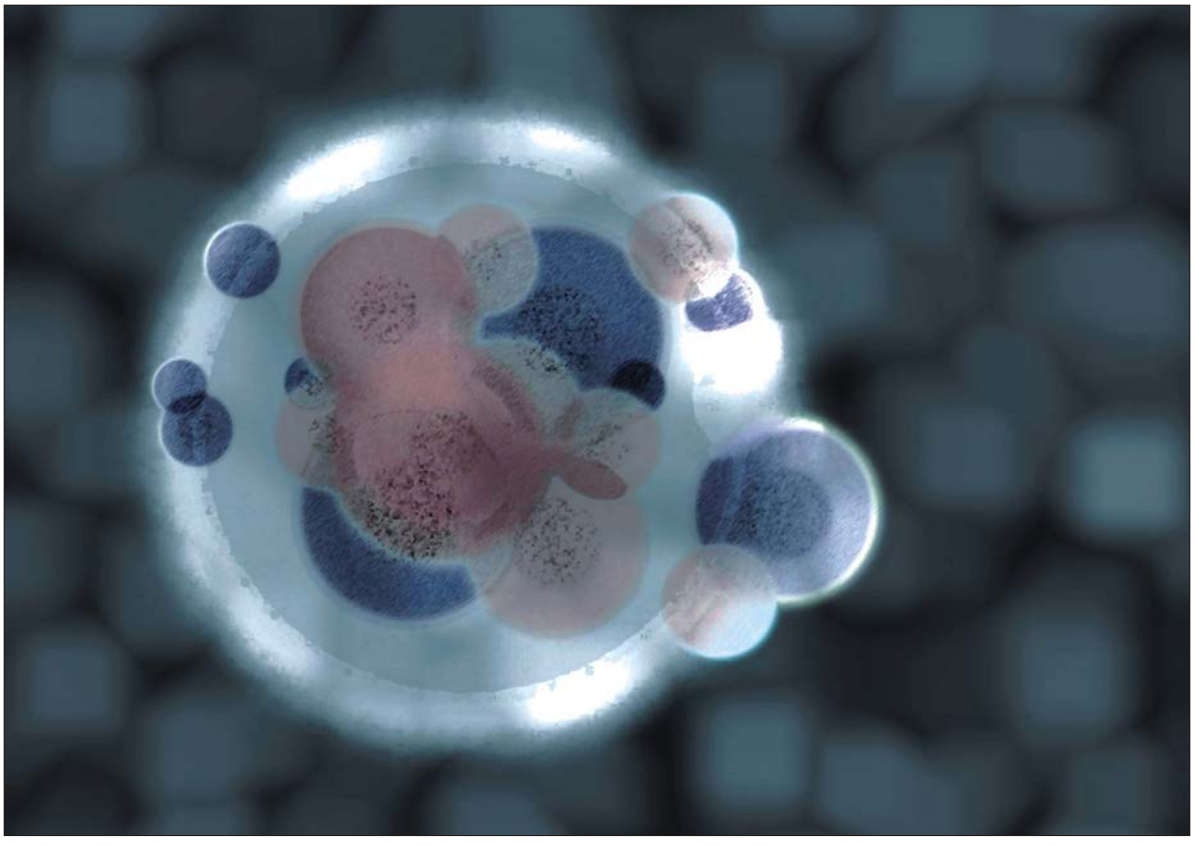

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(1→3)-β-D-Glucans from Libyan Dates (*Phoenix dactylifera* L.) and Their Anti-cancer Activities

[1]Omar Ishurd, [1]Faraj Zgheela, [1]Adel Kermagia, [1]Nansur Flefleaa,
[1]Mohamed Elmabruka, [2, 3]John F. Kennedy and [4]Ahmed Ashour
[1]Bio-technology Research Centre, Tripoli, Libya
[2]Birmingham Carbohydrate and Protein Technology Group,
School of Chemistry, The University of Birmingham, Birmingham, B15 2TT, UK
[3]Chembiotech Laboratories, Birmingham Research Park, 97 Vincent Drive, Birmingham, B15 2SQ, UK
[4]Department of Food Science, College of Agriculture, Altateh University, Tripoli, 12358, Libya

Abstract: A glucan of cellular origin has been isolated from Libyan dates (*Phoenix dactylifera* L.) and the structure of the purified glucan characterised using derivatisation methods including methylation, periodate oxidation and acetolysis. Glucans were found to exhibit potent antitumour activity; this activity could be correlated to their (1→3)-β-D-glucan linkages. This is the first report on studies of antitumour active compounds occurring in dates.

Key words: Data fruit, antitumor active compound, polysaceharides

INTRODUCTION

Dates fruits have been an important crop in arid and semiarid regions of the world since ancient times; they have always played an important role in the economic and social lives of the people of these regions. The fruit of the date palm is therefore well known as a staple food. It is composed of a fleshy pericarp and seed (Ahmed and Robinson, 1999; Ishurd *et al*., 2001; Shinwari, 1992). Polysaccharide material from dates has been used as a functional food and a source of active components in the development of drugs (Puri *et al*., 2000).

Some (1→3)-β-D-glucans have attracted attention because of their inhibitory action on the growth of certain tumours in animals (Misaki *et al*., 1981). Structural correlation to antitumour effects of these polysaccharides is not yet fully understood, except for the molecular-weight dependence of activity of some glucans (Sassaki *et al*., 1970; Usui *et al*., 1983). In a preliminary study we showed that water-soluble glucan exhibits potent antitumour activity against implanted Sarcoma-180 solid tumour growth in mice.

We previously reported the isolation of a novel β-D-glucan from dates (Ishurd *et al*., 2002). In our studies we are concerned with the data cell wall glucan introduced by microbial contaminants in ripe dates, which contain a high proportion of sugars, thereby creating a suitable environment for microbial growth. We have therefore studied the structure and anti activity of two polysaccharides (1→6)-branched, (1→3)-β-D-glucans, isolated from date fruits.

MATERIALS AND METHODS

Plant material: β-D-glucans were prepared from Libyan dates (*Phoenix dactylifera* L.) as done previously (Ishurd *et al*., 2002).

General: ^{13}C-Nuclear magnetic resonance (NMR) spectroscopy at 50°C was used for sample structural analysis (65 mg mL^{-1} in D_2O) recorded using a Bruker 500 MHZ instrument.

Smith degradations: Samples (25 mg) of date fruits were cut into small pieces immediately after harvesting and disintegrated in a blender; they were oxidized with 0.05 M $NaIO_4$ (10 mL) at 20°C in the dark for 48 h. The oxidation was stopped by the addition of 1, 2-ethanediol. The solution was then dialysed against distilled water. The dialysed material was reduced, by the addition of sodium borohydride (50 mg) in the dark, with stirring for 15 h at room temperature; and then neutralized with 50% v/v acetic acid, purified by repeated addition/evaporation of water and the residue (12 mg) freeze-dried. Second and third smith degradation sequences were performed under the same conditions.

Corresponding Author: John F. Kennedy, Chembiotech Laboratories, Birmingham Research Park, 97 Vincent Drive, Birmingham, B15 2SQ, UK

Selective hydrolysis: The native glucans were hydrolyzed with 1.0 M trifluoroacetic acid for 1 h at 100°C. Supernatant was neutralized by evaporation of the excess acid and fractionated by column chromatography on Sephadex G-15.

Assay of antitumour activity: Seven-day-old Sarcoma-180 ascites (0.1 mL, 2×10^6 cells) were transplanted subcutaneously into the right side of female CD1 mice (weighing ~22 g). The test samples, dissolved in saline solution and sterilized for 20 min at 120°C and then were injected intramuscularly every day for 10 days, starting 24 h after tumour implantation. At days 10, 20 and 30, the tumour diameter was determined with a caliber square. On day 30, the mice were sacrificed and the tumours were extirpated and weighed. Inhibition ratio (%) was calculated by comparing average weight of treated mice tumours with those of untreated controls. This work was done at the National Cancer Centre Research Institute of China.

RESULTS AND DISCUSSION

We have already described the isolation and characterised the structure of neutral polysaccharides from dates (Ishurd *et al*., 2002). Briefly, dates were extracted with hot water and the extract fractionated by sequential chromatography on columns of DEAE-cellulose, Sephadex 100 and concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden), followed by methylation, Smith degradation and acetolysis. It was concluded that these polysaccharides have a main chain of (1→3)-linked β-D-gluco-pyranosyl residues with (1→6)-linked branched saccharide residues (Fig. 1).

^{13}C-NMR spectroscopy: The ^{13}C-NMR spectra of the native date glucans showed multiple resonances (Fig. 2.), consistent with a branched (1→3)-β-D-glucan structure. The β-configuration of d-glucosyl residues was clearly evident by two anomeric peaks at δ103.5 and δ104 and branching at C-6 were shown by signals of *O*-substituted C-6 at δ70.8 and of unsubstituted C-6 at δ61.9. The predominance of the latter, together with the typical signal of *O*-substituted C-3 at δ85.6, supported the notion of a high proportion of (1→3) linkages in a linear arrangement as was previously demonstrated by chemical analysis.

The multiplicity of the signals and the broad C-3 signal at δ85.6 could be ascribed to the presence of linear (1→3,1→6) branch point and terminal β-D-glucopyranosyl residues.

Since the number of terminal residues equals the number of branched points, it was not possible to differentiate their signals on the basis of their relative intensity. However, the signals of the (1→3)-linked linear β-D-glucans could be assigned in the ^{13}C-NMR spectrum of the Smith-degraded glucan. After three sequential periodate oxidations, only six well-defined signals were left in the spectrum of the degraded polysaccharide. The assignment of the carbon resonances is shown in Table 1.

This confirmed that the linear-extended (1→3) side chains do not exceed three β-D-glucopyranosyl residues. By comparing differences between the spectra of the native and Smith-degraded polymer, it was possible to assign a few other signals in the spectrum of the branched glucan (Table 1). In particular, the second anomeric signal could be ascribed to branching on linear glucans, either the (1→3, 1→6) branch points or the terminal residues. Linear (1→3)-β-D-glucans with various (1→6) linkages could also be obtained by direct mild hydrolysis of parent material with 0.5 M trifluoroacetic acid for 15 h at 20°C, which is selective for (1→6)-linkages.

The spectrum of this non-Smith-degraded, partially hydrolyzed polysaccharide resembled that of the Smith-degraded glucans (Table 1), but still contained numerous extraneous peaks, indicating incomplete side chain hydrolysis. Incomplete hydrolysis of appending chains may explain why hydrolyzed glucan did not gel, whereas (1→3)-β-D-glucan scleroglucan (Rinaudo and Vincendon, 1982) and the antitumour β-D-glucan

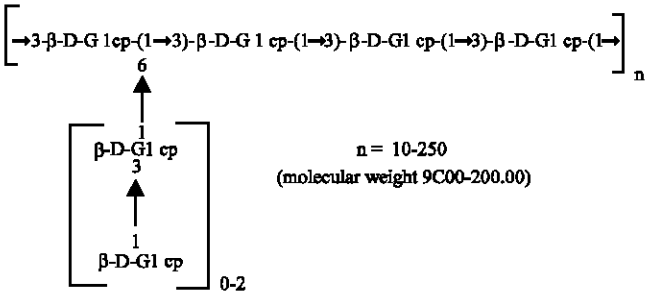

Fig. 1: Structure of D-glucans from Libyan dates (*Phoenix dactylifera* L.)

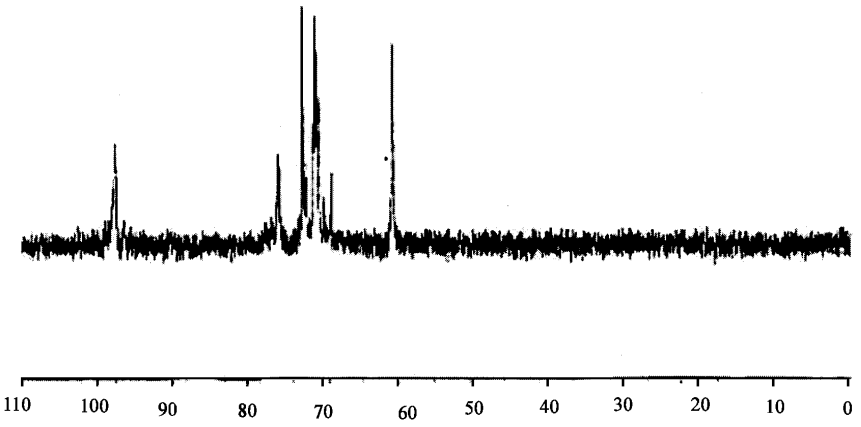

Fig. 2: ^{13}C-NMR spectrum of native D-glucan from Libyan dates (*Phoenix dactylifera* L.)

Table 1: ^{13}C-NMR assignments for D-glucans from Libyan dates (*Phoenix dactylifera* L.)

	Residues and linkages	Chemical shifts (δ)					
D-glucan	Linkages	C-1	C-2	C-3	C-4	C-5	C-6
	→3)-β-D-G1c*p*-(1→ 6 ↑ 1	103.5	74.6	85.9	69.3	76.7	70.8
Native	β-D-G1c*p* 3 ↑ 1	103.4	74.1	85.7	69.3	76.7	61.9
	(β-D-G1c*p*)$_{0\text{-}2}$	104.2	73.7	76.6	70.8	77.1[a]	61.9
Smith degraded	→3)-β-D-Glc*p*-(1→	103.4	74.1	85.6	69.3	75.6	61.9
Partially hydrolysed	→6)-β-D-Glc*p*-(1→	103.4	74.0	85.3	70.4	76.7	61.9
	→3)-β-D-Glcp-(1→	-[b]	-[b]	69.3	76.6	61.9	-[b]

{[a]Based on gentiobiose spectrum; [b]Not observable due to overlapping peaks}

Table 2: Antitumour effect of Libyan dates (*Phoenix dactylifera* L.) glucans on solid Sarcomo-180

Factor, Sample	Dose (mg kg^{-1})[a]	Mean tumour weight (g)	Inhibition (%)[b]	Complete regression[c]	Significance (p<)[d]
Dose dependence					
Control		4.50		0/10	
D-Glucans	0.2	0.35	92	8/9	0.02
	1	0.002	99	9/10	0.1
	5	0.01	98	7/8	0.01
Effect of pretreatment [e]					
Control		5.4		0/9	
D-Glucans	1[e]	0.31	95	8/10	0.001

*([a]D-Glucans dissolved in saline solution administered daily by intraperitoneal injections for 10 days, starting 24 h after inoculation; [b]$((C-T)/C)\times100$, where T is the average tumor weight of treated group and *C* is the average tumor weight of control group; [c]Number of tumor-free mice/number of treated mice, where treated mice were given 1 mg kg^{-1} daily for 10 consecutive days, starting 11 days prior to inoculation; Evaluated using. Student's *t* test with $p<0.05$ being a significant difference; Treatment with 1 mg kg^{-1} daily for 10 consecutive days, starting 11 days prior to inoculation)

isolated from *Volvariella volvacea* fruit (Misaki *et al*., 1986) do. Chromatographic analysis of dialysable material obtained during partial hydrolysis showed glucose to be the predominant sugar, with only traces of dimers and trimers, confirming side chains consist mainly of terminal d-glucopyranose (Ishurd *et al*., 2002).

Antitumour activity: Antitumour activity was tested on allogenic solid Sarcoma-180 in mice. This tumour model is useful for testing immunomodulating substances. In all experiments ~ 2×10^6 Sarcoma-180 tumour cells (ascites form) were subcutaneously transplanted in to the right side of female CD1 mice. Evaluation of antitumour activity was performed by measuring tumour diameter at 10-day intervals and determining the weight of excised tumours at day 30. Antitumour effects of date glucans was dose dependent, with an optimum activity at lmg/kg^{-1} (Table 2).

In all experiments, during the first 10 days after tumour inoculation the tumours of the glucan-treated mice increased at the same rate as the tumours of the control

group. After about 15 days, the tumour diameter in the treated group decreased and in many cases, the tumours showed complete regression by day 30. This delayed antitumour effect suggests an indirect mode of action of the date glucans.

To confirm an immunomodulating action, we examined the antitumour effect after pretreatment of the mice with the glucans; this pretreatment started 11 days before tumour inoculation and was continued for 10 consecutive days, by daily intraperitoneal injections of the polysaccharides. The results clearly indicated that pretreatment has almost the same effectiveness as treatment after tumour inoculation (Table 2). This indirect mode of antitumour action suggests an involvement of the immune system.

Our data are consistent with previous reports concerning a possible correlation between antitumour activity and (1→3)-β-D-glucan structure (Yoshioka *et al*., 1985). The mechanisms of the antitumour activity are under further investigation

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