Novel bioactive maloyl glucans from aloe vera gel: isolation, structure elucidation and in vitro bioassays

Keywords:
- Wound healing
- Anti-inflammatory
- Promotes cell division

Summary:
Aloe vera is known for its wound healing and anti-inflammatory properties. The study investigated which of the established active ingredients of aloe vera are responsible for the cell division-promoting (= wound healing) effect and which are responsible for the anti-inflammatory effect.

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Novel bioactive maloyl glucans from Aloe vera gel: isolation, structure elucidation and in vitro bioassays

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Abstract—In this study, three novel maloyl glucans were isolated at temperatures below 15 °C from Aloe vera gel (*Aloe barbadensis* Miller). These compounds were characterized using NMR spectroscopy, ESIMS, MALDITOF-MS and capillary electrophoresis. The compounds were characterized as 6-*O*-(1-L-maloyl)-α-,β-D-Glc*p* (veracylglucan A), α-D-Glc*p*-(1→4)-6-*O*-(1-L-maloyl)-α,-β,-D-Glc*p* (veracylglucan B) and α-D-Glc*p*-(1→4)-tetra-[6-*O*-(1-L-maloyl)-α-D-Glc*p*-(1→4)]-6-*O*-(1-L-maloyl)-α,-β-D-Glc*p* (veracylglucan C). These unusual malic acid acylated carbohydrates were then tested in vitro for effects on cell proliferation and gene expression of proinflammatory cytokines, IL-6, IL-8 and ICAM-1, using RT-PCR. Veracylglucan B demonstrated potent anti-inflammatory and anti-proliferative effects, while Veracylglucan C, on the other hand, exhibited significant cell proliferative and anti-inflammatory activities. Veracylglucan A could only be isolated in smaller quantities, and it proved to be very unstable. Thus no biological effects could be observed in this respect. The in vitro bioassays also indicated that Veracylglucan B and C are antagonistic and competitive in their effects on cell proliferation. The results of this work represent a major step forward in the research on aloe vera gel. This is the first time that two fully chemically characterized compounds are shown to be responsible for known biological activities of aloe vera gel.
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Keywords: Aloe vera gel; *Aloe barbadensis* Miller; CE; NMR spectroscopy; Maloyl glucans; Bioassays in vitro

1. Introduction

Aloe vera gel (AVG) from the leaves of *Aloe barbadensis* Miller (Asphodelaceae) has been used over the centuries in folk medicine as a therapeutic agent. This plant material is believed to posses potent therapeutic properties against a considerable number of diseases.[1] Some of these properties have been clearly demonstrated and confirmed in vitro[2,3] as well as in vivo.[4] AVG has been investigated many times clinically with remarkably positive outcomes in cases of genital herpes simplex infections,[5] Crohn's disease,[6] psoriasis[7] and several terminal cancer conditions,[8] just to name a few. After a comprehensive literature assessment referring to AVG as a therapeutic agent, it is quite striking to see that there are a number of discrepancies about its therapeutic properties. A considerable number of clinical reports have also shown, contrary to the above-mentioned studies, that AVG is not effective[9] and can even cause undesired effects like retardation of wound healing.[10] Moreover, there is no unambiguous chemical evidence about the main compounds responsible for the observed biological activities and healing effects.[1] The gel is believed to contain an arsenal of over 200 compounds, including many vitamins, minerals, organic acids, acetylated polymannose, etc., all of which work in synergy to bring about these manifold biological and healing effects. Many compounds and mixtures of compounds ranging from glycoproteins, carbohydrates and sterols, including aliphatic and aromatic carbonic acids have been mentioned as major biologically active constituents of AVG. Nevertheless, the chemical nature of none of these compounds has ever been confirmed with convincing

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analytical data.[1] It has also been reported that this gel has a pH which lies between 3.5 and 5.0 and can contain 95–99% water.[11] The present study was carried out in order to properly investigate the chemical constituents of AVG with modern analytical methods and to correlate these constituents to the reported biological activities. Special considerations were made during sample preparation with respect to the apparent instability of AVG active ingredients.

2. Results

2.1. Isolation and structure elucidation

The investigated AVG contained ca. 0.6 % (m/m) total maloyl glucans (Fig. 1). Veracylglucan B ($C_{16}H_{26}O_{15}$) (2); MW = 458; $[\alpha]_D$ +5.10 (H_2O); pH 3.8. Analyzing the one-dimensional ^1H, COSY ^1H–^1H, HMBC and HMQC (Fig. 2) ^1H NMR spectra of Vglc B (2), three anomeric protons can be seen at δ 5.07 (J 3.6 Hz), δ 4.95 and δ 4.49 (J 8.0 Hz) corresponding to two α ^1H and one β ^1H of glucopyranose residues, respectively. Also present in the ^1H NMR spectrum are maloyl protons H-2 at δ 4.21 (J 0 Hz), as well as H-3 and H-3′ at δ 2.63 (J 15.8 Hz) and δ 2.43 (J 14.0 Hz), respectively. The HMQC (Fig. 2I) reveals that the proton at δ 5.28 is bonded with the anomeric alpha carbon atom at δ 92, 28 and that the beta proton at δ 4.49 is bonded to the beta carbon atom at δ 96.10. The ^{13}C NMR spectrum reveals the presence of an anomeric carbon at δ 98.78 corresponding to an α-(1→4) linkage.[12] This spectrum also reveals the acylated (maloyl) C′-6 from glucopyranose at δ 64.07 and δ 63.56 (downfield shift) and the free C-6 glucopyranose at δ 60.79 and δ 60.94, respectively. The HMBC (Fig. 2III and IV) further revealed the following cross peaks between ^1H and ^{13}C. Around the α-(1→4) bond, α-C-1 (δ 98.78) interacts with α-H-2 (δ 3.41), α,β-H-6 (δ 3.60, δ 3.63). Interactions between the maloyl and the glucopyranose residues could also be revealed through the following interactions: maloyl C-1 (δ 180)/H-6′ (δ 3.60), α-H-1 (δ 5.05)/maloyl C-1 (δ 180.13) and C-4 (δ 177.78), maloyl C-2 (δ 67.75)/glucopyranose H-6′ (δ 3.60). All carbon and hydrogen atoms of the glucopyranose residues were also confirmed by comparison with NMR spectra of D-glucose and maltose. ESIMS of Vglc B revealed a molecular ion $[M-H+H^+]^+$ with m/z 458.09.

Veracylglucan C ($C_{56}H_{82}O_{51}$) (3): MW = 1570; $[\alpha]_D$ +11.13 (H_2O); pH 4.7. The ^1H NMR spectrum (not shown) and ^1H–^1H COSY NMR spectrum (Fig. 3I) of Vglc C (3) reveal the presence of two groups of alpha protons at δ 4.85 and δ 5.07 and one beta proton belonging to the corresponding glucopyranose residues. The very intensive cumulative proton signals of the maloyl residues are clearly seen at δ 4.2 and δ 2.5, respectively. Analyzing the ^{13}C NMR spectrum of Vglc C (Fig. 3II), three groups of methylene carbon atoms (–CH_2–), all belonging to the maloyl residues, can be seen at δ 41.77, 39.50 and 33.98. The signal cluster around δ 180.46 represents the carbonyl carbon atoms C-1 and C-4 of the maloyl residues. The anomeric region shows

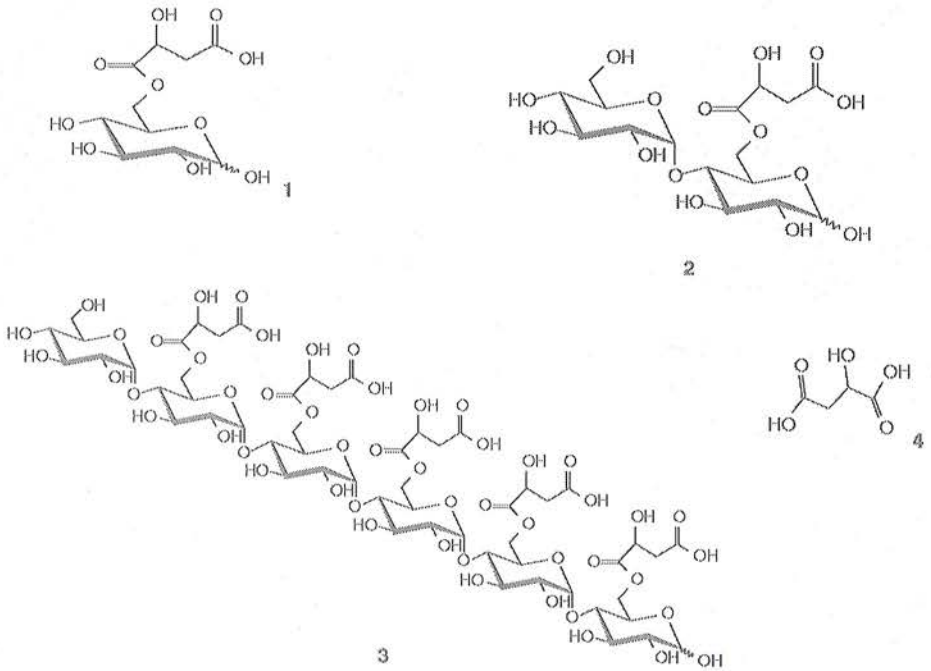

Figure 1. Structures of veracylglucan A (1), B (2), C (3) and malic acid (4).

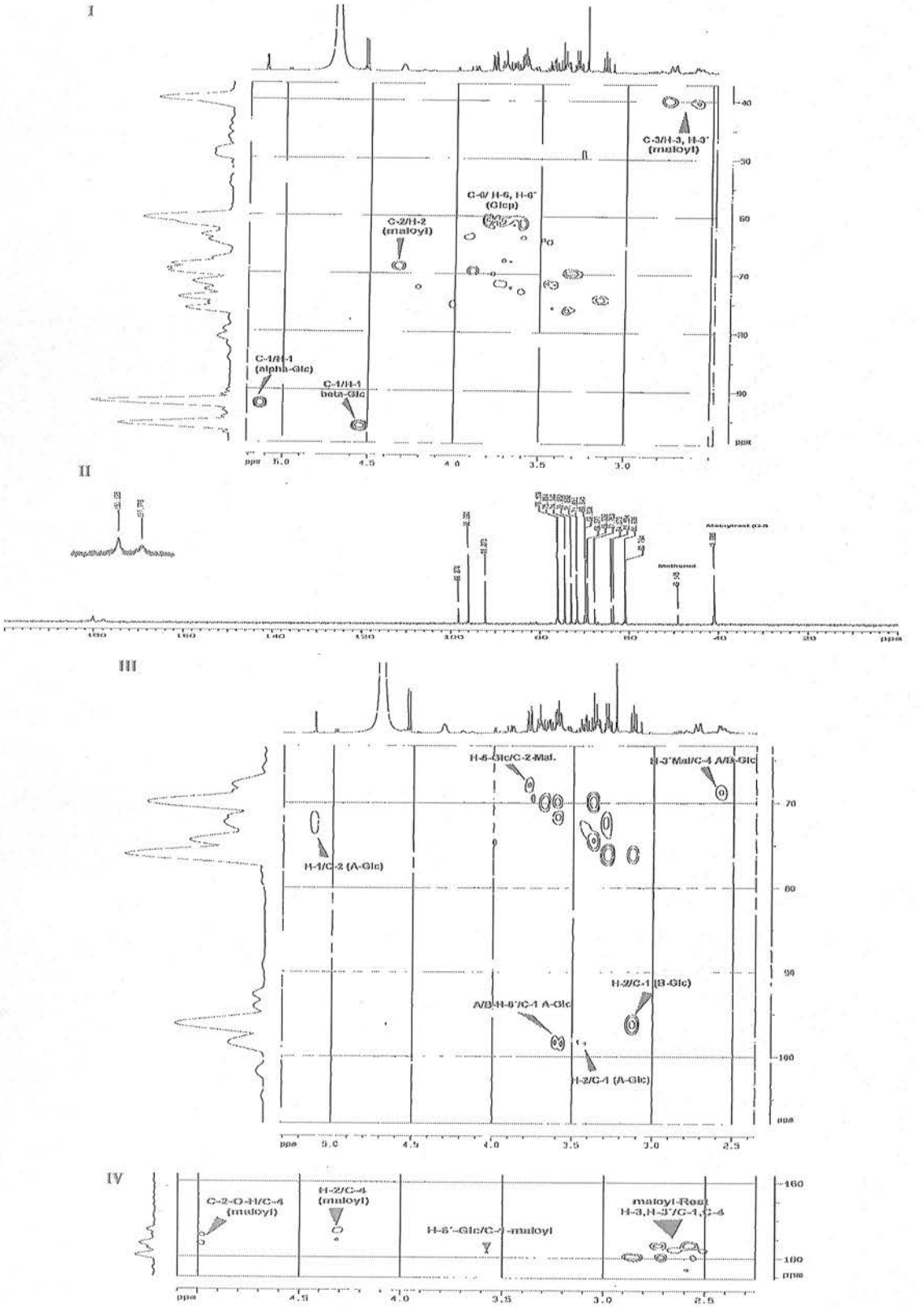

Figure 2. HMQC (I), ^{13}C NMR (75 MHz) (II) and HMBC (III and IV) spectra of veracylglucan B. Characteristic chemical shifts are indicated on the respective spectra.

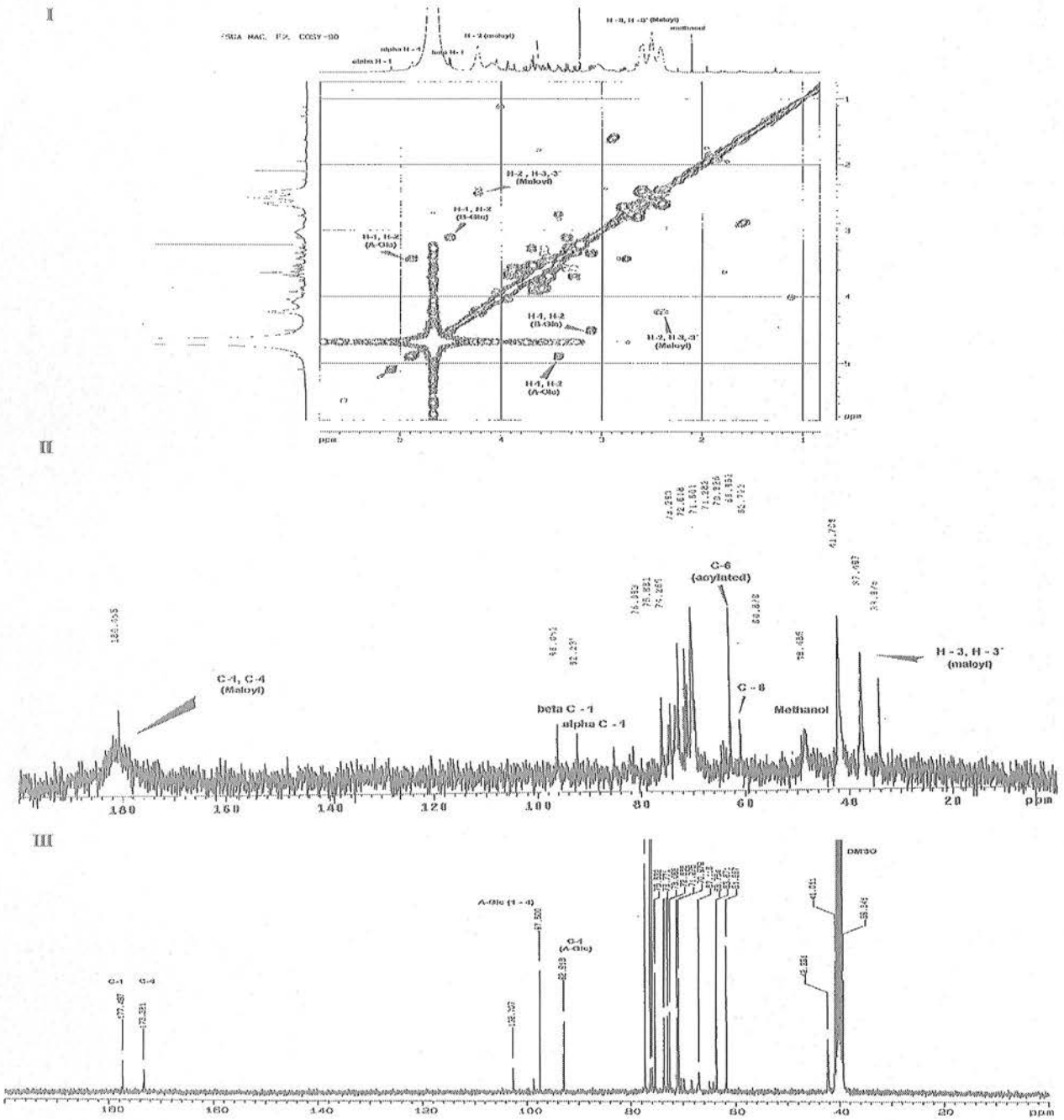

Figure 3. The ^{1}H–^{1}H NMR spectrum (600 MHz) of veracylglucan C indicated three anomeric D-glucopyranose protons (two α and one β) and the prominent protons of the maloyl residues (I); ^{13}C NMR (75 MHz) spectrum of Vglc C in D_2O (II) and its hydrolysate in DMSO (III) measured at rt. The hydrolysate reveals the α-(1→4) anomeric carbon atoms at δ 102.71 and δ 97.60, respectively.

only the alpha and beta carbons of the unbonded end glucopyranose residue. The other anomeric carbon atoms of the α-(1→4) linkages are not seen probably due to the long relaxation time of these atoms. After stripping the compound from some of its maloyl residues through partial acid hydrolysis, the ^{13}C NMR spectrum of the hydrolysate then revealed signals of the α-anomeric carbon atoms at δ 102.71 and 97.60 ppm (link between the Glc*p* and one maloyl-Glc*p* residue), respectively (Fig. 3III). The remaining carbon and hydrogen atoms of the glucopyranose residues were also confirmed here by comparisons with NMR spectra of D-glucose and maltose The MALDITOF-MS of Vglc C produced a spectrum containing a molecular ion $[M+H^{+}]^{+}$ with *m/z* 1571.7 and other ions, which are either multiply charged particles or fragments of Vglc

C. The following ions can be seen in the spectrum (Fig. 4); $[M+2H^+]^{2+}$ *m/z* 1572.7; $[M+3H^+]^{3+}$ *m/z* 1573.7; $[M+4H^+]^{4+}$ *m/z* 1574.8; $[M+5H^+]^{2+}$ *m/z* 1575.8; $[M+6H^+]^{6+}$ *m/z* 1576.7; $[M-OH+2H^+]^{2+}$ *m/z* 155.7; $[M-OH+4H^+]^{4+}$ *m/z* 1557.8; $[M+K+H^+]^{2+}$ *m/z* 1611.7; $[M+K+2H^+]^{3+}$ *m/z* 1612.7. The following molecular fragments MF1, MF2 and MF3 could all be assigned to the parent molecule ion (Fig. 4).

Capillary electrophoresis (CE) is based on the separation of charged molecules according to their charge/mass ratio in an electric field. The method is per se very suitable for the characterization of an AVG extract containing maloyl glucans. According to their molecular size and charge, Vglc A (1) migrated for 1.9 min, Vglc B (2) for 2.3 min, Vglc C (3) for 2.5 min and malic acid (4) for 6.2 min (Fig. 5).

Vglc A ($C_{10}H_{16}O_{10}$) (1), MW = 296, was only contained in smaller quantities in AVG. It also proved to be very unstable. After the isolation, no reasonable quantities of this compound could be obtained. Proof

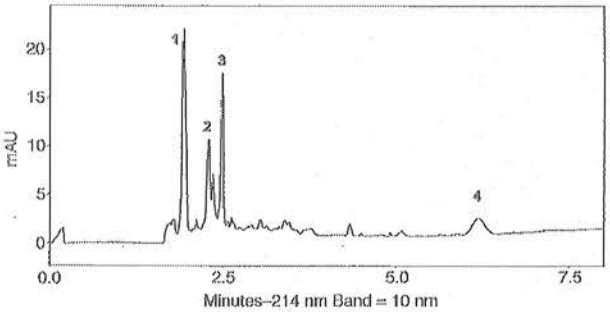

Figure 5. CE of 0.05% maloyl glucans from aloe vera gel special extract: pH 10.5 (borate buffer), photo-diodearray detection (λ 214 nm), 25 kV and 31 cm capillary length. Migration assignment; 1 = Vglc A (1.9 min), 2 = Vglc B (2.3 min), 3 = Vgl C (2.5 min) and 4 = malic acid (6.2).

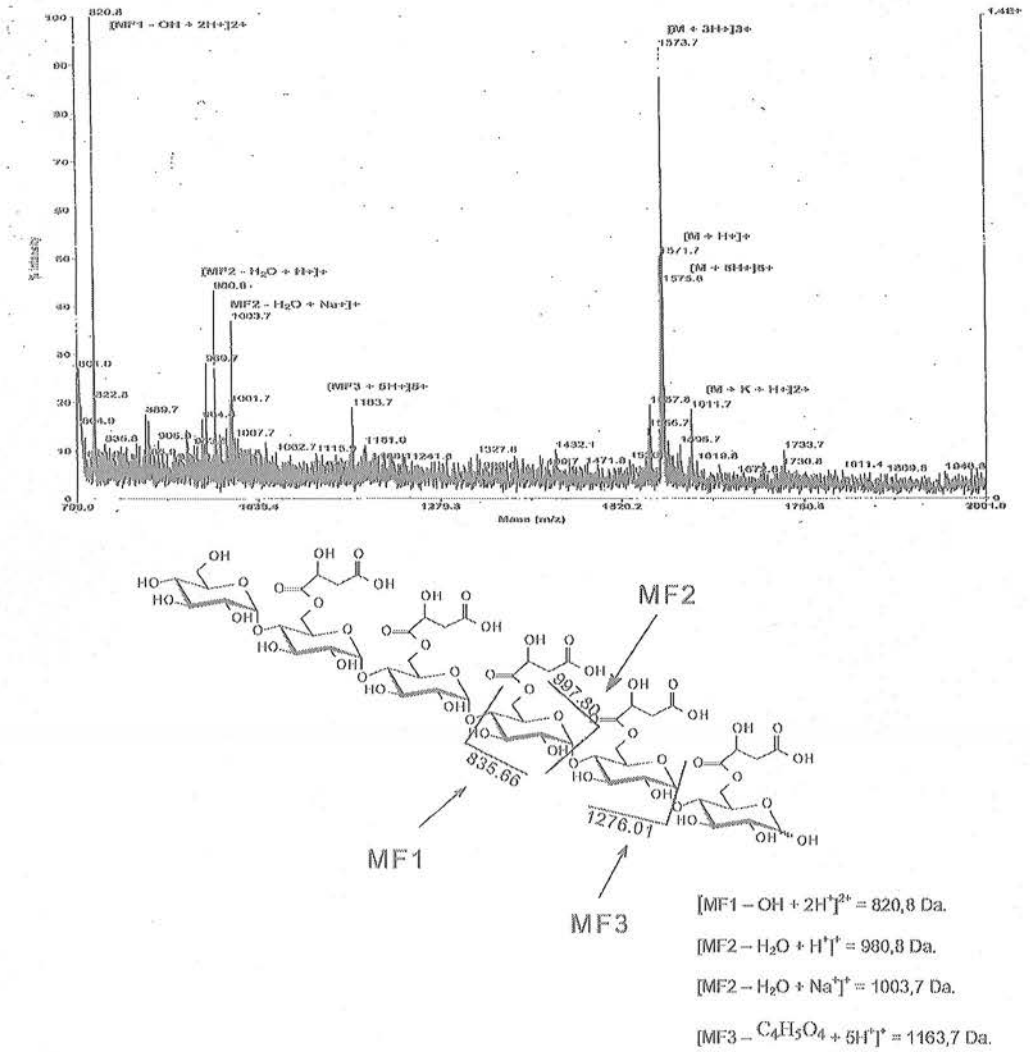

Figure 4. MALDITOF-MS (positive ion) of veracylglucan C under addition of trifluoroacetic acid and α-cyano 4-hydroxycinnamic acid as matrix. The formulae indicate molecular fragments recorded during the MALDI-process.

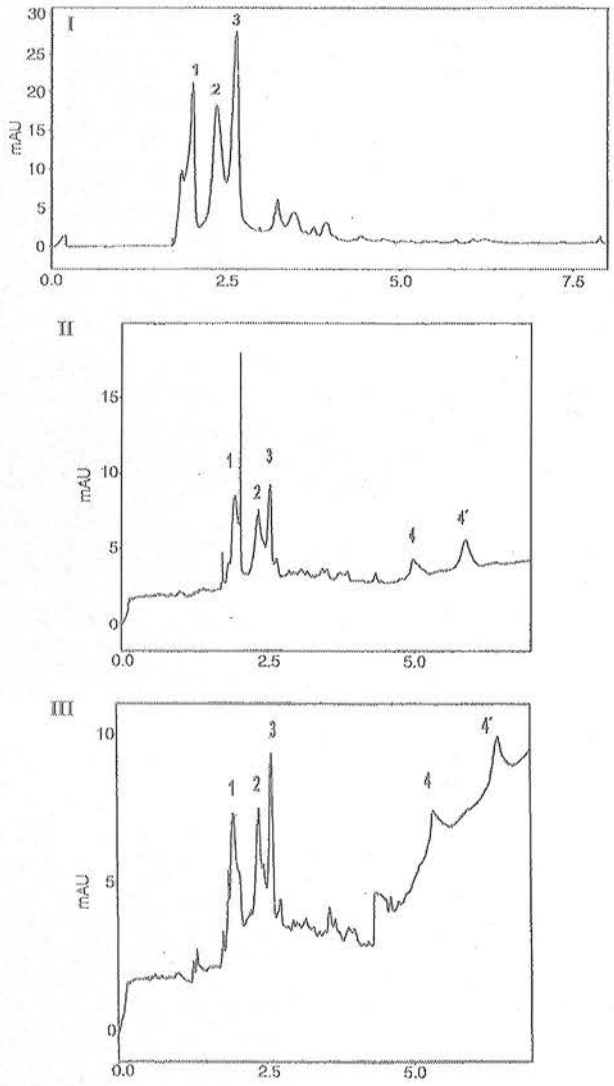

Figure 6. CE stability investigation of purified maloyl glucans from AVG in 1% aq solution (I) after 7 days at 7 °C (II) and after further 24 h (III) at rt.

of its presence was carried out using ^{13}C NMR spectroscopy, ESIMS and CE. The CE stability investigation of a 1% aq solution of maloyl glucans shows that the compounds are indeed very unstable. Figure 6II shows that after one week in a refrigerator at 7 °C, hydrolysis of the ester group [-*O*-6(1-L-maloyl)-Glc*p*-] easily occurs. This was further observed, continuing with the same solution, at room temperature after 24 h (Fig. 6III). The formation of monobasic malate (4) and dibasic malate (4′) could be observed at migration times beyond 6 min.

2.2. In vitro bioassay

The cell proliferation investigation on fibroblasts cultures (Fig. 7) showed that Vglc B is significantly antiproliferative (cytotoxic) at 1 mg/mL, and Vglc C significantly increases the cell proliferation at a concentration of 100 μg/mL. Vglc C at 1 mg/mL had no effect on cell proliferation when compared to the control. A concentration-dependent increase in cell proliferation through Vglc C after 24 h incubation was likewise observed. A remarkable antagonism of the proliferative effect of Vglc C through Vglc B resulted from this assay. Vglc C demonstrated a slight superiority by the increase in cell proliferation compared to PDGF at the same concentrations.

The anti-inflammatory effect of the isolated maloyl glucans showed that both Vglc B and C exercise anti-inflammatory biological effects on the tested keratinocytes. After the real-time RT-PCR gene expression analysis, it could be seen that Vglc B more or less significantly downregulates the expression of the inflammatory mediators IL-6, IL-8, and ICAM-1 (Fig. 8). Vglc C demonstrates throughout a stronger and significant downregulation of these mediators. A mixture of both compounds also significantly downregulated these mediators. The strongest biological effect was observed by the downregulation of IL-6. The gene expression of the housekeeping-protein *RPS26* from cells of all the test wells and controls were monitored. There were no significant differences observed, thus proving that the test compounds at the concentrations used had little or no toxic effect on the cells. In routine experiments, genes of the housekeeping proteins act as internal standards to qualitatively monitor the integrity of cultured cells under test conditions. This allows changes in gene expression to be considered specific.[13]

3. Discussion and conclusions

From the results of this study, one can now begin to understand why there are so many contradictions about the efficacy and chemical constituents of AVG. The hygroscopic, unstable and amorphic nature of Veracylglucan A, B and C surely accounted for the difficulties faced by many researchers when trying to investigate this aqueous mucilaginous plant material. Most of these problems usually lie in separation, quantification, reproduction and interpretation of chemical data during structure elucidation experiments and bioassays.

The wound-healing properties of AVG, as mentioned in the introduction, for example, are the subjects of many contradictions and ambiguities.[1] Considering that wound healing involves cell proliferation and that a recombinant PDGF (Becaplermin®) is available as a remedy for poor wound-healing by diabetic ulcers,[14] it can be concluded that Vglc C is the compound in AVG which is responsible for these properties. The anti-proli-

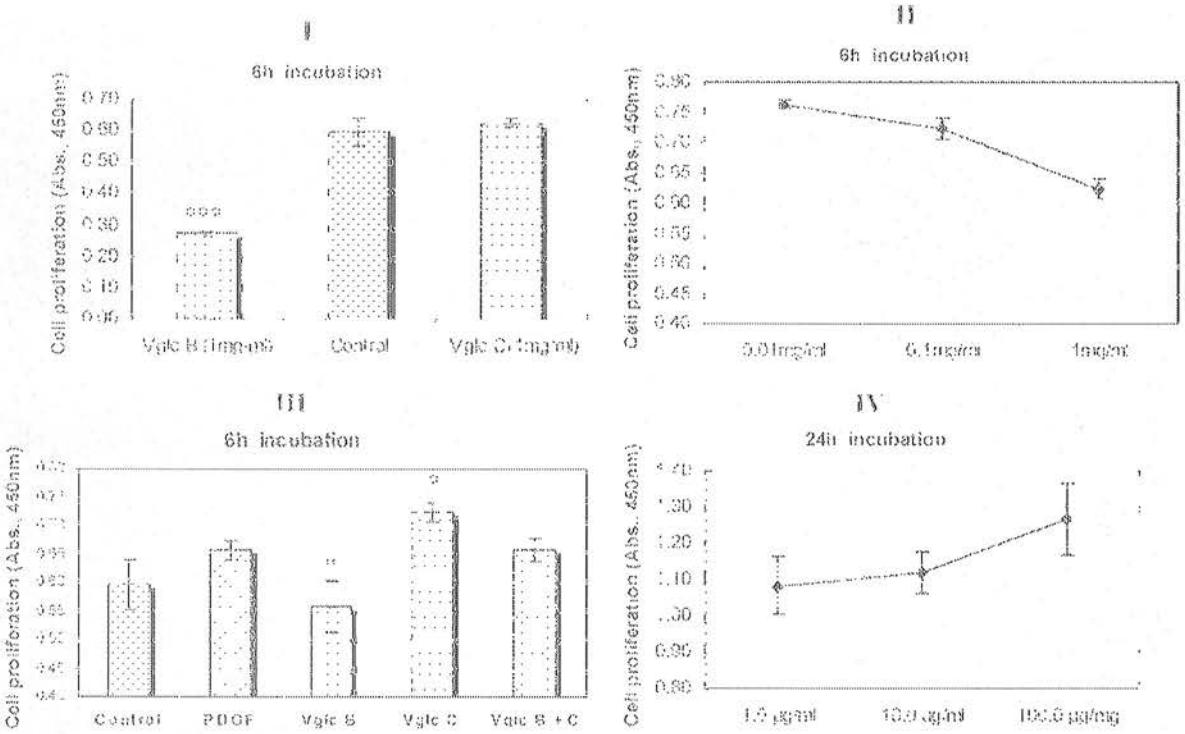

Figure 7. Activity of Vglc B and C on fibroblast cell proliferation using ELISA (BrdU colorimetric); after 6 h (I and II), 24 h (III), concentration-dependent cell proliferation activity of Vglc C after 24 h (IV) ($n = 6$, M ± SD, $*p \leqslant 0.05$, $***p \leqslant 0.009$).

ferative activities of Vglc B are, on the other hand, responsible for the retardation and cancellation of significant healing effects arising from Vglc C. The cultivation in different climatic zones, harvesting and processing (stability problems) of *Aloe barbadensis* Miller to AVG naturally leads to variations in the concentration of the bioactive maloyl glucans and contamination with anthranoids (e.g., aloin). AVG containing higher amounts of Vglc B or anthranoids will lead to retardation of wound healing, while higher quantities of Vglc C would definitely foster wound healing. Downregulation of proinflammatory mediators is an effective way to treat many autoimmune and chronic diseases caused by their overexpression.[15,16] The anti-inflammatory effects of AVG by Crohn's disease for example and a series of other chronic inflammatory diseases, can be correlated with Vglc B and C, taking into consideration that both compounds demonstrate the same effect against the gene expression of very important proinflammatory cytokines as well as counteracting the effect of TNF-α. ICAM-1 has been discovered to intensify HIV-1 infection.[17] Consequently, a downregulation of this cell-surface protein with AVG, for example, can lead to improvements by subjects suffering from AIDS. Common colds caused by the rhinovirus can as well be treated with fresh AVG, considering the fact that ICAM-1 is the biological receptor of this virus.[18]

Poorly processed and conserved AVG would loose its quality through breakdown of Vglc A, B and C into malic acid, glucose and simple oligosaccharides; therefore no reasonable biological activity can be anticipated from such a gel or products derived from it. In this era of evidence-based medicine, a proper characterization of AVG with respect to its purity and maloyl glucan content is consequently a very important step to rationalize the use of this medicinal plant material. The present study represents a major stepping stone to rationally utilize the therapeutic potential of AVG and the novel maloyl glucans. Further in vitro and in vivo experiments, as well as clinical studies, are hence necessary to establish these results.

4. Experimental

4.1. Isolation and structure elucidation

AVG (100 g, pH 3.5, from 4-year-old plants), supplied by Santaverde, Hamburg Germany, was homogenized at temperatures <15 °C with Ultra-Turrax T 50 and centrifuged for 30 min at 4 °C and 10,000*g*. The supernatant was then processed according to the method described in the international patent application.[19] The special extract obtained was stored at −30 °C before use. Capillary electrophoresis (CE) was performed using

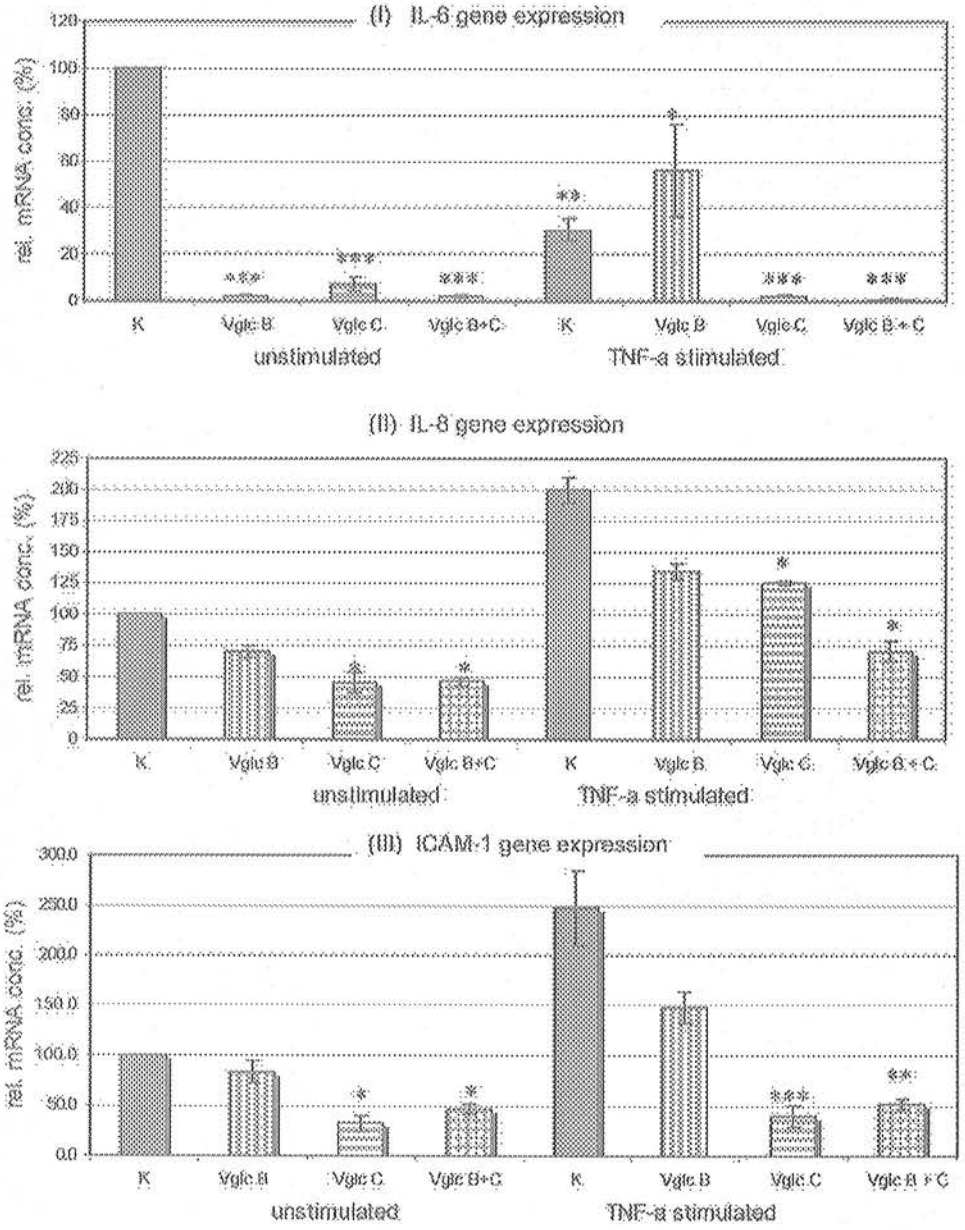

Figure 8. The anti-inflammatory effect of Vglc B and C (each 100 μg/mL and Vglc B + C; 100 μg/mL + 100 μg/mL) through downregulation of IL-6, IL-8 and ICAM-1gene expression in normal and TNF-a stimulated keratinocytes. Relative mRNA concentrations using real time RT-PCR after 24 h ($n = 3$, M ± SD, $*p \leqslant 0.05$, $***p \leqslant 0.009$).

the total extract (0.5%), a 31-cm effective capillary length filled with borate buffer (pH 10.5), and a voltage of 25 kV. Detection was carried out with a photo diode-array detector (P/ACE System MDQ, Beckman Coultard). The stability of the isolated compounds in 1% (m/v) aq solution was also investigated using CE, since many ambiguities about the effectiveness of AVG are related to the instability of its active constituents. UV–vis spectra of the compounds were recorded during detection. The structures of the isolated maloyl glucans were elucidated using 1D (^{1}H and ^{13}C NMR) and 2D NMR (^{1}H–^{1}H COSY, HMBC and HMQC) spectroscopy. NMR experiments (Varian Gemini 300 Hz and Bruker DRX 600 Hz) were performed in D_2O and DMSO, respectively. A Fourier-transformed ion-cyclotron resonance mass spectrometer (FTICR-MS) with ESI (electrospray ionization) source and Matrix-assisted laser desorption time of flight mass spectroscopy (MALDI-TOF-MS) (Applied Biosystems Voyager System 2022) were used to determine the molecular weights of these compounds. For the ESIMS, samples were prepared in 1:1 MeOH–H_2O. After adding trifluoroacetic acid to

the sample, α-cyano 4-hydroxycinnamic acid was used as the matrix for the MALDITOF-MS.

4.2. Bioassays

4.2.1. Cell-proliferation assay. The cell-proliferation assay was performed on clinically obtained human fibroblast culture (6th passage) by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody to bromodeoxyuridine. The commercially available ELISA-Kit, *Cell Proliferation ELISA, BrdU (colorimetric)-Kit*® (Roche Molecular Biochemicals), was used. The experiment was performed following the manufacturer's protocol (colorimetric, ELISA-Reader at λ 450 nm and ref. λ 690 nm). Cells (2×10^4) were suspended in 100 μL of culture medium [Dulbecco's minimum essential medium (DMEM) Biochrom AG, Germany, supplemented with fetal bovine serum, penicillin and streptomycin.] and introduced in the wells. The cultures were incubated at 37 °C until all the cells settled and stuck at the bottom of each well. The assay was then performed using human platelets-derived growth factor (PDGF), 10 μg/mL of medium, as positive control. The maloyl glucans were tested in the following manner: a mixture of Vglc B and C (1:1) 200 μg/mL, Vglc B 10,000, 1000 and 100 μg/mL, Vglc C 10,000, 1000 and 100 μg/mL. Vglc C was subsequently tested again at concentrations of 100, 10 and 1 μg/mL, due to the results obtained from the previous experiment. Cell cultures were incubated at 37 °C for 6 h and 24 h, respectively.

4.2.2. Anti-inflammatory assay. The anti-inflammatory effects of Vglc B and C were performed on keratinocytes in cell cultures in 6-well test plates (same culture medium as above). TNF-α stimulated and unstimulated cell cultures were investigated alongside with controls. For this assay, the expression of the intercellular adhesion molecule 1 (ICAM-1), a potent inflammatory mediator,[16] and the proinflammatory cytokines IL-6 and IL-8[20] were investigated using real-time reverse transcriptase polymerase chain reaction (RT-PCR). After a 6-h stimulation with TNF-α (tumour necrosis factor alpha), the medium was discarded, and fresh medium containing Vglc B and C in the following concentrations was introduced in all cultures, respectively: a mixture of Vglc B and C 300 μg/mL, (2:1), Vglc B (100 μg/mL) and Vglc C (100 μg/mL). After a 24-h incubation, the medium was discarded from the cultures, and the cells were trypsinzed to harvest the total messenger ribonucleic acid (mRNA). mRNA harvesting was performed using a commercially available chromatography kit, RNeasy® Mini (Qiagen). The harvested total mRNA (2 μg) was then transformed into complementary DNA (cDNA) using M-MLV reverse transcriptase (Promega) following the manufacturer's protocol. The PCR was performed using specific primer pairs for human mRNA of ICAM-1, IL-6 and IL-8, designed as previously described.[21] Real-time PCR was performed with a Light Cycler Fast Start DNA master SYBR Green 1 kit (Roche Diagnostics) with 4 mM $MgCl_2$ under primer-specific conditions according to the manufacturer's protocol. The following experimental protocol for the PCR (40 cycles) was used: denaturation for 10 min at 95 °C, followed by amplification cycles comprising denaturation for 15 s at 95 °C, annealing for 5 s at 68 °C, elongation for 20 s at 72 °C, and fluorescence measuring for 3 s at 84 °C. The relative amount of mRNA was determined using threshold cycle determination and normalization.

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