRs weight was calculated as follows:

$$\text{Pectin yield}_{\text{AIRs}} = (g_{(\text{extracted pectin})}/g_{(\text{dry AIRs})})100; \quad (4)$$

Finally, the pectin yield compared to the initial dry pulp was estimated as follows:

$$\text{Pectin yield}_{\text{PULP}} = (g_{(\text{extracted pectin})}/g_{(\text{dry pulp})})100 \quad (5)$$

**FT-IR Analysis.** The extracted pectin samples were characterized by FT-IR spectroscopy (Nicolet 6700, Thermo Electron Corporation) by use of a single-bounce Attenuated Total Reflectance (ATR) accessory equipped with a ZnSe crystal. Spectra of dried samples were acquired at a resolution of 4 $cm^{-1}$ (region of 4000–700 $cm^{-1}$) using the OMNIC software. In order to quantify the presence of esterified carboxylic groups and of the carboxylate anions, the areas of the bands at 1745 and 1630 $cm^{-1}$ were recorded by the use of the built-in software of the FT-IR instrument. The limits of the band at 1745 $cm^{-1}$ were set from 1800 and 1690 $cm^{-1}$ and those of the band at 1630 $cm^{-1}$ from 1690 and 1530 $cm^{-1}$. The area peaks were then normalized versus the area of the CH stretching at 2900 $cm^{-1}$ (limits set from 3000 and 2830 $cm^{-1}$).

Low methoxyl (LM) commercial pectin from citrus fruits (CU701, DE = 42%, Herbstreit & Fox Neuenbuerg), was used for comparison purposes.

**Molecular Weight Evaluation by Intrinsic Viscosity.** The intrinsic viscosity $[\eta]$ of the extracted pectin samples was measured using an Ostwald viscometer. The measurements were made at a temperature of 24°C ± 1°C. Pectin powders were dissolved in 0.1*M* NaCl (13423 Sigma) with five different concentrations (2, 1.33, 1, 0.8, 0.67 mg/mL); for all solutions, the efflux time was measured. Intrinsic viscosity $[\eta]$ was determined by Mark–Howkin–Sakurada equation:

$$[\eta] = K \times M^{\alpha} \quad (6)$$

where $K = 9.55 \times 10^{-4}$, $\alpha = 0.73$.[28]

**Measure of the Degree of Esterification.** The colorimetric method of McReady[29] was used to quantitatively evaluate the ester groups on pectin structure. The carboxylic acids derivatives, such as the esters, react in the presence of hydroxylamine to form hydroxamic acid complex that produces red/purple ions with Iron (III). In short, 1 mL of a 1% (w/v) aqueous solution of pectin was added to 1 mL of 13.9% (w/v) hydroxylamine solution (379921-5G Sigma) and to 1 mL of 3.5*M* NaOH. The resulting solution was left under stirring for 2 min, then 1 mL of 4.1*M* HCl (37% 25148 Sigma) was added, followed by 1 mL of 0.74*M* ferric chloride ($FeCl_3$, 157740 Sigma) solution in 0.1*N* HCl. A yellow-orange color was developed in the sample solutions, and the absorbance of the chromophore was measured at 520 nm by UV spectroscopy (6705 UV/Vis Spectrophotometer, Jenway). Before each acquisition, a spectrum of the blank solution containing all the reagents, pectin excluded, was acquired. To evaluate the pectin degree of esterification, the absorbance of the blank solution at 520 nm was subtracted to the absorbance of the unknown sample at the same wavelength.

The measured absorbance was fitted in a calibration curve previously developed[18] to obtain the degree of esterification.

### Gelling Behavior of the Extracted Pectin

Pectin gels were produced by using the pectin powder extracted from the E4 process. Before the assessment of the cytocompatibility tests, the pectin powder was disinfected with ethanol (5 mL of ethanol solution for 10 mg of pectin powder). In short,

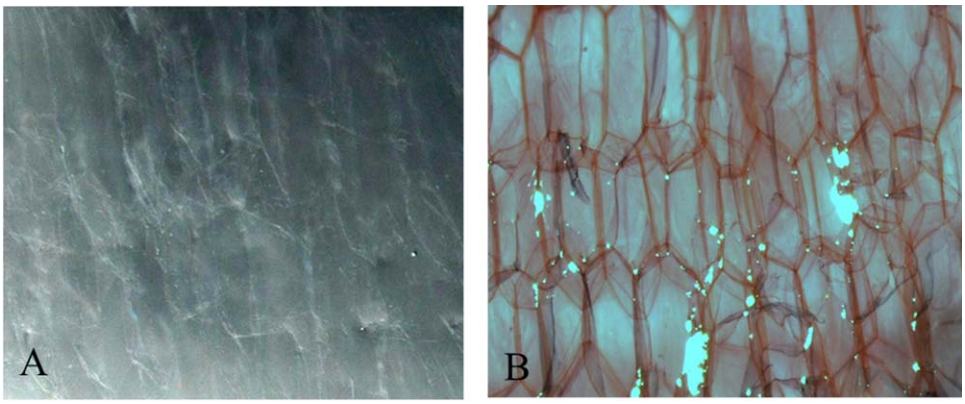


**Figure 2.** Optical microscope images of the pulp before (A) and after (B) ruthenium red staining: the red staining of pectin (B) shows the typical honeycomb structure of the plant cell walls. The inner zone remained transparent to the dye. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the pectin powder was suspended in ethanol solution (96% v/v) for 1 h under vigorous stirring. The ethanol solution was changed every 15 min. Then the powder was dried under a laminar flow hood until complete alcohol evaporation. The pectin powder was employed for producing gels with $Ca^{++}$ ions using the ionotropic method.[18] In details, 1 mL of 2% (w/v) pectin aqueous solution was put in each well of a 48-multiwell plate. The well plate was left overnight at −30°C, then 1 mL of 1.6% (w/v) aqueous solution of calcium chloride ($CaCl_2$, C1016 Sigma) was added, and the multiwell plates were left at 4°C overnight to allow the formation of homogeneous gel networks. Subsequently, the $CaCl_2$ solution in excess was discarded, and the gels were washed three times with 1 mL of distilled water, with changes of water every 10 min. All steps were performed in a laminar flow box.

### Swelling and Stability Pectin Gels

The swelling and the stability of pectin gels were investigate upon immersion of the samples ($n = 3$) in 15 mL of PBS up to 24 h at 37°C. The water uptake (WU%) and the weight loss (WL%) were determined according to the eq. (1) and (2).

### Cytocompatibility of Pectin Gels

The cytocompatibility of the disinfected gels was tested *in vitro,* using the osteosarcoma cell line SAOS-2 (American Type Culture Collection, Rockville, MD) by a direct contact test. Briefly, SAOS-2 cells were collected from a stock culture by trypsinization and seeded onto multiwell tissue culture plates (TCPS) with a cell density of $1 * 10^6$ cells/$ml_{gel}$. The cells were fed with culture medium (McCoy's 5A, Lonza) supplemented with 10% FBS and incubated at 37°C in a 5% $CO_2$ atmosphere. After the incubation time (24 h), the cell viability was evaluated by the MTT test. MTT assay is based on the use of the MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] that is reduced to a purple insoluble formazan product in living cells by mitochondrial succinate dehydrogenase.

The cells and MTT solution (5 $mg_{MTT}$/1,5 $mL_{PBS}$) were incubated at 37°C for 3 h. At the end of the incubation period, the MTT solution was removed and replaced with 0.5 mL/well of isopropanol in 0.04*N* HCl. The wells were stirred for 5 min until the color was uniform and the absorbance was evaluated at 595 nm. The analyses were performed in triplicate, using cells seeded on a TCPS as a control. All data were analyzed with one-way analysis of variance (Anova) test with Bonferroni post-hoc analysis. A $P_{value} < 0.0001$ was assumed as statistically significant.

## RESULTS AND DISCUSSION

Before pulp characterization and pectin extraction, *A. Vera* pulp underwent a purification step by deionized water washing at room temperature for 30 minutes. The water treatment effectively removed anthraquinones present in the exudate.[30] Previous studies reported that the anthraquinone-rich exudate is toxic to epithelial tissue cells, causing hypersensitivity skin reactions, and produces undesirable effects to the intestine, causing an inflammatory response of the mucous membranes.[30]

### Swelling and Stability of Lyophilized Pulp Samples

The swelling curves of Aloe pulp samples showed that after 5 minutes a mean plateau value of 5000% was reached (data not shown). The samples retrieved the entire amount of water that they lost during the lyophilization (about 98% of the whole pulp). The weight loss of lyophilized Aloe pulp samples was slightly increasing with time, reaching 25% of weight loss in 14 days.

### Pectin Staining in *A. Vera* Pulp

The presence of pectin in the cell walls of Aloe pulp was confirmed by clear pulp ruthenium red staining. The cell walls, rich in pectin, acquired a pink/red color, whereas the inner parts, mainly composed of water, did not absorb the color (Figure 2).

Pectin was extracted from the inner gel of *A. Vera* leaves by the procedure described in the scheme of Figure 3. The experimental parameters were varied to optimize the yield of the extracted product, leading to four different extraction processes (E1, E2, E3, E4).

Changing the experimental conditions resulted in four different extraction processes and in four extraction products with different physico-chemical characteristics, as shown in Table I.

### Optimization of the Extraction Process (E1)

To develop a method able to profitably extract pectin from *A. Vera* leaves, various procedures described in the literature[22,24,31] for pectin extraction from different sources have been considered.

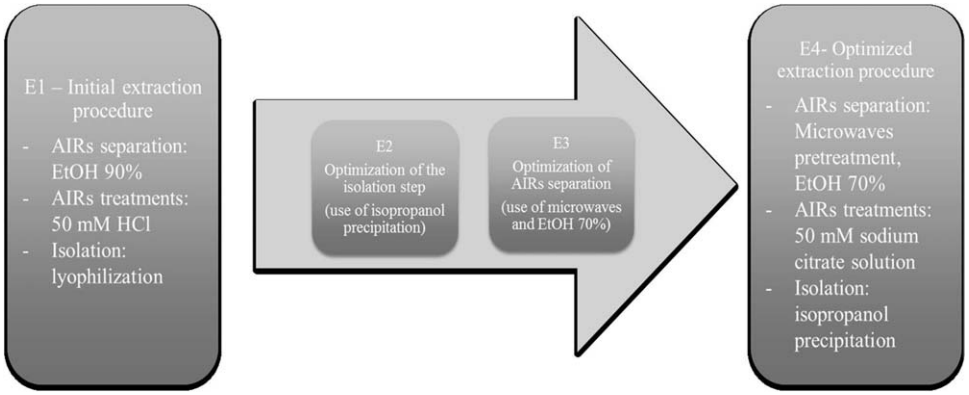


**Figure 3.** Scheme of the procedure optimization from extraction E1 to E4.

The extraction in severe acidic conditions allows obtaining a high amount of pectin from the hydrolysis of insoluble protopectin, formed by the combination of the pectin molecules with the cellulose.[32] However, acidic extractions lead to great amounts of effluents that needed to be removed.[22]

The initial extraction method (E1), which involved the AIRs separation in ethanol 90% (v/v), AIRs treatment in HCl solution and freeze-drying, led to a pectin with a high DE% (77.41%).

The chemical structure of the extracted pectins was evaluated by FTIR spectroscopy (Figure 4). The IR absorption in the O-H region is because of the inter- and intramolecular hydrogen bonding of the galacturonic acid chain.[33] In E1 spectrum, the peak at 1745 $cm^{-1}$, associated to the C=O stretching vibration of methyl esterified groups and COOH groups, and the peak of the asymmetric carboxylate ion stretching band ($COO^{-}$) at 1630 $cm^{-1}$ showed similar patterns to the corresponding peaks of the commercial pectin (Figure 4).

In spite of the high methoxy content detected by UV measurements, the *A*/*B* peak area ratio of E1 extract was comparable to that of the commercial low-methoxy pectin (Table I). As the band at 1745 $cm^{-1}$ is the sum of the contribution of both de-esterified, not salified, COOH and the COOR, the variation of this ratio cannot be considered a robust tool for the evaluation of the de-esterification when the differences in intensity are small.[34]

In the finger print region, the spectrum showed typical bands of homogalacturonans between 1200–1000 $cm^{-1}$ (C-O stretching at 1100 $cm^{-1}$ and C-C stretching at 1017 $cm^{-1}$) and the bands typical of the different monosaccharidic residues present in the branched regions (1070-1043 $cm^{-1}$) indicating the presence of the branching composed of rhamnagalactoronan regions (Figure 5).[35]

The extracted pectin did not allow forming ionotropic gels. The process required long times and an extensive step of purification, therefore it was gradually changed to optimize the aspects concerning both the procedure of extraction and the quality of the final product.

**Optimization of Isolation of Pectin (E2)**

Following extraction, pectin samples can be obtained by removing other compounds (i.e. free sugars, salts) by washing the precipitate with different concentrations of alcohol.[36] In E2 extraction, the precipitation in isopropanol, instead of lyophilization, allowed a reduction of the duration of the whole process from days to few hours. Furthermore, the precipitation in alcohol such as isopropanol increases pectin purity by removing alcoholic soluble salts and neutral sugars present in the cell walls.[36–39]

The introduced changes of the extraction process did not provide substantial physico-chemical differences detectable by FT-IR analysis in the E2 product (Figures 4 and 5), although minor differences can be observed in the intrinsic viscosity and degree of esterification (Table I).

**Optimization of AIRs Separation (E3)**

Pectin yield can be improved by a microwave pretreatment that inhibits the activity of pectin-esterase enzymes, which induce the depolymerization of the polysaccharide before the extraction.[26] For this reasons, a microwave pretreatment was introduced in E3.

On the other side, the rate of the depolymerization by acidic hydrolysis is known to increase with temperature and degree of esterification[40] and occurs at faster rates than neutral polysaccharides in the acid pH range[41] as in *A. Vera*.[42]

**Table I.** DE%, Intrinsic Viscosity, Molecular Weight, and Yields of the Four Different Extraction Procedures

| Sample | DE % | $\eta_i$ (mL/g) | MW (kDa) | AIR yield (%) | Pectin $yield_{AIRs}$ (%) | Pectin $yield_{PULP}$ (%) |
|---|---|---|---|---|---|---|
| E1 | 77.41 | 386.29 | 87.30 | 0.34 | 4.20 | 0.95 |
| E2 | 68.00 | 257.58 | 50.10 | 0.31 | 1.84 | 0.38 |
| E3 | 22.41 | 197.52 | 34.80 | 0.75 | 0.79 | 0.45 |
| E4 | 2.93 | 481.14 | 117.93 | 0.69 | 5.77 | 2.64 |

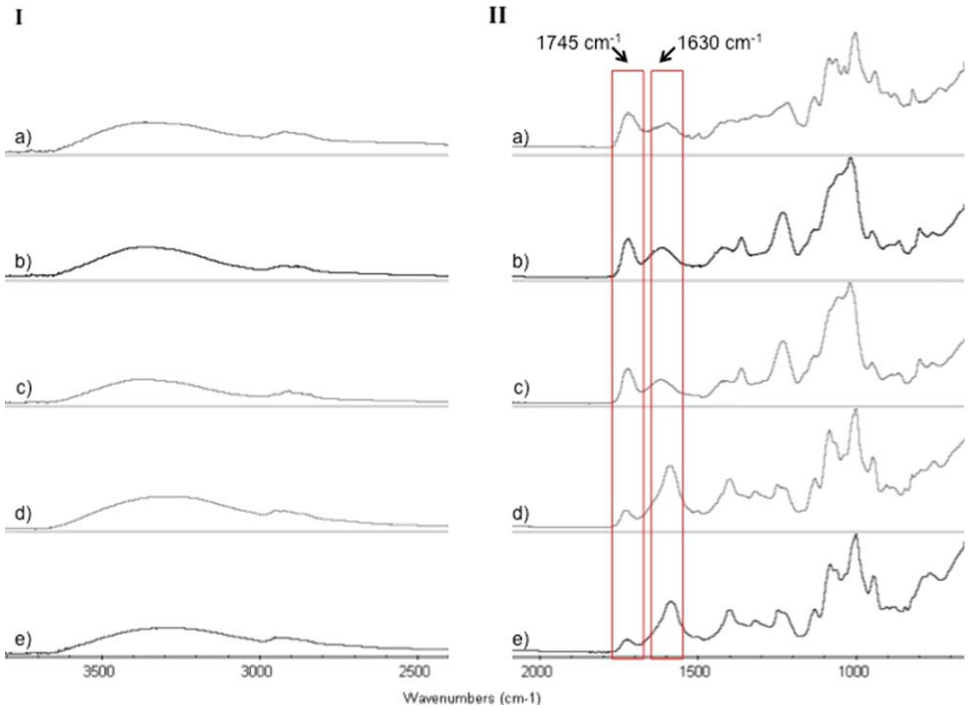


**Figure 4.** IR spectra of (I) 4000–2500 $cm^{-1}$ region and (II) 2000–800 $cm^{-1}$ region of a commercial LM pectin (a) and of pectins extracted following the four different extracting processes: (b) E1, (c) E2, (d) E3, and (e) E4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The combination of these two phenomena can explain the obtained results. Firstly, the partial yield (dry AIR weight compared to the fresh pulp weight) increased from 0.31% of E2 to 0.75% of E3 (Table I).

The lower intrinsic viscosity of the E3 extracts, if compared to the products obtained in E1 and E2 (Table I) can be correlated to different phenomena or a combination of them, such as the changes in the intermolecular bonds, strictly dependent upon the chemical structure. Another reason for a change in the viscosity properties of polyelectrolyte solutions is often found in the changes of the charge of the polymer, with a decrease of viscosity in the case of shielded charges or their lower content. In this case, however, the increase of carboxylate anions content, observed from the noticeable contribution of the asymmetric and symmetric stretching of carboxylate group at 1630 and 1411 $cm^{-1}$ in FT-IR spectra associated to the increase of the *A*/*B* ratio (Table II), should lead to an increase, other than a decrease, of the intrinsic viscosity.

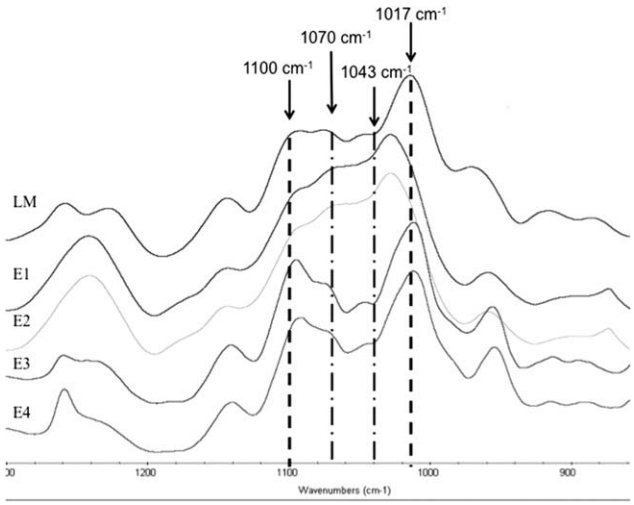


**Figure 5.** Magnification of IR spectra of pectins extracted: it highlighted the characteristic "fingerprint" region between 1220 and 800 $cm^{-1}$, showing the two homogalacturonic peaks at 1100 and 1017 $cm^{-1}$ and rhamnogalacturonan I and II peaks at 1070 and 1043 $cm^{-1}$.

Although no literature is available about the reduction of the molecular weight of pectins as a consequence of the microwave treatment, the heating induced by microwaves combined with the acidic pH could lead to a cleavage of the branched chains of rhamnogalacturonan I and II. This hypothesis, to be further investigated, is supported by FT-IR analysis: infrared spectrum of E3 pectin exhibited two well-defined peaks of homogalacturonan at 1094 and at 1011 $cm^{-1}$, indicating the predominance of the linear backbone chain over the side chain constituents.[35]

### Optimization of AIRs Treatment (E4)

The use of acidic solutions such as HCl enables the separation of pectin from the AIRs, as the acidic environment stimulated the hydrolysis of insoluble protopectin (formed by the natural combination of the pectin molecules with cellulose), leading to the dissolution of the polysaccharide.[32] The use of chelating agents such as sodium citrate, EDTA, or sodium hexametaphosphate represents an alternative mean of extraction. In the cell walls, the carboxyl groups of the homogalacturonan region of pectin form ionic bonds with calcium.[43,44] A single ion can

**Table II.** Ratio of Ester Carboxyl and Carbonyl Ion Peak Area to CH Peak Area of Pectin Samples

| Sample | COO-R peak area/CH peak area (*A*) | $COO^-$ peak area/CH peak area (*B*) | Ratio *B*/*A* |
|---|---|---|---|
| LM pectin | 1.49 | 1.45 | 0.97 |
| E1 | 2.44 | 2.39 | 0.98 |
| E2 | 2.36 | 2.12 | 0.89 |
| E3 | 9.60 | 87.81 | 9.15 |
| E4 | 1.67 | 35.00 | 20.95 |

bind two chains of pectin, creating a highly connected, interwoven and insoluble structure. When complexing agents bind to the calcium ions of AIRs, ionic bonds are broken and pectin becomes soluble in an aqueous solution. The use of sodium citrate as a sequestering agent, compared to other chelating agents, allows obtaining a final product free of amounts of toxic effluents that require treatment of purification before the *in vitro* tests for cytocompatibility.[27]

In E4, sodium citrate treatment was introduced instead of the HCl process.

The FT-IR analysis, performed on the product of the E4 extraction, was comparable to E3 extract, but with an evident presence of bands attributed to sodium citrate. Therefore, it was necessary to purify the extracts through ethanol washing (40% v/v), and to extend the dialysis time to remove the excess of sodium citrate (data not shown). The peak areas of carbonyl and carboxylate (Table II) showed that the contribution of carboxylate group was strongly increased in the case of E4 extract (Table II). The low degree of esterification was confirmed by UV measurements of DE% (Table I), indicating lower DE% for this sample if compared to the commercial analyzed one.

The sodium citrate treatment had a double effect on the extracts: firstly, a significant increase of the yield of pectin extracted with sodium citrate was observed if compared to the yield of HCl treatment (increase of pectin yield of 14 %, see the values of pectin $yield_{AIRs}$ in Table I); then the extracts had a lower DE% because of the higher pH of the treatment solution (pH 7.6 of sodium citrate solution compared to pH 2.9 of HCl solution) that led to a partial pectin de-esterification, as reported in literature.[45]

The procedure developed for extract E4 led to pectin with high molecular weight, as estimated by viscometry (Table I). Intrinsic viscosity is a characteristic of macromolecules and is a key property characterizing the frictional contributions of polymers in dilute solutions. For molecules which can exist with a variety of molecular weights, the relation between intrinsic viscosity and molecular weight is one of the most important properties [see eq. (1)]. Gelling abilities of pectins are related very closely to molecular weight (MW): a higher MW allows to form stronger and more entangled gel, as this polymer property is a direct indication of the number of reactive sites available for complexation.[46,47] However, there are several correlated factors (i.e. molar mass, polymer/solvent interactions and charge on the macromolecules[48]) that influence the intrinsic viscosity of a pectin diluted in a solution; hence, more studies have to be performed to explain the correlation between the intrinsic viscosity and the molecular weight of extracted pectins.

In the present work, the product obtained by extraction with sodium citrate (E4) showed the lowest degree of esterification and the highest MW, therefore it was selected for the production of gels for growth/delivery of cells.

### Gelling Evaluation of E4 Pectin

To evaluate the gelling ability of E4 extracts, the gels were obtained by crosslinking with $Ca^{2+}$ ions, using $CaCl_2$ solution (Figure 6).

**Swelling Studies.** The time dependence of swelling of the obtained gels is shown in Figure 7. The swelling behavior in PBS of E4 pectin gels was compared to the one of commercial LM pectin gels. The rate of swelling is influenced by the dimensions of the gel network affecting the rate at which water can diffuse into the polymer network. The swelling of Aloe pectin gels reached a value of about 2500% within 1 h and it retained this value up to 24 h of incubation. On the other hand, LM

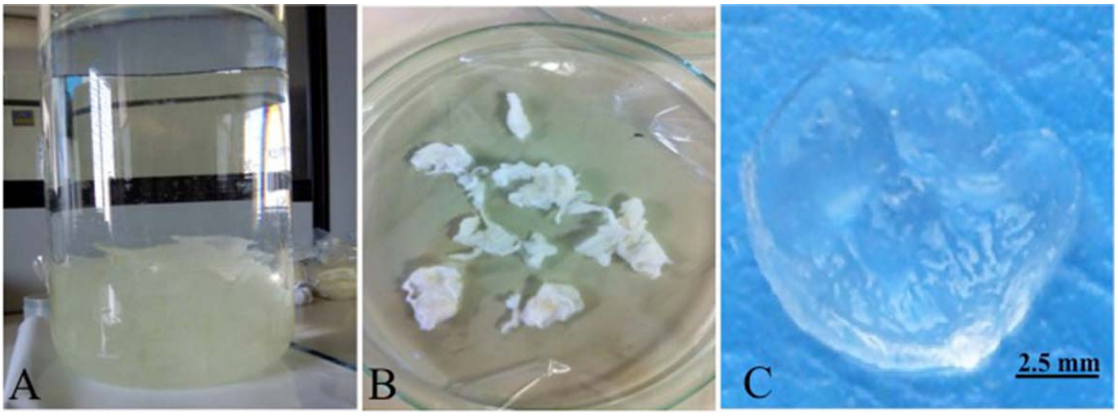


**Figure 6.** (A) *Aloe Vera* pulp wash, (B) pectin obtained by E4 extraction, and (C) pectin gel obtained by ionic interaction with $Ca^{2+}$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pectin gels reached a plateau of 1000% after 1 h and their weight was retained up to 24 h. LM pectin gels showed lower uptake, probably because of their cross-linked and entangled networks, that resulted in a lower swelling if compared to Aloe pectin gels. These data clearly demonstrated different structural characteristic of the two pectins.

**Stability Studies.** Pectin gels lost approximately 15% of their initial weight after 24 h of immersion in PBS, and then retained their weight up to 14 days (end of the experiment, data not shown).

The stability in PBS of Aloe pectin gels is significantly different than the stability of gels prepared with commercially available pectin. In fact, previous studies showed that pectin gels dissolved after few hours of incubation in PBS.[18] Different hypotheses could be made to explain the rapidly degradation occurred during the first 24 h. The fast weight loss of the initial stage of incubation could be related to a partial release of the residual citrate in the extracted pectin; the purification process by ethanol washing and the extension of the dialysis time may have to be optimized to exclude the presence of the salt. Furthermore, the initial weight loss could be because of the release of non-crosslinked pectin inside the network of the gel or to the degradation of the pectin chains that can be enhanced at 37°C. All of these phenomena could be possible and further investigations have to be performed.

**Cytocompatibility Tests.** To investigate the suitability of Aloe pectin gels for application in regenerative medicine, cytocompatibility tests were assessed *in vitro* by seeding and culturing human cell line onto the gels and evaluating the number of viable cells by MTT test. The viability of SAOS-2 cells seeded onto Aloe pectin samples, measured 24 h after cell seeding resulted similar (86%) to that of cells seeded on TCPS control plates (assumed 100%), indicating that cells were alive and proliferating. Cell viability on commercial LM pectin gels were lower (17%) than that on TCPS control (Figure 8). As SAOS-2 cells need to adhere for proliferation, these results indicate an improved adhesion onto Aloe pectin gels. Enzymatically cleaved oligosaccharides from pectin branched regions were used as coatings for different substrates. They are described to promote osteoblasts adhesion, e.g. SAOS-2[49] and MC3T3 cell line.[50] Thus, it could be hypothesized that the different monosaccharidic residues in the branched regions caused an improved cellular adhesion onto the Aloe pectin gel. This can be considered indirectly giving information on the extracted pectin structure.

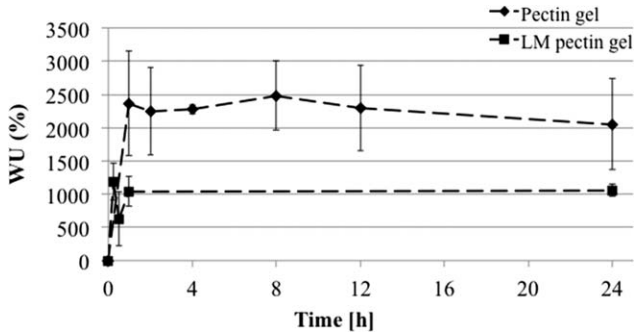


**Figure 7.** Swelling behavior of E4-Aloe pectin gels in PBS at 37°C up to 24 hours compared to commercial LM pectin.

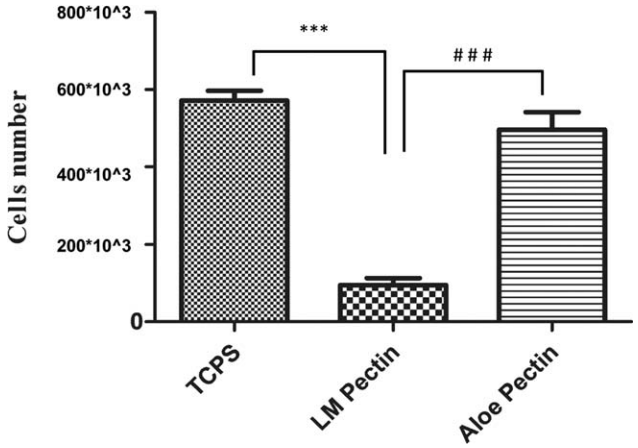


**Figure 8.** Cell viability on Aloe pectin and LM pectin gels. Data are compared with the control tissue culture polystyrene (TCPS) wells. Each bar represents the mean ± SD of three experiments. ***Statistical significance between LM pectin and TCPS ($P < 0.0001$) and ###statistical significance between LM pectin and Aloe pectin ($P < 0.0001$)

## CONCLUSIONS

In this work we developed a novel process for pectin extraction starting from a nonconventional pectin source, *A. Vera* leaves.

Although the *A. Vera* pulp is mainly composed of water (98.5% on the whole) in this work we prove that *A. Vera* pectin can be extracted with an easy and low-cost process. In particular, the optimization of the extraction process led to a fair yield of purified pectin, which has interesting gelling proprieties because of a low degree of esterification and high molecular weight.

In the present study, we put forward that the way of extraction influences the chemical composition and macromolecular features of pectins, in terms of DE%, molecular weight, structural characteristics, and ability to form gel. The use of calcium chelating agents, used in the E4 extraction pectin gels, demonstrated an improved cytocompatibility compared to commercial LM pectin gels. These results underline the potentiality of the Aloe pectin to be used as a biomaterial suitable for the production of matrices and gels for regenerative medicine applications.

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## REFERENCES

1. Davis, R. H.; Leitner, M. G.; Russo, J. M.; Byrne, M. E. *J. Am. Podiatr. Med. Assoc.* **1989**, *79*, 559.
2. Rodriguezbigas, M.; Cruz, N. I.; Suarez, A. *Plast. Reconstr. Surg.* **1988**, *81*, 386.

3. Chandu, A. N.; Santhosh, C.; Bhattacharjee, C.; Debnath, S.; Kannan, K. K. *Int. J. Appl. Biol. Pharm. Techonol.* **2011**, *2*, 19.
4. Hamman, J. H. *Molecules* **2008**, *13*, 1599.
5. Li, J.; Zhang, Y.; Liu, X. in *Bioinformatics and Biomedical Engineering, (iCBBE) 2011 5th International Conference on,* **2011**, p 1.
6. Rajasekaran, S.; Ravi, K.; Sivagnanam, K.; Subramanian, S. *Clin. Exp. Pharmacol. Physiol.* **2006**, *33*, 232.
7. Hu, Y.; Xu, J.; Hu, Q. *J. Agric. Food Chem.* **2003**, *51*, 7788.
8. Liu, L. S.; Fishman, M. L.; Hicks, K. B. *Cellulose* **2007**, *14*, 15.
9. Sriamornsak, P. *Expert Opin. Drug Deliv.* **2011**, *8*, 1009.
10. Kokkonen, H. E.; Ilvesaro, J. M.; Morra, M.; Schols, H. A.; Tuukkanen, J. *Biomacromolecules* **2007**, *8*, 509.
11. Bussy, C.; Verhoef, R.; Haeger, A.; Morra, M.; Duval, J. L.; Vigneron, P.; Bensoussan, A.; Velzenberger, E.; Cascardo, G.; Cassinelli, C.; Schols, H.; Knox, J. P.; Nagel, M. D. *J. Biomed. Mater. Res. A, 86A*, 597.
12. Liu, L. S.; Won, Y. J.; Cooke, P. H.; Coffin, D. R.; Fishman, M. L.; Hicks, K. B.; Ma, P. X. *Biomaterials* **2004**, *25*, 3201.
13. Coimbra, P.; Ferreira, P.; de Sousa, H. C.; Batista, P.; Rodrigues, M. A.; Corriea, I. J.; Gil, M. H. *Int. J. Biol. Macromol.* **2011**, *48*, 112.
14. Ni, Y.; Yates, K. M.; Zarzycki, R. Carrington Laboratories, Inc., Irving, Texas, US Patent, 1999.
15. McConaughy, S. D.; Stroud, P. A.; Boudreaux, B.; Hester, R. D.; McCormick, C. L. *Biomacromolecules* **2008**, *9*, 472.
16. Voragen, A. G. J.; Pilnik, W.; Thibault, J. F.; Axelos, M. A. V.; Renard, C. M. G. C., Eds. Pectins; Marcel Dekker: New York, **1996**.
17. Munarin, F.; Guerreiro, S. G.; Grellier, M. A.; Tanzi, M. C.; Barbosa, M. A.; Petrini, P.; Granja, P. L. *Biomacromolecules* **2011**, *12*, 568.
18. Munarin, F.; Petrini, P.; Tanzi, M. C.; Barbosa, M. A.; Granja, P. L. *Soft Matter* **2012**, *8*, 4731.
19. Munarin, F.; Petrini, P.; Bozzini, S.; Tanzi, M. C. *J. Appl. Biomater. Funct. Mater.* **2012**, *10*, e67.
20. Kurita, O.; Fujiwara, T.; Yamazaki, E. *Carbohydr. Polym.* **2008**, *74*, 725.
21. Liu, Y.; Shi, J.; Langrish, T. A. G. *Chem. Eng. J.* **2006**, *120*, 203.
22. Panouille, M.; Thibault, J. F.; Bonnin, E. *J. Agric. Food Chem.* **2006**, *54*, 8926.
23. Iglesias, M. T.; Lozano, J. E. *J. Food Eng.* **2004**, *62*, 215.
24. Zykwinska, A.; Boiffard, M. H.; Kontkanen, H.; Buchert, J.; Thibault, J. F.; Bonnin, E. *J. Agric. Food Chem.* **2008**, *56*, 8926.
25. Singthong, J.; Ningsanond, S.; Cui, S. W.; Douglas Goff, H. *Food Hydrocoll.* **2005**, *19*, 793.
26. Kratchanova, M.; Pavlova, E.; Panchev, I. *Carbohydr. Polym.* **2004**, *56*, 181.
27. Machado-Silveiro, L. F.; Gonzalez-Lopez, S.; Gonzalez-Rodriguez, M. P. *Int. Endod. J.* **2004**, *37*, 365.
28. Anger, H.; Berth, G. *Carbohydr. Polym.* **1986**, *6*, 193.
29. McCready, R. M.; Reeve, R. M. *Agric. Food Chem.* **1955**, *3*, 260.
30. McAnalley, B. H.. Carrington Laboratories, Inc.: Irving, Texas, **1990**.
31. Levigne, S.; Ralet, M. C.; Thibault, J. F. *Carbohydr. Polym.* **2002**, *49*, 145.
32. Yeoh, S.; Shi, J.; Langrish, T. A. G. *Desalination* **2008**, *218*, 229.
33. Gnanasambandam, R.; Proctor, A. *Food Chem.* **2000**, *68*, 327.
34. Fellah, A.; Anjukandi, P.; Waterland, M. R.; Williams, M. A. K. *Carbohydr. Polym.* **2009**, *78*, 847.
35. Kacurakova, M.; Capek, P.; Sasinkova, V.; Wellner, N.; Ebringerova, A. *Carbohydr. Polym.* **2000**, *43*, 195.
36. Garna, H.; Mabon, N.; Robert, C.; Cornet, C.; Nott, K.; Legros, H.; Wathelet, B.; Paquot, M. *J. Food Sci.* **2007**, *72*, C001.
37. Gusek, T. W.; Zullo, L.; Eyal, A. M. US Patent, 2009.
38. Crandall, P. G.; Braddock, R. J.; Rouse, A. H. Proceedings of the Florida State Horticultural Society, **1978**, p 109.
39. Liu, L.; Cao, J.; Huang, J.; Cai, Y. R.; Yao, J. M. *Bioresour. Technol.* **2010**, *101*, 3268.
40. Munarin, F.; Bozzini, S.; Visai, L.; Tanzi, M. C.; Petrini, P.; *Food Hydrocoll.* **2013**, *31*, 74.
41. Krall, S. M.; McFeeters, R. F. *J. Agric. Food Chem.* **1998**, *46*, 1311.
42. Zapata, P. J.; Navarro, D.; Guillen, F.; Castillo, S.; Martinez-Romero, D.; Valero, D.; Serrano, M. *Ind. Crops Prod.* **2013**, *42*, 223.
43. Habibi, Y.; Heyraud, A.; Mahrouz, M.; Vignon, M. R. *Carbohydr. Res.* **2004**, *339*, 1119.
44. Yapo, B. M.; Robert, C.; Etienne, I.; Wathelet, B.; Paquot, M. *Food Chem.* **2007**, *100*, 1356.
45. Joye, D. D.; Luzio, G. A. *Carbohydr. Polym.* **2000**, *43*, 337.
46. Vandeventerschriemer, W. H.; Pilnik, W. *Acta Aliment.* **1987**, *16*, 143.
47. Christensen, P. E. *Food Res.* **1954**, *19*, 163.
48. Yoo, S. H.; Fishman, M. L.; Hotchkiss, A. T.; Lee, H. G. *Food Hydrocoll.* **2006**, *20*, 62.
49. Gurzawska, K.; Svava, R.; Syberg, S.; Yu, Y. H.; Haugshoj, K. B.; Damager, I.; Ulvskov, P.; Christensen, L. H.; Gotfredsen, K.; Jorgensen, N. R. *J. Biomed. Mater. Res. A* **2012**, *100A*, 654.
50. Kokkonen, H.; Cassinelli, C.; Verhoef, R.; Morra, M.; Schols, H. A.; Tuukkanen, J. *Biomacromolecules* **2008**, *9*, 2369.
51. Munarin, F.; Tanzi, M. C.; Petrini, P.; *Int. J. Biol. Macromol.* **2012**, *51*, 681.