Structural Characterization and Cytotoxic Properties of a 4-*O*-Methylglucuronoxylan from *Castanea sativa*. 2. Evidence of a Structure−Activity Relationship

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Xylans were purified from delignified holocellulose alkaline extracts of *Castanea sativa* (Spanish chestnut) and *Argania spinosa* (Argan tree) and their structures analyzed by means of GC of their per-trimethylsilylated methylglycoside derivatives and ^{1}H NMR spectroscopy. The structures deduced were characteristic of a 4-*O*-methylglucuronoxylan (MGX) and a homoxylan (HX), respectively, with degrees of polymerization ranging from 182 to 360. In the case of MGX, the regular or random distribution of 4-*O*-methylglucuronic acid along the xylosyl backbone—determined by MALDI mass spectrometry after autohydrolysis of the polysaccharide—varied and depended both on the botanical source from which they were extracted and on the xylan extraction procedure. The MGX also inhibited in different ways the proliferation as well as the migration and invasion capability of A431 human epidermoid carcinoma cells. These biological properties could be correlated with structural features including values of the degree of polymerization, 4-*O*-MeGlcA to xylose ratios, and distribution of 4-*O*-MeGlcA along the xylosyl backbone, giving evidence of a defined structure−activity relationship.

Xylans, the most common hemicelluloses, besides accounting for the major noncellulosic cell-wall polysaccharide fraction, are heterogeneous with regard to composition and structure.[1] Although they have been used for pharmaceutical or agricultural purposes,[2] few of these have been proposed for applications such as gels, food additives, and biological films.[1,3,4] Xylans have also been studied for their possible medical use as ulcer protective,[5] antitussive,[6] immunostimulatory,[7] and antitumor agents.[8] Oligosaccharides derived from polysaccharides and especially from xylans can also have notable properties, such as antimicrobial[9] or mitogenic activities,[10] or can be used as substrates for the production of biologically active aldobiuronic acids.[11]

In a previous paper,[12] we have reported the structural characterization of a 4-*O*-methylglucuronoxylan (MGX) extracted from *Castanea sativa* Mill. (Fagaceae) (Spanish chestnut; sweet chestnut), a common tree of the Limousin region (France). This polymer represents up to 35% of the holocellulose fraction.[13] Its structure consists of a β-(1→4)-D-xylopyranosyl backbone with 4-*O*-methyl-D-glucuronic acid side chains at the 2-position of the xylose units and exhibits a specific Xyl to MeGlcA ratio estimated to be 5.9:1. MGX was found to inhibit proliferation as well as migration and invasion of A431 human squamous carcinoma cells. Preliminary results showed that chestnut tree MGX strongly inhibited the expression of the metalloproteinases MMP2 and MMP9, which are involved in cell basement membrane degradation and migration through extracellular matrices.

Establishing a precise relationship between structure and biological activity of an individual polysaccharide is a real challenge. In this context Ebringerová et al.[14] provided some significant experimental data that could help with an understanding of such structure−function relationships. In the case of β-(1→3)-D-glucans, Yanaki et al.[15] found that a decrease in weight-average molecular mass, correlated with triple-helix formation, led to the loss of cytotoxic activities, whereas Kulicke et al.[7] showed that helical structures are not essential for the expression of immunological activity. The 4-*O*-methylglucuronic acid content of glucuronoxylans and their distribution along the xylosyl backbone have been thought to regulate the expression of their immunomodulatory properties when tested with rat thymocytes (Ebringerová et al.[14]), but conclusive evidence of a structure−function relationship is still lacking. This is why this present study was undertaken with the aim of correlating the structural characteristics of several MGXs in terms of 4-*O*-methylglucuronic acid content and distribution as well as their molecular mass to influence on A431 cell proliferation, invasion, and migration. Toward this end, two MGX extracts from *Castanea sativa* were purified, and their molecular masses and monosaccharidic content were evaluated by means of colorimetric assays, gas chromatography, and NMR spectroscopy. Their bioactivities were compared to those of homoxylan (HX) and MGX obtained from *Argania spinosa* (L.) Skeels (argan tree) and then discussed in relation with the distribution of their 4-*O*-methylglucuronic acid substituents characterized after autohydrolysis of the polysaccharides and MALDI mass spectrometric analysis.

Results and Discussion

Strong interactions between xylans and lignin within the plant cell-wall hamper their extraction from wood. Preliminary delignification helps considerably in the extraction of cell-wall xylan from hardwood sawdust.[16] A dilute KOH pretreatment, by inducing the swelling of the cell wall, can likewise improve extractability of cell-wall polysaccharides. From a quantitative point of view, xylans were isolated according to the procedure described in Schemes S1 and S2 (Supporting Information) in mass yields ranging from 12% (MGX C_2) to 19% (MGX C_1) mass ratio (Table 1). The water-insoluble fraction from *A. spinosa* pericarp, with a xylosyl molar ratio higher than 98.5% (Table 1), presents the typical composition of a homoxylan-type polysaccharide (HX). With their xylosyl and 4-*O*-methylglucuronic acid contents ranging from 76% to 86% and 13% to 14.6%, respectively (Table 1), KOH extracts from *A. spinosa* pericarp (MGX A) and *C. sativa* sawdust (MGX C_1 and MGX C_2) were characteristic of 4-*O*-methylglucuronoxylan-type polysaccharides. The percentage of uronic acids is also confirmed by ^{1}H NMR spectroscopic analysis. Traces of Rha, Ara, Man, Glc, Gal, and GalA were also detected and could be considered as contaminants.

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Table 1. Extraction Yield and Monosaccharide Composition of Alkaline MGX and HX Extractives

	extractionyield (% w/w)	Rha	Ara	Xyl	Man	Glc	Gal	GalAU	GlcAU	4-*O* -Me GlcA
MGX C1	*19*	1.8	0.8	76.3	0.8	1.0	2.0	2.1	0.6	14.6
MGX C2	*12*	2	1.3	78.4	0.1	0.1	2.8	2.3	0	13
MGX A	*19*	0.9	0	85.8	0	0	0	0	0	13.3
HX	*18*	0.7	0	98.6	0	0	0	0	0	0.7

Table 2. ^{1}H NMR Chemical Shift (ppm) Assignments for Residues of *Castanea sativa* and *Argania spinosa* Xylans[a]

		(1→4)-*β*-D-Xyl*p* ^{1}H	(1→4)-*β*-D-Xyl*p*-2-*O*-(4-*O*-Me-Glc*p*A) ^{1}H	4-*O*-Me-α-D-Glc*p*A ^{1}H
	position	*δ* (ppm) (*J* Hz)	*δ* (ppm) (*J* Hz)	*δ* (ppm) (*J* Hz)
MGX C_1	1	4.48 d (7.5)	4.63 d (7.2)	5.29 d (2.0)
	2	3.29 t (8.2)	3.44 m	3.60 m
	3	3.55 t (9.0)	3.62 m	3.76 m
	4	3.79 m	3.81 m	3.22 t (9.7)
	5_{ax}	4.10 dd (4.5 ; 11.5)	4.15 m	4.33 d (10.1)
	5_{eq}	3.38 t (11.0)	3.42 m	
	6			
	O-CH3			3.46 s
MGX C_2	1	4.51 d (7.5)	4.66 d (7.0)	5.31 d (2.5)
	2	3.32 t (8.4)	3.57 m	3.61 m
	3	3.59 t (9.0)	3.68 m	3.77 m
	4	3.79 m	3.82 m	3.26 t (9.7)
	5_{ax}	4.14 dd (4.4 ; 11.5)	4.15 m	4.36 d (10.0)
	5_{eq}	3.41 t (11.0)	3.46 m	
	6			
	O-CH_3			3.50 s
MGX A	1	4.51 d (7.1)	4.66 d (6.4)	5.32 d (1.6)
	2	3.31 t (8.1)	3.57 m	3.61 m
	3	3.61 t (8.9)	3.67 m	3.77 m
	4	3.80 m	3.82 m	3.26 t (9.6)
	5_{ax}	4.14 dd (4.6 ; 11.0)	4.15 m	4.36 d (9.9)
	5_{eq}	3.41 t (11.0)	3.47 m	
	6			
	O-CH_3			3.50 s

[a] Ax = axial, eq = equatorial, $^3J_{H,H}$ in Hz.

Table 3. ^{1}H NMR Chemical Shift (ppm) Assignments for Residues of *Argania spinosa* Homoxylan[a]

position	(1→4)-*β*-D-Xyl*p* ^{1}H *δ* (ppm)
1	4.27
2	3.05
3	3.30
4	3.50
5_{ax}	3.88
5_{eq}	3.17

[a] Ax = axial, eq = equatorial, $^3J_{H,H}$ in Hz.

From a structural point of view, NMR spectroscopic analysis can provide useful information required to establish the structure of these xylans. The complete assignments of the proton NMR spectra (Tables 2 and 3) were conducted by performing 2D COSY experiments and were consistent with our previous report.[12] Examination of data relative to ^{1}H NMR analysis revealed three important groups of protons: major signals corresponding to the nonsubstituted D-xylose units of the backbone and two groups of minor signals, with the first one corresponding to 4-*O*-Me-d-GlcA residues and the second assigned to D-Xyl units substituted with 4-*O*-Me-D-GlcA. From the fact that the coupling constants of anomeric protons of substituted (4.5 ppm) or nonsubstituted (4.6 ppm) xylose units were larger than 7 Hz, the xylose residues were shown to be linked via *β*-glycosidic bonds, while the anomeric proton of 4-*O*-Me-α-D-GlcA exhibited a doublet with a coupling constant less than 2 Hz, corresponding to an α-configuration. In addition, the presence of the methyl group of MeGlcA was confirmed by a corresponding sharp singlet at 3.46 ppm. The linkage via (1→2) glycosidic bonds between Xyl and 4-*O*-MeGlcA was confirmed by the deshielding of the H-2 signal of the substituted Xyl (3.44 ppm), in comparison with the nonsubstituted one (3.29 ppm). The ^{1}H NMR spectrum of HX showed six signals at *δ* 4.28 (H-1), 3.05 (H-2), 3.30 (H-3), 3.50 (H-4), 3.17 (H-5 eq), and 3.88 (H-5 ax) (Table 3) corresponding to (1→4) linked *β*-D-Xyl residues and was consistent with data published by Habibi and Vignon[17] for the typical structure of a homoxylan.

The mass polydispersity of both the MGX and HX extractives was then studied by means of SEC-HPLC (Figure 1). For the chromatographic conditions used for filtration, *C. sativa* and *A. spinosa* MGX as well as *A. spinosa* HX were eluted as narrow peaks characteristic of polymers with good homogeneous molecular masses (Figure 1). On the basis of colorimetric estimation, the ratio of the reducing sugar to the total carbohydrate suggests that the average degree of polymerization (DP) of MGX ranges from 182 (*C. sativa*) to 340 (*A. spinosa*). HX from *A. spinosa*, with a DP value of 360, exhibited the highest molecular mass.

To investigate the effects of xylans on cell proliferation, A431 tumor cells were treated with xylan extracts at 0.7 and 50 *μ*M (Table 4). It was found that *C. sativa* MGX extractives inhibited tumor cell proliferation, but the concentration inducing 50% of maximal inhibition (IC_{50}) was reached with MGX C1 only. With respectively 29% and 19% inhibition of cell proliferation, MGX and HX extracts from *A. spinosa* exhibited lower bioactivity.

A431 human squamous carcinoma cells represent a good model of an aggressive, highly angiogenic, and invasive tumor.[18,19] They display an increase of epidermal growth factor receptors (EGFR) and produce large amounts of vascular endothelial growth factor (VEGF),[20] promoting neovascularization.[21] Increased EGFR expression renders A431 cells less dependent upon an exogenous source of epidermal growth factor (EGF) and enhances the EGF-induced mitogenic responses of squamous cell carcinoma cell lines compared with human epidermal keratinocytes, thus contributing to the invasiveness of malignant cells.[22]

In the presence of a chemotatic stimulus (FCS) in the lower part of the Boyden migration chamber, A431 cells migrated through

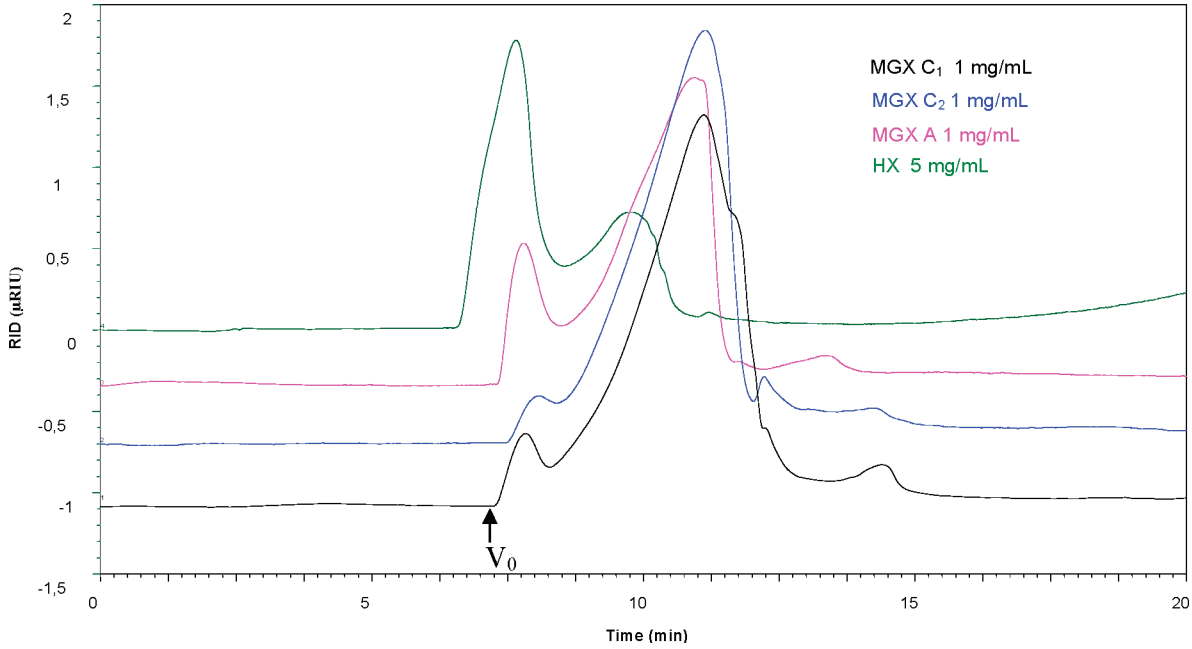

Figure 1. Superposition of elution profiles obtained on HPLC analysis of MGXs and HX. V_0: Void volume.

Table 4. Effects of *Castanea sativa* and *Argania spinosa* Xylans on Proliferation, Migration, and Invasion of A431 Cells and on MMP9, ProMMP2, and ProMMP9 Expression by A431 Cells

	proliferation inhibition (%)		migration inhibition (%)		invasion inhibition (%)		12.5 μM zymography		
xylan	0.7 μM	50 μM	5 μM	50 μM	5 μM	50 μM	MMP9 inhibition (%)	ProMMP2 inhibition (%)	ProMMP9 inhibition (%)
MGX C1	35 ± 1	51 ± 2	68 ± 2	99 ± 2	0	55 ± 2	100	56	50
MGX C2	18 ± 2	45 ± 3	50 ± 4	55 ± 2	72 ± 6	72 ± 6	10	39	0
MGX A	29 ± 1	29 ± 1	nd[a]	nd	nd	nd	nd	nd	nd
HX	0	19 ± 2	nd	nd	nd	nd	nd	nd	nd

[a] nd: not determined.

the pores to the lower surface of the membrane; this migration was found to be strongly inhibited by C_1 and C_2 MGX from *C. sativa*. Compared with untreated control cells, migration of A431 was significantly decreased up to 99% ($p < 0.05$) in the presence of 50 μM of MGX C_1 (Table 4). A Matrigel invasion assay was performed to study the effect of these MGX products on the invasive ability of A431 cells. At a concentration of 50 μM (corresponding to the IC_{50}), with up to 72% inhibition (in the case of MGX C_2), both extractives significantly reduced the invasion of A431 cells as compared with untreated control cells (Table 4).

Cell migration that takes place during angiogenesis requires the degradation of the extracellular matrix by proteases such as matrix metalloproteases (MMP).[23] Since MGX inhibited A431 cell migration and invasion, the influence of this compound was evaluated on the secretion of MMP9 and MMP2 gelatinases by A431 cells. Exposure of these cells to 12.5 μM MGX C_1 resulted in a significant inhibition of ProMMP9 and ProMMP2. Analysis by quantitative zymography indicated that the amount of ProMMP secreted in the medium and normalized to cell number decreased by 56% for ProMMP2 and by 50% for ProMMP9. The expression of MMP9 was totally abolished in 24 h-treated cells as compared to control cells. Therefore the antimigration and antiproliferative effects of MGX C_1 can be explained, at least in part, by a decrease of MMP9 and MMP2 expression. The MGX C_2 preparation at the same concentration induced lower or nonsignificant inhibition of MMP9, ProMMP2, and ProMMP9 expression (10%, 39%, and 0%, respectively).

Ebringerová and co-workers[14] have suggested that the structural variability of xylans may affect their biological properties. Such structural variability may originate from the degree of polymerization (DP) of the polysaccharide (hence its molecular mass), the monosaccharidic composition (and especially the typical Xyl:MeGlcA ratio), and the random or regular distribution of MeGlcA substituents along the xylosyl backbone. The latter feature, reflecting the fine structure of the macromolecular chains, may affect the intermolecular interactions between xylan molecules in solution, creating networks or microgels, as well as interactions with biopolymers located on the surface layers of the A431 cell coat.[14] This led us to investigate the distribution of MeGlcA units in MGX C_1, MGX C_2, and MGX A by means of MALDI-MS analysis, after their degradation by autohydrolysis. Autohydrolysis of polysaccharides, i.e., polysaccharide hydrolysis through their own acid groups, is a soft, selective, and easy method.[24] Yields of autohydrolysis depend on the concentration of acid groups. In contrast, its chemical mechanism depends on the distribution of these acid groups and their ability to interact with glycosidic linkages. Fragments obtained are then representative of the starting structure of the polymers. When applied to *C. sativa* and *A. spinosa* MGX extracts, autohydrolysis led to their degradation into characteristic oligosaccharides formed of 4-*O*-methylglucuronic acid (GA) and xylosyl (X) residues whose molecular masses, observed by MALDI-MS, could be assigned to typical X_n, X_nGA, or X_nGA_2 ($n = 1$ to 14) oligosaccharides (Figure 2). In this figure, the mass data are represented as graphs, in which the height of a given species is deduced from the relative abundance of the corresponding ion in the mass spectrum (about 250 laser shots were averaged in each mass spectrum, in order to reach a total ion current of around 2.5×10^3 and to get a representative view of the spot). The mass profiles of glucurono-xylooligosaccharides obtained from MGX C_2 and MGX A are quite similar: a Gaussian distribution is observed, centered on the DP 5 to 7 species. Two series of abundant species were observed in both cases, corresponding to nonsubstituted (X_n)

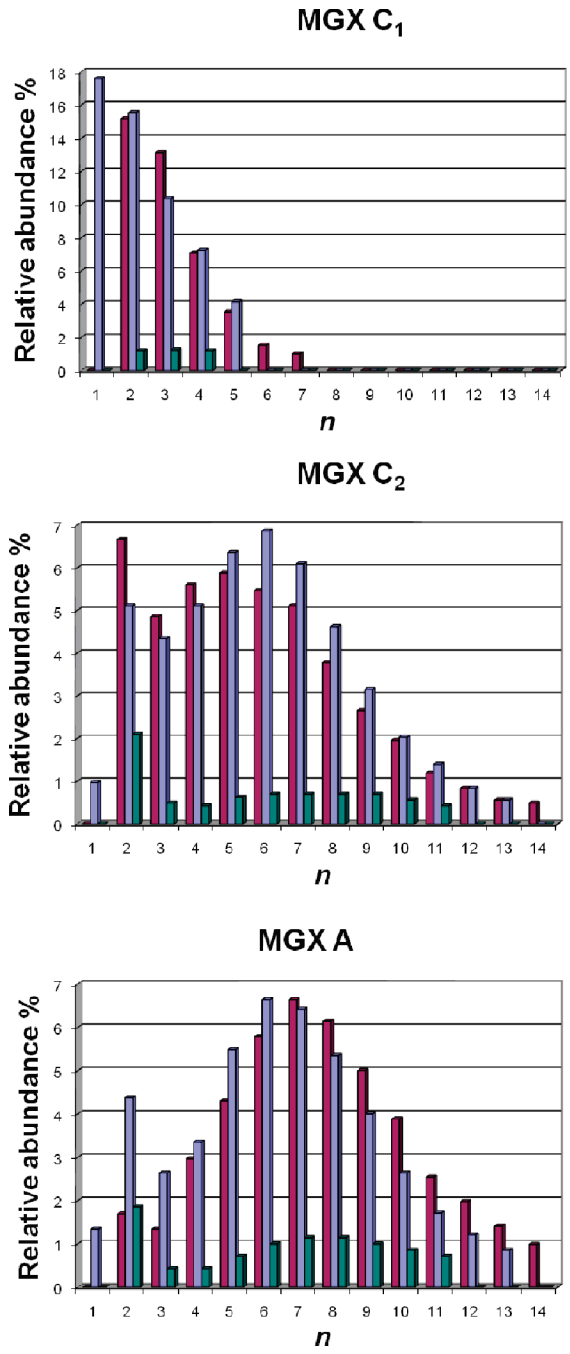

Figure 2. Distribution of uronic acids in xylans from *Castanea sativa* and *Argania spinosa* after autohydrolysis and MALDI-MS analysis. Mass data are represented as graphs, where the heights correspond to the relative abundance of the corresponding ions. Red: X_n. Lilac: X_nGA. Green: X_nGA_2.

or monosubstituted (X_nGA) forms. Additionally, the presence of large amounts of 4-*O*-methylglucuronic acid disubstitution, mainly of the X_2GA_2 types, as well as X_n, mainly of the X_2 to X_{12} forms, reveals an irregular distribution of GA, already suggested by Jacob et al.[25] for hardwood MGX. In contrast, in the case of MGX C_1 obtained from nonimpregnated *C. sativa* sawdust, the glucuronoxylooligosaccharides were found to be significantly shorter, with X_1, X_2, X_3, XGA, and X_2GA being the most abundant species. The X_nGA_2 series is also present but in very low amount, suggesting in this case a more regular distribution of GA units along the xylan backbone. Of interest is the structural difference deduced from the MALDI-MS data of MGX C_1 and MGX C_2 obtained from autohydrolysis of nonimpregnated or impregnated *C. sativa* sawdust, respectively. Such differences might be explained by the methods of extraction used. As suggested by Dahlman et al.,[26] xylan molecules are distributed from the inner to the outer layers of hardwood pulp, and their structural characteristics (molar mass, uronic acid content) differ according to their location and also the pulping process used (e.g., cooking, bleaching, and delignification). From our results, it could then be assumed that a preliminary KOH impregnation step would favor the extraction of a second class of MGX—*with an irregular distribution of 4-O-methylglucuronic acid units*—closely associated with the cell wall through ester linkage with residual phenolic compounds.[27]

Table 5. Contents and Distribution of 4-*O*-Methylglucuronic Acids Values, Degrees of Polymerization, and IC_{50} of Extracted Xylans

	Xyl/MeGlcA ratio (^{1}H NMR)	distribution of acids	DP	IC50
MGX C_1	5.9/1	regular	200	50 μM
MGX C_2	5.5/1	random	182	Nr[a]
MGX A	4.8/1	random	340	Nr
HX			360	Nr

[a] Nr: not reached.

The biological activity of polysaccharides is often correlated with their acidity, even though this characteristic is not an absolute determinant. On the other hand, a more complex structure (main glycosidic linkage, extent of branching and ramification, and DP) seems to have a positive influence on their capacity to trigger a biological activity.[28] Comparison of biological responses obtained from the tested neutral HX and acidic MGX revealed some interesting relationship between DP, xylosyl to MeGA ratio, MeGA distribution pattern through the xylosyl backbone, and cytotoxic properties of A431 cells. The *A. spinosa* neutral HX, characterized by a high DP and the absence of any MeGA substituents, was found to be devoid of cytotoxic activity against A431 cells (Table 5). This is also the case for its MGX acidic forms. So, the presence of MeGA cannot be considered as a key determinant. The cases of MGX C_1 and MGX C_2 extracted from *C. sativa* are of interest (Table 5). With similar DP values and xylosyl to MeGA ratios, these extractives did not exhibit the same level of A431 cytotoxic activity. The IC_{50} value could be reached only with the nonimpregnated MGX C_1 chestnut sawdust extract. The latter is the one that presents a regular distribution of its MeGA substituent through the xylosyl backbone of the molecule. In another direction, the monosaccharidic compositions obtained from *A. spinosa* MGX A and impregnated *C. sativa* MGX C_2 extracts as well as their characteristic MeGA distribution through the xylosyl backbone of the molecule are quite similar. Nevertheless, MGX A, while showing a lower A431 cytotoxic activity, is also characterized by a higher DP value. In this case, the high DP value seems to have a negative influence on its biological activity.

From the data obtained in the present study, it can be speculated that the distribution of MeGA and also the degree of polymerization are the structural key determinants of the biological activity of xylans of the type investigated. The degree of polymerization can influence the three-dimensional structure of xylans and especially their helical organization. It is now accepted that not only the primary structure but also the whole complex of chemical and physicochemical properties and supramolecular structural features of these polymers may all contribute to the expression of their biological properties. To enlighten this structure−function relationship, our group has initiated a study on three-dimensional structure, according to which xylans appear as highly organized helical macromolecules.[29] To study the effects of higher DP and the variability of their MeGA substitution pattern along the xylan helical conformation as well as the identification of the carbohydrate sequence responsible for the A431 cytotoxic properties are the next steps that could enhance our comprehension of the biological properties of hemicelluloses of the xylan type.

Experimental Section

General Experimental Procedures. All extracts (see below) were evaporated at a temperature of <40 °C under reduced pressure. The centrifugation conditions were 10 000 rpm for 15 min at 25 °C. Total carbohydrate was measured by the phenol sulfuric acid method.[30] Hexuronic acids were determined with the *meta*-hydroxydiphenyl method.[31] The content of reducing sugars was estimated according to the method of Lever.[32] The FT-IR spectra were recorded using a KBr pellet with a FT-IR Perkin-Elmer Spectrum 1000 in the frequency range 400 to 4000 cm^{-1}. Separation of the per-trimethylsilylated methylglycosides was done with a Perichrom gas chromatograph fitted with a flame-ionization detector. A capillary column, CPSIL-5CB (Chrompack, 0.32 mm × 50 m), was used with the following temperature program: 120−240 at 2 °C min^{-1}. Nitrogen was the carrier gas at 0.5 atm.

Plant Material. *Castanea sativa* Mill., identified by Dr. G. Saladin, botanist at Limoges University, was collected in the Limousin region of France in October 2004. Voucher specimens were deposited in the Life Sciences Department of the University of Limoges under the references CsM1-2004 and CsM2-2004 for *C. sativa*. Sawdust from the same plant were obtained from a local sawmill.

Seed pericarp of *Argania spinosa* fruit was donated by the society EFAS located in Morocco and was identified by Prof. A. Mouradi (Ibn Tofail, University of Kenitra, Morocco). A sample was stored at the herbarium of the Life Sciences Department of the University of Limoges under the reference AsL1-2006.

Isolation and Purification of the 4-*O*-Methylglucuronoxylans from *Castanea sativa*. Sawdust was dried 48 h at 60 °C in a ventilated oven and ground to pass a 500 μm size sieve. Ground sawdust was then extracted in a Soxhlet extractor with 80% EtOH and freeze-dried before further purification. The dewaxed sawdust was then sequentially depectinized using a 1% ammonium oxalate solution (2 h at 80 °C) and delignified by addition of an acidic sodium chlorite solution (0.47 g of sodium chlorite and 0.2 mL of glacial acetic acid per gram of sawdust) at 80 °C for 2 h. After filtration, the residue was washed with water and then air-dried at 60 °C for 16 h to give a holocellulose fraction. Last, MGX C_1 was extracted from the holocellulose for 24 h at room temperature with a 4.3 M KOH solution containing 3 mg of $NaBH_4$ per mL with continuous agitation and under nitrogen. The hemicellulose solution was neutralized by addition of glacial acetic acid and dialyzed against water (Spectrapor MWCO 6000−8000 Da). Hemicelluloses were finally precipitated by addition of three volumes of ethanol into the aqueous solution, with the pellet recovered after centrifugation, solubilized in water, and then freeze-dried for storage. Sawdust impregnation was effected in a 0.43 M KOH solution containing 3 mg of $NaBH_4$ per mL for 1 h at room temperature with continuous agitation and under nitrogen, before extraction leading to the MGX C_2 fraction.

Isolation and Purification of the Homoxylan (HX) and the 4-*O*-Methylglucuronoxylan (MGX-A) from *Argania spinosa*. Argan pericarps were ground to obtain a powder that was extracted in a Soxhlet extractor with 80% EtOH and freeze-dried before further purification. The dewaxed powder was then sequentially depectinized using distilled water (2 h at 100 °C) and delignified by addition of an acidic sodium chlorite solution (0.47 g of sodium chlorite and 0.2 mL of glacial acetic acid per gram of sawdust) at 80 °C for 2 h. After filtration, the residue was washed with water and then air-dried at 60 °C for 16 h to give the holocellulose fraction. Then, an extraction from holocellulose for 24 h at room temperature with a 4.3 M KOH solution containing 3 mg of $NaBH_4$ per mL with continuous agitation and under nitrogen was performed. The hemicellulose solution was then neutralized by addition of glacial acetic acid and dialyzed against water (Spectrapor MWCO 6000−8000 Da). The water-insoluble fraction contained HX. Glucuronoxylans present in the water-soluble fraction were precipitated by addition of 4 volumes of ethanol to the aqueous solution, with the pellet recovered after centrifugation, solubilized in water, and then freeze-dried for storage.

Monosaccharide Composition. . Monosaccharide determination was carried out after methanolysis (MeOH/HCl 0.5 N, 24 h, 80 °C) by gas−liquid chromatography of per-trimethylsilylated methylglycosides, according to Kamerling and co-workers,[33] and modified by Montreuil and co-workers.[34]

Mass Polydispersity. The mass polydispersity of MGX and HX was studied using HPLC with a Dionex P-480 system fitted with two PL Aquagel-OH mixed 8 μm columns (Polymer Laboratories, 300 × 7.5 mm). The system was equilibrated in MilliQ water at a flow rate of 1 mL min^{-1}. The refractive index of effluents was recorded with a detector sensitivity of 0.25 μRIU.

Autohydrolysis of Xylans. An aqueous solution of native glucuronoxylan (0.5% w/v) was converted into its acid form by passage through an Amberlite IR 120 (H^+) column and subsequently subjected to autohydrolysis by heating in a sealed tube at 100 °C for 24 h. Oligosaccharides were then separated by ion-exchange chromatography on Dowex 1 × 2 columns. A neutral fraction was removed by filtration. Acidic glucuronoxylooligosaccharides were desorbed by addition of a 50 mM ammonium formate solution. The purified oligosaccharides were desalted after five successive lyophilization cycles by steric exclusion chromatography on Biogel P2 and freeze-dried for storage before analysis.

MALDI Mass Spectrometry of Autohydrolyzed MGX. MALDI-MS were performed via the RIO platform "Biopolymers-Interactions-Structural Biology" located at the INRA Center of Nantes (UR1268, Biopolymères Interactions Assemblages, INRA, F-44300 Nantes). Hydrolyzed samples were dissolved in water at a concentration of 0.1 mg mL^{-1}. One microliter of the hydrolyzed sample was spotted on a stainless steel MALDI target plate. One microliter of the matrix, consisting of 2,5-dihydroxybenzoic acid (DHB) dissolved at 5 mg/mL in 0.1% TFA, was then added and allowed to dry. Mass data acquisitions were performed on a MALDI-LR instrument (Waters, Manchester, UK) equipped with a conventional nitrogen laser (wavelength 337 nm, frequency 5 Hz).

NMR Analysis. MGX samples were freeze-dried three times in D_2O and then dissolved into 600 μL of D_2O (99.97% purity, Euriso-top, Saclay, France). $CDCl_3$ (99.8% purity + 0.03% TMS, Euriso-top, Saclay, France) was used for the HX sample. The chemical shifts (ppm) were calibrated relative to the signals from acetone, used as an internal standard, at 2.22 and 31.5 ppm for the ^1H and ^{13}C NMR spectra, respectively. The samples were analyzed at 300 K in 5 o.d. mm BMS-005-B Shigemi tubes on a Bruker DPX-400 spectrometer operating at 400.13 MHz (^1H) and 100.62 MHz (^{13}C). ^1H spectra were acquired using a 5.7 KHz spectral width with 32 K data points, 2831 s acquisition time, and 152 scans were accumulated (^1H 90° pulse = 5.2 μs). The ^{13}C spectrum was obtained with a 26.2 KHz spectra width, 32 K data points, 0.62 s acquisition time, and 15154 scans. The pulse programs of the two-dimensional experiments were taken from the Bruker software library, and the parameters were as follows: for 2D $^1H-^1H$ correlated spectroscopy (COSY), relaxation delay, d_1 = 2 s, 90° pulse = 5.20 μs for ^1H, 2048 data points in t_2, spectral width 10.483 ppm in both dimensions, 512 experiments in t_1.

Xylan Solubilization. According to the solubility of the MGX, the stock solution was prepared at 0.5 mM in water. Dilutions of the stock solution were conducted with Eagle's medium, and the highest concentration tested corresponded to 0.05 mM.

Cell Lines and Cell Culture. Cell lines were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 U mL^{-1} streptomycin (all obtained from Life Technologies, Inc.), at 37 °C in a 5% CO_2 humidified atmosphere. Human A431 squamous carcinoma cells (vulvar epidermoid carcinoma) were obtained from the American Type Culture Collection.

Cell Viability Experiments. Cell viability was evaluated using the MTT microculture tetrazolium assay,[35−37] based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) into purple formazan crystals. Cells were seeded at a density of 5 × 10^3 cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 h. Then, the medium was removed and replaced by 2% FCS medium containing increasing concentrations of xylan varying from 0.8 to 50 μM. After 72 h incubation for A431 cells, the cells were washed with phosphate-buffered saline (PBS, Life Technologies) and incubated with 0.1 mL of MTT (2 mg/mL, Sigma-Aldrich) for an additional 4 h at 37 °C. The insoluble product was then dissolved by addition of 200 μL of DMSO (Sigma-Aldrich). Absorbances corresponding to the solubilized formazan pellet (which reflects the relative viable cell number) were measured at 570 nm using a Labsystems Multiskan MS microplate reader. Concentration−response curves were constructed, and IC_{50} values (the concentration of the compound inhibiting 50% of cell proliferation) were determined. All in vitro cell experiments (viability, migration, and invasion assays) were carried out at 37 °C in a 5% CO_2 incubator.

Cell Migration Assay. The influence of MGX on the migration of A431 cells was investigated as described previously[36] using Boyden invasion chambers with 8 μm pore size filters coated with 100 μL of fibronectin (100 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA), and the chambers were allowed to stand overnight at 4 °C. Then, 3×10^4 untreated or 24 h MGX (at 50 or 5 μM) pretreated A431 cells were added to each insert (upper chamber). A strong chemoattractant (10% FCS) for A431 cells was added to the lower chamber. After 24 h incubation at 37 °C in a 5% CO_2 incubator, nonmigrated cells were removed by scraping and migrated cells were fixed in methanol and stained with hematoxylin. Cells migrating on the lower surface of the filter were counted in 10 fields using a Zeiss microscope. Results were expressed as a percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

Cell Invasion Assay. Cell invasion experiments were performed with Boyden chambers as described above. The inserts were coated with a Matrigel membrane matrix (Falcon, Becton Dickinson Labware, Bedford, MA). The A431 cells (5×10^4) were seeded in the upper well of the Boyden chamber, and 10% FCS was added to the lower chamber. Before seeding in the upper chamber, the cells were pretreated with MGX at 50 or 5 μM for 24 h. After 24 h at 37 °C in a 5% CO_2 incubator, noninvaded cells in the upper chamber were wiped with a cotton swab, and the filters were fixed, stained, and counted. Results were expressed as a percentage relative to controls normalized to 100%. Experiments were performed in triplicate.

Zymography. A431 cells were seeded at a density of 50×10^4/well into six-well tissue culture plates in DMEM/10% FCS. Cells were allowed to adhere for 24 h and then incubated with 12.5 μM MGX. Conditioned media were collected 24, 48, or 72 h after treatment with MGX, normalized to cell number, mixed with nonreducing Laemmli sample buffer, and subjected to 10% SDS-PAGE containing 0.1% (w/v) gelatin. Gels were washed three times at room temperature in a solution containing 2.5% (v/v) Triton X-100 in H_2O and incubated at 37 °C for 24 h in 50 mM Tris/HCL, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.05% Brij 35. Each gel was stained for 60 min with 0.5% (w/v) R-250 Coomassie blue in 30% methanol (v/v)/10% acetic acid (v/v). ProMMP9, MMP9, and ProMMP2 detection proceeded by direct visualization of white zones on the gels, indicating the gelatinolytic activity of proteinases. Gelatinase activity was quantified using the NIH Image program.

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Supporting Information Available: Schemes for the extraction of xylans from *Castanea sativa* and *Argania spinosa*. This information is available free of charge via the Internet, at http://pubs.acs.org.

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