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Sedum telephium L. Polysaccharide Content Affects MRC5 Cell Adhesion to Laminin and Fibronectin

L. RAIMONDI, G. BANCHELLI, D. DALMAZZI, N. MULINACCI*, A. ROMANI*, F. F. VINCIERI* AND R. PIRISINO

*Department of Pharmacology and *Department of Pharmaceutical Science, University of Florence, Italy*

Abstract

In traditional medicine the fresh leaves and juice of *Sedum telephium L.* are used as wound-healing promoters. Cell adhesion represents a primary event in wound repair and in tissue homeostasis, and therefore we have investigated the effect of Sedum juice and its main fractions, polysaccharides and flavonols, on human fibroblast (MRC5) adhesion to fibronectin and laminin.

Our findings revealed that total Sedum juice strongly inhibited cell adhesion to laminin and fibronectin (EC50 $1{\cdot}03 \pm 0{\cdot}12\ mg\ mL^{-1}$). This anti-adhesive feature was concentrated mainly in the two polysaccharide fractions (EC50 values comprised between 0·09 and $0{\cdot}44\ mg\ mL^{-1}$). The flavonol fractions did not seem to contribute to this effect.

A first attempt to elucidate the polysaccharide-related anti-adhesive feature of Sedum juice was also performed.

The results confirmed that natural polysaccharides, with chemical structures different from heparin, were able to interfere with integrin-mediated cell behaviour and they contributed to the outstanding effects of Sedum juice and to the role of polysaccharides in cell–matrix interaction.

Sedum species (Crassulaceae) are widely diffused throughout Europe and *Sedum telephium L.* is used in traditional medicine for many kinds of inflammatory diseases of the skin. The leaves, without the external cuticle, or the fresh juice are usually applied topically to painful wounds, burns and eczema to promote healing and to reduce inflammation and pain. A large amount of evidence indicating the efficacy of this treatment has been registered at the Emergency Unit of the Torre Galli Hospital in Florence (Balatri 1981). However, little is known regarding the mechanism of action responsible for Sedum's effects.

Chemical analysis of *S. telephium L.* leaves revealed two main groups of components: flavonol glycosides of quercetin and kaempferol (Mulinacci et al 1995a; 1995b), and polysaccharides (Sendl et al 1993). These latter compounds were characterized as branched neutral rhamnogalacturonans, with a molecular weight between 13000–13500 Da. Due to their abundance in the juice, the polysaccharides and flavonols were suspected of participating in some of the clinical effects of Sedum.

Flavonol activities as modulators of vascular tissue functions (Duarte et al 1994) or as enzyme inhibitors (Havesteen 1983) have been extensively studied. The spectrum of action of polysaccharides has been mainly characterized for those that are sulphated which, interacting with proteins, are implicated in different cell processes (Salmivirta et al 1996). Less is known about the pharmacological features of non-sulphated natural polysaccharides. Many natural polysaccharides are commonly used as viscosity enhancers in pharmaceutical preparations where they can modulate drug availability or drug-related cell toxicity (Raimondi et al 1998). Thus it would be possible to hypothesize that exogenous polysaccharides can have their own pharmacological profile.

In this respect, recent evidence attributes anti-inflammatory properties to some Sedum polysaccharides (Sendl et al 1993). These findings could indicate the beneficial role of Sedum polysaccharides in the reorganization of cellular layers during tissue healing when a certain degree of local

Correspondence: L. Raimondi, Department of Pharmacology, University of Florence, 50134 Florence, Italy.
E-Mail: raimondi@server1.pharm.unifi.it

inflammation, supported by circulating cells, is often present.

The components of the extracellular matrices, most of which are produced by the cells then secreted and assembled in insoluble networks, have a key role in the wound-healing process (Mikami et al 1994; Nakanura et al 1994). Extracellular matrix components have specific receptors, integrins, transmembrane proteins belonging to the immunoglobulin superfamily. Integrins are distributed on cell membranes and, binding glycoproteins from the matrix, allow the firm assembly of the cell in-situ. Apart from adhesion, integrins mediate in other cell behaviour, such as chemotaxis and chemoinvasion, proliferation, transformation and wound-repair (Takashima & Grinnel 1985; Ruoslahti 1991; Inuoue & Katakami 1993).

It is well known that integrin binding to some substrates can be affected by natural polysaccharides among which are the sulphated ones, such as heparin. Many extracellular components have specific sites for heparin, and binding to substrates reduces their recognition by cells. However, other heparin-like features seem to be shared by other saccharides with different chemical structure (Kleinman et al 1979; Yamada et al 1980; Pall et al 1996).

Sedum contains polysaccharides, as do many other plants, and so we have investigated the effect of the Sedum juice and its main components on cell adhesion to fibronectin and laminin, two glycoproteins present, in their isoforms, in most of the cellular extracellular matrices.

Materials and Methods

Materials

Fresh leaves of *Sedum telephium L.*, from an experimental field in a hilly area near Florence, were collected in July 1996. The leaves were stored at −30°C. MRC5 cells were obtained from the European Cell Culture Collection (ECACC, Salisbury, UK). Human soluble fibronectin, laminin (from EHS tumour), heparin, non-sulphated heparin, phosphate-buffered saline (PBS), bovine serum albumin (BSA), Minimum Essential Medium (MEM), glutamin, trypsin, antibiotic mixtures, and other cell culture grade reagents were obtained from Sigma-Aldrich (St Louis, MO). Foetal bovine serum was obtained from Boehringer Manneheim (Germany). Tritiated amino acid mixture (56 mCi $mmol^{-1}$) and linear non-sulphated dextran (MW 10 000 Da, glucose units 95% α 1–6 linked), were purchased from Amersham-Pharmacia.

Preparation of Sedum fractions

Fresh leaves of *S. telephium L.* (constituted by 92–96% water) were collected and immediately frozen in liquid nitrogen. Approximately 700 g fresh leaves were ground and then centrifuged at 6000 *g* for 10 min. Different parts of this supernatant (first supernatant) were used to prepare the tested samples. A 100-mL sample of the supernatant was frozen and lyophilized to obtain the total juice. A 400-mL sample was placed at 4°C for 12 h to induce the precipitation of the less soluble polysaccharides. This latter precipitate was recovered after filtration, and then washed with ethanol and freeze-dried (SMG). The residual supernatant was diluted with 3 vols ethanol and solubilized by stirring for 12 h and left standing at 4°C for 8 h. After centrifugation (6000 *g* for 10 min), another polysaccharide fraction (SMUC) was collected, washed with ethanol, frozen and lyophilized. The corresponding clear supernatant was frozen and lyophilized to obtain the SFLA sample.

Portions of SMUC and SMG were dialysed for up to 36 h (membrane cut-off 1000 Da) against an excess of demineralized water. The dialysed portions (SMUCd and SMGd) were conserved lyophilized.

For biological evaluations, all the lyophilized fractions were used after reconstitution in culture medium devoid of serum.

HPLC/DAD analysis

The HPLC/DAD method of Mulinacci et al (1995a) was used for the quali-quantitative evaluation of polyphenol content in the SFLA fraction. Calibration curves at 350 nm obtained with the previously isolated kaempferol 3,7-O-dirhamnoside and quercetin 3,7-O-dirhamnoside (Mulinacci et al 1995b) were performed to quantify the flavonol content. To evaluate the content of gallic acid in the SFLA sample, a specific calibration curve at 280 nm was performed using the pure commercial standard. The data were expressed choosing 600 g mol^{-1} as medium molecular weight for all the flavonol glycosides and 170 g mol^{-1} for the gallic acid.

Adhesion assay

Well coating. Fifty microlitres of soluble fibronectin or laminin at different concentrations, or BSA (0·1%) or Sedum fractions (blanks) were added to each multiwell and incubated for at least 1 h at 37°C in an atmosphere of 5% CO_2. Wells were then washed twice with PBS and then 50 μL 0·1% heat

denatured BSA was added to the fibronectin- and laminin-coated wells and incubated again under the same conditions for an additional 1 h. All the plates, blanks or laminin/fibronectin-coated wells were finally washed twice with PBS and cells plated on them.

Cell adhesion evaluation. Confluent MRC5 cells were labelled for 3 h at 37°C, 5% CO_2 and 95% air, by the addition of 1 μCi mL^{-1} tritiated amino acid mixture to culture medium, devoid of serum. Cells were then washed, trypsinized, centrifuged at 1000 *g* for 5 min, resuspended in medium containing 10% heat-inactivated calf serum and incubated at 37°C for 15 min to allow recovery after trypsinization. Afterwards the cells were centrifuged, resuspended in the culture medium devoid of serum in the presence or in the absence of the tested compounds, and plated on previously coated bacteriological 96-multiwell plates. Cell density was maintained between 1000–1500 cells/well. Blanks, controls and Sedum-treated cells were incubated at 37°C as above and adhesion monitored by microscope observation.

Usually adhesion was complete after 90 min and at this time the medium was removed. Adherent cells were extensively washed with PBS and solubilized by the addition of 0·2% sodium dodecylsulphate (SDS) dissolved in 0·1 M sodium hydroxide. The radioactivity present in the medium (total radioactivity) and in solubilized cells was evaluated by a β-counter machine. Calculations were performed comparing the percentage of radioactivity found in the cell with the total radioactivity recovered. Each value presented was the mean of three to six experiments, each run in triplicate. Sedum fraction additions to the wells did not change cell adhesion to plastic, therefore adhesion to BSA (8·1 ± 1·5%) was considered blank for each sample and, unless otherwise stated, subtracted from the glycoprotein-induced value of adhesion. The percentage of cell adhesion to glycoproteins were monitored as control for cell senescence. Within cell passages used (from 20 to 31) the difference among cell adhesion to glycoproteins did not differ significantly.

Modified adhesion assay

To investigate the mechanism of action of Sedum components on cell adhesion, the glycoprotein coating of the well was modified. Fifty microlitres laminin (10 μg mL^{-1}) with 50 μL cell culture medium devoid of serum, or 50 μL total juice (10 mg mL^{-1}) or SMUCd (10 mg mL^{-1}), were added to wells and incubated for 1 h under the same conditions. In another series of wells, laminin was coated together with 50 μL heparin (0·1 mg mL^{-1}), non-sulphated heparin (0·1 mg mL^{-1}) and linear dextran (1 mg mL^{-1}) and treated as above. Blanks (BSA-coated wells) were run as previously described.

After 1 h, 50 μL BSA was added and incubated for a further 60 min. Several washings with PBS followed this last step. The adhesion assay was then carried out following the protocol described above.

Each substance was also tested as an adhesive molecule as described previously.

Determination of cell viability

Cell viability, after exposure to total juice (1% w/v), was measured using the method described by Mossman (1983). Confluent MRC5 cells were exposed to total juice at 37°C for 60 min. After that time MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) (0·5 mg mL^{-1}) was added and the cells were incubated for a further 3 h under the same conditions. The amount of oxidized formazan produced by viable cells was evaluated spectrophotometrically at 570 nm after extraction with isopropyl alcohol. For these experiments, total juice and MTT were prepared in serum- and phenol-red-free culture medium.

Results

Sedum fractions

The lyophilized fractions used for the biological assays gave the following yields expressed as percentage (w/w) compared with fresh leaves: total juice 2·8–2·9%, SMG 0·4–0·41%, SMUC 0·8–0·82%, and SFLA 0·15%.

The SMUCd showed 4·6–4·75% yield compared with dialysed SMUC and the yield of SMGd was 1·3% compared with non-dialysed SMG.

The elementary analysis performed on the SMUC and SMG samples showed the absence of nitrogen in these polysaccharide fractions.

HPLC/DAD analyses on reverse phase were carried out on SMG and SMUC to detect trace amounts of flavonols and gallic acid, and on SFLA to evaluate the concentrations of these compounds. The applied experimental conditions were previously described by Sendl et al (1993). The lyophilized polysaccharide samples were dissolved up to saturation (12–16 mg mL^{-1} in water) and injected as such. The residual content of flavonols in the SMUC and SMG was not more than 1×10^{-5} mmol g^{-1}, and the concentration of gallic acid was not more than 1×10^{-4} mmol g^{-1}.

The polyphenol content of the SFLA fraction was gallic acid 0·42% and flavonol glycosides 5·71%, expressed as weight of the products on weight of the total dry sample.

Cell viability

Cell viability following Sedum treatment was evaluated spectrophotometrically by the MTT test after 4-h exposure of cell monolayers to total juice. At the end of this assay, the measured values of MRC5 cell viability were $0{\cdot}648 \pm 0{\cdot}032$ and $0{\cdot}597 \pm 0{\cdot}039$ optical density (means ± s.e. of three different experiments run in triplicate) for control and total juice-treated cells, respectively. These findings suggested that total juice, at a concentration up to 1% w/v, did not modify MRC5 cell viability, also indicating that this fraction did not contain toxic compounds that reduced cell viability up to 4-h exposure.

Adhesion assay

Total juice and the other fractions tested did not produce any significant change in cell adhesion to BSA (blank). In addition, they did not alter cell adhesion to plastic confirming that no adhesive substrates seemed to be present in any of the fractions tested. On the other hand, total juice and polysaccharides produced a significant decrease of cell adhesion to fibronectin and laminin.

As shown in Figure 1, total juice, tested at concentrations not higher than $10\,mg\,mL^{-1}$, inhibited adhesion to fibronectin ($10\,\mu g\,mL^{-1}$), producing an apparent EC50 value of $1{\cdot}032 \pm 0{\cdot}120\,mg\,mL^{-1}$.

The same adhesion assay was performed with SMUC, SMG and SFLA samples to individuate the juice fraction(s) responsible for the anti-adhesive activity shown by total juice. The results obtained are shown in Table 1, from which it is possible to conclude that SMUC ($2\,mg\,mL^{-1}$) and SMG ($3{\cdot}5\,mg\,mL^{-1}$) significantly reduced cell adhesion to fibronectin. SFLA up to $1\,mg\,mL^{-1}$ did not produce any inhibition of cell adhesion.

To exclude interference by other compounds present as contaminants in SMUC and SMG, the fractions were dialysed against water. SMGd and SMUCd were still able to inhibit MRC5 cell adhesion. The ability of both fractions to inhibit glycoprotein-related cell adhesion was also monitored using laminin as substrate.

As shown in Figures 2 and 3, SMUCd and SMGd inhibited MRC5 adhesion to laminin and fibronectin in a concentration-dependent fashion.

A summary of the relative EC50 ($mg\,mL^{-1}$) for total juice, SMUCd and SMGd as inhibitors of cell adhesion to fibronectin and laminin is reported in Table 2. SMUCd seemed to be the most active fraction. In addition, while SMUCd had a lower EC50 on fibronectin ($87{\cdot}7\,\mu g\,mL^{-1}$), SMGd could work better on laminin ($139\,\mu g\,mL^{-1}$).

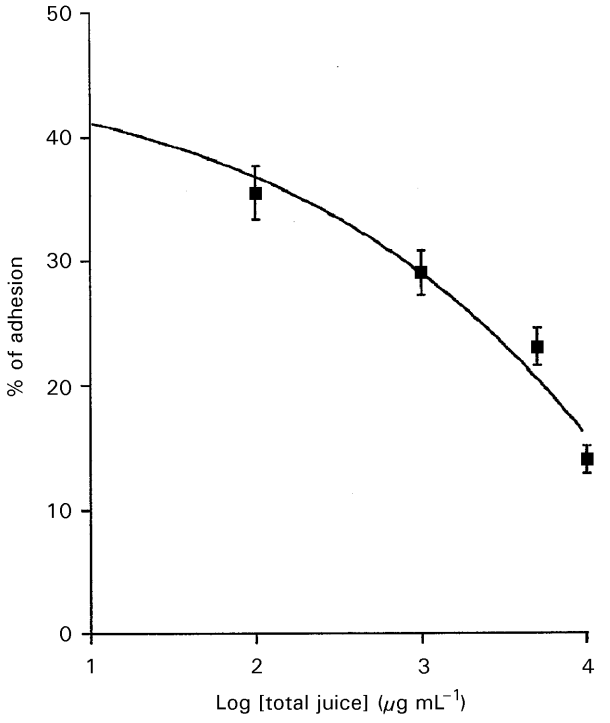

Figure 1. Effect of total juice on MRC5 cell adhesion to fibronectin. Total juice (10·0, 0·5 or $0{\cdot}1\,mg\,mL^{-1}$) was added to MRC5 cells prepared for the adhesion. A maximal fibronectin concentration ($10\,\mu g\,mL^{-1}$), previously determined, was used as a substrate. Results are the means ± s.e.m. of five different experiments run in triplicate.

Table 1. Effect of Sedum juice and Sedum juice fractions on MRC5 cell adhesion to fibronectin.

	% of adhesion
Fibronectin	$48{\cdot}05 \pm 0{\cdot}16$
Fibronectin + total juice	$25{\cdot}70 \pm 2{\cdot}10$*
Fibronectin + SFLA	$42{\cdot}03 \pm 2{\cdot}30$
Fibronectin + SMG	$20{\cdot}10 \pm 5{\cdot}10$*
Fibronectin + SMUC	$17{\cdot}25 \pm 4{\cdot}51$*

The samples total juice, SFLA, SMG and SMUC were tested as MRC5 cell adhesion inhibitors. Confluent cells were treated as described under Materials and Methods, and adhesion to fibronectin ($10\,\mu g\,mL^{-1}$) was evaluated after 90 min at 37°C in a humidified atmosphere. Total juice ($10\,mg\,mL^{-1}$), SFLA ($1\,mg\,mL^{-1}$), SMUC ($2\,mg\,mL^{-1}$) and SMG ($3{\cdot}5\,mg\,mL^{-1}$) were used as described under Materials and Methods. Results are expressed as the mean ± s.e. of four different experiments run in triplicate. *$P < 0{\cdot}05$ compared with values from cell adhesion to fibronectin. Student's *t*-test for grouped data.

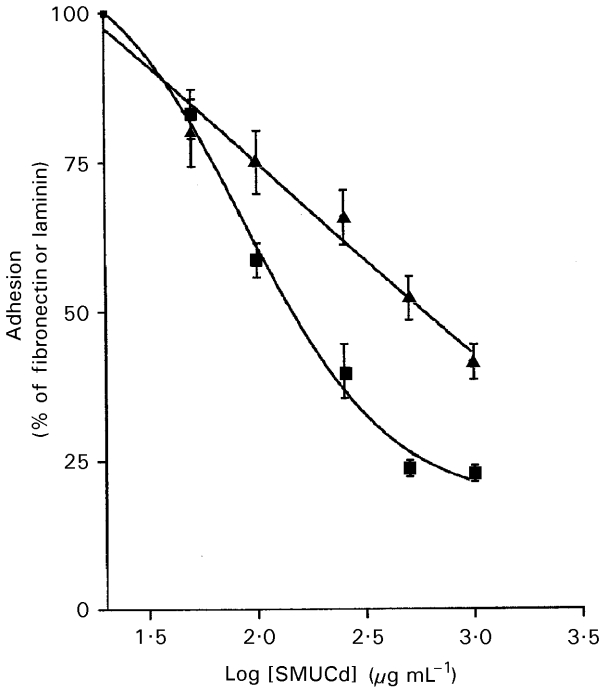

Figure 2. Effect of SMUCd on MRC5 adhesion to fibronectin and laminin. Results for SMUCd-treated cells are expressed as a percentage of adhesion over the value of fibronectin and laminin, considered as 100%, and are the mean ± s.e.m. of four different experiments run in triplicate. ▲, Laminin as substrate; ■, fibronectin as substrate.

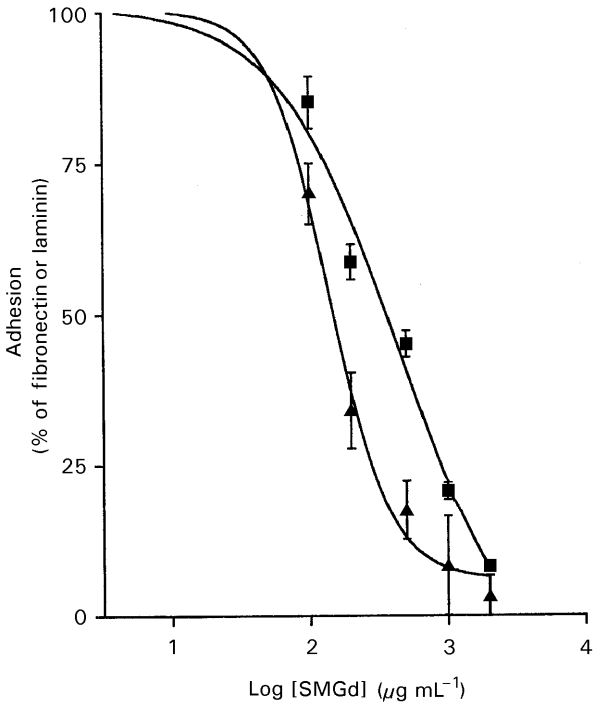

Figure 3. Effect of SMGd on MRC5 cell adhesion to fibronectin and laminin. SMGd reconstituted in culture medium devoid of serum was assayed as cell adhesion inhibitor on fibronectin and laminin (10 μg mL^{-1}). Results for SMGd-treated cells are expressed as a percentage of adhesion over the value of fibronectin and laminin, considered as 100%, and are the mean ± s.e.m. of four different experiments run in triplicate. ▲, Laminin as substrate; ■, fibronectin as substrate.

Possible mechanism of action

We investigated only the effect of the polysaccharide fraction SMUCd on laminin because of the low availability of SMGd and because the EC50 value for SMUCd on laminin could be considered representative of the SMGd EC50 on both glycoproteins.

As shown in Table 3, total juice and SMUCd coated with laminin were still able to inhibit cell adhesion. The same effect was observed with heparin, whereas dextran and non-sulphated heparin, tested at high concentration, were completely inactive (data not shown).

None of the studied fractions or reference molecules altered cell adhesion to BSA or to plastic.

Table 2. EC50 (mg mL^{-1}) for total juice, SMUCd and SMGd as MRC5 inhibitors of adhesion to laminin and fibronectin.

	EC50 (mg mL^{-1})	
	Fibronectin	Laminin
Total juice	1·032 ± 0·120	
SMGd	0·440 ± 0·081	0·140 ± 0·022
SMUCd	0·090 ± 0·008	0·322 ± 0·050

EC50 values were calculated using GraphPad Prism, 1999 (GraphPad Software Inc., San Diego, CA).

Discussion

Cell adhesion is a process that is initiated by the attachment of cells to specific domains of adhesive matrix proteins via cell surface receptors of the integrin family. This process is followed by a reorganization of cytoskeletal elements that produces the formation of focal adhesion plaques followed by cell spreading (Ruoslahati 1991). Cell adhesion is crucial for maintaining tissue homeostasis and remodelling after an injury. Interference in producing incorrect integrin recognition can be directed toward integrins, glycoproteins or to intracellular effectors. Among them, the ability of polysaccharides to interfere with cell–matrix interaction has been described (Nomoto et al 1986), confirming the importance of polysaccharides in cell signalling. After studying heparin and other sulphated polysaccharides Rapraeger et al (1991) and Soeda et al (1994) have deduced the possible use of exogenous polysaccharides as probes to interfere with cell adhesion and growth factor-mediated mitosis. However, the ability to reduce cell adhesion to matrix components is a feature not

Table 3. Effect of Sedum juice and Sedum juice fractions in the modified adhesion assay.

	% of adhesion
BSA	8·10 ± 1·5
Laminin	33·36 ± 4·6
Laminin + heparin	24·00 ± 5·5*
Laminin ± SMUCd	25·00 ± 4·8*
Laminin + total juice	17·25 ± 8·5*

SMUCd (10 mg mL^{-1}), heparin (0·1 mg mL^{-1}) and total juice (10 mg mL^{-1}) were tested as inhibitors of cell adhesion to laminin in the modified adhesion assay described under Materials and Methods. Results are the mean ± s.e.m. of four different experiments run in triplicate. *$P < 0·05$ compared with values from cell adhesion to laminin. Student's *t*-test for paired data.

restricted to sulphated polysaccharides (Inohara & Raz 1994).

Sedum leaves contain mainly polysaccharides and flavonols (Mulinacci et al 1995a; 1995b; Sendl et al 1993). Thus, we evaluated the effect of total juice, polysaccharide and flavonol fractions, all obtained from fresh leaves, on cell adhesion mediated by integrins, a key event in wound healing.

Total juice, even at high concentrations, did not produce any change in cell viability but it worked as a cell–matrix adhesion inhibitor. This feature was concentrated in the polysaccharide fractions; flavonols were completely inactive in this test. This finding was sustained having excluded unspecific effects of the fractions, such as the ability to build an artificial matrix or gel around the cells, or to interfere with basal cell adhesion to plastic or to BSA, by preparing opportune blank samples and several washings before plating cells.

The fact that SMUC and SMG were the active principles in the adhesion assay was confirmed using the dialysed fractions.

The different preferential inhibition of cell adhesion toward the two glycoproteins studied, shown by polysaccharides, could arise from the different chemical structures of the two fractions studied, even if we can not draw a conclusion on the specificity of each polysaccharide toward the different substrates used. However, SMUCd seemed more active on fibronectin than on laminin. SMGd preferentially inhibited cell adhesion to laminin (Figure 3) as shown in Table 2.

Regarding the possible mechanism of action of Sedum, our results suggested that the total juice, polysaccharide (SMUCd) and heparin were still able to reduce cell adhesion to laminin in the modified adhesion assay (Table 3). At present, we can not speculate on the nature of the laminin–Sedum juice interaction. Further studies are necessary to verify whether heparin and Sedum (total juice and SMUCd) share a common mechanism of action.

Fibronectin and laminin are present in many types of extracellular matrices and they participate strongly in the wound-healing process (Mikami et al 1994). Our findings could suggest a wide range of Sedum polysaccharide applications as cell adhesion inhibitors.

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