1. Polysaccharides from the South African medicinal plant

2. *Artemisia afra*: Structure and activity studies.

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11. Abstract

12. *Artemisia afra* (*Jacq. Ex. Willd*), is an indigenous plant in South Africa and other parts of the

13. African continent, where it is used as traditional medicine mostly for respiratory

14. conditions. The objective of this study was to investigate the structural features of the

15. polysaccharides from the leaves of this plant, as well as the biological activities of the

16. polysaccharide fractions against the complement assay. Leaves of *Artemisia afra* were

17. extracted sequentially with organic solvents (dichloromethane and methanol), 50%

18. aqueous ethanol, and water at 50 and 100 °C respectively. The polysaccharide extracts

19. were fractionated by ion exchange chromatography and the resulting fractions were

20. tested for biological activity against the complement fixation assay. Active fractions were

21. further fractionated using gel filtration. Monosaccharide compositions and linkage

analyses were determined for the relevant fractions. Polysaccharides were shown to be of the pectin type, and largely contain arabinogalactan, rhamnogalacturonan and homogalacturonan structural features. The presence of arabinogalactan type II features as suggested by methylation analysis was further confirmed by the ready precipitation of the relevant polysaccharides with the Yariv reagent. An unusual feature of some of these polysaccharides was the presence of relatively high levels of xylose as one of their monosaccharide constituents. Purified polysaccharide fractions were shown to possess higher biological activity than the selected standard in the complement assay. Digestion of these polysaccharides with an endo-polygalacturonanase enzyme resulted in polymers with lower molecular weights as expected, but still with biological activity which exceeded that of the standard. Thus on the basis of these studies it may be suggested that immunomodulating properties probably contribute significantly to the health-promoting effects of this medicinal plant.

1. Introduction

Artemisia afra is a plant which is used traditionally in South Africa against a variety of indications. Some of these include internal worm infestation, cough, cold, fever, colic, headache, loss of appetite, pain in the ear and malaria (Liu et al, 2009). The most common way to use the plant is to inhale the steam after boiling the leaves in water, although it may also be taken as a tea infusion (van Wyk et al. 2002). Other uses include putting the leaves in the socks to prevent sweating; they are also used against hemorrhoids, diabetes, digestive problems, on wounds and as insecticide (von Koenen, 2001). *Artemisia afra* is one of the most used medicinal plants in South Africa, and other African countries as well; the plant has been the object of various studies over the recent years, although focus has mainly been on essential oils and low molecular weight compounds, mostly for their

association with antimicrobial and antioxidant activities. As one of those multi-purpose medicinal plants, it has also been investigated for potential cardioprotective and antidiabetic cytotoxic properties, for which results were shown to be positive in animal experiments (Guantai and Addae-Mena, 1999; Issa and Bule, 2015; Sunmonu and Afolayan, 2013). Cytotoxicity studies of an ethanol extract against HeLa cancer cells followed by a bio-assay guided fractionation led to the isolation of a sesquiterpene lactone, known as isoalantolactone, as the main bioactive compound (Spies et al., 2013; Venables et al, 2016). The plant has also been shown to contain products that indicate it to have efficacy against experimental tuberculosis (Ntutela et al. 2009). Since water extraction is one of the most common ways of preparation of products used from the plant, it is interesting that little has been done on the compounds soluble only in water, among these, the polysaccharides. Recent studies by various groups have shown that polysaccharides may play an important role in illnesses related to the immune system. (Paulsen and Barsett, 2005; Wangensteen et al, 2015; Yamada and Kiyohara, 2007). The authors have also recently described the immunomodulating activity of pectic polysaccharides from the South African medicinal plant *Lessertia frutescens*, (syn. *Sutherlandia frutescens*), (Zhang et al. 2014). Thus it was of interest for us to study the polysaccharides present in the above-ground parts of *Artemisia afra* and also investigate possible effects in immunomodulating assays.

2. Material and methods

2.1 Plant material

The plant material, *Artemisia afra* Jacq. Ex Willd., Compositae, was collected from the botanical gardens of the Montague Museum (Western Cape, South Africa) and consists of the stalks and leaves of the plant; its identity was authenticated by Frans Weitz, a plant

systematist from the Department of Biodiversity and Conservation Biology, University of the Western Cape, South Africa. (Voucher specimen number 6639 which is deposited in the herbarium at the University of the Western Cape, Cape Town, South Africa.)

2.2 Preparation of the polysaccharide fractions.

Prior to the extraction of the polysaccharides, the plant material was extracted with organic solvents to remove lipophilic and low molecular weight substances. This was performed in a Soxhlet apparatus, successively with dichloromethane and methanol. The residue was then subjected to extraction with 50% ethanol in water, followed by extraction with water at 50^{o} C and finally water of 100^{o} C. The 50% ethanol extract was first evaporated to remove all ethanol present, and then filtered to remove material not soluble in water. This extract was then treated in the same way as the two water extracts described below. All fractions were subjected to ultrafiltration using a PALL suspended screen channel Ultrasett with cut off 5 kD. The high molecular weight fractions were then applied onto an anion exchange column, ANX Sepharose 4 Fast Flow, 5x20 cm, in chloride form. The neutral polysaccharides were eluted with distilled water, and the acidic ones with a NaCl- gradient, 0-1.5 M NaCl. The eluent flow was 2 ml/min. Fractions of 10 ml were collected in a Pharmacia LKB SuperFrac fraction collector, and they were monitored for the content of carbohydrate by the phenol-sulfuric acid method (Dubois et al., 1956). The fractions were pooled, dialyzed using a cut-off of 3500D, against distilled water in order to remove NaCl and then freeze-dried. All fractions were tested for bioactivity using the complement assay described below. The fractions that proved to be bioactive were all further fractionated by gel filtration using a Sephacryl S-200 matrix, Pharmacia Fine Chemicals, 3x70cm. The eluent was 0.3 M sodium acetate; elution speed 1 ml/min and fractions of 5 ml were collected and tested with the phenol-sulfuric acid method as

described above. Relevant fractions were pooled, dialysed against a cut off of 3500D, freeze-dried and analysed for bioactivity using the complement assay. All fractions obtained after gel filtration were objects for more detailed structural and bioactivity studies.

2.3. Determination of the monosaccharide composition

The monosaccharide compositions of the all extracts and purified fractions were determined by gas chromatography of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained after methanolysis with 3 M hydrochloric acid in anhydrous methanol for 24 h at 80°C (Austarheim et al., 2012; Barsett et al. 1992; Chambers and Clamp, 1971). Mannitol was used as an internal standard. The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy).

2.4. Molecular weight determination

The homogeneity and molecular weights of the purified polysaccharide fractions were determined by size exclusion chromatography on a Superose 6 prepacked column (Amersham Biosciences) combined with the Äkta system (FPLC, Pharmacia Äkta, Amersham Pharmacia Biotech). Dextran polymers (Pharmacia) B512 (5.6 kDa), T8360 (19 kDa), T250 (233 kDa) and T500 (475 kDa) were used as calibration standards. Approximately 5 mg of the samples were dissolved in 2 mL of 10 mM NaCl buffer, the resulting solution was filtered through a Millipore filter (0.45 μm) and applied to the column. The samples were eluted with 0.3 M sodium acetate at pH 5.2 at a flow rate of 0.5 mL/min, collecting 2 mL fractions. The eluate was monitored with a Shimadzu RI detector. The retention volume of the main peak of each sample was converted to

molecular weight based on a standard curve prepared on the basis of the standards given above.

2.5. Precipitation with the Yariv β-glucosyl reagent

Precipitation with the Yariv β-glucosyl reagent was performed on the samples as described by van Holst and Clarke (1985). The Yariv β-glucosyl reagent forms a colored precipitate with compounds containing certain types of AG-II structures. A solution of Arabic gum in water (1 mg/mL) was used as a positive control.

2.6. Determination of glycosidic linkages

Glycosidic linkage elucidation was performed by methylation studies. The fractions most active in the complement assay from each extract were selected for linkage determination. Prior to methylation, the uronic acids were reduced with $NaBD_4$ to their corresponding neutral sugars. After reduction of the polymers, methylation, hydrolysis, reduction and acetylation (Kim and Carpita, 1992) were carried out. The derivatives were analyzed by GC-MS using a GCMS-QP2010 (Shimadzu) attached to a Restek Rxi-5MS capillary column (30 m; 0.25 mm i.d.; 0.25 µm film). The injector temperature was 280 °C, the ion source temperature 200 °C and the interface temperature 300 °C. The column temperature was 80 °C when injected, then increased at 10 °C/min to 140 °C, followed by 4 °C/min to 210 °C and then 20 °C/min to 300 °C. Helium was the carrier gas (pressure control: 80 kPa.) The compounds represented by each peak were characterized by their retention times and interpretation of their characteristic mass

spectra. The estimation of the relative amounts of each linkage type was related to the total amount of each monosaccharide type as determined by methanolysis. Effective carbon-response factors were applied for quantification (Sweet et al., 1975).

2.7. Complement fixation assay

The complement fixation test is based on inhibition of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera as described by Michaelsen et al. (2000) (Method A), using PMII , a polysaccharide from *Plantago major*, as the positive control. Inhibition of lysis induced by the test samples was calculated by the formula [($A_{control}$-A_{test})/$A_{control}$] ×100%. From these data, a dose-response curve was created to calculate the concentration of test sample giving 50% inhibition of lysis (ICH_{50}). A low ICH_{50} value means a high complement fixation activity. The activity of all the polysaccharide fractions are given as the ICH_{50} value of the positive control BP-II divided on the ICH_{50} value of the sample.

2.8. Degradation of the samples with endo polygalacturonanase

5 mg polysaccharide was dissolved in 3 mL 0.1 M NaOH for de-esterification. The solution was left for 2 h and neutralized with acetic acid. 10 ml sodium acetate buffer, pH 4.2 and 1 drop of toluene was added followed by 60 µl endo-polygalacturonase M2 (Megazyme). The mixture was then kept at 37^{o} C for 72 h. After boiling to denature the enzyme, the mixture was filtered through a Millipore filter 0.45 µm and the filtrate concentrated to 1 ml prior to application on a PD-10 column. The PD-10 column was

then eluted as described by the manufacturer (Pharmacia), the high molecular weight material freeze dried and subjected to monosaccharide determination and the complement assay.

3. Results and discussion

The dried plant material (406 g) was extracted as described. The fractionation scheme is given in Fig 1 below: -

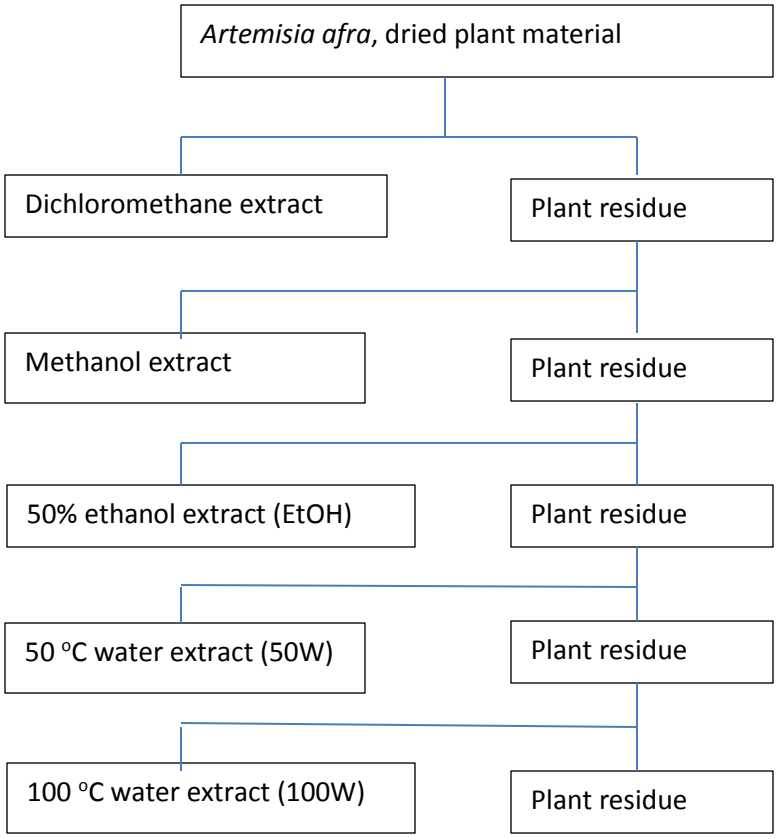

Figure 1: Fractionation scheme of *Artemisia afra.*

The yield obtained, of the fractions showing high activity in the complement assay, after anion exchange chromatography and gel filtration is given in Table 1. The bioactive, purified fractions were denominated as 50%EtOH, 50W, 100W1 and 100W2 and used

167. for further studies.

Original plant material, 406 g	Chromatography methods		
Fractions	Anion exchange	Gel chromatography	% of start material
50% EtOH	807 mg	207 mg	0.05%
50W	243 mg	60 mg	0.014%
100W1	1615 mg	355 mg	0.09%
100W2	1080 mg	712 mg	0.18%

168. Table 1. Yield based on dry plant material of the purified bioactive fractions obtained

169. after separation by anion exchange and gel chromatography

170. Structural analyses of the polysaccharides from *A. afra*

171. Monosaccharide analyses of the four fractions gave results typical for pectic

172. polysaccharides (Table 2).

Fraction	50%EtOH	50%EtOHE	50W	50WE	100W1	100W1E	100W2	100W2E
Monosaccharide								
Arabinose	19.0	15.9	12.6	12.2	10.6	9.5	3.5	4.7
Rhamnose	7.9	8.7	5.6	6.2	11.9	14.2	5.7	9.1
Fucose	0.4	0.2	0.4	0.4	0.6	0.6	0.6	1.2
Xylose	7.8	4.5	15.1	10.5	4.4	4.5	1.3	2.1
Mannose	3.2	3.1	3.1	2.2	4.7	1.3	7.5	2.8
Glucose	1.8	2.8	3.2	2.8	3.8	3.6	9.5	6.6
Glucuronic acid	4.4	4.3	8	5.7	5.7	4.2	4.5	2.2
Galactose	33.4	34.7	26.3	27.3	17.3	16.6	5.8	7.3
Galacturonic acid	22.1	22.9	25.7	30.4	41.0	4.8	61.6	63.2
4-O-Me-GlcA	tr.	2.9	tr.	2.3	tr.	0.7	tr.	0.8

172. Content of monosaccharides in the most bioactive fraction after separation by

173. anion-exchange chromatography and gel filtration, and the same after treatment with

174. endopolygalacturonase (E). All fractions, apart from 100W2 have high contents of

175. arabinose and galactose, indicating the presence of arabinogalactans, polymers

common in pectins as side chains to the main core. Galacturonic acid is present in fairly high amounts, and with the content of rhamnose found, the polymers may contain a main core consisting of a rhamnogalacturonan backbone linked with longer chains of homogalaturonans. The other monosaccharides found are also often present in pectins (Yamada and Kiyohara, 2007). The most striking of those is the relatively high amount of xylose present, which may have an impact on the bioactivity, and this will be discussed below. The molecular weights of these four fractions were 69, 88, 43 and 10 kD respectively. After degradation of the polymers with the enzyme endopolygalacturonase, the most bioactive fractions thus obtained also revealed the presence of small amounts of 4-0-methylglucuronic acid, also frequently found in pectins as terminal units (Yamada and Kiyohara, 2007).

The types of linkages in the four bioactive native fraction obtained were determined based on the methylation experiments (Table 3).

Fraction		50%EtOH	50W	100W1	100W2
Monosaccharide	Linkage type*	2. Ratio of the different linkage types[#]			
Arabinose					
	T*f*	10.1	4.6	5	1.4
	1,3*f*	1.5	0.6	0.6	tr.
	1,5*f*	5.9	4.3	2.8	1.3
	1,3,5*f*	0.5	0.4	2.2	0.8
	1,2,5*f*	1	2.7	n.d.	n.d.
Rhamnose					
	T*p*	2.3	3.4	1.9	3.5
	1,2*p*	tr.	n.d.	5.4	1.2
	1,2,4*p*	5.6	2.2	4.6	1
Xylose					
	T*p*	1.2	3.1	4.4	1.3
	1,4*p*	6.6	12	n.d	n.d.
Glucose					
	T*p*	tr.	tr.	1.1	1.9
	1,4*p*	1.4	3.1	2.5	7.6
	1,4,6*p*	0.4	0.1	0.2	n.d.
Glucuronic acid					
	T*p*	4.4	8	5.7	n.d.
Galactose					
	T*p*	7.1	7.7	6.4	tr.
	1,3*p*	3	2.3	1.9	1.9
	1,6*p*	10.4	8.4	5	n.d.
	1,3,6*p*	11.7	7.1	3.5	2.5
	1,3,4,6*p*	1.2	0.8	0.5	1.4
Galacturonic acid					
	T*p*	n.d.	tr.	n.d.	10.1
	1,4*p*	20.2	24.5	37.3	51.5
	1,3,4*p*	1.4	1.1	2.5	n.d.
	1,2,4*p*	0.5	0.1	1.2	n.d.

190. Table 3. Linkage analyses of the bioactive polysaccharides. The linkage types for the
191. monosaccharides not mentioned were not detected. The linkages for fucose and
192. mannose are not included.
193. *T = non-reducing terminal unit; *f* = furanose; *p* = pyranose
194. [#]tr = trace; n.d. = not determined

195. The fractions were all rich in 1,4-linked galacturonic acid residues, and all except Fr.

100W2 contained significant amounts of galacturonic acid residues, which additionally were branched in either position 2 or 3. These are features which have recently been shown to be present in polysaccharides from other medicinal plants, e.g. *Combretum glutinosum*, where it is believed that xylose units are appended to these branch positions (Glaeserud et al. 2011). It is thus interesting to note that terminal and 1,4-linked xylose units are also found in the polysaccharides from *A. afra*. Rhamnose, often part of the main RG I core as 1,2-linked units, is mainly found in Fr. 100W1 and 100W2, while in all fractions rhamnose units linked both in positions 2 and 4 are present, indicating that the 1,2-linkages are present in the RGI region while concomitantly carrying branches at position 4. These features are also common in RGI units from other plant polysaccharides (Yamada and Kiyohara, 2007).

Galactose normally constitutes the main core in the arabinogalactans I and II. Except in FR. 100W2, the galactose units were mostly 1,6-linked, with substantial branching at position 3, thus providing the important side chains often observed in AGII structures (Van Holst and Clarke, 1985). The arabinose units, in their furanose form, are largely present as non-reducing terminal and 1,5-linked units in the peripheral parts of these side chains.

The Yariv reagent has the ability to precipitate polysaccharides containing arabinogalactan type II. Depending on the length of the galactose chain, a precipitate will form if galactose units contain branches at positions 3 and 6 (Kitazawa et al. 2013; Paulsen et al, 2014, Van Holst and Clarke, 1985).

Since linkage analyses revealed the presence of galactose units which are linked at

positions 3 and 6, the four polysaccharide fractions were tested against the Yariv reagent. Fractions 50% EtOH, 50W and 100W1 precipitated with Yariv reagent, while fraction 100W2 did not, although 1,3,6-linkages were present. The specification of the Yariv reagent for binding to the AGPII containing the 1,3,6-linked galactoses, remained unknown for a long time. However recently, following a systematic investigation by Kitazawa et al. (2013), they concluded that the β-1,3 galactan chain should be at least 5 units long in order to bind with the Yariv reagent, and the presence of 7 units was needed to form cross linkages and thus produce a precipitate. They also concluded that 1,6-linked chains did not bind the Yariv reagent. The results of our studies related to the Yariv test are displayed in Fig 2.

Based on the above theory, it was concluded that samples 50% EtOH, 50W and 100W1 all contain sequences consisting of at least 7 units of β-1,3-linked galactose units in their arabinogalactan chains, while fraction 100W2, although containing 1,3-linked and 1,3,6-linked galactose units, probably had shorter chains of the β-1,3 galactose units, and hence was unable to form a precipitate.

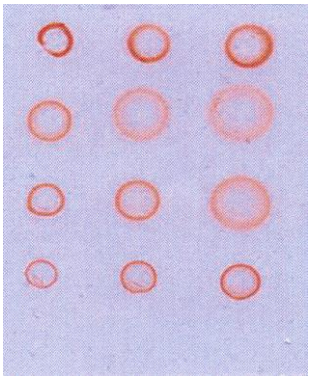

Fig.2 Yariv reagent precipitation of the fractions. Fr 100W2 did not precipitate. (for colour reproduction, both on web and in print)

Polysaccharides from other A*rtemisia* species have also been studied. Yamada et a l (1985, 1986, and 1987) showed that the polysaccharides from the leaves of the Japanese medicinal plant, *Artemisia princeps,* contain immunomodulatory polysaccharides of the pectic type. The polysaccharides were shown to have a typical rhamnogalacturonan type I backbone, in which position 4 of the 1,2 linked rhamnose units were substituted with arabinogalactan type II sidechains, i.e. have galactose being 1,3-; 1,6- and 1,3,6-linked, with arabinofuranose units attached at the peripheral parts of these side chains. Furthermore, the polysaccharides contained 1,4-linked xylose units which were attached to galacturonic acid, in addition to 1,4-linked galactose and terminal glucuronic acid units. Pectic type polysaccharides have been found in other Artemisia spp. *Artemisia absinthium* was recently studied (Correa-Ferreia et al., 2014), and the basic structure was determined to be of the arabinogalactan type II. The arabinose units were present as terminal, 1,2-, 1,5- and 1,3,5-linked units, which is common in arabinogalactan type II polymers (Togola et al., 2008). Methylation analysis of the polymer also provided evidence for the presence of 1,4-linked galacturonic acid residues, and 1,2,4- and 1,3-linked rhamnose units. On the basis of the foregoing discussion, it may be deduced that *Artemisia afra* contains polysaccharides with similar basic structural features as other *Artemisia* species reported to date.

Biological activities of the polysaccharides from *A. afra.*

One of the main methods for in vitro determination of the effect of chemical compounds on the immune system is the complement assay (Michaelsen et al., 2000). The method used in our laboratory is not influenced by possible contamination with lipopolysaccharides. Thus, the effects observed are an indication of an immunomodulating effect, which may be either stimulating or depressing. Following

extraction of the plant material, the effect on the complement assay was the measure by which fractions were selected for further studies. It was found that the biological activities of the selected fractions were higher than those of the standard used (Fig.3), and after purification using gel filtration the activity was still relatively high compared to both the standard and other polysaccharides previously studied in our laboratory thus far.

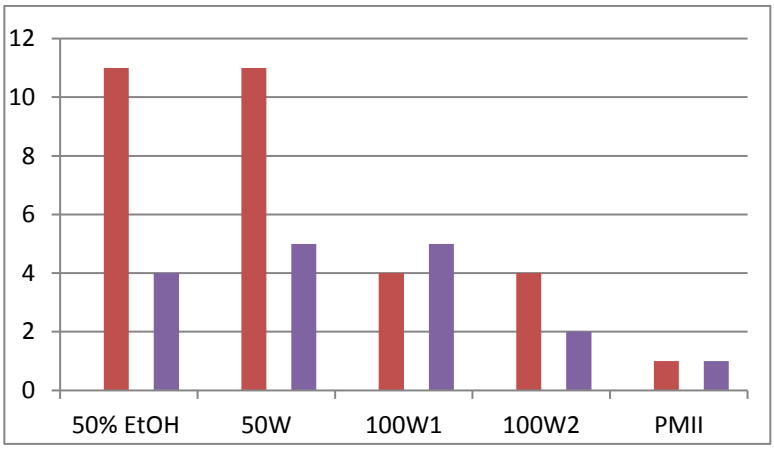

Figure 3. Complement effect of the fractions, ICH50 values, related to ICH50 value of the standard polysaccharide, PMII. The values are presented as ICH50 PMII / ICH50 sample. The red bars are the results after anion exchange chromatography and the blue after purification by gel filtration. (for colour reproduction, both on web and in print)

Fig 3 shows that the fraction 50W and 100W1, after gel filtration were slightly more active than 50%EtOH, and 100W2, and were still more active than the standard used. It is also interesting to note that the EtOH and 50W fractions both lost activity after gel filtration. This shows that it is not only the highest molecular weight compounds that are active, lower molecular weight (but still higher than 3,5kD) water soluble compounds are also of interest to investigate, but these were not the object of the

present study.

The higher molecular weight fractions, being the most active, were also subjected to enzymatic degradation with polygalacturonase, in order to find which part of the molecule were the most responsible for the bioactivity shown for the purified polymers. After the enzyme treatments, the samples were separated by gel filtration and the different fractions tested for effects in the complement assay. Again the effect was compared with the standard PMII and the results are displayed in Figure 4:

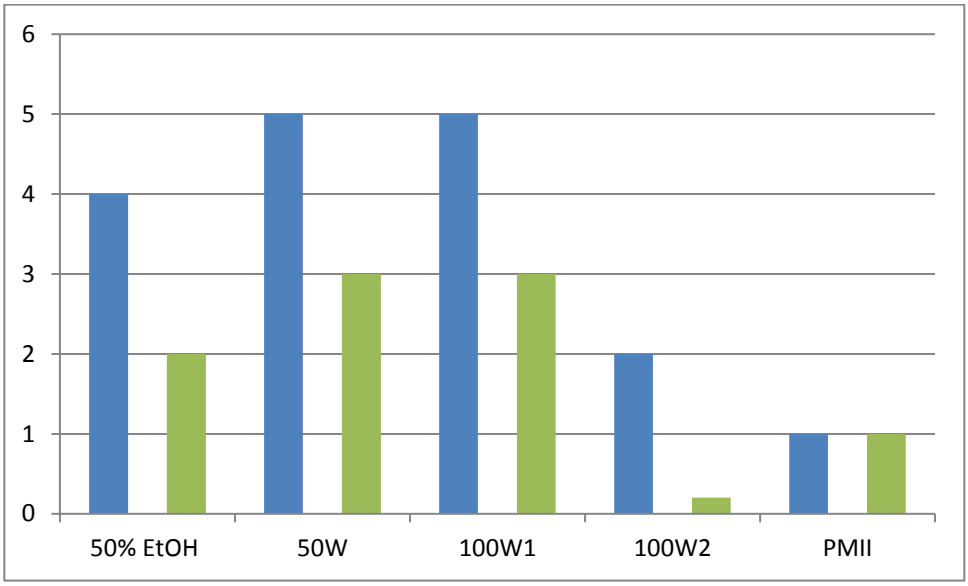

Figure 4. Complement effect of the fractions, ICH50 values, related to ICH50 value of the standard polysaccharide, PMII. The values are presented as ICH50 PMII / ICH50 sample. The blue bars are the results of the samples after gel filtration, and the green those with highest activity after separation of the polygalacturonase degraded samples. (for colour reproduction, both on web and in print)

The activity of fractions from samples 50% EtOH, 50W and 100W1 are still higher

compared to the standard, while the 100W2 is very low in activity (Fig. 4). The monosaccharide composition of the fractions obtained after enzyme degradation is given in Table. 1. There is relatively little change in the ratio between the monosaccharide compositions of the parent polymers compared with the most active fraction after enzyme degradation, but they are lower in molecular weight compared to the native polymers. The most striking difference is that 4-O-methyglucuronic acid is present in all fractions. When comparing the components of the four fractions, it is evident that the fraction 100W2 has a very different overall structure than the other three polymers. This is basically a polygalacturonan and has no branches on the galacturonic acid residues. This sample has also a much lower molecular weight than the other three fractions. Mw may have an influence on the biological activity as obtained in the complement assay. It is also of interest that the three other polysaccharides all contain xylose, and also have branch points on galacturonic acid. It was shown in the polysaccharides from the Malian medicinal tree, *Combretum glutinosum* that the presence of xylose on the galacturonic acid chain leads to higher effect in the complement assay than polysaccharides devoid of many of those substituted galacturonic acid units Glaeserud et al, 2011). The polysaccharides found with high bioactivity from *Lessertia frutescens* did also have the feature of xylose units linked to the galacturonic acid chain (Zhang et al. 2014). The three most active fractions all have a relatively large arabinogalactan type II part of the structure, and this is very small in fraction 100W2. This is evident both from the data in Table 2, linkage analyses, and Figure 3, the Yariv test, where this fraction gave no precipitation. Several other studies have shown that the AGII structure is important for the effect in the complement assay, and the structural requirements have been discussed above (Wangensteen et al, 2015).

It should also be noted that the polysaccharides present in the leaves of *Artemisia princeps* also have strong effects in the complement system (Yamada et al. 1985, 1986) These studies on the polysaccharides from *Artemisia afra* clearly show that the water-soluble fraction of this plant contains biologically active polysaccharides, and that these have an effect that may be of importance in immune modulation.

4 Conclusion

Artemisia afra is a plant with several traditional uses as a medicinal plant in South Africa, and some of these uses may influence the immune system. As the healers mainly use water extracts, the polysaccharides present in the water extract of the aboveground plant part were studied. The studies revealed the presence of pectin like polysaccharides with side chains consisting of arabinogalactan type II structures. Three of the polymers, 50%EtOH, 50W and 100W1 all contained substitution with xylose on parts of the polygalacturonan chain, which is probably important for the bioactivity. The polysaccharides were all tested in the complement assay for immunomodulating properties, and 4 fractions were obtained giving significant effect in the *in vitro* complement assay, thus indicating that the use of this plant as a remedy in traditional African medicine may be substantiated.

5 Acknowledgements.

We are indebted to the South African National Research Foundation for funding the research carried out in these studies.

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