

Cladonia furcata polysaccharide induced apoptosis in human leukemia K562 cells¹

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KEY WORDS *Cladonia furcata*; polysaccharides; apoptosis; K562 cells; DNA fragmentation; electrophoresis; flow cytometry

ABSTRACT

AIM: To study whether *Cladonia furcata* polysaccharide (CFP-1) might induce apoptosis in human leukemia K562 cells. **METHODS:** Inhibition of proliferation was measured by MTT assay. Morphological assessment of apoptosis was performed with fluorescence microscope and electron microscope. DNA fragmentation was visualized by agarose gel electrophoresis. The amount of apoptosis cells was measured by flow cytometry. **RESULTS:** CFP-1 (50 - 800 mg/L) inhibited K562 cell proliferation in a concentration-dependent manner. After incubation of K562 cells with CFP-1 300 mg/L for 5 d, morphological changes of typical apoptosis were observed and agarose gel electrophoresis of DNA revealed "ladder" pattern. **CONCLUSION:** CFP-1 induced apoptosis in human leukemia K562 cells.

INTRODUCTION

A great deal of experimental evidence has accumulated in the past several decades, suggesting that polysaccharides have wide bioactivities⁽¹⁾. Many plant polysaccharides, as biological response modifiers, have immunopotentiating and antitumor activities. *Cladonia furcata* belongs to lichen and has been used as folk medicine in Japan. A number of scholars have investigated that usnic acid, the most abundant constituent of several

lichen species such as *Cladonia* and *Cetraria*, has antibacterial, antimetabolic, and antiviral properties⁽²⁾. However, little is known about the effects of lichenins on apoptosis induction in tumor cells. Recently, a kind of water soluble lichenin, *Cladonia furcata* polysaccharide (CFP-1), was first extracted and purified from *Cladonia furcata* (Huds) distributed in Gansu province, China⁽³⁾. Briefly, CFP-1 was obtained by water extraction, alcohol precipitation, DEAE-cellulose, and Sephadex G-200 column chromatography. Gas chromatography (GC) of acid hydrolyzate of CFP-1 suggested that it was composed of glucose only. The homogeneity of CFP-1 was established by high voltage electrophoresis and gel filtration chromatography. Its mean molecular weight was estimated to be $M_r 9.9 \times 10^4$. Periodate oxidation, Smith degradation, IR, ¹H NMR and ¹³C NMR analysis showed that the possible structure of CFP-1 was $[\rightarrow 3)\text{-}\alpha\text{-D-Glc}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}(1\rightarrow)]_n$. Our experiments discovered CFP-1 inhibited the growth of human leukemia K562 cells. Hence, it was suggested that CFP-1 might be a potential anti-tumor drug. In this paper, we studied whether CFP-1 could induce apoptosis in K562 cells or not.

MATERIALS AND METHODS

Materials *Cladonia furcata* polysaccharide (CFP-1) was obtained from and identified by the State Key Laboratory of Applied Organic Chemistry, Lanzhou University. MTT and RPMI-1640 were purchased from Sigma.

Cell culture Human leukemia K562 cell line was obtained from Shanghai Institute of Cell Biology, and was maintained in RPMI-1640 supplemented with 10 % heat-inactivated calf serum, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L in a 5 % CO₂ atmosphere. CFP-1 was dissolved in 0.9 % NaCl solution.

MTT assay To determine cell viability, MTT as-

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say was performed as previously described⁽¹⁾. MTT was dissolved at a concentration of 5 g/L in sterile PBS and stored at 4 °C in a dark bottle equipped with a tight cap. Cells were placed in replicated 96-well microtiter plate at a density of 1×10^5 /L in a volume of 0.1 mL. CFP-1 (50 – 800 mg/L) was added immediately. The absorbance (*A*) was measured on DG-3022 ELISA microplate Reader at 570 nm.

Morphology K562 cells were collected and stained by acridine orange, then these cells were observed under OLYMPUS VANOX fluorescence microscope⁽⁵⁾. The nuclear chromatin was aggregated in dense masses beneath the nuclear membrane. The ultrastructural appearance was observed under a JEM-100CX electron microscope⁽⁶⁾. Exponentially growing cells (5×10^7 /L) were exposed to CFP-1 300 mg/L for 5 d. After the treatment, cells were harvested, fixed, and viewed.

DNA fragmentation Fragmentation detection of DNA was performed as previously described⁽⁷⁾. After incubation with CFP-1, fragmented DNA was analyzed by electrophoresis. The cellular DNA was extracted, dialyzed, and separated by electrophoresis in 1.5 % agarose gel, and visualized by ethidium bromide. The gel was photographed under UV light.

Apoptosis analysis by flow cytometry Flow cytometric DNA analysis was performed to evaluate the percentage of apoptotic cells whose DNA content was lower than that of diploid cells⁽⁸⁾. Cells were harvested and fixed. Distribution of cells with different DNA contents was determined by flow cytometer (EPICS XL; Coulter, USA) and the data were analyzed by multicycle DNA content and cell cycle analysis software (copyright: 1994 University of Washington).

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed by *t*-test.

RESULTS

Growth-inhibitory effect K562 cells exposed to CFP-1 (100 – 800 mg/L) revealed that cell proliferation was inhibited in a concentration-dependent manner at 4 d, 5 d, and 6 d (Tab 1).

Morphological changes When K562 cells were treated with CFP-1 300 mg/L, the morphological features of an apoptotic cell, such as cell surface protuberances and nuclear fragment, appeared in a large cell subpopulation under the fluorescence microscope (Fig 1). The ultrastructural feature of an apoptotic cell was shown in Fig 2. The cell volume reduced, which indicated shrinkage

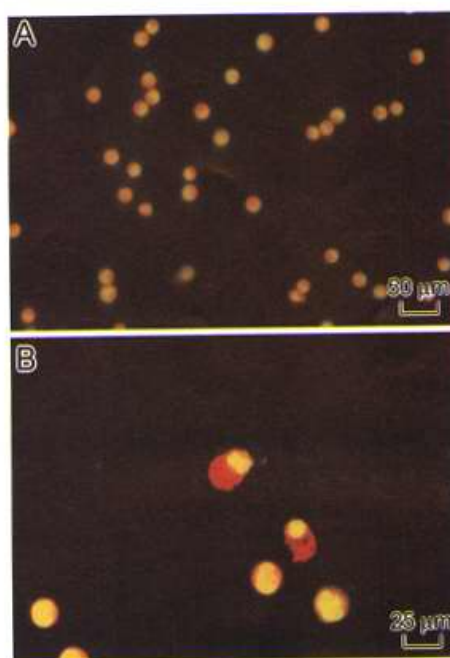


Fig 1. Fluorescence of K562 cells treated with CFP-1 for 5 d. Acridine orange stain. (A) Control, $\times 200$; (B) Cells treated with CFP-1 300 mg/L, $\times 400$.

Tab 1. Antiproliferative action of CFP-1 on K562 cells after treated with CFP-1 (50 – 800 mg/L). $n = 6$ experiments (1×10^4 cells per well). $\bar{x} \pm s$. ^a $P > 0.05$, ^b $P < 0.01$ vs control.

Drug/mg·L ⁻¹	d 4		d 5		d 6	
	<i>A</i> ₅₇₀	Inhibitory rate/%	<i>A</i> ₅₇₀	Inhibitory rate/%	<i>A</i> ₅₇₀	Inhibitory rate/%
Control	0.81 ± 0.03	0.0	0.93 ± 0.04	0.0	0.87 ± 0.03	0.0
50	0.797 ± 0.024 ^a	1.2	0.890 ± 0.020 ^a	4.3	0.85 ± 0.04 ^a	2.3
100	0.79 ± 0.05 ^a	2.5	0.85 ± 0.04 ^a	8.6	0.84 ± 0.05 ^a	3.4
200	0.715 ± 0.023 ^c	11.1	0.76 ± 0.03 ^c	18.3	0.74 ± 0.05 ^c	14.9
400	0.65 ± 0.04 ^c	19.8	0.64 ± 0.03 ^c	31.2	0.615 ± 0.024 ^c	28.7
800	0.59 ± 0.03 ^c	27.2	0.44 ± 0.05 ^c	52.7	0.45 ± 0.03 ^c	48.3

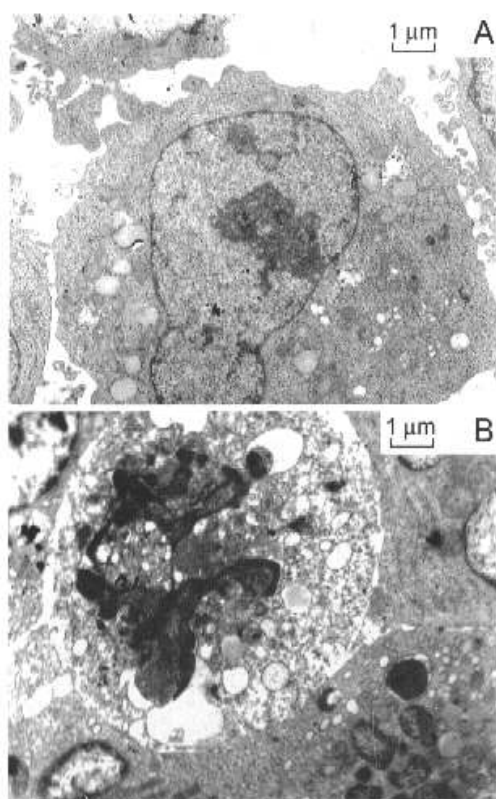


Fig 2. Electron micrographs of K562 cells treated with CFP-1 for 5 d. (A) Control; (B) Cells treated with CFP-1 300 mg/L. $\times 6600$.

of cytoplasm, while the plasma membrane remained well defined. The chromatin became condensed, and nucleus with the formation of apoptotic body was seen. Therefore, morphological changes suggested the occurrence of apoptosis in K562 cells treated with CFP-1.

DNA fragmentation After treatment with CFP-1 (100–300 mg/L) for 5 d, DNA electrophoresis of K562 cell showed a typical "ladder" of DNA. No DNA fragmentation of K562 cells was seen in the control group (Fig 3).

DNA degradation by flow cytometry After exposure of K562 cells to CFP-1 (300–900 mg/L), 13.6%–32.8% of apoptotic cells were located in sub- G_1 -phase position. Moreover, changes in cell cycle distribution of the nonapoptotic cell population occurred in the cells treated with CFP-1 (Fig 4).

DISCUSSION

The results demonstrated strongly the occurrence of

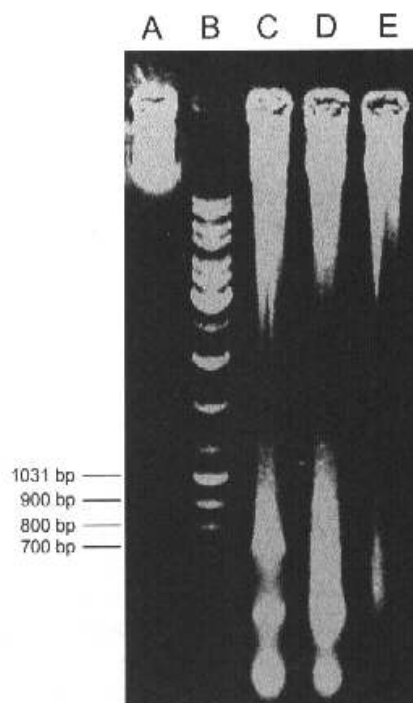


Fig 3. Agarose gel electrophoresis of DNA extracted from K562 cells treated with CFP-1 for 5 d. Lane A: control; Lane B: DNA marker; Lane C, D, E: CFP-1 300, 200, 100 mg/L.

apoptosis in K562 cells treated with CFP-1 *in vitro*. Apoptosis is a specific mode of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular change^[9]. This mode of cell death has recently become a focus of interest in oncology and may also shed light on cancer therapy^[10]. Changes in cell morphology, such as condensation of chromatin, nuclear fragmentation, and apoptotic bodies, were the most reliable markers of apoptosis^[11]. Apoptosis was also best characterized biochemically by the cleavage of DNA into nucleosomal size fragments of 180–200 bp, which were detected by gel electrophoresis as a DNA ladder^[12]. The appearance of a ladder-like DNA fragmentation pattern from CFP-1 treated K562 cells was shown in our experiment. In addition, the flow cytometric methods of identifying apoptotic cells were based on measurement of cellular DNA content. Our results showed that 5 d after CFP-1 (300–900 mg/L) treatment, 13.6%–32.8% of collected K562 cells were located in sub- G_1 -phase position.

Scientific and commercial interest in polysaccharides has been extremely active because of their higher bio-

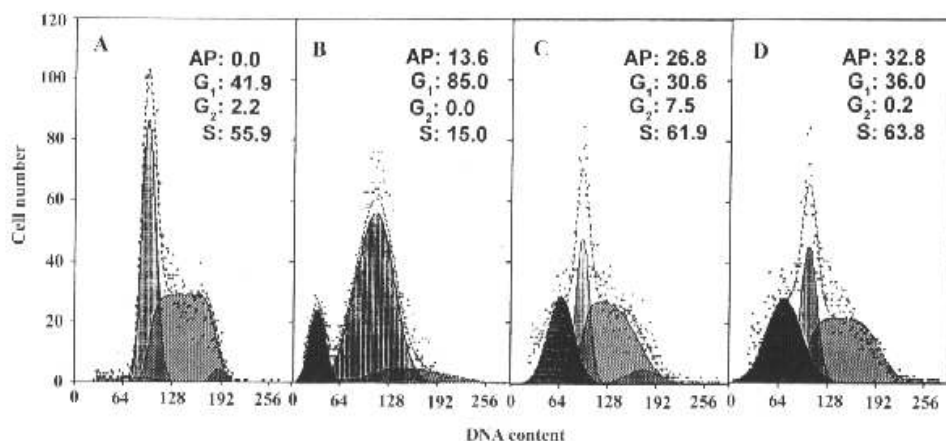


Fig 4. DNA contents of K562 cells treated with CFP-1 for 5 d. (A) Control; (B) CFP-1 300 mg/L; (C) CFP-1 600 mg/L; (D) CFP-1 900 mg/L.

activities and lower toxicity in recent decades. Many plant polysaccharides, as biological response modifiers, have immunopotentiating and antitumor activity. Our data indicate that CFP-1 induces apoptosis in K562 cells, which is one of its mechanisms by which the agent exhibits the antitumor activity. On the other hand, we also find CFP-1 increases the proliferation of mouse T lymphocytes activated by Con A and the specific antibody formation of mouse splenocytes *in vitro* (data not shown). CFP-1 might be a potential anti-tumor drug and immunopotentiator.

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分枝石蕊多糖诱导人白血病 K562 细胞凋亡¹

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关键词 分枝石蕊; 多糖类; 细胞凋亡; K562 细胞;
DNA 断片; 电泳; 流式细胞术

目的: 研究分枝石蕊多糖(CFP-1)是否能诱导 K562

细胞凋亡。方法：抑制细胞增殖的测定采用 MTT 法；用荧光显微镜和透射电镜观察细胞的形态学变化；采用琼脂糖凝胶电泳法观测 DNA 碎片；用流式细胞仪检测凋亡细胞数。结果：CFP-1 (50 - 800 mg/L) 明显抑制 K562 细胞增殖，并且呈浓度依赖

性。K562 细胞与 CFP-1 300 mg/L 共同培养 5 d 后，观察到典型的凋亡形态变化，电泳呈现梯形条带。结论：CFP-1 诱导人白血病 K562 细胞凋亡。

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重 要 新 闻

2000 年度美国科学引文索引 (SCI) 的期刊引用年度报告 (JCR) 日前揭晓。在所报道的中国 47 种期刊中，《中国药理学报》排在生命科学领域的第三位，仅次于《世界胃肠病学杂志》和《中国科学 B 辑》。《中国药理学报》继续保持在国际药学药理学核心期刊榜中，是其中唯一的中国期刊。影响因子 (IF) 首次达到 0.485，总引次 854，及时因子 0.105。相对 1999 年 0.196 的分数，影响因子翻了一倍多，取得了重大进步。这必将为我国药学和药理学工作者提供更便捷、更有价值的国际学术论著发表途径。

Notice to authors

The 2000 Impact Factors have just become available. The Impact Factor (IF), Total Cites, and Immediacy Index of our journal Acta Pharmacologica Sinica are 0.485, 854, and 0.105, respectively. The Impact Factor for 2000 shows an increase over the 1999 Impact Factor, which was 0.196.