British Journal of Pharmacology and Toxicology 5(6): 186-193, 2014
ISSN: 2044-2459; e-ISSN: 2044-2467

Submitted: August 13, 2014 Accepted: September 14, 2014 Published: December 20, 2014

Analysis of Toxicity and Hemostatic Properties of Polysaccharides from Plants Endemic to Gabon

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Abstract: Plants are commonly used throughout the world, most particularly in Africa for different purposes including use as drugs, food and cosmetics. Polysaccharides were extracted from the cell walls of seven plants endemic to Gabon, *Uvaria klainei*, *Petersianthus macrocarpus*, *Trichoscypha addonii*, *Aphanocalyx microphyllus*, *Librevillea kleaneana*, *Neochevalierodendron stephanii* and *Scorodophloeus zenkeri*. Pectins and hemicelluloses from these plants were extracted using ammonium oxalate and potassium hydroxide and their sugar compositions were determined using gas chromatography. These fractions were used to test their toxicity compared to standard products such as mercuric chloride ($HgCl_2$), 4-Nitro-quinoloine (4-NOQ), 2-Amino-Anthracene (2AA) and ivermectin. Their anticoagulant capacity was tested by measuring Activated Partial Thromboplastin Time (APTT), prothrombin time (PT/QT) compared to snake venom and the heparin response. The results showed that these extracts had no cytotoxicity since the IC_{50} of polysaccharide extracts and ivermectin varied from 456.62 to 892.42 µg/mL compared to standard toxic HgCl, 4-NOQ, 2AA: 1.8-22 µg/mL. No genotoxicity or pro-genotoxicity was found: the SOS test Inhibiting Factor (IF) from pectins and hemicelluloses varied from 0.4 to 1.2 compared to the standard genotoxic 4-NOQ, with an IF of 9.33. Acute toxicity was not observed, as indicated by the minimum inhibition at 20% (MIC_{20}) that varied from 25.45 to 65.85 for polysaccharides, while the standard toxic level was at a MIC_{20} of 0.0284. However, *in vitro* anticoagulant activities were detected. De-esterification and enzyme degradation of the homogalacturonan pectin chains did not change the hemostatic trend of pectic fractions.

Keywords: Gabon, hemicelluloses, hemostatic, plants, pectins, toxicity

INTRODUCTION

The use of plants and their derivatives is a common practice throughout the world. Despite their widespread use in traditional medicine, cosmetics and as a food source, the toxicity of plants is not well known and even less so for plant polysaccharides. In Gabon where the practice of traditional medicine based on plants is popular, there are no reports on the secondary effects of plant usage. A compound is designated as toxic when by contact or penetration of body it can cause dysfunction in cells or the organism characterized by modifications at the cellular level (cytotoxicity), the DNA level (genotoxicity), or immediate dysfunction (acute toxicity). The toxic properties of plants can forbid their use. For example, *Ambrosia maritime* appears to be toxic against molluscs (Alard *et al*., 1991). Toxic properties have been reported for an alimentary plant for sheep (Mihalka, 1982). Also, indigo has been widely use as dye and a drug in Morocco for several decades; however, its administration as an infusion of the *Indigofera* leaves as gastroenteritis treatment in children was fatal (Labib *et al*., 2012). All these examples show the necessity of evaluating the toxic potential of plants used by humans.

Hemostasis is the process that keeps the blood liquid. Excessive blood clots can induce thrombosis and fatal embolism (Pawlaczy *et al.*, 2009); in contrast, insufficiency in this process can lead to excessive or spontaneous bleeding (gastrointestinal, nasal, etc.) such as hemophilia (Pawlaczy *et al*., 2009). To prevent this risk, anticoagulants are sometimes necessary.

The anticoagulant activity of pectins and hemicelluloses isolated from higher plants has been demonstrated (Côte and Hahn, 1994). Polysaccharides isolated from *Paeonia suffructicosa* have presented antithrombin and thrombolytic activity (Liapina *et al*., 1997). Polyphenolic-polysaccharide macromolecules without sulfate groups, but with hexuronic acid were isolated from *Erigeron Canadensis L*. and have

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presented anticoagulant activity on both pathways (Pawlaczyk *et al*., 2011). In addition,anticoagulant activity has been demonstrated in polysaccharide acids isolated from *Porana volubilis* (Yoon *et al*., 2002) by a sulfated fucane isolated from *Echiniderm L. variagatus* (Pawlaczyk *et al*., 2009; 2011) and sulfated galactan from *Echiniderm E. lucunter* (Mourão and Periera, 1999; Pawlaczyk *et al*., 2011). Heparin is a sulfated polysaccharide acting as an anticoagulant on antithrombin III (Yang *et al*., 2012) and its cofactor heparin II acts on inactive thrombin and serine protease (Fan *et al*., 2012; Yang *et al*., 2012). Another study has demonstrated anticoagulant activity from polysaccharides isolated from higher plants due to the presence of hexuronic acid residues, such as GlcA and GalA and their derivatives (Yoon *et al*., 2002; Pawlaczyk *et al*., 2009). The higher plants cell wall components such as hemicelluloses and pectins are the polysaccharides containing hexuronic acids (Côte and Hahn, 1994; Pawlaczyk *et al*., 2009). In contrast, snake venom may actually shorten the coagulation process. Snake bites are an important public health problem: it is estimated that there are 5 million snake bites per year and 125,000 deaths per year, essentially in tropical countries (Chippaux, 2002; Larreéché *et al*., 2008). This study was undertaken to search for potential anticoagulant molecules that could be used safely in humans.

MATERIALS AND METHODS

Plant samples: Plant samples were collected in Gabon from the Estuaire and Ngounie regions. Identification of voucher specimens was confirmed by the National Herbarium of Gabon (HNG). These plants were: *Uvaria klainei* (Annonaceae), *Petersianthus macrocarpus* (Lecythidaceae), *Trychoscyphaaddonii* (Anacardiaceae) and four Fabaceae, *Aphanocalyx microphyllus*, *Librevilleakleaneana*, *Neochevalierodendron stephanii* and *Scorodophloeus zenkeri*.

Reagents: Oxalate ammonium, sodium borohydride, fetal bovine serum, penicillin/streptomycin, trifluoroacetic acid (TFA), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) kit, a Toxi-Chromotest kit (EBPI, Ontario, Canada), an SOS-Chromotest kit (EBPI, Ontario, Canada) and heparin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Endopolygalacturonase (EPG) from *Aspergillus niger*, alpha-amylase and ando-β(1,4)-xylanase were purchased from Megazyme International Ireland (Wicklow, Ireland). Other reagents were obtained from chemical and analytical reagents. Acidic pectin (DM0) and methylesterified pectin (DM85) were purchased from Sigma.

Extraction and characterization of cell wall: Powder of different organs (stems, leaves, or bark) of the plants studied were washed with solvents (water, methanol, ethanol 70%). These samples were then incubated in hot ethanol 70% for 1 h. The alcohol-insoluble residues mainly composed of cell wall polymers were submitted to sequential chemical extraction as previously described (Mengome *et al*., 2014). Briefly, the residues were boiled in 0.05% ammonium oxalate for 1 h. The soluble extracts called Oxa were separated by centrifugation, dialyzed against water and freeze-dried. The pellets were then incubated in potassium hydroxyl 1M (fraction K1) and 4M (fraction K4) containing 20 mM $NaBH_4$. The monosaccharide composition of these fractions was determined using gas phase chromatography analysis of the trimethylsilyl methylglycoside derivatives according to Ray *et al*. (2004). The pectic fraction was demethylesterified (Oxa+NaOH) in 0.1 M NaOH overnight and digested with endopolygalacturonase (EPG).

Cytotoxicity: $LLCMK_2$ cells (Vero) were thawed after cryopreservation in liquid nitrogen and then cultured in 6 mL of Eagles Minimum Essential Medium (MEM) supplemented with antibiotic (100 U/mL) and antifungal (100 µg/mL) 10% fetal bovine serum. These plates were incubated at 37°C with 5% CO_2 for 3days. Confluent cells were collected by trypsinization according the method proposed by our laboratory and subjected to the cytotoxicity assays as described below.

The colorimetric method described by Dubois *et al*. (1956) was used with several modifications. Briefly, 100 µL of cell suspension $LLCMK_2$ (Vero) was distributed in 96-well plate wells as a suspension of 5.10^5 cells/mL in a MEM medium containing 10% fetal bovine serum and (100 U/mL) penicillin and streptomycin (100 µg/mL). The plates were then incubated in an incubator at 37°C with 5% CO_2 atmosphere for 24 h before adding 100 µL of different polysaccharide extracts at 25-500 µg/mL concentrations and controls: Ivermectin, $HgCl_2$ at 4 µg/mL, 2-amino-anthracene (2AA) at 100 µg/mL and 4-nitro-quinoloine (4NOQ) at 50 µg/mL. Plates were again left to incubate for 4 days. At this stage, 20 µL of MTT was added to each well and left to incubate for 4 h in the same conditions. Then 50 µL of isopropanol was added and mixed with the cells. The plates were then passed through a spectrophotometer at 570 nm and the optical density (OD) of each well recorded. The percentage of proliferation was calculated as % = (OD sample-OD control) ×100, where the OD sample is the sample obtained 5 days after stimulation by polysaccharides and the control OD was that obtained with unstimulated cells after 5 days in culture. The color of the mixture changed from yellow to purplish blue. The intensity of the color was proportional to the number of living cells present in the test as well as their metabolic activity (Gosland *et al*., 1989). The inhibitory concentration (IC_{50}) was determined from GraphPad Prism 6 analysis

by the regression from the percentage of proliferation cells.

Acute toxicity: Acute toxicity was measured using the prokaryotic cell *Escherichia coli* PQ37 according to the procedure described by the manufacturer of the Toxic Chromotest (EBPI, Canada). The bacteria grow in presence of chromogenic substrate and 100 µL of different polysaccharides test and control ($HgCl_2$) chromogenic substrate hydrolysis while adding a toxic product that can interfere with the process and therefore the synthesis of the enzyme and the development of a blue color reaction. OD was measured at 615 nm. Mercury chloride ($HgCl_2$) (4 mg/mL) was used as the standard positive control. Acute toxicity was assessed by calculating the minimum inhibitory concentration (MIC_{20}) causing 20% toxicity. MIC_{20} is calculated using the following formula:

$$MIC_{20} = (OD\text{-}1 \text{ test/control OD}) \times 100$$

Analysis of genotoxicity and pro-genotoxicity: The SOS Chromotest (EBPI) was used following the manufacturer's suggested procedure. The test used *E. coli* PQ37 cells according to Quillardet *et al*. (1982). Briefly, the test principle is based on the bacterial strain *E.coli* PQ37, which was submitted to several changes; the outer membrane was modified to increase the permeability of several types of product (Quillardet *et al*., 1982). It consists in determining the ability of a chemical to induce DNA damage to be quantified indirectly by measuring the expression of a component of the SOS repair system, the *sfiA* gene. In this bacterial strain, the *lacZ* gene, responsible for the synthesis of β-galactosidase, was under the control of the promoter *sfiA*. Thus, when the bacterial DNA is damaged by genotoxicity, the SOS repair system is activated, which leads to the induction of the *lacZ* gene and the synthesis of the β-galactosidase enzyme whose activity is quantified by colorimetric assay (appearance of a blue-green color). The activity of β-galactosidase is compared to the activity of alkaline phosphatase (PAL) measured at the same time, as an internal standard since it is not inducible by genotoxic agents. We used 100 µL of polysaccharides in this experiment. Pro-genotoxicity was measured by adding S9, allowing S9 metabolic activation in the case of pro-genotoxicity. Quinoline 4-nitro oxide (4-NOQ) at 10 mg/mL was used as a positive control. For the assay using the S9 activation, 2-amino-anthracene (2AA) 1 mg/mL was added as a control. The genotoxic activity of a sample is determined by the induction factor (IF), which is calculated by the Rc/Ro ratio; with Ro = OD β-galactosidase/OD PAL and Ro= OD β-galactosidase negative control/OD PAL negative control. Samples are considered as genotoxic when their IF is greater than or equal to 2, moderately genotoxic when $1.5 \leq IF < 2$ and non genotoxic if $IF < 1.5$.

Hemostatic activity: Plasma of a human volunteer donor was used for examination of the intrinsic (Quick Time (QT), Prothrombin Time (PT)) and extrinsic pathways (Activated Partial Thromboplastin Time, APTT) of coagulation.

Plasma sample: The plasma of a human volunteer donor was obtained by centrifugation of citrated whole blood collected by free venipuncture. One hundred microliters of this plasma was placed in tubes and mixed either with an equal volume of water (control) or different concentrations of the polysaccharides (test samples). Then the procedure continued depending on the pathway to be explored and the test used. Since the result obtained during exploration of hemostasis expressed in seconds may vary from one laboratory to another, we used the more stable International Normal Ratio (INR). The clotting time of plasma was determined by the INR, which is calculated by the ratio between the clotting times obtained for a sample over the clotting time of plasma treated with water.

Exploration of the extrinsic pathway of coagulation: We explored this pathway with a test designed as PT when expressed as a percentage or QT when expressed in seconds. This technique is based on Quick *et al*. (1935). After the preparation of reagents according to the manufacturer's instructions (BioMérieux, France), the clotting time was determined at 37°C in the presence of tissue thromboplastin and calcium. The treated plasma (0.1 mL) was incubated for 2 min at room temperature and 0.2 ml of thromboplastin was added and incubated for 15 min under the same conditions. Time was measured by machine (BioMerieux Vitek System Option 4). Each sample was prepared in duplicate. The reference interval (Clinical Guide to Laboratory Test, 1995) was chosen as follows: Normal QT: usually between 11 and 15 s; PT: between 70 and 100%.

Exploration of the intrinsic pathway of coagulation: The APTT was used as follows: after the preparation of reagents, we recalcified the plasma containing the polysaccharides or control water in presence of a standardized amount of cephalin and an activator of factor XII (kaolin). Kaolin has the double advantage of easy and rapid reading. In this experiment, we preincubated calcium chloride ($CaCl_2$) 0.025M in a water bath at 37°C. This solution was circulated through the tubes according to the manufacturer's instructions (Biomérieux, France) as follows: 0.1 mL of homogenized kaolin was added to an equal volume of treated plasma after incubation for 3 min at 37°C, 0.1 mL of 0.025M $CaCl_2$was added immediately to the machine for timing. The reference interval (Clinical Guide to Laboratory Test, 1995) for normal APTT: 30-40 s depending on the method used. The INR was determined as above for PT.

Ethical considerations: This study obtained clearance from the National Ethical Board of Gabon under the reference PROT No. 0006/2013/SG/CNE.

Statistical analyses: The statistical analyses were determined using GraphPad Prism6 with three replicates of each sample. The results are presented as the mean±SD.

RESULTS

Plant samples: Four classes of endemic plants of Gabon were studied: Magnoliales, Ericales, Sapindales and Fabales. Magnoliale is represented by *Uvaria klainei*, one annonaceae only found in Gabon. Stems (UKS) and leaves (UKL) were used from the specimen. The second class is Ericale, represented by *Petersianthus macrocarpus*, a Lecythidaceae family widespread in tropical Africa (Guinea at Angola and Congo-kinshasa), barks were part used and name used (PMB). In Gabon, this species is found frequently in the north. It is used as an antiseptic, for cicatrizing and to treat lumbago and venereal disease. Sapindale is the third class represented by *Trichoscypha addonii*, one anacardiaceae spread out from the tropical Africa, their barks were usually used from treat dysentery, amenorrhea and name used (TAB). Four Fabaceae-Caesalpinoideae in the Fabales class were represented by *Aphanocalyx microphyllus*, which is found throughout central Africa, barks were part used and name used (AMB); *Librevillea kleaneana* only found in Gabon which treat veneral disease, barks were part used and name used (LKB); *Neochevalierodendron stephanii* found in equatorial forest, used as antibiotic, leaves were part used and name used (NSL); *Scorodophloeus zenkeri*, widespread in Africa, to be of as spice, treat high blood pressure, respiratory disease, barks were part used and name used (SZB).

The polysaccharide characterization: The stems, leaves, or bark of the seven plants endemic to Gabon were isolated and chemically analyzed according to Mengome *et al*. (2014). The samples were heated in 70% ethanol and the resulting insoluble materials were then successively treated with ammonium oxalate and KOH 1M and 4M. The fractions solubilized with ammonium oxalate (called oxa) mainly contained pectic polysaccharides. Their sugar compositions indicated that galacturonic acid (GalUA), rhamnose (Rha), galactose (Gal) and arabinose (Ara) are the main constitutive monosaccharides. As a consequence, these fractions contain homogalacturonan (HG), which is a polymer of repeated units of α(1-4)-D-GalUA that can be methylesterified and acetylesterified and rhamnogalacturonan-I (RG-I), which consists of the repeating disaccharide α (1-4)-D-GalUA-α (1-2)-L-Rha substituted with a wide variety of side chains attached to the rhamnosyl residues, ranging from monomers to large oligosaccharides such as β (1-4)-D-galactan and α (1-5)-L-arabinan. The ratio between GalUA and Rha in pectin extracts varied between 2 and 5, indicating that these fractions contained various proportions of HG and RG-I. To better characterize which part of these pectic polysaccharides were responsible for the observed activities, pectic fractions were either saponified with NaOH to remove methyl and acetylester groups linked to GalUA residues or saponified and then treated with an endopolygalacturonase (EPG) to remove HG chains (Mengome *et al*., 2014). The sugar composition of the resulting enzyme-treated fractions indicated that the GalUA/Rha ratio is about 1, as expected for a pure RG-I fraction (Mengome *et al*., 2014).

Fractions solubilized by KOH 1M (called K1) and 4M (called K4) were mainly composed of xylose (Xyl) residues, indicating that they contained xylan and/or xyloglucans. In a previous study, the main polysaccharides of hemicellulosic fractions isolated from the leaves and stems of *Uvaria klainei* and from the bark of *Petersianthus macrocarpus* and *Aphanocalyx microphyllus* were identified as XXXG-type xyloglucans and β (1, 4)-xylans substituted by 4-*O*-Me GlcUA residues (Mengome *et al*., 2014).

The cytotoxicity of polysaccharides extracts: The cytotoxicity was analyzed by measuring cell viability with Vero cells and Bromure of 3-[4, 5-dimethylthiazol-2yl]-2-5-diphenyltetrazolium (MTT), after contact with different concentrations of polysaccharide extract at 25 to 500 µg/mL. Ivermectin (IVM), a drug used for filarial treatment, was used here as a control (Fig. 1). Thus at 500 µg/mL the concentration of polysaccharide extracts capable of inhibiting 50% cellular activity (IC_{50}) varied between 445 µg/mL and 841µg/ml and the concentration of ivermectin was 892 µg/mL (Table 1). In contrast, control toxic molecule concentrations were 100 times lower (2AA, 4NQO and $HgCl_2$), with IC_{50} varying between 2 and 22 µg/mL.

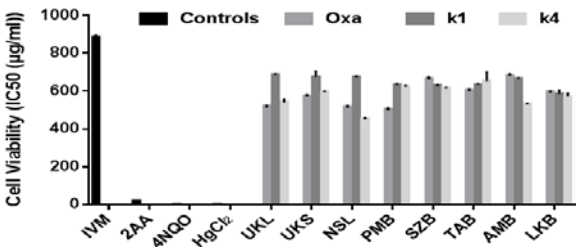

Fig. 1: Cytotoxicity of polysaccharide extracts on Vero cells as measured by MTT assays after contact with plants (UKL, UKS, NSL, PMB, SZB TAB, AMB, LKB), pectic (Oxa) and hemicellulosic (K1 or K4) extracts, ivermectin (IMV), or control toxic molecules (2AA, 4NQO, and $HgCl_2$). The concentration of samples capable of inhibiting 50% cellular activity (IC_{50}) was determined for each molecule.

Table 1: Toxicity of polysaccharides extracts and after treatment of pectin extracts on eukaryotic or prokaryotic cells

Samples		Cytotoxicity IC_{50} (µg/mL)	Acute toxicity MIC_{20} (µg/mL)	IF	
				Genotoxicity	Progenotoxicity (+ S9)
UKS	Oxa	576.84±4.7	47.5±1.0	0.9	1.0
	K1	695.35±24.9	46.05±8.5	1.0	1.0
	K4	598.35±0.8	41.05±4.5	0.7	1.0
	Oxa+PG	-*	33.15±4.5	0.5	1.0
	Oxa+NaOH	-	30.4±0.3	0.6	1.0
UKL	Oxa	520.34±11.0	53.95±7.5	1.2	1.0
	K1	687.99±34.6	32.15±4.5	1.1	1.0
	K4	551.77±24.1	64.85±2.5	0.5	1.0
	Oxa+PG	-	37.15±4.5	0.5	1.0
	Oxa+NaOH	-	27.5±0.2	0.6	1.0
NSL	Oxa	516.29±21.7	-	-	-
	K1	674.88±8.8	45.05±8.5	1.0	1.0
	K4	456.62±6.5	36.3±1.3	0.4	1.0
PMB	Oxa	500.87±7.9	31.2±3.5	0.5	1.0
	K1	637.13±114.2	34.65±0.9	0.5	1.0
	K4	628.73±1.8	65.85±3.5	1.0	1.2
	Oxa+PG	-	36.15±3.5	0.5	1.0
	Oxa+NaOH	-	53.95±2.5	0.6	1.0
SZB	Oxa	668.5±28.8	36.6±1.0	0.5	1.0
	K1	632.16±29.3	47.25±0.3	1.0	1.0
	K4	617.89±3.9	36.15±1.5	1.0	1.0
TAB	Oxa	601.37±27.9	26.35±0.4	0.5	1.0
	K1	637.15±22.4	36.3±0.3	0.9	1.0
	K4	685.81±18.3	37.35±0.3	0.9	0.9
AMB	Oxa	685.81±18.3	36.15±1.5	0.9	1.0
	Oxa+PG	-	33.65±2.0	0.5	1.0
	Oxa+NaOH	-	53.00±1.5	1.4	1.0
	K1	668.09±14.6	25.45±0.2	1.2	1.0
	K4	531.13±6.9	43.1±0.5	0.5	1.0
LKB	Oxa	596.57±25.8	-	-	-
	K1	598.87±6.8	36.35±0.3	0.8	1.0
	K4	558.83±23.2	38.35±0.3	0.6	1.0
Controls	Pectin DM85	-	32.15±0.1	0.8	1.0
	Pectin DM0	-	52.5±9.9	1.2	1.0
	IVM	892.42±186.6	-	0.8	1.0
	2AA	21.82±0.0	-	-	1.0
	4NQO	2.09±0.0	-	9.3	1.0
	$HgCl_2$	1.80±0.1	0.0284±0.0	-	-

(-* = not done)

The acute toxicity of polysaccharides on *Escherichia coli* PQ37: The acute toxicity was evaluated using the Toxi-Chromotest (EBPI), which uses *E. coli* PQ37 cells. Bacterial β-galactosidase activity was measured at 620 nm to determine the viability of the cells after contact with polysaccharides. Mercury chloride ($HgCl_2$) was used here as a positive control. The minimum concentration capable of inducing 20% toxicity (MIC_{20}) was determined for each molecule. The results show that the MIC_{20} of all polysaccharide samples varied between 25.45 and 65.85 µg/mL compared to the MIC_{20} of a toxic molecule ($HgCl_2$), which is 0.0284 µg/mL (Table 1). After treatment of pectin extracts, by either enzyme or saponification, the MIC_{20} of these pectins varied between 27.5 µg/mL and 53.95 µg/mL (Table 1).

The genotoxicity and pro-genotoxicity of polysaccharides on *Escherichia coli* PQ37 as measured by the SOS-Chromotest: Since polysaccharides may be toxic long term, we measured the effect of polysaccharides from a genetic point of view. This effect was measured by the SOS-Chromotest (EBPI). This test is based on the genotoxic or pro-genotoxic effect of polysaccharides on bacterial DNA (*E. coli* PQ37). The result was converted as the inhibition factor (IF), which was compared to that obtained with a patent genotoxic 4-nitro quinoline oxide (4NQO). The samples with an IF greater than 2 were considered genotoxic or pro-genotoxic. However, when analyzing the samples, their IF varied between 0.4 and 1.2, including ivermectin (0.8) compared to the toxic control 4NQO (9.3) (Table 1). Treatments of pectin extracts, by either enzyme or saponification, did not alter their inhibition factor (IF, 0.5 and 1.4, respectively; Table 1).

Hemostatic activity: The hemostatic activity of polysaccharides was evaluated by measuring the PT and APTT. The INR was calculated, which for PT was 1.7, whereas the QT gave 62.2% and the APTT was 2.4. The time of normal plasma with water was compared to the time generated by the following molecules: pectins, hemicelluloses, pectins treated with endopolygalacturonate (Oxa+EPG) or saponified (Oxa+NaOH). Commercially available non methylesterified (DM0) pectins or those exhibiting a

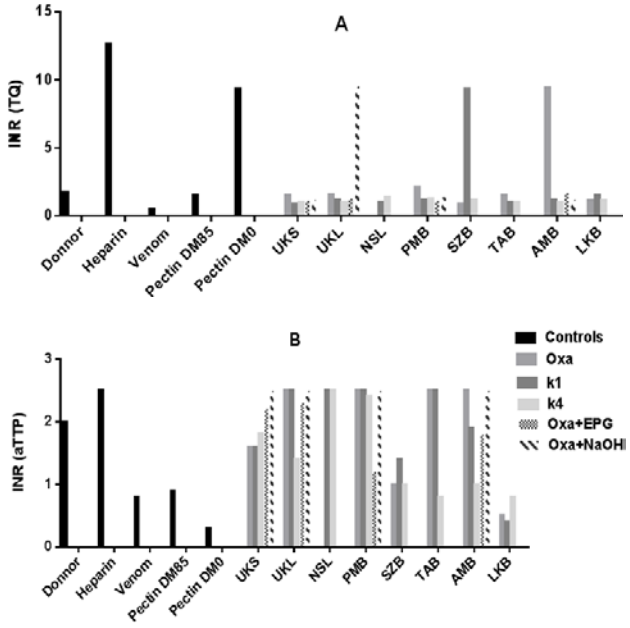

Fig. 2: Effects of plant (UKS, UKL, NSL, PMB, SZB, TAB, AMB, and LKB) pectin (Oxa) and hemicellulose (K1 and K4) extracts compared to different controls (donor, heparin, venom, DM0, DM85) on the extrinsic pathway (A) and intrinsic pathway (B) of coagulation. The different INRs of the PT and APTT tests are plotted against each molecule.

Table 2: Hemostatic activity of polysaccharides and after treatment of pectin extracts as determined by PT (PT/QT) and APTT tests

Pathway extrinsic					Pathway intrinsic	
Ref. extraits		QT (sec)	PT (%)	INR (PT)	APTT (sec)	INR (APTT)
UKS	oxa	34.8±2.9	78.0±42.0	1.5±0.7	194.5±0.7	1.6±1.0
	K1	23.5±3.7	110.5±29.6	0.9±0.3	200.45±3.1	1.6±1.0
	K4	25.2±1.3	104.0±36.0	1.0±0.4	220.55±1.1	1.8±1.1
Oxa+EPG		27.45±1.6	94.3±35.7	1.1±0.4	219.35±40.3	2.2±1.8
Oxa+NaOH		29.85±3.6	89.3±30.7	1.2±0.4	300±0.0	2.5±1.5
UKL	oxa	35.4±5.5	83.8±46.2	1.6±0.9	300±0.0	2.5±1.5
	K1	29.15±3.6	91.4±28.6	1.2±0.4	300±0.0	2.5±1.5
	K4	23.4±2.8	104.3±45.7	1.0±0.5	146.25±17.6	1.4±1.0
Oxa+EPG		29.05±5.9	91.9±48.1	1.3±0.8	232.3±33.9	2.3±1.7
Oxa+NaOH		171.5±128.5	55.0±44.9	9.5±8.5	300±0.0	2.5±1.5
NSL	oxa	-*	-	-	-	-
	K1	25.35±2.3	99.0±31.0	1.0±0.4	300±0.0	2.5±1.5
	K4	35.25±3.3	79.2±30.8	1.4±0.5	300±0.0	2.5±1.5
PMB	oxa	57.4±18.0	44.5±6.9	2.1±0.3	300±0.0	2.5±1.5
	K1	29.8±4.6	91.4±28.6	1.2±0.3	300±0.0	2.5±1.5
	K4	34.55±5.8	77.4±22.6	1.3±0.4	272.4±13.8	2.4±1.6
Oxa+EPG		27±4.4	99.0±31.0	1.1±0.3	200.25±49.9	1.2±0.2
Oxa+NaOH		29.8±4.6	104.0±36.0	1.0±0.4	176.65±61.7	2.1±1.9
SZB	oxa	28.55±8.2	111.9±38.1	0.9±0.4	120.2±3.4	1.0±0.5
	K1	35.1±5.3	75.1±64.9	9.3±8.7	167.55±1.6	1.4±0.9
	K4	22.85±0.9	106.9±33.1	1.2±0.6	114.85±1.5	1.0±0.6
TAB	oxa	23.85±1.6	106.9±33.1	1.5±0.1	300±0.0	2.5±1.5
	K1	42.7±3.3	71.6±38.4	1.0±0.3	300±0.0	2.5±1.5
	K4	26.95±0.4	99.3±40.8	1.0±0.3	109.35±1.2	0.9±0.5
AMB	oxa	26.05±3.0	99.0±31.1	9.4±8.5	300±0.0	2.5±1.4
	K1	28.7±2.3	94.3±35.8	1.2±0.4	251.1±24.5	1.9±0.9
	K4	24.55±6.7	109.7±20.3	1.0±0.4	122.85±7.6	1.0±0.5
Oxa+EPG		164.25±135.8	35.4±19.3	1.7±0.3	244±28.0	1.8±0.8
Oxa+NaOH		28.45±0.1	92.4±37.7	1.2±0.1	300±0.0	2.5±1.5
LKB	oxa	25.8±1.4	99.3±40.8	1.1±0.5	59.85±0.4	0.5±0.3
	K1	38.85±7.2	68.9±20..5	1.5±0.4	70.3±19.1	0.4±0.0
	K4	25.8±0.3	99.3±40.8	1.1±0.5	103.45±3.8	0.8±0.5
Heparin		300±0.0	13.2±2.9	12.6±5.4	300±0.0	2.5±1.4
Venum		8.2±0.1	150	0.5±0.1	33	0.8±0.1
Pectin DM85		40.95±10.4	66.2±14.8	1.5±0.3	103.3±0.3	0.9±0.5
Pectin DM0		163.5±136.5	75.1±64.9	9.3±8.7	48.75±11.8	0.3±0.0
Donnor + water		29.35±12.7	62.2±18.7	1.7±0.5	187.3±56.6	2.4±1.1

(-* = not done)

high degree of methylesterification (DM85) as well as snake venom were tested for comparison (Fig. 2).

Polysaccharide activity on the extrinsic pathway*:* When the effect of polysaccharides on the intrinsic pathway was evaluated using PT, only three extracts (Fig. 2A) out of the 24 tested induced a long PT compared to the normal (1.7) and heparin (12.6) values: PMBOxa (2.1), AMBOxa (9.4) and the hemicellulose extract SZB K1 (9.3). The other 21 extracts tended to shorten the time, such as UKSK1 and SZB Oxa (0.9) approaching the time obtained with snake venom (0.5), while nineteen others varied between 1 and 1.5 compared to normal (1.7) (Fig. 2A, Table 2).

Polysaccharide activity on the intrinsic pathway: The influence of polysaccharides on the intrinsic pathway was evaluated using the APTT test. Nine polysaccharide fractions out of the 24 tested induced an increase of the APTT with and INR value of 2.5 compared to snake venom (0.8), heparin (2.5) and normal (2.4) values. These were four pectic extracts (UKLOxa, PMBOxa, AMBOxa and TA Oxa) and five hemicellulose extracts (UKLK1, NSLK1, PMBK1, TABK1 and NSL K4). Other extracts tended to shorten the APTT: three pectic extracts (LKBOxa, 0.5; SZBOxa, 1; UKSOxa, 1.6) and 12 hemicellulose extracts UKSK1, 1.6; SZBK1, 1.4; AMB1, 1.9; LKBK1, 0.4; UKSK4, 1.8; UKLK4, 1.4; SZBK4, 1; and LKBK4, 0.8 (Fig. 2B, Table 2).

Impact of chemical and enzymatic treatment of polysaccharides on their hemostatic activity: Pectin extracts were submitted to saponification with NaOH and digestion with endopolygalacturonase (EPG). Only the extract of *Uvaria* leaves increased the coagulation time on both pathways after saponification. Four pectic extracts increased the APTT at 2.5 compared to snake venom (0.8). With the commercial pectins, the non methylesterified pectin (DM0) exhibited an increased PT compared to the methylesterified pectin (DM85) (ratio, 10:2). It was also shown that the APTT was shortened indifferently in both the methylesterified (0.9) and non methylesterified pectins (0.3) compared to the plasma (2.4) and heparin (2.5) controls.

DISCUSSION

Pectins and hemicelluloses were extracted from seven plants endemic to Gabon. The toxicity and hemostatic properties of these components were analyzed given their importance in human health: 6-7% of all hospitalizations and 100,000 deaths annually in the US have been shown to be due to toxicity (Eichelbaum *et al*., 2006; Edling *et al*., 2009). The numbers of reported serious adverse drug effects during recent years (1998-2005) have increased (Moore *et al*., 2007; Edling *et al*., 2009). There are few studies investigating the toxicity of plant extracts in general, most particularly their genotoxic aspect. It is commonly accepted that their long-term use proves the lack of toxicity. This study has investigated cytotoxicity, acute toxicity, genotoxicity and pro-genotoxicity of the polysaccharide extracts of several plants endemic to Gabon.

Cytotoxicity was analyzed with the MTT assay, a widely used test to measure cell proliferation, cell viability and drug toxicity. The study demonstrated that polysaccharides are not toxic. In fact, the evaluation of cytotoxicity using the IC_{50} showed that all samples had IC_{50} 100 times greater than toxic molecules ($HgCl_2$, 4NQO and 2AA) and within the same range as the IC_{50} generated by ivermectin, a drug currently in use for parasite treatment. Similarly, the *in vitro* evaluation showed that the dose of polysaccharides capable of generating acute toxicity is 1000 times higher than the dose of $HgCl_2$, a toxic molecule. Finally, none of the polysaccharides was capable of inducing a mutagenic effect on *E. coli* PQ37, as shown by the Inhibition Factor (IF) less than 1.5. This lack of toxicity warrants further study on the hemostatic properties of these polysaccharides. These analyses revealed diverse trends from the shortened coagulation times to increased time. These results suggest anticoagulant activity in these polysaccharides that can possibly be used in humans. In the present study, we observed that treatment of polysaccharides using either enzymes or saponification did not always abolish the anticoagulant properties, suggesting that the site of this activity is the polysaccharide backbone. Today heparin is widely used as an anticoagulant in the treatment of thrombolytic diseases, but it unfortunately induces severe side effects over the long term (Freedman, 1992; Johnell *et al*., 2002; Pawlaczyk *et al*., 2009). This molecule is extracted from mammalian cells with the risk of contamination by different pathogens, hence the value of searching for new anticoagulants in polysaccharides whose advantages include bioavailability and biodegradability and can potentially be used as snake bite treatment. It should be noted that a few polysaccharides considerably reduced the coagulation time compared to snake venom. This study shows the lack of toxicity of polysaccharides from these endemic plants and suggests that their hemostatic activity is due to the polysaccharide backbone.

ACKNOWLEDGMENT

The authors extend their thanks to The University of Rouen, France, for training, investigation in this study and access to technical platforms and to Muriel Bardor for recording the of MALDI-TOF mass spectra. Thanks are also due to the Centre International de Recherches Médicale de Franceville (CIRMF),

sponsored by the government of Gabon, Total Gabon and the Ministère Français des Affaires Etrangères for biology test platforms. We also acknowledge Raymonde Mboma and Mr. Raoul Niangadouma for botanical identification and collection of the plants used in the study.

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