International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 2 (5), pp. 088-093, August, 2013. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Urinary excretion of mannose and mannose related compounds in humans consuming Aloe saponaria pulp

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Accepted 17 June, 2013

The adhesion of *Escherichia coli* to uroepithelium can be altered by the interaction between specific carbohydrate molecules and the receptors on the bacterial surface. Mannose is one of the most potent inhibitors among carbohydrates. *Aloe saponaria*, currently used as a dietary supplement, contains polymannanes as main components. This work was designed to evaluate the mannose oligosaccharide metabolites excretion after oral intake of *Aloe saponaria* pulp in order to estimate its potential utility in preventing urinary infections. Five volunteers received a daily oral intake of *Aloe saponaria* pulp for 7 days. Urine samples were collected at time 0 and on the seventh day and assayed for their mannose contents by Dubois method, TLC, HPLC and ^{1}H-NMR. The results showed that the oral intake of *Aloe Saponaria* fresh pulp produced sugar excretion, composed mainly of mannose and mannose related compounds, suggesting that *Aloe saponaria* pulp could be a potential therapeutic agent for prevention of urinary tract infection.

Key words: *Aloe saponaria,* mannose, urinary excretion, urinary tract infection, anti-adhesion therapy, uropathogenic *Escherichia coli.*

INTRODUCTION

Infections of the lower urinary tract (urethra and bladder) are frequent among women – affecting as many as one in five women at some time during their lifetime (Head, 2008). Urine is normally sterile so, for disease to occur, pathogenic microorganisms must first enter the urethra and adhere to host tissue. Once attached, bacteria are able to proliferate and subsequently cause clinical symptoms of infection. Distinct adhesins located on the cell surface of pathogens mediate attachment to complementary glycoproteins or glycolipids on the host tissue (Zopf et al., 1996; The cranberry institute, 2012). Adhesins are found on the stiff, hair-like submicroscopic structures known as fimbriae (or pili) that form bonds with a host of cell receptor site. In uropathogenic bacteria, these bonds are strong enough to resist the cleansing action of urine flow (Sharon et al., 2002). Most infections are bacterial, the most common etiological agent being gram-negative bacilli. *Escherichia coli* (*E. coli*) accounts for 80 percent of urinary tract infections (UTIs), while other gram-negative bacilli, including *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterobacter aerogenes* contribute somewhat less to incidence. As a consequence, UTIs are most commonly treated with antibiotics during the acute episode. In addition, it is not uncommon to use antibiotics for long-term prophylaxis for individuals with recurrent infections (Head, 2008). However due to side effects resulting from the use of prophylactic antibiotics such as alterations in nasopharyngeal and gastrointestinal flora there is a need to explore new alternatives in the prevention of UTIs (De Leon-Jaén et al., 2009).

D-mannose is a simple sugar that prevents adhesion of certain bacterial strains to uroepithelial cells of the bladder. *In vitro* research has identified a mannose-specific lectin on the surface of adherent strains of *E. coli* and has elucidated the adhesion mechanism (Connell et al., 1996; Ofek et al., 2003). D-mannose is apparently the

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primary bladder cell receptor site for uropathogenic *E. coli*. The first step in adhesion involves the mannose-sensitive binding of FimH (adhesin at the tip of type 1 pili of *E. coli*, for example) to bladder epithelium (Kau et al., 2005; Wellens et al., 2008).

Also, small-molecular weight compounds termed "mannosides" that specifically inhibit the FimH type 1 pilus lectin of uropathogenic *E. coli* were developed. These molecules are bacterial adhesion antagonists based on α-D-mannose glycosides (Schaeffer et al., 1984; Han et al., 2010).

Aloes are succulent plants, xerophytes, members of Asphodelaceae family. Of over 300 Aloe species, *Aloe vera*, *Aloe saponaria* and *Aloe arborecens* are the most widely accepted and used for various medical, cosmetic and nutraceutical purposes (Grindlay et al., 1986; McAnalley, 1988; Yagi et al., 1984; Hamman, 2008). The plant is made of elongated and pointed leaves. Each leaf consists of two parts, an outer green rind and an inner clear pulp. The pulp, the major part of the leaf by volume, appears to be clear and mucilaginous. Various studies have revealed that substances isolated from Aloe pulp possess many pharmacological activities, including anti-inflammatory, anti-oxidative, anti-aging, anti-cancer, and immunomodulatory (Yagi et al., 2002; Yagi et al., 2003; Lim et al., 2003; Im et al., 2005; Yu et al., 2009; Reuter et al., 2008; Tudose et al., 2009). McAnalley (1988) has characterized *Aloe saponaria* pulp, which is an antraquinone-free gel, mainly composed of linear polymers of acetylated mannose monomers. Accordingly, Yagi et al., (1984) isolated the 1,4-linked β-D-mannopyranose and 1,4-linked α-D-mannopyranose from the pulp of *Aloe saponaria*. They are two neutral polysaccharides, partially acetylated, isolated by gel filtration. These were shown to be linear polymers of a 1,4-linked β-D-mannopyranose (mol. Wt 15,000) containing 18 % acetyl groups and a 1,4-linked α-D-mannopyranose polymer containing a single branch on the principal chain, consisting of D-glucose residues linked at C-2 and C-4 (mol. Wt. 66,000), with 10% acetyl groups.

An *in vitro* study showed that α and β links can be catabolyzed to lower molecular compounds (mannose or mannose oligosaccharides) by the human intestinal feces microflora (Yagi et al., 2001). Besides, tissue distribution of aloemannan after i.v. injection in mice showed accumulation of metabolites of aloemannan primarily in the kidneys (Yagi et al., 2001). Another *in vivo* study on mice with polymicrobial sepsis showed that the i.v. administration of *Aloe vera* fresh gel markedly enhanced bacterial clearance (Yun et al., 2009).

At present, no study in humans has demonstrated the presence of mannose or mannose related compounds in urine after Aloe intake, which could be the first step to establish the possible implementation of *Aloe saponaria* pulp oral intake in the prevention of UTIs.

The objective of this work was to evaluate whether the oral intake of *Aloe saponaria* pulp produces in volunteers (women) the excretion of mannose or mannose oligosaccharides. For this purpose, a systematic study of quantification and identification of mannose present in urine was performed.

MATERIALS AND METHODS

D (+) mannose was procured from Sigma Chemical Co. All other chemicals and reagents used were of analytical grade.

Plant materials and fingerprinting

Fresh mature whole leaves of *Aloe saponaria* were collected in a plantation from Calamuchita Valley, Córdoba, Argentina. The plant material was identified by Prof. Dr. Luis Ariza Espinar and a voucher specimen was deposited at the Herbarium of Museo Botánico de Córdoba (CORD 00019452).

The procedure previously described by Beneke et. al. (2012) to obtain the polysaccharides of *A. vera* and *A. ferox* was followed for fingerprinting. A suspension of *A. saponaria* pulp in D_2O (20 mg of pulp /ml of D_2O) was filtrated in a Pasteur pipette with a cotton wool. An aliquot of the filtrate was reserved to be subjected to ^{1}H-NMR and the rest was added with ethanol to the volume of four times of that of the aqueous solution. The polysaccharides precipitated were separated centrifugally at 4000 rpm for 10 min and washed with ethanol. The precipitate was washed 4 times after which the solid was separated by filtration, dried under vacuum to constant weight and further dissolved in 1 ml of D_2O. The ^{1}H-NMR spectra of both samples were recorded with a Bruker Advance II 400 UltraShield-TM spectrometer using 3-(Trimethylsilyl)-propionic acid-D4 (TPS) as reference compound. The residual water signal was reduced by using the PRESAT sequence.

Study design and methodology

The leaves were washed with distilled water and cut in portions of 32 ± 4 g each. On the first day of the trial period, the volunteers were provided with 7 (seven) portions (total weight 220 ± 28 g) which were kept in a refrigerator covered by a polyethylene film. Each day of the trial, the colorless gelatinous pulp was separated from the green cortical layer for oral intake. Preliminary studies showed that the pulp obtained after taking out the green cortical layer to the said portions weighed 18 ± 3 g. Therefore, total intake of pulp was 125 ± 28 g.

The study was designed as a single center, single branch trial. Five healthy women participated in the study, ranging in age from 25-40. The study was approved by the Ethical Committee of the School Hospital, National

University of Córdoba, according to Helsinki Declaration criteria (Book N° 1, Certificate number 89, page 82; August 5th, 2010). All the volunteers gave written informed consent prior to study inclusion. Every subject completed the study.

On the first day of the study, each volunteer supplied a urine sample, taken in the morning, after a light dinner the night before (t_0 sample). Next night and for the seven successive ones, they consumed, after a light dinner, a portion of *Aloe saponaria* pulp. Subjects were allowed to eat their normal lunch and evening meals. On the eighth day, each volunteer supplied a second urine sample (t_7 sample), taken in the same conditions than t_0. The t_0 samples of the 5 volunteers were filtered with Millipore filter 0.45 µm and frozen. The samples obtained at t_7 were also filtered and analyzed immediately.

Dubois Method

Total urinary sugar was measured using the phenol-H_2SO_4 Dubois method (Dubois et al., 1956) with slight modifications (García-Peña et al., 2008). Briefly, 0.5 mL of 5 % phenol solution and 2.5 mL of concentrated H_2SO_4 were consecutively added to 1 mL of 1/10 diluted t_0 and t_7 samples and placed in an ice-cooled bath. The mixture was vortexed and rested at room temperature for 20 min and afterwards spectrophotometrically assayed at 490 nm. The absorbance values were related to the solution concentrations using a calibration curve with mannose concentrations between 10–60 mg/L. According to Malinowska et al., (2010), this method accounts for a value of 3.3 µg/mL of glucose as the lower limit of quantification.

Enzymatic determination of glucose

Glucose was measured by the enzymatic method (detection limit: 5 µg/mL). Two mL of the reactive (AA Wiener lab) were added to 200 µL of the urine samples, incubated at 37 ºC for 10 min and then measured for their glucose content by visible spectrophotometry at 505 nm. This assay can differentiate glucose from other carbohydrates. Then, mannose and its related compounds levels can be inferred by discounting glucose levels from total carbohydrate obtained by Dubois method.

TLC

Urinary sugars were identified by TLC. In order to concentrate t_0 and t_7 samples, 30 mL of each one were filtered and freeze-dried (Freeze-Dry System LABCONCO) under a vacuum of 10×10^{-3} mBar after initial freezing with liquid air. The lyophilized urine powder was reconstituted in 1 mL of distilled water and applied on silica-gel plates (Merck). Chromatograms were developed with isopropanol: ethyl acetate: water mixture in the ratio 6:3:1 (v/v) which is known to produce the best separation of hexoses (Montreuil et al., 1997). A solution of 24 mg of mannose in 30 mL of urine was lyophilized and used as reference. The plates were air-dried, sprayed with H_2SO_4 5 % and heated 20 min at 105 °C to visualize the spots.

^{1}H-NMR spectroscopy

Lyophilized urine samples of t_0 and t_7, obtained as previously described, and mannose (24 mg) were assayed by ^{1}H NMR (Bruker Advance II 400 UltraShield-TM) at 25 °C. Approximately 25 mg of the solid residue was dissolved in 1 mL of D_2O. Chemicalshifts (δ ppm) were obtained from the residual signal of HDO present in D_2O.

HPLC

Mannose content in t_0 and t_7 samples was analyzed by HPLC. Samples t_0, t_7 and t_0 spiked with 2 % mannose were filtrated thorough a Millipore filter (0.45 µm) and injected. In addition, an aliquot 20 mL of the samples were added with 5 mL of H_2SO_4 1 N at 25 ºC, rested for 20 min, neutralized by an equimolar amount of NaOH 1N, filtered through 0.45 µm and analyzed by HPLC. This process produced the rupture of glycosidic bonds. All runs were done in a Konik KNK 500-A series HPLC chromatograph (Barcelona, Spain), equipped with refractive index detection and Konik Datajet recorder−integrator. Helium-degassed ultrapure water was used as mobile phase with a flow rate of 0.6 mL/min, and with a furnace temperature of 80 °C. The injection volume was 20 µL. The column was Supelco™ (Supelcogel-Ca, 9 µm particle size, 30 cm x 7.8 mm). Solutions of 2 % mannose in water and in urine and a solution of 2 % glucose in water were used as references.

RESULTS AND DISCUSSION

^{1}H-NMR fingerprinting of materials

The compositions of *A. saponaria* pulp and precipitated polysaccharides were determined by ^{1}H-NMR spectroscopy. As can be seen in Figure 1, they contained acemannan, whose signals appear at 2-2.5 ppm (Jiao et al., 2010). It is known that acemannan is the main component of *A. saponaria* pulp (McAnalley, 1988; Yagi et al., 1984).

In our study, urine samples were subjected to several

Figure 1. ^{1}H-NMR spectra of (up) *Aloe saponaria* precipitated polysaccharides and (down) *Aloe saponaria* pulp.

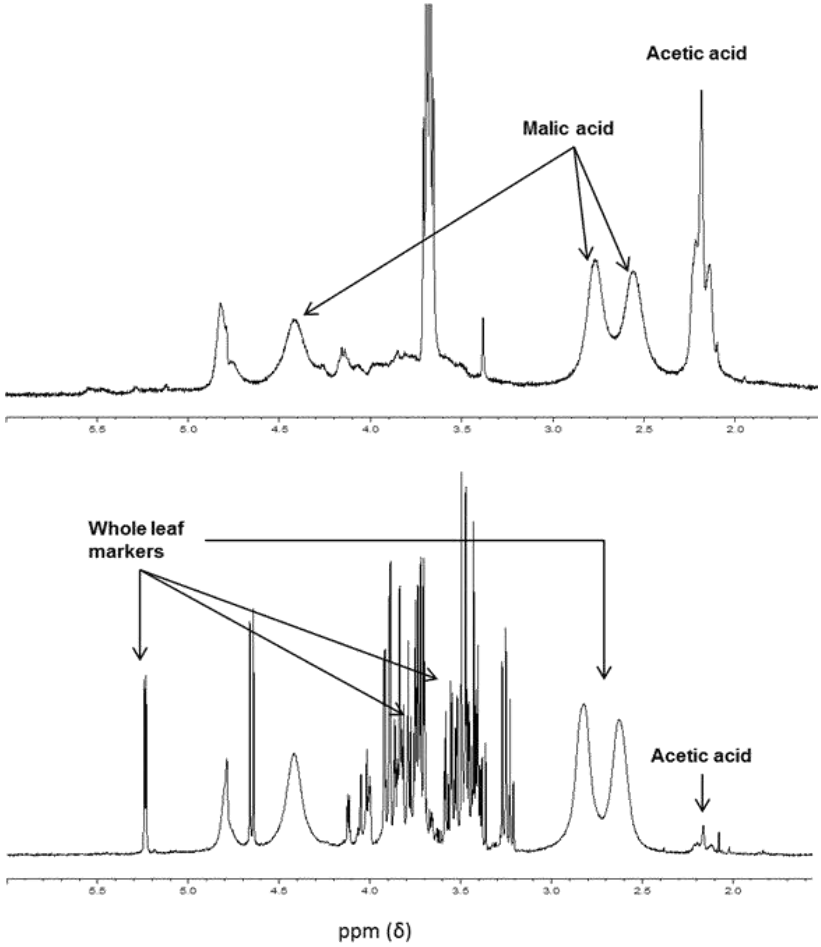


Table 1. Values of total sugars and glucose (mg/mL) in urine samples at t_0 and t_7.

Volunteer	Total sugars [a]		Glucose [b]	
	t_0	t_7	t_0	t_7
1	nd	622	8	13
2	nd	289	11	10
3	nd	480	15	18
4	nd	356	12	15
5	nd	287	6	7
Average ± SD		406.8 ± 143.6	10.4 ± 3.5	12.6 ± 4.3

[a] determined by Dubois method and expressed as mannose. [b] determined by enzymatic method. Samples t_0 and t_7 showed non-significant differences ($p< 0.05$, ANOVA). nd: not detected.

analyses to identify and/or quantify mannose and mannose-related compound contents after *Aloe saponaria* intake. Total sugar in urine at t_0 and t_7 was assayed through Dubois method and glucose levels in the same samples were determined by the current enzymatic method. As reported in table 1, glucose levels in urine did not exhibit significant differences between urine samples at t_0 and t_7. In addition, 1:10 diluted samples exhibited at t_7 an average sugar concentration of 406.8 mg/L (expressed as mannose) while they were below the detection limit in t_0 samples suggesting significant concentration of sugar different from glucose in urine after *Aloe saponaria* pulp intake. Notice that the Dubois method reduced not only sugars but also oligosaccharides, glycosides and polysaccharides, which may be hydrolyzed to sugars.

Figure 2. ^{1}H-NMR of (A) urine t_0 days, (B) urine t_7 days (C) mannose, in D_20. ■ mannose anomeric C1βH, ● and ▲ C1αH and C1βH of mannose and manosse olygomers. The presence of α and β H is related to the metabolization of acetyl-mannanes after the intake of *Aloe saponaria* pulp.

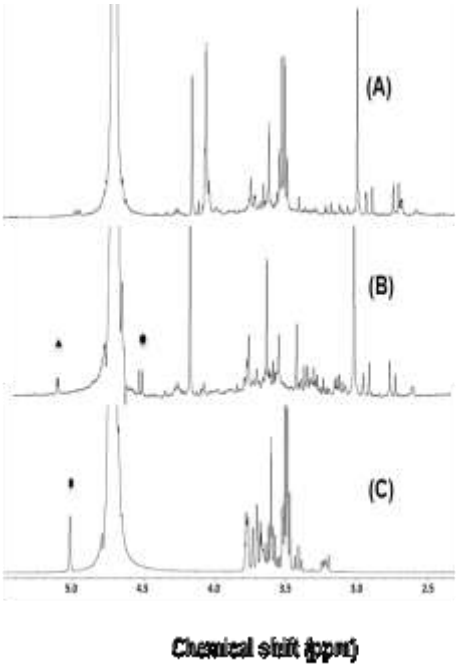
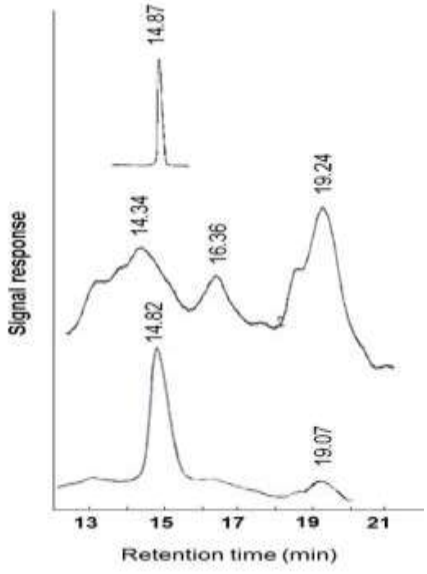


Figure 3. HPLC chromatograms with refractive index detector of (up): mannose 2%, (medium): t_7 urine sample and (down): t_7 sample after acid hydrolysis showing an increase of the mannose matching peak and a reduction of the accompanying overlapped peaks.



On the other hand, TLC of t_7 urine samples exhibited a spot at the same R_f (0.81) as the mannose reference that was not present in t_0 samples.

Solution ^{1}H-NMR spectra of the samples were obtained in D_2O. As can be seen in figure 2, mannose signals at 3.2-3.7 ppm cannot be easily differentiated from urine signals since they are partially overlapped. However, a well resolved signal ascribed to C_1 β H anomeric is clearly observed at 5.01 ppm in mannose. Samples obtained after seven days of *Aloe saponaria* intake showed signals

at 4.52 ppm (d, J=3.96 Hz) and 5.11ppm (d, J= 1.88 Hz) which can be ascribed to C_1 α H and C_1 β H of mannose and mannose oligomers, as previously informed for polysaccharides arising from *Aloe saponaria* fresh pulp (Yagi et al., 1999). The presence of α and β H is directly related to intestinal metabolization of acetyl-mannanes (Yagi et al., 1984). Although other ^{1}H-NMR signals have been reported as arising from Aloe, in our study, they cannot be differentiated from urine signals.

Additional evidence of the presence of mannose and related compound in urine was obtained by HPLC (Figure 3).

Samples t_7 present a peak with a retention time of 14.84 min that matches with that of the mannose reference. As can be seen in Figure 3, the peak is partially overlapped by other peaks on both sides which might correspond to related structures as observed by TLC. Then, the urine sample was subjected to an acid hydrolysis under conditions able to break glycosidic bonds. Such process increased the mannose matching peak and depleted the accompanying ones suggesting the concomitant urinary excretion of mannose and mannose oligomers.

Our results clearly showed that the oral daily intake of small portions of *Aloe saponaria* pulp for seven days produced in all volunteers a significant sugar excretion composed mainly of mannose, accompanied by mannose related compounds that yielded mannose after hydrolysis. High differences were observed in the levels of mannose among the volunteers, with a total sugar value of 622 and 287 in patients 1 and 5, respectively. This could be associated with inter-individual differences in the degree of Aloe absorption and metabolism. The excretion of these metabolites may arise from a metabolic depolymerization of Aloe polymannans in the volunteers' intestinal lumen followed by the absorption of the resultant mannose and mannose related compounds that are not further metabolized. In this context it has been informed that high molecular weight acemannan (> 600 KD) is metabolized by the mucosa in the large intestine following oral administration to a smaller size molecule (3 KD) that is easily absorbed in mice (Yagi et al., 2001).

It is known that bacteria adhere via their adhesins to cognate receptors on epithelial cells of the mucosal surfaces to withstand normal cleansing mechanisms aimed at eradication of the invading pathogen. Bacteria that lack the adhesins are swept away. In the presence of inhibitors of adhesion, the bacteria are eradicated by the normal cleansing mechanism. Then, the use of agents that interfere with the ability of the bacteria to adhere to tissues of the host has been proposed as an attractive approach, since such adhesion is one of the initial stages of the infectious process (Ofek et al., 2003). Besides, adhesion inhibition circumvents the conventional requirement for drug penetration of the outer membrane, minimizing the potential for the development of bacterial resistance.

Schaeffer et al (1980) measured *in vitro* the adherence of *E. coli* cells to voided uroepithelial cells from healthy women and discovered that a concentration of 250 mg/mL of D-mannose, D-mannitol, or α-methyl-D-mannoside completely inhibited *E. coli* adhesion and 5 mg/mL were required for 50 % inhibition. D-xylose, D-arabinose, D-fructose, and D-glyceraldehyde at a concentration of 250 mg/mL only partially inhibited adhesion.

In our study, the excretion of significant levels of mannose or mannose oligosaccharides observed after 1 week of oral intake of *Aloe saponaria* pulp was proved. The average level obtained was 406.8 mg/mL (expressed as mannose) which is higher than that informed by Schaeffer (1980). However, it is important to note that the weight of the aloe portions, the intake period as well as the sampling times used in this study were an arbitrary choice. Additional experiments and a higher number of volunteers are required to investigate the impact of the amount of Aloe consumed in the levels of mannose excreted as well as the urinary anti-adhesion effect.

Our results suppose a potential usefulness of *Aloe saponaria* pulp in clinical practice for the prevention of UTIs produced by pathogenic *E. coli* or as a complement of a pharmacotherapeutic treatment.

ACKNOWLEDGEMENT

This work was supported by CONICET, SECYT-UNC and FONCYT. The authors thank PhD. D. Murature for valuable discussions. We acknowledge G. Bonetto (PhD) and I. Tártara (MD) for their help, Prof. D. Wunderlin and MV Baroni for their help with HPLC measurements. Deuterated water was a donation from Nucleoeléctrica Argentina S.A. CNE.

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