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Isolation and structural elements of a water-soluble free radical scavenger from *Nyctanthes arbor-tristis* leaves

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ABSTRACT

The leaves of *Nyctanthes arbor-tristis* L. (Oleaceae) are used in Ayurvedic medicine for the management of a range of diseases, but reports on its phytochemicals and pharmacological properties are inadequate. Herein, we report purification of an antioxidative polysaccharide (F2) extracted from its leaves by water. The presence of a highly branched polysaccharide (75 kDa) containing esterified phenolic acids was revealed by chemical, chromatographic and spectroscopic analyses. Particularly, ESMS analysis of per acetylated oligomeric fragments derived by Smith degradation provides important structural information on a spectrum of glycerol tagged oligosaccharides. This polysaccharide showed dose dependent free radical scavenging capacity as evidenced by DPPH and Ferric reducing power assay. This pharmacologically active compound (F2) formed a water soluble complex with bovine serum albumin over pH 4.0–7.4. Accordingly, traditional aqueous extraction method provides a molecular entity that induces a pharmacological effect: this could epitomize a smart approach in phytotherapeutic management.

1. Introduction

Natural products have been invaluable as biologically validated platforms for drug development (Clardy and Walsh, 2004; Cragg et al., 2009; Harvey, 2008; Newman and Cragg, 2012). In a series of review articles, Newman and colleagues (Cragg et al., 2009; Newman and Cragg, 2007, 2012) have analyzed the sources of drugs in the last 30 years. The analysis revealed the continuing and valuable contributions of nature as a source of lead compounds that have imparted the basis and inspiration for the synthesis of new drugs (Cragg et al., 2009; Harvey, 2008). Many plant extracts contain vitamins, flavonoids, and polyphenols that are of great interest for use in complementary medicine as supplements (Stavric, 1994). However, it is fascinating to observe that an emerging class of bioactive compounds from plant extracts namely polysaccharides exhibit a large range of properties suitable for industrial application (Gross, 1986). Polysaccharides natural sources show promising antiviral (Ghosh et al., 2009; Ray et al., 2013) and *in vivo* antitussive (Nosalova et al., 2013; Raja et al., 2014) activities. They also elicit antioxidant activities *in vitro* (Aruoma, 2003; Ghosh et al., 2013). Low molecular weight carbohydrates such as agaro-oligosaccharides also exert *in vitro* and *in vivo* hepatoprotective effects by preventing oxidative damage provoked by reactive oxygen species (Chen et al., 2006). These species that are produced in consequence of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems (Finkel and Holbrook, 2000) attack target molecules (lipids, proteins and DNA) inducing oxidative modifications. Oxidative stress is involved in the pathology of many diseases, including atherosclerosis, Alzheimer's, diabetes, neurodegenerative and cancer (McBrian and Slater, 1982; Meyer et al., 1998).

Leaves of *Nyctanthes arbor-tristis* L. (Oleaceae) has been recognized to confer antioxidative and chemopreventive properties (Agrawal and Pal, 2013; Rathee et al., 2007; Rathore et al., 1990). Recently, a crude polysaccharide containing fraction (CP) possessing antitussive activity was isolated from *N. arbor-tristis* leaves (Ghosh et al., 2014). In the present study we have focused our attention on purification and detailed structural analysis of a polysaccharide (F2) present in the crude extract. Assessment of its antiradical ability has also been done. Additionally, interaction of this polysaccharide (F2) with bovine serum albumin (BSA), the most common carrier protein, was studied.

2. Result and discussion

2.1. Purification and chemical characterization of polysaccharide from *N. arbor-tristis* leaf

Extraction of leaves of *N. arbor-tristis* with water yielded a crude polysaccharide-containing fraction (named CP) having antitussive

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activity (Ghosh et al., 2014). This fraction (CP) was further purified by anion exchange chromatography. The major fraction F2, which consisted of 75% of the total material recovered from the column, contained Ara, Gal and Rha residues as neutral sugars in the molar ratio of 30:19:1. It also contained uronic acid (9%). TLC analysis of the acid generated monosaccharides indicates the presence of, *inter alia*, an uronic acid with R*f* values similar to that of GalA. GC analysis of the per-*O*-trimethyl-silylated methyl glycosides derivatives confirmed this result. Size exclusion chromatography of F2 on Sephacryl S-300 suggested that this polysaccharide is homogeneous. Based on calibration with standard dextrans, the apparent molecular mass of F2 would be 75 kDa. The other specialty of F2 is the presence of esterified phenolic acid residues. Indeed, the total phenolic content of F2 was 40 mg GAE/g of sample and it contained *p*-coumaric acid, sinapic acid, ferulic acid, and 8,5′-diferulic acid (diFA) in a molar ratio of 86:2:6:6.

Glycosidic linkage analysis (Table 1) showed that this polymer (F2) was highly branched. It contained, *inter alia*, terminal-, (1,3)-, (1,5)- and (1,3,5)-linked Ara*f* and terminal-, (1,3)-, (1,6)- and (1,3,6)-linked Gal*p* residues (Fig. S1, Supplementary data). Remarkably, (1,2,4)-linked Rha*p* unit, a component of rhamnogalacturonan I (RG I) was also present.

The signals in the NMR spectrum of fraction F2 were assigned according to sugar composition, glycosidic linkage makeup, 2D NMR technique and data in the literature (Capek et al., 2010; Dong and Fang, 2001; Ozaki et al., 2010; Ponder and Richards, 1997; Sims and Furneaux, 2003). The HSQC spectrum of F2 showed three groups of anomeric resonances with the following chemical shifts: δ 5.01–5.3, δ 4.42–4.59 and δ 4.65–4.69 (Fig. 1). Linkage analysis data showed that F2 contained terminal-, 1,5-, and 1,3,5-linked Ara*f* together with small amount of terminal Ara*p* residues. The strong signal at δ 109.4 was correlated with the H-1 (δ 5.05) of terminal Ara*f*. Hence, the resonances at δ 112.2 and 111.1 could be assigned to 1,5- and 1,3,5-linked Ara*f* residues. The low-field chemical shifts indicated Ara adopted an α-configuration and the furanose form. The C-1 resonance for the β-Ara*p* residue was at 102.0 ppm (Ponder and Richards, 1997; Sims and Furneaux, 2003). Thus, the C-1/H-1 resonance at δ 102.5/5.01 was deduced to be terminal-β-Ara*p*. The C-5 resonance of 1,5- and 1,3,5-linked α-Ara*f* had been assigned at δ 64.2 and 66.7, respectively, while that for terminal α-Ara*f* had been assigned around δ 63.8. The HSQC spectrum for F2 showed C-1/H-1 cross-peaks (δ 103.6/4.68, and δ 105.1–107.5/4.59–4.42) and hence the H-1 resonance at δ 4.42–4.75 originates from anomeric protons (H1) of different β-Gal*p* residues. Dong and Fang (2001) assigned signal at δ 69.6 to the C-6 of 1,6- and 1,3,6-linked Gal*p*. Hence, cross peaks at δ 71.3/3.97 and 72.2/4.08 were assigned to C6/H6 of 1,6- and 1,3,6-linked Gal*p* residues.

2.2. Periodate oxidation of polysaccharide

Smith degradation was performed to obtain information on fine structural details. The polysaccharide F2, on Smith degradation using standard protocol, yielded a periodate resistant fraction (named as PRF2) and an aqueous 80% ethanol soluble fraction (PRF2S) consisting of oligomeric fragments. The ethanol soluble fraction, which yielded Gal and Ara in 34:66 ratio, was further analyzed by ESMS, after chemical acetylation. The mass spectrum (Fig. 2) of acetylated product (PRF2SA) revealed the presence of five series of oligosaccharides, three of which were tagged with glycerol (*O*-Gly). The *O*-Gly tag at the reducing end of oligomeric fragments, $Ara_1Gal_{1-2}Gly_1Ac_{9-12}$, $Ara_{1-3}Gly_1Ac_{6-10}$ and $Gal_{1-4}Gly_1Ac_{7-19}$ present in 1st–3rd series (Table 1S, Supplementary data) was generated during Smith degradation of (1,6)-linked Gal units. Remarkably, the presence of $Ara_1Gal_{1-2}Gly_1Ac_{9-12}$ provided evidence for the occurrence of Ara and Gal residues in same chain confirming that the polymer was not a mixture of arabinan and galactan. Moreover, the presence of at least three consecutive (1,3,5)-linked Ara and four contiguous (1,6)-linked Gal*p* residues substituted at *O*-3 were confirmed. Methylation analysis of PRF2SA revealed the presence of terminal- and 1,6-linked Gal*p* units. The occurrence of other two series $Gal_{2-5}Ac_{8-17}$ and Ara_1Ac_{3-4} may be due to the removal of *O*-Gly tag by CF_3CO_2H during Smith degradation. In addition, ESMS analysis coupled with glycosidic linkage analysis revealed the existence four consecutive (1,6)-linked Gal units each substituted at C-3 in the parental F2.

Then again the periodate-resistant product (PRF2) contained Gal and Ara in a molar ratio of 93:7 indicating that the backbone of this polymer was made up of Gal residues. Comparison of glycosidic linkage analysis of F2 and PRF2 showed that the proportion of (1,6)-linked Gal*p* residues increased significantly (Table 1). Hence, the presence of chain containing (1,6)-linked Gal*p* residues substituted at C-3 was ascertained. Moreover, this results also points toward the existence of a chain containing (1,3)-linked Gal*p* residues substituted at C-6. Remarkably, major part of the Ara residues was either degraded or present in the ethanol soluble part (PRF2S) as monomer or oligomer. Modification of structure of F2 after Smith degradation (PRF2) was also evident from their ^{1}H NMR spectra (Fig. 3). Anomeric signals of Ara residues disappeared after Smith degradation (PRF2) in accord with the oxidation of terminal-Ara*f* and (1,5)-linked Ara residues in parental fraction F2 by periodic acid. The disappearance of (1,3,5)-linked Ara residue suggest that this unit is flanked by either (1,5)-linked Ara and/or (1,6)-linked Gal*p* residue(s).

On the basis of the data obtained, it was concluded that the polysaccharide is a rhamnogalacturonan I containing mainly branched arabinogalactan and phenolic acids. Notably,

Table 1
Linkage analysis data of polysaccharide (F2) from *N. arbor-tristis* leaves and its Smith degraded derivatives (PRF2 and PRF2SA).

Methylation products[a]	*m/z* Values	Peak area[b]		
		F2	PRF2	PRF2SA
2,3,5-Me_3-Ara*f*	43,45,102,101,118,129,161,162,205	12	7	18
2,3,4-Me_3-Ara*p*	43,101,102,117,118,161,162	5		33
2,5-Me_2-Ara*f*	43,118,173,233			14
2,3-Me_2-Ara*f*	43,102,118,129,162,189,233	30		
2-Me-Ara*f*	43,118,201,261	15		
3-Me-Rha	43,130,143,190,203	2		
2,3,4,6-Me_4-Gal*p*	43,45,101,102,118,129,145,161,162, 205	5	19	25
2,3,4-Me_3-Gal*p*	43,102,118, 129,162,189,233	6	23	10
2,4,6-Me_3-Gal*p*	43,45,101,118,129,161,174,234	11	25	
2,4-Me_2-Gal*p*	43,118,129,189,174,234	14	26	

[a] 2,3,5-Me_3-Ara*f* denotes 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.
[b] Percentage of total area of the identified peaks.

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